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Endoplasmic Reticulum Stress: An Arising Target for Metal-Based Anticancer Agents

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Abstract

The endoplasmic reticulum (ER) has recently emerged as a promising target for anticancer agents. Cytotoxic compounds that target the ER often exhibit selectivity for cancer cells over non-cancer cells. Furthermore, the induction of ER stress often leads to immunogenic cell death, providing another factor that contributes to the clinical efficacy of drugs that target this organelle. Among potential ER stress-inducing agents, metal complexes, which possess redox activity and modular structures, have arisen as promising candidates. In the last two decades, dozens of metal complexes have been reported that kill cancer cells via ER stress induction, and many of these complexes exhibit nanomolar activity in vitro as well as powerful tumor inhibition in vivo. In this review, we summarize the current state of investigations on the ER stress-inducing properties of metal complexes. This review starts with a description of the ER, its function, and its role in cancer progression and treatment. Following this discussion, a guide to experimental methods that can be used by researchers to detect ER stress is provided. The majority of this review summarizes previous studies on metal-based anticancer agents that cause ER stress. Finally, a discussion on the perspectives and significance of using metal complexes as ER stress-inducing agents for the treatment of cancer is provided, along with a summary of structural trends that contribute to this type of biological activity.

1. Introduction

The endoplasmic reticulum (ER) is responsible for the synthesis, folding, and trafficking of cellular proteins. Thus, proper ER function is essential for cell growth and survival. In cancer cells, however, ER function becomes dysregulated. The rapid, uncontrolled growth of cancer cells and the hostile, nutrient-deficient tumor environment lead to an increase in protein misfolding and demand for protein synthesis. These factors ultimately contribute to heightened levels of ER stress in cancer cells relative to healthy cells.[1–3] To cope with this stress, cancer cells upregulate a pathway known as the unfolded protein response (UPR), which increases their ability to survive under heightened ER protein-folding burdens. Induction of the UPR is generally cytoprotective, and cancers that upregulate the UPR are often more aggressive and resistant to chemotherapy.[4–6] However, under certain conditions of prolonged or acute UPR activation, this pathway can initiate apoptosis. As such, many cancers with upregulated UPR are hypersensitive to chemotherapeutics that interfere with ER function. Recently, this sensitivity has been leveraged to develop new drugs that disrupt the UPR, such as the clinically-approved proteasome inhibitors bortezomib and carfilzomib, which interfere with endoplasmic reticulum-associated degradation (ERAD).[7] The recent discovery of these drugs has led to a heightened interest in compounds capable of inducing ER stress as potential new anticancer drugs.[8–13]

Although the majority of known ER stress-inducing agents are organic compounds, in recent years there have been several reports that describe the potential of metal complexes as ER-targeting cancer therapeutics. These complexes range from first row transition metal complexes that operate via the catalytic production of reactive

oxygen species (ROS), to hydrophobic late transition metal complexes that interfere with ER Ca^{2+} storage, to targeted photodynamic therapy (PDT) agents that generate singlet oxygen ($^1O_2$) locally inside the ER. The wide range of accessible structures, tunable chemical reactivities, and targeting capabilities of coordination complexes make them ideal for the development of ER-targeting anticancer drugs. Despite the clear potential of the ER as a metallodrug target, this organelle is often overlooked in comparison to traditional intracellular targets, such as the nucleus and mitochondria. The vast majority of studies investigating ER stress induction by metal complexes have been conducted within the last 20 years, with only a handful performed prior to the 21st century. In this Review, we highlight the recent advances in developing metal complexes that target the ER and provide an overview of the relevant biological assays that can be used detect ER stress.

The objective of this Review is to provide a comprehensive guide for researchers seeking to design ER-targeting metallodrugs. We begin with a broad introduction of the relevant biological pathways, signaling processes, and therapeutic implications of ER stress induction, especially in relation to cancer treatment (**Section 2**). We then provide a brief summary of the common experimental methods that are used to detect ER stress induction and ER stress-mediated cell death (**Section 3**). After discussing this background, we explore the ER stress-inducing capacities of various metal complexes and discuss relevant structural features and mechanisms of action (**Section 4**). Finally, we provide a comparative overview, discussion, and perspective on the potential value of metal complexes for inducing ER stress in cancer cells. (**Section 5**). We note that an article on a similar topic was recently published elsewhere.[14] However, this Review

focuses exclusively on therapeutic anticancer applications of complexes that induce ER stress. Furthermore, this Review is more expansive and comprehensive, and we provide an in-depth analysis of the biological mechanisms of these compounds, thus filling an unmet gap in the literature.

2. Background

2.1 The Endoplasmic Reticulum: Structure and Function

The ER is the largest organelle in the cell. However, despite its size, it was one of the last organelles to be discovered.[15,16] This vast organelle consists of two main regions, the nuclear envelope that borders the cell nucleus and the peripheral ER that branches throughout the cell. Both the nuclear envelope and the peripheral ER contain a mixture of flat sheets and tubular structures. The sheets form flat membranes with a lumen between them, whereas the tubules connect the ER to other organelles and provide pathways for transport of lipids and proteins. The ratio of sheets to tubules varies in a manner that depends on the specific cell type and its environment, leading to wide variability in ER morphology. The peripheral ER connects directly to other organelles, including the mitochondria, Golgi apparatus, and cytoskeleton. The interactions between the ER and mitochondria are particularly extensive, and crosstalk between these organelles has been the topic of several recent reviews.[17–20] The ER's function, while still not fully understood, may be broadly divided into three categories: protein regulation, lipid biosynthesis, and intracellular Ca^{2+} storage. Each of these functions and some of the relevant regulatory pathways are described in greater detail below.

The ER's most well-understood purpose is its role in cellular protein regulation.[10,15,21] The ER directly synthesizes many integral membrane proteins and several cytosolic proteins. This synthesis occurs at ribosomes associated with the ER membrane in a portion of the ER known as the rough ER. Once synthesized, proteins are translocated into the ER lumen, where they are folded. After folding, cytosolic proteins are trafficked to their destination, and membrane proteins are incorporated into their respective membranes. Occasionally, the proteins do not fold properly or aggregate.[21] Once detected, misfolded or aggregated proteins are tagged with ubiquitin, most often by the SEL1L-HRD1 protein complex. After ubiquitination, proteins are translocated back through the ER to the cytosol, where they are degraded by the proteasome. This pathway, known as ERAD, is an essential component of the ER's protein homeostasis machinery, and it has recently received attention for its role in several diseases, including Alzheimer's disease[22] and cancer.[23,24]

Although the ER is most well-known for its role in protein metabolism, it also synthesizes and organizes the vast majority of cellular lipids, such as phosphatidyl choline, triacylglycerides, and cholesterol.[25] Unlike protein synthesis, lipid synthesis occurs in either the smooth or rough ER. Once synthesized, these lipids are transferred to their desired destinations via the secretory pathway. Lipid synthesis and organization by the ER is a dynamic process, which can be altered in response to external stimuli in order to change production or to regulate the size of the ER itself.

The final major role of the ER is to regulate and store intracellular Ca^{2+}.[15,26] The ER stores the majority of intracellular Ca^{2+}; the Ca^{2+} concentration within the ER lumen is approximately 1 mM, in contrast to the 100 nM concentration found in the cytosol.

Signaling molecules, such as inositol 1,4,5-triphosphate, induce the release of stored Ca^{2+} into the cytosol or mitochondria. Conversely, the ER may take up Ca^{2+} from the cytosol through sarco(endo)plasmic reticulum calcium ATPase (SERCA) transporters. By balancing levels of Ca^{2+} uptake and release, the ER ensures that the cytosolic Ca^{2+} remains in the nanomolar range.

2.2 Endoplasmic Reticulum Stress Pathways and the Unfolded Protein Response

When ER function becomes disrupted by insults such as toxins or environmental changes, ER stress occurs. ER stress may be broadly categorized as being due to a perturbation of one of the three major functions of the ER: protein folding/trafficking, lipid synthesis and processing, and Ca^{2+} homeostasis. These stresses result in a decreased capacity of the ER to fold proteins, leading to an increase in misfolded proteins inside the cell. The cell has machinery for resolving this stress, such as the UPR and the integrated stress response (ISR). If the cell cannot resolve the insult or reduce the resulting stress to acceptable levels, programmed cell death occurs, often via apoptosis or paraptosis.

The major hallmark of ER stress in a cell is an increase in the amount of misfolded proteins. If these proteins accumulate, they activate the UPR by binding to the ER chaperone binding immunoglobin protein (BiP/GRP78). In a normal, unstressed state, BiP binds to and inactivates the ER stress response proteins PERK, ATF6, and IRE1α. At higher levels of ER stress, BiP will bind to misfolded proteins, thereby leaving the three ER stress response proteins free to be activated. Once freed, PERK and IRE1α autophosphorylate to reach their active forms, whereas free ATF6 translocates to

the Golgi apparatus. All three sensors then activate their downstream pathways, as shown in **Fig. 1**. We have provided an abbreviated description of the UPR; in-depth analysis can be found in several recent reviews of the topic.[27–30]

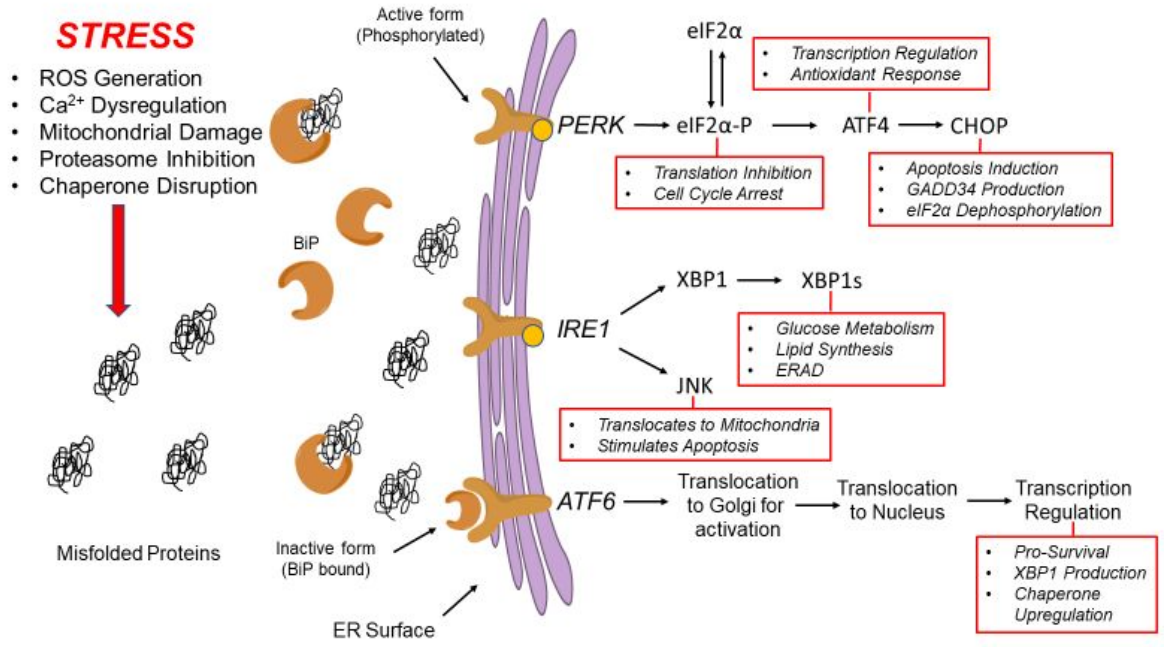


Fig. 1. Schematic diagram of ER stress-response pathways and activation of the UPR. A variety of stressors may lead to the accumulation of misfolded proteins, which bind to BIP, causing it to release from stress sensors PERK, IRE1, and ATF6 on the ER membrane. These sensors then activate their respective downstream processes to resolve the insult.

The most well-established arm of the UPR is the PERK pathway. The PERK arm begins with the phosphorylation of the eukaryotic initiation factor 2 alpha (eIF2α).[31] eIF2α is required for protein synthesis, initiating the process by forming a critical component of the ternary translation initiation complex. Upon phosphorylation, however, eIF2α cannot initiate translation, and a decrease in global protein synthesis in the cell occurs. This decrease helps reduce the protein folding load on the ER and allows the ER to devote its resources to refolding or eradicating misfolded proteins. Although

global translation decreases after eIF2α phosphorylation, some stress response proteins become upregulated, including activating transcription factor 4 (ATF4) and its downstream products. ATF4 induces the transcription of proteins that ameliorate ER stress by managing ROS, amino acid synthesis, and protein export.[32] If these efforts succeed in reducing ER stress, eIF2α phosphorylation levels decrease, and normal translation resumes. However, if ER stress remains unresolved for an extended time or is acutely elevated to unmanageable levels, ATF4 upregulates the proapoptotic C/EPB homologous protein (CHOP), which triggers apoptosis.[33]

The second arm of the UPR that may be activated by BiP dissociation is the IRE1α pathway.[29] Once BiP dissociates, IRE1α activates several downstream targets, including *c*-Jun N-terminal kinases (JNKs) and tumor necrosis factor receptor-associated factor 2 (TRAF2). It also cleaves part of X-box binding protein 1 (XBP1), activating this protein and allowing it to begin expression of its downstream transcripts, which regulate a variety of processes including glucose metabolism, lipid synthesis, redox homeostasis, and DNA repair. In cases of extreme ER stress, XBP1 and JNKs can also mediate apoptosis induction.[34]

The final, least well-understood branch of the UPR is mediated by ATF6. ATF6 coordinates largely pro-survival pathways.[35,36] After its activation, ATF6 translocates to the Golgi apparatus, where it is cleaved. The cleaved domain, a transcription factor, travels to the nucleus and begins regulating gene expression. The gene expression program induced by ATF6 includes upregulation of protein folding chaperones, such as BiP, and other ER stress-response proteins. ATF6 also causes production of antiapoptotic machinery that sequester and reduce the proapoptotic Bcl-2. ATF6

cleavage has been linked to some proapoptotic functions as well. Cleavage of ATF6 leads to increased CHOP expression, which may initiate apoptosis.[33,37]

2.3 ER Stress and Cancer Progression

Recent studies have found that ER signaling and ER stress pathways undergo drastic changes in cancer cells. As such, the ER plays an integral role in cancer aggressiveness, metastasis, and response to chemotherapy.[38,39] Each of these aspects of the ER's role in cancer may be modulated by chemotherapeutic drugs. The role of many ER stress responses in chemotherapy is still not fully understood, and there are often conflicting reports regarding the role of specific ER pathways in cancer. However, several key operational changes to the ER and their implications for cancer therapy have been established.

