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A new tellurium-containing amphiphilic molecule induces apoptosis in HCT116 colon cancer cells

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ABSTRACT

Background: Chalcogen-based redox modulators over the years have attracted considerable attention as anti-cancer agents. New selenium- and tellurium-containing compounds with a polar head group and aryl-groups of various lengths have recently been reported as biologically active in several organisms. In the present study, we used the most active of the tellurium compound DP41, and its selenium counterpart DP31 to investigate their effects on the human cancer cell line HCT116.
Methods: Cells were treated with DP41 or DP31 and the formation of superoxide radicals was determined using dihydroethidium. Cell cycle analysis and apoptosis was determined by cytofluorimetry. Proteins involved in ER signaling and apoptosis were determined by Western blot analysis and fluorescence microscopy.
Results: With 50 μM of DP41, we observed an increase in $O_2^{\bullet -}$ formation. There was, however, no such increase in $O_2^{\bullet -}$ after treatment with the corresponding selenium compound under the same conditions. In the case of DP41, the production of $O_2^{\bullet -}$ radicals was followed by an up-regulation of Nrf2, HO-1, phospho-eIF2α and ATF4. CHOP was also induced and cells entered apoptosis. Unlike the cancer cells, normal retinal epithelial ARPE-19 cells did not produce elevated levels of $O_2^{\bullet -}$ radicals nor did they induce the ER signaling pathway or apoptosis.
Conclusions: The tellurium-containing compound DP41, in contrast to the corresponding selenium compound, induces $O_2^{\bullet -}$ radical formation and oxidative and ER stress responses, including CHOP activation and finally apoptosis.
General significance: These results indicate that DP41 is a redox modulating agent with promising anti-cancer potentials.

1. Introduction

Organosulfur-containing compounds have had a prominent role in medicine since ancient times [1–3]. There is a comprehensive literature in particular on sulfur-containing compounds derived from garlic, onions and leek [4–7]. Against this background, the medicinal applications of other members of the chalcogen family, namely selenium and tellurium compounds, are clearly still in their infancy. Selenium is an essential trace element which is involved in a variety of different functions in the human body [8]. Furthermore, selenium seems to play a role in cancer prevention and treatment. In contrast to selenium, tellurium does not have any known natural biological function in the human body. One of the reasons for this difference may be that tellurium–carbon bonds are more labile than their selenium counterparts and so bond cleavage occurs much more readily [9]. There are many reports on efforts which have been directed towards the evaluation of synthetic selenium compounds for pharmaceutical applications [10], but only a very limited number of attempts are known for synthetic tellurium compounds. Some of the compounds studied so far exhibit anti-fungal, anti-bacterial and anti-malarial activities [11,12]. Many of these applications, but not all, rely on an inherent redox modulating ability of selenium and tellurium. Unfortunately, many of the most interesting organoselenium or organotellurium compounds are poorly water soluble and unstable and hence inappropriate to test in a biological context. On the other hand, highly water-soluble compounds have only a limited capability to cross the plasma membrane and to penetrate into cells. Thus, bioavailability of such agents is usually limited. About a decade ago, first attempts on the synthesis of amphiphilic selenium and tellurium compounds with an anionic head group and different hydrophilic tail lengths were published. These compounds were tested for their protective activity against oxidation and nitration reactions caused by peroxynitrite [13]. Furthermore, the catalytic activity of these compounds towards the peroxidation of the zinc-containing protein metallothionein was investigated. Recently, newly designed tellurium-

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containing compounds were used to monitor *in vivo* redox reactions between peroxynitrite and glutathione [14]. Other new tellurium compounds have also been used for the detection of reactive oxygen species [15]. Both these detection systems utilize the redox properties of the tellurium atom. On the other hand, new tellurium-containing pyrylium compounds were tested as modulators of the activity of multidrug resistance proteins [16,17]. It turned out that the electronegativity of the chalcogen determines whether these compounds act as inhibitors or activators of the multidrug resistance proteins. A whole series of new organoselenium and organotellurium compounds with different arylside chains have recently been synthesized and initially characterized [18]. At the same time, chalcogen nanoparticles have been used successfully providing further impetus for the development of more sophisticated self-assembling tellurium agents. In general, it was found that the tellurium compounds are more potent than the corresponding selenium compounds with regard to a reduction in cell viability and killing of bacteria. In the present study, we used the most potent compounds and tested their ability to induce cell death in cancer cells. Moreover, we analyzed cellular signaling pathways leading to growth arrest and eventually to apoptosis. We found that the organotellurium compound DP41 generates $O_2^{\bullet -}$ radicals in cancer cells. The production of $O_2^{\bullet -}$ radicals is followed by the activation of the ER signaling pathway which finally results in the induction of apoptosis. In contrast, normal retinal pigment epithelial (ARPE-19) cells failed to produce any significant amounts of $O_2^{\bullet -}$ radicals in response to DP41 treatment and consequently there was no induction of apoptosis in these cells.

2. Materials and methods

2.1. Reagents and antibodies

Protease inhibitor cocktail Complete™ (Roche Diagnostics, Mannheim, Germany), ascorbic acid (ASC) and anti-α-tubulin antibodies were obtained from Sigma-Aldrich (Munich, Germany). DMSO was from Merck (Darmstadt, Germany). Antibodies against CHOP (GADD153), cytochrome c, γH_2AX, GAPDH and Nrf2 were purchased from Santa Cruz Biotechnology (Heidelberg, Germany). Anti-poly(ADP-ribose) polymerase (anti-PARP), anti-ATF4, anti-eIF2α, anti-phospho-eIF2α (Ser 51) and anti-HO-1 antibodies were purchased from Cell Signaling Technology (Frankfurt, Germany), while the caspase 3 antibody was purchased from Promega (Mannheim, Germany). Goat, mouse and rabbit secondary antibodies were all bought from Dianova (Hamburg, Germany). Organic selenide DP31 and telluride DP41 compounds were synthesized and described recently [18]. They were dissolved in DMSO to a 100 mM stock solution which was freshly prepared before use. Ascorbic acid was dissolved in distilled water to a 100 mM stock solution and applied to the cell culture medium 0.5 h (unless stated otherwise) before treatment with chalcogen compounds.

