# The Role of Intracellularly Released Formaldehyde and Butyric Acid in the Anticancer Activity of Acyloxyalkyl Esters

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Previous studies described a family of anticancer histone deacetylase inhibitor prodrugs of formula $Me(CH_2)_2COOCH(R)OR^1$, which upon intracellular hydrolysis release acids and aldehydes. This study examines the mechanisms by which the prodrugs affect tumor cells and the contribution of the released aldehyde (formaldehyde or acetaldehyde) and acids to their anticancer activity. Type I prodrugs release 2 equiv of a carboxylic acid and 1 equiv of an aldehyde, and of Type II release 2 equiv of acids and 2 equiv of an aldehyde. SAR studied inhibition of proliferation, induction of differentiation and apoptosis, histone acetylation, and gene expression. Formaldehyde, measured intracellularly, was the dominant factor affecting proliferation and cell death. Among the released acids, butyric acid elicited the greatest antiproliferative activity, but the nature of the acid had minor impact on proliferation. In HL-60 cells, formaldehyde-releasing prodrugs significantly increased apoptosis. The prodrugs affected to a similar extent the wild-type HL-60 and MES-SA cell lines and their multidrug-resistant HL-60/MX2 and MES-Dx5 subclones. In a cell-free histone deacetylase (HDAC) inhibition-assay only butyric acid inhibited HDAC activity. The butyric acid and formaldehyde induced cell differentiation and increased p53 and p21 levels, suggesting that both affect cancer cells, the acid by inhibiting HDAC and the aldehyde by an as yet unknown mechanism.

## Introduction

Acyloxyalkyl ester prodrugs of biologically active agents possessing carboxylic acid functionalites have been extensively studied, including the $\beta$-lactam antibiotics pivampicillin, bacampicillin, and talampicillin.[1] Derivatization as acyloxyalkyl esters increased their stability, lipophilicity, and oral activity.[2] In previous studies we have described the synthesis and biological activities of butyric acid prodrugs having the general formula $Me(CH_2)_2COOCH(R)OR^1$, where R = H, Me, Pr, *tert*-Bu; $R^1$ = OC-alkyl, OC-Ar, and $P(O)(OEt)_2$.[3−6] These acyloxyalkyl prodrugs serve as molecular carriers for the efficient transport of butyric acid to the cells, leading to a significant increase in its potency.[7] The compounds studied, with the exception of **AN-186**, have been described earlier (Table 1).[8] Their expected intracellular hydrolytic products are two carboxylic acids and an aldehyde from prodrugs of Type I, and two aldehydes, three carboxylic acids, one of which is dicarboxylic, for prodrugs of Type II (Scheme 1).

Butyric acid is a pleotropic anticancer agent that has a specific effect on the inhibition of nuclear histone deacetylase enzyme(s), leading to an increase in the acetylation level of H3 and H4 histones.[9,10] However, in vivo it displays low potency due to rapid metabolism.[11] To overcome this problem, we synthesized and characterized a variety of butyric acid prodrugs, where the one most extensively studied is pivaloyloxymethyl butyrate, **AN-9**. The design of **AN-9**, as well as other early analogues, resulted in highly lipophilic derivatives with ability to cross cell membranes greater than butyric acid.[12] **AN-9** modulates gene expression, induces histone hyperacetylation, differentiation, and apoptosis of cancer cells, and affects cancer cells about 100 times faster and at a concentration of about 10-fold lower than butyric acid.[3−7,13] When evaluated in 76 primary solid human tumors (including colorectal, breast, lung, ovarian, renal, and bladder), it was found to be more active than butyric acid in inhibiting solid-tumor colony-forming units.[14] Twenty-one primary samples of acute leukemia, including chemoresistant cells, were also sensitive to the antiproliferative effects of **AN-9** with an average $IC_{50}$ of 45.8 ± 4.1 $\mu$M.[15] While **AN-9** displayed synergy with DNA interacting agents, butyric acid itself did not.[16,17] Its synergistic effects with DNA-disrupting agents have been observed in murine monocytic leukemia cells, and the combination of **AN-9** with daunorubicin led to a significant increase in survival of mice inoculated with acute monocytic leukemia cells.[16] **AN-9** was shown to dramatically increase the level of doxorubicin-DNA adducts and to affect doxorubicin inhibition of proliferation in neuroblastoma and breast cell lines, in a synergistic manner. The synergy can be attributed largely to the formaldehyde released by **AN-9** that activates doxorubicin, resulting in the potentiation of doxorubicin−DNA adduct formation.[17] The in vivo anticancer activity of **AN-9** has been demonstrated in syngeneic murine models.[3,4,16] In a Phase I clinical study, **AN-9** displayed low toxicity.[18] The fact that **AN-9**

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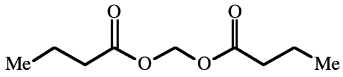
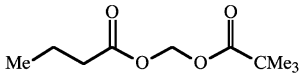
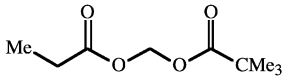
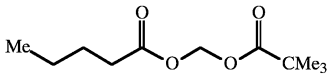
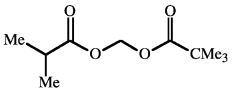
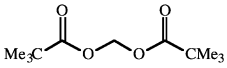
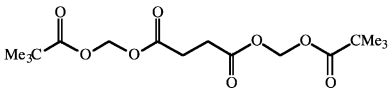
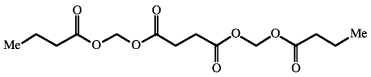
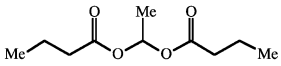
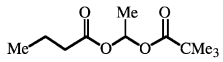
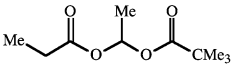
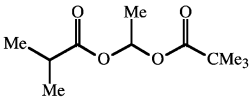
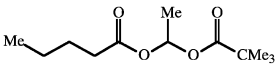
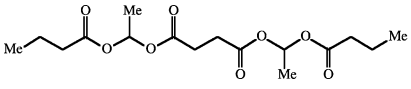
**Table 1.** Prodrugs That Release Formaldehyde and Acetaldehyde

| Prodrugs | *Structures* | *Hydrolysis products (No. eq)* | *Ref.* |
|---|---|---|---|
| **AN-1** |  | 2 Butyric Acid<br>1 Formaldehyde | 47 |
| **AN-9** |  | 1 Butyric Acid<br>1 Formaldehyde<br>1 Pivalic Acid | 47 |
| **AN-36** |  | 1 Propionic Acid<br>1 Formaldehyde<br>1 Pivalic Acid | 4 |
| **AN-38** |  | 1 Valeric Acid<br>1 Formaldehyde<br>1 Pivalic Acid | 4 |
| **AN-37** |  | 1 Isobutyric Acid<br>1 Formaldehyde<br>1 Pivalic Acid | 4 |
| **AN-212** |  | 2 Pivalic Acid<br>1 Formaldehyde | 49 |
| **AN-192** |  | 1 Succinic Acid<br>2 Pivalic Acid<br>2 Formaldehyde | 48 |
| **AN-193** |  | 2 Butyric Acid<br>1 Succinic Acid<br>2 Formaldehyde | 48 |
| **AN-11** |  | 2 Butyric Acid<br>1 Acetaldehyde | 4 |
| **AN-158** |  | 1 Butyric Acid<br>1 Acetaldehyde<br>1 Pivalic Acid | 14 |
| **AN-184** |  | 1 Propionic Acid<br>1 Acetaldehyde<br>1 Pivalic Acid | 48 |
| **AN-185** |  | 1 Isobutyric Acid<br>1 Acetaldehyde<br>1 Pivalic Acid | 48 |
| **AN-186** |  | 1 Valeric Acid<br>1 Acetaldehyde<br>1 Pivalic Acid | |
| **AN-191** |  | 2 Butyric Acid<br>1 Succinic Acid<br>2 Acetaldehyde | 48 |

is virtually devoid of water solubility, requiring non-aqueous media for clinical formulation, led to the design of a second generation of butyric acid prodrugs which display solubility ranging from highly lipophilic to hydrophilic.[5] The compounds possessing moderate water solubility in the range of 3−22 mg/mL were found most advantageous for drug formulation.

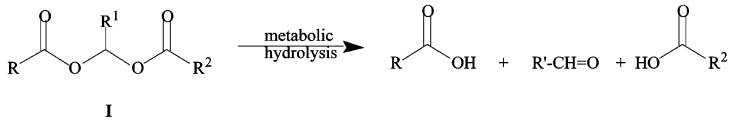
In this work, we sought to examine (a) the cellular mode of action of the histone deacetylase inhibitor (HDACI) prodrugs as single agents; (b) the contribution of the aldehyde (formaldehyde or acetaldehyde) and acids (released upon cellular hydrolysis) to induction of differentiation, apoptosis, and histone acetylation; (c) the mode by which they affect drug-resistant cells; (d) the levels of intracellular formaldehyde released in prodrug-treated cells; and (e) the effect of the released formaldehyde on cell-free doxorubicin−DNA adduct formation.

