Department of Biochemical Toxicology
Chemical Industry Institute of Toxicology
Research Triangle Park NC 27709, U.S.A.

JOHN G. DENT*
STEPHANIE R. SCHNELL

* All correspondence should be addressed to: Dr. John G. Dent, Department of Biochemical Toxicology, Chemical Industry Institute of Toxicology, P.O. Box 12137, Research Triangle Park, NC 27709, U.S.A.

REFERENCES

1. F. Oesch, *Xenobiotica* **3**, 305 (1973).
2. K. C. Leibman and E. Ortiz, *J. Pharmac. exp. Ther.* **173**, 242 (1970).
3. E. W. Maynert, R. L. Foreman and T. Watabe, *J. biol. Chem.* **245**, 5234 (1970).
4. F. Oesch, D. M. Jerina and J. Daly, *Biochim. biophys. Acta* **227**, 685 (1971).
5. D. R. Thakker, H. Yagi, W. Levin, A. Y. H. Lu, A. H. Conney and D. M. Jerina, *J. biol. Chem.* **252**, 6328 (1977).
6. D. M. Jerina, P. M. Dansette, A. Y. H. Lu and W. Levin, *Molec. Pharmac.* **13**, 342 (1977).
7. P. M. Dansette, V. B. Makedonska and D. M. Jerina, *Archs Biochem. Biophys.* **187**, 290 (1978).
8. A. Y. H. Lu, D. M. Jerina and W. Levin, *J. biol. Chem.* **252**, 3715 (1977).
9. F. Oesch, V. Kaubisch, D. M. Jerina and J. W. Daly, *Biochemistry* **10**, 4858 (1971).
10. T. Watabe and K. Akamatusu, *Chem. pharm. Bull. Tokyo* **22**, 2155 (1974).
11. J. G. Dent, K. J. Netter and J. E. Gibson, *Toxic. appl. Pharmac.* **38**, 237 (1976).
12. A. Y. H. Lu, D. Ryan, D. M. Jerina, J. W. Daly and W. Levin, *J. biol. Chem.* **250**, 8283 (1975).
13. H. U. Schmassman, H. R. Glatt and F. Oesch, *Analyt. Biochem.* **74**, 94 (1976).
14. W. Levin, P. E. Thomas, D. Korziniowski, H. Seifried, D. M. Jerina and A. Y. H. Lu, *Molec. Pharmac.* **14**, 1107 (1978).
15. F. P. Guengerich, P. Wang, M. B. Mitchell and P. S. Mason, *J. biol. Chem.* **254**, 12248 (1979).
16. F. P. Guengerich, P. Wang, P. S. Mason and M. B. Mitchell, *J. biol. Chem.* **254**, 12255 (1979).
17. G. C. DuBois, E. Appella, W. Levin, A. Y. H. Lu and D. M. Jerina, *J. biol. Chem.* **253**, 2932 (1978).
18. L. Ehrenberg, S. Osterman-Golkar, D. Segerback, K. Svensson and C. J. Calleman, *Mutation Res.* **45**, 175 (1977).

Biochemical Pharmacology, Vol. 30, No. 12, pp. 1714–1716, 1981.
Printed in Great Britain.

0006-2952/81/121714-03 $02.00/0

Effect of nonsteroidal anti-inflammatory drugs on sublethal retinoic acid toxicity in Swiss mice

(*Received* 8 *September* 1980; *accepted* 19 *December* 1980)

Administration of excessive amounts of retinol (vitamin A) and of certain derivatives and synthetic analogs (retinoids) produces a characteristic set of symptoms, including headache, vomiting, pruritus, skeletal pain, and bone resorption in experimental animals [1, 2] and man [3–6]. In animals, administration of retinyl esters [2] or retinoic acid (vitamin A acid) [7] at sufficiently high doses can result in lethality. The symptoms of retinoid toxicity are similar to reported physiologic effects of prostaglandins [8–11] so it seemed reasonable to us that retinoid toxicity may be mediated in some way by prostaglandins. If this were the case, co-administration of an inhibitor of prostaglandin synthesis might interfere with the toxicity resulting from retinoid administration. We have demonstrated that concurrent administration of aspirin, an inhibitor of prostaglandin synthesis [8, 12], protects mice against the toxicity resulting from administration of retinoic acid at the LD_{50} [13] or LD_{10} [14]. This study was designed to compare the abilities of nonsteroidal anti-inflammatory (NSAI) drugs of known potency as inhibitors of prostaglandin synthetase to protect mice against retinoic acid toxicity.