2.2. Cell culture

HCT116 cells were maintained at 37 °C and 5% CO_2 in McCoy's 5A medium (PromoCell, Heidelberg, Germany) with 10% fetal calf serum (FCS). ARPE-19 cells were maintained at 37 °C and 5% CO_2 in DMEM medium (PromoCell, Heidelberg, Germany) with 10% FCS. The androgen-sensitive prostate cancer cells, LNCaP, were maintained at 37 °C in RPMI 1640 media, supplemented with 10% FCS in an atmosphere enriched with 5% CO_2.

2.3. Tubulin immunofluorescence analysis

HCT116 cells were grown on coverslips until they were 70% confluent. They were washed with PBS (pH 7.4), treated with DP41 at 50 μM and with DMSO as solvent control. After incubation for various time periods, cells were washed with PBS, fixed in 3% (v/v) formaldehyde in PBS at room temperature for 20 min and permeabilized on ice for 5 min with 0.2% (v/v) Triton X-100. This was followed by another washing step with PBS, blocking with 2% (w/v) bovine serum albumin (BSA) for 3 × 10 min at room temperature on a shaker. Cells were then incubated with a primary antibody against α-tubulin for 30 min at 37 °C in a humidified chamber. They were further washed with PBS for 3 × 10 min, incubated with secondary antibody (ALEXA-Fluor™ 488) at 37 °C for 30 min in the dark, then washed under the same conditions as above. This was followed by another treatment with 0.5 μM 4′,6-diamidino-2-phenylindole (DAPI) at 37 °C for 15 min in a humidified chamber and another washing step with PBS on a shaker. Cells on the coverslips were fixed with a drop of mounting medium and were analyzed using the QLC-100 microscope equipped with a 60× oil immersion objective (NA 1.4; Nikon, Japan).

2.4. Cell cycle analysis

HCT116 cells (5×10^5) were seeded on a 10 cm Petri dish and grown overnight. The medium was changed and cells were treated with DMSO (solvent control) or 50 μM DP41 and incubated for 24 h. Media and trypsinized cells were subsequently collected from a Petri dish, and centrifuged with 200 ×*g* at 4 °C for 7 min. Cells were washed with cold PBS then centrifuged under the same conditions as above twice before being re-suspended in PBS and fixed with 70% ethanol. The cells were incubated further with 1 mg/ml RNase (Sigma-Aldrich, Taufkirchen, Germany) and 400 μM propidium iodide (1 mg/ml, Sigma-Aldrich) to label DNA. Cells were then analyzed in a flow cytometer (FACSCalibur, 4CS E4021, Becton and Dickinson, Heidelberg, Germany). 10,000 events were counted for each sample. Data were analyzed using FlowJo software (FlowJo, USA).

2.5. Analysis of apoptosis

HCT116 cells or ARPE-19 cells (5×10^5) were seeded and grown on a 10 cm Petri dish overnight. The medium was changed and cells were treated with 50 μM DP41 and incubated for 24 h. Cells were trypsinized and collected along with media from the Petri dish, then centrifuged with 200 ×*g* at 4 °C for 7 min. Cells were washed with binding buffer (BioLegend, Fell, Germany), centrifuged twice and then resuspended in 100 μl binding buffer. They were stained with 5 μl annexin V FITC (BioLegend) and 10 μl of 0.1 mg/ml propidium iodide (1 mg/ml, Sigma-Aldrich). Cells were then analyzed in a cytofluorimeter (FACSCalibur, 4CS E4021, Becton and Dickinson). 10,000 events were counted for each sample. The data were analyzed using FlowJo software (FlowJo).

2.6. ROS assay

HCT116 or ARPE cells (2×10^4) were seeded in 96 black/transparent wells from Becton and Dickinson overnight. Medium was removed and cells were washed 3 times with Tyrode's buffer (10 mM HEPES, 140 mM NaCl, 5 mM KCl, 10 mM glucose, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.4) for 3 times. For ROS staining, 3.2 mg dihydroethidium (DHE, Sigma-Aldrich) was dissolved in 100 μl DMSO as stock solution, and diluted with Tyrode's buffer to 25 μM as working solution. 100 μl DHE-working solution was added to each well. After cells were incubated with DHE for 30 min at 37 °C in the dark, they were washed again 3 times with Tyrode's buffer. 100 μl Tyrode's buffer containing 50 mM DP31 or DP41 was then added directly into each well. The fluorescence (Ex/Em: 518 ± 9/606 ± 20 nm) per each well was measured with a plate reader (Tecan Infinite M200, Crailsheim, Germany) every 5 min to determine the concentration of $O_2^{\bullet -}$ inside the cells.

2.7. Extraction of cellular proteins

Following incubation of HCT116 cells or ARPE-19 cells with the test compounds, cells were collected in cold PBS, pH 7.4 and centrifuged

together with the cell culture medium at 4 °C and 250 ×g for 7 min. After one washing step with cold PBS, cells were lysed with 100 μl of RIPA buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.5% sodium desoxycholate, 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS)) supplemented with the protease inhibitor cocktail Complete™ according to manufacturer's instructions (Roche Diagnostics, Mannheim, Germany). The cell lysate was left on ice for 15 min, subjected to sonification (3 × 1 min) at 4 °C and then cell debris was removed by centrifugation at 16,250 ×g at 4 °C for 30 min. The protein content of the supernatant was determined according to the Bradford method using the Bio-Rad protein assay reagent (Bio-Rad, Munich, Germany).

2.8. SDS polyacrylamide gel electrophoresis and Western blot analysis

Proteins were separated on either a 10 or 12.5% sodium dodecyl sulfate-polyacrylamide gel and transferred onto a polyvinylidene difluoride membrane (PVDF) by tank blotting using a transfer buffer containing 20 mM Tris–HCl, pH 8.8 and 150 mM glycine. The membrane was blocked with 5% dry milk in PBS containing 0.1% Tween-20 for 1 h at room temperature and then incubated with the specific antibody which was diluted in PBS with 0.1% Tween-20 containing 1% dry milk powder. The membrane was washed with PBS Tween-20 containing 1% skimmed milk (3 × 10 min), before being incubated with a peroxidase-coupled secondary antibody (anti-rabbit 1:30,000 or anti-mouse 1:10,000) for 1 h at room temperature. The membrane was washed again in PBS Tween-20 (3 × 10 min). Signals were developed and visualized by the Lumilight system from Roche Diagnostic (Mannheim, Germany).

2.9. Treatment with antioxidant

In order to estimate antioxidant effects on the cellular process triggered by the organotellurium compound DP41, and hence, to establish a possible redox-link between this compound and its biological activity, HCT116 cells were pretreated with 100 μM ascorbic acid for 0.5 h and then washed with PBS twice prior to treatment with the organochalcogen compounds as described above.

