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ORIGINAL PAPER

Chlorambucil conjugates of dinuclear *p*-cymene ruthenium trithiolato complexes: synthesis, characterization and cytotoxicity study in vitro and in vivo

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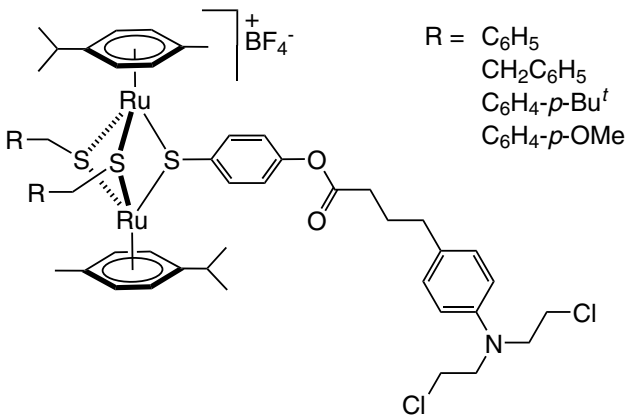
Abstract Four diruthenium trithiolato chlorambucil conjugates have been prepared via Steglich esterification from chlorambucil and the corresponding trithiolato precursors. All conjugates are highly cytotoxic towards human ovarian A2780 and A2780cisR cancer cell lines with IC_{50} values in the nanomolar range. The conjugates exhibit selectivity towards A2780 cells as compared to non-cancerous HEK293 cells, while being only slightly selective for RF24 and A2780cisR cells. In vivo, the conjugate [**10**]BF_4 suppressed the growth of a solid Ehrlich tumor in immunocompetent NMRI mice but did not prolong their overall survival. The reactivity of the chlorambucil conjugates with glutathione, a potential target of the dinuclear ruthenium motive, and with the 2-deoxyguanosine 5′-monophosphate (dGMP—a model target of chlorambucil) was studied by mass spectrometry and NMR spectroscopy. The conjugates did not show catalytic activity for the oxidation of glutathione nor binding to nucleotides, indicating that glutathione oxidation and DNA alkylation are not key mechanisms of action.

Graphical abstract Four highly cytotoxic diruthenium trithiolato chlorambucil conjugates have been prepared. All conjugates exhibit selectivity towards A2780 cells as compared to HEK293 cells, while being only slightly active in RF24 and A2780cisR cells. In vivo, the best candidate suppressed the growth of a solid Ehrlich tumor in immunocompetent NMRI mice but did not prolong their overall survival.



Keywords Arene ruthenium · Chlorambucil · Dinuclear complexes · Anticancer activity · In vivo study

Abbreviations

cabCOOH	Chlorambucil
CTG	CellTiter-Glo® luminescent cell viability assay
dGMP	2-deoxyguanosine 5′-monophosphate
dmso	Dimethylsulfoxide
ECACC	European Centre of Cell Cultures

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en	Ethylenediamine
GSH	Reduced glutathione
GSSG	Oxidized glutathione
ind	Indazole
imi	Imidazole
MTD	Maximum tolerated dose
SEM	Standard error of the mean
VUFB	Research Institute for Pharmacy and Biochemistry

Introduction

Inspired by the discovery of the anticancer properties of *cis*-$Pt(NH_3)_2Cl_2$ (cisplatin) by Rosenberg in 1970 [1], research on metal-based anticancer agents expanded to metals other than platinum [2–4]. Already in 1980, Clarke showed that certain ruthenium complexes are cytotoxic to cancer cells [5]. Moreover, in vivo they often possess a lower general toxicity than platinum complexes. Based on these encouraging biological properties and the fact that their physico-chemical properties can be easily tuned, ruthenium complexes are among the most studied non-platinum metal-based drugs [6–9].

Among ruthenium complexes, arene ruthenium derivatives exhibit promising anti-cancer activity. A wide and diverse range of arene ruthenium complexes has been studied [10–12], including $[(\eta^6\text{-arene})Ru(P\text{-pta})Cl_2]$ (pta = 1,3,5-triaza-7-phospha-tricyclo-[3.3.1.$1^{3,7}$]-decane) complexes termed RAPTA [13, 14] as well as $[(\eta^6\text{-arene})Ru(N,N\text{-en})Cl]^+$ (en = 1,2-ethylenediamine) complexes [15, 16]. Moreover, coupling of organic molecules to arene ruthenium complexes can increase their solubility, facilitate their transport into cells or inhibit unique cancer targets. For instance, Hartinger's group designed an arene ruthenium peptide conjugate by a click reaction of the ruthenium moiety containing a pyronato ligand with the neuropeptide [Leu^5]-enkephalin [17]. The resulting conjugate showed antiproliferative activity in human ovarian carcinoma cells with an IC_{50} value of 13 μM, whereas the peptide or the ruthenium moiety alone were hardly cytotoxic. Similarly, the ruthenium complex $[(\eta^6\text{-}C_6H_5CH_2NH_2)Ru(P\text{-pta})Cl_2]$ was coupled to ethacrynic acid, a compound that inhibits the enzyme glutathione-S-transferase. The resulting complex was found to bind to glutathione-S-transferase, whereupon the metal moiety was enzymatically cleaved and released into the sensitized cancer cells [18]. Very recently, RAPTA-type complexes were reported, in which the arene ligand was functionalized by chlorambucil [19], an anticancer drug routinely employed in the treatment of chronic myeloid leukemia, myeloma and chronic lymphocytic leukemia [20–22]. Some of these chlorambucil-functionalized RAPTA complexes show superior anticancer activity to cisplatin-resistant cancer cells as compared to chlorambucil, to the parent RAPTA complex or to the mixture of both. These conjugates are supposed to act by two distinct but complementary modes of action, namely by protein ruthenation and by DNA alkylation [19].

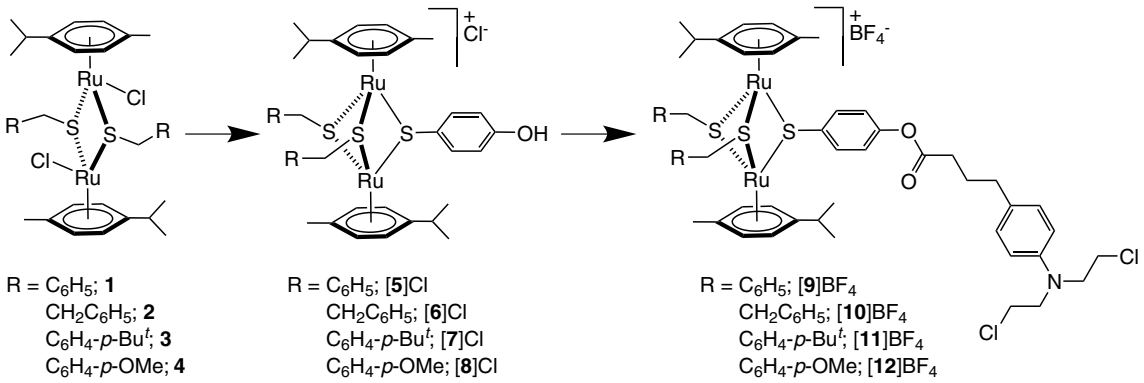
Complexes of the general formula $[(\eta^6\text{-arene})_2Ru_2(\mu\text{-SR})_3]^+$ are highly cytotoxic to human ovarian cancer cells, they are in fact among the most active arene ruthenium anti-cancer compounds reported so far, the IC_{50} values of these compounds being in the nanomolar range for A2780 human ovarian cancer cells and for the cisplatin-resistant A2780cisR cells [23–25]. Like other arene ruthenium complexes, thiolato-bridged arene ruthenium complexes were recently coupled to oligopeptides, the resulting complexes having significantly increased solubility in water while maintaining their high cytotoxicity [26]. These dinuclear arene ruthenium trithiolato complexes can also be functionalized at only one of the thiolato ligands, in particular since mixed complexes of the type $[(\eta^6\text{-arene})_2Ru_2(SR^{al})_2(SR^{ar})]^+$ are easily accessible with aliphatic (al) and aromatic (ar) substituents at the thiolato bridges [27].

