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New half-sandwich ruthenium(II) complexes as proteosynthesis inhibitors in cancer cells

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Half-sandwich ruthenium(II) complexes [(η^6-*p*-cymene)Ru(C^N)-(X)]$^{0/+}$ (X = Cl, py or 4-NMe$_2$-py) containing a cyclometalated 2-ppy or 1-ppz with a non-coordinated CHO group as a handle for further functionalization have been synthesized to achieve selective cytotoxicity to cancer cells, the more potent compounds acting as proteosynthesis inhibitors; this is a new mode of action for half-sandwich metal complexes.

Due to their high growth rate, cancer cells are exposed to a constant demand for newly synthesized proteins, which are required for proliferation.[1] Translation, one of the most energy-consuming activities within the cell, is the central regulator process that permits gene expression and the overproduction of the translation apparatus is commonly associated with tumorigenesis.[2] The prospect of using eukaryotic translation as a chemotherapeutic target is attractive since elevated protein synthesis rates and the increased ribosome function represent characteristic hallmarks of cancer cells.[3] In fact, FDA-approved omacetaxine, a translation inhibitor, has entered clinic trials for chronic myelogenous leukemia patients who are not responding to tyrosine kinase inhibitors.[4]

In the field of anticancer metallodrugs, cisplatin (CDDP) and other platinum-based compounds are still widely used as chemotherapeutic agents in clinic, the mode of action (MoA) of CDDP involving DNA crosslinking adducts and apoptotic induction.[5] However, drug resistance usually develops, either acquired through prolonged treatment with suboptimal doses or as inherent phenomena.[6] There is an urgent need for new chemotherapeutic candidates that cause tumor cell death through novel MoAs. The Ru(III) complex NKP-1339 is undergoing clinical trials for cancer treatment[7] and organometallic compounds have been studied as promising pharmacological tools.[8] Rational design of improved systems requires the identification of the biological target. Particularly, Ru^{II}(η^6-arene) and Ru^{II} polypyridyl complexes have been investigated for their tunability and novel MoAs, with Dyson's RAPTA-C and RM175 by the Sadler group having emerged as prime candidates in preclinical investigations.[9] On the other hand, over the last years, Ru(II) arene complexes bearing C,N-coordinating ligand systems have attracted considerable attention due to their promising anticancer activities.[10]

Very recently, three novel series of cationic octahedral metal complexes have been reported to be able to interfere with protein synthesis, namely one of the type [Ru(N^O)(N^N)$_2$]$^+$, containing substituted hydroxyquinolines reported by Glazer,[11] and two in-house families of compounds [Ru(C^N)(phen)$_2$]$^+$ and [Ir(C^N)$_2$(phen)]$^+$ (C^N = C,N chelating ligand).[12] These complexes have shown a markedly higher potency than conventional inhibitors of DNA translation; this is a new MoA for metal complexes, that has been found recently also for oxaliplatin.[13] Very Interestingly, this mechanism has not hitherto been described for half-sandwich metal compounds.

Herein we report a new series of selective cytotoxic Ru(II) arene complexes of the type [(η^6-*p*-cym)Ru(C^N)(X)]$^{0/+}$ (*p*-cym = *para*-cymene; C^N = ppy-CHO, ppz-CHO; X = Cl, py or 4-NMe$_2$-py) containing a C^N ligand with a non-coordinated CHO group as a handle for further functionalization. Replacement of Cl by py or 4-NMe$_2$-py could modulate the anticancer potency of Ru(II) complexes.[14] Those complexes with the electron-donating group NMe$_2$ substituent of the py act as proteosynthesis inhibitors.

