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Gold(III) Complexes in the Oncological Preclinical Arena: From Aminoderivatives to Peptidomimetics

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Abstract: In the last decade, we have been developing some gold(III) derivatives showing interesting antitumor properties and reduced systemic and renal toxicity, compared to the clinically-established reference drug cisplatin. Starting from the rationale at the base of our investigations, this review has been divided into two sections, with respect to our patented first- (aminoderivatives) and second-generation (peptidomimetics) potential drugs. Every section describes the *in vitro* and *in vivo* anticancer activity of the compounds, chosen as models, towards different types of tumor. In particular, we summarize the results achieved so far, in particular taking into account the latest in-depth studies related to their activity, mechanism of action and toxicological profile. Taken together, our data could open up new prospects for further advanced preclinical pharmacological testing.

Keywords: Antitumor drugs, apoptosis, dithiocarbamate, gold complexes, peptides, proteasome, thioredoxin, toxicity.

1. INTRODUCTION

The medical applications of metals have been widely documented in the treatment of various diseases, including cancer. The accidental discovery of the anticancer properties of cisplatin (*cis*-dichlorodiamminoplatinum(II), $\text{cis-[Pt}^{\text{II}}\text{Cl}_2(\text{NH}_3)_2]$) in the mid-1960s [1], has triggered the development of alternative platinum- [2] and other metal-based [3,4] compounds. As antitumor drugs, metal-based compounds have shown remarkable preclinical and clinical results, and platinum compounds are still widely used in the modern chemotherapy. Nevertheless, the platinum-based treatment of tumor diseases is seriously burdened by severe side-effects and onset of resistance [5]. Consequently, the development of new metallodrugs with a better pharmacological outline is the main objective of the current medicinal chemistry. The intrinsic nature of metal centers, gives rise to peculiar coordinations and kinetics and allows metallodrugs to work through mechanisms that cannot be mimicked by organic agents, *i.e.* they otherwise influence crucial cellular processes, such as cell division and cancer-related pathways. The modern methodologies are directed to synthesize complexes with tumor-targeting features, thus enhancing the effect on cancer cells and reducing the incidence of adverse and disabling side-effects [6,7].

In the field of new non-platinum based antitumor compounds, gold complexes have been obtaining increasing consideration due to their strong antiproliferative activity, usually attained by exploiting biochemical mechanisms different from that of cisplatin [8]. The use of gold complexes in medicine, mainly gold(I) derivatives, has been called

“chrysotherapy” [9]. In particular, in the past, late-stage rheumatoid arthritis was treated with several gold drugs, including aurothioglucose (solganol), aurothiomalate (myocrisin), aurothiosulfate (sanocrysin), aurothiopropanol sulfonate (allosocrysin), and triethylphosphinogold(I)tetraacetylthioglucose (auranofin) (Fig. 1). In addition, auranofin (Fig. 1e) is particularly interesting as it can be administered orally, contrary to the other marketed gold(I) compounds usually injected.

In the light of their traditional use in medicine for the treatment of rheumatoid arthritis, gold compounds could represent potential alternatives to platinum drugs. In this context, their anti-arthritis activity derives from the recognized immunosuppressive and anti-inflammatory properties. This may determine, in principle, a link between the two therapies. In recent years, many papers about the use of gold complexes as drugs, have highlighted the special interest in this class of metal complexes as new medicinal entities, mostly as anticancer agents [10-14].

The wide range of gold-based agents with stated antiproliferative properties, includes a great number of different ligands coordinated to the metal ion in the +1 or +3 oxidation states. Accordingly, a sole way of action for gold complexes seems to be highly improbable, and a number of intracellular targets (different from the DNA recognized for cisplatin and its analogues [15]) have to be taken into consideration when elucidating the pharmacology action of the gold-based agents [16, 17].

In this context, during the last decade, a number of gold(III)-dithiocarbamate derivatives have been designed and tested by our research group as anticancer agents, showing very promising activity [18, 19] thus highlighting the necessity to carry out detailed biological and mechanistic studies. Up-to-date results are here summarized and discussed.

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Fig. (1). Clinically-established gold(I) antiarthritic drugs: solganol (a), myocrisin (b), sanocrysin (c), allocrysin (d) and auranofin (e).

1.1. The Choice of Dithiocarbamic Ligands

Notwithstanding the therapeutic efficacy in the treatment of several types of tumors, some severe side-effects, such as nausea, alopecia, ototoxicity, neurotoxicity, myelosuppression, and nephrotoxicity, limit the use of cisplatin and subsequent platinum-based chemotherapeutics. In particular, nephrotoxicity seems result from platinum accumulation in the body. Even if the negative effects of cisplatin on renal functions are not entirely clarified, latest researches further elucidated the mechanism of cisplatin-induced nephrotoxicity. In particular, the signaling pathways associated with tubular cell death and inflammation seems to be involved. The hypothesis that renal failure may be induced by platinum binding to thiol-containing renal enzymes with the consequent their function inactivation, is the most accepted [20].

Sulfur-containing biomolecules, such as cysteine, methionine, glutathione, metallothionein and albumin, play a detoxifying and, at the same time, an antagonist role in platinum-based anticancer chemotherapy because of their high affinity towards platinum(II) ion. In fact, sulfur is involved in the entire metabolic process of platinum drugs, including the deactivation and chemical reactions occurring before entering the cell and bind to DNA. The interaction between sulfur-containing biomolecules and platinum drugs have negative effects on the therapeutic effectiveness. In fact, strong and irreversible binding of cisplatin to intracellular thiolato ligands is shown as a crucial inactivation stage, and reactions with sulfur-donor atoms pre sent in peptides and proteins are able to chnge their conformation, resulting in the alteration of their specific biological activity, especially when enzymatic processes are affected [21]. Thus, various sulfur-containing nucleophiles were tested as chemoprotectants to modulate cisplatin nephrotoxicity (Fig. 2), and some proved encouraging for clinical use [22]. In this regard, two main problems were considered for their development; namely the selective protection of human normal cells, and their possibly absent or low systemic toxicity. However, a selective protection of normal tissues without reduction of the anticancer activity of platinum drugs was challenging.

About that the chemoprotectant sodium diethyldithiocarbamate (NaDED, showed helpful effects (Fig. 2c). In fact, it proved protective against renal, gastrointestinal and bone marrow toxicity induced by cisplatin without reducing its antitumor activity. Its chemoprotective peculiarity arises

from the capability to remove platinum from the thiol groups of proteins without affect the formed platinum-DNA adducts, widely recognized as liable for its antitumor action. Remarkably, the treatment with NaDED soon after cisplatin administration, decreases by *ca.* 50% platinum-DNA adducts affecting the therapeutic effect. However, if NaDED is administered 3 h after the drug, there is no change in the antitumor activity. In fact, when the chemoprotectant is administered later, cisplatin (only 5-10%) is on his way to reach its target of choice (DNA) [22-23]. Then, NaDED can cleave platinum-sulfur adducts from a variety of sulfur-containing biomolecules, hence forming a Pt-DED derivative that can itself have some antiproliferative activity, not relying on DNA adducts. Therefore, the correct dosing of NaDED as chemoprotectant diminishes the nephrotoxicity connected to cisplatin chemotherapy without affecting its antitumor efficacy [22]. On the other hand, the general nephroprotective benefits of NaDED are to some extent limited by the effects of the acute toxicity showed by dithiocarbamates themselves. In fact, potential human health hazards associated with free (*i.e.*, not coordinated) dithiocarbamates have been investigated, including genotoxicity [24]. Nowadays, chemoprotectants are not clinically used anymore, being replaced by strong hydration and diuresis along with antiemetic prophylaxis during the cisplatin treatment. In the meantime, continuous efforts are still being made by researchers in order to reduce cisplatin-induced toxicity.

Taking into account the previous concerns, we have been designing a number of metal-dithiocarbamate (hereinafter, DTC) complexes with the potentiality to associate the cytotoxic activity of the metal centers (*e.g.*, Pt(II), Pd(II), Au(III), Ru(III), Zn(II), Cu(II)) with lack of nephrotoxicity due to the presence of the chemoprotectant DTC ligand (*i.e.*, -NCSS moiety).

The basis of our approach derived from the chemical features of the DTC moiety. In fact, dithiocarbamates are bidentate ligands that can coordinate soft metal ions in a chelating way allowing the resulting complexes to be quite stable thanks to the "chelate effect". In this kind of structure, the electronic and steric influences are able to impede the loss of the dithiocarbamate ligand. Additionally, the solubility properties of the resulting metal complexes, could be modulated through changing the organic backbone of the dithiocarbamate.

Fig. (2). Cisplatin toxicity modulator molecules (sulfhydryl and peptide derivatives): glutathione (GSH, (a)), the GSH-depleting antimetabolite L-buthionine sulfoximine (L-BSO, (b)), the sulfhydryl metabolite of disulfiram diethyldithiocarbamate (DEDT, (c)), the parent disulfide disulfiram (antabuse, (d)), *N*-acetyl-L-cysteine (L-NAC, (e)), S-mercaptoethane sulfonate sodium salt (mesna, (f)), sodium thiosulfate (g), the phosphothiolamifostine (WR-2721) and its active metabolite actifostine (WR-1065) formed by alkaline phosphatase activity (h), and the melanocortin-derived peptide ORG-2766 (i).

Concerning square-planar complexes, the presence of a chelating dithiocarbamate ligand should impede the coordination of an additional *S*-donor ligands (*e.g.*, methionine and cysteine residues), in *trans* to the $-\text{NCSS}$ moiety. This chemical process becomes less favorable because of the quite strong *trans* effect of the DTC sulfur atoms, that potentially avoids the interaction of the metal center with thiol-containing renal enzymes and, accordingly, reduces nephrotoxic side-effects.

2. FIRST GENERATION

In the light of the abovementioned issues, we first designed a number of platinum(II) and, given the strict similarity, palladium(II) derivatives with dithiocarbamate (DTC) ligands, some of which were endowed with a good chemotherapeutic index (high antitumor activity and lack of nephrotoxicity) compared to the reference drug cisplatin [25]. Unfortunately, despite these encouraging results, long synthetic routes, low water solubility and inadequate stability under physiological conditions (for pharmacological purposes) were the major drawbacks, so their advanced evaluation as potential anticancer agents was dismissed. Therefore, we explored alternative ways, the most promising being the design of dithiocarbamate derivatives of metals different from platinum and palladium. In particular, we synthesized a number of gold(III)-dithiocarbamate derivatives of the type $[\text{Au}^{\text{III}}\text{X}_2(\text{DTC})]$ ($\text{X} = \text{Cl}, \text{Br}$; DTC = various dithiocarbamate ligands, yield $\sim 70\text{--}80\%$) that were in principle designed to closely reproduce the main features of cisplatin (Fig. 3) [26, 27].

All the compounds were fully characterized by means of several techniques, confirming that the $-\text{NCSS}$ moiety coordinates the metal center in a bidentate symmetrical mode through the sulfur-donating atoms in a near square-planar geometry, the remaining coordination positions being occupied by two *cis*-halogen atoms. When crystals for a X-ray analysis were not obtained, we performed density functional calculations, for confirming the structural data of the complexes. [26].

2.1. Antitumor Activity *In vitro*

From comparative *in vitro* cytotoxicity studies on Pt(II)-, Pd(II)-, and Au(III)-MSDT derivatives (MSDT = methylsarcosinedithiocarbamate) involving human squamous cervical adenocarcinoma (HeLa) cells and human leukemic promyelocytes (HL60), the gold(III) complexes resulted to be significantly more active (in terms of IC_{50} values), than both cisplatin and the platinum(II) and palladium(II) counterparts under the same experimental conditions (IC_{50} *ca.* $1\ \mu\text{M}$ vs. *ca.* 2, >15, and $5\ \mu\text{M}$, respectively), inducing apoptosis especially in HL60 cells [28]. $[\text{Au}^{\text{III}}\text{X}_2(\text{MSDT})]$ -type compounds (Fig. 3) were also tested on a panel of acute myelogenous leukemia cell lines, representing different French-American-British subtypes, and toward the Philadelphia-positive (K562) cells [29]. Compared to the corresponding palladium(II) analogues [30], they were able to inhibit cell growth in all the tested myeloid cell lines with IC_{50} values *ca.* ten-fold lower than the reference drug. Besides, after short exposure (18 h), $[\text{Au}^{\text{III}}\text{X}_2(\text{MSDT})]$ -type compounds induced strong and rapid apoptosis paralleled by down-regulation of

Fig. (3). Selected gold-dithiocarbamate derivatives: $X = \text{Cl}, \text{Br}$; DMDT = *N,N*-dimethyldithiocarbamate ($(\text{CH}_3)_2\text{NCSS}^-$); MSDT = methylsarcosinedithiocarbamate ($\text{CH}_3\text{O}(\text{O})\text{CCH}_2\text{N}(\text{CH}_3)\text{CSS}^-$); ESDT = ethylsarcosinedithiocarbamate ($\text{CH}_3\text{CH}_2\text{O}(\text{O})\text{CCH}_2\text{N}(\text{CH}_3)\text{CSS}^-$)

the antiapoptotic molecule Bcl-2 and up-regulation of the proapoptotic molecule Bax, whereas cisplatin did not. After long treatment (72 h), they showed to be able inducing only modest cell cycle perturbations but high DNA fragmentation, whereas classical platinum(II) complexes are known to promote characteristic cell cycle alterations, resulting in increased G₂M cell fraction [31]. In addition, $[\text{Au}^{\text{I}}\text{X}_2(\text{MSDT})]$ -type complexes stimulated early apoptosis and membrane injury to a much greater extent than cisplatin, suggesting a different mechanism of action [29].

As a consequence of these positive results, we synthesized and developed other gold(III)-dithiocarbamate derivatives, namely $[\text{Au}^{\text{III}}\text{Cl}_2(\text{DMDT})]$, $[\text{Au}^{\text{III}}\text{Br}_2(\text{DMDT})]$, $[\text{Au}^{\text{III}}\text{Cl}_2(\text{ESDT})]$ and $[\text{Au}^{\text{III}}\text{Br}_2(\text{ESDT})]$ (from now on referred to as AuL10, AuL14, AuL13 and AuL12, respectively), where DMDT and ESDT are *N,N*-dimethyldithiocarbamate and ethylsarcosinedithiocarbamate, respectively. The *in vitro* tests towards human tumor cell lines sensitive and intrinsically resistant to cisplatin (such as Daudi, MeWo, LoVo and A549 cells) put in evidence the great cytotoxicity of these compounds with respect to cisplatin. (Table 1). Moreover, they were still active on cell lines made resistant to cisplatin treatment, compared to the corresponding cisplatin-sensitive parent cell lines, thus excluding the occurrence of cross-resistance. [27].

