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Mitochondria are primary targets in apoptosis induced by the mixed phosphine gold species chlorotriphenylphosphine-1, 3-bis(diphenylphosphino)propanegold(I) in melanoma cell lines

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ABSTRACT

Based on previous evidence indicating a selective cytotoxic activity of the mixed phosphine gold complex chlorotriphenylphosphine-1,3-bis(diphenylphosphino)propanegold(I) for melanoma cells, we investigated the cellular bases of its antiproliferative effect in a panel of human melanoma cell lines (JR8, SK-Mel-5, Mel-501, 2/60, 2/21 and GRIG). The drug consistently induced a dose-dependent inhibition of cell growth, with IC_{50} values ranging from 0.8 to 2.3 μM and, when tested under the same experimental conditions, its cytotoxic activity was higher than (from 2- to 5-fold) or comparable to that of cisplatin as a function of cell lines. The ability of the gold complex to activate programmed cell death was assessed in JR8 and 2/60 cells, and a dose-dependent increase in cells with an apoptotic nuclear morphology was observed in both cell lines (up to 40 and 66% of the overall cell population, for JR8 and 2/60 cell lines, respectively). Such an apoptotic response was mediated by a dose-dependent loss of mitochondrial membrane potential, cytochrome c and Smac/DIABLO release from mitochondria into cytosol and enhanced caspase-9 and caspase-3 catalytic activity. A reduced or completely abrogated expression of the anti-apoptotic proteins c-IAP1, XIAP and survivin in drug-treated cells was also observed. Overall, results from the study indicate that chlorotriphenylphosphine-1,3-bis(diphenylphosphino)propanegold(I) markedly inhibits melanoma cell growth by inducing mitochondria-mediated apoptosis and suggest it as a good candidate for additional evaluation as an anticancer agent against melanoma.

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Abbreviations: SRB, sulforhodamine B; LEHD-AFC, Leu-Glu-His-Asp-7-amino-4-trifluoromethylcoumarin; Ac-DEVD-AMC, *N*-acetyl-Asp-Glu-Val-Asp-aldehyde-7-amino-4-methylcoumarin; $\Delta\psi_{mt}$, mitochondrial membrane potential

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1. Introduction

Over the last two decades evidences have been collected that gold complexes exhibit significant cytotoxic effects on cancer cell lines and also display antitumor activity in some transplantable *in vivo* tumor models [1]. Specifically, the gold(I) monophosphine derivative "auranofin" induced *in vitro* antiproliferative effects against P388 murine leukemia, B16 murine melanoma and cultured human cancer cells whereas *in vivo* antitumor activity was only observed for the P388 leukemia [2–4]. Later, stable four-coordinate gold(I) complexes containing diphosphine donor ligands were found to induce *in vitro* and *in vivo* antitumor activity toward several tumor models including P388 and L1210 leukemias, M5076 reticulum cell sarcoma, B16 melanoma and Lewis lung carcinoma [5–7].

The exact mode of action of gold(I)-diphosphines derivatives is still largely unknown, although several possible mechanisms responsible for the cytotoxic activity of these compounds have been proposed. Gold seems to function as a carrier for the diphosphine ligand which can be released, for example, after interaction of gold(I) with thiols. That is, gold can protect the diphosphine ligand from oxidation reactions, enabling it to be delivered to cells and display its antiproliferative activity. In fact, it has been shown that diphospines are themselves antiproliferative agents at high doses [7–9]. An additional hypothesis suggests that the diphosphine can coordinate copper to form a Cu(I)-diphosphine species likely involved in the cytotoxicity mechanism [10]. However, a more direct involvement of gold in antitumor activity is possible, as some gold(III) compounds without any phosphine bound to the metal are also active against several tumor models [11].

It has been shown that gold(I)-monophosphine and -diphosphine complexes act on mitochondria that undergo uncoupled oxidative phosphorylation with consequent permeability to cations and protons through the inner mitochondrial membrane [12–15]. Mitochondrial membrane permeability transition and consequent loss of mitochondrial membrane potential ($\Delta\psi_{mt}$) are widely accepted as being central to the process of mitochondria-dependent apoptosis [16,17]. Loss of $\Delta\psi_{mt}$ results in decreased ATP synthesis and release of apoptogenic factors including cytochrome *c*, apoptosis inducing factor and Smac/DIABLO from the mitochondria into the cytosol, leading to cell death [18]. For this reason, there is considerable interest in the design and development of drugs that specifically compromise the structural and functional integrity of mitochondria as novel anticancer agents [16,19].

Gold(I) complexes containing simultaneously both mono- and diphosphine ligands have recently been synthesized with the aim to potentially increase their antitumor activity due to synergistic action [20]. Specifically, the mixed phosphine gold species chlorotriphenylphosphine-1,3-bis(diphenylphosphino)propanegold(I) (Fig. 1) showed appreciable *in vitro* antiproliferative activity against the National Cancer Institute (NCI) 60-cell line panel of tumors of different histotypes. IC_{50} values in the micromolar range were obtained for 29 cell lines, and melanoma was the most sensitive subgroup analyzed [20].