We therefore prepared the neutral dithiolato precursors $[(\eta^6\text{-}p\text{-Me-}C_6H_4\text{-}Pr^i)_2\text{-}Ru_2(SCH_2R)_2Cl_2]$ (R = Ph: **1**, R = CH_2Ph: **2**, R = C_6H_4-*p*-Bu^t: **3**, R = C_6H_4-*p*-OCH_3: **4**) from *p*-cymene ruthenium dichloride dimer and the corresponding thiol, as well as the cationic mixed trithiolato complexes $[(\eta^6\text{-}p\text{-Me-}C_6H_4\text{-}Pr^i)_2\text{-}Ru_2(SCH_2R)_2\text{-}(SC_6H_4\text{-}p\text{-OH})]^+$ (Ph: **5**, R = CH_2Ph: **6**, R = C_6H_4-*p*-Bu^t: **7**) by reaction with *p*-mercaptophenol according to published methods [27–29]. The new derivative $[(\eta^6\text{-}p\text{-Me-}C_6H_4\text{-}Pr^i)_2\text{-}Ru_2(SCH_2C_6H_4\text{-}p\text{-OMe})_2(SC_6H_4\text{-}p\text{-OH})]^+$ (**8**) was synthesized in an analogous way. The phenolic group of the mixed thiolato-bridged cationic complexes **5**–**8** can be subsequently functionalized with chlorambucil. Herein we report the synthesis, the characterization and the in vitro activity of four thiolato-bridged arene ruthenium chlorambucil conjugates and an in vivo evaluation of the most promising derivative.

Results and discussion

Synthesis and characterization

The three previously reported trithiolato complexes **5**–**7** were synthesized according to published methods [27–29]. The new complex **8** was synthesized in an analogous fashion, isolated as a chloride salt and fully characterized. To allow the conjugation of the cationic trithiolato complexes with chlorambucil (cabCOOH), compounds [**5**–**8**]Cl were reacted with sodium tetrafluoroborate to yield the corresponding tetrafluoroborate salts, which were reacted with chlorambucil under Steglich esterification reaction conditions



Scheme 1 Synthesis of the precursor complexes [**5**–**8**]Cl from complexes **1**–**4**, and the chlorambucil conjugates [**9**–**12**]BF_4 (see "Experimental part" for the conditions)

Table 1 IC_{50} values (nM) of complexes **1**–**4** and [**5**–**8**]Cl on human ovarian carcinoma cells

Compound	A2780	A2780cisR
1	2940 ± 600	3600 ± 800
2	200 ± 50	310 ± 80
3	>5000	>5000
4	235 ± 50	>5000
[**5**]Cl	47.8 ± 3.0	42.9 ± 1.0
[**6**]Cl	74.4 ± 2.8	49.9 ± 1.9
[**7**]Cl	163 ± 8.0	59.5 ± 1.3
[**8**]Cl	320 ± 80	109 ± 30
Cisplatin[a]	860 ± 60	>10,000

Values are given as the means ± the standard error of the mean

[a] Taken from Ref. [35]

Table 2 IC_{50} values (nM) of [**9**–**12**]BF_4 against human carcinoma cell lines A2780 and A2780cisR determined using the CTG assay after 72 h

Compound	A2780	A2780cisR	HEK293	RF24
[**9**]BF_4	75 ± 14	321 ± 44	335 ± 26	152 ± 15
[**10**]BF_4	55 ± 6	353 ± 10	448 ± 40	325 ± 45
[**11**]BF_4	183 ± 25	335 ± 50	451 ± 65	280 ± 36
[**12**]BF_4	600 ± 72	591 ± 62	1580 ± 300	770 ± 100
Cisplatin[a]	860 ± 60	>10,000	4400 ± 400	N.D.

Values are given as the means ± the standard error of the mean

[a] Taken from Ref. [35]

to give the chlorambucil conjugates [(η^6-*p*-MeC_6H_4-Pr^i)$_2$-Ru_2(SCH_2R)$_2$(SC_6H_4-*p*-OOCcab)]$^+$ R = C_6H_5: **9**, R = $CH_2C_6H_5$: **10**, R = C_6H_4-*p*-Bu^t: **11**, R = C_6H_4-*p*-OMe: **12**) (Scheme 1). The cationic complexes are isolated as the tetrafluoroborate salts in the form of orange crystalline powders. Compounds [**9**–**12**]BF_4 are well soluble in chlorinated solvents, alcohols, acetonitrile, THF and DMSO. All complexes have been fully characterized by ^1H and ^{13}C NMR spectroscopy, mass spectrometry and elemental analysis, the analytical data are given in the Experimental Part.

Cytotoxicity studies

The in vitro anticancer activity of the chlorambucil conjugates [**9**–**12**]BF_4 was evaluated against the human ovarian cancer cell lines A2780 and A2780cisR with acquired resistance to cisplatin using the CellTiter-Glo® luminescent cell viability assay (CTG) that determines the number of viable cells in culture based on quantitation of the ATP present, an indicator of metabolically active cells. The IC_{50} values are compared with values for the neutral dithiolato complexes **1**–**4** and cisplatin (Table 1). In general, the neutral dithiolato complexes are less cytotoxic than the cationic trithiolato complexes; the new derivative **8** confirms this tendency. The chloride salts of the cationic trithiolato complexes [**5**–**8**]Cl exhibit IC_{50} values in the nanomolar range.

Chlorambucil, *N,N*-bis(2-chloroethyl)-*p*-aminophenylbutyric acid, is a drug routinely employed in the treatment of chronic myeloid leukemia, myeloma and chronic lymphocytic leukemia [20, 21]. Its anticancer properties involve direct binding to DNA, specifically to the N7 of guanines. Chlorambucil can form either monofunctional nucleotide adducts, or interact with both of its electrophilic sites at once, forming either intrastrand or interstrand DNA cross-links [22].

The cytotoxicity of chlorambucil conjugates [**9**–**12**]BF_4 was studied on four cell lines: non-cancerous human embryonic kidney cells (HEK293), immortalized human endothelial cells (RF24) and the human ovarian cancer A2780 and A2780cisR cell lines. The IC_{50} values for the two ovarian cancer cell lines are in the nanomolar range (Table 2), albeit higher than those of the chloride salts of

