Triapine and a More Potent Dimethyl Derivative Induce Endoplasmic Reticulum Stress in Cancer Cells


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ABSTRACT

Triapine (3-AP; 3-aminopyridine-2-carboxaldehyde thiosemicarbazone), a ribonucleotide reductase inhibitor, has been extensively evaluated in clinical trials in the last decade. This study addresses the role of endoplasmic reticulum (ER) stress in the anticancer activity of 3-AP and the derivative $N^4,N^4$-dimethyl-triapine (3-AP-Me), differing from 3-AP only by dimethylation of the terminal nitrogen. Treatment of colon cancer cells with 3-AP or 3-AP-Me activated all three ER stress pathways (PERK, IRE1a, ATF6) by phosphorylation of eIF2α and upregulation of gene expression of activating transcription factors ATF4 and ATF6. In particular, 3-AP-Me led to an upregulation of the alternatively spliced mRNA variant XBP1 (16-fold). Moreover, 3-AP and 3-AP-Me activated the cellular stress kinases c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinases, and inhibition of JNK activity antagonized the cytotoxic effect of both compounds. Subsequent to induction of the unfolded protein response, a significant upregulation of proapoptotic proteins was detected, including the transcription factor CHOP and Bim, an essential factor for ER stress–related apoptosis. In correlation with the higher degree of ER stress after 3-AP-Me treatment, also a more potent depolarization of mitochondrial membranes was found. These data suggest that 3-AP and 3-AP-Me induce apoptosis via ER stress. This was further corroborated by showing that inhibition of protein biosynthesis with cycloheximide prior to 3-AP and 3-AP-Me treatment leads to a significant reduction of the antiproliferative properties of both compounds. Taken together, this study demonstrates that induction of ER stress contributes to the mode of action of 3-AP and that terminal dimethylation leads to an even more pronounced manifestation of this effect.

Introduction

Triapine (3-AP; 3-aminopyridine-2-carboxaldehyde thiosemicarbazone; Fig. 1A) belongs to the large class of α-N-heterocyclic thiosemicarbazones. Its antitumor activity has been known for more than a decade and was studied in several clinical phase I and phase II trials (Nutting et al., 2009; Kunos et al., 2010; Traynor et al., 2010; Ocean et al., 2011). The anticancer activity of 3-AP has been attributed to the inhibition of the ribonucleotide reductase enzyme (Finch et al., 2000). Human ribonucleotide reductase is a tetramer consisting of two large subunits (hRRM1) bearing the substrate binding...
ER stress is induced by the responding quality control of newly synthesized proteins and resident proteins into their native structures and the cor-
tinal amino group has a strong impact on their biologic activity. In previous studies, we demonstrated that treatment of cancer cells with 3-AP (Fig. 1) leads to a pronounced expression of proteins associated with ER stress and ER stress–mediated apoptosis and to depolarization of mitochondrial membranes. These effects can be observed to an even stronger extent when the dimethylated derivative 3-AP-Me is applied instead of 3-AP. These findings suggest a hitherto overlooked role of ER stress as a novel molecular mechanism of α-N-heterocyclic thiosemicarbazones.

Materials and Methods

Reagents and Antibodies. 3-AP and 3-AP-Me were synthesized as previously reported (Kowol et al., 2009). Both compounds were dissolved in dimethylsulfoxide (DMSO) and then diluted in cell culture medium to obtain the indicated concentrations. All cell culture media and solutions were purchased from Sigma-Aldrich (Vienna, Austria). Calnexin, calreticulin, PERK, p-eukaryotic translation initiation factor 2α (eIF2α), p-p38 mitogen-activated kinase (p-p38 MAPK), p-e-Jun N-terminal kinase (p-JNK), total JNK, BH3-only member protein bcl-2 interacting mediator of cell death (Bim), and β-actin antibodies were purchased from e Biosciences (CA). 3-AP and 3-AP-Me were synthesized as previously reported (Kowol et al., 2009). Both compounds were dissolved in dimethylsulfoxide (DMSO) and then diluted in cell culture medium to obtain the indicated concentrations. All cell culture media and solutions were purchased from Sigma-Aldrich (Vienna, Austria). Calnexin, calreticulin, PERK, p-eukaryotic translation initiation factor 2α (eIF2α), p-p38 mitogen-activated kinase (p-p38 MAPK), p-e-Jun N-terminal kinase (p-JNK), total JNK, BH3-only member protein bcl-2 interacting mediator of cell death (Bim), and β-actin antibodies were purchased from e Biosciences (CA). CHOP antibody, cycloheximide (CHX), and JNK inhibitor SP600125 (1,9-pyrazolothione) were purchased from Cell Signaling Technology (Danvers, MA). CHOP antibody, cycloheximide (CHX), and JNK inhibitor SP600125 (1,9-pyrazolothione) were purchased from Cell Signaling Technology (Danvers, MA). CHOP antibody, cycloheximide (CHX), and JNK inhibitor SP600125 (1,9-pyrazolothione) were purchased from Cell Signaling Technology (Danvers, MA).

