

Review

# Designing Ruthenium Anticancer Drugs: What Have We Learnt from the Key Drug Candidates?

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**Abstract:** After nearly 20 years of research on the use of ruthenium in the fight against cancer, only two Ru(III) coordination complexes have advanced to clinical trials. During this time, the field has produced excellent candidate drugs with outstanding *in vivo* and *in vitro* activity; however, we have yet to find a ruthenium complex that would be a viable alternative to platinum drugs currently used in the clinic. We aimed to explore what we have learned from the most prominent complexes in the area, and to challenge new concepts in chemical design. Particularly relevant are studies involving **NKP1339**, **NAMI-A**, **RM175**, and **RAPTA-C**, which have paved the way for current research. We explored the development of the ruthenium anticancer field considering that the mechanism of action of complexes no longer focuses solely on DNA interactions, but explores a diverse range of cellular targets involving multiple chemical strategies.

**Keywords:** ruthenium; anticancer; metal complex

## 1. Introduction

Cancer is a major health burden in the developed world. Although many breakthroughs in biological targeted approaches have occurred (specifically, immunological therapy), an unmet clinical need remains for a large section of the patient population, so wide-spectrum chemotherapy agents are still required. This space is currently addressed with the use of platinum(II) coordination complexes, namely Cisplatin (CDDP), Oxaliplatin and Carboplatin (worldwide approval), alongside Nedaplatin and Lobaplatin, which are restricted to selected markets. Such platinum agents lack cellular selectivity, so rapidly proliferating cells (hair follicles, bone marrow, and gastrointestinal tract) are particularly vulnerable to off-target effects. This lack of cellular selectivity can lead to the development of severe side effects not limited to nausea, vomiting, hair loss, nephrotoxicity, neurotoxicity, and hearing loss [1,2].

The prevalence of platinum resistance in the clinic, both intrinsic and acquired [3–5], is an escalating clinical concern, especially considering that platinum drugs are currently used in over half of all chemotherapy regimens [3]. This has sparked the development of a new generation of cytotoxics based on different metals, and corresponding coordination complexes, which excel in cellular selectivity and retain their use against a wide range of malignancies, while exhibiting unique mechanism(s) of action. Given the chemical similarity of the platinum group of metals (Pt, Pd, Rh, Ir, Ru, and Os), given the successes of platinum therapies, interest in ruthenium(II/III) complexes has increased considerably.

## 2. Ruthenium Complexes as Anticancer Agents

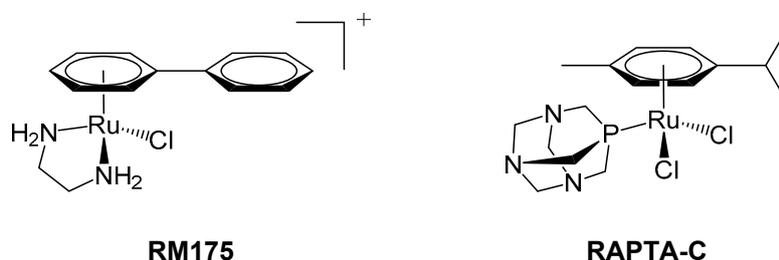
Ruthenium compounds have been proven to be a starting point in the search for alternatives to platinum drugs in the clinic, as these compounds have both comparable ligand exchange kinetics and a greater number of accessible coordination geometries. Such comparison considers that Pt(II) compounds are limited to square planar geometries, akin to cisplatin, and that octahedral geometries comparable to ruthenium complexes can be only be achieved by a higher oxidation state, e.g., Pt(IV). The investigation around such complexes has been intense, with highly-renowned and international-leading research groups pouring resources into this fast growing field. The chemistry of Ru(II) and Ru(III) complexes is well established in both the materials and medicinal chemistry fields, and research into their use as prospective anticancer agents is widely spread. The latter oxidation state complexes are typically considered to be more inert, which is, to some extent, attributable to the higher effective nuclear charge. In combination with the highly reducing environment of a cancer cell, an “activation by reduction” mechanism has been speculated for many Ru(III) complexes [6], which are reduced to their more active Ru(II) form by cellular reductants such as ascorbate [7].

The in-cell mechanism of action (MoA) for many ruthenium complexes differs from the DNA-binding mechanism typically associated with platinum drugs [8]. With a wider range of intracellular targets, ruthenium anticancer complexes are well established, and many examples have shown promise in chemical model systems, in vitro, and in vivo [9–11]. However, despite widespread efforts, only three ruthenium complexes (**NKP1339**, **NAMI-A**, and **TLD1433**) have reached clinical trials. **NKP1339**, and **NAMI-A** are both being developed as chemotherapeutic agents, while octahedral Ru(II) complex **TLD1433** has potential as a photosensitizer for photo-dynamic therapy [12]. Research into future anticancer agents should learn from both successes and difficulties encountered during the development of previous complexes in order to identify desirable physical, chemical, and biological properties associated with a successful future Ru drug candidate.

