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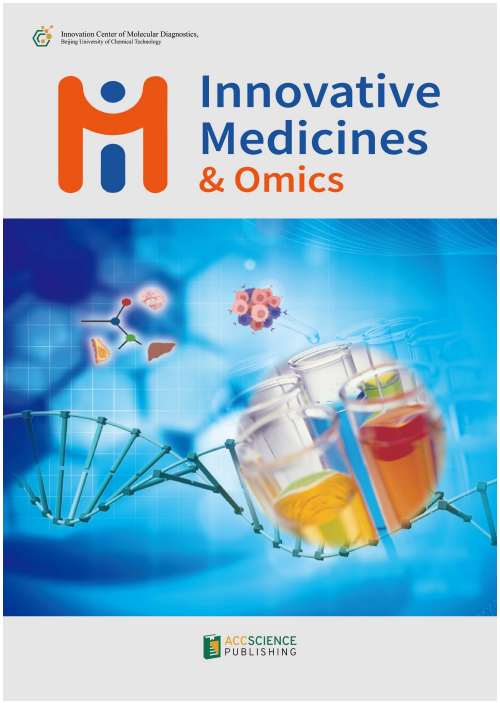

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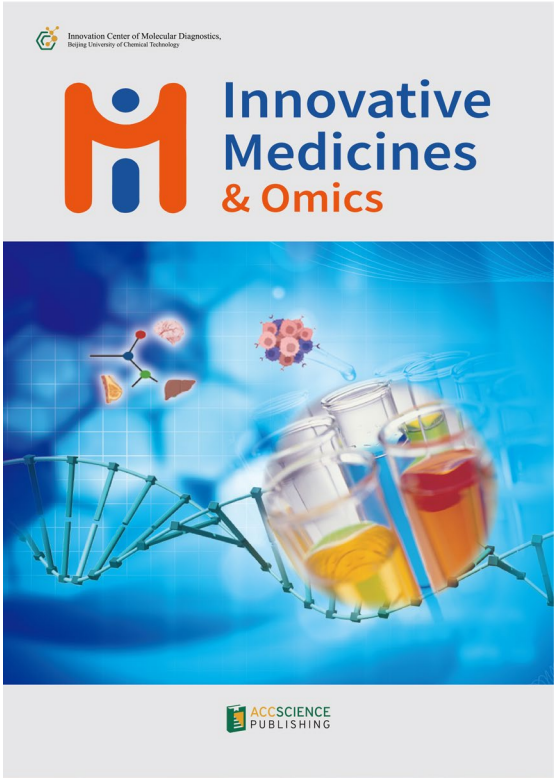

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Volume 2 | Issue 3 | August 2025 | Page 1 – 91 |

Innovative Medicines & Omics

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CONTENTS

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Innovative Medicines & Omics

REVIEW ARTICLE

Therapeutic potential of mesenchymal stem cell exosomes for tumors in the digestive system: From bench to bedside

Ying Liu[1,2,3]*, Tao Zhang[3], and Pengyun Lin[3]

[1]Sinocelltech Ltd., Beijing, China

[2]Department of Internal Medicine, Huailai Huaqing Ophthalmology Hospital, Zhangjiakou, Hebei, China

[3]General Laboratory, The Affiliated Hospital of Hebei Northern University, Zhangjiakou, Hebei, China

Abstract

Exosomes are small, bilayer lipid vesicles with diameters ranging from approximately 40–160 nm. These vesicles carry a diverse array of molecular cargo, including DNA, RNA, lipids, and proteins, which play a critical role in intercellular communication. Among the various cell types, mesenchymal stem cells (MSCs) are recognized as highly efficient producers of exosomes. MSC-derived exosomes (MSC-exo) have been demonstrated to play dual roles in cancer progression, either promoting or inhibiting tumor growth, depending on the specific context. This unique ability positions MSC-exo as a promising tool for cancer therapy. This review examines the multifaceted roles of MSC-exo in various types of digestive system tumors. It highlights the exosomes' potential to modulate tumor microenvironments, influence immune responses, and deliver therapeutic molecules, thereby offering new avenues for targeted cancer treatment. In addition, the review explores the clinical application value of MSC-exo as anti-tumor agents, emphasizing the exosomes' potential for drug delivery and personalized medicine. However, despite the exosomes' therapeutic potential, several challenges must be addressed before MSC-exo can be widely adopted in clinical settings. These include issues related to large-scale production, standardization, safety, and regulatory approval. By addressing these challenges, MSC-exo could emerge as a transformative approach in cancer treatment, offering innovative solutions for precision medicine and improved patient outcomes. This review underscores the importance of continued research to fully realize the potential of MSC-exo in oncology.

Keywords: Mesenchymal stem cells; Exosomes; Mesenchymal stem cell-derived exosomes; Intercellular communication regulators; Anti-tumor; Drug delivery

***Corresponding author:**
Ying Liu
(liuying2021@126.com)

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1. Introduction

The global burden of malignant tumors has been rising continuously. In 2022, an estimated 20 million new cases of malignant tumors were diagnosed globally, and 9.7 million people died from malignant tumors, with digestive system tumors contributing significantly to the global cancer burden. In 2022, digestive system tumors accounted for 23.9% of new global cancer cases and 33.2% of cancer deaths.[1] Treatment for advanced malignant

tumors of the digestive system includes surgical treatment, radiotherapy, and drug therapy. For most patients with metastatic malignant tumors, curative treatment is often no longer a viable option. Palliative chemotherapy has been the main systemic drug treatment, but its clinical application is limited by significant toxicity. In recent years, targeted therapy has further improved treatment efficacy; however, off-target toxicity remains a pressing issue in clinical practice.[2] As research into the mechanisms of mesenchymal stem cells (MSCs) and MSC-derived exosomes (MSC-exo) deepens, growing evidence suggests that both MSCs and MSC-exo hold great potential in treating malignant tumors.[3]

2. MSCs and their exos

2.1. MSCs

MSCs represent a class of pluripotent stem cells (iPSCs) capable of self-renewal and differentiation into various cell types. Under specific induction conditions, MSCs can differentiate into various tissue cells, including adipocytes, muscle cells, tendon cells, ligament cells, nerves cells, liver cells, cardiomyocytes, and endothelial cells, among others. MSCs typically exhibit spindle-shaped or stellate adherent growth, with high expression of CD73, CD90, and CD105, and low expression of CD34, CD45, CD14 or CD11b, CD79a or CD19, and HLA-DR. Even after repeated passage culture and cryopreservation, MSCs retain their multilineage differentiation potential, making them an ideal choice for cell therapy.[4] Clinically, MSCs have achieved significant breakthroughs in treating various diseases, including hematological diseases, cardiovascular diseases, liver cirrhosis, neurological disorders, and autoimmune diseases.[5] More importantly, MSCs can regulate tumor growth through various mechanisms.

2.2. Exosome formation and components

Exosomes are nanoscale vesicles with a closed membrane structure, typically ranging from 40 to 100 nm in diameter, and are encapsulated by a lipid bilayer. These vesicles contain many biologically functional molecules – such as proteins, nucleic acids, and lipids – and serve as an important medium for transmitting biological signals between cells.[6] In 1983, exosomes were discovered for the 1st time,[7] whereas in 2007, exosomes were found to contain microRNAs (miRNA) and mRNAs, confirming that genetic material can be exchanged between cells through exosomes, thereby altering cellular biological behavior.[8] Exosomal proteins generally encompass a variety of types, such as integral membrane proteins, peripheral membrane proteins, outer membrane proteins anchored by lipids, inner membrane proteins anchored by lipids, surface proteins, and enzymes associated with exosomes. Notably, most of the identified exosomal proteins, such as heat shock proteins and MHC molecules, are also found in other types of extracellular vesicles. However, a series of proteins is relatively specific to exosomes, including CD9, CD63, CD81, TSG101, Alix, HSP70, and HSP90. These proteins are considered markers for identifying exosomes. The lipid composition of exosomes is mainly divided into four categories: sphingolipids, phospholipids, glycolipids, and fatty acids. Thousands of RNA molecules, including miRNAs, long non-coding RNAs (lncRNAs), and circular RNAs, have been identified in exosomes.[9-13] The process of exosome formation (Figure 1) mainly involves the following steps:

(i) Endocytosis: The cell membrane invaginates, forming the endosome.
(ii) Transformation into multivesicular bodies (MVBs): The endosome further transforms into MVBs. During this process, the membrane of the endosome invaginates, forming multiple small vesicles with lipid bilayers.
(iii) Release of exosomes: The MVBs merge with the cell membrane, discharging their internal small vesicles into the extracellular environment as exosomes.[14]

3. Roles of MSC-exo in cancer

Tumor tissues are made up of tumor cells and the tumor microenvironment (TME). Tumor cells, having lost their normal regulatory mechanisms, can grow uncontrollably, invade nearby tissues, and spread to distant parts of the body. The TME includes various components, such as endothelial cells, T cells, natural killer T-cells, myeloid-derived suppressor cells, cancer-associated fibroblasts (CAFs), and tumor-associated stromal cells, among others.[15] MSC-exo plays a crucial role in tumor growth by transporting regulatory molecules. Interestingly, growing evidence suggests that MSC-exo can have dual effects in cancer, acting as a double-edged sword. Some studies indicate that MSC-exo can promote tumor growth. For example, Wang *et al.*[16] demonstrated that exosomes derived from bone marrow mesenchymal stem cells (BMMSC-exo) from both multiple myeloma patients and healthy donors can enhance the growth of multiple myeloma (MM) cells by activating several signaling pathways related to cell proliferation, such as *p38, p53*, and Akt. Further research by Deng *et al.*[17] confirmed that LINC00461 in BMMSC-exo from multiple myeloma patients increases BCL-2 expression by targeting miR-15a/16, thereby preventing apoptosis in multiple myeloma cells. Likewise, studies have also reported that MSC-exo inhibits tumor growth. For instance, BMMSC-exo can release miR-222-3p, which directly targets the *IRF2* gene, thereby negatively regulating the IRF2/INPP4B signaling pathway in THP-1 cells and

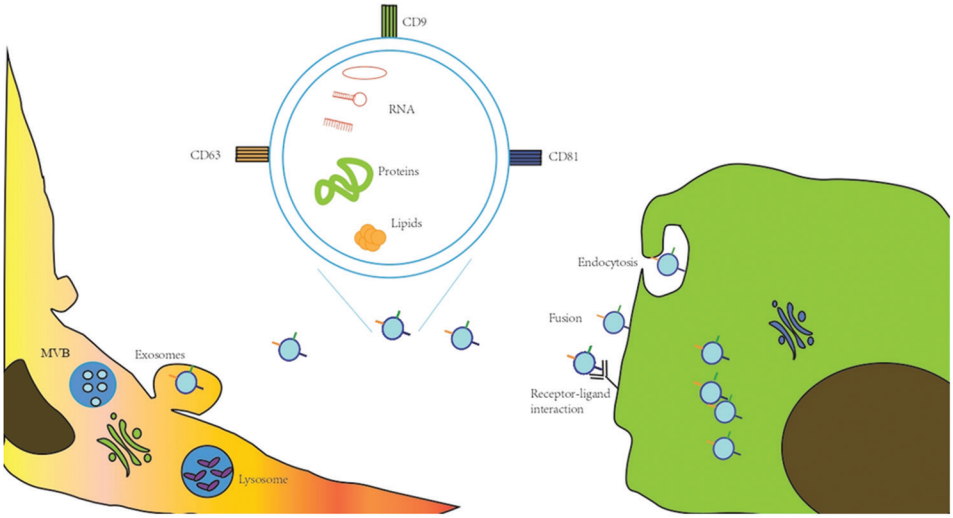

Figure 1. The process of exosome formation. The membrane of the endosome invaginates, forming multiple small vesicles with lipid bilayers. Late endosomes that encapsulate small vesicles are referred to as multivesicular bodies (MVBs). While some MVBs are directed to the Golgi complex or lysosomes for degradation, others fuse with the plasma membrane, releasing their internal small vesicles into the extracellular space, where they are identified as exosomes. MSC-exo can enter cancer cells via various mechanisms, such as membrane fusion, receptor-mediated endocytosis, and phagocytosis. The exosome is composed of three common surface markers (CD9, CD63, and CD81). These exosomes carry a diverse range of biologically active molecules, including proteins, nucleic acids, and lipids.
Abbreviations: MSC: Mesenchymal stem cell; MSC-exo: Mesenchymal stem cell-derived exosomes.

suppressing leukemia cell proliferation.[18] The following sections explore the role of MSC-exo in various types of malignant tumors of the digestive system.

3.1. Liver cancer

The primary pathological type of liver cancer (accounting for 75 – 85%) is hepatocellular carcinoma (HCC). Liver cancer has an insidious onset and a high post-operative recurrence rate, with an overall recurrence rate of up to 70% within 5 years.[19] Recently, MSC-exo has garnered wide attention for its potential in treating liver cancer. Most studies suggest that MSC-exo can inhibit HCC through various pathways, though a few studies indicate that MSC-exo may promote HCC development. Furthermore, MSC-exo may be bioengineered to enhance anti-tumor effects.

Research has demonstrated that exogenous MSC-exo exerts inhibitory effects on liver cancer. For instance, BMMSC-exo containing miR-15a suppressed HCC by downregulating the expression of *SALL4*.[20] Similarly, exosomes derived from umbilical cord mesenchymal stem cells (UCMSC-exo) inhibit HCC proliferation and angiogenesis by reducing the expression of various proteins, such as *SIRT-1, VEGF, SDF-1*, and *CXCR-4*, while simultaneously upregulating *TNF-α* and *caspase-3* levels.[21] UCMSC-exo facilitates the transfer of miRNA-451a from UCMSCs to HCC cells, leading to a reduction in ADAM10 expression. This process reverses the resistance of HCC cells to paclitaxel (PTX). Furthermore, the decreased expression of ADAM10 suppresses epithelial-mesenchymal transition (EMT) and HCC cell proliferation.[22] LncRNAs in MSC-exo also exhibit anti-tumor effects. UCMSC-exo containing lncRNA FAM99B reduces the proliferation, migration, and invasion of MHCC97L and MHCC97H cells. *In vivo*, UCMSC-exo significantly inhibited tumor growth, and exosomes overexpressing lncRNA FAM99B further enhanced this effect.[23] BMMSC-exo, when co-cultured with Hep3B and HuH7 cancer stem cells (CSCs), suppressed the proliferation, invasion, and angiogenesis of these tumor stem cells. *In vivo* experiments demonstrated that these exosomes also inhibited the growth of transplanted tumors. Further investigations revealed that BMMSC-exo facilitated communication between BMMSCs and HCC cells via lncRNA C5orf66-AS1. This lncRNA acted as a sponge, reducing the levels of the oncogenic miR-127-3p in HCC cells, which in turn activated the DUSP1/ERK signaling pathway, thereby curbing the malignant behavior of HCC cells.[24]

However, studies also suggest that MSCs, when stimulated externally, may secrete exosomes that promote HCC progression. For instance, hypoxia-induced BMMSC-exo containing miR-652-3p inhibits TNRC6A, thereby promoting HCC cell proliferation and metastasis[25] (Table 1).

Numerous studies have confirmed that MSC-exo, rich in various non-coding RNAs, can inhibit HCC

Table 1. The role of MSC-exo in HCC

MSC-exo	Loaded small molecule	Cell line	Animal model	Mechanism of action	Effect	References
BMMSCs	miR-15a	Hep3B; Huh7	Hep3B cell xenograft in BALB/c nude mice	Downregulated SALL4 expression	Inhibited HCC progression	[20]
UCMSCs	lncRNA FAM99B	MHCC97L; MHCC97H	MHCC97L cell xenograft in BALB/c nude mice	-	Enhanced cell cycle arrest and cell apoptosis while suppressing cell viability, migration, and invasion in HCC	[23]
BMMSCs	lncRNA C5orf66-AS1	Hep3B-CSCs; HuH7-CSCs	Hep3B-CSCs cell xenograft in BALB/c nude mice	Downregulated miR-127-3p; upregulated the DUSP1/ERK axis	Blocked malignant behaviors of HCC-sourced CSCs	[24]
BMMSCs	miR-652-3p	SMMC-7721; hepG2	-	Downregulated the TNRC6A expression	Promoted the proliferation and metastasis of HCC	[25]
AMSCs	miR-199a-3p	Huh7; SMMC-7721; PLC/PRF/5	PLC/PRF/5 cell xenograft in BALB/c nude mice	Downregulated the mTOR pathway	Increased the sensitivity of HCC cells to chemotherapeutic agents	[27]
BMMSCs	miR-338-3p	HepG2	-	Downregulated EST1 expression	Inhibited HCC cell proliferation, invasion, and migration; induced cell apoptosis	[28]

Abbreviations: AMSCs: Adipose mesenchymal stem cells; BMMSCs: Bone marrow mesenchymal stem cells; CSCs: Cancer stem cells; HCC: Hepatocellular carcinoma; UCMSCs: Umbilical cord mesenchymal stem cells; MSC-exo: mesenchymal stem cell-derived exosomes; HCC: Hepatocellular carcinoma.

cells through different mechanisms, suggesting their therapeutic potential for HCC. MSC-exo, in combination with other anti-tumor drugs, has also demonstrated more significant efficacy. Research indicates that miR-125a and miR-125b inhibit HCC cell growth and stemness via the CD90 pathway.[26] Adipose MSC-exo (AMSC-exo) can mediate the transfer of miR-199a-3p, miR-374c-5p, and miR-338-3p between AMSCs and HCC cells.[27-29] Overexpression of miR-199a-3p and miR-122 in AMSC-exo can alter downstream gene expression, enhancing HCC cell sensitivity to chemotherapy.[27] MiR-338-3p-overexpressing BMMSC-exo inhibits HCC cell proliferation, invasion, and migration by downregulating ETS1 and inducing apoptosis.[28] In addition, MSC-exo can serve as effective drug carriers for CSC-targeted therapy. For instance, BMMSC-exo delivering norcantharidin (NCTD) demonstrated greater anti-tumor effects compared to NCTD alone, promoting NCTD uptake by tumor cells, inducing cell cycle arrest, and enhancing apoptosis. BMMSC-exo-NCTD exhibited tumor-targeting effects at liver cancer sites and repaired liver cells without inducing systemic toxicity.[30] Moreover, BMMSC-exo modified with siGRP78, combined with sorafenib, targeted GRP78 in HCC cells, inhibiting cancer cell growth. The exosomal transfer of siGRP78 enhanced sorafenib sensitivity in chemoresistant HCC cells.[31]

3.2. Gastric cancer (GC)

East Asia is a hotspot for GC. In regions where routine early screening for GC has not been widely implemented, the early diagnosis rate is low. Most patients are diagnosed at advanced stages, often missing the optimal window for treatment. Therefore, there is an urgent need to explore novel therapeutic strategies for GC.

Research has demonstrated that MSC-exo has a certain inhibitory effect on GC. *In vitro*, UCMSC-exo co-cultured with the GC cell line BGC-823 significantly inhibited tumor cell activity.[32] In addition, BMMSC-exo can deliver miR-200a to TGF-β-treated AGS cells, reversing EMT, normalizing the expression of *ZEB1*, vimentin, and Snail1, and inhibiting tumor progression.[33] MiR-1228 is inversely correlated with the survival of GC patients, with lower levels observed in those diagnosed with stage III and IV GC. When BMMSC-exo, loaded with miR-1228, were co-cultured with GC cells, miR-1228 was found to function as a tumor suppressor by targeting and downregulating *MMP-14*, effectively inhibiting GC cell metastasis.[34]

However, some studies have indicated that MSC-exo may contribute to GC progression. BMMSC-exo promotes EMT in GC by upregulating *RHOXF2*. After oxaliplatin (OXA) treatment, the upregulation of miR-424-3p in BMMSC-exo inhibits *RHOXF2* expression in GC cells, thereby suppressing their proliferation, migration, and

invasion. This suggests that delivering miR-424-3p through BMMSC-exo is a promising therapeutic approach for GC.[35] In addition, MSC-exo conferred drug resistance to GC cells, primarily through their protein content. These proteins activate the *CaM-Ks/Raf/MEK/ERK* signaling pathway, increase multidrug resistance protein expression, and protect GC cells from chemotherapy-induced apoptosis.[36] Another study found that exosomes from p53-deficient BMMSCs enhance the proliferation and migration of GC cells and p53 wild-type BMMSCs.[37] Research has also reported that BMMSC-exo can transfer miR-374a-5p to GC cells, enhancing the expression of adhesion molecules in these cells by targeting *HAPLN1*, which in turn facilitates the migration of GC cells.[38] BMMSC-exo has been proposed to secrete miR-221, which acts as a tumor-promoting molecule by activating the Hedgehog and *PTEN/P27* signaling pathways, thereby promoting GC proliferation and progression.[39] Moreover, BMMSC-exo can promote tumor growth by triggering the Hedgehog signaling pathway[40] (Table 2).

3.3. Colorectal cancer (CRC)

For advanced-stage CRC, enhancing the efficacy of drug therapy is crucial. Most studies suggest that MSC-exo can inhibit CRC. AMSC-exo has been reported to suppress the expression of aquaporin 5 (*AQP5*) and *EGFR* genes, which are key molecules in tumor progression, within the HCT-116 tumor cell line.[41] BMMSC-exo can also deliver miR-100 into CRC cells, downregulating the mTOR/miR-143 axis and inhibiting CRC cell proliferation, migration, and invasion.[42] In addition, BMMSC-exo can inhibit tumor-associated macrophage activity. Research has demonstrated that UCMSC-exo mediates miR-1827, inhibiting *SUCNR1* in CRC cells. Furthermore, UCMSC-exo also suppresses M2 macrophage polarization and liver metastasis.[43]

Studies have confirmed that MSC-exo significantly inhibits overexpressed integrin family proteins in CRC cells. Xu *et al.*[44] demonstrated that integrin α2 (*ITGA2*) is overexpressed in CRC cells. It is indicated that miR-16-5p derived from BMMSC-exo inhibits CRC cells by downregulating *ITGA2*.[44] Some studies have reported that MSC-exo may promote CRC. AMSC-exo promotes the advancement of CRC by triggering the transformation of MSCs into CAFs (MSC-CAFs) via the *TRPC3/NF-KB* signaling pathway[45] (Table 3).

In recent years, MSC-exo-based therapies have become a key focus of research for treating CRC. Studies have reported that UCMSC-exo upregulates miR-431-5p, consequently inhibiting CRC progression by suppressing

Table 2. The role of MSC-exo in GC

MSC-exo	Loaded small molecule	Cell line	Animal model	Mechanism of action	Effect	References
UCMSCs	-	BGC-823	-	-	Enhanced GC cell proliferation, invasion, and migration	[32]
BMMSCs	miR-200a	TGF-β-treated AGS	-	Inhibited mesenchymal-epithelial transition	Inhibited GC cell proliferation and migration	[33]
BMMSCs	miR-1228	SGC-7901; MGC-823	-	Downregulated MMP-14 expression	Inhibited GC cell metastasis	[34]
BMMSCs	miR-424-3p	SGC-7901	SGC-7901 cell xenograft in BALB/c nude mice	Inhibited RHOXF2 expression	Inhibited GC cell proliferation, invasion, and migration	[35]
UCMSCs	-	HGC-27; MGC-803; SGC-7901	HGC-27 cell xenograft in BALB/c nude mice	Inhibited the CaM-Ks/Raf/MEK/ERK pathway	Enhanced drug resistance	[36]
$p53^{-/-}$ mBMMSCs	-	MFC	MFC cell xenograft in BALB/c nude mice	Inhibited the Wnt/β-catenin pathway	Enhanced GC progression	[37]
BMMSCs	miR-374a-5p	SGC-7901; MGC-823	SGC-7901 cell xenograft in BALB/c nude mice	Upregulated HAPLN1 expression	Enhanced GC cell proliferation and migration	[38]
BMMSCs	miR-221	SGC-7901; BGC-823	SGC-7901 cell xenograft in BALB/c nude mice	Activated the Hedgehog and PTEN/P27 pathways	Enhanced the oncogenic activity of GC cells	[39]
BMMSCs	-	MG63; SGC7901	-	Activated the Hedgehog pathway	Enhanced GC progression	[40]

Abbreviations: AMSCs: Adipose mesenchymal stem cells; BMMSCs: bone marrow mesenchymal stem cells; UCMSCs: umbilical cord mesenchymal stem cells; MSC-exo: Mesenchymal stem cell-derived exosomes; GC: Gastric cancer.

Table 3. The role of MSC-exo in CRC

MSC-exo	Loaded small molecule	Cell line	Animal model	Mechanism of action	Effect	References
AMSCs	-	HCT-116	-	Inhibited the expression of aquaporin 5 and EGFR	Inhibited HCT-116 cell polarization	[41]
BMMSCs	miR-100	HCT-116	-	Downregulated the mTOR/miR-143 axis	Suppressed CRC cell proliferation and metastasis	[42]
UCMSCs	miR-1827	HCT-116; SW480; Caco-2; HT-29	HCT-116 cell xenograft in BALB/c nude mice	Downregulating SUCNR1 expression	Inhibited macrophage M2 polarization; prevented colorectal liver metastasis	[43]
BMMSCs	miR-16-5p	Caco-2; SW480; SW620; LoVo; HT29	Caco-2 BALB/c cell xenograft in BALB/c nude mice	Downregulated ITGA2 expression	Inhibited CRC cell proliferation, migration, and invasion	[44]
AMSCs	-	HCT-116	HCT-116 cell xenograft in BALB/c nude mice	Upregulated TRPC3 expression	Accelerated CRC progression by inducing the MT-CAF cell phenotype	[45]
UCMSCs	miR-431-5p	Caco-2; SW480; SW620; LoVo; HCT 116	LoVo cell xenograft in BALB/c nude mice	Downregulated PRDX1 expression	Suppressed CRC cell growth	[46]
BMMSCs	miR-4461	DLD1; HCT116; SW480	-	Downregulated COPB2 expression	Inhibited CRC cell tumorigenesis	[47]

Abbreviations: AMSCs: Adipose mesenchymal stem cells; BMMSCs: Bone marrow mesenchymal stem cells; CAF: Cancer-associated fibroblasts; MT: Mesenchymal stem cell-transformed; UCMSCs: Umbilical cord mesenchymal stem cells; MSC-exo: Mesenchymal stem cell-derived exosomes; CRC: Colorectal cancer; DLD: Deterministic lateral displacement.

PRDX1.[46] Chen *et al.*[47] demonstrated that miR-4461 level is lower in CRC cells and tissues. BMMSC-exo-induced miR-4461 overexpression reduces CRC cell migration by downregulating *COPB2*. Pishavar *et al.*[48] developed an effective method to load SN38 into exosomes modified with *MUC1* aptamers to target MUC1-overexpressing cells, demonstrating good inhibitory effects on CRC. *In vivo* studies in a BALB/c mouse C26 ectopic model demonstrated that a single intravenous injection of doxorubicin (DOX)-loaded MUC1-modified UCMSC-exo (UCMSC-exo-DOX) significantly inhibited tumor growth compared to the group with conventional DOX.[49] Additional research indicated that UCMSC-exo-DOX exhibited greater accumulation in tumors and quicker clearance by the liver compared to the group treated with conventional DOX. Han *et al.*[50] constructed an iRGD-Lysosome-associated membrane protein 2 (Lamp2b) fusion gene-modified UCMSCs and isolated and purified exosomes. They loaded anti-mir-221 into exosomes through electroporation. The findings demonstrated that exosomes modified with iRGD effectively suppressed the colony formation of CRC cells. It was further revealed that these iRGD-modified exosomes were internalized by CRC cells from their interaction with the NRP-1. *In vivo* experiments also indicated a significant accumulation of iRGD-modified exosomes at the tumor site.[50] Taken together, MSC-exo holds great promise as drug delivery vehicles, but further research is warranted to fully realize the exosomes' potential.

3.4. Pancreatic cancer

The prognosis for pancreatic cancer is extremely poor, characterized by difficulties in early diagnosis, low surgical resection rates, and a high tendency for recurrence and metastasis. Studies have indicated that the impact of MSC-exo on pancreatic cancer varies and lacks consistency. Ding *et al.*[51] discovered that miR-100-5p is highly expressed in UCMSC-exo, promoting the growth of PANC-1 and BxPC3 cells. In addition, miR-145-5p is highly expressed in UCMSC-exo, but it suppresses pancreatic ductal cell carcinoma by inhibiting the *TGF-β/Smad3* signaling pathway.[52] Other studies have reported that BMMSC-exo carrying miR-1231 can inhibit pancreatic cancer by suppressing the *EGFR/cyclin E* pathway[53] (Table 4).

The TME of pancreatic cancer differs from that of other tumors. Beyond the typical cellular elements, pancreatic cancer features an abundant extracellular matrix (ECM) composed of collagen, matrix proteins, and a variety of soluble factors, including cytokines, chemokines, and growth factors. Essentially, the TME in pancreatic cancer is characterized by a dense stromal structure resulting from excessive fibrosis driven by active connective tissue proliferation and accumulation. This extensive fibrosis, along with a lack of vasculature, immune infiltration, and a hypoxic stromal environment, not only promotes tumor growth and invasion but also induces resistance to anti-tumor drugs. Therefore, studying the roles of different components within the TME of pancreatic cancer and

Table 4. The role of MSC-exo in pancreatic cancer

MSC-exo	Loaded small molecule	Cell line	Animal model	Mechanism of action	Effect	References
UCMSCs	miR-100-5p	BxPC-3; PANC-1	PANC-1 cell xenograft in BALB/c nude mice	-	Promoted pancreatic ductal adenocarcinoma growth	[51]
UCMSCs	miR-145-5p	PANC-1; BxPC; Capan-1; CFPAC-1	BALB/c PANC-1 cell xenograft in BALB/c nude mice	Downregulated the TGF-β/Smad3 pathway	Inhibited pancreatic cancer progression	[52]
BMMSCs	miR-1231	BxPC-3; MIA PaCa-2; PANC-1; SW1990	BxPC-3 cell xenograft in BALB/c nude mice	Downregulated the EGFR/cyclin E pathway	Inhibited pancreatic cancer cell proliferation	[53]

Abbreviations: BMMSCs: Bone marrow mesenchymal stem cells; UCMSCs: Umbilical cord mesenchymal stem cells; MSC-exo: Mesenchymal stem cell-derived exosomes.

targeting therapies is crucial for the future management of pancreatic cancer. The good tumor-targeting ability and deep tissue penetration capability of MSC-exo make them ideal carriers for drugs targeting pancreatic cancer.[54-56]

In an effort to address the chemoresistance of pancreatic cancer, Zhou *et al.*[57] encapsulated PTX, gemcitabine monophosphate, and an intermediate metabolite of gemcitabine into purified BMMSC-exo. Their findings revealed that the BMMSC-exo-based drug delivery system demonstrated excellent targeting and tissue penetration capabilities. This approach resulted in a promising anti-tumor effect while minimizing systemic toxicity.[57] Zhou *et al.*[58] employed BMMSC-exo to simultaneously deliver galectin-9 siRNA and OXA, effectively reversing the immunosuppressive TME. This was achieved by suppressing M2 macrophage polarization and promoting the recruitment of cytotoxic T cells, thereby improving the efficacy of immunotherapy in treating pancreatic cancer.

OXA is a critical component of the standardized FOLFIRINOX regimen for pancreatic cancer, capable of triggering immunogenic cell death (ICD) at the tumor site and killing tumor cells by inhibiting DNA synthesis and repair. To further enhance the anti-tumor effect, a research group used galectin-9 siRNA to block the *galectin-9/dectin-1* interaction, synergizing with OXA to reverse M2 tumor-associated macrophage-induced immunosuppression.[58] This delivery platform was developed by encapsulating galectin-9 siRNA via electroporation and functionalizing the surface with an OXA prodrug to act as an ICD inducer. In addition, the research group engineered siRNA-exosome-OXA (iEXO-OXA) nanoparticles and reported that siRNA EXO-OXA enhanced the drug concentration at the tumor site. EXO-OXA promoted anti-tumor immunity by driving the polarization of tumor-suppressive macrophages, recruiting cytotoxic T lymphocytes, and reducing regulatory T cells (Tregs), resulting in notable therapeutic outcomes in cancer treatment. The findings suggest that MSC-exo can be used as drug delivery vehicles to enhance immunogenicity and regulate the TME, providing a theoretical foundation for the advancement of novel pancreatic cancer treatments.[58]

3.5. Other malignant tumors of the digestive system

MSC-exo has also demonstrated certain potential for the treatment of esophageal cancer. For example, miR-375 in UCMSC-exo can inhibit the proliferation, invasion, and migration of esophageal squamous cell carcinoma cells while promoting apoptosis by suppressing *ENAH* expression and regulating the protein levels of *Bax* and *E-cadherin*.[59] Several studies have focused on using MSC-exo for treating biliary tract cancer. For example, miR-15a-5p in UCMSC-Exo can hinder the progression of cholangiocarcinoma by inhibiting *CHEK1* expression.[60] However, the role of MSC-exo in tumor cells remains controversial, likely due to the exosomes' inherent complexity and diversity, as well as variations in culture conditions.

4. MSC-exo as a vehicle for drug delivery

Drugs can be loaded into exosomes through pre-loading (before exosome isolation) and post-loading (after exosome isolation).[61] Common exogenous drug-loading methods include electroporation, co-incubation, sonication, freeze-thaw cycles, and extrusion.[62] The primary advantage of post-loading is its simple process. However, post-loading has certain drawbacks, such as the potential to damage the integrity of exosomes during the loading process. Moreover, some exosomes may fail to load the drugs successfully, necessitating additional purification steps to remove them. Pre-loading involves introducing or expressing target molecules (*e.g.*, nucleic acids, proteins, or drugs) into MSCs before exosome isolation. The target molecules are then incorporated into exosomes, which

are subsequently collected. The advantage of pre-loading is that it preserves the integrity of the exosomes, but the process is complex, time-consuming, and costly.[63] Despite substantial progress in the anti-tumor research of MSC-exo, several key unresolved issues and limitations hinder the exosomes' clinical application:

(i) Heterogeneity of MSC-exo: The heterogeneity of MSC-exo can be managed by standardizing the isolation, purification, storage, and characterization methods. Standardized procedures can maximize uniformity in exosome size, composition, and contents. In addition, different extracellular vesicle subpopulations derived from MSCs should be identified for their anti-tumor activity, as different isolation and purification methods can result in MSC-exo with varying contents, characteristics, and biological functions.[64,65]

(ii) Large-scale production and storage: The large-scale production and storage of MSC-exo present significant challenges to its translation into clinical practice, largely due to the limited availability of MSCs. Induced iPSCs, generated by reprogramming fully differentiated somatic cells through the introduction of specific transcription factors, offer a potential solution. IPSCs possess the ability to self-renew indefinitely and can differentiate into any cell type, including MSCs.[66] Therefore, iPSC-MSCs (iMSCs) could provide an unlimited source of iMSCs to overcome the limitations of primary MSCs, making them a primary source for large-scale applications. Studies have compared the efficacy of iPSC-derived MSCs with that of primary MSCs. The findings indicated that iPSC-derived MSCs and bone marrow-derived MSCs displayed no significant difference in their ability to promote collagen synthesis and angiogenesis, both effectively promoting skin wound healing.[67,68] In addition, JNKi- and DAC-programmed MSCs from human embryonic stem cells (hESC-MSCs) exhibit similar adipogenic, osteogenic, and chondrogenic differentiation abilities as tissue-derived MSCs. They also facilitate hematopoiesis and alleviate hind limb ischemia.[69] These findings suggest that iMSCs and hESC-MSCs are emerging as attractive alternatives to traditional MSCs.

