The thiosemicarbazone Me$_2$NNMe$_2$ induces paraptosis by disrupting the ER thiol redox homeostasis based on protein disulfide isomerase inhibition

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Abstract
Due to their high biological activity, thiosemicarbazones have been developed for treatment of diverse diseases, including cancer, resulting in multiple clinical trials especially of the lead compound Triapine. During the last years, a novel subclass of anticancer thiosemicarbazones has attracted substantial interest based on their enhanced cytotoxic activity. Increasing evidence suggests that the double-dimethylated Triapine derivative Me$_2$NNMe$_2$ differs from Triapine not only in its efficacy but also in its mode of action. Here we show that Me$_2$NNMe$_2$- (but not Triapine)-treated cancer cells exhibit all hallmarks of paraptotic cell death including, besides the appearance of endoplasmic reticulum (ER)-derived vesicles, also mitochondrial swelling and caspase-independent cell death via the MAPK signaling pathway. Subsequently, we uncover that the copper complex of Me$_2$NNMe$_2$ (a supposed intracellular metabolite) inhibits the ER-resident protein disulfide isomerase, resulting in a specific form of ER stress based on disruption of the Ca$^{2+}$ and ER thiol redox homeostasis. Our findings indicate that compounds like Me$_2$NNMe$_2$ are of interest especially for the treatment of apoptosis-resistant cancer and provide new insights into mechanisms underlying drug-induced paraptosis.

Introduction
α-N-Heterocyclic thiosemicarbazones (TSCs) are a promising class of therapeutics, which have been extensively investigated for their anticancer activity$^{1,2}$. The most prominent and best-studied drug candidate is 3-aminopyridine-2-carboxaldehyde TSC, also known as Triapine. Triapine displayed promising results in clinical phase I and II trials against hematological cancers$^{3-6}$ and has also been tested against diverse solid tumors$^7,8$. In addition, several new TSC derivatives have been developed over the last years. Two of them, namely Coti-2 and DpC, have recently entered clinical phase I trials (www.clinicaltrials.gov). Coti-2, DpC as well as the predecessor Dp44mT showed highly improved anticancer activities compared to Triapine with IC$_{50}$ values in the nanomolar concentration range (hence, called "nanomolar TSCs")$^9,10$. Our group has recently synthesized a new nanomolar TSC derivative, Me$_2$NNMe$_2$, characterized by dimethylation of both primary amino groups of the Triapine molecule(Fig. 1)$^2,11$.

Based on promising clinical trials, it is of interest to better elucidate the reasons for the greatly improved anticancer activity of nanomolar TSCs. There are several indications that nanomolar TSCs differ in their mode of...
Fig. 1 (See legend on next page.)

**A**

Time (h) & IC$_{50}$ (μM ± SD)
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| SW480 | HCT-116 |
|-------|---------|
| 24    | > 100   | > 100   |
| Triapine | 48     | 1.4 ± 0.7 |
|         | 72      | 0.6 ± 0.1 | 0.9 ± 0.2 |
| Me$_2$NNMe$_2$ | 24 | 60.0 ± 40.2 | 9.3 ± 10.1 |
|         | 48      | 1.8 ± 0.7 | 0.03 ± 0.02 |
|         | 72      | 0.02 ± 0.02 | 0.006 ± 0.002 |

**B**

**C**

Fig. 1 (See legend on next page.)
action from Triapine\textsuperscript{2,12,13}. In particular, their interaction with intracellular copper ions might be important, as intracellularly formed copper complexes have been suggested to be the active metabolites of nanomolar TSCs\textsuperscript{12–16}. In this regard, during our recent studies, we have discovered that treatment with Me\textsubscript{2}NNMe\textsubscript{2} as well as Dp44mT resulted in the formation of perinuclear cytoplasmic vesicles\textsuperscript{11} that are characteristic for paraptosis, a recently described new type of programmed cell death\textsuperscript{15,16}. Further hallmarks of paraptosis include mitochondrial swelling and damage, caspase-independent cell death and the absence of membrane blebbing/DNA condensation or fragmentation. Moreover, disruption of endoplasmic reticulum (ER) homeostasis, activation of MAPK signaling as well as protection by the thiol-containing radical scavenger N-acetylcysteine (NAC) and the MEK inhibitor U0126 have been reported\textsuperscript{15,16}. However, the exact molecular mechanisms underlying paraptosis induction are widely unexplored.

