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METAL COMPLEXES AS POTENTIAL ANTICANCER AGENTS

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Ai miei genitori

Abstract

Metal-based antitumor drugs play a relevant role in antineoplastic chemotherapy. Cisplatin is regarded as one of the most effective drugs, even if severe toxicities and drug resistance phenomena limit its clinical use. Therefore, in recent years there has been a rapid expansion in research and development of novel metal-based anticancer drugs to improve clinical effectiveness, to reduce general toxicity and to broaden the spectrum of activity.

The variety of metal ion functions in biology has stimulated the development of new metallodrugs other than Pt drugs with the aim to obtain compounds acting via alternative mechanisms of action. This work has been dedicated to the investigation of the behaviour, at cellular, subcellular and molecular level of copper(I) and gold(I) phosphine complexes.

Concerning gold(I) complexes, we highlighted their ability of interacting with one of the most important system involved in redox cellular homeostasis, thus creating the pathophysiological conditions stimulating mitochondria to undergo the way of apoptosis. Acting as potent inhibitors of the selenoenzyme thioredoxin reductase, gold complexes determine an alteration of the redox state of the cell leading to an increased production of hydrogen peroxide and oxidation of the components of the thioredoxin system, therefore creating the conditions for augmented apoptosis.

Differently, phosphine copper complexes were found to activate an atypical programmed cell death pathway. Indeed, they inhibit tumor cell growth through paraptosis, an alternative non-apoptotic cell death mechanism characterized by a massive cytoplasmatic vacuolization and a consequent ER stress. This different way to induce a programmed cell death appeared particularly interesting as these copper complexes may be utilized in inducing cell death even in tumors resistant to apoptosis-inducing therapies.

Riassunto

I complessi metallici ricoprono un ruolo rilevante nella terapia anticancro. Il cisplatino è riconosciuto come uno dei farmaci più attivi, anche se sia la sua tossicità che lo sviluppo di resistenza limitano fortemente il suo impiego clinico. Negli ultimi anni, quindi, la ricerca si è indirizzata verso lo sviluppo di nuovi complessi metallici come potenziali agenti antitumorali, al fine di ottenere composti che presentino maggior efficacia e posseggano una minore tossicità ed un più ampio spettro d'azione.

La varietà di funzioni biologiche svolte dagli ioni metallici ha notevolmente stimolato in particolare lo sviluppo di complessi metallici contenenti metalli diversi dal platino, allo scopo di ottenere composti che agiscano attraverso un meccanismo d'azione diverso. In tale ambito, questo lavoro è stato dedicato allo studio gli effetti a livello cellulare, subcellulare e molecolare indotti da complessi fosfinici di oro(I) e rame(I).

Per quanto concerne i complessi fosfinici di oro(I), è stato messo in luce la loro capacità di interagire a livello il selenoenzima tioredossina reduttasi, creando condizioni fisiopatologiche in grado di stimolare la via mitocondriale dell'apoptosi in cellule tumorali.

I complessi di oro(I), agendo come potenti e selettivi inibitori della tioredossina reduttasi determinano una alterazione dello stato redox cellulare che a sua volta porta all'aumento della produzione cellulare di perossido di idrogeno oltre che all'ossidazione dei componenti del sistema tioredossinico. Questo sbilancio dell'omeostasi cellulare determina induzione della morte cellulare per apoptosi.

Diversamente, l'attività antitumorale indotta dai complessi di rame(I) è stata correlata alla loro capacità di indurre una morte cellulare programmata atipica. Infatti, essi inibiscono la crescita cellulare tumorale mediante induzione di paraptosi, un meccanismo di morte cellulare caratterizzato dalla massiccia formazione di vacuoli e da un conseguente stress del reticolo endoplasmatico.

L'induzione di morte cellulare mediante un meccanismo diverso da quello dell'apoptosi appare particolarmente interessante poiché questi complessi di rame(I) potrebbero essere impiegati nel trattamento di cellule tumorali che hanno sviluppato numerosi meccanismi al fine di eludere l'induzione di apoptosi.

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1. Metals in medicine

The use of metals in medical practice is certainly not in and of itself anything new. In fact, medicinal applications of metals can be traced back almost 5000 years and have been used since the early days of civilization [1]. Furthermore, there has always been a curious connection between the discovery of a new precious element and its quick movement into the medicinal armamentarium [2].

The medical use of gold can be traced back to 2500 BC in China. The ancient Egyptians used copper to sterilize water, and the Greek physician Hippocrates used mercury in 400 BC. In the seventeenth century Swiss alchemist and physician Paracelsus pioneered the use of minerals in medicine using antimony, arsenic and mercury salts, such as mercurous chloride, as a diuretic. An English contemporary, the herbalist Nicholas Culpepper, advocated a gold elixir *aurum potabile* for ailments caused by a decrease in the vital spirits including melancholy, fainting, fevers and falling sickness. The beginning of the 20th century saw metals making an impact on modern medicine with Ehrlich's discovery of the arsenic organometallic drug Salvarsan for the treatment of syphilis [1].

However, as a discipline, medicinal inorganic chemistry has only existed for about the last 35–40 years, and its development, stimulated by the serendipitous discovery of cisplatin, has been facilitated by the inorganic chemist's extensive knowledge of the coordination and redox properties of metal ions. Orvig and Abrams [1] define this discipline as "a field of medicinal chemistry that aim to elucidate the mechanism of medicinal action of the inorganic compound and to optimize and improve its physiological activity". So, on the whole, medicinal inorganic chemistry is a multidisciplinary field combining elements of chemistry (synthesis, reactivity), pharmacology (pharmacokinetics, toxicology), biochemistry (targets, structure, conformational changes), and medicinal chemistry (therapeutics, pharmacodynamics, structure-activity relationships (SAR)).

Ninety elements occur naturally on earth. Of these, 9 are radioactive, and the remaining 81 could support life; 61 are metals. In 1991, Peter Sadler noted that most elements of the periodic table, both essential and non-essential, natural and man-made elements, have potential uses as drugs or diagnostic agents [3]. In particular, inorganic or metal-containing medicinal compounds may contain either chemical elements essential to life forms or nonessential/toxic elements that carry out specific medicinal purposes.

Today, broad array of medicinal applications of metal complexes have been investigated and therapeutic applications of inorganic chemistry in medicine are varied, encompassing many

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aspects of the introduction of metal ions into the body (or their intentional removal), from therapeutic to diagnostic effects [4–8]. Some selected compounds that are currently used in clinical diagnosis and treatment are summarized in Table I.

Table I. Some Examples of Inorganic Elements and Compounds with Medicinal Purposes

Element	Example of a product name	Active compound in the product	Medical usage
Li	Camcolit	Li_2CO_3	Manic depression
Mg	Magnesia	MgO	Antacid, laxative
Fe		Fe(II) fumarate or succinate	Dietary iron supplement
Co	Cobaltamin S	Coenzyme vitamin B_{12}	Dietary vitaminsupplement
Zn	Calamine	ZnO Zn undecanoate	Skin ointment Antifungal
Tc	TechneScan PYP	$^{99\text{m}}\text{Tc}$ -pyrophosphate	Bone scanning
Sb	Triostam	NaSb(V) gluconate	Antileshmanial (antiprotozoal)
Ba	Baridol	BaSO_4	X-ray contrast medium
Gd	Magnevist TM	$[\text{Gd(III)(DTPA)(H}_2\text{O)}]^{2-}$ DTPA= diethylenetriamine pentaacetic acid	MRI contrast agent
Pt	Cisplatin, platinol Carboplatin	Cis- $[\text{Pt}(\text{NH}_3)_2\text{Cl}_2]$ $[\text{Pt}(\text{NH}_3)_2(\text{CBDCA})]$ CBDCA= cyclobutanedicarboxylic acid	Anticancer agent Anticancer agent
Au	Auranofin	$\text{Au(I)(Pet}_3\text{)(acethylthioglucose)}$	Antiartthritic
Bi	De-Nol	$\text{K}_3[\text{Bi(III)(citrate)}_2]$	Antacid, antiulcer

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Inorganic compounds have found usage in chemotherapeutic agents such as anticancer agents (cis-[Pt(NH₃)₂Cl₂]), antiarthritic (Auranofin), metal-mediated antibiotics (bleomycin, which requires iron or other metals for activity), short-lived isotopes used as radiopharmaceuticals (Technetium-99m and others) in disease diagnosis and treatment, magnetic resonance imaging (MRI)-enhancing compounds (gadolinium) antibacterials, antivirals, antiparasitics, and radiosensitizing agents.

Despite almost all drugs placed in the market are organic non metal substances, medicinal chemistry is taking a growing interest in the development of metal complexes for the use as drugs or diagnostic agents. Based on their wide spectrum of coordination numbers and geometries as well as kinetic properties, metal compounds offer mechanisms of action that can not be realised by organic agents. Metals can coordinate ligands in a precise three-dimensional configuration thus allowing the tailoring of the molecule to recognize and interact with a defined molecular target. This is further enhanced by different chemical modifications of ligands. In fact, it should be recognised that both metal ion and its coordinated ligands contribute to metal complex induced biological activity. Despite the importance of ligands in modifying the biological effects of metal-based drugs cannot be overestimated, ligands can modify the oral/systemic bioavailability of metal ions, can assist in targeting specifically either a particular tissue or an enzyme; can deliver, protect, or sequester a particular metal ion, depending on the requirements, for therapy or diagnosis. Ligands are capable of ensure protection of tissues from toxic metal ions or, differently, enhance uptake of pharmacologically beneficial metal ions [9]. Addition of substituents that either bind preferentially to a definite receptor or mimic naturally-occurring hormones or humores, assist in rendering ligands with designed target specificity, to yield far-reaching diagnostic and therapeutic implications [10]. Transition metals, on the other hand, possess different oxidation states, which not only allow modification of the three-dimensional space into which the molecule can fit, but significantly allow them to participate in biological redox chemistry [11]. Metal ion oxidation state may be decisive in regulating the instantaneous *in vivo* response to metal-based pharmaceutical agents, frequently making the difference between a beneficial and a toxic response at the same administered dose, and as well in directing towards the metabolic pathways by which the compound will be integrated. The oxidation state of the metal ion also determines particular coordination geometries, hence limiting appropriate binding for different ligand sets.

In addition, the ability to undergo ligand exchange reactions offers opportunities for metals to interact and coordinate with biological molecules. While for diagnostic agents the whole molecule

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have to remain intact, from administration through to excretion, differently, concerning metal-based therapeutics, they would not even function if they remained intact indefinitely.

Taking together, these considerations allow to conclude that by optimizing the choice of ligands and the oxidation state, investigators can build in control of kinetic and thermodynamic properties of metallo-pharmaceuticals for specific therapeutic needs.

The pharmaceutical use of metal complexes therefore has an excellent potential. Developing metal complexes as drugs, however, is not an easy task. Body metal ion accumulation could lead to deleterious effects. Thus, biodistribution and clearance of the metal complex as well as its pharmacological specificity have to be considered. Physiological responses of the investigated drugs need to be demonstrated by *in vitro* study with targeted biomolecules and tissues as well as *in vivo* investigation with xenografts and animal models before they enter clinical trials. A mechanistic understanding of how metal complexes achieve their activities is crucial for clinical success and may contribute to the rational design of new compounds with improved potency.

1.1 Platinum-Containing Anticancer Agents

1.1.1 Cis-Dichlorodiammineplatinum(II)

Thousands of research papers have made platinum antitumor compounds the most studied inorganic drugs and probably the most studied drugs of any type. Much research attention has been paid to platinum complexes as potential anticancer drugs because of the success of cisplatin, cis-dichlorodiammineplatinum(II) (Fig. 1).

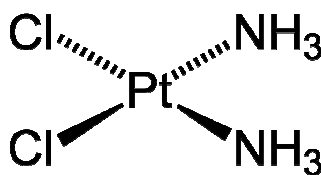


Fig. 1. Cisplatin

Cisplatin was first synthesized by an Italian doctor, Michele Peyrone in 1844 and has been called Peyrone's chloride [12]. Its structure was first elucidated in 1893 by Alfred Werner who correctly assigned to this compound a *cis*- geometry distinguishing it from its *trans*- isomer [13], even if its structure was eventually unambiguously confirmed in an X-ray crystal structure determination reported in 1966 [14].

In the middle 1960s, Barnett Rosenberg and his co-workers serendipitously discovered that cisplatin exhibited antitumor activity [15]. Rosenberg, a biophysicist which had been studying the growth of bacterial cells in solution in the presence of electrical fields, observed the elongation of the *Escherichia coli* cells, a phenomenon known as filamentous growth, rather than cell division. He discovered that the apparently inert platinum electrodes were able to directly react with ammonium and chloride ions in the nutrient broth in the electrical cell forming a number of platinum(II) and platinum(IV) compounds. These platinum compounds, later identify as *cis*-[Pt(NH₃)₂Cl₂] and *cis*-[Pt(NH₃)₂Cl₄] in particular, were found to cause filamentous growth in cells, despite being absent any electrical current. Faced with his discoveries, Rosenberg made a spectacular "leap of logic" postulating that platinum compounds causing filamentous growth rather than cell division could be possess antitumor activity. His reasoning was based on the well-known fact that one most accepted attribute of an ideal antitumor agent is to inhibit rampant and uncontrolled cell division characteristic of malignant tumor. Lately, his hypothesis was proven

correct and in 1972 cisplatin entered clinical trials leading, in 1979, to its approval by the U.S. Food and Drug Administration for treatment of genitourinary tumors [16]. Cisplatin, at the current time, is one of most widely used anticancer drugs. It is effective in treating a variety of cancers and the drug main targets are testicular, ovarian, bladder, and head and neck tumors [17-21]. Noticeably, it represent the first line chemotherapeutic agent for treatment of testicular cancer, for which it has a greater than 90% cure rate.

1.1.2 Mechanism of cisplatin antitumor activity

Cisplatin is administered to patients intravenously as a sterile saline solution. In the bloodstream, cisplatin retains its chloride ions because Cl^- concentrations are high (~ 0.1 M). Before any reaction can take place between cisplatin and DNA, the platinum drug must enter the cell. Cisplatin enters cells by passive diffusion and also, as recently discovered, by active transport mediated by different proteins [22]. Evidence for a role of active transporters in the uptake and efflux of cisplatin and other platinum compounds has been available since many years. Multiple studies demonstrated that reactive aldehydes inhibit cisplatin accumulation in cells, possibly by modifying membrane proteins [22, 23]. Recently, a series of experiments have indicated a direct link between copper transporters and the uptake/efflux of platinum compounds [24]. In particular, several studies considering ATP7B as well as ATP7A, two of the most important copper chaperone proteins, suggested that these proteins modulate cisplatin levels in cells, presumably by provoking drug efflux [25-28]. Furthermore, studies on embryonic fibroblast cells, confirmed that also copper transporter hCtr1 mediates platinum drugs uptake. In addition to copper transporters, the facilitated delivery of platinum compounds into cells has also been associated with the presence of organic cation transporters, or OCTs [22]. It has become thereby clear that platinum drugs enter the cell through multiple routes including both passive and active mechanisms. In addition, each of these cell-entry pathways is likely to influence differently the cellular accumulation of a platinum compound, depending on its chemical composition and structure. Additional work is needed to understand fully how the cellular levels of platinum are managed by passive diffusion, copper homeostasis proteins, OCTs, and other as yet unidentified transporters.

Inside the cell, chloride concentration is much lower (~ 0.004 M) and equilibria are established for the hydrolysis reactions shown in Fig. 2. Because the chloride ion is a better leaving group than the ammonia ligand, which forms a more thermodynamically stable covalent bond with metal ions, the ammine ligands remain while chloride ions are displaced by water. This aqueous chemistry for

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platinum(II) compounds is more complex than which shown in Fig. 2 as different OH⁻ as well as H₂O ligand combinations are possible at physiological pH.

Once the cisplatin has entered the cell and aquation has occurred, reactions with cellular components will take place. Reactive cellular components include proteins, RNA, DNA, membrane phospholipids, microfilaments, and thiol-containing molecules such as glutathione. The detailed nature of these interactions is not known because of the complexity of the intracellular milieu and the lack of methodologies to investigate these processes [16].

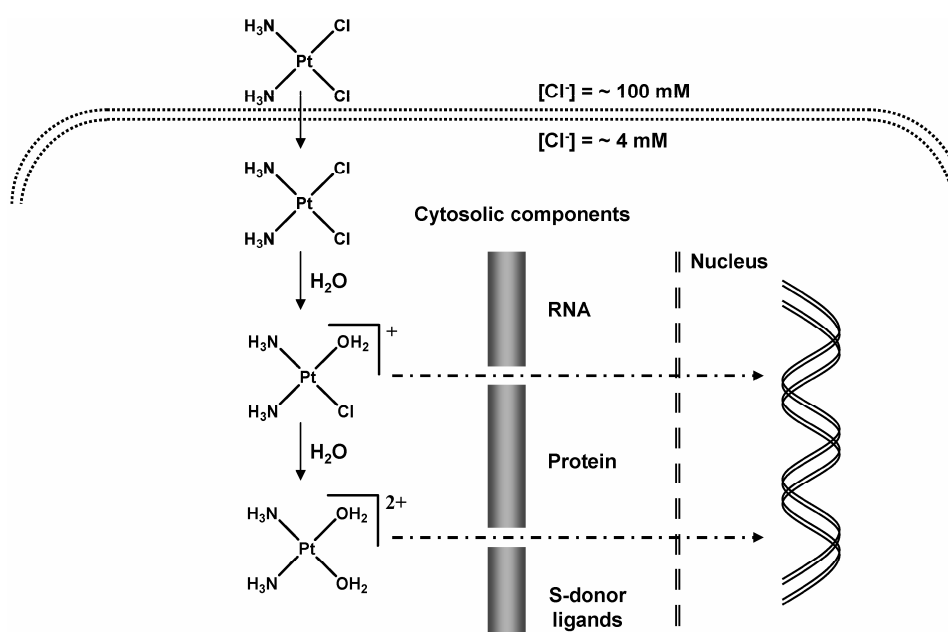


Fig. 2 .Cisplatin aquation reactions and interaction with intracellular targets

The effects of cisplatin on mitochondrial DNA are not well understood, but it is possible that this damage, resulting from cisplatin treatment, contributes to cell death. The interaction of cisplatin with sulphur-containing enzymes is better understood and is believed to be involved in promoting cell resistance toward cisplatin. The effects of cisplatin on RNA and DNA have been extensively studied. Although cisplatin can coordinate to RNA, this interaction is not believed to play an important role in cisplatin physiological mechanism of action. First, a single damaged RNA molecule can be replaced by newly synthesized material; studies have revealed that cisplatin does not affect RNA synthesis. Second, when cisplatin was administered *in vitro* at its lethal dose to a strain of cancer cells, only a small fraction (1 to 10%) of RNA molecules were damaged [16].

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The evidence that DNA is the primary target of cisplatin among many potential cellular possibilities has been extensively discussed. Convincing proof came from experiments with DNA repair-deficient cells, which are universally more sensitive to cisplatin. Moreover, a significant correlation is usually found between platinum-DNA adduct levels and the sensitivity of treated cells to the drug. In addition, the levels of platinum atoms bound to proteins and RNA are too low to exhibit significant inhibitory effects on the targets. Taking together, many studies confirm that cellular DNA is the cisplatin preferred target of the drug [21].

As seen in Fig. 3, cisplatin hydrolyzed form reacts with DNA and water ligands, hence, are replaced by nucleobase ligands from DNA strands and once formed, these nucleobase nitrogen attachments are thermodynamically stable. The preferred point of attachment of platinum(II) along DNA strands has been found to be at the guanine N7 position of the ring because it does not establish hydrogen bonds with any other DNA base and these nitrogen atoms are located in the DNA major groove thus being more easily exposed to platination [29].

The most likely sequence of events is the hydrolysis of one chloride ligand followed by reaction with a dsDNA guanine ligand, hydrolysis of the second chloride ion, and attachment of a second dsDNA guanine (reaction sequence (1), (2), (3) in Fig. 3).

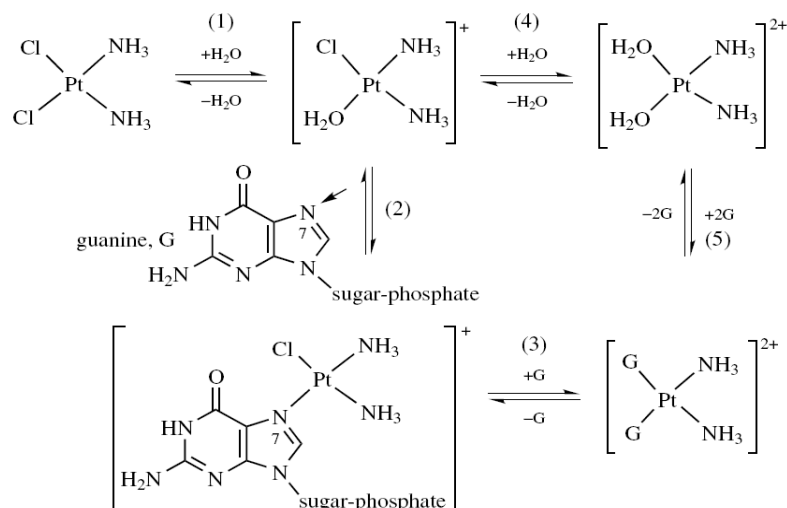


Fig. 3. Physiological behaviour of cisplatin *in vivo*. Aquation reactions followed by guanine N7 ligand attachment.

The reaction between cisplatin and DNA results in the formation of different kinds of adducts (Fig. 4). It is now known that 65% of DNA-cisplatin cross-links are intrastrand 1,2-d(GpG) cross-links with lesser contributions from 1,2-d(ApG) (20%), 1,3-d(GpXpG) (where X is any nucleobase, 9%),

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interstrand GG adducts and DNA-protein cross-links [30]. Moreover, thioether sulphur donors such as methionine or perhaps oxidized glutathione may be additional intermediates in hydrolysis reactions before the thermodynamic end product with nucleobases is reached. Monofunctional adducts are rapidly produced by both *cis*- and *trans*-platin, but these adducts do not show any biological activity, as they are labile, they determine only slight structural modifications on DNA and do not inhibit DNA synthesis [31, 32, 33].

In the formation of the bis-functional platinum(II)-DNA it is determinant the *cis*- or *trans*- geometry of the platinum(II) complex, as reactions with DNA occur with retention of stereochemistry [19]. Due to its geometry, transplatin cannot form d(GpG) intrastrand adducts with DNA. Since transplatin is inactive in killing cancer cells, it is believed that these intrastrand adducts formed between cisplatin and DNA are important for the anticancer activity of cisplatin itself [34, 35, 36].

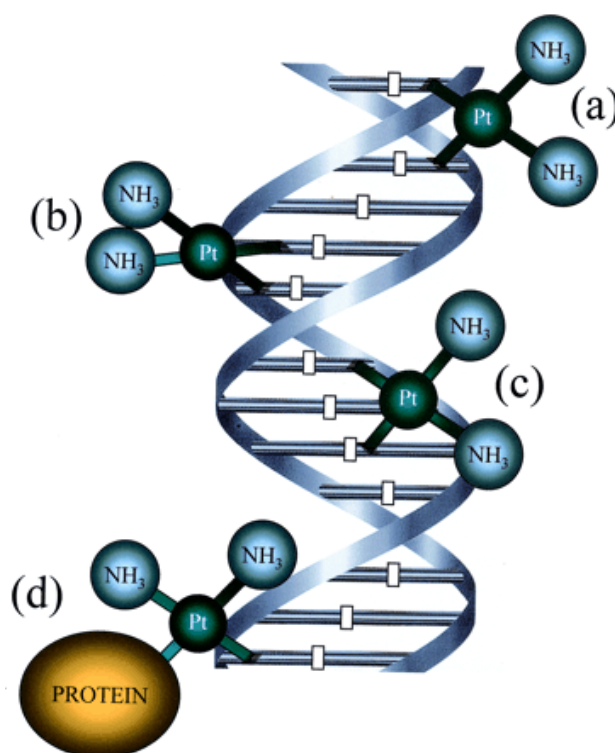


Fig. 4 Main adducts formed in the interaction of cisplatin with DNA. (a), interstrand cross-link. (b), 1,2-intrastrand cross-link. (c), 1,3-intrastrand cross-link. (d), protein-DNA cross-link.

The binding of cisplatin to DNA leads to significant distortion of DNA helical structure [37]. When cisplatin binds to DNA forming bifunctional adducts, it promotes superhelical unwinding and shortens the double helix [38]; the distortions induced in the DNA double helix by inter and

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intrastrand cross-links have been characterized by several techniques, as X-ray and 2D-NMR. It has been shown that in the interstrand d(GpG) cross-links, the platinum residue is positioned in the minor groove, and the axis of the double helix is bent of about 45° towards the minor groove, with a large unwinding of about 75° [39, 40]. On the other hand, the formation of intrastrand d(GpG) cross-links causes the purines to become destacked and the DNA helix to become kinked with a bending of 26-35° towards the major groove and the shorter unwinding of the double helix (~13°) [41]. This 'local distortion' leads to an impairment in the processing of DNA in tumor cells. The distorted, platinated DNA structure serves as a recognition binding site for cellular proteins [42]. Several types of proteins can interact with cisplatin-damage-DNA: NER (nucleotide excision repair), TFs (transcription factors), MMR (mismatch repair), p53 tumor suppressor gene (nuclear protein that exerts its effects through transcriptional regulation maintaining the genomic stability) and HMG (high mobility group) proteins [21].

Linked or bent DNA is recognized by high mobility group (HMG) proteins which, upon binding to the cisplatin cross-link, significantly increase the bending of the helix. The HMG-domain proteins constitute the largest and most extensively characterized group of non-histone chromosomal proteins and may contain multiple HMG domains which specifically recognize DNA structures or sequences [43, 44]. HMG protein binds to the DNA-cisplatin adducts in a 1:1 ratio and protects them from DNA repair enzymes. Thus, the normal excision of the major cisplatin-DNA adducts occurring primarily by the NER system is aborted [45]. Normally, DNA lesion is excised as a small, single-strand oligonucleoside fragment, and new DNA is synthesized to fill the resulting gap [46]. In this repair system, before the damaged portion of DNA is excised from the rest of the strand, it must be recognized by the cell, especially through HMG proteins. When HMG-domain proteins bind to the cisplatin-DNA adducts, the adducts cannot be recognized by the repair machinery. The cisplatin-DNA adducts are then be more persistent than they would in the absence of HMG-domain proteins, and DNA repair is definitely slower. This could interfere with the normal functions of the cell (among them, replication and transcription) and possibly trigger cell death [47]. So it is not simply the distortion of DNA that disrupts the function of the DNA, but also the ability of the platinated-DNA function to attract HMGs. Binding of the HMG-domain proteins to cisplatin-DNA lesions, thus, mediate the antitumor activity of the drug [47]. The anticancer efficacy of cisplatin is also influenced by the efficiency of cisplatin-DNA adduct removal by the human mismatch-repair proteins, hMSH. These proteins binds specifically to DNA containing cisplatin adducts and displays selectivity for the DNA adducts of therapeutically active platinum

complexes. In particular, the human mismatch binding factor hMSH2/hMSH6 seems to selectively bind the 1,2-GpG cisplatin adduct [48].

As mentioned before, various adducts are formed upon interaction of cisplatin with nucleotides, but contribution of individual adducts to antitumor activity and toxicity of platinum complexes still remains to be examined. Even now, it is not known which adduct is primarily responsible for the cytotoxicity (antitumor activity) of cisplatin; furthermore, other platinum compounds may induce completely different DNA lesions retaining a significant cytotoxic potency [49]. It appears that these differences in DNA lesion type depending on compound stereochemistry are critically important in determining the efficacy of a particular compound for the treatment of cancer. For this reason, a great deal of efforts has been placed on discovering the specific cellular proteins that recognize cisplatin-DNA complexes and then examining how the interaction of these proteins with the complexes might lead to programmed cell death of cancer cells [21, 49].

DNA damage caused by cisplatin modulates several apoptotic signal transduction pathways (Fig. 5):

- **c-ABL**: c-ABL is a member of the SRC family of non-receptor tyrosine kinases, which possess a large C-terminal region that contains nuclear localization motifs and nuclear export sequences. c-ABL tyrosine kinase activity can be stimulated by cisplatin and other DNA-damaging agents and acts to transmit DNA-damage signals from the nucleus to the cytoplasm [50]. c-ABL tyrosine kinase has a role in the activation of apoptosis that is induced by cisplatin and the transcription factors p73 and p53 seem to be possible downstream effectors of c-ABL pathway. c-ABL is also an upstream effector of MAPK/ERK kinase kinase 1 (MEKK1) and the stress-activated kinases JNK and p38 MAPK.
- **MAPK/JNK/ERK**. Cisplatin triggers the activation of the ERK, JNK and p38 MAPK cascades in tumour cells or transformed cell lines. These are the three major subfamilies of mammalian MAPK. A growing body of evidence indicates that activation of the p38 MAPK pathway has an important role in the cellular response to cisplatin [51]. This kinase regulates immediate-early gene expression and other cellular responses through phosphorylation of various substrates, including transcription factors, chromatin protein constituents and downstream serine/threonine effector kinases.
- **Cell-cycle checkpoints and arrest**: Interestingly, cisplatin does not cause in all cases G1 arrest through G1/S checkpoint, able to ensures that damaged DNA is not replicated.

Conversely, it has been proposed that G2 arrest is essential to the process of engaging cell death following cisplatin treatment [52].

- **DNA-repair.** Either NER and MMR pathways activate other cell-cycle regulators, such as p53, p73 and c-ABL, which mediate the induction of cell-cycle arrest and apoptosis [53]
- **Caspases:** Apoptosis results also from activation of the caspase family (aspartate-specific proteases): upstream initiator caspases, such as caspase-8 and caspase-9, are activated early in the apoptotic process and they then activate other downstream caspases, such as caspase-3, largely responsible for the cleavage of many other cellular proteins responsible for apoptosis induction [54].

However, cisplatin induces cell death both through necrosis and apoptosis and the activation of molecular determinants addressing cells preferentially to one of these different death modes seems to be affected by drug concentration [55]. Indeed, excessive DNA damage induces hyperactivation of poly(ADP-ribose)polymerase (PARP). PARP cleaves NAD⁺ and transfers ADP-ribose moieties (ADPR) to carboxyl groups of nuclear proteins. It thereby causes NAD⁺/ATP depletion, resulting in necrotic cell death if ATP depletion reaches lethal-inducing levels.

Although research has focused on cisplatin ability to modify genomic DNA (gDNA) in the cell nucleus, mitochondrial DNA (mtDNA) may also be a possible alternative target. The mtDNA-cisplatin adducts have been found to be persistent perhaps because mitochondria do not perform nucleotide excision repair [56], but it is not clear if these lesions may contribute to cisplatin anticancer activity and, in particular, for activating intrinsic mitochondrial apoptotic cell death pathway.

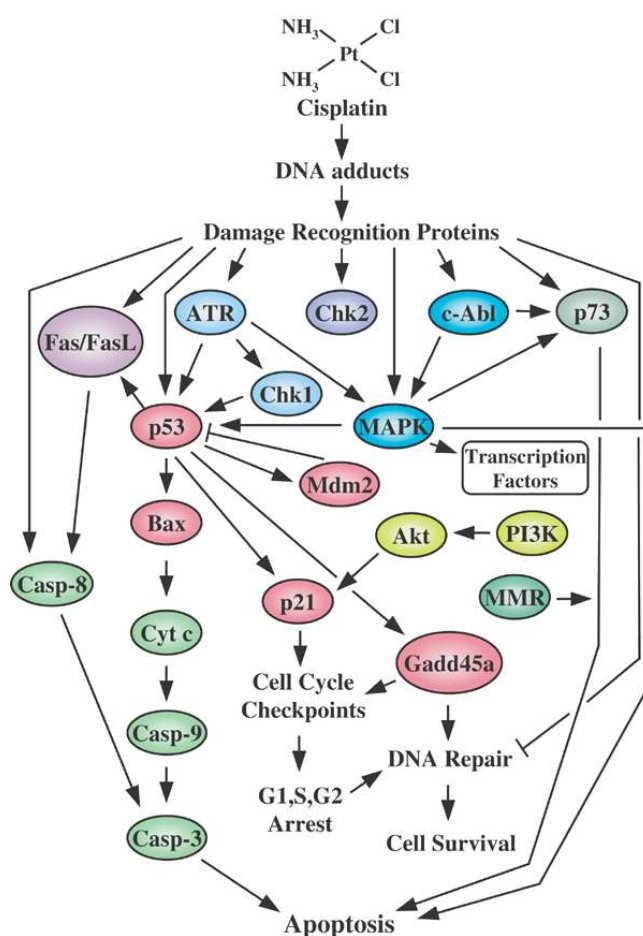


Fig. 5. Simplify overview of pathways involved in mediating cisplatin-induced cellular effects.

1.1.3. Drug Resistance

Drug resistance is a major complication in cancer chemotherapy. Innate resistance of malignancies (intrinsic resistance) or refractoriness upon subsequent treatment (acquired resistance) limits cisplatin curative potential [56]. Cellular resistance to platinum anticancer drugs is multifactorial and consists of mechanisms that limit the formation of lethal platinum-DNA adducts, alter or reduce drug transport, and inactivate mechanisms that enable a cell to repair or tolerate platinum-DNA damage once it occurs. These mechanisms, which may take place through the overexpression or inactivation of certain genes, have been identified mainly through the use of cell lines *in vitro* selected for their cisplatin resistance. Such model systems may exhibit high levels of resistance, thus facilitating the identification of molecular determinants involved in resistance and providing a

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system to study potential compounds that would increase the sensitivity of cancer cells to platinum drugs. The cellular mechanisms contributing to cisplatin resistance, which have been extensively studied, consisted of change in:

- **Membrane permeability and drug influx/efflux.** One of the major mechanisms of cisplatin resistance is represented by decreased effective concentration of drug in the cell. Reduction in cisplatin concentration of 20–70% has been observed in cell lines resistant to cisplatin and can be attributable either to decreased influx or increased efflux of drug from cells [57, 58]. It has long been supposed that cisplatin and carboplatin cellular uptake occur passively by the cell, as accumulation is not saturable, nor is it inhibited by structural analogues [56]. However, ouabain, an inhibitor of membrane sodium/potassium ATPase pumps, has been shown to block cisplatin import, thus indicating that cisplatin uptake may be dependent on the cellular membrane potential [59]. Recent studies evidenced that cisplatin may enter cells also by an active transport mediated by the copper transporter Ctr1 and various human cisplatin-resistant cancer cells have been found to downregulate protein expression of Ctr1. Besides most of cisplatin-resistant phenotypes present less than half of Ctr1 protein than its sensitive counterpart, experiment with hCtr1-protein transfected cells indicated that Ctr1 expression alone is not enough to revert cisplatin resistance [60]. Two other copper transporters have also been implicated in resistance to cisplatin, ATP7A and ATP7B. They have been shown to take a possible role in cisplatin export from cells. ATP7B was first proposed to be involved in cisplatin resistance when Komatsu *et al.*, overexpressed this transporter in a human epidermoid carcinoma cell line and observed that these cells increased cisplatin resistance [61]. Cisplatin uptake in ATP7B-transfected cells was approximately 60% than that observed in cells transfected with empty vector. Following studies underlined that among a series of human ovarian cell lines, SKOV3 cells, which exhibited the highest level of ATP7B expression, also showed the highest resistance to cisplatin [62]. Furthermore, additionally, 82 primary ovarian carcinomas were profiled for expression of several known resistance genes (including ATP7B, MDR1, MRP1, MRP2, LRP, and BCRP) and with the exception of ATP7B, none were indicators for resistance of ovarian cancer to cisplatin [63]. Increased expression of ATP7A in the 2008 line (2008/MNK) led to increased resistance to cisplatin; ATP7A overexpression led to increased sequestration of platinating agents and not to decreased total accumulation, as ATP7A seemed able to sequester cisplatin into vesicles [64]. Interestingly, ATP7A resulted overexpressed in some cisplatin-resistant ovarian carcinoma cell lines [65] and in particular, in two cell lines,

A2780/CisPt and C13*, overexpression of ATP7A was shown to be causative for resistance to cisplatin.

In addition to the copper transporters, the multi-drug resistance protein (MRP) is thought to function as an ATP-dependent pump for many drugs, including cisplatin [66]. In rat hepatocyte cell lines treated with cisplatin, MRP-2 protein amount and mRNA levels increased approximately 3-fold upon treatment with cisplatin [67]. Cisplatin also slightly increased the mRNA expression of MRP3, but did not affect MRP1 expression [67]. Furthermore, considering some sensitive and resistant human melanoma cell lines, MRP2 expression was shown to be upregulated in the cisplatin-resistant phenotypes as compared to the sensitive parental sublines [68]. However, additional studies on liver carcinoma cell lines showed conflicting results. So, despite MRP expression may play a role in cisplatin-resistance in some tissue types, it does not appear to be a global mechanism by which resistance to cisplatin occurs.

- **Detoxification pathways.** Resistance to cisplatin because of increased inactivation by intracellular proteins has also been reported [58]. In the cytoplasm, platinating agents can react with thiol-containing molecules, including glutathione (GSH) and metallothioneins (MT). These molecules should be good ligands for platinum compounds because they contain many soft sulphur ligands attractive to the soft Pt metal centre. Increased concentrations of these compounds are known to intervene in cisplatin resistance [57]. Glutathione, acting as an antioxidant, contributes to preserve the cellular redox homeostasis. Glutathione is also involved in platinum cellular detoxification. Reaction between cisplatin and GSH forms a 2:1 (GSH:Pt) complex which may therefore inactivate the drug and take part of cellular resistance mechanism [69]. Moreover, there is an over-expression of the pump for glutathione conjugates in cisplatin resistant cells, suggesting that Pt-GSH complexes are pumped out of the cell [70]. Several ovarian cancer cell lines known to be resistant to cisplatin showed a correlation between the degree of resistance and the levels of GSH, likely due to increased γ -glutamylcysteine synthetase [71]. In bladder carcinoma cell lines, endowed with a significant intrinsic cisplatin resistance, exposure to buthionine sulfoximine (BSO), which significantly depleted cellular glutathione concentration, resulted in a significant enhancement of cisplatin cytotoxicity [72]. However, this treatment does not sensitizes these cells to the level of their parent sensitive phenotypes, indicating that glutathione level is only one component of cisplatin resistance [72].

MT are low molecular mass cysteine-rich proteins and are believed to be involved in protecting cells from oxidative stress and toxicities associated with heavy metals [73]. Production of MT is triggered by the presence of heavy metal ions, glucocorticoids, interferon and stress and also cisplatin administration can lead to MT expression induction, particularly in head and neck cancers. MT bind to cisplatin in a protein : drug ratio of 1 : 10 leading to displacement of the amino ligands and giving rise to [PtS₄] cluster containing 7-10 platinum atoms per mole [74], thus affecting cellular sensitivity to the drug. Elevated levels of MT have been described in some cisplatin-resistant cell lines confirming that MT may contribute to cisplatin resistance. However, the results are inconclusive as in some cases the MT levels are unaffected in resistant phenotypes [75].

An additional member of the anti-oxidant defence system is thioredoxin (Trx), which contribute to the cellular redox environment maintenance [76]. Recently, in clinical samples, a strict correlation has been observed between Trx levels and cisplatin resistance in bladder, prostate, liver, gastric, and colon cancer cells [76].