AuL10 and AuL12 were investigated for their cytotoxic activity also against the androgen-resistant prostate cancer PC3 and DU145 cell lines [32]. Among all, AuL10 resulted to be the best performer, with IC₅₀ values about four and ten times lower than cisplatin on PC3 and DU145 cells, respectively. It proved cytotoxic also against the cisplatin-resistant PC3-R cells, with activity levels comparable to those detected for the parent cisplatin-sensitive PC3 cell line, excluding, again, the onset of any cross-resistance [32].

In addition to slightly affecting the cell cycle, treatment of PC3 cells with AuL10 induced mitochondrial membrane depolarization, cytochrome-c release and caspase-9 activation, pointing out that it exerts its activity through the mitochondrial intrinsic apoptotic pathway. In this regard, it considerably diminished the expression of the pro-survival pro-

tein Bcl-2 and also, led to the up-regulation of the proapoptotic Bax protein. These are two well-known regulators playing a major role in the mitochondrial apoptotic pathway [33, 34]. Likewise, other studies on PC3 cells confirmed that AuL10 causes powerful cytotoxic effects, inducing apoptosis, as assessed by Annexin-V assay and by the expression of APO2.7, a mitochondrial membrane protein exposed on the cell surface when cells undergo apoptosis [32].

With the aim of get further understandings into the ability of the investigated gold(III) complexes to overcome resistance to cisplatin, we tested their capability to inhibit DNA synthesis in tumor cells, that had acquired resistance to cisplatin *in vivo*, [35]. Remarkably, AuL12 induced a dramatic inhibition of DNA synthesis in both the tested cell lines (murine leukemia cisplatin-sensitive L1210 and -resistant L1210-R) at comparable levels, whereas cisplatin did not. These results are considerable as L1210-R cells, contrarily to the resistant sublines previously tested (see Table 1), acquired their resistance *in vivo* evading all the host defense mechanisms. Hence, AuL12 circumvented cisplatin resistance *in vivo* as well [35].

2.2. Antitumor Activity *In vivo*

Among all the gold(III)-dithiocarbamate derivatives, AuL10, AuL12 and AuL14 have been selected for subsequent *in vivo* studies, due to their general encouraging stability, solubility, and antiproliferative properties [32, 36-38].

2.2.1. Murine Tumor Models

In vivo antitumor activity of AuL12 was evaluated on Ehrlich solid-tumor-bearing mice. The test compound caused a significant tumor mass reduction (*ca.* two-fold) if compared with the animals treated with cisplatin [38]. Moreover, tumors excised from AuL12-treated mice (after 11-d treatment *via* i.p. at a daily dose of 10 mg kg⁻¹, being one third of LD₅₀) showed 74% inhibition of tumor growth compared to untreated animals. Analogous results were obtained for Lewis lung carcinoma (i.p. treatment at 10 mg kg⁻¹), for which AuL12 induced 80% inhibition of tumor growth [38].

Table 1. *In vitro* cytotoxic activity of Au(III)-dithiocarbamate derivatives [Au^{III}Cl₂(DMDT)] (AuL10), [Au^{III}Br₂(DMDT)] (AuL14), [Au^{III}Cl₂(ESDT)] (AuL13) and [Au^{III}Br₂(ESDT)] (AuL12), and the reference drug cisplatin tested under the same experimental conditions (over 24 h) towards established human tumor cell lines: human squamous cervical adenocarcinoma (HeLa), human leukemic promyelocytes (HL60), human Burkitt's lymphoma (Daudi), human malignant melanoma (MeWo), human colon adenocarcinoma (LoVo), human non-small lung adenocarcinoma (A549), human ovarian carcinoma cisplatin-sensitive (2008) and cisplatin-resistant (2008-R), human squamous cervix carcinoma cisplatin-sensitive (A431) and cisplatin-resistant (A431-R), human osteosarcoma cisplatin-sensitive (U2OS) and cisplatin-resistant (U2OS-R) cells. *In vitro* cytotoxic activity of AuL10, AuL12 and cisplatin tested over 72 h towards human prostate cisplatin-sensitive tumors PC3 and DU145, and cisplatin-resistant cancer R-PC3. Data are expressed in terms of IC₅₀ ± S.D. (standard deviation) values (μM).

Cell Line	Compound				
	AuL10	AuL14	AuL13	AuL12	Cisplatin
HeLa	2.10 ± 0.01	3.50 ± 0.01	8.2 ± 0.2	7.6 ± 0.2	15.6 ± 0.4
HL60	(0.80 ± 0.01)·10 ⁻²	(0.70 ± 0.01)·10 ⁻²	0.43 ± 0.09	0.14 ± 0.02	25.6 ± 0.3
Daudi	(0.10 ± 0.01)·10 ⁻²	(0.10 ± 0.01)·10 ⁻²	4.65 ± 0.09	5.8 ± 0.2	95 ± 1
MeWo	2.0 ± 0.3	(0.10 ± 0.01)·10 ⁻²	12.5 ± 0.9	10.0 ± 0.9	48 ± 2
LoVo	(2.40 ± 0.04)·10 ⁻²	3.8 ± 0.1	7.6 ± 0.2	7.9 ± 0.1	56 ± 2
A549	(0.35 ± 0.01)·10 ⁻²	0.41 ± 0.03	4.73 ± 0.04	9.6 ± 0.2	35 ± 1
2008	(0.20 ± 0.01)·10 ⁻²	30.0 ± 0.1	49.3 ± 0.1	16.5 ± 0.4	43.2 ± 0.4
2008-R	(0.10 ± 0.01)·10 ⁻²	21.8 ± 0.2	23.8 ± 0.1	(0.10 ± 0.01)·10 ⁻²	556 ± 3
A431	(1.20 ± 0.01)·10 ⁻²	1.8 ± 0.1	0.29 ± 0.01	(1.50 ± 0.01)·10 ⁻²	77.4 ± 0.4
A431-R	(0.20 ± 0.01)·10 ⁻²	2.8 ± 0.2	0.43 ± 0.03	(0.10 ± 0.01)·10 ⁻²	382 ± 3
U2OS	4.8 ± 0.3	18 ± 1	5.8 ± 0.4	0.49 ± 0.09	35 ± 2
U2OS-R	6.4 ± 0.1	13 ± 1	5.2 ± 0.2	0.24 ± 0.09	84 ± 3
PC3	0.7 ± 0.2	-	-	1.6 ± 0.4	> 2.5
DU145	0.25 ± 0.05	-	-	1.1 ± 0.3	2.5 ± 0.7
R-PC3	0.9 ± 0.3	-	-	-	10 ± 1

The antitumor potential was also evaluated on Ehrlich ascitic carcinoma-bearing mice (*via i.p.*), obtaining a remarkably greater efficacy than cisplatin (dose: 3.5 mg kg⁻¹ which is one third of LD₅₀) in increasing the life span of treated animals with %T/C values (average survival time of treated mice/average survival time of control mice) of 157 and 130%, respectively [38]. Moreover, a higher value of Long-Term-Survivors (LTS, treated mice alive after 30 d with total tumor suppression) was observed in the group of animals treated with AuL12 compared to cisplatin (37.5% *vs.* 25%, respectively). AuL12-treated mice well tolerated chemotherapy and quickly recovered the initial body weight loss. This proof was paralleled with the results of acute toxicity tests carried out on non-tumor-bearing Swiss mice. In that study, we recorded for AuL12 a LD₅₀ value (*i.e.*, the median lethal dose of a toxic substance required to kill half the members of a treated population) of 30 mg kg⁻¹, much higher than that recorded for cisplatin (11.4 mg kg⁻¹) [38].

2.2.2. Human Xenograft Tumor Models

Some gold compounds reported in literature have shown promising *in vitro* cytotoxicity that, unfortunately, was not confirmed by *in vivo* studies afterwards [11]. In contrast, the *in vivo* antitumor activity of our gold(III) derivatives is fully consistent with *in vitro* results. For example, treatment of MDA-MB-231 breast tumor-bearing nude mice with AuL14 resulted in significant inhibition of tumor growth (*ca.* 50% compared to control, after daily treatment with 1.0 mg kg⁻¹ for 29 days) (Fig. 4A), associated with massive apoptosis induction, inhibition of proteasome activity (Fig. 4B) and accumulation of protein p27. It has to be highlighted that during the administration of the test compound, the mice did not show signs of toxicity with decreased activity or anorexia [37].

With respect to the PC3 prostate tumor, administration of 1 mg kg⁻¹ every other day of AuL10 caused an overall 85% reduction of the corresponding xenografts in nude mice after

Fig. (4). The antitumor activity of AuL14 is linked with inhibition of the proteasomal chymotrypsin-like activity and stimulation of apoptosis *in vivo*. Female nude mice bearing MDA-MB-231 tumors were treated with either control vehicle or AuL14 at 1 mg/kg/d for 29 days. inhibition of MDA-MB-231 tumor growth by AuL14. Points, mean tumor volume in each experimental group; bars, SD. *, $p < 0.01$. (A) Tumors were collected after 29 days of treatment, and the prepared tissues were analyzed by the proteasomal chymotrypsin-like activity assay (B). *In vivo* anticancer activity of AuL10 on PC3 prostate tumor-bearing nude mice (xenografts). Tumor volume was measured after s.c. injection of either drug-free vehicle or containing 1 mg kg⁻¹ of AuL10 every other day. (C) (adapted from ref. [37] and [32]).

a 19-day treatment (compared to control untreated mice) (Fig. 4C). Once more, treated mice well tolerated chemotherapy and seemed not suffer from systemic toxicity. In addition, and histology showed no detectable damage to main animals' organs (Fig. 5) [32].

2.3. Toxicological Studies

In vivo nephrotoxicity, after treatment with both AuL12 and cisplatin, was explored by assessing some peculiar biomarkers in either urine or renal cortical slices of treated rats [38]. In this context, it is well known that a number of xenobiotic substances are harmful to the kidney, in particular to the renal proximal tubule, the portion of the nephron characterized by the greater sensitivity to nephrotoxic agents. It is subdivided into three segments [*i.e.*, S1–S2 (*pars convoluta*) and S3 (*pars recta*)] present in the cortical labyrinth and the medullar rays [39].

Increased levels of total urinary proteins (TUP) and of activity of *N*-acetyl- β -D-glucosaminidase (NAG) and glutamine synthetase (GS) in treated animals are widely recognized as a sign of a general kidney injury [40]. Moreover, reduced activity of GS in the kidney cortex of the sacrificed treated rats is a commonly accepted marker for renal damages occurring in the S3 segment of the proximal tubules [41]. As expected, cisplatin induced a significant increase of GS and NAG activity and TUP excretion in urine. Conversely, AuL12-treated rats showed very low changes (if

compared to control rats), in the urinary profile principally evident at the higher dose (20 mg kg⁻¹; Fig. 6). It has been demonstrated that reduced PAH (*p*-aminohippuric acid) uptake and GS activity in the renal cortical slices of sacrificed [41, 42] treated rats is related to specific damages touching S1–S2 and S3 segments, respectively. Significant inhibition of GS activity in renal cortical slices was detected in cisplatin-treated rats due to a severe diffuse tubular necrosis of the S3 segment of the proximal tubule in the outer stripe of the outer medulla, whereas PAH accumulation was not affected, in agreement with data previously reported [25]. On the contrary, rats treated with AuL12 did not display any particular change in the examined renal cortical biomarkers accounting for the almost total lack of nephrotoxic side-effects [36]. These results are extremely positive when compared to the few data reported in literature related to the renal toxicity induced by gold derivatives. For instance, the gold-based antiarthritic drugs auranofin and myochrysine are well known to give rise to proteinuria and kidney dysfunction [43]. In this context, also the gold nanoparticles, which received great attention in recent years for their potential applications in medicine [44–46], proved to have nephrotoxicity as a severe dose-limiting factor because of their ability to penetrate renal cells causing renal damages [47].

The favorable toxicity profile of the tested gold(III) complex was subsequently confirmed by histopathological investigations, detecting no important sign of toxicity in the

Fig. (5). The histologic examination of the AuL10-treated mice did not show any differences with respect to the control tissues. All tissues (A, bone; B, liver, C-D, spleen) were formalin-fixed and paraffin-embedded. Sections 4 micron thick were stained with Hematoxylin-Eosin.

Fig. (6). Urinary profiles of male albino Wistar rats on a single i.p. injection of cisplatin and AuL12. Evaluation of (a) total urinary proteins (TUP) excretion, (b) *N*-acetyl- β -D-glucosaminidase (NAG) activity, and (c) glutamine synthetase (GS) activity in urines collected over 24 h after administration (adapted from ref. [38]).

treated animals' heart, liver, spleen, kidneys, testicles, pancreas, lungs and brain. SEM data related to the surface of all examined tissues proved compatible with normal conditions when compared to control animals. Unexpectedly, atomic

adsorption analyses evidenced, in the investigated tissues, no traces of gold, thus excluding the accumulation of the metal in any of the organs taken into account [38]. Also the accumulation around the injection site (*i.e.*, peritoneal area) was

excluded. These results are completely in tune with our experiments demonstrating gold is rapidly cleared from the body (*i.e.*, within 48 hours), the large majority being excreted through the feces (>89%) and only about 10% *via* the urinary system [38].

2.4. Targeting and Delivery Strategies of Gold(III) Complexes through the Encapsulation in Micellar Systems

As previously assessed, our gold(III) dithiocarbamate complexes proved very promising for their biological activity as antiproliferative agents, but are characterized by very low water solubility. Efficient carriers can be used to enhance the therapeutic properties of not soluble drugs [48].

For the delivery of our compounds, we exploited micellar systems (Fig. 7A left), characterized by two well-defined regions: an external hydrophilic layer and a central core with hydrophobic properties (which can incorporate our lipophilic substances).

