Biochemical Pharmacology, Vol. 25, pp. 2479–2484. Pergamon Press, 1976. Printed in Great Britain.

INHIBITION OF PROSTAGLANDIN SYNTHETASE BY TOLMETIN (TOLECTIN, McN-2559), A NEW NON-STEROIDAL ANTI-INFLAMMATORY AGENT

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(*Received* 22 *December* 1975; *accepted* 9 *April* 1976)

Abstract—Tolmetin [1-methyl-5-*p*-toluoylpyrrole-2-acetic acid (McN-2559, Tolectin)] and several of its analogs were shown to be potent inhibitors of the synthesis of prostaglandin E_2 from arachidonic acid by bovine seminal vesicle prostaglandin synthetase *in vitro*. Kinetic studies indicated that tolmetin, like indomethacin and aspirin, inhibited the synthetase competitively with respect to substrate. Unlike most non-steroidal anti-inflammatory agents, however, tolmetin was a competitive reversible inhibitor, and did not promote a time-dependent inactivation of the prostaglandin synthetase. Tolmetin and these other 1-methyl-pyrrole acetic acids represent a new structural class of anti-inflammatory agents which inhibit prostaglandin synthetase.

Tolmetin [1-methyl-5-*p*-toluoylpyrrole-2-acetic acid (McN-2559, Tolectin)] is a new, non-steroidal anti-inflammatory agent which is clinically effective in rheumatoid arthritis [1–6]. In animals, tolmetin showed anti-inflammatory activity in the acute kaolin- and carageenan-induced rat hind paw edema tests, the subacute carageenan abscess test, and the chronic adjuvant arthritis test [7–9]. The compound also exhibited anti-nociceptive and anti-pyretic activities in appropriate models, and showed relatively low ulcerogenicity in rats [7].

Prostaglandins have been reported to evoke inflammatory and pyretic responses [10, 11], and the observed inhibition of prostaglandin synthesis by indomethacin, aspirin and other non-steroidal agents has been proposed as the mechanism of their anti-inflammatory and anti-pyretic activities [12, 13]. Several other non-steroidal agents with anti-inflammatory and anti-pyretic activities have been subsequently reported to inhibit prostaglandin biosynthesis [14–16]. It was, therefore, of interest to determine whether tolmetin, and anti-inflammatory agent from a new chemical class, also inhibits prostaglandin synthesis. The results of studies *in vitro* of the effects of tolmetin on bovine seminal vesicle microsomal prostaglandin synthetase are presented in this paper.

MATERIALS AND METHODS

Materials. Tolmetin and several analogs were synthesized in these laboratories by Dr. J. R. Carson, as previously described [8, 9], and tolmetin[^{14}C] was prepared by L. E. Weaner in a similar manner. The other drugs were generously supplied by the manufacturers indicated: indomethacin (Merck, Sharp & Dohme), mefenamic acid and flufenamic acid (Parke-Davis), diclofenac sodium and phenylbutazone (CIBA-Geigy), ketoprofen (Rhone-Poulenc), naproxen (Syntex), fenoprofen (Eli Lilly) and ibuprofen (Boots Pure Drug, Ltd.). Aspirin was purchased from Sigma Chemical Co., and 2.7-dihydroxynaphthalene from Aldrich Chemical Co. All compounds were dissolved in 50 mM Tris–HCl buffer (pH 8.3), in some cases with the aid of 0.1 N sodium hydroxide, just prior to use. Care was taken to keep the pH below 8.3.

Arachidonic acid [5, 6, 8, 9, 11, 12, 14, 15[^{3}H] (N)] (72 Ci/m-mole) was obtained from New England Nuclear, and was diluted with cold arachidonic acid (Analabs, Inc., 99% pure) in ethanol to a specific activity of 1.5 mCi/m-mole and stored at −20° under nitrogen until use. The purity of the arachidonic acid[^{3}H] was frequently checked by thin-layer chromatography using a hexane–ether–acetic acid (70:30:1) system.

Prostaglandins E_2 and $F_{2\alpha}$ were purchased from Analabs, Inc., 1-epinephrine bitartrate from CalBiochem, and glutathione from Sigma Chemical Co. Silica gel G thin-layer chromatographic plates were obtained from Analtech, Inc.