(iii) Exosome isolation technology: The isolation technology of exosomes warrants further optimization. At present, the commonly used exosome isolation techniques, such as ultracentrifugation and commercial kit-based methods, do not meet the needs of commercial production. Studies combining polyethylene glycol precipitation, dielectrophoresis, and deterministic lateral displacement (DLD) isolation methods indicate that integrating multiple isolation methods provides a viable approach for achieving efficient and high-purity exosome production, though further improvements in isolation efficiency are still required.[70]

(iv) Target specificity and reducing off-target toxicity: Although MSC-exo displays tumor-targeting properties, the exosomes can still bind to normal tissue cells, necessitating the optimization of exosome targeting to reduce off-target toxicity. The most commonly used method to enhance exosome targeting is genetically engineering tumor-specific ligands on the exosome membrane. Lamp2b, the transmembrane protein platelet-derived growth factor receptor, members of the tetraspanin superfamily, and lactoferrin can all serve as exosome membrane targeting ligands to improve exosome tumor targeting. RGD (Arg-Gly-Asp) peptides, present in various ECMs, are capable of precisely identifying and attaching to integrins present on the surfaces of tumor cells, acting as competitive inhibitors of RGD peptide-like substances *in vivo*. This can inhibit tumor cell adhesion and migration to the ECM, suppress tumor angiogenesis, and induce tumor cell apoptosis. Research has demonstrated that intravenous injection of DOX-containing iRGD-exosomes can effectively penetrate the blood-spinal cord barrier and deliver DOX to the spinal cord injury site, enhancing neurological function recovery.[71]

Although generating exosomes from modified MSCs offers new possibilities for cancer treatment, it also carries the risk of altering the biological properties of MSCs. Such alterations may affect the composition, function, and stability of exosomes, thereby impacting their therapeutic efficacy and safety.[72] Therefore, when developing MSC-exo-based therapeutic strategies, it is essential to fully consider the potential risks associated with modifications and to optimize the quality and function of exosomes through precise modifications and functional validation. Future research should further explore the mechanisms through which modifications affect MSCs and exosomes to advance their clinical applications.

5. Conclusion

MSC-exo is rich in various bioactive substances, including nucleic acids, proteins, and lipids. As carriers of intercellular communication, MSC-exo exhibits both tumor-promoting and tumor-inhibiting effects. This further highlights the potential of MSC-exo as novel anti-tumor drugs in clinical applications, providing new clinical options for anti-tumor therapy. However, since MSC-exo plays a critical role in tumors, further research is warranted to mitigate the tumor-promoting effects of MSC-exo and expand its application as anti-tumor therapeutics.

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Author contributions

Conceptualization: Ying Liu
Visualization: Pengyun Lin
Writing – original draft: Ying Liu
Writing – review & editing: Tao Zhang

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ACCSCIENCE PUBLISHING

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REVIEW ARTICLE

Advancements in antivenom therapy: Historical perspectives, current challenges, and ongoing clinical trials

Luis A. Roque*

Diabetes Research Institute, Miller School of Medicine, University of Miami, Miami, Florida, United States of America

Abstract

Snakebite envenomation remains a severe global health burden, particularly in impoverished, rural, and tropical regions where healthcare resources are sparse. Despite over 125 years of progress in antivenom therapy, numerous obstacles persist related to efficacy, specificity, cost, and availability. Conventional antivenoms, although life-saving, are associated with significant drawbacks, including species specificity and adverse immunologic reactions. This review explores the historical milestones in antivenom development, discusses present therapeutic limitations, highlights novel innovations through biotechnological approaches, and presents a list of ongoing clinical trials that aim to revolutionize the field. It emphasizes the pressing need for improved therapeutics and the critical role of translational research in mitigating the global impact of snakebite envenomation.

Keywords: Snakebite envenomation; Antivenom therapy; Recombinant antivenom; Monoclonal antibodies; Venom immunology; Neglected tropical diseases; Clinical trials; Toxicology

***Corresponding author:**
Luis A. Roque
(lroque@med.miami.edu)

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1. Introduction

Snakebite envenomation remains one of the most underrecognized and underfunded public health crises in the modern era, despite its classification as a high-priority neglected tropical disease by the World Health Organization (WHO) in 2017.[1] This categorization underscores the urgent need for expanded research, therapeutic innovation, and coordinated policy interventions. Globally, it is estimated that more than 5.4 million snakebites occur each year, resulting in up to 2.7 million cases of envenomation.[2] Of these, approximately 81,000 to 138,000 result in death, while an additional 400,000 individuals experience permanent disabilities, such as blindness, limb amputations, or disfigurement.[2] The actual burden is likely even higher, as many cases go unreported due to inadequate surveillance systems and the prevalence of traditional or informal medical care in affected regions.

The epidemiology of snakebite envenomation reveals a pronounced geographical and socioeconomic disparity. The majority of envenomation cases occur in sub-Saharan Africa, South Asia (particularly India, Bangladesh, and Sri Lanka), Southeast Asia, and Latin America—regions characterized by a high density of venomous snake

species and limited access to modern healthcare services.[3] Within these areas, the most vulnerable populations are impoverished agricultural laborers, herders, hunters, and children, who are frequently exposed to snake habitats during their daily activities. Rural isolation, deficient transportation infrastructure, and economic constraints often delay or prevent victims from receiving timely medical treatment. Consequently, snakebite envenomation is both a biomedical emergency and a disease of inequity, mirroring broader systemic failures in healthcare delivery and resource allocation.

The clinical course of snakebite envenomation varies significantly based on the offending species and the composition of its venom, which may contain neurotoxins, hemotoxins, myotoxins, cardiotoxins, or cytotoxins, often in complex mixtures. Envenomation can lead to rapid-onset systemic manifestations, such as hypotension, coagulopathy, renal failure, neuromuscular paralysis, and extensive local tissue necrosis. Without prompt administration of effective antivenom, the risk of irreversible organ damage or death increases dramatically. Compounding this challenge, diagnostic capabilities to identify the envenoming species are frequently absent in rural clinics, further complicating clinical decision-making.

Treatment status worldwide remains suboptimal. The mainstay of therapy is the administration of antivenom derived from the immunized plasma of horses or sheep, which contains polyclonal antibodies capable of neutralizing venom components. However, the efficacy of such preparations is often geographically limited to the venom profiles of specific snake populations used during the immunization process. In Africa and Asia, polyclonal antivenoms often lack adequate species coverage or exhibit poor cross-reactivity. Adverse reactions, such as early anaphylaxis or late-onset serum sickness, are relatively common due to the xenogeneic nature of these biologics. Furthermore, many antivenoms are prohibitively expensive and require cold-chain storage, rendering them inaccessible or impractical in remote regions. The unregulated proliferation of substandard or counterfeit products further erodes the trust of both clinicians and patients, jeopardizing treatment outcomes and contributing to therapeutic hesitancy.

Recognizing these challenges, the WHO launched its global strategy for the prevention and control of snakebite envenoming in 2019, with the goal of halving the number of deaths and disabilities by 2030.[4] This plan emphasizes four pillars: Community empowerment, accessibility of safe and effective antivenoms, strengthened health systems, and increased research and innovation. As part of this initiative, there is an urgent call to modernize antivenom production technologies, improve pharmacovigilance, and implement regulatory frameworks that ensure both product efficacy and equitable distribution.

In light of these realities, this review aims to provide a comprehensive examination of the historical development of antivenom therapy, analyze the present therapeutic limitations, and explore emerging innovations that promise to revolutionize the management of snakebite envenomation. In doing so, it highlights the interplay between scientific progress and global health policy and underscores the necessity for translational research that bridges laboratory breakthroughs with real-world impact.

2. Historical background

The genesis of antivenom therapy can be traced to the late 19th century, an era marked by burgeoning discoveries in immunology and the emerging germ theory of disease. Central to the early development of antivenom was the pioneering work of French physician and bacteriologist Albert Calmette. In 1891, Calmette was sent by the Institut Pasteur to establish a research facility in Saigon, then part of French Indochina, where he was confronted with high mortality rates due to cobra bites.[5] Recognizing the urgent need for a therapeutic countermeasure, Calmette embarked on experimental immunization of horses with sublethal doses of *Naja naja* (Indian cobra) venom. Through repeated injections, he induced the production of circulating neutralizing antibodies in the horses, which he subsequently extracted and purified from their serum. In 1894, he reported the successful production of the first anti-cobra antivenom, which could confer passive immunity in envenomated subjects.[6] Calmette's work marked a transformative moment in toxinology, heralding the birth of serotherapy—using serum-derived antibodies to neutralize exogenous toxins—as a viable and scientific approach to treating snake envenomation (Table 1).

Table 1. Historical timeline of antivenom therapy

Year	Milestone	Key contributor/ institution
1891	First experimental immunization with cobra venom	Albert Calmette, Institut Pasteur
1894	First anti-cobra antivenom developed	Albert Calmette
1901	Development of polyvalent antivenoms	Vital Brazil, Instituto Butantan
1916	Establishment of the Commonwealth Serum Laboratories	Australia
2019	Launch of the WHO global strategy for snakebite envenoming	WHO

Abbreviation: WHO: World Health Organization.

Shortly thereafter, significant advancements were made in South America by Brazilian physician, immunologist, and biomedical scientist Vital Brazil Mineiro da Campanha. Recognizing that the venoms of different snake species required targeted therapeutic strategies, Vital Brazil expanded on Calmette's work by developing polyvalent antivenoms—preparations capable of neutralizing the venoms of multiple species, especially those endemic to Brazil, such as *Bothrops*, *Crotalus*, and *Micrurus*.[7] Unlike Calmette, who initially produced monovalent antivenoms, Vital Brazil's research highlighted the antigenic specificity of venom components and the need for regionally adapted treatments. His meticulous experiments demonstrated that snake venoms were not universally interchangeable, even among species within the same family, and that effective antivenom production required immunization with locally relevant venom mixtures. These innovations culminated in the establishment of the Instituto Butantan in São Paulo in 1901, a premier institution that has since become one of the world's leading centers for venom research, antivenom production, and public health outreach.[8]

The early 20th century witnessed further expansion of antivenom research in other parts of the world. In Australia, systematic efforts were initiated to combat the medically significant elapids, particularly *Pseudonaja* (brown snakes) and *Oxyuranus* (taipans), leading to the foundation of the Commonwealth Serum Laboratories in 1916.[9] Similarly, India and Africa developed region-specific immunization protocols to address the "Big Four" snakes in South Asia and medically important vipers in Africa, respectively. These global initiatives contributed to the creation of a foundational infrastructure for snakebite management, with the shared understanding that regional ecological diversity required tailored immunobiological strategies.

Throughout much of the 20th century, antivenom therapy remained grounded in the principles established by Calmette and Vital Brazil, namely, passive immunization using animal-derived polyclonal antibodies. Horses (and occasionally sheep) were the preferred source animals due to their size, immunologic tolerance, and capacity to generate high antibody titers. The antibodies were often administered as whole immunoglobulin G (IgG) molecules, though subsequent innovations led to the enzymatic digestion of IgG into smaller fragments, such as $F(ab')_2$ and Fab, to reduce the incidence of adverse immunologic reactions and improve pharmacokinetics.

Despite its life-saving potential, traditional antivenom therapy has been historically hindered by limitations in production scalability, quality control, geographic specificity, and adverse reactions associated with heterologous proteins. These challenges laid the groundwork for 21st-century calls to modernize and innovate antivenom therapeutics through biotechnological and recombinant approaches.

In sum, the historical trajectory of antivenom therapy is a compelling example of translational medical science that spans over a century. The foundational contributions of Calmette and Vital Brazil not only saved countless lives but also galvanized international efforts to confront one of the most lethal yet neglected causes of injury and mortality in the Global South. Their legacy continues to shape contemporary research, underscoring the enduring importance of venom immunology as both a clinical and scientific frontier.

3. Present state of antivenom therapy

Modern antivenom production is fundamentally grounded in immunological principles established over a century ago by Calmette and Vital Brazil, yet the process has undergone considerable refinement to improve yield, purity, and safety. The most common production method involves hyperimmunization of large, domesticated animals—most often horses (*Equus ferus caballus*) or sheep (*Ovis aries*)—through the administration of gradually escalating doses of snake venom. These animals develop a robust humoral immune response, generating high titers of polyclonal IgG antibodies capable of neutralizing the toxic components of the venom.

After sufficient antibody production is confirmed through serological assays, blood is harvested from the animals, and plasma is separated for downstream processing. The resulting antibody preparations are subjected to fractionation and enzymatic digestion to enhance their pharmacological profiles and minimize adverse reactions. Three principal antivenom formulations are commonly used: Whole IgG; $F(ab')_2$ fragments, produced through pepsin digestion (which removes the Fc portion while preserving bivalent antigen-binding sites); and Fab fragments, derived through papain digestion (monovalent and rapidly cleared from circulation). The choice of fragment type influences both the efficacy and safety of the antivenom. While Fab fragments exhibit faster tissue penetration and reduced immunogenicity, they are also associated with shorter half-lives and an increased risk of venom recurrence, or "rebound" toxicity, particularly in envenomations involving tissue-depositing toxins.

Despite their proven life-saving capabilities, traditional antivenoms suffer from multiple limitations that impede their effectiveness and availability in real-world clinical settings. One of the most prominent challenges is venom specificity. Because antivenoms are typically raised against

venoms from particular species or genera, their efficacy is restricted to envenomations by those specific snakes or closely related taxa. Given that accurate snake identification is often impossible in emergency scenarios—particularly when the bite is unwitnessed or the snake escapes—there is a high risk of therapeutic mismatch. This is especially problematic in regions with high biodiversity or where multiple medically significant species co-occur.

Another serious limitation is the immunogenicity of heterologous antibodies. Since these products are derived from non-human animals, their administration can provoke adverse immune responses ranging from mild urticaria and fever to severe anaphylaxis and delayed serum sickness. This immunologic burden not only complicates clinical management but also deters some patients from seeking care due to fear or previous negative experiences. Although premedication with antihistamines and corticosteroids is common practice, these measures are not always effective in mitigating severe reactions.

Furthermore, logistical and economic barriers play a substantial role in restricting access to antivenom therapy in the regions where it is most urgently needed. Antivenoms are biologic products that require strict cold-chain storage conditions (typically 2–8°C) to maintain their stability and efficacy. Such requirements are difficult to meet in many rural or resource-poor settings, where electricity and refrigeration may be intermittent or nonexistent. The production process itself is also expensive and time-consuming, involving extensive quality control measures to ensure sterility, potency, and freedom from transmissible agents. These costs are often passed on to healthcare systems or patients, rendering the antivenom unaffordable in many low-income countries. As a result, supply-demand mismatches and commercial disincentives have led to the withdrawal of manufacturers from unprofitable markets, exacerbating global shortages.

In response to these systemic challenges, the WHO has intensified its efforts to coordinate a global strategy for snakebite envenomation, which it formally designated as a neglected tropical disease in 2017.[1] A landmark initiative—"Snakebite Envenoming: A Strategy for Prevention and Control"—was published by the WHO in 2019.[4] This strategy outlines ambitious goals to reduce snakebite deaths and disabilities by 50% by the year 2030. It emphasizes strengthening antivenom manufacturing capabilities in endemic countries, developing standardized preclinical testing protocols, and fostering international partnerships to subsidize and regulate the antivenom market. One of the key components of this initiative is the creation of a prequalification program, akin to those used for vaccines and essential medicines, which would evaluate the safety, efficacy, and quality of antivenoms to guide procurement decisions by national health agencies.

In addition to WHO efforts, academic research institutions, non-governmental organizations (NGOs), and public-private partnerships have begun investing in alternative therapeutic approaches, including recombinant antivenoms, monoclonal antibodies, and small-molecule inhibitors. These emerging technologies hold the promise of safer, more cost-effective, and broadly neutralizing antivenoms, but their widespread clinical adoption remains several years away.

In conclusion, while modern antivenoms have saved countless lives and represent a significant achievement in medical science, their present formulation and distribution remain suboptimal. A multifaceted approach—spanning scientific innovation, policy reform, and health systems strengthening—is required to overcome these barriers and ensure that antivenom therapy becomes a universally accessible and effective intervention.

4. Innovations in antivenom therapy

Innovations in antivenom therapy over the past two decades have sought to overcome the persistent limitations of traditional animal-derived antivenoms and to develop novel approaches that are safer, more effective, and more accessible to snakebite victims in endemic regions. While present manufacturing processes remain largely rooted in the historical principles established by Calmette and Vital Brazil, emerging biotechnological advancements are now reshaping the landscape of envenomation treatment.

One of the most promising avenues of innovation involves the development of recombinant and monoclonal antibody-based therapies.[10] Unlike conventional polyclonal antivenoms, which contain a complex and variable mixture of antibodies harvested from immunized animals, recombinant antivenoms can be engineered to contain specific human or humanized monoclonal antibodies that target the most toxic and medically relevant components of snake venom, such as metalloproteinases, phospholipase A_2 (PLA_2), and three-finger toxins. These monoclonal antibodies can be produced in controlled cell culture systems, eliminating the reliance on animal immunization and addressing key issues related to batch variability, immunogenicity, and contamination. Furthermore, because they can be precisely designed to recognize conserved toxin epitopes across different snake species, recombinant antivenoms hold the potential for broader cross-neutralization, thereby mitigating the clinical challenges posed by species misidentification.

Another area of advancement lies in the use of small-molecule enzyme inhibitors that directly target venom toxins.[11] Compounds, such as varespladib and batimastat have been shown in preclinical models to neutralize venom activity by inhibiting enzymatic components critical to tissue damage and systemic toxicity. Varespladib, in particular, is a potent inhibitor of secretory PLA_2 ($sPLA_2$), a major component of many elapid and viperid venoms. Its small molecular size and potential for oral or parenteral administration make it a promising candidate for field-based, first-line intervention before definitive antivenom administration. Such adjunctive therapies may not only improve survival rates but also reduce the required antivenom dose, thereby diminishing the risk of adverse reactions and treatment costs.

Advancements in nanotechnology have also opened new possibilities for the development of antivenom alternatives.[12] Nanoparticles, such as liposomes and dendrimers are being explored as vehicles for delivering toxin-binding ligands or as scaffolds for detoxifying agents. These nanoscale platforms can be tailored to enhance tissue penetration, prolong circulation time, and improve stability under ambient conditions—features particularly valuable in resource-limited settings where cold-chain logistics are a major barrier to effective care.

Synthetic epitope-based vaccines represent a preventive strategy that diverges from reactive treatment paradigms. By identifying conserved immunogenic regions across venom toxins, researchers aim to develop immunogens capable of eliciting protective immune responses in humans, thereby conferring immunity against envenomation. While still in early experimental phases, such vaccines could revolutionize snakebite management in high-risk occupational or geographic populations, especially in areas with limited access to healthcare.

Despite the promise of these innovations, several obstacles remain. Regulatory pathways for novel biologics, particularly in low- and middle-income countries, can be lengthy and inconsistent. Substantial investment is also needed to support clinical trials assessing the safety, efficacy, and cost-effectiveness of these emerging therapies compared to traditional antivenoms. Moreover, stakeholder coordination—including governments, manufacturers, academic institutions, and NGOs—is critical to ensure these technologies reach the populations most in need.

In response to these complex challenges, the WHO has launched a global strategy to halve the number of snakebite deaths and disabilities by 2030.[13] This strategy includes a commitment to supporting research and development of next generation antivenoms, promoting the use of standardized preclinical efficacy testing, and facilitating regulatory harmonization for new products. Furthermore, WHO has established a global antivenom prequalification program aimed at ensuring the quality, safety, and effectiveness of antivenoms through rigorous review and quality control standards. This initiative seeks not only to rebuild market confidence but also to ensure equitable access to high-quality products in affected regions.

In sum, while traditional antivenoms remain essential tools in snakebite management, ongoing scientific advancements are paving the way toward more rational, scalable, and patient-centered therapies. The integration of recombinant biotechnology, small-molecule pharmacology, nanomedicine, and synthetic vaccinology represents a paradigm shift that, if successfully translated into clinical practice, could dramatically improve outcomes for snakebite victims worldwide.

5. Ongoing clinical trials

Numerous clinical trials registered on ClinicalTrials.gov and other international platforms are actively investigating advanced therapeutic strategies to improve the treatment of snakebite envenomation. These efforts reflect a global commitment to modernizing antivenom therapy, expanding beyond traditional polyclonal antibody-based products to encompass a broad spectrum of innovative modalities. The diversity of these clinical studies highlights the growing complexity and scientific depth of the field.

One of the most notable advancements in this domain is the investigation of small molecule inhibitors, such as varespladib-methyl, an oral $sPLA_2$ inhibitor. The Phase 2 clinical trial, NCT04996264 is currently evaluating its safety, tolerability, and efficacy in envenomated patients.[14] This molecule has shown promise as a broad-spectrum neutralizer of venom activity in preclinical studies, offering the potential to be used as a pre-referral intervention in rural or remote settings where access to healthcare is limited and delays in treatment are common (Table 2).

Another interventional study, NCT04470791, conducted in Mexico, is examining the use of localized cryotherapy as a supplementary approach to standard antivenom treatment in patients with *Bothrops* envenomation.[15] Cryotherapy may reduce local inflammation and tissue necrosis, which are common complications associated with viperid bites. If successful, this strategy could be incorporated into clinical practice to improve functional outcomes and reduce the need for surgical interventions, such as debridement or amputation.

In terms of observational research, trial NCT04520282 aims to measure hemostatic parameters in patients suffering from venom-induced consumption coagulopathy,

Table 2. Summary of ongoing clinical trials in snakebite envenomation

Trial ID	Intervention	Target condition	Location	Status
NCT04996264	Varespladib-methyl	Broad-spectrum venom neutralization	Multinational	Completed
NCT04470791	Cryotherapy+standard antivenom	Bothrops envenomation	Mexico	Completed
NCT04520282	Hemostatic parameter monitoring	VICC	India	Completed
NCT03859154	Non-invasive waveform analysis	Hematotoxic envenomation	India	Completed
NCT00303303	CroFab®	Copperhead envenomation	USA	Completed
NCT00811239	Specific antivenom	Bungarus multicinctus envenomation	Vietnam	Completed

Abbreviation: VICC: Venom-induced consumptive coagulopathy.

a frequent and serious manifestation of envenomation, especially by vipers.[16] By improving the understanding of coagulation profiles in affected individuals, this study may contribute to the development of more targeted and effective supportive therapies, including the timing and dosing of clotting factor replacements.

Diagnostics are also a focal point in contemporary clinical research. Trial NCT03859154, for instance, is developing non-invasive waveform analysis tools to detect early signs of hematotoxic envenomation.[17] Accurate and rapid diagnostics are essential in low-resource environments where laboratory infrastructure may be limited and snake identification is often impossible. Such innovations can inform early triage and therapeutic decisions, thereby improving patient survival and outcomes.

In the United States, a Phase 4 clinical trial, NCT00303303, is assessing the efficacy of CroFab®—a crotaline polyvalent Fab antivenom—for the treatment of copperhead envenomation.[18] This study is particularly relevant given the frequent debate over the necessity of antivenom for copperhead bites, which are generally considered less severe. Data from this trial will inform regulatory and clinical decisions regarding when and how antivenom should be used in such cases.

In addition, trial NCT00811239 evaluates the clinical utility and safety of specific antivenoms in treating envenoming by *Bungarus multicinctus*, the many-banded krait, which produces a neurotoxic venom capable of causing respiratory paralysis.[19] Randomized controlled trials like this one are essential for validating the efficacy of targeted therapies and optimizing antivenom specificity.

Collectively, these trials demonstrate a multidimensional approach to snakebite envenomation, integrating pharmacologic, procedural, diagnostic, and supportive care innovations. They not only enrich the scientific understanding of envenomation pathophysiology but also provide essential data for shaping global guidelines, regulatory policies, and treatment algorithms. The future of antivenom therapy depends on sustained investment in such clinical research, which will be instrumental in achieving the WHO's strategic goal of halving snakebite-related deaths and disabilities by 2030.

6. Conclusion

Over a century after Albert Calmette's pioneering work laid the foundation for antivenom therapy, snakebite envenomation remains a persistently neglected yet urgent global health challenge. It disproportionately affects impoverished populations in sub-Saharan Africa, South and Southeast Asia, and parts of Latin America—regions where health infrastructure is often fragile, access to timely medical care is limited, and reliable supplies of quality-assured antivenom are inconsistent or entirely absent. Despite being classified as a high-priority neglected tropical disease by the WHO, snakebite envenomation continues to receive less attention, funding, and scientific engagement compared to other similarly burdensome diseases.[20] This disconnect has perpetuated a cycle of inadequate treatment access, delayed interventions, and high rates of morbidity and mortality, particularly among rural and agrarian communities.

While traditional polyclonal antibody-based antivenoms remain the cornerstone of clinical treatment, their limitations are increasingly apparent. Species-specific efficacy, the risk of hypersensitivity reactions, cold chain dependency, and complex manufacturing requirements hinder their utility, especially in precisely the areas where they are most needed. These challenges underscore the need for innovation not only in therapeutic design but also in systems of distribution, affordability, and global policy regulation.

Recent scientific advancements provide a promising outlook for transforming the therapeutic landscape. Monoclonal antibodies offer enhanced specificity and reduced immunogenicity, while phage display and recombinant technologies allow for the precise identification and production of neutralizing components against a broad spectrum of venom toxins. Small-molecule inhibitors, such as varespladib and metalloproteinase

blockers represent an entirely different pharmacological class of antivenom, one that holds promise as an orally available, broad-spectrum, and potentially pre-hospital therapy—especially critical in settings where immediate access to healthcare is not possible. Moreover, the parallel development of novel diagnostic tools and supportive care protocols aims to improve early detection and targeted intervention, ultimately enhancing patient survival and reducing complications, such as limb necrosis and coagulopathy.

Ongoing and emerging clinical trials serve as the necessary scientific bedrock for translating these experimental therapies into clinical practice. These trials are crucial not only for assessing safety and efficacy but also for informing treatment guidelines, facilitating regulatory approvals, and guiding future research investments. As the global health community continues to prioritize the elimination of preventable deaths and disabilities caused by snakebite, the importance of evidence-based, scalable, and context-appropriate interventions cannot be overstated.

Ultimately, to reduce the global burden of snakebite envenomation, sustained and coordinated action is required. This entails increased public and private investment in research and development, capacity-building for regional manufacturing, and international collaboration to harmonize regulatory standards and ensure equitable access to antivenom products. Public health campaigns aimed at education, prevention, and community engagement must also be integrated into broader health systems strengthening initiatives. Only through a comprehensive and sustained global response can we hope to overcome the challenges posed by snakebite envenomation and honor the scientific legacy that began over a century ago with the goal of saving lives.

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Availability of data

The availability of data utilized in this communication is grounded in the use of open-source information and data. Open-source data refers to information that is publicly accessible and can be freely used, modified, and shared by anyone. This approach ensures transparency, reproducibility, and verifiability of the information presented. By relying on publicly available sources, the communication adheres to principles of openness and accountability, enabling stakeholders to independently evaluate the data and its interpretations. Furthermore, the use of open-source data enhances the credibility of the communication, as it allows for a broader range of scrutiny and validation by the academic and professional community. In this context, all referenced data were obtained from reputable and publicly accessible platforms, ensuring that the findings and assertions made are based on verifiable and legally accessible information.

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REVIEW ARTICLE

Tyrosine kinases: Structural insights and mechanistic roles in cancer progression and therapeutics

Eswar Kumar Nadendla[1]*, Gangadhar P. Vadla[2], Manohar Radhakrishnan[3], and Raghavendra Sashi Krishna Nagampalli[1]*

[1]Department of Immunology, St Jude Children's Research Hospital, Memphis, Tennessee, United States of America

[2]Department of Veterinary Pathobiology, Bond Life Sciences Center, Columbia, Missouri, United States of America

[3]Department of Biochemistry and Molecular Biology, Indiana University School of Medicine, Indianapolis, Indiana, United States of America

Abstract

Protein tyrosine kinases (PTKs) are key enzymes of cellular signaling, regulating key processes such as proliferation, differentiation, migration, metabolism, and apoptosis. Tyrosine kinases (TKs) modulate protein functions in normal and disease states by phosphorylation of tyrosine residues on target proteins. In this critical role, dysregulation of TKs is directly linked with disease progression, particularly in cancer, therefore making TKs an attractive target for therapeutic intervention. The PTK family is broadly classified into receptor TKs (RTKs) and non-receptor TKs (NRTKs), having variation at both structural and functional levels. RTKs are membrane-bound kinases that initiate intracellular signaling when they react with extracellular ligands, whereas NRTKs within the cytoplasm or nucleus convey intracellular signaling upon receptor activation. This paper aims to review the organization, mechanistic activity, and therapeutic potential of PTKs, with a particular focus on epidermal growth factor receptor and proto-oncogene tyrosine-protein kinase (Src) as representatives of RTK and NRTK, respectively. In addition, this review also focuses on addressing emerging strategies to enhance tyrosine kinase inhibitor efficacy and overcome acquired resistance in cancer therapy.

Keywords: Protein tyrosine kinases; Epidermal growth factor receptor; Receptor tyrosine kinases; Non-receptor tyrosine kinases; Cancer; Src kinase; Exo-site

***Corresponding authors:**
Eswar Kumar Nadendla (nadenlagem@gmail.com)
Raghavendra Sashi Krishna Nagampalli (rsnagampalli@gmail.com)

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1. Introduction

Tyrosine kinases (TKs) are crucial enzymes involved in signal transduction that regulates key cellular processes such as proliferation, differentiation, migration, metabolism, and apoptosis.[1-3] By catalyzing the phosphorylation of tyrosine residues in target proteins, kinases mediate vital cellular communication and maintain homeostasis.[4] Phosphorylation functions as a post-translational modification that is essential for normal cellular processes, but its dysregulation can cause disease, including cancer.[5] Unusual activation of protein TKs (PTKs) is usually associated with disease progression and therapy resistance, while

making them critical targets for therapeutic interventions, particularly in cancer treatment.[6,7]

The PTK family is diverse, with members varying in structure and function.[8] These kinases are classified into two major subgroups: receptor TKs (RTKs) and non-receptor TKs (NRTKs).[7,9] RTKs are membrane-bound enzymes that transmit extracellular signals such as growth factors, cytokines, and hormones to the cytoplasm and nucleus, initiating a cascade of cellular responses.[4,7] The key function of RTKs is to rapidly and reversibly phosphorylate protein substrates, which leads to alterations in protein conformation and interaction, driving various cellular processes such as growth and survival.[10] On the other hand, NRTKs lack extracellular and transmembrane domains and are found in the cytoplasm or nucleus. These kinases are involved in mediating intracellular signals, often in response to receptor-dependent activation at the cell membrane.[7,11] While RTKs and NRTKs function similarly by regulating crucial cellular processes, including cell division, growth, and immune responses, their structures are strikingly distinct.[7,12] Due to their essential roles in cellular signaling, both RTKs and NRTKs are critical in the regulation of various physiological functions and are often implicated in the progression of cancers when their activation becomes dysregulated.[13] The discovery of the Src oncogene and the identification of the epidermal growth factor receptor (EGFR) as the first RTK laid the foundation for understanding the role of TKs in cancer.[14] So far, over 90 TKs have been identified, and these enzymes are now recognized as pivotal players in cellular signaling circuits that contribute to cancer development.[15] Hence, TKs represent a significant portion of oncoproteins, and targeting these for therapeutic development is a promising strategy in the treatment of cancers associated with their dysregulation.[6] This review focuses on a deeper structural and mechanistic understanding and therapeutic implications of PTKs, using EGFR and Src as representative models of RTKs and NRTKs, respectively. In addition, the current review also emphasizes recent developments aimed at overcoming resistance to tyrosine kinase inhibitors (TKIs).

2. Classification of PTKs

As described above, PTKs are primarily classified as RTKs and NRTKs.[9] Based on the composition of the extracellular regions, the 58 identified RTKs in humans are further categorized into 20 distinct families. A brief introduction and description of each of the distinct RTK families is presented in Table 1.

The epidermal growth factor family (EGF) includes epidermal growth factor receptor (EGFR), human epidermal growth factor receptor (HER)1, HER2, HER3, and HER4. These receptors are often overexpressed in epithelial tumors, such as colorectal, head and neck, non-small cell lung, breast, pancreatic, and renal cell cancers.[17] The insulin growth factor (IGF) and insulin receptor (InsR) family consists of the IGF1R and InsR receptors. Both

Table 1. RTKs' classification is based on the sequence of the kinase domain

Class	Family	Receptors
I	EGF/ ErbB	EGFR, ErbB2/HER2, ErbB3/HER3, ErbB4/HER3
II	Ins	InsR, IGF1R, InsRR
III	PDGF	PDGFRα, PDGFRβ, CSF1R, KIT, FLT3
IV	VEGF	VEGFR1/Flt1, VEGFR2/KDR, VEGFR3/Flt4
V	FGF	FGFR1, FGFR2, FGFR3, FGER4
VI	PTK7	PTK7/CCK4
VII	TRK	TRKA, TRKB, TRKC
VIII	ROR	ROR1, ROR2
IX	MuSK	MuSK
X	HGF	MET, MST1R (RON)
XI	TAM	AXL, MER, TYRO3
XII	TIE	TIE1, TEK (TIE2)
XIII	Eph	EphA1-8, EphA10, EphB1-4, EphB6
XIV	RET	RET
XV	RYK	RYK
XVI	DDR	DDR1, DDR2
XVII	ROS	ROS
XVIII	LMR	LMR1, LMR2, LMR3
XIX	ALK	LTK, ALK
XX	STYK1	STYK1

Note: Adapted and modified from ref.[16]

Abbreviations: RTKs: Receptor tyrosine kinases; EGF: Epidermal growth factor; EGFR: Epidermal growth factor receptor; HER2: Human epidermal growth factor receptor; Ins: Insulin; InsR: Insulin receptor; PDGF: Platelet-derived growth factor; PDGFR: Platelet-derived growth factor receptor; CSF1R: Colony-stimulating factor 1 receptor; KIT: KIT Proto-oncogene receptor; FLT3: FMS-like tyrosine kinase 3; VEGF: Vascular endothelial growth factor; VEGFR: Vascular endothelial growth factor receptor; FGF: Fibroblast growth factor; FGFR: Fibroblast growth factor receptor; PTK7: Protein tyrosine kinase-like 7; CCK4: Colon carcinoma kinase 4; TRK: Tropomyosin receptor kinases; ROR: RTK-like orphan receptor; MuSK: Muscle-specific kinase, HGF: Hepatocyte growth factor; MET: Mesenchymal-epithelial transition; MST1R: Mesenchymal-epithelial transition 1 receptor; RON: Recepteur d'Origine Nantais; TAM: TYRO3, AXL, MER; TIE: tyrosine kinase with immunoglobulin-like and EGF-like domains; Eph: Erythropoietin-producing hepatocellular; RYK: Receptor like tyrosine kinase; DDR: Discoidin domain receptor; ROS: Reactive oxygen species; LMR: Lemur receptor kinases; ALK: Anaplastic lymphoma kinase; LTK: Leukocyte tyrosine kinase; STYK: Serine/threonine/tyrosine kinase; ErbB: Erythroblastic leukemia viral oncogene homolog; RET: Rearranged during transfection.

