Europ. J. Cancer Vol. 15, pp. 1417–1423.
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0014-2964/79/1201-1417 $02.00/0

Activity of Retinoids Against Benzo(A)pyrene-induced Hyperplasia in Mouse Prostate Organ Cultures*

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Abstract—*The antihyperplastic activity of several retinoids (with ring, side chain or polar group modifications) against benzo(a)pyrene (BP)-induced hyperplasia in mouse prostate cultures was examined. Prostate explants were made hyperplastic by treatment with BP for* 8 days, *followed by simultaneous treatment with BP and different concentrations of a retinoid. The anti-mitotic activity of retinoids was compared with β-retinoic acid (RA). Different retinoids produced various degrees of mitotic inhibition in the hyperplastic prostate epithelium. Five retinoids:* 13-cis-*retinoic acid, the methylketo cyclopentenyl analog of retinoic acid, the* 1-*methoxyethyl cyclopentenyl analog of retinoic acid,* N-*retinoylglycine, and the* 14-*fluoro derivative of the trimethylmethoxyphenyl analog of retinoic acid ethyl ester, showed greater activity than RA. Seven other retinoids were as active as RA. Two retinoids were less active than RA, and one retinoid produced no mitotic inhibitory effect.*

INTRODUCTION

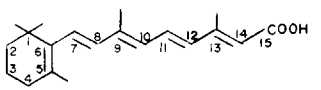
ANIMAL studies during the past several years have established that retinoids have prophylactic and therapeutic properties against a number of epithelial lesions [1–5]. The clinical application of vitamin A (retinol, retinyl palmitate, retinyl acetate), however, has been limited because of the toxic side effects it produces when applied in effective doses [6]. Recently extensive efforts have been made to synthesize new retinoids with low toxicity and high potency [6]. The amounts of new retinoids are often too limited to perform *in vivo* experiments but preliminary information on the activity of retinoids against epithelial lesions can be obtained by using tissue culture methods. For instance, in the absence of retinoids, the epithelium of tracheal tissues derived from vitamin A-deficient hamsters and explanted into organ culture, lose their normal columnar morphology, and develop lesions of squamous metaplasia while addition of retinoids to such cultures causes reversal of squamous metaplastic changes and replacement of squamous cells by ciliated, columnar and mucous cells [4]. Several retinoids were shown to reverse hyperplastic lesions in mouse prostate explants, induced by *N*-methyl-*N*-nitro-*N*-nitrosoguanidine (MNNG), a carcinogen not requiring metabolic activation for its activity [7]. The methylketo cyclopentenyl and 1-methoxyethyl cyclopentenyl analogs of retinoic acid and retinyl methyl ether were found to be significantly more active than β-retinoic acid (RA) in reversing MNNG-induced lesions [7]. In this paper we have compared the activity of retinoids in reversing benzo(a)pyrene (BP), (a carcinogen requiring metabolic activation) induced hyperplastic lesions in mouse prostate organ cultures.

MATERIALS AND METHODS

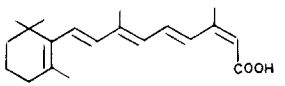
The organ culture method for mouse prostate has been previously described [7–9]. Briefly, ventral prostate from 8- to 12-week-old C3H mice are removed aseptically, teased into explants (1.5 × 1.5 mm) and pooled. The randomly selected explants were cultured on lens papers supported by stainless steel grids placed in 35 mm Petri dishes. The medium was CMRL 1066 supplemented with 10%

Accepted 28 May 1979.
*Supported by Public Health Service Contract NO1-CP-22064 from the Lung Cancer Segment, Division of Cancer Cause and Prevention, National Cancer Institute (NCI), U.S.A.

