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BRIEF REPORT

Platelet aggregation inhibition by fluorophenyl-substituted 2-isoxazoline-5-carboxylic acids and their derivatives

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Abstract

Platelets perform many important bodily functions, with their primary task being the prevention of bleeding by facilitating hemostasis. While platelets protect the body from blood loss, they also contribute to the development of serious diseases, such as atherosclerosis and its complications. Understanding the dual role of platelets is crucial for developing new treatments aimed at reducing thrombotic risk while improving the prognosis for patients with cardiovascular diseases. Specifically, elucidating the mechanisms underlying platelet activation may facilitate the development of selective agents that inhibit pathological platelet activity without compromising their protective function. In this context, the present study evaluated the antiplatelet activity of newly synthesized fluorophenyl-substituted 2-isoxazoline-5-carboxylic acids and their derivatives. Results showed that all compounds demonstrated the ability to suppress platelet aggregation. Increasing the concentration of the active substance from 1 to 25 mmol/L enhanced the inhibitory effect of the compounds. Methyl esters, compared to derivatives with a free carboxyl group, exhibited a stronger ability to suppress the activation of platelet receptors glycoprotein (GP) IIa/IIIb, thereby inhibiting their binding to fibrinogen and subsequent aggregation. The half-maximal inhibitory concentration values for two of the studied compounds were 7.5 mmol/L (methyl ester of 3-[3]-fluorophenyl]-2-isoxazoline carboxylic acid) and 12.5 mmol/L (methyl ester of 3-[2]-fluorophenyl]-2-isoxazoline carboxylic acid), respectively. In conclusion, the findings of this study indicate that 3-aryl-2-isoxazoline-5-carboxylic acids and their methyl esters, containing a single fluorine atom in the aryl group, effectively suppress the activation of platelet receptors GPIIa/IIIb.

Keywords: Antiplatelet agent; Heterocycle; 2-isoxazoline; Platelet; Flow cytometry

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1. Introduction

Platelets, or thrombocytes, are essential components of the hemostatic system, playing a key role in preventing blood loss during trauma. However, their function extends beyond merely protecting the body against bleeding. Platelets are also involved in pathological mechanisms, leading to thrombus formation, which can cause acute vascular diseases,

such as acute coronary syndromes, unstable angina, myocardial infarction, ischemic attack, and peripheral arterial disease.[1] 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, which are necessary to stop bleeding. However, in atherosclerotic conditions, platelets begin to aggressively interact with the vascular wall when an atheromatous plaque ruptures, leading to the formation of pathogenic thrombi that block blood flow and cause ischemic tissue damage.[2] In recent years, researchers have discovered that platelets are not only involved in thrombus formation but also play a role in the inflammatory processes associated with atherogenesis. For example, molecules such as soluble CD40 ligand, C-C chemokine ligand 5, and soluble P-selectin mediate platelet interactions with other cells, contributing to the progression of atherosclerotic disease.[3] These interactions highlight the versatility of platelet functions, which can both protect and harm the vascular wall. Following an atheromatous plaque rupture, platelets adhere to the damaged vessel area, releasing granule contents that activate platelets and form thrombi. This process can cause intermittent or persistent obstruction of blood flow, resulting in ischemic tissue injury and organ dysfunction with serious clinical consequences.[4]

Acetylsalicylic acid (aspirin) has traditionally been used to combat platelet hyperactivity and reduce the risk of serious ischemic events, such as stroke and myocardial infarction. This drug has become the standard of care for many patients with cardiovascular disease, including those who have undergone coronary artery bypass grafting. Acetylsalicylic acid acts by inhibiting the enzyme cyclooxygenase, which leads to a decrease in the synthesis of thromboxane A2, a potent agonist of platelet aggregation. However, despite its effectiveness, the use of aspirin is associated with some limitations and risks. For example, some patients may develop resistance to aspirin, which reduces its effectiveness. In addition, long-term use may lead to an increased risk of gastrointestinal bleeding. In this regard, new antiplatelet drugs, such as clopidogrel and ticlopidine, are being studied, which can be combined with aspirin to increase the effectiveness of treatment and reduce the risk of thrombosis.[5] In recent years, there has also been interest in studying the role of platelets in other pathological aspects, including their role in immune responses. Platelets can interact with immune cells, such as neutrophils and monocytes, promoting inflammation and modulating the immune response. This opens new horizons for understanding their role in the pathogenesis of various diseases, including infectious and autoimmune diseases. Thus, platelets are multifunctional cells that play a key role in maintaining hemostasis and the development of multiple pathologies. They can protect the body from blood loss and contribute to serious diseases like atherosclerosis and its complications. Understanding the mechanisms of platelet activation and their interactions with other cells and molecules is important for developing new therapeutic strategies to reduce the risk of thrombosis and improve the prognosis for patients with cardiovascular diseases.