IGF1 and IGF2 are capable of binding to and activating the IGF1R transmembrane receptor kinase. However, when IGF2 binds, it does not activate any downstream signaling pathways because the IGF2R lacks the kinase structural domain necessary for this activation.[18] Platelet-derived growth factor receptor (PDGFR), colony-stimulating factor 1 receptor (CSF1R), KIT proto-oncogene receptor, and FMS-like tyrosine kinase 3 receptors are critical for various cellular processes.[19] Platelet-derived growth factor (PDGF) is essential for tissue growth, division, and blood vessel formation. CSF1R, secreted by cancer cells to evade immune detection, promotes the growth and recruitment of tumor-associated myeloid cells, contributing to poorer survival in many cancers.[20] The vascular endothelial growth factor (VEGF) receptor (VEGFR) family—VEGFR-1, VEGFR-2, and VEGFR-3—regulates processes such as cell migration, angiogenesis, and metabolic homeostasis.[21] Likewise, the fibroblast growth factor receptor (FGFR) family, including FGFR1-4, plays a role in tissue repair, regeneration, and the growth and differentiation of cells during development and organ formation.[22] Protein tyrosine kinase-like 7 and colon carcinoma kinase 4 receptors are involved in epithelial cell polarization and brain structure formation.[23] These receptors are catalytically active protein kinases and play roles in the Wnt and VEGF signaling pathways.[24] The neurotrophin receptor kinases family includes tropomyosin receptor kinases (TRK) A, TRKB, and TRKC receptors, which are vital for the proliferation and migration of the nervous system.[25] TRKA responds to nerve growth factor, TRKB to brain-derived neurotrophic factor, and TRKC to neurotrophin-3.[26] The RTK-like orphan receptor (ROR) family includes ROR1 and ROR2 receptors. ROR1 acts as a substitute receptor and co-receptor for Wnt signaling, regulating cell division, polarity, and tissue maintenance.[27] In contrast, ROR2's role in tumor development varies depending on the tumor type or stage; it can either repress or activate tumor growth through atypical Wnt signaling.[28] The muscle-specific kinase receptor is essential for the formation and organization of neuromuscular junctions in skeletal muscle.[29] The hepatocyte growth factor (HGF) receptor family includes mesenchymal-epithelial transition (MET) factor and Recepteur d'Origine Nantais receptors. When HGF binds to MET, it activates the proliferation, migration, and morphogenesis of epithelial cells.[30] The TYRO3, AXL, and MER receptors are activated by the vitamin K-dependent proteins Gas6 and protein S, regulating cell proliferation, survival, adhesion, and migration.[31] They also have anti-inflammatory properties and are implicated in carcinogenesis in various malignancies.[32] The tyrosine kinase with immunoglobulin (Ig)-like and EGF-like domains (TIE) receptor family, consisting of TIE1 and TIE2, regulates angiogenesis and lymphangiogenesis.[33] The erythropoietin-producing hepatocellular (Eph) receptor family (EphA1–A10, EphB1–B6) controls angiogenesis, cell migration, patterning, and neuronal formation.[34] The RET receptor, activated by glial cell-derived neurotrophic factor ligands, is crucial for cell proliferation, neuronal navigation, migration, and differentiation.[35] The receptor tyrosine kinase is characterized by extracellular Wnt-binding domains and is closely associated with Wnt signaling.[36] The discoidin domain receptor (DDR) family, which includes DDR1 and DDR2, regulates cell adhesion, proliferation, and metalloproteinase expression.[37] DDR1 also promotes tumor cell invasion and enhances the survival of tumor stem cells in collagen-rich environments.[38] The reactive oxygen species (ROS) receptor family is present in various malignant tumors, making it a promising target for anticancer drugs.[38] Lemur receptor kinases are linked to cancer and influence multiple signaling pathways involved in cell proliferation, migration, and invasiveness.[39] The anaplastic lymphoma kinase (ALK) receptor family includes ALK and leukocyte tyrosine kinase.[40] *ALK* gene fusion is linked to the formation of various tumors.[41] In addition, the serine/threonine/tyrosine kinase receptor plays a role in cellular processes such as proliferation, differentiation, and survival.[12]

Non-receptor TKs (NRTKs) include Ack, Janus Kinase (Jak), feline sarcoma (Fes), focal adhesion kinase (Fak), Tec, sarcoma (Src), C-terminal Src kinase (Csk), Abelson (Abl), and spleen tyrosine kinase (Syk) kinases.[42] These NRTKs typically consist of the N-terminal kinase domain (KD), which is around 300 residues long, and the C-terminal region, which contains several functional domains.[43] NRTKs share significant sequence similarity within their KDs, and their catalytic domains are like those of Ser/Thr protein kinases.[44] In addition to their catalytic domains, NRTKs also feature non-catalytic domains that regulate their activity.[45] The classification of NRTKs into distinct families is based on molecular analysis of their domain structures, variations in amino acid sequences, and genomic organization of the KDs.[16] Below is a brief overview of the most common NRTK families.

The activated Cdc42-associated kinase (ACK) is a large protein of 120 kDa whose kinase activity can be mediated by the phosphorylation of its tyrosine residues.[46] Ack1 is a non-receptor tyrosine kinase with a unique multidomain structure, including an Src homology 3 (SH3) and Cdc42/Rac interactive binding (CRIB) domain that regulates cellular functions such as migration and adhesion and plays a critical role in cancer progression.[47] Furthermore, Ack1 promotes tumor growth, resistance to chemotherapy, and recurrence through gene amplification, mutations,

and epigenetic regulation.[48] The Jak/Janus family consists of four kinases (JAK1, JAK2, JAK3, and TYK2), each with two KDs, one functional and one pseudo-kinase.[49] These kinases are activated by cytokine receptor ligation, leading to transphosphorylation and downstream signaling.[50] JAKs play crucial roles in immune cell regulation and tumor development through the JAK-STAT pathway. JAK3 is primarily found in hematopoietic cells, while other JAKs are involved in diverse cytokine signaling processes.[51] Fes and Fes-related (Fer) kinases are a subgroup of NRTKs with similarities to viral oncogenes from Fes virus and avian Fujinami poultry Src virus.[42] Fes kinases have a unique Fes/CIP4 homology (FCH) domain, coiled-coil motifs, an Src homology 2 (SH2) domain, and a C-terminal KD.[52] Fes and Fer kinases are implicated in cancer progression, with Fes playing a role in cell signaling pathways that influence cell migration, proliferation, and survival, contributing to tumorigenesis.[53] The Fak family includes Fak, Pyk2, Cak-beta, Cadtk, Raftk, and Fak2, with varying expression in organs such as the brain, liver, and hematopoietic cells.[54] Fak family kinases feature a FERM domain that mediates interactions with integrins and RTKs and a C-terminal FAT region involved in focal adhesion targeting. Fak plays a crucial role in tumor cell signaling, including transcriptional regulation within the tumor microenvironment (TME). Overexpression of Fak is linked to aggressive cancers, including breast, colon, and ovarian, and is associated with metastasis and poor prognosis.[55] The Tec family consists of five NRTK members, including Tec, Itk, Btk, Txk, and Bmx, characterized by several conserved domains, such as a pleckstrin homology domain involved in membrane association, a Tec homology domain, which includes a zinc-binding region, a SH3 domain that regulates protein-protein interactions, an SH2 domain that interacts with phosphorylated tyrosine residues, and a catalytic KD.[56] Tec kinases are involved in immune cell signaling, with specific expression in T, B, and natural killer cells.[57] The Src family is one of the largest NRTK families that include eight members, such as Fyn, Yes, Fgr, and Lyn, divided into two subfamilies: Src-A (Fgr, Fyn, Src, Yes) and Src-B (Blk, Hck, Lck, Lyn).[58] These kinases share a similar structure with Src homology 4 (SH4), SH3, SH2, and KDs but differ in their C-terminal regulatory regions.[59] Src family kinases (SFKs) are involved in diverse cellular processes, with distinct expression patterns in hematopoietic and other tissues.[60] Fyn-related kinase (FRK) is a member of the breast tumor kinase family, closely related to SFKs.[61] FRN kinases feature an SH3, SH2, and KD but lack the N-myristoylation site, which prevents membrane localization and allows nuclear localization.[62] Unique to FRK and inhibitory tyrosine kinase (IYK) kinases is the presence of a nuclear localization signal (NLS) within the SH2 domain.[63] The NLS is a bipartite motif that enables nuclear targeting and functional regulation in the cell.[64] The Abl family includes Abl and Arg kinases, which are widely expressed, with high levels in the thymus, spleen, and brain.[65] Both kinases have structures similar to Src family members but feature a unique C-terminal actin-binding domain and nuclear localization signals.[66] Abl activation, through mutation or phosphorylation, is linked to leukemia and solid tumors such as brain, lung, and prostate cancers.[67] The Syk family includes Syk and zeta-chain-associated protein kinase 70 (ZAP70) kinases, which share a similar structure containing two SH2 domains followed by a catalytic KD.[68] These kinases are cytosolic proteins lacking fatty acid modification sites, and upon cell stimulation, Syk and Zap70 translocate to immune receptor complexes at the membrane to trigger downstream signaling.[69] A tabular representation of kinases that play a significant role in various cancer types is represented in Table 2.

3. Structural and regulatory mechanisms of TKs

PTKs play a critical role in cellular signaling pathways, and their catalytic activity is tightly regulated. Numerous atomic structures of PTKs reported in the literature have provided structural and mechanistic insights into the regulation of both receptor and non-receptor PTKs.[89] As several PTKs are available in the Protein Data Bank (PDB), the current review will focus on EGFR kinase as a representative of receptor PTK and Src for non-receptor PTKs.

3.1. PTK domain architecture

RTKs are composed of three main regions: a large extracellular region, which binds to polypeptide ligands, a transmembrane helix, and a cytoplasmic region, which possesses tyrosine kinase activity. The extracellular region of RTKs is classically composed of a diverse array of distinct globular domains, including Ig-like domains (domain-1), fibronectin type-III-like domains (domain-2), cysteine-rich domains (domain-3), and EGF-like domains (domain-4). In the case of EGFR kinase, the extracellular region includes amino acids 1–165 (domain-1), 166–310 (domain-2), 311–480 (domain-3), and 481–621 (domain-4). However, the cytosolic region of RTKs domain organization is simple, consisting of the juxtamembrane region (amino acids 643–685), immediately followed by the transmembrane helix, a tyrosine KD (amino acids 686–952), and a carboxy region (amino acids 953–1186) (Figure 1A-C). Unlike RTKs, the extracellular and transmembrane regions in NRTKs are absent, and most of the NRTKs are present in the cytosol. The NRTKs comprise intrinsically disordered regions (IDR) and folded domains. At the N-terminus IDR region, unique myristoylated SH4 fragments, a smaller SH3 domain (~60

Table 2. Summary of cancer-associated tyrosine kinases

Class of tyrosine kinase	Cancer type and mechanism
EGFR (HER1, HER2, HER3, HER4)	Epithelial tumors in lung, breast, and colon[17,70]
VEGFR-1 to -3	Regulate angiogenesis and cell migration in tumors[21,71,72]
FGFR-1 to -4	Tissue cancer[22]
TRKA, TRKB, TRKC (NTRK family)	Neuronal cancer[25,73]
RET	Implicated in multiple cancers[35,74]
RYK	Contributes to tumorigenesis[36,75]
DDR1, DDR2	Regulate adhesion, invasion, and survival in collagen-rich tumors[37,76,77]
ROS	Present in many cancer types[38]
LMTK/LMR	Cancer-linked; influences proliferation, migration[78,79]
ALK, LTK	Fusion-driven cancers (e.g., ALK fusions in lymphoma, lung cancer[40,41,80]
STYK	Involved in proliferation and survival; emerging as a cancer target[81]
Ack1	Promotes tumor growth, chemoresistance, gene amplification[45,82]
JAK1, JAK2, JAK3, TYK2	Crucial for immune modulation in cancers[83]
Fes, Fer	Signal for migration, survival; linked to oncogenesis[84]
FAK family	Adhesion, motility; high expression in aggressive tumors[85,86]
Src family	Major signaling mediators; upregulated in various tumors[60]
Abl, Arg	Leukemia[67,87,88]
Syk, Zap70	Hematologic cancers[69]

Abbreviations: EGFR: Epidermal growth factor receptor; HER: Human epidermal growth factor receptor; VEGFR: Vascular endothelial growth factor receptor; FGFR: Fibroblast growth factor receptor; NTRK: Neurotrophic tyrosine receptor kinases; TRK: Tropomyosin receptor kinases; RYK: Receptor tyrosine kinase; DDR: Discoidin domain receptor; ROS: Reactive oxygen species; LMTK/LMR: Lemur receptor kinases; ALK: Anaplastic lymphoma kinase; LTK: Leukocyte tyrosine kinase; STYK: Serine/threonine/tyrosine kinase; Fes: Feline sarcoma; Fer: Feline sarcoma-related; JAK: Janus Kinase; FAK: Focal adhesion kinase; Src: Sarcoma; Abl: Abelson; Syk: Spleen tyrosine kinase; RET: Rearranged during transfection; Ack1: Activated Cdc42-associated kinase; Arg: Abl-related gene; Zap70: Zeta-chain-associated protein kinase 70.

residues), a short (SH2 ~100 residues), SH2 kinase linker, catalytic tyrosine-protein KD (Src Homology 1 [SH[1]), and a short intrinsically disordered C-terminal tail. While the KD has a catalytic function, the SH2 and SH3 domains are commonly involved in non-catalytic regulatory properties. However, all three domains are essential in signal transduction[8,90-94] (Figure 1D and E).

3.2. Src structure and regulatory mechanism

The primary function of the Src is to transmit the external signal to the cell interior by phosphorylating tyrosine residues on substrates, mainly downstream of RTKs and integrins.[95] Src kinases are crucial in various cellular processes, such as cell proliferation, adhesion, migration, and more.[42] The Src kinase's complicated regulation is due to its complex structure. The structures of SH3, SH2, and SH1 KDs of Src kinases have been extensively studied and reviewed elsewhere. The Src KD features a characteristic bilobed architecture comprising a small N-terminal lobe and a large C-terminal lobe. The residues 267–337 and 341–520 make up these lobes, respectively. The N-lobe predominantly anchors and orients ATP, featuring a G-rich loop, which is a part of the nucleotide-phosphate binding site. The N-lobe is mostly composed of antiparallel β-sheet structures.[96] The C-lobe is predominantly composed of α helix, responsible for binding the protein substrates and contributing to the ATP-binding site. The catalytic site of Src is situated in a cleft between these two lobes; they open and close during ATP hydrolysis.[97] The dynamic conformational switch regulates ATP binding and ADP release; the open form is required to allow ATP to its catalytic pocket and release ADP; the closed form is important to bring residues into the catalytically active form. The Src kinase regulation precisely involved the coordination of non-regulatory SH2 and SH3 domains and a regulatory KD.[89,97] In the autoinhibitory conformation, the SH2 domain binds to phosphotyrosine-containing motifs, precisely, phosphorylated Tyr527 in the C-terminal tail of Src and stabilizes the conformation.[89,97] The SH3 domain interacts with a polyproline-rich motif situated between the SH2 and KDs. This interaction positions SH2–SH3 domains as a compact structural unit, which further prevents the movement of the KD and, consequently, locks the Src in its inactive state. The activation loop (residues 404–418) conformations in the KD dictate the active and inactive state of the Src kinase. In the inactive Src kinase, the activation loop forms a short α-helix between N- and C-lobes, known as the A-loop helix.[89,97] As a result, the Tyr416 residue side chain is buried between the N- and C-lobes, and this conformational switch leads to the prevention of the formation of a salt bridge between Lys295 and Glu310 required for enzyme activity. The autophosphorylation of Tyr416 disrupts the autoinhibitory state of the Src kinase, leading to an extended conformational switch in the activation loop and alignment of catalytic residues such as Asp386 and Asp404. Asp386 residue acts as a catalytic base for the tyrosine substrate, whereas Asp404 interacts with magnesium ions that stabilize ATP. Numerous studies on Src have revealed that SH2 and SH3 domains are critical

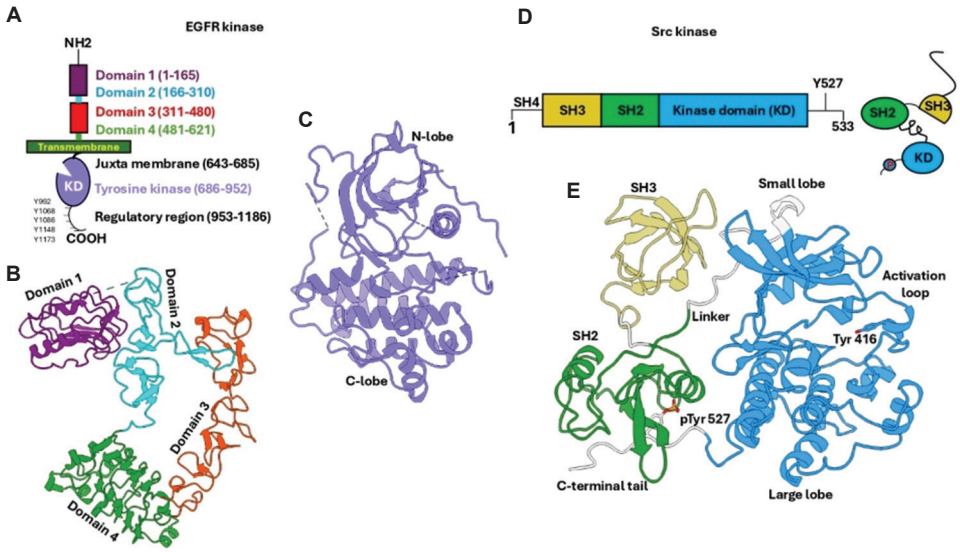

Figure 1. Structural architecture of EGFR and Src kinases. (A) The domain architecture of EGFR. (B) The extracellular region of EGFR is composed of four domains I–IV: domain I (red), domain II (cyan), domain III (green), and domain IV (orange) (PDB: 4KRP). (C) The EGFR kinase domain is displayed in medium purple (PDB: 4LQM). (D) The domain architecture of Src. The boundaries of domains are based on the chicken numbering system. (E) Ribbon diagram displaying the overall structure of Src (PDB: 2SRC). The SH3 (pale yellow) and SH2 (green) domains coordinate the linker and C-terminal tail regions, respectively. The kinase domain is colored in blue. Figures are generated using UCSF ChimeraX: tools for structure building and analysis.
Abbreviations: EGFR: Epidermal growth factor receptor; PDB: Protein Data Bank; SH3: Src homology 3; SH2: Src homology 2.

for maintaining the autoinhibited state of Src. However, the KD is involved in severe conformational changes to switch between active and inactive states. This structural equilibrium is disrupted when C-terminal Tyr527 is mutated. In the case of v-Src, a mutation at Tyr527 has been shown to impair the SH2-SH3 interaction between the KD and result in constitutive kinase activity.[4,89]

The Src protein-tyrosine phosphorylation levels are balanced by counteraction between CSK and protein-tyrosine phosphatases (PTPs). Okada and Nakagawa[98] were the first to demonstrate that CSK, a cytoplasmic PTK, controls the regulatory tyrosine phosphorylation in rat brains. They also highlighted its efficiency in phosphorylating Src at Tyr527, a key regulatory site for its activation. In contrast, PTPs such as PTPε and PTPε facilitate the dephosphorylation of phosphotyrosine 527 in the Src KD, thereby displacing it, leading to Src kinase activation (Figure 2A and B). Structural studies have revealed that the substrate recognition mechanism between Src and PTPs relies on the cysteine-dependent active site of PTPs and the phosphorylated tyrosine side chain of Src.[99] Recent findings have identified two additional key charge-charge interactions between rPTPε and phospho-Src beyond the active site interactions.[100] These biochemical and structural insights are extremely important for the development of novel therapeutic strategies for targeting kinases, particularly in cancer treatment.

3.3. EGFR structure and regulatory mechanism

EGFR regulates multiple functions involved in developmental, metabolic, and physiological processes.[101] When exposed to ligands like EGF, the EGFR binds to EGF, undergoing a conformational switch from an inactive monomer to an active dimer (Figure 2C). This conformational change leads to autophosphorylation of the receptor, which sequentially activates downstream signaling pathways to control cell proliferation and differentiation. EGFR, along with growth factor-α, amphiregulin, and other ligands, promotes either homodimerization of two EGFRs or heterodimerization of EGFR with other family members.[102] Upon activation of RTKs, there is a subsequent activation of the downstream Ras/mitogen-activated protein kinase pathway, the pI3K/Akt pathway, and transcription pathways.[103]

3.4. Extracellular structure of EGFR

The extracellular structural modules of all four EGFR members have been thoroughly studied both in the presence and absence of their respective ligands, as well as in complexes with antibodies.[104,105] Atomic structures reveal two key conformations that are important in the extracellular modules. One is an extended form that facilitates the conformation of one protomer in the active dimer, while the other is folded over or tethered conformation where dimerization elements are buried. Upon ligand binding, the extracellular domains display

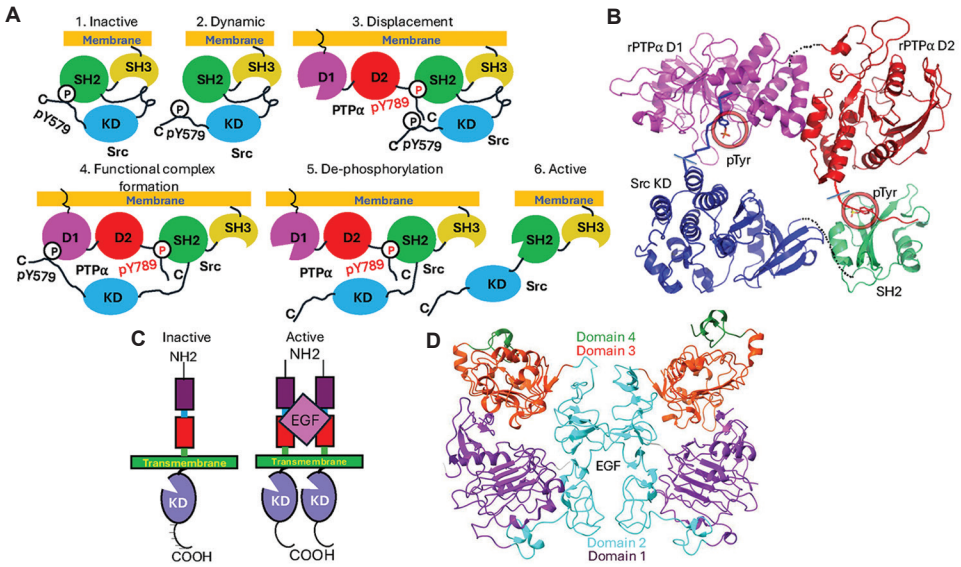

Figure 2. Structural transition of EGFR and activation mechanism of Src. (A). Schematic diagram of inactive EGFR, ligand-bound active dimeric extracellular EGFR, and an asymmetric dimer of kinase domain. (B) Structure of TGFα dimer of human EGFR (PDB ID: 1MOX). (C) Phosphotyrosine displacement by PTPα and activation mechanism of Src kinase.[99] (D) Haddock model of PTPα and Src kinase complex, displaying the phosphatase and tyrosine kinase interaction. Figures are generated using UCSF ChimeraX: tools for structure building and analysis.
Abbreviations: EFR: Epidermal growth factor; EGFR: Epidermal growth factor receptor; PTPα: Protein-tyrosine phosphatases; TGFα: Transforming growth factor alpha; PDB: Protein Data Bank; Src: Sarcoma.

a significant conformational change, transitioning the module from tethered to extended state, resulting in dimerization and activation of the EGFR (Figure 2D). This extended conformation is represented as a back-back dimer configuration, with the ligand positioned between domains I and III of each receptor subunit. The glycosylation of the EGFR extracellular region is critical for its activation; the sugar moiety around 40 kDa is known to play a role in EGFR maturation and cell-surface translocation. Mutation studies have identified that Asn579 is crucial for regulating receptor conformation and ligand binding affinity. Another mutation at Asn579, located on a specific glycosylation site, influences the structural conformation of EGFR and ligand binding. Furthermore, the N420D mutation in EGFR was shown to display ligand-independent activation through spontaneous oligomer formation.[105] Together, the biochemical and structural details underscore the complexity of these receptors' regulation and offer a base for therapeutic strategies targeting EGFR family members.

3.5. EGFR intracellular kinase structure activation

The intracellular region of EGFR mostly comprises its KD, which adopts a canonical kinase fold that exists in both active and inactive conformations. EGFR structure (unphosphorylated) in the presence of erlotinib from Genentech is the first atomic structure of the EGFR KD; this structure provides its unique structural features and activation mechanism.[103,106] The structure features the conserved Asp-Phe-Gly (DFG) motif at the base of the activation loop, which is a key activation/regulatory motif. In inactive conformation, the aspartic acid residue flips out of the catalytic center, making the kinase inactive and preventing the entry of ATP; this is observed in several kinases.[106] In the EGFR: erlotinib complex, the DFG motif is found in the 'in' conformation; in this, the activation loop is open and properly configured to bind its ligands. In addition, the active site element αC in the N-lobe switches inward and facilitates the ion pair interaction between Glu738 and Lys721, which is critical for catalytic activity.[105] In contrast to other kinases, EGFR does not require phosphorylation of its activation loop to transition to the active state. The atomic structure of EGFR in complex with lapatinib is captured in its inactive state; surprisingly, this structure resembles the inactive states of Src-family kinases.[105] In the structure, the αC-helix in the N-lobe switched outwards, and the activation loop formed a short helix, blocking its ATP binding. Mutations in the activation loop phosphorylation sites revealed that the phosphorylation is not an absolute requirement for EGFR's activation. Overall, the atomic details of these structures detailed the understanding of EGFR's regulatory flexibility and underlined its divergence from other RTKs, which rely on autoinhibitory interactions and activation loop phosphorylation for regulation.[105]

The activation mechanism of EGFR was revealed through the determination of the homodimer KD structure

determination. In this structure, one KD (activator) allosterically interacts with its partner (receiver) to activate the EGFR. This dimerization interaction occurs at the N-lobe of the receiver and the C-lobe of the activator, resembling the cyclin-mediated CDK type of activation. Unlike other RTKs, the EGFR activation mechanism is driven by protein-protein interactions at the dimerization interface. This mechanism is also observed in other members such as HER2, HER3, and HER4 (Figure 2A and B).[107] Further molecular dynamics simulation studies demonstrated how EGFR transitions between active and inactive conformations through local unfolding at the hinge region between N- and C-lobes. Together, these structural insights have significant clinical implications, helping in developing novel targeted antibodies like erlotinib and lapatinib, which exploit EGFR's conformational flexibility. For example, EGFR inhibitor Mig6 is known to block the asymmetric dimer interface and inhibit activation.[108] This understanding highlights the unique regulatory mechanism of EGFR and its critical role in cancer biology.

4. The role of TKs in cancers

TKs, a large family of kinases that include both RTKs and NRTKs, serve as critical molecular switches in regulating various cellular processes such as growth, survival, development, and differentiation.[83] Several studies have highlighted the role of PTKs in various cancers and their potential for drug discovery. The current review focuses on EGFR and Src's role as therapeutic targets for developing treatments against cancer cell-specific pathways.

4.1. Role of EFGR-tyrosine kinase in cancers

The EGFR family regulates developmental, metabolic, and physiological processes.[101] A key aspect of EGFR-driven cancers involves mutations in the tyrosine KD of the *EGFR* gene (exon 18–21), categorized into three classes: class I (in-frame deletions in exon 19), class II (single-nucleotide substitutions), and class III (in-frame duplications and or insertions in exon 20).[109,110] Class I accounts for approximately 44% of the activating EGFR-TK domain mutations, including deletion at LRE (Leu-747 to Glu-749), while class II mutations contribute ~41%, often affecting the KD C-helix. Class III mutations, constituting ~5%, are less frequent but still play a role in tumor progression.[103,111] Sigismund *et al.,* in 2018, best characterized the function of EGFR in ligand-and kinase-dependent activation, also known as the canonical EGFR signaling pathway.[112] Several of these stress pathways are activated in cancer cells to induce survival advantage as well as resistance to cancer therapy.[113,114] Casanova *et al.*[115] demonstrated that EGFR signaling is responsible for the Ha-ras-dependent activation in epidermal tumor cells. Recent publications support the activation of EGFR signaling pathways in epithelial cancers, including breast, ovarian, prostate, and non-small cell lung cancer (NSCLC).[116-118]

4.2. Role of Src-tyrosine kinase in cancers

SFKs play a crucial role in various cellular processes, such as cell proliferation, adhesion, and migration.[42] Their dysregulation is frequently implicated in tumors, where they are often overexpressed due to their role in cell-cell adhesion.[119,120] Particularly, Src is involved in activating STAT transcription factors, promoting tumorigenesis, and influencing cytokine signaling in hematopoietic cells.[121] It also plays a significant role in regulating the RAS/RAF/MEK/ERK MAPK and VEGF pathways in various tumors.[122] In addition, Src plays a vital role in facilitating tumor cell invasion by phosphorylating target substrates, aiding in the translocation of tumor cells through matrix barriers and tissue compartments. Invasion is a complex process, and tumor Src activation leads to the phosphorylation of targeted substrates, influencing the activity of cellular proteins to carry out this entire cellular process.[123] SFKs are activated in tumors through mutations of the Src allele, leading to a disorganized negative regulatory pathway or by binding to activating partners such as growth factors (Her 2/Neu, PDGF, EGFR). Oncogenic Src (v-Src) can activate Ras by recruiting the Grd 2/Sos complex, thereby stimulating Ras-mediated tumorigenic signals.[124] Furthermore, p120RasGAP-mediated activation of c-Src is important for Ras-induced tumor invasion.[125] The TME plays a crucial role in Src upregulation, leading to enhanced Src activity during cancer progression.[126] In addition, inhibitory phosphorylation of Tyr530 is mediated by the kinase Csk, which acts as a crucial regulator of Src activity.[127] Given the importance of Src/EGFR in tumor progression, the review will explore tyrosine kinase therapeutic targets and also provide insights into potential strategies for overcoming therapeutic resistance.

5. TKs as therapeutic targets

5.1. Development of TKIs

Cancer cell survival in the TME is challenging and highly influenced by external factors. Cancer treatment has advanced in developing TKIs. Discovery and development of imatinib (Gleevec, Inc.,) as the first effective TKI to treat chronic myeloid leukemia established it as a tumor-targeted therapy that acts specifically against the BCR-ABL fusion protein. Inhibitors such as sorafenib and sunitinib served as early examples of TKIs approved for solid tumors and renal cell carcinoma.[128] Over the past 20 years, robust and specific TKIs with single or multiple targets have been identified, including EGFR, ROS1, VEGFR, MEK,

Table 3. Tyrosine kinase inhibitors used in research

TKIs in EGFRm NSCLC (clinical and research data)	TKI	Clinical phase	Number of patients (%EGFRm+)	Response rate (%)
First/Second-generation EGFR TKI	Neratinib[132]	II	91 (100)	3
	XL647[133]	II	33 (53)	3
	Afatinib (A) vs. placebo (P)[134]	IIB/III	585 (16)	7 (A) < 1 (P)
	Afatinib[135]	II	62 (73)	8
	Dacomitinib[136]	II	62 (73)	8
	MM-121 + erlotinib[137]	II	50 (48)	9
	AP26113[138]	I	32 (35)	3a
Mutant-specific TKI	CO-1686[139]	I	40 T790M+ (100)	58
	AZD9291[140]	I	107 T790M+ (100)	64
	HM61713[141]	I	48 T790M+ (100)	29
EGFR antibodies	Cetuximab + erlotinib[142]	II	19 (84)	0
	Cetuximab + afatinib[143]	IB	126 (98)	29
Chemotherapy	Carboplatin/paclitaxel	III	52 (100)	28.8
	CE vs. C[144]	Retro	78 (100)	41 (CE); 18 (C)
	Pemetrexed + gefitinib or erlotinib[145]	II	27 (100)	25.9

Abbreviations: EGFR: Epidermal growth factor receptor; EGFRm+: Epidermal growth factor receptor mutation-positive; TKI: Tyrosine kinase inhibitor; CE: Chemo/erlotinib; C: Chemo.

FGFR, and PDGFR.[129] The known approved TKI is listed in Tables 3-5. IRIS trials (2000–2001) confirmed the long-term survival benefit of treating imatinib.[130] However, there has been concern over the emergence of resistance to imatinib. Nilotinib and dasatinib are two of the TKIs (second-generation) approved worldwide for the treatment of chronic myeloid leukemia after imatinib failure.[130] Developing TKIs is always a challenging endeavor because most patients develop acquired resistance against TKIs within a median period of 10–15 months.[131]

Two main approaches to therapeutically targeting EGFR rely on using mAbs and small molecules of EGFR-TKIs. Monoclonal antibodies (mAbs) specific to EGFR target the extracellular domain, whereas EGFR-TKIs block the binding of ATP to the intracellular catalytic domain of EGFR.[149] For example, panitumumab and cetuximab are two approved mAbs widely used in the treatment of colorectal cancer patients whose tumors express wild-type kirsten rat sarcoma viral oncogene homolog (KRAS), as KRAS mutations are associated with resistance to anti-EGFR therapies.[150] Erlotinib and gefitinib are two selective TKIs used in combination with mAbs in the treatment of NSCLC. Several preclinical and clinical studies were conducted to study the effect of these EGFR inhibitors alone and in combination with mAbs/chemotherapies.[151] Cetuximab and panitumumab have been studied in combination with anthracycline/taxane-based chemotherapy through pilot multicentric studies of neoadjuvant triple-negative breast cancers (TNBC).[152] Studies reported that using cetuximab in combination with either gefitinib or erlotinib has proven to enhance apoptosis and growth inhibition of neck cancer cell lines over using them alone in the treatment.[153] In addition, it is suggested that cetuximab and gefitinib showed a synergistic effect on EGFR downstream signaling pathways.[154] Trastuzumab, in combination with lapatinib, is used to treat HER2-overexpressed breast cancer; these two develop resistance in patients when treated alone.[155] One of the strong reasons to use combinational therapy including mAbs and selective EGFR-TKIs was to target different molecular domains of the EGFR. However, selective targeting of EGFR was limited to EGFR-driven cancers; in the case of EGFR- and KRAS- or STKs-driven cancers, one needs to be more selective in choosing combinational therapies.