So far, mainly diverse natural compounds have been identified as paraptosis inducers. Interestingly, the list also includes some copper complexes\textsuperscript{17–19}, supporting the idea that nanomolar TSCs could also induce this novel form of cell death. Therefore, in this study, we investigated the role of apoptotic and paraptotic cell death in the mode of action of Triapine and Me\textsubscript{2}NNMe\textsubscript{2}. Our experiments revealed that treatment with Me\textsubscript{2}NNMe\textsubscript{2} induces all of the main hallmarks of paraptotic cell death. In addition, we identified the inhibition of the ER-resident protein disulfide isomerase (PDI) as a potential target of the intracellularly formed Me\textsubscript{2}NNMe\textsubscript{2} copper metabolite.

**Results**

**Anticancer activity of Triapine and Me\textsubscript{2}NNMe\textsubscript{2}**

Cytotoxicity and morphological changes induced by Triapine and Me\textsubscript{2}NNMe\textsubscript{2} were investigated in SW480 and HCT-116 cells at different time points (Fig. 1a). In general, HCT-116 cells proved to be more sensitive to TSC treatment than SW480. Moreover, in accordance with previous results\textsuperscript{11}, double-dimethylation of Triapine resulted in markedly higher activity in a time-dependent manner. The two drugs had distinct effects on cell morphology, as shown in Fig. 1b, c. Especially, Triapine-treated cells were characterized by increased cell area (up to 500%) and flattening (Fig. 1c). In contrast, treatment with Me\textsubscript{2}NNMe\textsubscript{2} led to formation of cytoplasmic vesicles (see black arrows in Fig. 1b), which dose- and time-dependently increased in size and number (Fig. 1b, Suppl. Figure 1). These observations were consistent in both cell lines. Comparable vesicle formation was also observed with the other nanomolar TSCs, DpC, Dp44mT, and Cot-2 (Suppl. Figure 2).

**Me\textsubscript{2}NNMe\textsubscript{2} accumulation in the ER-derived vesicles**

Several groups have reported that paraptosis induction is associated with the appearance of cytoplasmic vesicles originating from the ER\textsuperscript{15,16}. To investigate whether the cytoplasmic vesicles seen in Me\textsubscript{2}NNMe\textsubscript{2}-treated cells also arise from the ER, transfection experiments with ER-localized YFP were performed (Fig. 2a). As visualized by live-cell microscopy, ER-derived vesicles formed around the nucleus and rapidly increased in size (by fusion) (Fig. 2b). Moreover, no overlap of these vesicles with mitochondria or lysosomes was found (Fig. 2c and Suppl. Figure 3). Consequently, we concluded that the observed cytoplasmic vesicles after Me\textsubscript{2}NNMe\textsubscript{2} treatment originated solely from the ER.

Mapping cells by Raman microspectroscopy and subsequent principal component analysis (PCA) revealed a unique biochemical composition of these vesicles compared to the rest of the cell (Fig. 2d). Component spectra suggested enrichment of lipids (bands at ~1295 cm\textsuperscript{-1}, 1435–1480 cm\textsuperscript{-1}, and ~1650 cm\textsuperscript{-1}) in these vesicles, while bands corresponding to nucleic acids (~715 cm\textsuperscript{-1}, ~775 cm\textsuperscript{-1}, ~1090 cm\textsuperscript{-1}, and ~1570 cm\textsuperscript{-1}) were weaker compared to the rest of the cell (Suppl. Figure 4A)\textsuperscript{20}. Furthermore, classical least squares (CLS) fitting of the spectrum of the pure substance (Suppl. Figure 4B) to the Raman map revealed that Me\textsubscript{2}NNMe\textsubscript{2} appears to accumulate in these vesicles (Fig. 2d), indicating that the compound might have its intracellular target in the ER.

**Impact of the TSCs on mitochondrial integrity**

Paraptotic cell death is frequently associated with changes of mitochondrial morphology and functionality\textsuperscript{21–27}. Consequently, JC-1 staining was conducted to evaluate the impact of both drugs on mitochondrial membrane potential. Upon treatment with Triapine, only slight, non-significant effects were detected in both cell lines (Fig. 3a), while Me\textsubscript{2}NNMe\textsubscript{2} had a profound impact. In detail, in SW480 cells, at all investigated concentrations ~10% of the cells displayed depolarized mitochondria. In
Fig. 2 Me₂NNMe₂ accumulation in the ER-derived vesicles. 

a Representative fluorescence microscopy images and overlaid differential interference contrast images of the ER lumen of ER-YFP-transfected SW480 cells treated with 10 µM Me₂NNMe₂ for 24 h (scale bar: 50 µm).

b Life-cell fluorescence imaging of ER-located YFP-transfected SW480 cells treated with 1 µM Me₂NNMe₂. Time after treatment is indicated as hh:mm (scale bar: 10 µm).

c Representative fluorescence microscopy images of mitochondria (MitoTracker) showing no overlap with vesicles in ER-YFP-transfected SW480 cells treated with 10 µM Me₂NNMe₂ (scale bar: 50 µm).