The environment of cancer cells has several characteristics that contribute to ER stress, including low nutrient availability, hypoxia, low pH, and limited blood flow.[1,40] Furthermore, the metabolic changes that lead to cancer, such as increased cell replication, also lead to an increased protein folding load and heightened ER stress. All these factors contribute to overactivation of the UPR in cancer cells relative to normal cells, and the upregulation of all three UPR branches is a common cancer phenotype. As in normal cells, the UPR protects cancer cells from the consequences of ER stress by managing protein translation and increasing the levels of protein-folding chaperones. As a result, UPR activation allows cancer cells to survive and proliferate even under highly unfavorable conditions. Perhaps unsurprisingly in this context, UPR activity levels have been directly linked to increased aggressiveness in tumors.[5,41]

UPR induction does not only help cancer cells survive under harsh environmental conditions; it also conveys resistance to chemotherapy and radiation.[4–6,42] Increased expression of ER stress regulators such as BiP and XBP1 have been correlated to cancer resistance both in vitro and in vivo.[43,44] This resistance may be two-pronged. UPR activation may protect cancer cells from the direct consequences of chemotherapy, such as increased ROS levels or DNA damage, by upregulating ROS decomposition or DNA repair mechanisms. Alternatively, UPR induction may indirectly protect cells by inducing pro-survival pathways such as senescence or autophagy.

In addition to the role of ER stress in cancer survival, ER stress also has a strong link to cancer cell metastasis and the epithelial to mesenchymal transition (EMT).[38,45,46] Although this link is still not fully understood, a large body of evidence points to BiP playing a significant role in metastasis during ER stress by interacting with the PI3K/AKT pathway.[47–49] BiP attenuates PI3K, a metastasis inhibitor. Therefore, increased BiP expression correlates with increased tumor metastasis rates and poorer prognoses. As a result, elevated ER stress levels have generally been linked to increased rates of EMT,[50,51] especially in response to chemotherapy.[52,53] However, there is some debate regarding the relationship between ER and EMT because other studies have found that EMT sensitizes cells to ER stress-induced apoptosis.[54]

2.4 Targeting the Endoplasmic Reticulum with Chemotherapy

Despite the UPR's role in promoting cancer progression and chemotherapy resistance, chemical induction of ER stress has recently garnered attention as an anticancer strategy.[8–13,55] The high basal ER stress level in many cancer cells makes

them particularly susceptible to chemotherapeutics that target the ER. This hypersensitivity has already been exploited by the FDA-approved proteasome inhibitors bortezomib and carfilzomib, which induce ER stress by preventing the degradation of unfolded proteins. The success of these drugs has led to the investigation of other ER stress-inducing agents, such as heat-shock protein 90 (HSP90) inhibitors like geldanamycin and 17-AAG,[56] as well as BiP inhibitors such as versipelostatin.[57] These drugs selectively target cancer cells by disrupting their ability to remedy ER stress, leading to apoptosis. Another recently discovered therapeutic agent with high anticancer potential, salinomycin, selectively kills stem-like cancer cells via ER stress induction.[58] There have also been several reports of metal-based complexes that target the ER via mechanisms as diverse as their organic counterparts. Broadly, most ER stress induction by therapeutics may be subdivided into five causes: direct interaction with UPR machinery, disruption of protein folding chaperones, inhibition of protein degradation, interference with Ca^{2+} trafficking, or production of ROS.

3. Detection of ER Stress

The detection of ER stress may be accomplished by several methods, including fluorescence microscopy, histochemistry, and flow cytometry. Mechanistic information about the cause of ER stress induction may also be determined through some assays with chemical modulators of ER stress pathways. This topic has been reviewed extensively elsewhere,[59–61] but we have provided a brief summary of some of the simplest and most common methods for the detection of ER stress induction, categorized by ER stress pathway. Researchers should bear in mind that ER stress

induction may be a secondary or downstream effect of a compound, rather than the main target, and many compounds cause multiple types of ER stress. In order to prevent false-positive ER stress detection that occurs after the primary insult, stress detection assays should be conducted at early time points. Also, there is no single assay to verify or negate the role ER stress plays in cell death. Verification of the compounds' localization to the ER may also provide strong support for ER stress as a primary anticancer mechanism, but compounds do not necessarily have to localize to the ER to elicit ER stress. For further information about ER stress detection, categorized by stressor type, see **Table 1**.

The activation of one or more of the three main UPR branches is perhaps the most obvious sign of ER stress. These pathways, which all rely on enhanced levels of specific protein marker or phosphorylation of signaling proteins, can be readily detected through Western blots.[61,62] BiP upregulation serves as a broad indicator of ER stress, usually due to an increase in the level of misfolded proteins in the cell, which may be associated with any of the three UPR branches. The UPR branches can also be detected individually. The PERK arm of the UPR is investigated by measuring the relative levels of eIF2α vs phosphorylated eIF2α, with higher levels of phosphorylation indicating ER stress. If heightened phosphorylation of eIF2α is confirmed, the role of this stress in cell death is determined by investigating increased expression of downstream pro-apoptotic proteins such as ATF4, CHOP, and NOXA. CHOP detection, in particular, is a key indicator of ER stress-mediated apoptosis. The activation of the IRE1α arm of the UPR can be investigated by measuring the splicing of its substrate, XBP1. ATF6 activation is more difficult to detect, as it requires gene-reporter assays or transfection

with fluorescent reporters for confocal microscopy.

Given the role of the proteasome in the ERAD process, measuring the proper function of this process is an important means to assay ER stress in cells. Several assays have been developed for evaluating the ubiquitin-proteasome system.[63,64] The simplest assay for confirming proteasomal inhibition is to perform a Western blot to measure the level of ubiquitinated proteins. As the proteasome is inhibited, its substrates, marked with ubiquitin, accumulate inside the cell. An increase in the level of ubiquitinated proteins indicates a general disturbance in the proteasome system but does not confirm direct proteasome inhibition. The buildup of ubiquitinated proteins may also occur if folding chaperones, deubiquitinase enzymes, or ubiquitin ligases are inhibited. Inhibition of the proteasome subunits may also be measured outside of cells using the purified enzyme.[65] Finally, fluorescent reporter systems that label proteasomal substrates can be used, enabling the implementation of fluorescence microscopy or flow cytometry to probe proteasome function.[66]

Because disruption of cellular Ca^{2+} trafficking may be related to ER stress, measurements of intracellular Ca^{2+} levels can provide insight on the mechanism of action of ER-targeting agents.[67,68] For example, cell-permeable Ca^{2+} chelators can be used to probe these mechanisms. If an ER stress-inducing agent is operating through Ca^{2+} dysregulation, the addition of cell-permeable Ca^{2+} chelators is expected to alter the cytotoxicity of the compound. To more specifically evaluate the role of ER Ca^{2+} release, the SERCA pump inhibitor thapsigargin can be applied in conjunction with an ER stress-inducer in a similar manner. Intracellular Ca^{2+} fluctuations at the organelle level may also be evaluated using fluorescent Ca^{2+} sensors such as Calcium Green-5N. Lastly,

Western blots can be carried out to analyze the expression levels of specific Ca^{2+} regulatory proteins, such as inositol 1,4,5-triphosphate receptors (IP3Rs).[69]

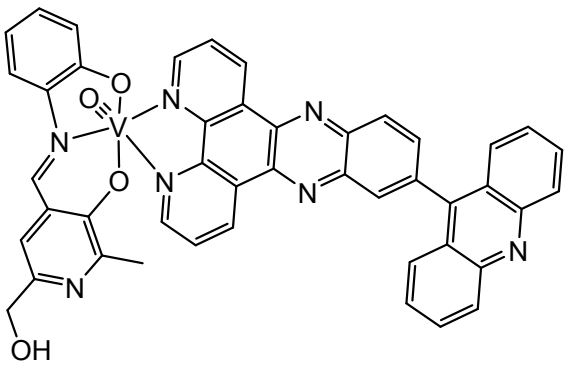
Chemical modulators of ER homeostasis can also be used to detect ER stress or identify the mechanism of action of ER stress-inducing agents. Co-treating cells with antioxidants such as *N*-acetylcysteine (NAC) will decrease cytotoxic effects of compounds that act by producing ROS but should have no effect on those that do not. Thus, this agent can be used to determine if a compound induces ER stress via ROS production.[70,71] The compound 4-phenylbutyrate aids in protein folding. If 4-phenylbutyrate decreases the cytotoxic effects of a compound, then a likely conclusion is that this compound acts via the induction of protein misfolding.[72] Chemical induction or downregulation of UPR pathways may also prove useful. For instance, the commercially available small molecules salubrinal and ISRIB stimulate and block the ER stress response, respectively. Salubrinal acts by preventing the dephosphorylation of eIF2α, activating the PERK arm of the UPR and protecting cells from unfolded protein accumulation.[73] ISRIB, on the other hand, decreases eIF2α phosphorylation levels and often sensitizes cells to ER stress.[74,75]

4. Anticancer Metal Complexes that Induce ER Stress

4.1 First Row Transition Metal Complexes

Relative to the reports of second and third row transition metal complexes that induce ER stress, first row complexes are comparatively rare. For this reason, first row transition metal complexes are grouped together, rather than by element, in this review. Generally, first row metal complexes have been shown to induce ER stress mediated by

one of three mechanisms: photodynamic generation of ROS, disruption of intracellular metal trafficking, and proteasome inhibition. Many of these compounds operate in response to light. For example, there are reports that document ER stress induction by phototoxic vanadium (**1**)[76,77] and bimetallic copper-ferrocene[78] (**2**) complexes. These complexes often act as photosensitizers for $^3O_2$, leading to the production of $^1O_2$ and other ROS. The fluorescent vanadium complex **1** was shown to colocalize with ER Tracker Red via confocal microscopy experiments, and the ability of **1** and other derivatives to generate $^1O_2$ was confirmed using spin-trap experiments and via the DCF-DA (dichlorofluorescein-diacetate) ROS-detection assay. Similarly, bimetallic complex **2** and related compounds exhibited colocalization with ER-tracking dyes and induced apoptosis via ROS generation. However, unlike the vanadium complex **1**, compound **2** generates ROS via a redox pathway rather than through energy transfer. All of these complexes bear extended aromatic structures such as acridine and dipyridophenazine, which likely engenders their observed ER localization due to hydrophobic interactions with the phospholipids of the ER membrane.



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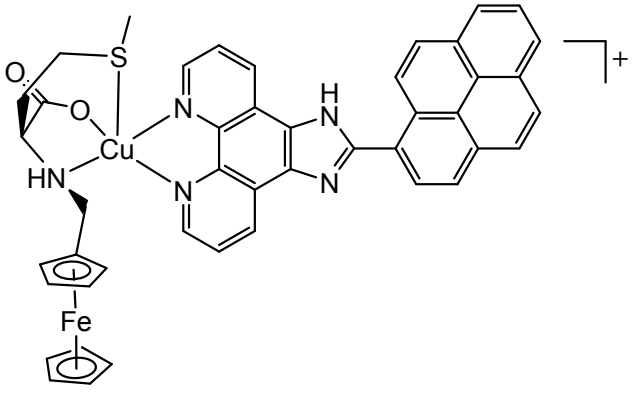

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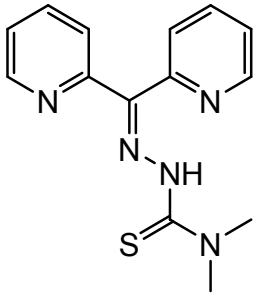
In addition to phototoxic ER stress mechanisms, there are several first row

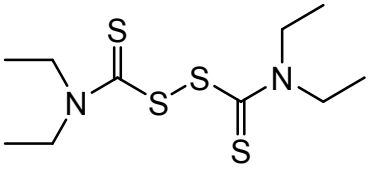
transition metal complexes that catalytically generate ROS via redox cycling. This family of anticancer agents includes copper and iron chelators that may be administered as the free ligand or as the pre-formed metal complex.[79,80,89,81–88] These types of compounds are often selective for cancer cells over normal cells. One of the most studied iron-chelating thiosemicarbazones, di-2-pyridylketone 4,4-dimethyl-3-thiosemicarbazone (Dp44mT) (**3**), has been shown to generate ROS and activate all three branches of the UPR, eventually leading to apoptosis.[79] In the same study, it was shown that the well-known iron-chelating siderophore deferoxamine (DFO) also induces ER stress with a subtly different phenotype, indicating that iron chelation may play a more general role in this phenomenon.



3

Similar ER stress response occurs when cells are treated with copper ionophores such as disulfiram (**4**), the Cu-complex of disulfiram (**5**), or pyrazole-pyridines and their copper complexes, such as compound **6**. These ionophores cause intracellular copper overload, which leads to paraptotic cell death.[87] Detailed biological analyses indicate that treatment with copper chelators induces several ER stress hallmarks, including eIF2α phosphorylation, CHOP expression, and polyubiquitinated protein accumulation. The authors conclude that these compounds share a similar mechanism in which the chelator brings extracellular Cu(II) into the cell and is then reduced to release cytotoxic

copper that leads to paraptosis, as shown in **Fig. 2**. The released copper also acts as a caspase inhibitor, as minimal increases in caspase activity were detected after treatment of cells with $CuCl_2$ or **5**. In vitro assays using isolated caspase-3 also show that $CuCl_2$ acts as an inhibitor, which may explain why cell death resulting from Cu complexes is generally caspase-independent paraptosis rather than apoptosis.



