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Sodium *n*-butyrate enhancement of prostaglandin D_2 antitumor efficacy

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While the cell growth inhibitory effects of prostaglandins of the E and A series have been well described [1, 2], the potent antiproliferative activity of prostaglandin D_2 (PGD_2) has been reported only recently. Fukushima *et al.* [3], for example, examined the *in vitro* growth inhibitory effects of PGD_2 against the murine leukemia L1210 as well as several human leukemia cell lines. They have suggested that PGD_2 may represent a new and promising antineoplastic agent due to its potency and reduced potential to produce adverse effects on the systemic circulation. Other recent preclinical studies have shown that PGD_2 reduces the metastatic potential of B16 melanoma cells [4] and significantly inhibits the growth of a mastocytoma cell line [5].

The potential for short chain fatty acids such as *n*-butyrate to result in apparent differentiation of certain leukemia cell lines *in vitro* and *in vivo* has been documented by several groups of investigators [6–9]. The ability of *n*-butyrate to induce fatty acid cyclooxygenase activity in specific cell culture lines *in vitro*, however, is a relatively new observation [10, 11]. Since activation of cyclooxygenase activity results in the endogenous production of prostaglandins including PGD_2, we reasoned that *n*-butyrate may augment the cytotoxicity of exogenously administered PGD_2. We, therefore, examined the ability of noncytotoxic and clinically achievable concentrations of *n*-butyrate to increase the antitumor activity of PGD_2 and PGE_2 against murine B16 melanoma as well as established human breast, uterine, and lung cancer cell lines.

Materials and methods

Prostaglandins D_2 and E_2 were purchased from the Cayman Chemical Co. (Denver, CO). [*Methyl*-^{3}H]Thymidine (5 ci/mmole) was purchased from the New England Nuclear Corp. (Boston, MA). Sodium *n*-butyrate, acetylsalicylic acid and MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] were purchased from the Sigma Chemical Co. (St. Louis, MO).

Cell culture. Maximally tolerated yet noncytotoxic concentrations of sodium *n*-butyrate were determined for each cell line utilizing a 72-hr drug exposure period. Cytotoxicity of prostaglandins D_2 or E_2 in the presence or absence of sodium *n*-butyrate was determined by a recently developed dimethylthiazol tetrazolium (MTT) dye method [12] or by uptake and incorporation of [^{3}H]thymidine into acid insoluble material. Since cytotoxicity determinations utilizing the MTT method produced results which were similar to that produced by measurement of [^{3}H]thymidine incorporation, the former method was routinely used throughout these studies.

Three human (NCI H-69, BT-475, and MES-SA) and one murine cell line (B16) were utilized for this study. The human small cell lung carcinoma cell line (NCI H-69) was obtained from Dr. Desmond N. Carney, NCI Navy Medical Oncology Branch, Bethesda, MD. The human breast carcinoma cell line (BT-475) was obtained from Dr. Helene S. Smith, Peralta Cancer Research Institute, Oakland, CA. The murine melanoma cell line (B16) was obtained from EG & G Mason Research Institute, Worcester, MA. The human sarcoma cell line (MES-SA) was developed in the laboratory of Dr. B. Sikic at Stanford University. The origin, characteristics and cytogenetics of this cell line have been described previously [4]. H-69 and BT-475 cells were grown in RPMI medium (GIBCO Laboratories, Grand Island, NY) supplemented with 10% fetal calf serum (FCS), insulin (5 μg/ml), penicillin (100 μg/ml), and streptomycin (100 μg/ml). MES-SA and B16 cells were maintained in a 1:1 mixture of McCoy's 5A and Waymouth's media supplemented with 15% newborn calf serum, insulin, penicillin, and streptomycin. H-69 cells, growing in suspension culture, were disaggregated by vigorous pipetting. The three other cell lines were grown in monolayer culture and harvested using 0.06 M EDTA. Cell number and viability were routinely determined by hemocytometer and trypan blue exclusion methods.

