# Ruthenium polypyridyl complexes with anticancer properties

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# 5. Explorations towards novel ruthenium anticancer drugs

Most of the compounds described in this thesis show a certain degree of activity in some selected cancerous cell lines. The research presented so far suggests that the mechanism of action of some of these compounds, namely the mononuclear ruthenium(II) complexes **1a-c**, **1e** and **1f**, might involve coordination to DNA. In this chapter, other alternative interaction modes with DNA are dealt with and a number of suggestions are presented for further development of this research line.

### *5.1. Alternative ways of interaction between metallodrugs and DNA*

#### *5.1.1. Introduction*

Anticancer therapy with classical ruthenium coordination compounds is based on the capability of the metal to coordinatively bind to DNA.[1] These ruthenium complexes are usually bifunctional and they mostly exert their action by forming intra- or interstrand crosslinks with the DNA molecule.[2] On the other hand, examples of monofunctional ruthenium complexes are also known that display an anticancer activity, such as some of the complexes described in this thesis (**1a**-**c** and **1e**). The cytotoxicity of these monofunctional complexes could also be related to coordination to DNA.

Other ways of interaction with DNA are known, including backbone binding[3] and recognition of DNA junction structures.[4] This chapter will focus on the interactions caused by intercalation between nucleic base-pairs and on groove recognition.

**Groove binding**

The dinuclear complex $[\{Ru(apy)(tpy)\}_2\{\mu\text{-}H_2N(CH_2)_6NH_2\}]^{4+}$ (**1g**), described in chapter 4, interacts with DNA presumably via electrostatic and especially via groove-binding interactions. The activity displayed by this compound in a number of cell lines is comparable to cisplatin.

Two strategies can be followed that are inspired by the above-described results. The first one consists on the synthesis of homodinuclear ruthenium(II) complexes that are first electrostatically attracted to DNA, subsequently form a coordinative interaction with the latter, and finally interact with the DNA in the same way **1g** does, *i.e.*, by groove binding (see Fig.5.1).

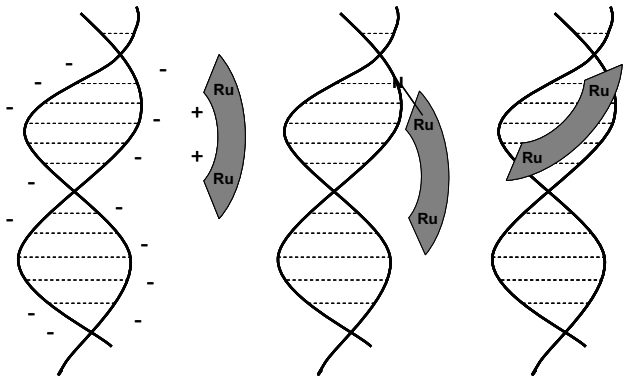


*Fig.5.1. Scheme depicting a homodinuclear, positively-charged Ru(II) complex being first electrostatically attracted to DNA (left), coordinated to a nucleic base (middle) and finally binding to a DNA groove (right).*

A second strategy deals with the synthesis of heterodinuclear Pt-Ru complexes using such ligands. The Pt moiety can be chosen such that it will form a coordinative interaction with DNA, like transplatin, or it could even be an intercalator, *vide infra*, such as $[Pt(tpy)]^{2+}$.

Following the first approach, the homodinuclear ruthenium(II) compound $[\{Ru(tpy)Cl\}_2(\mu\text{-paa})](BF_4)_2$ (**1h**) was obtained, where tpy is 2,2′-6′2”-terpyridine and paa is 2-pyridinealdazine (see Fig.5.2), and some cell tests were subsequently performed (as summarized in section 5.1.2, Table 5.1).

