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MECHANISM OF ACTION OF NOVEL ANTI-INFLAMMATORY DRUGS DIFLUMIDONE AND R-805*

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Abstract—Two novel nonsteroidal anti-inflammatory drugs (NSAID), R-805 (4-nitro-2-phenoxymethane sulfonanilide) and R-807 (3-benzoyldifluoromethane sulfonanilide, diflumidone), inhibit the biosynthesis of prostaglandin by bovine seminal vesicle microsomes and arachidonic acid-induced aggregation of human platelets in a concentration-dependent manner. Comparison of these sulfonanilides with NSAID indicates the following order of potency: (1) vs prostaglandin synthetase: indomethacin > flufenamic acid > R-807 > R-805 > phenylbutazone ≫ aspirin and (2) vs platelet aggregation: indomethacin > R-807 > R-805 > flufenamic acid > phenylbutazone ≫ aspirin. Time-dependent, irreversible inhibition of prostaglandin synthetase can be demonstrated for both R-805 and R-807. These compounds also inhibit equally the formation of PGE_2 and $PGF_{2\alpha}$, which suggests blockade of endoperoxide formation. This is the first report of inhibition of prostaglandin synthetase and platelet aggregation by drugs of this class.

Nonsteroidal anti-inflammatory drugs (NSAID) inhibit the synthesis of prostaglandins PGE_2 and $PGF_{2\alpha}$ from arachidonic acid [1]. It has been proposed that this property accounts for the activity of this class of drugs [2], and a correlation between anti-inflammatory activity *in vivo* and inhibition of prostaglandin synthesis *in vitro* has been demonstrated [3]. Formation of endoperoxide intermediates from arachidonic acid in human platelets induces their aggregation [4] and may trigger thrombus formation *in vivo* [5]. Several NSAID containing carboxylic acid functional groups (carboxyl-NSAID) are inhibitors of platelet aggregation induced by collagen or thrombin [6], and indomethacin, aspirin and salicylate have been reported to block platelet aggregation induced by arachidonic acid [7].

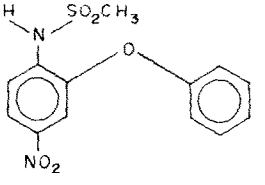
This paper describes the effects of two novel NSAID, which are acidic by virtue of a sulfonanilide rather than carboxyl group, on prostaglandin biosynthesis and arachidonic acid-induced platelet aggregation. The structures of 4-nitro-2-phenoxymethane sulfonanilide (R-805) and 3-benzoyldifluoromethane sulfonanilide (R-807, diflumidone) are shown in Fig. 1. Both compounds are anti-inflammatory in conventional animal models [8, 9], and R-805, the newest and most potent agent of its class, is currently undergoing clinical study.

MATERIAL AND METHODS

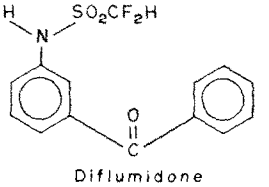
Chemicals. Arachidonic acid (grade 1, 99%) and *l*-epinephrine were purchased from Sigma Chemical Co. [^{3}H]arachidonic acid (6–10 Ci/m-mole) was purchased from New England Nuclear. GSH (glutathione) was obtained from CalBiochem, and Bio Sil A from Biorad Laboratories. Prostaglandins used in this study were purchased from Analabs, Inc. Anti-inflammatory drugs were gifts from the manufacturers, and R-805 and R-807 were synthesized in these laboratories.

Enzyme preparation. Bovine seminal vesicles were obtained from Pel Freeze Co. The microsomal fraction was prepared by the method of Flower *et al.* [10]. Microsomal pellets were suspended in 0.1 M Tris–HCl buffer, pH 8.2, and stored in small aliquots at −70°.

Prostaglandin synthetase assay. Synthesis of PGE_2 and $PGF_{2\alpha}$ from tritium-labeled arachidonic acid was measured by a modification of the assay developed by White and Glassman [11]. To glass conical centrifuge tubes, enzyme (10 μl, 15 mg/ml of protein) and cofactor solution (50 μl of a mixture of glutathione and *l*-epinephrine, 0.3 mg/ml each) were added, mixed and chilled on ice for 15 min. Just prior to incubation, 20 μl of test drug in 50% methanol was added and the reaction was initiated by the addition of 20 μl of substrate working solution. The substrate working



R-805: 4-nitro-2-phenoxymethane sulfonanilide



Diflumidone

R-807: 3-benzoyldifluoromethane sulfonanilide

Fig. 1. Structure of R-805 and R-807 (diflumidone).

