Research Article

Future Medicinal Chemistry

Preclinical activity of multiple-target gold(III)-dithiocarbamato peptidomimetics in prostate cancer cells and xenografts

Background: Recently, novel gold(III)-dithiocarbamato peptidomimetics, designed to target peptide transporters upregulated in several tumor cells have shown promise as anticancer agents. **Results:** The biological behavior of the most promising derivatives AuD8 and AuD9 was studied in PC3 and DU145 prostate cancer cells. They exert higher cytotoxicity *in vitro* than the reference drug cisplatin and induce apoptosis, promoting mitochondrial membrane permeabilization and stimulating reactive oxygen species generation. Moreover, they inhibit both selenoenzyme thioredoxin reductase and proteasome activity. Additionally, AuD8 effectively reduces tumor growth in prostate tumor-bearing nude mice with minimal systemic toxicity. **Conclusion:** Altogether, our results provide insights into the anticancer activity of these gold(III)-dithiocarbamato peptidomimetics and support their potential as new agents for prostate cancer treatment.

Marta Celegato[1], Dolores Fregona*[,2], Maurizio Mongiat[1], LucaRonconi[2,3], CinziaBorghese[1], Vincenzo Canzonieri[4], Naike Casagrande[1], Chiara Nardon[2], Alfonso Colombatti[1,5,6] & Donatella Aldinucci[1]

[1]Centro di Riferimento Oncologico, IRCCS, National Cancer Institute, Experimental Oncology 2, Via F. Gallini 2, Aviano (PN), 33081, Italy
[2]University of Padova, Department of Chemical Sciences, Via F. Marzolo 1, Padova, 35131, Italy
[3]National University of Ireland Galway, School of Chemistry, University Road, Galway, Ireland
[4]Centro di Riferimento Oncologico, IRCCS, National Cancer Institute, Division of Pathology, Via F. Gallini 2, Aviano (PN), 33081, Italy
[5]University of Udine, Department of Medical & Biological Science Technology, Piazzale Kolbe 3, Udine, 33100, Italy
[6]University of Udine, MATI (Microgravity, Ageing, Training, Immobility) Centre of Excellence, Italy
*Author for correspondence:
Tel.: +39 049 8275159
Fax: +39 02 700500560
dolores.fregona@unipd.it

Prostate cancer is a major cause of death in men, and bone metastasis is the primary cause of morbidity and mortality [1]. Most prostate tumors initially respond to androgen depletion therapy, but ultimately progress to androgen-independent disease with few therapeutic options [2], therefore new treatment strategies are required.

Cisplatin is amongst the first chemotherapeutic agents exhibiting broad effectiveness toward solid tumors and is still a widely used anticancer drug. In recent years, much effort has been made to design both platinum and nonplatinum metal complexes showing therapeutic outcomes, at least, comparable to cisplatin together with lower drawbacks (i.e., toxicity, tumor resistance and poor oral bioavailability) [3]. In this regard, some gold(III)-dithiocarbamato derivatives were reported to exert promising antiproliferative activity associated with reduced, or even no, systemic and renal toxicity [4]. Such complexes are characterized by the presence of a dithiocarbamato ligand stabilizing the metal center in the +3 oxidation state and likely acting as intrinsic chemoprotectant, thus limiting further interactions with biomolecules responsible for the onset of side effects and toxicity (in particular, nephrotoxicity), frequently accompanying metal-based chemotherapy [5], as widely demonstrated in animal models [6]. These gold(III)-dithiocarbamato derivatives were shown to overcome cisplatin resistance [7], displayed powerful antiproliferative and proapoptotic effects on a panel of acute myeloid leukemia cell lines [8], and were proved active both *in vitro* and *in vivo* in models of breast [9] and prostate cancer [10].

Unlike cisplatin, previous results indicated that DNA is not their main biological target, whereas they are able to activate the mitochondrial death pathways [11], inhibit proteasome activity both *in vitro* and *in vivo* [12,13], and affect the selenoenzyme thioredoxin reductase (TrxR) [14,15], recently identified as potential alternative targets for the development of novel chemotherapeutics for prostate cancer [2,16].

Subsequent to the positive outcomes achieved, we have extended our research toward 'second-generation' gold(III)-dithiocarbamato derivatives of oligopeptides as improved delivery and intracellular drug transfer systems, supported by transport pro-

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teins. Peptide transporters (PEPTs) are integral plasma membrane proteins that mediate the cellular uptake of di- and tri-peptides [17]. Two PEPTs, namely PEPT1 and PEPT2, have been identified in mammals. They are present predominantly in epithelial cells of the small intestine, mammary glands, lung, choroid plexus and kidney, but are also localized in other tissues to a lesser extent. Remarkably, they are overexpressed in some types of tumors [18], including prostate cancer [19]. A peculiar feature is their capability for sequence-independent transport inside the cells of most possible di- and tri-peptides, as well as peptide-like chemotherapeutics resembling the main structural scaffold of small peptides (i.e., peptidomimetics) [20]. Therefore, PEPTs represent an excellent target for the delivery of pharmacologically active compounds as their substrate binding site can fit a wide range of molecules of different size, hydrophobicity and charge [21].

On account of these considerations, we have been designing some novel gold(III)-dipeptidedithiocarbamato complexes that could combine both the antitumor properties and the favorable toxicological profile of the previously reported gold(III) analogs, along with an enhanced bioavailability and tumor selectivity owing to the exploitation of the dipeptide-mediated cellular internalization by targeting PEPT1 and PEPT2 via the dipeptide chain [22,23]. Although not yet demonstrated for our gold(III)-dithiocarbamato compounds, it was previously proved that peptidomimetics are able to target peptide transporters in prostate cancer [18], thus providing a reliable rationale for the design of these gold-based peptidomimetics. Among all, the gold(III) complexes [$Au^{III}X_2$(dtc-Sar-Aib-O[*t*-Bu])] (dtc = $SSCN^-$; sar: sarcosine [*N*-methylsarcosine]; Aib: α-aminoisobutyric acid [2-methylalanine]; X: Br [AuD8], Cl [AuD9]) (Figure 1) turned out to be among the best performers, reporting *in vitro* IC_{50} values in the low micromolar range toward a number of human tumor cell lines (up to fivefold lower than the reference drug cisplatin) [22].

Further to these extremely positive preliminary results, we here report on detailed *in vitro* and *in vivo* biological studies aimed at evaluating their anticancer activity against androgen-resistant prostate cancer, as well as at elucidating their mechanism of action by means of several assays focused on the inhibition of proteasome and thioredoxin, reactive oxygen species (ROS) accumulation and levels of expression of pro- and anti-apoptotic proteins.

Key terms

Gold-based peptidomimetics: Coordination compounds deriving from the binding of a peptideligand to a metal element (commonly a transition metal center, namely gold[III]).

Reactive oxygen species (ROS): Highly reactive oxygen containing molecules (free radicals) with unpaired electrons able to trigger oxidative chain reactions thus leading cell damages.

Proteasome: Multicatalytic enzyme complex that degrades ubiquitinated proteins via an ATP-dependent mechanism.

Thioredoxin reductase: Selenoenzyme recently identified as a potential alternative target for the development of novel anticancer chemotherapeutics.

Crossresistance: Some cell lines that show decreased sensitivity (resistance) to a chemotherapeutic also exhibit resistance to several other antitumor drugs.

Bcl-xl: B-cell lymphoma-extra large is a transmembrane molecule in the mitochondria and member of the Bcl-2 family of proteins, acting as a prosurvival protein by preventing the release of cytochrome c.

Bcl-2: B-cell lymphoma-2, encoded in humans by the ***BCL2*** gene, is the founding member of the Bcl-2 family of regulator proteins that regulate cell death (apoptosis). Bcl-2 is specifically considered as an important antiapoptotic protein and is thus classified as an oncogene.

