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Cellular resistance to a nitric oxide releasing glutathione S-transferase P-activated prodrug, PABA/NO

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Summary

PABA/NO is a diazeniumdiolate selectively activated by glutathione S-transferase P (GSTP) to release nitric oxide (NO) and is a potent inducer of protein S-glutathionylation, a redox-sensitive post-translational modification of cysteine residues. Using a procedure that incrementally increased exposure of cells to PABA/NO, an acquired drug resistant human promyelocytic leukemia HL60 cell line (HL60^{PABA}) that exhibited 1.9-fold resistance to the drug (IC₅₀ ~15 μM vs ~8 μM for wild-type) was created. HL60^{PABA} cells had a decreased growth rate attributable to altered cellular differentiation, as measured by increased expression of CD11b; decreased expression of CD14; decreased nuclear to cytoplasmic ratios and a condensation of nuclear chromatin. This was accompanied by alterations in both plasma and mitochondrial membrane potentials. Both GSTP expression and nitric oxide release were reduced two-fold, while increased expression levels of genes involved in the unfolded protein response (UPR) were evident in HL60^{PABA} cells. Wild type cells treated with PABA/NO had increased levels of protein S-glutathionylation and JNK activation, while JNK was constitutively active in HL60^{PABA} cells and these cells had reduced levels of S-glutathionylation. By removing PABA/NO from the growth medium, HL60^{PABA} cells reverted to sensitivity within 21 days suggesting that resistance was not genetically stable. Mechanistically, PABA/NO resistance is mediated through reduced levels of GSTP resulting in reduced NO release and its subsequent alterations in cellular response to nitrosative stress.

Keywords

Glutathione; Glutathione S-transferase; Nitric oxide; Oxidative stress; Nitrosative stress; Unfolded protein response

Introduction

Glutathione S-transferase P (GSTP1/2) is one of the members of a cytosolic GST super-family composed of alpha, mu, pi, omega, theta and zeta [1,2]. A single gene located on chromosome 11 encodes for proteins designated in the Pi class (GSTP1). The *GSTP1* gene spans ~3 kb, encodes 210 amino acids in seven exons [3]. Expression of GSTP1 occurs to varying extents in most tissues and cells [4] and is frequently increased in tumors. Moreover, a primary and reproducible characteristic of most tumor cells is an aberrant redox environment that expresses through various changes in glutathione homeostasis. As a consequence, there exists the potential to intervene in these pathways in a manner that can yield a beneficial therapeutic index. Increased GSTP expression patterns have been directly associated with ovarian, NSCLC, breast, liver, pancreas, colon cancers and lymphomas [5]. In addition, there are many examples of cancer cell lines that have acquired resistance to a range of cancer drugs that show high expression of GSTP. One of the conundrums in this field has been that in many instances, the drug used in selecting resistance is not a substrate for thioether conjugation with GSH via GST catalysis, raising the question of why such enhanced expression should occur [6]. This is particularly pertinent when considering that in some cases GSTP can be the most prevalent cytosolic protein in the cell. As a rationale for drug design, prodrugs activated by GSTP should provide an enhanced therapeutic index and preclinical and clinical experiences with Telcyta (TLK286) have provided a paradigm for this approach [7].

Nitric oxide (NO) is involved in a diverse number of physiological processes characterized in many previous publications, [8] but can also have cytotoxic consequences. Through interaction with metals, superoxides, oxygen and glutathione, nitric oxide can also lead to S-glutathionylation, a reversible, post-translational modification involved in cell signaling [9,10]. As a consequence, directed delivery (tumor cells with high GSTP) of a therapeutic concentration of nitric oxide is a relevant approach to drug design. A strategy to derivatize the O² position of a diazeniumdiolate with protective groups has been used to convert them into substrates for GST [11,12]. The resulting inactive prodrug can become cytotoxic when localized in a cell that has high GSTP concentrations. PABA/NO (O²- {2,4-dinitro-5-[4-(N-methylamino)benzoyloxy]phenyl} 1-(N,N-dimethylamino) diazen-1-ium-1,2-diolate) has N-methyl-p-aminobenzoic acid bound via its carboxyl oxygen as a 5-substituent on the 2,4-dinitrophenyl ring [13,14]. PABA/NO belongs to the O²-aryl diazeniumdiolates, electrophiles shown to transfer their aryl groups to attacking nucleophiles with simultaneous production of ions that release NO at physiological pH. In the presence of GSH PABA/NO is activated by GSTP, as shown in Fig. 1 [12]. This reaction results in the formation of a Meisenheimer-complex intermediate, and subsequently the leaving group of the reaction generates two molecules of NO. Elevated NO levels lead to cytotoxic effects by forming RNS/ROS intermediates. In our hands, PABA/NO-induced nitrosative stress results in limited levels of protein nitrosylation, but high levels of S-glutathionylation [9,15].

