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Evidence for human thromboxane receptor heterogeneity using a novel series of 9,11-cyclic carbonate derivatives of prostaglandin $F_{2\alpha}$

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1 The pharmacological activity of a novel series of 9,11-cyclic carbonate derivatives of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) was investigated in various isolated smooth muscle preparations possessing different prostanoid receptor subtypes as well as in human platelets. Since subdivision of thromboxane (TP-) receptors into vascular/smooth muscle and platelet subtypes is a controversial subject, our studies included a human smooth muscle preparation (myometrium) in addition to the widely used rat aorta and human platelets as TP-receptor preparations.

2 Two members of that series, AGN191976 and AGN192093 were found to be highly potent and selective thromboxane-mimetics. AGN191976 and AGN192093 contracted isolated tissues of the rat thoracic aorta with EC_{50} values of 0.32 ± 0.08 and 1.30 ± 0.53 nM, respectively. Both agonists were at least 10 times more potent than the benchmark TP-agonist, U-46619, in this preparation, whilst being at least 500 times less potent at other prostanoid receptors (DP, EP_1, EP_3, FP, IP) *in vitro*.

3 In human myometrial strips from pregnant and non-pregnant donors, both AGN191976 and AGN192093 were potent contractile agonists. The rank order of potency in myometrium of AGN191976 > AGN192093 > U-46619 correlated well with that in the rat aorta. In human platelet-rich plasma (PRP), however, AGN191976 had potent proaggregatory activity ($EC_{50} = 16.3 \pm 1.4$ nM), which is a TP-receptor-mediated event, whereas AGN192093 was a much weaker agonist ($EC_{50} = 37.9 \pm 2.0$ μM). AGN192093 did not behave as an antagonist in the platelets, since it did not antagonize platelet aggregation induced by ADP, arachidonic acid, U-46619 or AGN191976. In human washed platelets, the activity profile of AGN191976 ($EC_{50} = 4.15 \pm 0.52$ nM) and AGN192093 (no aggregation up to 10 μM) was similar to that obtained in PRP.

4 The involvement of TP-receptors was verified with the potent TP-antagonist, SQ29548. SQ29548 (0.1 μM in myometrium; 1 μM in aorta; 1 μM and 10 μM in platelets) antagonized responses to U-46619, AGN191976 and AGN192093 as expected.

5 In conclusion, AGN191976 and AGN192093, both 9,11-cyclic carbonate derivatives of $PGF_{2\alpha}$, were found to be highly potent and selective thromboxane-mimetics in rat vascular and human myometrial smooth muscle. However, only AGN 191976 was a potent agonist at TP-receptors in human platelets. The differential activity of AGN192093 on TP-receptor-mediated events in platelets and smooth muscle provides further evidence for a subdivision of TP-receptors. AGN192093 appears to be a useful tool for the pharmacological distinction of TP-receptor subtypes.

Keywords: TxA_2-mimetics; prostanoid receptors; TP-receptor heterogeneity; human platelets; smooth muscle; human uterus; rat aorta; AGN191976; AGN192093; U-46619

Introduction

Thromboxane A_2 (TxA_2) is a potent constrictor of airway and vascular smooth muscle and a potent mediator of platelet aggregation in several mammalian species. Whether these responses are mediated by one or more thromboxane receptor subtypes has been a highly debated issue since the development of a working classification for prostanoid receptors (Coleman *et al.*, 1984). According to this classification, thromboxane receptors, referred to as TP-receptors, have been characterized pharmacologically by a particular sensitivity to TxA_2, prostaglandin H_2 (PGH_2) and more stable analogues like U-46619 (9,11-dideoxymethanoepoxy-9α,11α-prostaglandin $F_{2\alpha}$) (Bundy, 1975; Coleman *et al.*, 1981). Since then, many different agonists and antagonists for TP-receptors have been described in the literature. An early study comparing a large number of full agonists, partial agonists and antagonists in the rabbit aorta, dog saphenous vein, and guinea-pig trachea found a generally good correlation between the tissues, which suggested TP-receptor homogeneity (Jones *et al.*, 1982). Similarly, Harris *et al.* (1992) found a good agreement between rat aorta and guinea-pig trachea contraction and human platelet aggregation using three structurally similar antagonists namely SQ29548, SQ33261 and SQ33552. A structurally different antagonist, GR32191, inhibited responses induced by U-46619 in human platelets, human pulmonary artery, rat aorta, and dog saphenous vein with similar potency in all four systems (Lumley *et al.*, 1989). Almost identical pA_2 values were obtained when GR32191 was tested against some other TP-agonists in the rat aorta and human platelets. The most compelling pharmacological evidence supporting homogeneity of TP-receptors was provided by Swayne *et al.* (1988). They compared the activities of nine structurally dissimilar prostanoid and non-prostanoid

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TP-antagonists in human and rabbit platelets, rabbit aorta and guinea-pig airway smooth muscle and reported significant correlations between assays and species.

However, evidence suggesting heterogeneity of TP-receptors cannot be dismissed. Binding studies with a large number of TXA_2-analogues in human platelets and canine saphenous vein revealed significant differences with respect to the rank order of potencies (Mais *et al.*, 1988). In human platelets and rat aorta the similarities in antagonist potencies for GR32191 against different TP-agonists contrasted with the differences in potencies obtained with different antagonists against U-46619 and it was proposed that not all TP-ligands may be suitable for detecting receptor heterogeneity (Lumley *et al.*, 1989). It was subsequently suggested by others that, with the pharmacological tools presently at hand, receptor heterogeneity can only be detected when comparing antagonist potencies whereas comparison of agonist potencies supports the single receptor concept (Tymkewycz *et al.*, 1991). Thus, by using various antagonists, differences in potencies between platelets and smooth muscle tissues from different species were found.

A substantial amount of all research on prostanoid receptors has involved the TP-receptor, resulting in the availability of a significant number of pharmacological tools, agonists as well as antagonists. Hence, it seems almost inconceivable that disagreement has been unresolved regarding the possibility of TP-receptor sub-division. The answer to the conflicting reports concerning TP-receptor heterogeneity seemed to be provided finally by Masuda *et al.* (1991) who took the suggestions by Tymkewycz *et al.* (1991) one step further with their findings that it is in fact the 13-azapinane TxA_2 analogues which discriminate between TP-receptors in platelets and rat aortic smooth muscle cells. Other, structurally different TP-antagonists did not provide such discrimination, which would answer the question as to why so many researchers failed to detect TP-receptor heterogeneity. Masuda *et al.* (1991) concluded from their experiments that the differences between platelets and aortic smooth muscle cells may reflect a subdivision of TP-receptors rather than a species difference, since the results obtained in platelets from human subjects and rats correlated rather well. This contention was further supported by a recent report on a non-prostanoid TP-antagonist that exhibited a good separation between activities in the rat aorta and human platelets, where a pA_2 value of 9.95 and a IC_{50} value of 2.4 μM respectively against U-46619 had been determined (Kosakai *et al.*, 1993).

