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Inhibition of prostaglandin biosynthesis by clidanac and related compounds: structural and conformational requirements for PG synthetase inhibition

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The inhibition of prostaglandin (PG) biosynthesis by clidanac (6-chloro-5-cyclohexyl-1-indancarboxylic acid, TAI-284), its metabolites and some analogues has been examined using various microsomal preparations as enzyme source. Clidanac and some analogues were among the most potent inhibitors. The (+)-isomer of clidanac was shown to be 1000 times more potent than the (−)-isomer in inhibiting PG synthetase activity. The *cis*-3′-hydroxyl metabolite which retains anti-inflammatory activity comparable to that of clidanac had much less inhibitory activity. Structure-activity studies with clidanac analogues showed that the position of halogen substitution in 1-indancarboxylic acid is of considerable significance for the conformational requirement for binding to the enzyme.

Clidanac (6-chloro-5-cyclohexyl-1-indancarboxylic acid, TAI-284) has anti-inflammatory, analgesic and antipyretic activity in animals (Kawai et al 1971) and man (Schichikawa et al 1977).

Inhibition of prostaglandin (PG) biosynthesis has been considered a mode of action of acidic non-steroidal anti-inflammatory agents (Vane 1971), and it has been documented that the anti-inflammatory activity follows to some degree the inhibitory activity against PG synthetase (Flower 1974).

We have compared the ability of clindanac and related compounds to inhibit the biosynthesis of PGs with that of several non-steroidal anti-inflammatory agents using rabbit renal medulla microsomes. The structural requirements for inhibition of PG biosynthesis by 1-indancarboxylic acid analogues were also examined. In guinea-pigs, the efficacy of (−)-isomer of clidanac was equivalent to those of (+)-isomer and racemate in the u.v.-induced erythema test (Kuzuna et al 1974b). For this reason, guinea-pig skin microsomes were also used as a source of PG synthetase.

Materials and Methods

Materials

Arachidonic acid was purchased from P-L Biochemicals (Milwaukee), [1-^{14}C]arachidonic acid (60 mCi mmol^{-1}) from the Radiochemical Centre (Amersham), $PGF_{2\alpha}$ and PGE_2 from Fuji Chemical (Tokyo), (−)-adrenaline bitartrate from Sigma Chemical (St. Louis) and reduced glutathione (GSH) from Wako Pure Chemical (Osaka). All other reagents and solvents were of reagent grade. Clidanac and several analogues were supplied by our laboratory. Drugs used were: indomethacin (Banyu), diclofenac sodium (Fujisawa), phenylbutazone (Fujisawa), aspirin (Yoshitomi) and ketoprofen. Ketoprofen was synthesized in this laboratory. Clidanac, its metabolites and diclofenac were used as Na salt. Other clidanac analogues, phenylbutazone and ketoprofen were solubilized with 0·1 M NaOH, and indomethacin and aspirin were dissolved with an aid of Na_2CO_3.

PG synthetase preparations

(a) *Rabbit and rat renal medulla.* Male albino rabbits, about 3 kg, and JCL-Sprague-Dawley rats, about 200 g, were decapitated, the kidneys removed, and medulla isolated from the cortex, and homogenized in 4 vol of ice-cold 0·1 M Tris-HCl buffer (pH 7·5, rabbit) or of ice-cold 1·15% KCl (rat). Homogenates were centrifuged for 10 min at 10 000 *g* and the supernatants centrifuged for 60 min at 105 000 *g* to yield a microsomal pellet. That from rabbit was lyophilized and stored at −20° C. That from rat was suspended in 0·1 M Tris-HCl buffer (pH 8·5) and immediately assayed.

(b) *Guinea-pig skin.* Male STD-Hartley guinea-pigs, about 350 g, were decapitated, and their shaved dorsal skins (mid-portion, 5 × 5 cm) were excised,

* Correspondence

trimmed of fat and subcutaneous tissue and cut into small pieces and homogenized in a Polytron homogenizer in 4 vol of ice-cold 0·1 M phosphate buffer (pH 8·0). Unbroken cellular debris was removed by filtration and the resultant homogenates were centrifuged for 10 min at 10 000 *g*. Microsomes obtained from the supernatants by further centrifugation as mentioned above were resuspended in the buffer (pH 8·0) and used for enzyme assay. Protein content was determined according to Lowry et al (1951).

