Defining the Anti-Cancer Activity of Tricarbonyl Rhenium complexes: Induction of G2/M cell cycle arrest and Blockade of Aurora-A Kinase Phosphorylation

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Abstract

Rhenium and ruthenium complexes containing N-heterocylic carbene (NHC) ligands and conjugated to indomethacin were prepared. The anticancer properties were probed against pancreatic cell lines, revealing a remarkable activity of the rhenium fragment as anticancer agent. The ruthenium complexes were found to be inactive against the same pancreatic cancer cell lines, either alone or in conjugation with indomethacin. An in depth biological study revealed the origin of the anticancer properties of the rhenium tricarbonyl fragment, of which a complete elucidation had yet to be achieved. It was found that the rhenium complexes induce cell cycle arrest at the G_2/M phase by inhibiting the phosphorylation of Aurora-A kinase. A preliminary study on the structure-activity relationship on a large family of these complexes revealed that the anticancer properties are mainly associated with the lability of the ancillary ligand, with inert complexes showing limited to no anticancer properties.

Cancer is one of the leading causes of death and, as such, the drive to discover new anti-cancer drugs is an expanding area of research. One class of frontline anti-cancer agents are the platinum-based drugs cisplatin, carboplatin, and oxaliplatin, which are extremely effective at eradicating cancer cells, but suffer from severe side-effects and poor activity against platinum-resistant cancers. Other organometallic and inorganic complexes have been studied extensively as an alternative to organic based drugs.[1-4] In this context, complexes of gold, platinum, and ruthenium have attracted considerable attention and have been shown to be particularly effective, often by targeting mitochondria or DNA.[5-9] Organometallic complexes of rhenium that contain the chemically robust $Re(CO)_3$ fragment have recently shown potential as cytotoxic agents, but they have been far less studied. Furthermore, their mechanism of action has not been established in details yet, which hinders the systematic design and investigation of targeted rhenium complexes as anticancer agents. All the reported cytotoxic rhenium complexes contain bidentate or tridentate ligands of nitrogen, oxygen, and phosphorus donors, a chemical design that seems to be predominantly inspired by the use of these species as luminescent cellular markers.[10] Other rare examples include rhenium complexes bearing cyclopentadienyl ligands.[11]

We and others have been recently interested in the investigation of the photochemical properties of rhenium complexes bound to N-heterocyclic carbene (NHC) ligands.[12-18] Surprisingly, this relatively new class of complexes has never been assessed in a biological context, either as luminescent cellular markers or anticancer agents. We therefore endeavored to evaluate rhenium NHC species against several pancreatic cell lines (one of the most aggressive cancers with a five-year survival rate around 5%) in direct comparison with ruthenium arene complexes, whose structural motif has shown anticancer properties in previous investigations.[19] For this scope, we decided to study the complexes alone or conjugated to the nonsteroidal anti-inflammatory drug (NSAID) indomethacin. This chemical design was inspired by the fact that molecular units comprising a metal fragment and NSAID have been previously reported to enhance anticancer properties.[20-26] Remarkably, it was found that the rhenium complexes were active against pancreatic cell lines, while the ruthenium complexes did not show any significant activity. Furthermore, conjugation to indomethacin did not seem to enhance the anticancer activity, which was traced back exclusively to the presence of the $Re(CO)_3$ fragment. With this study, for the first time, it was possible to elucidate that the anticancer properties of the rhenium tricarbonyl fragment originate from the lability of the monodentate ancillary ligand coordinated to the

rhenium center by inducing G2/M cell cycle arrest and blockade of Aurora-A Kinase phosphorylation.