Drug resistance remains a primary obstacle in the therapeutic treatment of cancer. In a preclinical setting, drug resistant cell lines can be useful experimental models for studying adaptive cellular responses to chronic drug induced stress. In turn, analysis of the adaptive changes can provide information regarding plausible mechanism(s) of action of a novel agent. The present study was designed to establish an in vitro model of chronic nitrosative

stress and to study those factors that might contribute to resistance to nitric oxide based therapies.

Methods

Materials

PABA/NO was prepared as previously described [12,15] and provided by Dr. Larry Keefer, Basic Research Program, SAIC, NCI, Frederick, MD. DAF-FM-DA and JC-9 fluorescent probes were from Invitrogen (Carlsbad, CA). ThioGlo-1 fluorescent probe was from Calbiochem (San-Diego, CA). Antibodies were obtained commercially from the following companies: anti-GSH (Virogen, Watertown, MA), anti-GSTP1 (MBL International Corporation, Woburn, MA), anti-GAPDH (Novus Biologicals, Littleton, CO), anti-JNK1/2 (BD Biosciences, San Diego, CA), anti-p-JNK, anti-p38, and anti-p-p38 (Santa Cruz Biotechnology, Santa Cruz, CA). Fluorescent conjugated antibodies CD11b-PECy7, CD14-PE, and Annexin V-FITC were all obtained from BD Biosciences.

Cell lines

Human promyelocytic leukemia HL60 cells were cultured in suspension in RPMI-1640 (Cellgro, Herndon, VA) supplemented with 10% fetal bovine serum (HyClone, Logan, Utah), 1% L-glutamine (Invitrogen, Carlsbad, CA), 1% Penicillin-Streptomycin (Cellgro), and 1% MEM non-essential amino acids (Invitrogen) at 37° C in 5% CO₂. HL60^{PABA} cells were created by exposing wild type cells to 1 μM PABA/NO for a period of at least two passages and through continuous selection in increasing doses (incremental increasing by 1 μM). PABA/NO is maintained in 100 mM stocks in 100% dimethyl sulfoxide (DMSO) (Sigma, St. Louis, MO), and drug dilutions were prepared fresh in medium immediately prior to treatment. 2×10⁵ cells were initially treated with 1 μM PABA/NO and assessed for viability every 48 h. Once viability reached greater than 80%, drug dosage was increased in 0.5 μM increments until growth continued to be exponential. In most cases, the incremental increases were administered approximately once every 2 to 3 weeks. Final selection of the HL60^{PABA} culture occurred after approximately 6 months. Experimental controls include vehicle treated HL60 cells (0.01% DMSO for 6 days) and HL60 cells treated with 1.5% DMSO for 6 days. An HL-60^{Revertant} line was created by withdrawing drug from the cells for at least 7 passages prior to analysis. For all experimental procedures, HL60^{PABA} cells were grown for 24 hrs without PABA/NO prior to experimental procedures.

Growth curve analysis

HL60, HL60^{PABA} and HL60^{Revertant} were plated at an initial density of 2×10⁵ cells/ml. The cell number and percent viability was assessed every 24 h for 5 days. Total cell number and percent viability was determined using an Auto T4 Cellometer (Nexcelom Biosciences, Lawrence, MA). To analyze cell growth in the presence of PABA/NO, every 24 h cells were counted and treated with a concentration of 8 μM. At each 24-hour interval following counting, cells were spun down and resuspended in new medium. Fresh drug was added each day for the PABA/NO treated groups. Total cell number was plotted as a function of time, and standard error is representative of three independent experiments.