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Ru(II) arene chlorido complexes **1a** and **1b** (Chart 1) were obtained by reaction of $[(\eta^6\text{-}p\text{-cym})RuCl_2]_2$ with two equivalents of the corresponding HC^N proligand (Scheme S1 in the ESI†). Compounds **2a,b** and **3a,b** were synthesized from **1a** or **1b**, respectively, as shown in Schemes S2 and S3 (ESI†). All the new complexes were fully characterized using ^1H and ^{13}C NMR spectroscopy (Fig. S1–S24 in the ESI†) as well as high-resolution (HR)-ESI-MS spectrometry. The complexes were detected as their $[M\text{-}X/X\text{-}H]^+$ ions in positive ion modes with the expected isotopic distribution pattern. Their purity was confirmed by elemental analysis (all complexes) and also by HPLC-MS (**2a**-**3b**). In addition, the HPLC chromatograms (Fig. S25-S36, ESI†) of **2a**-**3b** in RPMI culture medium remained unaltered after 24 h, with the ESI-MS spectra displaying the $[M - py\ (or\ NMe_2py)]^+$ peaks. In addition, as shown by NMR, **3a** and **3b** are stable at 37 °C at least for 24 h in the presence of N-acetyl-L-cysteine, glutathione and reduced nicotinamide adenine dinucleotide (Fig. S37-S42). Reversible hydrolysis of the Ru–Cl bond is relatively rapid in MeOD-d_4/D_2O mixtures of **1a** or **1b**, as observed by ^1H NMR (Fig. S43 and S44, ESI†). No hydrolysis was observed for **2a** and **3a** (Fig. S45 and S46, ESI†).

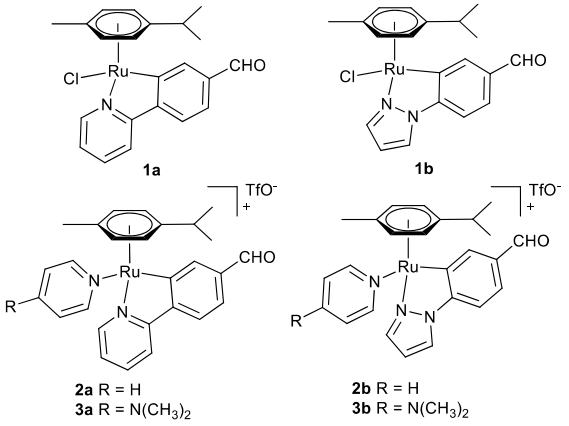

Chart 1 Structures of compounds included in this study.

The molecular structures of **1a**, **1b**, **2a** and **3a** are shown in Fig. S47 (ESI†). The solid-state structures confirmed their three-legged "piano stool" geometry in which the *p*-cym ring and C^N ligands are coordinated to the Ru centre via π-bonded η^6 and a bidentate chelate coordination modes (κ^2-C,N), respectively. Their $Ru\text{–}cym_{centroid}$ distances ranged from 1.701 to 1.719 Å, which were in the range typical for Ru arene complexes,[10] as occurred also with the Ru–Cl bond lengths found for **1a** and **1b**. The Ru–N(py/NMe_2py) distances observed are 2.1143(13) and 2.1224(15) Å for **2a** and **3a**, respectively. Crystallographic data and intermolecular interactions are shown in Tables S1-S6 and Fig. S48 (ESI†).

The interaction of **1a-3b** with HSA was studied by means of competition fluorescence spectroscopy. The values of K_{SV}, obtained from the lineal plots of F^0/F *vs. [M],* indicated that **3a** and **3b** were capable of quenching tryptophan fluorescence more strongly than **1a,b** and **2a,b** (Table S7 and Fig. S49-S52, ESI†).

The cytotoxicity of the six Ru compounds and CDDP was evaluated in several human cancer cell lines, including cells of the epithelial ovarian carcinoma A2780, CDDP-resistant ovarian cancer A2780cisR, breast cancer MCF-7 and also in the non-tumorigenic BGM and CHO cells. **3a** and **3b** exhibited potent activities, with IC_{50} values in the low micromolar range, in all cancer cells (see Table 1). The IC_{50} values for **1a**, **1b**, **2a** and **2b** were significantly higher (8.4 - 26.9 μM). So, it is evident that the effect of the 4-NMe_2-py ligand impacts on the antiproliferative effects of both **3a** and **3b** (py and 4-NMe_2-py free ligands were found to be inactive). All the compounds (except **1b**) were found to be inactive against non-cancerous BGM renal cells with an IC_{50}> 80 μM whereas toxicity against normal ovary CHO cells showed that the Ru complexes achieved high selectivity towards cancer cells and an improved potency against CDDP-resistant cancer cells, the resistance factors found for Ru complexes being below 1.4, whereas CDDP showed a remarkably higher value (23.5). This suggest that the MoA of the present complexes bypasses the molecular mechanisms underlying acquired resistance of CDDP in both A2780cisR and inherently-resistant MCF-7 cells.