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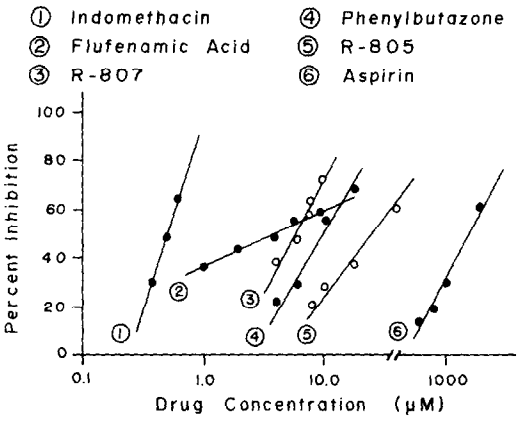

Fig. 2. Concentration-dependent inhibition of prostaglandin synthesis by R-805, R-807 and NSAID. Synthesis of PGE_2 and $PGF_{2\alpha}$ from arachidonic acid (3.0 μM) by bovine seminal vesicle microsomes (150 μg protein) was assayed radiochemically as described in Materials and Methods.

solution was prepared by the addition of 10 μl of 1.5 mM ethanolic [^{3}H]arachidonic acid (0.016 mCi/m-mole) and 10 μl of 1.9 mM Na_2CO_3 with 0.98 ml of 0.1 M potassium phosphate buffer, pH 7.4, which contained 3 mM $MgCl_2$. The solution was kept at 0° under nitrogen until it was added to the assay mixtures. The final concentration of substrate in the assay mixture was 3.0 μM for all experiments except determination of inhibitory constants where concentrations of 3.0 and 12.0 μM were employed. After mixing, the assay tubes were incubated at 37° for 10 min, during which time the reaction velocity was proportional to time of incubation. In control tubes, approximately 50 per cent of the substrate was converted to product during the 10-min incubation. There was no deleterious effect of methanol on the enzyme reaction at the final concentration (10%) employed. The reaction was terminated by quick freezing in dry ice–acetone. This procedure prevents formation of PGA_2 from PGE_2, which occurs when HCl is used as the terminating reagent. Blanks and controls contained all reactants except drug. Blanks were kept on ice during the incubation period.

Separation of products. A slurry of Bio Sil A in solvent A (hexane–dioxane–acetic acid, 70:30:1) was packed to a height of 4 cm in a sealed 9-in. micropipette. The tip was broken and several bed volumes of solvent A were flushed through each column. Solvent A (500 μl) was added to each frozen incubate. The incubates were thawed and immediately placed on the columns. Elution of unreacted substrate was accomplished by the addition of 5 ml of solvent A collected in liquid scintillation vials. The product prostaglandins were then eluted with 3 ml of solvent B (ethyl acetate–ethanol, 85:15) into fresh scintillation vials. Samples of unreacted substrate and products were collected from each tube and counted in a Packard model 3385 Tri-Carb liquid scintillation spectrometer.

Thin-layer chromatography. Duplicate samples of fractions eluted with solvents A and B were collected, taken to dryness under nitrogen and plated in Silica gel G with a thin-layer chromatography (t.l.c.) sample streaker (Applied Science). Plates were developed in a t.l.c. tank (lined with filter paper) to a height of 15 cm from the origin in benzene–dioxane–acetic acid (20:20:1). Each plate was scraped at increments of 0.5 cm from the origin to 4 cm, 0.25-cm increments from 5 to 9 cm, which was the area in which prostaglandin standards migrated, and in 0.5-cm increments from 9 to 15 cm from the origin. Each scraping was placed in a vial and suspended in 1.0 ml methanol, 10 ml aquasol was added and the suspension was counted. All scintillation counting data were captured on punched paper tape, processed and graphed with an H/P 9830 computer system.

Platelet aggregation. Human blood was obtained from volunteers and anti-coagulated with 0.1 vol. of 3.8% sodium citrate. Platelet-rich plasma was prepared by centrifugation of blood at 120 *g* for 15 min. Aggregation was measured by the method of Born and Cross [12] in a Chronolog aggregometer. Drugs used as inhibitors were initially dissolved in 0.05 M Tris buffered saline, pH 8.5, at 10^{-2} M and diluted to appropriate concentrations. Sodium arachidonate was prepared by the addition of 100 mM sodium carbonate to 33 mM arachidonic acid mixing under nigrogen to yield a 3.3 mM solution. A final concentration, usually 0.3 mM, was chosen to induce approximately 75 per cent of the maximal aggregation obtainable with sodium arachidonate.

