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INHIBITION OF THROMBOXANE A_2 BIOSYNTHESIS IN HUMAN PLATELETS BY BURIMAMIDE

G. ALLAN, K.E. EAKINS*, P.S. KULKARNI* & R. LEVI

Department of Pharmacology, Cornell University Medical College, New York, N.Y. and *Departments of Opthalmology and Pharmacology, Columbia University College of Physicians & Surgeons, New York, N.Y., U.S.A.

1 Burimamide selectively inhibited the formation of thromboxane A_2 from prostaglandin endoperoxides by human platelet microsomes in a dose-dependent manner ($IC_{50} = 2.5 \times 10^{-5}$ M). Burimamide was found to be equipotent to imidazole as a thromboxane synthetase inhibitor.

2 Metiamide, cimetidine and a series of compounds either bearing a structural or pharmacological relationship to histamine caused little or no inhibition of thromboxane A_2 biosynthesis by human platelet microsomes.

3 Burimamide (5×10^{-4} to 2.3×10^{-3} M) did not inhibit either the cyclo-oxygenase or the prostacyclin synthetase of sheep seminal vesicles or the prostacyclin synthetase of dog aortic microsomes.

4 Burimamide (2.5×10^{-5} to 1.2×10^{-4} M) inhibited sodium arachidonate-induced human platelet aggregation; the degree of inhibition was dependent upon the concentration of arachidonic acid used to aggregate the platelets.

Introduction

The prostaglandin endoperoxides (prostaglandin G_2, H_2), the first cyclo-oxygenated products of arachidonic acid metabolism can be converted enzymatically into either primary prostaglandins such as prostaglandin E_2, $F_{2\alpha}$ or D_2, the non-prostanoate thromboxane A_2 or prostacyclin (Moncada, Gryglewski, Bunting & Vane, 1976). Non-steroidal anti-inflammatory agents such as aspirin and indomethacin inhibit the initial cyclo-oxygenase reaction which converts arachidonic acid to the prostaglandin endoperoxides (Vane, 1971). Since there are great differences in the biological activities of the products generated from the prostaglandin endoperoxides, selective inhibitors of the formation of individual end products of endoperoxide metabolism would be desirable as pharmacological tools.

Several compounds have been found which inhibit selectively thromboxane generation from prostaglandin endoperoxides, these include: benzydamine (Moncada, Needleman, Bunting & Vane, 1976), imidazole (Needleman, Raz, Ferrendelli & Minkes, 1977; Moncada, Bunting, Mullane, Thorogood, Vane, Raz & Needleman, 1977), N-0164, a phenyl phosphonate derivative of phloretin phosphate (Eakins & Kulkarni, 1977), 9, 11-epoxy methanoprostanoic acid, (Sun, 1977) and 2-isopropyl-3-nicotinyl-indole (L8027) (Gryglewski, Zmuda, Korbut, Krecioch & Bieron, 1977).

Various imidazole derivatives were studied by Moncada *et al.* (1977) and of these, only one-methyl imidazole was found to be a potent inhibitor of thromboxane A_2 biosynthesis. Further studies by Tai & Yuan (1978) demonstrated one-alkyl substituted imidazoles to be the most potent thromboxane synthetase inhibitors, inhibitory activity being abolished when the substituents appeared in other positions of the imidazole ring. The histamine H_2-receptor antagonists, metiamide, cimetidine and burimamide, are also structural derivatives of imidazole, side chain substitution occurring at the four (five) position of the imidazole ring (Brimblecombe, Duncan, Durant, Emmett, Ganellin & Parsons, 1975).

