Cancer Chemother Pharmacol (2007) 59:527–535
DOI 10.1007/s00280-006-0295-5

ORIGINAL ARTICLE

Adaphostin cytoxicity in glioblastoma cells is ROS-dependent and is accompanied by upregulation of heme oxygenase-1

Jason Long · Tejas Manchandia · Kechen Ban · Shan Gao · Claudia Miller · Joya Chandra

Received: 14 March 2006 / Accepted: 12 July 2006 / Published online: 19 August 2006

Abstract
Purpose To delineate a role for reactive oxygen species (ROS) induction in adaphostin-induced apoptosis in glioblastoma cells.
Methods Three glioblastoma cell lines with different sensitivities to adaphostin were characterized for sensitivity to an oxidant, tert-butyl hydroperoxide. The degree and duration of the ROS levels was assessed in the three cell lines after adaphostin exposure. Antioxidant protein levels were evaluated by Western blotting.
Results Of the three glioblastoma cell lines, the U87 cells were least sensitive to adaphostin. These cells were also least sensitive to tert-butyl hydroperoxide, indicating that sensitivity to a direct oxidant stress mirrors the cells' adaphostin sensitivities. In addition, the antioxidant *N*-acetylcysteine, (NAC) was protective against adaphostin-induced apoptosis. Direct measurement of intracellular peroxides showed a transient increase in the two less sensitive cell lines (U87 and LN18) which diminishes by 24 h. In contrast, U251 cells, which are most sensitive to adaphostin, display a sustained increase in the ROS levels. After the initial increase in intracellular peroxides, the heat shock protein and antioxidant heme oxygenase-1 (HO-1) was upregulated. Levels of other antioxidant proteins, such as catalase and thioredoxin, however, were not altered by adaphostin in glioblastoma cell lines. NAC attenuated HO-1 upregulation, confirming the time course analysis.
Conclusions These results suggest a primary role for ROS in adaphostin-induced apoptosis in glioblastoma. Our data indicate that the duration of intracellular ROS levels is a key factor in mediating sensitivity to adaphostin. Furthermore, upregulation of HO-1 is a novel molecular marker of adaphostin's action. The kinetics with which adaphostin upregulates HO-1 correlates with sensitivity to the drug. Taken together, our data indicate that a cell's ability to cope with ROS dictates sensitivity to adaphostin and conceivably other chemotherapies that cause redox perturbations.

Keywords Adaphostin · Oxidative stress · ROS · HO-1 · Catalase · Thioredoxin

Introduction

Approximately 17,000 primary brain tumors are diagnosed every year, and of those, about 60% are gliomas. Glioblastoma multiforme is the most prevalent and malignant form of glioma. The median overall patient survival is less than 1 year after diagnosis with glioblastoma multiforme, even with treatment [20], and has remained relatively constant over the past 25 years. Current therapies employ surgery, chemotherapy, and radiation, but they lack the efficacy seen in other forms of cancer. New therapies are necessary; however, they can build on the strengths of current ones. Ionizing radiation has long been regarded as the most effective adjuvant therapy in glioblastoma [2] and

J. Long · T. Manchandia · K. Ban · S. Gao · C. Miller · J. Chandra (✉)
Department of Pediatrics Research, Division of Pediatrics, University of Texas M.D. Anderson Cancer Center, 1515 Holcombe Blvd., 853, Houston, TX 77030, USA
e-mail: jchandra@mdanderson.org

its cytotoxicity relies on the formation of reactive oxygen species (ROS) [21]. Since ROS induction appears to have a therapeutic benefit, a strategy for improving glioblastoma treatment might combine this feature with another mode of cytotoxicity, such as kinase inhibition.

A promising agent currently in preclinical evaluation is adaphostin, a tyrphostin kinase inhibitor that raises the intracellular ROS levels in leukemia cell lines and patient specimens [4]. Adaphostin arose as the adamatyl ester analog of the tyrphostin AG957 [13] and is in preclinical evaluation at the National Cancer Institute [25]. Although both AG957 and adaphostin were initially characterized as BCR/ABL kinase inhibitors, subsequent work from numerous investigators indicates that the adaphostin does more than simply inhibit $p210^{Bcr/abl}$. Firstly, adaphostin has a higher K_m value for $p210^{Bcr/abl}$ than AG957, however it is able to induce apoptosis in leukemia cells at lower concentrations than its parent compound [25]. This, in combination with the observation that cells which lack BCR/ABL are sensitive to adaphostin toxicity, [1, 4, 18, 26] indicates an entirely different mechanism than a simply kinase inhibition alone. In leukemia cell lines, as well as patient derived lymphocytes [4–6, 18, 24], ROS induction by adaphostin has been well documented and is necessary for the drug's cytotoxicity.

