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Anticancer Agents

Exploring Ovarian Cancer Cell Resistance to Rhenium Anticancer Complexes

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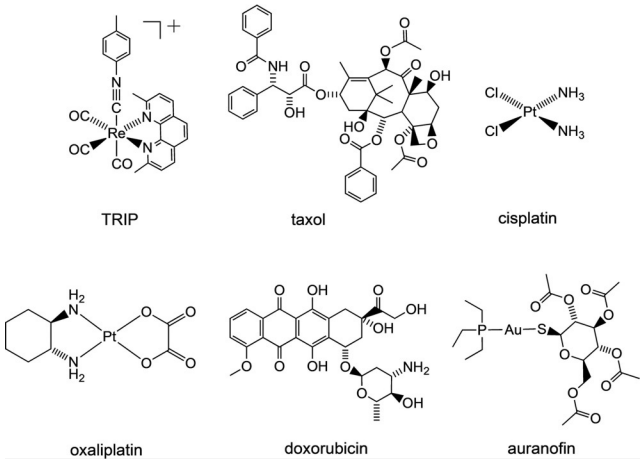
Abstract: *Rhenium tricarbonyl complexes have been recently investigated as novel anticancer agents. However, little is understood about their mechanisms of action, as well as the means by which cancer cells respond to chronic exposure to these compounds. To gain a deeper mechanistic insight into these rhenium anticancer agents, we developed and characterized an ovarian cancer cell line that is resistant to a previously studied compound $[Re(CO)_3(dmphen)(ptolICN)]^+$, where dmphen = 2,9-dimethyl-1,10-phenanthroline and ptolICN = para-tolyl isonitrile, called TRIP. This TRIP-resistant ovarian cancer cell line, A2780TR, was found to be 9 times less sensitive to TRIP compared to the wild-type A2780 ovarian cancer cell line. Furthermore, the cytotoxicities of established drugs and other rhenium anticancer agents in the TRIP-resistant cell line were determined. Notably, the drug taxol was found to exhibit a 184-fold decrease in activity in the A2780TR cell line, suggesting that mechanisms of resistance towards TRIP and this drug are similar. Accordingly, expression levels of the ATP-binding cassette transporter P-glycoprotein, an efflux transporter known to detoxify taxol, were found to be elevated in the A2780TR cell line. Additionally, a gene expression analysis using the National Cancer Institute 60 cell line panel identified the MT1E gene to be overexpressed in cells that are less sensitive to TRIP. Because this gene encodes for metallothioneins, this result suggests that detoxification by this class of proteins is another mechanism for resistance to TRIP. The importance of this gene in the A2780TR cell line was assessed, confirming that its expression is elevated in this cell line as well. As the first study to investigate and identify the cancer cell resistance pathways in response to a rhenium complex, this report highlights important similarities and differences in the resistance responses of ovarian cancer cells to TRIP and conventional drugs.*

Introduction

In the ongoing battle with cancer, tumor resistance to first-line chemotherapeutic agents has emerged as a key hurdle in the eradication of this disease. Among the different cancer types, ovarian cancers are particularly susceptible to this problem given that resistant relapse occurs in 75% of patients who were initially responsive to drug treatment.[1] A variety of mechanisms contribute to drug resistance. For example, in response to the platinum-based drugs, which are part of the first-line treatment for ovarian cancer, cells become resistant by increasing production of metal-binding agents like glutathione and metallothioneins and by upregulating DNA repair and anti-apoptotic pathways.[2–4] Acquired resistance to other drugs, like taxol and doxorubicin (Scheme 1), arises in part from changes in cellular uptake and export transporter levels, such as the ATP-binding cassette (ABC) transporters that are part of the multidrug resistance (MDR) pathway.[5–7] The diverse range of resistance mechanisms necessitates the identification of novel drug candidates that are not cross-resistant with currently approved chemotherapeutic agents.

In the context of novel drug candidates, rhenium-containing complexes have arisen as a promising and distinct class of anticancer agents.[8–11] Rhenium complexes possess several advantages over conventional organic and platinum-based



Scheme 1. Compounds explored in this study.

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Supporting information (dose-response curves, IC_{50} data table, primer sequences, multi-dose NCI-60 data, mRNA expression levels, gene analysis data (PDF)) and the ORCID identification number(s) for the author(s) of this article can be found under https://doi.org/10.1002/anie.202004883.

drugs that are currently in use for ovarian cancer. For example, these rhenium compounds generally require easily modified and modular syntheses that can facilitate access to large compound libraries.[12] Furthermore, many rhenium complexes have rich spectroscopic properties that can facilitate their direct in vitro and in vivo imaging in relevant biological systems. Lastly, these compounds tend to operate via unique mechanisms of action, which results in a lack of cross-resistance with the established platinum-based drugs. In pursuit of novel rhenium anticancer agents, our group has recently reported a tricarbonyl rhenium isonitrile polypyridyl complex (TRIP), $[Re(CO)_3(dmphen)(ptolICN)]^+$, where dmphen = 2,9-dimethyl-1,10-phenanthroline and *p*tolICN = *para*-tolyl isonitrile (Scheme 1), which induces apoptosis in cancer cells by triggering endoplasmic reticulum (ER) stress and the unfolded protein response (UPR) pathway.[13] Importantly, TRIP is not cross resistant with cisplatin, indicating that it may be useful for the treatment of relapsed ovarian cancer.

Although the resistance mechanisms that attenuate cisplatin cytotoxicity do not affect TRIP, there are likely alternative means by which cancer cells can become resistant to this novel compound. Gaining an understanding of how ovarian cancer responds to prolonged TRIP treatment will be important for developing long-term therapeutic strategies. Furthermore, cellular resistance pathways often convey information regarding the mechanism of action of a drug candidate.[14–17] To identify the resistance mechanisms that may attenuate the activity of TRIP, in this study we developed a TRIP-resistant A2780 ovarian cancer cell line, which we call A2780TR. These efforts have identified that TRIP resistance arises largely from increased expression of the ABCB1 transporter, or P-glycoprotein (Pgp), and the *MT1E* gene, which encodes for metal-binding metallothioneins. We have also tested the efficacy of other anticancer agents, including rhenium-based complexes, common organic chemotherapeutics, and well-known metal-based anticancer drugs, in this TRIP-resistant cell line, to determine the extent of cross resistance between these different compounds. Together, these results highlight the unexpected role of ABC transporters, which are generally known to act on organic substrates, to detoxify metal-based anticancer agents.

Results and Discussion

Development and Characteristics of the A2780 TRIP-Resistant Cell Line

Drug resistance is a primary cause of clinical failure of anticancer agents. Therefore, gaining an understanding of how cancer can become resistant is of significant importance at both the preclinical and clinical stages of drug development. Additionally, resistance pathways can often allude to the underlying mechanism or molecular target of the drug candidate.[14–20] Our lab has been studying the biological and anticancer activity of rhenium-based compounds, in part because they are not cross-resistant with the conventional and widely used platinum-based drugs.[12,13,21–23] Among the compounds that we have investigated, TRIP (Scheme 1) was found to be equally as effective as cisplatin in the A2780 ovarian cancer cell line. Furthermore, TRIP operates via a distinct mechanism of action by inducing ER stress, activating the UPR pathway, and subsequently initiating apoptosis. By contrast, the platinum-based drugs form covalent adducts on DNA and inhibit transcription. The ER stress induction by TRIP was also different from that of other ER stress-inducing agents, such as the proteasome inhibitors bortezomib and carfilzomib,[24] the heat-shock protein 90 inhibitor geldanamycin,[25] or the SERCA pump inhibitor thapsigargin.[26] In contrast to these compounds, TRIP induces rapid mitochondrial fission and protein aggregation leading to protein translation inhibition and expression of the ER stress marker CHOP. Although we found that TRIP rapidly causes intracellular protein aggregation, we have not yet discerned specific molecular targets of this compound. In an effort to glean more information about its mechanism of action and to anticipate cancer resistance that might arise during further preclinical development, we set out to develop TRIP-resistant cells derived from the A2780 ovarian cancer cell line, which is highly sensitive to TRIP and susceptible to acquired resistance.