## Results and Discussion

HDAC inhibitors are promising chemotherapeutic agents for the treatment of cancer.[19−23] In this work, new prodrugs of short chain fatty acids were synthesized and their anticancer activity was compared to our

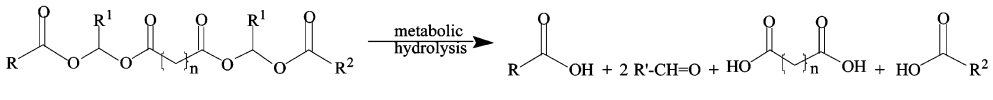
**Scheme 1**

**Type I Prodrugs**




I

**Type II Prodrugs**




II

R' = H; Me
n = 0-3

**Table 2.** Effect of Formaldehyde-Releasing Acyloxymethyl Esters on Cell Proliferation[a]

| cell line | MES-SA | MES-DX5 | PC-3 | HT-29 | HL-60 | HL-60 MX2 |
|---|---|---|---|---|---|---|
| AN-1 | 30 ± 2.3 | 32 ± 3.5 | 54 ± 4 | 39 ± 3.3 | 32 ± 0.8 | 44.3 ± 11.2 |
| AN-9 | 40 ± 0.9 | 46 ± 1.9 | 56 ± 2 | 52 ± 2.7 | 36 ± 0.5 | 49 ± 9.5 |
| AN-36 | 42 ± 0.65 | 37 ± 0.4 | 55 ± 4 | 46 ± 1.9 | 40 ± 2.6 | 48 ± 10.5 |
| AN-38 | 55 ± 5.5 | 54 ± 0 | 55 ± 3 | 56 ± 5 | 36 ± 2.3 | 47.7 ± 12.7 |
| AN-37 | 49 ± 1 | 47 ± 4.6 | 55 ± 4 | 64 ± 3 | 40 ± 1.5 | 54.7 ± 11.6 |
| AN-212 | 81 ± 5.3 | 133 ± 12 | >400 | >400 | 211 ± 44.6 | >400 |
| AN-193 | 19 ± 0.2 | 17 ± 1.5 | 23 ± 5 | 26 ± 4 | 18 ± 0.8 | 23.7 ± 5.0 |
| AN-192 | 19 ± 1.6 | 18 ± 3 | ND* | ND | 19 ± 0.8 | 25.7 ± 6.0 |

[a] Cells ($(2.5-5) \times 10^3$) were seeded in 96-well plates and treated with escalating concentrations of the prodrugs. After 4 days incubation, the cells were harvested as described in Materials and Methods. All $IC_{50}$ values are in $\mu$M. [b] ND, not determined.

**Table 3.** Effect of Acetaldehyde-Releasing Acyloxymethyl Esters on Cell Proliferation

| cell line | MES-SA | MES-DX5 | PC-3 | HT-29 | HL-60 | HL-60 MX2 |
|---|---|---|---|---|---|---|
| AN-11 | 456 ± 38 | 502 ± 14 | 475 ± 13 | 332 ± 8 | 550 ± 13.3 | 625 ± 5 |
| AN-158 | 525 ± 33 | 530 ± 27 | 462 ± 24 | 480 ± 36 | 550 ± 33.3 | 610 ± 9.4 |
| AN-185 | 542 ± 43 | 442 ± 47 | 363 ± 17 | 335 ± 49 | 720 ± 40 | 630 ± 19 |
| AN-186 | 204 ± 29 | 193 ± 24 | 268 ± 28 | 319 ± 8 | 495 ± 17 | 425 ± 51 |
| AN-184 | 956 ± 164 | 1246 ± 342 | 722 ± 107 | 530 ± 9 | >1000 | >1000 |
| AN-191 | 267 ± 19 | 349 ± 37 | 424 ± 24 | 207 ± 15 | 335 ± 50 | 480 ± 104 |

earlier prodrugs of butyric acid, **AN-9** and **AN-1**. The study describes the effect of the prodrugs on proliferation, differentiation, cell death, histone acetylation, gene expression, and direct inhibition of HDAC.

**Effect of the Prodrugs on Proliferation.** To assess the contribution of the released aldehyde and acids to the antiproliferative activity, the potency of prodrugs as proliferation inhibitors was measured in seven cancer cell lines using the Hoechst assay for solid tumors and the Alamar blue method for leukemic cell lines. The $IC_{50}$ values derived from detailed titrations of the prodrugs are shown in Tables 2 and 3.

**The Contribution of the Aldehyde (formaldehyde or acetaldehyde).** We have reported that the formaldehyde-releasing prodrugs (**AN-7**, **AN-40**, and **AN-46**) compared to their acetaldehyde releasing counterparts (**AN-88**, **AN-90**, and **AN-85**) showed significantly greater antiproliferative activity in prostate carcinoma cell lines.[5] This difference could be attributed to the activity of formaldehyde as a highly reactive compound that increases overall toxicity. However, in mice, both types of prodrugs displayed similar ip acute toxicity ($LD_{50}$ = 400−600 mg/kg). In an effort to better characterize the impact of the released aldehyde on cancer cell proliferation, we performed detailed SAR studies with formaldehyde-releasing prodrugs (Table 2) and their acetaldehyde-releasing analogues (Table 3). The $IC_{50}$ values obtained in more than three independent experiments were compared by *t*-tests. Formaldehyde releasing prodrugs had ~10-fold lower $IC_{50}$ values compared to the acetaldehyde-releasing analogues and were therefore significantly more effective ($p < 0.0001$). Moreover, Type II prodrugs that release 2 equiv of formaldehyde (i.e. **AN-193**) were notably more active ($p < 0.01$) than Type I prodrugs (i.e. **AN-1**) that release only one. Collectively, these observations suggest that the released formaldehyde has a pivotal role in inhibiting cancer cell proliferation.

While release of two formaldehyde units impacted substantially on the proliferation, the release of two acetaldehyde moieties had little or no effect. In general, comparison between Type I and Type II prodrugs showed no significant difference in their activities. The only exceptions were **AN-11** and **AN-191** that in HT-29 and MES-Dx5 cells differed significantly in their activity ($p < 0.02$). The two compounds release 2 equiv of butyric acid; however, while **AN-11** releases only one, **AN-191** releases 2 equiv of acetaldehyde as well as 1 equiv of succinic acid. It is unclear whether the additional acetaldehyde or the succinic acid or both contributed to the increased activity. However, from comparison between the high-potency (formaldehyde-releasing, Type II) **AN-193** and the low-potency (acetaldehyde-releasing, Type II) **AN-191**, it is apparent that

the contribution of the released succinic acid to inhibition of proliferation is minor. In the other cell lines a similar trend was observed; however, the differences were not significant.

**The Contribution of the Low Molecular Weight Acids**. The anticancer activity of butyric acid in HL-60 and HT-29 is well documented.[24−27] The contribution of the acid to the antiproliferative effect of the prodrugs releasing 1 equiv of formaldehyde was apparent in HT-29, HL-60, MES-SA, and MES-Dx5 cell lines (Table 2). While **AN-1** releases 2 equiv of butyric acid and **AN-9** 1 equiv, the other prodrugs release different acids. In these cell lines, **AN-1** was more potent than **AN-9**, **AN-36**, **AN-37**, **AN-38**, and **AN-212** ($p < 0.02$). In contrast, in PC-3 and HL-60/MX2 cell lines, the prodrugs affected proliferation in an indistinguishable manner. Thus, prodrugs that release 2 equiv butyric acid of (i.e. **AN-1**) had similar activity to those releasing 1 equiv (i.e. **AN-9**) or none ($p > 0.07$).

**AN-212** was the least effective of the formaldehyde-releasing prodrugs in all cell lines tested. This may be rationalized by the presence of two pivalate entities in the molecule that impart significant steric hindrance, expected to slow the rate of hydrolysis of this prodrug, similar to the reported effect of the ester moieties on the hydrolysis rate (acetate > propionate > butyrate > succinate > pivalate) of propranolol prodrugs.[28] The formaldehyde-releasing prodrugs **AN-193** and **AN-192** of Type II exhibited similar potency in inhibiting cell proliferation (Table 3). **AN-193**, in addition to two formaldehyde equivalents, also releases two butyric acid equivalents, while **AN-192** releases two pivalic acid equivalents. Comparison between the two compounds, suggests that formaldehyde and not butyric acid has a dominant effect on proliferation. Comparison between **AN-193** and **AN-191** suggests that the succinic acid does not affect proliferation yet it may affect the hydrolysis rate of the prodrug. **AN-192** and the low potency **AN-212** both possess two pivalate ester units. However, in **AN-192** the pivalate units are bound to the succinate bridge; it is conceivable that the hydrolysis takes place initially and rapidly at the succinate ester sites. Thus, it is expected that the inclusion of the succinic acid bridge (instead of the closely spaced pivalate moieties found in **AN-212**) improved the activity. This is consistent with the comparable potency of **AN-192** and **AN-193**. Unlike acetic, propionic, butyric, succinic, valproic, and isobutyric acids that are rapidly metabolized, catabolism of the liberated pivalic acid is limited. Pivalate can be activated to a coenzyme A thioester that conjugates with carnitine to generate pivaloylcarnitine, leading to a decrease in carnitine levels. Since the carnitine cellular pool is limited and it plays an important role in the bioenergy and detoxification of the cells, the released pivalate may lead to toxicity, especially when administered together with xenobiotics.[29]

No clear pattern of activity was observed among the different Type I acetaldehyde-releasing prodrugs, and in general their potency was low (Table 3). **AN-11**, **AN-158**, and **AN-185** (that respectively release 2 equiv of butyric acid; one equiv of pivalic acid and one equiv of butyric acid; one equiv of pivalic acid and one equiv of isobutyric acid) had similar potency. Unexpectedly, **AN-184** (that releases one pivalic acid and one propionic acid equivalent) was significantly less effective in inhibiting proliferation, while **AN-186** (that releases one pivalic and one valeric acid equivalent) was the most potent prodrug. It is unclear whether these differences are due to the released products, the rates of cellular penetration, or hydrolysis.