The cellular concentrations of metals in A2780 cells having been exposed to **1a**-**3b** or CDDP for 24 h were determined by ICP-MS in order to investigate the relationship between cellular uptake and cytotoxicity. The results (Table S8, ESI†) indicate that the cellular uptakes of the most active compounds **3a** and **3b** are both similar, and approximately 4-fold higher than those of **2a** and **2b**, and more than 10-fold higher than those of their parents **1a** and **1b.**

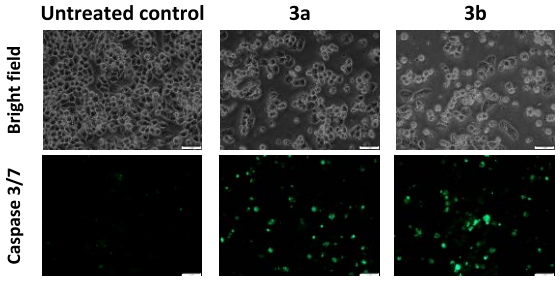

Fig. 1. Caspase 3/7 activation in A2780 cells after 24 h incubation with 10 μM of **3a** and **3b**.

First, the mitochondrial activity was examined as depolarization of the mitochondrial membrane potential (MMP) leads to activation of apoptotic cell death. We found that **3a** and **3b** caused a dose-dependent loss in MMP, as revealed by a decreased of rhodamine-123 fluorescence intensity in A280 cells (Fig. S53, ESI†), suggesting that perturbation of MMP by Ru complexes might trigger an apoptotic cell death. Next, the induction of apoptosis in A2780 cells exposed to **3a** and **3b** was investigated by flow cytometry using Annexin-V/propidium iodide (PI) dual-staining. Both **3a** and **3b** treatment at 10 μM considerably increased the percentage of early and late apoptosis after 24 h (Fig. S54, ESI†). To further confirm the effect of Ru complexes on apoptosis, the activation of key effector caspases on A2780 was determined using a caspase 3/7 detection reagent. A significant elevation of the active caspases was evidenced at 12 h drug-exposure and the activation continued until 48 h time point (Fig. 1 and Fig S55, ESI†).

As observed, the treatment with **3a** and **3b** displayed an activation of caspases 3 and 7, which indicated that apoptosis is induced via caspase-dependent death. On the other hand, the effect of Ru compounds on intracellular ROS levels after 2 h drug exposure was studied using 2'7'-dichlorodihydrofluorescein diacetate (DCFH-DA) staining. As shown in Fig. S56 (ESI†), both **3a** and **3b** induced no generation of ROS compared to untreated cells.

Impedance-based time-dependent cell-response profiling (TCRP) was used as the predictive method for the mechanism of biological action of the investigated Ru compounds. It was previously shown

that TCRP curves could be used to partially identify the MoA of small molecules.[12] The TCRP analysis showed (Fig. S57, ESI†) that the action of **3a** is characterized by an increment in cell population followed by a concentration-dependent decrease in the cell index compared to the control, indicating a notorious cytotoxic response and revealing a similar profile as [Ru(ppy-CHO)(phen)$_2$][PF$_6$], an in-house Ru-based proteosynthesis inhibitor.[12a] This result led us to investigate the inhibition of protein translation as the possible MoA of **3a** and **3b**.

