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Unprecedented collateral sensitivity for cisplatin-resistant lung cancer cells presented by new ruthenium organometallic compounds[†]

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Platinum-based therapies continue to be the main regimen used to treat non-small cell lung cancers (NSCLC), where multidrug resistance plays a key role in treatment failure and strategies to overcome this limitation are urgently sought for. In view to contribute to the development of this field, two sets of new organometallic Ru(^{II}) compounds with general formula [Ru(η^5 -C₅H₄R')(bipyR)(PPh₃)][CF₃SO₃], where R' = CHO or CH₂OH and bipyR = 2,2'-bipyridine (**1**, **5**), 4,4'-dimethyl-2,2'-bipyridine (**2**, **6**), 4,4'-di(hydroxymethyl)-2,2'-bipyridine (**3**, **7**) and 4,4'-dibiotin ester-2,2'-bipyridine (**4**), were synthesized and fully characterized. All compounds were tested against four types of NSCLC cell lines (A549, NCI-H228, Calu-3 and NCI-H1975), and four of them (**1**, **2**, **4** and **6**) presented a strong activity against cisplatin-resistant NSCLC cells. They were also able to increase cisplatin cytotoxicity up to 1390-fold (when administrated at non-toxic doses) by inhibiting MRP1 and P-gp transporters. Given the role of MRP1 in cisplatin resistance, in particular in lung cancer where cisplatin is the first-line treatment, the finding that these compounds are inducers of collateral sensitivity is of particular relevance. As far as we are aware, these are the first ruthenium-based compounds with such a mechanism of action, taking advantage of an "Achilles' heel" and acting as MDR-selective compounds.

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Introduction

Cancer is a leading cause of death worldwide, with an estimated 9.6 million deaths in 2018. Among all types of cancer, lung cancer is the most common and most lethal disease, due to late diagnosis and intrinsic and/or acquired resistance. In particular, non-small cell lung cancer (NSCLC) accounts for 85% of all lung cancers and its treatment relies on surgery, chemotherapy, radiation, immunotherapy or combinations thereof.¹ The first line treatment for locally advanced stage NSCLC is simultaneous chemotherapy with different che-

motherapeutics and radiation.¹ A multi-chemotherapeutics approach is frequently used and includes a platinum-based agent (such as cisplatin or carboplatin) and other drugs with different mechanisms of action such as a taxane or a vinca alkaloid.² Yet, due to drug resistance these therapeutic options have only limited success.

During the last years several research groups have developed new metallodrugs/metallodrugs formulations aiming at overcoming several forms of multidrug resistance.^{3–12} However, in what concerns lung cancer, promising examples are still scarce. One of the strategies used has been the co-delivery of valproic acid (VPA), a histone deacetylase inhibitor, with a metallodrug.^{13,14} For example, Mao and co-workers have developed VPA-functionalized cyclometalated iridium(^{III}) complexes through a hydrolysable ester bond.¹³ The results have shown a significant increased activity for the new conjugates (vs. VPA alone or a mixture of complexes + VPA) validating the strategy adopted. Also, the conjugates were able to overcome cisplatin resistance in human lung carcinoma cells (A549R). In another work, Du, Meng and co-workers, used L-cysteine decorated Zr-based metal-organic frameworks (MOFs) to co-deliver cisplatin and VPA.¹⁴ Immunoblot and immunofluorescence analyses showed that the new MOFs were able to downregulate the expression of vascular endothelial growth factor (VEGF)

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† Electronic supplementary information (ESI) available: Schemes for synthesis of compounds **1**–**7**; characterization of compounds **1**–**7** (ESI-MS and NMR spectra and UV-vis data); crystallographic data and structural refinement details for X-ray data for **1**, **2**, **3**, **4**, **5**, and **6**; stability curves in DMSO/DMEM; P-gp and MRP1 expression in non-small cell lung cancer cell lines (immunoblotting). CCDC 2042407–2042412. For ESI and crystallographic data in CIF or other electronic format see DOI: 10.1039/d0qi01344g



and improved the sensitivity to cisplatin of resistant A549/CDDP. The *in vivo* experiments confirmed that chemotherapy using these MOFs combined with microwave thermal therapy significantly improved the therapeutic effect of cisplatin-resistant lung cancer. In a different approach, a family of Co^{II} and Co^{III} tris(bipyridine) compounds has shown an important cytotoxicity against a panel of cancer cell lines that included taxol-resistant, cisplatin-resistant and p53-deficient cancer cells.¹⁵ Overall, there was no direct correlation between the oxidation state of the complexes and their cytotoxicity, yet the methyl substituent at the bipyridine (*vs.* non-substituted or methoxy) seemed to impart a favorable response in cisplatin-resistant (A2780, SGC-7901, OV2008 and C13 *vs.* resistant phenotype), in taxol-resistant (MCF-7, HCT-8, and A549 *vs.* resistant phenotype) and in p53-deficient apoptosis-resistant (HCT116 p53^{+/+} *vs.* HCT116 p53^{-/-}) cancer cells. It was further elucidated that compound [Co(4,4'-dimethyl-2,2'-bipyridine)₃]³⁺ was able to inhibit P-gp, which is associated to taxol-resistance, and suppressed ~50% of tumor growth in a lung cancer xenograft model. Indeed, one of the most important mechanisms of cell resistance is the overexpression of drug transporters, such as P-gp.² In this frame, there are a few reports on metal complexes that are able to inhibit ABC pumps. For example, Choudhuri and coworkers developed a series of [M(N-(2-hydroxy acetophenone)glycinate)(H₂O)_n] complexes, where the M is Fe^{III} (*n* = 3),¹⁶ Ni^{II} (*n* = 1),¹⁷ Cu^{II} (*n* = 3)¹⁸ or Zn^{II} (*n* = 1).¹⁹ In particular, the Cu^{II} complex was shown to directly interact with P-gp showing potential to reverse P-gp mediated drug resistance.¹⁸ Interestingly, it did not compete for the substrate binding or to verapamil-, vinblastine- and progesterone-binding sites.

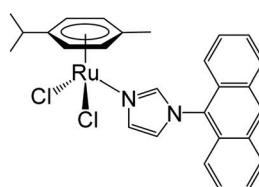
Ruthenium complexes are nowadays considered promising alternatives to the metal-based drugs in clinical use with some compounds still in clinical trials, namely KP1019 sodium salt NKP1339/IT-139 (sodium trans-[tetrachlorobis(indazole)ruthenate(III)])^{20,21} and TLD1433 (Ru(II) polypyridyl complex; ClinicalTrials.gov Identifier: NCT03053635).²² In the scope of

ruthenium MDR modulators, *i.e.*, compounds able to block the drug efflux, only a few examples are known. Among them, the compound [Ru(*p*-cymene)Cl₂(N-(Anthracen-9-yl)-imidazole)] (Fig. 1, left), developed by Juillerat-Jeanneret, Dyson and coworkers, showed good cytotoxicity against A549 lung, HT29 colon, and T47D breast carcinoma and inhibited P-gp close to the levels of the reference inhibitor verapamil.²³ In this regard, we have been also engaged in the last years in the development of ruthenium(II) compounds with ATP Binding Cassette (ABC) efflux pumps inhibitory properties. Notably, during structure-activity studies on compounds from the [Ru^{II}(*p*-C₅H₄R)(2,2'-bipyridine-4,4'-R')(PPh₃)]⁺ family, one compound (**LCR134** for R = H, R' = biotin ester, Fig. 1, right) has shown remarkable ability to inhibit P-gp (even better than the reference inhibitor verapamil)²⁴ and another stood out as a possible MRP1 inhibitor (**RT11** for R = CH₃ and R' = CH₃) (Fig. 1, bottom).²⁵ In addition, both compounds (**LCR134** and **RT11**) were also cytotoxic for breast (MCF-7 and MDA-MB-231) and ovarian (A2780/A2780cisR) cancer cells, respectively, exhibiting a rare dual behavior as both cytotoxic agents and ABC pump inhibitors. It was further observed that the substituent at the bipyridine is key to provide inhibitory properties to this family of compounds. Thus, in the continuation of these studies other substituents were added at the cyclopentadienyl ring (-CHO or -CH₂OH) allowing the synthesis of a new family of compounds. The results disclosed in this work will highlight the importance that specific chemical groups have on the inhibitory properties of ABC pumps and how this might contribute to improve the activity of the compounds and to sensitize NSCLC cells to cisplatin.

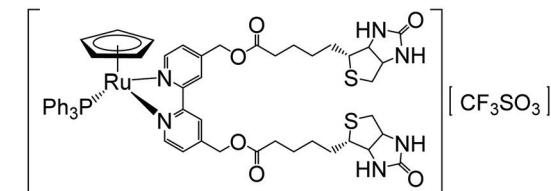
Results and discussion

Synthesis and characterization

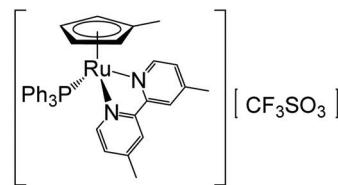
For the synthesis of the new compounds, we first optimized the method described by Bai Wei *et al.*²⁶ to prepare the precur-



[Ru(*p*-cymene)Cl₂(N-(Anthracen-9-yl)-imidazole)]



LCR134



RT11

Fig. 1 Ruthenium ABC pump inhibitors.

