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The (+)-enantiomer is responsible for the antiplatelet and anti-inflammatory activity of (±)-indobufen

CHIARA CERLETTI, STEFANO MANARINI, MONICA COLOMBO*, ALESSANDRA TAVANI*, *Istituto di Ricerche Farmacologiche "Mario Negri", Giulio Bizzozero Laboratory of Platelet and Leucocyte Pharmacology, Consorzio Mario Negri Sud, 66030 Santa Maria Imbaro, Italy, and *Opioid Neuropharmacology Unit, via Eritrea 62, 20157 Milano, Italy*

Abstract—The racemic compound indobufen and its (+)- and (−)-enantiomers have been compared for their effects on blood platelet function and rat carrageenan pleurisy. The antiplatelet properties were studied in-vitro in human platelets by measuring the inhibition of platelet aggregation and generation of serum thromboxane (Tx) B_2. In-vivo, the antiplatelet and anti-inflammatory properties were studied in rats by measuring the inhibition of serum TxB_2, the amount of 6-keto-$PGF_{1\alpha}$ in pleural exudate and pleural exudate volume. In all tests the (+)-enantiomer was slightly more potent than the racemate, while the (−)-enantiomer was far less potent. In the same rats, treatment with the lowest doses of the compounds giving 90% inhibition of serum thromboxane B_2 generation was associated with occasional macroscopic lesions of the gastric mucosa.

Indobufen, (±)-2-(*p*-(1-oxo-2-isoindolinyl) -phenyl)-butyric acid, with a chiral centre in the 2 position of butyric acid, is a reversible inhibitor of cyclo-oxygenase. Its anti-aggregatory activity has been investigated in-vitro and in-vivo in the platelets of animals (Bergamaschi et al 1979) and man (Fuccella et al 1979; Vinazzer & Fuccella 1980; Cattaneo et al 1987; Pinto et al 1987). As the enantiomers of the compound are available in pure form (> 98%), we have compared the antiplatelet effects of (±)-indobufen and its (+)- and (−)-enantiomers on human platelets in-vitro, by measuring the inhibition of serum thromboxane (Tx) B_2 generation and platelet aggregation induced by different stimuli. We also compared the in-vivo effects of the three preparations on platelet TxB_2 generation and on the carrageenan-induced inflammatory response in the pleural cavity of rats. Macroscopic lesions of gastric mucosa were also evaluated in the same group of rats.

Materials and methods

Materials. (±)-, (+)- and (−)-Indobufen provided by Farmitalia Carlo Erba (Milan, Italy): were dissolved in a small volume of 1 M NaOH (40 μL for each 10 mg of indobufen, mol. wt 295) and diluted with distilled water. Arachidonic acid (AA, 99% pure, sodium salt) and adenosine-5′-diphosphate (ADP) were purchased from Sigma (St. Louis, MO, USA), platelet activating factor (PAF) was from Bachem (Bubendorf, Switzerland) and carrageenan from Serva (Heidelberg, Germany). The antisera for specific radioimmunoassays of TxB_2 and 6-keto-$PGF_{1\alpha}$ were kindly donated by Professor Carlo Patrono (Catholic University, Rome, Italy).

Studies on human platelets in-vitro. Venous blood was collected from the antecubital vein of healthy volunteers, without anticoagulant, and immediately dispensed in glass tubes containing solvent or solutions of the test compounds. Blood was incubated at 37°C for 1 h and serum was separated by centrifugation. TxB_2 generation was measured in serum by a specific radioimmunoassay (Cerletti et al 1986).

For aggregation studies, blood was collected on sodium citrate (3·8%, 1/10; v/v), platelet-rich plasma (PRP) and platelet-poor plasma were obtained by differential centrifugation. Platelet aggregation was studied in a Born aggregometer (Elvi 840, Elvi Logos, Milan, Italy) (Di Minno et al 1979) and was induced by different agonists: arachidonic acid (AA), adenosine-5′-diphosphate (ADP) and platelet activating factor (PAF). For each subject and for each aggregating agent the threshold aggregating concentrations (TAC, defined as the smallest amount of aggregating agent producing 50% increase of light transmission at 3 min) was determined. The compounds (or solvent) were preincubated in the aggregometer with PRP at 37°C for 3 min (1 min with stirring at 1000 rev min^{-1}) before addition of the stimulus and the threshold inhibitory concentration (TIC, defined as the smallest amount of compound causing complete inhibition of aggregation induced by AA at its TAC or making fully reversible the aggregation induced by ADP or PAF at their respective TAC values (Di Minno et al 1979)), was found by progressively increasing the concentrations of the compounds in steps of 0·8 μM.