2.10. Statistics

GraphPad Prism software (GraphPad Inc., USA) was used for statistical analysis. Results were expressed as arithmetic mean ± s.d. Differences between the experimental groups were analyzed using one-way ANOVA or Student's *t*-test (two-tail, unpaired), and statistical significance differences were shown as follows: $p < 0.05$ (*), $p < 0.01$ (**) or $p < 0.001$ (***).

3. Results

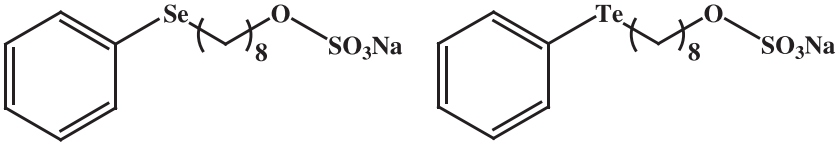
3.1. DP41 induces $O_2^{\bullet -}$ radical formation

Organoselenium and organotellurium compounds have been known for quite some time and their biological functions were tested in fungi, bacteria and some mammalian cells. One of the main disadvantages of many of these molecules, however, is the poor solubility in water while others, such as inorganic tellurium salts are poorly bioavailable. To circumvent these problems, novel amphiphilic selenium- and tellurium-containing compounds have been synthesized and initially characterized biophysically [18]. Here, we attempted to analyze two of these compounds, one selenium compound (DP31) and the corresponding tellurium compound (DP41) in mammalian cells. The structure of these two compounds is shown in Fig. 1. Since both, selenium as well as tellurium can act as redox modulators, we first asked whether treatment of the human cancer cell line HCT116 might induce the formation of $O_2^{\bullet -}$ radicals. We analyzed the generation of $O_2^{\bullet -}$ radicals with a dihydroethidium (DHE) assay according to the manufacturer's instructions. As shown in Fig. 2A, treatment of HCT116 cells with the selenium compound DP31 did not result in an increase in $O_2^{\bullet -}$ radical formation, whereas, the tellurium compound DP41 clearly induced the formation of $O_2^{\bullet -}$ radicals. As a control, cells were incubated with the solvent dimethyl sulfoxide (DMSO) alone and with H_2O_2 as a positive control. In order to confirm these results, we repeated the experiment with the active compound DP41 in the presence of the antioxidant ascorbic acid. As shown in Fig. 2B, ascorbic acid led to a down-regulation of the $O_2^{\bullet -}$ radicals after treatment of the cells with DP41. Thus, in contrast to the selenium compound the tellurium compound induces the formation of reactive oxygen species.

3.2. DP41 up-regulates the antioxidant response proteins Nrf2 and HO-1

Under normal physiological conditions, cells respond to reactive oxygen species (ROS) with enzymatic and non-enzymatic defense and signaling mechanisms. One of the key players in the antioxidant response in eukaryotic cells is the transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2), which is complexed with Keap1 in the cytoplasm [19]. ROS induces the liberation of Nrf2 from Keap1 repression with a subsequent translocation of Nrf2 into the nucleus where it binds to the antioxidant response element (ARE) for transcription of various genes. In order to analyze a possible activation of Nrf2 by DP41, cells were treated with these compounds for various periods of time. Afterwards, nuclear extracts were prepared and analyzed by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis followed by Western blot with an Nrf2 specific antibody. As shown in Fig. 3A, there was an increase in the nuclear Nrf2 protein starting at 1 h and reaching a maximum at 2 h after treatment with DP41. After 2 h Nrf2 was still present but at a much lower level than before.

One of the down-stream targets of Nrf2 is the heme oxygenase 1 (*HO-1*) gene whose gene product is a sensitive and reliable indicator of cellular stress [20]. Therefore, in the next step, we were interested in evaluating the effect of DP41 on the HO-1 protein levels following treatment of HCT116 cells. As shown in Fig. 3B, there was a steady increase in HO-1 levels after 2 h of treatment of HCT116 cells with DP41. Thus, from these results it is clear that treatment of HCT116 cells with the tellurium compound DP41 resulted in the formation of $O_2^{\bullet -}$ radicals, which is followed by an antioxidant response including Nrf2 and HO-1 activation.



DP31 DP41

Fig. 1. Chemical structures of selenium- and tellurium-containing compounds, DP31 and DP41, respectively.

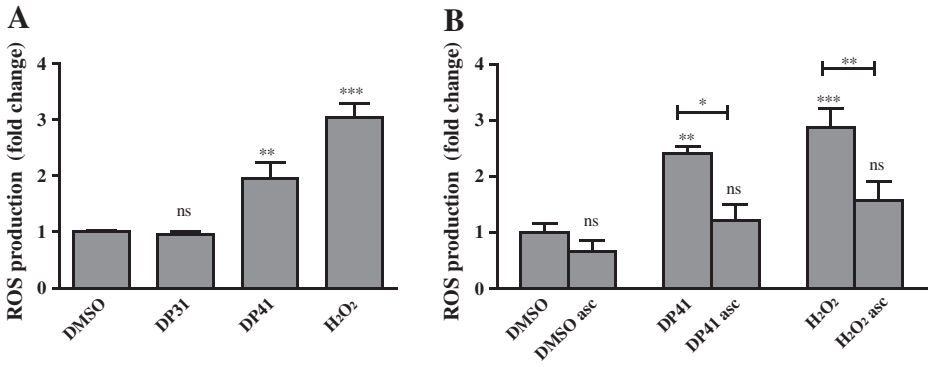


Fig. 2. DP41 induces generation of $O_2^{\bullet -}$. (A) Relative increases in DHE oxidation in HCT116 cells, indicative of $O_2^{\bullet -}$ radical formation following treatment with DMSO (solvent control), 50 μM DP31, 50 μM DP41 or 50 μM H_2O_2 for 45 min. (B) Pre-treatment of HCT116 cells with ascorbic acid reduces the DP41- or H_2O_2-induced formation of $O_2^{\bullet -}$. Data are expressed as a relative increase in DHE oxidation in relation to solvent control and depicted as means ± s.d. (n = 3). $^{**}p < 0.01$ or $^{*}p < 0.05$.

