Cytotoxic Activity of Metal Complexes of Biogenic Polyamines: Polynuclear Platinum(II) Chelates

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Several polynuclear Pt(II) chelates with biogenic polyamines were synthesized and screened for their potential antiproliferative and cytotoxic activity in different human cancer cell lines. To gather information regarding the structure–activity relationships underlying their biological activity, the complexes studied were designed to differ in geometrical parameters such as the nature of the ligand and the number and chemical environment of the metal centers. Distinct effects were found for different cell lines and different structural characteristics of the complexes; chelates II, III, and IV displayed specificity toward the HeLa and HSC-3 epithelial-type cells, while V, VI, and VII were clearly more effective against the THP-1, MOLT-3, and CCRF-CEM leukemia cell lines. The toxicity of these Pt(II) complexes on noncancer cells was, in all cases, found to be reversed upon drug removal.

Introduction

Platinum-based antitumor drugs have been the target of intense research since Rosenberg’s discovery of an unexpected inhibition of cell division by platinum complexes in the late 1960s. It is presently well known that the antitumor properties of these kinds of compounds are based on a selective interaction with DNA inside the cells. Despite its well-established anticancer properties, cisplatin (cis-diaminedichloroplatinum(II), CDDP), one of the most widely used anticancer drugs, presents several drawbacks, namely, severe toxicity and development of resistance in certain types of cancer cells. Therefore, the search for structurally novel platinum compounds displaying antitumor activity is crucial, aiming at the design of more efficient, less toxic drugs (by avoiding undesired reactions within the cell) that are non-cross-resistant with first- and second-generation agents such as cisplatin and carboplatin (cis-diamine(cyclobutane-1,1-dicarboxylato)platinum(II)).

Multinuclear Pt(II) complexes, containing two or three cisplatin-like metal centers and variable length biogenic polyamines as bridging linkers, constitute a new class of platinum-based, third-generation drugs of great potential clinical importance. These compounds, which have been the subject of intense study for the past few years, were found to be distinct from their mononuclear counterparts in terms of DNA binding features, yielding adducts containing “long-distance” intra- and interstrand cross-links not available to the conventional mononuclear platinum complexes. Moreover, these polynuclear metal complexes have often been reported to overcome acquired cisplatin resistance probably because of a distinct mechanism of DNA interaction that results in different types of drug-induced DNA lesions. The presence of more than one metal center in these Pt(II) multifunctional chelates is thus expected to lead to a higher efficacy and specificity regarding DNA binding (e.g., increased number of interstrand cross-links, possibly at more than one position). In addition, each type of polynuclear platinum structure (e.g., number and nature of the leaving groups and their geometry relative to the bridging amines) has been shown, in recent studies, to have its own particular cytotoxic characteristics (e.g., triplatinum BBR3464, currently in phase III of clinical trials). In fact, it is known that a simple chemical modification in the structure of a certain compound may alter its DNA binding properties and/or the relative amounts of interstrand cross-links in double-stranded DNA, thus governing its antitumor activity. It should then be possible to functionalize DNA-binding modes to obtain compounds displaying not only selective but also enhanced and unique anticancer properties.

The present work aims at screening for potential antiproliferative and cytotoxic activities of several di- and trinuclear Pt(II) complexes in different types of human cancer cells (leukemia and epithelial-like adherent cell lines), following a previous study on similar systems. These chelates, containing the biogenic polyamines spermidine (H3N(CH2)3NH(CH2)4NH2) and spermine (H3N(CH2)4NH(CH2)4NH(CH2)4NH2) as bridging linkers, differ in the metal coordination pattern, as well as in the number and geometry of their leaving groups. This is intended as an interactive structure–activity research work, the structural characteristics of the compounds tested (determined in a concurrent study...
through physical—chemical methods\textsuperscript{32–36}) being changed according to their antitumor activity evaluation. Thus, the results obtained from these studies may hopefully contribute to a better understanding of the molecular basis of toxicity (i.e., of the biochemical processes underlying the antiproliferative effect of these kinds of compounds), which is fundamental in order to design new cytostatic agents with improved therapeutical properties.