Bax: Bcl-2 associated x protein that promotes apoptosis by binding to and antagonizing the Bcl-2 protein. The ratio of Bcl-2 to Bax determines survival or death following an apoptotic stimulus.

Results & discussion

Cell growth inhibition & apoptosis induction

We first evaluated the *in vitro* cytotoxic effect of the gold(III)-dipeptidedithiocarbamato derivatives AuD8 and AuD9 (Figure 1) on the androgen-resistant prostate cancer PC3 and DU145 cell lines. For comparison purposes, cisplatin (Figure 1) was also evaluated under the same experimental conditions. As reported in Table 1, both AuD8 and AuD9 inhibited cell proliferation over 72 h in a dose-dependent way, showing IC_{50} values about up to fivefold lower than the reference platinum drug. To establish whether cancer cells could start proliferating again after removal of the gold compounds, we performed cell growth recovery experiments. After incubation for 24 h with either AuD8, AuD9 or cisplatin, the medium was replaced with a drug-free one and cells were incubated for an additional 72 h. Remarkably, the effect of the gold(III) compounds was not reversed, suggesting a cytotoxic rather than cytostatic activity, whereas cisplatin showed lower antiproliferative potency after the shorter incubation time of 24 h. Moreover, the gold(III) derivatives were proved significantly more cytotoxic than the reference platinum drug also toward the corresponding cisplatin-resistant parent cell lines PC3-R and DU145-R, with activity levels comparable with those induced in the cisplatin-sensitive counterparts (over 72 h), thus ruling out crossresistance phenomena.

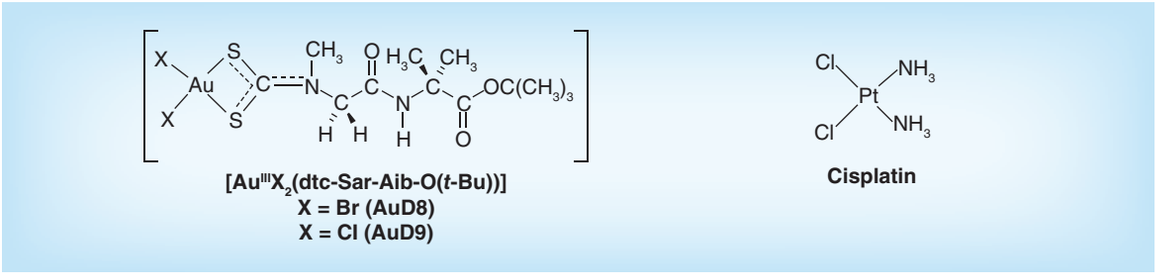

Figure 1. Chemical drawing of the compounds studied in this work. [$Au^{III}Br_2$(dtc-Sar-Aib-O*t*-Bu)] (AuD8), [$Au^{III}Cl_2$(dtc-Sar-Aib-O*t*-Bu)] (AuD9) and *cis*-[$Pt^{II}Cl_2(NH_3)_2$] (cisplatin).

Since PC3 cells are more invasive and more resistant to chemotherapy than DU145, the former cell line was selected for further in-depth investigations aimed at assessing the capability of the gold(III) compounds to induce apoptosis and cell cycle modifications. Following a 24 h incubation with a single cytotoxic dose (10 µM) of either AuD8, AuD9 or cisplatin, treatment with both complexes resulted in a substantial phosphotidylserine exposure together with a higher percentage of cells permeable to propidium iodide (PI) staining (whereas cisplatin had negligible effect under the same experimental conditions), thus supporting apoptosis as a major mechanism of cell death (Figure 2A).

Cell cycle progression and DNA fragmentation were measured by PI staining and flow cytometric analyses. Incubation for 12 h with the gold compounds caused an increase of the S phase and a decrease of the G_2M phase, the latter being again detected after 24 h together with a reversal of the S phase and a block in G_0/G_1 step with respect to the control (Figure 2B). DNA fragmentation was apparent after 48–72 h. On the other hand, after 72 h an equivalent amount of cisplatin caused a blockade in the G_2M phase of the cell cycle, with no significant DNA fragmentation (Figure 2B). DNA fragmentation induced by AuD8 and AuD9 was also confirmed by Apo-Direct analysis (Figure 3A).

Altogether, these results point out a quite different mechanism of action of our compounds if compared with the reference drug cisplatin. In this context, they seem to trigger cell death commitment in few hours, thus highlighting DNA is not their main target.

Inhibition of mitochondrial functions & activation of the caspases pathway

Incubation of PC3 cells with the investigated compounds promoted mitochondrial membrane permeabilization and cytochrome-c (Cyt-c) release from the mitochondria (Figure 3A). Additionally, treatment with either compound also induced activation of caspase 3 and caspase 9 after just 3 h (Figure 3B).

Since Bcl-2, Bcl-xL and Bax proteins play a key role in regulating the intrinsic apoptotic pathway, we analyzed whether AuD8 and AuD9 could affect their expression. Both gold derivatives increased the proapoptotic molecule Bax and markedly decreased the antiapoptotic molecule Bcl-xL (Figure 3C). Bcl-2 expression resulted significantly downregulated only upon incubation with AuD8. On the contrary, under the same experimental conditions, cisplatin reduced Bcl-xL expression (Figure 3C) but had negligible or even no effect on Bax and Bcl-2 expression.

Overall, these findings put in evidence that our gold complexes affected the expression of three essential regulators of the mitochondrial apoptotic pathway after a short incubation. On the contrary, over 24 h cisplatin was shown to not be able to affect the mito-

Table 1. *In vitro* growth inhibition of human prostate cancer cisplatin-sensitive (PC3 and DU145) and -resistant (PC3-R and DU145-R) cells by the gold(III)-dipeptidedithiocarbamato derivatives AuD8 and AuD9, and comparison with the reference drug cisplatin.

Cell line	Cisplatin		AuD8		AuD9	
	72 h†	24 + 72 h‡	72 h	24 + 72 h	72 h	24 + 72 h
PC3	3.3 ± 0.3§	15.0 ± 1.6	0.8 ± 0.1§	1.5 ± 0.1	1.1 ± 0.1§	1.6 ± 0.2
PC3-R	9.9 ± 1.1	–	1.8 ± 0.2	-	2.9 ± 0.3	–
DU145	4.5 ± 0.1§	12.0 ± 1.3	1.4 ± 0.1§	1.5 ± 0.1	2.2 ± 0.1§	1.8 ± 0.1
DU145-R	8.2 ± 0.8	–	2.7 ± 0.3	-	2.1 ± 0.1	–

IC_{50} (µM): Mean ± standard deviation ($p < 0.0001$).
†72 h: cells incubated with the tested compound for 72 h.
‡24 + 72 h: cells incubated with the tested compound for 24 h then washed and incubated for additional 72 h with fresh medium.
§See reference [22].