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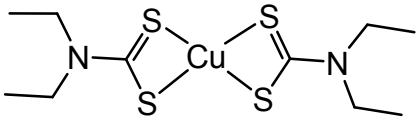

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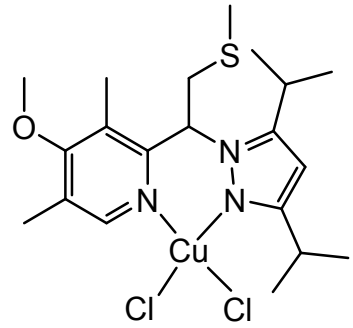

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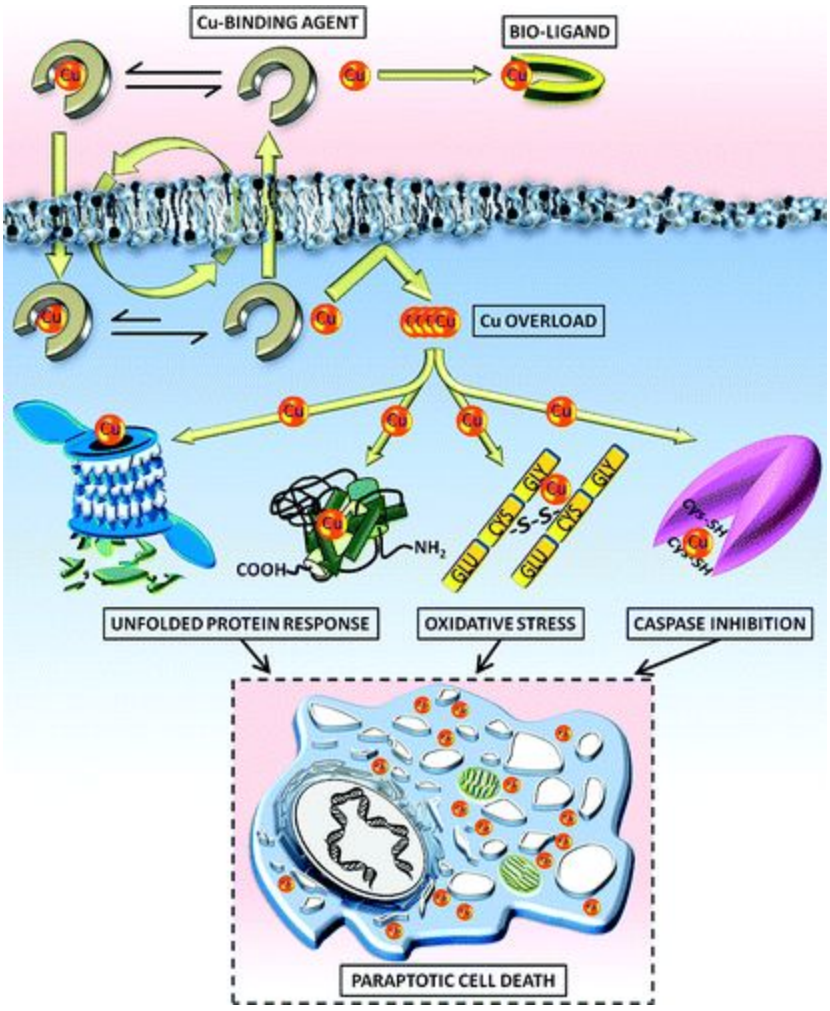


Fig. 2. Proposed mechanism of ER stress induction by pyridine-pyrazole-based copper chelators, like that of **6**. These ligands act as ionophores for Cu(II), ultimately inducing copper overload that triggers the UPR and paraptosis. Adapted from reference 88 with permission from the American Chemical Society, copyright [2011].

Due to their capacity to act as electrophilic warheads, several first row metal complexes inhibit the nucleophilic catalytic site of the proteasome[90–97] or ubiquitinase enzymes,[91] resulting in ER stress and apoptosis via the buildup of unfolded proteins. Due to the rapidly expanding interest in proteasome inhibitors as cancer therapeutics, the topic of metal-based proteasome inhibitors has recently been reviewed elsewhere.[98–100] Here, we will focus on only a few examples that are pertinent to ER

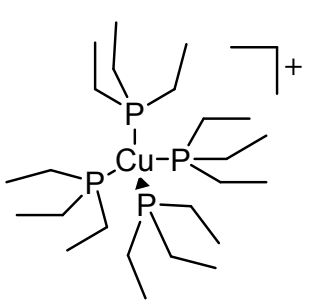
stress. One of the most relevant proteasome inhibitors is disulfiram (**4**), a drug clinically approved for alcoholism that is now being investigated as an anticancer agent. Several studies have shown that the copper complex of disulfiram and other dithiocarbamates inhibit proteasomal activity, leading to overaccumulation of misfolded proteins and apoptosis.[92,93,101] Together, these studies highlight the potential of metal complexes as proteasome inhibitors, and support efforts to further optimize them to generate more potent, selective complexes.

Another well-investigated copper complex that induces ER stress and inhibits the proteasome is the Cu-phosphine complex **7**.[102,103] This complex exhibits nanomolar activity against several colon and leukemia cancer cell lines, and it induces cell death by a combination of paraptosis and caspase-dependent apoptosis. Detailed mechanistic studies reveal that **7** causes increased expression of ER stress markers BiP and CHOP, as well as eventual PARP cleavage and caspase activation. The complex also effectively inhibits the ubiquitin-proteasome system and causes the accumulation of polyubiquitinated proteins. The cytotoxicity **7** is significantly attenuated in the presence of the translation inhibitor cycloheximide, indicating that protein synthesis, and possibly paraptosis, is required for this compound to exert its biological effects.

The Cu(II)-thioxotriazole complex **8** also triggers proteasome-mediated ER stress and paraptosis.[104] Thorough transcriptomics analysis of HT1080 human fibrosarcoma cells treated with **8** revealed a marked upregulation of ER stress-related genes, especially those associated with protein folding and unfolded protein binding. Analysis via quantitative polymerase chain-reaction (qPCR) revealed upregulation of ER stress markers BiP and CHOP. The authors also observed the phosphorylation of eIF2α, ATF4

upregulation, and XBP1 splicing, further confirming ER stress induction. Complex **8** triggers the accumulation of polyubiquitinated proteins and inhibits the activity of the proteasome in cell lysates, indicating that proteasomal inhibition may be its primary mechanism of action. It should be noted that the chelating thioxotriazolyl pyridine ligands similar to complex **8** may bind metal centers in two ways, either the S-N coordination mode shown, or via N-N coordination through the thioxotriazole and pyridine nitrogens.[105] An investigation of thioxotriazole complexes of both types: N-N coordinating and N-S coordinating, revealed that the N-N version, as in complex 8, is a much more potent inducer of paraptosis.[106]

Proteasome inhibition leading to ER stress induction has also been reported for Cu(I) complexes bearing tris(pyrazolyl)borate ligands, such as compound **9**.[95] Compound **9** exhibits sub-micromolar cytotoxicity against a broad panel of cancer cell lines and is selective for cancer cells over normal kidney (HEK293) cells. Treatment of ovarian cancer 2008 cells with **9** induced the accumulation of polyubiquitinated proteins and triggered increased phosphorylation of the UPR regulators PERK and IRE1. Like several other Cu complexes, the mode of cell death caused by this compound is paraptosis. Paraptosis was confirmed using cycloheximide, which attenuated the cytotoxicity of **9**, whereas caspase-dependent apoptosis was ruled out because caspase inhibitors had no effect on the activity of **9**. Compound **9** also significantly inhibited tumor growth in mice bearing murine Lewis lung carcinoma tumors without affecting their bodyweight, indicating that this compound does not give rise to acute toxicity. Given these favorable properties, compound **9** and related analogues have great promise as potential anticancer agents.



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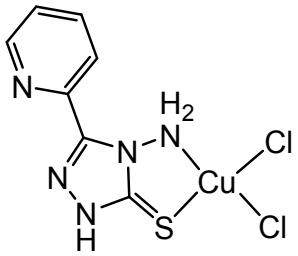

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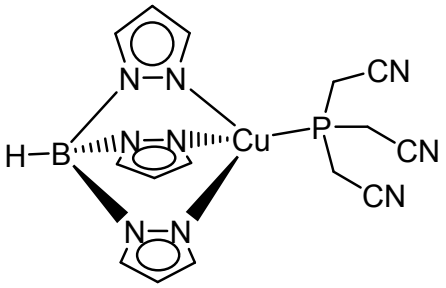

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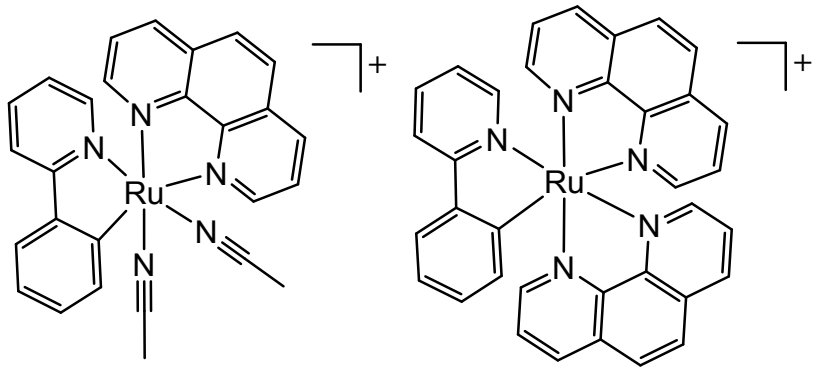
Another Cu(II) complexed to phenanthroline and the ER stress-mediator salubrinal has recently been reported.[107] This complex induces ER stress leading to cell death, and it has much higher toxicity than salubrinal alone. The complex also leads to upregulation of BiP and CHOP, further confirming ER stress as the root cause of cell death. Such conjugates represent and interesting new class of bifunctional anticancer agents.

4.2 Ruthenium

Of all transition metals, ruthenium complexes have by far the most reports of ER stress-mediated anticancer activity. The ligand frameworks of Ru-based ER stress-inducing compounds vary widely. These compounds assume a range of different structural archetypes, including trispolypyridyl complexes, cyclometalated compounds, organometallic piano-stool structures, and simple coordination complexes. Despite having the same metal center, these complexes exhibit diverse mechanisms of ER stress induction.

One particularly potent and well-investigated class of Ru-based ER stress inducers are mono-cationic, cyclometalated Ru(II) complexes.[108–110] A large library of ruthenium cyclometalated species, such as **10** and **11,** have been shown to induce ER stress and subsequent apoptosis. ER stress induction by these compounds has been extensively characterized by demonstrating hyperphosphorylation of eIF2α, splicing of XBP1, and expression of CHOP.[109] The role of CHOP in apoptosis induction was confirmed through the use of anti-CHOP siRNA. By silencing this pro-apoptotic factor, the cells were significantly less sensitive to treatment by **10** and **11**. The original lead compound, **10**, also significantly inhibited tumor growth in vivo without severe side effects. Further structural optimization of **10** led to an expanded library of cyclometalated complexes with increased solubility and higher potency, with complex **11**, for instance, exhibiting low-nanomolar anticancer activity in vitro.[111,112] Structure-activity studies revealed that complexes with relatively high lipophilicity were the most potent, and that complexes with intermediate redox potentials (0.4–0.6 V vs SCE) were most active. Based on this redox potential dependence, the authors concluded that electron transfer or oxidation of the Ru(II) center may play a role in their activity. Further investigation of the biological properties and cellular response to these compounds indicates that several of them induce both DNA damage and ER stress, whereas others act only via targeting the ER.[113] From a detailed analysis of cells treated with **10**, the authors found that this compound binds to histones, which ultimately lead to disruption of DNA replication.[114] The compound also downregulates hypoxia-inducible factor 1-α (HIF1α) by directly interacting with the regulatory protein prolyl hydroxylase domain-containing protein 2 (PHD2), leading to hypoxia-selective anticancer activity.[115] Further

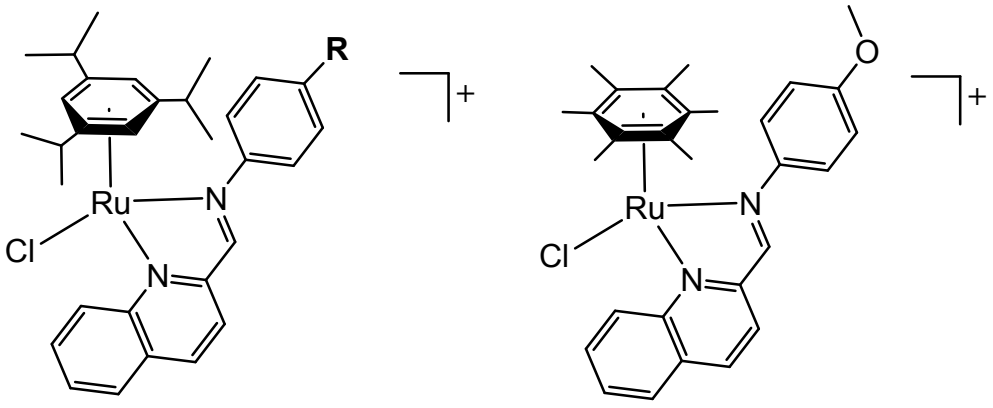
experiments will be needed to determine whether DNA damage or ER stress is the major cause of cytotoxicity of this compound. Cellular uptake experiments revealed that **10** enters cells through iron transporters and via amino acid transporters. Genomic experiments revealed that cellular export and resistance to these compounds are mediated by the multi-drug transporter ABCB1 and by the endothelial growth factor repair (EGFR) pathway.[110] Together, these studies provide a thorough picture of the ER stress-mediated anticancer properties of this class of compounds, which is further supported by promising in vivo activity and interesting insight on their resistance mechanisms.