The TRIP-resistant cell line (A2780TR) was obtained through the continuous treatment of the wild-type A2780 cell line with increasing concentrations of TRIP ranging from 1–14 μM over the course of one year. This chronic low-dose treatment strategy was previously used to generate other drug-resistant cell lines.[2,27,28] Initially, A2780 cells were incubated with 1 μM of TRIP in growth media for three days. After three days, the media containing TRIP was removed and fresh growth media with no TRIP was added, allowing the cells to grow and reach confluence. This process was repeated, and each month the cytotoxicity of TRIP in this cell line was evaluated. As the cell line became resistant to TRIP, the dose was escalated in 2 μM-increments until a concentration of 14 μM was reached at 15 months. At the end of 15 months, the 50% growth inhibitory concentration (IC_{50}) of TRIP in the A2780TR cell line was 13 μM, in comparison to 1.5 μM in the wild-type cell line. Thus, the ratio of IC_{50} values between the resistant and wild-type cells, or the resistance factor (RF), was found to be 9 (Figure 1, Table S1, Supporting Information or SI). For continuous culture of this cell line, 14 μM of TRIP is still included in the growth media to ensure that the resistant phenotype is not reversed. Alternatively, A2780TR cells can be frozen and stored in liquid nitrogen. Over multiple freeze-thaw cycles, this cell line retains its TRIP-resistant phenotype. The morphology of A2780TR is notably different than that of the wild-type A2780 cells; rounding and aggregation of cells is more apparent in the A2780TR cells by optical microscopy.

Cross-Resistance of Known Anticancer Agents and Rhenium Complexes

With the stable A2780TR cell line developed, we next evaluated its sensitivity to several established anticancer agents, namely cisplatin, oxaliplatin, doxorubicin, taxol, and auranofin, in comparison to the wild-type cell line (Table S1,

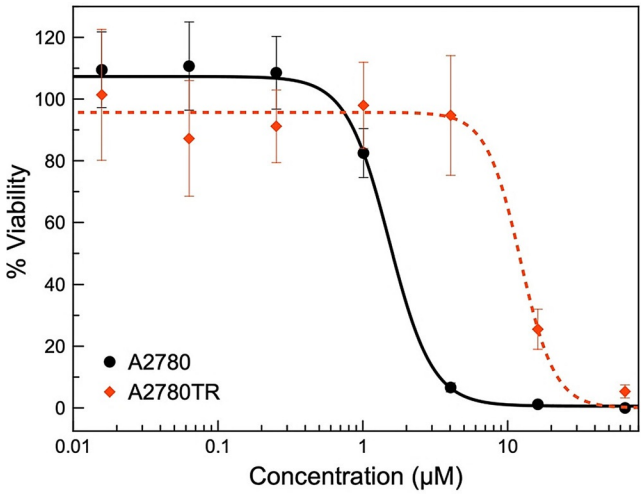


Figure 1. Dose-response curves of TRIP in A2780 (solid) and A2780TR (dashed) cells.

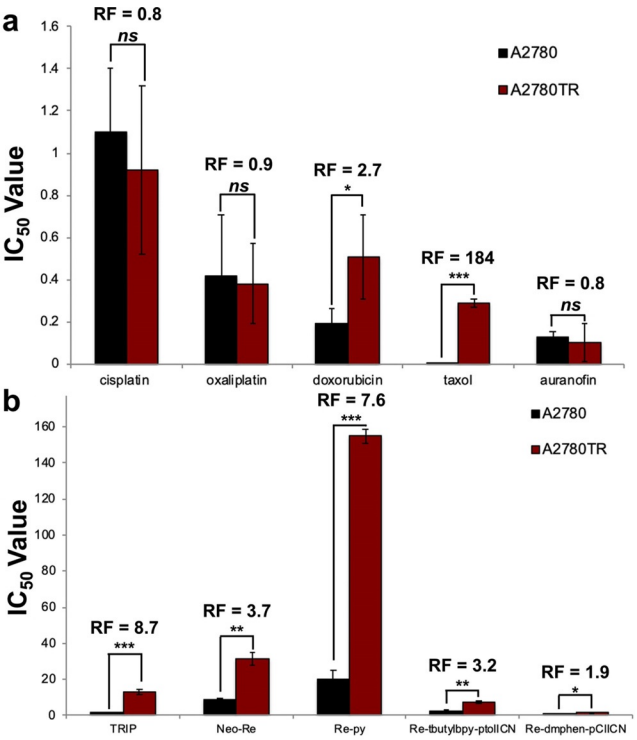


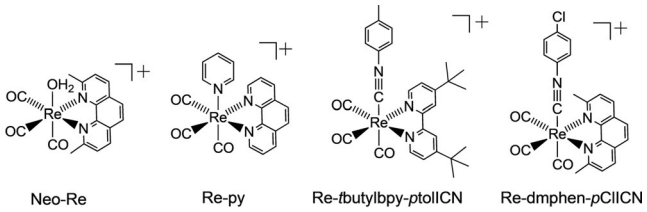
Figure 2. a) IC_{50} values of known anticancer agents and b) rhenium complexes explored for cross-resistance in A2780 (black) and A2780TR (red) cells. The *p* values represent the significance between A2780 and A2780TR cells treated with the indicated compounds, *ns* = non-significant, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.005$.

SI). These data provide information about the cross-resistance of these compounds with TRIP. If their cytotoxicity is diminished in the A2780TR cells compared to the wild-type A2780 cells, then it is likely that the mechanisms of TRIP resistance lead to their decreased potency. Furthermore, these data are valuable for establishing the potential clinical use of TRIP for patients that have been exposed to and may have resistance to these established drugs. The IC_{50} values of these compounds in A2780 and A2780TR cells and their RF values are shown in Figures 2a, S1–S5, and Table S1, SI. The RF values of the metal-based drugs, cisplatin, oxaliplatin, and auranofin, are 0.8, 0.9, and 0.8, respectively. These data thus indicate that these three metal-based drugs are not cross-resistant with TRIP. By contrast, the organic drug doxorubicin, was moderately cross-resistant with TRIP, as reflected by an RF of 2.7. Remarkably, taxol showed a significant decrease in potency in the A2780TR cell line, as characterized by an RF value exceeding 180 (Figures 2a and S5, SI). This RF value is significantly greater than that observed for TRIP, indicating that the A2780TR cell line is highly effective at detoxifying this organic drug. A common feature among taxol-resistant cells is the upregulation of the ABC transporter ABCB1, or Pgp. This protein is an active efflux transporter that is implicated in MDR pathways. It is efficient at removing hydrophobic molecules, like taxol and doxorubicin, from the cell.[6,7] Thus, it is likely that a key resistance mechanism of TRIP is upregulation of and efflux through Pgp.