In a preliminary series of experiments burimamide was found to inhibit thromboxane A_2 biosynthesis in human platelet microsomes (Allan & Eakins, 1978). In the present study we have compared this activity of burimamide with imidazole and the histamine H_2-receptor antagonists, metiamide and cimetidine, and some other chemically related compounds. We have also investigated the selectivity of this inhibitory action of burimamide: thus we have studied the effects of burimamide on cyclo-oxygenase and prosta-

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cyclin synthetase activities. In addition, because of the postulated involvement of thromboxane A_2 as the endogenous trigger of platelet aggregation, we have studied the effects of burimamide on arachidonic acid-induced human platelet aggregation.

Methods

Thromboxane synthetase activity

Thromboxane A_2-like activity was generated using the coupled enzyme preparation developed by Moncada *et al.* (1976a, b). Sheep seminal vesicle microsomes (200 to 300 μg protein) were incubated without co-factors with sodium arachidonate (1 μg) in a total volume of 100 μl Tris buffer (50 mM pH 7.5) at room temperature (22°C) for 1 min; 50 μl samples were then tested for prostaglandin endoperoxide-like activity by bioassay. The remainder of this suspension was incubated with indomethacin-treated human platelet microsomes (150 to 250 μg protein) for 2 min at 0°C; the thromboxane A_2-like activity thus generated was then tested by bioassay. Biological characterization of endoperoxides and thromboxane was made by bioassay on spiral strips of rabbit thoracic aorta continuously superfused at 10 ml/min with oxygenated Krebs solution at 37°C. The assay tissues were treated with a combination of antagonists, infused to give final concentrations of diphenhydramine, atropine and phenoxybenzamine of 0.1 μg/ml, propranolol 2 μg/ml and methysergide 0.2 μg/ml. These antagonists rendered the assay tissues insensitive to histamine, acetylcholine, catecholamines and 5-hydroxytryptamine.

Indomethacin 1 μg/ml was also added to the superfusion medium to prevent prostaglandin biosynthesis by the assay tissues. A series of compounds bearing a structural resemblance to imidazole were tested for their ability to inhibit the formation of thromboxane A_2-like activity from prostaglandin endoperoxides by human platelet microsomes. The test drugs were preincubated with the human platelet microsomes for 2 min at 0°C before the addition of the crude endoperoxide substrate (Allan & Eakins, 1978).

Cyclo-oxygenase and prostacyclin synthetase activity

Radiochemical determination of the formation of the cyclo-oxygenase product, prostaglandin E_2, and the stable degradation product of prostacyclin, 6-keto prostaglandin $F_{1\alpha}$ was carried out by the method of Cottee, Flower, Moncada, Salmon & Vane (1977). These workers had demonstrated that at low substrate/enzyme ratios, in the presence of adrenaline, the major prostaglandins biosynthesized by the sheep seminal vesicle cyclo-oxygenase from arachidonic acid were 6-keto prostaglandin $F_{1\alpha}$ and prostaglandin E_2. [^{14}C]-arachidonic acid (1 μg) was incubated with sheep seminal vesicle microsomes (6.1 mg protein) for 20 min at room temperature in the presence of 2 mM adrenaline in a total volume of 2 ml Tris buffer (50 mM pH 7.4). Acid lipid extraction was followed by paper chromatographic separation of products on Silica Gel G paper using the organic phase of ethyl acetate; isoctane: acetic acid: water (11:5:2:10). Radioscan of the products of paper chromatography was carried out with a Vanguard Autoscanner Model 930. Areas of radioactivity coinciding with the migration of authentic standards were located and radioactivity counted in a Packard Model 2405 Liquid Scintillation Counter. Burimamide was incubated for 5 min at 22°C with the sheep seminal vesicle microsomes before the addition of radiolabelled arachidonic acid and the inhibitory effect of this compound on product formation was determined.