3.3. DP41 induces other ER stress proteins but not CHOP

It is well established that $O_2^{\bullet -}$ radicals cause an endoplasmic reticulum (ER) stress response [21]. In ER stress response signaling, phosphorylation of the eukaryotic initiation factor 2α (eIF2α) is one of the early events. This specific phosphorylation is a signal for the attenuation of protein synthesis in ER stressed cells. In order to analyze whether treatment of HCT116 cells with the tellurium compound DP41 might induce this particular kind of ER stress signaling, cells were treated with DP41 for various time intervals. Cells were subsequently lysed and the cell extract analyzed on an SDS polyacrylamide gel followed by a Western blot with antibodies against total eIF2α or the phosphorylated form of eIF2α. As shown in Fig. 4A, the overall amount of eIF2α in the cells remained constant over the time period analyzed. In contrast, we noticed a transient increase in the level of phosphorylated eIF2α, commencing after 1 h of treatment of HCT116 cells with DP41 and remaining at an elevated level for up to 6 h after treatment.

Although ER stress signaling leads to a general repression of protein synthesis, it also induces the expression of the activating transcription factor 4 (ATF4). ATF4 is a transcription activator of genes which are involved in protein folding and assembly, metabolism and antioxidation [22]. Therefore, in the next step, we analyzed the expression of ATF4 after treatment of HCT116 cells with DP41. As shown in Fig. 4B, we observed an increase in the level of ATF4 after 4 h treatment.

One of the down-stream targets of ATF4 is the C/EBP homologous protein (CHOP) which is also known as growth arrest- and DNA damage-inducible gene 153 (GADD153) [23,24]. CHOP is considered an inducer of stress mediated apoptosis. After incubation of HCT116 cells with DP41 for various periods of time, we lysed the cells and the cell extract was subsequently analyzed on an SDS polyacrylamide gel followed by a Western blot with a CHOP specific antibody. Fig. 4C shows an increase in the level of CHOP 6 h after treatment of HCT116 cells with DP41. The level of CHOP remained elevated up to 16 h after treatment.

3.4. DP41 causes cell cycle arrest in the G_2/M-phase of the cell cycle and apoptosis

Ultimately, CHOP induction after treatment of HCT116 cells with DP41 may result in cell cycle arrest and/or apoptosis. Therefore, in the

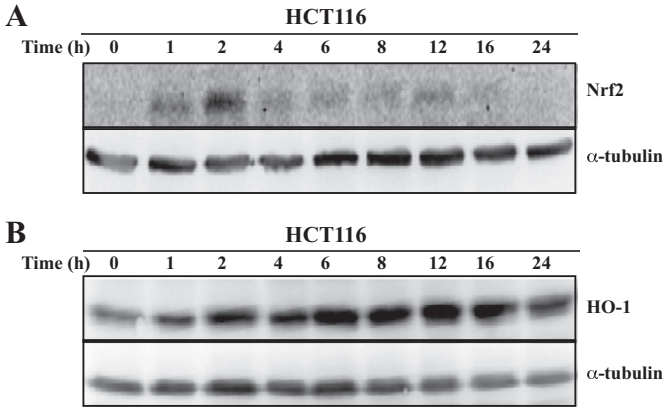


Fig. 3. DP41 induces Nrf2 and HO-1 protein expressions in HCT116 cells. Cells were untreated (0 h) or treated with 50 μM DP41 for the indicated period of time. Cell lysates were prepared and analyzed on a 10% (A) or a 12.5% (B) SDS-polyacrylamide gel followed by Western blotting using an anti-Nrf2 (A) or an anti-HO-1 (B) specific antibody. α-Tubulin was used as a loading control. One representative of at least 2 Western blots is shown here.

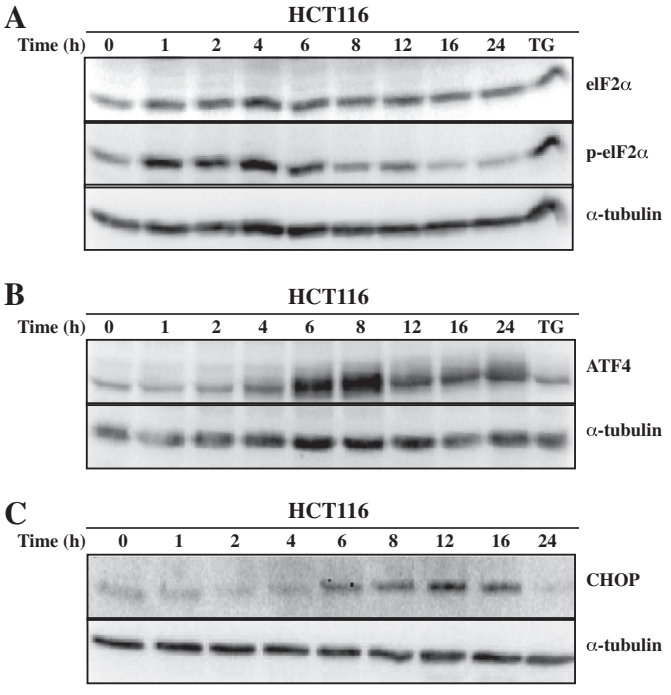


Fig. 4. DP41 induces the expression of proteins related to ER stress in HCT116 cells. Cells were untreated (0 h) or treated with 50 μM DP41 for the indicated period of time. For phospho-eIF2α and ATF4 positive controls, HCT116 cells were treated with 1 μM thapsigargin (TG) for 4 h. Cell lysates were prepared and analyzed on a 12.5% SDS-polyacrylamide gel followed by Western blotting using anti-p-eIF2 or anti-eIF2α (A), anti-ATF4 (B) or anti-CHOP (C) specific antibody. α-Tubulin was used as a loading control. One representative of at least three Western blots is shown here.