The in vivo efficacy of adaphostin in orthotopic mouse models for glioblastoma was reported by Avramis et al. [1]. Combining adaphostin with a Flt-1/Fc chimera which inhibits VEGF caused synergistic effects in vitro and in vivo, and was attributed to adaphostin's ability to inhibit VEGF-R1 initiated kinase signaling. However, a role for ROS in this mechanism of action was not addressed. The present study corroborates and extends the finding that adaphostin is cytotoxic to glioblastoma cells. We find that sensitivity to a pure oxidant, tert-Butyl hydroperoxide, and the duration of intracellular peroxide production are predictive of adaphostin's efficacy. Furthermore, we have identified upregulation of heme-oxygenase-1 (HO-1), also known as hsp32, as a molecular marker for adaphostin's action in glioblastoma.

Materials and methods

Chemicals

Adaphostin (initially called NSC680410) was kindly provided by Dr. Robert Schultz, Developmental Therapeutics Program, National Cancer Institute (Bethesda, MD, USA). *N*-acetylcysteine (NAC), Trypsin-EDTA, propidium iodide (PI), Triton X-100, and tert-Butyl hydroperoxide were obtained from Sigma (St. Louis, MO, USA). Dye for detection of intracellular peroxides (5-chloromethyl-2′,7′-dichlorofluorescein diacetate [CM-H_2DCFDA]) was purchased from Molecular Probes (Eugene, OR, USA).

Cell lines

All cells were grown in a humidified incubator at 37°C with 5% CO_2. LN18 and U87 glioblastoma cells were acquired from American Type Culture Collection (Manassas, VA, USA). U-251 glioblastoma cells were kindly provided by Dr. Vinay K. Puduvalli, Department of Neuro-Oncology, M.D. Anderson Cancer Center. The three glioblastoma cell lines were all grown in a DMEM/F12 50:50 mixture containing 10% heat-inactivated FBS, 50 U/ml penicillin, 50 μg/ml streptomycin, 1X MEM nonessential amino acids (Sigma), 1 mM Sodium Pyruvate, and 2 mM L-Glutamine.

Assessment of DNA fragmentation

Propidium iodide staining was used to quantitate the subdiploid population indicative of cells with fragmented DNA [19]. After indicated treatments in vitro, cells were harvested with Trypsin-EDTA, pelleted at 700*g*, and resuspended in phosphate-buffered saline (PBS) containing 50 μg/ml PI, 0.1% Triton X-100, and 0.1% sodium citrate. After at least 1 h of incubation in the PI solution, cells were vortexed gently and read on the FL-3 channel of a Becton Dickinson (Franklin Lakes, NJ, USA) FACSCalibur. The subdiploid population from a total of 10,000 events was quantitated using CellQuest software by measuring the percentage of cells below the G_1 peak.

Assessment of adaphostin effects on cell number

Cells were plated at a density of 150,000 per well and exposed to increasing concentrations of adaphostin or diluent. After 24 h, cells were stained with trypan blue, and live cells were counted on a hemacytometer.

Quantitation of intracellular peroxides

Cell-permeable CM-H_2DCFDA dye (Molecular Probes) was used to measure intracellular peroxide levels [22]. This dye diffuses into cells and is trapped there due to a de-esterification reaction. After reacting with intracellular peroxides, the fluorescent molecule

5-chloromethyl-2′-7′-dichlorofluorescein (DCF) is released. This fluorescence is measurable on the FL-1 channel of Becton Dickinson FACSCalibur. After treatment with adaphostin for the indicated time, cells were washed with PBS, incubated with DMEM/F12 media containing 10 μM CM-H_2DCFDA for 30 min at 37°C in darkness, harvested with Trypsin-EDTA, centrifuged at 700*g*, washed in PBS to remove excess dye, and then read on the FACSCalibur and analyzed using CellQuest software.

Western blotting

After indicated treatments, cells were harvested using Trypsin-EDTA, washed with PBS, resuspended in lysis buffer (1% Triton X-100, 150 mM NaCl, 5 mM EDTA, 20 mM Sodium Phosphate, pH 7.4) for 1 h at 4°C, and centrifuged at 20,000*g* for 15 min to remove debris. Fifty micrograms of protein from each lysate were separated on 12% acrylamide gels by SDS-PAGE and then transferred to nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). Incubations with 1:1,000 dilutions of antibodies against actin (Sigma), catalase (Calbiochem, EMD Biosciences, Inc., La Jolla, CA, USA), HO-1 (Stressgen, VIC, Canada), and thioredoxin (BD PharMingen, San Diego, CA, USA) were then performed overnight with subsequent incubation in ECL™ Anti-rabbit IgG Horseradish Peroxidase linked whole enzyme (Amersham Biosciences, Buckinghamshire, England) and band visualization with ECL plus Western Blotting Detection System (Amersham Biosciences).

