Transcriptional Profiling Identifies Altered Intracellular Labile Iron Homeostasis as a Contributing Factor to the Toxicity of Adaphostin: Decreased Vascular Endothelial Growth Factor Secretion Is Independent of Hypoxia-Inducible Factor-1 Regulation

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Abstract

Purpose: Adaphostin was developed as an inhibitor of the p210⁰bcd-abl tyrosine kinase, but as its activity is not limited to tumor cell lines containing this translocation, transcriptional profiling was used as a tool to elucidate additional mechanisms responsible for adaphostin cytotoxicity.

Experimental design: Profiles of drug-induced transcriptional changes were measured in three hematopoietic cell lines following 1 and 10 μmol/L adaphostin for 2 to 6 hours and then confirmed with real-time reverse transcription-PCR (2-24 hours). These data indicated altered iron homeostasis, and this was confirmed experimentally. Alteration of vascular endothelial growth factor (VEGF) secretion through hypoxia-inducible factor-1 (HIF-1) regulation was also investigated.

Results: Drug-induced genes included heat shock proteins and ubiquitins, but an intriguing response was the induction of ferritins. Measurement of the labile iron pool showed release of chelatable iron immediately after treatment with adaphostin and was quenched with the addition of an iron chelator. Pretreatment of cells with desferrioxamine and N-acetyl-cysteine reduced but did not ablate the sensitivity of the cells to adaphostin, and desferrioxamine was able to modulate adaphostin-induced activation of p38 and inactivation of AKT. VEGF secretion was shown to be reduced in cell lines after the addition of adaphostin but was not dependent on HIF-1.

Conclusions: Adaphostin-induced cytotoxicity is caused in part by a rapid release of free iron, leading to redox perturbations and cell death. Despite this, reduced VEGF secretion was found to be independent of regulation by the redox responsive transcription factor HIF-1. Thus, adaphostin remains an interesting agent with the ability to kill tumor cells directly and modulate angiogenesis.

Adaphostin, NSC 680410, is the adamantyl ester congener of the tyrphostin AG957. Both the parent compound and this analogue have been studied with regards to their mechanism of action and several cellular consequences of treatment with these agents have been determined; however, a comprehensive mechanism of action remains to be defined. Initial interest in the tyrphostins was generated from reports indicating that AG957 antagonized p210bcd-abl tyrosine kinase activity (1) and could alter tyrosine kinase signaling (2, 3). Further studies indicated AG957 activity was not specific for p210bcd-abl, with altered tyrosine kinase activity detected in cells not expressing p210bcd-abl leading to inhibition of mitogen-activated protein kinase (MAPK) activation (4) and furthermore could affect the phosphorylation state of phosphatidylinositol-3 kinase/Akt leading to apoptosis in a bcr-abl-independent manner (5).

In a study to compare AG957 and adaphostin, the adamantyl analogue was a less potent inhibitor of p210bcd-abl under cell-free conditions compared with AG957 but was significantly more potent in down-regulating p210bcd and inhibiting K-562 colony formation (6). Moreover, a comparison among the p210bcd-abl kinase inhibitor, imatinib mesylate, an ATP binding site-directed agent, and adaphostin showed the latter agent was mechanistically distinct and maintained activity in imatinib-resistant cell lines (7). These data indicated that although adaphostin had inhibitory effect on p210bcd-abl kinase activity; this was likely not its sole mechanism of action. As imatinib-resistant variants of p210bcd-abl kinase are being defined, interest in adaphostin as a means of decreasing p210bcd-abl signaling has reemerged.

In a series of further studies designed to clarify adaphostin’s mechanism of action in hematologic malignancies, Avramis et al. (8) showed that adaphostin was relatively toxic to a range of leukemia cell lines including p53-null and drug-resistant phenotypes and could inhibit secretion of vascular endothelial growth factor (VEGF) in those cell lines where it was measurable. When the U87 MG glioblastoma cell line was...
inoculated orthotopically into the caudate putamen, treatment with adaphostin resulted in smaller brain tumors at the site of inoculation and no extracranial tumors, whereas adaphostin treatment in combination with the Flt-1/Fc chimera, a specific inhibitor of VEGF, showed a more marked inhibition of tumor growth (8). More recent data has implicated oxidative stress in the toxicity of adaphostin, linking reactive oxygen species (ROS) with resulting DNA strand breaks (9) and triggering inactivation of the cytoprotective Raf-1/ERK kinase/extracellular signal-regulated kinase (ERK) and AKT cascades, culminating in mitochondrial injury, caspase activation, and apoptosis (10).

In light of these different potential mechanisms involved in adaphostin toxicity, transcriptional profiling was undertaken using cDNA microarrays to evaluate drug-induced gene expression changes and gain additional insight into the mechanism of action. As we show in the experiments described here, these data lead us to propose that adaphostin alters the size of the labile iron pool, which suggests an immediate basis for the development of ROS which could then contribute to adaphostin-induced cytotoxicity. Moreover, we also confirmed that VEGF secretion could be diminished by adaphostin and we extended those findings to document that decreased secretion of VEGF by adaphostin was independent of hypoxia-inducible factor-1 (HIF-1) regulation.

