RUTHENIUM CHEMOTHERAPEUTICS: DESIGN AND APPLICATION

by

ANNA MARIA WILLIAMS

(Under the Direction of Professor Jeffrey Urbauer)

ABSTRACT

Metallic anticancer compounds have generated increasing amounts of interest since the discovery of cisplatin. The chemical properties of ruthenium make it an ideal candidate for such studies. Organometallic Ru-arene complexes are attractive for both primary tumor and antimetastatic treatments; in particular, [(η^6-arene)Ru(XY)(Z)] complexes and RAPTA complexes have been studied intensely. Ligand variability of these compounds allows for detailed study of their structure-activity relationships, creating a valuable knowledge base for the educated design of ideal Ru anticancer complexes. In addition, a variety of different methods have been developed to mediate Ru compounds activity, to include the addition of targeting moieties and the development of Ru photodynamic therapeutic agents. The rate at which these compounds are being researched is currently increasingly, suggesting the possibility of their clinical use in the near future.

INDEX WORDS: Ruthenium; Anticancer treatments; Metallopharmaceuticals; Biomedicial organometallics; RAPTA complexes; DNA-interactions; Photodynamic therapy; Half-sandwich complexes

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LIST OF SYMBOLS AND ABBREVIATIONS

9EtG	ethyl bound to N9 of guanine
acac	acetylacetone
AMP	adenosine monophosphate
anth	anthracene
bip	biphenyl
bpy	bipyridine
cbdca	1,1-cyclobutanedicarboxylic acid
cGMP	cyclic guanosine monophosphate
chxn	cyclohexane-1,2-diamine
Clazpy	5-chloro-2-(phenylazo)-pyridine
ctDNA	calf thymus DNA
cym	cymene (isopropyltoluene)
Cys	cysteine
dab	1,2-diaminobenzene
DFT	density functional theory
dGMP	deoxyguanosine monophosphate
dha	dihydroanthracene
DNA	deoxyribonucleic acid
dmp	2,5-dimethylpyrrole

DMSO	dimethylsulfoxide
DMDPPZ	3,6-dimethyldipyrido[3,2-a:2′,3′-c]phenazine
dsDNA	double-stranded DNA
E. coli	*Escherichia coli*
en	ethylene diamine
Gln	glutamine
Glu	glutamic acid
Gly	glycine
GMP	guanosine monophosphate
GSH	glutathione
His	histidine
IC_{50}	50% inhibitory concentration
im	imidazole
ind	indazole
Lys	lysine
Met	methionine
MDR	multidrug resistant
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NCP	nucleosome core particle
PDT	photodynamic therapy
phen	phenyl
pta	1,3,5-triaza-7-phosphaadamantane
RNA	ribonucleic acid

ssDNA	single-stranded DNA
Tf	transferrin
tha	tetrahydroanthracene
Ub	ubiquitin

CHAPTER 1

INTRODUCTION

1.1 METALLIC ANTICANCER AGENTS

The founding moment of modern medicinal inorganic chemistry is often cited as the discovery of the chemotherapeutic potential of cisplatin [*cis*-$PtCl_2(NH_3)_2$] by Rosenberg and Van Camp in 1969 and its subsequent approval and widespread use.[1] Used for the treatment of a variety of solid tumors, cisplatin is particularly effective in testicular cancer.[2] Although initially approved by the FDA over thirty years ago, cisplatin has remained one of the most popular chemotherapeutics used in the United States. Unfortunately, there are a number of side effects associated with cisplatin, including nephrotoxicity, vomiting, and neurotoxicity.[2] In addition, many tumor types show inherent or acquired resistance to cisplatin.[3] Because the drug itself has a poor pharmacokinetic profile and exhibits systemic toxicity, its oncological use is limited.

In the past forty years, numerous labs have devoted their resources to developing thousands of different platinum-based and other metal-based chemotherapeutics in order to combat these deficiencies. Some second generation derivatives, such as carboplatin [*cis*-$Pt(NH_3)_2$(cbdca)] and oxaliplatin [Pt(oxalato)(1R,2R-chxn)], have found their niches in chemotherapeutics worldwide, while a few other derivatives have also been approved in select Asian countries: nedaplatin in Japan, lobaplatin in China, and heptaplatin in South Korea (Figure 1.1).[4]

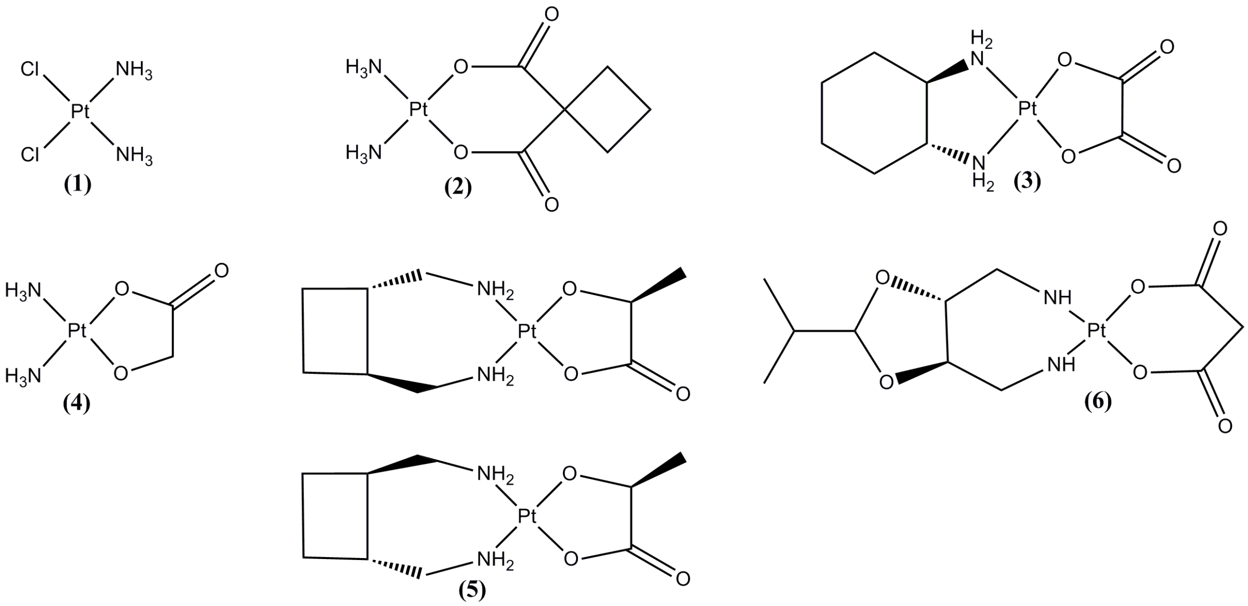


Figure 1.1 (1) Cisplatin. (2) Carboplatin. (3) Oxaliplatin. (4) Nedaplatin. (5) Lobaplatin (50:50). (6) Heptaplatin.

Despite the relatively small success rate when compared to organic compounds, metallic chemotherapeutics are advantageous for several reasons: 1) they can coordinate different quantities of ligands in different geometries; 2) these ligands can have a variety of different tunable structures and activities; 3) the metal centers are capable of accessing different redox states; and 4) the type of metal center itself can provide unique reactivity.[1] These factors are each individually adjustable so that, under the ideal circumstances, one would be able to merely select different options from a veritable "toolbox" of techniques and parts in order to custom design a metal-based chemotherapeutic for a particular tissue, genetic target, and/or cancer.

1.2 HISTORY OF RUTHENIUM ANTICANCER AGENTS

In 1931, a screen of multiple compounds discovered that a ruthenium compound, $K_4[Ru(CN)_6]$, showed some antitumor activity; however, no quantitative data was given.[5] The first significant interest in ruthenium (Ru) as an anticancer agent was the discovery that

$[Ru(DMSO)_4Cl_2]$ and [*fac*-$Ru(NH_3)_3Cl_3$] produced filamentation by arresting bacterial cell division in *Escherichia coli* (*E. coli*).[6,7] Induction of filamentation had been observed for other compounds with anti-tumor activity.[5,8,9] This indicates that a compound reacts with DNA, halting division but otherwise not affecting cellular pathways, meaning the compound possibly causes initiation of the DNA repair response. Rosenberg *et al.* had tested ruthenium compounds previously in order to develop a traceable metal compound with a long-lived radioactive isotope.[10] Some activity was observed but not enough to inspire further testing at the time. When testing for *E. coli* filamentation induction, Monti-Bragadin *et al.* found $Ru(DMSO)_4Cl_2$ to have activity comparable with cisplatin.[6] Durig *et al*., testing additional ruthenium compounds under the premise that second row transition metals are generally more reactive and less toxic than their third row counterparts, found that [*fac*-$Ru(NH_3)_3Cl_3$] also induced filamentation on par with that produced by cisplatin.[7] Contrary to Monti-Bragadin *et al.*'s findings, their concentrations were slightly less than those used for cisplatin. However, poor solubility and rapid decomposition prevented the further development of this compound.

