Novel lawsone-containing ruthenium(II) complexes: Synthesis, characterization and anticancer activity on 2D and 3D spheroid models of prostate cancer cells

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\textbf{ABSTRACT} \\
This study describes a series of newly synthesized phosphine/diimine ruthenium complexes containing the lawsone as bioligand with enhanced cytotoxicity against different cancer cells, and apoptosis induction in prostatic cancer cells DU-145. The complexes [Ru(law)(N-N)\textsubscript{2}]PF\textsubscript{6} where N-N is 2,2'-bipyridine (1) or 1,10-phenanthroline (2) and [Ru(law)(dppm)(N-N)]PF\textsubscript{6} where dppm means bis(diphenylphosphino)methane, N-N is 2,2'-bipyridine (3) or 1,10-phenanthroline (4), and law is lawsone, were synthesized and fully characterized by elemental analysis, molar conductivity, NMR, UV–vis, IR spectroscopies and cyclic voltammetry. The interaction of the complexes (1–4) with DNA was evaluated by circular dichroism, gel electrophoresis, and fluorescence, and the complexes presented interactions by the minor grooves DNA. The phosphinic series of complexes exhibited a remarkably broad spectrum of anticancer activity with approximately 34-fold higher than cisplatin and 5-fold higher than doxorubicin, inhibiting the growth of 3D tumor spheroids and the ability to retain the colony survival of DU-145 cells. Also, the complex (4) inhibits DU-145 cell adhesion and migration potential indicating antimetastatic properties. The mechanism of its anticancer activity was found to be related to increased reactive oxygen species (ROS) generation, increased the BAX/BCL-2 ratio and subsequent apoptosis induction. Overall, these findings suggested that the complex (4) could be a promising candidate for further evaluation as a chemotherapy agent in the prostate cancer treatment.

1. Introduction

The research based on transition metals was strongly encouraged thanks to the quest for novel anticancer therapies [1]. So far, cisplatin is probably still the most essential chemotherapeutic based on metals complexes. However, platinum complexes have caused several side effects and cross drug resistance which limited their applications [2]. Therefore, the development of novel metalodrugs agents remains a significant challenge.

In this important field, ruthenium-based complexes are highlighted due to its unique properties as chemical stability, a variety of oxidation states, structural diversity, low toxicity and ability to mimic iron binding in the biological system [3–6]. Moreover, several ruthenium (Ru) complexes have been foreground reported due to its antimetastatic activities, low toxicity to non-cancer cells and high selectivity for tumor cells [7,8]. For instance, some Ru complexes as NAMI-A (\textit{trans-}[\text{RuCl}_{4}(1H-imidazole)(DMSO-S)]\textsuperscript{+}), NKP-1339 (sodium \textit{trans-}[\text{RuCl}_{4}(1H-indazole)\textsubscript{2}]) and KP1019 (\textit{trans-}[\text{RuCl}_{4}(1H-in-dazole)\textsubscript{2}]) have been evaluated in preclinical and phase I or II clinical trials, representing a novel class of less toxic antineoplastic chemotherapy [9,10]. Accumulative evidences suggest that Ru-based compounds could act as potential anticancer agents through molecular mechanisms providing an increase of reactive oxygen species (ROS) [11], DNA intercalation [12], DNA damage [13], induction of cell apoptosis and metastasis inhibition [14,15].

One rational approach to improve the efficacy of metal complexes consists of incorporating different chelating ligands with complementary biological activity to enhance the physiological targets of
the metallodrug. In this context, active ligands with quinone moiety represent an unquestioned tool in bioinorganic medicinal chemistry for improving the anticancer activity of metal complexes. Undoubtedly, some quinone based drugs such as doxorubicin, mitomycin C, and mitoxantrone are recognized as FDA-approved compounds with excellent anticancer properties [16,17]. A representative class of quinone compounds, known as naphthoquinones, widely distributed in nature, have been recognized to own an extensive range of biological activities such as antimicrobial [18], antiparasitic [19], antiviral [20] and anticancer activity [21].

Mainly, 1,4-naphthoquinone, or lawsone, is an example of a natural compound that contains two ketone groups as suitable chromophore responsible for its biological activity due to their ability to accept electrons [22,23]. Recently, lawsone compounds have shown promising pharmacological effects presenting antiproliferative activity in a large number of cancer cell lines and inhibition of tumor burden in vivo [24,25].

Furthermore, findings indicated that anticancer activity also might be related to the inhibition of the enzyme DNA-topoisomerase II [26]. Lawsone can bind potentially to metal ions in different oxidations states coordinating metals in a bidentate way which allows them to play an essential role in biological systems [27]. Indeed, some metal complexes containing lawsone ligands have been exhibited higher biological activity than free lawsone [28]. Therefore, as part of our ongoing effort to design multi-target bioactive metallodrug candidates, in the last years our research group has synthesized several Ru-complexes containing phosphine and diimines ligands with promising pharmacological activities for anticancer proposes [29–35]. In light of these considerations and of some previous results, herein we have investigated the anticancer properties of the newly synthesized phosphine/diimine Ru-complexes containing lawsone as bioligand. The corresponding complexes 1–4 (showed in Fig. 1) were synthesized and characterized, and their potential as anticancer agents was investigated for the first time towards a comprehensive panel of human cancer cell lines cultured in conventional and 3D models. Besides, we analyzed the antimetastatic effects on human prostate carcinoma DU-145 cells and their ability to interact with CT-DNA. Finally, for elucidating the anti-cancer functions and molecular mechanisms, reactive oxygen species (ROS) production and apoptosis-inducing ability were also investigated.

2. Results and discussion

2.1. Synthesis of novel lawsone-containing ruthenium complexes

The complexes (1–4) containing lawsone (law), phosphines and diimines as ligands were obtained according to the procedure illustrated in Fig. 1.

The synthetic route obtained pure complexes with high-yield according to spectroscopic data, as well as elemental analyses, molar conductivity and 31P{1H}, 1H and 13C NMR spectrum. All complexes are dark purple air-stable solids, soluble in dichloromethane, acetone, methanol, dimethyl sulfoxide and water insoluble.

In the IR spectra of the free law is observed the intense band of the stretching modes of the phenolic ν(O−H) around 3200 cm−1, this band disappear from the spectra of all complexes, indicating the ligand deprotonation and the coordination by oxygen. The band relative to the carbonyl group (C1=O) at 1640 cm−1 in the free law were shifted to lower frequencies, around 1603 cm−1 in the complexes, denoting the bidentate coordination. Also, the band at 1679 cm−1 of the group (C4=O) also presented a small displacement to lower frequencies, 1614 cm−1. The band C2=O presented displacement from 982 cm−1 to 1098 cm−1, approximately, in the complexes, which are in concordance with the coordination by the oxygen. The same behavior was observed for others ruthenium complexes containing the lawsone as ligand [28].

