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Iminophosphorane-organogold(III) complexes induce cell death through mitochondrial ROS production

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Abstract

Gold compounds are being investigated as potential antitumor drugs. Some gold(III) derivatives have shown to induce cell death in solid tumors but their mechanism of action differs from that of cisplatin, since most of these compounds do not bind to DNA. We have explored cellular events triggered by three different iminophosphorane-organogold(III) compounds in leukemia cells (a neutral compound with two chloride ligands [Au{ κ^2 -C,N-C₆H₄(PPh₂=N(C₆H₅)-2)Cl₂] **1**, and two cationic compounds with either a dithiocarbamate ligand [Au{ κ^2 -C,N-C₆H₄(PPh₂=N(C₆H₅)-2)(S₂CN-Me₂)]PF₆ **2**, or a water-soluble phosphine and a chloride ligand [Au{ κ^2 -C,N-C₆H₄(PPh₂=N(C₆H₅)-2)(P{Cp(*m*-C₆H₄-SO₃Na)₂)}₃Cl]PF₆ **3**). All three compounds showed higher toxicity against leukemia cells when compared to normal T-lymphocytes. Compounds **1** and **2** induced both necrosis and apoptosis, while **3** was mainly apoptotic. Necrotic cell death induced by **1** and **2** was Bax/Bak- and caspase-independent, while apoptosis induced by **3** was Bax/Bak-dependent. Reactive oxygen species (ROS) production at the mitochondrial level was a critical step in the antitumor effect of these compounds.

Keywords

Antitumor agents; organogold(III) compounds; iminophosphorane ligands; ROS; mitochondria; Bcl-2 family; caspases

1. Introduction

Cisplatin combination chemotherapy is the cornerstone of treatment of many cancers. Cisplatin and the follow-on drugs carboplatin and oxaliplatin are used to treat 40–80% of cancer patients [1]. The high effectiveness of cisplatin in the treatment of several types of tumors is severely hindered by some clinical problems related to its use in curative therapy, such as normal tissue toxicity and the frequent occurrence of initial and acquired resistance to the treatment [1–4]. The subsequent successful development of antitumor platinum drugs has paved the way for studying other metal-based chemotherapeutic compounds [3]. During

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the last few years there has been a renewed interest in the application of gold(I) and gold(III) compounds in cancer chemotherapy [5, 6]. Attention was directed towards gold compounds for two reasons: (1) gold(III) centers are isoelectronic to Pt(II) compounds and adopt square-planar configurations similar to that of cisplatin, and (2) gold(I) compounds are well known pharmaceuticals, some of which are currently being used to treat rheumatoid arthritis. Gold compounds (Figure 1) have displayed strong tumor cell growth inhibition effects by a non-cisplatin-like mode of action. Antiarthritic linear gold(I)-phosphine *auranofin* and related derivatives **a** [7–13] and gold(I)-N-heterocyclic carbenes **b** [14] have been reported to induce apoptosis via selective inhibition of the mitochondrial isoform of thioredoxin reductase via induction of ROS formation. Antitumor effects of tetrahedral gold(I) complexes $[\text{Au}(\text{dppe})_2]^+$ and related compounds **c** are ascribed to their delocalized lipophilic cation properties [14–16].

Gold(III) complexes currently hold great potential to enter clinical trials since a few of them are highly cytotoxic to solid cancer tumors *in vitro* and *in vivo* while causing minimal systemic toxicity [17–21]. Most gold(III) compounds display reduced affinity for DNA and it seems reasonable that DNA is neither the primary nor the exclusive target for most gold(III) complexes. Recent studies have proposed different modes of action for these compounds. Gold(III)-porphyrin complexes **d**, known gold-based DNA intercalators, induce intracellular oxidation and apoptosis through both caspase-dependent and -independent mitochondrial pathways [21–23]. Gold(III) complexes with dithiocarbamate ligands **e** inhibit thioredoxin reductase activity, generate free radicals, increase ERK1/2 phosphorylation, affecting mitochondrial functions [18, 24]. These compounds have also been described to cause a strong inhibition of the proteasome system, via both redox-dependent and – independent processes [25]. Gold(III) derivatives with polydentate N,N ligands **f**, **g**, **h** are also potent inhibitors of thioredoxin reductase [8, 26]. Recently, histone deacetylases [27], mTOR, and cyclic-dependent kinases have been proposed as possible biochemical targets for some of the gold(III) complexes [27]. Moreover, a recent proteomic study of dinuclear oxo gold(III) compound **h** showed that its mode of action is strictly related to that of auranofin (gold(I)) that they induced changes in protein expression that are limited and selective, that both compounds trigger caspase 3 activation and apoptosis, and that a few affected proteins are primarily involved in cell redox homeostasis [28]. While some gold(III) compounds are reduced easily to gold(I) derivatives *in vitro* (and have therefore a similar mode of action), for gold(III) complexes with dithiocarbamate **e** or porphyrin ligands **d** the “activation by reduction” mechanism has been discarded [28].