- **Ability to remove cytotoxic lesions from DNA.** Cells can become resistant to cisplatin because they are endowed with an enhanced ability to remove cisplatin-DNA adducts and to repair cisplatin-induced lesions in DNA. Such an ability might result from the presence of certain DNA repair proteins. Cell lines selected for resistance to cisplatin after prolonged culture in the presence of cisplatin have significantly higher levels of repair than the corresponding parental cell lines [77, 78, 79], indicating that DNA repair is an important determinant of cisplatin resistance. Moreover, differential capacity to repair cisplatin adducts is postulated to be responsible for part of the variability in clinical response to cisplatin-based chemotherapy [80]. NER is the major pathway for platinum adduct removal and repair of DNA damage. Although the NER complex is composed of at least 17 different proteins, it appears that upregulation of only a few rate-limiting proteins is necessary to increase the excision repair capacity in resistant tumor cells [81]. For instance, cisplatin resistance is associated with increases in the excision repair crosscomplementing ERCC1 or ERCC1/XPF complexes, but not ERCC3 [75]. Similarly, both the gene encoding for ERCC1 and the NER-related XPA gene were found overexpressed in cisplatin-resistant cells compare with sensitive phenotypes [75]. Conversely, mammalian cells deficient in NER are generally more sensitive to cisplatin than the corresponding wild type cells [82, 83]. Complementary studies with mismatch repair deficient cells have shown that inactivation of MMR genes confers resistance to cisplatin. Indeed,

cisplatin resistant cell lines isolated after exposure to the drug *in vitro* frequently acquire the mutator phenotype associated with mismatch repair defects and are usually mutated in the *hMLH1* genes [84, 85, 86].

Tumors arising in testicular tissues are the tumors most successfully treated with cisplatin. Significantly, in testicular tissues MMR proteins are expressed in high levels while NER in low levels. Specifically, hMSH2 protein levels are high in testicular and ovarian tissues [87]. A screening of ovarian tumor samples prior to and after platinum-based chemotherapy reveals a significant increase in the proportion of tumors deficient in hMLH1 expression after treatment [88]. These studies indicate that exposure of cells to cisplatin can result in loss of MMR.

Besides to ensure genomic stability it is vital that repair of DNA occurs prior to DNA replication, most of resistant cells possess enhance capacity to replicate DNA past the adduct, and then initiate postreplication repair [89]. This in essence increases the ability of tumor cells to tolerate high levels of DNA adducts induced by cisplatin. Replicative bypass is usually increased in cells defective of MMR system. However, increased replicative bypass may also occur independently of MMR [90].

Another example of a DNA repair protein that has been shown to repair cisplatin lesions is a nuclear protein called XPE-BF (Xeroderma Pigmentosum sometime group E Binding Factor). Levels of XPE-BF were found to increase early in the development of cisplatin resistance. As with other mechanisms of cisplatin resistance, it appears that DNA repair is one of the several possible mechanisms [56]. On the other hand, studies showing an increase in levels of DNA repair proteins as tumor cells become less sensitive, and therefore more resistant, to treatment with cisplatin have suggested that DNA repair seems to be the mechanism activated first in cisplatin resistance.

- **Oncogene and protein kinase signal transduction pathways.** Alterations in the expression of oncogenes (such as *c-fos*, *c-myc*, *H-ras*, *c-jun*, and *c-abl*) have also been implicated in the cellular resistance to cisplatin. The expression of *c-fos* is higher in cisplatin resistant cells and in patients that have failed cisplatin-based chemotherapy and downregulation of *c-fos* expression by antisense DNA or by cleavage with a *fos* ribozyme (catalytic RNA that cleaves *c-fos* mRNA) reverses cisplatin resistance [91]. Moreover, cisplatin administration leads to *c-fos* induction and *c-Fos* modulates the expression of genes that have the AP-1 (Fos/Jun complex) binding domain, such as *c-myc*, metallothionein, and DNA polymerase β . Some cisplatin resistant cells express higher levels of the *c-Myc* protein and downregulation of *c-myc* by antisense DNA

increases their sensitivity to cisplatin [91]. It is noteworthy that tumors for which cisplatin is particularly effective, testicular and ovarian carcinomas, have a low frequency of mutated *ras* alleles, whereas cisplatin resistant tumors such as colon and renal cancers have a higher frequency of mutated *ras* alleles [92]. These results suggest that expression of *ras* might be involved in cisplatin resistance. H-Ras can modulate MT and DNA polymerase β expression levels and induction of *ras* expression results in resistance, decreased intracellular accumulation of cisplatin and increased MT content. However, no changes in cisplatin sensitivity were found following transfection of H-ras in two of the cisplatin sensitive cell lines and there is little correlation between total Ras protein expressed in the cells and resistance to cisplatin [93]. In summary, it appears that mutant *ras* expression is not a major determinant of cisplatin resistance. The *c-abl* gene encodes a non-receptor tyrosine kinase that can be activated in response to cisplatin damage. Cells deficient in *c-abl* are more resistant to cisplatin than wild type cells, suggesting that c-Abl may promote cisplatin induced apoptosis [94]. However, the mechanism by which DNA damage activates c-Abl is not completely understood and seems to involve the DNA-dependent protein kinase and the ataxia telangiectasia mutated (ATM) gene product [95]. Cisplatin treatment activates JNK kinase activity and induces *c-jun* expression [96]. Higher levels of c-Jun are expressed in cisplatin resistant cells and induction of *c-jun* mRNA by cisplatin is significantly lower in cisplatin resistant cells [96].

- **Gene expression.** The *p53* gene is a tumor suppressor gene encoding for a nuclear phosphoprotein that mediates cellular response towards genotoxic stress, including cisplatin treatment [93]. Activation of the normal *p53* gene due to DNA damage or other cellular stress can result in arrested cell cycles and apoptosis, helping to maintain gene stability. Specifically, *p53* activates cell cycle arrest at G1 or G2/M phases in response to DNA damage, thereby extending the time available to repair the damage. *p53* can also play a direct role in repair of the damage or it can initiate an apoptotic response to DNA damage. Consequently, a defect in *p53* can have pleiotropic effects in the cellular sensitivity to cisplatin. There is a contradictory character to the literature on the association of *p53* with resistance or susceptibility of tumor cell lines to cisplatin. A comparison on nine ovarian cancer cell lines and six testicular cancer cell lines indicated that *p53* is not a determinant of cisplatin induced toxicity [97, 98, 99]. A comparison of the sensitivity to cisplatin and *p53* status on eight breast and kidney cancer cell lines as well as on six central nervous system and leukaemia cancer cells indicated, taken

together all results, the mutant *p53* cell lines are only slightly more resistant to cisplatin than the wild type *p53* cell lines [100].

1.1.4 New Platinum Antitumor Agents

Despite possess high efficacy, cisplatin has several limitations. The major dose limiting toxicity of cisplatin is cumulative nephrotoxicity, which it has been correlated to platinum binding and inactivation of kidney thiol-containing enzymes which can lead to oxidative stress and subsequent cell injury [21]. Other considerable side effects are myelosuppression, peripheral neuropathy (may be due to hypomagnesaemia, hypocalcaemia, hypokalaemia, and hypophosphataemia), anemia, hepatotoxicity, irreversible ototoxicity and from moderate to severe nausea and vomiting. Furthermore, cisplatin has reduced applicability to few tumor types, has to be given as a large volume intravenous infusion and it is not orally bioavailable, as well as, as mentioned above, many cancer cells possess intrinsic or acquired resistance.

Hence, much attention has been focused on designing new less toxic platinum compounds with improved pharmacological properties and a broader range of antitumor activity. Thousands of platinum compounds have been tested for antitumor activity but less than 30 of these have entered clinical trials. The new drug should present some advantages in comparison with cisplatin: (1) the drug may be less toxic to the body while maintaining the same cytotoxicity; (2) the drug should have a wider or different spectrum of activity in treating malignancies; (3) the drug must overcome cisplatin drug resistance and be effective in tumors that have become refractory and (4) the drugs should have an administrative method other than the intravenous delivery now practiced. Advantage in the efficacy and toxicity profiles were obtained with second-generation analogs. Carboplatin (Fig. 6) $[\text{Pt}(\text{NH}_3)_2(\text{CBDCA})]$ (CBDCA: cyclobutane-1,1-dicarboxylate ligand), has been introduced into clinical usage and, being its activity spectrum rather similar to that of cisplatin, has replaced it in many drug protocols for treating ovarian cancers.

It is currently the second most widely used platinum anticancer drug in the world [101]. Despite maintaining the same spectrum of treatment of cisplatin, carboplatin is much less toxic to the kidneys and can be given in much higher dosages. Its lower toxicity is related to the slower intracellular hydrolysis undergo by the bidentate CBDCA ligand compared to the monodentate chloro ligands of cisplatin.

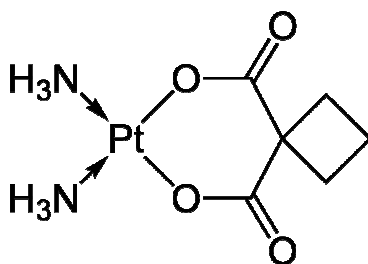


Fig. 6. Carboplatin

The mechanism of action of carboplatin is very similar to that of cisplatin, forming preferential cross-links with guanine in DNA, thus eventually causing cell death. Therefore, there is cross resistance with cisplatin.[102].

A different activity spectrum, instead, is shown by oxaliplatin (Fig. 7), the L isomer of [oxalato(trans-1,2-diaminocyclohexane)platinum (II)], ([Pt(II)(oxalato)(DACH)]).

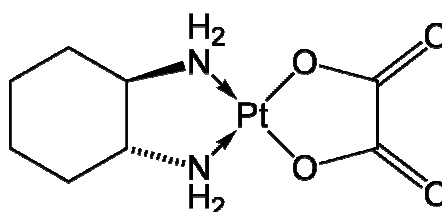


Fig. 7. Oxaliplatin

This platinum compound, used for secondary treatment of metastatic colorectal cancer [103], similarly to carboplatin, has a leaving group that undergo displacement slower and shows a lower nephrotoxic activity than cisplatin. The limiting toxicity of oxaliplatin is the peripheral sensory neuropathy, that increases in incidence and severity with repeated doses. The great difference in sensitivity profiles provided by oxaliplatin and cisplatin suggested that these two platinum compounds have a different target and mechanism of action. Studies indicated that it attaches onto proteins which are vital for DNA transcription, thus preventing cell division and causing cell death. Moreover, because oxaliplatin has a different spectrum of activity and low cross-resistance to cisplatin, it is utilized in patients who have developed resistance to platinum reference drug [104].

In addition to carboplatin, oxaliplatin and other second-generation cisplatin analogues, several third-generation drugs have been synthesized and tested.

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A platinum compound, *cis*-[dichloroammine(2-methylpyridine) platinum(II)], ZD0473 (picoplatin, Fig. 8) was designed to circumvent cisplatin resistance using a sterically-hindered bulky group at the platinum center in order to reduce the kinetics of substitution and avoid the reaction with glutathione and other cellular thiols.

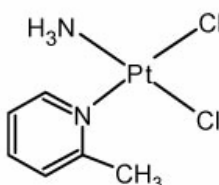


Fig. 8. ZD0473 (Picoplatin)

Hindrance is caused by the methyl group situated in the 2-position of the pyridine ring [105]. *In vitro* experiments have shown that picoplatin is indeed slower in reacting with DNA and with sulphur-containing ligands. In fact, picoplatin exhibited lower resistance than cisplatin in A2780 cisplatin resistant cells, in which detoxification due to elevated glutathione levels takes place, as well as in 41McisR and CH1cisR cell lines where cisplatin resistance is related to enhanced removal or increased tolerance to Pt-DNA adducts [105]. In 2001, picoplatin was being developed for both intravenous (i.v.) and oral administration, the i.v. drug was used in phase I clinical trials for the potential treatment of a range of solid tumors, including colorectal cancer, and in trials for patients with cisplatin-resistant ovarian tumors [105].

It is well known that platinum complexes with dissimilar DNA binding modes from that of cisplatin may produce a different spectrum of adducts lesions into DNA and hence an altered spectrum of antitumor activity including a superior antitumor activity against cisplatin-resistant cancer cells. In this field, compounds with an ammine ligand and an organic amine ligand, the mixed amines, were investigated with the hypothesis that the mixed amine ligands would give a different interaction with DNA. The combination of the mixed amine series with Pt(IV), led to the discovery of satraplatin, JM216 (Fig. 9), the first orally bioavailable platinum drug [106].

Satraplatin, with an ammine and cyclohexylamine as ligands, and axial acetate ligands, was found to be orally bioavailable whilst retaining antitumor activity. Satraplatin was found to have a similar activity profile to cisplatin and carboplatin in tumor models, but interestingly was also active against some acquired cisplatin-resistant tumor cell lines. This behaviour may be due to a different interaction with DNA, and/or susceptibility to different DNA repair mechanisms.

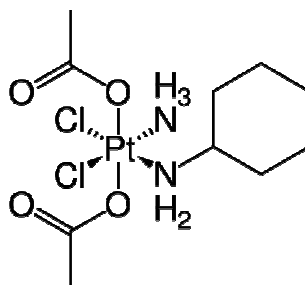


Fig. 9. Satraplatin

Furthermore, among platinum complexes with dissimilar DNA binding modes particularly interesting are those with amine ligands having *trans*-stereochemistry. The clinical inactivity of the *trans*-analog of cisplatin, transplatin, is considered a paradigm for the classical structure-activity relationships of platinum drugs [107]. However, recently several new transplatin analogs exhibiting a considerable *in vitro* antitumor activity have been identified. Discovery that transplatin inactivity partly originated from its kinetic instability and consequential susceptibility to inactivation has stimulated the development of new complexes with *trans*- geometry, obtained by substitution of one or both ammine with more bulky ligands. This substitution gave rise to more cytotoxic compounds, as the bulky ligands retard ligand exchange reactions of the two chloride ions, thereby reducing undesired and unspecific reaction between platinum and cellular components and facilitating its interaction with DNA. Among various classes of *trans*-derivatives that have been developed, several demonstrated a great cytotoxicity against cancer cells being generally more active than their *cis*-analogues, particularly against cisplatin-resistant cells [108]. The “spectator” ligands in these complexes can be classified into three groups: planar aromatic amines, alkylamines and iminoethers. Novakova *et al.* have shown that the replacement of amine ligands by iminoether in *trans*-platin (*trans*- [PtCl₂(NH₃)₂]) results in a marked enhancement of its antitumor activity, including activity towards cisplatin-resistant tumor cells [109]. The formation of stable monofunctional adducts on DNA and the high propensity for forming DNA-protein cross-links markedly enhances the efficiency of this adduct to terminate DNA polymerization by DNA polymerases *in vitro* and to inhibit removal of this adduct from DNA by NER. In addition, it has been suggested that DNA-protein ternary cross-links produced by P(II) complexes containing iminoether groups, of general formula *trans*-[PtCl₂(E-iminoether)₂] (*trans*-EE, Fig. 10), could persist considerably longer than the non-cross-linked monofunctional adducts, thus increasing its

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antitumor activity. These findings, hence, clearly distinguish these compounds from classic platinum drugs.

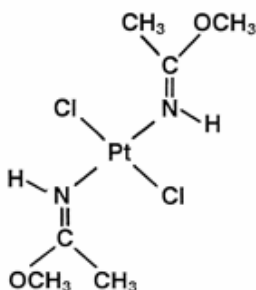


Fig. 10. *trans*-[PtCl₂(E-iminoether)₂]

In order to widen the knowledge on antitumor *trans*-[PtCl₂(E-iminoether)₂] complexes, Coluccia *et al.* have synthesised new derivatives, *trans*-[PtCl₂(NH₃){Z-HN=C(OMe)Me}] and *trans*-[PtCl₂(NH₃){E-HN=C(OMe)Me}] differing in the configuration of the iminoether ligands, Z or E, depending upon the *cis*- or *trans*- position of the alkoxy group and the metal atom with respect to the C=N double bond [110]. In a panel of human tumor cell lines, both mixed Z and mixed E show a cytotoxic potency higher than that of transplatin but *in vivo* mixed Z have been demonstrated more active and less toxic than mixed E.

Other following studies of platinum iminoether derivatives with different configuration at the iminoether ligands confirmed that the antitumor activity of *trans*-[PtCl₂(iminoether)₂] complexes may be affected by the configuration, Z or E, of the iminoether ligands [111]. Further studies aiming at comparing the biological activity of platinum(II) drugs with *cis* or *trans* geometry have been done by Marzano *et al.*. They have investigated a series of amidine complexes of the type *cis*- or *trans*- [PtCl₂{HN=C(NMe₂)R}₂] and [PtCl₂{HN=C(NHMe)R}₂] (R=Me or Ph) and the results confirmed *trans*-[PtCl₂{HN=C(NMe₂)Ph}₂] as the most effective derivative also able to circumvent acquired cisplatin resistance. Furthermore, studies concerning the cellular DNA damage on both parental chemosensitive and resistant sublines suggested for this new *trans*-amidine complex a different mechanism of action compared with that exhibited by cisplatin [112]

Because causative factors of *trans*-platinum complexes cytotoxic activity do not follow the same patterns as those found for cisplatin and its analogs, the differences in cellular and biochemical pharmacology between *trans*-platinum antitumor complexes and cisplatin need to be systematically exploited to support design of novel *trans*-platinum complexes with a clinical profile complementary to that of cisplatin and its related analogs. On the other hand, DNA adducts

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formed by antitumor-active *trans* platinum complexes are certain qualitatively and quantitatively different from those of cisplatin, indicating that cisplatin-like DNA adducts are not obligatory determinants for antitumor activity of platinum compounds.

Another class of platinum complexes that bind to DNA in a different manner from that of cisplatin are multinuclear platinum complexes. They contain many platinum centres, generally linked by a variable length polyamine chain, and possess both *cis* and/or *trans* configurations. Among them, the lead compound up to now is the trinuclear complex BBR3464 (Fig. 11).

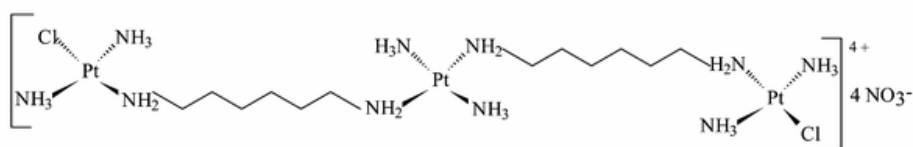


Fig. 11. BBR3464

BBR3464 is a highly charged species that binds to DNA rapidly, forming various long-range interstrand and intrastrand cross-links, in particular 1,4- and 1,6-interstrand and 1,5-intrastrand crosslinks. Recent mechanistic studies suggest that the interstrand crosslinks, rather than intrastrand adducts, are important to the antitumor activity [113]. Furthermore, it seems particularly effective against human tumor mouse xenografts containing mutant p53 gene [114], thus suggesting that BBR3463 is able to bypasses p53-mediated pathways. BBR3464, has presently entered a phase II clinical trial and has exhibited a considerable activity against lung and pancreatic cancer as well as melanoma.

Concluding, at this time, more is known about platinum-containing anticancer agents than about possibly any other known antitumor agent. Significant progress in platin-based anticancer agents has been achieved, based in part on a mechanistic understanding of the DNA-binding and pharmacological effects of cisplatin. Several new compounds with reduced toxicity and high specificity have been developed but, in spite of this, many questions remain concerning how platinum drugs carry out their antineoplastic (antitumor) activity.

1.2 Gold-Containing Anticancer Agents

1.2.1 Gold in medicine

The success of metal-based keystone cisplatin in anticancer chemotherapy as well as its unresolved disadvantages showed in clinical treatment has also raised great interest in the study of other metal complexes to be used as antitumor agents, stimulating research on novel non platinum metal-containing antitubercular therapeutics. Several families of non-platinum metal complexes have been studied intensely as potential cytotoxic agents. Among them, gold containing derivatives are under evaluation and present results are of particular interest.

Gold has been used for the treatment of disease in humans since the early days of civilization but its rational use in medicine has not begun until the early 1920s. It have had its genesis with the employ in rheumatoid arthritis (RA) treatment, when Robert Koch evaluated gold cyanide gas for inhibiting the growth of *tuberculosis bacilli*. Later, in 1927, Lande reported on the use of aurothioglucose for bacterial endocarditis and other conditions probably including rheumatic fever, suggesting a trial of gold compounds in painful arthritis. So, over the next 50 years, numerous studies were performed but only a few confirmed the efficacy of gold in the management of RA since Sutton and co-workers reported their findings on an oral gold compound, auranofin (Fig. 12), with antiarthritic activity in 1972, making an enormous progress in the history of chrysotherapy [115].

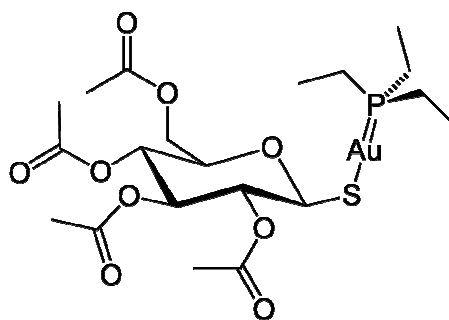


Fig. 12 Auranofin

This coordination complex, where a central atom of gold (I) is stabilized by phosphine and thiolate ligands, were shown to have optimal pharmacological activity in models of rheumatoid arthritis. After auranofin introduction in 1985, over 500 gold compounds have been used medically. Five are in current parenteral use as a second-line therapy for difficult cases of rheumatoid arthritis:

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aurothioglucose, aurothiomalate, aurothiopropanol sulphonate, aurothiosulphate and aurothioglycanide (Fig. 13).

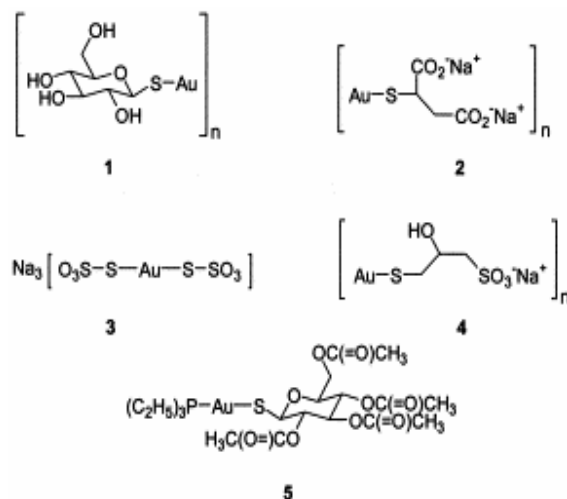


Fig. 13. Chemical structures of gold(I) thiolates used in the treatment of rheumatoid arthritis: (1) aurothioglucose (solganol); (2) aurothiomalate (myocrisin); (3) aurothiosulphate (sanocrysin); (4) aurothiopropanol sulphonate (allocrysin); and (5) triethylphosphinegold(I) tetraacetatothioglucose (auranofin).

The gold(I) thiolates, 1–4, being water soluble owing to the presence of solubilising groups and/or charge, are administered parenterally. This form of treatment suggests that that biodistribution is restricted, at least in the first instance, to the vascular fluids. By contrast, owing to its monomeric and lipophilic nature, 5 may be administered orally and is absorbed in the gut [115].

The investigation of the efficacy as anticancer agents of therapeutics typically used in RA treatment, as e.g. 6-mercaptopurine and cyclophosphamide, arose from their known immunosuppressive and antiinflammatory properties. These pharmacological characteristics, in fact, are also commonly displayed by anticancer drugs, therefore establishing, at least in principle, a connection between antiarthritic and anticancer therapies. Long-term studies on RA affected patients, moreover, evidenced that patients undergoing chrysotherapy were less likely to suffer from cancer disease [116]. This finding inevitably led to the investigation of anti-tumour activity of gold compounds used in the treatment of rheumatoid arthritis.

In the past years, a number of studies have shown that auranofin presented an *in vitro* antitumor activity similar to that of cisplatin toward different cell lines [117]. Given the wide range of sulfur-containing molecules with biological activity and the tendency of gold(I) to coordinate “soft” sulfur and phosphorus donor atoms, it is not surprising that lots of following investigation had been

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carried out to assess the antitumor activity of phosphine gold(I) thiolates. Different series of gold(I) coordination complexes of general structure P-Au(I)-SH (P=tertiary phosphine, S=thiolate), in which Au(I) has c.n.=2 and shows linear geometry, including auranofin and its analogs, were evaluated both *in vitro* and *in vivo* for their antitumor properties [118]. Among them, notably, the phosphine coordinated gold(I) thiosugar complex was underlined as the most potent cytotoxic agent against both cell lines *in vitro* and leukemia *in vivo*, despite being scarcely inactive against solid tumors [119]. These experiments, in particular, suggested that the phosphine ligand is necessary for antitumor activity as the same gold thiolates without phosphine had significantly reduced potency. The data obtained with these gold(I) derivatives, moreover, provided the basis for the following observations: (i) lack of potency *in vitro* correlates well with lack of antitumor activity; (ii) potent cytotoxicity *in vitro* is not necessarily predictive of antitumor activity *in vivo*; and (iii) *in vivo* antitumor activity is generally improved and optimized by ligation of Au(I) with a substituted phosphine and a thiosugar.

More promising *in vivo* activities against solid tumors were achieved with a series of di-phosphine gold complexes, such as gold(I) 1,2-bis(diphenylphosphine)ethane (DPPE), which were shown to possess a wide spectrum of *in vivo* activity especially in some cisplatin-resistant cell lines [120]. Unlikely, these compounds possess high cardiac, hepatic and vascular toxicity. Many other gold(I) compounds owing different ligands, such as chiral phosphines, bidentate thiolates, charged or biological active thiols and different amines have been evaluated for their anticancer activity but in many cases *in vitro* cytotoxicity was solely dependent on the presence of phosphine molecules and was not always confirmed by *in vivo* studies.

Among them, an Au(I) complex bearing both monophosphine and diphosphine ligands has also shown to possess significant *in vitro* antitumor activity [121]. Very recently, moreover, a hydrophilic tetrakis(trishydroxymethyl)phosphine gold(I) complex (Fig. 14) has been reported to induce cell growth inhibition in different cell lines as a result of G1 cell cycle arrest [122].

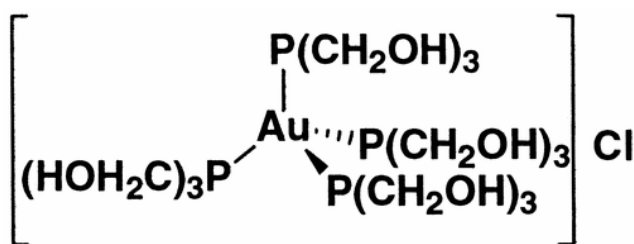


Fig. 14. Tetrakis((trishydroxymethyl)phosphine)gold(I) chloride

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On the other hand, the Au(III) complexes, with their metal centers being isoelectronic and isostructural to Pt(II), are also promising candidates as anticancer agents. With respect to Pt(II), however, the Au(III) ion shows greater charge, Lewis acidity, oxidizing power and speed of reaction of ligand exchange, all properties that make Au(III) difficult to use. Hence, despite gold(III) complexes exhibit interesting cytotoxicity and antitumor properties, their development has been heavily hampered by their poor stability under physiological conditions. Interestingly, it is possible to stabilize this metal ion by making a proper choice of the ligands, thus obtaining new derivatives with very appealing features. The first promising result in term of stability of gold in its 3+ oxidation state coupled with a significant antitumor activity, was obtained with a class of gold(III) complexes with 2-[(dimethylamino) methyl]phenyl] (damp) ligand. These complexes exhibited cytotoxic effects, against a wide panel of human cancer cell lines, comparable to or even greater than cisplatin retaining their cytotoxic activity also against cisplatin-resistant cells [123]. Cycloaurated N,N-dimethylbenzylamine complexes with either diphenylurea or salicylate [124a] and thiosalicylate [124b] as the dianionic ligands synthesized by Dinger et al. demonstrated a high *in vitro* cytotoxicity and an *in vivo* antitumor potency against the P388 leukemia, underlining the methoxy substituted N,N-dimethylbenzylamine cyclometallated gold(III) thiosalicylate complex as the most potent derivative (Fig. 15).

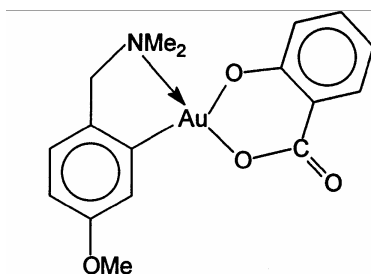


Fig. 15. $[[C_6H_3(CH_2NMe_2)-2-(OMe)-5]Au\{OC_6H_4(COO)-2\}]$

Recently, a series of gold(III) complexes with multidentate ligands ($[Au(en)_2]Cl_3$, $[Au(dien)Cl]Cl_2$, $[Au(cyclam)](ClO_4)Cl_2$, $[Au(terpy)Cl]Cl_2$, and $[Au(phen)Cl_2]Cl$) showed a relevant *in vitro* cytotoxic activity against A2780 ovarian cancer cells and their cisplatin-resistant variant A2780Pt cells, thus demonstrating their ability in overcoming cisplatin resistance [125, 126]. A number of other gold(III) complexes have been synthesized and their cytotoxic activities have been evaluated. Calamai et al. tested a group of square planar gold(III) complexes containing at least two gold-

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chloride bonds in cis-position [127]. Bruni et al. synthesized four gold(III) complexes, trichloro(2-pyridylmethanol) gold(III) $[\text{AuCl}_3(\text{Hpm})]$, dichloro(Nethylsalicylaldiminato) gold(III) $[\text{AuCl}_2(\text{esal})]$, trichlorodiethyl-endiamine gold(III) $[\text{AuCl}(\text{dien})]\text{Cl}_2$, and trichlorobisethylendiamine gold(III) $[\text{Au}(\text{en})_2]\text{Cl}_3$, showing a significant cytotoxic effect [128].

Among the compounds with sulphur ligands, those in which the Au(III) ion is stabilized by dithiocarbamic ligands proved to be particularly interesting (Fig. 16) [128, 129].

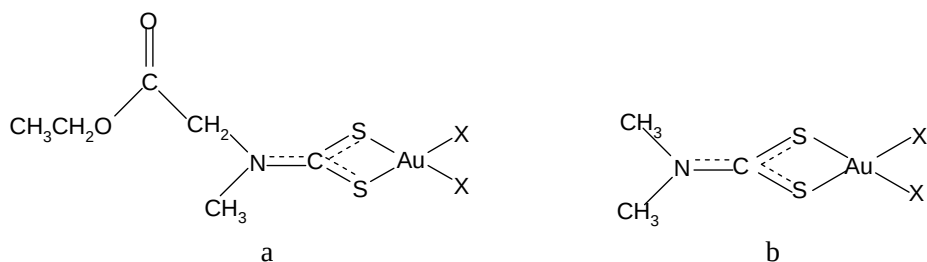


Fig. 16. Gold complexes: $[(\text{ESDT})\text{AuX}_2]$ (a); $[(\text{DMDT})\text{AuX}_2]$ (b); X = Cl, Br

These Au(III) complexes proved to be able to markedly reduce the viability of several tumor cell lines, showing a significantly higher *in vitro* antitumor activity with respect to cisplatin, even on cisplatin-resistant phenotypes. Moreover, preliminary evaluations of *in vivo* antitumor activity have shown that the treatment with gold(III)-dithiocarbamate complexes allows both to slacken the tumor growth (increasing host surviving time) in treated animals and to sometimes recover the tumor-bearing mice more effectively than cisplatin. The systemic toxicity of these complexes demonstrated also to be very low. These dithiocarbamic ligands, indeed, chelate the metal ion in such a robust way to prevent reaction with the renal thiol-containing enzymes, when passing through the renal tubules [128, 129].

Nowadays, although a number of structure/activity relations are known both for Au(I) and Au(III) derivatives, very little is known about the mechanism of action of these compounds. It seems to be ascertained that the mechanism of cytotoxic and antitumor activity of gold derivatives are in most cases different from platinum derivatives because, unlike the latter, the primary target of these compounds is not DNA. Studies of the interactions of gold(III) complexes with DNA, the classical target of platinum complexes, revealed, indeed, that binding to nucleic acids is not tight as in the case of platinum drugs, being in most cases electrostatic and reversible, thus suggesting the occurrence of a different mechanism for the observed antitumor activity [125].

Important evidence that proteins, instead of DNA, may be the main target for both gold(I) and gold(III) complexes came from studies of the interaction of such complexes with biological donor

ligands, in particular S-donor ligands as glutathione and cysteine. In line with these observation, these authors proposed that protein containing exposed Cys residues might be appropriate targets for gold complexes [119]. This has prompted a new search for gold-protein interactions in an attempt to identify possible targets responsible for the biological effects of gold compounds. Recent studies showed that mitochondria may play a major role in their mechanism of cytotoxicity and antitumor activity [130]. Production of hydrogen peroxide by gold compounds was already observed and due to the lack of effect of these complexes on respiration it is reasonable to conclude that the observed increase in hydrogen peroxide is essentially due to their inhibitory effect on thioredoxin reductase rather than to stimulation of the mitochondrial electron transport chain [131].

1.2.2 Thioredoxin system: structure and mechanism

The mammalian thioredoxins (Trx) are a family of 10–12 kDa proteins that contain a conserved – Trp-Cys-Gly-Pro-Cys-Lys – catalytic site [132–134]. These proteins undergo reversible oxidation/reduction of the two cysteine groups. The reduced dithiol form of Trx [Trx-(SH)₂] reduces oxidized protein substrates that typically contain a disulfide group, and the oxidized disulfide form of Trx [Trx-(S-S)] redox cycles back in an NADPH-dependent process mediated by thioredoxin reductase (TrxR) [132–134]. All Trx-mediated functions, indeed, depend on the disulfide reductase activity of thioredoxin, but also on the supply of NADPH and the activity of thioredoxin reductase (Fig. 17).

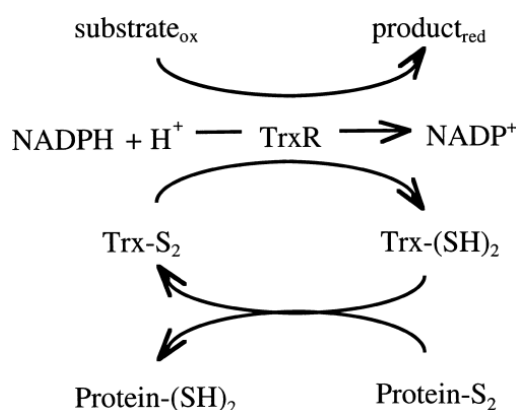


Fig. 17. Trx system

The mammalian TrxRs are selenium-containing flavoproteins having a conserved – Cys-Val-Asn-Val-Gly-Cys – catalytic site, an NADPH binding site, a C-terminal Cys-selenocysteine sequence

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that communicates with the catalytic site and is essential for its redox activity a dimerization interaction domain (Fig. 18) [132-133]. Indeed, these proteins exist as homodimers and undergo reversible oxidation/reduction.

The catalytic mechanism proposed for its reducing activity individuate the C-terminal relatively flexible tail of TrxRs as the responsible for the transport of electrons from the buried redox-center near the flavin to bulky substrates at the surface. The active site receives electrons from NADPH via the flavin adenine dinucleotide (FAD) molecule and transfers electrons to the selenocysteine redox active site present in the other subunit. The selenolate formed in the C-terminal active site region can then act to reduce the required substrate [134]. The more exposed position of the reduced C-terminus enables a broader range of substrates to be reduced, but small molecules can also take over reducing equivalents from the N-terminal active site.

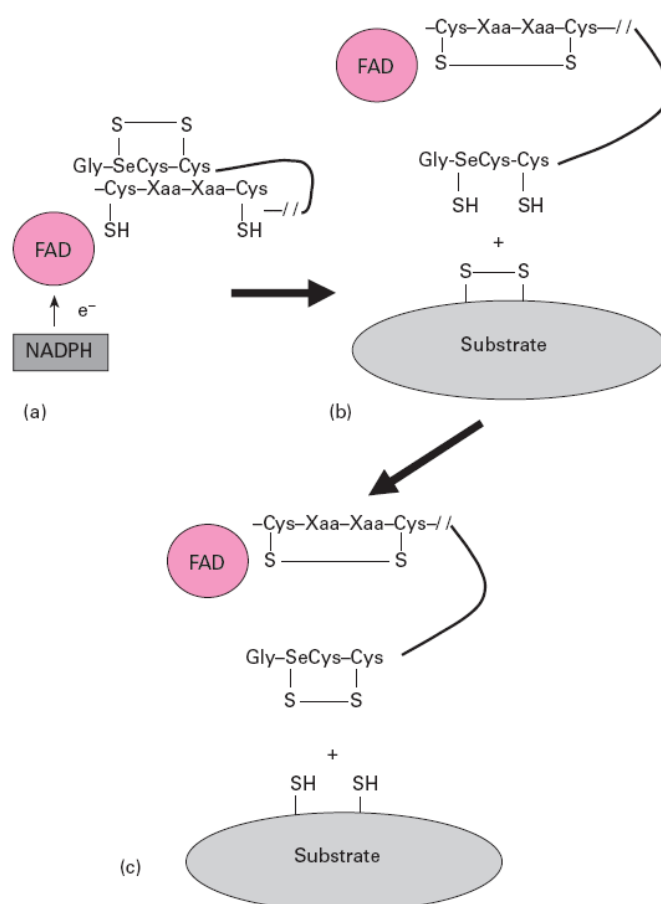


Fig. 18. Postulated mechanism for the action of Se-containing TrxR

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Two principal forms of Trx system have been identified and represented in Fig. 19. Trx1 is a 104-amino acid protein and TrxR1 a 54 kDa enzyme that are predominantly found in the cytoplasm of normal endometrial stromal cells, tumor cells, and primary solid tumors [132]. In contrast, Trx2 is a 166-amino acid protein and TrxR2 a 56 kDa enzyme that contains a mitochondrial N-terminal translocation sequence that directs them to the mitochondria [132, 135]. Both TrxR1 and TrxR2 are each also expressed in the form of several isoforms derived from alternative splicing, reflecting a highly complex and cell type-specific expression pattern [135-136].

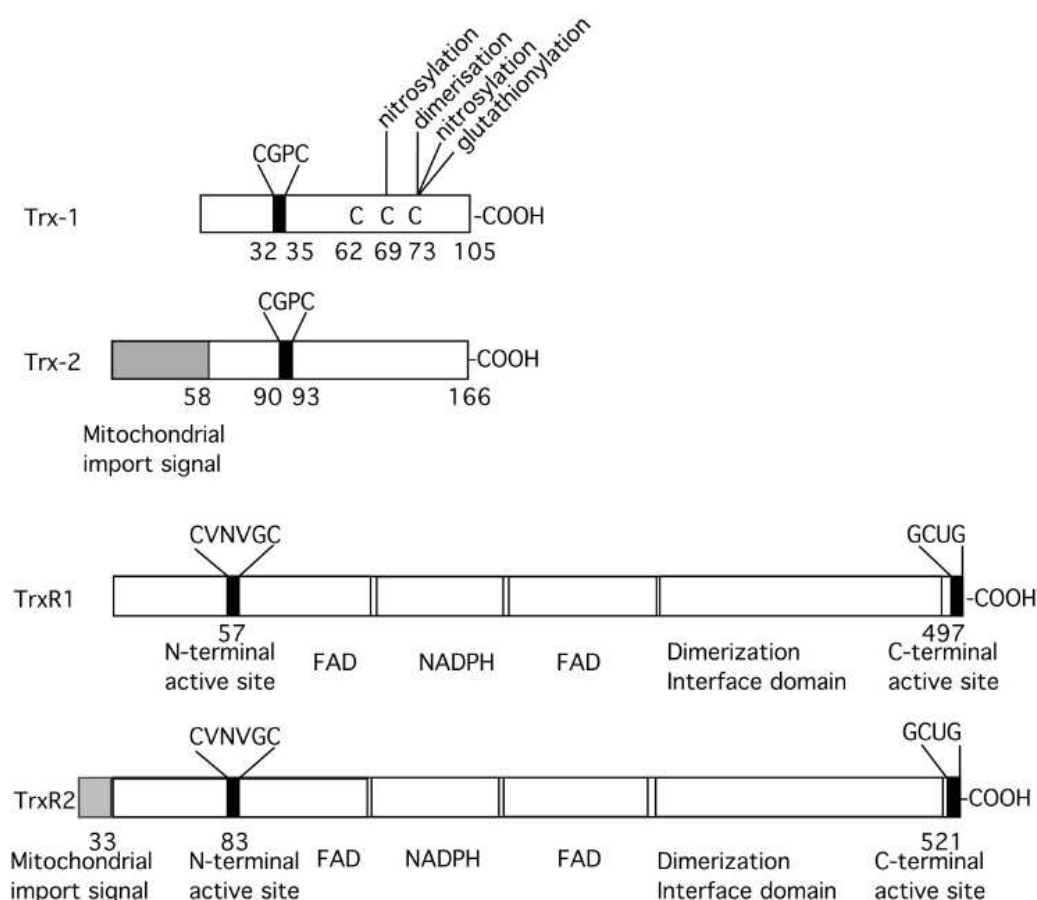


Fig. 19. Structure of cytosolic and mitochondrial Trx and TrxR proteins.

