

# Anticancer Derivative of Butyric Acid (Pivalyloxymethyl Butyrate) Specifically Potentiates the Cytotoxicity of Doxorubicin and Daunorubicin through the Suppression of Microsomal Glycosidic Activity

NOZOMI NIITSU, TAKASHI KASUKABE, AKIHIRO YOKOYAMA, JUNKO OKABE-KADO, YURI YAMAMOTO-YAMAGUCHI, MASANORI UMEDA, and YOSHIO HONMA

*Saitama Cancer Center Research Institute, Saitama, Japan (N.N., T.K., A.Y., J.O.-K., Y.Y.-Y., Y.H.) and The 1st Department of Internal Medicine, Toho University School of Medicine, Tokyo, Japan (N.N., M.U.)*

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## ABSTRACT

Pivalyloxymethyl butyrate (AN9) is an anticancer derivative of butyric acid. In this study, doxorubicin (DXR) and AN9 synergistically inhibited the growth of lymphoma and lung carcinoma cells, whereas there was no synergy between AN9 and antimetabolites. AN9 did not affect the intracellular uptake of DXR. Among anthracyclines and their derivatives, the synergistic effect was prominent in compounds with a daunosamine moiety, suggesting that AN9 may affect the catabolism of these compounds. The degradation of DXR in the extract from AN9-treated cells was much less than that in extract from untreated

cells. AN9 did not directly inhibit the enzyme activity but rather suppressed expression of the enzyme. With respect to the expression of drug resistance-related genes, there was no significant difference between untreated and AN9-treated cells. However, AN9 significantly down-regulated the levels NADPH-cytochrome P450 reductase and DT-diaphorase mRNA in the presence of DXR but not the level of xanthine oxidase mRNA. The enhancement of the sensitivity to anthracyclines was closely associated with the suppression of the mRNA expression.

The anthracycline antitumor antibiotics occupy an important position in the field of cancer chemotherapy. Doxorubicin (DXR) is a broad-spectrum drug that is particularly useful in the treatment of malignant lymphomas, acute leukemias, and sarcomas and solid tumors of the breast, lung, and ovary (Young et al., 1981). DXR binds to DNA, RNA, chromatin, and cell membrane, but its antitumor activity likely results from the inhibition of topoisomerase II. DNA intercalation and stabilization occurs in the drug-nucleic acid-topoisomerase II ternary complex, which is referred to as the cleavable complex (Tewey et al., 1984; Myers, 1986). The anthracycline can also undergo redox cycling to produce free radicals that cause DNA cleavage and membrane peroxidation. Free radicals may contribute to the antitumor activity of anthracyclines, but are involved primarily in cardiotoxicity (Sinha, 1989). The reactive glycosidic cleavage of anthracyclines is a unique enzymatic reaction that metabolizes free radicals to a biologically inactive 7-deoxyanthracyclinone (7-deoxyaglycone) by splitting off a biofunctional

glycosidic aminosugar. This cleavage also is a major pathway in the metabolism of anthracyclines in mammalian systems (Asbell et al., 1972). The reaction is catalyzed by NADPH-cytochrome P450 reductase, xanthine oxidase, and DT-diaphorase. When treatment is prolonged, however, cardiotoxic effects and multidrug resistance appear and become serious therapeutic problems. To reduce these adverse effects and improve the therapeutic effect, several compounds have been newly synthesized and are now being studied. Alternatively, attempts to increase the therapeutic index of anthracycline anticancer agents should be pursued.

The products of certain oncogenes suppress transcription of their target genes by recruiting histone deacetylases, which cleave acetyl groups from histones and block DNA conformational changes. Experimentally, this transcriptional blockade can be overcome by agents that inhibit these enzymes. Recently, clinical treatment with an inhibitor of histone deacetylase has been shown to induce histone hyperacetylation in target cells and to restore sensitivity to the antileukemic effect of all-*trans*-retinoic acid in acute promyelocytic leukemia (Warrell et al., 1998). Because butyric acid and its derivatives are known to inhibit histone deacetylases

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**ABBREVIATIONS:** DXR, doxorubicin; AN9, pivalyloxymethyl butyrate; DNR, daunorubicin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; RT, reverse transcription; PCR, polymerase chain reaction.

(Candido et al., 1978), these drugs could be used for "targeted transcription therapy" of several types of cancer (Warrell et al., 1998).

Butyric acid has been reported to be a potent differentiating and antiproliferation agent in a wide spectrum of neoplastic cells in vitro (Prasad, 1980). Clinical trials have been conducted with sodium butyrate on hematopoietic malignancies (Novogradsky et al., 1983). Butyrate induced a partial and temporary remission in a child with acute myeloid leukemia, but showed no clinical activity in nine adults with acute leukemia (Miller et al., 1987). This lack of clinical efficacy may be due to its rapid metabolism and, to a lesser extent, to its excretion. To overcome these disadvantages, a search for new butyrate prodrugs, which would reduce clearance rates, was undertaken, and some prodrug derivatives of butyrate were found that had better pharmacokinetic qualities than butyrate itself.

Pivalyloxymethyl butyrate (AN9) exhibited much greater anticancer activity than butyrate in mouse cancer models using Lewis lung carcinoma and Mm-A monocytic leukemia in vitro and in vivo (Nudelman et al., 1992; Kasukabe et al., 1997). AN9 and DXR or daunorubicin (DNR) synergistically inhibited the growth of mouse Mm-A leukemia cells, whereas there was no synergy between butyrate and these drugs, or between AN9 and antimetabolic agents in inhibiting the growth of these cells, suggesting that the synergistic effect is specific to AN9 and anthracyclines. AN9 as a single agent prolonged the survival of mice inoculated with Mm-A cells in a dose-dependent manner. Moreover, the administration of AN9 plus DNR markedly prolonged their survival (Kasukabe et al., 1997). These results suggest that the combination of AN9 and anthracyclines has clear therapeutic potential. Therefore, in the present study we sought to clarify the synergistic effect of these compounds on human lymphoma and lung carcinoma cells and to examine the mechanism of the increase in the sensitivity of the AN9-treated cells to anthracyclines.

## Materials and Methods

**Chemicals.** AN9 (Pyvanex) was obtained from Ansan, Inc. (South San Francisco, CA). DXR, DNR, aclarubicin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), RNase A, and propidium iodide were purchased from Sigma Chemical (St. Louis, MO). Other drugs were obtained from the following sources: idarubicin and epirubicin from Pharmacia Co., Ltd., Tokyo; pirarubicin from Meiji Seika Co. Ltd., Tokyo; MX-2 (KRN-8602) from Kirin Brewery Co., Ltd., Tokyo; and SM-5887-13-OH from Sumitomo Seiyaku, Tokyo.

**Cells and Culture.** Human nonsmall-cell lung carcinoma and B cell lymphoma cells were maintained in RPMI-1640 medium (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% fetal bovine serum at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> in air (Goto et al., 1996; Niitsu et al., 1998). The lung carcinoma cells were collected by treatment with 0.1% trypsin-0.04% EDTA (Life Technologies, Inc.) for 5 min with gentle shaking and were transferred every 3 to 5 days.

