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Drug Discovery

Half-Sandwich Ruthenium(II) Biotin Conjugates as Biological Vectors to Cancer Cells

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Abstract: Ruthenium(II)–arene complexes with biotin-containing ligands were prepared so that a novel drug delivery system based on tumor-specific vitamin-receptor mediated endocytosis could be developed. The complexes were characterized by spectroscopic methods and their in vitro anticancer activity in cancer cell lines with various levels of major biotin receptor (COLO205, HCT116 and SW620 cells) was tested in comparison with the ligands. In all cases, coordination of ruthenium resulted in significantly enhanced cytotoxicity. The affinity of Ru^{II}–biotin complexes to avidin was investigated and was lower than that of unmodified biotin. Hill coefficients in the range 2.012–2.851 suggest strong positive cooperation between the complexes and avidin. To estimate the likelihood of binding to the biotin receptor/transporter, docking studies with avidin and streptavidin were conducted. These explain, to some extent, the in vitro anticancer activity results and support the conclusion that these novel half-sandwich ruthenium(II)–biotin conjugates may act as biological vectors to cancer cells, although no clear relationship between the cellular Ru content, the cytotoxicity, and the presence of the biotin moiety was observed.

Introduction

The most frequently used chemotherapeutic agents indiscriminately target rapidly dividing cells, such as cancer cells, but also healthy epithelial cells. This leads to low selectivity and severe side effects, such as nausea, loss of hair, and development of ulcers. As a result, a number of patients refuse or stop adjuvant chemotherapy.[1,2] Therefore, a key feature of new chemotherapeutic agents is selectivity for cancerous cells over healthy tissue. This can be achieved by exploiting intrinsic differences between healthy and tumor cells. One strategy is to take advantage of the fact that cancerous cells overexpress various receptors on their surface that are less prevalent in healthy cells. If an anticancer agent can selectively interact with one of those receptors, a cytotoxin may be accumulated more effectively in tumor cells. Consequently, the therapeutic effect will be enhanced and adverse effects will be reduced.

Cancer cells require significant amounts of vitamins to sustain their rapid growth. Moreover, there is a strong correlation between the level of over-expression of vitamin receptors and the stage of tumor growth, with the highest levels for tumors at the terminal stage.[3] The attachment of vitamins, such as vitamin B12, folic acid, and biotin to anticancer prodrugs is therefore a worthwhile strategy to enhance tumor targeting.[4] Various cancer cell types express higher levels of biotin receptors than of folate or vitamin B12 receptors and consequently display higher levels of uptake of biotin-modified molecules. It was reported that biotin derivatives were also more effective in killing cancer cells, which makes biotin a particularly promising vector.[5]

The main biotin uptake system in human intestinal epithelial cells is the sodium-dependent multivitamin transporter (SMVT) system. SMVT is a protein (635 amino acids) encoded by the *SLC5A6* gene, which was found to be activated in various aggressive cancer cell lines.[6,7] Consequently, biotinylation of

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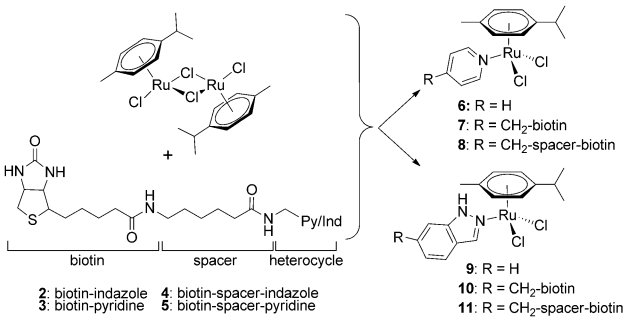
compounds converts them into biological vectors to SMVT-overexpressing cells. This has been the subject of numerous studies with organic drugs and drug candidates (camptothecin,[8] metothrexate,[5,9] doxorubicin,[5] paclitaxel,[10–12] gemcitabine,[13,14] polyamidoamine dendrimers (PAMAM),[15–17] and TAT peptides).[18,19] However, the targeted delivery of analogous metal-based compounds remains largely unexplored. Although a number of metal-based biotin compounds have been reported, only a few have been tested for their biological properties in cells. Lo and co-workers evaluated the anticancer activity of Re,[20,21] Ir,[22,23] and Rh[23] complexes with a range of polyaromatic diimine ligands with a differing number of attached biotin moieties. The cytotoxicity of the complexes was strongly dependent on the number of pendant biotin groups, with lower IC_{50} values corresponding to a lower number of biotin residues.[21] However, nonbiotinylated analogues with a similar structure exhibited the same order of cytotoxicity as the biotinylated derivatives. Similarly, the cytotoxicity of a biotin-appended RAPTA complex ([Ru(η^6-arene)Cl_2(PTA)], in which PTA is 1,3,5-triaza-7-phosphaadamantane) was not higher than of unmodified RAPTA-C, [Ru(η^6-*p*-cymene)Cl_2(PTA)].[24] In contrast, biotin–ferrocene conjugates[25] and biotinylated cisplatin-loaded nanoparticles[15] showed a significant increase of cytotoxicity compared with the respective nonbiotinylated derivatives. However, much of the data was collected on different cell lines with different affinity and capacity of the SMVT transporter.[26]

Herein, we present the results of a systematic investigation of novel biotin-conjugated half-sandwich Ru^{II} compounds in terms of their antiproliferative activity in cancer cells. We have, in particular, aimed to evaluate the correlation between the cytotoxicity, ruthenium compounds' uptake, and the *SLC5A6* gene (SMVT) expression, as the latter is an important parameter for biotin to function as a vector. These studies were complemented by the determination of the binding affinity of the biotin derivatives to avidin and by molecular docking experiments.