One of us have recently reported on the synthesis of apoptotic organogold(III) complexes containing iminophosphorane ligands whose stability in solution and oxidation state can be easily followed by ^{31}P NMR spectroscopy (selected compounds for the present study in Figure 2) [29]. The choice of secondary ligands like dithiocarbamate is related to their known chemoprotective effects. Other secondary ligands like water soluble phosphines (in **3**) were used to increase the solubility of the cycloaurated compounds in water. Cationic compounds (like **2**) containing dithiocarbamate ligands, are the most cytotoxic *in vitro* against HeLa human cervical carcinoma and Jurkat-T acute lymphoblastic leukemia cells [29]. Compounds **1** and **3** are mainly apoptotic but in the case of the more cytotoxic compound **2**, cell death is activated due to both apoptosis and necrosis. We confirmed by ^{31}P NMR spectroscopy that especially **1** and **2** do not get reduced to Au(I) derivatives in solution. Also, a possible interaction of these compounds with DNA has been discarded. Compound **2** manifests a high reactivity toward cytochrome c and thioredoxin reductase (as confirmed by spectroscopic methods) [29].

We report here on the cytotoxic effect of 3 of these iminophosphorane-organogold (III) complexes with different ligands (Fig 2) and we describe cell death pathways activated by

these compounds, focusing on the role of Bcl-2 proteins, caspases and ROS. Our present results indicate that these compounds induce intracellular oxidative stress that subsequently provokes mitochondrial dysfunction. Mitochondrial alterations induced by compounds **1** and **2** depend partially in Bax/Bak activation. Caspases make a limited contribution to the toxicity of compounds **1–3**. ROS production at mitochondria seems to be the key event for the toxicity of these compounds, as antioxidants can fully revert their killing activity and Jurkat rho0 cells are highly resistant to the toxicity of **1–3**.

2. Experimental

2.1. Drugs and chemicals

[Au{ κ^2 -C,N-C₆H₄(PPh₂=N(C₆H₅)-2)Cl₂}] (**1**), [29,30] [Au{ κ^2 -C,N-C₆H₄(PPh₂=N(C₆H₅)-2)(S₂CN-Me₂)}PF₆] (**2**) [29] and [Au{ κ^2 -C,N-C₆H₄(PPh₂=N(C₆H₅)-2)(P{Cp(*m*-C₆H₄-SO₃Na)₂}₃)Cl}PF₆] (**3**) [29] were prepared as described previously. The general caspase inhibitor z-VAD-fmk was from Bachem (Geneve, Switzerland). Any other chemicals were purchased from Sigma-Aldrich (Madrid, Spain).

2.2. Cell culture

The human T-cell leukemia Jurkat (clone E6.1) was from the ATCC collection. Jurkat cells lacking Bak were generated using RNA interference techniques, as described previously [31]. In all cases, cell lines containing the empty vectors were used as suitable controls. Jurkat- ρ^0 cell line (Dr. Ignacio Aguiló, University of Zaragoza, Spain), lacking normal mitochondrial DNA, was generated by the long-term culture in the presence of low concentrations (50ng/ml) of ethidium bromide. Cells were routinely cultured at 37°C in RPMI 1640 medium supplemented with 5% or 10% (Jurkat ρ^0) fetal calf serum (FCS), L-glutamine and penicillin/streptomycin (hereafter, complete medium). Jurkat- ρ^0 cultures were also supplemented with glucose (4.5mg/ml), sodium pyruvate (0.1mg/ml) and uridine (50 pg/ml).

