

# INHIBITORS OF THROMBOXANE SYNTHASE IN HUMAN PLATELETS

Ulf DICZFALUSY and Sven HAMMARSTRÖM

*Department of Chemistry, Karolinska Institutet, S-10401 Stockholm 60, Sweden*

Received 8 August 1977

## 1. Introduction

Thromboxanes A are a new group of compounds with extreme potency to induce platelet aggregation and smooth muscle contractions. They are derived from prostaglandin endoperoxides and have a half-life of only 30–40 s in aqueous solution (pH 7.4, 37°C). Thromboxanes B, the products of the spontaneous degradation, are biologically inactive [1]. Two enzymes are required to convert polyunsaturated fatty acids to thromboxanes: prostaglandin endoperoxide synthase (cyclooxygenase) and thromboxane synthase (fig.1). These enzymes from human platelets were recently solubilized and chromatographically resolved [2]. In the present report the ability of various compounds to inhibit thromboxane synthase from human platelets is described.

## 2. Materials and methods

[1-<sup>14</sup>C]Prostaglandin H<sub>2</sub> (1 Ci/mol) was prepared from [1-<sup>14</sup>C]arachidonic acid (Radiochemical Centre, 55 Ci/mol) [3]. The incubation mixture contained 5 mM L-tryptophan, a cofactor for the conversion of prostaglandin G<sub>2</sub> to prostaglandin H<sub>2</sub> [4]. Phtalazinol was kindly provided by Dr T. Shimamoto. 9 $\alpha$ ,11 $\alpha$ -Azo-15(S)-hydroxy-prosta-5(*cis*), 13(*trans*)-dienoic acid was a generous gift from Dr E. J. Corey. 9 $\alpha$ ,11 $\alpha$ -Epoxymethano-15(S)-hydroxy-prosta-5(*cis*), 13(*trans*)-dienoic acid, 9 $\alpha$ ,11 $\alpha$ -methanoepoxy-15(S)-hydroxy-

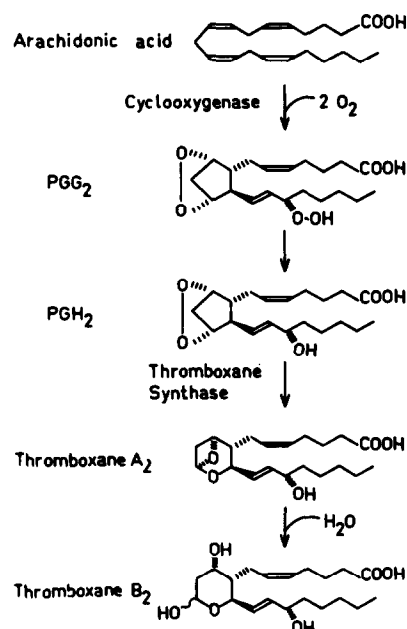


Fig.1. Enzymes required to convert arachidonic acid to thromboxane B<sub>2</sub>.

prosta-5(*cis*), 13(*trans*)-dienoic acid, prostaglandins and thromboxane B<sub>2</sub> were kindly provided by the Upjohn Company. 5,8,11,14-Eicosatetrayonic acid, 2-isopropyl-3-nicotinyl-indole and sodium *p*-benzyl-4-[1-oxo-2-(4-chlorobenzyl)-3-phenyl propyl] phenyl phosphonate were generously provided by Hoffman La Roche, Labaz and Nelson Research, respectively. Imidazole was purchased from Sigma.

### 2.1. Platelet microsomes

Washed platelets were prepared from human blood collected with 7.5% (v/v) of 77 mM sodium EDTA

*Abbreviation:* ETA, 5,8,11,14-eicosatetraynoic acid

This work was presented at the 11th FEBS Meeting, Copenhagen, August 14–19, 1977

[3]. The platelets from 400 ml blood were resuspended in 10 mM Tris-HCl, pH 7.4 to vol. 10 ml and sonicated at 0°C using a Branson sonifier model S-125 (setting no 4); six 5 s treatments separated by 1 min intervals for cooling. The fraction sedimenting between  $1500 \times g \times 15$  min and  $100\,000 \times g \times 60$  min was resuspended in 50 mM Tris buffer, pH 7.4, to vol. 20 ml. Platelet disruption and subsequent manipulations were performed at 0–4°C.

## 2.2. Enzyme assay

Resuspended microsomes, 100  $\mu$ l, (0.65 mg protein/ml) were preincubated with various presumed inhibitors at 24°C. After 15 s, 2.5  $\mu$ g [ $1\text{-}^{14}\text{C}$ ]prostaglandin  $\text{H}_2$  was added and the incubation was continued for another 60 s at 24°C. The reaction was stopped by adding 0.3 ml diethyl ether/methanol/0.2 M citric acid (30:4:1, v/v/v) precooled to –70°C. After rapid mixing and phase separation the lower phase was frozen. The ether phase was aspirated, mixed with 10  $\mu$ g unlabeled thromboxane  $\text{B}_2$ , methylated with ethereal diazomethane and chromatographed on silica gel G thin-layer chromatography with 2% methanol in diethyl ether as solvent system. The thin-layer chromatograms were scanned for radioactivity on a Berthold Dünnschichtsscanner II and sprayed with phosphomolybdic acid to detect reference compounds. Appropriate zones were then scraped off into scintillation vials and counted in a Packard Tri Carb 3385 liquid scintillation counter, after the addition of 10 ml Permablend® (4.4 g/l toluene/ethanol, 4:1 v/v) to each vial. Percent conversion to thromboxane  $\text{B}_2$  was obtained by relating the radioactivity which co-chromatographed with the internal reference thromboxane  $\text{B}_2$  to the total radioactivity recovered from the plate.

