Enantioselective Cytotoxicity of Chiral Diphosphine Ruthenium(II) Complexes Against Cancer Cells

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Abstract: The chiral cationic complex [Ru(η^1-OAc)(CO)((R,R)-Skewphos)(phen)]OAc (2°), isolated from reaction of [Ru(η^1-OAc)(η^1′-OAc)((R,R)-Skewphos)(CO)] (1°) with phen, reacts with NaOPiv and KSAc affording [RuX(CO)([(R,R)-Skewphos](phen))]Y (X = Y = OPiv, 3°; X = SAC, Y = OAc, 4°). The corresponding enantiomers 2°−4° have been obtained from 1° containing (S,S)-Skewphos. Reaction of 2° and 2° with (S)-cysteine and NaPF_6 at pH = 9 gives the diastereoisomers ([Ru(S)-Cys](CO)(PP)(phen)]PF_6 ([P = (R,R)-Skewphos 2°-(S)-Cys; (S,S)-Skewphos 2°-(S)-Cys). The DFT energetic profile for 2° with (S)-cysteine in H_2O indicates that aquo and hydroxo species are involved in formation of 2°-Cys. The stability of the ruthenium complexes in 0.9% w/v NaCl solution, PBS and complete DMEM medium, as well as their n-octanol/water partition coefficient (logP), have been evaluated. The chiral complexes show high cytotoxicity activity against SW1736, 8505 C, HCT-116 and A549 cell lines with EC_{50} values of 2.8−0.04 μM. The (R,R)-Skewphos derivatives show higher cytotoxicity compared to their enantiomers, 4° (EC_{50} = 0.04 μM) being 14 times more cytotoxic than 4° against the anaplastic thyroid cancer 8505 C cell line.

Introduction

The control of the configuration at the metal center is a key issue for the fine-tuning of the properties of transition metal complexes, which can find applications in catalysis and pharmacology. As a matter of fact, the use of suitable chiral ligands has been demonstrated a valuable strategy for achieving highly stereoselective catalytic reactions. Recently, great effort has been devoted to the search of efficient chiral anticancer complexes, a well-known example is oxaliplatin, bearing (R,R)-cyclohexane-1,2-diamine, which forms DNA adducts with a higher rate than its (S,S)-enantiomer.[1–3] Although ruthenium derivatives are considered promising candidates as chemotherapeutic agents in addition to the platinum ones,[4–5] only few examples of chiral ruthenium complexes have been investigated.[6] In 2006 Meggers and co-workers isolated the derivatives A and B (Figure 1), containing a bidentate staurosepin, which are inhibitors of the glycogen synthase kinase-3 (GSK-3) with 6 and 260-fold higher activity, compared to their enantiomers.[7–8] The poly(pyridyl) Λ-Ru(phen)_2(TPEP){}^{2+} derivative, described by Sun et al., can induce apoptosis of tumor cells showing an IC_{50} as low as 7.6 μM, a value 3-times lower than the Λ-Ru enantiomer (Figure 1).[9] Interestingly, Dyson and co-workers developed RAPTA derivatives containing a chiral acetamide functionalized arene, the oxalate (S)-enantiomer being almost 4-fold more cytotoxic with an IC_{50} of 8.7 μM against the A2780 cancer cell line, than the analogous (R)-enantiomer.[10] Sadler’s group investigated the arene ruthenium [Ru([η^5-arene](NN))]+ complexes with chiral iminopyridines, affording four stereoisomers of poor anticancer activity, with a less than 2-fold difference in cytotoxicity among the species (Figure 1).[11] Notably, the related osmium complexes [OsX([η^5-arene](NN))]^{+} (X = Cl, I) showed potent anticancer activity and similarities for the pair of enantiomers with IC_{50} as low as 0.6 μM.[12,13] Recently, chiral ruthenium complexes with phosphines,[14,15] diminoimidazolyl ligands,[16] and sulfur amino
Acids, have been reported to display cytotoxic activity against cancer cells. Chiral ruthenium complexes have been deeply investigated in the asymmetric hydrogenation and transfer hydrogenation of carbonyl compounds. Outstanding catalysts are the arene [RuCl(η-arene)(TsDPEN)]21–23, the diphosphine [RuCl2(PP)(NN)]24–27, as well as the carboxylate [Ru(η1-OCOR)2(PP)(en)]28 and [Ru(η2-OAc)(CO)(PP)(NN)]OAc29,30 (PP = diphosphine, NN = diamine, ampy) complexes.

Recently, we described that the cationic complexes [RuX(CO)(dppb)(phen)]Y (X, Y = Cl, carboxylate) display high cytotoxic activity against anaplastic thyroid cancer (ATC) cell lines with EC50 values much lower than that of Cisplatin, with an increment of apoptosis and reduction of cancer cell aggressiveness. It is worth pointing out that the arene ruthenium complexes which have been investigated as promising anticancer drugs can be involved in the disruption of the cellular redox homeostasis via NADH transfer hydrogenation, as well as GSH metal thiol binding and oxidation. Conversely, only few studies have been reported on the use of diphosphine ruthenium hydrogenation catalysts as efficient anticancer systems.

Herein we report the isolation of the cationic enantiomer complexes [RuX(CO)(PP)(phen)]Y (X, Y = carboxylates, thioacetate, PP = (R,R)- or (S,S)-Skewphos) and their behaviour with (S)-cysteine and GSH via formation of aquo complexes. Remarkably different and very promising cytotoxic activity toward several cell lines has been observed for the couples of enantiomers.

Results and Discussion

Synthesis and characterization of cationic chiral ruthenium complexes

Treatment of the acetate complex [Ru(η1-OAc)2(η2-OAc)((R,R)-Skewphos)(CO)] with phen (1 equiv) in methanol at 60 °C overnight affords the cationic derivative [Ru(η1-OAc)(CO)((R,R)-Skewphos)(phen)]OAc (2) in 91% yield, as a single stereoisomer (Eq. (1)).
The $^{31}$P($^1$H) NMR spectrum in CDCl$_3$ of the thermally stable complex $2^R$ displays two doublets at $\delta$ 42.9 and 41.0 ppm ($^2J_{PP}$ = 32.2 Hz) for the P atoms trans to N and acetate O atoms, respectively, as established by a $^{31}$P-$^1$H HMBC measurement, showing a long-range coupling between the P atom at $\delta$ 42.9 ppm with the ortho phenanthroline proton at $\delta$ 8.75 ppm, which points toward the CO ligand. The $^1$H NMR signals at $\delta$ 3.43 and 1.16 ppm are for the $\text{PCH-CH}_3$ moiety of the $\text{Ptrans}$ to N atom, while the other $\text{PCH-CH}_3$ resonances for the $\text{Ptrans}$ to the acetate are at $\delta$ 2.91 and 0.82 ppm (Figures S1–S3). NMR measurements show that the two ortho protons at $\delta$ 7.78 ppm of the phenyl bound to the $\text{Ptrans}$ to N show NOE effect with both the acetate methyl group ($\delta$ 1.19 ppm) and with the ortho phenanthroline proton at $\delta$ 7.18. In addition, the chiral CH proton at $\delta$ 2.91 ppm exhibits a NOE effect with the two up-field ortho protons at $\delta$ 6.79 ppm of the phenyl bound to the $\text{Ptrans}$ to OAc, consistent with the assigned configuration of the ruthenium center (Figure 2).

The pivalate derivative [Ru($\eta^1$-OPiv)(CO)((R,R)-Skewphos)(phen)]OAc ($3^R$) was easily prepared in high yield, by treatment of $2^R$ with NaOPiv (10 equiv) in methanol at 60°C for 24 h via displacement of OAc (Scheme 1). PUT SCHEME 1 HERE

The up-field shift of two ortho Ph hydrogens is due to the superimposition of one phenyl with the phenanthroline ring through $\pi$-$\pi$-interactions, as observed in related ruthenium complexes containing pyridine ligands cis to the PPh$_2$ moiety.[41] Interestingly, no formation of the other possible stereoisomers, namely trans-$2^S$ and the additional cis-$2^S$ complexes, has been observed upon heating, suggesting that cis-$2^S$ is the thermodynamically most stable species in agreement with the DFT calculations (see further part) and our previous studies on the trans-cis isomerization of phosphine-pyridine ruthenium complexes (Figure 3).[26] The $^{31}$P($^1$H) NMR spectrum of $3^R$ in CDCl$_3$ shows two doublets at $\delta$ 43.7 and 41.6 ppm ($^2J_{PP}$ = 31.4 Hz), whereas the $^1$H NMR singlets at $\delta$ 1.22 and 0.05 ppm correspond to the methyl groups of the free and coordinated pivalate, respectively. In the $^{13}$C($^1$H) NMR spectra, the CO carbon appears as a doublet of doublets at $\delta$ 204.7 ppm ($^2J_{CP}$ = 20.0 and 15.2 Hz), while the free and coordinated pivalate carbonyl moieties appear as singlets at $\delta$ 184.0 and 183.6 ppm, respectively. Similarly, the thioacetate [Ru($\eta^1$-SAc)(CO)((R,R)-Skewphos)(phen)]OAc ($4^R$) was obtained by treatment of $2^R$ with KSAc (10 equiv) in methanol at 60°C overnight, by displacement of the coordinated OAc (Scheme 1). The $^{31}$P($^1$H) NMR spectrum of $4^R$ in CDCl$_3$, exhibits a doublet at $\delta$ 41.0 ppm ($^2J_{PP}$ = 29.6 Hz).
for the P\textit{trans} to the N atom and an up-field shielded doublet at $\delta$ 31.0 ppm for the P\textit{trans} to S atom. Complex 4$^R$ has been isolated with acetate as counterion, as inferred from $^{13}$C\{\textit{1H}\} NMR measurements showing two CO singlets at $\delta$ 204.1 and 176.1 ppm for the coordinated SAc and free OAc moieties, in addition to the doublets of doublets at $\delta$ 205.5 ppm ($^2J_{CP}$ = 19.5 and 12.2 Hz) for the Ru-CO.

