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Design, synthesis and evaluation of aspirin analogues having an additional carboxylate substituent for antithrombotic activity

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ABSTRACT

Acetylsalicylic acid (aspirin) is an effective long-term prophylaxis of thrombotic events such as heart attacks and strokes. It covalently inhibits prostaglandin-H-synthase by interacting with Arg120 or Tyr385 at the active site allowing delivery of its acetyl group to Ser530. However the structure has not been optimized to fit the active site. We have designed acetylsalicylate analogues with an additional carboxylate substituent which allows simultaneous interaction with Arg120 and Tyr385 whilst positioning the acetyl group in close proximity to Ser530. One of these, an ester derivative which unlike acetylsalicylic acid is non-acidic, may act as useful lead compound for further exploitation of this approach.

Recognition of vessel rupture induces outside-in signalling in platelets, which leads to activation of phospholipases that release arachidonate from the lipid bilayer. Arachidonic acid is then converted to prostaglandin G_2 (PGG_2) in the cyclooxygenase (COX) site of the enzyme prostaglandin H_2 synthase (PGHS). Following this, PGG_2 is converted to prostaglandin H_2 at the peroxidase site (POX) of PGHS, and this in turn is processed to thromboxane A_2 (TxA_2) by synthases. TxA_2 is a potent stimulator of platelet recruitment and aggregation, resulting in clot formation and haemostasis. However, thrombus formation in at-risk individuals leads to vessel occlusion, disruption in blood flow, oxygen starvation of downstream tissues and ischaemic heart attack or stroke.

Acetylsalicylic acid (aspirin, **1**) shows antithrombotic, antipyretic, analgesic and antiproliferative effects due to the irreversible inhibition of PGHS, the rate limiting enzyme in the production of prostaglandins and thromboxane. Aspirin acetylates Ser530 found between the positively charged arginine at the mouth of the COX channel (Arg120), and a deeply buried tyrosine (Tyr385) that initiates the cyclo oxygenation of arachidonate.[2,3] This irreversible acetylation permanently blocks entry of arachidonate to the active site. Vascular endothelial synthesis of the antithrombotic prostaglandin, prostacyclin, is also blocked. Unlike endothelial cells, platelets are anucleate and are unable to replace the inactivated PGHS enzymes, thus giving aspirin a therapeutic advantage against thrombosis.[4] Long-term, low daily doses of aspirin remains an effective inhibitor of platelet function in cardiovascular patients.

The crystal structure of ovine PGHS-1 shows that the PGHS substrate arachidonic acid binds Arg120 by a salt linkage.[1] Most aromatic carboxylic acid containing non-steroidal anti-inflammatory drugs (NSAIDs) interact similarly, and orientate the aromatic functionality of the drug towards the cyclooxygenase site at Tyr385.[5,6] Interestingly, Tyr385 is critical for aspirin acetylation of Ser530, as confirmed by site-directed mutation of Tyr385 to phenylalanine which reduces aspirin action by over 90%.[7] The role of Tyr385 is to stabilize the negatively charged tetrahedral intermediate formed during acetylation. It follows that a more effective inhibitor might result by simultaneous interaction with both Arg120 and Tyr385, while orientating an acetyl group in close proximity to the hydroxyl oxygen of Ser530.

Docking using the MOE-Dock programme allowing receptor flexibility[8,9], accurately predicted the crystallographic placement of arachidonate in the crystal structure of ovine PGHS-1[1,10], PDB:1DIY (Fig. 1A), with a root-mean-squared deviation (RMSD) of 1.11 Å and a binding energy of −14.3 kcal/mol. Similar docking of acetylsalicylate showed carboxylate binding to either Arg120 or Tyr385, but not both simultaneously (Fig. 1B). The optimal binding energy was significantly higher at −8.8 kcal/mol, although this