An important feature to consider when selecting nanocarriers for drug delivery is the evaluation of the dimensions of the supramolecular aggregates, since variations in the nanoscale range strongly affect blood circulation times and the bioavailability of the particle-loaded chemotherapeutics within the body [49]. In particular, it was shown that the loading of drugs into micelles can diminish their extravasation into normal tissues while providing a passive drug targeting to tumors *via* the enhanced permeability and retention (EPR) effect [50]. Drug-encapsulating systems can selectively extravasate in tumor tissues due to their abnormal vascular nature and escape renal clearance [51].

For pharmaceutical applications, the preferred size range goes from 10 to 100 nm. Interestingly, particles ranging from 10 to 70 nm are known to offer effective distribution in certain tissues and are preferred to obtain faster drainage from the site of injection [52-53].

To date, we have investigated two nanoscale-sized formulations as delivery systems for our compounds. In particu-

Fig. (7). Schematic depiction of micelles and liposomes (A); chemical drawing of DSPE-PEG2000 (B); Pluronic® F127 (C); PC (D) and DOPC (E) phospholipids; MonY-BN-AA1 amphiphilic peptide (F); equilibrium of formation of non-targeted (G) and targeted micelles (H) loaded with the model compound AuL12 (depicted as yellow circle); (C18)₂-PEG1000-G-CCK8 (I).

lar, we have focused on the supramolecular aggregation of the amphiphilic polymers DSPE-PEG2000 (*i.e.*, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-*N*-[amino-(polyethylene glycol)-2000], Fig. 7B) and Pluronic® F127 (PF127, Fig. 7C) [54-55]. In both cases, the gold(III)-dithiocarbamate complex AuL12 was selected as a model compound to be encapsulated due to its good antiproliferative rates recorded both *in vitro* and *in vivo*.

Regarding the first studied formulation, we used pure micelles of DSPE-PEG2000, and mixed counterparts, containing PC (L- α -phosphatidylcholine, Fig. 7D) or DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine, Fig. 7E) phospholipids (5, 10 or 20% mol/mol with respect to DSPE-PEG2000), as delivery systems [54]. A functionalization of some mixed micelles was carried out in order to obtain a tumor-selective delivery, thus further improving the chemotherapeutic index of our compounds (which is defined as the ratio between the lethal dose [LD₅₀] and the effective dose [ED₅₀] of a drug). In particular, those involving 10% PC were labeled on the hydrophilic corona with a bombesin analogue at 5% (BN-AA1, sequence: D-Phe-Gln-Trp-Ala-Val-NMeGly-His-Sta-Leu-amide, Fig. 7F) [54]. Remarkably, the structural properties of empty or AuL12-filled micelles did not change after BN-AA1 insertion (diameter around 20 nm).

BN-AA1 should drive the final aggregates towards the gastrin-releasing peptide (GRP) receptors up-regulated by several cancer types such as prostate and ovarian malignancies [56]. Target-selective properties of these empty and loaded systems were tested on PC-3 human prostate cancer cell line, overexpressing the GRP/bombesin receptors. In these *in vitro* cytotoxic studies, a decrease of cell viability, *ca.* 50%, was observed in cells treated for 48 h with targeted AuL12-loaded micelles (10 μ M drug concentration, Fig. 7G) with respect to untargeted micelles (Fig. 7H). It should be underlined that the collected biological data are promising as these carriers ensure a very low drug release (about 10% after 72 hours) [54]. A so good activity despite the slow drug release could be in our opinion associated with an endocytic process.

With respect to the second investigated formulation, our gold derivative was loaded into two types of self-assembling supramolecular aggregates, namely consisting of Pluronic® F127 (PF127, Fig. 7C) or its combination with the amphiphilic peptide (C18)₂-PEG1000-G-CCK8 (Fig. 7I), thus yielding the formation of either non-targeted (Fig. 7H) and targeted micelles (Fig. 7G), respectively [55]. In fact, upon self-assembling, the second type of drug carrier is characterized by the presence of the octapeptide CCK8 (amino acid sequence: Asp²⁶-Tyr²⁷-Met²⁸-Gly²⁹-Trp³⁰-Met³¹-Asp³²-Phe³³-amide whose properties are described below) bound to the external hydrophilic corona of the resulting micelles (Fig. 7H).

Amphiphilic Pluronic molecules form a class of tri-block copolymers characterized by an A-B-A architecture of polyoxyethylene-polyoxypropylene-polyoxyethylene (PEO_x-PPO_y-PEO_x) units (Fig. 7C). For the encapsulation of our gold(III) dithiocarbamate complexes, the Pluronic F127 was chosen [55], endowed with the stoichiometry PEO₉₈-PPO₅₇-PEO₉₈ and an average molecular weight of 12600 g mol⁻¹.

Pluronic® F127 is one of the most used nanocarriers for biomedical applications, following its first use in clinical trials for the delivery of the anticancer drug doxorubicin (Dox) in a mixed formulation named SP1049C, which contains two different types of Pluronic, F127 and L61 [57-58]. Besides Dox, other anticancer drugs, such as Paclitaxel and Carboplatin, were encapsulated in this type of nanocarriers and tested *in vivo*, leading to enhanced drug activity, along with overcoming multidrug resistance (MDR) in cancer cells [50]. Concerning the biocompatibility, it is worth highlighting that this class of organic carriers remains metabolically intact until renal clearance and has proved to be little or no toxic [59].

The targeting peptide CCK8 belongs to the family of cholecystokinin (CCK) peptides whose receptors [60-61] are normally present on the cell surface and are known to be overexpressed after malignant transformation. CCK receptors were found up-regulated in medullary thyroid cancers (92%), in stromal ovarian cancers (100%), in small-cell lung cancers (57%), in astrocytomas (62%), in meningiomas (30%), in gastro-entero-pancreatic neuroendocrine tumors (38%), and in some neuroblastomas (19%) [61-64]. Remarkably, the octapeptide amide CCK8 binds with nanomolar affinity its receptors (CCK1-R and CCK2-R), also when modified at its *N*-terminus with chelating agents or alkyl chains [65-66]. Upon binding (K_D ~20 nM), CCK8 is internalized into the cell through receptor-mediated endocytosis, thus leading to a cascade of biological events [67]. After the preparation of both AuL12-loaded PF127 non-targeted micelles and PF127/CCK8 targeted micelles, various characterizations and studies were carried out, pointing out: i) an average micelle diameter of about 30 nm (dynamic light scattering measurements); ii) a good stability in saline solution up to 72 h; iii) a quite fast compound release from the micelle systems into the aqueous medium, reaching 50% after 2 h; iv) good solubilizing capabilities, thus increasing the water solubility; v) satisfying carrier properties as they keep or increase the antiproliferative activity of the model compound (compared to the DMSO vehicle) [55]. In fact, on passing from A431 cells (epidermoid carcinoma) to the CCK2-R-transfected counterpart, the cytotoxicity of the loaded AuL12 compound proved to be 10-fold higher [55].

2.5. Investigation of the Mechanism of Action

2.5.1. Solution Chemistry and DNA Binding Affinity

Although not still completely elucidated, the generally accepted mechanism of action of cisplatin considers its uptake into the cell followed by hydrolysis in the cytoplasm. This process is chemically permitted by the low intracellular Cl⁻ concentration (4-20 mM) and leads to the formation of positively charged aquo species that reach the nucleus and bind to DNA, universally accepted as crucial biological target of cisplatin [68]. In this regard, we investigated the behavior under physiologic-like conditions and the DNA-binding properties of some selected gold(III) complexes [35]. With respect to their solution chemistry, these gold derivatives were proved to hydrolyze in a physiological-like milieu by delivering two moles of halide per mole of starting compound, allowing the formation of

the corresponding gold(III)-diaquo counterparts within 30–40 min. The hydrolyzed species proved reasonably stable in aqueous environment, with reduction to gold(I) occurring after 12–24 h [35].

As the physiological milieu is generally reducing, gold(III) complexes, often characterized by high redox potentials [69], may easily undergo reduction to the corresponding less active gold(I) derivatives, a critical issue for their pharmaceutical advance. Therefore, we studied, the intrinsic electrochemical properties of our compounds by cyclic voltammetry, observing both DMDT (AuL10 and AuL14) and ESDT (AuL12 and AuL13) complexes undergo reduction processes through irreversible steps, resulting in the formation of the corresponding dinuclear gold(I) species $[\text{Au}^{\text{I}}(\text{DMDT})]_2$ and $[\text{Au}^{\text{I}}(\text{ESDT})]_2$, at *ca.* -300 mV and -180 mV (*vs.* saturated calomel electrode, SCE), respectively [35]. Remarkably, these reduction reactions take place at potentials considerably lower than the typical values for the Au(III)/Au(I) couple known for the corresponding $\text{K}[\text{AuX}_4]$ ($\text{X} = \text{Cl}, \text{Br}$) precursors (*ca.* +1.29 V) [70]. In fact, gold coordination by dithiocarbamates produces a great stabilization of the metal center in the +3 oxidation state, due to the stabilizing chelate effect and the great electron-donating capability of the DTC moiety.

Given the strict chemical and structural similarity of these gold(III) complexes to cisplatin, we firstly hypothesized their cytotoxic properties could spring from a direct interaction with intracellular DNA. Experimental results established their high affinity towards some biologically-relevant isolated macromolecules, resulting in a dramatic inhibition of both DNA and RNA synthesis in a non-dose-dependent fashion. On the contrary, as expected, cisplatin strongly inhibited DNA synthesis in a dose-dependent manner while it does not significantly affect RNA synthesis [35]. In addition, our gold(III) compounds showed to be able to very fast bind the purified DNA *in vitro*. Our experiments put in evidence that AuL10 has got a great affinity towards DNA (calf thymus) whose binding is observable even soon after contact (time zero) [35]. Reaction with DNA appears to be extremely rapid and dependent on both r_i (molar ratio of tested compound to nucleotides) value and contact time, achieving 100% binding after less than 3 h, compared to 51% reached by cisplatin, tested under the same experimental conditions after 24 h [35]. These compounds were also shown to form interstrand cross-links with a faster kinetics than cisplatin. In contrast with cisplatin, they do not seem to act as bifunctional agents as they are not able to induce DNA-protein cross-links [71]. This phenomenon is of paramount importance taking into account that the exposure to various DNA-protein cross-linking agents gives rise to genotoxic and carcinogenic effects, believed to be mediated *via* the formation of this type of lesion [72].

All the experiments, described in this last section, were performed *in vitro* with purified biological macromolecules. For this reason, they are not representative of the chemical reactivity of the Au(III) dithiocarbamate derivatives in physiological environment and the real target seems not to be the DNA or the RNA as demonstrated by our next *in vitro* (on intact cells) or *in vivo* (on animal model) experiments.

2.5.2. Inhibition of Thioredoxin Reductase

A number of reports have recognized the enzyme thioredoxin reductase (TrxR) as a effective target for anticancer gold compounds [73]. The thioredoxin (Trx) system plays a key role in regulating the cytosolic (Trx1) and mitochondrial (Trx2) redox balance. In particular, Trx protects cells from a variety of oxidative stresses by regulating the redox state and activity of many proteins that regulate cell growth. TrxR is a large homodimeric selenoenzyme regulating the redox state of Trx. TrxR was showed to be inhibited by a large number of gold derivatives able to cause modifications of the mitochondrial functions and to induce severe oxidative stress, ultimately leading to cell apoptosis. With the aim to elucidate their mechanism of action, the four gold(III) complexes AuL10, AuL12, AuL13 and AuL14 were underwent in-depth studies to assess their capability to inhibit the thioredoxin system mainly on HeLa cells [74]. All the tested compounds induced cancer cell death through both apoptotic and non-apoptotic mechanisms, favored formation of reactive oxygen species (ROS), modified some mitochondrial features (*i.e.*, membrane potential, thus promoting mitochondrial swelling), and inactivated both cytosolic and mitochondrial thioredoxin reductase [74]. The inhibitory effects on thioredoxin system are massive and are likely due to the interaction of the selenocysteine residue nearby the active site of the enzyme. Following these results, we initially suggested a possible working model where the deregulation of the TrxR/Trx system could be a key mechanism involved in their anticancer activity [74].

All the investigated complexes inhibited TrxR by irreversibly binding to the catalytic site, thus inactivating the enzyme that acts as a mediator of electron flow from nicotinamide adenine dinucleotide phosphate (NADPH) to peroxiredoxins through Trx, leading to the accumulation of ROS (especially hydrogen peroxide). We hypothesized the inhibition of the Trx/TrxR redox system stimulates the dissociation of the Trx-ASK-1 (apoptosis signal-regulating kinase-1) complex and the consequent activation of the MAPK (mitogen-activated protein kinase) system [74]. Both the increased levels of ROS and the stimulation of the MAPK system cause enhanced long-lasting levels of phosphorylated extracellular signal-regulated kinases-1 (phosphor-ERK-1) and -2 (phosphor-ERK-2) that should lead to cell death [74]. In that work, we assumed that a persistent ERK-1/2 activation generated initially by accumulation of hydrogen peroxide, and then by ASK-1 pathway dysregulation, might lead to cell death throughout both apoptotic and non-apoptotic ways. On the contrary, cisplatin is able to kill cells only through an apoptotic pathway (as determined by the poly-(ADPribose)-polymerase (PARP) cleavage) [74].

In many different types of tumor and transformed cell lines [75, 76], including prostate cancer [77], the levels of Trx and TrxR are found higher if compared to the healthy counterparts from the same patient. Also the resistance of prostate cancer to clinically used anticancer drugs, such as cisplatin [78] and docetaxel [79], was associated with increased levels of Trx.