**Effect of the Prodrugs on Proliferation of Drug-Resistant Cell Lines.** The two types of isogenetic pairs of sensitive and multi-drug-resistant cell lines examined were HL-60/MX2 cells, that display multidrug resistance associated with an altered topoisomerase II activity,[30] and MES-SA/Dx5 cells, that express high levels of mdr-1 mRNA and P-glycoprotein.[31] When the effect of the prodrugs (Tables 2 and 3) on the proliferation of drug-sensitive HL-60 and MES-SA cells and the above resistant subclones were compared, they displayed comparable activity. Therefore, it is suggested that the hydrolysis products of the prodrugs are not substrates for P-glycoprotein and they do not require fully functional topoisomerase II for their inhibitory effect on proliferation. These results suggest that the antiproliferative activity of most prodrugs is unaffected by the resistance mechanisms involving topoisomerase II or overexpression of P-glycoprotein. The exceptions are the pivalic acid-containing prodrugs **AN-212** and to lesser extent **AN-9** which were significantly less active against MES-Dx5 and HL-60/MX2 cells ($p < 0.05$). Whether in these cases the intact prodrugs or their hydrolytic metabolites are affected by the resistance mechanism remains to be determined.

**Induction of Cell Death.** Two methods for assessing apoptosis were used to examine the effect of the prodrugs on cell mortality: Annexin-V binding to phosphatidylserine (PS), to detect early stages of apoptosis, and increase in caspase-3 activity to detect downstream events of apoptosis. Increase in PS in the outer membrane as an early apoptotic event was visualized by annexin-V FITC binding and was evident by the appearance of green-stained cells. Advanced apoptotic or necrotic cells were identified by penetration of propidium iodide (PI) into cells and fragmented DNA. Treatment of the cells with **AN-1** induced a small increase in cell mortality at 50 $\mu$M and a significant rise in apoptosis at 100 $\mu$M, where 74% early apoptotic and 18% advanced apoptotic/necrotic cells were observed (Figure 1A). Treatment with **AN-193** caused a considerable increase in cell mortality at 50 $\mu$M, resulting in apoptotic and advanced apoptotic/necrotic cells (38% and 28%, respectively), while treatment with 100 $\mu$M **AN-193** resulted in 57% apoptotic and 43% advanced apoptotic/necrotic cells (Figure 1B). **AN-191** caused low cell mortality at 100 $\mu$M, and at 200 $\mu$M it increased somewhat to produce 30% apoptotic and 8% advanced apoptotic/necrotic cells (Figure 1C). Since increase in caspase 3 is a late event in apoptosis and is downstream to the increase in PS in the outer plasma membrane, the annexin binding is a more sensitive method for detection of early apoptosis events. Indeed, using this method considerable apoptosis was detected at 50 $\mu$M **AN-1** or **AN-193**. In **AN-1**-treated cells, 100 $\mu$M induced a significantly higher incidence of early apoptosis compared to advanced apoptosis/necrosis events ($p \sim 0.003$). However, in cells treated with 100 $\mu$M **AN-193**, the percentage of cells undergoing advanced apoptosis/

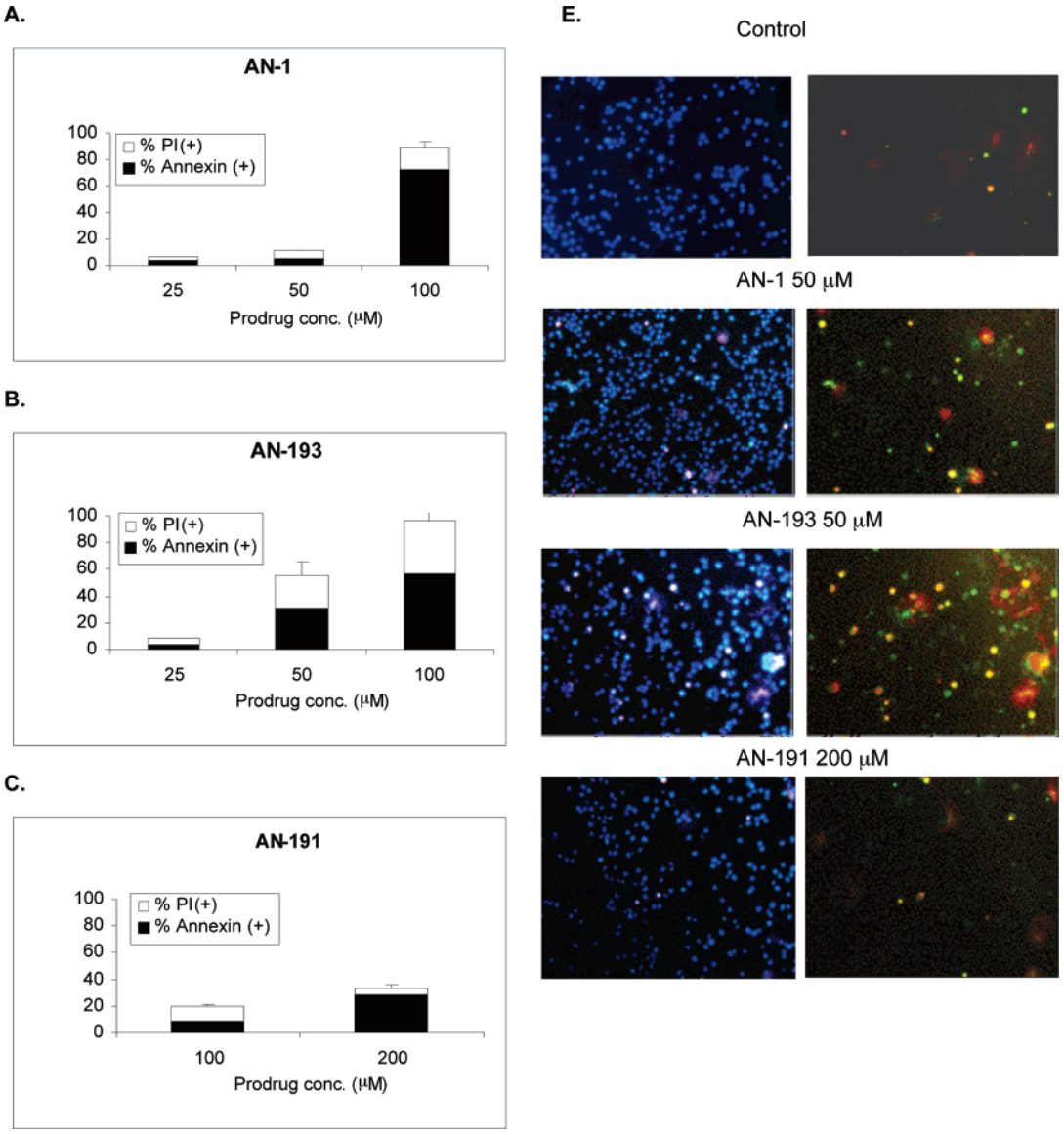



**Figure 1.** HL-60 cells were treated for 4 h with **AN-1**, **AN-193**, and **AN-191**, as specified, and stained with propidium iodide (PI), bis-benzimide (Hoechst), and AnnexinV−FITC. Cells were scored as described. The percentage of spontaneous cell death in the control cultures was <5% and was subtracted from the data.

necrosis did not differ significantly from those undergoing early apoptosis ($p \sim 0.3$). Treatment of cells with 200 $\mu$M **AN-191** resulted in 37% cell death, where most of the cells were in early apoptosis.

For detection of caspase 3 activity, HL-60 cells were treated for 4 h with **AN-1**, **AN-193**, **AN-192**, and **AN-191**, representing compounds that release different amounts of butyric acid and formaldehyde (Figure 2). Increase in caspase-3 activity was significant only at 100 $\mu$M concentrations, where **AN-193** was the most active compound. **AN-1** and **AN-192** caused a comparable induction of caspase-3 activity, whereas **AN-191** did not induce the activity even at higher concentrations (200 and 400 $\mu$M). Overall, the order of potency in induction of apoptosis was **AN-193** > **AN-192**, **AN-1** >>> **AN-191**. Butyric acid alone, at the tested concentrations, was insufficient to promote apoptosis (negligible activity of **AN-191**); however, it potentiated the apoptosis induction when formaldehyde is released as well (i.e. **AN-193** > **AN-192**). Therefore, it can be concluded that the released formaldehyde was the major factor of cell death (i.e. **AN-193** >>> **AN-191**).

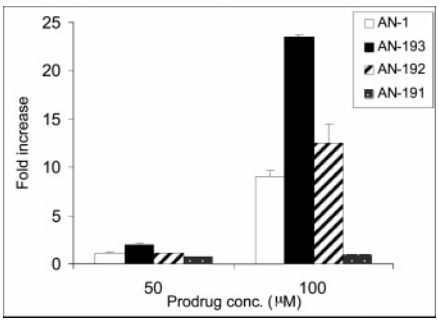



**Figure 2.** HL-60 cells treated for 6 h with **AN-1**, **AN-193**, **AN-192**, and **AN-191**, as specified, were harvested and the activity of caspase 3 was measured as OD/min/$\mu$g of protein. The "fold increase" was normalized relative to untreated cells.

