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Transferrin binding and transferrin-mediated cellular uptake of the ruthenium coordination compound KP1019, studied by means of AAS, ESI-MS and CD spectroscopy†

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Indazolium *trans*-[tetrachlorobis(1H-indazole)ruthenate(III)] (KP1019) shows particular promise as an antitumour agent against colorectal cancer. It is known that KP1019 reacts with human serum proteins, whereby the major amount binds to albumin (present in large excess) and a smaller amount to transferrin. It has been hypothesised that transferrin-mediated uptake by transferrin receptor expressing tumour cells may in part explain the apparent tumour selectivity of this compound. Circular dichroism spectroscopy and electrospray ionisation mass spectrometry studies demonstrate that two equivalents of KP1019 bind specifically to human apotransferrin, while additional amounts of the ruthenium complex bind unspecifically. Uptake studies in the transferrin receptor-expressing human colon carcinoma cell line SW480 revealed a higher cellular accumulation of KP1019 in comparison to a KP1019-transferrin adduct (2:1), while the uptake of a KP1019–Fe(III)-transferrin conjugate (1:0.3:1) significantly exceeded that of KP1019, suggesting that iron binding is necessary to obtain a protein conformation which favours recognition by the transferrin receptors on the cell surface. Our study showed that KP1019 is transported into the cell by both transferrin-independent and transferrin-dependent mechanisms. Transferrin-mediated uptake is more efficient when transferrin is saturated with iron to a physiological degree (~30%). Cell fractionation experiments demonstrated that after a 2 h treatment of human colon cancer cells with 10 μM KP1019 on average 55% of the intracellular ruthenium is located in the cellular nucleus, while 45% remain in the cytosol and other cellular components.

Introduction

Some ruthenium(III) complexes and, in particular, complexes with the general formula HL *trans*-[RuL_2Cl_4], in which L is an azole heterocycle, *e.g.*, indazole, imidazole, triazole, *etc.*, show antitumor activity both *in vitro* and *in vivo*.[1–3] Indazolium *trans*-[tetrachlorobis(1H-indazole)ruthenate(III)] (KP1019) (for structural formula see Fig. 1) proved to be an effective tumour-inhibiting drug in experimental therapy of autochthonous colorectal carcinomas in rats, which strongly resemble colon cancer of humans.[4] In this tumour model, highly effective doses of KP1019 could be repeatedly administered over several weeks without any signs of acute toxicity.

Transferrin is a single-chain glycoprotein, containing about 700 amino acids which are arranged in two similar lobes, each containing one iron binding site, consisting of two tyrosines, a histidine, an aspartate and a bicarbonate anion bound in an adjacent pocket, so that transferrin operates as an iron carrier.[5] The molecular mass of human serum transferrin is approximately 80 kDa. It is present in human serum at concentrations of 2.5–3.5 mg ml^{-1}.[6] Iron-loaded transferrin binds to the transferrin receptor and is taken up into the cells *via* endocytosis.[7,8] Upon acidification of the endosomes, Fe(III) is released again and transferrin is recycled back to the cellular surface. Malignant cells have a high iron requirement and, accordingly, express a large number of transferrin receptors on their cell surface. It has been suggested that this iron(III)-transport protein is responsible for the cellular accumulation of ruthenium(III) complexes *via* transferrin receptor-mediated endocytosis due to the fact that Ru(III)-complexes also bind to transferrin.[6] This hypothesis has been put forward to explain the apparent tumour selectivity of KP1019. Ruthenium-loaded albumin may also be interesting for tumour therapy due to the EPR-syndrome.