Accordingly, the interest in the development of drugs that might target the Trx system arises from its key role in regulating apoptosis and its high levels of expression detected in

different cancer histotypes [80]. In this regard, we investigated the biological activity and the molecular mechanisms of our gold(III) derivatives against prostate cancer [32], which represents, according to the last WHO data, the third cause of cancer-related death in males. In fact, a 12-h incubation of PC3 cells with AuL10 at 5 μ M strongly inhibited (90%) the TrxR enzyme activity whereas glutathione reductase was unaffected by the same treatment [32]. This is in agreement with the findings on HeLa cells above described [74]. In fact, although glutathione reductase is a protein structurally and functionally related to TrxR, and belongs to the same family of pyridine nucleotide oxidoreductase, it lacks selenium nearby the catalytic site, a sulfur cysteine being present (Fig. 8). In this regard, it should be underlined that, from the chemical point of view, selenium is a more soft element than sulfur and this can break down the trans effect of the DTC ligand bound to the gold(III) center. On the contrary, when sulfur cysteine is present the metal reactivity towards the active site is overthrown.

Besides TrxR system unbalance, another aspect of the androgen-independent prostate cancer is its greater invasive potential than its hormone-responsive counterpart. Notwithstanding 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 [81]. A successful therapeutic approach should suppress not only cell proliferation, but also the metastatic

potential of any cell escaping from the primary tumor site to colonize the bone marrow [82]. Our study showed AuL10 is able to both inhibit PC3 cell migration and down-regulate the surface-expressed and phosphorylated forms of the epithelial growth factor receptor (EGFR), whose ligands are paracrine and autocrine growth factors for prostate cancer and are involved in prostate cancer metastasis [83-85].

2.5.3. Induction of Permeability Transition Pore Opening

In a recent paper [86], we have studied additional targets affected by our gold(III) dithiocarbamate derivatives and associated with the unbalanced homeostatic redox equilibrium of tumor cells. In this regard, survival of malignant cells is favored by mitochondrial changes that make death induction more difficult under many stress conditions, including the treatment with chemotherapeutics. These changes have not been completely characterized yet in tumor mitochondria, but comprise the inhibition of the permeability transition pore (PTP) opening through kinase signaling pathways, the unbalance of the redox equilibrium and the modulation of members of the Bcl-2 protein family. On the contrary, opening of the mitochondrial PTP, a large channel located in the inner mitochondrial membrane, leads to mitochondrial depolarization, swelling, rupture of the outer membrane, Ca^{2+} release and delivery of proteins involved in apoptosis commitment [87].

Fig. (8). Effect on Thioredoxin and Glutathione Reductase of four gold(III) complexes (1=AuL10; 2=AuL14; 3=AuL13; 4=AuL12). Cytosolic (A), mitochondrial (B) and *E. coli* (C) thioredoxin reductases and glutathione reductase (D) were incubated with the gold compounds at the indicated concentrations. The final concentrations of the enzymes were 2.1 and 3.8 nM for TrxR1 and TrxR2, respectively. *E. coli* thioredoxin reductase was 40.2 μ g/mL while glutathione reductase was 4.3 nM. The inhibitory effect of each tested compound was evaluated in comparison with the corresponding control (untreated thioredoxin and glutathione reductase). Dose-response curves were calculated over the indicated range of concentrations to obtain the IC_{50} values (given as means $\pm$ SE of at least three independent experiments) (adapted from ref. [74]).

Remarkably, our compound AuL12 was able to strongly inhibit the activity of the respiratory chain Complex I and to cause a rapid raise in ROS levels, which in turn results in mitochondrial PTP opening and hence cancer cell apoptosis [86]. In particular, AuL12 treatment triggers the activation of the pro-apoptotic protein Bax of the Bcl-2 family, followed by its interaction with active GSK-3 α/β (glycogen synthase kinase) and translocation into mitochondria, resulting in PTP unlocking and tumor cell death [88-89]. These results provide evidence that targeting the redox equilibrium kept by mitochondria in malignant cells, allows hitting key mechanisms that shield tumors from the effect of several anticancer drugs.

Similarly to AuL12, we recently identified another compound with the general formula [Au^{III}Br₂(PDT)], where PDT stands for pyrrolidinedithiocarbamate, able to elicit oxidative stress with effects on the permeability transition pore [90]. [Au^{III}Br₂(PDT)] showed a marked cytotoxicity in a short treatment time (3 hours) towards different tumor cell types (*i.e.*, the highly aggressive SAOS-2 osteosarcoma cells, the HeLa cervix adenocarcinoma cells, the HCT116 colorectal carcinoma cells and the mouse embryonic fibroblasts MEF-NF1^{-/-}, obtained from mice where the *bona fide* tumor suppressor gene NF1 had been genetically ablated), recording IC₅₀ values in the range 3.6-17.7 μ M.

The great activity of this compound on MEF-NF1^{-/-} cells was shown to be associated with an increase of ROS levels, which in turn causes PTP opening. Interestingly, the presence of the scavenger Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; water-soluble analog of vitamin E) did not affect the ability of the investigated compound to induce the pore transition in this kind of cells, thus pointing out the oxidative stress can only partially account for the efficacy of our potential drug [90].

2.5.4. Proteasome Inhibition

It is known that cancerous cells are more sensitive to apoptosis-inducing signals than healthy ones. In this context, proteasome inhibition proved to be a winning strategy to treat neoplasms. The ubiquitin-proteasome system (UPS) plays a crucial role in many intracellular processes such as DNA damage and repair, apoptosis, endocytosis, cell cycle progression, angiogenesis, drug resistance and cell differentiation [91-92]. In cancer cells protein equilibrium is altered, making them more sensitive to inhibitors of the UPS than normal ones [93].

The 26S proteasome is a large multi-subunit protease, localized both in the nucleus and the cytosol, that identifies and hydrolyzes the proteins labeled with a chain of ubiquitin molecules. The proteolytic core of the 26S complex, the 20S proteasome, contains multiple peptidase activities (*i.e.*, chymotrypsin(CT)-, trypsin(T)- and caspase(PGPH)-like) and acts as a digestive machine. Ubiquitinated proteins are shuttled to the 26S proteasome where they undergo final degradation and the ubiquitin is released and recycled (Fig. 9). Aberrant proteasome-dependent proteolysis seems to be associated with the physiology of some illnesses, including cancer, in which some regulatory proteins are either somehow “up-regulated” due to their decreased degradation or “down-regulated” due to their accelerated degradation. Thus,

the ubiquitin-proteasome pathway has become a pharmaceutically-attractive target in oncological chemotherapy, following the FDA approbation of bortezomib (Velcade[®]) for the treatment of multiple myeloma [92-94].

Our aim to deepen the knowledge of the mechanism of action of our gold(III)-dithiocarbamate complexes, has lead us to test the possible proteasome-inhibiting properties of AuL14 in human breast cancer cell cultures and xenografts, so finding such a protease as a major *in vitro* and *in vivo* intracellular target [37, 95]. In this context, in addition to inhibiting cell proliferation of various breast cancer cell lines (pre-malignant MCF10AT1K.cl2, malignant MCF10dcis.com, estrogen receptor α -positive MCF-7 and estrogen receptor α -negative MDA-MB-231), AuL14 showed higher *in vitro* cytotoxic activity than cisplatin towards the highly metastatic and triple-negative MDA-MB-231 breast cancer cell line (85% vs. <20% inhibition, respectively, both at 5 μ M concentration) [37].

AuL14 proved a strong inhibitor of the proteasomal CT-like activity in MDA-MB-231 whole-cell extract in a dose-dependent way, and this outcome is particularly important as the inhibition of the chymotrypsin-like activity is associated with growth arrest and/or apoptosis induction in cancer cells [96-97]. However, in addition to the CT-like pocket inhibition of 20S proteasome, the simultaneous inhibition of the PGPH-like and/or T-like sites increases the activity of UPS inhibitors. In fact, recent studies demonstrated the growing importance in protein degradation of the two remaining catalytic sites when chymotrypsin-like pocket is blocked [98-99]. Inhibition of all the three catalytic activities was observed on purified rabbit 20S proteasome [37]. Proteasomal inactivation was detected also in intact MDA-MB-231 cells and further confirmed by western blot analysis in terms of increased levels of ubiquitinated proteins and the proteasome target proteins p27 and I κ B- α . Proteasome inhibition by AuL14 was correlated with tumor cell death *via* apoptotic mechanisms by different assays, identifying cellular and biochemical hallmarks, such as apoptotic morphological changes, the presence of apoptotic nuclei, and apoptosis-specific PARP cleavage [37].

2.5.5. Overall Considerations: Activity via Reduction?

In 2009 a group of thirteen gold(III) complexes, including our compound AuL12, was tested *in vitro* at Oncotest GmbH (<http://www.oncotest.de>) according to a specific comparative strategy for the development of new antitumor drugs [69]. Among all, AuL12 proved the second best performer with an excellent degree of selectivity (17%) and IC₅₀ values in the low micromolar range against all the screened 36 human cancer cell lines, in particular against ovary and brain malignancies. It should be highlighted that, from a comparative analysis involving 110 reference substances with known mechanism of action, AuL12 resulted to be the only gold(III) complex (covered by this screening study) whose mode of action was not sortable in a particular class of any of the reference drugs.

It is worth underlining that the biological activity of complexes containing metals, hence endowed with different oxidation states and rich coordination chemistry, can depend on coordination to different biomolecules and/or on redox

Fig. (9). The ubiquitin-proteasome pathway. A target protein degraded by the ubiquitin-proteasome pathway is first covalently modified by multiple ubiquitin (Ub) molecules in a three-stepped, highly regulated enzymatic process involving the Ub-activating (E1), the Ub-conjugating (E2), and the Ub-ligating (E3) enzymes. The ubiquitinated protein is then escorted to the proteasome, recognized by the 19S cap, de-ubiquitinated and then hydrolyzed by the catalytic 20S core into oligopeptides. The ubiquitin molecules are released and recycled.

reactions occurring under physiological conditions. In fact, in the second case, changes in the charge of the involved metal can result in death-inducing alterations of the cellular milieu.

Since the physiological environment is generally reducing, gold(III) complexes, characterized by high redox potentials [69], can undergo reduction to the corresponding gold(I) counterparts (or even to metallic gold). In order to demonstrate the importance of the metal oxidation state for the mechanism of action and, ultimately, the overall antitumor activity of our gold complexes, we carried out a comparative study on two gold-dithiocarbamate derivatives, namely AuL12 and AuL15 ($[\text{Au}^{\text{I}}(\text{ESDT})]_2$, Fig. 3), containing the metal center gold in the oxidation states +3 and +1, respectively [100]. First, we compared their antiproliferative activities against the human breast cancer cells MDA-MB-231, observing that AuL15 is less potent than AuL12 ($\text{IC}_{50} = 13.5$ and $4.5 \mu\text{M}$, respectively). In agreement with this finding, both compounds were shown to inhibit the proteasomal chymotrypsin-like activity of 26S proteasome in human breast cancer MDA-MB-231 cells and of purified 20S proteasome (*i.e.*, cell-free conditions), AuL15 being again less effective than AuL12 ($\text{IC}_{50} = 17.7$ and $1.13 \mu\text{M}$, respectively). Furthermore, contrary to the gold(I) complex AuL15, the treatment with the gold(III) analogue AuL12 was associ-

ated also with ROS production in intact breast cancer cells [100]. These results point out that the ROS species induced by the gold(III) compound, but not the gold(I) counterpart, could be, at least partially, responsible for the observed different proteasome-inhibitory properties. Similarly to AuL12, the gold(III) complex AuL14 could stimulate production of ROS that may oxidize and inactivate the proteasome [37], in the light of the well-known susceptibility of proteasome to oxidative modification and inactivation after exposure to free radical-producing systems [101].

The lack of proteasome inhibition after treatment with the investigated compounds in the presence of reducing agents, such as L-NAC or dithiothreitol (DTT), could be the result of a reduction process from gold(III) to gold(I) or metallic gold, with lower or no capability of producing ROS species. On the other hand, it is also possible that L-NAC or DTT are able to take the complex away from the proteasome, thus preventing proteasome binding and inhibition.

The identification of the proteasome as an important target of our gold(III) complexes is not in contrast with the previously discussed model related to the TrxR/Trx system, as key target. In fact, Colomer and co-workers reported that the proteasome inhibitor bortezomib induces apoptosis also through generation of ROS [102]. Since treatment with our

compounds proved to be associated with ROS production, the observed proteasome inhibition could favor the long-lasting persistence of phosphorylated ERK-1/2.

On the basis of the results collected so far, we can state that there is no sole mechanism involving, for instance, an unique target, responsible for the biological activity of our metallodrugs. Furthermore, to date, the dithiocarbamate ligand has proven to fulfill the chemoprotective role along with stabilizing the high oxidation state of gold enough to make possible crucial processes in terms of both redox reactions and coordination events to tumor-relevant biomolecules. Remarkably, since every couple of $\text{Au}^{\text{I}}/\text{Au}^{\text{III}}$ investigated compounds owns the same ligand, the superior activity of the latter is likely due to the different oxidation states (+3 vs. +1) and geometry (tetra-coordinate square-planar vs. dicoordinate linear) of the involved gold centers, thus strengthening our hypothesis of an “*activity via reduction*” mechanism for our gold(III) compounds.

3. SECOND GENERATION

Cellular uptake of therapeutic agents is a challenging task because of the plasma membrane, which constitutes an impermeable barrier for most of these molecules. In order to circumvent this issue, several delivery systems have been developed in the last decades for different applications, including drug delivery to solid tumors, antibiotic and pulmonary therapy, tissue repair and regeneration in the central nervous system [103-114]

In addition to nanocarriers (*see section 1.4*), our attention was recently focused on the use of particular biomolecules able to specifically deliver the cytotoxic metal agent within the tumor site. In this context, some peptide transporters (integral plasma membrane proteins) are able to recognize and mediate the cellular uptake of small peptides, and peptide-like drugs. Two peptide transporters, namely PEPT1 and PEPT2, have been identified in mammals. They are present mostly in epithelial cells of the small intestine, mammary glands, choroid plexus, lung and kidney, but are also found in other cell types [115-117] and are overexpressed in some types of malignancies [118-123]. Both PEPT1 and PEPT2 exhibit a similar substrate specificity but differ in structure, hence in binding affinity and transport capacity. PEPT1 is a low-affinity, high-capacity transporter whereas PEPT2 acts with high affinity and low capacity. Their substrate-binding site can accommodate a wide range of molecules of different charge, hydrophobicity and size [124]. Thus, functionalizing various peptide derivatives with transition metals seems to be a smart strategy to force the tumor cell uptake *via* the recognition by PEPT transporters, and hence to selectively induce cell death.

