Design, Synthesis, and Docking Studies of Arylhydrazone Compounds and Evaluation of Their Platelet Aggregation Inhibitory Effect and Cytotoxicity

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Abstract

In view of proven antiplatelet activity of hydrazone group containing compounds, two series of hydrazone derivatives were synthesized by coupling appropriate aldehydes with phenyl hydrazine and Isonicotinic acid in the presence of distilled water and a catalytic amount of glacial acetic acid. All synthesized compounds were screened for their antiplatelet activity against induced aggregation by adenosine diphosphate (ADP) and arachidonic acid (AA). The results indicate that compounds in arylhydrazone group had shown satisfactory activity. Among them, 1-(3-methoxybenzylidene)-2-phenylhydrazine (**1c**), 2-methoxy-4-(2-phenylhydrazono) methyl phenol (**1g**), and 2-((2-phenylhydrazono) methyl)-1H-pyrrole (**1h**) were found to be the most potent antiplatelet compounds with IC_{50} less than 39 μ M. Furthermore, the cell toxicity assay, (MTT test) indicates their noncytotoxic in various cell lines. None of the synthesized N-isonicotinohydrazide derivatives in this study excreted sufficient antiplatelet activity.

Introduction

Cardiovascular disorders (CVDs) are considered as the major causes of death among non-communicable diseases worldwide ^[1]. In 2017, 17.8 million people lost their lives due to cardiac and cerebral vascular diseases ^[2], and the mortality rate is expected to rise to 25million by 2030 ^[3]. It is generally proven that platelet plays a key role in vascular haemostasis and thrombosis. While platelet aggregation is a vital process in normal hemostasis and clot formation for impeding bleeding, dysregulated platelet aggregation results in pernicious CVDs like transient ischemic attack and myocardial infarction (MI) ^[4].

Amongst three classes of antithrombotic drugs (anticoagulant, antiplatelet, and thrombolytic), antiplatelet agents (such as aspirin, clopidogrel, or ticlopidine) are generally regarded as the first-line therapy for prophylaxis and treatment ^[5]. The most common antiplatelet drugs are categorized mainly into three groups; irreversible cyclooxygenase inhibitors, Glycoprotein IIB/IIIA inhibitors, and Adenosine diphosphate (ADP) receptor inhibitors ^[6]. Aspirin and triflusal impede cyclooxygenase I (COX-1) activity in transforming arachidonic acid to thromboxane A_2 (TXA₂). Clopidogrel, cangrelor, and prasugrel attach to P₂Y₁₂ ADP receptor and subsequently hinder the aggregation. Finally, abciximab, tirofiban, and eptifibatide that are IIB/IIIA antagonists and affect GP α II β (the most abundant receptor on the platelet surface), leading to inhibition of platelet aggregation ^[6].

Although, these drugs have a substantial effect on alleviating thromboembolic diseases, they are flawed with some limitations comprising their potency, safety, and patients' adherence to treatment. Some of these drawbacks include; adverse effect on the digestive system (e.g. aspirin, ticlopidine, and clopidogrel) ^[7, 8], reduction in the number of platelet and white cells (e.g. aspirin) ^[9], bleeding (e.g. tirofiban, clopidogrel, and aspirin) ^[10], and variable responses (e.g. aspirin and clopidogrel) ^[11–14].

Therefore, developing a new antiplatelet agent with little side effects and higher efficacy is an ongoing endeavor of researchers ^[10]. Compounds with hydrazone backbone have been reported to demonstrate

diverse biological properties including anticonvulsant, antidepressant, antimalarial, antimicrobial, analgesic, and anti-inflammatory^[15]. Interestingly, they can inhibit COX-1, which indicates that hydrazone derivatives are promising scaffold for designing an antiplatelet agent^[16].

The antiplatelet aggregation activity of heterocyclic acylhydrazone^[17] and 10-H-phenothiazine-1-acylhydrazone^[18] have been reported. 2-hydrazinyl-1,3,4-thiadiazole derivatives were also reported as platelet aggregation inhibitor and some of them demonstrated IC₅₀ values comparable to indomethacin when AA used as platelet aggregation inducer^[19]. We have reported the satisfactory antiplatelet activity of phenylhydrazones of indole-3-carbaldehyde (Figure 1, I) against platelet aggregation induced by AA^[20]. Considering this background, in this study two groups of hydrazones were designed and synthesized according to the general procedure of Schiff bases synthesis^[21, 22]. A series of phenylhydrazones (**1a-1j**) was designed based on phenylhydrazone of indole-3-carbaldehyde (Figure 1, I). In order to further study of antiplatelet structure-activity relationship of hydrazones, indole ring replaced by heterocyclic aromatic rings and substituted benzene rings. The compounds **2a-2l** were also designed by molecular simplification of N'-benzylidene-carbohydrazide-1H-pyrazolo[3,4-b]pyridines (Figure 1, II) which was designed and reported as antiplatelet agents by Lourenço et al.^[23]. Finally, the antiplatelet activities of synthesized compounds against ADP and AA-induced aggregation were evaluated and effective compounds were assessed for their cell toxicity.