In addition to testing established drugs, we also evaluated several other rhenium tricarbonyl complexes (Chart 2). These complexes were chosen to probe the effects of different structural modifications on their susceptibility to TRIP-resistance mechanisms. The compound Neo-Re, $[Re(CO)_3(dmphen)(OH_2)]^+$,[22] which contains an axial water ligand, is somewhat cross-resistant with TRIP; its RF is 4 (Figures 2b and S6, SI). Additionally, the compound Re-py, $[Re(CO)_3(1,10\text{-phenanthroline})(pyridine)]^+$, which was previously shown to have poor anticancer properties,[23] exhibits an RF of 8, comparable to that of TRIP (Figures 2b and S7, SI). Unexpectedly, two other rhenium compounds, $[Re(CO)_3(t\text{-butylbpy})(ptolICN)]_+$ (Re-*t*butylbpy-*p*tolICN), where *t*-butylbpy = 4,4′-Di-*tert*-butyl-2,2′-bipyridine, and $[Re(CO)_3(dmphen)(pClICN)]^+$ (Re-dmphen-*p*ClICN), where *p*ClICN = *para*-chlorophenyl isonitrile, which both contain axial isonitrile ligands like TRIP, exhibit only a 3-fold and 2-fold decrease in activity in A2780TR cells, respectively (Figures 2b, S8 and S9, SI). Collectively, these results show that subtle structural modifications on the rhenium tricarbonyl complexes can alter their sensitivity to the TRIP-resistance mechanisms in the A2780TR cell line. Although the direct relationship between these structures and cross-resistance to TRIP is not entirely clear, this observation does demonstrate that resistance to rhenium-based anticancer agents may be overcome by implementing minor structural modifications.

Reversal of TRIP Resistance with Verapamil

Based on the poor sensitivity of A2780TR cells to taxol, we hypothesized that overexpression of ABC transporters might be playing a key role in mediating resistance in these cells. To probe the role of ABC transporters on the TRIP and taxol resistance of the A2780TR cell line, we carried out cytotoxicity assays in the presence of the established Pgp inhibitor verapamil.[7] A2780 and A2780TR cells were pre-



Scheme 2. Structures of rhenium complexes tested in this study.

treated with 20 μM of verapamil for 1 h and then dosed with either TRIP, taxol, or cisplatin for 48 h. In the wild-type A2780 cell line, verapamil had no significant effect on the IC_{50} values of these three compounds (Table S2 and Figures S10–S12, SI). By contrast, verapamil significantly affected the cytotoxicity of TRIP (Figure 3a and Table S2, SI) and taxol (Figure 3b and Table S2, SI) in the A2780TR cell line. The IC_{50} values of TRIP and taxol were reduced by factors of 5 and 22, respectively, indicating that inhibition of Pgp recovers the potency of these compounds. Conversely, verapamil had no effect on the cytotoxic activity of cisplatin in this cell line (Table S2 and Figure S13, SI). This result is consistent with the fact that cisplatin is not an effective substrate for Pgp.[29] The use of verapamil in these experiments confirms the likely involvement of Pgp in the TRIP-resistance mechanisms of the A2780TR cell line.

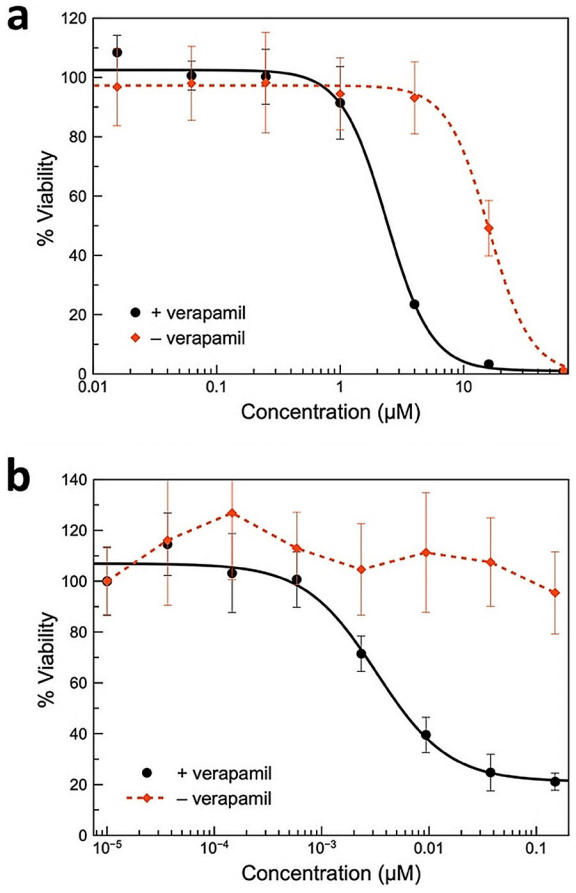


Figure 3. Dose-response curves of a) TRIP and b) taxol in the presence (solid) and absence (dashed) of verapamil (20 μM) in A2780TR cells.

Rhenium Uptake and Cellular Accumulation

Increased efflux or decreased uptake can often cause drug resistance, and several transporters have been implicated in resistance to metal-based anticancer agents.[30] Based on our hypothesis that the increased expression of the Pgp exporter contributes to TRIP resistance in the A2780TR cell line, we explored the intracellular concentration of this compound in the wild-type and resistant cell lines. If the transporter Pgp is overexpressed in the A2780TR cell line, we would expect to see low levels of intracellular rhenium content in this cell line. The cellular uptake of TRIP was evaluated by both confocal fluorescence microscopy and by inductively coupled plasma optical emission spectroscopy (ICP-OES). The intrinsic phosphorescent nature of TRIP makes it easy to track in cells via fluorescence microscopy. Therefore, A2780 and A2780TR cells were treated with 10 μM of TRIP for 2 h, prior to being imaged by confocal fluorescence microscopy. To control for effects due to background fluorescence, we also imaged untreated A2780 and A2780TR cells. Compared to untreated A2780 cells, those treated with TRIP exhibited a 2-fold increase in intracellular luminescence. Upon treatment of A2780TR cells, however, no significant change in the intracellular luminescence was detected compared to the untreated control. This result suggests that decreased cellular uptake of TRIP may be a mechanism of TRIP-resistance in the A2780TR cells (Figure 4a). Although fluorescence microscopy is an efficient way to probe uptake, the phosphorescence of rhenium tricarbonyl complexes is highly dependent on their local environment and may therefore not provide the best quantitative data regarding the quantity and localization of TRIP in the cells.[31–34] As an alternative, more precise measure, we turned to ICP-OES. Both the A2780 and A2780TR cells were treated as described above with 10 μM of TRIP for 2 h, prior to their digestion and analysis of Re content by ICP-OES. The Re content found in the wild-type A2780 cells is 5 times greater than that found in the A2780TR cell line (Figure 4b). Thus, these data are consistent with the fluorescence microscopy results and confirm that decreased cellular uptake is a key feature of TRIP resistance.

mRNA Expression Levels of Efflux Transporters

To confirm that Pgp overexpression in A2780TR cells is a contributing factor to their resistance to and decreased uptake of TRIP, we probed the mRNA expression levels of Pgp and 4 other ABC transporters, ABCC1, ABCC2, ABCC3, and ABCG2, using reverse transcriptase quantitative PCR (RT-qPCR) (Figure 5 and Table S3, SI). Each of these transporters have specificities for different classes of hydrophobic compounds. For example, Pgp recognizes taxanes, anthracyclines, and some antibiotics, whereas ABCG2 is more specific to the anthracycline mitoxantrone, organic dyes including BODIPY, and topoisomerase I inhibitors, such as topotecan.[7] Upon quantification of the mRNA levels of these 5 transporters, we found that Pgp had the largest degree of overexpression, with almost 800-fold higher mRNA levels in the A2780TR cells. Additionally, the ABCC1 transporter