The use of the precursor [Ru(\textit{1H}-OAc)(\textit{1H}-OAc)((S,S)-Skewphos)(CO)] (1$^R$), in place of the enantiomer 1$^S$, with phen and following the same procedures described above, leads to the acetate [Ru(\textit{1H}-OAc)(CO)((S,S)-Skewphos)(phen)]OAc (2$^S$) in 87\% yield (Scheme 2).

The derivative 2$^S$ reacts with NaOPiv and KSAc in methanol, affording the pivalate [Ru(\textit{1H}-OAc)(CO)((S,S)-Skewphos)(phen)]OAc (3$^S$) and thioacetate [Ru(\textit{1H}-SAc)(CO)((S,S)-Skewphos)(phen)]OAc (4$^S$) isolated in 78 and 75\% yield, respectively.

### Reactivity of the chiral Skewphos complexes

The cationic complexes [RuX(CO)(Skewphos)(phen)]Y (X, Y = OAc, OPiv, SAc) are highly soluble and stable in alcohols (MeOH, EtOH), acetone and DMSO under inert atmosphere, while in CH$_2$Cl$_2$ they slowly decompose (hours) by reaction of the counter ion. NMR studies carried out in CD$_3$OD at 60°C revealed that 2$^S$ promptly reacts with KSAc, affording the thioacetate derivative 4$^S$ by displacement of the acetate ligand, while 4$^R$ does not react with NaOAc or NaOPiv, indicating a stronger Ru–S vs. R–O bond, in line with the previous investigations on related complexes.\[31\] By difference to 4$^R$, the derivatives 2$^S$ and 3$^S$ are soluble in water, resulting in carboxylate displacement and formation of hydroxo species (Supporting Information, Figure S4). Thus, NMR measurements show that complex 2$^S$ (3 mM) in D$_2$O at 37°C leads to the
formation of the hydroxo species \([\text{Ru(OH)}(\text{CO})(\text{R},\text{R}\text{-Skewphos})(\text{phen})\text{OAc (2\text{2}^\circ\text{OH}})\ (\delta_p = 44.7 \text{ ppm and } 38.9 \text{ ppm, } \nu_{\text{vps}}(\text{H}_2\text{O}) = 35.5 \text{ Hz}) in the presence of 2\text{2}^\circ\ (2\text{2}^\circ\text{OH}/2\text{2} = 1/2 \text{ molar ratio). The derivative 2\text{2}^\circ\text{ in H}_2\text{O provides a pH of about } 4.5, which is a value close to that of a buffer solution of acetic acid-acetate (pK_a of acetic acid = 4.76 at 25 \degree \text{C}) consistent with the deprotonation of the dicationic aquo complex 2\text{2}^\circ\text{H}_2\text{O}, affording the hydroxo 2\text{2}^\circ\text{OH as the main species (Scheme 3).}

As a matter of fact, thermodynamic studies demonstrate that hydrate carboxylates lead to the formation of strongly stabilized RCOO\text{−}\text{HOOCR} species.\text{[43]} Similarly, the \(\text{31P}^\text{1}[\text{H}]\text{ NMR spectrum of pivalate 3\text{3}^\circ\text{ in D}_2\text{O shows the signals of 3\text{3}^\circ\text{OH in addition to those of 3\text{3}. Conversely, the thioacetate complex 4\text{4}, which shows poor water solubility and can be dissolved by addition of DMSO\text{−d}_6 \text{ (10\% v/v), shows no displacement of MeCO\text{−} with H}_2\text{O, in agreement with our previous studies on } \(\text{RuX}(\text{CO})(\text{dpbb})(\text{phen})\text{Y.}\text{[31]}\text{ Transition metal complexes are susceptible to interact with biological nucleophiles (i.e. nucleobases, glutathione, thiol-containing proteins) leading to modulation of their concentration and activity in the physiological media. Since the main reducing agent present in mammalian cells at mM concentrations is the tripeptide glutathione (GSH)\text{[43]} we studied the interaction of this class of ruthenium complexes with (S)-cysteine and GSH. The complex 2\text{2}^\circ\text{ promptly reacts with (S)-cysteine in phosphate buffer solution (PBS) at pH = 9 affording a single stereoisomer in which the amino acid is bound through a Ru−S bond, as inferred from NMR measurements, and it was isolated as complex 2\text{2}^\circ\text{Cys in 70\% yield by addition of NaPF}_6 \text{ (6 equiv) (Scheme 4).}

This reaction occurs easily in water media and involves the formation of the aquo 2\text{2}^\circ\text{H}_2\text{O / hydroxo 2\text{2}^\circ\text{OH complexes, according to the pH, and subsequent coordination of (S)-cysteine. It is worth noting that this reaction does not take place in common organic solvents, including methanol, indicating that water appears crucial for the acetate substitution, as also evidenced by DFT calculations (see further part). The \(\text{31P}^\text{1}[\text{H}]\text{ NMR spectrum of 2\text{2}^\circ\text{Cys in CD}_2\text{OD displays two doublets at } \delta 40.5 \text{ and } 28.9 \text{ ppm (} \nu_{\text{vps}} = 29.3 \text{ Hz}) for the P atoms trans to N and S ones, respectively. In the \(\text{1H} \text{ NMR spectrum, the doublet of doublets at } \delta 2.89 \text{ ppm corresponds to the cysteine CH group, while the signals at } \delta 1.75 \text{ and } 0.98 \text{ ppm are attributed to the diastereotopic CH}_2 \text{ protons of the amino acid. The attribution of P atoms is consistent with the } 2\text{D } \text{31P}^\text{1}[\text{H} \text{ HMBC spectrum, where the doublet at } \delta_p 28.9 \text{ ppm shows a long range coupling with the CH}_2 \text{ cysteine protons. In the } \text{13C}^\text{1}[\text{H} \text{ NMR spectrum, the CO carbon appears as a doublet of doublets at } \delta 205.6 \text{ ppm (} \nu_{\text{vps}} = 20.4 \text{ and } 11.6 \text{ Hz), whereas the doublet at } \delta 57.2 \text{ ppm (} \nu_{\text{vps}} = 3.3 \text{ Hz) and the singlet at } \delta 28.0 \text{ ppm are for the cysteine CH and CH}_2 \text{ carbon atoms, respectively, as inferred from } 2\text{D } \text{13C}^\text{1}[\text{H} \text{ HSQC spectrum. Similarly, treatment of 2\text{2}^\circ\text{ with (S)-cysteine in the presence of NaPF}_6 \text{ gives 2\text{2}^\circ\text{Cys isolated in 65\% yield, displaying two doublets at } \delta_p 40.3 \text{ and } 29.0 \text{ ppm, close to those of 2\text{2}^\circ\text{Cys (Eq. (2)).

Conversely, in \(\text{1H} \text{ NMR spectrum the cysteine signals of 2\text{2}-
\text{Cys are at } \delta 2.52 \text{ ppm for the CH and } \delta 1.54 \text{ and } 1.32 \text{ ppm for the CH}_2 \text{ protons, which significantly differ from the diastereoisomer 2\text{2}^\circ\text{Cys. The NOESY } \text{1H} \text{ 2D NMR spectra of both complexes exhibit NOE effect between the low field ortho phen and the CH}_2 \text{ protons of cysteine. By lowering the pH in the range 5.0–7.5 the } \text{31P}^\text{1}[\text{H} \text{ NMR measurements of 2\text{2}^\circ\text{Cys and 2\text{2}-

\begin{figure}
\centering
\includegraphics[width=\textwidth]{scheme3}
\caption{Scheme 3. Formation of the aquo and hydroxo complexes from 2\text{2}^\circ\text{ in water.}
\end{figure}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{scheme4}
\caption{Scheme 4. Reaction of 2\text{2}^\circ\text{ with (S)-cysteine in water.}
\end{figure}
Non-covalent interactions are observed between the \((\eta^1\cdot\text{OAc})(\text{CO})(\text{Skewphos})(\text{phen})\)^+ (2^+) (Figure 4). The intra-molecular non-bonded interactions are evidenced by the spatial distribution of the \(\delta g^\text{iso}\) descriptor.\(^{[44–45]}\)