**Differentiation Induction.** Differentiation induction was evaluated in two cell lines: human myeloid leukemia HL-60 cells, expressed by increase in super-

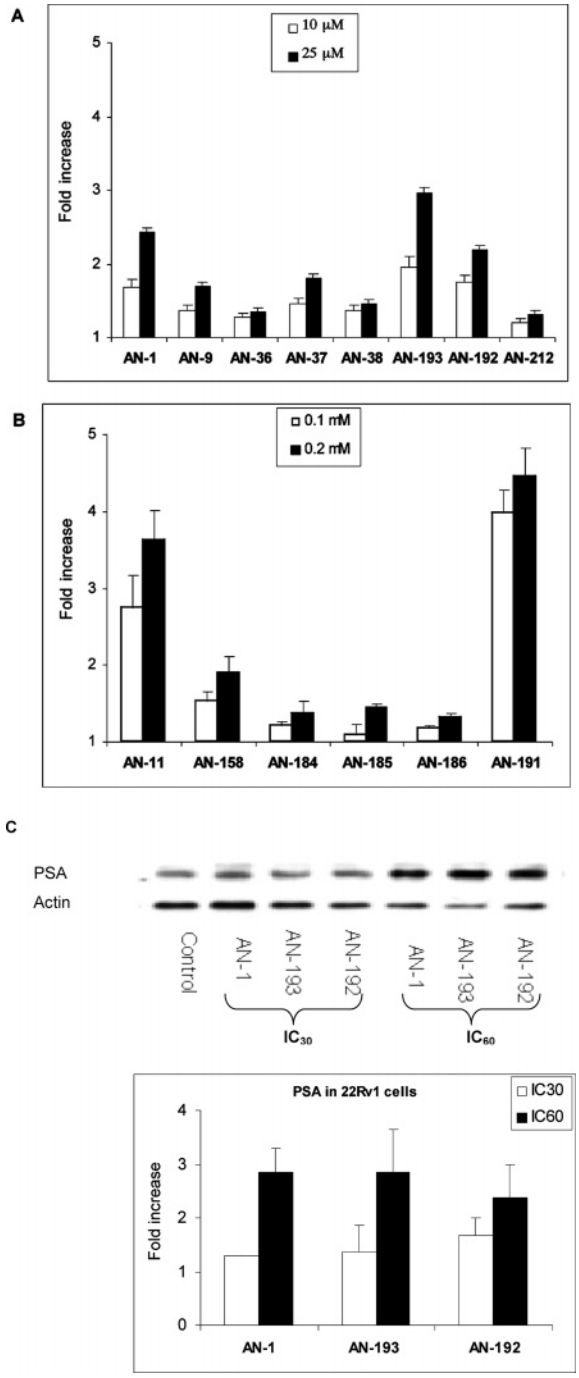



**Figure 3.** HL-60 cells were treated with the indicated prodrugs for 96 h, and differentiation was measured by NBT reduction as OD (450)/number of cells. The "fold increase" was normalized relative to untreated cells. Formaldehyde-releasing compounds (A) and acetaldehyde releasing compounds (B). 22Rv1 cells were treated with **AN-1**, **AN-193**, and **AN-192** at their $IC_{30}$ and $IC_{60}$ concentrations for 96 h, after which, cell differentiation was determined from the increase of PSA protein using Western analysis, as specified. The "fold increase" was normalized relative to actin levels and to control (C).

oxide production and detected by NBT assay (Figure 3A,B), and androgen-dependent prostatic carcinoma 22Rv1 cells, detected by an increase in PSA expression (Figure 3C). To allow a sufficient number of cells to enter differentiation rather than apoptosis, differentiation induction was examined at concentrations ranging between $IC_{25}-IC_{75}$. Formaldehyde-releasing prodrugs (**AN-1**, **AN-9**, **AN-36**, **AN-37**, **AN-38**, **AN-192**, **AN-193**, and **AN-212**) induced differentiation at 10-fold lower concentrations than the corresponding acetaldehyde-releasing ones (**AN-11**, **AN-158**, **AN-184**, **AN-185**, **AN-186**, and **AN-191**) (Figure 3). These two types of prodrugs could not be tested at the same concentrations for two reasons: the former induce differentiation at doses below 50 mM and above this dose they induce cell death, while the latter are inactive at these low concentrations. Induction of differentiation by **AN-193** showed that it was the most potent agent, significantly more so than **AN-1** ($p \sim 0.03$). Since both compounds release 2 equiv of butyric acid, but **AN-1** releases 1 equiv of formaldehyde and **AN-193** releases 2 equiv, it implies that the contribution of formaldehyde to the differentiation is additive and independent to that of the butyric acid. The fact that **AN-192**, that releases 2 equiv of formaldehyde and no butyric acid, induced differentiation further supports the notion that the intracellularly released formaldehyde is an inducer of differentiation. Therefore, it was important to demonstrate that reducing the concentration of cellular formaldehyde would attenuate differentiation. This was achieved by the use of semicarbazide, which sequesters formaldehyde.[32] While treatment with **AN-193** (25 $\mu$M) alone increased differentiation, cotreatment with semicarbazide (1 mM) reduced differentiation (>2-fold lower). Treatment with semicarbazide, in the absence of the prodrug, did not alter differentiation (data not shown).

The observation that the intracellularly released formaldehyde specifically induces differentiation in HL-60 cells was unexpected. To confirm this observation, another in vitro differentiation model was examined. In 22Rv1 prostate carcinoma cells PSA (prostate specific antigen) levels are regulated by the binding of androgen to DNA-responsive elements; however, it can also be induced in an androgen-independent mechanism by differentiation inducers such as butyric and retinoic acids.[33] To confirm that compounds that release formaldehyde can induce differentiation, the effect of **AN-1**, **AN-193**, and **AN-192** was evaluated by examining their ability to induce PSA expression. The selected concentrations for determining differentiation induction were based on the proliferation inhibition activity of the compounds, which displayed $IC_{50}$ values of $52.3 \pm 2.5$ (**AN-1**), $29.1 \pm 0.05$ (**AN-193**), and $30.2 \pm 3.2$ (**AN-192**). When the cells were treated for 96 h at $IC_{30}$ doses, the PSA level did not change significantly, whereas at $IC_{60}$ doses, all three drugs increased its expression to a similar >2-fold level (Figure 3C). The results demonstrate that the differentiation induced by **AN-192** (that releases formaldehyde but no butyric acid) can be attributed to the formaldehyde. In 22Rv1 cells, **AN-1** and **AN-193** exhibited similar activity, while in HL-60 cells **AN-193** was significantly more active than **AN-1**. The activity difference in the two cell lines could be attributed to their differential sensitivity or to reduction of actin expression by **AN-193**.

Collectively, the above observations suggest that intracellular formaldehyde triggers a specific signal that can direct the cells to undergo differentiation and plays a pivotal role in inducing cancer cell death. Although it is known that formaldehyde participates in important cellular functions such as DNA and protein methyla-

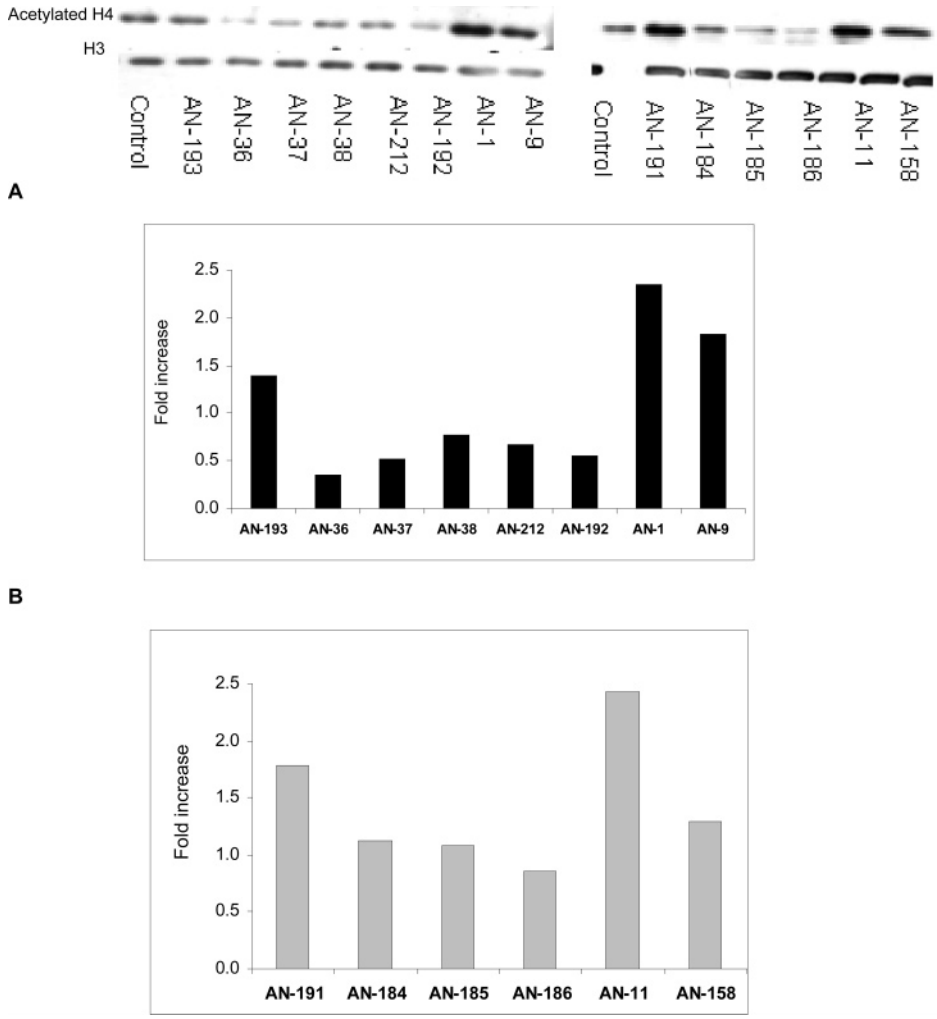



**Figure 4.** Representative experiment where HL-60 cells were treated with 100 $\mu$M of the indicated prodrugs for 4 h. Histones were extracted as described, and the level of the proteins (acetylated H4 and total H3) was determined with specific antibodies using Western blot analysis. The "fold increase" represents the relative level of acetylated H4/total H3 and relative to control. Formaldehyde-releasing compounds (A) and acetaldehyde-releasing compounds (B).

