Synthesis, Characterization and Antiplatelet Activity of Antithrombotic novel 2,5-substituted aryl-7-phenyl-1,3,4-oxadiazolo-[3,2-a]-1,3,5-triazine Derivatives

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Abstract

Synthesized fused heterocyclic compounds 1,3,4-oxadiazolo[3,2-a]-s-triazine derivatives based on microwave mediate multi-component reaction (MCRs). In general, multi-component reactions (MCRs) are economically and environmentally very advantageous because multi-step synthesis produce considerable amounts of waste mainly due to complex isolation procedures often involving expensive, toxic and hazardous solvents after each step. Simpler procedures, equipment, lower costs, time and energy and environmentally friendly. All the synthesized compounds were characterized using physical and spectral analysis. Antiplatelet Activity of Antithrombotic synthesized compounds was carried out. A low toxicity profile of this series was observed through hemolytic, genotoxic and mutagenic assays. The most active derivatives were able to reduce both PGE2 and TXB2 production in human platelets, suggesting a direct inhibition of COX-1. These results reinforce their promising profile as lead antiplatelet agents for further in vivo experimental investigations.

Keywords: *Triazine, Semicarbazone, Schiff's base, Microwave mediate multi-component reaction (MCRs), Antiplatelet Activity,*

1. INTRODUCTION

In general, antithrombotic drugs, including antiplatelet agents (e.g., clopidogrel, aspirin, tirofiban), are the primary treatment option for these diseases. However, they can lead to serious adverse reactions in some patients, including bleeding, neutropenia, thrombocytopenia and drug resistance[1]. Recent reports point to the highly expression of multidrug resistance protein 4 (MRP4) in platelets of patients with high incidence of transitory aspirin resistance. MRP4 is an ATP-binding transporter which acts as an unidirectional pump for organic anionic compounds such as acetylsalicylate (the organic anion of aspirin)

and is related to the extrusion of aspirin from platelets, reducing its pharmacological inhibition of cyclooxygenase-1 (COX-1). Suboptimal platelet inhibition by aspirin leads to an incomplete suppression of thromboxane generation, that is independently associated with increased risk of cardiovascular events[2]. Many non-steroidal anti-inflammatory drugs (NSAIDs) with antiplatelet activity due to COX-1 inhibition comprise the class of carboxylic acids, based on their ability to form strong salt-bridges with the guanidinium group of Arg120, located at the entrance of the cyclooxygenase site of COX-1[3]. The role of this anchoring mechanism exemplifies that the ability of an acidic functional group to become anionic at physiological pH is a valuable structural and physicochemical feature of NSAIDs[4-7]. Unfortunately, the stable carboxylate anions formed by such compounds have a known. Hemostasis is a widely studied topic due to the pathogenic nature of thrombotic and bleeding disorders. The inappropriate activation of the hemostatic system contributes to the development of severe pathophysiological disorders, including the thromboembolic diseases, such as atherothrombosis and venous thromboembolism[8]. A microwave is a form of electromagnetic energy that falls at the lower frequency at the end of electromagnetic spectrum[9]. Microwave heating is the best process due to the microwave couple directly with the molecule that are present in the mixture, leading to fast rise in temperature, faster reaction and cleaner chemistry[10-18]. The microwave is also called as green chemistry because it does not produce any hazardous material like gas fumes or heating using external energy source[19-25]. Microwave uses electromagnetic radiation that passes through material and causes oscillation of molecule which produces heat. Microwave heating produces heat in entire material in the same rate and the same time at a high speed and at a high rate of reaction. Microwave assisted synthesis has become an important tool to the medicinal chemist for rapid organic synthesis[26-28]. Conventional method of organic synthesis usually requires longer heating time, tedious apparatus setup which result in higher cost of process and the excessive use of solvents or reagents lead to environmental pollution. Growth of green chemistry holds necessary potential for the reduction of by product, a reduction in the waste production and a lowering of energy costs. Due to its ability to couple directly with reaction molecule and passing thermal conductivity leading to fast rise in the temperature microwave irradiation had used to improve many organic synthesis. The Principle behind the heating in microwave oven is because interaction of charged particle of reaction material with electromagnetic wavelength of particular frequency. The phenomena of the producing heat by electromagnetic irradiation are either by conduction or collision. Computational studies are the crucial steps in the drug designing. Docking study is the computational routine to determine probable binding manners of a ligand to the dynamic site of a receptor. It makes an image of the dynamic site with interaction points known as grid. Then it fits the ligand in the binding site either by grid search or energy search. Due to failure of ADME so it necessary to perform docking studies before pharmacological activity. An outbreak of coronavirus disease (COVID-19) caused by the novel severe acute respiratory syndrome coronavirus-2 (SARS-CoV-