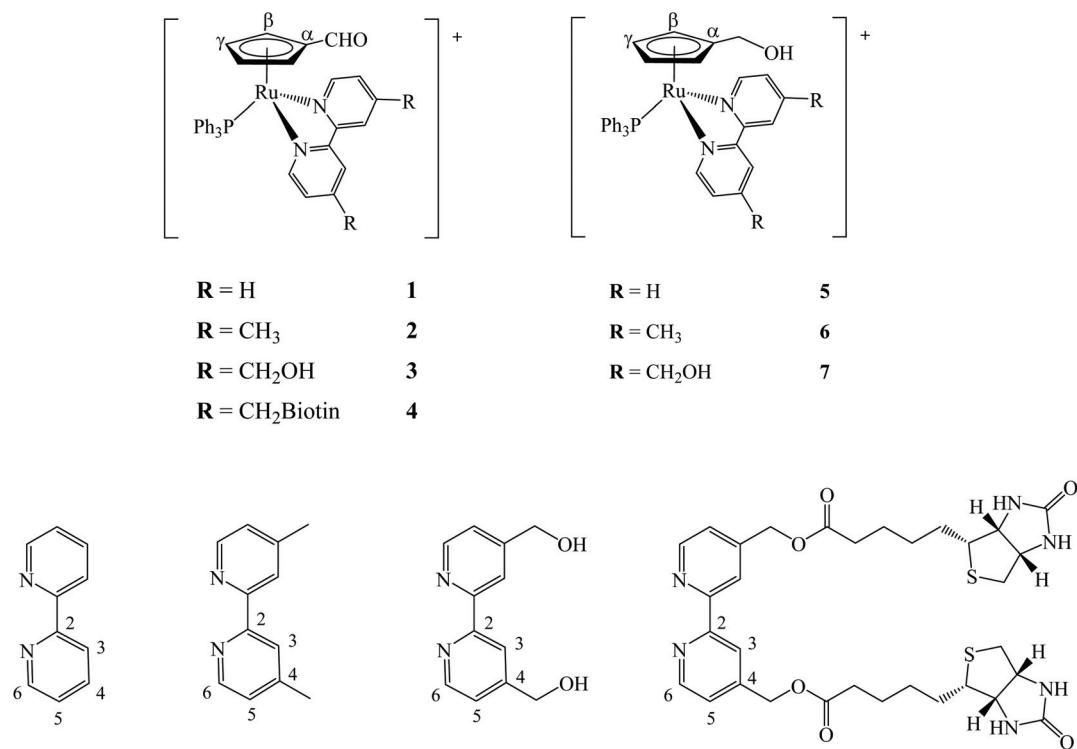


Fig. 2 Chemical structures of compounds 1–8. All compounds were isolated as CF_3SO_3^- salts.

sor $[\text{Ru}(\eta^5\text{-C}_5\text{H}_4\text{CHO})(\text{PPh}_3)_2\text{Cl}]$, by slightly changing the purification protocol avoiding chromatography (*cf.* Experimental section). Using this complex as starting material, three new cationic compounds with general formula $[\text{Ru}(\eta^5\text{-C}_5\text{H}_4\text{CHO})(\text{bipy})(\text{PPh}_3)][\text{CF}_3\text{SO}_3]$, where bipy is 2,2'-bipyridine (1), 4,4'-dimethyl-2,2'-bipyridine (2) and 4,4'-bis(hydroxymethyl)-2,2'-bipyridine (3), were synthesized in moderate to good yield by chloride abstraction with silver trifluoromethanesulfonate (Fig. 2, left). However, the reaction with 4,4'-dibiotin ester-2,2'-bipyridine (bipy-biotin) originated a secondary product very difficult to eliminate from the mixture even by column chromatography or repeated recrystallization. In order to overcome this difficulty, the ‘chemistry-on-the-complex’ concept²⁷ was used. Thus, complex 3 was used as a platform to directly conjugate the envisaged vitamin *via* carbodiimide-based activation method using *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (Scheme S1†).²⁸ This proved to be an efficient alternative method to afford complex $[\text{Ru}(\eta^5\text{-C}_5\text{H}_4\text{CHO})(\text{bipy-biotin})(\text{PPh}_3)][\text{CF}_3\text{SO}_3]$ (4) with a high yield (88%). Finally, we reduced the formyl group in the cyclopentadienyl ring of the previous four compounds into their hydroxymethyl analogues of general formula $[\text{Ru}(\eta^5\text{-C}_5\text{H}_4\text{CH}_2\text{OH})(\text{bipyR})(\text{PPh}_3)][\text{CF}_3\text{SO}_3]$ (where bipyR = 2,2'-bipyridine (5), 4,4'-dimethyl-2,2'-bipyridine (6), 4,4'-di(hydroxymethyl)-2,2'-bipyridine (7) and 4,4'-dibiotin ester-2,2'-bipyridine (8)) (Fig. 2, right). This reduction reaction was successfully performed for complexes 1–3 in THF in a one-step high yield procedure, using sodium borohydride as reduction agent (Scheme S2†). Yet, the reduction reaction of complex 4 did not lead to the

expected hydroxymethyl analogue and the main product of the reaction contained the methylated-bipyridyl counterpart (confirmed by NMR and single crystal X-ray diffraction). Thus, under the reduction reaction conditions, the bipy-biotin ester bonds were found to be very sensitive and ended by being cleaved. Therefore, another approach was to reduce the formyl group at the precursor prior to the coordination of the biotinylated ligand, since the presence of three equally reactive primary alcohol groups at compound 7 prevented the use of the previously proposed ‘chemistry-on-the-complex’ approach. Unfortunately, all purification attempts of complex 8 were unsuccessful and therefore the characterization and, consequently, biological activity of complex 8 was not evaluated in this study. Thus, the seven new compounds were fully analyzed by ^1H , ^{13}C and $^{31}\text{P}\{^1\text{H}\}$ NMR, FTIR and UV-vis spectroscopies, ESI-MS and their purity confirmed by elemental analysis. In addition, single crystals of six compounds (1, 2, 3, 5, 6 and 7) were successfully obtained and studied using single-crystal X-ray crystallography.

^1H , $^{13}\text{C}\{^1\text{H}\}$ and $^{31}\text{P}\{^1\text{H}\}$ NMR characterization of the new ruthenium-based compounds fully supported the proposed formulations (see ESI†). 2D NMR techniques (COSY, HMQC and HMBC) were used to assist on the assignment of all signals. Overall, analysis of the ^1H NMR spectra shows that Sigma coordination of the bipyridyl derivatives to the ruthenium-cyclopentadienyl scaffold induces a common deshielding effect of the monosubstituted η^5 -cyclopentadienyl protons along with a deshielding of H6 (ΔH_6 up to 0.82 ppm) and a shielding of H3 (ΔH_3 up to -0.41 ppm) bipyridyl protons, as it

was reported for related ruthenium cationic compounds.²⁹ Additionally, other resonances in the aromatic region ($6.95 \text{ ppm} < \delta < 7.42 \text{ ppm}$) were ascribed to the aromatic *ortho*, *meta* and *para* protons of the triphenylphosphane co-ligand. As expected, compounds **5**, **6** and **7** exhibit shielded resonances for the hydroxymethylcyclopentadienyl protons when compared to their formylcyclopentadienyl analogues, which is in good agreement with the electron donating character of the newly introduced pendant group. Detailed spectroscopic data concerning APT-¹³C{¹H} and ³¹P{¹H}-NMR experiments are included in the Experimental section and are in accordance with the effects discussed in this ¹H NMR analysis.

The solid state FTIR of the complexes (KBr pellets) of the novel organometallic ruthenium-cyclopentadienyl compounds **1–7** shows the bands for the $\nu\text{C-H}$ stretching vibration of the phosphane, cyclopentadienyl and bipyridyl ligands in the range $3100\text{--}3050 \text{ cm}^{-1}$ and bands for $\nu\text{C=C}$ ranging from 1489 to 1390 cm^{-1} . In addition, the presence of the triflate counter-ion was observed at $1250\text{--}1260 \text{ cm}^{-1}$. Characteristic stretching vibrations of the formyl and hydroxymethyl appended groups were also found at the expected region at $\sim 1670 \text{ cm}^{-1}$ and $\sim 3420 \text{ cm}^{-1}$, respectively.

Optical absorption spectra of complexes **1–7** were recorded at room temperature using $\sim 10^{-4}$ to $\sim 10^{-6} \text{ M}$ solutions in dichloromethane and dimethylsulfoxide. Table S1† presents molar absorptivity coefficient (ϵ) values and the correspondent wavelength (λ_{\max}) for the bands observed. Fig. 3A shows the spectra of compounds **1** and **5** which are representative of the behavior of formyl- or hydroxymethylcyclopentadienyl series of compounds (which exhibit very similar spectral features), whereas Fig. 3B illustrates the solvatochromic phenomenon observed for compound **3**. The same trend is observed in the electronic spectra of all complexes with two very intense absorption bands at high energy values ($\lambda = 240\text{--}330 \text{ nm}$) that are ascribed to electronic transitions occurring in the organometallic fragment ($\{[\text{Ru}(\eta^5\text{-C}_5\text{H}_4\text{R})](\text{PPh}_3)\}^+$ R = CHO or CH_2OH) and the coordinated ligands. In the visible range, one or two medium-strength absorption bands assigned to metal-to-ligand charge transfer transitions (MLCT) from Ru 4d orbitals to the π^* orbitals of the phosphane and bipyridyl ligands ($\lambda = 330\text{--}550 \text{ nm}$), as previously reported for related compounds.^{25,30} The charge transfer character of these bands is corroborated by solvatochromic studies in DMSO where a clear blue-shift is observed with the increase of polarity of the solvent for compound **3** (379 nm in CH_2Cl_2 vs. 366 nm in DMSO).

The crystal structures of complexes **1–3** and **5–7** have been solved by X-ray diffraction analysis. Fig. 4 displays the ORTEP diagrams of their molecular structures whereas the main crystallographic data and selected bond distances and angles can be found in the ESI† section (Tables S2–S3†). All compounds crystallize in the monoclinic system, space group $C12/c1$ (**1**), $P12_1/c1$ (**3**, **7**) or $P12_1/n$ (**5**, **6**) with exception of compound **2** which crystallizes in the triclinic system, space group $P\bar{1}$. In all cases the corresponding unit cells of the complexes display two enantiomers in the racemic crystal.