On the basis of these considerations, the rationale of our second approach of antitumor chemotherapy was the design of gold(III)-peptidodithiocarbamate complexes (Fig. 10) able to maintain both the antitumor properties and the lack of nephrotoxicity of the previously reported gold(III) analogues (first generation) [36], together with an enhanced bioavailability through the peptide-mediated cellular internalization by exploiting peptide transporters PEPT1 and PEPT2. Moreover, a number of modifications of the peptide chain of the dithiocarbamate ligands was brought in the formulas

with the aim to improve the water solubility of the resulting gold(III) complexes [125]. In particular, Fig. (10) shows the structures of our second-generation compounds with the general formula $[\text{Au}^{\text{III}}\text{X}_2(\text{pdtc})]$ (X = halogen, pdtc = peptidodithiocarbamate consisting of 2 to 5 amino acids).

Fig. (10). Chemical drawing of our gold(III) peptidomimetics ($n=2-5$). The mainly studied complexes ($n=2$) are AuD6 (X=Br; $\text{R}_1=\text{CH}_3$; $\text{R}_2=\text{R}_3=\text{H}$; $\text{R}_4=\text{R}_5=\text{H}$; $\text{R}_6=\text{OtBu}$), AuD8 (X=Br; $\text{R}_1=\text{CH}_3$; $\text{R}_2=\text{R}_3=\text{H}$; $\text{R}_4=\text{R}_5=\text{CH}_3$; $\text{R}_6=\text{OtBu}$) and AuD9 (X=Cl; $\text{R}_1=\text{CH}_3$; $\text{R}_2=\text{R}_3=\text{H}$; $\text{R}_4=\text{R}_5=\text{CH}_3$; $\text{R}_6=\text{OtBu}$). The ester moiety ($-\text{COOR}_6$) was also properly modified to enhance the water solubility of the final gold complexes, for instance by preparing the TEG (triethylene glycol monomethyl ether)-esterified gold(III) derivatives.

The up-to-date results of this class of gold(III) complexes are extremely promising and highlight their great deal of potential as anticancer agents.

3.1. Antitumor Activity *In vitro*

Preliminary *in vitro* cytotoxicity tests have been recently carried out on a panel of five human tumor cell lines in comparison with cisplatin over 72-h treatment. The investigated cell lines were related to the human prostate cancer PC-3 and DU-145 (androgen-resistant), the ovarian adenocarcinoma cisplatin-sensitive (2008) and -resistant (2008-R) and the Hodgkin lymphoma L540 [126-127].

On the whole, the compounds AuD8 ($\text{Au}^{\text{III}}\text{Br}_2(\text{dtc-Sar-Aib-O}(\text{t-Bu}))$) and AuD9 ($\text{Au}^{\text{III}}\text{Cl}_2(\text{dtc-Sar-Aib-O}(\text{t-Bu}))$) proved the most active with IC_{50} values of 0.8 ± 0.1 and $1.1 \pm 0.1 \mu\text{M}$ against the PC-3 cell line, respectively. Regarding the DU-145 cell line, they recorded IC_{50} values of 1.4 ± 0.1 and $2.2 \pm 0.1 \mu\text{M}$, respectively. AuD8 and AuD9 showed comparable efficacy against the ovarian adenocarcinoma cisplatin-sensitive (2008) and -resistant (2008-R) cell lines with IC_{50} values around $4.5 \mu\text{M}$. Again, they displayed similar antitumor activity towards the Hodgkin lymphoma L540 with IC_{50} values of about $1.6 \mu\text{M}$ [126]. It is worth highlighting that these two gold-based agents recorded an antitumor efficacy superior to cisplatin (IC_{50} values ranging from 3.3 to $117.2 \mu\text{M}$) against all the tested cell lines.

Then, AuD8 and AuD9 were tested also against the cisplatin-resistant PC3-R and DU145-R cell lines, showing antitumor efficacy comparable (IC_{50} ranging from 1.8 to $2.9 \mu\text{M}$) with that observed in the cisplatin-sensitive cell lines (over 72 h), thus overcoming cisplatin resistance phenomena [128]. In addition, cell growth recovery experiments were carried out to check whether tumor cells are able to proliferate again after removing the gold complex from the medium. In detail, following 24-h incubation with increasing concentrations of two anticancer agents, the cell medium was sub-

stituted with fresh drug-free one and cells were incubated for additional 72 h, detecting IC_{50} values comparable to those observed after 72 h treatment (in the low-micromolar range). These results suggest that the antiproliferative activity is not reversed, hence excluding a cytostatic effect of our compounds [128].

In another work, we tested the antiproliferative effects of the complexes AuD6 ($Au^{III}Br_2(dtc-Sar-Gly-O(t-Bu))$) and AuD8 on the triple-negative human breast cancer (TNBC) cell line MDA-MB-231, as these cells are highly metastatic and invasive [129]. Even though both compounds inhibited tumor cell growth in a concentration-dependent manner, AuD8 proved more potent than AuD6, with IC_{50} values $\pm$ SD of 6.5 ± 0.6 and 17 ± 1 μ M (24 hours of incubation), respectively. After 72-h treatment, the IC_{50} of AuD6 decreased to 13 ± 1 μ M. It should be underlined that MDA-MB-231 cells were resistant to cisplatin under the same experimental conditions in agreement with literature data [130].

It should be underlined that our peptidomimetics with longer ligands (Fig. 10, n ranging from 3 to 5) also showed good antiproliferative activity (in the low micromolar domain) towards the four evaluated human tumor cell lines (PC3, 2008, 2008-R, and L540). In addition, these oligopeptide-derivatives showed no cross-resistance with cisplatin and inhibited tumor cell proliferation by inducing almost exclusively late apoptosis/necrosis [127].

3.2. Antitumor Activity *In vivo*: Two Human Xenograft Tumor Models

Based on the preliminary cell-line screening [126-129], we selected the complexes AuD6 and AuD8 for *in vivo* key investigations against PC-3 prostate and MDA-MB-231 breast cancer human xenografts.

In the first case, the testing was addressed to PC-3 tumor model as these cells are more invasive and more resistant to chemotherapy than DU145 cells [128]. The anti-neoplastic activity of AuD8, chosen as a model compound, was investigated in nude mice. After s.c. injection of 2 mg/kg of AuD8 every other day for 19 days of treatment, a reduction of 70% in tumor volume was recorded with neither weight loss nor decreased activity, in contrast with animals treated with cisplatin. Moreover, no organ damage was histologically-detected in the spleen, liver and kidney of animals except for the observation of focal lung lymphocyte infiltration [128].

As previously mentioned, the human breast cancer MDA-MB-231 was taken into account since this cell line is a triple-negative breast cancer, frequently associated with poor prognosis and lack of benefit from both monoclonal antibody therapy (targeting the human epidermal growth factor receptor 2) and the hormonal therapies (based on estrogen and progesterone receptor antagonists) [131]. Accordingly, MDA-MB-231 tumors grow quickly and often yield early gastrointestinal metastases [132].

In this study, mice were treated five days a week by s.c. injection of vehicle containing 1.0 mg/kg of AuD6 or AuD8, or vehicle alone. All animals were sacrificed after 27 days of treatment, or when tumors reached *ca.* 1,800 mm³. Contrary to the *in vitro* results, the two investigated gold compounds showed comparable antitumor activity against the

xenografts, observing 53% inhibition compared to control treatment (Fig. 11A). Remarkably, 40% of mice displayed 85% inhibition and, in some cases, tumor shrinkage after 13 days of treatment (Fig. 11B). Mouse weights were monitored twice a week with no detected weight loss. It should be noted that mice appeared healthy and very active throughout the treatment [129]. In fact, subsequent toxicological studies on healthy animals detected no death in the treated animals at different doses, much higher than those used in chemotherapy, *via* two administration routes (endovenous and oral). In addition, no clinical issue was recorded in all the survivors (data non published yet).

3.3. Investigation of the Mechanism of Action

The ability of our second-generation compounds to induce apoptosis was investigated. In this context, one of the earliest characteristics of apoptosis is a morphological change in the plasma membrane, namely the translocation of the phosphatidylserine (PS) from the internal to the external layer of the cell membrane. Thus, the binding of Annexin V to cells with exposed PS is exploited as a sensitive method to detect apoptosis. However, a population of apoptotic cells may contain also necrotic cells, similarly able to bind Annexin V, due to their injured plasma membrane. The distinction between apoptotic and necrotic cells is carried out by cell staining with the fluorescent dye propidium iodide (PI) as it can cross damaged plasma membrane of necrotic cells only. For comparison purposes, also in these studies we considered for testing the MDA-MB-231 and PC-3 human tumor cell lines and evaluated the effects of the complexes AuD6, AuD8 and AuD9 by flow cytometry. All these Au(III) derivatives caused apoptosis, inducing a significant increase of Annexin V staining [128-129]. Concerning the first experiments, MDA-MB-231 cells were treated with AuD6 or AuD8 for 16 or 24 h at 20 μ M, observing that the amount of cells undergoing non-apoptotic cell death was comparable to the solvent control. After 16 h incubation with AuD8, the greater part of cell death (62.9%) occurred by apoptosis (late stage) and this percentage further enhanced after 24 h (74.1%). Even though both compounds showed similar percentages of cells in early stage apoptosis (*ca.* 7–9%), AuD6 turned out to be less potent than AuD8 as it triggered late-stage apoptosis in only 49% of cells after 24 h-treatment [129]. In the other study involving PC-3 cells, the antitumor agents AuD8 and AuD9 proved able to induce mainly apoptosis at 10 μ M after 24-h incubation. The majority of cells were found in early-stage apoptosis (about 58%) or in the late-stage (18%) [128].

Similarly, cell cycle modifications and DNA fragmentation were evaluated by PI staining and flow cytometric analyses. Incubation for 12 h with the gold compounds AuD8 and AuD9 caused a rise of the S phase population, with similar % loss (with respect to the control) from both the G₂/M and G₀/G₁ phases. After 24-h treatment, cell population in S phase was comparable to that of the control (upon percentage reduction with respect to 12 h incubation) paralleled with a block in G₀/G₁ phase (with a low percentage of cells in G₂/M stage with respect to the medium). This condition of block in G₀/G₁ was again detected after 48 and 72 h of incubation [128]. DNA fragmentation was significant

Fig. (11). Antitumor activity *in vivo* on MDA-MB-231 xenografts. Female nude mice bearing MDA-MB-231 tumors were treated with either vehicle (control) or the compounds AuD6 and AuD8 at 1 mg/kg. Inhibition of xenograft growth by both complexes. Tumor volumes were measured every other day using a caliper. Points represent the mean $\pm$ SD (bars) of seven mice per group (A). If only the most responsive mice are considered, the xenograft growth inhibition is greater (B). (adapted from ref. [129]).

(about 15-20%) only after 48 or 72 h treatment. On the contrary, after 72 h an equivalent amount of cisplatin induced a blockade in the G₂/M phase of the cell cycle, with no significant DNA fragmentation, thus pointing out a different mechanism of action for our compounds compared to Pt-based drugs [128].

In the light of the key role played by the proteins Bcl-x1 (prosurvival protein which prevents the release of cytochrome c), Bcl-2 (important antiapoptotic protein associated with the corresponding oncogene) and Bax (apoptosis-promoting protein) in regulating the intrinsic apoptotic pathway, we investigated whether our compounds AuD8 and AuD9 were able to affect their expression. After 24-h treatment with either AuD8/AuD9 or cisplatin (10 μ M), PC-3 cells showed a significant reduction of the levels of the pro-survival Bcl-x1 protein along with increasing the expression of the pro-apoptotic Bax protein. However, only the anticancer agent AuD8 was able to down-regulate the expression of the anti-apoptotic Bcl-2 protein. Under the same experimental conditions, cisplatin decreased the levels of expression of Bcl-x1 but had no effect on Bcl-2 and Bax counterparts [128].

Based on the results collected for our “first-generation” gold(III)-dithiocarbamate derivatives (see section 1), we aimed to check whether a deregulation of the thioredoxin (Trx)/thioredoxin reductase (TrxR) redox system and an induction of ROS accumulation are likewise involved in the mechanism of action of this new class of gold(III) complexes. Both gold peptidomimetics AuD8 and AuD9 caused a great increase of mitochondrial ROS, even after a short incubation time (12 h). Contrary to the reference drug cis-

platin, our complexes were able to promote production of ROS species that may be involved in the release of Cyt c and hence the overexpression of proapoptotic factors [128]. As previously mentioned, it is worth highlighting that higher levels of Trx and TrxR are found in many different malignant cells compared with healthy ones from the same patient [75-76, 133]. In addition, increased Trx levels have been associated with resistance to several anticancer drugs in prostate cancer, including cisplatin [78] and docetaxel [79]. Therefore, therapeutic agents able to selectively target the Trx system are required [79]. To date, only a few studies have reported inhibitors of TrxR in cancer cell lines [80-134] while other experiments are usually carried out *in vitro* only on the purified TrxR. In this context, a short incubation (12 h) with either AuD8 or AuD9 at 10 μ M resulted in a strong inhibition (~90%) of the TrxR activity in PC3 cells. It should be underlined that under the same experimental conditions, cisplatin displayed smaller effects in terms of ROS formation and TrxR activity inhibition [128].

Since first-generation compounds were able to inhibit the chymotrypsin-like catalytic pocket of the proteasome [37, 100], we hypothesized that these new complexes could target the tumor proteasome as well. To test this hypothesis, we investigated the effects of the compounds AuD8 and AuD9 in PC3 cells, observing a strong and comparable inhibition (> 90% compared to control) of the proteasome activity at 10 μ M after 12 hours. On the contrary, cisplatin had insignificant inhibiting activity under the same experimental conditions [128].