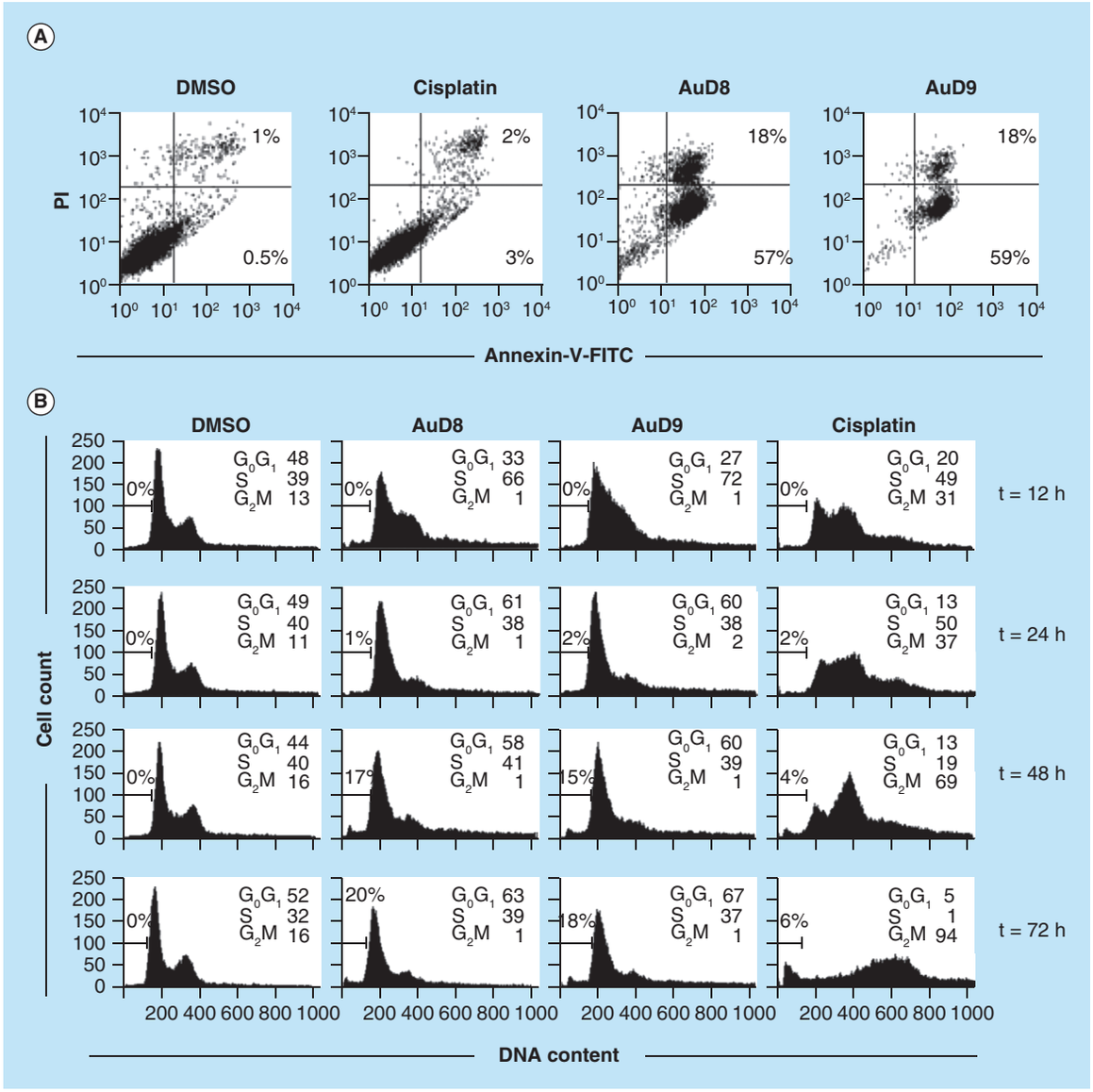

Figure 2. Induction of apoptosis, DNA fragmentation and changes in cell cycle progression by AuD8 and AuD9.
(A) Fluorescence-activated cell sorting analysis of PC3 cells in drug-free medium and after 24 h incubation at 37°C with either AuD8, AuD9 or cisplatin (10 μM), and double stained with Annexin-V-FITC and PI.
(B) Fluorescence-activated cell sorting histograms showing cell cycle progression and DNA fragmentation after 12, 24, 48 and 72 h. The percentages of cells in the G_0/G_1 phase, S phase and G_2M phase are reported, as well as the percentage of sub-G_1 cells. The same amount of DMSO necessary to dissolve the compounds was used as negative control. Fluorescence-activated cell sorting dot plots and histograms are representative of one of three different experiments.

chondrial functions and, hence, to lead to apoptotic upstream signals in agreement with its well-known long time required to target the cell nucleus.

Mitochondrial ROS accumulation & inhibition of the activity of the selenoenzyme TrxR

In our previous studies carried out on prostate cancer cells, 'first-generation' gold(III)-dithiocarbamato derivatives were shown to inhibit the activity of selenoenzyme TrxR and to promote ROS production, thus highlighting a potential major involvement of a deregulation of the thioredoxin/thioredoxin reductase redox system in their antiproliferative activity [10]. Therefore, we extended our investigations to assess the capability of these gold(III)-based peptidomimetics to induce ROS accumulation. Both gold complexes induced a dramatic increase of mitochondrial ROS, as evaluated by Mitosox Red assay, even after a short incubation time (12 h) (Figure 4A & 4B). In agreement with previously described results, AuD8 and AuD9, contrary to

the reference drug, promote ROS species production that may be involved in the release of Cyt C and so in the upregulation of proapoptotic factors.

In order to establish a possible relationship between ROS overproduction and decreased cell viability, PC3 cells were cotreated with the ROS scavenger *N*-acetyl-l-cysteine (l-NAC) and either AuD8 or AuD9. With reference to Figure 4C, pretreatment with l-NAC effectively blocked ROS production and, at the same time, completely neutralized the antiproliferative effect of the gold derivatives (Figure 4D), thus suggesting that their cytotoxic activity is somewhat connected with ROS generation. On the other hand, ROS scavenging could be also associated with a direct reaction of the thiol moiety of l-NAC with the metal center, leading to its reduction to a nonactive metallic gold.

We also investigated the effects on the thioredoxin (Trx) system which is involved in ROS detoxification, thus avoiding the formation of proapoptotic molecules, as well as in the maintenance of the intracellular redox balance [24,25]. Higher levels of Trx and TrxR are found in many different tumor types and in transformed cell lines compared with healthy cells from the same patient [24,26–27]. Prostate cancer tissues are in a state of redox imbalance, and the Trx/TrxR system is frequently upregulated [28,29]. Increased Trx levels have also been associated with resistance to several powerful anticancer drugs in prostate cancer, including cisplatin [30] and docetaxel [31]. The pivotal role of TrxR in regulating apoptosis [32] and the high levels of expression detected in different cancer histotypes have boosted interest in developing drugs that, on either their own or in combination with other therapeutic agents, can target the Trx system [33]. Many compounds, including our gold(III)-dithiocarbamato derivatives [14], were proved potent inhibitors of purified TrxR *in vitro*, but only a few studies have reported its inhibition in cancer cell lines [10,33–34]. In this regard, a short incubation (12 h) with either AuD8 or AuD9 (10 μM) was able to inhibit dramatically (~90%) the activity of TrxR in PC3 cells (Figure 4E).

Remarkably, under the same experimental conditions, cisplatin was much less effective in both inducing ROS accumulation and affecting TrxR activity.

Inhibition of proteasome activity

The proteasome is a multicatalytic enzyme complex that degrades ubiquitinated proteins via an ATP-dependent mechanism, and plays a central role in cell cycle progression and cell death [35]. Specific proteasome inhibitors have recently emerged as effective anticancer agents [13,36] and they are showing promise against several tumor types [37]. In this context, our 'first-generation' gold(III) compounds turned out to be proteasome inhibitors in breast cancer cells and xenografts [9,16]. In this study, we demonstrate that both compounds AuD8 and AuD9 significantly inhibit the proteasome activity also in PC3 cells (Figure 4F), whereas cisplatin had negligible effect under the same experimental conditions.