6. Resistance to TKIs and strategies to overcome resistance

TKIs are the most common and successful strategies for targeting cancer cells.[156] However, eventually, cancer cells develop resistance to these drugs. Multi-drug resistance

Table 4. U.S. FDA-approved tyrosine kinase inhibitors for use in cancer therapy[146,147]

TKI	Family targeted	Inhibitor name	Application	Adverse effects (cardio-related)	Extra-cardio adverse effects
First-generation TKI	EGFR/ERBB family	Gefitinib	NSCLC	MI	Skin rashes, nausea, diarrhea, anorexia, stomatitis, nausea
First-generation TKI	EGFR/ERBB family	Icotinib	NSCLC	HTN	Diarrhea, nausea, skin rashes, loss of appetite
First-generation TKI	EGFR/ERBB family	Lapatinib	Breast cancer	HF, LVD	Skin rashes, diarrhea, nausea
First-generation TKI	EGFR/ERBB family	Erlotinib	NSCLC and prostate cancer	Edema	Skin rashes, diarrhea, nausea, loss of appetite, fatigue, neuropathy, alopecia
Second-generation TKI	EGFR/ERBB family	Afatinib	NSCLC	HTN	Severe diarrhea, loss of appetite, paronychia, dry skin, rashes
Second-generation TKI	EGFR/ERBB family	Neratinib	Breast cancer	Low rates and decline in LVEF and QT prolongation	GI-related disorders, headache, fatigue, diarrhea
Second-generation TKI	EGFR/ERBB family	Dacomitinib	EGFR-mutated NSCLC	HTN	Dry skin, appetite loss, diarrhea, weight loss, alopecia, cough, hemorrhoids, wounds, back pain, headache
Third-generation TKI	EGFR/ERBB family	Osimertinib	NSCLC	MI, pericardial effusion, LVD, HF	Diarrhea, nausea, fatigue, stomatitis
Third-generation TKI	EGFR/ERBB family	Pyrotinib	HER2-postive	-	Diarrhea, hand-foot syndrome, leukopenia, neutropenia, GI disorders, increased ALT, anemia, asthenia
Third-generation TKI	EGFR/ERBB family	Mobocertinib	EGFR-mutated NSCLC	-	Acneiform dermatitis, GI disorders, rash, dry skin, stomatitis, fatigue, rash, paronychia, anemia

Abbreviations: ALT: Alanine transaminase; EGFR: Epidermal growth factor receptor; FDA: Food and Drug Administration; GI: Gastrointestinal; HER: Human epidermal growth factor receptor; HF: Heart failure; HTN: Hypertension; LVD: Left ventricular dysfunction; NSCLC: Non-small cell lung cancer; MI: Myocardial infarction; TKI: Tyrosine kinase inhibitor; ERBB: Erythroblastic leukemia viral oncogene homolog.

in cancer arises when tumors become nonresponsive to chemotherapeutic agents. Many factors contribute to multi-drug resistance, including enhanced drug efflux caused by overexpressed ABC transporters,[157] genetic mutations, the activation of specific signaling pathways, and intracellular-extracellular ATP.[158] Mutations in the EGFR and Src also contribute to drug resistance in cancers. To overcome multi-drug resistance, researchers have developed strategies emphasizing the use of mAbs that target specific receptors or signaling components of the pathway, or any protein that specifically promotes tumor oncogenesis. Here, we highlight the use of mAbs alone and in combination to achieve effective treatment against cancers.

Resistance to TKIs in *EGFR*-mutated NSCLC remains a challenge in cancer therapy. Studies have identified that, on average, 50% of resistance to first- and second-generation EGFR-TKIs is due to the EGFR T790M mutation. This amino acid substitution in EGFR leads to an increased affinity to ATP caused by a conformational change, resulting in steric hindrance and reducing drug efficacy.[159] Osimertinib, a third-generation EGFR-TKI, inhibits both EGFR T790M and EGFR-sensitizing mutations, demonstrating increased efficiency over gefitinib and erlotinib.[160] However, patients developed resistance to long-term usage of third-generation EGFR-TKIs, particularly EGFR C797S on exon 20, as the main cause for this acquired resistance.[161] Patients responded to a combination of first- and third-generation EGFR-TKIs when harboring C797S in trans with T790M, whereas those with C797S in cis with T790M did not respond to this combination.[162] EGFR T790 and Src-mediated resistance are two distinct mechanisms where tumor cells develop resistance to therapies, especially EGFR-targeted therapies. Most of the TKIs that target EGFR were less sensitive because of the specific mutation in the *EGFR* gene, whereas Src-mediated resistance involves the activation of Src kinase, which can also bypass the effects of EGFR inhibitors and drugs that target NTKIs.[163] To overcome this evolving resistance, researchers are developing fourth-generation

Table 5. List of approved monoclonal antibodies targeting EGFR[148]

mAbs	Nature of molecule	Binds to	Antibody-dependent cell-mediated cytotoxicity	Mechanism	Clinical approval
Nimotuzumab	Humanized, mouse mAb	Extracellular domain of EGFR	-	Prevents binding of EGF	Yes, phase III (approved for treating HNSCC in non-USA countries)
Zalutumumab	Humanized IgG1	Extracellular domain of EGFR	Yes	Prevents the binding of ligands such as EGF and TGFα, thereby inhibiting EGFR signaling	Yes, phase III
Trastuzumab	Humanized IgG1	Juxtamembrane domain IV	Yes	Inhibits HER2 homodimers and ligand-independent HER2–HER3 dimers	Yes
Pertuzumab	Humanized IgG1	Heterodimerization domain II	Yes	Inhibits ligand-induced HER2-containing heterodimers	Yes
Cetuximab	Humanized IgG1	Extracellular domain of EGFR	Yes	Prevents the binding of ligands like EGF and TGFα, thereby inhibiting EGFR signaling	Yes
Panitumumab	Humanized IgG1	Extracellular domain of EGFR	Yes	Prevents the binding of ligands such as EGF and TGFα, thereby inhibiting EGFR signaling	Yes, phase III

Abbreviations: EGF: Epidermal growth factor; EGFR: Epidermal growth factor receptor; HER: Human epidermal growth factor receptor; HNSCC: Head-and-neck squamous cell carcinoma; mAb: Monoclonal antibody; NSCLC: Non-small cell lung cancer; TGFα: Transforming growth factor alpha; IgG1: Immunoglobulin G1.

EGFR-TKIs and also exploring combination therapies. For instance, EGFR-TKIs combined with programmed death ligand 1 antibodies with chemotherapy have shown significant survival benefits to patients suffering from *EGFR* mutation-driven drug resistance in cancers.[164] The FDA-approved TKI and NTKI inhibitors used in cancer therapy are listed in Tables 3 and 4.

7. Resistance and the mechanism of developing resistance to therapy

Trastuzumab (Herceptin), a therapeutic antibody used to treat breast cancer, often encounters resistance in patients. It binds to an epitope in the juxtamembrane region of the HER2 RTKs. Upon binding, trastuzumab induces uncoupling of ligand-independent HER2-HER3 heterodimers and inhibits downstream signaling as well as antibody-dependent cell cytotoxicity.[165] The main reasons reported for resistance to trastuzumab in patients are decreased interactions with HER2 due to blockage by cell-surface proteins like mucin-4.[166] Consistent treatment with trastuzumab leads to decreased expression of the tumor suppressor *PTEN* gene and activation of the Akt signaling pathway. Another main reason for developing resistance is the activation of the phosphatidylinositol 3-kinase/Akt pathway, which can lead to decreased sensitivity to trastuzumab.[167] Another potential explanation for the development of trastuzumab resistance is its ability to bind to hyaluronan and CD44, a transmembrane receptor that can hinder trastuzumab's access to HER2.[168] A clinical study was conducted to analyze sensitivity to trastuzumab treatment and reported in the study on 46 patients with breast cancer, in which 11.1% of patients responded to trastuzumab (expressing p95HER2), with 51.4% of the patients who expressed p185HER2 achieving clinical response.[169] Lapatinib, a small molecule that can inhibit both HER2 and EGFR kinase, was tested in p95HER2 preclinical studies to prevent HER2 signaling loss of the trastuzumab binding site.[169] Coupled lapatinib with trastuzumab has been clinically shown to be effective in patients with stage IV HER-overexpressing breast cancer.[170]

Cetuximab is an mAb that treats metastatic colorectal cancer and squamous cell cancer (head-and-neck squamous cell cancer). The use of cetuximab and panitumumab in colorectal cancer patients is successful.[171,172] However, treatment with cetuximab and panitumumab as single agents was only 10% effective in clinical significance. This clearly explains the development of resistance to the therapy. Most patients develop resistance within 3–12 months of starting therapy.[173] The most probable explanation for developing resistance is, but not limited to, *RAS* mutations

(these mutations prevent patients from having a response to therapy). Acquired resistance is another important reason when using EGFR-targeted mAbs. Preclinical and molecular profiling of clinical specimens that developed resistance to EGFR-targeted mAbs have revealed genetic alterations of genes in the EGFR-RAS-RAF-MEK signaling pathway, and RTKs are the mechanism of acquired resistance to anti-EGFR mAbs.[174-176] Mutations in codons 12 and 13 of *KRAS* were the first identified mechanism of primary resistance to anti-EGFR therapy; later, patients were screened for *KRAS* mutations before mAb treatment. Researchers also reported that oncogenic Ras and wild-type p53 stimulate STAT non-cell autonomously and promote tumor radioresistance.[122] However, in some instances, RAS wildtype patients can be non-responders to anti-EGFR therapy, as it is well understood that additional mechanisms of intrinsic resistance are attributed to mutations in PI3KCA/BRAF.[177,178] The above genetic mutations leading to acquired resistance and escape from anti-EGFR blockade appear to converge on the activation of MEK-ERK/AKT signaling pathways. Considering that each mAb has distinct advantages and disadvantages in therapy, treatment selection should be guided by the molecular profile of the tumor and the patient's clinical context.

Pertuzumab (Omnitarg, 2C4) is an anti-HER2 mAb that binds to the domain II epitope of HER2 and is able to block a binding pocket essential for receptor dimerization and signaling. Pertuzumab is speculated to engage in a potential synergism with trastuzumab in HER2-overexpressing cell lines. Phase II clinical trials of pertuzumab in combination with trastuzumab have shown disease progression over trastuzumab in patients with HER2-overexpressing metastatic breast cancer.[179] Currently, clinical trials in different stages testing pertuzumab in combination with trastuzumab in different settings and as well as pertuzumab with chemotherapy, are ongoing.[180] Toxicity profiles of these new antibodies are comparable to that of cetuximab, even though they are associated with less hypersensitivity reactions. Mostly, mAbs administrations needed frequent clinical visits due to their mode of administration (intravenous infusions). Also, the proposed resistance to cetuximab can be applied to most EGFR-targeted mAbs. From these studies, it is well understood that mAbs targeting specific signaling molecules or receptors, in combination with other mAbs or chemotherapy, have shown progress in overcoming resistance in cancers.

7.1. Emerging strategies to enhance TKI efficacy

To further expand therapeutic options for overcoming resistance to mABs and TKIs, novel strategies such as antibody-drug conjugates (ADCs) and bispecific antibodies have emerged as promising alternatives that can deliver toxic payloads directly to tumor cells, potentially bypassing resistance mechanisms. Specifically, ADCs are designed to target cells expressing specific cancer antigens, thus releasing the cytotoxic chemotherapeutic payload while sparing normal tissues. For example, trastuzumab deruxtecan (T-DXd), an ADC that is an approved treatment for metastatic HER2+breast cancer, can be used even in those resistant to traditional HER-2 targeted therapies[181] (Figure 3). Likewise, the bispecific T cell engager (BiTE) is an alternative and promising approach, combining tumor-associated antigens (such as EGFR or HER2) with CD3 on T cells to initiate immune-mediated tumor cell killing (Figure 3). Nevertheless, major mechanisms of resistance to BiTE therapy involve antigen loss and immunosuppressive factors such as immune checkpoint upregulation. Thus, next-generation immunotherapies may be required to enhance treatment effectiveness and reduce toxicity, especially for solid tumors where responses to BiTE therapy are consistently poor.[182] However, both ADCs and bispecific antibody therapies are not without limitations, causing side effects such as interstitial lung disease (in the case of T-DXd) and cytokine release syndrome with BiTEs. Despite these major challenges, the current advancements and alterations of such molecules highlight the dynamic and adaptive nature of cancer therapy, with continued focus on overcoming drug resistance and maximizing patient benefit.

8. Combined targeting EGFR and Src as a potential therapeutic approach

TNBC is an aggressive subtype of breast cancer with limited therapeutic options. It is characterized by the absence of estrogen and progesterone receptors and a lack of EGFR2 (HER2) gene amplification and protein expression.[183] Notably, overexpression of EGFR is highlighted in TNBC, attracting significant research interest in evaluating EGFR-TKIs as potential treatments.[184] Despite overexpression of EGFR in TNBC, the EGFR-specific TKIs have shown limited efficacy due to their intrinsic or acquired resistance mechanisms.[185] Studies identified the association of SFKs as a key factor that contributes to EGFR resistance, which has been shown to increase HER-family receptor expression.[186,187] The overexpression of Src enhances HER2/HER3 dimerization, consequently delaying receptor internalization and hence prolonging its downstream oncogenic signaling.[188-190] This crosstalk between EGFR and Src kinases suggests that targeting EGFR alone may not be sufficient, suggesting a dual-targeted approach that can inhibit Src signaling.

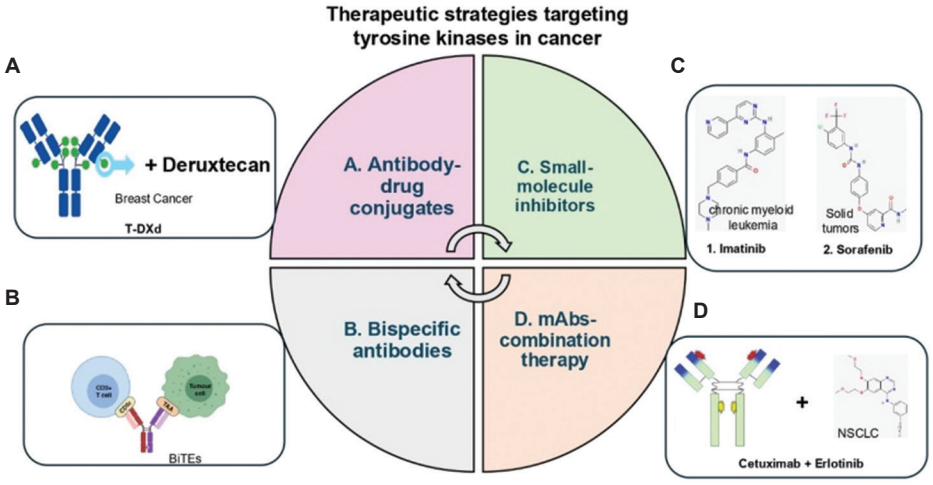

Figure 3. (A-D) Schematic illustrating the specific therapeutic strategies targeting tyrosine kinases in cancer. Image created by the authors. Abbreviations: BiTEs: Bispecific T cell engagers; mAbs: Monoclonal antibodies; NSCLC: Non-small cell lung cancer; T-DXd: Trastuzumab deruxtecan.

Dasatinib, an Src inhibitor, initially exhibits sensitivity in TNBC cells; however, resistance develops over time.[191,192] However, combining both EGFR and Src inhibitors has shown promising results.[193] For instance, afatinib (an irreversible pan-HER inhibitor) and Src inhibitors have shown synergistic effects in MDA-MB-468, TNBC cell lines. In addition, the combination of afatinib and dasatinib has also been shown to enhance apoptosis and growth suppression of NSCLC *in vitro* and *in vivo*.[194,195]

These preclinical research studies have progressed to phase I clinical trials evaluating the efficacy of these combination therapies (ClinicalTrials.gov identifier: NCT01999985). The cooperative interactions between these Src-TKs and HER family members in acquired resistance reveal the significance of developing novel combination drug therapies targeting both pathways. This combination of therapies may hold significant potential in overcoming multidrug resistance, improving treatment response, and increasing clinical benefits in TNBC and other EGFR-driven cancers.

8.1. Therapeutic challenges and limitations

The development of TKIs against cancer has significantly advanced in recent years. However, their clinical utility is often reduced by the extra-cardio adverse effects due to toxicity. It is well documented that older generations of TKIs can cause a wide range of cardiovascular issues such as hypertension, atrial fibrillation, and heart failure. These adverse effects highlight a critical therapeutic challenge: the necessity to design novel TKIs that maintain therapeutic efficacy with reduced off-target toxicities.[196] In addition to TKI toxicity, another limitation is the development of drug resistance, accompanied by postmenopausal symptoms, muscle/joint pains, and osteoporosis as common issues in prolonged usage of TKI therapy.[196] Both drug toxicity and the development of drug resistance attributed to long-term treatment necessitate the development of novel TKIs that strike a balance between specific target inhibition and favorable safety profiles. In general, the therapeutic design must prioritize both efficacy and reduction of toxicity to improve patient outcomes and long-term treatment sustainability.

9. Summary and conclusion

The current review highlights the crucial role of PTKs, with special emphasis on EGFR and Src, in regulating important cellular processes such as growth, differentiation, survival, and regulation underlying carcinogenesis. Furthermore, this review addresses the structural mechanism of EGFR and Src kinases that provides valuable insights into designing novel cancer therapies. Besides that, this review emphasizes the development of TKIs, including gefitinib and erlotinib, and the challenges posed by resistance in cancer treatment. We also outline and evaluate the existing clinical trials of combination therapy targeting EGFR and Src kinases, particularly in aggressive cancers like TNBC. In conclusion, EGFR and Src kinases are significant players in tumor development and therapeutic resistance. Hence, the development of inhibitors/combination treatment holds substantial promise in overcoming multidrug resistance and augmenting therapeutic response in a broad spectrum of cancers.

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Author contributions

Conceptualization: Eswar Kumar Nadendla, Raghavendra Sashi Krishna Nagampalli

Visualization: Eswar Kumar Nadendla

Writing–original draft: Eswar Kumar Nadendla, Gangadhar P. Vadla, Raghavendra Sashi Krishna Nagampalli

Writing–review & editing: Eswar Kumar Nadendla, Manohar Radhakrishnan, Raghavendra Sashi Krishna Nagampalli

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The HADDOCK-generated Src: PTPα complex model shown in the review is available from the corresponding authors on request.

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REVIEW ARTICLE

Biocompatibility of nanomaterials in medical applications

Marvellous O. Eyube[1,2]*, Courage Enuesueke[1,2], and Marvellous Alimikhena[1,2]

[1]Department of Chemistry, Faculty of Physical Science, University of Benin, Benin City, Nigeria

[2]Science and Technological Enhanced Laboratory for Advanced Learning and Research, Benin City, Nigeria

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Abstract

Biocompatibility is a critical factor in the application of nanomaterials in medical fields, as these materials must interact safely and effectively with biological systems to be viable for therapeutic and diagnostic use. This article investigates this feature, focusing on the interactions of nanomaterials with cells, tissues, and the immune system. Key properties such as surface chemistry, size, shape, and material composition are examined for their influence on the biological response. The article also explores the role of nanomaterials in medical applications, including drug delivery, diagnostic imaging, and tissue engineering, while discussing the challenges involved in enhancing their biocompatibility. A case study on the calcium oxide (CaO)–calcium phosphate (CaP) binary system is presented, showcasing its potential in bone tissue engineering, particularly its osteoinductive properties and ability to mimic the bone mineral content. The analysis underscores both its therapeutic potential and the biocompatibility concerns of CaO–CaP scaffolds. The article concludes by outlining strategies to optimize nanomaterial biocompatibility and future directions for their translation into medical applications.

Keywords: Biocompatibility; Calcium oxide–calcium phosphate system; Nanomaterials; Tissue-engineering; Osteointegration; Regeneration

***Corresponding author:**
Marvellous O. Eyube
(marvellous.eyube@physci.uniben.edu)

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1. Introduction

Modern medicine is witnessing a paradigm shift, shaped by the rise of precision medicine, implantable technologies, and patient-specific treatment regimens. These emerging approaches demand materials that can perform reliably within complex biological systems while enabling fine-tuned control over therapeutic or diagnostic outcomes. However, conventional biomaterials often fall short of these requirements. Their limited biological responsiveness, low adaptability, and potential to trigger immune reactions have created a critical gap in realizing next-generation medical solutions, as highlighted in foundational biomaterials research that emphasizes the limitations of traditional materials in dynamic physiological contexts.[1]

Nanomaterials have emerged as compelling candidates for addressing current biomedical challenges, largely due to their distinctive physicochemical characteristics. Their nanoscale dimensions facilitate interactions with biomolecules, cells, and tissues

at the molecular level, enabling enhanced biological integration. Key features—such as a high surface-area-to-volume ratio, modifiable surface chemistry, and responsiveness to external stimuli—confer functional advantages not typically observed in conventional materials. These advantages have been demonstrated in recent comprehensive analyses focusing on their synthesis, customizability, and wide-ranging biomedical applications of nanomaterials.[2]

The multifunctional nature of nanomaterials enables their incorporation into a wide array of biomedical platforms, including injectable drug carriers and surface-modified implants. Their amenability to large-scale production further enhances their translational potential for routine clinical use, although considerations remain regarding their toxicological profiles and biocompatibility. Prior studies have examined the safety, bioaccumulation, and immune responses of nanomaterials, highlighting both opportunities and challenges in their clinical deployment.[3]

These advantages make nanomaterials particularly attractive for addressing real-world medical demands. In cancer therapy, for example, liposomal formulations such as Doxil® have revolutionized chemotherapy by delivering doxorubicin directly to tumor sites, reducing systemic toxicity and improving therapeutic efficacy. This milestone—recognized as the first Food and Drug Administration (FDA)-approved nanodrug—demonstrates how rational nanodesign can overcome long-standing limitations in pharmacokinetics and safety, as detailed in case studies tracing the translation of nanoparticle-based formulations from bench to bedside.[4]

In diagnostic imaging, ferumoxytol, an iron oxide nanoparticle, has been successfully used off-label as a magnetic resonance imaging contrast agent in various clinical settings, enhancing vascular imaging in patients unsuitable for conventional gadolinium-based agents. This clinical adaptation highlights the flexibility of nanomaterials in addressing diagnostic limitations and expanding imaging capabilities in vulnerable patient populations.[5]

In orthopedic and dental applications, nanostructured coatings and scaffolds—such as those made from calcium phosphate (CaP) or calcium oxide (CaO)—promote bone regeneration and tissue integration due to their osteoconductive nature. These materials, with their nanoscale topographies and bioactive interfaces, offer significant improvements in implant performance and bone-anchoring efficiency.[6] More specifically, nano-hydroxyapatite scaffolds have shown great promise in clinical and pre-clinical bone regeneration efforts, offering high surface reactivity, biomineral mimicry, and superior compatibility with osteoblasts. Their use as bioinspired, osteoinductive matrices has been validated through recent studies examining their structural properties and biological responses *in vivo* and *in vitro*.[7]

Tissue engineering represents another critical domain in which nanomaterials play a transformative role. By mimicking the extracellular matrix, nanomaterial-based scaffolds support cellular activities necessary for tissue regeneration. Their high surface area and controllable porosity facilitate cell adhesion and enable the localized delivery of bioactive agents. Investigations into carbon-based and cellulose-derived nanomaterials have demonstrated their capacity to support tissue integration, promote vascularization, and guide targeted regeneration.[8]

Across these applications, nanomaterials are not only enhancing current medical practices but also enabling previously unachievable technological advances. Despite these promising developments, the clinical use of nanomaterials hinges on their ability to safely interact with biological environments. Their high reactivity, while beneficial for functionality, introduces risks of cytotoxicity, inflammation, or immune system activation. As such, biocompatibility has emerged as a core requirement for their medical use. Defined as a material's ability to perform its intended role without provoking adverse biological responses, biocompatibility ensures that nanomaterials are both effective and safe for clinical use.[1,2]

The next section delves into examining the criteria for assessing biocompatibility, the mechanisms by which nanomaterials interact with biological systems, and the strategies employed to mitigate risks. As the foundation of successful medical applications, biocompatibility serves as the critical bridge linking nanomaterial innovation to real-world patient outcomes.

2. The critical significance of biocompatibility in nanomedicine

2.1. The role of biocompatibility in clinical success

Biocompatibility is a key factor in determining whether a nanomaterial can be successfully translated into clinical applications. For any medical material, especially one designed to work at the molecular or cellular level, it must be able to interact with tissues and fluids in the body without causing harm. If a nanomaterial triggers toxicity, inflammation, or an unwanted immune reaction, it can compromise the therapy entirely. As highlighted in foundational research on the biomedical potential of nanomaterials, ensuring compatibility with the physiological environment is not just important—it is essential.[9]

Unfortunately, there have been many cases where promising nanomaterials performed well in the laboratory but failed *in vivo*. These failures often stem from poor biocompatibility. For example, nanoparticles not designed to avoid immune surveillance may be rapidly cleared from circulation, or, more concerningly, provoke dangerous responses. Studies on targeted delivery have shown the importance of anticipating such reactions during the design phase, underscoring that the clinical success of nanomaterials hinges on proactive biocompatibility assessment and modulation.[10]

2.2. Comparison with conventional biomaterials

Nanomaterials exhibit fundamentally different biological interactions compared to traditional biomaterials. While bulk materials such as metals and polymers are typically inert and used for their mechanical strength, nanomaterials are reactive, customizable, and operate at a scale that enables intimate interaction with biological structures. These features allow for exciting possibilities in medicine—such as targeted drug delivery or real-time monitoring—but they also introduce significant challenges. Studies on nanoparticle surface engineering have shown that poorly designed nanomaterials can adsorb proteins non-specifically, trigger immune responses, or cause cellular damage.[11]

Unlike conventional implants, which typically remain inert and static within the body, nanomaterials are often intended to move, respond dynamically, or break down after fulfilling their function. Their tiny size allows them to enter cells more easily, but it also increases their accumulation in tissues. For instance, particles smaller than 100 nanometers are great for intracellular delivery; however, if they are not biodegradable, they may accumulate and cause harm over time—a concern raised in earlier work on nanoparticle design.[12] Particle shape also significantly influences biological interactions. Spherical nanoparticles tend to be taken up more readily, while rod-shaped particles exhibit distinct uptake pathways and may interact with immune cells differently, potentially altering their safety profile.[13]

2.3. Regulatory emphasis on biocompatibility

Health regulatory agencies around the world—including the U.S. FDA and the European Medicines Agency (EMA)—place a strong emphasis on biocompatibility in the evaluation of nanomedicine products. Developers are expected not only to prove that a treatment works but also to provide detailed evidence about how the material behaves in the body. Key questions include whether the material exhibits toxicity, elicits immune responses, or how it is metabolized and cleared from the body. These concerns are particularly critical for nanomaterials due to their unique and complex properties. Environmental studies on nanoparticle degradation have underscored the importance of understanding material fate as a determinant of long-term safety.[14]

To meet these expectations, developers must prioritize the selection of biocompatible materials and implement surface modifications to improve safety. Organic nanomaterials, such as liposomes or biodegradable polymers, are favored in regulatory assessments due to their inherent capacity to break down into harmless byproducts. On the other hand, inorganic materials such as iron oxide or gold may offer advantages in imaging or durability, but usually require surface coatings to reduce toxicity and prevent accumulation in tissues. These strategies are supported by recent studies showing the feasibility of safely using inorganic nanoparticles in biomedical applications through engineered surface modifications.[15] Additional research has further pointed out the need to manage potential immune effects from inorganic particles, highlighting the value of immunomodulatory surface design.[16]

2.4. Foundation for engineering nanomedicines

Biocompatibility must be integrated into nanomaterial design from the outset—it is not a parameter that can be retroactively optimized. Every physicochemical characteristic of the nanomaterial, including size, shape, surface charge, texture, and chemical composition, plays a pivotal role in determining biological interactions. One widely used method to enhance biocompatibility is PEGylation, whereby polyethylene glycol (PEG) chains are grafted onto nanoparticle surfaces. This modification helps the material stay in the bloodstream longer and reduces detection by the immune system. PEGylation, along with hydrophilic surface coatings, has proven highly effective in improving compatibility, as noted in several design-focused studies.[10,11]

Surface charge also plays a delicate role. Positively charged particles are more likely to enter cells, thanks to their attraction to the negatively charged cell membranes. However, this benefit comes with a downside—a higher propensity for cytotoxicity and pro-inflammatory responses. On the other hand, neutral or slightly negative particles are usually better tolerated, though they might not be taken up as efficiently. Striking the right balance is critical, as highlighted in immunological studies focused on nanomaterial-host interactions.[17]

Another aspect that influences biocompatibility is surface energy. When a nanoparticle enters the bloodstream, it quickly gets coated with proteins, forming

a "protein corona" that changes its biological response. Uncontrolled corona formation may lead to unpredictable pharmacokinetics or off-target effects. Researchers have shown that tweaking surface energy can help control corona composition, thereby guiding biological interactions in a way that supports therapeutic goals.[18]

Finally, the intrinsic nature of the nanomaterial itself matters. Organic nanomaterials, such as liposomes or polymers, are generally safer because the body naturally breaks them down. They are often preferred for treatments requiring repeat dosing or prolonged systemic exposure.[14] In contrast, inorganic nanomaterials such as silica or gold may be preferred for their strength or imaging capabilities, but they often need to be coated or encapsulated to ensure safety. This is especially important in antiviral applications, where precise control over surface charge and hydrophilicity is required to avoid immune activation while preserving efficacy.[19]

3. Methodologies for biocompatibility assessment

Assessing the biocompatibility of nanomaterials involves a multidisciplinary framework, incorporating laboratory assays, animal studies, computational tools, and regulatory evaluation. This integrated approach is essential to understand the complex interactions between nanomaterials and biological systems, ensuring both safety and efficacy for clinical translation. As nano–bio interactions vary depending on material properties and intended application, using a combination of assessment methods helps to identify and mitigate potential risks early in the development process.

3.1. *In vitro* methods

In vitro techniques are typically the first step in evaluating the biological compatibility of nanomaterials. These cell-based assays offer a controlled environment to investigate how nanoparticles influence cellular health, behavior, and morphology. Standard protocols include MTT and resazurin reduction assays, which assess metabolic activity, along with tests for membrane disruption, oxidative stress, and programmed cell death.[19]

For example, Siller *et al.*[20] introduced a real-time live-cell imaging system that continuously monitors the cytotoxicity and morphology of cells in response to 3D-printed biomaterials. This approach enables high-throughput analysis with temporal resolution. Similarly, Wang *et al.*[21] explored the biosynthesis of zinc oxide nanoparticles using plant extracts and evaluated their effect on human osteoblast-like cells. The MTT assay results indicated improved cell proliferation and bone-forming potential.

To better simulate physiological conditions, advanced systems such as 3D cultures and co-culture platforms are increasingly used. These models provide insights into how nanoparticles affect cell signaling, differentiation, and inflammatory pathways in environments that more closely mimic actual tissue architecture. While *in vitro* methods offer speed, scalability, and cost-efficiency, they remain limited in representing the full complexity of a living organism. This limitation highlights the need for complementary *in vivo* evaluations.[19]

3.2. *In vivo* methods

In vivo testing remains a cornerstone of biocompatibility assessment, particularly for assessing systemic distribution, metabolism, excretion, and long-term toxicity. Animal models—especially rodents—enable comprehensive monitoring of biological responses at the organismal level, including immune responses, hematological changes, and potential organ-specific adverse effects.[22]

A practical example involves the implantation of CaO and CaP nanocomposite scaffolds in rat bone defects. Our ongoing *in vivo* studies demonstrated not only effective tissue regeneration but also favorable immune modulation at the site of implantation. Histopathological analysis, a key component of *in vivo* assessments, helped detect subtle tissue-level reactions such as fibrosis and inflammation. Kyriakides *et al.* further highlighted the value of *in vivo* testing in revealing immunological changes such as cytokine production and complement activation, offering critical insights into nanomaterial-host interactions.[23]

Despite their utility, *in vivo* models face limitations due to ethical concerns, regulatory scrutiny, and species-to-species differences, which complicate the extrapolation of animal data to human contexts. To address these challenges, alternative platforms—such as organ-on-chip devices and *ex vivo* perfusion systems—are being explored as more ethically sound and potentially more predictive options.[22]

3.3. Computational models

Computational modeling provides a predictive layer to biocompatibility evaluation using simulations and data-driven algorithms to estimate biological interactions. Molecular dynamics simulations, for example, allow scientists to explore how nanoparticles interact with cellular membranes or proteins at the atomic level, helping to anticipate toxic effects before physical experimentation.[24]

Cao *et al.*[25] demonstrated the utility of computational tools such as nano-quantitative structure–activity relationship models to estimate the toxicity of metal oxide nanoparticles. By analyzing properties such as surface

charge, energy band gaps, and hydration tendencies, they predicted biological outcomes with high reliability, thereby streamlining the screening process. Although these models are powerful, they rely heavily on the quality and diversity of the datasets used for training. Inconsistencies in data reporting and the lack of standardized descriptors can limit their predictive accuracy. As such, computational results are best viewed as complementary to experimental methods, with ongoing improvements necessary to achieve broader regulatory acceptance.[24,25]

3.4. Regulatory standards

The regulatory landscape for nanomaterials is shaped by the guidelines issued by leading authorities, including the FDA, EMA, the International Organization for Standardization (ISO), and the Organization for Economic Co-operation and Development (OECD). These bodies define the protocols and criteria that nanomaterials must meet before clinical application, with emphasis placed on safety, stability, pharmacokinetics, and immune compatibility.[26]

One of the earliest success stories is the approval of Doxil®, a liposomal formulation of doxorubicin, which underwent comprehensive biocompatibility testing for sterility, blood compatibility, and immune responses.[4] However, as nanomedicine continues to evolve rapidly, current regulatory frameworks often struggle to keep pace. The lack of standardized global guidelines poses challenges for developers seeking international approval and commercialization.

In response, regulatory bodies are moving toward greater harmonization, promoting validated *in vitro* and computational models as part of a robust evaluation pipeline. This push not only ensures safety but also facilitates the adoption of innovative technologies.[27]

3.5. Comparative metrics and evaluation criteria

Given the diverse methodologies employed in biocompatibility assessment, the establishment of standardized metrics is essential to ensure reproducibility and facilitate cross-study comparisons. Common evaluation parameters include cytotoxicity thresholds, quantification of pro-inflammatory cytokines such as interleukin (IL)-6 and tumor necrosis factor-alpha (TNF-α), cellular uptake rates, tissue biodistribution profiles, and histopathological scoring.

Efforts to integrate these variables into unified frameworks have led to the development of integrated testing strategies, which synthesize data from *in vitro*, *in vivo*, and *in silico* methods into a single evaluative framework. One example is the work by Schloemer *et al*.,[28] who used quantum-level modeling alongside biological tests to assess light-activated nano-upconversion systems, thereby demonstrating a comprehensive route from material design to functional testing. This integration of disciplines improves both predictive accuracy and translational potential. As noted by Seoane-Viaño *et al*.,[29] the successful implementation of 3D-printed nanomedicines hinges not only on their design but also on thorough biocompatibility assessment and regulatory alignment.

4. Scientific challenges in biocompatibility

The integration of nanomaterials into biomedical applications continues to drive innovation in diagnostics, therapeutics, and tissue engineering. However, their clinical translation remains impeded by unresolved challenges surrounding biocompatibility. These include immunological risks, long-term safety concerns, and regulatory uncertainties. This section examines key scientific barriers related to nanotoxicology, immune response, material degradation, and global safety standards.

