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COMMUNICATION

Identification of an Active Metabolite of PAR-1 Antagonist RWJ-58259 and Synthesis of Analogues to Enhance its Metabolic Stability

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The discontinuation of PAR-1 antagonist RWJ-58259 beyond use as a biological probe is most likely due to its short half-life *in vivo*. However, retention of significant *in vivo* activity beyond the point where most of the RWJ-58259 had been consumed implies the generation of an active metabolite. Herein we describe the biological activity of a predicted metabolite of RWJ-58259 and the synthesis of analogues designed to enhance the metabolic stability of RWJ-58259.

The serine protease thrombin plays a central role in the coagulation cascade - one of the earliest responses to tissue injury and a vital part of the normal wound healing process.¹ The coagulation cascade is a sequence of protease activations. It is initiated as factors circulating in the bloodstream come into contact with tissue factor in the extravasculature, as a consequence of tissue injury. Thrombin is activated in the cascade by the cleavage of its zymogen, pro-thrombin, and proceeds to convert soluble fibrinogen into insoluble fibrin. Thrombin also promotes the crosslinking of fibrin to form a preliminary clot, to which activated platelets can adhere. Through activation of various other components of the coagulation cascade, thrombin has both positive and negative feedback mechanisms, which regulate the activation of the cascade and ultimately the amount of fibrin generated. Thrombin is thus a key component of the coagulation cascade.

As well as its role in the coagulation cascade, thrombin is responsible for the activation of various cellular responses, the signalling for which has been identified as being mediated by transmembrane proteins known as protease-activated receptors (PARs).² As their name suggests, PARs are activated as a result of interaction with certain proteases; this leads to cleavage of their unusually long extracellular *N*-terminus chain at a specific location, uncovering a unique sequence of amino

acids, which in turn binds to the receptor activation site. This then triggers downstream cellular signalling mechanisms.

Four PARs (*i.e.* PAR-1-4) have been discovered to date with PAR-1 recognised as the major thrombin receptor. PAR-1 activation has been implicated in the progression of various diseases and as such has been investigated as a potential therapeutic target for antagonists.³ Since targeting PAR-1 allows for the blockade of thrombin's cellular actions without interfering with its wider role in the wound healing process, investigations have focused on the treatment of fibrosis and thrombosis where it is desirable to reduce, but not eliminate, the actions of thrombin. The result of investigations relating to thrombosis led to the development and FDA approval of PAR-1 antagonist Vorapaxar, the active ingredient in Merck's ZontivityTM.⁴

RWJ-58259, developed by Johnson & Johnson, was one of the first PAR-1 selective antagonists with potency sufficient to compete with the intramolecular activation mechanism of the receptor.⁵ Several groups have reported the use of RWJ-58259 to investigate the role of PAR-1 activation in various diseases and cell signalling mechanisms.⁶⁻¹¹ Although RWJ-58259 was demonstrated to be effective as a selective PAR-1 antagonist, the compound was not progressed into clinical trials by the developers. This is likely due to its poor oral bioavailability and short half-life ($t_{1/2}$). In a human liver microsomes assay RWJ-58259 was reported to have a $t_{1/2}$ = 9.2 min and *i.v.* administration in rats gave a $t_{1/2}$ = 19.2 min.¹² Despite the reported pharmacokinetic issues, the compound has been successfully utilised as a biological probe in numerous studies and continues to provide a significant biological response even in *in vivo* studies. This discrepancy between the observed and predicted activity led us to suspect that metabolites generated from the parent compound may be contributing towards the overall biological response.

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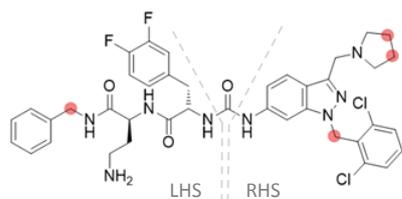
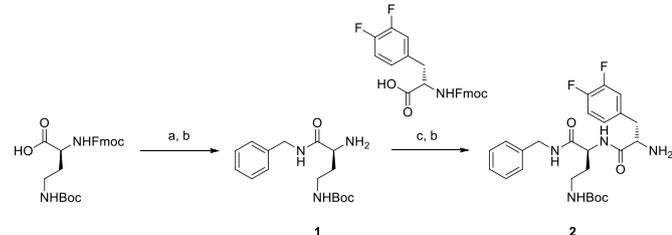


Figure 1 RWJ-58259 major sites of phase I metabolism as predicted by *in silico* screening using MetaSite; left-hand side (LHS) & right-hand side (RHS) fragments shown, which are referred to in the synthesis description

