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THE LONG DURATION, *IN VIVO*, INHIBITION OF PROSTAGLANDIN SYNTHETASE BY 2-METHYL-8-*CIS*-12-*TRANS*-14-*CIS*-EICOSATRIENOIC ACID

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Abstract—Competitive inhibition of prostaglandin synthetase by 8-*cis*-12-*trans*-14-*cis*-eicosatrienoic acid has been reported to occur *in vitro*. No *in vivo* effects were observed, possibly due to rapid metabolic degradation of this fatty acid by β-oxidation. The present study involved the evaluation of this compound *in vivo*, and the preparation and evaluation *in vivo* of its α and β methyl-substituted analogs which retain the carbon skeleton of the parent compound, and which might be expected to be resistant to β-oxidation. Using a newly developed radioimmunoassay for the total urinary metabolites of prostaglandin E, data were obtained that indicates that both the parent compound and its 2-methyl analog are prostaglandin synthetase inhibitors *in vivo*. The 2-methyl analog exhibited an unusually long duration of activity as compared to both indomethacin and the parent compound. The lengthened duration of action of the 2-methyl analog may be explained both by its possible resistance to β-oxidation, and to possible alteration in rates of either fatty acid transport, or incorporation/release from triglycerides (acylation/deacylation of triglycerides).

The inhibition of prostaglandin biosynthesis has been reported for a variety of substances [1]. Included among these are structural analogs of essential C-20 fatty acids which normally serve as substrates for the enzyme fatty acid cyclo-oxygenase (EC 1.14.99) (prostaglandin synthetase, PG-synthetase). Certain substrate analogs have been found to be potent competitive inhibitors *in vitro*, particularly 8-*cis*-12-*trans*-14-*cis*-eicosatrienoic acid, compound 1 [2]. The competitive inhibition exhibited by this compound may be due to its structural similarity to 11-hydroperoxy-8-*cis*-12-*trans*-14-*cis*-eicosatrienoic acid, compound 5, which is the first intermediate in the conversion of di-homo-γ-linolenic acid to prostaglandin E_1 (PGE_1) and $PGF_{1\alpha}$ [3, 4]. Although compound 1 was reported to have *in vitro* activity, no *in vivo* activity was reported. Possibly, this lack of *in vivo* activity might have been due to rapid metabolic degradation of the fatty acid by β-oxidation.

The investigations described in this paper involved the preparation, and the *in vitro* and *in vivo* evaluation of effects on prostaglandin synthesis of methyl-substituted analogs of compound 1, which might be expected to be resistant to β-oxidation while retaining the basic C-20 carbon skeleton of the parent compound, i.e. C-20 straight chain fatty acids containing the 8-*cis*-12-*trans*-14-*cis* triene system. For this purpose, we synthesized and tested the 2-methyl, the 2,2-dimethyl, and the 3-methyl eicosatrienoic analogs, compounds 2, 3, and 4, respectively.

MATERIALS AND METHODS

The α-substituted acids (Fig. 1), compounds 2 and 3, were prepared from compound 1§ by first converting compound 1 to its methyl ester, followed by α-alkylation of the ester with lithium *N*-isopropylcyclohexylamide and methyl iodide [5]. To prepare compound 3, this alkylation procedure was repeated a second time to give the α-α-dimethyl methyl ester. The methyl esters were then converted to the corresponding acids by using lithium iodide-collidine [6].

Compound 4, which is the 3-methyl eicosatrienoic acid, was prepared from 7-bromo-3-methyl-heptanoic acid [7], using the procedures described for the total synthesis of compound 1 [8]. All of the fatty acids thus prepared had NMR signals in the 5.0 δ and 6.7 δ region, which were identical to the NMR patterns observed for compound 1 in this same region, indicating that the requisite 8-*cis*-12-*trans*-14-*cis*-triene system was present in compounds 2, 3, and 4.‖

These fatty acids were then tested for *in vitro* inhibition of fatty acid cyclo-oxygenase prepared from ovine seminal vesicles per the protocol of Table

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§ A sample of compound 1 was provided by Dr. D. A. van Dorp, Unilever Laboratories, Vlarrdingen, The Netherlands.

‖ In addition to possessing the correct NMR characteristics, compounds 2, 3, and 4 gave correct elemental analysis (within ± 0.4%), and displayed homogeneity when examined by thin-layer chromatography on silica G in two different solvent systems: ethyl acetate–hexane–acetic acid [20:77:3]; ethyl acetate–water–2,2,4-trimethylpentane–acetic acid [11:10:6:4] upper phase. One hundred microliters of a 1 mg/ml toluene solutions of the compounds was spotted. It was visualized with UV-254 millimicrons.