d Raman microspectroscopy of SW480 cells treated with 10 µM Me₂NNMe₂ for 24 h. Principal component analysis (PCA) of Raman spectra can differentiate between background (black), cell (green) and vesicles (red). CLS fitting of Me₂NNMe₂ Raman spectrum to the spectral map of the cell revealed accumulation of the drug inside the vesicles.
Fig. 3 (See legend on next page.)
contrast, 30% of HCT-116 cells showed mitochondrial depolarization at 0.05 and 0.1 \mu M Me2NNMe2, which decreased to about 10% at higher concentrations. In parallel to mitochondrial depolarization, Me2NNMe2, but not Triapine, induced mitochondrial fragmentation or swelling (a main hallmark of paraptosis) already at 0.1 \mu M (Suppl. Figure 5). In order to investigate whether this observed swelling is accompanied by increased intramitochondrial Ca\(^{2+}\) levels, Rhod-2 AM stains were performed. Indeed, distinct accumulation of mitochondrial Ca\(^{2+}\) together with organelle swelling was observed in Me2NNMe2-exposed cells (Fig. 3b). In contrast, thapsigargin, a well-known SERCA (ER-localized Ca\(^{2+}\) ATPase) inhibitor and ER stress inducer, initiated mitochondrial Ca\(^{2+}\) accumulation but no organelle swelling. Together with the lack of organelle swelling, Triapine had also no impact on mitochondrial Ca\(^{2+}\) levels (Fig. 3b).

In agreement with the suggested contribution of mitochondria to Me2NNMe2 activity, HCT-116 cells with a BAX knockout\(^15,16\) were (in contrast to Triapine) significantly less sensitive to the methylated derivative (Fig. 3c). Interestingly, Me2NNMe2 activity was accompanied by a decrease of both pro-apoptotic BAX as well as anti-apoptotic Bcl-x\(_L\) protein levels in BAX wild-type cells, which argues against induction of apoptosis via the intrinsic (mitochondrial) pathway (Fig. 3d). Taken together, this indicates that Me2NNMe2 distinctly impacts on mitochondrial integrity already at very low drug concentrations and disruption of mitochondrial Ca\(^{2+}\) homeostasis is a key event in Me2NNMe2-induced paraptosis.

**Caspase independence of Me2NNMe2 anticancer activity**

As paraptosis is often described as a caspase-independent process\(^15,16\), as a next step the impact of the pan-caspase inhibitor z-VAD-FMK on the activity of the two TSCs was investigated. As shown in Fig. 4a, there was no relevant effect of z-VAD-FMK on the anticancer activity of the tested TSCs, in contrast to TRAIL, which was used as a positive control (Suppl. Figure 6). In addition, treatment with the pan-caspase inhibitor did not prevent the formation of cytoplasmic vesicles induced by Me2NNMe2 (Fig. 4b). To confirm the caspase independence of Me2NNMe2-induced cell death, annexin V (AV) stains were performed in the presence and absence of the pan-caspase inhibitor (Fig. 4c). Caspase inhibition had no significant impact (calculated to control by one-way ANOVA and Bonferroni’s multiple comparison test) on the AV\(^+\) cell fractions after Me2NNMe2 treatment in both cell lines. In contrast, Triapine-induced cell death in HCT-116 was strongly diminished upon addition of z-VAD-FMK, suggesting cell line-dependent apoptosis induction by this compound.

**The role of MAPKs in Me2NNMe2-induced paraptosis**

There are indications that MAPK signaling plays an important role in the execution of parapotic cell death\(^16,28\). However, whether and how Me2NNMe2 activity impacts on this pathway is so far unknown. Consequently, as a first step, we compared gene signatures of whole-genome gene expression arrays performed with 0.1 \mu M and 1 \mu M Me2NNMe2 treatment or untreated cells. Gene set enrichment analysis (GSEA) of these data showed significant upregulation of MAPK signaling pathway genes in treated as compared to untreated cells at both concentrations (Fig. 5a). A more detailed illustration of the genes up- (red) or down- (blue) regulated in this KEGG pathway is shown in Fig. 5b. When comparing these mRNA data with Western blot analysis of MEK and ERK, interestingly, both Triapine and Me2NNMe2 treatment had a tendency to stimulate the MAPK signaling at higher drug concentrations (Fig. 5c). However, at lower doses strongly reduced phosphorylation (especially of MEK1/2) was observed, indicating that stimulation of the MAPK pathway could be due to a compensatory feedback loop.