Results and discussion

B16 murine melanoma was chosen since the effects of PGD_2 on cell growth and macromolecular synthesis have been well characterized in this cell line [5]. Three established human cell culture lines were also examined to provide an estimate of the relative antitumor sensitivity to prostaglandins D_2 and E_2. In agreement with studies by Simmet and Jaffe [5], B16 cells were found to be very sensitive to PGD_2 (Fig. 1A) with an estimated IC_{50} value of 2.8 μg/ml (Table 1). In contrast, melanoma cells were far less sensitive to PGE_2; the IC_{50} for PGE_2 was greater than 50 μg/ml. Adding a noncytotoxic concentration (0.01 mM) of sodium *n*-butyrate to B16 cultures increased the inhibition of cell growth produced by PGD_2 by an average of 150–200%, resulting in an IC_{50} of 1.4 μg/ml. Sodium *n*-butyrate did not, however, significantly potentiate the cytotoxicity of PGE_2 in these melanoma cells.

Human uterine sarcoma and lung adenocarcinoma were both 6-fold less sensitive *in vitro* to the cytotoxic effects of PGD_2 than was melanoma (Table 1). The cytotoxic activity of PGD_2, however, could be significantly potentiated by exposure of the cells to a noncytotoxic concentration of sodium *n*-butyrate (Figs. 2 and 3). Neither human cell line was particularly sensitive to incubation with PGE_2. While

sodium *n*-butyrate increased the antitumor efficacy of PGE_2 against human uterine sarcoma, it did not enhance significantly the cytotoxicity of PGE_2 against the lung adenocarcinoma cell line.

Inhibition of the growth of breast carcinoma by PGD_2 could only be achieved using concentrations of prostaglandin D_2 that were 3-fold higher than that used to achieve a similar level of inhibition against human uterine or lung cell lines (Fig. 4A). In addition, the increase in the antitumor activity of PGD_2 achieved by coincubation with sodium *n*-butyrate was far smaller than that produced in the other cell lines. Growth of breast carcinoma was also found to be relatively insensitive to PGE_2 (Fig. 4B).

The mechanisms responsible for PGD_2-mediated inhibition of cell growth are not well understood. Inhibition of protein synthesis has been shown to occur prior to inhibition of RNA and DNA synthesis [6]; however, a 24-hr incubation with PGD_2 at 5 μg/ml totally inhibits incorporation of labeled precursors into DNA and RNA [3, 6]. More recently, PGD_2 has also been shown to result in inhibition of DNA polymerase α and β activities, although the relative importance of this phenomenon in producing cell death is unclear [13].

Treatment of cells *in vitro* with PGD_2 has shown that not all cell types are equally sensitive to the growth inhibition by this prostaglandin. While B16 melanoma [5], mastocytoma P-815 [6], murine leukemia L1210 and several human leukemia cell lines [3] are sensitive to low concentrations of PGD_2 (i.e. less than 5 μg/ml), the present report demonstrates that human lung, uterine, and breast malignant cell lines required 4- to 12-fold as much PGD_2 to provide a similar level of inhibition of cell growth as that produced in murine melanoma (Table 1).

Kawamura and Koshikara [14] have shown that treatment of cloned mastocytoma cells with sodium *n*-butyrate results in the synthesis of prostaglandins D_2, E_2, and $F_{2\alpha}$ that were not formed at detectable levels by the control cells. Sodium *n*-butyrate enhancement of PGD_2 formation, however, appears to be cell specific; prostaglandin-synthesizing activities of mouse hepatoma, HeLa, rat granu-

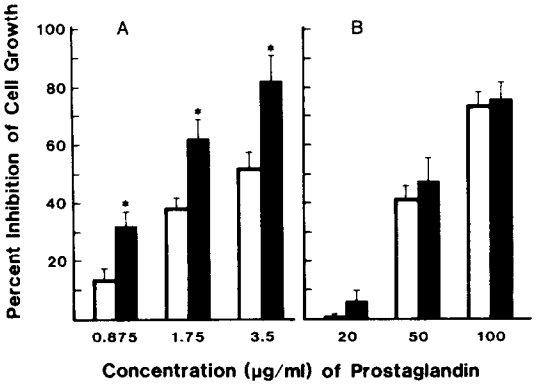

Fig. 1. Cytotoxicity of PGD_2 (A) and PGE_2 (B) to B16 melanoma *in vitro*. Cells were exposed to prostaglandin continuously for 72 hr in the absence (open columns) or presence (closed columns) of sodium *n*-butyrate (0.1 mM) Data are mean ± S.E.M. for eight to twelve determinations. An asterisk (*) indicates $P < 0.05$ compared to respective control.