Conclusive evidence that thromboxanes exert their effects through a typical G-protein coupled receptor, as had first been proposed from pharmacological studies (Kennedy *et al.*, 1982), was provided by cloning of the human thromboxane receptor (Hirata *et al.*, 1991). This triggered the subsequent cloning of several other prostanoid receptors. However, Northern blot analysis and sequencing of mRNA clones derived from different human tissues, including a platelet precursor cell line, did not reveal any differences. This prompted the conclusion that only one TP-receptor type exists in man. Cross-hybridization with rat tissues was unsuccessful, and this was ascribed to species rather than receptor differences, thereby reopening the question of TP-receptor heterogeneity from a new angle.

Recently, we reported the discovery of a series of dioxobicyclo-derivatives which included some highly potent thromboxane-mimetics (Burk *et al.*, 1994). In the present study, we detail the pharmacological profile of these compounds, which provide further conclusive evidence for the existence of thromboxane receptor subtypes.

Methods

Isolated tissue preparations

Smooth muscle tension of isolated tissues, suspended in jacketed 10 ml glass organ baths (Harvard Apparatus, South Natick, MA; Radnoti Glass Technology, Monrovia, CA), was measured isometrically with force displacement transducers (Grass FT03, Grass Instruments, Quincy, MA, U.S.A.) and recorded on a Grass Polygraph (Models 7G and 79E). The organ baths contained Krebs buffer maintained at 37°C and gassed with a mixture of 95% O_2 and 5% CO_2 to give a final pH of 7.4. The Krebs buffer had the following composition (mM): NaCl 118.0, KCl 4.7, KH_2PO_4 1.2, $CaCl_2$ 1.9, $MgSO_4$ 1.18, $NaHCO_3$ 25.0, glucose 11.7 and indomethacin 0.001.

Rat thoracic aorta

Adult Sprague-Dawley rats weighing 180–250 g were anaesthetized by CO_2 inhalation and then decapitated and exsanguinated. The thoracic portion of the aorta was removed and cleaned of any adhering tissue. The lumen was flushed immediately to remove any blood. The aorta was dissected into three small segments, 5 to 8 mm in length. Each segment was mounted under 2 g of tension in the jacketed organ bath with the aid of two wire hooks placed through the lumen of the vessel. This arrangement allowed for the measurement of contractile forces developed by the circular smooth muscle. The tissues were allowed to equilibrate for 1 h, then compounds were added cumulatively to the organ baths. The response to 1×10^{-7} M U-46619 was determined at the beginning and end of each experiment to give reference contractions. A 30 to 45 min recovery period was allowed after complete wash-out of a drug. Only one test compound was examined in each tissue. Generally, fluid contents of the organ baths were replaced with fresh buffer solution every 15 to 20 min during equilibration and wash-out periods.

Guinea-pig and chick ileum

Albino Hartley strain guinea-pigs weighing 350 to 500 g were pithed followed by exsanguination. Young chickens, 7 to 21 days old, were killed by cervical dislocation. Proximal ileal portions of 1.5 cm length were removed, cleaned of adhering connective tissue and flushed. The longitudinal strips were suspended under 1 g of tension. Contractile responses were obtained in a non-cumulative fashion. After a 1 h equilibration period, a standard dose-response curve to PGE_2 was obtained with 30 min wash-out periods between individual doses. Subsequently, one compound was tested. A maximal concentration of PGE_2 (1 μM) was given at the end to serve as a second reference standard and to verify viability of the tissues. Contractile activity at each concentration of test compound was calculated as percentage of the maximal PGE_2 response.

Guinea-pig vas deferens

Activity of prostanoids was determined by the ability to inhibit the electrically stimulated smooth muscle twitch response. A 1.5 cm portion of the vas deferens was suspended longitudinally under 1 g of tension and allowed to equilibrate for at least 30 min without electrical stimulation.

Tissues were then subjected to stimulation every 30 s by a train of electrical pulses. Each train consisted of ten 20 V pulses of 1 ms duration each with individual pulses separated by 100 ms. Electrical currents were generated with a Grass S48 Stimulator (Grass Instruments) and distributed to the individual organ baths with a Stimu-Splitter II (Med-Lab Instruments, Loveland, CO, U.S.A.) using glass tissue supports with stimulating platinum electrodes in parallel orientation to the tissues (Radnoti Glass Technology, Monrovia, CA, U.S.A.). After stabilization of the twitch response, PGE_2 was cumulatively tested resulting in a complete inhibition of the twitch response at a concentration between 1 and 10 nM. Two investigational compounds were then evaluated in a cumulative fashion as well. PGE_2 was once again tested at the conclusion of the experiment. Between compounds, tissues were

washed out and allowed to re-equilibrate for 1 h. Activity was calculated as percentage inhibition of the muscle twitch response.

Cat iris sphincter

Adult domestic cats were killed by intravenous injection of 1 ml of Eutha-6 C-II (pentobarbitone sodium, Western Medical Supply, Arcadia, CA, U.S.A.). The eyes were enucleated immediately and placed on ice. The iris sphincter from each eye was dissected to provide strips approximately 3 mm wide and 15 to 20 mm long. The sphincter muscle was mounted vertically under 50 to 100 mg tension. A 1 h stabilization period was allowed at the beginning of each experiment. Activity was determined as contractile responses. Compounds were added cumulatively to the organ bath and at least 30 min were allowed for recovery after complete wash-out and return to baseline tension. The response to 1×10^{-7} M $PGF_{2\alpha}$ was determined at the beginning and end of each experiment and between compounds as a reference. No more than two compounds were tested in each tissue.

Human myometrium

Human non-pregnant myometrium, sampled from the anterior wall of the corpus uteri, was obtained from premenopausal patients undergoing hysterectomy for benign disorders. Human pregnant myometrial samples were taken from the lower segment of the uteri of consenting donors during elective Caesarean section. Preparations were completed within 1 h after surgery and the experiments were conducted as previously described in detail (Senior *et al.*, 1991). Briefly, longitudinal tissue strips ($20 \times 3 \times 3$ mm), free from endometrium and serosa, were placed under initial tension of 2 g and superfused at a rate of 2 ml min^{-1} with Krebs solution. The strips were allowed to equilibrate for a minimum of 2 h and until spontaneous activity had become regular. Agonists were added as a bolus dose to the superfusate and the amounts reported do not represent actual concentrations in the buffer medium. The antagonist, on the other hand, was dissolved in the Krebs buffer at the concentration indicated, and the tissues were in contact with the antagonist for not less than 30 min before agonist dose-effect curves were constructed. Only one compound was tested per tissue whereby dose-effect curves were constructed sequentially. The response to 1×10^{-8} mol $PGF_{2\alpha}$ was determined at the beginning and end of each experiment as a reference. Comparisons were made between preparations in a non-paired manner. Responses were quantified as follows: agonist doses were added to the tissues at resting/baseline tension immediately after one spontaneous, rhythmic contraction had subsided and before the next spontaneous contraction was expected to start which could be reliably estimated due to the regularity of these rhythmic contractions. Thus, it is important to note that any contraction, smaller or larger in magnitude than a spontaneous contraction, occurring immediately after a drug was added is in response to the drug administration and not a spontaneous contraction. Responses to an agonist (T) were expressed relative to the intrinsic spontaneous background activity (B) as T/B ratio. Thus, a drug-induced response of equal magnitude to a spontaneous contraction was calculated as T/B = 1 and any T/B value of larger than zero represents an actual drug effect.