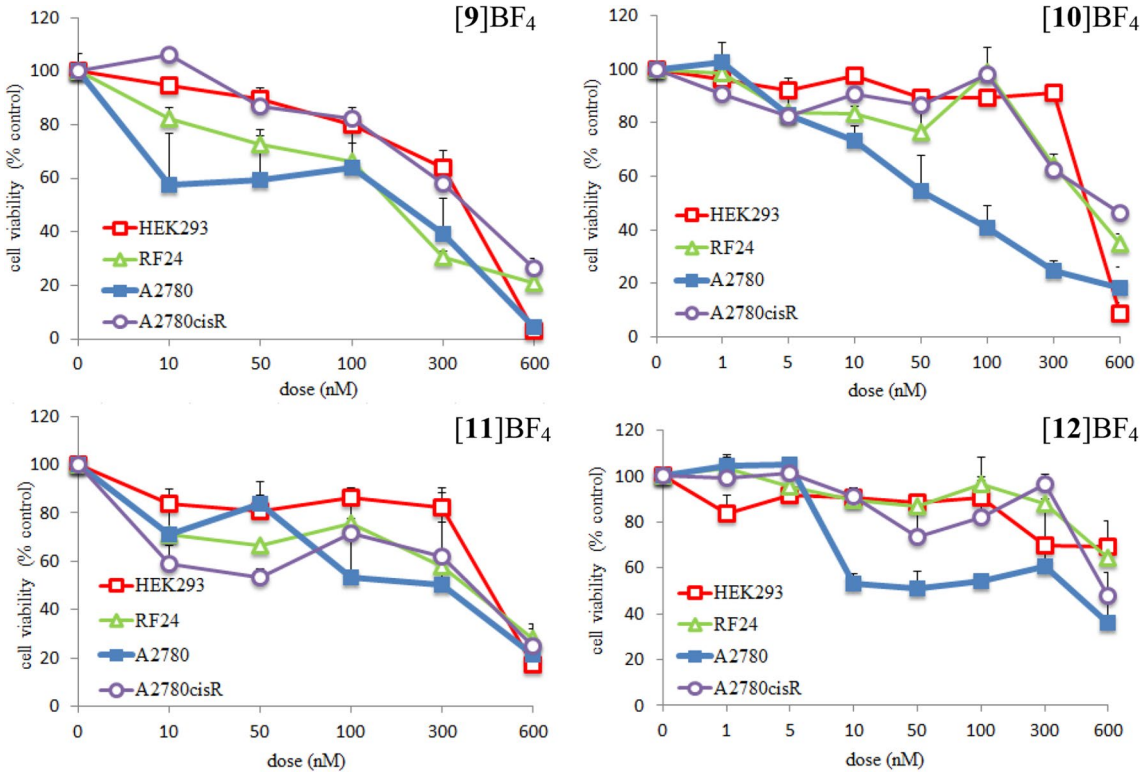


Fig. 1 Dose-dependent cell viability after administration of [**9**–**12**]BF_4. Values are presented as the means ± the standard error of the mean

the corresponding trithiolato complexes [**5**–**8**]Cl that are not conjugated to chlorambucil (Table 1).

The cell viability experiments conducted in the four cell lines (Fig. 1) show that the chlorambucil conjugates [**10**]BF_4 and [**12**]BF_4 exhibit selectivity for the A2780 cell line at the dose range 50–300 nM (complex **10**) or 10–100 nM (complex **12**) as compared to HEK293 cells. The IC_{50} value for the chlorambucil conjugate [**10**]BF_4 is 55 ± 6 nM for A2780 cells, similar to that of its precursor [**6**]Cl (74.4 ± 2.8 nM), while for chlorambucil alone it is approximately 22-fold higher (1200 nM) [30]. Complex [**11**]BF_4, at low doses (up to 70 nM), is the most selective one towards A2780cisR cells, which is not observed in the higher dose range. In general, the four conjugates are less cytotoxic to the RF24 and A2780cisR cells at lower doses, while at higher doses the difference in cytotoxic activity is not retained.

Interactions with 2-deoxyguanosine 5′-monophosphate and glutathione

In order to study the mechanism of action of the new chlorambucil conjugates, the conjugate with the highest activity in vitro, [**10**]BF_4, was incubated with the tripeptide glutathione and with the nucleotide 2-deoxyguanosine 5′-monophosphate. The catalytic oxidation of glutathione is reported to be at least partially responsible for the high in vitro cytotoxicity of trithiolato dinuclear arene ruthenium complexes [31]. On the other hand, the mode of action of chlorambucil is its covalent binding to DNA nucleotides, namely to the N7 position of guanine. Both of these targets were investigated in order to see if the chlorambucil conjugates retain the mode of action of its respective parts.

Interestingly, [**10**]BF_4 does not oxidize glutathione, although its precursor [**6**]Cl has a reported turnover frequency of 7.02 h^{-1} for the catalytic glutathione oxidation [27]. After 24 h of incubation at 37 °C, the NMR spectrum shows only the peaks of the reduced form of glutathione, no formation of the disulfide being detected.

The interaction of [**10**]BF_4 with 2-deoxyguanosine 5′-monophosphate (dGMP) was studied using mass spectrometry at two different pH values, at pH = 3.5 without the addition of any buffer or base, and after the addition of 0.1 M NaOH, which raised the pH to 8. According to biological studies of chlorambucil [32], the alkylation of guanines proceeds much faster at elevated pH. However, in our case the MS spectra did not show any adducts of 2-deoxyguanosine 5′-monophosphate and [**10**]BF_4 even at pH = 8. The only detected products aside from the original complex were the two hydrolysis products of the chlorambucil moiety of the conjugate, as shown in Fig. 2. When the same

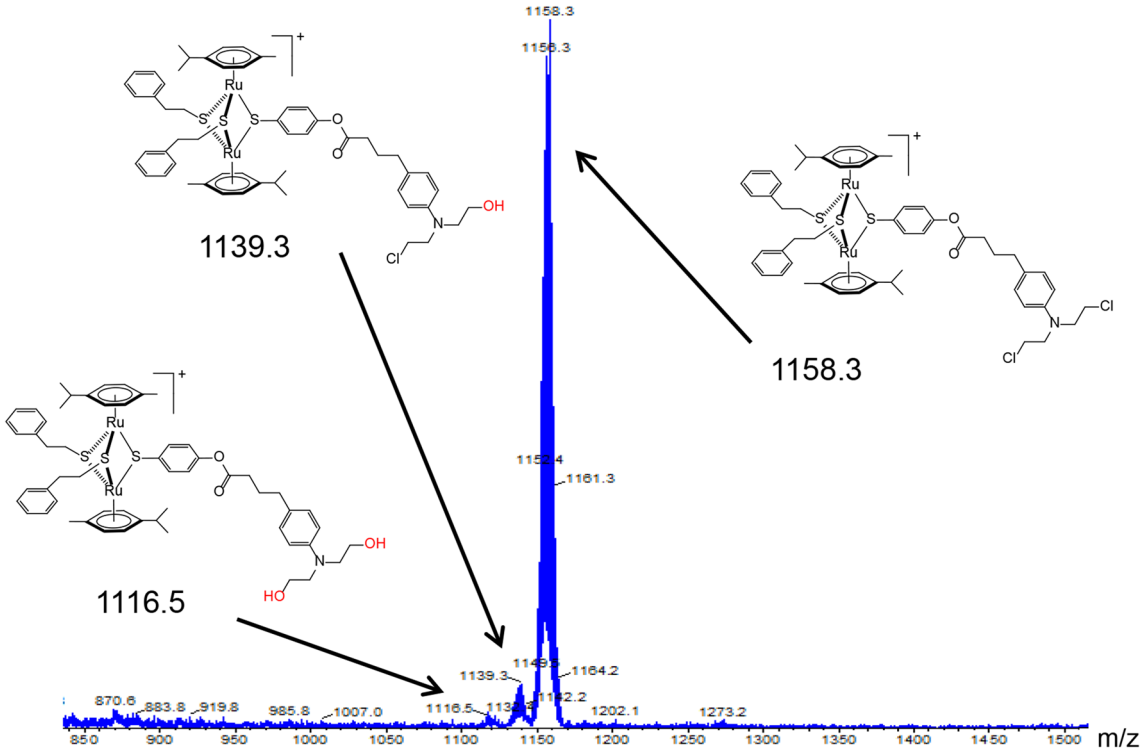


Fig. 2 Hydrolysis products of compound [**10**]BF_4 after 24 h incubation at 37 °C with four equivalents of 2-deoxyguanosine 5′monophosphate

experiment was followed by ^{1}H NMR spectroscopy, no adduct formation was observed (data not shown).