Blood samples from healthy donors were obtained from the 'Banco de Sangre y Tejidos de Aragón'. Leukemic blood samples from patients with the clinical diagnosis of B-cell Chronic Lymphocytic Leukemia (CLL) were obtained from the 'Hospital Clínico Universitario Lozano Blesa'. All subjects gave written informed consent and the Ethical Committee of Aragón approved the study. PBMC were freshly isolated by Ficoll-Paque density centrifugation, as previously described [32]. After isolation, normal PBMC and leukemic CLL cells were kept in complete medium. CLL cells were cultured in the presence of 20 ng/ml of IL-4 (Preprotech).

2.3. Cytotoxicity studies

To evaluate the toxicity of compounds **1–3**, Jurkat cells (3×10^5 cells/ml) or PBMC from healthy donors or CLL patients (3×10^6 cell/ml) were cultured in 96-well flat-bottom plates with different concentrations of each compound. The cytotoxic effect of **1–3** was assessed after 24 h by measuring simultaneously the mitochondrial membrane potential and the exposure of phosphatidilserine, as described below. For caspase inhibition assays the cells were preincubated with 100 μ M Z-VAD-fmk for 1h before the addition of compounds **1–3**. DMSO was added to controls in the experiments with compounds **1** and **2**.

2.4. Cell death analysis

Quantification of cell death was performed by the simultaneous analysis of phosphatidilserine (PS) exposure and mitochondrial membrane potential ($\Delta\psi_m$) in a flow cytometer (FACScan, BD Bioscience, Spain). In brief, 2.5×10^5 cells in 200 μ l were incubated in ABB (140mM NaCl, 2.5 mM CaCl₂, 10 mM Hepes/NaOH, pH 7.4), with either

5nM DiOC₆(3) or 60nM tetramethylrhodamine ethyl ester (TMRE) (both from Molecular Probes) at 37°C for 10 minutes. AnnexinV-PE or AnnexinV-FITC (Invitrogen) at a concentration of 0,5 µg/ml was added to samples and incubated at 37°C for further 15 minutes. In all cases, cells were diluted to 1 ml with ABB to be analyzed by flow cytometry.

To additionally assess cell viability after treatment with the compounds, $2,5 \times 10^5$ cells were harvested and incubated in 200 µl of PBS containing 50 ng/µl of 7-Amino-Actinomycin D (7-AAD, Immunostep). When analyzed simultaneously to either PS exposure or $\Delta\Psi_m$, 7-AAD was added to samples in ABB.

2.5. Nuclear morphology

Morphology of nuclei after the treatment with the different compounds was analyzed by staining cell cultures with Hoechst 33342 (Molecular Probes) at 25 ng/ml. Cells were visualized in a fluorescence microscope (Nikon Eclipse 50i) and ACT Software was used for the acquisition of the images.

2.6. Intracellular ROS quantification

Oxidative stress was analyzed by intracellular staining with the fluorescent probe 2-hydroxyethidium (2-HE, Molecular Probes). After 16h of culture in the presence of compounds **1–3**, cells were incubated with 2 µM 2-HE at 37°C for 15 min. Red fluorescence produced by reduction of 2-HE to ethidium was quantified in a flow cytometer.

3. Results

3.1. Leukemia cells are more sensitive to iminophosphorane-organogold (III) compounds **1–3** than normal PBMC

The sensitivity to compounds **1–3** of T-cell leukemia Jurkat cells was compared to that of normal lymphocytes (PBMC). As shown in Figure 3, Jurkat and CLL cells were more sensitive to the cytotoxic effect of compounds **1–3** than PBMC. The IC₅₀ of compound **1** was 2.57 µM for Jurkat cells and 6.0 µM for PBMC (Fig 3A). Compound **2** at 0.5 µM induced cell death in around 100% of Jurkat cells but only in 17% of PBMC (Fig 3B). Finally, compound **3** induced killing of 96% of Jurkat cells at a concentration of 25 µM (IC₅₀ = 16.1 µM) whereas only 33.5% of PBMC were dead at the same concentration (Fig. 3C). The IC₅₀ of the compounds for CLL cells ranged from 0.48 to 1.2 µM for **1**, 0.06 µM to 0.1 µM for **2** and from 2.7 µM to 14.2 µM for compound **3**.