### Materials and Methods

**Drugs and cell culture.** Adaphostin (NSC 680410), cisplatin (NSC 119875), and salicylaldehyde isonicotinoyl hydrazone (SIH; NSC 33760) were obtained from the drug repository of National Cancer Institute’s Developmental Therapeutics Program (Rockville, MD) and were prepared in 100% DMSO at a concentration of 40 mmol/L and stored at −70°C until required. Desferrioxamine and N-acetyl-cysteine (NAC) were purchased from Sigma (St. Louis, MO). Phen Green-SK (PG-SK) was purchased from Molecular Probes (Eugene, OR). K-562 and HL-60(TB) were obtained from the National Cancer Institute anticancer drug screening cell line panel (National Cancer Institute, Frederick, MD). Jurkat cells were obtained from American Type Culture Collection (Rockville, MD). All cell lines were maintained in RPMI 1640 supplemented with 5% fetal bovine serum and 2 mmol/L L-glutamine (Cambrex, Walkersville, MD) referred to herein as complete media.

**Transcriptional profiling of adaphostin-treated hematopoietic cells.** Human OncoChip (10K cDNA) arrays from the National Cancer Institute/CCR microarray center were used according to protocols published on the mAdam homepage (http://nciarray.nci.nih.gov). Brieﬂy, logarithmically growing hematopoietic cell lines [Jurkat, K-562, and HL-60(TB)] were treated with 1 and 10 μmol/L adaphostin for 2 and 6 hours. Equal amounts of total RNA (20 μg) extracted from the samples were reverse transcribed and amino-allyl-modified dUTP was incorporated into control- and drug-treated samples using the Fairplay kit (Stratagene, La Jolla, CA). Each cDNA sample was then chemically coupled to a Cy3 (control) or Cy5 (treated) fluorescently labeled dye (Amersham, Piscataway, NJ), purified, the two probes combined, filtered, blocked, and the remaining sample transferred to a prehydrized glass array under a coverslip. Arrays were hybridized at 42°C for 16 hours, washed thrice, and dried. Fluorescence was read on a GenePix Pro 4100A microarray scanner (Axon Instruments, Union City, CA) at a wavelength of 635 nm for the Cy5 (pseudocolored red) and 532 nm for the Cy3 samples (pseudocolored green). Data was analyzed through GenePix Pro 4.1 software, then data and image files were uploaded to the National Cancer Institute/CCR Microarray Center mADB Gateway for storage, analysis, and multiple array comparisons.

**Data from duplicate arrays and treatments were averaged and then genes were selected based on a 3-fold change in expression in any two of the different treatments. This led to a group of 202 genes, and from there, a robustly induced subset was selected that included ferritins, heat shock proteins, and ubiquitins.**

**Real-time reverse transcription-PCR.** Quantitative real-time reverse transcription-PCR reactions were measured using the ABI Prism 7700 Sequence Detection System and Taqman chemistries (Applied Biosystems, Foster City, CA). Total RNA was isolated from Jurkat, K-562, and HL-60(TB) control cells or cells treated with 1 or 10 μmol/L adaphostin for 2 and 6 hours using the Qiagen RNeasy mini kit (Qiagen, Valencia, CA), quantified using the absorbance at 260 nm, and purity was measured by the A260/A280 ratio. One microgram of total RNA was reverse transcribed in a 50-μL reaction using a Taqman Reverse Transcription Reagents kit (Applied Biosystems) and resulting cDNA was stored at −70°C until required. Primers for the genes were designed with Primer Express Software (Applied Biosystems) from the appropriate gene bank sequences for the human gene (Table 1). PCR reactions were done using Taqman SYBR Green master mix with 5 ng of cDNA per reaction in 50-μL reactions. Primer concentrations were 300 nmol/L for each of the genes and 100 nmol/L for glyceraldehyde-3-phosphate dehydrogenase (endogenous control). Samples were tested in triplicate wells for both the genes and glyceraldehyde-3-phosphate dehydrogenase, data was analyzed using the comparative C_{T} method (Perkin-Elmer User Bulletin 2), and expressed as fold induction of the relevant gene in adaphostin-treated cells compared with the untreated control cells.

