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(Arene)Ru(II) complexes of epidermal growth factor receptor inhibiting tyrphostins with enhanced selectivity and cytotoxicity in cancer cells

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ABSTRACT

Ru(η^6-arene) complexes of epidermal growth factor receptor (EGFR) inhibiting tyrphostins **1a** and **1b** were prepared, characterized and tested for DNA interaction and bioactivity in four human tumor cell lines. The intrinsic cytotoxicity and cell line selectivity of *o*-hydroxyanisol **1a** was greatly enhanced in its Ru(η^6-*p*-cymene) complex **2a** and in its Ru(η^6-toluene) complex **3a**. Complex **2a** was particularly efficacious against multi-drug resistant EGFR(+) MCF-7/Topo breast carcinoma cells and also against mTOR-dependent EGFR(−) HL-60 leukemia cells. Complex **3a** showed enhanced activity only against 518A2 melanoma cells and HL-60 cells, which are both known to express the mTOR protein. DNA was strongly metallated (ca. 1.7–2%) by all new Ru complexes without undergoing topological changes. Apparently, by complexation to Ru fragments tyrphostin derivatives can address additional biological targets in a manner instrumental to antitumoral strategies.

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1. Introduction

Ruthenium complexes are attracting considerable interest as potential anticancer agents [1]. Two classical coordination compounds of Ru(III) with anti-metastatic properties, NAMI-A [2] and KP1019 [1], are currently undergoing clinical trials. Their preferential accumulation in cancer cells and low toxicity are thought to originate from their ability to bind to the iron transporter transferrin. Receptors for this protein are overexpressed in tumor cells [3]. Other mechanisms were also discussed [4]. In addition, (arene)Ru(II) complexes were devised that contain the metal in the biologically active yet ligand-stabilized oxidation state +II and which also offer opportunities for efficacy optimization by modifying the arene ring. Noteworthy examples are complexes with pta (=1,3,5-triaza-7-phosphaadamantane) ligands [5] and a series of [(arene)Ru(en)Cl]$^+$ complexes that were efficacious even in cisplatin-resistant cancer cell lines [6]. More recently, the first (arene)Ru(II) complexes with bioactive natural product ligands such as CDK-inhibiting paullones were published [7] as were staurosporin mimics containing (arene)Ru(II) fragments [8].

Following a similar approach we now prepared the first (arene)Ru(II) complexes of compounds **1a** and **1b** (RG 13022 [9]), two established inhibitors of the tyrosine kinase activity of the epidermal growth factor receptors (EGFR) and tested them for in vitro anticancer activity (Fig. 1). EGFR, the products of *c-erbB* proto-oncogenes, are dimeric transmembrane proteins which trigger cancer-relevant downstream effectors such as Src kinase, PI3 kinase or Ras protein [10] and upregulate DNA repair mechanisms [11]. On the other hand, Ru complexes such as NAMI-A are also known to interfere with membrane localized proteins, e.g., protein kinase C and Ras [12]. Finally, the clinical benefit of combination regimes of metal complexes and small inhibitors or antibodies for EGFR was already demonstrated for various tumors [13,14].

2. Results and discussion

2.1. Chemistry

The tyrphostin derivatives **1a** and **1b** were obtained from Knoevenagel reactions of 3-(cyanomethyl)pyridine and the corresponding aryl aldehyde in hot ethanol in the presence of a little piperidine [15]. The products **1** were then reacted with the respective [Ru(arene)Cl$_2$]$_2$ complex in CH$_2$Cl$_2$/CH$_3$OH mixtures to give the dichloridoruthenium complexes **2a,b** and **3a** as light brown solids in high yields (Scheme 1). They were characterized by NMR, IR and mass spectrometry. The ^{1}H and ^{13}C NMR spectra of complexes **2a** and **2b** (in CDCl$_3$) and of **3a** (in DMF-d$_7$) showed downfield shifts for the protons and carbons at positions 2 and 6 of the pyridine ring when compared with the free ligands **1** due to the deshielding effect of the central metal.

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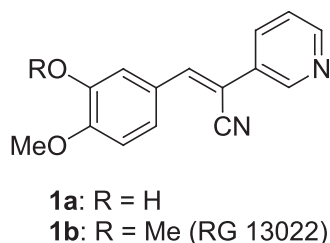
1a: R = H
1b: R = Me (RG 13022)

Fig. 1. EGFR inhibitors of the tyrphostin group.