3.2. Iminophosphorane-organogold (III) compounds **1–3** induce mitochondrial depolarization and apoptosis or necrosis

We have analyzed cell and nuclear morphology in Jurkat cells after 16 h in the presence of compounds **1–3**. Nuclear staining showed that only a fraction of cells exhibited chromatin condensation and fragmentation, typical features of apoptosis (Fig 4). However, a high percentage of cells, around 50%, had lost mitochondrial transmembrane potential ($\Delta\Psi_m$), in the same cultures. Since collapse of mitochondrial transmembrane potential is a feature of cell death in most models [33], we concluded that a significant fraction of cells were death without exhibiting apoptotic morphology. This discrepancy was more evident in the case of compound **2**. These results suggest that compounds **1–3** induced mitochondrial depolarization and later cell death that can proceed both through apoptosis and necrosis.

3.3. Implication of Bax/Bak in the toxicity of iminophosphorane-organogold (III) compounds 1–3

The toxicity of compounds **1–3** was analyzed in Jurkat-shBak cells, lacking both Bax and Bak. Control Jurkat cells were highly sensitive to these compounds and around 50% (**1** and **3**) or 80% (compound **2**) of cells bound annexin V-PE after 24 h, with doses of 10 μ M for compounds **1** and **3** and 0.5 μ M for compound **2** (Figs. 5A and B). Analysis of $\Delta\Psi_m$ loss indicated that the three compounds induced mitochondrial depolarization in a similar percentage of cells (Figure 5B). Cisplatin at 25 μ M caused death of around 60% of Jurkat control (CN) cells. Jurkat-shBak cells were completely resistant to cisplatin but still displayed moderate sensitivity (40–50%) to compound **2**. Also compound **1** exerted some toxicity on Jurkat-shBak cells, with around 20% annexinV-PE⁺ and $\Delta\Psi_m^{\text{low}}$ cells (Figs 5A and B). Cell death produced by compound **3** seemed to be dependent on the presence of Bax and Bak, since Jurkat-shBak cells were almost completely resistant to the toxic effect of this compound (Figs 5A and B). These results indicate that, whereas compound **3**-induced cell death is totally dependent on the presence of multidomain proteins of the intrinsic/mitochondrial route, some Au(III)-iminophosphorane compounds, such as **1** and **2**, activate both Bax/Bak-dependent and -independent cell death mechanisms.

We further analyzed the mechanism of cell death induced by Au(III)-iminophosphorane compounds in Bax/Bak-deficient Jurkat cells. As shown in Figure 5C, compounds **2** and **3** induced necrosis and apoptosis at similar levels in control Jurkat cells. Around a third of death cells analyzed after treatment with compound **1** were necrotic (Fig. 5C). In contrast, cell death induced by compounds **1–3** in Jurkat-shBak cells was mainly necrotic (Fig. 5C). In the case of compound **3**, both apoptotic and necrotic cell death were prevented in Jurkat-shBak cells. These results suggest that necrosis observed in Jurkat control cells after treatment with compound **3** could be a secondary event to apoptosis. However, necrosis induced by compounds **1** and **2** could be induced by a primary mechanism independent of Bax/Bak.

3.4. Caspase inhibition do not prevent cell death induced by iminophosphorane-organogold (III) compounds

The general caspase inhibitor z-VAD-fmk did not significantly reduce the loss of mitochondrial transmembrane potential and plasmatic membrane integrity (7-AAD⁺ cells) induced by compounds **1–3** (Fig. 6A). A limited protection was observed for compound **3**, probably indicating the implication of caspases in an amplification loop that accelerates the execution of apoptosis [31]. In the same way, cell death induced by compounds **1–3** in Jurkat-shBak cells was mainly caspase-independent, as z-VAD-fmk did not significantly reduced the percentage of annexin V-PE⁺ or 7-AAD⁺ cells (Fig. 6B). These results indicate that these iminophosphorane-organogold(III) compounds activate caspase-independent pathways that lead to cell death.