Experimental Section

Chemistry

Chemical were purchased from Merck Chemical Company. Melting points (°C) were determined by the Electrothermal IA9300 apparatus. (Cole-Parmer, Staffordshire, UK). Infrared spectra (IR) were recorded on a Perkin-Elmer 843 spectrometer employing potassium bromide (KBr) as the diluent (PerkinElmer, UK). Proton nuclear magnetic resonance (¹H-NMR) spectra were acquired using the Bruker AVance series; AV-300, AV-500, and AV-600 MHz Spectrometer and benchtop NMR spectrometer, the NMReady-60 NMRPro spectrometer. Dimethylsulfoxide (DMSO-d₆) solution and tetramethylsaline were utilized as an internal standard. Coupling constant values (J) are given in Hertz. Peak positions are expressed in parts per million (δ). Splitting patterns are as follows: s, singlet; d, doublet; t, triplet; q, quadruplet; dd, double doublet; m, multiplet. Mass spectra were obtained by Agilent 6410 Triple Quad LC MASS equipped with Electrospray Ionization (ESI) and Agilent 7000 Triple Quad GC MASS provided Electronic Ionization (EI). (Santa Clara, United States) The development of all reactions was monitored by thin-layer chromatography (TLC) performed on aluminium sheets coated with silica and visualized by UV irradiation.

Synthesis of arylhydrazones derivatives 1a-1j

Phenyl hydrazine (0.005 mole, 0.54 g) was added to a stirring solution of corresponding aldehyde (0.005 mole) in distilled water (10mL) and in the presence of 2mL glacial acetic acid (as the catalyst). The mixture stirred at room temperature (RT). Once the solid was formed, completion of the reaction was confirmed by carrying out the TLC sample. Next, the precipitate was filtered off, washed with cold water, dried under vacuum, and recrystallized with appropriate solvents.

Compound **1j** was synthesized in a different proportion of 0.005 moles of aldehyde (Glyoxal) and 0.01 mole of phenylhydrazine under the same condition described above.

N-(4-((2-phenylhydrazono)methyl)phenyl)acetamide (**1a**) Yield: 70%; m.p. 200°C; Recrystallization solvent: ethanol: water (1:1); ¹HNMR (600 MHz, [D₆] DMSO): δ = 10.194 (s, 1H; N-NH), 10.001 (s, 1H; NH-CO), 7.787 (s, 1H; C=N), 7.59 (d, ³J_{H,H} = 8.4 Hz, 2H; Ph2-H3, Ph2-H5), 7.548 (d, ³J_{H,H} = 8.4 Hz, 2H; Ph2-H2, Ph2-H6), 7.188 (t, ³J_{H,H} = 7.2-7.8 Hz, 2H; Ph1-H3, Ph1-H5), 7.03 (d, ³J_{H,H} = 7.8 Hz, 2H; Ph1-H2, Ph1-H6), 6.71 (t, ³J_{H,H} = 7.2, 1H; Ph1-H4), 2.037 ppm (s, 3H; CH₃); IR (KBr): ν̃ = 3265 (s) (NH), 3183 (m) (CH, aromatic), 3098 (m) (CH, aliphatic), 1657 (vs) cm⁻¹ (C=N); GC-MS (EI) m/z (%): 253 (100) [M+H]⁺; elemental analysis calcd (%) for C₁₅H₁₅N₃O: C 71.13, H 5.97, N 16.59; found: C 71.21, H 5.96, N 16.53.

1-(3-nitrobenzylidene)-2-phenylhydrazine (**1b**) Yield: 40%; m.p. 122-125°C; Recrystallization solvent: ethanol: water (1:1); ¹HNMR (300 MHz, [D₆] DMSO): δ = 10.64 (s, 1H; NH), 8.39 (s, 1H; Ph2-H2), 8.061 (m, 2H; Ph2-H4, Ph2-H6), 7.92 (s, 1H; CH=N), 7.624 (t, ³J_{H,H} = 8.1 Hz, 1H; Ph2-H5), 7.216 (t, ³J_{H,H} = 7.5 Hz, 2H; Ph1-H3, Ph1-H5), 7.082 (d, ³J_{H,H} = 7.8 Hz, 2H; Ph1-H2, Ph1-H6), 6.763 ppm (t, ³J_{H,H} = 7.2 Hz, 1H; Ph1-H4); IR (KBr): ν̃ = 3319 (s) (NH), 1528 (vs) (C=N), 1499 and 1343 (s) (NO₂), 692, 739 and 816 (m) cm⁻¹ (metha substituted phenyl); GC-MS (EI) m/z (%): 241 (100) [M+H]⁺; elemental analysis calcd (%) for C₁₃H₁₁N₃O₂: C 64.72, H 4.60, N 17.42; found: C 64.75, H 4.59, N 17.36.

1-(3-methoxybenzylidene)-2-phenylhydrazine (**1c**) Yield: 50%; m.p. 51-53°C; Recrystallization solvent: ethanol: water (1:1); ¹HNMR (300 MHz, [D₆] DMSO): δ = 10.32 (s, 1H; NH), 7.82 (s, 1H; CH=N), 7.27 (m, 3H; Ph2-H2, Ph2-H5, Ph2-H6), 7.18-7.2 (t, 2H; Ph1-H3, Ph1-H5), 7.06 (d, ³J_{H,H} = 7.5 Hz, 2H; Ph1-H2, Ph1-H6), 6.85 (d, 1H; ³J_{H,H} = 7.9 Hz, Ph2-H4), 6.738 (t, ³J_{H,H} = 7.2 Hz, 1H; Ph1-H4), 3.81 ppm (s, 3H; OCH₃); IR (KBr): ν̃ = 3310 (m) (NH), 1588 (vs) (C=N), 1263 and 1033 (s) (Ar-O-CH₃), 697, 760 and 792 cm⁻¹ (m) (metha substituted phenyl); GC-MS (EI) m/z (%): 226 (100) [M+H]⁺; elemental analysis calcd (%) for C₁₄H₁₄N₂O: C 74.31, H 6.24, N 12.38; found: C 74.3, H 6.23, N 12.4.

