The epithelial differentiating activity *in vivo* of (*E*)-4-[2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthylenyl)-1-propenyl]benzoic acid and 4,4-difluororetinoic acid*

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Female rats fed on a vitamin A-deficient diet from weaning were oophorectomized after introitus and used to test analogues of all-*trans*-retinoic acid for epithelial differentiation activity by the vaginal-smear assay. Several modifications have been made in the assay; housing facilities were modified, the diet changed and the existing scoring system for the assay altered. The arotinoid (*E*)-4-[2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthylenyl)-1-propenyl]benzoic acid was 12-fold more active than all-*trans*-retinoic acid, which had a 50% effective dose (ED₅₀) of 80 pmol/vagina. The fluorinated analogue 4,4-difluororetinoic acid had an ED₅₀ of 2.5 nmol/vagina and was therefore 30-fold less active than all-*trans*-retinoic acid.

Several investigators have examined the metabolism of vitamin A during the past twenty years (Dunagin *et al.*, 1964; Zachman & Olson, 1964; Roberts *et al.*, 1980; Silva & DeLuca, 1982; Stephens-Jarnagin *et al.*, 1983). The results of these studies have suggested that all-*trans*-retinoic acid may be the active form of vitamin A in both the maintenance of growth and epithelial differentiation. During the quest for the active form of the vitamin, the structures of several metabolites of all-*trans*-retinoic acid have been elucidated. Roberts *et al.* (1980) have shown, in tissues of the golden hamster, that primary metabolites of all-*trans*-retinoic acid degradation and/or inactivation *in vitro* are the 4-oxygenated derivatives (4-hydroxyretinoic acid and 4-oxoretinoic acid). Silva & DeLuca (1982) showed that these 4-oxygenated derivatives are minor metabolites *in vivo* after a physiological dose of retinoic acid to vitamin A-deficient rats.

There have been numerous reports that 5,6-epoxyretinoic acid is a significant metabolite of all-*trans*-retinoic acid in some target tissues in the rat (McCormick *et al.*, 1978; Zile *et al.*, 1980; Silva & DeLuca, 1982). Earlier reports (McCormick *et al.*, 1978) provided evidence that this epoxide is an

active metabolite of retinoic acid. More recent evaluations of this compound (Zile *et al.*, 1980; Sietsema & DeLuca, 1982) have shown the epoxide to be inactive in two assays *in vivo* of vitamin A activity, i.e. the rat vaginal-smear assay and the vitamin A-deficient-rat growth assay. Thus oxygenation of either the 4-position or the 5,6-positions has been shown to decrease biological activity (Newton *et al.*, 1980; Zile *et al.*, 1980; Sietsema & DeLuca, 1982) and are considered to be pathways of degradation and/or inactivation.

Recently, a bioassay *in vivo* has been redefined for examining the activity of small amounts of vitamin A derivatives, i.e. the rat vaginal-smear assay (Sietsema & DeLuca, 1982). The ability of retinoids to reverse the formation of a keratinized vaginal epithelium is assayed in oophorectomized female vitamin A-deficient rats. Retinoids are administered directly on to the vaginal epithelium to eliminate any dilution that can occur when compounds are given systemically.

The purpose of this study is two-fold: (1) to characterize more completely the vaginal-smear assay and to simplify the analysis of the assay to minimize the requirement for an in-depth knowledge of histology, and (2) to determine if synthetic analogues of all-*trans*-retinoic acid modified such that the 4- and 5,6-carbon oxygenations are prevented would either increase or prevent biological activity in the differentiation of vaginal epithelium.

Abbreviations used: TTNPB, (*E*)-4-[2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthylenyl)-1-propenyl]benzoic acid

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Materials and methods

Materials

all-*trans*-Retinoic acid was obtained from Eastman Organic Chemicals (Rochester, NY, U.S.A.). 4-Hydroxyretinoic acid was a gift of Dr. Michael Rosenberger from Hoffmann-La Roche (Nutley, NJ, U.S.A.) and TTNPB was provided by Dr. M. Sporn at the National Cancer Institute (Bethesda, MD). 4,4-Difluororetinoic acid was a gift from Dr. J. A. Olson and Dr. A. Barua (Iowa State University, Ames, IA, U.S.A.). all-*trans*-Retinoic acid was derivatized with diazomethane to obtain the methyl ester of retinoic acid, methyl retinoate. All other materials were obtained through commercial suppliers and were of analytical grade.

