Pharmacological Studies on Proglumetacin Maleate, a New Non-Steroidal Anti-Inflammatory Drug (4) Mode of Action on Anti-Inflammatory Activity

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Abstract—The possible mechanism of the anti-inflammatory activity of proglumetacin maleate (PGM), a new indomethacin (IND) derivative interacting with arachidonic acid (AA) metabolism, was investigated to elucidate the contributions of PGM itself and its two major metabolites, desproglumideproglumetacin maleate (DPP) and IND. PGM caused much less inhibition of PGE2 formation by sheep seminal vesicle microsomes (IC50=310 nM) and TXB2 formation by a washed rabbit platelet suspension (IC50=6.3 μM) than IND. DPP also caused less inhibition of cyclooxygenase than IND. Moreover, PGM had less effect on sodium arachidonate (SAA)-induced rat platelet aggregation ex vivo and AA-induced sudden death in rabbits than IND. These results show that PGM has anti-inflammatory activity after its conversion to the active metabolite IND. However, the inhibitory effects of PGM and DPP were as strong as that of IND on SAA- or collagen-induced rabbit platelet aggregation in vitro. These activities are considered to be associated with platelet membrane interaction. Moreover, unlike IND, PGM (IC50=1.5 nM) and DPP (IC50=16.3 μM) strongly inhibited 5-HETE formation by the cytosol of guinea pig polymorphonuclear leukocytes. This unique activity of PGM on 5-lipoxygenase may contribute to its anti-inflammatory activity.

Proglumetacin maleate (PGM), 3-[4-[2-(1-p-chlorobenzoyl-5-methoxy-2-methylindol-3-ylacetoxy)ethyl]-1-piperazinyl]propyl(±)-4-benzamido N, N-dipropylglutaramate dimaleate, is a new indomethacin (IND) derivative synthesized and developed by Rotta Research Laboratory.

The anti-inflammatory, analgesic and antipyretic activities of PGM were reported to be almost equal to those of IND on a molar basis in various experimental models (1, 2), while PGM caused much less damage of the gastrointestinal tract than IND (3). Pharmacokinetic studies showed that orally administered PGM was mainly converted to IND in rats (4); and in dogs, it was also converted to desproglumideproglumetacin, 2-[4-(3-hydroxypropyl)-1-piperazinyl]ethyl(1-p-chlorobenzoyl-5-methoxy-2-methyl)-3-indolyacetate, an intermediate from PGM to IND (5).

In this paper, we investigated the effects of PGM, desproglumideproglumetacin maleate (DPP) and IND on arachidonic acid (AA) metabolism to determine the contributions of these three drugs to the anti-inflammatory mechanism of PGM in vivo. Materials and Methods

Animals: Male Wistar rats were purchased from Tokushima Experimental Animal Laboratory; male Hartley strain guinea pigs were from Hitachi Animal Laboratory; male New Zealand White rabbits for preparation of platelets were from KEARI, and those for AA-induced sudden death were from Inoue Experimental Animal Center.

PGE2 formation by SSVM: The procedure

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used was based on the methods of Flower et al. (6) and Taylor and Salata (7). Lyophilized sheep seminal vesicle microsomes (SSVM, 0.5 mg) suspended in 1 ml of 50 mM Tris-HCl buffer (pH 8.3) containing 3 mM epinephrine and 3 mM reduced glutathione (GSH) were preincubated with test drugs at 37°C for 3 min in a shaking water bath. The reaction was started by adding 5 μl of [1-14C] AA (0.05 μCi, 5 μg) dissolved in ethanol as substrate. After incubation at 37°C for 5 min, the reaction was stopped by adding 20 μl of 2N-HCl. The reaction products were extracted twice with 3 ml of ethyl acetate with vigorous shaking and centrifugation, and the combined organic layers were evaporated under a stream of N2 gas. The residue was redissolved in 30 μl of chloroform/methanol (2:1, v/v), and 20 μl was applied to a TLC plate, and the TLC plate was developed with ethyl acetate/acetic acid (50:1, v/v). Radioactive zones were detected by autoradiography (ARG) with Sakura X-ray film, and the radioactivity in the zone corresponding to authentic prostaglandin (PG) E2 was determined in a liquid scintillation counter (Mark-III, Tracor Analytic).