**Drug combinations.** HL-60(TB), Jurkat, and K-562 cells were inoculated onto 96-well plates at a density of 25,000, 10,000, and 10,000 cells per well, respectively. Plates were incubated at 37°C for 24 hours before addition of the drugs. For drug addition, adaphostin was diluted from frozen aliquots (40 mmol/L) and added to the plates combined with each dose of either NAC or desferrioxamine (duplicate wells). Plates were incubated for 48 hours at time 20 μL of the metabolic dye, alamar blue (Sigma), was added to each well of the plates. The plates were incubated for an additional 6 hours at 37°C after which relative fluorescence was measured on a Tecan Ultra plate reader (509-nm excitation and 520-nm emission). Percent

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**Table 1. Real-time PCR primers**

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Accession no.</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
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<tr>
<td>FTH</td>
<td>NM.002032</td>
<td>AATTGGGTGACCACTGACACC</td>
<td>TTCCGCGCAAGCGATTC</td>
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<tr>
<td>TIL</td>
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<td>CGCGATGATGTGGCTCTG</td>
<td>CGTCTCGTCGGAAT</td>
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<td>HSP105B</td>
<td>NM.006644</td>
<td>TCAAAGTGCGAGTCAACACC</td>
<td>CAGTTGGACCTTCTCCACCA</td>
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<tr>
<td>HSPA6</td>
<td>X51757</td>
<td>CCGGTCGTGAACACC</td>
<td>AGCGGTTGGGCCGTCAG</td>
</tr>
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</table>
treated/control was calculated for adaphostin, NAC, and desferrioxamine either alone or in combination. Dose response curves were generated from the % treated/control data and graphed. Gene expression of selected genes was also measured in RNA collected from an aliquot of the samples.

**Measurement of the labile iron pool.** The fluorescent probe PG-SK, which is quenched in the presence of iron (Fe^{3+}), was used to measure the labile iron pool (11). Cells [HL-60(TB), Jurkat, and K-562] were loaded with 20 μmol/L PG-SK for 30 minutes at 37°C, washed 2× with PBS to remove free dye, and counted. PG-SK loaded cells were then inoculated onto 96-well Optiplates (Perkin-Elmer Life Sciences, Boston, MA) at a density of 50,000 cells per well in 100 μL of PBS. Immediately before fluorescent measurements, adaphostin and SIH were diluted in PBS and 100 μL of each was added to the plates to give a final concentration of 10, 5, and 1 μmol/L for adaphostin and 100 μmol/L for SIH. Control wells (cells loaded with PG-SK) were adjusted to the correct volume by addition of 100 μL of PBS. Triplicate wells were used for each condition. The plate was then read in 5-minute intervals over 70 minutes on a Tecan ultra fluorescent plate reader (488-nm excitation and 535-nm emission). At the end of the 70-minute time course, 10 μL of SIH were added to each of the adaphostin-treated wells (100 μmol/L final well concentration) to chelate-free iron, and fluorescent measurements were taken in 5-minute intervals for an additional 20 minutes. Fluorescent measurement at each time point for each treatment condition were averaged for the triplicate wells and graphed as a percent change in relative fluorescent units compared to untreated control cells.

**Transient transfections.** Logarithmically growing HL-60(TB) cells were grown to ~70% confluency on the day of transfection when 2 × 10^6 cells were transfected with hypoxia-responsive element (HRE) and pGL-3 (plasmids were a kind gift from Dr. Giovanni Melillo) promoter using the Amassa Nucleofector (program T-19) and the nucleofector kit V reagents (Amassa Technologies, Gaithersburg, MD). After 24 hours of incubation (37°C), 3 × 10^4 cells per well were inoculated onto a 96-well plate, and incubated for an additional 24 hours. Adaphostin (0-5 μmol/L) and the positive control topotecan (0.5 μmol/L) were then incubated with the cells for 16 hours followed by addition of 100 μL Bright-Glo reagent (1:2 dilution; Promega Corp., Madison, WI) and plates were read immediately on a TopCount luminometer (Packard Bioscience, Foster City, CA). Luminescence values were averaged and data was graphed.

**Western blot.** Cells treated with adaphostin were centrifuged, washed with ice-cold PBS, and the cell pellet was lysed in cell lysis buffer [20 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1% v/v Triton X-100, 1 mmol/L EGTA, 1 mmol/L EDTA, 1 mmol/L sodium orthovanadate, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L B-glycerophosphate, 10 μg/mL leupeptin, and 1 mmol/L phenylmethylsulfonyl fluoride]. Cell lysates were sonicated and cleared by centrifugation at 14,000 × g for 15 minutes. Protein concentrations of the clarified supernatants were determined and equal amounts of proteins were resolved by SDS-PAGE on 4% to 20% Tris glycine gels (Invitrogen, Carlsbad, CA). Proteins were transferred to polyvinylidene difluoride membrane (Millipore, Bedford, MA); blots were blocked and probed.
Fig. 2. A, a cluster of eight genes from microarray data after 2 and 6 hours of treatment with adaphostin (1 and 10 μmol/L). These genes, (UBC, ubiquitin C; FTL, ferritin light polypeptide; FTH, ferritin heavy polypeptide 1; HSPH1, heat shock 105/110-kDa protein 1; HSPA6, heat shock 70-kDa protein 6; HSPA5, heat shock 70-kDa protein 5; HSPA8, heat shock 70-kDa protein 8; UBB, ubiquitin B) are a subset of an original group of genes (n = 202) selected for a 3-fold increase or decrease in expression in at least two of the different treatments, then isolated based on a robust response from visual inspection of original array spots. Average of duplicate array measurements. Microarray data was generated by comparative hybridization of equal amounts of Cy3- and Cy5-labeled cDNA (control versus treated) to 10K cDNA arrays, and details are given in Materials and Methods. B, quantitative real-time reverse transcription-PCR confirmation of four genes of interest that were dysregulated in the microarray experiments. Cell lines were treated with 1 and 10 μmol/L adaphostin and gene expression was measured after 0 hour (○), 2 hours (□), 6 hours (▲) and 24 hours (■) and shown as the relative expression of adaphostin-induced genes compared with control (0 hour). C, cell lines were treated with 5 μmol/L cisplatin (a negative control for nonspecific induction of adaphostin-related genes) and gene expression was measured after 0 hour (○), 2 hours (□), 6 hours (▲) and 24 hours (■) and shown as the relative expression of cisplatin-induced genes compared with control (0 hour). Average of two independent experiments. Quantitative real-time reverse transcription-PCR was done using sequence-specific primers (Table 1).
overnight with pAKT, AKT, pERK, tERK antibody (Cell Signalling, Beverly, MA), and p-p38 and p38 (Biosource, Camarillo, CA). Proteins were visualized by chemiluminescence and imaged on Kodak Image station 2000 MM. Quantitation was done using Kodak software.