These results, although preliminary, suggest that the conjugation of the thiolato-bridged arene ruthenium complex to chlorambucil hampers the chlorambucil moiety to interact with nucleotides. Conversely, the conjugation also blocks the catalytic activity of the dinuclear trithiolato unit and the mode of action of this compound is therefore unclear.

In vivo study

To investigate the effects of [**10**]BF_4 on tumor development and survival of adult female immunocompetent NMRI mice, we used a solid Ehrlich tumor implanted subcutaneously as an experimental model for breast cancer. The compound doses were selected according to the dose-finding study, which had revealed a maximum tolerated dose (MTD) of 20 mg/kg. For the MTD assessment, two or three healthy mice per group were observed for weight loss (the limit was 10 %) over 14 days after treatment. Figure 3 shows the weight of resected tumors after treatment with saline in propane-1,2-diol, [**10**]BF_4 administered at 20, 15 or 10 mg/kg, or chlorambucil (5 mg/kg), respectively, measured on day 11. [**10**]BF_4 at the dose of 15 mg/kg had a significant inhibitory effect on tumor growth (TGI = 44.4 %, $P = 0.0474$). That was

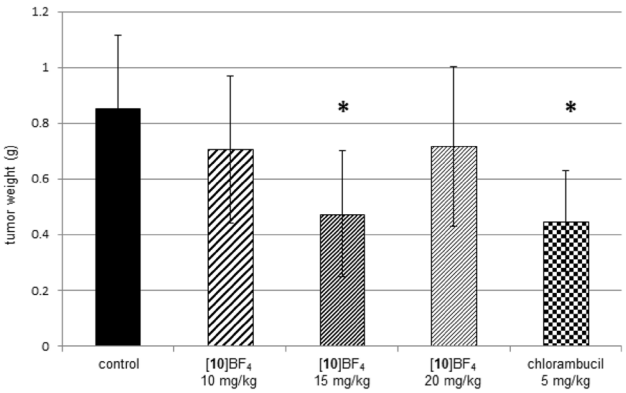


Fig. 3 Weight of the solid Ehrlich tumor (in grams) on day 11 of mice injected on days 1, 4 and 8 i.p. with [**10**]BF_4 in doses of 10, 15, or 20 mg/kg, or chlorambucil 5 mg/kg. Values are the means ± the standard error of the mean (SEM) ($n = 7$ in each group). Values that are significantly different from the control experiment are labeled by * (*$P < 0.05$, ***$P < 0.001$)

similar to the activity of the positive control, chlorambucil (TGI = 47.4 %, $P = 0.0268$).

The mean overall survival of sham-treated control tumor-bearing mice was 21.6 days. [**10**]BF_4 at both 10 and 15 mg/kg showed a tendency to prolong the survival albeit the effect was not significant ($P = 0.458$ and 0.693, respectively). The group receiving [**10**]BF_4 at 20 mg/kg could not

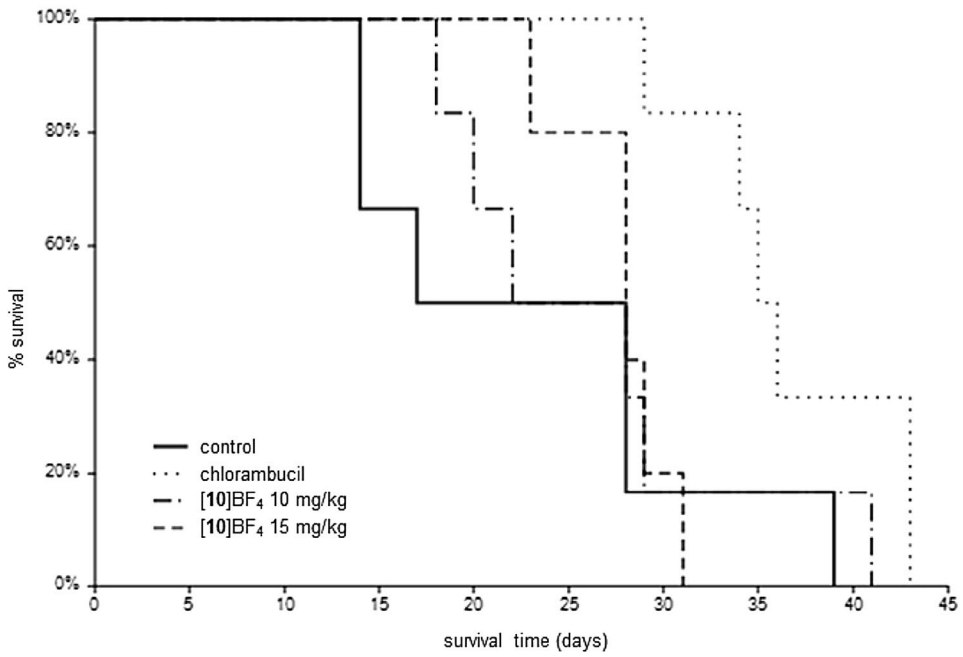


Fig. 4 Kaplan–Meier analysis of survival. Only the administration of chlorambucil at 5 mg/kg significantly prolonged the survival of tumor-bearing mice compared to tumor-bearing controls treated with saline i.p. (control). Neither 10 mg nor 15 mg of [**10**]BF_4/kg body weight had any statistically significant effect. The compounds were administered i.p. on days 1, 4 and 8 after tumor inoculation

be evaluated because of their bad general condition. Unlike the healthy mice in the MTD finding study, the tumor-bearing mice treated with 20 mg/kg of [**10**]BF_4 displayed severe symptoms of intoxication and had to be euthanized. Only chlorambucil prolonged the mean survival time significantly when compared with sham-treated tumor-bearing control mice ($P = 0.0401$). Chlorambucil was also more effective when compared with its conjugate, [**10**]BF_4, at both evaluated doses ($P < 0.01$). A Kaplan–Meier analysis of survival can be seen in Fig. 4.

The conjugate [**10**]BF_4 at any dose tested did not have a statistically significant therapeutic effect in comparison to chlorambucil alone. When the dose of 20 mg/kg was applied, which is stoichiometrically almost equivalent to 5 mg/kg chlorambucil, [**10**]BF_4 turned out to be more systemically toxic and less potent than chlorambucil itself. It is quite possible that, even if the conjugate was completely hydrolyzed into the putatively more active components, the summation of their toxicity might hinder the body's own defense mechanisms such as the anticancer immune surveillance. Studies in more cancer models are needed to determine whether or not the conjugation in question increases the anticancer potential of the components.

Conclusions

Four chlorambucil conjugates of trithiolato arene ruthenium complexes were synthesized, fully characterized and studied for their anticancer properties. In vitro studies showed the complexes to be highly cytotoxic, their IC_{50} values being comparable to those of the trithiolato precursors. The difference in the cytotoxicity towards the cisplatin-resistant cell line A2780cisR shows the clear influence of the chlorambucil moiety. The cisplatin-resistant cancer cells are known to be cross-resistant to chlorambucil [33], presumably due to the similar modes of action of the two agents. The difference in the spectrum of anticancer activity of the chlorambucil conjugates compared to their trithiolato precursors suggests that chlorambucil is cleaved after uptake into cells with both parts of the conjugate subsequently exhibiting the cytotoxic effects by their respective modes of action.

In a breast cancer model in immunocompetent mice, [**10**]BF_4 at the dose of 15 mg/kg shows a relevant effect on the inhibition of tumor growth. However, it did not show a statistically significant effect on the survival of tumor-bearing mice, possibly due to the high systemic toxicity of [**10**]BF_4. In conclusion, the studies show that conjugation of chlorambucil with dinuclear *p*-cymene ruthenium trithiolato complexes did not result in the intended synergistic enhancement of the anticancer properties of both components. The different modes of action do not seem to be cooperative.