The different sub-cellular locations of the TrxR and Trx subtypes suggest that the cytoplasmic and mitochondrial systems may play different roles within cells. Moreover, recently in mammals a third form of TrxR is also expressed, predominantly in testis, which in contrast to TrxR1 and TrxR2 can reduce glutathione disulfide in addition to Trx. This enzyme has therefore been named TGR, indicating its thioredoxin/glutathione reductase activity [133].

Expression in mammalian cells of TrxR lacking the penultimate SeCys-Gly residues, or substitution of SeCys with Cys, decreases enzyme activity of at least two orders of magnitude [137]. Selenium, therefore, is essential for the activity of TrxR, and adding Se at 1 μ M to the medium of cultured cells increases cellular TrxR activity by as much as 40-fold [138]. Furthermore, studies utilizing ^{75}Se have demonstrated that, as the ^{75}Se concentration in the medium increased, its incorporation into TrxR increased from a molar ratio of 0.01 Se unit per TrxR subunit at 27 nM Se to 0.98 Se unit per TrxR subunit at 1 μ M Se [138]. There was also an increase in both TrxR1 mRNA and TrxR1 protein levels as the concentration of Se in the medium increased, although these increases were less than the increase in TrxR activity [138]. Alterations in dietary Se intake affect TrxR activity also *in vivo*. Rats supplied with a Se-deficient diet for several weeks showed decreased TrxR activity in the liver, kidney and lung [138]. TrxR protein levels were also found to be decreased in these same rats. Both *in vitro* and *in vivo* results suggest that the increased TrxR activity is due to increased Se incorporation into the TrxR.

Trx system play a key role in redox regulation of enzymes and transcription factors by thiol redox control and in defence against oxidative stress or in control of apoptosis. As dithiol-disulfide oxidoreductases, all thioredoxins catalyze reduction of disulfides with the rates for proteins being orders of magnitude faster than those for dithiothreitol or GSH as the low redox potential of thioredoxin ensures that thioredoxin-(SH)₂ is the major dithiol reductant in the cytosol [132]. Moreover, recent analyses suggests that thioredoxin reductases of higher organisms as mammals can also reduce many substrates different than thioredoxin [131], carrying out functions unconnected with those of thioredoxin.

1.2.3 Thioredoxin system: biological functions and role in cancer

In mammals, the thioredoxin system is quite complex in terms of its proteins and its functions. The physiological functions of thioredoxin system in different types of organisms have evolved from a common fundamental reaction to a large number of different specialized functions. At present, more than 1900 references in Medline mention 'thioredoxin' and it has more than 600 entries in GenBank (excluding ESTs and high throughput sequences). It has thus become impossible to cover all research on the physiological functions of the thioredoxin system. However, it plays a central role in maintaining the cellular redox homeostasis and is involved in many signaling pathways. The thioredoxin system participates in several biological cell processes essentially by means of redox reactions. Main biological functions are summarized as follow.

TrxR/Trx as a cofactor in DNA synthesis. The TrxR/Trx-coupled system plays a critical role in the generation of deoxyribonucleotides, which are necessary in DNA synthesis and essential for cell proliferation [132]. Trx provides the electrons needed in the reduction of ribose mediated by ribonucleotide reductase, an enzyme that catalyzes the conversion of nucleotide diphosphates into deoxyribonucleotides [136].

TrxRX/Trx in protecting cells against apoptosis. Trx1 prevents apoptosis when added to the culture medium of lymphoid cells as well as when its gene is transfected into these cells [132]. Increased Trx1 cytoplasm and nucleus levels of Murine WEHI7.2 lymphoid cells stably transfected with human Trx1 resulted in a substantially lower tendency to undergo apoptosis when exposed to dexamethasone and the other agents normally capable of inducing apoptotic cell death in this cell population [139]. The pattern of apoptosis inhibition with Trx1 transfection was similar to that obtained following transfection with the *bcl-2* anti-apoptotic oncogene. Differently, the mechanism by which Trx2 protects against apoptosis remains unknown [132]. One may speculate that increases in cellular reductive power allow reduction of proteins damaged by oxidative stress, thus countering a known inducer of apoptosis.

Thioredoxin in stimulating cell proliferation. Exposure to Trx1 stimulates the growth of lymphocytes, fibroblasts, and a variety of non-tumor, leukaemic and solid tumor cell lines [132,139]. In contrast, the Cys^{32,35}→Ser^{32,35} redox mutant at 50-fold higher concentration does not stimulate cell growth. The mechanism for the proliferative effect is unclear but appears related to augmented production of cytokines (e.g., IL-1, IL-2 and TNF α) and enhance of growth factor activity (e.g., basic fibroblast growth factor [bFGF]) [132].

Antioxidant effects of thioredoxins. Even as there are other antioxidant systems within a cell, Trx has some specific protein substrates that give it some distinct functional roles for counteracting oxidative stress. Trx produces antioxidant effects primarily by serving as an electron donor for thioredoxin peroxidases. By reducing the oxidized peroxidases, Trx restores the enzyme to its monomeric form, thus allowing it to continue its oxyradical scavenging [140]. Trx may also increase the expression of thioredoxin peroxidase. For example, in MCF-7 human breast cancer cells stably transfected with Trx1, mRNA for thioredoxin peroxidase was doubled relative to wild-type and empty-vector transformed cells, and Western blots showed higher protein levels as well. Trx can also act directly on certain ROS through its action as a potent singlet oxygen quencher and hydroxyl radical scavenger [140].

Thioredoxin in stimulating transcription factor activity. Trx increases the DNA-binding activity of a number of transcription factors, including NF- κ B, AP-1, and AP-2, and nuclear receptors [134]. With NF- κ B, for example, Trx reduces the Cys⁶² residue of the p50 subunit in the nucleus, thus allowing it to bind to DNA [141]. The effect of Trx on some transcription factors is mediated via reduction of Ref-1, a 37 kDa protein that also possesses DNA-repair endonuclease activity [132]. For example, Trx reduces Ref-1, which in turn reduces cysteine residues within the fos and jun subunits of AP-1 to promote DNA binding [142].

Thioredoxin binding to cellular proteins. Reduced Trx1, but not its oxidized form or the Cys^{32,35}→Ser^{32,35} redox mutant, binds to a variety of cellular proteins and may regulate their biological activities [132]. In addition to NF- κ B and Ref-1, Trx binds to apoptosis signal-regulating kinase 1 (ASK1) an activator of the JNK and p38 MAP kinase pathways strictly required for TNF α -mediated apoptosis, different isoforms of protein kinase C (PKC), p40 phagocyte oxidase, the nuclear glucocorticoid receptor, and lipocalin.

Most attention has been recently focused on thioredoxin system as target for cancer therapy, since an increased evidence that this enzymatic system involved in fundamental biological functions is essential for survival of cancer cells has emerged. Many are reasons of this consideration:

Thioredoxin stimulates angiogenesis and tumor invasion. Transfection of several different cell lines, including human breast cancer MCF-7, human colon cancer HT-29, and murine WEHI7.2 lymphoma cells, with human Trx1 produced significant increases in secretion of vascular endothelial growth factor (VEGF) [143]. VEGF secretion was increased by 41%–77% under normoxic (20% oxygen) conditions and by 46%–79% under hypoxic (1% oxygen) conditions. In contrast, transfection with a redox-inactive Trx1 mutant (Cys^{32,35}→Ser^{32,35}) partially inhibited VEGF production. When Trx1-transfected WEHI7.2 cells were grown in SCID mice, VEGF levels were increased markedly and tumor angiogenesis measured by microvessel vascular density was increased by 2.5-fold relative to wild-type WEHI7.2 tumors.

It has been recently observed Trx may reduce disulfides in extracellular matrix proteins such as laminin and may stimulate both matrix metalloproteinase activity and tumor cell invasiveness [143]. It is not yet clear, however, if the Trx system is absolutely required for tumor cell tissue invasion.

Thioredoxin expression increase in aggressive tumor. Trx expression is increased in a variety of human malignancies, including lung, colorectal, cervical, hepatic, and pancreatic cancer

[139,143,144-148]. In pancreatic ductal carcinoma tissue, for example, Trx levels were elevated in 24 of 32 cases as compared to normal pancreatic tissue [149]. Similarly, tissue samples of primary colorectal cancer or lymph node metastases had significantly higher Trx1 levels than normal colonic mucosa or colorectal adenomatous polyps [146]. In two recent studies, Trx expression was associated with aggressive tumor growth and poorer prognosis. In a study of 102 primary non-small cell lung carcinomas, tumor cell Trx expression was measured by immunohistochemistry of formalin-fixed, paraffin-embedded tissue specimens. The absence of Trx expression was significantly associated with lymph node negative status and better outcome and was independent of tumor stage, grade, or histology. The investigators concluded that these results are consistent with the proposed role of Trx as a growth promoter in some human cancers, and overexpression may be indicative of a more aggressive tumor phenotype. Furthermore, a study of 37 patients with colorectal cancer, evidenced that Trx1 expression increase with higher Dukes stage and was significantly correlated with reduced survival [145]. The relationship between Trx activity and tumor growth is less clear. Tumor cells may not need to increase expression of the TrxR enzyme, although its catalytic activity may be increased functionally. It has been found that human colorectal tumors had 2-times higher TrxR activity than normal colonic mucosa, but a non-specific TrxR assay was used in this study [133]. Further evaluations relating TrxR enzyme levels and catalytic activity with cancer stage and outcome are needed.

Thioredoxin in stimulating hypoxia-inducible factor. Cancer cells are able to adapt to the hypoxic conditions found in almost all solid tumors [140]. Hypoxia leads to activation of hypoxia-inducible factor 1 (HIF-1), which is a transcription factor involved in development of the cancer phenotype. Specifically, HIF binds to hypoxia response elements (HRE) and induces expression of a variety of genes that serve to promote angiogenesis (e.g., VEGF), metabolic adaptation (e.g., GLUT transporters, hexokinase and other glycolytic enzymes), and cell proliferation and survival [140]. HIF is composed of two subunits: HIF-1a that is induced by hypoxia and HIF-1b that is expressed constitutively. Trx overexpression has been shown to significantly increase HIF-1a under both hypoxic and normoxic conditions, and this was associated with increased HRE activity as well as increased expression of HRE-regulated genes [143]. HIF may induce hexokinase and thus allow glycolysis to serve as the predominant energy source [151].

Trx as immunomodulator. Trx1 may be secreted from cells through a leaderless pathway and extracellularly it is found both as a full-length Trx1 and a shorter truncated form, called Trx80. These two extracellular forms of Trx1 have potent and from each other distinct immunomodulatory

functions, with co-cytokine or chemokine activities [152]. This is of importance not only in rare cases but in quite common diseases such as B- and T-cell leukemias.

TrxR/Trx system in cancer drug resistance. Several lines of evidence suggest that Trx may be necessary but not sufficient for conferring resistance to anti-cancer drugs [132]. First, the resistance of adult T-cell leukemia cell lines to doxorubicin and ovarian cancer cell lines to cisplatin has been associated with increased intracellular TrxR levels [153, 154]. Similarly, hepatocellular carcinoma cells with increased Trx1 levels were less sensitive to cisplatin (but not less sensitive to doxorubicin or mitomycin C) [155]. Second, Trx1 mRNA and protein levels were increased by 4- to 6-fold in bladder and prostate cancer cells made resistant to cisplatin, but lowering Trx1 and TrxR1 levels with an antisense plasmid restored sensitivity to cisplatin and increased sensitivity to several other cytotoxic drugs [156]. Trx and TrxR levels were also elevated in cisplatin-resistant gastric and colon cancer cells [154]. Third, stable transfection of fibrosarcoma cells with Trx1 resulted in increased cisplatin resistance [156]. However, stable transfection of ovarian and colon cancer cell lines with Trx1 did not increase resistance to cisplatin, doxorubicin, or mitomycin C [157]. The latter finding suggests that a factor in addition to increased Trx1 may be needed to confer resistance in these tumor cell lines.

As summarized, the Trx system seems to be involved in several separate aspects within the hallmarks of cancer. However, further studies must be conducted in order to elucidate the molecular mechanisms through which this occurs, and to understand how important for the cancer development these different interactions may be.

1.2.4 Thioredoxin system inhibition

The biological activities of TrxR/Trx and their apparent relevance to aggressive and drug-resistant tumor growth suggest that this system may be an attractive target for cancer therapy. Either individual enzymes or substrates can be altered to determine system block. Both Trx or TrxR can be targeted by a chemotherapeutic reagent since a non-functional TrxR will result in lower levels of reduced Trx in a cell, thus preventing the redox function of Trx.

Several attempts have been made to develop Trx inhibitors, with some promising results [159]. Further development and clinical trials may reveal if Trx inhibitors for cancer treatment could become used in a future clinical setting.

With a highly reactive selenocysteine residue positioned at an easily accessible C-terminal position, TrxR can be easily and irreversibly inactivated by reaction with electrophilic agents. Such targeting

of the active site of TrxR should give rise to inhibition of the Trx system and, similarly to direct Trx inhibition, thus have a theoretical anticancer effect. Fig. 20 summarises the effects resulting from interactions between TrxR and inhibitors and their relevance to cancer therapy.

Inhibition of TrxR results in enhanced levels of oxidized Trx and a reduction of reduced Trx. Trx is therefore unable to activate the substrates, resulting in inhibition of cell functions including a reduction in DNA synthesis, protein repair, transcription factor activity, and peroxidase function. An accumulation of hydrogen peroxide and free radicals subsequently occurs. This leads to oxidative stress conditions, which promote apoptosis. Indeed, these redox control systems have direct interactions with components of the apoptotic signaling pathways and a consequence of TrxR inhibition is that oxidized Trx can no longer repress ASK1, allowing it to initiate apoptosis [159]. Some drugs are also capable of converting TrxR into a ROS generating system, in direct contrast to its usual role as a defensive enzyme. If this redox system is affected, hence, it can lead to either an inability to get rid of ROS or to a redox imbalance, which are conditions that favour apoptosis. Thus, the inhibition of both cytosolic and mitochondrial thioredoxin system can shift the redox balance toward a more oxidized state and hence alter the mitochondrial membrane permeability conditions with the consequent release of the segregated pro-apoptotic factors [159].

There are a number of inhibitors that targetting the selenocysteine-containing active site of TrxR leads to an accumulation of oxidized Trx resulting in cellular conditions that promote apoptosis. These include gold compounds, platinum compounds, arsenic trioxide, motexafin gadolinium, nitrous compounds, and various flavonoids [160].

Among the TrxR inhibitors, gold compounds are very effective and act at nanomolar level. For instance, the coordinated gold compound auranofin is known to react with selenol-containing residues, and inhibits the thioredoxin reductase in near stoichiometric amounts with a formal K_i of 4 nM [161]. In addition, gold compounds, successfully used as antirheumatic drugs, were also shown to act as anticancer agents [162], to inhibit mitochondrial functions [163, 164], to stimulate the release of cytochrome c and to induce apoptosis [165]

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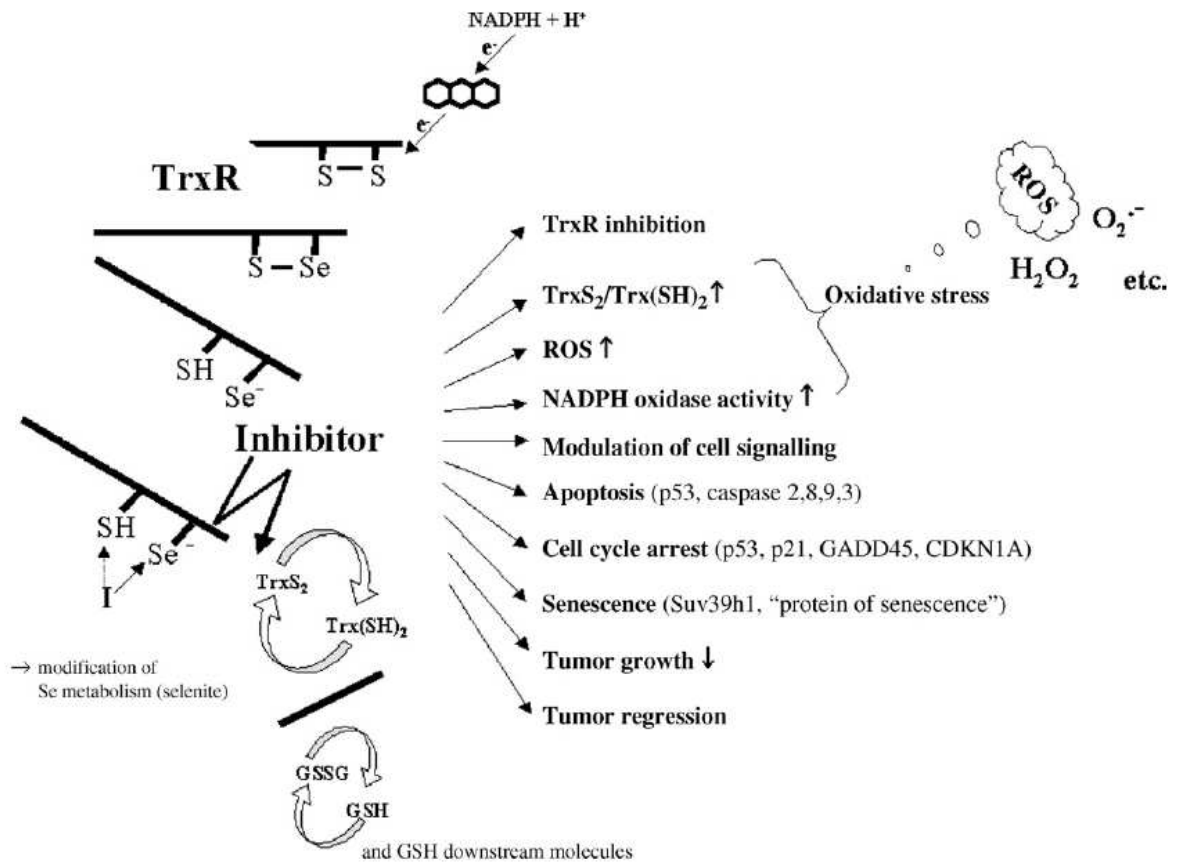


Fig. 20. Schematic representation of various effects of TrxR inhibition

1.3 Copper-Containing Anticancer Agents

Among extensive researches aiming to characterize other antitumor-active inorganic complexes with improved pharmacological properties compared to cisplatin an important field is focused on the use of biologically active complexes formed by essential ions. Indeed, since an essential metal which escapes its normal metabolic pathways can be very toxic to the organism, complexes of such metals may serve as effective cytotoxic agents.

1.3.1 Copper: homeostasis and related-diseases

Copper is one of the essential trace metals for all organisms living in oxygen-rich environments. It plays a crucial role in eukaryotic redox chemistry, growth and development being a redox catalytic cofactor of several enzymes and proteins involved, among others, in energy metabolism, mitochondrial respiration (cytochrome oxidase; Cco), antioxidation (Zn,Cu-superoxide dismutase; SOD), collagen cross-linking (lysyl oxidase), pigmentation (tyrosinase) and catecholamine biosynthesis (dopamine- β -monooxygenase) [166]. The main functions of copper compounds involve oxidation-reduction reactions in which copper containing biological molecules react directly with molecular oxygen to produce free radicals. Hence, Cu overload can be very toxic, due to generation of these hydroxyl free radical species that can damage DNA, proteins and other cellular components. This has required the development of quite sophisticated mechanisms able to control and regulate copper levels in the cell and in the different organs. For this reason, Cu homeostasis is tightly regulated and free cellular copper concentrations are maintained at extremely low levels by means of specific protein pathways that ensure that this essential enzymatic co-factor is delivered to its required targets [166].

Dietary copper absorption takes place primarily in the small intestine and stomach, mainly thought the action of ATP7A protein, and then it is regulated by the liver that distributes copper into the bloodstream. More of 80% of copper in normal human serum is carried by ceruloplasmin, an enzyme containing six copper atoms both in Cu(II) and Cu(I) state. While copper-binding ceruloplasmine is not transferable, the exchangeable form of copper is bound to albumin (12 %) and amino acids (7 %). Among the latters, copper-histidine complex was identified as the main copper-amino acid complex in human serum [167].

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Copper uptake is represented in Fig. 21. Cu uptake by cells is through 190 amino acid Cu transporter 1 (hCtr1), the main copper influx transporter in human cells, which is located predominantly on the plasma membrane and recognize only reduced Cu [168]. This assumption is principally based on the results obtained by competitive studies using various metal ions suggesting that hCtr1 is a selective monovalent metal transporter. No inhibition of copper transport is observed by addition of divalent cations such as Zn, Mn, Cd, etc., but monovalent Ag causes inhibition [169]. Therefore, in order to be transported in cells by hCtr1, Cu(II) has to be reduced to Cu(I) by a hypothetical membrane bound metalloreductase. Biochemical studies has suggested that hCtr1 binds copper through methionine residues and an histidine-rich N-terminal domain and transports it across cell membrane through pores [168]. Very recently, a different model proposed by Petris et al., suggests that hCtr1 acting as a copper coordinating endocytic complex trigger intracellular copper uptake with a mechanism of action similar to that describe for iron ion [170].

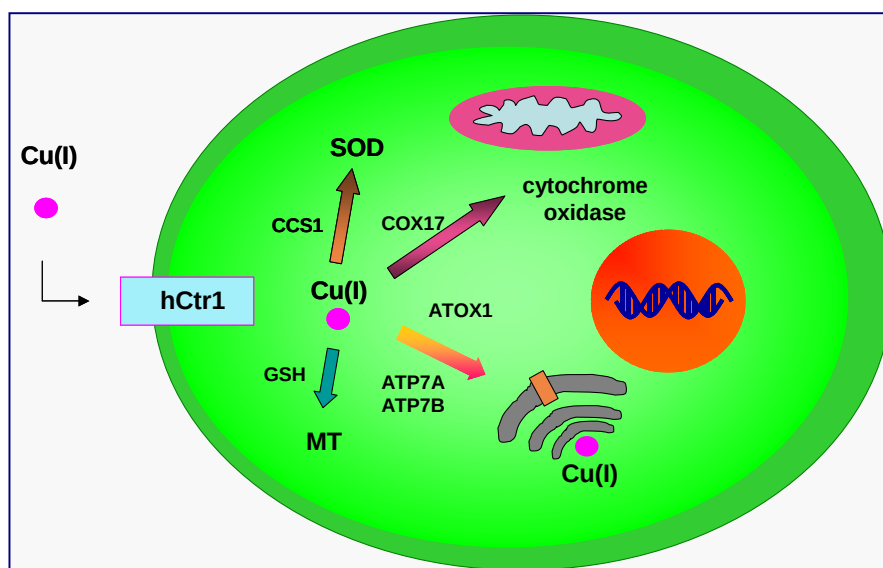


Fig. 21. Copper uptake.

Within a cell, various Cu-binding proteins may complex copper once it has entered the cytoplasm, thus preventing the interaction of free copper with cellular membranes, proteins, or DNA that can lead to oxidative damage. It is believed that the majority of cytoplasmic copper is complexed to GSH as Cu(I) [170]. The Cu(I)-GS complex may serve as a vehicle to deliver copper to various intracellular proteins such as MT, a family of proteins important for metal detoxification [171]. Another

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important class of proteins that are vital for copper delivery is copper chaperone group, a family of cytosolic peptides, including ATOX1 (HAH1), that delivers Cu to the P-type ATPase ATP7A and ATP7B at the *trans*-Golgi network, COX17 that delivers Cu to cytochrome-c oxidase in mitochondria and CCS1 which loads Cu onto cytoplasmic SOD [172]. Binding of copper to chaperones triggers highly regulated subcellular relocation to its appropriate cellular sites. Both cellular copper redistribution and efflux is mediated by Cu-transporting ATPases, ATP7A and ATP7B.

Copper intracellular excess, toxic to the human body, is therefore excreted through the two ATPases. Both proteins function as monomers and have 8 membrane spanning domains and 6 repeats of GMTCXXCIE motif that has very high affinity to copper [173].

Recent studies have demonstrated that copper transporters including hCtr1, ATP7A and ATP7B, are involved in the influx, subcellular distribution and efflux of cisplatin-related drugs, and consequently could be involved in regulating the sensitivity of human cancer cells to platinum drugs, despite the mechanisms by which these transporters shuffle platinum-based antitumor agents are largely unknown.

Disorder of copper homeostasis based on alteration of copper-trafficking protein functions results in many pathological conditions in individuals affected (Fig. 22). Genetic disorders of two proteins involved in the copper machinery have long been known, and related diseases caused by deficiency (Menkes disease) or accumulation (Wilson disease) of the metal are now under control if the pathologies are diagnosed in the first infancy. Mutations in the copper-transporter gene ATP7A, coding for a P-type ATPase ATP7A protein, responsible for excretion of copper from cells and delivery of the metal to enzymes in the trans-golgi network (TGN), leads to Menkes disease (MD) [174, 175]. MD is a fatal X-linked genetic recessive disorder of copper metabolism which causes an uncorrected intestinal absorption of copper. Dysfunction of ATP7A accounts for MD because it leads to a failure of copper efflux from intestinal cells. In individuals affected by MD, indeed, copper cannot leave the enterocytes, thus generating a systemic Cu deficiency. Symptoms of the disease are mostly associated with the lack of activity of copper-dependent enzymes leading to various clinical features such as weak muscle tone, seizures, mental retardation, developmental delay, and premature death [176]. The treatment of MD required subcutaneous and/or intravenous administration of copper salts [177].

On the other hand, mutation or deletion of ATP7B gene, that codes for a copper transport gene required for copper excretion via the bile, results in Wilson's disease (WD), an autosomal recessive genetic hepatic disease resulting from the excessive accumulation of copper in the liver and brain

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of affected individuals due to an impairment of biliary copper excretion [176]. When copper accumulates beyond the normal safe storage capacity of the liver, hepatocellular injury results. Furthermore, when the storage capacity of the liver for copper is exceeded, or when additional cellular copper is released because of hepatocellular damage, levels of non-ceruloplasmin-bound copper in the circulation become elevated and copper accumulates in multiple extrahepatic sites, notably in the brain [176]. Pathologic manifestations include liver failure, tremors, slurred speech and other neurological impairments. The most promising treatment consists on orally administration of drugs that are capable to remove copper from potentially toxic sites by formation of copper complexes which are then excreted via the urinary tract [177]. Recent trials with zinc salts revealed this therapy is also an effective way to remove excess copper from the body with no toxic side effects as zinc prevents absorption of endogenously secreted and dietary copper from the intestine [177].

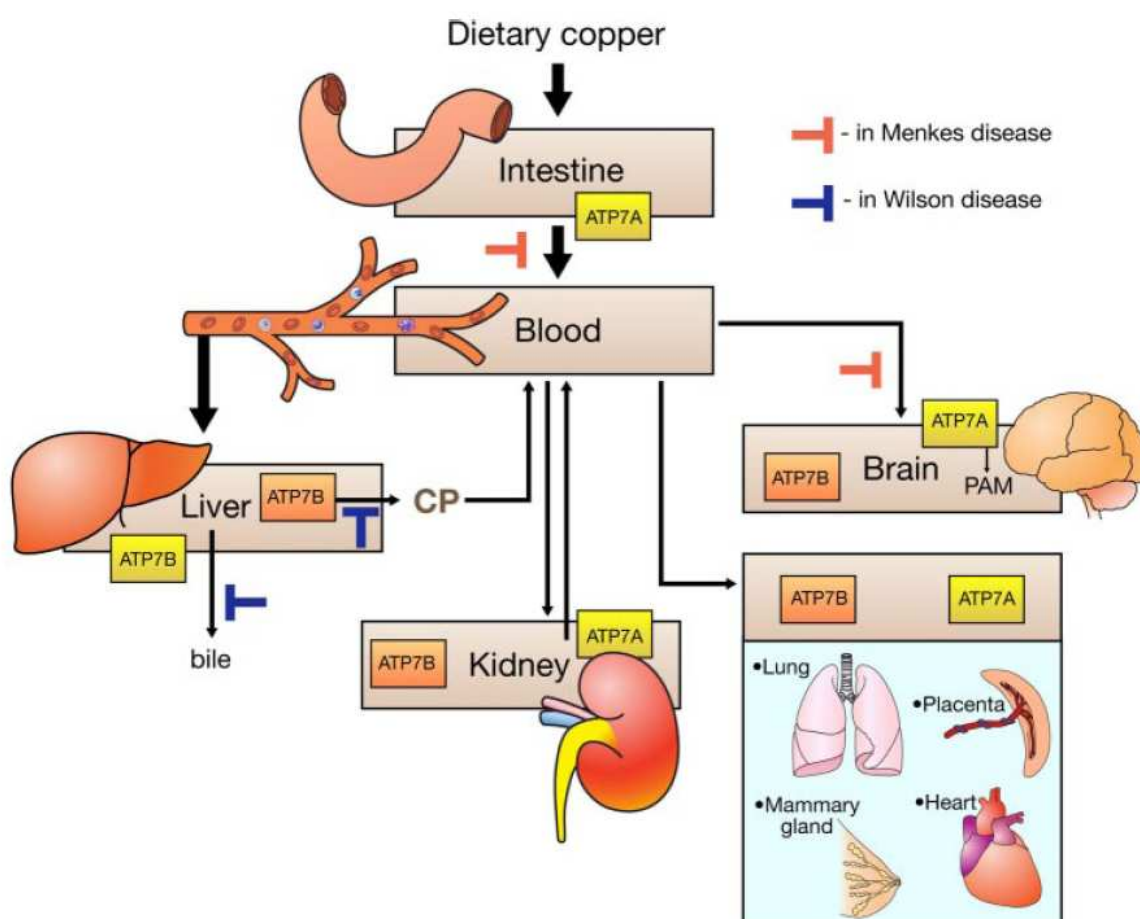


Fig. 22. Simplified scheme of copper distribution in the body and related disorders.

Numerous functions that lead to neurodegenerative diseases appear to be modulated by metals. Indeed, oxidative-stress induced injuries and protein aggregation which represent peculiar characteristics of neurodegeneration appeared to be connected to the involvements of metals in neurodegenerative disease etiology. Among neurodegenerative disorders, Parkinson's disease (PD) have been yet considered as a copper-involved disease, as recent evidence implicates that Cu could be a causative factor of this pathologic condition. Indeed, it has been demonstrated that cellular copper overload can induce aggregation of α -synuclein, a protein possibly involved in PD pathogenesis [179]. Remarkable evidence revealed that either Alzheimer disease (AD) may be a copper-related disease and, recently, copper chelators have been entered clinical therapy for patients affected by AD [180]. Copper-binding has been very recently shown to play a key role in prion protein stabilization. This copper-mediated action represents a molecular determinant in Human Prion disease pathogenesis.

1.3.2 Copper and cancer

The remarkable metabolic changes that have long been known to occur in cancer cells have been associated, among other factors, with copper handling and copper utilizing proteins.

A number of copper-dependent roles in angiogenesis have been proposed. Angiogenesis is a physiological process characterized by new blood vessels generation from the existing vascular bed [181]. Upregulation of this process happens in pathological condition as, for e.g., arthritis, atherosclerosis as well as in solid tumor development and metastasis. Tumors, indeed, are strictly dependent on angiogenesis for their growth, invasion and metastasis ability. The involvement of copper in angiogenesis has been known since 1980s, when McAuslan and Reilly discovered that copper salts stimulate *in vitro* proliferation and migration of endothelial cells of a tumor extract [182]. In particular, endothelial cells exposed to copper sulphate increase production of fibronectin. Subsequently, researches, aiming at understanding the role of copper in promoting angiogenesis, demonstrated that this metal interacts with several pro-angiogenic factors. Despite the functional significance of these interactions remains mostly unclear, it has been suggested that the role of copper in angiogenesis is referable to several mechanisms:

- copper may act through binding of angiogenic growth factors and increasing their affinity for endothelial cells, as with angiogenin and HIF-1
- copper may control the secretion of angiogenic cytokines, as demonstrated with NF κ B, FGF1 and IL-1 α

- copper may induce expression of angiogenic growth factors such as VEGF [183].

Thus, therapy aimed at depleting copper may be a successful anticancer strategy. Hence, several anti-copper chelators used in Wilson disease [184] have been evaluated as anti-angiogenic drugs in cancer models. It had first shown that three copper chelators, such as *D*-penicillamine, trientine, and tetrathiomolybdate (TM) [178], which quickly and effectively deplete copper stores, possess anti-angiogenic effects in murine cancer models [178]. These promising results *in vitro*, in pre-clinical animal models and those arising from an early clinical phase I trial have therefore led to ongoing phase II evaluation of TM in patients with advanced cancers [178].

Moreover, mixtures constituted by the combination of copper(II) salts and specific copper chelators have been shown to suppress proliferation and clonogenicity of different types of human cancer cells. In particular, Dou and coworkers described some 'copper mixtures', what they called 'organic copper compounds' as potent proteasome inhibitors.

1.3.3 Copper complexes as anticancer agents

Copper has a long history of medicinal application but its potential antitumor activity has been explored only in the last decades. With the aim to develop novel non platinum-based antitumor drugs with better pharmacological properties, several families of copper-based complexes have been investigated as potential antitumor agents. These researches have been motivated based on the assumption that endogenous metals may be less toxic than non-essential metals, as human cellular physiology count upon tightly regulated washout mechanisms concurring to maintain endogenous metal homeostasis. Moreover, since an essential metal can flees its normal metabolic pathways being toxic to the organism, copper complex-based therapy may provide an effective antineoplastic strategy. Although only a little understanding of the molecular basis of their mechanism of action has been documented, copper complexes have attracted attention based on modes of action different from that of cisplatin (covalent binding to DNA). Therefore, copper complexes may provide, at least in principle, a broader spectrum of antitumor activity and they might be effective against human cancers that are poor chemosensitive or have become resistant to conventional platinum drugs.

The properties of copper-coordinated compounds, whether in classical inorganic coordination complexes, in organometallic compounds, or in bioinorganic model compounds are mainly determined by the nature of ligands and donor atoms bound to the metal ion [185, 186]. Even though three oxidation states of the metal can be stabilized, (Cu(III), Cu(II) and Cu(I), very few

examples of copper(III) compounds are reported and coordination chemistry of copper is dominated by Cu(II) derivatives with little, but important, examples of Cu(I) compounds [186]. Due to the closed-shell d^{10} electronic configuration, Cu(I) complexes strongly prefer ligands having soft donor atoms such as P and aromatic amines. Although two-coordinated linear and three-coordinated trigonal arrangements are known, Cu(I) complexes are mostly four-coordinated species adopting a tetrahedral geometry. Conversely, in Cu(II) derivatives the coordination number varies from four to six, including four-coordinate square planar (sp), five-coordinate trigonal bipyramidal (tbp) and six-coordinate octahedral (oc) geometries. The variety of available arrays allows for a great assortment in the choice of the ligands (from mono- to hexa-dentate chelates), and of the donor atoms (N, O, S and halides). The redox potential of the physiologically accessible Cu(I)/Cu(II) couple varies dramatically depending upon the ligand environment due to the donor set, geometry, substituent electronic effect, substituent steric effect, and chelation [185]. Different class of copper complexes have been developed: copper complexes containing TSC (thiosemicarbazone), polydentate Schiff base ligands, aromatic heterocycles (such as imidazoles, pyrazoles and triazoles), aromatic heterocycles (phen and bipy), and tertiary phosphines [187].

In general, attempts to correlate the antitumor activity of copper compounds with copper oxidation state, coordination number and geometry is matter of debate and correlations, if exist, are far to be demonstrated.

Most of the investigated compounds belong to the family of copper(II) complexes and the greater part of these agents are mononuclear species, but there are few distinctive examples of dimeric compounds eliciting remarkable antitumor activity [187]. Noteworthy examples of tetrahedral copper(I) complexes having soft tertiary phosphines as donor ligands, display antiproliferative activity in the sub-micromolar range [187].

Copper phosphine complexes. After auranofin success as anticancer agents, that has represented the starting point for a series of investigations focused on the biological chemistry and anticancer properties of phosphine metal complexes, extensive research performed by Berners-Price, Sadler and co-workers in the 1980s [188, 189, 190] underlined that bisaryldiphosphine (P-P) metal complexes of the type $[M(P-P)_2][Cl]$ ($M = Au, Ag$ and Cu ; P-P = dppe: 1,2-bis(diphenylphosphino)ethane, dppp: 1,2-bis(diphenylphosphino)propane, dppey: 1,2-bis(diphenylphosphino)ethylene) own an elevated cytotoxic activity against P388 murine leukemia [190], B16 melanoma and M5076 reticulum cell sarcoma [188]. In particular, the activity of some Cu derivatives was at least 20-fold more higher than those shown by Au(I) analogues, suggesting that

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particular metal ions may enhance the arylphosphines cytotoxic properties [191]. Despite considerable efforts carried out in this field, the presence of several phenyl groups in these copper and gold compounds led in animal models to undesired side effects as nephrotoxicity [188] and cardiovascular toxicity [191], respectively, thus precluding clinical trials in humans. Among various synthetic strategies aiming at the enhancement of the antitumor efficacy, it has to be mentioned the attempts made to improve the hydrophilic character of copper compounds in order to get a suitable solubility in physiological conditions, thus enhancing the bioavailability and reducing the *in vivo* toxicity. The effect of exchanging phenyl substituents with more physiological ethyl groups in $[M(eppe)_2][Cl]$ species ($eppe = 1-(diethylphosphino),2-(diphenylphosphino)ethane$) was, unlikely, to lower significantly the cytotoxic activity [193]. Therefore, many efforts have been focused to design more hydrophilic copper derivatives as in the case of the partial substitution of the 'CuP₄' arylphosphine coordination sphere with heterocyclic thiones [193], acetonitrile [194] and N-heterocycles such as carbazole and benzotriazole producing 'mixed-ligand' type compounds. In particular, $[Cu_2(dppe)_3(CH_3CN)_2][ClO_4]_2$ complex (Fig. 23) exhibited a potent *in vitro* cytotoxicity in H460 human lung carcinoma cells, comparable to that displayed by adriamycin [194].

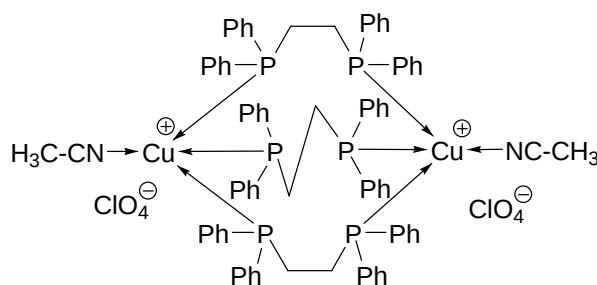


Fig. 23. $[Cu_2(dppe)_3(CH_3CN)_2][ClO_4]_2$

With double aim at increasing the water solubility and the kinetic inertness Marzano et al. synthesized the mixed copper complexes of (I) bis(nitrotriazolyl)borate $[H_2B(tz^{NO_2})_2]$ scorpionate co-ligands. These less hydrophobic copper(I) $\{[H_2B(tz^{NO_2})_2]Cu[(PR_3)_2]\}$ (Fig. 24) compounds were proved to be easier to handle during the *in vitro* tests, and they retained cytotoxic activity against a panel of human tumor cell lines including examples of ovarian (2008), cervix (A431), and lung (A549) cancer, melanoma (A375) and leukemia (HL60). IC₅₀ values indicated that both compounds were appreciably more effective against all cell lines compared to the reference metallodrug cisplatin, and that free phosphine ligands presented IC₅₀ values at least 15-fold higher.