2) raises an unparalleled challenge in the discovery of appropriate drugs for prevention and treatment. Given the rapid pace of scientific research and clinical data produced by the large number of people quickly infected with SARS-CoV-2, clinicians need reliable proof of successful medical care for this infection as in intial stage with help of molecular docking software it is easy to do in-silico study[29-31]. The chemical modification of drug delivery system for protein and peptide drugs is important in improving both enzymatic stability and membrane permeations can help to have good biological activity from any heterocyclic compound modification. Someday soon, you might be making your own medicines at home. That's because researchers have tailored a 3D printer to synthesize pharmaceuticals and other chemicals from simple, widely available starting compounds fed into a series. In addition we explored their potential molecular targets as well as their toxicological profile[32-38]. The present study report the synthesis of 2,5-substituted aryl-7-phenyl-[1,3,4]-oxadiazolo- [3,2-a][1,3,5]-triazine. Required semicarbazones were prepared by using the reported method as a reaction between aldehyde and semicarbazide (Vogel's,1996). Starting compound 2-amino-5-aryl-1,3,4-oxadiazole were synthesized by using Semicarbazone (0.01 M) and sodium acetate (0.02 M) were dissolved in 30–40 ml of glacial acetic acid taken in a round-bottomed flask equipped with a separating funnel for the addition of bromine. Bromine (0.7 ml in 5ml glacial acetic acid) was added slowly to it, while stirring magnetically. After half an hour stirring, the solution was poured on crushed ice. The resulting solid was separated, dried and recrystallized from aldehyde free ethanol. Respective Schiff's base were synthesized reaction between 2-amino-5-aryl-1,3,4-oxadiazole and aromatic aldehyde 26. By considering the above facts a novel series of 2,5-substituted aryl-7-phenyl-[1,3,4]-oxadiazole-[3,2-a][1,3,5]-triazine derivatives have been synthesized and screened for antimicrobial activity. The structures of the compounds were assigned on the basis of IR, 1HNMR and Mass spectral data[39-43].

2. MATERIAL AND METHODS

All the chemicals used were that of laboratory grade. Melting points were determined in open capillary tubes and were found uncorrected. The progresses of reactions were monitored by TLC. All compounds were purified by recrystallization with suitable organic solvents. The Purity of all the compounds was checked on Precoated silica gel-G plates using iodine vapour as detecting agent. IR spectra were recorded on FT-IR Spectrometer using KBr disc method. 1H-NMR spectra were recorded on Bruker avance-400 MHz NMR spectrometer in DMSO, CDCl3. Mass spectra were recorded.

CHEMISTRY

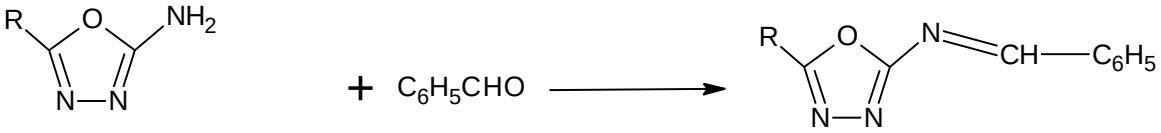
General Procedure for the Synthesis of Schiff's base

A solution of 2-amino-5-aryl-1,3,4-oxadiazole (0.01 M) was prepared in 30 ml alcohol in a round-bottomed flask. Benzaldehyde (0.01 M) then added to it. The mixture was refluxed for 5–6 hr. The volume of alcohol was reduced to half by distillation under reduced pressure. The resulting solution was poured on crushed ice. The precipitate which got separated was dried and recrystallized from alcohol.

