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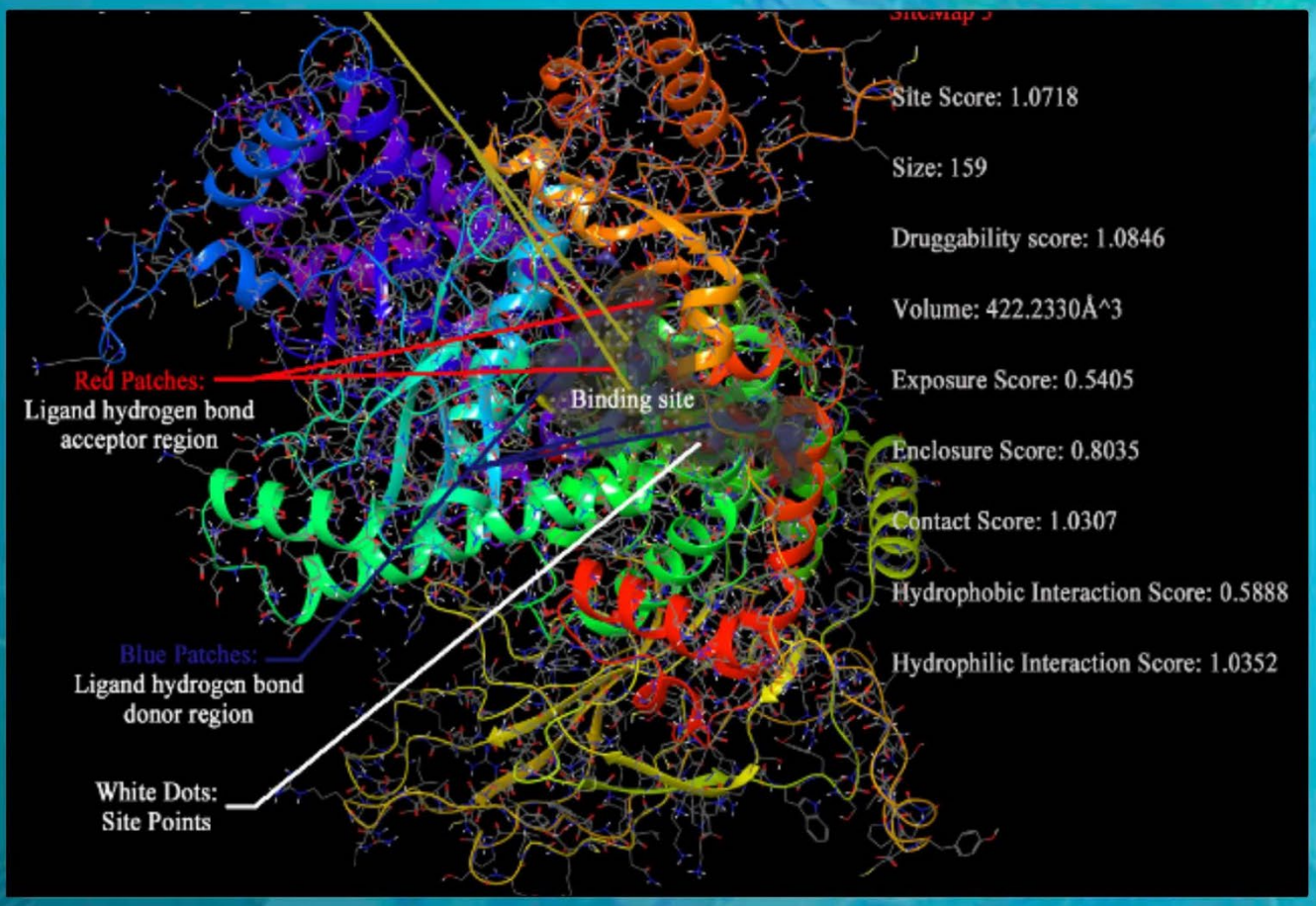

Rational drug design from phosphatidylinositol 3-kinase-α inhibitors through molecular docking and 3D-QSAR methodologies for cancer immunotherapy

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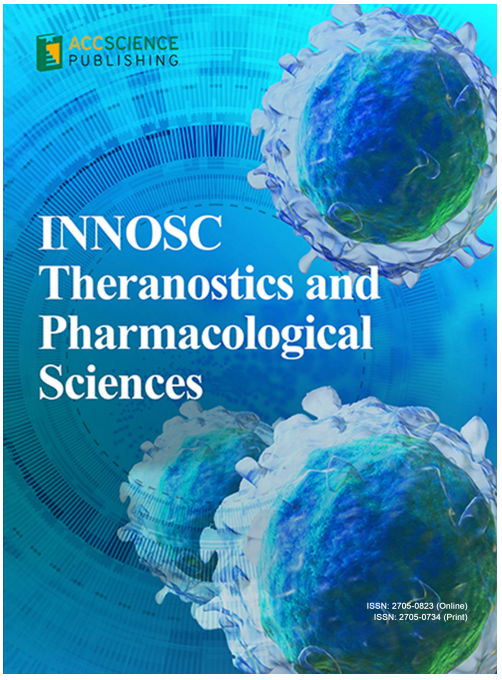

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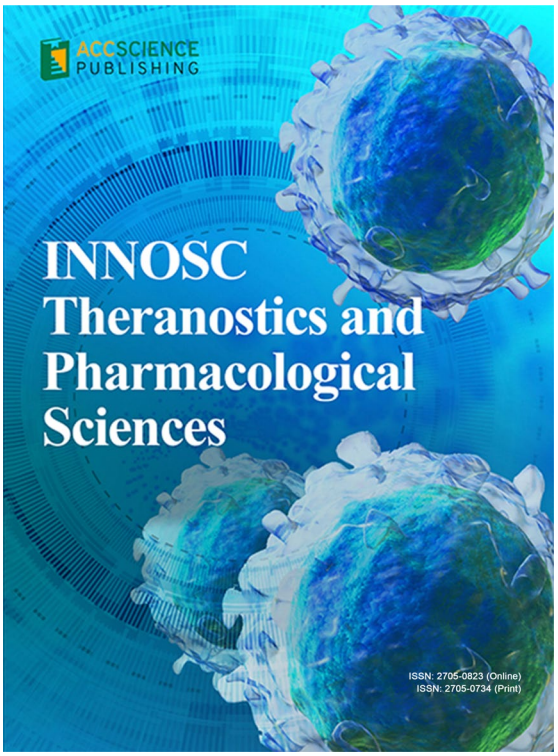

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INNOSC Theranostics and Pharmacological Sciences

REVIEW ARTICLE

Therapeutic small molecules in the development of treatment for subarachnoid hemorrhage

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Abstract

Subarachnoid hemorrhage (SAH) is a severe and often fatal condition characterized by the accumulation of blood beneath the arachnoid layer of the meninges. Predominantly affecting individuals in the 40–60 age range, it is commonly caused by head trauma from falls or car accidents. Ruptures of cerebral aneurysms also contribute significantly to SAH. Risk factors for SAH include hypertension and smoking, and symptoms typically include severe headache and neck pain. Diagnosing SAH typically involves a combination of medical history, physical examination, and imaging studies such as computed tomography angiography or magnetic resonance imaging angiography. Recent research suggests that pharmaceutical management of intracerebral hemorrhage (ICH) includes the administration of recombinant activated factor VII, tranexamic acid, and aggressive blood pressure reduction. For patients with significant SAH and ICH, minimally invasive surgical procedures for hematoma evacuation, as well as surgical evacuation of SAH and ICH, have proven to be highly beneficial. Furthermore, an emerging area of treatment involves therapeutic small molecules designed to interrupt the pathophysiological pathways leading to SAH. This novel approach holds promise for advancing our understanding and management of this complex medical condition.

Keywords: Subarachnoid hemorrhage; Neurosurgery; Therapeutic small molecules; Novel therapy; Pharmacological management

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1. Introduction

Subarachnoid hemorrhage (SAH) remains a significant contributor to both long-term morbidity and mortality. This review employs a variety of research articles to guide readers through the pathophysiology, risk factors, diagnosis, and treatment options for SAH. Of particular interest are the innovative therapeutics currently under exploration for SAH. Future directions in SAH, as well as controversial issues, current advancements, and prognostic factors, are comprehensively addressed.

The treatment of SAH can be approached on an individualized basis, incorporating combinations of newer therapies alongside previously available surgical or medical options tailored to address the specific needs of each patient. The selection of specific interventions

for individual patients may contribute to a reduction in the occurrence of complications and facilitate targeted treatments. This approach provides future researchers the opportunity to explore these novel therapies, aiming to manage diverse patient types with unique combinations tailored to the specific needs of each individual. Subsequently, this research can contribute to the development of an updated guideline for reference in the future.

The major limitation of this review lies in its inability to evaluate the combinations of novel therapies along with the currently available traditional therapies. The principal objective of this article is to furnish comprehensive information regarding the current treatment options for SAH, placing particular emphasis on the discovery and ongoing research of novel therapies. Another objective is to provide researchers with a further direction for evaluating the efficacy of these therapies, especially when used in combination with traditional therapy options.

This review was formulated based on the results of searches conducted on PubMed, Embase, Scopus, and conference abstracts up to the year 2023 for studies related to SAH and its treatment. The study adheres to the PRISMA guidelines for systematic review. Information was systematically retrieved from the aforementioned databases, and relevant studies were identified based on the crucial information they provided. Each article's information was thoroughly reviewed and extracted to construct a comprehensive database. This approach ensured that the authors had access to all pertinent information during the writing process of this review article, thereby minimizing the risk of overlooking important details.

1.1. Understanding SAH

Blood can infiltrate the cerebrospinal fluid due to cerebral insult, a burst intracranial aneurysm, and/or other severe head trauma, frequently resulting in a deadly condition known as SAH[1,2]. Failure to restore normal blood flow can result in fatal consequences, including death, hydrocephalus, stroke, and permanent disability. SAH, a form of cerebrovascular disease, is recognized as one of the most severe and fatal neurological emergencies[3]. It is estimated that up to 50% of patients experiencing SAH succumb within 30 days of onset, with an additional 30% suffering from moderate-to-severe morbidity[4-8].

1.2. Causes and risk factors of SAH

Ruptures of pre-existing intracranial aneurysms are the primary cause of SAH, affecting 1% to 5% of the general population[3,9]. In addition, other causes, such as cerebrovascular malformations, vascular abnormalities at the skull base, and head trauma, can also precipitate SAH[10-12]. Notably, spinal vascular pathologies and isolated spinal artery aneurysms are also identified as potential causes[13]. Risk factors associated with SAH include hypertension and smoking[14]. Symptoms commonly associated with SAH include severe headache, neck pain, nausea or vomiting, and photophobia[15]. In some cases, SAH may present with atypical symptoms such as back pain and lower-extremity weakness. Complications of SAH that may result in death include hemorrhage, cerebral edema, infection, pneumonia, external ventricular shunt, and ischemic damage[16-18]. A comprehensive understanding of the causes and risk factors of SAH is important for its prevention, diagnosis, and effective treatment.

1.3. Symptoms and diagnosis of SAH

Symptoms of SAH vary depending on the severity and location of the bleeding[19]. Common symptoms include sudden and severe headaches, neck pain or stiffness, nausea and vomiting, sensitivity to light (photophobia), changes in vision or double vision, seizures, and loss of consciousness[20-22]. The diagnosis of SAH typically involves a combination of medical history, physical examination, and imaging studies such as computed tomography angiography or magnetic resonance imaging angiography[23,24]. The pathophysiological mechanisms and symptoms of SAH are illustrated in Figure 1.

2. Pathophysiology of SAH

SAH is a complex and life-threatening condition characterized by blood infiltration into the subarachnoid space surrounding the brain. The pathophysiology of SAH involves a series of events that lead to both immediate and delayed complications. The following subsections provide a comprehensive overview that integrates findings from multiple references.

2.1. Rupture of intracranial aneurysm

The rupture of an intracranial aneurysm plays a pivotal role in the pathophysiology of SAH. When considering the pathogenesis of SAH, consulting these key sources yields valuable insights. Osgood[25] discusses the complex pathophysiology of aneurysmal SAH, highlighting the significance of aneurysmal rupture as the primary trigger for this devastating condition. Boling and Groves[26] delve into the management of SAH, emphasizing that the rupture of intracranial aneurysms leads to the sudden release of blood into the subarachnoid space. D'Souza[27] provides a comprehensive perspective on aneurysmal SAH, underlining how the rupture of an intracranial aneurysm disrupts the normal cerebral environment and initiates a cascade of events.

Furthermore, Sorrentino *et al.*[28] contribute a contemporary narrative review that specifically addresses

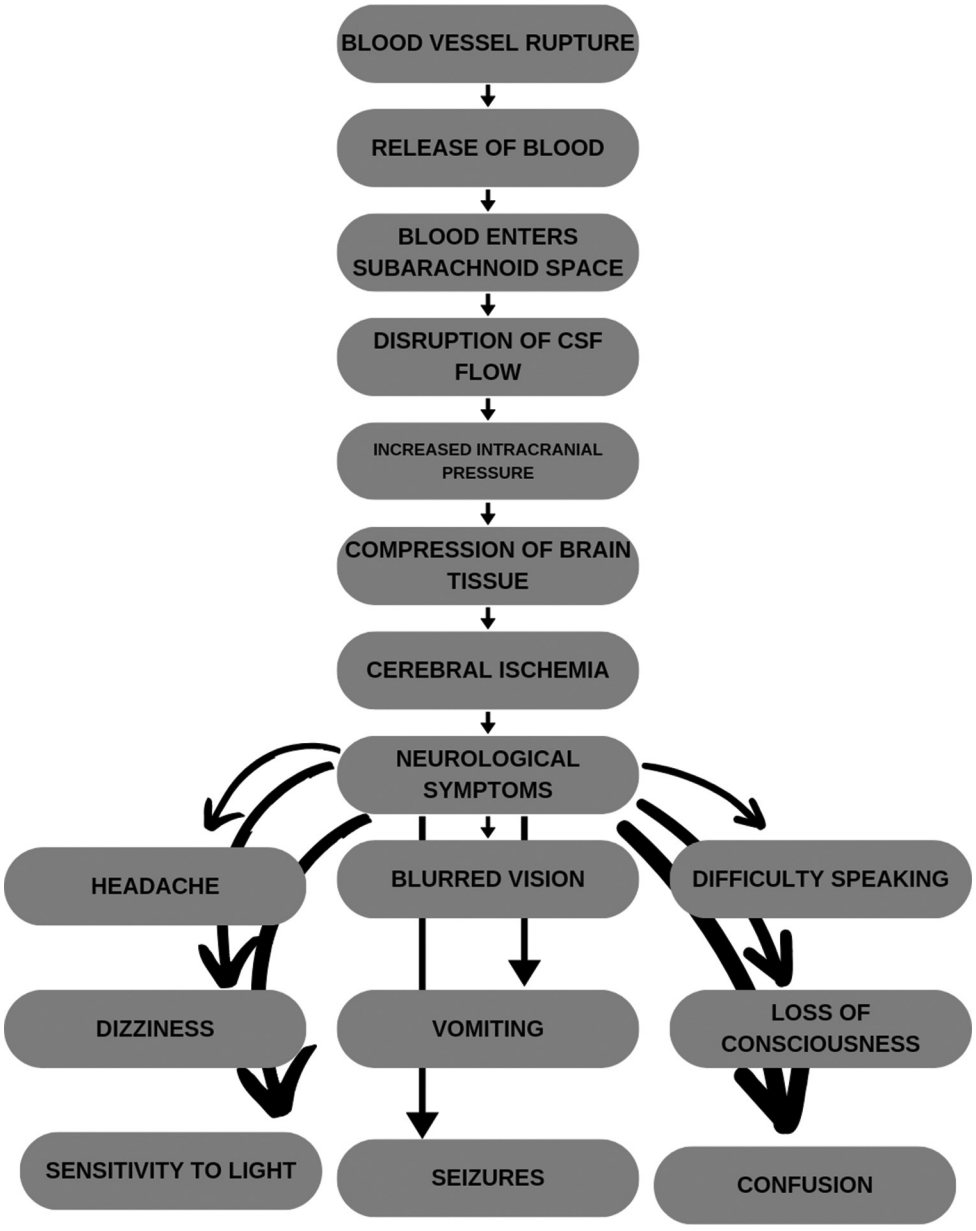

Figure 1. Pathophysiology and symptoms of subarachnoid hemorrhage.

the persistence of headaches following aneurysmal SAH, shedding light on the long-term consequences of aneurysmal rupture. Collectively, these sources underscore the critical role of intracranial aneurysm rupture in the pathophysiology of SAH. This highlights the need for early diagnosis and intervention to mitigate the potentially severe consequences associated with this event.

2.2. Toxic hemoglobin products

The breakdown of hemoglobin results in the production of toxic substances, leading to the following adverse effects:

(i) Oxidative stress: Hemoglobin breakdown releases heme, which generates reactive oxygen species (ROS). These ROS induce oxidative stress, damaging neuronal and vascular structures. Oxidative stress is associated with cerebral vasospasm, delayed cerebral ischemia (DCI), and brain injury following SAH. Sorrentino *et al.*[28] discuss the persistence of headaches after SAH, attributing them, in part, to the oxidative stress and inflammation resulting from toxic hemoglobin products.

(ii) Inflammation: Toxic hemoglobin products can trigger an inflammatory response within the subarachnoid space and brain parenchyma. This inflammation,

mediated by cytokines and immune cells, can exacerbate tissue damage.

(iii) Vasoconstriction: Hemoglobin and its degradation products, such as bilirubin, can induce vasoconstriction and impair cerebral blood flow regulation. This can result in complications like cerebral vasospasm, which is associated with poor outcomes in SAH patients. Towner *et al.*[29] discuss the use of mechanical ventilation in aneurysmal SAH, potentially touching upon the role of vasoconstriction in the context of ventilatory support.

2.3. Blood in subarachnoid space

Voldby[30] conducted a literature review highlighting the relationship between SAH and cerebral vasospasm. They emphasize that the initial insult, characterized by the presence of blood in the subarachnoid space due to aneurysmal rupture or other causes, triggers a cascade of events leading to vasospasm. The presence of blood components, particularly hemoglobin breakdown products, contributes to inflammation and dysfunction of cerebral blood vessels, ultimately resulting in vasospasm – a hallmark complication of SAH.

Voldby[30] provides insights into the pathophysiology of SAH based on both experimental and clinical data. The presence of blood in the subarachnoid space is identified as a primary event. This blood irritates the meninges and surrounding brain tissues, triggering an inflammatory response. In addition, breakdown products of hemoglobin, such as bilirubin, exert toxic effects on brain tissue, exacerbating the damage caused by SAH.

Collectively, these references emphasize the pivotal role of blood within the subarachnoid space in the pathophysiology of SAH. This presence triggers a cascade of events, including inflammation and cerebral vasospasm, which contribute to the clinical manifestations and complications associated with this condition[31].

2.4. Vasospasm and reduced cerebral blood flow

As mentioned earlier, the study conducted by Voldby[30] contributed experimental and clinical data on the pathophysiology of SAH. While not directly addressing vasospasm and reduced blood flow, these data likely enhance a broader understanding of the influence exerted by these factors on the development and progression of SAH.

In a related context, Hayman *et al.*[32] discussed the pathophysiology of acute intracerebral and SAH. They emphasized the impact of reduced blood flow on brain tissue, particularly in the context of SAH. Insufficient blood supply can result in ischemia, contributing to the development of SAH-related complications.

2.5. Seizure burden associated with clinical outcome

A study conducted by De Marchis *et al.*[33] focused on the clinical and functional outcomes in SAH patients with respect to seizures. The study included every patient with spontaneous SAH consecutively admitted and continuously monitored with an electroencephalogram in the neurological intensive care unit at Columbia University Medical Center. The number of hours with seizures recorded on continuous EEG (cEEG) was used to quantify the seizure load. Cognitive results were evaluated using the Telephone Interview for Cognitive Status. In 12% of SAH patients undergoing cEEG monitoring, whose primary purpose was to screen for cerebral ischemia, seizures occurred. Since none of the seizures were convulsive, cEEG would not have detected them. Three months after SAH, a significant correlation was found between the likelihood of a poor functional outcome and the occurrence of both non-convulsive seizures (NCSZ) and seizure load. However, the occurrence of NCSZ alone was not related to cognitive results at 3 months; only the seizure load showed a correlation. The more nuanced relationship between seizure load and cognitive outcome implies that seizure prophylactic measures should not be separated from therapeutic approaches aimed at reducing seizure burden[33].

2.6. Inflammation and immune response

SAH triggers an inflammatory response involving immune cells and the release of pro-inflammatory cytokines, potentially contributing to secondary brain injury[32]. The inflammatory and immune responses in SAH include several key processes:

(i) Blood–brain barrier (BBB) disruption: The presence of blood in the subarachnoid space can lead to the disruption of the blood–brain barrier, allowing immune cells and inflammatory molecules to enter the brain tissue.

(ii) Immune cell activation: Resident immune cells in the brain, such as microglia and macrophages, become activated in response to blood products. They phagocytize red blood cells and release pro-inflammatory cytokines, contributing to neuroinflammation.

(iii) Cytokine release: Inflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α) and interleukin-1 beta (IL-1β), are released in response to SAH. These cytokines can exacerbate inflammation and neuronal injury.

(iv) Oxidative stress: The breakdown of hemoglobin from the lysed red blood cells leads to the release of heme, generating ROS and causing oxidative stress. This oxidative stress further contributes to tissue damage.

(v) Immune cell infiltration: In some cases, peripheral immune cells may infiltrate the injured brain tissue, amplifying the immune response and potentially contributing to secondary brain injury.

(vi) Perivascular innervation: Cerebral blood vessel contractions are concentration dependent and occur when noradrenaline is administered *in situ* or *in vitro*. It seems that pial veins are more noradrenaline sensitive than pial arterioles. Specifically, α-adrenoceptor blockers can prevent these contractile responses. According to thorough receptor characterizations, the brain vasculature of certain animals (like dogs and cats) has post-junctional α2-adrenoceptors, while adrenoceptors of the α1-subtype are found in the brain vessels of other animals (such as rats, monkeys, and humans)[34].

(vii) Vasoactive neurotransmitters: The cerebral vasculature is densely innervated by sympathetic and parasympathetic nerve fibers, which release various neurotransmitters, including norepinephrine and acetylcholine, regulating vascular tone. In SAH, the balance between these neurotransmitters is disrupted due to the stress response and inflammation, leading to alterations in vascular tone and dysfunction that contribute to SAH. Cardiovascular and cardiac abnormalities are believed to result from sympathetic nervous system activation, which raises circulating catecholamine levels.

(viii) Sympathetic activation: SAH triggers a robust sympathetic nervous system response, resulting in increased norepinephrine release. Norepinephrine induces vasoconstriction of cerebral blood vessels, potentially contributing to DCI in SAH patients. This vasoconstriction can further reduce cerebral blood flow and exacerbate brain injury.

(ix) Inflammatory response: SAH initiates an inflammatory cascade that affects perivascular nerves. Inflammatory molecules, such as cytokines and chemokines, activate or sensitize nerve fibers, thereby enhancing their vasoactive effects. This inflammation-induced neuronal sensitization may result in sustained vasoconstriction, contributing to DCI.

(x) Altered neurotransmitter release: SAH disrupts the release and reuptake of neurotransmitters at perivascular nerve terminals. This dysregulation can lead to sustained vasoconstriction, increased vascular resistance, and impaired autoregulation of cerebral blood flow.

The pathophysiology of complications is illustrated in Figure 2.

2.7. Potential therapeutic targets

Understanding the role of perivascular innervation in SAH pathophysiology provides potential targets for therapeutic interventions. Modulating the activity of perivascular nerves or their neurotransmitters may help mitigate the vasoconstriction and inflammation associated with SAH, thereby improving cerebral perfusion and patient outcomes.

In addition, vascular abnormalities often arise from ruptured intracranial aneurysms or abnormal cerebral blood vessel dilations, leading to the sudden release of blood into the subarachnoid space. Inflammatory responses ensue from the introduction of blood into the subarachnoid space[35].

Hemodynamic disturbances lead to increased intracranial pressure (ICP) and reduced cerebral blood flow due to blood presence in the subarachnoid space, consequently initiating an inflammatory cascade with the

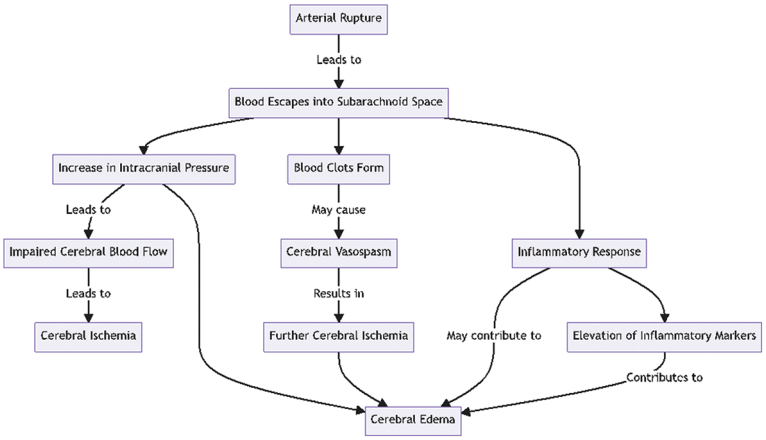

Figure 2. Pathophysiology of subarachnoid hemorrhage complications.

release of inflammatory mediators and contributing to complications such as cerebral vasospasm and DCI[36,37]. SAH leads to cerebral vasospasm, involving the narrowing of cerebral blood vessels due to breakdown products of blood and inflammation, potentially compromising blood flow to brain tissue. SAH primarily stems from ruptured intracranial aneurysms[38], as emphasized in Kellner *et al.*[39]. This rupture leads to an immediate disruption in hemodynamics, causing increased ICP and reduced cerebral blood flow, amplifying the impact of SAH. In addition, inflammatory responses, as highlighted in Cardentey-Pereda and Pérez-Falero[40], are initiated as blood enters the subarachnoid space, contributing to complications such as cerebral vasospasm. This narrowing of cerebral blood vessels can further compromise blood flow to the brain. In summary, the pathophysiology of SAH involves vascular rupture, hemodynamic disruption, and inflammatory processes, collectively contributing to its complex clinical manifestations.

3. Current treatment options

The two basic categories of treatment for SAH are surgical and pharmacologic. Non-contrast computerized tomography (NCCT) is a quick, reliable, and accurate method for identifying SAH[41]. Following an acute episode, a significant proportion of patients with SAH and intracerebral hemorrhage (ICH) showed a two-point decline on the Glasgow Coma Scale[42,43]. The primary goals of care for unstable patients are to secure the airway and continue resuscitative procedures before arriving at the hospital.

3.1. Pharmacologic management

3.1.1. Blood pressure control

During the acute phase, the majority of ICH patients present with high blood pressure when they first arrive. This high blood pressure might lead to hematoma development and a dismal prognosis[43]. The INTERACT2 trial, a major clinical trial that randomly assigned patients to either a blood pressure control group with systolic blood pressure <140 mmHg or a blood pressure control group of systolic blood pressure <180 mmHg for the first 24 h, has provided the most comprehensive data on blood pressure management.

3.1.2. Use of tranexamic acid and factor VII

Tranexamic acid is used to reduce bleeding-related mortality in trauma and postpartum hemorrhage situations. An investigation into the potential of tranexamic acid to reduce hematoma growth and enhance prognosis in adult ICH-related stroke patients was carried out. Adult participants with ICH took part in a randomized controlled trial, and the results demonstrated a significant improvement in patient prognosis, a reduced frequency of hematoma growth, and fewer serious sequelae. These outcomes corroborate the antifibrinolytic properties of tranexamic acid[44].

3.1.3. Antioxidants

One promising medication for the treatment of ICH is edaravone. While it has demonstrated efficacy in patients with ICH, the debate about its long-term advantages and prognosis persists[45]. This potent free radical scavenger[46-50] was initially licensed in Japan[51,52] for the management of acute ischemic stroke (AIS).

3.2. Surgical management

3.2.1. Craniopuncture

Craniopuncture has been the accepted method of treating ICH in China[53]. In a study comparing the results of craniopuncture and conservative management in 377 patients who experienced basal ganglia hemorrhage, researchers discovered that patients undergoing craniopuncture exhibited significantly improved neurological function by the end of 2 weeks, with no variation in the rates of rebleeding and no discernible difference in patient mortality[54].

3.2.2. Craniotomy

While the role of surgery in treating patients with ICH remains a subject of debate, craniotomy for hematoma drainage is the most commonly utilized method in hospitals[55,56]. The surgical study in ICH (STICH)[57] aimed to assess the benefits of prompt hematoma drainage combined with conservative treatment in a multifocal, multinational, randomized clinical study. The study concluded that prompt hematoma drainage did not improve the overall prognosis[57]. However, when significant hematomas impart a mass effect with midline shift causing altered awareness, or when the neurological decline occurs due to hematoma enlargement, a craniotomy becomes an essential life-saving procedure.

3.2.3. Hematoma aspiration and thrombolysis

After a minimum of 5 – 6 h following the computed tomography scan used for diagnosis in stereotactic aspiration with thrombolysis, patients undergo a follow-up scan to assess the stable nature of the clot. In the Minimally Invasive Surgery Plus Alteplase for ICH Evacuation (MISTIE) trials, stereotactic hematoma aspiration with thrombolysis and medical therapy were compared in three stages. The initial published results indicate that the MISTIE approach resulted in a greater reduction in clot volume compared to medical treatment[58].

3.2.4. Neuroendoscopy and evacuation

In the neuroendoscopy and evacuation procedure, the combination of an endoscope and an aspiration cannula is utilized. Research indicates that individuals undergoing endoscopic evacuation had a significantly better prognosis than those receiving medicinal therapy[59].

3.2.5. Surgical clipping and endovascular coiling

Surgical clipping and endovascular coiling are the two most frequently used procedures to lower the incidence rate of bleeding following a superficial arterial hematoma. Surgical clipping involves open surgery performed under general anesthesia. To visualize the aneurysm, the brain is gently retracted, and a tiny clip is positioned across its neck to prevent blood flow into the aneurysm, with titanium clips serving as a permanent fixture on the artery[60]. Despite advancements in endovascular therapy, surgical clipping of paraclinoid aneurysms remains a necessary therapeutic option with a manageable risk profile. The prediction and prevention of post-operative motor impairments during aneurysm clipping have demonstrated feasibility through intraoperative monitoring of motor and somatosensory evoked potentials[61,62].

In endovascular coiling, an initial catheter is used to implant a microcatheter into the femoral artery. The tip of the microcatheter is connected with a platinum coil, and an electrical current is utilized to detach the coil from the catheter once it enters the aneurysm lumen. The coil is left permanently within the aneurysm, inducing thrombosis[60].

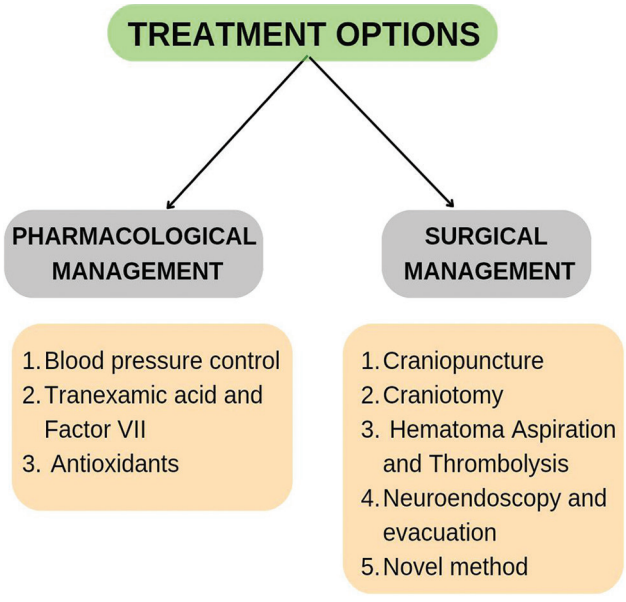

Figure 3. Current treatment options for the management of subarachnoid hemorrhage.

A summary of the current treatment options is illustrated in Figure 3.

3.2.6. Novel surgical method

A study on a case involving a large ICH evacuated utilizing a unique parafascicular BrainPath/Myriad procedure guided by diffusion tensor imaging suggests that this approach is a superior method for rapidly diminishing the hematoma[63].

4. Novel treatment options

SAH is a critical condition associated with a high risk of complications, including vasospasm, cerebral infarction, and poor outcomes. While traditional treatment strategies have limitations, ongoing research is exploring novel approaches to improve the management of SAH. Section 4 outlines several emerging treatment options supported by recent research.

4.1. Exosomes

With their ability to improve cognitive function, inhibit cell death, reduce inflammation, regulate autophagy, and protect the BBB, exosomes show great promise for the treatment of central nervous system (CNS) damage. Certain chemicals and pathways, including microRNA, NF-κB, PI3K/AKT, Notch1, and ERK, collaboratively contribute to these effects. The review by Zhang *et al.* provides an overview of the diverse applications of exosomes in CNS injury, highlighting their importance as therapeutic targets[64].

In comparison to the use of unmodified exosomes, a separate study[65] discovered that administering exosomes derived from human umbilical cord mesenchymal stem cells (hUCMSCs) with miR-206 knockdown confers superior neuroprotective benefits in the context of SAH-induced early brain injury (EBI). By selectively targeting brain-derived neurotrophic factor (BDNF), these miR-206-knockdown exosomes exhibit a considerable capacity to alleviate neurological impairments, reduce brain edema, and mitigate neuronal death. Moreover, *in vivo* administration of exosomes modified with miR-206 activates the BDNF/TrkB/CREB signaling pathway. Essentially, the study[65] confirms that exosomes produced from hUCMSCs with miR-206 knockdown are essential in preventing EBI after SAH by blocking apoptosis through the BDNF/TrkB/CREB signaling cascade. This discovery provides a novel and promising therapeutic target for treating SAH-induced EBI, potentially resulting in better treatment outcomes[66].

Exploring the potential of exosomal miR-3064-5p derived from dendritic cells to restore the BBB in the event of SAH. The results indicate that miR-3064-5p overexpression improves BBB integrity, reduces

inflammation, and mitigates apoptosis in SAH rats by suppressing iNOS expression, enhancing tight junction proteins, and inhibiting the SIRT6/PCSK9 pathway[67]. In SAH rats, the protective effects of dendritic cell exosomes are further reinforced, with miR-3064-5p playing a crucial role in the restoration of BBB following damage. These findings highlight the potential of exosomal miR-3064-5p as a treatment option for SAH, addressing BBB integrity and mitigating damage caused by SAH[67].

4.2. Inhibition of ferroptosis

A study[68] investigating the role of ferroptosis, an iron-dependent regulated cell death process, in SAH revealed that the ferroptosis inhibitor, liproxstatin-1, effectively protected HT22 cells from hemin-induced damage by preserving mitochondrial function and mitigating lipid peroxidation. In *in vivo* experiments, characteristic mitochondrial alterations in cortical neurons following SAH were demonstrated, and liproxstatin-1 treatment attenuated neurological deficits, brain edema, neuronal cell death, and redox imbalance[68]. Ferroptosis inhibition by liproxstatin-1 was linked to the preservation of glutathione peroxidase 4 and the down regulation of acyl-CoA synthetase long-chain family member 4 and cyclooxygenase 2. Furthermore, liproxstatin-1 reduced microglial activation and the release of pro-inflammatory cytokines (IL-6, IL-1β, and TNF-α), contributing valuable insights into SAH-related cell death mechanisms for future preclinical investigations[68].

NTN-1 was identified as a potent enhancer of peroxisome proliferator-activated receptor gamma (PPARγ), a key transcription factor governing lipid metabolism. In the context of NTN-1-mediated neuroprotection in SAH, a study investigated ferroptosis, a recently identified form of cell death linked to lipid metabolism[69]. The results demonstrated that NTN-1 treatment significantly improved survival rates, increased neuron survival, and enhanced neurological function, underscoring its role in inhibiting ferroptosis and mitigating neuron death. NTN-1 treatment also upregulated the expression of critical regulators of ferroptosis, including PPARγ, nuclear factor erythroid 2-related factor 2 (Nrf2), and glutathione peroxidase 4 (GPX4), thereby contributing to improved neurological outcomes in SAH. These findings suggest that NTN-1 exerts neuroprotective effects by mitigating neuronal ferroptosis through the PPARγ/Nrf2/GPX4 and coenzyme Q10-ferroptosis suppressor protein 1 (CoQ10-FSP1) pathways, offering valuable insights into potential therapeutic strategies for SAH-induced brain injury[69].

Peroxiredoxin 6 (PRDX6), a recognized antioxidant protein, has been previously linked to ferroptosis and lipid peroxidation, though its role in SAH remains elusive. Moreover, the potential involvement of PRDX6 in the neuroprotective effects of Fer-1, a selective ferroptosis inhibitor, in SAH has not been explored. The study employed endovascular perforation to induce a SAH model and administered Fer-1 and *in vivo* siRNA targeting PRDX6 to investigate the underlying mechanisms[70]. The research confirmed Fer-1's capacity to inhibit ferroptosis and provide neuroprotection in SAH. SAH induction resulted in reduced PRDX6 expression, which Fer-1 ameliorated. Furthermore, Fer-1 effectively addressed dysregulated lipid peroxidation, as indicated by glutathione and malondialdehyde levels, but this effect was reversed by si-PRDX6. Notably, the neuroprotective benefits of Fer-1 in SAH were compromised by PRDX6 knockdown and the use of a calcium-independent phospholipase A2 (iPLA2) inhibitor[70]. These findings underscore the involvement of PRDX6 in SAH-induced ferroptosis and its association with Fer-1's neuroprotective mechanism, particularly through its iPLA2 activity[70].

4.3. Nle^{4}DPhe^7-α-melanocyte-stimulating hormone

A study[71] investigated the potential protective effects of the α-MSH analog Nle^{4}DPhe^7-α- melanocyte-stimulating hormone (NDP-MSH) in experimental SAH in rats. Initial experiments demonstrated that intrathecal injection of low concentrations of NDP-MSH induced a tolerant phenotype in the basilar artery. Systemic treatment with NDP-MSH following SAH significantly reduced vasospasm on day 5. Transcript analysis revealed that SAH caused significant disruptions in the transcriptional profile of the basilar artery, affecting genes related to inflammation, stress response, apoptosis, and vascular remodeling[71]. NDP-MSH treatment mitigated most of these transcriptional changes and reduced the phosphorylation of extracellular-signal-regulated kinases (ERK1/2) and inhibitor protein IκBα. These findings suggest that melanocortins, including NDP-MSH, may serve as safe and effective therapeutic candidates for addressing SAH-related complications, including vasospasm[71].

In a separate study[72], the aim was to investigate the potential of NDP-MSH in reducing oxidative stress and neuronal apoptosis following ICH and to uncover the underlying mechanism. In a mouse ICH model, NDP-MSH was administered intraperitoneally after ICH induction. The results revealed that NDP-MSH treatment effectively mitigated neurological deficits, reduced brain water content, and inhibited oxidative stress and neuronal apoptosis 24 h after ICH[72]. Furthermore, NDP-MSH administration promoted the expression of melanocortin-1 receptor (Mc1r), as well as the phosphorylation of PI3K, Akt, and Nrf2, leading to increased Bcl-2 expression and decreased cleaved caspase-3 levels. Conversely, suppressing

Mc1r expression and PI3K phosphorylation reversed these effects. In conclusion, the study demonstrates that NDP-MSH activation of Mc1r ameliorates oxidative stress and neuronal apoptosis through the PI3K/Akt/Nrf2-signaling pathway in the context of ICH in mice[72].

NDP-MSH, an analog of α-melanocyte-stimulating hormone (α-MSH), was investigated for its anti-inflammatory potential in microglial cells through melanocortin receptor 4 (MC4R). In the pertinent study, NDP-MSH treatment induced the upregulation of the M2a/M2c marker Ag1 and reduced the M2b marker Il-4rα and Tlr4 expression in microglia[73]. Furthermore, it inhibited the nuclear translocation of NF-κB subunits p65 and c-Rel induced by lipopolysaccharide. NDP-MSH effectively reduced TNF-α release triggered by TLR2 and TLR4 agonists, while TLR-induced IL-10 release remained unaffected[73]. Moreover, NDP-MSH inhibited TLR2-induced high mobility group box 1 (HMGB1) translocation and phagocytic activity. These results highlight the potential of melanocortins, particularly NDP-MSH, in attenuating pro-inflammatory mechanisms and promoting M2-like polarization in microglia, offering a promising avenue for immunomodulation in neuroinflammatory disorders[73].

4.4. Glycyrrhizic acid

In a study involving a rat model of SAH[74], the aim was to investigate the therapeutic potential of glycyrrhizic acid (GA) in reducing cerebral vasospasm and to elucidate the underlying mechanisms. Three groups of male rats were established: GA, SAH, and control. The GA and SAH groups received intraperitoneal injections of GA (10 mg/kg) and normal saline (10 mg/kg), respectively, and underwent experimental cerebral vasospasm induction. Following SAH, GA therapy significantly enhanced cerebral function[74]. In comparison to the SAH group, the basilar artery in the GA group exhibited a considerable decrease in vascular wall thickness and a noticeable increase in diameter. Furthermore, in comparison to the SAH group, GA therapy resulted in a significant reduction in *Hmgb1* expression and a decrease in the mRNA expression of *Il-1β*, *Il-6*, and *Tnf-α* but an increase in *Il-10* expression. These results imply that GA may prevent cerebral vasospasm after SAH by inhibiting the expression of HMGB1 and the subsequent generation of inflammatory cytokines[75].

In a mouse model of SAH, a separate study[76] investigated the effects of glycyrrhizin on pro-inflammatory cytokines and peroxisome proliferator-activated receptors (PPARs). The study unveiled morphological alterations in the basilar arteries and decreased PPAR-γ and PPAR-δ protein levels in the SAH groups. Treatment with glycyrrhizin boosted the expression of *Ppar-γ* mRNA and protein, as well as *Ppar-δ* mRNA, which corresponded to lower TNF-α and IL-1β levels[76]. The administration of a PPAR-γ inhibitor prevented the decrease in TNF-α and IL-1β in the groups treated with glycyrrhizin. Glycyrrhizin was linked to the production of PPARs, especially PPAR-γ, and exhibited anti-inflammatory effects on SAH-induced vasospasm overall, indicating its potential use in treating inflammation and vasospasm in SAH[76].

4.5. Proteasomes (HMGB1 and Purpurogallin)

Research into the causes of SAH, specifically immediate and delayed neuroinflammation, has been spurred by the catastrophic consequences of SAH. This process involves a complex role played by T-cell infiltration during SAH. According to a study[77] examining this process, purpurogallin demonstrates potential in mitigating early inflammation and subsequent stimulation of HMGB1 in a mouse model of SAH. This suggests that the natural polyphenol purpurogallin may hold therapeutic promise for treating and preventing SAH-induced vasospasm. Nevertheless, the work is constrained by its inherent limitations, warranting the necessity for future research to consider variables such as SAH-induced vascular remodeling and the current paucity of comprehensive mechanistic evidence[77].

Rhinacanthin-C (RCT-C) demonstrates neuroprotective properties by decreasing cleaved caspase-3 and caspase-9a, mitigating apoptosis. Its anti-inflammatory effect is evident in the RCT-C groups, as indicated by the decreased expression of HMGB-1 mRNA and protein. Importantly, the administration of HMGB-1 recombinant protein counteracts the neuroprotective and immunosuppressive effects of RCT-C. This finding suggests that RCT-C influences the HMGB-1 pathway and mitigates brain apoptosis in the context of SAH pathogenesis[78].

In the basilar artery of SAH rat models, HMGB1 is released from smooth muscle cells through a dynamic process[78]. Anti-HMGB1 monoclonal antibodies (mAb) efficiently blocked this cascade of events, which included the migration of HMGB1, upregulation of receptors constricting blood vessels, and the presence of inflammation-linked chemicals. The use of anti-HMGB1 mAb contributed to the relaxation of blood vessel constriction in the context of SAH. This treatment approach successfully interrupted the sequence by neutralizing extracellular HMGB1, preventing inflammatory responses, alleviating severe blood vessel constriction in the basilar artery, and ultimately decreasing brain damage caused by insufficient blood flow, thereby improving neurological symptoms. These results suggest that anti-HMGB1 mAb therapy can effectively disrupt the series of events triggered by SAH[78].

Table 1. All available treatment options for subarachnoid hemorrhage

Treatment types	Pharmacologic treatment	Surgical treatment	Novel therapies
Treatments	Blood pressure control	Craniopuncture	Exosomes
	Tranexamic acid	Craniotomy	NDP-MSH
	Factor VIII	Hematoma aspiration and thrombolysis	Proteosomes-HMGB1, and purpurogallin
	Antioxidants	Neuroendoscopy and evacuation	Liproxstatin-1
			Glycyrrhizic acid
			Clazosentan and endothelin receptor antagonists

Abbreviations: HMGB1: high mobility group box 1; NDP-MSH: Nle4DPhe7-α-melanocyte-stimulating hormone.

4.6. Clazosentan and endothelin receptor antagonists

Clazosentan, an endothelin receptor antagonist, has demonstrated promise in preventing cerebral vasospasm and improving outcomes post-SAH. Clinical trials, such as CONSCIOUS-1 and CONSCIOUS-2, have substantiated their efficacy in reducing the incidence of vasospasm-related morbidity[79,80].

4.7. Calcium antagonists for SAH

A Cochrane systematic review explored the use of calcium antagonists in the management of aneurysmal SAH, with the aim of determining their effectiveness and safety[81]. The insights derived from these clinical trials and systematic reviews have contributed significantly to advancing our knowledge of treatment strategies for SAH, which helps to improve patient outcomes and reduce complications associated with this condition.

4.8. Triple-H therapy optimization

Triple-H therapy has long been used to prevent vasospasm. Recent research, however, emphasizes the importance of individualizing its components to achieve optimal cerebral perfusion while mitigating potential complications[82]. Triple-H therapy involves the manipulation of hemodynamic parameters, namely hypertension, hypervolemia, and hemodilution. The principles, controversies, and outcomes associated with triple-H therapy can be explored in scholarly articles[80,81,83-86], which cover topics including:

(i) The rationale behind inducing hypertension to improve cerebral perfusion.
(ii) The role of hypervolemia in preventing DCI.
(iii) The use of hemodilution to reduce blood viscosity and enhance microcirculation.
(iv) Challenges in achieving the delicate balance of these parameters.

4.9. Intracranial pressure monitoring

Intracranial pressure monitoring plays a crucial role in identifying and managing secondary insults post-SAH.

Table 2. Clinical trials on subarachnoid hemorrhage (SAH)

Clinical trials	Details of the clinical trials
CONSCIOUS-1 Trial	The CONSCIOUS-1 trial aimed to investigate the potential of clazosentan, an endothelin receptor antagonist, in preventing neurological ischemia and infarction following SAH[91]. This randomized, double-blind, placebo-controlled phase 2 trial assessed the drug's effectiveness and safety in improving outcomes for SAH patients.
CONSCIOUS-2 Trial	Building upon the findings of CONSCIOUS-1, the CONSCIOUS-2 trial expanded the investigation into clazosentan's efficacy in SAH patients undergoing surgical clipping[79]. This phase 3 trial continued to explore its potential in preventing adverse neurological outcomes post-SAH.
STASH Trial	The STASH trial investigated the use of simvastatin, a statin medication, in patients with aneurysmal SAH[80]. This multicenter, randomized phase 3 trial aimed to determine whether simvastatin could improve patient outcomes and reduce complications following SAH.

Recent systematic reviews highlight its importance in guiding treatment decisions and improving patient outcomes[87]. ICP monitoring is critical in SAH management, allowing continuous assessment of intracranial dynamics. Numerous scholarly articles explore relevant topics[81,82,88,89], covering topics including:

(i) The importance of ICP monitoring in identifying and managing elevated ICP.
(ii) Various monitoring techniques, including intraventricular and intraparenchymal monitors.
(iii) The correlation between elevated ICP and poor outcomes in SAH patients.
(iv) Controversies surrounding the threshold for intervention based on ICP values.

4.10. Statin therapy

Statins, particularly simvastatin, have exhibited potential in reducing the incidence of vasospasm and improving clinical outcomes post-SAH. The STASH trial demonstrated the safety and efficacy of statins in this context[81]. Table 1 provides a summary of all available treatment options.

4.11. Modified Fisher scale for risk prediction

Recent studies have emphasized the utility of the modified Fisher scale in predicting symptomatic vasospasm and cerebral infarction post-SAH. This scale enables risk stratification, allowing for more targeted interventions in high-risk patients[90]. The development of outcome prediction models, exemplified by the SAHIT multinational cohort study, aids in identifying patients at a higher risk of complications, allowing for targeted interventions and improved outcomes[91].

4.12. Clinical trials

Table 2 mentions the major clinical trials.

5. Conclusion

SAH is a severe and often fatal condition across all age groups, stemming from different causes. The treatment landscape for SAH is a subject of ongoing discussion, with a recognition of the need for tailored treatment options to address the specificity of each patient. SAH carries a high risk of complications, including vasospasm, cerebral infarction, and poor prognosis.

Traditional treatment strategies exhibit limitations, prompting the exploration of novel approaches to improve the management of SAH. The two basic categories of SAH treatment are surgical and pharmacologic. NCCT has emerged as a rapid, reliable, and accurate diagnostic method for SAH. Pharmacologic treatments mainly involve blood pressure control, the use of tranexamic acid, factor VIII, and antioxidants. Surgical treatments encompass craniopuncture, craniotomy, hematoma aspiration and thrombolysis, neuroendoscopy, and evacuation. Novel therapies, such as exosomes, demonstrate the ability to improve cognitive function, inhibit cell death, reduce inflammation, regulate autophagy, and protect BBB. The utilization of exosomes holds great promise for treating damage to CNS.