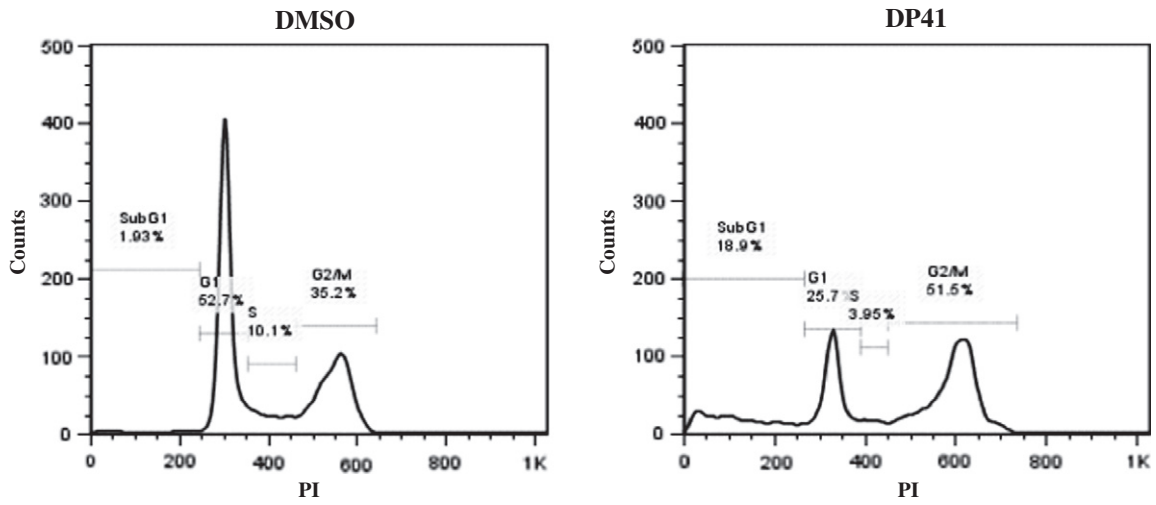


Fig. 5. Influence of DP41 on cell cycle distribution. HCT116 cells were treated for 24 h with DMSO (solvent control) or 50 μM DP41 and then analyzed by FACS. Cytofluorimetric analysis of treated cells. One representative of at least three independent experiments is shown here.

next step, we analyzed the cell cycle distribution after treatment of HCT116 cells with DP41 using flow cytometry. After treatment of the cells for 24 h, we observed a G_2-arrest (Fig. 5). In addition to the G_2-arrest, we also observed an increase in the number of cells in the subG_1-phase, which might indicate the beginning of apoptosis. In order to confirm a possible induction of apoptosis, we analyzed HCT116 cells with annexin V and propidium iodide staining (Fig. 6A). Quantification of apoptotic cells revealed that 24 h after treatment, around 20% of the cells were already present in the apoptotic fraction (Fig. 6B). To further confirm induction of apoptosis, we analyzed the expression of the anti-apoptotic bcl-2 protein. Here, cells were treated with DP41 or with DMSO (solvent control) alone for 24 h or 48 h. Cell extracts were analyzed on a SDS polyacrylamide gel followed by Western blot with a bcl-2 specific antibody. As shown in Fig. 6C, there was a significant down-regulation of bcl-2 after 24 h which became even more pronounced after 48 h treatment of HCT116 cells with DP41. In the next step, we analyzed the release of cytochrome c from mitochondria into the cytosol as an additional marker of the apoptotic process. Cells were treated as described above and cytosolic extracts were analyzed on an SDS-polyacrylamide gel followed by Western blot with a cytochrome c specific antibody. Fig. 6D shows an increase of cytosolic cytochrome c after treatment of the cells with DP41, whereas there was no such increase in DMSO treated control cells. Next, we analyzed the cells for caspase 3 cleavage as an even further proof for apoptosis. Cells were treated as described earlier and caspase 3 cleavage was analyzed with a caspase 3 specific antibody. The caspase 3 cleavage product was detected in DP41 treated cells but not in the control cells treated with DMSO alone (Fig. 6E). To further confirm this result, we looked for the cleavage of poly-ADP-ribose polymerase (PARP), which is a well known substrate for caspase 3/7. We again treated HCT116 cells with DP41 for 24 h or for 48 h. Cells were lysed and the cell extract was analyzed on an SDS polyacrylamide gel followed by Western blot with a PARP specific antibody. As shown in Fig. 6F, after 24 h and 48 h of treatment, there was a band for the PARP cleavage product. Thus, all these results show that treatment of HCT116 cells with DP41 results in a G_2-arrest of the cell cycle, and the induction of apoptosis.

Based on the finding that DP41 arrested HCT116 cells in the G_2-phase of the cell cycle, we next examined the effect of DP41 on the microtubule organization by immunostaining of α-tubulin. A normal microtubule distribution in HCT116 cells and network formation were observed in the cytoplasm of HCT116 cells treated with DMSO alone. In sharp contrast, taxol as a positive control caused a disruption of the microtubule network and a very similar result was obtained with DP41 (Fig. 7). Thus, DP41 destroys the microtubulin network in HCT116 cells.

All experiments described so far were performed with the cancer cell line HCT116. In order to evaluate whether the effects seen with DP41 are restricted to such cancer cells, we analyzed the effect of DP41 on normal retinal epithelium ARPE-19 cells. ARPE-19 cells were treated with DP41 and cells were analyzed for ROS production as described above for HCT116 cells. The results are shown in Fig. 8A. In the control experiment with H_2O_2, we found a clear induction of ROS production, whereas, with the tellurium compound DP41, ROS induction was notably absent in these cells. Since there is no notable increase in ROS levels in normal cells, we wonder whether normal cells would also avoid apoptosis under these conditions. Apoptosis was analyzed by annexin V and propidium iodide staining after treatment of ARPE-19 cells with DP41 for 48 h. Fig. 8B and the statistical analysis presented in Fig. 8C reveal no induction of apoptosis in ARPE-19 cells. In order to support these findings by another method, we treated ARPE-19 cells with DP41. As a positive control, HCT116 cells were treated with 5-fluorouracil (5-FU). The cell extract was analyzed on an SDS-polyacrylamide gel followed by Western blot with an anti-PARP antibody. As shown in Fig. 8D, there was a clear signal for the PARP cleavage product at a molecular weight of 89 kDa in 5-FU treated cells, whereas, no such signal was observed in DMSO or DP41 treated cells. Thus, these results demonstrate that in contrast to HCT116 cells, ARPE-19 cells are resistant against apoptosis after treatment with DP41.

Since CHOP is a critical factor in ER stress induced apoptosis in cancer cells (Fig. 4C), we next analyzed ARPE-19 cells for CHOP induction after DP41 treatment. Cell extracts were analyzed on an SDS-polyacrylamide gel followed by Western blot with a CHOP specific antibody. As shown in Fig. 8E, we could not detect the expression of CHOP in DP41 treated ARPE-19 cells, whereas, in the positive control, there was a clear signal for the CHOP protein.