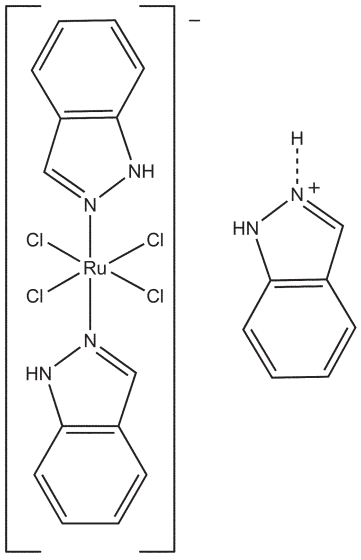

Fig. 1 The structural formula of indazolium *trans*-[tetrachlorobis-(1H-indazole)ruthenate] (HInd *trans*-[$RuCl_4Ind_2$], KP1019, FFC14a).

† Dedicated to Prof. Wolfgang Lindner on the occasion of his 60th birthday.

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The interaction of KP1019 with human serum transferrin has been investigated in solution by various techniques, including ^{1}H NMR, HPLC, spectrophotometry, and circular dichroism spectroscopy (CD). UV-VIS and HPLC studies demonstrated that KP1019 coordinates to human serum proteins, whereupon most of the ruthenium complex (80–90%) is bound to albumin and a much smaller amount to transferrin.[9] Binding of KP1019 to apotransferrin proceeds through the formation of two intermediates which then bind rapidly (within 5 min) to the protein—this process is observed only in the presence of bicarbonate. One molecule of transferrin is able to form adducts with at least ten KP1019 units.[10] In order to reach its target, the ruthenium complex anion has to be released after the protein complex adduct has entered the tumour cell. The release process may be followed by reduction of Ru(III) to Ru(II) in the hypoxic cell. This reduction is supposed to activate the complex for binding to nucleic acids.[3] In cell-free experiments, reversal of transferrin binding can be accomplished by citric acid or ATP at pH 4–5.[9] Crystallographic studies using apolactoferrin, which has a high sequence homology to apotransferrin and is available in a suitable crystalline form,[11] showed that KP1019 specifically binds at His 253 in the open binding cleft of the N-terminal lobe of the protein, with at least one of the indazole ligands still being attached to the ruthenium centre and the second indazole ligand being either disordered or present at lower site occupancy. It has been suggested that in the latter case the indazole ligand may no longer be bound directly to the Ru(III) ion, but may remain within the protein. Additionally, one or more chloride ligands are replaced by water molecules.[12]

Since Fenn *et al.* introduced electrospray ionisation mass spectrometry in 1989,[13] the technology and application of mass spectrometry in determining primary structures of proteins have been rapidly developed and are now well established. MS has nowadays attained the leading position as a standard analysis method for peptides, proteins and their covalent and non-covalent interactions in biochemistry.[14] Due to the fact that ESI-MS is the softest desorption/ionisation method, it induces only a low degree of fragmentation in case of proteins (unless induced actively in the atmosphere/vacuum interface) and furthermore allows the detection of intact biospecific non-covalent complexes.[15–18]

Therefore, ESI-MS has been used extensively to characterise the formation of protein–DNA, protein–peptide and protein–transition metal complex adducts.[14] The method proved to be useful for investigating interactions of anticancer metal complexes with proteins. Allardyce *et al.* described the application of ESI-MS for determination of the cisplatin-binding stoichiometry and human apotransferrin binding sites.[19] Najajreh *et al.* investigated the binding rate of bulky ligand bearing *cis*- and *trans*-Pt(II)-complexes to proteins.[20] The same group studied whether proteins that form methionine–Pt adducts are influencing the transfer to biological nucleophiles, *i.e.*, DNA.[21]

In this work, the exact molecular mass of human apotransferrin and human apotransferrin loaded with KP1019 as well as the stoichiometry of the formed complex was determined by means of ESI quadrupole ion trap mass spectrometry. In order to examine the consequences of transferrin binding for the cellular accumulation of the ruthenium complexes, cellular uptake studies were performed with free KP1019 and KP1019 bound to apotransferrin and Fe(III)-loaded transferrin. The presented results strongly support the hypothesis that KP1019 enters cells mainly *via* a transferrin-mediated mechanism.