In contrast with all other antitumor gold-phosphine compounds found in the literature chlorotriphenylphosphine-1,3-bis(diphenylphosphino)propanegold(I) did not show anti-leukemic activity. This marked difference may be of importance considering that the development of the promising compound bis[1,2-bis(diphenylphosphino)-ethane]gold(I) chloride was stopped due to liver toxicity in dogs [21]. Interest in this field is reemerging, however, as seen by two recent patents [22], and a study showing that lower lipophilicity of bis-diphosphine-gold(I) compounds is correlated to a decrease of liver toxicity [23]. Reduced lipophilicity in the title compound, compared to bis[1,2-bis(diphenylphosphino)-ethane]gold(I) chloride, is achieved by replacing a (diphenylphosphino)ethane ligand with a triphenylphosphine, thus removing one Ph and two CH_2 groups.

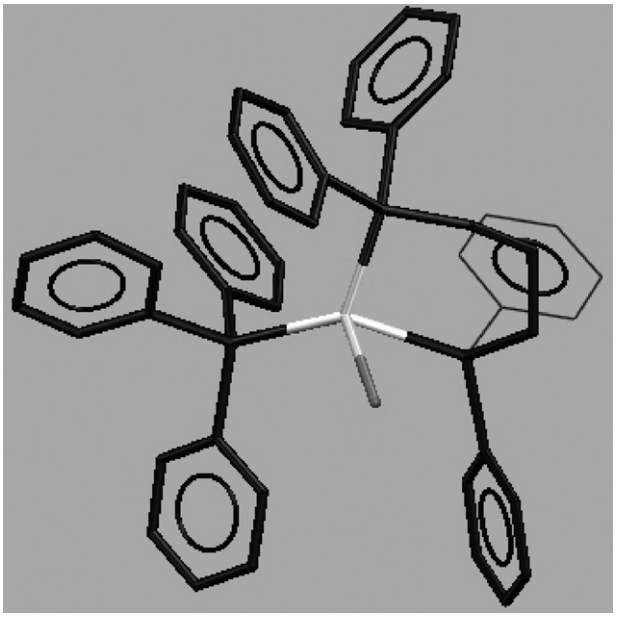

Fig. 1 – Chlorotriphenylphosphine-1,3-bis(diphenylphosphino)propanegold(I) 3-dimensional X-ray molecular structure [20], Au white, Cl grey, C black, one phenyl ring is thinner for clarity and H atoms are omitted.

The present study was undertaken to validate the selective antiproliferative effect of chlorotriphenylphosphine-1,3-bis(-diphenylphosphino)propanegold(I) in a panel of six human melanoma cell lines, other than those assayed in the NCI screening, and to evaluate the ability of the compound to activate a mitochondria-dependent apoptotic response in these cells.

2. Materials and methods

2.1. Chemicals and reagents

Chlorotriphenylphosphine-1,3-bis(diphenylphosphino)propanegold(I), synthesized as previously described [20], was reconstituted in sterile DMSO at a concentration of 5 mM and then diluted with sterile water to the desired concentrations, immediately before each experiment. Cisplatin (Bristol-Myers, Evansville, IL, USA) was diluted with fresh medium immediately before each experiment.

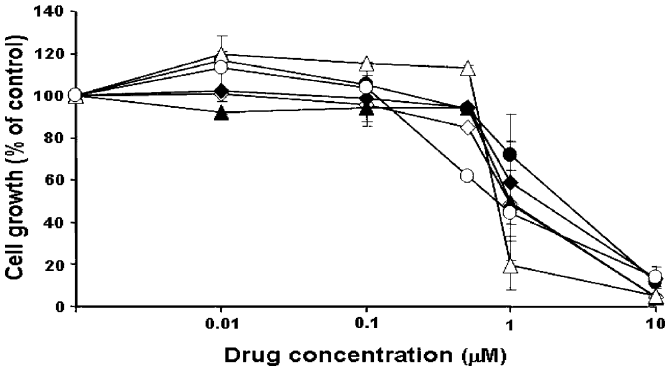

Fig. 2 – Dose–response survival curves of different cell lines ((○) JR8; (●) GRIG; (◇) 2/21; (◆) 2/60; (▲) SK-MEL-5; (△) MEL-501) exposed to chlorotriphenylphosphine-1,3-bis(diphenylphosphino)propanegold(I) for 48 h. Points represent mean values ± S.D. of three independent experiments.