2.2. Biological evaluation

The cytotoxicities of the tyrphostins **1a,b**, of their *p*-cymene complexes **2a,b** and of the toluene complex **3a** were evaluated by means of MTT assays with cells of human 518A2 melanoma, HL-60 leukemia, KB-V1/Vbl cervix carcinoma and MCF-7/Topo breast carcinoma (Table 1). The nature of both organic ligands, the tyrphostin and the η^6-bound arene, had a distinct influence on the anticancer activity of the new complexes. The 1,2-dimethoxyphenyl derivative RG 13022 (**1b**) and its Ru(*p*-cymene) complex **2b** were far less efficacious against all cell lines than the *o*-hydroxyanisyl analogues **1a**, **2a**, and **3a**. While coordination of **1b** to a ruthenium fragment afforded a complex **2b** of diminished cytotoxicity, ligation of the hydroxyanisol **1a** led to complexes **2a** and **3a** with significantly enhanced activity. The nature of the η^6-arene ligand also had an influence, apparent from the different cell line specificities of these complexes. The *p*-cymene complex **2a** was most cytotoxic in the multi-drug resistant MCF-7/Topo cancer cells at an IC$_{50}$ (48 h) of ca. 0.2 μM, which is a seventh of that of the free ligand **1a**. A similar increase in activity was observed for the toluene complex **3a** against 518A2 melanoma cells. In HL-60 cells both complexes **2a** and **3a** showed the same high activity (IC$_{50}$ (48 h) ca 1 μM), exceeding that of the uncomplexed tyrphostin **1a** by a factor of nine. Unspecific ruthenation is an unlikely reason for this activity boost as ruthenium complex **2b** was virtually inactive in these cells. An alternative rationale is based on the fact that HL-60 cells, while lacking endogenous EGFR [16], depend on Akt/mTOR signaling [17]. Possibly, tyrphostin **1a** and its complexes **2a** and **3a** interfere with this pathway by EGFR-independent mechanisms. Like HL-60 cells, 518A2 melanoma cells express mTOR and respond to rapamycin, the classical inhibitor of this protein. This matches with the observation that 518A2 and HL-60 cells responded much faster to **2a** and **3a** than the multi-drug resistant cells. Only upon 48 h of incubation complex **2a** surpassed tyrphostin **1a** in growth inhibition of both MDR(+) cells which are derived from parent lines possessing EGFR [18,19]. Complex **3a** remained inferior to **1a** even after 48 h. Both EGFR-inhibiting tyrphostins **1a** and **1b** used in this study are generally more cytotoxic in these multi-drug resistant cells than in 518A2 and HL-60 cells. The extraordinary increase in efficacy of complex **2a** against MCF-7/Topo cells [IC$_{50}$(48 h) = 200 nM] when compared with that of the parent tyrphostin **1a** [IC$_{50}$(48 h) = 1.5 μM] is nevertheless remarkable and possibly due to synergisms originating from the cymene ligand

Table 1
Inhibitory concentrations IC$_{50}$[a] [μM] of compounds **1**, **2** and **3a** when applied to cells of human 518A2 melanoma, HL-60 leukemia, Kb-V1/Vbl cervix carcinoma and MCF-7/Topo breast carcinoma.

	518A2 (24 h/48 h)	HL-60 (24 h/48 h)	Kb-V1/Vbl (24 h/48 h)	MCF-7/Topo 24 h/48 h
1a	94/16 ± 3.4	11 ± 2.4/9 ± 1.4	9 ± 2.4/6 ± 1.4	50/1.5 ± 0.3
1b	45 ± 1.5/50 ± 6.2	33 ± 4.2/32 ± 3.3	46 ± 5.6/20 ± 4.8	48 ± 5.8/11 ± 3.8
2a	31 ± 2.3/13 ± 4.2	2 ± 1.1/0.8 ± 0.2	50/3 ± 07	50/0.2 ± 0.14
2b	–/60 ± 1.7	–/95 ± 5	59 ± 3.1/31 ± 4.7	49 ± 3.9/29 ± 3.4
3a	3 ± 0.3/2.2 ± 0.2	1.3 ± 0.02/1 ± 0.2	20 ± 4.6/7 ± 1.5	75 ± 13.7/7 ± 2.7

[a] Values are derived from concentration–response curves obtained by measuring the percentage of grown cells relative to untreated controls after the indicated time of incubation using an MTT assay.

beneficially mediating the uptake or efflux of complex **2a**, or the specific metallation of certain bionucleophiles. In calcein-AM and mitoxantron assays no significant differences were observed in the interaction of the test compounds with the ABC-transporters of the resistant cells.