Human platelets

Platelet-rich plasma (PRP) Prostanoid activity in human platelets was determined by the ability to induce aggregation (TP-receptor activity) or to inhibit ADP-induced aggregation (DP- and IP-receptor activity) *in vitro*. Fresh whole blood was drawn from consenting healthy human volunteers who did not take any drug at that time, and mixed in a 9:1 ratio with acid citrate-dextrose (ACD) to prevent coagulation. The blood was centrifuged at 250 *g* for 15 to 20 min at room temperature in an Omnifuge RT (Heraeus Sepatech, Osterode, Germany) centrifuge to obtain platelet-rich plasma (PRP). The PRP was used within 2 h; 4.5 μl of prostanoid or vehicle was added to 450 μl of PRP, incubated for 2 min at 37°C in a Payton aggregometer and observed for any aggregatory activity. Aggregatory activity was recorded in this system as an increase in light transmission through the cuvette containing the platelet suspension. Information on platelet shape change could be obtained simultaneously in these experiments. Shape change responses were recorded as a transient decrease in light transmission which occurred within seconds after addition of an aggregatory agent and preceded the aggregatory response. Alternatively, certain concentrations of a compound could solicit only a shape change response without causing the platelets to aggregate. In this case, the shape change response was recorded and expressed as a negative aggregatory response. ADP 20 μM (final concentration) was then added to the cuvette to induce full aggregation. A fresh sample of PRP was used for each concentration of each agonist tested. Standard aggregatory responses to 20 μM ADP alone were obtained at the beginning and end of each experiment. Aggregatory activity was calculated as the percent aggregation induced by the prostanoid relative to the aggregation induced by 20 μM ADP alone. Inhibition of aggregation was calculated as the percentage difference between aggregation evoked by 20 μM ADP in the absence and presence of a drug.

Inhibitory (DP/IP-) activity of AGN191976 and AGN192093 was assessed differently, since AGN191976 was a potent TP-agonist which could potentially mask any anti-aggregatory activity and it was possible that the weak aggregatory activity of AGN192093 was a result of concomitant pro- and anti-aggregatory activities resulting in a lack of a net effect. The protocol was the same as described above except that TP-receptors were blocked by preincubating the PRP with the potent TP-antagonist, SQ29548 (10 μM) for 5 min prior to addition of AGN191976 or AGN192093 and then 20 μM ADP.

It was also determined whether AGN192093 was able to block platelet aggregation induced by U-46619 (10–1000 nM), AGN191976 (1–1000 nM), arachidonic acid (800 μM) and ADP (2, 20 μM). For those experiments the PRP was preincubated with either 0.1 or 1 μM AGN192093 or vehicle for 5 min prior to addition of the aggregating agent. Responses were compared to aggregation induced by 20 μM ADP alone.

Washed platelets Washed platelets were prepared as described by Mais *et al.* (1985). Briefly, PRP obtained as described above was centrifuged at 800 *g* for 20 min at room temperature. The supernatant (plasma) was aspirated, discarded and replaced with an equal volume of suspension buffer containing Tris-HCl 50 mM, NaCl 100 mM, glucose 5 mM and indomethacin 10 μM at pH 7.4 in which the platelet pellet was resuspended. $CaCl_2$ was then added to the platelet suspension to give a final concentration of 250 μM. Unlike its effect in PRP, 20 μM ADP did not give a reproducible aggregatory response in washed platelets over time. Therefore, U-46619 (1 μM) was used as a standard agonist in lieu of ADP in a similar fashion. Only aggregatory activities of AGN191976 and AGN192093 were determined and compared to aggregation obtained with 1 μM U-46619 alone.

Drugs

AGN191976 ((Z)-7-[(1α, 5α, 6α, 7β)-7-[(1E, 3S)-3-hydroxy-1-octenyl]-3-oxo-2,4-dioxobicyclo[3.2.1]oct-6-yl]-5-heptenoic acid), AGN192093 ((Z)-7-[(1α, 5α, 6α, 7β)-7-[(1E, 3S)-3-hydroxy-1-octenyl]- 3-oxo-2,4-dioxobicyclo[3.2.1]oct-6-yl]-5-heptenol), AGN192107 ((Z)-7-[(1α, 5α, 6α,7β)-7-hydroxymethyl-3-oxo-2,4-dioxobicyclo[3.2.1]oct-6-yl]-5-heptenoic acid), AGN192134 (N-isopropyl (Z)-7-[(1α, 5α, 6α, 7β)-7-[(1E, 3S)-3-

hydroxy-1-octenyl]-3-oxo-2,4-dioxobicyclo[3.2.1]oct-6-yl]-5-heptenamide), AGN192135 ((Z)-7-[(1α, 5α, 6α, 7β)-7-[(1E 3S)-3-hydroxy-1-octenyl]-3-oxo-2,4-dioxobicyclo[3.2.1]oct-6-yl]-5-heptenamide), AGN192181 ((Z)-7-[(1α, 5α, 6α, 7β)-7-(hexyloxy)methyl-3-oxo-2,4-dioxobicyclo[3.2.1]oct-6-yl]-5-heptenoic acid) were synthesized at Allergan. Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) tromethamine salt, PGE_2, U-46619 (9,11-dideoxymethano-epoxy-9α,11α-prostaglandin $F_{2\alpha}$), SQ29548 (7-[3-[[2-[(phenylamino)carbonyl]hydrazino]methyl]-7-oxabicyclo[2.2.1]hept-2-yl-, [1S-[1α,2α(Z),3α,4α]]-5-heptenoic acid) and I-BOP (7-[3-[3-hydroxy-4-(4-iodophenoxy)-1-butenyl]-7-oxabicyclo[2.2.1]-hept-2-yl]-, [1S-[1α,2α(Z),3β(1E,3S*),4α]]-5-heptenoic acid) were obtained from Cayman Chemical (Ann Arbor, MI). Iloprost and BW245C were gifts from Schering AG, Berlin, Germany and Wellcome Research Laboratories, Beckenham, UK respectively. All other chemicals were purchased from Sigma Chemical.

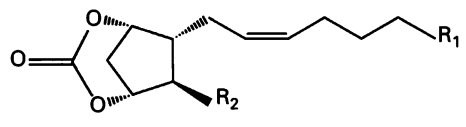
Calculations and statistical analysis

The data described in this study are expressed as arithmetic means ± standard error of the mean. Coefficients for linear regression and correlation and corresponding P values were obtained with the StatView II and Primer of Biostatistics software packages for MacIntosh computers. Differences were considered statistically significant at $P<0.05$.

Results

Effects of dioxobicyclo-derivatives on different smooth muscle preparations

We evaluated several dioxobicyclo-derivatives for their activity at different prostanoid receptors. The structures of these compounds are shown in Figure 1. Agonist activities in isolated tissues were determined in a cumulative (rat thoracic aorta, cat iris sphincter, guinea-pig vas deferens) or non-cumulative fashion (guinea-pig ileum, chick ileum, human myometrium) and expressed relative to a standard agonist (all smooth muscle preparations except human myometrium) or as T/B ratio relative to spontaneous contractions (human myometrium) as described in more detail in the Methods section.