4.1. Toxicity and immune response

A central challenge in nanomedicine is the potential toxicity and immunogenicity of nanomaterials. Their high surface area and physicochemical reactivity can lead to unintended biological interactions. For instance, nanoparticles may induce oxidative stress, disrupt cell membranes, or trigger inflammatory cytokine release.[3] Surface charge and hydrophobicity strongly influence these outcomes. For example, positively charged particles often facilitate enhanced cellular uptake but are also linked to increased membrane disruption and inflammation.[3]

Furthermore, the adsorption of proteins onto the nanoparticle surface—the "protein corona" effect—alters their biological identity and can lead to immune misrecognition. This dynamic interaction may affect circulation time, biodistribution, and therapeutic efficacy. Importantly, even formulations previously considered inert may provoke immune responses when administered *in vivo*, emphasizing the need for rigorous pre-clinical immunotoxicity testing.[3]

4.2. Long-term stability and degradation

The long-term fate of nanomaterials in the body is a growing concern, especially when they exhibit poor biodegradability. Inorganic nanoparticles, such as those used for imaging and targeted drug delivery, may lack efficient metabolic or excretory pathways. This can result in their accumulation in organs involved in clearance, such as the liver, spleen, and kidneys, potentially causing chronic toxicity over time.[4,5] For example, gold and iron oxide nanoparticles exceeding 50 nm often localize in

the reticuloendothelial system (RES), where they resist renal filtration and remain for prolonged durations. This sequestration may lead to oxidative damage, inflammation, or organ dysfunction.[5] To address these issues, various strategies—such as PEGylation, encapsulation, or size reduction below 10 nm—have been developed to enhance clearance and mitigate RES uptake.[5]

In contrast, biodegradable polymers such as polylactic acid (PLA) and polycaprolactone (PCL) degrade into biocompatible byproducts and offer greater safety for long-term use. Yet, achieving consistent degradation rates across various physiological environments remains challenging.[30] Tailoring polymer composition and nanostructure is essential to balance therapeutic performance with predictable *in vivo* clearance.

4.3. Regulatory and safety concerns

Regulatory pathways for nanomaterials often lag behind the pace of technological advancement. Existing frameworks—originally designed for bulk materials—fall short in addressing the unique risks posed by nanomaterials, particularly those related to long-term biodistribution, individual variability in physiological responses, and complex immunological interactions.[4] Agencies such as the FDA, EMA, and ISO have issued updated guidelines, but significant gaps remain in standardized testing protocols for nanomedicine.

Developers are increasingly expected to adopt a safety-by-design approach, including generating comprehensive toxicology profiles and conducting lifecycle assessments during early-stage development. The need for harmonized global standards is especially urgent for nanotherapeutics intended for systemic or repeat-dose administration.[4,29] Bridging this regulatory gap requires multidisciplinary collaboration and proactive engagement with policymakers.

5. Strategies for targeted improvement

To improve the clinical performance of nanomaterials, targeted design strategies must be integrated early in the development process. These strategies not only reduce adverse biological responses but also enhance the safety, specificity, and real-world usability of nanomedical tools. While many of these methods have proven successful in controlled laboratory environments, translating them into practical applications remains the true benchmark of success. This section outlines four primary approaches: surface engineering, biodegradable material selection, targeted delivery, and hybrid designs. Each strategy offers a framework for enhancing biocompatibility and functionality. These concepts are further illustrated through the case of the CaO–CaP binary system in Section 6.

5.1. Surface modifications

Surface engineering plays a pivotal role in enhancing nanomaterial biocompatibility. Among the most widely adopted techniques is PEGylation, which involves attaching PEG chains to the nanoparticle surface to minimize immune detection and extend systemic circulation.[3,10,18] This "stealth" property allows therapeutic particles to circulate longer, increasing the likelihood of target site delivery.

Modifying surface properties such as charge, hydrophilicity, and the presence of targeting ligands also influences how nanomaterials interact with biological components such as membranes, proteins, and cells.[11,18] These modifications help minimize protein corona formation, reduce immunogenicity, and promote selective uptake by desired cell types. As will be explored later with the CaO–CaP system, such tailoring becomes particularly important when adapting materials to specific physiological environments.

Despite its advantages, PEGylation has limitations. Repeated administration can result in accelerated clearance, and the immune system may develop anti-PEG antibodies. These concerns have prompted ongoing optimization efforts focusing on PEG chain density, molecular weight, and branching to balance stealth effects with immunological safety.[31]

Zwitterionic coatings present an alternative approach. Composed of molecules carrying both positive and negative charges—such as sulfobetaines and phosphorylcholines—these coatings form a densely hydrated shell that resists protein adsorption. Debayle *et al.*[32] demonstrated that zwitterionic polymers could completely inhibit corona formation, outperforming PEGylation in maintaining nanoparticle stealth and physiological stability.

Another innovative approach leverages biomimicry. For example, by incorporating CD47 peptides onto nanoparticle surfaces, researchers can mimic natural "do-not-eat-me" signals. These peptides engage the signal regulatory protein alpha receptor on macrophages, suppressing phagocytosis and allowing for prolonged circulation. However, caution is needed, as excessive immune suppression and potential blood-related toxicities remain concerns in clinical settings.[33]

Another emerging strategy involves cloaking nanoparticles with cellular membranes—harvested from red blood cells, leukocytes, platelets, or cancer cells—to form biomimetic coatings. These membrane-derived surfaces provide immune camouflaging and can even enable tissue-specific homing due to retained surface proteins and antigens. Such carriers have demonstrated promise in drug delivery, detoxification, and vaccine delivery,

offering enhanced biocompatibility and functionality.[34] Collectively, these surface modification techniques form a robust toolkit for tailoring nanomaterials to navigate immune challenges, improving both their safety and therapeutic potential in clinical scenarios.

5.2. Biodegradable nanomaterials

Biodegradable polymers such as PLA and PCL offer significant advantages in terms of biological compatibility and safety.[14] These materials naturally break down into non-toxic byproducts, lowering the risk of prolonged organ retention or chronic inflammatory responses.

Critically, their degradation profiles can be finely tuned to match specific therapeutic timelines, allowing for sustained or controlled drug release. This is particularly beneficial in applications such as tissue regeneration and chronic disease management, where timing and clearance are vital.[10,14] The CaO–CaP system highlights the importance of selecting biodegradable components when developing clinically translatable materials.

5.3. Targeted delivery

Precision targeting has become a cornerstone of effective nanotherapy. By functionalizing nanocarriers with ligands that bind to disease-specific receptors—such as those overexpressed in tumors—therapeutic agents can be concentrated at the site of interest while sparing healthy tissue.[10] In addition, stimuli-responsive platforms that react to environmental cues such as pH, temperature, or enzymatic activity have enabled on-demand drug release tailored to pathological conditions. These adaptive systems reduce off-target effects and enhance therapeutic efficacy, especially in diseases such as cancer, where site-specific intervention is essential.[10,29]

For example, in colorectal cancer treatment, multifunctional nanomaterials have been employed for integrated diagnosis, therapy, and monitoring. These platforms are engineered to selectively accumulate at tumor sites, improving therapeutic precision while reducing collateral damage. Such targeted approaches underscore the importance of using biocompatible materials—such as calcium-based carriers—to meet the safety demands of clinical deployment.[35]

5.4. Hybrid systems

Hybrid nanostructures, which combine organic and inorganic components, offer the best of both functional versatility and biocompatibility. Metallic cores—such as gold or calcium compounds—can be encapsulated within biodegradable or bioactive shells, creating platforms that are both structurally robust and biologically safer.[3,15]

These multifunctional systems are especially well-suited for theranostic applications, where imaging, diagnosis, and treatment are integrated into a single material. However, the complexity of hybrid materials necessitates precise control over properties such as surface chemistry, charge distribution, and degradation kinetics to ensure they are biologically harmonious.[3,18] The CaO–CaP binary system discussed in the next section exemplifies how such hybrid materials can be engineered for enhanced compatibility and targeted performance in a biomedical setting.

6. Case-based empirical analysis: The CaO-CaP binary system

The successful clinical translation of nanomaterials relies on their ability to balance functional performance with biocompatibility. As discussed in the previous section, targeted strategies such as surface modification, biodegradability, and composite design are foundational. The CaO–CaP binary system provides a compelling case study in this regard, illustrating both the promise and the challenges of deploying biocompatible nanomaterials in regenerative medicine. Based on empirical work and laboratory experience, this section explores the material's key features, *in vitro* and *in vivo* findings, clinical challenges, and future strategies in the context of bone tissue engineering.

6.1. Material properties and molecular mechanisms

The binary system comprising CaO and CaP leverages the individual strengths of both materials. CaO is known for its high alkalinity and rapid dissolution, facilitating a bioactive environment that promotes mineralization and bone induction. CaP, being structurally similar to the mineral phase of the bone, contributes to long-term mechanical stability and degradation. As shown in Figure 1, the structural and functional attributes of the CaO–CaP nanomaterial are closely linked to its molecular interactions and phase composition.

6.2. Functional synergy of CaO–CaP composites

The functional benefits of CaO and CaP composites stem from their complementary behavior in biological environments. CaO provides an early burst of calcium ions, initiating mineralization, while CaP maintains structural support for long-term cell attachment and tissue integration. This dual-phase release promotes hydroxyapatite formation and enhances interactions between the scaffold and native tissue—key goals in osteogenic material design.[2,36,37]

Research also highlights that adding trace elements such as magnesium to these systems can further

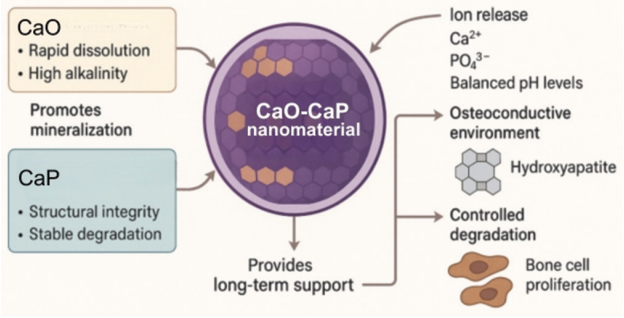

Figure 1. Diagram illustrating the material properties and molecular mechanisms of the CaO–CaP binary system. Image created by the author. Abbreviations: CaO: Calcium oxide; CaP: Calcium phosphate.

elevate performance. For instance, Qi *et al.*[38] found that incorporating magnesium into bioceramics significantly improved cell response and vascular development, both essential for bone healing. This supports the growing view that finely tuned ion release—including calcium, phosphate, and magnesium ions—helps replicate the bone's natural healing environment and bolsters the rationale for materials such as CaO–CaP in regenerative design.

Another valuable trait of CaO–CaP systems is their inherent antimicrobial potential, increasingly important for reducing post-surgical infection risks. The basic nature of CaO elevates the surrounding pH upon dissolution, disrupting bacterial membranes, denaturing proteins, and impairing enzyme function, ultimately killing harmful microbes.[39]

This effect has been demonstrated using CaO and calcium peroxide (CaO_2) nanoparticles. For example, Yu *et al.*[40] reported that polyacrylic acid-coated CaO_2 nanoparticles not only supported wound healing but also combated bacterial growth through the combined release of calcium ions and reactive oxygen species. Similarly, Levingstone *et al.*[41] found that CaP-based scaffolds not only promoted bone regeneration but also resisted bacterial colonization.

Although specific research on CaO–CaP systems remains nascent, our team at Science and Technological Enhanced Laboratory for Advanced Learning and Research (S.T.E.L.L.A.R) Laboratories is actively evaluating their antibacterial potential—especially against *Staphylococcus aureus*, a frequent cause of orthopedic infections. Initial *in vitro* results are promising, showing less bacterial adhesion and better scaffold sterility. This points to the dual functionality of CaO–CaP materials: supporting tissue repair while simultaneously offering protection against infection.

6.3. Biocompatibility studies (*in vitro* and *in vivo*)

A series of *in vitro* and *in vivo* evaluations confirms that CaO–CaP nanomaterials exhibit excellent biocompatibility. In cell culture studies using osteoblasts, these scaffolds supported healthy cell growth, attachment, and differentiation, with minimal toxicity. The controlled release of calcium and phosphate ions also encouraged robust matrix mineralization.[42]

These findings were backed by *in vivo* experiments in rodent models, where CaO–CaP scaffolds were implanted into critical-size bone defects. Tissue analysis showed strong new bone formation, seamless integration with host tissue, and tight bonding at the interface. Compared to conventional grafts, CaO–CaP composites accelerated healing and enhanced defect closure, reinforcing their suitability for clinical use.[43,44]

6.4. Clinical bottlenecks and inflammation response

Despite their clear advantages, CaO–CaP scaffolds face some hurdles in clinical translation, particularly regarding inflammation caused by rapid degradation. The high dissolution rate of CaO can cause spikes in calcium ion levels and increase local pH, which may irritate surrounding tissues and trigger immune responses.

In our own pre-clinical tests, areas where the scaffold degraded quickly showed signs of local inflammation and mild immune cell activation—likely a response to sudden changes in ion concentration and pH. To address this, we applied biodegradable polymer coatings such as poly(lactic-co-glycolic acid) (PLGA) and PEG to the scaffold surface. These coatings help regulate the ion release profile, minimize pH shifts, and reduce inflammatory responses. Our findings align with prior research showing that surface modification of CaO-based materials can delay degradation and mitigate adverse reactions while maintaining regenerative function.[45,46] As illustrated in Figure 2, PLGA-coated CaO–CaP scaffolds appear to activate a more controlled immune response, particularly in relation to cytokine release.

Further analysis of the inflammatory microenvironment revealed that early-stage responses (0–7 days post-implantation) were characterized by elevated levels of pro-inflammatory cytokines such as IL-6, TNF-α, and IL-1 beta. These mediators contribute to tissue swelling, leukocyte recruitment, and vascular changes. In the subacute phase, the inflammatory signal begins to subside, making way for reparative processes. A critical aspect of recovery is the phenotypic transition of macrophages from the pro-inflammatory M1 phenotype to the anti-inflammatory M2 phenotype. This shift is associated with increased secretion of anti-inflammatory cytokines like IL-10, which downregulate the immune response and promote tissue regeneration. Modulating this immune balance through material design and surface treatment is

key to minimizing long-term tissue damage and enhancing scaffold integration. As shown in Figures 3 and 4, PLGA modification alters the functional behavior of CaO–CaP nanomaterials, while subsequent scaffold degradation triggers a time-dependent inflammatory response marked by shifts in cytokine expression.

6.5. Emerging strategies for clinical translation

Translating CaO–CaP-based systems into clinical practice requires more than just demonstrating biological compatibility; it also demands innovations in material design, delivery mechanisms, and scalable fabrication methods. One notable advancement is the inclusion of osteogenic growth factors such as bone morphogenetic proteins (BMPs) and vascular endothelial growth factor (VEGF), which play key roles in promoting both osteoblast differentiation and vascularization of the implant site.[47]

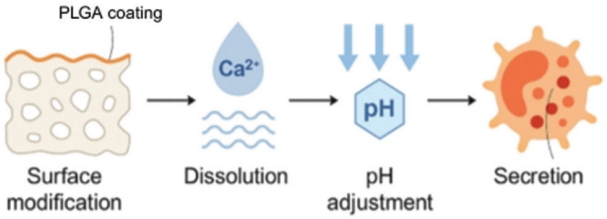

Figure 2. A series of reactions triggered by the surface modification of CaO–CaP. Adding PLGA coating changes the pH and affects the secretion of pro-inflammatory cytokines. Image created by the author.
Abbreviations: CaO: Calcium oxide; CaP: Calcium phosphate; PLGA: Poly(lactic-co-glycolic acid).

Beyond biochemical cues, gene-activated scaffolds have emerged as a powerful tool, enabling the localized delivery of therapeutic DNA or RNA to stimulate regenerative pathways directly within the defect area. These platforms are gaining attention for their potential in treating complex and non-healing bone injuries, where conventional scaffolds often fall short.

A transformative shift is also underway with the adoption of 3D printing technologies, allowing the fabrication of patient-specific scaffolds with precise anatomical conformity. This level of personalization improves not only implant integration and mechanical performance but also healing outcomes. As part of our ongoing investigations, CaO–CaP scaffolds are being combined with bioactive molecules and additive manufacturing techniques to boost regenerative efficiency while enhancing clinical adaptability.[48]

In parallel, ion-doped biodegradable systems—particularly those incorporating magnesium ions—are showing great promise for bone repair. A recent study by Tao *et al.*[49] described the successful development of porous PLA-based microspheres doped with magnesium, which exhibited enhanced biocompatibility, improved osteogenic potential, and controlled biodegradation. These findings align with our own data, underscoring the importance of controlled ionic release and scaffold adaptability in the clinical success of CaO–CaP materials.

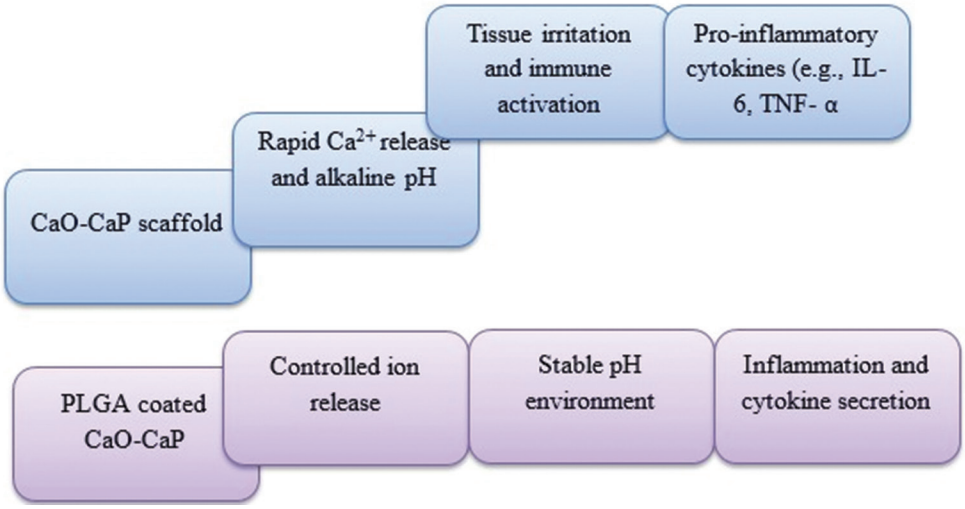

Figure 3. The effect of PLGA modification on the behavior of CaO–CaP nanomaterials. The uncoated pathway results in rapid calcium ion release and pH elevation, which may lead to tissue irritation and upregulation of inflammatory cytokines such as IL-6 and TNF-α. In contrast, the PLGA-coated pathway moderates ion release and stabilizes pH, thereby reducing inflammation and improving biocompatibility. Image created by the author.
Abbreviations: CaO: Calcium oxide; CaP: Calcium phosphate; IL-6: Interleukin-6; PLGA: Poly(lactic-co-glycolic acid); TNF-α: Tumor necrosis factor alpha.

Magnesium, in particular, appears to play a supportive role in modulating osteoblast responses and stimulating new bone matrix deposition. Its inclusion reflects a broader trend toward ion-enhanced strategies for fine-tuning scaffold performance. As highlighted in Tables 1 and 2, nanomaterials used in these systems vary widely in their biocompatibility and regenerative potential, emphasizing the importance of careful material selection and design optimization for translational success.

In pursuit of improved therapeutic outcomes, scaffold systems are increasingly being functionalized with biologically active molecules. Among the most promising are BMP-2 and VEGF, which are well known for promoting osteogenesis and angiogenesis, respectively.[46] These signaling molecules are often embedded within biodegradable carriers—such as PLGA microparticles—which facilitate controlled, localized release while preserving bioactivity over time. In parallel, gene delivery strategies have gained significant traction. For example, plasmid DNA encoding VEGF or BMP-2 has been immobilized within CaP-based scaffolds, forming gene-activated matrices that stimulate site-specific expression of regenerative signals. Although these approaches have demonstrated considerable potential, challenges remain—particularly with maintaining vector stability during scaffold fabrication, ensuring effective gene transfection, and avoiding unintended off-target effects.

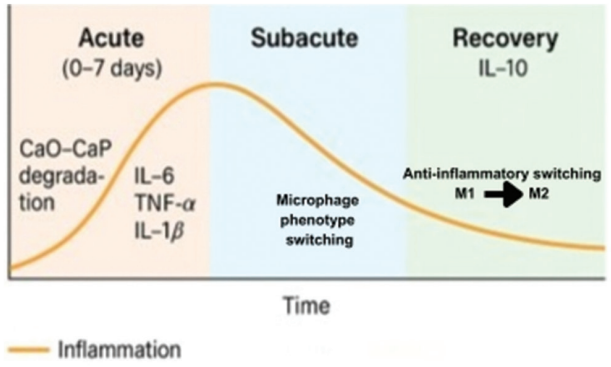

Figure 4. Inflammation and cytokine dynamics following CaO–CaP scaffold degradation. Image created by the author.
Abbreviations: CaO: Calcium oxide; CaP: Calcium phosphate; IL: Interleukin; PLGA: Poly(lactic-co-glycolic acid); TNF-α: Tumor necrosis factor alpha.

Additive manufacturing is also transforming the landscape of scaffold development. In 2022, patient-specific CaP-based craniofacial scaffolds achieved a clinical success rate of over 95%, demonstrating both feasibility and therapeutic promise.[47] Technologies such as fused deposition modeling and stereolithography are now commonly employed to produce custom-fit scaffolds tailored to a patient's anatomical features. These advanced fabrication methods offer superior control over porosity, mechanical strength, and spatial distribution of bioactive

Table 1. Comparative biocompatibility parameters of selected nanomaterials

Nanomaterial	Hemolysis rate	Complement activation	Circulation half-life	Cytotoxicity level	Remarks
AuNPs	Low (<5%)	Moderate (dose-dependent)	Moderate (~24 h)	Low	Excellent imaging agent; surface-dependent immunogenicity
SiNPs	Moderate (10–15%)	High (due to surface silanol)	Short (<12 h)	Moderate	High surface reactivity; surface passivation improves compatibility
LNPs	Very low (<2%)	Minimal	Long (up to several days)	Low	Used in mRNA vaccines; highly biocompatible
CaP	Low (<5%)	Minimal	Biodegradable	Very low	Excellent for bone integration and mineralization
CaO	High (>15%) uncoated	Moderate to high	Fast-degrading	High (alkalinity-induced)	Requires coating to reduce cytotoxicity (e.g., PLGA, PEG)
PLGA-coated CaO–CaP	Low (<3%)	Low	Controlled (tailored by design)	Very low	Reduced inflammation; enhanced osteointegration
CNTs	Variable (type-dependent)	High (can activate immune cells)	Long (>48 h)	Moderate to high	Requires functionalization to improve compatibility
QDs	High (>20%)	High	Long (up to several days)	High	Toxic elements (e.g., Cd); limited clinical use without shielding strategies

Abbreviations: AuNPs: Gold nanoparticles; CaO: Calcium oxide; CaP: Calcium phosphate; Cd: Cadmium; CNTs: Carbon nanotubes; LNPs: Lipid nanoparticles; PEG: Polyethylene glycol; PLGA: Poly (lactic-co-glycolic acid); QDs: Quantum dots; SiNPs: Silica nanoparticles.

Table 2. Comparative biocompatibility metrics of selected nanomaterials

Nanomaterial	Hemolysis rate (%)	Complement activation (C3a level)	Circulation half-life (hours)	Inflammatory response (IL-6/IL-1β)
CaO–CaP	4.5	Moderate	6–8	Low (with coating)
AuNPs	2.1	Low	12–24	Minimal
SiNPs	7.8	High	2–5	Elevated
PLGA nanoparticles	3.3	Low	8–12	Minimal
LNPs	1.2	Very low	24+	Negligible

Abbreviations: AuNPs: Gold nanoparticles; CaO: Calcium oxide; CaP: Calcium phosphate; IL: Interleukin; LNPs: Lipid nanoparticles; PLGA: Poly (lactic-co-glycolic acid); SiNPs: Silica nanoparticles.

components—design features that are challenging to replicate using traditional scaffold manufacturing. As shown in Figure 5, successful bone tissue engineering relies on synergistic integration of scaffold architecture, signaling cues, and responsive biomaterials to drive effective regeneration.

The CaO–CaP binary system exemplifies the delicate balance between biological reactivity and structural stability that defines successful nanomaterial applications in medicine. While its osteogenic potential and favorable integration are well-established, fine-tuning degradation rates and immune compatibility remains crucial. As ongoing innovations in surface coatings, biofunctionalization, and manufacturing techniques evolve, CaO–CaP composites stand poised for greater clinical relevance, offering a tangible example of how theoretical biocompatibility strategies can translate into real-world biomedical impact.

7. Emerging trends and future directions in nanomedicine

Nanomedicine is advancing rapidly, propelled by technological convergence and multidisciplinary collaboration. Next-generation nanomaterials—such as quantum dots, carbon-based structures, and multifunctional hybrid platforms—offer superior optical, electrical, and mechanical properties while maintaining biocompatibility. These attributes make them highly promising for precision drug delivery, targeted diagnostics, and image-guided therapy.[50,51]

Clinical applications of nanotechnology are expanding across therapeutic domains. Multifunctional nanoplatforms have shown significant promise in managing colorectal cancer, where they facilitate early detection, targeted drug delivery, and image-guided interventions, offering a synergistic approach to both diagnosis and treatment.[52] Likewise, surface-engineered nanomaterials have proven

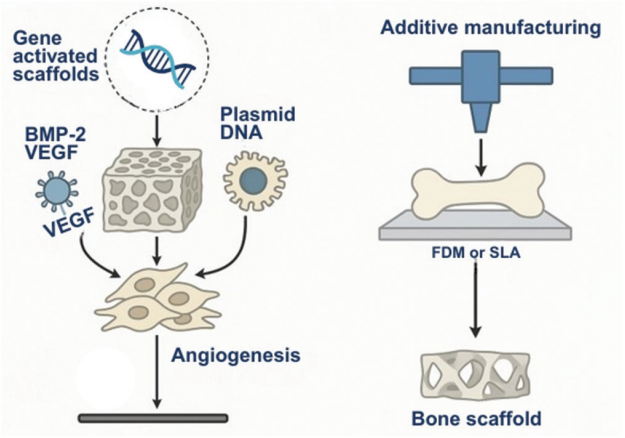

Figure 5. Synergistic strategies of scaffold architecture, signaling cues, and responsive biomaterials in bone tissue engineering. Image created by the author.
Abbreviations: BMP: Bone morphogenetic protein; FDM: Fused deposition modeling; SLA: Stereolithography; IL-6: Interleukin-6; VEGF: Vascular endothelial growth factor.

effective in enhancing immune compatibility and targeted antiviral drug delivery, particularly for managing viral infections.[19]

Addressing the challenges of scalability and reproducibility in nanomaterial production is critical for clinical translation. To this end, spray-drying methods have replaced traditional solvent evaporation techniques, significantly improving nanoparticle uniformity and yield.[51] Automated microfluidic platforms are also being developed to enable continuous nanoparticle synthesis with real-time process monitoring, minimizing batch-to-batch variability. At S.T.E.L.L.A.R. Labs, we are currently integrating AI-guided microfluidic synthesis and in-line spectroscopic monitoring to standardize particle morphology, size, and surface functionality—key parameters for regulatory compliance and clinical-grade manufacturing.

AI and machine learning are also transforming the design and validation of nanomaterials. AI-enabled modeling allows rapid optimization of physicochemical properties while predicting biocompatibility with high accuracy, thereby accelerating preclinical development and reducing experimental costs.[26,53]

In parallel, the field of personalized medicine is evolving, with nanotechnology enabling tailored treatment strategies based on individual genetic and physiological profiles. A landmark application is the use of lipid nanoparticles (LNPs) in delivering mRNA vaccines, as seen during the COVID-19 pandemic. LNPs have proven effective in protecting and transporting nucleic acids to target cells while minimizing systemic toxicity. This platform now

serves as a blueprint for future applications in oncology, genetic disorders, and rare diseases.[54] Nanocarriers are also being designed to function as both immune modulators and delivery systems, making them invaluable tools in managing cancers and infectious diseases.[19,34]

Despite these advancements, regulatory and translational barriers persist. Nanomaterials often evolve faster than existing regulatory frameworks can accommodate. In response, global entities such as the OECD, along with industry–academic consortia, have intensified efforts to harmonize safety assessments, standardize testing protocols, and streamline clinical translation. Progress in this area, fueled by international co-operation and public–private partnerships, is gradually easing these hurdles.[55,56]

As the field moves forward, the future of nanomedicine lies in integrative approaches: Combining smart biomaterials, AI-driven modeling, regulatory foresight, and personalized care. This holistic vision not only enhances the scientific rigor of nanomedicine but also paves the way for meaningful clinical impact in global healthcare.

8. Conclusion

Biocompatibility remains a foundational requirement for the effective use of nanomaterials in medical fields such as drug delivery, diagnostic imaging, tissue regeneration, and antimicrobial therapy. Key parameters—including surface chemistry, particle size, and material composition—critically determine biological responses, as illustrated by the promising performance of the CaO–CaP binary system in bone tissue engineering. The complementary properties of CaO and CaP, when combined with surface modifications, demonstrate strong potential for clinical osteointegration.

As the field progresses, innovations in materials science and cross-disciplinary collaboration are expected to overcome persistent challenges related to toxicity, immune compatibility, and large-scale application. The integration of computational tools and AI will further streamline the design and prediction of safer, high-performance nanomaterials. Although regulatory complexities continue to pose barriers, coordinated efforts among scientists, clinicians, and regulatory bodies will be vital in driving successful clinical translation. By embedding biocompatibility at the core of nanomaterial design and actively addressing translational gaps, nanomedicine is poised to reshape modern healthcare and unlock solutions once beyond reach.

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The authors declare that they have no competing interests.

Author contributions

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Visualization: Marvellous Eyube
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Ethics approval and consent to participate

Not applicable.

Consent for publication

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Availability of data

Not applicable.

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ACCSCIENCE PUBLISHING

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PERSPECTIVE ARTICLE

Exploring *Peganum harmala* as a natural alternative to semaglutide: A novel approach to glucagon-like peptide-1 stimulation and insulin sensitization

Maher Monir. Akl[1]* and Amr Ahmed[2]

[1]Department of Chemistry, Faculty of Science, Mansoura University, Mansoura, Egypt

[2]Department of Public Health, Riyadh First Health Cluster, Ministry of Health, Saudi Arabia

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Abstract

Glucagon-like peptide-1 (GLP-1) is a crucial incretin hormone that regulates glucose homeostasis by enhancing insulin secretion, suppressing glucagon release, and delaying gastric emptying. While synthetic GLP-1 receptor agonists such as semaglutide have demonstrated efficacy in managing type 2 diabetes mellitus and obesity, their high cost, limited accessibility, and adverse effects have limited their applicability, necessitating the search for alternative therapeutic strategies. *Peganum harmala* (harmal), a traditional medicinal plant, has gained attention for its bioactive alkaloids, harmine, and harmaline, which have been shown to modulate key molecular pathways involved in GLP-1 secretion and insulin sensitization. These alkaloids enhance Akt phosphorylation (pS473-Akt), facilitating glucose transporter type 4 translocation and glucose uptake, while concurrently activating the nuclear factor erythroid 2-related factor 2 pathway, leading to increased antioxidant defenses and reduced oxidative stress in pancreatic β-cells and enteroendocrine L-cells. Furthermore, *P. harmala* alleviates insulin resistance by suppressing IRS-1 serine phosphorylation (pS307-IRS-1) and improving phosphoinositide 3-kinase/Akt signaling, thereby optimizing insulin receptor sensitivity and metabolic homeostasis. Despite these promising pharmacological properties, the poor solubility and rapid metabolism of harmine and harmaline pose challenges to their clinical application. Nanotechnology-based drug delivery systems, including liposomal encapsulation and polymeric nanoparticles, offer a potential solution to enhance bioavailability, prolong systemic circulation, and enable targeted delivery to GLP-1-secreting cells. This paper delves into the molecular mechanisms by which *P. harmala* stimulates GLP-1 secretion and improves insulin sensitivity, compares its effects with semaglutide, and highlights the potential role of nanotechnology in optimizing its therapeutic applications. By integrating traditional medicine with modern pharmaceutical advancements, *P. harmala* represents a promising, cost-effective, and sustainable approach to metabolic disorder management, warranting further investigation through pre-clinical and clinical studies.

Keywords: *Peganum harmala*; Glucagon-like peptide-1; Semaglutide; Insulin sensitivity; Nanotechnology

***Corresponding author:**
Maher Monir. Akl
(maherakl555@gmail.com)

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1. Introduction

Glucagon-like peptide-1 (GLP-1) is an incretin hormone secreted by enteroendocrine L-cells in the distal small intestine and colon in response to nutrient ingestion, particularly carbohydrates and lipids. At the molecular level, GLP-1 exerts its effects through the GLP-1 receptor (GLP-1R), a G-protein-coupled receptor widely expressed in pancreatic β-cells, the central nervous system, and peripheral tissues.[1] Upon binding to its receptor, GLP-1 activates the cAMP/protein kinase A (PKA) signaling pathway, leading to enhanced glucose-stimulated insulin secretion, inhibition of glucagon release, and delayed gastric emptying. These actions collectively improve post-prandial glucose homeostasis, making GLP-1 a critical regulator of metabolic processes.[2]

In addition to its metabolic effects, GLP-1 demonstrates significant neuroprotective properties. Its receptor activation in the brain enhances neuronal survival, reduces oxidative stress, and inhibits neuroinflammatory pathways through modulation of intracellular signaling cascades such as phosphoinositide 3-kinase (PI3K)/Akt and MAPK.[3] These properties have positioned GLP-1 analogs as promising therapeutic agents for neurodegenerative disorders, including Alzheimer's disease (AD) and Parkinson's disease. Furthermore, GLP-1's ability to suppress appetite and promote weight loss highlights its role in addressing obesity-related metabolic dysfunctions.[4]

Peganum harmala, commonly known as harmal or Syrian rue, has been extensively utilized in traditional medicine across various cultures for its therapeutic properties, particularly in the management of neurological disorders. Historically, harmal has been employed as a remedy for conditions such as epilepsy, anxiety, and insomnia due to its notable psychoactive and neuroprotective effects. The medicinal potential of harmal is attributed to its bioactive alkaloids, primarily harmine, and harmaline, which belong to the β-carboline class of compounds.[5]

Harmine and harmaline exert their effects through multiple molecular mechanisms, including monoamine oxidase inhibition, which elevates neurotransmitter levels such as serotonin, dopamine, and norepinephrine in the central nervous system. This activity underpins harmal's antidepressant and anxiolytic properties. In addition, these alkaloids modulate GABAergic and glutamatergic pathways, contributing to their anticonvulsant effects. Recent studies have also highlighted their neuroprotective potential, which is realized by reducing oxidative stress and inflammation, as well as promoting neuronal regeneration.[6]

This perspective aims to explore the potential relationship between *P. harmala*, a plant with a rich history in traditional medicine, and the stimulation of GLP-1 secretion. Given the pharmacological significance of GLP-1 as a key regulator of glucose metabolism and its emerging role in metabolic and neurodegenerative disorders, identifying natural agents capable of enhancing GLP-1 secretion has garnered considerable scientific interest. *P. harmala*, known for its bioactive alkaloids, including harmine and harmaline, has demonstrated diverse metabolic and neuroprotective properties that may intersect with GLP-1 pathways. By examining existing evidence, this review seeks to provide a comprehensive overview of the molecular and biological mechanisms through which the herb's influence on GLP-1 secretion. In addition, the implications of this interaction in developing novel therapeutic strategies for diabetes, obesity, and related metabolic dysfunctions are highlighted. This synthesis aims to bridge the gap between traditional medicine and contemporary scientific research, paving the way for future investigations into the therapeutic potential of *P. harmala* in modulating GLP-1 activity.

2. Present *evidence on the effects of P. harmala on the brain*

Recent studies have shed light on the neurotherapeutic potential of *P. harmala*, particularly in enhancing GLP-1 levels in the brain and improving central insulin sensitivity. In a pre-clinical model of AD, *P. harmala* seed extract demonstrated significant efficacy in countering AD-related neurodegeneration, particularly within the hippocampus, a critical region for memory and cognition. The extract increased hippocampal GLP-1 and insulin levels while reducing insulin receptor substrate-1 phosphorylation at serine 307 (pS307-IRS-1), a key marker of insulin resistance. These findings highlight the ability of *P. harmala* to enhance insulin signaling through the activation of Akt phosphorylation at serine 473 (pS473-Akt) and upregulation of glucose transporter type 4 (GLUT4).