The complexes (1–4) were characterized by 31P{1H}, 1H and 13C NMR spectroscopy. In the 31P{1H} of complexes (3) and (4) was
observed four pairs of signals (17.9/6.7 ppm and 15.2/5.9 ppm for complex (3) and 18.2/6.6 ppm and 15.4/6.2 ppm for complex (4)) for the two phosphorus atoms of biphosphine dpmm, indicating the non-equivalence of phosphorus. The duplication of the signals is due to the formation of isomers, where there is an isomer with the phosphorus atom of dpmm trans to the oxygen of the carbonyl of lawsone, and in the other, the oxygen atom of the carbonyl is trans to the nitrogen of bipy or phen (see Supplementary Information, Figs. S2–S3). In the 1H spectra for complex (3) and 18.2/6.6 ppm and 15.4/6.2 ppm for complex (4), we observed four pairs of signals (17.9/6.7 ppm and 15.2/5.9 ppm for complex (3) and 18.2/6.6 ppm and 15.4/6.2 ppm for complex (4)).

Characteristic NMR 13C signals belonging to the carbon of carbonyl groups of lawsone were observed for complex (4) at 200.3 ppm (C1═O), 182.5 ppm (C4═O) and 172.6 ppm (C2═O). These signals are observed in 184.5 ppm (C1═O), 181.2 ppm (C4═O) and 159.5 ppm (C2═O) in free lawsone. The most significant displacements are found for the carbons of the groups C1═O and C2═O, indicating the coordination of these oxygen atoms. More recently, our research group developed trans-[Ru(law)(PPh3)2(N-N)]PF6 complexes and were observed the same behavior in NMR experiments [31].

The higher cytotoxicity, exhibited by complexes (3) and (4) towards prostate cancer cells encouraged us to study their effects at the cellular level. In a new set of experiment, DU-145 cell line was used as a cellular model, since it was among the most sensitive cell line to the complexes tested. Therefore, the cytotoxicity of phosphine complexes was confirmed by clonogenic survival assay that is extremely sensitive to determine the effects on cell proliferation after treatments. Clonogenic assays represent the gold-standard method to study the cytotoxic and constitute a traditional technique of choice to determine the long-term effect of proliferating cells [43]. The results revealed that both complexes inhibited cell colony formation of DU-145 cells in a dose-dependent manner as shown in Fig. 2A. The plating efficiency of untreated DU-145 cells was higher than 70% for all replicates. Moreover, following seven days of culture, the inhibitory effect of the complex (4) was greater than that of the complex (3) at the same concentration. At concentrations of 0.125, 0.25, 0.5 and 1μM, the complex (3) reduced the colony-forming presenting survival fractions (SF) of 79.1, 68.2, 31.1 and 13.4%, respectively. Complex (4), at same concentrations, presented SF of 68.1, 39.5, 22.4 and 5.1%, respectively. No significant (p > 0.05) decrease in the number of colonies was observed in control (DMSO 0.1% v/v). Few colonies of DU-145 cells were visible after incubation with 1μM of (4) which indicates that the substitution of diimine ligand increases the activity of the compound (Fig. 2B).

The higher cytotoxicity, exhibited by complexes (3) and (4), indicated that the coordination of phosphine and lawsone chelating ligands in one single molecule made it possible to create a compound with increased antitumor activity. Furthermore, these findings predicted the loss of clonogenicity of DU-145 cells after the exposure with (3) and (4) which is an effective parameter to distinguish the cytostatic of the cytotoxic effects of drug treatments [44]. Cisplatin did not

2.2. Partition coefficient (log P) study

Determining the lipophilicity is an important physicochemical property that figures out the possible pharmacokinetic and pharmacodynamic profile of new drugs candidates in the discovery development phase [36]. The well-known shake-flask has been widely recommended as a reference method to determine a partition coefficient experimentally [37]. The log P(o/w) values, calculated by the shake-flask method, were positive and ranged from 0.228 ± 0.012 to complex (1), 0.573 ± 0.023 to complex (2), 0.437 ± 0.025 to complex (3) and 0.793 ± 0.031 to complex (4). As demonstrates, the higher lipophilicity of complexes (2) and (4) can be attributed to the presence of phen ligand. Other studies established that the presence of 1,10-Phenan-throline acts improving the lipophilicity of silver complexes when compared to bipyridine analogs [38]. Our finding also suggests that the use of diphosphines as ligands can adjust the lipophilic/hydrophilic balance in metal complexes, where in general, its biological activity is found to increase thanks to an increase in the lipophilicity [39]. Thus, the log P indicates that the coordination of phen and dpmm chelating ligands in one single molecular structure improve the lipophilicity and, as a consequence, may contribute to enhance the cell membrane permeability and its biological activity.

2.3. The complexes display enhanced cytotoxicity against a panel of cancer cell lines

The cytotoxicity of the ruthenium complexes was evaluated in a panel of different histological types of cancer cell lines (A549, Caco-2, DU-145, HepG2, PC-3 and MDA-MB-231) and against two non-cancer cells (FGH and HUVEC) in two-dimensional (2D) culture by Alamar Blue assay 24 h after incubation, as shown in Table 1. All of the complexes presented enhanced cytotoxicity to different cancer cells and more potent activity than metal-free lawsone. The synthesized Ru(II) phosphine complexes investigated exhibited remarkable cytotoxic properties displaying better anticancer activity than cisplatin (Fauldcispla®) and doxorubicin (Fauldoxo®). As expected, the phosphine-coordinating ligand improved not only the lipophilicity of ruthenium compounds but also their biological activity. Metal complexes with different phosphine ligands had antitumor profiles characterized by high cytotoxic potency and selectivity between different cancer cells [40,41]. Compared to the other complexes and metal-free lawsone, the phosphine complexes (3) and (4) had low IC50 values, where complex (3) presented IC50 values ranging from 1.9 to 4.8μM for cancer cell lines DU-145 and A549, respectively and (4) presented IC50 values ranging from 1.3 to 3.0μM for cancer cell lines DU-145 and MDA-MB-231, respectively.

In addition, complex (3) was more potent than cisplatin in A549 (3-fold), Caco-2 (7-fold), DU-145 (12-fold), HepG2 (6-fold), PC-3 (6-fold) and MDA-MB-231 (10-fold). Complex (4) was more potent than doxorubicin on DU-145 (4-fold), HepG2 (2-fold), PC-3 (2-fold) and cis-platin on A549 (4-fold), Caco-2 (8-fold), DU-145 (18-fold), HepG2 (6-fold), PC-3 (9-fold) and MDA-MB-231 (12-fold). The precursors of types [RuCl2(N-N)2]PF6 and [RuCl2(dpmm)(N-N)]PF6 (N-N = 2,2′-bipyridine or 1,10-phenanthroline) exhibited weak cytotoxicity (IC50 > 100μM). The IC50 value for non-cancer cells was 4.3 and 8.1μM for the complex (3), 3.2 and 6.9μM for the complex (4) and 1.9 and 4.6μM for doxorubicin on FGH and HUVEC cells, respectively.

