

BRIEF REPORT

Inhibitors of platelet aggregation based on fluorinated 3-phenyl-2- isoxazoline-5-carboxamides

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Abstract

Platelets have many functions in the body but undeniably, their primary role is to stop bleeding (hemostasis). While this component can protect the body from blood loss, it can contribute to the development of other critical conditions, such as atherosclerosis and its complications. This knowledge is essential for the development of new treatments that reduce the risk of blood clots and improve the prognosis of patients with cardiovascular diseases. In particular, a good understanding of these mechanisms pave the way for the invention of drugs that selectively target platelets, thereby controlling their excessive activity without affecting the original protective function. In this study, the antiplatelet activity of newly synthesized fluorinated 3-phenyl-2-isoxazoline-5-carboxamides was investigated. All compounds demonstrated the ability to suppress the aggregation ability of platelets. Increasing the concentration of the active substance from 1 to 25 mmol/L led to an increase in the inhibitory power of the effectors. Thus, this study has, for the first time, revealed pronounced selectivity of certain 2-isoxazoline amide derivatives toward either adenosine diphosphate (ADP)- or collagen-induced aggregation, with the direction of selectivity determined by the position of fluorine atoms on the phenyl ring. It was also established that the functional group at the 5-position of the heterocycle influences activity, and the advantage of amides and esters over free acids was demonstrated. Among the studied compounds, the most active were 3-(3-fluorophenyl)-2-isoxazoline-5-carboxamide (in the ADP test) and 3-(3,5-difluorophenyl)-2-isoxazoline-5-carboxamide (in the collagen test).

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1. Introduction

Platelets are essential components of the hemostatic system, playing a key role in preventing blood loss following injury.¹ However, their function is not limited to protecting the body from bleeding. Platelets can also participate in pathological processes leading to thrombus formation, which, in turn, can cause acute vascular diseases such as acute coronary syndromes, unstable angina, myocardial infarction, ischemic attack, and peripheral artery disease.²

Platelet activation occurs under the influence of various agonists, such as arachidonic acid, adenosine diphosphate (ADP), thrombin, thromboxane A₂, and collagen. These substances activate platelets, leading to their aggregation and thrombus formation—a process necessary to stop bleeding. However, in the presence of atherosclerotic changes, when an atheromatous plaque ruptures, platelets begin to interact aggressively with the vascular wall, which can lead to the formation of pathogenic thrombi that block blood flow and cause ischemic tissue damage.^{2,3}

Consequently, the primary target for antiplatelet therapy is the various receptors on the platelet surface.⁴ Drugs acting as antagonists of these receptors prevent normal intercellular contacts and the formation of thrombotic masses by blocking membrane structures. The most common categories of such agents include P2Y₁₂ blockers (ticagrelor, clopidogrel), GP IIb/IIIa inhibitors (abciximab), and agents targeting PAR-1 (vorapaxar).⁵ These drugs are used both in the treatment of acute coronary syndromes and for the prevention of thrombus formation. For the most part, these are low-molecular-weight chemical compounds capable of selectively interacting with target receptors and slowing down the aggregation process.

Currently available antiplatelet drugs, despite their efficacy, are associated with a number of drawbacks: an increased likelihood of non-fatal hemorrhages, hepatotoxic effects, additional burden on the excretory system, and other adverse reactions. These limitations, coupled with the low efficacy of some compounds, are driving the scientific search for new molecules with antiplatelet activity that are free from these disadvantages. The greatest interest is directed at agents characterized by rapid onset of action, predictable patient response, and low bleeding risk.

Active screening of antiplatelet compounds of various chemical natures is being conducted in the world's largest pharmaceutical research centers. Remarkably, the vast majority of promising candidates belong to heterocyclic structures.⁶ Among these, isoxazole derivatives occupy a prominent place;^{7–9} however, their hydrogenated form—4,5-dihydroisoxazole (also called 2-isoxazoline)—has been studied to a much lesser extent. To date, two main mechanisms of action for such compounds have been described: inhibition of the GP IIb/IIIa receptor on the platelet surface^{10,11} or suppression of coagulation factor Xa.^{12,13} The active development of GP IIb/IIIa blockers, responsible for the final stage of aggregation through interaction with fibrinogen, occurred in the late 20th and early 21st centuries. It was during this period that a compound named roxifiban and its active metabolite, representing non-peptide GP IIb/IIIa receptor

antagonists containing a 4,5-dihydroisoxazole ring, were shown to demonstrate high efficacy in animal models.¹⁰ Concurrently, potent GP IIb/IIIa antagonists based on isoxazolinylacetamides were synthesized; the introduction of phosphoramidate or sulfonamide moieties into their structure improved receptor affinity and enhanced the antiplatelet effect.¹¹

Another approach to antithrombotic therapy is based on the inhibition of factor Xa (FXa), one of the key enzymes of the coagulation cascade.^{12,13} This pathway is considered safer compared to direct suppression of thrombin activity. Several publications^{12,13} report the production of isoxazoline derivatives as potent FXa blockers. For instance, a derivative was obtained for which the K_i value for human FXa was 0.52 nM, and the compound showed good selectivity and efficacy in *in vivo* thrombosis models.¹³ Simultaneously, pyridyl-substituted isoxazoles and their 4,5-dihydro analogues were synthesized; in the concentration range of 10^{–6}–10^{–3} M, they proved capable of inhibiting human platelet aggregation stimulated by arachidonic acid.^{14–16} Given that the action of widely known aspirin is based on the same mechanism, these data confirm the high potential of 2-isoxazolines as a scaffold for creating new antiplatelet agents.