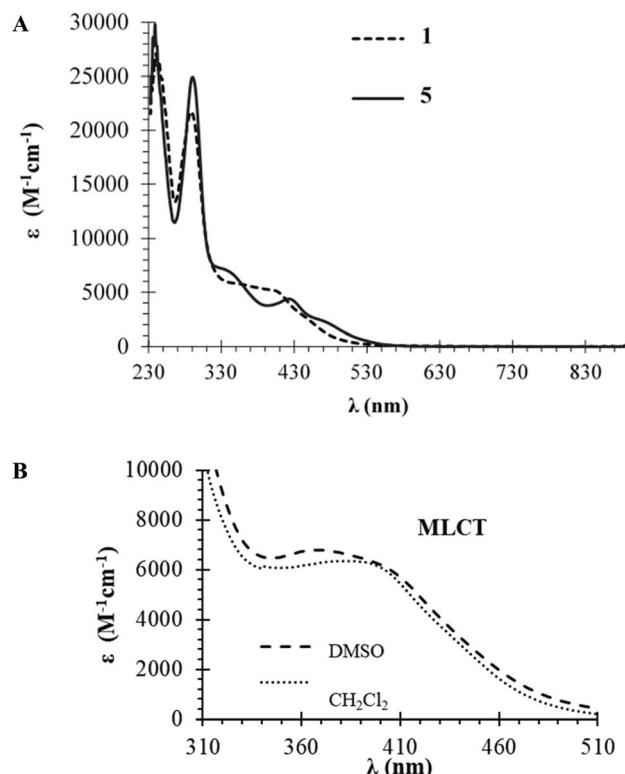


Fig. 3 Electronic absorption spectra: (A) UV-visible spectrum in dichloromethane for compounds **1** (—) and **5** (....) as representative of each series of compounds; (B) solvatochromic effect for compound **3**.

All complexes adopt the classical pseudo-octahedral three-legged piano stool geometry and, hence, the cyclopentadienyl arene rings exhibit the usual π -bonded η^5 coordination mode, whereas the bipyridyl ligands are coordinated in a bidentate fashion. The sixth coordination site is occupied by the triphenylphosphane ligands. The distances between Ru and the centroids of the cyclopentadienyl moiety in all the complexes, are within a narrow interval (1.828–1.841 Å), similar to other cyclopentadienyl ruthenium complexes described in the literature.²⁹

The Ru-P bond distances in the complexes are all in the same range (2.3086(11)–2.3351(3) Å), similar to the Ru-N_{bipy} bond distances, which are in the same order for all the compounds (2.0685(11)–2.1112(3) Å). The Ru-C distances are in general higher than Ru-N_{bipy}, probably due to the σ -donor character of the cyclopentadienyl rings. Moreover, the distances between Ru and the monosubstituted carbon on the η^5 -cyclopentadienyl ring in complexes **1–3** (Ru-C21 = 2.1901(13) **1**; Ru-C2 = 2.212(3) **2**; Ru-C21 = 2.1820(17) **3** Å) are in general slightly shorter than in complexes **5–7** (Ru-C21 = 2.230(2) **5**; Ru-C2 = 2.213(3) **6**; Ru-C21 = 2.235(3) **7** Å). This behavior could be due to the presence of the $-\text{CH}_2\text{OH}$ substituent onto the arene ring in the latter, which presents an electron-withdrawing character a lower than that of the $-\text{CHO}$ substituent. In the case of complex **1** (see Fig. S29†) an intramolecular hydrogen bond is formed between one pyridyl H atom and the



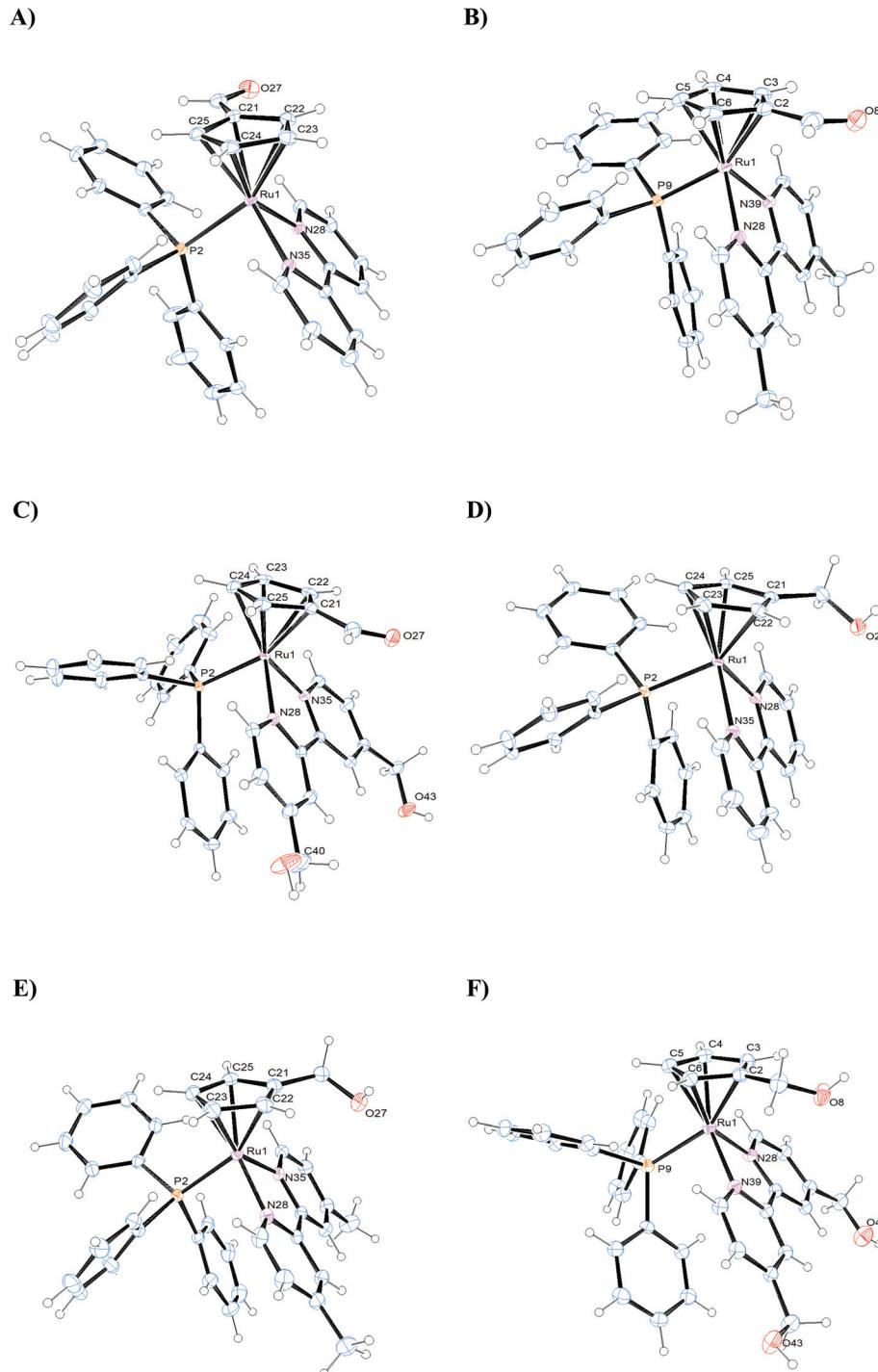


Fig. 4 ORTEP plots and labeling schemes for the cations of complexes 1–3 (A–C) and 5–7 (D–F).

O atom from the substituent $-\text{CHO}$ (H-bond distances are $\text{H}29\text{--O}27 = 2.580 \text{ \AA}$), since the O atom of $-\text{CHO}$ is orientated toward the bipyridine ligand.

The angles N--Ru--N and C--Ru--C in the complexes, show the geometrical restrictions imposed by the bipyridyl and cyclopentadienyl ligands, and the values found are similar to other compounds described in the literature.^{25,29}

It is worth mentioning the packing structure of the compounds, where additional intermolecular hydrogen bonds can be observed between oxygen atoms of the triflate anions and the hydrogen atoms of the triphenylphosphane (1) or the bipyridine ligands (2, 3); in addition, intermolecular hydrogen bonds are displayed between the oxygen atom of the $-\text{CH}_2\text{OH}$ substituent and the hydrogen atoms of the bipyridine ligand



of a neighboring molecule (5, 6, 7). In the case of 7 additional intermolecular hydrogen bonds are observed between O atom of the triflate anions and H atoms of the bipyridine ligand (Fig. S30†).

Stability in organic and aqueous media

All compounds were evaluated over time by monitoring their UV-Vis profile in 100% DMSO and 2–3% DMSO/DMEM in order to infer about their stability in conditions mimicking the biological assays. These studies were performed during 24 h at room temperature. UV-Vis absorption spectra of the complexes in cellular media exhibit one strong absorption band in the UV range and two broad absorption bands in the visible range, similarly to their correspondent spectra in organic solvents (Fig. S31†). No significant changes in the absorption spectra were observed over time, indicating adequate stability of all complexes for the biological assays. Additionally, we evaluated the behavior of compounds 1–4 by NMR to check the stability of the formyl substituent. The ¹H NMR spectra measured at 1 h, 2 h, 4 h and 24 h in 65% DMEM/35% DMSO (Fig. S32†) confirmed the stability of the formyl substituent over time.

Biological evaluation of the compounds

In order to access the biological activity of the new compounds, four non-small cell lung cancer cell lines (A549, NCI-H228, Calu-3, NCI-H1975 cells) with different expression levels of P-gp and MRP1 transporters were used (Fig. S33†), *i.e.* the two main transporters involved in cisplatin efflux.³¹ Cells were incubated with complexes 1–7 and cisplatin and cell viability was assessed after a 72 h period and their cytotoxicity (IC₅₀ values) was determined using the WST-1 assay.

Analyzing the different IC₅₀ obtained for cisplatin (Table 1), we observed that the higher the level of MRP1 in the cells analyzed (A549 and NCI-H228), the greater their resistance to cisplatin (*i.e.*, a higher IC₅₀ value is obtained), suggesting that MRP1 plays a key role in the efflux of cisplatin in our cellular models.

Based on the IC₅₀ values obtained, the resistance to cisplatin varies following the rank order: A549 > NCI-H228 > Calu-3 > NCI-H1975 cells.

The cytotoxicity of cisplatin, commonly evaluated with the MTT assay, often results in lower IC₅₀ values in NSCLC cells³² than those measured in the present work with the WST-1 assay. The different methods as well as the incubation time of

Table 2 Resistant factor (R_f) of the NSCLC cell lines treated with cisplatin alone *versus* cisplatin and Ru compounds

	1	2	4	6
A549	71.4	555.6	1250	243.9
NCI-H228	333.3	1389.9	588.2	344.8
Calu-3	26.3	126	33.2	78.7
NCI-H1975	2.9	0.6	1.6	0.7

Viability of cells measured after 72 h incubation with increasing concentrations (0–100 μ M) of cisplatin, co-incubated with 1 μ M of each compound, measured with a spectrophotometric assay. Values are data from $R_f = IC_{50}(\text{cisplatin})/IC_{50}(\text{cisplatin} + \text{Ru compound})$ ($n = 4$).

the dye in the MTT assay (ranging from 1 to 4 h in different experimental works) can explain such discrepancies.