Results and Discussion

To test the effect of using ruthenium–biotin conjugates on the viability of cancer cells and to assess the correlation with levels of *SLC5A6* gene expression, we synthesized biotin-containing ligands **2**–**5**, the corresponding half-sandwich ruthenium(II) complexes **7**, **8**, **10**, and **11**, and the structurally similar nonbiotinylated ruthenium(II) analogues **6** and **9** (Scheme 1). The SMVT transporter can recognize biotin when the thiophane and keto fragments of the latter are not modified.[25,27] Therefore, these moieties remained unaltered, and the valeric carboxylic acid residue of biotin was used for further derivatization. Consequently, **2** and **3** were prepared by standard amide coupling of the biotin targeting unit with primary amino groups of N-heterocycles to allow coordination to the metal center. 4-Aminopyridine and 6-aminoindazole were found to be inappropriate for such coupling and therefore commercially available 4-(aminomethyl)pyridine and 6-(aminomethyl)indazole from the reduction of 6-cyano-1*H*-indazole with $LiAlH_4$



Scheme 1. Biotinylation of half-sandwich Ru^{II} complexes (anhydrous DMF, ratio of [{Ru(η^6-*p*-cymene)Cl_2}$_2$] to ligand = 1:2, 3 h, RT).

were used. For comparison, the structurally similar ligands **4** and **5**, with an incorporated aminohexanoyl linker, were prepared. Published data on the effect of the spacer between the active component and targeting moiety on the biological activity of compounds is controversial. The incorporation of an aminohexanoyl spacer between biotin and a ferrocene complex had a detrimental effect on the cytotoxicity.[25] Other studies revealed that such spacers dramatically increase the stability of Ru^{II}(polypyridine)–biotin complexes and their interactions with cell membranes.[28] Moreover, it was demonstrated that spatial separation between biotin and the metal-based complex is vital for biorecognition of both units.[29]

Compounds of the biotin–linker–*N*-heterocycle type, such as **2**–**5**, have been used extensively for a range of purposes and their syntheses are well established. To synthesize ruthenium complexes with these ligands, it is essential that they do not contain any trace of unreacted precursors, which could also coordinate to the ruthenium fragment. Therefore, all ligands were additionally purified by reversed phase HPLC, and their purity was confirmed by analytical HPLC and elemental analysis. However, this resulted in low yield of the target compounds. 6-(Methylamino)indazole can be used for coupling with biotin derivatives without HPLC purification. All ligands were characterized by ^1H and $^{13}C\{^1H\}$ NMR (for the atom numbering scheme, see Figure 4 in the Experimental Section) as well as ESI-MS and elemental analysis.

To coordinate the biotin ligands to the Ru center, we adapted the procedure of Vock et al., who developed the synthesis of half-sandwich Ru^{II} complexes with a number of *N*-heterocyclic ligands.[30] By following this method, the nonbiotinylated [Ru^{II}(η^6-*p*-cymene)] complexes with pyridine (**6**) and indazole (**9**) ligands were obtained by heating [{Ru(η^6-*p*-cymene)Cl_2}$_2$] to reflux with the corresponding ligand in anhydrous toluene for 3 h. However, the analogous synthesis of Ru^{II}–biotin conjugates was seriously limited by the solubility of the biotin derivatives and by the stability of the corresponding complexes. Ligands **2**–**5** were moderately soluble in ethanol and effectively solvated only in dimethylformamide (DMF) and dimethyl sulfoxide. We have recently discovered that incubation of half-sandwich arene Ru^{II} complexes in dimethyl sulfoxide resulted in loss of the arene moiety.[24] Therefore, we investigated the reactions of [{Ru(η^6-*p*-cymene)Cl_2}$_2$] with 2 equiv of ligands **2**–**5** in ethanol or DMF by means of ESI-MS. In the present study, ethanol was

a less suitable solvent for such complexation reactions due to the marked formation of dinuclear Ru species, especially for **10** (for the mass spectrum see Figure 1) and **11** (Figure S1). The formation of ruthenium complexes **7**, **8**, **10**, and **11** was more selective in DMF (see Figure 1 and S1–S5), although either di-

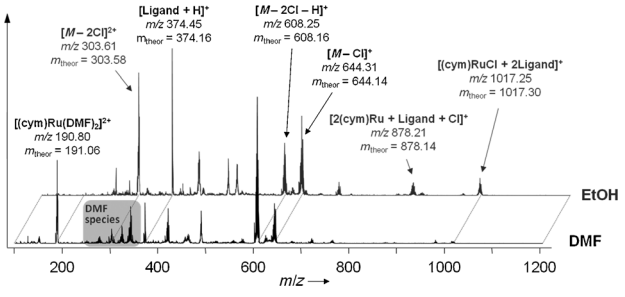


Figure 1. Full ESI-IT mass spectra of reaction mixtures giving **10** after 3 h. The reactions were carried out in DMF or in EtOH. The reaction using DMF as a solvent leads to more selective formation of **10**.

meric hydrolysis products or DMF adducts accompanied by ligand release were detected for the complexes without aminohexanoic spacers; that is, **7** and **10**, respectively. The detection of complexes with coordinated monodentate N-donor ligands can be challenging with ESI-MS and indicates limited complex stability under the ESI spraying conditions.[31,32] However, this does not reflect the situation in solution. Interestingly, such ions were not observed for the complexes with aminohexanoic spacers (i.e., **8** and **11**). These findings suggest that once the ligand is coordinated to the ruthenium center, the spacer is beneficial for the stability of the resulting complexes. Consequently, the complex stability increases when the ligand contains indazole and/or a spacer.

The reactions were analyzed by ESI-MS after 3, 6, and 24 h (Figures S2–S5). Whereas **8**, **10**, and **11** did not reveal any changes within 24 h of incubation, complex **7** was significantly less stable and started to decompose after 6 h (Figure S2). Based on the results of the ESI-MS experiments, we modified the procedure of Vock et al. to stirring a mixture of [{Ru(η^6-*p*-cymene)Cl_2}$_2$] with the corresponding ligand in anhydrous DMF at room temperature for 3 h with subsequent removal of DMF by freeze-drying. This generic method was used for the synthesis of biotinylated complexes **7**, **8**, **10**, and **11** (Scheme 1). Non-biotinylated **6** was also obtained by this method and its characterization was consistent with published data.[30] We assumed that lyophilization of the products did not affect their integrity, because the ESI-MS of the lyophilized and redissolved complexes did not change. The novel complexes **7**, **8**, **10**, and **11** were highly hygroscopic and moisture-sensitive, and prolonged exposure to air resulted in their decomposition. Therefore, for subsequent biological studies, the lyophilized complexes were incubated in situ with cell extracts. It was previously reported that addition of more than one biotin moiety to the substrate resulted in decreased cytotoxicity[21] and an aggravated SMVT transporter recognition.[19] Accordingly, only a single biotin-functionalized ligand was coordinated to the ruthenium center.