Preparation of microsomes. A microsomal prostaglandin synthetase was prepared from bovine seminal vesicles essentially according to the method described by Takeguchi *et al.* [17]. Frozen bull seminal vesicles (Pel Freeze Biologicals, Inc.) were thawed, trimmed free of excess fat and connective tissue, diced and blended for 2 min at 5° with 2 vol. of 100 mM Tris–HCl buffer (pH 8.3). The homogenate was centrifuged at 12,000 *g* for 20 min, and the supernatant was filtered through a double layer of cheesecloth. The filtered supernatant was then centrifuged at 80,000 *g* for 1 hr. The precipitated microsomes were resuspended in a small volume of 10 mM Tris–HCl (pH 8.3) and lyophilized. The lyophilized powder was pulverized and stored at −20°, with no loss of activity for at least 6 months. One mg of the powder was found to contain 0.68 mg protein as determined by the method of Lowry *et al.* [18].

Assay of PG synthetase. The assay of PG synthetase activity was based on methods reported by Takeguchi *et al.* [17] and Flower *et al.* [19]. The standard incubation mixture (total volume 1.0 ml) contained 0.33 mM arachidonic acid[^{3}H] (0.5 μCi), 3.0 mM

glutathione, 3.0 mM 1-epinephrine, and 5.0 mg of microsomal enzyme preparation in 50 mM Tris–HCl buffer (pH 8.3). The reaction was initiated by addition of the enzyme, and was incubated for 5 min at 37° during which period the rate of prostaglandin synthesis was found to be linear. The standard assay was used in all experiments unless otherwise indicated.

The incubation was terminated by the addition of 0.1 ml of 3 N hydrochloric acid and 3 ml ethyl acetate, and 10 μg each of PGE_2 and $PGE_{2\alpha}$ were added as carriers to facilitate extraction and chromatographic separation. The samples were extracted twice with ethyl acetate, the extracts combined and dried under nitrogen, and then redissolved in 0.1 ml methanol. An aliquot (0.02 ml) was spotted on a Silica gel G thin-layer chromatographic plate and developed in an ethyl acetate–acetic acid (100:2) solvent system. In this system, the R_f values were the following: $PGF_{2\alpha}$, 0.30; PGE_2, 0.47; and arachidonic acid, 0.83. These compounds were located by visualization in iodine vapor, the spots were scraped and placed in 10 ml toluene–ethanol (80:20) containing diphenyloxazole (0.4%) and 1,4-bis-2-(5-phenyloxazole) benzene (0.02%, and the radioactivity was determined using a liquid scintillation counter. The Silica gel remaining on the plate was also scraped and counted. The nmoles of PGE_2 and $PGE_{2\alpha}$ synthesized were calculated from the fraction of total plate radioactivity per PGE_2 or $PGF_{2\alpha}$ spot and the initial arachidonate concentration. At the arachidonate concentration used (0.33 mM), $PGF_{2\alpha}$ synthesis was low [19]. The K_m for arachidonate was found to be 0.25 mM.

RESULTS

Inhibition studies. Tolmetin was found to be an effective inhibitor of the synthesis of PGE_2 from arachidonate by bovine seminal vesicle microsomes *in vitro*, with an I_{50} of 11.7 μM, being similar in potency to indomethacin, which had an I_{50} of 10.5 μM in our system. A comparison of the potency of tolmetin with other non-steroidal anti-inflammatory agents is shown in Table 1. Mefenamic acid, flufenamic acid, ketoprofen and diclofenac sodium were found to be from two to three times more potent than tolmetin and indomethacin in our assay system. Naproxen, fenoprofen, ibuprofen and phenylbutazone were significantly less potent. Aspirin was a very weak inhibitor, with an I_{50} of 23,200 μM. The reported PG synthetase inhibitor 2,7-dihydroxynaphthalene [20] was slightly more potent than tolmetin.

Table 1. Inhibition of prostaglandin E_2 synthesis by tolmetin and other compounds*

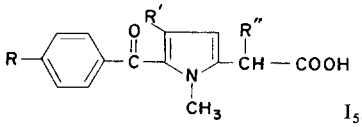
Compound	I_{50} (μM)	Relative potency
Tolmetin	11.7	100
Indomethacin	10.5	111
Mefenamic acid	4.0	292
Diclofenac sodium	4.6	254
Flufenamic acid	6.0	195
Ketoprofen	6.0	195
2,7-Dihydroxynaphthlene	7.6	154
Naproxen	32.0	37
Fenoprofen	62.0	19
Ibuprofen	120	10
Phenylbutazone	204	6
Aspirin	23,200	0.05

* Prostaglandin E_2 synthesis was measured as described in the text. I_{50} values were calculated from at least ten points on the linear portion (20–80 per cent inhibition) of a concentration curve, using the linear least-squares method. I_{50} = concentration (μM) producing 50 per cent inhibition.