Methods

Chemicals

Essentially iron-free human apotransferrin (MW 80 kDa) was purchased from Sigma. Indazolium *trans*-[tetrachlorobis(1H-indazole)ruthenate(III)] (KP1019) was prepared as described before.[1] For mass spectrometry studies, a 25 mM NH_4CO_3 solution was used and adjusted to pH 7.4 with 0.2 M HCl. For cell culture experiments, a physiological buffer containing 4 mM NaH_2PO_4, 100 mM NaCl and 25 mM Na_2CO_3 was prepared and adjusted to pH 7.4 with 0.2 M HCl. Ammonium hydrogencarbonate, sodium chloride, sodium dihydrogenphosphate and acetonitrile were acquired from Fluka and formic acid from Merck.

Transferrin conjugates

For mass spectrometry, a solution of 300 μM KP1019 and 100 μM transferrin in 25 mM ammonium bicarbonate (pH 7.4) was prepared and incubated for 10 min at 37 °C in order to obtain a threefold excess of the complex. The solution was stored at −4 °C in the dark. The sample solution, acetonitrile and formic acid (1% aqueous solution) were mixed in a 40:60:2 ratio—100 μl of the mixture were used for FPLC studies, the remaining solution was used for mass spectrometry.

For cellular uptake experiments, aqueous solutions of KP1019 (200 μM) and apotransferrin (100 μM) in physiological buffer were prepared. These solutions (2 ml of each) were mixed together, incubated for 15 min at 37 °C and diluted 1:20 with serum-free Minimal Essential Medium (MEM), containing 50 U ml^{-1} penicillin, 50 μg ml^{-1} streptomycin, 2 mM L-glutamine and 1 mM sodium pyruvate, to form the KP1019-transferrin (2:1) adduct. To prepare the KP1019-Fe(III)-transferrin (1:0.3:1) conjugate, 10 μl of a 9 mM $FeCl_3$ solution and 3 ml of an aqueous solution of KP1019 (100 μM) were added to 3 ml of the 100 μM apotransferrin solution and incubated at 37 °C for 15 min. This mixture was diluted 1:10 with serum-free MEM. FPLC studies were performed with these conjugates in order to verify complete binding of KP1019.

Fast protein liquid chromatography (FPLC)

FPLC studies were performed on an Amersham Biosciences ÄKTApurifier10 system applying two 5 ml HiTrap Desalting columns (Sephadex G-25) in series connected, with a size exclusion limit of 5 kDa. The chromatographic separations were monitored at 280, 360, and 425 nm in order to verify binding of KP1019 to apotransferrin.

Electrospray mass spectrometry

All analyses were carried out on a Bruker esquire$_{3000}$ ion trap mass spectrometer equipped with an orthogonal ESI ion source. For protein and conjugate measurements the mass spectrometer was operated in the positive ion mode and samples were continuously introduced into the source at a rate of 5 μl min^{-1}. A flow of nitrogen (130 °C, 11 l min^{-1}) assisted evaporation of the solvent. The spray needle potential was kept at 2.9 or 3.5 kV. Further mass spectrometric parameters important for complex and protein detection are depicted in Table 1.

Table 1 Operational ESI-MS parameters

Parameters	Protein and conjugate	KP1019
HV capillary/V	2900	3500
Dry gas flow/l min^{-1}	10	9
Dry gas temperature/°C	130	100
Nebuliser gas/psi	14	10
Skimmer 1/V	41.3	−11
Capillary exit offset/V	76.2	−77.2
Octopole/V	3.49	−2.61
Octopole Δ/V	2.4	−2.4
Trap drive/V	103.5	37.1
Injection rate/μl min^{-1}	5	5
Polarity	Positive	Negative
Scan range, *m*/*z*	1500–3450	50–1000

Circular dichroism spectroscopy

CD spectra were recorded with a Jobin Yvon CD6 spectrometer. KP1019 was added from a 5×10^{-4} M aqueous solution to human apotransferrin dissolved in physiological buffer (100 μM). The spectra were recorded from 300 to 650 nm (1 point nm^{-1}). A 100 μM apotransferrin solution (0.7 ml) was stepwise titrated with the KP1019 solution in a 1 ml cuvette with a path length of 1 cm to obtain molar ratios of 1 : 1, 1.5 : 1, 2 : 1, 3 : 1 and 5 : 1 of KP1019 to apotransferrin, respectively. Each data point is based on 5 measurements in one experiment, and the investigation was repeated twice.