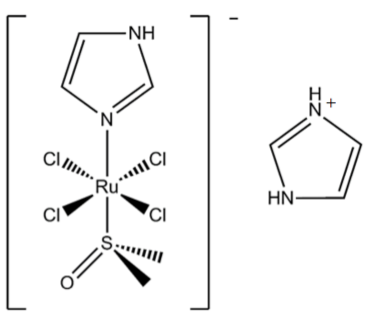
1.3 RUTHENIUM COMPLEXES

Ruthenium, a rare platinum group metal, has several unique properties that may aid it in overcoming the problems associated with cisplatin. For example, ruthenium complex structures and slow ligand exchange kinetics are often predictable due to the fact that the oxidation state of ruthenium complexes in aqueous solution is usually either 2^+ or 3^+. This characteristic often means these complexes are six-coordinate, octahedral, low-spin complexes.[11] In addition, many Ru(II) and Ru(III) compounds have shown selectivity towards cancer cell lines instead of normal human tissue cell lines, a tendency that may be enhanced through transferrin binding *in vivo*.[12] (Transferrin, a protein often overexpressed by cancer cells due to their increased need for iron,[13] has two Fe (III) binding sites. Called apotransferrin when no iron ions are bound, it can bind Ru (III) ions to the His residues surrounding the iron binding sites.[14]) In addition, the syntheses of Ru(II) and Ru(III) complexes are well known.[11] Finally, many ruthenium compounds exhibit different mechanisms from those of cisplatin and other platinum derivatives, increasing the probability that they will not be affected by the same resistance pathways that affect cisplatin.[1]

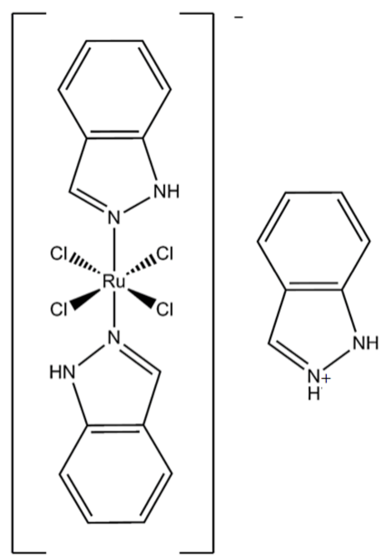
This last characteristic, differential resistance, is one of the most important. Most metal-based chemotherapeutics are designed with DNA as the target in mind, similar to cisplatin. With cisplatin, it is well-established that the platinum compound is reduced *in vivo* from Pt(IV) to Pt(II), at which point it intercalates between the base pairs of DNA, creating lesions that induce DNA repair), an error-prone process that eventually causes apoptosis.[2] Ideally, this reduction takes place in the hypoxic environment of the tumor. This limited oxygen supply results from the inability of angiogenesis to maintain the same growth rate as tumor cell growth,[15] causing glycolysis to become the primary energy source.[16]

Originally, potential ruthenium anticancer complexes were predicted and designed to behave in the same manner, with Ru (III) "prodrugs" that were analogous to cisplatin attracting much early attention.[11] The ability of single-electron transfer proteins in the mitochondrial and microsomal electro-transfer systems to reduce Ru (III) to Ru (II) has been demonstrated[17] and Ru(II) can be reoxidized in the presence of O_2 to give the inactive form.[18] This fact is important for compounds requiring activation by reduction, suggesting that these Ru(III) compounds should show a greater toxicity in the hypoxic environment of a tumor where they are easily reduced to Ru(II), rather than in the rest of the body. Not only is the rate of hydrolysis of ruthenium similar to that of platinum,[7] but these compounds also maintain their chemical properties: the reduction of Ru(III) to the "active" Ru(II) maintains it's ligand coordination number and interatomic bond distances, while the reduction of Pt(IV) to the "active" Pt(II) does not.[19]

Two Ru(III) compounds, NAMI-A [*trans*-$RuCl_4$(DMSO)(Im)]ImH and KP1019 [*trans*-$RuCl_4(Ind)_2$]IndH are currently in phase II clinical trials (Figure 1.2).[20] These agents show promise as antimetastatic and primary tumor treatments, respectively. While these compounds have been in clinical trials, other studies have revealed surprising information regarding their mechanisms: unlike cisplatin, protein-binding seems to be more important than DNA-binding. For example, in recent uptake studies, KP1019 is heavily concentrated in cell fractions containing particulate matter, such as mitochondria, while NAMI-A has a significant concentration in the cytoskeleton fraction.[21] These data support both compounds' activities: mitochondrial-induced apoptosis should abolish primary tumors while changes to cytoskeleton motility and adhesion should affect metastases.



(1)



(2)

Figure 1.2 (1) NAMI-A. (2) KP1019.

Recently, some of the most successful Ru compounds have been organometallic, rather than traditional coordination, compounds.[1] This thesis will address the structure and activity of some of the most important ruthenium complexes of this type.

CHAPTER 2

THE IMPORTANCE OF STRUCTURE: STRUCTURE-ACTIVITY RELATIONSHIPS IN RUTHENIUM ANTICANCER COMPOUNDS

The type of ligand attached to the metal center in a metal complex is a key structural component that can be adjusted in order to change the complex's lipophilicity, stereochemistry, rate of hydrolysis, pK_a of the hydrolyzed form, and specificity with regard to inter- and extracellular targets. Most Ru complexes possess at least one halide leaving group in order to facilitate covalent binding with intra- and extracellular targets. The most frequently employed halide is chloride.[22] However, the variety of ligands available means that a detailed study of their ability to influence the activity of different Ru complexes is of the upmost importance in order to be able to design effective anticancer therapeutics.

2.1 RU-ARENE-(XY)(Z) COMPLEXES

Following the success of Ru (III) compounds in clinical trials, Peter J. Sadler's group began looking into Ru (II) compounds as potential chemotherapeutics because the active form of Ru(III) is assumed to be the Ru (II) state. The compounds his group developed have the general formula [(η^6-arene)Ru(II)(X)(Y)(Z)].[1] Beyond stabilizing the 2^+ oxidation state of the metal ion, having a lipophilic arene as a ligand also opens up the possibility of π-π stacking and DNA intercalation, especially considering the arene-Ru(II) bonds' resistance to hydrolysis.[23] These half-sandwich complexes are characterized by a "piano-stool" geometry in which the π-bonded η^6-arene ring is the seat and the other ligands are the legs (Figure 2.1). Synthesis of these complexes usually requires the reaction of $RuCl_3 \bullet xH_2O$ with a cyclic 1,3- or 1,4-diene, forming a

Ru(II)-arene dimer that is then combined with the desired ligands.[1, 24] These types of complexes are now being developed for various other uses beyond chemotherapeutics such as antimalarials[25] and transfer-hydrogenation catalysts.[26]

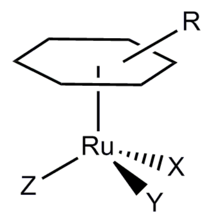
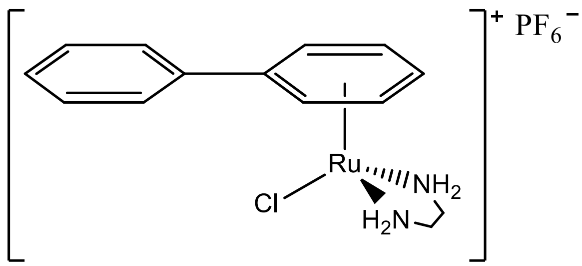


Figure 2.1 Example of "piano-stool" or half-sandwich geometry.

Although some of the first compounds had three monodentate ligands in addition to an arene, it was quickly discovered that reducing the number of coordination sites through substitution of a bidendate chelating ligand, usually with N and O electron-donating atoms for two of the monodentate ligands, enhanced the activity and stability of the compound by decreasing the likelihood that the drug would interact with media, extra-, or intracellular biomolecules.[27] The general formula for these compounds is $[(\eta^6\text{-arene})Ru(II)(XY)(Z)]$ in which (XY) is the bidentate ligand and (Z) is the anionic leaving group.[1] One of the most studied compounds in this class of complexes is $[(\eta^6\text{-bip})RuCl(en)]PF_6$, also known as RM175. RM175 undergoes rapid aquation and has a similar IC_{50} value to that of carboplatin in human ovarian carcinoma A2780 cells (Figure 2.2).[27,28] These results were supported by *in vivo* studies in which RM175 showed less activity than cisplatin in an A2780 xenograft, more activity than cisplatin in the cisplatin resistant A2780^{cis} xenograft, and no activity in the multidrug resistant (MDR) 2780^{AD} xenograft.[28] These, and other supporting *in vitro* data for the other first generation complexes of this type,[28] show that there is cross-resistance in MDR cells but not in cisplatin-resistant cells, indicating that it has potential as an alternative treatment to cisplatin.