This work aimed to study the ability of synthesized fluorophenyl-substituted 2-isoxazoline-5-carboxylic acids and their derivatives in inhibiting ADP-dependent platelet aggregation. The preparation of platelet aggregation inhibitors based on compounds containing an isoxazole ring with aromatic substituents in positions 3 and 5 of the heterocyclic fragment has been previously described.[6] Among the compounds obtained, some were found to slow down platelet aggregation. However, a limitation of the previously described compounds is the absence of functional groups in the aromatic substituents, which could increase the affinity of the inhibitor molecules to platelet receptors. Therefore, the present work investigated the activity of compounds containing only one aromatic fragment in positions 3 and 5, a carboxyl group either in a free state or in the form of its methyl ester. Compounds containing an aromatic fragment linked by bridging groups to the isoxazole ring have also been studied.[7] It was assumed that the absence of conjugation in the heterocyclic fragment of 2-isoxazoline and the rigidity imparted to the molecule by the second aromatic fragment would increase the inhibitory ability of the compounds under study. It should be noted that work is currently ongoing to obtain new isoxazole- and 2-isoxazoline-containing substances to assess their potential for medical use.[8-10] The novelty of the compounds studied in this work lies in their fluorine-substituted nature conjugated with the 2-isoxazoline cycle, which has not been explored as substances that promote the inhibition of platelet aggregation.

2. Materials and methods

2.1. Compounds of study

Infrared (IR) spectra were recorded using a Specord 75 IR instrument (Carl Zeiss Jena, Germany). Ultraviolet (UV) spectra of the solutions were obtained using a Specord M40 instrument (Carl Zeiss Jena, Germany). Nuclear magnetic resonance (NMR) spectra of solutions were recorded on a Bruker NMR spectrometer, Avance 400 (400 MHz) (Bruker, USA) in deuterochloroform, with chemical shifts (δ) reported relative to tetramethylsilane as the internal standard. Reaction progress and the characteristics of the obtained compounds were monitored using thin-

layer chromatography on Silufol UV-254 plates (Merck, Germany).

The target compounds 1 – 6 were synthesized using the following method (Figure 1). A solution of oxime (10 mmol) in dichloromethane (20 mL) at 0°C was added dropwise to a mixture of acrylic acid (or methyl acrylate) (10 mmol), 1.5 mL triethylamine (11 mmol), and 5% aqueous sodium hypochlorite solution (20 mL). The reaction mixture was stirred for 60 min and then extracted with dichloromethane (3 – 15 mL) in ice water. The aqueous layer was separated, and the combined organic extracts were dried over sodium sulfate. The solvent was distilled off under reduced pressure. The resulting solid was recrystallized from propan-2-ol. The purified products were then characterized using analytical techniques, as detailed below.