The molecular structure of the nonbiotinylated complex **6** was determined by X-ray diffraction analysis (Figure S6; for crystallographic data see Table S1). Single crystals were grown by slow diffusion of diethyl ether into a saturated dichloromethane solution at 277 K. The *p*-cymene ring is coordinated to the ruthenium center in a η^6-manner. The rest of the coordination sphere is filled by two chlorido and a pyridine ligand. The geometrical parameters of the complex are in agreement with previously determined structures of compounds, which vary only in the nature of the N-donor ligands.[32,33] The average Ru–C bond length in **6** is 2.186 Å, whereas Ru–Cl1, Ru–Cl2 and Ru–N distances are 2.4194(4), 2.4026(4), and 2.1368(12) Å, respectively (in comparison for [Ru(η^6-*p*-cymene)$Cl_2(NH_3)$], Ru–C_{av} 2.170(8), Ru–Cl1 2.4157(6), Ru–Cl2 2.4157(6), and Ru–N 2.130(3) Å).[32] The crystal structure of **6** was employed as the starting point for subsequent docking studies.

Cytotoxicity and cellular accumulation

The in vitro anticancer activity of the ruthenium complexes and ligands was determined in COLO205, SW620, and HCT116 colon carcinoma cells by means of the colorimetric MTT assay with an exposure time of 70 h (see Table 1 for the IC_{50} values;

Table 1. Cytotoxicity of the Ru complexes **6**–**11** in COLO205 (colon adenocarcinoma), HCT116 (colon carcinoma), and SW620 (colon adenocarcinoma) cells determined by means of the MTT assay. Calculations are based on results of three independent experiments. 95% confidence intervals are given in parentheses to enable better comparison of the results.

Compound		IC_{50} [μM]		
		COLO205 *SMVT* (low)	HCT116 *SMVT* (low)	SW620 *SMVT* (high)
pyridine	**6**	≥100	3.4 (2.2–5.1)	4.1 (2.3–5.5)
	7	4.6 (2.1–10.2)	6.7 (4.6–9.6)	1.1 (0.8–1.7)
	8	17 (9–36)	2.5 (1.4–4.5)	2.1 (1.3–3.4)
indazole	**9**	23 (18–31)	33 (18–60)[a]	14 (12–17)
	10	≥100	≥100	19 (15–24)
	11	≥100	6.7 (4.6–9.7)	29 (22–37)

[a] Extrapolated value.

concentration-effect curves are shown in Figure S7). These cell lines were chosen because of their different *SLC5A6* gene expression levels coding for SMVT. Therefore, the response of cancer cells to the Ru–biotin conjugates may be correlated with the relative level of SMVT expression. *SLC5A6* mRNA is most abundant in SW620 cells, and almost three times higher than in COLO205 and HCT116 cells, which have comparable *SLC5A6* gene expression levels.[25]

With the exception of **7**, COLO205 cells were markedly less chemosensitive than HCT116 and SW620 to **6**–**11** (Table 1). Complex **6** was reported to be inactive in TS/A murine adenocarcinoma cells (IC_{50} = 757 μM);[30] however, it was significantly more active in the human cell lines HCT116 and SW620, with IC_{50} values of 3.4 and 4.1 μM, respectively, although different conditions were used in these experiments. In general, cancer

cells were more sensitive towards the pyridine complexes **6**–**8** than their indazole analogues **9**–**11**. Whereas the activities of **6** and **8** were similar in cell lines with high and low levels of SMVT expression, complexes **7** and **10** were significantly more active in the SMVT-overexpressing SW620 cell line. In contrast, **11** was four times more active in the cell line with a low level of SMVT expression (6.7 and 29 μM in HCT116 and SW620, respectively). Interestingly, biotin derivatives with pyridine were more potent in the SW620 cell line ($IC_{50}=1.1$ μM and 2.1 μM for **7** and **8**, respectively) than the parent nonbiotinylated compound **6** ($IC_{50}=4.1$ μM). However, biotin complexes with an indazole fragment were less active in SW620 cells than their nonbiotinylated analogue **9**. From these results, no clear correlations between the cytotoxicity, the *SLC5A6* expression levels, and the structures of the complexes can be drawn. Notably, none of the corresponding ligands was toxic in the investigated concentration range (up to 30 μM). This is consistent with previous investigations that showed that there is no clear effect of biotin-conjugation on the antiproliferative activity.[15,21,24–26] It seems that the activity of the investigated drug itself also adds to the cytotoxicity of the conjugate.

The Ru content in the cancer cells was analyzed in dependence of the supplementation with biotin (Tables S2–S4). A similar picture was obtained to that found in the cytotoxicity studies: no clear relationship was identified between the presence of the biotin moiety and the concentration of Ru in the cells. This supports the assumption that alternative pathways must exist for cellular uptake of this compound class and that there is possibly more than one uptake mechanism in place.

Interactions with avidin

The relative affinity of compounds **7**, **8**, **10**, and **11** with avidin was determined by using Biotective Green reagent, which is a fluorescent derivative of avidin, complexed with 2-(4′-hydroxyazobenzene)benzoic acid as a quencher. The apparent equilibrium dissociation constants for the investigated compounds with avidin were found to be higher than for biotin, which was used as a reference. Accordingly, the affinity of all test compounds towards avidin was lower than for the natural ligand. The affinity for pyridine derivatives was higher than for the indazole analogues. Hill coefficients in the range of 2.012–2.851 indicate positive cooperativity in the binding event (Table 2). The values do not differ significantly among the compounds tested. This suggests no major steric hindrance was introduced by any of the investigated compounds because interactions among biotin-binding sites remain unchanged. The recognition of biotin-appended complexes by avidin and SMVT transporter indicates that thiophane and keto fragments of biotin ligands were not modified upon complexation.[25,27]

Table 2. Apparent equilibrium dissociation constants and Hill coefficients for investigated compounds. Calculations were based on results of at least three independent experiments. 95% confidence intervals are given in parentheses to enable better comparison of the results.

