Biochemical Pharmacology, Vol. 41, No. 10, pp. 1471–1477, 1991
Printed in Great Britain.

0006-2952/91 $3.00 + 0.00

GROWTH SUPPRESSION OF HUMAN BREAST CARCINOMA CELLS IN CULTURE BY *N*-(4-HYDROXYPHENYL)RETINAMIDE AND ITS GLUCURONIDE AND THROUGH SYNERGISM WITH GLUCARATE

RACHNA BHATNAGAR,* HUSSEIN ABOU-ISSA,*†‡ ROBERT W. CURLEY, JR.,§ ANTOINETTE KOOLEMANS-BEYNEN,*‖ MELVIN L. MOESCHBERGER¶ and THOMAS E. WEBB‖

Departments of *Surgery, †Human Nutrition, §Pharmacy, ‖Physiological Chemistry, and ¶Preventive Medicine and Biometrics Laboratory, The Ohio State University, Columbus, OH 43210, U.S.A.

(*Received* 10 *July* 1990; *accepted* 14 *November* 1990)

Abstract—The inhibitory effects of *N*-(4-hydroxyphenyl)retinamide (HPR) and its glucuronide derivative on the growth of MCF-7 human breast cancer cells *in vitro* were compared. The results indicate that the glucuronide had slightly greater potency and much less cytotoxicity than the free retinoid. At a concentration of 10^{-6} M, HPR inhibited MCF-7 cell growth by approximately 25%, whereas an equimolar concentration of the glucuronide caused a 40% growth inhibition. Higher concentrations of HPR were highly cytotoxic. At a 10^{-5} M concentration of the glucuronide, cell viability was 77%, and 65% of the cells were able to resume growth. On the other hand, at 10^{-5} M HPR, cell viability dropped to 49%, and only 15% of the cells were capable of resuming growth. The lower cytotoxicity and higher potency of the retinoid glucuronide compared to the parent retinamide suggest that the conjugate may have a chemotherapeutic advantage over the parent compound. The apparent higher efficacy of HPR in combination with glucarate (GT) compared to the single agents could be due to increased net formation of HPR glucuronide conjugate following conversion of GT to the β-glucuronidase inhibitor, D-glucaro-1,4-lactone. However, HPLC analysis of the cell metabolites did not show any detectable levels of the retinoid glucuronide upon treatment of MCF-7 cells with HPR and GT.

A number of studies have demonstrated that retinoids, in particular *N*-(4-hydroxyphenyl)retinamide (HPR**), a synthetic retinoid with trophism for the mammary gland, are effective in the chemoprevention of chemically induced mammary cancer in rodents [1–3]; high levels of 13-*cis*-retinoic acid (13-*cis*-RA) are also effective [4]. Retinoids reduce the proliferation of MCF-7 cells *in vitro* [5] and markedly inhibit the growth of chemically induced mammary tumors in rats [6]. Recently, HPR was shown to induce complete regression of small, chemically induced rat mammary tumors [7]. The sensitivity of the rat mammary gland model to the protective effects of retinoids and of the synthetic retinoid HPR plus the anti-estrogen tamoxifen has been reviewed recently [8].

Our recent studies established that low levels of dietary HPR, which alone are ineffective, interact synergistically with similarly low ineffective levels of dietary glucarate (GT) to inhibit the induction of rat mammary tumors [9] and the growth of pre-established 7,12-dimethylbenz[*a*]anthracene (DMBA)-induced rat mammary tumors [10]. Glucarate has been shown previously by us to be an effective chemopreventive and antitumor agent against cancer induction in several rodent organs, including the mammary gland [9, 10].

The synergistic interaction between retinoids and glucarate was of interest since it permits each component to be utilized at levels as low as one-half of their effective concentration as single agents. The nature of the interaction between glucarate and HPR has not been determined, but the possible involvement of HPR-*O*-glucuronide (HPROG) [11] is evaluated. Glucarate may undergo slow equilibrium conversion to D-glucaro-1,4-lactone, an inhibitor of β-glucuronidase, and the inhibition of β-glucuronidase can, in turn, lead to increased net glucuronidation of HPR and other biologically active endogenous and exogenous compounds [12]. Retinoyl-β-glucuronide, shown to be a natural vitamin A metabolite [13], is reported to have increased biological activity and less toxicity than the parent compound [14], to inhibit the growth of DMBA-induced mammary lesions in organ culture [15], and to induce the differentiation of leukemia cells in cell culture [16]. Recently, retinoyl-β-glucuronide was found to be taken up into a number of major organs after intraperitoneal injection, where it persisted as a major component for 24 hr [17].