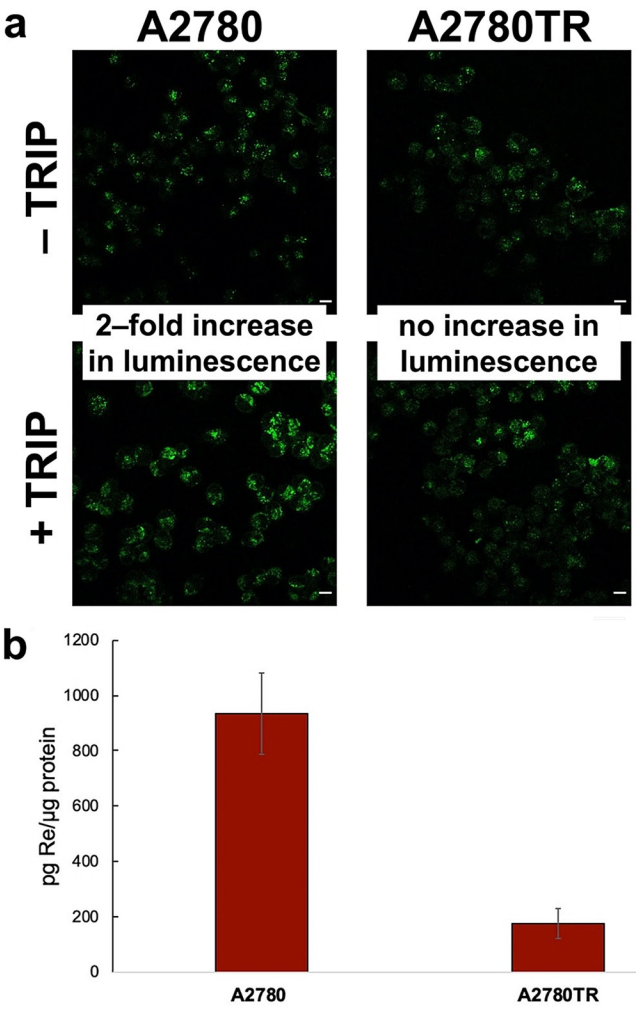


Figure 4. a) Intracellular luminescence of A2780 and A2780TR cell lines in the absence (top) and presence of TRIP (10 μM, 2 h, bottom). Scale bar = 10 microns. b) Amount of Re content after 2 h incubation of TRIP (10 μM) in A2780 and A2780TR cells as measured by ICP-OES.

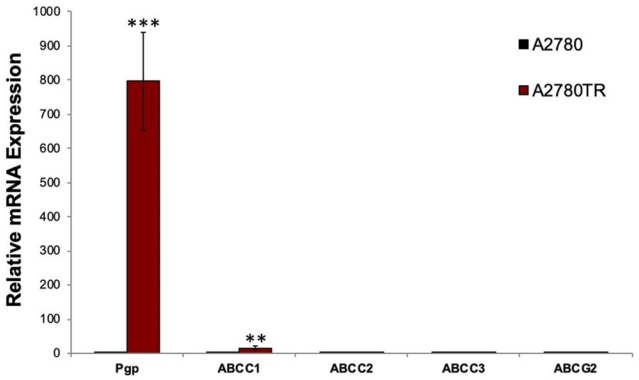


Figure 5. mRNA expression levels of ABC transporters in A2780 and A2780TR cell lines. The mRNA levels of ABCC2, ABCC3, and ABCG2 in both A2780 and A2780TR cells were below detectable levels. (** = $p < 0.01$, *** = $p < 0.005$).

had a 16-fold increase in mRNA levels in comparison to the wild-type cell line. The other transporters, ABCC2, ABCC3, and ABCG2 showed no detectable changes in mRNA expression levels, suggesting that their mRNA levels were not different in the wild-type and the resistant cell lines. In conjunction with the cytotoxicity, uptake, and verapamil data, these RT-qPCR results support our hypothesis that Pgp overexpression is a significant contributing factor to the resistance of the A2780TR cell line to TRIP and taxol. The elevated ABCC1 mRNA levels in this cell line also explain its moderate resistance to doxorubicin, which is a known substrate for the ABCC1 transporter.

In addition to these 5 ABC transporters, we also explored the mRNA expression levels for 3 organic cation transporters (OCTs), OCT1, OCT2, and OCT3. As their names imply, these transporters import organic cations, such as dopamine and serotonin, into cells. More recently, they have been implicated in the cellular uptake of inorganic complexes, such as platinum anticancer agents and ruthenium mitochondrial calcium uptake inhibitors.[35–41] Analysis of the mRNA levels of all 3 OCT transporters present within the wild-type and TRIP-resistant A2780 cells showed no differences in expression levels between these cell lines. As such, we conclude that the OCTs do not play an important role in modulating TRIP-resistance in these cells (Figure S14, SI).

Correlation of Nrf2 Activity and Pgp Overexpression

Elevated expression levels of ABC transporters, like Pgp, are a common phenotype of the MDR pathway. These transporters act to export cytotoxic species from the cells. The overexpression of these ABC transporters, however, is also directly linked to ER stress.[42–44] For example, colon cancer cells that are resistant to the ER stress-inducing agents thapsigargin and tunicamycin have increased expression levels of the ABC transporter ABCC1.[45] In the case of TRIP, the overexpression of Pgp in the A2780TR cells can be a consequence of overactivated PERK, a kinase in the UPR pathway that is activated upon induction of ER stress. Upon ER stress induction, PERK phosphorylates the eukaryotic initiation factor-2α (eIF2α) and the nuclear factor erythroid-derived 2-like-2 (Nrf2).[42,46] Phosphorylation of eIF2α causes global protein synthesis shutdown, initiation of specific transcription factors, such as ATF4, and eventually activation of CHOP, a proapoptotic protein.[47] However, phosphorylation of Nrf2 results in upregulation of various stress response proteins that can decrease ROS burdens, sequester heavy metal ions, and efflux xenobiotic species from the cell.[48] This activation results in translocation of this transcription factor from the cytoplasm to the nucleus. Nrf2 will then bind to the antioxidant response element (ARE), a gene promoter region on DNA, and activate transcription of genes that code for proteins that manage the stress. A portion of these Nrf2-regulated proteins are the ABC transporters.[49,50] Therefore, the overexpression of Pgp in the A2780TR cells may be a direct consequence of the ER stress-inducing properties of TRIP rather than a form of nonspecific drug resistance. To evaluate the role of PERK activation and ER stress induction in the acquired resistance to TRIP, we tested the Nrf2 activity in both wild-type and TRIP-resistant cell lines.

Because TRIP induces ER stress and activates the UPR via the PERK arm, we hypothesized that this compound would also upregulate and activate Nrf2, as described above. Both A2780 and A2780TR cells were treated with 10 μM TRIP for 18 h, after which these samples were subjected to RT-qPCR to determine Nrf2 mRNA expression levels (Figure 6a). Upon exposure of the wild-type A2780 cells to TRIP, we saw a 4-fold increase in mRNA levels of Nrf2, a result that is consistent with the known ability of this compound to induce ER stress. By contrast, in the resistant A2780TR cells, no significant increase (1.6-fold) in Nrf2 mRNA levels was observed. This result indicates that TRIP is less effective at upregulating the Nrf2 mRNA in resistant cells, potentially as a consequence of less TRIP accumulating in this cell line.