Evaluation of epithelial growth factor receptor phosphorylation & cell migration

Androgen-independent prostate cancer is characterized by a greater invasive potential than its hormone-responsive counterpart. A successful therapeutic approach should provide suppression of not only cancer cell proliferation, but also of the metastatic potential of any cell escaping from the primary tumor site to colonize the bone marrow [38]. Our study shows that both gold(III) complexes reduce EGFR expression, whose impaired endocytic downregulation contributes to oncogenic metastatic phenotype [39] and whose ligands are paracrine and autocrine growth factors [40–42]. Treatment of PC3 cells with AuD8 or AuD9 resulted in a significant decrease of the surface expression of both EGFR and its phosphorylated form (pEGFR), as assessed by flow cytometry (Figure 5A & 5B), whereas cisplatin was proved almost ineffective. EGFR phosphorylation is known to affect prostate cancer cells migration [43]. Accordingly, the effect of the gold complexes on PC3 cells migration was evaluated by means of the scratch wound healing assay [44]. Cells were cultured at low serum concentration so as to rule out that the differential migration rate subsequent to the treatment was attributable to decreased cell proliferation. After 24 h, incubation with either compound (4 μM) reduced PC3 cell migration rate (Figure 5C & 5D) and cells, contrary to the control counterparts, were not able to form a confluent monolayer.

Inhibition of tumor growth in prostate cancer xenografts

The anticancer activity of AuD8 was evaluated also *in vivo*. PC3 cells (3×10^6 cells) were implanted into the right flank of 6-week-old athymic nude mice and, once tumors reached a volume of about 28 mm^3, animals were treated every other day with intratumoral injections of either drug-free medium vehicle or containing 2 mg kg^{-1} of AuD8. Significant tumor growth inhibition induced by the gold complex was evident after 5 days of treatment. At the end of the experiment (19 days), the control tumors grew to an average 890 ± 79 mm^3 volume, whereas mice treated

Defined key terms

Scratch assay: Wound test that measures the expansion of a cell population on surfaces.

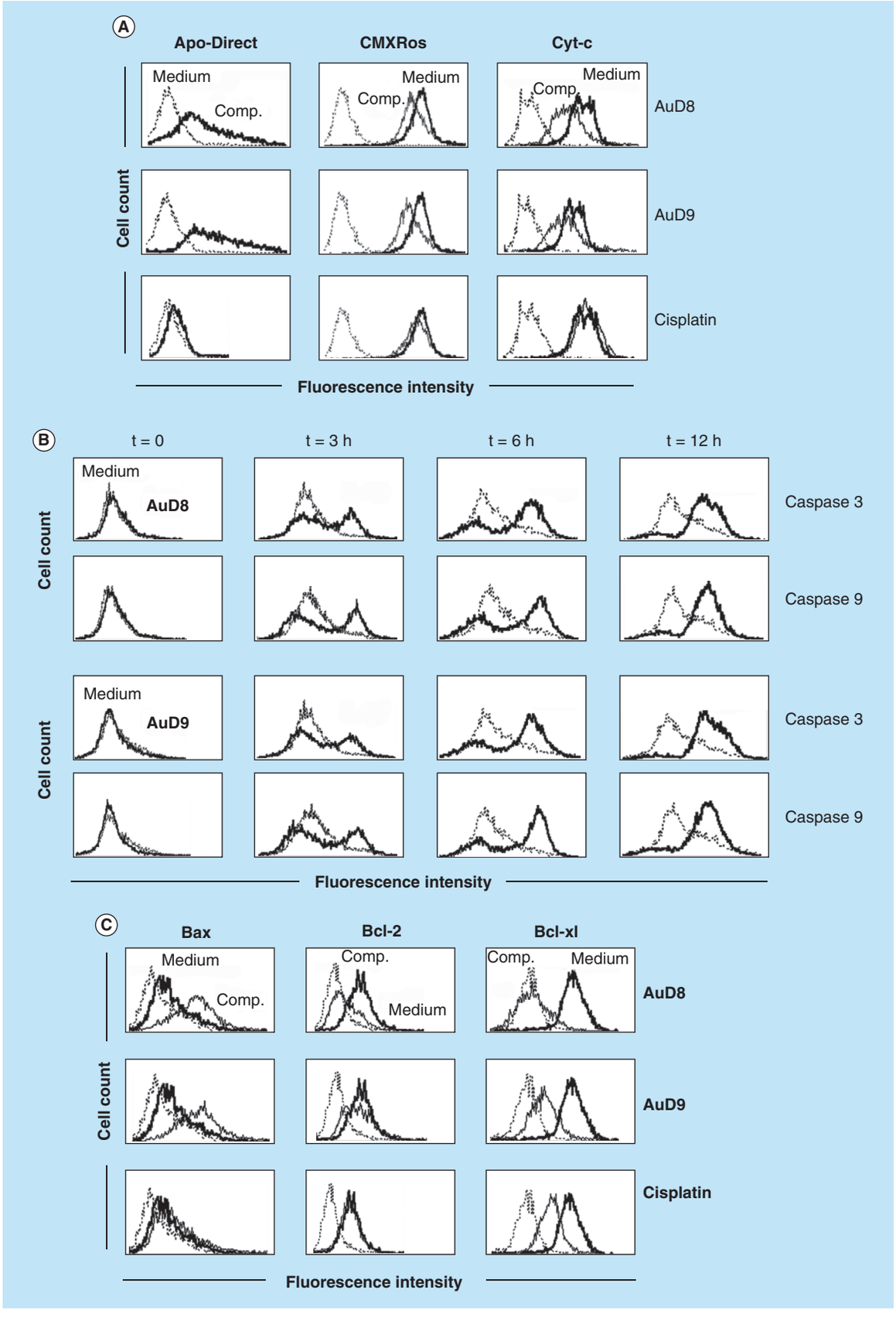
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Figure 3. Mitochondrial-mediated apoptosis and Bax, Bcl-2 and Bcl-xL modulation induced by AuD8 and AuD9 (see facing page). (A) Fluorescence-activated cell sorting (FACS) analysis of DNA fragmentation (Apo-Direct), mitochondrial membrane permeabilization (CMXros), and Cyt-c release as assessed by flow cytometry upon treatment of PC3 cells at 37°C for 24 h with either AuD8, AuD9 or cisplatin (10 μM). **(B)** FACS analysis of caspases activation upon incubation of PC3 cells at 37°C with either AuD8, AuD9 or cisplatin (10 μM) for 3, 6 and 12 h; cells were then harvested, washed and resuspended in warm complete medium supplemented with fluorochrome inhibitors of caspases for 1 h at 37°C under 5% CO_2, then washed again and analyzed by flow cytometry. **(C)** FACS analysis of Bax, Bcl-2 and Bcl-xL after treating PC3 cells at 37°C in complete medium containing either AuD8, AuD9 or cisplatin (10 μM). After 24 h, Bax, Bcl-2 and Bcl-xL expression was assessed by flow cytometry. FACS histograms are representative of one of three different experiments. Unless otherwise stated, dotted lines indicate background fluorescence of cells, as determined by isotype-matched immunoglobulins or autofluorescence. x- and y-axes indicate the logarithm of the relative intensity of fluorescence and the relative cell number, respectively. Cyt-c: Cytochrome-c.

with AuD8 experienced a 70% inhibitory effect, since tumors reached an average size of 270 ± 38 mm^3 (Figure 6A). Mice body weights were monitored every other day and no significant changes were detected upon treatment. Remarkably, no histologically detectable cytotoxicity involving animals' lung, liver, kidney or spleen was observed, except for focal and aspecific lung lymphocyte infiltration (Figure 6B).