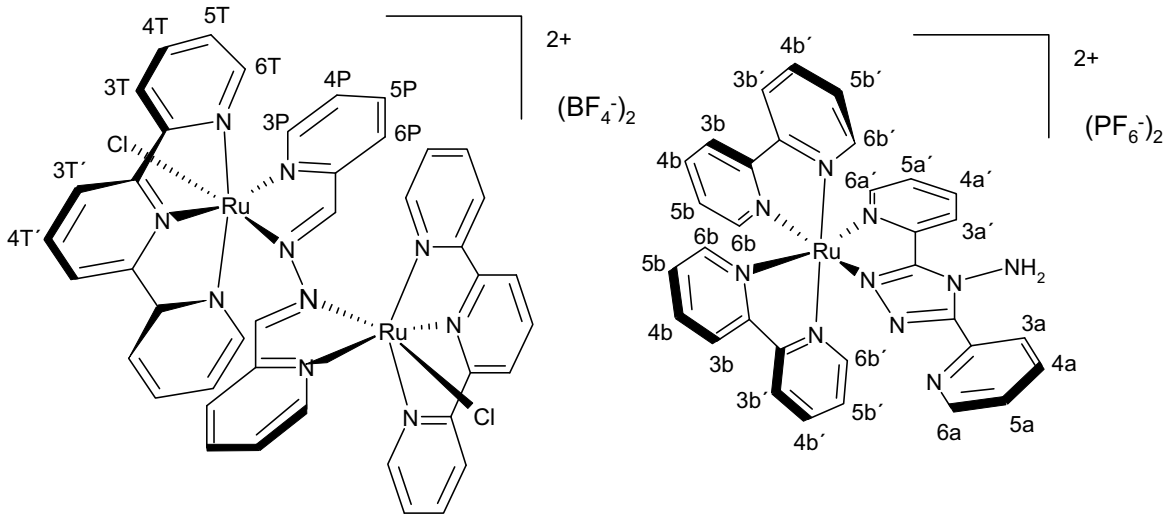


*Fig.5.2. Molecular structures of $[\{Ru(tpy)Cl\}_2(\mu\text{-paa})](BF_4)_2$ (**1h**, left) and $[Ru(abpt)(bpy)_2](PF_6)_2$ (**1i**, right). Proton numbering scheme as used in $^1H$ NMR spectra.*

**Intercalation**

Small, planar aromatic molecules can bind DNA through intercalation, as proposed already by Lerman in 1961.[5] The base pairs and helical backbone extend and unwind to accommodate the molecule, which inserts into the resulting hydrophobic pocket. The intercalating surface is stabilized electronically in the helix by π-π stacking with the bases, thus the intercalator is rigidly held and oriented with the planar moiety perpendicular to the helical axis.[6]

A decade later, the concept “metallointercalator” was introduced. The platinum(II) complexes $[Pt(tpy)(SCH_2CH_2OH)]^+$ and $[Pt(tpy)Cl]^+$, where tpy is 2,2′-6′,2”-terpyridine, were proven to bind strongly to DNA by intercalation between base pairs.[7] Subsequently,

other aromatic ligands were made to react with platinum to generate new compounds that interact with DNA in that same way.[8, 9]

Although in principle the square-planar geometry of platinum(II) was thought to be essential for a metallointercalator, octahedral metal centres with large planar aromatic ligands were synthesised afterwards, which also displayed intercalative interactions with the DNA helix.[10, 11] While one of the planar units inserts between base-pair planes, the metal and additional co-ligands interact in one of the DNA grooves.[10, 11] To date, many $[Ru(bpy)_2L]^{2+}$ and $[Ru(phen)_2L]^{2+}$ complexes have been described, where L is an aromatic bidentate ligand, which have been proven to interact with DNA via intercalation.[12-17] Even a dinuclear analogue with a large aromatic bridging ligand has been reported to very slowly bind to DNA via an intercalation process.[18]

It should be noted that distinguishing a groove binder from an intercalator is not straightforward, as illustrated by many discussions on the controversial case of $[Ru(phen)_3]^{2+}$, where phen is phenantroline.[6, 19-23]