RESULTS AND DISCUSSION

Inhibition of prostaglandin synthetase. Like the carboxylic acid-NSAID, the sulfonanilides R-805 and R-807 are potent inhibitors of bovine seminal vesicle

Table 1. Prostaglandin synthetase mode of inhibition and inhibitory constants

Drug	K_i(μM)	Mode of inhibition
Indomethacin	0.15	Competitive
Flufenamic Acid	0.30	Competitive
R-807	0.11	Competitive
Phenylbutazone	2.30	Competitive
R-805	3.00	Competitive
Aspirin	3.10	Competitive

Prostaglandin synthesis was measured as described in the text. K_i values were determined at substrate concentrations of 3.0 and 12.0 μM with varying inhibitor concentrations. Each K_i value represents the mean of two to four separate determinations.

Table 2. Comparison of *in vivo* and *in vitro* activity

Drug	Carrageenan bioassay* ED_{50} (mg/kg)	Prostaglandin synthetase inhibition IC_{50} (μM)†	Platelet aggregation inhibition IC_{50} (μM)‡
R-805	1.25	25.0	8.5
Indomethacin	2.95	0.5	0.7
Flufenamic Acid	14.7	4.0	30.0
Phenylbutazone	29.5	9.8	65.0
R-807	38.0	6.0	4.0
Aspirin	1.35	1700	>1000

* Reference [8].

† Drug concentration inhibiting 50 per cent of the conversion of arachidonic acid to PGE_2 and $PGF_{2\alpha}$.

‡ Drug concentration inhibiting 50 per cent of the platelet aggregation induced by 0.3 mM arachidonic acid in citrated platelet-rich plasma.

prostaglandin synthetase. Based on the concentration–inhibition curves illustrated in Fig. 2, the order of potency in this system is indomethacin > flufenamic acid > R-807 > phenylbutazone > R-805 ≫ aspirin.

Both sulfonanilides are competitive inhibitors of the enzyme (Table 1). Analysis of kinetic data by the method of Dixon [13] results in inhibitory constants (K_i) of 0.11 and 3.0 μM for R-807 and R-805 respectively. The affinity of R-807 for prostaglandin synthetase compares most closely to that found with indomethacin ($K_i = 0.15$ μM) and flufenamic acid ($K_i = 0.30$ μM), while R-805, with a K_i 30-fold higher than R-807, compares most favorably with phenylbutazone ($K_i = 2.3$ μM). Aspirin, previously reported to have low potency *in vitro* relative to other NSAID [3], also produced weak inhibition ($K_i = 310$ μM) in this study.

These results could be misleading as an indication of anti-inflammatory potential *in vivo*. Rome and Lands [14] have suggested that a time-dependent inhibitor may be more effective than would be predicted by its apparent K_i when this is determined after brief exposure to the enzyme *in vitro*. Indeed, in contrast to the carboxylic acid-NSAID tested in this study, the sulfonanilides R-805 and R-807 do not show a correlation between their inhibition of prostaglandin synthesis *in vitro* (IC_{50}) and anti-inflammatory potency as determined by the carrageenan-induced edema assay *in vivo* (ED_{50}, Table 2). In fact, R-805 *in vivo* is considerably more potent than R-807, whereas *in vitro*, the opposite is true. Assuming that inhibition of prostaglandin synthesis is in some way related to carrageenan-induced inflammation, this lack of correlation could be the result of differences in drug absorption, distribution or metabolism. Conversion of R-805 *in vivo* to a more active metabolite, for example, might account for the fact that it is the most potent drug examined in the carrageenan-induced edema test (Table 2). Such a metabolic conversion would not be anticipated *in vitro*. Sodium salicylate behaves in a similar manner in man [1, 15] and Willis *et al.* [16] have suggested active metabolite formation *in vivo* to account for this anomaly.

Relative synthesis of PGE_2 *and* $PFG_{2\alpha}$. Since E and F type prostaglandins often exert opposite effects *in vivo*, it was of interest to examine the effect of R-805 and R-807 on the relative synthesis of PGE_2 and $PFG_{2\alpha}$. At high concentrations ($IC_{50} = 1.3$ mM) benzydamine, also a non-carboxylic acid anti-inflammatory drug, selectively inhibits $PGF_{2\alpha}$ synthesis by bovine seminal vesicles but potentiates the synthesis of PGE_2 [10].

