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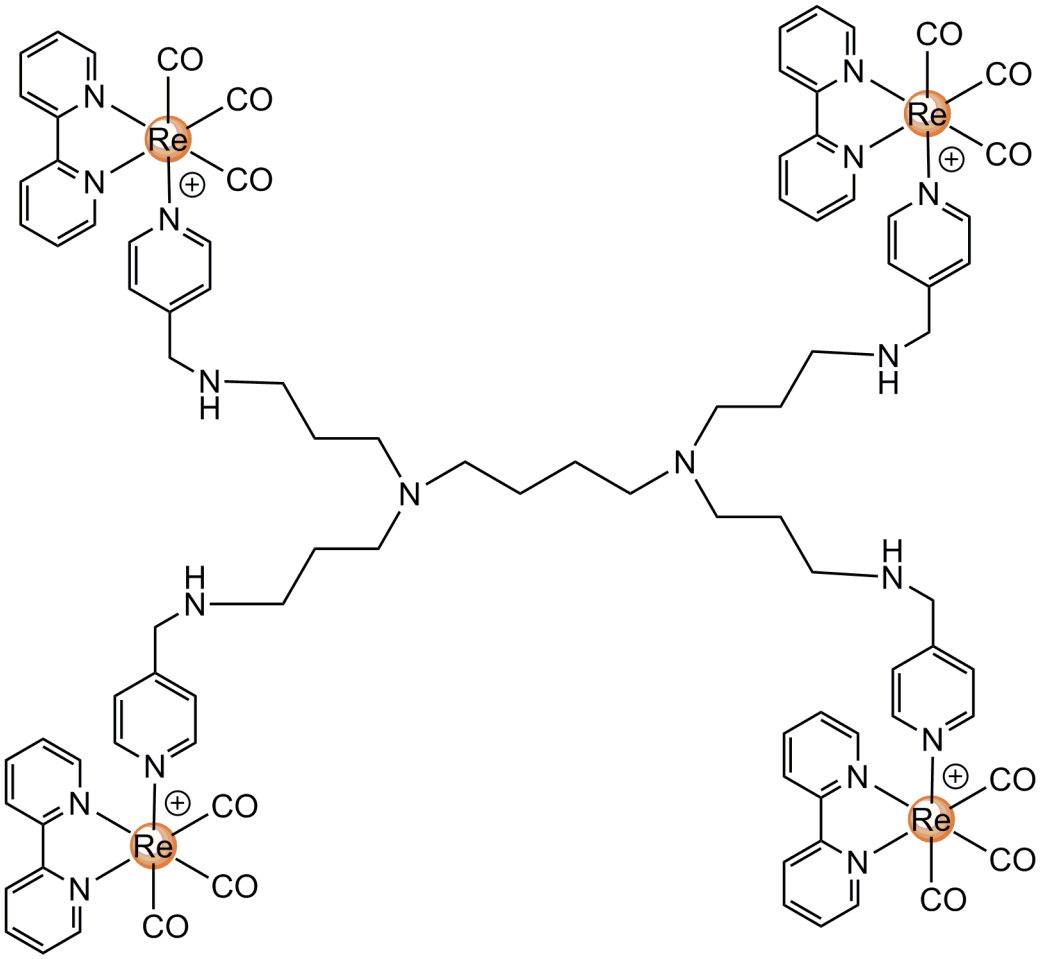

Synthesis and antitumour evaluation of mono- and multinuclear [2+1] tricarbonylrhenium(I) complexes

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Abstract

A series of mono- and multinuclear [2 + 1] Re(I) tricarbonyl complexes were synthesised and evaluated as antiproliferative agents against the epithelial carcinoma (A431), colon carcinoma (DLD-1) and ovarian cancer (A2780) tumour cell lines and a healthy fibroblast (BJ) cell line. The compounds have moderate to good activity against the tumour cell lines, with the trinuclear and tetranuclear complexes showing selective cytotoxicity towards the tumour cell lines. The Re(I) complexes were found to influence programmed cell death mechanisms *in vitro*. They were able to inhibit the soluble form of the Fas receptor in malignant cells, allowing the Fas domain to receive the apoptotic signal through an extrinsic pathway. The complexes augmented the pro-apoptotic Bax-α concentrations, with the tetranuclear complex more superior at modulating the Bax-α molecular target in all tested cell lines, which influenced its cell growth inhibitory activity. The tetranuclear complex has the best activity against all tumour cell lines.

1. Introduction

Since the discovery of the anticancer activity of the metallodrug, cisplatin and related platinum-based drugs, metal-based complexes have been at the forefront of rational cytotoxic drug design [1]. Metal-based complexes possess notable physicochemical properties when compared to their purely organic counterparts, many of which are somewhat unique and of interest in the context of medicinal chemistry. These interesting properties are due to the inclusion of a metal which allows structural diversity, redox properties, and ligand exchange, all of which can be advantageous in a biological setting [1]. However, cisplatin and related Pt complexes display physiological side-effects, such as toxicity towards healthy, non-tumourigenic cells, and inherent or developed chemoresistance [2-4]. New metal-based complexes are therefore needed to overcome the failings observed in current anti-cancer drug regimens and to meet the targeted drug discovery challenges [5].

Rhenium carbonyl complexes have been shown to display a wide range of biological properties, including anticancer [6-13], antimalarial [14] and antitrypanosomal [15] properties. Most noteworthy are Re(I) tricarbonyl complexes that show potent antiproliferative activity against a variety of cancerous cell lines. In particular, reports of a series of Re(I) tricarbonyl complexes have low micromolar IC_{50} values (1 – 5 μM) against HeLa cells [7, 16], while [2 + 1] Re(I) tricarbonyls show potent activity (IC_{50} = 2 – 10 μM) against the breast cancer MDA-MB-468 [10] and HeLa [8, 12, 17] cell lines. The Re(I) tricarbonyl complexes are also capable of triggering apoptosis in tumour cells [18] as a possible mechanism of action. It is therefore important to elucidate the *in vitro* cell death mechanisms by studying the interaction with intrinsic and extrinsic apoptotic targets. Two members of the Bcl-2 protein family of value to this study are the pro-apoptotic Bax-α (B cell leukemia/lymphoma-2 associated X), and the anti-apoptotic Bcl-xL (B-cell lymphoma-extra large) proteins. These molecules were first studied in lymphoma and later identified in almost all cell types, including solid tumours and normal cells. The proteins exert antagonistic (pro-apoptotic *versus* prosurvival) effects on intrinsic mitochondrial programmed cell death pathways, but also on invasion and metastasis [19] in tumour cell populations. Another participant in cell death mechanism is the Fas (first apoptosis signal) receptor, a member of the large group of tumour necrosis factor receptors on the cell membrane. Together with its ligand, FasL, is involved in the extrinsic regulation of cell death in tumours via the Fas/FasL pathway. This pathway is targeted by metal-based chemotherapy agents which act against the colon [20], ovarian [21] or epidermal [22] carcinoma.

