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Effect of Early vs. Late Administration of 4-Hydroxyphenylretinamide (4-HPR) on N-Methyl-N-Nitrosourea (MNU)-Induced Mammary Tumorigenesis

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Abstract Mammary tumors were induced in 48–52-day-old female Sprague-Dawley rats in metestrus or diestrus with a single jugular injection of MNU (50 mg/kg). Control rats received the saline vehicle (Group 4 n = 9). Rats were fed 4% Teklad diet containing either 0 (Group 3, n = 20) or 782 mg 4-HPR/kg diet. 4-HPR supplementation was initiated either 1 week prior to (Group 1, n = 14) or 4 weeks following MNU administration (Group 2, n = 19). Neither body weight nor food intake differed significantly between treatment groups. Feeding of 4-HPR 1 week prior to tumor induction reduced the number of tumors (0.8±.2) when compared to MNU control rats (2.1±.4). Immunohistochemical staining of mammary tumor sections for PCNA was quantitated by microdensitometry and expressed as an HSCORE. No differences in HSCORE were observed between tumor groups although the percentage of nuclear area occupied by intermediate and darkly stained nuclei was reduced in the late 4-HPR group. GC→AT transitions in codon 12 of the H-*ras* gene were detected in 50% (12/24) of MNU control tumors, 60% (6/10) of early 4-HPR tumors, and 38% (6/16) of late 4-HPR tumors. Mutation rates did not differ significantly between groups. 4-HPR appears to be a more effective chemopreventive when fed during the initiation period. J. Cell. Biochem. Suppl. 27:92–99.

Key words: chemoprevention; H-ras; PCNA; rat; retinoid

4-hydroxyphenyl retinamide (4-HPR) has been studied extensively in rat mammary carcinoma. Its demonstrated effects include decreased tumor incidence and multiplicity when fed 1 week prior to tumor induction in young (50-day-old) rats [1] or 60 days prior to induction in aged (120-day-old) rats [2], partial or complete regression in 40% of primary tumors [3], decreased recurrence following resection of primary tumor [4], and inhibition of in vitro mammary gland neoplastic transformation [5]. The chemopreventive effect of 4-HPR is reduced when dietary supplementation is delayed until 1 week following MNU induction in 50-day-old rats [6], and lost completely when dietary supplementation is terminated at the time of tumor induction [7]. Retinoids appear to act as nuclear transcription regulators modulating cell growth and differentiation [8,9]. The inhibitory effect of 4-HPR extends to other organ systems of the rat as well [10].

Hepatic toxicity, a common problem in prolonged therapy with RA appears to be alleviated by the use of 4-HPR [11]. Chronic administration results in accumulation of 4-HPR in human breast tissue and rat mammary gland where it is metabolized to the active N-(4-methoxyphenyl)retinamide metabolite; only minimal accumulation of parent compound and control levels of retinyl palmitate appear in the liver [12,13].

Animal age at time of carcinogen exposure is known to dramatically effect induction of tumors in Sprague-Dawley rats by 3-methylcholanthrene [14], DMBA [15], or MNU [16]. Rats at 35–50 days of age when mitotic activity is high in mammary terminal end buds appear

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most sensitive to initiating events caused by MNU exposure [16,17] . Expected incidence rates are greater than 90% in this age group but fall markedly (from 59 to 27%) when induction begins in rats aged 80 to 140 days [16]. Thus, sensitivity of mammary epithelium is effected more by the high proliferative activity present in younger animals than by O^6-methylguanine repair rate, which declines in mammary tissue with age [17].

The present experiment was designed to determine if 4-HPR supplementation could begin at a later time point when a high incidence of ductal carcinoma in situ is known to exist [18] and still show chemopreventive activity as has been demonstrated for RA [19]. We also sought to determine if the known antiproliferative activity of 4-HPR would alter the known involvement of H-*ras* mutation in this model system.

MATERIALS AND METHODS

Female Sprague Dawley rats were purchased (Fredrick Cancer Research Facility, Fredrick, MD) at 35 days of age, housed singly in polycarbonate cages at a room temperature of 70±2°F with ad libitum access to water and pelleted diet (Wayne Lab Blocs) and acclimated for 3 days to a 12 hr light/dark cycle under yellow light. All rats were then switched to a 4% Teklad powdered control diet for 3 days prior to randomization into treatment groups. 4-HPR was obtained from McNeil Pharmaceutical (Spring House, PA), and incorporated into the 4% Teklad diet at 786 mg/kg. All mixing of diet was done under yellow light, formulated diet was stored in the dark at 4°C. Food cups were changed 2 times per week.

