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ANTAGONISM OF PROSTAGLANDIN-MEDIATED RESPONSES IN PLATELETS AND VASCULAR SMOOTH MUSCLE BY 13-AZAPROSTANOIC ACID ANALOGS

EVIDENCE FOR SELECTIVE BLOCKADE OF THROMBOXANE A_2 RESPONSES

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Abstract—Studies were undertaken to examine the pharmacological properties and stereochemical requirements of a limited series of prostanoic acid analogs for inhibition of arachidonic acid (AA) and/or endoperoxide (U46619)-mediated responses in human platelets and rat aorta. To assess the role of stereochemistry, a set of *trans*- and *cis*-isomers of 13-azaprostanoic acid (APA) and 11a-homo-13-azaprostanoic acid (HAPA) were prepared. Each prostanoic acid analog blocked AA- or U46619-induced aggregatory and secretory responses in platelets, and U46619-mediated contractions of rat aorta in a concentration-dependent manner (0.1 to 100 μM). The azaprostanoic acid analogs blocked responses to both inducers of platelet activation with IC_{50} values ranging from 3.4 to 27.5 μM. *Trans*-APA was about 2- to 3-fold more active as an antagonist of serotonin release induced by AA or U46619 than the remaining analogs. The rank order of inhibitory potency (IC_{50}; μM) for these analogs against U46619-induced serotonin release in human platelets was *trans*-APA (3.4) > *cis*-APA (8.9) = *cis*-HAPA (8.7) = *trans*-HAPA (9.1). Concentrations of the prostanoic acid analogs required to block these responses to AA and U46619 were similar, and the highest concentration used (100 μM) did not modify AA-induced malondialdehyde production in human platelet preparations. In contrast, the isomers of APA and HAPA were equally active as antagonists of U46619-induced contractions of rat vascular tissue, possessing K_B values varying from 7.1 to 13.2 μM. Each azaprostanoic acid analog shifted the concentration–response curve of U46619 in rat aorta to the right, indicating a competitive-type inhibition. In addition, the azoprostanoic acid analog (U51605) was a more potent competitive antagonist of U46619 in this preparation and possessed an average pK_B value of 6.18. In summary, the results show that (1) expansion of the five-membered ring of APA to the six-membered ring analogs (HAPA) led to a retention of potent inhibitory activity against U46619 in human platelets and rat vascular smooth muscle, (2) the antiaggregatory and antisecretory actions of the azaprostanoic acid analogs were mediated by a blockade of the responses to AA and U46619, and not by an inhibition of AA metabolism, (3) the blocking activity for the APA isomers was stereoselective (*trans* > *cis*) whereas the isomers of HAPA were equally effective as inhibitors of platelet function; and (4) these azaprostanoic acid analogs act as selective endoperoxide (U46619)/thromboxane A_2 antagonists in these two tissues.

A release of arachidonic acid (AA) from plasma membrane phospholipids and conversion to bioactive metabolites are proposed to be responsible for the pathogenesis of bronchial asthma and cardiovascular and thromboembolic disorders [1–5]. Thromboxane A_2 (TXA_2) is a potent bioactive metabolite of AA which possesses antiaggregatory, vasoconstrictive and pulmonary hyperreactive properties [6, 7]. Therefore, we have initiated a program to develop TXA_2 receptor antagonists in both platelet and smooth muscle preparations which may be selectively useful for the treatment of these disease states.

To date, several structurally related prostanoids have been prepared and examined for their ability to block the biosynthesis and receptor-mediated actions of TXA_2 [8–11]. Among the prostanoids, the *trans*-isomer of 13-azaprostanoic acid (APA) has been reported to be a potent receptor antagonist of TXA_2 in human platelets [10, 12, 13]. In an attempt to determine the stereochemical requirements of this new class of TXA_2 receptor antagonists, we prepared the *cis*- and *trans*-isomers of APA, and a set of ring enlarged analogs of APA, which are *cis*- and *trans*-11a-homo-13-azaprostanoic acid (HAPA) (see Fig. 1). These methylene expanded homologs (HAPA) have a six-membered ring which resembles the natural endoperoxide/TXA_2 structure more closely than

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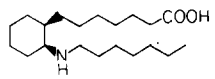
APA. Thus, the effect of ring size and stereochemical requirements on biological activity can be evaluated with the use of these two sets of compounds on TXA_2 responsive systems.