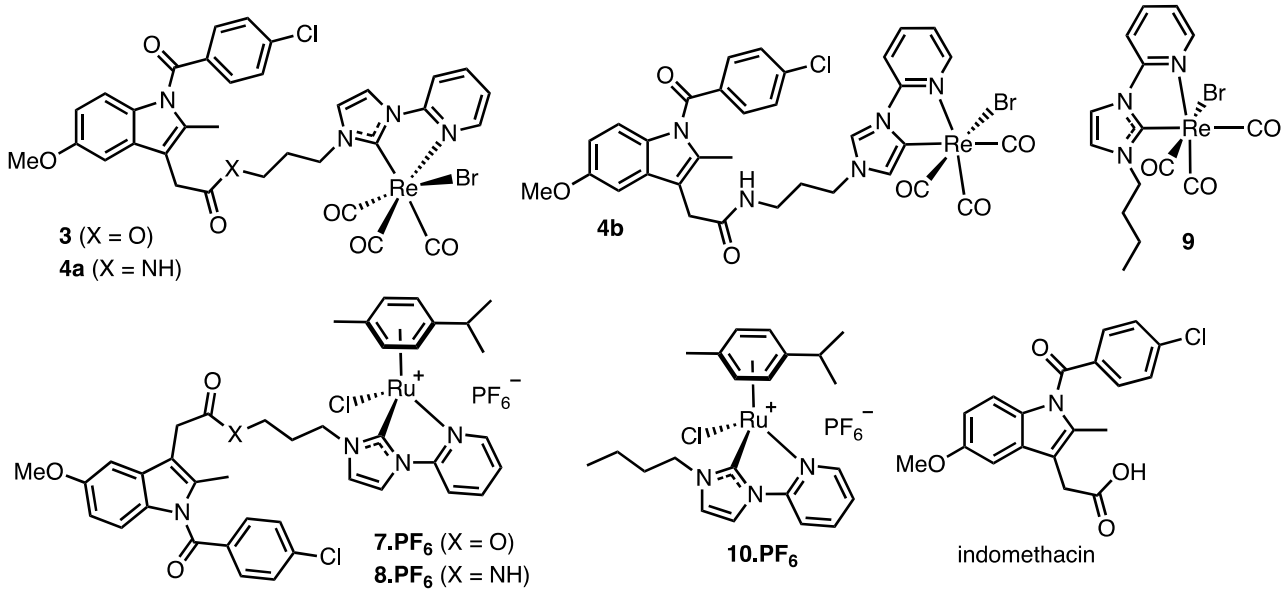

Figure 1. Structures of rhenium and ruthenium NHC complexes.

A detailed description of the synthesis of the complexes shown in Figure 1 is given in the Supporting Information. Briefly, indomethacin-functionalized azolium salts **1.Br** and **2.BPh$_4$** were prepared and then reacted with $Re(CO)_5Br$ to afford complexes **3**, **4a**, **4b**. To the best of our knowledge, this is the first example of an abnormal carbene complex of rhenium. The spectroscopic evidence for complex **4b** containing an abnormal carbene is discussed in the Supporting Information. The X-ray structure of complex **3** can be seen in Figure 2, with additional data found in the Supporting Information. Reaction of **1.Br** and **2.BPh$_4$** with Ag_2O gave complexes **5** and **6**, which were treated with $[Ru(cymene)Cl]_2$ (cymene = 1-methyl-4-(propan-2-yl)benzene) to afford complexes **7.PF$_6$** and **8.PF$_6$**.

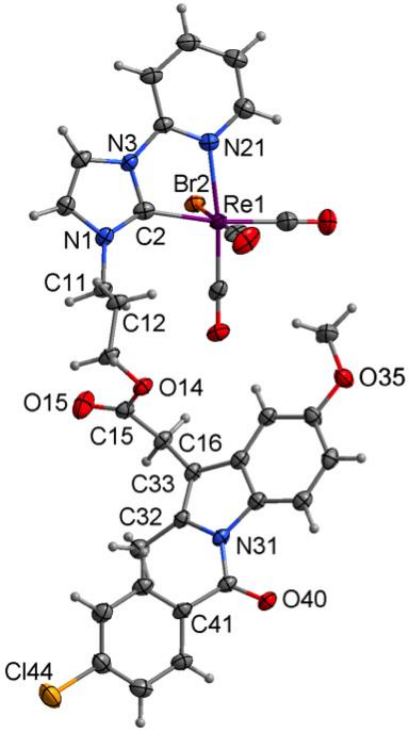

Figure 2. X-Ray structure of complex **3** with anion and disordered components omitted and displacement ellipsoids at the 50% probability level.

The biological activity of the rhenium and ruthenium complexes were tested on a panel of human pancreatic cancer cell lines comprising HPAF-II, ASPC1, and CFPAC. To understand if the metal-indomethacin conjugates possessed dual activity, complexes **9** and **10.PF$_6$** as well as indomethacin alone were tested against the same cells. Strikingly, complex **4b** and indomethacin were ineffective at concentrations up to 10 μM (Figure S11) while the ruthenium complexes were ineffective up to 75 – 100 μM (Figure S12). Contrarily, rhenium complexes **3**, **4a**, and **9** at 10 μM showed almost complete inhibition. To determine the effect of the rhenium compounds on cell growth, these cell lines were treated with increasing concentrations of specific rhenium compounds (Figure S13). We observed that treatment with each inhibitor significantly and dose-dependently reduced the number of cells, as assessed by cell counting after 72 hour incubation in complete media containing 10% FBS, with IC_{50} values for **3**, **4a**, **9**, and carboplatin as control shown in Table 1. The IC_{50} values indicate that the rhenium compounds are remarkably active given the robust nature of these particular pancreatic cancer cell lines, with similar or slightly higher activity than carboplatin. Importantly, while the rhenium compounds almost completely inhibit all pancreatic cancer cell lines at 10 μM, around 20% of all the pancreatic cancer cells in each line are resistant to carboplatin up to 50 μM. Furthermore, since compound **9** was as active, or more