General Procedure for the Synthesis of 2,5-substituted aryl-7-phenyl-[1,3,4]-oxadiazole-[3,2-a][1,3,5]-triazine

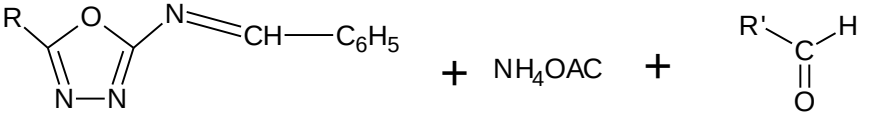
Schiff's base (compound-1) (0.01mol) was mix with ammonium acetate and various aromatic aldehyde treated in MW irradiation at 480 W for three times with minimum 2 min of intervals. Step-2 based on microwave mediate multi-component reaction (MCRs).

1st STEP:



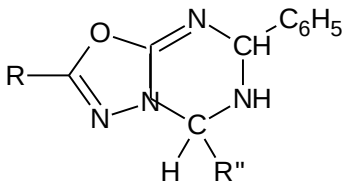
2-amino-5-aryl-1,3,4-oxadiazole Benzaldehyde Schiffs base

2nd STEP:



Schiffs base Ammonium acetate Various aldehyde

MW irradiation 480 W — 2-3 min 3-time



2,5-substituted aryl-7-phenyl-[1,3,4]-oxadiazole-[3,2-a][1,3,5]-triazine

Fig 1. Scheme of work

2-(4-hydroxyphenyl)-5-(2-nitrophenyl)-7-phenyl-6,7-dihydro-5H-[1,3,4]oxadiazolo[3,2-a] [1,3,5]triazine (2a)

M.P.-262°C, IR(KBr): 3434.67(O-H str.), 3312.78(N-H str.), 3117.92 (=C-H str.), 3048.93(C-H str.), 1547.56(C=C str.), 1608.26(C=N str.),1097.62(C-O-C str.), 1254.04(N-N=C str.), 1629.41(C-NO2 str.),1H-NMR(DMSO,δ ppm): 2.45(s, 1H, NH), 4.10 (s, 1H, CH), 5.27(s, 1H, OH), 5.6(s, 1H, CH), 7.01–7.78(m, 14H, Ar-H), MS: m/z-415.26

2-(4-hydroxyphenyl)-5-(3-nitrophenyl)-7-phenyl-6,7-dihydro-5H-[1,3,4]oxadiazolo[3,2-a] [1,3,5]triazine (2b)

M.P.-296°C, IR(KBr): 3434.65(O-H str.), 3370.23(N-H str.), 3068.65 (=C-H str.), 2917.59(C-H str.), 1531.55(C=C str.), 1657.18(C=N str.), 1075.56(C-O-C str.), 1202.31(N-N=C str.), 1603.97(C-NO2 str.), MS: m/z-415.04

2-(4-hydroxyphenyl)-5-(4-nitrophenyl)-7-phenyl-6,7-dihydro-5H-[1,3,4]oxadiazolo[3,2-a] [1,3,5]triazine (2c)

M.P.-279°C, IR(KBr): 3454.10(O-H str.), 3268.22(N-H str.), 2970.71(C-H str.), 1443.51(C=C str.), 1619.66(C=N str.), 1095.42(C-O-C str.), 1242.24(N-N=C str.), 1536.60(C-NO2 str.), MS: m/z-415.18

2-(4-hydroxyphenyl)-5-(2-chlorophenyl)-7-phenyl-6,7-dihydro-5H-[1,3,4]oxadiazolo[3,2-a] [1,3,5]triazine (2d)