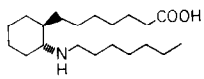
In this report, we have extended the pharmacological studies of LeBreton *et al.* [10] and Venton *et al.* [12] to determine the comparative effects of the geometric isomers of APA and the importance of ring expansion on the blockade of prostaglandin-mediated responses in rat thoracic aorta and human platelet aggregation and secretion. In our studies, we have included the use of U51605, an azo-prostanoid type agonist which is proposed to interact at TXA_2 receptor sites in vascular smooth muscle [9] (see Fig. 1). A preliminary report of this work has appeared [14].

MATERIALS AND METHODS

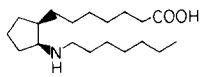
Drugs and chemicals. The *cis-* and *trans*-isomers of APA and HAPA were synthesized in our laboratory for use in these studies. U46619 (15*S*-hydroxy-11 α-,9α-epoxymethano-prosta-5Z,13E-dienoic acid) and U51605 (9,11-azoprosta-5,13-dienoic acid) were obtained from the UpJohn Co. (Kalamazoo, MI). [^{14}C]Serotonin (sp. act. 58 mCi/mmole; Amersham Corp., Arlington Heights, IL) was used without dilution in these experiments. Arachidonic acid was purchased from the Sigma Chemical Co. (St. Louis, MO). All other chemicals were of reagent grade. All prostaglandins were dissolved into ethanol and diluted with saline (0.9%, w/v).



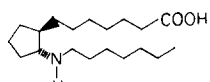
CIS-11α-HOMO-13-AZAPROSTANOIC ACID



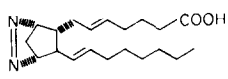
TRANS-11α-HOMO-13-AZAPROSTANOIC ACID



CIS-13-AZAPROSTANOIC ACID



TRANS-13-AZAPROSTANOIC ACID



9,11-AZOPROSTA-5,13-DIENOIC ACID
(U51605)

Fig. 1. Chemical structures of azoprostanoic acid (U51605) and the geometrical isomers of 13-azaprostanoic acid analogs used in this study. The *cis-* and *trans*-isomers of the six-membered ring homolog (11a-homo-13-azaprostanoic acid) and 13-azaprostanoic acid are given.

In vitro *aggregation and* [^{14}C]*serotonin secretion in human platelet preparations.* Blood was taken by venipuncture from normal human volunteers within the age of 25–30 years. These subjects were reported to be free of medication for at least 10 days prior to blood drawing. Venous blood samples were mixed with acid–citrate dextrose solution (9:1) and centrifuged at 120 *g* for 15 min at room temperature to obtain platelet-rich plasma (PRP) [15]. PRP was transferred to polypropylene tubes and stored under an atmosphere of 8% CO_2. The remaining blood sample was centrifuged at 1000 *g* for 10 min to obtain platelet-poor plasma. Platelets were counted using a Coulter counter (model ZF), and the aggregation studies were performed according to the method of Born [16] as modified by Mustard *et al.* [17] using a Payton aggregation module (model 600; Buffalo, NY). Platelet counts varied between 280,000 and 320,000 per cmm or were adjusted to 300,000 per cmm for the aggregation and secretion experiments. All studies were conducted within 2 hr after PRP recovery.

For aggregation studies, 0.5 ml of PRP was incubated with vehicle (1–5 μl) for 3 min at 37° prior to the initiation of aggregation with U46619. This time period also served as the incubation interval for analogs. In all experiments, the minimum concentration of U46619 (0.8 to 2 μM) and AA (0.5 to 1.0 mM) which caused maximal irreversible aggregation within each PRP preparation was used. Aggregation was monitored for another 3 min after the addition of the inducer. Data were analyzed as a percentage inhibition of the maximum light transmittance in the presence of various concentrations of each analog.

