Molecular Basis for the Synergistic Interaction of Adriamycin with the Formaldehyde-releasing Prodrug Pivaloyloxymethyl Butyrate (AN-9)\(^1\)

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ABSTRACT

The interaction of Adriamycin and pivaloyloxymethyl butyrate (AN-9) was investigated in IMR-32 neuroblastoma and MCF-7 breast adenocarcinoma cells. Adriamycin is a widely used anticancer drug, whereas AN-9 is an anticancer agent presently undergoing Phase II clinical trials. The anticancer activity of AN-9 has been attributed to its ability to act as a butyric acid prodrug, although it also releases formaldehyde and pivalic acid. Adriamycin and AN-9 in combination display synergy when exposed simultaneously to cells or when AN-9 treatment is up to 18 h after Adriamycin administration. However, the reverse order of addition results in antagonism. These interactions have been established using cell viability assays and classical isobologram analysis. To understand the molecular basis of this synergy, the relative levels of Adriamycin-DNA adducts were determined using various treatment combinations. Levels of Adriamycin-DNA adducts were enhanced when treatment combinations known to be synergistic were used and were diminished using those treatments known to be antagonistic. The relative timing of the addition of Adriamycin and AN-9 was critical, with a 20-fold enhancement of Adriamycin-DNA adducts occurring when AN-9 was administered 2 h after the exposure of cells to Adriamycin. The enhanced levels of these adducts and the accompanying decreased cell viability were directly related to the esterase-dependent release of formaldehyde from AN-9, providing evidence for the formaldehyde-mediated activation of Adriamycin.

INTRODUCTION

Adriamycin is a widely used drug in current chemotherapy regimes because it is effective against a broad range of neoplasms. It is used as a single agent but is more commonly used in combinations with other anticancer agents. The selection of these additional agents is not usually based on known synergistic interactions between the drugs but rather on complimenting mechanisms of action. The major drawbacks associated with the use of Adriamycin are its dose-dependent cardiotoxicity and the emergence of tumor resistance to the drug (1). Although Adriamycin is a known topoisomerase II inhibitor, this mechanism of action does not fully explain its broad-spectrum anticancer activity (1, 2). In recent years, it has been shown that Adriamycin induces adducts with DNA, and these occur predominantly at 5\(^\prime\)-GC sequences (3, 4). Chemical characterization of this structure has revealed that the 3\(^\prime\) aminosugar of Adriamycin is covalently bound to the N2 of guanine via a formaldehyde-derived bridge (5, 6). Two-dimensional NMR\(^3\) analysis of the structure showed that adducts at GC sequences are also virtual cross-links, because the Adriamycin monoaduct is stabilized by the complementary strand of DNA by intercalation and H-bonding (7). This structure of the virtual cross-link explains why the apparent Adriamycin cross-links are unstable. DNA cross-link formation by various anthracycline derivatives (including Adriamycin) has been correlated with cytotoxicity in HeLa cells (8), and more recently in MCF-7 cells, at sufficiently high levels to account for the cytotoxic response (9).

A new drug, doxoform, has been designed recently to take advantage of the fact that Adriamycin can be activated by formaldehyde (10). This complex of Adriamycin with formaldehyde is dramatically (200-fold) more cytotoxic than Adriamycin, and this appears to be attributable to enhanced formation of DNA adducts.

BA is an agent that induces differentiation primarily because of its ability to function as a histone deacetylase inhibitor (11). In human tumor cells in vitro, it displays growth arrest, decreased clonogenicity, and induction of morphological and biochemical changes resulting in antitumor activity (12, 13). However, BA is not clinically effective because of rapid metabolism and, to a lesser extent, excretion (14). To achieve a reduction in the clearance rate of BA, a panel of BA-releasing prodrugs were synthesized and screened for antitumor activity (15, 16). AN-9 is the best studied produg, and it affects cancer cells at ~10-fold lower concentrations and at least 100-fold faster than BA. Moreover, it penetrates 100-fold faster than BA into cancer cells in vitro (17). Derivatization of BA improves its permeability across cell membranes and enables efficient intracellular delivery of BA.

AN-9 belongs to a well-established family of acyloxyalkyl ester prodrugs of carboxylic acids (18–20) whose expected esterase-dependent intracellular hydrolytic degradation products are BA, pivalic acid, and formaldehyde (Fig. 1). Whereas pivalic acid does not contribute to the activity elicited by the produg, the role of the released formaldehyde remains unclear, and it also cannot be excluded that the intact AN-9 has some intrinsic activity. The pivaloyloxymethyl derivatives of propionic, valeric, and pivalic acids (analogues of AN-9 that lack a BA fragment) were found to have significantly lower antitumor activity in cancer cells (16). This suggests that the biological activity of AN-9 stems mostly from the released BA moiety.