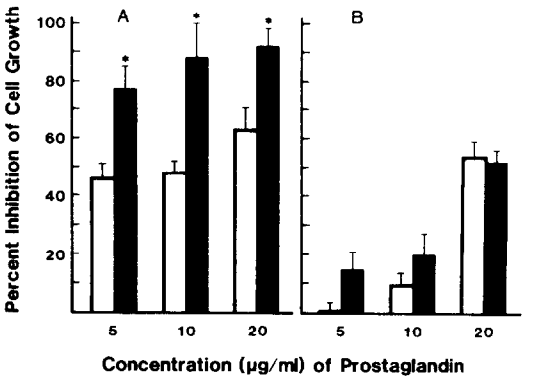

Fig. 3. Cytotoxicity of PGD_2 (A) and PGE_2 (B) to human small cell lung carcinoma cells (H-69) *in vitro*. Cells were exposed to prostaglandin continuously for 72 hr in the absence (open columns) or presence (closed columns) of sodium *n*-butyrate (0.1 mM). Data are mean ± S.E.M. for eight to twelve determinations. An asterisk (*) indicates $P < 0.05$ with respect to control.

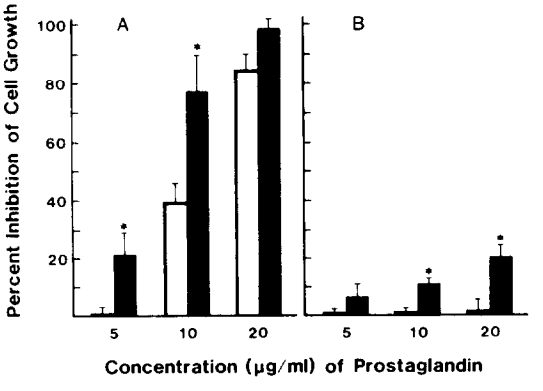

Fig. 2. Cytotoxicity of PGD_2 (A) and PGE_2 (B) to human uterine sarcoma cells (MES-SA) *in vitro*. Cells were exposed to prostaglandin continuously for 72 hr in the absence (open columns) or presence (closed columns) of sodium *n*-butyrate (0.1 mM). Data are mean ± S.E.M. for eight to twelve determinations. An asterisk (*) indicates $P < 0.05$ compared to control.

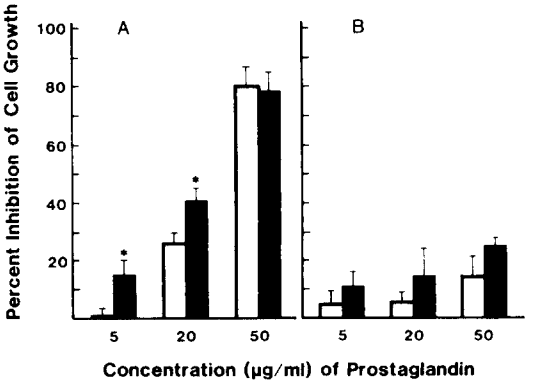

Fig. 4. Cytotoxicity of PGD_2 (A) and PGE_2 (B) to human breast carcinoma cells (BT-475) *in vitro*. Cells were exposed to prostaglandin continuously for 72 hr in the absence (open columns) or presence (closed columns) of sodium *n*-butyrate (0.1 mM). Data are mean ± S.E.M. for ten to twelve determinations. An asterisk (*) indicates $P < 0.05$ compared to control.

Table 1. Augmentation of PGD_2 cytotoxicity by sodium *n*-butyrate*

Cell line	IC_{50} (μg/ml)		Fold-increase in PGD_2 cytotoxicity
	PGD_2	PGD_2 + SnB	
B-16 melamona	2.84 ± 0.43	1.40 ± 0.21†	2.03
MES-SA	12.35 ± 1.15	6.49 ± 0.72†	1.90
H-69	13.35 ± 0.97	3.73 ± 0.49†	3.58
BT-475	34.86 ± 3.33	24.89 ± 1.90†	1.40

* Data are mean ± S.E.M. of three IC_{50} determinations in each cell line. Cells were incubated with PGD_2 continuously in the presence or absence of 0.1 mM sodium *n*-butyrate (SnB) which itself was noncytotoxic at this concentration. Cell survival was assessed by [^{3}H]thymidine incorporation and by the MTT assay as described in Materials and Methods.