Several research groups have revealed that using the [2 + 1] mixed-ligand approach, consisting of a metal ion with one bidentate ligand and one monodentate ligand, is a useful tool in the art of rational drug design. Mundwiler *et al*. described the [2+1] approach as comprising a bidentate ligand (displacing two of the aqua ligands of a *fac*-$[Re(CO)_3(OH_2)_3]^+$ entity) which can influence the overall lipophilicity of the tricarbonyl complex, whilst the third [+1] aqua site is occupied by a monodentate ligand (acting as a linker to a biomolecule) [20, 21]. The advantage of a [2+1] mixed-ligand complexation is that the apical [+1] site can be changed to modulate the physical characteristics of a compound, such as solubility, lipophilicity, and permeability. Cationic bipyridyl Re(I) tricarbonyl complexes display great potential in this regard, particularly, in the field of anticancer research [10, 12, 23]. With this approach, either of the two ligands can be modified to achieve the desired properties, such as introducing water-solubilising groups on the 2,2'-bipyridyl moiety. It is important to note that Re(I) tricarbonyl diimine complexes have been studied extensively as potential electrochemiluminescent probes, based on their lowest excited state properties and high quantum emission efficiencies from metal-to-ligand charge-transfer excited state.[17, 24, 25]

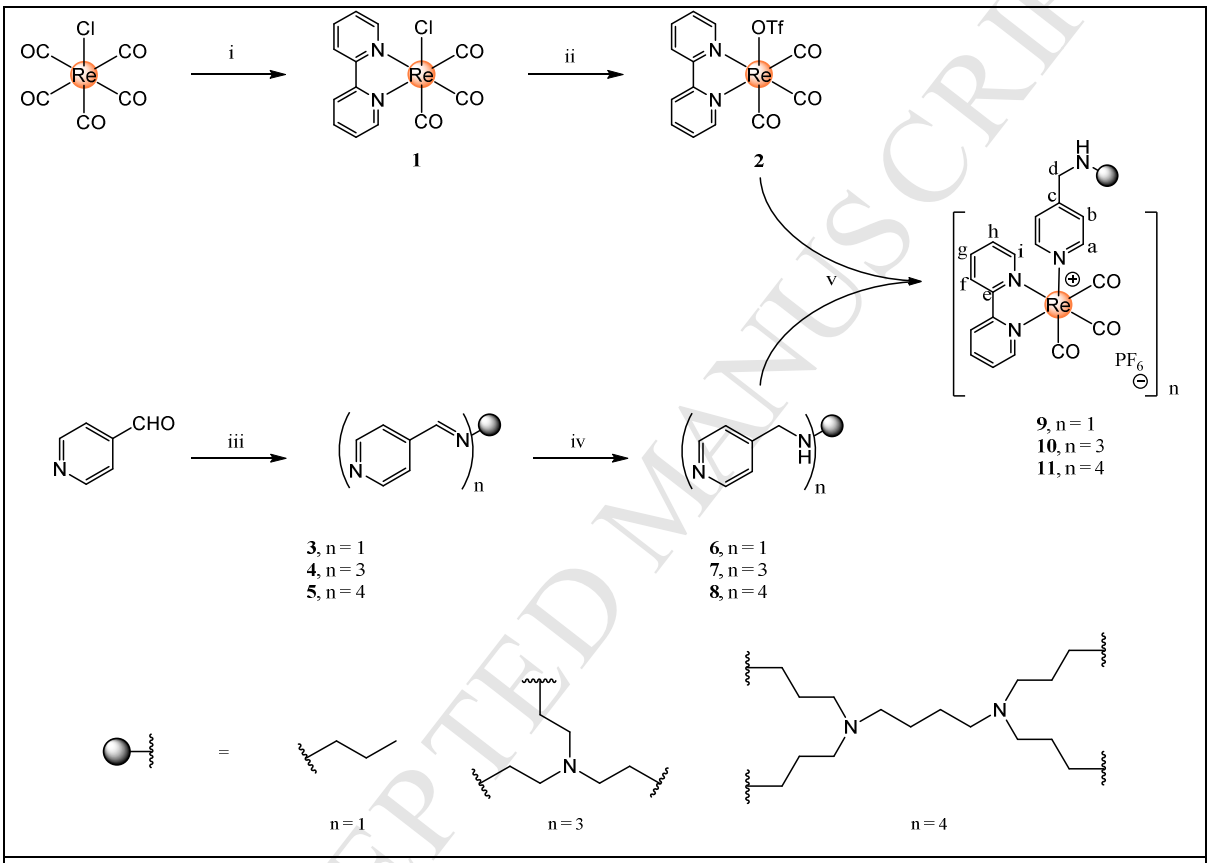
In this work, we designed, synthesised and characterised three different pyridine-based ligands with $[Re(bpy)(CO)_3]$ to yield mono-, tri- and tetranuclear [2 + 1] complexes. The dendritic nature allows for the exploitation of the multivalent effect exhibited by dendrimers, which may result in improved site-specific drug delivery and reduced systemic exposure [26, 27]. The complexes were evaluated *in vitro* as potential antitumour agents against A431 (epithelial carcinoma), DLD-1 (colon carcinoma), and A2780 (ovarian carcinoma) cancer cell lines, in addition to normal fibroblast cells (BJ). The mechanism of cell death was assessed quantifying their capacity to trigger apoptosis, by measuring the intracellular level of Bax-α and Bcl-xL, and by evaluating the influence of the complexes on extracellular Fas/FasL signalling.

2. Results and discussion

2.1 Synthesis and characterisation

Metal precursor **1** and ligand precursors **3-7** were prepared following published literature methods [28-31]. After a successful Schiff-base condensation reaction, ligands **6**, **7** and **8** were prepared (from **1-3** respectively) via reduction of the corresponding ligand precursors using sodium borohydride in methanol (Scheme 1). The corresponding complexes **9**, **10** and **11** were prepared in a one-pot reaction by first abstracting the halide using silver triflate,

followed by addition of the corresponding ligand in the appropriate stoichiometric ratio, to afford the corresponding [2 + 1] complex. The rhenium(I) complexes were isolated as the hexafluorophosphate salts through an exchange metathesis reaction using NH_4PF_6. The complexes **9** – **11** were isolated in very low yields, likely due to the need to use the metal precursor in excess to ensure functionalisation of all dendritic arms, and in part, due to the similar solubilities of the metal precursor and the products. The complexes are air-stable, and soluble in DCM, methanol, ethanol, acetone, and DMSO.



Scheme 1. (i) 2,2'-bipyridyl, toluene, 110 °C, 2 h. (ii) AgOTf, DCM, room temperature, 3 h. (iii) n-propylamine, tris-(2-aminoethyl)amine or DAB-AM-4, DCM, room temperature, 24 h. (iv) $NaBH_4$, MeOH, room temperature, 24 h. (v) DCM, 24 h, followed by addition of NH_4PF_6 and another hour of stirring.

The new cationic complexes **9** – **11** were fully characterised using standard analytical and spectroscopic techniques, such as ^{1}H, $^{13}C\{^{1}H\}$ and $^{31}P\{^{1}H\}$ NMR, infrared spectroscopy, high-resolution electrospray ionisation mass spectrometry, and elemental analysis. The ^{1}H NMR spectra of complexes **9** – **11** show similar signals for the aromatic peaks, notably with the main differences being in the aliphatic signals of the respective cores. Integration of the ^{1}H NMR spectra of complexes **9** – **11** show that the bipyridyl moiety has been added to the

ligand in the desired ratio. The characteristic peak attributed to the picolyl methylene protons (H_d) appear at *ca.* δ 3.80 ppm and display no significant shift upon coordination, suggesting that coordination occurs *via* the pyridyl nitrogen and not the secondary amine nitrogen. A downfield shift of the aromatic pyridyl signals (H_b) from *ca.* δ 7.30 ppm to *ca.* δ 7.43 ppm upon complexation further confirm the bonding modes of the complexes. The $^{13}C\{^1H\}$ NMR spectra of the complexes **9** – **11** all display signals for the carbonyls in the range of 190 – 196 ppm, aromatic carbon signals in the range of 120 – 160 ppm, and aliphatic signals in the range of 10 – 55 ppm. The infrared spectra show a distinct shift of the aromatic C=N stretching band from 1600 cm^{-1} to 1619 cm^{-1} upon coordination. This shift to higher wavenumbers is due to backdonation from the Re(I) centre to the pyridyl ring, increasing electron density in the C=N bond. This confirms coordination of the Re(I) centre to the ligand via the pyridyl nitrogen. In addition, high-resolution ESI-mass spectra for the complexes **9** – **11** confirm the presence of the desired structures. Peaks for complexes **9** and **10** correspond to the molecular ion in the absence of the PF_6^- counter ions ($[M\text{-}PF_6]^+$ and $[M\text{-}3PF_6]^{3+}$, respectively), *m/z* = 577.12 and 566.07, respectively. The tetranuclear complex displays a peak for the fragment $[M\text{-}CO\text{-}4PF_6]^{4+}$ with an *m/z* value of 590.08. Elemental analysis was used to confirm the structural integrity and purity of the complexes. All results were within reasonable limits, confirming high purity of the samples. Several solvent molecules were present in the sample of complex **11**, as confirmed by ^1H NMR, as dendrimers are known to incorporate solvent molecules and are notoriously difficult to dry [32].