10 **11**

In addition to cyclometalated compounds, several piano-stool ruthenium complexes have also been found to induce ER stress.[116–120] Notably, these complexes have highly modular structures that are amenable to combinatorial synthesis. A combinatorial screening method was used to identify complexes such as **12a** to be potent ER stress-inducing anticancer agents.[116] In a follow-up study, the role of the arene substituent of complex **12a** was investigated by comparing the activities of **12a**

and **13**, which contain the same pyridylimine ligand but different arenes. Both **12a** and **13** cause splicing of XBP1 and CHOP expression, indicating that they are ER stress inducers.[117] Surprisingly, these two compounds induce ER stress by different mechanisms of action. Compound **12a**, bearing the 1,3,5-triisopropylbenzene ligand, causes ROS-mediated ER stress, whereas **13**, which contains the hexamethylbenzene ligand, acts via an ROS-independent mechanism. Both compounds **12a** and **13**, along with a large number of related analogues, operate via p53-independent pathways and therefore retain their high cytotoxicity in p53-null cell lines.[118] By modifying the pyridylimine ligand of **12a**, other potent compounds like **12b-d** could be obtained. These compounds exhibit nanomolar activity against a broad panel of cancer cell lines.[120] The cytotoxicity of these compounds is directly related to the π-acidity of the pyridylimine ligand, with more π-acidic ligands giving rise to more potent compounds. The ligand π-acidity is also correlated to the ROS production capabilities of these complexes. This result indicates that these compounds induce ER stress and cell death via the production of ROS. This mechanism was confirmed by showing that the cytotoxicity of these compounds is significantly reduced in the presence of the antioxidant NAC, as shown in **Fig. 3**. Related dinuclear ruthenium arene complexes bearing diimine ligands also possess anticancer activity in the low micromolar range, and their activity arises from both DNA damage and ROS-mediated ER stress pathways.[121] Based on the powerful anticancer activity and unique anticancer mechanisms of this class of compounds, they represent a promising new class of metallodrugs.



12a, R = OMe; 12b, R = Et
12c, R = NMe_2; 12d, R = iPr

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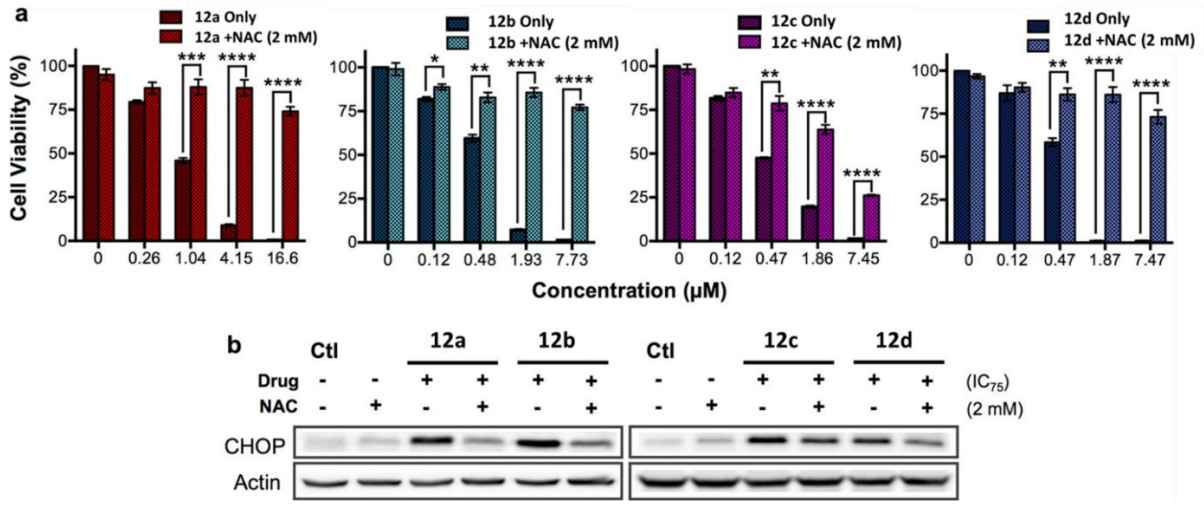
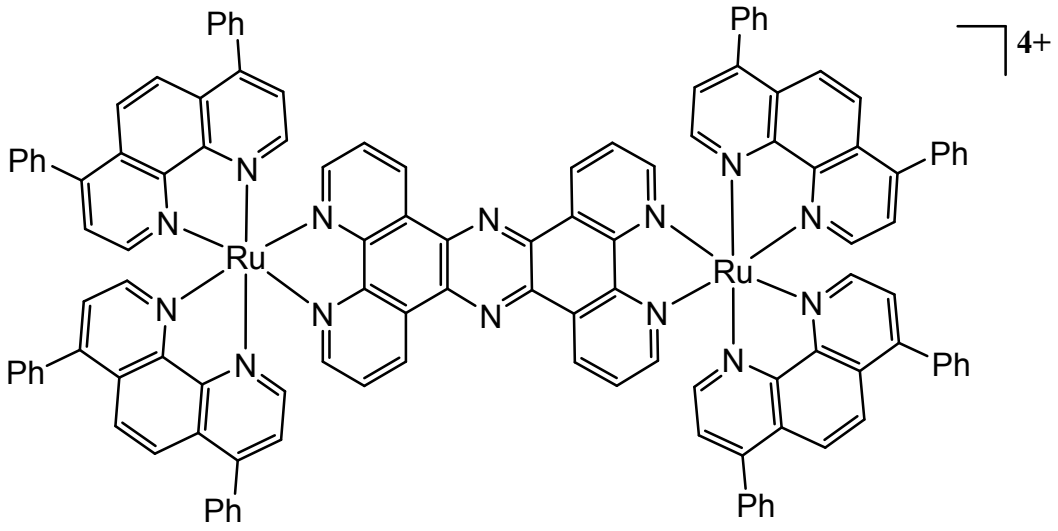


Fig. 3. Piano-stool ruthenium complexes, like **12**, induce their cytotoxic effects and ER stress by producing ROS. (a) Cell viability of cells treated with compounds **12a-d** in the presence and absence of NAC in HCT-116 cells (2 mM). (b) CHOP expression level in HCT-166 cells treated with **12a-d** in the presence or absence of NAC (2 mM). Adapted from reference 120 with permission from the American Chemical Society, copyright [2018].

Although polypyridyl Ru(II) complexes have well-established anticancer activity,[122] there are relatively few reports of ER stress-related anticancer mechanisms for these compounds.[123–126] One extremely hydrophobic, dinuclear complex bearing 4,7-

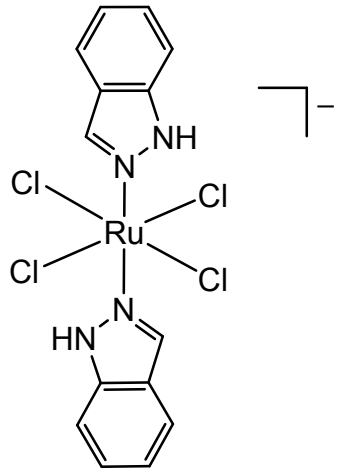
diphenyl-1,10-phenanthroline (DPP) ligands (**14**) exhibits low-micromolar anticancer activity. Its localization, tracked by fluorescence microscopy, confirmed that it is taken up by the ER.[123] The complex interacts strongly with liposomes and shows environment-dependent luminescence, with greatly increased luminescence in hydrophobic media. Despite the fact that this compound localizes to the ER, experiments to probe whether it induces ER stress were not carried out. A different family of ruthenium polypyridyl complexes bearing *p*-cresol groups has been reported to induce ROS-mediated ER stress, as evidenced by CHOP induction and phosphorylation of eIF2α.[125]



14

Ru(III) coordination complexes have also been linked to ER stress induction. The Ru(III)-indazole complex anion KP1019 or NKP-1339 (**15**), which has undergone clinical investigations for the treatment of cancer,[127,128] has recently been shown to induce ER stress.[129] This complex triggers several ER stress hallmarks, including phosphorylation of PERK and eIF2α, as well as upregulation of XBP1 and CHOP. The IRE1α pathway,

however, is unaffected. This complex also causes downregulation of BiP, a result that is unexpected for conventional ER stress-inducers. The authors attribute this downregulation to activation of the ERAD pathway because upregulation of ERAD has previously been shown to reduce BiP levels, and because no downregulation of BiP was found on the mRNA level. ER stress induction by **15** may arise from ROS generation, as previous reports have linked ROS generation to the anticancer activity of **15**.[130] Another recent study has reported that **15** inhibits SERCA transporters, which may induce ER stress by disruption of intracellular Ca^{2+} trafficking.[131] This mechanism explains the phenotype observed in response to this compound, as it is very similar to the thoroughly investigated organic SERCA inhibitor thapsigargin. ER stress induction by this compound may also result in immunogenic cell death (ICD), as a recent study reports that cells exposed to **15** exhibited several hallmarks of ICD, including calreticulin exposure and ATP release.[132] Taken together, this compound's in vivo activity, unorthodox mechanism of action, and potential to cause ICD in cancer cells make it a promising anticancer agent.



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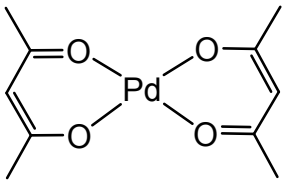
4.3 Palladium

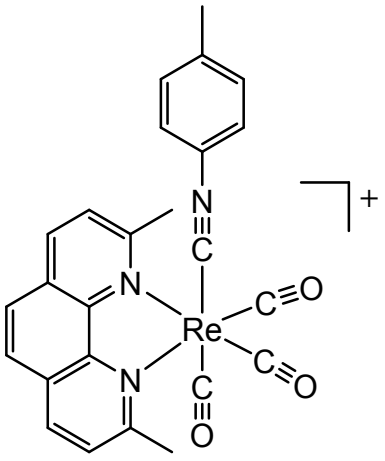
Unlike its heavier congener platinum, which has been widely investigated for anticancer activity, palladium complexes remain largely unexplored. To date, only one palladium complex, $[Pd(acac)_2]$ (**16**) has been shown to exhibit anticancer activity that is mediated by ER stress induction.[133] Unlike conventional platinum-based drugs, this compound does not bind closed circular DNA. Treatment of cancer cells with **16**, however, induced several markers of ER stress, including activation of ATF4 and XBP1, upregulation of BiP, and ER swelling. The complex also causes CHOP-dependent apoptosis, which was determined by showing that CHOP silencing with siRNA significantly rescues cells from apoptosis. Although the exact cause of ER stress induction by **16** is not confirmed, it is hypothesized to arise from interference with ER Ca^{2+} stores because treatment of cells with this compound gives rise to a dose-dependent release of stored Ca^{2+} from the ER. Complex **16** also shows activity in vivo. It significantly inhibited tumor growth in mice bearing H460 lung cancer xenografts, but no studies were performed to determine whether ER stress was also responsible for the in vivo activity of **16**. Further investigation is needed to determine whether this ER stress-inducing activity is characteristic of labile Pd(II) complexes or unique to $[Pd(acac)_2]$.



16

4.4 Rhenium

In recent years, rhenium complexes have arisen as a new promising class of metal-based anticancer agents.[134–138] Through research efforts to explore the anticancer activities of these compounds, an ER stress-causing rhenium compound was discovered.[139] Compound **17**, a Re(I) tricarbonyl complex bearing both a diimine and isonitrile ligand, induces ER stress by causing the accumulation of unfolded proteins. This buildup of unfolded protein results in increased phosphorylation of eIF2α, leading to the induction of ATF4 and expression of CHOP (**Fig. 4**). The complex also induces mitochondrial fragmentation and eventual depolarization, leading to apoptosis. Unlike similar late transition metal complexes, this complex apparently does not operate via ROS induction, interference with intracellular Ca^{2+} stores, or inhibition of the ubiquitin-proteasome system. The potency of this complex is enhanced in the presence of the eIF2α dephosphorylation inhibitor salubrinal. Because this compound does not operate via one of three canonical means of induction ER stress, further studies are needed to determine its mechanism of action to guide efforts to develop more potent analogues.



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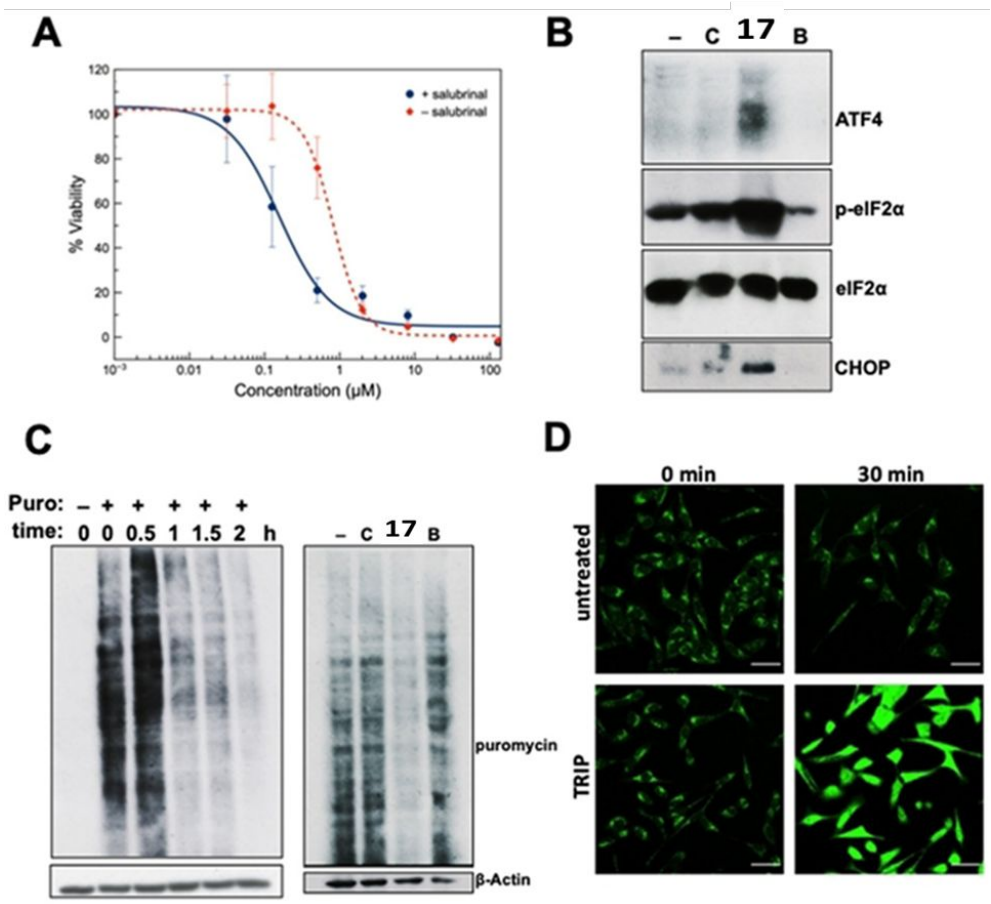


Fig. 4. Cellular response upon treatment with **17**. (A) Dose-response curves of HeLa cells treated with **17** in the presence or absence of the eIF2α dephosphorylation inhibitor salubrinal. (B) Western blot analysis of ER stress markers ATF4, p-eIF2α, and CHOP upon treatment of A2780 cells with vehicle control (**–**), cisplatin (**C**), **17**, or bortezomib (**B**). (C) Puromycin assay indicating a decrease in A2780 cellular translation levels upon treatment with **17** (left panel) or vehicle control (**–**), cisplatin (**C**), **17**, or bortezomib (**B**) (right panel). (D) Confocal fluorescence microscopy images of protein aggregates formed upon treatment of HeLa cells with **17**. Protein aggregates were stained with the thioflavin T dye. Adapted from reference 139 with permission from John Wiley and Sons, copyright [2019].