4. Discussion

The unique redox and catalytic properties of organoselenium and organotellurium compounds turn these compounds into attractive molecules for pharmaceutical studies. Previously, two major drawbacks of these compounds included the poor water solubility on the one hand and the limited penetration of the compounds through membranes into the cells on the other hand. These drawbacks were mainly solved by the recently published synthesis of amphiphilic organoselenium

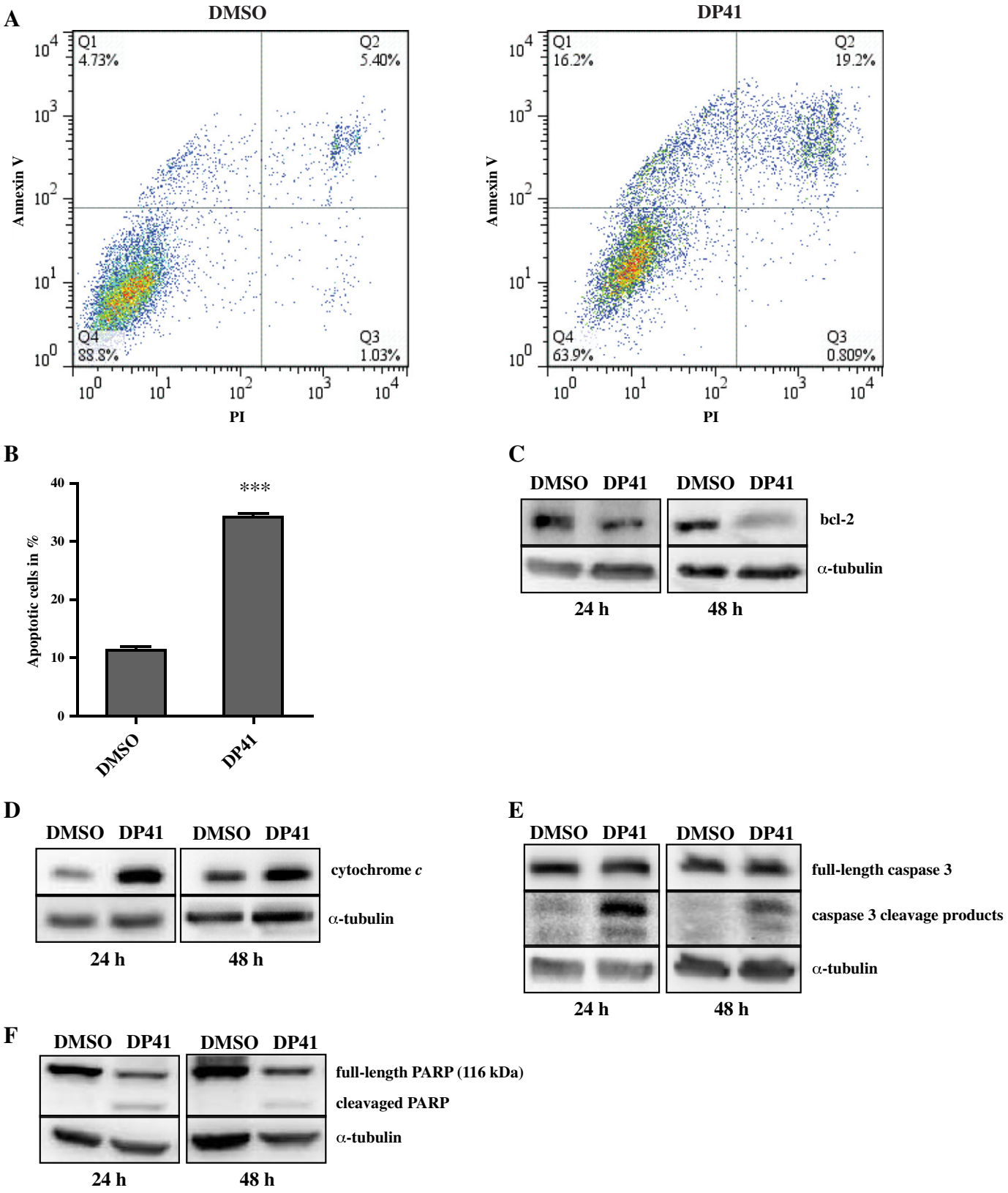


Fig. 6. DP41 induces apoptosis in HCT116 cells by modulating bcl-2 protein expression, inducing cytochrome c release and activation of both, caspase 3 and PARP cleavage. (A) Cells were treated for 24 h with either DMSO (solvent control) or 50 μM DP41 and then stained with annexin-V and propidium iodide (PI) before being analyzed by FACS for apoptotic cell death. Viable cells are presented as both annexin-V and PI negative. (B) Percentage distribution of DP41-induced apoptotic cell death relative to DMSO control. One representative of at least three independent experiments is shown here. Means ± s.d. (n = 3). $^{**}p < 0.01$ or $^{*}p < 0.05$. (C–F) HCT116 cells were treated with either DMSO or 50 μM DP41 for 24 and 48 h, and protein expressions, PARP cleavage and caspase 3/7 activation and cleavage were subsequently studied and analyzed by Western blot. Proteins were separated on a 12.5% SDS-polyacrylamide gel and blotted on a PVDF membrane. Bcl-2 (C), cytochrome c (D) or PARP cleavage (F) was visualized with the appropriate antibody, while full-length caspase 3/7 and its cleavage products (E) were detected with a caspase 3-specific antibody (8G10). α-Tubulin was used as a loading control. One representative of at least three Western blots is shown here.