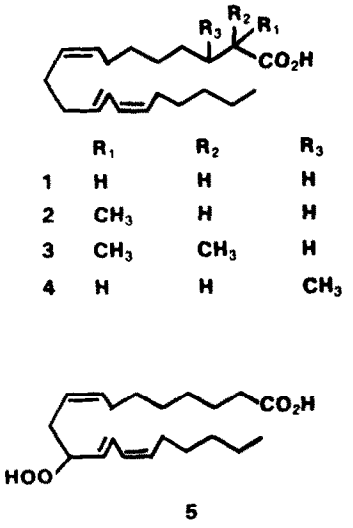

Fig. 1. Structures of eicosatrienoic acids (1–4) and prostaglandin intermediate (5).

1. Comparisons were made simultaneously with the known prostaglandin synthetase inhibitor indomethacin (Merck & Co.). Because of the low solubility of these compounds in water, stock solutions of the test compounds were prepared with dimethyl sulfoxide (DMSO) and diluted prior to use. Appropriate concentrations of DMSO were added to control solutions. The results, calculated from serial dilution studies, are presented as I_{50} values (μM concentration for 50% inhibition).

Single dose, long-term, *in vivo* determinations of prostaglandin synthetase inhibition were made using the newly described procedure of Fretland and Cammarata [9], which measures the mixed urinary metabolites of prostaglandins E_1 and E_2 by radioimmunoassay. Briefly, male Sprague–Dawley rats (six rats/compound), 180–250 g, were placed in individual metabolism cages, and 24-hr urine specimens were collected, volumes measured, and the urine samples stored frozen. Frozen samples were stable indefinitely. Control samples were collected for 3 days prior to drug treatment so that each animal could serve as its own control, as well as provide a basal excretion rate of metabolites.

Table 1. *In vitro* inhibition of PG-synthetase*

Compound	I_{50} (μM)
1	150
2	150
3	3000
4	>5000
Indomethacin	5

* Frozen ovine seminal vesicles were homogenized in 0.1 M potassium phosphate buffer, pH 8, containing 32 mM glutathione, 1 mM cysteine, and 0.1 mM hydroquinone. The supernatant fraction from a 10,000 *g* centrifugation step was used as the enzyme source with the addition of 2 mM isoproterenol as a stimulator. The PGE_2 produced from arachidonate (2.5 mM) was measured spectrophotometrically after alkali conversion to PGB_2 [10].

Between days 3 and 4, a single dose of the test compounds was injected subcutaneously, suspended in iso-osmotic, phosphate-buffered saline, pH 7.4. Phosphate-buffered saline was injected into concomitant control animals, and these animals were used for tests of significant difference of means using 'Student's' *t*-test. Twenty-four-hour urine samples were collected for 7 days after compound administration.

Quantitative dose–response studies, using compound 2, were conducted identically, except that four rats were used per dose of compound 2. Instead of absolute excretion values, $\Delta\%$ values for each rat, serving as its own control, were calculated [$\Delta\%$ = (test-control/control) × 100]. This was done in order to take into account the large range of basal excretion values of different groups of rats (see Fig. 3). For linear regression analysis, the means of $\Delta\%$ of four rats per dose, for seven doses, for the entire 7 days were calculated. Since these averages included the 2-day lag period in which metabolite excretion was not altered, they were the most conservative values we could use to test significance. Linear regression analysis using the 3 peak days for averaging as well as single days 6, 7, 8, 9 and 10 (data not shown) exhibited larger values of $\Delta\%$, but the correlation coefficients and slope values were nearly identical, and all regressions were significant.

RESULTS

The results of *in vitro* tests of cyclo-oxygenase inhibition are presented in Table 1. While the 2-methyl eicosatrienoic acid anlog was as active as the parent fatty acid, the 2,2-dimethyl analog was far weaker than the parent compound, and the 3-methyl analog was essentially inactive. Both compounds 1 and 2, *in vitro*, were less active than indomethacin, but this conclusion must be tempered by insolubility problems, as discussed later.