Assay of PG synthesis activity

The assay of PG synthetase activity from rabbit renal medulla microsomes was essentially according to Blackwell et al (1975), and Tachizawa et al (1977). The reaction mixture of GSH 2·5, adrenaline 2, [1-^{14}C]arachidonic acid 0·13 mM, 2 mg of lyophilized microsomes (1 mg of protein) and the test compound in 1 ml of 0·1 M Tris-HCl buffer (pH 7·5) containing 2 mM EDTA was incubated for 10 min at 37 °C.

The assay of PG synthetase activity from rat renal medulla microsomes was based on the method of Tachizawa et al (1977). The reaction mixture, GSH 1·7, adrenaline 0·5, [1-^{14}C]arachidonic acid 0·1 mM, freshly prepared microsomes (1·5–1·67 mg of protein) and the test compound, in 1 ml of 0·1 M Tris-HCl buffer (pH 8·5) containing 0·07 mM EDTA were incubated for 10 min at 37 °C.

The assay of PG synthetase activity from guinea-pig skin microsomes follows essentially the method of Ziboh et al (1975). The reaction mixture (2 ml), 0·1 M phosphate buffer (pH 8·0), EDTA 2, [1-^{14}C]-arachidonic acid 0·05, hydroquinone 0·5 and GSH 0·65 mM as cofactors, microsome suspension (1·6–2·1 mg of protein) and the test compound, was incubated at 37 °C for 30 min.

These reactions were initiated by addition of the enzyme and terminated by acidification with 1 M HCl to pH 3–4. The acidified media were extracted twice with 5 ml ether and evaporated to dryness under N_2 stream. After addition of PGE_2 and $PGF_{2\alpha}$ to the extract as chromatographic standards, each sample was spotted on a silica gel 60 thin layer plate (E. Merck, Darmstadt) and developed with ethyl acetate–water–isooctane–acetic acid (11:10:5:2, by vol). PGs and arachidonic acid were detected by I_2 vapour. The zones corresponding to PGs and other fractions were scraped into scintillation vials, and counted. The amounts of PGs synthesized were calculated from percent of PGs radioactivity to total plate radioactivity and initial substrate concentrations.

RESULTS

Clidanac inhibited the synthesis of PGE_2 from arachidonic acid by rabbit renal medulla microsomes in a dose-related manner, as did indomethacin, diclofenac, ketoprofen and phenylbutazone. The concentrations of compounds necessary to inhibit the PG synthesis by 50% (IC50) are shown in Table 1. Metabolites of clidanac in animals (Kanai et al 1973), 6-chloro-5-(*cis*-4′-hydroxycyclohexy)-1-indancarboxylic acid (Metabolite II_a), 6-chloro-5-(*cis*-3′-hydroxycyclohexyl)-1-indancarboxylic acid (Metabolite II_b) and 6-chloro-5-(*trans*-4′-hydroxycyclohexyl)-1-indancarboxylic acid (Metabolite III, Fig. 1), were significantly less potent than the parent compound in inhibiting the PGE_2 synthesis. The metabolites showed poor correlation between in vitro inhibition of PG synthesis and in vivo anti-inflammatory activity (Table 1). Metabolite II_b, one of the metabolites of clidanac in rat plasma, did not exert as much activity as clidanac in inhibiting the PG synthesis also in rat renal medulla despite its potent in vivo activity. Synthesis of $PGF_{2\alpha}$ from arachidonic acid was also inhibited by clidanac as well as indomethacin and diclofenac in dose-related manner. As shown in Table 1, IC50s for these compounds against $PGF_{2\alpha}$ synthesis were almost equal to those for PGE_2 synthesis. However, the metabolites II_a, II_b and III did not inhibit the $PGF_{2\alpha}$ synthesis in parallel with the PGE_2 synthesis.

Table 1. Comparison of the abilities of clidanac, its metabolites, indomethacin, diclofenac and aspirin to inhibit PGE_2 and $PGF_{2\alpha}$ syntheses in rabbit renal medulla microsomes, with the relative anti-inflammatory potencies. The microsome was incubated with 0·13 mM [1-^{14}C]arachidonic acid in the presence of the indicated compounds for 10 min at 37° C. The products formed were determined as described under 'Methods'.