TXB2 formation by platelets: The procedure used was based on the methods of Needleman et al. (8) and Vanderhoek et al. (9). Blood was withdrawn by cardiac puncture from rabbits weighing 3.2–4.8 kg into a plastic syringe containing 1/10 volume of 3.8% (w/v) trisodium citrate solution. Platelet-rich plasma (PRP) was obtained by centrifugation at 150Xg for 10 min at room temperature, and the resulting platelets were washed twice with 25 mM phosphate buffer (pH 7.4) containing 130 mM NaCl and 1 mM EDTA. Then they were resuspended in 50 mM phosphate buffer (pH 7.4) containing 1 mM EDTA and adjusted to approximately 5 x 10^8 cells/ml using an automatic cell counter (Celltac®: MEK-4500, Nihon Kohden Co.). Volumes of 1 ml of this washed platelet suspension (WPS) were preincubated with test drugs in the presence of 1 mM GSH at 37°C for 3 min, and then incubated with [1-14C] AA (0.1 μCi) at 37°C for 5 min. The reaction was terminated, and the mixture was extracted by addition of 3 ml of ethyl acetate/methanol/1 M citric acid (30:4:1, v/v). Radioactive metabolites from [1-14C] AA were separated by TLC in chloroform/methanol/acetic acid/H2O (90:8:1:0.8, v/v). The radioactive zone in the position of authentic thromboxane (TX) B2 was counted in a liquid scintillation counter.

Platelet aggregation in vitro: PRP was prepared from rabbits weighing 2.5–3.4 kg as described above. The residual precipitate of blood cells was centrifuged at 1,500 X g for 15 min to obtain platelet-poor plasma (PPP). The platelet count was adjusted to approximately 50 x 10^4 cells/μl by adding PPP. Platelet aggregation was measured by Born's turbidimetric method (10) in an aggregometer (Rikadenki Co.) at 37°C with stirring at 1,100 rpm. After preincubation with test drugs for 1 min, platelet aggregation was induced by adding the aggregating agent sodium arachidonate (SAA, 50 μg/ml) or collagen (10 μg/ml) to the PRP. Platelet aggregation was expressed as the maximum decrease in absorbancy and was compared with that of the control.

Platelet aggregation ex vivo: Rats weighing 240–320 g were used. Test drugs were administered orally 0.5, 1, 2, 4 or 8 hr before blood sampling. Blood was withdrawn into 3.13% (w/v) trisodium citrate solution (9:1, v/v) from the vena cava inferior of rats under ether anesthesia. PRP and PPP were prepared as described above. The platelet count was adjusted to approximately 110 x 10^4 cells/μl. Platelet aggregation induced by SAA at 80 μg/ml was measured optically, and the decrease in absorbancy was compared with that of control samples.

AA-induced sudden death: The methods by Silver et al. (11) and DiPasquale and Mellace (12) were used. Test drugs were administered orally to rabbits weighing 1.7–2.8 kg at 1 or 4 hr before injection of AA. AA dissolved in 100 mM Na2CO3 and diluted with saline was injected at a dose of 1.5 mg/0.5 ml/kg into the marginal ear vein at a rate of about 0.5 ml/10 sec. The rabbits were observed for sudden death, and their survival times were compared with that of the control group.

