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Original Paper

Metabolism of *N*-[4-hydroxyphenyl]retinamide (4-HPR) to *N*-[4-methoxyphenyl]retinamide (4-MPR) may Serve as a Biomarker for its Efficacy Against Human Breast Cancer and Melanoma Cells

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A clinical trial of *N*-[4-hydroxyphenyl]retinamide (4-HPR) has been in progress for the past 4 years to evaluate its role in chemoprevention of breast cancer. However, it is currently not known whether the effect of 4-HPR in breast cells is mediated by 4-HPR directly or through one of its metabolites. In this report, we investigated *in vivo* and *in vitro* effects of 4-HPR on three different breast carcinoma cells and two different melanoma cell lines. *In vitro*, the growth of all three breast carcinoma cell lines was inhibited by 4-HPR. Only one of two melanoma cell lines (UISO-Mel-1) showed growth inhibition to 4-HPR. The cell lines sensitive to 4-HPR *in vitro* also showed inhibition to 4-HPR in a xenograft model. Dietary 4-HPR (0.5 mmol/kg diet) reduced the growth of UISO-BCA-1 xenografts in female athymic mice, but had no effect on UISO-Mel-6 xenografts. Metabolism investigations of the 4-HPR-sensitive and insensitive cell lines indicated that *N*-[4-methoxyphenyl]retinamide (4-MPR), the major metabolite of 4-HPR, was detected only in cells sensitive to 4-HPR. Further *in vitro* studies with 4-MPR suggested that it is not an active metabolite of 4-HPR as it failed to inhibit growth of 4-HPR-resistant UISO-Mel-6 cells, and showed no dose-dependent inhibition of 4-HPR-sensitive breast carcinoma and melanoma cell lines. Our results in the present study indicate that, although 4-MPR is not an active metabolite of 4-HPR, detection of this metabolite in the malignant cells may serve as an indirect biomarker to predict response of cells to 4-HPR. © **1998 Elsevier Science Ltd. All rights reserved.**

Key words: retinoids, breast cancer, melanoma, 4-HPR, 4-MPR, metabolism
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INTRODUCTION

RETINOIDS HAVE been at the forefront of cancer prevention research for the past 20 years. The most crucial requirement for the selection of a retinoid in chemoprevention studies is that it must be efficacious in experimental models and should not have toxic side-effects at the effective concentration. Virtually hundreds of retinoids have been evaluated for their efficacy at non-toxic concentrations in a variety of screening assays [1, 2]. One such analogue, *N*-[4-hydroxyphenyl] retinamide (4-HPR, Fenretinide) was synthesised by Johnson and Johnson and extensively studied for its chemopreventive activity. Earlier studies from our laboratory showed that 4-HPR did not concentrate in the liver and, thus, was considerably less toxic than other retinoids examined, which included retinyl acetate, 13-*cis* retinoic acid, retinyl methyl ether, etc. [3]. As compared with all-*trans* retinoic acid, which is toxic at a concentration of less than 1 mmol/kg diet, 4-HPR could be tolerated at 3 mmol/kg diet [4]. In experimental mammary carcinogenesis models, 4-HPR was found to be effective against both 7,12,dimethylbenz(a)anthracene and *N*-methyl-*N*-nitrosourea-induced mammary cancers in rats [3–5]. This inhibitory activity was found to be synergistic with that of ovariectomy, tamoxifen and calcium glucarate treatments [6–8]. Furthermore, it was shown that the retinoid prevented development of additional primaries when the

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treatment was initiated after the removal of the first palpable tumour [4]. The majority of the effort is directed towards understanding the chemopreventive effects of 4-HPR. However, we reported that dietary treatment of 4-HPR suppressed progression of established mammary tumours and, in nearly 23% of the animals, the tumours regressed after 4-HPR treatment [9]. Clinically, 4-HPR is being evaluated for its efficacy in preventing contralateral primaries in patients already treated for breast cancer. Patients in intervention groups are treated with 200 mg/day 4-HPR for 5 years. The trial has been described in detail [10, 11]. Pharmacological studies showed that 4-HPR treatment lowers retinol level in these women [12]. This has resulted in providing a drug-holiday for the patients to bring back the retinol concentration in circulation.