Follow-up studies were performed using this compound and related analogs to further investigate their intracellular speciation, localization, and anticancer mechanism.[140] Intracellular localization and speciation of the rhenium isonitrile complexes were determined using X-ray fluorescence (XRF) experiments with an iodine-labeled isonitrile version of **17**. These experiments showed that the Re and I

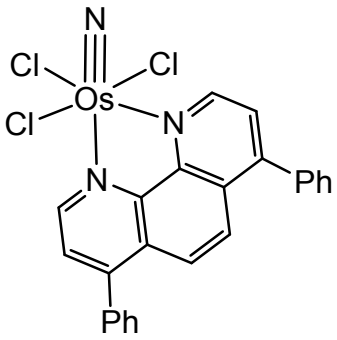
exhibit high colocalization, indicating that the complex remains intact inside cells.

A cell line resistant to **17** has also been developed by prolonged exposure of A2780 ovarian cancer cells to **17**, and the mechanism of resistance was investigated.[141] A series of experiments revealed that **17** exhibits lower accumulation in the resistant cell line due to increased expression of the ABCB1 transporter. This transporter is well-known to provide multi-drug resistance, and it is particularly important for the detoxification of organic therapeutic molecules such as paclitaxel. These results indicate that organometallic complexes serve as substrates for these transporters. Further experiments are needed to determine how these compounds may be modified to reduce their affinity for multi-drug transporters.

4.5 Osmium

Despite the increasing the use of osmium complexes for the treatment of cancer,[142,143] only two such complexes have been shown to induce ER stress.[144–146] These two ER stress-inducing compounds, **18** and **19**, both contain the high oxidation state Os(VI) center with a terminal nitrido ligand. Detailed mechanistic studies using varying shRNA sequences were performed on this class of compounds to probe their mechanisms of action. Based on the relative protective or sensitizing effects of the shRNA sequences, the cellular processes affected by the compounds could be determined. Surprisingly, the mechanisms of action of these Os-nitrido complexes depend heavily on the nature of the substituents on the supporting diimine ligand. For example, compound **18** with a DPP ligand operates via the induction of ER stress, whereas **19**, which bears 1,10-phenanthroline, causes both DNA damage and ER stress. Evidence for the fact that **18** causes ER stress was obtained by showing that

eIF2α phosphorylation and CHOP activation result when cells are treated with this compound. Furthermore, **18** gives rise to cellular apoptosis in a manner that is independent of p53. In contrast to the rhenium complex **17**, the activity of complex **18** is decreased in the presence of the ER stress mediator salubrinal. The less lipophilic complex **19**, which causes both ER stress and DNA damage, has cancer stem cell-selective activity, as shown in part by its ability to effectively reduce growth of breast cancer stem cell mammospheres (**Fig. 5**). This high activity may arise from the ability of the compound to trigger ER stress via the eIF2α pathway, which has been implicated as a target for selectively killing stem cells.[54] The stem cell selectivity exhibited by **19** makes it a particularly promising drug candidate because most conventional chemotherapeutic agents are ineffective against cancer stem cells.[147,148] In addition to its in vitro activity, **19** has high activity in vivo. In an orthotopic glioblastoma mouse model, mice treated at a dose of 0.5 mg/kg with complex **19** were able to prolong mouse survival by 130% in comparison to mice treated with the vehicle control. Given their promising in vitro and in vivo activity, these stem cell-selective, ER stress-inducing Os-nitrido compounds show great promise as anticancer agents. Because only a limited number of complexes have been analyzed thus far, more compounds should be synthesized and studied in order to develop a SAR to identify more potent analogues.



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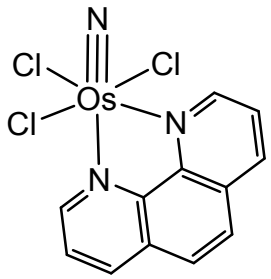

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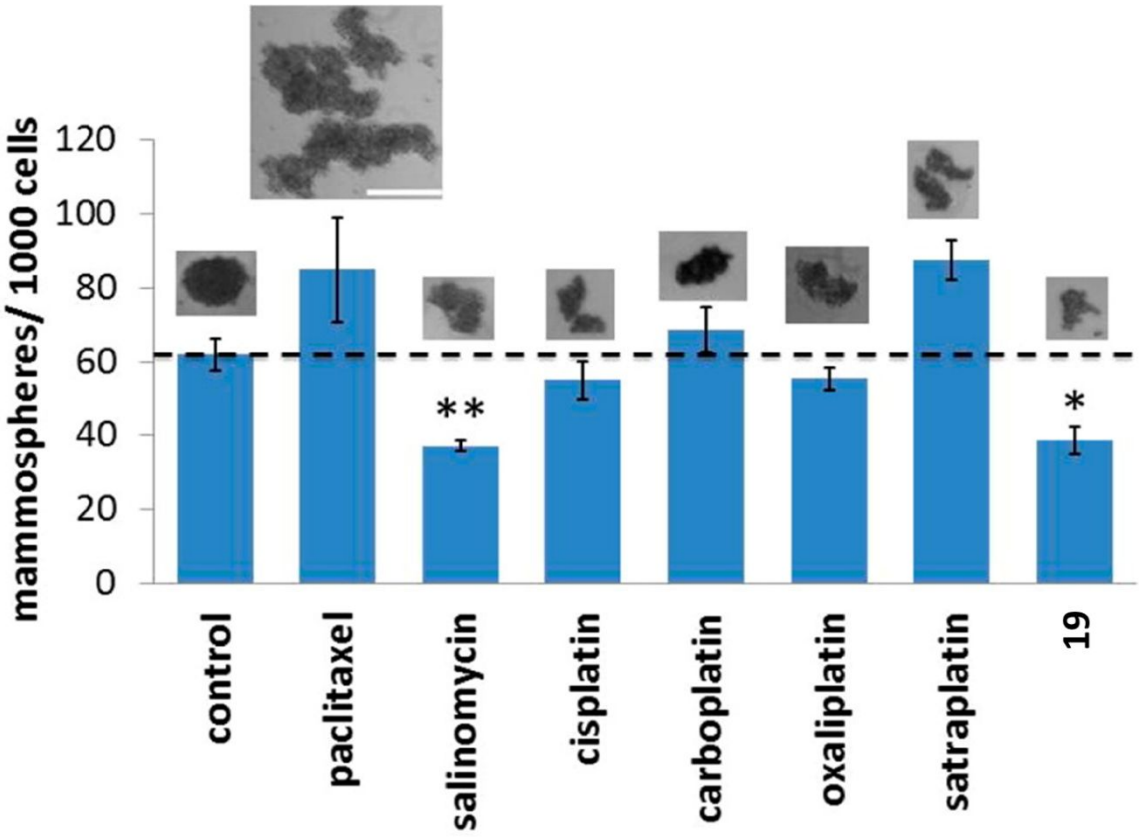
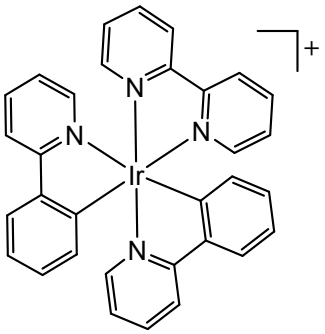


Fig. 5. Mammosphere formation of HMLER breast cancer stem cells in the presence or absence of the indicated anticancer compounds, including **19**, at their IC_{50} values after 5 days of treatment. Representative brightfield microscopy pictures of mammospheres treated with the indicated compounds are provided above the relevant columns. Adapted from reference 145 with permission from the American Chemical Society, copyright [2014].

4.6 Iridium

Although the majority of Ir(III) anticancer agents are based on the cyclopentadienyl piano stool structural type,[149] thus far only substitution-inert, cyclometalated polypyridyl complexes of iridium have been demonstrated to cause ER stress. Many of these complexes are luminescent with very high quantum yields, allowing determination of their intracellular localization to organelles such as the ER. Furthermore, the rich photophysical properties of this class of compounds often give rise to phototoxic effects that are mediated by the generation of ROS. If these compounds localize to the ER, the generation of ROS by light irradiation directly damages this organelle and gives rise to ER stress-mediated apoptosis.[150–153] Compound **20**, for example, is a representative phototoxic ER stress-inducing agent within this class of Ir(III) cyclometalated complexes that produces both $^1O_2$ and superoxide upon irradiation. This complex was demonstrated to photo-cross link and photo-oxidize proteins via both photoinduced electron transfer and energy transfer.[152] Proteomics experiments were performed on cell lysates to identify the most commonly damaged protein targets of this compound upon irradiation, as shown in **Fig. 6**. These targets span proteins associated with the ER, mitochondria, and membranes, indicating that widespread cellular damage is caused by the ROS released from **20**. Based on these results, the authors propose a dual mechanism of phototoxicity from these compounds, in which superoxide causes protein crosslinking and aggregation, and $^1O_2$ oxidizes proteins. Another ER-localizing photoactive cyclometalated Ir(III) complex bearing a terpyridine ligand, compound **21**, was investigated and shown to trigger ER stress upon irradiation.[153] This compound, which has an enhancement in cytotoxicity of nearly 100-fold in the presence of light, induces an increase in cytosolic Ca^{2+} levels

after irradiation, presumably due to release of Ca^{2+} from the ER. Thus, it is likely that the ROS generated by this compound directly attack the Ca^{2+}-trafficking machinery of the ER. The iridium *N*-heterocyclic carbene (NHC) complex **22** has also been shown to localize to the ER and generate ROS locally upon irradiation, but further studies are needed to characterize the full anticancer potential of this compound.[151]



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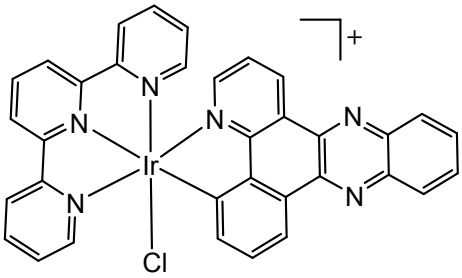

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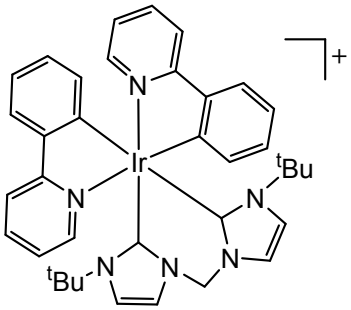