In this review, we critically evaluate four of the most significant Ru coordination complexes, **NAMI-A**, **NKP1339**, **RM175**, and **RAPTA-C**, and assess their contribution to the advancement of future ruthenium-based anticancer agents.

### 3. Case Study: RM175

**RM175** [Ru(biphenyl)Cl(en)]<sup>+</sup>, where en = 1,2-ethylenediamine, was amongst the first ruthenium(II) complexes to be explored for anticancer activity (Figure 1). This pseudo-octahedral organometallic complex was developed by the Sadler group in 2001 [13]. **RM175** is comprised of monodentate (chloride), bidentate (diamine), and arene (biphenyl) ligands, typical of a 3-legged “piano-stool” geometry. Originally synthesized with the aim of targeting DNA, it is intended to take advantage of the lower 2+ oxidation state that would not require activation by cellular reduction [14].



**Figure 1.** **RM175** (left) and **RAPTA-C** (right) are typical examples of 18-electron ruthenium arene “piano-stool” complexes, in which an  $\eta^6$ -arene ring stabilises the 2+ oxidation state of the ruthenium metal centre.

The arene substituent provides a hydrophobic surface, allowing for cellular diffusion through the lipophilic plasma membrane [14]. After entry into the cell, but prior to binding with DNA, complex activation is thought to occur by ligand exchange at the monodentate site [15,16]. The complex’s

design envisaged the halogen atom acting like a leaving group, drawing similarities with the activation of cisplatin. The aquation reaction would then generate a coordinative vacancy, which, in turn, would allow for covalent binding to the N7 of guanine in the DNA double helix [14]. Although Ru(II) complexes are known to bind to the guanine residues in DNA [17], it is thought that the extended arene in this complex enables hydrophobic interactions to occur between **RM175** and DNA via arene-intercalation between base pairs [18]. As a consequence of the free rotation of biphenyl about the Ru(II) centre, the structure is relatively flexible, which is anticipated to limit steric hindrance and increase the complex's DNA-binding affinity [19]. Conformational flexibility may allow the complex to intercalate into the base pairs and bind to guanine simultaneously, as has been shown possible with other ruthenium complexes [19]. This may explain why the resulting **RM175**-DNA adduct is more resistant to DNA repair than platinated DNA; therefore, such observation begins to explain the lack of cross-resistance with platinum [20].

Further biological studies into the mechanism of action of **RM175** revealed new cellular targets in addition to DNA binding, most notably the inhibition of matrix metalloproteinase-2 (MMP-2) [14]. MMPs are the most important members of the metalloproteinase family that contribute to tumor progression. They govern the conditions of the tumor microenvironment and use signalling pathways to assist in the modulation of cell growth and angiogenesis [21]. MMPs facilitate the migration and invasion of cancerous cells, through degradation of the extracellular matrix (ECM), suppression of the adaptive immune system, and other processes that promote cell survival and protect malignant cells from apoptosis [21,22]. Apoptosis, as a form of programmed cell death, does not procure an inflammatory response in principle; thus, MMPs are a noteworthy target for anticancer agents [23].

**RM175** also exerts activity by disrupting the processes of cell invasion and migration. In order to assess the effect of metallodrugs on cellular detachment and re-adhesion, several proteins have been investigated including collagen IV, fibronectin, and poly-L-lysine. All these proteins may come into contact with malignant cells *in vivo*, since poly-L-lysine attracts cells through electrostatic interactions, and both fibronectin and collagen IV are constituents of the extracellular matrix. Upon exposure to **RM175**, invasive MDA-MB-231 breast adenocarcinoma cells became more resistant to detachment from either fibronectin or poly-L-lysine, depending on the substance on which the cells were originally grown. This effect was absent in non-tumorigenic and non-invasive cell lines (HBL-100 and MCF-7, respectively), suggesting that the reduction in cellular detachment after exposure to **RM175** was selective for invasive cells that would otherwise metastasize. Adherence to cellular components is a vital stage in the formation of metastases, so the Ru(II) complex has the potential to expand this selective action across other cell lines. **RM175** significantly inhibits cellular contact-induced movement (haptotaxis) in MDA-MB231 and HLB-100 cells, preventing metastatic formation induced by this stimulus [14].