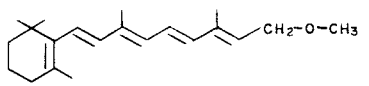
Table 1. Structure of retinoids



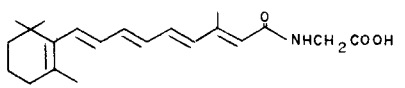
All — *trans* — retinoic acid



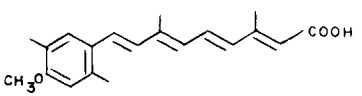
13 — *cis* — Retinoic acid



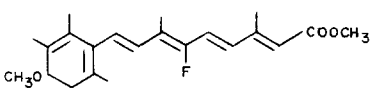
Retinyl methyl ether



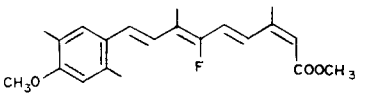
N—Retinoylglycine



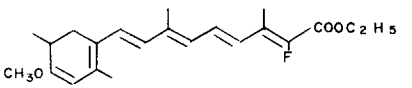
Trimethylmethoxyphenyl (TMMP) analog of retinoic acid



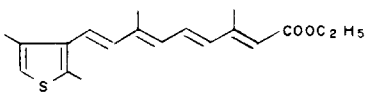
10—Fluoro derivative of TMMP analog of retinoic acid methyl ester



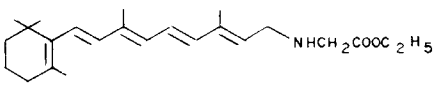
10—Fluoro derivative of TMMP analog of 13—*cis*—retinoic acid methyl ester



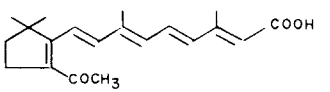
14—Fluoro derivative of TMMP analog of retinoic acid ethyl ester



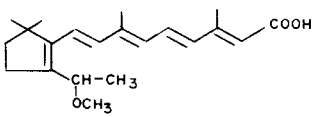
Trimethylthiophene analog of retinoic acid ethyl ester



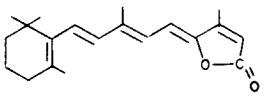
N—Retinoylglycine ethyl ester



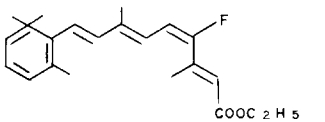
The methylketo cyclopentenyl analog of retinoic acid



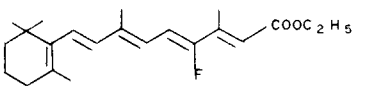
The 1—methoxyethyl cyclopentenyl analog of retinoic acid



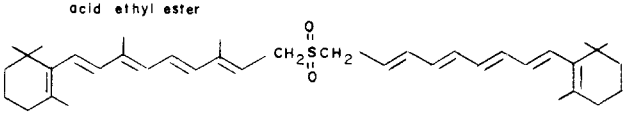
Lactone of 13—*cis*—retinoic acid



12—(*E*)—Fluororetinoic acid ethyl ester



12—(*Z*)—Fluororetinoic acid ethyl ester



β—Retinyl sulphone

horse serum, 3% chick embryo extract, and antibiotics. The cultures were incubated at 37°C in an atmosphere of 50% O_2 + 45% N_2 + 5% CO_2 and culture medium was changed every 48 hr.

BP (Sigma Chemical Co.) was dissolved in dimethylsulfoxide (DMSO) and added to the culture medium at a final concentration of 8 μg/ml. Retinoids (Table 1) were dissolved in ether or DMSO (4 mg/ml) and added to the culture medium to give the required concentrations. The final concentrations of the solvents in each dish were always less than 0.1%. Equal amounts of the solvents were added to the corresponding control cultures. For determining the activity of each retinoid, five groups of cultures (2–3 dishes per group; 4–6 explants per dish) were prepared. One group (group 1) was kept as the untreated control. The remaining four groups were allowed to develop hyperplastic lesions by treatment with BP for 8 days after which time these hyperplastic explants were treated with BP only (group 2) or simultaneously with BP and different concentrations of a retinoid (groups 3–5). The effects of RA at equimolar concentrations on untreated control explants was also examined. The explants were fixed in buffered formalin, processed for histology, serially sectioned, and stained in hematoxylin and eosin. The changes in rates of cell proliferation caused by BP or a retinoid were determined by the colcemid metaphase arrest technique [7, 8]. The number of arrested metaphases were counted in 1000–2000 cells per explant and the mitotic index (MI) determined for each explant. From each explant 2–3 sections were microscopically scanned and the mitotic index and standard deviation (MI ± S.D.) determined for each group (4–6 explants). Student's *t*-test was applied to the average logarithmic values of MI to test for statistical significance, the differences (a) between control and experimental groups and, also, (b) between the inhibitions of BP-induced hyperproliferation caused by the lowest concentrations (6.6×10^{-7}M) of RA and each of the analogs. A $P < 0.05$ was considered significant.