The ferroptosis inhibitor, liproxstatin-1, has proven effective in protecting HT22 cells from hemin-induced damage by preserving mitochondrial function and mitigating lipid peroxidation. These findings suggest that NTN-1 exerts neuroprotective effects. Systemic treatment with NDP-MSH following SAH significantly reduces vasospasm. Glycyrrhizin has been linked to the production of PPARs, especially PPAR-γ, and exhibits anti-inflammatory effects on SAH-induced vasospasm, suggesting its potential for treating inflammation and vasospasm in SAH. The use of anti-HMGB1 mAb contributes to the relaxation of blood vessel constriction in the context of SAH. This treatment method effectively interrupts the sequence by neutralizing extracellular HMGB1, preventing inflammatory responses, alleviating severe blood vessel constriction in the basilar artery, and ultimately decreasing brain damage caused by insufficient blood flow, thereby improving neurological symptoms. Clazosentan, an endothelin receptor antagonist, has demonstrated promise in preventing cerebral vasospasm and improving outcomes post-SAH.

The above-mentioned treatment options are some of the novel therapies available for the treatment of SAH. Predictably, numerous future therapies may target the pathophysiology mechanisms mentioned in this review. The treatment of SAH can be approached in an individualistic manner, involving the use of combinations of newer therapies with previously available surgical or medical options based on patient requirements. Tailoring treatment to each patient's specific needs could potentially reduce the incidence of complications and provide exclusively specific treatments. This approach provides a foundation for future research to explore these novel therapies and manage different types of patients with unique combinations indicated for specific patients. Ultimately, this may contribute to the development of updated guidelines for future reference.

The major limitation of this review lies in the challenge of evaluating the combinations of novel therapies mentioned alongside the traditional therapies currently available.

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The authors declare that they have no competing interests.

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REVIEW ARTICLE

Mitochondria: The master regulator of aging

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Abstract

Mitochondria are ATP-producing organelles in eukaryotic organisms that serve as the cell's power plants. Besides, mitochondria are integral to regulating cellular homeostasis and metabolism as a result of their essential roles in reactive oxygen species (ROS) production, bioenergetics, catabolism and anabolism, heme and iron-sulfur biosynthesis, iron and calcium homeostasis, apoptosis and signal transduction, as well as immunity and inflammation. It is well accepted that mitochondria are evolutionarily derived from endosymbiotic alphaproteobacteria within eukaryotic cells adapted for effective energy transduction. Although most of the mitochondrial DNA (mtDNA) is thought to have been transported to the eukaryotic nucleus during evolution, mitochondria may have preserved protein-coding genes within their own DNA. Accumulating data show that a progressive decline of mitochondria regulates aging. The present review aims to outline the role of mitochondria in various aspects of aging, including unfolded protein response, generation of ROS, and the contribution of somatic mtDNA mutations as well as inflammation in aging. Moreover, we propose mitochondria-targeted nanoparticles and mitochondrial genome editing as novel tools to modify mitochondrial genome aberrations.

Keywords: Aging; Heteroplasmy; Mitochondrial DNA; Mitochondrial genome editing; Mitochondrial quality control; Mitochondria-targeted nanoparticles; Mitochondrial unfolded protein response; Reactive oxygen species

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1. Introduction

Aging is an intricate process during which continual tissue and organ function loss occurs over time, leading to increased susceptibility to death along with a decline in mitochondrial function.[1-4] This debilitation is the major cause of substantial age-related disorders, including cardiovascular diseases, cancer, neurodegenerative disease, and diabetes.[3,5] Nevertheless, aging has several common features, such as (i) impotent intercellular communication, (ii) impairment of proteostasis, (iii) mitochondrial disorder, (iv) genomic alteration, (v) epigenetic modifications, (vi) cellular senescence, (vii) nutrient-sensing misfunction, (viii) stem cell debility, and (ix) telomere shortening.[3,6] The present review, however, focuses on the regulatory role of mitochondria in the aging process.

Mitochondria, specialized organelles with two distinct membranes and a unique genome, evolved through an endosymbiotic relationship of an alphaproteobacterium with a eukaryotic cell, which is protected in exchange for a supply of energy.[7,8] Like nuclear genetic material, mitochondrial DNA (mtDNA) can undergo mutations or damage that

can be passed on to offspring. Moreover, mtDNA is more prone to stress-induced damage owing to the deficiency of protective histones, reactive oxygen species (ROS) generation in the inner membrane, and limited repair mechanisms. Thus, it possesses a mutation rate expected to be 10–20 times higher than that of nuDNA.[9,10] These mtDNA modifications can have serious effects on ATP levels and other cellular processes associated with critical and debilitating diseases, including neurodegenerative disorders (NDs) encompassing Alzheimer's disease (AD), Huntington's disease (HD), multiple sclerosis, Parkinson's disease (PD), and amyotrophic lateral sclerosis. Each of these diseases is associated with distinct regions of the brain and abnormalities involving specific proteins.[10] Nevertheless, this organelle has developed multiple stress-response strategies that contribute to the re-establishment of cellular homeostasis through mitochondria-associated ubiquitination and proteasomal degradation systems, which usually prevent mitochondrial proteotoxicity and remove damaged elements, including protein turnover.[11] It is noteworthy that mitochondria, as a complex organelle, is controlled by both nuclear DNA (nuDNA) and its own DNA (mtDNA) and that cellular homeostasis depends on the dynamic interaction between the nucleus and the mitochondria. Furthermore, mitochondria have retained some of the original bacterial genomes that coevolved with the nuclear genetic material. However, they import over a thousand proteins that are essential for diverse mitochondrial functions encoded in the nucleus. Furthermore, the coordination of nuclear and mitochondrial genomes in a cell regulates metabolism, epigenetic alterations, and a variety of activities critical for the survival and activity of mammalian cells, reflecting their close relationship.[12-14] According to several studies, mitochondrial functions decline significantly throughout aging,[4,15] followed by a reduction in cell activity, which is associated with the development of a wide range of age-related diseases. Mitochondrial dysfunction is a broad term that encompasses various biological processes, including alterations in mitochondrial protein synthesis, mitochondrial morphology and content, mitochondrial metabolism, and degradation pathways, and changes in the functionality of electron transport chain (ETC) complexes.[16] The present review outlines the role of mitochondria in the aging process that occurs through multiple distinct pathways. Moreover, we propose new areas that could facilitate mitochondrial-targeted therapies for the treatment of age-associated diseases.

2. mtDNA structure and features

Mitochondria possess circular, supercoiled, and double-stranded DNA, which accounts for 0.1 – 2% of total DNA in most mammalian cells.[17,18] Mitochondrial genes are almost exclusively inherited from the maternal parent. However, several important exceptions have been reported.[19-21] The mtDNA is structured into distinct protein-DNA complexes known as nucleoids (mt-nucleoid), and these can be observed under the microscope as punctate structures located on the matrix surface of the mitochondrial inner membrane.[22-25] The mt-nucleoid is an mtDNA transmission unit that facilitates its accurate transfer into daughter cells during cell division and also serves as a platform for mtDNA replication.[26-30] Furthermore, transcriptional factor A mitochondrial, mitochondrial single-stranded DNA-binding protein (mtSSB), and Twinkle protein have been demonstrated to colocalize with mt-nucleoid in intramitochondrial foci in living cells.[31] Mammalian mtDNA is approximately 16.5 kb in length and comprises 37 genes required for optimal mitochondrial function.[32] It is previously known that mtDNA encodes two rRNAs, twenty-two tRNAs, and thirteen polypeptides that form the core components of ETC Complexes I, III, IV, and V, which are required for the oxidative phosphorylation process (OXPHOS).[33,34] The nuDNA encodes the majority of mitochondrial proteins (about 1500), which are produced in the cytosol and transported into the mitochondria.[35,36] Furthermore, mtDNA has no introns, no gaps between genes, and no 5' or 3' non-coding regions.[37,38] The mtDNA consists of two strands: the heavy (purine-rich) strand, which encodes most of the information, and the light (pyrimidine rich) strand, which encodes the genetic information for only one polypeptide and eight tRNAs.[39] Among the 14 known DNA polymerases in humans, DNA polymerase gamma (Pol γ) is responsible for the replication and repair of mtDNA and is encoded by the POLG gene.[40-42]

3. Damage and mutations to mtDNA contribute to aging

Although both nuDNA and mtDNA are constantly exposed to external agents such as ionizing radiation, radiation, environmental toxins, and many therapeutic drugs, mtDNA is more susceptible to toxic chemicals than nuDNA due to its proximity to OXPHOS sites, lack of histone protection, and low repair activity when damaged.[43-47] ROS-induced mtDNA damage is thought to be the principal cause of mutagenesis in mitochondria, resulting in both mtDNA mutations and deletions.[48] Furthermore, mtDNA is required for the maintenance and regulation of mitochondrial functions, and its mutation rate is believed to be 10 to 20 times higher than that of nuDNA.[9] Furthermore, lipophilic cations tend to accumulate in mitochondria, particularly in the mitochondrial membranes, due to the negative charge on

the matrix side of the inner mitochondrial membrane.[49] Thus, many biologically toxic compounds and lipophilic drugs that have positive charges are imported from the cytosol and concentrated in mitochondria up to 1000-fold, which can be a threat to mitochondrial components.[50,51] mtDNA is found in large quantities (hundreds to thousands of copies per cell). However, a subset of them are affected by mutations.[52] Thus, the proportion of mtDNA mutations can profoundly affect the cellular and clinical phenotype.

The ratio of mutant mtDNA copies to total mtDNA is sometimes referred to as the heteroplasmy level or heteroplasmy frequency.[53] The phenotypic threshold for pathogenic heteroplasmies is believed to be 60 – 90% of mitochondrial genomes inside a cell.[54-56] However, the outcome is still determined by the specific tissue in which they appear, the timing of their appearance, and the total mtDNA content of the cell.[18,57,58] The mtDNA mutations include mtDNA rearrangements such as inversions or duplications, deletions, and point mutations. Nevertheless, it is known that mitochondrial mutations contribute to multiple diseases. For example, the m.3243A > G DNA mutation in the mitochondrial *MTTL1* gene, which encodes a specific type of mitochondrial transfer RNA termed $tRNA^{Leu\ (UUR)}$, is the most frequent mtDNA point mutation and can cause a variety of symptoms depending on the extent of heteroplasmy.[59,60] This mutation was discovered in 1990 and was associated with a neurological phenotype known as mitochondrial encephalopathy lactic acidosis with stroke-like episodes.[61] Patients with this mutation typically exhibit signs of a multisystem illness, including stroke-like episodes, hyperglycemia, myopathy, intestinal immobility, deafness, headaches, and seizures. In general, mtDNA mutations increase with age and appear to differ among organs.[10,62-64] Despite mounting evidence that increased levels of mitochondrial mutations and their accumulations contribute to aging and age-related diseases,[53,65-69] other studies have questioned whether these mutations ever reach a significant level sufficient to contribute to the aging process.[65] According to studies, the production of a defective form of mtDNA polymerase γ, POLG, promotes early death in mice, as well as elevated levels of mitochondrial mutations and premature aging.[66-68] These studies vividly connect mitochondrial abnormalities to aging. However, the types and levels of mitochondrial mutations do not appear to replicate what is observed during normal aging.[69] Hence, it is uncertain whether the rise in mitochondrial mutations with age plays a key role in the aging process.

In 1956, Harman proposed the free radical theory of aging,[70] which suggests that organisms age as a result of the accumulation of oxidative damage over time, initiated by ROS produced in the mitochondria (Figure 1[iA–iB]). Furthermore, ROS can cause damage to specific macromolecules such as lipids, proteins, and, most crucially, mtDNA. The free radical theory of aging has been a popular concept in the area of aging for many years. Later, the free radical theory of aging incorporated other ideas and evolved into the mitochondrial 'vicious cycle' theory of aging.[71] It proposes that ROS formation and elevation in oxidative stress can induce damage to mtDNA, hence gradually giving rise to mtDNA mutations during life. The accumulation of mtDNA mutations, in turn, leads to increased mitochondrial ROS generation, which further increases oxidative stress and the rate of mtDNA damage and mutagenesis (Figure 1[iC]). Consequently, this "vicious cycle" of oxidative damage that is exponentially expanding causes tissue degradation and aging, which then leads to cell death.[72,73] Recently, the former free radical theory of aging has been contradicted by several studies.[74-82] Furthermore, another study found that the longevity of anaerobically grown yeast cells is shorter than that of aerobically produced yeast cells, demonstrating that aging still occurs under anaerobic conditions with little ROS.[77] These findings have prompted scientists to speculate that ROS may perform signaling activities that activate protective and adaptive responses.[83,84] Moreover, the latter 'vicious cycle' theory of aging claimed cautiously that not all mutations drive superoxide production, and in particular, mutations that block the synthesis of cytochrome b would actually abort any superoxide production at complex III that normal mitochondria may exhibit.[71,72] Although some studies endorsed the above statement,[85,86] other studies, however, did not support the idea that mtDNA mutations contribute to higher ROS generation and oxidative stress in mitochondria with age,[66,87,88] placing the mitochondrial "vicious cycle" theory of aging in question. Furthermore, several studies suggest that age-related mtDNA mutations are largely produced during mtDNA replication errors rather than unrepaired damage caused by ROS.[89,90] Nevertheless, multiple lines of evidence indicate that mtDNA point mutations and deletions can accumulate with age, which constantly increases the mutation load with metabolic consequences as well as gradual loss of cellular functions, which ultimately results in age-related phenotypes.[89-93] However, the precise role(s) mtDNA mutations play in aging and its related diseases is still being debated. In addition, the detection and exact physiological effects of mtDNA mutations, especially as they pertain to aging, have proven challenging.

Overall, the mitochondrial "vicious cycle" theory of aging has received both criticism and extensions since its introduction, and the relationship (correlative association) between ROS, mtDNA mutations, and aging seems to be

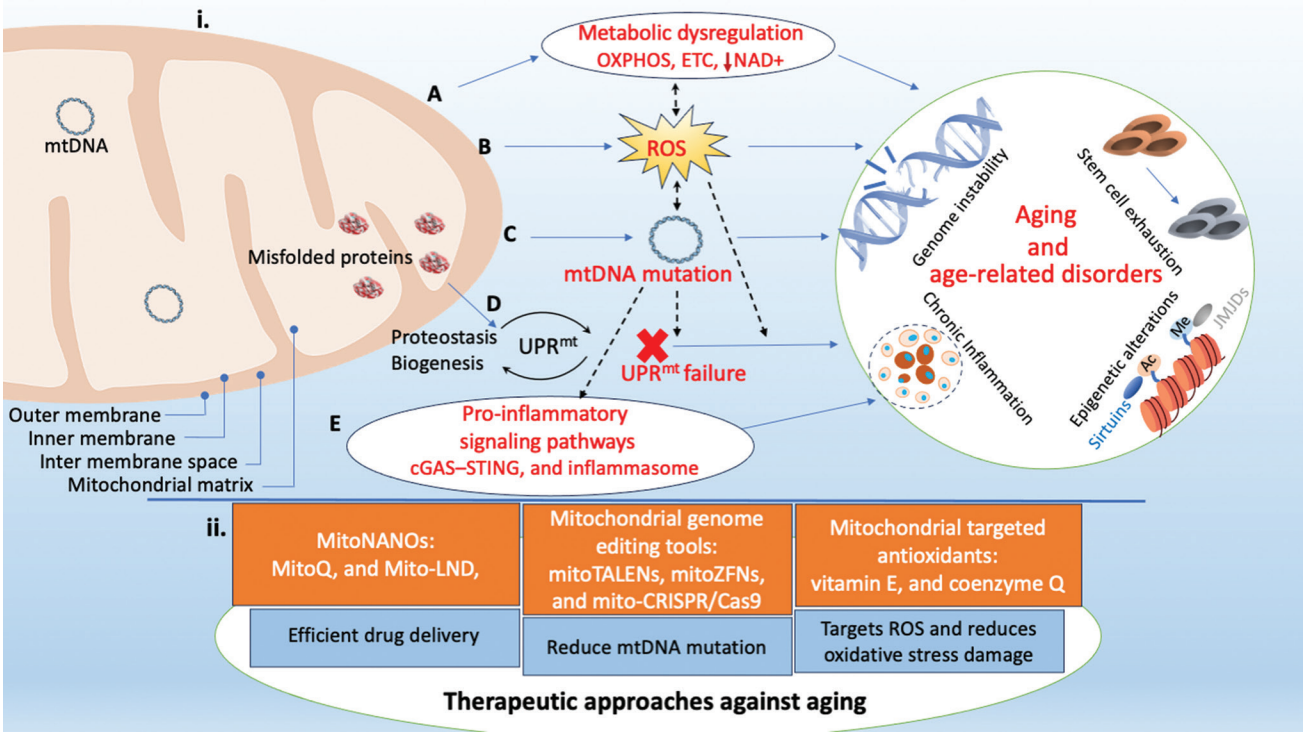

Figure 1. Mitochondria as a master regulator of aging and therapeutic approaches for aging treatment: (i) The illustration of mitochondria contribution to the aging process through distinct, but interconnected pathways—(A) oxidative stress due to ROS accumulation can cause dysregulation in mitochondrial metabolism including impairment of ETC enzyme complexes and impairment in OXPHOS which consequently promotes ROS production associated with aging and correlated with the development of age-related diseases; (B) consequently, ROS at high levels not only causes oxidative stress and damage to biological macromolecules (lipids, proteins, and nucleic acids) but also could directly damage the mtDNA, negatively affecting the nuDNA, and genome integrity which contributes to the aging phenotype; (C) mutations and accumulation of mtDNA can lead to ROS generation and elicit a coordinated alteration in the nuclear gene expression associated with aging; (D) mitochondria possess internal defense mechanisms, such as UPR^{mt}, which maintains mitochondrial proteostasis and biogenesis. However, excessive ROS generation and mtDNA damage result in the impairment or inhibition of these systems associated with aging; (E) oxidized mtDNA and its release into the cytosol can activate different PRRs and innate immune responses, including the recruitment of the cGAS-STING pathway and NLRP3 inflammasome, which can drive age-related pathologies such as neurodegenerative disorders; (ii.) therapeutic approaches for targeting aging and age-related diseases, which are discussed in the manuscript.
Abbreviations: cGAS-STING: Cyclic GMP-AMP synthase-stimulator of the interferon gene; ETC: Electron transport chain; Mito-CRISPR/Cas9: Mitochondria-targeted clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9; Mito-LND: Mitochondria-targeted lonidamine; MitoNANOs: Mitochondria-targeted nanoparticles; MitoQ: mitochondria-targeted ubiquinone; mitoTALENs: Mitochondria-targeted transcription activator-like effector nucleases; mitoZFNs: Mitochondria-targeted zinc-finger nucleases; mtDNA: Mitochondrial DNA; NAD^+: nicotinamide adenine dinucleotide; NLRP3: NACHT-, LRR-, and pyrin domain-containing protein 3; nuDNA: Nuclear DNA; OXPHOS: Oxidative phosphorylation process; ROS: Reactive oxygen species; TPP^+: Triphenylphosphonium cation; UPR^{mt}: Unfolded protein response.

more complex and involves more factors (environmental, nutritional, metabolic changes, the timing of their occurrence, tissue specificity, etc.), which need further vigorous studies. Moreover, further technological advancements are required to quantify the mtDNA mutation load in numerous single cells at different time points.

4. Mitochondrial unfolded protein response (UPR^{mt}) in aging

The aggregation, misfolding, and tissue accumulation of proteins seriously threaten cellular homeostasis and are associated with various human diseases.[94] In addition, various mechanisms have evolved individually in the highly compartmentalized eukaryotic cell to guarantee the integrity of the protein-folding environments in each compartment, including the cytosol, endoplasmic reticulum (ER), and mitochondria. Since all these three compartments are exposed to unfolded polypeptides, each has developed a dedicated repertoire of specific chaperones to promote efficient folding within each cellular compartment. As a result, any unfolded/misfolded protein stress is identified at each cellular compartment and conveyed to the nucleus for stimulation of the compartment-specific chaperone gene expression.[95,96] In addition, the complex organelle architecture of mitochondria, containing two membrane barriers, the harmful effects of ROS, and the susceptibility of mtDNA to acquiring mutations make the mitochondrial environment susceptible to the accumulation of unfolded

or unassembled proteins. Nevertheless, when misfolded or unfolded proteins build up beyond the capacity of the organelle's chaperones, cells initiate a UPR^{mt} (Figure 1[iD]). The UPR^{mt} is a mitochondrial-to-nuclear signal transduction pathway that culminates in the activation of mitochondrial protection genes such as chaperones and proteases to restore protein homeostasis within the mitochondrial protein-folding environment. Molecular chaperones are found in both the mitochondrial matrix and the intermembranous space (IMS).[97,98] For instance, HSP60 chaperonin, which is localized in the mitochondrial matrix, aids in the folding of tiny, monomeric proteins. Moreover, it comprises Hsp60 and Hsp10 subunits, which together form a barrel-shaped complex.[99,100] In addition to HSP60 chaperonin, there is mitochondrial HSP70 (mtHSP70), which is located in the matrix and is known to perform various functions. The mtHSP70 forms the core subunit of the machinery, called "import motors," which provides a driving force for the proteins imported into mitochondria.[98,101] Other studies show that mtHSP70 assists in the folding of imported polypeptides in the matrix while preventing their aggregation in the matrix.[102-104] In addition, mitochondria have a number of proteases that are positioned in the inner membrane and matrix, which mediate the destruction of misfolded or incorrectly assembled proteins. Inside the mitochondrial matrix, the protein-folding environment is monitored and protected. When accumulating unfolded proteins are detected, a mitochondria-to-nucleus signal transduction pathway sends a signal from the mitochondria to the nucleus via the cytosol. This frequently results in subsequent overexpression of the relevant chaperone-encoding genes and quality control proteases, guaranteeing protein-folding homeostasis at both the protein folding and removal levels.[95]

Besides, studies show that there are other factors that could activate UPR^{mt}, including deletion of mtDNA, the increase of ROS levels (Figure 1[iD]), alteration of mitochondrial dynamics, mitochondrial chaperone or protease inhibition, and impairment of the ETC.[105-108] The transcription factor associated with Stress-1' (ATFS-1) is a major regulator of UPR^{mt} in *C. elegans*[106] and its homolog ATF5 in mammals.[109] Studies show that this transcription factor is required for the regulation of almost half of mitochondrial stress-responsive genes, such as mitochondrial chaperones and peptidases, as well as immune response genes.[106,110] ATFS-1 is known to contain a nuclear localization sequence and a mitochondrion targeting sequence (MTS), which allows it to translocate between the nucleus and mitochondria, mediating mitochondria-to-nuclear communication.[111,112] In addition, under non-stress circumstances, this protein is imported into the mitochondria and degraded by the Lon protease.[106] Conversely, when the mitochondria are stressed, mitochondrial matrix protease ClpP cleaves misfolded or unfolded polypeptides, which are subsequently exported to the cytoplasm by HAF-1.[111] Consequently, the increased accumulation of peptides in the cytosol leads to a reduced capacity for mitochondrial protein import.[106,113] As a result, ATFS-1's mitochondrial import is inhibited, resulting in its buildup in the cytoplasm, thus letting ATFS-1 travel to the nucleus, where, in cooperation with ubiquitin-like protein 5 (UBL-5) and defective proventriculus 1 (DVE-1) upregulates the expression of mitochondrial chaperones, proteases, and different metabolic and detoxification enzymes.[114,115] In such a setting, cross-compartment synchronization seems to be an essential factor in maintaining protein homeostasis during UPR^{mt}. The activation of UPR^{mt} is thought to be one of the mitochondrial processes that protect against numerous types of aging-causing damage, with complex effects on longevity.[116] Notably, studies show that the activation of UPR^{mt} mechanisms by downregulation of ETC complexes I and IV promotes longevity.[117-119] Moreover, UPR^{mt} activation has been connected to the extension of lifespan caused by various types of bacteria,[120] possibly through enhanced production of the polysaccharide colanic acid (CA), which regulates mitochondrial dynamics and UPR^{mt} in the host *C. elegans*. Another study showed that ATFS-1 enhances longevity in long-lived nuo-6 mitochondrial mutants by activating a variety of stress response pathways.[121] Furthermore, the longevity of two long-lived mitochondrial mutants, namely *clk-1 and isp-1*, was likewise demonstrated to be decreased by the knockdown of *ubl-5* and *dve*-1 which are the regulators of UPR^{mt}.[119] In addition, studies suggest that histone H3 methylation is an essential epigenetic regulator of UPR^{mt} throughout the lifespan.[122,123] Intriguingly, Merkwirth *et al.* showed that histone demethylases, namely the Jumonji C domain-containing protein family (*jmjd-1.2* and *jmjd-3.1)* are required for activation of the UPR^{mt}-mediated longevity across species. These findings indicate that the epigenetic mechanism modulates the rate of aging downstream of mitochondrial perturbations.[124] Moreover, they demonstrated that the gain of function of demethylases is sufficient to increase lifespan in a UPR^{mt}-dependent manner, whereas their loss of function greatly reduces longevity and UPR^{mt}. In addition, increasing lines of evidence support the key role of epigenetic regulators namely the sirtuin deacetylase family in the mitochondrial stress response pathways; particularly, SIRT1, SIRT3, and SIRT7 which contribute to the UPR^{mt} via different axes.[117,125-129] These findings collectively suggest that UPR^{mt} plays a key role in specific longevity pathways. However, the relationship between UPR^{mt} and longevity seems more complex and requires further studies to ascertain this relationship.

5. Mitochondrial oxidative stress in aging

Mitochondria are known to generate various types of "reactive species" as side products of oxidative phosphorylation, including the reactive oxygen/nitrogen species (RONS). The increase in RONS production is linked to oxidative stress, which can cause oxidative damage to cells via apoptosis, autophagy, and inflammation if not eliminated efficiently by the cellular antioxidant defense system.[130,131] Moderate or low amounts of RONS, on the other hand, can operate as signaling molecules in the cell.[132-134] Even though mitochondrial RONS take part in numerous physiological processes, including epigenetic modifications[135] and disease progression, such as cancer,[136-141] the mechanisms of mitochondrial RONS have not been fully understood. Moreover, age-associated functional disorders can arise from RONS, which are by-products of oxygen and nitrogen originating from various sources, and their negative effects are compensated by antioxidant mechanisms.[2,142] Hence, RONS play a significant role in the development of age-associated diseases.[2,143] RONS can be derived from endogenous as well as exogenous sources. Endogenous sources consist of myeloperoxidase (MPO), nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, angiotensin II, and lipoxygenase.[143,144] O_2• is another form of RONS that is produced by the reduction of molecular oxygen with supplied electrons carried by NADPH during cellular respiration. O_2• can dismutate into hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD).[2,145] H_2O_2 is not considered a free radical since it does not contain unpaired electrons. However, it can produce highly reactive hydroxyl ions (OH•) through the Fenton or Haber-Weiss reactions.[2] Hydroxyl radicals are extremely reactive, particularly with phospholipids found in cell membranes and proteins.[2] On the other hand, H_2O_2, MPO, and chloride can be transformed into hypochlorous acid, a ROS-specific cellular damaging protein.[2,145] Nitric oxide (NO) originates from L-arginine via NO synthase (NOS), which forms three main isoforms: (i) Epithelial NOS involved in vascular regulation and vasodilation, (ii) neuronal NOS involved in intracellular signaling, and (iii) inducible NOS, which is released following numerous endotoxin or cytokine signaling.[2,146] Eventually, the interaction of O_2 with NO can lead to the formation of a rather reactive molecule called peroxynitrite ($ONOO^-$).[2,144,145]

The exogenous sources of RONS include alcohol, tobacco, water and air pollutants, drugs (e.g., cyclosporine, tacrolimus, gentamycin, and bleomycin), heavy or transition metals, cooking (e.g., smoked meat, waste oil, and fat), industrial solvents, and radiation, which are turned into free radicals after metabolization inside the body.[2,147] Both exogenous and endogenous forms of RONS conduct oxidative modulation of substantial macromolecules (lipids, proteins, carbohydrates, and DNA)[2,144] and can be considered as oxidative stress markers.[2,148] When there is an imbalance between the formation and clearance of RONS, oxidative stress occurs.[144] In this regard, antioxidants safeguard biological entities from deleterious free radicals comprising endogenous as well as exogenous molecules. Endogenous antioxidants consist of enzymatic and non-enzymatic forms.

The major enzymatic antioxidants are catalase (CAT), SOD, and glutathione peroxidase (GSH-Px).[2] As discussed before, SOD transforms O_2• into H_2O_2, which further breaks down to oxygen and water by CAT, inhibiting the production of hydroxyl radicals.[2] Furthermore, GSH-Px transforms hydroxyl radicals and peroxides into non-toxic compounds through the oxidation of reduced glutathione (GSH) into glutathione disulfide (GSSG), and then glutathione disulfide is reduced to the sulfhydryl form glutathione (GSH) by glutathione reductase.[2] Further antioxidant enzymes to be mentioned are glutathione-S-transferase and glucose-6-phosphate dehydrogenase.[2,149]

Non-enzymatic antioxidants are compounds that react with RONS and abate the free radical chain reactions: α-tocopherol (vitamin E), β-carotene, and bilirubin are present in the blood, whereas uric acid and albumin comprise 85% of antioxidants in plasma.[2,150] Exogenous antioxidants consist of ascorbic acid (vitamin C), which breakdown hydroxyl and superoxide radical anion, α-tocopherol (vitamin E), which plays a role in lipid peroxidation of cell membranes, phenolic antioxidants, selenium, oil lecitinas, zinc, and drugs such as acetylcysteine.[2,151] These molecules usually function as scavengers of free radicals or can even modulate the activity of enzymatic systems.[152] As already mentioned, the contribution of ROS to aging is controversial. Numerous studies have demonstrated that inhibition of oxidative stress corresponds to an increase in lifespan. On the other hand, some studies have questioned the possibility of ROS as a cause of an aged phenotype. Nevertheless, aged rats were shown to hold higher free radical levels.[153,154] On the other hand, reduced levels of antioxidants such as glutathione peroxidase (GPx), CAT, and SOD have been reported in aged rats as well as in humans.[155-161] The action of ROS is counteracted by antioxidant molecules. Studies suggest that fumarate, a metabolite of the mitochondrial TCA cycle, and its derivatives, specifically dimethyl fumarate, have antioxidant[162] and anti-inflammatory properties,[163-165] which can fight against age-related neurological disorders.[164,166-168] However, their exact molecular mechanism of action remains elusive despite their promising beneficial effects against neurological disorders.[168] The administration of antioxidants such as Vitamin E compounds, including

tocopherols and tocotrienols, was previously demonstrated to extend the life span and decelerate aging in different species.[169,170] Vitamin E compounds seem to extend life span in various ways, including ameliorating age-related decline in NO synthase and SOD2,[170] suppressing ROS production,[171] reducing ROS damage[172] (Figure 1[i–ii]), mitigating ethanol-induced accumulation of intracellular oxidants and counteracting the suppression of glutathione peroxidase/glutathione reductase,[173] providing protection against age-related hepatocytes polyploidization, and neuroprotection by reducing the quantity of p53-positive cells throughout the brain.[174] Furthermore, antioxidant-based therapy involving the use of natural sources of antioxidants such as Vitamin E and Coenzyme Q (CoQ, ubiquinone) appears to be effective in animal models of neurodegenerative disease, including mouse models of both PD and AD.[175] CoQ10 is naturally generated in the body and serves as an antioxidant agent, and its level declines in the body during aging.[176] CoQ10 is mostly found in the inner mitochondrial membrane of eukaryotic cells.[177] Furthermore, it acts as an electron shuttle between complexes I and II of the respiratory chain, as well as complex III in mitochondria.[178] As a result, it can exist in both oxidized (CoQ or ubiquinone) and reduced (CoQH2 or ubiquinol) forms.[179] Furthermore, CoQ10 protects against oxidative stress-induced cell death.[180] Studies show that CoQ10 protects the skin by combating free radicals, which were shown to damage collagen fibers through the activating MAPK pathway that produces matrix metalloproteinases (MMPs) such as collagenase.[181,182] Aberrations in CoQ10 biosynthesis genes cause primary CoQ10 deficiency, a mitochondrial syndrome associated with impaired OXPHOS and clinically heterogeneous diseases, including cerebellar ataxia, encephalomyopathy, infantile multisystemic form, isolated myopathy, and nephropathy.[177,178] Reportedly, some patients also developed retinopathy or optic atrophy, hypertrophic cardiomyopathy, and sensorineural hearing loss as a result of CoQ10 deficiency.[183] In addition, CoQ10 deficiency is connected with mtDNA point mutations, depletion, and deletions.[184] Nonetheless, Coenzyme Q supplementation was demonstrated to protect against age-related DNA double-strand breaks and prolong longevity in mice who consumed a polyunsaturated fatty acid (PUFA)-rich diet by attenuating oxidative alterations.[185] CoQ10 supplementation may improve human lymphocyte recovery from oxidative DNA damage due to the ordering and condensing impact of CoQ10 on cell membranes, resulting in a decrease in ROS formation and a protective benefit to DNA integrity.[186] Nonetheless, lifetime CoQ10 treatment had no effect on the lifespan of rats or mice,[187] indicating that CoQ10 might aid in preventing life span shortening as a result of cumulative oxidative insults by reducing oxidative stress damage (Figure 1[i–ii]). Paradoxically, some animal models, such as *C.elegans,* with CoQ biosynthetic deficiencies have shown an increase in life span.[188,189] This observed effect might be attributed to the maintenance of the efficiency of respiration together with an observed reduction in the production of superoxide anion in the mitochondrial electron change, which would cause lower damage to macromolecules in response to CoQ silencing. However, these data cannot be extrapolated to other biological systems, such as mammals.

6. Mitochondrial dysfunction leads to stem cell exhaustion

One of the hallmarks of aging is the reduction in stem cell numbers due to their impaired self-renewal capacity and function. Studies show that as hematopoietic stem cells (HSC) age, they exhibit increased mitochondrial OXPHOS and enhanced ROS production, indicating a direct role for mitochondria in the degenerative process. Studies show that ROS at low levels plays a positive role in stem cell biology, mainly by maintaining their stemness, quiescence, and self-renewal.[190-192] However, elevated ROS can activate stem cell differentiation, senescence, and apoptosis, resulting in their exhaustion, which is linked with the aging process and degenerative diseases.[190,193] In addition, low amounts of ROS can act as a signaling molecule to promote cardiovascular differentiation in mouse embryonic stem cells (mESCs). Meanwhile, high amounts of ROS can lead to the inhibition of cardiomyogenesis and vasculogenesis.[194] Similarly, ROS at low levels allows HSCs to keep up their normal functions, including proliferation, differentiation, and mobilization. Interestingly, several studies on POLG mtDNA mutator mice showed impairment of stem cell functions and found a wide range of defects, including impairment of neural stem cell (NSC) populations,[195] megaloblastic anemia, B-cell abnormalities,[196] and impaired reprogramming capacity into pluripotent stem cells due to increased ROS levels.[197] In addition, transplantation of HSCs from POLG mtDNA mutator mice into a normal host led to the same observed defect.[196] Another study demonstrated that the ability of POLG knockin cells to be reprogrammed into pluripotent stem cells is likewise significantly reduced; this deficiency is once again linked to an increase in the production of ROS by the mitochondria.[197] Taken together, studies on the POLG mtDNA mutator mice clearly linked mitochondria to stem cell functions, suggesting the POLG mtDNA mutator mice influence a wide range of cell types, including stem cells, their offspring, and the niche.[198]

Mitochondria can also help to maintain stem cells by regulating particular metabolites that act as secondary messengers for epigenetic regulation.[199] Numerous crucial

metabolic pathways, such as the one-carbon cycle, the tricarboxylic acid (TCA) cycle, and fatty acid oxidation (FAO), are known to be compartmentalized in the mitochondria.[200] Metabolites produced by these pathways can also serve as retrograde signals. Particularly, many of these metabolites are produced by the TCA cycle, such as acetyl-coenzyme A, succinyl-CoA, α-ketoglutarate (αKG), succinate, and nicotinamide adenine dinucleotide (NAD^+). For instance, it has been demonstrated that the ratio of αKG to succinate plays a crucial role in maintaining pluripotency in mouse mESCs through regulation of multiple chromatin modifications, including histone H3 lysin 27 tri-methylation (H3K27me3) and ten-eleven translocation (Tet)-dependent DNA demethylation.[201] In addition, NAD^+ is another important metabolite that links the mitochondria to stem cells, and its systemic decline has been reported during aging.[202,203] Furthermore, many NAD+-consuming enzymes use NAD^+ as a substrate, including the cyclic ADP-ribose synthase CD38, SARM1, poly-ADP-ribose polymerase (PARP), as well as a family of seven protein deacylases, namely sirtuins, which are present in the nucleus (SIRT1, SIRT6, and SIRT7), cytosol (SIRT2), and mitochondria (SIRT3 – SIRT5).[204-206] Alleviated age-dependent NAD^+ availability is associated with decreased activities of sirtuins, eventually disrupting the crosstalk between mitochondria and the nucleus during aging.[207,208] Reduced NAD^+ levels in NSCs were shown to recapitulate at least some of the phenotypes of stem cells during aging (Figure 1[iA]), while NAD^+ boosting through administration of precursor nicotinamide mononucleotide (NMN) could repair abnormalities induced by a decrease in NAD^+ levels in NSCs.[209] Hence, NAD^+ supplementation therapies are considered a therapeutic option to ameliorate age-related metabolic diseases.[210] Furthermore, NAD^+ boosting with the precursor NMN improved impaired glucose tolerance by restoring normal NAD^+ levels and enhancing either insulin sensitivity or insulin secretion in mice with diet- and age-induced diabetes.[211] The observed effects seem to be partially mediated by the sirtuin (SIRT) family of NAD^+-dependent histone deacetylases, which are known to regulate crucial metabolic pathways. For instance, SIRT3 was shown to regulate global mitochondrial lysine acetylation levels in an NAD^+-dependent manner.[212] Moreover, SIRT3 was shown to be abundant in HSCs, where it regulates stress responses.[213] In addition, the same study showed that SIRT3 is suppressed during aging, and its increased levels in aged HSCs improved their regenerative capacity, indicating that the plasticity of mitochondrial homeostasis controls stem cell and tissue maintenance during the aging process, and hence aging-associated degeneration can be reversed by a member of the sirtuin family.

Studies have uncovered that stem cell pool maintenance, expansion, or depletion are modulated via symmetric and asymmetric division events.[214-217] In addition, stem cells can exploit mitochondrial FAO during self-renewal along with glycolysis.[218] Inhibiting FAO in HSCs results in the loss of asymmetric division of HSC daughter cells, which is a vital process to maintain the stem cell pool during the simultaneous expansion of stem cell differentiation. Mechanistically, this process (HSC asymmetric division) is controlled by the PML-PPARδ-FAO pathway.[219] Moreover, the same study showed that PPARδ activation using PPARδ agonists increases asymmetric division and improves HSC functions. In addition, lipid metabolism is also an important player in NSC proliferation and maintenance.[220,221] Specifically, deletion of fatty acid synthase, the key enzyme of *de novo* lipogenesis, in mouse NSCs was shown to impair adult neurogenesis.[222] Taken together, mitochondria are key organelles that regulate the metabolic status of stem cells; consequently, maintaining their proper metabolic regulation is critical for lifelong health. Moreover, the modulation of pathways associated with mitochondrial metabolic dysfunction contributing to age-related stem cell exhaustion could potentially improve human health and prevent age-related diseases.

7. Mitophagy and age-associated diseases

Being extremely dynamic organelles, mitochondria go through various processes termed mitochondrial quality control (MQC). MQC mainly involves the coordination of multiple biological events, including constant fission and fusion, an endless transformation process occurring through biogenesis and mitophagy to affirm mitochondrial homeostasis, morphology, and inheritance.[223] Once facing challenges through oxidative or bioenergetic stress, mitochondria perform an arranged reaction containing morphological and dynamical transformation by triggering the specific molecular mechanism that synchronizes mitochondrial biogenesis, mitophagy, fusion, and fission.[224] Mitochondrial fusion and fission in mammalian cells are closely supervised by a number of proteins, including dynamin 1-like (DNM1L, recognized as Drp1), mitofusin 1 (MFN1), mitofusin 2 (MFN2), and optic atrophy protein 1 (OPA1).[225] The process of mitochondrial fission was discovered to participate in mitochondrial apoptosis and was proposed to be essential for mitophagy, while mitochondrial fusion is linked to an increase in mitochondrial metabolism.[226] The term "mitophagy," the selective mitochondrial autophagy, was first named by John Lemasters.[227] Mitophagy is a procedure during which depolarized, aged, or damaged mitochondria are selectively removed via double-membrane autophagosome for consequent lysosomal degradation.[228] The detection of this

subset of mitochondria is supported by the engagement of the mitochondrial kinase PINK1 (PTEN-induced putative kinase protein 1). When mitochondria lose membrane potential, PINK1 accumulates on the mitochondrial surface, resulting in the recruitment of the cytosolic protein Parkin, which regulates the mitochondrial protein ubiquitination and subsequently, engulfment of damaged mitochondria by membranes that then fuse with lysosomes,[229] a process known as mitophagy. Mitophagy was discovered to be a crucial process for preserving cellular health and homeostasis. Surprisingly, the BCL2 protein family was shown to take part in both mitophagy processes and mitochondrial dynamics, placing them in the spotlight of mitochondrial regulatory elements.[230] Studies reported that dysregulation of mitophagy and damage to mitochondria have been observed in several NDs correlated with aging, including PD,[231] AD, and HD.[232] Aging escalates the likelihood of the initiation of several chronic diseases, usually correlated with the build-ups in mtDNA mutations, impaired mitochondrial function, mitochondrial mass variation, enhanced cell death, and persistent immune activation[233] that are expected to happen due to compromised MQC machinery, resulting in the accretion of malfunctioned mitochondria, which consequently elevates immune activation (through ROS activation) as well as mitochondrial apoptosis (through the expression of apoptogenic factors). Several lines of the study indicate that impaired mitophagy promotes aging, while improved mitophagy, which is endorsed by reduced calorie intake and training, advocates a health-giving lifetime. For instance, the promotion of mitophagy has been shown to be connected to an increase in *C. elegans* longevity.[234] Lineage-specific expression of PGC-1 (master regulator of mitochondrial biogenesis) in *Drosophila melanogaster* results in a prolonged lifetime in this model.[235] Physical activity has been reported to trigger AMP-activated protein kinase (AMPK) activation, which in turn results in phosphorylation of the Unc-51-like autophagy activating kinase 1 (ULK1) and elevated mitophagy in skeletal muscle, which consequently stimulates mitochondrial biogenesis and enhances health conditions in murine models.[236] Besides, it has been notably reported that impaired mitophagy significantly affects PD development. Mutations in Parkin and PINK1 proteins participating in the induction of mitochondrial mitophagy were reported in PD.[237,238] Mitophagy was also reported to be linked with AD progression.[239,240] Studies have recently demonstrated that tau pathology, a hallmark of AD, disrupts mitophagy by preventing the translocation of Parkin protein to mitochondria.[241] These data underline the importance of mitophagy for proper mitochondrial function and dynamism, which in turn affect personal health conditions. In addition, a newly identified MQC mechanism, the mitochondrial-derived vesicle (MDVs) pathway, was shown to function during the early stages of cellular stress and has a key role in mitochondrial oxidative stress to maintain stable mitochondrial function.[242]

8. Dysfunctional mitochondria and inflammation during aging

As discussed, MQC maintains healthy mitochondria via the repair or selective elimination of damaged mitochondria in cells via a series of adaptive responses encompassing mitochondrial fusion and fission, mitophagy, and mitochondria-dependent cell death.[243] MQC dysfunction during aging causes an accumulation of damaged mitochondria, which contributes to aging and a variety of age-related diseases.[244] Studies reveal that when MQC is defective, mitochondrial-derived damage-associated molecular patterns (mtDAMPs) such as mtDNA and oxidized mtDNA accumulate in the cytosol, which can activate both intracellular and extracellular immune pathways affecting age-related disease progression[245,246] (Figure 1 [iA–iE]). According to studies, even in the absence of bacterial infection, mtDAMPs generated as a result of trauma might cause systemic inflammatory response syndrome.[247] Due to the lack of histone proteins, mtDNA is vulnerable to degradation and oxidation. Furthermore, mtDNA contains hypomethylated CpG patterns that are identical to those found in bacterial DNA and can be recognized as a pathogen-associated molecular pattern and hence can induce an inflammatory response.[248] Furthermore, these regions can interact with and activate membrane or cytoplasmic pattern recognition receptors (PRRs), such as the nucleotide-binding oligomerization domain-like receptor (NLR),[248] the toll-like receptor (TLR), and even the cytosolic cyclic GMP-AMP synthase (cGAS), which is a stimulator of interferon genes (STING).[249] For example, Oka *et al.* revealed that pressure-overload mtDNA release that escapes autophagic degradation causes TLR9-mediated inflammatory responses in cardiomyocytes and can cause myocarditis and dilated cardiomyopathy.[250]

In addition, the cytosolic release of oxidized mtDNA was shown to stimulate the NACHT-, LRR-, and pyrin domain-containing protein 3 (NLRP3) inflammasome, a large multi-protein complex that controls caspase-1 activation, which results in IL-1β and IL-18 secretion and an apoptotic activation cascade.[251] In contrast, deletion of the Nlrp3 inflammasome has been found to reduce age-related activation of the innate immune system, protecting animals from a variety of age-related diseases.[252] As a result, mitophagy plays an important role in inflammation prevention by boosting mtDNA clearance from damaged mitochondria. According to research, circulating mtDNA rises with age and is linked to higher levels of serum

inflammatory markers such as tumor necrosis factor α, interleukin 6 (IL-6), regulated upon activation, normal T cells expressed and secreted (RANTES), and IL-1 receptor antagonist protein (IL-1ra).[253] However, new research indicates that not every type of cell-free mtDNA is pro-inflammatory, nor does it seem to be sufficient to consistently cause inflammation.[254] Consequently, this belief may be the result of a misunderstanding of correlational clinical investigations, and it should be tested and re-evaluated in relevant biological systems.

9. Mitochondria-targeted drug delivery approaches in age-related diseases

Mitochondrial-targeted therapeutics are recognized as a revolutionary tool for diagnosing, preventing, and treating a wide range of age-related human diseases, including cardiovascular disease, metabolic disorders, cancer, and neurodegenerative diseases. Nonetheless, the mitochondria are made up of at least six compartments: the outer membrane, inner border membrane, IMS, cristal membranes, intracristal space, and matrix.[255] Hence, effective mitochondria-targeted drug delivery is challenging owing to the mitochondrial double-membrane and its complex structure, as well as the highly negative potential nature of the membrane. Therapeutic and tiny compounds, on the other hand, may diffuse through the outer membrane through passive diffusion and phospholipid cardiolipin.[256] It is noteworthy to mention that the pore in the outer membrane is wider, and hence, therapeutic molecules can easily traverse through this pore. The limited, highly folded inner mitochondrial membrane, on the other hand, has narrower transition slits that separate the mitochondrial matrix and IMS, making it difficult for many therapeutic compounds to cross the mitochondrial matrix. Nanotechnology has brought new hope over the years by bringing innovative compounds, such as nanoparticles (NPs), that can be employed for the clinical diagnosis and prognosis of a variety of illnesses, including numerous cancer kinds.[257,258] Furthermore, because of their deep tissue penetration capabilities, NPs not only increase drug half-life and increase drug accumulation in tumor tissues,[257-259] but they also provide a platform for weakly soluble medicines to be encapsulated and delivered more efficiently into circulation.[260] In addition, they can be rationally engineered to target certain intracellular organelles such as mitochondria,[261] ER,[262] Golgi apparatus,[262] and lysosomes.[263] Recently, multiple organic and inorganic NPs combined with conventional chemotherapeutic medicines to produce biocompatible, multifunctional mitochondria-targeted nanoplatforms.

Nevertheless, mitochondria-targeted NPs (mitoNANO) evolved to enhance the therapeutic targeting of mitochondria as well as overcome drug resistance and reduce the unwanted effects of the delivered drugs.[264] Mitolonidamine[265] and cisplatin[266] are two typical instances of mitochondria-targeted anticancer medications that promote programmed cell death and thereby prevent cancer cell survival, progression, and metastasis.[267] Interestingly, mitoNANOs loaded together with lonidamine showed 10-fold higher antitumor properties in comparison to pure lonidamine.[264] Furthermore, research indicates that mitochondria-targeted NPs have antiproliferative and cytotoxic impacts in tumor cells but not in healthy cells.[268] Moreover, mitoNANO-based therapies can accelerate mitochondrial blockades in cancer cells through multiple ways, including respiratory inhibition, modulation of the mitochondrial permeability transition pore, inhibition of the ETC, inhibition of anti-apoptotic protein family members, suppression of phenotypes linked to mutated DNA, and the promotion of mitochondrial-regulated cancer cell death.[264] In general, intracellular uptake is the first step for NPs to transport the drugs to the mitochondria. To complete this process, the cell membrane's negatively charged phospholipids must adhere to the positively charged, minute NPs, which leads to drug endocytosis and the formation of endolysosomes. Then, the endolysosomal membrane rupture occurs inside the cytoplasms, which results in the release of its contents, and the mitochondria to be targeted intracellularly.