1-phenyl-2-(3, 4, 5-trimethoxybenzylidene)hydrazine (**1d**) Yield: 30%; m.p. 130-133°C; Recrystallization solvent: ethanol: water (1:1); ¹HNMR (300 MHz, [D₆] DMSO): δ = 10.27 (s, 1H; NH), 7.77 (s, 1H; CH=N), 7.18-7.23 (t, ³J_{H,H} = 7.5-7.8 Hz, 2H; Ph1-H3, Ph1-H5), 7.06 (d, ³J_{H,H} = 7.8 Hz, 2H; Ph1-H2, Ph1-H6), 6.94 (s, 2H; Ph2-H2, Ph2-H6), 6.728 (t, ³J_{H,H} = 7.2 Hz, 1H; Ph1-H4), 3.82 (s, 6H; OCH₃, Meta, ortho), 3.67 ppm (s, 3H; OCH₃ para); IR (KBr): ν̃ = 3339 (m) (NH), 3300 (w) (CH, aromatic), 2975 (w) (CH, aliphatic), 1632 (vs)

(C=N), 1249 and 1024 cm^{-1} (s) (Ar-O-CH₃); GC-MS (EI) m/z (%): 286.1 (100) [M+H]⁺; elemental analysis calcd (%) for C₁₆H₁₈N₂O₃: C 67.12, H 6.34, N 9.78; found: C 67.2, H 6.33, N 9.77.

1-(2-chlorobenzylidene)-2-phenylhydrazine (1e) Yield: 80%; m.p. 81-85°C; Recrystallization solvent: ethanol: water (1:1); ¹HNMR (300 MHz, [D₆] DMSO): δ = 10.67(s, 1H; NH), 8.19(s, 1H; CH=N), 7.99-8.02(d, ³J_{H,H} = 7.8 Hz, 1H; Ph2-H3), 7.43(d, ³J_{H,H} = 7.65 Hz, 1H; Ph2-H6), 7.23(t, ³J_{H,H} = 7.5 Hz, 2H; Ph1-H3, Ph1-H5), 7.33(m, 2H; Ph2-H4, Ph2-H5), 7.08(d, ³J_{H,H} = 7.5 Hz, 2H; Ph1-H2, Ph1-H6), 6.77 ppm (t, ³J_{H,H} = 7.2 Hz, 1H; Ph1-H4); IR (KBr): $\tilde{\nu}$ = 3116 (w) (NH) 1607 (s) (C=N), 765 (s) cm^{-1} (C-Cl); MS (ESI) m/z (%): 231 (100) [M+H]⁺; elemental analysis calcd (%) for C₁₃H₁₁ClN₂: C 67.68, H 4.81, N 12.14; found: C 67.67, H 4.81, N 12.10.

4-((2-phenylhydrazono)methyl)benzoic acid (1f) Yield: 23%; m.p. 221-225°C; Recrystallization solvent: ethanol: water (1:1); ¹HNMR (300 MHz, [D₆] DMSO): δ = 12.86(s, 1H; COOH), 10.58(s, 1H; NH), 7.91 (d, ³J_{H,H} = 8.4 Hz, 2H; Ph2-H3, Ph2-H5), 7.886(s, 1H; CH=N), 7.73(d, ³J_{H,H} = 8.4 Hz, 2H; Ph2-H2, Ph2-H6), 7.23(t, ³J_{H,H} = 7.8 Hz, 2H; Ph1-H3, Ph1-H5), 7.09(d, ³J_{H,H} = 7.8 Hz, 2H; Ph1-H2, Ph1-H6), 6.77 ppm (t, ³J_{H,H} = 7.2 Hz, 1H; Ph1-H4); IR (KBr): $\tilde{\nu}$ = 3323 (br) (OH and NH), 3073 (w) (CH, aromatic), 1712 (m) (C=O), 1680 (s) (C=N) 767 and 699 (s) cm^{-1} (mono substituted phenyl); MS (ESI) m/z (%): 241 (100) [M+H]⁺; elemental analysis calcd (%) for C₁₄H₁₂N₂O₂: C 69.99, H 5.03, N 11.66; found: C 70.07, H 5.04, N 11.62.

2-methoxy-4-((2-phenylhydrazono)methyl)phenol (1g) Yield: 42%; m.p. 65-66°C (lit.^[26] m.p. 64-65°C); Recrystallization solvent: methanol: water (3:2); ¹HNMR (100 MHz, [D₆] CDCl₃): δ = 7.5-7.75 (s, 1H; CH=N), 6.75-7.40 (m, 10H; Ar H, CH=N and NH), 5.80-5.85 (s, 1H; OH), 3.85-4.00 (s, 3H; CH₃); IR (KBr): $\tilde{\nu}$ = 3532 (br) (OH), 3342 (m) (NH), 3083 (w) (CH, aromatic), 2969 (w) (CH, aliphatic), 1611 (vs) (C=N), 1276 and 1046 cm^{-1} (s) (Ar-O-CH₃); MS (ESI) m/z (%): 243 (100) [M+H]⁺; elemental analysis calcd (%) for C₁₄H₁₄N₂O₂: C 69.41, H 5.82, N 11.56; found: C 69.41, H 5.81, N 11.6.