It may be very interesting to synthesize ruthenium(II) polypyridyl ligands containing the ligands 4-amino-3,5-bis(2-pyridinyl)-1,2,4 triazole (abpt) and 3,5-bis(2-pyridinyl)-1,2,4 triazole (Hbpt), for several reasons. Firstly, some ruthenium complexes with π-deficient ligands behave as photo-oxidants, giving rise to photo-induced electron-transfer processes that lead to DNA cleavage.[24-27] Moreover, the strong σ–donor properties of the triazole/triazolate groups make these ligands optimal for use as bridges in the synthesis of dinuclear and polynuclear complexes.[28-31]

An especially interesting feature of this kind of complexes is the luminescence displayed by some of them.[32] Finally, the abpt and Hbpt ligands may behave as intercalators.

The ruthenium(II) complex $[Ru(abpt)(bpy)_2](PF_6)_2$ was synthesized and its anticancer activity was tested against some selected cell lines. Although this complex displayed an activity comparable to that of cisplatin in the cell line H226 and a reasonable activity in the cell line WiDR (see Tables 5.1 and 5.2), it was found to be virtually inactive in the rest of the tested cell lines. The interaction of this compound with DNA remains to be studied.

### *5.1.2. Experimental*

**Materials and reagents**

2-pyridinealdazine (paa), 4-amino-3,5-bis(pyridine-2-yl)-1,2,4-triazole (abpt), $Ru(tpy)Cl_3$ and *cis*-$Ru(bpy)_2Cl_2$ were synthesized following procedures described in literature.[33-36] 2-cyanopyridine, 2-pyridinaldehyde, hydrazine monohydrate, $NH_4PF_6$ and tpy (Aldrich), LiCl (Merck), $NaBF_4$ and bpy (Acros) and $RuCl_3 \cdot 3H_2O$ (Johnson & Matthey) were used as supplied. All other chemicals and solvents were reagent grade commercial materials and used as received, without further purification.

**Physical measurements**

C, H and N determinations were performed on a Perkin Elmer 2400 Series II analyzer. Mass spectra were obtained with a Finnigan MAT TSQ-700 mass spectrometer equipped with a custom-made electrospray interface (ESI). NMR spectra were recorded on a Bruker DPX-300 spectrometer operating at a frequency of 300 MHz. Chemical shifts were calibrated against tetramethylsilane (TMS).

**Synthesis and characterization of $[\{Ru(tpy)Cl\}_2(\mu\text{-paa})](BF_4)_2$ (1h)**

LiCl (500 mg, 11.80 mmol) was dissolved in 80 ml of ethanol-water (3:1). Triethylamine (0.160 ml, 1.135 mmol) was added, followed by $Ru(tpy)Cl_3$ (500 mg, 1.135 mmol) and paa (360 mg, 1.715 mmol). The mixture was vigorously refluxed for 90 minutes, and the hot solution was filtered to remove any insoluble material. The brown solution was evaporated to dryness. 15 ml methanol were used to dissolve the residue, to which 35 ml of a methanolic saturated solution of $NaBF_4$ were added. The flask was left for 3 days at 4 °C. A brown precipitate had then appeared, which was filtered, washed with little ice-cold ethanol and ether and dried *in vacuo* over silica Yield: 39 mg (3%). *Anal.* Calc. for $C_{42}H_{32}N_{10}B_2F_8Cl_2Ru_2$: C, 44.9; H, 2.9; N, 12.5. Found: C, 42.2; H, 2.9; N, 11.7. *m/z* (ESIMS) 580.1 ($[Ru(paa)(tpy)Cl]^+$); 475.0 ($[\{Ru(tpy)Cl\}_2(\mu\text{-paa})]^{2+}$). $^1H$ NMR (DMSO-$d_6$): δ (ppm): 9.71 (2H, d, 5.49 Hz, 6P); 8.44 (8H, m, 3T, 3T′); 8.22 (2H, t, 6.93 Hz, 4P); 8.12 (4H, t, 7.12 Hz, 4T); 8.00 (4H, m, 5P, 4T′); 7.92 (2H, d, 8.06 Hz, 3P); 7.46 (4H, t, 6.24 Hz, 5T); 7.11 (4H, d, 4.83 Hz, 6T); 6.97 (2H, s, CH=).