M.P.-307°C, IR(KBr): 3452.04(O-H str.), 3335.98(N-H str.), 2968.97 (C-H str.), 1467.73(C=C str.), 1615.89(C=N str.), 1037.88(C-O-C str.), 1249.27(N-N=C str.), 769.09 (C-Cl str.), 1H-NMR(DMSO,δ ppm): 2.56(s, 1H, NH), 4.12(s, 1H, CH), 5.18(s, 1H, OH), 5.47 (s, 1H, CH), 6.63 – 7.75(m, 14H, Ar-H), MS: m/z-404.36

2-(4-hydroxyphenyl)-5-(4-chlorophenyl)-7-phenyl-6,7-dihydro-5H-[1,3,4]oxadiazolo[3,2-a] [1,3,5]triazine (2e)

M.P.-284°C, IR(KBr): 3454.08(O-H str.), 3091.93(=C-H str.), 2938.27 (C-H str.), 1531.02(C=C str.), 1693.49(C=N str.), 1037.38(C-O-C str.), 1303.62(N-N=C str.), 719.82(C-Cl str.), MS: m/z-404.39

2-(4-hydroxyphenyl)-5-(4-dimethylaminophenyl)-7-phenyl-6,7-dihydro-5H-[1,3,4]oxadia zolo[3,2-a][1,3,5]triazine (2f)

M.P.-236^{o}C, IR(KBr): 3433.34(O-H str.), 3370.23(N-H str.), 3068.65(=C-H str.), 2917.59(C-H str.), 1445.26(C=C str.), 1470.73(C=N str.), 1075.56(C-O-C str.), 1202.31(N-N=C str.),1H-NMR(DMSO,δ ppm): 2.38(s, 1H, NH), 2.99(s , 6H, CH3), 3.80 (s, 1H, CH), 5.23(s, 1H, OH), 5.63(s, 1H, CH), 6.74-7.71(m, 14H, Ar-H), MS: m/z-413.23

2-(4-hydroxyphenyl)-5-(3,4,5-trimethoxyphenyl)-7-phenyl-6,7-dihydro-5H [1,3,4]oxadiazolo[3,2-a][1,3,5]triazine (2g)

M.P.-310^{o}C, IR(KBr): 3391.90(O-H str.), 3054.47(=C-H str.), 2970.64(C-H str.), 1493.62(C=C str.), 1573.84(C=N str.), 1082.15(C-O-C str.), 1229.27(N-N=C str.), MS: m/z-460.04

2-(4-hydroxyphenyl)-5-(4-methoxyphenyl)-7-phenyl-6,7-dihydro-5H-[1,3,4]oxadiazolo [3,2-a][1,3,5]triazine (2h)

M.P.-223^{o}C, IR(KBr): 3417.82(O-H str.), 3055.16(=C-H str.), 2981.81(C-H str.), 1560.09(C=C str.), 1643.39(C=N str.), 1036.90(C-O-C str.), 1333.39 (N-N=C str.), MS: m/z-400.14

2-(4-hydroxyphenyl)-5-(3-methoxy-4-hydroxyphenyl)-7-phenyl-6,7-dihydro-5H-[1,3,4]oxa diazolo[3,2-a][1,3,5]triazine (2i)

M.P.-231^{o}C, IR(KBr): 3444.06(O-H str.), 2979.75(=C-H str.), 1424.75(C=C str.), 1681.92(C=N str.), 1027.79(C-O-C str.), 1267.26(N-N=C str.), MS: m/z-416.12

2-(4-hydroxyphenyl)-5-(2,5-dichlorophenyl)-7-phenyl-6,7-dihydro-5H-1,3,4]oxadiazolo[3,2-a][1,3,5]triazine (2j)

M.P.- 215^{o}C, IR(KBr): 3208.14(O-H str.), 2938.62(C-H str.), 1451.85 (C=C str.), 1692.98(C=N str.), 1071. 64(C-O-C str.), 1264.72(N-N=C str.), 673.85(C-Cl str.), MS: m/z-439.07.