5-HETE formation by polymorphonuclear leukocytes (PMNL): The procedure used was based on the methods of Ochi et al. (13),
Jackschik and Lee (14) and Vanderhoek et al. (15). Guinea pigs weighing 580–1,030 g were treated i.p. with 2% casein in saline (5 ml/100 g body weight); and 16–18 hr later, they were treated with 50 ml of Dulbecco's phosphate-buffered saline (Ca²⁺- and Mg²⁺-free: PBS(−)) containing 5 units/ml of heparin. The peritoneal exudate was collected, filtered through 4 layers of cheese cloth, and centrifuged at 150 × g for 10 min. Cell pellets were suspended in a small volume of PBS(−) and treated with 3 volumes of 0.2% NaCl to lyse contaminating red blood cells. The cells (at least 80% PMNL as judged by Wright-Giemsa staining) were washed twice with 25 mM phosphate buffer (pH 7.4) containing 130 mM NaCl and 1 mM EDTA. The washed cells (1 × 10⁸/ml) were resuspended in 50 mM phosphate buffer (pH 7.4) containing 1 mM EDTA and 0.1% gelatin, sonicated in a Branson Sonifier® (Model 200) and centrifuged at 10,000 × g for 20 min. The supernatant solution was recentrifuged at 105,000 × g for 60 min to obtain the cytosol fraction. The cytosol fraction (500 μl) was preincubated with test drugs in the presence of 1 mM CaCl₂ and 1 mM GSH at 37°C for 3 min and then incubated with [1-14C] AA (0.1 μCi) at 37°C for 5 min. The reaction was terminated by precipitating the proteins with 1 ml acetone and adding 0.5 ml of ice-cold saline. The mixture was adjusted to about pH 3 with 150 μl of 2 N-formic acid and extracted twice with 2 ml of chloroform. The organic layer was evaporated, and the residue was applied quantitatively to TLC plates as described in the section of PGE₂ formation by SSVM and developed with petroleum ether/diethyl ether/acetone acid (50:50:1, v/v) for separation of 5-lipoxygenase metabolites. Radioactive zones on the plate were detected with a scanner (JTC-601, Aloka) or ARG. The radioactivity in the position corresponding to that of authentic 5-[^3]H]HETE was determined.

Test drugs and reagents: The chemical structures of PGM, DPP and IND are shown in Fig. 1. PGM was supplied by Rotta Research Laboratorium (Italy). DPP and benoxaprofen were synthesized in our laboratory. IND, caffeic acid and nordihydroguaiaretic acid (NDGA) were obtained from Sigma. [1-14C]AA (spec. act.: 56.0 mCi/mmol) and 5-[^3]H]HETE (5-D-[5, 6, 8, 9, 11, 12, 14, 15(n)-[^3]H]hydroxy-6, 8, 11, 14-eicosatetraenoic acid; spec. act.: 150.0 mCi/
mmol) were purchased from New England Nuclear; AA, SAA, PGE2, PGF2α, L-epinephrine bitartrate, GSH and adenosine diphosphate (ADP) were from Sigma; TXB2 was from Funakoshi Yakuhin Co.; collagen was from Hormon-Chemie; SSVM were from Ran Biochem.; casein and gelatin were from Difco; PBS(−) was from Nissui Seiyaku Co.; precoated thin-layer silica gel plates (Kieselgel 60F254; layer thickness 0.25 mm) were from E. Merck; ACS-II scintillation cocktail was from Amersham. All other reagents used were special grade commercial products.

Test drugs were suspended in 0.25% CM-Cellulose (Wako) for oral administration. For in vitro tests, they were dissolved in dimethylsulfoxide (DMSO), and the final concentration of DMSO was kept at 1%.

Statistical analysis: The values obtained were expressed as mean±standard errors. The concentrations of test drugs required for 50% inhibition (IC50 values) were calculated from the dose-response curves.

Results

Effect on PGE2 formation by SSVM: PGE2 was a major metabolite from AA under the present conditions. As shown in Fig. 2, formation of PGE2 was inhibited by PGM concentration-dependently at 30–1,000 μM and inhibited by DPP at 10–300 μM, but by IND at the lower concentration of 0.1–3 μM. The IC50 values of PGM, DPP and IND calculated from the dose-response curves were 310, 54.9 and 0.28 μM, respectively.

Effect on TXB2 formation by platelets: When WPS was incubated with AA, TXB2 and 12-hydroxyheptadecatrienoic acid (HHT) formed by the cyclooxygenase pathway and 12-hydroxyeicosatetraenoic acid (12-HETE) formed by the 12-lipoxygenase pathway were identified in the control. PGM at 1–100 μM decreased the formations of TXB2 and HHT concentration-dependently, with concomitant increase in 12-HETE to a maximum of about 6 times the control level (data not shown). DPP (1–100 μM) and IND (0.01–1 μM) had the same effects as PGM on the formation of these three metabolites. From the dose-response curves for TXB2 formation shown in Fig. 3, the IC50 values of PGM, DPP and IND were 6.3, 32.5 and 0.06 μM, respectively.

Effect on platelet aggregation in vitro: With PRP preparations preincubated in the absence of test drugs, the maximum decrease in absorbancy due to aggregations by SAA and collagen were 69.1% and 58.3%, respectively. PGM at 1–10 μM inhibited SAA-aggregations.

