Gold(III) Dithiocarbamate Derivatives for the Treatment of Cancer: Solution Chemistry, DNA Binding, and Hemolytic Properties

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Gold(III) compounds are emerging as a new class of metal complexes with outstanding cytotoxic properties and are presently being evaluated as potential antitumor agents. We report here on the solution and electrochemical properties, and the biological behavior of some gold(III) dithiocarbamate derivatives which have been recently proved to be one to 4 orders of magnitude more cytotoxic in vitro than the reference drug (cisplatin) and to be able to overcome to a large extent both intrinsic and acquired resistance to cisplatin itself. Their solution properties have been monitored in order to study their stability under physiological conditions; remarkably, they have shown to undergo complete hydrolysis within 1 h, the metal center remaining in the +3 oxidation state. Their DNA binding properties and ability in hemolyzing red blood cells have been also evaluated. These gold(III) complexes show high reactivity toward some biologically important isolated macromolecules, resulting in a dramatic inhibition of both DNA and RNA synthesis and inducing DNA lesions with a faster kinetics than cisplatin. Nevertheless, they also induce a strong and fast hemolytic effect (compared to cisplatin), suggesting that intracellular DNA might not represent their primary or exclusive biological target.

Introduction

Presently, there is a large interest in the research of metal complexes as potential anticancer agents. This field of investigation was opened by the success afforded by platinum(II) complexes in the treatment of selected malignancies. The introduction of cisplatin (*cis*-$[PtCl_2(NH_3)_2]$) in the clinic in 1979 has instigated the ongoing investigation of alternative metal-based anticancer drugs.[1] The rationale of these studies was that metal centers other than platinum might produce specific and/or improved anticancer effects in vitro and in vivo and may be developed as clinically useful drugs. Given their traditional use in medicine as antiarthritic agents,[2] gold compounds are obvious candidates as a possible alternative to antitumor platinum drugs. Already in pioneering times, some of the clinically established antiarthritic gold(I) compounds were found to exhibit significant antitumor properties, both in vitro and in vivo.[3] Analogously, the investigation of the efficacy of some anticancer drugs, e.g. 6-mercaptopurine and cyclophosphamide, in the treatment of rheumatoid arthritis arose from their known immunosuppressive and antiinflammatory actions, and this work established a connection, at least in principle, between the two therapies.[4] On the other hand, gold(III) complexes show chemical features that are very close to those of clinically employed platinum(II) complexes, such as the preference for square-planar coordination and the typical d^8 electronic configuration, making them very attractive for testing as antineoplastic drugs. Surprisingly, despite this strict similarity, little literature data exist on the use of gold(III) complexes as anticancer agents.[5,6] The paucity of data on gold(III) complexes probably derives from their high redox potential and relatively poor stability, which make their use rather problematic under physiological conditions. Given that the mammalian environment is generally reducing, compounds containing gold(III) may be expected to be reduced in vivo to gold(I) and metallic gold, a major drawback for possible clinical applications.

In recent years, owing to the contribution of a few research groups, new gold(III) compounds have been synthesized and characterized, and showed sufficient stability under physiologically relevant conditions. These stable compounds were generally achieved through an appropriate choice of the ligands, in most cases bearing nitrogen atoms as donor groups.[7] Intriguingly, some of these gold(III) compounds displayed in vitro cytotoxicity comparable to or even greater than cisplatin toward a series of established human tumor cell lines, and only a minimal cross-resistance with the reference drug was observed.[8]

From the few data available and by comparison with platinum(II) complexes, it might be hypothesized that the biological action of gold(III) complexes and, specifically, their antitumor activity is possibly mediated by direct interaction with DNA. The probable binding mode of gold(III) to DNA has been modeled by through crystallographic and spectroscopic investigations of gold(III) complexes with nucleosides and nucleotides.[9] In addition, a number of studies based on different physicochemical techniques suggest that probable binding sites for gold(III) are N(1)/N(7) atoms of adenosine, N(7) or C(6)O of guanosine, N(3) of cytidine, and N(3) of thymidine, which are analogous to the possible binding sites for the isoelectronic platinum(II) ion.[10] It still remains to be established to what extent gold(III) compounds are able to react intact with their presumed ultimate target, DNA. Remarkably, recent studies suggest that the interactions in vitro of some gold(III) complexes with calf thymus DNA are weak, whereas significant binding to model proteins takes place, implying that their mechanism of action

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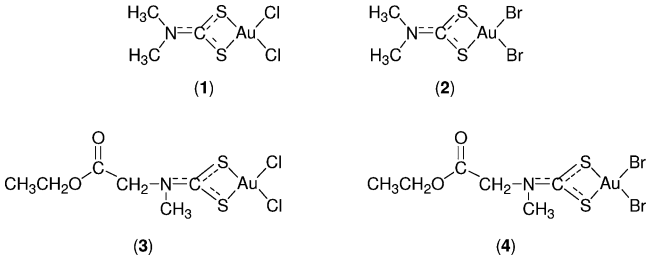
Chart 1. Sturctures of the Investigated Gold(III) Complexes: [Au(DMDT)Cl_2] (**1**), [Au(DMDT)Br_2] (**2**), [Au(ESDT)Cl_2] (**3**), [Au(ESDT)Br_2] (**4**)



might be substantially different from that of the clinically established platinum(II) compounds.[11]

In the present paper we report on the solution and electrochemical properties of the gold(III) dithiocarbamate derivatives [Au(DMDT)X_2] and [Au(ESDT)X_2] (DMDT = *N*,*N*-dimethyldithiocarbamate; ESDT = ethylsarcosinedithiocarbamate; X = Cl, Br. Chart 1). These compounds have been recently proved to be much more cytotoxic in vitro than cisplatin even toward human tumor cell lines intrinsically resistant to cisplatin itself. Moreover, they appeared to be much more cytotoxic also on cisplatin-resistant cell lines, with activity levels comparable to those on the corresponding cisplatin-sensitive cell lines, ruling out the occurrence of cross-resistance phenomena and supporting the hypothesis of a different mechanism of action.[12,13] The favorable cytotoxic properties observed in preliminary in vitro tests led us to investigate the behavior of these compounds under physiological conditions, to study the interaction with both DNA and red blood cells, and to identify their possible biological target. In addition, some chemosensitivity assays have been carried out on both L1210 murine leukemic cells and the parental cisplatin-resistant subline L1210-R, to confirm their ability to overcome resistance to cisplatin.

