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Cancer Chemopreventive Retinoids: Validation and Analysis of *in Vivo* and *in Vitro* Bioassay Results

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Abstract

Several natural and synthetic retinoids (vitamin-A derived analogies) were examined for their potential anti-cancer activity in both *in vivo* animal models and a novel *in vitro* human keratinocyte clonal growth bioassay system. The natural retinoids included all-*trans*-retinoic (RA), 13-*cis*-retinoic acid, 4-oxoretinoic acid, and retinol. Among the synthetic retinoids tested were all *trans N*-(4-hydroxy(phenyl)retinamide, 3-substituted oxoretinoic acids, and 13 *cis*-N-ethylretinamide. The animal models employed were: 1) vitamin A-deficient hamster tracheal organ assay (HTOC); 2) the benzo(*a*)pyrene-induced squamous metaplasia in a hamster tracheal organ system (BP-HTOC); 3) the mouse skin tumor promoter (TPA)-induced ornithine decarboxylase enzyme assay(ODC); 4) the mouse skin papilloma (MPA) assay; and 5) a novel retinoid bioassay in which retinoids display IC_{50} values to inhibit clonal growth of NHK. All-*trans*-RA, 4-oxoretinoic acid and retinol were consistently more active than any of the synthetic derivatives in all bioassays tested. A statistical model was developed and significant positive correlations were found between: 1) ED_{50} values in the HTOC system and reduction in TPA-induced ODC enzyme activity; 2) tumors per animal in the MPA bioassay and suppression of TPA-induced ODC activity; and 3) a positive correlation between suppression of tumors per animal in the MPA assay, and retinoid inhibition of keratinocyte clonal growth. Test retinoids, were tested for their capacity to inhibit the clonal growth of a squamous carcinoma cell line (SCC-25), which were found to be 2 - 3 logs less sensitive for each tested retinoid than the corresponding activity against NHK cells. Antineoplastic retinoid drugs were reviewed.

Keywords

Cancer Chemoprevention *in Vivo* Bioassays, *in Vitro* Bioassays, Retinoids

1. Introduction

Carcinogenesis is a multistep process that alters normal phenotype of cells into malignant counterparts, which acquire the ability to invade and metastasize, resulting in clinically frank cancers. Over the last several decades, significant progress has been made in our understanding of the physiological, genetic, environmental, and biochemical basis of cancer etiology [1]. Retinoids are a class of vitamin A analogs which display remarkable ability to promote the growth and differentiation of a variety of mammalian epithelial tissue [2]. Importantly, retinoids are highly active in the suppression of experimental carcinogenesis in both *in vivo* and *in vitro* animal models [3] [4]. Furthermore, with the finding that retinoids can arrest or reverse the transformed phenotype of cancer cell in *in vitro* experimental carcinogenesis [3], the promise of clinical remission of some cancers has seen some success particularly with the treatment of acute promelocytic leukemia (AML) [5]. Nevertheless, the problem of retinoid resistance [6] [7] has prompted the need to search for new retinoids that might overcome both the problem of toxicity and retinoid resistance. Derivatives of vitamin A, retinoids, have reported activity in treating specific premalignant lesions, and in reducing the incidence of second primary tumors in patients with prior head and neck, lung and liver cancers, but it remains to be proven that retinoids can prevent primary cancers at these sites [8]. With the discovery that retinoids activate nuclear retinoid receptors (RARα, RARβ, and RARγ, and RXR) which form heterodimers that act as transcription activators of specific target genes by modulating gene expression programs, retinoids and rexinoids have been extensively tested in many preclinical studies and for the treatment of malignancies as reviewed elsewhere [9] [10] [11] [12]. In the past, the development of new retinoids has been based primarily on existing data that indicate that substituent modifications could be made at either the nonpolar cyclohexanol ring or at the opposite free carbonyl terminus or at both ends of the vitamin A molecule [13]. Many of these have been found to have potential cancer chemopreventive activity [14] [15]. In this regard, it was reported that the bifunctional retinoids are ineffective as they do not have any binding affinity to the cytoplasmic retinoid receptor CRBPs; similarly, the class of retinoyl-amino acids has been reported to possess activity in the hamster tracheal organ culture (HTOC) bioassay [15] [16]. Close examination of these data, however, indicates that retinoyl-amino acids have anywhere from 3 - 5 logs less activity than t-RA. In support of these data, neither the bifunctional retinamides nor the retinoyl-amino acid binds to CRBP [17]. Studies on a series of conformationally-restricted retinoids showed biological activity in both the HTOC bioassay and the retinoid suppression of tumor promoter-induced ornithine decarboxylase (ODC) enzyme bioassays [18] [19] [20]. The focus of our study was to evaluate the potential chemo-preventive activity of selectively natural and synthetic retinoids using several different biological assays and to determine the validity of the methods by investigating whether significant positive linear correlations existed between two or more of the bioassay data. Earlier, we reported results showing that retinoids were active in reversing keratinization in the standard vitamin A-deficient HTOC correlated with the biological activity of retinoids active in reversing keratinization in the

HTOC-benzo(*a*)pyrene-induced squamous metaplasia [16]. We here, and others have shown a correlation between rank order of retinoid to suppress tumor promoter-induced ornithine decarboxylase (ODC) enzyme bioassay [21] [22] and retinoid rank order of potencies in the mouse skin two-stage carcinogenesis (MPA) bioassay. Here, we also report the development of a novel bioassay that employs a rapid and effective *in vitro* keratinocyte clonal growth method to yield a sensitive rank order of the retinoid potencies with both the ODC and the MPA bioassays.

2. Materials and Methods

Chemicals: RA was purchased from Sigma Chemical Co., St. Louis, MO. All other retinoids were prepared and supplied by Dr. Y. Fulmer Shealy of Southern Research Institute, Birmingham, AL.

Animals: Female CD-1 mice were purchased from commercial source (Charles River) and housed and fed as previously reported [16]. Syrian Golden hamsters were purchased from Charles River and housed and feed as previously reported [16].

Bioassay methods: 1) ODC Assay: retinoid-mediated suppression of tumor promoter-induced ornithine decarboxylase (ODC) enzyme activity in mouse skin epidermal extracts: Briefly, a group of 3 to 4 CD-1 mice were shaved and their backs painted with 0.2 mL of acetone (control) or 0.2 mL of 17 nm of 13-tetradecanoyl-phorbol-12-acetate (TPA). The area of treated skin was excised and epidermal extracts prepared and their ODC activity was determined by measuring the production of $^{14}C\text{-}CO_2$ formed by conversion of radiolabelled putrescine to ornithine as previously described [21] [22]. All ODC assays were performed on mice sacrificed 5 hours after TPA treatment. The protocol developed for testing retinoids always included two TPA only treatment groups, one at the start of the assay and one at the end of the assay to ensure that the inhibitory effect of the unknown test retinoid on ODC levels was bracketed in the window of maximal TPA induction. All test retinoids were stored in N_2(l) as 10^{-2} M stock solutions in dimethylsulfoxide (DMSO), and diluted into acetone in subdued yellow fluorescent lights immediately before application to the shaved backs of mice. The test retinoids were applied 30 minutes prior to TPA.

2) Retinoid-mediated suppression of tumors in mouse skin initiation promotion of tumorigenesis: the procedures employed in this assay are those previously described [23]. Briefly, the shaved backs of CD-1 mice were painted with a single application of 51.2 μg of 7, 12-dimethylbenzanthracene (DMBA) in 0.2 mL of acetone. Two weeks later, a group of 10 shaved mice which received either only acetone or which received only DMBA, were further treated biweekly correspondingly with either TPA only, or acetone only, while the test retinoid was applied 30 minutes before TPA. The mice were examined weekly thereafter and visually scored the production of papillomas. At the 15 week termination of the trial, all TPA-treated mice were necrotized by carbon dioxide, and an exact number of tumors per animal and the area size of each tumor recorded.

3) Vitamin A-deficient bioassay: the procedures for standard hamster tracheal organ culture (HTOC) have been previously described [16]. The procedure measures the abil-

ity of a given retinoid to reverse keratinization of tracheal explants derived from hamster in early stage of vitamin A deficiency. Briefly, tracheas are stripped of adherent fat and extraneous tissue, placed in 60 mm petri dish and cultured in 2 mL of serum-free medium, placed in humidified culture boxes and cultured for 3 days without retinoids, after which they are refed fresh medium containing retinoids dissolved in DMSO or DMSO alone. Tracheas are harvested, fixed, embedded in paraffin, sectioned, stained and scored for the presence of keratin, and keratohyaline granules. As a control, we employed RA at 1×10^{-9} M which suppressed keratinization by approximately 90%.

4) Benzo(*a*)pyrene (BP) bioassay: the BP-HTOC bioassay is similar to that described above, except that keratinization is induced by 5.0 μg/ml of benzo(*a*)pyrene (BP) in the explants of normal hamster tracheas as previously described [16].