2.2. *Cell lines*

Six human melanoma cell lines, JR8, SK-MEL-5, GRIG2211/1 (GRIG), MEL-501 and two melanoma clones, 2/21 and 2/60 (selected from the human melanoma cell line 665/2 by micromanipulation in soft agar), were used in the study. The biological characteristics of JR8, SK-MEL-5, MEL-501, 2/21 and 2/60 have been previously described [24,25]. GRIG has been recently established in the laboratory of Dr. A. Anichini (Istituto Nazionale Tumori, Milan). Cell lines were maintained in logarithmic growth phase at 37 °C in a 5% CO_2 humidified atmosphere in air. RPMI-1640 medium (Biowhittaker, Verviers Industries, Kibbutz Beth Haemek, Israel), containing 10% fetal calf serum, 2 mM L-glutamine and 0.1 mg/ml gentamycin was used for all cell lines, with the exception of SK-MEL-5 which was grown in DMEM-F12 medium (Biowhittaker) containing 10% fetal calf serum and 0.1 mg/ml gentamycin. For all cell lines, the experiments were performed within 10 passages from thawing.

2.3. *Cell survival assay*

The sulforhodamine B (SRB) assay was performed as described by Perez et al. [26] with minor modifications. Briefly, according to the growth profiles previously defined for each cell line, adequate numbers of cells in 0.2 ml culture medium were plated in each well of a 96-well plate and allowed to attach for 24 h. Cells were exposed to 0.01–10 μM of gold complex or cisplatin at 37 °C for 48 h. In each experiment, control samples were run with 0.2% DMSO. At the end of each treatment, cells were fixed and processed for SRB staining. The optical density (OD) was read at 550 nm. Each experimental point was run eight times. The results were expressed as the absorbance values of gold complex-treated samples compared with those of controls. The *in vitro* activity of the drug was expressed in terms of concentration able to inhibit cell proliferation by 50% (IC_{50}).

2.4. *Mitochondrial membrane potential ($\Delta\psi_{mt}$)*

Alterations in the $\Delta\psi_{mt}$ were studied by flow cytometry using the $\Delta\psi_{mt}$-sensitive dye JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide) (Molecular Probes Inc., Eugene, OR, USA [27]). In brief, after 24-h exposure to the gold complex JR8 and 2/21 cells were harvested, washed once with PBS, resuspended in complete medium and incubated with 10 μg/ml JC-1 at 37 °C for 15 min in the dark. Stained cells were then washed once in PBS and a minimum of 30,000 cells per sample was analyzed by a FACScan flow cytometer (Becton Dickinson, Sunnyvale, CA). Data were acquired in list mode and evaluated using Lysis II software. Forward and side scatter were used to gate the viable population of cells. JC-1 monomers emit at 527 nm (FL-1 channel), and J-aggregates emit at 590 nm (FL-2 channel). Duplicate samples of control cells were used for compensation (FL-1, FL-2), and flow cytometric profiles from these cells defined the 590 nm cut-off for drug-treated samples.

2.5. *Evaluation of apoptotic cell morphology by fluorescence microscopy*

Cells were harvested after a 24-h exposure to the gold complex or after a sequential treatment with the cell permeable pan-caspase inhibitor z-VAD-fmk (40 μM) or the caspase-9 specific inhibitor z-LEHD-fmk (50 μM) for 1 h followed by a 24-h exposure to the gold complex. Floating and adherent cells

Table 1 – Cytotoxic activity of gold complex and cisplatin in human melanoma cell lines

Cell line	IC50[a] (μM)	
	Gold complex	Cisplatin
JR8	0.8 ± 0.11	0.6 ± 0.08
MEL-501	0.8 ± 0.15	4.0 ± 0.13
SK-MEL-5	1.0 ± 0.56	1.8 ± 0.1
2/21	1.0 ± 0.28	0.9 ± 0.09
2/60	1.7 ± 0.47	3.5 ± 0.36
GRIG	2.3 ± 0.35	7.0 ± 0.31

Data represent mean values ± S.D. of three independent experiments.

[a] IC_{50} values were determined graphically from the growth inhibition curves obtained after a 48-h exposure of the cells to each drug.