In view of the synergism between glucarate and

‡ Address reprint requests to: Dr. Hussein Abou-Issa, Department of Surgery, College of Medicine, The Ohio State University, Columbus, OH 43210.

** Abbreviations: HPR, *N*-(4-hydroxyphenyl)retinamide; HPROG, HPR-*O*-glucuronide; GT, glucarate; DMBA, 7,12-dimethylbenz[*a*]anthracene; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; PBS, phosphate-buffered saline; MS, mass spectrometry; NMR, nuclear magnetic resonance; and P.E., plating efficiency.

retinoids and the growing evidence that retinoid glucuronides have biological activity, HPROG was synthesized and the activity of this analog was compared to that of the parent compound, HPR, in the MCF-7 cell culture system. The possibility that an observed synergism between HPR and glucarate against the MCF-7 cells was due to conversion or enhanced conversion of HPR to HPROG was also assessed.

MATERIALS AND METHODS

Materials. HPR was obtained from Dr. Schally, Southern Research Institute, Birmingham, AL, and more recently from McNeil Pharmaceuticals, Springhouse, PA. HPROG was synthesized by one of us (R.W.C.) as described below. D-Glucurono-6,3-lactone, 4-nitrophenol and retinoic acid, as well as other reagents necessary for the synthesis of HPROG, were obtained from the Aldrich Chemical Co., Milwaukee, WI. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and potassium glucarate were purchased from the Sigma Chemical Co., St. Louis, MO. Reagents not specified were of the highest purity available.

Synthesis of 4-hydroxyphenylretinamide-O-glucuronide. Methyl-(tri-*O*-acetyl-α-D-glucopyranosyl bromide)uronate was prepared by methanolysis and acetylation of D-glucurono-6,3-lactone, followed by treatment of the tetraester with HBr/acetic acid by the procedure of Bollenback and coworkers [18]. The silver oxide catalyzed reaction of 4-nitrophenol with the protected uronate [19], followed by catalytic hydrogenation [20], afforded the known methyl-(4-aminophenyl 2,3,4-tri-*O*-acetyl-β-D-glucopuranoside)uronate. Treatment of this aminophenoxy sugar with retinoyl chloride according to the procedure of Dawson and Hobbs [21] provided methyl-(4-retinamidophenyl 2,3,4-tri-*O*-acetyl-β-D-glucopyranoside)uronate in 73% yield after silica gel column chromatography (35% ethyl acetate/hexane). This tetraester-amide was saponified according to the above procedure [21].

Following this reaction, the methanol was removed leaving a gum suspended in an aqueous phase which was acidified (1 N HCl), and the resulting yellow emulsion was extracted with ethyl acetate. The ethyl acetate was dried over Na_2SO_4 and concentrated to a semi-solid. The solid was crystallized from ethyl acetate to afford a 92% yield of the HPROG. This product showed physical and spectroscopic properties (^{1}H- and ^{13}C-NMR, MS and HPLC) consistent with the assigned structure as well as those previously reported [21] for the sodium salt of HPROG.

Assay of β-Glucuronidase. β-Glucuronidase was assayed as previously described [12]. This assay is based on the hydrolysis of phenolphthalein glucuronide at 56° and quantitation of phenolphthalein by spectrophotometry in alkaline medium.

Culture of cells. The MCF-7 cells used in this study were obtained originally from the Michigan Cancer Foundation by the OSU Cell Culture Service. The cells were routinely grown as monolayers in T-25 plastic tissue culture flasks (Falcon, Oxnard, CA) containing 5.0 mL of B-10 medium consisting of Minimum Essential Medium (MEM, Earle's Base) supplemented with MEM non-essential amino acids (1.0 mM), L-glutamine (2.0 mM), 10% fetal bovine serum (FBS), and antibiotics. Culture conditions included a humidified atmosphere of 5% CO_2 at 37°. One-half of the medium was replenished twice a week. Cells were harvested by trypsinization upon confluency. All variables were evaluated in triplicate.

For the growth inhibition assay, the MCF-7 cells were plated in 24-well plates at a density of 1.5×10^4 cells/cm^2. After 24 hr and again on day 4, the medium was replaced with treatment medium containing the specified retinoids at 10^{-5} to 10^{-8} M in absolute ethanol and added to the cell cultures so that the ethanol concentration did not exceed 0.1%. Control cultures were treated with an equivalent volume of ethanol. Glucarate was dissolved in phosphate-buffered saline (PBS) to give stock solutions of 0.01 M. Subsequent dilutions were carried out in the medium. On day 7 the cells were harvested by trypsinization, and viability was assessed by trypan blue exclusion [5]. Total cell counts were obtained by hemocytometer as well as by the Coulter Counter. Growth inhibition (cell survival) was calculated for the various retinoid concentrations using the equation: % inhibition = $100 - R/C \times 100$ where R and C are the number of cells in retinoid-treated and control cultures, respectively.