**Synthesis and characterization of $[Ru(abpt)(bpy)_2](PF_6)_2$ (1i)**

The synthesis of $[Ru(abpt)(bpy)_2](PF_6)_2$ was carried out as described in the literature,[32] with slight modifications. *cis*-$Ru(bpy)_2Cl_2$ (75 mg, 0.18 mmol) and abpt (82 mg, 0.34 mmol) were dissolved in 15 ml of ethanol and refluxed for two hours. The mixture was evaporated under reduced pressure, and the obtained residue was dissolved in 2.5 ml methanol. 5 ml of a saturated solution of $NH_4PF_6$ were added. An orange-red solid was collected by filtration and dried *in vacuo* over silica. Yield: 32 mg (19%). *Anal.* Calc. for $C_{32}H_{26}N_{10}P_2F_{12}Ru$: C, 40.8; H, 2.8; N, 14.9%. Found: C, 39.9; H, 2.6; N, 14.9%. *m/z* (ESIMS) 797.1 ($[Ru(abpt)(bpy)_2][PF_6]^+$), 326.1 ($[Ru(abpt)(bpy)_2]^{2+}$). $^1$H NMR (MeOD-$d_4$): δ (ppm): 9.13 (2H, d, 7.87 Hz, 3a); 8.75 (1H, d, 4.75 Hz, 6a′); 8.68 (2H, m, 6b′); 8.61 (2H, m, 6b); 8.11 (6H, m, 4a, 3a′, 5b, 5b′); 7.93 (3H, m, 4a′, 3b); 7.82 (3H, m, 6a, 3b′); 7.52 (5H, m, 5a′, 4b, 4b′); 7.43 (1H, t, 6.45 Hz, 5a).

***In vitro* cytotoxicity assays**

The anticancer activity of $[Ru(abpt)(bpy)_2](PF_6)_2$ was tested *in vitro* in several selected cell lines, following the experimental procedure described in chapter 4 of this thesis. The results can be seen in Tables 5.1 and 5.2. Preliminary results are also given for the dinuclear complex $[\{Ru(tpy)Cl\}_2(\mu\text{-paa})](BF_4)_2$.

### *5.1.3. Results, discussion and concluding remarks*

The synthesis of groove-binder homodinuclear ruthenium(II) and heterodinuclear Pt-Ru complexes has been introduced. As a possible example of the former, the ruthenium(II) compound $[\{Ru(tpy)Cl\}_2(\mu\text{-paa})](BF_4)_2$ (**1h**) was obtained. This dinuclear compound has two leaving groups, one per ruthenium atom, therefore a coordinative interaction with DNA is also possible, and even the formation of intra- and interstrand adducts might be expected.

According to the results obtained in preliminary cell tests (see Table 5.1), complex **1h** is moderately active in the L1210/2 cell line, although it displays virtually no activity in the human ovarian cancer cell lines A2780 and A2780R, in which the homodinuclear complex **1g** was shown to be active (see Table 4.3).

The ruthenium(II) complex $[Ru(abpt)(bpy)_2](PF_6)_2$ (**1i**) was selected as the parent compound of a family of ruthenium(II) polypyridyl complexes to be tested for anticancer activity. Substitution of the bpy groups by other chelating polypyridyl ligands, such as 2,2′:6′,2”-terpyridine or phenantroline, or the more π-deficient 2,2′-bipyrazine, 1,4,5,8-

tetraazaphenanthrene or 1,4,5,8,9,12-hexaazatriphenylene, would yield a group of various related ruthenium(II) complexes. The cytotoxicity of all these compounds should be tested, as well as their ways of interaction with DNA and their DNA cleavage ability. Some structure-activity relationships could be extracted from the differences in their properties and anticancer activities.