(i) Compound 1: 3-(2-Fluorophenyl)-2-isoxazoline carboxylic acid. The compound has a yield of 83% and a melting point between 181 and 183°C. The IR spectrum (KBr, cm^{-1}) shows peaks at 3100, 3093 (C-H aromatic), 1807, 1260 (COO), 1649 (C = N), and 1600, 1508 (C = C aromatic). The UV spectrum (EtOH, λ_{max}, nm) is 277. The ^{1}H NMR shows the following shifts: δ 2.96 (2H, dd, J = 15.5, 7.4 Hz), 5.31 (1H, dd, J = 7.9, 6.8 Hz), 7.14 – 7.35 (2H, 7.20 (ddd, J = 8.3, 1.1, 0.5 Hz), 7.28 (ddd, J = 7.9, 7.3, 1.1 Hz), 7.43 – 7.59 (2H), 7.49 (ddd, J = 7.9, 1.5, 0.5 Hz), and 7.52 (ddd, J = 8.3, 7.3, 1.5 Hz). The ^{13}C NMR shows: δ 41.8 (1C, s), 81.2 (1C, s), 115.5 (1C, s), 126.7 (1C, s), 128.4 (1C, s), 129.3 (1C, s), 131.3 (1C, s), 155.5 (1C, s), 159.7 (1C, s), and175.2 (1C, s).

(ii) Compound 2: 3-(3-Fluorophenyl)-2-isoxazoline carboxylic acid. The compound has a yield of 85 % and a melting point between 191 and 193°C. The IR spectrum (KBr, cm^{-1}) shows peaks at 3095, 3085 (C-H aromatic), 1805, 1265 (COO), 1651 (C = N), and 1605, 1510 (C = C aromatic). The UV spectrum (EtOH, λ_{max}, nm) is 275. The ^{1}H NMR shows the following shifts: δ 2.79 – 3.04 (2H, 2.87 (dd, J = 15.5, 6.8 Hz), 2.96 (dd, J = 15.5, 7.9 Hz)), 5.26 (1H, dd, J = 7.9, 6.8 Hz), 7.04 – 7.27 (2H, 7.10 [ddd, J = 8.1, 1.4, 1.2 Hz], 7.22 [ddd, J = 1.7, 1.4, 0.5 Hz]), 7.45 (1H, ddd, J = 8.1, 7.7, 0.5 Hz), and 7.65 (1H, ddd, J = 7.7, 1.7, 1.2 Hz). The ^{13}C NMR shows δ 41.8 (1C, s), 81.2 (1C, s), 115.0 – 115.1 (2C, 115.0 [s], 115.1 [s]), 127.3 (1C, s), 130.2 (1C, s), 132.1 (1C, s), 155.7 (1C, s), 161.2 (1C, s), and 175.2 (1C, s).

(iii) Compound 3: 3-(4-Fluorophenyl)-2-isoxazoline carboxylic acid. The compound has a yield of 82% and a melting point between 217 and 219°C. The IR spectrum (KBr, cm^{-1}) shows peaks as 3105, 3085 (CH aromatic), 1800, 1255 (COO), 1645 (C = N), and 1605, 1,515 (C = C aromatic). The UV spectrum (EtOH, λ_{max}, nm) is 272. The ^{1}H NMR shows the following shifts: δ 2.94 (2H, dd, J = 15.5, 7.4 Hz), 5.30 (1H, dd, J = 7.9, 6.8 Hz), 7.04 (2H, ddd, J = 8.7, 1.0, 0.6 Hz), and 7.93 (2H, ddd, J = 8.7, 1.6, 0.6 Hz). The ^{13}C NMR shows δ 41.8 (1C, s), 81.2 (1C, s), 115.4 (2C, s), 125.3 (1C, s), 128.6 (2C, s), 155.7 (1C, s), 162.5 (1C, s), and 175.2 (1C, s).

(iv) Compound 4: Methyl ester of 3-(2-fluorophenyl)-2-isoxazoline carboxylic acid. The compound has a yield of 87% and a melting point between 158°C and 160°C. The IR spectrum (KBr, cm^{-1}) shows peaks at 3110, 3090 (C-H aromatic), 1798, 1271 (COO), 1653 (C = N), and 1603, 1504 (C = C aromatic). The UV spectrum (EtOH, λ_{max}, nm) is 275. The ^{1}H NMR shows δ 2.97 (2H, dd, J = 15.5, 7.4 Hz), 3.75 (3H, s), 5.29 (1H, dd, J = 7.9, 6.8 Hz), 7.14 – 7.35 (2H, 7.20 [ddd, J = 8.3, 1.1, 0.5 Hz], 7.28 [ddd, J = 7.9, 7.3, 1.1 Hz]), 7.52 (1H, ddd, J = 8.3, 7.3, 1.5 Hz), and 7.72 (1H, ddd, J = 7.9, 1.5, 0.5 Hz). The ^{13}C NMR shows δ 41.8 (1C, s), 52.2 (1C, s), 77.8 (1C, s), 115.5 (1C, s), 126.7 (1C, s), 128.4 (1C, s), 129.3 (1C, s), 131.3 (1C, s), 155.5 (1C, s), 159.7 (1C, s), and 170.2 (1C, s).