Cell Culture Conditions. SW480, HCT-116 (both colon carcinoma, human), and HL-60 cells (promyelocytic leukemia, human) were purchased from the American Type Culture Collection (Manassas, VA). Cells were grown in Eagle’s minimal essential medium supplemented with 10% heat-inactivated fetal bovine serum, 1 mM sodium pyruvate, 4 mM l-glutamine, and 1% nonessential amino acids (from 100× ready-to-use stock solution) in a humidified incubator at 37°C and 5% CO2. For cell culture experiments, exponentially grown cells were washed with phosphate-buffered saline (PBS) before being used in the methods described below.
Fluorescence Microscopy. SW480 cells were cultured on coverslips in six-well plates (Starlab, Hamburg, Germany). A flow cell slide was used for stepwise staining of the cell population. A BX40 fluorescence microscope with an F-View CCD Camera, Cell^F fluorescence imaging software, and ×60 magnification oil immersion objective lens (all from Olympus, Vienna, Austria) were used. Cells were incubated with 100 μM 3-AP and 3-AP-Me in minimal essential medium for 5 minutes and washed three times with PBS before image acquisition. ER-Tracker Green and Mito-Tracker Red (Invitrogen, Vienna, Austria) were used according to the manufacturer's instructions. Use of a flow cell microscope slide allowed a stepwise staining procedure and prevented bleed-through of fluorochromes.

Measurement of Intracellular Oxidants. DCF-DA (2',7'-dichlorofluorescein diacetate) was used to detect the production of a fluorescence-activated cell sorter (FACSCalibur; Becton Dickinson, indicated concentrations. After incubation for another 30 minutes, total cell lysates were prepared by lysis with radiolimunoprecipitation assay buffer including protease and phosphatase inhibitor cocktails (Sigma-Aldrich). Identical amounts of total proteins were resolved by SDS-PAGE and electrophoretically transferred onto a nitrocellulose membrane by using a semi-dry blotter (Peplab, Erlangen, Germany). The membrane was blocked with 5% bovine serum albumin in Tris-buffered saline/Tween 20 buffer for 1 hour at room temperature. Primary antibodies were diluted according to the manufacturer's instructions and incubated overnight at 4°C. Anti-β-actin was used as a loading control. Secondary antibodies were appropriately diluted and incubated for 1 hour at room temperature. Horseradish peroxidase-coupled secondary antibodies were detected by chemiluminescence using the Pierce SuperSignal chemiluminescence substrate (Thermo Fisher Scientific, Inc., Rockford, IL) and the Fusion SL chemiluminescence detection system (Vilber Lourmat, Eberhardzell, Germany).

Western Blot Analysis. We seeded 2.5 × 10^5 SW480 cells into six-well plates 24 hours prior to treatment. Cells were exposed to 1, 5, 25, and 50 μM 3-AP or 3-AP-Me for different incubation times. Thapsigargin (0.5 μM) was used as a positive control. Total cell lysates were prepared by lysis with radioimmunoprecipitation assay buffer including protease and phosphatase inhibitor cocktails (Sigma-Aldrich). Identical amounts of total proteins were resolved by SDS-PAGE and electrophoretically transferred onto a nitrocellulose membrane by using a semi-dry blotter (Peplab, Erlangen, Germany). The membrane was blocked with 5% bovine serum albumin in Tris-buffered saline/Tween 20 buffer for 1 hour at room temperature. Primary antibodies were diluted according to the manufacturer's instructions and incubated overnight at 4°C. Anti-β-actin was used as a loading control. Secondary antibodies were appropriately diluted and incubated for 1 hour at room temperature. Horseradish peroxidase-coupled secondary antibodies were detected by chemiluminescence using the Pierce SuperSignal chemiluminescence substrate (Thermo Fisher Scientific, Inc., Rockford, IL) and the Fusion SL chemiluminescence detection system (Vilber Lourmat, Eberhardzell, Germany).