Cellular uptake

SW480 cells (human adenocarcinoma of the colon) were kindly provided by Brigitte Marian (Institute of Cancer Research, University of Vienna, Austria). Cells were grown as adherent monolayer cultures in complete culture medium, *i.e.*, Minimal Essential Medium (MEM) containing 10% heat-inactivated fetal bovine serum (FBS), 1 mM sodium pyruvate, 2 mM L-glutamine, 50 U ml^{-1} penicillin and 50 mg ml^{-1} streptomycin (all purchased from Gibco). Cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO_2. 2 h before treatment, the cells were washed with HBSS and kept in serum-free culture medium. In order to study the uptake of free KP1019 in comparison with KP1019 bound to apotransferrin and transferrin, cells were incubated with solutions of KP1019, KP1019–apotransferrin adduct (2 : 1) and KP1019–Fe(III)–transferrin adduct (1 : 0.3 : 1), each containing 5 μM KP1019, for 2 h at 37 °C. After incubation, the cells were harvested by trypsinisation. The cell suspensions were centrifuged at 1000 rpm for 2 min. The cells were then resuspended in 5 ml ice-cold PBS, counted and centrifuged at 1000 rpm for 2 min. The cell pellet was dissolved in 100 μl of a 20% aqueous solution of tetramethylammonium hydroxide (TMAH), lyophilised, redissolved in 0.2 M HCl and stored at 4 °C for AAS measurements. Each experiment was performed in triplicate.

Cellular uptake and separation of nuclei

SW480 cells, grown in complete culture medium, were incubated with a 10 μM KP1019 solution for 2 h at 37 °C, harvested by trypsinisation, centrifuged at 1000 rpm for 2 min, resuspended in 5 ml ice-cold PBS, counted and centrifuged again at 1000 rpm for 2 min. The cell pellet was resuspended in 1 ml TBS (pH 7.5), containing 10 mM Tris/HCl, 150 mM NaCl, 1 mM phenylmethyl sulfonyl fluoride (PMSF) and 0.1% of a 1000× proteinase inhibitor solution (PI). The 1000× PI solution was stored at −20 °C and is composed of bestatin hydrochloride (20 mg ml^{-1}), *N*-α-tosyl-L-lysine chloromethyl ketone (25 mg ml^{-1}), *N*-tosyl-L-phenylalanine chloromethyl ketone (40 mg ml^{-1}) (all purchased from Sigma), pepstatin A from microbial source (3.5 mg ml^{-1}), leupeptin hemisulfate from microbial source (40 mg ml^{-1}) and aprotinin from bovine lung (10 mg ml^{-1}). The cells were disrupted ultrasonically three times for 30 s at 50% intensity and centrifuged in a Sorvall, HB-4 rotor at 1200 rpm (= 150 *g*) for 5 min to obtain the nondisrupted cells in the pellet. The supernatant was transferred to a new tube and centrifuged at 1800 rpm (= 350 *g*) to receive the nuclear pellet. The supernatant contained the cytosol. The nuclear pellet and the nondisrupted cell pellet were dissolved in 100 μl 20% TMAH. The dissolved pellets and the cytosol fraction were lyophilised, redissolved in 200 μl 0.2 M HCl and stored at 4 °C for AAS measurements. The study was repeated thrice. Statistical analysis was performed using SPSS software.