To gain more insight into the role of the MAPK pathway in the activity of our TSCs, several MEK inhibitors (U0126, PD98058, trametinib, and selumetinib) with different affinities for MEK1 and MEK2 were used. As seen in Fig. 6a and Suppl. Table 1, all inhibitors were able to protect cells against Me2NNMe2-induced cytotoxicity. However, only U0126 distinctly reduced vesicle formation in Me2NNMe2 (Fig. 6b, c). The effects of U0126 were also confirmed in HCT-116 cells (data not shown). In contrast to Me2NNMe2, Triapine activity was largely unaffected by the MEK inhibitors. As U0126 is the only inhibitor that
inhibits MEK1 and 2 to a similar extent (while the others have a stronger preference for MEK1), we hypothesized that MEK2 could have a special role in Me2NNMe2 activity. To further evaluate this hypothesis, knockdown experiments using siRNA against MEK2 were performed (Fig. 6d). Indeed, further analysis revealed that Me2NNMe2-induced vacuolization decreased upon MEK2 knockdown (Fig. 6e, f) confirming the importance of this protein in the formation of paraptotic vesicles by Me2NNMe2. Noteworthy, also induction of vesicles and anticancer activity of other nanomolar TSC (DpC, Dp44mT, and Coti-2) could be inhibited by U0126 (Suppl. Figure 7), indicating induction of paraptotic cell death also with these TSCs.

Me2NNMe2-induced ER stress based on disturbed ER thiol homeostasis

So far, there are only a few hypotheses on the exact mechanisms underlying paraptosis induction. In case of natural products, especially proteasome inhibition...
Fig. 5 (See legend on next page.)
resulting in (unfolded) protein stress has been suggested\textsuperscript{16,29}. Consequently, paraptosis induction by such drugs is often dependent on active protein synthesis. However, inhibition of protein synthesis (by cycloheximide) had no impact on the activity of Me\textsubscript{2}NNMe\textsubscript{2} and no difference was observed in the impact on protein ubiquitination levels between Triapine and Me\textsubscript{2}NNMe\textsubscript{2} (data not shown), suggesting another mode of action. Based on ER localization of Me\textsubscript{2}NNMe\textsubscript{2} in the Raman microscopy studies together with the profound ER blebbing, we hypothesized that Me\textsubscript{2}NNMe\textsubscript{2} might have a target in this organelle. In line with this hypothesis, subsequent experiments confirmed a specific form of ER stress especially in Me\textsubscript{2}NNMe\textsubscript{2}-treated cells. In more detail, Me\textsubscript{2}NNMe\textsubscript{2} (but not Triapine) treatment resulted in enhanced nuclear localization of CHOP, an ER stress-induced transcription factor, (Fig. 7a and Suppl Figure 8) together with increased phosphorylation of its upstream activator PERK (Fig. 7b). In contrast, no changes in other ER stress markers, such as BiP, IRE1\textalpha, calnexin, or changes in the phosphorylation of eIF2-\alpha were detected. Remarkably, in contrast to thapsigargin, CHOP-regulated ero1L-\alpha (an ER-specific thiol oxidase) as well as the ER-localized chaperone, isomerase and thiol oxidoreductase PDI were upregulated by both Triapine and Me\textsubscript{2}NNMe\textsubscript{2} (Fig. 7b). Moreover, our array data showed that the expression of these proteins was also increased on mRNA level upon Me\textsubscript{2}NNMe\textsubscript{2} treatment (Fig. 7c), indicating increased gene transcription of these CHOP-target genes. Interestingly, there are reports that PDI is able to bind and reduce copper (although the impact of copper binding on the enzymatic activity is not fully characterized)\textsuperscript{30}. As Me\textsubscript{2}NNMe\textsubscript{2} has strong copper-binding properties and our previous studies already indicated that addition of copper strongly increases the activity of Me\textsubscript{2}NNMe\textsubscript{2}\textsuperscript{11}, we hypothesized that our drug or its copper metabolite interferes with the functionality of PDI. Subsequently performed enzyme inhibition assays revealed that, indeed, the copper complex of Me\textsubscript{2}NNMe\textsubscript{2} (but not of Triapine) had strong PDI-inhibitory potential (Fig. 7d). Noteworthy, the metal-free drugs did not inhibit the enzyme, even at high concentrations, suggesting that prior (intracellular) copper chelation is necessary for PDI inhibition. Similar activity was also detected with the copper complexes of DpC and Dp44mT (Suppl. Figure 9).