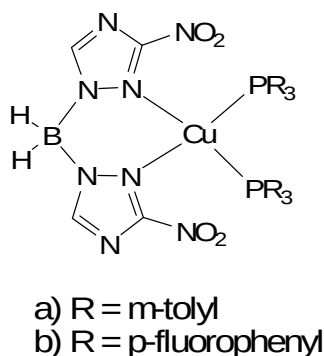


Fig. 24. $\{[H_2B(tz^{NO_2})_2]Cu[(PR_3)_2]\}$

In particular, on lung adenocarcinoma (A549) cells, with notoriously poor chemosensitivity to cisplatin, cytotoxicities shown by complexes exceeded that of cisplatin by a factor of about 20 [195].

1.3.4. Copper complexes: mechanism of action

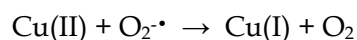
Copper complexes were subjected to a lot of studies concerning their chemical and biological behaviour. However, despite the enormous efforts in synthesizing different classes of copper complexes, very few data are available concerning their physiological processing.

Copper complexes were initially tested with a vision toward behavior analogous to cisplatin, and, consequently, their interaction with nucleic acids were largely investigated. Taken all together, the results of these studies suggested that DNA does not represent the main and/or unique biological target for this class of metal complexes. With the time it has become clear that copper compounds can act by different mechanisms. Besides DNA binding, intercalation and cleavage activity [196] also binding of copper complexes to specific protein sites, leading to a protein conformational structure modification have been described [197]. Moreover, as the major functions of copper-biological compounds involve oxidation-reduction reactions, similarly, the main mechanism have been proposed to explain copper-induced cellular toxicity comes from the propensity of free copper ions to participate in the formation of ROS. SOD mimetic activity as well as generation of ROS by redox-cycling, that both lead to an oxidative cell damage, have been therefore revealed as guilty effects determining copper compounds toxicity [198].

One of the main mechanisms proposed to explain copper-induced cellular toxicity comes from the propensity of free copper ions to participate in the formation of ROS. Cupric and cuprous copper

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intracellular ions participate in oxidation and reduction reactions. In the presence of superoxide ($\text{O}_2^{\bullet-}$) or reducing agents such as ascorbic acid or glutathione (GSH), Cu(II) can be reduced to Cu(I), which is capable of catalyzing the formation of hydroxyl radicals ($\text{OH}^{\bullet}$) from hydrogen peroxide (H_2O_2) via the Haber-Weiss reaction [198]:



The highly reactive hydroxyl radical is able to interact with biological molecule by abstracting the hydrogen from an amino-bearing carbon to form a carbon-centered protein radical and from an unsaturated fatty acid to form a lipid radical, resulting in an oxidative damage of cells [198].

Many authors provide evidence for the formation of $\text{O}_2^{\bullet-}$, H_2O_2 , and OH^- as reactive species that may play an important role in copper complex cytotoxicity. Also nuclease activity, characteristic feature often attribute to copper complexes has been shown in some cases to be related to ROS production [199, 200]. Copper complexes have been shown to cleave DNA both through an hydrolytic and an oxidative processes. In the latter instance, these complexes have been shown to react with molecular oxygen or hydrogen peroxide to produce a variety of active oxidative intermediates which react with DNA. Furthermore, experiments aiming at underline the influence of the radical scavengers on the DNA cleavage demonstrated that the hydroxyl radical scavengers diminished the nuclease activity of copper complexes, thus suggesting an active involvement of the ROS in the cleavage process [201].

Generally, copper molecular-induced events trigger cancer cell death through an apoptotic mechanism sometimes accompanied with down regulation of antiapoptotic proteins (Bcl-2 and Bcl-XL) [187].

On the other hand, more recently, a non apoptotic form of programmed cell death has been evidenced in human cancer cells treated with copper(II) complexes [202]. Furthermore, Dou and co-workers recently described some 'copper mixtures' as potent inhibitors of the proteasome activity [203].

The proteasome is a large multiprotein complex located both in the nucleus and the cytoplasm, selectively degrades intracellular proteins.

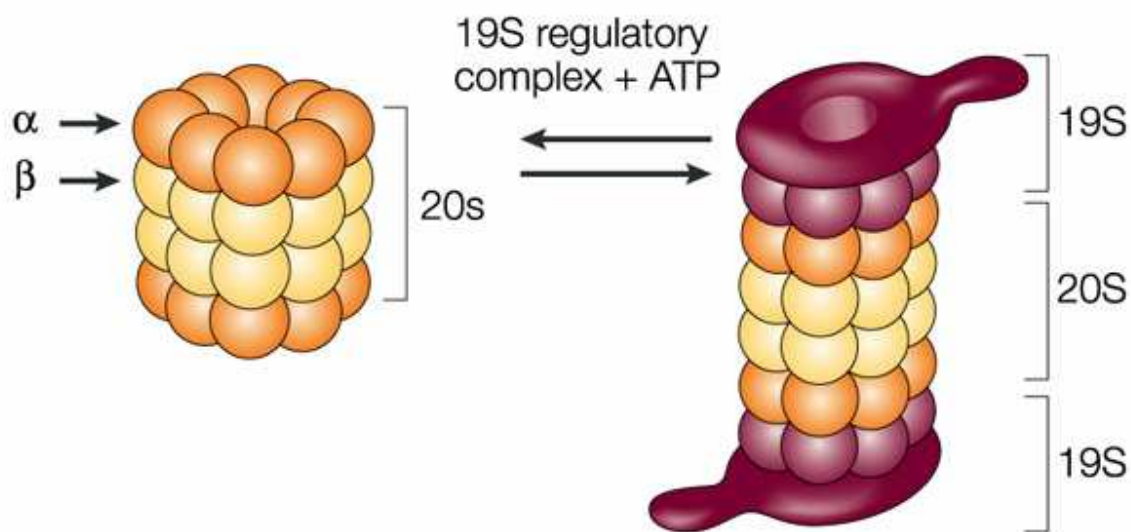


Fig. 25. A three-dimensional representation of the proteasome multi-enzyme complex.

As seen in Fig. 25, the 26S proteasome contains one 20S core particle structure and two 19S regulatory caps. The core is hollow and provides an enclosed cavity in which proteins are degraded; openings at the two ends of the core allow the target protein to enter. Each end of the core particle associates with a 19S regulatory subunit that contains multiple ATPase active sites and ubiquitin binding sites. In order to be recognized by the proteasome and be processed, hence, a protein substrate needs to be linked to a chain of the 76 amino-acid protein ubiquitin [204]. Transfer of ubiquitin to the target proteins is mediated by ubiquitin-activating enzymes (E1), conjugating enzymes (E2), and ligating enzymes (E3). This process requires energy, which is provided in the form of adenosine triphosphate (ATP). After polyubiquitinated proteins have been recognized by 19S subunit are transferred to the 20S catalytic core. 20S subunit consist of four stacked heptameric ring structures that are themselves composed of two different types of subunits; α subunits are structural in nature, whereas β subunits are predominantly catalytic. Each β subunit could possess at least one of the three distinct activities: chymotrypsin-like (cleavage after hydrophobic residues), trypsin-like (cleavage after basic residues), and caspase-like (cleavage after acidic residues) [205].

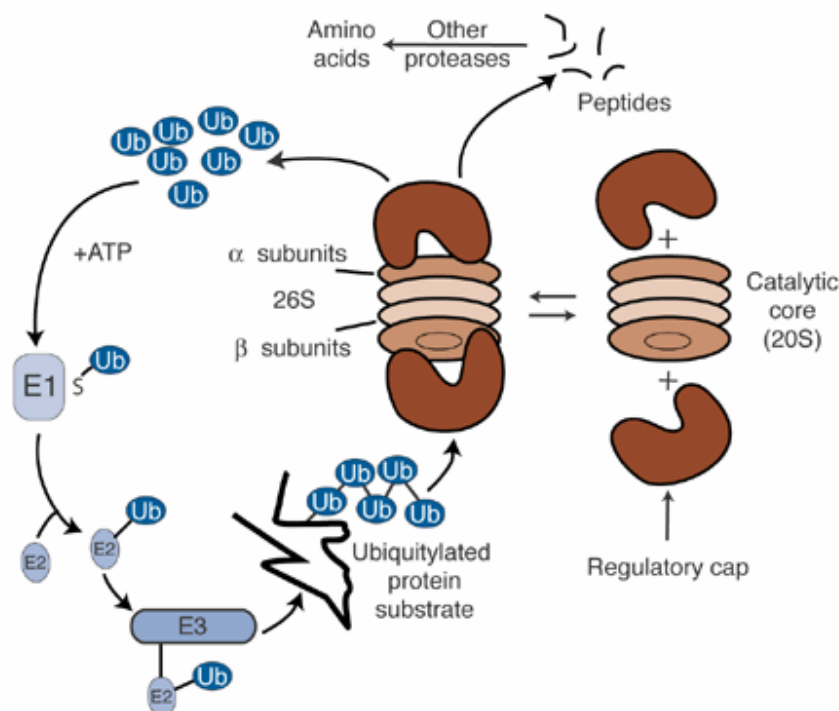


Fig. 26. Schematic representation of proteasome degradation pathway.

The main function of this massive multicatalytic protease is to degrade unneeded or damaged proteins by proteolysis. Proteasomes are part of a major mechanism by which cells regulate the concentration of particular proteins and decompose misfolded proteins. Hence, proteasome regulates the stability of hundreds of cellular proteins and thus, it is virtually implicated in all cellular functions, including processes of primary importance for carcinogenesis such as proliferation, apoptosis, angiogenesis and metastasis formation [207]. Due to its involvement in these processes, proteasome function is an essential requirement for the survival of normal and neoplastic cells and indeed during apoptosis proteasome is inhibited [207]. Many *in vitro* studies have demonstrated that proteasome inhibitors selectively kill cancer cells, and the strong clinical anti-tumor activity observed in refractory multiple myeloma and other malignancies establish that they are also cancer cells selective in patients [207]. Furthermore, proteasome inhibitors rapidly induce tumor cell apoptosis, by selectively activating programmed cell death in oncogene-transformed, but not normal or untransformed cells [207].

Although a concrete mechanistic explanation for this selectivity is not yet available, one attractive possibility, first advanced by Drexler, is that sensitivity is linked, besides to oncogene mutation, to proliferation and/or deregulated cell cycle progression [207]. As cancer cells usually proliferate

avoiding cell cycle checkpoints, they are more heavily dependent on proteasome-mediated degradation of cell cycle regulators (either positive regulators like cyclins or negative regulators like p21 and p27).

Moreover, it is possible that the increased requirement for protein synthesis in proliferating cells imposes an extra burden that makes them more vulnerable to proteasome inhibition. Indeed, many tumor cells are known to have very well developed ER-Golgi networks to handle their large secretory burden, and it is possible that this renders them particularly susceptible to agents that disrupt intracellular protein quality control mechanisms (mediated via proteasomal or lysosomal degradation) [208].

Finally, this copper-mediated proteasome inhibition seems to occur only in cells with augmented copper levels (typical of tumor cells), while copper complexes showed negligible toxicity toward normal or non-transformed cells, where the copper level is very low. This behaviour opens the interesting perspective to design new less toxic chemotherapeutic agents converting the essential element copper, both for life and for tumor cell proliferation, into an antitumor agent.

Although recent studies are trying to obtain more mechanistic information, it is evident that, up to now, very little understanding of the molecular basis of the mechanisms underlying the action of these classes of copper compounds is available. Efforts in this direction could provide additional information useful for the design of 'second generation' copper drugs having better selectivity and improved antitumor efficiency.

1.4 Aim of the present work

As previously discussed, the widespread clinical use of cisplatin is limited by several side effects including severe nephrotoxicity. In addition, cisplatin is not orally bioavailable, and both inherited and acquired resistance seriously limit its applications.

These drawbacks have motivated tremendous efforts in the research and development of new antitumour metallo-drugs with more favorable properties in terms of a broader spectrum of activity, reduced toxicity and better aqueous solubility.

Inorganic chemistry offers many opportunities in this field, and the development of metal-based drugs has moved on from chance discovery to rational drug design. Several families of metal complexes have been investigated for potential antitumor applications, including palladium, iridium, rhodium, ruthenium, copper, and gold [105]. Much of the early work on metal-based

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compounds focused on their interaction with DNA based on the platinum paradigm. However, it is now increasingly apparent that many non platinum metal complexes do not behave like cisplatin and “DNA-independent” mechanisms have been proposed. A systematic evaluation of the biological properties as well as the identification of molecular targets of these new non platinum metal complexes could contribute to achieving a preclinical and clinical development plan and to lay the basis for SAR’s rational design of future derivatives, having better selectivity and improved antitumor efficacy.

Novel targets for cancer could include thiol-based targets such as cysteine proteases including cathepsins and caspases, the thioredoxin reductase system, transcription factors, and redox and free radical chemistry such as superoxide dismutase mimics and NO scavengers.

In this field, there has been an ongoing interest in gold compounds for the treatment of cancer. Gold containing compounds have been widely used as chemotherapeutic agents in the treatment of rheumatoid arthritis. Recently, due to their cytotoxic effects on a variety of human tumor cell lines, many gold (I) and gold (III) compounds have been the focus of several investigations as potential new anticancer agents [115]. The increasing interest in gold complexes depends principally on their ability to induce cancer cell death targeting and inhibiting TrxR [161].

Trx enzymatic system is involved in several biochemical processes and plays a central role in maintaining the cellular redox homeostasis. Recently, TrxR has emerged as a new tumor-specific molecular target for anticancer drug development, since it is overexpressed in many tumor cells and may contribute to drug resistance toward different chemotherapeutic agents.

TrxR is a selenoprotein, containing a selenocysteine on its flexible C-terminal arm which is easily accessible for interaction with electrophilic compounds. Different classes of electrophilic compounds have been shown to inhibit TrxR possibly by interacting with the nucleophilic selenolate of reduced TrxR, including gold compounds, nitroaromatics and flavonoids [160]. Among inhibitors, gold containing drugs have been validated as potent and selective inhibitors of mammalian isolate TrxR, acting even at nanomolar concentration.

In particular, auranofin, a coordinated gold(I) compound, is known to inhibit TrxR in near stoichiometric amounts with a formal K_i of 4 nM [161].

In order to gain more information on biological effects in tumor cells deriving from Trx system inhibition and also aiming at providing new insights into a possible role of TrxR in cisplatin resistance, we have compared the effects of auranofin on human ovarian cancer cells sensitive (2008) and resistant (C13*) to cisplatin.

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In particular, our research focused on auranofin cytotoxicity and TrxR inhibition potency in these tumor cell lines. We tried to investigate cell death pathways involved, focusing attention on the auranofin effects in terms of modification of cellular redox homeostasis. Our results clearly indicated that auranofin, acting as a potent inhibitor of TrxR, determines an alteration of the redox state of the cell leading to an increased production of hydrogen peroxide and oxidation of the components of the thioredoxin system, therefore creating the conditions for enhanced apoptosis. Importantly, cisplatin resistant ovarian cancer cells easier undergo auranofin mediated cell death, as auranofin primary target, TrxR, is overexpressed in the resistant phenotype.

On this basis, we have subsequently investigated the cellular effects induced by further challenging tumor cells by a combination treatment with auranofin and selenite or tellurite. Despite selenium being, in the form of selenocysteine, an essential part of several important redox enzymes, such as GPx and TrxR and thus can be considered a physiological antioxidant, it is, however, a redox modulating factor of great importance for cell growth and differentiation and has, consequently, a high potential as a tumor selective drug [210]. The biological effects of selenium are, indeed, strictly concentration dependent with antioxidant properties at low concentrations and powerful pro-oxidant effects at moderate to high concentrations. In fact, at low concentrations selenium compounds are able to stimulate cell growth [211-213] and promote cytokine production [214]. In contrast, increasing levels inhibit cell proliferation [215, 216] and induce cell death [217] and apoptosis by the inhibition of transcription factors [218-220]. The effects of selenite either on the redox regulation of transcription factors or on the inhibition of cell growth appears to be associated with ROS production and alteration of thiol redox balance [210]. Tellurite on the other hand, has been far less investigated. However, tellurium, which has similar chemical properties to selenite, may function as catalysts in cellular reactive oxygen species production and consequently, may be detrimental in cancer cells even at very low concentrations [221]. The expected synergy between chalcogen metalloids and auranofin in inducing oxidative stress in tumour cells provides, at least in principle, a rational basis for the combination of these two drugs in our experiment.

Furthermore, various selenium compounds have recently entered phase II clinical trials for the treatment of several carcinomas at the Karolinska Institutet Hospital in Stockholm, and very promising results have been achieved in this regard. It has been described that many aggressive tumors are endowed with a marked TrxR overexpression. Therefore, it would be very interesting to thoroughly investigate the cellular effects determined by combining auranofin and selenium with the future goal of to achieving a preclinical and clinical development plan .

Thus, in collaboration with Prof. M.Björnstedt of the Pathology Department of the Karolinska Institutet Hospital in Stockholm, we investigated the effects of sodium selenite and potassium tellurite alone or in combination with auranofin in human ovarian cancer cells.

In particular, we thoroughly investigated the combined effects in terms of cytotoxicity and modification of TrxR activity or expression. The attention has been focused on the analysis of combined treatment effects on Trx, sulphidryl and glutathione redox states as well as on cell death induction.

Furthermore, recently other phosphine gold(I) complexes have been proposed as potential anticancer drug acting through TrxR inhibition. With the aim at obtaining new potent and selective TrxR inhibitors, we have studied different newly synthesized gold(I) complexes containing phosphine ligands.

The linear structure of auranofin and especially the lability of the thioglucose group appears to play a peculiar role both in the pharmacokinetic profile and in the cytotoxic effects of this molecule. It was also shown that phosphinegold(I) thiolates were more cytotoxic than corresponding gold(I) thiolates, indicating the importance of the phosphine ligand for cytotoxic activity. Consequently, a series of 'auranofin-like' gold(I) complexes maintaining the $[\text{Et}_3\text{P-Au}]^+$ moiety intact and replacing the thioglucose anion with other mono-dentate ligands having binding potential to gold(I) have been synthesized by the research group of Prof. F. Tisato of the ICIS-CNR of Padova [222]. This series comprises of common gold(I) precursors such as $[\text{Et}_3\text{P-Au-Cl}]$ and $[\text{Et}_3\text{P-Au-Br}]$ along with complexes containing diethyldithiocarbamate (dedc), xanthate (xant), thiocyanate (SCN), cyanate (CN) and thiourea (tu) (Fig. 27). The choice of these co-ligands was based on the rationale to optimize or customize TrxR inhibition (if any) by changing the thioglucose residue of auranofin, and on the availability of relatively simple synthetic procedures already reported in the literature for the preparation of linear phosphine gold(I) complexes. However, some gold(III)-DTC complexes have recently demonstrated an outstanding potential as anticancer agents able to overcome cisplatin resistance [130]. In addition, phosphine gold(I) dithiocarbamates and phosphine gold(I) xanthates have previously proved to possess anti-arthritis and in vitro antitumor activities [223]. Thiocyanate and cyanide represent gold ligands involved in human metabolism of auranofin like gold(I) complexes whose major pathway implicates myeloperoxidase [224].

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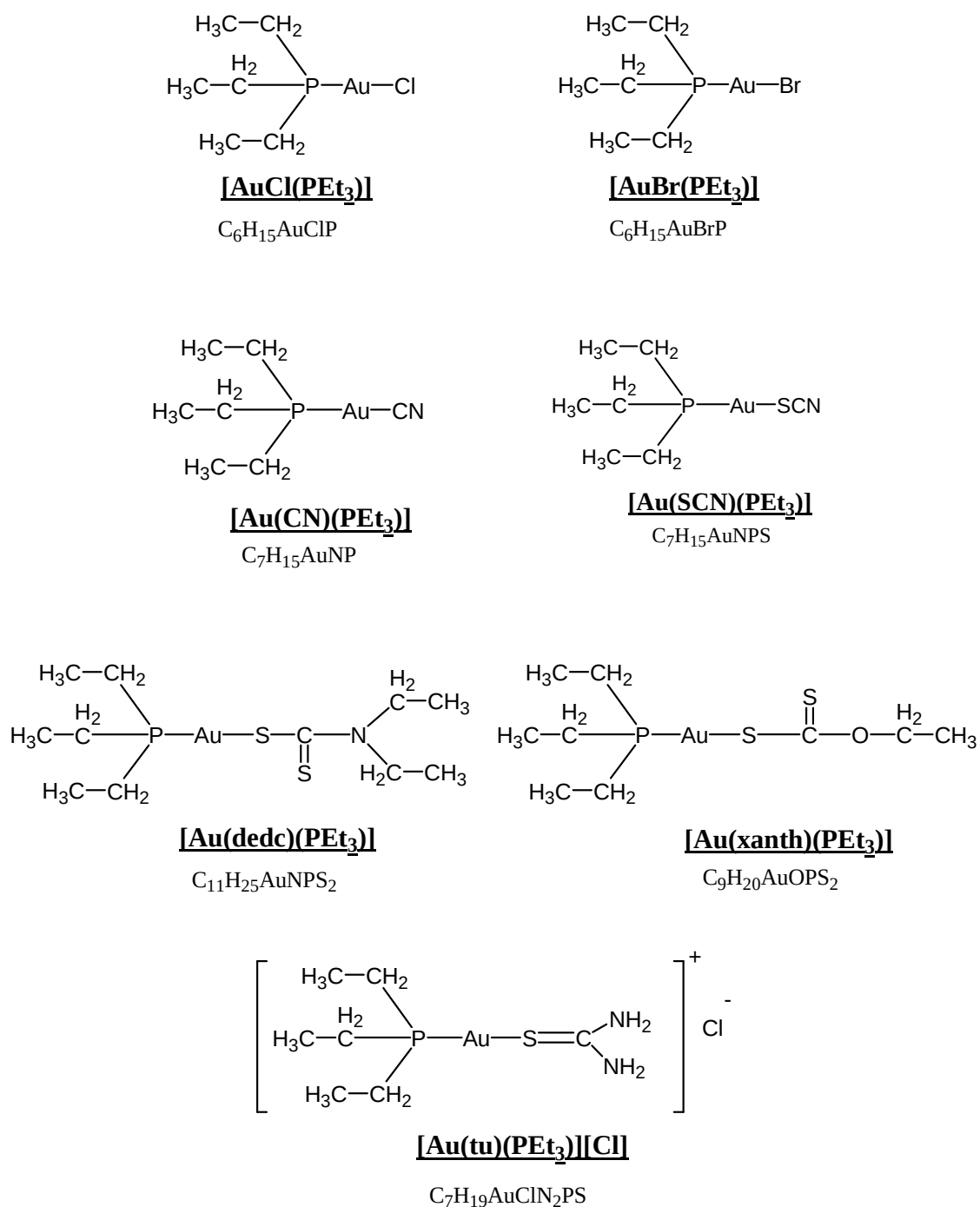


Fig. 27. New phosphine gold(I) complexes

The antitumor properties of these gold(I) compounds towards a wide panel of human cancer cell lines also including cisplatin resistant and multidrug resistance (MDR) phenotypes were assessed. Gold(I) complexes were evaluated for their capacity to inhibit cytosolic and/or mitochondrial isoform of TrxR, both *in vitro* (in a cell-free system) and in ovarian cancer cells. In addition, the inactivation of the closely related flavoenzymes glutathione reductase (GR) and glutathione peroxidase (GPx) was detected with the aim of assessing gold(I) complex selectivity. Finally, reactive oxygen species (ROS) production and triggering of apoptosis in treated cancer cells, consequent to cellular TrxR inhibition, have been investigated.

In the wake of the success gained by auranofin, the first phosphine complex introduced into the clinical practice, presently extensive investigations are focused on the biological chemistry and anticancer properties of phosphines and their related metal complexes. In particular, phosphine complexes of group 11 metals, other than gold, have been proposed as potential anticancer drugs [187].

A rational extension of phosphine gold(I) chemistry to the first row congener copper indicated that analogous copper(I) complexes of 'CuP₄' stoichiometry such as [Cu(dppe)₂][Cl] and [Cu(dppey)₂][Cl] (dppey = 1,2-bis(diphenylphosphino)ethylene) produced cytotoxic effects on cell metabolism comparable to those exhibited by Au(I) analogs. This behaviour was finally postulated to be the result of uncoupling of mitochondrial oxidative phosphorylation [190].

However, the presence of several phenyl groups appended to the phosphorus donors in both [Cu(dppey)₂][Cl] and [Au(dppe)₂][Cl] species caused undesired nephrotoxicity and cardiovascular toxicity [225] in animal models, respectively, thus precluding clinical trials in humans.

In an effort to overcome the biological drawbacks above described, while maintaining the stability of the Cu(I) moiety through the presence of residual Cu-P bonds, partial substitution of the CuP₄ coordination sphere with more hydrophilic ligands was attempted. Among these derivatives, [Cu₂(dppe)₃(CH₃CN)₂][ClO₄]₂ exhibited potent *in vitro* cytotoxicity in H460 human lung carcinoma cells, comparable to that displayed by adriamycin [226]

Following a similar 'mixed-ligand' approach, several N₂-scorpionate ligands have been attached to a coordination vacant lipophilic 'CuP₂' moiety (P₂ = bidentate dppe or two monodentate arylphosphines) [146]. The use of bis(nitrotriazolyl)borate [H₂B(tz^{NO2})₂] scorpionate co-ligands had the double aim of increasing the water solubility and the kinetic inertness of the resulting mixed-complex. These less hydrophobic copper(I) {[H₂B(tz^{NO2})₂]Cu[(PR₃)₂]} compounds (R = *m*-tolyl, *p*-

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fluorophenyl,) were proved to be easier to handle during the in vitro tests, and they retained cytotoxic activity against a wide panel of human tumor cell lines [227].

In this field, our attention has been focused onto new triazole-based scorpionate copper(I) complexes containing the tris(hydroxymethyl) phosphine coligand (Fig. 28).

These complexes were synthesised and characterized in collaboration with the research group of Prof. C. Santini of the Dept. of Chemistry of the University of Camerino.

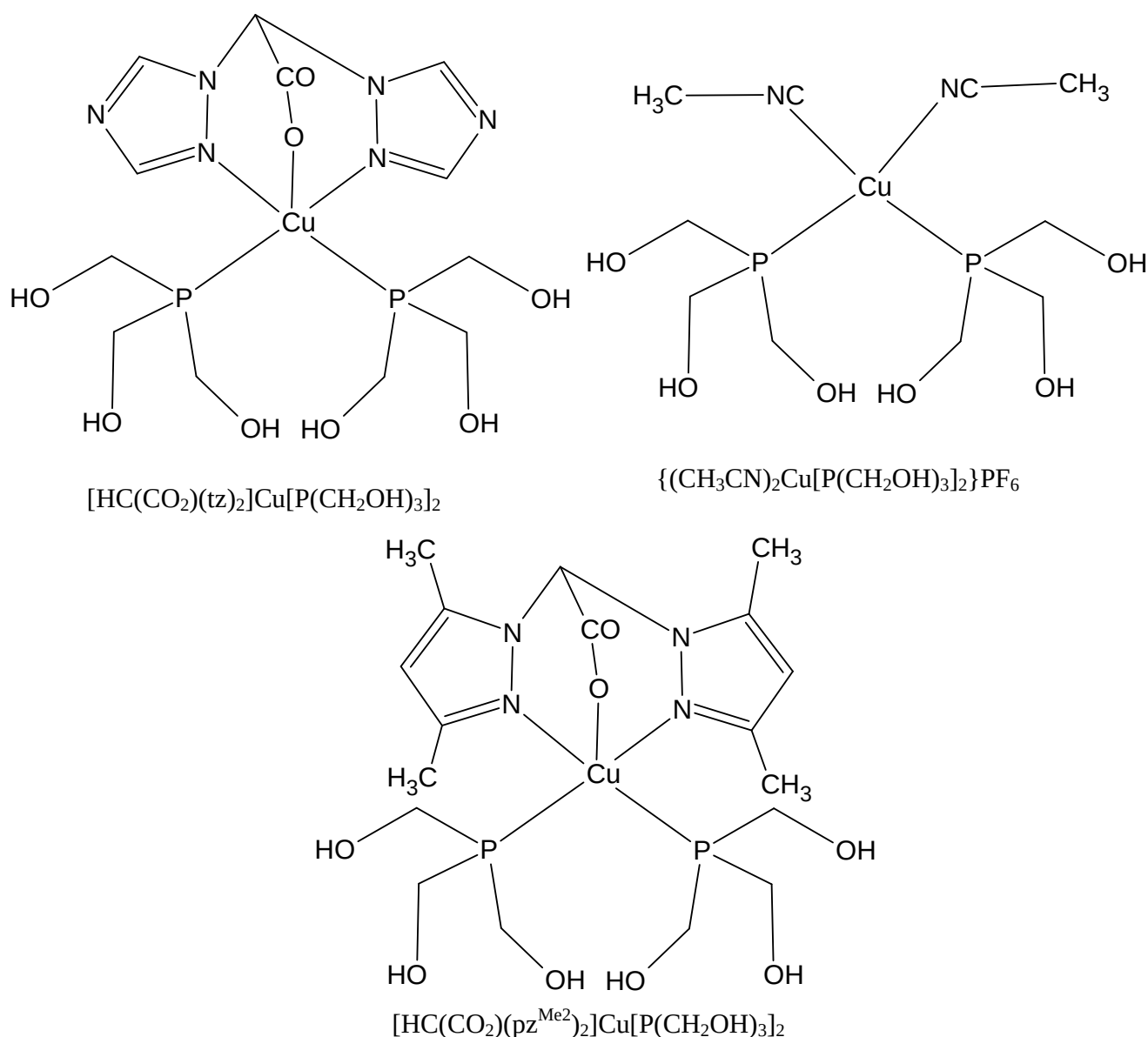


Fig. 28. Triazole-based scorpionate copper(I) complexes containing the tris(hydroxymethyl) phosphine coligand.

We have investigated the cytotoxicity profile of these copper(I) complexes against a panel of human tumor cell lines, also including cisplatin-resistant ovarian (C13*) and cervix (A431-Pt) carcinoma cells in which different molecular mechanisms underlie the resistance [59, 114]. Moreover, DNA flow cytometric analysis and biochemical investigations were performed to study the effects on the cell cycle and the induction of apoptosis.

Our results suggested that the substitution of hydrophobic phosphines (e.g., dppe or triphenylphosphine) with hydrophilic tris(hydroxymethyl)phosphines did not affect the stability of the resulting mixed Cu(I) complexes, and allowed for the preparation of water soluble species with improved cytotoxic properties.

Hence, taking advantage of the ideal aqueous solubility and stability toward disproportionation shown by tris(hydroxymethyl)phosphine (thp) containing copper(I) mixed compounds, we came back to the original 'CuP₄' type species preparing the fully hydroxymethylphosphine complexes [Cu(thp)₄][PF₆] and [Cu(bhpe)₂][PF₆] (bhpe = bis[bis(hydroxymethyl)phosphino]ethane) (Fig. 29).

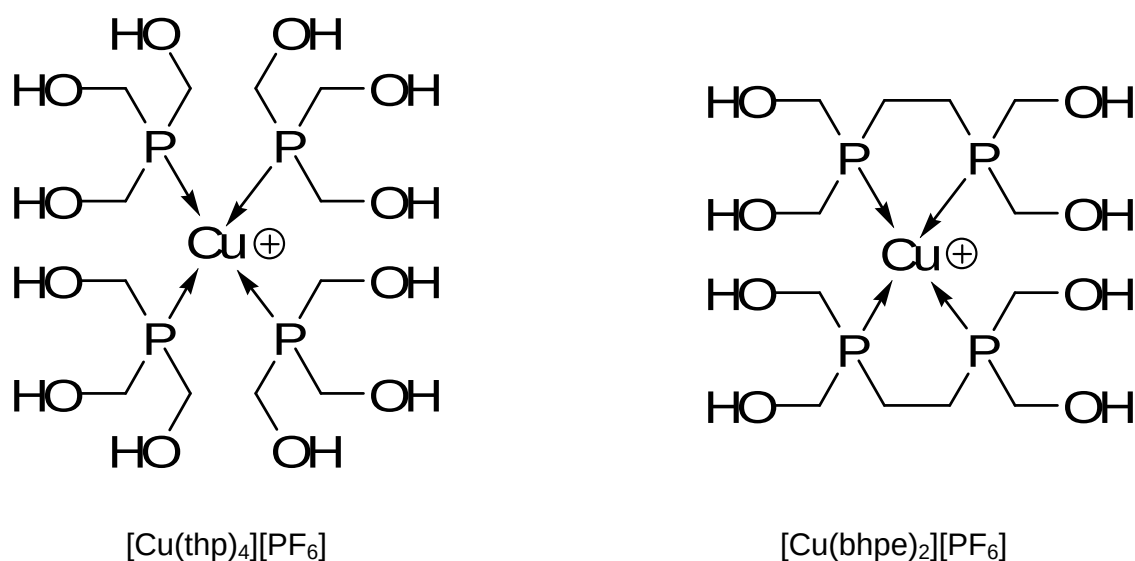


Fig. 29. Hydroxymethylphosphine copper(I) complexes.

They showed high solubility and stability in aqueous solutions. The excellent hydrophilic character of this class of copper(I) complexes represents a very interesting feature that may help to overcome the undesired cardiotoxicity exhibited by previously investigated bis(arylphosphine) copper(I) and gold(I) compounds [225]. We have assessed the antitumor properties of these copper complexes in

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a large panel of human cancer cell lines belonging to a variety of different tumor types and also including cisplatin and multidrug resistance (MDR) phenotypes.

In order to thoroughly explore the sequence of cellular events responsible for the antiproliferative action of these copper(I) complexes, we have investigated the effects on cell cycle modulation and the cell death mechanism by means of flow cytometric analysis.

Particular attention has been paid to cell death pathway triggered by cells treated with these new copper (I) derivatives.

2.1 Chemicals

Just before the experiments cisplatin, auranofin and gold(I) complexes were dissolved in DMSO while copper complexes and the corresponding uncoordinated ligands were dissolved in purified water. Calculated amounts of drug solution were then added to PBS or to the cell growth medium, to a final solvent concentration of 0.5%, which had no discernible effect on cell killing. Cisplatin, amtimycin, Na-selenite, K-tellurite, neutral red dye, ethidium bromide, trypan blue, DTNB, EGTA, Hepes buffer, MTT, NAC, SDS, Hoecsth 33342, propidium iodide, RPMI 1640, DMEM, MEM, Ham's and McCoy's media were obtained from Sigma. TMRM and Platinum SYBR Green qPCR super mix were procured from Invitrogen (Eugene, OR). Auranofin was obtained from Alexis Biochemicals (Lausen, Switzerland). Fetal calf serum, trypsin (0.25%), penicillin/streptomycin solution and L-glutamine were purchased from Euroclone (Berlin, Germany). Antiprotease cocktail Complete and the ELISApus cell death detection kit were procured from Roche (Penzberg, Germany). zVADfmk, CM-DCFDA, DHR and Ribogreen RNA Quantification Kit were obtained from Molecular Probes-Invitrogen (Eugene, OR). Focus™-Mitochondria kit were obtained from GENO-Technology Inc. (St. Louis, MO, USA). Rneasy Mini Kit and Omniscript Reverse Transcription Kit were obtained from Qiagen (Hilden, Germany). ApoAlert Caspase-3 Fluorescent Assay Kit was purchased from Clontech (Mountain View, CA, USA). All other reagents and solvents were of high purity and were used as purchased without any further purification.

2.2 *In vitro* studies

2.2.1 Enzyme inhibition

Isolation and purification of thioredoxin reductases from rat liver cytosol and mitochondria. TrxR1 was prepared according Luthman and Holmgren [228]. Rat liver cytosol was obtained after centrifugation of the liver homogenate at 45,000g for 1 hour. TrxR2 was purified from isolated liver mitochondria following the procedure of Rigobello et al. [229]. The fractions obtained after the 2', 5'-ADP-sepharose 4B affinity chromatography step were concentrated by ultrafiltration in the presence of 10% n-octyl- β -D-glucopyranoside and dialyzed with 50 mM Tris-HCl buffer (pH 7.5) containing 1 mM EDTA. Proteins of the purified isoforms of thioredoxin reductases were assayed with the Lowry et al. procedure [230].

TrxR1 and TrxR2 inhibition. The assay was performed in 0.2 M Na, K-phosphate buffer (pH 7.4) containing 2 mM EDTA, 0.25 mM NADPH and about 0.5-2 µg of TrxR protein. The reaction was initiated by the addition of 3 mM DTNB to both sample and reference and the increase of absorbance was monitored at 412 nm over about 5 min and at 25 °C. Enzyme activity was calculated taking into account that 1 mole of NADPH yields 2 moles of CNTP anion (reduced DTNB).

GR inhibition. GR activity was estimated at 25 °C in 0.1 M Tris/HCl (pH 8.1) containing 0.2 mM NADPH. Reactions were started by the addition of 1 mM GSSG and followed spectrophotometrically at 340 nm.

GPx inhibition. GPx was estimated at 25 °C in 50 mM Hepes/Tris (pH 7.0) and EDTA 3 mM, 0.3 mM NADPH, 5 mM GSH and 0.25 mM tert-butyl hydroperoxide according to Little *et al.* [230].

2.3 Experiments with mammalian cells

2.3.1 Cell cultures

The following established cell lines were grown in appropriate media supplemented with antibiotics penicillin (50 units ml⁻¹) and streptomycin (50 µM ml⁻¹), 25 mM Hepes buffer, 2 mM L-glutamine and 10 % socomplemented (that is, left at 57°C for 30 min to denaturize complement proteins) fetal calf serum. The growth of all the cell cultures was accomplished at 37 °C under continuous flux of a 5% carbon dioxide and moisture enriched atmosphere. For adherent cells trypsin (0.25 %) was routinely used for subculture.

Adherent tumor cells:

2008 and C13*cells. Cisplatin-sensitive human ovarian cancer cells (2008, Fig. 30a) were derived from a patient with serous cystadenocarcinoma of the ovary and its cisplatin-resistant variant, C13* cells (Fig. 30b), was generated by monthly selection with 1 µM cisplatin [231]. Both cell lines were kindly provided by Prof. Marverti (Dept. of Biomedical Sciences, University of Modena, Italy). Cells grow in RPMI medium and doubling occurs within 24 hours. The resistant factor, that is the ratio between IC₅₀ values calculated for the resistant cells and those arising from the sensitive ones (calculated for 24 hours treatment) is about 13.