Compound	IC_{50} (μM)
RM175	5 ± 0.4
Cisplatin	0.6 ± 0.06
Carboplatin	6.0 ± 0.7

Figure 2.2 Comparison of IC_{50} values in A2780 cells.[28]

2.1.1 STRUCTURE-ACTIVITY RELATIONSHIP

These complexes have three tunable sites: the arene, the leaving group, and the chelating ligand. As one would expect, increasing the size of the arene leads to an increase in hydrophobicity, a factor that influences the compound's bioavailability (i.e. uptake and efflux) and binding abilities.[28] This trend correlates with cytotoxicity; consequently, the tetrahydroanthracene (tha) analog of RM175 is as active as cisplatin in A2780 cells and, in later studies, more active than the smaller tetralin analog.[28,24] Conversely, this increase in hydrophobicity corresponds with a decrease in activity in 2780^{AD} cells, a cell line which overexpresses the efflux transporter P-glycoprotein, a protein that binds preferentially to hydrophobic, cationic molecules. This resistance can be eliminated with the concurrent administration of a P-glycoprotein inhibitor.[28] Despite this seeming correlation, a direct link between hydrophobicity and activity has yet to be substantiated,[29] indicating that interactions with biomolecules are, as expected, a more accurate predictor of activity than simple quantity of uptake.[24] Naturally, the choice of arene ligand affects biomolecular interactions, such as DNA ruthenation, as well. For example, hydrolysis of the leaving group, a crucial step in ruthenation (see section 2.1.1.3), was found to directly correlate with the ability of the arene ligand to accept electrons (Figure 2.3).[29]

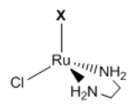
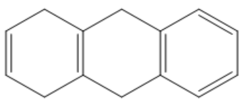
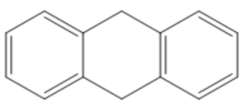
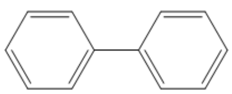
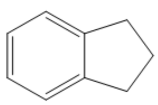
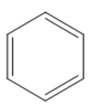
	**IC_{50} (μM)**	**Hydrolysis (k_{H_2O} 10^{-3} s^{-1})**
	0.5 ± 0.1	2.36 ± 0.02
	2 ± 0.4	2.23 ± 0.02
	5 ± 0.4	1.24 ± 0.13
	8*	2.29 ± 0.14
	17 ± 8.3	1.98 ± 0.02

Figure 2.3 Comparison of IC values in A2780 cells and hydrolysis values for various arene ligands. [28, 29] ***Standard deviation not reported.**

Density functional theory (DFT) calculations predict that hydrolysis of the leaving group in Ru-arene monodentate complexes goes through either an interchange associative or dissociative pathway, depending on the ligands involved.[29] The ability of the leaving group to hydrolyze was found to directly correlate with the complex's ability to induce cytotoxicity – in the order N_3 < I < Br < Cl, where Cl exhibits the most cytotoxicity.

Lastly, one may change or alter the chelating ligand. The effect of altering the chelating ligand backbone on the activity of the compound is unclear. For example, methylating the nitrogens in [(η^6-*p*-cym)RuCl(en)]PF_6 eliminated the compounds cytotoxicity[24] while methylating the nitrogens in RM175 had no effect.[28] Interestingly, substitution of *N'N'* – ethylenediamine (en) with diaminobenzene (dab) reversed the order of cytotoxicity of the arene compounds in A2780 cells, with tha (23 μM) < dha (7 μM) < bip (5 μM).[24] The same substitution, however, increased their activity in $A2780^{AD}$ cells, probably because their large, nonpolar dab ligands are unable to bind as well to the P-glycoprotein binding pocket as the smaller, positively charged en counterparts do.[24] This substantiates the idea that one might be able to customize these medicines for differential cancer treatment based on efflux protein expression.

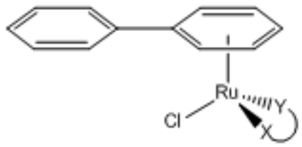
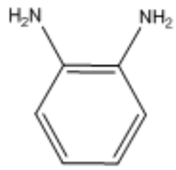
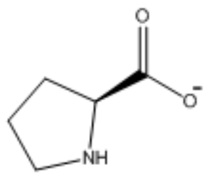
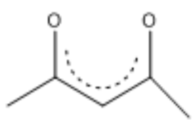
	**IC_{50} (μM)**
	5
	5
	> 100
	21

Figure 2.4 IC_{50} values for various chelating ligand analogs of RM175 in A2780 cells.[24]

The use of anionic N,O chelating ligands showed no activity towards A2780 cells, probably due to deactivation by rapid hydrolysis at high (500 mM) chloride concentrations.[24] Additional studies on a range of pyridinedicarboxylic acid *p*-cym analogs with chelating ligands such as 2,3-pyridinedicarboxylic acid showed no *in vitro* activity in six additional cell lines, despite their ability to quickly bind to 9-methyladenine.[30] More recent studies on the N,O chelating ligand in $[(\eta^6\text{-}p\text{-cym})RuCl(C_6H_5NO_2)] \bullet H_2O$ have shown a slight toxicity in the human mesothelioma MS1 and human ovarian HeLa cell lines and moderate toxicity in the human melanoma FemX cell line.[31,32] This compound's ability to halt the migration of HeLa cells and tube formation of MS1 cells means that it might be useful as an antimetastatic agent, as opposed to a treatment for primary tumors[32] and suggests that N,O ligands are worth another look, either in different cell lines or as potential antimetastatics.

The use of anionic O,O chelating ligands decreased the activity of the corresponding arene complex but not uniformly, changing the activity of the complexes such that dha analogs were less potent than bip analogs in A2780 cells.[24] Protonation and subsequent displacement of the acetylacetone (acac) ligand, made more difficult by the flexible bip or *p*-cym ligands, could lead to deactivation in this case.[24] In addition, O,O pyronato, pyridinato, and hydroxypyridone chelating ligand complexes were found to bind quickly with the amino acid methionine (Met) and the protein ubiquitin (Ub) in solution, suggesting that deactivation of O,O ligands is a result of non-specific reactivity.[33,34] In the last case, this is to be expected from the preliminary studies on the compound's rapid hydrolysis, both in the absence and presence of Cl^- ions.[34]

S,O ligands, although less studied than the others, have been found to show increased cytotoxicity due to increased stability resulting from their binding strength with the Ru metal ion as calculated through DFT.[33,35] These data have been supported by *in vitro* studies: when comparing maltol complexes with their thiopyrone analogs, it was found that the former increased the activity of the complex in human colon carcinoma SW480 by an order of magnitude – from >100 μM to 5.1 μM.[35] In addition, thiopyrone complexes are much more stable in the presence of amino acids such as Met,[36] increasing the likelihood that the complex will reach its active site if administered *in vivo*.

2.1.2 BIOMOLECULE-BINDING

Ru(arene)(XY)(Z) complexes are capable of binding with a variety of intra- and extracellular biomolecules. This binding may either increase the activity of the compound, as is the case with DNA ruthenation, or decrease the activity of the compound, as is the case with Ub.

a. DNA-BINDING

The DNA-binding ability of this class of compounds is extremely important; complexes in this family of compounds that are incapable of binding to DNA tend to show little to no activity.[32] It has long been known that Ru complexes preferentially coordinate with the readily available N-7 position of guanine (Figure 2.5), the most electron-rich site in dsDNA, rather than the interior N-1 and N-3 of adenine or the exterior N-cytosine sites.[11,27] $[(\eta^6$-*p*-cym)RuCl(en)]PF_6 covalently binds almost exclusively to N7 of guanine in ssDNA and destabilizes dsDNA.[37] This finding was later confirmed for RM175; crystallographic studies of the complex with guo (guanine with ribose attached) determined that there was additional stabilization due to hydrogen-bonding between an ethylene diamine hydrogen to O6 of guanine, with an N-O interatomic distance of 2.761 Å.[37] Also DFT studies show additional stabilization from hydrogen-bonding between the other ethylene diamine group to an O^- in the phosphate sugar backbone if these Ru complexes intercalate between the base pairs of DNA.[38]

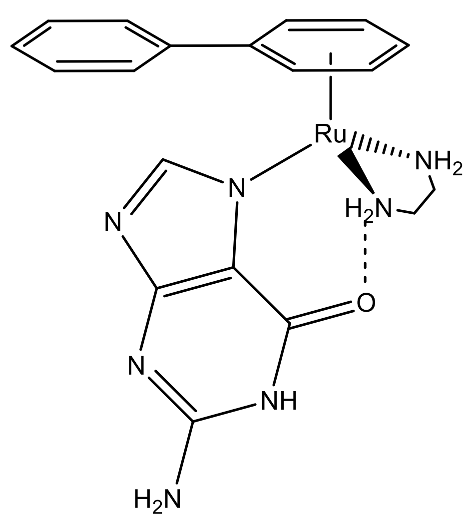


Figure 2.5 RM175 bound with guanine.[37]

Analogs with extended arenes such as $[(\eta^6\text{-tha})Ru(en)9EtG)]^{2+}$ (where 9EtG = ethyl bound at N9 of guanine) were seen to π-π stack with the guanine through their outer ring with centroid distances of 3.45 Å, suggesting that the hydrophobic interactions between the two might be the driving force for the binding activity of the Ru complex.[37] A DFT study on the binding energies of RM175, $[(\eta^6\text{-anth})Ru(en)Cl]^+$, and $[(\eta^6\text{-tha})Ru(en)Cl]^+$ with a GpC • CpG DNA fragment revealed that binding energies with the arenes as DNA intercalators are -67.63 kcal/mol, -72.45 kcal/mol, and -79.07 kcal/mol respectively.[38] RM175, while it has an extended phenyl ring, is nonplanar, with the outside ring twisted by 24.6 ^oC,[27] preventing it from intercalating as well as the other compounds. These data support Sadler's findings on the stacking properties of extended vs. non-extended arenes. In addition, these calculations indicate that the less saturated the extended arene, the more CH-π stacking interactions it would be able to have, increasing its stability and total number of π-stacking interactions.[39]

b. GLUTATHIONE

Glutathione, or γ-Glu-Cys-Gly, is a cellular tripeptide that exists at intracellular concentrations ranging from 0.5 mM to 10 mM.[40] In its reduced form (GSH), it has been shown to bind with Pt(II), making it a factor in cisplatin resistance in cells.[41] In addition, as with many thiols, GSH can be oxidized to its disulfide form (GSSG), indicating it is also able to reduce metal ions present within the cell.[42] Therefore, GSH can reduce Ru(III) and bind Ru(II), as is the case with $[Ru(NH_3)_5Cl]$.[43] This process prevents the compound from interacting with DNA or other biomolecules. Competitive reactions of RM175 with GSH and cGMP, however, showed that, even in the presence of excess GSH (250x GSH : 20x Ru-arene : 25x cGMP), the Ru-cGMP adduct formed.[44] The unexpected formation of an S-bound sulfonato complex, in addition to the thiolato product when RM175 reacts with only GSH under simulated physiological tumor conditions, suggests that oxidation of the thiolato product by residual molecular oxygen leads to the more labile sulfenato complex, which can then subsequently be displaced by guanine.[44] These data are additional support for the hypothesis that Ru-arene(XY)(Z) will not be affected by the same resistance mechanisms as cisplatin.