PDI plays a key role in the ER thiol redox homeostasis by forming and rearranging disulfide bonds during protein folding. In this process, PDI oxidizes unfolded target proteins with the help of oxidized thiol-containing molecules, such as GSSG or ero1L-\alpha, thereby resulting in the reduction of these molecules\textsuperscript{31}. To gain more insight into the role of the ER thiol redox homeostasis in the mode of action of Me\textsubscript{2}NNMe\textsubscript{2}, co-incubation experiments with the thiol-containing antioxidants NAC and 1-thioglycerol were performed. Indeed, both compounds protected the cells from Me\textsubscript{2}NNMe\textsubscript{2} (but not Triapine)-induced cytotoxicity (Fig. 7e and Suppl. Figure 10). In addition, NAC also reduced anticancer activity induced by DpC, Dp44mT and Coti-2 (Suppl. Figure 7C). Noteworthy, these Me\textsubscript{2}NNMe\textsubscript{2}-induced effects were not based on enhanced global superoxide (Fig. 7f) or ROS\textsuperscript{11} levels but coincided with increased glutathione and especially GSSG levels (Fig. 7g). This suggests that nanomolar TSCs induce either a very local, organelle specific form of ROS or ROS generation does not play a major role in their anticancer activity. Taken together, these results indicate that Me\textsubscript{2}NNMe\textsubscript{2} might form an intracellular copper metabolite with PDI-inhibitory properties, which then results in disturbed ER thiol redox balance and paraptosis induction. The proposed mode of action is shown in Fig. 8.

**Discussion**

In anticancer therapy, resistance of cancer cells to apoptosis is a major obstacle to successful treatment and the cause of many cancer-associated deaths\textsuperscript{32}. Targeting cancer cells by the induction of paraptosis, a recently discovered alternative caspase-independent cell death pathway\textsuperscript{15,16}, offers the opportunity to overcome apoptosis resistance. However, the mechanisms of paraptosis are still not fully understood (and sometimes even contradictory observations have been published\textsuperscript{16,33}), making the in-depth investigation of the underlying signaling pathways of high importance. In general, there are several main hallmarks of paraptosis that are widely accepted. Among these, cytoplasmic (ER-derived) vacuolization, mitochondrial swelling/damage, caspase independence together with absence of membrane blebbing as well as DNA condensation/fragmentation, disruption of ER homeostasis, activation of MAPK signaling, protection by
Fig. 6 (See legend on next page.)
NAC and U0126 as well as protein synthesis dependence are most prominent\textsuperscript{15,16}.

So far, mainly natural compounds, such as celastrol, curcumin or cyclosporine A, were found to induce paraptosis\textsuperscript{16}. In addition, a few synthetic drugs, including some copper complexes\textsuperscript{17,19}, have been studied. Here, for the first time, we report about paraptosis induction by TSCs. Initiated by the discovery that treatment with nanomolar TSCs, such as Me\textsubscript{2}NNMe\textsubscript{2} and Dp\textsubscript{44m}T, resulted in formation of prominent cytoplasmic vesicles\textsuperscript{11}, our aim in the here presented study was to investigate whether treatment with Me\textsubscript{2}NNMe\textsubscript{2} results in paraptosis or a paraptosis-like cell death. Therefore, we have investigated different pathways and organelles involved in (apoptotic) cell death and paraptosis. Through this approach, we found that indeed Me\textsubscript{2}NNMe\textsubscript{2} induced paraptotic cell death fulfilling several main hallmarks such as swelling of ER and mitochondria, caspase independence and MAPK activation (probably via MEK2 signaling).

Interestingly, Raman microscopy experiments revealed an accumulation of Me\textsubscript{2}NNMe\textsubscript{2} in the ER-derived vesicles, suggesting that this compound might directly interfere with ER-resident proteins. Subsequent investigations revealed that Me\textsubscript{2}NNMe\textsubscript{2} treatment indeed induced a specific form of ER stress. In detail, enhanced nuclear localization of CHOP and PERK phosphorylation were detected. Besides these typical ER stress markers, we additionally observed an upregulation of ero1L-\(\alpha\) and PDI, which are both involved in the ER thiol redox homeostasis\textsuperscript{34}. Here, especially PDI attracted our attention, as it has been recently described as a copper-binding and -reducing protein\textsuperscript{30}. This is of relevance as Me\textsubscript{2}NNMe\textsubscript{2} (and other nanomolar TSCs like DpC and Dp\textsubscript{44m}T) have been well characterized for their metal-chelating properties and especially formation of an intracellular copper metabolite has been suggested to be crucial for their anticancer activity\textsuperscript{2,11,12,35,36}. Thus, the PDI-inhibitory potential of Triapine, Me\textsubscript{2}NNMe\textsubscript{2} as well as their copper complexes was investigated. Indeed, the copper complexes of Me\textsubscript{2}NNMe\textsubscript{2} as well as those of DpC and Dp\textsubscript{44m}T were able to potently inhibit the enzyme, while the Triapine copper complex as well as the ligands alone were inactive in this assay. Further evidence connecting TSCs to PDI as a potential target can be seen in the overexpression of the PDI family member CaBP1 in a L1210 cell subline selected for resistance to 4-methyl-5-amino-1-formylsiquinoline TSC (MAIQ)\textsuperscript{32}. Although this suggests an important role of this protein class in the mode of action of at least some TSCs, no further studies on this topic have been performed so far. Consequently, the exact evaluation of the mechanisms resulting in the PDI inhibition by some copper TSCs is matter of currently ongoing investigations.