Animals and diet

Weanling female rats were obtained from the Holtzman Co. (Madison, WI, U.S.A.) and maintained under controlled environmental conditions (Biotron Facilities; University of Wisconsin). The animal facilities were maintained at 15–45% humidity (ambient) and 22°C. Approx. 2.8 room-air exchanges/min were made, with 10% of each air exchange being filtered fresh air and the remaining being filtered/recirculated air. A 12h-on/12h-off cycle of fluorescent light was used during the experiment. The specialized care taken in housing the rats was done to minimize the loss of animals during the course of the experiment due to naturally occurring infections, a symptom of vitamin A deficiency. The rats were maintained on a vitamin A-free diet (Sietsema & DeLuca, 1982). The diet used had the fat-soluble vitamins [calciferol (vitamin D₂) (87.5 µg/kg), α-tocopherol (159 mg/kg) and menadione (50 mg/kg)] added at Teklad (Madison, WI, U.S.A.).

Vaginal-smear assay

After introitus, approx. 7 weeks of age, the rats were oophorectomized. The vaginal samples were taken as previously described (Sietsema & DeLuca, 1982) and this was begun 1 week after oophorectomy. The scoring system for the assay was modified so as to simplify the analysis of the vaginal lavage. Each rat was sampled once daily at approximately the same time each day (to minimize any possible diurnal variation). The value recorded from the lavage was the average percentage of cells microscopically observed that were of a cornified-epithelial cell type. Intravaginal dosing of the test compounds as previously described (Sietsema & DeLuca, 1982) was begun once any rat maintained an average 3-day cornified cell score value of 90% or greater, over a 4-day period. A period of 4 days was chosen for normalization because periodically the lavage score would de-

crease to about 75% cornified cells for unknown reasons unrelated to the oestrous cycle. One possibility is that it may be related to the quantity of cells in the lavage. The activity of each retinoid tested at any time point was normalized to its 3-day predosing mean vaginal-smear score as follows:

$$\frac{\text{3-day predosing mean score} - \text{score at a given time}}{\text{3-day predosing mean score}} \times 100$$

Results are presented as the percentage of cells that are of a non-cornified epithelial type. Vaginal-lavage scores were determined for 5 days after the intravaginal dosing. The biological activity of a retinoid is evaluated as to its ability to mimic the vitamin A-replete state, where the vaginal lavage has a high percentage of non-cornified epithelial cells.

Results

Biological activity of retinoic acid and analogues

The synthetic retinoids tested were modified in several key positions (Fig. 1) analogous to important positions of the parent compound all-*trans*-retinoic acid. all-*trans*-Retinoic acid was examined in the vaginal-smear assay at doses ranging from 5×10^{-12} to 1×10^{-9} mol/vagina (Fig. 2). The ED₅₀ as determined by linear-regression analysis was 8×10^{-11} mol/vagina (Table 1, Fig. 2). Methyl retinoate was equieffective with retinoic acid on the vaginal epithelium over the dosage range examined (10^{-11} – 10^{-8} mol/vagina; Fig. 2). TTNPB showed the greatest activity of all the analogues tested, with an ED₅₀ of 6.8 pmol/vagina (Table 1).

all-*trans*-4-Hydroxyretinoic acid was relatively inactive over the 10^{-9} – 10^{-7} mol/vagina dose

Fig. 1. Structures of retinoids examined and assignment of atoms or functional groups at key positions

Table 1. Relative biological activities of several retinoids

	ED ₅₀ * (mol/vagina)	Relative activity†
Retinoic acid	8.3×10^{-11}	1.00
Methyl retinoate	7.0×10^{-11}	1.20
4,4-Difluororetinoic acid	2.5×10^{-9}	0.033
4-Hydroxyretinoic acid	1.3×10^{-8}	0.006
TTNPB	6.8×10^{-12}	12.20

* ED₅₀ as determined by linear-regression analysis of means. The precision of the assay as determined by the interassay variation of the ED₅₀ for all-*trans*-retinoic acid was $118 (\pm 54) \times 10^{-12}$ mol/vagina [mean $\pm$ S.D. from Skare *et al.* (1982), Sietsema & DeLuca (1982), Stephens-Jarnagin (1983) and above].

† Potency related to the standard (all-*trans*-retinoic acid) with the standard relative activity being 1.00.

Fig. 2. Dose-response curve for (Δ) all-*trans*-retinoic acid, ($\blacksquare$) methyl retinoate and ($\circ$) TTNPB. Error bars indicate the S.E.M. ($n = 6-9$).

Fig. 3. Dose-response curve for (Δ) all-*trans*-retinoic acid, ($\bullet$) 4,4-difluororetinoic acid and ($\blacktriangle$) 4-hydroxyretinoic acid. Error bars indicate the S.E.M. ($n = 6-9$).

range examined (Fig. 3), with an ED₅₀ of 1.3×10^{-8} mol/vagina. The fluorinated analogue of retinoic acid, 4,4-difluororetinoic acid, was only slightly more active than 4-hydroxyretinoic acid (Fig. 3), with an ED₅₀ of 2.5 nmol/vagina and was 30-fold less active than all-*trans*-retinoic acid (Table 1).