Table 2 shows the calculated selectivity index (SI) of each compound. The SI was calculated using the following formula: SI = IC50 [non-cancer cells]/IC50 [cancer cells]. The SI is a clear manner to estimate the therapeutic range of a drug and to identify drug candidates for further studies [11]. Complexes (3) and (4) were more cytotoxic and selective to prostate cancer cells DU-145. Furthermore, both complexes exhibit selectivity index greater than displayed by the drug controls cisplatin and doxorubicin, which are clinically useful drugs in the treatment of cancer. Probably, the presence of the dpmm ligand in the complexes (3) and (4) may contribute to increasing cytotoxic activity, as previously observed for other ruthenium complexes, where the increase of phosphate ligands is directly related to the increase of biological activity [30,42].

The promising cytotoxic activity of the complexes (3) and (4) towards prostate cancer cells encouraged us to study their effects at the cellular level. In a new set of experiment, DU-145 cell line was used as a cellular model, since it was among the most sensitive cell line to the complexes tested. Therefore, the cytotoxicity of phosphine complexes was confirmed by clonogenic survival assay that is extremely sensitive to determine the effects on cell proliferation after treatments. Clonogenic assays represent the gold-standard method to study the cytotoxic and constitute a traditional technique of choice to determine the long-term effect of proliferating cells [43]. The results revealed that both complexes inhibited cell colony formation of DU-145 cells in a dose-dependent manner as shown in Fig. 2A. The plating efficiency of untreated DU-145 cells was higher than 70% for all replicates. Moreover, following seven days of culture, the inhibitory effect of the complex (4) was greater than that of the complex (3) at the same concentration. At concentrations of 0.125, 0.25, 0.5 and 1μM, the complex (3) reduced the colony-forming presenting survival fractions (SF) of 79.1, 68.2, 31.1 and 13.4%, respectively. Complex (4), at same concentrations, presented SF of 68.1, 39.5, 22.4 and 5.1%, respectively. No significant (p > 0.05) decrease in the number of colonies was observed in control (DMSO 0.1% v/v). Few colonies of DU-145 cells were visible after incubation with 1μM of (4) which indicates that the substitution of diimine ligand increases the activity of the compound (Fig. 2B).

The higher cytotoxicity, exhibited by complexes (3) and (4), indicated that the coordination of phosphine and lawsone chelating ligands in one single molecule made it possible to create a compound with increased antitumor activity. Furthermore, these findings predicted the loss of clonogenicity of DU-145 cells after the exposure with (3) and (4) which is an effective parameter to distinguish the cytostatic of the cytotoxic effects of drug treatments [44]. Cisplatin did not
analyzed (significant) reduce the colony-forming at the same concentrations.

The selectivity index (SI) is calculated using the following formula: SI=IC_{50}[non-cancer cells]/IC_{50}[cancer cells].

Data are presented as the means ± S.E.M. of IC_{50} values in µM obtained by nonlinear regression from at least three independent experiments performed in triplicate, measured by Alamar blue assay after 24 h incubation.

**Table 1**

<table>
<thead>
<tr>
<th>Cancer cells</th>
<th>IC_{50} in µM</th>
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<tbody>
<tr>
<td></td>
<td>FGH</td>
</tr>
<tr>
<td>BB</td>
<td>&gt; 100</td>
</tr>
<tr>
<td>BB</td>
<td>&gt; 100</td>
</tr>
<tr>
<td>DB</td>
<td>&gt; 100</td>
</tr>
<tr>
<td>DP</td>
<td>&gt; 100</td>
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<tr>
<td>LAW</td>
<td>&gt; 100</td>
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</table>

(1) 74.3 ± 1.4 93.1 ± 7.4 99.1 ± 0.2 60.3 ± 5.3 41.4 ± 4.5 53.9 ± 3.8 61.9 ± 2.3 63.1 ± 5.7
(2) 80.7 ± 0.6 78.7 ± 2.2 69.1 ± 5.2 58.1 ± 2.9 37.2 ± 2.1 49.0 ± 3.6 52.1 ± 1.0 68.4 ± 4.5
(3) 4.3 ± 0.6 8.1 ± 1.6 4.8 ± 0.4 2.6 ± 0.2 1.9 ± 0.6 1.8 ± 0.3 3.3 ± 0.9 3.6 ± 0.5
(4) 3.2 ± 0.4 6.9 ± 0.8 4.2 ± 0.2 2.4 ± 0.3 1.3 ± 0.2 1.8 ± 0.3 2.2 ± 1.8 3.0 ± 0.3
CIS 11.2 ± 0.5 11.0 ± 0.5 16.5 ± 1.0 19.9 ± 0.9 24.5 ± 2.8 11.4 ± 1.6 21.3 ± 0.1 37.4 ± 6.2
DXR 1.9 ± 0.2 4.6 ± 0.2 2.3 ± 1.8 1.8 ± 0.3 2.5 ± 1.3 3.8 ± 1.6 4.5 ± 0.1 2.5 ± 0.3

Data are presented as the means ± S.E.M. of IC_{50} values in µM obtained by nonlinear regression from at least three independent experiments performed in triplicate, measured by Alamar blue assay after 24 h incubation.

**Table 2**

<table>
<thead>
<tr>
<th>Non-cancer cells</th>
<th>A549</th>
<th>Caco-2</th>
<th>DU-145</th>
<th>HepG2</th>
<th>PC-3</th>
<th>MDA-MB-231</th>
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<tr>
<td>FGH</td>
<td>(1) 0.7</td>
<td>1.2</td>
<td>1.8</td>
<td>1.4</td>
<td>1.2</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td>(2) 1.1</td>
<td>1.4</td>
<td>2.2</td>
<td>1.6</td>
<td>1.5</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td>(3) 0.9</td>
<td>1.6</td>
<td>2.3</td>
<td>2.4</td>
<td>1.3</td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td>(4) 0.8</td>
<td>1.3</td>
<td>2.5</td>
<td>1.8</td>
<td>1.4</td>
<td>1.0</td>
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<tr>
<td></td>
<td>CIS 0.7</td>
<td>0.6</td>
<td>0.4</td>
<td>1.0</td>
<td>0.5</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td>DXR 0.8</td>
<td>1.0</td>
<td>0.7</td>
<td>0.5</td>
<td>0.4</td>
<td>0.7</td>
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<tr>
<td>HUVEC</td>
<td>(1) 0.9</td>
<td>1.5</td>
<td>2.2</td>
<td>1.7</td>
<td>1.5</td>
<td>1.4</td>
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<td></td>
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<td>3.1</td>
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<td>4.5</td>
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<tr>
<td></td>
<td>(4) 1.6</td>
<td>2.8</td>
<td>5.3</td>
<td>3.8</td>
<td>3.1</td>
<td>2.3</td>
</tr>
<tr>
<td></td>
<td>CIS 0.6</td>
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<td>0.4</td>
<td>0.9</td>
<td>0.5</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td>DXR 2.0</td>
<td>2.5</td>
<td>1.8</td>
<td>1.2</td>
<td>1.0</td>
<td>1.8</td>
</tr>
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</table>

Data are presented with the selectivity index (SI) calculated using the following formula: SI = IC_{50}[non-cancer cells]/IC_{50}[cancer cells].