The binding affinities of the complexes for various forms of DNA were slightly different. According to ICP-OES analytics, the metal content of salmon sperm DNA was about 2% when treated for 24 h with 50 μM of the *p*-cymene complexes **2** and ca. 1.7% upon exposure to the toluene complex **3a**. This is ca. ten times the metallation degree typically achieved with platinum complexes [20]. However, in electrophoretic mobility shift assays (EMSA) with pBR322 plasmid DNA the new complexes did not give rise to any bandshift effects. This means they either did not bind to this DNA form or, if bound, did not cause topological changes. This resembles reports by Reedijk et al. for ruthenium polypyridyl complexes [21]. Insofar, these Ru(II) complexes differ sharply from cisplatin, and it is possible that DNA-binding contributes to the cytotoxicity of the active Ru complexes **2a** and **3a** in certain cancer cells.

Their uptake by 518A2 melanoma cells correlated with their sperm DNA-binding but not with their cytotoxicity in these cells. The Ru content of 518A2 cells was found by ICP-MS to be 1.43 ± 0.01 μg/L upon 24 h exposure to 10 μM cymene complex **2a** but only 1.22 ± 0.01 μg/L when treated with toluene complex **3a**. This difference is possibly a consequence of the more hydrophobic character of the cymene when compared with the toluene ligand. Apparently, the cellular complex concentration alone is not decisive for their cytotoxic potency.

3. Conclusion

Two new DNA-binding (η^6-arene)Ru(II) complexes **2a** and **3a** bearing a tyrphostin ligand were prepared and found to have enhanced cytotoxicities and cancer cell line specificities when compared with the free tyrphostin. The (*p*-cymene)Ru complex **2a** was particularly active in multi-drug resistant and EGFR(+) cancer cells, e.g., MCF-7/Topo breast carcinoma cells. The (toluene)Ru complex **3a** displayed a specific anti-melanoma activity which is possibly based on interference with mTOR signaling and obviously independent from EGFR inhibition. Unlike cytotoxic platinum compounds the new ruthenium complexes bound strongly to DNA without altering its topology. These findings open up new possibilities for the development of selective and multi-drug resistance breaking organometallic compounds consisting of a ligand specific for a certain biochemical target and a Ru fragment that confers selectivity to the whole complex for another target in a synergistic manner. The detailed mechanism of action of the new complexes is under investigation.

1a: R^1 = H
1b: R^1 = Me

2a: R^1 = H; R^2 = *i*-Pr
2b: R^1 = Me; R^2 = *i*-Pr
3a: R^1 = R^2 = H
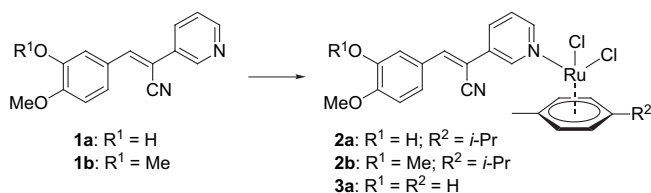
Scheme 1. Reagents and conditions: [Ru(arene)Cl$_2$]$_2$, CH$_2$Cl$_2$/CH$_3$OH, 3 h, r.t., 87–91%.

4. Experimental protocols

Melting points were determined with a Gallenkamp apparatus and are uncorrected. IR spectra were recorded on a Perkin–Elmer One FT-IR spectrophotometer. Magnetic resonance (NMR) spectra were recorded under conditions as indicated on a Bruker Avance 300 spectrometer. Chemical shifts (δ) are given in parts per million downfield from TMS as internal standard. Mass spectra were recorded using a Varian MAT 311A (EI). Elemental analyses were carried out with a Perkin–Elmer 2400 CHN elemental analyser. Satisfactory microanalyses (C, $\pm$0.2; H, $\pm$0.1) were obtained for the new complexes. [Ru(toluene)Cl$_2$]$_2$ was prepared according to a procedure for the preparation of [Ru(benzene)Cl$_2$]$_2$ [22]. Tyrphostin derivatives **1a** and **1b** were prepared according to a literature procedure [15].