Immunoblot

Equal amounts of protein were separated on 10% SDS-polyacrylamide gels and transferred overnight onto nitrocellulose membranes (Bio-Rad). Non-specific binding was reduced by incubating the membrane in 10% blocking buffer for 1 h containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10% bovine serum albumin, 0.1% Tween 20, 1× protease inhibitors. Protein expression was determined by incubating membranes with specific primary in 5%

blocking buffer according to manufacturer's recommendation. Briefly, the membranes were washed three times and incubated with the appropriate secondary antibodies, conjugated with horseradish peroxidase, in 5% blocking buffer for 1 h. The membranes were washed three times and developed with enhanced chemiluminescence detection reagents (Amersham Biosciences). The relative abundance of proteins was evaluated using ChemiDock XRS with Quantity One software (ver.4.5.2; Bio-Rad) and plotted as arbitrary units in relation to actin.

Protein preparation

Following PABA/NO treatment, cells were collected and washed twice with 1× phosphate-buffered saline. Cell pellets were lysed on ice in protein lysis buffer containing 20 mM Tris-HCl, pH 7.5, 15 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, and 1 mM-glycerophosphate freshly supplemented with complete protease inhibitor (Roche, Mannheim, Germany), 5 mM NaF and 1 mM Na₃VO₄. Cells were incubated on ice for 30 min, vortexed and sonicated twice for 10 s. Insoluble fraction was separated by centrifugation for 30 min at 14,000 rpm at 4°C, and supernatant protein concentrations were determined using the Bradford assay with IgG as a standard.

Flow cytometry

2×10⁶ cells per well were plated in 6-well plates containing 4 mls of complete medium. Five groups were represented: HL60, HL60 treated with 10 μM PABA/NO, HL-60^{PABA}, vehicle control, and 1.5% DMSO. Cells were collected after 24 h, washed and resuspended in Hank's balanced salt solution (HBSS). Replicates for each group were transferred to a 96 well plate, pelleted, and incubated for 30 min in the dark with antibody master mix containing CD14-PECy7, CD11b-PE, and FACS buffer (1× HBSS, 0.5% BSA, 0.1% sodium azide). Cells were pelleted and incubated for 15 min in the dark with Annexin-FITC antibody. Following incubation with Annexin V, cells were transferred to flow tubes and immediately analyzed using fluorescence-activated cell sorting analysis. Compensations were included in the analysis, and results were plotted as the relative percent gated population.

RNA isolation and quantitative real-time

RT-PCR Total RNA was isolated and DNase I treated using the RNeasy mini kit according to the protocol of the manufacturer (Qiagen, Valencia, CA). cDNA was prepared using either the iScript™ cDNA synthesis kit from BioRad (Hercules, CA) or the RT² First Strand Kit from SABiosciences (Frederick, MD) according to the protocols of the manufacturers. All quantitative real-time RT-PCR (qPCR) reactions were performed using the MyiQ™ system from BioRad, and iQ™ SYBR green supermix from BioRad or SYBR Green/Fluorescein PCR Master Mix from SABiosciences according to the protocols of the manufacturers. Actin mRNA levels were used to normalize differences in cDNA levels and the data are expressed using the $\Delta\Delta C_t$ method [16]. Primers were designed using Primer3 software [17] and previously reported [18] All reactions were performed in triplicate and data were analyzed using the $2^{-\Delta\Delta C_t}$ method [16]. GAPDH and actin was used as a gene control to normalize differences in cDNA between groups.

Protein sulfhydryl fluorescence detection

Supernatants from cell lysates (standard lysis buffer) were passed through Biospin-6 (BioRad) size-exclusion columns and diluted 1:100 with 20 mM PB (pH=7.4) in quartz cuvettes. After the addition of TG-1 (final concentration 5 μM, fluorescence at 513 nm (excitation at 379 nm) was recorded in real-time kinetics until saturation. The protein concentration of cell lysates was detected using the Bradford procedure (BioRad). The averaged emission saturation values were normalized for protein concentrations.