tions, the underlying molecular events of these pathways are undefined and will be the subject of future investigations.[35,43]

The contribution of the released carboxylic acid to the induction of differentiation was measured by NBT reduction activity in HL-60 cells and by comparing prodrugs that release different acids (Table 1). Among the prodrugs that release 1 equiv of formaldehyde, **AN-1** at 10 and 25 $\mu$M induced higher differentiation than **AN-9**, **AN-36**, **AN-38**, and **AN-212** ($p < 0.05$), but was not significantly better than **AN-37** (at 10 $\mu$M). When the prodrugs that release two formaldehyde equivalents, **AN-193** vs **AN-192**, were compared, the former, which releases two butyric acid equivalents, was more potent than the latter, which releases two pivalic acid equivalents. Among the acetaldehyde releasing prodrugs, **AN-11** and **AN-191**, that release 2 equiv of butyric acid, displayed similar differentiation induction and were significantly more potent than **AN-158** that releases only 1 equiv or **AN-185**, **AN-184**, **AN-186** that release other acids ($p < 0.05$). Therefore, among acetaldehyde-releasing prodrugs, only those that release 2 equiv of butyric acid induced significant differentiation (Figure 3B). These results further support the notion that the nature of the released acid plays an important role in differentiation and butyric acid is the most effective one.

**Histone Acetylation.** Butyric acid was shown to inhibit HDAC activity, resulting in the increase of the steady-state level of acetylated histones.[34] The link between histone hyperacetylation and transcription regulation has been established.[37] Histones were extracted from prodrug-treated HL-60 cells, and acetylation was determined by Western analysis. Following 4 h treatment with **AN-1** and **AN-193** (5−100 $\mu$M), acetylation increased in a dose-dependent manner the peak level being observed at 100 $\mu$M (data not shown). This concentration was thus selected to compare the effect of the different prodrugs on acetylation. Since only prodrugs that release butyric acid (**AN-1**, **AN-9**, **AN-193**, **AN-11**, and **AN-191**) increased acetylation of H4, we hypothesized that the butyric acid fragment of the molecules imparts the HDAC inhibitory effect (Figure 4). Prodrugs that release formaldehyde but not butyric acid (**AN-36**, **AN-37**, **AN-38**, **AN-212**, and **AN-192**) reduced acetylation to a level lower than in untreated cells (Figure 4A). In addition, a smaller elevation of histone acetylation was seen in cells treated with **AN-193** compared to those treated with **AN-1**. Moreover, the analogous prodrugs that release acetaldehyde (**AN-**

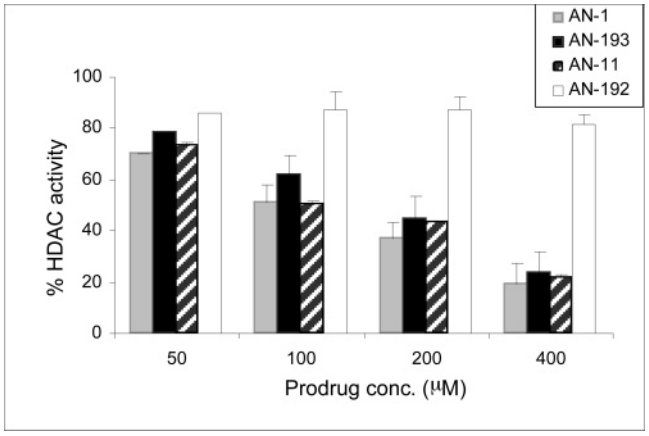



**Figure 5.** Inhibition of HDAC activity in nuclear extracts (0.5 $\mu$L/well) of HeLa cells, following incubation with different concentrations of **AN-1**, **AN-193**, **AN-11**, and **AN-192**, in the presence of an esterase for 30 min. The Fluor de Lys substrate (50 $\mu$M) was then added to the reaction and further incubated for 8 min (30 °C). The reaction was stopped by adding Fluor de Lys developer, and the fluorescence was measured (excitation at 390 nm and emission at 460 nm). Percent inhibition was calculated from the fluorescence ratio of treated/untreated samples.

**158**, **AN-184**, **AN-185**, and **AN-186**) did not alter the basal level of histone acetylation. Thus, the attenuation of histone acetylation may be attributed to the release of formaldehyde (Figure 4).

**Inhibition of HDAC Activity.** The effect of the prodrugs on HDAC activity was tested in a cell-free system to determine whether the increase in histone acetylation resulted from direct inhibition of HDAC. Prodrugs were preincubated with HDAC-enriched nuclear extracts for 30 min, and then the HDAC substrate was added (Biomol kit). The activity was monitored by an increase of fluorescence emitted by the deacetylated substrate. When esterase was added to the preincubation reaction mixture ($9 \times 10^{-3}$ units from porcine liver), the prodrugs containing butyric acid moieties inhibited HDAC activity (Figure 5). In the absence of an external esterase, no inhibitory effect was seen. The prodrugs that release one or 2 equiv of butyric acid (**AN-1**, **AN-193**, **AN-7**, and **AN-11**) inhibited HDAC activity. However, **AN-192** (that releases two formaldehydes and not butyric acid) had no effect on the activity, demonstrating that the butyric acid and not formaldehyde caused the inhibitory effect on HDAC. It can therefore be deduced that the products of hydrolysis, and not the intact prodrugs, are responsible for the inhibitory effect.

To examine whether the inhibition of histone acetylation by formaldehyde-releasing prodrugs resulted from a direct effect of the aldehyde on the enzymes (Figure 4), HDAC activity was tested in the presence of formaldehyde (50 $\mu$M to 50 mM) and was found to be unaffected. Therefore, it is unlikely that the inhibition of histone acetylation observed in cells treated with formaldehyde-releasing prodrugs (**AN-192)** is caused by a direct effect on HDAC. The inhibition could result from the competition for the same lysine residues on the histones that undergo methylations catalyzed by methyltransferases. Indeed, methyltransferases were reported to methylate multiple sites of histone lysine residues (such as lysines 4, 9, 27, and 36 in H3), as well as in nonhistone transcription factors.[35] The possibility that the formaldehyde released facilitates protein methylation will be tested in future investigations.

**Effect of the Prodrugs on Expression of p53 and p21.** Acetylation of nuclear histones is thought to play a crucial role in gene expression since transcriptionally activated genes have been found to be associated with highly acetylated chromatin, whereas inactive genes, with hypoacetylation loci.[36] These observations provide a molecular basis for the regulation of transcription through acetylation of histones. The tumor suppressor p53 and p21 genes play a key role in controlling cell fate. Their regulation in MCF-7 cells (possessing the wild-type p53 gene) has been described.[37,38] In response to cellular stress, p53 is stabilized mainly through posttranslational modifications including acetylation.[39]

The effect of the prodrugs on the expression of these genes was examined at concentrations effective in inhibiting cell proliferation. In MCF-7 cells, using the Hoechst assay, the following $IC_{50}$ values were obtained: $80 \pm 13$, $38 \pm 4$, $36 \pm 6$, $608 \pm 71$ $\mu$M for **AN-1**, **AN-192**, **AN-193**, and **AN-191**, respectively. Treatment with the drugs at their respective $IC_{50}$ concentrations for 4 and 24 h resulted in an increase of the p53 and p21 levels (Figure 6A,B). Apart from **AN-191**, the prodrugs increased p53 expression 2.5−5-fold already after 4 h, compared to the level of untreated cells. The expression of p53 after 24 h declined to >1.5 and <3-fold**. AN-191** that liberates two butyric acid units (and no formaldehyde) was the least effective; after 4 h, it did not change p53 expression, and after 24 h, it caused an increase of only 1.7-fold, while **AN-192** (that does not release butyric acid but releases two moieties of formaldehyde) increased the expression of p53 by 2.7-fold and 1.7-fold after 4 and 24 h, respectively.

The cyclin-dependent kinase inhibitor $p21^{WAF1}$ is an important regulator of cell cycle progression, senescence, and differentiation. HDAC inhibitors have been reported to consistently induce $p21^{WAF1}$ expression in a p53-dependent and -independent manner.[34,37,40,41] Here we show that prodrugs liberating butyric acid and/or formaldehyde increased p21 expression by 7−12-fold already 4 h posttreatment, and the protein remained at high level (Figure 6A,B).