Figures 2 and 3 present the results of single dose, long term, *in vivo* inhibition studies, using indomethacin, PGE_1, trienoic acids and saline. Prostaglandin E_1 was administered as a positive control. As expected, prostaglandin E_1 caused a large increase in excretion of metabolites, immediately post-injection, which quickly returned to baseline, while saline-injected animals demonstrated an essentially constant excretory rate during the 7-day test period. Again, as expected, the known prostaglandin synthetase inhibitor, indomethacin, caused a decrease in metabolite excretory rate. Indomethacin (10 mg/kg) decreased the secretory rate to 60 ng/24 hr on day 6, which returned to baseline on day 7. A possible rebound increase was observed on days 8 and 9.

Long term, single dose, *in vivo* inhibitory activity of the trienoic acids seemed to correlate, roughly, with *in vitro* activity. Both compound 1 (200 mg/kg) and compound 2 (180 mg/kg) significantly depressed the excretory rate, and both, at much higher doses, seemed to be more active than indomethacin. Compound 3, which was only weakly active *in vitro*, and compound 4, which was inactive *in vitro*, were both inactive *in vivo*.

It is of special interest that the onset and duration of inhibition produced by compound 2 differed sig-

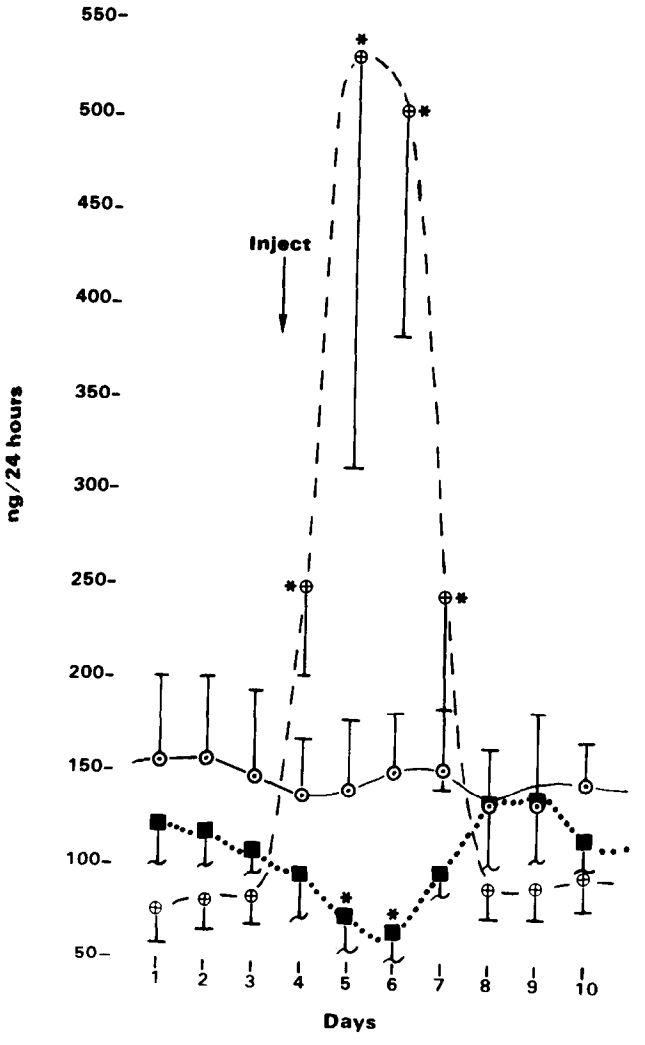

Fig. 2. Metabolite exeration rates due to control substances; single dose, *in vivo*, inhibition of prostaglandin synthetase. Control substances: (⊙——⊙) 0.9% NaCl, 0.25 cc; (⊕– –⊕) prostaglandin E_1, 3 mg/kg; and (■ · · · ■) indomethacin, 10 mg/kg. Each point is the mean of six rat determination. Key: (*) $P < 0.05$; ⊥ = S.E.M.

nificantly from that of compound 1, and from indomethacin. Although only one set of data are shown in Fig. 3, identical patterns were obtained in several other repeat studies. Compound 1 had a rapid onset of action which reached a minimum value (30 ng/24 hr) at day 6, followed by a gradual return to baseline (95 ng/24 hr). Indomethacin behaved similarly. Compound 2, on the other hand, did not produce a significant change in excretory rate until day 3 after compound administration. The depressed excretory rate of compound 2 did not return to baseline by day 10, at which time the experiment was terminated.

Multiple dose–response results *in vivo*, using only compound 2, are presented in Fig. 4. Using four rats per dose, it is evident, from the linear regression analysis results, that a linear dose–response curve is obtained in the range from 7 mg/kg to 175 mg/kg. All five doses produced responses which lie well inside, or on, the 95% confidence limit belt. The correlation coefficient ($r = 0.98$) and the slope ($b = -0.311$) were both highly significant. Overall regression was also significant ($P < 5 \times 10^{-5}$).