In a first experimental set, we evaluated the cell viability of the cell lines in the presence of increasing concentrations of the new ruthenium compounds (Table 1).

According to the IC₅₀ values obtained for the Ru compounds it can be noticed that four compounds – 1, 2, 4 and 6 – were more active. For the panel of compounds tested herein, the presence of the substituent –CH₂OH seems to be detrimental for the activity of the overall compound, whether placed in the cyclopentadienyl ring (in 5 and 7) or in the bipyridine ligand (in 3). The formyl substituent in the cyclopentadienyl ring seems advantageous, with compounds 1, 2 and 4 being active. The methyl substituent in the bipyridine co-ligand (complexes 2 and 6) is, in this panel of complexes, highly beneficial for the activity, rendering active complexes. Indeed, compounds 1, 2, 4 and 6, were stronger killers of A549 and NCI-H228 cells, *i.e.* the two cell lines showing the highest resistance to cisplatin among those analyzed, suggesting that they could be inducers of collateral sensitivity of drug resistant cells.

This is not the first time that metal-based compounds are reported to exert a selective cytotoxicity against drug resistant cells: for instance, cobalt-based complexes¹⁵ and ferrocene-based compounds³³ overcome resistance to taxol-resistant tumors. Ruthenium-based compounds have been described as potent anti-cancer drugs,^{34–37} but their potential as collateral sensitivity inducers and/or MDR-reversing agents remains poorly known.

To verify if the compounds can improve the sensitivity to cisplatin, in particular in the cell lines with the highest degree

Table 1 IC₅₀ (μ M) of the new ruthenium compounds and cisplatin in the NSCLC cell lines analyzed

	1	2	3	4	5	6	7	Cisplatin
A549	10.8 ± 1.3	12.4 ± 3.6	>100	15.4 ± 2.6	>100	12.5 ± 2.1	>100	>100
NCI-H228	4.3 ± 0.7	3.8 ± 1.4	>100	16.5 ± 1.3	>100	7.8 ± 1.2	>100	>100
Calu-3	24.7 ± 4.1	4.9 ± 1.6	>100	28.9 ± 0.8	>100	5.9 ± 1.2	>100	63.4 ± 8.7
NCI-H1975	91.8 ± 10.4	>100	>100	>100	>100	>100	>100	3.8 ± 1.1

Viability of cells measured after 72 h incubation with increasing concentrations (0–100 μ M) of each compound, measured with a spectrophotometric assay. Data are means ± SD ($n = 4$).



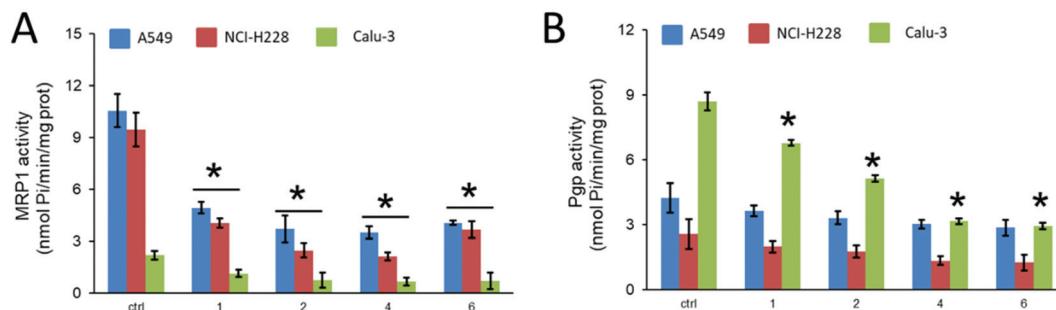


Fig. 5 MRP1 (A) and P-gp (B) ATPase activity, measured spectrophotometrically on the proteins immune-purified from cells treated without (ctrl) or with 1 μ M of compounds 1, 2, 4 and 6 for 24 h. Data are means \pm SD ($n = 3$). * $p < 0.05$: vs. ctrl.

of resistance to this drug, we incubated cells with increasing concentrations of cisplatin together with **1**, **2**, **4** and **6** at 1 μ M, a concentration equivalent to IC₂₅ of the Ru compounds for all cell lines. Working at these poorly toxic concentrations, it is indeed easier to evaluate the effect of the compounds as enhancers of cisplatin toxicity, not as cytotoxic drugs. The ratio IC₅₀(cisplatin)/IC₅₀(cisplatin + Ru compound), *i.e.* the Resistance factor (R_f) reported in Table 2, was considered an index of sensitization toward cisplatin³⁸ and indicates how many folds the compound enhances the efficacy of cisplatin.

All the compounds strongly sensitized A549 and NCI-H228 cell lines, showing a moderate sensitization effect in the mild cisplatin-resistant Calu-3 cells, and losing their effect on the cisplatin-sensitive NCI-H1975 cells.

Of note, A549 and NCI-H228 cells were the cell lines with the highest expression of MRP1, whereas in Calu-3 the most abundant transporter was P-gp. Thus, we decided to investigate if the Ru compounds can directly modulate the activity of these transporters by measuring the ATPase activity of cell extracts after incubation with the compounds (Fig. 5). This assay indicates if the compounds, once entered within the cells, bind the ABC transporters and interfere with their catalytic activity that is based on the hydrolysis of two ATPs associated to the efflux of the drug. Therein, a reduction in the ATPase activity of MRP1 or P-gp may suggest that the compounds inhibit the efflux activity of these transporters, *e.g.* by acting as inhibitors, substrates or negative allosteric modulators, as previously reported.³⁹

MRP1 and P-gp activities differed between the cell lines, in agreement with the expression levels of the protein, as observed for the controls. Interestingly, all Ru compounds inhibited MRP1 ATPase activity (Fig. 5A). They also inhibited P-gp activity in Calu-3 cells, that expresses P-gp to a greater extent (Fig. 5B). We could not detect any significant decrease of P-gp ATPase activity in A549 and NCI-H228 cells, most likely as a consequence of the lower amount of P-gp expressed. From the four complexes tested, compounds **4** and **6** seem to be the most efficient in inhibiting P-gp activity in Calu-3 cell extracts.

These results provided evidence that the selected ruthenium compounds increased the sensitivity to cisplatin in the resistant cell lines by directly impairing the catalytic activity of

the main drug efflux transporters expressed the panel of lung cancer cell lines chosen. We are now investigating if this inhibition is exerted on other ABC transporters and by which molecular mechanisms. Considering our previous results on anti-cancer ruthenium cyclopentadienyl compounds, it seems that a trend is gaining some consistency: concerning their cytotoxicity, hydroxylated [Ru(η^5 -C₅H₄R)(PPh₃)(bipy)]⁺ (R = H, CH₃, CHO or CH₂OH) based compounds are less active or inactive in any of the cell lines tested (lung, breast or ovarian). This could possibly be related to their behavior as substrates of ABC pumps as we have previously observed for [Ru(η^5 -C₅H₅)(4,4'-di(hydroxymethyl)-2,2'-bipyridine)(PPh₃)]⁺ (pmc79) and [Ru(η^5 -C₅H₄CH₃)(4,4'-di(hydroxymethyl)-2,2'-bipyridine)(PPh₃)]⁺ (RT12) compounds that were possibly P-gp substrates.^{25,28} RT12 was also a poor substrate for MRP1, MRP2 and BCRP. On the contrary, [Ru(η^5 -C₅H₅)(4,4'-dibiotin ester-2,2'-bipyridine)(PPh₃)]⁺ (LCR134) and [Ru(η^5 -C₅H₄CH₃)(4,4'-dimethyl-2,2'-bipyridine)(PPh₃)]⁺ (RT11) were found to be P-gp and MRP1/ MRP2 inhibitors, respectively.^{25,28} We will now focus on understanding how these structural changes influence the ability of the compounds to act as inhibitors at the molecular level.

Conclusions

Two sets of new piano-stool compounds with the general formula [Ru(η^5 -C₅H₄CHO)(bipyR)(PPh₃)][CF₃SO₃] and [Ru(η^5 -C₅H₄CH₂OH)(bipyR)(PPh₃)][CF₃SO₃], where bipyR = 2,2'-bipyridine (**1**, **5**), 4,4'-dimethyl-2,2'-bipyridine (**2**, **6**), 4,4'-di(hydroxymethyl)-2,2'-bipyridine (**3**, **7**) and 4,4'-dibiotin ester-2,2'-bipyridine (**4**), have been synthesized and completely characterized by analytical and spectroscopic techniques. In addition, the molecular structure of compounds **1**–**3** and **5**–**7** was unveiled by X-ray diffraction studies. All compounds were evaluated as prospective chemotherapeutics for lung cancer, and their activity assessed in a panel of different human lung cells. From this set of compounds, four of them (**1**, **2**, **4** and **6**) have shown strong activity against cisplatin-resistant non-small cell lung cancer cell lines, disclosing them as highly promising agents for the treatment of lung cancer.



These four complexes were further tested for their ability to sensitize cisplatin-resistant cells. Complexes **1**, **2**, **4** and **6** increased cisplatin cytotoxicity up to 1390-fold by inhibiting MRP1 and P-gp transporters. This is of particular interest since, besides exerting an important role in cisplatin resistance,⁴⁰ MRP1 has been proposed as a potential mediator of collateral sensitivity.^{41–43} Few compounds targeting MRP1 are inducers of collateral sensitivity⁴⁴ but the research in this field is in great expansion. In lung cancer, where cisplatin is the first-line treatment and MRP1 is one of the main responsible for resistance to this drug,⁴⁵ finding MRP1-targeting agents, inducing collateral sensitivity is absolutely preeminent. In this context, complexes **1**, **2**, **4** and **6** emerge as valuable prospective agents for lung cancer chemotherapy, specially compounds **4** and **6**, both quite active as cytotoxic agents and able to inhibit both MRP1 and P-gp, suggesting an important ability to overcome drug resistance mechanisms.