The antiproteasome activity was also observed in MDA-MB-231 human breast tumor model, both *in vitro* (purified

20S proteasome and cell extracts) and *in vivo*. In the first case, the complexes AuD6 and AuD8 were able to inhibit in the low micromolar range all the three proteasomal enzymatic activities described above in a concentration-dependent manner [129]. As previously discussed, co-targeting the PGPH-like and/or T-like sites has been suggested as a new approach in treating malignancies as it could enhance the efficacy of proteasome inhibitors [98-99]. Western blot analysis on cell extracts derived from cells treated with AuD8 allowed us to further confirm cell death occurs *via* apoptotic pathways and it is correlated with the proteasome inhibition. In fact, we observed accumulation of ubiquitinated proteins, p27 and I κ B α (proteasome substrates), caspase-3 activation, PARP cleavage and changes in levels of the proteasomal CT-like subunit. Treatment with AuD8 also caused greater levels of p36/Bax and subsequent reduction or complete disappearance of p21/Bax and p18/Bax fragments. This is usually followed by release of mitochondrial cytochrome c, activation of caspase-3 and cleavage of PARP (disappearance of intact PARP (116 kDa) and appearance of the characteristic fragments p65 and p85), leading to apoptotic cell death [135].

To validate the proteasome-inhibitory and apoptosis-inducing activities *in vivo*, IHC (immunohistochemical) staining was carried out on AuD6- and AuD8-treated tumors,

observing an increase in both p27 staining and TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) positivity, thus confirming proteasome inhibition and apoptotic cell death *in vivo*, respectively. It is worth underlining that apoptosis stimulation should not be merely due to the proteasome-inhibitory properties of these gold peptidomimetics. In fact, AuD8 treatment resulted in higher p27 staining than AuD6, whereas AuD6 caused more intense TUNEL staining. Therefore, their mechanism of action necessarily involves multiple targets which are responsible for cancer cell death [129].

In conclusion, a major point of the mechanism of action of successful therapeutics is associated with the research of drugs able to suppress not only cancer cell proliferation, but also the metastatic potential of all cells escaping from the primary tumor site. For instance, androgen-independent prostate cancer is endowed with a higher metastatic potential than its hormone-responsive counterpart. In this context, EGFR (epithelial growth factor receptor) phosphorylation is known to affect prostate cancer cells migration [136]. Thus, we tested the effects of AuD8 or AuD9 treatment on PC-3 cells, resulting in a great decrease of the surface expression of both EGFR and its phosphorylated form (pEGFR) (Fig. 12A-B). In contrast, cisplatin proved basically ineffective. In the light of these findings, the effect of both gold complexes

Fig. (12). 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 for 12 h at 37°C, with either AuD8/AuD9 or cisplatin (10 μ M). 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 $\pm$ SD of three independent experiments. (C) Analysis of PC3 cells migration by a scratch test after incubation at 37°C with either AuD8/AuD9 or cisplatin at 4 μ M 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 (original magnification 10 $\times$). $^{\circ}/^{\circ}$ AuD8 or AuD9 vs cisplatin ($^{\circ}$ p < 0.01, $^{\circ\circ}$ p < 0.0001) (adapted from ref. [128]).

on PC3 cell migration was studied by the scratch wound healing assay [137]. After 12-h and 24-h incubation with either compound at 4 μ M, the migration rate of PC3 cells dramatically decreased (Fig. 12C). In fact, contrary to the control samples, treated cells were not able to form a confluent monolayer [128].

CONCLUSION

Gold(III) complexes show chemical structures that are similar to those of clinically-established platinum(II) complexes. In particular, the favorite square-planar coordination and the characteristic d^8 electronic configuration, make them very enticing for testing as antineoplastic drugs. At the beginning, our gold(III) dithiocarbamate derivatives were designed to both reproduce the main features of the reference drug cisplatin and to minimize unwanted side-effects typical of the platinum-based chemotherapeutics. The first generation of Au(III) anticancer agents was conceived under this light. Then, our metallodrugs were made more selective towards cancer cells (second-generation) by enhancing the site-specific cell uptake. In fact, various short peptides were bound to the Au(III) center, so to work as carriers for a cancer-selective delivery of such noble transition metal, the whole system acting as a “smart bomb”.

Our decennial work on gold(III) dithiocarbamate derivatives as potential anticancer agents has been here summarized. On the whole, the data collected so far in terms of great *in vitro* and *in vivo* antitumor activity and low toxicity are beyond the expectations! In fact, the absolutely unconventional pattern of reactivity and mechanism of action of our gold(III) complexes, has highlighted: i) the capability to overcome the resistance showed by some types of tumors towards platinum-based drugs; ii) a good *in vivo* toxicological profile; and iii) the capability to cure some “orphan” tumors as well, including the triple negative breast cancer. In other words, our molecular design resulted in a tuned gold reactivity that makes this element a good cytotoxic agent but, at the same time, a biologically non-dangerous heavy metal. This behavior is also paralleled with the activation of completely different intracellular pathways, with respect to the other clinically-established chemotherapeutics.

Although the overall mechanism of action of our anticancer agents is still far from clear, some important biological targets have been identified (*e.g.*, the mitochondria, the proteasome and the selenoenzyme thioredoxin reductase) and their ability to trigger cell death was associated with apoptosis rather than necrosis.

These encouraging results prompted us to also design and develop micellar delivery systems in order to overcome the poor water solubility. To date, we loaded a model compound into two different supramolecular systems, involving the amphiphilic polymers DSPE-PEG2000 and Pluronic F127. In both cases, cancer-targeting and non-targeting micelles were prepared and biologically studied *in vitro*. The tumor-selective delivery was achieved by labelling the hydrophilic shell of the two amphiphilic polymers with two oligopeptides, bombesin and CCK8, respectively.

As advanced preclinical studies are required for entering phase I clinical trials, we have also investigated the acute

toxicity of the second-generation compounds (protected with a PCT patent [125]) under GLP (good laboratory practice) regulations. The obtained excellent outcomes (data not published yet) provide us with a solid starting point for their validation as suitable candidates for further pharmacological testing. The observed good toxicological profile could be beneficial in treating patients at a lower dosage if compared to the drugs already in clinical use, avoiding the onset of severe side effects and/or beneficial to cure “drugless tumors”.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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REFERENCES

- [1] Rosenberg, B. Fundamental studies with cisplatin. *Cancer*, **1985**, *55*, 2303-2316.
- [2] Kelland, L. The resurgence of platinum-based cancer chemotherapy. *Nat. Rev. Cancer*, **2007**, *7*, 573-584.
- [3] Sun, R.W.Y.; Ma, D. L.; Wong, E.L.-M.; Che, C.M. Some uses of transition metal complexes as anti-cancer and anti-HIV agents. *Dalton Trans.*, **2007**, *43*, 4884-4892.
- [4] Bruijninx, P.C.A.; Sadler, P.J. New trends for metal complexes with anticancer activity. *Curr. Opin. Chem. Biol.*, **2008**, *12*, 197-206.
- [5] Kostova, I. Platinum complexes as anticancer agents. *Recent Pat. Anti-Cancer Drug Discovery*, **2006**, *1*, 1-22.
- [6] Jakupec, M.A.; Galanski, M.; Arion, V.B.; Hartinger, C.G.; Keppler, B.K. Antitumor metal compounds: more than theme and variations. *Dalton Trans.*, **2008**, *2*, 183-194.
- [7] Wang, X.; Guo, Z. Towards the rational design of platinum(II) and gold(III) complexes as antitumor agents. *Dalton Trans.*, **2008**, *12*, 1521-1532.
- [8] da Silva Maia, P.I.; Deflon, V.M.; Abram, U. Gold(III) complexes in medicinal chemistry. *Future Med. Chem.*, **2014**, *6*(13), 1515-1536.
- [9] Glenna, A.; Kvien, T.K.; Andrup, O.; Clarke-Jenssen, O.; Karstensen, B.; Brodin, U. Auranofin is safe and superior to placebo in elderly-onset rheumatoid arthritis. *Br. J. Rheumatol.*, **1997**, *36*(8), 870-877.
- [10] Kostova, I. Gold coordination complexes as anticancer agents. *Anti-Cancer Agents Med. Chem.*, **2006**, *6*, 19-32.
- [11] Ott, I. On the medicinal chemistry of gold complexes as anticancer drugs. *Coord. Chem. Rev.*, **2009**, *253*, 1670-1681.
- [12] Tiekink, E.R.T. Anti-cancer potential of gold complexes. *Inflammopharmacology*, **2008**, *16*, 138-142.
- [13] Nobili, S.; Mini, E.; Landini, I.; Gabbiani, C.; Casini, A.; Messori, L. Gold compounds as anticancer agents: chemistry, cellular pharmacology, and preclinical studies. *Med. Res. Rev.*, **2010**, *30*(3), 550-580.
- [14] Nardon, C.; Pettenuzzo, N.; Fregona, D. Gold complexes for therapeutic purposes: an updated patent review (2010-2014). *Coord. Chem. Rev.*, **2015**, submitted for publication.
- [15] Johnson, N.P.; Butour, J.L.; Villani, G.; Wimmer, F.L.; Defais, M.; Pierson, V.; Brabec, V. Metal antitumor compounds: the mechanism of action of platinum complexes. *Prog. Clin. Biochem. Med.*, **1989**, *10*, 1-24.