There are various NP-based drug delivery methods used to treat mitochondrial disorders that aid in protecting drug payloads, including hydrophobic and hydrophilic compounds, from their elimination and degradation. NPs are classified into different groups according to their chemical and physical characteristics, surface area, shapes, or sizes, including liposomes, liposome-like vesicles (DQAsomes), MITO-porters, micelles, polymeric NPs, dendrimers, metal NPs such as gold NPs (AuNPs), quantum dots, or nanoscale semiconductor crystals, and each of them possesses specific features.[256] In addition, they can be accumulated in tumor cells utilizing either active or passive targeting techniques. The latter technique uses the enhanced permeability and retention phenomena and particular features of solid tumors, such as leaky vasculature and disrupted lymphatic drainage, which results in NP extravasation throughout the leaky blood arteries. On the other hand, active targeting is based on the ligand-receptor interaction system, which means that ligands attached to NPs can recognize molecules that are selectively overexpressed in tumor cells. The passive target technique for synthesizing mitoNANO provides some merits, such as simplicity and cost-effective synthetic procedure. However, the aggregation behavior of passively targeted NPs provides a reason for concern because it can result in their rapid clearance from the biological system. The active targeting of the mitoNANO approach requires

mitochondria-targeting ligands to be linked either to drug-loaded or free NPs. There are different ligands that can be linked to NPs depending on the prospective strategies and features connected with these ligands, including prices, synthesis complexity, off-target toxicity, immunogenicity, lengthy blood circulation time, and delayed clearance from the biological systems. Some of these ligands that are conjugated to therapeutic agents to improve their efficacy include tetramethylrhodamine-5-isothiocyanate,[269] triphenylphosphonium (TPP^+),[270,271] Vitamin E analogs,[272] dequalinium (DQA),[273] cationic N-heterocyclic carbenes,[274] and coumarin conjugate.[275] Among them, TPP^+ is probably one of the most extensively investigated ligands in targeting mitochondria for drug delivery. When TPP^+ is conjugated with NPs loaded with drugs, it increases their lipophilicity and enables them to escape from lysosomal compartments and easily penetrate the mitochondrial membrane bilayers. In addition, cancer cells facilitate the transfer of the TPP^+-labeled NPs from the cytoplasm into mitochondria, which can be up to a hundred times faster than non-functionalized NPs due to the mitochondria's higher negative plasma membrane potential in cancer cells compared to healthy cells.[264,276,277] For this reason, the mitochondria-targeted agents were developed by installing a triphenylphosphonium cation (TPP^+) joined to diverse bioactive molecules such as Mito-Met (metformin conjugated with TPP^+) and MitoQ (ubiquinone attached to TPP^+),[278] which exhibit enhanced lipophilicity and increased cellular uptake. A recent study shows the application of mitochondria-targeted lonidamine (Mito-LND) by coupling LND to TPP^+ through a connector aliphatic chain could potently inhibit primary lung cancer and suppress brain metastasis from primary lung cancer in an orthotopic animal model, which was more effective than LND in both cases.[267] Moreover, Mito-LND could induce autophagic cell death by inhibiting mitochondrial complexes I and II and stimulating ROS formation. In addition, curcumin-loaded nanostructured lipid carriers (NLCs) hold great potential to treat neurodegenerative diseases like AD.[279] NLCs are made up of a mix of solid and liquid lipids that can easily cross the blood–brain barrier (BBB),[280] one of the most challenging physiological barriers that exist between the central nervous system and peripheral circulation.[281] Curcumin is an herbal extract that has antioxidant as well as anti-inflammatory characteristics. When included with NLCs for targeted administration, curcumin can also reduce the formation of amyloid beta (Aβ) plaque by reducing the aggregation of amyloid β peptides and decreasing neurodegeneration.[279] In addition, silica NPs (SiNPs) were also utilized for BBB targeting due to their capacity for cellular absorption and accumulation in the intracellular amyloid cells (Aβ1-42), which showed decreased Aβ1-42 plaque formation and hyperphosphorylation.[282] Besides, conjugating the polyamidoamine dendrimers with an anti-inflammatory and antioxidant agent known as NAC (N-acetyl cysteine) was shown to target the activated microglial and macrophage (Mi/Ma) and astrocytes at the site of brain injury by penetrating the BBB. Thus, its application is a promising tool for treating mitochondrial dysfunction associated with neurological disorders.[283] Overall, the field of mitoNANO has sparked tremendous interest, and significant efforts are being made to develop mitochondrial-targeted nanomedicine. Preclinical and clinical investigations, on the other hand, are required to understand the safety of these drug delivery systems as well as their potential for a wide range of disorders.

10. Mitochondrial genome editing tool against aging

Genome editing is a powerful method for eliminating or replacing genetic abnormalities at specific loci to reprogram or modify their expression. Genome editing tools such as zinc-finger nucleases, transcription activator-like effector nucleases (TALENs), and CRISPR/Cas9 have already been utilized in a variety of scientific research to generate targeted changes across multiple species.[284-289] Nevertheless, mitochondrial genome editing studies began with the application of restriction endonuclease enzymes, including SmaI, ApaLI, and PstI, designed to be specifically utilized in mitochondria.[290-293] According to a number of studies, the use of mitochondria-targeted restriction endonucleases (mtREs) can change the ratio of mutant to wild-type mtDNA heteroplasmy in human somatic cells and mice models. The reports signified that mtREs notably reduce the amount of mutated mtDNA.[291,292] Nevertheless, the mtRE strategy has its limitations and challenges. For example, a single restriction site (XmaI) has been identified in around 200 mtDNA variants.[294] Further revolutionary strategies have been established to prevail over the restrictions and boundaries in the application of mtREs. The application of mitoZFNs or mitoTALENs, comprising a MTS, a specific DNA recognition site, and a FokI nuclease, has effectively changed the mitochondrial heteroplasmy ratio in earlier studies.[295-300] Zinc finger domains are the recognition motifs of the mitoZFN that can identify a 12-bp sequence. In contrast to mitoZFN, mitoTALEN takes advantage of TAL effector proteins as recognition motifs that can identify around 17 nucleotides.[301] As well as the established mitoTALEN strategy, a newer method called the mitoTev-TALE approach has been efficiently utilized to modulate the mutant mtDNA in cybrid cells.[302] In this method, instead of the FokI nuclease, the I-TevI nuclease is attached to TAL effector proteins. Nonetheless,

a few evident disadvantages have been reported for this approach, including demanding a CNNNG cleavage site for I-TevI nuclease.[290] Lately, a substantially unique method has been described for mtDNA editing, termed double-stranded DNA deaminase (DddA)-derived cytosine base editors (DdCBEs).[303-306] The DdCBE is comprised of mitoTALE proteins, the interbacterial toxin DddA, and also a uracil glycosylase inhibitor (UGI), which is designed to specifically generate C•G-to-T•A alterations in human mtDNA with extreme target accuracy.[301] Surprisingly, studies have confirmed the successful application of DdCBEs for mtDNA base editing in various species that can be mentioned as human embryos, mice, rats, zebrafish, and plants.[304,306-312] Regardless of the bright outcomes of applying mitoZFNs and mitoTALENs to mtDNA editing, these approaches have restrictions and boundaries. Either mitoZFN or mitoTALEN techniques should be meticulously aimed at and devised to identify a certain range of mtDNA sequences, which require a vast amount of effort and a costly assembly procedure.[301] Furthermore, the present viral-based delivery technology (adeno-associated virus, AAV) is insufficient for the size of the coding nuclease sequences.[313] The newly emerged programmable clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) genome engineering approach has facilitated the above issues as it benefits from a single guide RNA (sgRNA) to distinguish and target a specific 20 bp DNA sequence and a Cas9 nuclease to cleave this particular DNA sequence.[314-316] Notably, the Mito-CRISPR/Cas9 system has been published to easily cleave-targeted mtDNA in HEK293T cells[317] and zebrafish.[318] Despite the above, the crucial hurdle to this approach is introducing the exogenous sgRNA into mitochondria. While various studies have revealed substantial efforts to overcome this challenge, there are still unsolved drawbacks that demonstrate the ineffectiveness of this approach so far.[301,317,319] A visual summary of the mitochondrial contribution to the aging phenotype, alongside its therapeutic approaches targeting age-related disorders, is depicted in Figure 1.

11. Conclusion

Taken together, aging is a time-dependent deterioration in cell performance that is associated with the loss of cellular homeostasis. Mitochondria are central to various pathways in homeostasis because of their essential contribution to generating ROS, bioenergetics, apoptosis, catabolic and anabolic metabolism, and signal transduction. Accumulating evidence suggests that progressive mitochondrial impairment is associated with numerous aspects of aging, such as mtDNA mutation buildup, increased oxidative damage, and impaired respiratory capacity. However, the exact biological cause remains undetermined. Nevertheless, mitochondria contribute to the aging process through multiple distinct pathways that are responsive to environmental variations. However, the functional crosstalk between these pathways and their effects on the aging process remains largely uninvestigated. Moreover, the importance of nuclear and mitochondrial genes and their intricate cross-talk in regulating aging-related mitochondrial dysfunction adds more complexity to our understanding of the aging process. mtDNA is susceptible to accumulating mutations during the lifetime of the cell, resulting in increased heteroplasmy. Beyond a certain threshold, heteroplasmy of mtDNA mutations leads to the disruption of cellular homeostatic mechanisms, which can be translated into deleterious physiological consequences driving age-associated diseases.[320,321]

Increased ROS production has been associated with aging. Moreover, mitochondria are considered both producers and targets of ROS, which participate in cell homeostasis by acting as signaling molecules. However, ROS overproduction has harmful effects on cell homeostasis, resulting in age-related oxidative stress that can disrupt cell function, mtDNA mutations, the development of various pathologies, or even premature death. Regardless of the unanswered questions about the role of ROS in oxidative stress or as signaling molecules and their relationship with mitochondrial dysfunction in aging, antioxidant therapy, such as the administration of Vitamin E and CoQ10 compounds, seems quite promising for slowing down the aging process. However, further validation is required before their application in clinical trials against age-related diseases.

Furthermore, a growing number of studies have linked UPR^{mt} activation, mediated by epigenetic modifying enzymes, to the aging process and age-related disorders. However, it is important to highlight that the UPR^{mt} pathway has yet to be thoroughly described. Besides, mitochondrial perturbation can lead to complex cellular responses that can take various forms determined by the type of stressor and target tissue, going beyond the currently conceived model of UPR^{mt}. Hence, progress toward the complete characterization of UPR^{mt} is of great importance. As discussed, mitophagy is a highly evolutionarily conserved cellular process that specifically degrades damaged mitochondria in response to damage or stress to maintain a healthy mitochondrial population and, therefore, contributes to MQC. Nonetheless, mitophagy declines during aging and is linked to age-associated diseases, including cardiac dysfunction and neurological disorders. Thus, interventions that modulate mitophagy emerge as a promising therapeutic approach to counteract age-related disease development or progression. However,

the exact regulatory mechanisms of mitophagy and its role in age-related diseases remain elusive. Over the past decades, significant endeavors have been made to unravel the role of mitochondria in aging, along with targeted interventions. Yet, preventing mitochondrial dysfunction in age-related disorders is a major challenge.

Nevertheless, NPs (NPs) are capable of targeting particular cells and localizing within mitochondria, which facilitates treatments associated with age-related diseases and mitochondrial dysfunction disorders. Hence, NPs conjugated with mitochondriotropic ligands provide a promising tool for efficient mitochondrial delivery with proper nanoformulations. Although these nanopreparations have been demonstrated to efficiently transport a wide range of payloads to the mitochondria in both *in vivo* and *in vitro* systems, various clinical and pre-clinical investigations are needed to fully understand the safety of these drug delivery approaches. Recently, therapeutic gene editing techniques to correct DNA abnormalities, including mtDNA mutations and deletions, have offered the promise of curing human age-related diseases. Nevertheless, these techniques can help lower the mutation rate within impaired mitochondria, leading to a shift in mtDNA heteroplasmy, reversing mitochondrial defects, and hence improving mitochondrial function. However, the mitochondrial bilayer membrane is a significant obstacle to effective genetic manipulation. Furthermore, most heteroplasmic mtDNA mutations differ from the wild-type by a single nucleotide, and the ability of gene editing tools to detect single-nucleotide mutations is restricted, which can result in off-target editing or catastrophic mtDNA loss. Besides, efficient tissue-specific *in vivo* delivery of therapeutic genes that mostly rely on AAV as the delivery vehicle has proven challenging. Finally, a better comprehension of the role of mitochondria in aging remains a prerequisite for combating aging and age-related disorders.

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REVIEW ARTICLE

Navigating the complex landscape of cardiac metabolism in health and disease states

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Abstract

The intricate interplay between cardiovascular health and metabolic regulation forms a critical junction in understanding the complexities of heart-related conditions. Cardiometabolic regulation orchestrates a sophisticated network of factors governing energy utilization, substrate metabolism, and cellular processes within the cardiovascular system. Balancing these mechanisms is pivotal for optimal heart function, considering the substantial energy demands for both contractile and non-contractile activities. In a healthy heart, fatty acids (FAs) derived from FA β-oxidation contribute to approximately 70% of total energy production. However, emerging evidence sheds light on pathological changes in the heart that lead to profound metabolic alterations. These alterations involve a shift from predominant FA utilization to alternative substrates such as glucose and ketone bodies, accompanied by an increased reliance on FAs. This metabolic remodeling extends beyond substrate metabolism, encompassing changes in transporter expression, the activity of metabolic-related proteins, hormonal functions, and cardiac mitochondrial energetics. This comprehensive review article delves into the intricate web of cardiometabolic regulation, elucidating the multifaceted factors influencing cardiac metabolism across diverse states encompassing health, metabolic disorders, and heart diseases. Unraveling the molecular intricacies and interconnected pathways shaping cardiac metabolism in various physiological and pathological conditions provides critical insights into the adaptive mechanisms and dysregulations associated with heart-related conditions. Furthermore, the exploration of these regulatory mechanisms offers promising avenues for targeted therapeutic interventions and diagnostic strategies in cardiovascular medicine. Integrating multidisciplinary approaches and leveraging advanced technologies will facilitate a deeper understanding of cardiac metabolism, paving the way for innovative interventions to mitigate metabolic dysregulation and optimize cardiac health.

Keywords: Cardiac metabolism; Obesity; Metabolic syndrome; Diabetes mellitus; Myocardial ischemia; Cardiac hypertrophy; Heart failure

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1. Introduction

Mitochondrial fatty acid (FA) utilization, glycolysis, and glucose oxidation are pivotal processes responsible for generating almost all of the total adenosine triphosphate

(ATP) in healthy adult human hearts.[1,2] Typically, around 70% of the heart's total ATP is derived from FA β-oxidation (FAO) under normal physiological conditions.[3,4] Studies have indicated that heart diseases trigger changes in cellular mechanisms and metabolic regulations, leading to alterations in cellular morphology and damage to cellular structures. Considering the heart's high-energy demanding function, especially during contractions, metabolic control within the heart is of utmost importance for sustaining its high energy requirements.[3,4] However, under pathological conditions, the primary energy source in the cardiac metabolic pathway can undergo alterations, resulting in varied major energy sources among different types of heart diseases.

Currently, several concepts attempt to elucidate these metabolic changes in heart diseases. First, there is the observed shift from utilizing mitochondrial FAO to glycolysis and/or ketone bodies, documented in cardiac hypertrophy and heart failure.[5-7] Second, there is an elevated utilization of mitochondrial FAO, beyond normal physiological levels, noted in conditions such as obesity, diabetes, diabetic cardiomyopathy (DCM), and cardiac ischemia/reperfusion injury.[8-12] Various conditions, such as obesity, diabetes, and heart diseases, disrupt this equilibrium, leading to alterations in energy utilization, substrate preferences, and cellular metabolism within cardiac tissues.[3,4] These disruptions often manifest as changes in the utilization of primary energy sources, such as FA, glucose, and ketone bodies. In healthy states, the heart predominantly relies on FAO to meet its energy demands. However, under pathological conditions, such as heart failure or DCM, this preference may shift, leading to an increased reliance on glucose or alternative substrates.[8-12]

Moreover, hormonal imbalances, mitochondrial dysfunction, and alterations in enzyme activity further compound these pathological changes, impacting the heart's ability to generate sufficient energy for its functions. This dysregulation not only affects energy production but also influences cardiac contractility, efficiency, and overall performance.[13-15]

Understanding these intricate regulatory pathways and the factors influencing cardiac metabolism in health and disease is pivotal for developing targeted therapies. By elucidating the underlying molecular mechanisms, identifying genetic predispositions, and uncovering the complex network of metabolic alterations, researchers strive to refine therapeutic strategies. Ultimately, the goal is to mitigate the impact of metabolic disorders on heart health and improve the efficacy of treatments for various cardiac conditions.

This review focuses on elucidating metabolic regulation in healthy hearts and adaptive mechanisms in metabolic disorders and heart diseases. In addition, it examines influential factors affecting cardiac metabolisms, such as oxygen demand, myocardial substrate metabolism, cardiac mitochondrial performance, and energetics in health, metabolic disorders, and heart diseases.

2. Cardiac metabolism in health

The heart, with its high energy demands but limited energy reserves, heavily relies on specific substrates for myocardial energy metabolism. In a normal, healthy human heart, approximately 70% of ATP is derived from FAs, with glucose contributing about 25% and the remaining 5% originating from alternate substrates such as ketone bodies and pyruvate.[3,4,8,16-19] Transport mechanisms for FAs into the heart vary; short-chain and medium-chain FAs utilize passive diffusion, while long-chain FAs rely on plasma membrane-bound FA binding proteins (FABP) and FA transport proteins.[8,20-22] Subsequently, the acyl-coenzyme A (CoA) group is added to FAs before their entry into mitochondria through specific transporters. Carnitine palmitoyltransferase-1 (CPT-1), a crucial enzyme located on the outer mitochondrial membrane (OMM), acts as a rate-limiting step in this process, converting long-chain fatty acyl CoA to carnitine forms for mitochondrial entry, where they are reconverted back to fatty acyl CoA for FAO.[8,20] Key regulators of FA transporters include factors such as acetyl CoA carboxylase, malonyl CoA, malonyl CoA decarboxylase, and other enzymes, exerting direct and indirect effects on CPT-1 activity.[23-25]

In contrast, glucose, which contributes approximately 25% of total ATP in the basal state, cannot pass through the cell membrane by simple diffusion due to its hydrophilic nature. The human heart employs two classes of glucose transporters, namely glucose transporters (GLUTs) and sodium-glucose co-transporters (SGLTs).[26] GLUT1, GLUT2, and GLUT4 are three identified isoforms, with GLUT4 taking precedence in adult hearts, responsible for approximately 70% of glucose transport after birth.[27,28] GLUT1 serves as a basal cardiac glucose transporter primarily responsible for embryonic cardiac glucose transport. However, after birth, there is a rapid transition to utilizing GLUT4 for transporting glucose, which contributes to approximately 70% of glucose transport in the adult heart.[29] Insulin mediates GLUT4 translocation in cardiomyocytes, while SGLT1, abundantly expressed in the heart, is regulated by insulin and leptin, though its exact role remains unclear.[19] A previous study demonstrated that SGLT1 is an abundantly expressed isoform in the human heart, with expression levels approximately 10-fold higher than those in the kidney.[30] Surprisingly, another study demonstrated that cardiac SGLT1 expression and activity are regulated by insulin and leptin.[31] However, the crucial

mechanisms through which this regulation occurs, as well as the role of SGLT1 in the heart, are still unknown and were not examined in this study.[31]

Regarding ketone bodies, primarily generated within hepatocyte mitochondria during ketogenesis, they serve as an alternative fuel source, particularly in states such as fasting, exercise, pregnancy, and when following low-carbohydrate diets.[32,33] Acetoacetate and beta-hydroxybutyrate serve as energy sources, particularly beneficial for the brain and heart. While efficient, their contribution to total cardiac energy production under normal physiological conditions is relatively minimal, typically <5%. Despite their efficiency, their role in cardiac energy provision remains modest within the broader context of the heart's energy metabolism.[16,34] However, during specific physiological states such as fasting, post-exercise recovery, and pregnancy, ketone bodies play a more significant role in cardiac energy metabolism. They augment ATP synthesis by maintaining oxidized ubiquinone and widening the redox span in the electron transport chain (ETC).[16,34,35]

3. Cardiometabolic alteration in heart diseases

The shift in cardiac substrate utilization for ATP production to sustain cardiac contractile function signifies a notable change in the heart's biological activity, often linked to various heart diseases.[8,19-21] Recent evidence supports two distinct concepts indicating that metabolic changes occurring in heart diseases result from alterations in the primary substrates utilized for ATP generation within diseased cardiomyocytes.

The first concept involves a shift from mitochondrial FA utilization (via FAO) to alternative energy sources such as glucose and potentially other substrates such as ketone bodies, notably observed in cardiac hypertrophy and heart failure.[5-7] This transition also includes an observed increase in ketone body utilization.[36] While ketone bodies enhance cardiac metabolism by facilitating ATP synthesis through the maintenance of oxidized ubiquinone and extending the redox span in the ETC,[16,34,35] their oxidation concurrently elevates reactive oxygen species production, contributing to oxidative stress.[35] Despite increased hepatic ketogenesis in pathological heart conditions or metabolic disorders such as hormone resistance and diabetes mellitus (DM),[32,33] ketone body metabolism remains a contributor to heightened ATP production.[16,34,35] However, this process is also associated with acidosis and increased oxidative stress, potentially resulting in a redox imbalance,[35,37] subsequently elevating the morbidity and mortality risk among patients.[37]

The second concept revolves around the heightened utilization of mitochondrial FAs in pathological states such as obesity, diabetes, DCM, and cardiac ischemia/reperfusion, exceeding the normal physiological levels.[8-12] In these conditions, cardiomyocyte metabolism shifts to derive over 70% of cardiac energy from mitochondrial FAs, which, despite being a less efficient energy source (having a higher oxygen consumption-to-ATP production ratio compared to glucose and ketone bodies),[8,16-18,20] leads to compromised cardiac function.[38,39] Conversely, in other cardiac pathological states such as cardiac hypertrophy and heart failure, the metabolic profile of cardiomyocytes reverses to predominantly derive ATP from glucose rather than FAs, offering a more efficient energy source (with a lower oxygen consumption-to-ATP production ratio compared to FA),[8,16-18,20] potentially leading to improved cardiac function.[5] These observations underscore the difference between mitochondrial FAO and glycolysis in their respective oxygen consumption per ATP produced (differences in oxygen demands).[8,16-18,20] For instance, complete glucose oxidation consumes six oxygen molecules to yield 31 ATP molecules (Glucose oxidation: O_2:ATP = 1:5.167), while one palmitate molecule, in full mitochondrial FAO, requires 23 oxygen molecules to generate 105 ATP molecules (Palmitate: O_2:ATP = 1:4.565).[8,20] The comparatively lower ATP production per oxygen molecule consumed in the mitochondrial FAO system elucidates why heightened mitochondrial FAO diminishes cardiac efficiency.[8,20]

4. The reciprocal alteration of metabolism under the "Randle cycle" concept

Randle *et al.* demonstrated that elevated mitochondrial FAO disrupts mitochondrial glucose oxidation, establishing a reciprocal relationship between the two metabolic pathways.[11] This reciprocal interaction is articulated in the widely recognized "Randle cycle" or "glucose-FA cycle".[11] According to the "Randle cycle," heightened mitochondrial FAO can impede both glycolysis and mitochondrial glucose oxidation through several mechanisms: (i) increased mitochondrial FAO enhances nicotinamide adenine dinucleotide and acetyl CoA production, thereby inhibiting pyruvate dehydrogenase (PDH) activity;[38,39] (ii) elevated citrate levels resulting from increased FAO can inhibit phosphofructokinase 1 (PFK1) activity; and (iii) elevated glucose-6-phosphate levels can inhibit hexokinase enzymes in glycolysis oxidation.[10,20] These mechanisms collectively contribute to the suppression of glycolysis and glucose oxidation.[23] Conversely, reducing mitochondrial FAO levels can lead to an upsurge in glycolysis and glucose oxidation.[23]

5. Cardiac metabolism in obesity and diabetes

In obesity and DM, elevated levels of circulating FAs and/or glucose, alongside hormonal resistance, including

resistance to insulin, fibroblast growth factor 21 (FGF21), and leptin, have been observed.[12,40-49] These conditions correlate with heightened myocardial FAO and a predominant reliance on FAO as the primary myocardial metabolism. Moreover, the proportion of FAO is increased in these pathological states. The impairment of hormonal signaling pathways in the heart especially disturbs cardiac insulin signaling pathways and can also contribute to decreased GLUT4 vesicle trafficking to the plasma membrane, leading to decreased glucose uptake into cells,[48,49] since GLUT4 is the dominant glucose transporter in the human heart.[29] Therefore, in DM, increased plasma FA levels are attributed to elevated glucagon levels activating lipolysis and cholesterol synthesis.[23]

Cardiac insulin resistance in DM involves the overexpression of FA transporters such as CD36 and FABP on cardiomyocyte membranes, augmenting FA uptake.[50,51] In addition, high expression levels of peroxisome proliferator-activated receptor alpha and peroxisome proliferator-activated receptor-gamma coactivator 1-alpha (PGC-1α) in insulin resistance and DM contribute to elevated mitochondrial FAO levels by regulating genes involved in FAO, thereby reducing cardiac glycolysis and mitochondrial glucose oxidation (via the "Randle cycle").[52-54] Insulin resistance leads to impaired cardiac mitochondrial function, diminishing cardiac efficiency due to increased mitochondrial FAO, which elevates the cardiac mitochondrial workload and oxygen demands for substrate metabolism.[55,56]

In terms of SGLT1 activity and expression in DM, studies have revealed type-dependent effects: SGLT1 expression significantly increased in end-stage DCM in type 2 DM (T2DM) patients and animal models (db/db mice), while it decreased in type 1 DM (T1DM) animal models (streptozotocin-diabetic mice).[31] The elevated SGLT1 expression in T2DM might be influenced by chronic hyperinsulinemia, as SGLT1 activity is regulated by insulin. However, the contradictory findings regarding the effects of DM on SGLT1 expression necessitate further investigation, particularly considering the severe insulin resistance observed in end-stage cardiomyopathy.

6. Cardiac metabolism in myocardial ischemia and/or reperfusion

During myocardial ischemia, the myocardium experiences a decrease in oxygen supply, leading to reduced circulating FAs available for FAO. Conversely, there is an increase in glucose derived from glycogen breakdown, facilitated by enhanced glucose transport through GLUT1 and GLUT4 translocation to the plasma membrane, resulting in a metabolic shift toward glycolysis. This metabolic shift aligns with the concept described earlier, involving a shift from mitochondrial FAO to glycolysis or other substrates. In addition, during myocardial ischemia, SGLT1 expression increases approximately two to threefold, promoting increased glucose uptake into cardiomyocytes. These alterations are beneficial, protecting cardiomyocytes from irreversible injury, necrosis, and apoptosis.[57-59]

In clinical settings, a randomized controlled trial conducted with acute myocardial infarction (MI) patients using glucose–insulin–potassium (GIK) as a supplementary treatment alongside myocardial reperfusion showed no efficacy in cardioprotection. This lack of effectiveness in this trial might be attributed to delays in initiating therapy, which was administered only during reperfusion. However, findings from the IMMEDIATE study revealed that early intravenous GIK for acute coronary syndrome (ACS) in out-of-hospital emergency medical service settings demonstrated an 80% reduction in infarct size at 30 days. Although there was no significant reduction in serious endpoints at 1 year among patients with suspected ACS compared to placebo controls, individuals with ST-elevation MI treated with GIK exhibited reduced rates of cardiac arrest, 1-year mortality, or heart failure hospitalization within the year.[60-62]

During myocardial reperfusion following ischemia, reperfusion therapy elevates FA levels and reduces malonyl CoA, a potent inhibitor of CPT-1, leading to increased cardiac FAO. ATP production during this phase primarily arises from mitochondrial FAO. Moreover, there is a decrease in glycolysis, increasing cellular oxygen demand for ATP production, as described by the "Randle cycle," which outlines reciprocal changes between mitochondrial FAO and glycolysis. This aligns with the second concept mentioned earlier, which involves a shift toward a higher degree of FAO.[8,9]

In the context of myocardial reperfusion injury, reperfusion therapy contributes to this type of injury. Mitochondrial dysfunction triggers cellular apoptosis and ATP depletion. Despite hyperoxygenation at the onset of reperfusion, impaired cardiac mitochondrial function induces oxidative stress and mediates myocardial injury.[63,64] This impairment intensifies the cardiac mitochondrial workload due to a shift from glucose oxidation to FAO, increasing oxygen demands and potentially accelerating apoptosis, thereby decreasing cardiac efficiency and function.

7. Cardiac metabolism in cardiac hypertrophy and heart failure

Cardiac hypertrophy is an adaptive response of the myocardium to pressure or volume stress in the heart

chambers.[65,66] This response serves to decrease wall stress and oxygen consumption.[67-69] However, while it provides compensation, cardiac hypertrophy also significantly increases the risk of heart failure and malignant arrhythmias.[70,71] Previous studies have divided hypertrophic transformation of the heart into three stages: (i) hypertrophy with excessive load surpassing output, (ii) compensatory hypertrophy maintaining workload and cardiac output, and (iii) heart failure with ventricular dilation and a progressive decline in cardiac output.[66]

A previous study has demonstrated that in cardiac hypertrophy, there is a suppression of the mitochondrial FAO gene PGC-1α, resulting in a metabolic shift from mitochondrial FAO to glucose oxidation.[72] This compensatory shift decreases oxygen demand compared to mitochondrial FAO, enhancing cardiac efficiency.[20] However, in hypertrophic conditions, cardiac mitochondrial function is impaired, prompting a reduction in cardiac mitochondrial workload through a shift to anaerobic glycolysis.[19,73]

In heart failure, altered energy metabolism and reduced ATP production are well-documented, with levels dropping by approximately 30% compared to a normal adult myocardium.[74,75] Concurrently, cardiac mitochondrial function decreases, further contributing to a total ATP production drop to 30%–40% of normal physiological levels.[76,77] A majority of studies reveal reduced cardiac FAO during heart failure, prompting the myocardium to shift its energy metabolism from substrates that demand high oxygen consumption (FAO) to primary energy sources with lower oxygen demands (glucose and ketone bodies) to maintain cardiac function,[20,78,79] aligning with concepts described earlier.

While glycolysis increases in models of cardiac hypertrophy induced by abdominal aortic constriction, glucose oxidation remains unchanged.[79,80] Impaired glucose oxidation in heart failure is associated with mitochondrial dysfunction, reduced expression of glycolysis and glucose oxidation-related genes, and decreased abundance of pyruvate dehydrogenase complex, potentially contributing to cardiac dysfunction.[78,81] Intriguingly, elevated glycolysis coexists with diminished mitochondrial function and energetics in heart failure,[20] culminating in ATP depletion and apoptosis, consequently reducing cardiac efficiency and function.[20]

Controversial data suggest that high-plasma FA levels elevate cardiac mitochondrial FAO, potentially improving cardiac function under MI and heart failure conditions.[82,83] However, conflicting findings propose that decreased mitochondrial FAO levels could be detrimental, further reducing total ATP production, which is already diminished in heart failure.

8. Cardiac calcium homeostasis and signaling in health and diseases

Calcium signaling plays a crucial role in cardiac cellular function, particularly within mitochondria, influencing energy metabolism, redox balance, and cell fate determination. Disruptions in mitochondrial calcium handling significantly impact the progression of cardiac disease. In conditions such as ischemic heart disease, disturbed calcium homeostasis leads to mitochondrial dysfunction, reducing ATP production and triggering cell death pathways, thereby exacerbating tissue damage during ischemia–reperfusion injury.[83,84] Similarly, in heart failure, aberrant calcium handling contributes to pathological remodeling, affecting excitation-contraction coupling and prompting metabolic shifts. This dysfunctional signaling alters reliance on oxidative phosphorylation, favoring glycolysis, and affects mitochondrial dynamics, exacerbating cardiac dysfunction. While initially adaptive, mitochondrial changes can fuel chronic dysfunction, perpetuating cardiac pathology.[80]

A comprehensive understanding of the relationship between mitochondrial calcium dynamics and cardiac disease necessitates a detailed exploration of the molecular mechanisms governing calcium transport, buffering, and signaling within mitochondria. Identifying calcium-dependent effectors such as the mitochondrial calcium uniporter complex, mitochondrial permeability transition pore, and calcium-sensitive enzymes holds promise for developing therapeutic interventions aimed at restoring mitochondrial calcium balance and preserving bioenergetics in cardiac diseases.[84]

Mitochondrial calcium homeostasis serves not only as a secondary messenger but also as a feedback and feed-forward mechanism in the development of cardiac myopathy, particularly associated with ETC dysfunction.[85] Moreover, calcium plays an intricate role in cardiomyocyte function, particularly in excitation–contraction coupling, influencing various electrophysiological processes that impact cardiac metabolism and arrhythmias. While computational modeling has advanced our understanding, critical questions regarding macromolecular regulation, calcium-dependent pathways, and the interplay between electrophysiology and cardiac metabolism remain unanswered.[85] Addressing these uncertainties through *in vitro* and *in silico* studies could pave the way for improved therapeutic strategies.

9. Discussion

The investigation into cardiac metabolism unveils a multifaceted landscape, delineating intricate patterns of energy utilization in both physiological and pathological

Table 1. Substrate utilization for ATP production in cardiac metabolism across health and disease states

Conditions	Substrates			References
	Fatty acid	Glucose	Alternative substrates	
Normal heart	70% of total ATP production	25% of total ATP production	5% of total ATP production	1,2
Obesity	Increase	Decrease	Decrease	8-12
Diabetes mellitus	Increase	Decrease	Decrease	8-12
Ischemic heart	Decrease	Increase	Increase	8-12
Reperfusion heart	Increase	Decrease	Decrease	8-12
Cardiac hypertrophy	Decrease	Increase	Increase	5-7
Heart failure	Decrease	Increase	Increase	5-7

Notes: "Increase" signifies an increase exceeding a certain percentage from the normal condition; "Decrease" signifies a decrease exceeding a certain percentage from the normal condition.
Abbreviation: ATP: Adenosine triphosphate.

states. Central to this paradigm are the intersecting pathways of mitochondrial FAO, glycolysis, and glucose utilization, which establish the energetic framework of a healthy heart.[3,4] However, in pathological conditions, a noticeable divergence emerges: while cardiac hypertrophy and heart failure exhibit adaptations favoring more oxygen-efficient substrates such as glycolysis or ketone bodies, conditions such as obesity, diabetes, and DCM showcase heightened mitochondrial FAO. This juxtaposition underscores the complex interplay between substrate preferences and energy pathways, epitomizing the nuanced metabolic shifts characterizing cardiac pathology.

Hormonal imbalances, mitochondrial irregularities, and enzymatic disruptions add layers of complexity to cardiac metabolism in disease states. Diverse research perspectives propose varying degrees of reliance on glycolysis, alternative substrates, or increased mitochondrial FAO, presenting a mosaic of hypotheses. The exploration of these intricate pathways offers a multitude of potential therapeutic targets for understanding and intervening in metabolic dysregulation in various cardiac diseases.[13-15]

The "Randle cycle" concept, elucidating the reciprocal relationship between mitochondrial FAO and glycolysis, governs substrate utilization within the cardiac milieu. The adaptability of the heart during myocardial ischemia and reperfusion, adjusting substrate preferences based on oxygen availability, emphasizes the dynamic nature of cardiac metabolism under stress.[11]

The divergence between adaptive responses favoring glucose oxidation and heightened mitochondrial FAO in cardiac hypertrophy and heart failure necessitates further investigation to comprehend its impact on compromised cardiac energetics. Addressing this discrepancy is pivotal for understanding and mitigating metabolic dysregulation in diseased hearts.

Future strides in understanding cardiac metabolism entail elucidating regulatory mechanisms governing substrate preferences, exploring the "Randle cycle," and probing the molecular intricacies in disease states. Integrating diverse disciplines encompassing metabolomics, genetics, and systems biology with clinical data can facilitate tailored interventions and innovative diagnostic tools, revolutionizing cardiovascular medicine. Collaborative efforts leveraging advanced technologies promise to reshape the landscape of cardiac disease management by providing deeper insights into metabolic regulation in the heart.

10. Conclusion

Understanding the profound impact of metabolic disorders and heart diseases on myocardial metabolism is crucial, as these conditions influence various facets such as energy demand, substrate utilization, and cardiac mitochondrial function. These factors intricately regulate cardiac energy metabolism and efficiency. Unraveling the molecular mechanisms behind these metabolic alterations during such conditions holds promise in refining therapeutic strategies and pinpointing targets to treat heart diseases while bolstering cardiac efficiency. A schematic representation of substrate utilization for ATP production in cardiac metabolism across both healthy and diseased conditions is depicted in Table 1. Despite the potential benefits of modulating cardiac metabolism to enhance heart function, the intricate links between metabolic alterations and pathological conditions remain poorly elucidated. Clinical efforts to intervene and modulate cardiac metabolism have not provided comprehensive insights into this domain. Therefore, future investigations should aim at deeper exploration, unraveling the intricate molecular changes, genetic mutations, and complex networks involved in altering cardiac energy metabolism. Advancements in understanding these mechanisms hold the key to

enhancing the effectiveness of metabolic therapies for heart diseases.

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REVIEW ARTICLE

Addressing cortex dysregulation in youth through brain health check coaching and prophylactic brain development

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Abstract

The Carter Center has estimated that the addiction crisis in the United States (US), if continues to worsen at the same rate, may cost the country approximately 16 trillion dollars by 2030. In recent years, the well-being of youth has been compromised by not only the coronavirus disease 2019 pandemic but also the alarming global opioid crisis, particularly in the US. Each year, deadly opioid drugs claim hundreds of thousands of lives, contributing to an ever-rising death toll. In addition, maternal usage of opioids and other drugs during pregnancy could compromise the neurodevelopment of children. A high rate of DNA polymorphic antecedents compounds the occurrence of epigenetic insults involving methylation of specific essential genes related to normal brain function. These genetic antecedent insults affect healthy DNA and mRNA transcription, leading to a loss of proteins required for normal brain development and function in youth. Myelination in the frontal cortex, a process known to extend until the late 20s, delays the development of proficient executive function and decision-making abilities. Understanding this delay in brain development, along with the presence of potential high-risk antecedent polymorphic variants or alleles and generational epigenetics, provides a clear rationale for embracing the Brain Research Commission's suggestion to mimic fitness programs with an adaptable brain health check (BHC). Implementing the BHC within the educational systems in the US and other countries could serve as an effective initiative for proactive therapies aimed at reducing juvenile mental health problems and eventually criminal activities, addiction, and other behaviors associated with reward deficiency syndrome.

Keywords: Brain health check; Cognition; Dopaminergic dysregulation; Executive function; Reward deficiency syndrome; Genetics; Epigenetics

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1. Introduction

The purpose of the brain health check (BHC) is to integrate objective assessments across cognition, neurological imaging, psychiatry, and genomics to identify youths who are at risk for juvenile mental health problems, criminal activities, addiction, and other behaviors associated with reward deficiency syndrome (RDS). Identifying vulnerable youths through these assessments can provide insights into proper interventions, such as genome-matched amino acid therapies that can treat reward/dopamine dysregulation and prevent the inheritance of epigenetic insults associated with addiction to future generations. Amidst the increasing drug abuse crisis in the United States (US) and the potential for long-term enormous societal costs, a brain research consortium developed this approach. The group is comprised experienced teachers, educators, drug abuse counselors, psychiatrists, clinicians, scientists, neuroscientists, geneticists, and addiction medicine physicians, who encourage the adoption of the standardized BHC in K1–K12 education. In addition, they endorse basic and clinical scientific research into brain health prophylaxis for developing brains.

2. Understanding reward dysregulation and potential therapeutic approaches

As defined in the Sage Encyclopedia of Psychiatric Disorders (2017), there is emerging evidence of an over-representation of the antecedent to RDS, encompassing both substance- and non-substance-related addictive behaviors, within the general US population.

It is well established that dopamine resistance in individuals with food and drug addiction is caused by dysfunctional genetic neurotransmitter polymorphisms, such as the A1 allele of the *DRD2* gene, and epigenetic insults. A burgeoning line of evidence shows that a natural, non-addictive, and safe putative D2 agonist may aid in the treatment of and recovery from these RDS behaviors in patients addicted to substances. The impact of the patented KB220 nutrigenomic technology, known as "Synaptamine Complex," acts as an activator of the mesolimbic system, as observed through quantitative electroencephalography (qEEG) imaging. A published pilot study demonstrated that the intravenous administration of KB220 was observed to normalize the aberrant electrophysiological parameters of the reward circuitry site.[1] The study also revealed that the qEEG graphs of an alcoholic and a heroin abuser with existing abnormalities (widespread theta and alpha activity, respectively) during protracted abstinence were significantly normalized after the administration of a single intravenous dose of KB220® Synaptamine Complex Formulation.[1] Both patients were genotyped for several neurotransmitter reward genes to determine if they carried any putative dopaminergic risk alleles that may predispose them to alcohol or heroin dependence, respectively. The genes examined included the dopamine transporter (*DAT1*, locus symbol *SLC6A3*), dopamine D4 receptor exon 3 *VNTR* (*DRD4*), *DRD2* TaqIA (rs1800497), *COMT* val158 met *SNP* (rs4680), monoamine oxidase A upstream *VNTR* (*MAOA-uVNTR*), and serotonin transporter-linked polymorphic region (*5HTTLPR*, locus symbol *SLC6A4*). It

should be emphasized that these findings stem from case studies, and it is improbable for individuals to carry all putative risk alleles. Based on the previous research and our qEEG studies, we cautiously suggest that long-term activation of dopaminergic receptors may increase their proliferation, leading to enhanced "dopamine sensitivity" and a heightened sense of happiness, particularly in carriers of the *DRD2* A1 allele.[2]

The intravenous administration of the Synaptamine Complex Variant KB220 in >600 alcoholic patients resulted in a significant reduction in RDS behaviors; this effect was further supported by an expanded study involving oral KB220Z[3] and functional magnetic resonance imaging conducted on abstinent heroin addicts.[4] For a deeper understanding, future studies, including functional positron emission tomography scanning, are required to determine the acute and chronic effects of oral KB220Z on the number of D2 receptors and its interaction with the nucleus accumbens (NAc). In addition, further confirmation of these findings through large, population-based, and case-controlled experiments could ultimately lead to significant improvements in the treatment and recovery of patients with RDS and dopamine deficiency resulting from disruptions in the transduction of multiple neurotransmitter signals within the Brain Reward Cascade (BRC).[5]

Moreover, recent neuroimaging studies have highlighted the potent effects of KB220Z, underscoring the importance of Pro-dopamine regulation along the BRC (Figure 1).

It is also possible that ACH neurons at the NAc ACH can stimulate both muscarinic (red hash) and nicotinic (green hash) receptors. Finally, glutamate neurons in the VTA will project to dopamine neurons through NMDA receptors (green equal sign) to preferentially release dopamine at the NAc (shown as a bullseye), indicating euphoria or a "wanting" response. The result is that when dopamine release is low, there can be a state of unhappiness characterized by endorphin deficiency. At the same time, general (usual) happiness depends on the dopamine homeostatic tonic set point.[6] In addition to the coronavirus disease 2019 pandemic, there is a global addiction crisis. While being highest in the US, the devastation and deaths from drug overdose are global issues requiring "out of the box" thinking.[7] Even in the face of harm reduction, relying on opioids to treat issues caused by other potent opioids seems counterintuitive and perpetuates unwanted addictions.[8] Several investigative groups have been cognizant that addressing the root cause is one of the approaches to reducing harm.[9,10] Another approach is using a narcotic antagonist (like naltrexone) to induce "psychological extinction" through blocking D2 receptors.[11] The latter approach appears more acceptable; however, compliance remains a deterring issue.[12] The approved drug acamprosate, an NMDA receptor antagonist and a positive allosteric modulator of GABAA receptors, also disrupts dopaminergic signaling.[13] The growing acceptance of the RDS concept, introduced by Blum in 1995, facilitates the common mechanism hypothesis for substance and non-substance addiction. Understanding the in-common neuromodulating features of neurotransmission and its disruption through chronic exposure to substance and non-substance addictions requires the utilization of an approach that involves "dopamine homeostasis."[14]

3. Review of evidence

The "out of the box" approach involves coupling genetic risk polymorphic testing with a safe and well-researched complex, KB220Z. The KB220Z is customized to match the presence of resultant alleles and provide a precision nutraceutical with known prodopamine regulatory pharmacological properties.[2,15] High-tier publications strongly support a shared neuromechanism underlying both substance and non-substance addiction, such as alcohol, opioids, gambling, and food.

In the 1970s, Blum's laboratory developed an amino-acid-based enkephalinase inhibitory pro-dopamine regulator with the KB220 nutraceutical complex as its cornerstone ingredient, now validated by over 45 clinical studies published in peer-reviewed journals.[16,17] The basis of this complex is its ability to mimic the BRC,[17] an established model of reward processing. The most striking feature is the activation of BOLD by the KB220Z across the BRC,[18] including the NAc, anterior cingulate gyrus, anterior thalamic nuclei, hippocampus, prelimbic, and infralimbic parts of the prefrontal cortex (PFC). Evidence of genetic vulnerability as an antecedent to unwanted RDS behaviors may be a determining factor, which could be identified early in life. Based on previously published literature, the role of reward gene polymorphisms puts individuals at an increased risk for various forms of RDS behaviors, including anhedonia.[19,20] This insight spurred the development of the patented genetic addiction risk severity (GARS) test, aimed at identifying genetic risk for these behaviors. Specifically, published studies have illustrated the coupling of GARS with KB220Z formulations of semi-customized precision pro-dopamine regulators tailored to one's GARS profiles.[21] The biological approach of this system enhances the effectiveness of RDS treatment.[22]

Balancing the BRC or achieving "dopamine homeostasis" is generally preferred and considered a commendable objective, as opposed to interventions that involve blocking natural dopamine or administering potent opioids to overcome opioid addiction.[21] In the face

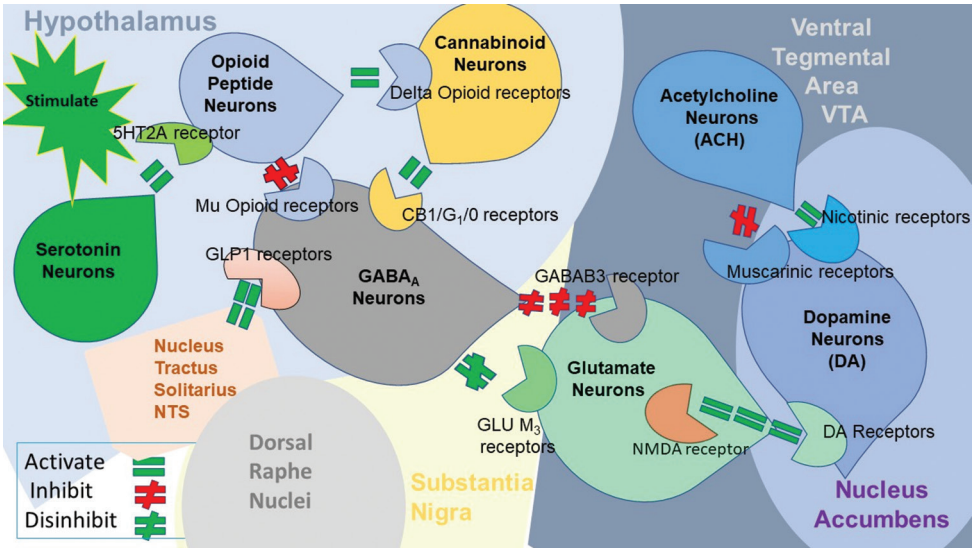

Figure 1. Interaction of at least eight major neurotransmitter-pathways involved in the brain reward cascade. In the hypothalamus, environmental stimulation triggers the release of serotonin, which, through receptors such as 5HT-2a, activates (green equal sign) the subsequent release of opioid peptides from opioid peptide neurons, also located in the hypothalamus. These opioid peptides, in turn, exert two distinct effects, possibly through two different opioid receptors. One effect inhibits (red hash sign) the mu-opioid receptor (possibly through enkephalin) and projects to GABAA neurons in the substantia nigra. The other effect stimulates (green equal sign) cannabinoid neurons (e.g., anandamide and 2-arachidonoylglycerol) through beta-endorphin-linked delta receptors, which further inhibit GABAA neurons in the substantia nigra. In addition, cannabinoids, primarily 2-arachidonoylglycerol, when activated, can indirectly disinhibit (red hash sign) GABAA neurons through the activation of G1/0 coupled to CB1 receptors in the substantia nigra. Not depicted in the figure, the dorsal raphe nuclei feature glutamate neurons that can indirectly disinhibit GABAA neurons in the substantia nigra through activation of GLU M3 receptors (red hash sign). When stimulated, GABAA neurons powerfully (red hash signs) inhibit VTA glutaminergic drive through GABAA neurons. It is also possible that stimulation of ACH neurons at the NAc can stimulate both muscarinic (red hash) and nicotinic (green hash) receptors. Glutamate neurons in the VTA project dopamine neurons through NMDA receptors (green equal sign) to preferentially release dopamine at the NAc, resulting in a sense of euphoria, or "wanting" response. Figure 1 also depicts that GLP1 from the nucleus tractus solitarius stimulates GABAA in the Substantia Nigra. As a result, dopamine release is low (endorphin deficiency), followed by feelings of unhappiness. On the other hand, overall (healthy) happiness depends on the optimal balance of dopamine, regulated by the dopamine homeostatic tonic set point.[6]

of the current addiction pandemic, we urge addiction neuroscientists and clinicians to embrace this innovative technology and establish a "standard of care" for treating and preventing addiction and all related RDS neurosequala.[23] While further research is required, it is crucial to establish a set of acceptable guidelines that include an understanding of the RDS concept. Understanding neurogenetics by utilizing a "systems biology" approach such as precision behavioral management, as outlined herein, seems prudent and represents a step forward in restoring well-being to the billions afflicted globally.[24-27] In terms of a system biology approach, Rosen *et al.* outlined the theory behind complex trait analysis and systems genetics. They describe web-accessible resources, including GeneNetwork, that facilitate rapid exploratory analysis and hypothesis testing. Moreover, GeneNetwork is a tightly bioinformatic integrated tool and data set, allowing investigation into complex networks of gene variants, molecules, and cellular processes that modulate complex traits such as behavior and disease susceptibility. This technique will enable scientists to analyze gene expression across various specific brain regions and tissues, explore genetic covariance among traits, and map loci that modulate these traits. Rosen *et al.* further suggested that these tools enable investigators to assess the complex interactions of gene networks, employing a systems approach.[28]

4. Neurogenetic and epigenetic correlates of adolescent predisposition to and risk for addictive behaviors as a function of PFC dysregulation

Within the medical community, especially among addiction professionals, there is growing concern about how preteens, adolescents, and young adults turn to substance abuse to cope with stress and anger. The turbulence of the underdeveloped central nervous system (CNS), especially the PFC, underscores the need for continued neuroimaging studies in both human and animal models, as well as encourages preventive measures and regulatory actions taken by governmental bodies.

The PFC is known to undergo significant developmental changes before individuals reach their 20s, impacting decision-making ability within this population.