(v) Compound 5: Methyl ester of 3-(3-fluorophenyl)-2-isoxazoline carboxylic acid. The compound has a yield of 86% and a melting point between 165 and 167°C. The IR spectrum (KBr, cm^{-1}) shows peaks at 3107, 3096 (C-H aromatic), 1802, 1263 (COO), 1653 (C = N), and 1607, 1509 (C = C aromatic). The UV spectrum (EtOH, λ_{max}, nm) is 273. The ^{1}H NMR shows the following shifts: δ 2.92 (2H, dd, J = 15.5, 7.4 Hz), 3.75 (3H, s), 5.28 (1H, dd, J = 7.9, 6.8 Hz), 7.04 – 7.27 (2H, 7.10 [ddd, J = 8.1, 1.4, 1.2 Hz], 7.22

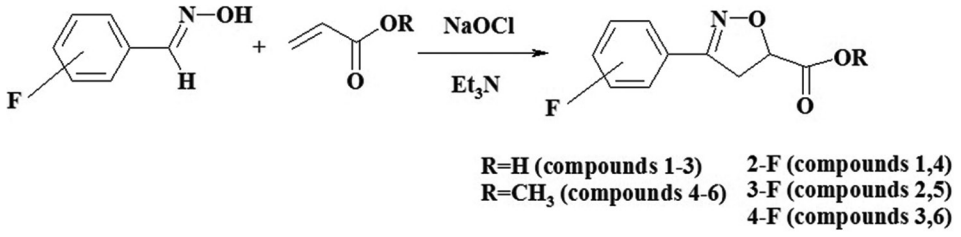

Figure 1. Synthesis of the compounds

[ddd, J = 1.7, 1.4, 0.5 Hz]), 7.45 (1H, ddd, J = 8.1, 7.7, 0.5 Hz), and 7.64 (1H, ddd, J = 7.7, 1.7, 1.2 Hz). ^{13}C NMR: δ 41.8 (1C, s), 52.2 (1C, s), 77.8 (1C, s), 115.0 – 115.1 (2C, 115.0 (s), 115.1 (s)), 127.3 (1C, s), 130.2 (1C, s), 132.1 (1C, s), 155.7 (1C, s), 161.2 (1C, s), and 170.2 (1C, s).

(vi) Compound 6: Methyl ester of 3-(4-fluorophenyl)-2-isoxazoline carboxylic acid. The compound has a yield of 84% and a melting point between 171 and 173°C. The IR spectrum (KBr, cm^{-1}) shows the following peaks: 3101, 3092 (C-H aromatic), 1806, 1263 (COO), 1651 (C = N), and 1603, 1507 (C = C aromatic). The UV spectrum (EtOH, λ_{max}, nm) is 271. The ^{1}H NMR shows the following: δ 2.94 (2H, dd, J = 15.5, 7.4 Hz), 3.75 (3H, s), 5.27 (1H, dd, J = 7.9, 6.8 Hz), 7.04 (2H, ddd, J = 8.7, 1.0, 0.6 Hz), and 7.93 (2H, ddd, J = 8.7, 1.6, 0.6 Hz). The ^{13}C NMR shows δ 41.8 (1C, s), 52.2 (1C, s), 77.8 (1C, s), 115.4 (2C, s), 125.3 (1C, s), 128.6 (2C, s), 155.7 (1C, s), 162.5 (1C, s), and 170.2 (1C, s).