In our recent investigation, we investigated the potential of compounds from the series of 3-(fluoro- or difluorophenyl)-2-isoxazoline-5-carboxylic acid and its methyl ester as platelet aggregation inhibitors.¹⁷ This paper describes the synthesis of similar compounds—3-(fluoro- or difluorophenyl)-2-isoxazoline-5-carboxamides, *i.e.*, compounds containing a carboxamide group, instead of a carboxyl group.

In medicinal chemistry, the approach of replacing a carboxyl group with a carboxamide group is well known, as it can improve the pharmacological properties of compounds. This method is a special case of bioisosteric replacement, a concept that plays a key role in modern drug design.¹⁸ Replacing a carboxyl group with an amide group primarily alters the ionization properties of the molecule. While the carboxyl group is predominantly ionized at physiological pH 7.4, the amide group remains neutral, which increases lipophilicity and improves cell membrane penetration.¹⁹ Studies have shown that such a replacement reduces the effective polar surface area of the molecule, which directly correlates with improved membrane permeability.¹⁹

From a pharmacokinetic perspective, the amide bond exhibits greater metabolic stability compared to the carboxyl group, which can be a substrate for conjugation reactions. As noted in review articles, the use of bioisosteres, including the amide group, improves parameters such as stability, oral

absorption, and distribution of drug candidates.¹⁸ From the standpoint of molecular recognition, the amide group can both accept and donate a proton, enabling more diverse hydrogen bonding with the target protein compared to the carboxylate ion.

It is known that replacing an ester or carboxyl group with a carboxamide group can significantly influence the antiplatelet activity of compounds. The literature describes examples where such modifications not only alter but also improve the inhibitory properties toward platelet aggregation. For example, a systematic comparison in the ability to inhibit platelet aggregation induced by adenosine diphosphate (ADP) and epinephrine between anilides and phenyl esters of piperidine-3-carboxylic acid (nipecotic acid) found that the amides generally exhibited approximately twice the activity of the corresponding esters.²⁰ In another study, 1-arylalkyl-5-phenylsulfonamido-imidazole-4-carboxylic acid esters and their carboxamides containing an additional secondary amino group were synthesized.²¹ Both types of compounds showed antiplatelet activity in the low micromolar range. However, the most important finding was that minor structural modifications, including the replacement of an ester group with a carboxamide group, could switch the activity profile of the compound between different platelet receptors. Specifically, the compound with an ester moiety exhibited antagonistic activity toward platelet-activating factor (PAF) with an IC_{50} of 1 μ M and inhibited cyclooxygenase-1 (COX-1) with an IC_{50} of 0.4 μ M. At the same time, the corresponding carboxamide demonstrated ADP-antagonistic properties with an IC_{50} of 2 μ M.²¹

Therefore, the examples provided confirm that the carboxamide group is often preferable to the ester or carboxyl group for synthesizing effective antiplatelet agents. This is because amides generally possess more optimal lipophilicity for interacting with platelet membranes, provide better metabolic stability, and allow for more precise tuning of interactions with specific receptors on the platelet surface.

In this study, the anti-aggregation properties of recently synthesized fluorophenyl- substituted 2-isoxazoline-5-carboxamides were investigated. In our previous study, platelet aggregation inhibitors based on monofluorophenyl-substituted 2-isoxazoline-5-carboxylic acid and its methyl ester were studied, which revealed their ability in slowing down platelet aggregation.¹⁷ The current investigation is an extension of the previous study, reporting the preparation and the anti-aggregation properties of mono- and difluoro-substituted compounds containing another functional group at position 5 of the heterocyclic fragment.

2. Materials and methods

2.1. Compounds under investigation

Infrared spectra of the studied compounds were recorded on a Specord 75 spectrometer (Carl Zeiss Jena, Germany) using the standard potassium bromide pellet technique. Ultraviolet (UV) absorption spectra were recorded on a Specord M40 instrument (Carl Zeiss Jena, Germany) in ethanol solution. Nuclear magnetic resonance spectra were recorded on a Bruker Avance 500 spectrometer operating at 500 MHz (Bruker, USA); chemical shifts (δ) are given relative to the tetramethylsilane signal, which was used as an internal standard. Samples were dissolved in deuterated dimethylformamide (DMF-d₇). The progress of chemical reactions and the purity of the synthesized products were monitored by thin-layer chromatography on Silufol UV-254 plates (Merck, Germany). High-resolution mass spectra, obtained by electrospray ionization, were recorded on an Agilent 6540 Q-TOF instrument.

The synthesis of compounds **1–9** was carried out according to the procedure schematically shown in Figure 1. A solution was prepared by dissolving 0.25 g of the corresponding oxime in 10 mL of dimethylformamide. The solution was subsequently cooled to 15 °C in a water bath, and 0.240 g of N-chlorosuccinimide (NCS) was added portion-wise. After stirring the reaction mixture for one hour, acrylamide was introduced. Then, 0.45 mL of triethylamine was added dropwise, and stirring was continued for 24 h. Upon completion of the reaction, the mixture was diluted with 70 mL of water, and the target product was extracted with dichloromethane (2 × 15 mL). The combined organic fractions were washed with water (30 mL), and the solvent was removed by evaporation. The resulting solid residue was recrystallized from 2-propanol. The purified compounds were subsequently characterized using the analytical methods listed above.