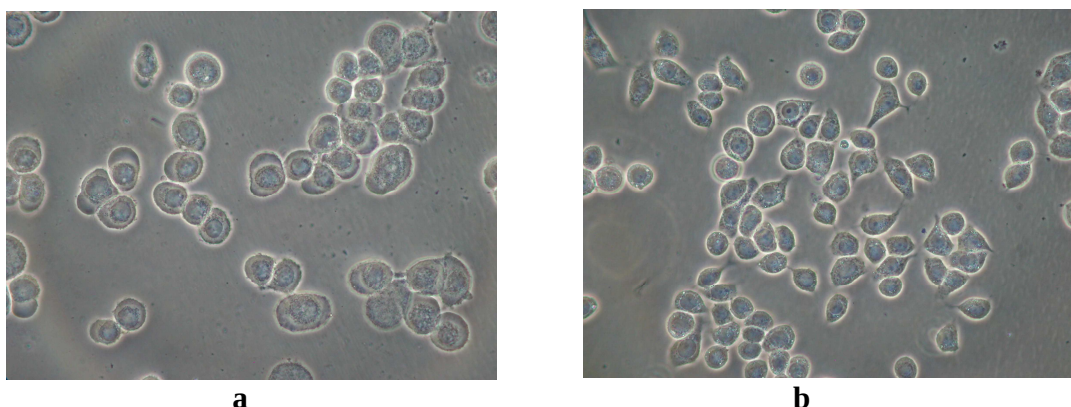


Fig. 30. Phase contrast microscope image of 2008 (a) and C13* (b) cells; same magnification in both pictures (40x)

RH4 cells. Human ovarian adenocarcinoma cells RH4 (kindly provided by Prof. Marverti, Dept. of Biomedical Sciences, University of Modena, Italy, are cisplatin-sensitive cells obtained from C13* after reverting cisplatin resistance. Cells grow in RPMI complete medium and have the same doubling time of parental C13* cells [232].

HeLa cells. Human squamous cervical adenocarcinoma HeLa cells were kindly provided by Prof. F. Majone (Department of Biology, University of Padova, Italy). Cells grow in Ham's-F12 medium and doubling occurs within 48 hours.

A549 cells. Human non-small lung adenocarcinoma A549 cells (American Type Culture Collection, ATCC) are adherent epithelial-type cells in which duplication occurs within 22 hours. This established cell line was cultured in DMEM medium.

A375 cells. Human melanoma cells A375 (ATCC) are cultured in complete DMEM and have a duplication time of 20 hours.

Hep-G2 cells. Human epatocellular carcinoma cells Hep-G2, derived from ATCC, were cultured in DMEM medium. Cells have a doubling time of 28 hours.

A431 and A431/Pt cells. Human squamous cervix carcinoma cisplatin-sensitive A431 (Fig. 31a) and cisplatin-resistant A431/Pt cells (Fig. 31b) were kindly provided by Prof. F. Zunino (Istituto Nazionale per lo Studio e la Cura dei Tumori, Milan, Italy). The cisplatin-resistant A431/Pt cell line was produced by repeated exposure to cisplatin of the sensitive parental A431 cell line [233]. Cell doubling occurs within 24 hours. Cells were maintained in RPMI complete media. R.F. value, calculated for 24 hours treatment, is about 2.4.

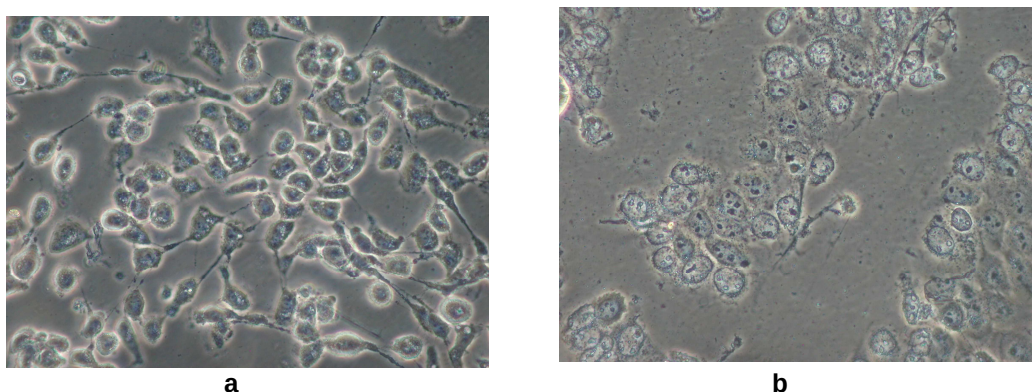


Fig. 31 Phase contrast microscope image of A431 (a) and A431/Pt (b) cells (magnification 40x).

LoVo and LoVo MDR cells. Human colon adenocarcinoma LoVo cells (ATCC, Fig. 32a) have a doubling time of 48 hours. They were cultured in Ham's-F12 complete medium. Derivative multidrug-resistant subline (LoVo MDR cells, Fig. 32b) was kindly provided by Prof. Franca Majone (Department of Biology of Padova University, Italy). LoVo MDR were grown in Ham's-F12 complete medium containing $0.1 \mu\text{g mL}^{-1}$ doxorubicin. R.F. value, calculated for 24 hours treatment, is about 47.12 [234].

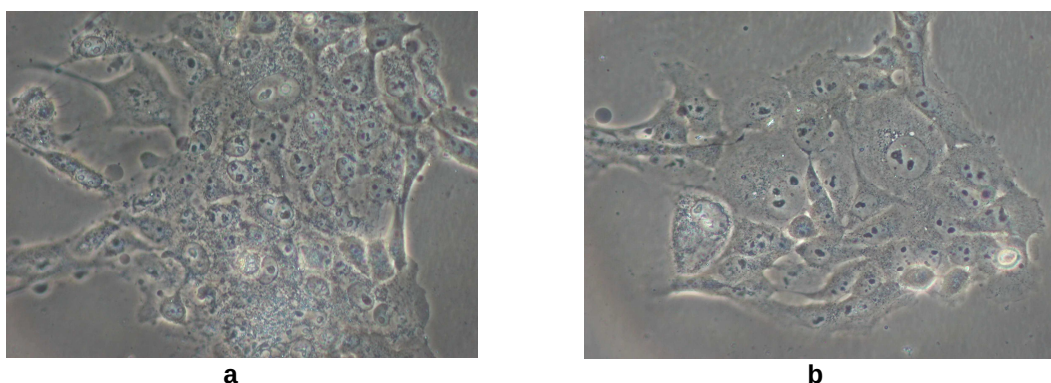


Fig. 32. Phase contrast microscope image of U2OS (a) and U2OS/Pt (b) cells (magnification 40x).

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MCF-7 and MCF-7-ADR cells. MCF-7 breast adenocarcinoma cells (ATCC) are epithelial-type cells in which doubling occurs within 29 hours. This established cell line was cultured in RPMI medium. Derivative acquired drug resistance (ADR) phenotype was kindly provided by Prof. N. Colabufo (Dept. Farmaco-chimico, University of Bari, Italy). ADR phenotype manifests a typical Multi Drug Resistance obtained culturing cells with 0.1 $\mu\text{g mL}^{-1}$ doxorubicin enriched RPMI medium [235].

HCT-15 cells. Colorectal adenocarcinoma cells HCT-15 (ATCC) were maintained in RPMI complete medium and have a doubling time of 20-25 hours.

CaCo2 cells. Colorectal adenocarcinoma cells CaCo2 (ATCC) are epithelial-type cells cultured in MEM complete medium in which doubling occurs within 62 hours.

U-2 OS and U-2 OS/r cell. Human osteosarcoma U-2 OS cells and MDR subline U-2 OS-r cells were kindly provided by Prof. Ruzzene, University of Padova, Italy. Both cells lines were grown in McCoy's medium and doubling time is almost 30 hours. The resistant U2OS-r cell line was produced by repeated exposure to doxorubicin [236].

A2780 and A2780 ADR cells. A2780 and its multidrug-resistant phenotype A2780 ADR are human ovarian adenocarcinoma cells and were kindly provided by Prof. S. Rosato (IOV, Padova, Italy). Doubling occurs in both cell line within 24hours.

Suspension tumor cells:

HL60 cells. Human laeukemic promyelocytes HL60 cells (ATCC) have a doubling time of 24-36 hours and were grown as suspension in RPMI complete medium.

Daudi cells. Human Burkitt's lymphoma Daudi cells (ATCC) have a duplication time of 30 hours and were cultured as above reported for HL60 cells.

U1285 and U1285dox cells. Human small-cell lung carcinoma cells U1285 and U1285dox were kindly provided by Prof. O. Brodin of Karolinka Institutet, Stockholm, Sweden. Cells have a duplication time of 18 hours and were cultured in RPMI complete medium. Resistant subline,

U1285dox cells, have been established from parental line after constant exposure to doxorubicin and were selected to tolerate 1.6 μ M doxorubicin as previously described [237]

Adherent non-tumor cells

CHO cells. Chinese hamster ovary CHO cells (kindly provided by Prof. F. Majone) are diploid adherent cells, with a duplication time of 18 hours. Cells were grown in Ham's-F12 complete medium.

V79 cells. Chinese hamster fibroblasts V79 (kindly provided by Prof. F. Majone) are triploid adherent cells in which duplication occurs within 16 hours, and they were cultured as above reported for HeLa cells.

HFF-1 cells. HFF-1 cells (ATCC) are human fibroblasts with a duplication time of 24 hours. Cells grow in DMEM complete medium.

2.3.2 Cellular *uptake* experiments

Cellular accumulation was detected differently for auranofin and other metal complexes.

Auranofin. 2008 or C13* cells ($3 \cdot 10^6$) were seeded in 10 cm Petri dishes in growth medium (10 ml). After 24 h the medium was replaced and the cells were incubated for 1, 3, 6, or 12 hours in the presence of increasing concentrations of auranofin. Cell monolayers were washed twice with cold PBS, harvested, and resuspended in bidistilled water. After 24 hours, cell suspensions were sonicated for 15 min and deproteinized with methanol and the supernatants were dried in a HETO VAC VR-1 apparatus (Denmark) and used for auranofin determination. The samples were treated with 50 mM phosphate buffer, 50 μ M DTNB, and 0.3 M potassium iodide at 60 °C for 30 min [238]. The increase of absorbance due to the interaction between acetylthiogluconate released from auranofin and DTNB was monitored by measuring the absorbance at 412 nm using a microplate reader. Aliquots were removed for the determination of protein content by the Lowry *et al.* assay [230]. Cellular auranofin levels were expressed as microgram acetylthiogluconate per milligram protein.

Other metal complexes. Briefly, $2 \cdot 10^6$ 2008 or C13* cells were seeded in 75 cm² flasks in growth medium (20 ml). After 24 hours, the medium was replaced and the cells incubated for different

time-points (6, 12, 18, 24 or 36 hours) in the presence of tested complexes. Cells monolayers were washed twice with cold PBS and harvested. Samples were subjected to three freezing/thawing cycles at -80 °C, and then vigorously vortexed. Aliquots were removed for the determination of protein content by the Lowry assay [230]. The samples were added of 5 ml 65% nitric acid and transferred into a microwave teflon vessel. Subsequently, samples were submitted to following procedure using a speed wave MWS-3 Berghof instrument (Eningen, Germany):

- o I step: 160°C x 5 min – slope 2 min – power 80%
- o II step: 190°C x 15 min – slope 1 min - power 80%
- o III step: 100°C x 20 min – slope 1 min - power 0%

After cooling, each mineralized sample was poured into plastic vial and diluted to 10 ml with ultra pure water. The determinations of Cu, Au, Ag and Zn were performed by using an axial ICP-OES Liberty, Series II, Varian (Palo Alto, CA, USA), at the wavelengths 324.754, 242.795, 328.068 and 213,856 nm, respectively. The calibration curve was obtained using known concentrations of standard solutions.

2.3.3 MTT test

The growth inhibitory effect toward tumor cell lines was evaluated by means of the MTT test [237]. This test is widely used for the evaluation of cell vitality in many drug-screening protocols and gives a precise indication on mitochondrial function, thus allowing to discriminate between living and dead cells [239]. The evaluation of the cell viability by MTT test is based on the ability of the mitochondria succinate-tetrazolium reductase enzyme to convert the yellow salt MTT to blue formazan crystals. After dissolution of these insoluble crystals, a spectrophotometric reading is made; formazan solution absorbs light at 550-570 nm and the obtained optical density is directly proportional to the number of living cells. Mean absorbance for each drug dose was expressed as a percentage of the control untreated well absorbance and plotted vs drug concentration. IC₅₀ values represent the drug concentrations that reduced the mean absorbance at 570 nm to 50% of those in the untreated control wells (concentration of chemical resulting in 50% inhibition of cell growth).

Briefly, 3-8 · 10³ cells/well, dependent upon the growth characteristics of the cell line, were seeded in 96-well microplates in growth medium (100 µL) and then incubated at 37°C in a 5% carbon dioxide atmosphere. After 24 hours, the medium was removed and replaced with a fresh one containing the compound to be studied at the appropriate concentration. Triplicate cultures were established for each treatment. After treatment, each well was treated with 10 µL of a 5 mg mL⁻¹

MTT saline solution, and after 5 hours of incubation, 100 μ L of a SDS solution in HCl 0.01 M was added. After overnight incubation, the inhibition of cell growth induced by the tested complexes was detected by measuring the absorbance of each well at 570 nm using a Bio-Rad 680 microplate reader (Hercules, CA, USA).

2.3.4 Trypan blue dye exclusion

Trypan blue is one of the stains recommended for use in dye exclusion procedure for viable cell counting; this method is based on the principle that alive cells do not take up the dye, whereas dead cells do [240]. In fact, trypan blue is a polar dye that cannot cross intact cell membranes but crosses the membranes of necrotic cells and apoptotic cells which are undergoing secondary necrosis.

Cells ($2 \cdot 10^5$ cells ml^{-1}) were seeded in 5 cm Petri dishes in RPMI growth medium (4 ml) and incubated for different time-points in presence of different concentrations of the compounds to be tested. Cells were then incubated for 2 min with 0.25 % trypan blue and 5 % fetal calf serum. Viable cells were identified by their ability to exclude dye. At least 100 cells were counted for each experimental point recorded. The controls were triplicate samples of untreated cells, and their viability ranged from 92 % to 98 %. Cell counting was performed by phase contrast microscope.

2.3.5 Neutral Red uptake (NR) assay

The NR assay, measuring the intracellular accumulation of a weak cationic dye, indicates damage to lysosomes and Golgi apparatus. This assay was performed as described by Borenfreund and Peuernner [241] with slight modifications. Briefly, 2008 cells ($3 \cdot 10^3$ cells/well) were seeded in 96-well microplates in growth medium (90 μ L) and then incubated at 37 °C in a 5 % carbon dioxide atmosphere. Twenty-four hours later, following incubation with increasing concentrations of tested compounds for different time-points the medium was removed and replaced with a fresh one containing 50 μ g/mL neutral red dye. After 3 hours of incubation, the dye-containing medium was discarded, each well was washed twice with PBS and 200 μ L of extracting solution (50 % ethanol in acetic acid, 1 %) were added. After 20 min the absorbance of each well at 540 nm using a Bio-Rad 680 microplate reader (Bio-Rad) was detected. Mean absorbance for each drug dose was expressed as a percentage of the control untreated well absorbance and plotted vs drug concentration. IC_{50}

values represent the drug concentrations that reduced the mean absorbance at 540 nm to 50 % of those in the untreated control wells.

2.3.6 Clonal growth test

2008 or C13* cells ($1.5 \cdot 10^5$) were seeded in 5 cm Petri dishes in growth medium (4 ml). After 24 hours the medium was replaced and the cells were incubated for 3 hours in the presence of increasing concentrations of the compounds examined. Triplicate cultures were established for each treatment. Aliquots of 200 cells were seeded in growth medium and incubated for 12 days. The colonies were then stained and counted, discarding colonies with less than 50 cells. The efficiency of clonal growth, that is the ratio between the number of colonies formed and the number of cells seeded, was then calculated.

2.3.7 Caspase-3 activity

Caspase-3 activity was detected with the ApoAlert Caspase-3 Fluorescent Assay Kit according to the producer's recommended procedures. The fluorescent kit detects the emission shift of 7-amino-4-trifluoromethyl coumarin (AFC). Upon proteolytic cleavage of the AFC-substrate conjugate, DEVD-AFC, by Caspase-3, free AFC fluoresces at 505 nm.

Briefly, 2008 cells were seeded in 25 cm² flasks in complete growth medium. After 24 hours cells were treated for 6, 12, 18 or 24 hours with tested compounds. Subsequently, in the amount of 10^6 , 2008 cells were collected, lysed on ice in 50 μ L of lysis buffer for 10 min and then treated with 50 μ L of reaction buffer containing dithiothreitol (DTT) and 5 μ L of caspase-3 substrate solution (Asp-Glu-Val-Asp-7-amino-4-trifluoromethyl-coumarin [DEVD- AFC]). Fluorescence was determined on a Perkin-Elmer 550 spectrofluorometer (excitation 440 nm, emission 505 nm). Caspase-3 activity was expressed as the increase in AFC-emitted fluorescence.

2.3.8 Nuclear DNA fragmentation

Mono- and oligo-nucleosome formation was detected with the ELISA^{plus} Cell Death detection Kit. This colorimetric assay gives a relative immunochemical determination of histone-complexed DNA fragment (mono- and oligonucleosomes) amount in cells after the induction of apoptosis.

2008 or C13* cells (seeded 24 hours before in the amount of 10^4 per well) were treated in a 96-well plate at 37°C for 24 hours with tested compounds. Afterward, the plate was centrifuged for 10 min,

the supernatant removed, and the pellet treated according to the manufacturer instructions of the ELISA^{plus} Cell Death detection Kit. The extent of nuclear fragmentation was measured in a plate reader (Bio-Rad) following absorbance at 405 minus 492 nm.

2.3.9 Cytochrome c release

2008 or C13* cells ($20 \cdot 10^6$) were seeded in 10 cm Petri dishes. After 24 hours cell were treated for different time points (4, 6, 18 and 24 hours) with tested compound at the appropriate concentration. Cells were then collected, washed twice with PBS, and centrifuged at 540 g per 10 min. Afterward, cells were treated with a hypotonic lysis buffer formed by 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 20 mM Hepes/Tris (pH 7.4) added with antiprotease cocktail. The suspension was centrifuged at 12,500 g for 10 min at 4°C and the obtained supernatants were treated with 0.5 mM EGTA and 2.5 mM PMSF and centrifuged for 1 hour at 100,000 g to obtain the cytosolic fraction. Aliquots of the supernatants were subjected to SDS-PAGE (gradient 8 to 18%) followed by Western blotting using an anticytochrome c monoclonal antibody (Biosource International). A peroxidase-conjugated secondary antibody and chemiluminescence were used to detect the immunoreactive bands. Protein content was estimated according to Lowry et al. [230].

2.3.10 Flow cytometric analysis

Cell Cycle analysis. Drug-induced cell cycle effects and DNA fragmentation were analyzed by flow cytometry after DNA staining with propidium iodide (PI), according to Nicoletti et al. [242]. Briefly, 2008 cells ($5 \cdot 10^5$ cells) were exposed for different time points to tested compounds. One mL of a PI solution, containing 50 µg/mL of PI, 0.1 % m/v of Triton X-100 and 0.01 % m/v of sodium citrate, was added to the cells and then incubated for 25 min at 4 °C in the dark. Induced cell death was determined as a percentage of hypodiploid nuclei counted out of the total cell population, measured on a FACSCalibur flow cytometer (Becton-Dickinson, Franklin Lakes, NJ) on a 550-600 nm filter. Analysis was performed by Cell Quest software (Becton-Dickinson).

$\Delta\Psi$ analysis. Mitochondrial membrane potential ($\Delta\Psi$) was determined by measuring the fluorescence of cells stained with the cationic mitochondrial selective dye TMRM. 2008 cells (10^6), following 12 or 24 hours of incubation with tested complexes, were washed once with PBS, harvested, and incubated for 15 min at 37 °C in PBS with TMRM at a final concentration of 10 nM,

freshly prepared from stock solution 10 mM in DMSO. The TMRM signal was analyzed in the FL2 channel, which was equipped with a bandpass filter at 560 ± 20 nm.

2.3.11 Fluorescence microscopy analyses

PI and Hoechst 33342 staining. 2008 cells were seeded into 8-well tissue-culture slides at $5 \cdot 10^4$ cells/well (0.8 cm^2). After 24 hours, cells were treated for an additional 24 hours with tested compounds. Following 20 min of incubation time with Hoechst 33342 at 37°C , cells were added of $1 \mu\text{M}$ PI solution and examined on a Olympus BX41 fluorescence microscope with DAPI filters. Cells were photographed on Olympus digital camera Camedia.

Monodansylcadaverine staining. 2008 cells were seeded and treated as described for PI/Hoechst 33342 staining. Subsequently, were washed three times with PBS and stained with $100 \mu\text{M}$ monodansylcadaverine (a specific marker for autophagic vacuoles) in PBS at 37°C for 30 min. After incubation, the cells were washed four times with PBS and immediately analyzed in an Olympus BX41 fluorescence microscope. Cells were photographed on Olympus digital camera Camedia.

2.3.12 Light microscopy analyses

Mitotic Index (MI) determination. MI was determined as previously described By Huang and Lee [243]. Briefly, 2008 cells were plated at a density of $1 \cdot 10^5$ cells in 5 cm Petri dishes. Next day, they were treated with tested compound. After 12 and 24 hours incubation, cells were treated with colchicine (10^{-4} M), that block cells in metaphase, for 4 h, harvested, washed with cold PBS, and then treated with 1 % sodium citrate for 12 min at room temperature. Cells were fixed with methanol/acetic acid solution (3:1 v/v), spread on clean slides, air-dried, and stained with a 10 % Giemsa solution. Slides were examined by light microscopy (Olympus BX41). At least 150 cells were counted for each concentration, and MI was calculated as the ratio of the number of dividing cells to the total number of cells multiplied by 100.

Hematoxylin-Eosin (HE) staining. Hematoxylin and eosin staining is one of the most widely used techniques in histology and allows for visualization of cellular morphology. Hematoxylin colors basophilic elements (i.e. nuclear chromatin) with a blue-purple hue and eosin colors acidophilic elements (i.e. cytosol, collagen, etc.) red-pink. Briefly, 2008 cells were seeded into 8-well tissue-culture slides at $5 \cdot 10^4$ cells/well (0.8 cm^2). After 24 hours, cells were washed twice with PBS and treated for an additional 24 h with tested compounds. Then, after being washed with PBS, cells

were fixed in ice-cold methanol and air-dried. Slides were then stained with hematoxylin (0.68 %) and eosin (0.5 %) solutions (see following procedure steps) before being examined by light microscopy (Olympus BX41). Cells were photographed on Olympus digital camera Camedia. Staining steps: Distilled water: 5 – 10 dips, Hematoxylin: 1 min, Distilled water 5 – 10 dips, 95 % Alcohol: 5 – 7 dips, Eosin-Y 15 seconds, 95 % Alcohol 5 – 7 dips, 100% Alcohol 5 – 7 dips, 11 Xylene 5 – 7 dips.

2.3.13 Reactive oxygen species (ROS) production

ROS production was detected by means of two fluorescent probe, CM-H₂DCFDA and DHR. Probes are non-fluorescent the oxidation occurs within the cell increasing their fluorescence.

The production of ROS was measured in 2008 or C13* cells (10⁴ per well) grown for 24 h in a 96-well plate in RPMI without phenol red. Cells were then washed in PBS and loaded with 10 μ M CM-H₂DCFDA or 50 μ M (DHR) for 25 min, in the dark. Afterward, cells were washed with the same medium and incubated with IC₅₀ concentration of tested compounds. Fluorescence increase was estimated utilizing the wavelengths of 485 nm (excitation) and 527 nm (emission) in a Fluoroskan Ascent FL (Labsystem, Finland) plate reader. Antimycin (3 μ M), a potent inhibitor of Complex III in the electron transport chain, was used as positive control.

2.3.14 Poly-ubiquitinated protein detection

Western blot analysis. 10⁶ 2008 cells were seeded in 10 cm Petri dishes. After 24 hours, cells were treated for 24 hours with tested compounds at the appropriate concentration. Then, cells were washed in cold PBS and lysed in RIPA buffer (1 % NP40, 0.5 % sodium deoxycholate, 0.1% sodium dodecyl (lauryl) sulfate), sonicated 10 s and centrifuged at 13 000 g for 15 min at 4 °C. Following, samples were electrophoresed on a 12 % sodium dodecyl sulfate-polyacrylamide gel and electroblotted to a nitrocellulose membrane. The membrane was incubated for 1 h in PBS-Tween 20 (0.05 %) containing 5 % non-fat milk. After incubation with anti-ubiquitin and anti- β -actin primary antibodies (Abcam, Cambridge, MA, USA), the membranes were stained with the corresponding peroxidase-conjugated secondary antibody and chemiluminescence were used to detect the immunoreactive bands. Protein content was estimated according to Lowry et al. [230].

Immunostaining. Determination of poly-ubiquitin aggresomes was assessed as previously described by Ding et al. [244]. Briefly, 2008 cells (5 · 10⁴ cells/well) were plated on 8-well slides.

Following designated treatments, cells were fixed with 4 % formaldehyde in PBS at room temperature for 10 min, washed three times with PBS, permeabilized in 0.2 % Triton X-100 in PBS at room temperature for 10 min, and placed in methanol at -20 °C for 30 min. Subsequently, samples were washed with PBS, incubated at 37 °C with 2 % bovine serum albumin (BSA) to block nonspecific sites for 1 hour, and then with a mouse monoclonal anti-ubiquitin antibody (diluted 1:500 in PBS) at 37 °C for 1 h. Slides were washed three times with PBS and incubated with a Tetramethyl Rhodamine Isothiocyanate (TRITC)-conjugated rat antimouse secondary antibody (diluted 1:200 in 2 % BSA) at 37 °C for a further 1 h. Slides were then washed repeatedly with PBS, mounted with mounting medium and analyzed in an Olympus BX41 fluorescence microscope equipped with an Olympus Camedia digital camera (excitation 550, emission 570).

2.3.15 Proteasome inhibition

10⁶ 2008 cells were seeded in 10 cm Petri dishes. After 24 hours cells were treated for 48 hours with tested compounds at the appropriate concentration. Subsequently, cell were lysed and supernatants were stained with fluorescent proteasome substrates, N-Suc-Leu-Leu-Val-Tyr-AMC or Z-Leu-Leu-Glu-AMC, specific for proteasomal chymotryptic and peptidylglutamyl-hydrolyzing activity, respectively. Free AMC fluorescence was recorded (ex: 355 , em: 460).

2.3.16 Cell subfractionation

2008 or C13* cells cultured in complete medium were grown to 80 % confluence in 75 cm² flasks and then treated with tested compounds. After incubation, cells were washed twice in PBS containing 10 mM glucose, and mitochondria were then isolated with the FocusTM-Mitochondria kit. To obtain the cytosolic fraction, supernatant from the mitochondrial step was centrifuged at 100,000 x g for 1 hour.

2.3.17 Cell determination of thioredoxin reductase, glutathione reductase and glutathione peroxidase activities.

2008 and C13* cells (8 · 10⁶) were seeded in 75 cm² flasks in RPMI medium. 24 hours later, cells were treated with tested compounds for 48 hours. The medium and reagents were refreshed after 24 hours. Cells were trypsinized, washed in PBS, centrifuged and the obtained pellet dissolved in RIPA buffer modified as follows: 150 mM NaCl, 50 mM Tris-HCl, 1 % Triton X-100, 1 % SDS, 1 %

DOC, 1 mM NaF, 1 mM EDTA, and immediately before use, an antiprotease cocktail (Roche) containing PMSF was added. - Thioredoxin reductase was assayed in 0.2 M Na, K-phosphate buffer (pH 7.4) containing 2 mM EDTA, 0.25 mM NADPH and about 70-80 µg proteins/ml protein. The reaction was initiated by the addition of 3 mM DTNB to both sample and reference and the increase of absorbance was monitored at 412 nm over about 5 min and at 25 °C. Enzyme activity was calculated taking into account that 1 mole of NADPH yields 2 moles of CNTP anion (reduced DTNB). Glutathione reductase activity was estimated on 40-50 µg protein/ml at 25 °C in 0.1 M Tris/HCl (pH 8.1) containing 0.2 mM NADPH. Reactions were started by the addition of 1 mM GSSG and followed spectrophotometrically at 340 nm. Glutathione peroxidase activity was estimated at 25 °C in 50 mM Hepes/Tris (pH 7.0) and EDTA 3 mM using 180 µg proteins/ml, 0.3 mM NADPH, 5 mM GSH and 0.25 mM tert-butylhydroperoxide.

2.3.18 Determination of the redox state of thioredoxin

Cells ($2.5 \cdot 10^5$) were incubated with selenite, tellurite or auranofin as indicated in the proper legend. Afterwards, the cells were washed with PBS and treated with 6 M guanidine, 0.5 % Triton, 50 mM Tris buffer (pH 8.3), 3 mM EDTA, 50 mM iodoacetic acid and incubated for 40 min at 37 °C. The excess of iodoacetic acid was removed by gel filtration through a MicroSpin G-25 column (GE Healthcare, Little Chalfont, UK). The oxidized and reduced forms of thioredoxin were separated by Western blotting with native, nondenaturing (15 %) PAGE in 25 mM Tris/0.192 glycine buffer (pH 8.3). Proteins were electroblotted with 0.1 M glycine, 20 % methanol buffered at pH 9.6 with ethanolamine and immunodetected with a mouse anti thioredoxin 1 antibody (Histoline, LabFrontier, Seoul, Korea).

2.3.19 Real-time reverse-transcriptase-polymerase chain reaction (RT-PCR)

RT-PCR, is a highly sensitive laboratory technique for amplifying a defined sequence of a ribonucleic acid (RNA) molecule. The RNA strand is first reverse transcribed into its complementary DNA, followed by amplification of the resulting DNA using polymerase chain reaction.

2008 and C13* cells were seeded in 75 cm² bottle in complete medium. After 24 hours cells were treated with tested compounds for 18 or 24 hours. After incubation, cells were washed with PBS and total RNA was isolated using the Rneasy Mini Kit, with optional on column DNase 1 digestion

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according to the supplied protocol. RNA quantification was performed using the Ribogreen RNA Quantification Kit. The synthesis of cDNA was performed through reverse transcription on 2µg of RNA using the Omniscript Reverse Transcription Kit with oligo(dT)₁₂₋₁₈ as a primer (final concentration 0.1µg/µl). Real-time quantitative PCR was then used to compare the total levels of TrxR1 and TrxR2 mRNA (for primers, see Table II). RT-PCR was performed on a BIO-RAD ICycler (Bio-Rad, Hercules, CA) with 20–50 ng of cDNA/reaction in triplicate on 96-well plates using Platinum SYBR Green qPCR super mix. The final volume for each reaction was 25 µl with a primer concentration of 300 nM. The PCR program used was 50° 2min, 95° 2min, 95° 15 s, 60° 1min (50 cycles) or 50° 2min, 95° 2min, 95° 15sec, and 64° 1min (50 cycles) for the γ primers. β-actin was used as an endogenous control. The results were analyzed using the 2^{-δδC_T} method. The C_T-value cut-off was set at 35 cycles. The efficiency of the different primer sets was 90% ± 5%.

Table II Primers used for real-time PCR

	Forward	Reverse
TrxR1	5'-GCCCTGCAAGACTCTCGAAATTA-3'	5'-GCCCATAAGCATTCTCATAGACGA-3'
TrxR2	5'-TCAGAAGATCCTGGTGGACTC C-3'	5'-TCGTGGGAACATTGTCGTAGTC-3'
β actin	5'-ACCTGACTGACTACCTCATGAAGA-3'	5'-GCGACGTAGCACAGCTTCTC-3'

2.3.20 Determination of total and oxidised glutathione

2008 and C13* cells (2 ·10⁵) were grown for 24 hours in a 6-well plate in complete medium and then incubated with tested compounds for 48 hours. After 24 hours treatment, the medium was replaced with fresh one. After incubation, cells were washed with PBS, treated with 6 % metaphosphoric acid, kept on ice for 15 min and centrifuged. Pellet was dissolved in RIPA buffer and utilized for protein determination according to Lowry *et al.* [230]. Aliquots of supernatant were neutralised with Na₃PO₄ and assayed for total glutathione following the procedure of Tietze [245], modified according to Anderson [243] for determination of oxidised glutathione.

2.3.21 Total thiol groups assay

2008 and C13* cells (2 ·10⁵), incubated as in glutathione estimation, were washed with PBS buffer and centrifuged. Supernatants were removed and cells treated with 7 M Guanidine in 0.2 M Tris-

HCl (pH 7.5) containing 10 mM EDTA. Samples were subjected to two freezing/thawing cycles in liquid nitrogen, and then vigorously vortexed and treated with 3 mM DTNB to determine the content of total thiol groups. The increase in absorbance was estimated spectrophotometrically at 412 nm.

2.3.22 Protein profile study

2008 and C13* cells were grown in 75 cm² flasks at 80% confluence and then scraped off from flask, washed with PBS and centrifuged at 1000 x g for 10 min. Cell pellets were kept at -80°C until use.

2D liquid chromatography protein fractionation

For each cell subline, about 10⁷ cells were solubilized in 2.5 ml lysis buffer (50 mM Tris-HCl, pH 8.0, 7 M urea, 2 M thiourea, 4 % (w/v) n-octyl -D-glucopyranoside, 10 mM TCEP and a cocktail of protease inhibitors). The buffer of solubilized proteins was then exchanged using a PD 10 desalting column equilibrated with PF 2D™ Start Buffer and, after centrifuging at 400,000xg for 15 min at 20 °C, samples were immediately injected onto the first dimension chromatographic column.

Total protein amount for each experiment was 4.3 mg ± 0.2 mg.

First dimension protein fractionation. PF 2D™ Start Buffer and PF 2D™ Eluent Buffer were adjusted to pH 8.5 with 1.0 M NH₄OH and to pH 4.0 with a saturated solution (50 mg/ml) of iminodiacetic acid, respectively. Chromatofocusing was carried out at room temperature, at a flow rate of 0.2 ml/min. The sample was loaded when a stable baseline pH (8.5-8.1) was recorded at 280 nm. Protein elution started by switching to the eluent buffer at pH 4.0 twenty minutes after the injection. At the end of this step, 1.0 M NaCl eluted proteins still retained on the column. Fractions were collected every 0.3 pH interval during the gradient, otherwise on the basis of time: however, one ml was the maximum collection volume.

Second dimension protein fractionation: reverse phase chromatography. Aliquots of 0.2 ml of each chromatofocusing fraction were resolved on a C18 reverse phase column by a 30-min gradient to 100% ACN containing 0.08 % (v/v) TFA at 50 °C. Elution absorbance profile was recorded at 214 nm and fractions were collected every 0.5 minute (0.375 ml).

Differential expression analysis

Alignment of corresponding peaks. Absorbance data from second dimension chromatographies were used for analysis with ProteoVue™ and DeltaVue™ programs.

Absorbance profiles of fractions of comparable pH (i.e. within 0.15 pH units' difference) of 2008 and C13* cells were matched peak-by-peak, so generating differential maps and sets of

MATERIALS AND METHODS

corresponding data. Variation of at least twice as much of the ratio between heights of corresponding peaks was considered consistent with a significant difference in protein expression between 2008 and C13* cells.

3. Gold Complexes

3.1. Auranofin

3.1.1 Auranofin effects in cisplatin sensitive and resistant cancer cells

Cytotoxicity of auranofin on human ovarian cancer cells sensitive (2008) and resistant (C13) to cisplatin.*

The cytotoxicity of auranofin on the ovarian cisplatin sensitive cell line (2008) and on its resistant subline (C13* cells) was studied by the MTT test. Fig. 33 (a–d) shows the dose response profiles and the corresponding IC_{50} values obtained incubating sensitive and resistant cells with increasing concentrations of auranofin or cisplatin for different exposure times (3, 12, 24, and 48 hours for a to d, respectively). The data indicate that auranofin was markedly more effective than cisplatin in reducing viability of both cell lines even at very low concentrations. By comparing the IC_{50} values, the cytotoxic potency of auranofin exceeded that of the reference drug by a factor of at least 30 in sensitive cells (Figs. 33, b) that increases to about 200 in resistant cells (Figs. 33, d). These results clearly demonstrate that auranofin is able to overcome the acquired cisplatin resistance. Noticeably, auranofin reduced cell viability at short incubation times (3 h, Figs. 33, a) while, on the contrary, cisplatin cytotoxicity became detectable only after at least 12 hours of incubation (Figs. 33, b). These findings strongly support the hypothesis of an action mechanism different from that of the reference drug. In fact, the time-dependent cellular sensitivity to cisplatin is mainly due to cellular DNA damage that requires a longer time interval to induce cell death. Furthermore, at longer incubation times, C13* cells appear to be more responsive to auranofin than 2008 cells (Figs. 33, b–d). This behaviour would be explained, at least in part, as a consequence of a different auranofin intracellular uptake. Indeed, it was previously noted that C13* cells, with respect to parental cells, are characterized by plasma membrane modifications that could be responsible for different drug accumulations [229].

To determine whether cancer cells would resume proliferation upon removal of auranofin, we performed cell growth recovery experiments in which, after incubation with different concentrations of auranofin for 12 hours, medium was removed followed by the addition of fresh growth medium. The obtained data (Fig. 34) indicate that the effect induced by auranofin was not reversed upon its withdrawal, thus suggesting the occurrence of a cytotoxic rather than cytostatic effect.

RESULTS AND DISCUSSION

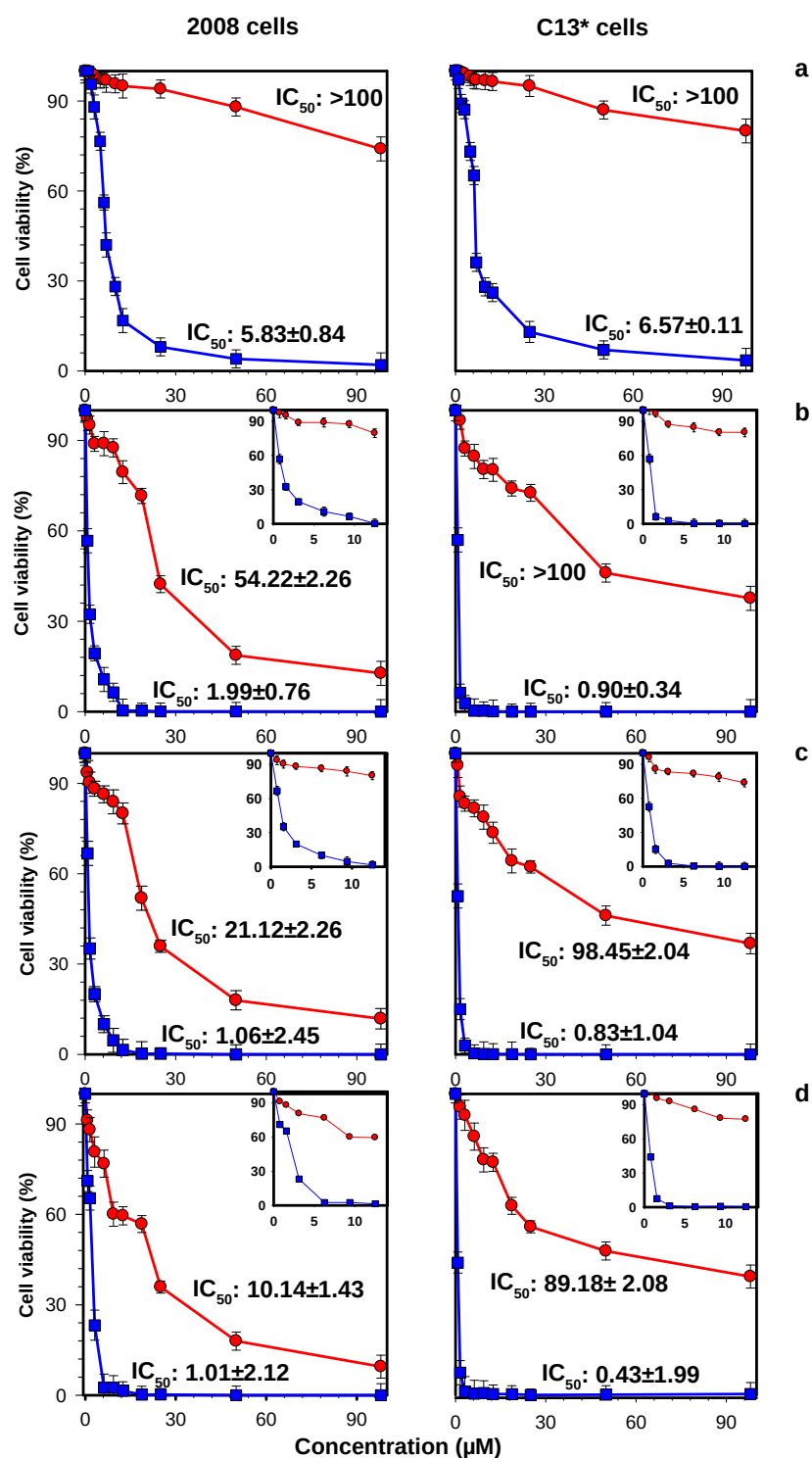


Fig. 33. Sensitivity of cisplatin-sensitive (2008) and resistant (C13*) cells to auranofin (■) or cisplatin (●). Drug exposure with the indicated compounds was for 3 (a), 12 (b), 24 (c), or 48 (d) hours. Cytotoxicity was evaluated by the MTT test.