Table 1. IC$_{50}$ values [μM] for **1a-3b** and CDDP after 48 h.[a]

Complexes	A2780	A2780cisR	MCF-7	BGM	CHO
1a	16.5 ± 0.5	12.8 ± 0.7 (0.8)	26.9 ± 1.4	> 80 [50.36 % ± 3.8]	37 ± 4
1b	12.8 ± 0.4	11.7 ± 0.8 (0.9)	18.2 ± 0.7	21.9 ± 0.4	13.9 ± 1.4
2a	19.1 ± 0.6	19.5 ± 0.7 (1.0)	8.4 ± 0.1	> 80	> 80
2b	20 ± 2	19.4 ± 1.8 (1.0)	9.1 ± 0.3	> 80	34 ± 4
3a	2.1 ± 0.2	4.6 ± 0.2 (2.2)	1.93 ± 0.06	> 80 [30.2 % ± 1.7]	14.6 ± 0.3
3b	3.3 ± 0.1	4.5 ± 0.1 (1.4)	2.88 ± 0.05	> 80	15.9 ± 0.4
CDDP	1.89 ± 0.05	44.5 ±0.5 (23.5)	36.4 ± 0.8	16.6 ± 0.1	8.6 ± 0.4

[a] Cell viability was determined by the MTT assay after 48 h treatment and IC$_{50}$ values were calculated as described in the Experimental Section. Each value represents the mean SD of three independent experiments. Resistance factors are given in parentheses. Resistance factor, defined as IC$_{50}$(resistant A2780cisR)/IC$_{50}$(sensitive A2780), is given in parentheses. The term ">80" indicates that no IC$_{50}$ value was reached up to 80 μM. If significant difference to the control at 80 μM is found, the respective percentage of inhibitory concentration value is given between brackets.

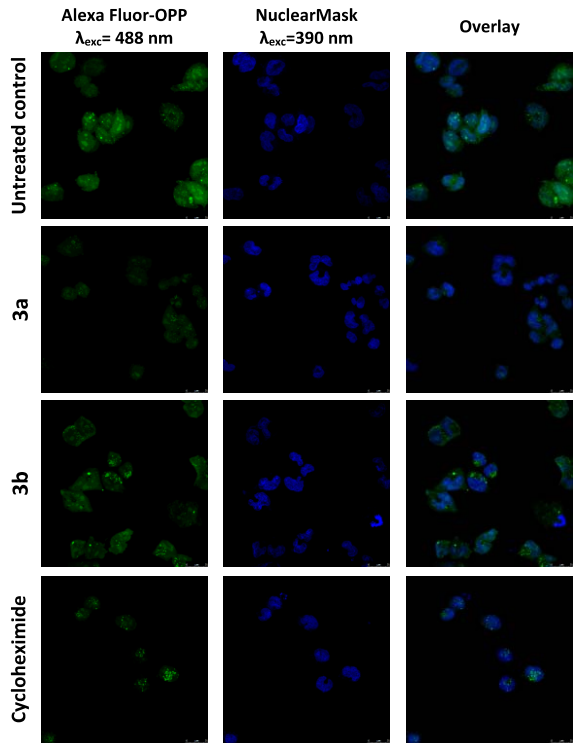

Fig. 2. Detection of protein synthesis using Click-iT®Alexa Fluor-OPP on A2780 cells after 6 h treatment with **3a** (20 μM), **3b** (20 μM) or CHX (500 μM) by confocal mycroscopy. NuclearMask™ was used for nuclear co-staining. Scale bar = 25 μM.

In order to verify this, newly nascent proteins were studied by using the click-iT Plus O-propargyl-puromycin (OPP) Alexa Fluor 488 protein synthesis assay kit (Invitrogen™)[15] in A2780 cells. As shown in Fig. 2, **3a** and **3b** interfered with translation process, cycloheximide (CHX) being used as positive control. In addition, fluorescence measurements indicated that both **3a** and **3b** are effectively capable of inhibiting up to 40% of the total protein synthesis after 6 h treatment (see Fig. S58, ESI†). This is consistent with the subsequent activation of caspases as protein synthesis blockade leads to caspase-dependent apoptosis (*vide supra*). The integrated stress response (ISR) constitutes a complex signaling pathway which involves the phosphorylation of the translation initiation factor eIF2αs, that ultimately leads to a decrease in global protein synthesis.[16] By co-treating A2780 cells with **3a** or **3b** and an ISR inhibitor ISRIB, it was demonstrated that translation inhibition occurred in an eIF2α-independent manner (Fig. 3A). Co-treatment with ISRIB did not restore translation to untreated control levels. Altogether, these results suggest that protein synthesis inhibition by **3a** or **3b** is not mediated by the ISR activation.