3.5. Iminophosphorane-organogold (III) compounds induce cell death through ROS production

Gold compounds have been described to induce intracellular oxidative stress [22]. ROS production in Jurkat cells treated with compounds **1–3** was analyzed by flow cytometry after staining with 2-hydroxyethidium. As shown in Figure 7, the three compounds induced ROS production in Jurkat cells (Fig. 7A). To evaluate the real contribution of ROS to the toxicity of these compounds, we treated Jurkat cells with **1–3** in the presence of the anion superoxide scavenger MnTBAP, the antioxidant glutathione (GSH) or its precursor N-acetylcysteine (NAC). MnTBAP almost completely protected Jurkat cells from the toxicity of compound **3** and it also offered a partial protection against compound **1**-induced cell death (Fig. 7B). The general antioxidants glutathione and NAC effectively inhibited cell death induced by **1–3**

(Fig. 7B). Taken together, these results indicate a role of ROS in the killing activity of the Au (III) compounds **1–3**, and particularly the implication of superoxide anion in the toxicity of **3** and **1**.

Mitochondria are in most cases the main source of intracellular ROS, due to electron flow in the respiratory chain. Rho0 cells, lacking mitochondrial DNA, show a very limited capacity to generate ROS at the mitochondrial level [34]. When we tested the sensitivity of Jurkat-rho0 cells to compounds **1–3**, we observed that these cells were completely resistant to them (Fig. 7C), even at a concentration twice the IC50 for Jurkat parental cells (Fig. 3).

In order to determine whether ROS were critical in the commitment of cells to death, we studied the growing potential of Jurkat cells transferred to fresh medium after 24h treatment with **1–3** in the presence of NAC. Total viable cells in cultures were analyzed 24 h and 48 h after removal of compounds **1–3** and NAC (Fig. 7D). Cells that had been treated with **1–3** in the presence of NAC fully recovered their growing potential, as compared with control cells, doubling their number from 24 to 48 h. Cells cultured only with **1** and **2** did not multiply. A fraction of cells treated with **3** alone were able to divide after being transferred to fresh medium.

4. Discussion

In the last years several gold compounds are being investigated as potential antitumour agents [5, 6]. We have studied the mechanisms of the antitumor effect of three organo-gold(III) compounds (**1–3**, Fig. 2) that we have previously reported to induce cell death due to both apoptosis and necrosis [29]. Necrosis has often been regarded as an unregulated process but new data indicate that a programmed necrotic death can be activated in some models [35]. Our more recent studies presented here, have first shown that these complexes are more sensitive to Jurkat-T acute lymphoblastic leukemia and B-CLL leukemia cells than to normal lymphocytes (PBMC), consistent to what some of us have found for those (**1–3**) and related iminophosphorane organo-gold(III) complexes in oral epithelial cells [36]. Mitochondrial depolarization preceded cell apoptotic or necrotic cell death induced by compounds **1–3**, suggesting that mitochondria could be the cellular target of these compounds. Similar results have been found for compound **3** in oral human cancer cells MDA686LN [36]. In this sense, it is accepted that most gold compounds do not interact with DNA and their mechanism of action could involve either direct mitochondrial damage or proteasome inhibition or modulation of specific kinases [6]. For instance, gold(I) N-heterocyclic carbenes accumulate selectively in the mitochondria of tumorigenic cells and cause loss of $\Delta\Psi_m$, depletion of the ATP pool and apoptosis [15, 16]. Mitochondrial permeabilization during cell death is regulated by proteins of the Bcl-2 family. Antiapoptotic members of this family, such as Bcl-2, Bcl-X_L or Mcl-1, block mitochondrial permeabilization and the release of apoptogenic proteins (cytochrome c, AIF, Smac/Diablo and others). Proapoptotic proteins of the Bcl-2 family classify in two groups, the “BH3-only” and the “multidomain” subfamilies. Multidomain proapoptotic proteins Bax and Bak are essential for the onset of mitochondrial permeabilization and apoptosis through the intrinsic pathway [37]. Gold(III) dithiocarbamate complexes (like **e**, Fig 1) are known to downregulate the antiapoptotic molecule Bcl-2, upregulate the proapoptotic molecule Bax and induce apoptosis on prostate cells and xenografts [20] and on human acute myeloid leukemia cells [38]. The implication of Bax/Bak in cell death induced by **1–3** was studied using Bax- and Bak-deficient Jurkat cells. Differences were found in the behavior of the compounds regarding the implication of Bax/Bak. The neutral compound **1**, containing two chloride ligands $[\text{Au}\{\kappa^2\text{-C}_6\text{H}_4(\text{PPh}_2=\text{N}(\text{C}_6\text{H}_5)_2)\text{Cl}_2]$, and the cationic compound **2**, with a dithiocarbamate ligand $[\text{Au}\{\kappa^2\text{-C}_6\text{H}_4(\text{PPh}_2=\text{N}(\text{C}_6\text{H}_5)_2)(\text{S}_2\text{CN-Me}_2)]\text{PF}_6$, activate both Bax/Bak-dependent and – independent cell death mechanisms. However, the