2.2 Antiproliferative studies

The Re(I) tricarbonyl complexes **9**, **10** and **11** were tested for their *in vitro* cytotoxicity against epithelial carcinoma (A431), colon carcinoma (DLD-1) and ovarian cancer (A2780) cancerous cell lines, in addition to normal fibroblast cells (BJ). The MTT assay was used to determine the cytotoxicity of the compounds. The extent of inhibition is displayed as an IC_{50} value, which is defined as the concentration required to inhibit cell growth to half. In parallel, three standard platinum-based cytotoxic drugs were tested, in the same concentration range as the Re(I) complexes; the results are presented in Table 1.

Table 1

IC_{50} values of Re(I) complexes **9**, **10** and **11** and reference platinum-based drugs against cancerous (A431, DLD-1, A2780) and normal (BJ) cell lines

Compound	n[a]	IC_{50} (μM)[b]			
		A431	DLD-1	A2780	BJ
9	1	57.84 ± 3.67	17.85 ± 1.79	26.61 ± 3.66	18.97 ± 1.76
10	3	46.64 ± 21.89	15.04 ± 2.20	36.45 ± 3.54	58.82 ± 7.65
11	4	14.09 ± 2.23	10.18 ± 0.47	6.38 ± 1.18	17.69 ± 1.50
Cisplatin[c]	1	17.17± 2.97	58.85± 6.55	37.30 ± 5.69	27.05 ± 4.14
Oxaliplatin[c]	1	106.61 ± 10.16	93.58 ± 14.79	81.57 ± 5.18	86.50 ± 14.53
Carboplatin[c]	1	138.73 ± 15.09	175.70 ± 13.01	101.13 ± 19.05	120.07 ± 21.99

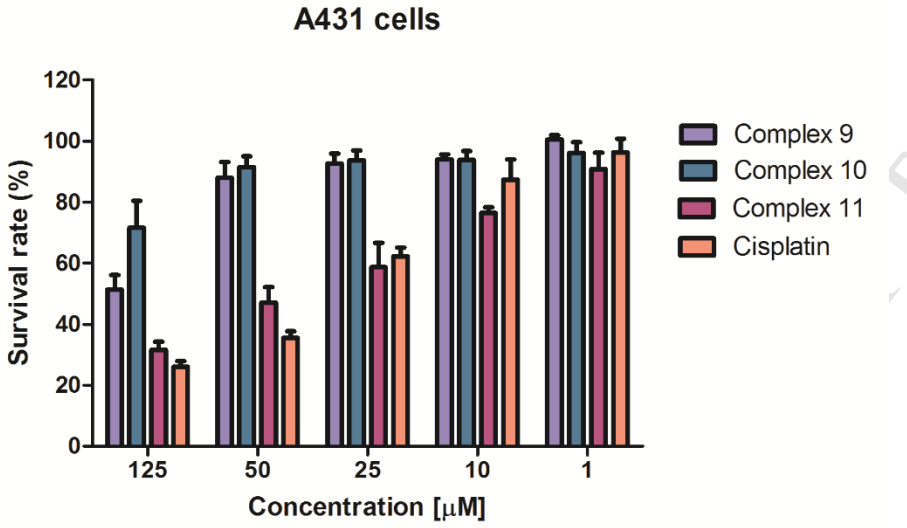
[a] *number of metal centres per molecule*

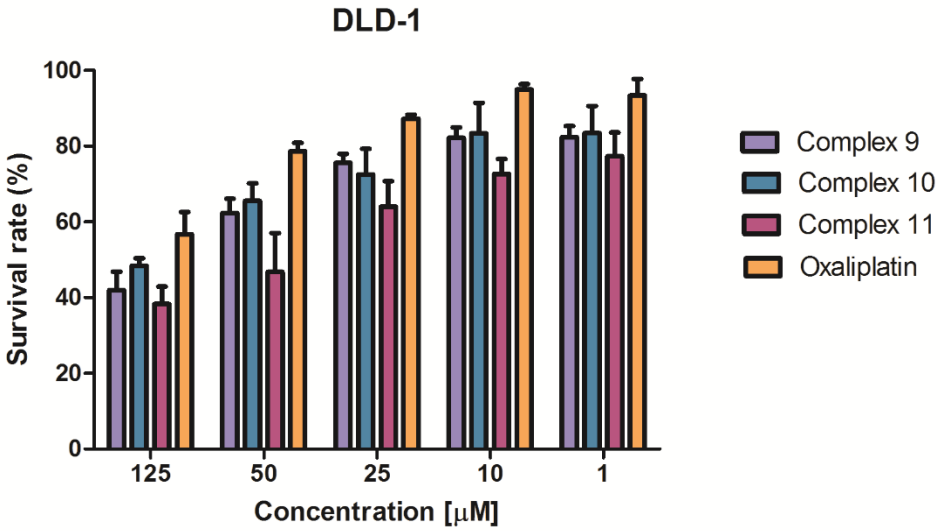
[b] *IC_{50} value ± standard error*

[c] *Standard clinically approved Pt(II) drugs*

The tested complexes show moderate to good cytotoxicity against the A2780, A431 and DLD-1 tumour cell lines, and are toxic to some extent against the normal BJ cells. Complex **11** show the best inhibitory effects against all the tested cell lines. No selectivity is observed in the case of complex **9**, as the IC_{50} values are similar against the tumour and normal cells. Complexes **10** and **11** showed selectivity, as significantly higher IC_{50} values were observed against the healthy BJ cell line, which indicates a lower toxicity towards normal cells. In the DLD-1 cells, complexes **10** and **11** revealed a good therapeutic window. The selectivity was moderated in A431 cells, and complex **11** was particularly selective against the A2780 cells as well. In general, the complexes are the most effective inhibitors against the colon carcinoma cell line (DLD-1) and have the least potent cytotoxicity against the epithelial carcinoma cell line (A431). In the treated DLD-1 tumour cell population, there is a discernible relationship between the IC_{50} values and the number of Re metal centres in the complex, i.e. complex **11** is the most cytotoxic, followed by complex **10**, while complex **9** is the least cytotoxic. In the A431 cell line, complex **10** showed a higher standard deviation from the best-fit IC_{50} value calculated by the software, indicating an inferior dose-relationship. In the A2780 cells and normal fibroblasts, complex **10** was not aligned with the correlation tendency between the IC_{50} values and the number of metal centres present per molecule, while complexes **9** and **11** seem to follow a trend; complex **11** with four metal centres having

a higher toxicity in A2780 as well. Both complexes **10** and **11** are more selective against normal BJ cells, in comparison with the standard drugs oxaliplatin and carboplatin.