PHARMACOLOGY

Platelet Aggregation Assays

The human blood samples were obtained from adult volunteers, who abstained from the use of drugs or other substances that could interfere with the experiment for at least 15 days prior. Local ethical committee approved the procedure under protocol 140/10 and 177/11. The platelet rich (PRP) and poor (PPP) plasma were prepared by differential centrifugation and platelet aggregation was monitored using the turbidimetric method described by Born and Cross with an aggregometer 4-Pack® (Helena Laboratories, Beaumount, TX, USA). Different concentrations of the synthetic derivatives and the vehicle

(DMSO 1%) were pre-incubated for 2 min before addition of the agonists (500 μM arachidonic acid and 5 μg/mL collagen). The platelet aggregation tests were performed in triplicate and the data were statistically analyzed. The concentration capable of inhibiting 50% of platelet aggregation was obtained through non-linear regression of the dose-response curve obtained for each compound (0, 10, 50 and 100 μM) performed in Matlab version 2014 (Mathworks©, Natick, MA, USA) with R2 = 0.99[44-46].

Clotting Assay

For tests of activated partial thromboplastin time (aPTT) and Prothrombin Time (PT) donor citrated plasma samples were obtained from Antônio Pedro University Hospital (pool = 6 donors). No disturbances to the hemostatic system were found, and the international normalized ratio (INR was expressed for coagulation less than or equal to 1.3. Assays were performed using the coagulation analyzer CoagLab®IV (Beijing Shining Sun Technology Co., Ltd., Beijing, China) as described by described by Sathler *et al*.

Measurement of Plasma TXB2 Levels

Plasma samples containing 100 μM of each thiourea with antiplatelet activity were used to determine the concentrations of thromboxane B2, an index of *in vitro* COX-1 activity [78]. Briefly, 10 μL of arachidonic acid (500 μM) were added to platelet-rich plasma samples containing each active derivative (100 μM) under stir conditions. After five minutes the suspensions were mixed with 3 μL Indomethacin (10 mM) stop-solution. By sonication for 15 min, platelets were disrupted, and the resulting homogenates were centrifuged at 2000 *g* for 10 min to allow complete release of TXB2. Plasma levels of TXB2 were measured in duplicate by competitive immunoassay using commercially available kits from EIA Cayman (TXB2 EIA Kit, Cayman Chemical Co, Ann Arbor, MI, USA) according to the manufacturer's instructions. A threshold of 25.75 ng/mL was defined as control plasma levels of TXB2after platelet disrupture.

Measurement of Plasma PGE2 Levels

Plasma samples containing 100 μM of each thiourea with antiplatelet activity were used to determine the concentrations of prostaglandin H2, an index of *in vitro* COX-1 activity [78]. Briefly, ozagrel (3 μL, 100 μM) were added to stop TXS activity and the aggregation were triggered with 10 μL of arachidonic acid (500 μM) in PRP samples containing each active derivative (100 μM) under stir conditions. After five minutes the suspensions were mixed with indomethacin (3 μL, 10 mM) stop-solution. By sonication for 1 min, platelets were disrupted and purification step were performed according to the manufacturer's instructions (Cayman Chemical Co.). Plasma levels of PGE2 were measured in duplicate by competitive

immunoassay using commercially available kits for EIA (PGE2 EIA Kit, Cayman Chemical Co.) according to the manufacturer's instructions. A threshold of 25.75 ng/mL was defined as control plasma levels of PGE2 after platelet disrupture[47-49].

Hemolysis Assay

Healthy erythrocytes were washed three times with PBS (pH 7.4) by centrifugation and suspended in the same buffer. All derivatives were incubated with the erythrocyte suspension for 3 h at 37 °C. The release of hemoglobin was determined by monitoring the optical density of the supernatant at 540 nm. The experiments were performed in triplicate and complete hemolysis (positive control) was determined using 1% Triton X-100. Hemolysis less than 10% indicated good hemocompatibility and non-toxicity of the molecules tested.