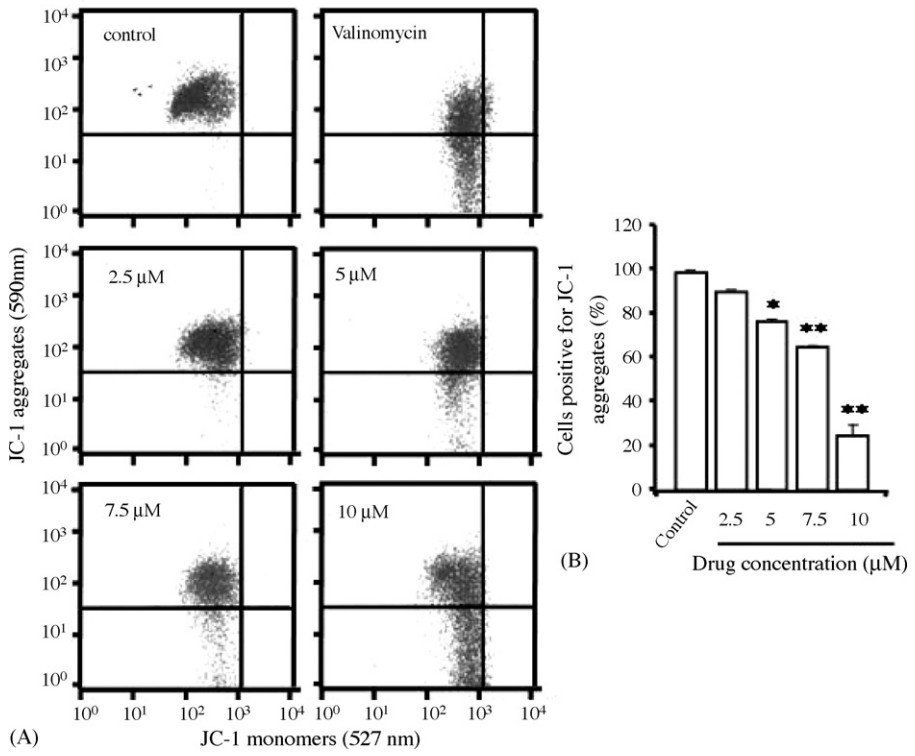

Fig. 3 – Effect of chlorotriphenylphosphine-1,3-bis(diphenylphosphino)propanegold(I) on the $\Delta\psi_{mt}$ in JR8 cells. After a 24-h treatment with different concentrations of the gold complex, cells were incubated in drug-free medium, stained with JC-1 and analyzed by flow cytometry. Cells treated for 30 min at 37 °C prior to JC-1 addition with 5 μM valinomycin were used as a positive control for $\Delta\psi_{mt}$ disruption. (A) Representative examples of the fluorescence pattern of cells incubated with the solvent (control) or with the gold complex (2.5–10 μM); (B) percentage of cells staining positive for JC-1-aggregates formation (emitting at 590 nm) in untreated (control) and the gold complex-treated cell populations. Data represent mean values ± S.D. of three independent experiments. $^{*}P < 0.01$; $^{**}P < 0.001$; Student's t-test.

were collected separately, washed in PBS and stained with a solution containing 50 μg/ml propidium iodide, 10 mg/ml RNAse, and 0.1% (v/v) Tween20 for 30 min at room temperature. After propidium iodide staining, an aliquot of the cell suspensions was spotted onto slides and assessed for typical apoptotic nuclear morphology (nuclear shrinkage, condensation and fragmentation) under a fluorescence microscope with appropriate filter combinations. Apoptotic cells were determined by scoring at least 500 cells in each sample by two independent observers.

2.6. *Assessment of caspase-9 and caspase-3 catalytic activity*

Caspase-9 and caspase-3 catalytic activity was determined by means of the Caspase-9/Mch6 Fluorometric Protease Assay Kit (MBL, LTD, Japan) and the Caspase-3 Assay Kit (BD Biosciences, Pharmingen, San Diego, CA), respectively. Briefly, total protein and the specific fluorogenic substrate (Leu-Glu-His-Asp-7-amino-4-trifluoromethylcoumarin, LEHD-AFC, for caspase-9 and *N*-acetyl-Asp-Glu-Val-Asp-aldehyde-7-amino-4-methylcoumarin, Ac-DEVD-AMC, for caspase-3) were mixed for 1 h at 37 °C. The hydrolysis of the specific substrates for caspase-9 and caspase-3 were monitored by spectrofluorometry at 505 and 440 nm, respectively.

2.7. *Western immunoblotting*

Cells (1×10^6) were washed once with ice-cold PBS and lysed on ice in lysis buffer. The cytosolic and mitochondria fractions were prepared using a Mitochondria Isolation Kit For Mammalian Cells according to the protocol provided with the kit (Pierce, Celbio, Milan, Italy). Total cellular lysates or cytosolic/mitochondria fractions were separated on a 12% SDS-polyacrylamide gel and transferred to nitrocellulose. The filters were blocked in PBS with 5% skim milk and incubated overnight with the primary antibodies specific for survivin (Novus Biologicals, Littleton, UK), cytochrome *c* (Santa Cruz Biotechnology, Santa Cruz, CA), XIAP, c-IAP1 and Smac/DIABLO (Abcam Ltd., Cambridge, UK). The filters were then incubated with the secondary peroxidase-linked whole antibody (Amersham Biosciences Europe, Freiburg, Germany). Bound antibody was detected using the enhanced chemiluminescence Western blotting detection system (Amersham Biosciences Europe). Anti-actin and anti-cytochrome c oxidase subunit IV (COX IV) monoclonal antibodies (Santa Cruz Biotechnology)