Next, to test the actual transcription activity of Nrf2, we used a dual luciferase reporter assay. This assay requires the transfection of cells with two plasmids. The first plasmid contains the ARE gene promoter region upstream of a gene encoding firefly luciferase (Fluc). Thus, if Nrf2 is actively promoting transcription, this activity will be related to the quantity of bioluminescence from Fluc. To provide an internal control for each cell line, a second plasmid, containing a gene for the complementary Renilla luciferase, was co-transfected. The bioluminescence of the Renilla luciferase thus serves to normalize the data to account for the overall transfection efficiency. Following the transfection of both plasmids in the A2780 and A2780TR cells, these cells were treated with 10 μM of TRIP for 18 h. Upon lysis and addition of luciferin and coelenterazine, the luminescence within the cell lysates was measured. After normalizing the Renilla luciferase emission to the Nrf2-regulated Fluc emission, the relative activities of Nrf2 were determined (Figure 6b). These data show that Nrf2 activity was almost 7-fold higher in TRIP-treated A2780 cells, compared to the untreated control. This result is consistent with the ability of TRIP to cause ER stress and activate Nrf2. By contrast, in the A2780TR cells, no difference in Nrf2 activity was observed between the TRIP-treated and untreated cells. In comparing untreated A2780 and A2780TR cells, it is also apparent that there is a 2-fold higher level of Nrf2 activity in the wild-type cell line. These data support the potential role of Nrf2 in mediating TRIP-resistance mechanisms. As shown, the treatment of wild-type A2780 cells results in enhanced expression and activity of Nrf2. As a transcription factor, Nrf2 will promote the expression of genes that code for the Pgp transporter. Thus, the conditions required to generate the A2780TR cell line, namely prolonged exposure to TRIP, could lead to enhanced Nrf2 activity and high expression of Pgp, which can effectively detoxify this compound. The lack of activity of Nrf2 in A2780TR could be due to several factors. For example, the lack of Nrf2 activity may simply arise as a consequence of the high Pgp levels of the cells that decrease the intracellular TRIP concentration to levels that cannot trigger Nrf2. Alternatively, Nrf2 activity could be decreased because the cell has found different ways to adapt to the stress of TRIP, in addition to Pgp overexpression. Overall, these results support the possibility that Pgp overexpression is a consequence of the ER stress-inducing capacity of TRIP, which in turn triggers activation of Nrf2.

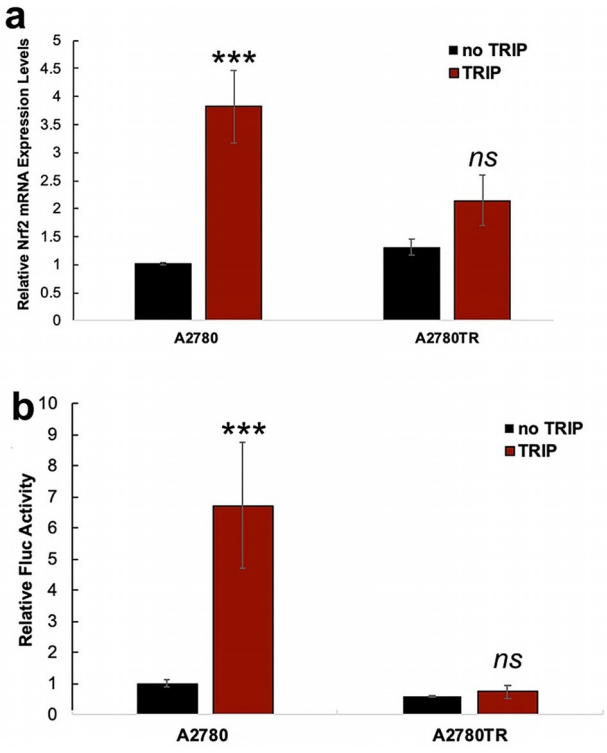


Figure 6. a) mRNA expression levels of Nrf2 in both A2780 and A2780TR cells in the presence (red) and absence (black) of TRIP (10 μM). b) Nrf2 activity as determined by the dual luciferase reporter assay in both A2780 and A2780TR cells in the presence (red) and absence (black) of TRIP (10 μM). (*ns* = non-significant, *** = $p < 0.005$).

Gene Expression Analysis of TRIP Using NCI-60 Screen

Gene expression analysis is a powerful technique that can be used to identify resistance mechanisms of drugs. To further investigate the mechanisms of TRIP resistance, we carried out a gene expression analysis using cytotoxicity data that was obtained from the National Cancer Institute (NCI)-60 Human Tumor Cell Line Screen.[51] Relative gene expression levels within the NCI-60 panel can be identified with CellExpress (http://cellexpress.cgm.ntu.edu.tw)[52] and The Cancer Genome Atlas (http://www.cbioportal.org). The use of gene expression analysis to identify resistance mechanisms of anticancer ruthenium- and osmium-based complexes that induce ER stress has recently been reported.[53] Based on the success of this study, we have adopted the same procedure to analyze the resistance mechanism of TRIP (Figure 7). First, we analyzed the multi-dose cytotoxicity data reported from the NCI-60 panel screen (Figure S15) for TRIP to identify the 4 most resistant (HCT-15, NCI/ADR-RES, ACHN, UO-31) and 4 most sensitive (KM12, OVCAR-3, T-47D, MDA-MB-468) cell lines spanning all cancer types. Using CellExpress, we compared the gene expression patterns within these two groups of cell lines. These results showed that 21 genes were differentially expressed between these groups to a statistically significant extent ($p < 10^{-3}$) (Figure 7a). Next, to further