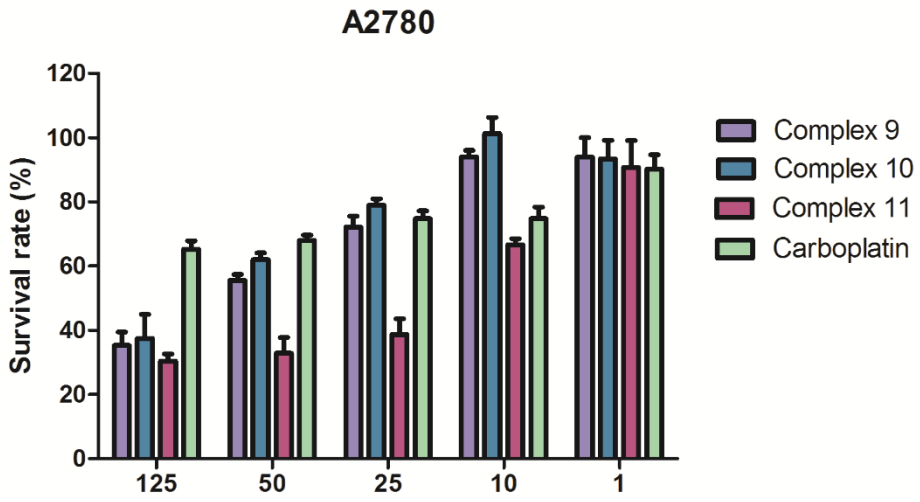


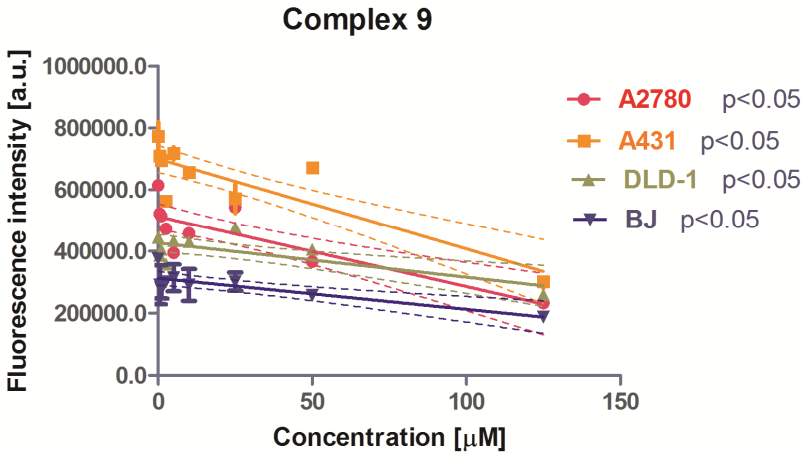
Fig. 1. Comparison between the cell survival rates of tumor cells subjected to different concentrations of complexes **9**, **10**, **11** and the reference platinum-based clinical drugs. For each cell line, the activity of the Re complexes was compared with the same concentration range of the platinum drug; oxaliplatin is the Pt(II) chemotherapeutic drug of choice in colorectal cancer, carboplatin in ovarian cancer, and cisplatin is the most used metal-based drug in epidermoid carcinoma.

In A431 epidermoid carcinoma, the parallel of survival rates at different concentrations proved once again that the toxicity of Complex 11 was comparable with cisplatin (Figure 1), and this most visible at higher concentrations. Despite complexes 9 and 10 being less toxic than cisplatin, *in vitro* studies reveal that they show higher toxicity than the platinum drugs oxaliplatin and carboplatin (Table 1). In the DLD-1 colon carcinoma, all complexes trigger cell death more than oxaliplatin, at each studied concentration, and this tendency was observed for the comparison of 9-11 with the other two standard drugs. For the A2780 ovarian carcinoma, above the concentration of 10 μM, carboplatin toxicity exceeds the inhibitory capacity of complex 11, and above 50 μM became more active than both complexes 9 and 10 as well.

The *in vitro* activity of the three platinum drugs was not consistent; there are significant differences between the IC_{50} values. This phenomenon was largely described in the literature [33,34] and although the antitumor activity of the rhenium compounds operates via an alternate mechanism compared to platinum-based drugs, the magnitude of their antiproliferative activity was not always convergent in the cell populations that originated from different tissues, namely, colon, ovary and epidermal. Nonetheless, in comparison with

the standard metal-based drugs, the *in vitro* activity of the compounds was remarkable, even in the resistant A431 cell line. The dose-effect relationship points towards the prodrug potential of complex **9** and especially complex **11**, while for complex **10** the dose/structure-activity relationship was less adequate.

In tumor cells, the ABCB1 and ABCG2 multidrug transporter molecules are responsible for the efflux of the drugs from the cell, which causes resistance to chemotherapeutic agents. These are widely expressed on many tumour populations, including A2780 [32], DLD-1 [35]) and A431 [36]. Nonetheless, there are some significant differences between the cell lines used in our experiment. For the A2780 cell line, there is a characteristic presence of ABCB4 and ABCA8 transporters [32], therefore these cells are more susceptible to a stronger drug resistance. For the A431 epidermal carcinoma, the role of ABCC6 multidrug transporter is also important [37], whilst in normal BJ cells, the above-mentioned transporter's role was not mentioned in previous studies. The differences between the multidrug resistance genetic patterns might allude to why the number of the Re metal centres is not directly related to the antiproliferative effect in the A2780 and BJ cell lines.



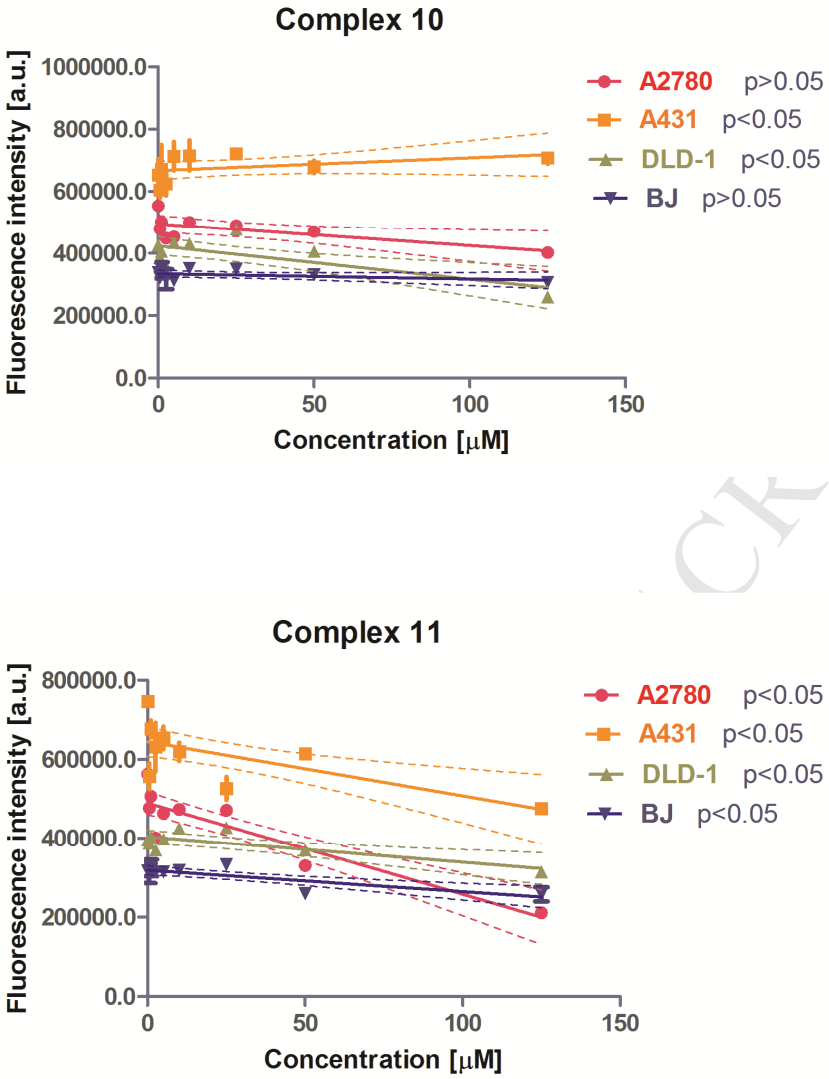


Fig. 2. The influence of Re compounds against the metabolic activity of tumor cells. The linear regression of dose-effect relationship data (provided by the GraphPad prism software) shows a decrease in the cells metabolism and proliferative capacity, where the hill slopes were negative in the 95% confidence interval; the dashed line indicates the standard error of the linear regression curve.