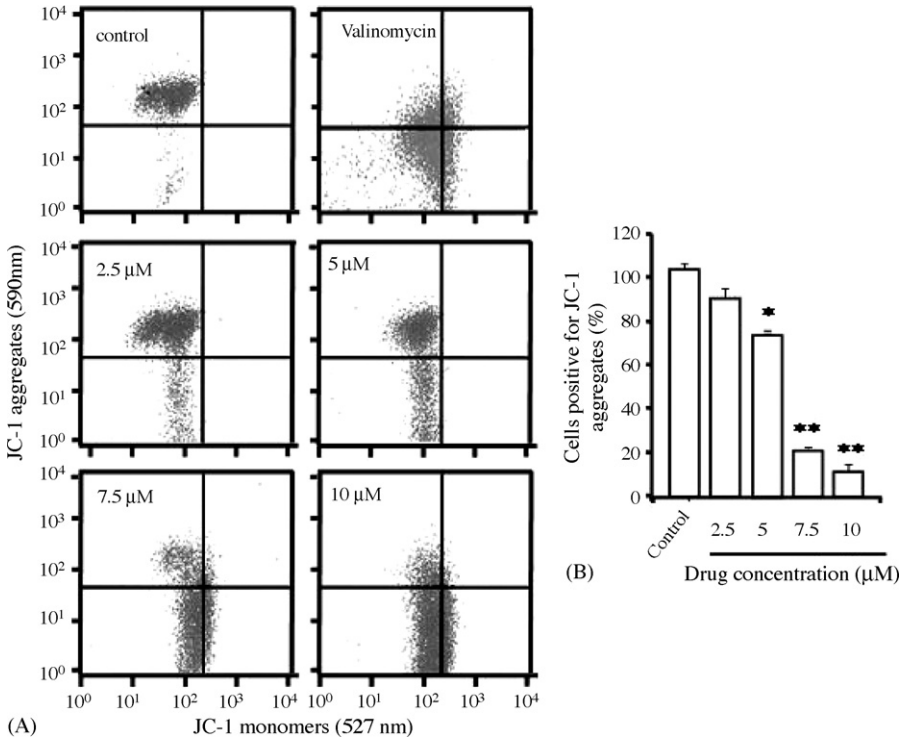

Fig. 4 – Effect of chlorotriphenylphosphine-1,3-bis(diphenylphosphino)propanegold (I) on the $\Delta\psi_{mt}$ in 2/60 cells. After a 24 h treatment with different concentration of the gold complex, cells were incubated in drug-free medium, stained with JC-1 and analyzed by flow cytometry. Cells treated for 30 min at 37 °C prior to JC-1 addition with 5 μM valinomycin were used as a positive control for $\Delta\psi_{mt}$ disruption. (A) Representative examples of the fluorescence pattern of cells incubated with the solvent (control) or with the gold complex (2.5–10 μM); (B) percentage of cells staining positive for JC-1-aggregates formation (emitting at 590 nm) in untreated (control) and the gold complex treated cell populations. Data represent mean values ± S.D. of three independent experiments. $^{*}P < 0.01$; $^{**}P < 0.001$; Student's *t*-test.

were used on each blot to ensure equal loading of protein on the gel.

2.8. *Statistical analysis*

Student's *t*-test was used to analyze the differences between control and gold complex-treated cells in terms of alterations in the $\Delta\psi_{mt}$, rate of apoptosis and catalytic activity of caspase-9. All tests were two-sided. *P*-values <0.05 were considered statistically significant.

3. Results

The cytotoxic activity of the gold complex chlorotriphenylphosphine-1,3-bis(diphenylphosphino)propanegold(I) was investigated in a panel of six human melanoma cell lines exposed to different concentrations (from 0.01 to 10 μM) of the compound for 48 h. A marked dose-dependent inhibition of cell growth was consistently observed in all cell lines (Fig. 2), with IC_{50} values ranging from 0.8 to 2.3 μM (Table 1). When the cytotoxic effect of the gold complex was compared to that of cisplatin under the same experimental conditions, we found a similar antiproliferative effect of the two drugs in three cell lines (JR8, SK-MEL-5 and 2/21) whereas in the remaining cell lines (MEL-501, 2/60 and GRIG) the gold complex was found to be from 2- to 5-fold more potent than cisplatin in inhibiting cell growth (Table 1).