Statistics

A minimum of three experiments was performed for each condition examined. Results are presented as the mean ± standard deviation of the indicated number of replicates. The *P* values reported result from paired student's *t* tests used to analyze the differences between groups studied.

Results

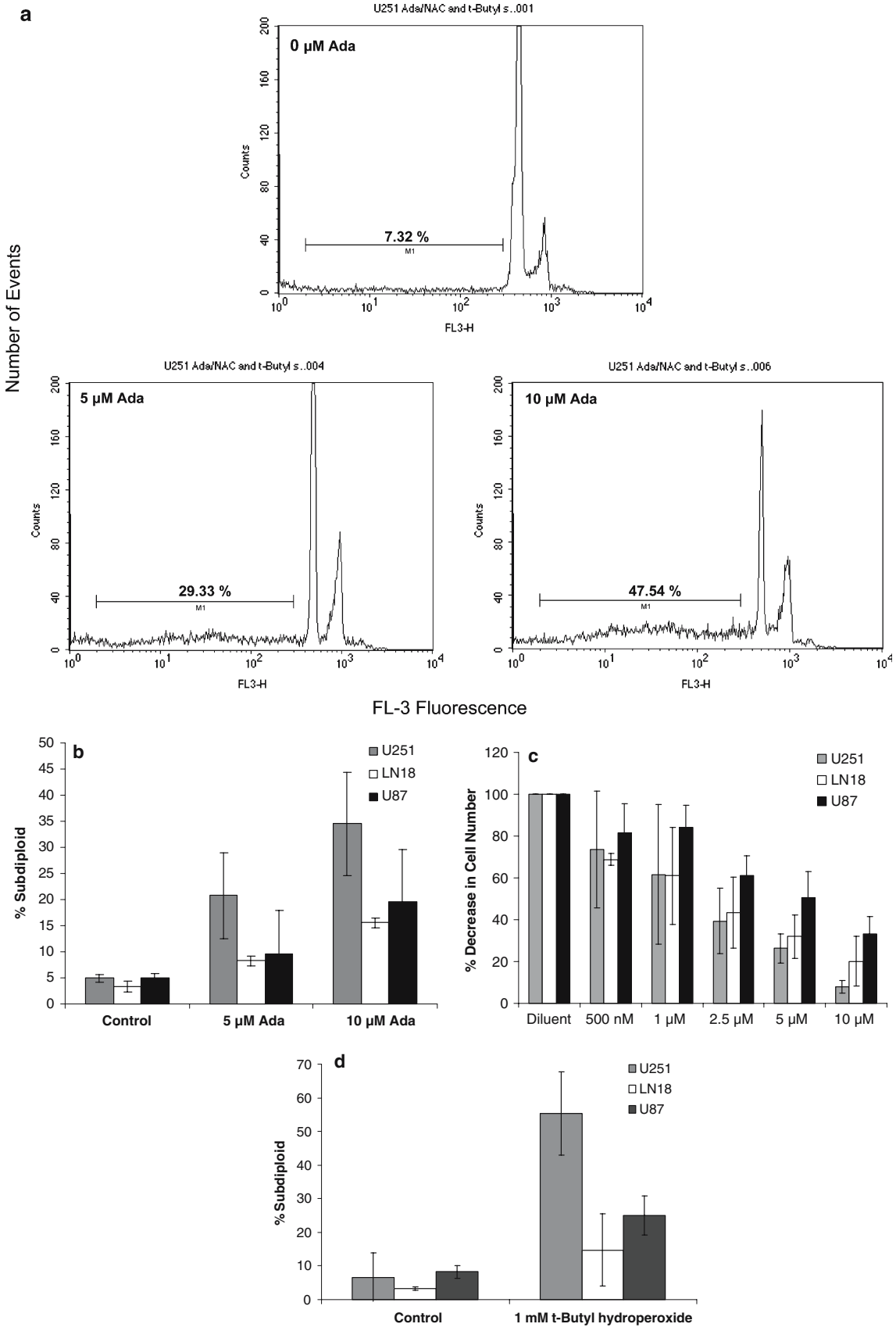
Differential sensitivity to adaphostin in three glioblastoma cell lines