Secretion of serotonin from dense granules was measured by monitoring the release of radioactivity from platelets preincubated with [^{14}C]serotonin (0.5 μCi/ml of PRP) for 15 min at 37°. Release of serotonin from platelets was measured by centrifugation of samples at 1200 *g* for 1 min in a microfuge, and determining the radioactivity present in an aliquot of the supernatant fraction. The ^{14}C in supernatant fractions of samples was determined by liquid scintillation spectrometry using an emulsion-type scintillation mixture (Thrift-Solve, KEW Scientific, Columbus, OH). The extent of quench in samples was monitored by internal standardization.

Secretion data were calculated as the net increase of serotonin released into the supernatant fraction by U46619 or AA and expressed as a percentage of the total radioactivity in platelets [15, 18]. The effect of compounds on serotonin release was expressed as the percentage inhibition of the maximum release by each inducer and plotted against the log molar concentrations of each agent.

Isolated smooth muscle preparations; Rat aortic strip. Sprague–Dawley male rats (Harlan Industries, Inc., Cumberland, IN) weighing 220–250 g were killed and a 4 cm segment of the thoracic aorta was excised and placed into a physiological salt solution.

Adhered fat and connective tissue were removed and sprial strips were prepared according to the method of Furchgott [19]. Each strip (20 × 3 mm) was mounted in a 12 ml water-jacketed muscle chamber maintained at 37° and bubbled with 95% O_2–5% CO_2 in a physiological salt solution of the following composition: NaCl, 118 mM; KCl, 4.7 mM; $CaCl_2 \cdot 2H_2O$, 2.5 mM; $MgCl_2 \cdot 6H_2O$, 5 mM; $NaHPO_4 \cdot H_2O$, 1 mM; $NaHCO_3$, 25 mM; and dextrose, 11 mM.

The initial load on each spiral strip was adjusted to 1 g with equilibration for 2 hr, and washing at 15-min intervals. Isometric contractions induced by U46619 were recorded on a Grass polygraph (model 7) via force-displacement transducers (FT-03) after the period of passive relaxation.

To examine the antagonism of U46619-induced contraction of rat aortic strips, the compounds were preincubated for 10 min, and cumulative concentration response effects of the agonist were obtained as described by van Rossum [20]. Concentration–response relationships were established for each agonist initially and subsequently, in the presence of the test compound after five washings and an equilibrium period of 1 hr. Control experiments with vehicle were performed to determine tissue desensitization, and data were corrected for desensitization within each experiment. Since the maximum contractility of tissues to U46619 was unchanged after pretreatment with the drugs, we normalized each concentration–response curve to 100% of the maximum response to U46619.

Statistical analyses and calculations. All median doses (ED_{50}, IC_{50} or pK_B values) were calculated from the normalized concentration–response curves obtained by linear regression analysis and transformed to the corresponding negative logarithmic values. Apparent dissociation (K_B) constants for antagonism were determined by the method of Furchgott [21]. Differences between control and drug-treated preparations were determined by Student's *t*- or paired *t*-tests.

RESULTS

Effects of 13-azaprostanoic acid analogs on human platelet aggregation and secretion. In preliminary experiments, we found that 0.5 to 1 mM and 0.8 to 2.0 μM concentrations of AA and U46619, respectively, were sufficient to produce maximal irreversible aggregation of platelets in platelet-rich plasma, and these concentrations were used to examine the effects of the isomers of APA and HAPA on platelet function. As shown in Figs. 2 and 3, each azaprostanoic acid analog blocked the aggregation responses to U46619 and AA in a concentration-dependent manner. In these experiments, the isomers of APA and HAPA produced both a time lag and progressive reduction in the maximum aggregation responses to both inducers in the concentration range of 1–100 μM. None of the azaprostanoic acid analogs produced any aggregatory response alone.