AN-9 was shown to inhibit the proliferation of a variety of cancer cell lines and primary human tumors (15, 16, 21). AN-9 displayed low toxicity in mice and was effective in prolonging survival of mice bearing melanoma, lung carcinoma, and mononcytic leukemia (15, 16, 22). It induced transient hyperacetylation of histones (23), leading to relaxation of the chromatin structure, which allowed access of transcription factors to the DNA (24). This activity is likely to be an important mechanism by which AN-9 exerts its effect on gene modulation. AN-9 modulates the expression of the early regulatory genes c-myc and c-jun and the tumor suppressor gene RB as well as the antiapoptotic gene bcl-2 in WEHI and HL-60 cells (20, 25–27). AN-9 induces differentiation and/or apoptosis depending on the concentrations and timing of the drug used (27). AN-9 formulated in lipid emulsion (PIVANEX), displayed low toxicity in a Phase I clinical study and was reported to have an estimated maximum tolerated dose of 2.7 g/m\(^2\)/day (28). It is presently in Phase II clinical trials with non-small cell lung carcinoma and hematoma patients.

Synergistic effects between AN-9 and DNA-disrupting agents have been observed in murine monocytic leukemia cells. Furthermore, it

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\(^3\) The abbreviations used are: NMR, nuclear magnetic resonance; BA, butyric acid; AN-9, pivaloyloxymethyl butyrate; AN-158, 1-pivaloyloxyethyl butyrate; DHFR, dihydrofolate reductase; CI, combination index.
has been shown that combination of the B/A/formaldehyde-releasing prodrug AN-9 with daunorubicin led to a significant increase in survival of mice inoculated with acute monocyctic leukemia cells (22). However, the molecular interactions responsible for this marked effect were unknown. We show here that AN-9 dramatically increases the level of Adriamycin-DNA adducts in IMR-32 and MCF-7 cells and that this effect is largely attributable to the release of formaldehyde. The formaldehyde is subsequently involved in the chemical activation of Adriamycin, resulting in the formation of Adriamycin-DNA adducts. The ultimate outcome of this work is improved understanding of the factors that govern the anticancer activity of Adriamycin. Exploration of this knowledge to improve the use of Adriamycin as an anticancer agent is discussed.

MATERIALS AND METHODS

Radiochemicals and Molecular Biology Reagents. [14-14C]Adriamycin hydrochloride (57 mCi/mmol) and the radionucleotides [α-32P]dCTP and [α-32P]UTP (2500 Ci/mmol) were obtained from Amersham Pharmacia Biotech UK Ltd., Buckinghamshire, United Kingdom. QIAamp blood kits for genomic DNA isolation were purchased from Qiagen, and restriction enzymes and random primed labeling kits were from Roche Molecular Biochemicals.

Cell Lines. IMR-32 neuroblastoma cells were kindly supplied by S. Bordow, M. Haber, and C. M. Ireland (Children’s Cancer Research Institute, Sydney Children’s Hospital, New South Wales, Australia). IMR-32 cells and MCF-7 breast adenocarcinoma cells were maintained in DMEM (Trace Scientific and 10% fetal bovine serum (Life Technologies, Inc.), supplemented with 0.1 mg/ml streptomycin and 100 units/ml penicillin.

Compounds. Adriamycin was a gift from Farmitalia Carlo Erba (Milan, Italy), and arminomycin was provided by Dr. Ken-ichi Kimura (Research Institute of Life Science, Snow Brand Milk Products Co. Ltd., Tochigi, Japan). Semicarbazide was purchased from Sigma Chemical Co. Aldrich. AN-9, isobutyryloxyethylpivalate (AN-37), and valeroyloxyethylpivalate (AN-38) were synthesized as described previously (15, 16). AN-158 was prepared as described for AN-9 from 1-chloroethylbutyrate and pivalic acid. 1H NMR (CDCl₃, δ): 0.92 (t, J = 7.5 Hz, 3H, MeCH₂), 1.16 (s, 9H, t-Bu), 1.45 (d, J = 5.5 Hz, 3H, MeCH), 1.67 (sexet, J = 7.5 Hz, 2H, MeCH₂), 2.3 (t, J = 7.5 Hz, 2H, CH₂CO), 6.86 (q, J = 5.5 Hz, 1H, OCH₃). Ethylenedipropionate (AN-188) was prepared from acetaceldehyde and propionic anhydride as described previously. 1H NMR (CDCl₃, δ): 6.86 (q, 1H, CH, J = 5 Hz), 2.32 (q, 4H, CH₂Me × 2, J = 7.54 Hz), 1.43 (d, 3H, CHMe, J = 5 Hz), 1.11 (t, 6H, MeCH₂ × 2, J = 7.54 Hz).