**Transferrin receptor expression.** To determine the transferrin binding at different time points, untreated and drug-treated cells were incubated with 0.5 µg of mouse IgG1 (negative control) or 0.5 µg of transferrin antibody (Ancell, Bayport, MN) in 0.1% bovine serum albumin in PBS, on ice for 30 minutes. Cells were washed thrice with 0.1% bovine serum albumin then incubated with 0.5 µg of R-phycocerythrin (Jackson ImmunoResearch, West Grove, PA) for additional 30 minutes on ice then washed thrice with PBS. Samples were analyzed on Guava PCA cytometer (Guava Technologies, Hayward, CA) using Guava Express software.

**Vascular endothelial growth factor secretion.** The levels of VEGF production in the culture supernatants were measured using standard sandwich ELISA methods (R&D Systems, Minneapolis, MN). Briefly, cells (2.5 × 10^4) were plated in a 6-well plate and after 24 hours, vehicle or drug were added in triplicates at twice the final concentration. Cells were incubated at 37°C for 24 hours. At the end of incubation, medium was removed and stored at −70°C and cells in each well were counted. VEGF quantitation was done on the samples using VEGF ELISA kit following manufacture’s specifications. VEGF secreted in the media was calculated from the standard curve. Data is representative of three experiments.

**Results**

**Structure and activity of adaphostin.** Figure 1 shows the structure of adaphostin, an adamantyl congener of the tyrphostin AG957. The mean graph representation (12) of adaphostin’s capacity to inhibit cell growth shows the relative toxicity profile of 60 human tumor cell lines measured at the GI50 (50% level of growth inhibition; Fig. 1B). The center line reflects the average GI50 of all cell lines and bars with the deflection to the right represents on a log scale the GI50 of those cell lines more sensitive, whereas bars with a deflection to the left reflects on a log scale the GI50 of those more resistant, than the average response. The leukemia cell lines are clearly the most sensitive as a group, with a few sensitive lines in the non–small cell lung cancer and renal panels. These data imply that on an empirical basis adaphostin might be expected to potently inhibit the growth of numerous hematopoietic as opposed to solid tumor cell lines. For the detailed screening information, see http://dtp.nci.nih.gov. The full adaphostin dose response curves (48-hour incubation) for the three leukemia cell lines selected for this study (Fig. 1C) illustrate that the bcr-abl expression is not critical for sensitivity to adaphostin, as the chronic myelogenous leukemia cell line K-562, the only bcr-abl expressing cell line, is the most resistant of the three cell lines, albeit with a GI50 of ~1.0 µmol/L.

**Transcription profiling in response to adaphostin.** Each of these three leukemia cell lines was treated with 1 and 10 µmol/L adaphostin (in duplicate) for 2 and 6 hours, and the data was averaged. Identification of genes altered >3-fold in any two of the different treatments indicated; ~200 genes met this criterium and were subjected to hierarchical clustering. Comparison of these genes indicated that the response of the two
most sensitive cell lines are most similar \((r = 0.720)\), whereas the more resistant line, K-562, shows a lesser number of responsive genes and is less well correlated \((r = 0.476)\). A subset of this cluster was selected based on the review of individual spots that made up this group and is shown in Fig. 2A, where green indicates down-regulation and red indicates up-regulation of genes and black spots represent no change in gene expression. These included genes encoding for both ferritin heavy and light, ubiquitins B and C, and several heat shock proteins. Selected genes were measured by real-time reverse transcription-PCR (Taqman) and confirmed that all were measurably up-regulated in the three cell lines after 1 and/or 10 \(\mu\text{mol/L}\) adaphostin treatment, usually between 6 and 24 hours (Fig. 2B). In contrast, 5 \(\mu\text{mol/L}\) cisplatin treatment did not result in induction of any of these genes under treatment conditions (Fig. 2C).