In agreement with the PDI inhibition, subsequent analysis showed that Me\textsubscript{2}NNMe\textsubscript{2} treatment led to an increase of total glutathione levels, especially of its oxidized form (GSSG) and co-incubation with thiol-containing antioxidants such as NAC or 1-thioglycerol had protective effects. A disrupted thiol redox homeostasis would also explain the enhanced levels of PERK phosphorylation and subsequent CHOP translocation into the nucleus, as seen upon Me\textsubscript{2}NNMe\textsubscript{2} treatment\textsuperscript{38,39}. CHOP in turn is a transcription factor, which can initiate the observed increased expression of (among others) PDI and ero1L-\(\alpha\)\textsuperscript{40–42}. In general, disruption of the ER thiol redox homeostasis has already been discussed as the cause of ER stress and dilution for other paraptotic inducers\textsuperscript{16,28,43}. To the best of our knowledge, this is the first report connecting the induction of paraptosis to the inhibition of ER-resident proteins. Thus, the role of ER enzyme inhibition definitely needs to be addressed in detail in further studies.

With regard to the paraptotic signaling process, the observed thiol-based ER stress is in good agreement with the mitochondrial changes observed after Me\textsubscript{2}NNMe\textsubscript{2} treatment, as it has already been shown that an altered thiol balance leads to Ca\textsuperscript{2+} release from the ER and its uptake by the mitochondria\textsuperscript{44,45}. Thus, mitochondria are proposed to function as a buffer system by absorbing released Ca\textsuperscript{2+}\textsuperscript{46}. However, prolonged occurrence of
Fig. 7 (See legend on next page.)
enhanced mitochondrial Ca$^{2+}$ levels ultimately results in organelle swelling and damage, which explains the excessive depolarization of mitochondria induced by Me$_2$NNMe$_2$ and many other paraptosis inducers.$^{25,27}$ Noteworthy, we found that BAX knockout resulted in reduced sensitivity to Me$_2$NNMe$_2$. This could be explained by previously observed lowered ER Ca$^{2+}$ stores in BAX-deficient cells, which led to reduced Ca$^{2+}$ uptake.
by mitochondria after release from the ER. In addition, also a link between PDI and BAX/BAK signaling has already been reported. Nevertheless, why this mitochondrial damage in the course of paraptosis does not activate the intrinsic (mitochondrial) pathway of apoptosis is still a matter of discussion and warrants further investigations.

Taken together, in the here presented study, we identified paraptosis induction via disruption of the ER thiol redox homeostasis as a new mode of action in the activity of the highly active nanomolar TSC Me$_2$NNMe$_2$ and possibly also for other nanomolar TSCs such as DpC, Dp44mT, and Coti-2. Moreover, we suggest the ER-resident PDI as possible new target for members of this compound class, which could make them interesting candidates for the treatment of cancers with deficiencies in apoptosis induction.

Materials and methods

Reagents

Triapine and Me$_2$NNMe$_2$ were synthesized as previously described. U0126 was purchased from Calbiochem, z-VAD-FMK from Enzo Life Sciences (New York, USA), 1-thioglycerol, thapsigargin, antimycin A, NAC, PD98059, trametinib and selumetinib from Selleck Chemicals (TX, USA). All other chemicals were from Sigma-Aldrich.

Cell culture

The following human cell models were used in this study: the colon carcinoma cell lines SW480 (obtained from the American Tissue Culture Collection) as well as HCT-116 and its respective subline with BAX knockout (obtained from B. Vogelstein, John Hopkins University, Baltimore). SW480 cells were cultured in MEME and HCT-116 cell lines in McCoy’s 5a Medium (from Sigma-Aldrich, MO, USA). The cells were cultivated in medium containing 10% fetal calf serum (FCS, PAA, Linz, Austria).

Transfection

SW480 cells were plated (3 × 10^5 cells/well) in 6-well plates and allowed to recover for 24 h. Transfection of pEYFP-ER expression plasmid (#632355, Clontech laboratories, USA) encoding a YFP fused to the ER-targeting sequence of calreticulin at the 5’-end and the ER retention sequence KDEL at the 3’-end or with a control plasmid was performed using Lipofectamine 2000 reagent (Invitrogen, CA, USA) according to the manufacturers’ instructions. Medium was changed after 5 h and selection medium containing 1.2 mg/ml G418 was added 24 h after transfection. Expression of YFP in the ER was investigated 48 h later.