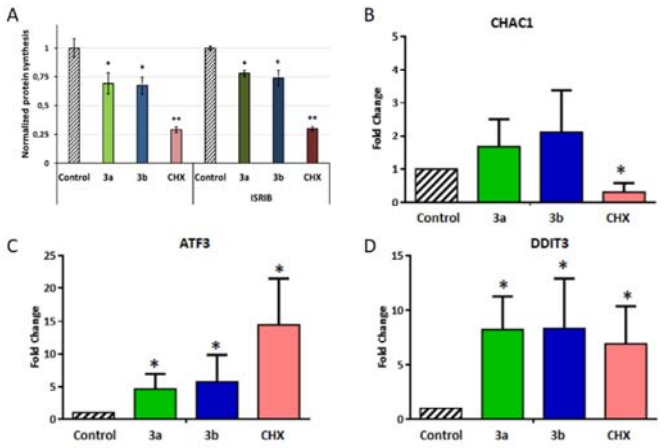

Fig. 3. Normalized translation levels measured by OPP incorporation in nascent proteins (A) and mRNA expression of CHAC1 (B), ATF3 (C) and DDIT3 (D) after 6 h treatment with either Ru complexes (20 μM) or CHX (500 μM) alone or in combination with integrated stress response inhibitor ISRIB (200 nM). Data are representative of at least three independent experiments and represented as mean ± SD. *p < 0.05 (unpaired *t*-test).

To further characterize the MoA of Ru complexes, A2780 treated with tested compounds or CDDP were analyzed by flow cytometry using PI staining. After incubation with 4 μM **3a** or **3b** for 24 h, the percentage of cells in G$_0$/G$_1$ phase increased (Fig. S59, ESI†) from 34.8 to 48.5 and 44.4, respectively. Exposure of Ru compounds could result in a dysregulation of cell cycle distribution possibly due to the inhibition of protein synthesis, a process that occurs in G$_1$ phase which would eventually lead to a G$_0$/G$_1$ arrest. Protein synthesis inhibition could be the result of activation of endoplasmic reticulum (ER) stress. We estimated by qPCR the mRNA levels of 3 genes induced by ER stress and that participate in the pro-apoptotic effects of the ATF4-ATF3-DDIT3/CHOP/CHAC1 cascade (Fig. S60, S61, ESI†). ATF3, DDIT3 were significantly increased but not CHAC1 perhaps due to CHAC1 is downstream of the ATF4-ATF3-CHOP pathway and 6 h incubation was insufficient to rise CHAC1 levels (Fig. 3B, 3C and 3D).

As a result, the mechanism of protein inhibition and cell demise could be then related to induction of ER stress.

In summary, a series of new half-sandwich Ru(II) complexes [(η^6-*p*-cym)Ru(C^N)(X)]$^{0/+}$ (X = Cl, py or 4-NMe_2-py) containing a modified 2-ppy/1-ppz ligand with a non-coordinated CHO group as a handle for further functionalization were designed and synthesized. The 4-NMe_2-py derivatives **3a** and **3b** were found to exert high anticancer activity *in vitro*. These compounds achieved high selectivity towards cancer cells over normal cells, displaying better antitumor activity than CDDP in either acquired or intrinsic CDDP-resistant cell lines. Flow cytometry analysis and fluorescence intensity measurements of nascent protein synthesis by Click-iT®Alexa Fluor-OPP (an alkyne analog of puromycin that is able to react bioorthogonaly with an a fluorogenic azide) in A2780 cancer cells pointed out the inhibition of the translation process at low micromolar concentration and subsequent G1/S arrest as the main MoA for **3a** and **3b**. Moreover, the loss of MMP, the increased Annexin V positive population produced by these compounds and the caspase 3/7 activation confirmed mitochondrial-mediated caspase-dependent apoptosis as a trigger for cell death. Further studies are required to explore the impact of the Ru complexes on the ribosome function and the induction of ER stress in detail.

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Conflicts of interest

There are no conflicts to declare.

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