5) Retinoid inhibition of normal and malignant keratinocyte clonal growth: the procedures employed in this bioassay are similar to those previously described [24]. Briefly, standard clonal growth assay were initiated with 500 cells per 60 mm Petri dish cultured in MCDB 153 serum-free medium supplemented with 5 ng/ml of epidermal growth factor and 5 mg/ml of porcine insulin. The dishes were gassed with 95% air and 5% carbon dioxide in a humidified incubator at 37˚C for 24 hours at which time serial dilutions of each retinoid were added to duplicate dishes and the dishes incubated for an additional 10 days, fixed, stained and the number of colonies per dish counted. The final concentrations of each retinoid were made by 10-fold serial dilutions from retinoid stock solutions (10^{-2} M) and ranged in concentration from 10^{-5} M to 10^{-11} M. Growth inhibition was calculated as an IC_{50} determined by plotting the log percent reduction of colonies per dish against concentration (M) of retinoid, and analyzed by performing a linear regression analysis to determine the significance of the result.

Statistical correlations: statistical correlations were performed by computer programs designed to test the significance of positive correlations sought between for data obtained between the different bioassays. A corresponding straight line formula was obtained for each type of correlation tested.

3. Results

Table 1 provides lists the retinoids and corresponding Southern Research Institute (SoRI) numbers. Figure 1 gives their corresponding chemical structural formulas. Additional chemical strictures are given for particular oxoretinoids and bifunctional retinoid analogs in the Results section.

3.1. Evaluation of Retinoid Suppression in Mouse Skin ODC Assay

Figure 2 presents the results obtained for the time course of ODC enzyme activity induced by a single application of TPA to the shaved backs of CD-1 mice. The peak activity routinely occurs between 4 - 5 hours post-treatment. Table 2 presents a summary of our data showing the comparative efficacy of 45 different retinoids to inhibit TPA-induced ODC enzyme activity. In this bioassay, each retinoid was tested by applying 17 nm of retinoid dissolved in acetone 30 minutes prior to applying 17 nm of TPA to the

Table 1. List of retinoids and their SoRI numbers.

Retinoid	SoRI#
t-Retinoic acid	6611
t-Retinol	6631
CIS-Retinol	6632
t-4-hydroxyphenylretinamide	6612
13-cis-Retinoic acid	6618
4-Oxoretinoic acid	6621
t-Rdetinyl methyl ether	6633
cis-Retinyl methyl ether	6634
t-Retinyl propyl ester	6635
t, cis-Dicarboxyretinoic acid	6637
t-Rerinoic acid methyl ester	6638
3-(2-propyl)-4-oxoretinoic acid methyl ester	6692
t-Retinyl butyl ether	6705
3-Methyl-4-oxoretinoic acidmethyl ester	6719
3-Ethyl-4-oxoretinoic acidethyl ester	6720
3-(phenylmethyl)-4-oxoretinoic acid	6776
4-Oxo-3-(2-propenyl)retinoic acid methyl ester	6777
3-Methyl-4-oxoretinoic acid	6800
N-all-trans Retinoyl-L = leucine	6623
n-4-Retinoyl-tyrsine	6624
N-t-retinoyl-alanine	6628
N-4-Retinoyl-phenylalanine	6629
4-Oxo-3-(2-propnyl)retinoic acid	6822
3-Ethyl-4-oxoretinoic acidethyl ester	6832
14[(Ethylamin)carbonyl]-13-cis-retinoic acid	6727
14[(Ethylamino)carboyl]retinoic acid	6728

same skin area, and the ODC enzyme activity determined. With only acetone applied post-TPA treatment applied an IC_{50} of 10 ODC units is consistently found, where 1 ODC value is defined as 1 nm CO_2 released/per 30 min/mg protein. This represents an 80-fold increase in stimulated activity above negligible background level in mice treatment only with acetone. As expected all-trans-retinoic acid (t-RA) was the most active (0.1 ± 0.1 SE) units; it suppressed TPA-stimulated ODC by >90%. The 13-cis-retinoid stereoisomer of t-RA was then next most active (2.2 ± 0.8 SE). Both t-RA and 13-cisRA inhibited ODC activity is a dose-dependent fashion and IC_{50} values of 2×10^{-11} M and 1.7×10^{-9} M, respectively. Other well-studied synthetic retinoids such as 13-cis-N-

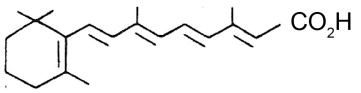
Table 2. ODC activity of select retinoids in mouse skin.

Retinoid	ODC	N	Retinoid	ODC	N
Acetone	0.1	7	t,cis-Dicarboxyl)retinoic acid	6.4	4
t-Retinoic acid	0.8	7	t,cis,Retinoic acid diethyl ester	8.3	2
13-cis-retinoic acid	2.2	4	t-N-Ethylretinamide,		
13-cis-Ethyl retinamide	4.5	2	cis-Retinoic acid ethyl ester	9.1	3
1-4-Hydroxy-phenylretinamide	6.8	5	t-Retinoic acid ethyl ester,		
t-Retinoyl-glycine	8	3	cis-N-Ethylretinamide	5.4	2
cis-Retinoyl-glycine	5.7	1	13-cis-14(Ethylamino carbonyl		
t-Retinoyl-leucine	8.7	1	Retinoic acid	3	1
cis-Retinoyl-leucine	8.9	3	14[Ethylamino)carbonyl]		
t-Retinoyl-alanine	8.8	4	Retinoic acid	8	1
t-Retinoyl-phenylalanine	8.2	3	N-(17-Oxoestra-1,3,5-(10)trien		
cis-Retinoyl-glycine	7.5	1	2-yl)Retinamide	3.1	1
cis-Retinoyl-glycine ethyl ester	5.1	1	4-Oxoretinoic acid	0.1	3
t-Retinyl methyl eater	5.2	3	4-Oxoretinoic acid methyl ester	0.2	3
t-Retinyl propyl ether	7.5	3	3-Methyl-4-oxoretinoic acid	1.6	3
t-Retinyl2,4-hexadienyl ether	3	1	3-(Phenylmethyl)-4-oxoretinoic acid	1.7	3
t-Retinyl butyl ether	13.6	1	3-(Phenylmethyl)-4-oxoretinoic acid		
t-Retinyl2-(1-aadamantyl)ethyl ether	4.6	1	Methyl ester	3.2	2
t-Retinylcinnamyl ether	9.8	1	4-Oxo-3)2-propenyl)retinoic acid	5	2
t-Retinyl-3-methyl-2-butenyl ether	5.6	1	4-Oxo-3(2-propenyl)retinoic acid methyl		
			Ester	5.6	1
			4-Oxo-3(propynl)-retinoic acid	2.2	1
			3-Ethyl-4-oxoretinoic acid	4.7	1
			3,3-Diethyl-4-oxoretinoic acid		
			Methyl ester	2.7	1
3-Ethyl-4-oxoretinoic acid	3.2	1			
3,3-Dimethyloxoretiboic acid					
Methyl ester	3.3	1			
(E)-4(2-(5,6,7,8-tatrahydro8,8					
Diethyl-2-napthyl)propenyl)					
Phenylmethanol	3.2	2			
(E)-4(2-(5,6,7,8-tatrahydro8,8					

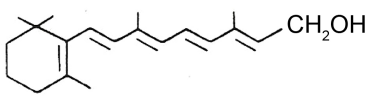
Continued

Dimethylpropenyl)benzoic acid	0.9	2
(E)-4(2-(5,6,7,8-Tatrahydro8,8		
Diethylpropenyl)benzoic acid	0.7	2
(w2E,4E,6E Methyl-7-(5,6,7,8-		
Tetrahydro-8,8-dimethyl-2-naphthyl-2-4,6-		
Octotrienoic acid	1	2
(6E,8E)-7-Dimethyl-9-(2,6,6)tri-		
Methylcyclohexanyl)34,6,8-		
Nonatetraen1-01	3.3	1
Trimethyl methoxyl phenyl		
Analog of retinal	2.8	1
Trimethyl methoxyl phenyl		
Analog of retinyl methyl ether	3.6	1

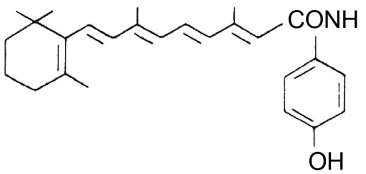
ethylretinamide13-cis-NER) and 4-hydroxyphenylretinamide (4-HPR) had ODC units of 4.5 and 6.8, respectively and IC_{50} values of 1×10^{-8} M and 2×10^{-7} M, respectively. A number of novel alkyl ethers of this series were synthesized in the ODC bioassay. Trans-retinylmethyl ester (t-RME) and the TMMP analogue of t-RME had about equal activity with an IC_{50} value of 17nm. None of the other retinyl esters showed significant activity. Both etretinate and the TMMP analogue of t-retinol were as active in inhibiting ODC as the TMMP analogues of t-RME. Four compounds of the arotinoid series were tested. Two displayed good activity comparable to t-retinol, *i.e.*, (E)-4-[2-5,6,7,8-tetrahydro-8,8-dimethyl-2-napthyl propenyl[phenylmethanol] and (6E,8E)-3,7-dimethyl-9-(2,6,6-trimethylcyclohexanol 0-3,4,6,8-nonatetraen-1-01. and two others displayed good activity that was equal in efficacy to RA, *i.e.*, (E)-4-[2-(5,6,7,8-tetrahydro-8,8-dimethyl-2-propenyl)benzoic acid and (2E, 4E, 6E)-3-methyl-7-(5,6,7,8-tetrahydro-6-dimethyl-2-napthyl)-2,4,6-octarienoic acid. **Figure 3** presents structural formulas for several different retinoyl amino acids. Including the *trans* and *cis* isomers of glycine and leucine and the L stereoisomers of the all *trans* isomers of alanine, phenylalanine, tyrosine and glutamic acid. Only the L-stereoisomers of glycine and leucine had significant activity. The addition of a second carbonyl group at the C12-C14 terminus of the retinoid skeleton was examined for possible bioactive retinoid. The dicarboxylic acid derivatives, 13 cis, 14-*trans* RA had sharply reduced activity relative to t-RA in the ODC bioassay. The mono-substituted *trans* ethyl ester of RA had moderate activity, but the diethyl ester of RA was without significant activity. Two other bifunctional derivatives were synthesized one with an ethyl ester at either the 13-*cis* or 14 *trans* positions and an N-ethyl amide at the corresponding C13-C14 position. The 13-*cis* ethyl ester of N-ethyl retinamide was inactive whereas the 13-*cis*-N-ethylretinamide of the ethyl ester of RA had moderate activity. Likewise, the 14[(ethylamino)-carbonyl]retinoic acid was



