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ORIGINAL ARTICLE

Formaldehyde-releasing prodrugs specifically affect cancer cells by depletion of intracellular glutathione and augmentation of reactive oxygen species

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Abstract Histone deacetylase inhibitory prodrugs that are metabolized to carboxylic acid(s) and aldehyde(s) possess antineoplastic properties. Formaldehyde-releasing prodrugs were shown to be the most potent. The objective of this study was to gain understanding on the mode of action of these prodrugs in cancer cells. HL-60 and MCF-7 cells in the presence of *N*-acetylcysteine or glutathione were protected from death induced by formaldehyde-releasing prodrugs but not from death caused by the homologous acetaldehyde-releasing ones. Cell death induced by the former was accompanied by depletion of intracellular glutathione and increased reactive oxygen species that were attenuated by *N*-acetylcysteine. At fourfold higher concentration, acetaldehyde-releasing prodrugs increased reactive oxygen species that were further augmented by *N*-acetylcysteine. In HL-60 cells, formaldehyde-releasing prodrugs dissipated the mitochondrial membrane potential and glutathione or *N*-acetylcysteine restored it. Although acetaldehyde-releasing prodrugs dissipated mitochondrial membrane potential, it occurred at 20-fold greater concentration and was unaffected by the antioxidants. Formaldehyde-releasing prodrugs abrogated c-myc protein expression and elevated c-Jun and H2AX phosphorylation, *N*-acetylcysteine partially reversed these changes. Herein, we show that formaldehyde-releasing prodrugs diminish the level of glutathione most likely by forming *S*-formylglutathione adducts resulting in increase of reactive oxygen species followed by signaling events that lead to cancer cells death.

Keywords Formaldehyde · Glutathione · HDAC-inhibitors · Prodrugs · Apoptosis

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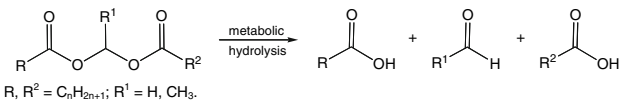
Introduction

Previously, we have studied anticancer acyloxyalkylester prodrugs of butyric acid (BA) that upon metabolic degradation release aldehyde together with BA (Scheme 1).



Scheme 1 Metabolic degradation of acyloxymethyl ester prodrug

Our extensive studies of these prodrugs have shown that although the released BA functions as a histone deacetylase (HDAC) inhibitor, the most active prodrugs are those that release formaldehyde [1–5]. Structure−activity relationship (SAR) studies of the anticancer activity, of acyloxyalkylester prodrugs that by metabolic hydrolysis release 0–2 equivalents of BA and 0–2 equivalents of formaldehyde, in several cancer cell lines, were conducted. The studies showed that formaldehyde released intracellularly, increase cellular formaldehyde level and plays a dominant role in the inhibition of proliferation and apoptosis. The contribution of BA to cancer cell death was secondary. The released BA inhibited HDAC and led to hyperacetylation of histones and consequently to an increase in differentiation and apoptosis of cancer cells

[2]. These prodrugs exerted their antiproliferative activity 100 times faster and at a concentration of about tenfold lower than BA [3, 6]. AN-9 was also shown to inhibit colony-forming units of solid-tumor and primary leukaemic cells [7, 8].

In vitro, the formaldehyde-releasing prodrugs (FARPs) increase intracellular formaldehyde concentration and anticancer activity in a manner proportional to the number of releasable formaldehyde equivalents [2]. The efficacy of the FARPs to the anticancer activity was evident in vivo by inhibition of lung metastasis in Lewis-lung carcinoma [4], murine Mm-A leukaemia [9], prostate [10] and glioma [11] xenografts. In the above animal studies as well as in Phase I and II clinical studies with AN-9 [12, 13] the prodrugs possessed overall low toxicity and the released formaldehyde did not contribute to systemic toxicity. Although the accepted paradigm views formaldehyde as a highly toxic substance [14], in living cells it plays a vital role in fundamental biological pathways. Intracellular formaldehyde is formed by oxidative demethylation of *N*-, *O*- and *S*-methyl compounds and xenobiotics and 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) [15–17]. SAM, the universal methyl donor, participates in the synthesis of essential molecules in cells. A recognized role for intracellular formaldehyde in potentiating the anticancer activity of anthracyclines and overcoming drug resistance by formation of adducts between DNA and the anthracyclines was extensively studied by others and us [2, 18–22].

In recent study we showed that the FARPs, AN-7 and AN-1 that synergize Doxorubicin (Dox) anticancer activity, protect the heart against Dox toxicity [23]. The cellular formaldehyde released from the prodrugs was shown to be a dominant factor in the cardio-protection activity. The observations that the FARPs display opposing biological roles toward cancer versus normal myocardial cells, demonstrate diversity of activity and specificity to particular cell types or tissues that are most desirable for an antineoplastic agent. The mechanism by which cellular formaldehyde elicits its cell type specific effects is unknown. In this study, we investigated the mode by which formaldehyde induces cancer cell death. The importance of intracellular redox level in mediating the cellular activity of formaldehyde in HL-60 and MCF-7 cell lines is described.

Material and methods

Cell cultures

Myelocytic leukaemia HL-60 (CCL-240) and the breast carcinoma MCF-7 (HTB-22), cell lines were obtained from the ATCC. HL-60 cells were grown in Roswell Park Memorial Institute medium (RPMI-1640) and MCF-7 cells in Dulbecco's modified Eagle's medium (DMEM) supplemented with penicillin (250 μg/mL) and streptomycin (125 μg/mL), L-glutamine (2 mM) and 10% fetal calf serum (FCS). The cells were transferred twice weekly and grown at 37°C in a 5% humidified CO_2 incubator. All cell culture reagents were purchased from Biological Industries (Beit Haemek, Israel).

Chemicals

Apoptosis kit (Annexin-V FITC and propidium iodide) was purchased from MBL. 5,5′,6,6′-Tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide (JC-1) from Calbiochem-Novabiochem (La Jolla, CA, USA). DMSO, *N*-acetyl–L-cysteine (NAC), glutathione (GSH), nitroblue tetrazolium (NBT), monobromobimane (mBBr), *N*-ethylmaleimide (NEM), trypan blue, catalase, 2′,7′-dichlorofluorescein diacetate (DCF-DA), Hoechst and all other chemicals used, when not specified, were obtained from Sigma (St Louis, USA).