Reverse Mutagenesis to Histidine Prototrophy (Ames Test)-"Spot Test"

This assay was performed as described by Maron and Ames using the histidine *Salmonellatyphimurium* auxotroph mutant strains TA97, TA98, TA100 and the wild type strain TA102 (Table 5).Each assay was conducted in duplicate and the results obtained show a comparison between the thioureaderivatives and the positive control 4-NQO. The negative results indicated that the thiourea derivativeshave no mutagenic properties

Table 1. Strains of *Salmonella typhimurium* used in the reverse mutagenesis to histidine prototrophy (Ames test)-"Spot test".

Designations	Relevant Genotype
TA97	*hisD6610/ hisO1242—ΔuvrB rfa pKM101 (ampR)*
TA98	*hisD3052—ΔuvrB rfa pKM101(ampR)*
TA100	*hisG46—ΔuvrB rfa pKM101 (ampR)*
TA102	*hisG428-wild type rfapKM101(ampR) pAQ1 (tetR)*

SOS Chromotest-"Spot Test"

The SOS chromotest (spot test) was performed according to Quillardet and Hofnung, using Escherichia coli strains PQ35 and PQ37. One hundred microliters of an overnight culture of the E. coli strains are diluted in 5 mL of LB medium and the culture is incubated at 37 °C in a gyratory incubator up to a concentration of 2 × 108 bacteria/mL. Fractions of 0.1 mL of the culture are then distributed into test tube with top agar, and the mixture is poured immediately on M63 medium plate. A sample of 10 µL of the thiourea derivatives is spotted onto the center of the plate. After overnight incubation at 37 °C, the presence of a blue ring around a zone of inhibition indicates genotoxic activity. Each assay was conducted in triplicate and the results obtained show a comparison between the derivatives and the positive control 4-NQO[50-54].

Table 2. Strains of *Escherichia coli* used in SOS chromotest—"Spot test".

Designations	**Critical Markers**	**Other Markers**
PQ35	*sfiA*::Mud(Ap*lac*) *cts* *lac*ΔU169 *mal+, uvr+, galEgalY*, PhoC, *rfa*	*Same markers as GC4436 rpoB*
PQ37	*sfiA*::Mud(Ap*lac*) *cts* *lacΔU169* *mal+, uvrA, galEgalY, PhoC, rfa*	*Same markers as GC4436 rpoB*

3. RESULTS AND DISCUSSION

CHEMISTRY

Schiff's base were prepeared by adding solution of 2-amino-5-aryl-1,3,4-oxadiazole was prepared in alcohol in a round-bottomed flask. Benzaldehyde then added to it. The mixture was irradiated for 30min. The volume of alcohol was reduced to half by distillation under reduced pressure. The resulting solution was poured on crushed ice. The precipitate which got separated was dried and recrystallized from alcohol. (Compound-1) and 2,5-substituted aryl-7-phenyl-[1,3,4]-oxadiazole-[3,2-a][1,3,5]-triazine (1a-1n) and

(2a-2n).perpared Schiff's base (compound-1) was mix with ammonium acetate and various aromatic aldehyde treated in MW irradiation at 480 W for three times with minimum 2 min of intervals. Step-2 based on microwave mediate multi-component reaction (MCRs). The proposed structures of all prepared compounds were confirmed by FTIR, 1H-NMR and Mass spectroscopy.