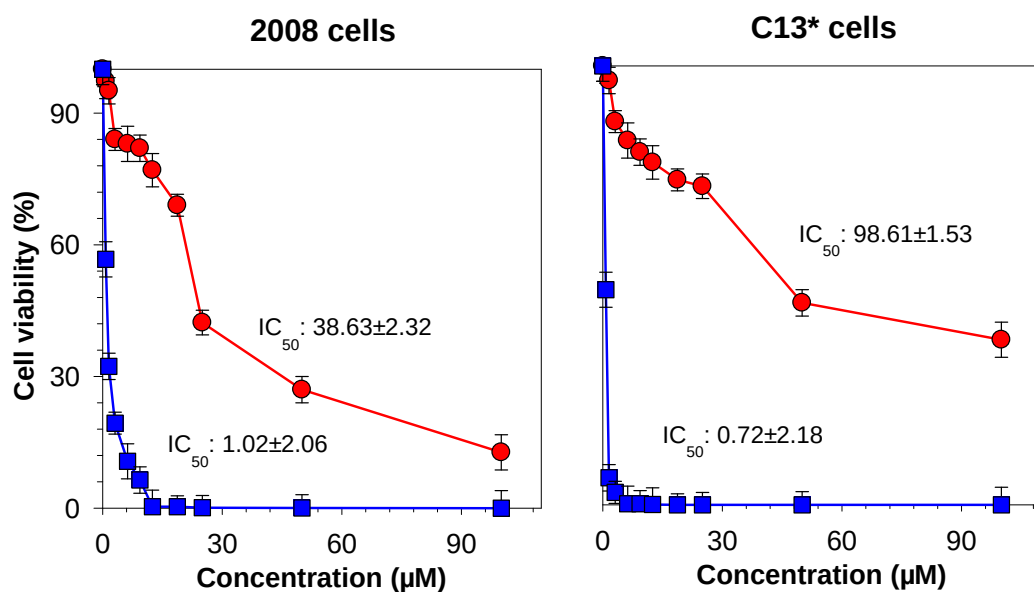


Fig. 34. Cell-growth recovery. Cells were treated for 3 hours with auranofin (■) or cisplatin (●), washed, and reincubated with fresh complete medium for further 48 hours before MTT determination.

Clonogenic assay and cross-resistance profile

In order to study the ability of auranofin to induce delayed reproductive cellular death, we carried out classical clonogenic assays. Cell survival curves, obtained after treating 2008 and C13* cells with increasing concentrations of auranofin or cisplatin for 3 hours, are reported in Fig. 35.

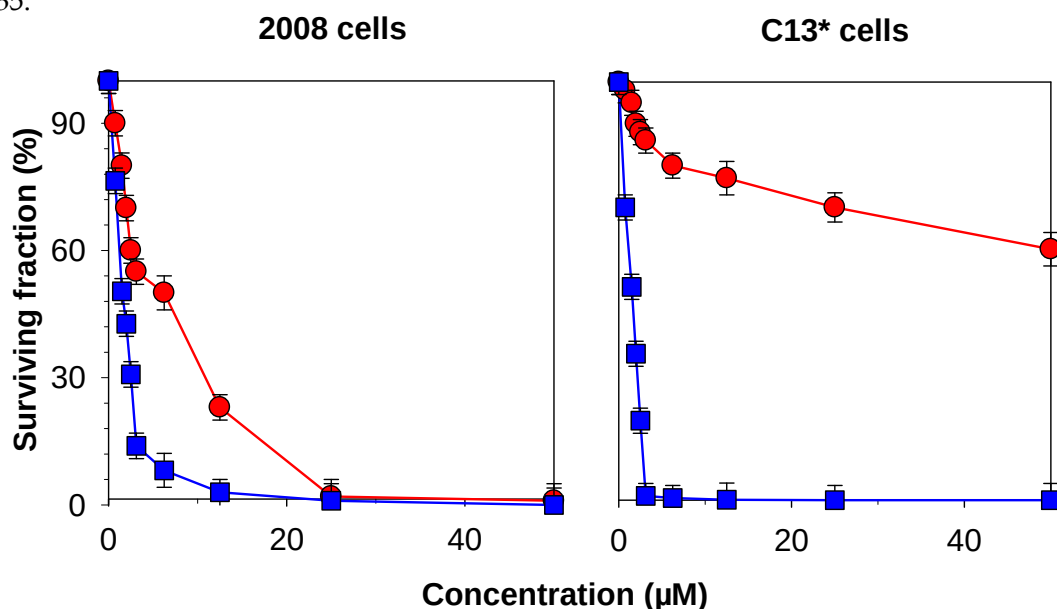


Fig. 35. Clonal growth in 2008 and C13* cells. Cells were treated for 3 hours with increasing concentrations of auranofin (■) or cisplatin (●) and the cell capacity to form clones was assayed.

RESULTS AND DISCUSSION

The inhibitory effect of auranofin on cell proliferation was concentration dependent and markedly higher than that of the reference drug even at low concentrations. In particular, auranofin dramatically reduced C13* cell colony forming ability even at lower concentrations than those required to abort indefinite proliferation in cisplatin sensitive 2008 cells. Table III summarizes the results in terms of IC₅₀ values, detected by probits analysis.

Table III Cross-resistance profiles

Compound	2008	C13*	R.F.
	IC ₅₀ ± SD (μM)	IC ₅₀ ± SD (μM)	
Auranofin	1.41±0.9	1.13±0.77	0.73
Cisplatin	6.51±0.75	114.8±0.84	17.51

SD = standard deviation.

IC₅₀ values were calculated by probit analysis ($P < 0.05$, χ^2 test).

Resistance factor (R.F.) is define as IC₅₀ resistant/parent line

The resistance factor estimated for auranofin was more than 20 times lower than that of cisplatin, clearly rouling out the occurrence of cross-resistance phenomena and suggesting the hypothesis of a different mechanism of action.

Cellular uptake

The cellular uptake of auranofin may play an important role in the observed cytotoxicity and on the intracellular effects of this drug. Fig. 30 shows the results obtained monitoring cellular accumulation of auranofin after 1, 3, 6, and 12 hours of exposure in sensitive (Fig. 36 A) and resistant (Fig. 36 B) cells.

Intracellular auranofin content was assayed by a color-developing reaction with DTNB and normalized to the cellular protein content. Intracellular accumulation was proportional both to the auranofin concentrations used (3 and 6 μM) and to the incubation time in the two cell lines. For short times of exposure (1 hour), cellular uptake was comparable in both parent and resistant sublines, whereas for longer times of exposure (3, 6, and 12 hours) it was noticeably higher in C13* cells. In particular, after 12 hours of exposure, auranofin cellular uptake in C13*

RESULTS AND DISCUSSION

cells was 1.5-fold higher than that detected in 2008 cells. These results correlate well with the cytotoxicity data, thus confirming the hypothesis that different auranofin cytotoxicity profile in the two phenotypes may be depend on its different intracellular accumulation.

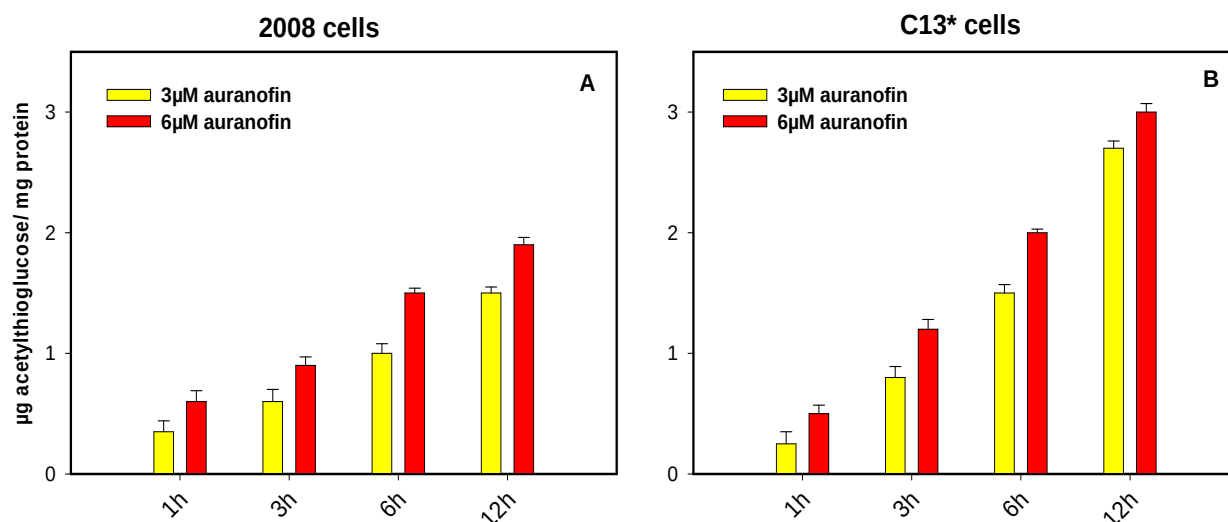


Fig. 36. Uptake of auranofin by 2008 (A) and C13* (B) cells. Cells were incubated for 1, 3, 6, and 12 hours with 3 or 6 μ M auranofin. Total intracellular auranofin content was measured by reaction with DTNB followed spectrophotometrically at 412 nm.

Cytocrome c release

According to Dong et al. [244] cisplatin-resistant cells undergo alterations of the respiratory chain accompanied by a decrease of ATP. These alterations make the cisplatin-resistant cells more easily damaged by the inhibitors of the mitochondrial functions and other agents such as gold compounds. Furthermore, it has been previously shown that mitochondria treated with auranofin at nanomolar concentrations undergo an extensive mitochondrial membrane permeability transition [246] accompanied by the release of cytochrome c [245] and possibly of other apoptogenic factors. Therefore, we have comparatively examined auranofin ability to stimulate in 2008 and C13* cells cytochrome c release (Fig. 37). Cells were treated with 20 μ M auranofin and 30 μ M cisplatin for 4 hours (2008) or 6 hours (C13*) and cytochrome c was estimated in the cytosolic fraction by Western blotting. It is apparent that both auranofin and cisplatin were able to stimulate cytochrome c release in 2008 cells, whereas cisplatin was ineffective on the resistant phenotype. In the latter, however, auranofin was still effective, thus overcoming C13* cell refractoriness in undergoing cytochrome c release and possibly, apoptosis.

RESULTS AND DISCUSSION

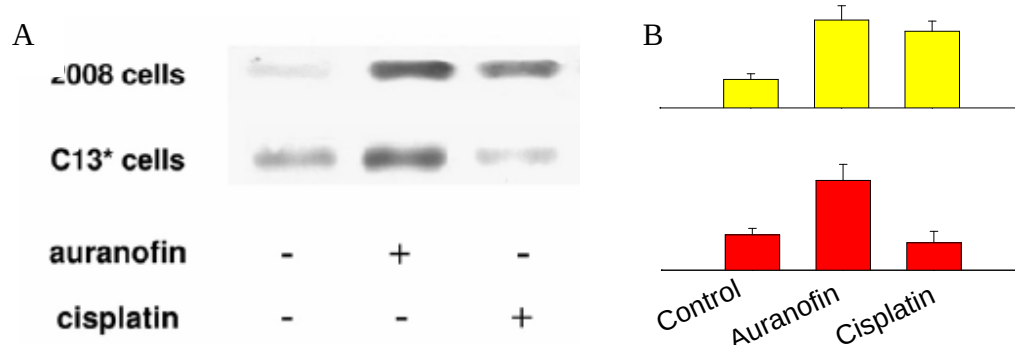


Fig. 37. Effect of auranofin and cisplatin on cytochrome c release in 2008 and C13* cells. Cells were treated with 20 μ M auranofin and 30 μ M cisplatin for 4 hours (2008) or 6 hours (C13*) and cytochrome c was estimated in the cytosolic fraction by Western blotting (A). The images of the immunoblot were analyzed for the intensity of the areas (B)

Nuclear DNA fragmentation

The release of cytochrome c from the mitochondrial intermembrane space to the cytosol, where it gives rise to the apoptosome complex, is a critical step in the apoptotic process [247]. Therefore the extent of apoptosis in 2008 and C13* cells treated with auranofin or cisplatin was measured.

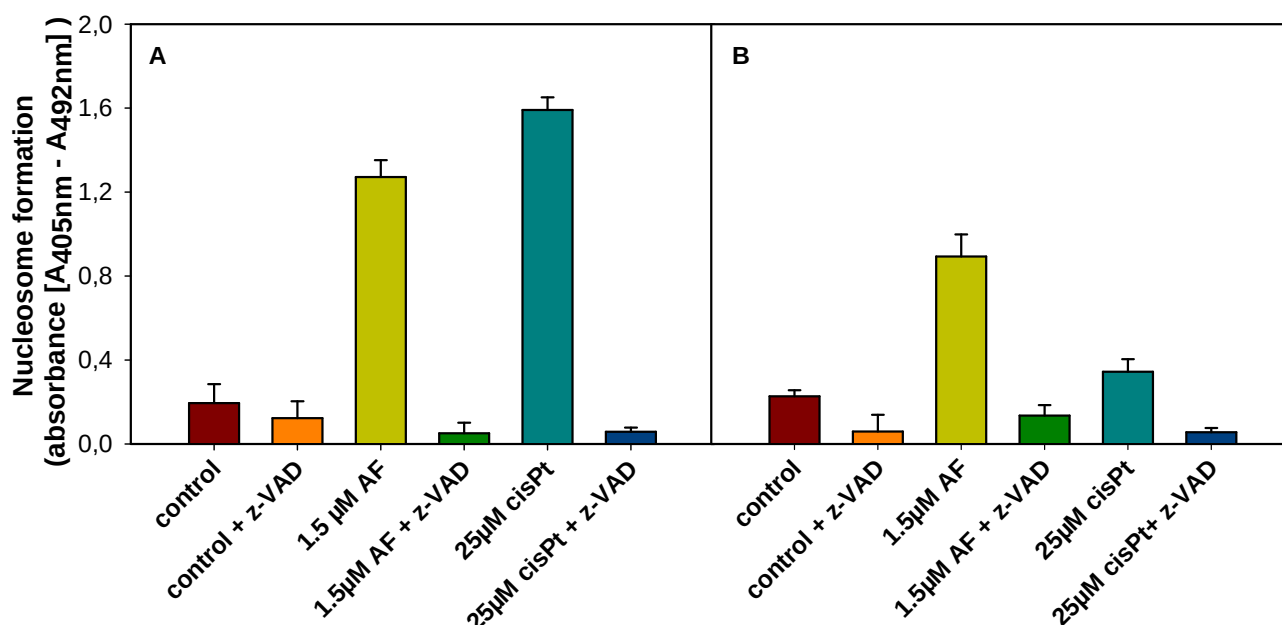


Fig. 38. Nuclear DNA fragmentation in 2008 and C13* cells treated with auranofin or cisplatin. 2008 (A) or C13* (B) cells were treated for 24 hours (2008) or 36 hours (C13*) with, when indicated, 1.5 μ M auranofin (AF), 25 μ M cisplatin (cisPt), and 100 μ M zVADfmk (zVAD). Cell processing and the quantitative estimation of DNA fragmentation with an ELISA test.

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In accordance with the results of cytochrome c release, apoptosis of cisplatin-sensitive 2008 cells, estimated as DNA fragmentation (mono- and oligo-nucleosome formation), was significantly stimulated by both auranofin and cisplatin and, as expected, the latter appears particularly efficient (Fig. 38 A). On the contrary, in resistant cells (Fig. 38 B), the effect of cisplatin is drastically reduced, whereas auranofin stimulates apoptosis to a slightly lower extent than that observed in 2008 cells (Figs. 38 A and B), thus confirming its capacity to avoid resistant cell ability in escaping the apoptotic cell death pathway.

Auranofin effect on 2008 and C13 thioredoxin reductase and glutathione reductase activities*

In general, the increase of the cell antioxidant potential is a characteristic of the chemotherapeutic-resistant cancer cell [248]. Among cellular antioxidant defenses, mitochondrial and cytosolic isoforms of thioredoxin reductase play an important role. Trx system, maintaining cellular redox homeostasis, regulate many cellular events, among which cell signalling, apoptosis, and cell proliferation. Gold complexes are recognised as extremely potent inhibitors of both isolated cytosolic and mitochondrial thioredoxin reductase (TrxR1 and TrxR2) [245, 246]. Among gold-containing drugs, auranofin has been found to inhibit TrxR in near stoichiometric amounts.

The activity of thioredoxin reductase was therefore estimated in both 2008 and C13* cells by means of western blot analysis and found remarkably higher in the latter (Fig. 39 A).

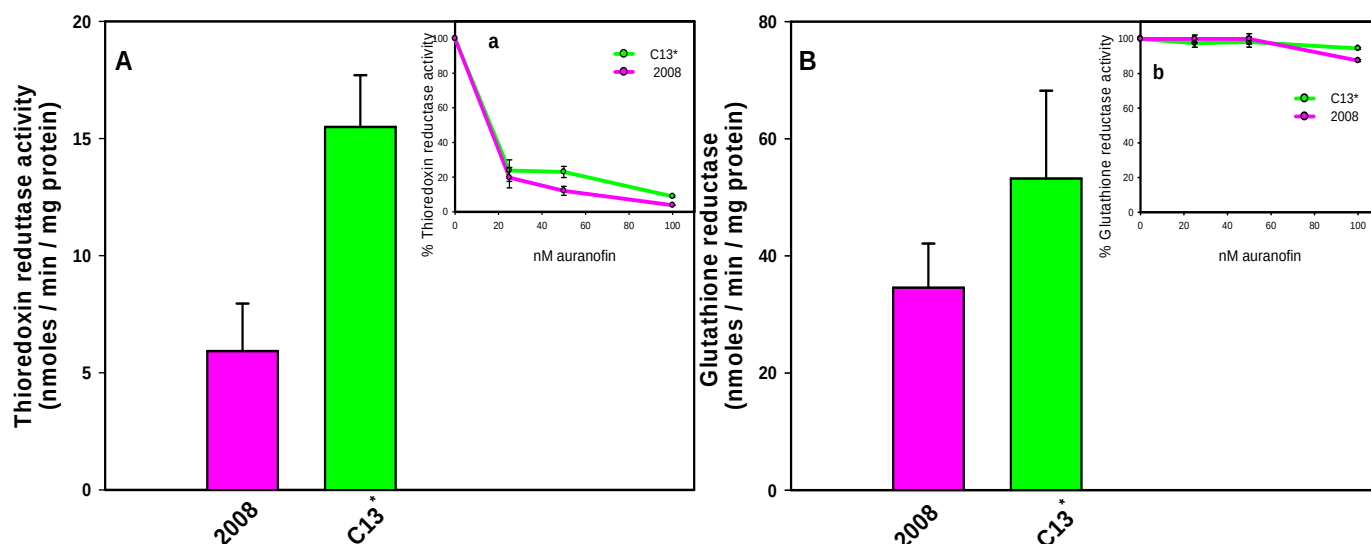


Fig. 39. Estimation of thioredoxin reductase (A) and glutathione reductase (B) activities in 2008 and C13* cells. 2008 and C13* cells were lysed and aliquots of proteins of the supernatant were utilized for TrxR and GR activity determination.

RESULTS AND DISCUSSION

Therefore, the overexpression of thioredoxin reductase appears as a defense strategy playing a critical role in cisplatin-resistant cell survival. Also activity of glutathione reductase, another important enzyme involved in maintaining cellular redox equilibria, was larger in C13* cells with respect to 2008 cells, but to a lower extent in comparison to thioredoxin reductase. Similar results were obtained from preliminary protein profile study performed by two-dimension liquid chromatography (Fig. 40). Indeed, differential expression analysis performed by DeltaVue™ software, underlined that Trx protein family resulted noticeably overexpressed in C13* cisplatin resistant cells.

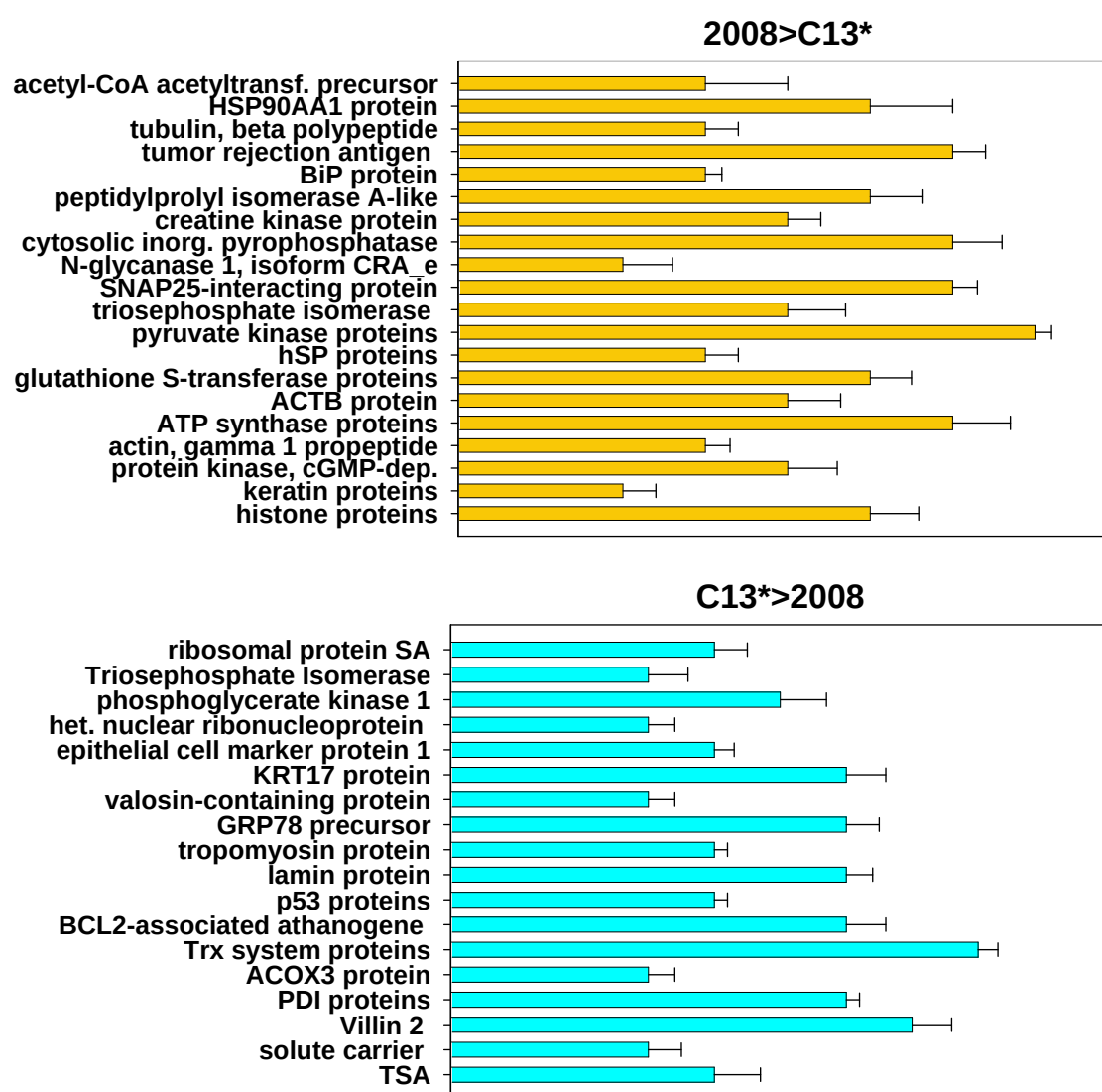


Fig. 40. 2008 and C13* protein profile comparison. Protein profile study was performed by two-dimension liquid chromatography separation on the ProteomeLab PF 2D platform (Beckman Coulter Inc., Ca, U.S), combined with differential expression analysis.

RESULTS AND DISCUSSION

However, as expected, in both cell lines, the thioredoxin reductase activity was inhibited by auranofin in the nanomolar range (Fig. 39 A, inset a), while glutathione reductase is almost totally unaffected at the indicated concentrations (Fig. 39 B, inset b).

Thioredoxin reductase and glutathione reductase activity in other cell lines

In order to gain more evidence about the connection between TrxR level and cellular type, the activities of thioredoxin reductase and GR have been estimated also in other types of tumor cells also including an MDR phenotype (U-2 OS-r cells). TrxR levels were found rather variable among cell hystotype (Fig. 41). However, the ratios between glutathione reductase and thioredoxin reductase are more consistent figures ranging from 2.58 for Jurkat cells to 5.90 for 2008 ovarian carcinoma cells with an average value of 3.58 ± 1.14 , indicating that glutathione reductase is roughly three times more active than thioredoxin reductase independent by the type of cancer cell.

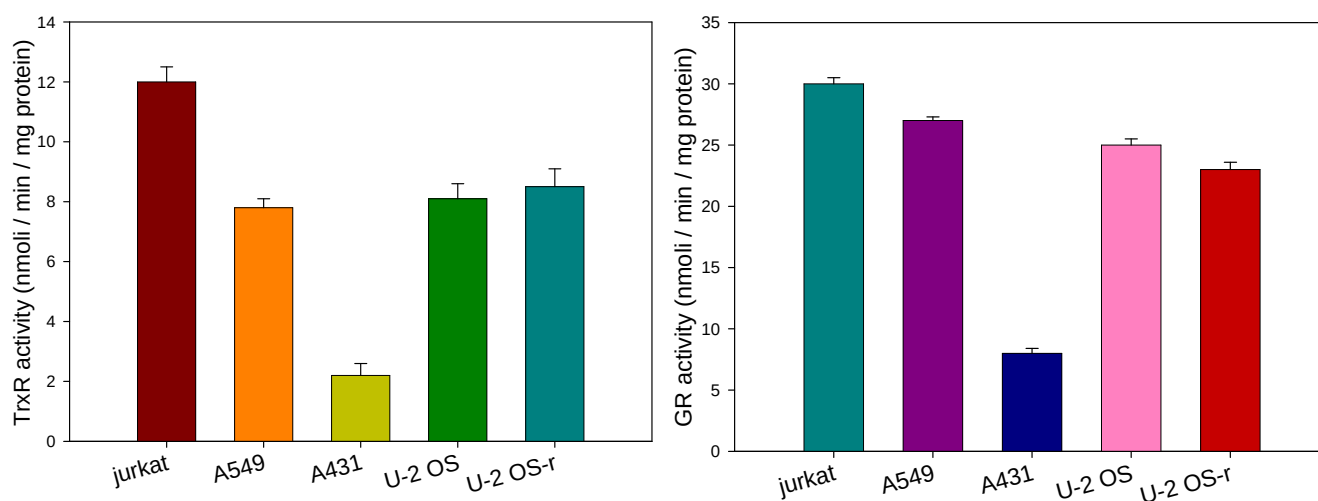


Fig. 41. TrxR (A) and GR (B) activities in different types of cancer cells. Cells were lysed and aliquots of proteins of the supernatant were utilized for TR and GR activity determination.

Thioredoxin reductase activity in C13* cells is higher than the average in the other considered cells and far higher than that in the corresponding sensitive cells pointing out the importance of this enzyme in the acquirement of cisplatin resistance, at least in this pair of cancer cells. Similarly, a slight increase of thioredoxin reductase activity of the doxorubicin-resistant MDR phenotype (U-2 OS-r), with respect to the sensitive cells is apparent.

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ROS production

The Trx redox system possesses a large variety of roles including the removal of hydrogen peroxide through peroxiredoxins [140]. Furthermore, in isolated mitochondria, Rigobello et al. have recently demonstrated that the major consequence associated with TrxR inhibition is the production of a large amount of hydrogen peroxide.

Fig. 42 reports the production of hydrogen peroxide measured with CM-H₂DCFDA in sensitive and resistant cells treated with auranofin or cisplatin. While the basal production of hydrogen peroxide is rather similar in both types of cells, an increase is apparent in the presence of auranofin further stimulated by antimycin, a classic inhibitor of the mitochondrial respiratory chain at the level of complex III.

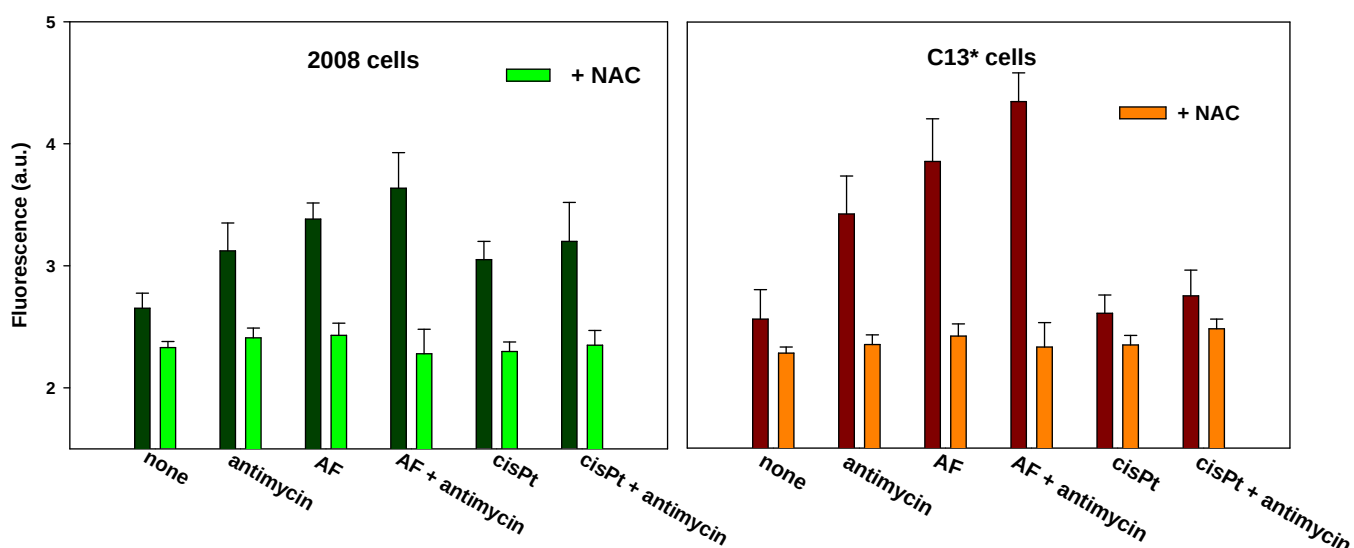


Fig. 42. Effect of auranofin and cisplatin on ROS production by 2008 and C13* cells. Cells were loaded with the fluorescent probe CM-H₂DCFDA and subsequently treated, when indicated, with 5 μ M auranofin (AF), 50 μ M cisplatin (cisPt), or 3 μ M antimycin. In the experiments with NAC, cells were pretreated with 3 mM NAC for 2 hours before adding the above reported substances.

Whereas antimycin is able to stimulate per se the production of hydrogen peroxide in both 2008 and C13* cells, Auranofin, as previously observed [248], at concentration adopted in our study, has no direct detectable effects on respiratory chain enzymes. Hence, the measured increase in hydrogen peroxide level does not depend on the increase of its production, but rather on the decrease of its removal due to auranofin inhibition of thioredoxin reductase.

The strengthening effect in hydrogen peroxide production exerted by antimycin in the presence of auranofin underlines the critical role played by mitochondria in the formation of reactive oxygen species.

Cisplatin is also able to stimulate ROS formation, although to a lower extent in comparison to auranofin. In addition, higher concentrations of cisplatin, with respect to auranofin, are required. ROS production observed in the presence of auranofin and cisplatin is completely abolished by NAC, a well-known and largely utilized reducing agent [249].

3.1.2 Treatment of human cancer cells with selenite or tellurite in combination with auranofin enhances cell death due to redox shift

Cytotoxic effects of selenite and tellurite on cisplatin sensitive (2008) and resistant (C13) cell lines*

Cisplatin sensitive (2008) and resistant (C13*) ovarian cancer cells were tested for their sensitivity towards selenite and tellurite. Cells were treated and analysed at different time points (3, 6, 18, 24 and 48 hours) with increasing concentrations of sodium selenite and potassium tellurite and cell viability was determined by means of MTT test. Fig. 43 shows the results obtained after 48 hour treatment. As apparent (Fig. 43, panel A) selenite, at low concentrations (below 0.05 μ M), stimulated cell proliferation in both sensitive and resistant ovarian cancer cells.

With concentrations higher than 2 μ M, a marked decrease of cell proliferation was induced. In particular, at 10 μ M, both 2008 and C13* cell surviving fraction did not exceed 10 % of the controls. This is in line with the well known properties of selenium that is a required micronutrient but becomes very toxic at high concentrations [214], confirming that at concentrations higher than the nutritional dose selenium compounds may be effective as anticancer agents.

In contrast to selenite, tellurite showed no detectable effect in stimulating cell growth in neither of the two phenotypes. However, at concentrations higher than 60 μ M, tellurite decreased 2008 and C13* cell viability of about 90 % (Fig 43, panel B).

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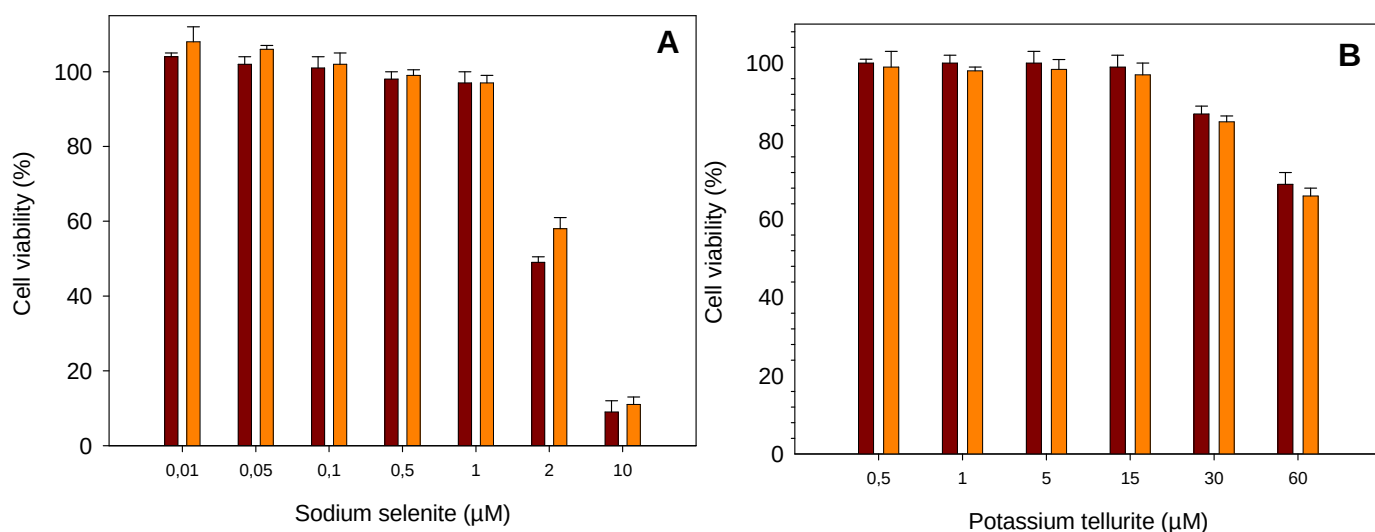


Fig. 43. Sensitivity of human ovarian 2008 (■) and C13* (■) cancer cells to selenite (panel A) or tellurite (panel B). Drug exposure with the indicated compounds was for 48 hours and cytotoxicity was detected by MTT test.

Measurements of total thiols and GSH redox state

The redox condition of glutathione and total sulfhydryl groups was also explored. 2008 and C13* cells were treated with selenite (0.5 - 2.5 μM) and tellurite (5 - 50 μM) for 48 hours and total and oxidised glutathione as well as total thiol groups (Fig. 44) were measured.

The results clearly showed that low concentrations of selenite did not alter the glutathione redox state, but instead, a slight increase of total glutathione was observed. However, at selenite concentrations higher than 1.5 μM , total glutathione concentration (GSH + GSSG) decreased, while a significant decline of reduced glutathione accompanied by an increase of oxidized glutathione was found (Fig. 44). This could be explained with an interaction of selenite with thiols has been previously described by Ganther [252] describing that at a ratio glutathione:selenious acid 4:1, the following reaction occurs:



Differently, treatment with tellurite induced a slight decrease of GSH only at concentrations higher than 30 μM , whereas it appeared totally ineffective at lower concentrations. Hence, besides the chemical properties of selenite and tellurite are considered similar; this indicates a differential chemistry between selenite and tellurite with biological systems.

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Total sulfhydryl groups markedly decreased after selenite treatment (about 70 % with respect to the controls, at 2.5 μM selenite), in accordance with literature data suggesting that several proteins, including redox-regulated signaling molecules, can be specifically oxidized by selenite at the level of their thiols [252].

Contrarily, tellurite at the reported concentrations only induced slight effects on total sulfhydryl groups redox state (Fig. 44).

On the basis of cytotoxicity test and glutathione and total sulfhydryl results as well as aqueous salt solubilities, 0.5 μM selenite and 5 μM tellurite have been selected as suitable, non toxic concentrations to be used in following studies.