Experimental part

Materials and methods

The dithiolato and trithiolato complexes **1**–**4** and [**5**–**7**]Cl were prepared according to published methods [27–29]. All other reagents were commercially available and were used without further purification. NMR spectra were recorded

with a Bruker 400 MHz spectrometer. Electrospray mass spectra were obtained in positive mode with an LCQ Finnigan mass spectrometer. The purity of the compounds was established by elemental analysis and was above 95 % for all compounds. The synthesis of chlorambucil conjugates [**9**–**12**]BF_4 was carried out using standard Schlenk techniques whereas all the other reactions were performed in air.

Synthesis and data for [(η^6-*p*-$MeC_6H_4Pr^i$)$_2$$Ru_2$($SCH_2C_6H_4$-*p*-OMe)$_2$($SC_6H_4$-*p*-OH)] [8]Cl

[(η^6-*p*-$MeC_6H_4Pr^i$)$_2$$Ru_2$($SCH_2C_6H_4$-*p*-OMe)$_2$$Cl_2$] (100 mg, 0.118 mmol) was dissolved in EtOH (50 mL). Then 46 mg (0.354 mmol) of 4-hydroxythiophenol, dissolved in 10 mL of EtOH, were added to this solution dropwise and the solution was refluxed over-night. The solution was concentrated to dryness, the residue dissolved in CH_2Cl_2 (1 mL) and subjected to column chromatography on silica gel (solvents CH_2Cl_2/EtOH 7:1). The reddish band was collected and evaporated to dryness to give the product as an orange powder. Yield: 110.6 mg (89 %). $C_{42}H_{51}ClO_3Ru_2S_3$: calcd. C, 53.80; H 5.48; found C, 53.42; H, 5.34. ESI MS: (MeOH + CH_2Cl_2): *m/z* = 903.1 $[M]^+$. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 10.30 (s, 1H, SC_6H_4-*p*-O*H*), 7.48 (d, 2H, ^3J = 8 Hz, SC_6H_4-*p*-OH), 7.42 (t, 4H, $SCH_2C_6H_4$-*p*-OCH_3), 7.22 (d, 2H, ^3J = 8 Hz, SC_6H_4-*p*-OH), 6.98 (t, 4H, $SCH_2C_6H_4$-*p*-OCH_3), 5.03 [d, 2H, ^3J = 6 Hz, *p*-$CH_3C_6H_4$-CH-$(CH_3)_2$], 4.91 [d, 2H, ^3J = 6 Hz, *p*-$CH_3C_6H_4$-CH-$(CH_3)_2$], 4.71 [t, 4H, *p*-$CH_3C_6H_4$-CH-$(CH_3)_2$], 3.88 (d, 6H, ^3J = 6 Hz, $SCH_2C_6H_4$-*p*-OCH_3), 3.53 (s, 2H, S$CH_2C_6H_4$-*p*-OCH_3), 3.34 (s, 2H, S$CH_2C_6H_4$-*p*-OCH_3), 2.02 [sept, ^3J = 7 Hz, 2H, *p*-$CH_3C_6H_4$-C*H*-$(CH_3)_2$], 1.69 [s, 6H, *p*-$CH_3C_6H_4$-CH-$(CH_3)_2$], 1.03 [d, ^3J = 7 Hz, 6H, *p*-$CH_3C_6H_4$-CH-$(CH_3)_2$], 0.97 [d, ^3J = 7 Hz, 6H, *p*-$CH_3C_6H_4$-CH-$(CH_3)_2$] ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 159.44, 159.32, 157.81, 133.88, 131.69, 130.6, 130.40, 115.38, 114.00, 113.85, 106.95, 99.97, 83.72, 83.59, 82.28, 55.99, 55.46, 39.51, 39.27, 30.87, 23.15, 22.42, 17.99 ppm.

General procedure for the synthesis of chlorambucil conjugates [9–12]BF_4

In a Schlenk tube, 30 mg of [(η^6-*p*-$MeC_6H_4Pr^i$)$_2$$Ru_2$($SCH_2R$)$_2$($SC_6H_4$-*p*-OH)]Cl (R = C_6H_5 0.032 mmol, R = $CH_2C_6H_5$ 0.032 mmol, R = C_6H_4-*p*-Bu^t 0.029 mmol, R = C_6H_4-*p*-OMe 0.030 mmol) were dissolved in distilled dichloromethane (10 mL), ten equivalents of $NaBF_4$ were added, and the mixture was stirred at room temperature for 12 h. Then the solution was filtered through a syringe filter (0.22 μm), passed through a small plug of silica gel (solvent system CH_2Cl_2/acetone 7:1) and transferred into another Schlenk tube containing a solution of chlorambucil (2.0 equivalents) and 4-(dimethyl-amino)pyridine (1.0 equivalent) in CH_2Cl_2 (5 mL). A solution of dicyclohexylcarbodiimide (2.0 equivalents) in CH_2Cl_2 (5 mL) was added dropwise to this mixture. The reaction was stirred at room temperature under nitrogen atmosphere for 16 h. The solvent was then removed under reduced pressure, the residue dissolved in cold acetonitrile, filtered through a syringe filter, evaporated to dryness and chromatographed on silica gel (solvent system CH_2Cl_2/acetone 7:1). The first orange band was collected and evaporated to give the products as orange powders.

Data for [9]BF_4

Yield: 29.7 mg (84 %). $C_{54}H_{64}BCl_2F_4NO_2Ru_2S_3$·0.75$CH_2Cl_2$: calcd. C, 51.42; H, 5.16; N, 1.10; found C, 51.64; H, 5.18; N, 1.15. ESI MS: (MeOH + CH_2Cl_2): *m/z* = 1128.4 $[M]^+$. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.82 (d, ^3J = 8 Hz, 2H, SC_6H_4-*p*-O), 7.59–7.35 (m, 10H, $SCH_2C_6H_5$), 7.15 [d, ^3J = 8 Hz, 2H, OCC_3H_6-*p*-$C_6H_4N(C_2H_4Cl)_2$], 7.05 (d, 2H, ^3J = 8 Hz, *p*-SC_6H_4O), 6.76 [d, ^3J = 8 Hz, 2H, OCC_3H_6-*p*-$C_6H_4N(C_2H_4Cl)_2$], 5.15 [d, ^3J = 5 Hz, 2H, *p*-$CH_3C_6H_4CH(CH_3)_2$], 4.99 [d, ^3J = 5 Hz, 2H, *p*-$CH_3C_6H_4CH(CH_3)_2$], 4.86 [d, ^3J = 5 Hz, 2H, *p*-$CH_3C_6H_4CH(CH_3)_2$], 4.71 [d, ^3J = 5 Hz, 2H, *p*-$CH_3C_6H_4CH(CH_3)_2$], 3.75 [m, 4H, OCC_3H_6-*p*-$C_6H_4N(C_2H_4Cl)_2$], 3.66 [m, 4H, OCC_3H_6-*p*-$C_6H_4N(C_2H_4Cl)_2$, 2H, $SCH_2C_6H_5$], 3.46 (s, 2H, $SCH_2C_6H_5$), 2.68 [t, ^3J = 7 Hz, 2H, OCC_3H_6-*p*-$C_6H_4N(C_2H_4Cl)_2$], 2.59 [t, ^3J = 7 Hz, 2H, OCC_3H_6-*p*-$C_6H_4N(C_2H_4Cl)_2$], 2.05 [quint, ^3J = 7 Hz, 2H, OCC_3H_6-*p*-$C_6H_4N(C_2H_4Cl)_2$], 1.92 [sept, ^3J = 7 Hz, 2H, *p*-$CH_3C_6H_4$-C*H*-$(CH_3)_2$], 1.76 [s, 6H, *p*-$CH_3C_6H_4$-CH-$(CH_3)_2$], 0.99 [d, ^3J = 7 Hz, 6H, *p*-$CH_3C_6H_4$-CH-$(CH_3)_2$], 0.92 [d, ^3J = 7 Hz, 6H, *p*-$CH_3C_6H_4$-CH-$(CH_3)_2$] ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 150.96, 139.73, 133.59, 129.93, 129.53, 129.35, 128.77, 128.61, 128.15, 122.30, 107.06, 100.27, 84.11, 83.62, 82.46, 40.09, 39.91, 30.88, 23.20, 22.36, 18.00 ppm.