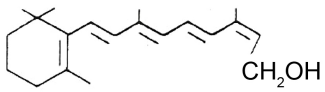
All-trans-Retinoic Acid



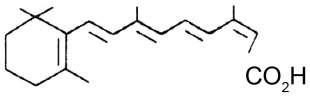
All-trans-Retinol
6631



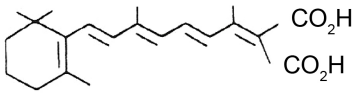
ll-trans-N-(4-Hydroxypheny)retinamide
6612



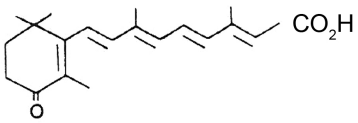
13-cis-Retinol
6632



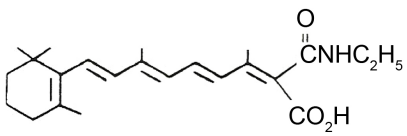
13-cis-Retinoic Acid
6618



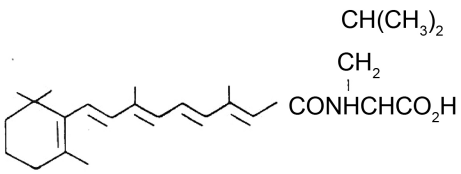
14-Carboxyretinoic Acid
6637



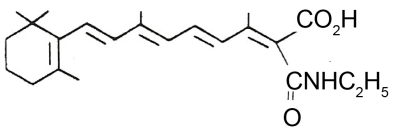
All-trans-4-Oxoretinoic Acid
6621



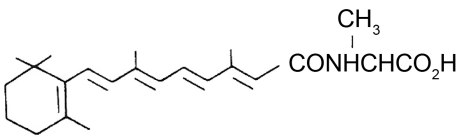
14-[(Ethylamino) carbonyl]-13-cis-retinoic Acid
6637



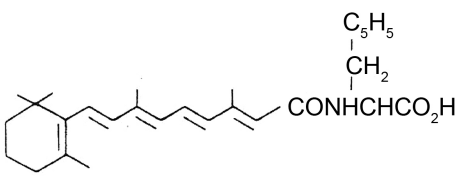
N-(All-trans-Retinoyl)leucine
6623



14-[(Ethylamino) carbonyl]retinoic Acid
6728



N-(All-trans-Retinoyl)alanine
6628



N-(All-trans-Retinoyl)phenylalanine
6629

Figure 1. Chemical structural formulas and SoRI numbers of select retinoids.

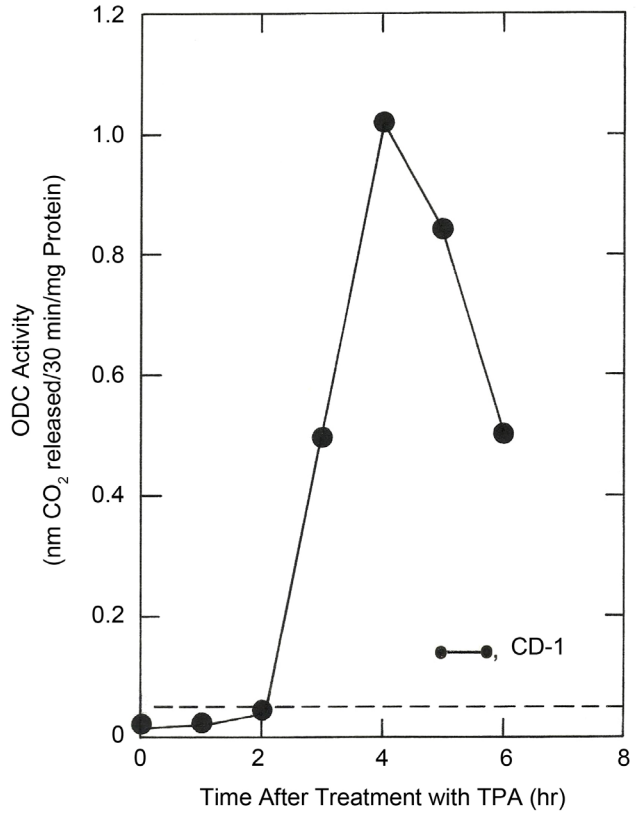


Figure 2. Kinetics of TPA-induced ornithine decarboxylase activity in CD-1 mouse epidermis.

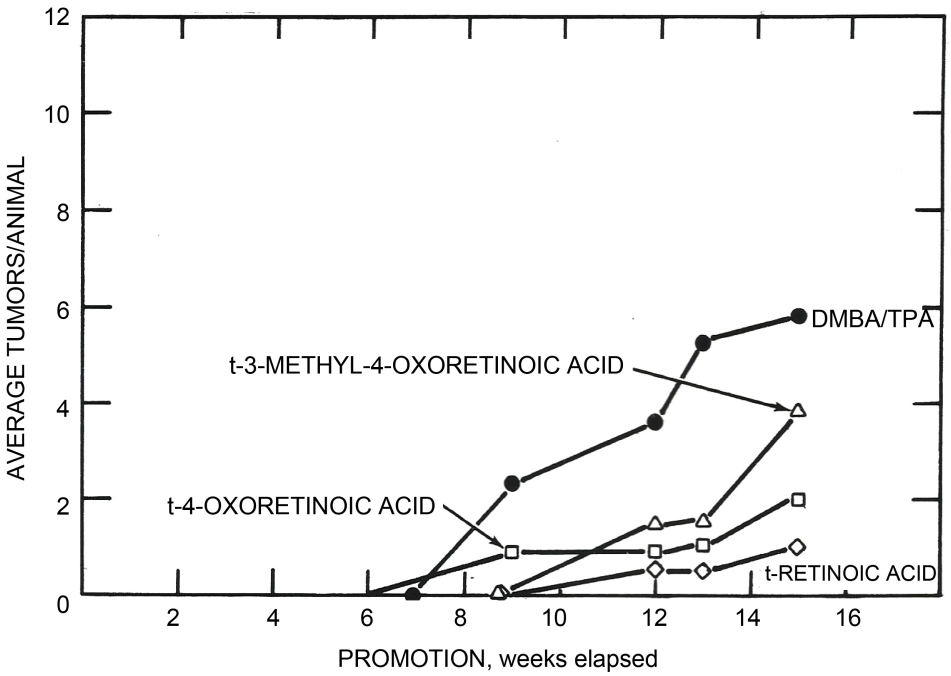


Figure 3. Time course of papilloma promotion in the MPA bioassay for: t-retinoic acid, t-RA (◊), 4-oxoretinoic acid (□), 3-methyl-4-oxoretinoic acid (Δ), and DMBA/TPA control (●).

inactive, whereas the 14[(ethylamino)-13] *cis*-retinoic acid had good activity. Earlier, it was reported [25] that 4-oxoretinoic acid, a natural metabolite of RA, is a biological active retinoid. We examined both 4-oxoretinoic acid and more than a dozen different 3-substitute 4-oxoretinoic acids and their corresponding methyl ester (see **Table 1**, #s:6, 23 - 26). The majority of the all-trans-4-oxoretinoic acids derivatives had good bioactivity in the ODC bioassay equal to RA or retinol. The 3-ethyl-, 3-(2-propenyl) and 3-(2-propenyl) derivatives of 4-oxoretinoic acid were less active.