The described effects on cell invasion and migration explain why the use of **RM175** was directed toward preventing and treating metastases as opposed to the reduction in primary tumor volume. Though *in vivo* studies demonstrated a 50% reduction in primary mammary carcinoma (MCa) tumor mass, upon withdrawal of **RM175**, tumor growth resumed, demonstrating the limited effect of **RM175** on primary tumors. MCa cells are also liable to spontaneously spread to lung tissue; therefore, the anti-metastatic activity was also assessed. The ruthenium complex was found to have greater potency against metastases over primary tumors, and the size of the administered dose was found to significantly impact the extent of metastatic growth. Although a high dose (10 mg/kg/day) resulted in an 85–95% reduction in metastatic mass, only 70% reduction was achieved at a lower dosage (7.5 mg/kg/day). Supplementation of the dose with human serum albumin (HSA) in ratios from 1:1 to 1:10 were found to elevate the drug's cytotoxic profile and reduce cell viability to a greater extent, indicating that efficacy of **RM175** may be greater *in vivo* than *in vitro* [14].

**RM175** has been reported to up-regulate the tumor suppressor *p53* and the pro-apoptotic protein *Bax* in HCT-116-wt cells, contributing to apoptosis. Significant apoptosis only occurred in cells that were not *p53/Bax*-null, demonstrating that both proteins are vital for early **RM175**-induced apoptosis

(<48 h). Treatment with **RM175** also induced long-term loss of cellular replication, independent of *p53*, *p21 waf1/Cip1*, and *Bax* [17].

**RM175** also induces G<sub>1</sub>/G<sub>2</sub>-phase cell cycle arrest in vitro [17]. G<sub>1</sub> inhibition of cell cycle progression was found to be dependent on tumor suppressors and cell cycle regulators, *p53* and *p21*, which binds to CDK1, as well as to, CDK2 [24], yet independent from the *Bcl-2* family member, *Bax* [17]. This highlights the vital role of both tumor suppressors in initiating cell cycle arrest in contrast to **RM175**-mediated apoptosis, which only requires *p53* and *Bax*. Exposure resulted in the accumulation of the aforementioned genetic components [17], particularly *p21*, as expression of this cell cycle regulator typically leads to cell cycle arrest [25]. Activation of *p53* initiates a cascade of anti-mitogenic signals, promoting the *p21*-induced inhibition of CDK2 activity. This consequently prevents expression of various genes (some of which are involved in DNA replication) [25] and inhibits progression to S phase. Alternatively, the cell cycle arrest in the G<sub>2</sub> phase could be attributed to *p53*-mediated activation of either the GADD45 protein or *p21* [24], both of which inhibit CDK1, thereby inducing G<sub>2</sub>/M cell cycle arrest [26]. **RM175** exerts its anticancer activity by both preventing progression through the cell cycle leading to apoptosis, and “traditional” disruption of DNA replication by strand intercalation and nucleobase binding.

#### 4. Case Study: RAPTA-C

A second notable piano-stool complex is **RAPTA-C**, a ruthenium(II) arene complex developed by the Dyson group that comprises an amphiphilic 1,3,5-triaza-7-phosphaadamantane (PTA) ligand and two labile chloride ligands additional to the η<sup>6</sup>-coordinated arene, which helps to stabilize the +2 oxidation state (Figure 1) [27]. In contrast to other phosphine ligands [28], PTA is relatively sterically undemanding and is anticipated to contribute to the increased water solubility of **RAPTA-C** relative to other Ru(II) arene complexes [29]. Similarly to cisplatin, **RAPTA-C** undergoes rapid hydrolysis of Ru–Cl at low (intracellular) chloride concentrations (4–5 mM), predominantly yielding the mono-aquated complex, [Ru(*p*-cymene)Cl(H<sub>2</sub>O)(PTA)]<sup>+</sup>. The Ru–Cl bond remains intact at higher chloride concentrations (100 mM, i.e., in blood) and, like cisplatin, **RAPTA-C** may be considered a pro-drug in its di-chlorido form [28]. Initial studies of **RAPTA-C** indicated that damage to supercoiled pBR322 DNA was pH-dependent (occurring only below physiological pH; <7.0), so it was hypothesized that this may invoke selective targeting of the typically more acidic environment of cancer cells [27].

Unlike cisplatin, preliminary in vitro studies found **RAPTA-C** to be remarkably inactive toward TS/A adenocarcinoma and non-cancerous epithelial (HBL-100) cell lines, as well as initially having limited activity against primary tumors in vivo. However, later in vivo studies using pre-clinical models (using chicken chorioallantoic membrane and, more recently, mice) found that **RAPTA-C** was able to inhibit tumor growth by 50–75% [30,31]. In both instances, analysis of the treated tumor identified the significant anti-angiogenic properties of the complex. Both **RAPTA-C** and structurally similar **RAPTA-B** (in which the arene unit has been changed to a benzene ring) showed promise for size reduction of solid lung metastases in vivo [30]. These pre-clinical studies clearly demonstrate the requirement for careful optimization and control of experimental conditions, and for scientists to be mindful of the fact that in vitro experiments often do not accurately predict in vivo activities. Complexes, such as **RAPTA-B** or HC11, that contain cyclic, non-substituted hydrocarbons in their structure have the potential to cause liver toxicity after P450 poly-hydroxylation; hence, in vivo experiments need to closely monitor phase I metabolism of the ruthenium drugs [32]. In the case of RAPTA complexes, even at the highest dose administered to mice, negligible side effects were observed. Ruthenium was found to be excreted rapidly via the renal system and did not significantly accumulate in vital organs [28,33].