The relative potencies of tolmetin and some of its structural analogs are shown in Table 2. A structure–activity analysis indicates that substitution of a *p*-chloro for a *p*-methyl of the benzoyl moiety decreased PG synthetase inhibition (i.e. I vs tolmetin, IV vs III). In one case, substitution of *p*-chloro with *p*-fluoro further decreased potency (i.e. VI vs V).

The importance of the *p*-position of the benzoyl moiety to inhibition of PGE_2 synthesis is suggested by the fact that substitution of a *p*-carboxyl group for the *p*-methyl (i.e. II vs tolmetin) markedly decreased potency by greater than 1000-fold. Interestingly, the *p*-carboxyl analog (II) has been found to be the major excreted metabolite of tolmetin in rats and man [21, 22].

Table 2. Inhibition of prostaglandin E_2 synthesis by tolmetin and other 5-benzoyl-1-methyl-pyrrole-2-acetic acids*



Compound	R	R'	R''	I_{50} (μM)	Relative potency
Tolmetin	CH_3	H	H	11.7	100
I	Cl	H	H	24.0	49
II	COOH	H	H	>10,000	<0.1
III	CH_3	CH_3	H	7.3	160
IV	Cl	CH_3	H	11.8	99
V	F	CH_3	H	26.0	45
VI	Cl	CH_3	CH_3	15.0	78

* Prostaglandin E_2 synthesis was measured as described in the text. I_{50} values were calculated from at least ten points on the linear portion (20–80 per cent inhibition) of a concentration curve, using the linear least-squares method.

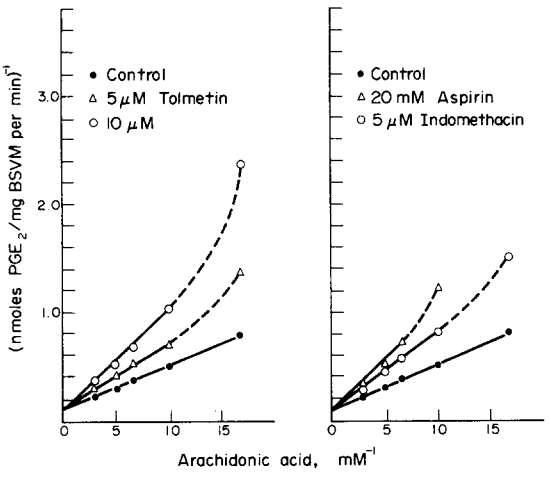


Fig. 1. Double-reciprocal plots of rate of PGE_2 synthesis vs arachidonate concentration. The incubation conditions were as described in the text.

The addition of a methyl group at position 4 on the pyrrole moiety increased potency (i.e. III vs tolmetin; IV vs I). The addition of an α-methyl group on the acetic acid moiety caused a slight decrease in activity (i.e. VI vs IV).

Effects of substrate concentration. The inhibition of PGE_2 synthesis by tolmetin appears to be competitive with substrate. Double-reciprocal plots of the rate of PGE_2 synthesis vs the concentration of arachidonic acid indicated competitive inhibition over the range of arachidonate concentrations from 0.06 to 0.33 mM (Fig. 1). At concentrations above 0.33 mM, the substrate inhibited PGE_2 synthesis, as has been observed by others [17, 19]. At arachidonate concentrations below 0.06 mM, the inhibition by tolmetin was found to be greater than that expected from normal Michaelis kinetics, and the double-reciprocal plot deviates from linearity. Indomethacin and aspirin also showed greater inhibition than the control at the low substrate concentrations (Fig. 1). This phenomenon has been observed by others with indomethacin [15, 23] and fenoprofen [15], and may be due to an effect of the inhibitors on the product-dependent activation reported to occur with vesicular gland fatty acid oxygenases [24].