The effect of burimamide on the prostacyclin synthetase activity of dog aorta microsomes was also studied by bioassay. Prostacyclin-like activity was produced by incubating prostaglandin H_2 (50 ng) with dog aorta microsomes (20 to 50 μg protein) for 1 min at 22°C (Kulkarni, Eakins, Saber & Eakins, 1977). Prostacyclin was extracted three times in ether, the ether was evaporated under nitrogen and the residue reconstituted in 50 mM Tris buffer and tested for biological activity. Prostacyclin was assayed by its ability to inhibit arachidonic acid-induced platelet aggregation. Washed rabbit platelets (WRP) were prepared by the method of Hamberg, Svensson, Wakabayashi & Samuelsson (1974). Aggregation of WRP was studied with 0.4 ml. samples of WRP in a chronolog platelet aggregometer. Aggregation was induced by the addition of sodium arachidonate; a concentration of sodium arachidonate yielding threshold aggregation was chosen for each batch of WRP. Extracts of prostacyclin-like activity were incubated with WRP for 2 min before the addition of the aggregating agent. Burimamide was allowed to incubate with dog aorta microsomes for 2 min at 0°C before the addition of prostaglandin H_2 and inhibition of prostacyclin formation determined.

The effect of burimamide on the cyclo-oxygenase activity of sheep seminal microsomes was also studied by bioassay. Prostaglandin endoperoxides were generated and characterized as described earlier. Burimamide was incubated with the sheep seminal vesicle microsomes for 2 min at 0°C before the addition of sodium arachidonate. Using the same experimental conditions, inhibition of the enzyme by indomethacin (Flower, 1974) was also studied.

Platelet aggregation studies

Human blood was collected by venipuncture from volunteers (who had not taken aspirin for at least 14

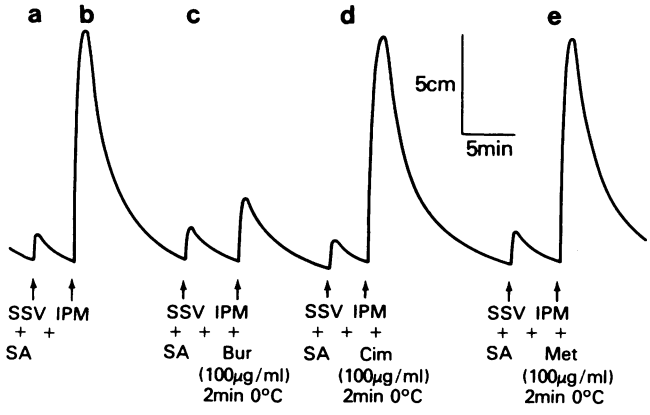


Figure 1 Effect of histamine H_2-receptor antagonists on the generation of thromboxane A_2-like activity. Bioassay of rabbit aorta contracting substance. (a) Sheep seminal vesicle microsomes (SSV, 246 μg protein) were incubated with 1 μg sodium arachidonate (SA) in 100 μl Tris buffer (50 mM, pH 7.4) for 1 min at 22°C and 50 μl tested to yield prostaglandin endoperoxide-like activity. (b) Further incubation of this sample at 0°C for 2 min with indomethacin-treated human platelet microsomes (IPM, 180 μg protein) resulted in the formation of thromboxane A_2-like activity. (c, d and e) Same as in (a) except that the IPM were incubated with burimamide (Bur), cimetidine (Cim) and metiamide (Met) (5×10^{-4} M) for 2 min at 0°C before the addition of the prostaglandin endoperoxide.

days) into 0.1 vol 3.2% sodium citrate. Platelet-rich plasma (PRP) was obtained by centrifugation at 200 *g* for 10 min at 22°C. Aggregation of PRP was studied in 0.4 ml samples in a chronolog platelet aggregometer. Threshold aggregation was induced by the addition of sodium arachidonate. In some experiments adenosine 5'-diphosphate (ADP) was used as an aggregating agent. For these studies PRP was prepared from blood obtained from donors who had ingested aspirin 12 to 24 h earlier, thus eliminating any contribution of cyclo-oxygenase products to the aggregatory response induced by ADP. Burimamide was incubated with the PRP for 5 min before the addition of the aggregating agent.