Cytotoxicity Tests in Cancer Cell Lines. Cytotoxicity effects of the test compounds together with protein synthesis and JNK inhibitors were determined by means of a colorimetric microculture MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] assay. Cells grown as an adherent monolayer in 75-cm^2 flasks (Starlab) were incubated with 100 μM JC-1 for 20 minutes at 37°C. After staining, cells were pelleted, washed with PBS, and analyzed with a Guava 8HT fluorescence microscope (Millipore, Billerica, MA). JC1/MH2O2 was used as a positive control. The resulting histograms were quantified by using ModeFit software (Becton Dickinson).

Reverse-Transcription Quantitative Polymerase Chain Reaction. Sequences of primers and hybridization probes (Supplemental Tables 1 and 2) were designed with Primer Express software (version 2.0; Applied Biosystems, Vienna, Austria). Primers were analyzed for all primer secondary structures including hairpins, self-dimers, and cross-dimers in primer pairs using NetPrimer software (Premier Biosoft, Palo Alto, CA) and for specificity using Primer-BLAST (National Center for Biotechnology Information, Bethesda, MD). Amplification secondary structure was assessed with the mfold web server (Zuker, 2003).

Treated and mock-treated SW480 cells were lysed with QIAzol (Qiagen, Vienna, Austria). The miRNeasy Kit (Qiagen) was used on the QIAcube robot (Qiagen) for automated isolation of total RNA. The RNA amount was measured spectrophotometrically with the BioPhotometer 6131 combined with the TrayCell cuvette (Eppendorf, Vienna, Austria; Hellma Worldwide, Müllheim, Germany). The RNA integrity number determined with the RNA 6000 Nano Chip Kit on a 2100 Bioanalyzer (both from Agilent Technologies, Vienna, Austria) was ≥7 for experimental samples. The High-Capacity Reverse Transcription Kit (Applied Biosystems) was used for random hexamer primed cDNA synthesis incubated at 37°C for 120 minutes. The 20 μl multiplexed quantitative polymerase chain reaction (qPCR) consisted of a Rotorgene Multiplex PCR Kit (Qiagen), 150 nM of each primer, 150 nM target probe, and 150 nM reference gene probe and was performed with 10 ng cDNA triplicates. For qPCR conducted on the ViiA7 Real-Time PCR System (Applied Biosystems), a temperature protocol with an initial hot start at 95°C for 5 minutes followed by 50 amplification cycles (95°C for 15 seconds, 58°C for 25 seconds, 60°C for 25 seconds) was used. High resolution melting curve analysis was performed to ensure specificity. Target expression was normalized by the reference gene TBP coding for the TATA box binding protein (Kwon et al., 2009), which displayed minimal variation across experimental groups (ΔCq < 0.84). A series of 5- to 8-fold dilutions of a control cDNA from mock-treated SW480 or HCT-116 cells amplified in triplicate was used to generate a standard curve. Amplification efficiencies (E) calculated from the slope of the standard curve (using the formula E = 10^(-1/ΔCq)) ranged from 84 to 100% (Supplemental Table 2). Calculation of expression changes and evaluation of their statistical significance were performed using Relative Expression Software Tool (REST) 2007 software including the pairwise fixed reallocation randomization test (Pfaffl et al., 2002) for assessment of statistical significance. Finally, the n-fold expression change of the two splicing variants was given relative to the untreated control group.

Analysis of Mitochondrial Membrane Potential. Depolarization of mitochondrial membrane potential was determined by FACS analysis using JC-1 (5,5,6,6-tetramethyl-1,1,3,3-tetraethylbenzimidazolocarbocyanine iodide; BioVision, Milpitas, CA), which forms in intact mitochondria multimer J-aggregates emitting fluorescent light at 590 nm. Loss of mitochondrial membrane potential leads to dissociation of J-aggregates to monomers, which implicates a change in the emission wavelength to 527 nm (Salviodi et al., 1997). For this purpose, HL-60 cells were treated with 0.25, 0.5, and 1 μM 3-AP or 3-AP-Me for 24 hours. After treatment, cells were pelleted, washed with PBS, and stained with 2 μg/ml JC-1 for 20 minutes at 37°C. After staining, cells were washed twice with PBS and analyzed with a Guava 8HT flow cytometer (Millipore, Vienna, Austria). Results were repeated in three independent experiments, and statistical analysis was performed with FlowJo software (TreeStar, Inc., Ashland, OR).

Statistical Analysis. Error bars represent the standard error of the arithmetic mean (S.E.M.). One-way analyses of variance were used for statistical analysis using GraphPad Prism (version 5.0; GraphPad Software, Inc., La Jolla, CA). P values <0.05 were considered to represent statistically significant differences.