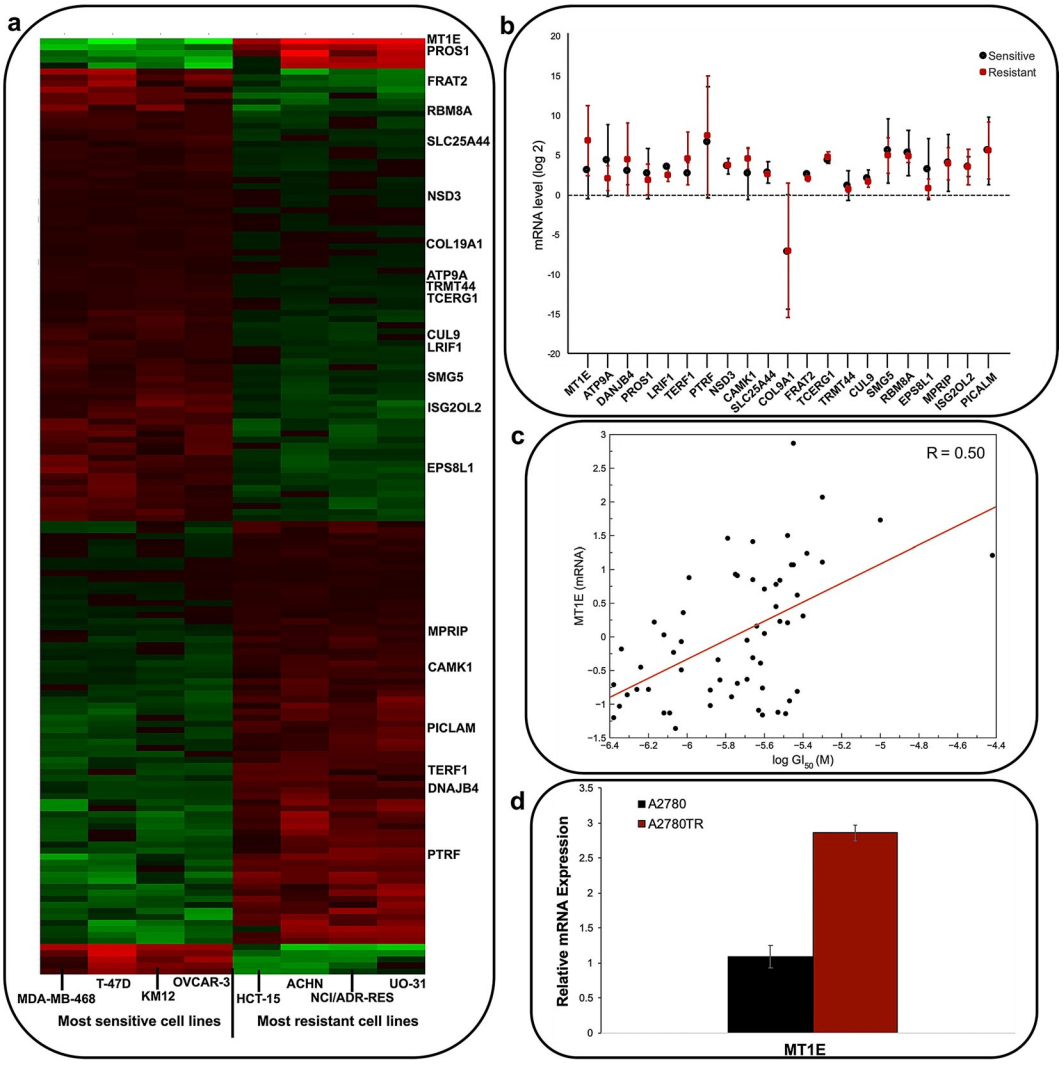


Figure 7. Selection process for resistant genes against TRIP. a) Heat map from CellExpress analysis that used 8 resistant and sensitive cell lines from NCI-60 cancer cell line screen. From the CellExpress analysis, 21 genes were identified exhibiting p values $<10^{-3}$. b) The cell lines were further divided into two groups, 4 sensitive and 4 resistant cell lines in ovarian, lung, colon, and breast cancer. The gene expression of these 8 cell lines was then validated using cBioPortal. Shown is the averaged mRNA levels, including standard deviations, for the 21 genes identified from the CellExpress analysis in both the 4 sensitive (black) and 4 resistant (red) cell lines. c) Each of the 21 genes were then explored for their expression levels of each gene against all 60 cell lines tested in the NCI-60 panel. The graph shows the log GI_{50} values of all cell lines in the panel vs. the mRNA levels of *MT1E*. The R value = 0.50. d) Functional validation of the analysis in steps (a)–(c) was carried out through RT-qPCR methods to determine the relative mRNA levels of *MT1E* in A2780 vs. A2780TR cells (*** = p value <0.005).

explore the significance of these genes, the cell lines from the NCI-60 panel were grouped in a different manner. Specifically, within 4 different cancer types, the most and least resistant cell lines were identified. Following this categorization approach, the most resistant cells were the non-small cell lung cancer NCI-H332M, colon cancer HCT-15, ovarian cancer NCI/ADR-RES, and breast cancer HS578T cell lines. The least resistant were found to be the non-small cell lung cancer NCI-H522, colon cancer KM12, ovarian cancer OVCAR-3, and breast cancer MDA-MB-468 cells. The use of these two new groups, which equally span 4 different cancer types, avoids bias in the sensitivity to TRIP that may arise from cancer type-specific properties. With these new 2 groups and the 21 genes identified in the gene analysis from the first step, we determined if the expression of these 21 genes correlated with the cytotoxicity of TRIP in these pairs of cancer cell lines (Figure 7b). Surprisingly, none of these 21 genes were found to be consistently differentially expressed between the sensitive and resistant cell lines. This result may reflect the fact that there is actually a cancer type-dependence on the role of these 21 genes in mediating TRIP sensitivity. Thus, we sought out correlations among all 60 cell lines in the panel between the efficacy of TRIP, as measured by its log GI_{50} value, and the expression of all of the 21 genes. Among these 21 genes, only 3 showed reasonable correlations with the cytotoxic activity of TRIP, as reflected by R values >0.4. These genes are *MT1E* ($R=0.50$, Figure 7c), *FRAT2* ($R=0.48$, Figure S16), and *EPS8L1* ($R=0.45$, Figure S17).

Metallothioneins, which are coded by *MT1E*, are small, cysteine-rich proteins that play a key role in the detoxification

of exogenous heavy metal ions.[54–56] The *FRAT2* gene is part of the GSK-binding-3 protein family and has been shown to act as a regulator for the WNT signaling pathway.[57] Lastly, *EPS8L1* is part of the epidermal growth factor receptor family and its function is currently unknown. In contrast to *MT1E*, both *FRAT2* and *ESP8L1* correlate with increased sensitivity to TRIP, indicating that these genes might play a role in accentuating the cytotoxic properties of this compound.

Among these correlations, the observation that the *MT1E* gene is a predictor for TRIP cytotoxicity is particularly interesting. In addition to detoxifying the cell, metallothioneins also play a key role in modulating the resistance to organic drugs[58,59] and other metal-based agents like those of platinum.[60] An additional, more indirect, role of metallothioneins is to help assist protein folding.[61] Because TRIP induces the formation of misfolded proteins, the ability of metallothioneins to reduce this cellular burden may be important with respect to their role in resistance. To determine if the *MT1E* gene was also overexpressed in the resistant A2780TR cell line, we carried out RT-qPCR on the resistant and wild-type cell lines. The expression levels of MT1E mRNA are approximately 3 times greater in the A2780TR cell line compared to the A2780 cell line (Figure 7d).

This significantly increased expression indicates that metallothioneins contribute to the TRIP resistance of the A2780TR cell line. Metallothioneins have been implicated in cancer cell resistance to a variety of metal-based anticancer agents, including complexes of platinum,[62] gold,[63] and ruthenium.[64] This gene expression analysis provides the first evidence connecting metallothioneins to the detoxification of rhenium compounds in living cells. Previous studies have shown that rhenium and technetium[65–67] complexes undergo transmetallation with four different isoforms of metallothionein, in a manner that removes their ligands in the process. Thus, metallothioneins are capable of converting rhenium-based anticancer agents to non-cytotoxic species, and enhanced expression of these small proteins is an effective resistance mechanism. Although the relative increase in MT1E mRNA levels in the resistant cell line is lower than those for Pgp, these data suggest that metallothioneins may play a role in mediating the resistance to TRIP. As observed for other anticancer agents,[68–70] the mechanisms of resistance to TRIP are most likely multifactorial in nature.

Conclusion

In this study, we have developed and characterized the first cancer cell line that is resistant to rhenium(I) tricarbonyl complexes. This resistant cell line exhibits a 9-fold decrease in sensitivity to TRIP and shows pronounced cross-resistance to the established anticancer drugs taxol and doxorubicin. We confirmed that the origin of this cross-resistance arises from increased expression of the Pgp transporter, which plays a role more broadly in MDR pathways. Although the Pgp transporter is well known to efflux organic molecules, like taxol, it has been less well-established as a means for removing inorganic complexes. There have been several key studies and examples, of metal complexes that cause overexpression of and are substrates for Pgp. For instance, the myocardial perfusion imaging agent Cardiolite, a homoleptic ^{99m}Tc isonitrile complex, was among the first metal complexes discovered to be a Pgp substrate.[71] Additionally, previous studies have illustrated that prolonged treatment of cancer cells with oxaliplatin[72] and auranofin[73] can also result in overexpression of ABC transporters, even though these compounds are not necessarily substrates for these transporters. The Ru-based anticancer agent KP1019, currently in clinical trials, has also been confirmed to be a Pgp substrate.[74] Together, these studies illustrate the importance of further understanding how Pgp and similar transporters effect metal drug detoxification. In the context of rhenium-based anticancer agents, specifically, a rhenium(I) tricarbonyl diselenoether complex was found to be effluxed efficiently from MDR MCF-7 breast cancer cells.[75] Although the mode of efflux was not investigated, Pgp is a likely candidate, based on our observation that this transporter works on several different classes of rhenium(I) tricarbonyl complexes.

This report describes the first study to date that investigates the in vitro resistance mechanisms of a cancer cell line to a rhenium anticancer agent. The key results of this study reveal the broad substrate scope of ABC transporters, which have typically been thought to primarily recognize hydrophobic organic compounds. This study also reinforces the potential role of metallothioneins in detoxifying a wide range of different metal ions. Importantly, resistance to rhenium-based anticancer agents differs from that of the clinically used platinum drugs. This result indicates that rhenium anticancer agents may find a role in treating platinum-resistant ovarian cancer.

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Conflict of interest

The authors declare no conflict of interest.