The metabolic rate of the treated cells, quantified using the Alamar Blue method, was proven to be dose-dependent and significantly decreased by complexes **9** and **11** in all cell lines (Fig. 2). Complex **10** was capable to slow down the metabolism of DLD-1 and A2780 cells but does not significantly influence the A431 and BJ populations. In these two cell lines the cytotoxicity of **10** was also less pronounced.

2.3 Effect of Re(I) Complexes on Cell Death Mechanisms

Complexes **9**, **10** and **11** influence the intracellular level of the two molecules implicated in programmed cell death pathways: the pro-apoptotic Bax-α and the pro-survival Bcl-xL (Fig. 3). The *in vitro* concentration of the pro-apoptotic Bax-α increases significantly in all tumour types following a 24-hour treatment with complex **11** ($p = 0{,}0185$ in DLD-1 cells, $p = 0{,}0050$ in A2780 cells and $p = 0{,}0148$ in A431 cells). In the normal BJ cells, no significant variation was observed. The anti-apotic Bcl-xL levels are not significantly influenced by complexes **9** and **10**.

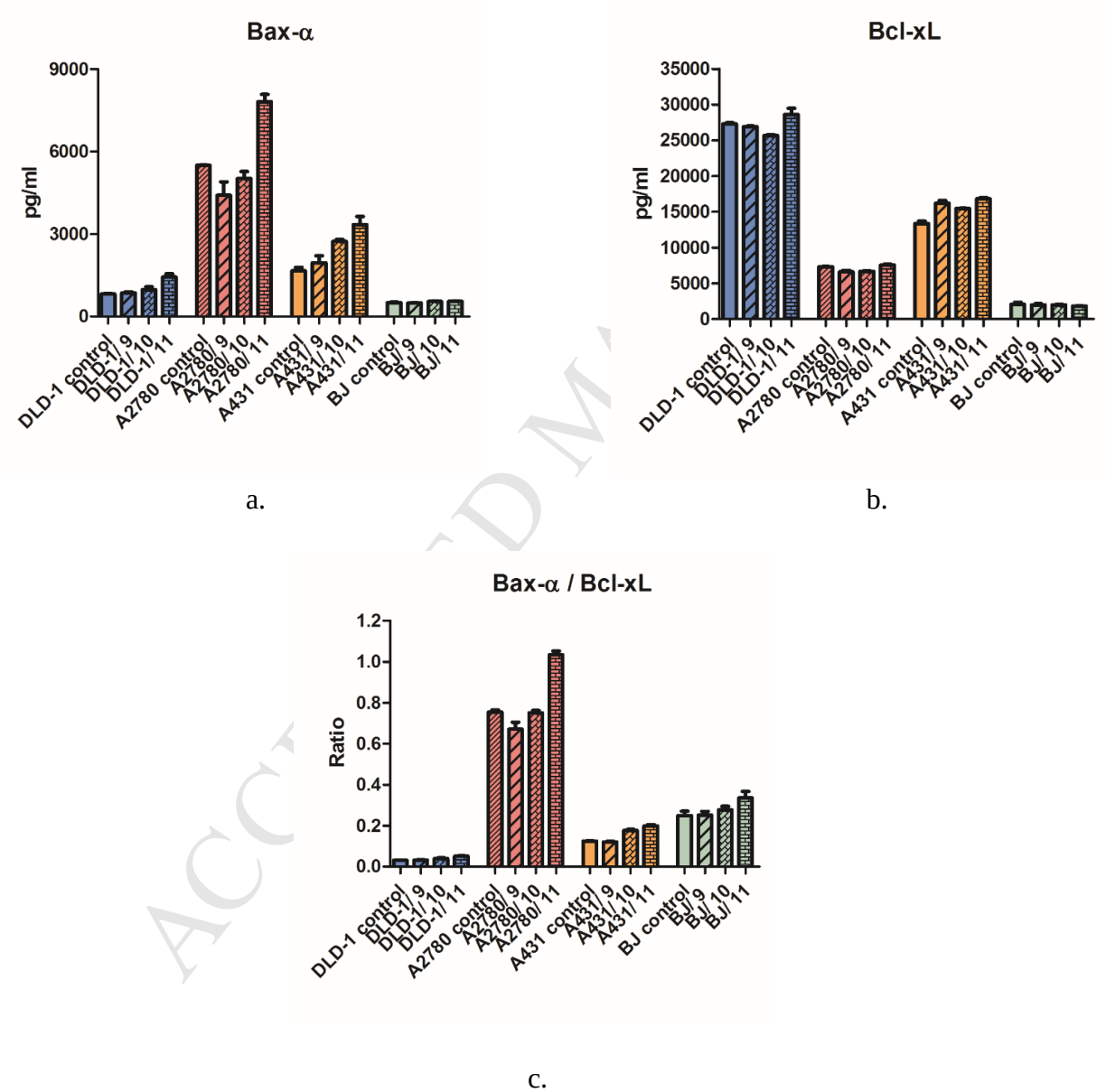


Fig. 3. Complexes **9**, **10** and **11** can modulate the apoptotic signal transduction through the soluble Bax-α and the anti-apoptotic Bcl-xL molecules. (a) The soluble pro-apoptotic Bax-α level inside the treated cells. (b) The intracellular anti-apoptotic Bcl-xL level. (c) The proportion between the two molecules, expressed as a ratio of Bax-α/Bcl-xL.

The anti-apoptotic Bcl-xL concentration was not modified significantly (one-way analysis of variance, $p > 0.05$), except in the A431 cell line where treatment with complexes **9**, **10** and **11** results in an increase of Bcl-xL (Fig. 3) at approximately the same intensity (one-way analysis of variance, Bonferroni post-test, $p = 0.0026$). The Bax-α/Bcl-xL ratio, a reliable apoptosis indicator, is higher following treatment with complex **11**, as for the untreated control cells, in all cell lines, except in the case of the normal BJ cells. An elevated Bax-α/Bcl-xL ratio indicates a sensitivity to treatment and a good transduction of the apoptotic streamline from extrinsic apoptotic signals such as the Fas/FasLigand towards mitochondrial functions [35].

It has been shown that overexpression of the anti-apoptotic Bcl-2 family does not influence the Re complexes activity [36], and these results confirm this assertion. Rhenium accumulates preferentially in the mitochondria [37] and lysosomes, and therefore the secretion of mitochondrial proteins which regulates the apoptotic processes will be strongly influenced by rhenium complexes. As described previously in the literature [37], the Re(I) tricarbonyl [2 + 1] multinuclear complexes are more cytotoxic than their mononuclear counterparts. This is very likely to be reflected in the apoptosis regulation. Bcl-xL is an inhibitor of the cell cycle progression and can arrest the cell cycle before the mitotic phase [38]. In A431 cells treated with complexes **9**, **10** and **11**, the lowest cytotoxicity values of the studied Re(I) series were recorded, and an increase of Bcl-xL secretion observed. Therefore, the augmentation of Bcl-xL observed in the A431 cells points to the assumption that, in this case, the cell death mechanism was triggered through a non-apoptotic pathway.

2.4 Influence of Re(I) Complexes on Fas/FasL Signalling

The Fas (First apoptotic factor) membrane protein or tumour necrosis factor receptor 6, is a receptor implicated in cell differentiation, proliferation and apoptosis. Fas receptors have some soluble isoforms, which lack the transmembrane domain. They bind the Fas ligand (FasL) before it reaches the Fas receptor on the cell membrane, and thereby exert an inhibitory effect on the Fas/FasL signalling pathway and inhibit apoptosis [39]. We measured the soluble form of both Fas and its ligand FasL (Fig. 4). The secreted Fas receptor level showed a decreasing tendency in DLD-1, A2780 and A431 tumour cell lines, following a 24-hours exposure to complexes **9**,**10** and **11**. The decline was statistically significant (one-way analysis of variance, in the 95% confidence interval, p value 0.0008

for DLD-1, 0.0109 for A431 and 0.0138 for A2780). In normal BJ cells the values are also reduced, but not significantly ($p > 0.5$).