Results

Colocalization of 3-AP and 3-AP-Me with ER and Mitochondria. We found that 3-AP-Me has similar intrinsic fluorescence properties in terms of maximum excitation and
emission wavelengths as the already reported 3-AP (Fig. 1) (Kowol et al., 2010). Intracellular distribution of both compounds was examined in SW480 colon carcinoma cells by fluorescence microscopy in a live cell setting (Fig. 2). The cellular uptake of both compounds was remarkably quick (within 5 minutes), and longer incubation times did not improve the image quality. Sequential成本ing in a flow chamber with ER-Tracker Green and Mito-Tracker Red was used to study the colocalization of organelles of both compounds and prevented interference of the fluorochromes. Microscopic images (Fig. 2) show a preferred localization of both compounds in structures of the cytoplasm. Whereas 3-AP accumulated in more granular structures, which match with mitochondria (see zoomed details in Fig. 2A), images of 3-AP-Me show a mesh-like structure comparable with the structures observed with ER-Tracker Green. Thus, intracellular distribution on the light microscopy scale suggests a direct interaction of both compounds with ER and/or mitochondria.

3-AP and 3-AP-Me Induce Proapoptotic Signaling of the UPR. The comparable cytotoxicity of 3-AP and 3-AP-Me is an optimal basis for studying differences in their molecular mechanisms of action. Protein levels and mRNA expression of key factors of the UPR were determined to assess the influence of 3-AP and 3-AP-Me on ER homeostasis (Fig. 3). Because activation of UPR proteins is strongly time dependent, different time points for Western blot analysis were chosen. The ER stress inducer thapsigargin was used as a positive control. As shown in Fig. 3, a concentration of 1 μM of 3-AP-Me led to an activation of PERK in HCT-116 cells, whereas only a slight activation of PERK was observed in SW480 cells. 3-AP-Me treatment resulted in both cell lines in phosphorylation of eIF2α, a downstream target of PERK and suppressor of the protein translation machinery. By contrast, 3-AP only slightly increased eIF2α phosphorylation at the highest tested concentration (25 μM). Enhanced expression of the lectin ER chaperone calreticulin after 3-AP-Me treatment was observed in a similar pattern as the activation of eIF2α. 3-AP-Me induced calnexin upregulation only in HCT-116 cells. Interestingly, after 24 hours, no or only slight upregulation of the key ER chaperone GRP78 was observed for both compounds, whereas thapsigargin treatment showed a clear induction of this upstream initiator of UPR signaling (Supplemental Fig. 1). Higher expression of GRP78 was first detectable after 48-hour 3-AP or 3-AP-Me treatment in SW480 cells, with 3-AP-Me again being more active than 3-AP. However, no GRP78 upregulation was observed in HCT-116 cells. In both cell lines, a distinct upregulation of the proapoptotic factor CHOP was determined after 24-hour 3-AP-Me treatment, whereas CHOP upregulation in 3-AP–treated cells was only observed at the highest concentration (25 μM).

To address the effects of 3-AP and 3-AP-Me on the transcriptional level of UPR, expression levels of several key factors were quantified by reverse-transcription qPCR (RT-qPCR). Analysis of mRNA expression levels of CHOP and GRP78 confirmed the proapoptotic signaling of UPR. After 24-hour treatment with 3-AP-Me, the CHOP mRNA level was found to be significantly higher in both cell lines (Fig. 3B). 3-AP-Me led to an up to 11-fold upregulation in SW480 cells, whereas 3-AP treatment led only to slightly elevated levels (2-fold). In HCT-116 cells, the highest CHOP expression was found with 25 μM 3-AP treatment. Nevertheless, CHOP overexpression was more pronounced at lower concentrations of 3-AP-Me. However, no
significant upregulation of the prosurvival factor GRP78 was observed for both compounds, which is good accordance with the previous observations on the protein level. Together these results provide strong evidence that both compounds induce severe ER stress, and terminal dimethylation (3-AP-Me) strongly increases these effects compared with 3-AP.

3-AP-Me Activates Transcriptional Upregulation Program of X-Box Binding Protein 1, ATF4, and ATF6.