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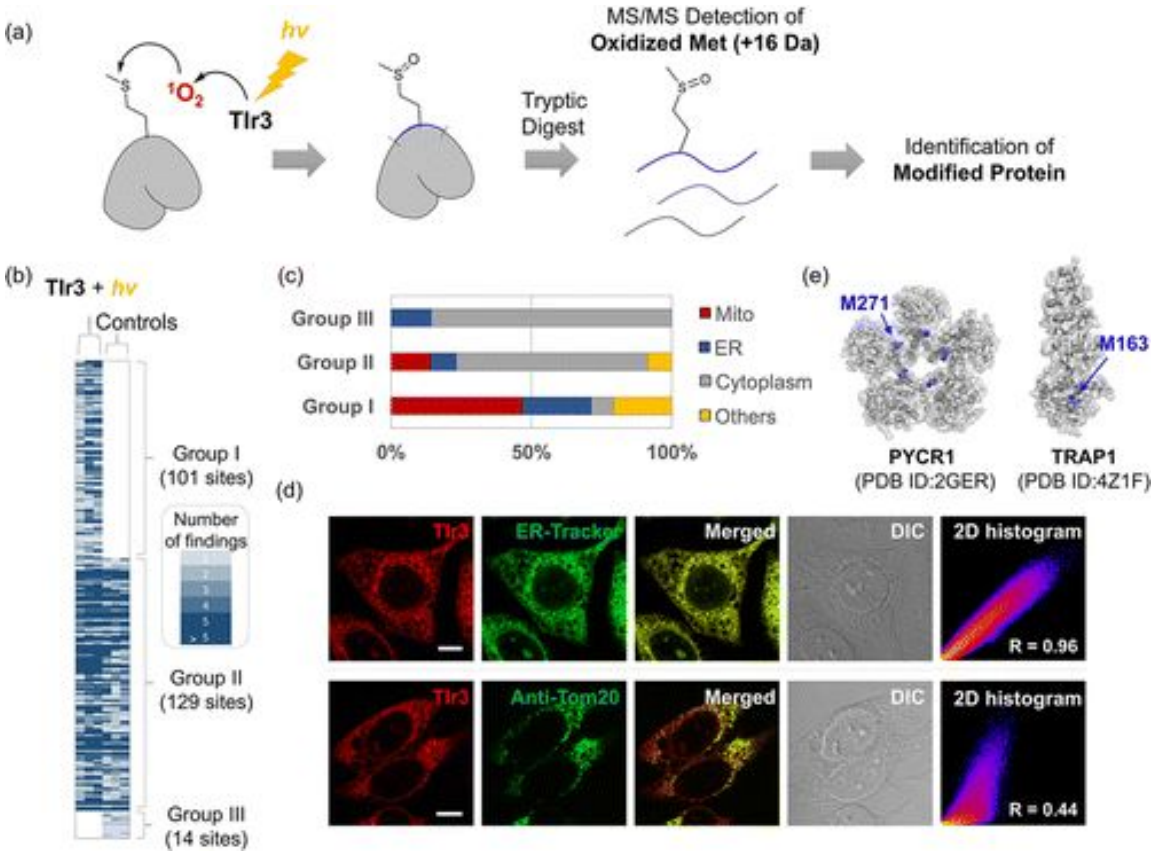
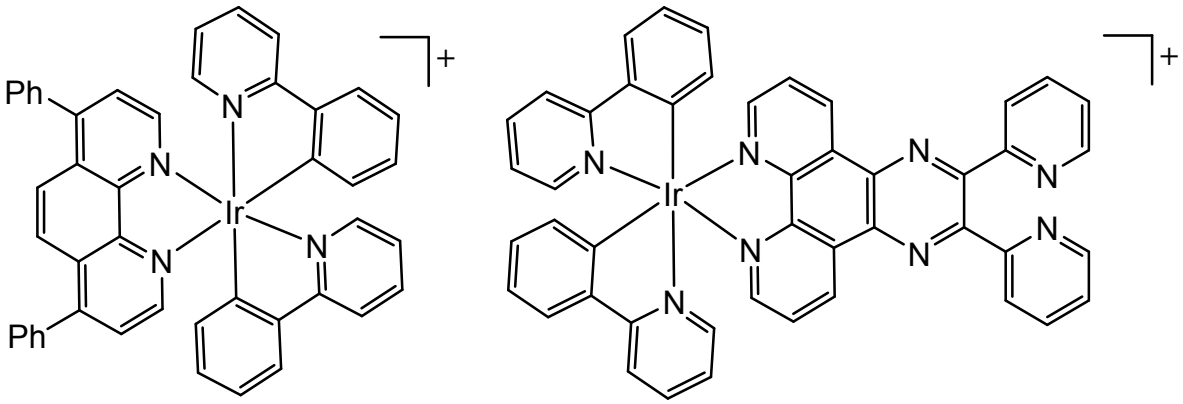


Fig. 6. Identification of intracellular protein targets of iridium photosensitizers. (a) Pictorial representation of the method used for MS/MS detection of oxidized methionine. (b/c) Distribution of proteins oxidized by **20** upon photoxidation (Group I), relative to those oxidized by endogenous processes (Groups II and III). (d) Confocal fluorescence microscopy imaging of iridium complexes showing colocalization with ER-Tracker dye and Anti-Tom20, a mitochondrial stain. TIr3 in the image corresponds to compound **20** (e) Crystal structures of selected oxidized proteins PYCR1 and TRAP1 responsible for mitochondrial function. Reproduced from reference 152 with permission from the American Chemical Society, copyright [2016].

Certain members of this class of cyclometalated Ir(III) complexes are also capable of inducing ER stress in the absence of light. Complex **23** and related structural analogues, for example, cause potent cytotoxic effects in the dark. These compounds were shown to localize to cell membranes, particularly the organelle membranes of the ER. Cell death induced by these agents was accompanied by mitochondrial

fragmentation, mitochondrial membrane depolarization, cytochrome *c* release from the mitochondria, and caspase 3 activation.[154] Complex **23** triggers expression of CHOP, indicating that ER-mediated apoptosis is operational. This compound also causes Ca^{2+} release from the ER to the cytosol, a process that precedes mitochondrial Ca^{2+} overload and the observed mitochondrial damage. Thus far, only the analog containing the DPP ligand has been studied for ER stress induction, so further studies will be needed to understand which structural properties are required to produce the observed phenotype. Complex **24**, which bears an expanded phenazine ligand, exhibits nanomolar cytotoxicity in MCF-7 breast cancer cells, which is mediated by ER stress.[155] This compound induces paraptotic cell death, as evidenced by activation of MAP kinases and abrogation of its anticancer activity by the protein synthesis inhibitor cycloheximide. Unlike the other cyclometalated Ir(III) ER stress inducers, this compound appears to accumulate preferentially in the mitochondria. Within the mitochondria, it has a secondary effect on the ER, due to the production of ROS that causes inhibition of the ubiquitin-proteasome system. Thus, this compound shows that ER localization is not a strict requirement for causing ER stress. A final, less well investigated class of Ir(III) compounds that deserves mention are a family of neutral complexes bearing phenylpyridine and tetrazolato ligands.[156] Despite not being cationic like the other Ir(III) complexes discussed, these agents still localize to the ER and trigger cell death. However, few mechanistic studies have been performed that verify them to cause ER stress. Their localization to this organelle, however, suggests that ER stress could be a likely mechanism.

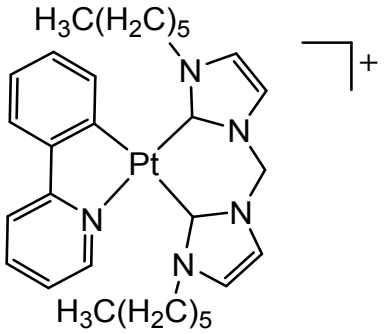


23 **24**

4.7 Platinum

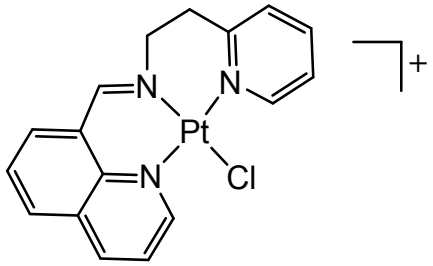
Based on their clinical success, Pt compounds are among the most thoroughly explored class of metal-based anticancer agents. Although the clinically approved Pt anticancer agents operate via DNA binding, recent studies have identified Pt complexes with novel mechanisms of action, which includes ER targeting. Within the latter category, a family of luminescent Pt(II)-NHC complexes, including complex **25**, localize to the ER and exhibit moderate phototoxicity.[157] Upon irradiation, these compounds induce ER stress, which was confirmed via the detection of phosphorylated PERK and eIF2α by Western blot. Following ER stress, the usual cascade of mitochondrial depolarization, caspase activation, and apoptosis occurs. Complex **25** was found to be the most selective agent within this class of compounds. It exhibits a greater than 30-fold increase in cytotoxicity upon irradiation, reflecting the potential of these complexes for use in PDT. Further mechanistic studies on **25** showed that this compound triggers ICD.[158] The ICD caused by **25** is likely a direct consequence of ER stress induction, as ER stress in general and especially eIF2α phosphorylation is often correlated with ICD.[159,160] The ICD triggered by **25** was detected by confirming that the “eat me” signal

calreticulin is translocated to the cell membrane. The presentation of calreticulin marks the cell for phagocytosis by immune cells. Flow cytometry provided further confirmation that macrophages will phagocytose cells treated with **25**. The potential of ER stress-inducing agents, like **25**, to trigger ICD highlights the value of these compounds in the clinic.



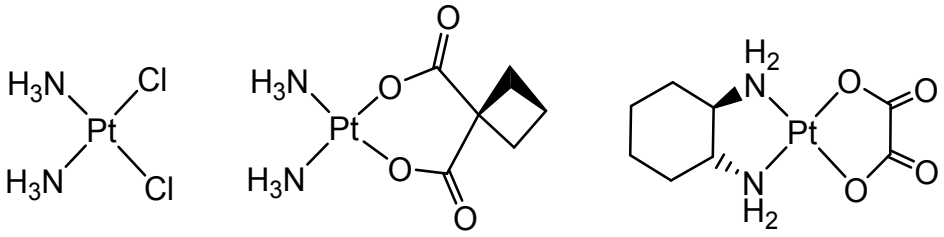
25

A Pt(II) complex bearing a tridentate quinoline-Schiff base ligand, **26**, has also been reported to induce ER stress.[161] This compound localizes to the mitochondria, triggering its depolarization. The ER is affected downstream of this process, as evidenced by the phosphorylation of PERK and eIF2α and induction of CHOP. Compound **26** was further evaluated in vivo. This compound was able to significantly reduce tumor growth in mice bearing A549 lung cancer xenografts. Despite its distinct mechanism, compound **26** was equally effective as cisplatin in this in vivo antitumor model.



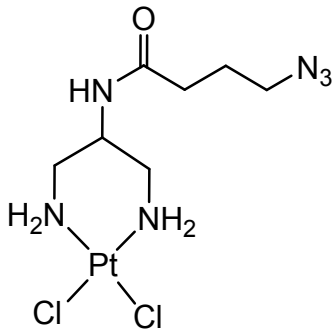
26

Recently, there has been some controversy regarding the mechanisms of action of the three FDA-approved platinum drugs cisplatin (**27**), carboplatin (**28**), and oxaliplatin (**29**). These compounds are known to act as DNA-damaging agents based on their abilities to form covalent adducts. However, recent studies have shown that ER stress can result from treatment with these drugs.[162–166] For instance, cisplatin can induce apoptosis even in anucleated cytoplasts, cells lacking a nucleus, where DNA damage-mediated cell death cannot occur.[162] The authors of this study showed using cell-permeable Ca^{2+} chelators that in these cells apoptosis induction via cisplatin was dependent on cytosolic Ca^{2+} accumulation. Cisplatin also induced upregulation of BiP and the Ca^{2+}-dependent protease calpain in these cells, confirming the role of ER stress and Ca^{2+} trafficking in apoptosis induction by cisplatin. These results support a growing body of literature indicating that platinum drugs induce ER stress in addition to DNA damage.[167]



27 **28** **29**

In a more recent study, pull-down methods were used to identify the protein targets of platinum anticancer agents in yeast cells.[164] Azidoplatin (**30**), a model for conventional DNA-binding platinum anticancer agents, contains an azide functional group for carrying out pulldown experiments via click chemistry. This compound was used to identify potential molecular targets of cisplatin-like Pt(II) compounds, as shown in **Fig. 7**. After treating yeast cells with **30**, click chemistry and biotin-streptavidin pull down was used to isolate covalent Pt-protein complexes. A large number of the proteins identified were related to ER stress, whereas relatively few proteins were related to DNA damage response. In particular, components of the ubiquitin-proteasome system and the protein-folding chaperone PDI were detected. Isolated PDI was used to confirm that compound **30** makes covalent adducts with this protein, leading to its inhibition. The authors also confirmed that both **30** and cisplatin induce the UPR pathway and ER stress in yeast. supporting the theory that ER protein binding is responsible for Pt-induced cell death. Further experiments are needed to elucidate whether these pathways are also operative in human cells.



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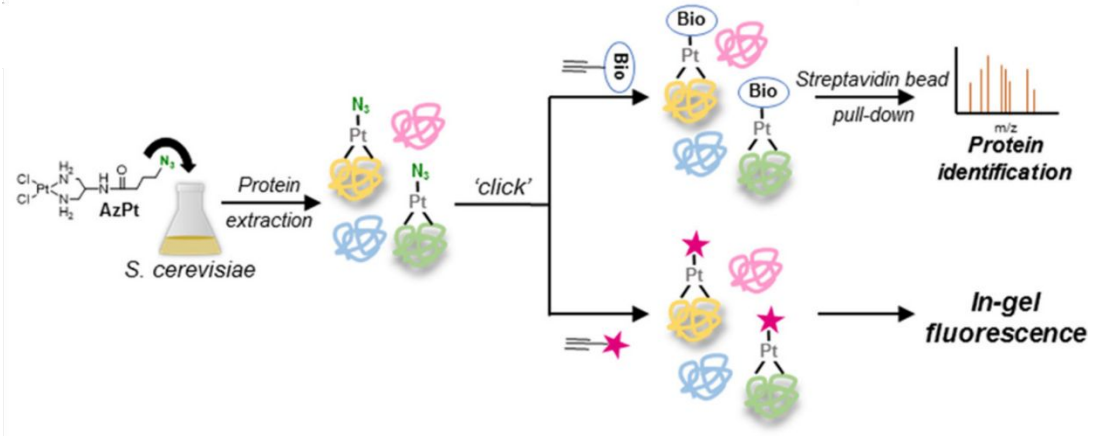
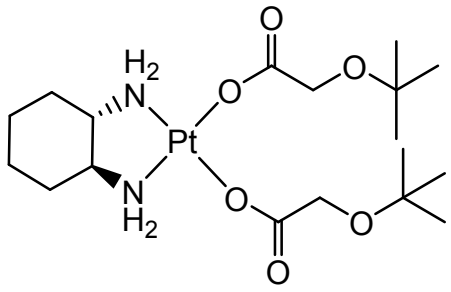


Fig. 7. Schematic representation of pull-down assays performed using **30**, abbreviated as AzPt in this figure, to detect protein targets of Pt(II) agents. Adapted from reference 164 with permission from the American Chemical Society, copyright [2017].

ER stress and UPR induction have also been found to occur following oxaliplatin treatment. In one study, the administration of oxaliplatin gave rise to elevated ROS levels that proceeded to activate the ER stress response, which led initially to cytoprotective autophagy followed by eventual apoptosis.[163] The knockdown of CHOP with siRNA decreased the cytotoxicity of oxaliplatin, supporting the role of ER stress in apoptosis induction. The antioxidant NAC also protected cells from oxaliplatin toxicity. The interpretation of this experiment, however, is challenging because thiols, like NAC, are known to directly bind to and deactivate Pt(II) compounds in a manner that is

independent of ROS.[168,169] Another study also investigated the role of ER stress in the anticancer activity of oxaliplatin. The authors also show that ER stress mediates apoptosis induction by oxaliplatin. Resistance to oxaliplatin was conferred by overexpression of the multi-drug transporter ABCG2, which decreases the ER stress response.[165] The idea that oxaliplatin may not have DNA as a primary target is also supported by recent work using an RNAi approach to identify the cell damage profile of oxaliplatin.[170] The authors found that, rather than damaging DNA, oxaliplatin may instead target ribosome biogenesis.