## 3. Results and discussion

Radiochromatograms of products from prostaglandin  $\text{H}_2$  incubations with native and boiled platelet microsomes are shown in fig.2. The positions of unlabeled thromboxane  $\text{B}_2$  (A), prostaglandin  $\text{E}_2$  (B) prostaglandin  $\text{F}_{2\alpha}$  (C) used as internal (A) and external (B and C) reference compounds are also shown. The radioactive material co-chromatographing with unlabeled thromboxane  $\text{B}_2$  was analyzed by gas-

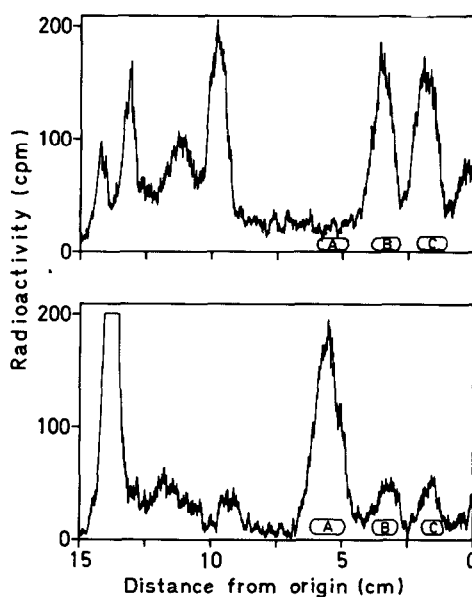


Fig.2. Thin-layer radiochromatogram of products isolated after incubations of [ $1\text{-}^{14}\text{C}$ ]prostaglandin  $\text{H}_2$  with native (lower panel) or boiled human platelet microsomes (upper panel). Positions of internal (A) and external (B, C) reference compounds are shown with striped areas. (A, thromboxane  $\text{B}_2$ ; B, prostaglandin  $\text{E}_2$ ; C, prostaglandin  $\text{F}_{2\alpha}$ ).

liquid chromatography–mass spectrometry and identified as thromboxane  $\text{B}_2$  [2].

Recently a number of stable analogues of the prostaglandin endoperoxides have been synthesized [5–7]. Three of these analogues were tested in the present investigation. One,  $9\alpha,11\alpha$ -azo-15(S)-hydroxy-prosta-5(*cis*), 13(*trans*)-dienoic acid (analogue I), was a potent inhibitor of thromboxane synthase with an  $ID_{50}$  of approximately 2  $\mu\text{M}$ . Another relatively potent inhibitor was  $9\alpha,11\alpha$ -epoxymethano-15(S)-hydroxy-prosta 5(*cis*), 13(*trans*)-dienoic acid (analogue II) which gave an  $ID_{50}$   $2 \times 10^{-5}$  M. The third analogue,  $9\alpha,11\alpha$ -methanoepoxy-15(S)-hydroxy-prosta-5(*cis*), 13(*trans*)-dienoic acid (analogue III) was not effective in inhibiting thromboxane synthase (figs. 3–4). The relative potency of these compounds as inhibitors of thromboxane synthase paralleled their binding to platelet microsomes\*, suggesting that the analogues inhibit

\*Diczfalusy, U., Powell, W. S., Hammarström, S. and Samuelsson, B., unpublished observations

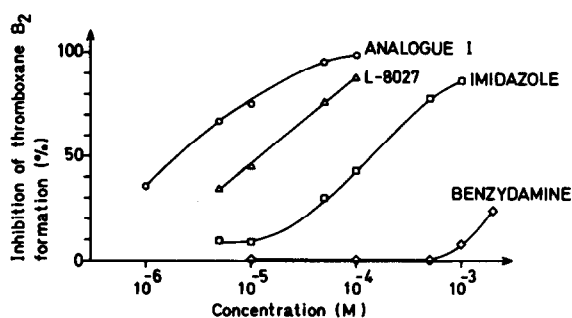


Fig. 3. Inhibition of thromboxane  $B_2$  formation from prostaglandin  $H_2$  as a function of inhibitor concentration.

thromboxane synthase by binding to the substrate site of this enzyme.