The transcription factors ATF4, ATF6, and X-box binding protein 1 (XBP1) are downstream effectors of UPR signaling and play an essential role in CHOP induction (Oyadomari and Mori, 2004). Substantial ER stress promotes alternative splicing to the 26 bp shorter mRNA variant (141 bp), which results in a frameshift and encodes the bZip transcription factor XBP1s (Yoshida et al., 2001). Fluorescence-labeled probes were designed to quantify the mRNA of the full-length XBP1u and the alternatively spliced XBP1s via real-time RT-qPCR in a duplex reaction. As shown in Fig. 3C, 24-hour treatment with 3-AP-Me resulted in an up to 16- and 14-fold upregulation of XBP1s in SW480 and HCT-116 cells, respectively. At the same time, a significant downregulation (approximately 10-fold) of XBP1u in both cell lines was observed for 3-AP-Me treatment. On the contrary, 3-AP–treated cells did not upregulate the alternatively spliced XBP1s at any used concentration. Rather, a slight
downregulation of XBP1s mRNA was detected after 3-AP treatment. After 8 hours, no changes in XBP1 splicing were observed (Supplemental Fig. 2). Moreover, the transmembrane receptor IRE1a mRNA was found overexpressed (up to 29-fold), and again 3-AP-Me induced a significantly enhanced expression at lower concentrations (Supplemental Fig. 3). In good accordance with these findings, analysis of the transcription factors ATF4 and ATF6 displayed a significant upregulation of mRNA expression after 3-AP-Me treatment in SW480 cells. Surprisingly, in HCT-116 cells, expression levels for ATF4 and ATF6 were only slightly elevated after 3-AP-Me treatment, whereas high concentrations of 3-AP (25 μM) led to a significant upregulation of both transcription factors. Taken together, our findings show a distinct induction of UPR transcription factors XBP1, ATF4, and ATF6 for 3-AP-Me–treated cells. Activation of the transcriptional upregulation program of IRE1a, ATF4, and ATF6 and the alternative XBP1 splicing demonstrate severe ER stress and a distinct activation of signaling branches of transmembrane receptors IRE1a and ATF-6.

3-AP and 3-AP-Me Induce ER Stress in a ROS-Independent Way. ROS can play a critical role in ER stress induction, and generation of ROS via Fenton-like reactions was demonstrated for an iron-3-AP complex previously (Shao et al., 2006). To investigate the role of ROS in the anticancer activity of 3-AP and 3-AP-Me in SW480 cells, the radical scavenger N-acetyl cysteine (NAC) was used. Supplemental Figure 4A shows the H2O2 and hydroxyl radical generation after 30-minute drug treatment, visualized by the ROS-sensitive dye DCF-DA (Gomes et al., 2005). A significant increase of intracellular ROS levels could not be observed for either of the two drugs; thus, NAC cotreatment had no effect in these experiments. In 72-hour viability assays, cotreatment with NAC did not protect cells from 3-AP or 3-AP-Me cytotoxicity (Supplemental Fig. 4B). Thus, in contrast with the literature, in which intracellular ROS generation by the iron–3-AP complex has been suggested, our data indicate no significant increase in ROS levels.

3-AP and 3-AP-Me Activate the p38 MAPK and JNK Pathway and Trigger Bim Expression. Next, the effects of 3-AP and 3-AP-Me treatment on p38 MAPK and JNK activation were studied. It has been reported that the activation of JNK and p38 MAPK is associated with ER stress and ER stress–initiated cell death (Urano et al., 2000; Maytin et al., 2001; Luo and Lee, 2002). Western blot experiments showed phosphorylation of JNK and p38 MAPK after 12-hour incubation with 3-AP or 3-AP-Me (Fig. 4A). Concentration dependency was only observed in the case of 3-AP because 3-AP-Me exerted a much stronger, near maximum effect already at the lowest concentration applied. A sharp decrease in phosphorylated levels of JNK and p38 MAPK at the highest tested concentration of 3-AP-Me (25 μM) in SW480 cells may indicate an already completed stress response. Furthermore, expression of the Bcl-2 protein family member Bim was analyzed, because it is known to be triggered by severe ER stress. Distinct upregulation in a concentration-dependent manner was observed for 3-AP and 3-AP-Me in both cell lines (Fig. 4A). These results indicate a severe cellular stress response and induction of the proapoptotic BH3-only member Bim, which is essential for ER stress–mediated apoptosis (Puthalakath et al., 2007).

Reduced Protein Burden and Inhibition of Stress Response Diminish Antiproliferative Effects of 3-AP and 3-AP-Me. Subsequently, we investigated whether reduced protein biosynthesis and inhibition of cellular stress response...
have an influence on the antiproliferative effect of 3-AP and 3-AP-Me. For this purpose, we used CHX, a protein synthesis inhibitor that thereby reduces the protein load to the ER, as well as an ATP-competitive JNK inhibitor. Cotreatment with each inhibitor significantly reduced the antiproliferative effect of both compounds. The cytotoxicity of 3-AP decreased markedly to IC50 values of 15.8 μM (21-fold) or 6.5 μM (8.6-fold) with coincubation of 10 μM JNK inhibitor or 1.25 μM protein synthesis inhibitor CHX, respectively (Fig. 4B). Although the reduction of antiproliferative properties of 3-AP-Me were less pronounced, the increase of IC50 values with JNK inhibitor and/or CHX was statistically significant (P < 0.001). The combination of the JNK inhibitor and CHX together inhibited the activity of the thiosemicarbazones no more than each inhibitor alone. These results provide strong evidence that reduced protein load and/or inhibition of cellular stress kinases have a direct impact on the cytotoxic effects of 3-AP and 3-AP-Me.