**Assay of Cell Growth.** The cells were seeded at a concentration of  $2 \times 10^4$ /ml in a multidish (Nunc, Roskilde, Denmark). After culture with or without the test compounds for 4 to 6 days, viable cells were examined by the modified MTT assay (Goto et al., 1996). Briefly, 100  $\mu$ l of MTT solution (5 mg/ml in saline PBS) was added to each well. After incubation with MTT for 4 h, the cells were centrifuged at 1000g for 10 min. The precipitates were dissolved in 1 ml of

dimethyl sulfoxide, and their absorptions at 560 nm were determined.

**Cell-Cycle Analysis.** The cell cycle was analyzed using propidium iodide-stained nuclei (Kanatani et al., 1996). Samples of  $2 \times 10^6$  cells were harvested at the time points indicated, washed in ice-cold PBS, fixed by the addition of 100% ethanol, and left for 30 min on ice. The cell pellet was washed and suspended in 200  $\mu$ l of 1.12% sodium citrate containing RNase A (250  $\mu$ g/ml) for 30 min at room temperature. Thereafter, the cells were stained with 50  $\mu$ g/ml propidium iodide in the presence of 1.12% sodium citrate and analyzed in a fluorescence-activated cell sorter.

**DXR Uptake and DNA Binding of DXR and Topoisomerase Activity.** Fluorimetric determination of DXR uptake and DNA binding were performed as described in the literature (Gieseler et al., 1994). Topoisomerase II activity was measured using an assay kit (TopoGen, Inc., Columbus, OH).

**Assay of Glycosidic Cleavage of Anthracyclines.** Microsomal fractions were prepared from cells homogenized in 50 mM potassium phosphate buffer (pH 7.8) containing 1 mM EDTA. The homogenate was centrifuged at 10,000g for 20 min, and the sediment was discarded. The microsomal sediment was obtained by centrifugation at 105,000g for 60 min. The packed microsomes were suspended in potassium phosphate buffer (0.1 ml containing 0.1–0.3 mg of protein). Nitrogen gas was bubbled into tubes containing 50  $\mu$ mol of Tris-HCl buffer (pH 7.8), 0.5  $\mu$ mol of NADPH, and 0.5  $\mu$ mol of anthracycline in 0.4 ml to displace oxygen. The microsomal fraction was added to each deoxygenated reaction mixture, and the tubes were again flushed with nitrogen gas before capping. The capped tubes were incubated at 37°C for 30 min, and the reaction was terminated by the addition of 0.5 ml of *n*-butanol. Saturating amounts of NaCl crystals were added to the reaction mixtures, which were then centrifuged to separate the phases (Loveless et al., 1978). Aliquots of the upper phase were applied to silica gel thin layer plates for chromatographic separation, and the aglycones were quantified as described in the literature (Bachur et al., 1974).

**Determination of mRNA Level by Reverse Transcription-Polymerase Chain Reaction.** RNA was extracted by a modification of the method of Chomczynski and Sacchi (1987). Quantitative reverse transcription-polymerase chain reaction (RT-PCR) was performed using a GeneAmp RNA PCR kit (Takara Shuzo Co., Tokyo, Japan), and the amplification reactions were performed as described in the literature (Yokoyama et al., 1996). Total RNA (0.2  $\mu$ g) was reverse-transcribed to synthesize cDNA using random hexamers at 4°C, then amplified by means of PCR using specific primers (4 pmol) and 0.11 MBq of [ $\alpha$ -<sup>32</sup>P]dCTP in 20- $\mu$ l mixtures consisting of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.2 mM MgCl<sub>2</sub>, and 0.2 mM dNTPs. The primers were prepared as described in the literature (Horikoshi et al., 1992; Shephard et al., 1992; Saksela and Raivio, 1996). After amplification, PCR products were analyzed on 6% polyacrylamide or 2% agarose gels.

**Oligonucleotides and Cell Treatment.** We designed 18-mers corresponding to the antisense sequences flanking the translation initiation region of the human mRNAs for NADPH-dependent cytochrome P450 reductase and DT-diaphorase. The sequences of the phosphorotioate oligonucleotides (Takara, Otsu, Japan) were as follows: P450 reductase antisense, 5'-CACCGGCTTCTTCATAGA-3'; DT-diaphorase antisense, 5'-ACCAAACCTCGCTCACAAG-3'. Cells were washed and suspended in 0.5 ml of serum-free RPMI1640 medium. Appropriate dilution of oligonucleotides in 0.5 ml of RPMI1640 medium were preincubated at room temperature for 15 min with 12  $\mu$ l of TransFast (Promega, Madison, WI). This mixture was added to the cell suspension and incubated for 1 h at 37°C. At the end of the incubation period, 5 ml of complete medium (containing serum) was added. Various concentrations of DXR were added 2 days later, and the cells were further cultured for 4 days.

**Western Immunoblot.** Cells were harvested and lysed in Laemmli buffer [60 mM Tris-HCl (pH 6.8), 10% glycerol, 2% SDS, 5% 2-mercaptoethanol, and 0.003% bromophenol blue]. The protein ly-

sate was electrophoresed on SDS-polyacrylamide gels and transferred to Immobilon-P transfer membranes (Millipore, Bedford, MA). The filters were blocked with 5% nonfat dried milk in 1× TBS buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl] and then incubated overnight with 1 µg/ml of rabbit polyclonal antibody against cytochrome P450 reductase (a gift from Dr. K. Kawajiri, Saitama Cancer Center Research Institute) (Kawajiri et al., 1979). Alkaline phosphatase-conjugated IgG (Bio-Rad Laboratories, Hercules, CA) was used as a secondary antibody (1:1000), and the bands were developed using the Immune-Lite II chemiluminescent protein detection system (Bio-Rad) as per the manufacturer's instructions.

## Results

**Combined Effects of AN9 and DXR on the Growth of Lung Cancer and Lymphoma Cells.** AN9 and DXR synergistically inhibited the growth of mouse leukemia Mm-A cells, but there was no synergy between butyrate and DXR, or between AN9 and antimetabolic agents in inhibiting the growth of these cells (Kasukabe et al., 1997). We examined whether this synergistic effect was extended to human lymphoma and lung cancer cells. The synergy between DXR and AN9 was observed in all of the human cell lines tested, although variations in the synergism were found among the cell lines, indicating that the combined treatment is effective in human hematopoietic and nonhematopoietic malignant