It was recently reported that an anti-inflammatory compound, 2-isopropyl-3-nicotinyl-indole (L-8027) selectively inhibits thromboxane  $A_2$  synthesis [8]. This compound was tested in our system and was found to be a potent inhibitor ( $ID_{50}$  approx.  $10 \mu M$ , fig. 3). Benzylamine, which was reported to inhibit thromboxane formation by horse platelet microsomes [9], was not effective in our system at concentrations below 1 mM. It is thus doubtful if this compound should be considered a specific inhibitor of thromboxane synthase. It has been reported that imidazole is a selective inhibitor of thromboxane formation [10]. Our results (fig. 3) confirm that imidazole inhibits the conversion of prostaglandin  $H_2$  to thromboxane  $B_2$ . The potency of imidazole was however 10–100-times less than that of the compounds

described above ( $ID_{50} 1.5 \times 10^{-4} M$ ). The acetylenic analogue of arachidonic acid, 5,8,11,14-eicosatetraenoic acid, was not effective in inhibiting thromboxane synthesis (fig. 4). Sodium *p*-benzyl-4-[1-oxo-2-(4-chlorobenzyl)-3-phenyl propyl] phenyl phosphonate (N-0164) has been reported to inhibit the formation of thromboxane  $A_2$ -like activity from prostaglandin endoperoxides by human platelet microsomes [11] as assayed on rabbit aorta. The reported  $ID_{50}$  ( $2.4 \times 10^{-5} M$ ) is identical to that obtained in our system (fig. 4). Phthalazinol (EG-626) did not inhibit the conversion of prostaglandin  $H_2$  to thromboxane  $B_2$  catalyzed by human platelet microsomes (fig. 4). This agrees with a proposal that EG-626 is an antagonist of thromboxane action [12] rather than an inhibitor of thromboxane synthase.

In conclusion, four compounds were found to be good inhibitors of microsomal thromboxane synthase from human platelets. These inhibitors (analogue I, L-8027, analogue II and N-0164, decreasing order of potency) had  $ID_{50}$  values of 2–24  $\mu M$ . The endoperoxide analogues I and II have biological activities similar to those of prostaglandin  $G_2$  and prostaglandin  $H_2$  [13]. L-8027 and N-0164, however, appear to be promising compounds for studies on the relative biological importance of prostaglandin endoperoxides and thromboxanes in platelets and other tissues.

#### Acknowledgements

This work was supported by a grant from the Swedish Medical Research Council (Project 03X-217).

#### References

- [1] Hamberg, M., Svensson, J. and Samuelsson, B. (1975) Proc. Natl. Acad. Sci. USA 72, 2994–2998.
- [2] Hammarström, S. and Falardeau, P. (1977) Proc. Natl. Acad. Sci. USA in press.
- [3] Hamberg, M., Svensson, J., Wakabayashi, T. and Samuelsson, B. (1974) Proc. Natl. Acad. Sci. USA 71, 345–349.
- [4] Miyamoto, T., Ogino, N., Yamamoto, S. and Hayaishi, O. (1976) J. Biol. Chem. 251, 2629–2636.
- [5] Corey, E. J., Shibasaki, M., Nicolaou, K. C., Malmsten, C. L. and Samuelsson, B. (1976) Tetrahedron Lett. 10, 737–740.

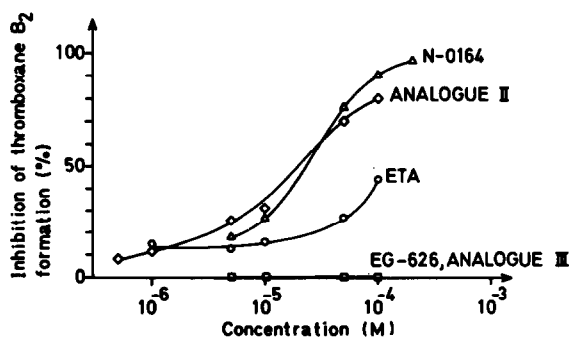


Fig. 4. Inhibition of thromboxane  $B_2$  formation from prostaglandin  $H_2$  as a function of inhibitor concentration.

- [6] Corey, E. J., Nicolaou, K. C., Machida, Y., Malmsten, C. L. and Samuelsson, B. (1975) *Proc. Natl. Acad. Sci. USA* 72, 3355–3358.
- [7] Bundy, G. L. (1975) *Tetrahedron Lett.* 24, 1957–1960.
- [8] Gryglewski, R. J., Zmuda, A., Korbut, R., Krecioch, E. and Bieron, K. (1977) *Nature* 267, 627–628.
- [9] Bunting, S., Higgs, G. A., Moncada, S. and Vane, J. R. (1976) *Brit. J. Pharmac.* 58, 269P.
- [10] Needleman, P., Raz, A., Ferrendelli, J. A. and Minkes, M. (1977) *Proc. Natl. Acad. Sci. USA* 74, 1716–1720.
- [11] Kulkarni, P. S. and Eakins, K. E. (1976) *Prostaglandins* 12, 456–469.
- [12] Shimamoto, T., Takashima, Y., Kobayashi, M., Moriya, K. and Takahashi, T. (1976) *Proc. Japan Acad.* 52, 591–594.
- [13] Malmsten, C. (1976) *Life Sci.* 18, 169–176.