Duration of action of retinoic acid and other retinoids

In order to determine both the onset and duration of action of retinoid reversal of vaginal cornification, four distinct groups of vitamin A-deficient rats were used. As previously stated, the rats were lavaged only once each day. Therefore, in order to examine the activity of the test compounds every 6 h, four groups of rats were used. Each group of rats was dosed at different times throughout the day with the time of day that all rats were lavaged kept constant in order to minimize any potential diurnal variation in the response. Each of the four groups represented different time points in the profile as follows: Group I, 0, 24, 48, 72 and 96 h; Group II, 0, 6, 30, 54, 78 and 102 h; Group III, 0, 12, 36, 60, 84 and 108 h; and Group IV, 0, 18, 42, 66, 90 and 114 h. The 6 h lavage for Group II was

not performed because there was concern for the length of time that the retinoid must remain in the vagina for a maximal response to be observed. Between six and twelve rats were tested in each group. No group examined in the duration-of-action study had 100% survival (Figs. 4 and 5). The time course of action was estimated by using the ED₈₀ dose for all compounds tested. These values were determined from regression analysis of the dose-response curves (results not shown). The ED₈₀ value for all-*trans*-retinoic acid was determined to be 3.9×10^{-10} mol/vagina. Time-course data in Fig. 4 shows that all-*trans*-retinoic acid appears to have near maximal activity from 30 to 72 h after intravaginal dosing, and that maximal response occurred at 54 h. The onset of activity was first observed at 30 h. 4,4-Difluororetinoic acid similarly showed maximal activity at 54 h after dosing (Fig. 4); however, the near-maximal plateau differed, occurring between 30 and 84 h, and it was the only compound to have biological activity at 12 h. These differences in activity at the initial time points between all-*trans*-retinoic acid and 4,4-difluororetinoic acid were statistically significant. These differences were $P < 0.1$ at 12 h, more

Fig. 4. Time course of action for (Δ) all-trans-retinoic acid ($ED_{80} = 390 \times 10^{-12}$ mol/vagina) and (●) 4,4-difluoro-retinoic acid ($ED_{80} = 33 \times 10^{-9}$ mol/vagina)

Error bars indicate S.E.M.; the numbers of rats per time point are given in parentheses.

Fig. 5. Time course of action for (■) methyl retinoate ($ED_{80} = 340 \times 10^{-12}$ mol/vagina) and (○) TTNPB ($ED_{80} = 37 \times 10^{-12}$ mol/vagina)

Error bars indicate S.E.M.; the numbers of rats per time point are given in parentheses.

significant at 18 h ($P < 0.05$) and not significant by the 24 h time point ($P < 0.2$).

Both TTNPB and methyl retinoate showed maximal activity from an ED_{80} dose at 54 h (Fig. 5). Overall, the time-course data from both of these retinoids were similar to the all-trans-retinoic acid response, although minor differences were observed.

All retinoids studied exhibited a secondary or rebound response (Figs. 4 and 5). The time of the secondary response varied with each retinoid, and the cause is unknown.

Discussion

In the present study, several modifications were made in the rat vaginal-smear assay. These alterations have decreased the number of parameters by which the assay provides assessment of vitamin A activity. However, these changes did not alter the estimation of biological activity for all-trans-retinoic acid. In this study, the ED_{50} was found to be 80 pmol/vagina, which is identical with that found in previous studies (Sietsema & DeLuca, 1982; Stephens-Jarnagin *et al.*, 1983), where the modified scoring system and the controlled environmental conditions were not utilized. The scoring system has been improved by decreasing the number of different epithelial cell types that must be measured. When determining the proportion of cells that are keratinized, the scoring system now expresses the degree of keratinization in a continuous manner as opposed to the discrete levels used previously (Sietsema & DeLuca, 1982). Consequently, it is considered that there will be a decrease in the potential scoring variability and that this will then result in decreases in the inter-assay variability in retinoid responses, thereby increasing the reliability of the assay.

To date no study has examined whether endogenous esterase activity would alter the response time or the effectiveness of retinoic acid esters in the rat vaginal-smear assay. Examination of the biological activity of a retinoid ester in this assay was performed with methyl retinoate. It was observed that methyl retinoate was equipotent with all-trans-retinoic acid (Table 1). Time-course data using equipotent doses of both retinoic acid and methyl retinoate showed that the peak response was at 54 h for both retinoids. We conclude that either the methyl ester is active without change or sufficient esterases are present to convert methyl retinoate into the free acid.