Compound		K_d [nM]	Hill coefficient
biotin		680 (610–751)	2.752 (1.985–3.520)
pyridine	**7**	895 (823–967)	2.851 (2.257–3.445)
	8	1124 (1050–1198)	2.785 (2.390–3.179)
indazole	**10**	1194 (1112–1275)	2.371 (2.085–2.658)
	11	1980 (1144–2816)	2.012 (1.030–2.994)

Docking studies with strept(avidin)

To explain the findings from the cytotoxicity assays and to estimate the likelihood of binding to the SMVT transporter, docking studies with crystal structures of avidin (PDB: 1LDQ) and streptavidin (PDB: 3RY2) were conducted using the GOLD software.[34] Goldscore (GS) was the only scoring function able to treat ruthenium complexes. GS gives arbitrary numbers with higher values predicting better binding. The docking studies were conducted with streptavidin after initial experiments with avidin. However, when docking **10** to avidin, the biotin moiety was found to be situated outside the binding pocket of avidin, thus giving no reason to suggest any binding between the ligand and protein. The docking experiments of biotin with the crystal structure of streptavidin gave a good overlap of modeled biotin and co-crystallized biotin (GS 71), therefore this was used as a model system.

Docking of pyridine derivatives **6**–**8** to streptavidin was compared with that of biotin. The biotin moieties of **7** and **8** showed good overlap with the co-crystallized biotin in the streptavidin crystal structure, reproducing also the hydrogen-bonding pattern. The top three results for **7** had scores of 70 ± 2 (Figure 2). This is comparable with the scoring of biotin, and indicates similar binding energies of **7** and biotin. Compound **8** gave top scores of 80 ± 1, predicting higher binding than for **7** and biotin. This may be related to the increase in the molecular size. The addition of the linker provides greater flexibility to the ruthenium moiety, whereas the biotin was anchored deep in the pocket. In contrast, the GS for the pyridine complex **6** was only 36 ± 1 and was found in the binding pocket where biotin is normally located (Figure S8). The low score coupled with the observed lack of hydrogen bonding suggests that the complex binding in the pocket is not favored. In addition, a model compound with the {Ru(η^6-*p*-cymene)Cl_2} fragment coordinated to the biotin-sulfur atom was studied (Figure S9) and a score of 55 ± 2 was obtained. The biotin moiety of the top three docking results showed some overlap with the co-crystallized biotin, resulting in a partial reproduction of the hydrogen-bonding pattern. The top three hits also showed good overlap with each other.

The same set of experiments was conducted with the indazole-substituted biotin derivatives. For **10**, the top three hits showed overlap between each other, but not in the expected position, resulting in no overlap with the co-crystallized biotin in the protein structure (Figures 3 and S10). The biotin head of the docked compounds was positioned outside the binding pocket. The scoring for these molecules was 57 ± 1, suggesting less favorable binding in comparison to biotin or analogue **7**. Even more surprising was the result of the docking studies featuring **11**, for which the top three scores from the screening showed no overlap at all. Due to the high level of flexibility,

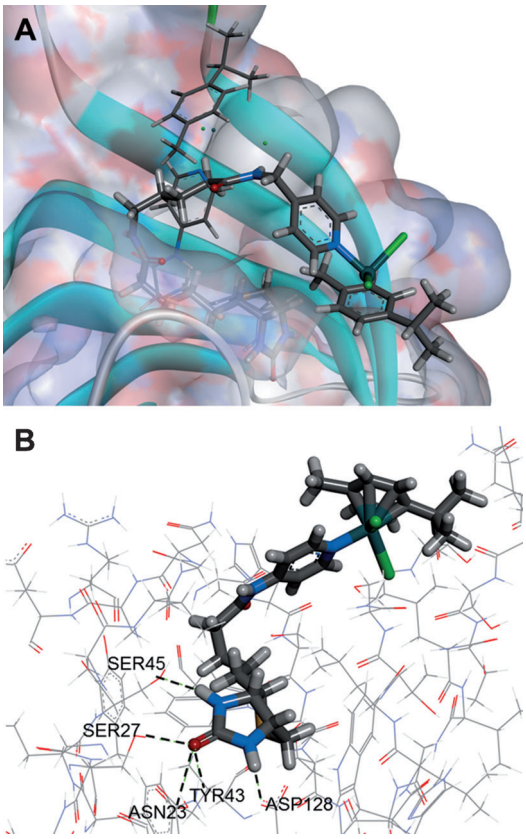


Figure 2. A) Overlay of the docking results of **7** and **8** in the binding site of streptavidin indicating overlap of the biotin moieties of both molecules, and B) the hydrogen-bond network formed between the biotin moiety of **7** and the amino acids Asp128, Asn23, Ser27, Tyr43 and Ser45 in streptavidin.

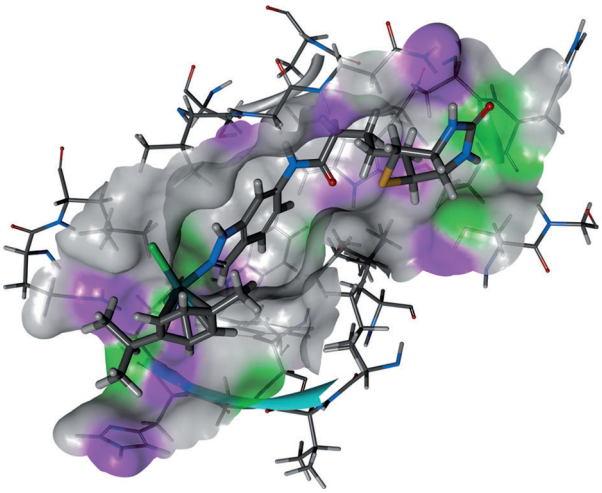


Figure 3. Complex **10** docked with streptavidin (the charge distribution surface of the protein is shown) was predicted to be on the surface rather than the biotin moiety in the biotin binding site.

the molecules were spread out in various positions. Similar to **6**, complex **9** occupied the binding pocket without any hydrogen bonding. The scores for the top three configurations were 40 ± 1, slightly higher than that of **6**.