Preparation of Probes for Southern Hybridization. The plasmid pBH31R1.8 was provided by Dr. V. A. Bohr (National Institute of Aging, NIH, Baltimore, MD). A 1.8-kb EcoRI fragment containing exons I and II of the human DHFR gene (29) was isolated from pBH31R1.8 and radio-labeled with [α-32P]dCTP using a random primed labeling kit. The mitochondrial probe pCR1I-H1 was a gift from Dr. C. A. Filburn (National Institute of Aging, NIH). The strand-specific human mitochondrial probe (corresponding to nucleotides 652-3226) was prepared by generating run-off transcripts from the T7 promoter in the presence of [α-32P]UTP.

Drug Treatment of Cells. Cells were seeded in 10-cm Petri dishes (Interpath) at a density of 1.5 × 10⁵ cells/dish (IMR-32 cells) or 2.5 × 10⁶ cells/dish (MCF-7 cells). Cells were incubated with differing concentrations of Adriamycin (dissolved in H₂O) or AN-9 (dissolved in DMSO) in 10 ml of complete medium, typically for 2–6 h. The final concentration of DMSO in the medium did not exceed 0.5%. IMR-32 cells were subsequently removed from Petri dishes by gentle mixing with medium and washing three times in PBS after pelleting. MCF-7 cells were washed twice in PBS, trypsinized, and then were pelleted and washed once more in PBS. Pellets were stored at −80°C until required. All experiments were performed at least in duplicate. Genomic DNA was isolated using a QIAamp blood kit with two modifications; cell lysis was conducted at 50°C for 30 min (to minimize the loss of heat-labile adducts), and an RNase A digestion step was included.

Detection of Adducts by Cross-Linking Assay. Genomic DNA was quantitated by agarose electrophoresis and subsequent comparison to genomic DNA of known concentrations. Amounts of 2.5 μg were restriction digested with BamHI to linearize the mitochondrial genome, whereas 7.5 μg amounts were restriction digested with HindIII for 90 min at 37°C (to produce a 22-kb genomic fragment of DHFR). The DNA was then subjected to a cleanup procedure consisting of one phenol extraction, one chloroform extraction, and subsequent ethanol precipitation using glycogen as an inert carrier of the DNA. Pellets were washed in 70% ethanol and vacuum dried at room temperature in a Speed Vac concentrator (Savant). The pellet was resuspended in 10 μl of Tris-EDTA and 20 μl of loading buffer containing 90% formamide, 10 mM EDTA, 0.1% xylene cyanol, and 0.1% bromphenol blue (final formamide concentration, 60%). Samples were denatured at 60°C for 5 min, quenched on ice, and resolved on 0.5% agarose gels in 1 × Tris-acetate EDTA by overnight electrophoresis at 30 V.

The DNA was then probed using a Southern hybridization procedure that involved transfer onto Hybond N⁺ nylon membranes in 0.4 M NaOH. For detection of the mitochondrial genome, membranes were hybridized overnight in 5 × Denhardt’s, 0.5% SDS, 5 × SSPE, and 100 μg/ml salmon sperm DNA. For detection of the DHFR fragment, membranes were prehybridized in 10 ml of Hybrisol I (Onco) containing 100 μg/ml salmon sperm DNA at 52°C. Membranes were washed and then exposed to Phosphor plates for 4 h (mitochondrial probe) or overnight (DHFR), and images were captured and quantitated using a PhosphorImager (model 400B; Molecular Dynamics, Sunnyvale, CA).

Detection of 14C Adducts. IMR-32 cells were seeded into 3.5-cm Petri dishes at a density of 7.5 × 10⁵ cells/dish. Cells were incubated with varying concentrations of [14C]Adriamycin and AN-9. Cells were harvested, and the genomic DNA was isolated as described above. Samples were then extracted twice with phenol and once with chloroform, and DNA was selectively precipitated from RNA by ammonium acetate precipitation. DNA pellets were washed in 70% ethanol and vacuum dried at room temperature in a Speed Vac concentrator (Savant). The pellet was resuspended in 1 ml of Optiphase Hisafe scintillation mixture, and the incorporation of 14C-labeled drug into the DNA was monitored using a Wallac 1410 Liquid Scintillation Counter.