Drugs

The following were used: adenosine 5'-diphosphate: sodium salt, arachidonic acid: sodium salt, imidazole, histamine, *N*-acetyl histamine, *N*-acetyl histidine, L-histidine (Sigma Chemical Co., St. Louis, Mo., U.S.A.), indomethacin (Merck, Sharp & Dohme, Rahway New Jersey, U.S.A.), methysergide maleate (Sandoz, Hanover, New Jersey, U.S.A.); diphenhydramine hydrochloride; atropine sulphate (Burroughs Wellcome Co., Research Triangle Park, North Carolina, U.S.A.); propranolol (Ayerst Laboratories, New York, N.Y., U.S.A.); phenoxybenzamine (Smith Kline and French, Philadelphia, Pa., U.S.A.). Burimamide, metiamide and cimetidine were generously supplied by Dr D. A. A. Owen of Smith Kline & French Laboratories Ltd., Welwyn Garden City, Hertfordshire. Prostaglandins used in the study were all generously supplied by Dr J. E. Pike of the Upjohn Co., Kalamazoo, Michigan, U.S.A.

All drug solutions were freshly prepared in 50 mM Tris buffer pH 7.4. Prostaglandin standards used in chromatographic procedures were prepared in absolute ethanol.

Results

Effects on thromboxane A_2 biosynthesis

When sodium arachidonate was incubated without co-factors with sheep seminal vesicle microsomes at room temperature for 1 min, a product was formed which resembled the prostaglandin endoperoxides. It contracted the rabbit aorta, had a half-life of 2 to 4 min in aqueous solution at 37°C and was converted to a more powerful, very labile thromboxane A_2-like activity when incubated at 0°C for 2 min with indomethacin-treated human platelet microsomes and immediately tested (Figure 1a, b).

Burimamide (2.5×10^{-6} to 5×10^{-4} M) when added to the human platelet microsomes for 2 min at 0°C before the addition of the crude endoperoxide, inhibited the subsequent generation of this thromboxane A_2-like activity in a concentration-dependent manner (Figure 1c, Figure 2). The concentration of burimamide required to produce 50% inhibition

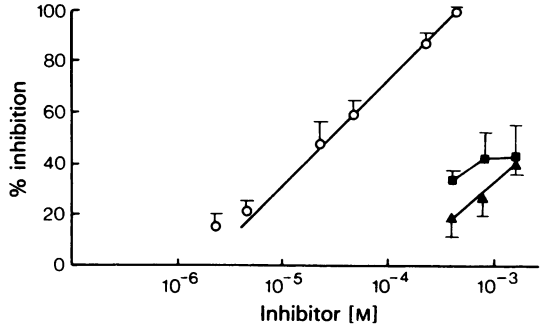


Figure 2 Inhibition of thromboxane A_2 biosynthesis in human platelet microsomes by burimamide (○), metiamide (■) and cimetidine (▲). Points (means, $n = 6$; vertical lines show s.e. mean) represent % inhibition of the formation of thromboxane A_2-like activity measured by bioassay. The dose-response curve for imidazole is superimposable upon that of burimamide and is therefore not shown.

(IC_{50}) of thromboxane A_2 biosynthesis was determined by linear regression from dose-response curves and found to be 2.5×10^{-5} M.

The other histamine H_2-receptor antagonists, metiamide and cimetidine, exhibited only weak activity as inhibitors of thromboxane A_2 biosynthesis (Figure 1 d, e, Figure 2). Concentrations up to 400 μg/ml (approximately 10^{-3}M) of both compounds failed to produce 50% inhibition of thromboxane A_2 biosynthesis (Figure 2).

A series of compounds possessing the imidazole ring and bearing either a structural or a pharmacological relationship to histamine was tested as inhibitors of thromboxane A_2 biosynthesis. Each compound was tested at a concentration of 100 μg/ml (Table 1), and none was found to exhibit appreciable activity. Under identical experimental conditions, imidazole was equipotent with burimamide as a thromboxane synthetase inhibitor in human platelet microsomes. The dose-response curve obtained for imidazole was identical to that for burimamide, with an IC_{50} calculated by linear regression from dose-response curves of 2.5×10^{-5} M.