\textbf{Results and Discussion}

The distinct complexes tested for their antitumor activity differ mainly in the number and chemical environment of the Pt(II) centers (number and geometry of the leaving groups), as well as in the nature and coordination mode of the amine linkers (Figure 1). The flexibility, charge, and hydrogen-bonding capabilities of these kinds of compounds determine their interaction with DNA. The present results may be interpreted as reflecting these structure–activity relationships, although they do not constitute direct evidence of a drug–DNA interaction. It should be emphasized that, apart from covalent bonding to the DNA double helix, both electrostatic interactions and formation of hydrogen bonds\textsuperscript{37} play a very important role in the mechanism of interaction of these kinds of cationic polyamine metal complexes with DNA, which determines their antiproliferative and cytotoxic action. According to Cleare and Hoeschele’s structure–activity relationships (SARs) for platinum(II) complexes,\textsuperscript{38,39} for such compounds to display significant antitumor activity they should have a cis geometry with the general formula \(\text{cis-PtX}_2(\text{Am})_2\), Am being an inert amine containing at least one NH moiety and X being an anionic leaving group with a weaker trans effect than the amine. All the complexes reported in the present work comply with these conditions except for the number of X groups (which is, in some of the chelates studied, only 1). However, it should be noted that conformity to these SAR rules is not essential for cytostatic activity to occur, since it was recently found that novel trans-dichloroplatinum(II) complexes also exhibit significant antitumor activity.\textsuperscript{40}

Among the complexes presently studied, both II and IV are dinuclear 2,2/c,c Pt(II) chelates;\textsuperscript{24} i.e., they comprise two difunctional moieties at each metal center, in a cis position relative to the bridging ligand(s). However, while II has two distinct polyamine (spermidine) molecules, each one coordinated to a different platinum ion, IV contains only one tetraamine ligand (spermine) acting as a linker for both metal centers, yielding two identical six-membered intramolecular rings in a trans orientation relative to each other (Figure 1). Compound III, in turn, is a trinuclear (2,2,2/c,c,c) chelate containing (as in species II) two spermidine units as ligands. However, while in complex II these molecules act as bidentate N,N’-donor ligands, yielding two six-membered rings through coordination to each of the two metal ions, in III all three polyamine nitrogen atoms coordinate to the three platinum(II) centers, which may somewhat affect the motional freedom of the whole compound. Thus, although both II and III are difunctional relative to each Pt(II), the former has two cisplatin-like structures (\(\text{PtCl}_2(\text{NH}_2\text{CH}_2\text{NH}_3)_2\)) \((n = 1 \text{ or } 2)\), as opposed to III which has three, yielding (upon hydrolysis) respectively four and six possible coordination sites to the DNA helix. Furthermore, the terminal protonated

![Figure 1. Schematic representation of the polynuclear polyamine Pt(II) complexes studied in this work.\textsuperscript{28}](image_url)
(cationic) amino groups of the spermidine linkers in II are free to eventually interact electrostatically with DNA. Compound III, in turn, displays a longer, stretched geometry, which may also be favorable for interacting with the DNA backbone. As for complexes V, VI, and VII, they share a particular conformational feature that renders them quite rigid; they comprise two identical units, where the metal center coordinates to all three nitrogen atoms of the bridging spermidine-like amine, resulting in a nearly cyclic structure defining two intramolecular rings whose dimensions are determined by the length of the \(-\text{N}-(\text{CH}_2)_n-\text{N}-\) moieties of the polyamine linker.

From the dose- and/or time-response curves (cell density and viability variation as a function of the incubation time with the drug) plotted for the distinct complexes and cell lines studied (for different drug concentrations), it is possible to relate the conformational preferences of these compounds to their antitumor activity and to learn from factors such as reversibility and specificity of their growth-inhibition and/or cytotoxic effect. The clinically used drug cisplatin was considered for comparison purposes in all the experiments carried out in this work except in the case of the HCS-3 cells, for which carboplatin (CBP) was used, since it is the most suitable chemotherapy agent for this particular line. The results obtained through both the MTT and the Alamar blue methods were found to be in very good agreement, as well as to sustain the conclusions yielded by the Trypan blue assays.