Plasma membrane potential measurements

Suspension of HL60 cells (0.5×10^6 cells/ml) in PBS with 100 μ M CaCl_2 (pH=7.4) was incubated under constant stirring in quartz cuvette at 37°C. BisOxanol fluorescent probe in DMSO was added to cell suspension (final concentration 5 μ M) and fluorescence kinetics at (at 520 nm excitation at 488 nm) was detected with resolution of 0.1 sec for 5 min. Experimental traces were averaged and smoothed using standard SigmaPlot 10.0 (Systat Software Inc., San Jose, CA) software.

Mitochondrial potential fluorescence detection

Fluorescent dye JC-9 (Invitrogen) was added (final concentration 0.3 μ g/ml) to the HL60 or HL60^{PABA} cell suspensions (1.0×10^6 cells/ml in PBS with 100 μ M CaCl_2 , pH=7.4). The cells were incubated in quartz cuvettes with dye at 37°C under constant stirring for 1 h. JC-9 fluorescence data were acquired in ratiometric mode (530/590 nm, excitation at 485 nm) of QM-4 (PTI) at 37°C under constant stirring.

Nitric oxide fluorescence detection

Suspensions of cells (0.5×10^5 cells/ml) were incubated with 5 μ M of DAF-FM-DA (3-Amino, 4-aminomethyl-2',7'-difluorofluorescein Diacetate (Invitrogen) for 1 h in complete media at 37°C. The labeled cells were washed 3 times with PBS, containing 100 μ M of CaCl_2 or 4 mM EGTA and used in quartz cuvettes (1.0×10^6 cells /ml) for real-time kinetic measurements in a QM-4 (PTI) spectrofluorometer at 37°C under constant stirring.

Cell morphology

5×10^5 cells were washed with $1 \times$ phosphate-buffered saline and smeared onto clean slides coated with poly-L-lysine (Sigma). Slides were left to air-dry overnight, then stained with Wright-Giemsa stain (Sigma) and imaged using a Nikon Eclipse E800 microscope.

Statistical analysis of experimental data

All experimental data were statistically treated using SigmaStat 3.5 (Systat Software Inc., San Jose, CA) and generally represented as the mean $\pm$ SD. We used *t*-test and ANOVA to evaluate the relevance of treatment differences.

Results

Effects of PABA/NO on HL60 Cell Growth and Viability

Chronic treatment of HL60 cells led to the development of resistant cells (HL60^{PABA}) with distinct growth characteristics. In the absence of drug for ~7 passages, the resistant cells showed a revertant, yet intermediate phenotype (HL60^{Revertant}). Growth curve analysis showed that HL60^{PABA} grew more slowly than HL60 or HL60^{Revertant} cells, $p < 0.001$ (Fig. 2a). The doubling time of the cells was calculated (HL60, 20 h; HL60^{PABA}, 50 h and HL60^{Revertant}, 28 h). These findings are consistent with changes in the cell cycle distribution (Fig. 2b) which showed HL60^{PABA} cells with a diminished number of cells in S and more in G2, values consistent with the slower proliferation rate, $p < 0.05$.

Figures 3a–b show the growth of HL60 and HL60^{PABA} cells when challenged on day 1 with 10 μ M or 20 μ M PABA/NO. There was a statistically significant difference in survival of HL60^{PABA} cells compared to HL60 cells, $p < 0.05$. At 24 h following 10 μ M PABA/NO treatment, only $32 \pm 4\%$ of HL60 cells survived versus $100 \pm 9\%$ of HL60^{PABA} cells. HL60 cells did recover from this treatment, however only the HL60^{PABA} cells continued growth following further drug exposure. Because response to both thapsigargin and cisplatin is also linked with GSTP expression and with S-glutathionylation [19], we assessed cell kill by

each of these drugs. In each case, collateral resistance of the HL60^{PABA/NO} cells was found, $p < 0.05$ (Fig. 2c and d).