Methods

Materials

All chemicals were purchased from commercial sources and used without further purification (unless otherwise stated).

Instrumentation and methods (Experimental section)

General procedures. All reactions and purification of compounds were performed under nitrogen atmosphere using Schlenk techniques. All solvents were used as purchased. Only dichloromethane, *n*-hexane and tetrahydrofuran used for synthetic procedures and work-up were dried using an MBRAUN solvent purification system (MB SPS-800, M Braun Inertgas-Systeme GmbH, Garching, Germany). NMR spectra were recorded on a Bruker Advance 400 spectrometer at probe temperature using commercially available deuterated acetone. Chemical shifts (δ) are reported in parts per million (ppm) referenced to tetramethylsilane (δ 0.00 ppm) using the residual proton solvent peaks as internal standards. $^{31}\text{P}\{\text{H}\}$ NMR chemical shifts were reported downfield from external standard 85% H_3PO_4 . The multiplicity of the peaks is abbreviated as follows: s (singlet), d (doublet), t (triplet), m (multiplet), comp (complex). Coupling constants (J) are reported in Hertz (Hz). All assignments were attributed using COSY, HMBC, and HMQC 2D-NMR techniques. Infrared spectra were recorded on KBr pellets using a Mattson Satellite FT-IR spectrophotometer. Only considered relevant bands were cited in the text. Electronic spectra were recorded at room temperature on a Jasco V-660 spectrometer from solutions of 10^{-4} – 10^{-6} M in quartz cuvettes (1 cm optical path). Elemental analyses were performed at *Laboratório de Análises*, at *Instituto Superior Técnico*, using a Fisons Instruments EA1 108 system. ESI-MS data acquisition, integration and handling were performed using a PC with the software package EAGER-200 (Carlo Erba Instruments).

Synthesis of the ruthenium complexes

Synthesis of the precursor complex $[\text{Ru}(\eta^5\text{-C}_5\text{H}_4\text{CHO})(\text{PPh}_3)_2\text{Cl}]$. The synthesis of $[\text{Ru}(\eta^5\text{-C}_5\text{H}_4\text{CHO})(\text{PPh}_3)_2\text{Cl}]$ is already described²⁶ but in this work another purification method was applied. A mixture of $\text{RuCl}_2(\text{PPh}_3)_3$ (307 mg, 0.32 mmol), 6-dimethylaminopentafulvene (77 mg, 0.63 mmol) in 10 mL of THF/water (v/v = 7 : 3) was refluxed for 3 h. After that period, the reaction mixture was cooled to room temperature and concentrated to dryness and the aqueous phase discarded. The brown residue was washed with water (5 mL \times 2), light petroleum ether (5 mL \times 3), a mixture of light petroleum ether/diethyl ether (v/v = 3/3 \times 3) and hexane (5 mL \times 3). The experimental data obtained is in agreement with the previous literature.

Synthesis of the complexes $[\text{Ru}(\eta^5\text{-C}_5\text{H}_4\text{CHO})(\text{bipy-R})(\text{PPh}_3)][\text{CF}_3\text{SO}_3]$ (1–3). Complexes of general formula $[\text{Ru}(\eta^5\text{-C}_5\text{H}_4\text{CHO})(\text{bipy-R})(\text{PPh}_3)][\text{CF}_3\text{SO}_3]$ (1–3) were prepared from the parent neutral complex $[\text{Ru}(\eta^5\text{-C}_5\text{H}_4\text{CHO})(\text{PPh}_3)_2\text{Cl}]$ (150 mg, 0.2 mmol) by halide abstraction with silver trifluoromethanesulfonate (55 mg, 0.22 mmol) in degassed methanol and in the presence of a slight excess (1.2 equivalents) of the 2,2-bipyridine ligands (for **1**, 38 mg, 0.24 mmol), 4,4'-dimethyl-2,2'-bipyridine (for **2**, 44 mg, 0.24 mmol) or 4,4'-hydroxymethyl-2,2'-bipyridine (for **3**, 52 mg, 0.24 mmol), at reflux under nitrogen atmosphere for 13–15 h. After cooling to room temperature, filtering and removing the solvent, the compounds were treated with a mixture of propan-2-ol/water (v/v 1 : 2, 15 mL) and filtered. The filtrate was concentrated to dryness and the residues obtained were washed with *n*-hexane (15 mL \times 3) and recrystallized from acetone/*n*-hexane (**1** and **2**) or dichloromethane/ether (**3**), to give dark orange-brown crystalline solids.

$[\text{Ru}(\eta^5\text{-C}_5\text{H}_4\text{CHO})(2,2'\text{-bipy})(\text{PPh}_3)][\text{CF}_3\text{SO}_3]$ (1). Yield: 75% (114 mg). Orange-brown single crystals were obtained from slow diffusion of *n*-hexane into acetone solution.

^1H NMR $[(\text{CD}_3)_2\text{CO}, \text{Me}_4\text{Si}, \delta/\text{ppm}]$: 9.35 (broad, 2H, H6), 9.24 (s, 1H, $(\eta^5\text{-C}_5\text{H}_4\text{CHO})$), 8.20 (d, 2H, $^3J_{\text{HH}} = 8.0$, H3), 7.97 (t, 2H, $^3J_{\text{HH}} = 7.6$, H4), 7.46 (m, 5H, $\underline{\text{H}}_o\text{PPh}_3 + \underline{\text{H}}_5$), 7.36 (m, 6H, $\underline{\text{H}}_m\text{PPh}_3$), 7.12 (t, 6H, $^3J_{\text{HH}} = 8.4$, $\underline{\text{H}}_o\text{PPh}_3$), 5.80 (broad, 2H, $\underline{\text{H}}_\beta$ - $\eta^5\text{-C}_5\text{H}_4\text{CHO}$), 4.93 (broad, 2H, $\underline{\text{H}}_\gamma\eta^5\text{-C}_5\text{H}_4\text{CHO}$).

$\text{APT}^{13}\text{C}\{\text{H}\}$ NMR $[(\text{CD}_3)_2\text{CO}, \delta/\text{ppm}]$: 189.1 ($\eta^5\text{-C}_5\text{H}_4\text{CHO}$), 156.8 (C6), 138.1 (C4), 133.9 (d, $^2J_{\text{CP}} = 11$, $\underline{\text{CH}}_o\text{PPh}_3$), 131.8 (d, $^1J_{\text{CP}} = 43.8$, $\underline{\text{C}}_q\text{PPh}_3$), 131.4 (d, $^4J_{\text{CP}} = 1.9$, $\underline{\text{CH}}_p\text{PPh}_3$), 129.5 (d, $^3J_{\text{CP}} = 9.8$, $\underline{\text{CH}}_m\text{PPh}_3$), 126.7 (C5), 124.4 (C3), 100.8 ($\underline{\text{C}}_\alpha\eta^5\text{-C}_5\text{H}_4\text{CHO}$), 85.2 ($\underline{\text{C}}_\beta\eta^5\text{-C}_5\text{H}_4\text{CHO}$), 79.1 ($\underline{\text{C}}_\gamma\eta^5\text{-C}_5\text{H}_4\text{CHO}$).

$^{31}\text{P}\{\text{H}\}$ NMR $[(\text{CD}_3)_2\text{CO}, \delta/\text{ppm}]$: 48.81 (s, PPh_3).

FTIR [KBr, cm^{-1}]: 3105–3055 ($\nu_{\text{C}-\text{H}}$ aromatic rings), 1667 ($\nu_{\text{C}=\text{O}}$, $\eta^5\text{-C}_5\text{H}_4\text{CHO}$), 1433 ($\nu_{\text{C}=\text{C}}$ aromatic rings), 1262 ($\nu\text{CF}_3\text{SO}_3$ counter ion).

UV-vis [DMSO, $\lambda_{\text{max}}/\text{nm}$ ($\epsilon \times 10^3/\text{M}^{-1} \text{cm}^{-1}$)]: 289 (24.49), 362 (6.46), 415 (Sh).

UV-vis [CH_2Cl_2 , $\lambda_{\text{max}}/\text{nm}$ ($\epsilon \times 10^3/\text{M}^{-1} \text{cm}^{-1}$)]: 289 (21.71), 357 (Sh), 409 (Sh), 457 (Sh).

Elemental analysis calc. for $\text{C}_{35}\text{H}_{28}\text{F}_3\text{N}_2\text{O}_4\text{PRuS}$ (761.71): C, 55.2, H, 3.7; N, 3.7; S, 4.2. Found: C, 54.9; H, 3.5; N, 3.6; S, 4.3.

ESI-MS: $[\text{1-CF}_3\text{SO}_3]^+$ calc. for $[\text{C}_{34}\text{H}_{28}\text{N}_2\text{OPRu}]^+$: 613.10 found: 613.05.

$[\text{Ru}(\eta^5\text{-C}_5\text{H}_4\text{CHO})(4,4'\text{-CH}_3\text{-2,2'-bipy})(\text{PPh}_3)][\text{CF}_3\text{SO}_3]$ (2). Yield: 64% (101 mg). Orange-red single crystals were obtained by slow diffusion of *n*-hexane into acetone solution.

^1H NMR $[(\text{CD}_3)_2\text{CO}, \text{Me}_4\text{Si}, \delta/\text{ppm}]$: 9.25 (s, 1H, $(\eta^5\text{-C}_5\text{H}_4\text{CHO})$), 9.16 (broad, 2H, H_6), 8.04 (s, 2H, H_3), 7.47 (m, 3H, H_pPPh_3), 7.36 (m, 6H, H_mPPh_3), 7.30 (br s, 2H, H_5), 7.12 (t, 6H, $^3J_{\text{HH}} = 8.4$, H_oPPh_3), 5.75 (broad, 2H, $\text{H}_\beta\text{-}(\eta^5\text{-C}_5\text{H}_4\text{CHO})$), 4.85 (broad, 2H, $\text{H}_\gamma\text{-}(\eta^5\text{-C}_5\text{H}_4\text{CHO})$, 2.48 (s, 6H, CH_3).