Double-reciprocal plots of activity vs arachidonate concentration for aspirin and indomethacin (Fig. 1) indicated that these compounds were also competitive inhibitors in our system.

Reversibility of inhibition. To evaluate reversibility, both enzyme and inhibitor were pre-incubated at 37° in the absence of substrate for 5 min, and the mixture was then diluted with 4 vol. buffer and centrifuged at 80,000 *g* for 1 hr. The pellet was resuspended in a fresh incubation mixture with the substrate, and PGE_2 synthesis was measured. The results are shown in Table 3. The high concentration of tolmetin used (about twenty times the I_{50}), which completely inhibited PGE_2 synthesis after a 5-min pre-incubation (Table 3, Expt. A), showed no inhibition after the dilution–centrifugation step. Indeed, tolmetin appeared to protect the enzyme complex from the loss in activity due to the experimental procedure, as shown by the differences in activity between control A and control B. When this study was repeated using tolmetin[^{14}C], more than 99 per cent of the radioactivity was recovered in the supernatant after centrifugation. These results further indicate that tolmetin is a readily reversible inhibitor of PGE_2 synthesis. On the other hand, a concentration of 20 mM aspirin, which inhibited PGE_2 synthesis after pre-incubation, also inhibited it after centrifugation, indicating the irreversibility of this inhibitor. Similarly, indomethacin (10 μM) blocked synthesis after pre-incubation and also after centrifugation. Its inhibition after centrifugation was, however, considerably less, suggesting that indomethacin may be more reversible than aspirin.

Inhibitors of PG synthetase have been classified as reversible or irreversible on the basis of their effects on extended incubations [16]. The effects of an incubation time of 2 hr (at 37°) on the inhibition of PGE_2 synthesis by tolmetin concentrations of 10, 20 and 100 μM are shown in Fig. 2. Under our assay conditions, non-inhibited net PGE_2 synthesis tapered off after 60 min. In the presence of tolmetin at concentrations approximating one and two times the I_{50}, net synthesis approached that of the control. Even at 100 μM, a concentration about ten times the I_{50} of tolmetin, net PGE_2 synthesis continued to increase. Thus, tolmetin behaved as a reversible inhibitor.

In contrast, at approximate I_{50} concentrations, aspirin and indomethacin caused PGE_2 synthesis to taper off after 10 and 75 min respectively (Fig. 2). In both cases, the net synthesis was markedly depressed, indicating these compounds to be irrevers-

Table 3. Reversibility of inhibition of prostaglandin synthetase activity

	Control A	Control B	Tolmetin (200 μM)	Indomethacin (10 μM)	Aspirin (20 mM)
Expt. A*					
nmoles PGE_2 ± S. E.	56 ± 1		4 ± 1	8 ± 1	2 ± 1
% of control A	100		7	14	4
Expt. B†					
nmoles PGE_2 ± S. E.		33 ± 2	56 ± 2	22 ± 1	2 ± 1
% of Control B		100	170	67	6

* Experiment A: activity after pre-incubation of inhibitor for 5 min at 37°, then 5 min standard incubation after addition of arachidonate. Control A had no inhibitor.

† Experiment B: activity after pre-incubation of inhibitor for 5 min at 37°, diluted with 4 vol. buffer, centrifuged at 80,000 *g* for 1 hr, reconstitution of enzyme in standard incubation medium, and incubated for 5 min. Control B had no inhibitor.

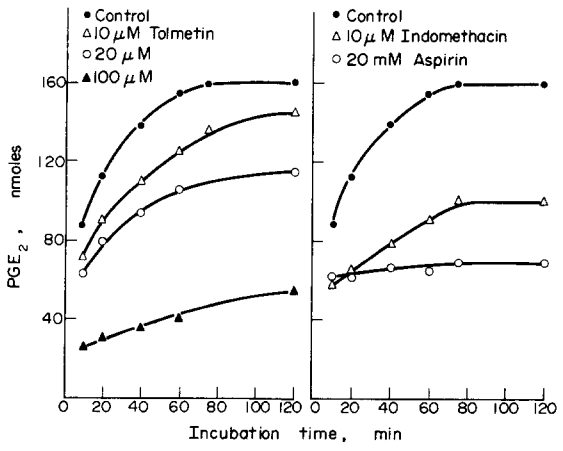


Fig. 2. Time-course of inhibition of PGE_2 synthesis by tolmetin and other compounds. The incubation conditions were described in the text.

ible inhibitors. Similar observations have been reported previously for indomethacin [25].

