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Antitumour Synergism between Non-toxic Dietary Combinations of Isotretinoin and Glucarate

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Dietary calcium glucarate (CGT) increased the activity of non-toxic levels of dietary isotretinoin against pre-established tumors in the chemically-induced rat mammary tumour model. In the range of 1.0–1.5 mmol/kg diet, isotretinoin enhanced tumour growth by 20% over a 4 week course of treatment. Tumour growth inhibition not exceeding 15% was observed only at dosages as high as 2.0 mmol/kg, i.e. in the cumulative toxicity range. Growth inhibition by 64 mmol/kg diet of CGT alone was marginal, varying from zero to 8%. In contrast, the combination of 1.0 mmol/kg of isotretinoin and 64 mmol/kg of CGT caused a reversible inhibition of tumour growth, culminating in a net decrease in tumour volume of 20%. This study documents the marginal enhancement of tumour growth by high sub-optimal concentrations of isotretinoin alone, and describes conditions for inhibition of tumour growth by sub-optimal concentrations of the natural retinoid. Related *in vitro* studies on retinoid sensitive and insensitive cell lines suggest that the anticancer activity of the combination is dependent on sensitivity of the cells to retinoids.
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INTRODUCTION

RETINOIDS HAVE received considerable attention both as cancer chemopreventive and as antitumour agents. Various natural and synthetic retinoids have been evaluated as inhibitors of induction of skin papillomas, mammary tumours and bladder tumours [1]. Vitamin A is an essential micronutrient for differentiation of tissues of mesodermal, endodermal and ectodermal origin [2]. Natural retinoids have been shown to be effective at toxic levels in the rat mammary tumour model [3, 4]. The development of the synthetic analog fenretinide [*N*-(4-hydroxyphenyl)retinamide] circumvented many of the toxicity problems associated with chemoprevention of mammary carcinogenesis [5]. Retinoic acid or retinyl acetate have been used topically [6]. However, the toxicity of vitamin A, its metabolites and cogeners has restricted their general use in prevention or treatment of cancer. More recent reports that retinoids may enhance carcinogenesis in certain organs [7, 8] further indicated the need for additional approaches in the use of these agents for the treatment and prevention of cancer. Some retinoids, all-trans retinoic acid, in particular, is embryotoxic and teratogenic [9]. Despite these drawbacks, the retinoids remain one of the most versatile and potentially useful group of chemopreventive and anticancer agents which act through normal biological response pathways.

The enhancement of carcinogenesis or tumour growth observed in some *in vivo* systems upon treatment with retinoids may be related, in part, to the use of sub-optimal doses to avoid host toxicity. It would be possible to circumvent this problem by combination of low doses of the retinoids with another agent with which it interacts additively, or better still synergistically, to inhibit carcinogenesis or tumour growth. Our earlier studies showed that dietary glucarate is an effective chemopreventive and antitumour agent against cancer induction/and growth in several rodent organs, including the mammary glands [10, 11]. The purpose of the present study was, therefore, to determine whether low ineffectual dietary levels of isotretinoin (13-cis-retinoic acid) and of CGT interact synergistically to inhibit the growth of pre-established rat mammary tumours. The dependence of this phenomenon on the sensitivity of human breast tumour cells to retinoids was also evaluated *in vitro* to determine whether the combination was effective on cells normally resistant to retinoids alone.