APT- $^{13}\text{C}\{^1\text{H}\}$ NMR $[(\text{CD}_3)_2\text{CO}, \delta/\text{ppm}]$: 189.0 ($\eta^5\text{-C}_5\text{H}_4\text{CHO}$), 156.5 (C2), 156.0 (C6), 150.5 (C4), 134.0 (d, $^2J_{\text{CP}} = 11$, CH_oPPh_3), 132.2 (d, $^1J_{\text{CP}} = 43$, C_qPPh_3), 131.3 (d, $^4J_{\text{CP}} = 2$, CH_pPPh_3), 129.5 (d, $^3J_{\text{CP}} = 9$, CH_mPPh_3), 127.7 (C5), 125.1 (C3), 103.0 ($\text{C}_\alpha\text{-}\eta^5\text{-C}_5\text{H}_4\text{CHO}$), 84.8 ($\text{C}_\beta\text{-}\eta^5\text{-C}_5\text{H}_4\text{CHO}$), 78.6 ($\text{C}_\gamma\text{-}\eta^5\text{-C}_5\text{H}_4\text{CHO}$), 20.8 (CH3).

$^{31}\text{P}\{^1\text{H}\}$ NMR $[(\text{CD}_3)_2\text{CO}, \delta/\text{ppm}]$: 48.94 ppm (s, PPh_3).

FTIR [KBr, cm^{-1}]: 3071–3050 cm^{-1} ($\nu_{\text{C-H}}$ aromatic rings), 2922 cm^{-1} ($\nu_{\text{C-H}}$ alkanes), 1670 (ν_{CO} , $\eta^5\text{-C}_5\text{H}_4\text{CHO}$), 1440 ($\nu_{\text{C=C}}$ aromatic rings), 1258 cm^{-1} ($\nu\text{CF}_3\text{SO}_3$ counterion).

UV-vis [DMSO, $\lambda_{\text{max}}/\text{nm} (\epsilon \times 10^3/\text{M}^{-1} \text{ cm}^{-1})$]: 289 (26.27), 371 (7.21), 412 (Sh).

UV-vis [CH_2Cl_2 , $\lambda_{\text{max}}/\text{nm} (\epsilon \times 10^3/\text{M}^{-1} \text{ cm}^{-1})$]: 290 (26.10), 366 (6.91), 412 (Sh).

Elemental analysis calc. for $\text{C}_{37}\text{H}_{32}\text{F}_3\text{N}_2\text{O}_4\text{PRuS}$ (789.76): C, 56.3; H, 4.1; N, 3.6; S, 4.1. Found: C, 54.8; H, 3.9; N, 3.4; S, 4.0.

ESI-MS: $[\text{2-CF}_3\text{SO}_3]^+$ calc. for $[\text{C}_{36}\text{H}_{32}\text{N}_2\text{OPRu}]^+$: 641.13 found: 641.02.

$[\text{Ru}(\eta^5\text{-C}_5\text{H}_4\text{CHO})(4,4'\text{-CH}_2\text{OH-2,2'-bipy})(\text{PPh}_3)][\text{CF}_3\text{SO}_3]$ (3). Yield: 62% (103 mg). Orange single crystals were obtained by slow diffusion of diethyl ether into dichloromethane solution.

^1H NMR $[(\text{CD}_3)_2\text{CO}, \text{Me}_4\text{Si}, \delta/\text{ppm}]$: 9.26 (m, 3H, $(\eta^5\text{-C}_5\text{H}_4\text{CHO}) + \text{H}_6$), 8.09 (s, 2H, H_3), 7.43 (m, 5H, $\text{H}_p\text{PPh}_3 + \text{H}_5$), 7.35 (m, 6H, H_mPPh_3), 7.12 (t, 6H, $^3J_{\text{HH}} = 8$, H_oPPh_3), 5.78 (broad, 2H, $\text{H}_\beta\text{-}\eta^5\text{-C}_5\text{H}_4\text{CHO}$), 4.87–4.80 (m, 8H, $\text{H}_\gamma\text{-}\eta^5\text{-C}_5\text{H}_4\text{CHO} + \text{CH}_2\text{OH} + \text{CH}_2\text{OH}$).

APT- $^{13}\text{C}\{^1\text{H}\}$ NMR $[(\text{CD}_3)_2\text{CO}, \delta/\text{ppm}]$: 189.0 ($\eta^5\text{-C}_5\text{H}_4\text{CHO}$), 156.5 (C2), 156.2 (C6), 154.9 (C4), 134.0 (d, $^2J_{\text{CP}} = 11$, CH_oPPh_3), 132.1 (d, $^1J_{\text{CP}} = 44$, C_qPPh_3), 131.3 (d, $^4J_{\text{CP}} = 2$, CH_pPPh_3), 129.5 (d, $^3J_{\text{CP}} = 10$, CH_mPPh_3), 123.9 (C5), 121.3 (C3), 103.0 ($\text{C}_\alpha\text{-}\eta^5\text{-C}_5\text{H}_4\text{CHO}$), 89.4 ($\text{C}_\beta\text{-}\eta^5\text{-C}_5\text{H}_4\text{CHO}$), 78.7 ($\text{C}_\gamma\text{-}\eta^5\text{-C}_5\text{H}_4\text{CHO}$), 62.4 (CH2OH).

^{31}P NMR $[(\text{CD}_3)_2\text{CO}, \delta/\text{ppm}]$: 48.94 (s, PPh_3).

FTIR [KBr, cm^{-1}]: 3464 ($\nu_{\text{O-H}}$), 3084–2916 ($\nu_{\text{C-H}}$ aromatic rings); 2884–2857 ($\nu_{\text{C-H}}$ alkanes), 1678 (ν_{CO} , $\eta^5\text{-C}_5\text{H}_4\text{CHO}$), 1440 ($\nu_{\text{C=C}}$ aromatic rings), 1246 cm^{-1} ($\nu\text{CF}_3\text{SO}_3$ counter ion), 1221 cm^{-1} ($\nu_{\text{C-O}}$).

UV-vis [DMSO, $\lambda_{\text{max}}/\text{nm} (\epsilon \times 10^3/\text{M}^{-1} \text{ cm}^{-1})$]: 290 (27.15), 366 (7.27), 416 (Sh).

UV-vis [CH_2Cl_2 , $\lambda_{\text{max}}/\text{nm} (\epsilon \times 10^3/\text{M}^{-1} \text{ cm}^{-1})$]: 277 (Sh), 289 (28.43), 379 (7.43), 451 (Sh).

Elemental analysis calc. for $\text{C}_{37}\text{H}_{32}\text{F}_3\text{N}_2\text{O}_6\text{PRuS}$ (821.76): C, 54.1; H, 3.9; N, 3.4; S, 3.9. Found: C, 54.1; H, 3.9; N, 3.4; S, 3.9.

ESI-MS: $[\text{3-CF}_3\text{SO}_3]^+$ calc. for $[\text{C}_{36}\text{H}_{32}\text{N}_2\text{O}_3\text{PRu}]^+$: 673.12 found: 673.02.

$[\text{Ru}(\eta^5\text{-C}_5\text{H}_4\text{CHO})(4,4'\text{-CH}_2\text{Biotin-2,2'-bipy})(\text{PPh}_3)][\text{CF}_3\text{SO}_3]$ (4). $[\text{Ru}(\eta^5\text{-C}_5\text{H}_4\text{CHO})(4,4'\text{-CH}_2\text{OH-2,2'-bipy})(\text{PPh}_3)][\text{CF}_3\text{SO}_3]$ (3, 100 mg, 0.12 mmol) was combined with 5-[3aS,4S,6aR]-2-oxohexahydro-1*H*-thieno[3,4-*d*] imidazol-4-yl]pentanoic acid (biotin) (68 mg, 0.28 mmol), DMAP (14 mg; 0.12 mmol) and EDC-Cl (55 mg, 0.28 mmol) in a Schlenk and DMF (8 mL) was added. The orange mixture was stirred for 14 h at room temperature. After that, the solvent was removed under vacuum and the residue obtained washed with water (10 mL \times 3) and light petroleum ether (10 mL \times 3), to yield an orange solid.

Yield: 88% (135 mg).

^1H NMR $[(\text{CD}_3)_2\text{CO}, \text{Me}_4\text{Si}, \delta/\text{ppm}]$: 9.35 (broad, 2H, H_6), 9.26 (s, 1H, $\eta^5\text{-C}_5\text{H}_4\text{CHO}$), 8.15 (s, 2H, H_3), 7.48 (m, 3H, $^3J_{\text{HH}} = 7.6$, H_4), 7.46 (m, 5H, $\text{H}_p\text{PPh}_3 + \text{H}_5$), 7.36 (m, 6H, H_mPPh_3), 7.12 (t, 6H, $^3J_{\text{HH}} = 8$, H_oPPh_3), 6.11 (broad s, 2H, NH) 5.90 (d, 2H, NH), 5.83 (broad, 2H, $\text{H}_\beta\text{-}\eta^5\text{-C}_5\text{H}_4\text{CHO}$), 5.28 (m, 4H, bipy-CH₂Biotin), 4.93 (broad, 2H, $\text{H}_\gamma\text{-}\eta^5\text{-C}_5\text{H}_4\text{CHO}$), 4.50 (SCH₂-CHBiotin), 4.32 (m, 2H, CHBiotin), 3.22 (m, 2H, S-CHBiotin), 2.94 (m, 2H, SCH₂Biotin), 2.67 (m, 2H, H), 2.52 + 1.75–1.49 (3 \times m, 16H, CH₂CH₂CH₂CH₂Biotin).