Effects on cyclo-oxygenase and prostacyclin synthetase

The selectivity of this action of burimamide was assessed by studying the effect of burimamide on prostaglandin endoperoxide and prostacyclin generating systems. When burimamide (5×10^{-4} to 2.3×10^{-3} M) was incubated with sheep seminal vesicle microsomes (246 μg protein) for 2 min at 0°C it did not inhibit the conversion of sodium arachidonate (1 μg) to bioassayable prostaglandin endoperoxide (Figure 3). However, when the known cylo-oxygenase inhibitor, indomethacin, was incubated with sheep vesicle microsomes under the same experimental conditions, the conversion of arachidonic acid to prostaglandin endoperoxide was inhibited in a concentration-dependent manner (Figure 3). The concentration of indomethacin required to produce 50% inhibition (IC_{50}) of prostaglandin endoperoxide formation was determined graphically and found to be 3 μg/ml (8.4×10^{-6} M).

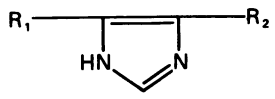
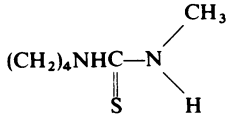
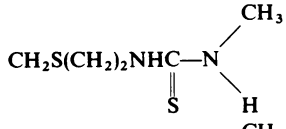
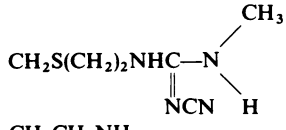
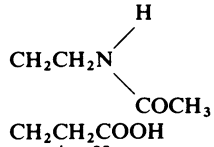
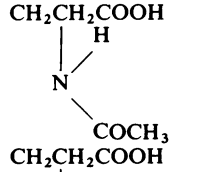
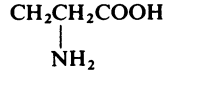
When prostaglandin H_2 (50 ng) was incubated with dog aortic microsomes (28 μg protein) for 1 min at 22°C, the ether-extracted product inhibited arachidonic acid-induced aggregation of washed rabbit platelets. This product had the characteristics of prostacyclin. Anti-aggregatory activity slowly diminished within 15 to 20 min at room temperature whereas acidification of the extracts (pH 5.5) resulted in total loss of anti-aggregatory activity within 10 min. When burimamide (9×10^{-4} M) was preincubated with the dog aortic microsomes for 2 min at 0°C, the conversion of prostaglandin H_2 to prostacyclin-like activity was not inhibited (not shown).

Incubation of radiolabelled arachidonic with sheep seminal vesicle microsomes in the presence of 2 mM adrenaline for 20 min at room temperature, followed by acid-lipid extraction, and paper chromatographic separation of the products, resulted in major peaks of radioactivity coinciding with the migration of authentic prostaglandin E_2 and 6-keto prostaglandin $F_{1\alpha}$ (Figure 4). When burimamide (5×10^{-4} M) was incubated with the sheep seminal vesicle microsomes for 5 min at room temperature, no inhibition of the formation of prostaglandin E_2 or 6-keto prostaglandin $F_{1\alpha}$ occurred (Figure 4).

Effects on platelet aggregation

Addition of sodium arachidonate (0.375 to 0.75 mM) to human PRP resulted in irreversible platelet aggregation. When burimamide was incubated with PRP for 5 min before the addition of sodium arachidonate, the subsequent aggregatory response was inhibited dose-dependently. The degree of inhibition of aggregation caused by burimamide was dependent on the concentration of sodium arachidonate used to induce platelet aggregation. Thus platelet aggregation induced by addition of 0.375 mM sodium arachidonate was completely prevented by burimamide at a concentration of 1.2×10^{-4} M whereas when platelet aggregation was induced by addition of 0.75 mM sodium arachidonate, a 10 fold higher concentration of burimamide was required to inhibit platelet aggregation (Figure 5). Burimamide, at this higher concentration (1.2×10^{-3} M) did not inhibit the primary wave of platelet aggregation induced by ADP (0.5 to 2 μM) in aspirin-treated platelets.

