Dear Sirs,

Protease-activated receptors (PARs) are a family of four G protein–coupled receptors expressed on the surface of a wide variety of cells (1). PAR function has been thoroughly studied and the use of pharmacological agents and mouse genetic models have facilitated the discovery of numerous functions for these receptors over the past decade or so (for review, see [2, 3]). PAR1 is the prototypical receptor in this family and is by far the most extensively studied-in large part because PAR1 is the major thrombin receptor on human platelets and antagonists of PAR1 have been developed as novel anti-platelet agents for the prevention of arterial thrombosis (4, 5). Both atorvapax (formerly E5555) and vorapaxar (formerly SCH530348) are small, competitive, orthosteric PAR1 antagonists under clinical investigation for the prevention of arterial thrombosis (4, 5). As a result of this clinical advancement, numerous PAR1 antagonists have been developed over the last ~15 years. However, few of these exhibit sufficiently useful potency and selectivity and, of those, only one – SCH79797 (6) (a predecessor to SCH530348) – is commercially available. For these reasons, SCH79797 has become the most commonly used experimental PAR1 antagonist over the last decade, and has been used to study the role of this receptor in a number of settings, including cell proliferation (7), metastasis (8), placentation (9), steroidogenesis (10), kidney function (11), ischaemia reperfusion (12), inflammation (13), and thrombosis (14), and in cell types as broad as neurons (15), smooth muscle cells (16), endothelial cells (17), fibroblasts (18), macrophages (19), cancer cells (20), and platelets (21). Here we report that SCH79797, at concentrations required to achieve meaningful PAR1 antagonism, exerts an important off-target effect on the structure and function of platelets independently of PARs.

We first confirmed that SCH79797 displayed the potency and selectivity for PAR1 inhibition as previously reported (6). SCH79797 (10 μM; 10 minutes [min] pre-treatment) abolished aggregation of human washed platelets induced by a PAR1-activating peptide (PAR1-AP; 30 μM TFLLR) but had no effect on responses to any other PAR-dependent event. Finally, platelets pretreated with SCH79797, but not E5555, and then stimulated with a PAR4-activating peptide (PAR4-AP; 100 μM AYPGKF), collagen-related peptide (CRP) and ADP (Figure 1A). However, platelets pretreated with SCH79797 in these experiments lacked the initial shape change response (recorded as a decrease in light transmission immediately following agonist stimulation) always observed in un-treated or vehicle- (DMSO, 0.2% v/v) treated platelets in response to any agonist examined (Figure 1A). The initial shape change response is caused by activated platelets becoming more spherical and forming pseudopods. Since the loss of this response is often due to prior shape change, we next examined the morphology of SCH79797-pretreated human platelets and saw these cells appeared more spherical than vehicle-treated platelets (Figure 1B). In line with these observations, the mean platelet volume (MPV) was significantly increased in SCH79797-pretreated human platelets compared with vehicle-treated platelets (9.3 ± 0.3 vs. 7.4 ± 0.1 fl; p < 0.05, t-test). A chemically distinct PAR1 antagonist, E5555 (atopaxar, 0.1 μM), also functioned as a selective inhibitor of PAR1-AP induced aggregation of human platelets (Figure 1A), but had no effect on the shape change response (Figure 1A), platelet morphology (Figure 1B), or MPV (7.4 ± 0.1 fl) when used at concentrations required to abolish PAR1-mediated platelet aggregation.

We next examined whether the altered platelet morphology induced by SCH79797 affected platelet function beyond an impairment of shape change. The plasma membranes of strongly activated platelets ‘flip’ to externalise phosphatidylserine (PS) not normally exposed on the platelet surface. Since altered platelet morphology suggested changes in membrane structure and/or function, we assessed the impact of SCH79797 on PS exposure via the binding of fluorescently labelled annexin V to the platelet surface. Strikingly, human platelets treated with SCH79797, but not E5555, externalised significant amounts of PS even in the absence of agonist stimulation (Figure 1C). PS externalisation occurred rapidly: ~20% of SCH79797-treated human platelets were PS-positive after 5 min (data not shown) and ~50% were PS-positive by 15 min compared with < 5% in vehicle- or E5555-treated platelets (Figure 1C). An equivalent rate and extent of PS exposure was observed in wild-type mouse platelets, which do not express PAR1, and in platelets isolated from PAR4+/− mice, which lack all PARs, indicating that the observed effect occurred independently of PAR binding and was therefore not due to partial agonist activity or any other PAR-dependent event. Finally, mouse or human platelets pretreated with SCH79797, but not E5555, and then stimulated with any of CRP (Figure 1C), ADP or thrombin (not shown) almost all expressed PS externally within 5 min (data not shown). Of note, and as previously reported (22, 23), none of the platelet agonists examined stimulated robust (> 20%) PS externalisation in vehicle-pretreated platelets (Figure 1C and data not shown). All of the PAR-independent effects