Previous studies using leukemia cell lines and patient specimens have shown that adaphostin induces ROS-dependent apoptosis characterized by DNA fragmentation [4, 5], whereas in glioblastoma cells, cell death has solely been assessed ex vivo by MTT assay [1]. In order to determine if DNA fragmentation accompanies adaphostin-induced cell death in glioblastoma, three widely used glioblastoma cell lines—U251, U87, and LN18—were employed. Sensitivity to adaphostin-induced cell death was first assessed by treating these three lines with 5 and 10 μM concentrations of adaphostin for 24 h. Staining with PI and subsequent fluorescence-activated cell sorting (FACS) analysis determined the extent of DNA fragmentation by quantitating the percentage of cells composing the subdiploid population (Fig. 1a). When net DNA fragmentation was calculated for each cell line by comparing untreated cells to the 10 μM dose of adaphostin, the U251 cells showed a higher absolute increase in DNA fragmentation ($P = 0.03$) than either LN18 ($P = 0.05$) or U87 ($P = 0.14$) cells, indicating that the U251 cells are the most sensitive to adaphostin-induced apoptosis (Fig. 1b).

Adaphostin also caused a net decrease in cell number in all three cell lines which was dose dependent (Fig. 1c). Comparison of values across cell lines for percent decrease in cell number after exposure to 10 μM adaphostin revealed that there was a significant difference between U251 and U87 cells ($P = 0.01$) and between U251 and LN18 cells ($P = 0.01$). However, comparison of effects of the 10 μM dose of adaphostin in LN18 versus U87 showed no significant difference ($P = 0.21$). Taken together, these data indicate that the U251 cells display significantly more sensitivity to adaphostin at the 10 μM dose as compared to the other two cell lines (U87 and LN18).

Differential sensitivity to a direct oxidant

In order to determine if sensitivity to general oxidative stress is a factor in the apparent disparate sensitivity to adaphostin observed across the glioblastoma cell lines, tert-Butyl hydroperoxide, a strong oxidant, was administered to the cells. Dose response and time course analysis indicated that no significant DNA fragmentation occurred in any of the three cell lines below an 8 h exposure to 1 mM tert-Butyl hydroperoxide (data not shown). At this time-point and concentration, the net levels of DNA fragmentation normalized to control values show that the U251 cells experience a much greater absolute increase in DNA fragmentation than both LN18 ($P = 0.05$) and U87 ($P < 0.05$) cells (Fig. 1c). This correlation between the cell lines' sensitivities to adaphostin and tert-Butyl hydroperoxide suggests that the two agents may share a common mechanism.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U251 Ada/NAC and t-Butyl s..001
0 μM Ada
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U251 Ada/NAC and t-Butyl s..006
10 μM Ada
47.54 %
Number of Events
FL-3 Fluorescence
b
U251
LN18
U87
% Subdiploid
Control
5 μM Ada
10 μM Ada
c
% Decrease in Cell Number
Diluent
500 nM
1 μM
2.5 μM
5 μM
10 μM
d
% Subdiploid
Control
1 mM t-Butyl hydroperoxide

◀**Fig. 1** Differential sensitivities to adaphostin in three glioblastoma cell lines. **a** U251 cells undergo DNA fragmentation as assessed by PI staining. In all experiments, cells were treated at indicated doses, fixed and stained with PI and the percent subdiploid population quantitated by flow microfluorimetry. Representative histograms from one experiment in U251 cells either untreated or treated with 5 or 10 μM adaphostin for 24 h. The percentage of subdiploid cells is indicated within each histogram. **b** Adaphostin sensitivity varies in glioblastoma cells. Glioblastoma cell lines U251 (*grey bars*), LN18 (*white bars*) and U87 (*black bars*) were either untreated or exposed to 5 or 10 μM adaphostin concentrations for 24 h. *P* values for untreated versus 10 μM adaphostin for U251 was 0.03; for LN18 was 0.05; and for U87 was 0.141. **c** Adaphostin diminishes cell number in all three glioblastoma cell lines. Wells containing 150,000 of the U251 (*gray bars*), LN18 (*white bars*) or U87 (*black bars*) cells were treated with the indicated doses of adaphostin for 24 h. Cells were harvested and counted after staining with trypan blue (to exclude dead cells) using a hemacytometer. *P* values comparing percent decrease in live cell number after treatment with 10 μM adaphostin indicated that the U251 and LN18 cells were statistically different ($P = 0.01$). U251 and U87 cells were also statistically different ($P = 0.01$). U87 and LN18 were not statistically different ($P = 0.21$). **d** Sensitivity to t-Butyl hydroperoxide varies in glioblastoma cells. Cells were either untreated or exposed to 1 mM t-Butyl hydroperoxide for 8 h, stained with PI and examined for subdiploid population percentage by flow microfluorimetry. Absolute increase in DNA fragmentation was $P > 0.05$ for U251; $P = 0.05$ for LN18 and $P < 0.05$ for U87. Data are shown as mean ± SD from three independent experiments