Prodrugs

The structure and metabolic products of the investigated prodrugs are shown (Table 1). The synthesis of these prodrugs was performed as described [2, 3].

Cell treatment

The prodrugs were dissolved in DMSO followed by dilution with the medium, to a final DMSO concentration of ≤0.1%. In all tests appropriate controls of 0.1% DMSO medium were run. The solutions of the prodrugs were handled using Hamilton syringes and Teflon or glass vials. The antioxidants (NAC and GSH) were dissolved in PBS and neutralised to pH 7.45, and added to the growth medium 30 min prior to treatment with the prodrugs. Catalase at 500 U/mL was added as indicated.

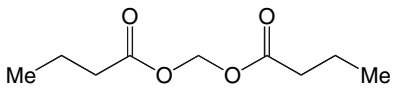
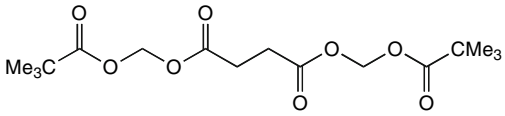
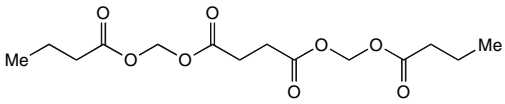
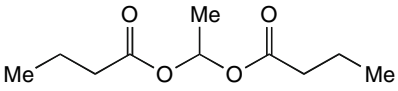
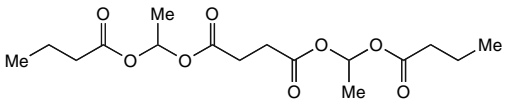
Antibodies

Mouse monoclonal anti-phosphorylated H2AX at ser139 (BioLegend, San Diego, USA); anti-phosphorylated c-Jun at ser63 (Santa Cruz, CA, USA); actin (MP Biomedicals, OH, USA), and rabbit polyclonal anti-human c-Myc (Cell Signaling, MA, USA) were used. The secondary antibodies for all the described procedures were Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG or goat anti-mouse (Jackson ImmunoResearch Labs Inc., West Grove, PA, USA).

Proliferation assays

MCF-7 cells in growth medium were seeded (5×10^3 cells/well) in 96-well plates (in triplicate) for 24 h and then

Table 1 FARPs and AARPs and their metabolites

Prodrugs	Structures	Hydrolysis products (No. equivalents)
AN-1		2 BA 1 Formaldehyde
AN-192		1 Succinic Acid 2 Pivalic Acid 2 Formaldehyde
AN-193		2 BA 1 Succinic Acid 2 Formaldehyde
AN-11		2 BA 1 Acetaldehyde
AN-191		2 BA 1 Succinic Acid 2 Acetaldehyde

exposed to different concentrations of the prodrugs for additional 72 h. Viability of the cells was measured by Hoechst assay [2].

HL-60 cells in growth medium were seeded (5×10^3 cells/well) in 96-well plates (in triplicate) and exposed to different concentrations (titration) of the prodrugs. After incubation of 72 h, MTT reagent (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) was added according to the manufacturer's instructions (Sigma St Louis, MI, USA). Absorbance at 570 nm was measured using a Bio-Tech Instruments spectrophotometer (IN, USA).

Apoptosis assay by FACS analysis

Cell viability was determined using Mebcyto apoptosis kit (MBL-Japan). HL-60 cells were seeded in six-well plates (4×10^5 cells/well) and treated with the prodrugs for 24 h. MCF-7 cells (4×10^5) were seeded in 60 mm^2 plates and after 24 h treated with the prodrugs for additional 24 h. The cells were harvested (MCF-7 were trypsinized) double-stained with annexin V-FITC and propidium iodide according to the manufacturer's instructions and subjected to flow cytometry analysis (FACSCalibur cytometer, Becton Dickinson). The percentage of cells was defined by their distribution in a fluorescence dot plot using WinMdi 2.8 software (Scripps Research Institute, La-Jolla, US).

Assessment of changes in mitochondrial membrane potential ($\Delta\psi m$)

The fluorescent mitochondrial-specific cationic dye JC-1 undergoes potential-dependent accumulation in the mitochondria [24]. HL-60 cells (1×10^6) were treated for 24 h with the corresponding prodrugs, centrifuged and resuspended in 0.5 mL of 40 mM HEPES buffer, pH 7.4, supplemented with 0.65% NaCl and 4.5 g/L glucose (Buffer A) at 37°C containing 1 μg/mL JC-1. The cells were incubated for 15 min at 37°C, washed and resuspended in 200 μL of dye free Buffer A and added to a 96-well black plate (Greiner Bio-one, Germany) and the fluorescence was

measured immediately with a fluorescent plate reader (FluoStar fluorometer) at excitation/emission filters of 485/540 nm (green); 540/590 nm (red). The ratio of red/green fluorescence was calculated.

Differentiation: NBT reduction

Differentiation activity in the human myeloid leukemia cell line HL-60 was determined by a modified NBT reduction assay [25]. Cells were pre-treated with NAC (1 mM) for 30 min and then exposed for 4 days to the prodrugs and NBT was determined as described [2].

Measurements of GSH

Changes in the content of cellular GSH were determined with monobromobimane (mBBr) staining based on the described procedure [26]. This probe turns fluorescent after conjugation with GSH catalyzed by glutathione-*S*-transferase (GST). HL-60 cells (1×10^5) were seeded in a 96-well plate and treated with the prodrugs as specified. Following the treatment, cells were loaded with 40 μM mBBr for 30 min at 37°C in the dark. Fluorescence was measured at excitation/emission filters of 360/460 nm. As a positive control for full deletion of GSH, the addition of mBBr cells were treated with NEM (100 μM) simultaneously. The percent of fluorescence compared to untreated cells was calculated and normalized to the number of viable cells as determined by MTT method. From the dose–response, ED50 values (concentration depleting 50% of total GSH) were derived by linear regression of the adjusted *Y* (% mBBr fluorescence) and *X* (concentration of the compounds in μM).