The calculated energies in methanol show that the isomer 2^+ is more stable than 2^− and trans-2^++, being \(G_2^+–G_2^+ = –4.6\) kcal mol\(^{-1}\) and \(G_2^{++} – G_\text{trans-2}^{++} = –7.2\) kcal mol\(^{-1}\). These results agree with the NMR spectra, which show the formation of the thermodynamically most stable 2^+ as a single stereoisomer. Non-covalent interactions are observed between the (R,R)-Skewphos phenyls and the phen ligand (\(\delta g^\text{iso}\) peaks at low sign(\(\lambda_\text{C}\)) in Figure S30) corresponding to CH–C interactions for the three species, while the cis isomers 2^+ and 2^+ display additional \(\pi–\pi\) interactions (Figure 4 and Figure S30). The bond lengths Ru–P1 and Ru–P2 are nearly equal in 2^+ (2.374 and 2.379 Å) while for trans-2^+ and 2^+ the difference between the Ru–P1 and Ru–P2 distance are 0.05 and 0.01 Å, respectively (Table S1 and Figure S31). The Ru–N1 and Ru–N2 (trans to CO and P, respectively) distances are 2.219 and 2.144 Å for 2^++, similar to those of 2^++, in agreement with strong trans influence of CO with respect to the phosphine, whereas for trans-2^+ the Ru–N1 and Ru–N2 are 2.180 and 2.187 Å. DFT calculations show that the diphosphine adopts a distorted boat conformation, with axial and pseudo-equatorial CH\(_2\) groups in the three isomers (Figure S32).

### DFT Calculations

#### Stability of 2^+,

Experimental data show that the reactivity of 2^+ is enhanced by the interaction with water through the formation of the aquo 2^+H\(_2\)O and hydroxo 2^+OH species (Scheme 3). The minimum energy structure of the 2^++H\(_2\)O reactant adduct (RA) presents the water molecule interacting via hydrogen bond with the acetate group that stabilizes the system with G\(_\text{2^+–H\(_2\)O} = –2.0\) kcal mol\(^{-1}\), as reported in Figure 5 (see also Figure S33 and Table S2).

The vibrational mode, with imaginary frequency in the transition state (TS), corresponds to the rupture of the Ru-OAc bond and the simultaneous formation of Ru-OH\(_2\) bond, with an activation energy \(\Delta G^* = G_{\text{TS}}–G_{\text{3}} = 18.4\) kcal mol\(^{-1}\) (Table S2 and Figure S33). This \(\Delta G^*\) is similar to that observed for the hydrolysis of [[\(\eta^1\cdot\text{p-cymene}\)]RuCl(methyl 1-butyl-2-arylbenzimidazolecarboxylate)] (18.8 kcal mol\(^{-1}\))\(^{[46]}\) and lower compared to...
leading in both cases to strong hydrogen-bond interactions (Figure 6). The reaction profile shows that $2^8\text{H}_2\text{O}^{++}$ and $2^8\text{OH}^+$ are isoenergetic ($G_{2^8\text{OH}^+} - G_{2^8\text{H}_2\text{O}^{++}} \sim 0.1 \text{ kcal mol}^{-1}$, Table S2), indicating that these two species coexist in solution. The overall reaction results thermodynamically slightly disfavored ($\Delta G = +2.3$ and $+2.4 \text{ kcal mol}^{-1}$, Table S2), in line with the NMR data of $2^8$ in water, as observed for the hydrolysis of related complexes.[46]

Reactions of $2^8+$, $2^8\text{H}_2\text{O}^{++}$ and $2^8\text{OH}^+$ with (S)-cysteine

In the case of $2^8+$ (Figure 7), the $\Delta G^+$ is significantly higher with respect to the acetate replacement by water ($\Delta G^+ = 32.9$ vs. $18.4 \text{ kcal mol}^{-1}$). This result supports a reaction model where the coordination of (S)-cysteine proceeds after the hydrolysis of $2^8+$ (Figure 6, Table S2). As reported above, $2^8$ reacts with (S)-cysteine in water at pH 9.0 giving $2^8\text{Cys}$ where the thiol group is coordinated to the metal. The comparison of the energy profile for the reaction of the Ru complexes with the thiol-deprotonated (S)-cysteine (Scheme S1) are shown in Figure 7. The formation of RA is thermodynamically favored in both cases, due to the formation of H-bonds between thiolate group of (S)-cysteine and H$_2$O or OH ligand (Figure S34, Table S2). The greater stabilization of RA between $2^8\text{H}_2\text{O}^{++}$ and (S)-cysteine compared to $2^8\text{OH}^+$ is likely related to the different charge of the complexes, +2 and +1, respectively. The TS of $2^8\text{H}_2\text{O}^{++}$ presents two H-bonds between the H$_2$O and (S)-cysteine (Table S2), while in the TS of $2^8\text{OH}^+$ a proton transfer from -NH$_3^+$ to OH occurs (Figure S34). The formation of Ru--S-(S)-cysteine-NH$_3^+$ product from $2^8\text{H}_2\text{O}^{++}$ and the thiol is the kinetically ($\Delta G^+ = 11.2 \text{ kcal mol}^{-1}$) and thermodynamically favored reaction path.

The reaction energy profiles for the ligand replacement in $2^8\text{H}_2\text{O}^{++}$ and $2^8\text{OH}^+$ by the zwitterionic form of (S)-cysteine which is prevalent at acidic-neutral pH (Scheme S1)[50] are shown in Figure 8 (Table S2) and the optimized structures of RA, TS and PA species are reported in Figure S35.

Figure 5. Free energy profile for the hydrolysis reaction leading to the formation the aquo $2^8\text{H}_2\text{O}^{++}$- species. The data for the reaction leading $2^8\text{OH}^+$ are reported in Table S2. The energies of the separated reactants (R) are taken as reference.

Figure 6. Minimum energy structures for the PA of aquo $2^8\text{H}_2\text{O}^{++}$+ OAc and the hydroxo $2^8\text{OH}^+$+ HOAc complexes. $\delta G^{\text{iso}}$ isosurfaces (isovalue 0.0055 a.u.) in green.

Figure 7. Left: free energy profiles in basic condition for the reactions of the thiol-deprotonated (S)-cysteine with $2^8\text{H}_2\text{O}^{++}$ (light blue); $2^8\text{OH}^+$ (blue). The energy profile for the reaction between zwitterionic (S)-cysteine and $2^8+$ is also reported (green dashed line). Right: structures of the RA, TS and PA (some atoms were removed for clarity, complete structures in Figure S34).
The formation of RA species with (S)-cysteine is thermodynamically favored for $2^\text{a}$-OH$^+$, while no significant stabilization is observed in the case of $2^\text{b}$-H$_2$O$^{++}$ (Figure 8, Table S2). The substitution of water in $2^\text{b}$-H$_2$O$^{++}$ by the SH group of (S)-cysteine is kinetically favored with respect to the same reaction for OH$^-$ in $2^\text{c}$-OH$^+$ ($\Delta G^\circ = 10.1$ kcal mol$^{-1}$ and 17.4 kcal mol$^{-1}$, respectively). It is worth pointing out that in the TS of $2^\text{b}$-OH$^+$ a proton transfer from SH group of (S)-cysteine to OH group occurs, with water as leaving group (Figure S35, Table S2). However, the formation of the Ru-S-OOC$^-$-cysteine is thermodynamically favored (Figure 8, Table S2).

The path leading to the formation of Ru-OOC-(S)-cysteine-NH$_3^+$ from $2^\text{b}$-H$_2$O$^{++}$ is kinetically favored with respect to that starting from $2^\text{c}$-OH$^+$ ($\Delta G^\circ = 9.7$ kcal mol$^{-1}$ and 15.5 kcal mol$^{-1}$, respectively, Table S2 and Figure S36) while the relative stability of the final PAs is very similar, consistent with presence of multiple species at lower pH observed by NMR. The Ru–S–(S)-cysteine product from $2^\text{a}$-OH$^+$ is observed in the case of (S)-cysteine with respect to (R)-cysteine-$2^\text{c}$-OH$^+$ species ($\Delta G^\circ = 0.5$ kcal mol$^{-1}$). Conversely, a slightly different interaction has been observed for $2^\text{a}$-OH$^+$ with the deprotonated (S)-cysteine with respect to (R)-species ($\Delta G^\circ = 1.2$ kcal mol$^{-1}$).

The relative stability of the two diastereoisomers obtained by reaction of $2^\text{a}$-OH$^+$ with protonated and deprotonated (R)- and (S)-cysteine (Figure S37) has been evaluated. The complex $2^\text{a}$-OH$^+$ presents similar interaction with HS-(S)-cysteine and HS-(R)-cysteine, the structures being almost isoenergic ($\Delta G^\circ = 2^\text{a}+\text{cysteine} - 2^\text{a}+\text{cysteine} = 0.5$ kcal mol$^{-1}$). Conversely, a slightly different interaction has been observed for $2^\text{a}$-OH$^+$ with the deprotonated (S)-cysteine with respect to (R)-species ($\Delta G^\circ = 2^\text{a}+\text{cysteine} - 2^\text{a}+\text{cysteine} = 1.2$ kcal mol$^{-1}$).