An antioxidant prevents DNA fragmentation in U251 and LN18 cells

Experiments in several leukemia models, including peripheral blood mononuclear cells isolated from CLL [24], CML, and Ph + ALL patients [4], indicate that the cytotoxic and growth inhibitory effects of adaphostin can be blunted with antioxidants. In order to extend this finding to the glioblastoma cell lines, we set out to determine if an antioxidant could inhibit cell death caused by adaphostin. *N*-acetylcysteine (NAC) is an antioxidant thought to work by stimulating production of the endogenous antioxidant glutathione (GSH) [23]. In all three glioblastoma cell lines, a duplicate of every adaphostin treatment received a 15 min pretreatment with 24 mM NAC (Fig. 2). At the 10 μM dose, both U251 and LN18 show protection from overall levels of DNA fragmentation when NAC is present in combination with adaphostin versus when the drug is given without NAC. This protection was statistically significant in the U251 cells when comparing cells treated with 10 μM adaphostin alone versus 10 μM adaphostin and NAC ($P = 0.05$). However, the same analysis using identical doses in the LN18 cells was not statistically significant ($P = 0.11$), reflecting adaphostin's inability to elicit high levels of DNA fragmentation in these cells, which is concordant with data presented in Fig. 1. The ability of NAC to significantly protect the U251 cells from adaphostin-induced apoptosis suggests that ROS induction is a major mechanism in this tumor model.

Intracellular peroxide levels vary after adaphostin administration

The observations that (1) variance in adaphostin sensitivity in the glioblastoma cell lines correlates to differences in sensitivity to oxidative stress and that (2) apoptosis caused by adaphostin could be blunted by an

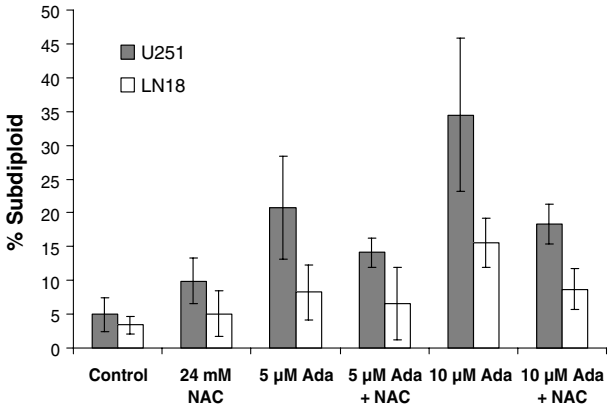

Fig. 2 Antioxidant protection from adaphostin-induced apoptosis. Cells were treated with 5 or 10 μM adaphostin in the presence or absence of 24 mM NAC for 24 h, fixed and stained with PI, and subdiploid population quantitated by microfluorimetry. Data are shown as mean ± SD from three independent experiments. U251 cells displayed a significant decrease in adaphostin induced apoptosis when NAC was used in combination with 10 μM adaphostin ($P = 0.05$). LN18 cells did not ($P = 0.11$)

antioxidant, led us to identify and quantitate intracellular ROS levels after administration of the drug. The CM-H_2DCFDA-staining was used to measure intracellular peroxide levels at a variety of time-points between 4 and 24 h after adaphostin treatment. Figure 3 shows that there is no significant difference in the intracellular peroxide levels of untreated samples between U251 and U87 or between U251 and LN18. Furthermore, after 4 h of adaphostin exposure, intracellular peroxide levels peaked in LN18 and U87 cells, with no significant difference in the average fluorescence across all three cell lines at this time point. By 8 h after adaphostin treatment, each cell type had an increase in intracellular peroxide levels to similar magnitudes, but this increase subsided in U87 and LN18 by 24 h post-treatment whereas peroxide levels continued to climb in U251 cells. At 24 h after adaphostin treatment, the U251 cells had significantly higher levels of