Furthermore, early genetic testing for addiction risk alleles will provide valuable information that could potentially be utilized by parents and caregivers before any psychoactive drug use begins. Beyond genomic testing, a more straightforward approach could be the widespread adoption of a standard BHC, such as school fitness programs.

Family history, parenting styles, and relationship attachments, modified by various reward genes, including the well-known bonding substances oxytocin/vasopressin, may affect dopaminergic function. In addition, well-characterized neuroimaging studies indicate region-specific differential responses to drugs, food, and non-substance-addictive behaviors via either "surfeit" or "deficit."[29,30] Therefore, a "reward deficiency solution system" that combines early genetic risk assessment, medical monitoring, including a BHC, and nutrigenomic dopamine agonist modalities to combat reward deficiency risk may help address the global crisis that is hindering youth from leading normal, productive, and happier lives.[31]

Unlike fully developed adults, preteens transitioning into adolescence may lack adequate decision-making capacity due to incomplete brain development and myelination. The PFC area, known as the "braking/inhibitory system," supports executive function and decision-making but can be hijacked by subcortical structures in the midbrain. Impairments in the midbrain region, which regulates social and emotional responses, may lead to deficits in neurotransmitter function.

We must be cognizant of the impact of stress on the brain's developmental process and how substance abuse, such as alcohol, cocaine, and opioids, alters the integrity of white and gray matter volume.[32] Furthermore, it is well known that myelination in the PFC begins when people are in their early 20s.[33-40] Myelination regulates brain speed and can be compromised by stress and drug exposure, especially during prenatal and other developmental phases.[37-39] During the turbulent years before adulthood, youth may encounter stressful situations, resulting in frustration that could trigger epigenetic changes that exacerbate genetic antecedent risk for drug abuse.[40,41] The D2 dopamine receptor (*DRD2*) is the most extensively investigated gene in diverse neuropsychiatric disorders. Numerous international studies have been performed since the first association of the TaqI A DRD2 minor (A1) allele with severe alcoholism in 1990. As of October 10, 2022, there are 5351 articles listed in PUBMED, with 120 meta-analyses yielding mixed results. In our opinion, negative reports on the association of various *DRD2* gene polymorphisms are due to poorly screened controls, resulting in the non-elimination of many hidden RDS behaviors. Moreover, pleiotropic effects of *DRD2* variants have been observed in neurophysiologic, neuropsychologic, stress response, social stress defeat, maternal deprivation, and gambling disorders, whereby epigenetic DNA methylation and histone post-translational negative methylation have been identified in many citations.[14,42-56] Methylation of *DRD2* has been observed in many facets of addiction, including increased striatal response to reward cues in alcoholics,[54] decreased functional connectivity of the executive control network,[43] and withdrawal.[44,46] Blum and Noble characterized the *DRD2 Taq A1* allele as a generalized reward gene rather than one specific to alcoholism. This underscores the need for the field to find ways to either use effector moieties to edit the neuroepigenetic insults or possibly harness the idea of potentially removing negative mRNA-reduced expression by inducing "dopamine homeostasis."

It is important to consider oxytocin as a crucial element in inducing dopamine balance within the brain. Evidence suggests an important interaction between oxytocin/vasopressin and dopamine function, as demonstrated by Modestino *et al.*[57] This important interaction should not be ignored, especially in instances of antisocial behavior in youth, including those with conditions such as autism spectrum disorder.[58]

5. Opting for immediate satisfaction relative to delayed higher reward value in you

According to Volkow and Baler,[41] it is imperative and critical for survival to learn how to balance behaviors that provide a reward NOW versus behaviors that provide an advantage LATER. Specifically, Volkow's group proposed a model in which dopamine can favor NOW processes through phasic signaling in reward circuits or LATER processes through tonic signaling in control circuits. At the same time, through modulation of the orbitofrontal cortex, which processes salience attribution, dopamine enables shifting from NOW to LATER. In addition, modulation of the insula, which processes interoceptive information, influences the probability of selecting actions NOW versus LATER based on an individuals physiological state. Disruptions along these circuits contribute to diverse pathologies, including obesity, excessive reward-seeking behaviors, and various types of addiction.[59]

It is noteworthy that adolescents with a family history of substance use disorder (SUD) are at a greater risk for SUD. Rodriguez-Moreno *et al.*[60] suggested that this may be partly attributed to the inheritance of behavioral impulsivity. They employed a delay discounting task to compare impulsivity in decision-making and its associated brain functioning among adolescents with and without a family history of substance abuse. During the task, subjects had to choose between

"smaller, sooner" or "larger, later" rewards. The group with a family history of substance abuse displayed greater impatience by responding to "smaller, sooner" rewards more frequently compared to those without a family history of abuse. Behavioral impulsivity is ascribed to the differential developmental trajectories of two brain systems in young individuals. To provide clarity for those unfamiliar, it is known that children can be described with regard to how closely they are functioning to age-expected development in the three early childhood outcomes measured for federal reporting purposes. This is evaluated by collecting a variety of formative assessment data and using it to rate the child's functioning on a 1 – 7 Likert scale, with 6 and 7 being the age-expected functioning level. In fact, the aim is to link performance with age expectation by comparing the functioning of children with disabilities to those developing according to age expectation. Specifically, Steinberg[61] reported on the dominating role of the socioemotional brain systems in driving reward-seeking behavior in the face of an underdeveloped self-regulatory system. Casey's group[62,63] suggested that adolescent developmental changes are hierarchical in subcortical and cortical regions and their interconnections. For clarity, a hierarchy (from Greek: ἱεραρχία, *hierarkhia*, "rule of a high priest," from *hierarkhes*, "president of sacred rites") is an arrangement of items (objects, names, values, categories, etc.) represented as being "above," "below," or "at the same level as" one another.

Most importantly, it is plausible that in adolescence, over-activation of the brain's reward system and under-activation of the cognitive control brain mechanisms can lead to unwanted substance-seeking behavior driven by impulsivity and sensation-seeking tendencies.[64] Others suggested that choosing Now versus Later involves developmental changes that load onto poor decisions due in part to an undeveloped reward and cognitive control system, unlike their adult counterpart.[65-69]

6. Cognitive impairment in youth

In terms of cognitive impairment, especially concerning deficient executive cognitive functioning (ECF) in children, Aytaclar *et al.*[70] reported that early adolescents at high risk for addictive behavior due to fathers with SUD demonstrated significantly poorer performance on ECF compared to lower risk adolescences. High-risk individuals in early adolescence displayed an earlier initiation of cannabis use and a greater prevalence of lifetime cannabis and tobacco use. Importantly, the level of ECF activity was predictive of the severity of drug involvement, including conduct problems and the number of drugs ever tried.

Several contributing factors are associated with cognitive impairment in youth, including but not limited to excessive opioid/alcohol intake in mothers during pregnancy,[71,72] substance abuse, food addiction, and neuropsychiatric illnesses such as attention deficit hyperactivity disorder (ADHD) and attention deficit disorder.[73] Bihlar Muld *et al.*[74] highlighted that the clinical characteristics of patients with both ADHD and SUD differed from those with only SUD or ADHD and other psychiatric conditions, indicating the disabling nature of ADHD when combined with SUD. Specifically, the combination of severe substance abuse and ADHD resulted in poor general cognitive ability, including antisocial behavior. In addition, disruptions in the nascent synaptic networks and glia induced by opioids can impact brain connectivity and cognition after the opioid supply is abruptly stopped after birth.[75] Neuroimaging has revealed abnormalities in brain structure, including cortical development, white matter microstructure, and functional connectivity, in newborns with fetal alcohol syndrome. These impairments in brain development modify developmental trajectories, leading to deficits in cognition, executive function, memory, behavior, and social adaptation.[72] These catastrophic deficits in brain development pose risks for impending RDS behaviors, including SUD.

Undoubtedly, the prevalence of sugar in food and beverages has led to excessive consumption across all age groups, especially children and adolescents. It is staggering to note that over 60 countries consume sugar more than 4 times (>100 g/person/day), exceeding the World Health Organization's (WHO) recommendations (25 g/person/day). Utilizing a validated mouse model, Beecher *et al.*[73] reported that prolonged sugar overconsumption induces an abnormal response to novelty and changes both episodic and spatial memory. Their findings revealed that hippocampal-dependent learning and memory deficits accompany altered hippocampal neurogenesis. Specifically, there was an overall reduction in the proliferation and differentiation of neurons, especially within the dentate gyrus of newborns.

While the global obesity epidemic has been widely publicized in the media, understanding the evolution of sugar addiction could shed light on this dilemma. Avena's group[76] highlighted that the dopaminergic system in the mesolimbic region of the human brain is involved in hedonic rewards as a function of eating highly addictive, palatable foods like sugar. Particularly interesting is the role of acetylcholine in counteracting the dopaminergic surge as a plausible mechanistic action to help curb uncontrollable sugar cravings.

7. Proposing BHC as a novel program in the US's educational system

In 2021, over 100,000 individuals died prematurely from an opioid overdose. Neuropsychiatric and cognitive

impairments are underreported comorbidities of reward dysregulation due to genetic antecedents and epigenetic insults. Recent genome-wide association studies involving millions of subjects revealed frequent comorbidity with SUD in a sizeable meta-analysis of depression.[77] Significant associations were identified between the expression of *NEGR1* in the hypothalamus and *DRD2* in the NAc, among other genetic factors. However, despite the rise in SUD and neuropsychiatric illness, especially in youth, routine standard objective assessments of brain function remain absent.

The importance of exercise programs in the global educational system was emphasized in 2020 by the release of updated global guidelines by the WHO on physical activity and sedentary behavior for children, adolescents, adults, older adults, sub-populations such as pregnant and postpartum women, and those living with chronic conditions or disabilities. According to Chaput *et al.*,[78] increased and higher intensities of physical activity, as well as a diversity of physical activity (i.e., aerobic, muscle, and bone strengthening activities), are associated with improved health outcomes (primarily intermediate outcomes), as supported by various systematic reviews. Similarly, Thanos's group[79] reported that exercised rats had 18% and 21% lower dopamine D1R-like binding levels than sedentary rats within the olfactory tubercle and NAc shell, respectively. In addition, there was greater dopamine D2R-like binding in the NAc core (24%) and shell (25%) of exercised rats compared with sedentary rats. These observations support the hypothesis that aerobic exercise results in changes in the mesolimbic pathway that could mediate exercise-induced attenuation of drug-seeking behavior. The role of exercise, especially in the educational system, may have potential benefits for assisting school-age children with a positive family history of SUD, for example, through formal fitness programs.[80]

We propose that integrating existing education-based fitness programs with a standard BHC could synergistically not only improve the health of individuals but could also facilitate early identification of cognitive impairments. For early identification of cognitive abilities, DNA analysis through genetic testing, such as the GARS test, could provide important information, reflecting students' brain neurotransmitter function at a genetic level.[19,21,78,80,81]

The rationale for encouraging a standard objective BHC is to acquire an extensive dataset to treat clinical syndromes in psychiatric patients and high-risk populations. While we advocate for implementing a generalized BHC across all K1–K12 students, its importance is especially pronounced for high-risk children attending "recovery high school (RHS)." Spearheaded by one of us (AJF) and others is the needed development of RHSs that provide a supportive educational and therapeutic environment for students following SUD treatment. According to Weimer *et al.*,[82] most students served by RHSs have concurrent mental health disorders and are at risk for school failure, dropout, and substance use relapse. Fairly recently, RHS student high school graduation rates were 21 – 25 percentage points higher compared to students not attending RHS.[82] This finding was statistically significant, albeit with limitations related to non-randomized design, selection bias in the study conditions, and uncertainty in calculating school costs. In another study by Tanner-Smith *et al.*,[83] students attending RHS exhibited less frequent delinquent behavior while intoxicated and fewer days of substance use after discharge from SUD treatment than students attending non-RHS. Therefore, we propose RHS students as suitable candidates to test out the utilization of the BHC.

The proposed BHC comprises a set of reliable, accurate, and cost-effective objective assessments involving the following domains: (i) episodic and general memory; (ii) processing speed; (iii) attention; (iv) neuropsychiatry; and (iv) neurological imaging. After a review of over 36 years of computerized and written assessments primarily from PUBMED of memory, attention, psychiatric, and neurological imaging, the following recommendations have been selected for inclusion in the BHC: (i) MemTrax (episodic memory and processing speed); (ii) CNS vital signs (general and remote memory); (iii) test of variables of attention (attention); (iv) millon clinical multiaxial inventory III (neuropsychiatric); and (v) quantitative electroencephalogram/P300/evoked potential (neurological imaging). Continued research aims to simplify the BHC by including qEEG/P300/evoked potentials and genetically guided precision induction of "dopamine homeostasis."[84] This approach allows the assessment and treatment of reward deficiency and helps prevent dopamine dysregulation from being epigenetically transmitted to future generations.

During adolescence, developmental changes in the neural circuitry of reward processing, motivation, cognitive control, and stress may contribute to vulnerability to increased engagement in substance use and nonsubstance addictive behaviors.[85] It has been suggested that the adolescent's liability for addictions involves changes in the function and structure of the midbrain dopaminergic system, genetic antecedents, and epigenetic insults such as stress-induced neuroplasticity, contributing to imbalances between cognitive control and reward response.

Potenzas' group[85] suggests that leveraging genetics, epigenetics, and intermediate phenotypes/endophenotypes may help identify children and adolescents at risk. Once identified, it is crucial for these individuals to participate in a guidance program, essentially brain health coaching

(BHCo). The advent of molecular neurobiological tools to uncover neurotransmitter cascade surfeits or deficits and possibilities for restoring dopamine balance across these brain regions, including the PFC, can improve screening of cognitive abilities, which would enhance prevention and intervention approaches. However, implementing changes in educational programs requires top-down public policy strategies. A detailed description of our proposed BHC can be found in Braverman *et al.*[86]

8. Epigenetics of reward processing in adolescence

It is widely acknowledged that the adolescent brain matures through a prolonged reorganization of gray matter, white matter, and associated neurochemical systems. Interestingly, this period of enhanced cognitive ability in adolescents coincides with a reduction in cortical gray matter thickness, resulting from epigenetic experience-dependent loss of synapses and a concomitant strengthening of the remaining connections.[87-89] In addition, during adolescence, gray matter volume and density decrease in the brain, specifically in the parietal cortex, PFC, and basal ganglia, all of which are critical for executive function, motivated behaviors, and sensory processing.[34,90,91] Furthermore, Paus[89] demonstrated that there were corresponding increases in white matter, potentially reflecting augmented myelination and axonal diameter, leading to enhanced efficiency of impulse transduction. Notably, Gogtay *et al.*[92] observed that phylogenetically older brain regions mature earlier than the newer ones. This delayed, uneven maturation of subcortical, emotional, and reward-focused systems, including cortical executive and impulse control systems, could underlie many RDS behaviors, including SUD.[93-95]

The prevalence of mental health disorders, including addictive behaviors, in children and adolescents has increased at least two- to three-fold from the 1990s to the present day.[87] According to Monaco,[95] one plausible mechanistic reason for this increase may be the transmission of altered brain circuits epigenetically across generations through non-DNA-based mechanisms (intergenerational and transgenerational effects). These epigenetic insults to the developing brain may be due to a family history of SUD, obesity, or a poor diet (e.g., processed, palatable foods). These insults may cause intergenerational and transgenerational effects for at least up to 2 years, influencing set points in neuropathways integrating sensory-motor, reward, and feeding behaviors.

In line with this, Hurd's group linked parental THC exposure in rats to reduced proenkephalin mRNA expression in the NAc during early development, along with elevated expression during adulthood. Perinatal THC exposure also resulted in shorter latency to the first active lever press, greater responses to low heroin doses, and more heroin-seeking during mild stress and after extinction.[96] Studies by Yuan *et al.*,[97] and others[98] reveal that persistent alterations in neuronal signaling and cognitive ability result from chronic nicotine exposure, likely due to altered dopamine function in the brain. Dopamine D_2 receptor activation of fast-spiking interneurons in the PFC does not occur until late adolescence, along with the recruitment and maturation of local GABAergic activity.[99,100] In addition, Tseng and O'Donnell[99] point out that D_1-NMDA receptor interactions in cortical pyramidal neurons that are necessary for mature cognitive and attentional processing continue to develop during this period. Flores-Barrera *et al.*[101] discovered that ventral hippocampal input to the medial PFC is strengthened during late adolescence due to the D_1 receptor-mediated emergence of NMDA receptor GluN2B subunit function. Unfortunately, in the mesolimbic system, particularly in the NAc, D_1 and D_2 receptor responses are immature, leading to reduced synaptic interaction between NAc and the PFC.[102] Furthermore, the stimulation of the D_2 receptor has an age-specific influence on AMPA-evoked cell excitability, and interactions between D_2 and AMPA receptors elicit the activation of GABA interneurons, primarily in adults but not adolescents.[103] In summary, these observations suggest a functional switch in reward processing during adolescent development mediated by dopamine regulation of GABA interneurons. It is well known that enhanced GABA transmission following chronic alcohol intake significantly reduces dopamine release at the NAc.[104] In addition, stimulation of GABAB receptors inhibits dopaminergic VTA neurons.[105] However, Pandey's group demonstrated that the inhibition of VTA neuronal firing by bath-applied GABA is primarily mediated by GABAA receptors.[106]

The risk of all addictive drug and non-drug behaviors, especially in the unmyelinated PFC of adolescents, is both critical and complex. Many animal and human studies have highlighted the epigenetic impact on the developing brain in adolescents compared to adults. Some studies reveal an underlying hyperdopaminergia, which predisposes young individuals to risky behaviors by inducing high quanta presynaptic dopamine release at reward site neurons. In addition, altered reward gene expression in adolescents caused by epigenetically transferred social defeat, such as bullying, can persist into adulthood. However, there is also evidence that overstimulating epigenetic events can elicit adolescent hypodopaminergia. This complexity (Figure 2) suggests that neuroscience cannot definitively claim that all adolescents carry a hyperdopaminergic trait. To help dissect these seemingly opposing views, Blum's laboratory reported

a high risk for any addictive behavior (hypodopaminergia), especially drug-seeking (95%) and alcohol-seeking (64%) based on GARS testing of 24 Caucasians, ages 12–19 (derived from families with RDS). These results, although from a small cohort, should encourage further extensive studies in this area.

Mental disorders are widespread globally, influencing every community and age group, and contribute substantially to the overall disease burden, with major economic and social consequences as well as effects on human health and rights. Alarmingly, the largest inequities exist across nations, with 80% of people affected by mental disorders living in low- and middle-income countries, which benefit from scarcely 10% of global mental health resources. Unfortunately, poor rural areas in the US experience a significantly higher rate of mental disorders, including RDS behaviors such as SUD. Furthermore, due to low income and high juvenile delinquency in rural communities, possibly linked to cognitive inabilities such as poor decision-making, the recommendation of a standard BHC seems prudent. While globally accepted diagnostic

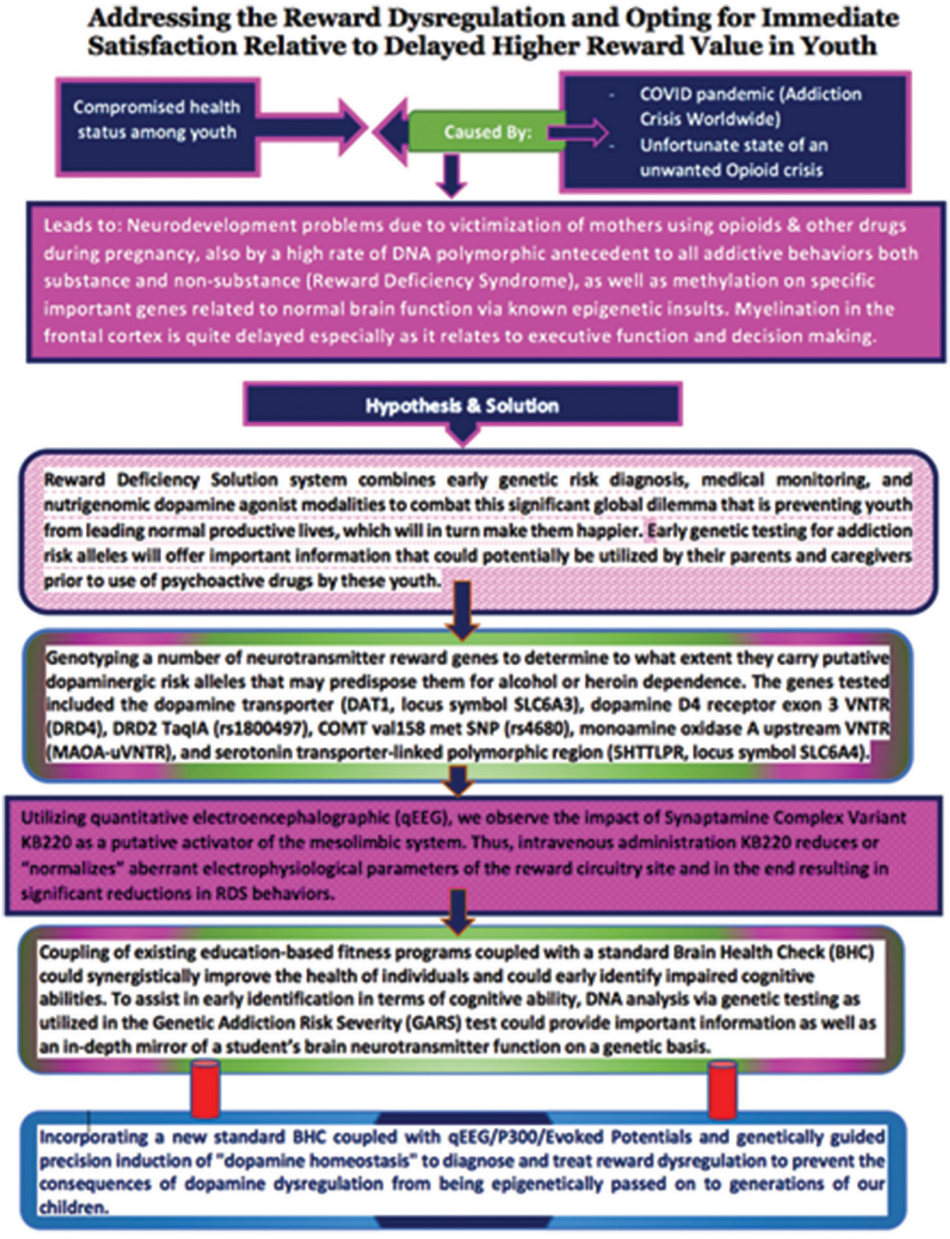

Figure 2. A conceptual schematic that summarizes reward dysregulation in youth and how the reward deficiency solution and brain health check can be used to diagnose and treat reward dysregulation.

categories and classifications, such as the Research Domain Criteria project, WHO International Statistical Classification of Diseases-11, or DSM-5, can help overcome global mental health challenges, our concern is that anomalous brain activity is not being adequately considered within the context of "systems biology," neglecting educational, economic, and behavioral consequences that require appropriate and effective interventions. The best approach to achieving positive clinical outcomes is to initiate novel strategic alternative modalities targeting the etiology rather than just the symptoms.[107,108]

9. Positive thinking in adolescence

Positive emotions and cognition have been widely recognized for their beneficial effects on overall mental health and well-being, particularly when people focus on positive thought processes. In positive psychology, the goal is typically to engender character traits such as optimism and hope, which reduce anxiety and depression while fostering strong social interactions.[109] There are many interventions aimed at developing and adjusting emotional and social skills in school, such as social and emotional learning programs[110] or positive youth development interventions.[111] However, research concerning positive thinking in adolescents has been relatively limited.[112] Data indicate that negative emotions, such as anxiety or depression, are associated with the dysregulation of the amygdala – PFC circuitry.[113] Positive emotional words are associated with increased activation in the ventral medial PFC.[114] Other studies have uncovered important connections between positive emotions and brain processes relevant to prosocial behaviors. For example, a study of the positive emotion of professional pride revealed a relationship to empathy, reward, and emotion regulation, as well as the theory-of-mind network.[115]

From a neurotransmitter perspective, positive emotions are associated with increases in dopamine function within the reward network.[116-119] Altered activity in serotonin modulates negative emotional responses.[120] Oxytocin, which supports affiliative behaviors, may also play a role in responses to positive versus negative emotional processes.[121] Thus, fostering positive emotions and implementing interventions that support them lead to substantial changes in the brain, involving various areas associated with reward, positive self-image, prosocial behaviors, and empathy. Working toward instilling positive emotions in adolescents is likely to yield short- and long-term benefits regarding their overall mental health and well-being.

10. Conclusion

Importantly, initial engagement in rehabilitation and detoxification bears similarities to experiencing a first stroke or heart attack in that the brain has already been impacted by pathological events that led to the manifestations of SUD and the need for treatment. The tools to prevent the progression of SUD are available and must be implemented urgently because deaths attributed to SUD have continued to increase unabated. Therefore, a reexamination of approaches to brain health and addiction and novel perspectives needs to be implemented by the medical community.

The clinical evidence accumulated during the past three decades underscores the necessity for establishing a BHC focused on precision neuropsychiatric testing, including episodic memory and processing speed (MemTrax),[122-124] general memory (CNSVS),[125-130] attention (T.O.V.A),[131-137] neuropsychiatric (MCMI-III),[138-140] and neurological imaging (qEEG/P300/EP),[131,137,141-154] for patients at risk of or presenting with problematic drug misuse. Since addiction is related to learning mechanisms, refocusing on learning and memory may change the perspective on the beneficial use of these brain mechanisms. For example, the online program MemTrax (www.memtrax.com) can help individuals monitor their memory as frequently as needed and observe how it is being impacted by substance abuse. Such feedback can lead to behavioral improvements and serve as a valuable tool for those providing therapeutic interventions, such as BHCo.

One of the basic neurochemical mechanisms in the brain, the midbrain dopamine system, participates in pacing critical cognition functions, including reward and the facilitation of addictive behaviors.[155-158] A study conducted by Rouhani and Niv,[155] through fitting reinforcement learning models to behavior, demonstrated that both signed (cue predicting the reward) and unsigned (unexpected, surprising reward) prediction errors (RPEs) contribute to learning by modulating the learning rate. They further characterized the effects of these RPE signals on memory, demonstrating that both signed and unsigned RPEs augment memory, aligning with midbrain dopamine and locus-coeruleus modulation of hippocampal plasticity. Further research by this group supports the complex nature of reward and learning involving dopaminergic mechanisms.[156] Finally, Katzman and Hartley's work indicates that both children and adults tend to remember past events more when the value of choice is beneficial compared to non-beneficial.[157] These proposed BHC/BHCo can be used as a standardized approach for school-aged children, akin to fitness programs. Our suggestion introduces a set of objective brain assessments parallel to those used in cardiology for diagnosing and following the clinical course of cardiac diseases. The coaching approach, including close evaluation and management guidance (including GARS testing and subsequent KB220z variant matching), could easily be adapted for implementation throughout the US and global educational systems. We understand that this initiative would require a substantial and bold approach to the care of

the general US population. This commission believes that the BHC/BHCo would synergize with current fitness programs, particularly in addressing co-occurring RDS behaviors. Thanos *et al.*[158] underscore the role of exercise in preventing the initiation of cocaine use in adolescence, suggesting that the implementation of exercise programs might be an important preventive measure and significantly improve students' mental health. Based on the reviewed research, this appeal promises to stop/prevent the increased prevalence of SUD through early detection utilizing robust brain screening[159-209] as recently proposed by the Society of Brain Mapping and Therapeutics,[84] as well as psychological and pharmacological treatment approaches espoused herein.[210-257]

Notably, each year, over a million adolescents globally succumb to preventable or treatable causes. Psychosocial factors are the strongest factors associated with drug abuse, bullying, attempted suicide, and sleep deprivation resulting from bullying.[258-316] The Carter Center has estimated that the addiction crisis, if continues to worsen at the same rate, may cost the US approximately 16 trillion dollars by 2030. Furthermore, the neurodevelopment of children could be compromised by maternal usage of opioids and other drugs during pregnancy. A high rate of DNA polymorphic antecedents compounds the epigenetic insults involving the methylation of specific essential genes related to normal brain function. Myelination in the frontal cortex, a process known to extend until the late 20s, delays proficient executive function and decision-making abilities. Understanding this delay in brain development, along with the presence of potential high-risk antecedent polymorphic variants or alleles and generational epigenetics, provides a clear rationale to mimic fitness programs with an adaptable BHC. Implementing the BHC within the educational systems in the US and other countries might be a good starting point for proactive therapies aimed at reducing juvenile mental health problems and, eventually, criminal activities, addiction, and other behaviors associated with RDS.

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Conflict of interest

Kenneth Blum is the holder of both USA and foreign patents related to kb220 and gars. Other authors declare no conflicts of interest.

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ACCSCIENCE PUBLISHING

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ORIGINAL RESEARCH ARTICLE

Sujok as an alternative therapy to reduce dyspnea in patients with respiratory problems

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Abstract

The management of dyspnea has received little attention as compared to other potentially severe symptoms of the disease, such as acute and chronic pain. The previous case reports indicated that *Sujok* therapy can alleviate dyspnea in a short time. This study aimed to determine whether *Sujok* therapy could reduce dyspnea symptoms in patients with oxygen saturation of less than 96%. *Sujok* originated from the Korean language, which consisted of the words *Su* and *Jok*, denoting hand and foot, respectively. *Sujok* therapy involves manipulating the hands or feet through massaging, coloring, or attaching seeds, magnets, or needles. This quasi-experimental study involved 34 males and 26 females with oxygen saturation of < 96% and experienced dyspnea with a grade of more than 2 on the Likert scale (1 – 5). Respondents were divided into an intervention group (IG) ($n = 30$) and a control group (CG) ($n = 30$), where IG was given the *Sujok* therapy. Measurements were taken for both groups at 0, 5, 15, and 30 min. The study reported mean ages of 55.6 ± 13.49 and 60.63 ± 9.26 in CG and IG, respectively. The increase in oxygen saturation was statistically significant in the overall measurement time in IG ($P < 0.01$). After 30 min, the average grade of dyspnea was 3 (moderate) for CG and 2 (mild) for IG. In CG, dyspnea decreased significantly at 30 min by 0.185 ($P = 0.001$; $P < 0.05$), whereas in IG, dyspnea decreased significantly at 5 min by 0.649 ($P < 0.01$). In conclusion, *Sujok* therapy can increase oxygen saturation and reduce the dyspnea grade in patients with respiratory problems.

Keywords: *Sujok* therapy; Dyspnea; Respiratory problem

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1. Introduction

Dyspnea is a complex symptomatic condition and is influenced by several factors, such as environmental, physiological, and psychological factors,[1] inclusive of previous experiences, emotions, beliefs, values,[2] and diseases.[3] According to the American Thoracic Society, dyspnea is defined as a subjective experience associated with discomfort in breathing that causes qualitatively different sensations with varying intensities.[1] There are different dyspneic sensations, such as tachypnea, rapid breathing, increased work of breathing, chest tightness, and air hunger.[2]

Dyspnea is commonly caused by heart and lung diseases, such as pneumonia, chronic obstructive pulmonary disease (COPD), asthma, heart failure, and coronary heart disease,[3] which account for up to 85% of dyspnea cases.[4] Other common causes

of dyspnea are obesity,[5] exercise-induced dyspnea,[6] pregnancy,[7] and psychological pressure such as anxiety.[3,8] Likewise, dyspnea is considered to be the most severe symptom experienced by patients with lung cancer.[9]

While it may be difficult to gauge the prevalence of dyspnea, several studies have reported that up to 8.4% of visits to the emergency department and 2.5% of all doctor visits require treatment for dyspnea.[10] In addition, it was reported that about 30% of patients older than 65-years-old had difficulty breathing when walking.[11] Likewise, the prevalence of dyspnea is as high as 32% for those over 70 years old.[12]

Healthcare workers are responsible for relieving the symptoms of dyspnea through comprehensive management of the disease,[13] which involves identifying the cause of dyspnea, treating it appropriately, and optimizing the recovery and improvement of dyspnea symptoms.[3]

Recent research has focused on alternative therapies to treat dyspnea (e.g., fan therapy, traditional Chinese medicine [TCM], music therapy, hypnosis, yoga, tai-chi, and Qigong), but most of these therapies require a long duration, and the corresponding studies lack the utilization of objective measurements (e.g., oxygen saturation measurement) to determine the effect of therapy on dyspnea.

Nonetheless, the management of dyspnea is often overlooked as compared to other potentially severe symptoms of respiratory diseases, such as acute and chronic pain, which could be attributed to the patients' low likelihood of reporting the dyspnea symptoms, as well as the complex mechanism of terminal dyspnea.[2] In addition, studies often exclude patients with dyspnea,[2] and dyspnea is not considered an outcome in various interventions.[14] It was also reported in a study that the palliative care team in an intensive care unit rarely consulted patients with respiratory problems.[15] A recent case study reported that *Sujok* therapy could remarkably reduce dyspnea in patients with COVID-19 within 27 min, and it warranted further studies with a larger sample size and a more rigorous research method to validate its findings. Herein, the present study aimed to evaluate the efficacy of *Sujok* therapy to reduce dyspnea symptoms in patients with an oxygen saturation of <96%.

2. Methods

2.1. Study design

This quasi-experimental study was conducted with a control group (CG) in a hospital in Indonesia. The primary and secondary objectives of this study were to evaluate the effects of *Sujok* therapy on the severity of dyspnea in patients and on oxygen saturation levels, respectively. This study received an ethical approval number (KE/FK/0118/EC/2021) from the Medical and Health Research Ethics Committee of the Faculty of Medicine, Public Health, and Nursing, Universitas Gadjah Mada-Dr. Sardjito General Hospital, Yogyakarta, Indonesia. This study was registered for a clinical study (ID: 202103010) in the research repository at Universitas Gadjah Mada on March 24, 2021. A research assistant was assigned to collect patient data after obtaining patient consent from all patients before the study.

2.2. Participants

The participants of this study were selected from two different wards: one ward for the CG and the other for the intervention group (IG). The inclusion criteria were oxygen saturation < 96%, at least one breathing-related symptom (e.g., dyspnea, cough, excess sputum production, and chest tightness), and a grade > 2 on the Likert scale (i.e., 1 – 5; 1 = No symptoms; 2 = Mild symptoms; 3 = Moderate symptoms; 4 = Severe symptoms; and 5 = Very severe symptoms). The exclusion criteria were those who could not respond to the researcher. Patients from these two wards were approached for recruitment based on the inclusion and exclusion criteria, and the recruitment process continued until the number of respondents in each group reached 30. The recruitment process was performed by members of the research team and a lung specialist, and the patients were prospectively considered if they had been in these wards for treatment. No patients were excluded from the recruitment process.

2.3. Study procedures

A Likert scale was used to measure the severity of dyspnea as one of the symptoms of respiratory problems. This instrument was tested with five respondents to determine whether they understood the statement. Subsequently, 52 respondents were involved in the validity and reliability tests in August 2021 (i.e., $r = 0.268$ and Cronbach's alpha = 0.65). The Likert scale was then used to collect data from September 2021 to February 2022 at a hospital in Central Java, Indonesia.

During the data collection process, oxygen saturation and symptoms were measured at 0, 5, 15, and 30 min in the CG. In the IG, after oxygen saturation and symptoms were measured at 0 min, the respondents were given *Sujok* therapy, which took approximately 3 min. Subsequently, oxygen saturation and symptoms of respiratory problems were measured at 5, 15, and 30 min post-therapy. The *Sujok* therapy was performed as follows:[16]

(i) Massage the areas marked in red and green according to the direction of the arrows (i.e., from inside to the top, from bottom to top, and from outside to the top) (Figure 1) 30 times with a roller-shaped probe (Figure 2);
(ii) Color areas of the hands according to Figure 1.
(iii) Attach fenugreek seeds to the colored areas with tape.

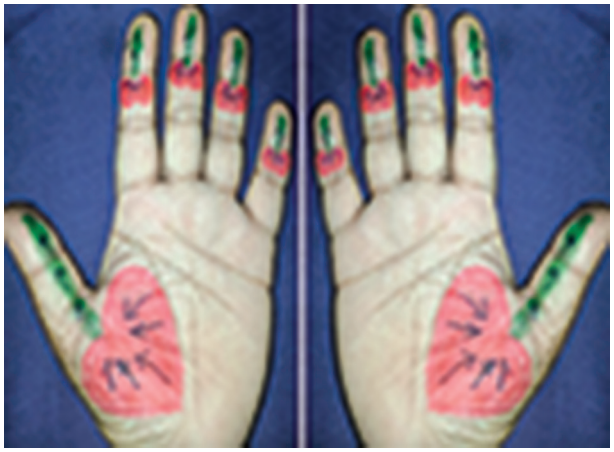

Figure 1. The area of the hand where the *Sujok* therapy is performed. Permission was obtained for reusing the figure.

Figure 2. Probe for massage.

An oximeter was then placed on the finger of each patient at 0, 5, 15, and 30 min. Positioning the oximeter in the same area is important in this study to avoid measurement bias. Likewise, the accuracy of the oximeter could decrease due to the presence of skin pigmentation, high light intensity, excessive patient movement, decreased perfusion, the presence of hemoglobin or carboxyhemoglobin, intravascular dyes, a decrease in saturation below 83%,[17] and the presence of nail polish.[18]

All respondents, both in the control and IGs, received standard treatment as defined in the hospital's care management protocol.

2.4. Statistical analysis

The collected data were verified and subsequently analyzed using paired *t*-test.

3. Results

3.1. Characteristics of respondents

The number of respondents in the control and IGs was 30 people each. The mean age of respondents in the intervention and CGs was 60.63 ± 9.26 and 55.6 ± 13.49 years, respectively. There were no differences in age, gender, and job status between the intervention and CGs (Table 1). Further characteristics related to the medical diagnosis of each respondent are displayed in Table 2.

Table 1. The respondents' age, gender, and job status (n=30)

Variable	Frequency		Intervention group		Control group		Sig (2-tailed)
	n	%	n	%	n	%	
Age							0.336
21 – 30	1	1.7	-	-	1	3.3	
31 – 40	3	5.0	1	3.3	2	6.7	
41 – 50	9	15.0	4	13.3	5	16.7	
51 – 60	19	31.6	9	30.0	10	33.3	
61 – 70	23	38.3	14	46.7	9	30.0	
71 – 80	4	6.7	2	6.7	2	6.7	
81 – 90	1	1.7	-	-	1	3.3	
Gender							0.605
Female	26	43.3	12	40.0	14	46.7	
Male	34	56.7	18	60.0	16	53.3	
Job-status							0.676
Housewife	22	36.6	11	36.7	11	36.7	
Entrepreneur	19	31.6	11	36.7	8	26.7	
Labor	10	16.7	4	13.3	6	20.0	
Public servant	1	1.7	1	3.3	-	-	
Others	4	6.7	1	3.3	3	10.0	
Unemployed	4	6.7	2	6.7	2	6.7	

3.2. The result of *Sujok* therapy

The results indicated that the average oxygen saturation in the IG increased at 5, 15, and 30 min until the normal range of oxygen saturation (>95%) (Figure 3). Meanwhile, in the CG, the average oxygen at 30 min was still not in the normal range (< 96%) (Figure 3).

Statistical analysis revealed that there was a significant increase in oxygen saturation in the IG for each measurement ($P < 0.05$) (Table 3). Meanwhile, a significant increase in the CG only occurred at the 15-min measurement ($P < 0.05$).

Measurements with the dyspnea scale (Figure 4) revealed a decreasing grade in the IG for each consecutive measurement. In contrast, the CG displayed an approximate grade of 3 (mild) for measurements at 5, 15, and 30 min.

Statistical analysis of dyspnea for both the control and IGs is displayed in Table 4. The grade reduction was statistically significant at 5 min for the IG ($P < 0.05$) and at 30 min for the CG ($P < 0.001$).

Table 2. Medical diagnosis of the respondents (n=30)

Control group	Intervention group
Lung cancer and pleural effusion (sinistra)	Pulmonary tuberculosis and dyspnea
ST-elevation myocardial infarction (STEMI) post-Streptase, pulmonary edema-mixed pneumonia, and diabetes mellitus type II	Congestive heart failure, diabetes mellitus, and pneumonia
Bacterial pneumonia, septic shock, disseminated intravascular coagulation (DIC), and thrombocytopenia	Fever, suspected leptospirosis, hypokalemia, and hyponatremia
Chronic kidney disease, pleural effusion, and pulmonary edema	Pneumonia and pleural effusion
Pneumonia and diabetes mellitus	Asthma, atrial fibrillation, rapid ventricular response, and a history of breast cancer
Lung cancer and pleural effusion	Chronic kidney disease, fever, and vomitus
Acute decompensated heart failure, chronic kidney disease, and pneumonia	Intestinal tuberculosis and hepatitis
Bradycardia and cephalgia	Chronic kidney disease and pleural effusion
Respiratory failure (Type 1) and hypertension	Neurogenic shock, spinal cord injury, atrial fibrillation, normal ventricular response, and congestive heart failure
Pulmonary tuberculosis	Suspected pulmonary tuberculosis and Differential diagnosis pneumonia
Mediastinal tumor	Dyspnea and chronic kidney disease
Lung cancer	Ascites and hepatocellular carcinoma
Anasarca edema and congestive heart failure	Post-laparotomy exploration Et causa gastric perforation
Respiratory failure (Type 1) and hypertension	Hemorrhagic stroke, diabetes mellitus, and mild head injury
Lung cancer	Congestive heart failure, dyspnea, and diabetes mellitus
Acute asthma	Vomitus and hyperglycemia
Hematochezia and asthma	Open fracture femur sinistra and fracture on clavicula
Pleural effusion, ileus obstruction, electrolyte imbalance, and renal insufficiency	Asthma
Post-laparotomy, liver repairment, and anemia	STEMI, history of supraventricular tachycardia, and diabetes mellitus
Suspected leptospirosis and chronic kidney disease	Hemiparese dextra and cephalgia
Atrioventricular (AV) block and electrolyte imbalance	Post-proximal femoral nail anti-rotation
Decubitus ulcer, hypertension, and cerebrovascular accident	Hemorrhagic stroke and post-craniotomy
Supraventricular tachycardia and suspected abscess cerebra	Abdominal pain and melena
Observation dyspnea, pneumonia, and COVID-19	Abscess on the mandibula and COVID-19
COVID-19, pneumonia, non-ST-segmental elevation myocardial infarction (NSTEMI), and diabetes mellitus	COVID-19, pneumonia, and a history of depression
Discharged COVID-19, acute kidney injury, and a history of cerebrovascular accident	COVID-19 and suspected mass in the right lung
COVID-19, sepsis, anorexia, and diabetes mellitus	COVID-19, diabetes mellitus, ischemic heart disease, and dyspnea
Diabetes mellitus, hypoglycemia, vertigo, and COVID-19	COVID-19, chronic kidney disease, and pleural effusion
COVID-19, rectal cancer, pneumonia, and diabetes mellitus	COVID-19, pulmonary tuberculosis, and pneumonia
COVID-19 and Parkinson's	COVID-19, chronic kidney disease, and cellulitis

Abbreviations: AV: Atrioventricular; DIC: Disseminated intravascular coagulation; NSTEMI: Non-ST-segmental elevation myocardial infarction; STEMI: ST-elevation myocardial infarction.

4. Discussion

Our results indicated that there was no difference between the two groups in terms of age, gender, and job status. Since this study did not focus on a specific diagnosis as an inclusion criterion but rather on whether the patient had respiratory problems characterized by oxygen saturation below normal standards (<96%), the patient's medical diagnoses varied greatly, where the respondents had more than one medical diagnosis. The multiple diagnoses for each respondent validated that dyspnea is a multidimensional symptom due to various mechanisms that occur.[2]

This research observed that most respondents (approximately 84%) had heart- and/or lung-related problems, which was consistent with the results of a systematic review involving 10 longitudinal studies that stated the involvement of dyspnea in heart and lung problems.[19] Another study stated that asthma, COPD, heart failure, pneumonia, and coronary arteries were among the 85% most common causes of dyspnea.[4] Severe dyspnea also occurs in patients with lung cancer.[9]

Our results displayed a statistically significant decrease in the dyspnea scale for both the CG and IGs. However, the statistically significant decrease occurred more rapidly in the IG (5 min) than in the CG (30 min). Although both groups exhibited a significant decrease, the average severity of dyspnea symptoms in the IG was lower at grade 2 (mild), while the CG was at grade 3 (moderate). Notwithstanding, the Likert scale measurement was considered subjective because variations in sensation and intensity were noted in the patient reports.[14]

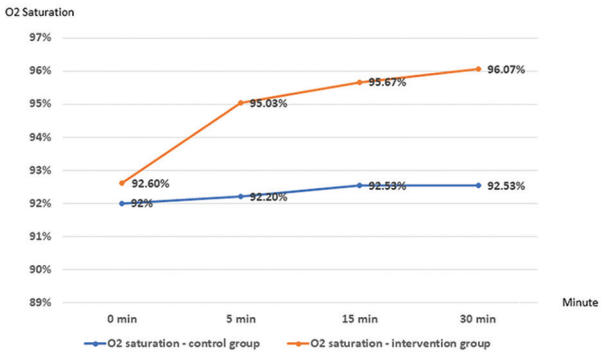

Figure 3. Graph of the average oxygen saturation in the control and intervention groups.

Therefore, dyspnea was subsequently measured objectively with an oximeter. The oximeter has a sensitivity of 92% and a specificity of 90% when detecting hypoxia with a 92% oxygen saturation limit.[20] Notably, dyspnea is also one of the symptoms that indicate the presence of hypoxemia. A recent study reported that dyspnea is not implicated in all hypoxemia, and this type of hypoxemia is called "silent hypoxemia".[21] Oxygen saturation is often considered a fifth vital sign measurement.[17] Oxygen saturation is considered abnormal if it is <95%,[22] while other references indicated that normal oxygen saturation ranges around 96 – 100% at sea level.[23]

Meanwhile, another reference stated that hypoxemia is defined by oxygen saturation of less than 90%.[23] For more severe conditions, cyanosis, a form of hypoxemia, may occur when the oxygen saturation decreases to 67%, but cyanosis is not always visible during physical examination. In this regard, the use of oximetry may be beneficial.[17]

Table 3. Comparison of oxygen saturation between control and intervention groups

Group	Paired differences					*t*	*df*	Sig (2-tailed)
	Mean	Standard deviation	Standard error mean	95% Confidence interval of the difference				
				Lower	Upper			
Control								
Pair 1								
O_2 (%) 0 – 5 min	0.200	1.375	0.251	−0.313	0.713	0.797	29	0.432
Pair 2								
O_2 (%) 0 – 15 min	0.533	1.332	0.243	0.036	1.031	2.193	29	0.036*
Pair 3								
O_2 (%) 0 – 30 min	0.533	1.525	0.278	−0.036	1.103	1.915	29	0.065
Intervention								
Pair 1								
O_2 (%) 0 – 5 min	2.433	1.478	0.270	1.881	2.985	9.016	29	0.000*
Pair 2								
O_2 (%) 0 – 15 min	3.067	1.596	0.291	2.471	3.663	10.525	29	0.000*
Pair 3								
O_2 (%) 0 – 30 min	3.467	1.833	0.335	2.782	4.151	10.357	29	0.000*

Note: *Denotes significance (P<0.05).

In the present study, the oxygen saturation of the IG increased and reached the normal range after 30 min. In contrast, the oxygen saturation remained below the normal range after 30 min for the CG. The results demonstrated that *Sujok* rapidly increased oxygen saturation, as evidenced 5 min after the first measurement or 2 min after completion of therapy in the IG. Therefore, despite the subjective assessment of dyspnea by the patient, the increase in oxygen saturation as measured by the oximeter was an objective measurement and provided strong evidence that *Sujok* therapy can increase oxygen saturation in a short time.

However, there was also the possibility of errors when reading the oximeter. For example, the presence of pigmentation or nail polish where the oximeter is installed could lower the oximeter reading, which corresponds to a decreased oxygen saturation.[24-26] To prevent this, we placed the oximeter on the same finger and used the same instrument as earlier literature stated that the estimated value of oxygen saturation is based on the type of instrument used to measure.[21]

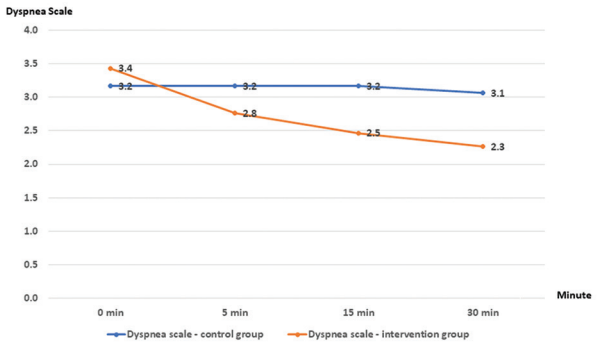

Figure 4. Dyspnea scale in the control and intervention groups.

In addition, there were no patients with oxygen saturation below 83%, which validated the accuracy of the oximeter because inaccurate oximeter readings may typically indicate oxygen saturation below 83%.[17] Other references have also reported that pulse oximeter readings of 70% may not be accurate as compared to the gold standard of using blood gas measurements.[23]

Other studies have investigated the use of fan therapy to reduce dyspnea symptoms.[27,28] Fan therapy is applied directly at the feet and face, but it was reported to only lower the dyspnea score to baseline with no reduction in dyspnea after 60 min of therapy. Another similar study involved a larger sample of 20 patients who underwent fan therapy to the face or feet for 5 min. The results revealed that the reduction in dyspnea was significantly higher in fan-to-face than in fan-to-leg therapy. Although both treatment methods reported improvements on the dyspnea scale, there was no significant difference between the SpO_2 (peripheral oxygen saturation levels) before and after treatment.[28] Notably, SpO_2 measurements may have higher values in the presence of bias in the data collected from the respondents.