Biological activity of the ruthenium complexes

**Solution stability of the complexes $2^\text{a}$-4$^\text{d}$ and $2^\text{a}$-4$^\text{d}$ over time**

Prior to in vitro cell testing, the stability of each chiral compound (S enantiomers taken as a model) has been checked in different media, starting from the organic solvent DMSO (vehicle approved for clinical use, if properly diluted). The analysis has been carried out in aqueous media endowed with increasing complexity and biocompatibility (i.e., deionized water, 0.9% NaCl w/v saline solution and PBS). Afterwards, the complete DMEM (Dulbecco’s Modified Eagle Medium supplemented with 10% v/v FBS) has been considered for all the six complexes, in light of the chiral nature of the components (e.g., amino acids, vitamins, growth factors, saccharides). Each obtained scanning kinetics is reported in Supporting Information (Figures S38–S53). The Figures S38–S40 report a selection of UV-Vis spectra collected in DMSO, overlapped over 72 h. The most intense band at ca. 270 nm and the shoulder at about 300 nm have been ascribed to intraligand $\pi-\pi^*$ transitions (Figures S54 and S55 display the UV-Vis spectra of the free ligands in DMSO and in water). The band at around 375 nm is likely due to a M–L charge transfer.$^{[52]}$ On passing from the spectra collected in pure DMSO (where all compounds are stable) to those in water, the complexes $2^\text{a}$-4$^\text{d}$ and the related aquo/hydroxo species have proved stable over 72 h, whereas $3^\text{d}$ has been associated with an hyperchromic effect, occurring after 5 h. No bathochromic or hypochromic shift has been detected, thus highlighting no significant change of the compound structure. Interestingly, the spectrum of $3^\text{d}$ shows no changes in saline solution (NaCl 0.9% w/v) over 72 h (Figure S45). By contrast, $4^\text{d}$ has showed an absorbance decrease in saline solution after the first 5 h with a slight precipitate, as also observed in PBS after 3 h (Figure S49), as a result of the counterion or ligand (carboxylate, OH, H$_2$O) replacement. A hypochromic shift has been observed also for $2^\text{a}$ and $3^\text{d}$ in PBS but to a lesser extent. It is worth pointing out that in complete DMEM (25% v/v) the aquo/hydroxo species of the (S)-enantiomers are stable over time (Figures S50-52), while the spectra of the (R)-enantiomers in the same medium do not overlap during the kinetics. As a matter of fact, under these physiological conditions $3^\text{a}$ and $4^\text{a}$ show a hyperchromic effect, while $2^\text{a}$ displays a hypochromic effect, pointing out a change in the electronic transition probability, due to the formation of new species (Figure 9 and Figure S53).
In this regard, the higher cytotoxicity of the R enantiomers, namely complexes 2\(^{\text{S}}\) and 4\(^{\text{S}}\) and the corresponding hydroxo and aquo complexes, compared to the S derivatives (Table 1), may be related to their larger reactivity in the cell culture medium (Figure 9), leading to adducts with biomolecules (i.e. sugars, vitamins, serum albumin and growth factors), thus highlighting the key role of the transition metal complex chirality.

**Effects of the enantiomeric ruthenium complexes on cell viability**

The effectiveness of the three pairs of enantiomeric ruthenium complexes 2\(^{\text{S}}\)/2\(^{\text{R}}\), 3\(^{\text{S}}\)/3\(^{\text{R}}\) and 4\(^{\text{S}}\)/4\(^{\text{R}}\) was first evaluated in anaplastic thyroid cancer (ATC) cell lines (SW1736 and 8505 C), and their results have been compared with those of cisplatin. To test the effects of the chiral complexes on the viability of ATC cells, an MTT assay has been performed after the administration of different doses of the complexes for 24, 48 and 72 h. The EC\(_{50}\) values (the concentration of the test complex inducing 50% reduction in cell number compared with control cultures) have been calculated at 72 h and reported in Table 1. Regarding the incubation time, 72-h treatments are commonly carried out for comparison reasons, the pivalate enantiomers do not exhibit a great difference in activity compared to 3\(^{\text{R}}\) with EC\(_{50}\) values of 0.35 and 0.68 \(\mu\)M in the 8505 C cell line, respectively. The chiral complexes have been further investigated against the HCT-116 and A549 cancer cell lines and the obtained EC\(_{50}\) values are collected in Table 1. It is worth pointing out that all compounds are more active than cisplatin and 2\(^{\text{S}}\) and 4\(^{\text{S}}\) are 4-to-7-fold more cytotoxic against A549 cells and 15-to-24-fold more active against HCT-116 cells. Interestingly, as for the ATC cell lines, the (R,R)-enantiomers display the most promising antitumor activity, with the 2\(^{\text{S}}\) and 4\(^{\text{S}}\) derivatives exhibiting 3-to-5-fold lower EC\(_{50}\) values, compared to the (S,S) counterparts in both the HCT-116 and A549 cell lines. Conversely, the pivalate enantiomers 3\(^{\text{S}}\)/3\(^{\text{R}}\) showed comparable EC\(_{50}\) data, in line with those obtained with the ATC cells. For comparison reasons, the ligands phen and the (R,R)- and (S,S)-Skewphos were tested in vitro under the same experimental conditions. Briefly, the NN ligand phen does not exhibit a great difference in activity compared to cisplatin, and the (R,R)- and (S,S)-Skewphos were tested in vitro under the same experimental conditions. Briefly, the NN ligand phen does not exhibit a great difference in activity compared to cisplatin, and the (R,R)- and (S,S)-Skewphos were tested in vitro under the same experimental conditions.

![Figure 9. Selection of electronic spectra of the complex 2\(^{\text{S}}\) (left) and 4\(^{\text{S}}\) (right) in DMEM (25 % v/v) acquired during a 72-h scanning kinetics.](image)

**Table 1.** EC\(_{50}\) (\(\mu\)M \(\pm\) SD) of the complexes 2\(^{\text{S}}\)/2\(^{\text{R}}\), 3\(^{\text{S}}\)/3\(^{\text{R}}\), 4\(^{\text{S}}\)/4\(^{\text{R}}\) and cisplatin in SW1736, 8505 C, HCT-116 and A549 cell lines.

| Complex | Human cancer cell lines\(^{[a]}\) |
|---------|----------------------------------|
|         | SW1736 | 8505 C | HCT-116 | A549 |
| 2\(^{\text{S}}\) | 0.29 ± 0.03 | 1.4 ± 0.2 | 0.24 ± 0.05 | 0.9 ± 0.1 |
| 2\(^{\text{R}}\) | 2.0 ± 0.1 | 2.3 ± 0.3 | 1.2 ± 0.1 | 2.8 ± 0.6 |
| 3\(^{\text{S}}\) | 1.35 ± 0.04 | 0.35 ± 0.02 | 0.81 ± 0.08 | 1.9 ± 0.8 |
| 3\(^{\text{R}}\) | 2.3 ± 0.2 | 0.7 ± 0.1 | 0.9 ± 0.1 | 1.66 ± 0.04 |
| 4\(^{\text{S}}\) | 0.7 ± 0.1 | 0.04 ± 0.01 | 0.37 ± 0.09 | 0.54 ± 0.05 |
| 4\(^{\text{R}}\) | 1.28 ± 0.09 | 0.58 ± 0.05 | 1.1 ± 0.3 | 2.51 ± 0.01 |
| Cisplatin | 6 ± 2 | 5 ± 2 | 5.7 ± 0.2 | 3.6 ± 0.7 |

\([a]\) Each value represents the mean value of at least three-fold determinations after a 72-h treatment.
when studying biological systems. Thus, the (S,S)-Skewphos ligand has been found more cytotoxic against A549 cells (50 \( \mu \)M < \( EC_{50} < 100 \) \( \mu \)M) compared to the (R,R) one, which shows an \( EC_{50} \) value higher than 100 \( \mu \)M. By contrast, the (R,R)-enantiomer diphosphine is more active against HCT-116 cells (10 \( \mu \)M < \( EC_{50} < 25 \) \( \mu \)M) compared to the (S,S)-ligand (50 \( \mu \)M < \( EC_{50} < 100 \) \( \mu \)M), clearly indicating that chirality affects the cytotoxicity and that the ruthenium complexes display higher activity compared to the free ligands.

**Effects of enantiomeric ruthenium complexes on cell colony forming ability**

To assess the effects of the enantiomeric ruthenium complexes on marker of aggressiveness in the two ATC cell lines, its influence on the ability of cells to form colonies in an anchorage-independent manner was analyzed using a soft-agar colony formation assay. As shown in Figure 10, we have observed a significant reduction in the number of colonies in cells treated with the ruthenium complexes, each used at its own \( EC_{50} \), except for 3\( \text{R} \), compared to those treated with DMSO alone. A trend can be observed, according to which the (S,S)-enantiomers have a less effectiveness in reducing the number of colonies, although still used at their \( EC_{50} \). In SW1736 cells the enantiomers 2\( \text{R} \) and 2\( \text{S} \) show significantly different effects, with the 2\( \text{R} \) enantiomer inducing a significantly smaller reduction in the number of colonies compared to 2\( \text{S} \).