Later structural iterations of **RAPTA-C** included tethering of organic substituents on the coordinated arene. Planar anthracene groups were investigated to enhance DNA interaction by intercalation with concurrent fluorescence to map intracellular localization, with limited success [34–36]. In contrast, similar naphthalimide (a DNA intercalator) complexes were found to possess modest

potencies against A2780 ovarian cancer cells (and a platinum-resistant-derived cell line, A2780Cis) ( $IC_{50}$  2.3–9.1  $\mu$ M) and displayed some selectivity over non-cancerous (HEK-293) cells [37]. Conjugation of ethacrynic acid, a glutathione transferase inhibitor, to **RAPTA-C** yielded a new ruthenium complex that was found to bind to the enzyme's H-site. Importantly, the ruthenium center is involved in the inhibition of glutathione transferase through cysteine residue binding [38]. After an extended incubation time, the ethacrynic acid moiety remained in the H-site of the enzyme but the ruthenium center had been released, which was also found to occur in vitro [39,40].

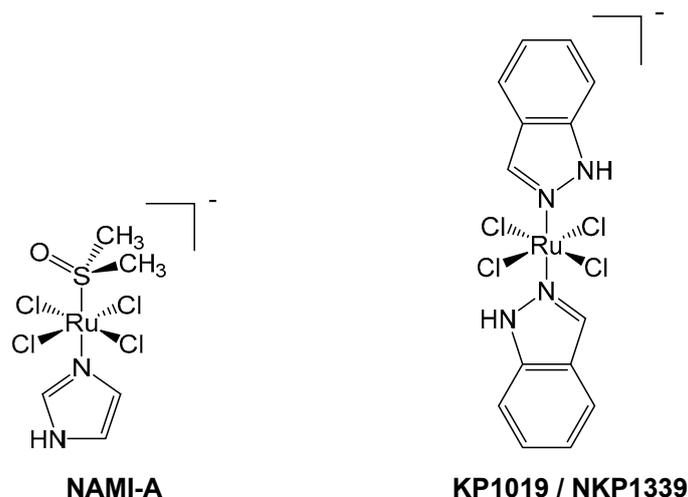
Most recently, a comparative metallomic study of the metabolism of **RAPTA-C** and cisplatin was reported. Despite **RAPTA-C** and cisplatin having similar labile chloride ligands with comparable exchange kinetics, **RAPTA-C** was found to be more inert to extracellular reactions despite being administered at a dose 40-fold higher than cisplatin. **RAPTA-C** was found to predominantly bind albumin, though extracellular metal speciation was found to be time-dependent [41]. This highlights the need to better understand speciation, and consequently drug pharmacokinetics and dynamics, to form a well-rounded pre-clinical research portfolio.

Various combination therapies involving **RAPTA-C** have also been explored in vivo. When administered alongside erlotinib (epidermal growth factor receptor (EGFR) inhibitor) and BEZ-235 (Phosphoinositide 3-kinase PI3K/mTOR inhibitor), the combination was found to synergistically inhibit tumor growth (up to 11-fold relative to single-drug experiments) [42]. An alternative combination therapy involving **RAPTA-C** and axitinib (vascular endothelial growth factor receptor (VEGFR) targeting tyrosine kinase inhibitor) led to a ca. 90% inhibition of tumor growth with a dose of only 0.4 mg/kg, despite negligible toxicity observed at doses of 100 mg/kg [43].

## 5. Case Study: NAMI-A

**NAMI-A**, originally synthesized by the groups of Alessio and Sava (Figure 2), differs from the two previously described complexes, being composed of chloride, imidazole, and dimethylsulfoxide (DMSO) in an octahedral arrangement around a Ru(III) center. Ruthenium(III) complexes are often considered to be pro-drugs, since they are typically less reactive than Ru(II) congeners and require activation via reduction to the 2+ oxidation state [44,45]. Such activation improves tumor targeting as the hypoxic cellular environment favors reduction of the metal center, thereby generating antiproliferative selectivity for cancerous cells compared to healthy cells [45,46]. Reduction of ruthenium from +3 to +2 also leads to kinetic lability; hence, the hydrolysis of chlorido ligands occurs at a faster rate, which may facilitate the reaction of ruthenium complexes with DNA [47]. In highly reactive cases, such substitution process, like hydrolysis, can effectively occur before the activation via reduction [48].