Cytotoxicity assays. For evaluation of cytotoxicity, cells were harvested and seeded in siliconized glass vials at 0.5×10^5 cells per vial (10^4 cells/mL). The medium used was B-10 medium containing 10% FBS, with or without drug. Each variable was set up in triplicate. Cell suspensions were preincubated for 1 hr at 37° in the presence of 5% CO_2, then sealed and placed in a shaking water bath at 37°. At T = 24 hr the cell suspensions were incubated for 1 hr again at 37° and 5% CO_2 for re-equilibration, then sealed tightly once more and returned to the shaking water bath. After 48 hr the cells were harvested by centrifugation, and cell counts and viabilities were determined by trypan blue exclusion. To assess the effect of each drug on subsequent plating efficiencies, approximately 10^4 cells were removed from each vial following treatment and seeded into wells of a 24-well plate and incubated at 37° and 5% CO_2. The next day all non-adherent cells were collected from each well and counted. Plating efficiencies (P.E.) were calculated using the equation $(N_{cp} - C_{na})/N_{cp} \times 100 =$ % P.E. in which N_{cp} is the total number of cells plated and C_{na} is the number of nonadherent cells removed from each well.

For the 7-day experiments, cytotoxicity was evaluated using the tetrazolium salt assay described by Mosmann [22]. In this method, cells were incubated for 4 hr at 37° with tetrazolium salt, following the 7-day incubation with drug exposure as described previously. Each variable was assessed in 4 wells. The metabolically active cells reduced the dye to purple formazan. Optical densities were measured using a test wavelength of 570 nm and a reference wavelength of 630 nm, and compared against a standard curve of known numbers of viable MCF-7 cells. Experiments were performed a minimun of three times, and values are expressed as percent of control.

HPLC analysis of cells. MCF-7 cells (approx. 10^6)

Table 1. Effects of GT, HPR and their combination on the growth of MCF-7 cells in culture

GT concn (μM)	% Cell survival* at HPR concn (μM) 0	0.01	0.1	1.0
0	100.0 ± 5.0	99.2 ± 5.0	84.5 ± 4.0†	71.1 ± 3.5†
0.01	100.0 ± 10.0	96.5 ± 3.5	75.0 ± 1.0	ND‡
0.1	94.0 ± 10.0	85.6 ± 10.7	75.5 ± 6.5	56.4 ± 3.2
1.0	94.5 ± 6.5	76.4 ± 15.6	59.3 ± 0.5	53.7 ± 2.3
10.0	87.8 ± 9.0	77.8 ± 15.3	55.5 ± 1.0	60.0 ± 11.0

* Each value has been corrected for approximately 7% inhibition by the 0.1% alcohol (vehicle).

† $P < 0.05$ compared to HPR control without GT.

‡ Not done.

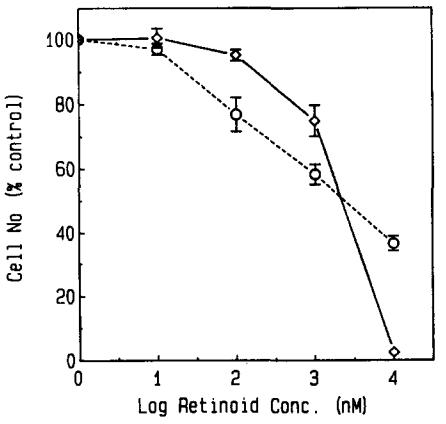

Fig. 1. Concentration–response curves for HPR (◇) and HPROG (○) against MCF-7 cell growth. MCF-7 cells (10^4) were treated with the indicated concentrations of retinoids in ethanol. Control cultures received an equivalent volume of ethanol. The final concentration of ethanol was 0.1%. The cells were harvested on day 7 and counted as described under Materials and Methods. Each value is the mean of five experiments ±SEM.

were treated with 10^{-7} M HPR with and without 10^{-6} M GT for 12 hr, then separated from the medium, lyophilized, and extracted twice with 1.0 mL of methanol [cf. Ref. 23]. The combined methanol extracts were concentrated under argon, and the residue was redissolved in 200 μL of methanol. For analysis, 100 μL was injected into a Beckman (model 332) HPLC equipped with a UV detector (model 164) and a 250 mm × 4.6 mm Zorbax-ODS column (Dupont Instruments, Wilmington, DE). Elution was first with methanol:water 50:50, 0–10 min; followed by a methanol:water gradient 50:50 to 100:0, 10–30 min; and finally 100% methanol, 30–45 min. The flow rate was 1.0 mL/min, and the eluate was monitored at 350 nm. In a separate experiment, MCF-7 cells (approx. 5×10^6 cells) were treated as above for 24 hr with 10^{-7} M HPROG. After centrifuging, the cells and medium were processed separately as above and analyzed using the HPLC protocol described above.