The concentration-dependent effects of each isomer of APA and HAPA are summarized in Fig. 4. Only small quantitative differences in anti-aggregatory activity are seen with these isomers. *Trans*-APA was 1.8- and 3.4-fold more potent as an inhibitor of AA- and U46619-mediated platelet aggregation, respectively, than the corresponding *cis*-isomer. Whereas both isomers of HAPA were equally active as antagonists of U46619-mediated aggregation responses, *cis*-HAPA was about 2-fold more active than *trans*-HAPA against AA-induced aggregation. Against U46619-induced aggregation, the rank order of inhibitory potency (IC_{50}, μM) for

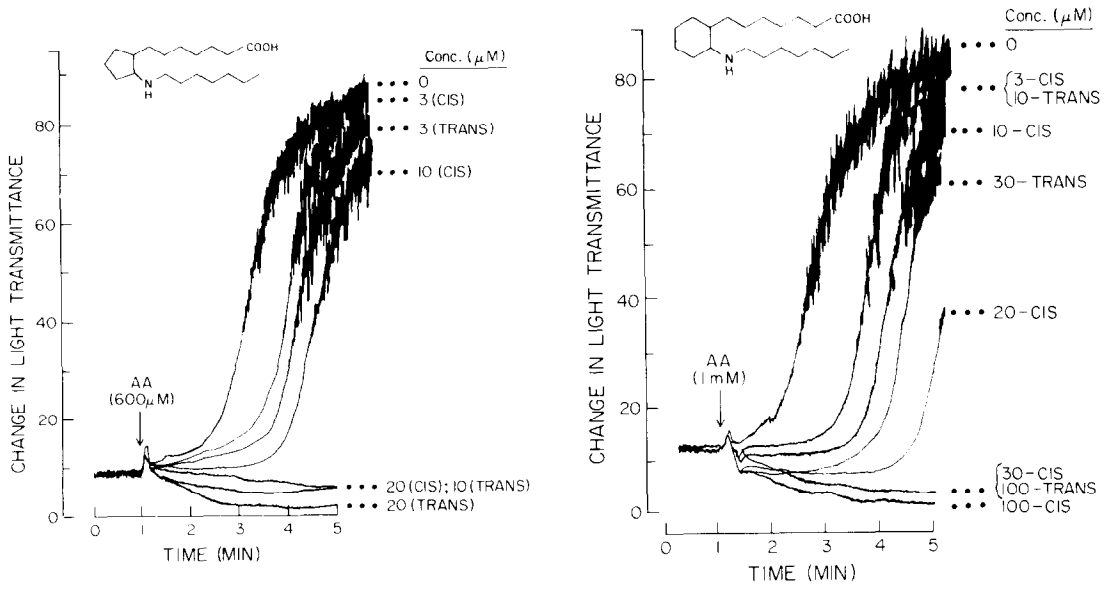


Fig. 2. Comparative concentration-dependent effects of the isomers of 13-azaprostanoic acid (left panel) and 11a-homo-13-azaprostanoic acid (right panel) on arachidonic acid-induced platelet aggregation. The tracings are representative of data taken from four platelet-rich plasma preparations. The concentration of arachidonic acid (AA) used was between 0.6 and 1.0 mM.