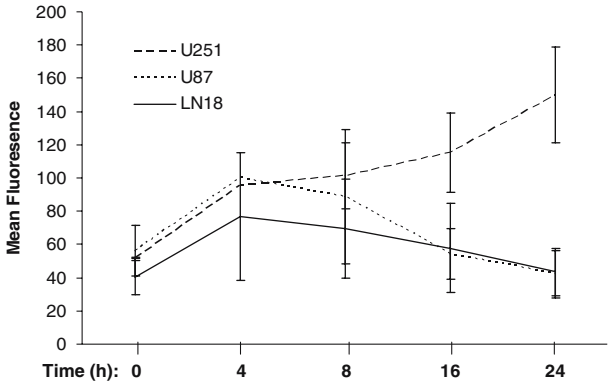

Fig. 3 Sustained versus transient intracellular peroxide production. Intracellular peroxide levels were measured in U251 (*dashed line*), U87 (*dotted line*), and LN18 (*solid line*) cells during a time course treatment of 10 μM adaphostin for 4, 8, 16, and 24 h. Cells were stained with CM-H_2DCFDA for 30 min after treatment and then FL-1 fluorescence was measured on a flow cytometer. No significant difference in the intracellular peroxide levels of untreated samples between U251 and U87 ($P > 0.06$) or between U251 and LN18 ($P > 0.16$) was apparent. By 8 h after adaphostin treatment, each cell type showed an increase in intracellular peroxide levels to similar magnitudes (U251 vs. U87, $P > 0.06$; U251 vs. LN18, $P > 0.20$). The U251 cells showed an increase in intracellular peroxide levels after 24 h of adaphostin exposure as compared to untreated cells ($P = 0.026$). Data are shown as mean ± SD from three independent experiments

intracellular peroxides than both U87 ($P < 0.05$) and LN18 ($P < 0.02$). Comparison of the increase in intracellular peroxide production from time 0 to 24 h of exposure to adaphostin indicated that there was a significant increase in the U251 cells ($P = 0.026$).

HO-1 upregulation with adaphostin exposure

Alterations in antioxidant enzyme defenses may cause or be a consequence of any general oxidative stress; however, this possibility has not been carefully addressed in adaphostin's mode of action. While lowered GSH levels have been cited as a consequence of adaphostin treatment in CML cell lines [4], the decrease was less than that seen with buthionine sulfoximine, which specifically depletes GSH but is not cytotoxic. In order to further characterize adaphostin-induced oxidative stress, we screened several cellular antioxidants after adaphostin exposure. HO-1 is an antioxidant and heat shock protein family member indicated to be downstream of BCR/ABL [16], suggesting that it may be a putative target for adaphostin action. Western blotting using whole-cell lysates of cells treated with adaphostin alone and adaphostin in combination with 24 mM NAC was employed to examine how the HO-1 expression varied with both dose (Fig. 4a) and time (Fig. 4b). Strong upregulation of HO-1 after adaphostin treatment appears dose dependent in all three glioblastoma cell lines (Fig. 4a–c). Time course analysis of HO-1 upregulation indicates that it follows heightened intracellular peroxide levels, occuring 8 h after administration of adaphostin in U251 (Fig. 4b), whereas ROS levels increase as early as 4 h after adaphostin treatment (Fig. 3). Interestingly, the U87 cells also upregulate HO-1 in response to adaphostin exposure; however, with slower kinetics (at 16 h) as compared to the U251 cells.

Because this HO-1 upregulation could be part of a general increase in oxidant-related proteins, we examined other relevant proteins as well. Neither thioredoxin, a ubiquitious antioxidant, nor catalase, an enzyme that breaks down hydrogen peroxide to water and molecular oxygen, was altered after multiple adaphostin exposures up to 24 h in U251 cells (Fig. 4d). Similar data, confirming that adaphostin does not affect thioredoxin or catalase protein expression, were also obtained in K562 and Jurkat leukemia cells (data not shown). These results indicate that adaphostin-induced oxidative stress selectively affects HO-1 protein levels rather than altering all cellular antioxidant mechanisms.

Discussion

The potential utility of adaphostin in glioblastoma has been demonstrated by Avramis et al. [1] in vivo. Our data, presented herein, reconcile that observation with studies conducted in leukemia cells indicating that adaphostin cytotoxicity is ROS dependent [4, 5]. We show that adaphostin raises levels of intracellular peroxides in glioblastoma cell lines (Fig. 3) and the antioxidant, NAC, blunts adaphostin's cytotoxicity (Fig. 2). Furthermore, sensitivity to adaphostin correlates with sensitivity to tert-Butyl hydroperoxide (Fig. 1c), a direct oxidant. These results confirm that the mode of action of adaphostin in glioblastoma cells is comparable to that observed in leukemia lines.