Statistical analysis. Two separate methods of statistical analysis, Student's *t*-test and the two-way analysis of variance (ANOVA), were performed to assess the main effect of the GT or HPR concentration levels as well as their possible interaction together on growth inhibition of MCF-7 cells *in vitro*. Because we wished to assess a multiplicative interactive effect, the logarithm of the response (cell survival) was taken as the dependent variable in the ANOVA analysis. To compare the effects of HPR and HRPOG concentration levels on cell viability and P.E., a randomized complete block design was used as the blocking level [24].

RESULTS

Effects of GT, HPR, and HPR + GT on the growth of MCF-7 cells. Summarized in Table 1 are the individual and combined effects of GT, HPR, and HPR + GT on the growth of MCF-7 cells. Both GT and HPR were evaluated over a 1000-fold concentration range. GT alone had only a slight effect, causing a 5% inhibition of cell growth at 1.0 μM. HPR alone was moderately inhibitory, the inhibition being approximately 15% at 0.1 μM. This effect was statistically significant ($P < 0.05$). In contrast, 1.0 μM GT and 0.1 μM HPR together inhibited cell growth by approximately 40%. Other combinations within this concentration range were equally effective, indicating a synergistic interaction between HPR and GT similar to the one observed against primary rat mammary tumors *in vivo* [10]. Statistical analysis by Student's *t*-test showed that the interaction involving GT at 10^{-6} M and HPR at 10^{-7} M was statistically significant ($P < 0.05$). However, using the two-way analysis of variance, taking into account the overall variability of the experiment, the results indicate only borderline significance at $P = 0.06$.

Concentration–response curves for HPR and HPROG. The glucuronide conjugate of HPR was synthesized according to published procedures. However, the final step of the synthesis was modified to give *ca.* 500 mg of the compound in high yield as its free acid, as opposed to the sodium salt form previously prepared [20]. The crystallized retinoid was not purified further, since spectroscopic and chromatographic results showed little or no contamination with the 13-*cis* retinoid or α-anomeric glucuronide.

Table 2. Viablilities and subsequent plating efficiencies of MCF-7 cells after 48-hr treatment with HPR and HPROG

Concn (μM)	HPR			HPROG		
	% Viability*	% P.E.*	Growth rate	% Viability*	% P.E.*	Growth rate
0	100	100	1.9	100	100	1.9
0.01	82.0 ± 3.2	76.7 ± 3.5	3.7	83.8 ± 1.8	79.5 ± 5.5	ND†
0.1	80.0 ± 3.9	71.0 ± 1.5	2.3	84.2 ± 2.6	74.7 ± 2.3	ND
1.0	72.6 ± 6.6	63.3 ± 3.2	2.8	78.4 ± 3.2	71.3 ± 2.9	ND
10.0	49.4 ± 4.9‡	15.0 ± 1.5‡	3.3	77.2 ± 4.2‡	65.3 ± 7.2‡	6.6

* Values of viability and plating efficiency (P.E.) are the means ± SEM of five experiments done in triplicate.

† Not done.

‡ P = 0.03 compared to the controls.

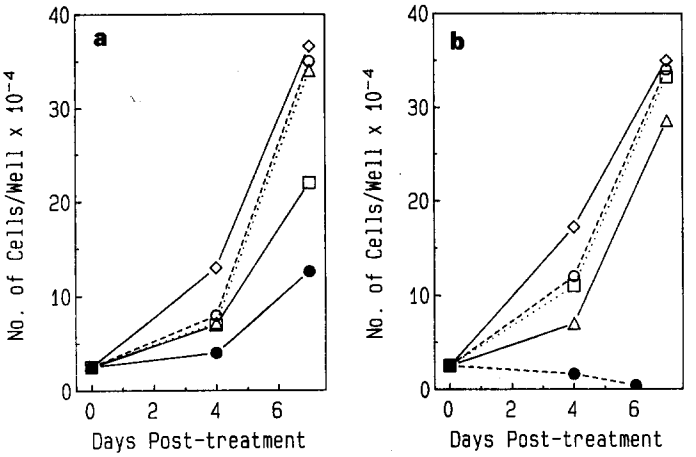

Fig. 2. Time course of inhibition of MCF-7 cell growth by (a) HPROG and (b) HPR at 0 μM (◇), 0.01 μM (○), 0.1 μM (△), 1.0 μM (□), and 10 μM (●). MCF-7 cells were plated in 24-well plates (2–3 × 10^{-4} cells/well). At 1 day after seeding, the cells were treated with the indicated concentrations of retinoids in ethanol; the control cultures received an equivalent volume of ethanol. Cells were harvested on day 4 and day 7 as described under Material and Methods. Each point is the mean of triplicate determinations ± SEM. The data represent one of five experiments that gave essentially the same result.