c. UBIQUITIN

Ubiquitin (Ub) is a prominent intracellular protein. It has two major binding sites, Met1 and His68, both of which are easily capable of binding Ru compounds.[33,34] Once bound to Ub, Ru-arene(XY)(Z) adducts are extremely stable, acting to sequester the drug from interactions with prominent biomolecules. For example, displacement studies of [(η^6-*p*-cym)Ru(20-methyl-3-oxo-*4H*-pyrano-4-onato)Cl]-Ub adducts with dGMP showed no dGMP adduct formation after one week in aqueous solution.[33] Therefore, Ub's behavior as a biomolecular sink should be carefully monitored with regard to Ru anticancer complexes.

2.1.3 MECHANISM

Kinetics and ligand exchange rates play a large role in the activity of Ru-arene compounds. In order for any of these compounds to coordinate with a biomolecule, a coordination site must open up first. Usually, this step involves hydrolysis of the complex to the active mono-aqua species.[29] For chloride species, this is usually in the cell when the Cl concentration changes from ~100 mM in the extracellular fluid to 20-55 mM in the cell.[45] One study showed that, in the unlikely event that the chelating ligand in [(η^6-*p*-cym)Ru(XY)Cl] is hydrolyzed at pH~5, such as that found in a tumor which has become hypoxic from the production of lactic acid during glycolysis, the predominant species would be the chloride species, not the fully hydrolyzed $[\{Ru(\eta^6\text{-}p\text{-cym})\}_2(\mu^2\text{-}OH)_3]^{2+}$ species,[39] which is more difficult to substitute. This indicates that the compound might retain activity. It is, therefore, reasonable to expect that most of the uptaken compound would be activated and capable of creating lesions in DNA. The specific mechanism of action of these compounds is still a subject of debate,[30] but most reviews on the topic focus on the DNA-binding capabilities of the Ru-arene complexes after hydrolysis.[1,46]

Interestingly, the substitution of a thiol ligand seems to change the coordination mechanism of the complex. For instance, substitution of SPh for Cl causes the first mechanistic step to change from hydrolysis to possible direct coordination with guanine.[29] Finding that $[(\eta^6$-hmb)Ru(en)SPh$)]^{2+}$, which undergoes no aquation and only slow anation with Cl^- ($t_{1/2}$=107 min), could bind to GMP, Sadler and coworkers surmised that the thiolate ligand was oxidized by oxygen and subsequently displaced by guanine, eliminating the hydrolysis step completely.[29]

Recent studies suggest that the interactions with biomolecules may play a more prominent role in the activity of these complexes than originally thought. The amino acid Cys, for example, shows preferential displacement of $[(\eta^6$-*p*-cym)Ru(20-methyl-3-oxo-*4H*-pyrano-4-onato)Cl] from its Ub adduct when compared to dGMP, implying a preference for protein binding over that of DNA-binding.[33]

Since the initial interest in Ru-arene complexes, a variety of different complexes have been synthesized. Recently, two neutral Ru-arene complexes were synthesized in imitation of cisplatin, $[(\eta^6$-*p*-cym)Ru$(Cl)_2NH_3]$ and $[(\eta^6$-bip)Ru$(Cl)_2NH_3]$.[47] Studies have shown that both compounds form adducts with 9-EtG, proving them both capable of forming mono- and diguanine adducts. However, neither compound showed any cytotoxicity in A2780 cells, providing further evidence of the importance of tuning ligand substitution reactivity so that compounds do not become deactivated during transit into the cell.

2.2 RAPTA COMPLEXES

RAPTA, or Ru-arene-1,3,5-triaza-7-phosphaadamantane (pta), complexes were initially investigated by Paul J. Dyson and his colleagues. The pta ligand was designed as a solubility trigger; normally extremely soluble, its intracellular protonated form moves less swiftly through biological tissues, presumably becoming trapped within the cell.[48] The first RAPTA complex, [(η^6-*p*-cym)$RuCl_2$(pta)], known as RAPTA-C (see Figure 2.6),[49] was found to remain neutral at physiological pH, only to bind to pBR322 DNA as the pH was gradually lowered from 7.5, presumably after the complex became protonated.[48] However, it was later found that this complex has a pK_a of 3.13, indicating this as an unlikely mode of action.[49] While RAPTA-C was proven to be almost nontoxic in cells, it displayed remarkable *in vivo* antimetastatic activity that has encouraged interest in this group of compounds.[49]

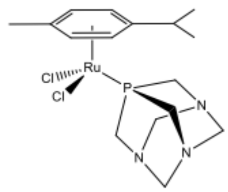
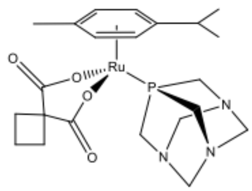
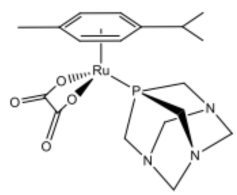
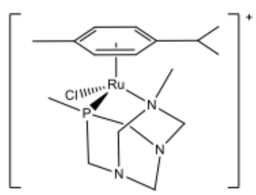
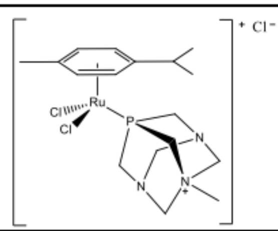
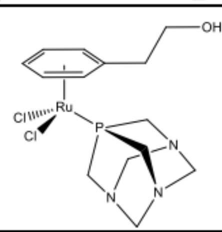
Name	Complex	Cathespin B IC_{50} (μM)
RAPTA-C		2.5 ± 0.5
carbo-RAPTA		5 ± 0.6
oxali-RAPTA		>200
ptn-RAPTA		ND
RAPTA-Me^+-C		6.5 ± 0.4
RAPTA-OH		1.6 ± 0.4

Figure 2.6 Examples of RAPTA complexes and their ability to inhibit the cathespin B.[64] ND indicates the experiment was not done.

2.2.1 STRUCTURE-ACTIVITY RELATIONSHIP

Several structure-activity relationship studies have been completed on RAPTA-C complexes. Substitution of a bidentate carboxylate ligand such as oxalate (as is used to modify cisplatin to form oxaliplatin) or 1,1-cyclobutanedicarboxylate (as is used to modify cisplatin to form carboplatin) for the two monodentate chloride ligands in RAPTA-C not only makes the compounds more soluble but also reduces the pKa of the complex from 3.13 to 2.35 and 2.64, respectively.[50] Their stability and resistance to hydrolysis in water were also increased. Furthermore, the complexes were able to bind ssDNA as the original RAPTA-C can,[51] indicating either that the ligands remained labile enough to be displaced or that the arene was capable of slipping.[50] However, the use of the pta ligand as a bidendate P, N, ligand, the first P,N chelating ligand to be studied with regard to organometallic Ru complexes, completely negated the ability of the corresponding RAPTA analog to bind with ssDNA, likely because of a decrease in hydrolysis rate.[52] The cytotoxicity of the compound increased relative to the original RAPTA-C complex, changing the IC_{50} value from 353 μM to 278 μM in A2780 cells.[52] Overall, however, bidentate substitutions do not improve this class of compounds' cytotoxicities much beyond that of RAPTA-C; the best IC_{50} value obtained out of a series of cells was still only 265 μM, i.e. virtually nontoxic.[50] In addition, modification of the pta ligand with a methyl group was found to change the activity of the RAPTA complexes such that they were more selective towards healthy human breast epithelial HBL-100 cells than metastatic mouse mammary adenocarcinoma TS/A cells, probably because of increased DNA interactions from the now positively charged complex.[49]

One study that focused on the fact that majority of metallic drugs are designed to target DNA, attempted to increase the DNA-binding ability of the RAPTA complexes by adding a hydrophilic hydroxyl group to the arene ligand.[51] While it did increase the compound's ability to bind to DNA, it simultaneously increased its general toxicity and decreased its specificity for tumorigenic cells. For example, the hydroxyl analog of RAPTA-C, RAPTA-OH, was found to increase the cytotoxicity in healthy HBL-100 cells from >1000 μM to 778 μM. While RAPTA-OH's cytotoxicity is still negligible, it is obviously unfavorable to decrease the specificity of the RAPTA compounds for cancerous cells.