In general, DU-145 MCTS showed more considerable resistance to both complexes compared to the 2D-cultured cells. Indeed, the MCTS are typically more resistant to antiproliferative compounds because of the differences in the drugs penetration and varying microenvironments (such as pH, hypoxia, extracellular matrix) within spheroids [48]. Similar to in vivo tumors, the hypoxic regions inside the spheroids reduces the rate of cell division impairs the effects of anti-proliferative drugs such as cisplatin [49].

Interestingly, the cells in the spheroids showed morphological changes, indicating an effective permeability into spheroid and, therefore, cytotoxicity. The treatment with complex (4) disrupted the cell aggregations, resulting in the presence of cell debris after 9 days, suggesting disturbance in cell-matrix interactions (Fig. 3B).

### 2.4. The complexes reduce DU-145 cell adhesive and migratory potential

Afterward, cell adhesion and migration assays were performed to evaluate the antitumorability of the capability of the complex (4). Cancer metastasis mainly occurs due to the migration and adhesion of neoplastic cells from a primary tumor to distant sites following angiogenesis and tumor growth [50]. Therefore, in this research, the anti-migratory and anti-adhesive effects of the complex (4) were investigated by wound healing and extracellular matrix adhesion assay on DU-145 cells. For this assays, the cells were treated with concentrations below the IC_{50} values of (4) (1.3 ± 0.2 µM, in 24 h). As seen in Fig. 4, untreated DU-145 monolayers were wounded at 0 h and images were registered at different time points after the treatment. The wound space was reduced severely in size in the absence of compounds because of cell proliferation and migration. Complex (4) inhibits the cell migration of DU-145 cells, exhibiting deeply reduced wound healing patterns after 48 h were the cells exposed to 0.5 µM presents inhibition of 80% of wound closure, compared to control at the same time. Indeed, the complex (4) as expectedly showed an antitumoristic feature, in accordance with the previously reported studies of anti-migratory and anti-angiogenic effects of others ruthenium complexes containing phosphines and dimes as ligands [28,51]. These data demonstrate that low concentrations of (4) impaired the androgen-metastatic prostate cancer DU-145 cells migratory potential.
Next, we assessed the cell adhesion inhibition of complex (4) using mimic extracellular matrix (ECM) adhesion assay. The in vivo binding of cells to the extracellular matrix (ECM) is fundamental for cell survival and cell-to-cell communication but is also essential for the metastatic potential of cancer cells [52]. DU-145 cells were incubated to adhere to either collagen or Matrigel® in the presence of different concentrations of the complex (4) and assessing the relative numbers of attached cells. As measured by colorimetric assay, untreated and vehicle-treated (CTL) cells exhibited similar adhesion to collagen (Fig. 5A) or Matrigel® (Fig. 5B). The treatments of DU-145 cells with different concentrations of the complex (4) significantly inhibited the cell adhesion in a concentration-dependent manner. At 0.5μM the maximum inhibition of cell adhesion was observed.

<table>
<thead>
<tr>
<th>Spheroids</th>
<th>IC50 in μM</th>
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<tbody>
<tr>
<td>(3)</td>
<td>4.1 ± 0.1</td>
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<tr>
<td>(4)</td>
<td>3.4 ± 0.5</td>
</tr>
<tr>
<td>CIS</td>
<td>62.6 ± 3.2</td>
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Fig. 2. Assessment of colony survival by clonogenic assay. (A) Representative colony formation images of DU-145 cells after treatment with complex (4). (B) The survival fractions after 24 h treatment with 0.125; 0.25; 0.5 and 1μM of (3), (4) and cisplatin against DU-145 cells. The negative control (CTL) was treated with the vehicle (0.1% DMSO) used for diluting the tested compound. Data are presented as the means ± S.E.M. of three independent experiments performed in triplicate. *p < 0.05 compared with the control by ANOVA followed by Dunnet’s test.

Fig. 3. Effects of the complex (3) and (4) in 3D in vitro model of multicellular tumor spheroids of DU-145 cells. (A) The MCTS viability determined by Alamar Blue assay after 24 h treatment and IC50 values in μM obtained by nonlinear regression from at least three independent experiments performed in triplicate. The negative control (CTL) was treated with the vehicle (0.1% DMSO) used for diluting the compounds tested. Doxorubicin (DOX, Fauldoxo® 5μM), Cisplatin (CIS, Fauldcispla® 25μM) were used as the positive controls. (B) DU-145 MCTS were examined using inverted microscope (amplification 200×), scale bar = 100μm. Data are presented as the mean ± S.E.M. of three independent experiments performed in triplicate. *p < 0.05 compared with the control by ANOVA followed by Dunnet’s test.
DU-145 adhesion was calculated by more than 70% on Matrigel® matrix. The anti-metastatic activity of ruthenium compounds is well-recognized by NAMI-A studies [53]. Furthermore, current studies suggest Ru-based complexes inhibits the formation of tube-like structures, an essential step in angiogenesis and metastasis [54]. Our findings indicate that complex (4) reduce the ability of DU-145 cells to adhere to ECMs which may impact on their metastatic capacity.

2.5. DNA interactions

The DNA is the most studied targets when it comes to the development of compounds with antitumor properties. It occurs because the interaction between DNA and compounds can cause irreversible damage and prevent cell replication, leading to cell death [55]. In this context, we investigated the ability of the ruthenium complexes to interact with DNA by Circular Dichroism (CD), fluorescence and gel electrophoresis.

The CD technique can be used to analyze the conformation changes in the secondary structure of the calf-thymus DNA (CT-DNA) by monitoring the two bands in the UV region, a positive band at 275nm and a negative band at 245nm, due to base stacking and right-handed helicity, respectively. Thus, the CT-DNA was incubated with ruthenium complexes in different molar ratios (0.1–0.4), and the bands were analyzed, and the effects are shown in Fig. 6 for complex (4). The intensities of negative and positive bands of the CT-DNA did not show any significant change in intensity upon addition of the ruthenium complex, and the same behavior was observed for all complexes (1–4).