Results

Solution Properties Analyzed by UV/Vis Absorption Spectroscopy. The evolution with time of complexes **1**−**4** was followed by UV/vis spectroscopy in water for 48 h. The compounds were initially dissolved in dimethyl sulfoxide (DMSO) followed by water, giving a final concentration of complexes and DMSO of 100 μM and 0.5% (v/v), respectively. All the complexes behave similarly and are quite inert in water at 298 K, as revealed by the rather slow spectral changes with time. Each complex is characterized by two strong bands at around 270 and 312 nm, attributed to intramolecular intraligand $\pi^* \leftarrow \pi$ transitions located in the NCS and CSS moieties, respectively.[14] No new spectral features appear, and only a very small decrease in spectral intensity has been detected with time (less than 2% after 48 h).

On the other hand, when dissolved in phosphate buffered saline (PBS, pH 7.4) solution at 298 K, two different behaviors may be observed for [Au(DMDT)X_2] and [Au(ESDT)X_2] (X = Cl, Br) complexes respectively (see Supporting Information). For DMDT derivatives, the position of the previously mentioned bands is essentially unchanged, pointing out that the gold center remains in the +3 oxidation state. The increase in spectral intensity, mainly observed within the first 2 h, may be ascribed to the progressive hydrolysis of gold(III)-bound halogen ions, leading to the more water soluble hydroxo derivative [Au(DMDT)$(OH)_2$]. In fact, the dominant species in solution at physiological pH generally contain coordinated hydroxo groups, the gold(III) center being strongly acidic and drastically lowering the pK_a of coordinated water molecules.[15−17] This process is accompanied by a general increase of the background absorption, and a new low intensity band appears at around 380 nm, assignable to a metal-to-ligand charge-transfer transition due to the presence of gold(III)-bound hydroxo groups.[18]

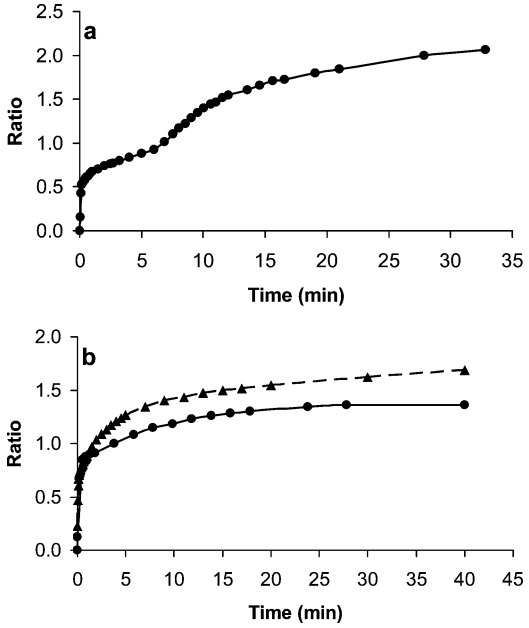


Figure 1. Patterns of halide release in DMSO/phosphate buffered solution (pH 7.4) expressed as the molar ratio delivered halide/starting complex amount vs time, monitored by potentiometric measurement of halide ion concentration with halide ISE. (a) Hydrolysis profile of **4** at 310 K. (b) Hydrolysis profile of **1** at 298 K (●) and 310 K (▲).

Conversely, the absorption spectra in the UV/vis region of the ESDT derivatives show a dramatic change soon after addition of the complexes to the physiological medium. The position of the bands does not substantially change, but their intensities strongly decrease within the first hour, due to the immediate hydrolysis of the halogen atoms bound to the gold(III) metal center, followed by the precipitation of a yellow water-insoluble residue which has been isolated, dried, and spectroscopically identified as the hydroxo gold(III) dithiocarbamate derivative [Au(ESDT)$(OH)_2$] (see Supporting Information). If the first yellow precipitate is maintained in PBS solution for other 24 h, it undergoes a reduction process leading to the formation of a brown-violet residue whose characterization is completely consistent with the gold(I) binuclear species [Au(ESDT)]$_2$, which we have previously synthesized and characterized.[13]

Hydrolysis Kinetic Studies. Supplementary information on the solution chemistry of these gold(III) complexes have been obtained by potentiometric techniques. All the complexes were previously dissolved in 0.2 mL of DMSO and then added to 25 mL of 0.01 M phosphate buffered solution (pH 7.4), the final concentrations being 80−160 μM. Hydrolysis of the compounds was then monitored by following the release of the halide species with time at 310 K with suitable ion-selective electrodes (ISEs).

The bromide release profile with time for complex **4** (90 μM) is reported in Figure 1a; results are expressed as the molar ratio delivered bromide/starting complex amount vs time. The process appears to occur in two fairly separate stages, with a fast phase of a few minutes in which one mole of bromide per mole of complex is detected, thus leading to the formation of the monohydrolyzed adduct [Au(ESDT)Br(OH)]. The second phase, involving the complete hydrolysis of the gold(III) precursor, requires about 30−40 min to reach completion. It is accompanied by the precipitation of a yellow solid which has been

isolated, dried, and spectroscopically identified as the hydroxo gold(III) dithiocarbamate derivative [Au(ESDT)$(OH)_2$], in agreement with that previously reported on the study of the behavior of complex **4** under physiological conditions carried out by means of UV/vis absorption spectroscopy.

A similar behavior was observed for the other bromo derivative **2** (see Supporting Information). These hydrolysis studies pointed out that the release of the first bromide ion per molecule occurs slightly faster than the ESDT analogue **4**, hydrolysis being complete within about 40 min.

Potentiometric monitoring of hydrolysis of the chloro derivative **3** was apparently affected by the interference of traces of sulfide slowly set free by the complex itself. The interference of sulfide ion on the halide-ISEs is indeed very strong, being 100 times stronger over chloride- than over bromide-ISE.[19] By consequence, hydrolysis delivered an apparent amount of chloride sensibly (about 30%) larger than stoichiometric. Despite this, the shape of the experimental plot was similar to the one recorded for compound **4**, showing that the chloride analogue **3** follows the same hydrolysis pattern characterized by two fairly separate stages occurring at similar times. The correctness of this deduction was proved by performing experiments in dilute phosphate buffered solution (0.001 M) and monitoring at 298 K by pH-static titration[20] of the set free hydrogen ions simultaneously with potentiometric measurement of released chloride. The amount of strong base required to neutralize the acidity set free by the hydrolysis reaction, being unaffected by sulfide interference, did correspond to the effected amount of chloride ions.

On the other hand, the behavior of complex **1** was somewhat different. As shown in Figure 1b, while the first chloride atom was set free within less than 2 min, the delivery of the second one did not reach completeness even after 2 h, also being sensibly dependent on temperature in the investigated interval (298−310 K). With this compound, the sulfide interference was apparently absent.