Measurement of ROS

ROS were measured in live cells as intracellular peroxides by monitoring the oxidation of DCF-DA. The membrane-permeable dye undergoes deacetylation by intracellular esterases and oxidation by ROS [27]. Cells (8×10^5) were treated with the specified prodrugs, incubated with 10 μM DCF-DA for 15 min at 37°C, washed twice with PBS and analysed (10^4 cells) by flow cytometry using a 488 nm excitation beam. The percentage of cells producing ROS [% DCF (+)] was determined with CellQuest software (BD Biosciences, USA).

Western blot analysis

HL-60 cells (10^6/mL) were plated in 6-well plates and treated with the corresponding prodrugs. The samples were subjected to Western blot analysis as previously described [2].

Data analysis

The IC50 values were derived from linear or exponential regression of the adjusted *Y* (% control viability) and *X* values of the concentration of the compounds. The data represent averages of three or more experiments and the standard error was calculated for each group. The analysis of variance followed by *t* tests to determine whether differences observed were significant.

Results

The antioxidants NAC and GSH protect HL-60 and MCF-7 cell lines against cytoxicity induced by FARPs

To test whether the cytotoxic effects of the prodrugs on cancer cells are mediated by ROS, the potential protective effect of exogenous NAC and GSH on the cells was evaluated. HL-60 and MCF-7 cells were treated with various concentrations of the prodrugs in the absence or the presence of the antioxidants NAC or GSH (100 and 400 μM). IC50 values were derived from dose–response studies (Table 2). In HL-60 cells, it was evident that the FARPs, AN-1, AN-193 and AN-192 cytotoxicity was drastically reduced (four to five-fold) by GSH and NAC and in contrast that of acetaldehyde-releasing prodrugs (AARPs) AN-11 and AN-191 were either unaffected by antioxidants or significantly augmented by them, as evident by the reduced IC50 values (Table 2A). Similarly, in the breast carcinoma cell line MCF-7, the cytotoxicity of the FARPs was dramatically reduced by the antioxidants while that of the AARPs increased significantly by 1 mM of NAC or GSH (Table 2B).

Hydrogen peroxide that diffuses freely across the cell membrane can be degraded by extracellular catalase. Addition of 500 U/mL catalase to HL-60 cells reduced the toxicity of AN-193 by ~20% in three independent experiments (Table 2A).

The prodrugs effect on HL-60 and MCF-7 cells mortality was analysed also by FACS. Treatment of the cells with FARPs (25–200 μM) or AARPs (0.5–2 mM) for 24 h in the absence or presence of NAC was followed by staining with Annexin-V (FITC) and propidium iodide (PI). The average ED50 values calculated for total cell death from dose–response studies (Table 3) and representative dot plots depicting the flow cytometry analysis are shown (Fig. 1). The ED50 values showed that the toxicity of FARPs was inhibited by NAC, in HL-60 by 10–50 and in MCF-7 cells by 8–10-folds. Moreover, in HL-60 cells, 1 mM and in MCF-7 cells, 2 mM NAC abolished the cytotoxicity (ED50 > 2 mM) of FARPs. In both cell lines, the mortality, induced by the AARPs AN-11 and AN-191 at significantly higher concentrations,

Table 2 The antioxidants NAC and GSH, protect HL-60 (A) and MCF-7 (B) cell lines against the toxic effects of FARPs but not against those of AARPs

Treatment	Single agent IC50 (μM)	+NAC (0.1 mM) IC50 (μM)	+NAC (0.4 mM) IC50 (μM)	+GSH (0.1 mM) IC50 (μM)	+GSH (0.4 mM) IC50 (μM)	Catalase (500 U/mL)
A						
AN-1	32 ± 0.3	56 ± 4.4	144 ± 12	41 ± 3.3	76 ± 0.8	*ND
AN-193	17.8 ± 0.9	32 ± 2.9	104 ± 4.6	33.0 ± 2.7	99 ± 0.5	21.8 ± 0.9
AN-192	21 ± 0.5	33 ± 0.4	130 ± 2	35 ± 1.9	177 ± 2.6	*ND
AN-191	208 ± 14	245 ± 27	228 ± 12	208.0 ± 5	184 ± 2.3*	*ND
AN-11	306 ± 26	380 ± 53	329 ± 26	311 ± 13	280 ± 25*	ND

Treatment	Single agent IC50 (μM)	+NAC (0.4 mM) IC50 (μM)	+NAC (1 mM) IC50 (μM)	+GSH (0.4 mM) IC50 (μM)	+GSH (1 mM) IC50 (μM)
B					
AN-1	47 ± 1	83 ± 5	129 ± 5	70 ± 6	137 ± 4
AN-193	24.6 ± 0.5	62.4 ± 1.8	136 ± 5	59 ± 4	132 ± 5
AN-192	25 ± 1.4	53 ± 2	148.5 ± 3	56 ± 7	147 ± 4
AN-191	525 ± 19	410 ± 13	375 ± 8*	416 ± 31	373 ± 22*
AN-11	655 ± 15	625 ± 12	456 ± 8.5*	636 ± 12.3	432 ± 14.2*

MCF-7 and HL-60 cells (5×10^3per well) were seeded in 96 well plates. HL-60 cells immediately and MCF-7 cells after 24 h were treated with various concentrations of AN-1, AN-193, AN-192, AN-191 and AN-11 for 72 h in the presence of the indicated concentrations of NAC and GSH. Viability was determined by the MTT assay for HL-60 cells and by the Hoechst assay for MCF-7 cells. The IC50 values for HL-60 (A) and MCF-7 (B) cell lines were determined by linear regression. The data in the table represents average of three or more independent experiments and standard error (SE)

Table 3 NAC abrogates cell death induced by FARPs in HL-60 and MCF-7 cell lines but did not affect AARPs toxicity