Considering the ability of other gold derivatives to induce mitochondrial membrane permeability transition and alteration of $\Delta\psi_{mt}$ [28], we investigated whether $\Delta\psi_{mt}$ was

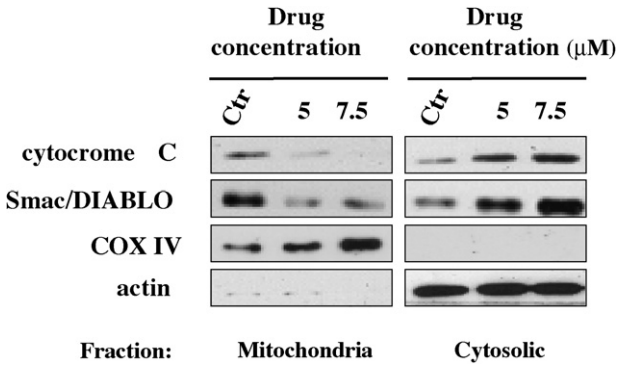

Fig. 5 – Representative experiments illustrating cytochrome c and Smac/DIABLO translocation from mitochondria into the cytosol of 2/60 cells. Cytosolic extract from cells exposed to the gold complex (5–7.5 μM) for 24 h were subjected to SDS-PAGE followed by Western blotting. Actin and COX IV were used as controls for correct loading of cytosolic and mitochondrial fractions, respectively.

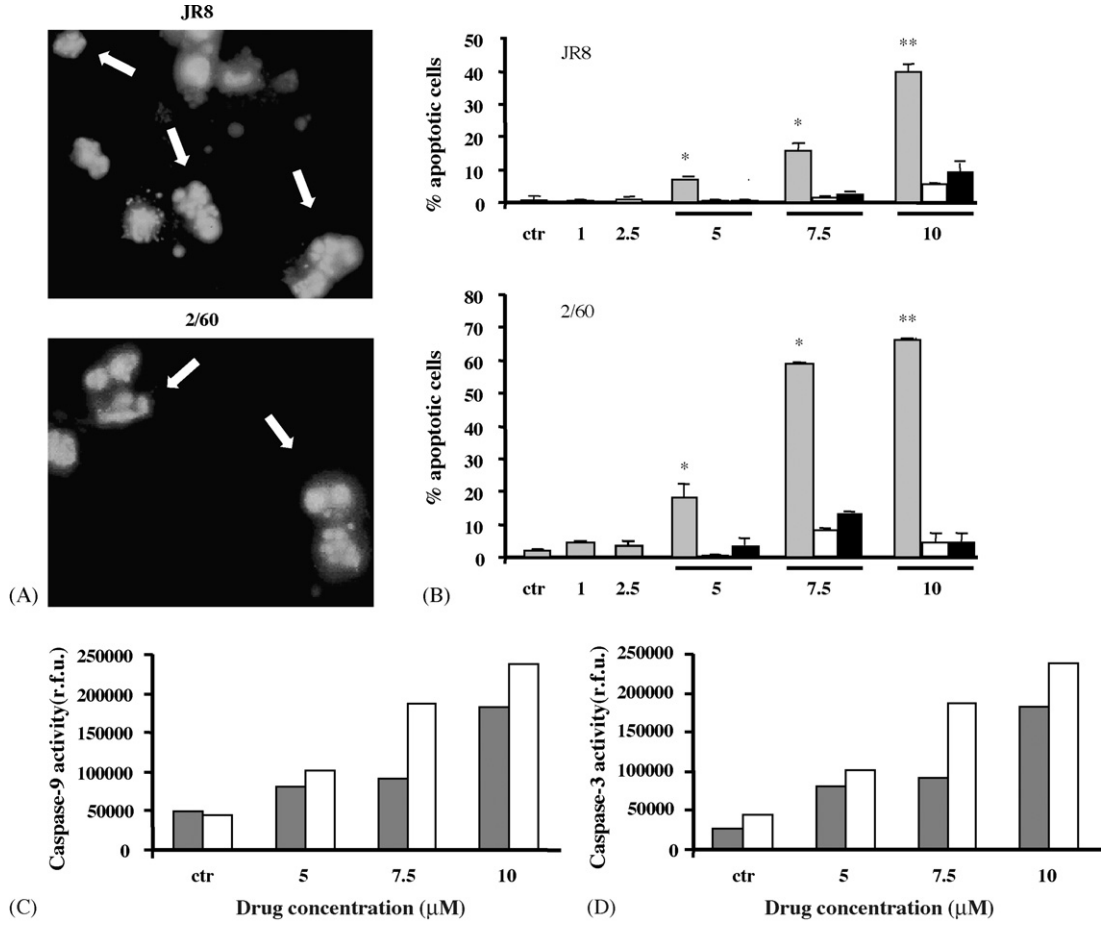

Fig. 6 – (A) Propidium iodide staining of JR8 and 2/60 cells exposed to 7.5 μM of 1,3-bis(diphenylphosphino)propanegold(I)chloride for 24 h. (B) Quantification of cells with an apoptotic morphology with respect to the overall population as assessed by fluorescence microscopy in JR8 and 2/60 cells exposed to different concentrations of the gold complex alone for 24 h (gray column) or with a 1-h pretreatment with 40 μM of the pan-caspase inhibitor z-VAD (empty column) or 50 μM of the caspase-9 inhibitor z-LEHT-fmk (black column). Data represent mean values ±S.D. of three independent experiment. $^{*}P < 0.01$; $^{**}P < 0.001$; Student's t-test. (C) Caspase-9 catalytic activity as determined by hydrolysis of the fluorogenic substrate LEHD-AFC in JR8 (gray column) and 2/60 (empty column) cells exposed to different concentrations of the gold complex. Data are expressed as relative fluorescence units and represent mean values of two independent experiments. (D) Caspase-3 catalytic activity as determined by hydrolysis of the fluorogenic substrate Ac-DEVD-AMC in JR8 (gray column) and 2/60 (empty column) cells exposed to different concentrations of the gold complex. Data are expressed as relative fluorescence units and represent mean values of two independent experiments.