**Effects in terms of cell death, morphology and migration**

To check whether cell death occurs via apoptosis, we carried out an Annexin V/Propidium Iodide (PI) assay. Considering 0.5 \( \mu \)M as \( EC_{50} \) cut-off value, we have chosen the HCT-116 cell line for this investigation. As a matter of fact, Table 1 points out that only against colon carcinoma cells the complexes 2\( \text{R} \) and 4\( \text{R} \) have comparable antiblastic activity. HCT-116 cells have been hence treated at 0.5 \( \mu \)M with the selected compounds for 72 h. Then, cells have been harvested and labeled with Annexin-V FITC and PI prior to flow cytometry, aimed at evaluating the percentage of apoptotic cells. In these experiments, apoptotic cells at early stage occur in the lower right quadrant, while those at late stage set in the up-right part. The percentage in the lower left quadrant represents viable cells whereas the upper left part corresponds to cells undergoing non-apoptotic cell death. Remarkably, the number of cells undergoing non-apoptotic cell death is comparable for both treatments to the vehicle (DMSO) control. Both complexes trigger apoptosis with similar percentages between the early-stage-apoptosis cell population and the late-stage one (Figure 11).

The 2\( \text{R} \)-treated sample is associated with the highest percentage of apoptotic cell death (total 62.9%), confirming its greater potency when compared to 4\( \text{R} \) (total 27.5% of cells undergoing apoptosis) in this cell line.

Regarding cell morphology, treatment of HCT-116 cells with 2\( \text{R} \) and 4\( \text{R} \) complexes (72 h at 0.5 \( \mu \)M) decreased cell proliferation. In both cases cells change shape as well as apoptotic bodies and cell debris are visible. In addition, 2\( \text{R} \) induced cell disaggregation whereas the 4\( \text{R} \) compound did not (Figure S56). Cell migration and invasion are key phenomena in physiologic and pathologic processes, such as wound healing and cancer metastasis. To test whether the 2\( \text{R} \) complex affects cell migration, cells have been seeded in a Petri dish and allowed to attach, spread and form a confluent monolayer. A pin tool or needle is usually exploited to scratch and remove cells from a discrete area of the confluent monolayer so to form a cell-free zone.\(^{34,58}\) We examined cell migration in response to the mechanical scratch wound, carried out after treatment (or not, control) with the model compound 2\( \text{R} \) (Figure 12). Additional microscope images pointed out – after a 24-h treatment at 3 \( \mu \)M of HCT-116 cells – a reduction of about 30% of the cell migration rate on the fifth day (Figure S57).

Therefore, the cells previously treated with the selected Ru(II)-based compound migrated at the edges of the wound to a lesser extent compared to the control.

**Log P evaluation**

In light of the very promising data collected in vitro, it was of paramount importance to evaluate the n-octanol-water partition coefficient (log P) to shed light on the capability of this class of compounds to pass through the biological barriers.


In addition, the collected values point out possible scenarios in terms of future nanoformulation for advanced preclinical testing. We considered the (S,S)-enantiomers as model compounds and the results are collected in Table 2.

Despite the same molecular design, metal-to-ligand stoichiometry and ionic character, the studied complexes display quite different log \( P \) values. In fact, the positive recorded value for the compound 3 shows its hydrophobic nature. Conversely, the complex 2 bearing acetate as a ligand and counterion is associated with a negative log \( P \) value. A value around zero was recorded for the compound 4. Taken together, the nature of the counterion/ligand reflects an increasing lipophilic character in the order \( 2 < 4 < 3 \). These results underline the possibility for this new class of complexes to show affinity to both the phospholipidic layer of cell membrane and hydrophilic physiological conditions, *conditio sine qua non* for future biological applications.

**Conclusions**

In summary, we have described the preparation of the chiral cationic complexes of formula [RuX(CO)(diphosphine)(phen)](Y), bearing (R,R)- and (S,S)-Skewphos as diphosphine, carboxylate, thioacetate and thiolate as X ligand and RCO\(_2\) and PF\(_6\) as Y counterion. These derivatives have been easily obtained as single stereoisomers in high yield from [Ru(η\(^1\)-OAc)(η\(^2\)-OAc)(diphosphine)(CO)] and phen, followed by acetate substitution, their structure being established by NMR and DFT studies. The carboxylate complexes display facile displacement of the RCO\(_2\) ligand with (S)-cysteine in water due to the formation of reactive aquo/hydroxo species via hydrogen bond interactions as established by DFT calculations which also provide rather similar energy for the two cysteine diastereoisomers [Ru((S)-Cys)(CO)(Skewphos)(phen)]PF\(_6\). The derivatives [Ru(X)(diphosphine)(phen)]Y inhibit cancer cell proliferation and colonization and display high cytotoxic activity in the range of 2.8–0.04 μM against the SW1736, 8505 C, HCT-116 and A549 cell lines, strongly depending on the chirality at metal center. As a matter of fact, the thioacetate complex [Ru(η\(^1\)-SAc)(CO)(R,R-Skewphos)]OAc (4) shows an EC\(_{50}\) value of 0.04 μM for the anaplastic thyroid cancer cell line 8505 C, for which no effective treatments are available. This value is significantly lower than that of Cisplatin and it is 14 times lower with respect to its enantiomer 4. On account of this straightforward synthetic protocol, entailing the use of commercial chiral diphosphines, this class of air stable [Ru(X)(PP)(phen)]Y complexes appears attracting for applications as metallodrugs. Ongoing studies are focused on improv-

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**Table 2. Log \( P \) values of the chiral ruthenium complexes 2\(^\circ\), 4\(^\circ\) and 3\(^\circ\), as \( n \)-octanol/water partition coefficient.**

| Complex | Log \( P \) (pH 7; 25 °C) |
|---------|--------------------------|
| 2\(^\circ\) | −0.15 ± 0.02 |
| 3\(^\circ\) | +0.56 ± 0.06 |
| 4\(^\circ\) | +0.04 ± 0.01 |

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**Figure 11.** Flow cytofluorimetry assay of the complexes 2\(^\circ\) and 4\(^\circ\). Percentages of viable (lower left), apoptotic (lower and upper right), and necrotic (upper left) cells are reported in the corner of each quadrant.

**Figure 12.** Cell migration - scratch assay: collection of microscope (Leica DMi1; inverted phase contrast) images (4X) concerning day 0 and day 5 after treatment with and without (Control) complex 2\(^\circ\).
ing their antitumor activity through the combination of suitable ligands and investigating the mechanism of action.