Experimental

Chemicals

Cisplatin (Ebewe Pharma, Unterach, Austria), *N*-acetyl-L-cysteine, DMSO, propidium iodide, RPMI medium, penicillin, streptomycin, L-glutamine, digitonin, fetal bovine serum (FBS) (Sigma-Aldrich, Milan, Italy) were used as received. All other reagents and solvents were of high purity and were used as purchased without any further purification. The gold(III)-dithiocarbamato complexes [$Au^{III}Br_2$(dtc-Sar-Aib-O[*t*-Bu])] (AuD8) and [$Au^{III}Cl_2$(dtc-Sar-Aib-O[*t*-Bu])] (AuD9) were synthesized as previously described [22]. Before use, the gold(III) complexes were dissolved in DMSO, aliquoted (10 mM) and stored at -80°C. Just before the experiments, calculated amounts of either gold compounds DMSO solutions or cisplatin aqueous isotonic solution were then added to RPMI medium and filter sterilized, to a final organic solvent concentration lower than 0.5% (v/v). In particular, taking into account that the logP (partition coefficient) of AuD8 and AuD9 is approximately 1, we are able to achieve 100-μM concentrations in aqueous media by previous dissolution with DMSO. The culture medium added with the same amount of drug-free DMSO was used as negative control in all experiments. All the tested gold(III) complexes were proved by ^{1}H NMR studies to be stable in DMSO over 48 h [22].

Cell lines & culture conditions

Androgen-resistant human prostate cancer PC3 and DU145 cell lines were obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany) and further authenticated by BMR Genomics (Padova, Italy). The parent cisplatin-resistant PC3-R and DU145-R cell lines were obtained by weekly treatment with 1 μM cisplatin. All cells were cultured at 37°C in 5% CO_2 and moisture-enriched atmosphere in RPMI medium supplemented with 10% heat-inactivated FBS, 0.1% (w/v) L-glutamine and antibiotics (0.2 mg mL^{-1} penicillin and streptomycin).

Cell proliferation assay

Before testing, 2.5 × 10^3 cells were seeded in 96-well flat-bottomed microplates in RPMI medium (100 μl) and incubated at 37°C in a 5% CO_2 atmosphere for 24 h to allow cell adhesion. The medium was then removed and replaced with fresh one containing the compounds to be studied at increasing concentrations (0.3, 0.6, 1.25, 2.5, 5 and 10 μM) at 37°C for 72 h. Alternatively, cells were exposed to increasing concentrations (0.5–10 μM) of the investigated compounds at 37°C for 24 h, then washed and incubated for additional 72 h with fresh medium. Each treatment was performed in triplicate in three independent experiments. Cell proliferation was assayed using the Bromodeoxyuridine (BrdU) Cell Proliferation ELISA kit (Roche Diagnostics, Mannheim, Germany) according to manufacturer's instructions. IC_{50} values (representing the concentration of a substance required for 50% growth inhibition *in vitro*) were calculated using the CalcuSyn software (Biosoft, MO, USA) [45].

Evaluation of cell cycle progression & apoptosis

A total of 5 × 10^5 PC3 cells were incubated in Petri dishes with complete medium containing 10 μM of either AuD8, AuD9 or cisplatin at 37°C. After 12, 24, 48 and 72 h, DNA fragmentation and cell cycle progression were evaluated by PI staining, as described elsewhere [8]. Data analysis was performed using ModFit LT software (Verity Software House, Topsham (ME), USA).

Annexin-V binding, DNA fragmentation, changes in mitochondrial membrane potential & production of mitochondrial ROS

A total of 2.5 × 10^5 PC3 cells were incubated in six-well plates with RPMI medium supplemented with

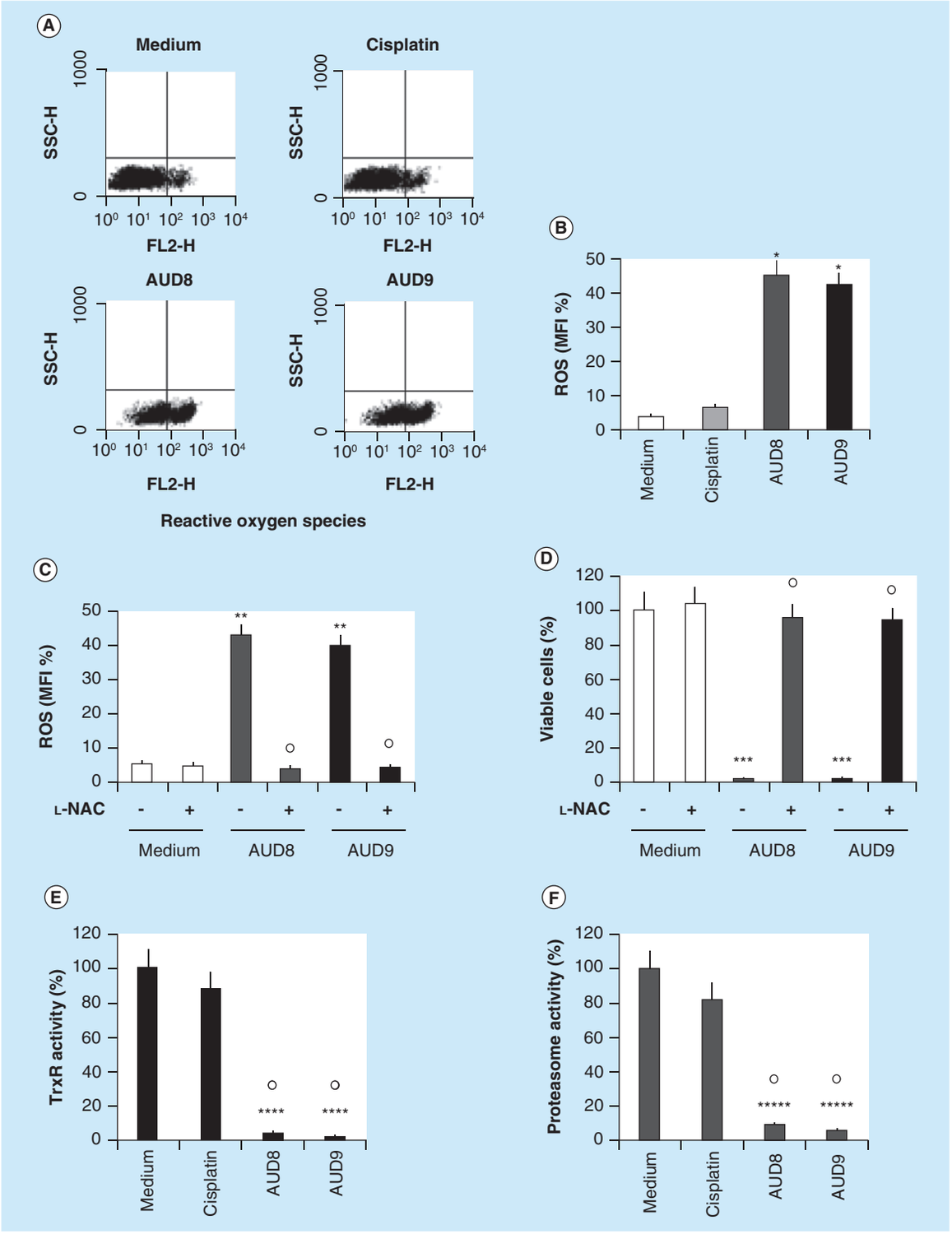

Figure 4. Reactive oxygen species accumulation, inhibition of thioredoxin reductase and proteasome activity induced by AuD8 and AuD9. (A) Fluorescence-activated cell sorting analysis of mitochondrial reactive oxygen species (ROS) production in PC3 cells treated with either AuD8, AuD9 or cisplatin (10 μM) at 37°C for 12 h by flow cytometry after incubation for 30 min at 37°C with MitoSox reagent. FACS dot plots are representative of one of three different experiments. **(B)** The histogram represents the percentage of ROS as the mean ± standard deviation (SD) of three different experiments. **(C)** Effect on ROS production in PC3 cells upon cotreatment with either AuD8 or AuD9 (10 μM) and the antioxidant l-NAC (5 mM) at 37°C for 24 h.