RESULTS

The histological apperance of prostate explants has been described in detail [7, 8]. Eight to 12 days after incubation the alveolar epithelium of control explants consisted of 1–2 cell layers that exhibited a low rate of cellular proliferation. Treatment of explants with BP for 8 days stimulated proliferation, and extensive hyperplasia was present at 12 days after treatment. However, when explants were first treated with BP for 8 days, followed by simultaneous treatment with BP and an active retinoid, the hyperproliferation was reversed (Table 2). For instance, in an experiment in which the activity of RA was examined, the MI in control explants at 12 days after incubation was 87 ± 47, while the corresponding value in BP-treated explants was 416 ± 56. The values of MI in explants that were first treated with BP for 8 days followed by simultaneous treatment with BP and different concentrations of RA were 109 ± 49, 144 ± 54, and 253 ± 55 at 1.7×10^{-5}M, 3.3×10^{-6}M and 6.6×10^{-7}M respectively.

The antihyperplastic activities of other retinoids were compared to RA at equimolar concentrations (Table 2). The following five retinoids were found to be more active than RA: 13-*cis*-retinoic acid, the methylketo cyclopentenyl analog, the 1-methoxyethyl cyclopentenyl analog, the 14-fluoro derivative of trimethylmethoxyphenyl (TMMP) analog of retinoic acid ethyl ester and *N*-retinoylglycine. These retinoids at concentrations of 6.6×10^{-7} exhibited significantly ($P < 0.05$) greater mitotic inhibition than RA in the epithelium of BP-treated explants.

The lactone of 13-*cis*-retinoic acid, retinyl methyl ether, *N*-retinoylglycine ethyl ester, the 10-fluoro derivative of TMMP analog of all-*trans*-RA methyl ester, the trimethylthiophene analog of retinoic acid ethyl ester, the 12-(*E*)-fluoro retinoic acid ethyl ester, and the 10-fluoro derivative of TMMP analog of 13-*cis*-retinoic acid methyl ester, were as active as RA.

The 12-(*Z*)-fluororetinoic acid ethyl ester and the TMMP analog of RA were probably less active than RA. β-Retinyl sulfone showed no activity in reversing BP-induced hyperproliferation in mouse prostate explants.

RA at these concentrations had no detectable effect on cell proliferation in untreated control explants (Table 3).

It should be noted that although the prostate organ culture system provides reliable information on the comparative mitotic inhibitory activity of the various retinoids, the effect is not necessarily dose-dependent at the concentrations tested. This probably is one of the limitations of this system. It is possible that the treatment of explants with the carcinogen produces a heterogenous cell population that contains cells in different stages of the cell cycle. The variations in the degree of

Table 2. Reversal of BP-induced hyperproliferation (MI±S.D.) by different concentrations of retinoids*