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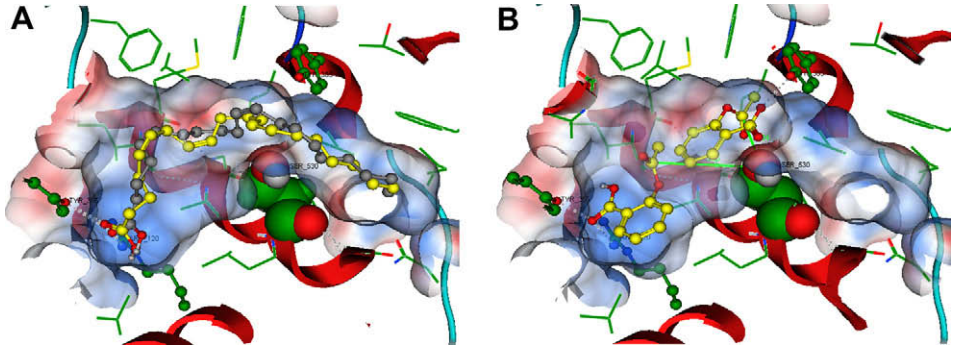


Figure 1. Docked conformations of arachidonate and aspirin in the PGHS-1 cyclooxygenase site. MOE-Dock was used to place arachidonate (A) and aspirin (B) in the active site of the ovine PGHS-1 COX site crystal structure 1DIY.[1] The PGHS-COX site isoelectric surface is shown semi-transparent, where negative regions are red, positive regions blue, and neutral regions white. Receptor carbons are green, the crystallographic arachidonate carbons are grey, and the binding pose found using MOE-Dock has yellow carbons. Nitrogens are blue, oxygens are red. Non-polar hydrogens (white) were removed for clarity. Ser530 is shown in space fill, Arg120, Tyr355 and Tyr385 are shown in ball-and-stick, and other Cox site residues are shown in line notation. The isolated backbone alpha helix is shown in red ribbon, while the loop regions are depicted with cyan. Half the site has been cropped for visualization. Light green lines indicate distance measures mentioned in the text. In (B), aspirin bound to Arg120 is shown with yellow carbons.

is compensated for by irreversible inhibition. If bound to Arg120, the distance between the acetyl group and Ser530 is 5.88 Å, which is too far for acetyl group transfer. This is corroborated by the crystallographic position of salicylate in PDB:1PTH[11] which is bound to Arg120 and is separated from Ser530 by 5.54 Å (Fig. 2).

To allow the acetylsalicylate moiety to advance further down the COX active site channel, while retaining a salt bridge with Arg120, we introduced an *n*-pentanoate chain at the *meta* position, giving **2** as a target molecule. In **2** the *n*-pentanoate group is capable of interacting with the active site Arg120, while the salicylate carboxylate interacts with Tyr385, and the acetyl group is in close proximity to Ser530. MOE-docking showed that **2** could indeed form a salt bridge with Arg120, and a hydrogen bond to Tyr385, while allowing the acetyl group to interact favourably with Ser530 (Fig. 3A). The binding energy of −13.7 kcal/mol is similar to that of the substrate, and significantly lower than that for aspirin.

Compound **2** was obtained in a 6-step process from salicylic acid **6**, in overall 13% yield as shown in Scheme 1. Esterification of **6** gave **7** (99% yield), which was converted by Fries rearrangement with glutaric anhydride to **8** (29% yield).[12] Reduction of the ketone **8** with triethylsilane/trifluoroacetic acid at room temperature[13] afforded **9** (79% yield), hydrolysis of which gave **10** (93% yield). Acetylation of the phenolic group in **10** with acetic anhydride and base in dry DMF afforded the target compound **2** in 60% yield.[14]

Compound **2** was found to be inactive in platelet aggregation assays at the highest concentration tested (1000 μM). In this assay, arachidonate is used to stimulate human platelets to form aggregates, as would occur at sites of vascular injury, atherosclerotic plaque rupture and thrombosis formation. As PGHS-1 is the main form of prostaglandin H_2 synthase in anucleate platelets, it is a convenient and physiologically relevant model for PGHS-1 activity.[15]