Retinoids have been recommended for use in man as cancer chemopreventive agents, that is, to arrest or reverse the development of cancer [15]. As such, retinoids would be used for long periods in individuals who are generally healthy but who have a greater than average risk of developing cancer [15,16]. Because clinical use of retinoids is anticipated, studies that examine interference with the effects of lethal doses of retinoid are less practical than those concerned with sublethal doses of these drugs. We have shown that retinoid-induced bone fractures in mice are dose-related and that they reliably indicate the comparative toxicity of retinoids at lethal and sublethal doses [7]. Consequently, we have chosen the occurrence of bone fractures as a useful end point for sublethal retinoid toxicity.

NSAI drugs that are more potent inhibitors than aspirin of prostaglandin synthesis *in vitro* [17] are currently available. In addition to these, ascorbic acid decreases the weight loss and lethality resulting from excess vitamin A [18] and exhibits weak anti-inflammatory properties [19]. Moreover, ascorbic acid inhibits prostaglandin synthestase *in vitro* [20, 21]. We report here the effect of concurrent administration of NSAI drugs or ascorbic acid on bone fractures occurring in mice treated with a sublethal dose of retinoic acid.

Methods

Materials. All-*trans*-retinoic acid (RA) was suspended in an aqueous solution of 8% Cremophor EL (Sigma Chemical Co., St. Louis, MO) and 10% propylene glycol; these

Table 1. Effects of retinoic acid alone and in combination with NSAI drugs in Swiss mice

NSAI drug	NSAI drug dose (μmoles/kg) Orally	Group*	Number of fractures per survivor 0	1	2+
Aspirin	833	RA alone	7/39	14/39	18/39
		Combination	18/37†	14/37	5/37
Ibuprofen	436	RA alone	16/39	10/39	13/39
		Combination	26/39†	8/39	1/39
Calcium ascorbate	128	RA alone	11/39	16/39	12/39
		Combination	24/38†	9/38	5/38
Indomethacin	1.1	RA alone	21/39	14/39	4/39
		Combination	36/40‡	4/40	0/40
m-Hydroxybenzoic acid	833	RA alone	9/20	5/20	6/20
		Combination	10/20	6/20	4/20

* Groups of twenty Swiss mice were treated as described in the text with RA (14 mg/kg) alone or in combination with the NSAI drug at the dose indicated.

† The number of animals with no fractures was significantly higher in the group receiving the combination compared to the concurrent group receiving RA only ($P < 0.01$).

‡ The number of animals with no fractures was significantly higher in the group receiving the combination compared to the concurrent group receiving RA only ($P < 0.001$).

suspensions were protected from light. The stability of RA in these suspensions has been reported [7]. NSAI drugs were suspended in a mixture of physiologic saline and 0.3% hydroxypropylcellulose. Suspensions were prepared in concentrations appropriate to deliver the desired dose in a volume equivalent to 1.0% of body weight. Aspirin and indomethacin were obtained from the Sigma Chemical Co. and *m*-hydroxybenzoic acid (HBA) from the Aldrich Chemical Co., Atlanta, GA. Ascorbic acid dissolved in water produces solutions of pH 2–3. To avoid administration of acid solutions, calcium ascorbate (Sigma Chemical Co.), which produces solutions of approximately neutral pH [22], was used.

Animal treatment. Adult male and female Swiss mice (Charles River Laboratories, Wilmington, MA) weighing approximately 25 g (range 20–30 g) were used in all experiments. Dose groups of twenty mice (ten/sex) were treated daily with RA alone or in combination with an NSAI drug for 21 days. RA was administered i.p. at 14 mg/kg, which approximates the LD_{10} for this treatment schedule [7]. NSAI drugs were administered p.o. Indomethacin and ibuprofen were administered as two divided doses per day at approximately 9:00 a.m. and 4:00 p.m. for total doses of 0.4 and 90 mg/kg/day respectively. Calcium ascorbate, aspirin, and HBA were administered, respectively, as single daily doses of 50, 105 and 115 mg/kg/day.

Mice were weighed daily and X-rayed on the day after the last treatment. X-ray films were evaluated to determine the number of fractured bones in each mouse. Two experiments were performed with each NSAI drug. The frequency of fractures among survivors on the day after the last treatment was arranged as a contingency table for each experiment. Chi-square analysis [23] indicated that the data from each pair of experiments (using a particular NSAI drug) were sufficiently homogeneous to justify pooling them. The pooled data were then tested for statistical significance [23].