Data for [10]BF_4

Yield: 31.5 mg (81 %). $C_{56}H_{68}BCl_2F_4NO_2Ru_2S_3$: calcd. C, 54.10; H, 5.51; N, 1.13; found C, 53.71; H, 5.53; N, 1.23. ESI MS: (MeOH/CH_2Cl_2): *m/z* = 1158.0 $[M]^+$. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.73 (d, ^3J = 8 Hz, 2H, SC_6H_4-*p*-O), 7.45–7.27 (m, 10H, $SC_2H_4C_6H_5$), 7.13 [d, ^3J = 8 Hz, 2H, OCC_3H_6-*p*-$C_6H_4N(C_2H_4Cl)_2$], 7.01 (d, 2H, ^3J = 8 Hz, SC_6H_4-*p*-O), 6.68 [d, ^3J = 8 Hz, 2H, OCC_3H_6-*p*-$C_6H_4N(C_2H_4Cl)_2$], 5.23 [d, ^3J = 5 Hz, 2H, *p*-$CH_3C_6H_4CH(CH_3)_2$], 5.19 [d, ^3J = 5 Hz, 2H, *p*-$CH_3C_6H_4CH(CH_3)_2$], 5.15 [d, ^3J = 5 Hz, 2H, *p*-$CH_3C_6H_4CH(CH_3)_2$], 5.10 [d, ^3J = 5 Hz, 2H, *p*-$CH_3C_6H_4CH(CH_3)_2$],

3.72 [m, 4H, OCC_3H_6-*p*-$C_6H_4N(C_2H_4Cl)_2$], 3.65 [m, 4H, OCC_3H_6-*p*-$C_6H_4N(C_2H_4Cl)_2$, 2H, $SCH_2C_6H_5$], 3.08 (m, 4H, $SC_2H_4C_6H_5$), 2.88 (m, 2H, $SC_2H_4C_6H_5$), 2.66, [m, 4H, OCC_3H_6-*p*-$C_6H_4N(C_2H_4Cl)_2$], 2.59 (t, ^3J = 8 Hz, 2H, $SC_2H_4C_6H_5$), 2.06 [m, 2H, OCC_3H_6-*p*-$C_6H_4N(C_2H_4Cl)_2$, 2H, *p*-$CH_3C_6H_4$-C*H*-$(CH_3)_2$], 1.83 [s, 6H, *p*-$CH_3C_6H_4$-CH-$(CH_3)_2$], 1.05 [t, ^3J = 7 Hz, 12H, *p*-$CH_3C_6H_4$-CH-$(CH_3)_2$] ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 171.72, 150.92, 139.85, 133.53, 129.75, 128.85, 128.77, 128.72, 126.82, 122.15, 112.49, 107.03, 100.37, 84.18, 83.95, 83.19, 41.17, 40.41, 40.19, 38.67, 38.55, 33.87, 33.54, 30.88, 26.51, 23.21, 22.38, 17.80 ppm.

Data for [11]BF_4

Yield: 28.0 mg (79 %). $C_{62}H_{80}BCl_2F_4NO_2Ru_2S_3$: calcd. C, 56.10; H, 6.07; N, 1.06; found C, 56.19; H, 6.17; N, 1.05. ESI MS: (MeOH + CH_2Cl_2): *m/z* = 1240.6 $[M]^+$. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.80 (d, ^3J = 8 Hz, 2H, SC_6H_4-*p*-O), 7.45 [m, 8H, $SCH_2C_6H_4$-*p*-$C(CH_3)_3$], 7.14 [d, ^3J = 8 Hz, 2H, OCC_3H_6-*p*-$C_6H_4N(C_2H_4Cl)_2$], 7.04 (d, ^3J = 8 Hz, 2H, SC_6H_4-*p*-O), 6.73 [d, ^3J = 8 Hz, 2H, OCC_3H_6-*p*-$C_6H_4N(C_2H_4Cl)_2$], 5.11 [d, ^3J = 6 Hz, 2H,, *p*-$CH_3C_6H_4CH(CH_3)_2$], 4.97 [d, ^3J = 6 Hz, 2H, *p*-$CH_3C_6H_4CH(CH_3)_2$], 4.89 [d, ^3J = 6 Hz, 2H, *p*-$CH_3C_6H_4CH(CH_3)_2$], 4.60 [d, ^3J = 6 Hz, 2H, *p*-$CH_3C_6H_4CH(CH_3)_2$], 3.74 [m, 4H, OCC_3H_6-*p*-$C_6H_4N(C_2H_4Cl)_2$], 3.66 [m, 4H, OCC_3H_6-*p*-$C_6H_4N(C_2H_4Cl)_2$], 3.61 [s, 2H, $SCH_2C_6H_4$-*p*-$C(CH_3)_3$], 3.41 [s, 2H, $SCH_2C_6H_4$-*p*-$C(CH_3)_3$], 2.67 [t, ^3J = 7 Hz, 2H, OCC_3H_6-*p*-$C_6H_4N(C_2H_4Cl)_2$], 2.58 [t, ^3J = 7 Hz, 2H, OCC_3H_6-*p*-$C_6H_4N(C_2H_4Cl)_2$], 2.05 [quint, ^3J = 7 Hz, 2H, OCC_3H_6-*p*-$C_6H_4N(C_2H_4Cl)_2$], 1.89 [sept, ^3J = 7 Hz, 2H, *p*-$CH_3C_6H_4$-C*H*-$(CH_3)_2$], 1.76 [s, 6H, *p*-$CH_3C_6H_4$-CH-$(CH_3)_2$], 1.37 [s, 9H, $SCH_2C_6H_4$-*p*-$C(CH_3)_3$], 1.33 [s, 9H, $SCH_2C_6H_4$-*p*-$C(CH_3)_3$], 0.94 [d, ^3J = 7 Hz, 6H, *p*-$CH_3C_6H_4$-CH-$(CH_3)_2$], 0.89 [d, ^3J = 7 Hz, 6H, *p*-$CH_3C_6H_4$-CH-$(CH_3)_2$] ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 171.66, 151.57, 150.87, 136.68, 133.53, 129.88, 129.06, 125.51, 125.34, 122.25, 106.89, 100.54, 84.17, 83.47, 82.46, 39.94, 39.38, 34.74, 33.88, 33.45, 31.34, 30.79, 26.36, 23.05, 22.52, 18.07 ppm.