DISCUSSION

These studies indicate that both 8-*cis*-12-*trans*-14-*cis*-eicosatrienoic acid, compound 1, and 2-methyl-8-*cis*-14-*trans*-eicosatrienoic acid, compound 2, are prostaglandin synthetase inhibitors both *in vitro* and *in vivo*. Compound 1 resembled indomethacin in that it had a rapid onset on inhibited of excretion, which returned to baseline by day 10. Compound 2, on the other hand, did not demonstrate inhibitory activity until 2 days after injection, and it did not return to baseline by day 10. Presumably, this prolonged duration of action was due to the α-methyl substitution, which might be expected to block β-oxidation and degradation. The delayed onset of activity, as a result of α-methyl substitution, con-

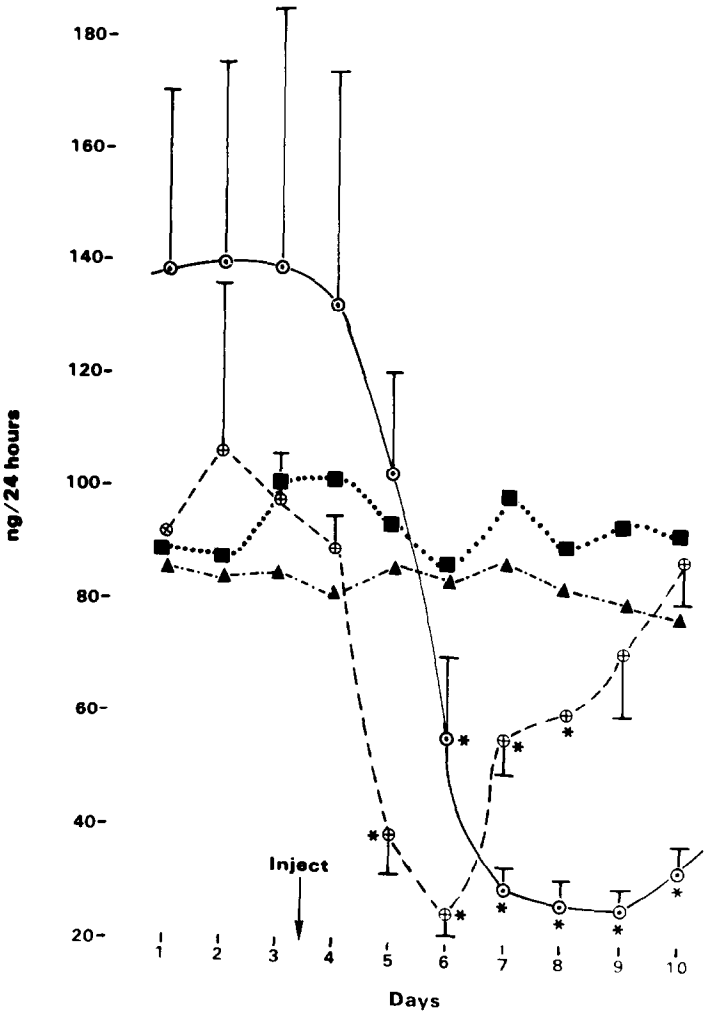

Fig. 3. Metabolite exeration rates due to test substances. Test substances: (⊕--⊕) compound 1,200 mg/kg; (⊙——⊙) compound 2, 180 mg/kg: (■·····■), compound 3, 190 mg/kg; and (▲·—·—·▲) compound 4, 200 mg/kg. Each point is the mean of six rat determinations. Key (*): $P < 0.05$; ⊥ = S.E.M.

ceivably could also be due to alteration of fatty acid transport and incorporation into triglycerides, or release from triglycerides.

The *in vivo* inhibition produced by the fatty acid analogs roughly correlated with the *in vitro* results, but this was not true of indomethacin. This anomaly in correlation could be partially a reflection of the insolubility of these compounds, and the necessity to use in these studies either suspensions, or DMSO as solvent, which would obscure the availability of these compounds in the *in vitro*, as well as in the *in vivo* studies.