modulated by our gold complex in two cell lines characterized by a slightly different sensitivity profile to the drug, JR8 and 2/60 cells. For this purpose, control and gold complex-treated cells were stained with the $\Delta\psi_{mt}$-sensitive dye JC-1 [27]. Representative flow cytometry data are shown in Figs. 3A and 4A for JR8 and 2/60 cell lines, respectively. Both cell lines showed a similar behavior. Specifically, in untreated JR8 and 2/60 cells, JC-1 exists predominantly in a highly aggregated form. Conversely, in cells exposed to valinomycin, a K+ ionophore that uncouples oxidative phosphorylation, JC-1 was largely present in its monomeric state, thus indicating a low $\Delta\psi_{mt}$. Following a 24-h treatment with the gold complex, a dose-dependent decrease in $\Delta\psi_{mt}$ was observed in both cell lines (Figs. 3A and 4A). Specifically, such a decrease was already appreciable after exposure to 5 μM of the compound (23 and 31% reduction of cells positive for JC-1 aggregates compared to control for JR8 and 2/60 cell lines, respectively; $P < 0.01$) and reached its maximum after treatment with the highest (10 μM) drug concentration (75 and 90% reduction of cells positive for JC-1 aggregates compared to control for JR8 and 2/60 cell lines, respectively; $P < 0.001$) (Figs. 3B and 4B). Results from immunoblotting experiments carried out in mitochondria and cytosolic fractions showed that decrease of $\Delta\psi_{mt}$ in gold complex-treated cell lines was accompanied by release of cytochrome c and the apoptogenic Smac/DIABLO protein from mitochondria into the cytosol (Fig. 5).

Since the disruption of $\Delta\psi_{mt}$ has been linked to the induction of apoptosis by different stimuli [29], we tested whether the $\Delta\psi_{mt}$ reduction observed in our cell lines following treatment with the gold complex was associated

to an apoptotic response. For this purpose, we assessed the presence of cells with an apoptotic nuclear morphology by fluorescence microscopy after cell staining with propidium iodide (Fig. 6A). Spontaneous apoptosis was observed in a negligible fraction of control cells (1 and 2% of the overall cell population for JR8 and 2/60 cell lines, respectively). However, in both cell lines the rate of apoptosis increased in a dose-dependent fashion following treatment with the gold complex (Fig. 6B). Such an enhancement was already appreciable after cell exposure to 5 μM of the gold complex (7 and 18% of the overall cell population for JR8 and 2/60, respectively; $P < 0.01$) and reached its peak at the highest (10 μM) drug concentration (40 and 66% of the overall cell population for JR8 and 2/60, respectively; $P < 0.001$) (Fig. 6B).

At the molecular level, the increased apoptotic response was accompanied by an enhanced catalytic activity of caspase-9 and caspase-3, as assessed by *in vitro* hydrolysis of the specific fluorogenic substrates. Such an enhancement was appreciable after exposure to 7.5 and 10 μM of the gold complex in 2/60 cells and only at the highest drug concentration in JR8 cells. Moreover, after treatment with 10 μM of the gold complex, the extent of enhancement of caspase-9 catalytic activity was greater in 2/60 than in JR8 cells (2.3-fold versus 3.7-fold increase for caspase-9 and 2.7-fold versus 3.6-fold increase for caspase-3, respectively) (Fig. 6C and D). In both cell lines gold complex induced apoptosis was almost completely prevented by a 1-h pre-incubation with 40 μM of the pan-caspase inhibitor z-VAD-fmk or with 50 μM of the caspase-9 specific inhibitor z-LEHD-fmk.

Exposure to 10 μM of the gold complex also induced a decrease in the expression of the anti-apoptotic proteins survivin and c-IAP1 and a complete abrogation of XIAP in both cell lines (Fig. 7).