Estrus stage was determined in all rats from vaginal smears. Between 48–50 days of age, rats in either metestrus or diestrus received an intrajugular injection of MNU (Sigma, St. Louis, MO, 50 mg/kg, pH 5.0, 12.5 mg/ml) prepared immediately before use in 3% acetic acid/distilled water. Group 1 animals (n = 14; Early 4-HPR) were fed the 4-HPR diet beginning at 41 days of age, 1 week prior to MNU injection. Group 2 (n = 19; Late 4-HPR) was fed the control diet for 28 days following MNU injections, then switched to the 4-HPR diet. Rats in group 3 (n = 20; MNU controls) received MNU but were fed the control diet throughout the experiment. Group 4 animals (n = 9) served as vehicle controls and were also fed the control diet.

Four weeks following administration of MNU, animals were palpated weekly for the remainder of the experiment. Location, date of appearance, and measurements of all tumors were recorded. Animals were observed twice daily to monitor general health and weighed weekly.

Invasive tumors were saved as both formalin-fixed paraffin blocks and frozen tissue. Formalin fixation was accomplished using 10% neutral buffered formalin for 15 min, after which the tissue was stored in 70% ethanol until embedding. Overfixation can seriously compromise antigen reactivity. Tumors to be saved as frozen specimens were mounted on steel chucks equilibrated to −35°C, coated in OCT (Miles Inc., Kankekee, IL), and stored at −70°C.

Immunohistochemistry

Proliferating cell nuclear antigen (PCNA) protein was identified in 6 µm paraffin sections that were first heated at 57°C for 30 min, deparafinized in 3 changes of xylene, and hydrated through graded alcohols to phosphate buffered saline (PBS, pH 7.4). Slides were then exposed to microwave irradiation in citrate buffer (50 mM, pH 6.0) that had first been heated to boiling, for 4 successive 5-min periods at 10–20% power, sufficient to hold the buffer temperature between 95–100°C. The slides were then allowed to cool in the buffer for 30 min and washed 3 times in PBS. Endogenous peroxidase activity was blocked by incubation in 0.1% H_2O_2 in methanol/PBS (1:1) for 30 min. Nonspecific antibody binding was blocked with goat serum (diluted 1:15) prior to addition of primary antibody (Coulter Immunology, Hialeah, FL) for overnight incubation at 4°C. Following 45 min binding of goat anti-mouse secondary antibody (1:400), the complex was detected by avidin peroxidase (Vector Laboratories, Burlingame, CA) with diaminobenzidine as substrate. Density of immunostaining was quantified by image analysis (CAS 200, Becton Dickenson, San Jose, CA). Using uniformly applied optical density cutoffs, nuclei were partitioned into light, intermediate, and darkly stained categories representing early G1, late G1 to early S-phase cells, and mid to late S-phase cells, respectively [20]. Tumor nuclei within random microscopic fields were evaluated to provide a total nuclear area of 32,000 µm^2. With typical nuclei having areas between 16–20 µm^2, analysis of each section corresponds to more than 1,600 nuclei. A quantitative HSCORE was de-

rived based on percentage of total nuclear area and calculated as HSCORE = $\Sigma(i+1)Pi$ where i= 1, 2, 3, and P is the fractional nuclear area varying from 0 to 100%.

DNA Extraction

Frozen tumor was pulverized at −80°C to a fine powder DNA and was isolated by overnight incubation with nuclease-free proteinase K at 37°C in 10 mM Tris, pH 7.5, 150 mM NaCl, 2 mM EDTA and 0.5% sodium dodecyl sulfate. Saturated NaCl was used to extract the DNA followed by ethanol precipitation [21].