Cytotoxicity Assays. IMR-32 cells, 100 μl at a density of 5 × 10⁴ cells/ml were seeded in tissue culture 96-well plates (in triplicate) for 48 h. They were exposed to different concentrations of the drugs at the specified ratio and times. The viability was measured after 48 h by neutral-red assay as described (30). When drugs were added sequentially, the second drug was added 18 h after the first, and viability was assessed 30 h later. The mean value obtained from three wells was calculated, and IC₅₀s were derived from linear regression of the adjusted Y (% control viability) and X values of log concentration of the compounds.

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RESULTS

Synergy/Antagonism. Initial experiments established that AN-9 and Adriamycin were more cytotoxic in combination than either agent alone. This interaction has been established previously to be synergistic (22). The effect of treatment of IMR-32 cells with Adriamycin and AN-9 as single agents and in combination was analyzed according to the classical isobologram equation:

\[
CI = \frac{(D_1 / D_{x1}) + (D_2 / D_{x2})}{2}
\]

where \( D_x \) is the dose of one compound alone required to produce an effect, and \( D_1 \) and \( D_2 \) are the doses of both compounds that produce the same effect. From this analysis, the combined effects of two drugs can be assessed as either additive (or zero) interaction indicated by \( CI = 1 \), synergism as indicated by \( CI < 1 \), or antagonism indicated as \( CI > 1 \). CI values that describe the interaction between Adriamycin and AN-9 in IMR-32 cells are shown in Table 1. It is apparent that synergy is observed where both Adriamycin and AN-9 are administered simultaneously or where AN-9 is administered 18 h after Adriamycin. The most profound synergy is obtained in the case of simultaneous drug treatment. In contrast, when AN-9 is administered 18 h prior to Adriamycin, antagonism is observed.

Detection of Adducts. On the basis of this synergistic interaction and the knowledge that Adriamycin induces DNA adducts, we sought to establish whether the levels of adducts in human tumor cells could account for this synergy. A concentration-dependent study was therefore initiated. A ratio of AN-9:Adriamycin of 25:1 was chosen on the basis of the IC50 data because this ratio showed good synergy (Table 1). Exposure of both MCF-7 and IMR-32 cells to Adriamycin alone (0–10 \( \mu M \)) resulted in barely detectable levels of adducts (Fig. 2).

![Fig. 2. Potentiation of Adriamycin-DNA adducts by AN-9. IMR-32 cells (A, C, and D) and MCF-7 cells (B, E, and F) were treated with Adriamycin alone for 4 h (0–10 \( \mu M \) as shown) or Adriamycin followed by an additional 2-h incubation with a 25-fold excess of AN-9 (Adriamycin/AN-9) or AN-9 for 2 h, followed by an additional 4-h incubation with Adriamycin (AN-9/Adriamycin). Genomic DNA was isolated and treated for Southern analysis as described. A and B are representative Southern blots for IMR-32 cells and MCF-7 cells, respectively, where BamHI-digested DNA has been probed for mtDNA. DS, double-strand DNA; SS, single-strand DNA. Phosphorimaging analysis was used to quantitate Adriamycin adducts in the mtDNA of IMR-32 cells (C) and MCF-7 cells (E) and also in the DHFR gene of IMR-32 cells (D) and MCF-7 cells (F). Adducts formed in the Adriamycin/AN-9 incubation (■) are compared with the levels formed with the AN-9/ Adriamycin incubation (□). Data were derived from each of two separate blots of two biological experiments, and the values are the means; bars, SE.](image-url)
where adducts were detected as virtual interstrand DNA cross-links using a gene-specific interstrand cross-linking assay (9). However, when in combination with AN-9, high levels of adducts were induced, and in many of the treatments, these levels were the maximum that can be detected by the gene-specific cross-linking assay (i.e., 100% cross-linked DNA). Results are presented as percentage of cross-links rather than adducts per 10 kb because the Poisson distribution used to calculate the levels of adducts is not meaningful for values approaching 100% cross-linked DNA (31). Table 2 provides a conversion guideline that relates the percentage of cross-linked DNA (20–80%) to the approximate number of adducts per 10 kb.

In both the mitochondrial genome and DHFR gene of IMR-32 cells, 50% cross-linked DNA for the Adriamycin/AN-9 combination (i.e., Adriamycin added 2 h prior to AN-9) was observed at ~3.5 µM Adriamycin but at ~5.5 µM for the AN-9/Adriamycin combination (i.e., AN-9 added 2 h prior to Adriamycin). Similarly, in MCF-7 cells, 50% cross-linked DNA was detected in mitochondrial and nuclear genomes at ~4 µM Adriamycin for the Adriamycin/AN-9 treatment but at 7 µM for the reverse sequence of addition. In both cell lines and for both DNA probes, the level of maximal cross-linking was achieved at low Adriamycin concentrations (~6 µM) for the Adriamycin/AN-9 treatment; however, this did not occur, even at 10 µM for the reverse schedule. When AN-9 was used as a single compound, cross-links were not observed, even at the highest concentrations used (250 µM; see for example Fig. 4, last lanes of A and B).