**Figure 2.** Time dependence of the antiproliferative effect of compounds I–VII (100 \(\mu\)M) toward all the cell lines tested. Cells \((3.0 \times 10^5\) cells/mL) were incubated with the drugs for periods of 24–72 h. Every 24 h, aliquots of the cell suspensions were removed and the cell density was evaluated by the Trypan blue exclusion method (as described in the Experimental Section). In addition, the drug was removed 72 h after seeding and the cell density was assessed following a further incubation of 72 h. The data represent the average \(\pm\) mean standard deviation from experiments carried out in triplicate: (A) HeLa; (B) HSC-3; (C) THP-1; (D) MOLT-3; (E) CCRF-CEM; (F) L-132. For intergroup comparison between the different compounds, \(p < 0.05\). For intergroup comparison between cell lines, the asterisk (*) indicates \(p < 0.01\). Values for cisplatin (CDDP) and carboplatin (CBP) are included for comparison. Cell densities for the control are as follows: 24 h, \(6.7 \times 10^5\) cells/mL; 48 h, \(9.8 \times 10^5\) cells/mL; 72 h, \(15.4 \times 10^5\) cells/mL; 144 h, \(25 \times 10^5\) cells/mL.)
general, it was verified that chelates IV–VII were particularly effective against leukemia cells MOLT-3 and THP-1, while the trinuclear complex III displayed a rather high antiproliferative and cytotoxic activity toward the epithelial-like lines HeLa and (to a smaller extent) HSC-3. Thus, a selectivity of action was observed, related to the conformational characteristics of the compounds studied versus the kind of cancer cells tested; the cyclic-like, more rigid chelates displayed a clear preference for leukemia, as opposed to the linear, cis-oriented, flexible complexes. Concerning the results on fibroblasts, all the complexes studied displayed a reversible effect and consequently a rather low toxicity.

Table 1 comprises the values of 50% inhibitory concentration (concentration of drug yielding a 50% decrease in either cell density or cell viability, IC_{50}) measured for the distinct complexes investigated. Com-

Table 1. Cytotoxic Potency (IC_{50}, M) of the Pt(II) Complexes I–VII on the Different Cell Lines Tested.

<table>
<thead>
<tr>
<th>complex</th>
<th>HeLa</th>
<th>HSC-3</th>
<th>THP-1</th>
<th>MOLT-3</th>
<th>CCRF-CEM</th>
<th>L-132</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>20.0</td>
<td>96.0</td>
<td>130.0</td>
<td>128.0</td>
<td>125.0</td>
<td>100.0</td>
</tr>
<tr>
<td>II</td>
<td>13.0 (13.0&lt;sup&gt;a&lt;/sup&gt;)</td>
<td>20.0</td>
<td>97.0</td>
<td>90.0</td>
<td>83.0</td>
<td>104.0</td>
</tr>
<tr>
<td>III</td>
<td>5.0  (5.0&lt;sup&gt;a&lt;/sup&gt;)</td>
<td>11.0</td>
<td>30.0</td>
<td>50.0</td>
<td>78.0</td>
<td>120.0</td>
</tr>
<tr>
<td>IV</td>
<td>15.0</td>
<td>20.0</td>
<td>35.0</td>
<td>37.0</td>
<td>30.0</td>
<td>145.0</td>
</tr>
<tr>
<td>V</td>
<td>45.0</td>
<td>10.0</td>
<td>6.0</td>
<td>50.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VI</td>
<td>137.0</td>
<td>12.0</td>
<td>15.0</td>
<td></td>
<td></td>
<td>83.0</td>
</tr>
<tr>
<td>VII</td>
<td>62.00</td>
<td>14.0</td>
<td>18.0</td>
<td></td>
<td>135.0</td>
<td></td>
</tr>
<tr>
<td>cisplatin</td>
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<td>2.0</td>
<td>4.0</td>
<td>1.0</td>
<td>2.0</td>
</tr>
<tr>
<td>carboplatin</td>
<td>3.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Only the values corresponding to an irreversible cytotoxic effect are presented. IC_{50} values were calculated from dose–response curves for a 72 h drug exposure. Data were obtained through independent measurements of cell density and cell viability by both the Trypan blue method and the MTT and Alamar blue assays. From ref 31.
chelate II yielded an IC50 of 13 μM against HeLa cells and of 20 μM for the HSC-3 line (Table 1).