Having entered clinical trials in 2008, **NAMI-A** constitutes one success story in the development of ruthenium anticancer complexes. **NAMI-A** was first used in a phase I/II study in 32 patients suffering from an advanced form of non-small cell lung cancer (NSCLC) [49]. The metallodrug was administered intravenously in combination with gemcitabine, which is regularly used alongside cisplatin in this type of cancer [44]. Trial results indicated that such combination of **NAMI-A** and gemcitabine affected the quality of life of the patients, with side effects including gastrointestinal (GI) disturbances, neutropenia, and elevated liver enzymes [44]. Although the regimen was deemed to be “insufficiently effective for further use” [49], this problem may yet have a solution.



**Figure 2.** NAMI-A (left) and KP1019/NKP1339 (right) are octahedral Ru(III) pro-drugs, which are hypothesized to undergo an “activation by reduction” mechanism inside cells to form more active Ru(II) species.

NAMI-A has a synergistic ability to prevent cell invasion and hinder neo-angiogenesis [50], making it selective for metastasis rather than fully formed tumors [46]. Compared to cisplatin, NAMI-A has a wide variety of biological targets, most of which are extracellular rather than DNA-based [44]. One of the main mechanisms through which NAMI-A exerts its anti-angiogenic effects is thought to be the scavenging of nitric oxide [51]. The nitric oxide synthetase (NOS) pathway stimulates angiogenesis and endothelial cell migration and also includes VEGF-activated enzymes that catalyze the generation of NO—a signaling molecule involved in these processes [51,52]. The ruthenium center of NAMI-A, as well as its albumin adducts, have been proven to bind strongly to NO through displacement of the DMSO ligand [53]. This reaction is thought to be irreversible in vivo as NO release is unfavorable, even in the presence of glutathione or other reducing agents. Nitric oxide is a downstream mediator of VEGF and is implicated in endothelial cell migration [52]. NO scavengers, including NAMI-A, are reported to cause potent blockade of VEGF-mediated endothelial cell processes related to angiogenesis, including cell migration [51]. This may be due to NAMI-A’s observed effects on NO, since NAMI-A has no effect on the phosphorylation status of the downstream proteins, PKB (more commonly known as *Akt*) and ERK $\frac{1}{2}$  [54]. NAMI-A does not affect VEGF itself but instead is thought to enable cells to overcome its inductive properties, supporting the theory that NAMI-A prevents VEGF-mediated activity through NO scavenging [54].

Another angiogenic process effected by NAMI-A is inhibition of endothelial cellular proliferation [51], since NO is also a signaling molecule in the mitogen-activated protein kinase (MAPK) pathway [55]. This pathway includes a cascade of kinases that partake in cellular signaling, which coordinate various biological processes including cellular proliferation, migration, and survival [56]. After treatment with NAMI-A, cellular proliferation was inhibited for at least 48 h [51], an effect that could be attributed to NO’s role in the MAPK pathway. Angiogenesis is essential for tumor progression, since oxygen and nutrients are needed to sustain malignant growth. Angiogenic-inhibition provides another means of suppressing metastatic development. NAMI-A also reduces the VEGF-dependent migration of cancerous cells [57]. Inhibition of VEGF activity hence provides a selective mechanism through which NAMI-A can prevent neo-angiogenesis and the formation of metastases.

NAMI-A has also been reported to act on cells via selective inhibition of the Ca<sup>2+</sup>-activated potassium ion channel, *KCa3.1* [58]. In most cells, the concentration of potassium ions regulates the membrane potential, which, in turn, governs cell cycle progression [59]. However, the gene that encodes *KCa3.1* (KCNN4) is overexpressed in many cancer cells [58]. The downstream biological processes governed by *KCa3.1* differ depending on the cell type: (1) proliferation in leukemia and

lymphoma cells or (2) migration in epithelial and glial cancer cells [58,60]. Such cell-type-dependent function of the *KCa* ion channel may explain why the activity of **NAMI-A** is typically limited in primary tumors, yet the complex remains potent against leukaemia and metastases. In leukemia cells, **NAMI-A**-induced inhibition of *KCa3.1* was found to induce G<sub>2</sub>/M cell cycle arrest, leading to apoptosis [58]. Ion channels such as *KCa3.1* are considered to be indispensable in relation to cellular migration [61]; hence, **NAMI-A** is also able to inhibit cell motility. Proliferation and migration of NSCLC cells is also thought to be dictated by *KCa3.1*, as more aggressive cancers typically display up-regulation of this ion channel [62]. **NAMI-A**-mediated inhibition of *KCa3.1* effects malignant cell migration, either by diminishing or completely preventing the migration of epithelial cancer cells [61].