Polymerase Chain Reaction

Oligo primers flanking the H-ras codon 12 were synthesized using the solid phase phosphoramidite method (DNA model 391B synthesizer; Applied Biosystems, Foster City, CA). Upstream and downstream primer sequences were (P1U) 5′-GTAGAAGCCATGACAGAATACAAG-CTTGT-3′ and (P1L) 5′-CAGAGCTCACCTC-TATAGTGGGATCATACT-3′, respectively. One hundred microliters of reaction mixture containing approximately 100 ng of genomic DNA in 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 100 µM of each of the 4 deoxyribonucleoside triphosphates (dATP, dCTP, dGTP, and dTTP), 2.5 U of Taq DNA polymerase, and 40 pmol of each primer were overlaid with 100 µl of mineral oil and subjected to 30 cycles of amplification using a thermal cycler (Barnstead Thermolyne, Dubuque, IA). Each cycle consisted of 1 min denaturation at 94°C, 2 min annealing at 60°C, and 2 min of extension at 72°C. Ninety microliters of the reaction product was loaded onto a 0.8% agarose gel to isolate the 1,120 bp expected fragment. DNA was extracted from the excised gel block using a commercial kit (Qiagen, Inc., Chatsworth, CA). A second round PCR and agarose gel purification step yielded the final product for direct sequencing.

Direct Sequence Analysis

H-*ras* mutations were identified by direct sequencing of the purified PCR product [22]. Purified P1L downstream primer was end-labeled with [γ-^{32}P]ATP by T4 polynucleotide kinase and annealed to H-*ras* DNA at 0°C for 5 min. The mixture was then divided into 4 tubes containing 3 µl of 80 µM dideoxyribonucleoside triphosphates, 1.5 U of sequenase (USB, Cleveland, OH) was added, and the reaction allowed to proceed for 5 min at 37°C. The reaction was terminated by heating at 95°C for 5 min and the labeled fragments displayed by electrophoresis on an 8% polyacrylamide gel. The gel was dried and exposed to X-ray film for 24 hr.

Statistical Analysis

Tumor-free survival functions were estimated by the Kaplan-Meier product-limit for censored data. The three survival functions were then compared by log rank and Wilcoxon tests (SAS Institute Inc., Cary, NC). Tumor incidence was compared using the χ^2 test. Tumor multiplicity was compared using Jonckhere-Terpstra test for ordered alternatives [23]. Percent distribution of nuclei positively stained for PCNA was analyzed following arcsin square root transformation by analysis of variance using the Bonferroni t-test for individual group means (Jandel Scientific, San Rafel, CA). Injection and final body weights were compared by analysis of variance.

RESULTS

At 782 mg/kg diet, 4-HPR had no effect on measured food intake (data not shown) or weight gain when compared to the MNU control group (Fig. 1). Mean body weight of rats receiving MNU fell significantly below that of vehicle controls at 5 and 8 weeks post tumor induction but not at other time points. At termination of the experiment the coefficient of variation for body weight of all rats was less than 10% (Table I). Early death in 4/5 rats resulted from aggressive tumor growth; the other had no detectable tumor present. These early deaths did not result in significant differences for survival among treatment groups. The first palpable tumors appeared at 11, 12, and 13 weeks post MNU injection in Groups 2, 3, and 1, respectively, indicating that 4-HPR treatment was without effect on latency. Tumor incidence at 26 weeks post injection was similar to results previously reported for this model system, with all tumors being diagnosed as adenocarcinomas. Administration of 4-HPR in the diet 1 week prior to tumor induction at 48–52 days of age reduced tumor multiplicity by nearly 50% when compared to rats whose treatment was delayed until 4 weeks after tumor induction and by 60% when compared to MNU control rats.