2-((2-phenylhydrazono)methyl)-1H-pyrrole (1h) Yield: 70%; m.p. 137-140°C; Recrystallization solvent: ethanol; ¹HNMR (600 MHz, [D₆] DMSO): δ = 11.09 (s, 1H; NH pyrrole), 7.71 (s, 1H; CH=N), 9.87 (s, 1H; NH), 7.157 (t, ³J_{H,H} = 7.8 Hz, 2H; Ph1-H3, Ph1-H5), 7.027 (d, 2H; ³J_{H,H} = 7.8 Hz, Ph1-H2, Ph1-H6), 6.799(s, 1H; H3 pyrrole), 6.66 (t, 1H; ³J_{H,H} = 7.2 Hz, Ph1-H4), 6.23(s, 1H; H4 pyrrole), 6.05 ppm (d, 1H; H5 pyrrole); IR (KBr): $\tilde{\nu}$ = 3432 (m) (NH), 3327 (w) (CH, aromatic), 1600 (vs) (C=N) 749 and 695 (s) cm^{-1} (mono substituted phenyl); MS (ESI) m/z (%): 185 (100) [M+H]⁺; elemental analysis calcd (%) for C₁₁H₁₁N₃: C 71.33, H 5.99, N 22.69; found: C 71.29, H 5.99, N 22.72.

3-((2-phenylhydrazono)methyl)-1H-indole (1i) Yield: 70%; m.p. 200-204°C; Recrystallization solvent: methanol: water (3:2); ¹HNMR (300 MHz, [D₆] DMSO): δ = 11.29 (s, 1H; NH indole), 9.79(s, 1H; NH), 8.244(d, 1H; J=6, H5 indole), 8.10(s, 1H, C=N), 7.61(d, 1H; H6 indole), 7.41(d, ³J_{H,H} = 6.15 Hz, 1H; H4 indole), 7.17(m, 4H; Ph1-H3, Ph1-H5, H3 indole, H9 indole), 7.03(d, J=7.8 Hz, 2H, Ph1-H2, Ph1-H6), 6.67 ppm (t, ³J_{H,H} = 7.2 Hz, 1H; Ph1-H4); IR (KBr): $\tilde{\nu}$ = 3456 (s) (NH), 3312 (m) (CH, aromatic), 1610 (vs) (C=N),

1490 (m) (NH, indole ring), 782 (vs) cm^{-1} (CH out of plane bending, indole ring); GC-MS (EI) m/z (%): 236 (100) $[\text{M}+\text{H}]^+$; elemental analysis calcd (%) for $\text{C}_{15}\text{H}_{13}\text{N}_3$: C 76.57, H 5.57, N 17.86; found: C 76.58, H 5.58, N 17.84.

1,2-bis(2-phenylhydrazono)ethane (1j) Yield: 13%; m.p. 179-179.9°C; Recrystallization solvent: ethanol; ^1H NMR (600 MHz, $[\text{D}_6]$ DMSO): δ = 10.35 (s, 1H; NH), 7.63(s, 1H; CH=N), 7.19(t, $^3J_{\text{H,H}}$ = 7.2-7.8 Hz, 2H; Ph1-H3, Ph1-H5), 6.97(d, $^3J_{\text{H,H}}$ = 7.8 Hz, 2H; Ph1-H2, Ph1-H6), 6.73 ppm (t, $^3J_{\text{H,H}}$ = 7.2 Hz, 1H; Ph1-H4); IR (KBr): $\tilde{\nu}$ = 3319 (m) (NH), 3043 (w) (CH, aromatic), 1604 (vs) (C=N) 767 and 698 (s) cm^{-1} (mono substituted phenyl); GC-MS (EI) m/z (%): m/z = 238 (60) $[\text{M}+\text{H}]^+$; elemental analysis calcd (%) for $\text{C}_{14}\text{H}_{14}\text{N}_4$: C 70.57, H 5.92, N 23.51; found: C 70.55, H 5.92, N 23.53.

Synthesis of N-isonicotinohydrazide derivatives 2a-2l

Isonicotinic acid hydrazide (0.005 mole, 0.68 g) was added slowly to a stirring solution of appropriate aldehyde (0.005 mole) in distilled water (10mL) and in presence of 2mL glacial acetic acid (as catalyst). The mixture stirred at room temperature (RT). Once the solid was formed, completion of the reaction was confirmed by carrying out the TLC sample. Next, the precipitate was filtered off, washed with cold water, dried under vacuum, and recrystallized with appropriate solvents.

N-(4-((2-isonicotinoylhydrazono)methyl)phenyl)acetamide (2a) Yield: 91%; mp 288-292°C; Recrystallization solvent: ethanol; ^1H NMR (60 MHz, $[\text{D}_6]$ DMSO): δ = 12.0 (s, 1H; N-NH), 10.2(s, 1H; NH amide), 7.5–8.5 (m, 9H; phenyl and pyridine H, and CH=N), 2 ppm (s, 3H; CH₃); IR (KBr): $\tilde{\nu}$ = 3303 (s) (NH, amide), 3035 (m) (CH, aromatic), 2926 (w) (CH, aliphatic), 1659 (vs) (C=O amide), 1582 (vs) (C=N), 843 (s) cm^{-1} (CH out of plane bending, 4-substituted pyridine); MS (ESI) m/z (%): 283 (100) $[\text{M}+\text{H}]^+$; elemental analysis calcd (%) for $\text{C}_{15}\text{H}_{14}\text{N}_4\text{O}_2$: C 63.82, H 5.00, N 19.85; found: C 63.81, H 4.99, N 19.84.