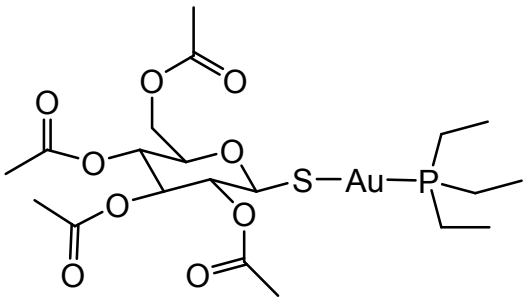
A structural analogue of oxaliplatin, compound **31**, was also recently shown to exhibit potent in vitro and in vivo anticancer activity.[171] This compound induces mitochondrial damage and ROS generation, leading to Ca^{2+} release from the ER. The resulting cytosolic Ca^{2+} overload triggers apoptosis via the intrinsic mitochondrial pathway. This compound activates several ER stress markers, including upregulation of BiP, phosphorylation of eIF2α, and expression of CHOP. The role of Ca^{2+} in cell death induction was confirmed by the simultaneous upregulation of IP3R and downregulation of ERp44, essential Ca^{2+} regulatory proteins associated with the ER. The activity of **31** decreased when CHOP was silenced with siRNA, confirming the role of ER stress in cell death initiation. The compound also significantly inhibited tumor growth in vivo with minimal side effects. Importantly, Western blot analysis confirmed that ER stress was responsible the in vivo antitumor activity of **31**.



31

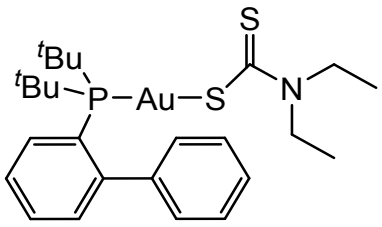
4.8 Gold

Several gold complexes exhibit ER stress-mediated anticancer activity, including the well-known rheumatoid arthritis drug auranofin (**32**), which has also been evaluated as a potential anticancer agent.[172,173] Compound **32** triggers multiple ER stress markers in cancer cells, including XBP1 splicing, eIF2α phosphorylation, and expression of CHOP.[174] The underlying cause of ER stress induced by **32** likely arises from ROS generation that results in the accumulation of misfolded proteins. This hypothesis is supported by the observation of increased levels of polyubiquitinated proteins and folding chaperones like the heat shock proteins. It has also been shown that **32** directly inhibits proteasomal deubiquitinases, a property that may be responsible for the in vivo and in vitro detection of excess polyubiquitinated proteins.[175,176] **32** and other gold complexes also inhibit thioredoxin reductase (TRX), a selenium-containing enzyme responsible for redox homeostasis.[177–180] TRX inhibition disrupts disulfide formation in the ER, further contributing to ER stress.[181]



32

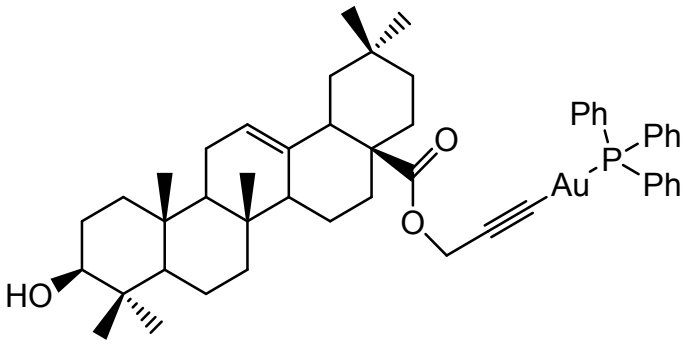
There has recently been a report of Au(I) complexes containing both phosphine and DTC ligands that trigger ER stress with nanomolar potency.[182] The lead complex, **33**, induces ROS-mediated ER stress, leading to the expression of calreticulin on the surface of treated cells, which is a key indicator of ICD. ER stress induction was confirmed by the detection of BIP upregulation as well as phosphorylation of PERK and eIF2α. The complex was active against both wild type and cisplatin-resistant A2780 ovarian cancer cells, indicating that it may circumvent platinum resistance. Furthermore, apoptosis induction by **33** did not affect p53 expression levels, indicating that this complex may induce p53-independent apoptosis. Collectively, these results indicate that these compounds are a promising class of ER stress-inducing anticancer agents.



33

Another family of Au complexes conjugated to oleanolic acid such as **34** has been shown to induce ER stress-mediated apoptosis.[183] Like auranofin, **34** inhibits TRX,

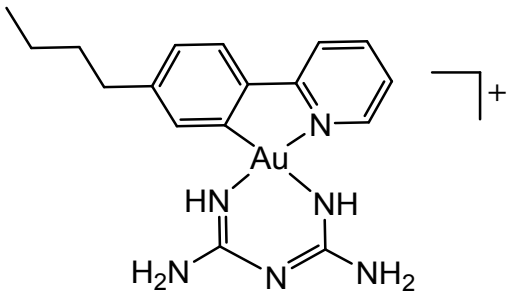
leading to increased ROS production and eventually apoptosis. ER stress induction was confirmed via Western blot analysis, for compound treatment caused increased levels of ATF4, BiP, calnexin, PERK, and CHOP. Apoptosis triggered by **34** could be significantly inhibited by treatment with salubrinal, indicating that activation of the eIF2α pathway is cytoprotective in this case.



34

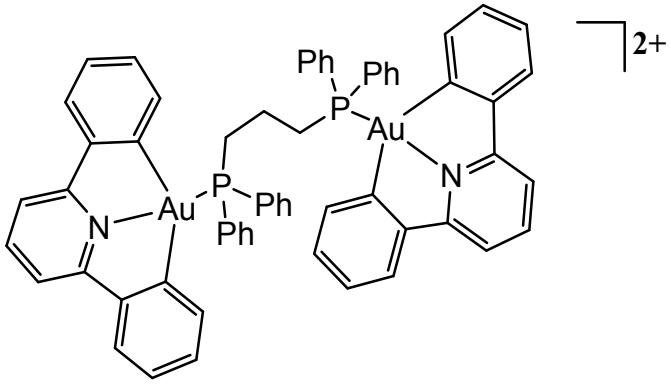
Although there are not many Au(I) complexes reported to induce ER stress, several anticancer complexes of Au(III) target the ER.[184–187] For example, the cyclometalated Au(III) complex **35** induces cancer cell death and prevents angiogenesis by causing ER stress.[184] Confocal fluorescence microscopy experiments of cells treated with this compound revealed ER swelling. Furthermore, upregulation of BiP and CHOP, as well as eIF2α phosphorylation, caused by **35** was confirmed by Western blot. Unlike other ER stress inducers discussed above, the activity of compound **35** was not diminished in the presence of caspase inhibitors, indicating that it does not trigger caspase-dependent apoptosis. Instead, the translation inhibitor cycloheximide was

shown to decrease the cytotoxic effects of **35**, suggesting that paraptosis is operational.



35

The dinuclear compound **36** is another example of an ER stress-inducing Au(III) complex.[185] Compound **36** is extremely potent in vitro, with IC_{50} values in the low-nanomolar range in a variety of cancer types. Western blot analyses showed BiP upregulation, hyperphosphorylation of eIF2α, and CHOP expression in response to this compound, confirming that it induces ER stress. The cause of ER stress triggered by **36** was attributed to the inhibition of TRX. Assays using both purified enzyme and within living cells confirmed that **36** is a potent TRX inhibitor. Compound **36** was also tested for in vivo anticancer activity in mice bearing hepatocellular carcinoma cancer xenografts. The results of these studies (**Fig. 8**) showed that this compound is more effective at inhibiting tumor growth than both cisplatin and doxorubicin. These results further support ER targeting as a strategy for the development of anticancer agents.



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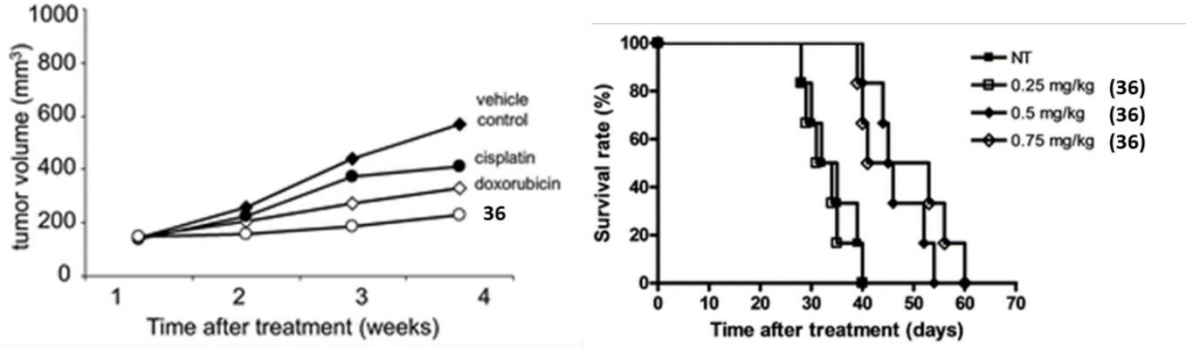
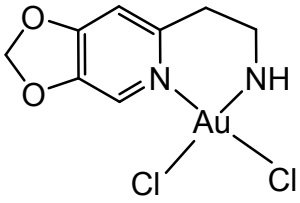


Fig. 8. (Left) Tumor volume versus treatment time for mice bearing hepatocellular carcinoma cancer xenografts. Mice were treated with **36** and other control compounds twice weekly. (Right) Mouse survival rate versus treatment time for mice bearing orthotopic hepatocellular carcinoma tumors. Adapted from reference 185 with permission from the Royal Chemical Society, copyright [2013].

Compound **37** is another example of a Au(III) complex that has potent in vitro and in vivo anticancer activity.[186] Cellular uptake and organelle fractionation experiments revealed that **37** localizes preferentially to the mitochondria. Furthermore, mitochondrial ROS induction was confirmed via the DCF-DA assay. The cytotoxic effects of these mitochondrial ROS were validated by co-treatment with a mitochondria-specific superoxide scavenger, which decreased the potency of **37**. This mitochondrial damage subsequently engenders ER stress, which was evidenced by phosphorylation of PERK and eIF2α and CHOP expression, and ultimately apoptosis. Compound **37**

was also evaluated in vivo. This compound exhibited striking in vivo activity, with greater efficacy than cisplatin in mice bearing A549 lung cancer xenografts (**Fig. 9**).



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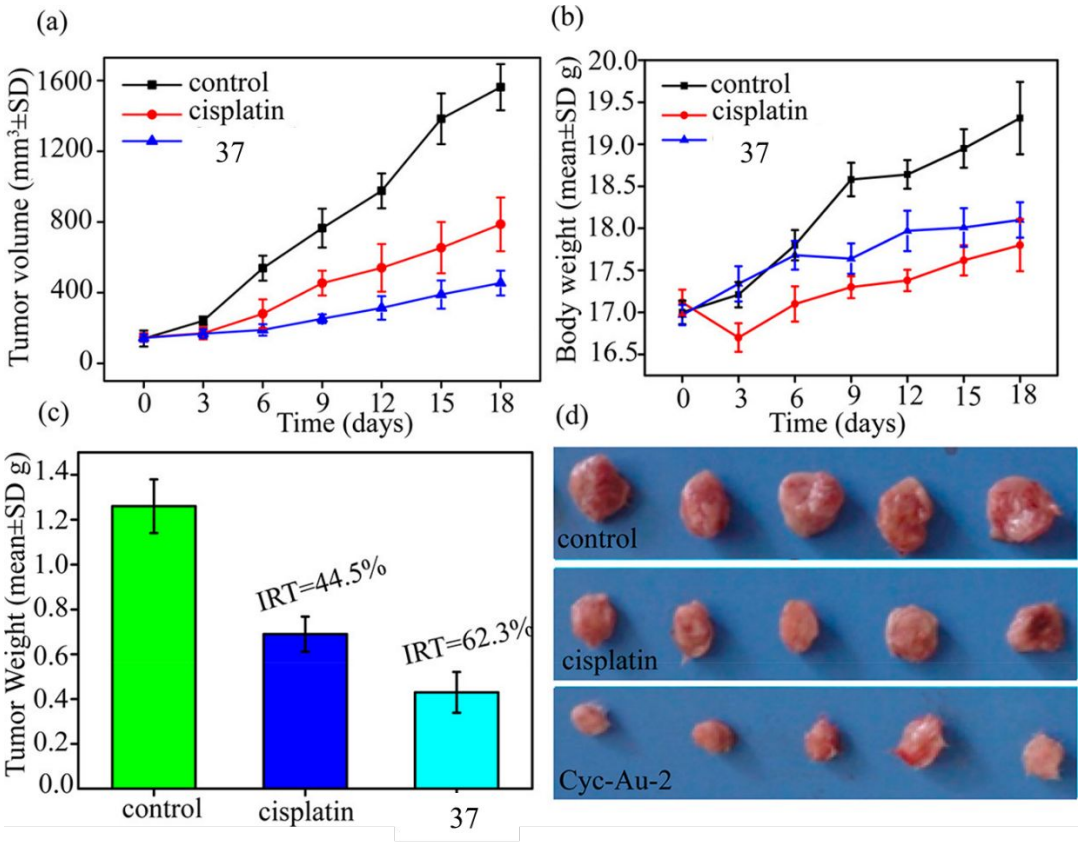
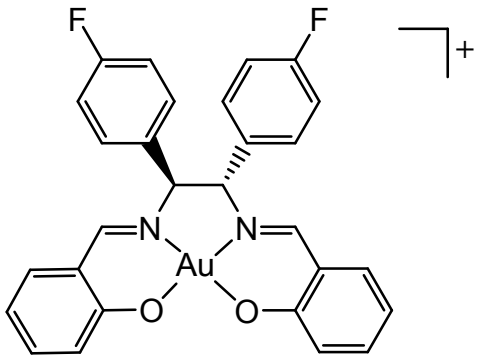


Fig. 9. In vivo activity of **37**. (a) Tumor volume versus treatment time for mice bearing A549 lung cancer xenografts treated with **37**, cisplatin, or the vehicle control. (b) Mouse body weight versus treatment time for mice. (c) Tumor weights for mice treated with **37** or cisplatin at the end of the study. (d) Representative pictures of excised tumors from these experiments. Cyc-Au-2 in this figure corresponds to **37**. Adapted from reference 186 with permission from the American Chemical Society, copyright [2018].