† $P < 0.05$ with respect to PGD_2 treatment.

Table 2. Inhibition by acetylsalicylic acid of sodium butyrate enhancement of PGD_2 antitumor activity

Drug*	Concn (μg/ml)	Percent inhibition of control B-16 cell growth†
Acetylsalicylic acid (ASA)	100	0 (9)
Sodium *n*-butyrate (SnB)	10	0 (12)
Prostaglandin D_2 (PGD_2)	1.75	36 ± 4 (12)
PGD_2 + SnB		65 ± 8‡ (12)
PGD_2 + SnB + ASA		41 ± 5^{NS}§ (12)

* B-16 melanoma cells were exposed continuously to drugs or drug combinations for 3 days prior to determination of cell viability as described in Materials and Methods.

† Data are presented as mean ± S.E.M. for three separate experiments; number of observations are given in parentheses.

‡ $P < 0.05$ compared to PGD_2-treated cells.

§ NS > P 0.05 compared to PGD_2-treated cells.

loma, and human embryonic fibroblast cell lines are only marginally increased by incubation with *n*-butyrate [15]. In the present study, we also observed the enhanced sensitivity of B16 melanoma cells to PGD_2 in the presence of *n*-butyrate. While no direct determinations of prostaglandin synthetic activity were made, incubation of B16 melanoma cultures with a noncytotoxic concentration of acetylsalicylic acid blocked the augmentation of PGD_2-mediated inhibition of cell growth produced by *n*-butyrate (Table 2). This suggests, but does not prove, that *n*-butyrate-enhanced cytotoxicity in B16 cells may also occur by an increased endogenous formation of cytotoxic prostaglandins. This observation is in agrement with that of Kawamura *et al.* [14, 15], who have suggested that *n*-butyrate-enhanced prostaglandin synthesizing activity is brought about by an induction of prostaglandin cyclooxygenase activity.

The enhanced cytotoxic activity of PGD_2 produced by *n*-butyrate in human lung adenocarcinoma (H-69 cells) is of particular interest. Likewise, the relative ineffectiveness of *n*-butyrate in enhancing the sensitivity of a human breast cancer cell line to PGD_2 would suggest that not all human cell lines respond in a similar manner to the many effects shown to be produced by *n*-butyrate [7]. A continued exploration of the relative sensitivities of human cell lines to cytotoxic prostaglandins and potentiation of their cytotoxicity by *n*-butyrate is indicated by the present study.

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Effects of benzene and pyridine on the concentration of mouse striatal tryptamine and 5-hydroxytryptamine

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We have shown recently that the parenteral administration of benzene or some other organic solvents produces significant increases in the striatal concentrations of β-phenylethylamine, *p*-tyramine and *m*-tyramine [1]. These findings are in good agreement with early reports that the addition of benzene to guinea pig kidney aromatic-L-aminoacid decarboxylase preparations produces marked increases in the *in vitro* (decarboxylation of phenylalanine and tyrosine (presumably the *p*-isomer) [2]. The increase in the decarboxylation of *p*-tyrosine has also been observed with rat [3] or mouse brain [1] extracts. In addition, an increase in the decarboxylation of tryptophan by guinea pig kidney aromatic-L-aminoacid decarboxylase preparations [2] was observed in the presence of benzene.

In this communication, we report the effects of benzene and some other organic solvents on the decarboxylation of tryptophan and 5-hydroxytryptophan by partially purified brain aromatic-L-aminoacid decarboxylase and the effect of the parenteral administration of benzene or pyridine on the striatal concentration of tryptamine, 5-hydroxytryptamine (5-HT) and 5-hydroxyindole acetic acid (5-HIAA).