Studies in the rat in-vivo. Overnight fasted Sprague-Dawley CD-COBS rats, 180–200 g, (Charles River, Calco, Italy) were used. For studies on serum TxB_2 generation, rats were dosed orally 1 h before blood collection. Carrageenan pleurisy was induced by an intrapleural injection between the 3rd and the 4th rib of 0·5 mL of a suspension of 0·4% carrageenan in 0·1% Evans Blue in 0·9% NaCl (saline) to rats under light ether anaesthesia (Whittle et al 1980; Vinegar et al 1982; Chiabrando et al 1989).

After 5 h, rats were killed with ether anaesthesia and the pleural exudate collected by aspiration and its volume measured. The cavities were then washed with 2 mL cold saline. This wash was immediately combined with the exudate for prostaglandin determination. Exudates which appeared to contain blood were discarded. The exudate was rapidly centrifuged at 4°C to remove cells and the supernatants frozen until assayed for 6-keto-$PGF_{1\alpha}$, the stable metabolite of prostacyclin, by specific RIA (Cerletti et al 1986). Immediately thereafter blood was collected by heart puncture and incubated for 1 h at 37°C in glass tubes. The serum obtained was stored at −20°C until assayed for TxB_2.

For evaluating the presence of gastric lesions, the stomach was dissected, everted and rinsed with saline. Gastric lesions were macroscopically observed and counted by a person unaware of the treatment given. Solvent corresponding to doses below 300 mg kg^{-1} of drug had no obvious damaging effect on gastric mucosa.

Results

Studies on human platelets in-vitro. Table 1 shows the in-vitro effect of (±)-indobufen and its enantiomers on human serum TxB_2. (±)- and (+)-Indobufen inhibited serum TxB_2 dose-dependently and the former was slightly less potent than the latter. Conversely, (−)-indobufen was virtually inactive up to a concentration of 12·8 μM.

Table 2 reports the results of in-vitro studies on human platelet aggregation. The concentrations of (±)-indobufen inhibiting the aggregation induced by threshold aggregating

Correspondence to: C. Cerletti, Consorzio Mario Negri Sud, 66030 Santa Maria Imbaro, Italy.

Table 1. In-vitro effect of (±)-, (+)- and (−)-indobufen on human serum TxB_2 production.

Concentration (μM)	Serum TxB_2 (% of control) (±)	(+)	(−)
0·4	71·3±7·2	69·0±5·2	—
0·8	63·6±6·8	48·7±5·5	—
1·6	48·0±2·0	35·0±4·9	109·6 (2)
3·2	30·3±1·4	27·9±4·7	113·1 (2)
6·4	13·9±0·2	10·2±1·2	90·5 (2)
12·8	9·0±0·9	5·7±0·8	86·9±7·8

Values are means±s.e.m. of 3–7 observations, except where specified. Control values were 566±97 pmol mL^{-1}. Serum was obtained by incubation of blood without anticoagulant at 37°C for 1 h.

Table 2. Effect of (±)-, (+)- and (−)-indobufen on in-vitro human platelet aggregation induced by arachidonic acid (AA), adenosine 5′-diphosphate (ADP) and platelet activating factor (PAF).

Aggregating agent*	Threshold inhibitory concentration (μM) (±)	(+)	(−)
AA (mM)			
0·4	8·0	3·2	>32
0·5	3·2	1·6	>32
0·5	3·2	1·6	>32
0·4	4·8	2·4	>32
ADP (μM)			
2·6	4·8	2·4	>32
2·0	3·2	1·6	32
3·2	11·2	1·6	>32
2·6	3·2	1·6	>32
PAF (nM)			
200	1·6	1·6	32
20	3·2	1·6	>32
40	1·6	1·6	32
90	0·8	0·8	>32

*The concentration used for each aggregating agent was the threshold aggregating concentration (TAC), defined as the minimal concentration of aggregating agent inducing irreversible aggregation producing 50% increase of light transmission at 3 min. TAC were calculated for each platelet rich plasma. The threshold inhibitory concentration (TIC) is the minimal concentration of inhibitor ((±)-, (+)- or (−)-indobufen) suppressing (in the case of AA) or making reversible (in the case of ADP or PAF) the aggregation obtained at the corresponding TAC. The inhibitory compounds were incubated in platelet-rich plasma at 37°C for 3 min before the addition of the aggregating stimulus.

concentrations (TAC) of AA, ADP and PAF varied from 0·8 to 11·2 μM, with a median value of 3·2 μM; the concentrations of (+)-indobufen ranged between 0·8 and 3·2 μM, with a median of 1·6 μM; the threshold inhibitory concentrations (TIC) of (−)-indobufen was 32, or in most cases, >32 μM. The inhibitory effect of these compounds was comparable when tested against the different inducers of aggregation.