Adjusting the leaving ligand seems to have a greater affect on the RAPTA complexes. PPh_3 has been substituted for one of the chloride ligands on RAPTA-C and RAPTA-OH complexes, rendering them more hydrophobic, less able to bind to biomolecules, and more cytotoxic.[53] Although the authors did not comment on it, PPh_3, due to its positive charge, has been used as a targeting agent for mitochondria in delivery of small molecules.[54] It is possible that this increase in cytotoxicity is due to mitochondrial targeting and the subsequent increase in apoptosis, rather than simply as a result of a decrease in hydrophobicity.

2.2.2 BIOMOLECULE-BINDING

a. DNA-BINDING

DNA-binding has been shown to be less relevant to the mechanism of these compounds than with Ru(arene)(XY)(Z) compounds. When made more soluble with the substitution of a PPh_3 ligand for a Cl ion, the complex was found by ESI to have increased DNA binding activity when compared with its RAPTA-C analog.[53] This increase in binding increased the general toxicity of the complex, as would be expected, rendering it less useful for clinical studies, despite also simultaneously limiting its reaction with intracellular proteins.[53] This increase in general toxicity due to an increase in DNA-binding ability is also seen when comparing the activity of

RAPTA-OH to RAPTA-C (see previous section).[51] As with Ru(arene)(XY)(Z) complexes, RAPTA-type complexes also show preferential binding with N7 of guanine.[55]

b. HISTONES

Histones are proteins that make up half of the mass of a human chromosome. Eight histones form the core around which DNA 145bp in length wraps, forming ordered structures called nucleosome core particles (NCPs).[56] These proteins are therefore important intracellular targets for studying potential interactions with drug candidates. Reactions of RAPTA-C with crystalized NCPs, showed three binding sites on the same face of the NCP: one with Gln and Lys side chains, one with two Gln side chains, and one with one His side chain with possible stabilization from a nearby (4.0 Å) Glu carboxyl.[57] Additional binding sites are highly likely: the crystal packing arrangement sterically hindered certain binding sites, and, under saturating conditions in solution, eight RAPTA-C adducts formed. Although not mentioned in the paper, it seems unlikely that it is a coincidence that the number of adducts formed and the number of histones in a NCP are equal. It is interesting to note, however, that no binding was seen with DNA, in contrast to the studies of RAPTA-C with 14-mer DNA strands mentioned previously.[51]

c. UBIQUITIN

As with Ru(arene)(XY)(Z) compounds, Ub can bind with RAPTA-C completely, forming monoruthenated adducts and sequestering the complex from further activity.[53] This action can be mediated by substituting the more sterically hindered PPh_3 for the Cl (see section 2.1.2.1), but unfortunately there is a general increase in toxicity as well.[53] Interestingly, Ub-binding with RAPTA-C can be reversed through the addition of proteins such as GSH[58] and rabbit metallothionein-2,[59] meaning these complexes, if administered in excess, may be able to be removed before creating excessive cellular toxicity from nonspecific protein-binding.

2.2.3 MECHANISM

Although RAPTA-C undergoes hydrolysis of both chloride ligands at 4 mM NaCl and not at 100 mM NaCl, it showed no primary cytotoxicity in TS/A cells.[49] However, *in vivo* studies of RAPTA-C on a MCa mammary carcinoma showed that, with 200 mg/kg doses (2x), there was a reduction in the average number of lung metastases from 14.6 ± 4.5 to 6.3 ± 3.5 and a lesser change in metastases weight,[49] hinting at the notion that this class of compounds operates under a different mechanism than ruthenation of DNA. Some RAPTA complexes have displayed some moderate cytotoxic activity, such as $[(\eta^6\text{-}C_7H_8)RuCl_2(PTA)]$ and $[((\eta^6\text{-}C_6H_5)(CH_2)_2Im)RuCl_2(PTA)][BF_4]$, which have shown selectivity for cancerous TS/A over healthy HBL-100 cells (IC_{50} = 74 μM vs. >300 μM and 66 μM vs. >300 μM, respectively).[49] Admittedly, they do not approach the activity of cisplatin with an IC_{50} value of 0.53μM.[49]

As with Ru(arene)(XY)(Z) compounds, RAPTA-C's mechanism is not well understood. When given in 40 mg/kg doses once a week for four weeks, it was seen to promote apoptosis in EAC (esophageal adenocarcinoma) cells.[60] In addition, the expression of tumor suppressor protein p53, p53 regulator JNK, cyclin E suppressor protein p21, proapoptotic protein Bax, and cytosolic caspase-cleaving protein Cytochrome *c* were increased, while the expression of Cyclin E (a protein involved in activating the transition from G1 to S phase), antiapoptotic protein Bcl-2, and cytosolic protein Procaspase-9 decreased.[60] Bcl-2 inhibits the ability of Bax to induce cytosolic release of Cytochrome *c*, which in turn cleaves Procaspase-9, triggering the caspase cascade and apoptosis (Figure 2.7).[61] Multiple types of stress stimuli stimulate JNK, which activates p53 in turn.[62] DNA damage also increases p53 concentrations, inducing the transcription of p21, which inhibits the activity of Cyclin E/G1-S/CDK complexes, halting the cell cycle at the G1 checkpoint.[63] An increase in p53 concentrations also activates transcription of the bax gene.[96] This evidence indicates that RAPTA-C is capable of both inducing apoptosis and cell cycle arrest.

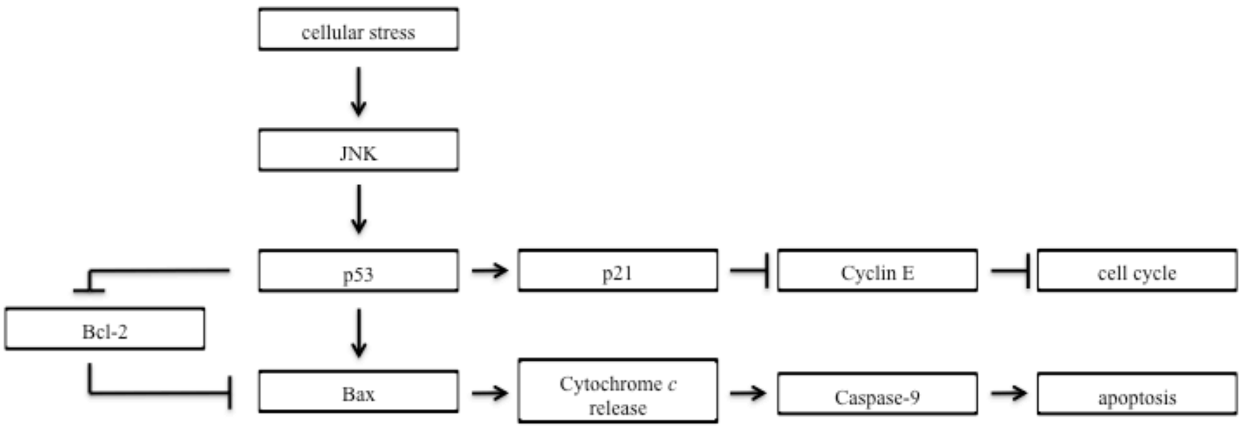


Figure 2.7 Simplified diagram of p-53 apoptosis and cell cycle arrest pathways relevant to the RAPTA-C mechanism.[60]

Localization studies using anthracene, a well-studied fluorescent molecule, attached to the arene of either RAPTA-C or a sugar-derived 3,5,6-bicyclophosphite-1-2,*O*-isopropylidene-α-D-glycofuranoside RAPTA-C analog via an amide linker, showed no localization of these compounds in the nucleus.[63] No *in vivo* studies were completed using these compounds, therefore, whether or not they truly behave like their parent compounds is unknown. While their cytotoxic profile in 12 different human cell lines resembled that of the parent compounds[63] – meaning they were virtually nontoxic – this alone is not a guarantee that that the complexes behave similarly, as the parent compounds are known to produce metastases *in vivo*. Consequently, there is still the possibility that the parent compounds may localize in the nucleus.

While some possible binding targets are known (see section 2.1.2.2), the actual cellular targets for RAPTA compounds have yet to be identified. It has been proposed that proteins are a more vital target for RAPTA complexes than DNA due to a lack of correlation between DNA-binding ability and cytotoxicity.[52] RAPTA-C's ability to inhibit cathespin B (IC_{50} = 2.5 ± 0.5 μM), a prominent protein associated with tumor migration, suggests that it is at least one of the probable targets of the complex, thereby creating its antimetastatic effect (see Figure 2.6).[64] In order to develop a tool to help elucidate the mechanism further, acetal-functionalized-benzene RAPTA complexes have been synthesized.[65] Capable of undergoing acid activation to form an aldehyde and then oxime-coupling to link with hydroxylamine-functionalized fluorophores, these complexes show promise as a tracking agent for biomolecular binding.[65] However, no biological activity data has been recorded for the new compound, so further studies on its similarities, or lack thereof, with traditional RAPTA complexes will have to be performed before it can be used in this manner.