Results and Discussion

The effects of concurrent administration of NSAI drugs on the number of fractures produced by RA are presented

Table 2. Relative potencies of NSAI drugs

NSAI drug	Inhibition of prostaglandin synthetase IC_{50} (μM)	Carrageenan-induced paw edema ED_{50} (μmoles/kg)	NSAI dose (μmoles/kg) (% Inhibition)*
Indomethacin	0.65†	14†	1.1 (78)
Calcium ascorbate	‡	128‡	128 (49)
Ibuprofen	10†	150†	436 (69)
Aspirin	2500†	538†	833 (38)

* Per cent inhibition in parentheses was the per cent inhibition of fractures induced by sublethal RA intoxication in our studies. Per cent inhibition was determined by the formula:

$$1 - \frac{\%\text{ mice with fractures (RA + NSAI)}}{\%\text{ mice with fractures (RA only)}} \times 100$$

and is included to provide an assessment of the effectiveness of the dose used.

† Data taken from Procaccini *et al.* [17].

‡ The IC_{50} for calcium ascorbate was not reported from *in vitro* inhibitory studies [20, 21]; the dose used in the carrageenan-induced paw edema test resulted in 12 per cent inhibition (ED_{12}) [19].

in Table 1. Treated mice receiving either RA alone or RA plus an NSAI drug were assigned to one of three columns based on the number of fractures observed (none, one, or more than one). For each NSAI drug the number of mice with no fractures was significantly higher in the group that received the combination than in the group that received RA alone. HBA, however, did not appear to affect the number of fractures produced by RA, although only a single experiment was performed.

The results clearly indicate that NSAI drugs are capable of protecting mice from the development of fractures associated with sublethal retinoid intoxication. HBA is structurally similar to aspirin, but it is only approximately 8 per cent as potent as aspirin in inhibition of prostaglandin synthetase [12]. HBA was tested at the same molar dose as that of aspirin. No protective effect of HBA could be demonstrated in the single experiment performed. The lack of a protective effect of HBA suggests that inhibition of the enzyme is required for protection rather than, for example, nonspecific binding to plasma or cellular proteins.

In Table 2, the NSAI drugs used in these studies are arranged in order of their reported abilities to inhibit prostaglandin synthetase *in vitro*, and the relative potencies of the drugs in the carrageenan-induced rat hind paw edema test, a standard screening method for anti-inflammatory activity, are also compared. That the drugs show the same order of potency in the two systems has been cited by some investigators as evidence that inhibition of prostaglandin synthetase is the mechanism of the anti-inflammatory activity of the NSAI drugs [12, 24, 25]. It is apparent from Table 2 that the order of potency of NSAI drugs for protecting mice from sublethal RA toxicity is the same as that for inhibition of prostaglandin synthetase. These results provide further support for our suggestion that prostaglandin biosynthesis is involved in the expression of retinoid toxicity [13]. More substantial support for this suggestion would require quantitative demonstration that prostaglandins are produced in response to RA.

Studies with cells in culture have demonstrated that RA stimulates release of prostaglandins by cells of Lewis lung carcinoma [26], an experimental tumor of mice, and by MDCK cells, an epithelial-like dog kidney cell line [27]. Needleman [28] has presented five criteria that should be met to establish that endogenous prostaglandins mediate a physiologic or pathologic event. The first of these requires that the concentration of prostaglandins produced should be proportional to the dose of the stimulus. We have demonstrated that RA induces dose-related release of prostaglandins by cells of Lewis lung carcinoma [26]. Another of Needleman's criteria requires that abolition of prostaglandin synthesis should abolish the physiologic action of the stimulus. The results reported here clearly indicate that compounds that inhibit prostaglandin synthetase significantly reduce the production of bone fractures by RA. Further support of our hypothesis would require demonstration that (a) RA administration increases prostaglandin biosynthesis *in vivo*, and (b) concurrent NSAI drug administration, at a dose that reduces the incidence of fractured extremities in RA-treated mice, also reduces prostaglandin biosynthesis in these mice. These additional studies would contribute to a better understanding of the mechanism of retinoid toxicity. An appreciation of this mechanism could influence the future clinical use of retinoids as chemopreventive agents by providing a means of controlling retinoid toxicity.