It thus seems that the number of metal centers within the drug determines its antiproliferative and cytotoxic activity, which was found, for the cell lines tested, to increase with the number of Pt(II) ions coordinated to the amine ligand(s) (III vs II and IV; Figure 1, Table 1). The degree of flexibility of the chelate also appears to be important, as well as the orientation of its DNA potential binding sites (either cis or trans relative to the polyamine backbone), a higher flexibility, and a cis localization of the cisplatin-like groups within the complex corresponding to a more effective agent (II vs IV; Figure 1, Table 1). Furthermore, the results obtained for leukemia nonadherent cells and epithelial-like adherent cells reflect a specificity of complex III toward the latter, a quite small antiproliferative and cytotoxic activity being measured for THP-1, MOLT-3, and CCRF-CEM cells, even after prolonged drug exposure (Table 1, Figures 2 and 3), while for HeLa and HSC-3 cells an evident cytotoxicity was observed at early times of drug incubation. Figure 4 illustrates the cytotoxic behavior of chelate III against HeLa cells compared to that toward THP-1 and MOLT-3 lines.

In contrast, the macrocyclic-like complexes V–VII were found to display a clear specificity as antiproliferative and cytotoxic agents toward the leukemia cell lines (Table 1, Figures 2, 3, and 5). Moreover, in most of the cancer cells tested, compound V was shown to be more effective than its analogues VI and VII, which is most probably a consequence of its higher flexibility, since the length of the (CH2)n chains between Pt(II) centers decreases from V to VII (Figure 1).

The degree of reversibility of both the antiproliferative and cytotoxic effects displayed by compounds aimed for use as antitumor drugs is, understandably, of the utmost importance. In fact, consideration of a recovery period following drug exposure, apart from allowing the determination of the reversibility of the drug effect, as well as the measurement of a possible delayed cytotoxicity, avoids over- or underestimation of the level of cell killing achieved. The present results indicate that the antiproliferative and cytotoxic activities of complex III...
are irreversible for all epithelial cell lines tested for concentrations higher than 50 \( \mu \text{M} \), as opposed to the leukemia cells (Figures 2–4). Also, chelate IV displayed irreversible anticancer properties against HeLa and HSC-3 lines (for concentrations equal to or higher than 50 \( \mu \text{M} \)). However, while its cytotoxic effect was found to be reversible toward THP-1 and MOLT-3 leukemia cells (Figure 3), its growth-inhibition properties were shown to be irreversible for these cells (Figure 2). In turn, compound II presented a reversible effect against both HeLa and MOLT-3 cells, even at 100 \( \mu \text{M} \) (this factor having not been assessed in a previously reported work on the cytotoxic activity of II). Chelates V–VII, at both 75 \( \mu \text{M} \) (data not shown) and 100 \( \mu \text{M} \) concentrations, displayed an irreversible antitumor effect against THP-1 and MOLT-3 lines (Figures 2, 3, and 5).

A significant disadvantage of the presently used chemotherapeutic agents is their rather high toxicity, mainly due to side reactions with other biomolecules apart from DNA (e.g., proteins). To evaluate this factor for the complexes now investigated, cytotoxicity experiments were carried out in non-neoplastic cells (human embryonic fibroblasts from lung tissue (L-132 cell line)). The results obtained allowed us to conclude that all the chelates studied present quite a low toxicity against healthy cells (Table 1). Complex V displayed the higher cytotoxic effect toward noncancer cells (IC\(_{50} = 50 \mu \text{M} \)), which was, however, found to be almost totally reversed upon drug removal (Figure 3).

Farrell and co-workers have recently reported the synthesis and antitumor properties of several dinuclear Pt(II) complexes with bridging diamine linkers of varying chain lengths\(^{24,28}\) displaying active participation both in vitro and in vivo even against cisplatin-resistant cancer cell lines. These compounds, containing two PtCl(NH\(_3\))\(_2\) or PtCl\(_2\)(NH\(_3\)) moieties linked by polyamine groups in a cis orientation, can be compared to complex IV presently studied, which comprises two cisplatin-like PtCl\(_2\)(NH\(_3\))(NH\(_2\)) fragments in a trans configuration instead. The IC\(_{50}\) values reported by Farrell for those dinuclear chelates against leukemia cell lines are lower than 1 \( \mu \text{M} \), as opposed to the very high values now obtained for lines THP-1, MOLT-3, and CCRF-CEM cells (Table 1). As previously referred to above, the trans position of the Cl leaving groups relative to the amine backbone and the lower flexibility of complex IV relative to the ones studied by Farrell’s group are probably responsible for a significant decrease of the corresponding antitumor activity. The effect of the degree of flexibility of the compounds tested as anticancer drugs is clearly reflected in the results presently obtained for compound III (IC\(_{50}\) value toward HeLa cells equal to 5 \( \mu \text{M} \) (Table 1)) when compared to the ones reported for the cationic trinuclear Pt(II) chelate BBR3464 (a bifunctional (1,0,1/ttt) type complex of formula [Pt(NH\(_3\))(NH\(_2\))\(_2\)Cl\(_2\) \( \times \) H\(_2\)N(CH\(_2\))\(_6\)NH\(_2\)]\(_2\))\(_2\)Cl\(_2\), which displays a higher (and equally irreversible) cytotoxic potency in similar types of cancer cells (e.g., 0.012 \( \mu \text{M} \) against the A2780 ovarian carcinoma line).\(^{16,17,19,29,30}\)