N'-(3-nitrobenzylidene)isonicotinohydrazide (2b) Yield: 70%; m.p. 227-230°C; Recrystallization solvent: ethanol; ^1H NMR (500 MHz, $[\text{D}_6]$ DMSO): δ = 12.32 (s, 1H; NH), 8.79-8.80(d, $^3J_{\text{H,H}}$ = 4.5 Hz, 2H; pyridine-H3, H5), 8.573(s, 2H; CH=N, Ph-H2), 8.27-8.29(d, $^3J_{\text{H,H}}$ = 8 Hz, 1H; Ph-H4), 8.17-8.19(d, $^3J_{\text{H,H}}$ = 8 Hz, 1H; Ph-H6), 7.82-7.83(d, $^3J_{\text{H,H}}$ = 4.5 Hz, 2H; pyridine-H2, H6), 7.75-7.81 ppm (t, $^3J_{\text{H,H}}$ = 8 Hz, 1H; Ph-H5); IR (KBr): $\tilde{\nu}$ = 3423 (s) (NH, amide), 3208 (m) (CH, aromatic), 3034 (m) (CH, aliphatic), 1663 (vs) (C=O amide), 1525 (vs) (C=N), 1500 and 1350 (m) (NO_2) 850 (w) cm^{-1} (CH out of plane bending, 4-substituted pyridine); MS (ESI) m/z (%): 271 (100) $[\text{M}+\text{H}]^+$; Anal.Calcd for $\text{C}_{13}\text{H}_{10}\text{N}_4\text{O}_3$: C 57.78, H 3.73, N 20.73; found: C 57.79, H 3.72, N 20.74.

N'-(3-methoxybenzylidene)isonicotinohydrazide (2c) Yield: 32%; m.p. 218-222°C; Recrystallization solvent: ethanol; ^1H NMR (60 MHz, $[\text{D}_6]$ DMSO): δ = 12.2 (s, 1H; N-NH), 7.5 – 8.5 (m, 9H; phenyl and pyridine H, CH=N), 3 ppm (s, 3H; OCH₃); IR (KBr): $\tilde{\nu}$ = 3549 (s) (NH, amide), 3182 (w) (CH, aromatic), 3058 (w) (CH, aliphatic), 1659 (vs) (C=O, amide), 1588 (s) (C=N), 1290 and 1053 (s) (Ar-O-CH₃), 859 cm^{-1} (CH

out of plane bending, 4-substituted pyridine); MS (ESI) m/z (%): 260 (100) $[M+H]^+$; elemental analysis calcd (%) for $C_{14}H_{13}N_3O_2$: C 65.87, H 5.13, N 16.46; found: C 65.88, H 5.12, N 16.47.

N'-(2-nitrobenzylidene)isonicotinohydrazide (2d) Yield: 70%; m.p. 225-229°C (Lit.^[27] m.p. 226-228°C); Recrystallization solvent: ethanol; 1H NMR (60 MHz, $[D_6]$ DMSO): δ = 12.1 (s, 1H; N-NH), 7.5 – 8.5 ppm (m, 9H; phenyl and pyridine H, CH=N); IR (KBr): $\tilde{\nu}$ = 3431 (s) (NH, amide), 3167 (m) (CH, aromatic), 2994 (m) (CH, aliphatic), 1655 (vs) (C=O, amide), 1570 (s) (C=N), 1513 and 1346 (s) (NO_2), 844 (w) cm^{-1} (CH out of plane bending, 4-substituted pyridine); MS (ESI) m/z (%): 271 (100) $[M+H]^+$; elemental analysis calcd (%) for $C_{13}H_{10}N_4O_3$: C 57.78, H 3.73, N 20.73; found: C 57.79, H 3.72, N 20.8.

N'-(4-hydroxy-3-methoxybenzylidene)isonicotinohydrazide (2e) Yield: 30%; m.p. 217-220°C; Recrystallization solvent: 1-propanol; 1H NMR (300 MHz, $[D_6]$ DMSO): δ = 11.86 (s, 1H; -NH-N=), 8.76 (d, $^3J_{H,H}$ = 4.3 Hz, 2H; pyridine), 8.34 (s, 1H; -N=C-H), 7.80 (d, 2H; pyridine, J = 3.9 Hz), 7.32 (s, 1H; benzylidene), 7.09 (d, $^3J_{H,H}$ = 8.2 Hz, 2H; benzylidene), 5.08 (s, 1H; OH, D_2O exchangeable), 3.82 ppm (s, 3H; CH_3);^[28] IR (KBr): $\tilde{\nu}$ = 3527 (m) (OH), 3459 (m) (NH amide), 3075 (m) (CH aromatic), 1669 (s) (C=O, amide), 1593 (s) (C=N), 1285 (s) cm^{-1} (Ar-O-C); MS (ESI) m/z (%): 272 (100) $[M+H]^+$; elemental analysis calcd (%) for $C_{14}H_{13}N_3O_3$: C 61.99, H 4.83, N 15.49; found: C 61.97, H 4.82, N 15.51.