Another pathway thought to be involved in the mechanism of action of **NAMI-A** is the ATM/ATR (Ataxia telangiectasia mutated / RAD3-related) kinase pathway, which is activated after DNA damage and works to regulate the cellular response [63]. **NAMI-A** activates both kinases, though appears more selective for phosphorylation of ATR [54]. The downstream effects include phosphorylation of the tumor suppressor *p53* and kinases CHK1/CHK2, which are normally activated in response to genetic irregularities to coordinate cell cycle arrest [54]. **NAMI-A** also increases the expression of tumor suppressors such as *p15INK4b*, *p21*, and *p27Kip1*—CDK inhibitors that contribute to cell cycle arrest [54,64].

The anti-metastatic properties of **NAMI-A** arise from its ability to inhibit certain processes that are vital for metastatic formation and survival, including the adhesion and migration of cells. In addition to the mechanisms explained above, **NAMI-A** can inhibit these processes through protein interference, such as via  $\alpha 5\beta 1$  integrin [65]. Integrins are transmembrane-bound proteins that bind to other cellular components to facilitate the adhesion and migration of cells. Studies indicate that **NAMI-A** not only blocks  $\alpha 5\beta 1$  but significantly reduces the number of integrin receptors by modulating the expression of *ITGA5* and *ITGB1*—the coding genes for the integrin subunits  $\alpha 5$  and  $\beta 1$ , respectively. This reduces downstream phosphorylation of focal adhesion kinase (FAK) and both of these processes are thought to contribute to decreased cellular adherence. Pre-treatment of HCT-116 colon cancer cells with **NAMI-A** significantly reduced adherence to Fibronectin and collagen I; an effect that was less apparent in  $\alpha 5\beta 1$  integrin-null cells. Notably, the observed effects on cellular adhesion and pFAK occurred in a manner that was inversely correlated with **NAMI-A** concentration, further supporting the hypothesis that lower doses of **NAMI-A** have greater efficacy [65].

MMP targeting is another anti-metastatic mechanism associated with **NAMI-A**. It inhibits the production of MMP-2 and the activity of MMP-2 and MMP-9 [66]. Within the functions of these MMPs, those of particular interest include chemotaxis and the activation of transforming growth factor (TGF  $\beta 1$ ) [67]. Since **NAMI-A** inhibits endothelial chemotaxis, this may be mediated via MMP inhibition [66]. The MMPs inhibited by **NAMI-A** and TGF- $\beta 1$  partake in a feedback mechanism as TGF- $\beta 1$  is also responsible for regulating MMP expression [68], which may explain the effect of **NAMI-A** on MMP-2 generation. TGF- $\beta 1$  is a growth factor that selectively targets cancerous cells, inducing the epithelial–mesenchymal transition (EMT), invasion and migration; all integral processes needed for metastatic growth. **NAMI-A** counteracts this stimulus as it supposedly shares some of the pathways used by TGF- $\beta 1$  but leads to opposing outcomes. Therefore, a metastatic response to TGF- $\beta 1$  is prevented in tumor cells by hindering the processes mentioned above, leaving non-cancerous cell lines unaffected [69].

## 6. Case Study: KP1019/NKP1339

Another success story in the development of Ru(III) compounds that have reached clinical trials is **NKP1339**, reported by the Keppler group (Figure 2). The original form, **KP1019**, was modified to increase its aqueous solubility, generating the sodium salt equivalent, **NKP1339** [46]. This Ru(III) complex has some structural similarity to **NAMI-A** due to its octahedral geometry and chloride ligands [46]. **NKP1339** is a pro-drug and relatively inert compared to **NAMI-A**, as ligand loss/exchange does not occur as readily [44,47].

Both **NAMI-A** and **NKP1339** bind non-covalently with proteins in the blood, likely via hydrophobic interactions [44,46]. **NKP1339** undergoes rapid binding with albumin, so this is an important factor to consider when the metal complex is administered intravenously. The metal-protein adduct does account for the low side effect profile documented during the phase I trial of **NKP1339**, as the complex remains in its pro-drug form until it undergoes activation by reduction, after internalization by cells and release from albumin [70]. Albumin binding may also improve the selective accumulation of **NKP1339** through two possible mechanisms: the EPR effect or by binding to the glycoprotein, gp60 [44]. The former occurs due to leaky vasculature within malignant cells, resulting in a greater uptake of albumin and other substances, which are then prevented from leaving by poor lymphatic drainage. In contrast, gp60 is present along the tumor endothelium and allows receptor-mediated transcytosis of the drug-albumin adduct to occur through to the extracellular tumor matrix [44]. Adduct formation with blood proteins is more significant for **NKP1339** than **NAMI-A** since cellular uptake for the latter is limited [44], whereas uptake of **NKP1339** is considered significantly more efficient [71].