**Summary and Conclusions**

The time- and dose-dependent growth-inhibition and cytotoxic effects determined for the distinct Pt(II) complexes presently tested against the human epithelial-type and leukemia cancer cell lines HeLa, HSC-3, THP-1, MOLT-3, and CCRF-CEM, as well as toward the L-132 noncancer cells, allowed us to gain a better insight into the structure–activity relationships governing this antitumor activity.

Some of the chelates studied were found to behave as rather efficient antiproliferative and cytotoxic agents, their effect on healthy cells being reversible upon drug removal. Complexes II–IV revealed a distinct specificity toward adherent cancer cells, while V–VII exhibited this behavior against leukemia cells. In fact, complex III was particularly effective on HeLa cells (corroborating previously reported results\(^{31}\)), as well as against the HSC-3 cancer cell line.

From the results now obtained, it is possible to conclude that the conformational preferences of the complexes studied (under physiological conditions), namely, the nature of the amine ligand(s) (e.g., the number of N atoms), the characteristics of the leaving groups at platinum (chlorine atoms in the compounds now tested), the number and coordination mode of the metal ion(s), and their chemical environment (which basically contains a di- or triamine and a mono- or dichloro moiety), determine their antitumor activity, most probably through induction of DNA structural rearrangements. Thus, the design of new, more effective anticancer drugs should be governed by these crucial factors, since slight changes in either the polyamine conformation or the metal coordination are sufficient to significantly change the in vitro anti proliferative and/or cytotoxic properties of these kinds of compounds. Furthermore, some relevant criteria for future, third-generation Pt(II) drugs should also be considered, namely, good water solubility, stability under physiological conditions, absence of undesired reactions, and optimized hydrolysis reactions within the cell. Tissue specificity and low toxicity toward healthy cells are also goals expected to be achieved in the near future.

**Experimental Section**

**Chemicals.** Culture media (DMEM-HG and RPMI-1640), antibiotics (penicillin–streptomycin 100× solution), carboplatin, cisplatin, EDTA (ethylenediaminetetraacetic acid, disodium salt, dihydrate), glutamine, HEPES, MITT, phenol red (phenolsulfonphthalein), isopropanol alcohol, Trypan blue (0.4% solution, prepared in 0.81% sodium chloride and 0.06% dibasic potassium phosphate), trypsin, trisodium citrate, inorganic salts, and acids (of analytical grade) were purchased from Sigma-Aldrich Chemical Co. (Madrid, Spain). Fetal calf serum was obtained from Biolog KM & Berlin. Alamar blue was acquired from Accurate Chemical & Scientific Corporation, Westbury, NY.

**Cells.** The following human cell lines were used: epithelial-like adherent cell lines—human cervix adenocarcinoma (HeLa) and squamous tongue epithelioma (HSC-3); leukemia—lymphoblastic leukemia (CCRF-CEM), acute lymphoblastic leukemia (MOLT-3), and acute monocytic leukemia (THP-1); fibroblasts from embryonic lung tissue—L-132. HeLa, THP-1, and MOLT-3 were purchased from the European Collection of Cell Cultures (ECACC, U.K.). HSC-3 and CCRF-CEM were obtained from the American Type Culture Collection (ATCC, MD). L-132 was kindly offered by the Centre of Experimental Medicine and Surgery of the University General Hospital Gregorio Marañón, Madrid, Spain.

**Physical Measurements.** Synthesized compounds were identified by FTIR spectroscopy. Spectra were recorded on an ATI Mattson Genesis series FTIR spectrometer, using potassium bromide disks. The elemental analysis of the complexes.
were carried out at the Microanalysis Laboratory, Chemistry Department, University of Manchester and at the Butterworth Laboratory, Middlesex, U.K.