behavior of **3**, a cationic compound with a water-soluble phosphine and a chloride ligand $[\text{Au}\{\kappa^2\text{-C}_6\text{H}_4(\text{PPh}_2=\text{N}(\text{C}_6\text{H}_5)_2)\}(\text{P}\{\text{Cp}(m\text{-C}_6\text{H}_4\text{-SO}_3\text{Na})_2\}_3)\text{Cl}]\text{PF}_6$ is totally dependent on the presence of the multidomain proteins Bax/Bak. Moreover, experiments in Bax/Bak-deficient Jurkat cells indicate that necrosis observed in Jurkat control cells after treatment with compound **3** could be a secondary event to apoptosis, while necrosis induced by compounds **1** and **2** could be induced by a primary mechanism independent of Bax/Bak. These differences between compounds **1**, **2** and **3** might derive from the reduction of **3** to gold(I) complexes. Thus while **1** and **2** are very stable in solution *in vitro*, we found compound **3** to be stable only for 24 h [29]. Again, the observed differences between our compounds may be due to the fact that **1** and **2** are reduced to gold(I) derivatives in the cells *in vitro* while **3** is reduced and its cytotoxicity and cell death pathway may be that of the plausible gold(I) decomposition product $[\text{AuCl}\{\text{Cp}(m\text{-C}_6\text{H}_4\text{-SO}_3\text{Na})_2\}_3]$. This compound, recently reported by one of us, resulted an efficient antibacterial agent and induced a moderate rate of apoptosis against Jurkat cells [39]. Some gold(III) compounds like $[\text{Au}_2(\text{bipy}^{\text{Me,Me}})_2(\mu\text{-O})_2](\text{PF}_6)_2$ (**h**) are reduced easily to gold(I) derivatives *in vitro* (and have therefore a similar mode of action to gold(I) compounds) [28]. For gold(III) complexes with dithiocarbamate **e** or porphyrin ligands **d** the “activation by reduction” mechanism has been discarded [28]. In our case the behavior of iminophosphorane-organogold (III) compounds **1** and **2** (with chloride and with dithiocarbamate ligands respectively) may be very similar to that for the gold (III) compounds with only dithiocarbamate ligands of the type **e**. The crystal structures of **1** [40] of **2** [29] confirmed that the coordination geometry around the gold atom is slightly distorted from square-planarity, with C(12)-Au(1)-N(1) angles of $84.9(2)^\circ$ (**1**) and $85.9(3)^\circ$ (**2**) and suggesting a rigid ‘bite’ angle [29], [40]. Besides, the coordination plane for gold and the metallocyclic plane from the ligand iminophosphorane are slightly twisted to give an angle of 14° (**1**) 5.66° (**2**) between them. For the gold(III) compound with dithiocarbamate ligand $[\text{Au}(\text{ESDT})\text{Br}_2]$ a similar near square-planar geometry around the gold atom is proposed [24]. Apart from other considerations, the distortion from square-planarity for our iminophosphorane derivatives or compounds of the type **e** may be an explanation of the different behavior observed for gold(III)-porphyrin complexes like **d** which are lipophilic planar cations with less distortion from square-planarity (N-Au-N angles average $89.8\text{--}90.1^\circ$) [41] especially in their interaction with DNA.

Caspases are in many models the executors of cell dismantlement. In the intrinsic pathway, caspases are activated after release of cytochrome c from mitochondria and formation of the apoptosome. Experiments with the general caspase inhibitor z-VAD-fmk on cells treated with compounds **1–3** showed that these iminophosphorane-organogold(III) compounds induce caspase-independent cell death. Activation of caspase-3 and/or caspase-9 have been reported for gold(I) and gold(III) compounds in general [6]. However, it has also been described that caspase inhibition with z-VAD-fmk only confers a partial protection against gold(III) porphyrin **1a** [22], showing that gold compounds can also exert their killing activity through caspase-independent mechanisms. These mechanisms could include apoptogenic factors such as AIF [22] or ROS generation. The production of ROS leading to apoptosis has been reported for some gold(I) compounds [8, 11, 12, 42, 43] and for gold(III) derivatives with porphyrin (**d**) or dithiocarbamate (**e**) ligands [17–19, 21, 24, 25]. Most recently, the cytotoxicity of gold(I)-carbene derivatives has been reported to be related to the induction of ROS formation, finally resulting in apoptosis and necrosis of the cells as well as to direct effects on mitochondrial integrity [44].