Since 4-HPR is currently being investigated in a clinical trial for breast cancer patients, its mechanism of action has been examined by several investigators. In order to understand the mechanism of action of 4-HPR, it is important to determine whether 4-HPR or one of its metabolites is the active chemopreventive agent. Previous studies, including reports from our laboratory, have shown that one of the principal metabolites of 4-HPR is *N*-[4-methoxyphenyl]retinamide (4-MPR) [13]. In addition to 4-MPR, there is a minor, more polar metabolite found in all the tissues examined. Hultin and colleagues [13] conducted pharmacokinetic experiments and showed that after a single intravenous injection, the distribution of 4-HPR was completed in 4 h followed by the first order of elimination kinetics. The $t_{\frac{1}{2}}$ for 4-HPR was observed to be 9–12 h for liver and serum, whereas it was 44 h for mammary glands. Moreover, in both mice and rats, 4-MPR was found to be a major metabolite of 4-HPR. These results, however, did not indicate whether the retinoid was metabolised mainly in the liver, with the metabolites subsequently distributed to various organs, or if 4-HPR by itself was distributed to various organs and then metabolised by the target organ(s). Using mouse mammary gland organ culture, we showed that 4-HPR is metabolised by the mammary gland and again 4-MPR is the main metabolite [14]. We showed that there was a differential distribution of 4-HPR and 4-MPR in epithelial cells and fat components in normal and cancerous breast tissues. 4-HPR was mainly concentrated in the epithelial cells, whereas 4-MPR was largely located in the adipose tissues [15]. It is currently not known whether the metabolism occurs in the adipose tissue or epithelial cells. Although the metabolism of 4-HPR into 4-MPR has been well established, the role of 4-MPR is not defined. In mammary gland organ cultures, 4-MPR inhibited growth of carcinogen-induced mammary lesions only at 10^{-5} M concentration. Similarly, for many cells types, including human breast cancer cells in culture, 4-MPR has been ineffective in inhibiting cell proliferation [16, 17]. In the present study, we evaluated the effects of 4-HPR and 4-MPR in breast cancer cells and melanoma cells *in vitro* and characterised their metabolism patterns. Furthermore, we correlated the extent of metabolism of 4-HPR into 4-MPR and the effectiveness of 4-HPR *in vitro* and *in vivo*.

MATERIALS AND METHODS

Cell lines

Three different human breast carcinoma cell lines (MCF-7, UISO-BCA-1, UISO-BCA-4) and two different human melanoma cell lines (UISO-Mel-1, UISO-Mel-6) were used for the experiments. All UISO cell lines were established and characterised in our own laboratory [18–20]. MCF-7 human breast carcinoma cell line was obtained from American Type Culture Collection (ATCC, Rockville, Maryland, U.S.A.). All cell lines were maintained in culture in our laboratory in culture medium (minimum essential medium with Earle's salt (MEM-E)) containing 2 mM glutamine 15% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml Fungizone and 0.5 mM non-essential amino acids (Biologos, Naperville, Illinois, U.S.A.).

In vitro *effect of* 4-*HPR and* 4-*MPR on breast carcinoma and melanoma cells*

Cells were seeded in 24-well culture plates (10^4 cells/well) in MEM-E culture medium containing 5% charcoal stripped heat inactivated fetal bovine serum and then incubated overnight at 37°C in an atmosphere containing 95% air and 5% CO_2. Following incubation, the cells were fed with culture medium containing increasing concentrations of 4-HPR and 4-MPR (ranging between 0.01 and 10 μM). Controls received basal culture medium without the retinoid supplementation. The medium was changed on days 4 and 7 post-seeding. The number of cells was counted in each well 10 days postincubation using a Coulter counter. The number of cells obtained in the controls was considered as 100%. Results obtained in the treated cells were calculated as the percentage of controls. Each observation was run in triplicate to obtain mean values of each observation.

In vivo *effect of* 4-*HPR supplemented in diet*

The effects of dietary 4-HPR supplement were evaluated in selected cell lines with proven tumorigenic potential using an *in vivo* athymic mice model. UISO-BCA-1 was selected as a representative 4-HPR responsive cell line, and UISO-Mel-6 cells were selected as 4-HPR non-responsive cells. For *in vivo* studies, a cell suspension made in Hanks balanced salt solution (HBSS; 10^6 cells/0.1 ml) was injected (0.1 ml) subcutaneously into the dorsal flank region of 3–4 week old female athymic mice (Frederick Cancer Research Facility, Frederick, Maryland, U.S.A.). Animals were divided into two groups, a control group receiving a placebo diet and an experimental group receiving the 4-HPR diet. Each group consisted of five animals. Animals received water and diet *ad libitum*.