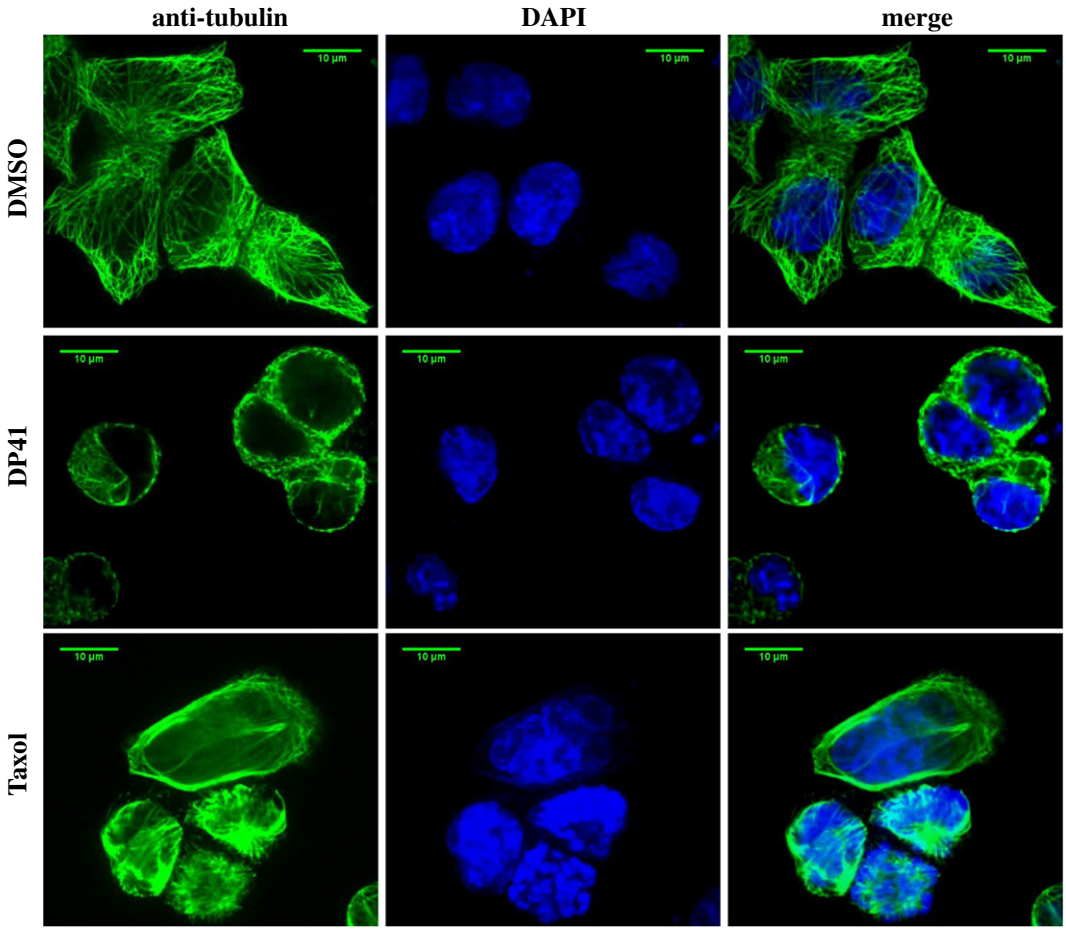
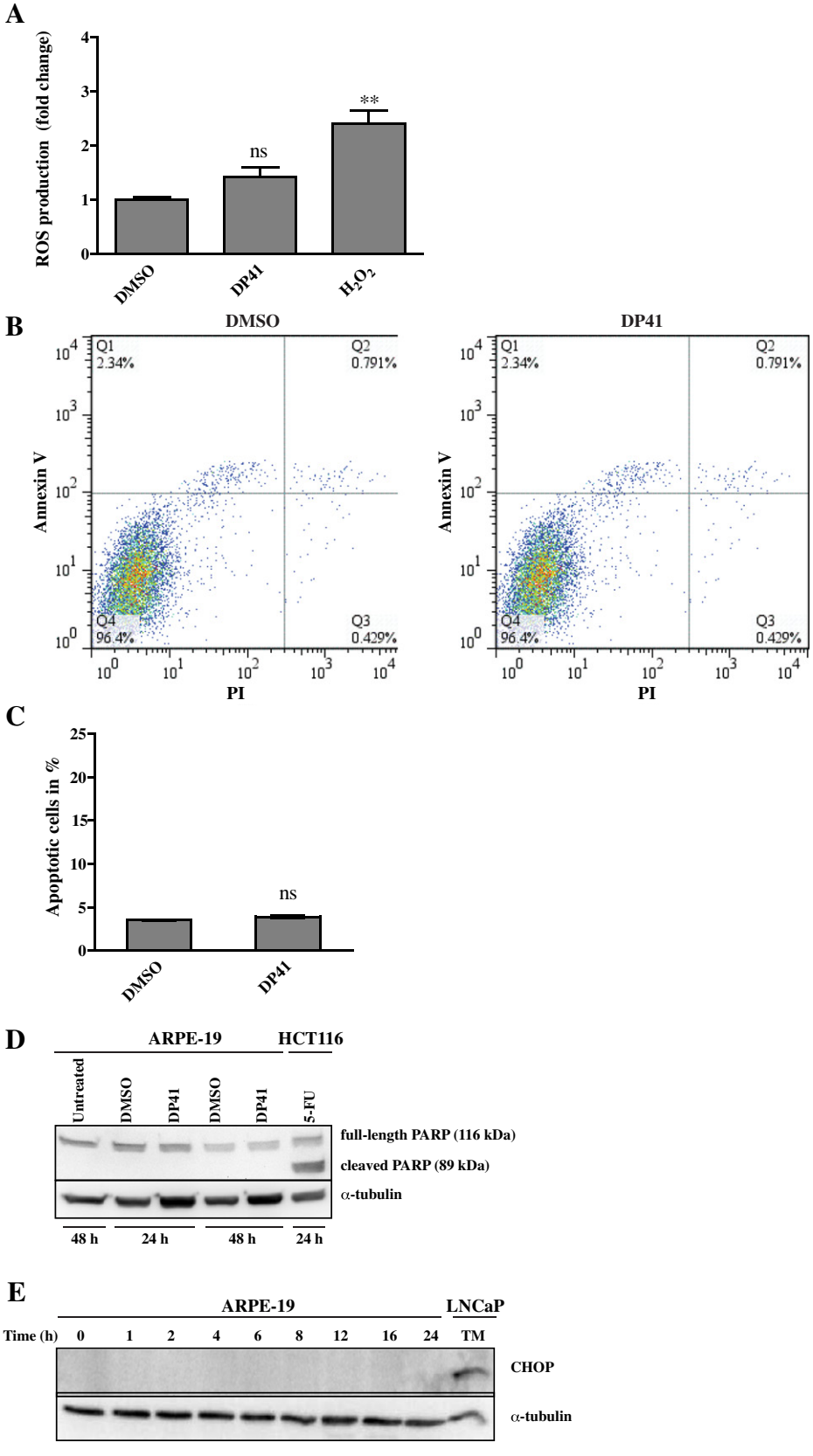


Fig. 7. Treatment of HCT116 cells with DP41 affects the organization of the cells' microtubule network. HCT116 cells were cultured overnight before incubation with DMSO (solvent control), 50 μM DP41 or 50 μM taxol (positive control) for 24 h. Cells were fixed with formaldehyde before being incubated with monoclonal anti-α-tubulin for 30 min at 37 °C followed by 30 min incubation at 37 °C with the ALEXA-Fluor™ 488 secondary antibody. After three washing steps, the cells were briefly stained with DAPI. The organization of the microtubule network (green) and nucleus (blue) was visualized with a Zeiss Axioskop fluorescence microscope.