### Labile iron pool after adaphostin treatment

An implication of altered ferritin heavy- and light-chain gene expression induced by adaphostin relates to the tight control of ferritin gene expression by the ambient iron concentration. Ferritins are known to be regulated transcriptionally by iron response elements that respond to increased cellular iron with increased transcriptional activity of ferritin and other genes related to the management of iron in the cellular economy \((13–17)\). Our findings raised the hypothesis that adaphostin in some way was increasing the actual level of iron in the cell or altering the cells' perception of endogenous iron by the transcriptional regulatory apparatus.

To address this issue directly, we measured the change in the labile iron pool immediately after adaphostin treatment using the fluorescent probe PG-SK, which is quenched in the presence of labile iron. To graphically visualize the increase in labile iron over the first hour after adaphostin treatment, the data in Fig. 3 are expressed as the percentage change in the fluorescent signal of PG-SK, indicating the decrease in signal that reflects an increase in the labile iron pool. The higher concentrations of adaphostin \((5, 10 \mu\text{mol/L})\) release free iron in all three cell lines over the entire 70 minutes, whereas 1 \(\mu\text{mol/L}\) adaphostin causes a measurable change in iron only in the two most sensitive cell lines. When the iron-chelating agent SIH was added to all the samples after 70 minutes...
of adaphostin treatment, PG-SK fluorescence returned to levels similar to control within 15 minutes. Moreover, 20 to 25 minutes after SIH was given to untreated cells, the labile iron pool (measured by an increase in PG-SK fluorescence) was reduced to a plateau 20% to 30% lower than the control cell lines, indicating the sensitivity of this dynamic assay.

Modulation of adaphostin-induced toxicity, gene expression, and signaling proteins following iron chelation or antioxidants. If labile iron contributes functionally to the cytotoxicity of adaphostin, then reduction of the adaphostin-induced increase in the labile iron pool should modulate adaphostin-induced inhibition of cell growth. Figure 4 shows the toxicity of adaphostin was diminished in all three cell lines after cells were pretreated with increasing concentrations of the iron-chelating agent desferrioxamine. Moreover, the desferrioxamine treatment was able to significantly reduce the induction of the two heat shock proteins (with the exception of K-562 where desferrioxamine alone induced more HSPA6 than adaphostin), and ferritins in response to adaphostin treatment, indicating that chelation of free iron reduces toxicity, stress responses, and the iron response. In Fig. 5, similar data are shown for the antioxidant peroxide scavenger, NAC. This agent reduces adaphostin toxicity even more effectively than desferrioxamine and ablates the induction of stress response genes, HSPA6 and HSPA1. However, in Jurkat and HL60, the adaphostin-induced FTH gene was not significantly altered by NAC, whereas FTL was reduced by <50%. In contrast, in K-562 cells, the adaphostin-induced FTL was not altered by NAC, whereas the FTH was reduced by <25%, indicating different cell lines may use the two ferritin subunits differently in the regulation of iron. These data are in accord with the findings of Chandra et al. and Yu et al. (9, 10) with respect to cytoprotection by the free radical scavengers but extends their findings by emphasizing that the free radical scavenger NAC, whereas attenuating cytotoxicity does not affect the functional consequences of an increase in the labile iron pool.

In Jurkat cells, we have confirmed that adaphostin induces activation of the proapoptotic p38 (MAPK14) MAPK and down-regulation of activated AKT (p-AKT; Fig. 6A-D) and ERK (p-ERK; data not shown), whereas levels of the total proteins
(p38, AKT, and ERK) were not significantly altered (Fig. 6E). Moreover, we showed that desferrioxamine pretreatment inhibited both adaphostin-induced p-p38 activation and the loss of activated p-AKT (Fig. 6) in a manner similar to that shown by Yu et al. (10) for NAC. In contrast, we were only able to measure a very limited expression of p-ERK in these cells, and although p-ERK expression was totally inhibited by adaphostin, there was no measurable modulation by desferrioxamine pretreatment (data not shown).

These data are concordant with the model that the free iron released by adaphostin would be “upstream” of the generation of ROS through reactions with H2O2 exemplified by Fenton’s reaction (H2O2 + Fe2+ → Fe3+ + OH− + hydroxyl radical [−OH]; ref. 18) to generate hydroxyl radicals that cause lethal DNA damage and death, in accord with previous observations (9, 10). When the cellular peroxide is scavenged and unavailable for interaction with the Fe2+ to create hydroxyl radicals, then the toxicity is reduced, although the induction of ferritin, as a surrogate marker of iron increase, indicates there is indeed release of free iron. Moreover, the prevention of free iron release by desferrioxamine was able to inhibit adaphostin triggered activation of the proapoptotic p38 MAPK signaling pathway and also inactivation of AKT in terms of a decrease in p-AKT expression (phosphatidylinositol-3 kinase pathway), supports the hypothesis that altered iron homeostasis is a critical element of adaphostin toxicity.