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[1] L. Norouzi-Barough, M. R. Sarookhani, M. Sharifi, S. Moghbelinejad, S. Jangjoo, R. Salehi, *J. Cell. Physiol.* **2018**, *233*, 4546 – 4562.
[2] B. C. Behrens, T. C. Hamilton, H. Masuda, K. R. Grotzinger, J. Whang-Peng, K. G. Louie, T. Knutsen, W. M. McKoy, R. C. Young, R. F. Ozols, *Cancer Res.* **1987**, *47*, 414 – 418.
[3] M. Ohmichi, J. Hayakawa, K. Tasaka, H. Kurachi, Y. Murata, *Trends Pharmacol. Sci.* **2005**, *26*, 113 – 116.
[4] G. Damia, M. Broggini, *Cancers* **2019**, *11*, 119.
[5] M. M. Gottesman, T. Fojo, S. E. Bates, *Nat. Rev. Cancer* **2002**, *2*, 48 – 58.
[6] G. Szakács, M. D. Hall, M. M. Gottesman, A. Boumendjel, R. Kachadourian, B. J. Day, H. Baubichon-Cortay, A. Di Pietro, *Chem. Rev.* **2014**, *114*, 5753 – 5774.
[7] R. J. Kathawala, P. Gupta, C. R. Ashby, Z. S. Chen, *Drug Resist. Updates* **2015**, *18*, 1 – 17.
[8] A. Leonidova, G. Gasser, *ACS Chem. Biol.* **2014**, *9*, 2180 – 2193.
[9] C. C. Konkankit, S. C. Marker, K. M. Knopf, J. J. Wilson, *Dalton Trans.* **2018**, *47*, 9934 – 9974.
[10] P. Collery, D. Desmaele, V. Veena, *Curr. Pharm. Des.* **2019**, *25*, 3306.
[11] E. B. Bauer, A. A. Haase, R. M. Reich, D. C. Crans, F. E. Kühn, *Coord. Chem. Rev.* **2019**, *393*, 79 – 117.
[12] C. C. Konkankit, B. A. Vaughn, S. N. MacMillan, E. Boros, J. J. Wilson, *Inorg. Chem.* **2019**, *58*, 3895 – 3909.
[13] A. P. King, S. C. Marker, R. V. Swanda, J. J. Woods, S.-B. Qian, J. J. Wilson, *Chem. Eur. J.* **2019**, *25*, 9206 – 9210.
[14] S. A. Wacker, B. R. Houghtaling, O. Elemento, T. M. Kapoor, *Nat. Chem. Biol.* **2012**, *8*, 235 – 237.
[15] C. Kasap, O. Elemento, T. M. Kapoor, *Nat. Chem. Biol.* **2014**, *10*, 626 – 628.
[16] T. M. Kapoor, R. M. Miller, *Trends Pharmacol. Sci.* **2017**, *38*, 1100 – 1109.
[17] R. Pisa, T. Cupido, J. B. Steinman, N. H. Jones, T. M. Kapoor, *Cell Chem. Biol.* **2019**, *26*, 1263 – 1273.
[18] R. L. Indorato, S. DeBonis, F. Kozielski, I. Garcia-Saez, D. A. Skoufias, *Biochem. Pharmacol.* **2013**, *86*, 1441 – 1451.
[19] S. Kapoor, H. Waldmann, S. Ziegler, *Bioorg. Med. Chem.* **2016**, *24*, 3232 – 3245.
[20] J. M. Povedano, J. Liou, D. Wei, A. Srivatsav, J. Kim, Y. Xie, D. Nijhawan, D. G. McFadden, *Cell Chem. Biol.* **2019**, *26*, 1315 – 1321.
[21] S. C. Marker, S. N. MacMillan, W. R. Zipfel, Z. Li, P. C. Ford, J. J. Wilson, *Inorg. Chem.* **2018**, *57*, 1311 – 1331.
[22] K. M. Knopf, B. L. Murphy, S. N. MacMillan, J. M. Baskin, M. P. Barr, E. Boros, J. J. Wilson, *J. Am. Chem. Soc.* **2017**, *139*, 14302 – 14314.
[23] B. L. Murphy, S. C. Marker, V. J. Lambert, J. J. Woods, S. N. MacMillan, J. J. Wilson, *J. Organomet. Chem.* **2020**, *907*, 121064.
[24] L. Kubiczkova, L. Pour, L. Sedlarikova, R. Hajek, S. Sevcikova, *J. Cell. Mol. Med.* **2014**, *18*, 947 – 961.
[25] K. Jhaveri, S. O. Ochiana, S. M. P. S. Dunphy, J. F. Gerecitano, A. D. Corben, R. I. Peter, Y. Y. Janjigian, E. M. Gomes-DaGama, J. Koren III, S. Modi, G. Chiosis, *Expert Opin. Invest. Drugs* **2014**, *23*, 611 – 628.
[26] P. Sehgal, P. Szalai, C. Olesen, H. A. Praetorius, P. Nissen, S. B. Christensen, N. Engedal, J. V. Møller, *J. Biol. Chem.* **2017**, *292*, 19656 – 19673.
[27] A. K. Godwin, A. Meister, P. J. O'Dwyer, C. S. Huang, T. C. Hamilton, M. E. Anderson, *Proc. Natl. Acad. Sci. USA* **1992**, *89*, 3070 – 3074.
[28] M. McDermott, A. J. Eustace, S. Busschots, L. Breen, J. Crown, M. Clynes, N. O'Donovan, B. Stordal, *Front. Oncol.* **2014**, *4*, 1 – 16.
[29] A. Breier, L. Gibalova, M. Seres, M. Barancik, Z. Sulova, *Anti-Cancer Agents Med. Chem.* **2012**, *13*, 159 – 170.
[30] S. Spreckelmeyer, C. Orvig, A. Casini, *Molecules* **2014**, *19*, 15584 – 15610.
[31] G. Gasser, S. Neumann, I. Ott, M. Seitz, R. Heumann, N. Metzler-Nolte, *Eur. J. Inorg. Chem.* **2011**, 5471 – 5478.
[32] S. Imstepf, V. Pierroz, P. Raposinho, M. Bauwens, M. Felber, T. Fox, A. B. Shapiro, R. Freudenberg, C. Fernandes, S. Gama, G. Gasser, F. Motthagy, I. R. Santos, R. Alberto, *Bioconjugate Chem.* **2015**, *26*, 2397 – 2407.
[33] S. Imstepf, V. Pierroz, R. Rubbiani, M. Felber, T. Fox, G. Gasser, R. Alberto, *Angew. Chem. Int. Ed.* **2016**, *55*, 2792 – 2795; *Angew. Chem.* **2016**, *128*, 2842 – 2845.
[34] C. C. Konkankit, A. P. King, K. M. Knopf, T. L. Southard, J. J. Wilson, *ACS Med. Chem. Lett.* **2019**, *10*, 822 – 827.
[35] S. Zhang, K. S. Lovejoy, J. E. Shima, L. L. Lagpacan, Y. Shu, A. Lapuk, Y. Chen, T. Komori, J. W. Gray, X. Chen, S. J. Lippard, K. M. Giacomini, *Cancer Res.* **2006**, *66*, 8847 – 8857.
[36] K. S. Lovejoy, R. C. Todd, S. Zhang, M. S. McCormick, J. A. D'Aquino, J. T. Reardon, A. Sancar, K. M. Giacomini, S. J. Lippard, *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 8902 – 8907.
[37] H. Burger, A. Zoumaro-Djayoon, A. W. M. Boersma, J. Helleman, E. M. J. J. Berns, R. H. J. Mathijssen, W. J. Loos, E. A. C. Wiemer, *Br. J. Pharmacol.* **2010**, *159*, 898 – 908.
[38] S. S. More, S. Li, S. W. Yee, L. Chen, Z. Xu, D. M. Jablons, K. M. Giacomini, *Mol. Cancer Ther.* **2010**, *9*, 1058 – 1069.
[39] I. Buß, A. Hamacher, N. Sarin, M. U. Kassack, G. V. Kalayda, *Metallomics* **2018**, *10*, 414 – 425.
[40] A. Hucke, G. Y. Park, O. B. Bauer, G. Beyer, C. Köppen, D. Zeeh, C. A. Wehe, M. Sperling, R. Schröter, M. Kantauskaitè, Y. Hagos, U. Karst, S. J. Lippard, G. Ciarimboli, *Front. Chem.* **2018**, *6*, 180.
[41] J. J. Woods, J. Lovett, B. Lai, H. H. Harris, J. J. Wilson, *Angew. Chem. Int. Ed.* **2020**, *59*, 6482 – 6491; *Angew. Chem.* **2020**, *132*, 6544 – 6553.
[42] S. Ledoux, R. Yang, G. Friedlander, D. Laouari, *Cancer Res.* **2003**, *63*, 7284 – 7290.
[43] C. Riganti, J. Kopecka, E. Panada, S. Barak, M. T. Rubinstein, *J. Natl. Cancer Inst.* **2015**, *107*, 1 – 14.
[44] M. Hano, L. Tomášová, M. Šereš, L. Pavlíková, A. Breier, Z. Sulová, *Molecules* **2018**, *23*, 337.
[45] I. C. Salaroglio, E. Panada, E. Moiso, I. Buondonno, P. Provero, M. Rubinstein, J. Kopecka, C. Riganti, *Mol. Cancer* **2017**, *16*, 91.
[46] S. B. Cullinan, D. Zhang, M. Hannink, E. Arvisais, R. J. Kaufman, J. A. Diehl, *Mol. Cell. Biol.* **2003**, *23*, 7198 – 7209.
[47] Y.-P. Vandewynckel, D. Laukens, A. Geerts, E. Bogaerts, A. Paridaens, X. Verhelst, S. Janssens, F. Heindryckx, H. van Vlierberghe, *Anticancer Res.* **2013**, *4694*, 4683 – 4694.
[48] T. W. Kensler, N. Wakabayashi, S. Biswal, *Annu. Rev. Pharmacol. Toxicol.* **2007**, *47*, 89 – 116.
[49] F. Jeddi, N. Soozangar, M. R. Sadeghi, M. H. Somi, M. Shirmohamadi, A. T. Eftekhar-Sadat, N. Samadi, *Biomed. Pharmacother.* **2018**, *97*, 286 – 292.
[50] X. Wang, C. R. Campos, J. C. Peart, L. K. Smith, J. L. Boni, R. E. Cannon, D. S. Miller, *J. Neurosci.* **2014**, *34*, 8585 – 8593.
[51] R. H. Shoemaker, *Nat. Rev. Cancer* **2006**, *6*, 813 – 823.
[52] Y. F. Lee, C. Y. Lee, L. C. Lai, M. H. Tsai, T. P. Lu, E. Y. Chuang, *Database* **2018**, *2018*, bax101.
[53] C. Licona, J.-B. Delhorme, G. Riegel, V. Vidimar, R. Cerón-Camacho, B. Boff, A. Venkatasamy, C. Tomasetto, P. da Silva Figueiredo Celestino Gomes, D. Rognan, J.-N. Freund, R. Le Lagadec, M. Pfeffer, I. Gross, G. Mellitzer, C. Gaiddon, *Inorg. Chem. Front.* **2020**, *7*, 678 – 688.
[54] P. Coyle, J. C. Philcox, L. C. Carey, A. M. Rofe, *Cell. Mol. Life Sci.* **2002**, *59*, 627 – 647.
[55] M. Vašák, *J. Trace Elem. Med. Biol.* **2005**, *19*, 13 – 17.
[56] N. Sakulsak, *Int. J. Morphol.* **2012**, *30*, 1007 – 1012.
[57] R. van Amerongen, M. C. Nawijn, J.-P. Lambooij, N. Proost, J. Jonkers, A. Berns, *Oncogene* **2010**, *29*, 93 – 104.