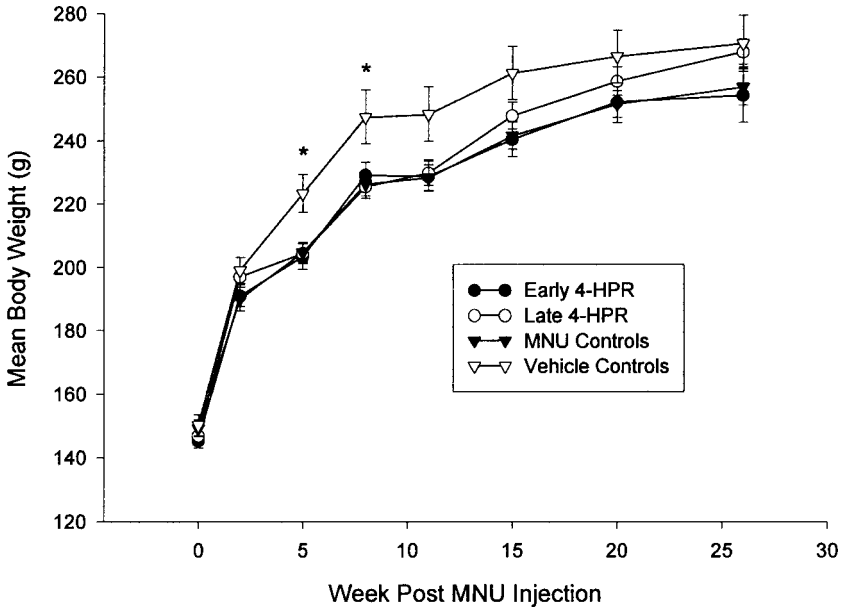

Fig. 1. Effect of 4% Teklad diet supplementation with 4-HPR on weight gain in female Sprague Dawley rats following a single intrajugular injection of MNU or saline at 48–52 days of age. Mean body weight of vehicle control group is significantly different from MNU treated rats only at weeks 5 and 8 ($P < 0.05$).

TABLE I. Effect of Early Vs. Late Administration of 4-Hydroxyphenylretinamide (4-HPR) on Methylnitrosourea (MNU) Induced Mammary Tumorigenesis†

4-HPR diet supplement (782 mg/kg)	No. of rats	MNU dose (mg/kg)	Injection wt. (g)	Final wt. (g)	Survival (%)	Incidence (%)	Tumors per rat	Latency (days)
Early (−7 days)	14	50	145 ± 2	254 ± 9	100	71	0.8 ± .2*	120 ± 5
Late (+28 days)	19	50	147 ± 2	268 ± 4	84	74	1.5 ± .3	131 ± 11
None	20	50	149 ± 3	256 ± 6	90	84	2.1 ± .4	138 ± 8
None	9	0	150 ± 3	271 ± 9	100	0	0	

†Values are means ± SEM.
*Significantly different from MNU control group ($P < 0.05$).

Immunostaining for PCNA within tumor nuclei was focal in nature, with dark staining characteristic of S-phase cells present to some extent in all tumors examined. Quantitation of staining intensity expressed as an HSCORE, which included lightly stained and presumably G1 phase nuclei, was not significantly different between tumor groups (55±13, early 4-HPR; 31±6, late 4-HPR; and 42±6, MNU controls). The intermediate + darkly stained fraction of PCNA positive cells as a percentage of total nuclear area was decreased significantly in the late 4-HPR group (Fig. 2).

All tumors with mutations identified in the H-*ras* gene were GC→AT transitions in codon 12. With the exception of two tumors from the early 4-HPR group, all mutations occurred at the second base pair position (Table II). Although there was a tendency for fewer mutation events from tumors where 4-HPR supplementation was delayed, this difference was not significant.

DISCUSSION

The present results are consistent with previous work demonstrating that retinoic acid (RA) and 4-HPR decrease tumor multiplicity when fed during the initiation and promotion phases of MNU or 7,12-dimethylbenz(a)-anthracene (DMBA) induced tumor formation [24, 25]. McCormick and Moon [19] have demonstrated that a treatment window for RA chemopreventive effectiveness exists dependent upon the initiating carcinogen dosage. Reduction in tumor incidence was achieved when 1 mmol/kg RA administration was delayed by 4 or 16 weeks after