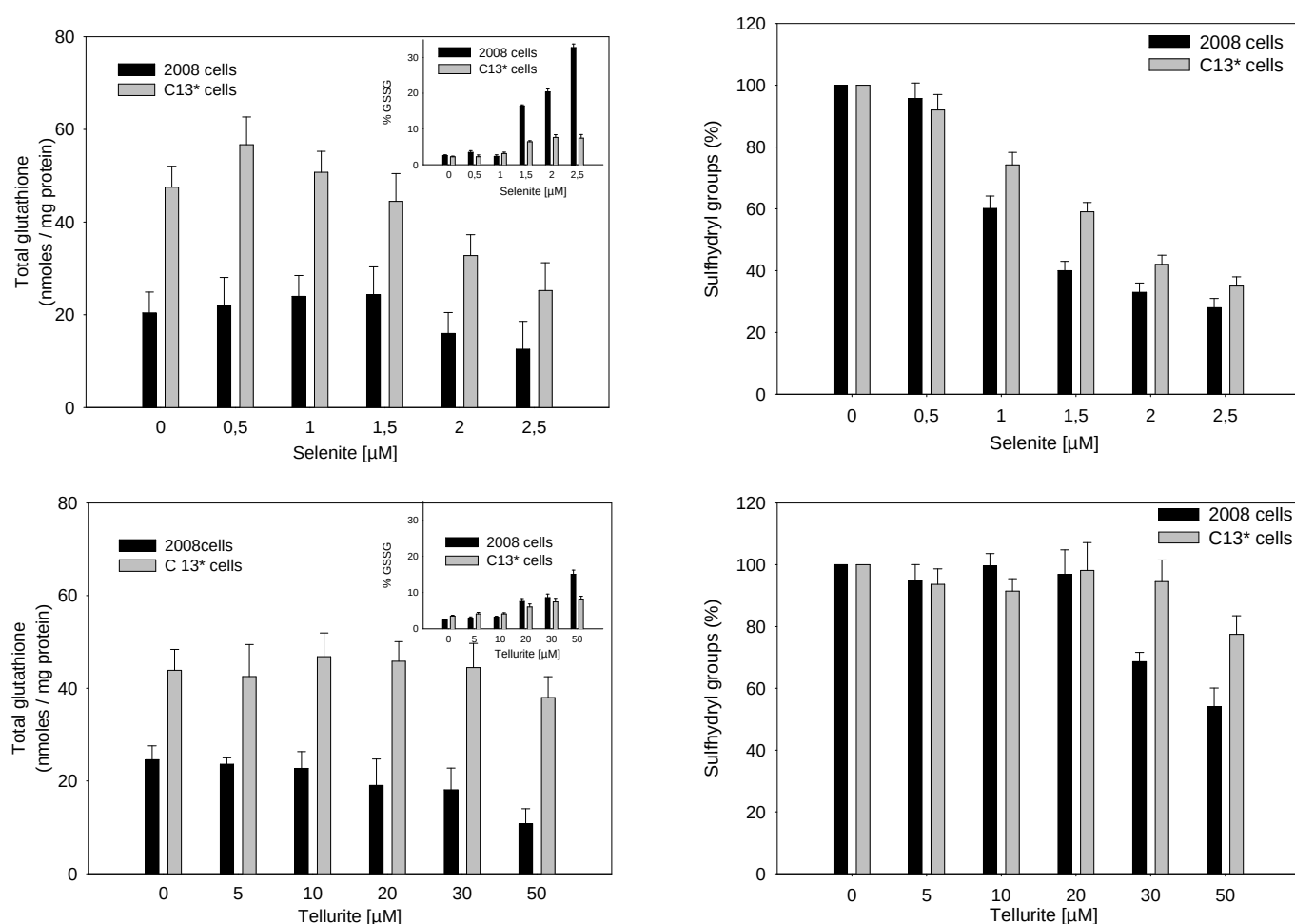


Fig. 44. Estimation of glutathione and total thiol groups in ovarian cancer cell treated with selenite and tellurite. 2008 and C13* cells were incubated for 48 h with increasing concentrations of selenite or tellurite. Total (A, B), oxidized (a, b) glutathione and total thiol groups were estimated in cell lysates.

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TrxR, GR and GPx activity after treatment with selenite or tellurite

Selenium compounds, by contributing to the formation of selenoenzymes, act in cancer prevention as reviewed by Ganther [252]. Selenoproteins have been proposed to follow a hierarchy for selenium supply as the amounts of certain selenoenzymes decrease more rapidly under selenium-deficient conditions. Gasdaska *et al.* [253] demonstrated that the components of TrxR system were highly active under selenium deficiency. Therefore, TrxR levels should be better preserved at low selenium concentrations.

Our experiments, aiming to measure the activity of some essential enzymes controlling cellular thiol redox state, showed that enriching cellular growth medium with moderate concentrations of selenite (0.5 μ M) or tellurite (5 μ M) induced a noticeable increase of TrxR activity. As apparent in Fig. 39, panel A and A', the activity of TrxR measured in cell lysates was markedly enhanced by selenite and tellurite in cisplatin resistant C13* cell (approximately 135 % and 90 %, respectively), while both these agents were far less effective in the corresponding sensitive cell line (2008 cells).

On the contrary, glutathione reductase was only slightly affected by both compounds (Fig. 45, panel B and B'). Glutathione peroxidase appeared markedly increased but either only by selenite (Fig. 45, panel C and C') and in resistant C13* cells (increase of about 85 %, with respect to the control).

Therefore, only the selenium dependent enzymes in the cisplatin resistant phenotype appeared to be induced by selenite and tellurite.

Furthermore, comparing the data regarding cytotoxicity and TrxR activity, it is apparent that selenite exerted a biphasic action since at high concentrations it decreased cell viability, whereas at low concentrations it increased both TrxR activity and cell proliferation. This is in line with the well known properties of selenium that is a required micronutrient but becomes very toxic at high concentrations.

RESULTS AND DISCUSSION

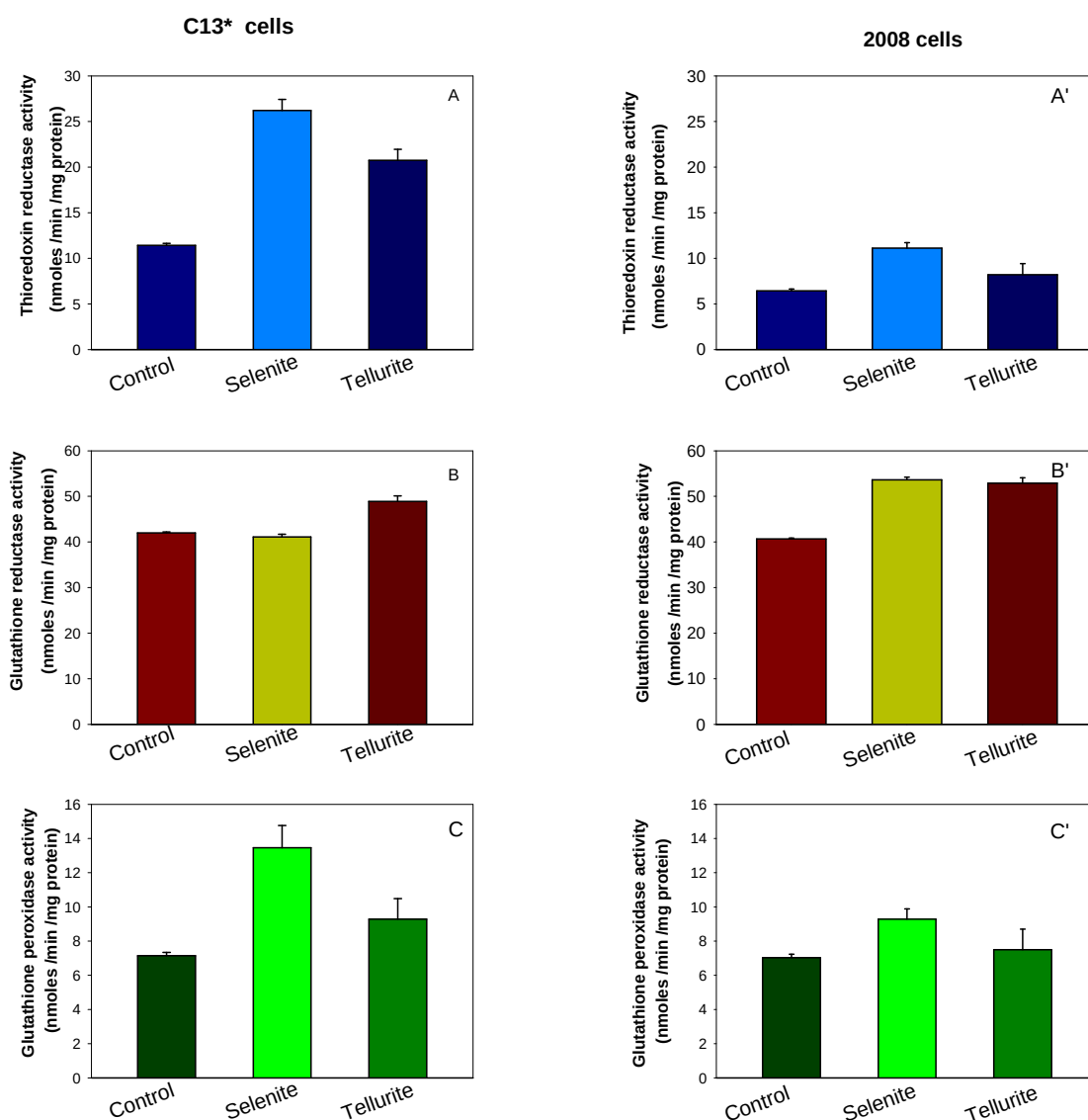


Fig. 45. Thioredoxin reductase, glutathione reductase and glutathione peroxidase activity in ovarian cancer cells after treatment with selenite or tellurite. 2008 or C13* cells were incubated for 48 hours with 0.5 μ M selenite or tellurite. Cells were lysed and aliquots of proteins of the supernatant were utilized for TrxR and GR activity determination.

Activities of TrxR1 and TrxR2 in subcellular fractions after treatment with selenite or tellurite in combination with auranofin

Gold complexes are potent inhibitors of both mitochondrial and cytosolic thioredoxin reductases and they induce a large production of oxygen radicals. In particular, as stated before,

RESULTS AND DISCUSSION

that auranofin is able to exert a marked cytotoxic effect due to thioredoxin inhibition both in 2008 and C13* cells .

In order to evaluate the combined effect of auranofin and the two chalcogen metalloids, cancer cells were pre-treated with selenite or tellurite and further challenged with auranofin. In Fig. 46, the estimation of cytosolic and mitochondrial TrxR activities are reported.

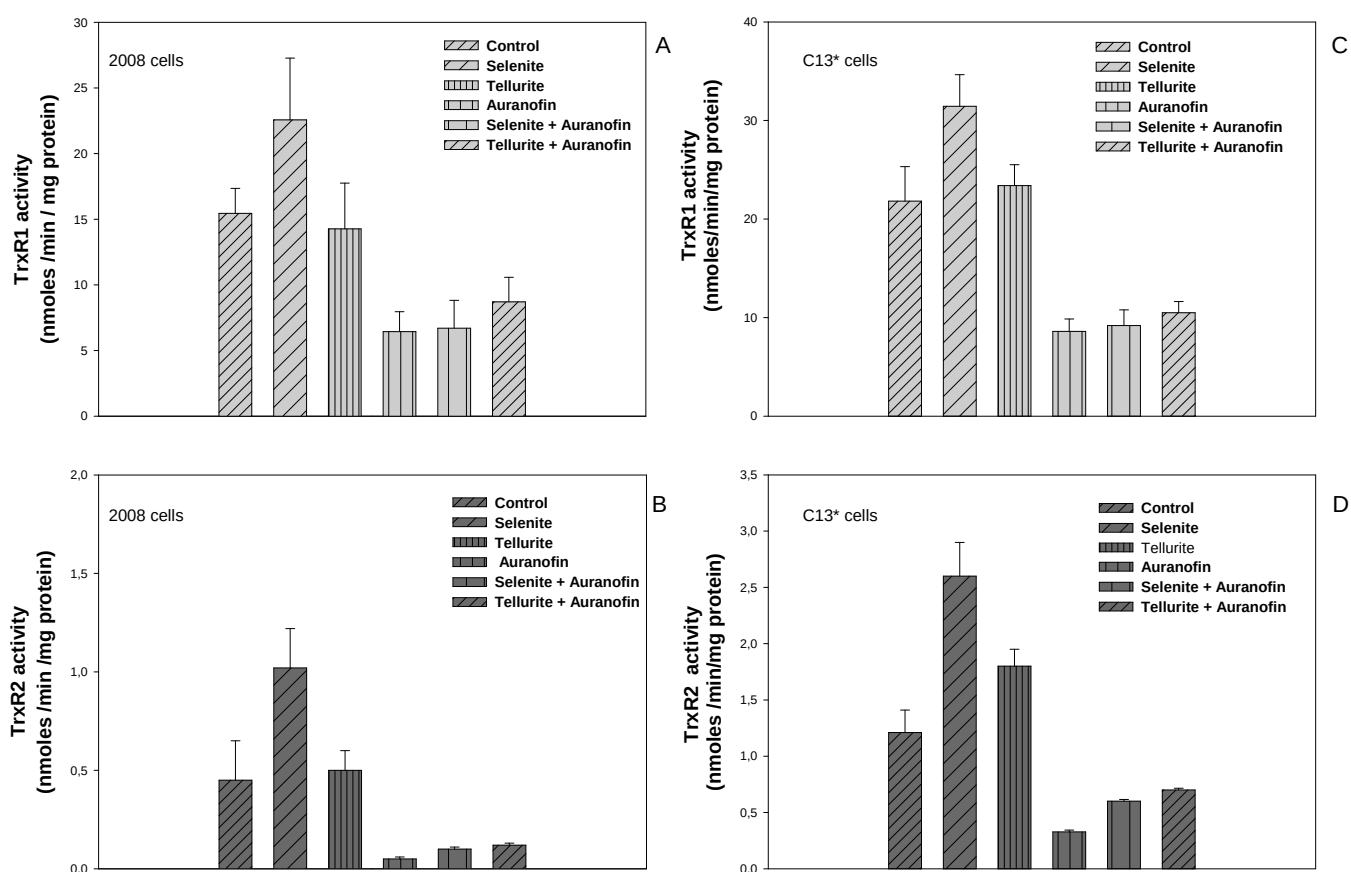


Fig. 46. TrxR1 and TrxR2 activity in ovarian cancer cells after treatment with selenite, tellurite and auranofin. 2008 or C13* cells were incubated for 18 hours with 0.2 μ M selenite or 5 μ M tellurite. Afterwards, 2 μ M auranofin was added where indicated and the incubation carried on for another hour. Cells were washed, lysed and subfractionated. Cell subfraction were used for enzyme determination.

The activities of TrxR1 and TrxR2 were measured in the separated fractions obtained after disruption of cells. Therefore, the specific activities of the two isoforms are referred to the cytosolic and mitochondrial proteins, respectively. As apparent, treatment of cells with selenite and tellurite, increased the activities of both TrxR1 and TrxR2. Furthermore, the combined

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treatment of C13* and 2008 cells with selenite and auranofin completely depressed the stimulation of these enzyme activities induced by selenite.

Effect on cell viability by combined treatment of selenite or tellurite with auranofin in 2008 and C13 cells.*

Exploring cell viability after 48 hours of combined treatment of selenite (0.5 μ M) or tellurite (5 μ M) with auranofin, a marked synergism in decreasing cell viability of selenite and auranofin, particularly on C13* cells, was revealed (Fig. 47).

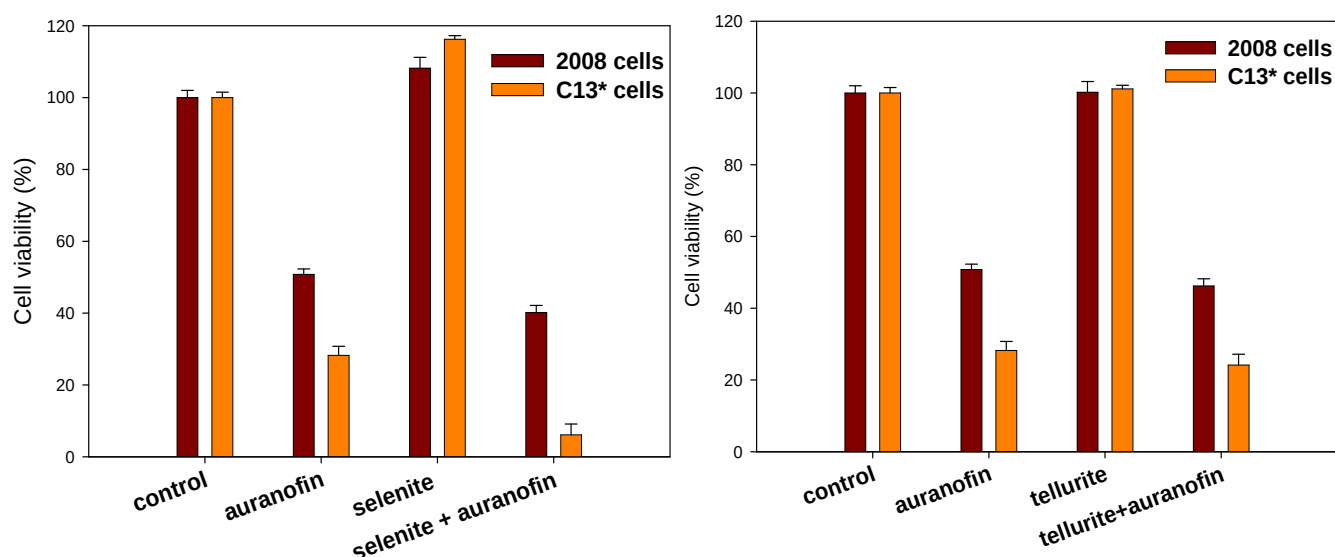


Fig. 47. Cells were treated with selenite (0.5 μ M), tellurite (5 μ M) or auranofin (1 μ M) alone or by combining selenite or tellurite pre-treatment with a subsequent 6 h treatment with auranofin. Total cell treatment lasted for 48 hours.

In particular, the results attested that the combined treatment of 2008 or C13* cells with selenite and auranofin dramatically increased auranofin cytotoxicity. Differently, tellurite presence did not enhance auranofin potency on cell viability.

Apoptosis and cell cycle modifications

In order to further investigate the cellular effects induced by selenite, tellurite and by their combination with auranofin in terms of cell cycle modifications and induction of apoptosis, a time-dependent evaluation of cell cycle profile was performed by FACS on 2008 and C13* cells.

RESULTS AND DISCUSSION

Table IV reports the percentages of cancer cells in different cell cycle phases. Differently, the treatment with gold(I) complex for only 6 hours markedly increased the percentage of cells with hypodiploid DNA; in particular, the percentages of 2008 and C13* cells with fragmented DNA were about 10- and 13-fold higher compared to the control samples, respectively. These results agree well with those obtained by measuring mono- and oligo-nucleosome formation and cytochrome c release, and previously described. After 48 hour treatment, selenite induced in both cell lines a noticeable decrease of the G1 phase as well as a concomitant increase of sub-G1 cell subpopulation (about 2.4- and 2.1-fold higher than controls in 2008 and C13* cells, respectively). Moreover, the combination of selenite and auranofin, magnificated the sub-G1 cell shift, increasing about 12- and 16-fold the amount of cells with DNA fragmentation with respect to controls, in 2008 and C13* cells, respectively.

Table IV Cell Cycle Phases

C13						
	Ctr	Auranofin	Selenite	Tellurite	Se+Au	Te+Au
Apoptosis %	1.4±1.12	18.6±0.98	3.0±1.18	1.4±0.03	22.04±1.97	3.04±1.46
G1 %	72.13±1.29	42.23±1.40	58.34±1.67	65.35±1.33	59.86±2.46	65.43±1.11
S,G2/M%	26.21±1.27	38.21±1.32	38.35±1.31	32.64±1.97	19.23±1.48	31.57±2.93
2008						
	Ctr	Auranofin	Selenite	Tellurite	Se+Au	Te+Au
Apoptosis %	1.7±0.43	16.1±0.65	4.1±0.78	1.1±0.89	19.64±0.94	1.13±1.12
G1 %	71.24±1.56	44.65±1.54	56.19±1.77	67.35±1.02	47.03±2.46	64.53±1.45
S,G2/M%	27.12±1.65	39.34±1.64	39.70±1.31	31.61±1.31	32.15±1.39	34.15±0.56

Percentages of cells in different cell cycle phases.

After 48 hours of tellurite treatment, no increase of sub-G1 phase was detectable and only co-treatment with auranofin induced a slight increase of cells with fragmented DNA in both cell lines (about 1.2- and 1.5-fold higher than controls in 2008 and C13* cells, respectively).

RESULTS AND DISCUSSION

Expression of TrxR mRNA after auranofin treatment in combination with selenite or tellurite

The effect on the expression of TrxR1 and TrxR2 in response to non-toxic doses of selenite and tellurite, alone or in combination with auranofin, was analyzed by means of Real-time qPCR. Treatment with 0.3 μ M auranofin for at least 6 hours modestly increased TrxR1 mRNA levels in both human ovarian cancer cell lines (Fig. 48). In both cell lines treatment with 0.5 μ M sodium selenite resulted in a modest up-regulation of TrxR1 mRNA levels, whereas selenite significantly decreased the expression of TrxR2. TrxR2 expression in both cell lines was significantly decreased (about 90 %) as consequence of the combined treatment with selenite and auranofin. On the contrary, a significant up-regulation of TrxR1 activity, mainly in cisplatin resistant phenotype, was found.

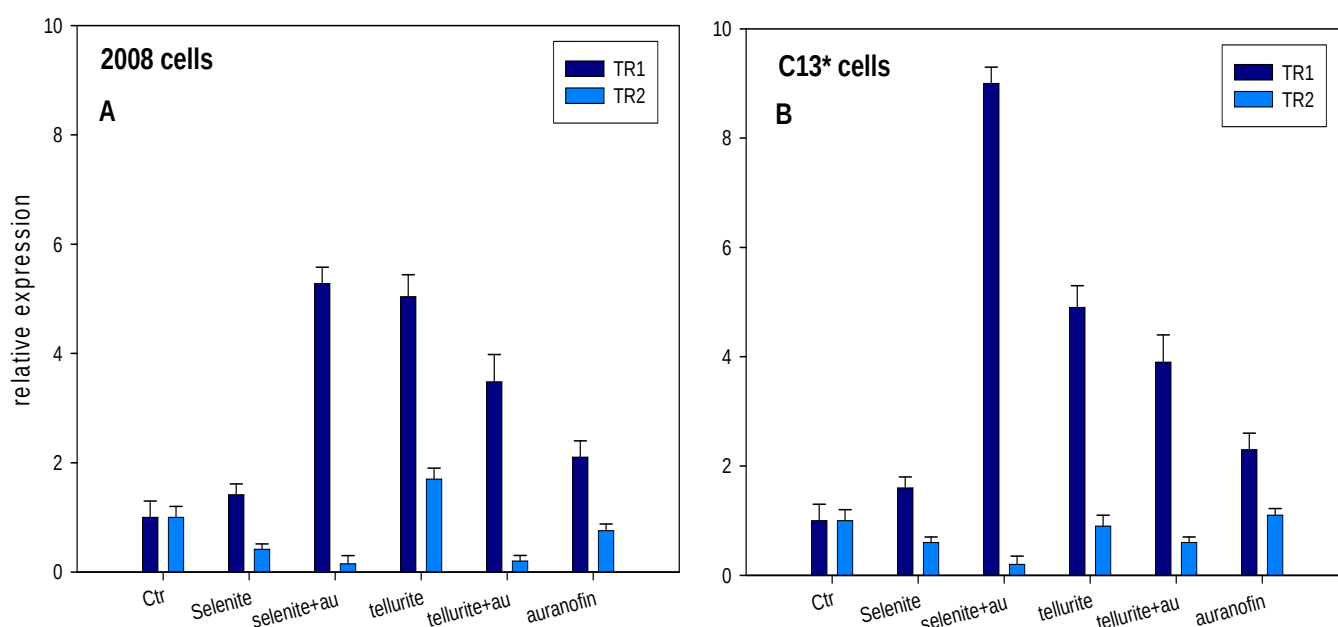


Fig. 48. TrxR1 and TrxR2 mRNA expression patterns after selenite, tellurite or auranofin treatment. 2008 and C13* cells were treated with 0.5 μ M selenite, 5 μ M tellurite and/or auranofin (1 μ M) for totally 48 hours before harvesting. Relative expressions of mRNA isoforms were measured with real-time qPCR. The data was analysed using the $2^{-\Delta\Delta CT}$ method with β -actin as an endogenous control and the C_T -value cut-off set to 32 cycles. Results are expressed as relative amounts with untreated cells.

On the other hand, tellurite treatment induced a marked change in mRNA levels of TrxR1 in both cell lines without exhibiting any significant effect on TrxR2 levels. Contrary to the effect of the combination of selenite and auranofin, tellurium and gold(I) compound induced TrxR1

expression to a lower extent than tellurium alone. Furthermore, this effect was slightly more pronounced in the cisplatin-resistant subline (Fig. 48).

ROS production

As the molecular mechanism underlying both selenite and gold complexes toxicity also depends on the generation of ROS, particularly of hydrogen peroxide, the production of these species was estimated after treatment of ovarian cancer cells with selenite, tellurite and the gold complex auranofin. 2008 and C13* cells, after incubation with CM-DCMDF probe, were treated with increasing concentrations of selenite (0.25 to 1 μM), tellurite (2.5 to 5 μM) alone or in combination with auranofin (1 μM).

As reported in Fig. 49, only a slight increase of fluorescence was induced by selenite and tellurite treatment in both cell lines.

As previously stated, auranofin has been found to determine an alteration of the redox state of the cell leading to an enhanced production of hydrogen peroxide. The combined presence of auranofin and selenite or tellurite moderately enhanced the production of ROS both in sensitive and resistant cells.

However, hydrogen peroxide formation detected after treating 2008 and C13* cells with auranofin both in the absence and in the presence of the two chalcogen metalloids was quite similar.

Therefore, the cooperative effect provoked by selenium and auranofin on cell viability and TrxR inhibition as well as on apoptosis induction cannot be explained only considering the increase of cellular ROS levels.

RESULTS AND DISCUSSION

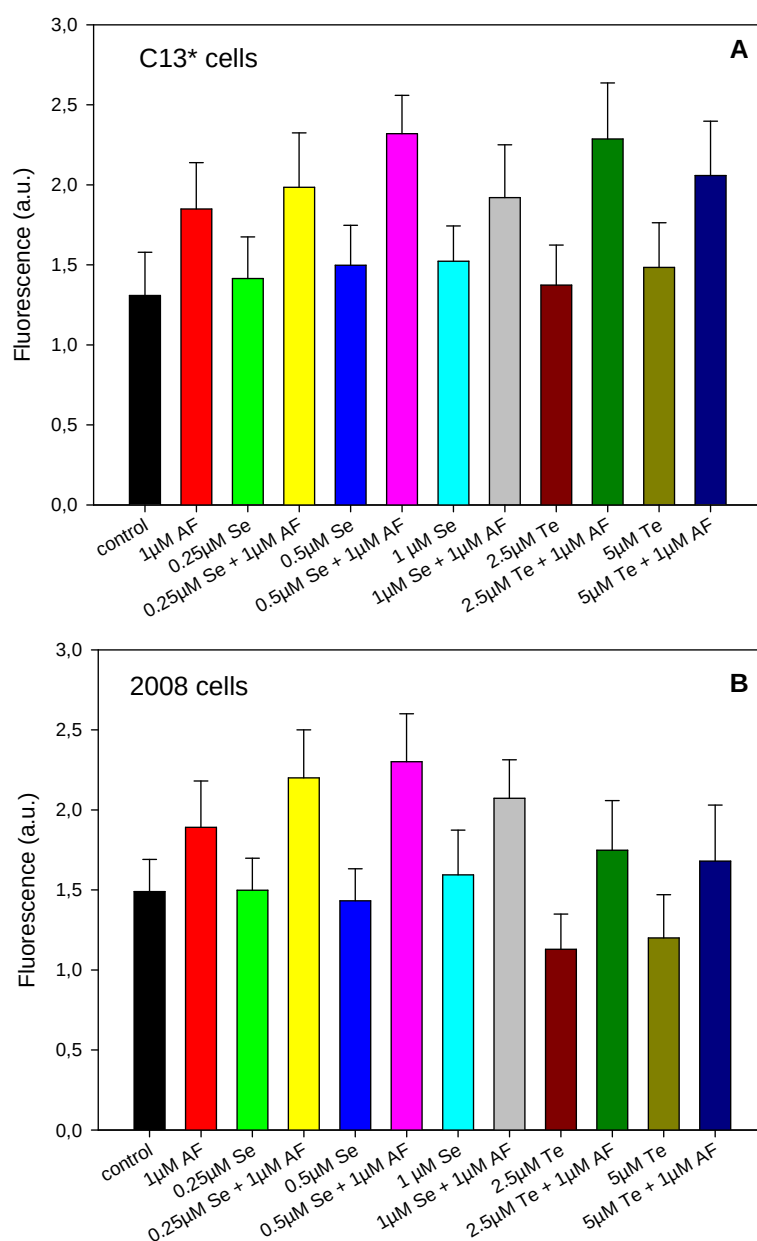


Fig. 49. Effect of auranofin on ROS production in ovarian cancer cells treated with selenite or tellurite. 2008 and C13* cells were pretreated for 48 hours with selenite and tellurite. After washing and cells were treated with 10 μ M CMDCFDA for 20 min in the dark. In combined experiments, cells were washed again and treated with 1 μ M auranofin. Fluorescence was measured at ex 495- em 515.

Redox state of Trx1 after separate treatment with selenite or tellurite alone or in combination with auranofin

Oxidative stress has been related to the balance between formation/removal of ROS and to thiol state. In particular, sulfur switches function as sensors in redox signaling pathways and

RESULTS AND DISCUSSION

thioredoxins are one of the major redox control nodes involved in sulfur switches regulation [252]. Therefore, the redox state of Trx1 in sensitive and resistant cancer cells was examined after cell treatment for 24 hours with selenite or tellurite (Fig. 50 A). 1.5 μ M selenite completely oxidized Trx in 2008 cells, whereas tellurite was less effective and caused only a partial oxidation of thioredoxin. As apparent in Fig. 50 B, in cells treated for 30 min with 2 μ M auranofin, thioredoxin undergoes oxidation that was further enhanced, particularly in 2008 cells, by the combined addition of 0.5 μ M selenite. In 2008 cells, also 5 μ M tellurite reinforced auranofin action in inducing the oxidation of Trx.

While the increased oxidation of Trx elicited by auranofin might be related to TrxR inhibition, the effect of selenite and tellurite on Trx redox state seems to be due to a direct oxidation. Consequently, combining selenite with auranofin, thioredoxin could be directly oxidised by chalcogen and cannot be reduced back by thioredoxin reductase, which is inhibited by gold(I) complexes. Consequently, the gold(I) complex and chalcogens share a similar pathophysiological action as both ultimately prevent the cyclic reduction of thioredoxin [].

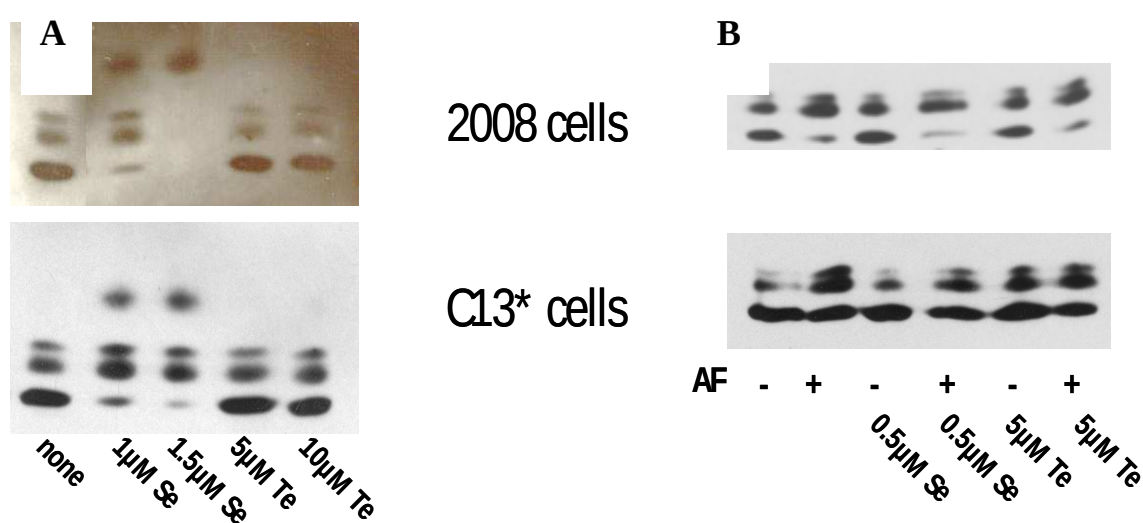


Fig. 50. Redox western blot analysis of thioredoxin 1 after cell treatment with selenite, tellurite in combination with auranofin (AF). 2008 and C13* cells were treated with the indicated concentrations of selenite or tellurite for 24 hours (Panel A). Auranofin (2 μ M) was added at the end of the treatment with selenite or tellurite and the incubation lasted for additional 30 minutes (Panel B).

3.2 Mixed Phosphine Gold(I) Compounds

3.2.1 *In vitro* antitumor effects and thioredoxin reductase inhibition induced by mixed phosphine gold(I) compounds

Cytotoxicity Studies.

Newly synthesized gold(I) complexes and the corresponding uncoordinated ligands were examined for their cytotoxic properties against a panel of human tumor cell lines containing examples of lung (A549), colon (HCT-15), breast (MCF-7) and cervix (HeLa) cancer along with leukemia (HL60) and melanoma (A375). Under the same experimental conditions, cisplatin and auranofin were also evaluated, the former as the most widely used anticancer metallodrug and the latter as the reference phosphine gold(I) compound. IC₅₀ values, calculated from the dose-survival curves obtained after 72 h of drug treatment from the MTT test, are shown in Table V.

Whereas uncoordinated ligands proved to be quite ineffective against all tumor cell lines, all gold(I) derivatives showed IC₅₀ values lower than cisplatin.

The less active compound was Au(tu)PEt₃. Indeed, although Au(tu)PEt₃ complex showed a cytotoxic potency higher than that of cisplatin, it proved to be roughly 5-times less effective than auranofin in decreasing cell viability over 6 cancer cell types.

Au(dedc)PEt₃, Au(Cl)PEt₃ and Au(Br)PEt₃ complexes showed a significant cytotoxic activity in the sub-micromolar range against all cancer cell lines, with IC₅₀ average values from about 15- to 20-fold lower than those obtained with cisplatin and comparable with those exhibited by the phosphine gold(I) reference drug.

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Table. V Cytotoxicity of gold(I) compounds

Compound	IC ₅₀ (μM) ± S.D.					
	HL60	A549	MCF-7	A375	HCT-15	HeLa
AuCl(PEt ₃)	0.52±0.13	0.85±0.01	0.84±0.01	0.78±0.31	0.97±0.17	0.90±0.21
AuBr(PEt ₃)	0.62±0.06	1.21±0.54	1.44±0.41	1.02±1.00	1.08±0.82	0.63±0.32
Au(CN)(PEt ₃)	0.19±0.07	0.41±0.06	0.45±0.12	0.21±0.23	0.08±0.01	0.09±0.02
Au(SCN)(PEt ₃)	0.21±0.12	0.71±0.02	0.65±0.11	0.37±0.29	0.32±0.02	0.18±0.01
Au(dedc)(PEt ₃)	0.96±1.21	1.22±0.96	1.63±0.58	1.47±0.77	0.92±0.01	0.54±0.07
Au(xant)(PEt ₃)	0.19±0.06	0.33±0.47	0.41±0.21	0.13±0.27	0.61±0.13	0.13±0.61
Au(Tu)(PEt ₃)	2.84±1.29	3.01±1.01	3.28±1.12	2.23±1.03	2.75±1.43	1.84±0.25
Auranofin	0.23±0.11	0.75±0.05	0.98±0.32	0.34±0.21	0.11±0.02	0.15±0.03
Cisplatin	4.56±1.13	29.21±1.9	19.04±1.51	2.37±1.23	20.34±1.3	8.50±1.51

S.D.= standard deviation

IC₅₀ values were calculated by probit analysis ($P < 0.05$, χ^2 test). Cells ($5-8 \cdot 10^4 \cdot \text{mL}^{-1}$) were treated for 72 hours with increasing concentrations of tested compounds. Cytotoxicity was assessed by MTT test

The most effective compounds were Au(xant)PEt₃, Au(CN)PEt₃ and Au(SCN)PEt₃. Xantogenate, cyanate and thiocyanate gold(I) complexes showed IC₅₀ values about 80-times lower than that of cisplatin and the cytotoxic potency was greater than that of auranofin by a factor of about 2. In particular, their *in vitro* antitumor potency was significantly higher against human non-small lung A549 and colon HCT-15 cancer cells, notoriously poor sensitive to cisplatin, thus reflecting their ability to overcome intrinsic cisplatin resistance.

Cross-resistance profiles

The encouraging results obtained against the in-house panel of cell lines prompted us to test the cytotoxic activity of these gold(I) complexes onto three additional cell line pairs, two of which (2008/C13* human ovarian cancer cells and A431/A431-Pt human cervix carcinoma cells) had

been *in vitro* selected for their resistance to cisplatin and two (U1285/U1285dox human lung cancer cells and LoVo/LoVoMDR human colon adenocarcinoma cells) for their resistance to doxorubicin, (MDR phenotypes). Cytotoxicity of tested compounds in cisplatin sensitive and resistant cells was assessed after a 48-hours drug exposure by the MTT test (Table VI).

Tested gold(I) complexes, as well as auranofin, exhibited a different cross-resistance profile than that of cisplatin. RF values calculated for all gold(I) complexes were about 2- and 10-times lower than those calculated for cisplatin against human cervix and ovarian cancer cell line pairs, respectively.

These results attested their ability to overcome cisplatin resistance in both phenotypes strongly supporting the hypothesis of a different pathway of action of these gold(I) complexes from that of cisplatin.

Table VI also reports data obtained in RH4 cisplatin-revertant cells, a cisplatin-sensitive cell line obtained from C13* subline after reverting cisplatin resistance, that retained a number of the phenotypic features related to cisplatin resistance [253]. As a whole, against RH4 cells, the behavior of all gold complexes was super imposable to that elicited in cisplatin sensitive 2008 cell line.

Acquired multidrug resistance (MDR), whereby cells become refractory to multiple drugs, poses a very important challenge to the success of anticancer chemotherapy [232]. The resistance of U1285-doxo cells to doxorubicin, a drug belonging to the MDR spectrum, is an atypical multidrug-resistance characterised by the absence of P-gp overexpression and atypical cross-resistance [235]. Recently U1285-doxo cells have been found to overexpress TrxR. [235]. On the contrary, in LoVo/MDR cells, resistance to doxorubicin is associated with an overexpression of the multispecific drug transporters, such as the 170 kDa P-glycoprotein (P-gp) [232]. Cytotoxicity of tested compounds in wild-type and MDR cells was assessed after a 48-hours drug exposure by the MTT test (Table VII).