The results of t.l.c. of product fractions from incubates of control, R-805 and R-807 are shown in Fig. 3. Two peaks of radioactivity were found which corresponded to PGE_2 and $PGF_{2\alpha}$. R-805 and R-807 inhibit the formation of both products and only minor variations were noted in the ratio of PGE_2:$PGF_{2\alpha}$ in the presence of either drug. Thus, the sulfonanilides, like several carboxylic acid-NSAID, appear to block the formation of endoperoxide intermediate rather than inhibit subsequent steps leading to reduction of endoperoxide to $PGF_{2\alpha}$ or isomerization to PGE_2.

Time-dependent inhibition. The time that prostaglandin synthetase is exposed to NSAID can influence the calculation of relative potency. Indomethacin and aspirin are among several carboxylic acid-NSAID which promote irreversible destruction of the enzyme in a time-dependent manner [17]. When assayed at concentrations equal to their inhibitory constants, both R-805 and R-807 exhibit a similar mode of inhibition (Fig. 4). The rate of synthesis tapered off after 15–20 min of incubation and the total product synthesized was markedly depressed after 30 min. The results are again consistent with inhibition of cyclooxygenase activity. In contrast, phenylbutazone assayed under the same conditions does not produce time-dependent inhibition, i.e. the final yield of prostaglandins approached the control value after 30 min

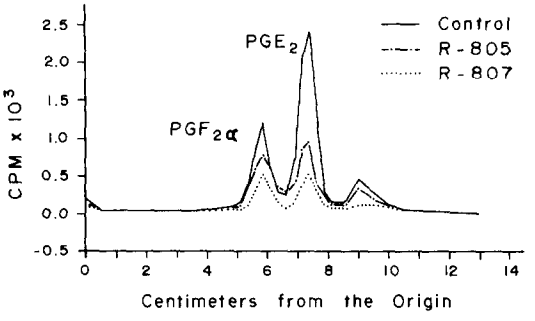

Fig. 3. Synthesis of PGE_2 and $PGF_{2\alpha}$: effect of R-805 and R-807. Formation of PGE_2 and $PGF_{2\alpha}$ was assessed by t.l.c. after separation of product and substrate fractions on Biosil A columns as described in Material and Methods.

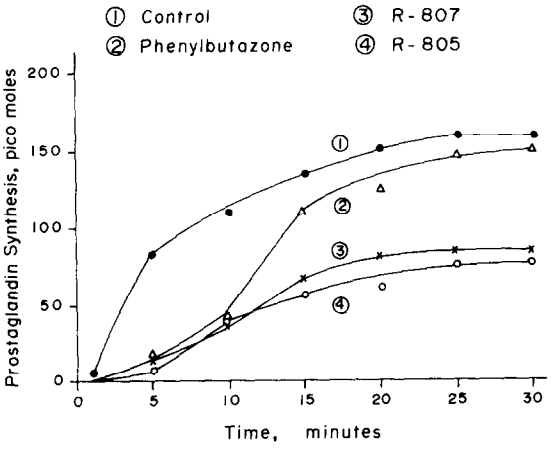

Fig. 4. Time-dependent inhibition of prostaglandin synthesis by R-805, R-807 and phenylbutazone. Formation of prostaglandins from [^{3}H]arachidonic acid (3.0 μM) was determined as described in Material and Methods. Microsome concentrations were adjusted so that approximately 50 per cent of the substrate was consumed in 30 min in control mixtures.

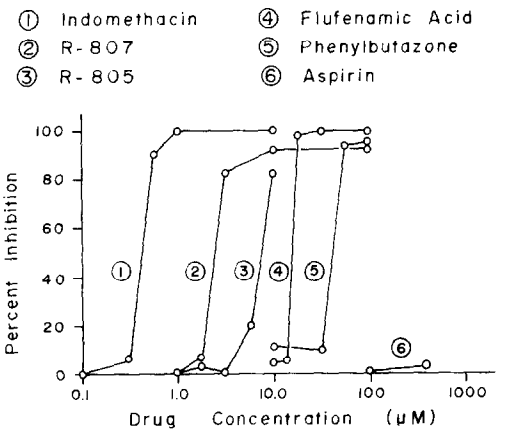

Fig. 5. Concentration-dependent inhibition of arachidonic acid-induced platelet aggregation by R-805, R-807 and NSAID.

except when the drug was present at several times its K_i.