Work in these compounds has not gone yet any further than the synthesis and testing of the chosen parent compound against some selected cancer cell lines. The activity displayed by $[Ru(abpt)(bpy)_2](PF_6)_2$ was disappointing in most of the cell lines (see Tables 5.1 and 5.2). Considering that this compound is structurally very different from the other compounds described in this thesis, no conclusions can be extracted by comparison of the results listed in Tables 5.1 and 5.2 with the results described in chapter 4.

*Table 5.1. $IC_{50}$ values (μM) of $[\{Ru(tpy)Cl\}_2(\mu\text{-paa})](BF_4)_2$ (**1h**), $[Ru(abpt)(bpy)_2](PF_6)_2$ (**1i**) and the reference compound cisplatin in some selected cell lines*

| Tested compound | A2780 | A2780R | L1210/0 | L1210/2 |
|---|---|---|---|---|
| $[\{Ru(tpy)Cl\}_2(\mu\text{-paa})](BF_4)_2$ (**1h**) | 61 | > 100 | 36 | 53 |
| $[Ru(abpt)(bpy)_2](PF_6)_2$ (**1i**) | > 200 | > 200 | > 200 | 75 |
| Cisplatin | 6 | 25 | 2 | 24 |

*Table 5.2. $IC_{50}$ values (μM) of $[Ru(abpt)(bpy)_2](PF_6)_2$ (**1i**) and the reference compound cisplatin in some selected cell lines*

| Tested compound | A498 | EVSA-T | H226 | IGROV | M19 | MCF-7 | WiDR |
|---|---|---|---|---|---|---|---|
| $[Ru(abpt)(bpy)_2](PF_6)_2$ (**1i**) | 43 | 43 | 14 | >65 | 44 | 44 | 27 |
| Cisplatin | 7 | 1 | 11 | 1 | 2 | 2 | 3 |

### *5.2. Interactions between metallodrugs and other biological molecules*

#### *5.2.1. Introduction on serum proteins*

**Albumin**

Serum albumin is the most abundant plasma protein. It plays a key role in a number of physiological functions, such as the control of osmotic blood pressure; transport, metabolism and distribution of various compounds; radical deactivation, and delivery of amino-acids after hydrolysis for the synthesis of other proteins.[37]

**Transferrin**

The transferrins are a class of iron-binding and transporting proteins, widely distributed in the extracellular fluids of vertebrates. Most of the transferrins consist of a single polypeptide chain with a molecular weight of around 80 kDa, constituted by two remarkably similar amino acid sequences, each accounting for half of the molecule and each carrying an iron-binding site.[38]

Binding of iron is dependent on concomitant binding of carbonate, hydrogencarbonate or some other synergistic anion, which serves as a bridging ligand between protein and metal. The role of the bridging anion may be to prevent water from binding in the coordination sphere of the metal, locking it tightly to the protein and avoiding hydrolysis. Iron binding is strong enough to resist hydrolysis in the extracellular fluids, but still allows iron to be released within specific intracellular compartments. The metal binding site with its associated anion-binding site is a characteristic of all transferrins.[38]

The iron-binding cleft in the C-lobe is closed, both in the presence and in the absence of the metal. However, the cleft in the N-lobe is wide open in apotransferrin, exposing three basic side chains, which are buried within it in the iron-loaded transferrin. These side chains are Arg 121, Arg 120 and Lys 301; they may serve to attract the carbonate anion as the first step in binding.[38]

Transferrin receptors are present in all dividing cells, in a number varying from several tens of thousands to almost a million. This number increases when a cell is in need of iron. Transferrin receptors are continuously traveling between the surface and the interior of the cell.[38]

At the slightly alkaline extracellular pH of 7.4, transferrin can bind 1 or 2 ferric ions, and 2 iron-bearing transferrin molecules can bind the dimeric transferrin receptor. Iron-free transferrin is not recognized by the receptor at this pH.[38]