Autoclavable powdered diet specific for athymic mice, obtained from Tek Lad (Madison, Wisconsin, U.S.A.), was mixed with 4-HPR (1 mmol/kg diet) dissolved in ethanol using diet mixer. The control diet received a comparable amount of ethanol without 4-HPR. All diets were stored at 4°C in containers protected from direct light exposure. All diets were autoclaved prior to being placed in the food cups. The concentration of 4-HPR was determined by HPLC analysis prior to and after, autoclaving the diet. The stability of 4-HPR mixed in the diet and stored at 4°C was also determined periodically. 4-HPR mixed in the diet and stored at 4°C was stable for more than 3 months. After autoclaving, only 50% (0.5 mmol) of the originally added 4-HPR was detected in the diet. Both control and experimental animals were observed daily for the signs of gross toxicity. Tumours developing at the original cell inoculation site were monitored for their growth weekly. Tumour size (cm) in three different planes was determined using calipers and tumour volume was calculated using the mathematical formula $l \times h \times w \times \pi/6 = \text{cm}^3$

volume. Animals were sacrificed by CO_2 asphyxiation, 30 days postdietary treatment or if they appeared moribund or tumours became necrotic in any of the animals. At the termination of the experiment, tumours and liver tissues were dissected and quickly frozen in liquid nitrogen. The tissues were stored at −70°C until used for HPLC analysis. Data for tumour volume are represented as mean tumour volume obtained in the 5 mice in the group.

Analysis of 4-HPR and its metabolites

4-HPR and its metabolites were determined in cells and tissues by the procedure established by our group and described in detail previously [21]. The procedure separates both *trans* and *cis* forms of 4-HPR from other retinamides, such as 4-MPR, retinyl esters and more polar forms such as *trans* and its retinoic acid. The retention times for 4-HPR and 4-MPR are 24 and 27 min, respectively. In brief, tissues were lyophilised and extracted twice with 1:10 volume of chloroform:methanol (2:1 v/v). The extraction mixture was centrifuged at 800 *g* for 10 min. Following centrifugation, the organic phase, obtained as a supernatant, was separated from the tissue debris and was evaporated under a gentle stream of nitrogen. The residue was reconstituted in 0.5 ml methanol and was used for HPLC analysis. Retinoid metabolites in the cells were determined directly, without lyophilising. The extracts were chromatographed on a Whatman C18 Particil, 10ODS-2 reversed HPLC column using the Hitachi HPLC system. The retinoids were separated with a linear gradient of methanol:water (70:30) to 100% methanol in 30 min at a flow rate of 1.2 ml/min. The retinoids were detected at 350 nm wavelength using a Hitachi detector. Along with the sample, various known concentrations were processed as known standards. 4-HPR concentration in the tissues/cells was calculated from the standard curve generated. The metabolite of 4-HPR was identified from known standards of 4-MPR processed along with the samples. Other metabolites detected as peaks on the chromatogram were referred to as unidentified peaks. For each cell line/tissue and each treatment group, assays were performed in triplicate.

RESULTS

Antiproliferative effects of 4-HPR and 4-MPR

The effect of 4-HPR on breast cancer and melanoma cells was evaluated *in vitro* at various concentrations ranging between 1 nM and 10 μM. The cells were exposed *in vitro* to 4-HPR/4-MPR for 10 days. As shown in Figure 1, 4-HPR inhibited cell proliferation at 1 μM (10^{-6} M) concentration in all breast carcinoma cell lines studied. At a higher concentration (10 μM), very few cells survived and inhibition was more than 90%. These results suggest that 10 μM 4-HPR exposure may be toxic to these cells. In human breast carcinoma cells, growth inhibition was evident between 0.1 and 1 μM concentration. At 1 μM concentration, growth inhibition varied between 38 and 77% in different breast carcinoma