The results of the docking experiments explain to some extent the in vitro anticancer activity data. Compounds **9**–**11** showed much lower scores than especially **7** and **8** in the streptavidin docking experiments, which is consistent with the higher in vitro activity of the latter derivatives in the SMVT-high cell line SW620. The same set of compounds showed much lower activity in the SMVT-low cells and therefore in this case the (strept)avidin binding ability may be indicative of an SMVT-mediated mode of action. On the other hand, the indazole derivatives also show significant cytotoxicity in some of the cell lines, which may be explained with a mode of action not involving the SMVT transporter. The favorable interaction of the indazole derivatives with avidin in the in vitro experiment may be related to the extended π-system of indazole rather than the biotin moiety.

Conclusions

The high demand of tumors for vitamin H (biotin), which is required to sustain their rapid growth,[3,4] means that the tumor cells overexpress biotin receptors (e.g., SMVT) on the cell surface. This provides an opportunity to selectively accumulate chemotherapeutics with high affinity for SMVT or other biotin receptors by designing novel drug delivery systems. We linked a biotin-receptor-targeting moiety through a spacer to a biologically active metal fragment. Biotin derivatives featuring the natural binding moiety to the biotin receptor were used as the basis for the vector to exploit tumor-specific vitamin-receptor mediated endocytosis. As a proof of concept, a series of half-sandwich ruthenium(II) complexes with ligands functionalized with biotin were prepared. Their biological activity was confirmed by cytotoxicity assays in cell lines with differing SMVT expression profiles. Whereas the ligands were not toxic in the investigated concentration range, the complexes exhibited marked cytotoxicity, and **7** and **10** showed cell-specificity based on the level of SMVT expression, although no direct correlation with the cellular uptake was identified. The relative binding affinity of the complexes to avidin was determined and it was shown that all complexes were recognized by avidin, although to a lower extent than biotin but still in the same dimension. This indicates that the structural components of biotin that are essential for recognition were not significantly altered upon complexation. The interactions of the complexes with (strept)avidin were investigated by docking studies and considering the antiproliferative activity of the complexes confirmed the likelihood of binding of the complexes to SMVT transporter. However, other transport mechanisms might contribute to Ru accumulation in the cell. Overall, the findings suggest that the complexation of these biotin-functionalized ligands to Ru centers results in cytotoxic compounds with some degree of selectivity for cancer cells with higher biotin receptor levels.

Experimental Section

Synthesis

6-(Methylamino)indazole (1): $LiAlH_4$ (12 mmol, 456 mg) was suspended in anhydrous THF (80 mL) at 0 °C under argon. A solution

of 6-cyano-1*H*-indazole (3 mmol, 430 mg) in anhydrous THF (5 mL) under argon atmosphere was cautiously added to this suspension at 0 °C. After the color of the reaction mixture changed from light yellow to dark brown, it was allowed to warm to RT and heated at reflux for 4 h. The solution turned almost colorless and a light yellow precipitate formed. The reaction mixture was cooled to 0 °C and a minimum amount of water was added in 50 μL aliquots with the last aliquot following termination of gas evolution. The suspension was filtered and the pale yellow solution was evaporated to dryness and dried in vacuo. The crude product was purified by reversed-phase HPLC (H_2O/MeOH/0.1% CF_3COOH, gradient 5–95% MeOH, 20 min) followed by evaporation under reduced pressure to give **1** (243 mg, 54%) as a white solid. The purity was assessed by analytical HPLC (retention time 11.6 min). ^{1}H NMR (500.10 MHz, [D$_6$]DMSO): $\delta = 13.24$ (s, 1H; NH-indazole), 8.22 (br s, 3H; NH_3^+), 8.11 (s, 1H; H_u), 7.82 (d, ^3J(H,H) = 8.4 Hz, 1H; H_s), 7.66 (s, 1H; H_w), 7.20 (d, ^3J(H,H) = 8.4 Hz, 1H; H_r), 4.19 ppm (s, 2H; H_p); MS (ESI+): *m/z*: 130.8 $[M-NH_2]^+$ (calcd 131.1), 148.4 $[M+H]^+$ (calcd 148.1).

Biotin(6-methylamido)indazole (2): Biotin *N*-hydroxysuccinimidyl ester (240 mg, 0.7 mmol) and 6-(methylamino)indazole (**1**; 103 mg, 0.7 mmol) were dissolved in a minimum amount of DMF (ca. 10 mL). Triethylamine (292 μL, 212 mg, 2.1 mmol) was added to the solution and the color changed to light yellow. The reaction mixture was stirred overnight at RT. DMF was removed under reduced pressure and the residue was dissolved in a minimum amount of methanol and filtered. Diethyl ether (50 mL) and petroleum ether (50 mL) were added to the filtrate. The formed precipitate was collected by filtration, washed with petroleum ether, and dried in vacuo to give a pale yellow powder. The crude product was purified by reversed-phase HPLC (H_2O/MeOH/0.1% CF_3COOH, gradient 5–95% MeOH, 20 min) followed by evaporation under reduced pressure to obtain **2** (191 mg, 73%) as a white solid. The purity of the product was confirmed by analytical HPLC (retention time 17.8 min). ^{1}H NMR (500.10 MHz, [D$_6$]DMSO): $\delta = 12.97$ (s, 1H; NH-indazole), 8.36 (t, ^3J(H,H) = 6.1 Hz, 1H; NH), 8.02 (s, 1H; H_u), 7.69 (d, ^3J(H,H) = 8.4 Hz, 1H; H_s), 7.38 (s, 1H; H_w), 7.01 (d, ^3J(H,H) = 8.4 Hz, 1H; H_r), 6.41 (s, 1H; NH-biotin), 6.36 (s, 1H; NH-biotin), 4.39 (d, ^3J(H,H) = 6.0 Hz, 2H; H_p), 4.31 (m, 1H; H_g), 4.13 (m, 1H; H_f), 3.10 (m, 1H; H_e), 2.83 (dd, ^2J(H,H) = 12.5 Hz, ^3J(H,H) = 5.0 Hz, 1H; H_h), 2.59 (d, ^2J(H,H) = 12.6 Hz, 1H; $H_{h'}$), 2.17 (t, ^3J(H,H) = 7.4 Hz, 2H; H_a), 1.68–1.28 ppm (m, 6H; $H_{b,c,d}$); ^{13}C NMR (125.75 MHz, [D$_6$]DMSO): $\delta = 172.5$ (C_i), 163.2 (C_x), 140.6 (C_v), 138.4 (C_q), 133.7 (C_u), 122.3 (C_t), 120.8 (C_r), 120.8 (C_s), 108.5 (C_w), 61.5 (C_f), 59.7 (C_g), 55.9 (C_e), 40.4 (overlap with DMSO) ($C_{h,h'}$), 42.8 (C_p), 35.7 (C_a), 28.7 ($C_{b/c/d}$), 28.5 ($C_{b/c/d}$), 25.8 ppm ($C_{b/c/d}$); ^{19}F NMR (470.56 MHz, [D$_6$]DMSO): $\delta = -76.0$ ppm (s, CF_3COOH); MS (ESI+): *m/z*: 374.45 $[M+H]^+$ (calcd 374.16); elemental analysis calcd (%) for $C_{18}H_{23}N_5O_2S \cdot 1.25\,CF_3COOH$ (516.00): C 47.72, H 4.74, N 13.57, S 6.21; found: C 47.58, H 4.89, N 13.58, S 6.42%.