N'-((1H-pyrrol-2-yl) methylene)isonicotinohydrazide (2f) Yield: 26%; m.p. 232-233°C (Lit.^[29] m.p.. 230-232°C); Recrystallization solvent: 1-propanol; 1H NMR (60 MHz, $[D_6]$ DMSO): δ = 12 (s, 2H; N-NH), 7.5 – 8.5 ppm (m, 8H; pyrrole and pyridine H, CH=N); IR (KBr): $\tilde{\nu}$ = 3401 (m) (NH, amide), 3211 (m) (CH, aromatic), 2973 (m) (CH, aliphatic), 1660 (s) (C=O, amide), 1610 (s) (C=N), 845 (w) cm^{-1} (CH out of plane bending, 4-substituted pyridine); MS (ESI) m/z (%): 215 (100) $[M+H]^+$; elemental analysis calcd (%) for $C_{11}H_{10}N_4O$: C 61.67, H 4.71, N 26.15; found: C 61.68, H 4.72, N 26.14.

N'-((E)-3-phenylallylidene)isonicotinohydrazide (2g) Yield: 40%; m.p. 199-203°C; Recrystallization solvent: ethanol; 1H NMR (60 MHz, $[D_6]$ DMSO): δ = 12 (s, 1H; N-NH), 7-9 ppm (m, 12H; phenyl and pyridine H, CH=N, $CH_2=CH_2$); IR (KBr): $\tilde{\nu}$ = 3495 (m) (NH, amide), 3236 (m) (CH, aromatic), 2987 (m) (CH, aliphatic), 1672 (s) (C=O, amide), 1645 (s) (C=N), 843 (w) cm^{-1} (CH out of plane bending, 4-substituted pyridine); MS (ESI) m/z (%): 252 (100) $[M+H]^+$; elemental analysis calcd (%) for $C_{15}H_{13}N_3O$: C 71.70, H 5.21, N 16.72; found: C 71.7, H 5.21, N 16.74.

N'-(pyridin-3-ylmethylene)isonicotinohydrazide (2h) Yield: 34%; m.p. 239-243°C; Recrystallization solvent: 1-propanol; 1H NMR (60 MHz, $[D_6]$ DMSO): δ = 12.2 (s, 1H; N-NH), 7.5 – 8.5 ppm (m. 9H; pyridine H, CH=N); IR (KBr): $\tilde{\nu}$ = 3192 (m) (NH, amide), 3009 (m) (CH, aromatic), 2936 (m) (CH, aliphatic), 1678 (vs) (C=O, amide), 1594 (m) (C=N), 849 (m) (CH out of plane bending, 4-substituted pyridine), 822 (w) cm^{-1} (CH out of plane bending, 3-substituted pyridine); MS (ESI) m/z (%): 227 (100) $[M+H]^+$; elemental analysis calcd (%) for $C_{12}H_{10}N_4O$: C 63.71, H 4.46, N 24.76; found: C 63.83, H 4.45, N 24.67.

N'-(pyridin-4-ylmethylene)isonicotinohydrazide (2i) Yield: 34%; m.p. 231-235°C; Recrystallization solvent: 1-propanol; ¹H-NMR (60 MHz, [D₆] DMSO): δ = 12.2 (s, 1H; N-NH), 7.5 – 8.5 ppm (m, 9H; pyridine H, CH=N); IR (KBr): ν̃ = 3480 (m) (NH, amide), 3196 (m) (CH, aromatic), 3017 (m) (CH, aliphatic), 1669 (s) (C=O, amide), 1574 (s) (C=N), 836 (w) cm⁻¹ (CH out of plane bending, 4-substituted pyridine); MS (ESI) m/z (%): 227 (100) [M+H]⁺; elemental analysis calcd (%) for C₁₂H₁₀N₄O: C 63.71, H 4.46, N 24.76; found: C 63.72, H 4.47, N 24.76.

N'-(4-nitrobenzylidene)isonicotinohydrazide (2j) Yield: 93%; m.p. 195-200°C; Recrystallization solvent: ethanol; ¹H-NMR (60 MHz, [D₆] DMSO): δ = 12.2 (s, 1H; N-NH), 7.5 – 8.5 ppm (m, 9H; phenyl and pyridine H, CH=N); IR (KBr): ν̃ = 3196 (w) (NH, amide), 3000 (w) (CH, aromatic), 2904 (w) (CH, aliphatic), 1684 (vs) (C=O, amide), 1570 (s) (C=N), 1514 and 1342 (vs) (NO₂), 856 (m) cm⁻¹ (CH out of plane bending, 4-substituted pyridine); MS (ESI) m/z (%): 271 (100) [M+H]⁺; elemental analysis calcd (%) for C₁₃H₁₀N₄O₃: C 57.78, H 3.73, N 20.73; found: C 57.77, H 3.73, N 20.72.

N'-benzylideneisonicotinohydrazide (2k) Yield: 60%; m.p. 198-200°C; Recrystallization solvent: ethanol; ¹H-NMR (60 MHz, [D₆] DMSO): δ = 12.06 (s, 1H; N-H), 8.80 (d, ³J_{H,H} = 6 Hz, 2H; aromatic H), 8.48 (s, 1H; azomethine), 7.84–7.74 (m, 4H; aromatic), 7.49–7.46 ppm (m, 3H; aromatic);^[30] IR (KBr): ν̃ = 3408 (m) (NH, amide), 3197 (m) (CH, aromatic), 3031 (m) (CH, aliphatic), 1668 (s) (C=O, amide), 1575 (m) (C=N), 847 (w) (CH out of plane bending, 4-substituted pyridine), 707 and 690 (m) cm⁻¹ (mono substitution phenyl group); MS (ESI) m/z (%): 226 (100) [M+H]⁺; elemental analysis calcd (%) for C₁₃H₁₁N₃O: C 69.32, H 4.92, N 18.66; found: C 69.33, H 4.91, N 18.65.