Studies in the rat in-vivo. Table 3 shows that serum TxB_2 generation in rats was inhibited dose-dependently by all three treatments. The (+)-enantiomer was the most potent, being slightly more potent than the racemic form and at least 10 times more potent than the (−)-form.

The effect of higher doses of (±)-, (+)- and (−)-indobufen was tested on serum TxB_2 generation, production of 6-keto-$PGF_{1\alpha}$ in pleural exudate on exudate volume, and the number of gastric lesions in rats with carrageenan-induced pleurisy (Table 4).

Serum TxB_2 was inhibited by more than 90% at all doses of

Table 3. Effect of (±)-, (+)- and (−)-indobufen on rat serum TxB_2 generation.

Treatment (mg kg^{-1})	Serum TxB_2 (% of control)
(±)	
0·50	78·4±14·5
1·50	44·8±15·7
3·00	18·8±6·5
(+)	
0·38	38·6±7·3
0·75	28·5±3·5
1·50	8·4±1·4
(−)	
7·50	66·8±16·7
15·00	22·9±5·7
30·00	10·7±1·6

Values are means±s.e.m. of 4 observations. Control values of serum TxB_2 were 108±26 pmol mL^{-1}.

Table 4. Effect of (±)-, (+)- and (−)-indobufen on serum TxB_2, exudate 6-keto-$PGF_{1\alpha}$ production, exudate volume and gastric lesions in rats with carrageenan-induced pleurisy.

Treatment (mg kg^{-1})	% of control Serum TxB_2	Exudate 6-keto-$PGF_{1\alpha}$	Exudate Volume	Lesioned rats/ total rats
(±)				
7·5	5·9±2·2	25·6±3·4	50·7±4·0	2/8
15·0	3·0±0·7	—	47·3±5·3	9/11
30·0	1·2±0·3	17·9±1·5	24·7±2·0	8/9
(+)				
3·75	9·4±1·7	40·8±5·9	61·3±7·9	1/9
7·5	4·4±1·4	—	49·3±8·7	8/11
15·0	2·4±0·8	27·7±6·5	29·3±5·3	7/9
(−)				
15·0	50·0±17·5	39·9±1·5	72·0±6·0	1/7
75·0	3·5±0·3	23·5±3·8	40·0±7·3	4/9
300·0	3·2±0·8	—	30·7±6·0	9/9*

Values are means±s.e.m. Control values were 189·4±31·8 and 78·0±2·9 pmol mL^{-1}, respectively, for serum TxB_2 and exudate 6-keto-$PGF_{1\alpha}$. The exudate volume in controls was 1·5±0·08 mL and no gastric lesions were reported in 13 control rats.

*The number and extent of the lesions in this group could in part be due to the large volume of NaOH required to dissolve the compound at this dose.

(±)- and (+)-indobufen. (−)-Indobufen (at 15 mg kg^{-1}) was less potent than corresponding doses of the other two compounds. The inhibition of 6-keto-$PGF_{1\alpha}$ in pleural exudate ranged from 60 to 82% of control at the doses used, (−)-indobufen being less potent than the (+)- and (±)-forms. The volume of pleural exudate induced by carrageenan was reduced by pretreatment with (±)-indobufen or either enantiomer; the effect was dose-dependent, ranging from 25 to 72% of controls. In this test (+)-indobufen was slightly more potent than the racemate and about one order of magnitude more active than the (−)-enantiomer. The occurrence of gastric lesions was proportional to the doses of the compounds used. The lesions were only occasionally found in rats treated with the lowest doses tested of (±)- or (+)-indobufen which suppressed serum TxB_2 by more than 90% (data not shown).

Discussion

The enantiomeric purity of the (−)-enantiomer (lot. no. A02003; 3-12-87), determined by HPLC, was 98·6%, with 1·4%

contamination with the (+)-enantiomer. It was calculated that this extent of contamination could account for part of the pharmacological effects reported in our study for (−)-indobufen. In fact, 1·4% of 30 mg kg^{-1} (the dose of (−)-indobufen reducing by 90% TxB_2 generation in rat serum) corresponds to 0·42 mg kg^{-1} of (+)-indobufen, a dose which could inhibit the same parameter by 70%. Similarly, as far as the study on pleural exudate is concerned, 1·4% of 300 mg kg^{-1} (the dose of (−)-indobufen reducing pleural exudate volume by 70%) corresponds to 4·2 mg kg^{-1} of (+)-indobufen, a dose which exerted an inhibitory activity of about 50%.

Although the possibility of indobufen racemization in-vivo cannot be excluded, we conclude that the (+)-enantiomer alone is responsible for the antiplatelet and anti-inflammatory activity of (±)-indobufen.

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