We have found that compounds **1–3** induce cell death through ROS production. The general antioxidants glutathione and NAC effectively inhibited cell death induced by **1–3** (Fig. 7B). MnTBAP almost completely protected Jurkat cells from the toxicity of compound **3** and it also offered a partial protection against compound **1**-induced cell death (Fig. 7B). Taken

together, these results indicate a role of ROS in the killing activity of the Au (III) compounds **1–3**, and particularly the implication of superoxide anion in the toxicity of **3** and **1**. More importantly, we have demonstrated that ROS are critical for the commitment to death, since cells treated with compounds **1–3** in the presence of NAC started to proliferate when they were transferred to fresh medium. Mitochondria are in most cases the main source of intracellular ROS, due to electron flow in the respiratory chain. For instance, superoxide can be produced by Complex I and Complex III as a byproduct of mitochondrial respiration. Rho0 cells, lacking mitochondrial DNA, show a very limited capacity to generate ROS at the mitochondrial level [34]. When we tested the sensitivity of Jurkat-rho0 cells to compounds **1–3**, we observed that these cells were completely resistant to them, even at a concentration twice the IC₅₀ for Jurkat parental cells. These results point to mitochondria as the main target of compounds **1–3**, causing ROS generation (all three compounds) and/or Bax/Bak activation (compound **3** and partially for compound **1**).

In summary, we have demonstrated here that iminophosphorane-organogold(III) compounds are highly cytotoxic to Jurkat T-cell acute lymphoblastic leukemia cells and B-CLL cells, showing a higher toxicity against these cells than to normal T-lymphocytes. We have found significant differences in the mechanisms activated by **1–2** and **3**. Superoxide anion and Bax/Bak seem to be highly implicated in the toxicity of **3**, inducing predominantly apoptotic cell death. However, the role of superoxide and Bax/Bak is only partial for **1** and very limited for **2**, which induce necrotic cell death. As previously pointed out, we believe that the different behavior displayed by **1** and **2** *versus* **3** comes from the fact that **1** and **2** are not reduced to gold(I) derivatives *in vitro* while compound **3** may be reduced to AuCl(PR₃) species. We are currently working on the design of new iminophosphorane ligands with different hydrophylic/lipophilic ratios and their coordination to d⁸ metal centers for the preparation of novel more potent cytotoxic compounds with a lower systemic toxicity in humans.

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Abbreviations

ABB	annexin binding buffer
AIF	apoptosis inducing factor
auranofin	2,3,4,6-tetra-O-acetyl-1-thio-β-D-glucopyrasonato-S-triethylphoshine gold(I)
bipy^{dmb}	6-(1,1-dimethylbenzyl)-2,2'-bipyridine
bipy^{Me,Me}	6,6'-dimethyl-2,2'-bipyridine
CLL	B-cell chronic lymphocytic leukemia
DMSO	dimethylsulfoxide
dppe	1,2-bis(diphenylphosphino)ethane
ERK	extracellular signal-regulated kinase
ESDT	ethylsarcosinedithiocarbamate
FCS	fetal Calf Serum
2-HE	2-hydroxiethidium

GSH	glutathione
HDAC	histone deacetylase
Hepes	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
L-NAC (or NAC)	N-acetyl-L-cysteine
MAPK	mitogen-activated protein kinase
MnTBAP	Mn(III)tetrakis (4-benzoic acid) porphyrin
mTOR	mammalian target of rapamycin
PBMC	peripheral blood mononuclear cells
PS	phosphatidylserine
ROS	reactive oxygen species
terpy, 2,2'	6',2''-terpyridine
TMRE	tetramethylrhodamine ethyl ester
TrxR	thioredoxin reductase

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Figure 1.