active, than the indomethacin conjugates **3** and **4a**, we can confidently conclude that it is the rhenium complex that is responsible for pancreatic cancer cell inhibition. To the best of our knowledge, this is the first example of rhenium-NHC complexes displaying anti-cancer activity. While there are a number of rhenium complexes of diimine ligands that display moderate to high anti-cancer activity, as recently reviewed by Gasser, only two reports describe their activity against pancreatic cancer, although no explanation of the mechanism was given.[27,28] Interestingly, the active compounds, and in particular **9**, seem to only be effective at higher concentrations (above 10 μM) when tested on healthy human embryonic kidney 293T cells (HEK293T) (Figure S14), indicating a moderate selectivity toward pancreatic cancer cells over healthy cells. To further validate these results, pancreatic cancer cells were treated with **3**, **4a**, and **9** and cell viability assessed by the Alamar blue assay.[29] The three compounds almost completely impair viability of all pancreatic cancer cell lines tested, while being less active against HEK293T (see Figure S15-17). Overall, these results demonstrate the great efficacy of the novel rhenium compounds in blocking proliferation/viability of different pancreatic cancer cells.

Table 1. IC_{50} (μM) values for compounds **3**, **4a**, and **9** against pancreatic cancer cell lines.

Compound	ASPC1	HPAF-II	CFPAC	HEK293T
3	7.9 ± 1.4	6.0 ± 2.0	6.0 ± 1.8	11.8 ± 2.3
4a	9.4 ± 3.5	4.8 ± 0.8	5.4 ± 1.4	8.6 ± 0.3
9	4.0 ± 1.2	5.6 ± 0.6	5.7 ± 2.8	14.8 ± 2.4
Carboplatin	6.8 ± 2.0	8.7 ± 4.3	7.4 ± 1.2	45[a, 30]

[a] Literature data.

The effect of **3**, **4a**, and **9** on the growth rate of the pancreatic cancer cells was determined by the Incucyte Zoom real-time video imaging system. Treatment with **3**, **4a**, and **9** had a significant inhibitory effect on the growth of pancreatic cancer cells in a dose and time dependent manner compared to the vehicle-treated cells. Representative video of ASPC1 control cells and treated with 10 μM **9** are shown (Suppl. video). Moreover, quantification of viable cells after 72 hours of treatment showed that **3**, **4a**, and **9** significantly decreased the percentage of viable pancreatic cancer cells. When pancreatic cancer cells nuclei were probed for active caspase 3 or 7 (#4440 IncuCyte), and cell membrane integrity assessed via Red CytoTox permeabilization (#4632

Incucyte), no major differences were observed in cells treated with **3** and **9** compared to the vehicle control (Figure S18 and S19). In contrast, compound **4a** induced cell apoptosis as confirmed using a different assay (Caspase-Glo® 3/7 Assay System). Taken together, these results provide evidence that the rhenium compounds **3** and **9** do not induce apoptosis or disrupt the cell membrane in pancreatic cancer cells, but act as cytostatic drugs.