In all cases, gold remains in the +3 oxidation state due to the stabilization effects played by the chelating dithiocarbamate ligands.[16]

Cyclic Voltammetry Studies. The inherent electrochemical properties of these gold(III) dithiocarbamate derivatives have been preliminarily studied through cyclic voltammetry soon after dissolution. Dithiocarbamates are versatile ligands toward gold ions,[21−23] but the electrochemical behavior of the pertinent complexes is not straightforward, as their electron transfer processes are generally complicated by difficulties in defining the involved chemical reactions.[24] Complex **1** in DMSO undergoes two irreversible reduction processes at $E_P = +0.08$ and −0.18 V, respectively, which are interposed by a spurious peak likely arising from byproducts generated in correspondence of the first reduction process. In fact, at a glassy-carbon electrode, such a spurious peak tends to disappear (See Supporting Information). Since it is well-known that the reduction of protons at the glassy-carbon electrode is shifted at notably negative potential values with respect to platinum or gold electrodes,[25,26] this finding proves that the first reduction process generates a byproduct which releases protons. Controlled-potential coulometry (at a platinum gauze) at the first cathodic step (potential of the working electrode (E_W) = 0.0 V) consumed about 0.7 electrons per molecule, and the cyclic voltammetric profile recorded on the resulting solution was dominated by the peak previously assigned to the protons reduction. Further exhaustive reduction at the second process ($E_W = -0.3$ V), involving about one electron per molecule, led to the progressive precipitation of a brown solid residue, whose spectroscopic and preliminary crystallographic characterization is consistent with the gold(I) binuclear species [Au(DMDT)]$_2$.[13,27] As a matter of fact, this result rules out the simple reduction to metal gold with release of free ligand,[16] as no metallic deposit was detected on the macroelectrode surface. As a further confirmation no redox process due to free DMDT ligand was detected ($E_P = +0.15$ V). A qualitatively similar redox pathway has been exhibited by complex **2** ($E_P = +0.23$ and −0.17 V respectively).

Complex **4** exhibits a different reduction pathway (See Supporting Information), as, at the platinum electrode, it undergoes two irreversible and largely separated reduction processes ($E_P = -0.02$ and −1.39 V, respectively), again interposed by the minor reduction peak, assigned to proton reduction (once again confirmed by the response at the glassy-carbon electrode).

Finally, the chloro analogue (compound **3**) exhibits a redox profile similar to that of **4** ($E_P = -0.04$ and −1.46 V, respectively).

Vital Dye Exclusion Test. Before use, all the tested compounds were dissolved in DMSO just before the experiments; calculated amounts of drug solutions were then added to PBS solution or to the growth medium containing cells, to a final organic solvent concentration of 0.5% (v/v), which had no discernible effect on cells killing. All the tested complexes have been proved, by ^{1}H NMR studies, to be stable in DMSO over 48 h.

Cytotoxicity toward human leukemic promyelocites HL60 cells has been studied by trypan blue dye exclusion test.[28] The results of this set of experiments are reported in Figure 2, in which the drug sensitivity profiles of HL60 cells toward complexes **2** and **4**, representative of the two types of the gold(III) DMDT and ESDT derivatives respectively, are shown. The results are expressed as the percentage of cell viability; for comparison purposes, cisplatin was also tested under the same experimental conditions. Noticeably, the cellular damage induced by cisplatin is dose- and, in particular, time-dependent; in fact, cell viability after 3 h incubation is greater than 60% even at the highest concentration, decreasing to ∼20% after a 24 h contact. This behavior is consistent with the mechanism of action of cisplatin, whose target is cellular DNA, and thus requiring a longer time interval to induce cell death. Conversely, the treatment with both the investigated gold(III) complexes leads to a remarkable reduction of cell viability (∼50%) ever after only 3 h incubation at the lowest concentration, being less than 20% after 6 h incubation.

Antiproliferative Activity on L1210 and L1210-R Cells. To obtain more information concerning the ability of the tested compounds to overcome resistance to cisplatin, we have studied their effect in inhibiting DNA synthesis in tumor cells that had acquired resistance to cisplatin in vivo. Murine leukemia L1210-R cells (a cisplatin-resistant subline of the parental cisplatin-sensitive L1210 cell line) were established by treatment with a single dose of cisplatin according to literature procedure,[29] causing in tumor-bearing mice a rapid development of drug resistance. In Figure 3, a comparison of the inhibitory effect of complex **4** and cisplatin toward cisplatin-sensitive L1210 (Figure 3a) and -resistant L1210-R (Figure 3b) cells is reported. It is apparent that compound **4** induces a dramatic inhibition of DNA synthesis in both cell lines, the effect being much more evident than that induced by cisplatin. These results, confirming the circumvention of cisplatin resistance, are particularly interesting as L1210-R cells, despite the resistant sublines previously

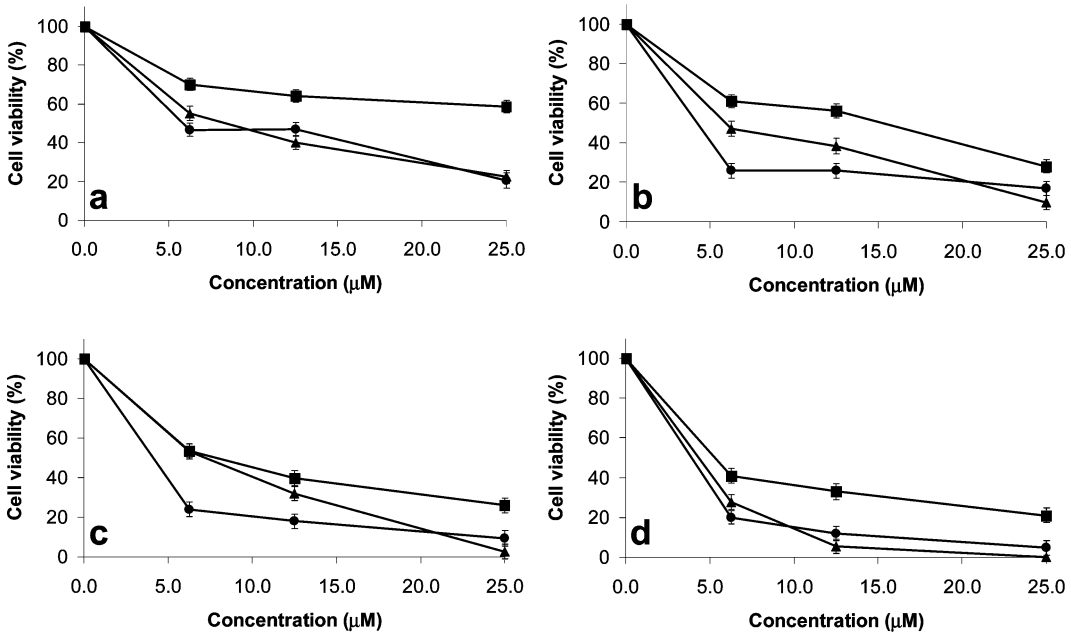


Figure 2. Drug sensitivity profiles of HL60 cell line evaluated by trypan blue dye exclusion test: cisplatin (■), complex **2** (▲), and complex **4** (●). Bars represent the corresponding standard deviations. (a) After 3 h incubation. (b) After 6 h incubation. (c) After 8 h incubation. (d) After 24 h incubation.