Figure 4. Reactive oxygen species accumulation, inhibition of thioredoxin reductase and proteasome activity induced by AuD8 and AuD9 (cont.). The histogram represents the percentage of ROS as the mean ± SD of three different experiments. **(D)** Percentage of PC3 cells viability upon cotreatment with either AuD8 or AuD9 (10 μM) and the antioxidant l-NAC (5 mM) at 37°C for 24 h, counted after additional 72 h of incubation with fresh medium. The histogram represents the percentage of ROS as the mean ± SD of three different experiments. **(E)** Inhibition of TrxR in PC3 cells treated at 37°C with either AuD8, AuD9 or cisplatin (10 μM) at 37°C for 12 h, evaluated by Thioredoxin Reductase Assay kit. Values represent the mean ± SD of three different experiments. **(F)** Inhibition of proteasome chymotrypsin-like activity in PC3 cells treated AuD8, AuD9 or cisplatin (10 μM) at 37°C for 12 h, evaluated by Proteasome Assay kit. Values represent the mean ± SD of three different experiments.
*$p < 0.0001$ AuD8 or AuD9 vs control.
**$p < 0.0001$ AuD8 or AuD9 vs control; °$p < 0.0001$ AuD8 or AuD9 vs AuD8 + l-NAC or AuD9 + l-NAC.
***$p < 0.0001$ AuD8 or AuD9 vs control; °$p < 0.0001$ AuD8 or AuD9 vs AuD8 + l-NAC or AuD9 + l-NAC.
****$p < 0.0001$ AuD8 or AuD9 or cisplatin vs control; °$p < 0.0001$ AuD8 or AuD9 vs cisplatin.
*****$p < 0.0001$ AuD8 or AuD9 or cisplatin vs control; °$p < 0.0001$ AuD8 or AuD9 vs cisplatin.

10% FBS containing 10 μM of either AuD8, AuD9 or cisplatin at 37°C for 24 h.

Annexin-V binding was detected by flow cytometry, as described elsewhere [8]. DNA fragmentation within cells was assessed using the Apo-Direct kit (Becton-Dickinson Pharmigen, CA, USA) according to manufacturer's instructions.

To evaluate the dissipation of the mitochondrial membrane potential, 200 nM CMXros (Molecular Probes, OR, USA) was added to the cell culture for 30 min, then cells were washed twice and analyzed by flow cytometry, as described elsewhere [46].

For Bcl-2, Bcl-xL and Bax analysis, fixed and permeabilized PC3 cells were incubated with fluorescein isothiocyanate (FITC)-conjugated mouse antihuman Bcl-2 (clone 124) (DAKO Citomation, Milan, Italy), or with rabbit anti-human Bcl-xL (Cell Signaling, MA, USA) followed by goat antirabbit immunoglobulin G (IgG)-FITC (Becton-Dickinson Pharmigen), or with 1 μg ml^{-1} of mouse anti-Bax generated from Bax-alpha (Becton-Dickinson Pharmigen), followed by phycoerythrin (PE)-conjugated goat anti-mouse IgG (Becton-Dickinson Pharmigen) [8].

Cyt-c release was assessed, with minor modifications, as explained elsewhere [47]. Briefly, PC3 cells were permeabilized with 100 μg ml^{-1} digitonin and fixed with 4% paraformaldehyde in phosphate buffered saline (PBS) solution for 20 min at room temperature. After washing twice with PBS, cells were incubated with labeling medium (2% FBS, 0.2% sodium azide, 0.5% TritonX-100 in PBS) for 15 min, then with 1 μg ml^{-1} of the mouse anticytochrome-c antibody (Becton-Dickinson Pharmigen), followed by PE-conjugated goat anti-mouse IgG.

For intracellular ROS evaluation, cells were incubated with 5 μM of MitoSox reagent working solution (Molecular Probes Invitrogen, Milan, Italy) for 15 min at 37°C. Red fluorescence was immediately analyzed by flow cytometry. In another series of experiments, 25×10^3 PC3 cells were seeded in 24-well plates and exposed to either AuD8 or AuD9 (10 μM) in the presence or absence of the antioxidant l-NAC (5 mM) at 37°C for 24 h. Cells were then washed, medium replaced with fresh one, and viable cells were counted after 72 h using the trypan blue dye exclusion assay.

Caspase activity was evaluated using the fluorochrome inhibitors of caspases CaspaTagTM caspase-3/7 (FAM-DEVD-FMK), caspase-9 (FAM-LETD) (Chemicon International, Milan, Italy). PC3 cells were treated with either AuD8, AuD9 or cisplatin (10 μM) at 37°C for 3, 6 and 12 h, then harvested, washed and resuspended in warm complete medium supplemented with fluorochrome inhibitors of caspases for 1 h at 37°C in a 5% CO_2 atmosphere, and then analyzed immediately by flow cytometry. Viable antibody-labeled cells were identified according to their forward and right-angle scattering, electronically gated and analyzed on a FACScalibur flow cytometer (Becton-Dickinson Pharmigen), using CellQuest software (Becton-Dickinson Pharmigen).

Thioredoxin reductase enzyme activity assay

TrxR activity in cultured cells was assessed using the Thioredoxin Reductase Assay Kit (Sigma-Aldrich, Milan, Italy), according to manufacturer's instructions: 2.5×10^5 cells were treated with 10 μM of either AuD8, AuD9 or cisplatin and, after 12 h, cells were lysed with lysis buffer (50 mM Tris-HCl, 0.1% Triton X-100, 0.9% NaCl, pH 7.6) on ice for 30 min. Total protein content was analyzed with the protein assay dye reagent (Bio-Rad Laboratories, CA, USA). Cell lysates were then incubated in 100 mM of potassium phosphate with 10 mM ethylenediaminetatraacetic acid (EDTA) and 0.24 mM nicotinamide adenine dinucleotide phosphate (NADPH) with and without a TrxR inhibitor. The reaction was started by adding dinitrothiocyanobenzene (DNTB) and it was monitored spectrophotometrically at 412 nm.

Evaluation of proteasome activity

Cytosolic extracts were assayed to measure proteasome activity using 20S Proteasome Activity Assay kit (Chemicon International) according to manufacturer's