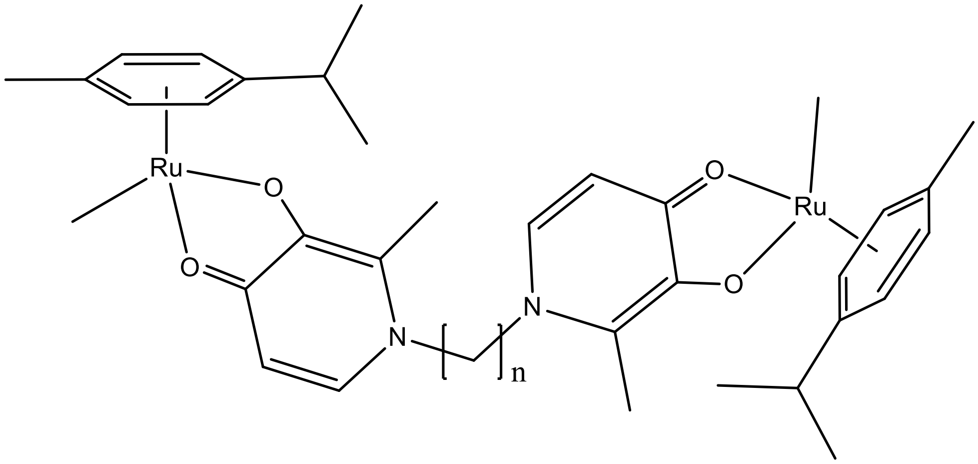
2.3 OTHER RUTHENIUM COMPLEXES

2.3.1 MULTI-NUCLEAR COMPLEXES

As previously stated, one of the most pervasive concepts in ruthenium chemotherapeutics has been to create compounds that target DNA. One problem with this particular methodology is the steric constraint imposed by the octahedral geometry found in Ru compounds, making it difficult to form traditional crosslinks. To enhance the ability of some Ru compounds to form both inter- and intrastrand crosslinks, some groups have made multinuclear complexes.[66]

The idea of forming multi-nuclear complexes to enhance activity is not novel; a trinuclear platinum compound, Triplatin or BBR3464, that showed 15-20x more activity than cisplatin *in vivo* went to phase II clinical trials before being rejected because of lack of response.[4,67] Studies in this field have been few when compared to other Ru-based complexes, likely due to early studies in which multinuclear complexes exhibited 6x less activity than their respective mononuclear complexes and 36x less than cisplatin.[22,68]

There are several variables that should be taken into consideration when designing multi-nuclear Ru complexes. Not only can one change the nature of the ligands, but one can also change the number of metal nuclei, the framework for the nuclei, and the distance between the nuclei. In one of the first studies to look at multi-nuclear complexes and their structures with regard to activity, Mendoza-Ferri *et al.* coupled two Ru(II)-arene complexes together with bis(3-hydroxy-2-methyl-4-pyridone) ligands of varying chain lengths to determine the effect of spacer length on activity.[69] In human colon adenocarcinoma SW480 cells, the longest spacer made the compound equally as active as oxaliplatin, 15x more active than cisplatin, and 170x more active than KP1019 (Figure 2.8). The mononuclear analog shows no cytoxicity. In both the SW480 and A2780 cell lines, an increase in chain length from 3 to 12 methylene groups ($PyRu_2^3$ – $PyrRu_2^{12}$) improved the cytotoxicity of the compound, most likely due to an increase in lipophilicity, making it easier for the compound to cross the cell membrane. A more recent study on rigid, supramolecular tetra-nuclear rectangular Ru-arene complexes supports the general trend that with increased spacer length one can enhance the cytotoxicity of a complex.[70]



Compound	IC_{50} (μM)
$PyrRu_2^3$	62 ± 14
$PyrRu_2^{12}$	0.29 ± 0.05
cisplatin	4.5 ± 1.7
oxaliplatin	0.30 ± 0.08
KP1019	49 ± 11

Figure 2.8 IC_{50} values in SW480 cells of multinuclear Ru complexes based on spacer length.[69]

It was later found that differential interaction with biomolecules, such as Ru(12)'s increased binding to DNA and ability to cause interhelical and DNA-protein crosslinking, may also cause differences in activity. In this case, the length of the chain allows additional arene π-π stacking, causing an increase in DNA unwinding and forming different DNA lesions than those formed by cisplatin, thus decreasing the chance of cross-resistance.[71] A more complete screen of these compounds indicated that the trend held in 9 out of 12 different cell lines.[72] This family of compounds is capable of binding to transferrin, GMP, *and* AMP in a 2:1 ratio,[72] proving them to be less specific with regard to nucleotide binding than traditional Ru organometallic complexes.

It is important to note that the complex chosen from this family of compounds for their protein studies only had 3 methylene groups "…because it had good aqueous solubility while still having a considerable spacer length…".[72] This statement highlights the fact that, while longer chain lengths may improve cytotoxicity by increasing lipophilicity,[22] they also decrease solubility, making some of them impractical for *in vivo* testing.

Additional studies have been performed on Ru(II)arene multinuclear complexes in which the arene ligand, leaving group, and number of metal centers have been varied systematically.[22] Variation of the leaving group led to complexes with instantaneous aquation whose cytotoxicity negligibly decreased as the ligand changed from Cl to I to Br. Changing the arene from *p*-cym to bip kept the activity almost exactly the same in the SW480 cell line and only decreased it slightly in A2780 cells, despite probable increased π-π stacking with DNA and decreased solubility. Previously, this lack of significant change in activity had also been seen with the same arene substitution in the mononuclear $[(\eta^6\text{-}p\text{-cym})RuCl(en)]PF_6$ compound.[27] Increasing the number of metal nuclei led to an increase in solubility from the presence of a tertiary amine, but a simultaneous decrease in activity, possibly from the decrease in lipophilicity. This find contrasts with a separate study on $[(\eta^6\text{-}arene)Ru(XY)(Z)]$ complexes attached to dendrimer scaffolds through their chelating ligands in which the number of nuclei increased the cytotoxicity of the compounds in A2780 cells due to increased DNA-binding ability.[73]

One group using $[(\eta^6\text{-}p\text{-cym})Ru(XY)(Z]$ complexes as a base synthesized a variety of different supramolecular hexanuclear trigonal prismatic cages and tetranuclear rectangles, linking sides together through a shared tetradentate, O-donor as the chelating ligand.[70,74] These O-donor ligands either had an arene of varying size or no arene at all. One might expect that the complexes with the largest lipophilic linker would show the most cytotoxicity, as has been the case with most other Ru-arene complexes. However, in both types of supramolecular structure, the second largest lipophilic face, with a $\mu\text{-}C_{10}H_4O_4$ connector, showed the greatest cytotoxic activity in five different cell lines, indicating that lipophilicity is not always an accurate predictor of activity in multinuclear complexes.

Unfortunately, while copious *in vitro* data on these compounds abound, to the best of my knowledge, no *in vivo* research has yet to be reported on organometallic Ru compounds.

2.3.2 POLYPYRIDYL RUTHENIUM COMPLEXES

Polypyridyl Ru(II) complexes are among the more traditional Ru anticancer agents studied. Although resistant to susbstitution and therefore unlikely to covalently bond with biomolecules, they are capable of either photosensitizing or intercalating into DNA.[75] Additional uses for this class of compounds abound, such as their use as cellular imaging agents[76] and catalysts.[77]

The ability of Ru(II) polypyridyl ligands to bind DNA relies closely on the ligands chosen for the complex. The relationship between DNA-cleavage ability and the ligand structure closely parallels that of Ru-arene complexes. For example, a comparison study between $[Ru(Clazpy)_2bpy]Cl_2 \bullet 7H_2O$ and $[Ru(Clazpy)_2phen]Cl_2 \bullet 8H_2O$ showed the latter to have better cleavage and intercalation abilities due to the increased hydrophobicity, π-stacking ability, planarity, and lack of steric hindrance of the phenyl group when compared to the bpy ligand.[78] Another comparative study on pBR322 binding of $[Ru(phen)_2(DMDPPZ)](ClO_4)_2$ and $[Ru(dmp)_2(DMDPPZ)](ClO_4)_2$ also found the less bulky first compound to be more efficient at single stranded DNA cleavage because of better intercalation, due to the absence of methyl groups on the phenyl ligands.[79] The same study, however, noticed there was an inverse relationship between DNA binding ability and cytotoxicity, suggesting an alternate target for these complexes other than DNA.

CHAPTER 3

TRANSLATION: HOW RUTHENIUM ANTICANCER DRUGS ARE ADDRESSING TRANSLATIONAL CHALLENGES

"Finally, we ought not to underestimate the alarming side effects of these 'anticancer' drugs. In fact, the drugs are less specific than the term implies and are really antigrowth reagents." – David R. Williams[80]

The quotation above is from a 1972 paper entitled "Metals, Ligands, and Cancer" – one of the many early 1970s review papers to discuss metals as both carcinogen and treatment in the post-cisplatin era. Without much fuss, Williams has stated the largest and most obvious problem with regard to chemotherapeutics: their lack of selectivity. Fast-growing healthy tissues, such as bone marrow, can be attacked by supposed "anticancer" drugs as if they were tumors. This imprecision in targeting, as well as other administrational challenges, has caused a flurry of research into improving the translational potential of Ru complexes.