Another recent study stated that the duration of fan therapy is generally 5 min. Although as many as six studies (60%) reported improvements in dyspnea symptoms with fan therapy, results from fan therapy were considered subjective data from patients and not objective measurements (e.g., SpO_2 measurements through blood gas analysis or using oximetry).[29]

Table 4. Comparison of the scale of dyspnea between the control and intervention groups

Group	Paired differences					*t*	*df*	Sig (2-tailed)
	Mean	Standard deviation	Standard error mean	95% confidence interval of the difference				
				Lower	Upper			
Control								
Pair 3								
Dyspnea at 0 – 30 min	−0.18463	0.28774	0.05253	−0.29208	−0.07719	−3.515	29	0.001*
Intervention								
Pair 1								
Dyspnea at 0 – 5 min	−0.64860	0.70693	0.12907	−0.91257	−0.38463	−5.025	29	0.000*
Pair 2								
Dyspnea at 0 – 15 min	0.17950	0.86919	0.15869	−0.14506	0.50406	1.131	29	0.267
Pair 3								
Dyspnea at 0 – 30 min	−0.00030	0.96380	0.17597	−0.36019	0.35959	−0.002	29	0.999

Note: *Denotes significance ($P<0.05$).

Other researchers have also studied the use of TCM in dyspnea, such as herbs, acupuncture, and exercise. One respondent with a diagnosis of asthma and COPD received TCM therapy for 4 weeks, which yielded satisfactory results, with the patient no longer experiencing symptoms of dyspnea.[30] Unfortunately, the case study did not explain the changes in the dyspnea scale with regard to the intervention period, and the study also did not use oxygen saturation parameters. Therefore, in terms of the dyspnea and oxygen saturation scales, the results could not be compared to this study. In addition, TCM therapy requires more time, effort, and complex materials than *Sujok* therapy, which was performed for approximately 3 min in this study.

There was another study that investigated the use of music to reduce dyspnea. Results of this study also reported that music could statistically reduce dyspnea in the IG, but the hemodynamic measurements of the respondents were not significant.[31] The result was the same as the fan therapy study, where the subjective results did not correlate with the objective measurements. Similar studies, without objective measurements, reportedly improved dyspneic symptoms (based on subjective data) with glutathione (i.e., oral and intravenous)[32] and yoga.[33]

Notably, an intervention study utilizing hypnosis therapy has reportedly succeeded in reducing dyspnea, as evidenced by objective (e.g., respiration rate [RR] and SpO_2/arterial oxygen saturation) and subjective (e.g., Borg Score – Visual Analog Scale) measurements, as well as anxiety evaluation (i.e., with STAI-6 questionnaires).[34] However, hypnosis therapy took a longer time (15 min) than *Sujok* therapy (~3 min).

Several other alternative therapies have been studied, especially regarding pulmonary rehabilitation in patients with COPD, using mind-body therapy (e.g., yoga, tai-chi, and Qigong)[35] and acupuncture, which yielded promising results related to the sensation of shortness of breath.[36]

In this study, *Sujok* therapy was able to improve oxygen saturation levels (objective data) and dyspnea (subjective data). *Sujok* is a multimodal therapy on the hands and feet, including meditation (Triorigin Smile Meditation [TSM]), massages, applying color, attaching seeds, leaves, or other parts of plants, using needles (e.g., acupuncture), twisting, and magnets. Even when a combination is used, it should not take a long time, as evidenced by the intervention in this study that took only about 3 min to perform.[37]

One study suggested that the use of a combination of methods for a particular target could have a better effect than using only one method[38-41] and could even improve emotional problems by including twists and putting seeds.[41] The method used in this study consisted of massages, coloring, and seeds. This method was used in dyspnea patients who were suffering from COVID-19, and this method is called therapy for lung correspondence (TKP) and therapy for airway correspondence (TSN).[42] In this case study, many symptoms improved except dyspnea, and many other targets were treated with different *Sujok* protocols. TKP and TSN methods have reportedly improved dyspnea in patients from grade 10 (the most severe) to 0 (no dyspnea) within 27 min. Unfortunately, this study only involved one respondent, and no measurement of oxygen saturation (objective data) was used to complement the subjective data. Another study reported that TKP and TSN could reduce symptoms in the respiratory tract of COVID-19 patients.[43] A total of four male patients with COVID-19 with oxygen saturation of <96% and experienced dyspnea displayed restoration of oxygen saturation to normal and improvement of dyspnea symptoms after therapy within the first 5 min after the first measurement. The findings from these two studies (i.e., Nurjannah[16] and Nurjannah *et al.*[42]) were consistent with the results of the present study. These reports further validated dyspnea symptom improvement by *Sujok* therapy, with both objective and subjective data.

The first step of *Sujok* therapy involves massages. The stimulation of an affected area will not only relieve the pain but also address the causes of the pain and stabilize the flow of energy in the affected area.[37] Furthermore, identifying a particular area for therapy is considered an important step of therapy.[37] For an effective treatment, the area of therapy requires proper stimulation,[36] and the massage can be either anticlockwise or clockwise and should continue until the pain subsides.[37] In this study, the researchers modified the shape and direction of the massage to the outside (from the lungs to the nose). This is essential because dyspnea patients may have excess sputum production, and inward massages (to draw air into the lungs) may accumulate phlegm (and pathogens) in the lower lungs. Likewise, outward massages can expel phlegm (and pathogens) from the respiratory tract.

The second part of *Sujok* therapy is the use of color. Color is a manifestation of cosmic forces that are responsible for life and its healing properties.[43] For example, the color red has a strengthening or tonification effect.[44-46] Other references also mentioned that red is associated with blood and blood flow and has the property of warmth. If a person is imbued with the color red, the pituitary gland becomes active and increases adrenaline in the blood, thereby stimulating the sensory nerves and increasing the sense of smell, sight, hearing, taste, and touch.[43]

The color red represents heat energy.[44] This heat energy is placed in the lung area, which corresponds to the area under the thumb (standard system of *Sujok*) and under the upper joints of each finger on the hands (insect system of *Sujok*). The color red can also stimulate the lungs into hyperactive conditions. The color red in Six Ki is the color of the heart, but the axis is the brown color, representing the lungs.[44] Although the color red increases the warmth of the chest area and strengthens the heart organ, it becomes a debilitating sedative color for the lungs. In summation, the color red provides warmth, strengthens the heart, and stimulates the lungs.

The color green represents the respiratory tract in both the basic (thumb) and insect (fingers) systems of *Sujok*. Likewise, the color green in Six Ki represents the color of wind energy,[44] the energy of movement. Imbuing the color green will stimulate movement and, expectedly, reduce the severity of the breathing process. In addition, green is at the axis of the color yellow, which indicates the presence of humidity energy.[44] Conditions with excess humidity energy encompass the presence of excess sputum or phlegm, especially in respiratory problems. Therefore, the color green not only reduces humidity and prevents phlegm but also increases movement of the respiratory tract for better functionality.

The last step of *Sujok* therapy is the use of seeds. A previous study used green beans in place of seeds,[16] whereas the present study used fenugreek seeds. Green beans require more time to prepare because the buds from the green beans must stick to the skin of patients during seed attachment. In contrast, fenugreek seeds do not have buds, making it easier to attach to the patient's hand. Seeds contain latent energy power that is ready to be used,[47] and seeds can transfer biological waves of energy to the target area of therapy and also absorb negative energy.[47]

The seeds are applied after the massage and can be left for 24 h for better effects. Ideally, the seeds are massaged again periodically every 3 – 4 h by pressing with a circular motion. The common signs indicating the natural energy effect of the seeds are itching, tingling, reduced pain, and a feeling of warmth in the target areas. After 24 h, the seeds can be replaced with new ones, and the procedure is repeated. This method is effective for long-term chronic diseases that are followed by persistent pain.[37]

Seeds can be placed at any point of pain or cover all areas of the affected organ. The seed used here must be undamaged, fresh, and capable of germinating.[37] Another reference mentioned that seeds can be used to get rid of energy blockages because seeds have a large amount of energy, similar to the natural growth of seeds into big trees with little water and sunlight. It is also recommended that patients use self-seed therapy at home for at least 2 weeks.[48]

The limitation of this study was that not all respondents who experienced dyspnea had the same medical diagnosis.

5. Conclusion

Sujok therapy can restore oxygen saturation to normal levels and reduce the severity of dyspnea symptoms.

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Conflict of interest

The authors declare no competing interests in this study.

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All authors equally contributed to this work.

Ethics approval and consent to participate

This study received an ethical approval number (KE/FK/0118/EC/2021) from the Medical and Health Research Ethics Committee of the Faculty of Medicine, Public Health, and Nursing, Universitas Gadjah Mada-Dr. Sardjito General Hospital, Yogyakarta, Indonesia. Written consent was obtained from each of the subjects to participate in the study before data collection.

Consent for publication

Participates gave consent to publish their data.

Availability of data

Data used in this work are available from the corresponding author on reasonable request.

Further disclosure

Part of the findings has been presented in an oral presentation at the International Online Conference

"Ethical Lessons Learned During Epidemic Outbreaks" in 2021.

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ACCSCIENCE PUBLISHING

INNOSC Theranostics and Pharmacological Sciences

ORIGINAL RESEARCH ARTICLE

A pilot study assessing the feasibility and effectiveness of amniotic bladder therapy in patients with neurogenic detrusor overactivity

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Abstract

Neurogenic detrusor overactivity (NDO) is characterized by involuntary detrusor contractions that often occur following spinal cord injury (SCI). In addition, patients with SCI above T6 are at risk for autonomic dysreflexia (AD). Amniotic membranes (AM) are used for the management of wound healing in multiple medical disciplines. Thus, this study aims to evaluate the efficiency of amniotic bladder therapy (ABT) in managing NDO, specifically in patients with SCI. The patients received intra-detrusor injections under general anesthesia of 100 mg micronized AM (Clarix Flo) diluted in 10 mL 0.9% preservative-free sodium chloride. Clinical evaluations, including maximum detrusor pressure, maximum cystometric capacity, and frequency of AD, were conducted, alongside the completion of questionnaires (Qualiveen questionnaire) preoperatively and postoperatively at weeks 2, 4, 8, and 12. Eight consecutive patients with an average age of 39.6 ± 13.6 years were included. After ABT, a significant decrease in the severity of urinary tract symptoms was observed based on the Qualiveen questionnaire: 3.9 ± 0.17 at baseline to 2.9 ± 0.21 at week 2, 2.1 ± 0.53 at week 4, and 1.4 ± 0.20 at week 8 ($P < 0.01$). Improved clinical symptoms were associated with a decreased maximum detrusor pressure, increased maximum cystometric capacity, and reduced frequency of AD. In conclusion, we investigated ABT as a potential treatment option for NDO associated with SCI. Further investigations are warranted to validate the effectiveness of ABT in this patient population and determine treatment durability.

Keywords: Neurogenic bladder; Neurogenic detrusor overactivity; Autonomic dysreflexia

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1. Introduction

Neurogenic detrusor overactivity (NDO) after spinal cord injury (SCI) is a common complication, generally characterized by urinary urgency and incontinence, significantly impacting normal daily activities and reducing the patient's quality of life (QoL). In patients with NDO, achieving a state of low storage bladder pressure along with an increase in maximum cystometric bladder capacity can improve symptoms of incontinence.

However, the loss of supraspinal neuronal connections to the bladder after SCI can induce a series of complex reactions, namely urothelial cell death, inflammation, urinary tract infection (UTI), urinary incontinence, bladder hypertrophy, and fibrosis, which impair the contractile properties of the bladder and make it difficult to maintain low bladder pressures while storing urine.[1] Subsequently, as the bladder pressure increases, the risk of more serious complications (e.g., upper urinary tract deterioration and autonomic dysreflexia [AD] incidence) increases. Hence, the treatment of NDO primarily aims to protect the upper tract from damage by decreasing bladder pressure, which can be achieved by impeding the processes of bladder inflammation, fibrosis, urothelial cell death, neural sensitization, detrusor dyssynergia, incontinence, UTI, and AD. NDO after SCI is often managed with intermittent catheterization as well as indwelling catheters (often the q4 to q6 hour regimen) and potentially reflex voiding.

The amniotic membrane (AM) is the inner part of the placenta that forms a sac around the fetus during pregnancy. This biological tissue is inherently known to provide therapeutic properties for the treatment of different pathologies. It has been used clinically over the last century for applications such as dermal wound covering and ocular surface reconstruction to promote apoptosis of pro-inflammatory cells, prevent differentiation of pro-fibrotic cells, and promote expedited wound healing.[2] Based on these properties, the use of AM in amniotic bladder therapy (ABT) may overcome the limitations in the current management of NDO. We have previously evaluated ABT in patients with interstitial cystitis/bladder pain syndrome, idiopathic detrusor overactivity (IDO), and radiation cystitis (RC), and we observed symptomatic improvement as early as 2-week post-injection without any complications.[3] This observed that symptomatic improvement was associated with an improvement in urodynamic assessments, including a significant increase in maximum cystometric capacity. In addition, we evaluated the use of ABT in the management of NDO, specifically in patients with SCI, based on the safety profile and potential benefits in recalcitrant patients with limited treatment options.[2,4] Herein, ABT was applied intraoperatively directly into the detrusor based on the anti-inflammatory and anti-fibrotic properties of AM and the inflammatory and fibrotic processes in the pathophysiology of NDO.

2. Methods

The patients enrolled in this study had terminal NDO, defined as those who experienced involuntary detrusor contractions (IDC) near or at the maximum cystometric capacity (MCC) in the setting of a clinically relevant neurologic disease and who had failed previous treatment modalities, including oral therapies with anticholinergics, beta-3 adrenergic agonists, and intravesical therapy with botulinum toxin injections.[5] All patients had traumatic SCI and were using intermittent self-catheterization to empty the bladder. The study was approved by the local institutional review board committee (IRB-22-12-5277). We excluded patients with evidence of upper urinary tract damage, pregnancy, prior radiotherapy, intravesical stones, acute UTI, and a history of bladder and/or pelvic cancers. We also excluded the patients who had used intravesical botulinum toxin (Botox) within 6 months before the commencement of the study to avoid interference with the results. The patients did not receive additional anticholinergics or beta-3 agonists after ABT.

Baseline evaluation included history, physical examination, serum chemistries, urinalyses, urine culture, urine cytology, cystoscopy, renal ultrasound, urodynamic examination (i.e., cystometry by filling the bladder at a rate of 20 mL/min and recording storage pressures), and symptom assessment as measured by the Qualiveen questionnaire. The urodynamic parameters were analyzed according to the standardization report of the International Continence Society. The assessments conducted included maximum detrusor pressure (MDP; cmH_2O) and volume (mL) for the first IDC, as well as MCC corresponding to the volume (mL) at which involuntary voiding occurred and/or filling was stopped. In the absence of involuntary voiding bladder and without spontaneous bladder contractions, filling was stopped at 500 mL. To calculate detrusor compliance (as measured by the change in volume over the change in detrusor pressure), detrusor pressure was measured when the bladder was empty and at MCC. Clinical evaluation and questionnaires were repeated at weeks 2, 4, 8, and 12. Urodynamics were repeated at weeks 4 and 12. Local or systemic side effects were evaluated during and after treatment. AD was evaluated at baseline and repeated at weeks 2, 4, 8, and 12 by asking the patients the following questions: "do you have episodes of AD (a condition where blood pressure rises very fast, usually because of a painful stimulus below the level of your lesion, resulting in symptoms such as headaches, sweating, goosebumps)? If so, how many?"

ABT was standardized among all patients. In brief, patients were given intra-detrusor injections of 100 mg micronized AM (Clarix Flo; BioTissue, USA) diluted in 10 mL 0.9% preservative-free sodium chloride. Injections were performed through a cystoscope using a 23-gauge Williams needle into the lateral and posterior bladder walls, sparing the dome (to avoid intraperitoneal injection) and the trigone (because of the possible risk of reflux) under general anesthesia. A standardized procedure was followed for all patients, with twenty 0.5 mL injections delivered into two rows of ten throughout the lateral and posterior bladder

walls. Clarix Flo is a sterile, micronized human AM product that is derived from the placenta and umbilical cord and is aseptically processed and manufactured from donated human birth tissues according to regulations established by the US Food and Drug Administration. The micronized AM is terminally sterilized through gamma irradiation and does not contain live cells but retains the natural extracellular matrix components innate to the AM tissue.[6]

Descriptive statistics for continuous variables are reported as the mean ± standard deviation (SD), and statistical analysis was performed using the R software version 4.1.3 (R Core Team, Austria). The differences between parameters before and after treatment were analyzed with paired *t*-tests. A *P*-value < 0.05 was considered statistically significant.

3. Results

Eight consecutive patients (female [n = 5] and male [n = 3]) with an average age of 39.6 ± 13.6 years (23 – 65 years) met the inclusion criteria and were included in the current study. On average, the patients experienced symptoms of the neurogenic bladder (NGB) for 9.4 (6.2 – 13.8) years and had tried multiple therapies, including anticholinergics (n = 7), beta-3 adrenergic agonists (n = 8), and Botox injection (n = 8). All patients initially responded to intravesical botulinum toxin A but had a decreased response after 3 – 4 years. All patients had SCI with lesions at or above the T-6 level (Table 1). In addition, before treatment with AM, none of the included patients were completely continent on clean intermittent catheterization (CIC), although all used self-catheterization to empty their bladders. After ABT, all patients were completely continent between CIC and continued to use self-catheterization to empty their bladders every 4 – 6 h. The severity of the injury, as evaluated by the American Spinal Injury Association impairment scale, was grade A in 100% of patients.

Before micronized AM treatment, all the patients reported a high symptomatic impact on their daily lives based on their responses to the Qualiveen questionnaire (Figure 1). After injection, all eight patients reported a significant decrease in the severity of their urinary tract symptoms as the average Qualiveen score decreased from 3.9 ± 0.17 at baseline to 2.9 ± 0.21 at post-ABT week 2 (P < 0.01), 2.1 ± 0.53 at post-ABT week 4 (P < 0.01), and 1.4 ± 0.20 at post-ABT week 8 (P < 0.01). A considerable decrease in symptomatic impact on their daily lives was noted in all four categories of the Qualiveen. Furthermore, their symptoms remained relatively stable when measured at post-ABT week 12 with a reported embarrassment score of 1.39 ± 0.08, a constraint score of 1.42 ± 0.09, a fear score of 1.45 ± 0.09, and a QoL score of 1.3 ± 0.19 (Figure 1, all P < 0.01 as compared to baseline).

In addition, all eight patients included in the current study reported a significant increase in their first volume IDC and MCC and a decrease in MDP for at least 3 months following treatment. The patient's pre-treatment MCC was 208.63 ± 34.98 mL, and MDP was 67.38 ± 2.88 cmH_2O. At 4 weeks following micronized AM treatment, MCC was increased to 430.25 ± 32.4 mL (P < 0.01), and MDP was decreased to 33 ± 1.2 cmH_2O (P < 0.01). At 12 weeks following treatment, MCC and MDP remained relatively the same at 427.25 ± 24.9 mL and 33.38 ± 1.85 cmH_2O, respectively. The post-treatment mean detrusor compliance increased from 27.5 ± 10.6 mL/cm water to 65 ± 15.2 mL/cm water at week 4 and subsequently to 63 ± 14.4 mL/cm water at week 12 (Table 2). Furthermore, at 12 weeks following injection, all patients were completely continent on CIC.

We also evaluated the frequency of AD in all eight patients at baseline and weeks following treatment with micronized AM. Before treatment, all eight patients experienced symptoms either daily or every other day. All patients reported improvements in their symptoms by week 2, and at week 12, all eight patients reported no symptoms of AD (Table 3).

Table 1. Patient demographics

Patient	Age (years)	Neurological level	Upper motor neuron bladder dysfunction
1	37	T6	Complete
2	29	T3	Complete
3	31	T6	Complete
4	35	C7	Complete
5	47	C6	Complete
6	23	T4	Complete
7	50	C5/6	Complete
8	65	C2	Complete

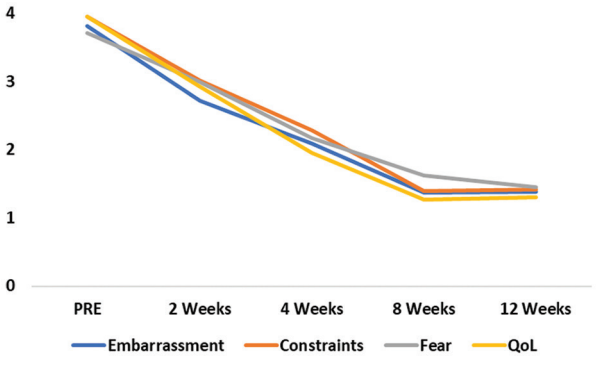

Figure 1. Qualiveen questionnaire scores before and after amniotic bladder therapy.

Table 2. MCC, MDP, and volume at first IDC (mL) at baseline, week 4, and week 12

Patients	MCC (mL)			MDP (cmH_2O)			Volume at first IDC (mL)		
	Baseline	Week 4	Week 12	Baseline	Week 4	Week 12	Baseline	Week 4	Week 12
NGB #1	217	402	422	66	34	33	75	165	175
NGB #2	260	442	404	71	32	30	88	170	190
NGB #3	226	451	472	63	35	32	96	185	180
NGB #4	236	472	450	69	32	34	79	182	180
NGB #5	180	390	400	69	32	36	68	130	140
NGB #6	160	405	410	67	33	33	62	120	140
NGB #7	220	470	440	64	32	34	81	165	170
NGB #8	170	410	420	70	34	35	58	158	160

Abbreviations: IDC: Involuntary detrusor contraction; MCC: Maximum cystometric capacity; MDP: Maximum detrusor pressure; NGB: Neurogenic bladder.

Table 3. Frequency of autonomic dysreflexia at baseline and weeks following treatment

Patients	Baseline	Week 2	Week 4	Week 8	Week 12
NGB #1	Daily	Once in week 2	None	None	None
NGB #2	Daily	None	None	None	None
NGB #3	Every other day	Twice in week 2	None	None	None
NGB #4	Daily	None	None	None	None
NGB #5	Daily	Every other day	Once a week	Once a week	None
NGB #6	Every other day	Once a week	None	None	None
NGB #7	Daily	None	None	None	None
NGB #8	Daily	Every other day	None	None	None

Abbreviations: NGB: Neurogenic bladder. Two patients had an acute UTI 2-week post-injection and were successfully treated with oral antibiotics. No other adverse events related to micronized AM injections occurred throughout the study.

4. Discussion

NDO is caused by a neurologic lesion, such as SCI, which results in aberrant and disorganized neuronal pathways controlling micturition. These patients generally have abnormal alterations of the bladder tissue, including disruption of the uroepithelium, decreased transepithelial resistance, and increased urea permeability. This can further lead to urothelial tissue inflammation, urothelial cell apoptosis, and the release of a cascade of pro-inflammatory cytokines that increase the excitability of bladder nerve fibers. Subsequently, this affects the ability of the patient to store urine and to empty the bladder efficiently, thereby resulting in urinary urgency, retention, and incontinence. With the progression of the disease, NGB patients exhibit varying degrees of thickening and hardening of the bladder wall caused by fibrosis, thereby further impacting bladder functions, which include decreased bladder capacity, increased bladder pressure during the storage phase, and poor compliance.[7] The high bladder pressure may then lead to reflux of urine into the kidney and cause renal scarring and chronic renal insufficiency.

The primary aim of treating NDO is to protect the upper tract from damage by decreasing bladder pressure to <40 cmH_2O in the storage phase. The secondary aim is to maintain urinary continence and improve QoL.[8] Current pharmacological NDO treatments, such as antimuscarinics or beta-3 adrenergic receptor agonists, induce bladder muscle relaxation, thereby improving bladder storage parameters.[9] However, these treatments have reported side effects, including constipation and dry mouth. Intra-detrusor injections of botulinum toxin A are the gold standard option for refractory patients to conventional medications, which aim to induce muscle paralysis and inhibit urothelial sensory nerve function.[10] However, the available therapies often carry significant side effects and may lose efficiency over time.[11] Furthermore, these treatment options may relieve the clinical symptoms of patients but have limited effects on the underlying pathophysiology and prevention of recurrence. Notably, there is currently no effective treatment for lower urinary tract symptoms associated with bladder fibrosis. To enhance the management of NDO, it is paramount to target the molecular mechanisms involved in NDO, including the perpetuating cycle of inflammation, fibrosis, urothelial cell death, and neural sensitization.[12,13]

AM is known to contain anti-scarring and anti-inflammatory properties and has been used in the treatment of many clinical indications.[14] The therapeutic applications of AM are diverse, and the mechanisms of action are multimodal. The immunomodulatory function is related to the apoptosis of pro-inflammatory cells and the suppression of pro-inflammatory cytokines such as

IL-12, TNF-α, and NO synthase 2.[15] The anti-scarring effect stems from preventing the expression of α-smooth muscle actin (α-SMA) by pro-scarring myofibroblasts through the suppression of TGF-β1 promoter activity and canonical TGF-β signaling.[2] Recent studies have also displayed that the key component within AM, i.e., HC-HA/PTX3, can reprogram and de-differentiate pro-scarring myofibroblasts, which was mediated by SDF1-CXCR4 signaling followed by the activation of canonical BMP signaling.[16] These functions are especially relevant in patients with NDO wherein pathologic processes, such as chronic inflammation, unabated fibroblast proliferation, and persistent myofibroblast activation, coupled with excessive extracellular matrix deposition, can disrupt the normal healing cascade and promote the development of contractile fibrosis.[17] Hence, the use of ABT and other similar modalities in patients with NDO is a promising therapeutic concept to reduce pathologic bladder inflammation and scarring in SCI patients.

In this study, we evaluated the ability of ABT to promote rapid symptomatic improvements and long-term benefits in recalcitrant NGB patients with high-level SCI. We have previously evaluated ABT in patients with interstitial cystitis/bladder pain syndrome, IDO, and RC and found symptomatic improvements as early as 2 weeks post-injection.[3] Specifically, the RC and IDO patients also exhibited increased bladder capacity based on voided volume and MCC up to 12 weeks after ABT. Similarly, in the current study, we reported the promising effects of ABT for NDO with improvements in patient-reported outcomes and functional data. The severity of urinary tract symptoms improved by 32% at 2-week post-injection and 64% at 3-month post-injection based on the total Qualiveen score. This was associated with a 50% reduction in MDP and a 105% improvement in MCC at 3-month post-injection. More importantly, the MDP was <40 cmH_2O in all patients after treatment, thereby reducing the potential for upper urinary tract damage. The study also reported long-term implications as the patients were predominantly young, with an average age of 39.6 ± 13.6 years, and had a full life ahead of them.

Aside from preserving renal function, urinary continence is also a significant factor affecting the patient's QoL and long-term independence. The decreased bladder compliance and increased pressure in these patients can lead to persistent incontinence that ultimately damages the tissue integrity of the skin, which leaves patients susceptible to skin breakdown, such as pressure injuries and incontinence-associated dermatitis.[18] Hence, increasing bladder compliance in these patients can directly affect their overall QoL. Likewise, a better understanding and promotion of long-term psychological and social well-being may facilitate advances in medical care to further extend the individual life spans of people with SCI.[19]

An additional problem frequently encountered by these patients is AD, a potentially life-threatening condition of the autonomic nervous system with an exaggerated reflexive increase in blood pressure. This occurrence is associated with severe headaches, bradycardia, facial flushing, lower extremity sweating, and a significantly increased risk of stroke by 300% to 400%.[20] It is well known that NDO can increase afferent stimulation and trigger AD episodes in patients with high-level lesions.[21] Thus, the peripheral stimulation after high bladder pressures must be resolved to prevent AD occurrence. Although the effect of constipation on AD was not investigated in our study, it is well known that this can also be a factor leading to AD and its consequences in patients. In future studies, we will exclude patients with heavy constipation or document methods of bowel evaluation. In our study, we found all patients had decreased episodes of AD at 12 weeks post-ABT, likely due to their high bladder pressures being resolved. Patients progressed from experiencing ADs daily before injection to none after injection. While the immediate impact on QoL is clear, future studies are warranted to monitor the prevention of longer-term complications (e.g., stroke) through the utilization of ABT.

This was the first study to investigate ABT for the management of NDO in SCI patients, and this study was not without limitations, which included the lack of randomization, the small number of participants, and the absence of a control group. Further studies to investigate the physiologic mechanism and duration of improvement, as well as randomized placebo-controlled trials, should be performed to validate the promising results obtained in this study for ABT in neurogenic patients with SCI.

5. Conclusion

We investigated ABT as a potential treatment option for SCI-associated NDO. Further investigations are needed to validate the effectiveness of ABT in this patient population and determine treatment durability. More research is required to develop a better understanding of the mechanisms through which ABT treats these complex disorders.

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Conflict of interest

The authors have no relevant conflict of interest to disclose.

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Ethics approval and consent to participate

This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Wayne State University Institutional Review Board (WSU IRB# 22-12-5277). Informed consent was obtained from participants included in the study.

Consent for publication

Verbal consent and permission were obtained from participants included in the study to publish their data.

Availability of data

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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ACCSCIENCE PUBLISHING

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ORIGINAL RESEARCH ARTICLE

Evaluating the SARS-CoV-2 spike glycoprotein as a molecular target for therapeutic development

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Abstract

The SARS-CoV-2 virus gains entry into host cells by binding its spike glycoprotein (S-glycoprotein) to the angiotensin 2 receptor. This viral protein contains several conserved regions, such as the receptor binding domain region, making it an ideal target for treating COVID-19. Notably, the majority of existing vaccines elicit antigenic reaction by targeting this protein epitope. This study evaluated the binding affinities of 44 different drugs against the SARS-CoV-2 S-glycoprotein, considering their toxicity profiles and previous clinical studies at different testing stages. Our results revealed that maraviroc and estradiol benzoate exhibited high affinities (−7.7 and −7.6 kcal mol^{-1}, respectively), while other ligands, such as indinavir and ritonavir, showed affinity at lower levels. Among the drugs with high affinity, toxicity levels ranged from harmful if swallowed (300 mg/kg < LD50 < 2000 mg/kg) to non-toxic (LD50 > 5000 mg/kg), with only three having undergone clinical testing, yielding promising or controversial results. Furthermore, emtricitabine and docosanol, previously explored as COVID-19 treatments, exhibited the lowest affinities (−4.7 and −3.9 kcal mol^{-1}, respectively), with associated harmful effects if swallowed. These results provide essential information about drug interaction against the SARS-CoV-2 S-glycoprotein and potential treatment pathways for COVID-19.

Keywords: SARS-CoV-2; Coronavirus spike glycoprotein; Molecular docking simulation; Pharmacology; Cluster MCMC

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1. Introduction

On March 11, 2020, the World Health Organization declared COVID-19, caused by the SARS-CoV-2 virus, a pandemic due to its rapid spread. This virus belongs to the *Coronaviridae* family, *Coronavirinae* subfamily, and *Betacoronavirus* genus. Coronaviruses derived their name from the multiple spikes (S-glycoproteins) surrounding their spherical lipoprotein capsule, giving them a crown-like appearance, which is recognized by receptor proteins on host cells susceptible to infection.[1,2] The S-glycoprotein is responsible for the attachment of the virus to host cells through angiotensin-converting enzyme 2 (ACE2), followed by binding to the transmembrane serine protease 2 (TMPRSS2), which allows cell membrane fusion and viral entry.[3,4] On

entering the host cell, the virus releases its RNA genome, which undergoes transcription through protein cleavage and assembly of the replicase-transcriptase complex. Viral RNA replication occurs, and structural viral proteins are synthesized, assembled, and packaged within host cell membranes before virions release.[5,6]

Understanding the variability of the S-glycoprotein is necessary for developing new vaccines, guaranteeing specificity, broad reactivity, and persistent immunity through cross-reactive responses.[7] Any change in the receptor binding domain (RBD) region of the spike protein can impact its binding affinity, necessitating close monitoring of mutant strains. Molecular docking studies of the spike virus and the ACE2 receptor junction are often utilized to assess potential changes in binding affinity.[8] However, a comprehensive understanding of S-glycoprotein binding does not equate to knowledge of its pharmacological interactions.

The pandemic underscores the critical need for effective medicines to prevent and treat viral infections, driving the urgency for the development of new treatments. However, given that the drug development process is both lengthy and costly, *in silico* evaluation through molecular simulation docking is employed to expedite this process.

At present, therapeutic options for COVID-19 are limited, primarily comprising antiviral medications such as molnupiravir, nirmatrelvir + ritonavir (Paxlovid), and remdesivir. In addition, monoclonal antibody therapies, exemplified by bamlanivimab/etesevimab and casirivimab/imdevimab, along with anti-inflammatory medications such as dexamethasone and immunomodulatory agents such as baricitinib and tocilizumab, constitute available treatment options. These treatments have been made available following clearance from the Food and Drug Administration (FDA). The majority have undergone testing in cell cultures, with a few demonstrating promising results in clinical trials.[9-14] In this context, a drug repositioning study has been carried out using *in silico* tools targeting the delta spike protein/ACE2 interface, employing a virtually screened library of 4388 approved drugs. The results suggest that several antihistaminic drugs form stable complexes against the spike RBD (with binding affinities between −32.92 and −53.78 kcal mol^{-1}), while practically all currently used antiviral drugs are not included in Spike-Antihistamine interaction.[13]

The primary objective of this study is to propose and evaluate various antiviral drugs with potential therapeutic effects, selecting those most promising for treating COVID-19 through serial simulations of molecular docking and a Markovian approach.

2. Methods

An exhaustive search was conducted across scientific databases and publications such as PubMed, Google Scholar, FEBS Press, Springer Link, and Nature to identify literature pertaining to chemical compounds or drugs capable of interacting with the SARS-CoV-2 S-glycoprotein. The A chain of the S-glycoprotein (6VSB, EM Map EMD-21375)[15] was chosen using the UCSF Chimera Software v.1.16 developed by the University of California.[16] Using the Swiss-Model server provided by Biozentrum, University of Basel,[17] homology modeling of the protein structures was carried out to predict the missing structures in the protein and complete it. Subsequently, the Protox-II server provided by the Charité Universita Medizin Berlin[18] filtered out innocuous compounds by applying a score greater than four on the toxicity scale. Following this, the 3D molecular representation of the glycoprotein with 6VSB identification was downloaded in PDB format from the Protein Data Bank provided by the National Centre for Biotechnology Information,[19] and it was subjected to cleaning and preparation for further analysis.

To prepare and simulate ligand docking, we used the software PyRx v.0.8 developed by Sargis Dallakyan.[20] This software enabled us to obtain the binding free energy (ΔG) of the compounds and identify the most promising drugs. This modeling process involved 10 resampling steps to obtain the statistical parameters necessary for grouping medications with the highest affinity. Subsequently, a cluster analysis based on Euclidean distances was conducted using Ward's method. This grouping was achieved through 1000 bootstrap–MCMC, facilitated by the Past v.4.2 program developed by Hammer.[21]

3. Results

Forty-four drugs with therapeutic potential were identified within the consulted data sets. The binding affinity of these drugs ranges between −3.76 and −7.22 kcal mol^{-1}, as measured by their critical energy cost values (ΔG). The cytotoxicity predicted by the Protox-II assay ranges from 4 to 6 toxicity points (Table 1).

Overall, the clustering analysis revealed two groups of drugs, with a high-affinity subgroup consisting of estradiol benzoate, itraconazole, maraviroc, indinavir, vicriviroc, dasabuvir, dolutegravir, and telaprevir (Figure 1). Within this high-affinity subgroup, estradiol benzoate and indinavir exhibited the lowest toxicity values. Hence, based on this study, they may be considered as the drugs with the most significant therapeutic potential. On the other hand, docosanol and valaciclovir comprised the lowest affinity subgroup. It is worth noting that while some low-affinity antiviral drugs were identified, they have proven effective

Table 1. Binding affinity of drugs tested against the spike protein of SARS-COV-2

Molecule	Binding affinity (kcal mol^{-1})	Predicted toxicity level	Clinical trials
Estradiol benzoate*	−7.22±0.58	5	Tested, controversial results[23–26]
Itraconazole*	−7.14±0.42	4	Tested, potential effect[25,28]
Maraviroc*	−7.07±0.41	4	Tested, controversial results[31,32]
Indinavir*	−7.04±0.32	5	Untested
Vicriviroc*	−7.03±0.4	4	Untested
Dasabuvir*	−6.87±0.47	4	Untested
Dolutegravir*	−6.84±0.45	4	Untested
Telaprevir*	−6.67±0.43	4	Untested
Nelfinavir	−6.55±0.31	4	Untested
Remdesivir	−6.47±0.33	4	Tested, potential effect[32,38]
Ritonavir	−6.44±034	4	Tested, controversial results[32,38,39]
Delavirdine	−6.38±0.38	4	Untested
Lopinavir	−6.37±0.49	5	Tested, controversial results[38,39]
Ceftazidime	−6.22±0.29	6	Tested, partial results[40]
Sofosbuvir	−6.08±0.34	6	Tested, potential effect[41]
Nevirapine	−8.89±0.32	4	Untested
Rilpivirine	−5.89±0.56	4	Tested, potential effect[42]
Tenofovir alafenamide	−5.67±0.29	6	Tested, potential effect[43,44]
Efavirenz	−5.64±036	4	Untested
Nitazoxanide	−5.46±0.36	4	Tested, potential effect[45]
Viramidine	−5.35±0.3	5	Tested, adverse effects[38]
Zanamivir	−5.33±0.37	5	Untested
Valganciclovir	−5.31±0.4	5	Untested
Tenofovir disoproxil	−5.27±0.49	6	Tested, unsupported[44]
Peramivir	−5.27±0.3	4	Tested, unsupported[46]
Ribavirin	−5.25±0.34	5	Tested, adverse effects[38,46]
Zidovudine	−5.19±0.31	6	Tested, unsupported[47]
Methisazone	−5.17±0.35	5	Untested
Telbivudine	−5.11±0.37	6	Untested
Famciclovir favipiravir	−5.09±0.49	4	Tested, potential effect[48]
Edoxudine	−5.08±0.36	6	Untested
Stavudine	−5.04±0.43	4	Untested
Didanosine	−4.92±0.45	4	Tested, potential effect[49]
Cidofovir	−4.84±0.27	4	Tested, potential effect[47]
Arbidol	−4.81±0.48	4	Tested, potential effect[38]
Zalcitabine	−4.8±0.38	5	Untested
Emtricitabine	−4.65±0.38	4	Tested, potential effect[42]
Lamivudine	−4.65±0.37	4	Tested, potential effect[50]
Penciclovir	−4.62±0.42	4	Untested
Aciclovir	−4.51±0.43	5	Tested, potential effect[51]
Ganciclovir	−4.46±0.47	5	Tested, potential effect[52]
Moroxydine	−4.41±0.36	4	Untested
Valaciclovir	−4.33±0.44	5	Tested, potential effect[47]
Docosanol	−3.76±0.42	4	Untested

Notes: *Drugs with high affinity, according to Ward's grouping. Predicted toxicity level: 4: Harmful if swallowed (300 mg/kg<LD50<2000 mg/kg); 5: May be harmful if swallowed (2000 mg/kg<LD50<5000 mg/kg); 6: Non-toxic (LD50>5000 mg/kg). Potential effects in co-infection treatment, pilot, phase I, II, III, or IV clinical trials. Controversial results are based on conclusions that contradict those of some publications.

against COVID-19 in auxiliary roles rather than as primary treatments. These drugs are often used in conjunction with antihistamines or in the treatment of comorbidities such as human immunodeficiency virus (HIV) and Epstein-Barr virus infections (Table 1).

4. Discussion

Estradiol benzoate, a female sex hormone of the estrogen group, exhibited the highest affinity among the drugs studied. Commonly prescribed for conditions such as menopause, female hypogonadism, vaginal atrophy, and osteoporosis in postmenopausal women, its therapeutic potential has garnered interest in the context of COVID-19. Recent studies have suggested a lower risk of severe COVID-19 complications in women, hypothesized to be linked to higher estradiol levels in women compared to men.[22] Estradiol benzoate inhibits the interaction between the spike protein and ACE2, thereby preventing intercellular fusion mediated by the SARS-CoV-2 protein.[23,24] However,

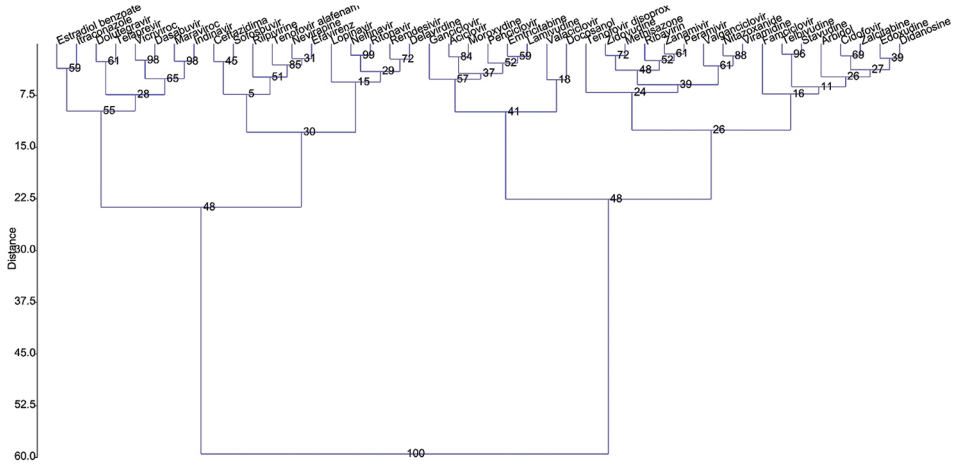

Figure 1. Clustering analysis based on Euclidean distances by Ward's method of resampling (1000 steps) binding free energy of the tested drugs.

in vitro studies have demonstrated an IC50 range between 1.02 and 1.75 μM for this drug, which is much higher than the physiological concentration of estrogen. Despite the disparity, these doses have proven effective against viral infection.[25,26] These studies propose that the drug binds to the spike protein or ACE2 in a non-competitive manner outside the binding region, potentially inducing conformational changes in the protein and disrupting the spike–ACE2 interaction.[25] Therefore, it is suggested that future *in vitro* tests delve into these mechanisms in greater detail.

Itraconazole, a lipophilic compound drug with high bioavailability, is approved for the treatment of fungal infections. It inhibits viral entry by targeting the six-helix furoin core of the SARS-CoV-2 S-glycoprotein, as evidenced by cell culture tests.[27] Furthermore, investigations have explored its interaction with the heptad repeat 1 (HR1) region of the spike protein.[25] Our results place itraconazole as having the second highest affinity among the group of drugs with significant treatment potential. Despite its demonstrated *in vitro* activity, its clinical utility in hospitalized COVID-19 patients remains limited, having only been used in a pilot test. Consequently, further clinical trials are warranted.[25,28]

Maraviroc and vicriviroc[29] are antiretroviral drugs primarily used to treat CCR5-tropic HIV-1 infection. The pharmacological blockade of CCR5 has demonstrated promise in improving symptoms in severe COVID-19 patients and alleviating post-COVID-19 symptoms.[30] This effect is attributed to their mechanism of action, which involves blocking the entry of the virus into cells by binding to the CCR5 chemokine receptor, constituting a consistent and statistically significant inhibitory effect against SARS-CoV-2. This encouraging attribute makes these drugs promising candidates for COVID-19 treatment, as they not only prevent immune cell exhaustion but also redirect immunoreactive cells away from sites inflamed by COVID-19. Consequently, this effect reduces viral load and minimizes damage to organs such as the lungs.[31] However, it is crucial to note that these findings are subject to controversy, as other studies have failed to demonstrate any anti-SARS-CoV-2 activity of maraviroc.[32] Therefore, it is of the utmost importance to carry out additional studies and clinical tests to validate these findings.

Numerous antiviral drugs are currently under investigation for their efficacy against COVID-19, but only a selected few have demonstrated encouraging results in clinical and preclinical studies, even when there has been a significant reduction of viral load in cell cultures. Notable examples include remdesivir and ritonavir.[33] Our analysis grouped both of them with nelfinavir and delavirdine in a second cluster, exhibiting a binding affinity lower than -6.38 kcal mol^{-1}. However, clinical studies on remdesivir and ritonavir are lacking. These two drugs have been suggested solely as adjuvants due to their antiretroviral and protease inhibitory action, which could improve the efficacy of treatments involving molnupiravir, nirmatrelvir, or ensitrelvir[34-36] without directly interacting with the spike protein. Therefore, *in silico* studies do not substitute clinical treatments but rather offer potential treatment routes and drug interactions that could improve patient outcomes.

The effectiveness of a combination of an antiviral (−7 kcal mol^{-1}) and an antihistamine (−53.78 kcal mol^{-1})[14] in binding to the spike–ACE2 complex may potentially mislead our understanding of optimal treatment strategies, particularly when considering that antihistamines alone have shown promising outcomes. Antihistamines belonging to the histamine-1 receptor binding class are very effective

for treating COVID-19. Studies have demonstrated that the use of diphenhydramine, hydroxyzine, and azelastine correlates with a reduced incidence of SARS-CoV-2 positivity in individuals older than 61. While these drugs exhibit direct antiviral activity against SARS-CoV-2 *in vitro*, the specific mechanisms underlying their antiviral effects remain unclear. Hydroxyzine and potentially azelastine are known to bind to the ACE2 and sigma-1 receptors as unintended targets, which may explain their exceptional efficacy. Therefore, it is recommended that antivirals serve as adjuvants in combined treatments alongside these antihistamines.[37]

5. Conclusion

Within the realm of COVID-19 treatment, numerous other aspects, such as viral replication, enzymatic processes within infected cells, and molecular aspects of cellular response, serve as potential targets for therapeutic intervention. Molecular docking models, such as amixin, an antiviral approved by the FDA for treating MERS-CoV, offer promising avenues. While amixin has demonstrated partial success in treating COVID-19, its action does not rely on binding to the S-glycoprotein but on targeting the enzymatic complex involved in viral replication.[38]

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Conflict of interest

The authors declare no conflicts of interest.

Author contributions

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Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data

Data used in this work are available from the corresponding author on reasonable request.

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ACCSCIENCE PUBLISHING

INNOSC Theranostics and Pharmacological Sciences

ORIGINAL RESEARCH ARTICLE

Rational drug design from phosphatidylinositol 3-kinase-α inhibitors through molecular docking and 3D-QSAR methodologies for cancer immunotherapy

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Abstract

Dysregulation or aberrant activation of the phosphatidylinositol 3-kinase (PI3K) signaling pathway is commonly observed in various cancers and is associated with tumor growth, metastasis, and resistance to therapy. Targeting PI3K-α with appropriate inhibitors can disrupt this pathway, hindering cancer progression, and potentially enhancing the immune system's ability to recognize and eliminate cancer cells. In this study, we aimed to design a novel and potent inhibitor of PI3K-α for cancer immunotherapy using rational drug design techniques, including virtual screening, molecular docking, and 3D-QSAR. We obtained the human PI3K-α protein (6PYS) complexed with (3S)-3-benzyl-3-methyl-5-[5-(2-methylpyrimidin-5-yl)pyrazolo[1,5-a] pyrimidin-3-yl]-1,3-dihydro-2H-indol-2-one (PJ5) from the RCSB Protein Data Bank. Virtual screening of ligands, integrated with predictive computational molecular docking and 3D-field-based-QSAR, was implemented using appropriate Schrödinger Maestro modules. Rational drug design was also carried out, and its clinical relevance was validated across several ADMET descriptors. Docking results suggested that a hybrid of sulfonamide and pyridine-based heterocyclic compounds, functionalized with potent moieties derived from alkaloids, exhibited adequate synergistic biological effects capable of enhancing sufficient biological activity against PI3K-α. A field-based 3D-QSAR model was built on four partial least squares factors, and five statistical metrics were employed to validate the model. The newly designed ligand from this approach, named 6'-amino-5'-(2-fluoro-1,3-oxazol-5-yl)-N-{[3-(hydroxymethyl)oxetan-3-yl]methyl}-3-methyl-[2,3'-bipyridine]-6-sulfonamide or T85, exhibited a predicted bioactivity (pIC_{50}) of 8.25. The predicted ADMET properties of T85 fell reasonably within the range of recommended standards, especially adhering to Lipinski's rule of five and Jorgensen's rule of three. In conclusion, the results of this study offer significant insights into *in silico* drug design using a rational approach, which could expedite the discovery and development of new drug molecules.

Keywords: Cancer; PI3K-α; Molecular docking; 3D-QSAR; ADMET

†These authors contributed equally to the work.

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1. Introduction

Human physiology is governed by intricate cellular metabolisms, which exemplify how cell-specific programs efficiently regulate overall health and well-being. The body initiates cascade mechanisms of cellular signaling pathways to control physiological functions such as cell proliferation, growth, survival, differentiation, and metabolism.[1] Cells serve as the building blocks of life; however, genetic mutations or alterations can lead to anomalies, disrupting the mitotic and cytokinetic regulatory mechanisms of the cells. Over time, dysregulation of cellular signaling progresses to carcinogenic effects, affecting various enzyme functions, such as phosphoinositide 3-kinase alpha (PI3K-*α*), an isoform of the phosphatidylinositol-3-kinase (PI3K) family of enzymes.