These same insolubility problems make valid comparisons of potency values, both *in vivo* and *in vitro*, somewhat questionable. Subcutaneous injection of insoluble suspensions would be expected to alter the kinetics of distribution of these compounds, as compared to results using DMSO as solvent, in a manner not necessarily related to their intrinsic potencies. However, taken at face value, *in vitro* compound 2 appears to be only 1/30 as active as indomethacin. From the dose–response results, *in vivo*, compound 2 appears to be roughly as potent as indomethacin, even if one does not take into consideration the long duration of activity. Since four to six animals were averaged to determined each point, and each point showed significant differences from concomitant control animals as well as from the values in which each animal was used as its own control, the long duration of activity and the dose–response results are real, and they require further study to resolve these somewhat anomalous results.

These results are the first demonstration that structural analogs of an intermediate in prostaglandin biosynthesis, which *in vitro* act as competitive inhibitors of prostaglandin synthesis, can act *in vivo* as inhibitors of prostaglandin synthesis. It remains for future studies to determine if they possess other significant, *in vivo*, biological activities. In particular, the uncertainties with regard to relative potency values, and the use of a single species in these preliminary studies, dictate the view that these com-

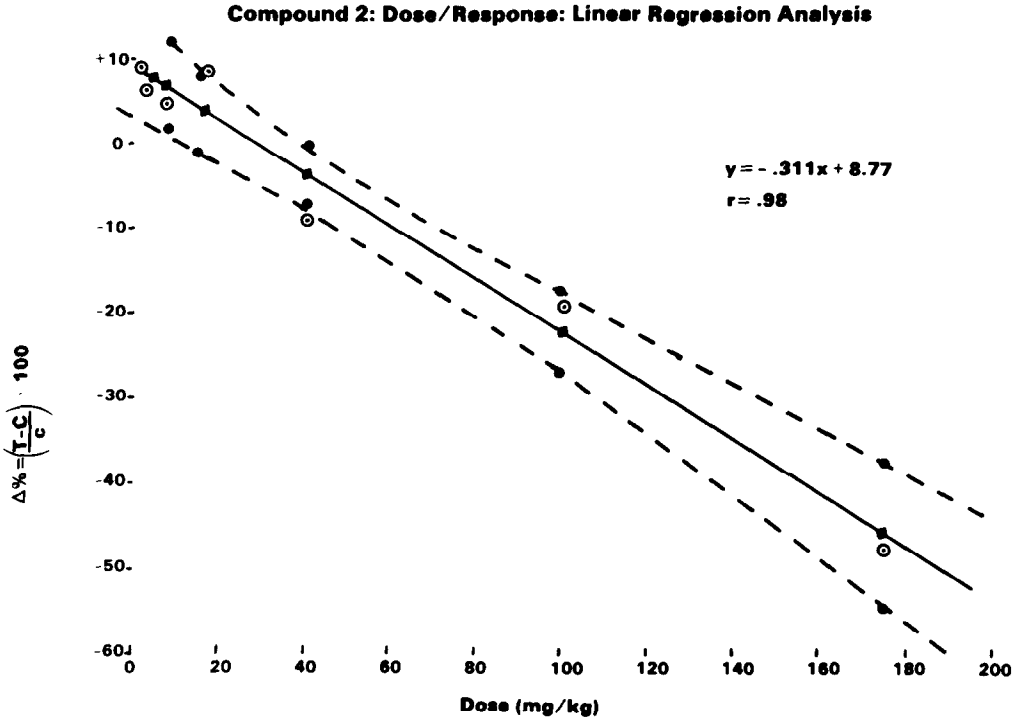

Fig. 4. Dose–response curve for compound 2. Doses were 175, 100, 40, 16, 7, 2.8 and 0.9 (mg/kg). Key: (⊙) experimental, (◆——◆) predicted regression line, and (●– –●) 95% confidence belt. Significance of slope ($P < 0.001$). Significance of intercept ($P < 0.01$). Significance of overall regression ($P < 5 \times 10^{-5}$). $\Delta\%$ = percent change of U-PGE-M from control.

pounds must be regarded as prototype compounds, with possible clinical utility unpredictable at this time. Extensive studies, under uniform solubility conditions, with known concentrations, will be required to resolve these uncertainties.

In conclusion, using a newly developed assay for the mixed urinary metabolites of PGE_2, data were obtained that indicate that both 8-*cis*-12-*trans*-14-*cis*-eicosatrienoic acid and its 2-methyl analog, are prostaglandin synthetase inhibitors *in vivo*. The 2-methyl analog exhibited an unusually long duration of activity, as compared to indomethacin. The lengthened duration of action of the 2-methyl analog is attributed to both its possible resistance to β-oxidation, and to possible alteration of either fatty acid transport, or incorporation/release from triglycerides.

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