Transferrin is thought to release its iron within the cell in an endosomal compartment which has a pH of 5.5. Then the apotransferrin-transferrin receptor complex travels back to the membrane, and the apotransferrin is released again in the extracellular medium.[38]

**Cytochrome *c***

Cytochrome *c* is a mitochondrial peripheral membrane protein. Its function in the respiratory chain in the inner mitochondrial membrane consists on electron transfer from cytochrome *c* reductase to cytochrome *c* oxidase.[39] In 1996 it has been reported that, when released into the cytosol, cytochrome *c* activates a programmed cell death cascade (apoptosis).[40]

**Other proteins**

Haemoglobin is a globular tetrameric protein consisting of four subunits (two α- and two β-polypeptide chains) bound through non-covalent interaction. Each protein subunit carries a haeme group including a Fe(II) as the central atom.[37] Haemoglobin is in charge of $O_2$ and $CO_2$ transport in the blood.

Ubiquitin is a small cytoplasmic protein which has two potential binding sites for cisplatin. It was chosen as a model protein to study the formation of protein-cisplatin adducts.[41, 42]

Another familiy of essential metal-transporting serum proteins are the γ-globulins.[37]

### *5.2.2. Interactions between metallodrugs and serum proteins*

Protein interactions with platinum drugs, amongst which cisplatin and carboplatin, have been studied thoroughly, using various techniques. The influence of these interactions in the distribution and pharmacokinetics of the drugs has been recognised.[37, 39]

**Albumin**

Cisplatin binds preferentially to haemoglobin, followed by albumin.[37] The efficient binding to the latter can be explained by the high affinity of platinum to sulfur. Hence, the most likely binding point of cisplatin to albumin is the cysteine-34 residue. Cisplatin

irreversible binding leads to cleavage of albumin disulfide bonds, inducing changes in the structure of the protein, thus affecting its activity. Other platinum compounds, such as oxaliplatin (see chapter 1, section 1.3), display the same behaviour with albumin; the interaction between albumin and transplatin is reported to be not very significant.[37]

**Transferrin**

In an analogous way, cisplatin binds to sulfur-containing residues of transferrin, although the exact interaction position is a subject of debate.[43, 44] This interaction was proven to be determinant of properties such as cytotocixity, *in vivo* distribution of the drug and tumour-specificity.[45]

Certain anticancer ruthenium(III) complexes, such as indazolium *trans*-[tetrachloridobis(indazole)ruthenate(III)], KP1019, were also proven to bind to both albumin and transferrin. Particularly the interaction of KP1019 with the latter suggested the theory that this ruthenium(III) complex could act as a virtually non-toxic prodrug that enters the cell when it is bound to transferrin. This prodrug would then be activated by intracellular reduction to a ruthenium(II) complex, which would be the actual cytotoxic drug.[46] This mechanism would also account for a selective entrance of the drug in the tumorous cells, which express an increased number of transferrin receptors in their membranes, due to their higher iron requirements.[39]

A study of the ability of ruthenium(III) cytotoxic compounds to bind to transferrins was carried out in 1996.[47] The presence of a large water-filled cavity in the interdomain cleft of each transferrin lobe, in which the metal- and anion-binding site is found, apparently allows some flexibility in the species that can be bound, while domain closure is still possible. Cell-culture experiments have given evidence that the antitumour capacity of some ruthenium(III) complexes is enhanced by binding to transferrin,[47] and so the role of serum transferrin in the accumulation of ruthenium(III) complexes in tumours is suspected to be important. The ruthenium complex binds via a coordinative interaction with a histidine residue in the N-lobe of transferrin. The heterocyclic ligands remain bound to ruthenium, and this is presumably essential for antitumour activity following the release of the complex.[37, 47]

**Cytochrome *c***

The results obtained with various techniques indicate that the binding of the ruthenium(III) complex KP1019 to cytochrome *c* induces conformational changes in the protein. A loss of tertiary structure is experienced, together with changes in the haeme group and an increase in the α–helical content of apocytochrome *c*.[48] These conformational changes are expected to have an influence in the biological activity of cytochrome *c*, and subsequently, in its ability to induce cell apoptosis.