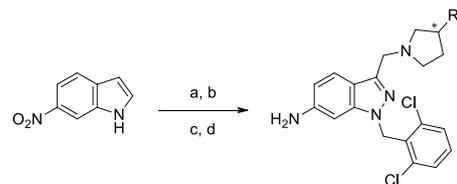
To probe potential metabolic sites, the structure of RWJ-58259 was analysed by *in silico* screening using MetaSite software (Molecular Discovery Ltd) to determine the compound's susceptibility to metabolism by the major cytochrome P450s involved with first-pass xenobiotic modification.¹³ Highlighted atoms in Fig. 1 show the predicted major sites of metabolism, these include the benzylic positions of the terminal benzylamine, the substituted indazole N-1 position, and the 3-position of the pyrrolidine ring. The latter of these sites was deemed to be of particular interest since it has been reported in the literature that the major metabolites formed in a human liver microsomes assay involved hydroxylation and oxidation of the pyrrolidine ring.¹²

As a result of these findings, the pyrrolidin-3-ol derivative of RWJ-58259 was proposed as a likely metabolite and consequently synthesised on the basis of a published route for the synthesis of RWJ-58259 (Schemes 1-3).^{14,15} To begin, amide coupling of Fmoc-DBu(Boc)-OH with benzylamine, and subsequent Fmoc-deprotection afforded amine **1**. Next, amine **1** was coupled to Fmoc-3,4-diFpHe-OH, which following Fmoc-deprotection generated left-hand side fragment **2** in 65% overall yield over the 4 steps (Scheme 1).

For the right-hand side fragment, nitrosation of 6-nitroindole with sodium nitrite under aqueous acidic conditions resulted in rearrangement to form 6-nitro-1*H*-indazole-3-carbaldehyde. Reductive amination of this intermediate with the relevant pyrrolidine moiety, *i.e.* pyrrolidine for RWJ-58259 and pyrrolidin-3-ol for the metabolite, was achieved in the presence of a mild reducing agent. Subsequent *N*-alkylation followed by nitro reduction afforded the targeted substituted indazoles (**3** & **4**) in an overall yield of 18% & 28%, respectively (4 steps, Scheme 2). Urea coupling of dipeptide fragment **2** with indazole fragments

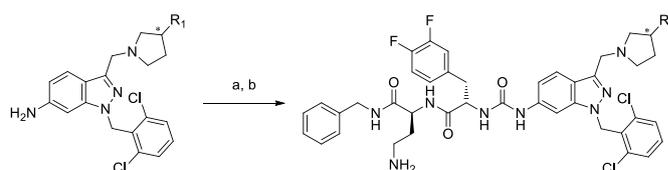


Scheme 1 Reagents and conditions: a) HBTU, HOBT, DIPEA, DMF, BnNH₂, 3 h; b) Et₂NH, DMF, 2 h; c) HBTU, HOBT, DIPEA, Fmoc-3,4-diF-Phe-OH, DMF, 16 h



- 3** R₁ = OH
4 R₁ = H
5 R₁ = (R)F
6 R₁ = (S)F

Scheme 2 Reagents and conditions: a) NaNO₂, HCl (6 M), 5 h; b) pyrrolidine or 3-OH/(S)-3-F/(R)-3-F derivative, NaB(OAc)₃H, CH₂Cl₂/DMF/AcOH (90:9:1), 3 h; c) 2,6-diClBnBr, Cs₂CO₃, THF, 20 h; d) FeCl₃·6H₂O, activated charcoal, Me₂NNH₂, MeOH, reflux, 5 h



- 3** R₁ = OH
4 R₁ = H
5 R₁ = (R)F
6 R₁ = (S)F
7 R₁ = OH
8 R₁ = H
9 R₁ = (R)F
10 R₁ = (S)F

Scheme 3 Reagents and conditions: a) triphosgene, DMAP, THF, 0-20 °C, 2 h; b) HCl (4 M) in 1,4 dioxane, 20 °C, 2 h

3 & **4**, followed by Boc-deprotection gave target compounds **7** & **8** (RWJ-58259) in 19% & 53% overall yield, respectively (2 steps, Scheme 3), after purification by preparative HPLC and lyophilisation from dilute HCl (aq).

To evaluate the biological activity of the proposed metabolite in human lung fibroblasts, an intracellular calcium ion mobilisation downstream of thrombin-mediated PAR-1 activation was measured. Following incubation of the cells with compound **7**, PAR-1 was agonised by addition of thrombin (10 nM). A FLIPR instrument was utilised for the detection of fluorescence generated as a result of complexation of released cytosolic Ca²⁺ with the Fluo-4 NW dye.¹⁶ RWJ-58259 (**8**), a full antagonist of PAR-1, was used as the standard in the assay.