Selected gold(I) (**a–c**) and gold(III) (**d–h**) complexes which displayed cytotoxicity against solid cancer tumors *in vitro* (**a–h**) and *in vivo* (**d, e**) and whose mode of action has already been studied: *auroanofin* and related compounds (**a**), gold(I)-carbene derivatives (**b**), $[\text{Au}(\text{dppe})_2]^+$ and related complexes (**c**), gold(III)-meso-tetraarylporphyrin complexes (**d**), gold(III) dithiocarbamate derivatives containing N,N-dimethyldithiocarbamate ligands (**e**), $[\text{Au}(\text{terpy})\text{Cl}]\text{Cl}_2$ (**f**), $[\text{Au}(\text{bipy}^{\text{dmb}} - \text{H})(\text{OH})][\text{PF}_6]$ (**g**) and dinuclear gold(III) $[\text{Au}_2(\text{bipy}^{\text{Me,Me}})_2(\mu_2\text{O})](\text{PF}_6)_2$ (**h**).[7–32]

Figure 2. Selected cytotoxic organogold(III) complexes with iminophosphorane ligands [29, 30] object of the present study (**1–3**).

Figure 3.

Jurkat cells (3×10^5 cells/ml) and PBMC from normal donors or CLL patients (2×10^6 cells/ml) were cultured for 24 in the presence of the indicated doses of each compound (**1–3**). Cell death was evaluated by annexinV-PE labeling and quantification of fluorescent cells by flow cytometry. Results are mean $\pm$ SD of three independent experiments.

Figure 4.

Jurkat cells were cultured for 24h in the presence of compounds **1–3** or left untreated. Nuclei were stained with Hoechst 333248 (10 μ g/ml) and cells were photographed under UV light (left panels). Bright-field pictures are shown (right panels). Loss of transmembrane potential was assessed in a portion of each sample by flow cytometry after staining with DiOC₆(3) as indicated in Materials and methods. Figures in left panels indicate the percentage of $\Delta\Psi_m^{\text{low}}$ cells.

Figure 5.

Jurkat-CN or Jurkat-shBak cells were treated with cisplatin or **1–3** for 24h and PS exposure (**A**) and $\Delta\Psi_m$ (**B**) were evaluated by flow cytometry after labeling with annexinV-PE and TMRE, respectively. (**C**) Cell viability and apoptotic morphology were evaluated after staining with Trypan blue in Jurkat-CN or Jurkat-shBak treated with cisplatin or **1–3** for 24h. Trypan blue stained cells were scored as necrotic and Trypan blue negative cells with blebbing and/or condensation were scored as apoptotic. Results are mean $\pm$ SD of three independent experiments.

Figure 6.

(A) Jurkat cells were treated with compounds 1–3 for 24h in the presence or in the absence of the general caspase inhibitor z-VAD-fmk. Mitochondrial transmembrane potential and membrane integrity were analyzed by flow cytometry after staining with DiOC₆(3) and 7-AAD, respectively, as indicated in Materials and methods. Results are mean $\pm$ SD of three independent experiments. (B) Jurkat-CN and Jurkat-shBak cells were treated with compounds 1–3 for 24h in the presence or in the absence of the general caspase inhibitor z-VAD-fmk. Mitochondrial transmembrane potential and membrane integrity were analyzed as in (A). Results are mean $\pm$ SD of three independent experiments.

Figure 7.

(A) Cells were cultured for 24h in the absence or in the presence of compounds 1–3. At the end of incubations, cells were labeled with annexinV-FITC and 2-HE as indicated in Materials and methods. FITC and ethidium fluorescence were analyzed in a flow cytometer. The percentage of total cells in each quadrant is indicated. Dot plots are representative of two independent experiments. (B) Cells were treated for 24h with 1–3 and/or the indicated antioxidants. Collapse of mitochondrial transmembrane potential was evaluated at the end of experiments. Results are mean $\pm$ SD of 4 independent experiments. (C) Jurkat-p0 cells lacking mitochondrial DNA were treated with the indicated doses of 1–3 and $\Delta\Psi_m$ was analyzed by flow cytometry. Results are mean $\pm$ SD of three independent experiments. (D) After 24h culture in the presence NAC and/or each of the three compounds 1–3, cells were washed and transferred to fresh medium. Total cell number and viability were determined 24 and 48h after removal of compounds and NAC. Cells were stained with Trypan blue and counted in a Neubauer hemocytometer. Results are mean $\pm$ SD of three independent experiments.