To compare the rate of AN-9 facilitated adduct formation to that observed using Adriamycin alone in previous studies, a time course of adduct formation was conducted (data not shown). Both drugs were added to cells simultaneously, and at various times DNA was isolated and then analyzed for adduct formation. Adriamycin was used at a concentration of 4 µM with the same 25-fold excess of AN-9 as used previously. The number of adducts reached a plateau at between 5 and 8 h for both the mtDNA and DHFR gene and showed that maximal cross-linking attainable was ~60% under these conditions of simultaneous treatment with both Adriamycin and AN-9.

**Optimal Timing of AN-9 Addition.** To further understand the complexity of the relative time of addition of Adriamycin and AN-9, a subsequent time course experiment was performed. However, in this case, the time of exposure of cells to 4 µM Adriamycin was constant (a short incubation of 4 h to ensure that the cells did not have sufficient time to undergo replication, because this would result in underestimation of total cross-links obtained). The time of addition of AN-9 was varied to establish the optimal time for maximal cross-link formation, and this is shown in Fig. 3. For a 4-h Adriamycin treatment, maximal cross-links (80%) were obtained when AN-9 was administered 2 h after Adriamycin. Therefore, when compared with simultaneous treatment, it is clear that the potential maximum of Adriamycin cross-links is >60% because the optimal time of addition of AN-9 can actually significantly elevate the level of cross-links formed.

Another time course was then initiated to establish conditions where adduct formation was severely compromised. As for previous studies, the level of Adriamycin was held constant, and the time of addition of AN-9 was varied. However, in this experiment, the addition of AN-9 was from 24 h prior to and up to 2 h after treatment by Adriamycin (Fig. 4). The time course established that adduct levels did not increase significantly until AN-9 was administered ~3 h prior to Adriamycin and increased for up to 2 h after Adriamycin addition.

**Involvement of Formaldehyde and Butyric Acid.** It was then critical to establish which of the components of AN-9 was responsible for the dramatic enhancement of these DNA adducts. Because AN-9 has been shown previously to release formaldehyde, BA, and pivalic acid, an alternative prodrug, AN-158, was used as a control. This prodrug, which releases acetaldehyde, BA, and pivalic acid, was used with Adriamycin in a variety of combinations and was shown to have no effect on the adduct levels induced in mtDNA and DHFR gene by Adriamycin (data not shown), consistent with the assumed requirement for formaldehyde.
probing of the DHFR gene and the condition chosen were identical to those presented in Fig. 4. The results are also shown for Adriamycin treatment alone (Ad) and AN-9 treatment alone (AN9). Genomic DNA was extracted from cells and then processed for Southern analysis. A, probing of mtDNA; B, probing of the DHFR gene. Phosphorimage analysis was used for quantitation of the adducts in mtDNA (C) and the DHFR gene (D). Bars, SE.

Fig. 4. Conditions that antagonize adduct formation. IMR-32 cells were exposed to 6 μM Adriamycin for 4 h. However, 125 μM AN-9 was added at varying times from 24 h prior to Adriamycin addition (∼24) to 2 h after Adriamycin. Results are also shown for Adriamycin treatment alone (Ad) and AN-9 treatment alone (AN9). Genomic DNA was extracted from cells and then processed for Southern analysis. A, probing of mtDNA; B, probing of the DHFR gene. Phosphorimage analysis was used for quantitation of the adducts in mtDNA (C) and the DHFR gene (D). Bars, SE.

To further confirm the mechanism of enhanced Adriamycin cross-linking by AN-9, it was relevant to investigate the effect of AN-9 on cross-links induced by barminomycin. Barminomycin is an anthracycline compound that does not require activation by formaldehyde to induce DNA cross-links (32). Cells were therefore exposed to barminomycin and AN-9 in a series of combinations. Despite incubating barminomycin with up to a 12,500-fold excess of AN-9, there was no effect on the level of barminomycin-induced cross-links in either the mtDNA or DHFR gene. Varying the time of addition of AN-9 also had no effect on cross-linking in either the mtDNA or DHFR gene (data not shown).