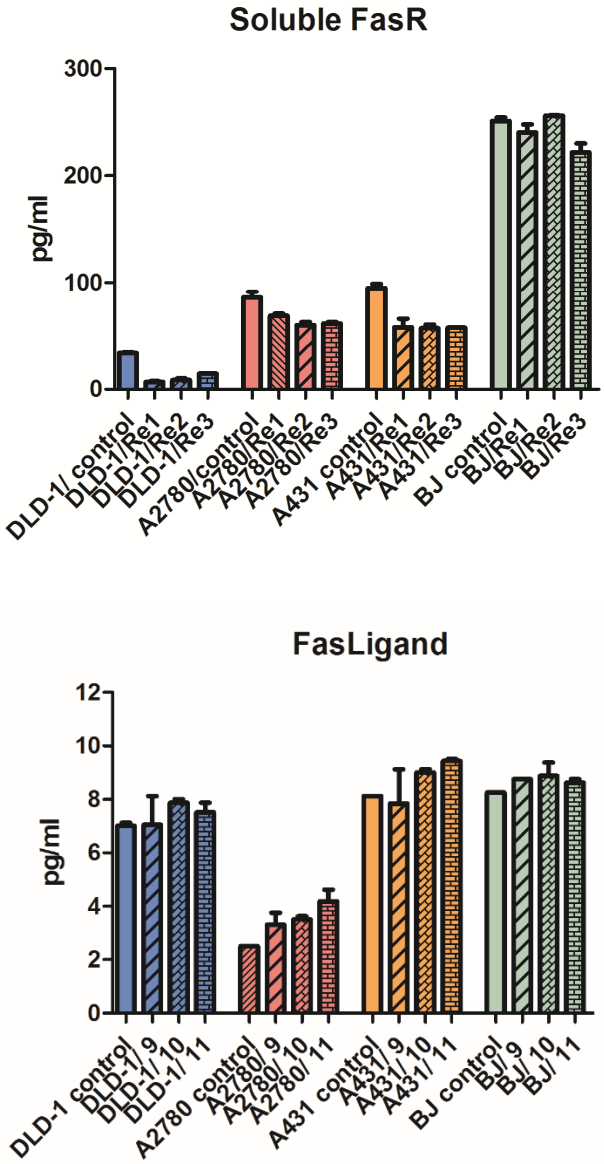


Fig. 4. The modulation of soluble FasReceptor and FasLigand concentration following the treatment of DLD-1, A2780, A431 tumor cells and BJ normal cell line with complexes **9**, **10** and **11**.

The Fas receptor is ubiquitously expressed in normal or tumour human cells, whereas its ligand, FasL, is expressed mainly in activated immune players such lymphocytes. The soluble FasL levels were very low in the studied cells, close to 5 pg, the sensitivity limit of

the reagent kit we used. The variations were not significant following the treatment with the rhenium compounds in any of the studied cell lines.

The Fas-mediated extrinsic apoptotic pathway can crosstalk to the intrinsic pathway through caspases, cleavage of BID (a BH3-ONLY member of the BCL2 family of proteins), which results in Bcl family protein cleavage, and the activation of Bax proteins in mitochondria. In the DLD-1 and A2780 cells, no correlation was found between the level of soluble Fas receptor and the other molecules linked to apoptosis: Bax-α, Bcl-xL or their ratio. Instead, in A431 cells, the Fas receptor modulation was inversely correlated to Bcl-xL (Pearson correlation, r value -0.925; p value 0.0374). Therefore, the cell death signal exerted through Fas receptor can be counterbalanced by the increase of Bcl-xL, indicating once again that in this cell line, the cell death mechanisms were directed in a non-apoptotic manner. In the DLD-1 and A2780 cell populations, the Bax/Bcl signalling and the soluble Fas receptor decline or converge to apoptosis induction, with each Re(I) treatment.

3. Conclusions

Three new mono- and multinuclear Re(I) tricarbonyl [2 + 1] complexes (**9** – **11**) were prepared and characterised using various spectroscopic and analytical techniques. Complexes **9** – **11** were evaluated for their antiproliferative activity against three tumour cell lines and one normal cell line. All of the complexes displayed moderate biological activity against the tested cancer cell lines (IC_{50} < 60 μM). The tetrameric complex **11** displayed the best activity against all cancer cell lines, as well as significant selectivity towards inhibiting malignant over normal cell lines, while complex **10** needs further structural improvements before further testing. *In vitro*, the rhenium complexes **9**, **10** and **11** can influence programmed cell death mechanisms in three different types of tumors. The Re complexes inhibit the soluble form of the Fas receptor, leaving the membrane Fas domain available to capture the apoptotic signal. The cells are driven towards cell death through pro-apoptotic Bax-α augmentation, with the influence on anti-apoptotic Bcl-xL being less important. In this study, complex **11** was the more prominent Bax-α modulator in all cell lines, which is linked to its cell growth inhibitory activity.

4. Experimental

4.1 General information

All solvents, reagents and deuterated solvents were purchased from Sigma-Aldrich and used without further purification. Metal precursor (**1** [28]) and ligands (**3** [29], **4** [30], **5** [31], **6** [29] and **7** [30]) were prepared using literature or modified literature procedures.

4.2 Instruments

All reagents were purchased from Sigma-Aldrich and were used as received unless otherwise stated. Solvents were purchased from KIMIX and were used as received. Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Topspin GmbH (^{1}H at 400.22 MHz, ^{13}C{^{1}H} at 100.65 MHz, ^{31}P{^{1}H} at 162.01 MHz) or a Varian Mercury 300 (^{1}H at 300.08 MHz) spectrometer, with a Bruker Biospin GmbH casing and sample injector at 30 °C. Tetramethylsilane was used as the internal standard for the chemical shift reports. Infrared (IR) absorption spectra were recorded using a Perkin-Elmer Spectrum One FT-IR spectrometer using attenuated total reflectance (ATR). Mass spectrometry was carried out using a JEOL GC Mate II single magnetic mass spectrometer in the positive-ion mode. High resolution mass spectrometry was carried out using a Waters Synapt G2 ESI probe. Elemental analyses were carried out using a Fission EA 110 CHNS analyser or an Elementar Vario EL Cube Analyser. Melting points were determined using a Buchi B-540 apparatus.

4.3 Chemistry

*4.3.1 Synthesis of tetrameric DAB-G1 ligand (**8**)*

Ligand precursor **5** (0.063 g, 0.093 mmol) was stirred in methanol (30 mL) in an ice bath, under argon for ten minutes. $NaBH_4$ (0.057 g, 1.51 mmol) was added and the ice bath removed. The mixture was stirred at room temperature for 22 hours. The reaction was quenched with cold distilled water (40 mL), and the solvent volume reduced to 30 mL. The product was extracted with DCM (40 mL), and the organic layer was washed with distilled water (2 × 20 mL). The organic layer was then dried with $MgSO_4$, which was removed by gravity filtration before removal of the solvent to afford the product **8** as a brown oil. Yield: 0.0464 g, 0.0681 mmol, 72 %. ^{1}H NMR (acetone-d_6): δ (ppm) = 8.48 (dd, ^{3}J = 4.4, ^{4}J = 1.5 Hz, 8H, H_a), 7.33 (d, ^{3}J = 5.9 Hz, 8H, H_b), 3.77 (s, 8H, H_d), 2.63 (t, ^{3}J = 6.7 Hz, 8H, H_f), 2.47 (t, ^{3}J = 6.9 Hz, 8H, H_h), 2.38 (t, ^{3}J = 5.7 Hz, 4H, H_i), 1.63 (p, ^{3}J = 6.8 Hz, 8H, H_g), 1.50 – 1.39 (m, 4H, H_j). ^{13}C{^{1}H} NMR (acetone-d_6): δ (ppm) = 150.4 (C_c), 149.5 (C_a), 122.9 (C_b), 54.1 (C_i), 52.4 ($C_{d/h}$), 47.8 (C_f), 27.6 (C_g), 25.1 (C_j). FTIR ν (cm^{-1}) = 3270 (br, NH), 1602 (s, C=$N_{aromatic}$). Elemental analysis for $C_{40}H_{60}N_{10}\cdot 5H_2O$: Found C, 61.87 %, H, 8.90 %, N, 18.23

%; calcd. C, 62.31 %, H, 9.15 %, N, 18.17 %. MS (EI, *m/z*): 121.07 (100 %, $[M\text{-}C_{33}H_{51}N_8]^+$), 149.05 (78.0 %, $[M\text{-}C_{31}H_{47}N_8]^+$).