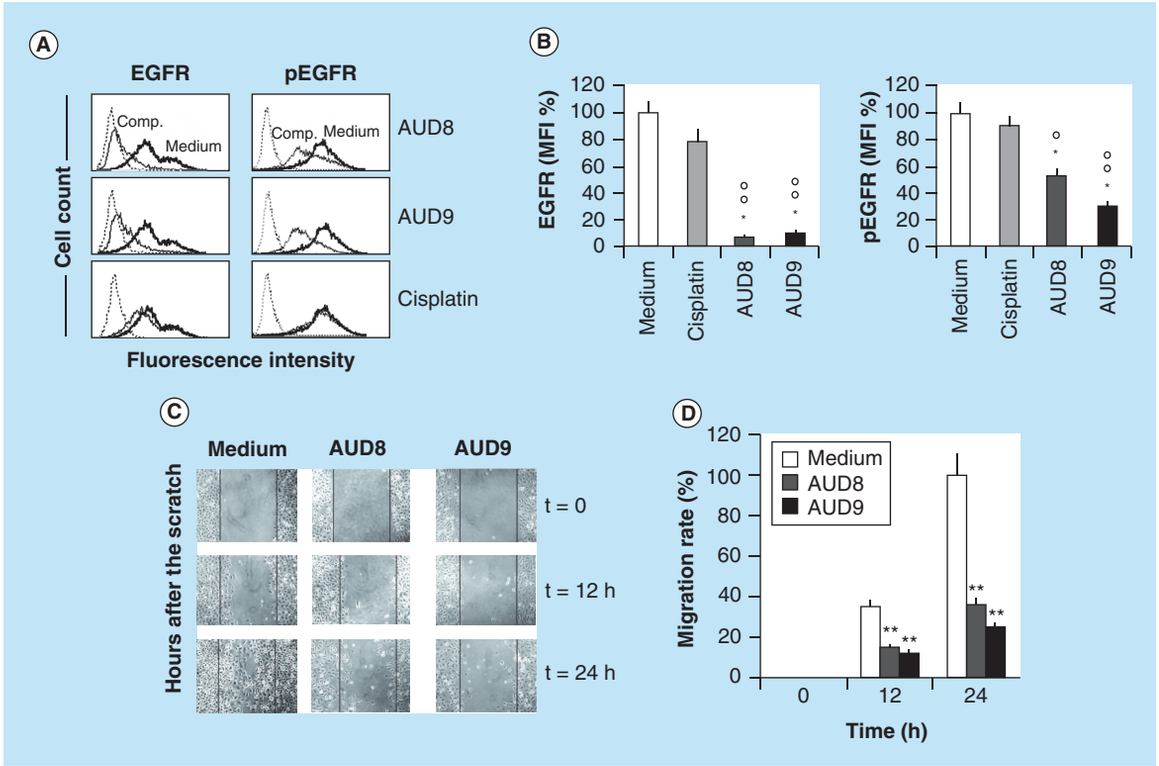

Figure 5. Inhibition of EGFR expression and cell migration induced by AuD8 and AuD9. (A) Fluorescence-activated cell sorting analysis of EGFR and the corresponding phosphorylated form pEGFR after treating PC3 cells at 37°C for 12 h, with either AuD8, AuD9 or cisplatin (10 μM). EGFR and pEGFR were analyzed using the anti-EGFR mAb 528 and the anti-pEGFR PY1197, respectively. Fluorescence-activated cell sorting histograms are representative of one of three different experiments. Dotted lines indicate background fluorescence of cells, as determined by isotype-matched immunoglobulins or autofluorescence. x- and y-axes indicate the logarithm of the relative intensity of fluorescence and the relative cell number, respectively. **(B)** Quantification of EGFR and p-EGFR protein levels as MFI. Data are expressed as mean ± standard deviation of three separate experiments. **(C)** Scratch test analysis of PC3 cells migration after incubation at 37°C with either AuD8, AuD9 or cisplatin at 4 μM for 24 h. Cells were then washed, scraped up three times in the confluent monolayer, and then cultured in low serum medium for additional 24 h. Pictures are representative of one of three different experiments (original magnification 10×). **(D)** The migration rate is represented by the distance between the edges of the wound (defined by the lines), indicating the surface area occupied by the migrating cells after 12 and 24 h. Values in the bar graphs represent the mean ± standard deviation of three different experiments.
*p < 0.0001 AuD8 or AuD9 or cisplatin vs control; °/°°AuD8 or AuD9 vs cisplatin (°p < 0.01, °°P < 0.0001).
**p < 0.0001 AuD8 or AuD9 vs control.
MFI: Mean fluorescence intensity; pEGFR: Phosphorylated EGFR.

instructions. The assay is based on the detection of the fluorophore 7-amino-4-methylcoumarin (AMC) after cleavage from the labeled substrate LLVY-AMC. Levels of released AMC were measured using an excitation wavelength of 380 nm and an emission wavelength of 460 nm with an automatic multiwell plate reader. The relative activity was standardized by protein concentration, determined using the protein assay dye reagent (Bio-Rad laboratories, Milan, Italy).

Cell migration assay

Cell migration was assessed using the scratch wound healing assay, as described elsewhere [44]. Cells were grown to confluence in tissue culture dishes, then either 4 μM of AuD8, AuD9 or drug-free medium were added. After 24 h, cells were washed twice with PBS and scraped up using a sterile pipette tip, then washed again and cultured in RPMI medium with 2% FBS for 24 h. The migration rate was evaluated by the Image Tool Software measuring the area of the scratch covered by the migrated cells after 12 and 24 h [48] (TX, USA).

Evaluation of EGFR phosphorylation

In total, 2 × 10^5 PC3 cells were incubated in six-well plates with RPMI medium supplemented with 10% FBS containing 10 μM of either AuD8, AuD9 or cisplatin at 37°C for 12 h. After fixing cells in 2% paraformaldehyde (for 15 min at 4°C), the surface expression of the EGFR was analyzed by flow cytometry using the

anti-EGFR monoclonal antibody (mAb) 528 (Santa Cruz Biotechnology, CA, USA). To detect tyrosine phosphorylated EGFR (pEGFR), cells were fixed in 2% paraformaldehyde in PBS for 15 min at 4°C, then permeabilized with 1% Tween 20 for 30 min at 4°C, and, eventually, incubated with mouse anti-pEGFR (PY1197) (DAKO Citomation, Milan, Italy) [49]. The PE-conjugated goat anti-mouse IgG (Becton-Dickinson Pharmigen) was used as secondary antibody.

Human prostate tumor xenograft experiments

Six-week-old athymic nu/nu (nude) mice were purchased from Charles River (Lecco, Italy). In total, 3 × 10^6 PC3 cells suspended in 0.1 mL of matrigel (1:3 in PBS) were inoculated subcutaneously into the right flank of each mouse. When tumors reached sizes of approximately 28 mm^3, mice were divided randomly into two groups of eight mice each, and treated every other day with intratumoral injection of either drug-free vehicle or medium containing 2 mg kg^{-1} of AuD8. This kind of administration reduces side effects and pain and prevent recurrence, in addition to shrinking the tumor. Tumor size was measured over time using a caliper and tumor volumes were calculated according to the standard formula (width^2 × length × π)/6. Mice were sacrificed after 19 d of treatment when control tumors had reached about 900 mm^3. Organs were then excised and fixed in formalin for tissue toxicity analyses. Sections were cut and counterstained with hematoxylin and eosin according to standard procedures. Images were taken using a Leica DFC495 camera on a Leica DM4000B microscope at 10× magnification. Image processing was conducted by Leica Acquisition Suite v4.0 acquisition software (Leica Microsystems, Wetzlar, Germany).

Statistical analyses

Graphs were generated using Microsoft Office Excel (Microsoft Italia, Milan, Italy) and SigmaPlot software (Systat Software, San Jose (CA), USA). Values are reported mean ± standard deviation of no less than three measurements (unless otherwise stated). Statistical analysis was performed using GraphPad Prism 6 software (GraphPad, CA, USA). Statistical comparisons were drawn using Student's t-test for comparing two groups. Analysis of variances was used to evaluate the correlation of data among three or more groups; consecutive multiple comparison analysis was performed using Dunnett's or Tukey's tests. Differences were considered statistically significant at $p < 0.05$.

Conclusion

Prostate cancer is among the most common malignancies, as well as a major cause of cancer-related death worldwide [50]. Despite the benefits of androgen ablation in the early stages of the disease, many tumors recur in an androgen-independent form with a dramatic increase in the mortality rates [1], thus making the development of more active and less toxic chemotherapeutics a major goal of medicinal chemistry.

In this work, we report on the biological activity and molecular mechanisms involved in the anticancer properties of 'second-generation' gold(III)-dithiocarbamato complexes, focusing on prostate cancer. In this regard, two gold(III)-dipeptidedithiocarbamato derivatives, namely [$Au^{III}Br_2$(dtc-Sar-Aib-O[*t*-Bu])] (AuD8) and [$Au^{III}Cl_2$(dtc-Sar-Aib-O[*t*-Bu])] (AuD9), have been selected owing to the promising results recently obtained in preliminary *in vitro* cytotoxicity studies [22].