TABLE 1

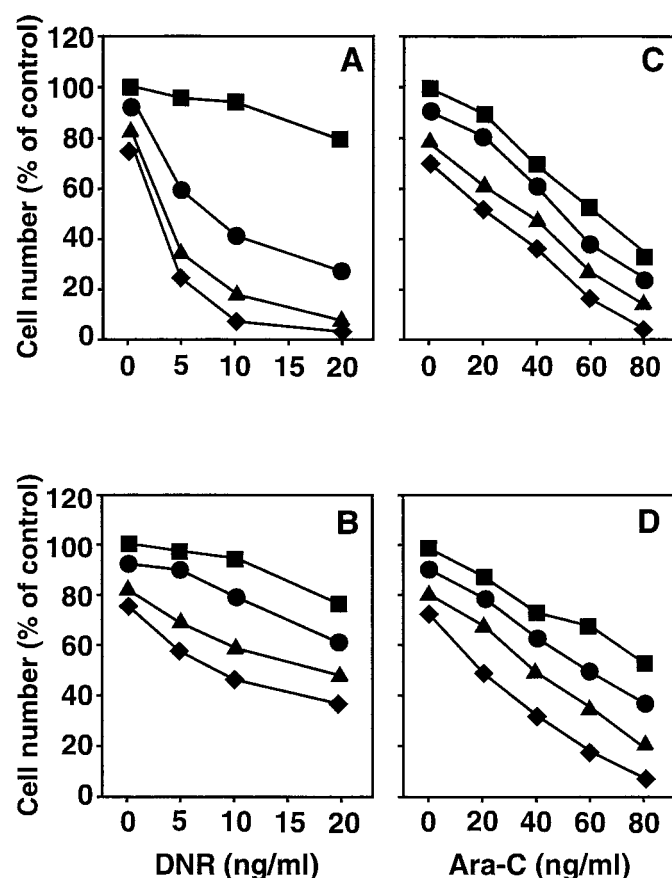
Synergy between AN9 and various anticancer drugs in growth inhibition of human lung carcinoma and lymphoma cells

Cells were cultured with various concentrations of antineoplastic agents in the presence or absence of 32 µM AN9 for 4 days.

Drug	IC <sub>50</sub>		
	−AN9	+AN9	CI <sup>a</sup>
×10 <sup>−7</sup> M			
Lung carcinoma EBC-1 cells			
DXR	1.02 ± 0.1 <sup>b</sup>	0.43 ± <0.1	0.48
DNR	2.17 ± 0.1	1.26 ± 0.2	0.52
Cytosine arabinoside	4.32 ± 0.5	4.15 ± 0.3	0.98
Methotrexate	2.61 ± 0.2	2.93 ± 0.3	1.04
6-Mercaptopurine	0.84 ± 0.1	0.87 ± 0.1	1.02
Lung carcinoma PC9 cells			
DXR	1.33 ± 0.2	0.51 ± <0.1	0.47
Lung carcinoma A549 cells			
DXR	1.97 ± 0.3	0.67 ± <0.1	0.50
Lymphoma BALM3 cells			
DXR	0.52 ± <0.1	0.23 ± <0.1	0.44
Lymphoma SKW4 cells			
DXR	0.63 ± <0.1	0.31 ± <0.1	0.48

<sup>a</sup> Combination index at IC<sub>50</sub>. CI = 1 indicates summation (additive or zero interaction), CI < 1 synergism.

<sup>b</sup> Mean ± S.D. for four determinations.



**Fig. 1.** Combined effect of AN9 or butyrate with DXR on the growth of human lung carcinoma EBC-1 cells. Cells were cultured with various concentrations of DXR or cytosine arabinoside (Ara-C) in the presence of 0 (■), 32 (●), 48 (▲), or 64 (◆) µM AN9 for 4 days (A and C). Cells were cultured with DXR or Ara-C in the presence of 0 (■), 0.1 (●), 0.2 (▲), or 0.3 (◆) mM sodium butyrate for 4 days (B and D). Means are representative of four determinations.

cells (Fig. 1 and Table 1). On the other hand, there was no synergy between butyrate and DXR, or between AN9 and anti-metabolic agents, as reported in mouse leukemia cells.

**Cell-Cycle Analysis of AN9-Treated Cells.** To understand the effect of AN9 on cell growth, we exposed EBC-1 cells to 32 µM AN9 and 30 ng/ml DXR, then measured the changes in the cell-cycle distribution after 3 days. This concentration of DXR did not affect the cell cycle, and the percentage of cells in G<sub>2</sub> phase was slightly increased in cells incubated with AN9. DXR with AN9 induced growth arrest of cells at G<sub>2</sub> phase (Fig. 2). Similar results were obtained when BALM3 lymphoma or PC9 lung carcinoma cells were treated with AN9 and DXR or DNR. Butyrate had less of an effect than AN9 on the cell-cycle distribution (data not shown).

**Uptake of DXR and DNA Binding by AN9-Treated Cells.** We incubated cells with DXR, extracted DXR from whole cells and nuclei, and determined the concentration fluorimetrically. Within 10 min, DXR crossed the outer membrane and the cytoplasm, and arrived in the nucleus. Lung carcinoma EBC-1 and lymphoma BALM3 cells were treated with or without 64 µM AN9 for 0.5 to 4 days and then suspended for various durations up to 120 min (Fig. 3). Neither intracellular nor nuclear uptake of DXR was affected by pretreatment with AN9. Similar results were obtained when the cells were treated with a higher concentration of AN9. The DNA binding rates of DXR were evaluated in PC9 and BALM3 cells by determining the fluorescence resonance energy transfer between DXR- and DNA-bound Hoechst dye 33342. The DNA bindings were not significantly affected by AN9 (data not shown). These results suggest that the enhanced sensitivity to DXR caused by AN9 is not associated with the increased uptake and DNA binding of DXR.

Cytotoxic activity of DXR is mediated through interaction of the drug with the target enzyme DNA topoisomerase II (Tewey et al., 1984). Thus, we examined the effect of AN9 on the DNA topoisomerase II activity of AN9-treated EBC-1