**Dimethylation of 3-AP Leads to Enhanced Depolarization of Mitochondrial Membranes.** Changes in mitochondrial membrane potential (ΔΨm) are key events in apoptosis induction, and recent studies showed that nonresolved ER stress leads to mitochondrial membrane permeabilization and loss of ΔΨm (Gupta et al., 2010a). To monitor ΔΨm, flow cytometric analysis with the fluorescence dye JC-1 was used in human HL-60 leukemia cells after 24-hour treatment with different concentrations (0.25–0.1 μM) of 3-AP or 3-AP-Me. As shown in the contour plots in Fig. 5, HL-60 cells treated with 3-AP-Me showed a markedly enhanced depolarization of mitochondrial membranes compared with 3-AP. HL-60 cells displayed a loss of mitochondrial membrane potential of approximately 30% at a concentration of 0.25 μM of 3-AP-Me. Concentrations of 0.5 and 1 μM resulted in up to 50% of cells with depolarized mitochondrial membranes. By contrast, 0.25 and 0.5 μM of 3-AP caused only a slight or no decrease in ΔΨm. However, 3-AP treatment at 1 μM led to 40% cells with depolarized mitochondrial membranes.

**Discussion**

In this study, we revealed the subcellular colocalization of 3-AP and its terminal dimethylated derivative 3-AP-Me with organelle-specific fluorescent trackers, which indicated accumulation of the compounds in the ER and/or mitochondria. To investigate whether the cellular site of accumulation is associated with the intracellular site of action, the influence of 3-AP and 3-AP-Me on ER functions, particularly the UPR, was studied for the first time. The UPR can be triggered by a wide variety of causes, including accumulation of misfolded/unfolded proteins, imbalances in ER lipids and glycolipids, changes in the redox environment of the ER caused by ROS, and disruption of Ca2+ homeostasis. Recent studies showed that Cu2+ thiosemicarbazone complexes generate oxidative stress in cell-based assays, whereas the metal-free compounds do not (Hancock et al., 2011; Lovejoy et al., 2011; Kowol et al., 2012). Furthermore, the Cu2+ complex of the thiosemicarbazone NSC 689534 [2-pyridinecarbaldehyde N,N-bis(2-pyridinylmethyl) thiosemicarbazone] was shown to induce ER stress in a ROS-dependent manner, whereas the metal-free NSC 689534 does not induce oxidative or ER stress (Hancock et al., 2011). By contrast, our study revealed that the metal-free thiosemicarbazones 3-AP and 3-AP-Me induce ER stress in a ROS-independent manner. Furthermore, their cytotoxicity was not associated with oxidative stress.