Biotin(4-methylamido)pyridine (3): Biotin *N*-hydroxysuccinimidyl ester (512 mg, 1.5 mmol) and 4-(aminomethyl)pyridine (243 mg, 2.25 mmol) were dissolved in a minimum amount of DMF (ca. 10 mL). Triethylamine (418 μL, 303 mg, 3 mmol) addition to the solution caused a color change to light yellow. The reaction mixture was stirred overnight at RT. DMF was removed under reduced pressure and the residue was subsequently washed with diethyl ether, dissolved in a minimum amount of methanol, and filtered. Diethyl ether (50 mL) and petroleum ether (50 mL) were added to the filtrate, and the precipitate was removed by filtration, washed with petroleum ether, and dried in vacuo to give a pale yellow powder. The crude product was purified by reversed phase HPLC (H_2O/MeOH/0.1% CF_3COOH, gradient 5–95% MeOH, 20 min) followed by evaporation under reduced pressure to give **3** (331 mg, 66%) as a white solid. The purity of the product was confirmed by analytical HPLC (retention time: 12.8 min). ^{1}H NMR (500.10 MHz, [D$_6$]DMSO): $\delta = 8.50$ (dd, ^3J(H,H) = 4.4 Hz, ^5J(H,H) = 1.6 Hz, 2H; $H_{v,s}$), 8.41 (t, ^3J(H,H) = 5.9 Hz, 1H; N*H*), 7.24 (dd, ^3J(H,H) = 4.4 Hz, ^5J(H,H) = 1.5 Hz, 2H; $H_{w,r}$), 6.43 (s, 1H; N*H*-biotin), 6.37 (s, 1H; N*H*-biotin), 4.32 (m, 1H, H_g), 4.29 (d, ^3J(H,H) = 5.9 Hz, 2H; H_p), 4.14 (m, 1H; H_f), 3.12 (m, 1H; H_e), 2.84 (dd, ^2J(H,H) = 12.6 Hz, ^3J(H,H) = 5.1 Hz, 1H; H_h), 2.59 (d, ^2J(H,H) = 12.5 Hz, 1H; $H_{h'}$), 2.19 (t, ^3J(H,H) = 7.4 Hz, 2H; H_a), 1.68–1.28 ppm (m, 6H; $H_{b,c,d}$); ^{13}C NMR (125.75 MHz, [D$_6$]DMSO): $\delta = 172.9$ (C_i), 163.2 (C_x), 150.0 ($C_{s,v}$), 149.2 (C_q), 122.5 ($C_{r,w}$), 61.5 (C_f), 59.7 (C_g), 55.9 (C_e), 41.5 (C_p), 40.4 (overlap with DMSO) ($C_{h,h'}$), 35.6 (C_a), 28.7 ($C_{b/c/d}$), 28.5 ($C_{b/c/d}$), 25.7 ppm ($C_{b/c/d}$); MS (ESI+): *m/z*: 335.14 $[M+H]^+$ (calcd 335.15); elemental analysis calcd (%) for $C_{16}H_{22}N_4O_2S \cdot 1.1\,H_2O$ (354.25): C 54.25, H 6.89, N 15.82, S 9.05; found: C 54.47, H 6.52, N 15.55, S 8.89

Biotinyl-(*N*-ε-amidocaproic)-(6-methylamido)indazole (4): Biotin-amidohexanoic acid *N*-hydroxysuccinimidyl ester (**3**; 545 mg, 1.2 mmol) was dissolved in a minimum amount of DMF (ca. 20 mL). Triethylamine (334 μL, 2.4 mmol, 242 mg) and 6-(methylamino)indazole (**1**; 176 mg, 1.2 mmol) in DMF (10 mL) were added. The mixture was stirred for 20 h at RT, filtered, and evaporated under reduced pressure. The residue was dissolved in a minimum amount of methanol and filtered. Diethyl ether (50 mL) was added to the filtrate, and the precipitate was removed by filtration, washed with diethyl ether, and dried in vacuo to give a pale yellow solid. The crude product was purified by reversed phase HPLC (H_2O/MeOH/0.1% CF_3COOH, gradient 5–95% MeOH, 20 min) followed by evaporation under reduced pressure to give **4** (128 mg, 22%) as a white solid. The purity of the product was confirmed by analytical HPLC (retention time 18.7 min). ^{1}H NMR (500.10 MHz, [D$_6$]DMSO; Figure 4): $\delta = 12.98$ (s, 1H; N*H*-indazole),

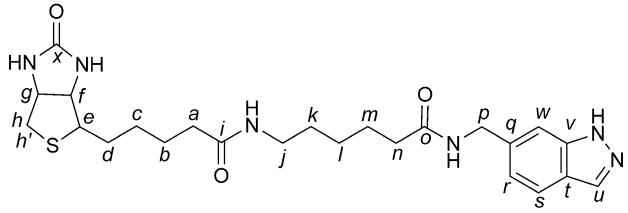


Figure 4. NMR numbering scheme used for **4**.