Interestingly, compound **7** - a predicted metabolite of RWJ-58259 - demonstrated potency in a similar range to the parent compound; however, the activity was significantly

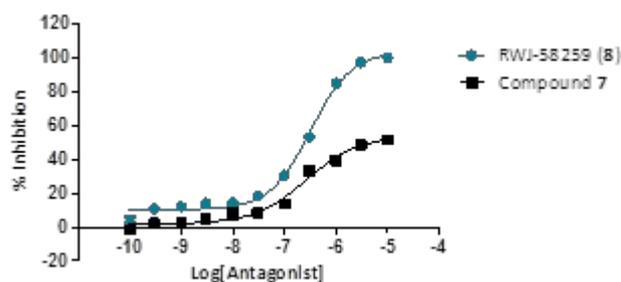
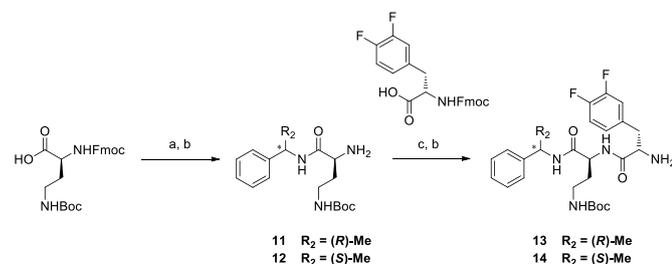


Figure 2 Dose-inhibition curves for compound **7** and RWJ-58259 (**8**) versus thrombin (10 nM) in a Ca²⁺ mobilisation assay

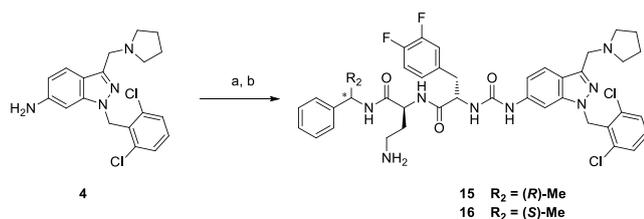
reduced with only 50% inhibition achieved compared to RWJ-58259 (Figure 2). This result, to the best of our knowledge, is the first example of a proposed metabolite of RWJ-58259 showing significant PAR-1 antagonist activity and confirms the possible role for metabolites in the overall biological activity demonstrated by RWJ-58259 in *in vivo* studies.

It was reasoned, however, that increasing the metabolic stability of the parent compound would be favourable in order to maintain the full antagonist activity demonstrated in *in vitro* studies over a longer period. Our initial focus remained on the 3-position of the pyrrolidine ring whereby a fluorine atom, a classical bioisostere for hydrogen, was incorporated to take advantage of the strong C-F bond with the intention of reducing oxidative metabolism occurring at this position.¹⁷ Analogues **9** and **10** were synthesised following the same synthetic route as described for the synthesis of **7** except in that 3-F-pyrrolidine was used in the reductive amination step (Scheme 2).

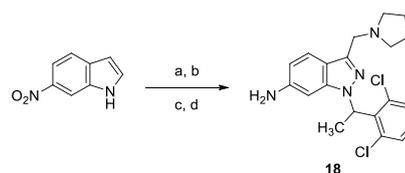
At this juncture, we also took the opportunity to expand our approach of blocking predicted sites of metabolism to include the benzylic positions highlighted in Fig. 1. Introduction of groups (*e.g.* Me, *t*-Bu) to increase steric hindrance at susceptible locations is a common strategy used to improve a molecule's metabolic stability and increase $t_{1/2}$.¹⁸ As such, analogues were synthesised with a methyl group incorporated in the terminal benzylamine by using the relevant 1-phenylethanamine for the first amide coupling reaction (Scheme 4), ultimately affording analogues **15** and **16** following urea coupling to indazole **4** (Scheme 5). This strategy was also used at the other benzylic position predicted as a major site of metabolism. To do this, 2,6-dichlorobenzaldehyde was reacted with a methyl Grignard reagent and the resulting secondary



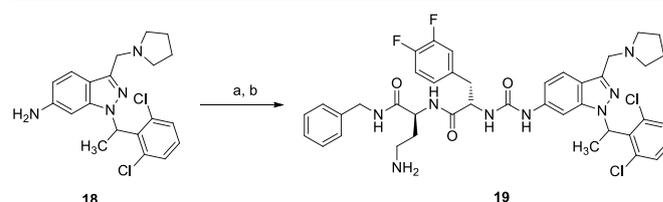
Scheme 4 Reagents and conditions: a) HBTU, HOBT, DIPEA, DMF, (R)- or (S)-1-phenylethanamine, 3 h; b) Et2NH, DMF, 2 h; c) HBTU, HOBT, DIPEA, Fmoc-3,4-diF-Phe-OH, DMF, 16 h