Fig. 6. Effect of desferrioxamine (DFX) on adaphostin-induced perturbations of p38 and AKT signaling proteins in Jurkat cells. A, p-p38 protein expression from Jurkat cells treated with 1 to 10 μM adaphostin, + or − pretreatment (4 hours) with 100 μM/L desferrioxamine (4 hours). B, quantitation of the p-p38 protein blot, showing adaphostin increased expression of p-p38 in the absence of desferrioxamine (−) and inhibition of this increase in the presence of desferrioxamine (+). C, p-AKT protein expression from Jurkat cells treated with 1 to 10 μM/L adaphostin, + or − pretreatment (4 hours) with 100 μM/L desferrioxamine. D, quantitation of the p-AKT protein blot, showing adaphostin increased expression of p-AKT in the absence of desferrioxamine (−) and inhibition of this increase in the presence of desferrioxamine (+). E, total AKT and p38 protein expression from Jurkat cells treated with 1 to 10 μM/L adaphostin, + or − pretreatment (4 hours) with 100 μM/L desferrioxamine. Proteins were resolved by western analysis as indicated in Materials and Methods.
Transferrin receptor expression. Regulation of the transferrin receptor is inversely related to ferritin regulation to maintain iron homeostasis in the cell (reviewed in ref. 14). Figure 7A shows a decrease in cell surface expression of the transferrin receptor after adaphostin treatment. Interestingly, the most drug resistant cell line, K-562, shows the greatest decrease in expression (25%) after 1 μmol/L adaphostin, whereas the more sensitive cell lines require up to 10 μmol/L to show such a change in expression after 24 hours. A more detailed evaluation of the K-562 response (Fig. 7B) indicated that treatment for 24 hours with 5 μmol/L adaphostin resulted in a maximal 40% decrease in cell surface expression of the transferrin receptor.

Effect of adaphostin on the hypoxia-responsive element. Published data (8) has shown that adaphostin can inhibit VEGF secretion in hematopoietic cell lines. We have confirmed that 0.1 to 10 μmol/L adaphostin could reduce VEGF secretion >50% within 72 hours in HL-60(TB) cells (Fig. 8A) but required 10 μmol/L to inhibit secretion to this level in K-562 cells (Fig. 8B). In contrast, these Jurkat cells did not secrete detectable levels of VEGF (data not shown). As adaphostin-induced perturbations of iron homeostasis and redox systems have been identified, we investigated the effect of adaphostin on the redox-responsive transcription factor, HIF-1, which has been implicated in the regulation of VEGF transcription (19). We examined the possibility that the inhibition of VEGF secretion resulted from an effect of adaphostin on HIF-1 transcription. Evaluation of adaphostin in the National Cancer Institute’s high throughput screen for inhibition of hypoxia-induced HIF-1 transcription (20), which uses a genetically engineered U251 (human glioma) cell line, indicated adaphostin had no effect in this screen (data not shown). However, to eliminate the possibility that adaphostin might act differently on hematopoietic cell lines, which seem more sensitive to its toxicity, an HL-60(TB) cell line was genetically engineered to transiently express a recombinant vector in which the luciferase reporter gene is under control of three copies of a canonical HRE, expressing luciferase in a hypoxia- and HIF-1-dependent fashion (Fig. 9A). The control for this assay is the luciferase reporter gene under the control of a constitutively active promoter, which is highly expressed under normoxia and not induced by hypoxia nor inhibited by the positive control drug, topotecan (21). Figure 9A shows that topotecan inhibited the hypoxia-induced luciferase expression ~50%, but adaphostin treatment (1-5 μmol/L) was unable to alter the hypoxia-induced HIF-1 response, in HL-60(TB), as measured by luciferase induction concordant with the idea that the mechanism for diminished VEGF secretion by adaphostin does not involve HIF-1 regulation. The pGL-3 reporter was unaffected by any treatment indicating the specificity for the HRE. These data are complimented by Fig. 9B showing that the expression of the HIF1α gene was unaffected by treatment with 1 and 10 μmol/L adaphostin for up to 24 hours.
Adaphostin Alters Iron Homeostasis

Adaphostin, the adamantyl ester of the tyrphostin AG957, was originally developed as an inhibitor of the p210bcr-abl tyrosine kinase, but several lines of evidence have indicated that additional mechanisms for cytotoxicity are necessary to consider, as adaphostin inhibited the growth of cells not expressing p210bcr-abl, caused altered activation of MAPK kinase/RAF signaling (9) with evidence of ROS formation (9, 10) and DNA damage (10).