Inhibition of platelet aggregation. Arachidonic acid can induce platelet aggregation both *in vitro* [7] and *in vivo* [18]; it was of interest to examine the effects of R-805, R-807, phenylbutazone and the carboxyl-NSAID on this process. With the exception of aspirin, human platelet aggregation induced by 0.3 mM arachidonic acid as described in Material and Methods, is inhibited in a concentration-dependent manner by all drugs which inhibited prostaglandin synthesis in this study (Fig. 5). The order of potency varies somewhat from that observed in the synthetase inhibition studies, most notably in that both sulfonanilides are more active inhibitors of aggregation than flufenamic acid.

Silver *et al.* (7) have reported arachidonic acid-induced platelet aggregation and platelet synthesis of PGE_2 and $PGF_{2\alpha}$ to be completely blocked by as little as 0.2 μM indomethacin. Much higher concentrations of aspirin, however, were required for complete inhibition. Similarly, our results indicate that indomethacin is completely inhibitory at 0.5 μM whereas aspirin was inactive at concentrations as high as 0.5 mM. The IC_{50} values, as determined graphically and shown in Table 2, indicate R-807 (4.0 μM) and R-805 (8.5 μM) do compare favorably as platelet aggregation inhibitors with phenylbutazone and the carboxyl-NSAID. However, their relative activities, as also found in synthetase inhibition studies, do not reflect their potency *in vivo*.

Evidence has accumulated that inhibition of prostaglandin synthesis is a major, but most likely not the only, mechanism of anti-inflammatory activity. We consider it likely that both R-805 and R-807 owe at least part of their demonstrated anti-inflammatory effects to this mechanism. Furthermore, in view of the well-documented involvement of platelet aggregation in the inhibition of arterial thrombosis [19] and the evidence that aspirin-like drugs may be effective anti-thrombogenic agents [20], further evaluation of R-805, R-807 and other sulfonanilides for this indication may be warranted.

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REFERENCES

1. J. R. Vane, *Nature New Biol.* **231**, 232 (1971).
2. S. H. Ferreira and J. R. Vane, *Seminaire Inserm Prostaglandins*, pp. 345–57. Editions Inserm, Paris (1973).
3. S. H. Ferreira and J. R. Vane, *A. Rev. Pharmac.* **14**, 57 (1974).
4. M. Hamberg, J. Svensson, T. Wakabayashi and B. Sammuelsson, *Proc. natn. Acad. Sci. U.S.A.* **71**(2), 345 (1974).
5. A. L. Willis, *Prostaglandins* **5**(1), 1 (1974).
6. M. J. Zucker and J. Peterson, *J. Lab. clin. Med.* **76**, 66 (1970).
7. M. J. Silver, J. B. Smith and J. J. Kocsis, *Prostaglandins* **4**(6), 863 (1973).
8. K. F. Swingle, R. R. Hamilton, J. K. Harrington and D. C. Kvam, *Archs int. Pharmacodyn. Ther.* **189**(1), 129 (1971).
9. T. J. Grant, G. G. I. Moore and K. F. Swingle, *Fedn Proc.* **34**, 759 (1975).
10. R. J. Flower, H. S. Cheung and D. W. Cushman, *Prostaglandins* **4**, 325 (1973).
11. H. L. White and T. Glassman, *Prostaglandins* **7**(2), 123 (1974).
12. G. V. R. Born and M. J. Cross, *Nature, Lond.* **197**, 974 (1963).
13. M. Dixon, *Biochem. J.* **55**, 170 (1953).
14. L. H. Rome and W. E. M. Lands, *Fedn Proc.* **34**, 790 (1975).
15. H. O. J. Collier, *Adv. Pharmac. Chemother.* **7**, 333 (1969).
16. A. L. Willis, P. Davison, P. W. Ramwell, W. E. Brocklehurst and B. Smith, in *Prostaglandins in Cellular Biology* (Eds. P. W. Ramwell and B. B. Pharris), pp. 227–59. Plenum, New York (1972).

17. W. E. M. Lands, P. R. LeTellier, L. H. Rome and J. Y. Vanderhoek, in *Advances in the Biosciences* (Eds. S. Bergstrom and S. Bernhard), Vol. 9, pp. 15–28. International Conference on Prostaglandins, Vienna, Pergamon Press, Vieweg, Braunschweig (1973).
18. M. J. Silver, W. Hoch, J. J. Kocsis, C. Ingerman and J. B. Smith, *Science, N.Y.* **183,** 1085 (1974).
19. J. F. Mustard, *Hospital Pract.* **7**(1), 115 (1972).
20. H. J. Weiss, in *Progress in Hemostasis and Thrombosis* (Ed. T. H. Spaet), pp. 199–232. Grune & Stratton, New York (1972).