In summary, mice treated with retinoic acid at a dose that induced bone fractures in concurrent control groups were protected from this effect by co-administration of inhibitors of prostaglandin synthesis. The abilities of the NSAI drugs to protect mice correlated with their reported abilities to inhibit prostaglandin synthesis *in vitro*. These results strengthen our suggestion that prostaglandin activity is involved in expression of retinoic acid toxicity.

Acknowledgements—RA was provided by Hoffmann-LaRoche, Inc., Nutley, NJ; ibuprofen was supplied by The Upjohn Company, Kalamazoo, MI. This work was supported by Contract N01-CP-22064, Division of Cancer Cause and Prevention, National Cancer Institute, NIH.

Southern Research Institute
Birmingham
AL 35205, *U.S.A.*

E. JANE HIXSON*
STEADMAN D. HARRISON, JR.

* Address all correspondence to: E. Jane Hixson, PhD., Mobay Chemical Corp., 17745 South Metcalf, Stilwell, KS 66085, U.S.A.

REFERENCES

1. W. Bollag, *Chemotherapy* **21**, 236 (1975).
2. D. Leelaprute, V. Boonpucknavig, N. Bhamarapravati and W. Weerapradist, *Archs. Path.* **96**, 5 (1973).
3. H. G. Mandel, in *The Pharmacological Basis of Therapeutics* (Eds. L. S. Goodman and A. Gilman), 5th Edn, p. 1570. MacMillan, New York (1975).
4. N. S. Scrimshaw, in *Textbook of Medicine* (Eds. P. B. Beeson and W. McDermott), 14th Edn. p. 1368. Saunders, Philadelphia (1975).
5. G. Stuttgen, *Acta Derm.-vener Stockh.* (Suppl.) **74**, 174 (1975).
6. R. Happle, W. Kastrup and E. Macher, *Dermatologica* **155**, 200 (1977).
7. E. J. Hixson and E. P. Denine, *Toxic. appl. Pharmac.* **44**, 29 (1978).
8. J. R. Vane, *Nature New Biol.* **231**, 232 (1971).
9. P. V. Sacks, *Lancet* ii, 1385 (1974).
10. E. R. Smith and M. M. Mason, *Prostaglandins* **7**, 247 (1974).
11. J. P. Minton, D. G. Bronn and W. E. Kibbey, *New Engl. J. Med.* **294**, 340 (1976).
12. R. J. Flower, *Pharmac. Rev.* **26**, 33 (1974).
13. S. D. Harrison, Jr., E. J. Hixson, J. A. Burdeshaw and E. P. Denine, *Nature Lond.* **269**, 511 (1977).
14. E. J. Hixson and E. P. Denine, *Toxic. appl. Pharmac.* **45**, 317 (1978).
15. M. B. Sporn, N. M. Dunlop, D. L. Newton and J. M. Smith, *Fedn. Proc.* **35**, 1332 (1976).
16. T. H. Maugh II, *Science* **186**, 1198 (1974).
17. R. L. Procaccini, R. D. Smyth and N. H. Reavey-Cantwell, *Biochem. Pharmac.* **26**, 1051 (1977).
18. E. B. Vedder and C. Rosenberg, *J. Nutr.* **16**, 57 (1938).
19. F. A. Dolbeare and K. A. Martlage, *Proc. Soc. exp. Biol. Med.* **139**, 540 (1972).
20. D. M. Pugh, S. C. Sharma and C. W. M. Wilson, *Br. J. Pharmac.* **53**, 469P (1975).
21. S. C. Sharma, S. K. Garg and C. W. M. Wilson, *IRCS Med. Sci.* **4**, 573 (1976).
22. *The Merck Index*, (Eds. M. Windholz, S. Budavari, L. Y. Stroumtsos and M. N. Fertig), 9th Edn, p. 209. Merck, Rahway, NJ (1976).
23. J. H. Zar, *Biostatistical Analysis*, p. 47. Prentice-Hall, Englewood Cliffs, NJ (1974).
24. R. J. Flower and J. R. Vane, *Biochem. Pharmac.* **23**, 1439 (1974).
25. S. H. Ferreira and J. R. Vane, *A. Rev. Pharmac.* **14**, 57 (1974).
26. E. J. Hixson, R. B. Boozer, D. P. Chopra, S. D. Harrison, Jr. and E. P. Denine, *Pharmacologist* **20**, 234 (1978).
27. L. Levine and K. Ohuchi, *Nature, Lond.* **276**, 274 (1978).
28. P. Needleman, *Biochem. Pharmac.* **27**, 1515 (1978).