3.2. Evaluation of 4-Oxoretinoids in the Mouse Skin Papilloma Bioassay

As expected from previous HTOC results 4-oxoretinoic acid (see **Table 1**, SoRI 6621) was one of the best natural retinoids in suppressing tumors in the MPA bioassay. In several independent studies, there were an average of 2.0 ± 3 S.E. tumors per animal compared with RA (1.0 tumors per animal) at 15 weeks. **Figure 3** presents a plot comparing the average number of tumors per animal (ordinate) against weeks of TPA promotion (abscissa) comparing the results of 4-oxoretinoic acid, and a oxoretinoic derivative, t-methyl-4-oxoretinoic acid, compared with the positive control (t-RA) and DMBA/TPA. Clearly, 4-oxoretinoic acid is nearly as effective over the time course TPA promotion as was the 3-methyl derivative relative to t-RA even as early as 12 weeks relative to DMBA/TPA. For comparison, **Figure 4** presents a composite plot comparing the average number of tumors per animal (ordinate) against weeks of TPA promotion

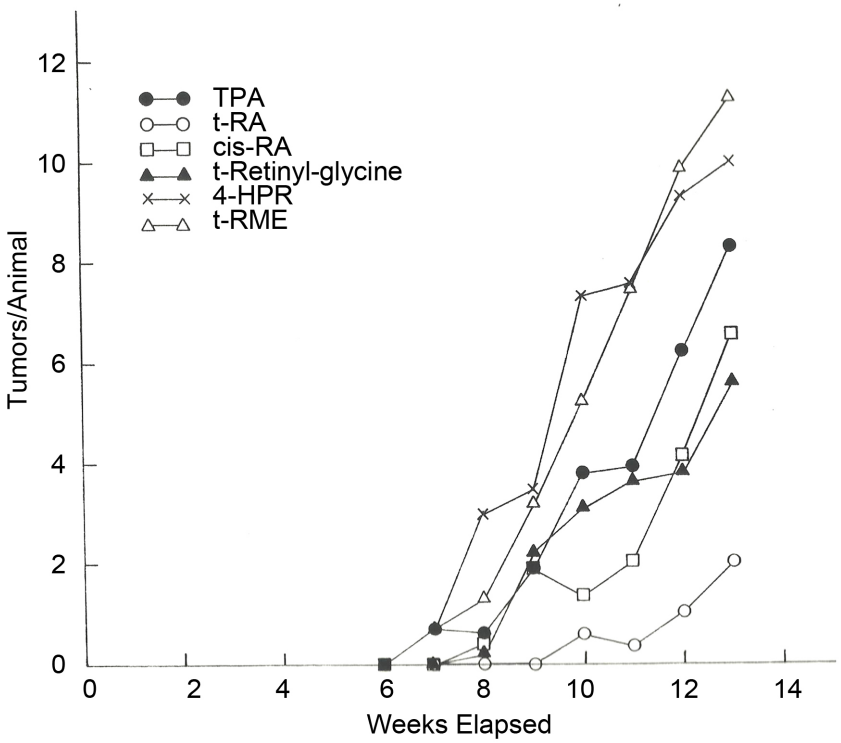


Figure 4. Comparison of time course of papilloma promotion (weeks) in the MPA bioassay for 5 different retinoids: t-RA (○), 13-cis-retinoic acid, cis-RA (□), t-retinoyl-glycine (▲), 4-hydroxyphenylretinamide, 4-HPR (x), t-retinyl methyl ether, t-RME (Δ), and DMBA/TPA control (●).

(abscissa) for several well-studied retinoids: t-RA, 13-*cis*-RA, t-RME, t-RPE. 4HPR and DMBA/TPA. Interestingly, both t-RME and 4HPR actually produced more tumors over the time course of TPA-promotion than DMBA/TPA suggesting that they are themselves tumor promoters relative to the classic chemopreventive retinoids t-RA and 13-*cis*-RA. Both of the 3-substituted ethyl derivatives of 4-oxoretinoic acid were only marginally active. The N-17-oxoestra-1,3,5[10]-trien-2-yl retinamide had some marginal activity, while no activity was detected for the 13-*cis*-N-ethylretinamide indicating that retinamides are not equally active is suppressing mouse skin tumors. The TMMP analogues of retinol had no anti-tumor activity nor did any of the compounds tested in the retinyl ether and dicarboxyl retinoic acid series (Table 3).

3.3. Evaluation of Some Retinoyl-Amino Acid in the Mouse Skin Papilloma Bioassay

Figure 5 shows the general structural formula of retinoyl amino acid compounds [26],

Table 3. Effect of many different 4-oxoretinoic acid derivatives and some bifunctional compounds on the suppression of tumorigeneis nesis in the mouse skin papilloma bioassay.

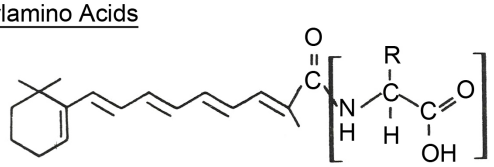
		Tumors/Animal ±S.E.	
Retinoids	**N**	**Week 15**	**Tumor Size (mm^2)**
DMBA/TPA	15	9.2 ± 6.8	313.5
t-Retinoic acid	8	1.0 ± 1.0	14.5
N-(Oxoestra-1,3,5(10)-trien-yl-retinamide	8	6.4 ± 4.0	128
4-Oxo-3(2-propenyl)retinoic acid	7	4.3 ± 4.3	61.5
4-Oxo-3-(2-propenyl)retinyl methyl ester	7	3.9 ± 3.2	54.3
4-Oxo-3-(2-propnyl)retinoic acid	8	5.9 ± 5.2	126
3-Ethyl-4-oxoretinyl methyl ester	8	6.0 ± 5.8	276
3,3-Dimethyl4-oxoretinyl methyl ester	7	2.0 ± 4.0	40
3-Ethyl 4-oxoretinyl methyl ester	8	7.1 ± 5.8	94
4-Oxo-3-(2-propenyl)retinyl methyl ester	7	3.7 ± 3.5	72.5
TMMP analog of retinol	7	13.7 ± 10.2	504.5
TMMP analog of retinol Log of retinyl methyl ether	8	11.9 ± 7.1	228
(E)-4[-2-(5,6,7,8-Tetra hydro-8,8-dimethyl 2-Naphthyl)propenyl]phenylmethanol	8	1.0 ± 1.3	4
(E)-4[-2-(5,6,7,8-Tetra hydro-8,8-dimethyl 2-Naphthylpropenyl]benzoic acid	7		2
(2E,4E,6E)-3-Methyl-7(5,6,7,8-tetrahydro[-8,8-Dimethyl)2-naphthyl)2,4,6-oxoretinoic acid	7	1.7 ± 2.0	12
Acetone	15	0	
Acetone/TPA	15	0	
DMBA/Acetone	7	0	

and a list of those synthesized and tested in the MPA assay. Figure 6 presents a plot of TPA-promotion of retninoyl-glycine in comparison to t-RA, cis-RA and DMBA/TPA. Among the retinoyl-L-amino acid tested only retinoyl-L-glycine and t-retinoyl-l-leucine (see Figure 4) displayed marginal antitumor activity.

3.4. Evaluation of Retinoids in the HTOC Bioassay

The HTOC bioassay is widely used one of the standard procedures to assay new retinoids. Retinoids are evaluated by their ability to reverse squamous metaplasia and keratinization of the tracheal epithelium in organ explants from vitamin A-deficient hamsters.

Retinoylamino Acids



NAME:	ABBREVIATION:
N-(All-trans-retinoyl)-L-leucine	(t-RA-leu)
N-(All-trans-retinoyl)-L-phenylalanine	(t-RA-ala)
N-(All-trans-retinoyl)-L-alanine	(t-RA-ala)
N-(All-trans-retinoyl)-L-leucine	(cis-RA-leu)
N-(All-trans-retinoyl)-L-glycine	(t-RA-gly)

Figure 5. Chemical structural formula for general retinoyl-amino acids and a list of 5 different retinoyl-amino acids and their abbreviations.

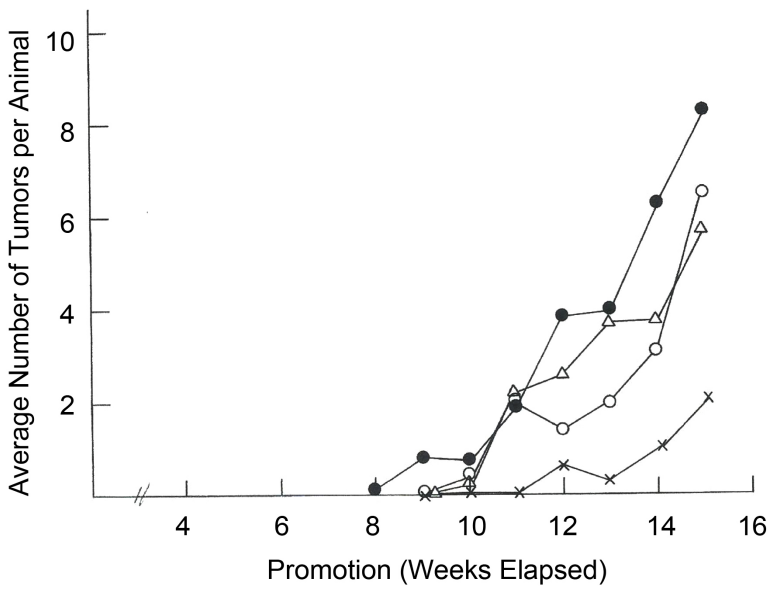


Figure 6. Comparison of the time-course of papilloma formation (weeks) in the MPA bioassay for: t-RA (x), 13-cis-RA (○), t-RA-gly-cine (Δ), and DMBA/TPA control (●).