Since one possible mechanism of action of GT is to enhance HPROG formation, the efficacies of HPR and HPROG were evaluated in the MCF-7 line. Shown in Fig. 1 are the concentration–response curves for the inhibition of the growth of MCF-7 cells by HPR and HPROG following exposure for 7 days. The results indicate that the growth inhibitory activity of the glucuronide is similar to, or slightly higher than, the parent compound, except at concentrations between 1.0 and 10.0μM where HPR was highly cytotoxic. These results were supported by the data in Table 2 listing cell viabilities and plating efficiencies of MCF-7 cells following treatment in suspension for 48 hr. A randomized complete block analysis was performed to compare the various concentration levels of HPR and HPROG on the percent viability and P.E. values. At a concentration of 10^{-5} M HPR or HPROG, there was a statistically significant difference (P = 0.03), while at 10^{-6} M the results showed borderline statistical significance (P = 0.07) and at 10^{-7} M the difference was not statistically significant (P = 0.14). Although at 10 μM HPR, 50% of the cells still appeared viable after this limited period of exposure, only 15% were capable of resuming growth. In view of the low plating efficiency after treatment with higher concentrations of HPR, a measure was made of the growth rate of the surviving cells which resumed growth. This measurement was made 5 days after initial seeding of the cells and 48 hr after the initiation of the P.E. determination. As indicated in Table 2, the cells in the cultures which resumed growth grew at a rate 1.3 to 2.0-fold greater than the untreated cells. The growth rate of cells exposed to HPROG was even greater. The growth rate represents the ratio of the number of adherent cells at 2 days post-plating to the number of cells originally plated.

The time-dependent inhibition of MCF-7 cell

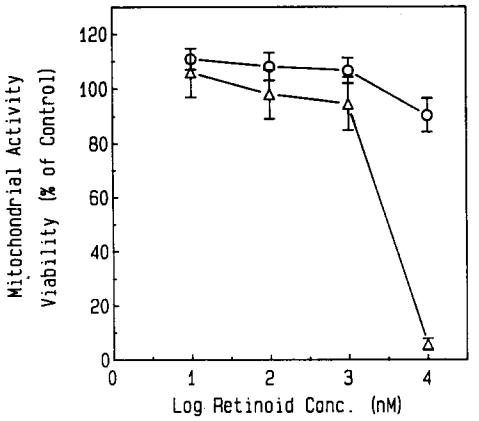

Fig. 3. Viability of MCF-7 cells after treatment with HPR and HPROG. MCF-7 cells were cultured at the indicated concentrations of HPR (△) or HPROG (○) for 7 days as described in Fig. 1. On day 7, cell viability was assayed by the MTT assay. The optical density of control cultures (O.D. 0.619) was considered as 100%, and percent viability was calculated accordingly. Results are the means ± SEM of three experiments, each done in quadruplicate.

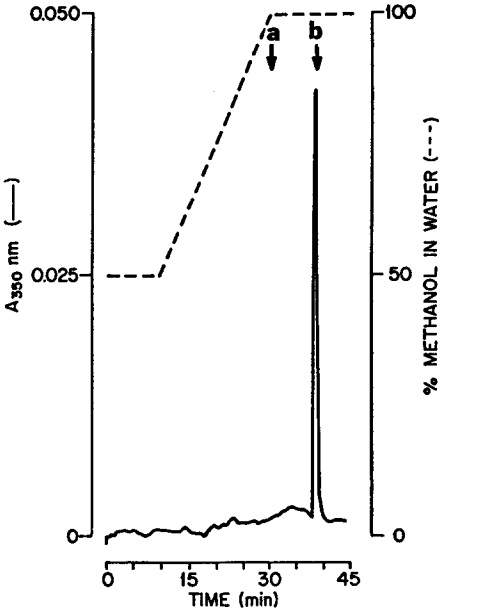

Fig. 4. HPLC profile of methanol extracts of MCF-7 cells incubated with HPR (10^{-7} M) and GT (10^{-6} M). Details are given in Materials and Methods. The elution profiles of HPROG and of HPR are shown by arrows (a) and (b), respectively.

growth by HPROG and HPR is shown in Fig. 2, a and b. The major difference in toxicity between HPR and HPROG occurred at 10 μM retinoid. After approximately 6 days, essentially no cells survived in the presence of HPR, whereas over 40% remained viable in the case of HPROG. Further, there appeared to be a sharp demarcation or threshold between cytostatic and cytotoxic activity for HPR, while there was a broad range of cytostatic activity of HPROG.