3.1 TARGETING

As mentioned previously, the most common monodentate ligand used in Ru anticancer complexes is chloride. Beyond being a good leaving group, the chloride ligand also allows for a type of semi-selective targeted release: at physiological concentrations of NaCl (100 mM) many Ru complexes resist aquation, only becoming hydrolyzed after entering the cell.[27] However, one problem in this field of research involves the hydrolysis of compounds under "physiological" conditions. Many studies assume the textbook intracellular Cl^- ion concentration as 4 mM and extracellular Cl^- concentration as 100 mM and then perform hydrolysis studies that suggest selective, specific, strong activation of their complexes.[49] However, these values are calculated for passive diffusion as applied to the Nernst equation for normal muscle and nerve cells,[45] completely disregarding the presence of transporters, which in many cases, is extremely high for tumor cells. In fact, according to one study, A2780 cells were reported to have an intracellular Cl^- concentration of approximately 55 mM in culture,[45] meaning researchers are potentially wasting time and/or resources gathering inaccurate anation data and pursuing "dead-end" compounds whose primary tumor cytotoxicity is remarkably non-existent.[49]

3.1.1 TRANSFERRIN/APOTRANSFERRIN

It was established early that KP1019 can bind reversibly to both iron-binding sites on apotransferrin.[81] The compound is released from the protein in acidic environments (pH 4-5), such as that of an hypoxic tumor, while in the presence of intracellular chelating agents, like citrate. At low concentrations binding to transferrin (Tf) was found to increase the cytotoxicity of KP1019 in the human colon cancer cell line SW707 when compared to the free complex, suggesting enhanced uptake.[82] Calculations support this theory by noting that the reduction potential of Tf-$Ru^{III/II}$ should be biologically accessible, leading to the release of the Ru.[66] It is a commonly cited attribute of Ru complexes that Ru can imitate Fe and are less systemically toxic than cisplatin due to Tf-binding.[12]

3.1.2 ALBUMIN

Some ruthenium compounds can bind with human serum albumin, an ubiquitous blood plasma protein.[81] This protein has 23 possible sulfur and nitrogen electron donating binding sites associated with three residues: Cys, Met, and His.[33] Interestingly, Ru compounds have been attached to albumin in order to enhance their cytotoxicity through the "enhanced permeability and retention", or EPR, effect. This effect is a result of leaky vasculature within tumors caused by the inability of angiogenesis to proceed at the same rate as tumor growth. In addition, albumin, like transferrin, is also taken up to a greater extent by cancerous cells than by healthy cells. This targeting technique seems to have promise; conjugation of RAPTA-C[83] and various $[(\eta^6\text{-arene})Ru(XY)(Cl)]$[84] compounds to recombinant human serum albumin greatly increased their cytotoxicity in A2780 cells in comparison to their parent compounds. For example, the IC_{50} value of RAPTA-C changed from >300 μM to 11 μM once conjugated to albumin.[83] However, mass spectrometric analysis of the conjugates did reveal that multiple metal centers were attached to each protein, meaning it is unknown whether the enhancement in cytotoxicity is due to albumin targeting or simple multinuclearity.

3.1.3 PHOTODYNAMIC THERAPY

Photodynamic therapy (PDT) is the use of light of a particular wavelength in conjugate with an excitable metal complex to generate reactive oxygen species from molecular oxygen in order to induce necrosis and apoptosis in cells. Once excited, the metal complex should transfer its energy to triplet oxygen, exciting it and forming the reactive singlet oxygen. Successful photosensitizers should exhibit no cytotoxicity until irradiated, not only confining the area of cell death to the area of interest (i.e. the tumor) but also to the point in time in which it is irradiated. The preferred therapeutic window for irradiation is light of 650-800 nm wavelengths because of low absorptivity in tissues.

Since one of the known mechanisms of action for Ru-arene species is hydrolysis of the leaving ligand, it is reasonable to assume that control over this process should allow some control over the time and place of action. By photochemically breaking the bond between a pyridine or pyridine derivative leaving group and the Ru ion in {$[(\eta^6\text{-arene})Ru(N,N')(L)]$, where N,N' = a bpy or phen derivative}, selective hydrolysis can be induced by both UVA (320-400 nm) and white light (400-600 nm) (Figure 3.1a).[85] These complexes, which are stable in the dark for at least 2 months, bind exclusively to guanine residues in plasmid DNA, inhibiting RNA synthesis. Unfortunately, these complexes exhibit IC_{50} values as low as 7.4 μM in A2780 cells under ambient light conditions and dark/irradiation studies were not completed. These data indicate that there is another mechanism of action other than hydrolytic DNA binding at work and that, while the selective photodissociation of the ligand is interesting, these compounds are not yet capable of PDT activity.

Another type of Ru anticancer drug is the Ru(II) polypyridyl anticancer complex. Due to their extended aromatic networks, many are capable of acting like photosensitizers for PDT applications. For instance, irradiation of $[Ru(Clazpy)_2phen]Cl_2 \bullet 8H_2O$ at 325 nm or $[Ru(dmp)_2(DMDPPZ)](ClO_4)_2$ (Figure 3.1b) at 365 nm increases their ssDNA-cleavage efficiency.[78,79]

Some groups have combined the photosensitizer effects of porphyrins with those of Ru-polypyridyl complexes.[86-89] One group, looking to capitalize on a possible synergistic effect, synthesized a complex that was able to completely degrade ctDNA in under 10 minutes.[88] The insertion of zinc in the porphyrin frame redshifts the Soret band from 413 to 422 nm Figure 3.1c),[64] which has been seen for other Zn-porphyrin-Ru-polypyridyl photosensitizers.[87] This shifts the compound's photochemical excitation more towards the ideal PDT therapeutic window and modulates the cytotoxicity of the compound so that irradiation of ctDNA bound by the complex only shows dsDNA breaks after 30 min. of irradiation with a 100W mercury arc lamp in which wavelengths below 400 nm are blocked out.[89] This group did perform tests on cells that supposedly showed preferential apoptosis towards melanoma instead of healthy cells; however, they only used morphological characteristics to quantify apoptosis which is necessarily not as quantitative as flow cytometry or a MTT assay.

Combining multiple types of drug activation control is currently a hot topic in drug delivery. One way to more precisely control PDT drug placement (and therefore activation) is to attach an additional targeting moiety to the complex so that the drug is concentrated by selective uptake at the tumor site. Recently, the first compound combining the targeting ability of peptides with the triggering function of Ru-PDT complexes was synthesized.[90] $[(\eta^6\text{-}p\text{-}cym)Ru(bpm)]^{2+}$ was created with a pyridyl-bound octreotide peptide as the third ligand and was found to release the peptide only after irradiation at 420 nm (Figure 3.1d). Interestingly, the hydrolyzed form of the complex not only preferentially bound to guanine, but it also also did not interact with His, Gly, or Met, increasing the probability that the complex would be able to interact with DNA instead of another biomolecule *in vitro* or *in vivo*. However, the actual differential targeting ability of these complexes has yet to be reported.

While the control over the time and placement of Ru-PDT agents is an attractive goal with regard to moderating systemic toxicity, there are a few areas that must be improved first before these compounds reach their full potential. Firstly, more irradiated/dark cytotoxicity studies need to be completed and optimized and secondly, the Ru complexes need to be tuned in such a way that their maximum absorbance occurs within the therapeutic window of 650-800 nm.

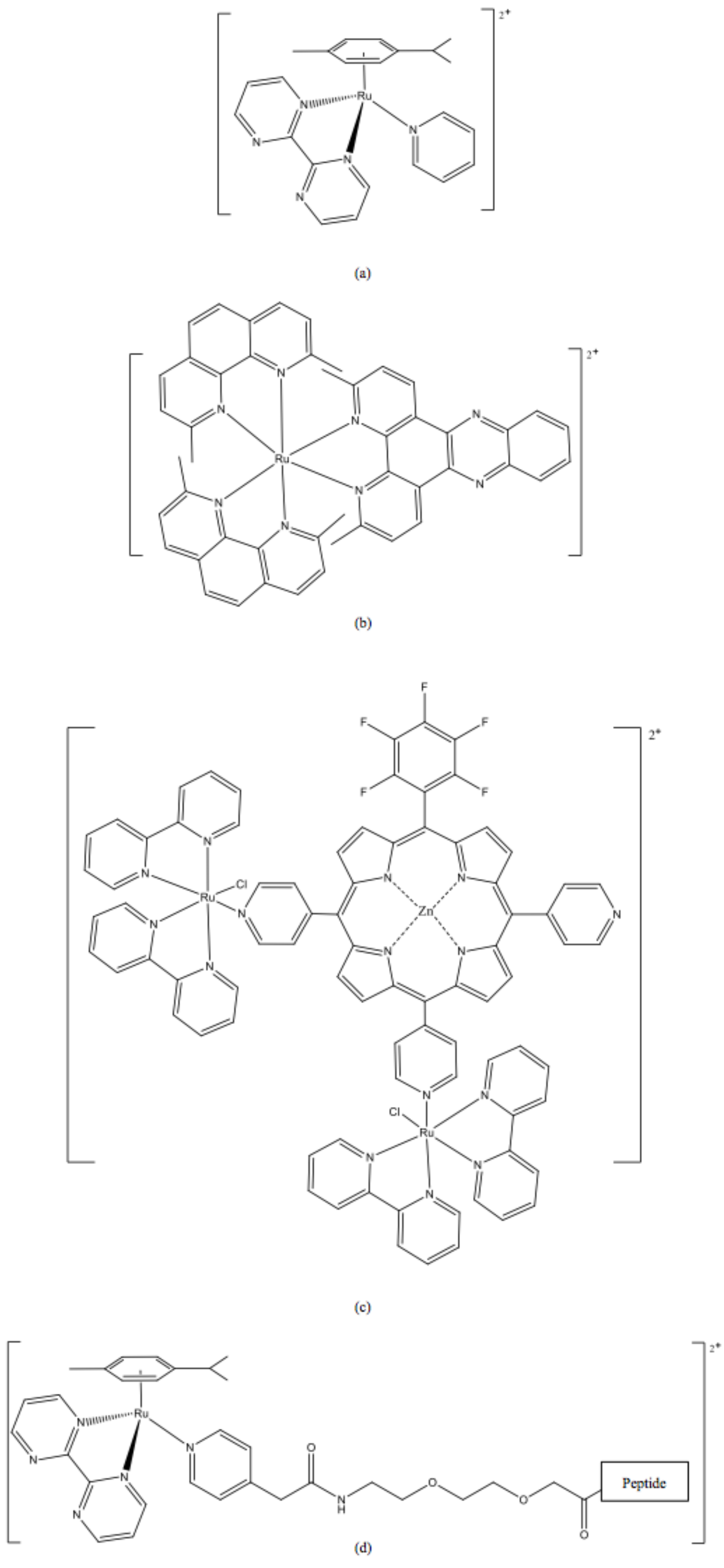


Figure 3.1 Representative Ru-PDT complexes. (A) $[(h^6\text{-}p\text{-cym})Ru(bpm)(py)]^{2+}$.[85] (B) $[Ru(dmp)_2(DMDPPZ)]^{2+}$.[79] (C) ZnPor-Ru_2.[89] (D) $[(h^6\text{-}p\text{-cym})Ru(bpm)(py)]^{2+}$-peptide.[90]