[58] S. L. Kelley, A. Basu, B. A. Teicher, M. P. Hacker, D. H. Hamer, J. S. Lazo, *Science* **1988**, *241*, 1813 – 1815.
[59] S. E. Theocharis, A. P. Margeli, A. Koutselinis, *Int. J. Biol. Markers* **2003**, *18*, 162 – 169.
[60] R. J. Schilder, L. Hall, A. Monks, L. M. Handel, A. J. Fornace, R. F. Ozols, A. T. Fojo, T. C. Hamilton, *Int. J. Cancer* **1990**, *45*, 416 – 422.
[61] S. Atrian, M. Capdevila, *Biomol. Concepts* **2013**, *4*, 143 – 160.
[62] G. Zhang, W. Hu, Z. Du, S. Lv, W. Zheng, Q. Luo, X. Li, K. Wu, Y. Han, F. Wang, *Int. J. Mass Spectrom.* **2011**, *307*, 79 – 84.
[63] B. P. Monia, T. R. Butt, C. K. Mirabelli, D. J. Ecker, E. Sternberg, S. T. Crooke, *Mol. Pharmacol.* **1987**, *31*, 21 – 26.
[64] A. Casini, A. Karotki, C. Gabbiani, F. Rugi, M. Vašák, L. Messori, P. J. Dyson, *Metallomics* **2009**, *1*, 434 – 441.
[65] M. M. Morelock, T. A. Comier, G. L. Tolman, *Inorg. Chem.* **1988**, *27*, 3137 – 3140.
[66] W. B. Jones, T. E. Elgren, M. M. Morelock, R. C. Elder, D. E. Wilcox, *Inorg. Chem.* **1994**, *33*, 5571 – 5578.
[67] J. Lecina, Ò. Palacios, S. Atrian, M. Capdevila, J. Suades, *J. Biol. Inorg. Chem.* **2015**, *20*, 465 – 474.
[68] M. Kartalou, J. M. Essigmann, *Fundam. Mol. Mech. Mutagen.* **2001**, *478*, 23 – 43.
[69] R. Z. Yusuf, Z. Duan, D. E. Lamendola, R. T. Penson, M. V. Seiden, *Curr. Cancer Drug Targets* **2003**, *3*, 1 – 19.
[70] M. Volm, T. Efferth, *Front. Oncol.* **2015**, *5*, 1 – 14.
[71] D. Piwnica-Worms, M. L. Chiu, M. Budding, J. F. Kronauge, R. A. Kramer, J. M. Croop, *Cancer Res.* **1993**, *53*, 977 – 984.
[72] H.-H. Hsu, M.-C. Chen, R. Baskaran, Y.-M. Lin, C. H. Day, Y.-J. Lin, C.-C. Tu, V. V. Padma, W.-W. Kuo, C.-Y. Huang, *J. Cell. Physiol.* **2018**, *233*, 5458 – 5467.
[73] I. Landini, A. Lapucci, A. Pratesi, L. Massai, C. Napoli, G. Perrone, P. Pinzani, L. Messori, E. Mini, S. Nobili, *Oncotarget* **2017**, *8*, 96062 – 96078.
[74] P. Heffeter, M. Pongratz, E. Steiner, P. Chiba, M. A. Jakupec, L. Elbling, B. Marian, W. Körner, F. Sevelda, M. Micksche, B. K. Keppler, W. Berger, *J. Pharmacol. Exp. Ther.* **2005**, *312*, 281 – 289.
[75] P. Collery, G. Bastian, F. Santoni, A. Mohsen, M. Wei, T. Collery, A. Tomas, D. Desmaele, J. D′Angelo, *Anticancer Res.* **2014**, *34*, 1679 – 1690.

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