We have shown that PABA/NO treatment leads to dose- and time- dependent increases in NO generation and activation of endoplasmic reticulum (ER) stress in HL60 and SKOV3 cells [18]. Basal levels of NO were approximately two-fold lower in HL60^{PABA} compared to wild type cells, $p < 0.001$ (Fig. 4). Addition of PABA/NO produced an increase of NO in each cell line, while the lower levels of NO in HL60^{PABA} cells remained equivalent. These results suggest that the resistant cells may be activating less of the drug. However, even though the resistant cells are able to grow in PABA/NO, our data suggest that they are in a state of continuous ER stress. Elevated levels of PDI, ERO1, BiP and XBP-1 are coordinate with ER stress and activation of the unfolded protein response (UPR) [18,20]. Table 1 shows by RT-PCR that HL60^{PABA} cells have elevated transcript levels for seven distinct gene products that have been associated with regulating the UPR. These data are consistent with adaptation of HL60^{PABA} to chronic ER stress as a consequence of drug-induced nitrosative stress.

We established previously that PABA/NO induces protein S-glutathionylation in a dose- and time-dependent manner in HL60 cells [9]. However, the influence of chronic exposure to PABA/NO on protein S-glutathionylation is not known. To examine this, we evaluated dose-dependent PABA/NO effects on S-glutathionylation patterns. Figure 5 confirmed the dose-dependency of PABA/NO increases in protein S-glutathionylation. Although the drug did induce S-glutathionylation in HL60^{PABA} cells, this was less dose-dependent and total levels were reduced. Interestingly, the baseline levels of S-glutathionylation were the same in both cell lines. The forward reaction of S-glutathionylation can be catalyzed by GSTP [21], the same enzyme that can activate PABA/NO. Figure 5a (second panel) shows that basal GSTP expression levels were >2-fold lower in HL60^{PABA} when compared to wild type, $p < 0.05$. Such data are consistent with the observed S-glutathionylation patterns. Moreover, the expression of JNK1 and its phosphorylated form is constitutively higher in the HL60^{PABA} cells ($p < 0.05$) and following PABA/NO treatment is diminished. However, these levels are not below those of the stimulated HL60 cells.

To show PABA/NO-mediated effects on cell surface and intracellular thiol redox status, we used a fluorescent (ThioGlo-1) detection technique. Figure 6a shows that HL60^{PABA} cells have decreased levels of cell surface protein sulfhydryls compared to wild type, $p < 0.001$. Additional treatments with PABA/NO further decreased cell surface protein-sulfhydryl content in both wild type and HL60^{PABA} cells, although there was no apparent dose response. These effects were insensitive to treatment with the disulfide reducing agent TCEP (data not shown). These data suggest that development of resistance to PABA/NO is accompanied by cell surface modification(s) that alter redox properties. This was supported by data for the plasma membrane potential following a single dose of PABA/NO (Fig. 6b). The membrane potential in the HL60^{PABA} cells was diminished compared to wild type.

Original efforts to assess collateral resistance using the MTT survival assays suggested that the HL60^{PABA} cells had some mitochondrial dysfunction, insofar as survival was incompatible with the growth curves. Accordingly, to measure the changes in the metabolism, we plated equivalent numbers of cells and immediately assessed mitochondrial reductase mediated conversion of the tetrazolium salt to formazan [22]. The MTT assay data indicated impairment of mitochondrial reductase activity in HL60^{PABA} cells resulting in a diminished conversion (per cell) relative to HL60 cells, $p < 0.05$ (Fig. 6c). To extend these results, we employed fluorescent detection of the mitochondrial membrane potential using the ratiometric dye JC-9. Our data showed that the initial trans-membrane potential in HL60^{PABA} cells was higher than in wild type, Fig. 6d. Moreover, the response of

mitochondrial membrane potential to additional PABA/NO was steeper in wild type compared to resistant cells (Fig. 6d). These data are consistent with drug induced partial impairment of mitochondrial function in resistant cells and indicate that PABA/NO may have direct effects on mitochondria that may be important to its mechanism of action.