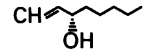
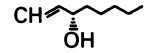
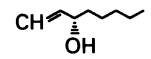
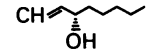
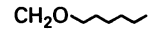
AGN No.	R_1	R_2
191976	COOH	
192093	CH_2OH	
192107	COOH	CH_2OH
192134	CONH 	
192135	$CONH_2$	
192181	COOH	

Figure 1 Chemical structure of 9,11-cyclic carbonate derivatives of prostaglandin $F_{2\alpha}$.

Two members of the series, AGN191976 and AGN 192093, were found to be highly potent and selective thromboxane-mimetics. The rank order of potency for the rat aorta TP-receptor was AGN191976 > AGN192093 > > AGN192135 > AGN192134 > AGN192181 > > AGN192107 (=0) (Figure 2 and Table 1). In fact, AGN191976 and AGN192093 were two of the most potent TP-agonists ever reported, with EC_{50} values in the rat thoracic aorta of 0.32 ± 0.08 nM and 1.30 ± 0.53 nM, respectively (Table 1). Thus, they are respectively approximately 50 and 10 times more potent than the benchmark TP-agonist, U-46619 (Tables 1 and 2). While AGN191976 retained some activity at EP and FP-receptors, AGN192093 was virtually devoid of EP-activity, but retained residual FP-activity as shown in Table 1. Compared to the activity at EP and FP-receptors, AGN191976 and AGN192093 showed at least 1000 fold and 500 fold selectivity for the vascular TP-receptor, respectively.

AGN192107, which by itself was inactive in the rat aorta (Table 1), failed at a 10 μM concentration to antagonize contractions elicited by U-46619 in the same preparation, suggesting that AGN192107 was devoid of any agonist or antagonist activity (data not shown).

AGN191976 as well as AGN192093 were yet more potent stimulators of isolated strips from human myometrium than U-46619. The dose-response relationship in non-pregnant myometrium is depicted graphically in Figure 3. Comparing at the level of a T/B ratio of 2.0 (for details see the Methods section) AGN191976 was approximately 6 and 100 times more

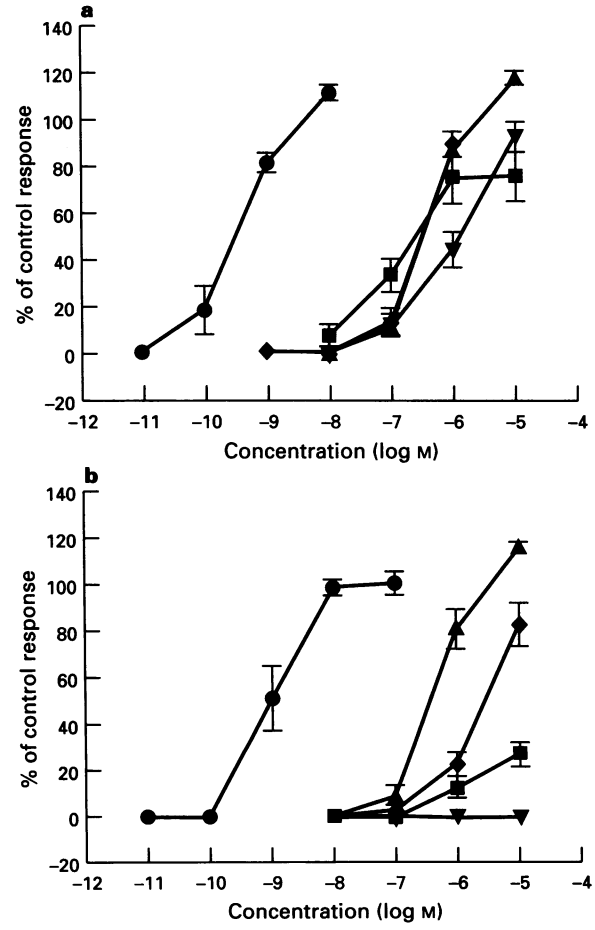


Figure 2 Effect of AGN191976 (a) and AGN192093 (b) on isolated smooth muscle preparations from rat aorta (●), cat iris sphincter (▲), chick ileum (■), guinea-pig vas deferens (◆) and guinea-pig ileum (▼). Each point represents the mean ± s.e.mean of 4 separate experiments. The activity of AGN191976 and AGN192093 is expressed relative to a standard agonist for each preparation as described in the Methods section.

Table 1 Potencies of various dioxobicyclo compounds at different prostanoid receptors

AGN No.	TP_{RA}	TP_{HP}	DP/IP_{HP}	EP_1	$EP_{3/CI}$	$EP_{3/GP}$	FP
191976	0.32±0.08	16.3±1.4	NA	1,420.3±503.7	393.8±207.8	308.0±47.7	440.5±35.3
192093	1.30±0.53	37,900±2,000	NA	NA	$>10^4$	3,241.3±808.0	558.3±127.8
192134	493.5±118.7	NA	NA	ND	ND	ND	3,188.7±642.7
192135	69.7±21.2	3,120.0±0.0	NA	ND	ND	ND	443.0±124.3
192107	NA	NA	NA	ND	ND	ND	NA
192181	1,582.0±415.5	NA	NA	ND	ND	ND	$>10^4$
Standard agonist	14.9±1.9 (U-46619)	538.3±2.8 (U-46619)	1.79±0.36 (BW245C) 3.44±0.32 (Iloprost)	5.34±1.67 (PGE_2)	4.31±1.09 (PGE_2)	0.57±0.10 (PGE_2)	8.11±0.75 ($PGF_{2\alpha}$)

Mean EC_{50} values±s.e.mean (nM) derived from 4 to 8 separate experiments. NA and ND denote 'not active at 10 μM' and 'not determined' respectively. Assay systems are abbreviated as follows: TP_{RA}, rat aorta; TP_{HP}, human platelet (PRP) aggregation; DP/IP_{HP}, inhibition of aggregation of human platelets (PRP); EP_1, guinea-pig ileum; $EP_{3/CI}$, chick ileum; $EP_{3/GP}$, guinea-pig vas deferens; FP, cat iris sphincter.

potent than AGN192093 and U-46619, respectively. Despite differences in potencies, AGN192093 and U-46619 were about equally efficacious, with maximal T/B ratios of 2.68±0.26 and 3.21 ±0.29, respectively. AGN191976, on the other hand, was not only more potent but was also more efficacious. Its T/B ratio did not appear to have peaked at the highest concentration investigated (T/B=4.62±0.23 at 10 nM) and was similar to the previously reported maximum for the human non-pregnant myometrium of 4.3 for $PGF_{2\alpha}$ (Senior *et al.*, 1992). A similar profile of activities for these agonists was obtained in myometrial strips from pregnant donors (data not shown).