N'-([1,1'-biphenyl]-4-ylmethylene)isonicotinohydrazide (2l) Yield: 70%; m.p. 218-221°C; ¹H-NMR (60 MHz, [D₆] DMSO): δ = 12 (s, 1H; N-NH), 8.5 (m, 3H; pyridine-H3, -H5, CH=N), 7.5 ppm (m, 11H; pyridine-H2, -H6, phenyl groups H); IR (KBr): ν̃ = 3413 (m) (NH, amide), 3022 (m) (CH, aromatic), 2930 (m) (CH, aliphatic), 1666 (s) (C=O, amide), 1597 (m) (C=N), 839 (w) cm⁻¹ (CH out of plane bending, 4-substituted pyridine); MS (ESI) m/z (%): 302 (100) [M+H]⁺; elemental analysis calcd (%) for C₁₉H₁₅N₃O: C 75.73, H 5.02, N 13.94; found: C 75.74, H 5.03, N 13.92.

In vitro study of anti-platelet aggregation activity

The anti-platelet aggregation activity was determined *in vitro* using the human plasma. Blood was withdrawn from healthy volunteers (non-smoker) with no history of drug- consumption within fifteen days before the blood drawn. Platelet-rich plasma (PRP) was obtained by centrifugation of citrated whole blood (9:1) at 1000 rpm for 8 minutes. The platelet-poor plasma (PPP) was prepared by centrifugation of the remained layer at 3000 rpm for 15 minutes and was collected the upper layer (used as blank). In order to adjust the platelet, count to an appropriate amount, PPP was added to PRP and diluted to the number of 250000 plt/μL. Test compounds dissolved in DMSO were added to PRP samples and incubated for 5 minutes at 37°C. Following the addition of ADP (5μL) or AA (18μL) (1.25mg/mL), the variation and changes in aggregation pattern and platelet shape and aggregation were observed.

DMSO was utilized as negative control while indomethacin or aspirin were the standard drugs. The formula stated below was used to calculate the platelet aggregation inhibition (%):

$$\text{Inhibition (\%)} = [1 - (D/S)] * 100$$

In the preceding formula D is defined as the platelet aggregation in the presence of the test compound, while S is the platelet aggregation in solvent^[31, 32]. IC₅₀ was defined as the concentration that inhibits the platelet aggregation by 50% and all of the synthesized compounds were screened at the concentration of 1 mM.

Cell viability test (MTT Test)

To measure cell viability in the presence of the effective synthesized compounds, the MTT test was performed by methods previously described^[33, 34]. To prepare the MTT solution, first 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was dissolved in Dulbecco Phosphate Buffer Saline (DPBS) with pH equals to 7.4 then the solution was filtered off (0.2µM filter size). Subsequently, the cells and selected derivative were placed in 96-well plates and incubated. Afterward, 10 µL of MTT solution was added to each cell and finally incubated for 1 to 4 hours at 37°C. To dissolve formazan crystals 100 µL of solubilization solution (Dimethylformamide 40% V/V solution in Glycol Acetic Acid 2% V/V solution, mixed with Sodium Dodecyl Sulphate (SPS) 16% solution, with final pH of 4.7) was added to the mixture. Ultimately, the absorbance was measured at 570 nm^[35]. Fibroblast cells and three types of cancerous cells including (MCF-7, HeLa, and HepG2) were used in this test. The cytotoxicity of compounds was computed as a percentage of controlled cells (untreated cells) and the presented values in the table are the inhibitory concentration (IC₅₀) of the three compounds compared to control.

Docking study

The PDB file (PDB ID: 2OYU) of crystal structure of cyclooxygenase-1 was downloaded from the RCSB Protein Data Bank. The co-crystallized ligand was removed and the receptor was prepared by Autodock Tools (ver 1.5.6rc3)^[36]. The structure of ligands were sketched, minimized and saved as pdbqt format. AutoDock Vina (version 1.1.2)^[37] was used for docking study. A box with 20 x 20 x 20 dimension used as search space. The centre of grid box was adjusted to be the centre of co-crystallized ligand. The results of docking were analysed by Pymol.

Results And Discussion

Chemistry

The hydrazone (**1a-1j**) and acylhydrazone derivatives (**2a-2l**) were synthesized in a single step route (Scheme 1). Phenyl hydrazine and isonicotinic acid hydrazide reacted with different aldehydes in distilled water using acetic acid as catalyst^[24]. The final products obtained in satisfactory yields and purity. The

structure of compounds was characterized by different spectroscopic techniques such as IR, ¹HNMR and MS.

Biological evaluation

All the synthesized compounds were evaluated for their antiplatelet activity in AA and ADP-induced aggregation of human plasma using light transmission aggregometry. The results are disclosed in Table 1 and aspirin and indomethacin were used as positive controls.

Based on the obtained results, the phenylhydrazone derivatives (**1a-j**) exhibited considerable activity when AA used as platelet aggregation inducer and showed no activity against ADP-induced platelet aggregation.

On the other hand the antiplatelet activity of pyridine acylhydrazone derivatives (**2a-l**) indicates that none of them exhibit acceptable activity against neither ADP nor AA-induced aggregation. Therefore, it can be concluded that the introduction of a carbonyl group is deleterious to the antiplatelet activity of hydrazone compounds. This has been confirmed by the previous study which reported loss of antiplatelet activity in aroylhydrazones of indole-3-carboxaldehyde ^[20].