**Experimental Section**

**General** All reactions were carried out under an argon atmosphere by using standard Schlenk techniques. The precursors [Ru(η-1-OAc)(η-2-OAc)(PP)(P)(Co)] (PP = (R,R)-Skeophos, 1; (S,S)-Skeophos, 1) were prepared according to literature procedures, whereas (R,R)-Skeophos, (S,S)-Skeophos, phen and all other chemicals and solvents were purchased from Aldrich and Stem and used without further purification. NMR measurements were performed using a Bruker Advance I II NMR 400 MHz spectrometer and the chemical shifts, in ppm, are internally referred to TMS for 1H and 13C (1H) NMR and 85% H2PO4, for 31P(1H) NMR. Elemental analyses (C, H, and N) were carried out with a Carlo Erba 1106 elemental analyzer, whereas IR analyses were performed with a Bruker Vector 22 FTIR spectrometer. The electronic spectra of the ligands and the compounds (evaluation of the n-octanol/water partition coefficient, log P) were recorded by the Evolution 201 (Thermo Fisher Scientific, Inc.) UV-Visible spectrophotometer. Adopted conditions: wavelength range: 190–500 nm, dual beam mode, scan rate 400 nm/min, integration time 0.30 sec, data range: 1 nm. The solution stability tests of the compounds were performed by serial acquisition of electronic spectra in the UV-Vis domain, generally from 265 nm to 500 nm, by means of the spectrophotometer Cary 60 (Agilent Technologies). Experimental set up: scanning time 0.088 sec, scan rate 102.9 nm/min, slit 1 nm, 149 total cycles of which 9 in the first 2 h and 140 in the next 70 h. For the spectrophotometric experiments of the 96-well plates (cell viability tests) the INFINITI M PLEX (Tecan) equipment was used. Synthesis of [Ru(η-1-OAc)(CO)(η-2,Skeophos)(phen)]OAc (2): Complex [Ru(η-1-OAc)(η-2-OAc)(η-2,Skeophos)(Co)] (1) (100.0 mg, 0.145 mmol) and 1,10-phenanthroline (26.1 mg, 0.145 mmol) were dissolved in methanol (1.5 mL) and the mixture was stirred at 60 °C overnight. The solution was evaporated under reduced pressure and the residue was dissolved in dichloromethane (0.5 mL). Addition of diethyl ether (5 mL) afforded a pale-yellow precipitate, which was filtered off and dried under reduced pressure. Yield: 115 mg (91%). Elemental analysis (%) calc for C2H8N2O2P2Ru: C 63.66, H 5.11, N 3.23; found: C 63.50, H 4.99, N 3.31. 1H NMR (400.1 MHz, CDCl3, 298 K): δ = 8.75 (t, JHH = 4.3 Hz, 1H; phen), 8.68 (d, JHH = 8.1 Hz, 1H; phen), 8.47 (d, JHH = 7.9 Hz, 1H; phen), 8.16 (s, JHH = 8.8 Hz, 1H; phen), 8.04 (d, JHH = 8.8 Hz, 1H; phen), 7.95-7.74 (m, 7H; Ph), 7.47 (m; 4H, Ph), 7.37 (m; 2H, aromatic protons), 7.28 (m), 7H; phen), 7.18 (m; 3H, Ph), 7.06 (m; 4H, aromatic protons), 6.79 (t, JHH = 8.6 Hz, 2H; Ph), 3.43 (br m, 1H; PCH), 2.91 (br m, 1H; PCH), 2.69 (m, 1H; PCH), 2.35-2.08 (m, 1H; CH3), 2.02 (s, 3H; COCH3), 1.19 (s, 3H; COCH3), 1.16 (dd, JCC = 15.1 Hz, JHH = 7.5 Hz; 3H; CH3CH2), 0.82 (dd, JCC = 13.0 Hz, JHH = 6.8 Hz; 3H; CH3CH2). 31P(1H) NMR (100.6 MHz, CDCl3, 298 K): δ = 204.7 (dd, JCC = 20.0 Hz, JHH = 15.2 Hz; CO), 184.0 (br s; COCH3), 183.6 (s; COCH3), 153.9-123.6 (m; aromatic carbon atoms), 65.9 (s; COCH3), 37.3 (br s; CH3), 32.7 (d, JCC = 30.3 Hz; PCH), 28.9 (br s; COCH3), 27.3 (s; COCH3), 23.1 (d, JCC = 31.2 Hz; PCH), 18.5 (d, JCC = 5.8 Hz; CH3CH2), 16.9 (s; CH3CH2). 31P(1H) NMR (162 MHz, CDCl3, 298 K) δ 43.7 (JCC = 31.4 Hz, 41.6 ppm (d, JCC = 31.4 Hz). IR (Nujol): 3000 cm−1. Synthesis of [Ru(η-1-OAc)(CO)(η-2,Skeophos)(phen)]OAc (3′): Complex [Ru(η-1-OAc)(η-2-OAc)(η-2,Skeophos)(Co)] (3′) was prepared following the procedure described for 3, starting from 2′ (50.0 mg, 0.058 mmol), in place of 2, and NaOPiv (66.0 mg, 0.576 mmol, 10 equiv). Yield: 43 mg (78%). Elemental analysis (%) calc for C10H8N2O2P2Ru: C 65.60, H 5.93, N 2.94; found: C 65.42, H 5.86, N 2.78. Synthesis of [Ru(η-1-SAC-O)(CO)(η-2,Skeophos)(phen)]OAc (4′): Complex [Ru(η-1-SAC-O)(η-2-OAc)(η-2,Skeophos)(Co)] (4′) was prepared following the procedure described for 4, starting from [Ru(η-1-OAc)(η-2-OAc)(η-2,Skeophos)(Co)] (1′) (100.0 mg, 0.145 mmol), in place of 1, and 1,10-phenanthroline (26.1 mg, 0.145 mmol). Yield: 110 mg (87%). Elemental analysis (%) calc for C50H47N6O5P2Ru: C 63.66, H 5.11, N 3.23; found: C 63.52, H 4.97, N 3.09. Synthesis of [Ru(η-1-O OPiv)(CO)(η-2,Skeophos)(phen)]OOPiv (3′): Complex 2′ (50.0 mg, 0.058 mmol) was dissolved in degassed methanol (2 mL) and then NaOPiv (71.5 mg, 0.576 mmol, 10 equiv) was added to the solution. The reaction mixture was stirred for 48 h at 60 °C and the solvent was evaporated. Dichloromethane (2 mL) was added and the excess of salt was filtered off, obtaining a solution that was concentrated at almost 0.5 mL evaporating the solvent under reduced pressure. Addition of diethyl ether (2 mL) afforded an orange product that was filtered, washed with diethyl ether (2 x 2 mL) and dried under reduced pressure. Yield: 45.5 mg (82%). Elemental analysis (%) calc for C10H8N2O2P2Ru: C 65.60, H 5.93, N 2.94; found: C 65.47, H 5.82, N 2.75. 1H NMR (400.1 MHz, CDCl3, 298 K): δ = 8.88 (dd, JHH = 8.2 Hz, JCC = 0.5 Hz; 1H; phen), 8.60 (t, JHH = 3.5 Hz; 1H; phen), 8.51 (dd, JHH = 8.2 Hz, JCC = 1.2 Hz; 1H; phen), 8.27 (d, JHH = 8.8 Hz; 1H; phen), 8.06 (d, JHH = 8.1 Hz; phen), 7.93-7.67 (m; 6H; Ph), 7.49-7.33 (m; 7H; aromatic protons), 7.31-7.18 (m; 3H; aromatic protons), 7.16-7.04 (m; 5H; Ph), 6.96 (t, JHH = 8.8 Hz; 2H; Ph), 3.48 (br m, 1H; PCH), 2.91 (m; 1H; PCH), 2.80 (m; 1H; CH3), 2.41-2.17 (m; 1H; CH3), 1.22 (s; 9H; CH3), 1.20 (dd, JHH = 15.0 Hz, JCC = 7.4 Hz; 3H; CH3), 0.89 (dd, JHH = 12.9 Hz, JCC = 6.8 Hz; 3H; CH3CH3), 0.05 ppm (s; 9H; CH3). 31P(1H) NMR (100.6 MHz, CDCl3, 298 K): δ = 204.7 (dd, JCC = 20.0 Hz, JHH = 15.2 Hz; CO), 184.0 (br s; COCH3), 183.6 (s; COCH3), 153.9-123.6 (m; aromatic carbon atoms), 65.9 (s; COCH3), 37.3 (br s; CH3), 32.7 (d, JCC = 30.3 Hz; PCH), 28.9 (br s; COCH3), 27.3 (s; COCH3), 23.1 (d, JCC = 31.2 Hz; PCH), 18.5 (d, JCC = 5.8 Hz; CH3CH2), 16.9 (s; CH3CH2). 31P(1H) NMR (162 MHz, CDCl3, 298 K) δ 43.7 (JCC = 31.4 Hz, 41.6 ppm (d, JCC = 31.4 Hz). IR (Nujol): 3000 cm−1.
Obtained solid was filtered, washed with cold water (2 x 2 mL) and the solvent was completely evaporated and water (2 mL) was added to the residue. The obtained solid was filtered, washed with cold water (2 x 2 mL) and dried under reduced pressure. Yield: 41 mg (70%). Elemental analysis (%) calcld for C_{72}H_{4}F_{8}N_{3}O_{2}P_{2}Ru: S 59.36, H 4.14; found: C 58.23, H 4.38, N 4.11. H NMR (400.1 MHz, CD_{2}OD, 298 K): δ = 8.63 (d, J_{HH} = 8.2 Hz, 1H, phen), 8.60 (m, 1H, phen), 8.46 (d, J_{HH} = 8.0 Hz, 1H, phen), 8.25 (t, J_{HH} = 8.3 Hz, 2H, phen), 8.11 (d, J_{HH} = 8.8 Hz, 1H, phen), 8.01 (d, J_{HH} = 8.8 Hz, 1H, phen), 7.99-7.89 (m, 3H, Ph), 7.85 (td, J_{HH} = 7.6 Hz, J_{CP} = 2.1 Hz, 2H, phen), 7.71-7.48 (m, 4H, aromatic protons), 7.44 (td, J_{HH} = 7.5 Hz, J_{CP} = 3.5 Hz, 1H, Ph), 7.34 (dd, J_{HH} = 8.1 Hz, J_{CP} = 5.4 Hz, 1H, phen), 7.31-7.17 (m, 4H, Ph), 7.10-6.98 (m, 4H, Ph), 6.75 (t, J_{HH} = 8.4 Hz, 2H, phen), 3.48 (br m, 1H, PCH), 1.36 (m, 1H, PCH), 2.89 (dd, J_{HH} = 9.9 Hz, J_{CP} = 3.5 Hz, 1H, CH Cys), 2.67 (m, 1H, CH), 2.36-2.11 (m, 1H, CH), 1.75 (dt, J_{HH} = 12.8 Hz, J_{CP} = 3.2 Hz, 1H, CH Cys), 1.09 (dd, J_{HH} = 15.0 Hz, J_{CP} = 7.4 Hz, 3H, CH(CH)_{3}), 0.98 (ddd, J_{HH} = 14.4 Hz, J_{CP} = 10.2 Hz, J_{CP} = 1.4 Hz, 1H, CH, Cys), 0.77 (dd, J_{HH} = 12.7 Hz, J_{CP} = 6.8 Hz, 3H, CH(CH)_{3}). \[\text{1}^{3}\text{C}(\text{H})\text{NMR (100.6 MHz, CD}_{2}\text{OD, 298 K)}: \delta = 205.6 (dd, J_{C} = 20.4 Hz, J_{CP} = 11.6 Hz, CO), 171.1 (s; Cys COOH), 152.6-122.4 (m; aromatic carbon atoms), 75.2 (d, J_{CP} = 2.8 Hz; Cys CH), 36.6 (dd, J_{CP} = 5.0 Hz, J_{CP} = 3.1 Hz; Cys CH), 33.8 (dd, J_{CP} = 29.6 Hz, J_{CP} = 2.5 Hz; PCH), 28.0 (s; Cys CH), 27.3 (dd, J_{CP} = 28.5, J_{CP} = 1.8 Hz; PCH), 17.7 (d, J_{CP} = 6.5 Hz; CH(CH)_{3}), 16.3 (3d, J_{CP} = 1.6 Hz; CH(CH)_{3}). \[\text{P}^{2}\text{H}\text{NMR (162 MHz, CD}_{2}\text{OD, 298 K); \delta = 24.5 (d, J_{CP} = 29.4 Hz), 28.9 ppm (d, J_{CP} = 29.4 Hz), 144.5 (hept, J_{CP} = 706.3 Hz).\]