Effects of dioxobicyclo-derivatives on human platelet aggregation

Aggregation of human platelets was investigated in platelet-rich plasma, PRP. In human PRP, AGN191976 was a powerful aggregatory agent (EC_{50}=16.3±1.4 nM) and exceeded U-46619 and I-BOP in potency by one order of magnitude (Table 2). Although a very potent TxA_2-mimetic at vascular TP-receptors, AGN192093 elicited platelet aggregation only at a high concentration of 100 μM, yielding an EC_{50} of 37.9±2.0 μM (Table 2). The rank order of potency at the platelet TP-receptor for the 9,11-cyclic carbonate derivatives of $PGF_{2\alpha}$ was AGN191976>>AGN192135>AGN192093> AGN192134, AGN192107, AGN192181 (=0).

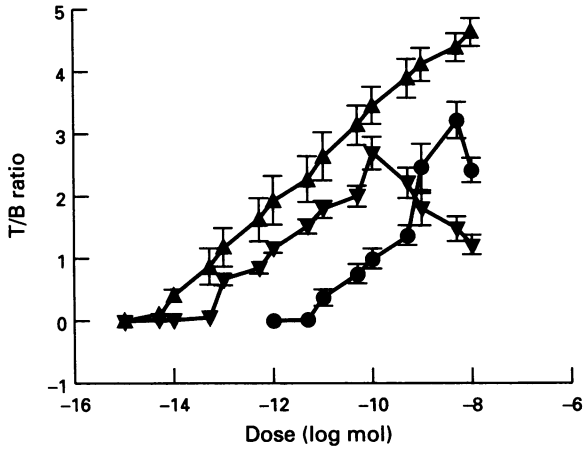


Figure 3 Activities of U-46619 (●), AGN191976 (▲) and AGN192093 (▼) in human non-pregnant myometrium. Each point represents the mean±s.e.mean of 5 separate experiments. The activity is expressed as T/B ratio as explained in the Methods section.

Table 2 TP-receptor potency of different thromboxane-mimetics

	Aorta	*Uterus*	*Platelets*
I-BOP	0.47±0.11	ND	222.5±57.5
U-46619	14.9±1.9	1.12±0.51	538.3±2.8
AGN191976	0.32±0.08	0.010±0.007	16.3±1.4
AGN192093	1.30±0.53	0.058±0.016	37.9±2.0 μM

Mean EC_{50} values±s.e.mean (nM) for rat aorta (contraction) and human platelet-rich plasma (aggregation) are derived from 4 to 8 separate experiments. Values for human non-pregnant myometrium represent the mean dose±s.e. mean (nmol) at a T/B ratio of 2.0 for 5 separate experiments. ND, not determined.

At concentrations approximately one order of magnitude lower than those required to produce full aggregation, U-46619, AGN191976 and AGN192093 caused a shape change in platelets. The rank order of potency for the shape change response in PRP was AGN191976>U-46619>>AGN192093. The concentrations that elicited a maximal shape change response were 3 nM, 100 nM and 10 μM respectively. Platelet shape changes are reflected in Figures 4 and 6 as negative aggregatory values as explained in the Methods section.

To ascertain that the weak aggregatory activity of AGN192093 in PRP was not due to excessive plasma protein binding, AGN192093, and AGN191976 for comparative purposes, were also investigated in washed human platelets. The effects of both compounds on aggregation of PRP and washed human platelets were almost identical. AGN192093 tested at a concentration range of 0.1 to 10 μM, did not cause any aggregatory response in washed platelets, whereas AGN191976 was again a potent mimetic (EC_{50}=4.14±0.52 nM, n=4) (data not shown). This suggests that the weak aggregatory activity of AGN192093 was not a result of excessive plasma protein binding.

To determine if AGN192093 behaved as an antagonist at the platelet TP-receptor, human PRP was preincubated for 5 min with either 0.1 μM or 1.0 μM AGN192093 prior to stimulation of aggregation with either U-46619 or AGN191976 in a separate set of experiments. AGN192093 was completely devoid of any inhibitory activity against U-46619 and AGN191976, as graphically depicted in Figure 4. As Table 3 shows, AGN192093 did not antagonize aggregation induced by arachidonic acid or ADP.

The lack of potency of AGN192093 in platelets could potentially have been due to similar pro- (TP) and anti-aggregatory (DP/IP-) activities, with no resulting net effect. In addition, AGN191976 was a potent TP-mimetic which did not

allow proper determination of DP/IP-receptor activity in the platelets with our experimental protocol. Thus, the effects of both compounds on platelet DP/IP-receptors were examined in a separate set of experiments in PRP in the presence of a high concentration of the potent TP-antagonist, SQ29548 (10 μM) to block any aggregatory effects linked to TP-receptor stimulation. Potential drug-induced inhibition of aggregation could then be observed since ADP-induced aggregation does not involve TP-receptors. Under these conditions, neither AGN191976 (0.1–100 μM, $n=4$) nor AGN192093 (0.1–100 μM, $n=4$) prevented ADP-induced aggregation suggesting that both $PGF_{2\alpha}$-analogues lack DP- or IP-receptor activity (data not shown). DP- and IP-receptor agonists, like BW245C and iloprost respectively, on the other hand, potently antagonize ADP-induced aggregation of PRP with EC_{50} values of 1.79 ± 0.36 and 3.44 ± 0.32 nM, respectively (Table 1).

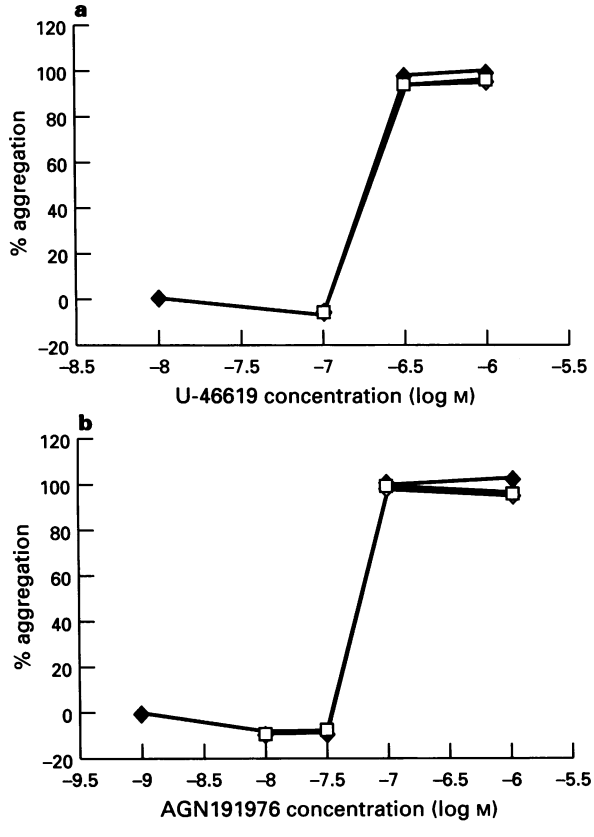


Figure 4 Platelet aggregation induced by U-46619 (a) and AGN191976 (b) in the absence (◆) and presence of 10^{-7} M (◇) or 10^{-6} M (□) AGN192093. Each point represents the mean ± s.e.mean from at least 3 separate experiments.