Similar results were obtained when cell function was determined by the MTT colorimetric assay as shown in Fig. 3. After 6 days of drug exposure, surviving MCF-7 cells treated with any concentration of HPROG still had intact mitochondrial activity, whereas cells treated with 10 μM HPR retained only 8% of the original activity.

HPR activity as a function of HPROG formation. HPROG appears to act directly on the cells since breakdown in the MCF-7 culture system is minimal due to normally low β-glucuronidase activity (see below). As noted above, one possible mode of action of the parent compound HPR in the presence of GT could be conversion to HPROG. However, several lines of evidence indicate that this mechanism may not be involved in the MCF-7 cell system. Shown in Fig. 4 is an analysis of an HPLC extract of cells incubated for 12 hr with 10^{-7} M HPR and 10^{-6} M GT. This elution profile, which shows only HPR, was identical to that obtained when the cells were incubated only with 10^{-7} M HPR. These results are in contrast to the significant conversion of HPR to HPROG observed in the rat, and the enhancement of the conjugation by co-administration of glucarate [9]. Furthermore, although there is a significant drop in blood and tissue β-glucuronidase activity in the rat after feeding glucarate [12], this inhibition was not seen in tissue culture. For example, the β-glucuronidase activity in the cells incubated for 24 hr with or without 10^{-6} M glucarate was 283 units/mg protein in control cells and 331 units/mg protein in the cells incubated with glucarate (Abou-Issa H, Koolemans-Beynen A and Webb TE, unpublished data). Thus, unlike the *in vivo* system where equilibrium conversion of glucarate to D-glucaro-1,4-lactone occurs at the acid pH of the stomach [cf. Ref. 12], the conversion was extremely slow at neutral pH. The results indicate that glucarate may not act by enhancing glucuronidation in MCF-7 cells as a result of inhibition of β-glucuronidase activity. In fact, MCF-7 cells appear incapable of glucuronidating retinoids. Furthermore, analysis of the medium from cells exposed to HPROG for 24 hr showed no evidence that the conjugate was hydrolyzed. Also, no free HPR was found in extracts of these treated cells (Curley R, unpublished data).

DISCUSSION

Consistent with the *in vivo* chemopreventative and anti-tumor studies utilizing the DMBA-induced rat mammary carcinogenesis system [9, 10], concentrations of GT and HPR have been identified in the present investigation which interact synergistically to inhibit the growth of MCF-7 mammary tumor cells. GT and 13-*cis*-retinoic acid may exhibit similar effects [25]. The anti-cancer effects of HPROG are also consistent with the effects observed by others with the HL-60 leukemia cell line [16]. The fact that this conjugate of HPR has cytostatic activity equal to, or greater than, the parent compound while showing little or no cytotoxicity suggests that this compound may be a highly desirable anti-cancer or chemo-

preventative agent. The discovery of retinoid glucuronides in vitamin A target tissues as well as their polar nature and greater amphipathic properties [23] may explain the lower cytotoxicity of the high concentrations of HPROG as compared to HPR. Further, retinoid glucuronides were shown recently to have a long half-life in rats [16]. It is very unlikely that the activity of HPROG observed in this study is the result of hydrolysis to the parent compound by β-glucuronidase released from the cells to the medium. The β-glucuronidase levels in the culture medium were essentially non-detectable at all time periods of culture, when the enzyme was assayed at its pH optimum of 4.5 (Webb T and Schooley W, unpublished data). In addition, HPLC analyses showed only intact HPROG in the culture medium of MCF-7 cells after a 24-hr exposure. The conclusion that the glucuronide itself is acting on the cells to inhibit their growth is further supported by clear differences in the biological activity, especially cytotoxicity between HPR and HPROG and the lack of activity of HPR at low concentrations.

A component of the present data, not always apparent but utilized in calculations when appropriate, was the cells in the medium (supernatant) of cultures treated with GT, HPR, GT + HPR, or HPROG. Although in all cases these cells exhibited 85% or greater viability (with the exception of those treated with 10 μM HPR) and the proportion unattached was concentration responsive, it appeared that these cells were incapacitated in some manner, being unable to resume growth in the monolayer culture system. In contrast, the growth rate of the cells which did resume growth was greater than that of the controls. This difference may reflect heterogeneity in the phenotype of the cell population itself. Although the appearance of viable, non-proliferating cells in the supernatant medium of the treated cells would appear inconsistent with differentiation, it is consistent with the induction of apoptosis, one model which has been proposed for regression of mammary cancer [26]. On the other hand, there was a slight increase in tetrazolium salt reduction and cell functional capacity in response to treatment with the retinoid glucuronide which could be taken as an indication of an increase in the cell differentiation index as previously seen with leukemia HL-60 cells [27].