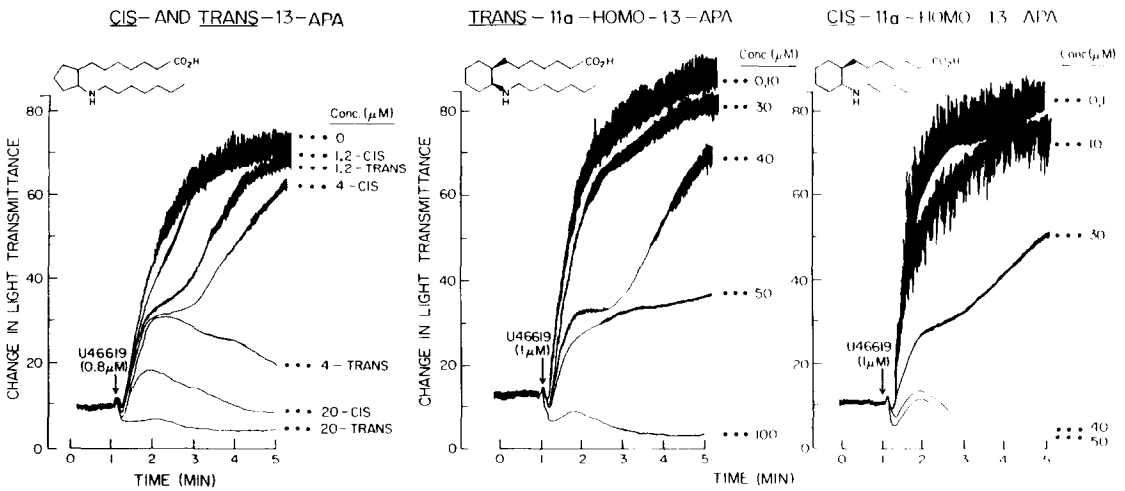


Fig. 3. Comparative concentration-dependent effects of the isomers of 13-azaprostanoic acid (left panel) and 11a-homo-13-azaprostanoic acid (middle and right panels) on U46619-induced platelet aggregation. The tracings are representative of data from three to four platelet-rich preparations. The concentration of U46619 used was between 0.8 and 1 μM.

these compounds was *trans*-APA (4.8) > *cis*-HAPA (11.8) = *trans*-HAPA (13.0) = *cis*-APA (16.5).

The comparative inhibitory potency for these azaprostanoic acid analogs on AA- and U46619-induced release of serotonin is presented in Table 1.

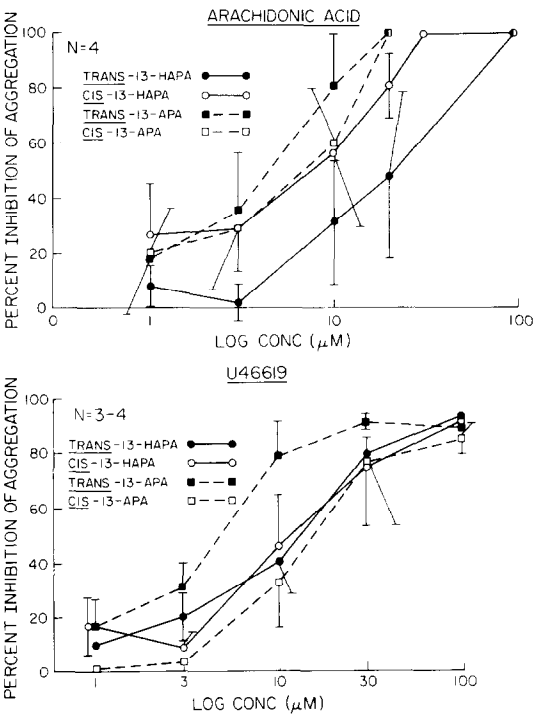


Fig. 4. Comparative concentration–inhibition curves of the isomers of 13-azaprostanoic acid and 11a-homo-13-azaprostanoic acid on platelet aggregation induced by arachidonic acid (upper panel) and U46619 (lower panel). In each experiment, the compounds were tested on the same platelet-rich plasma preparation. The symbol key is given in each panel. Values represent the mean ± S.E. of N = 3–4.

In the case of the APA isomers, *trans*-APA was 1.8- and 2.7-fold more potent as an inhibitor of AA and U46619 responses than *cis*-APA. In contrast the *cis*- and *trans*-isomers of HAPA were equally actve as antagonists of serotonin release to these two inducers, and the rank order of inhibitory potency for these compounds was *trans*-APA > *cis*-APA = *cis*-HAPA = *trans*-HAPA.

In other experiments, none of the azaprostanoic acid analogs (10, 30 to 100 μM) modified the production of malondialdehyde in AA-stimulated platelets (data not presented).