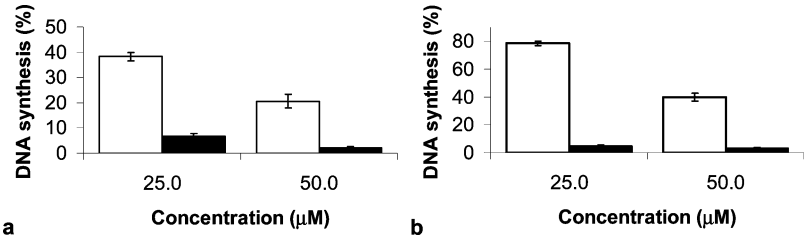


Figure 3. DNA synthesis inhibition in murine leukemia L1210 cells treated for 3 h with cisplatin (□) and compound **4** (■). Bars represent the corresponding standard deviations. (a) Cisplatin-sensitive L1210 cell line. (b) Cisplatin-resistant L1210-R cell line.

tested,[13] acquired their resistance in vivo evading all the host defense mechanisms.

Inhibition of the Macromolecular Synthesis. To study the inhibition of the macromolecular synthesis induced by these gold(III) dithiocarbamate derivatives, the incorporation of tritiated thymidine and uridine into the acid-insoluble fraction of human leukemic promyelocites HL60 cells, exposed for 3 h to increasing concentrations of the tested compounds, has been evaluated (see Supporting Information). Concerning the inhibition of DNA synthesis, cisplatin promotes a strong dose-dependent ^{3}H-thymidine incorporation decrease, and this is not surprising since DNA is its main biological target. Conversely, all the tested gold(III) complexes totally inhibit DNA synthesis even at the lower concentration (6.25 μM), showing a non-dose-dependent mode of action. Regarding the ^{3}H-uridine incorporation data, cisplatin does not directly affect RNA synthesis, having been proved that RNA is not its primary biological target;[30] on the contrary, gold(III) complexes dramatically inhibit RNA synthesis in a non-dose-dependent way.

DNA Binding Affinity. Binding affinity for gold(III) complex−DNA interactions were studied in a qualitative way using purified calf thymus DNA by means of a membrane filtration method.[31] As example, the DNA-binding profile with time of compound **1** is reported in Figure 4. The quantification of the gold(III) complex bound to DNA has been carried out by measuring the absorbance of the absorption band at ∼311 nm, ascribed to an intraligand $\pi^* \leftarrow \pi$ transition located in the CSS moiety, which is observable in both free and DNA-bound gold-

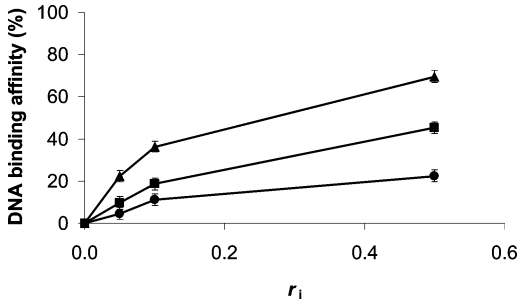


Figure 4. Binding affinity of compound **1** to calf thymus DNA at 0 min (●), 15 min (■), and 30 min (▲). Bars represent the corresponding standard deviations.

(III) complex. The results are expressed in terms of percentage of DNA-bound gold at the different r_i (molar ratio of tested compound to nucleotides) values (0.05, 0.1, and 0.5). It is apparent that this gold(III) compound has a great affinity toward DNA with evidence of binding observable even soon after contact (time zero). Reaction with DNA appears to be extremely rapid and dependent to both r_i value and contact time. Unfortunately, it was not possible to perform the same experiment on cisplatin because it does not present any relevant absorption band in the UV/vis region. Nevertheless, this technique has been successfully applied to cisplatin in previous studies (unpublished data), and the amount of calf thymus DNA-bound platinum(II) was determined by inductively coupled plasma atomic emission (ICP-AE) spectroscopy. Actually, it was found that in such a case DNA-binding percentages were

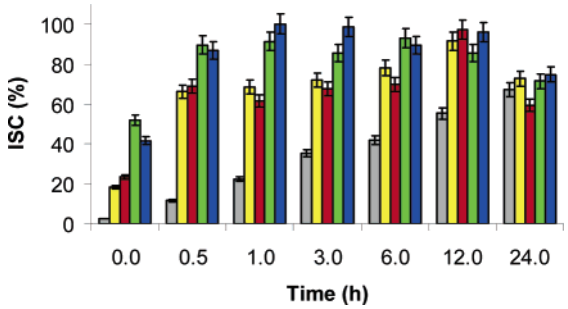


Figure 5. ISC detection with time in purified calf thymus DNA treated with cisplatin (gray), **1** (Yellow), **2** (red), **3** (green), **4** (blue). Bars represent the corresponding standard deviations.

significant at least after 3 h contact (about 10% with $r_i = 0.135$). These results suggest that the DNA-binding rate of the tested gold(III) complex is much faster than cisplatin.

Detection of Interstrand Cross-Links (ISC) in Calf Thymus DNA. The major site of platination induced by cisplatin in double-stranded DNA derives from intrastrand cross-links between neighboring deoxyguanosines, thus exerting its antitumor activity.[32] Anyway, it is well-known that cisplatin can also produce ISC when reacting with DNA in vitro, but this requires a major contortion of the DNA structure and may occur when an alternate purine is not in close proximity on the same strand. This presumably explains why ISC occur at less than 1% of the total platination of DNA.[32] Nevertheless, ISC are among the critical molecular lesions able to inactivate the DNA as template for replication, and the role played by this kind of lesions in the cytotoxic activity of cisplatin is still under debate.[33] The ability of the investigated gold(III) complexes to form DNA ISC was evaluated on purified calf thymus DNA by the ethidium bromide (EB) fluorescence technique following a literature procedure.[34,35] The reaction mixtures were incubated for different times, as cisplatin forms the bifunctional adduct by a double-step process, thus requiring the initial induction of the monofunctional adduct, subsequently evolving into bifunctional adduct. Experimental results (Figure 5) revealed that all the tested gold(III) complexes are more efficient in inducing ISC than cisplatin, in particular ESDT derivatives. This behavior might be correlated to their rapid hydrolysis under physiological conditions. The observation that these complexes induce ISC more rapidly than cisplatin well correlates with the previously discussed DNA binding data and suggests for these new gold(III) compounds a different mechanism of action. Remarkably, after 24 h incubation, the percentage of induced ISC decreases, allowing us to hypothesize that the gold(III) compound−DNA adducts formed with a faster kinetic mechanism are, nevertheless, less stable than those induced by the reference drug. This behavior is in agreement with some recently reported data claiming that gold(III) compounds interact with DNA double helix in a weak and reversible manner, this interaction being of predominantly electrostatic nature.[17]