Very little is known concerning the mechanism of action of retinoids although their effects are mediated, at least in part, through changes in cAMP-dependent protein kinase activity [4, 5] and through interaction with specific cellular receptors [28]. Similarly, the mechanism by which glucarate enhances the anti-cancer activity of retinoids is not clear. Glucarate is an end product of glucuronic acid metabolism and glucuronic acid, in turn, is derived by β-glucuronidase hydrolysis of glucuronide conjugates synthesized by an inducible phase II enzyme system (i.e. the UDP-glucuronyl transferase isozymes). It therefore appears that glucarate has itself a protective effect and additionally inhibits growth of neoplastic cells in combination with retinoids. The protective effect of glucarate alone at high concentrations has been demonstrated recently in an *in vitro* transformation assay [29]. Glucarate appears, therefore, to exert a direct effect on cells, in addition to the enhanced net glucuronidation demonstrable *in vivo*. In this regard it should be emphasized that glucarate can induce other glucuronidation-dependent effects *in vivo* which are not evident in cell culture systems lacking appropriate UDP-glucuronosyl transferase. Thus, if, as seems possible, the administration of HPROG as a chemotherapeutic regimen for other than superficial pre-neoplastic or neoplastic lesions is impractical, then the co-administration of HPR and glucarate may offer a distinct advantage. Further studies appear warranted to establish the relationship between HPROG, and HPR and GT *in vivo*.

Acknowledgements—This study was supported by Grant CTR 2262 and NCI Grant P30CA 16058-15. The authors thank Dr. F. Schally, Southern Research Institute, Birmingham, AL, and McNeil Pharmaceuticals, Springhouse, PA, for their supply of *N*-(4-hydroxyphenyl)retinamide. The authors thank Webster Schooley, Department of Surgery, for running the β-glucuronidase essays.

REFERENCES

1. Moon RC and Itri LM, Retinoids and cancer. In: *The Retinoids* (Eds. Sporn MB, Roberts AB and Goodman DS) Vol. 2, pp. 327–371. Academic Press, New York, 1984.
2. Moon RC, Thompson HJ, Becci PJ, Grubbs CJ, Gander RJ, Newton DL, Smith JM, Phillips SL, Henderson WR, Mullen LT, Brown CC and Sporn MB, *N*-(4-Hydroxyphenyl)retinamide, a new retinoid for prevention of breast cancer in the rat. *Cancer Res* **39**: 1339–1346, 1979.
3. Sporn MB and Roberts AB, The role of retinoids in differentiation and carcinogenesis. *Cancer Res* **43**: 3034–3039, 1983.
4. Abou-Issa H and Duruibe VA, Anticarcinogenic effect of retinoids on 7,12-dimethylbenz(a)anthracene-induced mammary tumor induction, and its relationship to cyclic AMP-dependent protein kinase. *Biochem Biophys Res Commun* **135**: 116–123, 1986.
5. Ueda H, Takenawa T, Millan JC, Gesell MS and Brandes D, The effects of retinoids on proliferative capacities and macromolecular synthesis in human breast cancer MCF-7 cells. *Cancer* **46**: 2203–2209, 1980.
6. Abou-Issa H and Duruibe V, *In vitro* and *in vivo* inhibition of breast cancer growth by retinoids via cyclic AMP-dependent protein kinase. *FASEB J* **2**: A859, 1988.
7. Dowlatshali K, Mehta RG, Thomas CF, Dinger NM and Moon RC, Therapeutic effect of *N*-(4-hydroxyphenyl)retinamide on *N*-methyl-*N*-nitrosourea-induced rat mammary cancer. *Cancer Lett* **47**: 187–192, 1989.
8. Welsch CV, Rodent models to examine *in vivo* hormonal regulation of mammary gland tumorigenesis. In: *Cellular and Molecular Biology of Mammary Cancer* (Ed. Medina D), pp. 495–508. Plenum Press, New York, 1987.
9. Abou-Issa HM, Duruibe VA, Minton JP, Larroya S, Dwivedi C and Webb TE, Putative metabolites derived from dietary combinations of calcium glucarate and *N*-(4-hydroxyphenyl)retinamide act synergistically to inhibit the induction of rat mammary tumors by 7,12-dimethylbenz[*a*]anthracene. *Proc Natl Acad Sci USA* **85**: 4181–4184, 1988.
10. Abou-Issa H, Webb TE, Minton JP and Moeschberger