Effect of time-dependent inactivation. The ability of tolmetin to promote a time-dependent inactivation of PG synthetase was investigated by measuring the effects of various times of pre-incubation of tolmetin with the enzyme in the absence of arachidonate as suggested by Rome and Lands [26]. The reaction was then initiated by addition of arachidonate after either 5 or 10 min of pre-incubation of the inhibitor with the enzyme in the presence of cofactors and buffer at 37°, and incubating for the standard 5 min. Under these conditions, tolmetin (10 μM) had no significant effect after 5 or 10 min of pre-incubation, although it inhibited 33 per cent with no pre-incubation (Table 4). Indeed, tolmetin also appeared to protect the enzyme from the slight loss of activity which occurred with the control after 10 min of pre-incubation. Indomethacin and aspirin, however, both caused greater inhibition after 5 or 10 min of pre-incubation (Table 4) confirming their reported time-dependent inactivation of PG synthetase [16, 27].

Indeed, in an additional study we found that the I_{50} for indomethacin markedly decreased from 10.5 to 1.5 μM after 5 min of pre-incubation with the enzyme. Similarly, pre-incubation with aspirin caused a decrease in its I_{50} from 23,200 to 1700 μM. However, the I_{50} of tolmetin increased slightly from 11.7 to 35 μM after 5 min of pre-incubation with PG synthetase, further confirming its inability to promote inactivation.

Since tolmetin did not promote the inactivation of PG synthetase, its ability to protect the enzyme complex from indomethacin-induced inactivation was determined. After a 10-min pre-incubation with tolmetin, no significant difference in PGE_2 synthesis was observed (Table 5) compared to the pre-incubated control. The addition of indomethacin to the enzyme after 5 min of pre-incubation caused 90 per cent inhibition. However, when the indomethacin was added after 5 min of pre-incubation of the enzyme with tolmetin, and the pre-incubatton continued to 10 min, PGE_2 synthesis was inhibited less than 50 per cent. In this preliminary study, the apparent decrease in the indomethacin-induced inactivation by tolmetin suggests that tolmetin may compete with indomethacin for the site involved in the time-dependent inactivation of PG synthetase.

Table 4. Effect of pre-incubation time on the inhibition of PGE_2 synthesis by tolmetin, indomethacin and aspirin

Inhibitor	nmoles PGE_2* ± S. E. at min pre-incubation 0	5	10
None	65 ± 4	62 ± 5	51 ± 5
Tolmetin (10 μM)	44 ± 2†	54 ± 5	63 ± 8
Indomethacin (10 μM)	40 ± 1†	10 ± 1†	9 ± 1†
Aspirin (10 mM)	54 ± 3†	4 ± 1†	2 ± 1†

* The enzyme was pre-incubated with the inhibitor in the absence of arachidonate at 37° for the indicated time prior to the standard assay. Each value represents the mean of four determinations.

† Significant ($P < 0.05$) compared to no inhibitor at the same pre-incubation time.

Table 5. Protective effect of tolmetin on the time-dependent inactivation of PG synthetase by indomethacin

Inhibitor(s)	Time of pre-incubation prior to adding arachidonate (min)	nmoles PGE_2* ± S. E.	Per cent of control
None		59 ± 2	100
Tolmetin (20 M)	10	55 ± 2	93
Indomethacin (10 μM)	5	6 ± 1	10
Tolmetin (20 μM) plus	10		
Indomethacin (10 μM)	5	25 ± 1	42

* Activity after 10 minutes of pre-incubation as described, plus 5 min of standard incubation.

DISCUSSION

The results presented in this report indicate that tolmetin is a potent inhibitor of bovine seminal vesicle prostaglandin synthesis *in vitro*. In addition, several tolmetin analogs also inhibited PGE_2 synthesis from arachidonic acid (Table 2). These 5-benzoyl-1-methylpyrrol-2-acetic acids represent a new structural series of aryl acetic acids which both inhibit PG synthesis and possess anti-inflammatory activity, giving further support to the proposal of Vane [12] that PG synthesis inhibition may be the mechanism of action of anti-inflammatory agents.