8.36 (t, ^3J(H,H) = 5.9 Hz, 1H; N*H*), 8.02 (m, 1H; H_u), 7.76 (t, ^3J(H,H) = 5.6 Hz, 1H; N*H*), 7.69 (d, ^3J(H,H) = 8.3 Hz, 1H; H_s), 7.37 (s, 1H; H_w), 7.01 (d, ^3J(H,H) = 8.3 Hz, 1H; H_r), 6.41 (s, 1H; N*H*-biotin), 6.35 (s, 1H; N*H*-biotin), 4.39 (d, ^3J(H,H) = 5.9 Hz, 2H; H_p), 4.31 (m, 1H; H_g), 4.13 (m, 1H; H_f), 3.40 (m,1H; H_e), 3.02 (dt, ^3J(H,H) = 12.8 Hz, ^3J(H,H) = 6.8 Hz, 2H; H_j), 2.82 (dd, ^2J(H,H) = 12.4 Hz, ^3J(H,H) = 5.0 Hz, 1H; H_h), 2.58 (d, ^2J(H,H) = 12.4 Hz, 1H; $H_{h'}$), 2.16 (t, ^3J(H,H) = 7.4 Hz, 2H; H_a), 2.05 (t, ^3J(H,H) = 7.4 Hz, 2H; H_n),1.67–1.28 (m, 12H; $H_{b,c,d,m,l,k}$); ^{13}C NMR (125.75 MHz, [D$_6$]DMSO; Figure 4): $\delta = 172.6$ (C_o), 172.4 (C_i), 163.2 (C_x), 140.6 (C_v), 138.4 (C_q), 133.7 (C_u), 122.3 (C_t), 120.8 (C_r), 120.8 (C_s), 108.4 (C_w), 61.5 (C_f), 59.7 (C_g), 55.9 (C_e), 40.4 (overlap with DMSO) ($C_{h,h'}$), 42.7 (C_p), 38.8 (C_j), 35.8 (C_a), 35.7 (C_n), 29.5 ($C_{b/c/d/m/l/k}$), 28.7 ($C_{b/c/d/m/l/k}$), 28.5 ($C_{b/c/d/m/l/k}$), 26.6 ($C_{b/c/d/m/l/k}$), 25.8 ($C_{b/c/d/m/l/k}$), 25.6 ppm ($C_{b/c/d/m/l/k}$); ^{19}F NMR (470.56 MHz, [D$_6$]DMSO): $\delta = -75.2$ ppm (s, CF_3COOH); MS (ESI+): *m/z*: 486.93 $[M+H]^+$ (calcd 487.25); elemental analysis calcd (%) for $C_{24}H_{34}N_6O_3S \cdot H_2O \cdot 0.3\,CF_3COOH$ (538.85): C 54.83, H 6.79, N 15.60, S 5.95; found: C 54.51, H 6.90, N 15.91, S 5.76.

Biotinyl-(*N*-ε-amidocaproic)-(4-methylamido)pyridine (5): The procedure of Lo et al. was used with additional purification steps.[35] Biotinamidohexanoic acid *N*-hydroxysuccinimidyl ester (182 mg, 0.4 mmol) was dissolved in a minimum amount of DMF (ca. 5 mL). Triethylamine (226 μL, 164 mg, 1.6 mmol) and a solution of 4-(aminomethyl)pyridine (65 mg, 0.6 mmol) in DMF (5 mL) were added. The mixture was stirred overnight at RT and evaporated to dryness under reduced pressure to give a pale yellow oil, which was suspended in methanol and the insoluble residue was removed by filtration. The filtrate was concentrated under reduced pressure and dichloromethane (7 mL) and diethyl ether (50 mL) were added. The precipitate was removed by filtration, washed with dichloromethane and diethyl ether, and dried in vacuo to give an off-white solid. The crude product was purified by reversed phase HPLC (H_2O/MeOH/0.1% CF_3COOH, gradient 5–95% MeOH, 20 min) followed by evaporation under reduced pressure to give **5** (66 mg, 37%) as a white solid. The purity of the product was confirmed by analytical HPLC (retention time 17.8 min). ^{1}H NMR (500.10 MHz, [D$_6$]DMSO): $\delta = 8.59$ (dd, $^3J(H,H) = 6.0$ Hz, $^5J(H,H) = 1.4$ Hz, 2H; $H_{v,s}$), 8.46 (t, $^3J(H,H) = 5.8$ Hz, 1H; N*H*), 7.74 (t, $^3J(H,H) = 5.6$ Hz, 1H; N*H*), 7.39 (dd, $^3J(H,H) = 6.1$ Hz, $^5J(H,H) = 1.4$ Hz, 2H; $H_{w,r}$), 6.42 (s, 1H; N*H*-biotin), 6.36 (s, 1H; N*H*-biotin), 4.35 (d, $^3J(H,H) = 6.0$ Hz, 2H; H_p), 4.31 (m, 1H; H_g), 4.14 (m, 1H; H_f), 3.11 (m, 1H; H_e), 3.02 (dt, $^3J(H,H) = 12.8$ Hz, $^3J(H,H) = 6.9$ Hz, 2H; H_j), 2.83 (dd, $^2J(H,H) = 12.5$ Hz, $^3J(H,H) = 5.1$ Hz, 1H; H_h), 2.59 (d, $^2J(H,H) = 12.5$ Hz, 1H; $H_{h'}$), 2.19 (t, $^3J(H,H) = 7.5$ Hz, 2H; H_a), 2.05 (t, $^3J(H,H) = 7.4$ Hz, 2H; H_n), 1.67–1.28 ppm (m, 12H; $H_{b,c,d,m,l,k}$); ^{13}C NMR (125.75 MHz, [D$_6$]DMSO): $\delta = 173.0$ (C_o), 172.3 (C_i), 163.2 (C_x),152.3 (C_q), 147.9 ($C_{s,v}$), 123.2 ($C_{r,w}$), 61.5 (C_f), 59.7 (C_g), 55.9 (C_e), 41.7 (C_p), 40.4 (overlap with DMSO) ($C_{h,h'}$), 38.8 (C_j), 35.7 (C_a), 35.7 (C_n), 29.5 ($C_{b/c/d/m/l/k}$), 28.7 ($C_{b/c/d/m/l/k}$), 28.5 ($C_{b/c/d/m/l/k}$), 26.6 ($C_{b/c/d/m/l/k}$), 25.8 ($C_{b/c/d/m/l/k}$), 25.6 ppm ($C_{b/c/d/m/l/k}$); ^{19}F NMR (470.56 MHz, [D$_6$]DMSO): $\delta = -75.15$ ppm (s, CF_3COOH); MS (ESI+): *m/z*: 448.20 $[M+H]^+$ (calcd 448.24); elemental analysis calcd (%) for $C_{22}H_{33}N_5O_3S{\cdot}0.9\,CF_3COOH$ (550.22): C 51.95, H 6.21, N 12.73, S 5.83, O 13.96; found: C 51.86, H 6.50, N 12.81, S 5.56, O 14.31.