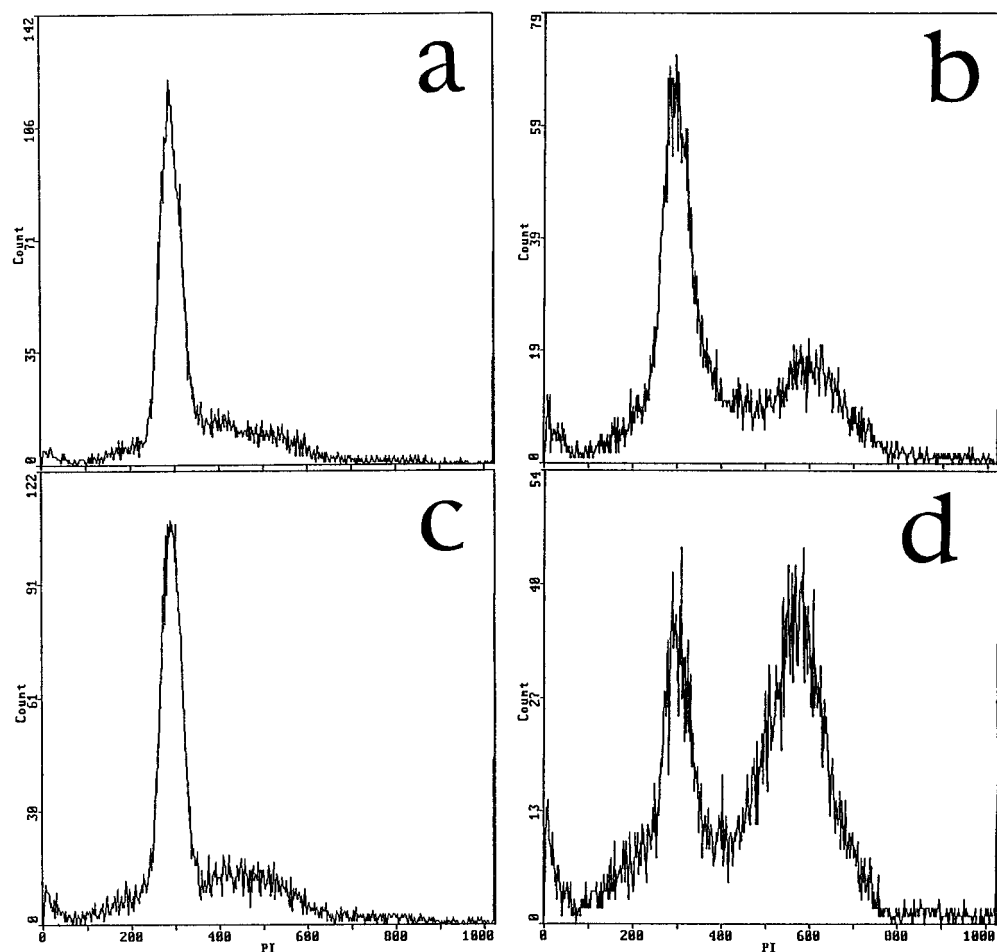
cells. DNA topoisomerase assays were carried out by measuring the decatenation of kinetoplast DNA to minicircles. Decatenation of the DNA by serial dilution of the nuclear extracts from AN9-treated cells showed activities similar to those of untreated cells (data not shown).

**Structure-Activity Relationships of Various Anthracyclines in AN9-Treated Cells.** We examined the combined effects of various anthracyclines (Fig. 4) with AN9 on the growth of BALM3 cells. Although the synergistic inhibition was observed in 3-day cultures with DXR, DNR, or idarubicin (Fig. 5), cooperation between AN9 and some other anthracyclines was observed in the growth inhibition of cells cultured for 5 days (Fig. 6). These effects were classified into three different categories: DXR, DNR, and idarubicin were effective in the synergistic inhibition of growth in the presence of AN9, whereas AN9 showed less synergy in combination with epirubicin, pirarubicin, and aclarubicin, and no synergy in combination with SM-5887-13-OH, MX-2, or mitoxantrone (an anthraquinone). Anthracyclines with a daunosamine moiety most effectively cooperated with AN9 in inhibiting cell growth. Similar results were observed when lung carcinoma EBC-1 or PC9 cells were treated with these anthracyclines and AN9.

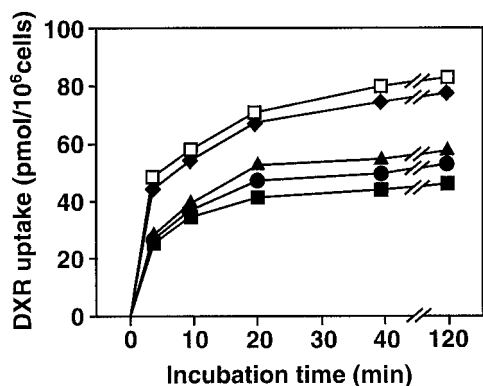
**Effect of AN9 on Glycosidase Activity.** The finding that sensitivity to anthracyclines with a daunosamine moiety was greatly enhanced by AN9 prompted us to determine the glycosidase activity, which inactivates the anthracycline glycosides. The reactions for microsomal glycosidase activity

were linear for at least 45 min, and NADPH was a strict cofactor. The glycosidase(s) from microsomes of BALM3 cells preferred DXR, DNR, and idarubicin over the other anthracyclines tested as substrates. There were no significant differences in cleavage rates and substrate preference among the microsomes of BALM3, EBC-1, and PC9 cells. The substrate preference was closely correlated with the synergism with AN9 in the growth inhibition. Microsomes from AN9-treated BALM3 cells contained less glycosidase activity, and down-regulation of the glycosidase activity by AN9 was significant after 24 h (Fig. 7). The reduction of the glycosidase activity by butyrate was much less than that by AN9. The metabolites of DXR were extracted from the reaction mixtures, and their cytotoxic activities were examined. The metabolites incubated with microsomes from AN9-treated cells were much more effective than those incubated with microsomes from untreated or butyrate-treated cells with regard to growth inhibition (data not shown). Incubation of microsomes with AN9 did not affect the glycosidase activity, even at a high concentration or for a long time (up to 150 min), suggesting that AN9 did not directly inhibit the enzyme activity.