In addition to modulating insulin pathways, *P. harmala* reduced the accumulation of pathological markers associated with AD, including beta-amyloid (Aβ42), phosphorylated tau, and glycogen synthase kinase-3β (GSK-3β). These effects were further augmented by the activation of the nuclear factor erythroid 2-related factor 2 (Nrf2) antioxidant pathway, leading to reduced oxidative stress and replenishment of hippocampal glutathione levels. Collectively, these molecular mechanisms underline the dual role of *P. harmala* in mitigating insulin resistance and enhancing GLP-1 signaling, which synergistically improves neuronal glucose uptake and reduces the burden of amyloid pathology in the brain.[7]

Harmine and harmaline, the primary bioactive alkaloids in *P. harmala*, modulate key signaling cascades involved

in neuronal glucose metabolism and insulin sensitivity.[5,7] Both compounds influence the PI3K/Akt pathway, a critical mediator of cellular survival, growth, and glucose uptake. By enhancing the phosphorylation of Akt at serine 473 (pS473-Akt), harmine and harmaline promote the downstream activation of GLUT4, a transmembrane protein essential for neuronal glucose uptake.[8]

This process begins with the binding of insulin or GLP-1 to their respective receptors, leading to the recruitment and activation of PI3K. PI3K catalyzes the conversion of phosphatidylinositol 4,5-bisphosphate (PIP2) to phosphatidylinositol 3,4,5-trisphosphate (PIP3), which acts as a docking site for Akt. Harmine and harmaline potentiate this signaling cascade, ensuring sustained activation of Akt.[9] The activated Akt phosphorylates downstream targets, including AS160, which facilitates the translocation of GLUT4 vesicles to the neuronal plasma membrane, enhancing glucose uptake into neurons.[10]

Harmine and harmaline exert potent antioxidant effects through the activation of the Nrf2 pathway, a master regulator of cellular redox homeostasis.[5,11] Oxidative stress, caused by an imbalance between reactive oxygen species (ROS) production and antioxidant defenses, is a major contributor to neuronal damage and neurodegeneration.[12] Both harmine and harmaline enhance the nuclear translocation of Nrf2, which is normally sequestered in the cytoplasm by its inhibitor, Kelch-like ECH-associated protein 1 (Keap1).[5,12]

Upon activation, Nrf2 dissociates from Keap1 and translocates to the nucleus, where it binds to antioxidant response elements in the promoter regions of target genes.[13] This interaction leads to the upregulation of antioxidant enzymes such as glutathione peroxidase, superoxide dismutase, and catalase. By boosting the production of glutathione and neutralizing ROS, harmine and harmaline reduce oxidative damage to lipids, proteins, and DNA, thereby protecting neurons from oxidative stress-induced apoptosis.[5,14]

An additional layer of molecular interaction exists between the Akt and Nrf2 pathways, as harmine and harmaline enhance Akt-mediated phosphorylation of GSK-3β, an inhibitor of Nrf2. This phosphorylation inactivates GSK-3β, preventing it from targeting Nrf2 for degradation. As a result, harmine and harmaline indirectly amplify Nrf2 activity, creating a synergistic effect that strengthens antioxidant defenses while improving glucose metabolism.[5,15]

Through the coordinated regulation of the Akt/GLUT4 and Nrf2 pathways, harmine and harmaline exhibit a dual mechanism of action. They not only optimize neuronal glucose uptake and energy utilization but also alleviate oxidative stress, which is central to the pathophysiology of neurodegenerative diseases. These mechanisms position *P. harmala* as a potential therapeutic agent for disorders characterized by impaired glucose metabolism and elevated oxidative stress, such as AD and other forms of cognitive decline.[5,7,12,14,15]

3. The systemic importance of GLP-1: Physiological roles and mechanisms

GLP-1 is a multifunctional hormone that plays a central role in maintaining glucose homeostasis and regulating metabolic processes at the systemic level. Synthesized and secreted primarily by enteroendocrine L-cells of the small intestine in response to nutrient ingestion, GLP-1 exerts its effects through the GLP-1R, which is widely expressed in pancreatic islets, the brain, and peripheral tissues.[16]

GLP-1 is a critical regulator of blood glucose levels through its glucose-dependent actions on pancreatic β-cells. By binding to GLP-1R, it activates the cAMP/PKA and phospholipase C signaling pathways, leading to enhanced calcium influx and insulin granule exocytosis. This mechanism ensures a precise, nutrient-driven increase in insulin secretion without causing hypoglycemia. Simultaneously, GLP-1 suppresses glucagon secretion from pancreatic α-cells, thereby reducing hepatic gluconeogenesis and further stabilizing blood glucose levels.[17]

GLP-1 significantly amplifies the insulinotropic response by increasing the sensitivity of β-cells to glucose. This effect is mediated by upregulating key transcription factors, such as pancreatic and duodenal homeobox 1, which enhances the transcription and translation of insulin.[18]

In addition, GLP-1 promotes β-cell proliferation and inhibits apoptosis, contributing to the long-term preservation of pancreatic function, particularly in conditions of metabolic stress such as type 2 diabetes mellitus (T2DM).[19] Beyond its effects on glucose metabolism, GLP-1 plays a pivotal role in the regulation of appetite and body weight. GLP-1R activation in the hypothalamus and brainstem modulates neuronal circuits involved in satiety and hunger. By activating pro-opiomelanocortin neurons and inhibiting neuropeptide Y and agouti-related peptide neurons, GLP-1 reduces food intake and increases feelings of fullness.[20] Furthermore, GLP-1 slows gastric emptying through vagal afferent signaling, prolonging the presence of nutrients in the gastrointestinal tract and enhancing satiety signals. These effects collectively contribute to reduced caloric intake and weight loss, making GLP-1 analogs valuable therapeutic agents for obesity and its associated metabolic dysfunctions.[21]

The physiological actions of GLP-1 extend far beyond glucose regulation. Its ability to enhance insulin secretion, suppress glucagon release, and regulate appetite underscores its systemic importance in maintaining energy balance and metabolic health. The therapeutic exploitation of these pathways through GLP-1R agonists (GLP-1RAs) has revolutionized the treatment of T2DM and obesity, while ongoing research continues to uncover its broader roles in cardiovascular and neurological health.[22]

4. Present therapeutic applications of GLP-1: Overview and challenges

GLP-1RAs have revolutionized the management of T2DM and obesity, offering significant benefits in glycemic control, weight management, and cardiovascular risk reduction. Agents, such as semaglutide, liraglutide, and dulaglutide mimic the physiological effects of endogenous GLP-1 by enhancing insulin secretion, suppressing glucagon release, and delaying gastric emptying, leading to improved blood glucose levels and weight loss. Semaglutide, in particular, has emerged as a model GLP-1RA due to its long-acting profile and once-weekly administration, which improve therapeutic efficacy and patient compliance.[23] Landmark trials such as SUSTAIN and STEP have demonstrated its efficacy in reducing Hemoglobin A1c, achieving substantial weight loss, and providing cardioprotective benefits by lowering the incidence of major adverse cardiovascular events (MACE) in high-risk populations. Despite these transformative outcomes, the clinical use of GLP-1RAs faces several challenges that hinder their widespread adoption.[24]

One major challenge is the high cost of GLP-1RAs, attributed to complex manufacturing processes and the substantial investments required for clinical development. This financial barrier disproportionately affects patients in low- and middle-income countries, limiting access to these life-changing therapies. In addition, gastrointestinal side effects, such as nausea, vomiting, and diarrhea, are common, particularly during the early phases of treatment, and may lead to discontinuation for some patients. Rare but severe adverse events, including pancreatitis and gallbladder disease, further necessitate careful patient selection and monitoring. The injectable nature of most GLP-1RAs, such as semaglutide, poses another limitation, as injection-related discomfort or needle phobia can deter treatment adherence. While oral formulations of semaglutide have been developed to address this issue, their slightly reduced bioavailability and efficacy compared to injectable forms present additional clinical considerations.[25,26]

To address these challenges, ongoing research aims to develop cost-effective manufacturing techniques, improved formulations to reduce side effects, and innovative delivery systems, such as transdermal patches or advanced oral technologies. Furthermore, the exploration of combination therapies that enhance the efficacy of GLP-1RAs while reducing required doses holds promise for mitigating adverse effects and lowering costs, thereby improving accessibility.

5. Potential development of *P. harmala* extract to stimulate GLP-1 systemically

The therapeutic potential of *P. harmala* in enhancing GLP-1 levels has been primarily explored in the context of brain research, particularly its effects on the hippocampus.[7] Translating these findings to systemic applications presents a promising avenue for metabolic disease management, such as T2DM and obesity. Studies have demonstrated that the bioactive alkaloids harmine and harmaline in *P. harmala* play pivotal roles in modulating molecular mechanisms critical for GLP-1 stimulation.[5,7] These mechanisms include the activation of the Akt/GLUT4 pathway, which enhances glucose uptake by promoting Akt phosphorylation at serine 473 and increasing GLUT4 translocation. Furthermore, *P. harmala* significantly reduces oxidative stress by activating Nrf2, a master regulator of antioxidant responses, thereby increasing glutathione levels and reducing lipid peroxidation. This antioxidant effect creates a conducive environment for GLP-1 synthesis and secretion. In addition, *P. harmala* exhibits potent anti-inflammatory effects by mitigating GSK-3β activity and reducing beta-amyloid accumulation, thereby alleviating neuroinflammation and cellular stress.[7] These mechanisms, observed in the hippocampus, suggest that harmine and harmaline could similarly stimulate GLP-1 secretion in enteroendocrine L-cells in the gut, the primary site of GLP-1 synthesis. By activating glucose-sensing pathways and protecting L-cells from oxidative and inflammatory stress, *P. harmala* could enhance GLP-1 production and stability systemically. Moreover, the plant's insulin-sensitizing effects, observed in brain studies, could extend to peripheral tissues, improving GLUT4 translocation and reducing insulin resistance.[7] However, challenges remain in translating these findings into clinical applications, including the need for standardized extracts to ensure consistent alkaloid content, elucidation of gut-specific pathways to confirm their therapeutic potential, and optimization of dosing to balance efficacy and safety given the potential toxicity of *P. harmala* at high doses. With antioxidant, anti-inflammatory, and GLP-1-enhancing properties, *P. harmala* holds significant promise for systemic metabolic regulation and the development of novel therapies targeting GLP-1 pathways.[5,7,27]

The development of *P. harmala* as an accessible, low-cost natural therapeutic holds significant potential for addressing metabolic disorders by targeting GLP-1 pathways. Unlike synthetic GLP-1RAs such as semaglutide, which are costly and require advanced manufacturing processes, *P. harmala* offers a plant-based alternative with bioactive alkaloids, such as harmine and harmaline that could modulate similar molecular mechanisms. Semaglutide functions by directly mimicking GLP-1, activating its receptor to promote insulin secretion, reduce glucagon release, and delay gastric emptying, thereby improving glycemic control and reducing appetite. However, its systemic distribution requires subcutaneous injection and advanced pharmacokinetic modifications to extend its half-life, increasing production complexity and cost.[28]

In contrast, *P. harmala* can indirectly enhance GLP-1 levels and activity by targeting upstream pathways. Studies demonstrate that harmine and harmaline stimulate GLP-1 secretion by modulating glucose-sensing mechanisms and reducing oxidative and inflammatory stress in cells. These compounds activate the Akt/GLUT4 pathway, which improves glucose uptake and metabolism, and enhances Nrf2-mediated antioxidant defenses, preserving cellular integrity and functionality. Furthermore, harmine and harmaline mitigate hyperinsulinemia by improving insulin sensitivity, reducing serine phosphorylation of IRS-1, and enhancing downstream signaling pathways.[7,29] These mechanisms make *P. harmala* a promising natural therapeutic for hyperinsulinemia and GLP-1 stimulation. To enhance the bioavailability and systemic distribution of *P. harmala* alkaloids, nanotechnology offers an innovative solution. Techniques such as encapsulating harmine and harmaline in biodegradable nanoparticles or liposomes could improve their solubility, protect them from enzymatic degradation in the gastrointestinal tract, and allow for controlled release. This approach could enable oral administration with enhanced absorption and targeted delivery to GLP-1-producing L-cells in the gut or pancreatic beta cells, mimicking the localized action of semaglutide.

Moreover, *P. harmala* could be developed as an oral supplement or capsule, with optimized doses of standardized extracts. Pre-clinical studies suggest a dose range of 150 – 200 mg/kg per day for achieving therapeutic effects in metabolic disorders.[30] By comparison, semaglutide typically requires weekly doses of 0.5 – 1 mg subcutaneously.[31] While semaglutide has a longer duration of action due to its synthetic modifications, *P. harmala* could benefit from nanotechnology to achieve sustained release and comparable efficacy with daily oral administration. The scalability and accessibility of *P. harmala* provide further advantages.

6. Comprehensive evaluation of *P. harmala* in GLP-1 modulation

While the theoretical mechanisms underlying *P. harmala*'s impact on GLP-1 secretion are well-explored, experimental validation remains crucial. Pre-clinical evidence suggests that harmine and harmaline interact with intracellular signaling pathways critical for GLP-1 synthesis and release. In particular, harmine has been shown to promote pancreatic β-cell proliferation by inhibiting dual-specificity tyrosine-regulated kinase 1A, a key regulator of cellular apoptosis.[32] Moreover, its influence on incretin-secreting enteroendocrine L-cells remains under investigation, emphasizing the need for targeted *in vivo* studies and controlled clinical trials. Despite promising pre-clinical insights, clinical trials assessing *P. harmala*'s efficacy in metabolic disorders are lacking. Future research must focus on conducting randomized controlled trials to determine its therapeutic potential in managing insulin resistance, obesity, and T2DM. Establishing standardized dosing regimens and long-term safety profiles will be essential for translating these findings into clinical practice.

Harmine and harmaline are believed to modulate GLP-1 secretion through ATP-sensitive K^+ (KATP) channels and calcium-mediated exocytosis.[7] These alkaloids likely enhance intracellular Ca^{2+} influx through L-type voltage-gated calcium channels, which, in turn, trigger vesicular GLP-1 release.[33] In addition, harmine's interaction with cAMP-response element-binding protein may potentiate *GLP-1* gene expression by upregulating proglucagon transcription, reinforcing its role in incretin hormone synthesis.[7] High doses of *P. harmala* have been associated with neurotoxic and hepatotoxic effects, largely attributed to excessive β-carboline alkaloid accumulation. *In vivo* toxicity studies reveal that harmine can induce mitochondrial dysfunction through oxidative stress-mediated cytochrome c release, necessitating careful dose optimization. Future studies should aim to delineate the therapeutic window for safe human consumption while implementing nanoparticle-based drug delivery systems to mitigate toxicity risks.[34]

Nanotechnology holds immense promise in improving the bioavailability of *P. harmala*-derived alkaloids. Liposomal and polymeric nanoparticle formulations can protect harmine and harmaline from enzymatic degradation, ensuring prolonged systemic circulation. Encapsulation techniques utilizing polyethylene glycol (PEG)-modified nanoparticles have demonstrated increased cellular uptake and sustained release, facilitating

targeted GLP-1 stimulation in L-cells and pancreatic β-cells.[35] Semaglutide, a synthetic GLP-1RA, directly mimics incretin activity, leading to robust glucose homeostasis regulation. However, its high cost and injectable administration limit widespread accessibility. On the other hand, *P. harmala* offers a plant-based, cost-effective alternative with broader metabolic benefits, including neuroprotection and antioxidative stress reduction. Nevertheless, its lower bioavailability and lack of regulatory approval necessitate further refinement before clinical adoption.

Recent discoveries highlight the interplay between GLP-1 and the gut microbiome, suggesting that modulating microbial composition may enhance endogenous incretin secretion. In addition, novel GLP-1R isoforms with tissue-specific functions are being investigated; presenting opportunities to refine targeted therapies.[36] Exploring *P. harmala*'s influence on these pathways could expand its therapeutic scope. The high manufacturing costs of GLP-1RAs pose accessibility challenges, particularly in low-income regions. *P. harmala*'s widespread availability and ease of cultivation position it as a scalable alternative.

Oral semaglutide formulations face significant bioavailability hurdles due to gastrointestinal degradation. Research on absorption enhancers such as sodium N-(8-[2-hydroxybenzoyl]amino) caprylate with the aim to improve uptake efficiency is currently underway.[37] Applying similar advancements to oral *P. harmala* formulations may enhance its pharmacokinetic properties, ensuring clinical viability. Beyond metabolic disorders, GLP-1 signaling is implicated in neurodegenerative and cardiovascular conditions.[38] Investigating *P. harmala*'s impact on AD, atherosclerosis, and gut-brain axis regulation could unveil novel therapeutic applications.[7] Furthermore, combinatory approaches involving *P. harmala* and other natural compounds warrant exploration for synergistic metabolic benefits. Notably, *P. harmala* has the potential to revolutionize various treatments by mimicking semaglutide's mechanism of action. This is particularly relevant given the emerging paradigm shift in utilizing semaglutide not only for T2DM but also as a promising therapy for type 1 diabetes, highlighting its broader metabolic and therapeutic implications.[39]

7. Discussion

The growing body of evidence suggests that *P. harmala*, through its bioactive alkaloids harmine and harmaline, holds significant potential in modulating key molecular pathways associated with glucose homeostasis and insulin sensitivity. Unlike synthetic GLP-1RAs such as semaglutide, which directly activate the GLP-1R, *P. harmala* appears to exert its effects by stimulating endogenous GLP-1 secretion and enhancing insulin signaling. Harmine and harmaline have been shown to influence GLP-1 synthesis by modulating cellular glucose-sensing mechanisms in enteroendocrine L-cells. Activation of the Akt/GLUT4 pathway plays a pivotal role in this process. By enhancing Akt phosphorylation at serine 473 (pS473-Akt), *P. harmala* facilitates the translocation of GLUT4 to the cell membrane, promoting glucose uptake and increasing intracellular ATP levels. This, in turn, triggers calcium-dependent exocytosis of GLP-1 granules, thereby enhancing the secretion of this incretin hormone. In addition, *P. harmala*'s effects extend beyond direct glucose sensing. The alkaloids modulate oxidative stress and inflammation, two factors that influence GLP-1 secretion. By activating Nrf2, harmine and harmaline upregulate antioxidant enzymes such as glutathione peroxidase and superoxide dismutase, reducing oxidative damage to L-cells and preserving their function. This antioxidant effect contributes to sustained GLP-1 production, making *P. harmala* a promising candidate for enhancing incretin-based therapies. One of the primary mechanisms by which *P. harmala* improves insulin sensitivity is through its interaction with the PI3K/Akt signaling pathway. Insulin resistance, a hallmark of T2DM, is often characterized by impaired Akt activation and GLUT4 translocation. Harmine and harmaline counteract this dysfunction by enhancing PI3K-mediated phosphorylation of Akt, leading to improved downstream insulin signaling, reducing IRS-1 serine phosphorylation (pS307-IRS-1), a key marker of insulin resistance, thereby enhancing insulin receptor sensitivity, and promoting GLUT4 vesicle mobilization, facilitating glucose uptake into peripheral tissues, particularly muscle and adipose cells.[40] The combined effects of these pathways position *P. harmala* as a potent insulin-sensitizing agent, capable of improving metabolic flexibility and reducing hyperinsulinemia. Beyond its role in insulin signaling, Akt activation indirectly enhances the Nrf2 antioxidant response, creating a synergistic effect that strengthens cellular defense mechanisms. Harmine and harmaline have been shown to inhibit GSK-3β, an upstream regulator that negatively modulates Nrf2 activity. By inactivating GSK-3β, *P. harmala* prevents the degradation of Nrf2, thereby amplifying its transcriptional activity and increasing the expression of cytoprotective genes. This interplay between Akt and Nrf2 pathways establishes *P. harmala* as a dual-action therapeutic, simultaneously improving insulin sensitivity and mitigating oxidative stress, both of which are crucial in the pathophysiology of diabetes and neurodegenerative disorders. Despite its promising pharmacological effects, the bioavailability of harmine and harmaline remains a critical challenge.

These alkaloids exhibit poor water solubility and rapid hepatic metabolism, limiting their systemic efficacy. Nanotechnology-based drug delivery systems offer a viable solution by encapsulating harmine and harmaline in liposomes, polymeric nanoparticles, or lipid-based carriers, improving their stability and controlled release, enhancing intestinal permeability, ensuring targeted delivery to enteroendocrine L-cells for GLP-1 stimulation and pancreatic β-cells for insulin secretion, and reducing systemic toxicity, a key consideration given the dose-dependent effects of *P. harmala* alkaloids. By leveraging nanotechnological advancements, the therapeutic potential of *P. harmala* can be optimized, allowing for oral administration with enhanced bioavailability and a more sustained pharmacokinetic profile. While pre-clinical data suggest that *P. harmal*a possesses significant metabolic benefits, several challenges must be addressed before its clinical application. One key issue is the standardization of alkaloid content, as variations in harmine and harmaline concentrations across plant extracts necessitate rigorous protocols to ensure consistent therapeutic effects. In addition, long-term safety and toxicity studies are crucial. Despite its historical use in traditional medicine, P. harmala exhibits dose-dependent neurotoxic and hepatotoxic risks, requiring comprehensive evaluations. Furthermore, comparative efficacy studies with GLP-1RAs are essential. Direct clinical trials comparing *P. harmal*a to semaglutide and other GLP-1 analogs are needed to establish its relative efficacy and pharmacodynamic properties. With ongoing advancements in phytochemical standardization, nanotechnology-based delivery systems, and metabolic disease modeling, *P. harmal*a represents a compelling natural candidate for the future of incretin-based and insulin-sensitizing therapies[41]

8. Conclusion

P. harmala presents a compelling natural alternative for enhancing GLP-1 secretion, improving insulin sensitivity, and mitigating oxidative stress, offering a novel therapeutic avenue for metabolic disorders. Across its bioactive alkaloids, particularly harmine and harmaline, *P. harmala* exerts multifaceted pharmacological effects, modulating key molecular pathways involved in glucose metabolism, pancreatic β-cell protection, and systemic insulin regulation. Furthermore, advancements in nanotechnology provide a promising strategy to overcome bioavailability limitations, ensuring its therapeutic efficacy. While the potential of *P. harmala* as a metabolic therapeutic is evident, further research is required to bridge the gap between pre-clinical findings and clinical application. Rigorous investigations, including randomized controlled trials, standardized dosing studies, and long-term safety assessments, are imperative to establish its clinical viability. Moreover, exploring its synergy with existing GLP-1RAs and its broader implications in neurodegenerative and cardiovascular diseases could unlock additional therapeutic opportunities.

By integrating traditional medicine with modern biomedical innovations, *P. harmala* holds the promise of transforming metabolic disorder management. Its affordability, accessibility, and natural origin make it a valuable candidate for global healthcare strategies, particularly in regions where conventional GLP-1RAs remain financially and logistically restrictive. Moving forward, interdisciplinary collaborations between pharmacologists, endocrinologists, and nanotechnology experts will be crucial in realizing the full therapeutic potential of *P. harmala*, paving the way for its integration into evidence-based clinical practice.

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ACCSCIENCE PUBLISHING

Innovative Medicines & Omics

ORIGINAL RESEARCH ARTICLE

Synthesis, spectroscopic characterization, density functional theory analysis, and molecular docking studies of diorganotin (IV) complexes with sterically congested ligands

Shama Chauhan[1], Harlal Singh[2], Venkatanarayana Pappula[3] and Rupa Madyal[1]*

[1]Department of Chemistry, National Defence Academy, Pune, Maharashtra, India

[2]Department of Chemistry, School of Liberal Arts and Sciences, Mody University of Science and Technology, Lakshmangarh, Rajasthan, India

[3]School of Sciences, Woxsen University, Hyderabad, Telangana, India

Abstract

Diorganotin (IV) complexes have attracted considerable attention due to their diverse structural features and promising biological properties. The investigation into diorganotin (IV) compounds as potential antimicrobial agents is an active and captivating area of research, particularly emphasizing the synthesis and characterization of diorganotin (IV) complexes with bioactive and sterically hindered ligands. In this study, novel diorganotin (IV) azomethine chelates were synthesized from sterically hindered 4-(2′-mercapto-phenyl-iminoaryl/alkyl)-2,4-dihydro-5-methyl-2-phenyl-3H-pyrazol-3-ones, characterized, and evaluated for their antimicrobial potential. These complexes were obtained by reacting dimethyltin dichloride with the corresponding disodium salts in benzene and characterized through infrared, ^{1}H, ^{13}C, and ^{119}Sn nuclear magnetic resonance spectroscopy, along with molecular weight determination. Structural optimization and electronic property analyses were performed using density functional theory (DFT) at the B3LYP/LanL2DZ level. Conceptual DFT descriptors indicated subtle variations in reactivity, with Chelate-4 exhibiting the highest softness and the lowest energy gap, suggesting enhanced electron-accepting capability. Molecular docking studies were conducted on the ligand moieties (L-1 to L-4) against proteins from Gram-positive and Gram-negative bacteria using cephalosporin and sulfamethoxazole as reference drugs. Ligand L-4 displayed superior binding affinities across all targets, aligning with its DFT-predicted reactivity. Absorption, distribution, metabolism, and excretion analysis revealed that while L-1 and L-2 showed favorable drug-likeness and oral bioavailability, L-4 demonstrated higher lipophilicity and possible metabolic concerns despite its potent antibacterial potential.

Keywords: Dimethyltin dichloride; Infrared; Nuclear magnetic resonance; Azomethines; ADME; Molecular docking; Density functional theory

***Corresponding author:**
Rupa Madyal
(chemasstprof2nda.ids@gov.in)

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1. Introduction

There has been an increasing interest in research in the field of organotin (IV) chemistry due to the ease with which modern physical techniques can be applied to organotin compounds, as well as their fascinating structural and toxicological properties.[1] Numerous studies have been conducted on the synthesis of organotin derivatives, which often include ligands such as alkyl, carboxylates, xanthates, Schiff bases, phenanthroline, and dipeptides.[2-18] Many of these derivatives have also been investigated for their antimicrobial, antitumor, and anti-inflammatory properties.[4,9-18] Organotin-phenanthroline complexes have demonstrated promising biological activities, including antimicrobial and anticancer properties, as the metal-nitrogen coordination enhances the stability and reactivity of the complex.

Medicinal chemistry and drug design have advanced significantly through the use of computational methods for the virtual screening of molecules as potential drug candidates, as they reduce both costs and time. Molecular docking and density functional theory (DFT) methods are efficient computational approaches widely used in the drug development process.[19,20] Molecular docking assesses the binding compatibility of molecules (ligands) with their target (receptor), while DFT calculations enable the evaluation of several electronic descriptors that characterize molecular reactivity.[21] These include measurements of electron distribution properties (electronegativity), resistance to electronic changes (chemical hardness), electron flow tendencies (chemical potential), and electron-accepting capacity (electrophilicity index). Numerous studies have employed two key computational approaches, namely DFT and molecular docking, to unveil potential biomedical applications of tin (Sn) complexes.[14-18] These significant applications of organic derivatives of Sn complexes have stimulated further studies in this field. However, neither experimental nor theoretical studies of pentacoordinated diorganotin (IV) azomethine chelates are available. Our work provides new experimental and theoretical data that could reveal unique structural features and reactivity profiles. These data provide a foundation for future studies on similar compounds.

In this paper, we report the preparation, spectral elucidation, structural aspects, molecular docking, and DFT study of several organotin complexes resulting from sterically overcrowded 4-(2'mercapto-phenyl-iminoaroyl/alkyl)-2,4-dihydro-5-methyl-2-phenyl-3H-pyrazol-3-ones. It is of interest to investigate the interaction of these sterically congested ligands to examine their microbicidal activity. Therefore, molecular docking was used to determine the binding affinity of these ligands with the bacterial protein. In addition, absorption, distribution, metabolism, and excretion (ADME) prediction analysis was performed for ligands L-1 to L-4 to further assess their drug-likeness potential.

2. Methodology

2.1. Experimental methodology

The reaction was carried out under anhydrous conditions, and the solvent was dried using standard methods. The molecular weight (MW) was determined osmotically on a vapor pressure osmometer (Model K-7000, Knauer, Germany) in chloroform ($CHCl_3$) solution at 45°C. Sulfur was estimated using the Messenger's method.[22] Dimethyltin dichloride and dibutyltin dichloride were distilled before use. The synthesized organotin (IV) chelates, resulting from Schiff bases, follow a similar procedure. Nuclear magnetic resonance (NMR) spectra (^1H and ^{13}C) were recorded on an NMR spectrometer (DELTA-2, JEOL, Japan), using tetramethylsilane (TMS) as the internal reference. With the help of potassium bromide (KBr) pellets, the infrared (IR) spectrum was recorded from 4,000 to 400 cm^{-1} using a spectrophotometer (SP-2, PerkinElmer, USA).

For the synthesis of 4-(2'-mercapto-phenyl-iminobenzoyl)-2,4-dihydro-5-methyl-2-phenyl-3H-pyrazol-3-onato dimethyltin (IV), sodium metal weighing 0.143 g (6.2 mmol) was dissolved in about 10.0 mL of methanol. A benzene solution of 4-(2'-mercapto-phenyl-iminobenzoyl)-2,4-dihydro-5-methyl-2-phenyl-3H-pyrazol-3-ones (5 mL, 0.62 M) was then added dropwise, followed by a 5-h reflux. A benzene solution of dimethyltin dichloride (5 mL, 0.62 M) was then added dropwise, and the reaction mixture was refluxed for about 5 h. The reaction mixture was then allowed to cool, and the resultant sodium chloride (NaCl) precipitate was filtered off. The precipitated NaCl was decanted, and the fraction of volatile matter was removed. White to dark brown-colored solid products were obtained. The chelated products were soluble in common organic solvents, such as benzene and chloroform, but insoluble in petroleum ether and n-hexane. The complex compounds were subjected to recrystallization from a chloroform/petroleum ether mixture. Osmometric MW analysis in chloroform solution at 45°C showed that the substances were monomeric. Further characterization of the complexes was carried out using spectral techniques (^1H, ^{13}C, ^{119}Sn NMR, and IR).

Similarly, the remaining three diorganotin (IV) complexes were synthesized through the reaction of 4-(2'-mercapto-phenyl-iminobenzoyl)-2,4-dihydro-5-methyl-2-phenyl-3H-pyrazol-3-one with dimethyltin dichloride in the presence of sodium methoxide. The formation of the desired chelates was confirmed based

on their physical properties, percentage yields, MW estimations, and elemental analyses.

2.2. Computational methods

The geometrical parameters of the ligands (L-1, L-2, L-3, and L-4) and their corresponding Sn complexes, referred to as chelates (Chelate-1, Chelate-2, Chelate-3, and Chelate-4), were optimized using the DFT/B3LYP method with the LanL2DZ basis set, performed through the Gaussian 03W software package (Revision 03, Gaussian, Inc., USA).[23] No initial symmetry constraints were applied during the optimization process. The normal frequencies for the optimized structures were calculated through Hessian analysis.

2.2.1. Molecular docking

Molecular docking simulations predicted ligand-protein interactions and binding energies for Sn complexes, investigating their antibacterial activity. Two protein targets, one from Gram-positive (*Staphylococcus aureus*) and one from Gram-negative (*Escherichia coli*) bacteria, were selected to represent different antibacterial mechanisms. DFT-optimized ligands (L-1 to L-4), along with control molecules (cephalosporin and sulfamethoxazole), were analyzed to provide a comparative assessment. Docking was performed using the standard operating procedure and default settings of AutoDock (Version 4.2.6, Scripps Research, USA), which employs an empirical free energy function. All water molecules were removed from the bacterial protein structures (protein database codes: 5TW8, 6NTW, 1AD4, and 5V7A) during preparation in AutoDock tools, and only polar hydrogens were added to the proteins. The different ligand conformers were generated using a Lamarckian genetic algorithm with an adaptive search method in AutoDock, and the interactions between the ligands and the target receptor were analyzed using Discovery Studio Visualizer (Discovery Studio® 24.1, BIOVIA, USA).

2.2.2. Ligands, control ligands, and protein preparation

DFT-optimized structures of ligands (chelates devoid of central metal ion, i.e., Ti^{2+} ion), named as L-1, L-2, L-3, and L-4, were further subjected to molecular simulation to inspect the antibacterial activity of ligands. The chemical structures of control molecules, cephalosporin (CID: 25058126), and sulfamethoxazole (CID: 5329), were retrieved from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/).

Bacterial targets, including dihydropteroate synthase (1AD4 and 5V7A)[24,25] and transpeptidase (5TW8 and 6NTW),[26,27] were chosen to represent well-known bacterial targets of antibiotics and are summarized in Table 1. These proteins are also relevant for exploring potential avenues for minimizing microbial resistance to antimicrobial agents.[28] Cephalosporins act as bacterial transpeptidase inhibitors,[29,30] while sulfamethoxazole targets the bacterial enzyme dihydropteroate synthase by acting as a competitive inhibitor.[31,32] Accordingly, molecular docking of cephalosporins was performed with target proteins 5TW8 and 6NTW, and sulfamethoxazole was docked with 1AD4 and 5V7A. The docking analysis utilized automated docking procedures with Gasteiger charge assignments applied to all molecular structures. A visualization software package enabled detailed examination of the interactions between the ligands and their protein targets. The docking region was defined by a cubic grid with dimensions of 60 units in each direction and a grid spacing of 0.375 Å, centered on the protein's active site (Table 1).[28]

3. Results and discussion

Dimethyltin dichloride reacts with the Schiff bases in its disodium salt form derived from substituted pyrazolones in an equal molar ratio in a solution of benzene (Figure 1). The formation of the desired chelates was confirmed through the analysis of physical properties, percentage yields, MW estimations, and elemental content. A summary of these data is presented in Table 2. Melting points of the complexes ranged from 108°C to 130°C, indicating moderately high thermal stability, which is characteristic of organotin (IV) complexes. Yields varied from 68% to 79%, with Chelate-4 showing the highest yield, suggesting efficient complexation under the adopted reaction conditions. The experimental values of Sn% and sulfur (S)% were in good agreement with the calculated values, further confirming the proposed compositions.