Detection of DNA−Protein Cross-Links (DPC). In cells, DNA is tightly associated with a variety of proteins that serve both to maintain the structural organization of the genetic material and to coordinate cellular processes including replication, repair, recombination, and transcription. Many endogenous compounds as well as environmental agents are reactive with both DNA and proteins and can produce covalent linkage between these two types of macromolecules. Cisplatin has also been shown to form cross-links between deoxyguanosine and a protein or a glutathione molecule; this adduct can be produced when DNA is first platinated for a short time to give monofunctional adducts, and then a protein or glutathione coordinates the metal center.[32]

Table 1. Percentage of RBC Hemolysis Induced by Complexes **1**−**4** and Cisplatin (as reference)

		RBC hemolysis (%)			
compound	concentration (μM)	15 min	30 min	60 min	100 min
1	6.25	32.9	82.7	100	100
	12.5	79.1	100	100	100
	25	100	100	100	100
2	6.25	33.9	72.5	100	100
	12.5	89.1	100	100	100
	25	100	100	100	100
3	6.25	59.8	100	100	100
	12.5	100	100	100	100
	25	98	100	100	100
4	6.25	56.9	98	100	100
	12.5	97	100	100	100
	25	98	100	100	100
cisplatin	6.25	7.1	8.2	12.8	20.8
	12.5	7.1	9.3	14.3	25.9
	25	7.3	10.1	15.6	32.1

The formation of DPC in HL60 cells, induced by the representative gold(III) dithiocarbamate derivative **4**, was detected by alkaline elution technique.[36] Cisplatin was also tested under the same experimental conditions as reference (see Supporting Information). The rate of elution depends on the molecular size of polynucleotide chains. Cellular DNA, after γ-ray exposure, is rapidly eluted, but if DPCs are formed, elution kinetics decreases. Incubation of cells for 3 h in complete growth medium with cisplatin led to a marked retention of DNA on the filter, suggesting the formation of a remarkable amount of DPC. On the contrary, the elution profile of DNA from cells treated with compound **4** was almost superimposable to that obtained from cells exposed to γ-rays only, showing that it is unable to induce this kind of DNA lesion.

Determination of Red Blood Cell (RBC) Hemolysis. Some compounds are able to damage the membrane of RBCs, as they can damage the cytoskeleton proteins and/or lipids, or as they can provoke a structural destabilization of the membrane by the insertion of the molecule into the phospholipid bilayer. As a consequence of these effects, the lysis of RBCs occurs. Cell membrane damage was studied using albino mouse erythrocytes as a model system.[37] The obtained results, expressed as percentage of hemolysis as a function of the incubation time and drug concentration, are summarized in Table 1; for comparison purposes, cisplatin was also tested under the same experimental conditions. It is clear that cisplatin has a negligible effect even at the highest concentration (25 μM) and after 100 min incubation. After 18 h, cisplatin did not induce a significant hemolysis level (data not shown). On the other hand, all the tested gold(III) dithiocarbamate complexes exert an immediate and dramatic hemolytic dose-dependent effect; in fact, even after only 15 min the percentage of hemolysis reaches 70−100%.

Discussion

The complexes [Au(DMDT)X_2] (X = Cl (**1**), Br (**2**)) and [Au(ESDT)X_2] (X = Cl (**3**), Br (**4**)) have been selected in such a way to reproduce very closely the main features of cisplatin. Conclusions reached upon application of several spectroscopic techniques suggest that coordination of both DMDT and ESDT ligands takes place in a near square-planar geometry through the sulfur-donating atoms, the NCSS group coordinating the metal center in a bidentate symmetrical mode; in addition, the remaining coordination positions are occupied by two *cis*-gold(III)−halogen bonds that may undergo easy hydrolysis (Chart

1).[13] This structural hypothesis is also supported by density functional calculations previously carried out for some analogous gold(III) dithiocarbamate complexes.[38] Recently, these complexes have been tested for their in vitro cytotoxic activity toward a panel of human tumor cell lines including HeLa (squamous cervical adenocarcinoma), HL60 (leukemic promyelocites), Daudi (Burkitt's lymphoma), MeWo (malignant melanoma), LoVo (colon adenocarcinoma), and A549 (nonsmall lung adenocarcinoma) cells, showing, in all cases, to be much more potent than cisplatin, even at nanomolar concentration, with IC_{50} values about 1 to 4 orders of magnitude lower than those of the reference drug.[13] In particular, data regarding their in vitro antiproliferative activity against colon and non small cell lung adenocarcinoma cell lines, which are notoriously not very sensitive to cisplatin,[39] are extremely interesting because these new gold(III) complexes seem to overcome intrinsic resistance to cisplatin. Further experiments have been also performed toward a panel of six human tumor cell lines sensitive and resistant to cisplatin, such as 2008/C13* (ovarian carcinoma), A431/A431-R (squamous cervix carcinoma), and U2OS/U2OS-R (osteosarcoma) cells. Remarkably, these gold(III) complexes appeared to be much more cytotoxic than the reference drug on both cisplatin-sensitive and -resistant cell lines with activity levels, in the latter case, comparable to those induced on the parental sensitive cells, ruling out the occurrence of cross-resistance phenomena and supporting the hypothesis of a different antitumor activity mechanism of action.[13] These encouraging results prompted us to carry out further investigations aimed at elucidating both their solution behavior and the mechanism of action.

Essential requirements for the pharmacological evaluation of new metal complexes as cytotoxic agents are an appreciable solubility in water and a sufficient chemical stability under physiologically relevant conditions. Unfortunately, the reported gold(III) complexes are not soluble in water; thus, before any further biological investigation, they needed to be previously dissolved in DMSO and then added to PBS solution or to the growth medium containing cells. For this reason we studied their electrochemical behavior in DMSO through cyclic voltammetry. Both DMDT and ESDT gold(III) derivatives undergo irreversible stepwise reduction processes leading to the corresponding gold(I) binuclear species $[Au(DMDT)]_2$ and $[Au(ESDT)]_2$, respectively. Remarkably, reduction processes occur at potentials considerably below the typical value of the Au(III)/Au(I) couple known for the corresponding $K[AuX_4]$ (X = Cl, Br) halide precursors ($E° \sim +1.29$ V).[24] Indeed, coordination by dithiocarbamates induces a large stabilization of the +3 oxidation state of the gold center, owing to the electron-donating ability and the chelate effect of the dithiocarbamate moiety.[16] These data are in substantial agreement with those obtained for the same complexes in a different solvent (acetonitrile),[13] confirming that their electrochemical properties do not change with the solvent, and these results are of paramount importance, as their poor solubility in water forced us to dissolve them in DMSO before carrying out any further biological test. It is worth noting that their somewhat easy reduction ($E_P \sim +0.2$ V vs normal hydrogen electrode (NHE)) supports the hypothesis that they could enforce their antitumor activity also through the so-called "activation by reduction" mechanism (i.e. the contact with the cellular cytosol can spontaneously induce electron-transfer processes in the drug, thus triggering side reactions such as complexation of either the gold fragments or the released byproducts by cellular components, and preventing cell replication[40]). Thus, a crucial aspect is the redox potential of the drug, as the reducing power of the cell environment is evaluated around −0.2 V vs NHE at pH 7.0 (corresponding to the reduction potential of the most abundant redox buffer in proliferating cells, namely the couple glutathione disulfide/glutathione).[41]