Atomic absorption spectroscopy

The AAS measurements were performed on a PerkinElmer Zeeman 5100 (graphite furnace atomic absorption spectrometer). Each measurement was repeated at least thrice. Furnace parameters were: pretreatment temperature: 1400 °C; atomisation temperature: 2500 °C; wavelength: 349.9 nm.

Results and discussion

Circular dichroism spectroscopy

The interaction of KP1019 with apotransferrin was studied by CD spectroscopy. The CD spectra of HInd *trans*-$[RuCl_4Ind_2]$-transferrin complexes in the spectral range 300–650 nm are shown in Fig. 2a. The titration of apotransferrin with KP1019 was followed by monitoring three negative and one positive dichroic bands at about 360, 448, 492 and 605 nm, correspondingly. As seen from the titration curves (see Fig. 2b), the CD changes at the three peaks at 448, 492 and 605 nm show a well defined break at a KP1019 to transferrin molar ratio of about 2 : 1, indicating specific binding of two ruthenium species per molecule of apotransferrin. Upon addition of KP1019, the circular dichroic changes at 605 nm show a saturation of the CD peak. Only a marginal increase is detected above approx. 2 mol of KP1019 added per mol of transferrin. At the shorter wavelength band CD_{448}, however, simple saturation of the CD peak is not observed. Instead, a further though more monotonic decrease of the peak intensity at a molar ratio >2:1 has been observed, indicating additional binding of KP1019 to sites other than the specific binding sites.

Mass spectrometry

In addition to CD spectroscopy, the stoichiometry of KP1019 binding to human apotransferrin was determined applying

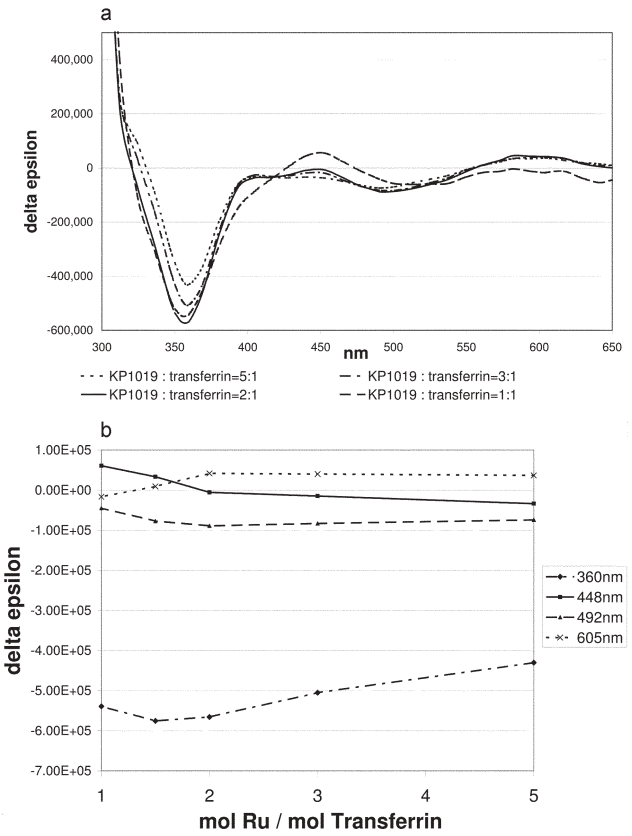

Fig. 2 (a) CD spectra of the KP1019-apotransferrin conjugates and (b) circular dichroic titration of apotransferrin with HInd *trans*-$[RuCl_4Ind_2]$. Conditions: protein concentration 100 μM at the beginning of the titration, pH 7.4, 25 °C, light path 10 cm.

ESI-MS. Two bound complex anions per transferrin molecule were detected (FPLC experiments with a mixture of KP1019 and transferrin in a molar ratio of 3:1 revealed that the whole amount of KP1019 was bound to the protein). It is supposed that the complex anions are specifically bound in the iron binding pockets of the protein, taking into account the crystal structure data obtained by Smith *et al.*[12] and the CD results mentioned above.