Other ruthenium complexes, including **RM175** [17], have been previously shown to interact with DNA through binding to guanine bases after reduction to their active hydrolyzed form [72], and **NKP1339** is no exception. Due to the ability of **NKP1339** to accumulate within the nucleus after activation [47], DNA may be one of the drug's intracellular targets [46]. During activation, the metal ion is reduced, and the Ru(II) species is responsible for the DNA-adduct formation. Only weak adduct signals were emitted when **NKP1339** was incubated with oligonucleotides, indicating low levels of interaction. This may be due, at least in part, to the slow ligand exchange rate of **NKP1339**, and a significant proportion of the drug remains intact [47]. In contrast with cisplatin, neither drug uptake nor genetic mutations, such as those involving *p53* and *k-ras*, are correlated with the efficacy of **NKP1339**. This *p53*-independent mechanism implies that DNA binding is not likely to be the primary mechanism of action of **NKP1339** [70], which may account for the lack of platinum-cross resistance observed with this Ru(III) complex in pre-clinical studies [73].

**NKP1339** induces cell cycle arrest through mechanisms attributed to its redox activity in cancer cells [46]. Intracellular reactive oxygen species (ROS) concentrations (such as hydroxide radicals and superoxide) are already elevated in neoplastic cells compared with healthy cells [74] and this is thought to increase the vulnerability of cancer cells to further ROS fluctuations [75]. As such, redox-targeting metal complexes have attracted significant attention in recent years, and often exhibit significant selectivity for cancer cells over healthy cells [74,76–78]. **NKP1339** has been shown to increase the intracellular ROS concentration [79] and upregulate the pro-apoptotic *p38* MAPK pathway [80]. This biological cascade is normally activated in response to cellular stress, such as cytokines, DNA damage and ROS, and is implicated in cell cycle progression [81]. Activation of this pathway leads to downstream regulation of gene expression, including those that encode for cytokines, transcription factors, and cellular receptors [82]. More importantly, this pathway also regulates the  $G_1/S$  and  $G_2/M$  check points within the cell cycle [81]. By generating ROS and altering the cellular redox balance, **NKP1339** induces  $G_2/M$  cell cycle arrest [46].

Treatment of sensitive cell lines with **NKP1339** induced cell death, typically within 20–30 h [70]. Accumulation of ROS prompts the loss of the mitochondrial membrane potential by inducing membrane permeabilisation, leading to activation of the intrinsic apoptosis pathway [83,84], which is typically caspase-dependent, and involves the cleavage of the zinc finger protein, poly-(ADP-ribose)-polymerase (PARP). [70,85] However, in the case of **NKP1339**, the extent of mitochondrial membrane depolarization is limited, suggesting another mechanism of apoptosis may be at work. Caspase-8 was cleaved as part of **NKP1339**-induced apoptosis [70], a key feature of the extrinsic apoptosis pathway. Combination treatment with **NKP1339** and a caspase-8 inhibitor increased the extent of apoptosis observed within less responsive cell lines. The researchers deduced that the majority of apoptosis occurs not by the mitochondrial-intrinsic pathway, but via the extrinsic pathway [70]. Though mitochondria are a target of **NKP1339**, the effect of this interaction is limited as

the majority of apoptotic activity is mediated by either death receptors on the cell surface or via other extrinsic pathways [86]. Since **NKP1339** does not enhance the expression of these death receptors or their ligands, the Ru(III) complex may act via an alternative extrinsic pathway involving endoplasmic reticulum (ER) homeostasis [70].

Since the anticancer activity of **NKP1339** is predominantly mediated through changes in redox homeostasis [46], another notable target is the endoplasmic reticulum (ER), which is also influenced by the cellular redox environment. This organelle controls the maturation, folding, and release of proteins, and initiates the unfolded protein response (UPR) as part of a feedback system, suspending the cell cycle either temporarily until the proteins are restored or, permanently, prior to apoptosis [79]. The protein *Nrf2* is able to trigger the expression of antioxidant genes to reduce the cell's exposure to oxidative stress [87]. After treatment with **NKP1339**, *Nrf2* translocates to the nucleus to commence its protective activity. This observation may be ROS-mediated and demonstrates that drug-induced hyper-oxidation is sufficiently substantial to initiate a cellular response. The redox properties of **NKP1339** lead to dysregulation of various other proteins that affect the ER. **NKP1339** increases ROS levels in vitro in colon carcinoma cell lines (HCT116 and SW480), causing upregulation of *CHOP* mRNA [79]. The targets of this transcription factor include (1) *GADD34*, implicated in cell cycle arrest; (ii) *DR5*, which promotes cell death through caspase activation and initiation of the extrinsic pathway; and (3) *Ero1 $\alpha$* , which leads to ER hyper-oxidation and stimulates cell death [88]. *CHOP* also reduces the expression of the anti-apoptotic protein *Bcl2*, and consequently, excess *CHOP* generation favors the induction of apoptosis [79].