3.1. Spectroscopic studies

3.1.1. IR spectra

The IR spectra of the complexes, recorded using KBr pellets in the 4,000 – 400 cm^{-1} range, showed significant changes upon coordination. The broad band observed between 3,440 and 3,295 cm^{-1} in the free ligand, attributed

Table 1. Molecular docking targets of selective bacteria with their protein database ID and coordinates

Bacteria target	Gram	Protein database ID	Coordinate		
			X	Y	Z
Transpeptidase	Gram+ve	5TW8	21.390	−62.210	36.200
	Gram−ve	6NTW	21.480	−32.370	42.150
Dihydropteroate synthase	Gram+ve	1AD4	33.106	8.125	41.463
	Gram−ve	5V7A	−17.836	7.522	103.740

Table 2. Synthetic, physical, and elemental data of the diorganotin (IV) complexes, with calculated values given in parentheses

No.	Compound	Melting point (°C)	Yield (%)	Molecular weight (g/mol) analyzed (calculated)	Sodium chloride filtered (g) (calculated)	Elemental analysis (%) (calculated)	
						Tin (Sn)	Sulfur (S)
1	Chelate-1	110	78	470.00 (470.18)	0.19 (0.20)	25.18 (25.24)	6.75 (6.82)
2	Chelate-2	130	68	482.00 (484.18)	0.22 (0.24)	24.48 (24.51)	6.58 (6.62)
3	Chelate-3	108	72	530.00 (532.23)	0.35 (0.36)	22.38 (22.30)	5.95 (6.02)
4	Chelate-4	112	79	570.00 (566.67)	0.38 (0.39)	20.86 (20.95)	5.63 (5.66)

to –OH and > NH groups, disappeared in the spectra of the corresponding organotin (IV) chelates. In addition, the appearance of a new band in the 1,625 – 1,645 cm^{-1} range indicates the presence of the stretching frequency (ν) > C=N–group.[33] In addition, a band detected in the range of 660 – 625 cm^{-1} is assigned to ν S–oxygen (O)[13] (asymmetric ν). The presence of the Sn-S bond is supported by the appearance of the ν Sn-S[6,7] absorption band in the region 422 – 402 cm^{-1}. A weak absorption band of low intensity in the region 456 – 425 cm^{-1} is associated with Sn–N bonds.[2] The presence of these bands indicates the formation of Sn–O, Sn–S, and Sn–N bonds, supporting the bifunctional tridentate coordination mode of the Schiff bases in the organotin (IV) chelates.

3.1.2. ^{1}H NMR

The ^{1}H NMR spectra were recorded in $CDCl_3$/DMSO-d_6 using TMS as the internal standard. A summary of the chemical shifts observed is presented in Table 3. The broad signal at δ 4.96 ppm, attributed to >NH/–SH protons in the Schiff bases,[3] is absent in the spectra of the organotin (IV) chelates, indicating deprotonation of these functional groups. The signal for ring methyl protons is observed at δ 1.69 – 2.46 ppm as a singlet.

No significant shifts were detected in their position compared with their positions in parent ligands. In Chelate-1, the signal for the terminal proton appears at δ 2.42 ppm as a broad singlet. In contrast, for Chelate-2 and Chelate-4, the terminal protons resonate as a quartet at δ 2.78 – 2.86 ppm, corresponding to > CH_2 protons, and as a triplet at δ 1.05 – 1.11 ppm, attributed to –CH_3 protons. The methyl protons bonded to the Sn atom are observed as a singlet in the range of δ 0.83 – 1.45 ppm, while the butyl protons attached to the Sn atom exhibit a complex pattern between δ 0.61 and 2.48 ppm. The value of Sn-hydrogen (H) J coupling, ^{2}J(Sn-H) for Chelate-1 was found to be 100.59 Hz.[34] The proton signals from the phenyl ring (C_6H_5/C_6H_4), as well as those from the thiophenol ring, are merged, and the aromatic protons appear as a complex pattern in the range δ 6.38 – 8.33 ppm. Thus, ^{1}H NMR spectral studies also support the bifunctional tridentate nature of the ligand.

Table 3. The ^{1}H nuclear magnetic resonance spectral data of organotin (IV) complexes (in δ ppm)

Chelate	Ring methyl	Ring/terminal C_6H_5/C_6H_4Cl thiophenol ring	Terminal protons		Sn-R	^{2}J(Sn-H)
			>CH_2	–CH_3		
Chelate-1	2.42 bs	6.54 – 7.99 m	-	2.42 bs	0.83 bs	100.59 Hz
Chelate-2	2.45 s	6.90 – 8.28 m	2.86 q	1.05 t	1.05 bs	-
Chelate-3	1.88 s	6.84 – 8.24 m	-	-	1.08 s	-
Chelate-4	1.97 s	7.26 – 8.22 m	-	-	1.45 s	-

Abbreviations: bs: Broad singlet; q: Quartet; R: functional group; s: Singlet; t: Triplet.

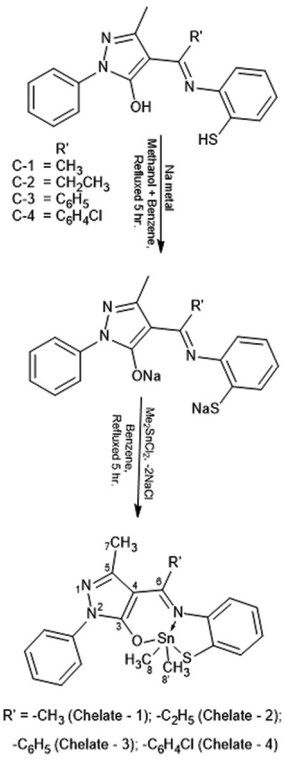

Figure 1. Synthetic pathway for the preparation of diorganotin (IV) chelates (Chelate-1 to Chelate-4)

3.1.3. ^{13}C NMR

The ^{13}C NMR spectra of the complexes were obtained in chloroform using TMS as the reference standard. The

chemical shift values are summarized in Table 4. Comparing the position of the C_6 carbon signal in organotin (IV) chelates with its position in the Schiff bases,[4] an up-field shift is observed. Further, a downfield shift is observed for the C_3 and C_4 carbon signals. The signal for the ring methyl carbon resonates in the range of δ 15.20 – 17.51 ppm. In Chelate-2 and Chelate-4, the terminal carbon signals are observed at δ 9.17 – 11.87 ppm for the $-CH_2$ group and δ 32.84 – 33.32 ppm for the $-CH_3$ group. The carbon atom of the methyl group bonded to the Sn atom shows a signal in the range of δ 7.87 – 8.87 ppm. The butyl carbon signals attached to the central Sn atom are observed at δ 13.52 – 29.30 ppm. The terminal C_6H_5/C_6H_4Cl, ring phenyl, and thiophenol ring carbon atoms are observed at δ 115.09 – 149.09 ppm. Therefore, ^{13}C NMR spectral evidence is also in agreement with IR and ^1H NMR spectral studies.

3.1.4. ^{119}Sn NMR spectra

The ^{119}Sn NMR spectra of these chelates were recorded in chloroform, with tetramethyltin used as an external reference. ^{119}Sn NMR spectroscopy is one of the specialized techniques[34] that provide necessary information on the structures of organotin compounds. Values of chemical shift observed in the case of phenyl and chlorophenyl substituents are δ −116.2 and −105.9, respectively, whereas for ethyl group is observed at δ −143.6.

Table 4. The ^{13}C nuclear magnetic resonance spectral data of organotin (IV) complexes (in δ ppm)

Parameter	Chelate 1	Chelate 2	Chelate 3	Chelate 4
C_3 (C=O)	161.68	160.5	162.2	162.09
C_4 (C=N)	104.56	103.85	104.4	104.29
C_5 (C=N–N)	138.22	138.8	138.58	138.1
C_6 (C=NR')	192.68	197.7	191.9	190.65
C_7 (CH_3 on pyrazolone)	17.34	15.2	16.1	16.31
$-CH_2$ (R' in chelate 2)	–	11.87	–	–
$-CH_3$ (R' in chelate 1 and 2)	27.82	33.32	–	–
$-C_6H_5$ (R' in chelate 3)	–	–	137.72	–
$-C_6H_4Cl$ (R' in chelate 4)	–	–	–	128.78
Ring phenyl (N1-Phenyl, C-ipso)	148.56	148.34	149.09	148.77
Ring phenyl (C-ortho)	128.8	128.83	128.89	128.94
Ring phenyl (C-meta)	125.43	126.89	126.12	126.39
Ring phenyl (C-para)	121	121.04	121.03	121.09
Thiophenol ring (C-ipso, C–S–Sn)	115.16	115.29	115.44	115.24
Thiophenol ring (C-para)	118.67	119.08	119.63	118.62
Thiophenol ring (C-ortho)	118.14	117.68	118.9	116.68
Thiophenol ring (C-meta)	121	121.04	120.73	120.88
Sn–R (aliphatic)	8.87	7.87	8.78	8.41

Abbreviation: R: Functional group.

The MW analysis conducted in chloroform solution at 45°C demonstrated that these compounds exist as monomers. Spectroscopic analysis revealed that the Schiff bases act as bifunctional tridentate ligands. Analysis of the ^{119}Sn NMR spectra indicated that the Sn atom exhibits pentacoordinate geometry.[35]

3.2. DFT calculations

Using DFT, we analyzed the comparative reactivity patterns of various Sn complexes. The analysis incorporated multiple reactivity descriptors, examining both global parameters (electrophilic and nucleophilic character) and their localized counterparts.

The study included calculations of fundamental electronic properties, such as the highest occupied electron orbital (HOMO) and lowest unoccupied molecular orbital (LUMO) energy levels. These molecular orbital energies were used to derive key chemical descriptors, including the molecule's ability to donate (ionization potential) and accept (electron affinity) electrons, chemical hardness and electronegativity, electrophilic behavior, and electronic chemical potential. These correlations provide insights into the molecular characteristics of the complexes.

3.2.1. Geometrical parameters

The DFT-optimized geometry of the Sn chelates is depicted in Figure 2, and the corresponding bond lengths and bond angles for the Sn complexes are listed in Table 5. In the Sn^{2+} complexes, Mulliken charge analysis was performed for Sn and the coordinating atoms (O, S, nitrogen [N]), as well as the two methyl groups. This analysis indicates that electron transfer from the ligands to Sn is consistent across all chelates, regardless of the substituent. Upon complexation, Sn experiences a loss of approximately 0.7e, resulting in a charge of +1.325 (down from +2). The coordinating atoms, O and N, possess similar Mulliken charges (−0.55 and −0.60, respectively), while S carries a slightly smaller charge (−0.2).

In the optimized structures, the bond distances between Sn and the heteroatoms O and N are 2.1 Å and 2.2 Å, respectively, whereas the Sn–S bond distance is longer, at 2.6 Å. The bond lengths for the two methyl groups covalently bonded to Sn (Sn–C8 and Sn–C8') are shorter, measuring 2.1 Å. Notable differences in coordinating bond angles are observed as a result of steric effects, particularly when substituting methyl groups with chlorophenyl rings. The bond angles between the two methyl groups attached to the Sn ion (C8'–Sn–C) fall within the range of 126° – 127°, while the ∠O–Sn–N angles range from 80° to 81°, and

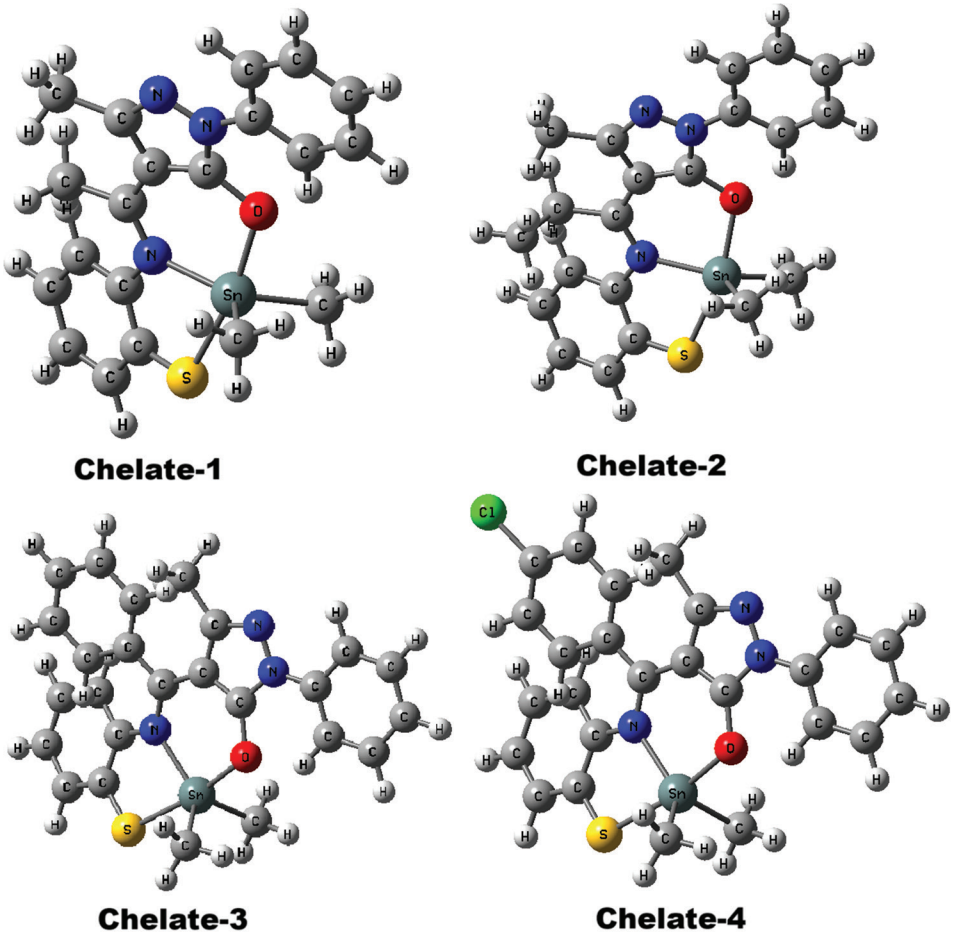

Figure 2. The optimized structure of Sn-complexes calculated using the B3LYP/LanL2DZ method

the ∠N–Sn–S angles are approximately 78°. In addition, a wider and more irregular range (96° – 120°) is observed for bond angles between Sn and the methyl carbon atoms (∠C–Sn–S and ∠C–Sn–C).

As the bulkiness of the substituent increases, a slight decrease in the ∠O–Sn–N angle is observed: from methyl to ethyl, the reduction is 0.36°, from ethyl to phenyl, 0.07°, and from phenyl to chlorophenyl, 0.14°. A similar trend is seen in the ∠N–Sn–S angle (0.27° → 0.20° → 0.12°). Overall, the DFT-optimized structures reveal no significant variations in Mulliken charges or bond lengths across chelates with different substituents, except for differences in bond angles. A separate table (Table 6) presents the computed electronic parameters, including the electron donation and acceptance tendencies (ionization potential and electron affinity), electronic distribution characteristics (electronegativity and chemical hardness), and reactivity indicators (electrophilicity index and chemical potential).

3.2.2. Global reactivity descriptors

Frontier orbital theory explains molecular behavior through two key electronic states: the HOMO and the first empty level above it (LUMO). The energy difference between these frontier orbitals serves as a valuable indicator of the molecule's stability and potential reaction pathways. This energetic separation helps predict how readily the molecule can participate in chemical transformations. A small HOMO-LUMO gap indicates that the molecule requires less energy to excite electrons, suggesting higher reactivity, while a large gap implies greater chemical stability.

The HOMO reflects a molecule's ability to donate electrons, corresponding to ionization potential, while the LUMO reflects its ability to accept electrons, corresponding to electron affinity. Ionization potential represents the energy needed to remove an electron from the molecule, with a high value indicating greater stability and inertness, while lower values suggest increased reactivity. Electron

affinity refers to the energy released when an electron is added to the molecule, and higher values imply a greater tendency to accept electrons.

According to calculations performed using the B3LYP/LanL2DZ method, the predicted HOMO and LUMO values for the Sn chelates are −0.20 eV and −0.08 eV, respectively. From these values, the HOMO-LUMO energy gap (ΔE) is calculated to be 0.12 eV, indicating the molecule's electronic properties. As observed in Figure 3, analysis of the frontier orbital distributions revealed distinct electronic patterns: the highest occupied state showed significant electron density around the S-containing portion of the molecule, whereas the lowest unoccupied state exhibited electron density spread across both the imidazole moiety and S region. We employed Koopman's theorem,[36] which applies to closed-shell systems, to compute global reactivity parameters.

Table 5. Selected molecular parameters and dipole moment (debye) of organotin (IV) complexes

Parameter	Chelate-1	Chelate-2	Chelate-3	Chelate-4
Dipole moment	3.010	2.856	3.440	2.129
Mulliken charge (e)				
Sn	1.326	1.325	1.325	1.325
O	−0.557	−0.555	−0.546	−0.546
N	−0.568	−0.590	−0.584	−0.581
S	−0.192	−0.198	−0.196	−0.193
Bond angle (°)				
O–Sn–N	81.390	81.034	80.969	80.833
N–Sn–S	78.248	77.973	77.772	77.656
S–Sn–C25	120.754	95.626	100.919	101.067
S–Sn–C26	101.030	101.445	96.515	96.654
C25–Sn–C26	126.164	125.688	126.612	126.499
Bond distance (Å)				
O–Sn	2.134	2.135	2.148	2.148
N–Sn	2.185	2.177	2.177	2.180
S–Sn	2.611	2.617	2.612	2.611
Sn–C26	2.118	2.118	2.118	2.117
C25–Sn	2.128	2.128	2.127	2.127

Our findings from these calculations were quantitatively documented in Table 5. The ionization potential of the complexes is 0.20 eV, and the electron affinity (A) is in the range of 0.076 – 0.089 eV. The hardness of the complexes is calculated to be approximately 0.06 eV across all chelates. However, the softness values for the methyl (Chelate-1) and ethyl (Chelate-2) substituents are around 7.8, while for phenyl (Chelate-3) and chlorophenyl (Chelate-4) substituents, they are slightly higher, at 8.4 and 8.6, respectively, indicating increased reactivity in the phenyl-substituted compounds. The electrophilicity index of the complexes is calculated to be 0.001 eV, which is relevant for describing their biological activity. The chemical potential of the complexes is −0.14 eV, a negative value, suggesting that these complexes are chemically stable.

3.3. Docking analysis

We performed molecular docking simulations on the above DFT-optimized structures. The docking analysis utilized cephalosporin and sulfamethoxazole as reference ligands for transpeptidase and dihydropteroate synthase, respectively, to benchmark the binding affinities of the ligands (L-1 to L-4) against selected bacterial target proteins (5TW8, 6NTW, 1AD4, and 5V7A), as shown in Table 7. Molecular docking was conducted using only the ligand moieties of the organotin (IV) complexes, with the

Table 6. Global reactivity descriptors and energies of organotin (IV) complexes on the B3LYP/LanL2DZ basis set

Molecular properties	Expression	Chelate-1	Chelate-2	Chelate-3	Chelate-4
E_{HOMO}, ev	Energy of H_{OMO}	−0.204	−0.203	−0.202	−0.207
E_{LUMO}, ev	Energy of L_{UMO}	−0.076	−0.077	−0.083	−0.090
E_{Gap}, ev	$E_{HOMO}-E_{LUMO}$	0.128	0.127	0.119	0.117
Ionization potential, ev	$-E_{HOMO}$	0.204	0.203	0.202	0.207
Electron affinity, ev	$-E_{LUMO}$	0.076	0.077	0.083	0.090
Chemical hardness (η), ev	$1/2\ (E_{LUMO}-E_{HOMO})$	0.064	0.063	0.059	0.058
Softness, S	$1/2\eta$	7.829	7.883	8.408	8.559
Chemical potential (μ), ev	$1/2\ (E_{LUMO}+E_{HOMO})$	−0.140	−0.140	−0.143	−0.148
Electronegativity (χ), ev	$-1/2\ (E_{LUMO}+E_{HOMO})$	0.140	0.140	0.143	0.148
Electrophilicity index (ω), ev	$\mu^2/2\eta$	0.001	0.001	0.001	0.001
Optimized energy, au	E	−1,027.7	−1,066.9	−1,219.4	−1,233.7

Abbreviations: HOMO: Highest occupied electron orbital; LUMO: Lowest unoccupied molecular orbital.

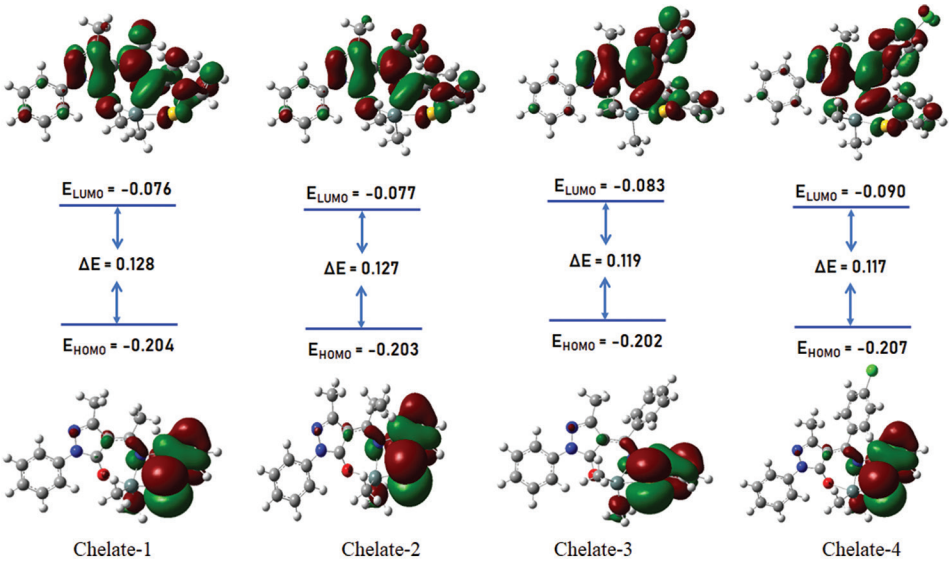

Figure 3. Highest occupied electron orbital plot of Sn-complexes performed by DFT/B3LYP level with LanL2DZ basis set

Sn center deliberately excluded. This decision was made to minimize computational expense and simplify the docking simulations, as the inclusion of a heavy metal such as Sn significantly increases system complexity due to its relativistic effects and lack of consistent parameterization in many docking software packages. Since the Sn atom remains constant across all complexes studied and the primary variations in binding affinities are attributed to the structural differences in the organic ligand frameworks, the docking results are expected to reflect the influence of ligand architecture on biological target affinity, independent of the Sn center.

Figure 4 depicts key residue interactions of target proteins (5TW8, 6NTW, 1AD4, and 5V7A), with test ligands (L-1, L-2, L-3, and L-4). We also considered key residue interactions of Gram-positive (5TW8, 6NTW) and Gram-negative (1AD4, 5V7A) strains with reference ligands for comparison of their corresponding binding abilities. The docking results are categorized into two distinct sections. The first section evaluates the antibacterial activity of the test ligands along with the cephalosporin reference ligand against transpeptidase enzymes. Binding values presented in Table 7 indicate that the binding order for 5TW8 is L-4 > L-3 > L-1 > L-2 > cephalosporin. Among them, ligand L-4 demonstrates the most favorable binding energies, with −8.50 kcal/mol for 5TW8, which is significantly higher than that of the cephalosporin (−5.72 kcal/mol). In 5TW8, cephalosporin interacts with several key residues, including Ser139, Ser262, Arg186, Glu183, Ser75, Ser116, Lys78, and Ala074, highlighting a diverse range of polar and charged contacts. The test ligands also share several of these crucial interactions, notably with Ser262, Ser075, and Ala074 residues. L-1 and L-2 maintain similar interaction profiles, while L-3 and L-4 form additional contacts with Phe241 and Ala182 residues, which likely contribute to their enhanced binding affinity.

In case of 6NTW, the order is L-4 > cephalosporin > L-3 > L-2 > L-1. Among them, ligand L-4 demonstrates the most favorable binding energies, with −9.48 kcal/mol for 6NTW, which is significantly higher than that of the cephalosporin (−7.53 kcal/mol). Here, cephalosporin interacts with a broad set of residues, including Pro501, Ser502, Met500, Glu504, Lys497, Asn426, Tyr507, Ala505, Pro428, and Trp425. L-1 shares four common binding residues with cephalosporin and additionally interacts with Ser526 and Cys528. L-2 displays a similar interaction profile but also engages with Ile506, His509, and Leu431. L-3, which nearly matches cephalosporin in binding strength, interacts with His509, Cys528, Ala505, Ser526, and Leu431, indicating that these residues may play a critical role in stabilizing the ligand-protein complex. L-4 establishes interactions with multiple critical residues, including His509, Tyr507, Ala505, Pro428, Leu431, Cys528, and Trp425, offering both polar and hydrophobic contacts that contribute to stable binding. Notably, specific residues such as Ser262, Ala074, and Ser075 in 5TW8, along with Tyr507, Ala505, Cys528, and Trp425 in 6NTW, are consistently involved in the binding of both cephalosporin and the test ligands, indicating conserved hotspots within the binding pockets of the transpeptidase enzyme.

The second section deals with the interactions of the test ligands along with the sulfamethoxazole reference ligand against dihydropteroate synthase enzymes. Table 7 indicates

Table 7. Comparative binding energies and key residue interactions of test ligands (L-1 to L-4) and control drugs (cephalosporin and sulfamethoxazole) with bacterial target proteins: transpeptidase (5TW8, 6NTW) and dihydropteroate synthase (1AD4, 5V7A)

Ligands	Target protein	Binding energy	Active amino acid and hydrogen bond interaction
Cephalosporin	5TW8	−5.72	Ser 139, Ser 262, Arg 186, Glu 183, Ser 075, Ser 116, Lys 078, Ala 074
L-1	5TW8	−6.77	Ser 262, Ala 074, Ala 182, Ser 075, Ser 262
L-2	5TW8	−6.66	Leu 115, Ala 074, Ser 075, Ser 262
L-3	5TW8	−7.27	Phe 241, Ser 262, Ser 260, Ser 075, Ala 074, Ala 182
L-4	5TW8	−8.50	Ser 075, Ser 139, Ala 074, Ala 182, Phe 241
Cephalosporin	6NTW	−7.53	Pro 501, Ser 502, Met 500, Glu 504, Lys 497, Asn 426, Tyr 507, Ala 505, Pro 428, Trp 425
L-1	6NTW	−6.78	Ser 526, Cys 528, Tyr 507, Pro 428, Trp 425, Ala 505
L-2	6NTW	−6.88	Pro 428, Trp 425, Ile 506, Cys 528, His 509, Leu 431, Ser 526
L-3	6NTW	−7.50	His 509, Cys 528, Ala 505, Ser 526, Leu 431
L-4	6NTW	−9.48	His 509, Tyr 507, Ala 505, Pro 428, Leu 431, Cys 528, Trp 425
Sulfamethoxazole	1AD4	−6.20	Ala 199, Asn 103, Met 128, Asp 84, Arg 239, Phe 172, Asp 167
L-1	1AD4	−6.98	Lys 203, Ala 199 Arg 239, Phe 172, Met 128, Asp 84, Ser 201, His 241, Asn 103
L-2	1AD4	−6.16	Lys 203, Arg 239, Phe 172, Asn 103, Asp 084, Ala 199, Met 128
L-3	1AD4	−6.89	Lys 203, Arg 239, Asp 084, Asn 103, Ser 201, Ala 199, Phe 172, Met 128
L-4	1AD4	−9.00	Phe 172, Asp 167, Met 128, Arg 239, Lys 203, His 055, Ile 009, Val 049
Sulfamethoxazole	5V7A	−6.43	Asn 022, Arg 063, Lys 221, Thr 062, Arg 255, Met 139, Phe 190, Ser 061
L-1	5V7A	−6.67	Asp 096, Lys 221, His 257, Ile 020, Thr 062, Phe 190
L-2	5V7A	−6.79	Arg 063, Pro 064, His 257, Phe 190, Thr 062, Ser 061, Glu 060
L-3	5V7A	−7.48	Arg 063, Thr 062, Phe 190, Lys 221, Arg 255, His 257, Ser 219, Asp 096
L-4	5V7A	−9.10	Glu 060, Ser 061, Met 148, Phe 190, Gly 189, Arg 220, His 257, Ile 020, Thr 062, Arg 063, Arg 255

(Cont'd...)

Table 7. ***(Continued)***

that the order of binding affinity of the ligands against the 1AD4 protein is L-4 > L-1 > L-3 > sulfamethoxazole >L-2. The standard drug sulfamethoxazole showed interactions with the Ala199, Asn103, Met128, Asp84, Arg239, Phe172, and Asp167 residues of 1AD4 protein. L-1 retains most of these interactions and further establishes additional contacts with Lys203, Ser201, and His241, which likely contribute to its enhanced stability within the active site. Similarly, L-3 shares all major binding residues of sulfamethoxazole and also forms supplementary interactions with Lys203 and Ser201. In contrast, L-2, despite sharing several binding residues with the control, shows no significant gain in binding strength. L-4 exhibits a significantly stronger binding affinity (−9.00 kcal/mol) compared to the reference drug (−6.20 kcal/mol). Both the L-4 and sulfamethoxazole share common interactions with Phe172, Asp167, Met128, and Arg239. In addition to these, L-4 establishes interactions with Lys203, His055, Ile009, and Val049, expanding its binding network and likely contributing to its enhanced stability and inhibitory potential.

In the case of the 5V7A protein, the binding affinity follows the order: L-4 > L-3 > L-2 > L-1 > sulfamethoxazole. Sulfamethoxazole forms interactions with several key residues of the 5V7A protein, including Asn022, Arg063, Lys221, Thr062, Arg255, Met139, Phe190, and Ser061, indicating its engagement with both polar and hydrophobic sites. L-1 retains interactions with Lys221, Thr062, and Phe190, which are also involved in sulfamethoxazole binding, and introduces additional contacts with Asp096, His257, and Ile020. L-2 also shares common residues, such as Arg063, Thr062, Phe190, and Ser061, while forming new interactions with Pro064, His257, and Glu060, contributing to a more diverse binding profile. Notably, both L-3 and L-4 exhibit a shared interaction network that significantly overlaps with that of sulfamethoxazole, particularly at key residues such as Arg063, Thr062, Phe190, and Arg255. In addition to these common interactions, L-3 establishes further contacts with His257, Ser219, and Asp096, enhancing its binding profile. Meanwhile, L-4 engages with several new residues,

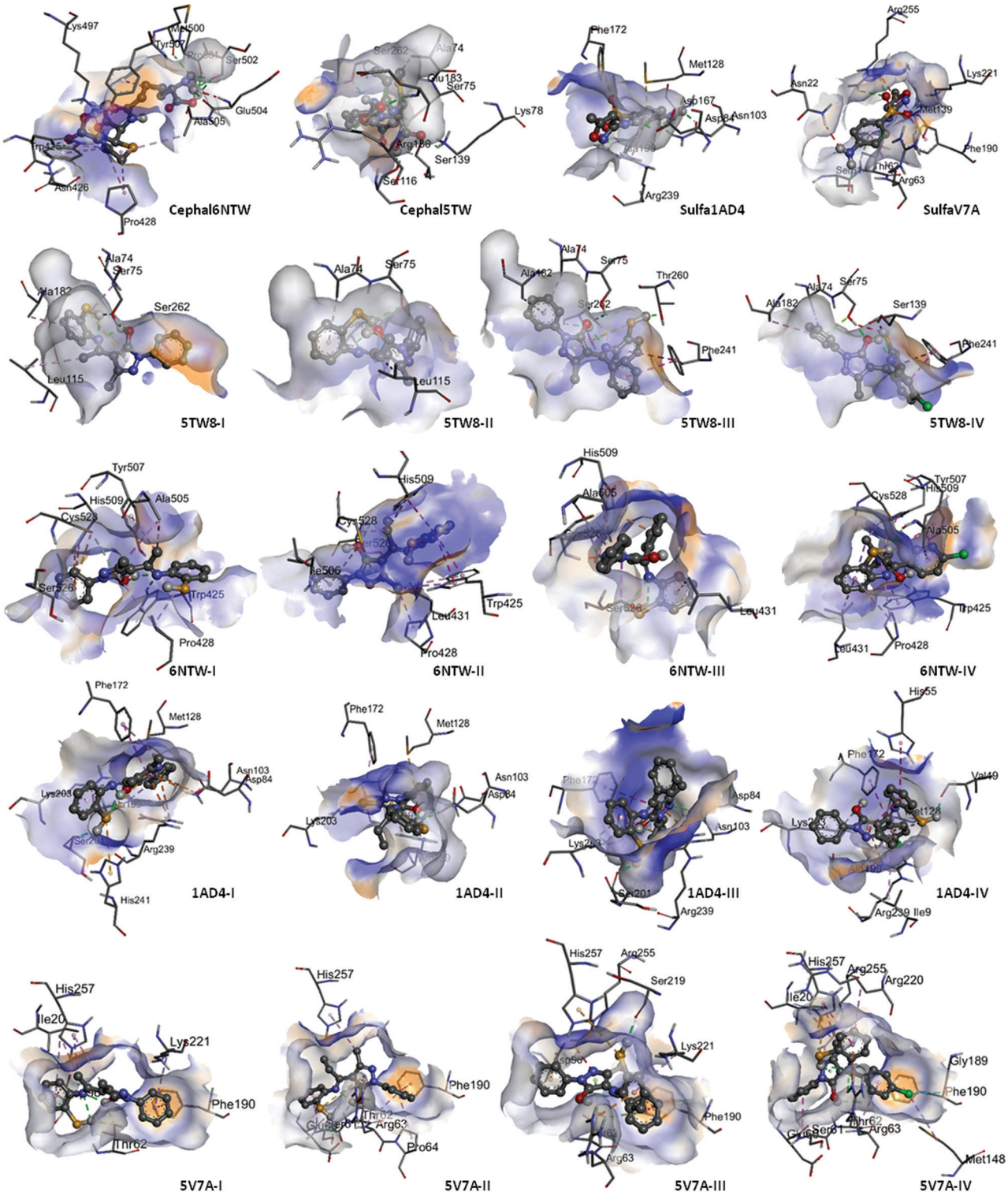

Figure 4. 3D representations of ligand binding within the active sites of transpeptidase (protein database IDs: 5TW8 and 6NTW) and dihydropteroate synthase (protein database IDs: 1AD4 and 5V7A). Ligands are depicted in ball-and-stick style with element-specific colors, while interacting protein residues are shown as sticks colored by residue type.
Notes: Ligands I-IV correspond to ligands L1-L4, respectively. "Cephal" refers to cephalosporin, and "Sulfa" refers to sulfamethoxazole.

including Glu060, Met148, Gly189, Arg220, His257, and Ile020, thereby considerably expanding its interaction network. These additional contacts are likely to strengthen binding affinity through a combination of hydrogen bonding, electrostatic interactions, and hydrophobic effects. Overall, these findings suggest that ligand L-4, followed by L-3, holds significant potential as a promising antibacterial agent targeting dihydropteroate synthase and transpeptidase enzymes.

3.4. Prediction of drug-likeness of ligands by the SwissADME tool

The SwissADME tool (https://www.swissadme.ch/) was employed to evaluate the *in silico* pharmacokinetic properties of ligands L-1 to L-4, with each molecule converted into its canonical Simplified Molecular Input Line Entry System format. The drug-likeness of these ligands, referring to their potential suitability as orally active drugs, was assessed using Lipinski's rule of five, a widely accepted guideline for predicting oral bioavailability based on key physicochemical properties.[37] The physicochemical properties relevant to ADME profiling include several key parameters (Table 8). An ideal drug candidate typically has a MW of 500 g/mol or less. The log *p*-value, representing lipophilicity, should not exceed 5, as higher values may lead to poor aqueous solubility. The topological polar surface area (TPSA) should be 140 Å^2 or less for adequate absorption, with values below 90 Å^2 being particularly favorable for blood-brain barrier (BBB) penetration. Furthermore, compounds should possess no more than five hydrogen bond donors and 10 hydrogen bond acceptors to ensure good membrane permeability. The number of rotatable bonds should ideally be 10 or fewer, which contributes to conformational flexibility and oral bioavailability. Finally, zero to one violation of Lipinski's rules is generally acceptable for a compound to be considered drug-like.