Fig. 3 shows the negative ion mass spectrum of KP1019, the positive ion mass spectra of apotransferrin (100 μM) and the protein incubated with a threefold excess of KP1019 in 25 mM bicarbonate buffer, acetonitrile and formic acid in a ratio of 40:60:2.

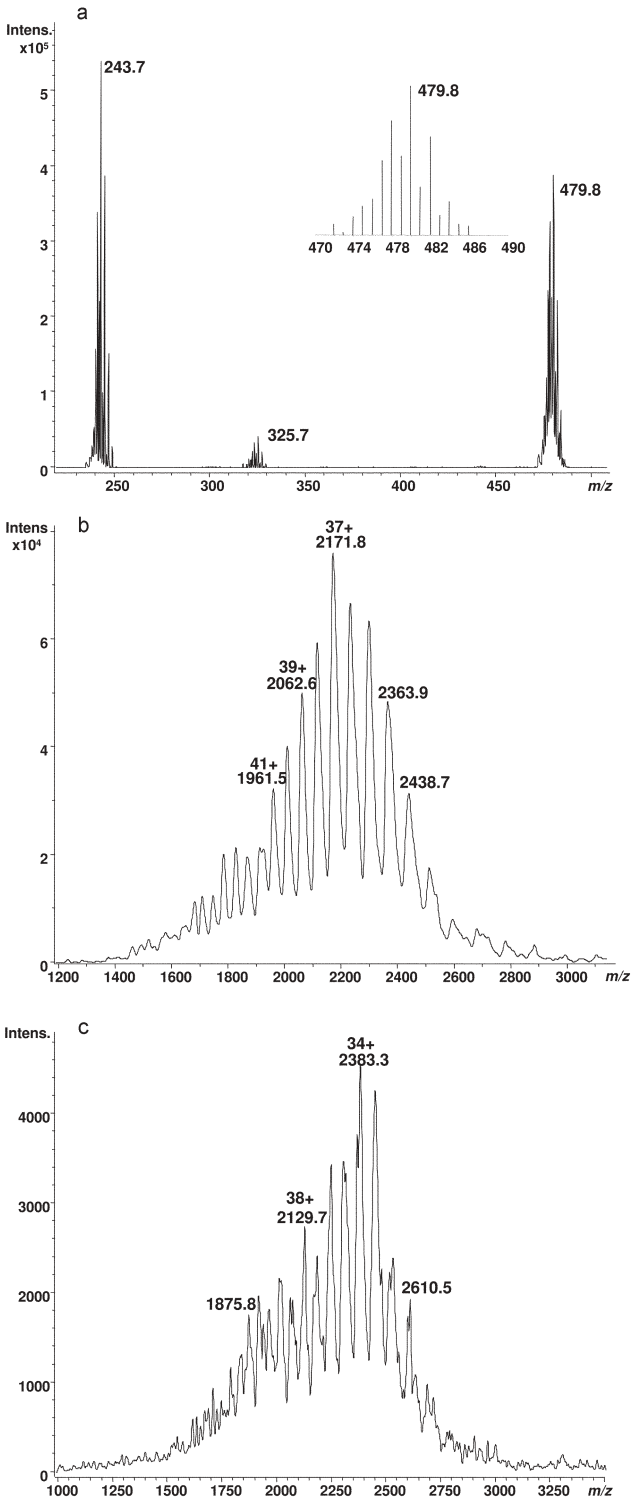

Fig. 3 The mass spectra of (a) KP1019, (b) human apotransferrin, and (c) transferrin-KP1019 conjugate.

In Fig. 3a, the mass spectrum of KP1019 is shown, exhibiting the molecular ion with its characteristic isotopic pattern. In the inset, the theoretical isotopic pattern of KP1019 is presented, which turned out to be in good agreement with the measured pattern. Furthermore, the major product of this ruthenium coordination compound at the applied spray conditions exhibited a major fragment ion at *m*/*z* 244 corresponding to $[RuCl_4]^-$ which was formed by loss of the two indazole ligands and a peak with *m*/*z* 326 assignable to $[RuCl_3Ind\text{-}H]^-$.