Methods

Male albino Swiss mice (18–22 g body wt) were killed by decapitation. The brain was removed quickly, and the striatum consisting mainly of the head of the caudate nucleus and including some of the underlying putamen (approximate weights 28–35 mg) was dissected out. Tryptamine concentrations were determined in the pooled tissues of three mice that were homogenized in 1 ml of 0.1 HCl containing ethylene diamine tetracetic acid disodium salt (EDTA, 1 mg/ml) and ascorbic acid (5 mg/ml), and 50 ng of tetradeutero tryptamine internal standard was added. The amines in the tissue homogenate were derivatized with 5-dimethylamino-1-naphthalene sulfonyl (dansyl) chloride, and the resultant derivatives were extracted into toluene–ethylacetate (9:1, v/v), evaporated to a small volume, and separated chromatographically in two different unidimensional systems [4]. Tryptamine was estimated by the high resolution mass spectrometric selected ion monitoring (integrated ion current) technique using deuterated tryptamine as an internal standard. Blanks of 0.1 N HCl, to which was added 50 ng of deuterated tryptamine, were carried out throughout the procedure. These gave blank values of 200 pg and thus enabled quantification of as little as 200 pg of tryptamine in the tissue samples. Complete details concerning these procedures have been described [5].

Brain regional concentrations of 5-HT and 5-HIAA were determined by high performance liquid chromatography with electrochemical detection [6]. The apparatus consisted of a solvent delivery system (model M45, Waters Associates, Inc., Mississauga, Ontario, Canada) equipped with a WISP automatic injector set with a fixed 20 μl loop (model 710B, Waters Associates, Inc., Mississauga, Ontario, Canada). The separations were achieved in a column (Altex Ultrasphere ODS) with the following characteristics: length, 250 mm; internal diameter, 4.6 mm; and particle size, 5 μm. Also, a 170 mm long precolumn (Whatman Inc., Clifton, NJ, U.S.A) was used. The 5-hydroxyindole compounds were detected on a carbon paste electrode (model TL-3, Bioanalytical Systems, West Lafayette, IN, U.S.A.) set at 0.75 V versus a Ag/AgCl reference electrode. Standards and test curves were displayed on a dual channel recorder (BD41, Kipp & Zonen, Holland). The mobile phase, consisting of 0.1 M NaH_2PO_4, 1 mM sodium octyl sulfate, 1 mM disodium EDTA and 12% acetonitrile adjusted to pH 3.3 with phosphoric acid, was filtered through a Buchner funnel (fritted glass pore, diameter 10–15 μM) and degassed by vacuum. Tissues obtained from one rat were homogenized in 0.1 M $HClO_4$ containing 0.67 mM EDTA and 100 ng/ml of isoproterenol as internal standard. Following centrifugation (Eppendorf centrifuge, model 5412), the supernatant fraction was injected directly into the system. The corresponding calibration curves were prepared daily and the correction factor for 5-HT and 5-HIAA with respect to the internal standard (2 ng isoproterenol) was determined. The minimum detectable amount was 100 pg for both 5-HT and 5-HIAA, with a signal to noise ratio <3.

Aromatic-L-aminoacid decarboxylase was prepared from rat whole brain. The animals were killed by decapitation, and the brain was dissected out and rinsed with cold saline. The brain was homogenized in 0.01 M phosphate buffer (pH 7.2) and centrifuged at 24,000 *g* for 20 min. Ammonium sulfate was added to the supernatant fraction to make up 30% saturation, and it was again centrifuged and to the supernatant more ammonium sulfate was then added until 50% saturation was reached and the resultant precipitate isolated by centrifugation. The pellet was dissolved in 0.01 M phosphate buffer (pH 7.2) and dialyzed. After dialysis, the enzyme was kept frozen at −20° and was stable for at least a month.

The aromatic-L-aminoacid decarboxylase activity towards 5-hydroxytryptophan was determined by the use of a radioenzymatic method with ^{14}C-labeled substrate and amberlite CG-50 ion exchange resin to separate the labeled product as previously described [1, 7]. The tryptamine formed *in vitro* by tryptophan decarboxylation was determined by a mass spectrometric method as indicated above. The incubation mixtures contained 0.1 M phosphate buffer (pH 7.5), 2.5×10^{-5} M pyridoxal-5′-phosphate,