Effects of azaprostanoic acid analogs and U51605 on U46619-induced contraction of rat thoracic aortic strips. The *cis*- and *trans*-isomers of APA and HAPA did not produce any contraction of rat aorta in concentrations up to 3×10^{-4} M, and experiments were then done to examine their ability to block the contraction of this tissue by U46619. As shown in Fig. 5, each of the isomers of APA and HAPA produced a parallel shift to the right in the concentration–response curve of U46619. The *cis*-isomers gave a concentration-dependent shift in the concentration-response relationship, and the calculated pK_B values for each azaprostanoic acid analog are summarized in Table 2.

In preliminary studies, U51605 was found to produce a partial contractile response in only 25% of the rat thoracic aortic strip preparations. In non-responsive preparations, we examined the concentration-dependent ability of U51605 to antagonize the action of U46619 in rat aorta and this azoprostanoid gave a progressive shift to the right in the concentration–response curve of U46619 (Table 2). The rank order of inhibitory potency (pK_B) for each of these compounds was U51605 (6.18) > *trans*-HAPA (5.15) = *cis*-HAPA (5.09) = *cis*-APA (4.91) = *trans*-APA (4.88). With the exception of U51605, all of the prostanoic acid analogs were effective antagonists of U46619 with the K_B values in the concentration range of 7.1 to 13.2 μM. U51605 was

Table 1. Inhibition of U46619- and arachidonic acid (AA)-induced release of serotonin by the isomers of 13-azaprostanoic acid (APA) and 11a-homo-13-azaprostanoic acid (HAPA) in human platelets

Compound	IC_{50} (μM) Inducer U46619	IC_{50} (μM) Inducer AA	Potency ratio* (*cis/trans*) Inducer U46619	Potency ratio* (*cis/trans*) Inducer AA
13-APA isomers.				
Cis-APA	8.9 ± 1.7†	12.3 ± 1.3	2.7	1.8
Trans-APA	3.4 ± 0.6	7.3 ± 1.5		
13-HAPA isomers				
Cis-HAPA	8.7 ± 1.5	20.3 ± 1.7	0.95	0.78
Trans-HAPA	9.1 ± 1.4	27.5 ± 1.4		

* IC_{50} Ratio = IC_{50} (*cis*)/IC_{50} (*trans*) for 13-APA and 13-HAPA against U46619 or AA.

† Values are means ± S.E.M. of N = 4.

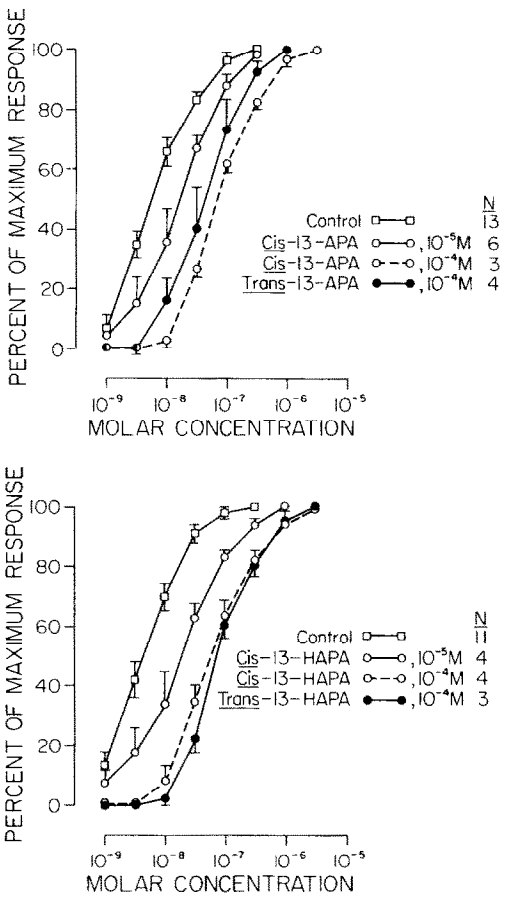


Fig. 5. Comparative concentration–response curves of U46619 in the absence or presence of various concentrations of the isomers of 13-azaprostanoic acid (upper panel) and 11a-homo-13-azaprostanoic acid (lower panel) on rat aorta. Values represent the mean ± S.E. of N = 3–13.

about 10 to 20-fold more potent than the isomers of APA and HAPA.