3.1.4 SUGAR MOIETIES

As has been discussed above, tumors tend to be oxygen deficient because of the inability of angiogenesis to maintain the same pace as tumor cell growth. The tumor cells must use glycolysis in order to fulfill their energy demand and, therefore, have an increased glucose uptake. Some scientists have used this mechanistic pathway as a target for cancer cells by using sugar moieties as targeting ligands.[63,91] However, attachment of sugar moieties does not guarantee enhanced uptake. Substitution of the pta ligand on anthracene-tethered RAPTA-C (see section 1.1.2) with the sugar-derived 3,5,6-bicyclophosphite-1-2,*O*-isopropylidene-α-D-glycofuranoside did not increase the uptake of the compound (305 ± 48 to 332 ± 16 pmol/10^6 A2780 cells of ruthenium), possibly due to the increase in lipophilicity associated with the anthracene moiety.[63] This particular type of targeting has yet to become prominent in Ru complex development. To the best of my knowledge this is the only case in which a carbohydrate moiety was intentionally attached to a Ru compound in order to affect its uptake, although carbohydrates have been substituted for the pta ligand in RAPTA complexes in order to affect their hydrolytic properties;[92,93] it was found the their cytotoxicities in a variety of cell lines were negligible.

3.2 DRUG ADMINISTRATION

One of the main issues with these types of compounds in regard to clinical development is that they are extremely susceptible to hydrolysis during storage, therefore, they must be administered intravenously in saline solution. KP1019, one of the drugs currently undergoing clinical trials, is an example of a drug that must be administered intravenously.[19] Ruthenium complexes can be made more clinically attractive by designing them so that they can be administered orally instead. For oral administration to be a valid option, the complex must not only be resistant to saliva and stomach enzymes, but also must have the ability to be taken up by the transporters in the small intestine.

One characterization of the RAPTA complexes is their sensitivity to pH changes.[48] RAPTA-C can be modified by exchanging the chloride ligands for oxalate or 1,1-cyclobutanedicarboxylate to have low pKa's of 2.35 and 2.64, respectively.[50] Gastric acid pH can range from 1.5-3.5, suggesting that these complexes may survive oral administration.[50]

3.2.1 RUTHENIUM COMPLEXES AS CARRIERS

The usefulness of a drug is a combination of a variety of factors such as lipophilicity, solubility, and molecular weight, all of which impact bioavailability, as well as cellular uptake and excretion. Many of these factors have been mentioned above with regard to the design of Ru-based drugs, and it goes without saying that they are necessarily applicable to organic molecular drugs as well. Some groups have taken a decidedly less traditional approach to Ru-based chemotherapeutic drug design by using the rigid structural characteristics of Ru coordination compounds to design and synthesize macromolecules capable of "delivering" other problematic chemotherapeutics to tumor cells. In particular, Dyson and coworkers have take a hexanuclear trigonal prismatic cage, encapsulated otherwise insoluble $[Pt(acac)_2]$ and $[Pd(acac)_2]$, and shown that the cage is capable of release hydrophobic molecules intracellularly,[94] greatly enhancing the encapsulated compounds activity.[95] Although more widely explored with regard to nanoparticulate delivery of hydrophobic drugs, this field will likely see an increase of activity in the near future as more research is completed on Ru multinuclear complexes.

CHAPTER 4

FUTURE DIRECTIONS

In Figure 4.1, the number of times different metallochemotherapeutic anticancer concepts have been published has been recorded as the number of hits per year in the Scifinder Scholar database. A similar figure in a 2006 review by Bernhard K. Keppler illustrated how, up until 2005, most research on anticancer compounds had focused on platinum compounds. However, interest in ruthenium was growing.[19] As can be clearly seen from the graph, the rate has substantially increased in the years since then.

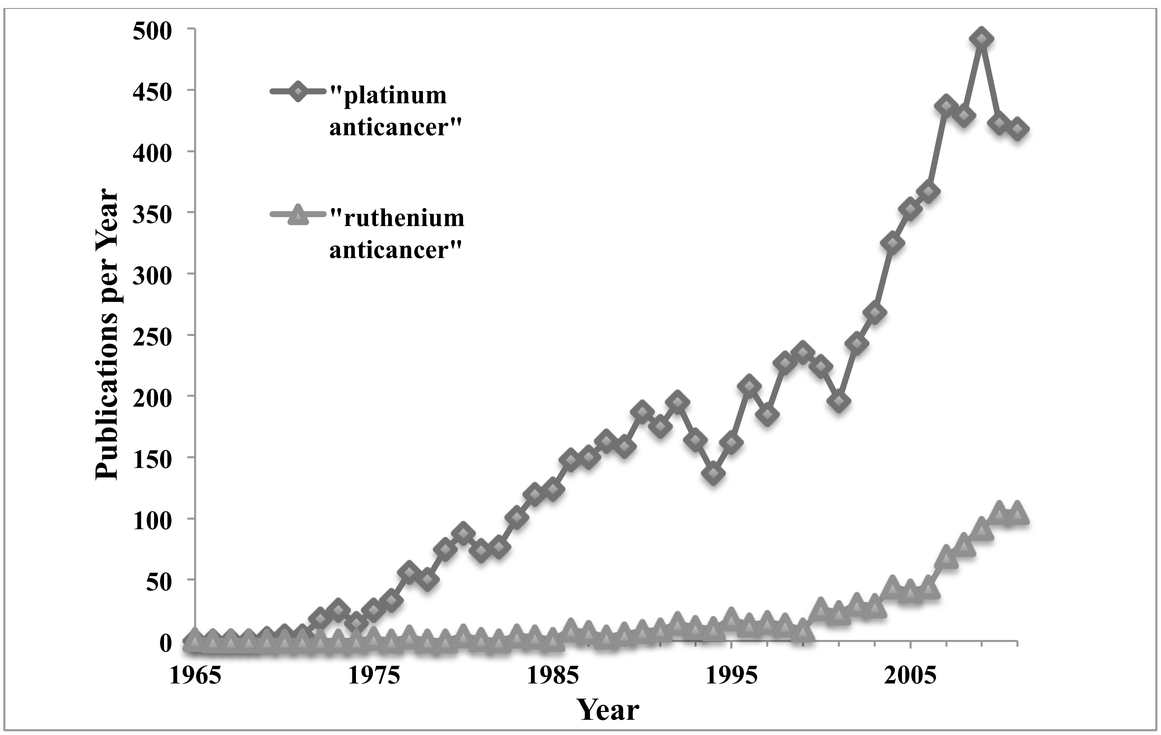


Figure 4.1 Graph of number of hits according to Scifinder Scholar for "platinum anticancer" and "ruthenium anticancer" according to year.

Consequently, a slew of compounds has been tested, declared cytotoxic, and therefore must be “potential anticancer agents”. As has been cautioned from the early days of transition metal chemotherapeutics, cytotoxicity data does not necessarily coincide with drug effectiveness.[5] Unfortunately, the costs of obtaining *in vivo* data are often prohibitive, severely limiting the quantity of data available in this regard. Three-dimensional cell culture, however, may only require the purchase of matrix gel, making it an excellent alternative for those who are unable to fully finance mouse studies. In addition, 3D cell culture can aid in narrowing the therapeutic window in order to reduce the number of mice required to obtain the desired data. Even an increase in selectivity studies of healthy versus cancerous cells, as recommended by Dyson with regard to platinum therapeutics,[53] would be more useful in choosing which compounds show the most promise. More recently, studies have begun to look for "characteristics" of metastases hindrance, such as adhesion, migration, invasion, and degradation of the extracellular matrix in order to determine whether of not their compound has potential as an antimetastatic agent.[32] This is also an excellent method for determining the activity spectrum of a potential drug.

Bearing all this in mind, it is difficult to predict which Ru anticancer agents seem to have the most promise, due to discrepancies between scientists in determining anticancer potential. In general, Ru organometallic chemotherapeutics seem to hold the most promise in preclinical studies due to the predictability of their properties. Additional research focused on increasing the efficiency of these compounds through some type of targeting method, whether it is receptor- or PDT-mediate, will only increase the probability that these compounds will enter clinical trials.

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