Cell viability assay

The cells were plated (2 × 10^3 cells/well) in 96-well plates and allowed to recover for 24 h. Then, cells were treated with Triapine or Me$_2$NNMe$_2$. In combination treatments, the modulator was always added 1 h in advance. Cell viability was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)-based vitality assay (EZ4U; Biomedica, Vienna, Austria) as published. GraphPad Prism software was used to calculate cell viability expressed as IC$_{50}$ values calculated from full dose-response curves.

Fluorescence staining and microscopy

Cells were seeded into 8-well μ-slides (ibidi GmbH, Germany) with 2 × 10^5 cells/well and left to recover for 24 h. For organelle tracking, the medium was replaced with serum- and phenol red-free medium with 50 nM MitoTracker Red CMXRos, MitoTracker Green FM or LysoTracker Red (Life technologies, Vienna, Austria). For calcium imaging, cells were incubated with 0.5 μM Rhod-2 AM (Abcam, Cambridge, UK) in serum- and phenol red-free medium for 30 min at 4 °C. After 1 h, cells were washed and imaged with the Nikon Eclipse Ti-e fluorescence microscope with differential interference contrast and RFP or GFP filter settings and a sCMOS pco.edge camera. Life-cell imaging was performed in an environmental chamber pre-heated to 37 °C with 5% CO$_2$. For non-fluorescence imaging, phase-contrast pictures were taken with the Nikon Eclipse Ti inverted microscope with a Nikon DS-Fi1c camera. Contrast and brightness were adjusted with ImageJ. Cell area was calculated as mean occupied area per cell from at least two different sections in one well at the end of life-cell imaging (48 h) using Imagel and then normalized to control.

CHOP immunofluorescence

Cells (2 × 10^5/well) were seeded in 8-well chamber slides (ibidi GmbH). After 24 h recovery, cells were treated with indicated drug concentrations and fixed with 4% paraformaldehyde for 15 min at room temperature and (after washing with PBS) blocked and permeabilized with 5% FCS, 0.3% Triton X-100 in PBS for 1 h. The primary antibody CHOP (Cell Signaling Technology) was added 1:3200 in 1% BSA and 0.3% Triton X-100 in PBS overnight at 4 °C. After washing with PBS, the cells were incubated with anti-mouse secondary antibody conjugated to AlexaFluor488 (Thermo Fisher, 1:500 in 1% BSA and 0.3% Triton X-100 in PBS) for 1 h. Cells were again washed and counterstained with 4’,6-diamidino-2’-phenyldiokol dihydrochloride (DAPI; 1 μg/ml) and wheat germ agglutinin (WGA, 10 μg/ml, Vector Laboratories,
CA, USA) in PBS for 10 min. The dyes were removed, and the cells were incubated in Vectashield mounting medium (Vector Laboratories, CA, USA) with a coverslip. Images were taken with a Zeiss LSM 700 Olympus (Carl Zeiss AG, Oberkochen, Germany) confocal microscope and CHOP fluorescence intensities per nucleus were measured using ImageJ.

Annexin V/PI stain and detection of mitochondrial membrane potential

Briefly, 2 x 10^5 cells/well were seeded in 6-well plates. After 24 h recovery, cells were treated for another 24 h with the indicated drug concentrations. Then, cells were either stained with annexin V-APC (AV) and propidium iodide (PI) or with 10 µg/ml JC-1 as previously described51,52.

Protein expression

After drug treatment, total protein lysates were prepared, separated by SDS-PAGE and transferred onto a polyvinylidene difluoride membrane for Western blotting as described previously50. The following antibodies were used: Cell Signaling Technology (MA, USA): BAX (#5023), Bcl-xL (#2764), PERK (#5683), phospho-PERK (Thr980, #3179), Calnexin (#2679), eIF2-α (#5324), phospho-eIF2-α (Ser51, #3398), PDI (#3501), eIF2-α (#3264), BiP (#3177), IRE1α (#3294), MEK1/2 (#9126), phospho-MEK1/2 (Ser217/221, #9154), MEK2 (#9125), ERK1/2 (#4695), phospho-ERK1/2 (Thr202/Tyr204, #4370), Sigma-Aldrich: β-actin (AC-15; #A1978). Primary antibodies were used 1:1000. Secondary, anti-mouse (#7076) and anti-rabbit (#7074) horseradish peroxidase-labeled antibodies from Cell Signaling Technologies were used in working dilutions of 1:10,000.