Table 1 Inhibition of thromboxane A_2 biosynthesis in human platelet microsomes by various imidazole derivatives

Name	*Structure*		*% inhibition*
			
Imidazole	H	H	100
Burimamide	H		100
Metiamide	CH_3		33 ± 3
Cimetidine	CH_3		19 ± 8
Histamine	H	$CH_2CH_2NH_2$	4 ± 4
4-Methyl histamine	CH_3	$CH_2CH_2NH_2$	0
N-acetyl histamine	H		21 ± 13
N-acetyl histidine	H		5 ± 5
L-Histidine	H		6 ± 5

Percentage (%) inhibition of the formation of thromboxane A_2-like activity, as measured by bioassay. Compounds were studied at a concentration of 100 μg/ml.

Discussion

The results of the present study demonstrate that the known histamine H_2-receptor antagonist, burimamide, is also an inhibitor of thromboxane A_2 biosynthesis in human platelet microsomes. Burimamide inhibited the conversion of crude prostaglandin endoperoxide to thromboxane A_2 by human platelet microsomes in a concentration-dependent fashion (IC_{50}, 2.5×10^{-5} M), and was equipotent with imidazole, an inhibitor of thromboxane A_2 biosynthesis (Moncada *et al.*, 1977). The histamine H_2-receptor antagonists, metiamide and cimetidine, exhibited only low activity as inhibitors of thromboxane A_2 formation (Figure 2) and other structurally or pharmacologically related compounds containing the imidazole ring had little or no inhibitory activity (Table 1).

The order of potency of the histamine H_2-receptor antagonists as thromboxane A_2 biosynthesis inhibitors was found to be burimamide > metiamide > cimetidine (Figure 2), whereas the order of potency as histamine H_2-receptor antagonists is cimetidine > metiamide > burimamide (Brimblecombe *et al.*, 1975). Thus, the inhibitory effect of burimamide described in these experiments would not appear to be related to histamine H_2-receptor antagonism.

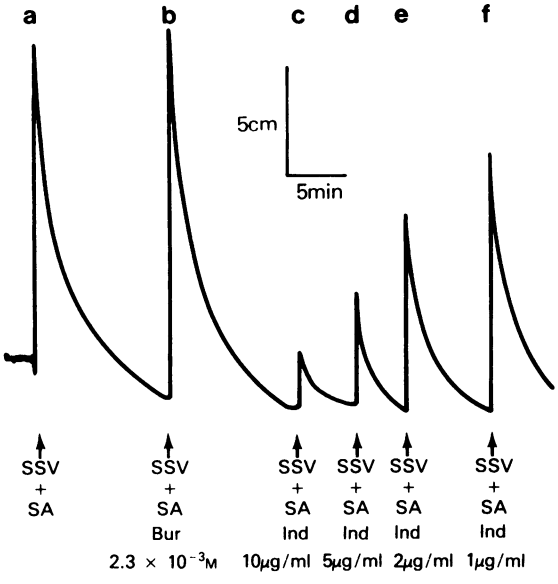


Figure 3 Effect of burimamide and indomethacin on the cyclo-oxygenase activity of sheep seminal vesicles. Bioassay of prostaglandin endoperoxide-like activity on isolated superfused strips of rabbit aorta. (a) Sheep seminal vesicle microsomes (SSV, 246 μg protein) were incubated with 1 μg sodium arachidonate (SA) in 100 μl Tris buffer (50 mM, pH 7.4) for 1 min at 22°C and 50 μl tested to yield prostaglandin endoperoxide-like activity; (b) same as (a) except that the SSV were incubated with burimamide (Bur, 2.3×10^{-3} M) for 2 min at 0°C before the addition of the SA. (c, d, e and f) Same as in (a), except that the SSV were incubated with indomethacin (Ind, 10, 5, 2, 1 μg/ml) for 2 min at 0°C before the addition of the SA.