Treatment (HL-60) ED50 (μM)	Single agent ED50 (μM)	+0.4 mM NAC ED50 (μM)	+1 mM NAC ED50 (μM)
AN-1	56 ± 0.4*	524 ± 52	>2,000
AN-193	33 ± 2.5	1,427 ± 278	>2,000
AN-192	30 ± 1	655 ± 223	>2,000
AN-191	1,483 ± 491	1,408 ± 650	1,523 ± 521
AN-11	>2,000	>2,000	>2,000

Treatment (MCF-7) ED50 (μM)	Single agent ED50 (μM)	+ 1 mM NAC ED50 (μM)	+ 2 mM NAC ED50 (μM)
AN-1	178 ± 38	1,643 ± 102	>2,000
AN-193	103 ± 23	1,251 ± 245	>2,000
AN-192	126 ± 26	813 ± 103	>2,000
AN-191	>2,000	>2,000	>2,000
AN-11	>2,000	>2,000	>2,000

HL-60 (4×10^5) and MCF-7 (2×10^5) cells were pre-treated with NAC for 30 min followed by the addition of various doses of AN-1, AN-193, AN-192, AN-11 and AN-191. After 24 h, the cells were stained with PI and annexin V-FITC, and subjected to FACS analysis. The ED50 of the prodrugs for cell death in HL-60 and MCF-7 cells were determined by the linear regression of the percent of viable cells versus prodrug concentration. NAC (1 and 2 mM) caused 102% of viability compared to control

was unaffected by NAC. These observations imply that only cell death induced by FARPs was dependent on ROS.

Effect of FARPs and AARPs on mitochondrial membrane potential

Loss of mitochondria membrane potential ($\Delta\psi_m$) is considered to be an early marker of apoptosis [28]. We examined the effect of the prodrugs on $\Delta\psi_m$ in HL-60 cells. The changes in mitochondrial $\Delta\psi_m$ were detected using the specific fluorescence dye, JC-1 that exhibits potential-dependent accumulation in the mitochondria. AN-193 and AN-192, prodrugs that release two equivalents of formaldehyde, caused the greatest change in $\Delta\psi_m$ at a concentration of 50 μM (Fig. 2a). Yet, AN-193 that releases two BA equivalents in addition to the formaldehyde was significantly more potent than AN-192 that releases two equivalents of pivalic acid. At the same concentration, AN-1, AN-191 and AN-11 had small or no effect. AN-191 only at 2 mM induced dissipation of $\Delta\psi_m$ (Fig. 2b). To assess the involvement of ROS in the collapse of $\Delta\psi_m$, HL-60 cells were pretreated for 30 min with 1 mM NAC or GSH and then with the prodrugs for 24 h. The antioxidants abrogated the collapse of $\Delta\psi_m$ by 50 μM of AN-193 and had no effect on $\Delta\psi_m$ induced by 2 mM AN-191 (Fig. 2b). These results demonstrate that while the collapse of $\Delta\psi_m$ by AN-193 is dependent on increase of ROS that induced by AN-191 was ROS-independent.