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MATERIALS AND METHODS

In vivo system

Mammary tumours were induced by treating 50 day old female Sprague–Dawley rats (Harlan Industries, Indianapolis, Indiana) by gavage with a single dose of 15 mg DMBA (75 mg/kg bw; Sigma Chemical Co., St Louis, Missouri) in 1.0 ml of sesame oil. The rats were maintained on semipurified AIN-76A diet (Dyets, Bethlehem, PA) and allowed food and water *ad libitum*. At 4 months post-DMBA treatment, when approximately 70% of the rats had at least one tumour of measurable size, the tumour-bearing rats were randomly assigned to four groups of 8–10 rats per group. Rats in the four groups were fed AIN-76A diets supplemented with (i) low isotretinoin (Grp RA; 1.0 mmol/kg diet); (ii) low calcium CGT (Grp CGT; 64 mmol/kg diet); (iii) the combination 1.0 mmol/kg isotretinoin + 64 mmol/kg CGT [Grp retinoic acid (RA) + CGT] or (iv) control (Grp C, no supplementation). These diets were fed for 4 weeks. The combination group was then shifted to and maintained on control diet for an additional 2 weeks. In a second experiment the food was supplemented with 1.0, 1.5 or 2.0 mmol/kg diet of isotretinoin to establish the dose response relationship at high dosages. The isotretinoin was added to the diet in a vehicle (1.0 mmol per 25 ml per kg diet) consisting of ethanol:tricaprylin (1:4, v/v) plus 6% (w/v) α-tocopherol. The vehicle was added

to all four diets. The supplements were blended into the AIN-76A diet using a Hobart food mixer. The isotretinoin was obtained from Hoffman-LaRoche, (Nutley, New Jersey) and the CGT from Gallard-Schlessenger, (Carle Place, New York). All other additives were purchased from Sigma. The diets were fed in stainless steel feeders designed with food hoppers. The food was replaced twice weekly.

Tumour specimens were fixed in 10% buffered formalin then processed for histopathology as previously described [10]. All tumours which had developed by 4 months and which were used in this experiment were adenocarcinomas. Tumours developing later usually include fibroadenomas but this was not confirmed. Tumour measurements were done twice weekly, beginning at the time the rats were placed on the supplemented diets. Tumour volume was calculated using the formula $4/3\ \pi\tau^3$ where τ is one-half the mean of the sum of the longest diameter and the axis at right angles to the latter diameter as determined with a micrometer caliper. The average tumour volume of each group was normalised to the average of the controls, then compared with its initial size on day zero to obtain the percentage change in tumour volume. Weights of rats in each group were recorded weekly.

Statistical significance of the differences between the means in each group was calculated by the t test.

In vitro-system

The efficacy of both isotretinoin and fenretinide (McNeil Labs, Springhouse, Pennsylvania) in combination with GT (Sigma Chemical Co.) was evaluated against the human MCF-7 and MDA MB-231 cell systems.The MCF-7 breast carcinoma cells [12] were obtained from the Ohio State University Cell Culture Service. They were grown at 37°C in a humidified atmosphere in monolayers in T-25 plastic tissue culture flasks (Falcon, Oxnard, California) containing 5.0 ml of B-10 medium. The latter consisted of minimal essential medium (MEM, Earles Base) supplemented with MEM non-essential aminoacids, L-glutamine (2.0 mmol/l), and 10% fetal bovine serum. One-half of the medium was replenished twice a week. Cells were harvested by trypsinisation upon reaching confluency. The oestrogen receptor negative, retinoid insensitive MDA MB-231 cell line [13], was obtained from the American Type Tissue Culture Collection (Rockville, Maryland). It is a highly anaplastic human breast adenocarcinoma. The cells were grown in L-15 culture medium with 10% fetal bovine serum.

For the growth inhibition assay the cells were plated in 24 well plates at a density of 1.5×10^4 cells/cm^2. At 24 h the medium was replaced with treatment medium containing retinoid, glucarate or their combinations at the specified concentrations. The retinoids were dissolved in absolute ethanol to give stock solutions of 10^{-2}mol/l and stored in the dark at −20°C until use, which was within 3 months. Control cultures were treated with an equivalent volume of ethanol. All variables were evaluated in triplicate. The treatment protocol was repeated on day 4, then on day 7 the cells were harvested by trypsinisation and viability was assessed by trypan blue exclusion [12].