4.1. Chemistry

4.1.1. Dichlorido(p-cymene)[3-(1′-cyano-2′-(3″-hydroxy-4″-methoxyphenyl)(Z)-ethenyl)pyridine]ruthenium(II) 2a

A mixture of compound **1a** (58 mg, 0.23 mmol), CH$_2$Cl$_2$ (5 mL), a few drops of methanol and [Ru(p-cymene)Cl$_2$]$_2$ (70 mg, 0.11 mmol) was stirred at room temperature for 3 h. A mixture of ethyl acetate and *n*-hexane (50 mL, 1:4) was added and the resulting suspension was stirred for 5 min. The precipitate was collected, washed with *n*-hexane and dried in vacuum. Yield: 119 mg (0.21 mmol, 91%); yellow solid of m.p. 190 °C (dec.); ν_{max} (ATR)/cm^{-1} 3236, 2949, 2207, 1602, 1573, 1535, 1437, 1374, 1320, 1310, 1269, 1211, 1192, 1137, 1033, 937, 890, 873, 821, 794; ^{1}H NMR (300 MHz, CDCl$_3$/CD$_3$OD) δ 1.27 (6H, d, $J = 6.9$ Hz), 2.04 (3H, s), 2.8–3.0 (1H, m), 3.90 (3H, s), 5.29 (2H, d, $J = 6.0$ Hz), 5.50 (2H, d, $J = 6.0$ Hz), 6.89 (1H, d, $J = 8.5$ Hz), 7.3–7.4 (2H, m), 7.47 (2H, s), 7.9–8.0 (1H, m), 8.89 (1H, d, $J = 5.6$ Hz), 9.20 (1H, s); ^{13}C NMR (75.5 MHz, CDCl$_3$/CD$_3$OD) δ 17.9, 22.0, 30.6, 55.7, 81.9, 82.8, 97.4, 102.5, 103.4, 111.0, 115.3, 123.6, 124.3, 125.9, 132.2, 134.8, 145.8, 146.1, 150.6, 151.4, 153.8; MS (EI) *m/z* 252 (19), 233 (4), 223 (6), 205 (5), 134 (26), 119 (100), 115 (42), 105 (31), 91 (22).

4.1.2. Dichlorido(p-cymene)[3-(1′-cyano-2′-(3″,4″-dimethoxyphenyl)(Z)-ethenyl)pyridine]ruthenium(II) 2b

Analogously to complex **2a**, compound **2b** was obtained from **1b** (61 mg, 0.23 mmol) and [Ru(p-cymene)Cl$_2$]$_2$ (70 mg, 0.11 mmol). Yield: 112 mg (0.20 mmol, 86%); yellow solid of m.p. 120 °C (dec.); ν_{max} (ATR)/cm^{-1} 2961, 2868, 2208, 1588, 1567, 1515, 1465, 1428, 1369, 1327, 1276, 1243, 1166, 1147, 1038, 1020, 907, 852, 803, 689; ^{1}H NMR (300 MHz, CDCl$_3$) δ 1.30 (6H, d, $J = 7.0$ Hz), 2.10 (3H, s), 2.9–3.1 (1H, m), 3.92 (3H, s), 3.93 (3H, s), 5.25 (2H, d, $J = 6.0$ Hz), 5.46 (2H, d, $J = 6.0$ Hz), 6.90 (1H, d, $J = 8.5$ Hz), 7.2–7.5 (2H, m), 7.52 (1H, s), 7.65 (1H, s), 7.8–7.9 (1H, m), 8.89 (1H, d, $J = 5.6$ Hz), 9.23 (1H, s); ^{13}C NMR (75.5 MHz, CDCl$_3$) δ 18.2, 22.3, 30.7, 56.0, 82.3, 82.7, 97.1, 102.8, 103.7, 111.0, 111.1, 124.6, 125.6, 125.9, 132.1, 134.7, 145.6, 149.0, 151.5, 152.1, 154.1; MS (EI) *m/z* 577 (6), 306 (14), 266 (100), 179 (36), 119 (26).