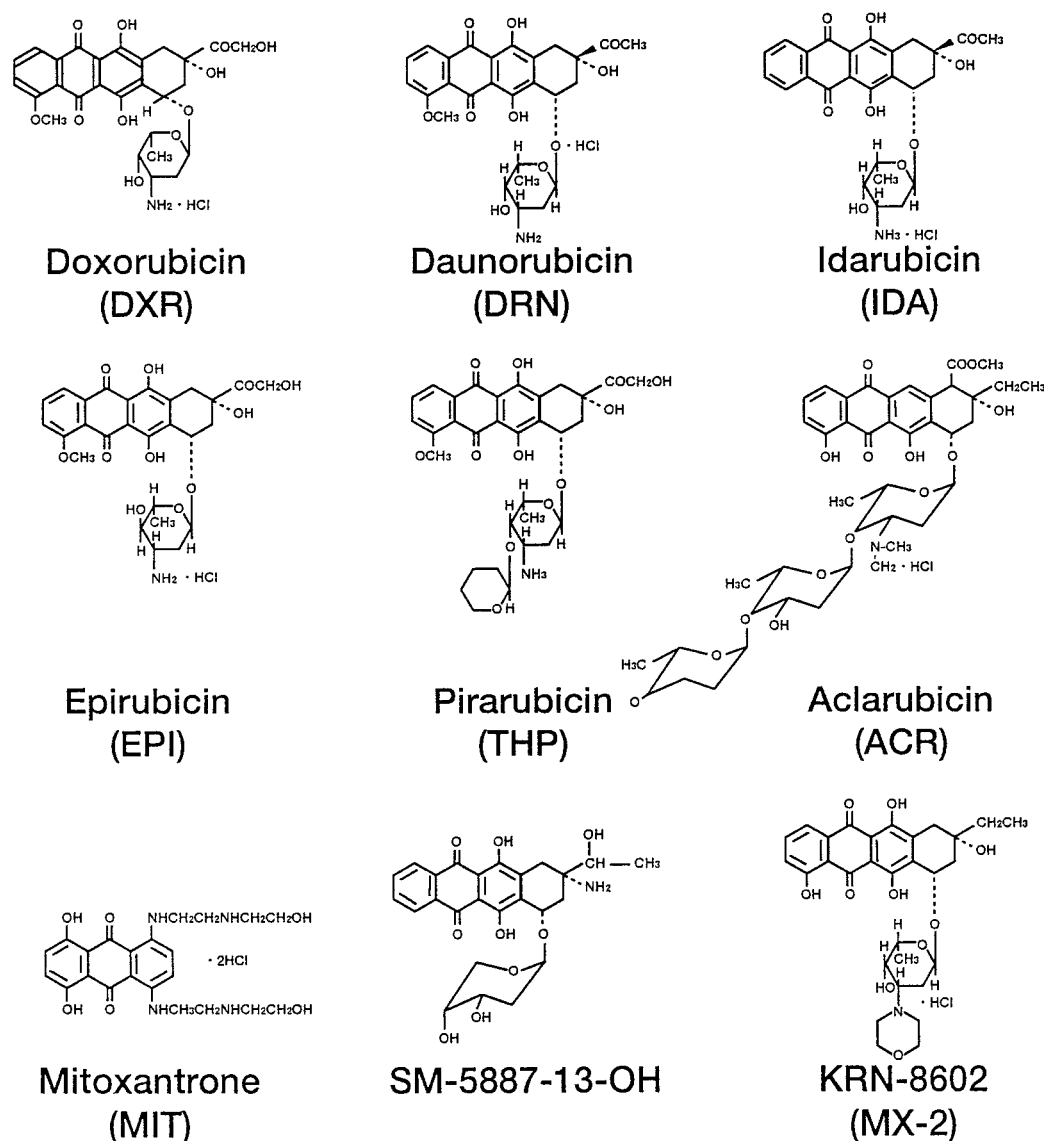
**Effect of AN9 on the Expression of Genes Related to Drug Resistance and Metabolism.** To determine whether AN9 affected mRNA levels, quantitative RT-PCR analysis was carried out on lung carcinoma EBC-1 cells. The amounts of *mdr-1*; *mrp*; glutathione *S*-transferase  $\pi$ ; DNA topoisomerases I and II ( $\alpha$  and  $\beta$ ) mRNA were not essentially affected



**Fig. 2.** Induction of  $G_2$  arrest in EBC-1 cells treated with AN9 plus DXR. Cells were cultured without (a) or with 32  $\mu$ M AN9 (b), 30 ng/ml DXR (c), or AN9 plus DXR (d) for 3 days, and DNA histograms were then analyzed.



**Fig. 3.** Time course for the intracellular accumulation of DXR in EBC-1 and BALM3 cells. Cell suspensions were exposed to 100 ng/ml DXR at 37°C. Following the indicated period of incubation, aliquots of cells were taken and the intracellular amounts of DXR were measured using a fluorescence spectrophotometric analysis. Points represent the average of two independent experiments within about 10% of variability. ●, untreated EBC-1 cells; and EBC-1 cells pretreated with 32  $\mu$ M AN9 for 0.5 (▲) or 2 (■) days. BALM3 cells pretreated with (□) or without (◆) 32  $\mu$ M AN9 for 2 days.



**Fig. 4.** Structures of anthracyclines.

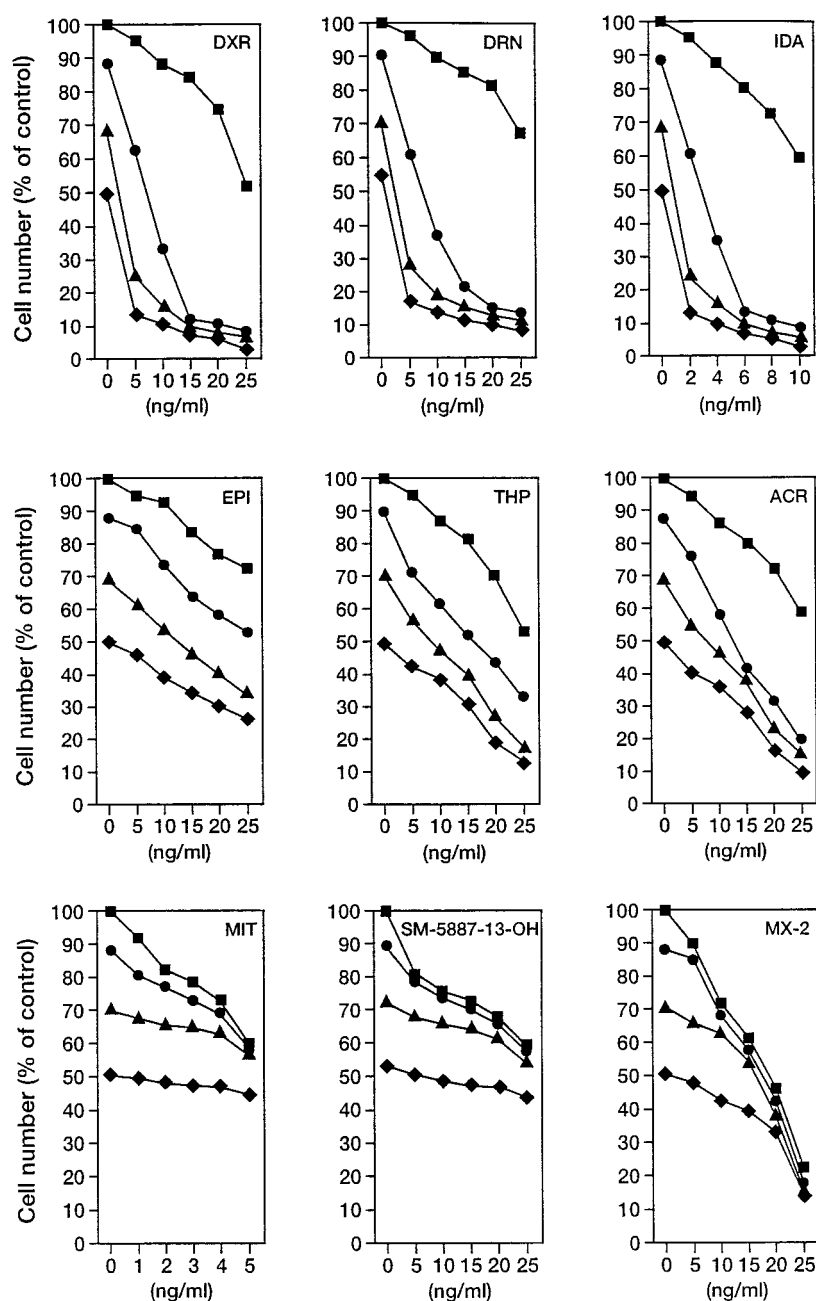
by AN9 (Table 2). Next, we examined the expression of mRNA for enzymes involved in anthracycline deglycosylation. The expression of cytochrome P450 reductase mRNA in BALM3 cells was slightly lower than that of DT-diaphorase. The levels of xanthine reductase mRNA were extremely low in BALM3 cells (Fig. 8A). The expression of xanthine reductase mRNA was not significantly affected by AN9, whereas cytochrome P450 reductase and DT-diaphorase mRNAs were slightly down-regulated by AN9 alone (Fig. 8A). The expression of these mRNAs was hardly down-regulated by DXR alone, whereas the expression was greatly down-regulated by the combined treatment with AN9 and DXR. This down-regulation was observed within 24 h after treatment with AN9 in BALM3 cells, and AN9 had a much greater effect than butyrate (Fig. 8B). On the other hand, the down-regulation was much less when the cells were treated with SM-5887-13-OH or MX-2 in the presence of AN9, suggesting that the down-regulation of cytochrome P450 reductase and DT-diaphorase mRNAs was selectively induced by DXR and AN9. The down-regulation of cytochrome P450 reductase protein was confirmed by Western blot analysis (Fig. 8C).