As concerns their solution properties, these gold(III) complexes hydrolyze in a physiological-like environment delivering two moles of halide and two moles of hydrogen ions per mole of starting complex. The processes are clearly biphasic with a fast phase of a few minutes in which ∼1 mol of released halide per mole of starting complex is detected, leading to the formation of the mono-hydrolyzed adduct. The second phase, involving the complete hydrolysis of the gold(III) precursors, requires at least 30−40 min. Despite the similarity of the hydrolysis reactions, a different behavior has been observed for DMDT and ESDT derivatives. Under physiological conditions, gold(III) DMDT derivatives (**1** and **2**) progressively increase their solubility due to the hydrolysis of gold(III)-bound halogen ions, leading to the more water-soluble hydroxo derivative $[Au(DMDT)(OH)_2]$, the metal center remaining in the +3 oxidation state due to the stabilization effects played by the chelating dithiocarbamate ligand. In the same physiological environment, ESDT derivatives (**3** and **4**) are completely hydrolyzed, leading to the corresponding hydroxo derivative $[Au(ESDT)(OH)_2]$, but, intriguingly, this hydrolysis product appears to be less soluble in such an aqueous medium, and, if maintained in PBS solution, it undergoes a subsequent reduction process within 24 h, leading to the formation of the gold(I) binuclear species $[Au(ESDT)]_2$. Anyway, this reduction process should not interfere with the actual cytotoxic properties of ESDT derivatives, since they have been proved to exert their cytotoxic activity mainly within the first 12 h even at nanomolar concentrations. Moreover, the binuclear gold(I) complex $[Au(ESDT)]_2$ has already shown to be much less cytotoxic than the gold(III) analogues **3** and **4**,[13] ruling out its involvement in the antitumor mechanism of action of its gold(III) counterparts.

In conclusion, the chemical characterization carried out in aqueous solution allowed us to establish that the four investigated gold(III) dithiocarbamate derivatives are sufficiently stable within a physiological-like environment, this representing the essential prerequisite for any further pharmacological evaluation.

Given the strict structural and chemical analogy of these gold(III) complexes with cisplatin, we started from the hypothesis that their cytotoxic properties should have derived from a direct interaction with DNA. Experimental results allowed us to establish their high affinity toward some biologically important isolated macromolecules, resulting in a dramatic as well as aspecific inhibition of both DNA and RNA synthesis in a non-dose-dependent way and extremely fast rates of DNA-binding. They have been also proved to induce ISC with a faster kinetics than cisplatin, even if the long-term stability of the resulting gold−DNA adducts appears to be dependent on subsequent hydrolytic processes. To asses the possible formation of DNA−protein cross-links, preliminary cellular DNA alkaline elution experiments have been performed on cells treated with complex **4**, and it was found to be totally unable to induce this kind of lesion, contrary to cisplatin that acts as a known bifunctional agent.[42] This behavior is, besides surprising, extremely interesting, as exposure to several DPC-inducing agents gives rise to genotoxic and carcinogenic effects, and for some agents their primary mutagenic effects are believed to be mediated through the formation of DPC.[43] Works in progress testing bacterial mutagenicity of complex **4** on *Salmonella Typhimurium* are in agreement with this observation, detecting no genotoxic effects.

According to data obtained processing cells, a plausible explanation of the high cytotoxic potency shown by these gold(III) complexes could be that their mechanism of action is different from that of cisplatin, as they bind DNA to a greater extent, and the resulting DNA lesions are more efficient in killing cells. Moreover, the lack of cross-resistance with cisplatin (tested on cells that acquired their resistance both in vitro[13] and in vivo) suggests that DNA lesions induced by compounds **1**−**4** are repaired less efficiently in comparison to those induced by cisplatin.

On the other hand, RBC hemolysis studies, carried out for both cisplatin and these gold(III) complexes, might open up new prospects concerning their mechanism of action. Mature RBCs have a cytoskeleton consisting of several proteins (spectrin, actin, ankyrin, etc.), and the loss of nucleus and internal cell organelles during hemopoietic process allows RBCs to carry more hemoglobin. The negligible hemolysis induced by cisplatin, even at the highest concentration (25 μM) and after 18 h incubation, is consistent with the fact that intracellular DNA is its main biological target. On the contrary, the immediate and dramatic hemolytic effect exerted by these gold(III) complexes would support their capability of irreversibly damaging the RBC membrane, thus leading to cell lysis. As far as we are concerned, no study about the interaction between RBCs and gold(III) complex/salts is reported in the literature, whereas some experiments have been carried out for gold(I) derivatives, due to their use as antiarthritic drugs. It has been shown that in RBCs $[Au(Ph_2P(CH_2)_2PPh_2)_2]Cl$ binds to membrane phospholipids, leading to the apparent disruption of lipoprotein structure.[44] Smith et al. suggested that gold may react with the sulfhydryl groups available on the surface of cells, damaging the cytoskeleton and, thus, inducing RBC hemolysis.[45] On the other hand, it has been recently reported that some gold(I) complexes, including $[Au(CN)_2]^-$, can cross the RBC membrane intact and bind to a protein inside the cell that is not hemoglobin but a not well-specified "higher molecular weight species (about 330 kDa)".[46] At this early stage of our research, we are not able to state for certain what type of mechanism is involved in the hemolytic activity of these gold(III) complexes, but their different behavior compared to cisplatin allows us to hypothesize that DNA might not represent their primary or exclusive biological target. For a long time it was believed that the mechanism of action of metal-based antitumor agents should essentially reproduce that of platinum complexes, thus relying on a direct damage to nuclear DNA originated by metal coordination to DNA nucleobases.[47] However, on the ground of the experimental results collected in the last 10 years, it is increasingly evident that the biological properties and the mechanisms of cytotoxicity of metal-based compounds are extremely varied.[48]

The encouraging chemical and biological properties of these investigated gold(III) dithiocarbamate derivatives warrant further studies to assess their pharmacological properties in vivo and to elucidate the actual mechanism of their biological activity.