Synthesis of [Ru(S)-Cys](CO)(S,S-Skewphos)(phen)IPSE (2^2-Cys): Complex [Ru(S)-Cys](CO)(S,S-Skewphos)(phen)IPSE (2^2-Cys) was prepared following the procedure described for 2^2-Cys, starting from 2^2 (50.0 mg, 0.058 mmol), in place of 2^2. Elemental analysis (%) calcld for C_{72}H_{4}F_{8}N_{3}O_{2}P_{2}Ru: S 59.36, H 4.14; found: C 58.23, H 4.38, N 4.11. H NMR (400.1 MHz, CD_{2}OD, 298 K): δ = 8.63 (d, J_{HH} = 8.2 Hz, 1H, phen), 8.60 (m, 1H, phen), 8.46 (d, J_{HH} = 8.0 Hz, 1H, phen), 8.25 (t, J_{HH} = 8.3 Hz, 2H, phen), 8.11 (d, J_{HH} = 8.8 Hz, 1H, phen), 8.01 (d, J_{HH} = 8.8 Hz, 1H, phen), 7.99-7.89 (m, 3H, Ph), 7.85 (td, J_{HH} = 7.6 Hz, J_{CP} = 2.1 Hz, 2H, phen), 7.71-7.48 (m, 4H, aromatic protons), 7.44 (td, J_{HH} = 7.5 Hz, J_{CP} = 3.5 Hz, 1H, Ph), 7.34 (dd, J_{HH} = 8.1 Hz, J_{CP} = 5.4 Hz, 1H, phen), 7.31-7.17 (m, 4H, Ph), 7.10-6.98 (m, 4H, Ph), 6.75 (t, J_{HH} = 8.4 Hz, 2H, phen), 3.48 (br m, 1H, PCH), 1.36 (m, 1H, PCH), 2.89 (dd, J_{HH} = 9.9 Hz, J_{CP} = 3.5 Hz, 1H, CH Cys), 2.67 (m, 1H, CH), 2.36-2.11 (m, 1H, CH), 1.75 (dt, J_{HH} = 12.8 Hz, J_{CP} = 3.2 Hz, 1H, CH Cys), 1.09 (dd, J_{HH} = 15.0 Hz, J_{CP} = 7.4 Hz, 3H, CH(CH)_{3}), 0.98 (ddd, J_{HH} = 14.4 Hz, J_{CP} = 10.2 Hz, J_{CP} = 1.4 Hz, 1H, CH, Cys), 0.77 (dd, J_{HH} = 12.7 Hz, J_{CP} = 6.8 Hz, 3H, CH(CH)_{3}). \[\text{1}^{3}\text{C}(\text{H})\text{NMR (100.6 MHz, CD}_{2}\text{OD, 298 K); \delta = 205.6 (dd, J_{C} = 20.4 Hz, J_{CP} = 11.6 Hz, CO), 171.1 (s; Cys COOH), 152.6-122.4 (m; aromatic carbon atoms), 75.2 (d, J_{CP} = 2.8 Hz; Cys CH), 36.6 (dd, J_{CP} = 5.0 Hz, J_{CP} = 3.1 Hz; Cys CH), 33.8 (dd, J_{CP} = 29.6 Hz, J_{CP} = 2.5 Hz; PCH), 28.0 (s; Cys CH), 27.3 (dd, J_{CP} = 28.5, J_{CP} = 1.8 Hz; PCH), 17.7 (d, J_{CP} = 6.5 Hz; CH(CH)_{3}), 16.3 (3d, J_{CP} = 1.6 Hz; CH(CH)_{3}).\]

Materials for biological testing: DMEM (Dulbecco’s Modified Eagle Medium) w/ GluMAX-AM(1 (pyruvate 1 mM) cell growth medium was purchased from Thermo Fisher Life Technologies while fetal bovine serum, sterile DMSO, cis-diammineplatinum(II) dichloride (herein-after, cisplatin) and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) were from Merck. Penicillin-streptomycin solution (5000 U/mL) were acquired from Thermo Fisher Life Technologies. FITC Annexin V apoptosis detection kit I was purchased from Thermo Fisher Scientific, (Waltham, MA, USA). All chemicals were of high-grade purity and used as purchased without any further purification.
Finally, the stability in the complete cell culture medium DMEM (Dulbecco’s Modified Eagle Medium), supplemented with 10% fetal bovine serum. The cell lines were validated using short tandem repeat analysis and confirmed to be mycoplasma-free.

Cells were grown in a humidified incubator with 5% CO<sub>2</sub> at 37°C. Cells were seeded in 96-well plates (volume = 100 μL; 4000 cells/well for SW1736 and 8505 C cells; 5500 cells/well and 7000 cells/well for HCT-116 and A549, respectively) and grown to 70–75% confluence, followed by treatment with DMSO (control) or each chiral compound (dissolved in DMSO) in fresh medium at different concentrations in the micromolar or sub-micromolar domain (both in control and in treatment wells a final DMSO concentration of 0.1% v/v; quadruplicate conditions). Likewise, cells were seeded in quadruplicate in 96-well plates and grown to the same confluence to be treated with cisplatin (dissolved in 0.9% w/v NaCl(aq)) in fresh medium at different concentrations, for comparison purposes.

After 72-h incubation at 37°C, inhibition of cell proliferation was measured by MTT assay, as previously described. The cytotoxicity of the compounds was quantified as the percentage of surviving cells compared to untreated cells. At least three MTT tests for each compound were carried out in order to evaluate the corresponding EC<sub>50</sub> values.

Soft agar assay: The clonogenic ability of the SW1736 and 8505C cells after treatment with chiral ruthenium complexes each used at its own EC<sub>50</sub> was evaluated using a soft agar assay. Briefly, 48 h after treatment, cells were collected, and 1 × 10<sup>5</sup> cells were suspended in 4 mL complete medium containing 0.25% agarose (Sigma-Aldrich), then seeded to the top of a 1% agarose complete medium layer in 6-cm plates. The colonies were counted by eye in four different fields, under a Leica DMI-600B inverted microscope (Leica Microsystems Ltd.). Data are representative of three independent experiments.

Apoptosis assay: Apoptosis indexes were measured using the Annexin-V fluorescein isothiocyanate (FITC) apoptosis detection kit I from by Thermo Fisher Scientific. HCT-116 cells were grown to approximately 75% confluence, treated with the most promising chiral complexes 2<sup>4</sup> and 4<sup>4</sup> (at 0.5 μM) or DMSO vehicle as a control (0.1% v/v) for 72 h, harvested by trypsinization and centrifugation. Cells were rinsed twice with ice cold PBS (1X) and re-suspended in binding buffer (1X) at a concentration of 3×10<sup>5</sup> cells/mL. The suspension (200 μL) was then transferred to a 5 mL flow cytometry tube. Cells were incubated with 5 μL of annexin V-FITC for 10 minutes in the dark. Propidium iodide (PI; 10 μL) was added in each tube just before the acquisition of the sample on the flow cytometry instrument. For annexin V/PI assay analysis, approximately 1.0×10<sup>5</sup>-gated events were acquired for each sample by a FACSAn<sup>c</sup>to<sup>c</sup> flow cytometer (Becton Dickinson). Flow cytometry data were processed using FlowJo software (v10 TreeStar). The excitation wavelength was 488 nm and the detection wavelengths were 530±15 and 620±21 nm for Annexin V and PI, respectively.

Data (Figure 11) are shown as density plots of Annexin-V- (x-axis) and propidium iodide (PI, y-axis) staining. Viable cells were defined as annexin V-negative and PI-negative. Early apoptotic cells were defined as annexin V-positive and PI-negative, late apoptotic cells were defined as annexin V- and PI-positive whereas cells positive for PI only were considered dead by necrosis. Percentages of viable, apoptotic, and necrotic cells are reported in the corner of each quadrant.

**Cellular morphology and cell migration assay:** An Olympus IX70 inverted tissue culture microscope was used for evaluating cellular morphology changes upon treatment and microscopic imaging with phase contrast. Cell migration was assessed using the scratch wound healing assay, as described elsewhere. Cells were grown to confluence in tissue culture dishes, then the very promising compound 2<sup>4</sup> (final concentration = 3 μM) or drug-free medium were added. After 24 h, cells were rinsed twice with PBS and scraped up using a sterile, 1,000 μL pipette tip, then cultured in the abovementioned medium. The migration rate is associated with change of the distance between the edges of the wound (defined by the lines), indicating the cell-free surface area. Pictures here reported are representative of one of three different experiments (original magnification 4X; scale bar = 100 μm).