4.1.3. Dichlorido(toluene)[3-(1′-cyano-2′-(3″-hydroxy-4″-methoxyphenyl)(Z)-ethenyl)pyridine]ruthenium(II) 3a

Analogously to cymene complex **2a**, the complex **3a** was obtained from **1a** (58 mg, 0.23 mmol) and [Ru(toluene)Cl$_2$]$_2$ (60 mg, 0.11 mmol). The crude precipitate was collected, washed with CH$_2$Cl$_2$ and dried. Yield: 101 mg (0.20 mmol, 87%); yellow solid of m.p. 220 °C (dec.); ν_{max} (ATR)/cm^{-1} 3259, 3025, 2210, 1599, 1590, 1571, 1528, 1507, 1437, 1375, 1269, 1211, 1193, 1134, 1024, 857, 821, 695; ^{1}H NMR (300 MHz, DMF-d$_7$) δ 2.18 (3H, s), 3.95 (3H, s), 5.57 (2H, d, $J = 5.5$ Hz), 5.7–5.9 (3H, m), 7.18 (1H, d, $J = 8.5$ Hz), 7.5–7.7 (3H, m), 7.97 (1H, s), 8.3–8.4 (1H, m), 9.08 (1H, d, $J = 5.9$ Hz), 9.41

(1H, s), 9.69 (1H, s); ^{13}C NMR (75.5 MHz, DMF-d$_7$) δ 18.4, 55.9, 80.0, 81.5, 87.4, 100.5, 103.1, 112.2, 115.7, 117.8, 123.8, 124.4, 126.8, 131.8, 134.9, 145.9, 147.6, 151.5, 152.6, 155.2; MS (EI) *m/z* 252 (90), 223 (24), 205 (26), 91 (100).

4.2. Biological studies

4.2.1. Cytotoxicity assays

HL-60 [23], KB-V1/Vbl and MCF-7/Topo cells were obtained from the German National Resource Center for Biological Material (DSMZ), Braunschweig. HL-60 cells were incubated in RPMI (Roswell Park Memorial Institute) media 1640 with 10% FBS (fetal bovine serum), 0.55% antibiotic–antimycotic and 0.3% gentamycin (all Gibco). KB-V1 cells were cultured in D-MEM (Dulbecco's modified eagle medium, Invitrogen, Carlsbad, CA) with 10% FBS, 0.55% antibiotic–antimycotic, 0.34 μM vinblastine and 0.3% gentamycin. MCF-7/Topo cells were grown in E-MEM (Eagle's minimum essential medium, Sigma–Aldrich) supplemented with 5% FBS and 0.55 μM topotecane. 518A2 cells [24,25] were obtained from the department of oncology and hematology of the Martin-Luther-University, Halle, and cultured like the KB-V1 cells.

Cultured cancer cells were grown in the presence of the test compounds in 96-well plates. After 24 h and 48 h treatment, the cell growth rates were determined by an assay with MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] as described in detail elsewhere [26]. These experiments were carried out in triplicate; the percentage of viable cells quoted was calculated as the mean $\pm$ S.D. with respect to the controls set to 100%.

4.2.2. DNA-binding via ICP-OES

Salmon sperm DNA (10 mg/mL) was incubated with Ru-complexes at a final concentration of 50 μM in TE buffer (Tris–HCl, EDTA, pH 8.0) supplemented with 10 mM NaClO$_4$ at 37 °C for 24 h. The stock solutions (10 mM) of the complexes were prepared in DMSO and freshly diluted in TE buffer with 10 mM NaClO$_4$ before using, while the DMSO content in every sample was 0.5%. DMSO was used as negative control. To precipitate the DNA, equal volumes of ethanol were added and incubated at −20 °C overnight. Following centrifugation at 12,000 *g* for 10 min the supernatants were discarded, the pellets were washed twice with 70% ice-cold ethanol, and finally DNA was lyophilized. The Ru content of the DNA samples was ascertained with a Varian "Vista Pro" ICP-OES.

4.2.3. Cellular uptake

518A2 cells (0.5×10^6/mL) were seeded into 6-well plates and grown overnight. The medium was replaced with medium containing 10 μM ruthenium complex (**2a** or **3a**; 0.1% DMF w/v) and cells were incubated for 24 h. Trypsinated cells were centrifuged and washed twice with PBS. The cell pellets were weighed and resuspended in 10 mL of water. The Ru content was measured with a Varian ICP-MS. Experiments were carried out in triplicate.

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