[14C]Adriamycin was then used to confirm that the adducts formed in the presence of AN-9 actually contained the Adriamycin chromophore and also used to accurately estimate the levels of adducts induced in the various treatment schedules (Fig. 5). Drug treatment conditions chosen were identical to those presented in Fig. 4. The adduct levels follow the same trend indicated by the gene-specific cross-linking assay. For example, there was a high level of adducts when AN-9 and Adriamycin treatments were simultaneous (∼41 adducts/10 kb) but an even higher level where AN-9 was added 2 h after Adriamycin (∼63 adducts/10 kb), and adduct levels decreased with longer preincubation with AN-9 (∼1.7 adducts/10 kb for the 16-h pretreatment). The adduct levels induced by Adriamycin as a

It is interesting to note that adduct levels obtained by the [14C] analysis method is ~60-fold higher than the gene-specific cross-linking assay. A similar difference was noted in a previous study and shown to be attributable mainly to the loss of adducts in the additional procedures required for the preparation of genomic DNA for the gene-specific cross-linking assay, compared with the quicker and more direct [14C] assay (9). Overall, the use of [14C] Adriamycin confirms the enhancement of drug-DNA adduct levels by AN-9 and also provides direct evidence for the incorporation of the Adriamycin molecule into these adducts. Control experiments (Fig. 5) showed that there was no potentiation of adducts by DMSO or sodium butyrate when added at various times. A series of prodrugs related to AN-9, which upon intracellular metabolic hydrolysis released either formaldehyde or acetaldehyde, and low molecular weight fatty acids were used in conjunction with [14C] Adriamycin (Table 3). Of the four formaldehyde-releasing drugs assessed, all significantly increased adduct levels above that observed with Adriamycin alone (Fig. 6). In contrast, all of the acetaldehyde-releasing drugs yielded only background levels of adducts, demonstrating that the ability to enhance adduct formation was limited to those prodrugs that release formaldehyde.

**Abrogation of Responses by Sequestering Formaldehyde.**

Because formaldehyde has been shown to be a critical element in the formation of DNA adducts, it was important to establish whether these adducts were responsible for the enhanced cytotoxicity displayed by the Adriamycin/AN-9 combination. Formaldehyde was sequestered by the addition of high concentrations of semicarbazide (33). As expected, the level of DNA adducts was dramatically reduced by incubation of cells with increasing ratios of semicarbazide (Table 4). Little difference was observed between adding semicarbazide at the same time as Adriamycin or at the same time as AN-9 (2 h later). Cell viability assays were used to measure the effect of semicarbazide inclusion on the interaction displayed by the Adriamycin/AN-9 combination. Incubation of Adriamycin and AN-9 with increasing concentrations of semicarbazide resulted in increasing levels of protection from the drug combination, and at the highest semicarbazide concentration (1

![Fig. 5. [14C] Adriamycin incorporation into adducts. IMR-32 cells were exposed to 6 μM [14C] Adriamycin alone for 4 h (Adr) or together with 125 μM AN-9 at varying times: 16 h prior (∼16), 2 h prior (∼2), simultaneously (0), and 2 h after Adriamycin addition (2). The remaining treatments were Adriamycin with 0.5% DMSO (DM), 250 μM AN-158 (b-158), or with sodium butyrate (1 mM) at varying times: 16 h prior (b-16), 2 h prior (b-2), simultaneously (b), and 2 h after Adriamycin (b+2). Genomic DNA was extracted from the cells, and incorporation of radiolabeled drug was determined by scintillation counting as described in “Materials and Methods” to determine the level of [14C] adducts per 10 kb. Bars, SE.

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cell viability was similar to that displayed by Adriamycin alone.

It should be noted that because the drug reaction conditions used for adduct formation and cytotoxicity experiments were quite different, adduct formation cannot be directly compared with relative survival in Table 4. For instance, the AN-9:semicarbazide ratios differ, drug treatment times were markedly different, and in cell survival experiments, the Adriamycin concentrations were varied at a fixed concentration of AN-9 and semicarbazide.