Data for [12]BF_4

Yield: 21.7 mg (53 %). $C_{56}H_{68}BCl_2F_4NO_4Ru_2S_3$: calcd. C, 52.75; H, 5.37; N, 1.10; found C, 52.61; H, 5.40; N, 1.11. ESI MS: (MeOH + CH_2Cl_2): *m/z* = 1189.6 $[M]^+$. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.80 (d, ^3J = 8 Hz, 2H, SC_6H_4-*p*-O), 7.44 (m, 4H, $SCH_2C_6H_4$-*p*-OCH_3), 7.12 [d, ^3J = 8 Hz, 2H, OCC_3H_6-*p*-$C_6H_4N(C_2H_4Cl)_2$], 7.02 (m, 2H, SC_6H_4-*p*-O, 2H, $SCH_2C_6H_4$-*p*-OCH_3), 6.95 (d, ^3J = 8 Hz, 2H, $SCH_2C_6H_4$-*p*-OCH_3), 6.67 [d, ^3J = 8 Hz, 2H, OCC_3H_6-*p*-$C_6H_4N(C_2H_4Cl)_2$], 5.14 [d, ^3J = 5 Hz, 2H, *p*-$CH_3C_6H_4CH(CH_3)_2$], 5.00 [d, ^3J = 5 Hz, 2H, *p*-$CH_3C_6H_4CH(CH_3)_2$], 4.87 [d, ^3J = 5 Hz, 2H, *p*-$CH_3C_6H_4CH(CH_3)_2$], 4.77 [d, ^3J = 5 Hz, 2H, *p*-$CH_3C_6H_4CH(CH_3)_2$], 3.86 (d, ^3J = 11 Hz, 6H, $SCH_2C_6H_4$-*p*-OCH_3), 3.72 [m, 4H, OCC_3H_6-*p*-$C_6H_4N(C_2H_4Cl)_2$], 3.65 [m, 4H, OCC_3H_6-*p*-$C_6H_4N(C_2H_4Cl)_2$], 3.59 (s, 2H, $SCH_2C_6H_4$-*p*-OCH_3), 3.39 (s, 2H, $SCH_2C_6H_4$-*p*-OCH_3), 2.67 [t, ^3J = 7 Hz, 2H, OCC_3H_6-*p*-$C_6H_4N(C_2H_4Cl)_2$], 2.58 [t, ^3J = 7 Hz, 2H, OCC_3H_6-*p*-$C_6H_4N(C_2H_4Cl)_2$], 2.05 [m, 2H, OCC_3H_6-*p*-$C_6H_4N(C_2H_4Cl)_2$], 1.92 [m, 2H, *p*-$CH_3C_6H_4$-C*H*-$(CH_3)_2$], 1.76 [s, 6H, *p*-$CH_3C_6H_4$-CH-$(CH_3)_2$], 0.99 [d, ^3J = 6 Hz, 6H, *p*-$CH_3C_6H_4$-CH-$(CH_3)_2$], 0.93 [d, ^3J = 6 Hz, 6H, *p*-$CH_3C_6H_4$-CH-$(CH_3)_2$] ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 204.31, 171.29, 159.36, 150.94, 144.45, 134.95, 133.56, 131.64, 130.61, 130.40, 130.18, 123.70, 122.19, 114.05, 113.88, 112.24, 109.93, 107.10, 100.09, 83.87, 83.65, 82.40, 55.50, 55.44, 53.59, 40.55, 33.86, 33.55, 30.87, 26.53, 23.16, 22.34, 17.98 ppm.

Cells and cell viability assays

Human A2780 and A2780cisR ovarian carcinoma cells were obtained from the European Centre of Cell Cultures (ECACC, UK). Non-cancerous human embryonic kidney HEK293 cells were provided by the Institute of Pathology, CHUV, Lausanne, Switzerland. Immortalized human endothelial cells RF24 were kindly provided by the Angiogenesis Laboratory of VU Medical Center, Amsterdam, The Netherlands. A2780 and A2780cisR cells were routinely grown in RPMI 1640 medium supplemented with GlutaMAX (Gibco), 10 % fetal calf serum and 1 % antibiotics (Penicillin/Streptomycin, Sigma), while HEK293 cells were grown in DMEM medium, both containing heat-inactivated fetal calf serum (FCS, Sigma, USA) (10 %) and 1 % antibiotics at 37 °C and CO_2 (5 %). RF24 cells were maintained in RPMI 1640/DMEM (1:1) supplemented as above.

10^5 cells/well were seeded in 96-well cell culture plates as described previously [34]. Briefly, 24 h after seeding, culture medium with or without compounds was added and cells were grown for an additional 72 h. Cell viability was assessed using the CellTiter-Glo®. Luminescent Cell Viability Assay (Promega, Madison, WI, USA) according to the manufacturer's instructions. Evaluation is based on means from two independent experiments, each comprising three microcultures per concentration level. 0.1 % DMSO in 0.9 % NaCl was used as the control.

Catalytic oxidation of glutathione

To evaluate the catalytic performance of the complexes for the oxidation of the reduced form of GSH to the disulfide form (GSSG), [**10**]BF_4 (1 mg) was dissolved in 0.3 mL

of D_2O and 0.3 mL of CD_3CN, and 100 equiv. of GSH (24.7 mg) were added to the solution. The sample was subsequently analyzed by ^1H NMR spectroscopy. The ^1H NMR spectra were recorded immediately after sample preparation, and then every 30 min over the period of 24 h.

Interaction with 2-deoxyguanosine 5′-monophosphate

1 mg of [**10**]BF_4 was dissolved in 0.3 mL of MeCN-d_3 and a solution of 1.1 mg of 2-deoxyguanosine 5′-monophosphate (4 equivalents) in 3 mL of D_2O was added. The solution was incubated at 37 °C. NMR and MS spectra were recorded immediately after mixing and after 24 h.

Animals and tumor model

Due to the poor solubility of [**10**]BF_4 in water, propane-1,2-diol (0.77 ml/kg of body weight) had to be added to saline, for which reason the i.p. route of administration was chosen rather than i.v. Female outbred mice (NMRI) were used for this study, they were obtained from Masaryk University (Brno, Czech Republic). Animal care was conform to EU recommendations and in accordance with the European convention for the protection of vertebrate animals used for experimental and other scientific purposes; it was approved by the Ethical Commission of the Medical Faculty in Hradec Králové (Nr. MSMT-56249/2012-310). For the in vivo activity study, 98 NMRI female mice weighting in the average 32.7 g (SD = 1.55 g) were fed a standard diet and water ad libitum. A solid Ehrlich tumor was purchased from the Research Institute for Pharmacy and Biochemistry (VUFB) in Prague, and then maintained in NMRI mice by periodical transplantations. The homogenized tumor tissue was inoculated subcutaneously into all mice on day 0, using 0.2 ml of 1/1 (v/v) homogenate freshly prepared in isotonic glucose solution. The tumor-bearing mice were then divided into five groups of 14 animals as follows: a control group treated with saline in propane-1,2-diol, 3 groups of animals treated with [**10**]BF_4 at doses of 10, 15, and 20 mg/kg i.p. and a positive controls receiving chlorambucil 5 mg/kg (purity 99.5 %; Sigma-Aldrich, MO, USA). The drugs were administered on days 1, 4 and 8 post tumor transplantations (0.2 ml/20 g body weight). The survival was followed until the last mice died.

Statistical analysis

One-Way Analysis of Variance with post hoc Dunnetts's multiple comparison test was used to detect differences in tumor weight. Kaplan–Meier curves and log-rank tests were used to compare survival times in groups. Here, the level of significance was $\alpha = 0.05$. MS Excel 2003 and NCSS software were used for the calculations and statistical evaluations. To calculate the tumor growth inhibition (TGI), the following formula was used: THI (%) = [(mean tumor weight in sham-treated controls − mean tumor weight in treated mice)/mean tumor weight in sham-treated controls] × 100 %.