M, Chemotherapeutic evaluation of glucarate and *N*-(4-hydroxyphenyl)retinamide alone and in combination in the rat mammary tumor model. *J Natl Cancer Inst* **81**: 1820–1823, 1989.
11. Swanson BN, Newton DL, Roller PP and Sporn MB, Biotransformation and biological activity of *N*-(4-hydroxyphenyl)retinamide derivative in rodents. *J Pharmacol Exp Ther* **219**: 632–637, 1981.
12. Dwivedi C, Downie AA and Webb TE, Net glucuronidation in different rat strains; Importance of microsomal β-glucuronidase. *FASEEB J* **1**: 303–307, 1987.
13. Dunagin PE Jr, Zachman RD and Olson JA, The identification of metabolites of retinal and retinoic acid in rat bile. *Biochim Biophys Acta* **124**: 71–85, 1966.
14. Zile MH, Inhorn RC and DeLuca HF, Metabolism *in vivo* of all-*trans*-retinoic acid. Biosynthesis of 13-*cis*-retinoic acid and all-*trans*- and 13-*cis*-retinoyl glucuronides in the intestinal mucosa of the rat. *J Biol Chem* **257**: 3544–3550, 1982.
15. Mehta RG, Barua AB, Olson JA and Moon RC, Effect of retinoid glucuronides on the mouse mammary gland development *in vitro*. *Proc Am Assoc Cancer Res* **28**: 50, 1987.
16. Gallup J, Barua A, Furr A and Olson JA, Effects of retinoid β-glucuronides and *N*-retinoyl amines on the differentiation of HL-60 cells *in vitro*. *Proc Soc Exp Biol Med* **186**: 269–294, 1987.
17. Barua AB and Olson JA, Chemical synthesis of all-*trans*-[11-^{3}H]retinoyl β-glucuronide and its metabolism in rats *in vivo*. *Biochem J* **263**: 403–409, 1989.
18. Bollenback GN, Long JW, Benjamin DG and Lindquist JA, The synthesis of aryl-D-glucopyranosidic acids. *J Am Chem Soc* **77**: 3310–3315, 1955.
19. Kato K, Yoshida K, Tsukamoto H, Nobunaga M, Masuya T and Sawada J, Synthesis of *p*-nitrophenyl β-D-glucopyranosiduronic acid and its utilization as a substrate for the assay of β-glucuronidase activity. *Chem Pharm Bull (Tokyo)* **8**: 239–242, 1960.
20. Shibasaki J, Sadakane E, Konishi R and Koizumi T, Drug absorption, metabolism and excretion. VI. Preparation of ether-type glucuronide of 4-hydroxyacetanilide. *Chem Pharm Bull (Tokyo)* **18**: 2340–2343, 1970.
21. Dawson MI and Hobbs PD, Synthesis of the D-glucuronic acid conjugates of *N*-(4-hydroxyphenyl)-retinamides and *N*-(2-hydroxyethyl)retinamides. *Carbohydr Res* **85**: 121–129, 1980.
22. Mossman T, Rapid colorimetric assay for cellular growth and survival application to proliferation and cytotoxicity assays. *J Immunol Methods* **65**: 55–63, 1983.
23. Zile MH, Cullum ME, Simpson RU, Barua AB and Swartz DA, Induction of differentiation of human promyelocytic leukemia cell line HL-60 by retinoyl glucuronide, a biologically active metabolite of vitamin A. *Proc Natl Acad Sci USA* **84**: 2208–2212, 1987.
24. Winer BJ, *Statistical Principles in Experimental Design*. McGraw-Hill, New York, 1971.
25. Abou-Issa H, Koolemans-Beynen A, Minton JP and Webb TE, Synergistic interaction between 13-*cis*-retinoic acid and glucarate: Activity against rat mammary tumor induction and MCF-7 cells. *Biochem Biophys Res Commun* **163**: 1364–1369, 1989.
26. Cho-Chung YS, Models of tumor regression in endocrine-related cancer. In: *Review on Endocrine-Related Cancer* Vol. 28, pp. 13–18. Stuart Pharmaceuticals, Division of ICI Americas, Inc., Wilmington, DE, 1988.
27. Breitman TR and He R, Combinations of retinoic acid with either sodium butyrate, dimethyl sulfoxide or hexamethylene bisacetamide synergistically induce differentiation of the human myeloid leukemia cell line HL60. *Cancer Res* **50**: 6268–6273, 1990.
28. Mangelsdorf DJ, Ong ES, Dyck JA and Evans RM, Nuclear receptor that identifies a novel retinoic acid response pathway. *Nature* **345**: 224–229, 1990.
29. Steele VE, Kelloff GJ, Wilkinson BP and Arnold JT, Inhibition of transformation in cultured rat tracheal epithelial cells by potential chemopreventative agents. *Cancer Res* **50**: 2068–2074, 1990.