Compound	Inhibition of PG synthesis IC50 (μM) PGE_2	$PGF_{2\alpha}$	Paw oedema inhibition MED[1] (mg kg^{-1} orally)
Clidanac	2·7	2·9	0·75
Metabolite II_a	150	300	3
II_b	64	140	0·75
III	1000	5000	15
Indomethacin	6·6	8·8	1
Diclofenac	2·4	3·2	0·75
Aspirin	>5 mM[2])	>5 mM[2])	100[3])

1) MED = minimum effective dose against the carrageenan-induced oedema of rats, cited from our previous reports (Kawai et al 1971; Kuzuna et al 1978).
2) Aspirin inhibited PGE_2 and $PGF_{2\alpha}$ syntheses by approx. 20% at a concentration of 5 mM, respectively.
3) Unpublished observation.

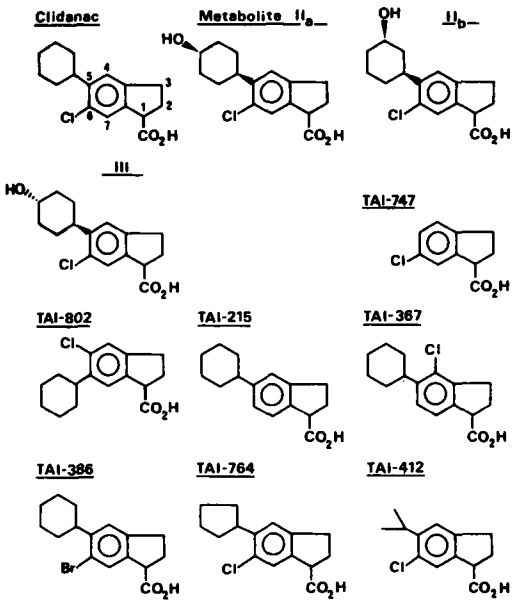

FIG. 1. Chemical structures of clidanac, its metabolites and the analogues used in this study.

The inhibitory efficacy of clidanac analogues and other compounds for PGE_2 synthesis, and their potency ratios expressed on a molar basis relative to clidanac are listed in Table 2. A positional isomer of clidanac, TAI-802, was much less potent than clidanac, suggesting that the replacement of the C-5 cyclohexyl by Cl apparently decreased the potency. Introduction of halogen at C-6 of TAI-215 markedly increased the potency. Thus, compounds with halogen at C-6 and with bulky alkyl groups such as cyclohexyl or cyclopentyl at C-5, clidanac, TAI-386, and TAI-764, showed extremely potent inhibitory activities on PG synthesis in vitro.

Table 2. Inhibition of PGE_2 synthesis by clidanac analogues and other compounds in rabbit renal medulla microsomes. Experimental conditions were the same as those in Table 1. Potency ratios were calculated by the parallel line assay method in comparison with clidanac.

Compound	IC50 (μM)	Potency ratio
Clidanac	2.7	1
TAI-367	38	0.07
TAI-802	190	0.01
TAI-386	1.2	2.2
TAI-764	1·1	2·4
TAI-412	82	0·03
TAI-215	60	0·04
TAI-747	>10 mM	—
Ketoprofen	3·4	0·78
Phenylbutazone	110	0.02

In guinea-pig skin microsomes, the inhibitory effects of clidanac and enantiomers, metabolite II_b, indomethacin and diclofenac on PGE_2 synthesis are illustrated in Fig. 2. All these compounds tested showed a dose-dependent inhibition, but the dose-response curve for the (−)-isomer differed from that for the (+)-isomer which was 1000 times more potent as an inhibitor of PG synthetase in vitro; it was also 2·5 times more potent than its racemate.

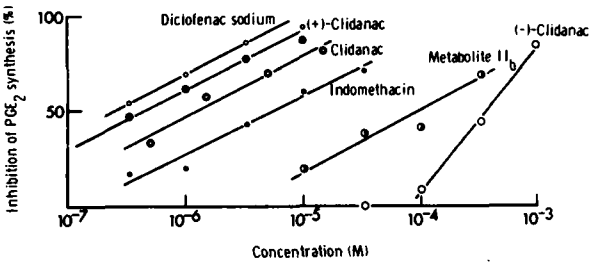

FIG. 2. Inhibitory effects of enantiomers of clidanac and other compounds on PGE_2 synthesis by guinea-pig skin microsomes. The microsomes were incubated with 0·05 mM [1-^{14}C]arachidonic acid in the presence of the indicated compounds for 30 min at 37° C. The PGE_2 formed was determined as described under 'Methods'. Each point represents the mean of 2–3 determinations.

DISCUSSION

The inhibitory activity of clidanac on the PG biosynthesis entirely resided in the (+)-isomer, (*S*-form, Noguchi et al 1974). This stereospecific requirement for the inhibition has been reported (Flower 1974).