Fig. 3b illustrates a positive ion ESI mass spectrum of apotransferrin. The average molecular mass determined for apotransferrin was 80233 Da based on seven datapoints, which is slightly higher than the reported value 79557 Da. This could be due to a different degree of glycosylation and/or due to alkali adduct formation of the protein product.[19,22]

The ESI-mass spectrum of the ruthenium complex anion bound to the protein is shown in Fig. 3c. It was found that an incubation of the protein solution with KP1019 (in threefold excess) at 37 °C for 10 min resulted in a molecular mass increase of 783 Da, consistent with the binding of two equivalents of the complex anion of KP1019 accompanied by a loss of chloride and an exchange of chloro- for hydroxo-groups. This is in good agreement with the known ability of KP1019 and other octahedral tetrachlororuthenate(III) complexes to undergo hydrolysis.[12] Additionally, a shift to peaks with lower *m*/*z* values was found, suggesting a loss of one or even both indazole ligands (see Fig. 3c).

The rather broad peaks in the mass spectrum of the transition metal bearing protein result from the rich isotope pattern of ruthenium.

Cellular uptake

Cell culture experiments on the human colon carcinoma cell line SW480 showed that 5.2 ng ruthenium are taken up into 10^6 cells, if serum is removed 2 h prior to incubation with a 5 μM KP1019 solution. Treatment of the cells with 5 μM KP1019 bound to apotransferrin in a molar ratio of 2 : 1 under the same conditions resulted, contrary to our expectation, in a *ca.* 47% lower ruthenium accumulation, the difference being not significantly different ($p = 0.064$). This finding suggests that loading of both iron binding pockets with KP1019 induces a protein conformation, which is unfavourable for transferrin receptor binding. It is well known that the affinity of transferrin for its receptor depends on the extent of metal loading due to the conformational changes induced to the transferrin molecule: diferric transferrin exhibits the highest affinity, monoferric transferrin an intermediate affinity, and apotransferrin shows the lowest affinity.[7,8] Under physiological conditions transferrin is saturated with iron only from 20 to 45% (on average ~33%).[23] In order to simulate physiological conditions and to provide the opportunity for KP1019 to bind to the vacant iron binding pocket of the protein, apotransferrin was loaded to 30% with iron. Incubation with KP1019 (5 μM) bound to Fe(III)-transferrin at a ratio of 1 : 0.3 : 1 (transferrin : Fe(III) : KP1019) resulted in an accumulation of 11 ng ruthenium per 10^6 cells, which is about the twofold amount when compared with free KP1019 and about the four- to fivefold amount of transferrin-bound KP1019 (1:2) in the absence of iron, the differences being statistically significant in both cases (see Fig. 4). These results suggest that iron binding plays a major role in obtaining a protein conformation which favours recognition by the transferrin receptors on the cell surface. These findings may explain the results of cytotoxicity experiments performed by Kratz *et al.*, who could not find increased activity of apotransferrin-bound KP1019 as compared to free KP1019.[24] The fact that the same authors could not unequivocally demonstrate increased activity when

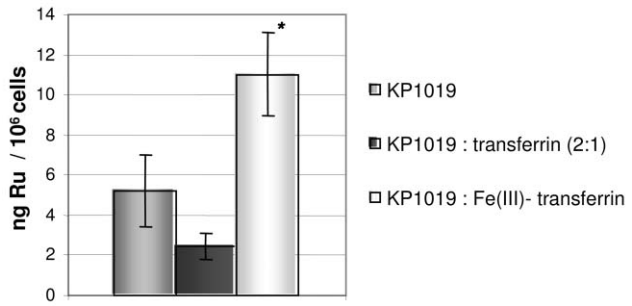

Fig. 4 The cellular uptake of ruthenium into the human colon carcinoma cells SW480 exposed to KP1019, KP1019 bound to human apotransferrin (2:1) and KP1019 bound to Fe(III)-loaded transferrin (1:1). In every case, the concentration of KP1019 was 5 μM. Each data point is the mean ± SD of at least three separate experiments. *Significantly different from KP1019, $p = 0.021$ and from KP1019: transferrin, $p = 0.003$ (unpaired *t*-test).