Synthesis. The Pt(II) complexes studied in this work were the following: (H₂spd)₂[PtCl₂(I)], (H₂spd)[PtCl₂]₄(II), (PtCl₂)₂(spd) (III), (PtCl₂)[spd] (IV), [PtCl₂(NH₂CH₃)₂(NH₂CH₃)₂]₄[V] (V), and [PtCl₂(NH₂CH₃)₂(NH₂CH₃)₂]₄[VII] (VI) (Figure 1) (spd = spermidine, NH₂CH₃CH₂NH₂CH₃NH₂; sp = spermine, NH₂CH₃CH₂NH₂CH₃NH₂). They were synthesized as reported earlier (I, II, III, IV, V, and VII) or following experimental procedures developed in our laboratory (VI). In a 100 mL round-bottom flask, K₂PtCl₄ (1.00 g, 2.41 mmol) was dissolved in water (30 mL), and to this solution dipropylethenediamine (1.29 mL, 9.20 mmol) was added. The pH of the solution was adjusted to ca. 3 by the addition of 1 M HCl, which was followed by further refluxing for 6 h. The red solution was filtered, concentrated to 4 mL, and left overnight in a refrigerator. The crystals were removed by filtration, washed with ethanol, and dried under vacuum. Recrystallization of this product in a small amount of water afforded red-orange crystals (0.127 g, 0.32 mmol), which were dissolved in water (3 mL). This solution was added under continuous stirring to a solution of K₂PtCl₄ (0.06 g, 0.16 mmol) in water (5 mL). A white-brown precipitate formed almost immediately after mixing. The mixture was stirred at room temperature for 1 h. The pale green solution was filtered, washed with water and diethyl ether, and finally vacuum-dried for 2 days (0.055 g, 32.5% yield). Anal. (PtC₈H₂₆N₄) (I) C, H, N, Cl. Anal. (PtC₄Cl₄H₁₂N₄O₂) (II) C, H, N, Cl. Anal. (PtC₄Cl₄H₁₂N₄O₂) (III) C, H, N, Cl. Anal. (PtC₆Cl₄H₁₆N₆) (IV) C, H, N, Cl. Anal. (PtC₆Cl₄H₁₆N₆) (V) C, H, N, Cl. Anal. (PtC₆Cl₄H₁₆N₆) (VI) C, H, N, Cl. Anal. (PtC₆Cl₄H₁₆N₆) (VII) C, H, N, Cl.

Preparation of Solutions. All compounds studied were water-soluble. Solutions were prepared at concentrations ranging from 5.0 × 10⁻⁵ to 1.5 × 10⁻⁴ M in phosphate-buffered saline solution (PBS): 132.0 × 10⁻³ M NaCl, 4.0 × 10⁻³ M KCl, 1.2 × 10⁻³ M NaH₂PO₄, 1.4 × 10⁻³ M MgCl₂, 6.0 × 10⁻³ M glucose, 1.0 × 10⁻² M HEPES (N-[2-hydroxyethyl]piperazine-N'-(4-ethanesulfonic acid)). Trypan blue was used as a 0.04% (w/v) solution in PBS. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was prepared at a concentration of 0.5 mg/mL in PBS solution containing 1.0 × 10⁻³ M CaCl₂.

Biological Assays. Cell Culture. Stock cultures of cells were maintained at 37 °C under 5% CO₂. HeLa, HSC-3, and L-132 cells (growth in monolayers was kept in Dulbecco's modified Eagle's high glucose (4500 mg/mL) medium (DMEM-HG) supplemented with 10% heat-inactivated fetal calf serum, glutamine (1.168 g/L), and antibiotics (100 units of penicillin and 100 mg of streptomycin). THP-1, MOLT-3, and CCRF-CEM cells were cultured as a suspension in Roswell Park Memorial Institute medium (RPMI-1640), containing 25 mM HEPES and 25 mM sodium bicarbonate supplemented with 10% heat-inactivated fetal calf serum, glutamine (0.3 g/L), and antibiotics (100 units of penicillin and 100 mg of streptomycin).