RESULTS

In vivo studies

Tumour incidence was 72% at 4 months post-DMBA treatment at which time the 60 rats with tumour volumes 0.20 cm^3 or larger (i.e. which could be measured with accuracy) were randomised into four groups. The average tumour multiplicities (number of tumours per rat) at initiation of rats on the four diets and again after 4 weeks of treatment were as follows: (a) controls: [mean (S.D.)] 1.64 (0.17), 2.31 (0.14; (b) isotretinoin: 1.73 (0.26), 2.43 (0.22); (c) calcium glucarate (CGT): 1.43 (0.22), 2.33 (0.19); and (d) CGT + isotretinoin, 1.43 (0.31), 1.74 (0.32). Although the location and size of all tumours were monitored and recorded, only the tumours which were present when the animals were randomised to the four dietary groups (i.e. pre-established tumours) were included in the experimental data analysed in this study.

Shown in Fig. 1 is the change in tumour volume in each group (S.E.) as a function of time on the control or supplemented diets. At the lower dosage levels employed, CGT alone, i.e. (64 mmol/kg diet) did not significantly inhibit tumour growth, while isotretinoin, i.e. (1.0 mmol/kg diet) appeared to have a slight enhancing effect compared with the controls. In contrast, the combination of isotretinoin + CGT not only completely inhibited the growth of the tumours, but caused a significant ($P < 0.05$) net shrinkage of 20% after 4 weeks on the supplemented diet. This growth suppression was rapidly reversed upon transfer of the rats from the combination (isotretinoin + CGT) to the control (C) diet, the average tumour volume attaining that of the control group within 7 days. The reversal was tested at 4 weeks so that the larger tumours in the control, isotretinoin and CGT groups did not become necrotic and thereby compromise the outcome.

The average tumour volumes in cm^3 (S.E.) at zero time (start of experimental diets) and at 4 weeks on the diets were as follows: (a) Controls: 0.35 (0.01) and 0.77 (0.04), (b) RA: 0.33 (0.06) and 0.74 (0.10); (c) CGT: 0.37 (0.07) and 0.70 (0.08) and (d) RA + CGT: 0.36 (0.02) and 0.29 (0.02). The mean tumour volume of the RA + CGT group was significantly different ($P < 0.05$) from those of the control group both before and after normalisation of tumour volume of the experimental groups to the control group. The RA group was also significantly different from the controls ($P < 0.05$).

Not all tumours responded to the treatments in the same manner. In the control rats 11% remained stable, 5% decreased in size and 84% increased in size. Similarly, in the rats on isotretinoin 7% showed no change, 26% decreased and 67% increased in volume, some of them quite rapidly. Approximately 80% of the tumours in rats on the glucarate-supplemented diet showed moderate increases in volume, while only 16% showed

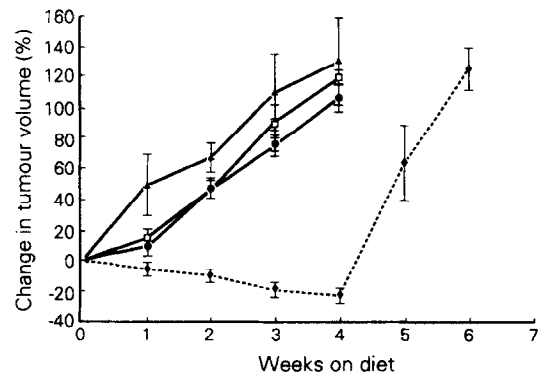

Fig. 1. Time-course change in tumour volume of rats fed (i) control diet (-□-); (ii) 1.0 mmol/kg 13-cis-retinoic acid (-△-); (iii) 64 mmole/kg calcium glucarate (-●-); or (iv) 13-cis-retinoic acid + calcium glucarate (-▲-). Rats on the combination diet were transferred to the control diet at week 4. The standard errors were based on 24, 25, 21 and 21 tumours in the control, RA, CGT and RA + CGT groups, respectively.