Details of compounds **1–9** are presented as follows:

Compound 1 — 3-(2-fluorophenyl)-2-isoxazoline-5-carboxamide 1. Yield 56%. Mp 185–187 °C. UV spectrum ($\lambda_{\max}$, nm): 259. IR spectrum (cm⁻¹): 3341, 3194 (N-H), 1675, 1598 (O=C NH₂). ¹H (δ , ppm): 3.69 (1H, ddd, J1 2.1 Hz, J2 6.8 Hz, J3 17.4 Hz, 4-CH), 3.80 (1H, ddd, J1 1.7 Hz, J2 11.6 Hz, J2 17.4 Hz, 4-CH), 5.19 (1H, dd, J1 6.8 Hz, J2 11.6 Hz, 5-CH), 7.31–7.38 (2H, m, aromatic protons), 7.50 (1H, br. s, NH), 7.57 (1H, m, aromatic proton), 7.73 (1H, br. s, NH), 7.83 (1H, m, aromatic proton). ¹³C NMR (δ , ppm): 40.9 (CH₂), 86.9 (CH), 116.1 (CH), 124.4 (C), 125.6 (CH), 127.7 (CH), 131.2 (CH), 155.5 (C), 158.5 (C), 175.1 (C). HRMS (ESI) m/z: calcd for C₁₀H₉N₂O₂F [M+H]⁺ 209.0726, found 209.0721.

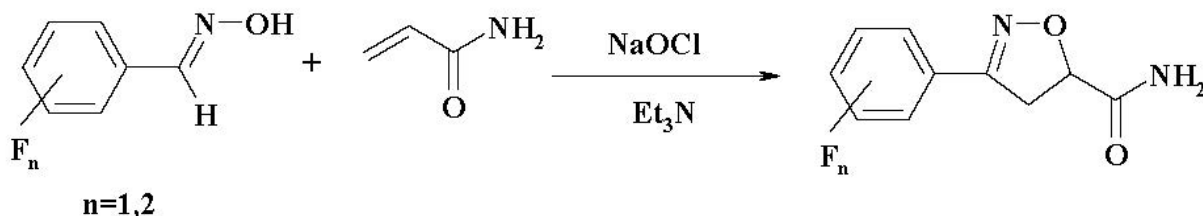


Figure 1. Synthesis of the compounds

Compound 2 — 3-(3-fluorophenyl)-2-isoxazoline-5-carboxamide 2. Yield 68%. Mp 193–195 °C. UV spectrum ($\lambda_{\max}$, nm): 262. IR spectrum (cm^{-1}): 3410, 3215 (N-H), 1660, 1610 ($\text{O}=\text{C}$ NH₂). ¹H (δ , ppm): 3.67 (1H, dd, J1 6.8 Hz, J2 17.2 Hz, 4-CH), 3.78 (1H, dd, J1 12.0 Hz, J2 17.2 Hz, 4-CH), 5.21 (1H, dd, J1 6.8 Hz, J2 12.0 Hz, 5-CH), 7.34 (1H, m, arom. proton), 7.50 (1H, br s, NH), 7.53–7.58 (2H, m, arom. protons), 7.60 (1H, t, J 8.2 Hz, arom. proton), 7.73 (1H, br s, NH). ¹³C NMR: δ 40.9 (CH₂), 86.9 (CH), 115.0 (CH), 116.0 (CH), 126.9 (CH), 129.4 (CH), 130.6 (C), 155.7 (C), 161.8 (C), 175.1 (C). HRMS (ESI) m/z : calcd for C₁₀H₉N₂O₂F [M+H]⁺ 209.0726, found 209.0723.

Compound 3 — 3-(4-fluorophenyl)-2-isoxazoline-5-carboxamide 3. Yield 90%. Mp 227–229 °C. UV spectrum ($\lambda_{\max}$, nm): 260. IR spectrum (cm^{-1}): 3401, 3193 (N-H), 1658, 1605 ($\text{O}=\text{C}$ NH₂). ¹H (δ , ppm): 3.66 (1H, dd, J1 6.7 Hz, J2 17.1 Hz, 4-CH), 3.77 (1H, dd, J1 11.6 Hz, J2 17.1 Hz, 4-CH), 5.18 (1H, dd, J1 6.7 Hz, J2 11.6 Hz, 5-CH), 7.33 (2H, m, arom. protons), 7.49 (1H, br s, NH), 7.70 (1H, br s, NH), 7.83 (2H, m, arom. protons). ¹³C NMR: δ 40.9 (CH₂), 86.9 (CH), 115.9 (2 × CH), 122.0 (C), 128.6 (2 × CH), 155.7 (C), 162.4 (C), 175.1 (C). HRMS (ESI) m/z : calcd for C₁₀H₉N₂O₂F [M+H]⁺ 209.0726, found 209.0721.

Compound 4 — 3-(2,3-difluorophenyl)-2-isoxazoline-5-carboxamide 4. Yield 65%. Mp 171–173 °C. UV spectrum ($\lambda_{\max}$, nm): 258. IR spectrum (cm^{-1}): 3439, 3214 (N-H), 1655, 1609 (CO NH₂), 1510, 1421 (C-C arom.). ¹H (δ , ppm): 3.65 (1H, ddd, J1 2.1 Hz, J2 6.8 Hz, J3 17.4 Hz, 4-CH), 3.75 (1H, ddd, J1 1.6 Hz, J2 11.6 Hz, J3 17.4 Hz, 4-CH), 5.18 (1H, dd, J1 6.8 Hz, J2 11.6 Hz, 5-CH), 7.20 (1H, ddd, J1 1.3 Hz, J2 2.6 Hz, J3 8.7 Hz, arom. proton), 7.21 (1H, ddd, J1 2.6 Hz, J2 9.3 Hz, J3 11.7 Hz, arom. proton), 7.51 (1H, br s, NH), 7.73 (1H, br s, NH), 7.76 (1H, dt, J1 6.6 Hz, J2 8.7 Hz, aromatic proton). ¹³C NMR: δ 40.9 (CH₂), 86.9 (CH), 112.8 (CH), 125.6 (C), 127.7 (C), 129.4 (CH), 144.5 (C), 150.6 (C), 155.5 (C), 175.1 (C). HRMS (ESI) m/z : calcd for C₁₀H₈N₂O₂F₂ [M+H]⁺ 227.0632, found 227.0627.