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References

1. Rosenberg B, VanCamp L (1970) The successful regression of large solid sarcoma 180 tumors by platinum compounds. Cancer Res 30:1799–1802
2. Ott B, Gust R (2007) Non platinum metal complexes as anti-cancer drugs. Arch Pharm 340:117–126
3. Fricker SP (2007) Metal based drugs: from serendipity to design. Dalton Trans 4903–4917
4. Gianferrara T, Bratsos I, Alessio E (2009) A categorization of metal anticancer compounds based on their mode of action. Dalton Trans 7588–7598
5. Clarke MJ (1980) Oncological implications of the chemistry of ruthenium. Met Ions Biol Syst 11:231–283
6. Clarke MJ (2003) Ruthenium metallopharmaceuticals. Coord Chem Rev 236:209–233
7. Kostova I (2006) Ruthenium complexes as anticancer agents. Curr Med Chem 13:1085–1107
8. Allardyce CS, Dyson PJ (2001) Ruthenium in medicine: current clinical uses and future prospects. Platin Metals Rev 45:62–69
9. Süss-Fink G (2010) Arene ruthenium complexes as anticancer agents. Dalton Trans 39:1673–1688
10. Romero-Canelón I, Sadler PJ (2013) Next-generation metal anticancer complexes: multitargeting via redox modulation. Inorg Chem 52:12276–12291
11. Nazarov AA, Hartinger CG, Dyson PJ (2014) Opening the lid on piano-stool complexes: an account of ruthenium(II)arene complexes with medicinal applications. J Organomet Chem 751:251–260
12. Smith GS, Therrien B (2011) Targeted and multifunctional arene ruthenium chemotherapeutics. Dalton Trans 40:10793–10800
13. Scolaro C, Bergamo A, Brescacin L, Delfino R, Cocchietto M, Laurenczy G, Geldbach TJ, Sava G, Dyson PJ (2005) In vitro and in vivo evaluation of ruthenium(II)-arene PTA complexes. J Med Chem 48:4161–4171
14. Murray BS, Babak MV, Hartinger CG, Dyson PJ (2016) The development of RAPTA compounds for the treatment of tumors. Coord Chem Rev 306:86–114
15. Bruijnincx PCA, Sadler PJ (2008) New trends for metal complexes with anticancer activity. Curr Opin Chem Biol 12:197–206
16. Bergamo A, Masi A, Peacock AFA, Habtemariam A, Sadler PJ, Sava G (2010) In vivo tumour and metastasis reduction and in vitro effects on invasion assays of the ruthenium RM175 and osmium AFAP51 organometallics in the mammary cancer model. J Inorg Biochem 104:79–86

17. Meier SM, Novak M, Kandioller W, Jakupec MA, Arion VB, Metzler-Nolte N, Keppler BK, Hartinger CG (2013) Identification of the structural determinants for anticancer activity of a ruthenium arene peptide conjugate. Chem Eur J 19:9297–9307
18. Ang WH, Parker LJ, De Luca A, Juillerat-Jeanneret L, Morton CJ, Lo Bello M, Parker MW, Dyson PJ (2009) Rational design of an organometallic glutathione transferase inhibitor. Angew Chem Int Ed 21:3912–3915
19. Nazarov AA, Meier SM, Zava O, Nosova YN, Milaeva ER, Hartinger CG, Dyson PJ (2015) Protein ruthenation and DNA alkylation: chlorambucil-functionalized RAPTA complexes and their anticancer activity. Dalton Trans 44:3614–3623
20. Catovsky D, Else M, Richards S (2011) Chlorambucil-still not bad: a reappraisal. Clin Lymphoma Myeloma Leuk 11:S2–S6
21. Rai KR, Peterson BL, Appelbaum FR, Kolitz J, Elias L, Shepherd L, Hines J, Threatte GA, Larson RA, Cheson BD, Schiffer CA (2000) Fludarabine compared with chlorambucil as primary therapy for chronic lymphocytic leukemia. New Engl J Med 343:1750–1757
22. Mohamed D, Mowaka S, Thomale J, Linscheid MW (2009) Chlorambucil-adducts in DNA analyzed at the oligonucleotide level using HPLC-ESI MS. Chem Res Toxicol 22:1435–1446
23. Gras M, Therrien B, Süss-Fink G, Zava O, Dyson PJ (2010) Thiophenolato-bridged dinuclear arene ruthenium complexes: a new family of highly cytotoxic anticancer agents. Dalton Trans 39:10305–10313
24. Giannini F, Furrer J, Ibao A-F, Süss-Fink G, Therrien B, Zava O, Baquie M, Dyson PJ, Štěpnička P (2012) P. Highly cytotoxic trithiophenolatodiruthenium complexes of the type $[(\eta^6\text{-}p\text{-}MeC_6H_4Pr^i)_2Ru_2(SC_6H_4\text{-}p\text{-}X)_3]^+$: synthesis, molecular structure, electrochemistry, cytotoxicity, and glutathione oxidation potential. J Biol Inorg Chem 17:951–960
25. Giannini F, Paul LEH, Furrer J, Therrien B, Süss-Fink G (2013) Highly cytotoxic diruthenium trithiolato complexes of the type $[(\eta^6\text{-}p\text{-}MeC_6H_4Pr^i)_2Ru_2(\mu^2\text{-}SR)_3]^+$: synthesis, characterization, molecular structure and in vitro anticancer activity. New J Chem 37:3503–3511
26. Giannini F, Bartoloni M, Paul LEH, Süss-Fink G, Reymond J-L, Furrer J (2015) Cytotoxic peptide conjugates of dinuclear arene ruthenium trithiolato complexes. Med Chem Commun 6:347–350
27. Giannini F, Furrer J, Süss-Fink G, Clavel CM, Dyson PJ (2013) Synthesis, characterization and in vitro anticancer activity of highly cytotoxic trithiolato diruthenium complexes of the type $[(\eta^6\text{-}p\text{-}MeC_6H_4^iPr)_2Ru_2(\mu^2\text{-}SR_1)_2(\mu^2\text{-}SR_2)]^+$ containing different thiolato bridges. J Organomet Chem 744:41–48
28. Ibao A-F, Gras M, Therrien B, Süss-Fink G, Zava O, Dyson PJ (2012) Thiolato-bridged arene–ruthenium complexes: synthesis, molecular structure, reactivity, and anticancer activity of the dinuclear complexes $[(arene)_2Ru_2(SR)_2Cl_2]$. Eur J Inorg Chem 9:1531–1535
29. Stíbal D, Süss-Fink G, Therrien B (2015) Crystal structure of bis-[μ-(4-methoxyphenyl)methanethiolato-κ^2S:S]bis-[chlorido-(η^6-1-isopropyl-4-methylbenzene)-ruthenium(II)] chloroform disolvate. Acta Cryst E71:1216–1218
30. Clavel CM, Zava O, Schmitt F, Kenzaoui BH, Nazarov AA, Juillerat-Jeanneret L, Dyson PJ (2011) Thermoresponsive chlorambucil derivatives for tumour targeting. Angew Chem Int Ed 50:7124–7127
31. Giannini F, Süss-Fink G, Furrer J (2011) Efficient oxidation of cysteine and glutathione catalyzed by a dinuclear areneruthenium trithiolato anticancer complex. Inorg Chem 50:10552–10554
32. Kundu GC, Schullek JR, Wilson IB (1994) The alkylating properties of chlorambucil. Pharmacol Biochem Behav 49:621–624
33. Panasci L, Paiment J-P, Christodoulopoulos G, Belenkov A, Malapetsa A, Aloyz R (2001) Chlorambucil drug resistance in chronic lymphocytic leukemia: the emerging role of DNA repair. Clin Cancer Res 7:454–461
34. van Beijnum JR, Nowak-Sliwinska P, van den Boezem E, Hautvast P, Buurman WA, Griffioen AW (2013) Tumor angiogenesis is enforced by autocrine regulation of high-mobility group box 1. Oncogene 32:363–374
35. Gupta G, Nowak-Sliwinska P, Herrero N, Dyson PJ, Therrien B (2015) Increasing the selectivity of biologically active tetranuclear arene ruthenium assemblies. J Organomet Chem 796:59–64