[Ru(η^6-p-cymene)Cl$_2$(κN2-H1-indazole)] (9): The synthetic method was adapted from a reported procedure.[30] Indazole (35 mg, 0.3 mmol, 2 equiv) was added to a suspension of [{Ru(η^6-*p*-cymene)Cl$_2$}$_2$] (92 mg, 0.15 mmol) in toluene (15 mL) at RT (an orange precipitate formed immediately). The resulting mixture was heated to reflux for 3 h. After the mixture cooled, the precipitate was filtered, washed with petroleum ether (3×10 mL) and diethyl ether, and dried in vacuo, affording **9** (93 mg, 73%) as an orange microcrystalline solid. ^{1}H NMR (500.10 MHz, $CDCl_3$): $\delta = 11.78$ (s, 1H; NH-indazole), 8.41 (s, 1H; H_u), 7.71 (d, $^3J(H,H) = 8.4$ Hz, 1H; H_s), 7.41 (m, 2H; $H_{r,w}$), 7.19 (m, 1H; H_q), 5.59 (d, $^3J(H,H) = 6.0$ Hz, 2H; CHcym), 5.41 (d, $^3J(H,H) = 6.0$ Hz, 2H; CH_{cym}), 3.00 (sept, $^3J(H,H) = 7.0$ Hz, 1H; $CHMe_2$), 2.28 (s, 3H; $C_6H_4(CH_3)$), 1.29 ppm (d, $^3J(H,H) = 7.0$ Hz, 6H; $CH(CH_3)_2$); elemental analysis calcd (%) for $C_{17}H_{20}Cl_2N_2Ru$ (424.33): C 48.12, H 4.75, N 6.60; found: C 48.19, H 4.69, N 6.54.

Preparation of complexes 7, 8, 10, and 11; general procedure: [{Ru(η^6-*p*-cymene)Cl$_2$}$_2$] (4.72 mg, 0.01 mmol) and **2**–**5** (2 equiv, 0.02 mmol) were dissolved in anhydrous DMF (2 mL). The reaction mixture was stirred for 3 h, freeze-dried, and incubated in situ with the cancer cells.

[Ru(η^6-*p*-cymene)(biotin(4-methylamido)-κN1-pyridine)Cl$_2$] (7): MS (ESI-IT, pos. mode): *m/z*: 285.06 $[M-2Cl]^{2+}$ (calcd 285.08); *m/z*: 605.12 $[M-Cl]^+$ (calcd 605.13).

[Ru(η^6-*p*-cymene)(biotinyl(*N*-ε-amidocaproic)-(4-methylamido)-κN1-pyridine)Cl$_2$] (8): MS (ESI-IT, pos. mode): *m/z*: 341.58 $[M-2Cl]^{2+}$ (calcd 341.62); *m/z*: 682.20 $[M-2Cl-H]^{2+}$ (calcd 682.24); *m/z*: 718.14 $[M-Cl]^+$ (calcd 718.21).

[Ru(η^6-*p*-cymene)(biotin(6-methylamido)-κN2-H1-indazole)Cl$_2$] (10): MS (ESI-IT, pos. mode): *m/z*: 608.25 $[M-2Cl-H]^{2+}$ (calcd 608.16); *m/z*: 644.31 $[M-Cl]^+$ (calcd 644.14).

[Ru(η^6-*p*-cymene)(biotinyl(N-ε-amidocaproic)-(6-methylamido)-κN2-H1-indazole)Cl$_2$] (11): MS (ESI-IT, pos. mode): *m/z*: 360.33 $[M-2Cl]^{2+}$ (calcd 360.62); *m/z*: 721.40 $[M-2Cl-H]^{2+}$ (calcd 721.25); *m/z*: 757.35 $[M-Cl]^+$ (calcd 757.22).

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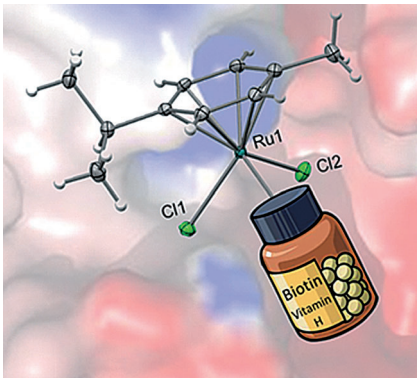
FULL PAPER

On target: Ruthenium(II) arene complexes with biotin-containing ligands were prepared so that drug delivery through tumor-specific vitamin-receptor mediated endocytosis could be exploited (see figure). Complex formation with a {Ru(η^6-*p*-cymene)} fragment resulted in significantly enhanced cytotoxicity in comparison with free ligands. These novel half-sandwich ruthenium(II) biotin conjugates may act as biological vectors to cancer cells.



Drug Discovery

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Half-Sandwich Ruthenium(II) Biotin Conjugates as Biological Vectors to Cancer Cells