Taken together, the data suggest that the tested prodrugs affect p53 and p21 expression by two distinct mechanisms, one activated by formaldehyde and the second by butyric acid. The marked effect was that of formaldehyde, resulting in rapid elevation of both. The modulation of p53 and p21 expression brought about by the prodrugs that release either formaldehyde and/or butyric acid is manifested downstream by the induction of differentiation and apoptosis.

Formaldehyde plays a vital role in living cells and participates in fundamental biological pathways. It is captured in the cell mainly by homocysteine that is converted to methionine (where the methyl donor is 5′-methyl-tetrahydrofolate) and further to *S*-adenosyl-L-methionine (SAM), the universal methyl donor participating in synthesis of essential molecules in the cells. Intracellular formaldehyde is generated by P-450 enzymes (CYP) from endogenous sources or from xenobiotics by oxidative demethylation of MeN, MeO, and MeS

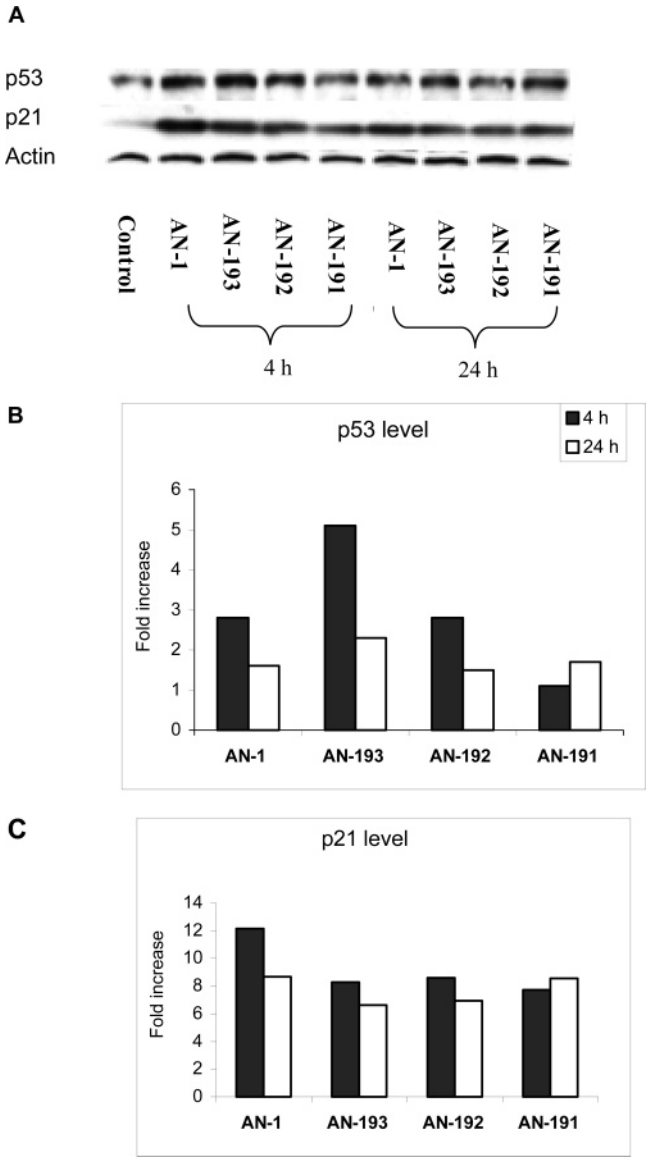
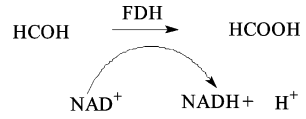



**Figure 6.** MCF-7 cells were treated with the $IC_{50}$s of the indicated prodrugs for 4 and 24 h. The lysates were subjected to Western blot analysis and the levels of p53 and p21 proteins were determined using a polyclonal rabbit anti-human p53 and p21 primary antibodies and an anti-rabbit secondary antibody (A). The "fold increase" represents the relative level of p53 (B) and p21 (C) normalized to actin and relative to control.

moieties as well as by demethylation of histones.[42] Exogenous formaldehyde was suggested to affect cell proliferation of normal and cancer cells selectively and in a dose-dependent manner.[43,44] We propose that a specific threshold of cellular formaldehyde concentration, achieved following treatment with formaldehyde-releasing prodrugs, is necessary to trigger inhibition of proliferation, apoptosis, and differentiation. For this to occur, it is necessary to demonstrate that treatment of cells with the formaldehyde-liberating prodrugs results in an increase in intracellular formaldehyde level.

**Detection of Intracellular Formaldehyde.** A quantitative assay to measure the concentration of intracellular formaldehyde, after treatment of cells with the formaldehyde-releasing prodrugs, was based on a formaldehyde dehydrogenase (FDH) coupled reaction. FDH oxidizes formaldehyde to formic acid, while $NAD^+$ is reduced to NADH (Scheme 2). The kinetics of the

**Scheme 2.** The Enzymatic Reaction Catalyzed by FDH




enzymatic reaction was monitored by the increase in optical density (OD) of NADH at 340 nm.[45]

The signal obtained using standard amounts of formaldehyde in solution or in lysates of Jurkat cells is shown in Figure 7A. The average OD values obtained in three experiments exhibited linearity between 0 and 15 $\mu$M formaldehyde. The standard curves of formaldehyde titrations with and without lysate overlapped, indicating that intracellular, endogenous formaldehyde was not present in the lysate at detectable levels. Lysate without exogenous formaldehyde produced a negative slope (OD/min = −0.234), suggesting that low activity of endogenous NADH was present. From the linear range of the calibration curve, the intracellular formaldehyde concentration was determined. The activity (OD at 340 nm/min/5 × $10^6$ cells) as a function of treatment time with the indicated concentrations of **AN-193**, in the absence or presence of the esterase inhibitor PMSF, is shown in Figure 7B. The highest formaldehyde concentrations were seen after 30 min, and the level was 2-fold higher when cells were treated with 4 mM compared to 2 mM **AN-193**, illustrating the expected concentration dependency. In cells treated with PMSF a very low level of formaldehyde was detected, indicating that active esterase is essential for intracellular release of formaldehyde. The intracellular formaldehyde level in the lysate of cells treated with **AN-1**, **AN-193**, and **AN-11**, expected to release 0, 1, and 2 equiv of formaldehyde, respectively, depended on drug concentration and the number of formaldehyde equivalents in the molecules (Figure 7C). As expected, treatment with **AN-11** did not produce an increase in formaldehyde, while treatment with **AN-1** and **AN-193** produced an approximately 2-fold higher increase in **AN-193** compared to **AN-1**. When cells were treated with **AN-193** and semicarbazide, no activity was detected in the lysate, since the formaldehyde released intracellularly was sequestered by semicarbazide.

**Effect of Formaldehyde-Releasing Prodrugs on DNA−Doxorubicin Adduct Formation.** It has been established that the formation of doxorubicin−DNA adducts is potentiated by formaldehyde-releasing prodrugs.[17] Additional support for the notion that cellular formaldehyde concentration is proportional to the number of formaldehyde equivalents expected to be released was obtained from the formation of doxorubicin−DNA adducts, as described below (Table 4).

Prodrugs that release 1 or 2 equiv of formaldehyde yielded adducts, while **AN-11**, which releases only acetaldehyde yielded none (Table 4). Moreover, adduct levels were proportional to the number of formaldehyde equivalents released. Previous findings support the notion that in order to function, the prodrugs undergo intracellular esterase-catalyzed hydrolysis. This was indirectly inferred by the observation that inhibition of intracellular esterases decreased the biological activities of HDACI esters.[7,13] However, the expected hydrolytic products, carboxylic acid and aldehyde, were not directly measured. In light of the central role that formaldehyde