**Other proteins**

The binding of different platinum complexes to the serum proteins haemoglobin, ubiquitin and γ-globulins has been widely studied and a review of these interactions is available.[37] On the other hand, the studies involving ruthenium(III) complexes have been mainly focused on the interactions between these drugs and transferrin or cytochrome *c*.

### *5.2.3. Interactions between Ru(II) polypyridyl complexes and serum transport proteins*

Some ruthenium(III) complexes are hypothesised to act as inactive prodrugs, which may get activated by reduction to ruthenium(II) once they entered the cells, *vide supra*. Serum transport proteins, such as transferrin, might be involved in this cellular uptake process. Hence the interest in studying the interactions between these proteins and the anticancer active ruthenium(III) complexes. However, while a number of ruthenium(II) complexes are known that display a considerable activity in cell tests, to the best of my knowledge no studies have been reported of the interaction between these complexes and transferrin. Therefore, a preliminary experiment was carried out to explore whether or not such interactions could occur.

Two 5 μM solutions of $[Ru(apy)(tpy)(H_2O)](ClO_4)_2 \cdot 2H_2O$ in phosphate buffered saline (PBS) were prepared. Human serum transferrin (Invitrogen) was added to one of them to give a 1 μM concentration. Both solutions were incubated for 3 hours at 37 °C. Both samples were ultrafiltered (Millipore centricon 10,000 MWCO) and the filter was washed four times with PBS. The unbound ruthenium complex should have been recovered after going through the filter in both cases. The portion that did not go through the filter should contain no ruthenium in the control experiment, and the transferrin-bound ruthenium, in the sample containing the protein. The four portions were analysed for ruthenium by inductively coupled plasma (ICP).

70% of the initial ruthenium was recovered in the portion of the control experiment that went through the membrane filter. The detected ruthenium in the portion that did not pass through the filter was negligible. From the sample that contained transferrin, the portion that went through the filter contained 34% of the initial ruthenium (unbound ruthenium), while the portion that did not go through the filter contained 35% of the initial ruthenium. This implies that after just 3 hours in PBS at 37 °C, at least 35% of the initial ruthenium was bound to transferrin.

The results obtained clearly encourage further studies of the interactions between transferrin and other ruthenium(II) polypyridyl complexes, such as those described in this thesis. Important questions still remain unanswered, such as whether this interaction has an influence in the cytotoxicity and tumour-selectivity of the compounds, or to what extent the results obtained in the performed cell tests are valid, without the involvement of serum transferrin in them.

### *5.3. Ruthenium complexes and metastasis*

The existence of ruthenium drugs which, despite showing no significant activity against the primary tumour (and no *in vitro* cytotoxicity), do yield an important activity against metastases,[49, 50] illustrates the importance of testing ruthenium complexes not only against cancerous cell lines, but also for antimetastatic activity.

Well-known *in vitro* methods for antimetastatic ability determination are migration and invasion assays. However, since apoptotic cells do not migrate and not all cancerous cells are invasive, cytotoxic compounds are not susceptible to these studies, nor is every type of cell lines.

The ability of a drug to diminish migration of a malignant cell from the initial tumour to another tissue can be measured in experiments involving Boyden chambers.[51] On the other hand, the invasion of basement membranes by tumour cells, a property which is characteristic of metastatic cells, can be studied by using Matrigel, a reconstituted membrane.[52-54]

In conclusion, a new testing routine is necessary for potential anticancer/antimetastatic ruthenium complexes. Not only should the interactions of these compounds with proteins be studied, which could lead to both selective apoptosis and a decrease in resistance to the drug, but also the antimetastatic ability of these drugs should

be tested. A broader knowledge of all these factors is expected to lead to a better understanding of the mechanism of action of ruthenium anticancer agents.

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