These data taken together suggest that AGN192093 behaves as a very weak agonist of human platelet aggregation, unlike AGN191976 which is a highly potent aggregating agent.

Comparison of effects of different thromboxane-mimetics on rat aorta and human platelets

When EC_{50} values for I-BOP, U-46619 and the two dioxobicyclo derivatives AGN191976 and AGN192135 were compared, a strong correlation (coefficient for linear regression and correlation $r=0.9964$, $P=0.0036$) was found between rat aorta and human platelets. Inclusion of EC_{50} values for AGN192093 in the analysis, however, eliminated any correlation between the data points (Figure 5, $r=-0.2271$, $P=0.71$). It has to be emphasized that these calculations of statistical correlation are based on a rather small number of data points and have to be viewed with some caution. However, these results lend support to the notion that either differences or similarities between agonist potencies in rat aorta and human platelets may be found depending on the structures of the compounds used.

Inhibition of TP-receptor-mediated responses in rat aorta, human myometrium and human platelets by SQ29548

In a separate series of experiments, U-46619, AGN191976 and AGN192093 were tested in the absence and presence of the TP antagonist, SQ29548, in rat aorta, human uterus and human

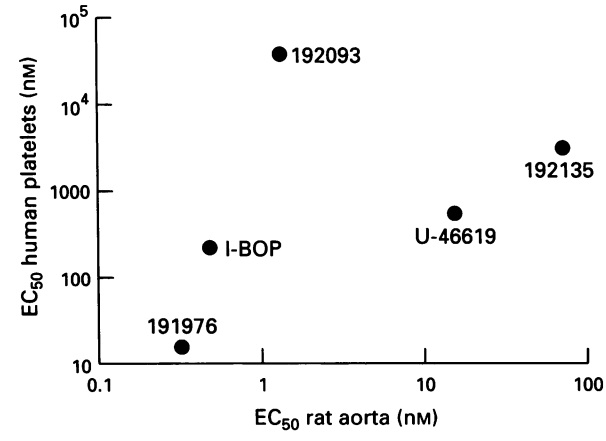


Figure 5 Comparison of TP-agonist potencies for human platelets and rat aorta. EC_{50} values for I-BOP, U-46619, AGN191976, AGN192093 and AGN192135 have been plotted on a logarithmic scale. Coefficients and P-values for linear correlations are $r=-0.2271$, $P=0.71$. If AGN192093 is excluded from the calculations, the potencies of the remaining 4 agonists are highly correlated ($r=0.9964$, $P=0.0036$).

Table 3 Effect of AGN192093 on agonist-induced aggregation of human platelets (platelet-rich plasma)

		Platelet aggregation AGN192093		
		–	10^{-7} M	10^{-6} M
Arachidonic acid	800 μM	101.6 ± 1.3	98.0 ± 1.8	98.8 ± 1.1
ADP	20 μM	100 ± 0	99.9 ± 1.4	97.6 ± 1.8
ADP	2 μM	73.8 ± 11.2	68.3 ± 12.9	73.1 ± 14.3

Values are means ± s.e.mean expressed as percentage aggregation relative to a maximally effective standard (20 μM ADP). Platelets had been preincubated with AGN192093 or vehicle for 5 min before stimulation of an aggregatory response through addition of arachidonic acid or ADP. Each agonist was tested in PRP preparations from at least 3 different donors in the absence and presence of AGN192093.

platelets. Pretreatment times with SQ29548 of 30 and 5 min were selected for smooth muscle tissues and platelets, respectively. As illustrated in Figure 6, SQ29548 potently blocked agonist stimulated contractions in the rat aorta (1.0 μM SQ29548) and platelet aggregation (1.0 μM and 10 μM SQ29548). Similarly, SQ29548 employed at a concentration of 0.1 μM inhibited the stimulation of myometrial strips from non-pregnant human donors (Figure 7). This was the highest concentration which could be used on human myometrium, since at 1.0 μM the spontaneous myogenic activity was affected.

These effects are consistent with the involvement of TP-receptors in the responses elicited by U-46619, AGN191976 and AGN192093 in rat aorta, human myometrium and human platelets.

Discussion and conclusions

The novel series of 9,11-cyclic carbonate derivatives of $PGF_{2\alpha}$ described here included two highly potent thromboxane-mimetics, AGN192093 and AGN191976, with approximately one

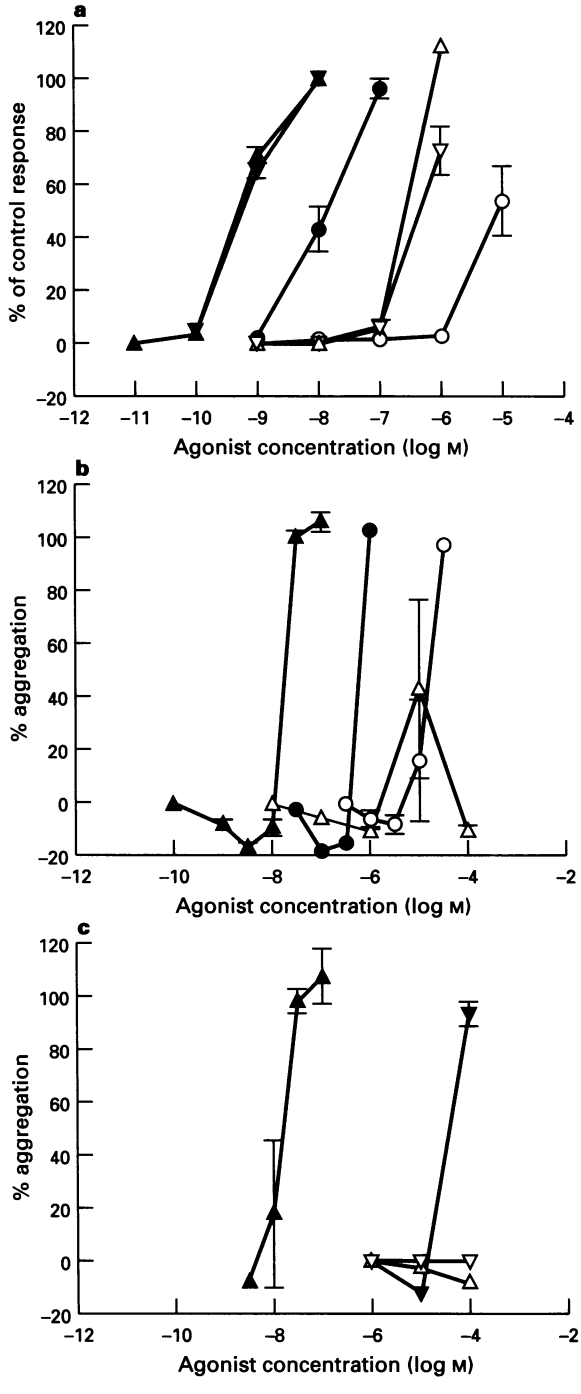


Figure 6 Inhibition by SQ29548 of responses in rat aorta (a, 1 μM SQ29548) and human platelets (b and c, 1 μM and 10 μM SQ29548, respectively) induced by U-46619 (circles), AGN191976 (triangles) and AGN192093 (inverted triangles). Responses obtained in the absence and presence of SQ29548 are depicted as solid and open symbols, respectively. Each point represents the mean ± s.e.mean of 4 separate experiments.