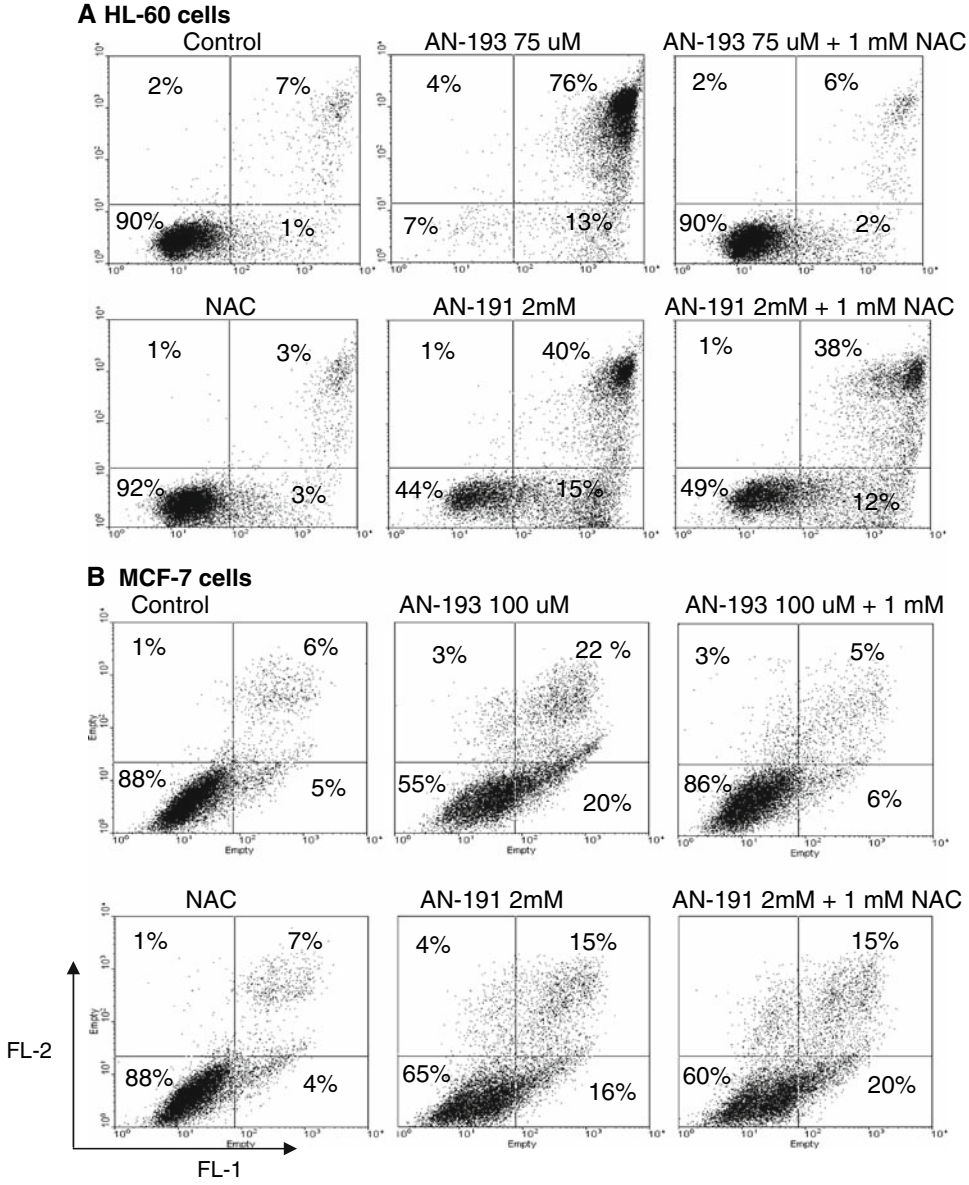


Fig. 1 NAC diminished HL-60 (**a**) and MCF-7 (**b**) cell death induced by the FARP An-193 but did not affect cell death induced by the AARP AN-191. HL-60 (4×10^5) and MCF-7 (2×10^5) cells were pre-treated with 0.4–2 mM of NAC for 30 min followed by the addition of various doses of AN-1, AN-193, AN-192, AN-11 and AN-191. After for 24 h the cells were stained with PI and annexin V-FITC, and subjected to FACS analyses. The dot-plots of HL-60 (**a**) and MCF-7 (**b**) cells treated as specified represent in the *X*-axis annexin FITC fluorescence and in the *Y*-axis the PI fluorescence. The *left lower panel* of each graph represents viable cells (negatively stained). The *left upper panel* represents necrotic cell (positively stained for PI). The *right lower panel* represents early apoptotic cells (positively stained for annexin). The *right upper panel* represents late apoptotic and/or necrotic cells (positively stained with both dyes)

FARPs reduce intracellular levels of GSH

The effect of FARPs and AARPs on intracellular GSH level was examined with the mBBr probe, which becomes fluorescent after conjugation with GSH. Treatment of HL-60 cells with the FARPs, AN-193, AN-1 and AN-192 caused a marked reduction of intracellular GSH after 1 h (data not shown) and a greater dose–dependent decrease after 4 h (Fig. 3). The calculated ED50 values for reduction of intracellular GSH, after 4 h of treatment, showed that the most effective prodrugs were AN-192 (45.0 ± 2 μM) and AN-193 (49.2 ± 0.2 μM) that release two equivalents of formaldehyde followed by AN-1 (67 ± 4.8 μM) (Fig. 3b). On the contrary in three independent experiments ED50 was unattainable for AARPs. Concentrations of up to 1 mM of the AN-11 and AN-191 (the solubility of these lipophilic prodrugs did not permit to exceed 1 mM) yielded average cellular GSH levels of 75 ± 3 and 58 ± 6%, respectively.

Formaldehyde-releasing prodrugs induce ROS release in HL-60 cells

The intracellular production of ROS in HL-60 cells was evaluated by FACS analysis using the cell-permeable probe, DCF-DA that upon oxidization becomes fluorescent. In a time course study the prodrugs induced ROS reached a peak level after 2 h of treatment (data not shown). Figure 4a depicts a representative plot portraying the changes in ROS signal after 2 h of treatment with 50 μM of AN-193. AN-193 increased ROS, as visualized by the shift to the right, was attenuated by NAC. A summary of independent experiments (>3) demonstrated that the ROS

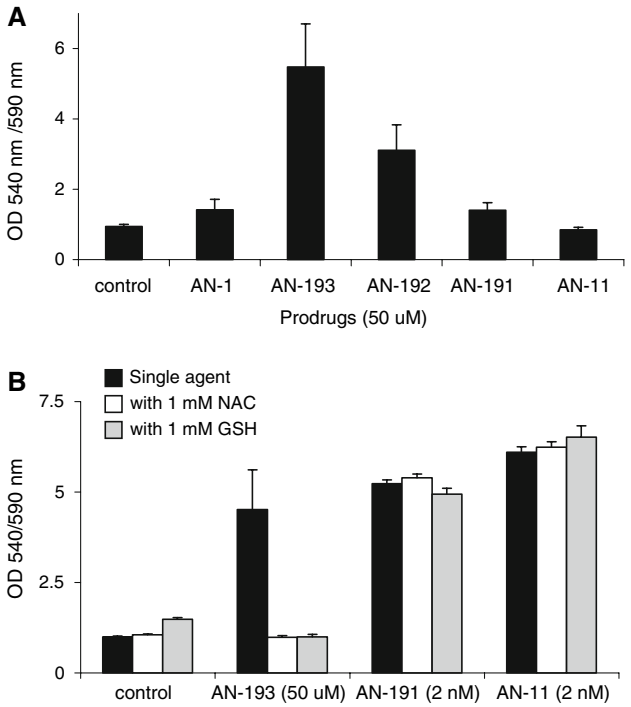


Fig. 2 Collapse of the mitochondrial membrane potential of HL-60 cells by the FARPs and AARPs. HL-60 cells (1×10^6 mL^{-1}) were treated with 50 μM of the corresponding prodrugs for 24 h and the mitochondrial membrane potential of the cells was determined by staining with JC-1 (**a**). The cells ($1 \times 10^6 mL^{-1}$) were pre-treated with 1 mM of NAC or GSH for 30 min, followed by the addition of 50 μM of AN-193 or 2 mM of AN-191. After 24 h the cells were stained with JC-1 (**b**). The data represent mean values of three or more independent experiments each performed in triplicate