The cell lines were subcultured twice a week. HeLa cells were harvested using a dissociation medium composed of 136.9 × 10⁻³ M NaCl, 2.7 × 10⁻³ M KCl, 8.2 × 10⁻³ M Na₂HPO₄, 1.5 × 10⁻³ M KH₂PO₄, 4.0 × 10⁻₄ M EDTA (pH 7.4) and containing 0.0004% (w/v) phenol red. HSC-3 and L-132 cells were harvested upon addition of trypsin/EDTA (0.05% trypsin, 0.35 mM EDTA-4Na reconstituted in free Ca²⁺ and Mg²⁺ balanced salt solution).

Toxicity and Cell Growth Inhibition Evaluation. Cytotoxicity and cell density evaluation following drug exposure (from 5 to 50 µM) by MTT assay were assessed with use of standard assays. Cells were plated at 3 × 10⁴ cells/mL on 12-well dishes. At 24 h hours after seeding, drug solutions were added to the medium and the cultures were incubated at 37 °C. Cells were harvested and analyzed (both in controls and in drug-treated cultures) every 24 h for a total period of 3–5 days (depending on the cell line and the compound used). Reversibility of the drug effect was tested by removing the drug and adding fresh culture medium in the last day of incubation with the drug and assessing the cell viabiliy following 3 more days of incubation. Cell density and viability were determined by Trypan blue exclusion on single-cell suspensions obtained from the monolayer cultures. Cell viability was further assessed by both mitochondrial dehydrogenase activity (MTT assay) and the Alamar blue colorimetric test.

The MTT assay for cell injury is based on the ability of mitochondrial dehydrogenases of viable cells to reduce MTT to a purple formazan product (insoluble in water) which can be quantified spectrophotometrically (ε₅₇₀ = 2.6 × 10⁴ M⁻¹ cm⁻¹, after solubilization in isopropanol containing 40 mM HCl). For each time point, the culture medium was aspirated and 1 mL of MTT solution (5 mg/mL in PBS) was added to the cells (both control and drug-treated cells) followed by a 4 h incubation at 37 °C. After this period, the formazan crystals formed were dissolved upon addition of 1 mL of 0.04 M HCl in 2-propanol and the absorbance of these solutions was measured at 570 nm (against a blank containing MTT and HCl/2-propanol in a 1:1 ratio). The results were expressed as the percentage of MTT reduction, setting the absorbance of control cells as 100%.

The Alamar blue assay is a fluorometric/colorimetric test based on the detection of metabolic activity. It incorporates an oxidizer–reducer indicator that changes from the oxidized form (nonfluorescent, blue) to the reduced state (fluorescent, red) in response to chemical reduction of growth medium, as a result of cell growth. For each time point, Alamar blue was added to the cells (both control and drug-treated) and to culture medium (blank) in an amount equal to 10% of the total culture volume. The samples were incubated for 8 h at 37 °C, and the absorbance was then measured at both 570 nm (A₅₇₀, A₅₇₀) and 600 nm (A₆₀₀, A₆₀₀) (against the blank). Cell viability was expressed as a percentage of the nontreated control cells and was calculated according to the following equation:

\[
\% \text{ reduced} = \left( \frac{\text{ε}_{\text{ox}} A_{\text{ox}} - \text{ε}_{\text{red}} A_{\text{red}}}{\text{ε}_{\text{red}} A_{\text{red}} - \text{ε}_{\text{red}} A_{\text{red}}} \right) \times 100
\]

ε₅₇₀ and ε₆₀₀ represent the molar absorptivity coefficients of the oxidized and reduced forms of Alamar blue, respectively, at 570 and 600 nm: (ε₅₇₀) = 80.856, (ε₆₀₀) = 155.677, (ε₅₇₀) = 117.216, and (ε₆₀₀) = 4.652.

Overall, 50% inhibitory concentration (concentration of drug required to inhibit cell growth by 50%, IC₅₀) was calculated for each drug tested from dose–response curves (for incubation periods of both 48 and 72 h).

All the results presented were obtained by three independent methods: cell density measurement with Trypan blue exclusion method and cell viability assessment with MTT and Alamar blue colorimetric assays.

Statistical Analysis. All experiments were performed in triplicate. The results are expressed as mean values ± SD (the corresponding error bars being displayed in the graphical plots). Statistical analyses were performed using ANOVA, followed by a post hoc test of Fisher’s protected least significant difference. Statistical comparison between the data was based on the Pearson correlation coefficient, values less than 0.05 being considered as significant.

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