*4.3.2 Synthesis of monomeric Re(I) complex (**9**)*

$[Re(bpy)Cl(CO)_3]$ **1** (0.105 g, 0.228 mmol) and silver triflate (0.075 g, 0.291 mmol) were stirred in DCM (30 mL) for 3 hours. The resulting precipitate (AgCl) was removed by gravity filtration. Ligand **6** was dissolved in DCM (5 mL) and added to the solution. The mixture was stirred at room temperature for 20 hours. Excess NH_4PF_6 was added to the solution, after which the solution was stirred for a further hour. Gravity filtration was employed to remove insoluble particles, and the solution was washed with distilled water (2×20 mL). The organic layer was dried with $MgSO_4$, which was subsequently removed by gravity filtration, and the solvent removed. The remaining residue was dissolved in acetone (5 mL) and distilled water (10 mL) added to the solution. The solvent was reduced until it became cloudy and placed in the fridge for 3 hours. The resulting precipitate was removed by gravity filtration and the product extracted from the aqueous solution with DCM (2×20 mL). The organic layer was dried with $MgSO_4$, which was subsequently removed by gravity filtration. The solvent was removed, and the product **9** dried *in vacuo* as an oily residue. Yield: 0.031 g, 0.0429 mmol, 19 %. Melting point: 104 – 107 °C. ^{1}H NMR (acetone-d_6): δ (ppm) = 9.49 (ddd, ^3J = 5.5, ^4J = 1.4, ^5J = 0.7 Hz, 2H, H_m), 8.77 (d, ^3J = 8.2 Hz, 2H, H_j), 8.52 – 8.45 (m, 2H, H_k), 8.44 (dd, ^3J = 5.2, ^4J = 1.4 Hz, 2H, H_a), 8.08 – 7.97 (m, 2H, H_l), 7.46 (d, ^3J = 6.6 Hz, 2H, H_b), 3.80 (s, 2H, H_d), 2.47 (t, ^3J = 7.0 Hz, 2H, H_f), 1.51 – 1.36 (m, 2H, H_g), 0.86 (t, ^3J = 7.4 Hz, 3H, H_h). $^{13}C\{^1H\}$ NMR (acetone-d_6): δ (ppm) = 195.7 (C_{CO}), 191.7 (C_{CO}), 156.2 (C_i), 155.9 (C_c), 154.0 (C_m), 151.5 (C_a), 141.5 (C_k), 129.1 (C_l), 125.6 (C_b), 124.9 (C_j), 51.4 (C_d), 51.1 (C_f), 22.8 (C_g), 11.0 (C_h). $^{31}P\{^1H\}$ NMR (acetone-d_6): δ (ppm) = -144.30 (hept, J = 708.2 Hz) FTIR ν (cm^{-1}) = 2025 (s, CO), 1899 (br, CO), 1619 (s, $C{=}N_{pyridyl}$) , 1604 (s, $C{=}N_{bpy}$). Elemental analysis for $C_{22}H_{22}N_4O_3Re{\cdot}PF_6$: Found C, 36.98, H, 3.02, N, 7.47 %; calcd. C, 36.62, H, 3.07, N, 7.76 %. MS (HR-ESI, *m/z*): 577.12 (100 %, $[M\text{-}PF_6]^+$, calcd. 577.12), 427.01 (33 %, $[M\text{-}L1\text{-}PF_6]^+$, calcd. 427.01).

*4.3.3 Synthesis of trimeric Re(I) complex (**10**)*

$[Re(bpy)Cl(CO)_3]$ **1** (0.131 g, 0.284 mmol) and silver triflate (0.080 g, 0.311 mmol) were stirred at room temperature in DCM for 3 hours. The resulting precipitate (AgCl) was filtered and ligand **7** (0.036 g, 0.0863 mmol) was added to the flask. The reaction mixture was stirred at room temperature for 19 hours, after which the solvent was removed and the resulting residue taken up in minimum ethanol (4 mL) and ammonium hexafluorophosphate (excess in H_2O) was added to precipitate the crude product as a yellow powder. The powder was

redissolved in minimum acetone and added dropwise to isopropanol (80 mL, 75 °C). The mixture was filtered through Celite and the product obtained by washing with DCM. Removal of the solvent afforded product **10** as a brown powder. Yield: 0.022 g, 0.0103 mmol, 12 %. Melting point: 145 – 150 °C. ^{1}H NMR (acetone-d_6): δ (ppm) = 9.44 (d, ^{3}J = 5.6 Hz, 6H, H_l), 8.78 – 8.62 (m, 6H, H_i), 8.51 – 8.33 (m, 12H, $H_{j/a}$), 8.07 – 7.91 (m, 6H, H_k), 7.41 (d, ^{3}J = 6.5 Hz, 6H, H_b), 3.79 – 3.70 (m, 6H, H_d), 3.33 – 3.23 (m, 6H, H_f), 2.94 – 2.79 (m, 6H, H_g). ^{13}C{^{1}H} NMR (acetone-d_6): δ (ppm) = 195.5 (C_{CO}), 191.5 (C_{CO}), 155.8 (C_h), 155.4 (C_c), 154.0 (C_l), 151.7 (C_a), 141.4 (C_j), 129.1 (C_k), 125.9 (C_b), 124.8 (C_i), 53.1 (C_f), 50.4 (C_d), 42.2 (C_g). ^{31}P{^{1}H} NMR (acetone-d_6): δ (ppm) = -144.26 (hept, J = 708.7 Hz). FTIR ν (cm^{-1}) = 2032 (s, CO), 1907 (s, CO), 1619 (s, C=$N_{pyridyl}$) , 1605 (s, C=N_{bpy}). MS (HR-ESI, *m/z*): 566.07 (15 %, $[M\text{-}3PF_6]^{3+}$, calcd. 566.10). Elemental analysis for $C_{63}H_{57}N_{13}O_9 \cdot 3PF_6$: Found C, 35.37, H, 2.89, N, 8.18 %; calcd. C, 35.46, H, 2.69, N, 8.35 %.