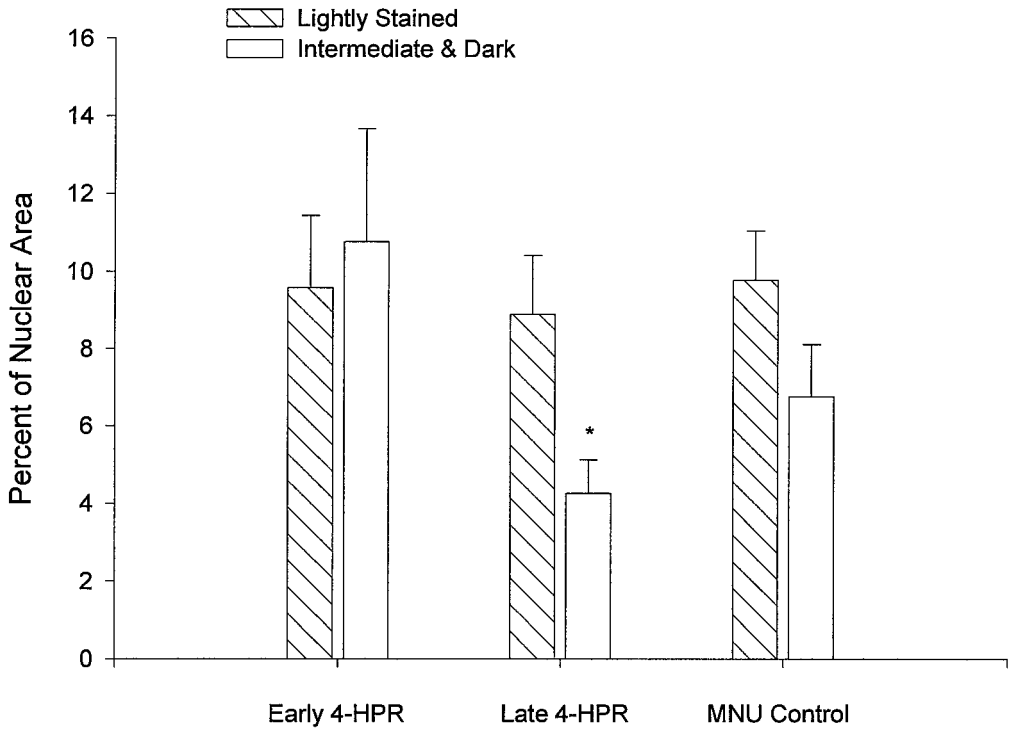

Fig. 2. Effect of early vs. late administration of 4-hydroxyphenylretinamide (4-HPR) on percent distribution of light vs. intermediate + darkly stained mammary tumor cells positive for proliferating cell nuclear antigen (PCNA). Percentage of intermediate + darkly stained cells was significantly reduced in late 4-HPR mammary tumors.

TABLE II. Effect of Early Vs. Late Administration of 4-Hydroxyphenylretinamide (4-HPR) on H-Ras Codon 12 Mutations in Methylnitrosourea (MNU) Induced Mammary Tumors

				Codon 12	
Treatment	Diet	No. of tumors	Activated H-*ras*	GGA → GAA	GGA → AGA
MNU	Early 4-HPR	10	6 (60%)	4	2
MNU	Late 4-HPR	16	6 (38%)	6	0
MNU	MNU control	24	12 (50%)	12	0

tumor induction with 50 or 25 mg MNU/kg, respectively, although a rather large group size (n = 50) was necessary to demonstrate significance at the lower dosage. Beyond these limits, RA is unable to suppress premalignant lesion development and formation of a palpable tumor. 4-HPR in combination with dehydroepiandrosterone (DHEA) shows maximal reduction in incidence when present in the diet from initiation (1 week prior to MNU) to termination [26]. Chemopreventive activity is less pronounced when feeding is started during the promotion phase (1 week post MNU), and is further limited to reduction only in tumor multiplicity when 4-HPR + DHEA feeding is restricted to the initiation phase (week −1 to week +1). Similar results have been obtained with 4-HPR alone in DMBA-induced tumors [25]. Feeding of RA or 4-HPR for 2 months prior to initiation followed by feeding of a control diet is without protective effect and has actually increased incidence of MNU induced mammary carcinomas or DMBA induced benign mammary tumors [7].

Ability of chemopreventive agents to reduce appearance of new tumors following excision of the primary lesion is of significant clinical interest. Moon et al. have demonstrated that 4-HPR alone or in combination with tamoxifen given from the time of first tumor removal reduced appearance of new tumors by 33 and 68%, respectively [4,27] . The ductal hyperplasia and non-palpable lesions they observed in mammary whole mounts taken at the time of pri-

mary tumor excision are similar to those we have observed at 4 weeks following treatment of 50-day-old rats with 50 mg/kg MNU .