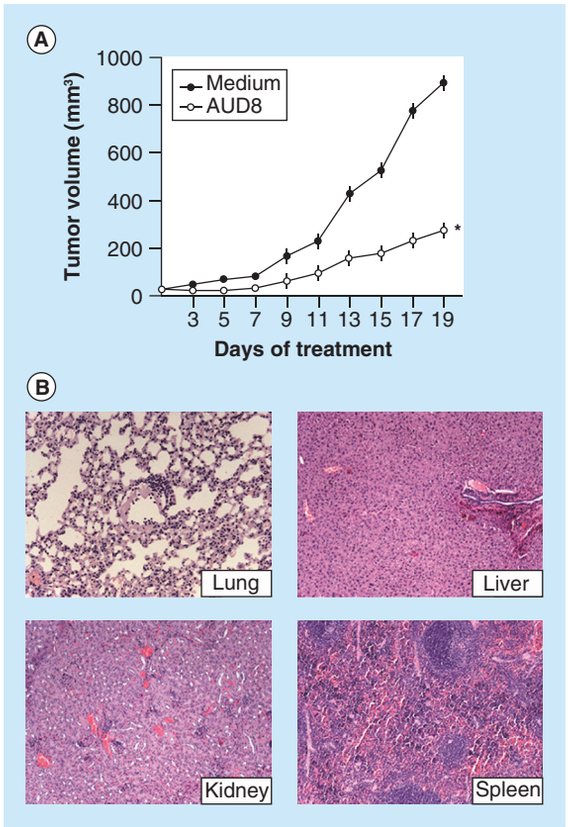

Figure 6. *In vivo* anticancer activity of AuD8 (xenograft). (A) Tumor volume was measured in athymic nude mice after intratumoral injection of either drug-free medium or containing 2 mg kg^{-1} of AuD8 every other day. Tumor volumes were measured three-times a week using a caliper. Points represent the mean ± standard deviation of eight animals per group. **(B)** Representative pictures of mouse lung, liver, kidney and spleen with no evidence of tissue toxicity. Lung parenchyma with focal lymphocyte infiltration (original magnification 10×).
*$p < 0.0001$ AuD8 vs control.

Both complexes were proved significantly more cytotoxic *in vitro* than cisplatin in human prostate cancer PC3 and DU145 cells. Moreover, they were able to overcome cisplatin resistance, showing comparable IC_{50} values in the corresponding cisplatin-resistant PC3-R and DU145-R cell lines. Such potent cytotoxic effect resulted from the induction of apoptosis in androgen-resistant cells, causing early cell damage and DNA fragmentation while affecting cell cycle progression in a different way compared with cisplatin.

From a mechanistic point of view, AuD8 and AuD9 promoted mitochondrial membrane depolarization, Cyt-c release and caspase 9 activation, suggesting that their anticancer activity involves the mitochondrial intrinsic apoptotic pathway. In this regard, they were shown to significantly reduce the expression of the pro-survival Bcl-2 and Bcl-xL proteins and, simultaneously, to increase the expression of the proapoptotic Bax protein, three fundamental regulators of the mitochondrial apoptotic pathway [51,52]. Additionally, treatment of PC3 cells with both gold(III) complexes induced a massive inhibition of TrxR activity accompanied by the generation of large amounts of mitochondrial superoxides, in agreement with the involvement of the selenoenzyme in ROS detoxification [28].

An additional feature of the gold(III) peptidomimetics here reported is the capability to strongly inhibit proteasome activity, likely accounting for a multiple-target antiproliferative action. AuD8 and AuD9 were also shown to inhibit cell migration and to reduce the expression and functional activity of EGFR and its phosphorylated form, likely owing to the occurrence of an oxidative stress [53].

Remarkably, the outcomes outlined above for the investigated gold(III) derivatives resulted largely different from those observed for the reference drug cisplatin tested under the same experimental conditions, thus suggesting a different mechanism of action compared with clinically established platinum chemotherapeutics.

As the two complexes showed similar biological activity *in vitro*, AuD8 was selected as a model compound and tested for *in vivo* anticancer activity in prostate cancer-bearing nude mice. Administration of 2 mg kg^{-1} every other day induced an overall promising 70% reduction of tumor xenografts after 19-day treatment (compared with control untreated mice). Remarkably, chemotherapy was well tolerated by mice, which did not display any sign of sufferance. Moreover, histology showed no detectable damage to main animals' organs.

Future perspective

Peptide transporters are overexpressed to a good extent in prostate cancer PC3 and DU145 cells, thus providing a potentially preferential uptake way to selectively accumulate anticancer peptidomimetics. In this regard, the rationale behind the design of the object gold(III)-

Executive Summary

Background

- Prostate cancer is a major cause of death in men, and bone metastasis is the primary cause of morbidity and mortality.
- Cisplatin is among the first chemotherapeutic agents exhibiting broad effectiveness toward solid tumors and is still a widely used anticancer drug.
- Peptide transporters are integral plasma membrane proteins that mediate the cellular uptake of di- and tri-peptides.
- Gold(III)-dipeptidedithiocarbamato complexes combine the antitumor properties and a favorable toxicological profile along with an enhanced bioavailability and tumor selectivity owing to the exploitation of the dipeptide-mediated cellular internalization by targeting PEPT1 and PEPT2.

***In vivo* anticancer activity**

- The gold complex AuD8 was administered to nude mice bearing prostate cancer xenograft.
- Every other day 2 mg kg^{-1} induced the 70% grow inhibition of tumor after 19-day treatment.

Cytotoxicity

- Our gold complexes were proved significantly more cytotoxic *in vitro* than cisplatin in human prostate cancer PC3 and DU145 cells.
- The investigated gold complexes were also able to overcome cisplatin resistance.

Mechanism of action

- Gold(III) dithiocarbamato derivatives seem to trigger cell death commitment in few hours.
- DNA is not their main target.
- They increase the proapoptotic molecule Bax and decrease the expression of antiapoptotic molecule Bcl-xL and Bcl-2.
- They promote mitochondrial membrane permeabilization and cytochrome-c release.
- They induce activation of caspase 3 and 9 after just 3 h.
- They promote reactive oxygen species production and inhibit the proteasome activity.

dithiocarbamato peptidomimetics relies on the potential recognition of the whole metal complex that, once recognized, transported and delivered by PEPTs inside the tumor cell, can exert its anticancer activity without affecting healthy tissues. In this context, we have recently investigated acute toxicity in mice by two administration routes (intravenous and oral) recording outstanding results [Nardon C, Unpublished Data].

Remarkably, the investigated compounds were proved to hold back the 'normal' development of cancers *in vivo*, by inducing up to 70% tumor mass decrease in prostate cancer, together with negligible (or even no) organ toxicity. The real breakthrough is not simply the use of gold compounds to treat cancer but the rational design of gold-based drugs that may be very effective, nontoxic and potentially selective towards cancer cells. Their enormous potential impact relies on the possible site-specific delivery, strongly improving the cellular uptake and minimizing unwanted side effects.

Altogether, the results here reported are promising and should encourage further studies in order to demonstrate the potential of these gold-based peptidomimetics as a novel therapeutic strategy for treating prostate cancer. It is worth pointing out that these outcomes along with others, allowed us to file an international patent for their use in cancer chemotherapy (just extended in several countries worldwide) [54].

Financial & competing interests disclosure

The EU (Marie Curie European Re-Integration Grant 204828-PEPMIDAS), Ministero della Salute (Ricerca Finalizzata FSN, I.R.C.C.S., Italy), MIUR (PRIN-2009) and A.R.TE.M.O. Association are acknowledged for their financial support. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The authors state that all studies involving animal testing were carried out in accordance with the ethical guidelines for animal research acknowledging the Italian Regulation and the European Directive 86/609/EEC as to the animal welfare and protection, and the related codes of practice.

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