Gene knockdown by siRNA

Cells were transfected with Xfect™ RNA Transfection Reagent (Clontech Laboratories, CA, USA) using siRNA against MEK2 (Dharmacon, #M-003573-03-0005) or non-targeting siRNA (Dharmacon, #D-001206-13-05) following the manufacturer’s recommendations. Briefly, 3 x 10^5 SW480 cells/well or 4 x 10^5 HCT-116 cells/well were seeded in 6-well plates. After 24 h cells were incubated with the siRNAs and transfection polymer in serum-free medium for 4 h. Then, the medium was exchanged and after another 24 or 48 h cells were collected for experiments. Efficacy and specificity of gene silencing was verified at the protein level by Western blot following 48 h siRNA transfection.

Total-RNA isolation and whole-genome gene expression array

Total RNA from SW480 cells (either untreated or treated for 15 h) was isolated using RNeasy Mini kit (Quiagen) following the manufacturer’s instruction. Transcriptional profiles of cells were determined performing a 4 x 44 K whole-genome oligonucleotide gene expression array (Agilent) as described previously53. Normalization was performed in R using the Bioconductor (version 3.7) package “limma”54. Whole-genome gene expression array and gene set enrichment analysis (GSEA) were performed as previously described51. Visualization of differentially expressed genes in the KEGG database-derived “MAPK signaling pathway” was conducted using the Bioconductor package “pathview”55.

Raman microspectroscopy

Cells (2 x 10^4 /well) were seeded into 8-well µ-slides with glass-bottom (ibidi GmbH, Germany) and left to recover for 24 h followed by 24 h drug treatment. Subsequently, samples were fixed with 2% formaldehyde in PBS for 5 min. Cells were mapped in PBS using an XploRA INV Raman microscope (Horiba Jobin Yvon, Bensheim, Germany) equipped with a 532 nm solid state laser at 100 mW, 1800 gr/mm grating and CFI Plan APO ×100 NA 1.4 Oil objective (Nikon). Two spectra per pixel were acquired with an integration time of two seconds in steps of 0.5 µm in X and Y. Cosmic rays were removed automatically. The spectral fingerprint region of 600–1800 cm⁻¹ was extracted from raw spectra, the 1st derivative (size = 5, degree = 1) was calculated and unit vector normalization was performed. Principal component analysis (PCA) with three components was computed and displayed as a spectral map. Component spectra were shifted on the intensity scale for better visualization. The spectrum of Me2NNMe2 powder was acquired using the 532 nm laser at 100 mW, 2400 gr/mm grating and CFI Plan APO ×100 NA 1.4 Oil objective (Nikon) with 4 x 5 s integration and processed as described above. The processed spectrum of Me2NNMe2 was fitted to the spectral map of the cells by using the CLS function. All calculation and visualization steps were performed in LabSpec 6 (Horiba, Jobin Yvon, Bensheim, Germany).

PDI reduction activity measurement

PDI reduction activity was measured using PROTEOSTAT PDI assay kit (#ENZ-51024, Enzo Life Sciences, Lausen, Switzerland). Experiments were performed according to the manufacturer’s instructions. Briefly, drugs alone or preincubated with CuCl2 (1:1) were added to a prepared insulin PDI solution. Then, DTT was added to start PDI reduction activity. After 30 min the reaction was stopped by the Stop reagent and the insulin precipitate was fluorescently labeled with Proteostat PDI detection reagent for 15 min. Fluorescence intensity was measured at 500 nm excitation and 603 nm emission...
using the spectrophotometer Tecan infinite 200Pro (Tecan Group, Männedorf, Switzerland).

**Glutathione measurement**

Cells were plated (4 × 10^3 cells/well) in 96-well plates and allowed to recover for 24 h. Then, cells were treated in sextuplicates with Triapine or Me₂NNMe₂ for another 24 h. Cells were lysed and levels of total and oxidized glutathione were measured in triplicates with GSH/GSSG-Glo™ Assay (#V6611, Promega, Madison, USA) according to the manufacturer's instructions. Fold increase in relative luminescence units (RLU) was calculated compared to untreated control after subtraction of cell-free blank.

**Detection of intracellular superoxide**

Dihydroethidium (DHE, #D7008, Sigma-Aldrich, MO, USA) was used to detect the production of intracellular superoxide. Briefly, 5 × 10^5 HL-60 cells per sample in 500 μl of PBS (78.1 mM Na₂PO₄ × 2 H₂O, 14.7 mM KH₂PO₄, 26.8 mM KCl, 1.37 M NaCl) were incubated with indicated concentrations of Triapine and Me₂NNMe₂ for 45 min. Subsequently, DHE (10 μM) was added 15 min after the compounds. After incubation, the mean fluorescence intensity was measured by flow cytometry using a FACSCalibur instrument (Becton Dickinson, Palo Alto, CA, USA). Antimycin A (AMA, 10 μM) was used as positive control.

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**Conflict of interest**

The authors declare that they have no conflict of interest.

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**Supplementary Information**

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