**Effect of Antisense Oligonucleotides on the Sensitivity to DXR.** AN9 and DXR synergistically inhibited the growth of BALM3 cells and decreased expression of cytochrome P450 reductase and DT-diaphorase mRNAs. To understand whether the expression of these mRNAs was directly correlated with the sensitivity to DXR, we treated BALM3 cells with antisense oligonucleotides for cytochrome P450 reductase and/or DT-diaphorase genes. Treatment with antisense oligonucleotide for cytochrome P450 reductase could greatly augment the growth-inhibitory effect of DXR, but the treatment with DT-diaphorase antisense oligomer was less effective (Fig. 9). Antisense oligomers for xanthine oxidase hardly affected the growth-inhibitory effect of DXR (data not shown). The antisense oligomers for cytochrome P450 reductase and DT-diaphorase significantly decreased intracellular levels of cytochrome P450 reductase and DT-

diaphorase RNA, respectively. However, the antisense oligomers for cytochrome P450 reductase did not significantly affect the level of DT-diaphorase RNA. Moreover, microsomal glycosidase activity for DXR was reduced by treatment with the antisense oligomer for cytochrome P450 reductase but not with that for DT-diaphorase (data not shown).

## Discussion

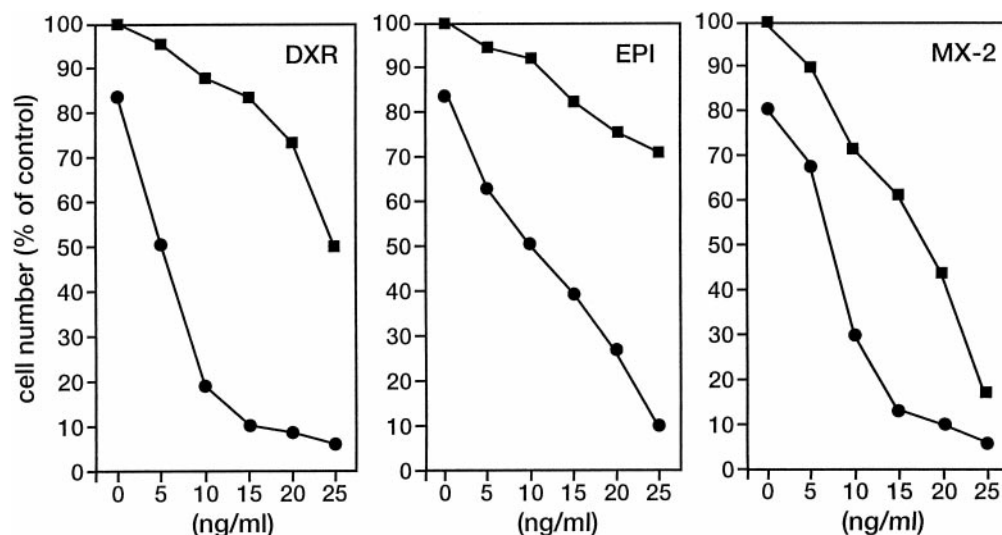
AN9 is a butyric acid prodrug, but its effect on the growth of hematopoietic and nonhematopoietic malignant cells is different from that of butyrate. Butyrate and AN9 modulate the expression of the early regulating genes, *c-myc* and *c-jun*, but AN9 elicits this effect at least 100 times faster than butyrate (Rabizadeh et al., 1993). This may be caused by a faster rate of intracellular penetration by the lipophilic AN9



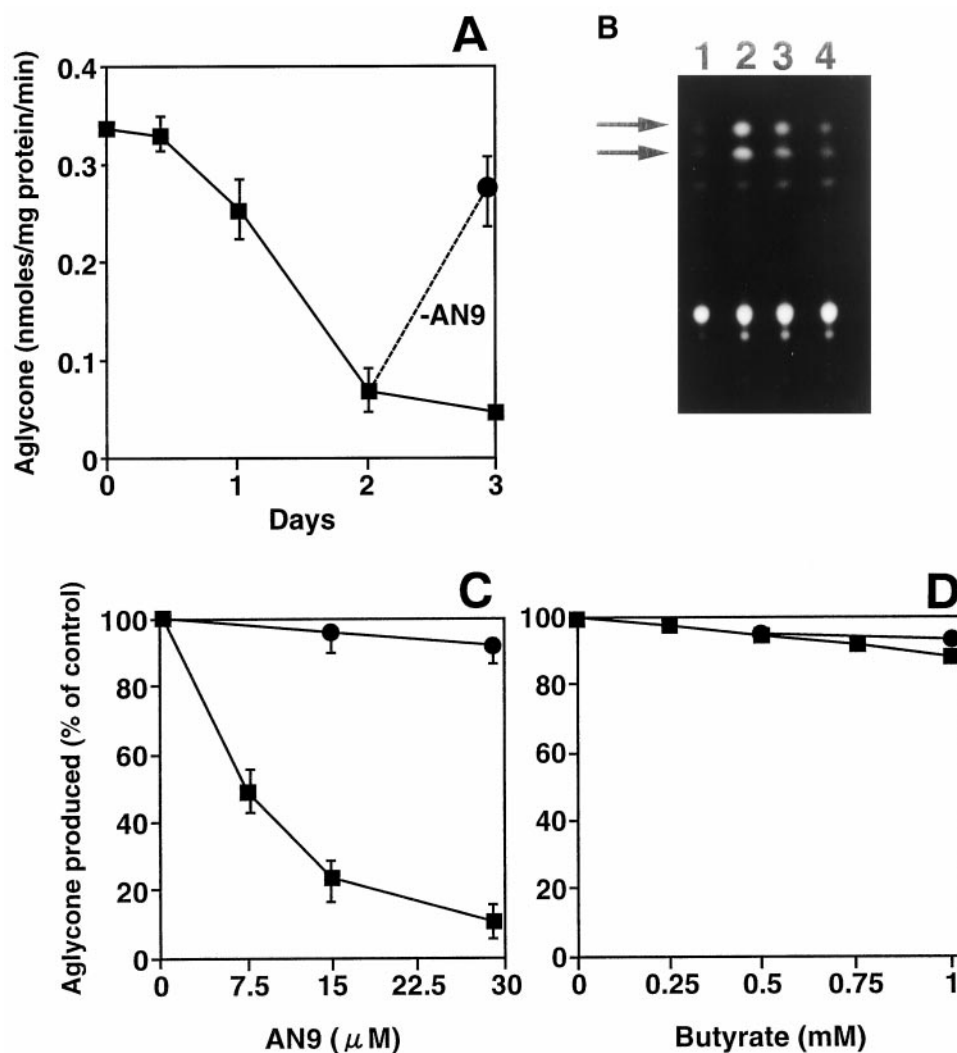
**Fig. 5.** Growth-inhibitory activities of various anthracyclines in the presence of AN9. BALM3 cells were cultured for 3 days with various concentrations of anthracyclines in the presence of 0 (■), 16 (●), 32 (▲), or 48 (◆)  $\mu$ M AN9. Points represent the average of four independent experiments.