Sensitivity to adaphostin in glioblastoma cells also correlates with the ability to clear intracellular peroxides. As this is likely a function of cellular antioxidants, we examined a series of antioxidant proteins (Fig. 4). Increased expression of antioxidant enzymes has been noted in more radioresistant variants of glioblastoma [12], which are consequently more resistant to oxidative stress. Interestingly, recent work in two of the cell lines examined in this study shows U87 to be more radioresistant than U251 [10]. Given the ROS mechanism of radiation cytoxicity, the striking correlation of these two cell lines' sensitivities to adaphostin supports

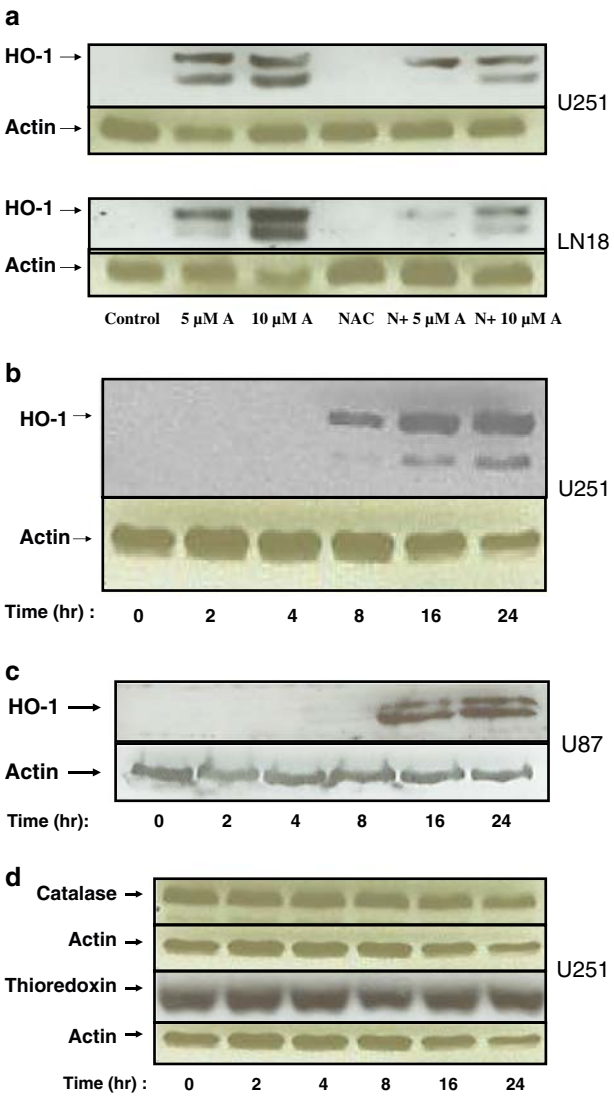

Fig. 4 Selective upregulation of HO-1 with adaphostin. **a** HO-1 protein is upregulated in U251 and LN18 cells. Cells were either untreated, treated with 5 or 10 μM adaphostin alone, or treated with adaphostin after a 15 min pretreatment of NAC and the product probed for HO-1. Whole cell lysates were made from cells with indicated treatments, run by SDS-PAGE, and probed with primary antibodies against indicated proteins. **b** Time course of HO-1 protein upregulation in U251 cells. The U251 cells were either untreated or treated with 10 μM adaphostin alone for 2, 4, 8, 16, or 24 h as indicated and lysates probed for HO-1. The same blot was reprobed with an anti-actin antibody to demonstrate equal loading. **c** Time course of HO-1 protein upregulation in U87 cells. The U87 cells were either untreated or treated with 10 μM adaphostin alone for 2, 4, 8, 16, or 24 h as indicated and lysates probed for HO-1. The same blot was reprobed with an anti-actin antibody to demonstrate equal loading. **d** Catalase and thioredoxin are not upregulated with adaphostin treatment in glioblastoma cells. Cells were either untreated or treated with 10 μM adaphostin alone for 2, 4, 8, 16, or 24 h as indicated and lysates probed for catalase, thioredoxin or actin

our findings of the primary role of ROS in adaphostin's cytoxicity and suggests further that antioxidant enzymes may be important in both cases. Adaphostin and its parent compound were originally developed for their activity against BCR/ABL. Therefore, we chose to examine antioxidant proteins putatively downstream of BCR/ABL that might be commonly expressed in glioblastoma. Levels of thioredoxin are driven by NFkB [8], a transcription factor downstream of BCR/ABL [11]. Western blotting indicates that levels of this ubiquitous antioxidant are not changed by adaphostin in glioblastoma or leukemia cells (Fig. 4d, and data not shown). Catalase, an antioxidant that inactivates hydrogen peroxide, is phosphorylated by c-abl, which may target it for proteasomal degradation [3]. However, protein levels of catalase also remained constant with adaphostin treatment of both glioblastoma and leukemia cell lines (Fig. 4d and data not shown). A third antioxidant, HO-1 (also known as hsp32), was reported to act as a survival factor for CML cells [16]. Interestingly, rather than deplete HO-1 levels, adaphostin caused a robust upregulation of HO-1 protein in all three glioblastoma cell lines (Fig. 4a–c). Time course analysis and NAC attenuation of the HO-1 protein increase indicate that this upregulation is a response to adaphostin-induced heightened peroxide levels. This finding is in line with results obtained by transcriptional profiling of adaphostin's effects in leukemia cell lines [9]. In K562, Jurkat and HL-60 cells, gene expression of hsp70 and hsp110 are increased, suggesting that adaphostin may cause a stress response characterized by upregulation of several heat shock proteins [9]. This same study addresses the mechanism by which adaphostin causes ROS induction and shows that adaphostin raises levels of free intracellular iron which could aid in the generation of hydroxyl radical via the Fenton reaction [9]. However, examination of the chemical structure of adaphostin reveals that it is a quinone and may undergo redox cycling, which may also account for its ability to raise ROS levels.