In the phenylhydrazone group, methoxyphenyl, 4-Hydroxy-3-methoxyphenyl (**1c, 1g**) and pyrrole-substituted derivatives (**1h**), demonstrated acceptable antiplatelet activity against AA-induced platelet aggregation with IC₅₀ values around 34-39 μM. This finding is in agreement with previous studies ^[19] showing that hydrazone compounds with electron-rich heterocyclic rings could be potent inhibitors of AA-induced platelet aggregation.

Table 1 Antiplatelet activity of synthesized compounds against AA and ADP.

<div style="display: flex; justify-content: space-around; align-items: center;"> <div style="text-align: center;"> <p>1a-1j</p> </div> <div style="text-align: center;"> <p>2a-2l</p> </div> </div>			
Compound	R	Antiplatelet activity, IC ₅₀ (μM) AA	aggregation ADP
1a	4-Acetamidophenyl	87.36 ± 2.3	>100
1b	3-Nitrophenyl	>100	>100
1c	3-Methoxyphenyl	34.6 ± 1.7	>100
1d	3,4,5-trimethoxyphenyl	>100	>100
1e	2-Chlorophenyl	85 ± 1.3	>100
1f	4-Carboxyphenyl	>100	>100
1g	4-Hydroxy-3-methoxyphenyl	38.9 ± 1.9	>100
1h	2-Pyrrolyl	34.1 ± 0.9	>100
1i	3-Indolyl	>100	>100
1j		47.4 ± 2.2	>100
2a	4-Acetamidophenyl	>100	>100
2b	3-Nitrophenyl	>100	>100
2c	3-Methoxyphenyl	>100	>100
2d	2-Nitrophenyl	>100	>100
2e	4-Hydroxy-3-methoxyphenyl	>100	>100
2f	2-Pyrrolyl	>100	>100
2g		>100	>100
2h	3-Pyridyl	>100	>100
2i	4-Pyridyl	>100	>100
2j	4-Nitrophenyl	>100	>100
2k	phenyl	>100	>100
2l	4-biphenyl	>100	>100
Indomethacin	-	3.0	>100
Aspirin	-	30.3	>100

Cytotoxicity assay

Furthermore, the cell viability assessment of the most effective compounds (**1c**, **1g**, and **1h**) using the MTT test revealed that these compounds are fairly safe. (Table 2) Accordingly, **1h** showed no toxicity, while **1c** and **1g** only reached a toxic level at concentration 3 to 4 times more than their IC₅₀ for antiplatelet activity. Moreover, **1g** has shown toxicity on cancerous cell lines with an IC₅₀ even less than its pharmacological effects. This observation indicates that **1g** can be an area of research for developing an anticancer agent.

Table 2
Cell cytotoxicity (MTT test results) of compounds with highest antiplatelet activity in normal cell (Fibroblast) and 3 different cancer cell lines (HepG2, HeLa, and MCF-7).

Compound	MTT assay, IC ₅₀ (μM)			
	Fibroblast	HepG2 ^a	HeLa ^b	MCF-7 ^c
1c	214.5	129.6	220.6	157.6
1g	163.9	21.00	17.79	43.17
1h	>1000	>1000	>1000	>1000
Cisplatin^d	9.76	26.96	9.3	23.89
^a Human liver cancer cell line.				
^b Human cervical carcinoma cell line.				
^c Human breast cancer cell line.				
^d Obtained from our previous studies [25].				

Docking studies

Docking studies of the most active compounds against AA-induced platelet aggregation (**1c,1g,1h**) were also achieved to check the binding interactions of compounds with the active site of cyclooxygenase 1 (COX-1) using Autodock Vina. For validation of used docking parameters, the co-crystallized ligand was docked into the active site. The obtained RMSD value (0.839) was acceptable for the validity of the docking procedure. Based on the results it was observed that hydrophobic interactions with Val349 and Gly524 are involved in the attachment of **1h** to the active site of COX-1. In compound **1c** and **1g** in addition of hydrophobic interactions with Gly526 and Val116, hydrogen bond is also formed between Ser530 and the oxygen atom of the methoxy group of compounds **1c** and **1g**. The structure of the best poses of compounds **1c** and **1g** in the active site is shown in figure 2.

Conclusion

In this study, two groups of arylhydrazone derivatives (phenylhydrazone and Isonicotinic acid hydrazone) were synthesized and their anti-platelet activity was evaluated. Among them, arylhydrazone group demonstrates adequate activity against AA-induced aggregation with no cell toxicity at therapeutic levels. However, N-isonicotinohydrazide derivatives were inactive in inhibiting neither ADP nor AA-induced aggregation. Developing a clear correlation between the activity and structural features requires a larger library of compounds. This is probably due to the presence of multiple receptors on the platelet surface and divergent mechanisms in charge of modulating platelet aggregation. Moreover, the studied

compounds could have exerted the observed antiplatelet activity characteristics by interacting with multiple receptor; thus, no obvious structure activity relationship could be concluded. Additional studies are essential for clarifying the mechanism of action and developing a more potent antiplatelet agent.

Declarations

Conflict of Interest

The author declares no conflict of interest.

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Scheme

Scheme 1 is available in supplementary section.

Figures

Figure 1

General structure of designed hydrazone and N-acylhydrazone compounds.

Figure 2

The binding interaction of the most active compounds 1c (A) and 1g (B) in the active site of COX-1. Hydrogen bond is shown by yellow dashed line.

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