Recently, we developed a second HTOC assay which is rapid, reliable and more relevant to antitumor activity of retinoids. In this BP-HTOC bioassay, the carcinogen, benzo(*a*)pyrene is used to induce keratinizing lesions of the tracheal epithelium of normal tracheas organs cultured in Vitro. Normal tracheas cultured in control medium do not develop lesions. Table 4 presents a comparison of ED_{50} (M) values for 12 natural and synthetic retinoids. As previously reported [16] all of the natural retinoids t-RA, 13-*cis*-RA, t-retinol, 13-*cis*-retinol and 4-oxoretinoic acid are more effective than any of the synthetic retinoids tested. The 3-methyl -4-oxoretinoic acid had ED_{50} values of 4×10^{-10} M and 2×10^{-9} M, respectively in the BP-HTOC and vitamin-A deficient HTOC bioassays. The dicarboxy t-RA, 13-*cis*-RA had ED_{50} values of 3.3×10^{-9} M and 1.0×10^{-8} M, respectively in the BP-HTOC, and vitamin-A-deficient HTOC bioassays, respectively. The retinoyl-amino acids in these assay displayed only marginal effective activity as did the bifunctional methylamino carbonyl derivatives of retinoic acid. Note that the relative rank order of efficacy is the same in both assays. For other synthetic retinoids, among the retinyl esters tested in the vitamin-A-deficient HTOC bioassay, t-retinyl methyl ether (t-RME), t-retinyl propyl ether (t-RPE), t-retinyl carbonylmethyl ether (t-RCME), and 13-*cis*-rethyl methyl ether (*cis*-RME) had a ED_{50} value of 2.9×10^{-9} M, 5×10^{-7} M, 2.8×10^{-8} M and 1.5×10^{-8} M, respectively.

Table 4. Comparison of the BP-HTOC and the standard HTOC bioassay.

		Inhibition of Keratinization (ED-50)[1]	
Retinoid	SoRI No.	BP-HTOC Assay	Standard HTOC Assay
t-Retinoic acid		4.0 × (E−12)	2.0 × (E−11)
13-cis-Retinoic acid	6618	1.0 × (E−11)	3.3 × (E−10)
t-4-Oxoretinoic acid	6621	4.0 × (E−11)	1.0 × (E−9)
t-Retinol	6631	3.5 × (E−10)	2.4 × (E−9)
13-cis-Retinol	6632	1.0 × (E−10)	4.0 × (E−9)
t-4-Hydroxyphenylretinamide	6612	1.5 × (E−10)	3.5 × (E−9)
14-Carboxyretinoic acid	6637	3.3 × (E−9)	1.0 × (E−8)
N-(t-Retinoyl)-leucine	6623	4.0 × (E−9)	2.0 × (E−8)
N-(t-Retinoyl)-alanine	6628	5.0 × (E−9)	2.5 × (E−8)
14-[Ethylamino)-carbonyl]-retinoic acid	6728	1.0 × (E−8)	2.7 × (E−8)
14[(Ethylamino)-carbonyl] 13-cis retinoic acid	6727	1.0 × (E−8)	3.5 × (E−8)
N-(t-Retinoyl)phenylalanine	6729	3.5 × (E−7)	1.0 × (E−6)

[1]ED-50 values were estimated graphically from best-fitting straight lines obtained by standard methods of linear regression analysis using data derived from typical experiments.

3.5. Evaluation of Retinoids by Determining the Dose-Dependent *in Vitro* Inhibition of Clonal Growth of NHK and Squamous Carcinoma Cells (SCC) Bioassay

A model system has been developed to screen retinoids for antitumor potential. It involves a quantitative assay of the dose-dependent inhibition of the proliferative potential of normal human keratinocytes (NHK) cultured in a serum-free medium in the presence of increasing concentrations of the test retinoids. The rationale for this bioassay is the need for a rapid and reliable assay that correlates with antineoplastic activity in other bioassays (ODC, MPA and HTOC), and is relevant to chemoprevention in human cells. Previous studies [27] reported that all-*trans*-RA inhibited batch culture growth of HeLa cells and arrested their growth in the G1 phase of the cell cycle. **Figure 7** shows that RA treatment of NHK cultures with 0.1 μm, 0.5 μM, 1 μM and 2 μM for 2 days inhibited growth by 20%, 23%, 33% and 40%, respectively. Flow microfluorimetry analysis showed that RA arrests NHK cells in the G_1 phase of the cell cycle (41% in G_1 for untreated NHK compared to 58% for RA-treated). By contrast, **Figure 8** shows that 0.1 μM RA treatment of SCC-25, a squamous carcinoma cell line, led to 60% inhibition of culture growth. Flow microfluorimetry analysis of cell cycle distributions of SCC-25 treated for 2 days of culture with 2 μM RA showed a G1 arrest at 57%. This compares with 41% of cell in G_1 in untreated SACC-25 cells. **Figure 9** presents the results of a concentration-dependent inhibition of NHK clonal growth by t-RA. The calculated IC_{50} value for RA was 2×10^{-9} M. IC_{50} ,defined as the molar (M) concentration that inhibits 50% of the total number of colonies per dish relative to untreated control (no retinoid). **Figure 10** presents the results of a concentration-dependent inhibition of SCC-25 clonal growth by t-RA. The calculated IC_{50} value for RA was 1×10^{-6} M. This represents an almost 3 log fold reduction in sensitivity of clonal growth inhibition by SCC-25 tumor cell line relative to normal keratinocytes. Four select retinoids, t-RA, 13-cis-RME 4-HPR and N-ER, were compared for their relative inhibitory effect on clonal growth of

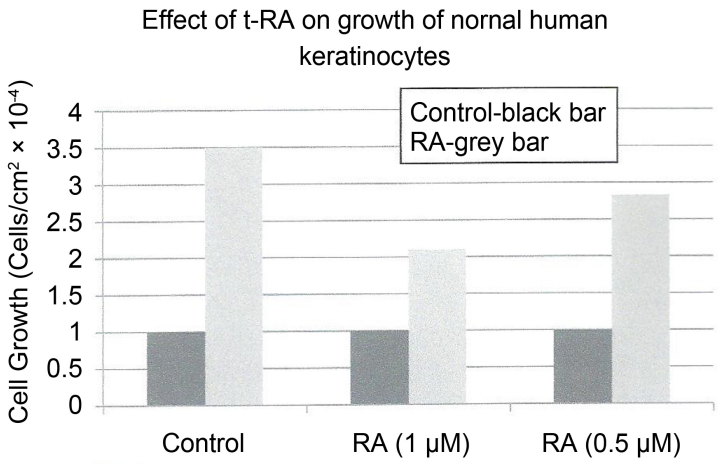


Figure 7. Effect of 2 days treatment with all-*trans*-RA on NHK cell growth. Control untreated (black bar); Abscissa: 1 μM and 0.5 μM RA-treated cultures (grey bar). Ordinate (cell/$cm^2 \times 10^{-4}$).

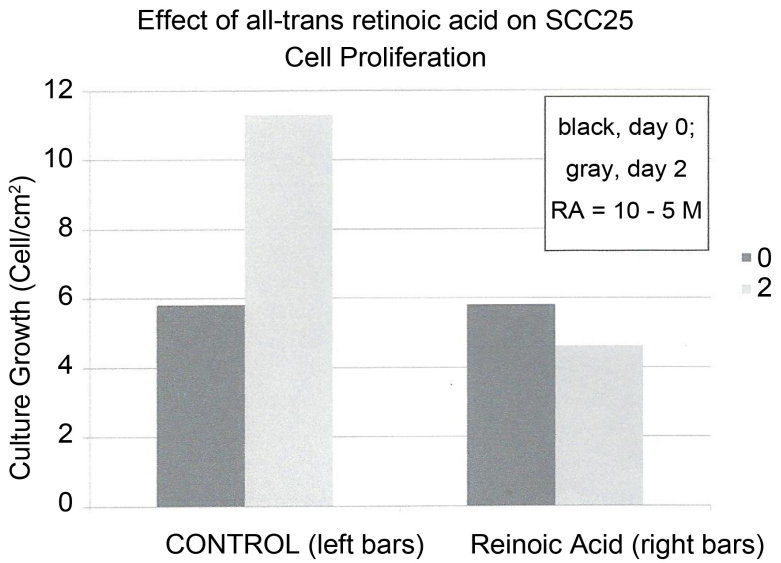


Figure 8. Effect of 2 days treatment with 1×10^{-5} M all-*trans*-RA on NHK cell growth. Control (left bars black-day 0; grey bar-day 2); Retinoic acid, RA (right bars (black bar-day 0, grey bard-day 2).

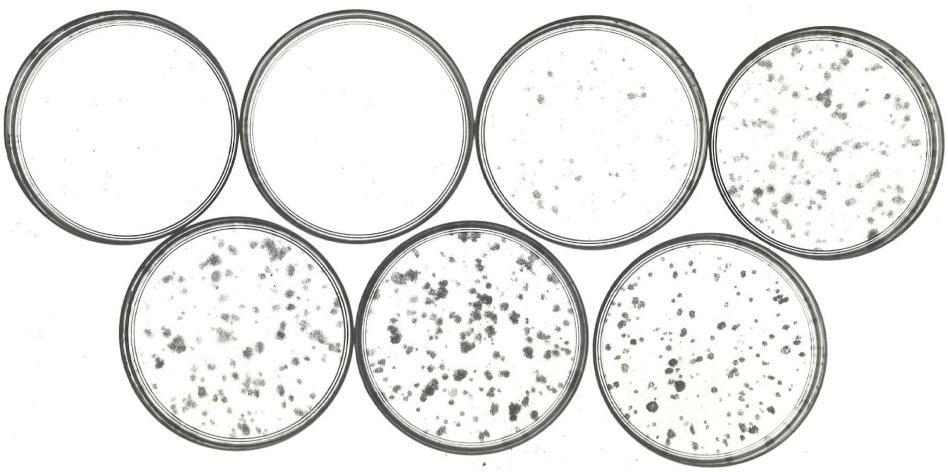


Figure 9. Photograph of a panel of culture dishes showing the effect of varying concentration of all-trans-RA on the inhibition of NHK clonal growth. Top row left to right: (A) 1×10^{-5} M, (B) 1×10^{-6} M, (C) 1×10^{-7} M, (D)1×10^{-8} M; bottom row left to right: (E) 1×10^{-9} M, (F) 1×10^{-10} M, and (G) 1×10^{-11} M. Total magnification: 3/4×.