The (+)-isomer of clidanac was 5 to 12 times more potent in vivo than the (−)-isomer in rats and mice, whereas in guinea-pigs the racemate and the isomers had equivalent anti-inflammatory activities (Kuzuna et al 1974b). Therefore, it is conceivable that in guinea-pigs the (−)-isomer is converted to the (+)-isomer almost completely as has been found with ibuprofen (Kaiser et al 1976), or to the racemic mixture.

Of the metabolites of clidanac, II_b was almost equivalent to the parent in the anti-inflammatory activity, and it was more potent in analgesic activity and in ulcerogenicity (Kuzuna et al 1974a). Considering its pharmacological profile and Vane's theory (Vane 1971), the biological action of II_b may be related closely to its inhibition of PG biosynthesis. The inhibitory activities of II_b in different tissues or species in vitro were much less than expected from its biological efficacy. Ceserani et al (1977) demonstrated that the potency in inhibiting PG synthetase

as well as the plasma protein binding ability of a series of indoprofen derivatives were, partly, ascribable to the lipophilicity of the compounds. Over 90% of a therapeutic dose of clidanac is bound to plasma proteins, but the protein binding of the metabolites was much less (Tanayama et al 1973). Thus, the apparent lack of correlations between the anti-inflammatory potencies of clidanac and II_b and their inhibitory potencies on the PG biosynthesis may result from differences in their plasma protein binding. The order of potency of the metabolites in inhibiting PG synthetase can be correlated with their in vivo activities (Table 1), supporting this view. Another explanation is that the metabolites may exert influence upon the breakdown of the endoperoxide intermediate to PGE_2 and $PGF_{2\alpha}$, exemplified by their differential inhibitory effects on PGE_2 and $PGF_{2\alpha}$ syntheses (Table 1). It is possible that these metabolites exert an action in vivo by shifting the ratios of PGE_2 and $PGF_{2\alpha}$, since PGE_2 and $PGF_{2\alpha}$ have different biological actions (Vane 1976).

A series of 1-indancarboxylic acids also inhibited PG synthesis activity. Alkyl moieties at C-5 play a

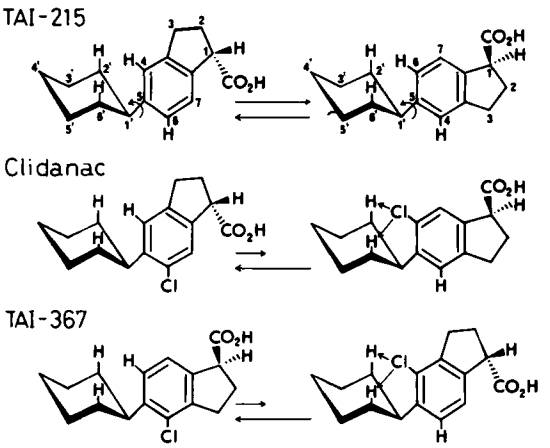

FIG. 3. Conformational equilibria of TAI-215, clidanac and TAI-367.

role in binding to the enzyme (e.g. clidanac vs TAI-747) and halogen at C-6 markedly increased activity (clidanac vs TAI-215), the position of the halogen substitution seeming to be more important, since the 4-chloro compound (TAI-367) was less potent than the corresponding 6-chloro compound (clidanac). Kamiya et al (1975) have demonstrated by X-ray crystallographic study that the cyclohexyl ring of clidanac is deviated away from the Cl at C-6 and out of the plane of the indan ring, and its spatial feature is common to that of indomethacin. Gund & Shen (1977) have also compared the conformations of indomethacin and some anti-inflammatory compounds and indicated that *S*-(+)-clidanac is superimposable with *S*-α-methyl indomethacin. According to their proposal, the bioactive conformations of some anti-inflammatory arylacetic acids reside in '*anti*-form' in which the conformation of the functional group (cyclohexyl ring) and carboxyl group are directed to the opposite side. Clidanac has little conformational flexibility because of the steric interaction between Cl at C-6 and 2'- and 6'-axial hydrogens (Fig. 3), and 4-chloro-5-cyclohexyl compound (TAI-367) also appears to have a similar conformational rigidity. In this case, the conformation may have the cyclohexyl ring and carboxyl group on the same side of the plane of the indan ring, i.e. '*syn*-form'. Consequently, the results of the experiments with these positional isomers show that the '*anti*-form' is more favourable in binding to the enzyme than '*syn*-form'.

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