Compound 5 — 3-(2,4-difluorophenyl)-2-isoxazoline-5-carboxamide 5. Yield 62%. Mp 168–169 °C. UV spectrum ($\lambda_{\max}$, nm): 257. IR spectrum (cm^{-1}): 3437, 3213 (N-H), 1654, 1608 (CO NH₂), 1509, 1423 (C-C arom.). ¹H

(δ , ppm): 3.68 (1H, ddd, J1 2.1 Hz, J2 6.8 Hz, J3 17.4 Hz, 4-CH), 3.79 (1H, ddd, J1 1.6 Hz, J2 11.6 Hz, J3 17.4 Hz, 4-CH), 5.19 (1H, dd, J1 6.8 Hz, J2 11.6 Hz, 5-CH), 7.24 (1H, ddd, J1 1.3 Hz, J2 2.6 Hz, J3 8.7 Hz, arom. proton), 7.39 (1H, ddd, J1 2.6 Hz, J2 9.3 Hz, J3 11.7 Hz, arom. proton), 7.51 (1H, br s, NH), 7.74 (1H, br s, NH), 7.90 (1H, dt, J1 6.6 Hz, J2 8.7 Hz, aromatic proton). ¹³C NMR: δ 40.9 (CH₂), 86.9 (CH), 105.0 (CH), 111.0 (CH), 127.2 (C), 127.7 (CH), 155.5 (C), 160.2 (C), 163.5 (C), 175.1 (C). HRMS (ESI) m/z : calcd for C₁₀H₈N₂O₂F₂ [M+H]⁺ 227.0632, found 227.0626.

Compound 6 — 3-(2,5-difluorophenyl)-2-isoxazoline-5-carboxamide 6. Yield 59%. Mp 167–168 °C. UV spectrum ($\lambda_{\max}$, nm): 260, 293 (shoulder). IR spectrum (cm^{-1}): 3447, 3214 (N-H), 1671 (C=N), 1653, 1610 (CO NH₂), 1180 (C-F). ¹H (δ , ppm): 3.70 (1H, ddd, J1 2.2 Hz, J2 6.9 Hz, J3 17.5 Hz, 4-CH), 3.81 (1H, ddd, J1 1.8 Hz, J2 11.7 Hz, J3 17.5 Hz, 4-CH), 5.22 (1H, dd, J1 6.9 Hz, J2 11.7 Hz, 5-CH), 7.41–7.45 (2H, m, arom. proton), 7.52 (1H, br s, NH), 7.60 (1H, m, arom. proton), 7.76 (1H, br s, NH). ¹³C NMR: δ 40.9 (CH₂), 86.9 (CH), 116.8 (CH), 117.2 (CH), 117.4 (CH), 127.7 (C), 155.5 (C), 156.3 (C), 158.5 (C), 175.1 (C). HRMS (ESI) m/z : calcd for C₁₀H₈N₂O₂F₂ [M+H]⁺ 227.0632, found 227.0627.

Compound 7 — 3-(2,6-difluorophenyl)-2-isoxazoline-5-carboxamide 7. Yield 51%. Mp 143–144 °C. UV spectrum ($\lambda_{\max}$, nm): 255. IR spectrum (cm^{-1}): 3379, 3184 (N-H), 1710 (C=N), 1674, 1623 (CO NH₂), 1596, 1460 (C-C arom.). ¹H (δ , ppm): 3.68 (1H, dd, J1 6.9 Hz, J2 17.5 Hz, 4-CH), 3.77 (1H, dd, J1 11.6 Hz, J2 17.5 Hz, 4-CH), 5.22 (1H, dd, J1 6.9 Hz, J2 11.6 Hz, 5-CH), 7.27 (2H, m, arom. protons 3,5'-CH), 7.53 (1H, br s, NH), 7.64 (1H, m, arom. proton 4'-CH), 7.76 (1H, br s, NH). ¹³C NMR: δ 40.9 (CH₂), 86.9 (CH), 115.4 (2 × CH), 122.6 (C), 129.3 (CH), 155.5 (C), 157.2 (2 × C), 175.1 (C). HRMS (ESI) m/z : calcd for C₁₀H₈N₂O₂F₂ [M+H]⁺ 227.0632, found 227.0626.

Compound 8 — 3-(3,4-difluorophenyl)-2-isoxazoline-5-carboxamide 8. Yield 61%. Mp 170–172 °C. UV spectrum ($\lambda_{\max}$, nm): 261. IR spectrum (cm^{-1}): 3396, 3220 (N-H), 1660, 1613 (CO NH₂), 1583, 1528 (C-C arom.). ¹H (δ , ppm): 3.67 (1H, dd, J1 6.9 Hz, J2 17.2 Hz, 4-CH), 3.78 (1H, dd, J1 11.6 Hz, J2 17.2 Hz, 4-CH), 5.21 (1H, dd, J1

6.9 Hz, J2 11.6 Hz, 5-CH), 7.50 (1H, br. s, NH), 7.56 (1H, m, arom. proton), 7.64 (1H, arom. proton), 7.73 (1H, br. s, NH), 7.79 (1H, ddd, J1 2.1 Hz, J2 7.8 Hz, J3 11.6 Hz, arom. proton). ¹³C NMR: δ 40.9 (CH₂), 82.0 (CH), 116.0 (CH), 117.2 (CH), 128.2 (C), 130.6 (CH), 149.8 (C), 152.5 (C), 155.7 (C), 175.1 (C). HRMS (ESI) m/z: calcd for C₁₀H₈N₂O₂F₂ [M+H]⁺ 227.0632, found 227.0629.