Scheme 5 Reagents and conditions: a) triphosgene, DMAP, THF, **13** or **14**, 0-20 °C, 2 h; b) HCl (4 M) in 1,4 dioxane, 20 °C, 2 h



Scheme 6 Reagents and conditions: a) NaNO₂, HCl (6 M), 5 h; b) pyrrolidine or 3-OH/(S)-3-F/(R)-3-F derivative, NaB(OAc)₃H, CH₂Cl₂/DMF/AcOH (90:9:1), 3 h; c) 2,6-dichlorobenzaldehyde or 1-(2,6-dichlorophenyl)ethanone, CsCO₃, THF, 20 h; d) FeCl₃·6H₂O, activated charcoal, Me₂NNH₂, MeOH, reflux, 5 h



Scheme 7 Reagents and conditions: a) triphosgene, DMAP, THF, **2**, 0-20 °C, 2 h; b) HCl (4 M) in 1,4 dioxane, 20 °C, 2 h

alcohol converted to the desired mesylate (**17**, see ESI for details) following treatment with methanesulfonyl chloride. This alkylating reagent was then used for the synthesis of indazole **18** (Scheme 6), ultimately leading to target compound **19** (Scheme 7).

Compounds **9**, **10**, **15**, **16** & **19** were then tested for biological activity and pleasingly were mostly found to be of similar potency to RWJ-58259 (**8**, pIC₅₀ of 6.5) with pIC₅₀ values in the range of 6.3-6.6 (Table 1); compound **19** even demonstrated a slight increase in potency with a pIC₅₀ of 7.1. Analogues **15**, **16** & **19**, with substituted benzylic positions, were shown to maintain full antagonist activity when compared to the activity of RWJ-58259. Interestingly, however, incorporating a fluorine atom at the 3-position of the pyrrolidine ring resulted in less than full antagonism for compounds **9** and **10**. The reduced activity demonstrated for compounds **7**, **9** & **10** infers that substitution of the pyrrolidine ring has a significant effect upon the compounds ability to fully block the binding of the sequence of amino acids uncovered following exposure to thrombin. Since a fluorine atom is of comparable size to that of a hydrogen atom, this trend is unlikely to be as a result of a steric effect. Instead this trend is likely to be a consequence of the influence of having an electronegative atom (F or O) near the nitrogen of the pyrrolidine ring, *i.e.* reducing the basicity of the nitrogen lone pair.¹⁹

Table 1 pIC₅₀ and activity data for antagonists of PAR-1

Compound	pIC ₅₀	Antagonist activity ^a
RWJ-58259 (8)	6.5	Full
7	6.6	54%
9	6.4	79%
10	6.5	63%
15	6.3	Full
16	6.4	Full
19	7.1	Full

a – compared to RWJ-58259 as standard

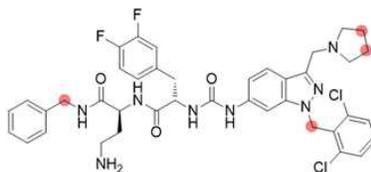
To conclude, we have shown that a predicted metabolite of PAR-1 antagonist RWJ-58259 demonstrates similar potency to the parent compound in an *in vitro* Ca²⁺ mobilisation assay, though intrinsic activity was significantly reduced. To the best of our knowledge, this is the first time a proposed metabolite of RWJ-58259 has been proven to demonstrate PAR-1 antagonist activity of similar potency to RWJ-58259. This result sheds some light on the reported *in vivo* activity of RWJ-58259 despite its incredibly poor bioavailability. Analogues were synthesised with the intention of maintaining the potency and activity demonstrated by RWJ-58259, whilst increasing the metabolic stability of the administered compound using well known strategies for reducing metabolism. Several compounds were tested and shown to be full antagonists of PAR-1 and equipotent to RWJ-58259. We believe our findings suggest that analogues of RWJ-58259 targeted at blocking metabolism, such as compounds **9**, **10**, **15**, **16** & **19**, may lead to significantly enhanced bioactivity as a result of the administered compound remaining the major active ingredient for a longer period rather than a less efficacious metabolite.

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Table of Contents Entry

How to improve PAR-1 antagonist RWJ-58259:



Understanding the pharmacokinetic behaviour of PAR-1 antagonist RWJ-58259 and the synthesis of analogues to enhance metabolic stability.