APT- $^{13}\text{C}\{^1\text{H}\}$ NMR $[(\text{CD}_3)_2\text{CO}, \delta/\text{ppm}]$: 189.3 ($\eta^5\text{-C}_5\text{H}_4\text{CHO}$), 173.5 (CO, bipy-Biotin ester), 164.1 (CO, bipy-Biotin, urea), 157.1 (C1), 156.6 (C2), 148.7 (C4), 133.9 ($^2J_{\text{CP}} = 11$, C_{ortho}-PPh₃), 131.9 ($^1J_{\text{CP}} = 40$, C_q, PPh₃), 131.5 ($^4J_{\text{CP}} = 1$, C_{para}-PPh₃), 129.6 ($^3J_{\text{CP}} = 10$, C_{meta}-PPh₃), 123.9 (d, $^4J_{\text{CP}} = 6$, C5), 122.6 (d, $^4J_{\text{CP}} = 8$, C3), 107.5 ($\text{C}_\alpha\text{-}\eta^5\text{-C}_5\text{H}_4\text{CHO}$), 85.1 (2 signals, s, C_β- $\eta^5\text{-C}_5\text{H}_4\text{CHO}$), 79.1 (2 signals, s, C_γ- $\eta^5\text{-C}_5\text{H}_4\text{CHO}$), 64.0 (2 signals, s, bipy-CH₂Biotin), 62.6 (2 signals, s, CHBiotin), 60.9 (2 signals, s, SCH₂-CHBiotin), 56.7 (2 signals, s, SCHBiotin), 41.1 (2 signals, s, SCH₂Biotin), 34.3 + 29.2* + 25.6 (sets of 2 signals, CH₂CH₂CH₂CH₂Biotin).

^{31}P NMR $[(\text{CD}_3)_2\text{CO}, \delta/\text{ppm}]$: 48.70 (s, PPh_3).

FTIR [KBr, cm^{-1}]: 3073 ($\nu_{\text{C-H}}$ aromatic rings), 2928–2859 ($\nu_{\text{C-H}}$ alkanes), 1736–1620 ($\nu_{\text{C=O}}$, $\eta^5\text{-C}_5\text{H}_4\text{CHO}$, urea, ester), 1433 ($\nu_{\text{C=C}}$ aromatic rings), 1260 cm^{-1} ($\nu\text{CF}_3\text{SO}_3$ counter ion).

UV-vis [DMSO, $\lambda_{\text{max}}/\text{nm} (\epsilon \times 10^3/\text{M}^{-1} \text{ cm}^{-1})$]: 293 (47.69), 358 (11.94), 414 (Sh) 468 (Sh).

UV-vis [CH_2Cl_2 , $\lambda_{\text{max}}/\text{nm} (\epsilon \times 10^3/\text{M}^{-1} \text{ cm}^{-1})$]: 291 (12.59), 345 (Sh), 416 (2.39), 479 (Sh).

Elemental analysis calc. for $\text{C}_{57}\text{H}_{60}\text{F}_3\text{N}_6\text{O}_{10}\text{PRuS}_3$ (1274.35): C, 53.7; H, 4.8; N, 6.6; S, 7.6. Found: C, 52.8; H, 5.2; N, 6.7; S, 7.0.

ESI-MS: $[\text{4-CF}_3\text{SO}_3]^+$ calc. for $[\text{C}_{56}\text{H}_{60}\text{N}_6\text{O}_7\text{PRuS}_2]^+$: 1125.27 found: 1125.09.

Synthesis of the complexes $[\text{Ru}(\eta^5\text{-C}_5\text{H}_4\text{CH}_2\text{OH})(\text{bipy-R})(\text{PPh}_3)][\text{CF}_3\text{SO}_3]$ (5–7). To a mixture of $[\text{Ru}(\eta^5\text{-C}_5\text{H}_4\text{CHO})(2,2'\text{-bipy})(\text{PPh}_3)][\text{CF}_3\text{SO}_3]$ (1, 100 mg, 0.13 mmol), $[\text{Ru}(\eta^5\text{-C}_5\text{H}_4\text{CHO})(4,4'\text{-CH}_3\text{-2,2'-bipy})(\text{PPh}_3)][\text{CF}_3\text{SO}_3]$ (2, 100 mg, 0.13 mmol) or $[\text{Ru}(\eta^5\text{-C}_5\text{H}_4\text{CHO})(4,4'\text{-CH}_2\text{OH-2,2'-bipy})(\text{PPh}_3)][\text{CF}_3\text{SO}_3]$ (3, 100 mg, 0.12 mmol) and NaBH₄ (586 mg, 10.1 mmol) in THF (2 mL) was slowly added MeOH (10 mL) over 20 minutes. Following the addition, the mixture was stirred at room temperature until gas bubbles were no longer detected (60–90 min). The solvents were removed under vacuum and the residue obtained was extracted with dichloromethane (20 mL \times 2) and acetone (10 mL) and filtered through



Celite®. The filtrate was concentrated to dryness, the residue was washed with water (5 mL × 3), hexane (5 mL × 3) and then recrystallized from methanol/diethyl ether (5) or dichloromethane/n-hexane (6 and 7), to afford bright orange crystalline solids.

[Ru(η^5 -C₅H₄CH₂OH)(2,2'-bipy)(PPh₃)][CF₃SO₃] (5). Yield: 81% (80 mg). Orange single crystals were obtained by slow diffusion of diethyl ether into a methanolic solution or slow evaporation of dichloromethane solution.

¹H NMR [(CD₃)₂CO, Me₄Si, δ /ppm]: 9.49 (broad, 2H, H₆), 8.17 (d, 2H, J_{HH} = 8, H₃), 7.88 (t, 2H, J_{HH} = 8, H₄), 7.41–7.32 (m, 11H, H_pPPh₃ + H_mPPh₃ + H₅), 7.11 (t, 6H, J_{HH} = 8, H_oPPh₃), 4.94 (broad, 2H, H_p- η^5 -C₅H₄CH₂OH), 4.62 (broad, 2H, H_γ- η^5 -C₅H₄CH₂OH), 4.11 (s, 3H, η^5 -C₅H₄CH₂OH + η^5 -C₅H₄CH₂OH).

APT-¹³C{¹H} NMR [(CD₃)₂CO, δ /ppm]: 157.0 (d, J_{CP} = 2, C₆), 156.7 (C₂), 136.9 (C₄), 133.8 (d, J_{CP} = 11, C_H_oPPh₃), 132.6 (d, J_{CP} = 41, C_qPPh₃), 130.9 (d, J_{CP} = 2, C_H_pPPh₃), 129.3 (d, J_{CP} = 10, C_H_mPPh₃), 126.1 (C₅), 124.1 (C₃), 104.9 (C_α- η^5 -C₅H₄CH₂OH), 76.7 + 76.5 (C_p- η^5 -C₅H₄CH₂OH + C_γ- η^5 -C₅H₄CH₂OH), 57.7 (η^5 -C₅H₄CH₂OH).

³¹P NMR [(CD₃)₂CO, δ /ppm]: 51.25 (s, PPh₃).

FTIR [KBr, cm⁻¹]: 3412 (ν_{O-H}), 3075–3055 (ν_{C-H} aromatic rings), 2962–2853 (ν_{C-H} alkanes), 1435 ($\nu_{C=C}$ aromatic rings), 1256 (ν CF₃SO₃ counter ion).

UV-vis [DMSO, λ_{max} /nm ($\epsilon \times 10^3/M^{-1}$ cm⁻¹)]: 292 (16.59), 344 (Sh), 411 (2.93), 483 (Sh).

UV-vis [CH₂Cl₂, λ_{max} /nm ($\epsilon \times 10^3/M^{-1}$ cm⁻¹)]: 291 (24.89), 348 (Sh), 423 (4.38), 486 (Sh).

Elemental analysis calc. for C₃₅H₃₀F₃N₂O₄PRuS (763.73): C, 55.0; H, 4.0; N, 3.7; S, 4.2. Found: C, 53.6; H, 3.9; N, 3.5; S, 4.2.

ESI-MS: [5-CF₃SO₃]⁺ calc. for [C₃₄H₃₀N₂OPRu]⁺: 615.11 found: 615.02.

[Ru(η^5 -C₅H₄CH₂OH)(4,4'-CH₃-2,2'-bipy)(PPh₃)][CF₃SO₃] (6). Yield: 65% (62 mg). Orange single crystals were obtained from slow diffusion of n-hexane into dichloromethane solution.

¹H NMR [(CD₃)₂CO, Me₄Si, δ /ppm]: 9.29 (broad, 2H, H₆), 8.01 (s, 2H, H₃), 7.41 (m, 3H, H_pPPh₃), 7.32 (m, 6H, H_mPPh₃), 7.20 (broad, 2H, H₅), 7.12 (t, 6H, J_{HH} = 8, H_oPPh₃), 4.88 (broad, 2H, H_p- η^5 -C₅H₄CH₂OH), 4.56 (broad, 2H, H_γ- η^5 -C₅H₄CH₂OH), 4.10 (s, 3H, η^5 -C₅H₄CH₂OH + η^5 -C₅H₄CH₂OH), 2.45 (s, 6H, CH₃).

APT-¹³C{¹H} NMR [(CD₃)₂CO, δ /ppm]: 156.4 (C₂), 156.1 (d, J_{CP} = 2, C₆), 149.1 (C₄), 133.9 (d, J_{CP} = 11, C_H_oPPh₃), 133.0 (d, J_{CP} = 41, C_qPPh₃), 130.8 (d, J_{CP} = 2, C_H_pPPh₃), 129.6 (d, J_{CP} = 9, C_H_mPPh₃), 127.1 (C₅), 124.6 (C₃), 104.5 (d, J_{CP} = 6, C_α- η^5 -C₅H₄CH₂OH), 76.2 (C_p- η^5 -C₅H₄CH₂OH), 76.1 (d, J_{CP} = 2, C_γ- η^5 -C₅H₄CH₂OH), 57.8 (CH₂OH), 20.8 (CH₃).

³¹P NMR [(CD₃)₂CO, δ /ppm]: 51.28 [s, PPh₃].

FTIR [KBr, cm⁻¹]: 3415 (ν_{O-H}), 3071–3050 cm⁻¹ (ν_{C-H} aromatic rings), 2922 cm⁻¹ (ν_{C-H} alkanes), 1440 ($\nu_{C=C}$ aromatic rings), 1258 cm⁻¹ (ν CF₃SO₃ counterion).

UV-vis [DMSO, λ_{max} /nm ($\epsilon \times 10^3/M^{-1}$ cm⁻¹)]: 291 (4.11), 351 (Sh), 418 (6.86), 472 (Sh).

UV-vis [CH₂Cl₂, λ_{max} /nm ($\epsilon \times 10^3/M^{-1}$ cm⁻¹)]: 287 (29.20), 345 (Sh), 417 (5.28), 470 (Sh).