Analog	Days after treatment	BP		Explants treated with BP for 8 days followed by simultaneous treatment with BP and the retinoids (M)		
		Control	Treated	1.7×10^{-5}	3.3×10^{-6}	6.6×10^{-7}
All-*trans*-retinoic acid	8	166±62	451±169†			
	12	87±47	416±56†	109±49‡ (74)	144±54‡ (65)	253±55‡ (39)
13-*cis*-retinoic acid	8	60±15	247±79†			
	12	62±29	399±110†	ND	36±2‡ (91)	43±13‡§ (89)
Methylketo cyclopentenyl analog of retinoic acid (RO8-7699)	8	408±116	1026±293†			
	12	128±44	365±67†	75±25‡ (79)	80±32‡ (78)	95±3‡§ (74)
1-Methoxyethl cyclopentenyl analog of retinoic acid	8	161±62	451±169†			
	12	87±47	416±56†	78±14‡ (81)	152±67‡ (63)	114±36‡§ (73)
N-Retinoylglycine	8	154±56	432±79†			
	12	241±76	684±224†	167±70‡ (76)	326±37‡ (52)	122±33‡§ (82)
14-Fluoro derivative of TMMP analog of retinoic acid ethyl ester	8	138±46	346±112†			
	12	63±18	380±72†	143±51‡ (62)	140±40‡ (63)	92±35‡§ (76)
Lactone of 13-*cis*-retinoic acid	8	138±46	346±112†			
	12	63±18	380±72†	84±24‡ (78)	71±30‡ (81)	167±57‡ (66)
Trimethylthiophene analog of retinoic acid ethyl ester	8	108±47	547±177†			
	12	96±17	486±122†	127±53‡ (74)	ND	239±60‡ (51)
Retinyl methyl ether	8	150±44	328±75†			
	12	58±45	371±72†	81±2‡ (78)	141±41‡ (62)	233±57‡ (37)
12-(*E*)-fluororetinoic acid ethyl ester	8	101±35	387±110†			
	12	114±47	246±88†	164±55 (33)	102±37‡ (59)	131±66‡ (47)
N-retinoylglycine ethyl ester	8	154±56	432±79†			
	12	241±76	684±224†	216±149‡ (68)	205±74‡ (70)	387±32‡ (43)
10-Fluoro derivative of TMMP analog of all-*trans*-retinoic acid methyl ester	8	89±39	447±110†			
	12	114±69	437±75†	279±91 (36)	209±43‡ (52)	250±57‡ (43)
Trimethylmethoxyphenyl (TMMP) analog of retinoic acid	8	150±44	328±75†			
	12	58±45	371±72†	229±5‡ (38)	285±89 (23)	239±56‡ (36)

Continued

Table 2—continued

Analog	Days after treatment	BP		Explants treated with BP for 8 days followed by simultaneous treatment with BP and the retnoids (M)		
		Control	Treated	1.7×10^{-5}	3.3×10^{-6}	6.6×10^{-7}
10-Fluoro derivative of TMMP analog of 13-*cis*-retinoic acid methyl ester	8	89 ± 39	447 ± 110†			
	12	114 ± 69	437 ± 75†	158 ± 47‡ (64)	183 ± 35‡ (58)	340 ± 94 (22)
12-(*Z*)-Fluororetinoic acid ethyl ester	8	101 ± 35	387 ± 110†			
	12	114 ± 47	246 ± 88†	117 ± 31‡ (52)	105 ± 50‡ (57)	219 ± 49 (11)
β-Retinyl sulphone	8	123 ± 48	440 ± 159†			
	12	53 ± 19	205 ± 32†	190 ± 61 (7)	325 ± 130 (−59)	319 ± 99 (−56)

*MI is the number of arrested metaphases per 10^5 cells during a 4-hr period of colcemid treatment.
†Mitotic stimulation significant as compared to the corresponding control value ($P<0.05$).
‡Mitotic inhibition significant as compared to the corresponding BP-treated group. Numbers in parentheses represent percent inhibition ($P<0.05$).
§Significantly higher activity as compared with β-retinoic acid at the corresponding concentration ($P<0.05$).
ND: not determined.

Table 3. Effects of different concentrations of RA on mouse prostate organ cultures at various intervals after treatment

Days after treatment	Control	MI ± S.D.* in untreated control explants receiving RA (M)		
		1.7×10^{-5}	3.3×10^{-6}	6.6×10^{-7}
4	68 ± 24	87 ± 49	78 ± 38	81 ± 52
8	100 ± 64	79 ± 28	71 ± 21	109 ± 50
11	101 ± 60	91 ± 48	96 ± 71	92 ± 50

*MI ± S.D. is the number of arrested metaphases per 10^5 cells during a 4-hr period of colcemid treatment.

mitotic inhibition at different retinoid concentrations may be related to the variable sensitivities to retinoid of these different cell cycle stages.