Experimental Section

Chemicals. Cisplatin, calf thymus DNA (cat. D-1501), ethidium bromide, and trypan blue (Sigma Chemical Co.), tetra(*n*-butyl)-ammonium hexafluorophosphate (TBAHFP, electrochemical grade) (Fluka), ^{3}H-thymidine (4.77 TBq mM^{-1}), ^{14}C-thymidine (2.2 GBq mM^{-1}), and ^{3}H-uridine (1.1 TBq mM^{-1}) (Amersham International Plc.) were used as received. Gold(III) complexes **1**−**4** were synthesized as previously described.[13] All other reagents and solvents were of high purity and were used as purchased without any further purification.

Instrumentation. Electronic spectra were recorded at 298 K in the range (190−900) nm with a Perkin-Elmer Lambda 15 double been spectrophotometer, using fresh 100 μM solutions of the samples (previously dissolved in DMSO, to a final DMSO concentration of 0.5% (v/v)) in both water and PBS solution. FT-IR spectra were recorded at room temperature in Nujol between two polyethylene tablets on a Nicolet Vacuum Far FT-IR 20F spectrophotometer for the range (600−50) cm^{-1}, and in solid KBr on a Nicolet FT-IR 55XC spectrophotometer for the range (4000−400) cm^{-1}. ^{1}H NMR spectra were recorded at 298 K in DMSO-d_6 on a Bruker Avance DRX400 spectrophotometer equipped with a Silicon Graphics workstation operating in Fourier transform, using tetramethylsilane (TMS, $\delta = 0.00$ ppm) as external standard.

Potentiometric Measurements. The delivery of bromide or chloride ions by hydrolysis was potentiometrically monitored at 310 K after adding to a known volume of phosphate buffered solution (25 mL, pH 7.4) and a measured volume (0.12 to 0.20 mL) of DMSO in which a known amount (2−4 μmol) of the investigated compounds had been dissolved. The electrochemical sensors were, respectively, a bromide ISE Selectrode F1022Br (Radiometer) and a home-assembled chloride ISE with an AgCl/Ag_2S solid membrane, coupled with an Ag/AgCl double junction reference electrode (Orion, cat. N° 900200) with1 M KNO_3 as the bridge solution. The cell electromotive force (EMF) was measured with a PHM84 Research pH-meter (Radiometer) having a resolution of 0.1 mV and recorded versus time. After the completion of the kinetic processes, the total amount of delivered halide and the ISE's calibration parameters were determined by multiple additions of standard solutions of the relevant potassium halide using the Gran linear transformation.[49,50] The recorded EMF vs time plot was then converted into halide concentration vs time and, finally, to delivered halide to total compound molar ratio vs time graphs.

In simultaneous potentiometric/pH-static measurements, a diluted (0.001 M phosphate buffered solution had to be used, and the ionic strength of the measured solution was stabilized by addition of 0.1 M $NaNO_3$; the measured solution was fluxed with carbon dioxide-free nitrogen. During the whole process, the pH was kept constant by neutralizing the hydrogen ions produced during hydrolysis with 0.01 M NaOH standard solution added by means of a pH-stat apparatus. The instrumental setup[51] consisted of a microprocessor controlled programmable potentiometric titrator MicroTT 2050 (Crison) connected to an Epson LX-400 printer and equipped with a syringe buret Microbur 2030 (Crison) fitted with an 1 cm^3 syringe 1001 TLL (Hamilton). The pH unbalance signal was provided by a double flush sleeve junction combination glass electrode (Hamilton, mod. 238190.01) with 1 M KNO_3 bridge. The delivered volume of base was recorded versus time every 10 s. Unfortunately, the response speed of both the halide electrodes and the pH-stat apparatus was slow compared to the initial rate of the hydrolysis processes, even when the experimental temperature was lowered to 298 K, and did not allow precise monitoring of the initial part of the process, thus preventing the determination of kinetic parameters.

Cyclic Voltammetry. All the electrochemical experiments were performed in anhydrous DMSO (dehoxygenated by bubbling ultrapure N_2 for 15 min.) solutions containing 0.2 M TBAHFP as supporting electrolyte at 298 K, and using an apparatus whose details have been reported elsewhere.[52] All the potential values are referred to the saturated calomel electrode (SCE), unless otherwise stated.

Cell Lines and Culture Conditions. Human leukemic promyelocites HL60 cells (American Type Culture Collection, ATCC) were grown as suspension in RPMI-1640 medium (Celbio) containing 10% fetal calf serum (Biochrom-Seromed GmbH Co.) and supplemented with the antibiotics penicillin (50 units mL^{-1}) and streptomycin (50 μg mL^{-1}). Cell lines were maintained in logarithmic phase at 310 K in a 5% carbon dioxide atmosphere. Murine leukemia cisplatin-sensitive L1210 cells were maintained by serial intraperitoneal transplantation in Balb/c x DBA/2 F1 syngenic mice of 10^5 cells per animal. The parental cisplatin-resistant L1210-R cell subline was established by intraperitoneal treatment of a single

dose of cisplatin (6−8 mg kg^{-1}) administered 2 days after the passage of 106 L1210 cells over successive generations in Balb/c x DBA/2 F1 syngenic mice29. In both cases, cells cultured in vivo were explanted from mice 10 days after the treatment.

Trypan Blue Dye Exclusion Test. HL60 cells (2×10^5 cells mL^{-1}) were seeded in 60-mm Petri dishes in RPMI-1640 growth medium (10 mL) and incubated for 3, 6, 8, and 24 h in the presence of different concentrations (6.25/12.5/25 μM) of the compounds to be tested, previously dissolved in the minimum required amount of DMSO (to a final organic solvent concentration of 0.5% (v/v)). Cells were then incubated for 2 min with 0.25% trypan blue and 5% fetal calf serum. Viable cells were identified by their ability to exclude dye: dead cells take up the blue stain of trypan blue, whereas alive cells have yellow nuclei. Cell counting was performed by phase contrast microscope.