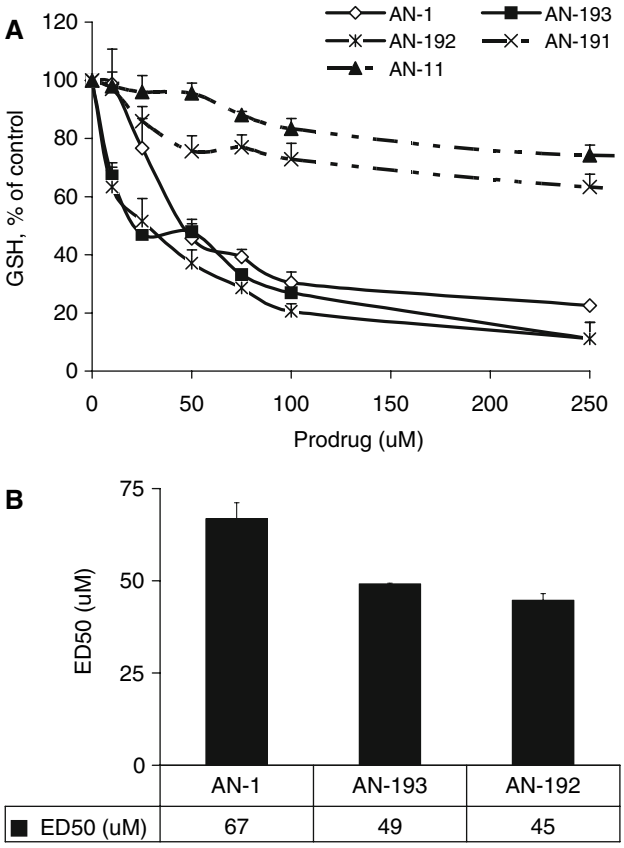


Fig. 3 FARPs reduce intracellular levels of GSH. HL-60 cells (5×10^5/mL), seeded in a 96-well plate, were treated with the indicated prodrugs for 4 h. Their GSH content was determined by monobromobimane (mBBr) staining. The percent of cells positive for mBBr fluorescence was determined relative to the untreated cells and normalised to the percent of viable cells as determined by MTT after 4 h of treatment with the prodrugs (**a**). The ED50 values, derived from linear regression of dose–response effect, after 4 h of treatment with the prodrugs are shown in the lower table (**b**). *N*-Ethylmaleimide at 100 μM was used as a positive control for complete inhibition

increased by FARPs was significantly reduced by NAC (Fig. 4b). The maximal increase in ROS (+) cells by AN-193 and AN-192 was 50% and it was lowered by NAC to 14–19%. AN-191 at 50 μM had a negligible effect on the percent of ROS (+) cells and at 200 μM increased it to 40%. However, addition of NAC, in sharp contrast to its inhibitory effect of FARPs, stimulated ROS production by the AN-191. In the presence of NAC, 50 or 200 μM AN-191 raised ROS (+) cells by 20% (to 20 and 60%, respectively).

HL-60 cells capacity to release oxygen burst is augmented by FARPs and inhibited by the antioxidant NAC

Differentiation of HL-60 cells toward the myelomonocytic lineage is accompanied by increase capability to generate, upon stimulation with PMA, ROS through the NADPH oxidase [29]. Previously we showed that intracellularly released formaldehyde specifically induces differentiation of HL-60 cells [2]. Herein, we examined whether formaldehyde induced differentiation will be affected by NAC. The differentiation of HL-60 cells treated with the prodrugs in the absence or presence of 1 mM NAC for 4 days was monitored by NBT reduction. NAC significantly diminished the NBT reduction induced by the FARPs, AN-1, AN-193 and AN-192 but did not significantly affect differentiation induced by AN-191 (Table 4).

Modulation of gene expression

Changes in the level of modification and expression of proteins after treatment of HL-60 cells with 100 μM of the prodrugs for 1, 4 and 24 h were evaluated. The prodrugs increased phosphorylation of ser139 on histone H2AX which is a marker of double-strand breaks (DSBs) [30]. Compared to AARP, FARPs caused a greater increase in the level of H2AX phosphorylation that remained high after 24 h of treatment (Fig. 5). AN-193 induced the highest phosphorylation level among the FARPs. The AARP AN-191 at 100 μM induced low, and at 500 μM higher levels of H2AX phosphorylation, even so, it was substantially lower

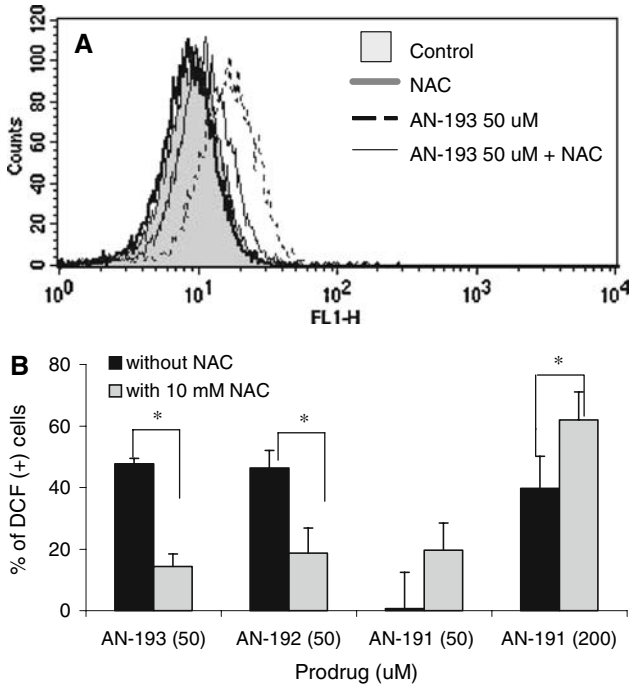


Fig. 4 FARPs increase ROS level in HL-60 cells. HL-60 cells (8×10^5 mL^{-1}) were exposed to 10 μM of DCF-DA for 15 min followed by the addition of 5 mM NAC for a further 15 min and then by addition of the prodrugs in the dark. At the end of 2 h incubation with the prodrugs the cells were washed, resuspended in PBS and subjected to FACS analysis. The representative histogram of cells treated with 50 μM of AN-193 is shown (**a**). The percent of cells stained positively with DCF was determined by subtraction of the percent DCF positive cells in untreated cultures from that of the prodrugs treated ones. The average values obtained in three independent experiments are shown (**b**). The *brackets* link between cells treated with the prodrugs to those treated with the prodrugs in the presence of NAC and are compared by *t* test. The *asterisks* indicate significant differences ($p < 0.05$)

Table 4 FARPs induce differentiation of HL-60 cells in a ROS-dependent manner: fold increase compared to untreated control

Treatment	Single agent fold increase	+1 mM NAC fold increase
Control (untreated)	1.0 ± 0.1*	1.0 ± 0.06
AN-1 (25 μM)	2.4 ± 0.1	1.1 ± 0.02
AN-193 (25 μM)	3.0 ± 0.1	1.3 ± 0.01
AN-192 (25 μM)	2.2 ± 0.1	1.0 ± 0.17
AN-191 (200 μM)	4.5 ± 0.4	3.5 ± 0.7

The differentiation of HL-60 cells treated with the indicated prodrugs for 96 h in the presence or absence of NAC (1 mM) was measured by NBT reduction as OD 450 nm/L × 10^6 cells. The "fold increase" was normalised relative to untreated control cells

than the phosphorylation instigated by the FARPs. Addition of NAC reduced significantly the intensity of FARPs induced phosphorylated H2AX but did not substantially affect the one induced by AN-191.