As shown in Table 8, the four ligands exhibit distinct drug-likeness profiles, determined by their compliance with Lipinski's rule of five and core physicochemical properties. The first ligand, L-1, demonstrates excellent drug-like properties, with a MW of 325.43, a log P of 4.3, a TPSA of 88.88 Å^2, four rotatable bonds, and two hydrogen bond donors and acceptors, resulting in zero Lipinski violations. The L-2 also complies fully with Lipinski's criteria, showing a slightly higher log P of 4.83 and MW of 339.45, while remaining within acceptable limits for oral bioavailability. In contrast, L-3 exceeds the recommended log P threshold, with a value of 5.59, resulting in one Lipinski violation. While other parameters, such as MW of 387.50 g/mol, TPSA of 88.88 Å^2, and hydrogen bonding characteristics, are within desirable ranges, the elevated lipophilicity may negatively affect its aqueous solubility and absorption. The fourth ligand, L-4, has a MW of 421.94 g/mol and a high log P of 6.22, leading to one Lipinski violation as well. The inclusion of a chlorine atom likely contributes to this increased lipophilicity, which may impair its solubility and pharmacokinetic profile despite acceptable TPSA and

Table 8. Physicochemical, pharmacokinetic, and drug-likeness properties of ligands (L-1 to L-4)

Ligand	L-1	L-2	L-3	L-4
Formula	$C_{18}H_{19}N_3OS$	$C_{19}H_{21}N_3OS$	$C_{23}H_{21}N_3OS$	$C_{23}H_{20}ClN_3OS$
Molecular weight (g/mol)	325.43	339.45	387.5	421.94
Log *p*-value	4.3	4.83	5.59	6.22
Topological polar surface area (Å^2)	88.88	88.88	88.88	88.88
Rotatable bonds	4	5	5	5
Hydrogen bond donors/acceptors	2/2	2/2	2/2	2/2
Lipinski violations	0	0	1	1
Gastrointestinal absorption	High	High	High	High
Blood-brain barrier permeant	No	No	No	No
P-glycoprotein substrate	No	No	Yes	Yes
CYP1A2 inhibitor	Yes	Yes	No	No
CYP2C19 inhibitor	Yes	Yes	Yes	Yes
CYP2C9 inhibitor	Yes	Yes	Yes	Yes
CYP2D6 inhibitor	Yes	Yes	Yes	Yes
CYP3A4 inhibitor	Yes	Yes	Yes	Yes
Log K*p* skin permeation (cm/s)	−5.23	−4.94	−4.69	−4.69

Abbreviation: CYP: Cytochrome P450 enzyme.

hydrogen bonding features. Overall, ligands 1 and 2 appear highly favorable for further development, while ligands 3 and 4, though structurally promising, may require additional formulation or optimization efforts due to their higher lipophilicity.

In drug discovery, several ADME-related parameters are crucial for assessing a compound's pharmacokinetic profile. Gastrointestinal absorption reflects the ability of a drug to be absorbed through the digestive tract, which is crucial in achieving effective oral bioavailability. BBB permeability determines whether a compound can cross into the central nervous system; while this is desirable for central nervous system (CNS)-targeted drugs, it may be avoided in non-CNS drugs to reduce neurological side effects. The status of a compound as a P-glycoprotein (P-gp) substrate influences its distribution and bioavailability, as P-gp actively transports substances out of cells, potentially lowering intracellular drug concentrations.

According to the *in-silico* ADME analysis, all four ligands presented in Table 8 exhibit high gastrointestinal absorption, implying good oral bioavailability potential. However, none of the ligands are predicted to be BBB permeant, suggesting limited potential for central nervous system activity, which may be advantageous in reducing neurological side effects for non-CNS targets. Notably, the L-1 and L-2 are not substrates of P-gp, implying lower chances of efflux-related bioavailability reduction. In contrast, L-3 and L-4 are identified as P-gp substrates, which may lead to reduced intracellular concentrations due to active efflux, particularly in the intestine or BBB regions.

The inhibition of cytochrome P450 (CYP) enzymes, such as CYP1A2, CYP2C19, CYP2C9, CYP2D6, and CYP3A4, can lead to significant drug–drug interactions (DDIs) by affecting the metabolism of co-administered therapies. Notably, CYP3A4 metabolizes a large proportion of clinically used drugs. All four ligands are predicted to inhibit multiple major cytochrome P450 enzymes, namely CYP2C19, CYP2C9, CYP2D6, and CYP3A4, while the first two ligands additionally show inhibition of CYP1A2 (Table 8). Such broad-spectrum inhibition suggests a high potential for DDIs, which could complicate co-administration with other medications metabolized by these enzymes. Skin permeability, measured as log K_p, estimates a compound's ability to penetrate the skin; higher negative values suggest lower transdermal absorption, which is important for evaluating the potential of dermal exposure or suitability for topical drug delivery. As shown in Table 8, the skin permeability values (log K_p) progressively increase from ligand L-1 to L-4 (from −5.23 to −4.46 cm/s), indicating a gradual increase in their potential to permeate the skin across the series. Despite this trend, all values remain within the range, indicating moderate to low skin permeability. Overall, while the compounds show strong oral absorption profiles, caution is warranted due to their CYP inhibition profiles and P-gp substrate status, particularly for L-3 and L-4. These factors may influence both efficacy and safety during drug development, requiring further ADME profiling and possible structural refinement to mitigate risks.

In several cases, increased steric hindrance may prevent hydrolysis or metabolic degradation, allowing the complex to reach the target intact and potentially increasing its selectivity or prolonging its activity.[38] Bulkier groups generally increase lipophilicity, which may enhance membrane permeability, potentially increasing bioactivity in lipophilic environments (e.g., tumor cell membranes). Steric hindrance around the Sn (IV) center often increases kinetic stability by physically blocking access to reactive sites. This can protect against ligand exchange, hydrolysis, or oxidation, which is especially important in physiological environments (aqueous, neutral pH).[39] Moreover, Schiff base-derived diorganotin (IV) complexes with bulky substituents (e.g., Ph_2SnL vs. Me_2SnL) exhibited a greater antimalarial and antioxidant activity.[40]

4. Conclusion

In the present work, sterically hindered 4-(2'mercapto-phenyl-iminoalkyl/aroyl)-2,4-dihydro-5-methyl-2-phenyl-3H-pyrazol-3-ones ligands were employed for the synthesis of organotin complexes under the specified reaction conditions. Molecular docking studies revealed the extensive interactions of ligands with key active-site residues of proteins. These findings suggest that L-4 possesses strong potential as a broad-spectrum antibacterial agent. In contrast, ligands L-1 and L-3 showed moderate binding affinities, with occasional performance comparable to the reference ligands, while L-2 consistently exhibited the weakest binding across all targets.

DFT calculations suggest that all four chelates display similar electronic properties with only slight variations. Among them, Chelate-4 is the most reactive, characterized by the smallest energy gap, highest electron affinity, and greatest softness, indicating enhanced electron-accepting ability and chemical reactivity. In contrast, Chelate-1 shows the greatest stability with the widest energy gap. These subtle electronic differences suggest that while the chelates share similar molecular frameworks, variations in their reactivity could influence their interactions with biological targets. Although both L-3 and L-4 possess

aromatic rings, L-4 exhibits slightly superior electronic characteristics across all molecular descriptors, such as a lower energy gap, higher softness, and greater electron affinity. These properties render L-4 more reactive and better equipped for strong and flexible interactions within the protein binding pocket, which is consistent with its enhanced binding affinities observed in docking studies.

From a drug-likeness and pharmacokinetic context, L-1 and L-2 emerged as the most promising candidates. Both fully adhered to Lipinski's rule of five, with MWs under 340 g/mol, optimal log *p*-values (4.30 and 4.83, respectively), suitable TPSA of <140 Å^2, and no Lipinski violations, suggesting good oral bioavailability. On the other hand, L-3 and L-4 each had one Lipinski violation due to higher log *p*-values (5.59 and 6.22, respectively), indicating increased lipophilicity, which may affect solubility and absorption. For L-4, the presence of a chlorine atom likely contributes to this elevated lipophilicity.

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Conflict of interest

The authors declare that they have no competing interests.

Author contributions

Conceptualization: Shama Chauhan, Rupa Madyal
Data curation: Harlal Singh, Venkatanarayana Pappula
Formal analysis: Rupa Madyal, Harlal Singh, Venkatanarayana Pappula
Investigation: Rupa Madyal, Shama Chauhan
Methodology: Rupa Madyal, Harlal Singh, Venkatanarayana Pappula
Supervision: Shama Chauhan, Rupa Madyal
Validation: Harlal Singh, Venkatanarayana Pappula
Writing–original draft: Shama Chauhan, Harlal Singh
Writing–review & editing: Rupa Madyal, Venkatanarayana Pappula

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Data will be made available on request.

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ACCSCIENCE PUBLISHING

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ORIGINAL RESEARCH ARTICLE

Evaluation of antimicrobial, analgesic, and hypoglycemic activities of *Commelina diffusa* (Commelinaceae)

Tasnia Islam Anika[1], Md. Monirul Islam[2], and Mohammad Shawkat Ali[1]*

[1]Department of Clinical Pharmacy & Pharmacology, University of Dhaka, Dhaka, Bangladesh

[2]Department of Pharmacy, Noakhali Science and Technology University, Noakhali, Bangladesh

Abstract

Commelina diffusa, also known as the climbing dayflower or spreading dayflower, is an herbaceous plant from the Commelinaceae family, found throughout tropical regions, including Bangladesh. The crude methanol extract and different fractions of *C. diffusa* were evaluated for their antimicrobial, analgesic, and hypoglycemic activities. The whole plant was extracted with methanol by the cold extraction method. The concentrated extract was then partitioned into petroleum ether- and chloroform-soluble fractions. The antimicrobial test was performed using the disc diffusion method. The analgesic effects were evaluated through both writhing and tail-flick tests at doses of 100 and 200 mg/kg body weight. The oral glucose tolerance test (OGTT) was performed to observe the hypoglycemic effect. The chloroform and methanol soluble fractions showed potent antimicrobial activity against Gram-positive bacteria and fungi. Statistical evaluation of the tail-flick test confirmed that the chloroform soluble fraction (100 mg/kg body weight) of *C. diffusa* had a significant amount of central analgesic activity ($p<0.001$). The petroleum ether soluble fraction showed significant central analgesic activity only at higher doses ($p<0.01$; 200 mg/kg body weight). The acetic acid-induced writhing test also confirmed the peripheral analgesic activity of the samples. The maximum inhibition was noted for the chloroform soluble fraction (64.56%), followed by crude methanolic extract (56.96%) and petroleum ether soluble fraction (53.16%). However, all the extracts showed no significant hypoglycemic activity in the OGTT. This observation, derived from an acute model of non-diabetic animals, does not preclude the possibility of antidiabetic effects in disease-related conditions. Further investigation is warranted to explore the specific metabolites and their pharmacological activities in relevant disease models.

Keywords: *Commelina diffusa;* Methanol extract; Antimicrobial activity; Hypoglycemic activity; Analgesic activity

***Corresponding author:**
Mohammad Shawkat Ali
(drshawkat@du.ac.bd)

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1. Introduction

Commelina diffusa (Family: Commelinaceae) is a herb traditionally used as a diuretic, blood coagulant, antidote for different poisons, heart tonic, anti-diarrheal, anti-inflammatory, and antipyretic across the world.[1] The Commelinaceae, or the spiderwort family, is one of the five families within the order Commelinales and comprises about

40 genera and 640 species.[2] *C. diffusa* is typically an annual herb with a wide range of medicinal applications.[3]

The increasing prevalence of antimicrobial resistance poses a significant global health threat, rendering many conventional antibiotics ineffective against resistant bacterial strains. Overuse and misuse of antibiotics in medicine and agriculture have accelerated the emergence of multidrug-resistant pathogens, leading to higher treatment failures, prolonged illnesses, and increased mortality rates.[4,5] The slow pace of new antibiotic development further exacerbates this crisis, necessitating the exploration of alternative therapeutic agents. Natural products, particularly medicinal plants, offer a promising source of novel antimicrobial compounds due to their diverse bioactive constituents, such as alkaloids, flavonoids, and terpenoids; these compounds often act through multiple mechanisms, reducing the likelihood of resistance.[6] In addition, plant-derived antimicrobials often exhibit synergistic effects, enhancing therapeutic efficacy while reducing side effects compared to synthetic drugs.[7,8]

Pain is an unpleasant sensory and emotional experience that can originate from various parts of the body and different causes. Usually, opioids and non-opioid drugs are used for pain relief. In most instances, these analgesic drugs can only relieve half of the pain in only 30% of patients. Furthermore, many of these drugs may cause serious side effects.[9] Medicinal plants may serve as a natural source of potent analgesic drugs with fewer side effects.[10] Diabetes mellitus is an endocrine disorder characterized by aberrant blood glucose levels. The complexity of its pathophysiology necessitates numerous approaches for effective management.[11] Traditional antidiabetic medications work well, but they also have inevitable adverse effects. Medicinal plants might serve as an alternative source of antidiabetic substances.[12]

Several species of the Commelinaceae family have demonstrated significant bioactive potential, including antimicrobial, anti-inflammatory, and antidiabetic properties.[13] Despite the extensive traditional use of *C. diffusa*, scientific validation of its pharmacological effects remains limited. Previous studies have reported the presence of flavonoids, alkaloids, tannins, and phenolic compounds in *C. diffusa*, all of which are associated with diverse biological activities.[14] This study aims to evaluate the antimicrobial, analgesic, and hypoglycemic activities of *C. diffusa* to provide scientific evidence supporting its ethnomedicinal uses. The findings could contribute to the development of alternative treatments for infections, pain management, and diabetes, addressing the need for safer and more accessible therapeutic options.

2. Materials and methods

2.1. Chemicals

All solvents used in this study, including methanol (Merck, Germany), petroleum ether (Merck, Germany), chloroform (Merck, Germany), and Tween-80 (Merck, Germany), were of analytical grade. Morphine (Morphine G) was purchased from Gonoghahsto Pharmaceuticals (Bangladesh), while diclofenac sodium and glibenclamide were gifts from a renowned pharmaceutical industry.

2.2. Preparation of plant extract and partitioning

The whole plant of *C. diffusa* was thoroughly cleaned, chopped into tiny pieces, and allowed to air dry for a few days. Following a 24-h oven drying, the fragments were ground into a coarse powder using a high-capacity grinding machine. Approximately 800 g of the powdered material was cold-extracted using methanol, yielding 65 g of crude extract. Solvent-solvent partitioning was subsequently carried out utilizing the Kupchan method as modified by Van Wagenen.[15] Five grams of the crude methanolic extract were dissolved in 10% aqueous methanol and successively extracted using petroleum ether and chloroform.

2.3. Antimicrobial activity

The antimicrobial activity was assessed by evaluating the ability of the test agents to stop the microbial growth surrounding the discs, resulting in distinct zones of inhibition.[16] Kanamycin (30 μg/disc; Bioprom, Greece) was used as the reference antibiotic.[17] After incubation, the diameter of the zones of inhibition was measured in mm using a scale and compared to the zones generated by the reference discs.

2.4. Experimental animal

Swiss albino mice of either sex, aged 4–5 weeks, were acquired from Jahangirnagar University. They were fed with water and rodent food formulated by ICDDR, B, and maintained in standard environmental conditions. The study was reviewed and approved by the Institutional Ethical Review Board (Reference number: DoP/RC/EC/2022/01/01).

2.5. Central analgesic activity

Using morphine as a positive control, the tail flicking method was used to assess the central analgesic activity.[18,19] In this method, thermal stimulus is applied to the mouse tail, and the response time until tail withdrawal is recorded as a measure of analgesic effect.[20] Forty mice were randomly assigned into eight groups ($n = 5$ per group). The groups received the following treatments: control (normal

saline, 10 mL/kg body weight, orally), standard (morphine, 2 mg/kg body weight, intraperitoneally), crude methanolic extract of *C. diffusa* (100 and 200 mg/kg), chloroform-soluble fraction (100 and 200 mg/kg), and petroleum ether-soluble fraction (100 and 200 mg/kg). At time zero, the test samples and control treatments were administered orally using a feeding needle. Tail-flick latency was recorded at 30 and 60 min post-administration for each mouse, and the average latency for each group was calculated.

2.6. Peripheral analgesic activity

The acetic acid-induced writhing method was used to assess the peripheral analgesic efficacy.[21] Pain was induced in the experimental animals via intraperitoneal injection of acetic acid, leading to writhing behavior, defined as contraction of the abdomen and elongation of the body. The analgesic effect of the test samples and the control was assessed by comparing their writhing inhibition relative to the standard drug. A total of 40 mice were randomly divided into `treatment and control groups as described in Section 2.5. However, the standard group received diclofenac sodium 10 mg/kg BW. At time zero, the mice received the respective treatments orally through a feeding needle. After 40 min, all animals were administered 1% acetic acid intraperitoneally. Five minutes post-injection, the number of writhes was recorded for each mouse over a 10-min period. The inhibition of writhing was calculated as a percentage using Equation I.

$$\%\,\text{Inhibition} = \frac{\begin{matrix}\text{Average}\\ \text{writhing of}\\ \text{control}\end{matrix} - \begin{matrix}\text{Average}\\ \text{writhing of}\\ \text{sample}\end{matrix}}{\begin{matrix}\text{Average}\\ \text{writhing of control}\end{matrix}} \times 100 \quad \text{(I)}$$

2.7. Oral glucose tolerance test (OGTT)

OGTT is one of the best ways to assess hypoglycemic activity. The test measures the rate at which glucose is removed from the circulation following administration.[22] A total of 25 experimental animals were chosen at random and split into five groups (n = 5 per group): Control, standard, methanol extract, petroleum ether-soluble fraction, and chloroform-soluble fraction. Each mouse was accurately weighed before treatment to ensure accurate dosage adjustment. The standard group received glibenclamide (5 mg/kg body weight),[23] the control group received 1% Tween-80 in normal saline, and the other three groups were given 200 mg/kg body weight of crude methanol extract, petroleum-ether soluble fraction, and chloroform soluble fraction of *C. diffusa*. All the doses were administered orally at zero hour. After 30 min, all animals were treated with a 10% glucose solution (2 mg/kg body weight; Eskayef Pharmaceuticals Ltd., Bangladesh). After 30, 90, and 150 min, blood was collected by pricking the tail vein, and the glucose level was measured using a glucometer (Get Well Limited, Bangladesh).[24]

2.8. Statistical analysis

Data were analyzed using one-way analysis of variance followed by Tukey's *post hoc* test. All statistical analyses were performed using GraphPad Prism version 8.0.2 (GraphPad Software, USA).

3. Results

3.1. Antimicrobial results

The methanol, petroleum ether, and chloroform-soluble fractions of *C.diffusa* exhibited low to moderate antimicrobial activity against most of the tested organisms (Table 1). At a concentration of 200 µg/disc, the zone of inhibition generated by each of these four extracts varied from 7.0 mm to 14.0 mm, while the standard disc of kanamycin (30 µg/disc) produced 17–27 mm zones. The highest antibacterial activity was noted for the chloroform extract against *Bacillus subtilis*.

These results suggest that the chloroform and methanol extracts of *C. diffusa* have antimicrobial activity, supporting its traditional medicinal use. Our result aligns with previous findings on the antimicrobial potential of this plant.[25] While the inhibitory effects were not as potent as standard antibiotics, the observed zones of inhibition suggest that the plant possesses bioactive compounds with

Table 1. Zone of inhibition (mm) exhibited by different fractions of *Commelina diffusa*

Test microbes	ME (200 µg/disc)	PEF (200 µg/disc)	CF (200 µg/disc)	Kanamycin (30 µg/disc)
Gram-positive bacteria				
Bacillus cereus	7	11	8	19
Bacillus subtilis	10	11	14	27
Staphylococcus aureus	8	10	9	22
Gram-negative bacteria				
Escherichia coli	7	9	9	19
Pseudomonas aeruginosa	8	8	9	17
Fungi				
Aspergillus niger	9	10	10	22
Candida albicans	-	10	9	17
Saccharomyces cerevisiae	9	8	9	25

Abbreviations: CF: Chloroform fraction; ME: Methanol extract; PEF: Petroleum ether fraction.

antimicrobial properties. However, the relatively lower efficacy compared to conventional antibiotics may be due to variations in extraction efficiency, the concentration of active compounds, or synergistic interactions among phytochemicals that were not fully optimized in this study.

3.2. Central analgesic activity

Tail flick latency was recorded at 30 and 60 min after sample administration, and the results are presented in Table 2.

The petroleum ether and chloroform-soluble fractions of *C. diffusa* significantly increased tail flick latency, indicating central analgesic activity. A consistent increase in latency was observed across the test groups, suggesting dose-dependent analgesic effects. These findings support the hypothesis that *C. diffusa* possesses central analgesic properties, in line with earlier studies on the plant.[26] The analgesic effect is likely due to the presence of bioactive constituents such as flavonoids, alkaloids, and terpenoids, which have been previously reported in *C. diffusa*.[14] The time-dependent response implies progressive absorption and distribution of active compounds, resulting in sustained analgesic effects. Further investigations are warranted to elucidate the exact mechanisms of action and therapeutic potential.

3.3. Peripheral analgesic activity

The peripheral analgesic activity of the various extracts was evaluated using the acetic acid-induced writhing test. Test samples were administered at doses of 100 and 200 mg/kg body weight. Five minutes after acetic acid administration, each mouse was observed for a 10-min period, during which the number of writhes was recorded. The results are shown in Figure 1.

Table 2. Tail flicking time for evaluating central analgesic activity of *Commelina diffusa* fractions in mice

Group	Treatment	Dose (mg/kg)	Tail flicking time (mean±SEM) (s)	
			After 30 min	After 60 min
CTL	Normal saline	10 mL	0.698±0.039	0.762±0.097
STD	Morphine	2	1.616±0.223*	1.688±0.201**
ME I	Methanol extract	100	1.006±0.120	0.914±0.109
ME II	Methanol extract	200	0.850±0.034	1.064±0.096
PEF I	Petroleum ether soluble fraction	100	1.128±0.195	0.896±0.064
PEF II	Petroleum ether soluble fraction	200	1.878±0.353**	0.888±0.097
CF I	Chloroform soluble fraction	100	1.262±0.228	1.998±0.250***
CF II	Chloroform soluble fraction	200	1.342±0.167	1.320±0.116

Note: Statistical significance was calculated using one-way ANOVA followed by *post-hoc* Tukey's HSD test. *p<0.05, **p<0.01, and ***p<0.001 compared to CTL; n=5 per group.
Abbreviations: BW: Body weight; CF: Chloroform fraction; CTL: Control; ME: Methanol extract; PEF: Petroleum ether fraction; SEM: Standard error of the mean; STD: Standard.

A remarkably strong analgesic effect was demonstrated by all *C. diffusa* extracts tested, with higher inhibition of writhing compared to the standard reference drug, diclofenac sodium. The crude methanol extract and the chloroform-soluble fractions at 200 mg/kg produced maximum analgesic activity, showing inhibition rates of 56.96% and 64.56%, respectively. The petroleum ether fraction also demonstrated analgesic activity comparable to the standard. This finding is particularly significant, as diclofenac is a well-established non-steroidal anti-inflammatory drug (NSAID) used for pain management. The superior analgesic activity of *C. diffusa* suggests the presence of potent bioactive compounds that may act through mechanisms distinct from or complementary to those of conventional NSAIDs. The strong analgesic effect could be mediated through multiple pathways, including inhibition of prostaglandin synthesis, modulation of opioid receptors, and influence on both peripheral and central pain pathways.[27,28] Similar analgesic effects have been reported for *Commelina benghalensis* using both the tail flicking and writhing methods.[29,30]

While the methanol extract showed less potency than morphine, its effect was comparable to NSAIDs. This indicates that *C. diffusa* may offer a safer alternative for pain management with fewer side effects, such as gastrointestinal irritation or addiction risk.[10]

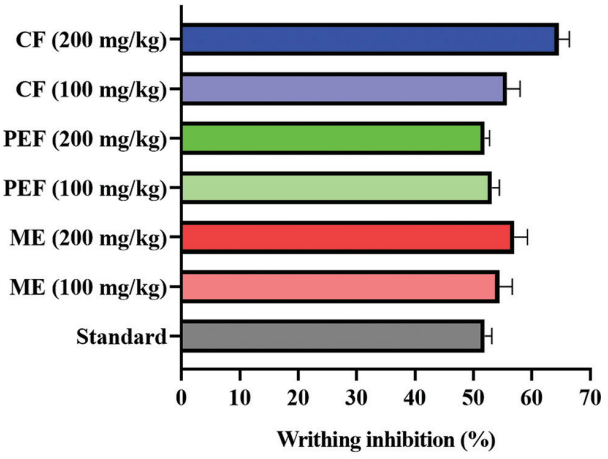

Figure 1. Peripheral analgesic activity of *Commelina diffusa*, expressed as percentage inhibition of writhing in mice. Data presented as mean±SEM (n = 5 per group).
Abbreviations: CF: Chloroform fraction; ME: Methanol extract; PEF: Petroleum ether fraction; SEM: Standard error of the mean.

3.4. Hypoglycemic activity

The hypoglycemic efficacy of *C. diffusa* was assessed by measuring blood glucose levels in treated mice following glucose administration. The results are illustrated in Figure 2.

Despite growing interest in natural products for managing metabolic disorders,[31] the crude methanol extract and the various fractions of *C. diffusa* did not produce a significant reduction in blood glucose levels in this study. This contrasts with reports of *Commelina benghalensis* extracts showing significant hypoglycemic effects in alloxan-induced diabetic rats.[32] The discrepancy may be attributed to interspecies variation and differences in phytochemical composition between species. Moreover, the use of a single dose in non-diabetic (normoglycemic) animals limits the interpretation of hypoglycemic efficacy.

4. Discussion

Bangladesh is endowed with rich biodiversity, particularly in medicinal plants that have been used traditionally for centuries. According to the Bangladesh National Herbarium, over 5,000 plant species exist in the country, of which around 500 are recognized as medicinal.[33,34]

C. diffusa, commonly known as climbing dayflower or spreading dayflower, is a herbaceous plant belonging to the family Commelinaceae. Morphologically, *C. diffusa* is a prostrate or ascending annual herb characterized by succulent stems, ovate to lanceolate leaves, and distinctive blue flowers borne in spathes.[35] The plant reproduces both sexually (via seeds) and vegetatively (through rooting at nodes), contributing to its invasive potential. *C. diffusa* has a pantropical distribution and is especially common in Asia (including India, China, Bangladesh, and Southeast Asia), the Caribbean, Africa, and South America. Traditional medicine and animal studies suggest that *C. diffusa* has a relatively low toxicity profile at therapeutic doses. Comprehensive toxicological evaluations, including chronic toxicity and genotoxicity assessments, are necessary before clinical use. Given its wide geographical distribution, phytochemical richness, and diverse pharmacological activities, *C. diffusa* is a promising candidate for further investigation in natural product drug discovery. With systematic scientific validation, *C. diffusa* could offer sustainable solutions in healthcare and environmental management.[26,35,36]

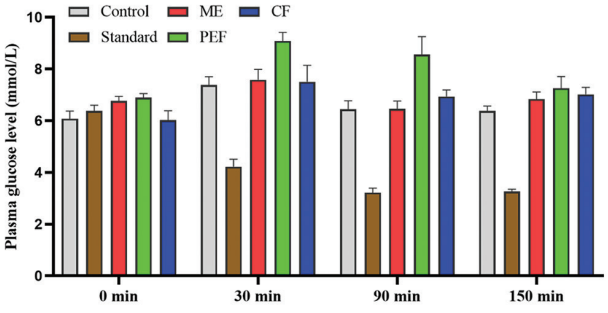

Figure 2. Plasma glucose level of the mice after administration of *Commelina diffusa* extracts at different time intervals. Data are shown as mean±SEM (n = 5 per group).
Abbreviations: CF: Chloroform fraction; ME: Methanol extract; PEF: Petroleum ether fraction; SEM: Standard error of the mean.

Antimicrobial resistance is a growing global health concern characterized by the ability of microorganisms, such as bacteria, viruses, fungi, and parasites, to resist the effects of antimicrobial agents. The World Health Organization has declared antimicrobial resistance as one of the top ten global public health threats.[37] The rise in resistance is driven by a complex interplay of factors, including the misuse and overuse of antibiotics, poor infection control practices, and inadequate surveillance and regulation. Natural products have historically served as a primary source of antibiotics.[6] Many widely used antimicrobials, such as penicillin, streptomycin, and tetracycline, were originally derived from microbial sources. The structural complexity and evolutionary refinement of these compounds allow them to target bacterial cells effectively, often through unique mechanisms. For example, actinomycetes and *Bacillus* species produce a wide array of secondary metabolites, including aminoglycosides, polyketides, and lipopeptides, which exhibit strong antimicrobial activity.[38,39] Curcumin, a polyphenol from *Curcuma longa*, also exerts antimicrobial effects and has been shown to disrupt bacterial cell membranes and inhibit quorum sensing. Similarly, essential oils (e.g., thymol, carvacrol, eugenol) exhibit bactericidal properties through membrane disruption and inhibition of bacterial enzymes.[40,41] Importantly, many of these compounds synergize with conventional antibiotics, enhancing their efficacy and reversing resistance mechanisms.

In the present study, the *C. diffusa* extracts showed potent antimicrobial activity, likely due to the presence of bioactive compounds. These results warrant further phytochemical investigation, including the isolation and characterization of the active compounds, for the development of novel antimicrobial agents.[42] Despite encouraging *in vitro* and *in vivo* findings, the clinical development of natural compounds faces several hurdles. These include poor solubility, low bioavailability, toxicity, and difficulties in standardization and large-scale production. To ensure effective translation, further research must focus on elucidating mechanisms of action, optimizing pharmacokinetics, and conducting rigorous clinical trials. Interdisciplinary collaborations and

sustainable sourcing strategies are also vital for realizing the therapeutic potential of natural products, especially in addressing antimicrobial resistance.[43,44]

Pain management remains a critical concern in modern medicine due to the limitations and adverse effects associated with conventional analgesic drugs, including opioids and NSAIDs. Alkaloids, flavonoids, terpenoids, and polyphenols are the principal classes of plant secondary metabolites investigated for analgesic activity.[45] In traditional medicine, many plants and their parts are also used for treating itching and rash.[46]

Several clinical and preclinical studies support the analgesic efficacy of flavonoids, such as quercetin, kaempferol, and rutin, which exhibit both central and peripheral analgesic effects by modulating oxidative stress, inflammatory pathways, and opioid receptors.[47] In our study, the *C. diffusa* exhibited potent analgesic activity in both hot-plate (tail-flick) and acetic acid-induced writhing methods. The observed effect may be mediated, at least in part, through anti-inflammatory pathways. Several phytochemical constituents reported in the genus Commelina, such as flavonoids and phenolic compounds, are known to possess anti-inflammatory properties. Further investigations are necessary to isolate and identify the compounds responsible for the analgesic activity.

4.1. Limitations and future research directions

The current study presents preliminary findings on the antimicrobial, analgesic, and hypoglycemic activities of *C. diffusa*. However, the study has some limitations. Notably, detailed phytochemical characterization of the bioactive extract was not conducted due to the lack of advanced analytical instrumentation such as liquid chromatography-tandem mass spectrometry, gas chromatography-mass spectrometry, or nuclear magnetic resonance at our current facility. As a result, the specific compounds responsible for the observed biological activities remain unidentified. This limits our ability to elucidate the underlying mechanisms of action and to propose a standardized phytochemical profile for potential therapeutic use. Future studies should focus on isolating and identifying the key active constituents using advanced chromatographic and spectroscopic techniques. Such investigations will be essential for understanding the mechanisms of action, ensuring batch-to-batch consistency, and progressing toward the development of standardized herbal formulations or drug leads. The study also employed a small sample size for the *in vivo* tests, which may limit the statistical power and generalizability of the findings.

Furthermore, although the antimicrobial activity was assessed using the disc diffusion method, determining the minimum inhibitory concentration is essential for quantifying the antimicrobial strength and enabling a more precise comparison with standard antibiotics. The poor solubility or limited diffusion of the extract components within the agar medium could contribute to the relatively weak antimicrobial effect observed in our assay. Future studies may include broth microdilution and alternative solvents to better assess the true antimicrobial potential of the extract under conditions that allow more homogeneous dispersion. In addition, the hypoglycemic potential was assessed using a single-dose OGTT in healthy animals. To fully evaluate the antidiabetic potential of *C. diffusa*, future studies should utilize diabetic models.

5. Conclusion

This study investigated the antimicrobial, analgesic, and hypoglycemic activities of the methanol extract of the whole *C. diffusa* plant, along with its petroleum ether- and chloroform-soluble fractions. Strong antimicrobial properties were demonstrated by the methanol and chloroform fractions against a variety of microorganisms. Central analgesic activity, assessed using the tail-flick test in mice, revealed potent efficacy for both fractions. The extracts also showed strong peripheral analgesic efficacy in the acetic acid-induced writhing test. However, the extract did not show significant hypoglycemic effects in a single-dose OGTT conducted in healthy mice. It is important to note that this finding is based on an acute model in normoglycemic animals and does not rule out potential antidiabetic effects under pathological conditions. Further investigations using diabetic animal models are warranted to comprehensively evaluate the plant's hypoglycemic activity and its possible mechanisms of action in metabolically compromised states. Overall, *C. diffusa* shows promising pharmacological potential and warrants further investigation for its possible therapeutic applications.

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None.

Conflict of interest

The authors declare that they have no competing interests.

Author contributions

Conceptualization: Tasnia Islam Anika, Mohammad Shawkat Ali,
Investigation: Tasnia Islam Anika, Md. Monirul Islam

Methodology: Tasnia Islam Anika, Mohammad Shawkat Ali
Writing–original draft: Tasnia Islam Anika, Md. Monirul Islam
Writing–review & editing: Md. Monirul Islam, Mohammad Shawkat Ali

Ethic approval and consent to participate

The animal study was reviewed and approved by the ethical review board of Northern University Bangladesh (reference number: DoP/RC/EC/2022/01/01).

Consent for publication

Not applicable.

Availability of data

Data are available from the corresponding author on reasonable request.

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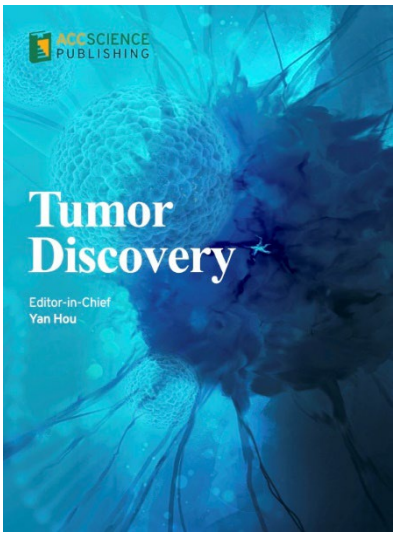

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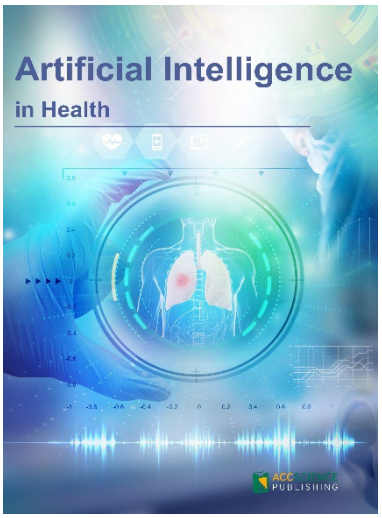

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