BIOLOGICAL EVALUATION

Platelet Aggregation Assays

In physiological conditions, the release of arachidonic acid (AA) from the platelet membranes generates thromboxane A2 (TXA2), a potent platelet agonist, leading to shape-change, granule release and platelet aggregation. The main enzymes responsible for the production of TXA2 in platelets are COX-1 and thromboxane synthase (TXS), both whose inhibition is known to prevent platelet aggregation. Aggregation of healthy human platelets induced by arachidonic acid revealed the antiplatelet activity of compounds 2b, 2d, 2h and 2j, that were able to inhibit AA-induced platelet aggregation in a range of 96%–98%, statistically similar to aspirin (97.5%) at the same concentration (100 μM). These results pointed to the efficacy of our proposal on the production of novel antiplatelet thioureas. The concentrations that caused 50% inhibition of platelet aggregation revealed compound 2b (IC_{50} = 28.2 μM ± 5.8) as the most potent of the series followed by 2d (IC_{50} = 32.1 μM ± 7.0), 2h (IC_{50}= 71.8 μM ± 3.2) and 2j (IC_{50} = 52.10 μM ± 8.5). These data revealed the potency of these antiplatelet 2,5-substituted aryl-7-phenyl-1,3,4-oxadiazolo-[3,2-a]-1,3,5-triazine derivatives derivatives to inhibit the arachidonic acid pathway of platelet aggregation and suggests their ability to reduce the TXA2 production as a mechanism to impair normal platelet aggregation.

Table 3. Antiplatelet profile of 2,5-substituted aryl-7-phenyl-1,3,4-oxadiazolo-[3,2-a]-1,3,5-triazine derivatives (100 μM) and aspirin (ASA) on *in vitro* platelet aggregation of human citrated platelet-rich plasma induced by arachidonic acid (AA).

Compound code	IC_{50}
2a	184.2 ± 6.2
2b	28.2 ± 5.8
2c	304.1 ± 7.1
2d	32.1 ± 7.0
2e	171.2 ± 9.2
2f	209.4 ± 8.3
2g	158. 2 ± 0.4
2h	71.8 ± 3.2
2i	89.5 ± 9.8
2j	52.10 ± 8.5

Measurement of Plasma PGE2 and TXB2 Levels

The stable direct metabolite of PGE2, 13,14-dihydro-15-keto-Prostaglandin E2, is a known marker of the COX-1 activity [32,33]. By the measurement of total levels of this PGE2 metabolite in human platelet-rich plasma, we evaluated the feasibility of antiplatelet *N,N'*-disubstituted thioureas to inhibit COX-1 activity in healthy platelets.

Table 4. Effects of novel antiplatelet *N,N'*-disubstituted thiourea derivatives (100 μM) on PGE2 and TXB2 production determined by Enzyme Immunoassay (EIA) in comparison to dimethylsulfoxide (DMSO, 1%), Ozagrel (100 μM), Aspirin (100 μM) and Indomethacin (100 μM).

Compound code	Platelet Inhibition of PGE2 (%)	Platelet Inhibition of TXB2 (%)
2b	28.4 ± 4.1	79.1 ± 8.4
2d	22.3 ± 8.5	72.6 ± 3.1
2h	30.1 ± 6.4	82.3 ± 2.9
2j	11.4 ± 5.1	78.1 ± 5.4
Aspirin	45.8 ± 1.9	80.3 ± 4.1
Indomethacin	29.1 ± 6.2	94.1 ± 6.5
Ozagrel	-	92.1 ± 6.0
DMSO	0.0 ± 1.2	0.0 ± 0.0

Similar inhibition rates observed for indomethacin for PGE2 production were obtained by these antiplatelet thioureas at the same concentration (100 μM) suggesting a mechanism of action equally efficient to a known clinical drug. Aspirin (100 μM) showed higher inhibition of PGE2 production (47.5%) in comparison to indomethacin and the thiourea derivatives, which might be related to the non-competitive inhibition of COX-1 by aspirin through selective acetylation of Ser530. Previous studies have demonstrated the anti-inflammatory role of thiourea derivatives in xylene-induced ear swelling in mice, with mild COX-1 inhibition in comparison to aspirin, which suggested a mechanism of action that diverges from that of acetylsalicylic acid. Thromboxane B2 (TXB2), a stable TXA2 metabolite, is widely used as a prognostic risk marker of platelet activation in cardiovascular disease, which is closely related to COX-1 and Thromboxane Synthetase activity**.**

Table 5. Mutagenic and genotoxic activity of thiourea derivatives without metabolic activationevaluated by Ames test and SOS chromotest. The thiourea derivatives were dissolved in dimethylsulfoxide (DMSO) to perform assays. As positive control 4-NQO was used. Results of three different concentrations (10 μM, 100 μM and 500 μM).