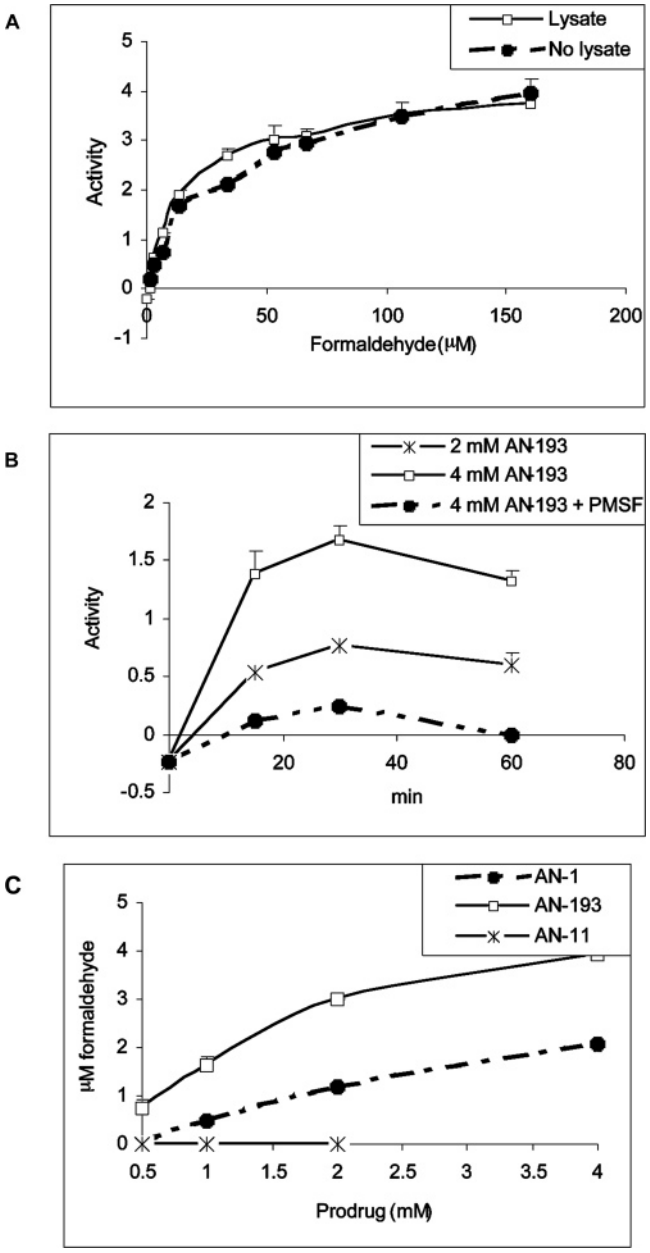



**Figure 7.** (A) Calibration curve of the enzymatic reaction of three independent experiments using increasing concentrations of formaldehyde with or without Jurkat cell lysate initiated by addition of FDH and $NAD^+$. The kinetics of the reaction was followed spectrophotometrically at 340 nm. The slope (OD/s) of the linear range was derived and plotted as a function of exogenous formaldehyde concentration. The "activity" represents OD (340 nm)/min/$5 \times 10^6$ cells. (B) Jurkat cells were treated with 2 and 4 mM of **AN-193** and incubated for 0, 15, 30, and 60 min. Time course measurement of cellular formaldehyde release from **AN-193** was performed in the presence and absence of the esterase inhibitor PMSF (1 mM). (C) Jurkat cells were exposed to 0.5, 1, 2, and 4 mM concentrations of **AN-1**, **AN-193**, and **AN-11** for 30 min and formaldehyde concentration was measured.

plays in increasing the prodrug potency and in its interaction with doxorubicin, we demonstrated that formaldehyde is released and that it activates doxorubicin−DNA adduct formation.[17] Formaldehyde release was demonstrated by quantifying its intracellular concentration (following exposure to formaldehyde-releasing prodrugs) and by formation of doxorubicin-DNA adducts (after treatment with formaldehyde-releasing prodrugs in a cell-free systems, in the presence of an esterase). The results shown in Table 4 are consistent with the findings obtained from direct measurements of intracellular formaldehyde levels and with the observation that only prodrugs capable of releasing formaldehyde stimulate formation of doxorubicin-DNA adducts. Moreover, prodrugs that release 2 equiv of formaldehyde, stimulate adduct formation to at least twice the level of those that release only 1 equiv.

**Table 4.** Doxorubicin DNA Adducts Formation in Cell-Free System[a]

| prodrug | equiv of released formaldehyde | adducts per 10 kb |
|---|---|---|
| AN-11 | 0 | 0.18 ± 0.06 |
| formaldehyde | 1 | 19.6 ± 1.0 |
| AN-1 | 1 | 24.3 ± 0.3 |
| AN-193 | 2 | 55.0 ± 4.6 |

[a] Adduct formation was measured in a cell-free system using radioactive doxorubicin as described in the Experimental Section.

## Conclusions

In this work we investigated a family of acyloxyalkyl esters of low molecular weight aliphatic acids and conducted SAR studies of their effect on proliferation, differentiation, cell death, histone acetylation, and direct inhibition of HDAC. The objective of the study was to elucidate the contribution of each of the intracellularly released metabolic products to the above activities. Formaldehyde was found to play a dominant role in the inhibition of proliferation and apoptosis, while the influence of butyric acid was secondary. The butyric acid released from prodrugs inhibited HDAC, leading to histone acetylation and induced cellular death and differentiation. For induction of differentiation and histone acetylation, butyric acid was significantly better than the other acids. An additional property of the prodrugs as anticancer agents is their ability to affect drug-resistant cancer cells.

Formaldehyde was shown to upregulate p53 and p21 expression and to be a critical antiproliferative factor that induces differentiation and cell death. Since formaldehyde does not inhibit HDAC and has no effect on histone acetylation, its specific effects on cell viability and differentiation are elicited by an as yet unclear mechanism that will be the subject of future studies.

## Experimental Section

**Chemistry.** $^1$H NMR spectra (200, 300, and 600 MHz) were obtained on Bruker AC-200, AM-300, and DMX-600 spectrometers, respectively. Chemical shifts are expressed in ppm downfield from $Me_4Si$ (TMS) as internal standard. The values are given in $\delta$ scale. Mass spectra were obtained on a Varian Mat 731 spectrometer (CI = chemical ionization, DCI = desorption chemical ionization, FAB = fast atom bombardment). TLC-monitored progress of the reactions was carried out on silica gel (Merck, Art. 5554). Most commercially available chemicals were purchased from Aldrich Co., Sigma Co., and Fluka Co. and were used without further purification.

The prodrugs examined, Table 1, were prepared as previously described: **AN-1**;[46] **AN-9**, **AN-11**, **AN-36**, **AN-37**, **AN-38**;[4] **AN-158**;[17] **AN-184**, **AN-185**, **AN-191**, **AN-192**, **AN-193**;[8] **AN-212**.[47]

**General Procedure for the Preparation of Acyloxyalkyl Esters.** To a mixture of an acid (1 equiv) and a haloalkyl ester (1 equiv) in dry acetone or DMF, was added $Et_3N$ (1 equiv). The temperature rose to ca. 40−50 °C, and a white precipitate began to form immediately. After 4 h of stirring at

40 °C, the precipitate was filtered, washed with 100 mL of ether, and dried to give $Et_3N{\cdot}HCl$. The filtrate was evaporated, and the residue was further stirred overnight at 40 °C. Usually, an additional small amount of precipitate was obtained, which was filtered, washed with ether, and dried. The filtrate was washed with 5% $NaHCO_3$, water, dried over $MgSO_4$, filtered, and evaporated. The residue was purified by distillation or by silica gel chromatography.

**1-Pentanoyloxyethyl Pivalate (AN-186).** Compound **AN-186** was prepared from valeric acid (10.2 g, 0.1 mol) and 1-butyroyloxyethyl chloride (16.45 g, 0.1 mol), as described for **AN-158**,[5] and was purified by fractional distillation (0.1 Torr) to give the product 50−80 °C (6.9 g, 30% yield). $^1$H NMR ($CDCl_3$) ppm $\delta$ 0.91 (t, Me, 3H, $J$ = 7.3 Hz), 1.2 (s, *t*-Bu, 9H), 1.34 (sext, $CH_2$Me, 2H, $J$ = 7.4), 1.48 (d, Me, 3H, $J$ = 5.5 Hz), 1.56 (quint, $CH_2CH_2$Me, 2H, $J$ = 7.4), 2.3 (t, $CH_2CO$, 2H, $J$ = 7.5 Hz), 6.63 (q, CH, 1H, $J$ = 5.5 Hz). $^{13}$C NMR ($CDCl_3$) ppm $\delta$ 13.51 (Me), 19.3 (Me), 21.96 ($CH_2$Me), 24.94 ($CH_2CH_2$Me), 26.73 (*t*-Bu), 33.7 ($CH_2CO$), 38.5 (C), 88.36 (OCHO), 171.6 ($CO_2$), 176.3 (*t*-Bu$CO_2$). MS (Cl, $NH_3$): *m/z* MW 248 ($MNH_4^+$, 100%), 204 ($C_{10}H_{19}O_4$, 98%). Anal. ($C_{12}H_{22}O_4$) C, H.

**Biology. Reagents.** Tissue culture media and serum were obtained from Biological Beith Haemek (Israel). $NAD^+$, formaldehyde dehydrogenase (FDH), MeCN, semicarbazide, porcine liver carboxyl esterase, caspase-3 reaction kit, 4-α-PMA (4-α-phorbol 12-myristate 13-acetate), bis-benzimide (Hoechst), phenylmethanesulfonyl fluoride (PMSF), DMSO, trypan blue, and all other chemicals used were obtained from Sigma; NBT (nitro blue tetrazolium) reagent and apoptosis kit (Annexin-V FITC and propidium iodide) were purchased from MBL.

For tissue culture studies, the prodrugs were dissolved in DMSO followed by dilution with medium, to a final DMSO concentration ≤ 0.1%. Solutions of the prodrugs were handled using Hamilton syringes and Teflon or glass vials. In parallel to the test compounds, appropriate controls were run using 0.1% DMSO in medium.

**Antibodies.** Rabbit anti-acetyl-histone H4 Lys-12 (Santa Cruz, Santa Cruz, CA), rabbit anti-histone H3, and rabbit anti-human p53 (Cell Signaling), rabbit anti-human prostate specific antigen, PSA (Dako), rabbit anti-human p21 (Delta Biolabs) and rabbit anti-human actin (H-300, Santa Cruz) were used for Western blot analysis. Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch, Cambridgshire, UK) was the secondary antibody for all the described procedures.