The ability of U51605 to antagonize U46619-induced contractions of rat aorta was of interest because this azoprostanoid was also active as a stimulant. In responsive tissues, U51605 was found to possess a maximum contractile response of 0.64 ± 0.07 and an ED_{50} value of 251 ± 44.0 ng/ml (EC_{50} = 0.75 μM; mean and S.E.; N = 7). The comparatively similar affinity values of U51605 as an antagonist (K_B = 0.66 μM) of U46619 and as an agonist (EC_{50} = 0.75 μM) suggests that this compound is interacting at common sites as a partial agonist in this tissue.

DISCUSSION

Coleman *et al.* [22] showed that the qualitative activities of the stable PGH_2 analog (U46619) and TXA_2 were the same in a variety of bioassay systems, and suggested that U46619 is a TXA_2-mimetic. The aggregation responses to U46619 were blocked in the same stereoselective fashion by the isomers of APA, and LeBreton *et al.* [10] and Venton *et al.* [12] suggested that *trans*-APA is a receptor antagonist of TXA_2. They have confirmed recently the existence of high affinity, saturable and stereoselective binding sites for radiolabeled *APA* as being similar to TXA_2 and U46619 receptor sites in platelets [13]. Thus, U46619 presents a useful experimental tool to examine the interaction of APA and six-membered ring homologs (HAPA) with putative TXA_2 receptor sites in vascular smooth muscle and human platelet preparations.

To characterize the structural requirements of TXA_2 receptor antagonists, we examined the interaction of the isomers of APA and HAPA in human platelets. As reported by LeBreton *et al.* [10] and Venton *et al.* [12], *trans*-APA was more effective as

Table 2. Inhibitory effect of U51605 and the isomers of 13-azaprostanoic and 11a-homo-13-azaprostanoic acids on U46619-induced contraction of rat thoracic aorta

Compound (M)	pD_2*		ΔpD_2*	pK_B†
	Absence	Presence		
I. 13-Azaprostanoic acid				
Cis-isomer (10^{-5})	8.14 ± 0.15	7.78 ± 0.15	0.35 ± 0.14	4.90
Cis-isomer (10^{-4})	8.29 ± 0.06	7.14 ± 0.06	1.15 ± 0.03	4.92
Trans-isomer (10^{-4})	8.28 ± 0.11	7.21 ± 0.14	1.11 ± 0.05	4.88
II. 11a-Homo-13-azaprostanoic acid				
Cis-isomer (10^{-5})	8.17 ± 0.15	7.68 ± 0.14	0.51 ± 0.19	5.15
Cis-isomer (10^{-4})	8.22 ± 0.16	7.00 ± 0.09	1.22 ± 0.10	5.02
Trans-isomer (10^{-4})	8.45 ± 0.13	7.08 ± 0.09	1.37 ± 0.15	5.15
III. U51605				
U51605 (10^{-5})	7.92 ± 0.09	6.36 ± 0.07	1.55 ± 0.11	6.34
U51605 (3×10^{-5})	7.55 ± 0.13	5.86 ± 0.07	1.68 ± 0.14	5.99

* Values represent the mean ± S.E.M. of N = 3–6.
† All values are corrected for a tissue desensitization of 0.2 log units.

an inhibitor of U46619- or AA-mediated aggregation and serotonin release than the *cis*-isomer. However, the isomers of the six-membered homolog of APA, *cis*- and *trans*-HAPA, were equally active as inhibitors of serotonin release by U46619. Similarly, the profiles of activity and potency for the blockade of AA-induced aggregation and secretory responses in the platelet preparations were identical to the results seen with U46619. It was possible that these compounds could interfere with the metabolism of AA and produce an inhibition at the level of TXA_2 biosynthesis in platelets. However, AA-induced formation of malondialdehyde was unaltered by the presence of the isomers of APA and HAPA. Thus, we propose that these isomers of HAPA, like that proposed for APA [10, 13] are acting as selective antagonists of U46619 and TXA_2 action in this pharmacological system.