### DISCUSSION

The objective of this study was to quantitate the level of DNA adducts induced by Adriamycin in the presence of AN-9 and to establish whether increased adduct formation was a critical component of the Adriamycin/AN-9 synergy. The rationale for measuring adducts as an indicator of increased cytotoxicity comes from the studies of Skladanowski and Konopa (8), who showed a strong correlation between the cytotoxicity of a series of anthracycline derivatives and their ability to cross-link DNA. From the time course data with varying times of administration of Adriamycin and AN-9 (Fig. 3–5), in comparison with the IC$_{50}$ data (Table 1), it is clear that drug treatments that are most synergistic parallel the conditions where the highest levels of adducts are observed. This is evidenced by good synergy when Adriamycin and AN-9 are administered simultaneously, where there are 15-fold more adducts than with Adriamycin alone and 20-fold more adducts when AN-9 treatment is 2 h after Adriamycin. The critical role of formaldehyde was also confirmed by reversal of formaldehyde-mediated effects by semicarbazide, which reduced adduct formation and also abolished the cytotoxicity resulting from the interaction of AN-9 with Adriamycin. It is therefore clear

#### Table 3 Structure of prodrugs

<table>
<thead>
<tr>
<th>Prodrug</th>
<th>Structure</th>
<th>Hydrolysis products</th>
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<tbody>
<tr>
<td>AN-9</td>
<td></td>
<td>1) butyric acid</td>
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<tr>
<td>Pivaloyloxymethyl butyrate</td>
<td><img src="image" alt="Structure" /></td>
<td>2) formaldehyde 3) pivalic acid</td>
</tr>
<tr>
<td>AN-7</td>
<td></td>
<td>1) butyric acid</td>
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<tr>
<td>Butyroyloxymethyl-diethyl phosphate</td>
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<td>2) formaldehyde 3) phosphoric acid</td>
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<td>1) butyric acid</td>
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<tr>
<td>Butyroyloxyethyl-diethyl phosphate</td>
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<td>2) acetaldehyde 3) phosphoric acid</td>
</tr>
<tr>
<td>AN-158</td>
<td></td>
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<td>1) propionic acid</td>
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<td>2) acetaldehyde 3) propionic acid</td>
</tr>
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</table>

It is therefore clear
that the formation of adducts is at least in part associated with (or responsible for) the synergy displayed by the AN-9/Adriamycin combination.

Because the $[^{14}C]$ Adriamycin chromophore is present in adducts and AN-9 alone does not induce the formation of cross-linked DNA, the adducts (detected as inter strand-cross-links) were likely to be of a similar composition to those induced by Adriamycin in cell-free systems (3–5). Furthermore, in IMR-32 cells, AN-9-induced Adriamycin-DNA adducts demonstrated 5’-GC sequence specificity, were unstable at elevated temperatures, and decayed slowly at 37°C. These characteristics are the same as those observed with formaldehyde-mediated adducts in cell-free systems (3–5, 34).

It should be noted that the Adriamycin concentrations used in our cell culture assays exceeded intracellular concentrations that are routinely achieved in the clinic. It is unknown whether sufficient levels of adducts (to induce a cytotoxic response) can occur when Adriamycin is used as a single agent. However, because the use of formaldehyde-releasing prodrugs significantly elevates adduct levels, it is likely that the clinical use of these prodrugs would facilitate high levels of adducts at clinically relevant Adriamycin concentrations. Because of the instability of adducts, the only sensitive means of detection at present is the $[^{14}C]$ assay (9).

Previously, it was shown that a combination of AN-9 and daunorubicin (but not BA and daunorubicin) exhibited synergy against mouse Mm-A cells. When mice were inoculated with these cells, the combined treatment was responsible for a 16-day increase in the median survival time of animals (22). However, this observation could not account for the synergy that occurred with AN-9 but was not observed with BA. It was therefore difficult to attribute the synergistic effect to the inhibition of histone deacetylase by BA released from AN-9, leading to transient hyperacetylation of histones and a corresponding “open” configuration of chromatin, thereby potentially modulating the accessibility of drugs to DNA (11). A recent study has attributed the potentiation of Adriamycin by AN-9 to suppression of microsomal glycosidic activity, leading to inhibition of metabolic degradation of Adriamycin (35). However, this may also be because of fixation of Adriamycin to DNA by formaldehyde, thus protecting the drug from degradation. The finding that the synergistic effect was prominent for anthracyclines with a daunosamine moiety (35) is consistent with the present results because formaldehyde-mediated adduct formation has an absolute requirement for this structural element (36). From the present study, it is apparent that a major part of the synergistic effect is attributable to a dramatic increase in the level of anthracycline-DNA adducts. Ultimate confirmation of this in mouse models is still required.