Heme oxygenase-1 catalyzes the NADPH, O_2, and cytochrome P450 reductase dependent oxidation of heme groups to carbon monoxide, free iron, and biliverdin [15]. Biliverdin is immediately reduced to the antioxidant bilirubin. Down-regulating biliverdin with specific siRNA has been shown to increase arsenic-dependent apoptosis fourfold in kidney cells, demonstrating its central importance in protection from ROS [17]. Hara et al. [7] found that the HO-1 mRNA was expressed at higher levels in many brain tumors compared to brain tissue. In addition, Li et al. [14] found that the HO-1 is one of a set of genes upregulated in neuroblastoma by the antioxidant response element (ARE) that can protect cells from oxidative stress. In order to connect the observed relatively higher sensitivity of U251 to both adaphostin and oxidative stress,

and the involvement of HO-1, we propose that the timing of HO-1 upregulation may be critical. In the U87 cells, the HO-1 upregulation occurs after 16 h of exposure to 10 μM adaphostin whereas in the U251 cells, the HO-1 upregulation occurs after 8 h of drug exposure. This data further validates a model in which the HO-1 upregulation is a response to adaphostin-induced oxidative stress.

The increased intracellular peroxide levels observed in adaphostin treated cells lead to a loss of cellular glutathione in leukemia cells [4]. In the current study, administration of NAC, which is thought to increase intracellular glutathione levels [23], significantly prevented adaphostin-induced apoptosis (Fig. 2), suggesting that GSH levels are also likely relevant to adaphostin action in glioblastoma cells.

Given adaphostin's development as a BCR/ABL directed tyrosine kinase inhibitor, we cannot rule out effects that adaphostin may be exerting on kinases present in glioblastoma cells. Reduced VEGF secretion and a reduction in VEGF receptor protein levels have been observed after adaphostin treatment [1, 9] and have been suggested as a potentially significant mechanism of adaphostin's cytotoxicity. This VEGF downregulation may be one of the multiple events that occur independently of adaphostin's effects on ROS. For example, in BCR/ABL containing cells, adaphostin degrades BCR/ABL protein within 8 h of treatment, but this function appears unrelated to ROS, as pretreatment with NAC does not prevent this degradative effect [18].

Drug sensitivity within the glioblastoma cell lines we have utilized has not been addressed in the past. In comparison to other cell lines in the 60-cell-line panel used for drug screening by the National Cancer Institute, the U251 cells have a slightly higher than average sensitivity to adaphostin [9]. Avramis et al. [1] identified U87 as sensitive to adaphostin-induced cytotoxicity as well, but prior to this study, no comparison to other glioblastoma lines has been conducted. Our data suggest that the differences are significant and that the cell lines' ability to clear intracellular peroxides may be responsible for the increased sensitivity of U251 cells to adaphostin as compared to LN18 and U87. From this result, we propose that the U251 cells are lacking a protective mechanism against ROS that seems to still be intact in LN18 and U87.

The knowledge that ROS and a stress response dependent mechanism are involved in adaphostin-mediated cytotoxicity in glioblastoma is useful when considering how to make the drug more effective. Adaphostin resistant cells can be generated through repeated exposure to the drug (S. Kaufmann, personal communication). Given the data presented in this study, it appears that adaphostin sensitivity relates to the cells' ability to remove intracellular oxidants. By targeting these specific antioxidants, it is likely that the sensitivity differences to the drug will diminish, rendering it effective against a much broader range of cell types.

Acknowledgements We are grateful to Scott Kaufmann, M.D., Ph.D., and Eugenie Kleinerman, M.D., for helpful critical discussions. We also thank the M.D. Anderson Institutional Research Grant Program for support of studies examining adaphostin's cytotoxicity.

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