The protein c-myc plays an important role in cell growth and differentiation [31]. After 4 h of treatment of HL-60 cells with 100 μM of FARPs, c-myc expression was very low (AN-1) or undetectable (AN-193 and AN-192) and remained that way after 24 h (Fig. 5). Treatment of the cells with of 100 and 500 μM AN-191 caused only a small decrease in c-myc, detectable at the higher dose. The changes in c-myc expression induced by FARPs treatment were restored by addition of NAC. In contrast, in cells treated with AN-191, NAC had no effect or it caused a small reduction in the expression of c-myc (Fig. 5). FARPs induced a transient phosphorylation of c-Jun on ser63 detectable only after 1 h and NAC reduced it. Phosphorylation of c-Jun, following treatment with AN-191, increased to a low level that was visible only after 1 h and was unaffected by NAC.

Discussion

In this study we explored the cellular events critical for the activity of FARPs in human myeloid HL-60 and breast carcinoma MCF-7 cells, and compared their effects to that of their homologous AARPs. The importance of FARPs as anticancer agent stem from their dual activity, they display anticancer activity and act in synergy with Dox as anticancer agents as well as protect cardiomyocytes against Dox induced toxicity.

Formaldehyde-releasing prodrugs reduced the viability of HL-60 and MCF-7 cell lines in a ROS-dependent manner, which was partially restored by NAC or GSH. In contrast, AARPs only at significantly higher concentrations (≥tenfold) reduced the viability of these cell lines. Moreover, their activity was unaffected or was even augmented by the antioxidants (Table 2). ROS-inducing agents were shown to trigger apoptosis by acting on the mitochondria [28, 32]. Herein, we showed that the FARP AN-193 induced $\Delta\psi_m$ collapse in a ROS-mediated mechanism, whereas the analogous AARP AN-191 at a 20-fold higher concentration dissipated $\Delta\psi_m$ in a ROS-independent manner. AN-193 was significantly more potent in disrupting $\Delta\psi_m$ than AN-192, probably due to the added contribution of the released BA.

Formaldehyde was shown [33, 34] to bind GSH covalently in a reaction catalysed by glutathione-dependent formaldehyde dehydrogenase, yielding *S*-formylglutathione adduct (Scheme 2). This enzyme that belongs to Class III of ADH, displays selectivity to formaldehyde and it catalyses the oxidation of *S*-hydroxymethylglutathione adduct much more effectively than ethanol and plays a major role in formaldehyde metabolism of the cell [34, 35].

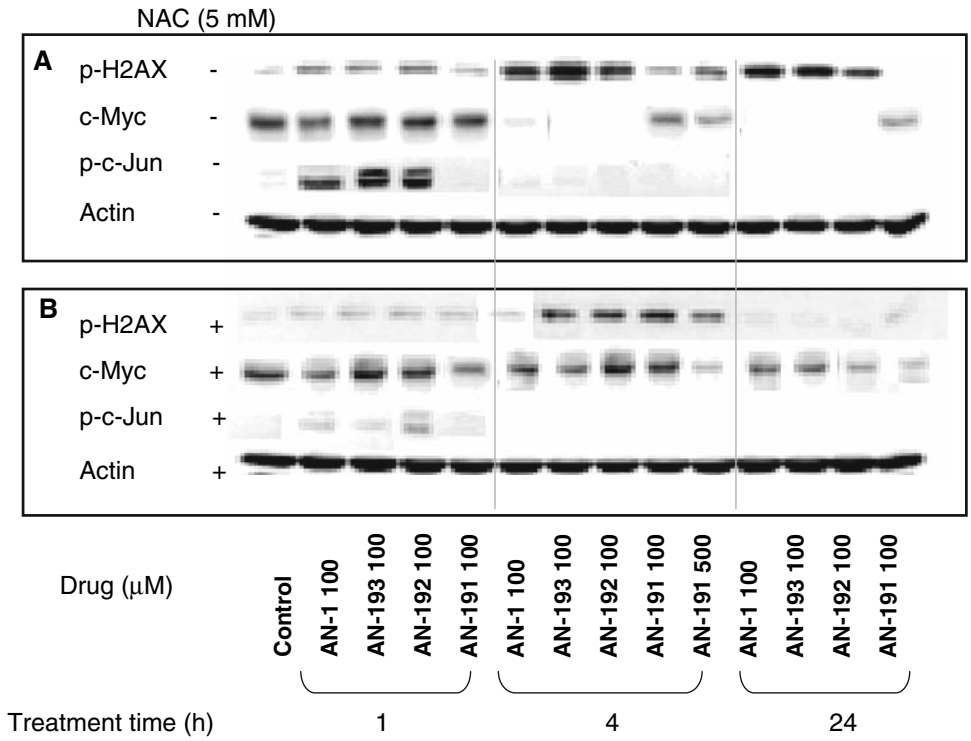
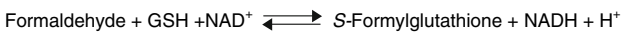


Fig. 5 The levels of c-Myc and phosphorylation of H2AX and c-Jun by FARPs was diminished by NAC. HL-60 cells (5×10^6) were treated with μM AN-1, AN-193 and AN-192 and 100 and 500 μM of AN-191 in the absence (**a**) or presence (**b**) of 5 mM NAC. The cells were harvested, lysed and 40 μg protein samples were subjected to Western blot analysis



Scheme 2 Formation of *S*-formylglutathione adduct

The steady state that exists between formaldehyde and GSH in the cells is also supported by the attenuation of FARPs induced cell death by GSH and by our recent observation that GSH abrogated formaldehyde mediated doxorubicin-DNA adducts formation induced by the FARP AN-9 [36].