Because NO can cause terminal differentiation of leukemic cells [14,23], we considered that the resistant phenotype observed in HL60^{PABA} may be linked with cellular differentiation. To test this, we performed flow cytometric analyses to measure the expression of cell surface antigen markers for myeloid differentiation, as well as Wright-Giemsa staining to examine cell morphology. Figure 7a shows the presence of cell surface antigen markers CD11b and CD14 expressed as a relative percent of the gated populations. Less than 1% of HL60 wild type cells expressed CD11b. However, HL60^{PABA} cells had a ~10-fold increase in CD11b compared to HL60 cells, $p < 0.001$. This increase was not seen following a single treatment of PABA/NO or vehicle control cells indicating that a portion of the HL60^{PABA} population was differentiated. A >2-fold increase in expression of CD14 was seen in both HL60 cells following a single PABA/NO treatment and HL60^{PABA} cells compared to vehicle and HL60 cells, $p < 0.001$. Wright-Giemsa staining illustrated a morphological difference between HL60 and HL60^{PABA} cells. Figure 8 shows that both HL60^{PABA} (panel D) and 1.5% DMSO treated cells (positive control; panel B) exhibited features characteristic of terminal differentiation including a marked change in size, decreased cytoplasm to nucleus ratio, reduction in number of nucleoli and nuclear segmentation. The morphology of the HL60^{Revertant} cells (panel C) showed a heterogeneous population that contained cells morphologically similar to HL60 (panel A) as well as differentiated cells.

Discussion

Tumor cell lines with acquired drug resistance frequently adapt cellular properties in response to the selection conditions. As a consequence, resistance models can provide information useful in understanding the drug's mechanism(s) of action. In the present study, an approximately two-fold resistance was achieved, even after 6–8 months of exposure to PABA/NO. Despite the extensive selection period, this low degree of resistance expressed is similar to another GSTP activated prodrug, where selection of a resistant HL60 cell line with Telcyta (TLK286; γ -glutamyl- α -amino- β (2-ethyl-N,N,N',N'-tetrakis (2-chloroethyl) phosphorodiamidate)-sulfonyl-propionyl-(R)-(-) phenylglycine]) was both unstable and difficult to achieve [24]. PABA/NO differs from Telcyta in that the latter produces an electrophilic aziridinium and a vinyl sulfone species. Conversely PABA/NO releases NO causing concomitant downstream nitrosative and oxidative stress. Resistance to each drug is similar in being accompanied by a reduced expression of GSTP. In each case this contributes to reduction in the kinetics of drug activation, a commonality that is unusual in that the majority of drug resistant cell lines over-express GSTP [5]. Since we have shown that GSTP catalyzes the forward S-glutathionylation reaction [21], the lower protein S-glutathionylation in HL60^{PABA} cells is also consistent with the adaptive down-regulation of GSTP. Treatment of wild type cells with PABA/NO increases JNK expression and phosphorylation. In HL60^{PABA} cells, there are constitutively high levels of JNK1 and its phosphorylated form, consistent with the chronic nature of the drug-induced stress.

The resistant cells have a significantly slower growth rate than the wild type, reflected by the smaller fraction of cells in G2/S phases. At least one contributing factor is the capacity of NO to cause HL60 cell differentiation. The proportion of HL60^{PABA} cells that differentiate as a consequence of drug selection provide an end stage population that may no longer contribute towards the overall proliferative index. Our results show that approximately 10% of the cells express differentiation markers that implicate movement into granulocyte/monocyte lineage. In principle, these cells could remain viable and because they

are not dividing, will be less sensitive to cytotoxic agents. They could represent end stage cells that will die off with time and might be less likely to contribute to repopulation since they will not re-enter the proliferative pool. When cells are passaged in the absence of PABA/NO for greater than 2 weeks the resistant cells revert to sensitivity and their growth characteristics become intermediate between the wild type or HL60^{PABA} cell lines. This implies that resistance is more a function of adaptive stress responses rather than genetic selection of a sub-population of resistant cells.