A recent study also reports a Au(III) complex (**38**) with powerful anticancer activity related to ER stress induction.[187] Notably, the complex reacts quickly with biological thiols such as glutathione and NAC, indicating that it may be reduced rapidly to Au(I) in the cellular environment. Like many other Au complexes, **38** inhibits TRX, leading to increased ROS in the cell leading to mitochondrial dysfunction and ER stress-mediated apoptosis. Treatment with **38** caused increased expression of ER chaperones CHOP and calnexin, confirming ER stress induction. Furthermore, administration of **38** was cytoprotective in mice chronically dosed with the liver-damaging agent CCl_4, indicating that this compound may have useful clinical applications in cancer prevention as well as therapy.

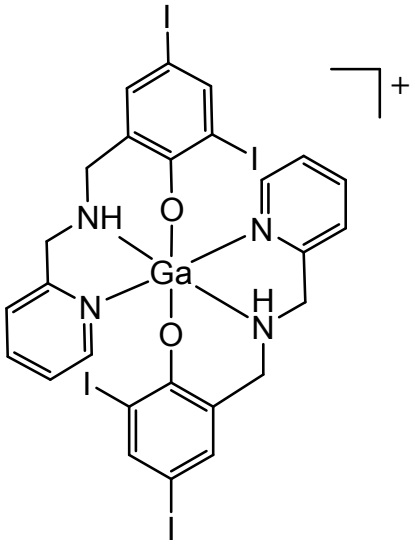


38

4.9 p- and f-Block Compounds

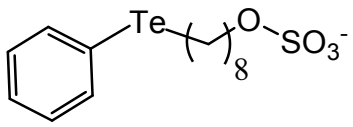
Another important family of metal-based proteasome inhibitors are Ga^{3+} complexes.[188,189] In one study, gallium complexes bearing pyridine/polyphenol ligands like compound **39** exhibited powerful anticancer activity in vivo toward prostate cancer xenografts, and the complexes were not cross-resistant with cisplatin.[188] Proteasome inhibition by the lead compound was confirmed both in vitro and in vivo. Although it is

likely that this compound induces ER stress via this mechanism, this possibility has not yet been investigated.



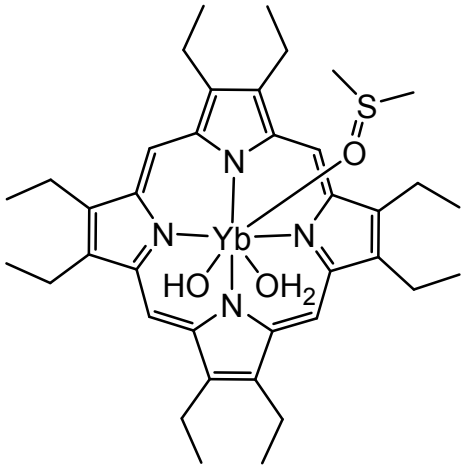
39

Although compounds containing tellurium have scarcely been explored for anticancer activity, there is one report of a tellurium-containing compound (**40**) that induces ER stress.[190] Compound **40** generates ROS, specifically superoxide, leading to ER stress-mediated apoptosis. This compound activates the ER stress marker ATF4 and phosphorylates eIF2α, ultimately triggering expression of CHOP. Surprisingly, the analogous selenium complex is inactive in cancerous cells, highlighting the importance of tellurium in the mechanism of action of this compound. Further structure-activity relationship (SAR) experiments and mechanistic work are needed to determine whether this ER stress induction may be a more general feature of tellurium-containing compounds.



40

Within the f-block, there has been a report describing the ER stress-inducing properties of an Yb(III) porphyrin anticancer agent (**41**).[191] Notably, this complex has sub-micromolar activity against several cancer cell lines. It also induces apoptosis by activating ER stress pathways. ER stress in response to **41** was confirmed via the detection of increased CHOP expression, eIF2α phosphorylation, and ER swelling. This ER stress leads to mitochondrial swelling and dysfunction. Although the specific cause of ER stress was not conclusively determined, this compound was found to inhibit the ubiquitin-proteasome system, which provides a likely hypothesis for its observed activity. Lastly, the effect of **41** on the gene expression profile of HeLa cells was investigated. These studies revealed that **41** causes changes in gene expression that were very similar to those found for the established ER stress-inducing agents thapsigargin and gossypol. This study highlights how relatively simple metal-containing compounds can have biological properties that match those of more complex organic natural products. Furthermore, compound **41** is a rare example of a lanthanide-containing anticancer agent. Given the chemical similarity of the lanthanide ions, further studies to probe the anticancer and ER stress-inducing properties of other lanthanide porphyrin complexes is warranted.

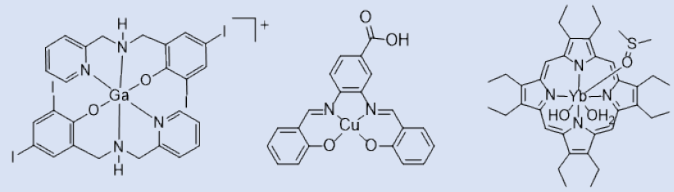
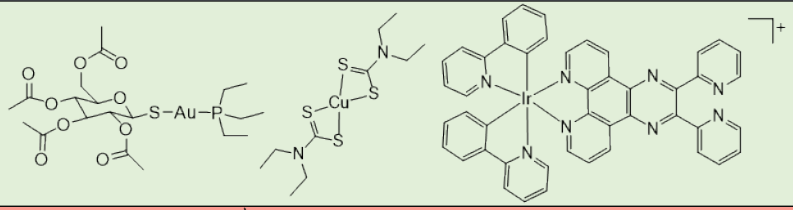
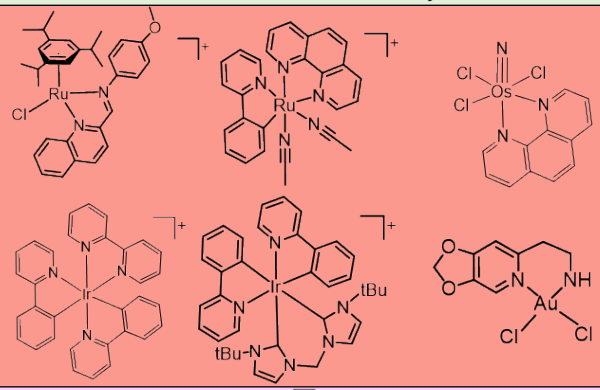
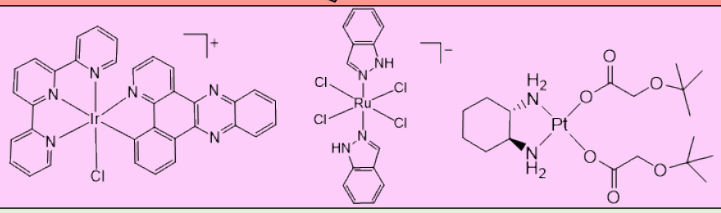
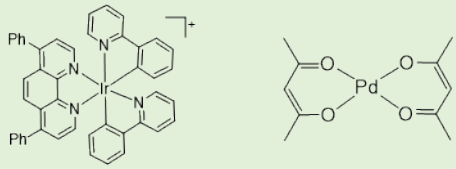


41

5. Trends in and Opportunities for Metal Anticancer Agents Targeting the ER

As summarized here, a diverse range of metal complexes have been demonstrated to trigger ER stress-mediated anticancer activity. To date, dozens of different metal complexes have been shown to possess this type of biological activity. The means by which these complexes cause ER stress are equally diverse; among many different possible mechanisms, these complexes can act as photosensitizers, proteasome inhibitors, modulators of Ca^{2+} trafficking, and inhibitors of essential enzymes for ER homeostasis. Despite the wide variance of ER stress induction mechanisms, some trends with respect to ligand types and isostructural compounds have become apparent. A broad overview of the types of ER stress induction, relevant metal complexes, detection methods, and an exhaustive list of references within each category is provided in **Table 1**.

Table 1. Phenotypes and Examples of Induction of ER Stress by Metal Complexes

Mode of ER Stress Induction	Representative Compounds Inducing this Phenotype	Assays for Detection	Reports of Induction by Metal Complexes
ERAD Inhibition		• Ubiquitin Western blot[a] • In vitro assays with isolated proteasome • Fluorescent tracking of proteasome substrates	91, 93, 98, 179, 188, 189, 191
ERAD Inhibition and ROS Generation		See relevant assays for ERAD inhibition and ROS generation	94–97, 174–180
ROS Generation		• DCF/DA assay[b] • Co-treatment with NAC • Co-treatment with MitoSox • Confocal microscopy with localized ROS indicators • Nrf2-activation	77–80, 87, 108, 109, 111–121, 125, 144–146, 150–153, 157, 159, 185, 186, 189
Ca^{2+} Disruption and ROS Generation		See relevant assays ROS generation and disruption of Ca^{2+} Trafficking	129–131, 153, 171
Disruption of Ca^{2+} Trafficking		• Measurement of ER Ca^{2+} levels[c] • Co-treatment with Ca^{2} chelators • Quantification of Ca^{2+} chaperones	133, 154

[a]For reviews on detection of proteasome inhibition, see references 192 and 193. [b]For detailed experimental description of ROS detection, see references 120, 186, and 194. [c]For experimental protocols to detect Ca^{2+} signaling disruption, see references 131 and 154.

Two ligand scaffolds have appeared frequently among these ER stress-inducing compounds. The first are polypyridyl phenanthroline derivatives. In particular, the DPP ligand appears in multiple ER stress-inducing compounds discussed here, including **10**, **11**, **14**, **17–20**, **23**, and **24**. These compounds show a proclivity for ER localization, especially for those that are mono-cationic. Despite the structural similarities between these complexes, they cause ER stress via different mechanisms, ranging from ROS generation to Ca^{2+} trafficking disruption. Thus, it is likely that these ligands and complex charge are factors that direct these complexes to the ER, whereas more subtle structural and electronic differences affect the activity of the compounds within this organelle. Another common scaffold among many ER-targeting complexes are phenylpyridine ligands, which form cyclometalated complexes. These ligands are found in compounds **10**, **11**, **20–25**, **33**, and **34**. Similarly, hydrophobicity of these ligands combined with their ability to counterbalance the high cationic charge on the metal often leads to hydrophobic, monocationic complexes. In the case of both phenanthroline and phenylpyridine, the resulting metal complexes are often saturated with inert ligands, indicating that the geometry or redox activity of the complexes, rather than their ligand substitution reactivity, may give rise to their anticancer properties.

A separate class of ER stress-inducing metal complexes comprises species with labile coordination sites. In these complexes, ligand substitution will occur readily under biological conditions, enabling the metal center to act as a potent electrophile. Complexes in this class arise from first row transition metals such as copper complexes **5–9**, as well as platinum- and gold-containing compounds **24**, **25**, and **30–38**. When ER stress induction occurs in response to these compounds, common targets are enzymes

in the ubiquitin-proteasome system or redox regulatory enzymes like TRX or PDI. Furthermore, many of these complexes exhibit reduced toxicity in the presence of NAC, which can attenuate their activity via neutralizing ROS or by binding directly to the central metal atom. In the case of these complexes, the reactivity of the metal center is essential for toxicity, and the ligands likely play a supporting role by modulating the metal's reactivity or localization.

Disruption of redox homeostasis is also a common feature of metal-based anticancer agents, particularly those in which the metal can easily access multiple oxidation states, such as first row transition metal or ruthenium complexes. ROS generation by photoexcited metal complexes, for example, is also a means of inducing ER stress. Furthermore, these ROS-generating complexes do not necessarily need to be localized to the ER in order to induce an ER stress phenotype, as mitochondrial damage often leads to ER stress-mediated apoptosis. Thus, researchers seeking to investigate the anticancer phenotypes caused by metal anticancer agents should investigate whether the compounds induce ER stress, even if they do not localize to the ER or if another type of cell damage is identified.

Overall, ER-targeting complexes exhibit remarkable potency, which is reflected by the large number of reports on this topic that describe compounds with nanomolar anticancer activity and significant in vivo tumor-reduction capabilities. These compounds also have generally high selectivity for cancer cells, which arises from the increased ER protein load and higher basal levels of ROS in the tumor microenvironment. The unorthodox mechanisms of action of these compounds allows them to circumvent traditional resistance mechanisms, such as upregulation of DNA-

repair pathways and mutation of p53. The ability of many ER-targeting complexes to induce ICD is also promising, as ICD induction has gained increased recognition as a critical hallmark of successful activity in the clinic.[195–197] Together, these results, most of which are from only the last five years, demonstrate the tremendous potential of metal complexes as ER-targeting anticancer agents.

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Conflict of Interest

The authors declare no competing financial interests.

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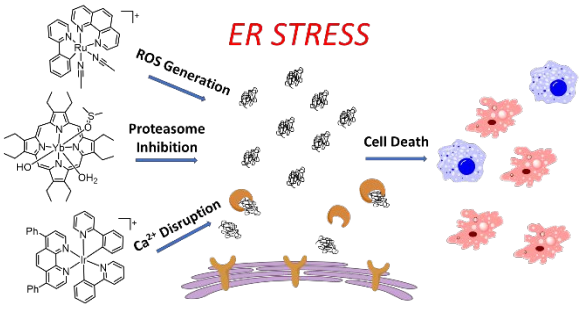

Synopsis: Metal anticancer agents are rapidly emerging as selective, potent therapeutics that exhibit anticancer activity by inducing endoplasmic reticulum stress.