Since, we have previously shown that the FARPs AN-193 or AN-1 increase the cellular level of formaldehyde in HL-60 cells [2], the sharp decline in cellular GSH can thus be attributed to the formation of *S*-formylglutathione. AN-193 and AN-192 that release two equivalents of formaldehyde, exhibited a greater potency in reducing the GSH level than AN-1 that releases only one equivalent of the aldehyde. This is consistent with our previous observation showing that AN-193 increased cellular formaldehyde by an approximately twofold higher level than AN-1 [2]. The AARPs, AN-11 and AN-191, were significantly less effective in reducing GSH (Fig. 3).

Since GSH plays a central role in maintaining redox homeostasis, its reduction inevitably leads to oxidative stress that activates numerous cellular events leading to cell death [32, 37–39]. Therefore, it can be deduced that reduction of cellular GSH by FARPs constitutes a major mechanism by which these prodrugs promote cell death.

While increased GSH level in cells has been implicated in resistance to chemotherapy, agents that reduce GSH were reported to reverse the resistant phenotype [39, 40]. The depletion of cellular GSH by FARPs is thus likely to contribute to the reduced tumor cell resistance seen previously upon exposure to these prodrugs [2, 5].

Formaldehyde-releasing prodrugs elevated the intracellular level of ROS and NAC reduced it while the AARP AN-191 only at a fourfold higher concentration lead to a similar elevation of ROS that was unexpectedly further increased by NAC. This observation is consistent and may explain the augmentation of AN-1 and AN-191 toxicity toward HL-60 and MCF-7 cells by NAC. Since AN-193 and AN-191 differ only by the nature of the released aldehyde, the opposite effects of NAC on their stimulatory effects on ROS release are difficult to fathom. The reason for this unusual observation is likely to be concealed in the multiple events governing the level of ROS in the cells; where some are linked to enhanced survival while others are associated with cell death [37, 38]. It was reported that low doses of ROS, particularly hydrogen peroxide, are mitogenic and promote cell proliferation, induce signal transduction and thus regulate gene expression while intermediate doses result in either temporary or permanent growth arrest. Very severe oxidative stress ultimately causes cell death via either apoptotic or necrotic mechanisms.

Differentiation induced by AN-192 was abrogated and that induced by AN-193 was attenuated by NAC while, differentiation induced by the AARP AN-191 was unaffected by it. These observations suggest that the formaldehyde released intracellularly induces differentiation by ROS-dependent and the BA by ROS-independent

mechanisms. These differences imply that intracellular BA and formaldehyde each induce cell differentiation by discrete mechanisms. BA was demonstrated to induce apoptosis of cancer cells by a mechanism associated with collapse of mitochondrial membrane potential [41]. Therefore, the greater activity of AN-193 as compared to AN-192 in the disruption of mitochondrial membrane potential can be attributed to the released butyric acid.

The dissimilar modes of action by which FARPs and AARPs affect cancer cells are also supported by the observation that the former increase the apoptotic (sub-G_1) population, whereas the latter, at higher concentrations induce G_0-G_1 cell cycle arrest (supplementary data).

The dissimilarity between the activities of FARPs versus AARPs was also evident in the protection of cardiomyocytes against Dox toxicity by the former and not by the latter [23]. The protective activity is in contrast to the synergism between Dox and FARPs in killing cancer cells [4, 5]. The dichotomy in activities exhibited by FARPs attest to the advantage of these prodrugs as selective anticancer agents. The high metabolic rate of cancer cells, compared to normal cells, is associated with increased electron transport chain activity and excess ROS production [42]. Therefore, it can be expected that cancer cells compared to normal ones will be more sensitive to GSH depletion. Such selectivity was also substantiated by the observation that HL-60 and Jurkat cells, compared to their normal mononuclear counterparts, were substantially more sensitive to treatment with FARPs (supplementary data).

Based on our findings it is likely that FARPs evoked ROS-release leading to the oxidative damage of DNA [30, 43]. Indeed, this was confirmed by the remarkable increase in the level of p-H2AX that was significantly attenuated by NAC. The AARP AN-191 at a fivefold higher concentration increased the phosphorylation of H2AX although to a lower level than that elicited by FARPs.

The protein c-myc is highly expressed in HL-60 cells due to gene amplification [44], and is directly involved in promoting cellular proliferation and inhibition of differentiation [45]. Its downregulation by ROS in these cells was reported previously [46]. FARPs abrogated the expression of c-myc after 4 h, whereas, at the same time AN-191 affected it to a substantially lesser extent. While NAC partially restored repression of c-myc caused by FARPs, it did not change significantly the effect of AN-191 on c-myc expression.

Phosphorylation of the c-Jun protein on ser63 and ser73, catalysed by c-Jun *N*-terminal kinase, inhibits cell cycle progression, promotes differentiation and apoptosis as well as other biological processes [47]. FARPs, transiently and in a ROS-dependent manner, increased the phosphorylation of c-Jun while AN-191 in the presence or absence of NAC had a minimal effect on the level of this protein.

Further the support of the role of intracellularly released formaldehyde in anticancer and cardio protective activities is provided by studies with the FARP sobuzoxane. Sobuzoxane is a topoisomerase inhibitor that when administered together with Dox synergizes its anticancer activity and attenuate Dox cardiotoxicity [48]. Recently we showed that sobuzoxane upon metabolic degradation releases formaldehyde and enhances Dox-DNA adduct formation. The latter suggest that sobuzoxane shift Dox activity from inhibition of topoisomerase II to induction of cytotoxicity by forming adducts with the DNA [36].

Collectively, our observations suggest that FARPs increase the level of ROS by forming *S*-formylglutathione adducts and thus diminishing the concentration of GSH, the ROS scavenger, triggering signals for differentiation and death of cancer cells. Other cellular changes triggered by FARPs associated with the induction of cancer cells death, such as increase in reactive nitrogen species (RNS) are currently under investigation.

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