Sustained ER stress may result in cellular dysfunction or death [88], so alterations in the UPR could be exploited for antiproliferative activity. In vitro treatment with **NKP1339** was shown to downregulate various ER-based proteins, including signaling molecules *PERK* and *Ire1 $\alpha$*  and the chaperone proteins calnexin and GRP78 [70]. **NKP1339** is an effective inhibitor of the last one by preventing its activation [70,89]. These proteins all have an active role in UPR regulation: calnexin assists with protein folding, whereas the signaling molecules *PERK* and *Ire1 $\alpha$*  are two of the main transmembrane receptors bound to the heat shock protein, GRP78 [79]. When GRP78 senses unfolded proteins, it simultaneously binds them and liberates the signaling molecules that drive the UPR [79]. GRP78 is often viewed as the "master regulator" of the UPR [89] and functions to protect the cell with the aim of restoring the ER to its original condition [90]. Downregulation or inhibition of ER proteins results in a lack of control over protein folding and accumulation of damaged proteins. Drug-induced ROS generation damages proteins, further enhancing the ER's exposure to stress [79]. Another advantage of this mechanism is that GRP78 is associated with chemo-resistance, so inhibition of this protein may lead to a better prognosis and prevent the occurrence of GRP78-mediated resistance [89]. In vitro use of **NKP1339** is also able to increase drug-sensitivity in cells that are already resistant; a promising indication that drug-resistance might be reversed.

Another reactive oxygen species, nitric oxide, can be scavenged by **NKP1339**, though its effect on NO-mediated processes is weak in comparison to **NAMI-A** [51]. Therefore, there is little evidence to support its interference with VEGF/NO-stimulated angiogenesis or cell migration [51], explaining why **NKP1339** is more effective against solid tumors than **NAMI-A**. By unsettling redox homeostasis, **NKP1339** is able to inhibit DNA synthesis, induce G<sub>2</sub>/M cell cycle arrest and initiate both intrinsic [46] and extrinsic apoptosis [70]. Owing to its multi-targeting mechanism of action, overexpression of proteins associated with multi-drug resistance (MRP1, BCRP, LRP, and the transferrin receptor) little hinders the drug's efficacy, which also explains the lack of cross-resistance [73,85]. The phase I trial of **NKP1339** was used as a dose-escalation study to assess its use for the treatment of advanced solid tumors. The study included patient tolerability as well as pharmacodynamic and pharmacokinetic studies of the drug (Niiiki Pharma Inc. and Intezyne Technologies Inc., 2017). The trial (NCT0145297) was completed in 2016 and was deemed successful [46]. **NKP1339** was also shown to be particularly effective against neuroendocrine tumors (and exhibited limited side effects in trial participants [46,73]).

## 7. Current Developments in Ruthenium Anticancer Agents and Future Perspectives

The complexes in these four case-studies have paved the way for future research, much of which is now being completed by not only by well-established researchers but also by internationally recognized new and upcoming leaders in the field. Pushing the boundaries, new ruthenium anticancer complexes show a wider diversity of coordination spheres and a vast range of ligands that include, but are not limited to, extended intercalating aromatic units, polypyridyl rings, fluorescent derivatives, bio-active molecules, ferrocifen analogues, and carbenes, all of which exploit donor atoms such as C, N, O, S and P.

Even more interesting is observing the escalation of novel approaches regarding targeting strategies and MoA involved in the anticancer activity of Ru complexes, all of which are fueled by our increased understanding of the complexes' fates at the cellular level. Disruption of protein-protein interactions, [91] enzymatic inhibition [92,93], and redox modulation [94], as well as chromatin [95] and histone [96,97] targeting, are only a few examples of cellular events being used as means for antiproliferative activity, with in-cell catalysis taking advantage of well-established reactions in the chemistry of materials field [77,78]. Crucially, the understanding of metal anticancer complexes as efficient multi-targeting agents is starting to have a positive outlook, if not yet for funding bodies, then at least from an academic perspective. Hence, the attempts to locate a single drug target may yet develop into the search for a majoritarian cellular event in a field that seems to move at a highly fast pace.

Such advances and new trends in thought should not forget how far **NKP1339**, **NAMI-A**, **RM175**, and **RAPTA-C** have advanced the field, and how critical these complexes have been in the development of the field. As a community, we may benefit from more closely examining these four compounds to learn, amongst other things, that small structural changes generate large variations in the MoA at the cellular level [98]. Although structure activity relationships are not easily established, current and further developments of analytical and cellular techniques will provide more investigative tools and, subsequently, a better understanding of cellular behavior. The key for the new generation of anticancer complexes may well be just round the corner.

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