- [16] Casini, A.; Messori, L. Molecular mechanisms and proposed targets for selected anticancer gold compounds. *Curr. Top. Med. Chem.*, **2011**, *11*(21), 2647-2660.
- [17] De Almeida, A.; Oliveira, B.L.; Correia, J.D.G.; Soveral, G.; Casini, A. Emerging protein targets for metal-based pharmaceutical agents: An update. *Coord. Chem. Rev.*, **2013**, *257*(19-20), 2689-2704.
- [18] Nagy, E. M.; Ronconi, L.; Nardon, C.; Fregona, D. Noble metal-dithiocarbamates precious allies in the fight against cancer. *Mini-Rev. Med. Chem.*, **2012**, *12*(12), 1216-1229.
- [19] Nardon, C.; Boscutti, G.; Fregona, D. Beyond platinum: gold complexes as anticancer agents. *Anticancer Res.*, **2014**, *34* (1), 487-492.
- [20] Pabla, N.; Dong, Z. Cisplatin nephrotoxicity: mechanisms and renoprotective strategies. *Kidney Int.*, **2008**, *73*, 994-1007.
- [21] Wang, X.; Guo, Z. The role of sulfur in platinum anticancer chemotherapy. *Anti-Cancer Agents Med. Chem.*, **2007**, *7*, 19-34.
- [22] Dorr, R.T. *Platinum and Other Metal Coordination Compounds in Cancer Chemotherapy*, Ed. Plenum Press: New York, **1996**, pp. 131-154. Pinedo, H.M and Schornagel, J.H. eds
- [23] Fuertes, M.A.; Alonso, C.; Pérez, J. Biochemical modulation of cisplatin mechanisms of action: enhancement of antitumor activity and circumvention of drug resistance. *Chem. Rev.*, **2003**, *103*, 645-662.
- [24] Segovia, N.; Crovetto, G.; Lardelli, P.; Espigares, M. *In vitro* toxicity of several dithiocarbamates and structure-activity relationships. *J. Appl. Toxicol.*, **2002**, *22*, 353-357.
- [25] Marzano, C.; Trevisan, A.; Giovagnini, L.; Fregona, D. Synthesis of a new platinum(II) complex: Anticancer activity and nephrotoxicity *in vitro* (Conference Paper), *Toxicology in vitro*, **2002**, *16*(4), 413-419.
- [26] Ronconi, L.; Maccato, C.; Barreca, D.; Saini, R.; Zancato, M.; Fregona, D. Gold(III) dithiocarbamate derivatives of N-methylglycine: an experimental and theoretical investigation. *Polyhedron*, **2005**, *24*, 521-531.
- [27] Ronconi, L.; Giovagnini, L.; Marzano, C.; Bettio, F.; Graziani, R.; Pilloni, G.; Fregona, D. Gold dithiocarbamate derivatives as potential antineoplastic agents: design, spectroscopic properties, and *in vitro* antitumor activity. *Inorg Chem.* **2005**, *44*, 1867.
- [28] Giovagnini, L.; Ronconi, L.; Aldinucci, D.; Lorenzon, D.; Sitran, S.; Fregona, D. Synthesis, characterization and comparative *in vitro* cytotoxicity studies of platinum(II), palladium(II) and gold(III) methylsarcosinedithiocarbamate complexes. *J. Med. Chem.*, **2005**, *48*(5), 1588-1595.
- [29] Aldinucci, D.; Lorenzon, D.; Stefani, L.; Giovagnini, L.; Colombatti, A.; Fregona, D. Antiproliferative and apoptotic effects of two new gold(III) methylsarcosinedithiocarbamate derivatives on human acute myeloid leukemia cells *in vitro*. *Anti-Cancer Drugs*, **2007**, *18*, 323-332.
- [30] Aldinucci, D.; Cattaruzza, L.; Lorenzon, D.; Giovagnini, L.; Fregona, D.; Colombatti, A. Antiproliferative and apoptotic effects of two new Pd(II) methylsarcosinedithiocarbamate derivatives on human acute myeloid leukemia cells *in vitro*. *Oncol. Res.*, **2008**, *17*, 103-113.
- [31] Eastman, A. *Cisplatin. Chemistry and Biochemistry of a Leading Anticancer Drug*, Wiley-VCH: Weinheim, **1999**, pp. 111-134. Lipert, B., Ed.
- [32] Cattaruzza, L.; Fregona, D.; Mongiat, M.; Ronconi, L.; Fassina, A.; Colombatti, A.; Aldinucci, D. Antitumor activity of gold(III)-dithiocarbamate derivatives on prostate cancer cells and xenografts. *Int. J. Cancer*, **2011**, *128*, 206-215.
- [33] Brunelle, J.K.; Letai, A. Control of mitochondrial apoptosis by the Bcl-2 family. *J. Cell. Sci.*, **2009**, *122*, 437-441.
- [34] Kang, M.H.; Reynolds, C.P. Bcl-2 inhibitors: targeting mitochondrial apoptotic pathways in cancer therapy. *Clin. Cancer Res.*, **2009**, *15*, 1126-1132.
- [35] Ronconi, L.; Marzano, C.; Zanello, P.; Corsini, M.; Miolo, G.; Maccà, C.; Trevisan, A.; Fregona, D. Gold(III) dithiocarbamate derivatives for the treatment of cancer: solution chemistry, DNA binding, and hemolytic properties. *J. Med. Chem.*, **2006**, *49*, 1648-1657.
- [36] Fregona, D.; Ronconi, L.; Marzano, C. Complessi ditiocarbammici di oro(III) e loro impiego come antitumorali. *Italian Patent 0001347835*, October 2, **2008**.
- [37] Milacic, V.; Chen, D.; Ronconi, L.; Landis-Piowar, K.R.; Fregona, D.; Dou, Q.P. A novel anticancer gold(III) dithiocarbamate compound inhibits the activity of a purified 20S proteasome and 26S proteasome in human breast cancer cell cultures and xenografts. *Cancer Res.*, **2006**, *66*, 10478-10486.
- [38] Marzano, C.; Ronconi, L.; Chiara, F.; Giron, M.C.; Faustinelli, I.; Cristofori, P.; Trevisan, A.; Fregona, D. Gold(III)-dithiocarbamate anticancer agents: activity, toxicology and histopathological studies in rodents. *Int. J. Cancer*, **2011**, *129*(2), 487-496.
- [39] Khan, K.N.M.; Alden, C.I. Kidney. In: Haschek, W. M.; Rousseaues, C.G.; Wallig, M.A. *Handbook of toxicologic pathology*, 2nd edition, vol. 2. San Diego: Academic Press, **2002**, 2255-330.
- [40] Trevisan, A.; Cristofori, P.; Fanelli, G. Glutamine synthetase activity in rat urine as sensitive marker to detect S3 segmentspecific injury of proximal tubule induced by xenobiotics. *Arch Toxicol*, **1999**, *73*, 255-62.
- [41] Cristofori, P.; Zanetti, E.; Fregona, D.; Piaia, A.; Trevisan, A. Renal proximal tubule segment-specific nephrotoxicity: an overview on biomarkers and histopathology. *Toxicol. Pathol.*, **2007**, *35*, 270-275.
- [42] Trevisan, A.; Giraldo, M.; Borella, M.; Bottegali, S.; Fabrello, A. Tubular segment-specific biomarkers of nephrotoxicity in the rat. *Toxicol. Lett.*, **2001**, *124*, 113-20.
- [43] Cheriathundam, E.; Alvares, A.P. Species differences in the renal toxicity of the antiarthritic drug, gold sodium thiomalate. *J. Biochem. Toxicol.*, **1996**, *11*, 175-81.
- [44] Salata, O.V. Applications of nanoparticles in biology and medicine. *J. Nanobiotechnol.*, **2004**, *2*, 3-6.
- [45] Mieszawska, A.J.; Mulder, W.J.M.; Fayad, Z.A.; Cormode, D.P. Multifunctional Gold Nanoparticles for Diagnosis and Therapy of Disease. *Mol. Pharmaceutics*, **2013**, *10*(3), 831-847.
- [46] Bowman, M.C.; Ballard, T.E.; Ackerson, C.J.; Feldheim, D.L.; Margolis, D.M.; Melander, C. Inhibition of HIV Fusion with Multivalent Gold Nanoparticles. *J. Am. Chem. Soc.*, **2008**, *130* (22), 6896-6897.
- [47] Sereemasun, A. Effect of gold nanoparticle on renal cell: an implication for exposure risk. *Ren Fail.*, **2008**, *30*, 323-325.
- [48] Kabanov, A. V. Polymer genomics: an insight into pharmacology and toxicology of nanomedicines. *Adv. Drug Deliv. Rev.*, **2006**, *58*, 1597-1621.
- [49] Torchilin, V. P. In: *In vitro and in vivo availability of liposomes* Kabanov, A. V.; Felgner, P. L.; Seymour, L. W. Eds.; Self-assembling Complexes for Gene Delivery: From Laboratory to Clinical Trial; Wiley: Chichester, UK, **1998**, 277-293.
- [50] Batrakova, E. V.; Kabanov, A. V. Pluronic block copolymers: evolution of drug delivery concept from inert nanocarriers to biological response modifiers. *J. Control. Release*, **2008**, *130*, 98-106.
- [51] Greish, K. In *Enhanced Permeability and Retention (EPR) Effect for Anticancer Nanomedicine Drug Targeting*; Grobmyer, S. R.; Moudgil, B. M. Eds.; Humana Press, **2010**, *624*, 25-37.
- [52] Stolnik, S.; Illum, L.; Davis, S. S. Long circulating microparticulate drug carriers. *Adv. Drug Deliv. Rev.*, **1995**, *16*, 195-214.
- [53] Hawley, A. E.; Davis, S. S.; Illum, L. Targeting of colloids to lymph nodes: influence of lymphatic physiology and colloidal characteristics. *Adv. Drug Deliv. Rev.*, **1995**, *17*, 129-148.
- [54] Ringhieri, P.; Iannitti, R.; Nardon, C.; Palumbo, R.; Fregona, D.; Morelli, G.; Accardo, A. Target selective micelles for bombesin receptors incorporating Au(III)-dithiocarbamate complexes, *Int. J. Pharm.*, **2014**, *473* (1-2), 194-202.
- [55] Nardon, C.; Boscutti, G.; Dalla Via, L.; Ringhieri, P.; Di Noto, V.; Morelli, G.; Accardo, A.; Fregona, D. CCK8 peptide-labeled Pluronic® F127 micelles as a targeted vehicle of gold-based anticancer chemotherapeutics, *MedChemComm*, **2015**, *6*, 155-163.
- [56] Accardo, A.; Salsano, G.; Morisco, A.; Aurilio, M.; Parisi, A.; Maione, F.; Cicala, C.; Tesaro, D.; Aloj, L.; De Rosa, G.; Morelli, G. Peptide modified liposomes for selective targeting of bombesin receptors overexpressed by cancer cells: a potential theranostic agent. *Int. J. Nanomed.*, **2012**, *7*, 2007-2017.
- [57] Valle, J. W.; Lawrence, J.; Brewer, J.; Clayton, A.; Corrie, P.; Alakhov, V.; Ranson, M. A phase II, window study of SP1049C as first-line therapy in inoperable metastatic adenocarcinoma of the oesophagus. In: *ASCO Meeting Abstracts*, **2004**, *22*, 4195.
- [58] Alakhov, V.; Klinski, E.; Li, S.; Pietrzynski, G.; Venne, A.; Batrakova, E.; Bronitch, T.; Kabanov, A. Block copolymer-based formulation of doxorubicin. From cell screen to clinical trials. *Colloids and Surfaces B: Biointerfaces*, **1999**, *16*, 113-134.
- [59] Chiappetta, D. A.; Sosnik, A. Poly(ethylene oxide)-poly(propylene oxide) block copolymer micelles as drug delivery agents: improved

- hydrosolubility, stability and bioavailability of drugs. *Eur. J. Pharm. Biopharm.*, **2007**, *66*, 303-317.
- [60] Reubi, J. C. Peptide receptors as molecular targets for cancer diagnosis and therapy. *Endocr. Rev.*, **2003**, *24*, 389-427.
- [61] Reubi, J. C.; Schaer, J. C.; Waser, B. Cholecystokinin(CCK)-A and CCK-B/gastrin receptors in human tumors. *Cancer Res.*, **1997**, *57*, 1377-1386.
- [62] Wank, S. A.; Pisegna, J. R.; de Weerth, A. Brain and gastrointestinal cholecystokinin receptor family: structure and functional expression. *Proc. Natl. Acad. Sci. U. S. A.*, **1992**, *89*, 8691-8695.
- [63] Wank, S. A. G protein-coupled receptors in gastrointestinal physiology. I. CCK receptors: an exemplary family. *Am. J. Physiol.*, **1998**, *274*, G607-13.
- [64] Reubi, J. C.; Waser, B. Concomitant expression of several peptide receptors in neuroendocrine tumours: molecular basis for *in vivo* multireceptor tumour targeting. *Eur. J. Nucl. Med. Mol. Imaging*, **2003**, *30*, 781-793.
- [65] Benedetti, E.; Morelli, G.; Accardo, A.; Mansi, R.; Tesauro, D.; Aloj, L. Criteria for the design and biological characterization of radiolabeled peptide-based pharmaceuticals. *BioDrugs*, **2004**, *18*, 279-295.
- [66] Vaccaro, M.; Mangiapia, G.; Paduano, L.; Gianolio, E.; Accardo, A.; Tesauro, D.; Morelli, G. Structural and relaxometric characterization of peptide aggregates containing gadolinium complexes as potential selective contrast agents in MRI. *Chemphyschem*, **2007**, *8*, 2526-2538.
- [67] Roettger, B. F.; Rentsch, R. U.; Pinon, D.; Holicky, E.; Hadac, E.; Larkin, J. M.; Miller, L. J. Dual Pathways of Internalization of the Cholecystokinin Receptor. *J. Cell Biol.*, **1995**, *128*, 1029-1041.
- [68] Arnesano, F.; Natile, G. Mechanistic insight into the cellular uptake and processing of cisplatin 30 years after its approval by FDA. *Coord. Chem. Rev.*, **2009**, *253*, 2070-2081.
- [69] Casini, A.; Kelter, G.; Gabbiani, C.; Cinellu, M.A.; Minghetti, G.; Fregona, D.; Fiebig, H.H.; Messori, L. Chemistry, antiproliferative properties, tumor selectivity, and molecular mechanisms of novel gold(III) compounds for cancer treatment: a systematic study, *J. Biol. Inorg. Chem.*, **2009**, *14*(7), 1139-1149.
- [70] Koelle, U.; Laguna, A. Electrochemistry of Au-complexes. *Inorg. Chim. Acta*, **1999**, *290*, 44-50.
- [71] Marzano, C.; Baccichetti, F.; Trevisan, A.; Giovagnini, L.; Fregona, D. Cytotoxicity and DNA damage induced by a new Pt(II) complex with pyridine and dithiocarbamate. *Chem.-Biol. Interact.*, **2002**, *140*, 215-219.
- [72] Minko, I. G.; Zou, Y.; Lloyd, R.S. Incision of DNA-protein crosslinks by UvrABC nuclease suggests a potential repair pathway involving nucleotide excision repair. *Proc. Natl. Acad. Sci. U.S.A.*, **2002**, *99*, 1905-1909.
- [73] Bindoli, A.; Rigobello, M.P.; Scutari, G.; Gabbiani, C.; Casini, A.; Messori, L. Thioredoxin reductase: a target for gold compounds acting as potential anticancer drugs. *Coord. Chem. Rev.*, **2009**, *253*, 1692-1707.
- [74] Saggioro, D.; Rigobello, M.P.; Paloschi, L.; Folda, A.; Moggach, S.A.; Parsons, S.; Ronconi, L.; Fregona, D.; Bindoli, A. Gold(III)-dithiocarbamate complexes induce cancer cell death triggered by thioredoxin redox system inhibition and activation of ERK pathway. *Chem. Biol.*, **2007**, *14*, 1128-1139.
- [75] Lincoln, D.T.; Ali Emadi, E.M.; Tonissen, K.F.; Clarke, F.M. The thioredoxin-thioredoxin reductase system: overexpression in human cancer. *Anticancer Res.*, **2003**, *23*, 2425-2434.
- [76] Sun, Y.; Rigas, B. The thioredoxin system mediates redox-induced cell death in human colon cancer cells: implications for the mechanism of action of anticancer agents. *Cancer Res.*, **2008**, *68*, 8269-8277.
- [77] Singh, S.S.; Li, Y.; Ford III, O.H.; Wrzosek, C.S.; Mehendint, D.C.; Titus, M.A.; Mohler, J.L. Thioredoxin reductase 1 expression and castration-recurrent growth of prostate cancer. *Transl. Oncol.*, **2008**, *1*, 153-157.
- [78] Yamada, M.; Tomida, A.; Yoshikawa, H.; Taketani, Y.; Tsuruo, T. Increased expression of thioredoxin/adult T-cell leukemia-derived factor in cisplatin-resistant human cancer cell lines. *Clin. Cancer Res.*, **1996**, *2*, 427-432.
- [79] Kim, S.J.; Miyoshi, Y.; Taguchi, T.; Tamaki, Y.; Nakamura, H.; Yodoi, J.; Kato, K.; Noguchi, S. High thioredoxin expression is associated with resistance to docetaxel in primary breast cancer. *Clin. Cancer Res.*, **2005**, *11*, 8425-8430.
- [80] Powis, G.; Kirkpatrick, D.L. Thioredoxin signaling as a target for cancer therapy. *Curr. Opin. Pharmacol.*, **2007**, *7*, 392-397.
- [81] Jemal, A.; Siegel, R.; Ward, E.; Hao, Y.; Xu, J.; Thun, M.J. Cancer statistics. *CA Cancer J. Clin.*, **2009**, *59*, 225-229.
- [82] Bonaccorsi, L.; Marchiani, S.; Muratori, M.; Forti, G.; Baldi, E. Gefitinib ('IRESSA', ZD1839) inhibits EGF-induced invasion in prostate cancer cells by suppressing PI3 K/AKT activation. *J. Cancer Res. Clin. Oncol.*, **2004**, *130*, 604-614.
- [83] Di Lorenzo, G.; Tortora, G.; D'Armiento, F.P.; De Rosa, G.; Stai-bano, S.; Autorino, R.; D'Armiento, M.; De Laurentiis, M.; De Placido, S.; Catalano, G.; Bianco, A.R.; Ciardiello, F. Expression of epidermal growth factor receptor correlates with disease relapse and progression to androgen-independence in human prostate cancer. *Clin. Cancer Res.*, **2002**, *8*, 3438-3444.
- [84] Schlomm, T.; Kirstein, P.; Iwers, L. Daniel, B.; Steuber, T.; Walz, J.; Chun, F.H.K.; Haese, A.; Koller mann, J.; Graefen, M.; Huland, H.; Sauter, G.; Simon, R.; Erbersdobler, A. Clinical significance of epidermal growth factor receptor protein overexpression and gene copy number gains in prostate cancer. *Clin. Cancer Res.*, **2007**, *13*, 6579-6584.
- [85] Bratland, A.; Boender, P.J.; Hoifødt, H.K. Osteoblast-induced EGFR/ERBB2 signaling in androgen-sensitive prostate carcinoma cells characterized by multiplex kinase activity profiling. *Clin. Exp. Metastasis*, **2009**, *26*, 485-496.
- [86] Chiara, F.; Gambalunga, A.; Sciacovelli, M.; Nicolli, A.; Ronconi, L.; Fregona, D.; Bernardi, P.; Rasola, A.; Trevisan A. Chemotherapeutic induction of mitochondrial oxidative stress activates GSK-3 α/β and Bax, leading to permeability transition pore opening and tumor cell death. *Cell Death and Disease*, **2012**, *3*, e444.
- [87] Rasola, A.; Bernardi, P. The mitochondrial permeability transition pore and its involvement in cell death and in disease pathogenesis. *Apoptosis*, **2007**, *12*, 815-833.
- [88] Yip, K.W.; Reed, J.C. Bcl-2 family proteins and cancer. *Oncogene*, **2008**, *27*, 6398-6406.
- [89] Walensky, L.D.; Gavathiotis, E. BAX unleashed: the biochemical transformation of an inactive cytosolic monomer into a toxic mitochondrial pore. *Trends Biochem Sci*, **2011**, *36*, 642-652.
- [90] Nardon, C.; Chiara, F.; Brustolin, L.; Gambalunga, A.; Ciscato, F.; Rasola, A.; Trevisan, A.; Fregona, D. Gold(III)-pyrrolidinedithiocarbamate derivatives as antineoplastic agents, *ChemistryOpen*, **2015**, *4*, 193-191.
- [91] Groll, M.; Ditzel, L.; Löwe, J.; Stock, D.; Bochtler, M.; Bartunik, H. D.; Huber, R. Structure of 20S Proteasome from Yeast at 2.4 Å Resolution. *Nature*, **1997**, *386*, 463-471.
- [92] Dalla Via, L.; Nardon, C.; Fregona, D. Targeting the Ubiquitin-Proteasome Pathway with Inorganic Compounds to Fight Cancer: A Challenge for the Future. *Future Medicinal Chemistry*, **2012**, *4*, 525-543.
- [93] Adams, J. Potential for Proteasome Inhibition in the Treatment of Cancer. *Drug Discov. Today*, **2003**, *8*, 307-315.
- [94] Milacic, V.; Dou, Q.P. The tumor proteasome as a novel target for gold(III) complexes: implications for breast cancer therapy. *Coord. Chem. Rev.*, **2009**, *253*, 1649-1660.
- [95] Milacic, V.; Fregona, D.; Dou, Q.P. Gold complexes as prospective metal-based anticancer drugs. *Histol. Histopathol.*, **2008**, *23*, 101-108.
- [96] Lopes, U.G.; Erhard, P.; Yao, R.; Cooper, G.M. p53-dependent induction of apoptosis by proteasome inhibitors. *J. Biol. Chem.*, **1997**, *272*, 12893-12896.
- [97] An, B.; Goldfarb, R.H.; Siman, R.; Dou, Q. P. Novel dipeptidyl proteasome inhibitors overcome bcl-2 protective function and selectively accumulate the cyclin-dependent kinase inhibitor p27 and induce apoptosis in transformed, but not normal, human fibroblasts. *Cell Death Differ*, **1998**, *5*(12), 1062-1075.
- [98] Kisselev, A.F.; Callard, A.; Goldberg, A.L. Importance of the different proteolytic sites of the proteasome and the efficacy of inhibitors varies with the protein substrate. *J. Biol. Chem.*, **2006**, *281*(13), 8582-8590.
- [99] Kisselev, A.F.; Van Der Linden W.A.; Overkleeft, H.S. Proteasome inhibitors: An expanding army attacking a unique target. *Chem.Biol*, **2012**, *19*(1), 99-115.
- [100] Zhang, X.; Frezza, M.; Milacic, V.; Ronconi, L.; Fan, Y.; Bi, C.; Fregona, D.; Dou, Q.P. Inhibition of tumor proteasome activity by gold dithiocarbamate complexes via both redox-dependent and -independent processes. *J. Cell. Biochem.*, **2010**, *109*, 162-172.