![Fig. 2. Effects of proglumetacin maleate (○), desproglumideproglumetacin maleate (△) and indomethacin (●) on PGE2 formation by SSVM. Points and bars are means±S.E. of 3 to 4 experiments.](image1)

![Fig. 3. Effects of proglumetacin maleate (○), desproglumideproglumetacin maleate (△) and indomethacin (●) on TXB2 formation by platelets. Points and bars are means±S.E. of 3 to 4 experiments.](image2)
induced aggregation concentration-dependently. IND was also inhibitory at the same concentrations as PGM. DPP was slightly less active than PGM and IND, and it caused complete inhibition at 30 μM. From the dose-response curves shown in Fig. 4A, the IC50 values of PGM, DPP and IND were 3.7, 8.3 and 5.4 μM, respectively. These three test drugs caused less inhibition of collagen-induced aggregation than of SAA-induced aggregation. From the dose-response curves shown in Fig. 4B, the IC50 values of PGM, DPP and IND were 9.0, 14.0 and 6.4 μM, respectively.

Fig. 4. Effects of proglumetacin maleate (○), desproglumideproglumetacin maleate (△) and indomethacin (●) on rabbit platelet aggregation induced by sodium arachidonate (A) or collagen (B) in vitro. Points and bars are means±S.E. of 3 to 4 experiments.

Fig. 5. Time courses of effects of proglumetacin maleate and indomethacin on rat platelet aggregation induced by sodium arachidonate ex vivo. Points are means of 3 experiments. ---○--- PGM, 0.3 mg/kg, p.o. ---●--- IND, 0.1 mg/kg, p.o. ---○--- PGM, 1 mg/kg, p.o. ---●--- IND, 0.3 mg/kg, p.o. ---○--- PGM, 3 mg/kg, p.o. ---●--- IND, 1 mg/kg, p.o.
Effect on platelet aggregation ex vivo: When platelet aggregation was induced by SAA, the absorbancy of the PRP preparation from control rats decreased to about 80% at each time. As shown in Fig. 5, PGM (0.3, 1, 3 mg/kg) caused similar dose-dependent inhibition of rat platelet aggregation to IND (0.1, 0.3, 1 mg/kg). The inhibitions by PGM at 0.3, 1 and 3 mg/kg rose gradually to peak values of 48.2%, 74.7% and 82.8% of the control, respectively, after 4 hr. With IND, except at the lowest dose, the inhibition was maximal in 2–4 hr.

Effect on AA-induced sudden death: Intra-venous AA at a dose of 1.5 mg/kg was enough to cause the death of all rabbits in the control group. Rabbits were in agony and died in 2–3 min after injection of AA. The protective effects of PGM and IND on this sudden death are summarized in Table 1. PGM (0.3, 0.9 mg/kg) had little effect in preventing death when given 1 hr previously, but was slightly effective when given 4 hr previously. On the other hand, IND (0.1, 0.3 mg/kg) was effective when given 1 or 4 hr previously. There was no difference in the survival times of control rabbits and rabbits treated with PGM or IND. All surviving rabbits were still alive the next day.

Effect on 5-HETE formation by PMNL: When the cytosol of PMNL was incubated with AA, 5-lipoxygenase metabolites were formed and detected on TLC plates. Figure 6 shows typical chromatograms in the absence (control) and presence of PGM. As shown in Fig. 6A, in the control, 5-hydroxyeicosatetraenoic acid (5-HETE) was identified as the main metabolite, while the minor metabolite at around the origin seemed to be 5,12-dihydroxyeicosatetraenoic acid (5,12-di-HETE) judging from its Rf value (15). PGM at 1 μM inhibited the formation of 5-HETE, causing almost complete inhibition at 3 μM (Fig. 6B and C). The dose-response curves of 5-HETE formation by PMNL are shown in Fig. 7. DPP inhibited 5-HETE formation.