KP1019 was bound to iron-saturated transferrin (diferric transferrin) can presumably be explained by the lack of available specific binding sites. In conclusion, the degree of iron saturation is a paramount factor for effective transferrin mediated cellular uptake and for the antiproliferative activity of KP1019.

Further experiments revealed that on average 55% of KP1019 (range: 51–59%) taken up into the cells after 2 h are located in the nucleus, while on average 45% (range: 42–49%) remain in the cytosol and other cellular components. Taking into account the relatively short exposure time of only 2 h with KP1019, the amount of ruthenium detected in the nuclei fraction seems to be very high.

A survey of the literature shows that in experiments with gallium citrate only 2–14% of the intracellular gallium was found associated with the nuclei of tumour cells and normal cells of various tissues isolated after *in vivo* exposure of at least 24 h.[25,26] Reports on the nuclear cisplatin uptake are rather divergent. Shirazi and co-workers found about 52%, 72% and 85% of the total cellular content of platinum in the nucleoplasm and DNA fractions of ovarian cancer cells following incubation with cisplatin, cisplatin in plasma ultrafiltrate and aquated cisplatin, respectively.[27] Experiments in oesophageal cancer cell lines showed that platinum was distributed between the nucleus and the cytosol at a ratio of approximately 3 : 1.[28] In contrast, Lindauer and Holler found only about 10% platinum located in the nuclei fraction when the human breast cancer cell line MCF-7 was treated with various platinum complexes, *e.g.*, cisplatin.[29] Experiments testing the accumulation of cisplatin *in vivo* in kidney and liver of rat for 24 h showed that 14% of the total platinum amount were located in the nuclear fraction.[30]

Comparing the subcellular distribution of KP1019 with gallium and platinum complexes, our experiments revealed a much higher accumulation in the nucleus than reported for gallium compounds but comparable with some reports on the distribution of platinum drugs.

Conclusion

ESI quadrupole ion trap mass spectrometry with an orthogonal sprayer was shown to be a powerful tool for the determination of molecular weights of proteins and the analysis of non-covalent protein–drug interactions. Due to the high mass precision and accuracy of the applied ESI mass spectrometer, it was possible to determine the exact biospecific stoichiometry of the KP1019 binding to human apotransferrin (1 : 2), which turned out to be in good agreement with the CD spectroscopy experiments. Two of the complex anions of KP1019 bind specifically in two different lobes of apotransferrin in the presence of a synergistic bicarbonate anion. In addition, an *m*/*z* decrease being consistent with an exchange of chloro ligands for hydroxo groups could be monitored—an observation conforming with the hydrolytic properties of the complex. KP1019 appears to be quite unstable in the gas phase, resulting in a loss of one or both indazole ligands. A loss of indazole is also observed in the KP1019 anion–protein conjugate—a protein complex anion signal with a lower *m*/*z* was found next to the protein–compound adduct.

The cellular uptake experiments confirm the assumption that transferrin promotes the transport of KP1019 into the cell. In the presence of iron the cellular accumulation of Fe(III)-transferrin-bound KP1019 is higher than that of the free ruthenium complex. In contrast, the uptake of KP1019 bound to apotransferrin was only about 22% of the Fe(III)-loaded KP1019-protein adduct, suggesting the necessity of iron(III) for the protein–receptor interactions.

Furthermore, cell fractionation studies showed that after only 2 h exposure as much as 55% of the intracellular KP1019 was found in the nuclear fraction. This nuclear localisation is a favourable precondition for the interaction with DNA, the likely target of this ruthenium complex.

Acknowledgements

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