PI3Ks are lipid kinases responsible for phosphorylating the -OH moiety at the 3' position of the inositol ring, generating phosphatidylinositol-3,4,5-triphosphate (PIP3).[2,3] They play a central role in regulating cell cycle, apoptosis, DNA repair, cellular senescence, angiogenesis, cellular metabolism, and motility. Besides, they serve as intermediate signaling molecules that are well known for their roles in the PI3K/serine-threonine protein kinase (AKT)/mammalian target of rapamycin (mTOR) (PI3K/AKT/mTOR) signaling pathway.[2,4-6] Furthermore, the PI3K/AKT/mTOR pathway is involved in oncogenesis and tumorigenesis,[5,7,8] which is frequently dysregulated in human cancers, such as pancreatic cancer,[2] breast cancer,[5] colorectal, and ovarian cancer.[6] At the molecular level, alterations at numerous nodes are described in different tumor types, including activating mutations and/or the amplification of phosphatidylinositol-4,5-biphosphate 3-kinase catalytic subunit alpha (PIK3CA), mutation or overexpression of upstream receptor tyrosine kinases (RTKs), or inactivating mutations or deletions of phosphatase and tensin homolog (*PTEN*) from chromosome 10.[4,7]

In the recent decade, several studies have described the role of the PI3K pathway,[2-5,9,10] which is a complex cascade of signal transduction that regulates proliferation, growth, differentiation, protein synthesis, glucose metabolism, migration, apoptosis, and other intracellular metabolisms activated in diverse types of cancer.[3,9] In humans, PI3K exists in three classes – Classes I, II, and III,[3,4] and is enzymes with a molecular weight of about 200 – 300 kDa.[4] Class I of the PI3K subfamily is primarily linked to oncogenesis.[4,11-13] The PI3K is a heterodimer comprised both the catalytic and regulatory subunits. The phosphorylation of the regulatory subunit leads to the activation of p110, the catalytic subunit of PI3K. The genes *PIK3CA, PIK3CB, PIK3CG,* and *PIK3CD* encode the four isotypes of p110: alpha, beta, gamma, and delta, respectively.[14] These p110 isotypes activate downstream signaling pathways, including binding corresponding ligands to tyrosine kinase receptors (RKTs).[9] However, the p110-alpha isotype is involved in insulin-like signaling, p110-beta plays a role in platelet-derived aggregation, thrombosis, and insulin signaling, while p110-gamma and p110-delta isotypes are expressed in lymphocyte activation, mast cell degranulation, and chemotaxis.[15] Furthermore, the catalytic p110 subunit forms a complex with one of the three p85-related regulatory subunits: p85-alpha, p85-beta, and p55-gamma,[14] encoded by the genes *PIK3R1, PIK3R2,* and *PIK3R3*, respectively.[14,16,17] Activation of the p85 regulatory subunit occurs through the stimulation of the receptor tyrosine kinase, where it binds to phosphotyrosine residues in the receptor tyrosine kinase. Such a process unleashes the p110 catalytic subunit from inhibition of the p85 regulatory subunit.[3] Progressively, PI3K localizes to the cellular membrane, where the p110 subunit converts phosphatidylinositol 4,5-biphosphate (PIP2) into PIP3, a lipid secondary messenger, which then activates the downstream effector protein kinase B (also known as AKT), promoting its translocation to the inner membrane of cells, where it is phosphorylated and activated by phosphoinositide-dependent protein kinase (PDK)-1, PDK2, and mTor-rictor.[3,5] AKT, a serine/threonine kinase, together with mammalian target of rapamycin complex 1 (mTORC1), further modulates the activities of downstream biomacromolecules, such as B-cell lymphoma-2 (BCL-2), BCL-2 antagonist of cell death (BAD), forkhead box O (FOXO), p53, p27, transcription factor, tuberous sclerosis complex 2 (TSC2), glycogen synthase kinase-3β (GSK3β), insulin-like growth factors, cyclin D1, C-MYC (cellular myelocytomatosis) oncogene, nuclear factor kappa B (NF-κB), caspase-3 and caspase-9, and murine double minute 2 (MDM2) (Figure 1).[3,5] These biomacromolecules regulate protein synthesis, cell survival, cell cycle progression, cellular growth, proliferation, motility transformation, DNA repair, glucose metabolism, and drug resistance.[3,18,19]

PI3K classes II and III also play significant roles in cell-specific functions and metabolism. There are three isoforms of PI3K class II, including PI3KC2-alpha, PI3KC2-beta, and PI3KC2-gamma.[3-5] These isoforms of PI3K class II are monomers with a high molecular weight.[16,20] Moreover, class II monomers have no regulatory subunits[3,5] but possess individual catalytic moieties that interact directly with phosphorylated adapter proteins.[16,20] In contrast to PI3KC2-beta, PI3KC2-alpha appears to be involved in cell migration and neuronal cell survival, as well as clathrin-mediated vesicle trafficking, insulin signaling, neurosecretory granular exocytosis, and smooth muscle contraction.[15] On the other hand, the role of PI3KC2 is

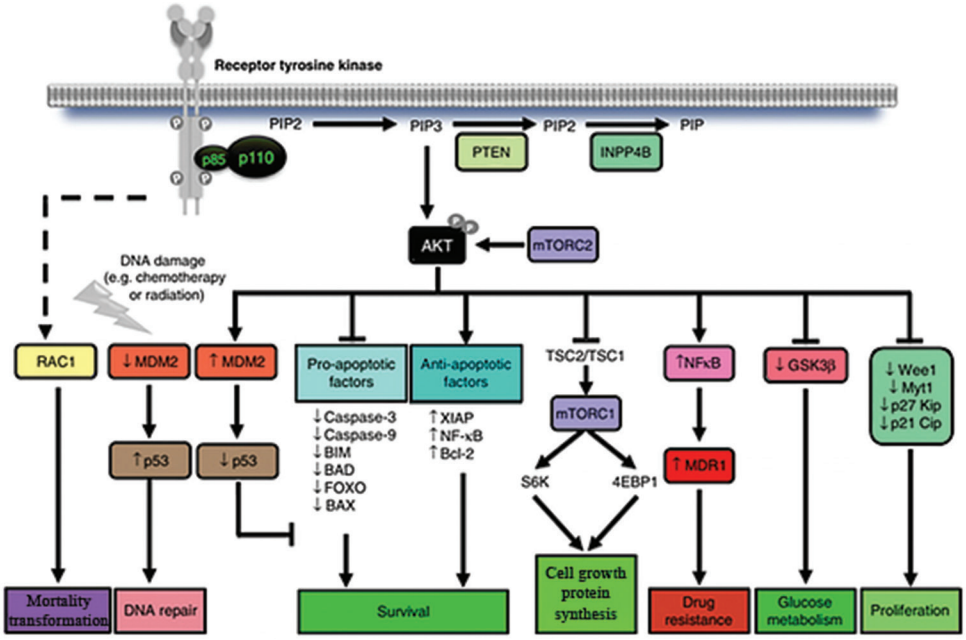

Figure 1. PI3K/AKT/mTOR signaling pathway in human cancer. Exposure of cells to DNA damage reduces intracellular proportions of MDM2, which enables p53 accumulation and stabilization, leading to the initiation of DNA repair pathways.[5]
Abbreviations: BAX: BCL-2-associated X protein; BIM: BCL-2-interacting mediator of cell death; INPP4B: Inositol polyphosphate-4-phosphatase type II B; MDR1: Multidrug resistance protein 1; Myt1: Myt1 kinase; S6K: Ribosomal protein S6 kinase; Wee 1: Wee 1 kinase; XIAP: X-linked inhibitor of apoptosis protein; 4EBP1: Eukaryotic translation initiation factor 4E-binding protein 1.

not established. The PI3KC3 gene produces the class III PI3K, also known as vacuolar protein sorting 34, which is a potential serine/threonine protein tyrosine kinase regulatory subunit. Vacuolar protein sorting 34 is also linked to mTOR signaling and plays a part in the vesicular transport of membrane proteins to the lysosome.[15] Furthermore, the PI3K/AKT/mTOR pathway plays a critical role in the pathogenesis and provides survival advantages in hematologic malignancies such as leukemia, lymphoma, and myeloma.[3]

PI3K-α is a member of the Class 1A PI3K family of enzymes and is activated by various upstream signaling biomacromolecules, such as growth factor receptors, which phosphorylate phosphatidylinositols in the cell membrane. It is linked to several tumorigeneses through amplification, overexpression, and mutation of the PIK3CA gene,[21] following the PI3K/AKT/mTOR pathway. In addition, PI3K-α specifically consists of heterodimers of a p110-α catalytic subunit and a p85 regulatory subunit.[22] The PI3K-α isoform transmits various extracellular stimuli through signaling pathways that regulate numerous cellular processes, including cell proliferation, motility, cell death, and cell invasion, thereby playing a crucial role in the physiology of cells.[23,24]

Recently, it has been demonstrated that selective inactivation of the PI3K-α isoform, which is the most frequently up-regulated isoform of PI3K in human cancer, effectively blocks PI3K/AKT/mTOR signaling in response to diverse growth stimuli.[25] Consequently, PI3K-α has emerged as a key target that is primarily affected by cancer mutations, gene rearrangement, and gene amplification, making it an attractive focus for drug development.[23,26] Ongoing efforts aim to exploit and develop novel selective inhibitors for PI3K-α as a promising drug target for anti-inflammation and anti-cancer therapy. Moreover, several kinase inhibitors for PI3K-α have been discovered and synthesized to regulate the proliferation of cancer cells. These small-molecule drugs exhibit unique potency. Notable examples include buparlisib (BKM120), pilaralisib (XL147), pictilisib (GDC-0941),[27] alpelisib (BYL719), umbralisib,[28] and taselisib (GCD-0032),[4,24] serving as PI3K isoform inhibitors utilized in the treatment of pancreatic cancer,[2] breast cancer,[2,5,29] or ovarian cancer.[6] Despite their development, most inhibitors encountered challenges in clinical trials, displaying poor efficacies due to nanotherapeutic-associated toxicity resulting from on-target and off-target effects.[21,24] To maximize effectiveness and minimize negative side effects, medicinal chemists focus on identifying isoform-selective PI3K-α inhibitors.[21] This discovery of isoform-selective PI3K-α inhibitors remains a priority to engineer therapeutic drugs with enhanced efficacy and reduced side effects[21]

through various scientific approaches. Furthermore, various essential metabolic pathways and key factors serve as a pool of associated therapeutic targets for anticancer therapy development.[30]

The drug discovery process is known for its time-consuming nature and high costs.[28] However, advancements in molecular biology, genomics, and computational technologies have accelerated the understanding of cancer development, leading to the discovery of novel biomarkers, targeted therapies, and immunotherapies. These advancements have significantly improved cancer diagnosis and treatment outcomes. Three-dimensional quantitative structure-activity relationship (3D-QSAR) in combination with molecular dynamics simulations is a hybrid *in silico* approach for the design and synthesis of drugs and is instrumental in identifying new compounds with superior activity.[31,32] The combination of 3D-QSAR and molecular docking approaches continues to demonstrate how *in silico* pharmacological systems can work together to produce novel therapeutics using structural data. The hybrid offers a more comprehensive insight into the structure-activity relationship of a compound (ligand)[33] and its interaction with the target protein. This understanding enables drug designers to modify the compound's structure to enhance its activity, selectivity, and binding affinity, ultimately leading to a rational design of more potent and effective drugs.

In the present study, the drug discovery process using PI3K-*α* inhibitors involved ligand preparation based on predefined descriptors by assessing the drug-like properties of the ligands and assessing their binding affinity to the target through molecular docking. In addition, the study considered pharmacophore screening with the least energy core to prioritize the binding of ligands to the active site of PI3K-*α*, predicting the most energetically favorable conformations and estimating the binding affinities. Moreover, the study included the prediction of absorption, distribution, metabolism, excretion, and toxicity (ADMET) profiles. These approaches aided in the rational design and optimization of PI3K-*α* inhibitors, contributing to the development of potential therapeutic agents for cancer and other diseases.

Current preclinical and clinical evidence suggests that inhibitors targeting the PI3K/AKT/mTOR pathway are utilized in combination with other anticancer therapies to combat resistance in cancer cells. Multiple ongoing clinical studies are investigating this approach. However, most targeted anticancer therapies, as well as cytotoxic and radiation therapies, are complicated by secondary resistance in cancer cells. Resistance is an intricate phenomenon involving numerous mechanisms, comprising the activation of signaling pathways such as PI3K/AKT/mTOR.[5] Drug discovery and development represent a lengthy and costly process, relying heavily on *in vitro* assays, animal models, and clinical trials for reliable testing of drug molecules. Hence, our objective is to contribute to the discovery of a novel PI3K-*α* inhibitory compound through a rational drug design approach with potential immunomodulatory, immunobiological, and clinical implications. We aim to explore a series of clinically approved selective PI3K-*α* inhibitors with excellent pharmacokinetic properties obtained from the public web-accessible molecular recognition database, BindingDB (http://www.bindingdb.org).[34,35] Rational drug design holds the potential to discover novel drugs or drug combinations and repurpose existing drugs for new indications. This work seeks to introduce innovative methods for discovering potential drug molecules through molecular docking and 3D-QSAR methods, coupled with robust rational drug design techniques focused on selected PI3K-*α* inhibitors.

2. Methods

2.1. Dataset

The dataset, comprised ligands, was retrieved from BindingDB. These cogeneric ligands were represented in three-dimensional (3D) coordinate structures, all sharing common orientation codes. Initially, this exploratory dataset contained 3994 rows of different inhibitory molecules and 56 columns, providing information on the inhibitory molecules targeting the phosphatidylinositol-3 kinase regulatory subunit alpha in *Homo sapiens* (human). Furthermore, the dataset contained some important column attributes, such as the half-maximal inhibition concentration (IC_{50}), BindingDB ligand name, UniProt (SwissProt) primary and secondary IDs of the target chain, molecule ID, and ROMol information. The UniProt serves as a focal point for collecting functional information on proteins with precise, dependable, and extensive annotations.[33] It integrates biologically significant data obtained from selected resources and the manual curation of protein features, such as functional domains and active sites, amino acid variations, ligand binding sites, and post-translational modifications (PTMs). UniProt records provide mechanistic insights into disease-drug relationships.[36] On the other hand, ROMol (the Read-Only molecule) is a representation of a molecule or a chemical structure that is strictly read-only. It is an object within RDKit, an open-source cheminformatics library written in C++ with Python bindings, enabling operations with chemical structures and data.

2.2. Data preprocessing

Data preprocessing is a crucial step in preparing and transforming datasets to improve raw data quality,

considering the inherent complexity and imperfections in data preparation operations.[37,38] It serves as a basis for valid data analysis.[37] Preprocessing includes various techniques such as cleaning, integration, transformation, imputation of missing values, and reduction.[33,38]

In this study, lists of PI3K-α inhibitory molecules were obtained from the binding databank, resulting in a dataset comprising 3994 inhibitory molecules in 3D geometry. Furthermore, the dataset included columns containing IC_{50} values of the molecules, molecule IDs, ligand names, ROMol object information of ligands, etc. The IC_{50} values column contained affinity information, indicating the potency of each molecule against the PI3K-α target. The dataset was in SDF (structure-data file) format, and data preprocessing was performed using the Python programming language.

The IC_{50} values of compounds, expressed in nanomolar (nM) units and ranging from 0.07 – 7200 nM, helped capture a broader chemical space, enhancing the identification of novel ligands. In addition, the IC_{50} column was also used as a reference column, in which duplicate rows sharing the same IC_{50} were dropped. The governing code syntax was specific to maintaining the first entries, as it was assumed that two or more ligands with the same IC_{50} value exhibited similar potency or affinity, pharmacological effects, and functional activities toward the target protein or receptor. Dropping duplicate entries of IC_{50} values offered a normal distribution of values that made the dataset more amenable to statistical analysis. However, docking ligands of similar half-maximal inhibitory concentrations may not provide significant additional insight. Later, the IC_{50} values were converted to pIC_{50} values to enable dataset standardization and consistency.

The IC_{50} values depicted in multiple units can complicate the analysis of results across different concentrations. Hence, it was necessary to convert the IC_{50} values in the dataset to pIC_{50} values. Data presentation in pIC_{50} values, which represent the values as the negative logarithm of the molar concentration of the IC_{50} values, is considered a better approach. This method enhances data clarity, minimizes potential errors in data representation, and improves reproducibility with standardization, linearity, normal distribution, and precision as additional attributes. Relevant columns were selected and preserved for further analysis. The data preprocessing stage functions as a preliminary filtering technique to minimize the compound selection size before executing virtual screening campaigns.

2.3. Protein complex refinement

The PI3K-α protein structure was obtained from the RCSB Protein Data Bank (rcsb.org). The architecture of the human PI3K-α protein, encoded as 6PYS, is a protein complex composed of a ligand and several water molecules. The structure of 6PYS, obtained through X-ray diffraction, exhibits a resolution of 2.19Å, with associated R-values of free, work, and observed, numerically presented as 0.259, 0.2243, and 0.225, respectively. The composition of 6PYS includes a total structural weight of 110.61 kDa, an atom count of 7558, modeled residue counts of 890, deposited residue counts of 945, and one unique protein chain A. Furthermore, no mutations were associated with the 6PYS polymer sequence that was engineered from the reference sequence.

The protein preparation involved isolating the ligand from the 6PYS protein-ligand complex, followed by protein content modification using the protein preparation and refinement wizard embedded in Schrödinger Maestro (Schrödinger Release 2020-3: Maestro, Schrödinger, LLC, United States, 2023). The Maestro software is an intuitive molecular modeling environment for various scientific discoveries based on material science, as well as an integrated predictive computational modeling and machine-learning platform for small-molecule drug development. During refinement, simulation settings were configured for a pH of 7.0, which allowed small molecules (HETs) to detect ligands, metals, and ions. In addition, the refinement process incorporated various measures, such as the assignment of bond orders the employment of a chemical component dictionary (CCD) database to help identify and characterize the ligand present in the protein structure in connection with its binding modes and their potential functional or therapeutic roles; inclusion of missing hydrogens in the protein; the addition of terminal oxygens to the protein; the conversion of selenomethionines to methionines; the filling of missing loops; cap termini; the deletion of water molecules beyond HETs of 0 Å; and the generation of HET state within 7.0 ± 2.0 pH value. The Kabat antibody annotation scheme was employed to facilitate the design and analysis of antibody-based therapeutics by comparing the protein sequences and structures of antibodies. Furthermore, to mimic the natural environment of the protein and prevent unwanted interactions or structural distortions that may arise from exposed termini, the termini of the protein were capped with small fragments of peptides.

Hydrogen bond assignment was carried out in the refinement stage to assign hydrogen bonds to the right geometry. The optimization of the hydrogen bond assignment scheme was carried out using PROPKA, a molecular dynamics program in Maestro that facilitated a quantitative analysis of the protein pKa values of ionizable groups. More specifically, PROPKA was utilized

in identifying the interactions of ionizable residues in the protein together with their structural determinants along with significantly perturbed pKa values that contributed to the stability of the protein. The last step of protein refinement was energy minimization and the deletion of water molecules around the ligand. In this step, the root mean square deviation (RMSD) for heavy atom coverage was set to 0.30 Å. This was carried out while pushing the minimization using the OPLS3 (optimized potentials for liquid simulations-3) force field and concurrently deleting water molecules within 3 Å from the ligand (HETs). The primary objective of protein minimization was to remove steric clashes,[39] reorganize structural features such as bond angles, bond lengths, and torsional angles, and remove strained conformations arising from crystal packing artifacts, experimental errors, or inaccuracies in computational modeling.[40]

2.4. Binding site/pocket identification

With a large amount of protein data available in the UniProt database, only a fraction of it is functionally annotated.[41] Consequently, understanding the biological or biochemical role of protein with an interacting partner in a binding relationship remains a fundamental challenge in the fields of medicinal chemistry, genomic bioinformatics, and pharmacology. This underscores the importance of automated sequencing tools. Protein function is closely correlated with the small molecules that attach to them. These small molecules may function as substrates or products of an enzyme reaction, cofactors that are crucial for catalysis,[41,42] or they may provide crucial structural or regulatory functions.[43] Hence, protein-ligand interaction in the context of binding sites is critical for drug discovery,[44] and understanding responses to drugs.[45]

Binding site identification was conducted following a predictive approach using the SiteMap wizard in Maestro. To conduct binding site prediction, the ligand in the 6PYS protein-ligand complex was masked. To find, visualize, and evaluate protein binding sites, the identification of top-ranked potential receptor binding sites was prioritized. This necessitated the search of at least 15 site points per reported site, from which up to five site-point groupings were to be reported. The strategy for identifying binding pockets involved employing a more restrictive definition of hydrophobicity and having a standard grid and crop site maps at 4 Å from the nearest site point. Furthermore, the binding site attributes such as druggable sites, docking space (site score), and desirable ligand size,[46] as well as other parameters including pocket volume, exposure score, contact score, and hydrophobic interaction score, were computed using the SiteMap algorithm. Furthermore, the respective values of these binding site attributes were evaluated to determine the optimal site for binding. The mathematical correlations depicted in Equations I and II were used by Maestro to compute the druggability and site scores, respectively. These procedures culminated in detecting deep binding sites/pockets for potential receptors, specific to a protein-ligand complex.

$$Druggability\ (D)\ score = 0.094\sqrt{n} + 0.60\delta - 0.34\omega \quad (I)$$

$$Site\ score = 0.0733\sqrt{n} + 0.6688\delta - 0.20\omega \quad (II)$$

Where, n is the number of site points (capped at 100); δ is the enclosure score; and ω is the hydrophilic score.

2.5. Ligand refinement

The idea behind ligand refinement is to transform two-dimensional (2D) or three-dimensional (3D) structures into corresponding low-energy 3D structures in the structure-data file (.sdf) format, with the option to expand each input structure by creating variations on ionization state, tautomers, stereochemistry, and ring confirmations, thereby generating broad chemical and structural diversity from a single input structure. In this study, the LigPrep package in Schrödinger Maestro (Schrödinger Release 2020-3: LigPrep, Schrödinger, LLC, United States, 2023) was employed to prepare the ligands.

The 6PYS is a co-crystallized protein with a ligand, P5J (Figure 2). The ligand P5J was isolated from the protein complex and refined using LigPrep in Maestro. The ligand states were generated at pH 7 ± 2.0 using the Hamette and Taft methodology encoded in the

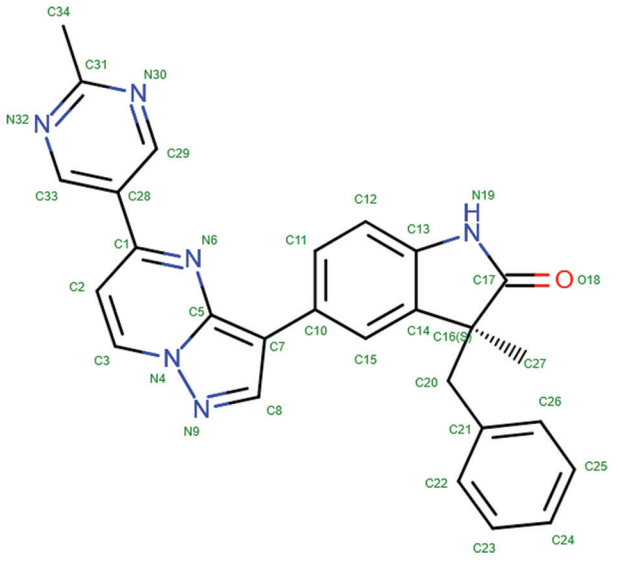

Figure 2. 2D representation of ligand P5J in 6PYS human protein complex. P5J refers to (3S)-3-benzyl-3-methyl-5-[5-(2-methyl pyrimidine-5-yl) pyrazolo[1,5-a]pyrimidin-3-yl]-1,3-dihydro-2H-indol-2-one; chemical structural formula: $C_{27}H_{22}N_6O$; molecular weight: 446.503 g/mol.

Epik wizard of Maestro to improve virtual screening enrichment. Moreover, the ligand was desalted to remove extra molecules, such as water molecules and counter-ions, present in the ligand file from certain structure databases. To define the stereoisomers, the computation was restrained to determine chirality from the 3D structure of P5J to account for keto-enol tautomerization, analogous sulfur and nitrogen tautomerizations, as well as histidine- and DNA-base tautomerizations. However, the generation of tautomeric forms of P5J was avoided to prevent tautomeric duplicates while maintaining accuracy, computational efficiency, quality experimental validation, reliable structure-activity relationship, consistency, and simplifying analysis. Similarly, refined PI3K-α ligand molecules obtained from the binding database (bindingdb.org) were refined for docking using a similar 6PYS protein-ligand complex LigPrep approach.

The LigPrep settings for refining the inhibitory molecules excluded tautomer generation. However, stereoisomer computation was carried out to determine chirality based on the 3D structure with the objective of having the internally produced stereoisomers filtered to remove any structures, fused ring systems, or chirality that were incompatible with that of natural products to generate the desired enantiomers. Ligand alignment was performed on all refined inhibitory molecules, including the P5J cocrystallized ligand. This option aligned structures with similar orientations, facilitating the identification of the ligand pose that maximizes beneficial interactions, such as hydrogen bonding, hydrophilic interactions, and electrostatic interactions, while minimizing detrimental interactions or clashes.

2.6. Ligand virtual screening-molecular docking

A structure-based, *in silico* virtual screening approach using Schrödinger Maestro was applied to predict the interaction, favorable binding orientations, and conformation of the refined ligands within the active site of the target PI3K-α protein. Essentially, for a molecule to tightly bind to a receptor, both geometric (shape) and electrostatic (charge) complementarities must exist.[47,48] These complimentary aspects define the molecular dynamics of the ligand-receptor relationship by incorporating interaction maximization while minimizing the total energy of the complex.

Typically, most *in silico* docking programs are built to predict binding mode and binding affinity between protein and ligand using a hybrid search algorithm and scoring function. While the search algorithm robustly generates multiple poses for a ligand in the binding site of the receptor, the scoring function ranks or orders the conformations to distinguish the experimental binding pose from the rest of the predicted poses. However, in Schrödinger Maestro, the binding affinity was calculated as (Equation III):

$$Binding\ affinity = \vartheta_{L/VdW} + \vartheta_{E} + \vartheta_{Hb} + \vartheta_{SM} + \vartheta_{\pi} + \vartheta_{Halogen} \quad \text{(III)}$$

where $\vartheta_{L/vdW}$ is the energy contribution associated with lipophilic pair concerning total Van der Waals force of interaction, ϑ_{E} is the energy contribution associated with electrostatic interactions, ϑ_{Hb} is the energy contribution associated with hydrogen bond interaction, ϑ_{SM} is the energy contribution associated with site map interactions, ϑ_{π} is the energy contribution associated with pi-cation interaction, and $\vartheta_{Halogen}$ is the energy contribution associated with halogen bond interactions.

In this study, a receptor grid was initially generated around the region occupied by P5J in the 6PYS protein, aiming to map the properties of the binding site onto a grid. Next, the refined ligands were docked into the minimized 6PYS protein through Schrödinger's Virtual Screening Workflow panel, with customized settings that automated a virtual screening-molecular docking workflow. The virtual screening workflow was automated to screen the ligands through successive stages, starting from high-throughput virtual screening mode (HTVS) to standard precision (SP) mode, and lastly, through extra-precision (XP) mode. The submission ratio of screened ligands at each stage was set to 70%, 60%, and 8%, respectively, as depicted in Figure 3. A trade-off between the speed and accuracy of the virtual screening served as the basis for selecting this ratio. By filtering out a large fraction of compounds in the early stages, the workflow could save time and resources, considering that docking and post-processing are computationally intensive and time-consuming compared to ligand preparation. However, by retaining a sufficient

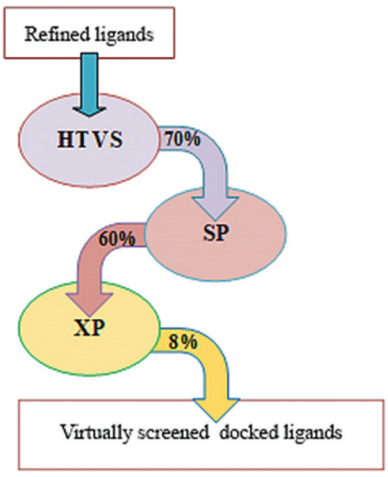

Figure 3. Schematics of submission ratio for virtual screening workflow for docking in Glide.
Abbreviations: HTVS: High-throughput virtual screening; SP: Standard precision; XP: Extra-precision.

fraction of compounds in the later stages, the workflow could ensure that the final hits are diverse, relevant, and reliable, as docking and post-processing are more accurate and informative than ligand preparation. Therefore, this ratio was chosen as a default value that balances the speed and accuracy of the virtual screening workflow. In addition, the virtual screening workflow aided in the computation of performance scores, including Glide, docking, interaction, and penalty scores, as well as similarity scores, to validate the refined ligands. Furthermore, during virtual screening, interaction scores for residues within 12 Å of the grid center were considered.

2.7. Implementation of 3D-QSAR

The computational modeling technique employed in this study was field-based 3D-QSAR to analyze and predict the relationship between the 3D structure of the refined ligands based on their alignment, similarity to a known pharmacophore, and biological activity. The pIC_{50} values served as a measure of the potency or biological activity of the ligands.

The pIC_{50} values of the refined ligands ranged from 4.866 to 9.398. In Schrödinger Maestro, structural alignment was deployed to identify similar ligand structures, focusing on identifying the core for each structure to align the molecules effectively. The field-based model was built on the Gaussian field domain that utilized a training set of 70% of the total input of refined ligands and a random seed set to 0, with a maximum of four partial least square factors. Both the steric and the electrostatic force fields were set truncated at 30.0 kcal/mol, and the cross-validation was performed by leaving out just one ligand.

Statistical analysis methods, such as comparative molecular field analysis (CoMFA) or comparative molecular similarity indices analysis (CoMSIA), as well as partial least squares (PLS), were applied to correlate the calculated descriptors with the activity values of the ligands in the training set. The generated correlation was utilized to predict the activity of new compounds based on their 3D structures. In addition, five Gaussian field fractions, including steric, electrostatic, hydrophobic, hydrogen bond acceptor, and hydrogen bond donor, were evaluated to provide insight into the field interactions of the ligands within the binding pocket of the receptor. An optimal number of PLS factors that can balance the trade-off between data fitting and model prediction was chosen.

2.8. Rational design of a new ligand

The rational design of a new ligand in this study involved a robust approach to iteratively modifying the skeletal structure of a lead compound, considered the reference ligand. The reference ligand was obtained after virtual screening-molecular docking, and the interactive pose prediction (IPP) panel offered in Schrödinger Maestro was employed for a new ligand design. The IPP operated under the maximum common substructure (MCS) constrained docking type, which simultaneously docked the compounds into the binding site of proteins using a grid-based approach. The GlideScore value and predicted biological activity from the 3D-QSAR model were the metrics employed for performance verification between the newly designed compound and the reference lead compound. Furthermore, the interaction pattern of the designed ligand within the protein was also studied. In addition, the ADMET-related indices of the new ligand were assessed using the QikProp program in Schrödinger Maestro at normal mode to evaluate its pharmacokinetics, efficacy, and safety profiles. All ADMET-related indices for the new compounds were evaluated using a total of 50 descriptors with a #star parameter as an indicator of several property descriptors computed by QikProp that violate a given optimum range of values for 95% of known drugs.

3. Results and discussion

3.1. Data preprocessing

Several ligands obtained from the database were dropped during the data preprocessing step due to missing column information, inconsistencies, and ambiguities in the data structure. This step resulted in reducing the initial dataset size from 3994 rows of ligands to 2972 rows and 48 columns, ensuring a clean dataset for analysis. In addition, the computed pIC_{50} values (activity) for each ligand ranged from 4.54 to 10.15 throughout the dataset. Using pIC_{50} as a measure of activity guarantees that the potency of different compounds can be precisely compared, facilitating the evaluation of their efficacy and the selection of the most promising candidates for further research or drug development.

3.2. Protein complex refinement

In mechanistic studies involving drug design, molecular docking, and prediction of protein functions, refining and minimizing protein structures play a significant role in improving their utility in pharmaceutical applications.[49] In Figure 4A, the 6PYS protein structure representing the human PI3K-α protein complex possesses inherent local and global errors, including irregular contacts or hydrogen bonds, chain breaks and atomic clashes, and unusual bond angles and lengths.[50] However, refining a protein obtained from a database before docking improves the accuracy and reliability of docking results.

Figure 4B illustrates the schematics of the refined 6PYS protein complex with the necessary side-chain

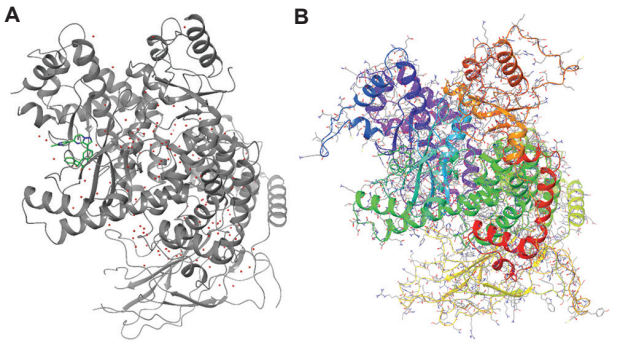

Figure 4. The human PI3K-α protein complex (Protein Data Bank ID.: 6PYS) with its cocrystallized ligand, (3S)-3-benzyl-3-methyl-5-[5-(2-methylpyrimidin-5-yl)pyrazolo[1,5-a]pyrimidin-3-yl]-1,3-dihydro-2H-indol-2-one (P5J). (A) Cartoon representation of the raw form; and (B) minimized-refined form. Red dots in (A) represent water molecules.

conformations, addressed chain breaks, added missing atoms or residues, assigned bond orders, converted selenomethionines, and deleted far-water molecules. In addition, refinement of the cocrystallize ligand enables the optimization of its positional and thermal parameters, resulting in a more precise representation of its interactions within the active site of the receptor molecule. This optimization accounted for the spatial arrangement of atoms, bond lengths, bond angles, and torsional angles of the ligand. Moreover, ligand refinement allows for the assessment of ligand-receptor interactions, such as hydrogen bonding, Van der Waals contacts, and electrostatic interactions. Precise refinement of these interactions provides insights into the binding affinity, specificity, and structural basis of ligand recognition. Furthermore, ligand refinement evaluates the underlying principles of binding affinity, specificity, and structural basis of the ligand concerning optimized ligand refinement parameters, including the assignment of proper bond orders, generation of accessible tautomers and ionization states, and prior virtual screening.[50]

3.3. Protein-ligand binding pocket identification

The binding pockets/sites of several therapeutic targets are significantly impacted by protein dynamics.[51] Moreover, the structural information of the protein-ligand complex can accelerate the optimization process of potential lead compounds and help solve problems related to compound selectivity, pharmacokinetics, and patentability.[52] To ensure specificity and further relay information between active and allosteric sites, protein-ligand binding is enhanced by physical interactions between the binding site residues of the protein and the ligand.[53]

Identifying binding sites that predict the concavity where the core scaffold can bind with the protein is essential for rational drug design. Binding pocket identification for

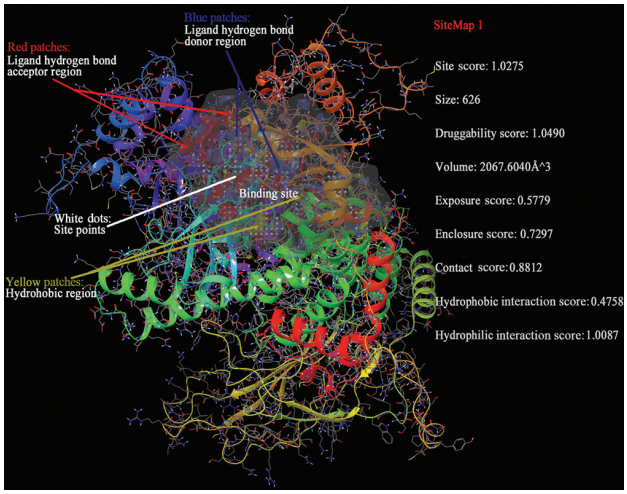

Figure 5. A visual representation of potential 6PYS (human PI3K-α protein complex) binding site (SiteMap 1).

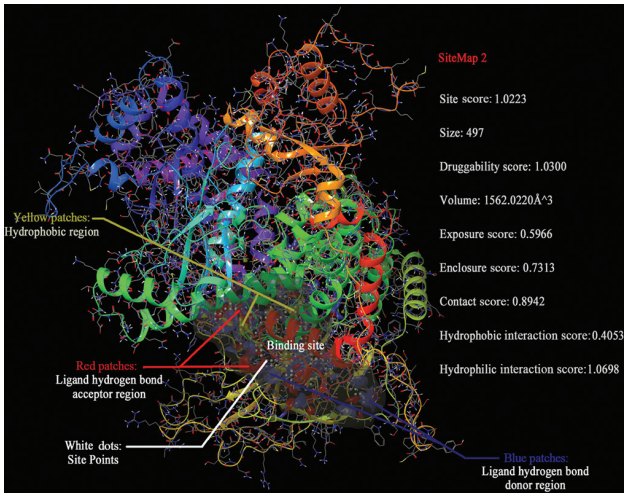

Figure 6. A visual representation of potential 6PYS (human PI3K-α protein complex) binding site (SiteMap 2).

6PYS protein and P5J ligand is depicted in Figures 5-9. In each figure, the binding site constituted some ligand hydrogen bond donor and acceptor regions, along with sites where hydrophobic interactions could occur. These specific properties of the protein significantly influence ligand binding and interaction.

The binding site score is a metric used to assess the likelihood or potency of an interaction between a small-molecule ligand and a protein at a specific binding site. It quantifies the propensity of the ligand to bind to the local site of the protein, facilitating firm ligand binding and the formation of a stable complex.[54] The score is usually presented as a numerical value and can be used to rank or prioritize ligands based on their potential binding affinity to the protein. It is computed based

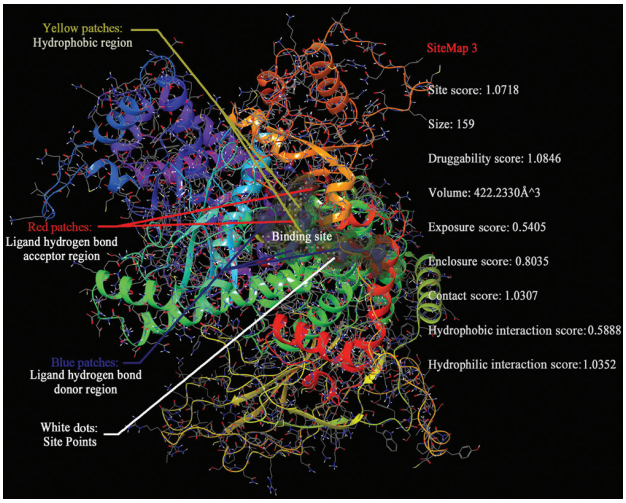

Figure 7. A visual representation of potential 6PYS (human PI3K-α protein complex) binding site (SiteMap 3).

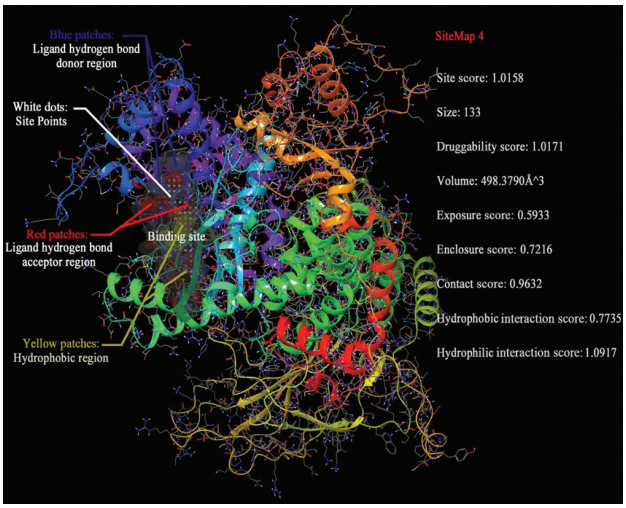

Figure 8. A visual representation of potential 6PYS (human PI3K-α protein complex) binding site (SiteMap 4).

on factors such as the exposure of the site to solvent, the degree of enclosure by the protein, and the degree of hydrophilicity/hydrophobicity. A higher binding site score indicates a stronger predicted interaction, suggesting a higher likelihood of optimized binding and potential biological activity. Conversely, a lower score indicates a weaker predicted interaction. A site score above 1 indicates a highly druggable protein pocket, while a score of 0.8 distinguishes between drug-binding and non-drug-binding sites.[54] The average site score value for sub-micromolar binding sites is 1.01.[55] In Figures 5-9, the computed site scores were mostly above 0.95. However, despite the nearly identical site score values, the site score value of SiteMap 3 (Figure 7) ranked highest at 1.0718.

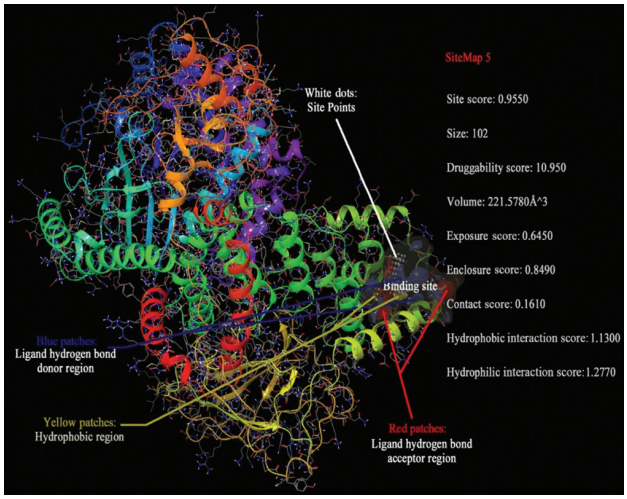

Figure 9. A visual representation of potential 6PYS (human PI3K-α protein complex) binding site (SiteMap 5).

These results suggested that while the site score may aid in deciding the target site, it may not provide sufficient information about the drug-binding state of the site,[55] as it relies on computational estimations and is primarily useful for predicting ligand-protein interactions.

The size of the binding site corresponds to the size of the interacting ligand, determining the maximum ligand size that can fit and interact with the protein. However, ligand binding pockets varied widely in size, as shown in Figures 5-9. Comparatively, a binding site size of 102 (Figure 9**,** SiteMap 5) would typically hold a smaller ligand than a binding site of size 162 (Figure 8**,** SiteMap 4). Moreover, the binding of ligands of different sizes, shapes, and compositions implies a different geometry of the receptor binding sites that allow the movement of backbone and side-chain atoms, resulting in differences in the details of residue interactions.

Druggability refers to the likelihood of a drug-like compound or molecule modulating or exhibiting protein interaction[54] at a therapeutically useful level of affinity. Here, the druggability of the protein was evaluated using SiteMap. Interestingly, Figures 5-9 revealed a wide range of druggability scores (0.9504 – 1.0846) for the refined protein. Among the identified druggable pockets, SiteMap 3 (Figure 7) ranked the highest, with a score of 1.0846 as compared to others. It is noteworthy that a druggability score over 0.5 and closer to 1.0 indicates good druggability. The druggability score is computed based on the enclosure and hydrophilicity (hydrophilic interaction score) of the tested pocket (Equation I).

In Figures 5-9, the enclosure score ranged from 0.6314 – 0.8035. There is a positive correlation between the

enclosure score and the druggability score, indicating that a rise in the druggability score corresponds to a higher degree of cavity enclosure, represented by the enclosure score. A higher enclosure score suggests an envelope with a tighter barrier that would prevent ligands from releasing once it is enclosed. Hence, an enclosure score of 0.8035 depicted a high-affinity enclosure (potentially stimulus-sensitive) for the 6PYS human PI3K-*α* protein to envelope a ligand and act as a diffusion barrier, providing an excellent slow release.[56]

Conversely, the pocket hydrophilicity (hydrophilic interaction score) of the protein depicted an inverse correlation with the druggability score, as illustrated in Figures 5-9. The hydrophilicity of the binding pockets primarily favors polar ligands,[57] which have a strong affinity for water and are soluble in water. Although the results shown in Figures 5-9 are approximately identical, SiteMap 5 obtained the best hydrophilic interaction score of 1.1301, indicating that the binding site provided a better hydrophilic environment with a higher affinity for interacting with polar ligands and water compared to other binding sites.

In addition, Figures 5-9 present the computed results of the volume of potential binding sites from the respective SiteMaps. The volume of the binding pocket plays a significant role in the process of rational drug discovery. The size and shape of the drug molecule that can successfully bind to the protein depend on the volume of the binding pocket. While a smaller binding pocket might be better suited for tiny molecules, a larger binding pocket might be able to accommodate larger medication molecules. In Figures 5-9, binding SiteMap 1 suggested a larger binding volume of 2067.6040 $Å^3$ when compared to the other binding SiteMaps. In any case, a well-fitted ligand into the receptor pocket can trigger signaling transduction, while a mismatch in volume fitting may decrease responses.

Figures 5-9 display the result of the ligand-receptor complex contact score. The contact score is a property that evaluates the strength of the average site-point interactions, such as hydrogen bonding, electrostatic interactions, hydrophobic interactions, and non-bonded Van der Waals interactions with the receptor.[55] These interactions contribute to the stability and strength of the ligand-receptor complex and are essential for effective binding. The contact score provides a numerical value representing the quality of the ligand-receptor interaction, calibrated such that the average score for the sub-micromolar sites is 1.0, facilitating comparison between sites, where a higher value indicates better interaction. In Figures 5-9, the contact score ranged from 0.8492 – 1.0307. SiteMap 5 possessed the highest contact score value (1.0307), indicating a strong and favorable interaction of the ligand with the target receptor, suggesting a higher potential for binding and therapeutic activity of optimal efficacy.

Figures 5-9 depict the computed results for the binding site exposure score, which explains the degree of accessibility or availability of the binding site to interact with a ligand, as well as its openness to solvents. However, in Figures 5-9, the exposure scores of the SiteMaps range from 0.5405 – 0.6446. A lower exposure score indicates a deeper or well-encapsulated site favorable for excellent ligand-protein binding. More so, for a favorable binding, the Van der Waals contact with the receptor should lie at least 4 Å from the nearest protein atom. Hence, the numeric value of the exposure score of SiteMap 5 (Figure 9) suggested that better molecular mechanotransduction could occur, due to a deeper and better-encapsulated site location in the receptor for ligand binding.[55]

The dynamics around the hydrophobicity of a binding site is another important property that ensures the presence of hydrophobic characters necessary for high binding affinity. Hydrophobicity is a characteristic that measures the extent to which a molecule is able to repel water. Proteins tend to bury hydrophobic residues within their core during the folding process to stabilize the protein structure and prevent aggregation. However, the hydrophobic interaction score measures how effectively hydrophobic molecules interact with each other in a solvent environment. Figures 5-9 depict the results of the respective SiteMaps' hydrophobic interaction scores. The hydrophobic interaction score for SiteMap 4 (Figure 8) was 0.7735, ranking top among all the SiteMaps assessed. This result depicted a region with a higher propensity for stronger hydrophobic binding sites to bind to ligands with higher affinity. In general, entropic phenomena cause hydrophobic molecules to interact with each other in water.[57]

3.4. Molecular docking analyses

In this study, we report a comprehensive workflow involving three stages of virtual screening processes: HTVS, SP, and XP modes that simultaneously incorporated molecular docking at each stage to discover potential top-performing PI3K-*α* inhibitors for the suppression of human PI3K-*α*-related problems.

Table 1 reveals four potential candidates identified as top-ranked inhibitory molecules from the virtual screening and docking of several ligands on the human 6PYS protein complex. The molecular docking results of these compounds revealed that the structural motifs of the top-ranked potential inhibitory molecules that could bind to the 6PYS human PI3K-*α* protein consisted of cyclic sulfonamide derivatives attached to pyridine cores,

Table 1. Molecular docking result from the three-stage virtual screening workflow

Reordered ligand candidacy number	Candidate 1	Candidate 2	Candidate 3	Candidate 4
Ligand digital nomenclature	00310001.cdx	00254001.cdx	00137001.cdx	00199001.cdx
Ligand nomenclature	3-(6-amino-5-(2-methyl oxazol-5-yl) pyridin-3-yl)-N-((3-hydroxy oxetan-3-yl) methyl)-4-methylbenzenesulfonamide	3-(6-Amino-5-(isoxazol-5-yl) pyridin-3-yl)-N-(2-hydroxy-2-methylpropyl) -4-methylbenzenesulfonamide	3-[6-Amino-5-(2-methyl-oxazol-5-yl)-pyridin-3-yl]-N-(3-hydroxy-3-methyl-butyl)-4-methylbenzenesulfonamide	3-(6-Amino-5-(3-methyl-1,2,4-oxadiazol-5-yl) pyridin-3-yl)-N-((4-(hydroxymethyl) tetrahydro-2H-pyran-4-yl) methyl)-4-methylbenzenesulfonamide
Activity (pIC_{50})	6.5086	6.4685	6.8539	6.2518
glide gscore	−12.8675	−12.6943	−12.7015	−12.6736
glide evdw	−38.4417	−41.9224	−43.5924	−45.2563
glide energy	−49.8733	−49.7553	−54.6716	−53.5503
glide einternal	5.0994	6.2456	5.8060	4.5817
glide emodel	−80.7610	−77.5800	−84.7264	−81.8777
XP HBond	−2.1467	−1.9817	−1.9926	−1.7768
XP PhobEn	−2.0000	−1.9500	−1.9500	−2.0750
XP LowMW	−0.0651	−0.1584	−0.0649	0.0000
XP RotPenal	0.1931	0.2181	0.2253	0.1894
XP LipophilicEvdW	−5.8921	−6.2412	−5.9585	−6.1663
XP Electro	−0.8574	−0.5875	−0.8309	−0.6220
XP Sitemap	−0.1494	−0.1468	−0.1799	−0.3769
XP ExposPenal	0.0000	0.1032	0.0000	0.1040
XP ClBr (halogen contribution)	0.0000	0.0000	0.0000	0.0000
XP PiCat	0.0000	0.0000	0.0000	0.0000
glide eff state penalty	0.0206	0.0008	0.0206	0.0008
Binding affinity	−9.0456	−8.9569	−8.9619	−8.9420
Computed RMSD against reference ligand structure (Å)	2.98	2.98	2.97	2.94

along with several other substituent moieties. While these moieties vary, they may be derived from alkaloids.

Surprisingly, sulfonamide derivatives represent a class of intriguing compounds with a diverse range of pharmacological activities,[58-60] including anti-cancer, anti-bacterial,[59,61-66] anti-fungal,[59,67] anti-oxidant,[59,68,69] anti-inflammatory,[59,62,70,71] and anti-diabetic[60,72] activities. However, the results presented in Table 1 suggest that a hybrid of sulfonamide and pyridine-based heterocyclic compounds functionalized with other potent moieties exhibits adequate synergistic biological effects capable of enhancing sufficient biological activity against PI3K-*α*. The binding affinity values for the respective candidates demonstrated a reasonable reflection of a spontaneous and favorable free energy change toward the bound complex. In addition, in Table 1, the RMSD values between the docked compounds and the cocrystallized ligand (P5J) were within the threshold of 2 – 3Å, suggesting a successful docking pose prediction with appreciable biological relevance, attributed to the chemostructural similarities of the candidates. Furthermore, in Table 1, the values of other crucial parameters for the respective candidates offer significant insights into key mechanistic interactions between a ligand and the target, PI3K-*α*.