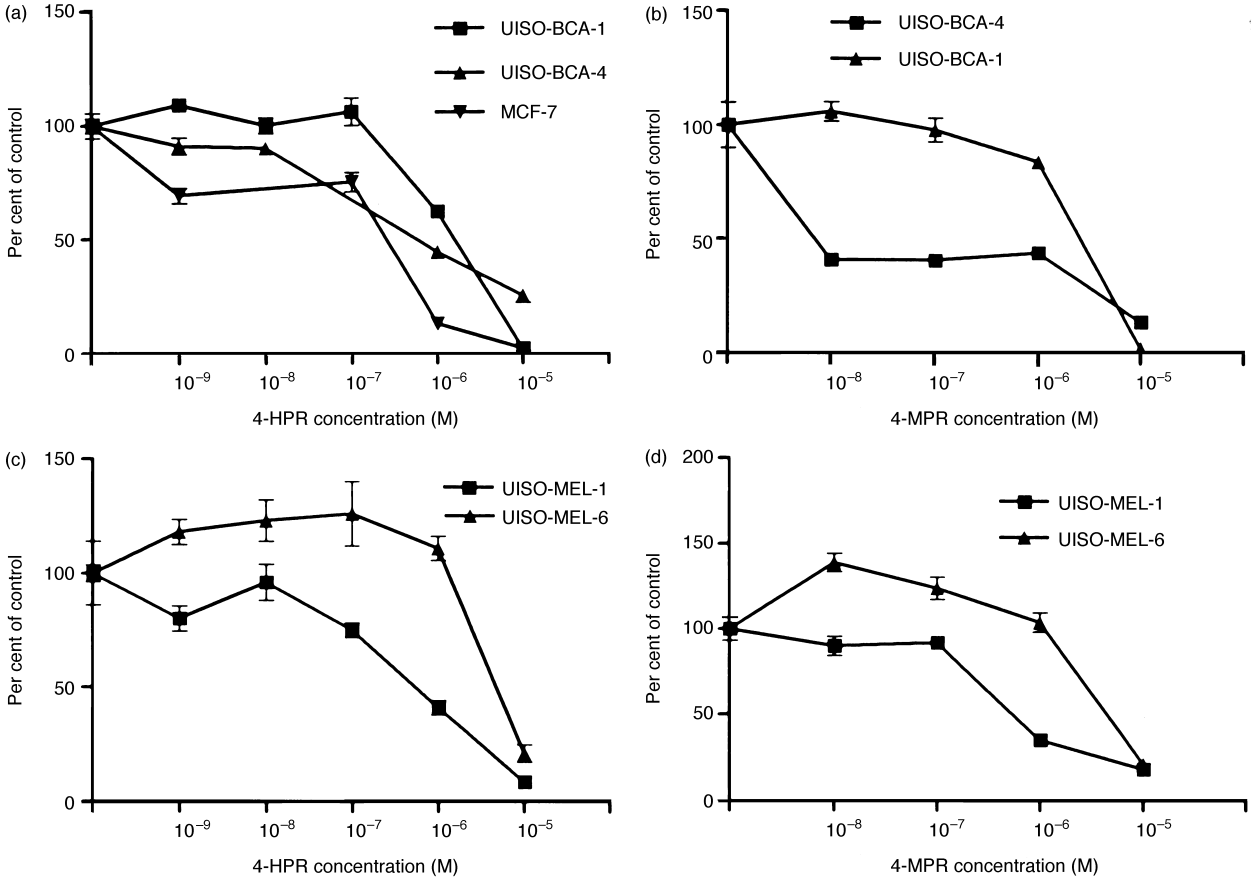

Figure 1. Effects of *N*-[4-hydroxyphenyl]retinamide (4-HPR) and *N*-(4-methoxyphenyl]retinamide (4-MPR) on breast and melanoma cell lines. Three human breast cancer cell lines, BCA-1, BCA-4 and MCF-7 and two melanoma cell lines were incubated with increasing concentrations of either 4-HPR (a) and (c) or 4-MPR (b) and (d) ranging from 0.001 to 10 μM for 10 days. At the termination of incubation, the cells were counted using a Coulter counter. Each data point represents the mean % ± standard error value of three independent observations.

lines. Only UISO-Mel-1 cells showed growth inhibition at 1 μM 4-HPR, with 59% growth inhibition. UISO-Mel-6 cells were only inhibited by 10^{-5} M 4-HPR (Figure 1c).