*4.3.4 Synthesis of tetrameric Re(I) complex (**11**)*

[Re(bpy)Cl(CO)$_3$] **1** (0.090 g, 0.194 mmol) and silver triflate (0.059 g, 0.229 mmol) were stirred in DCM (30 mL) for 3 hours. A precipitate (AgCl) was formed and removed by gravity filtration. Ligand **8** (0.033 mg, 0.0479 mmol) was dissolved in DCM (5 mL) and the solution added to the reaction mixture. The solution was stirred for 24 hours at room temperature, before the addition of NH_4PF_6 (excess) and subsequent stirring for one hour. The solvent was removed by rotary evaporation and the residue dissolved in minimum DCM. The solution was added to boiling isopropanol and stirred for five minutes. The solution was allowed to cool to room temperature, precipitating a brown powder. The powder was filtered using Celite and washed with isopropanol before being solubilised with DCM. The DCM was removed to afford the product **11** as a brown powder. Yield: 0.016 g, 0.00527 mmol, 11 %. Melting point: 134 – 137 °C. ^{1}H NMR (acetone-d_6): δ = 9.47 (ddd, ^{3}J = 5.4, ^{4}J = 1.4, ^{5}J = 0.7 Hz, 8H, H_o), 8.74 (d, ^{3}J = 8.1 Hz, 8H, H_l), 8.53 – 8.37 (m, 16H, $H_{a/m}$), 7.99 (ddd, ^{3}J = 7.6, ^{3}J = 5.5, ^{4}J = 1.2 Hz, 8H, H_n), 7.46 (d, ^{3}J = 6.6 Hz, 8H, H_b), 3.83 (s, 8H, H_d), 3.38 – 3.06 (m, 16H, $H_{f/h}$), 2.77 – 2.64 (m, 4H, H_i), 1.98 – 1.77 (m, 12H, $H_{g/j}$). ^{13}C{^{1}H} NMR (acetone-d_6): δ = 195.6 (C_{CO}), 191.6 (C_{CO}), 155.8 (C_k), 154.0 (C_o), 151.7 (C_a), 151.7 (C_c), 141.4 (C_m), 129.0 (C_n), 125.8 (C_b), 124.8 (C_l), 52.0 (C_f), 51.1 (C_d), 47.1 (C_h), 46.72 (C_i), 23.9 (C_g), 23.7 (C_j). ^{31}P{^{1}H} NMR (acetone-d_6): δ = -144.26 (hept, J = 708.9 Hz). FTIR ν (cm^{-1}) = 2028 (s, CO), 1901 (s, CO), 1619 (s, C=$N_{pyridyl}$), 1605 (s, C=N_{bpy}). MS (HR-ESI, *m/z*): 590.08 (26 %, $[M\text{-}CO\text{-}4PF_6]^{4+}$), 547.05 (19 %, $[M\text{-}7CO\text{-}4PF_6]^{4+}$). Elemental analysis for $C_{92}H_{92}N_{18}O_{12}Re_4 \cdot 4PF_6 \cdot 5H_2O \cdot 2DCM$: Found C, 35.28, H, 3.68, N, 7.26 %; calcd. C, 34.80, H, 3.36, N, 7.77 %.

4.4 Cytotoxicity and Antiproliferative Studies

The ovary carcinoma A2780, the epidermoid carcinoma A431 and the colon carcinoma DLD-1 cell lines were acquired from the European Collection of Authenticated Cell Cultures (ECACC) through Sigma Aldrich, St. Louis, USA. The normal human fibroblast cell line (BJ) was acquired from the American Type Culture Collection (ATCC, Manasses, VA, USA). The cells were cultured under conditions recommended by the cell bank using cell culture media and supplements from Sigma Aldrich. The MTT reagent and the Hank's media to dissolve the salt (5 mg/mL final concentration) were purchased from Sigma Aldrich. The cells were seeded on 96-well Nunclon Delta surface assay plates (from Thermo Scientific, Waltham, USA) in wells containing 200 µL cell suspension. The compounds **9**, **10** and **11** were diluted in DMSO, and serial dilutions were prepared using 0.01 M PBS solution, to obtain concentrations in the range of 10 – 2500 µM. Cisplatin and oxaliplatin were received from Actavis, through Sindan-Pharma Srl, Bucharest, Romania; carboplatin was provided from Teva UK Limited, Castleford, United Kingdom. The platinum-based drugs were diluted in physiological serum, and serial dilutions were made in PBS, to obtain the same concentration range as for Re(I) compounds: 10 – 2500 µM. For testing, each well containing cells was treated with the respective compound in a proportion of 1:20 (compound:cell culture media), to obtain a final concentration range of 0.5 – 125 µM of the compound in the cell culture media. To assess the cytotoxicity of the compounds, the cells were subjected to the treatment for 24 hours, than the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction assay was performed, as described before [41]. Untreated cells were used as a reference, and cell culture media without cells was used as a blank. Three independent experiments were performed, each in duplicate. Measurements were made at 492 nm and 570 nm using a Synergy 2.0 microplate reader (from BioTek Company, Winooski, USA), and the absorbance data were implemented using GraphPad Prism 5 software (from GraphPad Software, La Jolla, USA) to obtain the sigmoidal curves and the IC_{50} values. The antiproliferative effect was evaluated using the cell-permeable fluorescent Alamar Blue dye (from Invitrogen, acquired through Thermo Fisher Scientific, Waltham, MA, USA) which indicates the intracellular reducing potential of the cells. The plates were loaded with tumor cells; they were incubated and treated with the compounds in the same way as for MTT testing. After 24 hours treatment, to each sample 20µL dye was added, and incubated for three hours. The fluorescence of each well was measured at the 620nm emission (excitation wavelength 54nm), using the Synergy2 microplate reader. Three independent measurements were made,

in duplicates. The data were processed with the GraphPad Prism 5 software to obtain a linear regression between the doses and the degree of the metabolic activity reduction.

4.5 Cell Death Mechanistic Studies

The concentration of Bax-α and Bcl-xL were measured using the DuoSet IC Elisa kits from R&D Systems (Minneapolis, MN, USA), for each molecule. The cells were cultivated were seeded on 6-well plates at a density of $1.5x10^5$ cells/mL, and they were treated for 24 hours with the Rhenium compounds at the concentration of 10 μM in the cell culture media. Than the cell culture media was removed, the plates were washed twice with cold PBS. The wells were covered with 1 mL cell lysis solution (from MBL International Corporation, Woburn, MA, USA) and kept 20 minutes on ice. The lysates were harvested from the cell culture plates, centrifuged; the supernates aliquotes were placed in ultrafrezer. The total protein level was measured in the lysates, and all samples were diluted with lysis buffer to normalize the protein concentration. The Elisa plates were prepared by treating the each well with Bax-α and respectively Bcl-xL capture antibody overnight at room temperature, than the plates were washed 3 times with the automated plate washer (from DIAsource, Louvain-la-Neuve, Belgium) using the buffer provided by the manufacturer. In each well block buffer was added for two hours, and the washing steps were repeated. In each well samples (duplicates), blank values or standard proteins were added: 8 different concentrations and one blank, in the detection range of the kit. As reference we used untreated cells for each cell line. After two hours the plates were washed, incubated with 125 ng/mL detection antibody for another two hours. The plates were washed and filled with 100 μL Streptavidin-HRP for 20 minutes, than washed and incubated with substrate solution for 20 minutes. Acid stop solution was added to each well after the blue coloration development, and the samples color turned into yellow, the optical density of each well was determined at 450 nm, with correction at 540nm, using a Sunrise microplate plate reader (Tecan Group, Männedorf, Switzerland). The Magellan software provided measurement data and the single concentrations for each sample.

4.6 Fas/FasL Signalling Studies

The soluble Fas receptor and FasLigand were evaluated using the Quantikine Elisa kits from R&D Systems. The cells seeded for 24 hours on 6-well plates were treated with 10 μM **9**, **10** or **11**, then the cell culture media was harvested from the wells, centrifuged, and stored at -80

°C. The 96-well plates precoated with anti-Fas or anti-FasL monoclonal antibodies were filled with 100 μL of assay diluent provided by the kit, and 100 μL of samples were added to each well, in duplicate. For quantitative measurements, the standard recombinant human Fas or FasL proteins were used; 8 serial dilutions were prepared and added into the wells. The samples and standards were incubated for 2 hours, washed 4 times with the wash buffer provided by the kit, then horseradish peroxidase-conjugated polyclonal antibody was added to each well and incubated at room temperature for 2 hours. After 3 wash steps 200 μL HRP substrate solution was added into the wells, and the plates were incubated for 30 minutes. Stop solution was added, and the optical density was measured immediately, in similar condition and with the same instrument as described before. The soluble Fas and FasL concentrations were calculated using the standard curves.

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Highlights

- Mono- and multinuclear [2+1] Re(I) tricarbonyl complexes have been prepared.
- The complexes were characterized using several spectroscopic and analytical techniques.
- The complexes were evaluated as antiproliferative agents against A431, DLD-1 and A270 tumour cell lines.
- The Re(I) complexes all influence programmed cell death mechanisms.