3.5. Field-based 3D-QSAR analysis

To establish the structure-activity relationships of the selected human PI3K-*α* inhibitory compounds, a robust field-based 3D-QSAR model was employed via an *in silico* technique. The field-based 3D-QSAR model was a hybrid model composed of a force field model and a Gaussian

model. While the force field model is similar to the comparative molecular field analysis (CoMFA) models, the Gaussian model is similar to the comparative molecular structure analysis (CoMSIA). However, the hybrid model is built on the OPLS-3e force field, which requires a set of aligned compounds in a 3D space with known activities for predictive operation.

Table 2 summarizes the performance of the robust field-based 3D-QSAR model, organized into four different partial least squares (PLS) regression sub-models. Each row illustrates the application of special multivariate statistical analysis routines – PLS factors – used in building the model. The PLS factors are linear combinations of the original predator variables that are used to fit a linear regression model. The number of PLS factors or latent variables was employed to extract the most relevant information from the molecular descriptors and the biological activity data of the inhibitors. Based on the pIC_{50} as the activity property of the inhibitory compounds, the model performance was validated across several statistical indices, such as standard deviation (SD), coefficient of determination (R^2), R^2 cross-validation for the training set, R^2 scramble (R^2 *Scr*), model stability, *F*-value, *p*-value, root mean square error (RMSE), cross-validated coefficient of determination (Q^2) for the test set, and the Pearson-*r* correlation with the test set of the model. Hence, the PLS factors are compared with the best performer selected to depict the field-based 3D-QSAR model.

The SD is a descriptive statistic used in the majority of clinical and experimental studies, and it illustrates how the mean represents sample data while also evaluating the variation in a dataset that follows a normal distribution.[73-75] A low SD indicates more stable and consistent sample data whose values are close to the mean, while a high standard deviation indicates that the values are spread out (scattered) over a wide range, away from the mean. Thus, as shown in Table 2, the order of significance of the SD for the four PLS factors is illustrated by the following trend:

SD: PLS factor 4 ⟹ *PLS factor* 3 ⟹ *PLS factor* 2 ⟹ *PLS factor* 1

The coefficient of determination (R^2) is the measure of the proportion of variance in the observed activity that is explained by the model.[76] Statistically, the R^2 value ranges from 0 to 1, with 1 indicating the excellent explanatory power of a model. Furthermore, a high R^2 value indicates a good fit of the model data. Hence, in Table 2, the hierarchy of importance in the value of R^2 across each PLS factor is depicted in the following trend:

R^2: *PLS factor* 4 ⟹ *PLS factor* 3 ⟹ *PLS factor* 2 ⟹ *PLS factor* 1

Emphatically, it is important to note that a high value of R^2 does not necessarily translate to a better model when interpreting the R^2 statistic of a QSAR model for novel drug discoveries, since a high R^2 may be achieved by overfitting the model to the training data. Furthermore, it does not imply a causality nor provide a mechanistic interpretation of the QSAR model,[77,78] which is often misconceived among many researchers.

The coefficient of determination obtained by cross-validation (R^2 cross-validation) is a statistical method used to assess the predictive performance of the model on new data. In addition, the R^2 cross-validation values were computed from the predictions obtained by a leave-one-out (LOO) approach as a measure of the predictability of the CoMFA mode. According to the CoMFA approach, changes in the biological activities or binding affinities of sample compounds correlate with the variations in the steric and electrostatic fields of the molecules since drug-receptor interactions are typically non-covalent. In a typical CoMFA procedure, the steric and electrostatic fields around each molecule under study are sampled using probe atoms, typically sp3 carbon atoms with +1 charge, on a rectangular grid that includes the structurally aligned molecules.[79] The R^2 cross-validation value greater than 0.3 is considered significant. However, in Table 2, only PLS factor 1 deviated from significance in that its R^2 cross-validation value was 0.2157. Hence, the following trend depicted the order of significance among the four PLS factors:

R^2CV: *PLS factor* 4 ⟹ *PLS factor* 3 ⟹ *PLS factor* 2 ⟹ *PLS factor* 1

Table 2. Summary of Field-based 3D-QSAR model performance

# PLS factors	Standard deviation	R^2	R^2 cross-validation	R^2 scramble	Stability	*F*-value	*P*-value	RMSE	Q^2	Pearson-*r*
1	0.7356	0.2815	0.2157	0.0499	0.994	37.2	2.28E-08	0.67	0.3583	0.6035
2	0.5484	0.6049	0.4424	0.1749	0.963	72.0	1.11E-19	0.50	0.6486	0.8062
3	0.4370	0.7517	0.4643	0.2736	0.875	93.9	4.94E-28	0.45	0.7185	0.8488
4	0.3622	0.8313	0.4502	0.3674	0.721	113.3	1.11E-34	0.40	0.7776	0.8825

Abbreviations: PLS: Partial least squares; RMSE: Root mean square error.

The coefficient of determination obtained by scrambling, R^2 scramble (R^2Scr), is one of the statistical methods to test the significance of a 3D-QSAR model. It is the average value of R^2 from a series of models built using scrambled activities and measures the degree to which the molecular fields can fit meaningless data. Table 2 shows the values of R^2 scramble for the respective PLS factors, reveals that PLS factor 1 scored lowest with a value of 0.0499, while PLS factor 4 scored a value of 0.374. Moreover, a high R^2 scramble indicates that the model is not meaningful and may be overfitting the data. Hence, the model containing a PLS factor emerged as the most significant model with meaningful data fitting. Concerning R^2Scr values for various PLS factors in Table 2, the following trend summarizes the order of meaningfulness of models as obtained by random shuffles of the values of the bioactivity response variable:

R^2Scr: PLS factor 4 ⟹ *PLS factor* 3 ⟹ *PLS factor* 2 ⟹ *PLS factor* 1

Table 2 also shows the value of stability of respective PLS factors. Stability accounts for how stable the PLS factors are when different subsets of data are used to fit the model. This statistic ranges from 0 to 1, where 1 means that the factors are identical for all subsets (stable), and 0 means that they are completely different. However, it is inferred from Table 2 that PLS factor 1 is more stable at model predictions to changes in the training set composition than other PLS factors, as highlighted in the following trend:

Stability: PLS factor 4 ⟹ *PLS factor* 3 ⟹ *PLS factor* 2 ⟹ *PLS factor* 1

F-value and *p*-value are also statistical indices used to validate the 3D-QSAR model. *F*-value statistic tests whether adding a new PLS factor to the model significantly improves its fit or not, while *p*-value is a probability that measures how likely it is to obtain *F*-value as large or larger than the observed one by chance alone, assuming that adding a new PLS factor does not improve the fit of the model. *F*-value is computed by comparing the sum of square errors (SSE) of two nested modes: one with k PLS factors and one with k+1 PLS factors. A higher *F*-value means that adding a new PLS factor reduces the SSE significantly and improves the fit of the model. Conversely, a lower *p*-value means that adding a new PLS factor is more significant and not due to chance. Table 2 suggested that PLS factor 4 had a higher *F*-value (113.3) when compared with the other PLS factors, which means that its addition to the model would significantly improve its fit and reduce the SSE. The hierarchy of *F*-value significance is depicted below:

F-value PLS factor 4 ⟹ *PLS factor* 3 ⟹ *PLS factor* 2 ⟹ *PLS factor* 1

PLS factor 4 had the lowest *P*-value (1.11E-34) among all four PLS factors, and adding it to the model would improve the model significantly, but not due to chance. Hence, the following trend is the case for the order of *P*-value significance among the PLS Factors:

P-value PLS factor 4 ⟹ *PLS factor* 3 ⟹ *PLS factor* 2 ⟹ *PLS factor* 1

The remaining aspects of Table 2 were statistics typically associated with the test set of the input data used to build the 3D-QSAR model. The RMSE is the statistic that describes how close the predicted values of the dependent variable are to its actual values. A lower RMSE means that the predictions are more accurate and have fewer errors. However, Table 2 revealed the lowest RMSE value for PLS factor 4, while the RMSE value of 0.67, which represented PLS factor 1, suggested a slightly weaker model prediction with somewhat more errors than other PLS factors. The trend for the order of significance is presented as follows:

RMSE: PLS factor 4 ⟹ *PLS factor 3* ⟹ *PLS factor 2* ⟹ *PLS factor 1*

The predictive squared correlation coefficient of the test set, Q^2, is a statistical property that reinforces the validity of a QSAR model based on quantifying the predictive ability of the model in the aspects concerning reliability, accuracy, and applicability domain of the model. The Q^2 is obtained from methods based on simple reuse, such as leave-one-out and leave-many-out cross-validation.[80] This parameter is important and has become well-known because it takes values in a normalized range (i.e., ≤1), thereby permitting a trivial understanding of its values and easy comparison of different QSAR models and the different performance of fitting and predictive capabilities of a model. However, a higher Q^2 means that the predictions are reliable and have less uncertainty. Hence, in Table 2, it is revealed that the computed value for Q^2 for PLS factor 4 exceeded the Q^2 values of other PLS factors. This implied a more confident final result valid both for internal validation, such as cross-validation or bootstrap, as well as external validation[80] and the following trend depicts the reliability order of Q^2 on model:

Q^2: PLS factor 4 ⟹ *PLS factor* 3 ⟹ *PLS factor* 2 ⟹ *PLS factor* 1

The Pearson correlation coefficient, or Pearson-*r* statistic, assesses the strength of the correlation between two continuous variables, ranging from 0 to 1. A value of 1 indicates a stronger correlation, while a value of 0 represents a weaker correlation. Table 2 illustrates the Pearson correlation coefficient, which estimates the degree of correlation between the respective PLS factors and the predicted activities of the model using the test set. It is therein in Table 2 that PLS factor 4, with a Pearson-*r*

value of 0.8825, held a stronger correlation with the model than other PLS factors, even as PLS factor 3 ranked next. However, the rank which the degree of correlation is depicted in the following trend:

Pearson–r: *PLS factor* 4 ⟹ *PLS factor* 3 ⟹ *PLS factor* 2 ⟹ *PLS factor* 1

The table can be used to compare different models with different numbers of factors and select the best one based on the given statistical criteria. However, a good QSAR model should not only have a high R^2 value but also have high predictive power, robustness, interpretability, applicability, etc. It is logical to choose a model that has high R^2, Q^2], and Pearson-*r*, and a low RMSE and *p*-value, while avoiding overfitting (indicated by a large gap between R^2 and R^2 CV or a high R^2 *Scr*) and instability (indicated by a low stability). It is inferred from Table 2 that PLS factors 4 and 3 were the best performers among other PLS factors. To compare, PLS factor 4 performed better than PLS factor 3 in terms of fit, significance, accuracy, reliability, and correlation, but worse in terms of variation, prediction, generalization, and stability. Based on these critical criteria, PLS factor 3 was considered the optimal PLS factor, which offered the safest approach that accounted for model performance and quality.

Table 3 displays the percentage contribution of different Gaussian descriptors in the QSAR model for each number of PLS factors used in the model. The Gaussian descriptors encode the mean and covariance information of local features in a graphical object.[81] In Table 3, PLS factor 3 comprised 27.3% steric, 10.5% electrostatic, 19.4% hydrophobic, 25.8% hydrogen bond acceptor, and 17% hydrogen bond donor descriptors. These values indicate the contribution of each Gaussian descriptor to PLS factor 3 in analyzing the relationship between the predictor variables and the response variable in the regression model. The result suggests that most of the binding energy predominantly emanated from steric (27%) and hydrogen bond acceptor (25.8%) interactions. Steric and hydrogen bond acceptor interactions influence protein-ligand binding affinity and specificity. Steric interactions depict the shape complementarity and spatial fit of the ligand, as well as influence the entropy of the binding process of the protein-ligand complex.[82] On the other hand, hydrogen bond acceptor interactions illustrate how ligands accept electrons from a protein donor, stabilize the complex, and enhance ligand recognition within the protein,[83] in which the hydrogen bond paring influences shifts in pKa values of interacting groups and also affects charge distribution and electrostatic potential.[84]

In conjunction with Table 3, the visualization of the interactive contributions of the different Gaussian descriptors in the context of PLS factor 3 under study is graphically presented in Figure 10.

3.6. Rational design of a new ligand

3.6.1. Skeletal modifications

Medicinal chemistry continues to be impacted by innovative *in silico* methods, especially at the drug discovery stage using QSAR models. In this study, we attempted to design a new compound with enhanced binding affinity and specificity by enacting the desired chemical transformations on a reference ligand in a concise and chemospecific fashion. The concept of "molecular editing" involves building onto, modifying, or pruning molecules atom by atom, utilizing transformations that are adequately mild and selective for application in the later stages of drug synthesis and sequencing.[85]

The skeletal editing of the top-ranked hit compound (Candidate 1, in Table 1), which was obtained from the robust virtual screening-molecular docking workflow, involved some heterocyclic modifications that were in conformation with the congeneric series of molecules used in docking. The molecular modification between the reference compound and the resulting T85 is illustrated in Figure 11. The skeletal editing of the reference hit compound was associated with molecular modifications in the heterocycles of its oxazole and the oxetane-pyridine sub-cores, with fluorination and bipyridine formation dispatched, respectively. However, the fluorination involved the replacement of the 2-methyl group on the oxazole ring by a fluorine atom, and the pyridine ring was fused with another pyridine ring at the 3-position. The resulting putative ligand, referred to as T85, holds promise as the next synthetic hit compound.

Table 3. Molecular field fraction analysis

# PLS factors	Gaussian steric (%)	Gaussian electrostatic (%)	Gaussian hydrophobic (%)	Gaussian hydrogen bond acceptor (%)	Gaussian hydrogen bond donor (%)
1	0.428119	0.056828	0.172533	0.217674	0.124847
2	0.315070	0.093522	0.197463	0.237846	0.156099
3	0.273183	0.105146	0.194088	0.257736	0.169848
4	0.312171	0.093195	0.190332	0.260085	0.144218

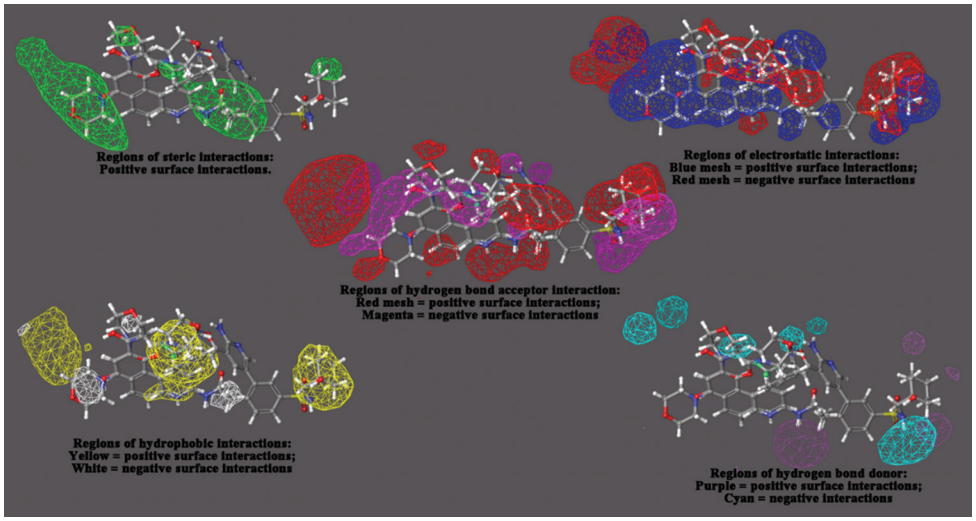

Figure 10. Field-based QSAR visualization of Gaussian descriptors for PLS factor 3.

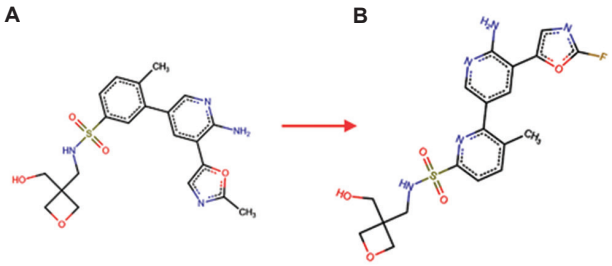

Figure 11. T85 (B) from the structural modification of Candidate 1 (reference hit compound [A]). The nomenclature of (A) and its SMILES notation is 3-[6-amino-5-(2-methyl-1,3-oxazol-5-yl)pyridin-3-yl]-N-{[3-(hydroxymethyl)oxetan-3-yl]methyl}-4-methylbenzene-1-sulfonamide and Cc1ncc(o1)-c1cc(cnc1N)-c1cc(ccc1C)S(=O)(=O)NCC1(CO)COC1, respectively, while the nomenclature of T85 (B) and its SMILES notation is: 6'-amino-5'-(2-fluoro-1,3-oxazol-5-yl)-N-{[3-(hydroxymethyl)oxetan-3-yl]methyl}-3-methyl-[2,3'-bipyridine]-6-sulfonamide and Cc1ccc(nc1-c1cnc(N)c(c1)-c1cnc(F)o1)S(=O)(=O)NCC1(CO)COC1, respectively.

The concept of incorporating a fluorine atom in the compound was to furnish T85 with unconventional and distinctive properties. Although fluorine addition is not a panacea, its presence in the drug molecule is intended to improve the drug's biological activity, affecting both the pharmacokinetics and dynamic properties,[86] ultimately aiding in saving lives. For example, the production of fluorine-associated novel therapeutic drugs approved by the FDA in 2021 was utilized for controlling the COVID-19 pandemic and treating various diseases.[87,88]

The low metabolic stability of drugs is one of the critical problems in drug development. However, this could easily be circumvented by blocking the metabolically labile sites with fluorine substituents. Moreover, the carbon-fluorine (C-F) bond is one of the strongest single bonds due to the high electronegativity of fluorine, giving the bond a significant dipole moment or polarity. In addition, the C-F bond is relatively short, enacted by its partial ionic character, which also affects the strengths of other bonds, rendering other parts of the drug harder to degrade.[89,90] Hence, the drug persists longer, exerting a prolonged effect on the targeted disease or condition. We anticipate this phenomenon to hold true for T85.

Improving the lipophilicity of the intended drug was another design consideration in line with drug development protocols. For effective passive transportation across the cell membrane, the drug must readily traverse the lipid membrane without any hindrance.[87]Therefore, for a better drug compound, moderate lipophilicity is necessary. Fluorination is often a better option due to its high lipophilicity, which increases drug absorption.[87,91] In light of the reported findings, the deliberate inclusion of fluorine during the design of T85 can productively influence its intrinsic potency and membrane permeability, thereby fostering effective metabolic pathways and pharmacokinetic properties against human PI3K-α. Moreover, the addition of fluorine to a molecule to inhibit a protein kinase involves modulating the binding affinity and selectivity of the drug to its target receptor by altering the electronic and steric properties of the molecule, influencing the conformation and solubility of the drug.

The asymmetrical combination of pyridines – bipyridine – in the design of T85 is believed to confer various advantages, such as enhanced binding affinity, selectivity, stability, and drug solubility. This modification can also alter the electronic and steric properties of the molecule, facilitating interactions with different molecules through non-covalent interactions and potentially resulting in the formation of supramolecular structures with interesting

properties. Furthermore, bipyridine is capable of inducing chirality through ring functionalization or restricted rotation (atropisomerism), thus increasing its relevance in asymmetry-based applications. However, the inclusion of fluorine in the design of T85 is still a limited study, and its full potential has yet to be clinically verified.

Table 4 summarizes the performance comparison between the reference hit compound and T85 obtained from the IPP under the MCS-constrained docking type and 3D-QSAR model prediction. To compare docking scores, a docking score of -9.88 was better than -9.51. This meant that T85 was predicted to have a stronger binding affinity or interaction with the target protein compared to Candidate 1. In addition, Table 4 indicates that Candidate 1 possessed a lower biological activity (6.51) than T85 (8.25) in inhibiting the target protein.

3.6.2. T85-6PYS interactions

In this work, we studied the interactive behavior of T85 within the receptor grid of the target protein in a 2D space. Figure 12 illustrates the amino acid residues of the 6PYS protein that enveloped T85 in the target binding site. The interaction diagram revealed both hydrogen bonds and non-bonded contacts, presenting a list of amino acid residues along with their corresponding positions in the protein sequence. Together, these elements formed the characteristic binding pocket of the 6PYS protein in complex with the T85 compound. The diagram revealed hydrogen bond interactions between T85 and the 6PYS protein complex, particularly in the regions where LYS 802 and VAL 851 amino acid residues were located. Furthermore, π-π bonded interactions occurred between the pyridine ring of T85 and the amino acid residue TYR 836, as well as between the oxazole ring of T85 and the amino acid residue TRP 780. The π-π bonded interactions are non-covalent interactions among the residues of proteins and nucleic acids, between ligands and proteins, which have extraordinary significance in interpreting the dynamics of intricate biological systems and the biological activity necessary for drug discovery.[92] These interactions contribute to the stability and selectivity of the protein-ligand complexes by providing additional attractive forces and complementarity between the binding partners. These suggest that T85 exhibits an adequate number of interactions, indicating optimal complementarity between the ligand and the receptor.

Figure 13 describes the non-covalent interaction of the hydrophobic type established in the enclosure of T85 by several hydrophobic amino acid residues. Interestingly, the result revealed that 11 hydrophobic amino acid residues enveloped T85, with more isoleucine (ILE) amino acid residues involved in the ligand-receptor hydrophobic interaction network at different positions in the protein sequence. However, the presence of multiple hydrophobic

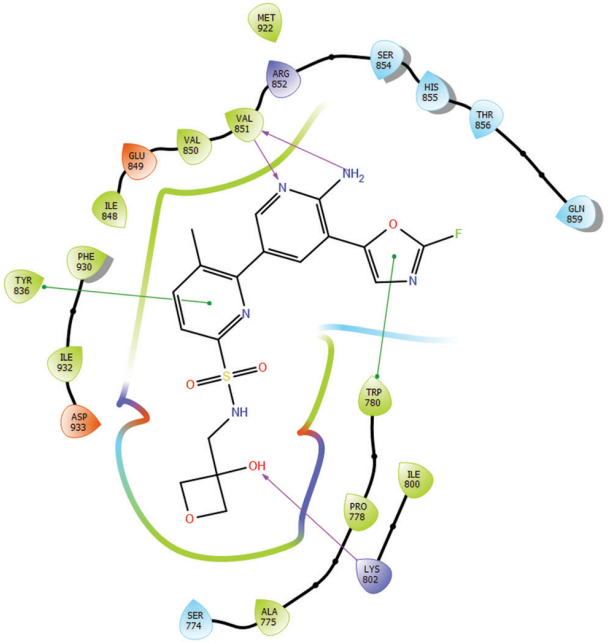

Figure 12. Two-dimensional schematics of T85 in the binding cavity 6PYS, surrounded by amino acids in their respective positions in the protein sequence.
Abbreviations: ALA: Alanine: ARG: Aginine; ASP: Aspartic acid; GLN: Glutamine; GLU: Glutamic acid; HIS: Histidine; ILE: Isoleucine; LYS: Lysine; MET: Methionine; PHE: Phenylalanine; PRO: Proline; SER: Serine; THR: Threonine; TRP: Tryptophan; TYR: Tyrosine; VAL: Valine.

Table 4. Performance comparison data between hit reference ligand and T85

Compound	Nomenclature	MCS- constrained docking score	Activity (pIC_{50})
Candidate 1 (hit reference compound)	3-[6-amino-5-(2-methyl-1,3-oxazol-5-yl) pyridin-3-yl]-N-{[3-(hydroxymethyl) oxetan-3-yl] methyl}-4-methylbenzene-1-sulfonamide	–9.51	6.51
T85	6'-amino-5'-(2-fluoro-1,3-oxazol-5-yl)-N-{[3-(hydroxymethyl) oxetan-3-yl] methyl}-3-methyl-[2,3'-bipyridine]-6-sulfonamide	–9.88	8.25

Abbreviation: MCS: Maximum common substructure.

amino acid residues around a ligand is crucial to driving coherent functional control of biomolecules.[93] Hence, from our simulation result, the formation of a hydrophobic core by the numerous hydrophobic amino acid residues around T85 demonstrated a strong interaction that indicated adequate ligand-receptor stability, binding specificity, and selectivity.

In addition, Figure 13 reveals the synergy between hydrophobic interactions and hydrogen bonds within the binding pocket of the 6PYS PI3K-α protein. The diagram depicted a hydrophobically packed correlated hydrogen bond, which is regarded as a signature type of interaction that explained the coexistence of polar (hydrogen bond) and non-polar (hydrophobic) interactions at certain locations of the binding cavity of the simulated T85-6PYS complex. The schematic revealed that hydrophobic interactions emanated from several unique hydrophobic amino acid residues of 6PYS, including VAL 851, TRP 780, VAL 850, TYR 836, PHE 930, ILE 800, ILE 848, LYS 802, and ILE 932.

The complexity of the interactions also involved the presence of hydrogen bonds within the hydrophobic vicinity of VAL 851. At a distance of 1.88 Å, a favorable intermolecular hydrogen bond interaction was formed between the polar hydrogen atom of T85 and an oxygen atom of the carbonyl functional group of the VAL 851 amino acid side chain (Figure 13). In addition, in the binding cavity of the protein, the nitrogen atom in the pyridine ring of T85 established another hydrogen bond with a hydrogen atom of the VAL 851 side chain with an interactive distance of 2.12 Å. These hydrogen bonds illustrated a strategic molecular recognition of T85 in its tautomeric state, signaling substantial therapeutic effects. The entrapment of T85 within the binding cavity of the 6PYS protein further illustrated that for T85 to be released, it would need to simultaneously break both hydrogen bonds with VAL 851 and all hydrophobic interactions between the hydrophobic amino acid residues of the receptor, implying a strong binding of the ligand to the receptor.

All hydrophobic amino acid residues are depicted as CPK representation, while the portions of the T85 that formed hydrophobic interaction are revealed in ball-and-tube representation.

Figure 14 describes the details of non-covalent interactions, especially hydrogen bonds and π-π (pi-pi) stacking interactions within the receptor-binding domain of the target 6PYS. Non-covalent interactions, such as hydrogen bonds, are ubiquitous in nature and serve a variety of significant roles in protein folding, protein-ligand interactions, catalysis, and maintaining specific

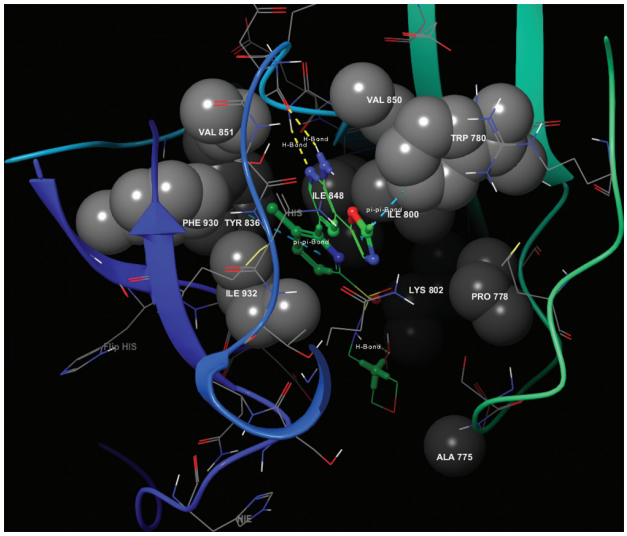

Figure 13. Hydrophobic encapsulation of T85 among several 6PYS protein residues.

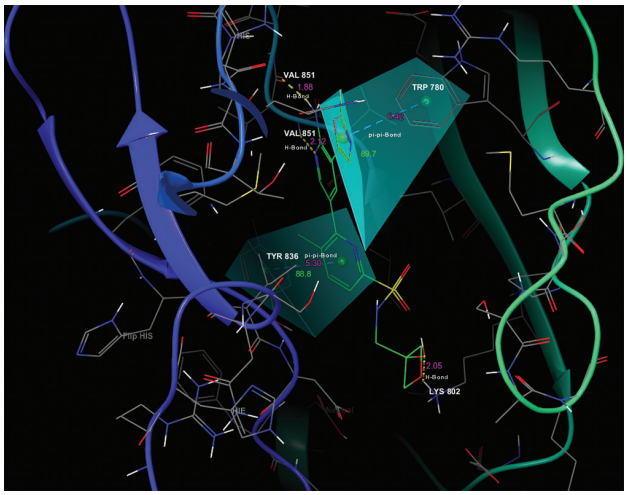

Figure 14. Hydrogen bond and π-π stacking interactions.

molecular conformations. Therefore, they are particularly important in biological systems.[94] Hence, numerous biological, chemical, and physical systems and processes are impacted by hydrogen bond interactions, which are essential in controlling the structure, characteristics, and activities of biomolecules.[95] On the other hand, π-π stacking interactions are a form of attractive and non-destructive non-covalent interactions between aromatic rings, which encompass π bonds, and whose contributions in a supramolecular assembly are vital for understanding the intrinsic nature of receptor-ligand binding domain.[96,97]

The hydrogen bond contributions between T85 and 6PYS are shown in Figure 14. The hydrogen bond interactions developed in the binding pocket of the receptor were

characterized by bond distances between certain atoms of T85 and side chains of the target, including VAL 850, VAL 851, and LYS 802. The architecture of the hydrogen bond interactions includes the H-N•••H between T85 and VAL 851 residue at a bond distance of 2.12 Å; the C-O•••H between VAL 851 residue and T85 at a bond distance of 1.88 Å; and the N-H•••O between amino acid residue LYS 802 and T85 at a bond distance of 2.05 Å. The architecture suggests that the hydrogen bonds formed within the computed bond distances indicate strong hydrogen bonds necessary for the effective binding of T85.

Figure 14 also presents another type of non-covalent interaction in our simulation, revealed as π-π stacking interactions. The distance and angle of the intermolecular contribution of π-π stacking interaction between the planes of the fluorine-associated oxazole aromatic ring of T85 and the indole ring of TRP 780 side-chain was computed at 5.42 Å and 89.7°. More so, another π-π stacking interaction existed at a different space within the binding site. This interaction manifested between the hydroxyphenyl ring along the side-chain of TYR 836 and the pyridine ring of T85 at a distance of 5.30 Å and an angle of 88.8°. Both π-π stacking interactions were coordinated in T-shaped configurations within the binding site of the 6PYS protein complex. This type of configuration involves quadrupole interactions among delocalized electrons in π-orbitals, leading to enhanced intermolecular electrostatic interactions.[97,98] Therefore, having multiple π-π stacking interactions between aromatic compounds between T85 and 6PYS suggested increased binding affinity and specificity of the ligand-protein interaction, which provided additional stabilizing forces and shape complementarity.

3.6.3. ADMET Investigations

To investigate the metabolic stability and safety profiles of T85, we assessed its clinical relevance using the QikProp program in Schrödinger Maestro. For this, we utilized all generated descriptors by QikProp, relevant for ADMET predictions to evaluate the pharmacokinetic profiles of T85. Table 5 displays a host of descriptors[99] computed by QikProp with their optimal tolerance ranges following

Table 5. Evaluation of the pharmacokinetic attributes of T85 ligand from Schrödinger's ADMET descriptors[99]

Descriptors	Description	Range or recommended values	T85
#stars	The quantity of attribute or descriptor values for known medications that are outside the 95% range of comparable values. A molecule with more stars than a few indicates that it is less drug-like than the other molecule. The quantity, #rotor, donorHB, accptHB, glob, QPpolrz, PlogPC16, QPlogPoct, QPlogPw, QPlogPo/w, logS, QPLogKhsa, QPlogBB, #metabol, and the following attributes and descriptors are taken into account while determining the #stars.	0 – 5	0
#amine	Quantity of amine groups that are not conjugated.	0 – 1	0
#amidine	Quantity of amidine and guanidine groups.	0	0
#acid	Count of groups of carboxylic acid.	0 – 1	0
#amide	Count of non-conjugated amide groups.	0 – 1	0
#rotor	Number of rotatable bonds that are non-trivial (not CX3) and non-hindered (not amide, tiny ring, or alkene).	0 – 15	7
#rtvFG	Reactive functional group count. These groups have the potential to cause toxicity, decomposition, or reactivity issues *in vivo,* as well as false-positive results in HTS assays.	0 – 2	0
CNS	CNS is measured on a scale ranging from -2 (indicating no activity) to+2 (indicating full activation).	−2 (inactive), +2 (active)	-2
Mol_MW (in Daltons, Da)	The molecule's molecular weight.	130.0 – 725.0	435.429
dipole†	The molecule's calculated dipole moment.	1.0 – 12.5	10.969
SASA	SASA (total solvent accessible surface area), measured in square angstroms with a 1.4 Å radius probe.	300.0 – 1000.0	648.546
FOSA	The SASA's hydrophobic component (saturated carbon and attached hydrogen).	0.0 – 750.0	208.350
FISA	The SASA's hydrophilic component (SASA on N, O, H on heteroatoms, and carbonyl C).	7.0 – 330.0	205.152

(Cont'd..)

Table 5. ***(Continued)***

Descriptors	Description	Range or recommended values	T85
PISA	The SASA's π (carbon and attached hydrogen) component.	0.0 – 450.0	182.467
WPSA	Weakly polar SASA component (halogens, P, and S).	0.0 – 175.0	52.577
Volume	The volume of solvent accessible in cubic angstroms, measured with a 1.4 Å radius probe.	500.0 – 2000.0	1179.394
donorHB	The approximate quantity of hydrogen bonding that the solute would provide to the molecules of water in an aqueous solution. The values represent averages calculated across multiple combinations.	0.0 – 6.0	3
accptHB	The approximate quantity of hydrogen bonding that a solute in an aqueous solution would accept from water molecules.	2.0 – 20.0	10.75
dip^2/V†	The molecule volume is split by the square of the dipole moment. This is the crucial term in the Kirkwood-Onsager equation, which calculates the free energy of solvation of a dipole with volume V.	0.00 – 0.13	0.102009
AC×DN^0.5/SA	Cohesive interaction index in solids. This term embodies the correlation (accptHB($\sqrt{donerHB}$))/(SA)	0.0 – 0.05	0.028710
glob	Globularity descriptor, $(4\pi r^2)$/(SASA); r=Radius of a sphere; volume equal to the molecular volume. Globularity is equal to 1.0 for a spherical molecule.	0.75 – 0.95	0.832396
QPpolrz	Polarizability prediction in cubic angstroms.	13.0 – 70.0	38.293
QPlogPC16	Predicted hexadecane/gas partition coefficient.	4.0 – 18.0	12.254
QPlogPoct‡	Predicted octanol/gas partition coefficient.	8.0 – 35.0	24.625
QPlogPw	Predicted water/gas partition coefficient.	−2.0 – 6.5	17.373
QPlogPo/w	Predicted octanol/water partition coefficient.	8.0 – 35.0	1.122
QPlogS	Aqueous solubility prediction, log S; S in mol dm^{-3} is the concentration of the solute in a saturated solution that is in equilibrium with the crystalline solid.	−6.5 – 0.5	-3.633
CIQPlogS	Conformation-independent predicted aqueous solubility, log S. S in mol dm^{-3}=concentration of the solute in a saturated solution that is in equilibrium with the crystalline solid.	−6.5 – 0.5	-4.59
QPlogHERG	Predicted IC_{50} value for blockage of HERG K^+channels.	Concern ≤- 5	-5.114
QPPCaco	Anticipated apparent permeability of Caco-2 cells in nm/sec. A model for the gut-blood barrier is provided by Caco-2 cells. The QikProp predictions pertain to passive transportation.	<25 poor, >500 great	112.317
QPlogBB	Estimated brain/blood partition coefficient. Note: QikProp predictions are for orally delivered drugs.	−3.0 – 1.2	-1.703
QPPMDCK	Estimated apparent permeability in nm/sec for MDCK cells. It is thought that MDCK cells are an excellent representation of the blood-brain barrier. For non-active transport, QikProp predictions are made.	<25 poor, >500 great	90.367
QPlogKp	Predicted skin (dermal) permeability, log Kp	-8.0 –-1.0	-3.986
IP (eV)†	PM3 calculated ionization potential (negative of HOMO energy).	7.9 – 10.5	8.667
EA (eV)†	PM3 calculated electron affinity (negative of LUMO energy).	−0.9 – 1.7	1.343
#metab‡	The quantity of possible metabolic reactions.	1 – 8	3
QPlogKhsa	Prediction of binding to human serum albumin.	−1.5 – 1.5	-0.44
HumanOralAborption	Human oral absorption predicted at the qualitative level: 1, 2, or 3 for low, medium, or high. The evaluation employs a set of knowledge-based guidelines that include determining appropriate values for logP, solubility, cell permeability, metabolite count, number of rotatable bonds, and percent human oral absorption.		3

(Cont'd..)

Table 5. ***(Continued)***

Descriptors	Description	Range or recommended values	T85
PercentHumanOralAbsorption	On a 0 – 100% scale, the predicted oral absorption by humans. An analysis of multiple linear regression data provides the basis for the forecast. HumanOralAbsorption and this attribute measure the same thing, hence their correlation is typically good.	>80% is high, <25% is poor	70.217
SAFluorine	The solvent-accessible surface area of fluorine atoms.	0.0 – 100.0	51.967
SAamideO	The solvent-accessible surface area of amide oxygen atoms.	0.0 – 35.0	0
PSA	Van der Waals surface area of polar nitrogen and oxygen atoms and carbonyl carbon atoms.	7.0 – 200.0	141.097
#NandO	Number of nitrogen and oxygen atoms.	2 – 15	10
RuleOfFive	Number of violations of Lipinski's rule of five. The rules are: mol_MW<500, QPlogPo/w < 5, donorHB ≤ 5, accptHB ≤ 10. Compounds that satisfy these rules are considered drug like. (The "five" refers to the limits, which are multiples of 5.).	Maximum is 4	0
RuleOfThree	Number of violations of Jorgensen's rule of three. The three rules are QPlogS > -5.7, QP PCaco>22 nm/s, # Primary Metabolites<7. Compounds with fewer (and preferably no) violations of these rules are more likely to be orally available.	Maximum is 3	0
#ringatoms	Number of atoms in a ring.		21
#in34	Number of atoms in 3- or 4-membered rings.		4
#in56	Number of atoms in 5- or 6-membered rings.		17
#noncon	Number of ring atoms not able to form conjugated aromatic systems (e.g., sp^3 C).		3
#nonHatm	Number of heavy atoms (non-hydrogen atoms).		30
Jm	Predicted maximum transdermal transport rate, Kp×MW×S ($\mu g\ cm^{-2}\ hr^{-1}$). The parameters (K_p and S) are obtained from the aqueous solubility and skin permeability, QPlog K_p and QPlogS.		0.01

95% of known drugs gleaned from a retrospective evaluation of drugs by the World Drug Index (WDI) to serve as a baseline for molecules. The drug-likeness and pharmacokinetic attributes of a compound are important considerations in drug development to guarantee that the molecule exhibits favorable characteristics for absorption, distribution, metabolism, excretion, and toxicity properties while possessing appropriate physicochemical properties for potential therapeutic use. Interestingly, T85 complied with the ADMET descriptors put forward by QikProp, and results were within optimal tolerance bounds, especially Lipinski's rule of five, Jorgensen's rule of three, the blood/brain barrier (BBB) penetration, access to the central nervous system, dermal penetration parameter ($QP\log K_p$), blockage of human ether-a-go-go-related gene potassium ion (hERG K^+), and so on, even the Verber rule.[100] The result culminated in the suitability of T85's development as a promising inhibitor for PI3K-*α*.

4. Conclusion

The enzyme PI3K-*α* plays a crucial role in regulating the growth, division, and survival of cells by phosphorylating specific lipids in the cell membrane, thereby activating downstream signaling pathways. It is connected to the frequently dysregulated PI3K/AKT/mTOR pathway in cancer. Increased signaling through this route can result from abnormal activation or mutations in PI3K-*α*, which supports cell survival, proliferation, and resistance to cell death. Such an imbalance may contribute to the development and spread of cancer. Mutations in the *PIK3CA* gene, which codes for the PI3K-*α* catalytic subunit, are frequently observed in cancer types such as lung, ovarian, colorectal, and breast cancers, contributing to increased PI3K activity and abnormal signaling that promotes cancer. To effectively target PI3K-*α* and other members of the PI3K/AKT/mTOR pathway, it is critical to rationally design potent multitarget inhibitors to address the heterogeneity and complexity of cancer cells, as well as the emergence of drug resistance and toxicity. In this study, we obtained the human PI3K-*α* protein (6PYS) co-crystallized with a ligand (PJ5) and selected PI3K-*α* inhibitors from protein and binding databases, respectively. The dataset comprised a congeneric series of 3D structures of selected PI3K-*α* inhibitors, which were

refined to improve the quality, accuracy, consistency, and completeness of the data while reducing noise, errors, outliers, and inconsistencies. A virtual screening workflow coupled with molecular docking evaluated the binding potentials of the refined ligands within the binding pocket 6PYS. Furthermore, the field-based QSAR technique elucidated the potential interactions between the ligands and probe atoms having specific attributes such as hydrophobic, electrostatic, hydrogen bond donor, or acceptor at each point in a 3D grid, highlighting key features influencing ligand activity, potency, and selectivity within the target binding site. In addition, the field-based QSAR model captured the shape, size, and flexibility of the ligands, along with their complementarity with the target. Multivariate PLS regression was employed to evaluate the relationship between the ligand molecular fields (independent variables) and the biological activity (dependent variable). Four PLS factors revealed important information from the data, indicating the influence of variance from the data on the 3D-QSAR model. A rational drug design approach was employed to design a molecule that could bind to the target and modulate its functions. The clinical relevance and ADMET properties of the newly designed molecule were predicted and evaluated according to recognized standards, including Lipinski's rule of five and Jorgensen's rule of three. Overall, the study proposes the use of advanced and reliable computational techniques to design novel and potent inhibitors of PI3K-α, a potential target for cancer immunotherapy, along with the repurposing of existing drugs for new indications.

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Conflict of interest

The authors declare that they have no competing interests.

Author contributions

Conceptualization: Kevin Tochukwu Dibia, Sandra Nneka Van-Dibia
Data curation: Kevin Tochukwu Dibia
Formal Analysis: Kevin Tochukwu Dibia
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Methodology: Kevin Tochukwu Dibia
Writing—Original Draft: Kevin Tochukwu Dibia
Writing—Review & Editing: Kevin Tochukwu Dibia, Sandra Nneka Van-Dibia

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data

Python codes for data preprocessing are available on request.

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ACCSCIENCE PUBLISHING

INNOSC Theranostics and Pharmacological Sciences

ORIGINAL RESEARCH ARTICLE

The effect of dapsone on skin flap survival depends on modulation of inflammatory response and VEGF expression

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Abstract

The random-pattern skin flap is a common method used for reconstructing skin defects. However, flap ischemia necrosis remains a significant challenge in plastic surgery. Strategies aimed at reducing persistent inflammation and promoting blood supply through angiogenesis have been identified as crucial for improving flap survival. Dapsone, a chemotherapeutic agent known for its anti-inflammatory properties through multiple pathways, is of interest in this regard. This study aims to investigate the effect of dapsone on random-pattern flap survival in rats, along with its impact on inflammation and angiogenesis. The ischemia/reperfusion (I/R) injury rat models were created using a caudal-based dorsal skin flap with delayed I/R. Twenty-four male Sprague Dawley rats were divided into control, sham, and two treatment groups receiving dapsone at doses of 12.5 mg/kg/day and 5 mg/kg/day, respectively. On the 7th post-operative day, flap survival was evaluated. Neutrophil infiltration and ulceration were measured through microscopic examination, and interleukin (IL)-8 levels through enzyme-linked immunosorbent assay. Expression levels of vascular endothelial growth factor (VEGF) and tumor necrosis factor-alpha (TNF-α) were determined using an immunohistochemistry (IHC) array. The findings revealed an increased flap survival on day 7 post-operation following systemic administration of dapsone for 5 consecutive days. Dapsone at both dosages significantly reduced the ulcer thickness, neutrophil infiltration, and IL-8 levels. The IHC results revealed that VEGF expression was significantly higher in the treatment groups compared to the control group. Moreover, TNF-α expression was significantly lower in the treatment groups compared to the control group. In conclusion, we confirmed that treatment with dapsone promotes skin flap survival, and this effect aligned with a reduction in persistent inflammation and the enhancement of VEGF. Nonetheless, more studies are required to elucidate the precise anti-inflammatory mechanism of dapsone in I/R injuries.

Keywords: Dapsone; Inflammation; Ischemia/reperfusion injury; Skin flap; VEGF

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1. Introduction

The random-pattern skin flap is commonly used to reconstruct skin defects in plastic surgery. Contrary to an axial flap, which is vascularized by a specifically identified artery, a random-pattern skin flap is designed without consideration for specific vessels and instead relies on subdermal plexuses and unnamed musculocutaneous arteries for blood supply. Following elevation from its bed, the flap attempts to restore its hemodynamic balance. Disruption in tissue blood perfusion can lead to metabolic changes that may result in distal necrosis.[1] Despite admirable advances in flap surgeries, flap necrosis remains a frequently encountered issue, posing complications for patients.[2-4] Various techniques, including surgical delay and the application of vascular endothelium growth factor (VEGF), have been clinically used to increase the flap survival rate. However, these methods are associated with high costs, time consumption, and inconvenience for patients.[5-8] In experimental contexts, the administration of diverse stem cell types or the implementation of advanced drug delivery methods, such as microneedles containing hyaluronic acid, has demonstrated promising outcomes in enhancing vasculogenesis within skin flaps.[9] Therefore, a proper pharmacologic agent represents an alternative therapy to increase the flap survival rate. The previous studies have indicated that flap necrosis primarily arises from inadequate blood supply and subsequent ischemia/reperfusion (I/R) injury, oxidative stress, inflammatory reactions, and possible surgical site infections.[10-12] Consequently, numerous potential pharmacologic agents targeting these contributing factors are being investigated clinically and experimentally to improve flap survival.

Inflammation plays a crucial role in the process of wound healing. However, during necrosis, an intense inflammatory response, characterized by excess neutrophil infiltration, exerts an inverse effect on healing. Thus, controlling inflammation is instrumental in enhancing flap survival. It is worth mentioning that while endogenously regulated neutrophil infiltration within the initial days following injury is necessary for the healing process, prolonged and persistent neutrophilia would lead to an impaired healing process.[13,14] Interleukin (IL)-8, known as a potent chemotactic agent, mediates the infiltration and activation of neutrophils. Moreover, it is well-established that secretion of IL-8 mediates the production of cytokines, including tumor necrosis factor-alpha (TNF-α).[15,16] Recent studies have suggested that, in addition to IL-8, TNF-α also mediates neutrophil recruitment to the injured tissue and induces inflammatory responses. It has been suggested that reduction in TNF-α levels and neutrophil migration alleviates the inflammatory response and increases the flap's viable area.[17,18] The previous studies have shown that the formation of new vessels in the flap is essential for increasing the survival area of random-pattern skin flaps through an improvement in tissue blood supply. Moreover, vascular endothelial growth factor (VEGF) promotes angiogenesis and significantly reduces distal flap necrosis.[19,20] Studies have shown that increased VEGF expression correlates with enhanced angiogenesis and increased flap survival area.[21] VEGF secretion is stimulated by hypoxia and inflammatory responses to an injury. Following injury, activated platelets and neutrophils release VEGF.[22,23] However, it is crucial to note that an excessive and persistent inflammatory response as a result of an I/R injury can potentially disturb angiogenesis and the healing process. It has been confirmed that inhibition of persistent inflammatory elements is accompanied by enhancement of angiogenesis and prevention of tissue necrosis.[24]

Dapsone (4,4'-diamino-diphenyl sulfone) is an antibiotic agent belonging to the group of synthetic sulfones. It possesses both antimicrobial and anti-inflammatory properties. Its bacteriostatic function arises from its ability to inhibit dihydrofolate synthesis in bacteria.[25] The US Food and Drug Administration-approved indications for dapsone include leprosy, dermatitis herpetiformis, and acne vulgaris.[26] In addition, dapsone exhibits multiple anti-inflammatory, anti-oxidative, and anti-apoptotic properties. Dapsone functions by inhibiting beta-2 integrin-mediated adherence of neutrophils, thereby preventing their chemotactic migration.[27,28] In addition, it can interfere with the function of enzymes and proteins in the integrin family and demonstrates an inhibitory effect on IL-8 and TNF-α production, both crucial in neutrophil-mediated inflammation and regulation of lymphocyte and monocyte.[29,30]

Given its multiple functions in inflammation and angiogenesis, dapsone emerges as a potential candidate for preventing flap necrosis. In the present study, we aimed to investigate the random-pattern flap survival in rats treated with dapsone, along with exploring possible underlying inflammatory mechanisms. Furthermore, we aimed to assess the VEGF expression levels as an indicator of angiogenesis and flap survival,[31] and TNF-α as a key representative of the inflammatory response, respectively. An overview of the study is illustrated in Figure 1.

2. Materials and methods

2.1. Animals

Twenty-four male Sprague Dawley rats (weighing 270–300 g) were obtained from the animal house of the Department of Pharmacology, Tehran University of Medical Sciences. To attain a constant experimental

condition, the animals were individually housed in rat cages at room temperature of 24 ± 1°C and a relative humidity of 50–60%. They were subjected to a 12-h light/dark cycle and provided *ad libitum* access to food and fresh water. All procedures conducted in this study adhered to the National Institute of Health Guide for the Care and Use of Laboratory Animals (National Academy of Sciences, revised in 1996) and were approved by the ethics committee of Tehran University of Medical Sciences (ethics approval ID: IR.TUMS.MEDICINE.REC.1399.867).

2.2. Drugs

Dapsone was procured from Sigma-Aldrich (United Kingdom) and was dissolved in 10% dimethyl sulfoxide (DMSO). The dapsone solution was freshly prepared before administration and delivered through oral gavage using 18-gauge stainless steel feeding tubes with rounded tips for rats. For anesthesia during surgical procedures, ketamine HCl and xylazine HCl were administered intraperitoneally. These anesthetic agents were purchased from Gedeon Richter Ltd. (Hungary) and Bayer AG (Germany), respectively.