2.2. Light transmission method

To assess platelet aggregation in our study, we used a Solar 2111 aggregometer (Solar, Republic of Belarus), which allows monitoring platelet aggregation using the light transmission method. This technique is based on the change in light transmission in the sample when platelets aggregate and form larger structures, leading to a decrease in the light intensity passing through the sample. The experiment used 480 µL of platelet-rich plasma (PRP) with a concentration of 200 × 10^9 platelets per liter. In the reaction vessel, the plasma was pre-incubated with 20 µL of physiological saline, which served as a control sample, and with the test substance. After 3 min of incubation, 20 µL of ADP solution with a concentration of 8 µM was added to the mixture. ADP is a potent activator of platelet aggregation, and its addition initiated the aggregation process, which was then measured for 6 min.

2.3. Flow cytometry method

The ability to inhibit platelet aggregation was studied using flow cytometry according to the method proposed by Vinholt *et al.*[11] A solution of ADP (final concentration 12 µmol/L) and the corresponding synthesized substance in 10 µL of dimethyl sulfoxide (DMSO) was added to 100 µL of PRP, prepared with the integrity PRP Kit (PRP-62621) (Integrity PRP, United States). A control sample was prepared by adding 10 µL of DMSO without the test compound to PRP. The activation of platelets by ADP induces conformational changes in glycoprotein (GP) IIa/IIIb receptors, which are responsible for further aggregation. Samples of PRP with added effectors were kept at room temperature for 15 min, followed by the addition of labeled antibodies: CD41a-fluorescein isothiocyanate (FITC) and CD61-phycoerythrin (PE). Further analysis of platelet surface markers GPIIa (CD41a) and GPIIIb (CD61) was performed using a flow cytometer Perlong FC2060 (Perlong Medical Equipment, China). The gating strategy was based on determining the number of double positive ($CD41a^+$ $CD61^+$) cells on the cytogram. Methyl ester of (+)-(S)-alpha-(o-chlorophenyl)-6,7-dihydrothieno[3.2-c]pyridine-5(4H)-acetic acid (Clopidogrel), which is currently used as an antiplatelet agent (final concentration 5 and 10 mmol/L, respectively) was used as a positive control.

2.4. Statistical analysis

To ensure the reliability of the results, all measurements were performed in triplicate, which eliminated random errors and increased the reliability of the data. The platelet aggregation results were determined as the maximum amplitude expressed as a percentage. This indicator was calculated using specialized software (version 2.1, MedCalc) that analyzed the data obtained during the experiment. Inhibition of maximum platelet aggregation was estimated as a percentage compared to the control, which made it possible to determine to what extent the test substance affected aggregation compared to saline. The data were expressed as mean ± standard error of the mean.

3. Results and discussion

All the studied compounds showed the ability to suppress the aggregation ability of platelets. Increasing the concentration of the substance from 1 to 25 mmol/L led to an increase in the inhibitory effect of the compounds. We studied the ability of the obtained compounds to inhibit platelet aggregation in two ways. The first is the classical method of determining the ability of platelets to aggregate using the light transmission method (Figure 2).[11]

The second method was based on flow cytometry. Specific GP receptors GPIIa/IIIb expressed on the platelet surface are known to be involved in platelet aggregation.[5]

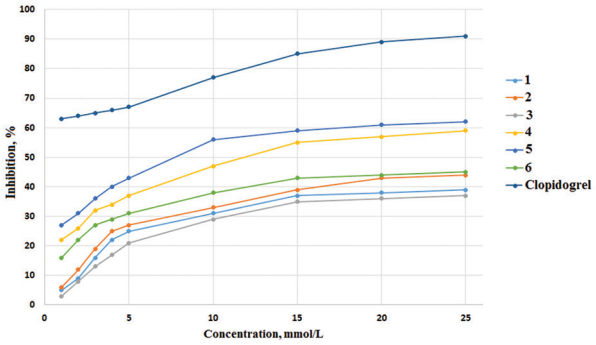

Figure 2. Inhibition of platelet aggregation (light transmission method)

The activation of platelets by adding ADP leads to a change in the conformation of membrane receptors, and GPIIa/IIIb is responsible for further aggregation. Samples of PRPR with added effectors were kept at room temperature, followed by an addition of a solution of labeled antibodies CD41a-FITC (antibodies to the GPIIa receptor) and CD61-PE (antibodies to the GPIIIb receptor). Subsequently, an analysis was performed, and the number of double-positive cells ($CD41a^+$ $CD61^+$) was counted and compared with the number of the same cells in the control sample, which did not contain any inhibitors. The obtained data on the ability to inhibit platelet aggregation is shown in Figure 3.