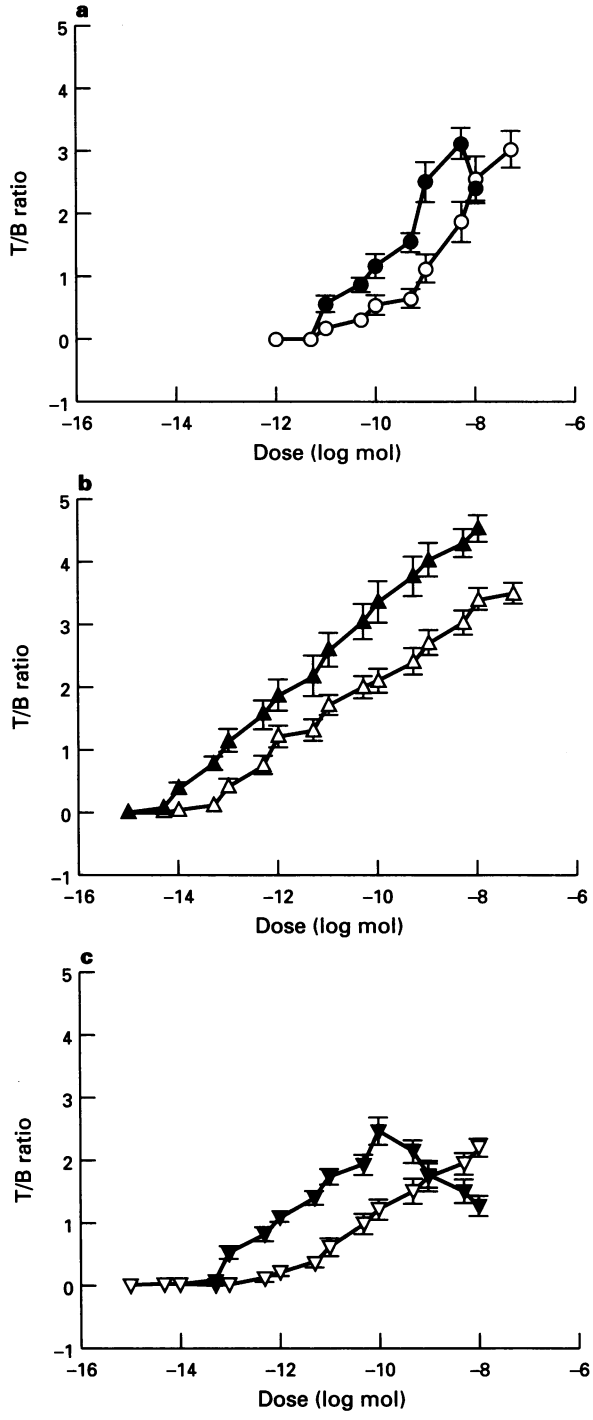


Figure 7 Inhibition by SQ29548 of responses in human non-pregnant myometrium induced by U-46619 (a), AGN191976 (b) and AGN192093 (c). Responses obtained in the absence and presence of SQ29548 (0.1 μM) are depicted as solid and open symbols, respectively. Each point represents the mean ± s.e.mean of 5 separate experiments. The activity is expressed as T/B ratio as explained in the Methods section.

to two orders of magnitude higher potency in the vasculature than the widely used benchmark agonist U-46619 (Bundy, 1975). AGN191976 and AGN192093 with EC_{50} values of 0.32 ± 0.08 nM and 1.30 ± 0.53 nM respectively, in the rat thoracic aorta contraction assay, were of a similar potency to the most potent TP-agonists reported thus far, I-BOP, EP171 and STA_2 (Table 2 and Katsura *et al.*, 1983; Mais *et al.*, 1985; Toda *et al.*, 1986; Jones *et al.*, 1989; Tymkewycz *et al.*, 1991). Both AGN191976 and AGN192093 proved to be very selective TP-mimetics with at least 500 fold selectivity for TP-receptors over other prostanoid receptors.

Our most interesting finding was that AGN192093 discriminated between TP-receptors in smooth muscle and platelets. AGN192093 was a highly potent TxA_2 mimetic in rat aorta and human myometrium having only residual pro-aggregatory activity in human platelets. In contrast, AGN191976 was a highly potent agonist in all three TP-receptor preparations. This suggests that AGN192093 may represent an important pharmacological tool for the characterization of TP-receptor subtypes.

The literature dealing with a possible subdivision of TP-receptors is confusing. A number of reports have previously suggested such a subdivision. The claims were based on the differences in activity or potency of TP-receptor ligands between platelets and smooth muscle (Nicolaou *et al.*, 1979 in conjunction with Jones *et al.*, 1982 and Armstrong *et al.*, 1985; Lefer *et al.*, 1980; Mais *et al.*, 1985; 1988; Lumley *et al.*, 1989; Tymkewycz *et al.*, 1991; Masuda *et al.*, 1991; Furci *et al.*, 1991; Kosakai *et al.*, 1993) or between different smooth muscle preparations, within the same species or across species (Lumley *et al.*, 1989; Tymkewycz *et al.*, 1991). Clear evidence for receptor heterogeneity between platelets from different species, on the other hand, is absent from the literature. However, TP-receptor heterogeneity based on pharmacological studies, is not undisputed. Depending on the compounds tested, activities or rank orders of potencies in human and rat platelets correlated either well (Lumley *et al.*, 1989; Masuda *et al.*, 1991; Harris *et al.*, 1992) or poorly (Tymkewycz *et al.*, 1991; Masuda *et al.*, 1991; Kosakai *et al.*, 1993) with rat aorta preparations. It appears that the selection of compounds for studies on TP-receptors and not just the selection of tissue preparations, significantly influences the outcome with respect to receptor homogeneity or heterogeneity. The best evidence for the potential to discriminate between platelet and vascular TP-receptors was provided by extensive radioligand binding studies, and this distinction has been ascribed to a group of 13-azapinane antagonists (Masuda *et al.*, 1991).