2.3. Study design

To investigate the effect of dapsone on flap survival, 24 rats were randomly assigned to four equal groups: a sham group, a control group, and two treatment groups. The sham group included rats that underwent anesthesia while their whole skin remained intact. Dorsal skin, in accordance with our flap design, was acquired and utilized as the baseline for our immunohistopathological assessments. Rats in the remaining groups underwent dorsal skin flap procedures. Rats in the control group received vehicle (10% DMSO) orally, while one treatment group received dapsone at 5 mg/kg/day, and the other treatment group received dapsone at 12.5 mg/kg/day, both dissolved in 10% DMSO. Both vehicle and dapsone treatments were administered for 5 consecutive days postoperatively, with the first dose administered three hours following the operation. The dose selection of dapsone was based on our previous studies on the anti-inflammatory properties of this reagent.[32,33]

2.4. Surgical procedure

The surgical procedures were performed by an author blinded to the treatment conditions under sterile conditions. Before surgery, all rats underwent a 12 h fasting period. General anesthesia was induced by intraperitoneal injection of a solution containing ketamine HCl (90 mg/mL) and xylazine HCl (10 mg/mL). Maintenance of deep anesthesia was achieved through intermittent administration of ketamine alone. Dorsal hair was removed using an electric razor, and this surgical site was prepared using a povidone-iodine solution. To prevent hypothermia, a heat-emitting light was located next to the rats during the operation. The random-pattern skin flap model described by McFarlane *et al.*[12,34,35] was employed. The procedure involved creating a bipedicled dorsal skin flap measuring 8 cm in length and 2 cm in width, positioned on the midline of the dorsum of each rat, with the iliac crest serving as a constant anatomic landmark defining the caudal margin of the flap. Each flap was cleaved beneath the panniculus carnosus and elevated, with the cranial pedicle clamped to allow for the cauterization of perforating vessels, establishing a random ischemic pattern. Afterward, the clamp was removed, and the cranial pedicle was cut to ensure blood perfusion solely

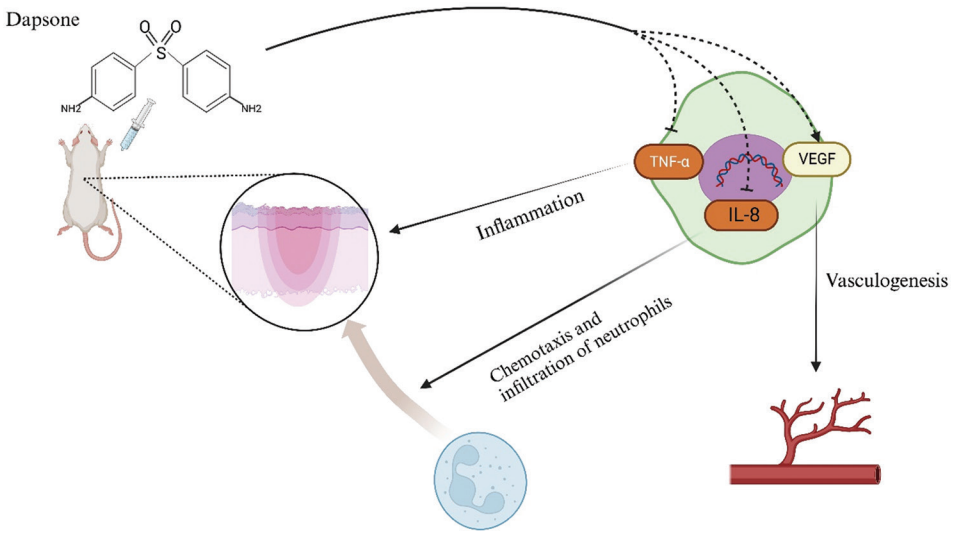

Figure 1. Overview of the study

from the caudal section. Flaps were maintained in moist, sterile gauzes during the operation. Subsequently, each flap was sutured back into its innate position using 4-0 reversed-cut nylon sutures. Corner stitches were employed to suture the cranial angles, while simple cutaneous stitches were used for all other margins. The surgical site was covered with sterile wound dressing, and each rat was housed individually in clean cages to facilitate recovery. All of the rats survived the operation and lived through 7 days of recovery.

2.5. Macroscopic evaluation

Each day, dorsal skin flaps underwent careful post-operative inspection and examination. On the 2nd day following the surgery, distal free edge necrosis was observed in a rat treated with dapsone 5 mg/kg/day. The necrotic area extended more than 1 mm from the surgical line, potentially attributed to inadvertent trauma during the procedure. Moreover, on the 5th post-operative day, infection-related necrosis was observed in another rat from the control group, likely stemming from inadequate sterilization control during the operation. No signs of circulatory failure were observed among the rats. To ensure the evaluation of skin flaps based on a comparable equal quality of procedure, these rats were excluded from the study. On the 7th post-operative day, rats were anesthetized, and the flaps were assessed by an author blinded to the treatment condition. Macroscopic changes, including appearance, skin color, texture, and hair condition of each flap, were carefully noted. Digital images were captured from the dorsal skin flaps to evaluate flap viability. Necrotic areas were characterized by dark color, edema, and eschar formation, while the total flap area was delineated by the surgical borders. ImageJ software (U.S. National Institutes of Health, USA) was used to demarcate and analyze the viable surface area. Flap survival was calculated and reported as follows:

$$\text{Flap survival (\%)} = (\text{Area of necrotic tissue}/\text{Total area of the flap}) \times 100 \quad \text{(I)}$$

2.6. Histopathological analysis and enzyme-linked immunosorbent assay

Histopathological evaluations were performed to examine the effect of dapsone on flap survival rate at a microscopic level. After the macroscopic assessments on the 7th post-operative day, the rats were anesthetized through intraperitoneal administration of ketamine HCl (90 mg/Kg) and xylazine HCl (10 mg/Kg). The necrotic tissue from the flap's cranial part and the viable tissue from the caudal part were dissected, with the obtained specimens divided into two halves: one half was fixed in 10% formalin solution for 24 h, while the other half was placed in liquid nitrogen at −70°C. Immediately after obtaining the specimens, the rats were sacrificed using a carbon monoxide (CO) chamber. The first half of the specimens were processed, embedded in paraffin blocks, sectioned into 5 μm thick slices, and prepared for hematoxylin and eosin (H&E) staining. An expert pathologist, blinded to the groups and study design, examined all H&E-stained sections for neutrophil infiltration, blood vessel distribution and angiogenesis, and ulcer thickness using an optical microscope. The second half of the specimen was homogenized using lysis buffer (2X Lysis Buffer; RayBio®, USA) and then centrifuged for 15 min (13,000 rpm, 4 °C). Subsequently, the IL-8 levels were measured using the enzyme-linked immunosorbent assay (Abcam, UK) method, adhering to the manufacturer's guidelines.

2.7. Immunohistochemistry (IHC)

The paraffin-embedded flap specimens were processed for IHC staining. These sections were then incubated with anti-TNF-α (ab6671, Abcam®, USA, 1:100) and anti-VEGF antibodies (ab46154, Abcam®, USA, 1:100). After washing with phosphate-buffered serum (PBS), specific binding to primary antibodies was visualized by enzymatic conversion of the chromogenic substrate 3,3-diaminobenzidine (DAB Substrate Kit, ab64238, Abcam®, USA) into a brown precipitate using horseradish peroxidase (ab6721, Abcam®, USA), in accordance with established protocols. Subsequently, the sections were mounted, cleared, and dehydrated. The slides were subjected to double staining using annexin-V fluorescein isothiocyanate (FITC) (Abcam, USA) and were evaluated using a fluorescence microscope. Expression levels of the desired markers were assessed and analyzed using ImageJ software (U.S. National Institutes of Health, USA).

2.8. Statistical analysis

Data are expressed as mean ± standard deviation (SD) and were analyzed using one-way and two-way analyses of variance (ANOVA), followed by Tukey's *post hoc* test for multiple comparisons between groups. All statistical analyses were performed using GraphPad Prism software version 6.07. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Assessment of skin flap survival

Figure 2 illustrates the percentage of necrosis as the survival rate of the skin flap among the rats. A one-way ANOVA confirmed a significant difference between study groups ($F[3, 13] = 28.01$, $P < 0.001$). Tukey's test revealed that treatment with dapsone at 12.5 mg/kg significantly reduced the necrosis percentage compared with the control group

(*MD* = 35.21, *P* < 0.001). However, no significant difference was observed between the control and dapsone 5 mg/kg groups (*P* = 0.620). These results indicate that dapsone at 12.5 mg/kg exhibited statistically higher effectiveness than dapsone at 5 mg/kg (*MD* = 26.48, *P* < 0.01) (Figure 2).

3.2. Microscopic ulceration and neutrophil infiltration

Figure 3 illustrates the microscopic evaluation of flap injury and ulceration using H&E staining in our histopathologic assessment. Results revealed variations in ulcer thickness among the study groups (*F*[3,8] = 113.9, *P* < 0.001). *Post hoc* analysis revealed significant effects of dapsone administration at both doses on ulcer thickness (*MD* = 88.67, *P* < 0.001; *MD* = 178.3, *P* < 0.001, respectively) (Figure 3B). Moreover, significant differences were observed among the four groups regarding neutrophil infiltration (*F*[3,8] = 29.20, *P* < 0.00) (Figure 3A). Tukey's analysis confirmed that the I/R injury in the random-pattern flap model promoted neutrophil infiltration at the injury site (*MD* = −146.00, *P* < 0.001). Notably, systemic administration of dapsone in both the 5 and 12.5 mg/kg groups resulted in significantly reduced neutrophil infiltration (*MD* = 98.33, *P* = 0.002; *MD* = 128.330, *P* < 0.001, respectively). This effect did not exhibit significant dose dependence (*P* = 0.357) (Figure 3A).

3.3. Assessment of IL-8 level

As depicted in Figure 4, IL-8 levels were measured and compared among groups. A one-way ANOVA confirmed a statistically significant difference between study groups (*F*[3,20] = 20.54, *P* < 0.001). The induction of I/R injury in our skin flap model led to an increase in IL-8 levels, as expected (*MD* = 80.5, *P* < 0.001). Administration of dapsone significantly reduced IL-8 levels, regardless of the dose administered (*MD* = 30.17, *P* = 0.039; *MD* = 38.67, *P* = 0.006; for doses of 5 and 12.5 mg/kg, respectively). However, the effect of dapsone on the IL-8 levels did not appear to be dose-dependent (*P* = 0.84) (Figure 4).

3.4. Skin flap expression of VEGF and TNF-α

Figure 5 presents the expression of VEGF and TNF-α in the skin flap using IHC staining. We observed significant differences in VEGF expression among the four groups (*F*[3,8] = 83.04, *P* < 0.001) (Figure 5A), with the highest mean VEGF expression observed in the sham group. Moreover, the results revealed that the induction of I/R injury in our skin flap model led to a decrease in the expression of VEGF (*MD* = 35.45, *P* < 0.001). In addition, the IHC assessment revealed higher VEGF expression in rats receiving dapsone at either 5 or 12.5 mg/kg compared with the control group (*MD* = 13.72, *P* = 0.002; *MD* = 22.08, *P* < 0.001; respectively)

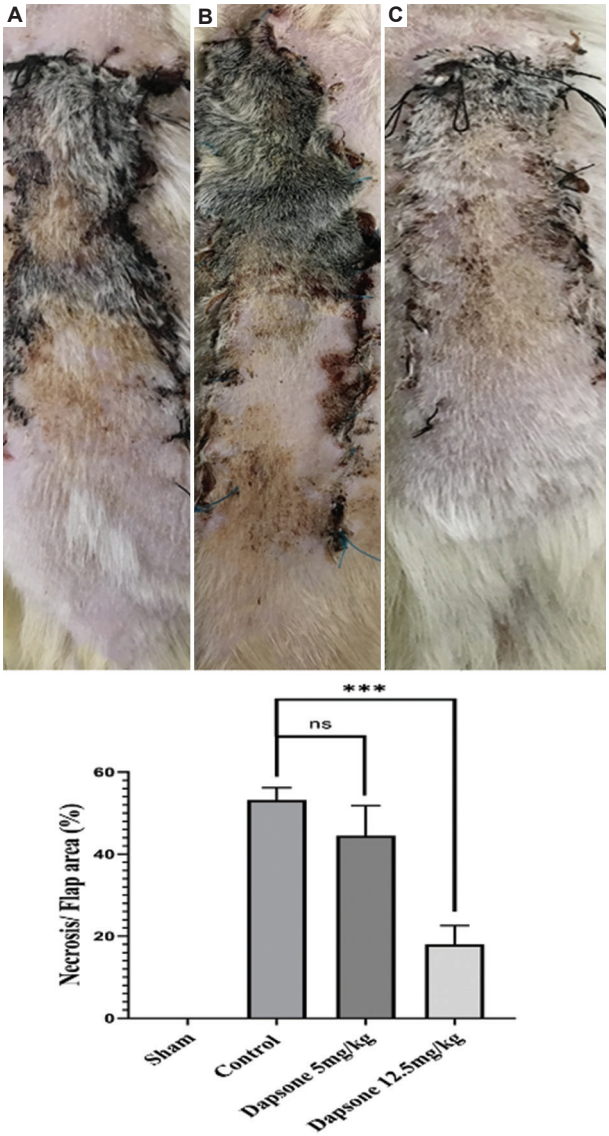

Figure 2. Flap survival investigations following an ischemia/reperfusion (I/R) injury. Upper panel: Dorsal skin flaps on day 7 following surgery: (A) Control group; (B) dapsone 5 mg/kg group; and (C) dapsone 12.5 mg/kg group. The necrotic area was defined by a dark color, edema, and eschar formation, and the total flap area was delineated by the surgical borders. Lower panel: The survival rate of the skin flap is quantified as the percentage of necrosis over the area of the surgical flap. Administration of dapsone 12.5 mg/kg had a significant effect on the flap necrosis (*P* < 0.001). Notes: ns: Non-significant; ***P < 0.001.

(Figure 5A). ANOVA analysis also confirmed statistically significant differences in TNF-α expression between the study groups (*F*[3,8] = 16.06, *P* = 0.001) (Figure 5B). As expected, induction of I/R injury increased the expression of TNF-α in the skin flap (*MD* = 26.48, *P* < 0.01). Notably, both doses of dapsone significantly reduced the expression level of TNF-α (*MD* = 19.35, *P* = 0.008; *MD* = 24.38, *P* = 0.002; respectively) (Figure 5B). Interestingly, the effect

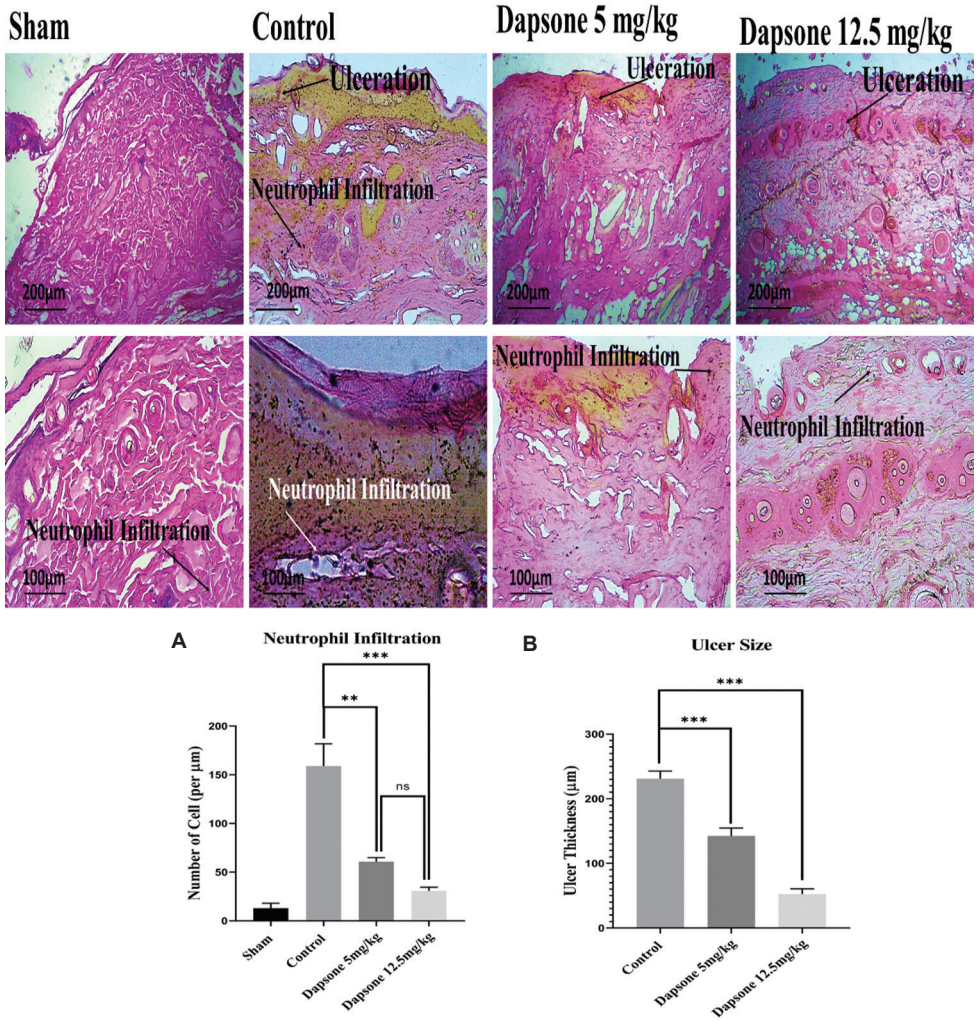

Figure 3. Neutrophil infiltration and ulceration following an ischemia/reperfusion (I/R) injury. Upper panel: Histopathological evaluation of skin flaps using hematoxylin and eosin staining on day 7 following surgery. The ulcerated area and infiltration of neutrophils are indicated by arrows. Scale bars: 200 μm, magnification ×40; 100 μm, magnification ×100. Lower panel: (A) Comparison of neutrophil infiltration among the groups, quantified as the number of cells per μm. As expected, I/R injury increased neutrophil infiltration. Both doses of dapsone inhibited neutrophil infiltration. (B) Comparison of ulceration among the groups. The mean of ulceration thickness (μm) in each group is compared between groups. Administration of dapsone in both doses ameliorated ulceration thickness significantly ($P < 0.001$).
Notes: ns: Non-significant; $^{**}P < 0.01$, $^{***}P < 0.001$.

of dapsone on the inhibition of TNF-α did not exhibit dose dependence ($P = 0.65$).

4. Discussion

This study, for the first time, evaluates dapsone as a possible therapeutic approach to flap survival in a well-established experimental model of a random-pattern skin flap. Interestingly, we observed that dapsone promoted flap survival following induction of I/R injury. Although the decrease in flap necrosis was not statistically significant at the dose of 5 mg/kg regarding macroscopic evaluation, a histopathologic study of dermal ulceration suggested favorable outcomes at both doses. Moreover, we employed inflammatory and angiogenetic examination on flap survival, following the trend among experimental skin flap models,[36] in an effort to explore the therapeutic mechanisms of dapsone. In summary, our results suggested inhibition of neutrophil infiltration, TNF-α production, and an increase in VEGF expression following 5 days of treatment with dapsone after the initial I/R injury in our skin flap model.

The employment of pharmacological strategies serves as a pivotal methodological avenue for researchers seeking to augment their comprehension of the fundamental mechanisms governing the viability of skin flaps subsequent to I/R injury. Various pharmacological agents

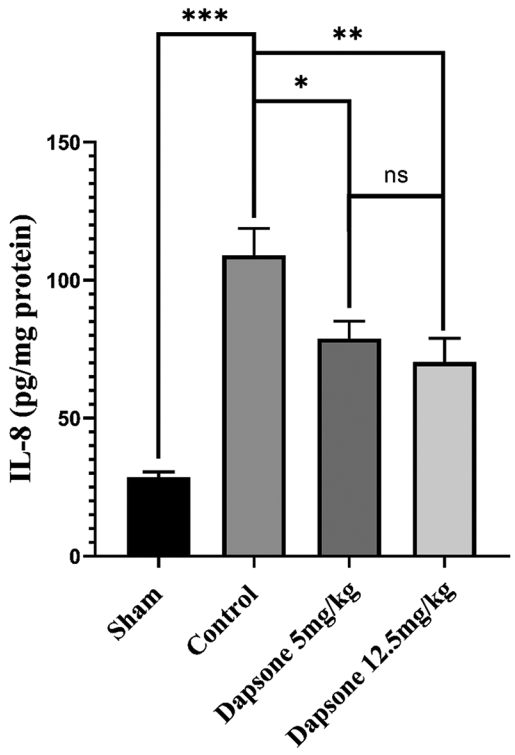

Figure 4. Assessment of interleukin (IL)-8 levels in the skin flaps using enzyme-linked immunosorbent assay. The mean level of IL-8 (pg/mg protein) is compared among the study groups. Performing a random-pattern skin flap increased the levels of IL-8. Administration of dapsone in both doses decreased the levels of IL-8 significantly. The effect of dapsone on IL-8 was not dose-dependent.
Notes: ns: Non-significant; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

have been suggested as exhibiting promising outcomes in promoting skin flap survival. Notably, modafinil has been demonstrated to mitigate necrotic manifestations within this context by activating ATP-sensitive potassium channels, thereby enhancing the anti-inflammatory properties inherent to the nitric oxide (NO) pathway.[37] In a parallel vein, an alternative investigation has propounded the favorable efficacy of ivermectin through modulation of gamma-aminobutyric acid receptors, subsequently leading to the downregulation of the TNF-α/IL-1β inflammatory cascade.[38] It has been demonstrated that the survival of skin flaps following I/R injury depends on factors including oxidative stress, neutrophil infiltration, angiogenesis, and a proinflammatory cytokine response. It is worth mentioning that sometimes it is hard to differentiate the causational outcomes of these factors from one another as one may also contribute to other pathways as well.[9,39] Neutrophil infiltration or influx is widely known as a critical feature of I/R injuries in general and skin flaps in particular. The initial release of reactive oxygen species in the skin flap I/R injury leads to chemotaxis and activation of neutrophils and, therefore, the production of free radicals and additional apoptosis.[36,40] Moreover, experimental studies of I/R injury have revealed that TNF-α is a key component of inflammation-mediated neutrophil migration, activation, and, consequently, the progression of the inflammatory response.[41-43] Expression of TNF-α not only leads to more cumulative apoptosis following I/R injury but also potentially enhances the expression of adhesion markers, chemokines, and cytokines, which lead to more infiltration of neutrophils into the injured site.[44]

On the other hand, the application of dapsone has exhibited anti-inflammatory properties over recent decades. Conventionally, dapsone is well-known for its anti-microbial and anti-protozoa effects. However, its clinical application in autoimmune settings has spurred the exploration of anti-inflammatory properties targeting both molecular and cellular elements of inflammation.[45] Indeed, the potential of dapsone to affect specific elements of inflammation led us to formulate our hypothesis. Modschiedler *et al.* have the first to conclude that dapsone could suppress the adhesion of neutrophils to epidermal binding sites.[46] Subsequent research revealed that dapsone not only inhibits the chemotaxis of neutrophils but also reduces neutrophil activity and functionality through an intracellular calcium-dependent signaling pathway.[47,48] As mentioned earlier, IL-8 is widely recognized as a potent chemotactic agent that facilitates neutrophil infiltration and activation. IL-8 also mediates the production and release of other cytokines, including TNF-α, by activated neutrophils. Previous experimental and clinical studies have revealed that dapsone inhibits the secretion of IL-8, resulting in the suppression of neutrophil infiltration.[44,49] In this study, dapsone exhibited a similar effect on the IL-8 levels, consistent with predictions from previous studies. Our results indicated that IL-8 levels were in alignment with neutrophil infiltration and TNF-α expression among study groups.

Moreover, the administration of dapsone for treating cutaneous lupus erythematosus patients has been observed to significantly inhibit the production of TNF-α.[29,50,51] Subsequently, it was also revealed that dapsone exhibits anti-TNF-α properties in other inflammatory dermal conditions.[52,53] For instance, although the etiology of necrobiosis lipoidica remains unclear, clinical research suggests that the administration of dapsone exhibits effectiveness and is accompanied by a decrease in dermal ulceration.[54,55] In addition, an *in vitro* assessment of lipopolysaccharide-induced inflammation has confirmed that dapsone inhibits the production of TNF-α in isolated bone marrow cells.[56] Interestingly, in alignment with the aforementioned underlying inflammatory mechanism of

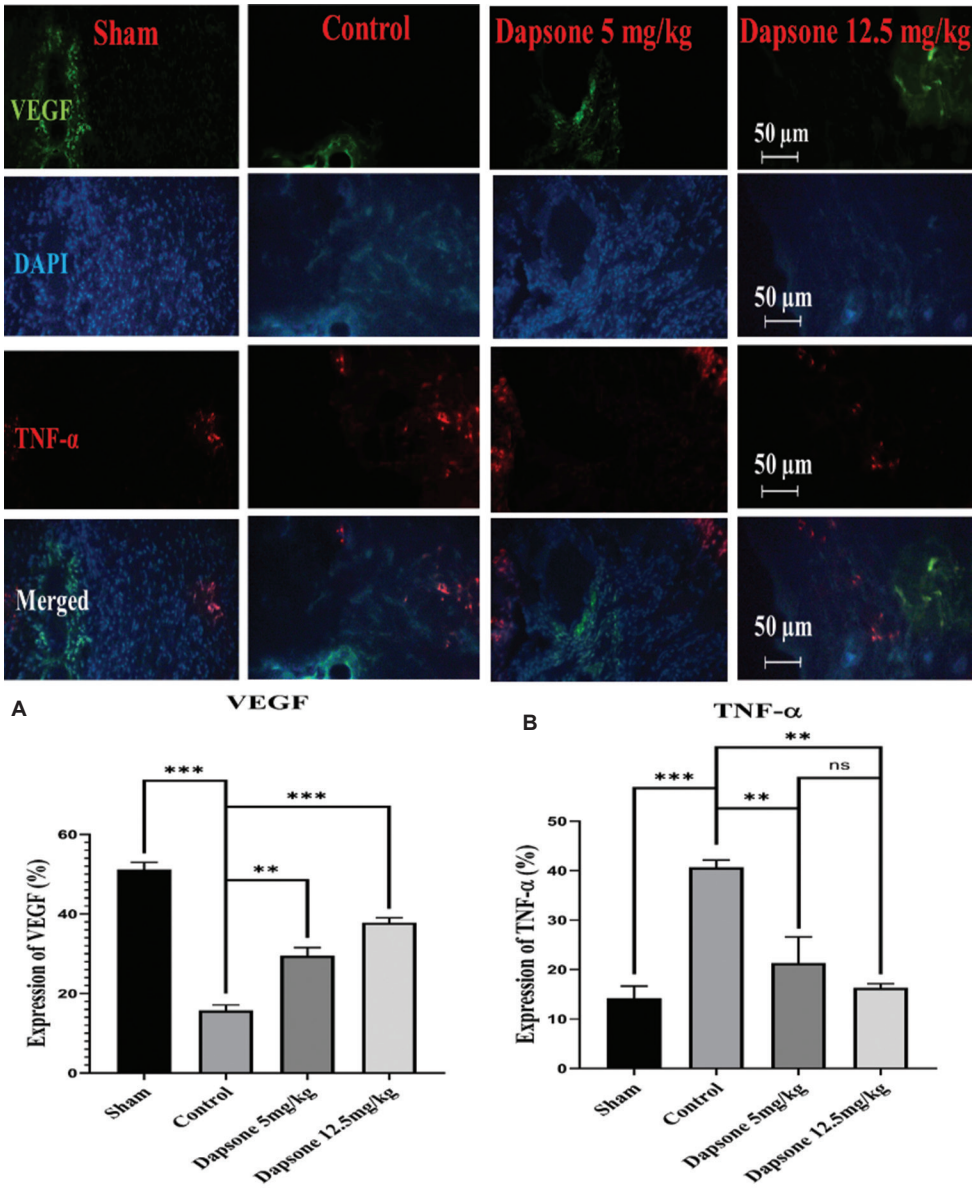

Figure 5. Expressions of vascular endothelial growth factor (VEGF) and tumor necrosis factor-alpha (TNF-α) in skin flaps following an ischemia/reperfusion (I/R) injury. Upper panel: Immunohistochemical assessment of VEGF and TNF-α on day 7 post-operation. Scale bars: 50 μm, magnification ×400. Lower panel: Mean VEGF and TNF-α expression comparisons for each group. As expected, induction of I/R injury and the ensuing inflammation reduced VEGF levels (A) while promoting TNF-α levels (B) in the skin flap. Oral administration of dapsone at either 5 or 12.5 mg/kg significantly ameliorated these trends in both markers. However, the effect of dapsone on TNF-α did not exhibit dose dependency.
Notes: ns: Non-significant; $^{**}P < 0.01$, $^{***}P < 0.001$.

I/R injury in skin flaps, it has been suggested that dapsone may provide protective effects against inflammation in kidney[57] and cardiac[58] I/R injury settings through the downregulation of TNF-α, oxidative stress, and neutrophil activity. In our study, we observed that the effects of dapsone on TNF-α levels, neutrophil infiltration, and, correspondingly, dermal necrosis and flap survival are consistent with previous findings. However, the precise mechanism of dapsone's anti-inflammatory properties remains unclear. Other studies have concluded that the anti-inflammatory effects of dapsone correspond to the inhibition of NF-κB and substantial downregulation of downstream cytokine production.[32]

In addition, our histopathological and IHC assessments indicated an increase in angiogenesis and VEGF expression

levels in our treatment groups. Although the precise impact of dapsone on angiogenesis remains poorly understood, our results suggest a dual effect: inhibition of excess inflammation and neutrophil infiltration on the one side, and enhancement of angiogenesis on the other. There is solid evidence supporting the role of dapsone in the inhibition of progressive inflammation and cytokine production.[59-61] Numerous studies have sought to explore the underlying mechanisms of wound healing and the formation of scar tissue and fibrosis as undesirable outcomes of impaired healing. Various factors contribute to regulating the process of appropriate healing and minimizing scar formation. The previous research has indicated that a balanced interplay between inflammation and angiogenesis plays a critical role in preventing impaired wound healing. For instance, persistent neutrophil infiltration and overexpression of TNF-α not only impede angiogenesis and downregulate VEGF expression but also decrease the viability and survival of keratinocytes.[24,62,63] Similarly, our results also suggest a correlation between prolonged inflammation and the status of angiogenesis.

Before drawing a conclusion, several factors should be taken into consideration. First, dapsone is associated with adverse side effects, including hematologic (methemoglobinemia and hemolysis), dermatologic (exfoliative dermatitis and erythema multiform), neurologic (peripheral neuropathy), and gastrointestinal (anorexia and nausea/vomiting) effects.[45] Second, patients with peripheral vascular disease (e.g., Buerger's disease) are known to possess a higher risk of flap surgery failure. It has been suggested that diabetic or atherosclerotic patients may benefit from preconditioning to mitigate their risk of flap failure. Pharmacologic or ischemic preconditioning, alone or in combination, could serve as a therapeutic approach for patients with compromised perfusion and, consequently, an increased risk of flap failure.[64-66] However, to translate pharmacologic agent studies into clinical practice, large-scale animal studies involving various flap types and sizes are required.

5. Conclusion

In this study, we explored the effects of dapsone on skin flap survival, focusing on its anti-inflammatory properties. Our results revealed that systemic administration of dapsone effectively prevented skin flap necrosis and ulceration. Subsequent assessments demonstrated that dapsone decreased neutrophil infiltration, IL-8 levels, and TNF-α expression, as well as enhanced the VEGF expression. However, future investigations are warranted to fully elucidate the promising anti-inflammatory effects of dapsone.

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Conflict of interest

The authors declare no conflicts of interest.

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Ethics approval and consent to participate

The study protocol was executed in agreement with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals (NIH publication No. 86-23, Eighth Ed.) and institutional and governmental concerns for animal care and use (Approval ID: IR.TUMS.MEDICINE.REC.1398.922).

Consent for publication

Not applicable.

Availability of data

The datasets used during this study are available on reasonable request from the corresponding author.

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CASE REPORT

Progression of pediatric febrile seizure to status epilepticus: A case report

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Abstract

Managing pediatric febrile seizures progressing into status epilepticus (SE) presents challenges due to diagnostic complexities and the evolving nature of symptoms. This case report presents the management of a 1-year-old pediatric patient who experienced a febrile seizure progressing into SE. Laboratory findings reflected common variations observed in febrile illnesses and seizures. Treatment comprised a multidrug regimen involving antibiotics, anticonvulsants, and supportive care, primarily focused on seizure control, infection management, and symptomatic relief, aligning with established protocols for SE management in pediatric patients. However, the potential for drug interactions, particularly with carbapenems, underscores the importance of medication selection, especially in patients predisposed to seizures or neurological complications. This case report emphasizes the necessity for a multidisciplinary approach involving pediatricians, neurologists, and clinical pharmacists in optimizing treatment strategies for SE. The involvement of clinical pharmacists in medication review, dosage adjustments, monitoring for drug interactions, and patient education played a pivotal role in achieving positive outcomes in this critical scenario. This report sheds light on the complexities and challenges inherent in managing pediatric febrile seizures advancing to SE and underscores the significance of collaborative, multidisciplinary care in such cases.

Keywords: Febrile status epilepticus; Pediatric seizures; Multi-drug regimen; Neurological complications

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1. Introduction

Febrile seizures manifest as sudden convulsions triggered by a rapid elevation in body temperature, typically surpassing 38°C (100.4°F), without underlying seizure-inducing conditions such as central nervous system infections, electrolyte imbalances, drug withdrawal, trauma, genetic predisposition, or known epilepsy. These seizures are categorized as either simple or complex febrile seizures, with each subclassification requiring distinct approaches and assessments.[1-3]

Febrile seizures often arise in conjunction with a temperature exceeding 38°C (100.4°F) and in the absence of other seizure-provoking factors, although the convulsive temperature threshold varies among individuals. They frequently coincide with the rise in body temperature, serving as an early indication of an underlying illness, with

viral infections, notably HHV-6 in the United States and European countries, and influenza A virus in Asian regions, commonly associated with febrile seizures. Nonetheless, any substantial fever can incite a febrile seizure.[3,4]

Febrile seizures are prevalent among children aged 6 months to 5 years, affecting up to 4% of this demographic. While some children may experience a single febrile seizure, others may endure multiple occurrences during early childhood. However, the precise pathophysiology of febrile seizures remains elusive. A hereditary predisposition exists, with 10 – 20% of first-degree relatives of affected patients also experiencing febrile seizures, although the mode of inheritance remains undefined.[5]

No specific treatment exists for simple or complex febrile seizures other than addressing underlying febrile illnesses. Antipyretics have not demonstrated efficacy in preventing recurrent febrile seizures. Studies examining the use of benzodiazepines as a short-term measure during subsequent febrile events in patients with frequent recurrences have been conducted. Febrile status epilepticus (FSE), occurring in <10% of initial febrile seizures, warrants immediate intervention using rectal diazepam or intranasal midazolam if the event exceeds 5 min. Such patients face an increased risk of future episodes.[6-8]

While most febrile seizure cases do not necessitate hospitalization or extensive intervention, prolonged complex febrile seizures may result in Todd's paralysis, which is characterized by focal weakness that typically resolves within hours to days. Notably, patients with febrile seizure status, defined as seizures lasting over 30 min, require immediate treatment akin to prolonged seizures from other causes. It is essential to promptly broaden the differential diagnosis if a patient fails to regain consciousness or exhibits unexpected neurological abnormalities post-seizure.[9,10]

These cases warrant evaluation for ongoing seizure activity or other intracranial abnormalities, often necessitating prolonged electroencephalogram (EEG) studies and further investigations for potential underlying pathologies. Collaborative management involving pediatricians and neurologists is essential for diagnosing and managing febrile seizures. Patient education plays a crucial role in mitigating unnecessary emergency room visits and avoiding unverified remedies. Parents should be informed about when to seek emergency care and cautioned against using aspirin for fever management. The unified approach of the interprofessional team ensures comprehensive care for patients experiencing febrile seizures. The prognosis for most children with febrile seizures is favorable, with approximately 30% experiencing subsequent seizures. While there is a slightly elevated risk of future epilepsy, febrile seizures typically do not impair cognition or intellect or induce neurological dysfunction.[11,12]

2. Case presentation

FSE represents a critical manifestation of febrile seizures, characterized by episodes lasting 30 min or more. This case delves into a 1-year-old baby boy admitted to the pediatric department weighing 12 kg, presenting a 5-day history of fever, vomiting, cough, and a severe seizure episode lasting 20 – 25 min. This seizure episode was marked by ocular deviation, frothing of the mouth, and generalized tonic-clonic features. Upon admission, clinical examination revealed a temperature of 102°F, a heightened heart rate of 162 bpm, and a respiratory rate of 24/min. Over 3 days, hematological and biochemical assessments as shown in Table 1 revealed fluctuating levels, including hemoglobin readings (12 – 11.8 g/dL; normal range: 11 – 15.5 g/dL); red blood cell counts (4.7 and 4.8 million/cumm; normal range: 4 – 5.2 million/cumm); white blood cell counts (40.9 – 9.6 thousand cells/cumm; normal range: 5 – 13 thousand cells/cumm); and platelet counts (3.24 and 2.19 lakhs cells/cumm; normal range: 1.8 – 4.5 lakhs cells/cumm) as shown in Table 2. In addition, electrolyte imbalances were observed with sodium levels of 137 – 141 mmol/L (normal range: 136 – 145 mmol/L), potassium levels of 3.3 – 4.3 mmol/L (normal range: 3.5 – 5 mmol/L), and chloride levels of 99 – 105 mmol/L (normal range: 95 - 105 mmol/L) as shown in Table 3. Renal function tests reflected normal blood urea nitrogen (BUN) and serum creatinine (Sr. Cr) levels within standard ranges as shown in Table 4. The diagnostic process included a magnetic resonance imaging (MRI) scan that exhibited normal myelination. Treatment encompassed a multidrug approach involving antibiotics (injection ceftriaxone, injection meropenem, and injection linezolid), anticonvulsants (injection levetiracetam, tablet clobazam, and syrup levetiracetam), and supportive care as shown in Table 5. Throughout the hospital stay, the patient experienced varying symptoms, including high-grade fever spikes, vomiting, decreased cough, and cold. Adjustments to the medication regimen resulted in reduced fever spikes, cessation of vomiting, and the absence of new complaints. Ultimately, the patient remained afebrile and active for 48 h before discharge. The subjective assessment on admission outlined a 1-year-old male admitted to the pediatric intensive care unit due to fever, seizures, vomiting, and loose stools, with objective findings indicating normal laboratory parameters. The tailored treatment approach led to discharge upon symptom reduction and minimized fever spikes. Notably, the patient had potential severe drug interactions with carbapenems, warranting caution in

Table 1. Biochemistry test

Biochemistry	Day 2	Normal values
Serum Ca^{+2}	9.9	3.8 – 10.6
Serum Mg^{+2}	2.5	1.6 – 2.6

Table 2. Complete blood picture

Hematology	Day 1	Day 3	Day 5	Normal values
Hemoglobin	12	12	11.8	11 – 15.5 g/dL
Red blood cells	4.7	4.8	4.7	4 – 5.2 million/cumm
White blood cells	40.9	18.5	9.6	5 – 13 thousand cells/cumm
Platelets	3.24	2.19	2.35	1.8 – 4.5 hundred thousand cells/cumm

Table 3. Electrolytes test

Electrolytes	Day 1	Day 3	Normal values
Na^{+}	137	141	136 – 145 mmol/L
K^{+}	3.3	4.3	3.5 – 5 mmol/L
Cl^{-}	99	105	95 – 105 mmol/L

Table 4. Renal function test (RFT)

RFT	Day 3	Normal values
BUN	14	10.8 – 38.4
Sr.Cr	0.3	0.2 – 0.4
Uric acid	1.1	3.5 – 7.2

Abbreviations: BUN: Blood urea nitrogen; Sr.Cr: Serum creatinine.

patients prone to seizures or neurological disturbances. Patient counseling focused on understanding febrile seizures, recognizing signs, adopting preventive measures, and knowing when to seek medical attention.

3. Discussion

FSE represents a critical end of the spectrum in febrile seizures, characterized by seizures lasting over 30 min. It poses significant challenges, especially in pediatric patients, necessitating immediate and tailored interventions.[1-3] A 1-year-old baby boy was admitted with a 5-day history of high-grade fever, vomiting, cough, and a seizure lasting 20 – 25 min, displaying ocular deviation and generalized tonic–clonic features. Upon examination, he presented with a temperature of 102°F, an elevated heart rate of 162 bpm, and a respiratory rate of 24/min. Hematological and biochemical investigations revealed fluctuating parameters, including variations in hemoglobin, red blood cell, white blood cell, and platelet counts. In addition, electrolyte imbalances were observed. Imaging through MRI revealed normal myelination. Treatment encompassed a combination of antibiotics, anticonvulsants, and supportive care, leading to a gradual resolution of symptoms. The patient achieved a 48-h afebrile state and was discharged with modified medication. The case highlights the complexity of managing FSE in pediatric patients. The prolonged seizure episode, along with the constellation of symptoms, posed diagnostic and therapeutic challenges. Laboratory findings indicated fluctuations in hematological parameters and electrolyte

Table 5. Treatment chart

Drug given	Dose	Roa	Frequency	Pediatric dosing
Injection ceftriaxone	500 mg	Intravenous	Bd	50 mg/kg/day
Injection pantoprazole	10 mg	Intravenous	Od	10 – 20 mg/day
Injection ondansetron	1 cc	Intravenous	Bd	0.1 mg/kg
Injection levetiracetam	200 mg (S)/50 mg	Intravenous	Bd	10 mg/kg/day
Tablet clobazam	5 mg	Oral	Bd	10 mg/kg/day
Syrup Paracetamol	3 ml	Oral	Qid	10 – 15 mg/kg
Syrup Cetirizine+Ambroxol	3 ml	Oral	Bd	-
Paracetamol suppository	170 mg	Rectal	Sos	-
Iv infusion of ½ dextrose normal saline	40 mL/h	Intravenous		-
3% Normal saline nebulization	2 drops E/S 3 cc	Nasal	Tid	-
Injection meropenem	200 mg	Intravenous	Tid	20 mg/kg
Injection linezolid	100 mg	Intravenous	Bd	10 mg/kg
Bacillus clausii capsule	1 capsule	Oral	Bd	-
Syrup levetiracetam	0.5 ml	Oral	Bd	10 mg/kg/day

Abbreviations: Bd: Twice daily; E/S: Each side; Iv: Intravenous; ns: Normal saline; Od: Once daily; Roa: Route of administration; Sos: Whenever required; Syp: Syrup; Tid: Thirce daily.

imbalances, common in febrile illnesses and seizures. The treatment regimen aimed at controlling seizures, managing infection, and providing symptomatic relief, aligning with established protocols for FSE management.

The dynamic nature of the patient's symptoms throughout the hospital stay underscores the importance of closely monitoring these cases. Adjustments in medication were crucial in mitigating fever spikes, controlling seizures, and reducing associated symptoms. The normalization of laboratory parameters and the resolution of clinical signs contributed to the decision for discharge. However, the case also revealed potential drug interactions, particularly with carbapenems, emphasizing the need for careful consideration of medication choices, especially in patients predisposed to seizures or neurological complications. This underscores the significance of a multidisciplinary approach involving pediatricians, neurologists, and pharmacists in optimizing treatment strategies.

In cases of FSE involving pediatric patients, the role of clinical pharmacists is indispensable in optimizing medication regimens and ensuring treatment effectiveness while mitigating potential risks associated with drug interactions. These professionals conduct comprehensive medication reviews, critically evaluating prescribed drugs, including antibiotics, anticonvulsants, and supportive care medications, to prevent adverse reactions and enhance therapeutic outcomes.[4-6] Clinical pharmacists provide valuable insights into dosing regimens, aligning medications with the patient's age and weight and suggesting alternative therapies to minimize drug interactions and potential complications. The complex nature of administering multiple medications, especially in critical scenarios like FSE, heightens the risk of drug interactions, particularly with compounds such as carbapenems. Clinical pharmacists excel in identifying potential interactions and guiding necessary adjustments or alternate medication options. Their vigilance extends to actively monitoring patients for signs of adverse reactions or drug interactions throughout the treatment trajectory, ensuring timely intervention to prevent complications. Education and counseling are fundamental aspects of a clinical pharmacist's role. They educate caregivers about medication usage, potential side effects, and the significance of adhering to the treatment plan. In cases like FSE, where managing seizures and fever is paramount, pharmacist-led guidance empowers caregivers to recognize warning signs, respond during emergency seizures, and adhere strictly to the prescribed medication regimen. The collaborative efforts of clinical pharmacists within the healthcare team, involving pediatricians, neurologists, and other specialists, are instrumental. They contribute invaluable perspectives in multidisciplinary discussions, aligning therapeutic plans with the patient's clinical condition to minimize adverse events and optimize treatment strategies.

FSE, where precise medication management is critical, underscores the pivotal role of clinical pharmacists in ensuring safe and effective pharmacotherapy. Their involvement significantly contributes to improved patient outcomes and reduced likelihood of medication-related complications, highlighting their indispensable role in pediatric care. FSE poses a serious health concern in pediatric patients, necessitating prompt recognition, appropriate interventions, and meticulous monitoring.[7-9] This case report emphasizes the challenges encountered in managing FSE, ranging from diagnosis to treatment modifications. Tailored therapeutic strategies, vigilant monitoring, and an understanding of potential drug interactions are pivotal for achieving favorable outcomes in such cases.

The evaluation and management of febrile seizures in children involve a nuanced approach to determine the underlying cause and ensure appropriate interventions.[1,13]

3.1. Diagnostic investigations

The two sources highlight varying recommendations for diagnostic investigations in the context of febrile seizures. The first source discourages routine tests such as full blood count, electrolytes, and glucose levels unless clinically indicated, emphasizing that these parameters do not alter the management course of febrile seizures. In contrast, the second source presents a more detailed approach, suggesting lumbar puncture in specific scenarios, especially for young infants and cases with suspected meningitis.[14-16]

3.2. Neuroimaging and EEGs

The recommendations on neuroimaging and EEGs also differ. The first source advocates against routine neuroimaging for febrile seizures, suggesting it is rarely needed for simple cases.[13,17] On the other hand, the second source acknowledges specific situations where neuroimaging and EEG might be considered, especially for children with recurrent or complex febrile seizures.[18,19]

3.3. Treatment and prevention

Both sources discuss the use of benzodiazepines for seizures, but the second source provides a more nuanced perspective on their efficacy in febrile seizures. It emphasizes the uncertain efficacy of benzodiazepines in febrile seizures while acknowledging their accepted use in FSE.[20-22] In addition, the second source introduces the consideration of antipyretics and their limited impact on febrile seizure recurrence.[22,23]

3.4. Evaluation

The second source introduces the concept of "red flags" for febrile seizures, emphasizing specific signs that warrant further evaluation, such as complex febrile seizures,

meningeal signs, and abnormal neurological findings. This adds a practical dimension to identifying high-risk cases.[24]

3.5. Management

While both sources touch upon the management of febrile seizures, the second source provides additional insights into the psychological impact on families and emphasizes the importance of reassurance and education. It also details the acute phase treatment, including considerations for prolonged seizures and when to seek medical assistance.[25] The comparison highlights variations in recommendations and perspectives in the evaluation and management of febrile seizures. While both sources provide valuable insights, the nuanced approach presented in the second source, along with specific considerations and red flags, offers a more comprehensive guide for clinicians dealing with this common childhood condition. These differences underscore the evolving nature of clinical guidelines and the need for a case-specific, patient-centered approach in managing febrile seizures in children.

4. Conclusion

This case report of FSE in a pediatric patient illuminates the intricate landscape and challenges associated with managing critical conditions in children. The multidimensional aspects of diagnosis and treatment, as detailed in the report, underscore the indispensable role of clinical pharmacists in optimizing medication strategies. The achievement of a 48-h afebrile state signifies the effectiveness of the optimized treatment protocol implemented. Notably, the report emphasizes the pivotal contribution of clinical pharmacists, showcasing their expertise in medication review, dosage adjustment, drug interaction assessment, and patient counseling. The collaborative health-care approach involving pediatricians, neurologists, and clinical pharmacists proves essential in navigating the complexities of pediatric febrile seizures progressing to status epilepticus (SE). The rationale behind the choice of anti-seizure medications, specifically levetiracetam and clobazam, is elucidated, with evidence-based support. The dynamic nature of the patient's symptoms underscores the necessity for close monitoring and adaptive medication adjustments. Furthermore, the recommendation for a follow-up in the pediatric outpatient department post-discharge is highlighted as crucial for ongoing evaluation and potential regimen adjustments, aligning with standard care practices. Together, these key points convey the profound significance of collaborative healthcare, the vital role of clinical pharmacists, evidence-based decision-making, and the nuanced complexities involved in managing pediatric febrile seizures evolving into SE.

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Conflict of interest

The authors declare that they have no conflict of interest.

Author contributions

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Consent for publication

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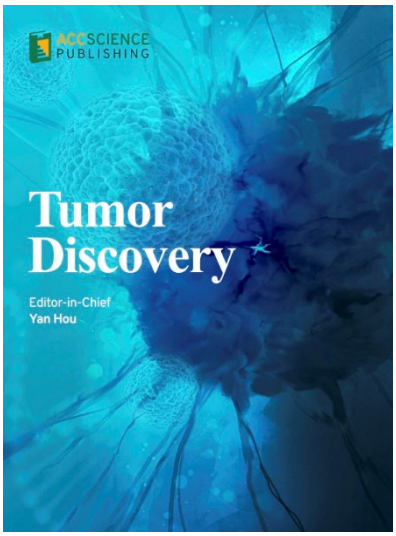

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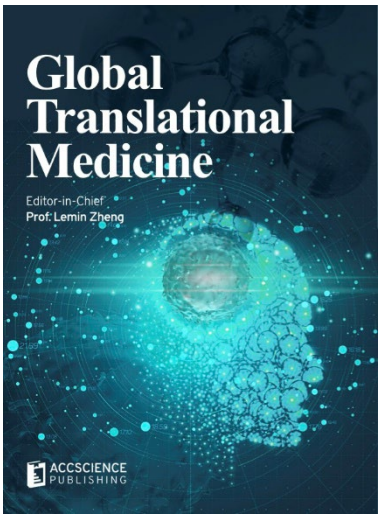

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