In the present study, we extended the earlier work of LeBreton *et al.* [10] with the *trans*- and *cis*-isomers of APA in human platelets by demonstrating that these compounds also antagonize the action of U46619 in vascular smooth muscle preparations. Although the inhibitory potencies of these isomers were similar to that in human platelets, the interaction of APA with U46619 in rat aorta was not stereodependent (Table 2). Moreover, the HAPA isomers also retained significant inhibitory activity and both isomeric forms were equally active against U46619. Therefore, the range of concentrations and rank order of inhibitory potencies required to block the pharmacological actions of U46619 in both preparations were nearly the same for the isomers of APA and HAPA. The only difference between the two preparations for these compounds was the absence of a stereoselective inhibition by *trans*- versus *cis*-APA in rat aorta. From these findings, we conclude that the receptor binding sites for the isomers of azaprostanoic acid analogs in human platelets and rat thoracic aorta are similar.

Gorman *et al.* [9] reported that the azoprostanoic acid analog, U51605, was both a TXA_2 synthetase inhibitor and agonist in human platelets and rat aorta respectively. In this study, we found that U51605 was a partial agonist for the contraction of rat aorta and our EC_{50} value (0.75 μM) was similar to that reported by Gorman *et al.* [9]. In most vascular preparations, U51605 was inactive as an agonist and was a competitive antagonist of U46619-induced contractile responses. In these non-responsive rat aortic strip preparations, U51605 behaved in the same way as the azaprostanoic acid analogs. However, U51605 was more potent as an U46619/TXA_2 antagonist than the isomers of APA and HAPA.

The development of therapeutic agents which can modulate the release and/or metabolism of arachidonic acid is a subject of current interest. The existence of an organ selective TXA_2 receptor antagonist may have considerable application. Toward this goal, Lefer *et al.* [23] prepared a carbocyclic TXA_2 which was a selective agonist for the contraction of rat aortic systems. In other reports [8, 9] using epoxy-imino- and azo-prostanoic acid analogs, a differentiation of pharmacological responses were observed for these compounds in rat aorta and human platelets. Analogous to that proposed originally by Lands *et al.* [24] in *beta*-adrenoceptor systems, Gorman *et al.* [9] suggested that two subtypes of TXA_2 receptor populations may exist in rat aorta and human platelets. Our results of the rank order of inhibitory potencies for the prostanoic acid analogs against U46619 support the view that the putative TXA_2 receptor binding sites in rat aorta and human platelet are the same. The nearly equivalent inhibitory potencies of the azaprostanoic acid isomers against U46619-mediated responses in rat aorta and human platelets are evidence for such a proposal. However, a structurally related analog of APA, 2-(carboxyhexyl)cyclopentanone hexylhydrazone, mediates antiplatelet properties by a blockade of cyclooxygenase activity, and not by an antagonism of TXA_2 receptor sites [25]. In our studies, the nearly identical affinities of U51605 as an agonist and antagonist in rat aorta suggest that this compound is acting at a common receptor system in this tissue, presumably by an interaction at the same binding sites

as U46619 and the isomers of APA and HAPA. As reported by Gorman *et al.* [9], U51605 also is a TXA_2 synthetase inhibitor and agonist in platelets and aorta. Thus, more work is necessary to fully understand the action of azaprostanoic acids (APA, HAPA) and azoprostanoids (U51605) since it is clear that prostanoic acid analogs interact at multiple sites in U46619 responsive systems. This ability of prostanoic acid analogs to interact with multiple stimulatory and inhibitory sites explains why they may exhibit agonist, partial agonist or antagonist activity in these preparations.

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