Table 1. Effect of high dietary concentrations of isotretinoin on the net growth of DMBA-induced rat mammary tumours

Isotretinoin (mmole/kg diet)	% Change in tumour volume*
1.0	+60 (8.0)
1.5	+20 (3.5)
2.0	−15 (2.0)†

* Values represent mean (S.E.) of 15 rats per group after 2 weeks of treatment.
† $P < 0.05$.

a decrease and 4% remained stable. Finally, the diet containing the combination of glucarate and retinoid caused a decrease in tumour size in approximately 60% of the rats, while 22% showed no change and a few (18%) showed continued moderate growth. The reason for this heterogeneity in response of the first tumours to these agents is not clear at present.

No significant differences in the average weights of rats occurred between groups during the course of the tumour measurements, i.e. at 1, 2, 3 and 4 weeks of treatment. For example, the average weight changes in gm/rat over the 4 week treatment period were +5.6 for controls, −0.4 for RA, +3.6 for CGT and −0.7 for the RA + CGT group. Some of these small changes might be secondary to tumour growth or regression. The initial weights of the rats at the beginning of the experiment was 264.4 (7.5), 262.1 (8.8), 266.3 (3.5) and 266.8 (3.6), respectively for the control, RA, CGT and RA + CGT groups.

Using a short-term treatment modality it is possible to evaluate the effects of dietary dosages of isotretinoin in excess of 1.0 mmol/kg without encountering observable toxicity, i.e. weight loss or reduced food intake of the tumour bearing rats. Rats bearing DMBA-induced mammary tumours were fed diets containing zero (controls), 1.0, 1.5 and 2.0 mmol/kg diet of isotretinoin acid and measurements were taken during the first 2 weeks after starting treatment. The results of the 2 week treatment summarised in Table 1 indicate that up to 1.5 mmol/kg diet, the isotretinoin group exhibits a significant enhancing effect. However, in the group receiving 2.0 mmol/kg a significant inhibition ($P < 0.05$) of tumour growth is observed. During these short-term treatments, no toxic effects, including reduced weight gain, were noted.

In vitro studies

Shown in Table 2 are the results of the *in vitro* experiments which attempt to duplicate the *in vivo* experiments on the MCF-7 and MDA MB-361 cell lines. Isotretinoin (1.0 μmol/l) is known to inhibit the proliferation of a number of human breast carcinoma cells, which are oestrogen receptor positive, including the MCF-7 cell line. It does not inhibit the proliferation of the oestrogen receptor negative human breast carcinoma cell line MDA MB-231 [12, 13]. As shown by the present study 0.1 μmol/l HPR inhibited the growth of MCF-7 cells by 17% while 1.0 μmol/l GT inhibited growth by approximately 12%. In contrast, together these two agents inhibited MCF-7 cell growth by 34%, instead of the expected inhibition of 27% if each agent were to act independent from each other. With the same MCF-7 cell line 1.0 μmol/l isotretinoin inhibited cell growth by 20% while the combination of 1.0 μmol/l isotretinoin and 1.0 μmol/l GT resulted in 45% growth inhibition, a 1.5-fold increase over the expected 30%. Evaluation by t test indicated that these differences are statistically significant at $P < 0.05$.

When similar concentrations of fenretinide, isotretinoin, GT or GT in combination with either retinoid are evaluated using the MDA MB-231 cell line, the effects of the retinoids alone are marginal varying from 5 to 12%. Furthermore, the combination of the retinoids with glucarate shows no increase in efficacy. Thus, the combination of glucarate with retinoids does not circumvent the innate resistance of the cells to retinoids. It should be noted that in both *in vivo* and *in vitro* experiments, HPR is much more effective against mammary tumour cells and therefore is used at a much lower concentration than isotretinoin acid.