Compound 9 — 3-(3,5-difluorophenyl)-2-isoxazoline-5-carboxamide 9. Yield 58%. Mp 176–178 °C. UV spectrum (λ_{max}, nm): 264. IR spectrum (cm⁻¹): 3393, 3220 (N-H), 1664, 1605 (O=C NH₂), 1126 (C-F). ¹H (δ, ppm): 3.69 (1H, dd, J1 7.0 Hz, J2 17.3 Hz, 4-CH), 3.79 (1H, dd, J1 11.7 Hz, J2 17.3 Hz, 4-CH), 5.24 (1H, dd, J1 7.0 Hz, J2 11.7 Hz, 5-CH), 7.37 (1H, tt, J1 2.3 Hz, J2 9.3 Hz, arom. proton 4'-CH), 7.46 (2H, m, arom. protons, 2',5'-CH), 7.51 (1H, br s, NH), 7.76 (1H, br s, NH). ¹³C NMR: δ 40.9 (CH₂), 82.0 (CH), 116.0 (2 × CH), 117.2 (CH), 128.2 (C), 130.6 (2 × C), 149.8 (C), 152.5 (C), 155.7 (C), 175.1 (C). HRMS (ESI) m/z: calcd for C₁₀H₈N₂O₂F₂ [M+H]⁺ 227.0632, found 227.0627.

2.2. Cytotoxicity assessment

After the washing procedure, the platelet concentration was adjusted to 3 × 10⁸ cells/mL. The cell suspension was incubated for 10 minutes at 37 °C with each test compound at a dose of 25 mM (the highest concentration tested). After incubation, platelets were pelleted by centrifugation at 800 × g for 8 min. From the resulting supernatant, 100 μL were taken for analysis using a commercial kit (Abcam Inc., AB65393) designed to measure lactate dehydrogenase (LDH) activity as a marker of cytotoxicity. Measurements were performed on a microplate photometer (Thermo Scientific Multiskan Go) at a wavelength of 490 nm. A sample treated with 10% Triton X-100 was used as a control for complete cell lysis.

2.3. Light transmission aggregometry

All procedures involving human participants complied with the ethical standards set forth in the Declaration of Helsinki. Venous blood was collected from the cubital vein of healthy volunteers who had abstained from taking acetylsalicylic acid and other nonsteroidal anti-inflammatory drugs for at least 14 days prior to the study. Informed consent was obtained from each participant before the procedure. Blood was placed into 50 mL plastic tubes containing 3.8% sodium citrate solution (blood to anticoagulant ratio of 9:1 by volume). To obtain platelet-rich plasma (PRP), the tubes were centrifuged at 300 rpm for 20 minutes at room temperature. The resulting supernatant (PRP) was carefully transferred to a new container. The remaining blood was centrifuged again at 900 rpm for 10 minutes to obtain platelet-poor plasma

(PPP), which was subsequently used as a control medium.

Platelet aggregation ability was assessed using a Solar 2111 aggregometer (Solar, Republic of Belarus). According to a previously reported method,¹⁷ 480 μL of PRP (final cell concentration adjusted to 200 × 10⁹/L) was placed into the measurement cuvette. The sample was then pre-incubated with 20 μL of either prostaglandin E1 solution (0.02 mM)—to prevent spontaneous aggregation and to serve as a control sample—or a solution of the test compound. After 3 min of incubation, 20 μL of an aggregation inducer (8 μM ADP or 1.5 μM collagen) was added to each cuvette. The aggregation curve was recorded over the following 6 minutes. Clopidogrel at concentrations identical to those of the newly synthesized compounds was used as a reference drug (positive control).

2.4. Statistical analysis

All experiments were performed in triplicate to minimize random errors and increase the reliability of the results. Platelet aggregation values were recorded as the maximum amplitude in percent. Aggregation inhibition was calculated as a percentage relative to the control (sample with prostaglandin E1 added), which allowed us to assess the effect of the test compound. Data are presented as mean ± standard error of the mean (SEM).

3. Results

Structural identification of the obtained products was performed using infrared (IR) and nuclear magnetic resonance (NMR) spectroscopy. For example, the IR spectrum of compound 2 shows characteristic absorption bands corresponding to the amide group in the regions of 1660 cm⁻¹ and 1610 cm⁻¹. The ¹H NMR spectrum of this compound exhibits characteristic signals indicating the occurrence of the [3+2]-dipolar cycloaddition reaction and the formation of the 2-isoxazoline ring: two doublets of doublets at δ 3.67 and 3.78 ppm, as well as a doublet of doublets at δ 5.21 ppm. The first two signals correspond to the two protons at C-4, while the latter signal belongs to the proton at C-5. This fact unequivocally confirms that compound 2 is a 3-aryl-5-carboxamide-2-isoxazoline, rather than its regioisomeric form, 3-aryl-4-carboxamide-2-isoxazoline. Characteristic signals in the ¹³C NMR spectrum at δ 40.9 and 86.9 ppm correspond to the C-4 and C-5 atoms of the isoxazoline ring, while the signal at δ 175.1 ppm belongs to the carbon atom of the carboxamide group.

It should be noted that when a fluorine atom is present at the *ortho*-position of the phenyl ring, the aforementioned signals of the 2-isoxazoline ring in the ¹H NMR spectrum undergo additional splitting due to interaction with the

fluorine atom through the double bond system; the spin-spin coupling constant is 1.5–2.0 Hz (see the ^1H NMR spectra of compounds **1**, **4**, **5**, **6**).^{22,23}

Prior to evaluating the ability of the obtained compounds to inhibit platelet aggregation, their cytotoxicity was assessed by measuring lactate dehydrogenase activity. Prolonged incubation of platelets in the presence of the new compounds did not reveal any noticeable cell lysis.