and/or a slower rate of metabolic degradation. Both AN9 and butyrate caused a transient hyperacetylation of histone, and AN9 induced this effect at a concentration one order of magnitude lower than butyrate (Aviram et al., 1994). The kinetics of AN9-induced histone acetylation were faster than those

of butyrate. Histone acetylation loosens the chromatin structure (Lee et al., 1993), and this may improve the accessibility of DXR to nucleosomal DNA. However, the present investigation revealed that AN9 did not affect intracellular and nuclear DXR uptake. Moreover, the DNA topoisomerase II



**Fig. 6.** Combined effect of AN9 and anthracyclines on the growth of BALM3 cells in a 6-day culture. Cells were cultured with various concentrations of DXR, epirubicin (EPI), or MX-2 in the presence (●) or absence (■) of 32  $\mu$ M AN9. Means are representative of three determinations.



**Fig. 7.** Effect of AN9 on microsomal glycosidase activity of EBC-1 cells. A, time course for the reduction of microsomal glycosidase activity. Cells (■) were treated with 32  $\mu$ M AN9 for the indicated periods. Cells were treated with 32  $\mu$ M AN9 for 2 days, washed, and further incubated without AN9 for an additional 1 day (●). Means  $\pm$  S.D. of three determinations. B, degradation of DXR by microsomal glycosidase(s) of EBC-1 cells treated without (lane 2) or with 0.5 mM butyrate (lane 3) or 32  $\mu$ M AN9 (lane 4) for 2 days. Lane 1, DXR sample without microsomes. Arrows indicate DXR metabolites (aglycones). Effects of AN9 (C) and butyrate (D) on microsomal glycosidase activity. Cells were treated with various concentrations of AN9 or butyrate for 2 days. Microsomal fractions were incubated with DXR (■) and aclarubicin (●) as substrates. Means  $\pm$  S.D. of three determinations.



activity was not affected by AN9. These results suggest that one or more other mechanisms are mainly involved in the synergistic interaction between DXR and AN9 in the growth inhibition.

The structure-activity relationships of various anthracyclines revealed that the synergistic effect of AN9 was pronounced in the combination with anthracyclines having a daunosamine moiety. The reduction of microsomal glycosidic activity by AN9 was closely associated with the synergy of AN9 and anthracyclines in the growth inhibition. The mechanisms responsible for converting DXR into a nontoxic metabolite are not yet well understood.

The expression of bioreductive enzyme activities showed considerable heterogeneity among the various tumor cell lines. In the case of DT-diaphorase, a relatively high degree of expression was seen in nonsmall-cell lung and colon carcinoma cell lines (Fitzsimmons et al., 1996). Down-regulation of DT-diaphorase mRNA expression was closely associated with the enhancement of DXR cytotoxicity in AN9-treated cells. Mitomycin C-resistant lung carcinoma PC9/MC4 cells was four times more sensitive to DXR than was the parent PC9 cells, and DT-diaphorase activity in the resistant cells showed an approximately 200-fold decrease than that in the parent PC9 cells, suggesting that DT-diaphorase is a determinant of sensitivity to DXR in lung cancer cells (Kasahara et al., 1994). However, there is no indication of an involvement in DXR deglycosylation in either cytosol or microsome, although DT-diaphorase is the major quinone reductase in tumor cells (Cummings et al., 1992). Although the present results indicate strongly that NADPH-cytochrome P450 reductase is the principal enzyme for determinant of sensitivity to DXR, we cannot exclude the possibility that DT-diaphorase is also involved in the augmentation of DXR sensitivity by AN9.

AN9 induced hyperacetylation of histones, which returned to basal levels after 6 h (Aviram et al., 1994). Histone acetylation has been implicated in changes in transcriptional regulation (Van Lint et al., 1996; Vettese-Dadey et al., 1996). There are several reports on the effect of acetylation status on gene suppression. Cyclin D1 expression is inhibited by butyrate at the transcriptional level (Lallemant et al., 1996) and IL-2 gene expression is selectively inhibited by trichostatin A, a potent inhibitor of histone deacetylase (Takahashi et al., 1996). AN9 might directly and/or indirectly modulate the transcription of some genes, including cytochrome P450

TABLE 2

Effect of AN9 on the expression of genes related to drug resistance in lung carcinoma EBC-1 cells

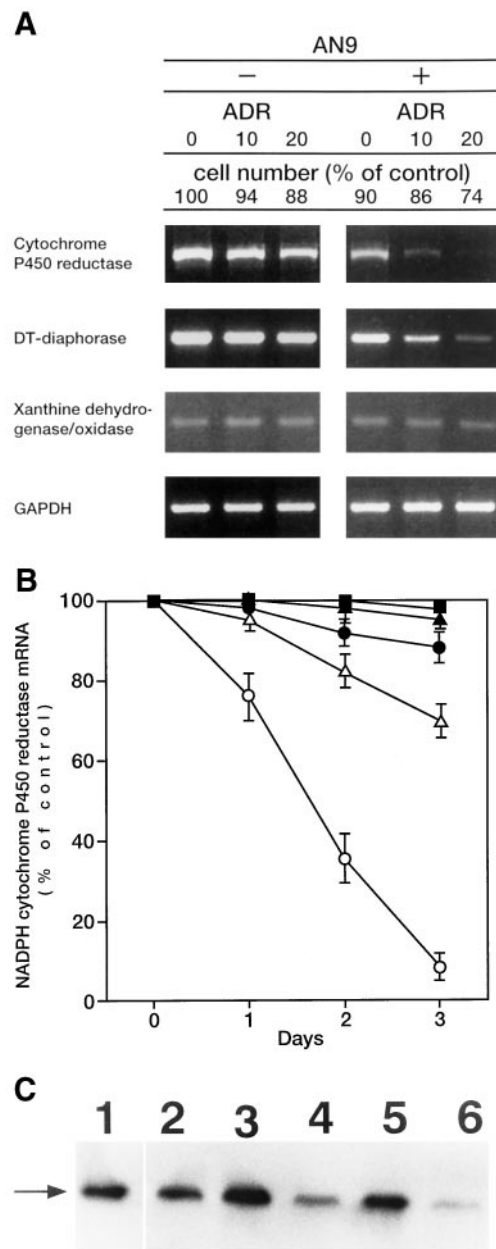
EBC-1 cells were cultured with AN9 for 3 days. The control (the index = 100) is represented by RNA extracted from the human erythroleukemia HEL cell line, which is known to be multidrug-resistant. Mean  $\pm$  S.D. of three determinations.