Following this result which may have been due to suboptimal polarity in **2** or in the length of the alkycarboxylate chain it was decided to synthesize the more polar **3**, the less polar **5** and the shorter chain alkyl carboxylate containing **4**. Compound **3** was obtained by hydrolysis of **8** to give **11** (95% yield) followed by acetylation (81% yield), Scheme 1.[16] Compound **5** was obtained by esterification of **2** (55% yield).[17] Synthesis of target compound **4** was carried out as illustrated in Scheme 2. Reaction of **7** with maleic anhydride using 2 equiv of $AlCl_3$ in tetrachloroethane gave **12** (44% yield) which upon catalytic hydrogenation gave **13** (97% yield), followed by hydrolysis gave **14** (90% yield) and finally acetylation gave target compound **4** (40% yield).[18]

Compounds **3** and **4** were found to be inactive in platelet aggregation assays at the highest concentration tested (1000 μM). The platelet aggregation assay measures the increased light transmission through platelet rich plasma (PRP), freshly isolated from healthy volunteers, in response to addition of 1.5 mM arachidonic acid as agonist. Various concentrations of each compound (0.1–1000 μM; final DMSO concentration <0.4%) were added to stirred PRP for 5 min prior to arachidonate addition.[19] However **5**, was found to inhibit platelet aggregation, with a 50% maximal inhibition concentration (IC_{50}) of 720 ± 85 μM which is 15-fold less potent than aspirin (47 ± 27 μM). Molecular modelling shows that **5** is capable of interacting simultaneously with Arg120 and Tyr385 by hydrogen bonding while correctly positioning the acetyl group near Ser530 for acetyl transfer to occur (Fig. 3B). Furthermore, the binding energy of −13.3 kcal/mol is similar to that of the dicarboxylate form **2.**

Importantly, **5** had no effect on platelet aggregation stimulated by 2 μM of the thromboxane analogue U46619, at the highest concentration of inhibitor used (1000 μM). This implies that inhibition is not due to non-specific destruction of human cells, and that its

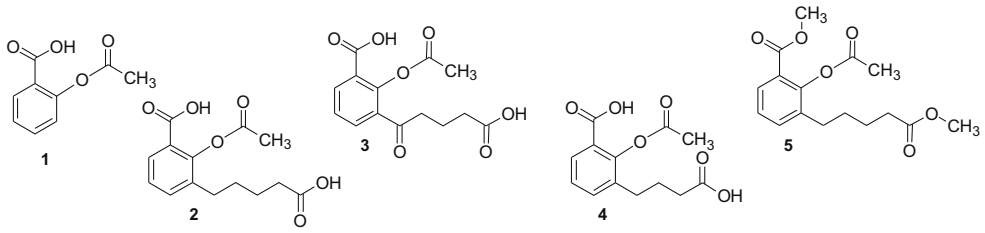


Figure 2. Compounds synthesized and tested for anti-platelet activity.

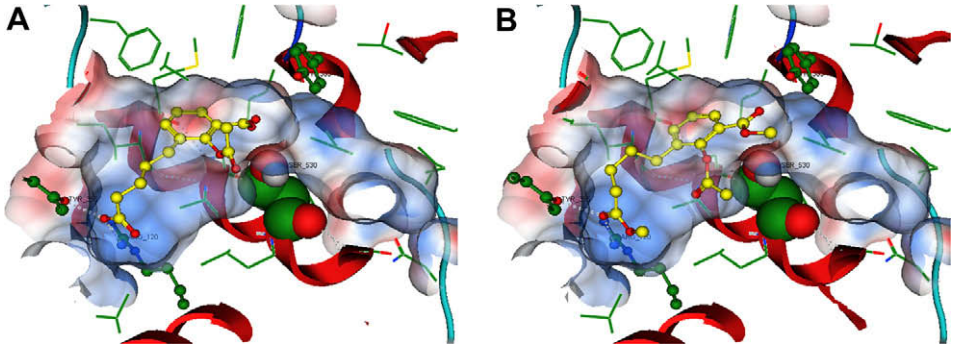
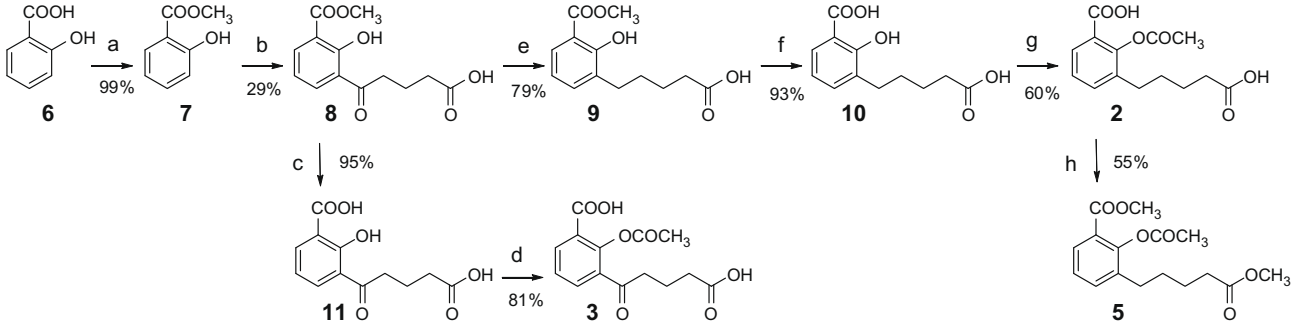
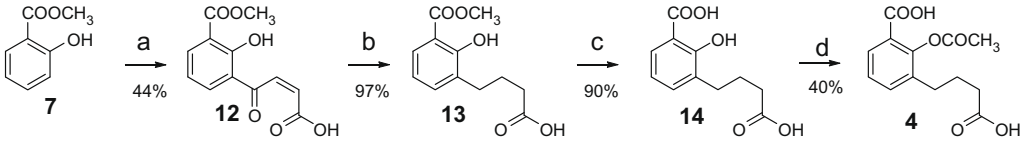