The inhibitory activity of the series of newly synthesized carboxamide derivatives of 2-isoxazoline against ADP-induced platelet aggregation is shown in Figure 2. All studied compounds exhibited a dose-dependent effect in the concentration range of 1–25 mM. The highest activity among the amides was demonstrated by 3-(3-fluorophenyl)-2-isoxazoline-5-carboxamide **2**, reaching 69% inhibition at a concentration of 25 mM. Close values were shown by 3-(2-fluorophenyl)-2-isoxazoline-5-carboxamide **1** (65% at 25 mM). The lowest activity in this series was recorded for 3-(3,5-difluorophenyl)-2-isoxazoline-5-carboxamide **9** (38% at 25 mM) and the 3-(2,6-difluorophenyl) derivative **7** (40% at 25 mM). The reference drug clopidogrel under the same conditions inhibited aggregation by 88% at 25 mM.

The inhibitory activity of the same carboxamide derivatives of 2-isoxazoline against collagen-induced platelet aggregation is shown in Figure 3. As with ADP,

all compounds exhibited a concentration-dependent inhibition pattern. However, the activity ranking differed significantly. The highest efficacy in this test was shown by 3-(3,5-difluorophenyl)-2-isoxazoline-5-carboxamide **9**, reaching 68.0% inhibition at 25 mM, which is only 18% lower than clopidogrel (86%). High activity was also demonstrated by the 3-(4-fluorophenyl) derivative **3** (63%) and the 3-(2,6-difluorophenyl) derivative **7** (62%). In contrast, the least active under collagen stimulation were the 3-(2,5-difluorophenyl) derivative **6** (41% at 25 mM) and the 3-(2,3-difluorophenyl) derivative **4** (43%).

4. Discussion

This study presents a comparative analysis of the inhibitory activity of a series of carboxamide derivatives of 3-phenyl-2-isoxazoline, which differ in the number and position of fluorine atoms on the phenyl substituent, against ADP- and collagen-induced platelet aggregation. Efficacy assessment was performed over the same concentration range (1–25 mM), allowing direct comparison of the activity of each compound depending on the activator type. Clopidogrel was used as a reference drug in all experiments.

Analysis of the data presented in Figures 2 and 3 (ADP- and collagen-stimulated platelet aggregation, respectively) shows that most of the studied compounds exhibit concentration-dependent inhibitory activity under both

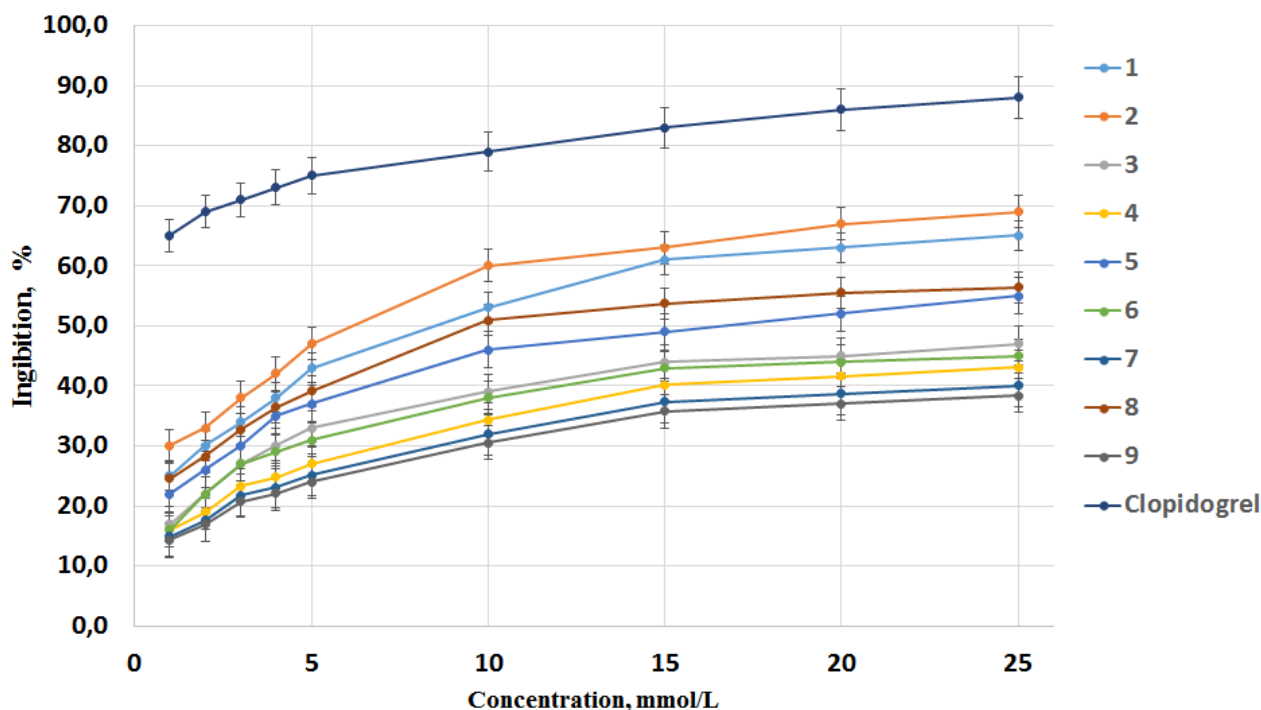


Figure 2. Inhibition of platelet aggregation by adenosine diphosphate (ADP) activation