Our next objective was to further characterize the cell death process upon **3**, **4a**, and **9** treatment. Since treatment with **3** and **9** did not result in significant increase in caspase-3 activity, we wanted to assess the mechanism of action of rhenium compounds. Microscopic analysis of the pancreatic cancer cells after treatment with **3** and **9** did not reveal morphological changes associated with apoptosis, instead we observed the presence of multinucleated cells. The presence of multinucleated cells suggests cell cycle arrest. Cell cycle analysis after **3**, **4a**, and **9** treatment revealed a significant decrease in the number of cells at G_1 with high percentage of cells that were arrested in G_2/M phase (see Figure S20). Aurora-A plays a crucial role in mitotic entry and G2 checkpoint control. Dysregulation of Aurora-A induces abnormal G2-M transition in mammalian cells leading to chromosome instability and eventually in the development and progression of malignant tumors. In addition, it has been shown that Aurora A is overexpressed in different tumors including pancreatic cancer. Therefore, we tested whether the rhenium compounds were able to inhibit the phosphorylation of Aurora-A in pancreatic cancer cells. As shown in Figure 3, 10 μM of **3**, **4a**, and **9** completely inhibit the phosphorylation of Aurora A. Several studies have shown amplification and overexpression of Aurora-A kinase gene (AURKA) in several cancers. Increasing evidence demonstrate that Aurora-A plays a key role in regulating cell cycle and mitosis, as well as a number of important oncogenic signaling pathways. Therefore, there is an increasing interest in developing novel Aurora-A inhibitors, and some have moved to phase III clinical trials in lymphomas. However, despite the increasing evidence of the role played by Aurora-A in many tumors, there has been a little progress in the clinical development of Aurora A inhibitors in solid tumors. Therefore, it is imperative to identify novel Aurora-A inhibitors to progress to clinical trials. To assess the tumorigenicity of the rhenium compounds in vitro against a three dimensional tumor model, we performed a three-dimensional soft agar colony formation assay. Consistently, treatment of ASPC1, HPAF-II and CFPAC with **9** inhibited anchorage-independent growth as it reduced the number of colonies assessed by soft agar assays (Figure S21).

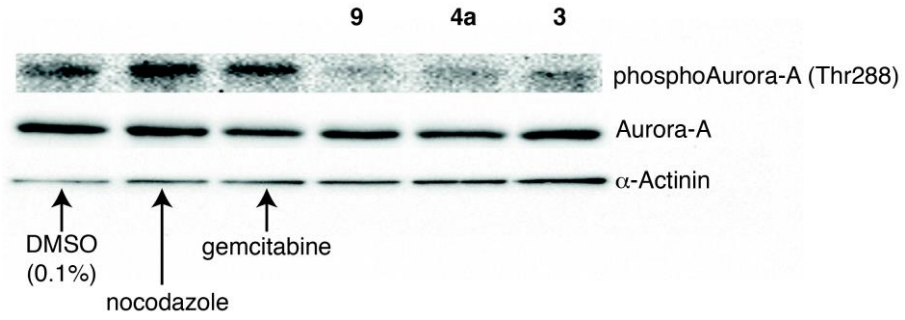

Figure 3. Regulation of Aurora-A kinase activity by Rhenium complexes compared to blank (0.1% DMSO) and positive controls (nocodazole and gemcitabine). Western blot analysis of activated phosphoAurora-A (Threonine 288) in AsPC1 cells treated for 24 hours with **3**, **4a** and **9**. Samples were analyzed for (top to bottom) Aurora-A phosphorylation (Thr-288), total Aurora-A protein (Aurora-A) and α-Actinin. The same blot used for phospho-Aurora-A was stripped and then reprobed with Anti-Aurora-A antibody. Results are representative of three independent experiments.

A preliminary structure-activity relationship (SAR) investigation was conducted by screening 17 complexes of the rhenium NHC family along with the precursor ligand imidazolium salt and the benchmark luminescent rhenium complex *fac*-[$Re(CO)_3$(phen)Cl] (phen=1,10-phenanthroline). The study clearly demonstrates that the anticancer activity originates from the degree of lability of the ancillary ligand, with inert complexes displaying limited to no anticancer activity (see Supplementary Information and Figure S22-24 for a more detailed discussion). Consistently, the two most active rhenium carbene complexes identified from the SAR were found to inhibit the phosphorylation of Aurora-A in AsPC1 cells, while *fac*-[$Re(CO)_3$(phen)Cl], which contains a significantly less labile chloro ligand, was inactive (Figure S25).

Our results reinforce the potential usefulness of tricarbonyl rhenium complexes as potent anti-cancer agents against extremely robust pancreatic cancer cells. Most importantly, we have demonstrated, for the first time, that this class of complexes acts as cytostatic drugs by inducing a cell cycle arrest at the G_2/M phase associated with inhibition of the phosphorylation of Aurora-A kinase. For design purpose, our study indicates that the anticancer activity of the rhenium complexes originates from the lability of the ancillary ligand. These results shed some light into the mechanism of anticancer activity of tricarbonyl rhenium complexes, which was previously largely unknown due to a lack of systemic studies. This class of complexes could have broad application as

anti-cancer drugs. It will be interesting to investigate both *in vitro* and *in vivo* the cytotoxic properties of these complexes and to assess *in vivo* their antitumor activity alone or in combination with other known chemotherapeutic agents.

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Rhenium tricarbonyl complexes bound to N-heterocyclic carbene ligands exhibit antitumor properties against pancreatic cancer, revealing a mechanism of action based on induction of G_2/M cell cycle arrest and blockade of Aurora-A Kinase phosphorylation.