A comparison of both methods based on the graphs obtained for the dependence of the inhibition capacity indicates the similarity of the results obtained. It should be noted that methyl esters, compared to substances with a free carboxyl group, showed a stronger ability to suppress the transition of platelet GPIIa/IIIb receptors to an active state, in which they are then able to bind to fibrinogen and, subsequently, undergo further aggregation. This is evident from the position of the inhibition curves in the graphs in Figures 2 and 3. Noticeably, at a concentration of 1 mmol/L of the studied compounds, the degree of inhibition is 15 – 27% and gradually increases thereafter. Unlike methyl esters 4 – 6, compounds with a free carboxyl group at a concentration of 1 mmol/L exhibit the ability to inhibit platelet aggregation in the 5 – 7% range. A further increase in concentration also leads to an improvement in the ability to suppress platelet fusion. However, the growth is more gradual, and even at a concentration of 25 mmol/L, it does not reach 50%.

A comparison with the currently used antiplatelet agent clopidogrel indicates the need for further research into new compounds, which may result in modifying the aromatic substituent in the structure of new substances. The activity of compounds containing the isoxazole ring[7] turned out to be higher (≥60%) than the inhibitory capacity of the compounds synthesized in this study. A comparison of our results with other compounds containing a similar heterocycle[6] showed a relatively equal ability to inhibit platelet aggregation. The degree of inhibition of the compounds we obtained was 21 – 43% at 5 mmol/L and 29 – 56% at 10 mmol/L. The inhibitory ability of the previous compounds was in the range of 35 – 65% at a concentration of 4.4 – 4.5 mmol/L.

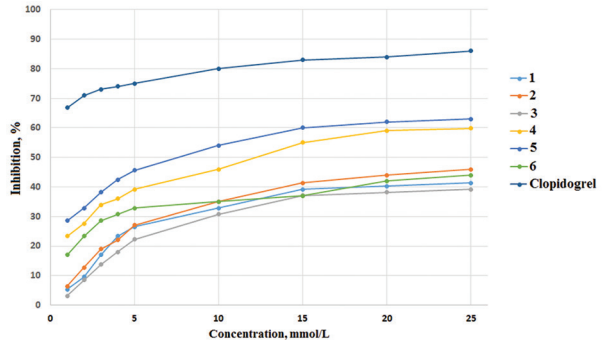

Figure 3. Inhibition of platelet aggregation (flow cytometry method)

While the simplicity of the obtained compounds may not suggest the presence of exceptional properties in platelet aggregation, we believe the data hold significant value. They provide insights into the establishment of a "structure-properties" relationship in the obtained compounds.

4. Conclusion

It has been established that 3-aryl-2-isoxazoline-5-carboxylic acids and their methyl esters containing one fluorine atom in the aryl group effectively suppress the activation of platelet receptors GPIIa/IIIb. This opens up prospects for using these compounds as synthetic fragments in designing the structure and synthesis of new antiplatelet agents. Ongoing research focuses on synthesizing new derivatives of 2-isoxazoline, particularly those incorporating an N-substituted amide group in position 5 of the heterocyclic fragment. The results of this work indicate that fluorine atoms located in the ortho- and meta-positions of the aromatic substituent (compounds 4 and 5) are more effective than fluorine in the para-position. The current efforts are directed toward synthesizing compounds with this arrangement of substituents, with results to be reported in the future.

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Conflict of interest

The authors declare that they have no competing interests.

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Ethics approval and consent to participate

This research involved the use of platelet-rich plasma from healthy donors. All donors gave written voluntary consent to participate in the study.

Consent for publication

Donors consented to the publication of their data.

Availability of data

Data will be made available upon request to the corresponding author.

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