The HL60^{PABA} cells developed collateral resistance to thapsigargin and cisplatin. While this could be related to the generally reduced sensitivity of cells with slower doubling times, the altered thiol homeostasis found in the resistant cells may also be of consequence. Thapsigargin is an inhibitor of the Ca²⁺ dependent ATPase SERCA1 [25] and its metabolism does not obviously produce any electrophilic metabolites [26]. Nevertheless, we have found that thapsigargin can cause protein S-glutathionylation and that this may be a consequence of a linkage between S-glutathionylation and intracellular Ca²⁺ homeostasis (Manevich et al. unpublished data). In this regard SERCA is regulated by S-glutathionylation [27]. These results may imply similar downstream effects of these drugs on thiol and calcium regulatory pathways. Changes in cell surface thiols and cell membrane potential accompanying the resistance phenotype may have impact on drug uptake or efflux. In addition, the modified mitochondrial membrane potential could influence a number of pathways that regulate cell survival and these might contribute to the observed collateral response patterns.

In summary, PABA/NO is in preclinical development as a possible anticancer drug and has shown promising in vitro and in vivo activity [9,28]. The present data suggest that a number of cellular adaptations underlie resistance. At least one adaptive response is decreased expression of GSTP with accompanied lower levels of intracellular nitrosative stress and a concomitant lower protein S-glutathionylation. The fact that resistance was difficult to achieve and unstable may be favorable factors in continued drug development plans.

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Abbreviations

PABA/NO	(O ² -{2,4-dinitro-5-[4-(N-methylamino)benzoyloxy]phenyl} 1-(N,N-dimethylamino) diazen-1-ium-1,2-diolate)
GSH	Glutathione reduced
GSSG	Glutathione oxidized
ER-stress	Endoplasmic reticulum stress
GSTP	Glutathione S-transferase P
PDI	Protein disulfide isomerase
Tg	Thapsigargin
JNK	c-Jun N-terminal kinase
ROS-RNS	Reactive oxygen and nitrogen species
NO	Nitric oxide

TCEP	Tris(2-carboxyethyl) phosphine
UPR	Unfolded protein response

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Fig. 1.
Activation of PABA/NO. Modified from Reference [11]

Fig. 2.

PABA/NO resistant cells have a slower growth rate. **a** Cellular growth rates were assessed for (■) HL60, (■) HL60^{PABA}, (●) HL60^{Revertant}. Cells were plated at a density of 2×10^5 cells/ml and the total cell number was recorded at regular intervals and plotted as a function of time. **b** Cell cycle distribution was assessed in the Flow Cytometry Facility at MUSC. Data represent the mean $\pm$ SE for 3 independent experiments. Student's *t* test was used to determine statistically significant differences indicated by (*) *p*-value < 0.001

Fig. 3.

a PABA/NO resistant cells are less sensitive to the cytotoxic effects of PABA/NO. 2×10^4 cells (■) HL60 or (■) HL60^{PABA} were treated with **a** 10 uM or **b** 20 uM PABA/NO. Collateral resistance was assessed by treating the cells with **c** 300 nM thapsigargin or **d** 5 uM cisplatin. The total number of cells was counted and cell viability plotted as a function of time. Statistically significant differences were determined using Student's *t* test and are indicated by, *p*-value <0.05

Fig. 4.

NO generation is decreased in PABA/NO resistant cells. To measure NO levels cells were labeled with 5 μ M DAF-FM. **a** Basal NO levels ($-$ bars) and NO levels resulting from an additional 25 μ M PABA/NO ($+$ bars) were assessed in HL60 (black) and HL60^{PABA} (grey). **b** Dose-dependent (PABA/NO) increases in NO generation were measured for HL60 (■) and HL60^{PABA} (□). Data represent the mean $\pm$ SE for 3 independent experiments. * Statistically significant differences were determined using *t*-test (SigmaStat 3.5), *p*-value <0.001

Fig. 5. S-glutathionylation, GSTP expression and JNK activation are diminished in PABA/NO resistant cells. HL60 and HL60^{PABA} cells were treated for 1 hr with 0–50 μM PABA/NO. **a** 50 μg protein lysate was separated by SDS-PAGE under non-reducing conditions and immunoblotted using specific antibodies for protein S-glutathionylation, GSTP and total and phosphorylated JNK levels. The corresponding relative abundances of **b** S-glutathionylated proteins; **c** GSTP levels; and **d** activated JNK were plotted as the relative ratio to actin for HL60 (■) and HL60^{PABA} (◻)

Fig. 6.