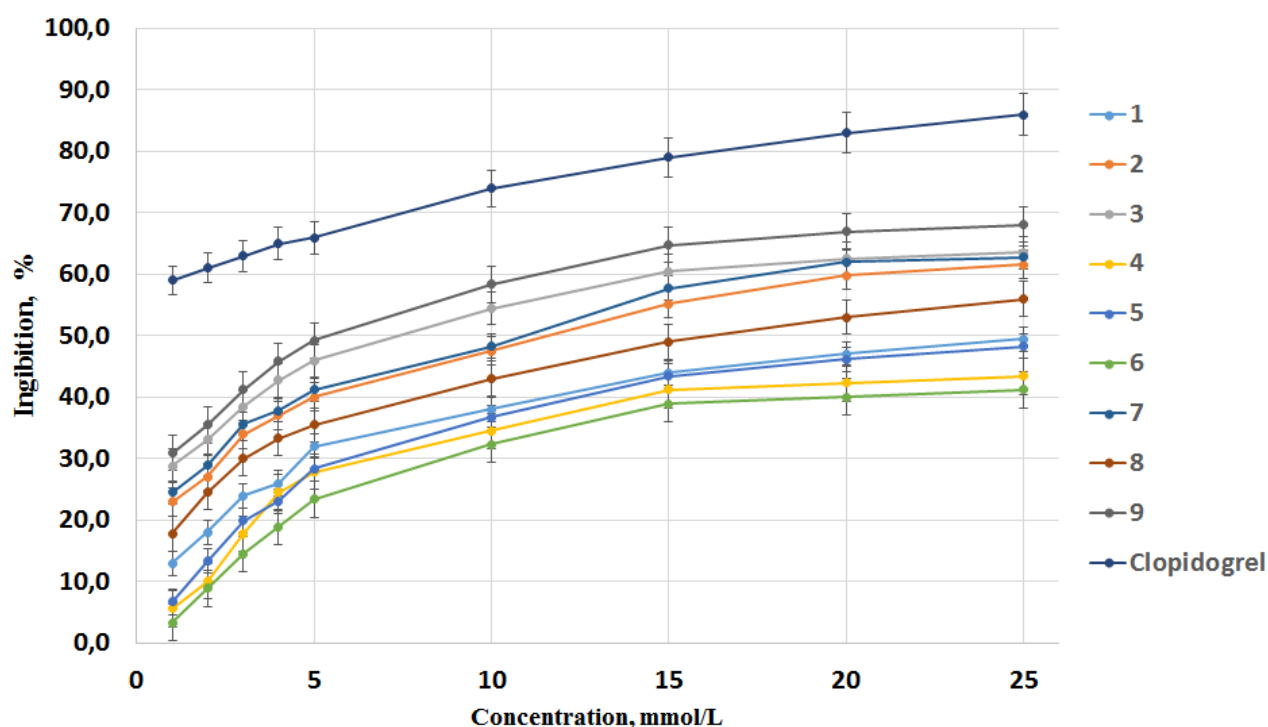


Figure 3. Inhibition of platelet aggregation by collagen activation

activation types. However, the nature of the dependence and the absolute efficacy values differ significantly depending on the activator. Clopidogrel, as expected, demonstrated high activity in both tests, reaching 88% inhibition with ADP and 86% with collagen activation (at 25 mM concentration), confirming the correctness of the experimental conditions.

Several compounds showed higher activity in the ADP test compared to the collagen test. This group includes compounds with 2-fluorophenyl (65% vs. 49% at 25 mM), 3-fluorophenyl (69% vs. 62%), 2,4-difluorophenyl (55% vs. 48%), and 2,5-difluorophenyl (45% vs. 41%) substituents. The difference in favor of ADP ranges from 4% to 15% at the maximum concentration. This activity distribution suggests that these compounds act primarily through mechanisms involving inhibition of the P2Y₁₂ receptor pathway, similar to clopidogrel.

The opposite pattern is observed for compounds with 4-fluorophenyl (47% vs. 63% at 25 mM), 2,6-difluorophenyl (40% vs. 63%), and most notably, 3,5-difluorophenyl substituents (38% vs. 68%). For the latter compound, the difference in favor of collagen reaches almost 30%, representing the most pronounced selectivity effect in the entire studied series. Such a substantial difference allows us to state with high confidence that the mechanism of action of these compounds is fundamentally different

from that of clopidogrel. These derivatives likely interact with molecular targets specific to the collagen-dependent platelet activation pathway, such as the GPVI receptor, integrin $\alpha 2\beta 1$, or affect intracellular signaling cascades activated specifically by collagen.

Compound **9** with the 3,5-difluorophenyl substituent deserves special attention: it shows minimal activity among all amides in the ADP test (38% at 25 mM), whereas under collagen stimulation, its efficacy (68%) is second only to clopidogrel (86%) and the 3,4-difluorophenyl derivative **8** (56% — non-selective). This compound represents a valuable tool for studying collagen-specific aggregation mechanisms and may be considered a promising lead for further optimization.

Some studied amides demonstrate similar activity in both tests. These include the 2,3-difluorophenyl derivative **4** (42% vs. 43% at 25 mM) and the 3,4-difluorophenyl derivative **8** (56% vs. 55%). These compounds likely act on signaling mechanisms common to both activation pathways, such as intracellular calcium homeostasis or integrin activation at the final stages of aggregation.