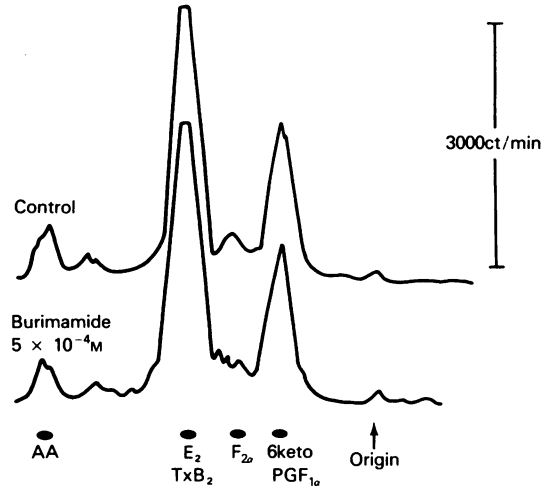


Figure 4 Radiochromatogram of the products obtained from the reaction of [^{14}C]-arachidonate (AA 1 μg) with sheep seminal vesicle microsomes (6.1 mg protein) in the presence of adrenaline (2 mM). The reaction was allowed to proceed for 20 min at 37°C and then acidified by the addition of 0.1 ml 1 N HCL. The products were extracted twice with ethyl acetate. Ethyl acetate was evaporated under vacuum and the residue reconstituted in chloroform and chromatographed on silica gel G paper in the organic phase of ethyl acetate:isooctane:acetic acid:water (11:5:2:10). When burimamide (5×10^{-4} M) was incubated with the sheep seminal vesicle microsomes for 5 min before the addition of the AA, no inhibition of the formation of the products occurred. The following abbreviations are used: AA, arachidonic acid: E, prostaglandin E_2; Tx B_2, thromboxane B_2; $F_{2\alpha}$, prostaglandin $F_{2\alpha}$; 6 keto $PGF_{1\alpha}$, 6 keto prostaglandin $F_{1\alpha}$.

The selectivity of burimamide as an inhibitor of thromboxane A_2 biosynthesis was assessed by studying the effectiveness of burimamide as an inhibitor at three enzyme sites in the pathways of arachidonic acid metabolism. Thus, the inhibitory effect of burimamide was determined on the conversion of arachidonic acid to prostaglandin endoperoxide by the fatty acid cyclo-oxygenase present in sheep seminal vesicles (Hamberg & Samuelsson, 1973), on the formation of thromboxane A_2 from the endoperoxide by thromboxane synthetase present in platelet microsomes (Hamberg, Svensson & Samuelsson, 1975; Needleman, Moncada, Bunting, Vane, Hamberg & Samuelsson, 1976) and finally on the conversion of prostaglandin endoperoxides to prostacyclin by the prostacyclin synthetase present in aortic microsomes (Salmon, Smith, Flower, Moncada & Vane, 1978).

Burimamide at concentrations 20 to 100 times greater than those required to cause 50% inhibition of thromboxane A_2 formation did not inhibit the cyclo-oxygenase and prostacyclin synthetase of sheep seminal vesicle microsomes or the prostacyclin synthetase of dog aortic microsomes. Thus, with respect to various enzymatic steps of arachidonic acid metabolism, burimamide is a selective inhibitor of thromboxane A_2 biosynthesis. Furthermore, we have previously found that burimamide is not an antagonist of the contractile action of preformed thromboxane A_2 on isolated preparations of rabbit aorta (Allan & Eakins, 1978).