Within this new series of compounds, slightly greater potency for inhibiting PGE_2 synthesis was seen when a methyl group was added to the pyrrole moiety (Table 2). In the anti-inflammatory tests, the potency also increased slightly with the methyl-pyrrole derivative [8, 9]. When the *p*-methyl group on the benzoyl moiety was substituted with chloride or flouride, inhibition of PG synthetase decreased, but potency in the anti-inflammatory tests was markedly increased [8, 9]. This lack of correlation between PG synthetase inhibition *in vitro* and the potency in the anti-inflammatory tests *in vivo* may be explained by the possible metabolic oxidation of the *p*-methyl to a *p*-carboxyl group. In fact, the *p*-carboxyl analog of tolmetin (compound II, Table 2) has been found to be the major urinary metabolite of the drug [22]. The presence of the *p*-chloro group would presumably block this metabolic oxidation. Although there appears to be a poor correlation between inhibition of PG synthesis and the potency in anti-inflammatory tests *in vivo* for many of the compounds in this series, these differences may be explained by differences in metabolism of the analogs, and therefore may still be consistent with Vane's hypothesis.

Kinetic studies indicated that tolmetin is a reversible, competitive inhibitor of bovine seminal vesicle PG synthetase. Like indomethacin and aspirin, tolmetin demonstrated competitive inhibition with respect to arachidonate (Fig. 1). Our studies with indomethacin confirm its competitive inhibition as reported by Vandenberg *et al.* [23] with bovine seminal vesicle microsomes and by Ku and Wasvary [25] with sheep seminal vesicles.

Unlike indomethacin and aspirin, tolmetin was found to be a reversible inhibitor of PG synthetase. On extended incubation, net PGE_2 synthesis was not greatly inhibited by tolmetin, but was markedly depressed by indomethacin and aspirin (Fig. 2). A further indication of the reversibility with tolmetin was seen by the complete restoration of activity and ready removal of the compound after centrifugation (Table 3). In fact, not only was enzyme activity restored to the control level, but tolmetin appeared to protect the enzyme against the slight loss of activity occurring as a result of the experimental conditions.

After pre-incubation with PG synthetase in the absence of arachidonate, tolmetin did not show greater inhibition of PGE_2 synthesis (Table 4); in fact its I_{50} was slightly increased. Indeed, again tolmetin appeared to protect the enzyme from the loss of activity resulting from the experimental pre-incubation conditions. In addition, tolmetin appeared to block the inactivation of PG synthetase induced by pre-incubation with indomethacin (Table 5).

These observations indicate that tolmetin does not promote a time-dependent inactivation of PG synthetase as do indomethacin and aspirin [16, 27], but competes with indomethacin for the substrate site or another site involved in the inactivation. Whether the observed protection by tolmetin of the loss of enzyme activity due to experimental conditions was due to protection of the same site has not been ascertained.

Some other compounds have recently been reported which inhibit PG synthetase in a manner similar to tolmetin. The anti-inflammatory agent pirprofen [25] and several anti-oxidants [28] have been reported to be competitive, reversible inhibitors which do not promote time-dependent inactivation of PG synthetase *in vitro*. It remains to be determined how these compounds differ from the competitive, irreversible inhibitors such as indomethacin in regard to the net synthesis and release of prostaglandins in tissues.

Some investigators have observed that I_{50} values for inhibitors of PG synthetase decrease at low substrate concentrations by a greater degree than would be expected by normal Michaelis kinetics for a competitive inhibitor [23]. We also made this observation for tolmetin, as well as aspirin and indomethacin (Fig. 1). This phenomenon may be due to the product-dependent activation reported to occur with fatty acid oxygenases [24]. At this time, however, the role of this observed activation to *in situ* PG synthesis is not clear. Indeed, an evaluation of the effects of non-steroidal anti-inflammatory agents on this activation step (i.e. using suboptimal substrate concentrations) might be relevant. The purpose of the present study, however, was to evaluate tolmetin as a PG synthetase inhibitor using classical Michaelis kinetics, as done in most other similar investigations [14, 16, 19, 20, 23, 25, 28, 29].

Based on the studies reported in this paper, and the increasing evidence that blockade of prostaglandin synthesis may be the mechanism of action of most non-steroidal anti-inflammatory agents, we conclude that the pharmacological and clinical effects of tolmetin may also be due in part to inhibition of prostaglandin synthetase.

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