Table 1. Preventive effects of proglumetacin maleate and indomethacin on arachidonic acid-induced sudden death in rabbits

| Drugs          | Dose (mg/kg, p.o.) | Mortality |
|----------------|--------------------|-----------|
|                | 1 hr pretreatment  | 4 hr pretreatment |
| Control        | vehicle            | 4/4        | 5/5        |
| Proglumetacin maleate | 0.3     | 4/4        | 3/4        |
|                | 0.9                | 3/4        | 2/4        |
| Indomethacin   | 0.1                | 4/5        | 3/5        |
|                | 0.3                | 1/4        | 1/4        |

Fig. 6. Thin layer chromatogram scan of the metabolites produced by incubation of [1-14C]AA with guinea pig polymorphonuclear leukocyte cytosol in the absence (A) or presence of proglumetacin maleate at 1 μM (B) or at 3 μM (C). I: AA, II: 5-HETE.
in the same concentration range as benoxaprofen (16) and caffeic acid (17) did. In contrast, IND was inhibitory at high concentrations, and the antioxidant NDGA (18) was markedly inhibitory at low concentrations. The IC50 values of PGM, DPP, IND, benoxaprofen, caffeic acid and NDGA were 1.5, 16.3, 237, 24.7, 19.4 and 0.02 µM, respectively.

Discussion

PGs and other metabolites of AA are thought to be important mediators of inflammation (19). Acidic non-steroidal anti-inflammatory drugs (NSAIDs) are thought to inhibit PG synthesis (20).

In the present study, we investigated the possible influences of PGM on AA metabolism in vitro, ex vivo and in vivo.

A high concentration of PGM was required to inhibit PGE2 formation by SSVM as a cell-free system: PGM and DPP were about 1/1,100 and 1/200 times as active as IND, respectively. Similarly, PGM and DPP were about 1/100 and 1/500 times as inhibitory as IND on TXB2 formation by platelets as an intact-cell assay system. Thus the inhibitory effects of PGM and DPP on cyclooxygenase were much weaker than that of IND. These results suggest that PGM shows anti-inflammatory activity after its conversion to the active metabolite IND. The weak effects of PGM in damaging the gastrointestinal tract (3) may be due to its weak inhibitory activity on PG biosynthesis in the gastrointestinal mucosa.

However, PGM inhibited platelet aggregations induced by SAA and collagen in the same concentration range as IND. Under these conditions, PGM was scarcely converted to IND in the PRP preparation (data not shown). Thus the inhibitory effect of PGM on platelet aggregation was caused by PGM itself, not its metabolite IND. IND is known to be a strong inhibitor of platelet aggregation concerned with AA metabolites, and it is known especially to inhibit TXA2 formation. Thus the effective concentration range for the inhibitions on preceding TXB2 formation and platelet aggregation are closely parallel in the case of IND but not of PGM (Figs. 3 and 4). So it is impossible to explain the effect of PGM on platelet aggregation only by its effect on AA metabolism. Like IND, even at 100 µM, PGM did not inhibit the aggregation by ADP (data not shown), so the possibility that PGM increased the cyclic AMP (cAMP) level in platelets can be excluded. Since PGM shows much higher affinity to rat and rabbit leukocytes in vitro
than IND (21). PGM may interact with platelet membranes and impair membrane-linked functions as chlorpromazine does (22). Since PGM has little effect on cyclooxygenase or on accumulation of cAMP, it may inhibit platelet aggregation at low concentrations because of such an interaction with platelet membranes and other reasons.

On the other hand, PGM was less inhibitory on both platelet aggregation ex vivo and AA-induced sudden death than IND. AA-induced sudden death reflects the blockade of the microcirculation of lungs by platelet aggregations in vivo (11). PGM could not express its potent anti-aggregating actions in vitro on these platelet aggregations ex vivo and AA-induced sudden death. These weaker activities ex vivo and in vivo are thought to result from the lower blood levels of IND formed from PGM than IND (23).

In addition, unlike common acidic NSAIDs containing IND, PGM strongly inhibited 5-lipoxygenase at low concentrations. DPP also inhibited 5-lipoxygenase as strongly as benoxaprofen (16) and caffeic acid (17), which are known 5-lipoxygenase inhibitors. We found previously that PGM reduced accumulation of leukocytes in carrageenin-induced pleurisy (1). This pharmacological effect may be partly due to its inhibitory effect on 5-lipoxygenase.

In conclusion, these studies show that PGM itself has little inhibitory effects on cyclooxygenase, while its metabolite, IND, causes cyclooxygenase inhibition, and they show that PGM itself inhibits platelet aggregation in vitro and particularly inhibits 5-lipoxygenase.

Further investigations on the in vivo effect of PGM in allergic inflammation are in progress.

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