It has been argued that the discrepancies reported from pharmacological studies may merely reflect species differences and not heterogeneity of TP-receptors (Narumiya *et al.*, 1986). These arguments were fuelled by Hirata and coworkers (1991) who cloned the human thromboxane receptor from a human placenta cDNA library using an oligonucleotide probe based on the amino acid sequence of a purified human platelet TP-receptor (Ushikubi *et al.*, 1989). Their subsequent findings suggested TP-receptor homogeneity between human vascular-rich tissues and a platelet precursor cell line. However, the human receptor was not found to be expressed in rat tissues since northern blot analysis of mRNA expression gave no clear signals with the human TP-receptor probe (Hirata *et al.*, 1991). Chromosomal mapping of the thromboxane A_2 receptor gene, carried out by the same group, lent further support to the concept of a single TP-receptor type in man (Nüsing *et al.*, 1993). Pharmacological demonstration of the presence of TP-receptors in human tissues, other than platelets, usually involved the use of U-46619 and a TP-antagonist. In this manner, TP-receptors were characterized in human airways with the aid of AH23848 (McKenniff *et al.*, 1988; Coleman & Sheldrick, 1989), EP092 (McKenniff *et al.*, 1988), Bay u3405 (Norel *et al.*, 1991), ICI192605 (Al Jarad *et al.*, 1994) and in human pulmonary arteries with Bay u3405 (Norel *et al.*, 1991), EP169 (Qian *et al.*, 1994) and GR32191 (Lumley *et al.*, 1989; Qian *et al.*, 1994). These studies were, however, of limited scope and did not allow speculation regarding TP-receptor heterogeneity in man. Good comparative studies between human platelets and human smooth muscle preparations do not exist to support or dispute the molecular biology-based concept of a homogeneous TP-receptor population in man. The pharmacological evidence for TP-receptor heterogeneity which stems from studies comparing animal smooth muscle with human platelets does not necessarily contradict the claim of a mere species difference made by Narumiya *et al.* (1986) and Hirata *et al.* (1991). In fact, cloning of the mouse and rat TP-receptors revealed only 76% and 72% identity to the human TP-receptor, respectively (Namba *et al.*, 1992; Kitanaka *et al.*, 1995). The pharmacological characterization of the cloned TP-receptors is limited at this point and does not allow any speculation about differences in receptors between species beyond the fact that differences in amino acid sequence exist.

However, findings with AGN192093 are difficult to reconcile with molecular biology. AGN192093 was a potent stimulator of human myometrial smooth muscle, but only a weak agonist in human platelets. The human myometrium contains a highly heterogeneous population of prostanoid receptors (Senior *et al.*, 1991; 1992) and it could be argued that AGN192093 contracted the myometrium by stimulating one of the other prostaglandin-receptor types present. The strong evidence against this argument includes *(i)* the potency and selectivity of AGN192093 for TP-receptors and *(ii)* the fact that the contractile activity of AGN192093 in myometrium and aorta was susceptible to the TP-antagonist, SQ29548 (Ogletree *et al.*, 1985). Another possible explanation could be that AGN192093, albeit a full agonist in smooth muscle, is only a partial agonist in platelets, like CTA_2 (Lefer *et al.*, 1980). Partial agonism in this context was defined as inducing shape change without causing aggregation, but inhibiting TP-receptor-mediated aggregation. However, the functional separation of shape change and aggregatory responses in platelets with CTA_2 may be due to the ability of CTA_2 to elevate platelet cyclic-AMP levels (Armstrong *et al.*, 1985), thus potentially inhibiting its own pro-aggregatory effect. For this reason, we investigated whether AGN192093 behaved as an antagonist in human platelets. AGN192093 did not affect aggregation induced by ADP, arachidonic acid or the TP-agonists U-46619 and AGN191976. All these studies were carried out using platelet-rich plasma. It could be argued that the apparent lack of activity of AGN192093 in platelets was due to excessive plasma protein binding. But even in human washed platelets, in the absence of plasma proteins, AGN192093 had no pro-aggregatory effect at concentrations up to 10 μM. These results suggest that AGN192093 is simply a very weak agonist in human platelets and, thus strikingly different from CTA_2.

Another explanation for the functional separation of shape change and aggregation in platelets, which was also observed with PTA_2 (Nicolaou *et al.*, 1979; Jones *et al.*, 1982; Armstrong *et al.*, 1985) and 8-epi $PGF_{2\alpha}$ (Banerjee *et al.*, 1992; Morrow *et al.*, 1992; Takahashi *et al.*, 1992), could be the involvement of two distinct TP-receptor subtypes, one subtype mediating shape change and the other subtype mediating aggregation, as proposed by Takahara *et al.* (1990). The subtype mediating shape change was reported to be similar to the one present in vascular smooth muscle (Furci *et al.*, 1991). In this case, AGN192093 should have caused a platelet shape change response at low nanomolar concentrations. But AGN192093 did not elicit a shape change until a concentration of 10 μM was achieved. This does not necessarily disprove the dual-TP-receptor concept in platelets but provides strong evidence that AGN192093 is not only a weak agonist in the platelets with respect to the induction of aggregation but shape change as well.

Claims by Hirata *et al.* (1991) of TP-receptor homogeneity in man certainly conflict with the purported presence of (at least) two subtypes in man based on evidence obtained in pharmacological studies (our studies; Takahara *et al.*, 1990; Furci *et al.*, 1991). A possible explanation for the discrepancy

may be the fact that the human TP-receptor gene contains two introns (Nüsing *et al.*, 1993), and one splice variant has already been detected in human vascular endothelium (Raychowdhury *et al.*, 1994). The authors of the latter report detected the presence of both variants in human placenta but only one in the vascular endothelium. Moreover, the vascular splice variant could not be detected by use of a primer for the 'placenta' receptor and, therefore, may have been missed by Hirata and coworkers (1991). If this splice variant relates in any way to one of the purported subtypes in the human platelets, or if other splice variants exist, remains to be determined. To our knowledge, mRNA from human uterus has not been subjected to northern blot analysis. It remains to determined whether the myometrial receptor which potently responds to AGN192093 is one of the splice variants reported thus far or is different altogether. In addition, the question of TP-receptor heterogeneity from a molecular biology standpoint was reopened in a recent report dealing with the chromosomal sublocalization of prostanoid receptors confirming the assignment of the TP-receptor to the 19p13.3 region by Nüsing *et al.* (1993) but detected two other minor loci on chromosomes 12 and 15 (Duncan *et al.*, 1995). The authors did not, however, provide conclusive evidence that the two minor loci are indeed different TP-receptor genes.

The acidic moiety of the natural ligands has been widely considered as a crucial feature for prostaglandin-receptor binding. An arginine residue in position 295 of the human TP-receptor was thought to provide the required countercharge for the carboxyl group (Hirata *et al.*, 1991) and, in fact, this arginine residue is highly conserved among all cloned prostanoid receptors (for comparison refer to Toh *et al.*, 1995). The structural difference between AGN191976 and AGN192093 resides in the C-1 position of the molecule. The introduction of an alcohol group in this position had a striking effect on the activity in platelets without affecting the potency at the smooth muscle receptor. The introduction of primary or substituted amide groups at C-1 resulted in a significant decrease in potency at the smooth muscle receptor as well. The structure-activity relationship of the 9,11-cyclic carbonate $PGF_{2\alpha}$ derivatives suggests that the smooth muscle TP-receptor may accept either an alcohol or a carboxyl group at the C-1 position, whereas the platelet TP-receptor requires the presence of a carboxyl group. Alternatively, certain smooth muscle preparations may express a mixed subpopulation of TP-receptors, which differ in their structural requirements at the C-1 position.

In conclusion, AGN192093 is a unique thromboxane-mimetic in that it is highly potent at TP-receptors in smooth muscle but virtually devoid of any TP-activity in human platelets. Since our investigations included a human smooth muscle preparation, myometrium, this distinction cannot be explained as a mere species difference but provides further support for the concept of TP-receptor heterogeneity.

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