We also evaluated the effect of 4-MPR, a major metabolite of 4-HPR. As shown in Figure 1(b), UISO-BCA-4 showed 60% growth inhibition at 0.1 nM (10^{-8} M) 4-MPR concentration, but at higher concentrations no dose-related inhibition of cell growth was observed. At 10 μM concentration, more than 80% growth inhibition was observed. UISO-BCA-1 cells, weakly responsive to 4-HPR, were not inhibited by 4-MPR at 0.1–1 μM. 4-MPR at 1–10 μM inhibited UISO-Mel-1 cells, but at lower concentrations no significant growth inhibition was observed. In UISO-Mel-6 cells, 0.1 nM 4-MPR resulted in a small increase in cell growth. Only at 10 μM concentration, was growth inhibited (90%) in these cells (Figure 1d).

Metabolism of 4-HPR in breast cancer carcinoma and melanoma cells

All breast cancer cell lines evaluated showed the presence of a distinct peak at 24 and 27 min representing 4-HPR and 4-MPR, respectively. The concentration of 4-HPR in different cell lines varied between 1.79 μg/10^6 cells and 3.59 μg/10^6 cells (Table 1). The presence of serum in the medium did not affect retention or metabolism of 4-HPR in the cells. In addition to 4-HPR and 4-MPR, we also observed other distinct unidentified peaks of polar and non-polar retinoids. In all breast carcinoma cells studied, 4-MPR was detected and it represented 8–10% of 4-HPR in the cells. The ratio of 4-MPR to 4-HPR was in the range of 0.12–0.19. In melanoma cells, the concentration of 4-HPR varied between 1.6 and 5.35 μg/million cells. In contrast to breast carcinoma cells, 4-MPR was detected only in UISO-MEL-1 cells, with no 4-MPR detected in UISO-Mel-6 cells. The ratio of 4-MPR/4-HPR in UISO-Mel-1 cells was 0.03 (Table 1).

Effect of dietary 4-HPR supplementation on the growth of UISO-BCA-1 and UISO-BCA-6 cells in athymic mice

We evaluated the effect of 4-HPR supplemented in the diet on the growth of UISO-Mel-6 and UISO-BCA-1 cells. *In vitro* UISO-BCA-1 cells were inhibited by 4-HPR, whilst UISO-Mel-6 cells were resistant. Both these cell lines are highly tumorigenic in athymic mice. In both control and experimental animals, 100% of animals showed growth of palpable tumours within 2 weeks. The xenografts from UISO-BCA-1 cells achieved exponential growth phase at 16 days postinoculation, both in control and experimental groups. However, at day 24 postinoculation, the mean tumour volume was 0.06 cm^3 in UISO-BCA-1 controls, which was reduced by 50% in animals receiving 4-HPR-supplemented diet (Figure 2a). 4-HPR in the diet failed to influence growth of UISO-Mel-6 cells (Figure 2b).

Table 1. *Metabolism of* N-*[4-hydroxyphenyl]retinamide (4-HPR) in breast cancer and melanoma cells*

Cell lines	4-HPR (μg/10^6 cells)	4-MPR	Peak height 4-MPR:4-HPR
MCF-7	3.59	+	0.19
BCA-1	1.59	+	0.12
BCA-4	1.79	+	0.17
Mel-1	5.35	+	0.03
Mel-6	1.60	ND	–

4-MPR, *N*-[4-methoxyphenyl]retinamide; ND, not detected.

DISCUSSION

In recent years, considerable attention has been directed towards understanding the role of 4-HPR in chemoprevention. This surge in 4-HPR research is attributed to current ongoing clinical trials that employ 4-HPR as a chemopreventive agent [10, 11]. In addition to a large-scale breast cancer chemoprevention trial on breast cancer patients, 4-HPR has also been evaluated against oral leucoplakia and in combination with tamoxifen against breast cancer [22]. The mechanism of 4-HPR action thus far has largely been speculative. Several attractive hypotheses have been discarded and some are still far from conclusive. For example, 4-HPR by itself does not bind to cellular retinoic acid binding proteins [23] or nuclear retinoic acid receptor β [24]. Some reports have speculated that 4-HPR is a retinoic acid receptor γ selective retinoid [16], but more recent studies have not confirmed this [25]. In addition, it induces transforming growth factor-β and downregulates insulin-like growth factor [26, 27]. Recent studies have also shown that 4-HPR induces apoptosis in several cell types [27]. Moreover, 4-HPR-induced programmed cell death is correlated with downregulation of survival factors, such as bcl-2 [27]. Yet none of these studies have focused on whether 4-HPR is the active retinoid or if it needs to be metabolised to an active metabolite.