Drugs and other chemicals are commonly fluorinated at key positions to behave as either suicide substrates of enzymes (Wang & Walsh, 1981; Stubbe & Kozarich, 1980) or to inhibit oxidations or other modifications at that or

neighbouring positions (Tanaka *et al.*, 1982, 1983; Barnette, 1984). 4,4-Difluororetinoic acid was synthesized in an attempt to inhibit the known oxidative inactivation occurring at the 4-position of retinoic acid (Roberts *et al.*, 1980). This fluorinated retinoid was not as effective as all-*trans*-retinoic acid (Table 1). However, when both compounds are compared using equiactive doses (ED_{80}), the fluorinated analogue showed a similar time course of action. Two minor differences in the time courses were (i) the 4,4-difluororetinoic acid showed statistically greater biological activity at the earliest time points evaluated, 12 and 18 h (Table 2), and (ii) that the duration of activity appeared to be extended for 12 h longer than retinoic acid.

The relative inactivity of the fluorinated compound may be due to several factors: (1) the decreased stability of the compound; (2) the change in steric bulk at the C-4 position; or (3) the change in electrodensity at the 4-position due to the highly electronegative fluorine atoms. Barua & Olson (1981) have recently stated that the similar compounds 4,4-difluororetinol and its esters were relatively stable ($<1\%$ degradation/day) at 37°C in neutral aqueous methanol. However, if the analogues are dissolved in basic solutions, there is a loss of fluorine to produce 4-oxoretinol, and under acidic conditions there was loss of fluorine to produce anhydrotretinol. Therefore it is possible that 4,4-difluororetinoic acid may not have been sufficiently stable at the site of action and had been degraded. The activity observed may have been caused by either the activity of some unknown quantity of 4,4-difluororetinoic acid or the activity of a breakdown product. This might explain why the compound was only slightly more active than 4-hydroxyretinoic acid (Table 1).

It is not likely that the decreased activity of this compound is entirely related to the replacement of a 4-position hydrogen atom with an atom of sufficiently greater size so as to sterically hinder its activity. In Table 2 the van der Waal's radii for several atoms and functional groups is given. A methyl group shows a 67% increase in size over a hydrogen atom, whereas fluorine only increases the radial displacement by 16%. However,

TTNPB, an analogue with methyl substituents on a position that corresponds to the 4-position of retinoic acid (Fig. 1), is 12-fold more active than all-*trans*-retinoic acid.

Assuming that the fluorinated retinoids are sufficiently stable, the decreased effectiveness of this compound may be due to the relatively higher electronegativity of the fluorine atoms (Table 2) as compared with the hydrogen atoms of the parent compound, retinoic acid. If the 4-position is important for the biological activity of retinoic acid, then compounds having comparable electronegative functional groups or atoms at that position might be expected to have similar activities. The hydrogen atoms on retinoic acid and the methyl groups on TTNPB have electronegativity values at 2.1 and 2.5 respectively, and these compounds were highly active in the rat vaginal-smear assay (Fig. 2). 4-Hydroxyretinoic acid and 4,4-difluororetinoic acid both have highly electronegative atoms at the 4-position (Table 2) and have similar activity. These differences in electron density at the 4-position may alter cellular transport of the compounds or may interfere with its interaction at critical sites.

The most effective retinoid tested to date in the rat vaginal-smear assay has been TTNPB, which is 12-fold more active than all-*trans*-retinoic acid, with an ED_{50} of 6.8 pmol/vagina. This was not unexpected, since TTNPB has been shown to be highly potent in several other assays *in vitro* (Newton *et al.*, 1980; Strickland *et al.*, 1983) and *in vivo* (Stephens-Jarnagin *et al.*, 1984; Loeliger *et al.*, 1980). The increase in biological activity may be due to a decrease in metabolic inactivation. The methyl groups at the position corresponding to the 4-position of retinoic acid and the presence of an aromatic ring at the analogous 5,6-position may decrease the activity of known oxidation enzymes that affect those positions (Roberts & DeLuca, 1968; McCormick *et al.*, 1978; Roberts *et al.*, 1980). Therefore the increase in biological activity may in part be due to an increase in metabolic half-life. The biological half-life of TTNPB and its metabolism is not known. However, Stephens-Jarnagin *et al.* (1984) showed that in the rat-growth assay when TTNPB was no longer administered

Table 2. *Physical properties of 4-position elements and functional groups*

Element or function group	van der Waal's radii (nm) (Pauling, 1960b)	Electronegativity (Pauling, 1960a)
H	0.12	2.1
F	0.135	4.0
O	0.14	3.5
C	—	2.5
CH ₃	0.20	—

the vitamin A effect (weight gain) was maintained for longer periods of time than for all-*trans*-retinoic acid, possibly indicating a decrease in clearance.

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