NHK (Figure 11) and SCC-25 (Figure 12). The rank order of NHK sensitivity to clonal growth inhibition for these select retinoids was RA > 13-cis-RA > 4HPR > N-ER. Correspondingly, the rank order of sensitivity to inhibition of SCC-25 clonal growth for these select retinoids was identical with 2 - 3 $\log_{10}$ less sensitivity for SCC-25 cell line. The effect of five other retinoids (*trans*-retinol, *cis*-retinol, *trans*-RME, *trans*-retinoyl-glycine and *trans*-TPE) gave similar results with a rank order of sensitivity to inhibition of clonal growth identical in both NHK and SCC-25, again, with a 1 - 2 log less sensitivity for SCC-25 relative to NHK (see Table 5).

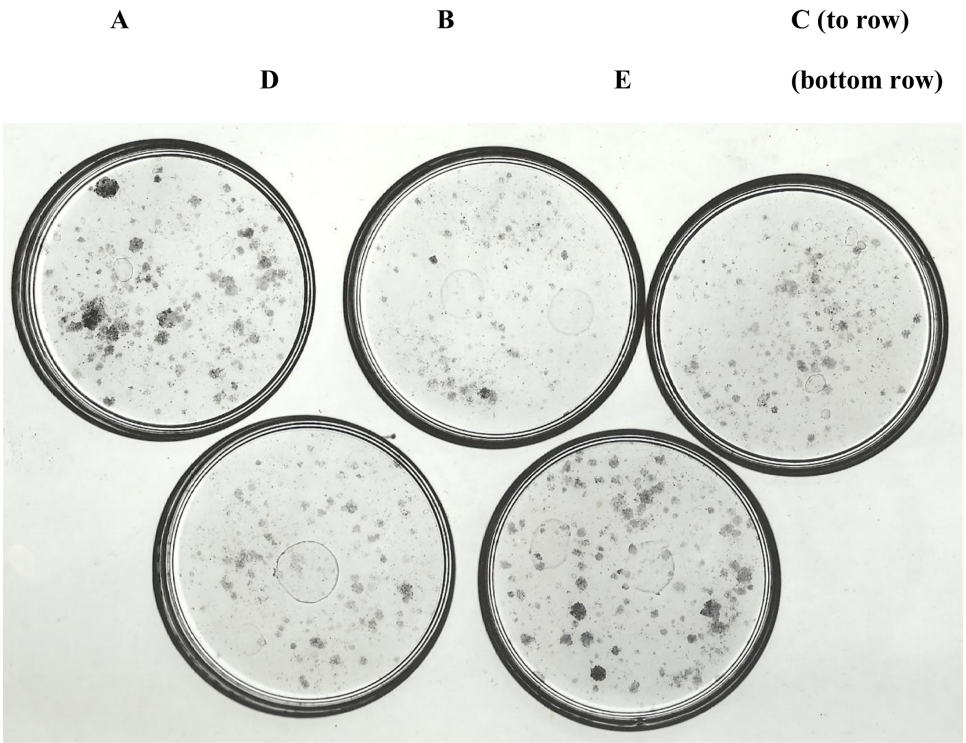


Figure 10. Photograph of a panel of culture dishes showing the effect of varying concentrations of all-trans-RA on the inhibition of SCC-25 clonal growth. Top row from left to right: (A) control RA-untreated, (B) 1×10^{-5} M, (C) 1×10^{-6} M; bottom row from left to right: (D) 1×10^{-7} M, and (E) 1×10^{-8} M. Total magnification, 1×.

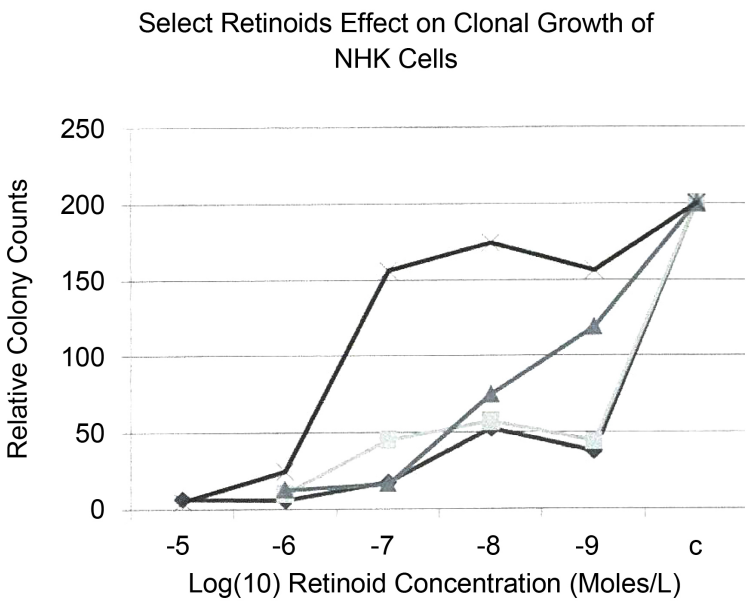


Figure 11. Effect of varying concentrations of 4 select retinoids on NHK clonal growth. Ordinate: relative colony counts; abscissa: $\log_{10}$ M retinoid concentration; c, control untreated. t-RA (solid ◊), 13-cis-RA (▬), 4-HPR (▲), and NER (X).

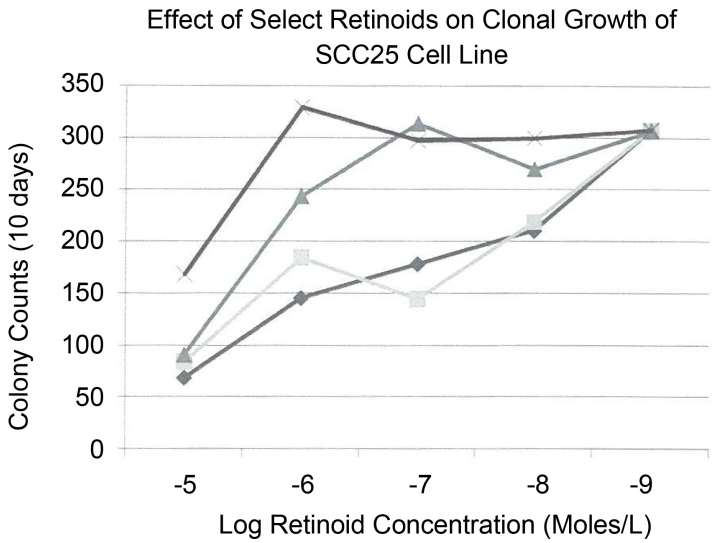


Figure 12. Effect of varying concentrations of 4 select retinoids on clonal growth of SCC-25 cells Ordinate: colony count per dish; abscissa: $\log_{10}$M retinoid concentration: c, control untreated. t-RA (solid ◊), 13-cis-RA (▬), 4-HPR (▲), and NER (X).

Table 5. Effect of select retinoids on the clonal growth of NHK and SCC-25 cells.

Retinoid	NHK (IC-50, M)	SCC-25 (IC-50, M)
trans-Retinoic acid	2.0 × (E−9)	3.0 × (E−6)
13-cis-Retinoic acid	1.0 × (E−8)	1.0 × (E−7)
4-Hydroxyphenylretinamide	1.0 × (E−8)	1.0 × (E−7)
cis-Retinol	2.5 × (E−8	5.0 × (E−6)
trans-Retinol	4.0 × (E−8)	9.0 × (E−7)
trans-Retinyl methyl ether	1.0 × (E−7)	1.0 × (E−5)
trans-Retinoyl-glycine	3.0 × (E−7)	3.0 × (E−6)
N-Ethylretinamide	3.6 × (E−6)	1.8 × (E−6)
trans-Retinylpropyl ether	8.0 × (E−7)	5.0 × (E−6)
trans-Retinylpropyl ether	8.0 × (E−7)	5.0 × (E−6)

3.6. Statistical Analysis of Retinoid Bioassays

We have examined whether there are strong positive correlations between the several different *in vivo* bioassay and between the different *in vivo* and in vitro clonal growth bioassays. Figure 13 documents a positive linear correlation for 13 different retinoids (#s:1-13) between the log ED_{50} (M) of the HTOC bioassay over a 5-log range and for the log reduction in TPA-induced ODC enzyme bioassay over a 95% range in suppression of ODC activity. Linear regression analysis confirms a significant positive correlation (r = 0.81. Figure 14 plots retinoid data showing a positive linear correlation exists