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Figure 1: The PAR1 antagonist, SCH79797, alters platelet morphology and function independently of PARs. A) Two distinct PAR1 antagonists, SCH79797 (10 μM) and E5555 (0.1 μM) selectively inhibit aggregation of human isolated platelets induced by a PAR1-activating peptide (P1AP; 30 μM TFLLR) but not a PAR4-activating peptide (P4AP; 100 μM AYPGKF), collagen-related peptide (CRP, 10 μg/ml), or ADP (10 μM). However, note that platelets pre-treated with SCH79797 failed to undergo the initial shape change (recorded as a decrease in light transmission immediately following agonist addition) that was always observed prior to aggregation in vehicle- (DMSO, 0.2% v/v) or E5555-treated platelets. B) Human isolated platelets treated with SCH79797, but not E5555, exhibited a more spherical morphology compared with platelets treated with vehicle (DMSO, 0.2% v/v). Two representative cells are shown for each sample, although the altered morphology was virtually uniform across a treated platelet sample. C) Externalisation of phosphotidylserine (PS) on the plasma membrane was assessed via annexin-V binding in platelets isolated from humans, wild-type mice (PAR4+/+), and PAR4-/- mice. Shown is the number of platelets expressing PS (%) in the absence and presence of SCH79797 or E5555 and following 15 min stimulation with CRP (10 μg/ml) or ionophore (iono, 10 μM), as well as in the absence of agonist stimulation (-). Note the significant PS exposure induced by SCH79797, but not E5555, even in the absence of agonist stimulation. D, E) Direct comparison of the concentration-response curves to SCH79797 and E5555 for D) the specific PAR1 inhibitory effect, as assessed by inhibition of aggregation to a PAR1-activating peptide (30 μM TFLLR), and E) the non-specific, PAR-independent, PS exposure effect. In A) and B), representative traces and images of N≥4 experiments are shown. In B), scale bar = 5 μm. In C-E), data are expressed as mean ± SEM of N=4-6 experiments; * = p < 0.05, ** = p < 0.01, *** = p < 0.001 (unpaired, two-tailed, t-test).
of SCH79797 reported here were observed across multiple batches of the drug and from two distinct commercial sources (Tocris Bioscience, Bristol, UK; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Perhaps most importantly, the PAR-independent effects of SCH79797 were always observed when the drug was used at concentrations required to abolish PAR1-mediated platelet aggregation (≥10 μM). Therefore we next performed a direct and detailed comparison of the specific PAR1 inhibitory effect and the non-specific, PAR-independent, PS exposure effect of SCH79797 versus E5555 (Figure 1D and E). In these experiments, in which each antagonist was preincubated for 10 min, a similar concentration-response profile was observed for both SCH79797 and E5555 for drug-induced PS exposure (Figure 1E). However, in marked contrast, the PAR1 inhibitory effect of E5555 was ~100-fold greater than that of SCH79797, as assessed by either IC50 or by concentration required to abolish PAR1-mediated aggregation (Figure 1D). These direct comparison studies show that PS exposure induced by SCH79797 occurs in the same concentration range as that required for meaningful PAR1 inhibition by this agent. In contrast, a ~100-fold difference was observed in the concentration of E5555 required for maximum PAR1 inhibition (0.1 μM) over which that elicits any PAR-independent effects on platelet structure and function (≥10 μM).

Together, these findings suggest that SCH79797 exerts important off-target effects on platelets when used at concentrations required to achieve significant levels of PAR1 antagonism. The spherical platelet morphology and rapid PS externalisation induced by SCH79797 in the present studies are both indicative of early apoptotic events in platelets (24). On this note, SCH79797 has previously been observed to induce apoptotic signalling events in NIH3T3 cells in culture. Specifically, Di Serio et al. showed that prolonged incubation of NIH3T3 cells with SCH79797 induced PS externalisation and caspase 3 activation (25). We did not observe a “complete” apoptotic response in platelets treated with ≥10 μM SCH79797 because the platelets remained functionally intact, as evidenced by the ability to aggregate (Figure 1A), adhere to fibrinogen (not shown), and participate in thrombus formation (not shown), and – at least in the case of aggregation – even after > 1 hour of exposure to 10 μM SCH79797. Furthermore, when we examined other early markers of platelet apoptosis following incubation with 10 μM SCH79797 (e.g. surface glycoprotein expression [24]), no effects were observed. We used the B3 mi-metric, ABT-737 (1 μM; 60 min), as a positive control for the drug-induced apoptosis in platelets (24). As previously observed (24), a similar change in morphology and increase in PV were observed in human platelets treated with ABT-737 as were observed in SCH79797-treated cells (not shown). However, in addition to these effects, ABT-737-treated platelets also shed significant levels of surface glycoprotein (GP)Ibα and GPVI (~50%) and almost completely lost their ability to adhere to fibrinogen and participate in thrombus formation ([24] and data not shown). These findings suggest that, at least under the conditions examined here, SCH79797 fails to elicit a “complete” apoptotic response in platelets.

Therefore, we have examined the current gold-standard commercially available PAR1 antagonist, SCH79797, and confirmed that it is a potent and selective PAR1 inhibitor, as assessed by platelet aggregation. However, we also show that significant off-target effects – most notably externalisation of PS on the platelet membrane and a disruption of the overall platelet morphology – were induced following exposure of cells to this drug at concentrations required to achieve PAR1 antagonism. Importantly, the same effects were not observed with either of the two PAR1 antagonists in clinical development, atopaxar (Figure 1) and vorapaxar (not shown), at PAR1-inhibiting concentrations, suggesting these drugs are more suitable agents for experimental use. Furthermore, a previous study showing similar effects in NIH3T3 cells in culture indicates that these effects of SCH79797 are not specific to platelets. As a result, we recommend caution in the ongoing use of SCH79797 as a PAR1 antagonist and suggest a need for more careful analysis of studies using this drug.

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Conflicts of interest
None declared.

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