The question arises as to why burimamide inhibits thromboxane A_2 synthetase and the other structurally closely-related histamine H_2-receptor antagonists do not. Metiamide and cimetidine, unlike burimamide, both possess a methyl group in the four position of the imidazole ring and a thioether linkage in the side chain. These structural modifications appear to alter the electronic and steric properties of the drug mol-

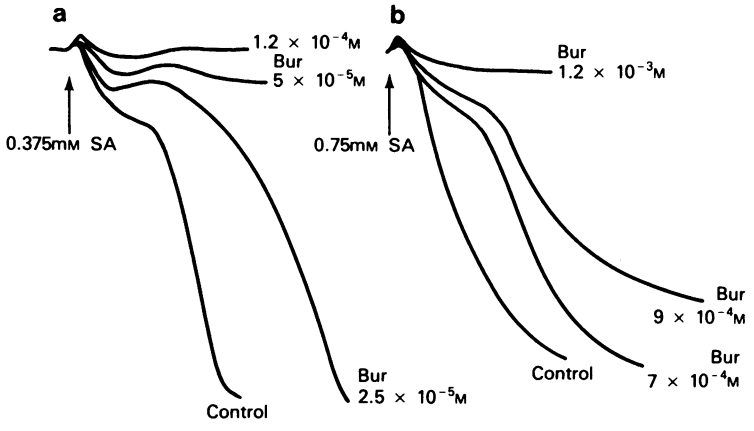


Figure 5 Superimposed platelet aggregation traces. In (a) aggregation of human platelet rich plasma by the addition of 0.375 mM sodium arachidonate (SA), in the absence and presence of burimamide (Bur) (2.5×10^{-5} to 1.2×10^{-4} M). In (b) aggregation of human platelet rich plasma by the addition of 0.75 mM SA, in the absence and presence of burimamide (Bur) (7×10^{-4} to 1.2×10^{-3} M). Burimamide was incubated with stirred platelet preparations for 5 min at 37°C before the addition of the SA.

ecule (Ganellin, Durant & Emmett, 1976). Furthermore, burimamide possesses a different profile of pharmacological action from that of either metiamide or cimetidine. For example, at effective histamine H_2-receptor blocking concentrations burimamide possesses an appreciable α-adrenoceptor blocking activity which is not shared by metiamide (Glover, Carroll & Latt, 1974; Brimblecombe, Duncan, Owen & Parsons, 1976). Also, burimamide, but not metiamide, increases blood pressure and heart rate in anaesthetized cats as a result of catecholamine release (Brimblecombe *et al.*, 1976).

Ganellin & Owen (1977) demonstrated that this difference between burimamide and metiamide in causing pressor responses may be related to the more basic nature of burimamide. Similarly, the difference between burimamide on the one hand, and metiamide and cimetidine on the other, with respect to thromboxane synthetase inhibition, may also be due to these same electronic and steric differences between the molecules.

Burimamide, at concentrations which inhibited thromboxane A_2 biosynthesis in human platelet microsomes, also inhibited platelet aggregation in human PRP. The degree of platelet aggregation by burimamide as dependent upon the amount of sodium arachidonate used to induce aggregation, since the inhibition was overcome when higher concentrations of substrate were used. Burimamide did not inhibit the primary wave of aggregation induced by ADP in aspirin-treated platelets, suggesting that the inhibitory effect of burimamide on arachidonic acid-induced platelet aggregation is a direct consequence of thromboxane inhibition rather than a non specific effect on platelet aggregability.

In conclusion, we have demonstrated that burimamide is a selective inhibitor of thromboxane A_2 biosynthesis and that this action of burimamide is unrelated to its histamine H_2-receptor blocking properties. Furthermore, burimamide also inhibited arachidonic acid-induced aggregation of human platelets thus supporting the concept that thromboxane A_2 biosynthesis within platelets is a prerequisite for the aggregatory response (Hamberg *et al.*, 1975).

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