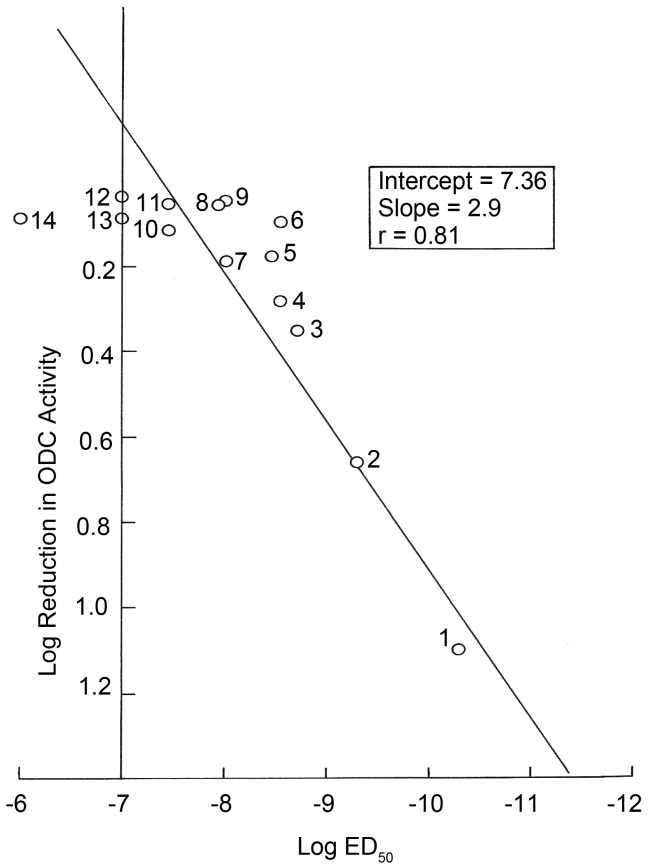


Figure 13. Linear correlation between vitamin A-deficient HTOC ED_{50}, M values (abscissa) and log reduction in ODC activity for select retinoids (ordinate):1) t-RA, 2) 13-cis-RA), 3) 13-cis-NER, 4) t-RME, 5) 4-HPR, 6) t-RPE, 7) dicarboxy, t-RA/cis RA, 8) t-RA-glycine, 9) t-REE, 10) t-RA-leucine, 11) t-RA-alanine, 12) t-RA-phenylalanine, 13 13-cisRA-leucine, and 14) t-NER/cis-NER,. Best-fitting line determined by linear regression analysis.

between log reduction in papillomas per mouse over an approximately 6 log_{10} range and log reduction in ODC activity over a range of 95% suppression of IDCA activity. Linear regression analysis confirmed a significant positive correlation (r = 0.85). **Figure 15** plots retinoid data showing a positive linear correlation exists between log reduction in papillomas per mouse and HTOC ED_{50} (M) values. Linear regression analysis confirmed a significant positive correlation (r = 0.88). Lastly, **Figure 16** displays a linear correlation exists between log reduction in ODC activity and log IC_{50} (M) values for 7 different retinoids (#s:1, *trans*-RA; 2, 13-*cis*-RA; 3, 4-HPR; 4, *trans*-retinoyl-glycine; 5, 13-*cis*-NER; 6, trans-*RME*; 7, *trans*-RPE) in the inhibition of clonal growth 6 presents of both NHK and SCC-25 cells. Linear regression analysis confirms a significant positive correlation NHK (r = 0.86). **Table 6** presents data for 7 retinoids (t-Ra, cis-RA, t-RME, 4-HPR, 13-cis-NER *(excluded as an outlier), t-retinoyl-glycine, and t-RPE) showing that there is a positive correlation in the rank order of retinoid sensitivity

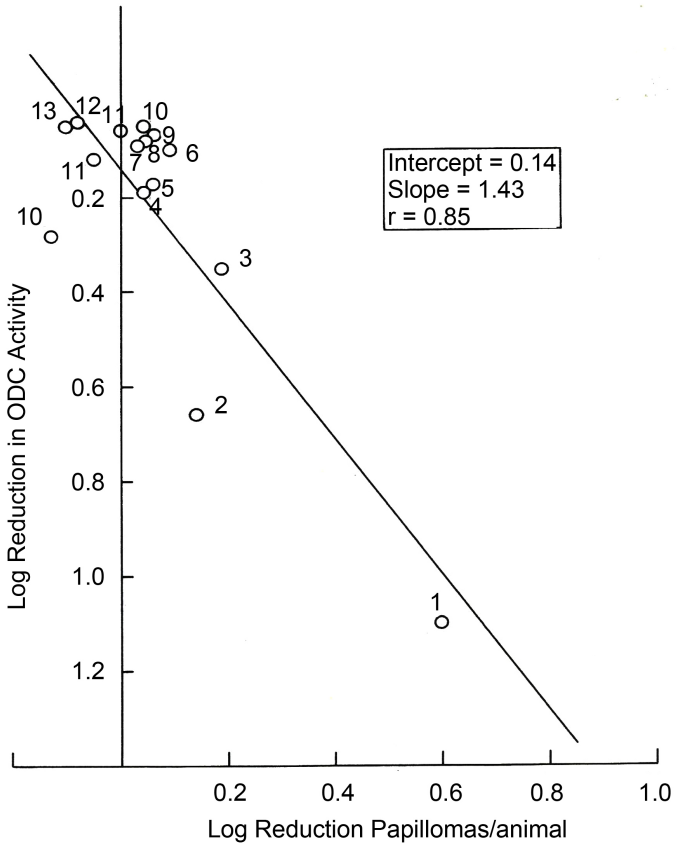


Figure 14. Linear correlation between log reduction of papillomas per animal (abscissa) and log reduction in ODC activity for select retinoids (ordinate): 1) t-RA, 2) 13-cis-RA), 3) 13-cis-NER, 4) t-RME, 5) 4-HPR, 6) t-RPE, 7) dicarboxy, t-RA/cis RA,, 8) t-RA- glycine, 9) t-REE, 10) t-RA-leucine, 11) t-RA-alanine, 12) t-RA-phenylalanine, 13 13-cisRA-leucine, and 14) t-NER/cis-NER. Best-fitting line determined by linear regression analysis.

between log reduction in papillomas per mouse in the MPA bioassay and the log IC_{50} inhibition of NHK clonal growth. Linear regression analysis of the above data had a significance (r = 0.9) and p = 0.001). Remarkably, this correlation yields the same rank order of retinoid sensitivity as shown above for the correlation between log reduction in ODC activity and log IC_{50} inhibition of NHK clonal growth, suggesting that the NHK bioassay is by far the best method to assess new chemopreventive retinoids.

3.7. Statistical Model

The statistical analyses performed for the above bioassay data preceeded in two general directions: 1) search for statistical correlations among the assays, and 2) development of a statistical screening model. For this purpose, we examined a sample of 37 retinoids in the mouse papilloma assay and the mouse ODC enzyme induction assay for significant correlations, Using the Pearson correlation coefficient, r, we found a significant correlation r = 0.72, p = 0.0001; and using the Spearman rank correlation coefficient, R, we

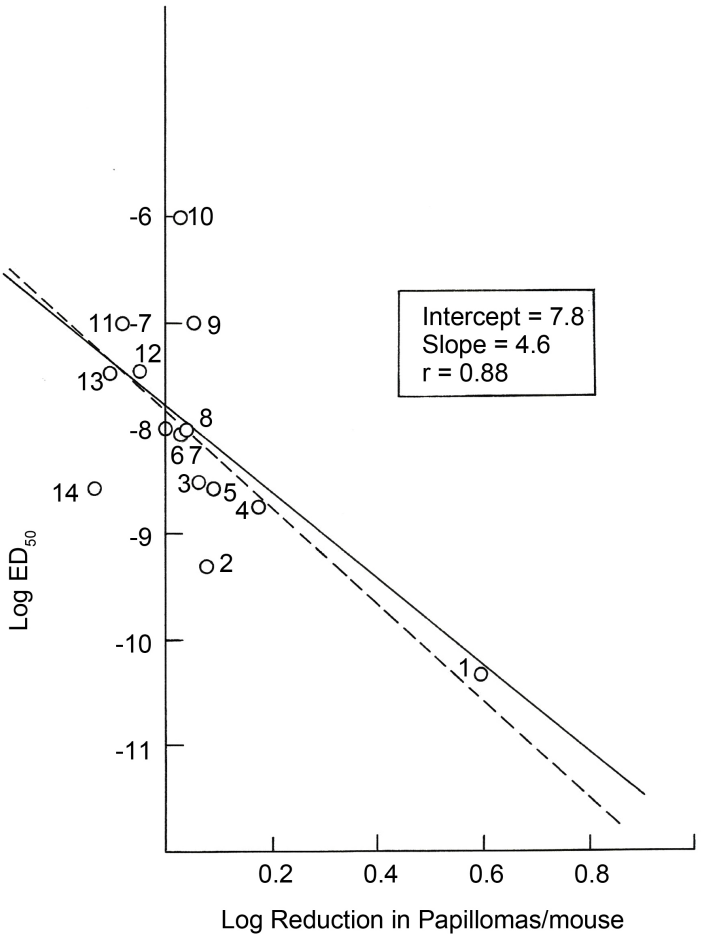


Figure 15. Linear correlation between log reduction in papillomas per mouse (abscissa) and log ED_{50}, M of the vitamin A-deficient HTOC bioassay for select retinoids (ordinate). 1) t-RA, 2) 13-cis-RA), 3) 13-cis-NER, 4) t-RME, 5) 4-HPR, 6) t-RPE, 7) dicarboxy-t-RA/cis RA, 8) t-RA-glycine, 9) t-REE, 10) t-RA-leucine, 11) t-RA-alanine, 12) t-RA-phenylalanine, 13 13-cisRA-leucine, and 14) t-NER/cis-NER. Best-fitting line (-) and second best fit line (--) excluding 13-NER as determined by linear regression analysis.

observed a correlation of R = 71, p = 0.0001. Further we developed a linear regression model to predict percentage suppression in the mouse papilloma assay using percent suppression in the ODC assay (y = −0.39 + 1.14x). To assess the predictive ability of the model, the split-half method of cross validation was used. This analysis gives a predictive root mean square error (RMSE) if 0.31 and a predictive mean bias of 0.10 for the above model. In addition, the PRESS statistic for the model was 4.8455. Correlations were also obtained for 24 retinoids on which ED_{50} values were available from published [16] and unpublished HTOC experiments. The correlations between suppression of ODC and log ED_{50} were r = −0.65, and R = −0.69, p = 0.0002 and the correlations between percentage suppression in the mouse papilloma assay and log ED_{50} were r = 0.59, p = 0.0024 and R = −0.65 and p = 0.0006.