Consequently, we launched an effort to use transcriptional profiling to probe for alternative mechanisms of action responsible for adaphostin-induced cytotoxicity. Several gene families were induced in all three hematopoietic cell lines investigated, including heat shock proteins and ubiquitins, but an intriguing response involved the induction of both ferritin light and heavy subunits. Ferritin is an important regulator of iron homeostasis (18, 22). Intracellular iron levels are tightly regulated through the action of iron-responsive proteins, which belong to the aconitase family of enzymes (reviewed in refs. 13, 14). When intracellular iron levels are low, iron-responsive protein displays a high affinity for the iron-responsive element, found in the 5′-noncoding region of ferritin mRNAs and in the 3′-noncoding region of the transferrin receptor mRNA. Binding of iron-responsive protein to these iron-responsive elements represses ferritin synthesis and stabilizes transferrin receptor mRNA. In contrast, in the presence of free iron, iron-responsive proteins are unable to bind the iron-responsive elements and allow for both ferritin translation and degradation of transferrin receptor mRNA, providing for a coordinated regulation of iron uptake and storage (reviewed in refs. 15–17). The transferrin receptor’s role is to internalize ferric-transferrin complexes and transport iron into the cell, and it has been reported to be responsible for doxorubicin-mediated toxicity in endothelial cells (23). The ferritin molecule has a vast capacity to sequester and store large amounts of iron, in a nontoxic form, and has been reported to protect cardiomyocytes against iron-mediated doxorubicin toxicity (24). In contrast, ferritin-bound iron can be mobilized and released by certain reducing agents making it available to catalyze the generation of active oxygen species (25, 26). Thus, the relative toxic and cytoprotective roles of iron, ferritin, and transferrin are complex and remain to be fully characterized for different biological circumstances.

Confirmation of the contribution of the array-identified gene families in the adaphostin response was made by quantitative reverse transcription-PCR (Fig. 2) allowing us to postulate the involvement of altered iron homeostasis leading to a stress response in drug-treated cells. This was consistent with recent investigations that revealed the involvement of reactive oxygen species in adaphostin toxicity (9, 10). However, it was not a generalized stress response as treatment of the cells with 5 μmol/L cisplatin did not induce the ferritins or the two heat shock proteins.

Direct evaluation of the labile iron pools confirmed the release of chelatable free iron immediately after drug treatment that continued for 70 minutes of the experiment, and could then be diminished by the introduction of a chelating agent (Fig. 3). Pretreatment with the iron-chelating agent, desferrioxamine and the antioxidant, NAC both reduced but did not completely ablate adaphostin-induced toxicity (Figs. 4 and 5). Moreover, desferrioxamine pretreatment prevented all, or most of the up-regulation of the ferritin and heat shock protein genes indicating that chelation of the released iron prevented the specific iron response and the stress response associated with it. The antioxidant, NAC, ablated the heat shock/stress response, presumably by scavenging one of the substrates (H2O2) of the Fenton reaction and thereby preventing hydroxyl radical formation, but the ferritin response remained essentially intact indicating release of the free iron despite reduced overall cytotoxicity. As adaphostin may be regarded as a hydroquinone with reducing potential, the rapidity with which labile iron could be measured suggests the likelihood that adaphostin causes release of iron from within ferritin stores and does not accumulate as a result of increased iron transport into the cell. We cannot however, rule out the possibility that

Discussion

Adaphostin, the adamantyl ester of the tyrphostin AG957, was originally developed as an inhibitor of the p210bcr-abl tyrosine kinase, but several lines of evidence have indicated that additional mechanisms for cytotoxicity are necessary to consider, as adaphostin inhibited the growth of cells not expressing p210bcr-abl, caused altered activation of MAPK kinase/RAF signaling (9) with evidence of ROS formation (9, 10) and DNA damage (10).

Consequently, we launched an effort to use transcriptional profiling to probe for alternative mechanisms of action responsible for adaphostin-induced cytotoxicity. Several gene families were induced in all three hematopoietic cell lines investigated, including heat shock proteins and ubiquitins, but an intriguing response involved the induction of both ferritin light and heavy subunits. Ferritin is an important regulator of iron homeostasis (18, 22). Intracellular iron levels are tightly regulated through the action of iron-responsive proteins, which belong to the aconitase family of enzymes (reviewed in refs. 13, 14). When intracellular iron levels are low, iron-responsive protein displays a high affinity for the iron-responsive element, found in the 5′-noncoding region of ferritin mRNAs and in the 3′-noncoding region of the transferrin receptor mRNA. Binding of iron-responsive protein to these iron-responsive elements represses ferritin synthesis and stabilizes transferrin receptor mRNA. In contrast, in the presence of free iron, iron-responsive proteins are unable to bind the iron-responsive elements and allow for both ferritin translation and degradation of transferrin receptor mRNA, providing for a coordinated regulation of iron uptake and storage (reviewed in refs. 15–17). The transferrin receptor’s role is to internalize ferric-transferrin complexes and transport iron into the cell, and it has been reported to be responsible for doxorubicin-mediated toxicity in endothelial cells (23). The ferritin molecule has a vast capacity to sequester and store large amounts of iron, in a nontoxic form, and has been reported to protect cardiomyocytes against iron-mediated doxorubicin toxicity (24). In contrast, ferritin-bound iron can be mobilized and released by certain reducing agents making it available to catalyze the generation of active oxygen species (25, 26). Thus, the relative toxic and cytoprotective roles of iron, ferritin, and transferrin are complex and remain to be fully characterized for different biological circumstances.