- [101] Szweda, P.A.; Friguet, B.; Szweda, L.I. Proteolysis, free radicals, and aging. *Free Radical Biol. Med.*, **2002**, *33*, 29-36.
- [102] Perez-Galan, P.; Roue, G.; Villamar, N.; Montserrat, E.; Campo, E.; Colomer, D. The proteasome inhibitor bortezomib induces apoptosis in mantle-cell lymphoma through generation of ROS and Noxa activation independent of p53 status. *Blood*, **2006**, *107*, 257-264.
- [103] Khawar, I.A.; Kim, J.H.; Kuh, H.J. Improving drug delivery to solid tumors: Priming the tumor microenvironment. *J. Control. Release*, **2015**, *201*, 78-89.
- [104] Zhu, Y.Y.; Liao, L.M. Applications of nanoparticles for anticancer drug delivery: a review. *J. Nanosci. Nanotechnol.*, **2015**, *15*(7), 4753-4773.
- [105] Kalhapure, R.S.; Suleman, N.; Mocktar, C.; Seedat, N.; Govender, T. Nanoengineered drug delivery systems for enhancing antibiotic therapy. *J. Pharm. Sci.*, **2015**, *104*(3), 872-905.
- [106] Sosnowski, T.R. Nanosized and nanostructured particles in pulmonary drug delivery. *J. Nanosci. Nanotechnol.*, **2015**, *15*(5), 3476-3487.
- [107] Anselmo, A.C.; Mitragotri, S. An overview of clinical and commercial impact of drug delivery systems. *J. Control. Release*, **2014**, *190*, 15-28.
- [108] Sao, R.; Vaish, R.; Sinha, N. Multifunctional drug delivery systems using inorganic nanomaterials: a review. *J. Nanosci. Nanotechnol.*, **2015**, *15*(3), 1960-1972.
- [109] Wicki, A.; Witzigmann, D.; Balasubramanian, V.; Huwyler, J. Nanomedicine in cancer therapy: Challenges, opportunities, and clinical applications. *J. Control. Release*, **2015**, *200*, 138-157.
- [110] Kozlovskaya, L.; Stepsky, D. Quantitative analysis of the brain-targeted delivery of drugs and model compounds using nano-delivery systems. *J. Control. Release*, **2013**, *171*(1), 17-23.
- [111] Chen, D.Q.; Wang, H.B. Novel pH-Sensitive Biodegradable Polymeric Drug Delivery Systems Based on Ketel Polymers. *J. Nanosci. Nanotechnol.*, **2014**, *14*(1), 983-989.
- [112] Moghassemi, S.; Hadjizadeh, A. Nano-niosomes as nanoscale drug delivery systems: an illustrated review. *J. Control. Release*, **2014**, *185*, 22-36.
- [113] Donaghue, I.E.; Tam, R.; Sefton, M.V.; Shoichet, M.S. Cell and biomolecule delivery for tissue repair and regeneration in the central nervous system. *J. Control. Release*, **2014**, *190*, 219-227.
- [114] Van Oudheusden, Thijs. Targeting the peritoneum with novel drug delivery systems in peritoneal carcinomatosis: a review of the literature. *Anticancer res.*, **2015**, *35*(2), 627-634.
- [115] Rubio-Aliaga, I.; Daniel, H. Peptide transporters and their roles in physiological processes and drug disposition. *Xenobiotica*, **2008**, *38*(7-8), 1022-1042.
- [116] Biegel, A.; Knutter, I.; Hartrodt, B.; Gebauer, S.; Theis, S.; Luckner, P.; Kottra, G.; Rastetter, M.; Zebisch, K.; Thondorf, I.; Daniel, H.; Neubert, K.; Brandsch, M. The renal type H⁺/peptide symporter PEPT2: Structure-affinity relationships. *Amino Acids*, **2006**, *31*(2), 137-156.
- [117] Terada, T.; Shimada, Y.; Pan, X.; Kishimoto, K.; Sakurai, T.; Doi, R.; Onodera, H.; Katsura, T.; Imamura, M.; Inui, K. Expression profiles of various transporters for oligopeptides, amino acids and organic ions along the human digestive tract. *Biochem Pharmacol*, **2005**, *70*(12), 1756-1763.
- [118] Mitsuoka, K.; Miyoshi, S.; Kato, Y.; Murakami, Y.; Utsumi, R.; Kubo, Y.; Noda, A.; Nakamura, Y.; Nishimura, S.; Tsuji, A. Cancer detection using a PET tracer, ¹¹C glycylsarcosine, targeted to H⁺/peptide transporter. *J. Nucl. Med.*, **2008**, *49*(4), 615-622.
- [119] Inoue, M.; Terada, T.; Okuda, M.; Inui, K. Regulation of human peptide transporter 1 (PEPT1) in gastric cancer cells by anticancer drugs. *Cancer Lett.*, **2005**, *230*(1), 72-80.
- [120] Knutter, I.; Rubio-Aliaga, I.; Boll, M.; Hause, G.; Daniel, H.; Neubert, K.; Brandsch, M. H⁺-peptide cotransport in the human bile duct epithelium cell line SK-ChA-1. *Am J Physiol Gastrointest Liver Physiol*, **2002**, *283*, G222-G229.
- [121] Nakanishi, T.; Tamai, I.; Takaki, A.; Tsuji, A. Cancer cell-targeted drug delivery utilizing oligopeptide transport activity. *Int. J. Cancer*, **2000**, *88*(2), 274-280.
- [122] Gonzalez, D.E.; Covitz, K.Y.; Sadée, W.; Mrsny, R.J. An oligopeptide transporter is expressed at high levels in the pancreatic carcinoma cell lines AsPc-1 and capan-2. *Cancer Res.*, **1998**, *58*(3), 519-525.
- [123] Nakanishi, T.; Tamai, I.; Sai, Y.; Sasaki, T.; Tsuji, A. Carrier-mediated transport of oligopeptides in the human fibrosarcoma cell line HT1080. *Cancer Res.*, **1997**, *57*(18), 4118-4122.
- [124] Brandsch, M. Transport of drugs by proton-coupled peptide transporters: Pearls and pitfalls. *Expert Opin Drug Metab Toxicol*, **2009**, *5*(8), 887-905.
- [125] Fregona, D.; Ronconi, L.; Formaggio, F.; Dou, Q.P.; Aldinucci, D. Gold(III) complexes with oligopeptides functionalized with sulfur donors and use thereof as antitumor agents, *PCT Patent* **2010**, WO2010105691A1.
- [126] Kouodom, M.N.; Ronconi, L.; Celegato, M.; Nardon, C.; Marchiò, L.; Dou, Q.P.; Aldinucci, D.; Formaggio, F.; Fregona, D. Toward the selective delivery of chemotherapeutics into tumor cells by targeting peptide transporters: tailored gold-based anticancer peptidomimetics. *J. Med. Chem.*, **2012**, *55*, 2212-2226.
- [127] Kouodom, M.N.; Boscutti, G.; Celegato, M.; Crisma, M.; Sitran, S.; Aldinucci, D.; Formaggio, F.; Ronconi, L.; Fregona, D. Rational design of gold(III)-dithiocarbamate peptidomimetics for the targeted anticancer chemotherapy. *J. Inorg. Biochem.*, **2012**, *117*, 248-260.
- [128] Celegato, M.; Fregona, D.; Mongiat, M.; Ronconi, L.; Borghese, C.; Canzonieri, V.; Casagrande, N.; Nardon, C.; Colombatti, A.; Aldinucci, D. Preclinical activity of multiple-target gold(III)-dithiocarbamate peptidomimetics in prostate cancer cells and xenografts. *Future Med. Chem.*, **2014**, *6*(11), 1249-1263.
- [129] Nardon, C.; Schmitt, S.M.; Yang, H.; Zuo, J.; Fregona, D.; Dou, Q.P. Gold(III)-Dithiocarbamate Peptidomimetics in the Forefront of the Targeted Anticancer Therapy: Preclinical Studies against Human Breast Neoplasia. *PLoS ONE*, **2014**, *9*(1).
- [130] Hong, Y.; Yang, J.; Wu, W.; Wang, W.; Kong, X.; Wang, Y.; Yun, X.; Zong, H.; Wei, Y.; Zhang, S.; Gu, J. Knockdown of BCL2L12 leads to cisplatin resistance in MDA-MB-231 breast cancer cells. *Biochim Biophys Acta Mol Basis Dis*, **2008**, *1782*(11), 649-657.
- [131] Yang, L.; Wu, X.; Wang, Y.; Zhang, K.; Wu, J.; Yuan, Y.; Deng, X.; Chen, L.; Kim, C. C. H.; Lau, S.; Somlo, G.; Yen, Y. FZD7 has a Critical Role in Cell Proliferation in Triple Negative Breast Cancer. *Oncogene*, **2011**, *30*, 4437-4446.
- [132] Kassam, F.; Enright, K.; Dent, R.; Dranitsaris, G.; Myers, J.; Flynn, C.; Fralick, M.; Kumar, R.; Clemons, M. Survival Outcomes for Patients with Metastatic Triple-Negative Breast Cancer: Implications for Clinical Practice and Trial Design. *Clinical Breast Cancer*, **2009**, *9*, 29-33.
- [133] Cadenas, C.; Franckenstein, D.; Schmidt, M.; et al. Role of thioredoxin reductase 1 and thioredoxin interacting protein in prognosis of breast cancer. *Breast Cancer Res.*, **2010**, *12*(3), R44.
- [134] Rackham, O.; Nichols, S.J.; Leedman, P.J.; et al. A gold(I) phosphine complex selectively induces apoptosis in breast cancer cells: implications for anticancer therapeutics targeted to mitochondria. *Biochem. Pharmacol.*, **2007**, *74*(7), 992-1002.
- [135] Gao, G.; Dou, Q.P. N-terminal cleavage of bax by calpain generates a potent proapoptotic 18-kDa fragment that promotes bcl-2-independent cytochrome C release and apoptotic cell death. *J. Cell. Biochem.*, **2000**, *80*(1), 53-72.
- [136] Bonaccorsi, L.; Marchiani, S.; Muratori, M. et al. Signaling mechanisms that mediate invasion in prostate cancer cells. *Ann. NY Acad. Sci.*, **2004**, *1028*, 283-288.
- [137] Liang, C.C.; Park, A.Y.; Guan, J.L. In vitro scratch assay: a convenient and inexpensive method for analysis of cell migration in vitro. *Nat. Protoc.*, **2007**, *2*(2), 329-333.