Despite these similarities, we were unable to show reduction in tumor incidence from the present study when 4-HPR feeding was delayed until 4 weeks following tumor initiation. It follows that while chemopreventive activity is most likely a continuous process related more to later events in neoplasia as suggested by McCormick and Moon [19], some activity must be directed against the very early events surrounding the initiation phase to explain the greater effectiveness when chemopreventive is fed prior to carcinogen administration and continuously thereafter. These data are relevant for women in clinical trials at higher risk for recurrence following excision of a primary lesion. Exposure to initiating events in the human population is continuous and events that occur early following initiation at high carcinogen dosage in the rat mammary gland may develop much more slowly in breast tissue subject to environmental exposure.

The absence of protection by early 4-HPR feeding 2 months prior to initiation [7] argues against a mechanism directed towards forced differentiation of susceptible terminal end bud epithelium. Retinoids are, however, active in both transcriptional regulation and cellular proliferation. Retinoid transcriptional activity is mediated by various retinoic acid receptor proteins that direct promoter binding at target genes [28–30]. 4HPR, although at concentrations 10-fold higher than required for retinoic acid, activates transcription through all three receptor proteins in addition to marked-up regulation of retinoblastoma protein [31]. Antiproliferative effects of 4-HPR have been demonstrated in vivo, suppressing mammary gland development [12] and expansion of aberrant colonic crypt foci in azoxymethane-treated F344 rats [32], and in vitro, suppressing c-*myc* expression and causing accumulation of human prostate adenocarcinoma PC3 cells in the Go/G1 and S phase of the cell cycle [33]. The antiproliferative effect may also result from activation of apoptosis as demonstrated for several hematopoietic [34] and human breast cancer cell lines [35].

Consistent with 4-HPR's antiproliferative activity, treatment of human prostatic cell lines causes down-regulation of PCNA, cyclin D, and E expression [36]. PCNA is an accessory protein for DNA polymerase δ[26,37]. Normally its mRNA is found to accumulate only in actively cycling cells with protein levels increasing late in G1, being maximal during S-phase and returning to low levels during G2/M [38]. Focal areas of strongly positive immunostaining for PCNA were seen in tumors from all three groups treated with MNU in the present study. Positively stained areas were more diffusely distributed in the late 4-HPR tumors, yielding a lower percentage of total nuclear area. Although tumor multiplicity was not significantly reduced in this group compared to MNU control rats, it is possible that appearance of new tumors would have declined over successive weeks of treatment. Alternatively, it is possible that this apparent inconsistency reflects deregulation in the control of PCNA expression as has been demonstrated for quiescent BALB/c3T3 cells stimulated by serum or epidermal growth factor [39], and for histologically normal tissue adjacent to breast or pancreatic carcinomas. It has been suggested in this case that dysregulation results from influence of paracrine growth factors [40].

Reduction in tumor incidence as a consequence of drug toxicity and loss of body weight has not been a complication associated with 4-HPR treatment [2,7] and was not observed here. Body weights of the three groups given MNU (191 ± 3 g, early 4-HPR; 197 ± 2 g, late 4-HPR; 190 ± 4 g, MNU control) were not different from controls (199 ± 4) after 2 weeks of feeding and were slightly reduced among all MNU groups at 5 weeks (203 ± 4 g, early 4-HPR; 204 ± 3 g, late 4-HPR; 205 ± 3 g, MNU control; 223 ± 6 g, vehicle control). Body weights were not different among any of the groups at termination of the experiment (Table I).

The tendency for fewer tumors having activated H-*ras* in the late 4-HPR group is interesting in this regard although the differences among groups was not significant. Mutational activation of H-*ras* following exposure to MNU is a relatively early event in mammary tumorigenesis, observable as early as 2 weeks following administration to neonatal rats [41] and within 30 or 60 days following MNU treatment of Buf/N or Copenhagen rats, respectively [42]. Early treatment with 4-HPR did not modulate MNU induced H-*ras* mutation in this model system.

In summary, reduction in tumor multiplicity due to dietary 4-HPR supplementation was sig-

nificant only when fed prior to initiation until termination of the study. No differences were found in proliferation status or H-*ras* mutation spectrum consistent with the lower observed tumor numbers. Further work to characterize expression level differences in tumors due to 4-HPR treatment is in progress.

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