DISCUSSION

The vitamin A molecule is composed of three parts: a ring, side chain and terminal polar group [6]. Modifications in the different parts of the molecule have resulted in retinoids with increased therapeutic activity [10], increased activity in preventing epithelial tumors in experimental animal studies [1, 2], and increased activity in modulating epithelial differentiation [11, 12]. In the present study several retinoids with modifications in either the ring, side chain or polar group were more active than RA in reversing BP-induced lesions in mouse prostate explants (Table 2).

The two cyclopentenyl retinoids (in which the six-carbon ring is substituted by a five-carbon ring, Table 1) were significantly more active than RA in reversing BP-induced lesions in mouse prostate explants (Table 2). Other reports have indicated that these cyclopentenyl retinoids are more active than RA or retinol in altering epithelial differentiation [11, 12]. In the present study, other ring

modifications resulted in retinoids which exhibited different degrees of activity. Of the several TMMP analogs tested, the TMMP analog of RA appeared to be less active than RA (Table 2). For instance at 3.3×10^{-6}M, RA produced a 65% inhibition of mitotic stimulation whereas the TMMP analog of RA at this concentration produced only a 23% inhibition. A recent report has indicated that this analog was more effective than RA in reversing MNNG-induced hyperplasia [7]. Other reports have demonstrated that the TMMP analog is effective in controlling tracheal cell differentiation [4, 13] and in modulating epithelial differentiation of chick embryo skin explants [11]. The other TMMP analogs with side chain modifications and esterified terminal carboxyl groups were either as active or more active than RA (Table 2). The trimethylthiophene analog of RA ethyl ester exhibited about the same degree of activity as RA.

Among the retinoids with side chain modifications, only 13-*cis*-RA was more active than RA (Table 2). Sporn *et al.* [5] have reported that this retinoid was very active in preventing hydroxybutyl-butyl-nitrosamine-induced lesions in the rat bladder. Recently, Hixson *et al.* [14] have shown that 13-*cis*-RA was less toxic than RA in mice. Other retinoids with side chain modifications tested in the present study were either as active or less active than RA.

Bard and Lasnitzki [15] have suggested that the toxic effects of RA are associated with the terminal carboxyl group. Thus retinoids with less polar terminal groups were synthesized. Retinyl methyl ether, that was significantly less toxic than retinol or retinyl acetate, was also active in controlling tracheal cell differentiation [13], and in the preventiation of mammary tumors in 7,12-dimethylbenz(a)anthracene-treated rats [3]. In the present study retinyl methyl ether was as active as RA in reversing BP-induced hyperplasia of the explants (Table 2). *N*-Retinoylglycine, however, was substantially more active than RA in reversing the BP-induced hyperplasia. *N*-retinoylglycine ethyl ester was as active as RA while β-retinyl sulphone was devoid of any activity (Table 2).

Since tissues have different thresholds of response to vitamin A [16], retinoids probably should be tested in more than one experimental system. For instance, studies with retinoids in several *in vitro* and *in vivo* systems have demonstrated that certain alterations in the molecule may result in an increased biological activity as defined by either its effect on preventing an epithelial lesion induced by a carcinogen or its effect on modulating epithelial differentiation [7, 11]. The degree of biological activity of a particular retinoid may vary with the test system, and this difference in activity may be associated, in part, with different tissue sensitivities to vitamin A.

Synthetic retinoids have potential value in the prophylaxis and treatment of epithelial lesions [6, 17]; and experimental evidence indicates that structural modifications of the retinoid molecule may enhance activity with a concomitant decrease in toxicity.

Acknowledgements—Retinoids were obtained from Hoffmann La Roche Inc. (Nutley, N.J.) and from Dr. Y. Fulmer Shealy, Southern Research Institute, Birmingham, Alabama, U.S.A. through the courtesy of the Lung Cancer Segment of the National Cancer Institute, Bethesda, Maryland, U.S.A.

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