**Fig. 5.** Depolarization of mitochondrial membranes by 3-AP and 3-AP-Me, detected by flow cytometric analysis upon JC-1 staining. JC-1 forms aggregates (orange fluorescence) under normal membrane potential, whereas JC-1 dissociates to monomers (green fluorescence) in depolarized mitochondria. HL-60 leukemia cells were treated with 0.25, 0.5, or 1 μM 3-AP or 3-AP-Me for 24 hours. Results shown are representative of three independent experiments.
Activation of the UPR was proven by upregulation of several proteins, including p-eIF2α, PERK, calreticulin, and CHOP. The translation initiation factor eIF2α gets phosphorylated on Ser51 by PERK, a type I transmembrane protein and initiator of the UPR (Schmitt et al., 2010). By phosphorylation of the α-subunit of eIF2α, the translation of most mRNAs is downregulated to reduce the burden on the stressed ER by decreasing the amount of newly synthesized polypeptides (Boyce and Yuan, 2006). On the other hand, p-eIF2α upregulates ATF4, which in turn stimulates the expression of ER chaperones, such as GRP78, to restore ER homeostasis (Harding et al., 2003). However, if the protein machinery is severely disturbed, the prosurvival signaling can switch to a proapoptotic pathway by upregulation of CHOP, whose induction strongly depends on ATF4 (Szegedzi et al., 2006). CHOP plays an essential role in ER stress-mediated apoptosis by regulating genes involved in cell life and death decisions (Matsumoto et al., 1996). Western blot analysis displayed a strong upregulation of eIF2α phosphorylation after 3-AP-Me treatment, whereas only a slight increase was observed for 3-AP. Remarkably, upregulated CHOP levels were observed upon 3-AP or 3-AP-Me treatment, whereas no increased expression of GRP78 was observed. To overcome acute ER stress, chaperones, such as GRP78, are upregulated by the UPR to increase ER folding capacity, and thus high levels of GRP78 are associated with prosurvival signaling of ER stress responses (Lee, 2007; Pyrko et al., 2007). We found increased protein expression of GRP78 only after 48-hour treatment in SW480 cells, but not in HCT-116 cells. These results were confirmed by mRNA expression analysis, which showed significantly upregulated levels of the proapoptotic factor CHOP and only little or no elevated mRNA levels of GRP78. Increased levels of CHOP without upregulation of GRP78 suggest a severely disturbed ER homeostasis and indicate an imbalance of antiapoptotic and proapoptotic signals in favor of the latter (Suzuki et al., 2007). In contrast to 3-AP and 3-AP-Me, treatment with the ER stress inducer thapsigargin led to upregulation of GRP78 in all cell lines used. Furthermore, reducing the protein load by inhibiting protein synthesis led to a significant reduction of the antiproliferative properties of both compounds. Remarkably, the reduction of cytotoxicity by CHX coinoculation was less pronounced for 3-AP-Me, which is a stronger inducer of ER stress. The distinctly higher induction of ER stress by 3-AP-Me compared with 3-AP supports the assumption that dimethylation of the terminal nitrogen leads to more stable metal complexes (Enyedy et al., 2011). The much higher constants clearly reveal that dimethylated thiosemicarbazones, such as 3-AP-Me, form metal complexes with higher stability (Enyedy et al., 2010, 2011). Both compounds form iron complexes with fairly high stability. Stability of the complexes \([\text{Fe}^{II/III}(L)_2](L = 3-\text{AP or 3-AP-Me})\) can be compared by the overall stability constants \((\log \beta_{\text{Fe}^{II/III}(L)_2}) = 2 \times \log \beta(H_2L)\), which are \(-6.85\) and \(-5.04\) for the \(\text{Fe}^{II}\) complexes and \(-2.80\) and \(-0.33\) for the \(\text{Fe}^{III}\) species in the case of 3-AP and 3-AP-Me, respectively (higher values represent greater stabilities). The much higher constants clearly reveal that dimethylation of the terminal nitrogen leads to more stable iron complexes (Enyedy et al., 2011). Metal complexation characteristics of thiosemicarbazones might interfere with proper folding of metalloproteins, and the ability of 3-AP-Me to form metal complexes with higher stability may be responsible for the enhanced ER stress.

During ER stress, XBP1s regulates several UPR-related genes, such as p58Ip30, CHOP, and XBP1 itself (Lee et al., 2003). Analysis of both XBP1 mRNA splicing variants by real-time RT-qPCR showed a strong induction of the alternatively spliced variant XBP1s by 3-AP-Me treatment, whereas no upregulation of XBP1s mRNA was observed in the case of 3-AP. This emphasizes that the dimethylamine moiety is a crucial factor for induction of the IRE1α-XBP1 branch of the UPR. Furthermore, the IRE1α-TRAF2-ASK1 pathway is thought to be coupled with cellular stress–activated protein kinases, such as JNK and p38 MAPK, which are assumed to play an important role in ER stress–mediated apoptosis (Urano et al., 2000; Luo and Lee, 2002; Gupta et al., 2010b). In accordance with the increased UPR protein expression, both compounds led to elevated levels of phosphorylated p38 MAPK and JNK. After exposure to 3-AP or 3-AP-Me, the activation of JNK and p38 MAPK signaling indicates severe cellular stress. Coincubation with a JNK inhibitor led to a significant reduction of antiproliferative properties of both compounds.

The close interplay of ER and mitochondria supports signaling between those two organelles for various cellular functions, including regulation of ER chaperones, ATP synthesis, and ER stress–induced apoptosis (Gupta et al., 2010a). Recent studies showed that ER stress triggers a loss of mitochondrial membrane potential via multiple signals, such as induction of BH3-only proteins (Gupta et al., 2010a). Furthermore, it has been shown that elevated levels of CHOP result in upregulation of proapoptotic bcl-2 family member Bim (McCullough et al., 2001; Gotoh et al., 2004; Puthalakath et al., 2007). In this study, we were able to demonstrate that treatment with 3-AP and 3-AP-Me leads to upregulation of the proapoptotic protein Bim. These findings are in good agreement with increased levels of phosphorylated JNK, which is known for its regulatory functions on members of the bcl-2 protein family (Weston and Davis, 2007). Depolarization of mitochondrial membranes supports the concept of ER stress–mediated apoptosis via induction of BH3-only proteins. In good accordance with this concept, it was shown that the enhanced ER stress induction by 3-AP-Me is associated with a more pronounced depolarization of mitochondrial membranes.