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adaphostin induces mitochondrial damage resulting in iron release from the rich stores in mitochondria, but little is known about the regulation of mitochondrial iron. Regardless of the iron source, these data are concordant with a mechanism where adaphostin-induced mobilization of iron from ferritin provides a cocatalyst for the Fenton reaction. This would be one basis for the production of cell-damaging hydroxyl radicals. Our observations therefore provide a mechanistic basis for the observations of Chanda et al. (9) and Yu et al. (10), where ROS were observed after exposure of cells to adaphostin as well as induction of DNA breakage. Notably, we observe that aspects of this response which could be attenuated by preincubation of cells with an iron-chelating agent, or an antioxidant. The adaphostin-induced increase in free iron would signal the iron-sensing system to respond with an increase in ferritin and a decrease in transferrin gene expression (15–17), as was seen in Figs. 2 and 7. Moreover, the prevention of free iron release by desferrioxamine inhibited adaphostin-triggered activation of the proapoptotic p38 MAPK signaling pathway and also inhibited inactivation of AKT by impeding loss of p-AKT (Fig. 6), which supports the hypothesis that that release of free iron into the cell milieu is a critical element of adaphostin-induced ROS-generated toxicity.

There is no question that both AG957, the parent compound of adaphostin, and adaphostin can inhibit tyrosine kinase activity. Whereas capacity to inhibit p210
\textsuperscript{bcr-abl} activity was a major criterion for initial interest in these drugs, AG957 for example can inhibit the tyrosine phosphorylation of p120
\textsuperscript{cbl} in Jurkat T lymphoblasts and affect activation of the phosphatidylinositol-3 kinase/Akt pathway in other non-p210
\textsuperscript{bcr-abl}–expressing hematopoietic cells. Therefore, in addition to acting as a means of generating free radical–mediated cytotoxicity, adaphostin may also compromise the function of the signaling systems related to promoting cell survival and resistance to apoptosis, as well as having a direct antiproliferative effect as a kinase inhibitor. The exact contribution of adaphostin’s free radical generating capacity in contrast to its kinase inhibitory potential conceivably could depend on the cellular context, availability of iron stores, and spectrum of kinases present in the target cell population. Further clarification of the spectrum of kinase inhibition obtained with adaphostin will be of value in considering this issue further and is in progress.

HIF-1 is a redox responsive transcription factor, consisting of a redox/oxyen sensitive \( \alpha \) subunit, and the constitutively expressed, promiscuous, \( \beta \) subunit (ARNT; refs. 26, 27). Recently, the Fenton reaction at the endoplasmic reticulum has been linked to the redox control of hypoxia-inducible gene expression, via HIF-1 (28). HIF-1 is a primary regulator of VEGF expression (19, 28). VEGF is a growth factor that regulates angiogenesis and vasculogenesis and has been implicated in tumor progression (20–33) and provides an exciting target for therapeutic intervention (34). It has been reported (8) and we have confirmed (Fig. 8) that adaphostin treatment reduced VEGF secretion from hematopoietic cell lines. Thus, we investigated the effect of adaphostin on the hypoxia dependent induction of HIF-1. When HL-60(TB) was transiently transfected, or U251 (human glioma) permanently transfected, with a recombinant vector in which the luciferase reporter gene was under control of three copies of a canonical HRE, these cells expressed luciferase when exposed to hypoxia, in an HIF-1-dependent fashion. Adaphostin (1-5 \( \mu \)mol/L) did not inhibit the hypoxia-induced expression of HIF-1-dependent luciferase (Fig. 9A), indicating that reduced VEGF secretion in response to adaphostin was independent of HIF-1 regulation. This was corroborated by the fact that adaphostin treatment over 24 hours (1 and 10 \( \mu \)mol/L) did not alter the expression of the HIF1\( \alpha \) gene (Fig. 9B).

In summary, adaphostin induced cellular cytotoxicity at least in part by causing a rapid release of free iron, leading to redox perturbations and generation of reactive oxygen species leading to cell death. However, neither iron chelation nor antioxidants was completely able to ablate toxicity, indicating there may be some additional mechanism contributing the adaphostin mechanism of action. Despite the redox perturbation, the ability of adaphostin to reduce VEGF secretion was found to be independent of regulation by the redox-responsive transcription factor, HIF-1. Thus, adaphostin remains an interesting preclinical agent with the dual ability to kill tumor cells directly and modulate angiogenesis.

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Transcriptional Profiling Identifies Altered Intracellular Labile Iron Homeostasis as a Contributing Factor to the Toxicity of Adaphostin: Decreased Vascular Endothelial Growth Factor Secretion Is Independent of Hypoxia-Inducible Factor-1 Regulation

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