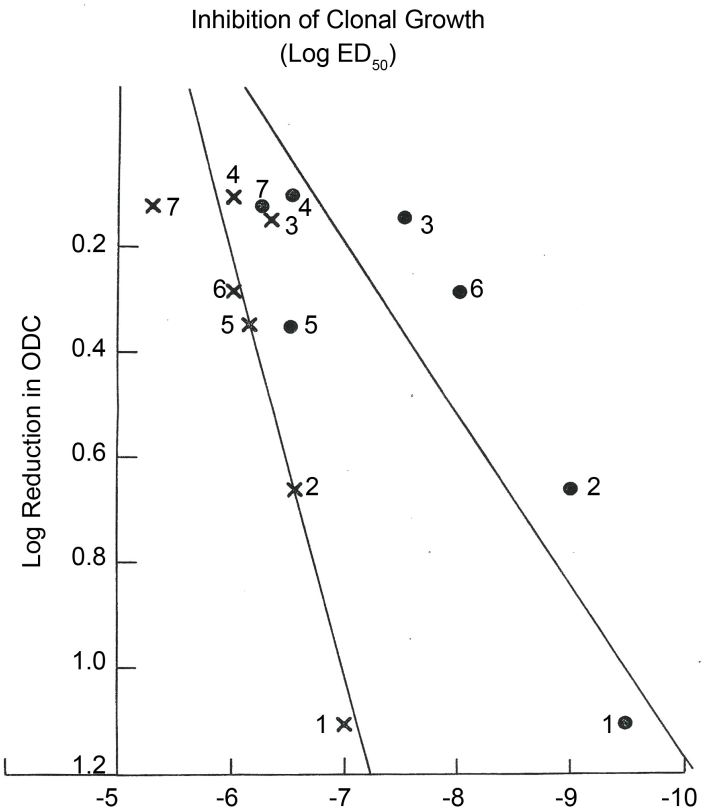


Figure 16. Linear correlation beteen log reduction in ODC activity (ordinate) and -log IC_{50}, M of NHK(●) and SCC-25(X) clonal growth bioassay (abscissa). 1) t-RA, 2) 13-cis-RA, 3) 4-HPR, 4) t-retinoyl-glycine, 5) 13-cis-NER, 6) t-RME, and 7) t-RPE.

3.8. Antineoplastic Retinoids

Retinoic acid, the most potent natural retinoid, is essential for normal cell growth and differentiation [28]. Aberrations in retinoid signaling cascade is often associated with abnormal cell growth and tumorigenesis. Currently, there are several retinoids and one rexinoid approved for treatment of specific cancers [29]. The rexinoid, 9-*cis*-retinoic acid is approved for the treatment of acute promyeolcytic leukemia (APL). 9-*cis*-retinoid (Alitretinoin) is a form of vitamin A (**Figure 17** for structural formula). It was first developed by *Ligand Pharmaceutical* as an antineoplastic agent. *Ligand Pharmaceutical* gained Food and Drug Administration approval for alitretinoin in February 1999. In gel form, Pancretin (alitretinoin) is indicated for treatment skin lesion in AIDS-related Kaposi sarcoma and to treat cutaneous T-cell lymphoma. The drug also has immune-modulating and anti-inflammatory properties. Another retinoid, bexarotene (4-[1-(5,6,7,8-tetrahydro-3,5,5,8,8-pentamethyl-2-npthalenyl)ethyenyl]benzoic acid, licensed under the trade name Targretin, is indicated for the treatment of cutaneous T-cell lymphoma (CTCL) in patient refractory to at least one prior systemic oral therapy and for topical treatment of cutaneous lesions with CTCL. Bexarotene selectively activates retinoid X receptors (RXRs) [30] [31] [32]. It induces cell differentiation and apoptosis.

Table 6. Relative effectiveness of select retinoids in suppression of TPA-induced tumors on mouse skin tumorigenesis correlated with IC-50 values in the NHK clonal growth bioassay.

Retinoid	Log Reduction Papilloma/mouse	NHK Clonal Growth (IC-50)
trans-Retinoic acid	0.6	−9.5
cis-Retinoic acid	0.42	−9
trans Retinyl methyl ether	0.13	−8
4-Hydroxyphenylretinamide	0.06	−7.5
13-cis-N-Ethylretinamide	0.19	−6.5
trans -Retinoyl-glycine	0.09	−6.5
trans-Retinyl propyl ether	0.05	−6.25

Significant straight-line correlation (r = 0.9) found graphically by linear regression analysis. N.B. 13-cis-N-Ethylretinamide was excluded from analysis as an outlier.

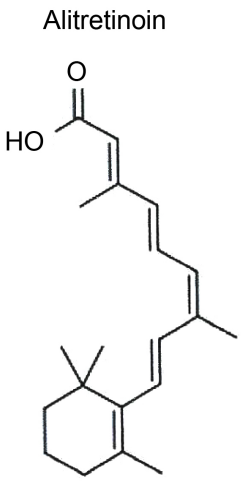


Figure 17. Chemical structural formula for 9-cis-retinoic acid (Alitretinoin) and its chemical name abstracted from www.wikipedia.com.

As with most other vitamin-A related products Alitretinoin and Bexarotene are contraindicated for pregnant women. Alitretinoin is a substrate for cytochrome CYP3A4) detoxification enzyme system. Biochemically, 9-*cis*-retinoic acid is the ligand for the nuclear RXR retinoid receptor, and it also activates the retinoic acid receptor. The natural vitamin A metabolite, t-RA, also known as tretinoin has been commercialized for the topical treatment of neoplastic skin lesions, and for treatment of APL. Under the trade name Vesnoid®. The natural retinoid, 13-*cis*-retinoic acid has been commercialized under the trade name Isotretinoin for treatment for abnormal hyperproliferative epidermal keratinocytes and to reduce the potential for malignant degeneration. It is given as a treatment for arsenical keratosis and as an effective treatment of keratocanthomas a disease related to squamous carcinomas.

4. Discussion

Our search for bioactive retinoids as effective cancer chemopreventive agents was focused on developing and validating several different reliable bioassays. Historically, this search led to the vitamin-A deficient hamster tracheal organ culture (HTOC) method [14] [33] and was later supplemented by a more rapid, less time-consuming and yet just as reliable BP-HTOC bioassay [16]. With the development of the ornithine decarboxylase (ODC) enzyme bioassay method, an entirely independent bioassay method became available. Each of these bioassay methods has been employed to assess new classes of retinoids for their potential anti-cancer activity. An important limitation of these bioassay methods has been their failure to predict their clinical outcome in human cancer clinical trials. In particular, the therapeutic index, a measure of the ratio of beneficial dose of retinoid to risks such as toxicity, has excluded all-trans retinoic acid (t-RA) and other promising retinoids. Nevertheless, several anti-neoplastic retinoids are in use today (reviewed here). In order to continue the effort to discover new potent and safe anticancer retinoid drugs, we have developed a rapid, reliable, and more relevant bioassay method based on the ability of select retinoids to inhibit the clonal growth of normal human keratinocytes. This bioassay method was validated here by demonstrating its ability to rank order a retinoid's sensitivity to inhibit clonal growth that correlated with that same test retinoids ability to suppress ODC activity. In this regard, we showed that the rank order of sensitivity of a given retinoid to inhibit clonal growth extends to SCC-25, a human epidermoid carcinoma cell line. Of interest was the finding that for each retinoid tested the tumor cells were less sensitive to retinoid inhibition of clonal growth. A note of caution is the possibility of toxicity and cell death at retinoid concentrations above 1×10^{-6} M, which could limit the detection of retinoid bioactivity. Earlier, we reported [34] that all-*trans*-RA prevents super-induction of benzo(*a*)pyrene-induced aryl hydrocarbon hydroxylase enzyme in serum-free NHK cultures, indicating that RA suppresses metabolic activation of arylhydrocarbon carcinogenesis. Further, we reported that RA effectively inhibits phosphoprotein kinase activity regulating the proliferation of both NHK and SV-40 transformed keratinocytes [35]. These latter findings could lead in the future to more specific biochemical retinoid bioassay methods.

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