**Cell Cultures.** Human prostate carcinoma PC-3 (CRL-1435), 22Rv1 (CRL-2505), myelocytic leukemia HL-60 (CCL-240) and HL-60/MX2 (CRL-2257), colon cancer HT-29 (HTB-38), uterine sarcoma MES-SA (CRL-1976), MES-SA/Dx5 (CRL-1977), breast carcinoma MCF-7 (HTB-22), and T-cell leukemia Jurkat (TIB-152) cells were obtained from the ATCC. The cells were grown in the following media: RPMI-1640 for PC-3, HT-29, 22Rv1, HL-60 and HL-60/MX2 cells; McCoy medium for Jurkat, MES-SA and MES-SA/Dx5 cells; and DMEM medium for MCF-7 cells. The medium was supplemented with 10% fetal calf serum (FCS), penicillin (250 μg/mL) and streptomycin (125 μg/mL). 1% HEPES was added for 22Rv1 cells with the above supplements. Cells were transferred into fresh medium twice weekly and incubated at 37 °C in a humidified 5% $CO_2$ incubator. All tissue culture materials were obtained from Biological Industries Beth-Haemek, Israel.

**Proliferation Assays.** Cells in 200−250 μL growth medium at a density of (2.5−5) × $10^4$ cells/mL were seeded in tissue culture 96-well plates (in triplicate), for 24 h, without FCS. They were then exposed to different concentrations (titration) of the prodrugs (in medium containing 10% serum) for the indicated period under sterile incubation conditions. Two methods were used for proliferation measurements: (a) The Hoechst assay was used to examine the proliferation of the solid tumor cell lines HT-29, PC3, MES-SA, and MES-SA DX5. After 4 days of incubation with the test compounds, the samples were rinsed with PBS and fixed by the addition of 100 μL of 70% ethanol. After 0.5 h, the ethanol was decanted and 200 μL (10 mg/mL) of the DNA binding dye Hoechst reagent, solubilized in PBS, was added. The fluorescence emitted by the samples was measured at 390−460 nm; (b) The Alamar blue reagent (20 μL), used for HL-60, HL-60/MX2, and Jurkat cells, incubated at 37 °C, was added 24 h prior to termination of treatment, and the fluorescence was measured at 390 nm excitation and 460 nm emission (FluoStar fluorometer).

**Cell Death Assays. FITC-Annexin Cytostaining.** HL-60 cells were plated in 24-well plates for 24 h to achieve 70% confluency and then exposed to different concentrations of **AN-1**, **AN-193**, or **AN-191** for 4 h. Cells were rinsed, stained with propidium iodide, annexin V−FITC (apoptosis kit, MBL, Japan), and Hoechst dye (20 μg/mL), and incubated for 15 min in darkness at room temperature. The stained cells were examined under a fluorescent Olympus microscope (I × 70) equipped with an Olympus digital camera (DPS50) using a UV excitation/emission filter at 370−460 nm for Hoechst and propidium iodide staining and 470−490 nm for FITC staining. Viable cells were visualized as blue intact nuclei; early apoptotic cells by fragmented blue nuclei (370−460 nm) or as green staining on the outer membrane (470−490 nm). Necrotic and late apoptotic cells were visualized as pink nuclei (370−460 nm) or green outer membrane and orange nuclei (470−490 nm). The number of apoptotic and necrotic cells were scored in four different fields for each sample. **Caspase-3 Activity.** HL-60 cells were treated with the specified prodrugs for 6 h at 37 °C. Following incubation the cells were collected, washed, resuspended in HEPES buffer (50 mM, pH 7.4), and lysed by three freeze−thaw cycles. Cell lysates were centrifuged at 14 000*g* for 5 min, the protein concentration in the supernatants was determined (BCA kit, Pierce), DTT (5 mM) was added, and the lysates were stored at −70 °C. To each protein sample in assay buffer (25 μg), caspase-3 fluorogenic substrate (Ac-DEVD-AMC, 17 μM) was added and the activity was monitored at 37 °C for 60 min by the release of the fluorescent product (7-amino-4-methylcoumarine, AMC) using a fluorimeter at excitation and emission wavelengths of 360 and 460 nm, respectively.

**Differentiation.** The differentiation activity in the human myeloid leukemia cell line HL-60 was determined by a modified NBT reduction assay.[48] Cells were grown in 12-well plates at 8 × $10^4$ cells/mL, exposed for 4 days to $IC_{25}$ and $IC_{10}$ of the test compounds. Following the treatment, cells were spun down and resuspended in 20% of the initial volume in PBS containing 1 mg/mL NBT and 0.5 μg/mL 12-*O*-tetradecanoylphorbol-13-acetate (PMA) and incubated for 30 min at 37 °C. At the end of the incubation, the cells were centrifuged (200*g*), and the pellet obtained was dissolved in 300 μL of DMSO, transferred to 96-well plates, and analyzed by an ELISA reader at 550 nm. For differentiation of 22Rv1 cells, 1 × $10^6$ cells were seeded in 100 mm culture dishes, supplemented with RPMI medium containing 10% FCS and 1% HEPES pH 7.5, and treated with the described prodrugs for 96 h. The cells were centrifuged and washed with PBS, and the pellet obtained was resuspended in 50 μL of cold double distilled water. The samples were loaded on 12% SDS−PAGE and subjected to Western analysis using an Ab against human PSA (Dako), followed by incubation with an HRP-goat anti-rabbit IgG secondary antibody (Jackson).

**Histone Acetylation.** HL-60 cells (3 × $10^6$) seeded on 100 mm culture dishes, supplemented with RPMI medium containing 10% FCS, were treated with the following compounds: **AN-1**, **AN-193**, **AN-11**, and **AN-191** for the indicated periods of time. The medium was discarded, and the cells were rinsed with ice-cold PBS, harvested with a rubber policeman, centrifuged twice (200*g*), and resuspended in 1 mL of lysis buffer (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 1.5 mM PMSF). Histones were extracted in acid solution and dialyzed against water and lyophilized, and the protein content was determined with a BCA kit (Pierce, Rockford, IL). The samples were loaded on 15% SDS−PAGE and subjected to Western analysis, using an Ab against human acetylated H4-lys 12 (Santa Cruz, CA), followed by incubation with an HRP-goat anti-rabbit IgG secondary antibody.

**Western Blot Analysis.** Whole cells or acid-extracted histones were solubilized in a 4× loading buffer (62.5 mM Tris-HCl pH 6.8, 1% SDS, 10% glycerol v/v, 1% DTT, 0.1 mg/mL bromophenol blue). Proteins (5−30 $\mu$g) were run on SDS−PAGE, transferred to a nitro-cellulose membrane (Schleicher & Schuell, Germany), and probed with the primary antibodies as indicated, followed by incubation with an HRP-goat anti-rabbit IgG secondary antibody. Visualization of the bound HRP-goat anti-rabbit IgG was performed using the enhanced chemoluminescence method. The intensity of the bands was measured with a Versa Doc instrument using a Quantity1 program (Bio-Rad). The fold increase of each specific protein was determined by the ratio of band intensity obtained from treated and untreated cells, normalized to actin for cell extract and to H3 for acetylated H4.

**HDAC Inhibitory Activity.** Inhibition of HDAC in a cell-free system was performed using a fluorescent kit (AK-500, Biomol, Plymouth Meeting, PA). HeLa nuclear extract (0.5 $\mu$L/well) was preincubated (30 °C) with specified prodrugs at different concentrations (50−400 $\mu$M) in the presence of esterase ($9 \times 10^{-3}$ units, porcine liver) for 30 min. The Fluor de Lys substrate (50 $\mu$M) was added for 8 min. The reaction was terminated by the addition of Fluor de Lys developer and 10 nM trichostatin A (TSA). The percent of inhibition was calculated from the ratio of the fluorescence (measured at 390 nm excitation and 460 nm emission) in the prodrug treated to untreated control samples.

**Formaldehyde Quantification.** Jurkat cells ($(4-5) \times 10^7$) were treated with the specified prodrugs at 37 °C. Cells were centrifuged, washed with PBS, and dissolved in 20 $\mu$L of cold acetonitrile, diluted with cold double distilled water to a final volume of 1 mL, lysed by three freeze−thaw cycles in liquid nitrogen, and centrifuged, and the supernatant was analyzed for formaldehyde content. The reaction was initiated by the addition of formaldehyde dehydrogenase (FDH, 2 U/mL) to a mixture of the lysed cells (200 $\mu$L) and $NAD^+$ (10 mg/mL). The kinetics of the enzymatic reaction were followed spectroscopically at 340 nm for 5−10 min until a plateau was reached, and the activity was derived from the linear slope of the reaction (OD/s). The standard curve, obtained by adding formaldehyde solution to the cell lysate, was repeated in every experiment. The formaldehyde concentration was derived from the linear range of the curve.

**DNA−Doxorubicin Adduct Formation in a Cell-Free System.** Plasmid DNA (110 $\mu$M bp) was reacted with 2 $\mu$M [$^{14}$C]-doxorubicin (Amersham Biosciences, 54 mCi/mmol) in the presence of porcine esterase (100 $\mu$g/mL) and 1 mM prodrug (or formaldehyde) in transcription buffer (40 mM Tris pH 8.0, 3 mM $MgCl_2$, 100 mM KCl, 0.1 mM EDTA) at 37 °C for 2 h. Samples were extracted twice with Tris-saturated phenol and once with chloroform and were subsequently precipitated with ethanol. The samples were resuspended in Tris-EDTA buffer and subjected to $^{14}$C scintillation counting. Counts were normalized to the amount of DNA present and converted to adducts per 10 Kb.

**Statistical Analysis.** $IC_{50}$ values were derived from linear regression of the adjusted $Y$ (% control viability) and $X$ values (concentration of the compounds). Data represents averages of three or more experiments. Analyses of variance were followed by $t$-tests.

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