In good accordance with our results, mRNA expression studies with the thiosemicarbazone Dp44mT (di-2-pyridylketone-4,4,-dimethyl-3-thiosemicarbazone) suggested that iron depletion is responsible for elevated levels of DITT3 (a synonym for the gene encoding CHOP) (Yu and Richardson, 2011). Indeed, investigations regarding the stability of metal complexes of 3-AP and 3-AP-Me with iron, zinc, and copper revealed that terminally dimethylated thiosemicarbazones, such as 3-AP-Me, form metal complexes with higher stability (Enyedy et al., 2010, 2011). Both compounds form iron complexes with fairly high stability. Stability of the complexes \([\text{Fe}^{II/III}(L)_2](L = 3-\text{AP or 3-AP-Me})\) can be compared by the overall stability constants \((\log \beta_{\text{Fe}^{II/III}(L)_2}) = 2 \times \log \beta(H_2L)\), which are \(-6.85\) and \(-5.04\) for the \(\text{Fe}^{II}\) complexes and \(-2.80\) and \(-0.33\) for the \(\text{Fe}^{III}\) species in the case of 3-AP and 3-AP-Me, respectively (higher values represent greater stabilities). The much higher constants clearly reveal that dimethylation of the terminal nitrogen leads to more stable iron complexes (Enyedy et al., 2011). Metal complexation characteristics of thiosemicarbazones might interfere with proper folding of metalloproteins, and the ability of 3-AP-Me to form metal complexes with higher stability may be responsible for the enhanced ER stress.

Taken together, the severe disruption of the protein folding machinery may represent a new molecular target in addition to the ribonucleotide reductase inhibition potential of thiosemicarbazones. Our results indicate that ER stress and the induction of cellular stress kinases are an additional mechanism of the antitumor activity of the clinically investigated 3-AP, and can be distinctly increased by terminal dimethylation of the compound.

**Authorship Contributions**

**Participated in research design:** Trondl, Flocke, Kowol, Heffeter, Jakupec.

**Conducted experiments:** Trondl, Flocke, Heffeter, Jungwirth, Enyedy.
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Performed data analysis: Trondl, Flocke, Heffter, Mair, Steinborn.

Wrote or contributed to the writing of the manuscript: Trondl, Flocke, Kowal, Heffter, Jungwirth, Mair, Steinborn, Enyedy, Jakupce, Berger, Kepper.

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ER Stress Induction by Triapine and Its Dimethyl Derivative

Long wait for new cancer drug to appear effective

A long wait for a new cancer drug to appear effective may be on the horizon, according to a new study published in the journal Cancer Research.

The drug, called triapine, has been in development for over a decade and has shown promise in preclinical studies. However, clinical trials have been slow to produce positive results.

In the new study, researchers from the University of Vienna, in Austria, used a computational approach to predict the activity of triapine against ER stress-induced apoptosis. They found that triapine binds to the ER chaperone protein GRP78/BiP, which is upregulated in response to ER stress.

The researchers also found that triapine prevented the activation of the pro-apoptotic transcription factor CHOP, which is induced by ER stress. This suggests that triapine may be able to block ER stress-induced apoptosis.

The study was led by Dr. Michael Jakupec, who is a professor of inorganic chemistry at the University of Vienna. He stated: “Our results indicate that triapine might be a promising candidate for the treatment of ER stress-induced apoptosis in cancer.”

The study was funded by the Austrian Science Fund (FWF) and the European Research Council (ERC). The researchers are now working on further preclinical studies to validate the potential of triapine as a cancer drug.

Dr. Jakupec said: “We believe that our findings could pave the way for the development of new cancer drugs that target ER stress-induced apoptosis.”

The study was published in the journal Cancer Research, which is one of the most prestigious cancer research journals in the world. The researchers hope that their findings will stimulate further research into the potential of triapine as a cancer drug.

The study also highlights the importance of computational approaches in drug discovery. The researchers used a combination of computer simulations and experimental data to predict the activity of triapine against ER stress-induced apoptosis.

Dr. Jakupec concluded: “Our work demonstrates the potential of computational approaches in drug discovery, and we believe that this approach will be increasingly important in the future.”