The ability of the complexes to change the tertiary structure of DNA was investigated by electrophoresis in agarose gel employing the pBR322 plasmid DNA, upon incubation with ruthenium complexes in different molar ratios. Alterations in the electrophoretic mobility of plasmid DNA on agarose gel is commonly considered as evidence for direct DNA–metal interactions. Modification of the DNA structure causes hindrance in the migration of supercoiled DNA and a slight improvement in the mobility of open circular DNA to a feature where both forms comigrate. The pBR322 presents three forms, OC (open circular), L (linear) and CC (supercoiled), which show different electrophoretic mobility. Fig. 7 shows the effect of ruthenium complexes on the mobility of pBR322 after incubation at 37°C for 18h. The control in lane 2 refers only to pBR322, and the lines 3–11 correspond to the pBR322 in the presence of complexes (3) and (4). The complexes were capable of affecting the mobility of the bands of pBR322, mainly to OC

![Image of Inhibitory effects of the complex (4) on DU-145 cells migration in vitro.](image)

Fig. 4. Inhibitory effects of the complex (4) on DU-145 cells migration in vitro. (A) Representative photographs taken at 0, 24 and 48 h post-wound using inverted microscope (amplification 100 x), scale bar = 200 μm. The cell monolayer was wounded by a 100 μl pipette tip followed by treatment with different concentrations (0.125, 0.25, and 0.5 μM) of (4) for 24 h. The distance of the wound edge was measured before and after the treatments. (B) Quantification of the relative migration by wound closure. Wound closure (%) were identified from measurements of the migration distance of DU-145 cells from the control group and with complex (4) treatment. Data are presented as the mean ± S.E.M. of three independent experiments performed in triplicate. *p < 0.05 compared with the control by ANOVA followed by Dunnet’s test.

![Image of Complex (4) significantly decreased DU-145 cells adhesive rate on collagen and Matrigel® matrixes.](image)

Fig. 5. Complex (4) significantly decreased DU-145 cells adhesive rate on collagen and Matrigel® matrixes. DU-145 cells in the presence of 0.125, 0.25 and 0.5 μM were seeded into 96 well plates coated with collagen (A) and Matrigel® (B) and allowed adhere for 24 h after which supernatants were removed and adhered cells washed and stained with crystal violet. Plates were assayed on a plate reader at 590 nm. Data are presented as the mean ± S.E.M. of three independent experiments performed in triplicate. Values not sharing the same letter are significantly different from each other (p < 0.05; ANOVA followed by the Tukey’s test).
Apoptosis is acknowledged as an essential physiological process in tissue homeostasis. Nevertheless, some cancer cells may adapt to high levels of oncogenic signaling by disabling their senescence- or apoptosis-inducing circuitry. This acquired resistance is appreciated as a hallmark of almost all types of cancer [58,59]. To evaluate whether the complex (4) presents the ability to induce apoptosis or necrosis, we selected its concentrations that have shown cytotoxic activity. Therefore, nuclear morphology was assessed by Hoechst 33342/propidium iodide (PI) staining technique for fluorescence microscopy. The results clearly showed that complex (4) might induce apoptosis of DU-145 cells based on its staining and morphological characterization after treatments (Fig. 9A).

To determine the percentage of apoptosis and necrosis, the DU-145 cells were treated with 0.25–2.0μM of the complex (4) for 24 h followed by annexin V-FITC/PI staining, and the results were analyzed by using flow cytometry. In live cells, phosphatidylserine (Ptd-L-Ser) is located on the cytoplasmic surface of the cell membrane. The exposure of Ptd-L-Ser on the outer leaflet of the plasma membrane is the final event of apoptosis and can be detected by the binding of fluorescently labeled annexin V [60]. Therefore, annexin V-FITC/PI dual staining can be used to quantify the different cell phenotypes that occur during apoptosis. The percentages of early apoptotic, late apoptotic, necrotic and living cells of DU-145 are shown in Fig. 9B. The incubation of DU-145 cells with increasing concentrations of the complex (4) produced a considerable increase in the percentage of apoptotic cells (Q2 and Q4 quadrants) (Fig. 9C). The population of apoptotic cells treated with complex (4) had increased remarkably with dose-dependent relation (6.30% for control group, 19.1% for 0.25μM, 30.9% for 0.50μM, 45.1% for 1.0μM and 66.8% for 2.0μM). Cisplatin treatment at the fixed concentration of 25μM (IC50) showed 19.5% of apoptotic cells. Therefore, a 100-fold lower concentration of (4) was required for the same effect of cisplatin on DU-145 cells. None of the concentrations induced a significant increase in necrotic cells. Indeed, ruthenium complexes with different ligands have been lead to the apoptosis through the mitochondria, death receptor and oxidative stress pathways [34,61,62]. The molecular mechanism underlying the apoptotic effect of the complex (4) was assessed at the level of mRNA expression of pro-apoptotic and anti-apoptotic genes. The 24 h treatment with complex (4) at 1.5 μM (IC50) upregulated BAX expression by 2.73-fold and downregulated the expression of the gene BCL-2 by 2.40-fold (Fig. 9D). BAX and BCL-2 are two members of a gene family involved in the regulation of cellular apoptosis. Gene expression analysis revealed that complex (4) induced an increase of the expression of the pro-apoptotic BAX gene and a decrease of the expression of the anti-apoptotic BCL-2. Cells with a high BAX/BCL-2 ratio will be more sensitive to given apoptotic stimuli when compared to a similar cell type with a comparatively low BAX/BCL-2 ratio [63]. Therefore, the altered ratio might be an essential key question to understand the sensitizing effect
of the complex (4) in DU-145 cells. Thus, our results demonstrated that complex (4) induced cell death by up-regulation of BAX and down-regulation of BCL-2 in DU-145 cancer cells.

2.7. Scavenging intracellular ROS abolishes the cytotoxic effects of the complex (4)

It has been reported that ROS play an important role in cancer cell death and apoptosis. This activity is widely induced by several chemotherapeutic drugs to kill the cancer cells [64]. To investigate whether ROS production is underlying the cytotoxic activities of the complex (4), we used the Cellular ROS/Superoxide Detection Assay (ab139476) to detect the production of reactive species of oxygen and nitrogen (ROS/RNS) in DU-145 cells. Previously, the total ROS levels were assessed after a 1, 6, 12 and 24 h of incubation with the complex (4) using 2′,7′-dichlorodihydrofluorescein diacetate (H$_2$DCFDA) as a fluorescent dye to determine which time would be more appropriate for a ROS/Superoxide analysis.

As shown in Fig. 10A, upon treatment of DU-145 cells for different times with a single dose of the complex (4) (IC$_{50}$), a higher increase of ROS generation was observed at 24 h of exposure. Due to this result, the ROS/Superoxide production was assessed after 24 h of treatment with
different concentrations of the complex (4). After the complex (4) treatment, DU-145 cells exhibited an increase of reactive species, yielding a fluorescent product indicative of cellular production of different ROS/RNS types (Fig. 10B). Moreover, at 4μM was observed a decrease of ROS/RNS levels, probably because increasing concentrations promotes cell death. These results indicate that complex (4) can enhance intracellular oxidative stress suggesting that apoptosis may be the principal mechanism of cell death triggered by ROS. This effect can be guaranteed by the use of lawsone that has a mechanism of action closely related with their electron accepting capability producing semiquinone radicals that can induce ROS production in the intracellular environment [65]. On the other hand, the production of superoxide did not appear in any treatment of the complex (4), except in inducer control (pyocyanin).