Resistance to PABA/NO was concurrent with altered plasma- and mitochondrial membrane potential. **a** Measurement of extracellular free thiols in HL60 (black) and HL60^{PABA} (gray) cells using the molecular probe, TG-1. TG-1 (final concentration 5 μ M) was added to the suspension of 1.0×10^6 cells/ml, in PBS with 100 μ M CaCl₂ before and after addition of indicated amounts of PABA/NO and incubated for 30 min at 37°C under constant stirring. Emissions at 513 nm (excitation at 379 nm) were recorded in real-time kinetics until saturation. The average emission values were normalized for cell number. **b** To determine if changes in sulfhydryl content at the cell surface alter membrane potential we monitored the plasma membrane potential (V_m) changes using emission detection of the fluorescent dye bis-oxanol (5 μ M; Ex. at 488 nm, Em. at 520 nm). Original traces (resolution 0.1 sec) were averaged and smoothed using standard Sigma Plot10 Software; Experimental data were fitted with exponential decay using standard SigmaPlot10 Software, $R^2 \geq 0.97$. **c** Mitochondrial function was assessed by measuring the conversion of tetrazole to formazan in living cells via mitochondria reductases. **d** The mitochondrial membrane potential (Ψ) in HL60 cells and HL60^{PABA/NO} are different. Cell suspensions in PBS (with 100 μ M CaCl₂, pH 7.4) were incubated with JC-9 (final concentration 0.3 μ g/ml) at 37°C under constant stirring for 1 h. JC-9 emission was recorded in ratiometric mode (530/590 nm, excitation at 485 nm) before and after (500 sec) addition of 20 μ M PABA/NO. The experimental points were fitted with standard sigmoid curves (SigmaPlot 10.0), $R^2 \sim 0.98$. Data are representative for 3 independent experiments

Fig. 7. PABA/NO resistant cells have cell surface markers consistent with differentiation. Flow cytometry was used to measure the expression of cell surface antigens **a** CD11b and **b** CD14 in HL-60, HL60 (10 μ M PABA/NO), HL60^{PABA}, vehicle control and 1.5% DMSO treated. Results are plotted as the relative percent gated population

Fig. 8. Cell morphology of PABA/NO resistant cells was studied using Wright-Giemsa stain. **a** HL60, **b** DMSO 1.5% treated (induced differentiation), **c** HL60^{Revertant}, **d** HL60^{PABA}. Cells were collected on 4 independent days, and the images are representative of all images captured

Fold-induction of UPR responsive genes. The right hand columns show the quantitative expression levels of each of the genes in wild type (HL60) and resistant cell lines HL60^{-PABA}

Table 1

Name	Sequence	HL60	HL60 ^{PABA}
PDI	F 5' TCC TTG CCC TGT ATC AAA TCT T 3' R 5' TGA CCA GTG GCA AAA TTA AAA A 3'	1.0±0.2	1.89±0.2 *
ERO1	F 5' TAA ACC TGA AGA GGC CGT GT 3' R 5' TGA CAT GGT TTG ACA GCA CA 3'	1.01±0.2	1.42±0.1 *
CHOP	F 5' CTG AAT CTG CAC CAA GCA TGA 3' R 5' AAG GTG GGT AGT GTG GCC C 3'	1.00±0.1	1.54±0.3 *
Bip	F 5' AAG AAG CTA TTC AGT TGG ATG GA 3' R 5' TTC TGT TAA CTT CGG CTT GGA 3'	1.01±0.1	8.65±0.9 *
HSP70	F 5' TGC AGC AGG ACA TCA AGT TC 3' R 5' ATG TCT TTG TTT GCC CAC CT 3'	1.02±0.2	3.04±0.7 *
GADD34	F 5' CTG GGG ACT TTT GGA TGA TG 3' R 5' ATT GAC TTC CCT GCC CTC TA 3'	1.04±0.2	3.41±0.6 *
XBP-1	F 5' CCA AAA ACT TTT GCT AGA AAA TCA GC 3' R 5' CAT CCC CAA GCG CTG TCT TA 3'	1.01±0.2	1.80±0.5 *

* Statistically significant differences were determined using *t*-test (SigmaStat 3.5), *p*-value <0.05