AN9 ( $\mu$ M)	mRNA Level		
	0	32	64
	Index $\pm$ S.D.		
<i>mdr1</i>	6.4 $\pm$ 0.8	5.3 $\pm$ 0.6	4.9 $\pm$ 0.6
<i>mvp</i>	93.1 $\pm$ 6.4	90.1 $\pm$ 8.7	89.3 $\pm$ 9.2
GST- $\pi^a$	22.4 $\pm$ 2.4	25.8 $\pm$ 3.1	23.6 $\pm$ 2.2
Topo I <sup>b</sup>	64.5 $\pm$ 6.3	50.9 $\pm$ 6.5	59.6 $\pm$ 5.3
Topo II $\alpha$	48.6 $\pm$ 4.1	32.4 $\pm$ 4.1	37.5 $\pm$ 3.4
Topo II $\beta$	54.4 $\pm$ 5.2	58.9 $\pm$ 5.4	50.5 $\pm$ 5.8

<sup>a</sup> GST- $\pi$ , glutathione S-transferase.

<sup>b</sup> Topo I, DNA topoisomerase I; Topo II $\alpha$ , DNA topoisomerase II $\alpha$ ; Topo II $\beta$ , DNA topoisomerase II $\beta$ .

reductase and DT-diaphorase by inhibiting histone deacetylases. AN9 may stimulate one or more genes that may suppressively regulate both of the reductive enzymes. Consequently, AN9 down-regulates gene expression of cytochrome P450 reductase and DT-diaphorase. Treatment with DXR at a therapeutic concentration decreases the levels of cytochrome P450 reductase and DT-diaphorase activities, and the down-regulation of the P450 reductase was more pronounced than that of DT-diaphorase (Cummings et al., 1992).



**Fig. 8.** Effects of AN9 and butyrate on the expression of drug-metabolizing enzyme genes in BALM3 cells. **A**, cells were treated with or without 32  $\mu$ M AN9 in the presence of DXR (0, 10, or 20 ng/ml, ADR) for 2 days. After 35 amplification cycles, PCR products were analyzed on 2% agarose gels. **B**, cells were treated with 32  $\mu$ M AN9 or 1 mM butyrate in the presence or absence of 10 ng/ml DXR. Means  $\pm$  S.D. of three determinations. ■, DXR; ●, AN-9; ▲, butyrate; ○, AN-9 + DXR; △, butyrate + DXR. **C**, changes in cytochrome P450 protein in the cells treated with AN9 and/or DXR for 2 days. Lane 1, untreated; lane 2, 32  $\mu$ M AN9; lane 3, 10 ng/ml DXR; lane 4, 32  $\mu$ M AN9 + 10 ng/ml DXR; lane 5, 20 ng/ml DXR; lane 6, 32  $\mu$ M AN9 + 20 ng/ml DXR.



Therefore, it is quite possible that the P450 reductase and DT-diaphorase mRNAs are preferentially and synergistically degraded in addition to the effect on gene transcription when the cells are treated with AN9 and DXR. Alternatively, butyrate may activate the expression of gene-specific repressors such as histone H1<sup>0</sup> (Khochbin and Wolffe, 1993; Dimitrov and Wolffe, 1996). Therefore, the repression of genes involved in metabolism could well be an indirect effect of AN9.

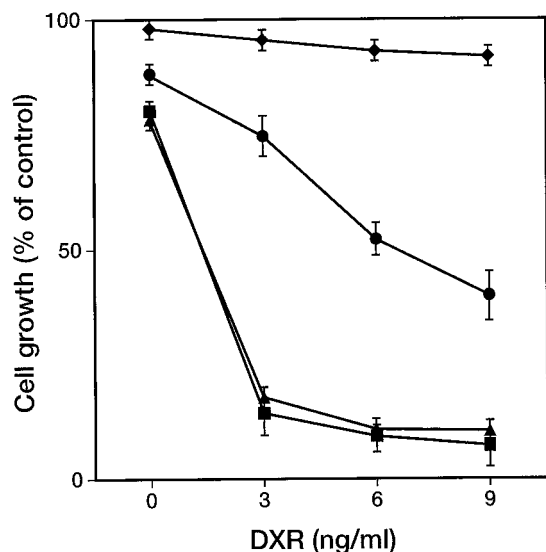
A cell-cycle analysis indicated that AN9-treated cells accumulated at the G<sub>2</sub> phase, and this G<sub>2</sub> accumulation was pronounced in the cells treated with AN9 plus DXR. Some inhibitors of histone deacetylase such as butyrate and trichostatin A have previously been shown to inhibit the cell cycle at the G<sub>2</sub> phase (Fallon and Cox, 1979; Yoshida and Beppu, 1988). The association between G<sub>2</sub> accumulation and the hyperacetylation of histones with the enhancing effect of AN9 on the sensitivity to DXR remains to be elucidated.

The cardiotoxic effects of DXR and DNR are serious therapeutic problems in cancer chemotherapy. The tissue levels of DXR reached a maximum concentration immediately after administration, and the concentration thereafter decreased in all tissues with time thereafter. In tumor tissue, however, a maximum concentration was maintained for several hours, although the peak concentration was lower than those in normal tissues, including heart. Sinkai et al. (1996) reported that the mean residence times of DXR in heart and tumor were 25.30 and 48.62 h, respectively. Glycosidic activity was reduced for more than 24 h after treatment with AN9. Pharmacokinetically, the effect of AN9 might be more prominent in tumor than heart tissue after 24 h. These results suggest that combined treatment with DXR and AN9 may be preferentially effective against some tumors with the less cardiotoxic effects. In the murine model system, the administration of AN9 plus DNR markedly prolonged the survival of mice inoculated with leukemia Mm-A cells (Kasukabe et al., 1997). These results suggest that the combination of AN9 and DXR

offers clear therapeutic potential against human lymphoma and lung carcinoma.

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**Fig. 9.** Enhancement of sensitivity to DXR by antisense oligomer of cytochrome P450 reductase. BALM3 cells were cultured with 10  $\mu$ M antisense oligomer of cytochrome P450 reductase (▲) or DT-diaphorase (●) for 2 days and then treated with various concentrations of DXR for 4 more days. ◆, control (transfection reagent alone); ■, 10  $\mu$ M antisense oligomers of cytochrome P450 reductase and DT-diaphorase. Means  $\pm$  S.D. of three determinations.

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**Send reprint requests to:** Yoshio Honma, Saitama Cancer Center Research Institute, 818 Komuro, Ina, Saitama 362-0806, Japan. E-mail: honma@cancer-c.pref.saitama.jp

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