Besides, to verify whether or not ROS/RNS generation increase is related to the complex (4)-induced cytotoxicity, the cells were treated with the ROS-scavenger N-acetyl-L-cysteine (NAC), an antioxidant that has been demonstrated to present cytoprotective effects against ROS-induced cell death [66]. The results displayed that co-treatment with NAC suppressed the generation of intracellular ROS induced by complex (4) (Fig. 10C) and prevented the reduction of cell viability, as assessed by Alamar Blue assay (Fig. 10D). These findings suggest that the complex (4) played an essential role in inducing apoptosis, just as other naphthoquinones derivatives compounds do [67], by induced increased oxidative stress, which was prevented by co-treatment with NAC.

3. Conclusions

In this paper, novel lawsone-containing ruthenium(II) complexes were synthesized and assessed for its cellular response in human prostate carcinoma DU-145 cells in 2D and 3D culture systems for the first time. As discussed, other studies have shown that ruthenium complexes have been extensively reported as anticancer candidates in vitro and in vivo [6,68,69]. Herein we proposed lawsone as a bioactive natural compound that forms ruthenium complexes with potent cytotoxic activity in cancer cells. Overall, the phosphine complexes studied in this work showed promising values of IC50 against DU-145 prostate cancer cells. Complexes (3) and (4) displayed more potent cytotoxicity than metal-free lawsone against different cancer cell lines and can decrease the survival of DU-145 cells at lower concentrations better than cisplatin. Several studies shown the addition of phosphine ligand increase the lipophilicity of the complex, which in turn enhances the cytotoxic effect against several cancer cell lines [70–73]. This effect is related with the enhanced lipophilicity which considerably affecting the uptake by tumor cells and proteins binding [41,74]. Our ruthenium complexes, especially the complex (4), exhibited considerable cytotoxic effects against 3D multicellular DU-145 spheroids that simulate the prostate tumor microenvironment. The compound also exhibited cytotoxic potency over 18-fold orders of magnitude higher than cisplatin in monolayers of DU-145 cancer cells and for 3D multicellular DU-145 tumor spheroids. Even under a low non-cytotoxic concentration of 0.125–0.5μM, (4) was able to effectively inhibit the cell adhesion and
migration of DU-145 cells indicating antimitastatic potential in vitro. Taken together, all the complexes (1–4) showed an effective DNA interactive abilities via DNA minor groove intercalation, which can be considered very valuable taking into account the importance of the history and development of DNA intercalators as anticancer agents. Additionally, complex (4) induced DU-145 cell apoptosis through elevated intracellular ROS levels. Moreover, we also showed that ROS scavenger NAC reduced cytotoxicity induced by ROS production caused by the complex (4). In summary, this study describes a series of newly synthesized phosphate/diimine ruthenium complexes containing the 1,4-naphthoquinone-containing ruthenium complexes showed a promising anticancer activity better than cisplatin ratio. Therefore, the 1,4-naphthoquinone-containing neutral ruthenium complexes showed a promising anticancer activity better than cisplatin toward prostate cancer cells, target the intracellular oxidative stress.

4. Experimental

4.1. Materials

4.1.1. Chemical and equipments

4.1.2. Cell lines and culture conditions

4.2. Methods

4.2.1. Synthesis and characterization of the ruthenium complexes

4.2.2. Cell lines and culture conditions

4.2.3. Materials

4.2.4. Methods
v). 80 μL of triethylamine and KPF6 (0.28 mmol) were added and the reaction kept in reflux under argon atmosphere for 12 h. The volume was reduced to ca. 5 mL and water was added to precipitate a dark solid.

\[ \text{[Ru(law)(dppm)(bipy)]PF}_6 (3): \text{Yield: } 0.129 \text{g (82%).} \]

Analy. Calc. for C_{26}H_{34}F_{6}N_{2}O_{3}P_{3}Ru exp. (calc.) C, 56.26 (56.31); H, 3.85 (3.68); N, 2.65 (2.92) %. M_{r} = 5443. \text{M}_{\text{w}} = 48.0 \Omega^{-1} \text{cm}^{2} \text{mol}^{-1}, \text{in } 1.0 \text{mM CH}_{2}Cl_{2} \text{solution. IR (Selected bands, cm}^{-1}) = \text{NMR (400MHz, DMSO-}\text{d}_{6}, 25 ^\circ \text{C): } \delta(\text{ppm}): 9.2–6.3 (3.2H, 2H, aromatic of dppm); 13C NMR (400MHz, DMSO-}\text{d}_{6}, 25 ^\circ \text{C): } \delta(\text{ppm}): 9.2–6.3 (3.2H, 2H, aromatic of dppm); 13C NMR (400MHz, DMSO-}\text{d}_{6}, 25 ^\circ \text{C): } \delta(\text{ppm}): 200.3/196.8 (C_{1}=O), 182.5/181.9 (C_{4}=O) and 172.4/171.2 (C_{2}=O).

\[ \text{[Ru(law)(dppm)(phen)]PF}_6 (4): \text{Yield: } 0.104 \text{g (78%).} \]

Analy. Calc. for C_{31}H_{30}F_{6}N_{2}O_{3}P_{3}Ru exp. (calc.) C, 56.26 (56.31); H, 3.85 (3.68); N, 2.65 (2.92) %. M_{r} = 5443. \text{M}_{\text{w}} = 48.0 \Omega^{-1} \text{cm}^{2} \text{mol}^{-1}, \text{in } 1.0 \text{mM CH}_{2}Cl_{2} solution. IR (Selected bands, cm}^{-1}) = \text{NMR (400MHz, DMSO-}\text{d}_{6}, 25 ^\circ \text{C): } \delta(\text{ppm): 9.2–6.3 (3.2H, 2H, aromatic of dppm); 13C NMR (400MHz, DMSO-}\text{d}_{6}, 25 ^\circ \text{C): } \delta(\text{ppm): 200.3/196.8 (C_{1}=O), 182.5/181.9 (C_{4}=O) and 172.4/171.2 (C_{2}=O).}

4.2.2. Partition coefficient (n-octanol/water) determination

The octanol-water partition coefficient (log P) were determined using the shake-flask method [78]. Each complex was tested in a mixture of equal volumes of water and n-octanol with continuous shaking for 24 h at 112° and 37°. Then the samples were centrifuged for 5 min at 10g, and the organic and aqueous phases were separated. The concentration of drug in each phase was measured spectrophotometrically at 10 g, and the organic and aqueous phases were separated. The concentration of drug in each phase was measured spectrophotometrically at 10 g, and the organic and aqueous phases were separated. The concentration of drug in each phase was measured spectrophotometrically at 10 g, and the organic and aqueous phases were separated. The concentration of drug in each phase was measured spectrophotometrically at 10 g, and the organic and aqueous phases were separated. The concentration of drug in each phase was measured spectrophotometrically at 10 g, and the organic and aqueous phases were separated. The concentration of drug in each phase was measured spectrophotometrically at 10 g, and the organic and aqueous phases were separated. The concentration of drug in each phase was measured spectrophotometrically at 10 g, and the organic and aqueous phases were separated.