Inhibition of the Macromolecular Synthesis. 4×10^5 cells mL^{-1} HL60, or 1×10^6 cells mL^{-1} L1210 and L1210-R cells, were seeded in 60-mm Petri dishes in RPMI-1640 growth medium (10 mL). After 24 h, the medium was removed and replaced by a fresh one containing the compound to be studied, previously dissolved in the minimum required quantity of DMSO (to a final organic solvent concentration of 0.5% (v/v)) at the appropriate concentration; cells were then incubated for 3 h at 310 K in the dark under controlled atmosphere. Triplicate cultures were established for each treatment. Monolayers were centrifuged, and the harvested precipitate was resuspended in an aqueous solution constituted by 0.5 mL of PBS solution, 0.1 mL of physiological solution and 35−40 kBq of tritiated thymidine or uridine, and reincubated for 30 min at 310 K. The acid insoluble fraction was then precipitated by adding 5% ice-cold trichloroacetic acid and filtered through Whatman GF/C glass microfiber filter (1.2 μm pore size) using a Sample Manifold apparatus (Millipore). After several washings with cold 1% trichloroacetic acid, the filters were dried and counted. The radioactivity measurements were performed by dipping the dried filters into 5 mL of a toluene-based scintillator (5 g of 2,5-diphenyloxazole, 0.25 g of 1,4-bis(4-methyl-5-phenyloxazol-2-yl)benzene, and toluene addition up to 1 L of solution). The results were calculated as the percentage of radioactivity incorporated in treated cells to untreated control cells (about 3−6 MBq). Counting was accomplished by a Packard Tri-Carb 1900TR spectrometer.

DNA Binding Affinity. DNA binding affinity was determined by a membrane filtration method as reported in the literature.[31] Calf thymus DNA was purified by ultracentrifugation (40000*g* for 30 min) in 10 mM tris-HCl buffer (pH 7.9) containing 1 mM EDTA. Purified calf thymus DNA (7.5×10^{-4} M) in phosphate buffered solution (pH 7.4) was incubated with different concentrations of the tested compounds, previously dissolved in the minimum required quantity of DMSO (to a final organic solvent concentration of 0.5% (v/v)), at 310 K for 0, 15, and 30 min. The amount of each compound added to DNA solutions was expressed in terms of r_i (molar ratio of tested complex to nucleotides). The mixture thus obtained was filtered through UFC3LCC filters (Bedford) using a Millipore Centrifree micropartition device and spun at 5000*g* for 90 min at 277 K. These filters are provided with cellulose membrane with a cutoff level of 5000 Da; as the molecular weight of calf thymus DNA is greater than 5000 Da, substances bound to DNA cannot pass through the membrane. The absorbance of free drugs was measured with a Perkin-Elmer Lambda 15 spectrophotometer, and the concentration of the free drug and the percentage of the bound drug to DNA were calculated.

Detection of ISC. DNA ISC were detected according to a literature method.[34] Calf thymus DNA (20 μg) was incubated with different amounts of tested drugs ($r_i = 0.05$, where r_i is drug-to-nucleotide ratio), previously dissolved in the minimum required quantity of DMSO (to a final organic solvent concentration of 0.5%), in a 400 μL volume of phosphate buffered solution (pH 7.4) for 0, 0.5, 1, 3, 6, 12, and 24 h at 310 K. The reaction mixtures were divided into two aliquots, and both were added to a phosphate buffered solution (3 mL) containing EB (1 μg mL^{-1}), 0.4 mM EDTA, and 20 mM potassium phosphate (pH 11.8). One sample was kept on ice, whereas the other was incubated at 373 K for 15 min and then chilled in ice for 5 min. The amount of EB fluorescence in both samples was determined at room temperature (excitation wavelength 525 nm; emission wavelength 600 nm) using a SFM-25 KONTRON Instruments spectrofluorimeter. All reactions were performed in triplicate. The percentage of interstrand cross-linked DNA (% ISC) was determined by the formula

$$\% \text{ ISC} = \left[\frac{f_t - f_n}{1 - f_n}\right] \times 100$$

where f_t and f_n represent fluorescence after denaturation divided by fluorescence before denaturation of treated (f_t) and control (f_n) samples.[33]

Detection of DPC. DNA−protein cross-links were detected by alkaline elution technique according to Kohn.[36] Each experiment was carried out using an internal standard, i.e. untreated cells labeled with ^{3}H-thymidine, whereas treated cells were labeled with ^{14}C-thymidine. HL60 cells in exponential growth were labeled by overnight incubation with ^{3}H-thymidine (7.4 kBq ml^{-1}) or ^{14}C-thymidine (3.7 kBq ml^{-1}).

For the experiments, the radioactive medium was removed and replaced, for ^{14}C-labeled cells, by fresh RPMI-1640 growth medium containing the compound to be studied (previously dissolved in the minimum required amount of DMSO, to a final organic solvent concentration of 0.5% (v/v)) at 25 μM concentration, whereas ^{3}H-labeled cells (control) where treated with 0.5% (v/v) DMSO only. After 3 h incubation at 310 K in the dark under controlled atmosphere, cells were washed twice with PBS solution.

For DPC determination, about $0.5-1.0 \times 10^6$ ^{14}C-labeled treated cells were mixed with an equal amount of ^{3}H-labeled control cells. The mixture was then cooled in ice, and the cell suspension was irradiated with 30 Gy γ-rays. Once deposited on a poly(vinyl chloride) filter (5 μm pore size, Nucleopore Corp.) in a Swinnex-25 filter holder (Millipore), cells were immediately lysed with 5 mL of a 2% sodium dodecylsulfate aqueous solution containing 0.1 M glycine and 0.025 Na_2EDTA (pH 10), and the solution was allowed to flow down by gravity. Elution was carried out with a Gilson Miniplus peristaltic pump at a flow rate of 0.04 mL min^{-1}, and in the fractions collected with a Gilson fraction collector (about 3.5 mL per fraction) the radioactivity of both isotopes was determined.

Hemolysis Determination. Whole blood, collected from untreated albino mice using heparin as anticoagulant, was washed with PBS solution and centrifuged (2500 rpm for 15 min at room temperature), and the supernatant and the buffered coat were discarded. The procedure was repeated until the supernatant was colorless. RBCs were then resuspended in PBS solution and used within 48 h. RBCs (10^6 cells mL^{-1}, corresponding to $A_{650} = 0.6-0.7$) were incubated with increasing concentrations (6.25/12.5/25 μM) of tested compounds, previously dissolved in the minimum required quantity of DMSO (to a final organic solvent concentration of 0.5%), for 15, 30, 60, and 100 min at 310 K. Hemolysis was determined by spectrophotometric measurements at 650 nm of light scattered by impact cells.[53] The percentage of hemolysis of mouse RBC was determined by the formula

$$\% \text{ hemolysis} = \left[\frac{(C - B) - (A - B)}{(C - B)}\right] \times 100$$

in which A = hemolysis of the sample, B = 100% hemolysis = RBCs in water 1:1000, and C = 0% hemolysis = untreated RBC in PBS solution (A_{650} from 0.6 to 0.7).

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Supporting Information Available: FT-IR spectral data of the hydrolysis derivatives of complex **4**; UV/vis spectra over time of **1** and **4** in DMSO/PBS solution; hydrolysis profile of **2** at 310 K; cyclic voltammograms of **1** and **4** in DMSO; inhibition of DNA and RNA synthesis; detection of DPC induced by **4** (alkaline elution experiments). This material is available free of charge via the Internet at http://pubs.acs.org.

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