The obtained data allow us to formulate a pattern that the position of fluorine atoms on the phenyl ring is a key determinant of selectivity towards the aggregation activator. Mono-2- and mono-3-fluoro-substituted amides, as well as 2,4- and 2,5-difluoro derivatives, are

predominantly active against ADP. In contrast, mono-4-fluoro-, 2,6- and 3,5-difluoro-substituted amides exhibit pronounced selectivity toward collagen. Thus, ortho- and meta-positions of fluorine (or their combinations that do not include the para-position) favor ADP activity, whereas the presence of fluorine in the para-position or symmetrical 2,6- and 3,5-difluoro substitution switches selectivity in favor of collagen.

In our previously published studies, we characterized 2-isoxazoline derivatives with a free carboxyl group ($-\text{COOH}$) and a methyl ester ($-\text{COOMe}$) at the 5-position of the heterocycle.¹⁷ In this work, we present for the first time data for the corresponding carboxamide derivatives ($-\text{CONH}_2$), allowing a direct comparison of the influence of the functional group nature on antiplatelet activity under ADP stimulation. All three series contain the same fluorophenyl substituents (2-F, 3-F, 4-F), ensuring the validity of the comparison. It was found that for each of the three fluorine positions, the activity of the compounds increases in the series: $-\text{COOH} < -\text{COOMe} \leq -\text{CONH}_2$

This trend is particularly evident from the values at the maximum concentration of 25 mM. For 3-fluorophenyl derivatives: acid — 44%, ester — 63%, amide — 69%. For 2-fluorophenyl derivatives: acid — 39%, ester — 59%, amide — 65%. For 4-fluorophenyl derivatives: acid — 37%, ester — 45%, amide — 47%. Thus, replacing the free carboxyl group with an ester group increases activity by an average of 15–20%, while the further transition to the amide gives an additional increase of 3–6% for the studied compounds. The explanation for this phenomenon likely involves several factors. First, the free carboxyl group at physiological pH 7.4 is predominantly in the ionized form ($-\text{COO}^-$), which hinders passive diffusion of the compound across the cell membrane and may reduce its intracellular concentration. Esters and amides are neutral, more lipophilic molecules, which facilitates their penetration to the site of action.^{19–21} Second, the carboxamide group can form additional hydrogen bonds with amino acid residues in the active site of the target (presumably the P2Y₁₂ receptor or associated proteins), which may increase the compound's affinity for the receptor. The methyl ester lacks this ability, explaining its somewhat lower activity compared to the amide. Third, different metabolic stability of the studied compounds under experimental conditions cannot be ruled out. It is possible that amides and esters are inactivated more slowly by plasma or platelet enzymes compared to the free acids. It is important to note that upon transitioning from acids to esters and amides, the ranking of activity depending on the fluorine position is preserved. In all three series, maximum activity is observed for the 3-fluorophenyl substituent, followed by the 2-fluoro and, with a notable

lag, the 4-fluorophenyl derivative. This indicates that the influence of fluorine position on target binding is dominant and is not negated by changing the functional group at the 5-position. Thus, the optimal combination for ADP-inhibitory activity is the 3-fluorophenyl substituent in combination with a carboxamide or ester group at the 5-position of the isoxazoline ring.

Comparison with our previously published data¹⁷ allows us to draw two important conclusions. First, the results obtained in this work are fully consistent with our previous observations and confirm the previously identified structure–activity relationship patterns. Second, introducing a carboxamide group at the 5-position of the heterocycle is an effective strategy for enhancing antiplatelet activity compared to free acids and, to a lesser extent, methyl esters. This finding may facilitate the optimization of existing platelet aggregation inhibitors and the development of novel inhibitors based on the 2-isoxazoline scaffold.

Our results are consistent with the work of other researchers and demonstrate that isoxazoline derivatives can exhibit antiplatelet activity through various mechanisms, including blockade of the fibrinogen receptor GPIIb/IIIa,²⁴ and antagonism at the PAR-1 thrombin receptor.²⁵ However, it has been shown for the first time that within the same structural scaffold (2-isoxazoline-5-carboxamides), variation in the position of fluorine atoms allows switching selectivity between two different activation pathways, namely the ADP- and collagen-dependent pathways. This opens new prospects for the targeted design of inhibitors with a desired mechanism of action. Furthermore, the pattern we obtained regarding inhibitory activity ($-\text{COOH} < -\text{COOMe} \leq -\text{CONH}_2$) is supported in the literature. For example, in studies of isoxazolines as GPIIb/IIIa antagonists, it was shown that replacing the carboxyl group with amide derivatives often leads to increased antiplatelet activity, attributed to improved membrane permeability and optimized receptor interaction.²⁴ Also, our previous results demonstrating the advantage of the 3-fluorophenyl substituent over the 2- and 4-fluorophenyl ones in all three series (acids, esters, amides) are fully consistent with literature data on the importance of the meta-position of electron-withdrawing substituents for binding to P2Y₁₂-like receptors.²⁵

4. Conclusion

This study has, for the first time, revealed pronounced selectivity of certain 2-isoxazoline amide derivatives toward either ADP- or collagen-induced aggregation, with the direction of selectivity determined by the position of fluorine atoms on the phenyl ring. It was also

established that the functional group at the 5-position of the heterocycle influences activity, and the advantage of amides and esters over free acids was demonstrated. Among the studied compounds, the most active were 3-(3-fluorophenyl)-2-isoxazoline-5-carboxamide (in the ADP test) and 3-(3,5-difluorophenyl)-2-isoxazoline-5-carboxamide (in the collagen test).

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Conflict of interest

All authors declare no conflicts of interest.

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Ethics approval and consent to participate

Not applicable. This research involved the use a platelet-rich plasma from healthy donors. All donors gave written voluntary consent to participate in the study.

Consent for publication

All blood donors gave written voluntary consent for publication.

Availability of data

Data used in this work can be made available to the readers by contacting the corresponding author.

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