Compound code	**Ames Test *S. typhimurium***				**SOS Chromotest *E. coli***	
	TA97	**TA98**	**TA100**	**TA102**	**PQ35**	**PQ37**
2a	-	-	-	-	-	-
2b	-	-	-	-	-	-
2c	-	-	-	-	-	-
2d	-	-	-	-	-	-
2e	-	-	-	-	-	-
2f	-	-	-	-	-	-
2g	-	-	-	-	-	-
2h	-	-	-	-	-	-
2i	-	-	-	-	-	-
2j	-	-	-	-	-	-
4-NQO	+	+	+	+	+	+
DMSO	-	-	-	-	-	-
ASA	-	-	-	-	-	-

The reduced production of TXB2 in platelet-rich plasma induced by the presence of the thiourea derivatives 2b, 2d, 2h and 2j presented similar inhibition rates in comparison to aspirin (78.5%), including

the most active molecules. These data prompt the potential of this series of compounds as new non anionic antiplatelet agents against human plasma. Studies suggest that the reduced TXB2 production by platelets after treatment with derivatives occurs due to the scavenging properties of hydroxyl radicals, a common property for thiourea compounds, which display antioxidant properties. However, Scholz and collaborators suggested that COX-1 inhibition has no correlation to the inactivation of hydroxyl radicals but rather to structural properties of the ligands, able to improve binding affinity.

Coagulation Assays

To verify whether the plasmatic phase of blood coagulation was affected by these new compounds, we investigated the influence of these novel thiourea compounds 2a-j in human pooled plasma (n = 6) on routine coagulation assays (activated partial thromboplastin time—aPTT, and prothrombin time—PT). Neither the extrinsic nor intrinsic pathways of the coagulation cascade were influenced by the presence of the compounds, suggesting that the antithrombotic profile of the compounds described herein relies on the direct impairment of platelet aggregation, thus differing from dual action molecules. This result reveals a small bleeding risk in comparison to dual acting molecules whose mechanism often lead to severe bleeding disorders as described in the literature.

In-vitro Toxicity Assays

The literature shows that the interaction between chemical derivatives and erythrocytes may accelerate cell aging or lead to a mechanical premature destruction of these cellular integrity with release of hemoglobin. According to our erythrocyte lysis assay no significant hemolytic profile was observed for thioureas 2a-j after incubation period (0%–9%). They were similar to aspirin (2%) ($p \leq 0.05$) and not comparable to Triton X-100 (100%), used as a positive control. Fisher and collaborators reported that hemolysis values below 10% are considered non-hemolytic, which frame these compounds within an acceptable toxicity profileThis safe profile is reinforced by further toxicological studies which revealed no mutagenicity profiles against *Salmonella typhimurium* auxotroph mutant strains for all thiourea derivatives through reverse mutagenesis and histidine prototrophy (Ames test), compared to the positive control 4-nitro-quinoline 1-oxide (4NQO), nor genotoxicity profiles, that was evaluated against *Escherichia coli* through the SOS chromotest. This low risk profile is maintained even at the highest concentrations tested (500 μM), revealing the safety profile of the series as promising for lead prototypes.

4. CONCLUSIONS

In this work we present a series of 2,5-substituted aryl-7-phenyl-1,3,4-oxadiazolo-[3,2-a]-1,3,5-triazine derivatives that were designed and synthesized in high yields (82%–97%) as non-anionic antiplatelet agents against the arachidonic acid platelet aggregation pathway. The most active antiplatelet agents (compounds 2b, 2d, 2h and 2j) were able to reduce both PGE2 and TXB2 production in human platelets, suggesting a direct inhibition of COX-1. This series of compounds shows low mutagenic and genotoxic profiles according to Ames test and SOS chromotest, and good hemocompatibility toward healthy human erythrocyte, which reinforce their promising lead profile as antiplatelet agents for further *in-vivo* experimental investigations.

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