Figure 3. Docked conformations of **2** and **5** in the PGHS-1 cyclooxygenase site. MOE-Dock was used to place **2** (A) and **5** (B) in the active site of the ovine PGHS-1 COX site crystal structure 1DIY.[1] Key as per Fig. 1. In (A), the interatomic distances between **2** and Arg120, Ser530 and Tyr385 are 2.34 Å, 2.53 Å and 3.60 Å, respectively. In (B), the interatomic distances between **5** and Arg120, Ser530 and Tyr385 are 2.51 Å, 3.21 Å, and 2.53 Å, respectively. Arg120 forms hydrogen bonds with either the *n*-pentanoate or ester groups, while the salicylate carboxylate or ester groups interact with Tyr385. The acetyl group is in close proximity to Ser530 in both **2** and **5**.



Scheme 1. Reagents and conditions: (a) CH_3COCl, MeOH, reflux 12 h; (b) glutaric anhydride 1.0 equiv $AlCl_3$, 2.1 equiv tetrachloroethane (TCE), 1 h rt then 130 °C 4 h; (c) NaOH 2.0 equiv H_2O, reflux 2 h; (d) Ac_2O 10 equiv pyridine 100 equiv DMF dry, 24 h rt; (e) $(C_2H_5)_3SiH$ 3.0 equiv TFA, 48 h. rt; (f) NaOH 4.0 equiv H_2O, reflux 2 h; (g) Ac_2O 10 equiv pyridine 100 equiv DMF dry, 3 h rt; (h) K_2CO_3 3.0 equiv MeI 10 equiv DMF dry, 12 h rt.



Scheme 2. Reagents and conditions: (a) maleic anhydride 1.0 equiv $AlCl_3$ 2.1 equiv tetrachloroethane (TCE), 1 h rt then 130 °C 2 h; (b) Pd/C 0.1 equiv, MeOH, 12 h rt; (c) NaOH 2.0 equiv H_2O, reflux 12 h; (d) Ac_2O 4.0 equiv pyridine 3.3 equiv DMF dry, 24 h rt.

mechanism of action is specific to the cyclooxygenase pathway, downstream of arachidonate and upstream of thromboxane.

Despite the poor correlation between docking studies and data, compound **5** is sufficiently diverse from aspirin to constitute a new starting point for investigation. In particular, compound **5** contains an extra aliphatic chain and two ester groups, offering the possibility of further derivatization at each of these sites. An added benefit of this type of derivative is that it is non-acidic and would not cause topical irritation as aspirin does due to the presence in its structure of an acidic carboxylic acid group. Finally, compound **5** has been shown by us to specifically inhibit the COX pathway of platelet activation.