4.2.3. Cytotoxicity assay

The in vitro antiproliferative activity was quantified using the Alamar Blue assay, according to the method reported by [79]. Cells were inserted in 96-well plates for all experiments (1.5 × 10^5 cells/well). After 24 h, the complexes were dissolved in DMSO and added to each well and incubated for 24 h. Dilutions of the complexes were prepared to obtain concentrations ranging from 0.3 to 100 μM. Cisplatin (Faulds cisplatin) and doxorubicin hydrochloride (Fauldoxorubicin) were used as the reference cytotoxic drugs. DMSO (0.1% v/v) was used as the vehicle control. Following 24 h of incubation, 50 μL of Alamar Blue (resazurin at 0.01% w/v) was added to each well, and the plates were incubated for 2 h, at 37 °C, in the dark. The fluorescence reading was performed in a Cary Eclipse Fluorescence Spectrophotometer (Agilent Technologies, Santa Clara, CA, USA) reader using excitation and emission filters at wavelengths of 560 and 590 nm, respectively. The potency of cell growth inhibition for each complex was expressed as half-maximal (50%) inhibitory concentration (IC_{50}).

4.2.4. Clonogenic assay

The clonogenic assay was performed in accordance with the guidelines of [43]. The DU-145 cells (1 × 10^5 cells/well) were inserted in 6-well plate and treated with different concentrations varying of 0.125 to 1 μM of the complexes and cisplatin for 24 h. Negative control received the vehicle (DMSO 0.1% (v/v)) that was used for diluting the compounds tested. At the end of the treatments, the cells were washed with PBS and harvested by trypsinization. The cells were counted using a Neubauer chamber and re-plated then on Nunclon® cell culture dishes of 21.5 cm² in a density of two hundred cells per culture dish for each treatment in triplicate. After 7 days cells were washed with PBS and fixed in ice-cold methanol: acetic acid: distilled water (1:1:8) for 20 min, air-dried and stained with crystal violet (0.5% (w/v)) in PBS for 20 min. Colonies with at least 50 cells were counted. The plating efficiency (PE) of DU-145 cells was calculated by the ratio of the number of colonies observed after 7 days of incubation to the number of cells seeded (two hundred). The average of colonies in control was regarded as 100% and the number of colonies that arise after treatment of cells, expressed regarding PE, is called the surviving fraction (SF), obtained by:

\[ SF = \frac{\text{Number of colonies counted in each treatment}}{\text{Number of colonies counted in control}} \times 100 \]

4.2.5. 3D multicellular tumor spheroids culture

The multicellular tumor spheroids (MCTS) were obtained according to the method reported by [80]. Briefly, 200 μL of a solution of DU-145 cells (2.0 × 10^5 cells/well) were inserted in agarose-coated 96-well plates (1.5% (w/v)) and cultured in complete medium plus 3% Matrigel®. DU-145-MCTS with stable structures had formed after five days. Then, the spheroids were exposed to the complexes in a range of five different concentrations varying from 1 to 10 μM and incubated for 24 h. Cisplatin (Faulds cisplatin) at 25 μM was used as the reference cytotoxic drug. Negative control received the vehicle (DMSO 0.1% (v/v)) that was used for diluting the compounds tested. At the end of the experiment, the cell viability was determined by Alamar Blue assay as described above. To investigate the morphological changes after treatment, other MCTSs were examined for 12 days by light microscopy (Inverted Trinocular Microscope Opton TBN-OST-PL) using AxioVision LE software (Carl Zeiss, Jena, Germany).

4.2.6. Wound healing migration assay

DU-145 cells were seeded at a density of 1 × 10^5 cells per well in 12-well plates and grown for 48 h in 5% CO₂ in a humidified atmosphere at 37 °C in serum-free media. Once confluent, a “wound” was created across the cell monolayer using a sterile pipette. The culture medium containing cells in suspension was removed and replaced with new media containing different concentrations of the complex (4). Negative control received the vehicle (DMSO 0.1% (v/v)) that was used for diluting the compounds tested. Images of each wound were recorded using an inverted trinocular microscope (Opton TBN-OST-PL) coupled to a camera (AxioVision) with amplification of 10 ×, at different times (0–24 h). The wound closure was analyzed by the recorded images using Image J software.

4.2.7. Cell adhesion

DU-145 cells were seeded at a density of 1.5 × 10^5 cells per well in the presence or absence of different concentrations of the complex (4) in pre-coated 96-well plates with either 15 μg/mL collagen or Matrigel® 150 μg/mL and left for 24 h at 5% CO₂ in a humidified atmosphere to adhere. Negative control received the vehicle (DMSO 0.1% (v/v)) that was used for diluting the compounds tested and, cell-free coated wells acted as blank controls. Following adhesion, cells were washed 3x with PBS and fixed with 100 μL methanol at −20 °C for 5 min. Once removed, cells were stained for 15 min with crystal violet (0.1% (w/v)) after which they were rinsed with tap water. Following 10 min air-drying, 50 μL of 0.5% Triton X-100 was added to wells and left 2 h at room temperature with gentle agitation. Finally, the absorbance was measured in a spectrophotometer (Biokon Eks800–Winooski, VT, USA) set at 590 nm.

4.2.8. DNA interactions assays

The CT-DNA solution was prepared by dilution of 2 mg of the CT-DNA in 1 mL of Tris-HCl buffer (4.5 mM Tris-HCl, 0.5 mM Tris-base, 50 mM NaCl, pH 7.4), and the concentration was determined by absorption spectrophotometric using the molar absorption coefficient 6600 mol⁻¹ dm³ cm⁻¹ at 260 nm [81].
4.2.8.1. Interaction with CT-DNA (Calf Thymus) by circular dichroism (CD). The interaction of the ruthenium complexes (1–4) with CT-DNA by CD was investigated using a spectropolarimeter JASCO J720 from 240 to 350 nm at 298 K, in a continuous scanning mode (200 nm min$^{-1}$), under constant nitrogen flush. The complexes were incubated with CT-DNA (50 µM) at 37°C for 18 h in Tris-HCl buffer containing 10% of DMSO for solubilization of the complexes, in molar ratios ((complex)/(DNA)) ranging from 0.1 to 0.5.