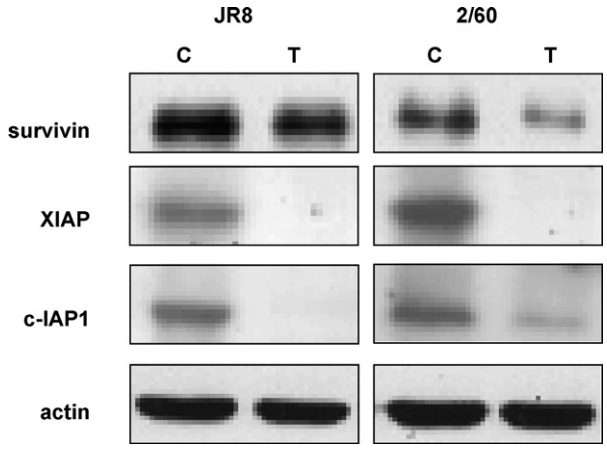

Fig. 7 – Representative Western blotting experiments illustrating survivin, XIAP and c-IAP1 expression in JR8 and 2/60 cells untreated (C) or treated (T) with 7.5 μM of the gold complex for 24 h. Actin was used as control for correct loading.

4. Discussion

Mitochondria are at the cross-road of many cellular pathways. In fact, these organelles generate 80–90% of ATP needed for cell respiration and survival by oxidative phosphorylation [30], regulate calcium flux [31] and play a major role in mediating programmed cell death, with particular reference to the integration of pro- and anti-apoptotic stimuli [32]. Drugs which specifically compromise the functional integrity of mitochondria could be of significant relevance in anticancer therapy considering that (i) continuous growth of tumor cells is highly energy-dependent, and (ii) tumor cells often develop drug resistance as a consequence to the inability to respond to pro-apoptotic stimuli.

Melanoma is the most aggressive of skin tumors, and disseminated disease is characterized by a very poor clinical outcome, which is not modified by conventional anticancer treatments [33]. Although not yet fully elucidated, the basis of melanoma resistance to chemotherapy seems to rely on dysregulation of apoptosis [34–37]. In fact, defects at multiple levels of the two major apoptotic pathways have been found in melanoma cell lines and surgical specimens [38,39].

New agents and novel approaches are under preclinical evaluation in this malignancy. In this context, it has been recently shown that chlorotriphenylphosphine-1,3-bis(diphenylphosphino)propanegold(I), a mixed phosphine gold species which is highly cytotoxic against several human tumor cell lines, displays a peculiar activity in melanoma cells [20]. The present challenge has been to elucidate the mechanisms responsible for the antiproliferative effect of this compound in melanoma cells, with particular reference to its ability to alter mitochondrial function, as previously reported for gold(I)-monophosphine and -diphosphine complexes [14,40,41].

In vitro proliferation studies confirmed a significant cytotoxic activity of the gold complex, with IC_{50} values ranging from 0.8 to 2.3 μM in melanoma cells. Moreover, a comparison with cisplatin, the most widely used metal complex in cancer therapy, showed similar or even superior (from 2- to 5-fold) antiproliferative activity of the gold complex in the different cell lines. Since cisplatin has a definite, although modest, activity in the therapy of advanced melanoma patients [42], this observation is of great interest since it would suggest a possible relevant antitumor activity of the gold complex in the clinical setting.

The gold complex caused a dose-dependent induction of apoptosis in melanoma cells, primarily through a caspase-9-mediated pathway, as suggested by the ability of the specific caspase-9 inhibitor z-LEHD-fmk to almost completely abrogate the apoptotic response in drug-treated cells. The activation of caspase-9 and caspase-3, as detected by increased *in vitro* catalytic activity, apparently resulted from drug-induced mitochondrial damage leading to collapse of mitochondrial trans-membrane potential and release of cytochrome *c* and the apoptogenic factor Smac/DIABLO, as a consequence of the osmotic swelling of the mitochondrial matrix and rupture of the outer membrane [43]. Cytochrome *c* is known to bind and activate the apoptotic protease activating factor-1 (Apaf-1), causing assembly of a multiprotein caspase-activating complex (apoptosome) and leading to activation of caspase-9 and initiation of a protease cascade [44]. Although this pathway is primarily governed by proteins of the Bcl-2 family, which include anti-apoptotic molecules and pro-

apoptotic molecules able to differentially affect mitochondrial homeostasis and cytochrome *c* release, other proteins belonging to the inhibitor of apoptosis protein (IAP) family are able to block a common step downstream of mitochondrial cytochrome *c* release by inhibiting terminal effector caspase-3 and caspase-7, and interfering with caspase-9 processing and activity [45].

The gold complex induced a decreased or completely abrogated expression of anti-apoptotic proteins belonging to the IAP family such as survivin, XIAP and c-IAP1. It is well recognized that these proteins counteract mitochondrial-death pathway by inhibiting caspases through direct/indirect binding [46,47] and are also able to physically interact with Smac/DIABLO thus preventing its pro-apoptotic function [48,49].

Overall, results from this study indicate that chlorotriphenylphosphine-1,3-bis(diphenylphosphino)propanegold(I) markedly inhibits human melanoma cell growth because it induces apoptosis via a caspase-9-dependent pathway and we conclude that chlorotriphenylphosphine-1,3-bis(diphenylphosphino)propanegold(I) is a good candidate for additional evaluation as anticancer agent against melanoma.

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