Elemental analysis calc. for C₃₇H₃₄F₃N₂O₄PRuS (791.78): C, 56.1; H, 4.3; N, 3.5; S, 4.1. Found: C, 55.4; H, 4.1; N, 3.3; S, 4.0.

ESI-MS: [6-CF₃SO₃]⁺ calc. for [C₃₆H₃₄N₂OPRu]⁺: 643.15 found: 643.00.

[Ru(η^5 -C₅H₄CH₂OH)(4,4'-CH₂OH-2,2'-bipy)(PPh₃)][CF₃SO₃] (7).

Yield: 58% (62 mg). Orange single crystals were obtained from slow diffusion of n-hexane into a dichloromethane solution.

¹H NMR [(CD₃)₂CO, Me₄Si, δ /ppm]: 9.39 (broad, 2H, H₆), 8.07 (s, 2H, H₃), 7.41 (m, 3H, H_pPPh₃), 7.32 (m, 8H, H_mPPh₃ + H₅), 7.12 (t, 6H, J_{HH} = 8, H_oPPh₃), 4.91 (broad, 2H, H_p- η^5 -C₅H₄CH₂OH), 4.79 (s, 6H, CH₂OH + CH₂OH), 4.60 (broad, 2H, H_γ- η^5 -C₅H₄CH₂OH), 4.11 (m, 3H, η^5 -C₅H₄CH₂OH + η^5 -C₅H₄CH₂OH).

APT-¹³C{¹H} NMR [(CD₃)₂CO, δ /ppm]: 156.4 (C₂), 156.3 (d, J_{CP} = 2, C₆), 153.7 (C₄), 133.8 (d, J_{CP} = 11, C_H_oPPh₃), 132.8 (d, J_{CP} = 40, C_qPPh₃), 130.8 (d, J_{CP} = 2, C_H_pPPh₃), 129.3 (d, J_{CP} = 10, C_H_mPPh₃), 123.4 (C₅), 120.9 (C₃), 104.5 (C_α- η^5 -C₅H₄CH₂OH), 76.3 + 76.2 (C_p- η^5 -C₅H₄CH₂OH + C_γ- η^5 -C₅H₄CH₂OH), 62.5 (CH₂OH), 57.8 (H₅-C₅H₄CH₂OH).

³¹P NMR [(CD₃)₂CO, δ /ppm]: 51.27 (s, PPh₃).

FTIR [KBr, cm⁻¹]: 3486–3470 (ν_{O-H}), 2922–2864 (ν_{C-H} alkanes), 1440 ($\nu_{C=C}$ aromatic rings), 1261 (ν CF₃SO₃ counterion), 1234 (ν_{C-O}).

UV-vis [DMSO, λ_{max} /nm ($\epsilon \times 10^3/M^{-1}$ cm⁻¹)]: 292 (5.67), 348 (Sh), 416 (1.02), 478 (Sh).

UV-vis [CH₂Cl₂, λ_{max} /nm ($\epsilon \times 10^3/M^{-1}$ cm⁻¹)]: 291 (4.11), 351 (Sh), 418 (6.86), 472 (Sh).

Elemental analysis calc. for C₃₇H₃₄F₃N₂O₆PRuS (823.78): C, 53.9; H, 4.1; N, 3.4; S, 3.9. Found: C, 52.3; H, 4.0; N, 3.1; S, 3.0.

ESI-MS: [7-CF₃SO₃]⁺ calc. for [C₃₆H₃₄N₂O₃PRu]⁺: 675.14 found: 675.01.

X-ray structure analysis

The X-ray intensity data were measured on a D8 QUEST ECO three-circle diffractometer system equipped with a Ceramic X-ray tube (Mo K α , λ = 0.71076 Å) and a doubly curved silicon crystal monochromator, using APEX3⁴⁶ software package. The frames were integrated with the Bruker SAINT.⁴⁷ Data were corrected for absorption effects using the Multi-Scan method (SADABS).⁴⁸ The structures were solved and refined using the Bruker SHELXTL.⁴⁹

The crystallographic data as well as details of the structure solution and refinement procedures are reported in ESI.† CCDC 2042407–2042412† contain the supplementary crystallographic data for this paper.

Stability studies

For the stability studies, all complexes were dissolved in 100% DMSO and a sample containing each compound in 2% DMSO/DMEM at 90–180 μ M was prepared. Their electronic spectra were recorded in the range allowed by the solvent mixture at set time intervals. Samples were stored at room temperature and protected from light between measurements. The vari-



ation percentage between measurements were calculated by the following expression:

$$\% \text{variation} = \frac{\text{Abs}(\lambda, t_{mix}) - \text{Abs}(\lambda, t_{mix} + i)}{\text{Abs}(t_{mix})} \times 100$$

Cell lines

Human non-small cell lung cancer cells A549, NCI-H228, Calu-3, NCI-H1975 were purchased from ATCC (Manassas, VA). Cells were grown in RPMI-1640 medium, supplemented with 10% v/v FBS and 1% penicillin–streptomycin, at 37 °C, 5% CO₂, in a humidified atmosphere.

Immunoblotting

Cells were rinsed with ice-cold lysis buffer (50 mM Tris, 10 mM EDTA, 1% v/v Triton-X100), supplemented with the protease inhibitor cocktail set III (80 µM aprotinin, 5 mM bestatin, 1.5 mM leupeptin, 1 mM pepstatin; Calbiochem, San Diego, CA), 2 mM phenylmethylsulfonyl fluoride and 1 mM Na₃VO₄, then sonicated (10 bursts of 1 s, amplitude 40%; Hielscher UP200S, Ultrasound Sonicator, GmbH, Teltow, Germany) and centrifuged at 13 000g for 10 min at 4 °C. 20 µg protein extracts were subjected to SDS-PAGE and probed with the following antibodies: anti-P-gp (1 : 250, rabbit polyclonal #sc-8313, Santa Cruz Biotechnology Inc., Santa Cruz, CA), anti-MRP1 (1 : 500, mouse clone MRPm5, Abcam, Cambridge, UK), anti-β-tubulin (1 : 1000, mouse clone D10, Santa Cruz Biotechnology Inc.). This procedure was followed by the exposure to a peroxidase-conjugated secondary antibody (Bio-Rad Laboratories). The membranes were washed with Tris-buffered saline-Tween 0.1% v/v solution, and the proteins were detected by enhanced chemiluminescence (Bio-Rad Laboratories).

Cytotoxic activity

Cells were seeded in 96 well plates. In a first experimental set, cells were incubated for 72 h with DMSO as a solvent or cisplatin at the following concentrations: 1 nM, 10 nM, 100 nM, 1 µM, 10 µM, 100 µM. In a second experimental set, cells were incubated with DMSO or each compound at the following concentrations: 1 nM, 10 nM, 100 nM, 1 µM, 10 µM, 100 µM. In the third experimental set, cells were incubated for 72 h with 1 µM of the selected compound plus cisplatin at the following concentrations: 1 nM, 10 nM, 100 nM, 1 µM, 10 µM, 100 µM. Cell viability was evaluated using the WST-1 assay (Sigma-Merck), as per manufacturer's instructions, using a Packard EL340 microplate reader (Bio-Tek Instruments, Winooski, VT). The absorbance units of the untreated cells were considered 100%; the absorbance units of the other experimental conditions were expressed as percentage *versus* untreated cells. IC₅₀ and IC₂₅ were defined as the concentrations of cisplatin of compound that killed 50% and 25% cells, respectively. The Resistance Factor was the ratio between IC₅₀ of cisplatin alone/IC₅₀ of cisplatin + compound.

ATPase activity

The P-gp ATPase activity was measured in membrane vesicles as described previously.³⁹ Cells were washed with Ringer's solution (148.7 mM NaCl, 2.55 mM K₂HPO₄, 0.45 mM KH₂PO₄, 1.2 mM MgSO₄; pH 7.4), lysed on crushed ice with lysis buffer (10 mM Hepes/Tris, 5 mM EDTA, 5 mM EGTA, 2 mM dithiothreitol; pH 7.4) supplemented with 2 mM phenylmethylsulfonyl fluoride, 1 mM aprotinin, 10 µg ml⁻¹ pepstatin, 10 µg ml⁻¹ leupeptin, and subjected to nitrogen cavitation at 1200 psi for 20 min. Samples were centrifuged at 300g for 10 min in the pre-centrifugation buffer (10 mM Tris/HCl, 25 mM sucrose; pH 7.5), overlaid on a sucrose cushion (10 mM Tris/HCl, 35% w/v sucrose, 1 mM EDTA; pH 7.5) and centrifuged at 14 000g for 10 min. The interface was collected, diluted in the centrifugation buffer (10 mM Tris/HCl, 250 mM sucrose; pH 7.5) and subjected to a third centrifugation at 100 000g for 45 min. The vesicle pellet was re-suspended in 0.5 ml centrifugation buffer and stored at -80 °C until the use, after the quantification of the protein content. 1 mg of total protein were immunoprecipitated with the anti-P-gp or anti-MRP1 antibody. 100 µg of each immunopurified protein was incubated for 30 min at 37 °C with 50 µl of the reaction mix (25 mM Tris/HCl, 3 mM ATP, 50 mM KCl, 2.5 mM MgSO₄, 3 mM dithiothreitol, 0.5 mM EGTA, 2 mM ouabain, 3 mM NaN₃; pH 7.0). The reaction was stopped by adding 0.2 ml ice-cold stopping buffer (0.2% w/v ammonium molybdate, 1.3% v/v H₂SO₄, 0.9% w/v SDS, 2.3% w/v trichloroacetic acid, 1% w/v ascorbic acid). After 30 min incubation at room temperature, the absorbance of the phosphate hydrolyzed from ATP was measured at 620 nm, using a Packard EL340 microplate reader. The absorbance was converted into nmoles hydrolyzed phosphate (Pi) per min per mg proteins, according to the titration curve previously prepared.

Statistical analysis

All data in the text and figures are provided as means ± SD. The results were analyzed by a one-way analysis of variance (ANOVA) and Tukey's test. *p* < 0.05 was considered significant.

Conflicts of interest

There are no conflicts to declare.

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