DISCUSSION

As noted in the Introduction, two issues related to the use of retinoids in cancer chemotherapy and cancer chemoprevention are host toxicity and enhancement of carcinogenesis or tumour growth. These two factors may be related. Thus, sub-optimal doses of natural retinoids employed to avoid toxicity may enhance rather than inhibit tumour induction. An exception is fenretinide, a synthetic retinoid which has trophism for the mammary gland, and has proven very effective at subtoxic doses in prevention and treatment of mammary and bladder carcinogenesis [14]. However, because of their activity in a wide variety of organs, the vitamin A metabolites, all-trans and isotretinoin, are still of great interest. In particular, isotretinoin has been shown to be effective against cancers as diverse as mammary [4] and skin [15] on the one hand and leukaemia [16] on the other. Unlike fenretinide, isotretinoin is effective as a chemopreventive or chemotherapeutic agent against mammary cancer at very high and toxic dosages [4, 17]. This may explain why several investigators [1, 12] failed to produce mammary tumour inhibition with low nontoxic doses of isotretinoin. This is mainly due to certain pharmacokinetic limitations in the use of low dosages of isotretinoin which has a short half-life *in vivo* [18]. The rapid elimination of administered isotretinoin from the body offers a possible explanation for its low biological activity [19, 20]. Thus, for isotretinoin to be more effective, it has to be given at high and more frequent doses. This would, however, provoke cumulative and toxic effects during its prolonged or chronic use. In this study, the problem of toxicity has been circumvented by the combination of low nontoxic doses of isotretinoin with low doses of CGT with which isotretinoin interacts synergistically to inhibit mammary tumour growth. Thus, isotretinoin which shows activity against mammary tumours at near toxic levels, can be converted to an effective inhibitor of tumour growth at low dosages through combination with CGT. These results are consistent with our earlier studies showing synergism between CGT and the synthetic retinoid, HPR, in the *in vivo* inhibition of mammary carcinogenesis [21] or tumour growth [10]. Recently isotretinoin was shown to be a normal component of human serum, its level being subject to dietary modulation [22]. It is also the subject of a number of clinical and preclinical trials designed to cause differentiation extinction of tumours [23].

One means of reducing the cytotoxicity of retinoic acids is to form the glucuronide conjugate. Thus, although both retinoic acid and its glucuronide are equal in activity when tested for ability to induce differentiation of cultured HL-60 leukaemia cells, the glucuronide is 50% less cytotoxic [24]. Moreover, retinoid glucuronides have been shown recently to have a long

Table 2. Differential effect of retinoids, glucarate or their combination on the MCF-7 and MDA-MB-231 cell lines

Agent(s) (μmol/l)	Cell number (% of Control)*	
	MCF-7 Cells	MDA-MB-231 Cells
None (Control)	38 250 (140) (100)	23 200 (150) (100)
HPR (0.1)	32 512 (1300) [82.9 (3.6)]	20 419 (633) [87.8 (3.1)]
GT (1.0)	36 337 (2180) [88.4 (2.9)]	22 739 (1205) [97.5 (5.2)]
HPR + GT	15 682 (78) [66.4 (4.6)]†	18 331 (1649) [83.2 (8.7)]
Isotretinoin (1.0)	34 425 (1721) [79.5 (4.2)]	21 950 (654) [94.6 (3.1)]
Isotretinoin + GT	21 802 (1417) [55.4 (4.3)]†	21 881 (547) [94.3 (2.5)]

* Values represent means (S.E.) of 3–7 replicate samples.
† $P < 0.05$.

half-life compared to the free retinoid [25]. The nature of the interaction between glucarate and isotretinoin has not been determined, but the possible involvement of retinoyl-β-glucuronide is considered. 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 the retinoid [26]. Although the glucuronide can be used topically *in vivo* and has been given intraperitoneally [25], dietary administration would appear impractical. The retinoid: glucarate combination does offer an alternative approach to obtaining maximal biological activity at low concentrations of retinoic acid. Thus, these results demonstrate for the first time the potential use in synergistic combination of two normal metabolites in nontoxic chemotherapy. Based on the tissue culture studies, it does appear that glucarate cannot reverse the resistance of cells to retinoids, although it cannot be ruled out that this failure is due to other phenotypic changes which accompanied the acquisition of resistance to the retinoids. It has been emphasised that the ability of retinoids to inhibit carcinogenesis in experimental animals is subject to wide variability depending on site, species and strain [27]. Since glucarate alone appears to be effective in a diverse number of organs in at least two species [10] it will be of interest to determine whether the retinoid:glucarate combination also exhibits a wide range of effectiveness *vis-a-vis* carcinogenesis and tumorigenesis. Finally, glucarate is not the first agent evaluated in combination with retinoids on rat mammary tumours. For example, the response of *N*-methyl-*N*-nitrosourea-induced rat mammary cancer to dietary fenretinide has been evaluated in combination with tamoxifen and other agents [28].