**Log P evaluation:** When evaluating the partition coefficient P<sub>n-octanol</sub> was pre-saturated with milli-Q water for 24 h under vigorous stirring, followed by equilibration at 25°C for 24 h. After that, weighted amounts of the (S,S)-enantiomers were dissolved in a defined volume of the organic phase (final concentration in the 50–100 μM range), then evaluated their actual concentration by measuring the absorbance at the maximum wavelength of the electronic band at about 270 nm. The solution was mixed with water and let to stir for 2 h at 25°C. Later, the mixture was left to equilibrate for at least 30 min. The concentration of every complex in the organic phase before (C<sub>org</sub>) and after partitioning (C<sub>ac</sub>) was evaluated by UV-Vis spectrophotometry, resulting in the calculation of the corresponding n-octanol/water partition coefficient (P) as log P = log (C<sub>org</sub>/C<sub>ac</sub>). The procedure was repeated at least three times for each compound.

**Statistical analysis:** Data are presented as the mean±standard deviation. All results were analyzed using the unpaired Student’s t-test or one-way ANOVA in GraphPAD Prism version 6 (GraphPAD Software, Inc.). After one-way ANOVA, the Dunnett’s post hoc test was performed. P < 0.05 was considered to indicate a statistically significant difference.

**Supporting Information**

NMR data, DFT calculated thermochemical data and structures, solution stability data, electronic spectra of free ligands, microscope images.

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**Conflict of Interest**

The authors declare no conflict of interest.

**Data Availability Statement**

The data that support the findings of this study are available in the supplementary material of this article.

**Keywords:** antitumor agents · chirality · cytotoxicity · N Ligands · P Ligands · ruthenium

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[1] J. Graham, M. Muhsin, P. Kirkpatrick, Nat. Rev. Drug Discovery 2004, 3, 11–12.

[2] H.-Y. Zhang, Y.-R. Liu, C. Ji, W. Li, S.-X. Dou, P. Xie, W.-C. Wang, L.-Y. Zhang, P.-Y. Wang, PolO One 2013, 8, e71556–e71566.

[3] F. Piccinno, C. Nardon, M. Bettinelli, A. Melchior, M. Tolazzi, F. Zinna, L. Di Barri, ChemPhotoChem 2022, 6, e20210143.

[4] A. Bijelic, S. Theiner, B. K. Keppler, A. Rompel, J. Med. Chem. 2016, 59, 5894–5903.

[5] R. Trondli, P. Heffert, C. R. Kowol, M. A. Jakupec, W. Berger, B. K. Keppler, Chem. Sci. 2014, 5, 2925–2932.

[6] Y. Wang, H. Huang, Q. Zhang, P. Zhang, Dalton Trans. 2018, 47, 4017–4026.

[7] G. E. Atilla-Gokcumen, N. Pagano, C. Streu, J. Maksimoska, P. Filippakopoulos, K. S. M. Smalley, R. Contractor, N. K. Haass, A. N. Kulp, G. E. Atilla-Gokcumen, D. S. Williams, H. Bregman, K. T. Flaherty, M. S. Soengas, E. Meggers, M. Herlyn, Cancer Res. 2007, 67, 209.

[8] W. W. Zhang, Y. Sun, J. Y. Wang, X. Y. Ding, E. D. Yang, L. L. Martin, D. D. Sun, J. Inorg. Biochem. 2021, 216.

[9] K. J. Kilpin, S. M. Cammack, C. M. Clavel, P. J. Dyson, Dalton Trans. 2013, 42, 2008–2014.

[10] M. J. Romero, P. J. Sadler, Chirality in Organometallic Anticancer Complexes 2015, 85–115.

[11] J. P. C. Covredal, J. Romero-Canelón, C. Sanchez-Cano, G. J. Clarkson, A. Habtemariam, M. Wills, P. J. Sadler, Nat. Chem. 2018, 10, 347–354.

[12] Y. Fu, R. Soni, M. J. Romero, A. M. Pizarro, L. Salassa, G. J. Clarkson, J. M. Hearm, A. Habtemariam, M. Wills, P. J. Sadler, Chem. Eur. J. 2013, 19, 15199–15209.

[13] O. Tokgum, D. E. Karakis, S. Tan, E. R. Karagü, B. Inal, H. Akca, F. Durap, A. Baysal, M. Aydemir, Chem. Bio. 2020, 74, 2833–2892.

[14] M. Pernar, Z. Kokan, J. Kraji, S. Glavasoc, L. M. Tumir, I. Plantianida, D. Eljuga, I. Turel, A. Brozovic, S. I. Kirin, Bioorg. Chem. 2019, 87, 432–446.

[15] P. G. Nandi, P. P. Jadi, K. Das, S. J. Prathapa, B. B. Mandal, A. Kumar, Inorg. Chem. 2021, 60, 7422–7432.

[16] M. C. Leite, H. J. de Araujo-Salvo, S. R. Corrêa, L. Colina-Vegas, D. Martinez-Otero, R. P. Martins, G. C. Silva, A. A. Batista, Anti-Cancer Agents Med. Chem. 2021, 21, 1172–1182.

[17] X. Xie, B. Lu, W. Li, Z. Zhang, Coord. Chem. Rev. 2018, 355, 39–53.

[18] D. Wang, D. Astruc, Chem. Rev. 2015, 115, 6621–6686.

[19] W. Baratta, P. Rigo, Eur. J. Inorg. Chem. 2008, 4001–4053.

[20] dppb = 1,4-bis(diphenylphosphino)butane: (R,R)- or (S,S)-5,5′-diphenyl-2,2′-bipyridine: (R,R)- or (S,S)-2,2′-bipyridine: (R,R)- or (S,S)-2-(ethylenedi-amine) ampy = 2-aminomethylpyridine; phen = 1,10-phenanthroline; TsDPEN = N-p-toluenesulfonyl-1,2-diphenylethylenediamine; Piv = pivalate.

[21] K.-J. Haack, S. Hashiguchi, A. Fuji, T. Kikumi, R. Noyori, Angew. Chem. Int. Ed. Engl. 1997, 36, 285–288.

[22] S. Hashiguchi, A. Fuji, K. J. Haack, K. Matsumura, T. Ikariya, R. Noyori, Angew. Chem. Int. Ed. Engl. 1997, 36, 288–290.

[23] W. Baratta, G. Chelucci, E. Herdtweck, S. Magnolia, K. Siega, P. Rigo, Angew. Chem. Int. Ed. 2007, 46, 7651–7654; Angew. Chem. 2007, 119, 7795–7798.

[24] T. Ohkuma, C. A. Sandoral, R. Sinivasan, Q. Lin, Y. Wei, K. Muriz, R. Noyori, J. Am. Chem. Soc. 2005, 127, 8288–8289.

[25] W. Baratta, E. Herdtweck, K. Siega, M. Toniutti, P. Rigo, Organometallics 2005, 24, 1660–1669.

[26] H. Doucet, T. Ohkuma, K. Murata, T. Yokozawa, M. Kozawa, E. Katakaya, A. F. England, T. Ikariya, R. Noyori, Angew. Chem. Int. Ed. 1998, 37, 1703–1707; Angew. Chem. 1998, 110, 1792–1796.

[27] P. Dupaš, L. Bonomo, L. Kermorvan, Angew. Chem. Int. Ed. 2013, 52, 11347–11350; Angew. Chem. 2013, 125, 11557–11560.

[28] S. Baldino, S. Giboulot, D. Lovision, H. G. Nedden, A. Pothig, A. Banotti, Angew. Chem. Int. Ed. 2021, 40, 1086–1103.

[29] S. Giboulot, C. Cunozzi, A. Del Zotto, R. Figliolla, G. Lippe, D. Lovision, P. Strazzolini, S. Susmel, E. Zangrando, S. Baldino, M. Ballico, W. Baratta, Dalton Trans. 2019, 48, 12560–12576.

[30] D. Lovision, L. Allegri, F. Baldan, M. Ballico, G. Damante, C. Jandl, W. Baratta, Dalton Trans. 2020, 49, 8375–8388.

[31] I. Sugitani, N. Onoda, K.-I. Ito, S. Suzuki, J. Nippon Med. Sch. 2018, 85, 18–27.

[32] P. Zhang, J. P. Sadler, J. Organomet. Chem. 2017, 839, 5–14.

[33] B. S. Murray, M. V. Babak, C. G. Hartinger, P. J. Dyson, Coord. Chem. Rev. 2016, 306, 86–114.

[34] C. G. Hartinger, N. Metz-Nolte, P. J. Dyson, Organometallics 2012, 31, 5677–5685.

[35] H. Petzold, P. J. Sadler, Chem. Commun. 2008, 4413–4415.

[36] S. A. Mirzaeifard, D. C. Tavares, J. Nutr. Biochem. 2004, 15, 215–228.

[37] S. A. Mirzaeifard, D. C. Tavares, J. Nutr. Biochem. 2004, 15, 215–228.