and organotellurium compounds with an anionic head group and different hydrophobic tail lengths [13,18]. In these studies, it turned out that the selenium compounds were less cytotoxic than the corresponding tellurium compounds. Yet at the time, the intracellular process responsible for this activity remained mostly speculative. We, therefore, selected the most active tellurium compound DP41 to study its influence on some of the most relevant cellular signaling events. We found that the organotellurium compound DP41 induced the production of reactive oxygen species $O_2^{\bullet -}$, which was prevented by the simultaneous application of ascorbic acid. In contrast, the corresponding organoselenium compound DP31 failed to induce $O_2^{\bullet -}$ radical production. Thus, the organotellurium compound DP41 is more redox active in cancer cells than in the corresponding organoselenium compound DP31, which supports previous findings [18]. Based on these results, we decided to analyze the cellular signaling pathways, which are triggered by DP41. Due to the fact that DP41 induced the production of reactive oxygen species, it was not surprising that DP41 also activated the Keap1–Nrf2 pathway, which is the major regulator of the cytoprotective response to endogenous and exogenous stresses caused by ROS and electrophiles [25]. Nrf2 is a transcription factor that binds to the antioxidant response element (ARE) in the regulatory regions of target genes. One of the Nrf2 target genes is heme oxygenase1 (*HO-1*), whose protein was here found to be up-regulated as well after treatment of HCT116 cells with DP41. Gene transcription of HO-1 is inducible by a broad spectrum of stimuli. Such agents include a large number of pharmacological agents as well as heat shock and other forms of extracellular and intracellular stresses [26]. A number of studies have demonstrated that HO-1 can confer protective *i.e.* anti-apoptotic and anti-proliferative effects on various cellular systems, where the underlying mechanisms, however, have not been completely elucidated. Yet, interestingly, the ER stress signaling pathway can also activate an antioxidant program by preferentially inducing the expression of the mRNA encoding ATF4 and by phosphorylation of Nrf2 [27]. Furthermore, Nrf2 activation has been implicated in the promotion of cell survival following ER stress [28]. Interestingly, we also observed an induction of the ER signaling pathway by an elevated level of phosphorylated eIF2α and an elevated level of ATF4 which is

Fig. 8. Impact of DP41 on non-cancerous ARPE-19 cells. (A) DHE oxidation in ARPE-19 cells, indicative of $O_2^{\bullet -}$ radical formation as a result of treatment with 0.05% DMSO, 50 μM DP41 or 50 μM H_2O_2 for 45 min. Data are expressed as a fold increase in DHE oxidation rate in relation to solvent control and are depicted as means ± s.d. (n = 3). *$p < 0.05$, **$p < 0.01$ or ***$p < 0.001$. (B) Cells were treated for 24 h with either DMSO or 50 μM DP41 and then stained with annexin-V and propidium iodide (PI) before being analyzed by FACS for apoptotic cell death. Viable cells are presented as both annexin V and PI negative. (C) Percentage distribution of DP41-induced apoptotic cells relative to DMSO control. One representative of at least three similar independent experiments is shown here. Means ± s.d. (n = 3). *$p < 0.05$, **$p < 0.01$ or ***$p < 0.001$. (D) ARPE-19 cells were either left untreated, treated with 0.05% DMSO or treated with 50 μM DP41 for 24 and 48 h. As a positive reference, HCT116 cells were treated with 50 μg/μl 5-fluorouracil (5-FU) for 24 h and PARP cleavage was then studied by Western blotting. Cells were untreated (0 h) or treated with 50 μM DP41 for the indicated period of time. For a positive control, LNCaP cells were treated with 2 μg/ml tunicamycin (TM) for 24 h. Cell lysates were prepared and analyzed on a 12.5% SDS-polyacrylamide gel followed by Western blotting using anti-CHOP specific antibody (E). α-Tubulin was used as a loading control in both (D) and (E). One representative of at least three Western blots is shown here.


A
ROS production (fold change)
ns
**
DMSO
DP41
H2O2
B
DMSO
DP41
Q1
2.34%
Q2
0.791%
Q4
96.4%
Q3
0.429%
Annexin V
PI
C
Apoptotic cells in %
ns
DMSO
DP41
D
ARPE-19
HCT116
Untreated
DMSO
DP41
DMSO
DP41
5-FU
full-length PARP (116 kDa)
cleaved PARP (89 kDa)
α-tubulin
48 h
24 h
48 h
24 h
E
ARPE-19
LNCaP
Time (h)
0
1
2
4
6
8
12
16
24
TM
CHOP
α-tubulin

followed by an activation of CHOP. In mice liver, it has been shown that CHOP expression in Nrf2 deficient cells is constitutively higher than in wild-type cells, whereas, Nrf2 over-expression attenuates CHOP accumulation during ER stress [29]. It is also known that the ATF4 gene product binds to the amino acid response element (AARE) in the CHOP promoter after severe ER stress and activates CHOP transcription. Induction of CHOP is reported to be essential for the apoptotic response after extended ER stress [30]. According to our present results, HCT116 cells start to go into apoptosis after treatment with DP41. Thus, ER stress induced by DP41 on the one hand seems to be so severe that the antioxidant mechanism leading to a HO-1 mediated down-regulation of CHOP resulting in cell survival fails in the cells, while the pro-apoptotic ER stress signaling *via* p-eIF2α/ATF4 and CHOP activation dominates leading to apoptosis on the other hand.

All of the experiments discussed above were performed with HCT116 colon carcinoma cells. It was therefore a very interesting observation that normal ARPE-19 cells did not respond with an increase in the level of $O_2^{\bullet -}$ radicals and also did not induce apoptosis when subjected to DP41 under the same experimental conditions as with HCT116 cells. Thus, we conclude that normal cells are more resistant to the organotellurium compound DP41 compared to cancer cells. This resistance is probably due to the fact that normal cells do not respond with an increased level of ROS when treated with DP41, or alternatively, keep such an increase under control, for instance by a strong antioxidant response. It has been shown earlier that red blood cells were comparably lysed by the organoselenium and by the organotellurium compound which seems to be independent from the redox activity of the compound and is more likely due to the amphiphilic character of these compounds which is similar to that of sodium dodecylsulfate [18]. HCT116 cells and also ARPE-19 cells are clearly resistant to this lytic function. The fact that cancer cells go into apoptosis and normal cells are apparently resistant to treatment with DP41 might indicate a future potential application of this organotellurium compound in cancer therapy.

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