We had predicted that the introduction of an *n*-pentanoate group *meta* to the carboxylate group of acetylsalicylic acid would form a salt bridge with Arg120 at the active site of PGHS, whilst the salicylate carboxylate group would interact with Tyr385 and allow delivery of the acetyl group to Ser530. Our results show that an esterified *n*-pentanoate group forms a sufficiently strong interaction with Arg120 for this to occur and we have produced a novel compound that inhibits PGHS and can be further developed to give a new type of non-steroidal anti-inflammatory drug.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.05.120.

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8. Docking MOE-Dock 2008.10 function was used for docking. Ligand placement was performed using alpha sphere triangle matching, with London dG scoring. The top 30 hits were retained and refined using MMFF94× forcefield energy minimization with Generalized Born solvation model, allowing the receptor residues within 6 Å to relax around the mobile ligand. The receptor side chains were tethered with a force constant of 1.0 kcal/(mol Å^2). Energy minimization was stopped when the root-mean-squared gradient cutoff of 0.01 kcal/(mol Å) was reached. Final poses were ranked using the London dG method to calculate the free energy of binding.
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14. *2-Acetoxy-3-(4-carboxy-butyl)-benzoic acid* (**2**): ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.59–1.51 (4H, m), 2.24 (5H, t, J = 7.2 Hz), 2.63 (2H, t, J = 7.2 Hz), 7.12–7.07 (1H, m, aromatic), 7.45 (1H, dd, J = 8.4, J = 2.4 Hz, aromatic), 7.73 (1H, d, J = 2.4 Hz, aromatic), 12.59 (2H, br, COOH). ^{13}C NMR (400 MHz, DMSO-d_6) δ 20.8, 24.0, 30.2, 33.4, 33.9, 123.5, 123.6, 130.9, 133.5, 139.9, 148.2, 165.7, 169.3, 174.4. IR (KBr disc) ν_{max} 3435 br, 2919, 1754, 1695, 1434, 1304 cm^{-1}. Elemental Anal. Calcd for $C_{14}H_{16}O_6$: C, 59.99; H, 5.75. Found: C, 60.54; H, 5.69. HRMS found: 279.0882 [M−H$^+$] $C_{14}H_{15}O_6$ requires 279.0869.
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17. *2-Acetoxy-3-(4-methoxycarbonyl-butyl)-benzoic acid methyl ester* (**5**): ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.59–1.54 (4H, m), 2.27 (3H, s, CH_3), 2.34 (2H, t, J = 6.8 Hz), 2.64 (2H, t, J = 7.2 Hz), 3.58 (3H, s, OCH_3), 3.80 (3H, s, OCH_3), 7.14 (1H, d, J = 8.4 Hz, aromatic), 7.50 (1H, dd, J = 8.4, J = 2.4 Hz, aromatic), 7.75 (1H, d, J = 2 Hz). ^{13}C NMR (400 MHz, DMSO-d_6) δ 20.7, 23.9, 30.1, 33.0, 33.7, 51.2, 122.5, 123.8, 130.7, 134.0, 140.1, 148.0, 164.5, 169.3, 173.3. HRMS found: 309.1341 [M+H$^+$] $C_{16}H_{21}O_6$ requires 309.1338.
18. *2-Acetoxy-3-(3-carboxy-propyl)-benzoic acid* (**4**): ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.80 (2H, quintet, J = 7.6 Hz), 2.23 (5H, s), 2.64 (2H, t, J = 7.6 Hz), 7.10 (1H, d, J = 8 Hz, aromatic), 7.46 (1H, dd, J = 8.4, J = 2 Hz, aromatic), 7.74 (1H, s, aromatic), 12.60 (2H, br, COOH). ^{13}C NMR (400 MHz, DMSO-d_6) δ 21.3, 26.6, 33.4, 33.9, 124.2, 124.1, 131.5, 134.1, 140, 148.9, 166.2, 169.8, 174.7. IR (KBr disc) ν_{max} 3121, 1763, 1686, 1609, 1488, 1449 cm^{-1}. HRMS found: 265.0720 [M−H$^+$] $C_{13}H_{13}O_6$ requires 265.0712.
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