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Direct Effects of Tamoxifen on Growth Hormone Secretion by Pituitary Cells *in vitro*

Suzanne A. Malaab, Michael N. Pollak and Cynthia G. Goodyer

There is now strong evidence to suggest that insulin-like growth factor I (IGF-I) plays an important role in breast cancer proliferation. Recently we observed that tamoxifen-treated stage I breast cancer patients have serum IGF-I levels significantly lower than placebo-treated patients. Since IGF-I is growth hormone (GH) dependent, we have tested the hypothesis that tamoxifen alters serum IGF-I levels through direct inhibition of GH secretion. Immature lamb pituitary cultures were examined for acute (3 h) or chronic (1–6 day) effects of the drug, using doses (0.1–10 μmol/l) based on known steady state levels in patients on tamoxifen therapy (0.31–3.1 μmol/l). Tamoxifen had a direct, dose-related, inhibitory effect on GH release from pituitary somatotropes, during acute as well as chronic treatment. The 10 μmol/l dose consistently decreased both basal and growth hormone releasing factor stimulated GH release. These *in vitro* data are consistent with our hypothesis that tamoxifen suppresses serum IGF-I levels by acting at the pituitary to inhibit GH release.
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INTRODUCTION

THERE IS now strong evidence to suggest that insulin-like growth factor I (IGF-I) plays an important role in breast cancer [1–5]. Recently, we observed that circulating IGF-I levels were significantly lower in patients with stage I breast cancer on tamoxifen treatment as compared with placebo-treated patients (0.9 vs. 1.4 U/ml; $P < 0.002$) [6]. Our study confirmed the preliminary findings from a smaller, non-randomised study (0.48 vs. 1.03 U/ml; $P < 0.01$) and supported the suggestion that the anti-neoplastic effects of tamoxifen might be, at least in part, due to suppression of circulating IGF-levels [7].

The mechanism by which tamoxifen, a synthetic nonsteroidal antioestrogen [8], exerts this inhibitory effect is not clear. However, the bulk of the evidence, from clinical as well as experimental studies, indicates that the major influence of oestrogens on serum IGF-I levels is indirect, by modulating growth hormone (GH) secretion and biological activity [9–13]. Therefore, we decided to test the hypothesis that tamoxifen is suppressing serum IGF-I levels by acting at the level of the pituitary to inhibit GH release.

Our laboratory has been using cultures of immature lamb pituitary cells to investigate the mechanisms that regulate GH secretion [14, 15]. We have chosen this test system to examine both acute and chronic effects of tamoxifen, on basal as well as growth hormone releasing factor (GRF) stimulated and somatostatin inhibited GH release. To determine the specificity of tamoxifen actions, prolactin (PRL) and luteinising hormone (LH) were also assayed. The doses tested (0.1–10 μmol/l) were based on known steady state drug levels in breast cancer patients receiving 20–40 mg of tamoxifen per day (0.31–3.1 μmol/l) [16].

MATERIALS AND METHODS

Materials

Collagenase (Type IV) was purchased from Cooper Biomedical (Mississauga, Ont., Canada), DNase from the Sigma Chemical Co. (St Louis, Missouri), 24-well (2 cm^2) tissue culture plates and Ham's F-10 culture medium from Flow Laboratories, Inc. (Mississauga, Ontario, Canada), defined fetal bovine serum (FBS) from Hyclone Sterile Systems (Logan, Utah), penicillin-

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