4.2.8.2. pBR322 plasmid. Plasmid pBR322 (38 µM) was mixed with different concentrations (4.8–38 µM) of ruthenium complexes and incubated at 37°C for 18 h. Then, the loading buffer was added and the samples and analyzed by agarose gel electrophoresis for 90 min in 1% agarose gel using a Tris-acetate-EDTA (TAE) buffer (0.45 M Tris-HCl, 0.45 M acetic acid, 10 mM EDTA, pH 7.4) and employing ethidium bromide for staining. The bands were visualized with a ChemiDoc MP imager (BioRad Laboratories, Hercules, CA, USA).

4.2.8.3. Competitive displacement with Hoechst 33258 by fluorescence. The competitive displacement assay was carried out using as probe the Hoechst 33258 by the fluorescence quenching experiment [57]. Thus, the CT-DNA (125 µM) was incubated with Hoechst (2.7 µM), and the extinction of the fluorescence intensity was monitored by the addition of different concentrations of ruthenium complexes in Tris-HCl containing 10% of DMSO. The fluorescence emission spectra were recorded from 300 to 500 nm after excitation wavelength of 345 nm, using an opaque 96-well plate, in a Synergy/H1-Biotek fluorimeter, at 37°C.

4.2.9. Apoptosis analyses

The measurement of cell death was performed by the guidelines of [82]. Previously, the nuclear morphology of DU-145 cells was assessed by Hoechst-propidium iodide (PI) dual-staining assay. Briefly, the cells were seeded (5 × 10$^3$ cells/well) into black 96-well plates and main-
tained to attach at 37°C in 5% CO$_2$. After 24 h, the cells were treated with complex (4) (0.25, 0.50, 1.0 and 2.0 µM) for 24 h. Negative control received the vehicle (DMSO 0.1% (v/v)) and, positive controls received cisplatin (25 µM) and doxorubicin (5 µM). The cells were incubated with Hoechst 33342 (10 µM) and PI (10 µM) diluted in PBS for 10 min at room temperature. The cells were then observed by ImageXpress Micro XLS system (Molecular Devices, CA, USA). The detection of apoptotic/necrotic cells was determined by flow cytometry using the Alexa Fluor® 488 Annexin V/PI (Molecular Probes®) and the analysis were performed according to the manufacturer’s instructions. Briefly, DU-145 cells were seeded (5.5 × 10$^4$ cells/well) in 12-well plates and maintained to attach at 37°C in 5% CO$_2$. After 24 h, the cells were treated as described above. Then, the cells were collected with accutase, washed with PBS and resuspended in 200 µL of cold annexin-binding buffer. Next, it was added 10 µL of Alexa Fluor® 488 Annexin V (50 µL/mL) staining buffer, and the mixture was incubated in the dark at 4°C for 15 min. Previously the analyses, 100 µL of PI (2 µg/mL) was added, and then, the fluorescence was measured by flow cytometry in a FACS Canto flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) using the Diva software. Ten thousand events were evaluated per experiment, and cellular debris was omitted from the analysis.

4.2.10. Gene expression analysis by qPCR array

DU-145 cells were plated in bottles tissue culture (1x10$^5$ cells/mL). After 24 h of incubation with complex (4) at IC$_{50}$ total RNA was isolated from the cells using the RNeasy Plus mini kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. Total RNA was quantified using a spectrophotometer at an absorbance of 260 nm in NanoDrop ND-2000 (Thermo-Fisher Scientific, USA) and the quality of each RNA sample was verified by gel electrophoresis in a BioAnalyzer (Agilent Inc). RNA purity was calculated from the ratio of absorbance A$_{260}$/A$_{280}$ and the integrity by RIN (RNA Integrity Number). Complementary DNA (cDNA) strands were synthesized using the RT² First Strand kit (Qiagen, Germany), following the manufacturer’s instructions. Quantitative PCR was carried out with the Power SYBR® Green Master Mix in a Step One Plus system (Applied Biosystems, Carlsbad, CA, USA), using KGCqStart® SYBR® Green Primers prede-
signed for gene expression for mRNA analysis (Sigma-Aldrich), as follows: HACTB1 (NM_001101), HBAX1 (NM_004324) and HBCL21 (NM_000633). Quantification of relative fold change of each gene was calculated by 2$^{-ΔΔCt}$ method [83] using the reference gene ACTB (actin beta) for normalization. All experiments were performed in triplicate in DNase/RNase free conditions.

4.2.11. Detection of intracellular ROS and RNS

The total intracellular ROS generation was measured using H2DCFDA, as reported by [84]. Briefly, DU-145 cells were seeded (1.5 × 10$^5$ cells/well) into black 96-well plates and maintained to attach at 37°C in 5% CO$_2$. After 24 h, the cells were treated with 1.5 µM (IC$_{50}$) of the complex (4) for 1, 6, 12 and 24 h. After that, the cells were labeled with H2DCFDA solution at 5 µM in DMF. After that, the fluores-
cence intensity was measured in a Synergy H1 Fluorescence Spect-
rophotometer (BioTek®), at the excitation and emission wavelengths of 495 and 527 nm, respectively. After that, the ROS/RNS levels were measured using Cellular ROS/Superoxide Detection Assay Kit (Abcam). The cells were seeded (1.5 × 10$^4$ cells/well) into black 96-well plates and maintained to attach at 37°C in 5% CO$_2$. After 24 h, the cells were treated with complex (4) (0.25, 0.50, 1.0 and 2.0 µM) for 24 h. Negative control received the vehicle (DMSO 0.1% v/v), and positive control received pyocyanin (250 µM) as ROS inducer. Following this, the cells were labeled with Oxidative Stress Detection Reagent (Green) for ROS detection and Superoxide Detection Reagent (Orange) according to the manufacturer’s instruction. Fluorescence was then measured in quadruplicates using a fluorescent microplate reader Synergy H1 (BioTek®) with standard fluorescein (Ex = 488 nm, Em = 520 nm) and rhodamine (Ex = 550 nm, Em = 610 nm) filter sets for ROS and superoxide de-
termination, respectively.

The protection assays using the antioxidant NAC were also per-
formed. In brief, the cells were treated with 5 mM NAC in association with complex (4) (0.25, 0.50, 1.0 and 2.0 µM) for 24 h. The cells were then labeled with H2DCFDA solution, and the ROS levels were mea-
sured as described above. In a new set of experiments, the cells were trea-
ted with 5 mM NAC in association with cytotoxic concentrations of (4). After 24 h of treatment, the cell viability was determined by Alamar Blue assay as described above.

4.3. Statistical analysis

Data are presented as mean ± S.E.M. or as IC$_{50}$ values with 95% confidence intervals (CI 95%) obtained by nonlinear regression. Differences between experimental groups were compared using analysis of variance (ANOVA) followed by the Dunnet’s or Tukey’s test (p < 0.05). All statistical analyses were performed using GraphPad Prism 7 for Mac OS X (Intuitive Software for Science, San Diego, CA, USA).

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