The release of BA by AN-9 is significant because the expression of BA causes accumulation of multiacetylated forms of histones H3 and H4, leading to an alteration of chromatin structure (11). This altered chromatin structure is more sensitive to DNase I and is a favorable configuration for transcription, and as a consequence gene regulation is changed at this level. This is accompanied by an increased accessibility to DNA by agents such as acridine orange, actinomycin D, and cisplatin (37, 38). Conversely, acetylation of histones can also lead to a greater exposure of damaged DNA to repair enzymes (39). AN-9 has been shown to induce histone acetylation in HL-60 cells, and this effect is transient because the basal level of acetylation is reestablished 6 h after the exposure to AN-9 (23). A similar scenario in the present experiments would mean that inhibition of histone deacetylation must occur within the time frame of the drug treatments. However, it is also not known at this stage what effect Adriamycin has on the ability of AN-9 to release BA or on the inhibition of histone deacetylase itself. Early studies suggested that sodium butyrate and Adriamycin were synergistic in mouse neuroblastoma cells (40), although experiments by Kasukabe et al. (22) showed that the interaction is additive. Determining the nature of the interaction of Adriamycin and sodium butyrate (if any) is the subject of ongoing work.

There is now a wide range of evidence from the present results to show that formaldehyde (and not BA) plays a major role in potentiating Adriamycin-DNA adduct formation in cells.

(a) Prodrugs that release acetaldehyde (rather than formaldehyde) did not enhance adduct formation (Fig. 6).

(b) Sequestration of formaldehyde by semicarbazide diminished adduct formation and reversed AN-9 induced cytotoxicity (Table 4).

(c) Formaldehyde-releasing prodrugs had no effect on histone acetylation and Adriamycin, a drug that functions as a preactivated form of Adriamycin.

(d) Sodium butyrate did not lead to any increases in the level of $[^{14}C]$ Adriamycin adducts, regardless of the time of addition of this drug (Fig. 5).

(e) Similar levels of adducts form in both mitochondrial and nuclear genomes, indicating that the status of histone acetylation in nuclear chromatin does not play a major role in adduct formation.

The mechanism of Adriamycin adduct formation is inherently

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**Table 4 Interaction of semicarbazide with Adriamycin/AN-9**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Adducts** (lesions/10 kb)</th>
<th>Relative survival**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adriamycin</td>
<td>0.7 ± 0.01</td>
<td>1.00 ± 0.12</td>
</tr>
<tr>
<td>Adriamycin + AN-9</td>
<td>11.0 ± 0.5</td>
<td>0.35 ± 0.06</td>
</tr>
<tr>
<td>Adriamycin + AN-9 + semicarbazide (250 $\mu$m)</td>
<td>6.2 ± 1.1 (6.7 ± 0.6)**</td>
<td>0.37 ± 0.11</td>
</tr>
<tr>
<td>Adriamycin + AN-9 + semicarbazide (500 $\mu$m)</td>
<td>4.9 ± 1.0 (4.6 ± 0.2)**</td>
<td>0.71 ± 0.08</td>
</tr>
<tr>
<td>Adriamycin + AN-9 + semicarbazide (1 mm)</td>
<td>3.9 ± 0.3 (3.2 ± 0.2)**</td>
<td>0.88 ± 0.22</td>
</tr>
<tr>
<td>Adriamycin + semicarbazide (1 mm)</td>
<td>0.7 ± 0.01</td>
<td>1.2 ± 0.16</td>
</tr>
</tbody>
</table>

**Adducts were determined by incubating IMR-32 cells in the presence of 2 $\mu$m $[^{14}C]$ Adriamycin for 2 h, followed by an additional 2 h in the presence or absence of 100 $\mu$m AN-9.

**Cells were seeded at a density of 1 x 10⁶ per well and exposed to increasing concentrations of Adriamycin in the presence or absence of 50 $\mu$m AN-9 and/or semicarbazide (0.25, 0.5 or 1.0 mM as indicated) for 72 h. IC₅₀ values for various combinations are shown as relative survival compared to Adriamycin alone.

**Values in parentheses indicate that the semicarbazide addition was at the same time as AN-9 treatment (i.e., 2 h after Adriamycin), whereas the values preceding the parentheses indicate the semicarbazide addition at the same time as Adriamycin (i.e., 2 h before AN-9).
dependent on formaldehyde as a critical step (5). The most likely scenario is that Adriamycin accumulation in the nucleus and mitochondria and subsequent DNA intercalation represent the rate-limiting steps in this process. A 2-h incubation of cells with Adriamycin overcomes this limitation. Subsequent addition of AN-9 leads to rapid esterase-dependent release of formaldehyde that can complex to Adriamycin closely associated (reversibly bound) with DNA. AN-9 is esterase-dependent release of formaldehyde that can complex to Adriamycin in nuclear and mitochondrial DNA of MCF-7 cells. Nuclear Acids Res., 28: 1019–1025, 2000.


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Molecular Basis for the Synergistic Interaction of Adriamycin with the Formaldehyde-releasing Prodrug Pivaloyloxymethyl Butyrate (AN-9)


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