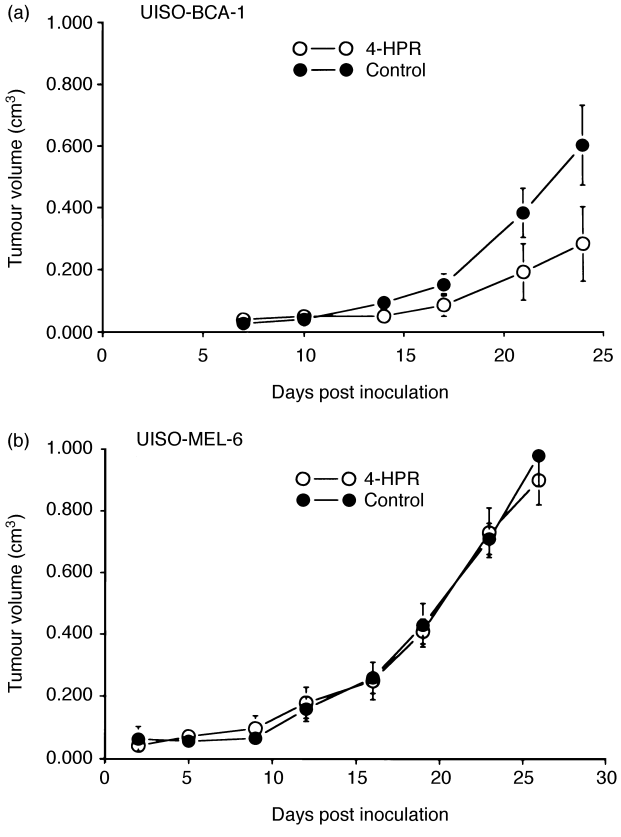

Figure 2. The effect of dietary *N*-[4-hydroxyphenyl]retinamide (4-HPR) on xenograft tumour growth from UISO-BCA-1 (a) or UISO-Mel-6 (b) cell lines. Animals received either the basal diet or the diet containing 0.5 mmol 4-HPR/kg diet.

Previous metabolism studies have consistently demonstrated that 4-MPR is the major metabolite of 4-HPR. In addition to 4-MPR, another unidentified polar metabolite has also been reported in both liver and mammary glands [13, 14]. We reported that 4-MPR is the major metabolite of 4-HPR in the breast tissue and tumour obtained from women receiving 4-HPR treatment in the Milan trial [10, 11]. Interestingly, we also observed that 4-MPR was largely localised in the adipose tissue, whereas 4-HPR and another metabolite, which has not yet been identified, were found in the epithelial cells. This raises the question of whether 4-HPR, 4-MPR, or an as yet unidentified metabolite is the active retinoid. In the present study, we observed that the cell lines that responded to 4-HPR treatment also contained 4-MPR as a metabolite, indicating that the metabolism of 4-HPR is essential for its action. Moreover, the 4-MPR:4-HPR ratio was directly related to the extent of 4-HPR inhibition of growth. For example, UISO-MEL-1 melanoma cells responded to 4-HPR and contained 4-MPR as a metabolite. Similarly, both UISO-BCA-1 and UISO-BCA-4 exhibited 4-MPR peaks and responded to 4-HPR treatment *in vitro*. In contrast, UISO-MEL-6 did not respond to 4-HPR and did not metabolise it, with no 4-MPR or other metabolites detected. It is also interesting to note that the xenografts of cells that metabolised 4-HPR in culture, also responded to dietary 4-HPR treatment and showed reduced tumour burden, whereas xenografts from cells that were not able to metabolise the retinoid were unaffected by dietary 4-HPR.

The importance of 4-MPR as a major active metabolite of 4-HPR has been reported in a study in which a decline of insulin-like growth factor was seen in plasma [27]. However, it does not suggest that 4-MPR is the active metabolite. In order to rule out the possibility of 4-MPR as an active metabolite, we evaluated effects of 4-MPR on both breast cancer and melanoma cells. The results clearly indicated that 4-MPR was ineffective in a dose-related manner. At the highest concentration, 4-MPR inhibited growth of all cell types. However, this effect can not be dissociated from the toxicity of 4-MPR. Thus, 4-MPR may not be an active metabolite of 4-HPR. Our results indicate that the presence of 4-MPR in tissues from 4-HPR-treated animals may serve as a biochemical marker for the effectiveness of 4-HPR on tumour xenografts.

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