Table VI Cisplatin cross-resistance profiles

Compound	Human ovarian adenocarcinoma cells			
	2008 IC ₅₀ (μM) ± S.D.	C13 IC ₅₀ (μM) ± S.D.	R.F.	RH4 IC ₅₀ (μM) ± S.D.
AuCl(PEt ₃)	1.12±0.29	1.05±0.43	0.93	0.91±0.53
AuBr(PEt ₃)	1.61±0.71	1.06±0.68	0.66	0.93±0.47
Au(CN)(PEt ₃)	0.99±0.01	0.96±0.25	0.96	0.98±0.12
Au(SCN)(PEt ₃)	0.95±0.02	0.94±0.04	0.93	0.12±0.59
Au(dedc)(PEt ₃)	0.86±1.38	0.87±0.98	1.00	0.81±0.45
Au(xant)(PEt ₃)	0.67±0.65	0.66±0.02	0.98	0.68±0.58
Au(Tu)(PEt ₃)	4.21±2.30	4.13±0.74	1.08	4.09±2.11
Auranofin	1.01±0.97	0.43±0.09	0.43	0.89±0.72
Cisplatin	10.14±1.43	89.18±2.08	8.79	26.61±1.53

Compound	Human cervix carcinoma cells		
	A431 IC ₅₀ (μM) ± S.D.	A431/Pt IC ₅₀ (μM) ± S.D.	R.F.
AuCl(PEt ₃)	0.95±0.11	1.08±0.91	1.14
AuBr(PEt ₃)	1.75±0.002	1.77±0.61	1.10
Au(CN)(PEt ₃)	0.97±0.17	0.98±0.13	1.01
Au(SCN)(PEt ₃)	0.95±0.52	0.97±0.16	1.02
Au(dedc)(PEt ₃)	2.02±0.75	2.06±0.72	1.02
Au(xant)(PEt ₃)	0.53±0.76	0.27±1.44	0.94
Au(Tu)(PEt ₃)	2.01±0.42	1.87±0.12	0.98
Auranofin	2.01±1.54	1.87±0.50	0.93
Cisplatin	22.06 ± 2.21	57.76 ± 3.94	2.61

S.D.= standard deviation

IC₅₀ values were calculated by probit analysis ($P < 0.05$, χ^2 test). Cells ($3\text{--}5 \cdot 10^4 \cdot \text{mL}^{-1}$) were treated for 48 hours with increasing concentrations of tested compounds. Cytotoxicity was assessed by MTT test. Resistant factor (R.F.) is defined as IC₅₀ resistant/parent line

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Table VII MDR cross-resistance profiles

Compound	Human small cell lung adenocarcinoma cells		
	U1285	U1285dox	R.F.
	IC ₅₀ (μM) ± S.D.	IC ₅₀ (μM) ± S.D.	
AuCl(PET ₃)	1.29±0.31	1.18±0.11	0.92
AuBr(PET ₃)	1.43±0.17	1.41±0.36	0.99
Au(CN)(PET ₃)	0.73±0.29	0.72±0.51	0.98
Au(SCN)(PET ₃)	0.62±0.23	0.61±0.36	0.98
Au(dedc)(PET ₃)	2.33±1.04	2.46±0.93	1.06
Au(xant)(PET ₃)	0.79±1.56	0.43±1.43	0.54
Au(Tu)(PET ₃)	6.62±1.98	6.69±1.39	1.01
Auranofin	0.92±1.43	0.67±0.45	0.73
Doxorubicin	1.01 ± 0.34	87.07 ± 2.97	86.21
Compound	Human colon adenocarcinoma cells		
	LoVo	LoVo MDR	R.F.
	IC ₅₀ (μM) ± S.D.	IC ₅₀ (μM) ± S.D.	
AuCl(PET ₃)	1.79±0.56	1.47±0.59	0.82
AuBr(PET ₃)	1.85±0.77	1.21±0.86	0.65
Au(CN)(PET ₃)	0.67±0.08	0.65±0.07	0.97
Au(SCN)(PET ₃)	0.69±0.33	0.61±0.65	0.88
Au(dedc)(PET ₃)	1.33±1.54	1.04±1.50	0.78
Au(xant)(PET ₃)	0.69±1.56	0.57±1.59	0.82
Au(Tu)(PET ₃)	6.06±1.98	6.28±1.67	1.04
Auranofin	1.12±0.74	1.11±0.45	1.01
Doxorubicin	1.46± 2.30	44.89 ± 0.90	30.75

S.D.= standard deviation N.D.= not detectable

IC₅₀ values were calculated by probit analysis ($P < 0.05$, χ^2 test). Cells ($5-8 \cdot 10^4 \cdot \text{mL}^{-1}$) were treated for 72 hours with increasing concentrations of tested compounds. Cytotoxicity was assessed by MTT test. Resistant factor (R.F.) is defined as IC₅₀ resistant/parent line

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The calculation of an RF average value from about 35 to 88 times lower than that obtained with doxorubicin, in LoVo/MDR and U1285dox, respectively, thus suggesting that gold(I) complexes are not potential MDR substrates.

In vitro thioredoxin reductase, glutathione peroxidase and glutathione reductase inhibition.

Gold complexes have recently gained attention because of their strong inhibition of TrxR enzyme [160]. In particular, as stated before, auranofin inhibit both cytosolic and mitochondrial rat TrxR at nanomolar level. As these 'auranofin-like' gold(I) complexes have been synthesized as putative selective TrxR inhibitors, their inhibitory effects toward cytosolic or mitochondrial forms of rat TrxR were measured according to established procedures. Gold(I) complexes were tested at increasing concentrations within the 1-200 nM range.

As seen in Fig. 51 A, the cytosolic isoform (TrxR1) appeared markedly inhibited by all gold(I) derivatives at nanomolar concentrations. In particular, Au(CN)PEt₃ and Au(Tu)PEt₃ showed an inhibitory potency just alike to that obtained with auranofin, whereas, interestingly, the IC₅₀ values calculated with Au(xant)PEt₃ and Au(dedc)PEt₃ were about 1.2 and 3 times lower than that determined with auranofin exposure, respectively. Among tested complexes, only Au(Cl)PEt₃, Au(SCN)PEt₃ and AuBrPEt₃ appeared slightly less effective than auranofin in decreasing thioredoxin reductase activity, with IC₅₀ averages roughly from 1.2 to 2.5 times higher.

The mitochondrial enzyme isoform (TrxR2) appeared to be less sensitive to the inhibitory effect promoted by tested complexes and at least 4-times higher concentrations were required for a reduction of 50% of TrxR2 (Fig. 51 B). Moreover, the IC₅₀ values obtained with all gold(I) derivatives were higher than that obtained with auranofin, unless for those calculated with Au(CN)PEt₃, Au(xant)PEt₃ and Au(dedc)PEt₃ which were about 1.2-, 1.5- and 5-fold lower compare to that obtained with gold(I) reference compound, respectively.

Cellular hydrogen peroxide levels and thiol redox state are controlled both by Trx system and by another essential enzyme system which depend on glutathione, glutathione reductase and peroxidase. Both glutathione peroxidase and thioredoxin reductase are endowed with a selenol group at their active site. It has already described that some phosphine gold(I) compounds are also able to inhibit glutathione peroxidase at very low concentrations [248]. With the aim to attest the selectivity of our gold(I) compounds toward thioredoxin reductase isoenzymes, we have also performed glutathione reductase and peroxidase inhibition tests (Fig. 45 C, D).

RESULTS AND DISCUSSION

Interestingly, glutathione reductase and glutathione peroxidase were slightly affected by all tested complexes. Among all, Au(Tu)PEt₃ appeared the most effective in affecting Gpx activity even if it is worthy gold(I) derivatives showed IC₅₀ values in the micromolar range, three orders of magnitude greater than those obtained with TrxR, suggesting that they act mainly as TrxR inhibitors.

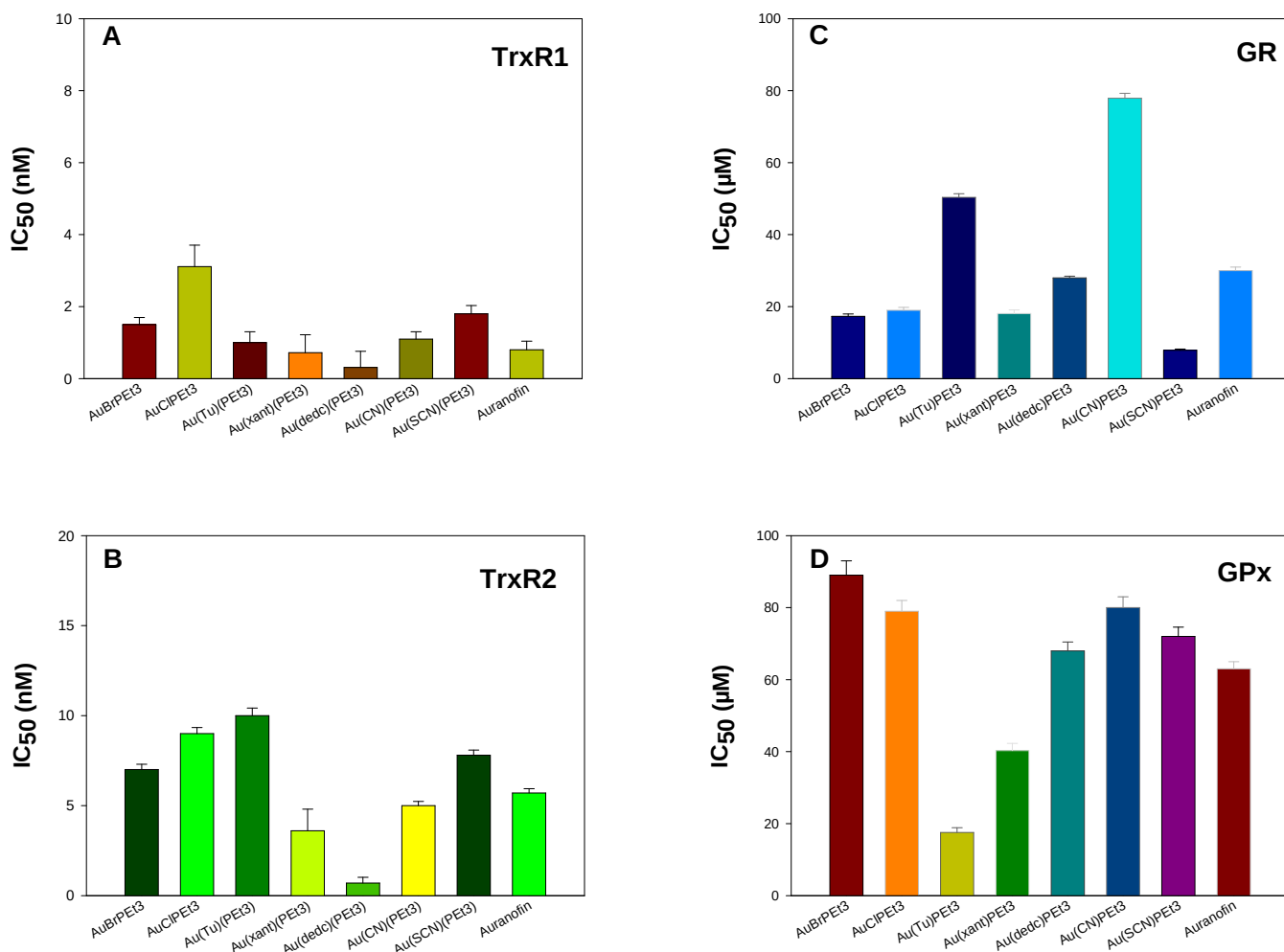


Fig. 51. Inhibitory effect of gold(I) compounds on rat cytosolic (TrxR1) and mitochondrial (TrxR2) thioredoxin reductases, on Glutathione Reductase (GR) and Glutathione peroxidase (GPx).

Thioredoxin reductase inhibition in human ovarian cancer cells.

In our previous studies we have compared the cellular response of human ovarian cancer cells to auranofin with that evoked by cisplatin, the first line drug against ovarian cancer. Auranofin,

acting as a potent TrxR inhibitor, provoked an alteration of the cellular redox state leading to an oxidation of the components of the Trx system thus triggering apoptosis.

TrxR inhibition by the phosphine gold(I) complexes was therefore evaluated in 2008 cells. In particular, all tested complexes showed IC_{50} values comparable with that obtained with auranofin. In particular, Au(dedc)PEt₃, Au(SCN)PEt₃ and Au(xant)PEt₃ were the most active derivatives, being able to decrease cellular TrxR activity of about 90, 92 and 94 % respect to untreated cells (Fig. 52). These results strongly support the hypothesis they act mainly as TrxR inhibitors and suggest that all gold(I) tested compounds are able to cross plasma membrane and, consequently, inhibit thioredoxin reductase in its own environment.

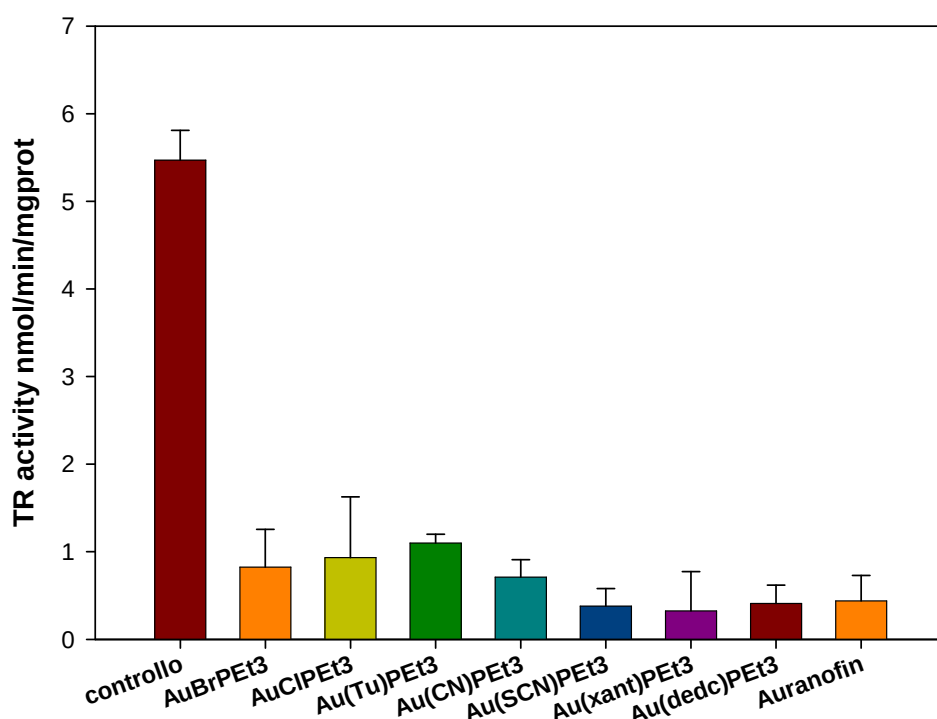


Fig. 52. Estimation of the inhibitory effect of gold(I) compounds on thioredoxin reductase activity in 2008 cells. 2008 cells were incubated for 24 hours with IC_{50} of tested compounds. Subsequently, cells were washed twice with PBS and lysed. Aliquots of 0.1 mg proteins of the supernatant were utilized for TrxR activity determination.

Cellular uptake

In our previous studies we highlighted the relationship between auranofin cytotoxicity and cellular accumulation. Cellular uptake of phosphine gold(I) complexes was quantified, by

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means of ICP analysis, in 2008 cells after a continuous treatment with IC_{50} concentrations lasting 6, 12 and 18 hours. The results revealed that intracellular accumulation was time dependent and reached maximum values after 18 hour exposure. Most probably, for longer times of exposure, the detection may be affected by the strong cytotoxicity induced by gold derivatives. As seen in Fig. 53, which reports the results after 18 hour treatment, Au(xant)PEt₃ cellular uptake was roughly 1.3 times higher with respect to auranofin intracellular accumulation. Furthermore, Au(dedc)PEt₃, Au(CN)PEt₃ and Au(SCN)PEt₃ entered cancer cells with a similar efficiency to auranofin, whereas AuBrPEt₃ and AuClPEt₃ cellular uptake was found roughly 2-fold lower. Differently, Au(Tu)PEt₃ seemed to hardly cross cellular plasmalemma giving rise to a cellular gold amount about 4 times lower compared with reference gold(I) compound. It seems clear that the uptake behavior for all gold derivatives correlates well with the cytotoxicity data.

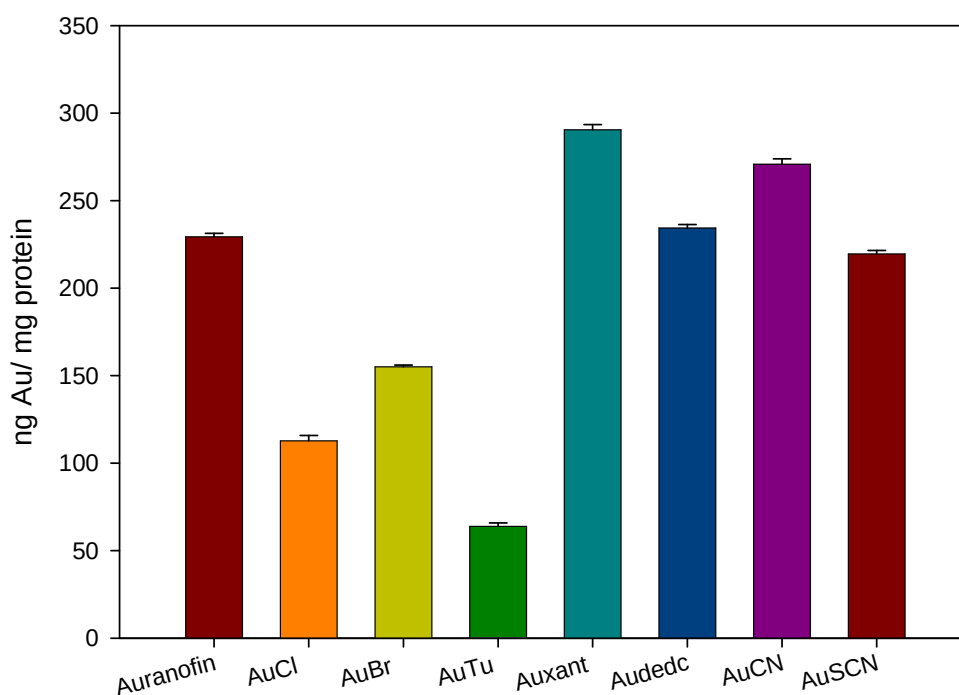


Fig. 53. Gold intracellular accumulation. Cells were treated for 18 hours with IC_{50} of tested compounds. Aliquots of lysate were submitted to ICP analysis. Results are expressed as ng Au/ mg of proteins.

ROS production.

It is well-known that mitochondria can generate hydrogen peroxide and, thus, play a crucial role in the apoptotic process [254]. Moreover, it has been previously shown that TrxR inhibition

creates dramatic consequences in cells, as the prevention of hydrogen peroxide removal and the consequent increase of its concentration causes an unbalance in cell redox conditions, thus leading to a mitochondrial membrane permeabilization and swelling [159]. On these basis, we thought of interest to measure the modification of H_2O_2 basal production in 2008 cancer cells treated with IC_{50} concentrations of gold(I) complexes by using CM-DCFDA as a fluorescent probe (Fig. 54).

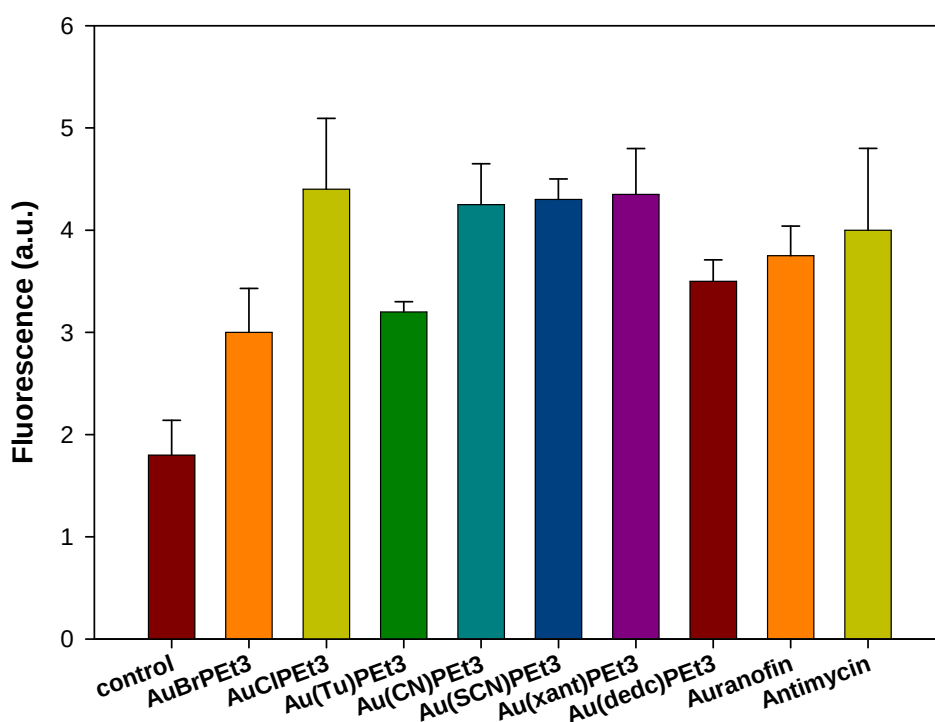


Fig. 54. Effect of gold(I) compounds on hydrogen peroxide formation in 2008 cells. 2008 cells (1×10^6) were pre-incubated in PBS/10 mM glucose medium for 20 min at 37°C in presence of $1.5 \mu\text{M}$ CM-DCFDA and then treated with IC_{50} concentrations of gold(I) compounds.

Antimycin, a classic inhibitor of the mitochondrial respiratory chain at the level of complex III, used as a positive control, was able to stimulate the production of hydrogen peroxide. Despite all phosphine gold(I) complexes were able to increase the basal production of hydrogen peroxide in treated cells, $\text{Au}(\text{CN})\text{PET}_3$, $\text{Au}(\text{SCN})\text{PET}_3$, $\text{AuCl}(\text{PET}_3)$ and $\text{Au}(\text{xant})\text{PET}_3$ were the most effective whereas the remaining derivatives stimulated ROS production in a similar or slightly lower manner than antimycin.

Apoptosis studies.

It has been previously reported that auranofin and other gold(I) derivatives induce apoptosis in cancer cells through the activation of caspases [165]. Moreover, the TrxR inhibition impairs control of apoptosis signal-regulating kinase 1 (ASK-1) activity exerted by Trx, resulting in the activation of downstream signaling cascades such as MAP kinase apoptotic cell death pathway [165]

To characterize the cell death pathway triggered by tested gold(I) compounds and with the aim to correlate TrxR inhibition to apoptosis induction, we have assessed the ability of these compounds to activate the apoptotic cell death pathway in ovarian cancer cells.

Caspase-3 activity. As Caspase-3 is a well-known executor enzyme in apoptosis pathway, we have investigated the ability of gold(I) derivatives to activate Caspase-3 in 2008 cells. Figure 55 shows that all derivatives markedly stimulated caspase-3 activity with respect to the control. The most active compounds were the gold(I) complexes bearing the cyanate, the thiocyanate and the xantogenate ligands, with an apoptosis induction about 1.2-, 1.4- and 1.6-times greater with respect to that induced by auranofin treatment, respectively.

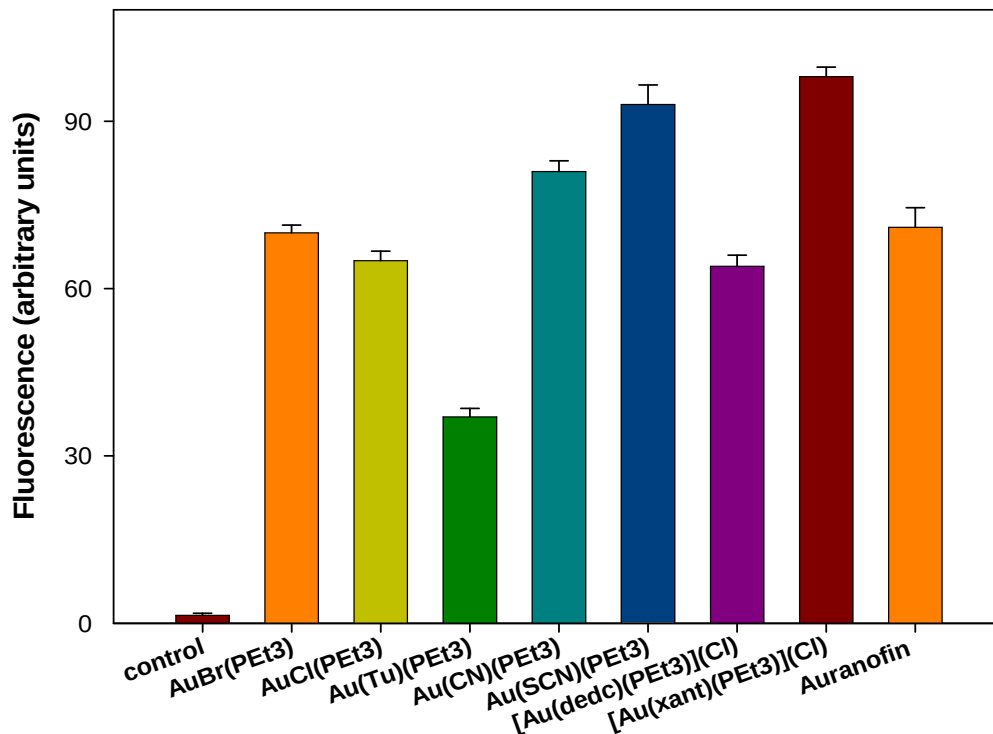


Fig.55 . Effect of gold(I) compounds on Caspase-3 activity. 2008 cells were treated for 24 hours with IC_{50} concentration of tested compounds. Cell were processes and Caspase-3 activation was estimated by AMC fluorescence

On the contrary, Au(Tu)PEt₃ was the less effective, with a Caspase-3 activation roughly 2-times lower compared to that exerted by auranofin.

Nuclear DNA fragmentation. DNA fragmentation tests performed on 2008 cells showed the ability of all gold(I) derivatives in increasing mono- and oligo-nucleosome formation, although to a different extent (Fig. 56). In particular, Au(xant)PEt₃ appeared the most efficient in inducing DNA fragmentation, being able to increase of 277 % nucleosome formation, and resulting 1.3-times more effective than auranofin in inducing apoptotic cell death. Au(Tu)PEt₃ and AuCl(PEt₃) were the less effective, with a DNA fragmentation about 3 and 8 times lower compared to that exerted by auranofin, respectively.

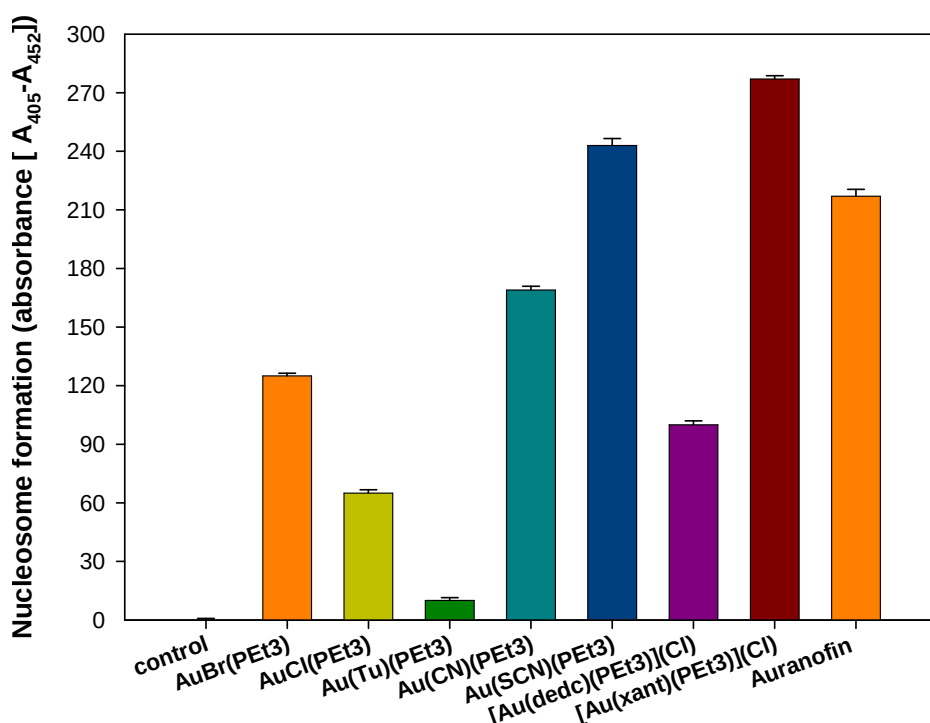


Fig. 56. Effect of gold(I) compounds on nucleosome formation. 2008 cells were treated for 24 h with IC₅₀ concentration of tested compounds. Cell were processed and the quantitative estimation of DNA fragmentation was obtained with an ELISA test.

Cell cycle analysis

In order to further investigate the cellular effects induced by gold(I) derivatives in terms of apoptosis induction as well as of cell cycle effects, a time dependent evaluation of cell cycle profiles was performed by FACS analyses on 2008 cells. Figure 57 reports the cell cycle profiles after 24 h treatment with IC₅₀ concentrations of tested gold(I) complexes. Cytofluorimetric

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results correlated well with those obtained with DNA fragmentation and Caspase-3 activation assays. In particular, Au(xant)Pet₃, Au(CN)Pet₃ and Au(SCN)Pet₃ were the most effective compounds, being able to stimulate a sub-G1 population increase of about 1.2, 1.4 and 1.6 times compared with control.

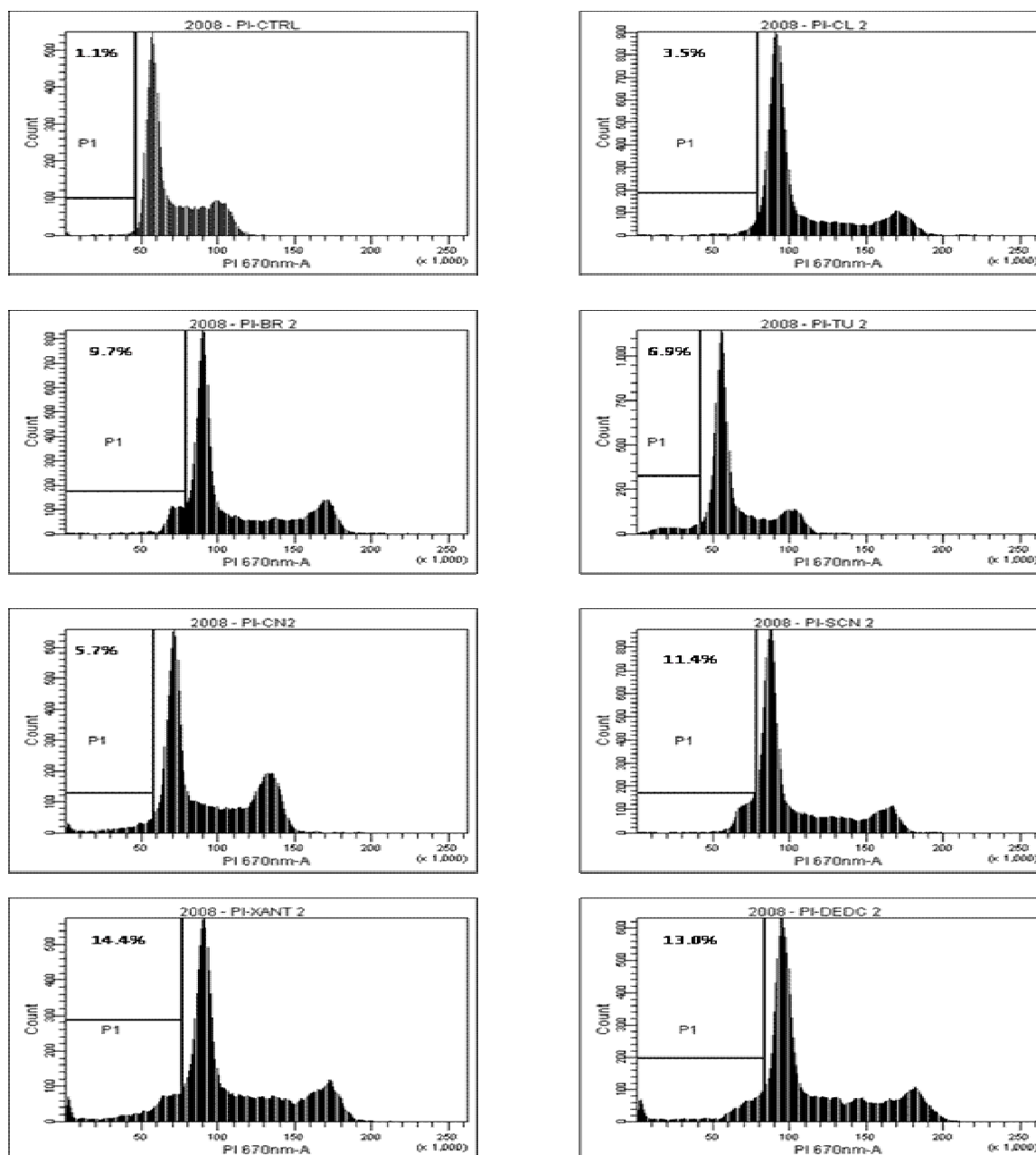


Fig. 57. Cell cycle analysis. 2008 cells were treated with gold(I) derivatives for 24 hours. Cell cycle profiles were analyzed by flow cytometry after DNA staining with propidium iodide.

RESULTS AND DISCUSSION

From this systematic analyses $\text{Au}(\text{xant})\text{PEt}_3$ and $\text{Au}(\text{SCN})\text{PEt}_3$ have been identified as the most promising agents and were selected to further investigations.

4.1 Copper Complexes

4.1.1 Tris(hydroxymethyl)phosphine copper(I) complexes containing the bis(1,2,4-triazol-1-yl)acetate ligand

Cytotoxicity Studies.

[HC(CO₂)(tz)₂]Cu[P(CH₂OH)₃]₂, [HC(CO₂)(pz^{Me2})₂]Cu[P(CH₂OH)₃]₂, and [(CH₃CN)₂Cu[P(CH₂OH)₃]₂]PF₆, and the corresponding uncoordinated ligands were examined for their cytotoxic properties against a panel of human tumor cell lines containing examples of lung (A549), colon (LoVo), and breast (MCF-7) cancer, leukaemia (HL60), and melanoma (A375). For comparison purposes, the cytotoxicity of cisplatin was evaluated under the same experimental conditions. IC₅₀ values, calculated from the dose-survival curves obtained after 48 hours of drug treatment from the MTT test, are shown in Table VIII.

Table VIII Cytotoxicity of copper(I) complexes

Compound	IC ₅₀ (μM) ± S.D.				
	HL60	A549	MCF-7	A375	LoVo-MDR
[HC(CO ₂)(tz) ₂]Cu[P(CH ₂ OH) ₃] ₂ (1)	34.3±3.9	25.1±1.1	11.3±1.7	26.7±2.0	32.3±3.6
{[(CH ₃ CN) ₂ Cu[P(CH ₂ OH) ₃] ₂]PF ₆ } (2)	21.2±2.8	14.8±0.9	16.7±2.7	21.5±1.7	47.2±2.9
[HC(CO ₂)(pz ^{Me2}) ₂]Cu[P(CH ₂ OH) ₃] ₂ (3)	10.6±2.5	2.1±1.3	1.6±0.2	2.6±0.9	7.8±1.3
HC(CO ₂ Na)(tz) ₂	ND	ND	ND	ND	ND
P(CH ₂ OH) ₃	58.7±2.3	73.0±2.4	64.2±4.2	88.7±3.8	65.2±3.2
HC(COOH)(pz ^{Me2}) ₂	ND	ND	88.8±3.9	ND	ND
Cisplatin	19.9±2.5	29.3±1.9	30.2±1.5	20.3±1.3	25.0±1.5

S.D.= standard deviation

N.D.= not detectable

IC₅₀ values were calculated by probit analysis ($P < 0.05$, χ^2 test). Cells ($5-8 \cdot 10^4 \cdot \text{mL}^{-1}$) were treated for 48 hours with increasing concentrations of tested compounds. Cytotoxicity was assessed by MTT test

Uncoordinated ligands proved to be quite ineffective in all tumor cell lines; $P(CH_2OH)_3$ gave detectable IC_{50} values that were, anyway, markedly higher than those of the corresponding copper(I) complexes. Compounds **1** and **2** showed IC_{50} values comparable with those exhibited by cisplatin against HL60, A375, and LoVo cell lines, whereas their cytotoxic activity was significantly higher against A549 and MCF-7 cell lines. This general behavior is magnified in the case of complex **3**, whose cytotoxic potency exceeded that of the parent drug by a factor ranging from about 2 to 15.

Cross-resistance profiles.

The encouraging results obtained against the in-house panel of cell lines prompted us to test the cytotoxic activity of our Cu(I) complexes onto two additional cell line pairs, which were selected for their resistance to cisplatin, 2008/C13* ovarian cancer cells, and A431/A431-Pt cervix carcinoma cells. Cytotoxicity of tested compounds in sensitive and resistant cells was assessed after a 48-hours drug exposure by the MTT test (Table IX). Cross-resistance profiles were evaluated by means of the resistance factor (RF).

Remarkably, all the tested copper(I) complexes exhibited a different cross-resistance profile than that of cisplatin. Against the ovarian cancer, RF values were about from 6 to 18 times lower than those calculated for cisplatin, while against the cervix cell line pair, they were roughly from 3 to 5 times lower. The overcoming of cross-resistance phenomena in both cell line pairs strongly supports the hypothesis of a different pathway of action of these copper(I) complexes from that of cisplatin. Also, in this set of experiments, complex **3** appeared to be the most effective derivative, giving IC_{50} values 2-3 times lower than the reference drug on the parental sensitive cell lines and 10 times lower on the cisplatin-resistant ones.

The lower cytotoxicity displayed by complexes **1** and **2** might be related to their moderate stability, as revealed by mass spectrometric and IR and NMR spectroscopic data [], which likely arises from partial *in vitro* hydrolysis of these species, thus diminishing their availability for cellular uptake and intracellular trafficking.

Table IX Cross-resistance profiles

Compounds	Human ovarian adenocarcinoma cells		
	2008	C13*	R.F.
	IC ₅₀ (μ M) $\pm$ S.D.	IC ₅₀ (μ M) $\pm$ S.D.	
[HC(CO ₂)(tz) ₂]Cu[P(CH ₂ OH) ₃] ₂ (1)	26.4 $\pm$ 1.9	12.4 $\pm$ 1.2	0.47
{(CH ₃ CN) ₂ Cu[P(CH ₂ OH) ₃] ₂ }PF ₆ (2)	24.0 $\pm$ 1.8	19.1 $\pm$ 1.9	0.79
[HC(CO ₂)(pz ^{Me2}) ₂]Cu[P(CH ₂ OH) ₃] ₂ (3)	6.6 $\pm$ 1.1	8.2 $\pm$ 2.5	1.23
Cisplatin	12.7 $\pm$ 1.7	89.2 $\pm$ 4.5	7.02
Compounds	Human squamous cervix carcinoma cells		
	A431	A431/Pt	R.F.
	IC ₅₀ (μ M) $\pm$ S.D.	IC ₅₀ (μ M) $\pm$ S.D.	
[HC(CO ₂)(tz) ₂]Cu[P(CH ₂ OH) ₃] ₂ (1)	49.3 $\pm$ 2.3	32.4 $\pm$ 2.0	0.65
{(CH ₃ CN) ₂ Cu[P(CH ₂ OH) ₃] ₂ }PF ₆ (2)	43.6 $\pm$ 1.4	23.1 $\pm$ 1.9	0.52
[HC(CO ₂)(pz ^{Me2}) ₂]Cu[P(CH ₂ OH) ₃] ₂ (3)	7.0 $\pm$ 0.9	5.7 $\pm$ 1.1	0.81
Cisplatin	22.1 $\pm$ 1.0	57.8 $\pm$ 1.8	2.62

S.D.= standard deviation

N.D.= not detectable

IC₅₀ values were calculated by probit analysis ($P < 0.05$, χ^2 test). Cells ($5-8 \cdot 10^4 \cdot \text{mL}^{-1}$) were treated for 48 hours with increasing concentrations of tested compounds. Cytotoxicity was assessed by MTT test. Resistant factor (R.F.) is defined as IC₅₀ resistant/parent line

Cell Cycle and Apoptosis Studies

We have consequently studied further the cellular effects induced by the representative complex **3**, in terms of cell cycle modifications and induction of apoptosis, aiming at the understanding of a possible mechanism of action. Up to now, there are very few mechanistic studies dealing with

the anticancer activity of copper(I) compounds. Cu(I) complexes of the type $[\text{Cu}(\text{dppey})]\text{Cl}$ (where dppey denotes *cis*- $\text{Ph}_2\text{PCH}=\text{CHPPh}_2$) have been supposed to act on mitochondria by uncoupling the oxidative phosphorylation by inner mitochondrial membrane depolarization [188]. More recently, the potent cytotoxic activity promoted by $[\text{Cu}_2(\text{DPPE})_3-(\text{CH}_3\text{CN})_2](\text{ClO}_4)_2$ (DPPE= $\text{Ph}_2\text{PCH}_2-\text{CH}_2\text{PPh}_2$) against human lung cancer cells was found to be due to a p53-mediated cell cycle arrest and apoptosis [193].

Caspase-3 activity. Caspase-3 is a well-known executor enzyme in apoptosis and cisplatin may cause apoptosis associated with increased caspase-3 activity [21]. Complex **3** was tested for its ability to induce Caspase-3 activation in lysates of 2008 cells over a period of 24 hours (Fig. 58). Treatment with IC_{50} concentrations of complex **3** did not result in a significant enhancement of enzyme activity with comparison to untreated cells at any incubation time tested. In contrast, in cisplatin-treated cells, Caspase-3 activity became significantly higher than that detected in control cells already at 12 hours, being about 6 times the control value.

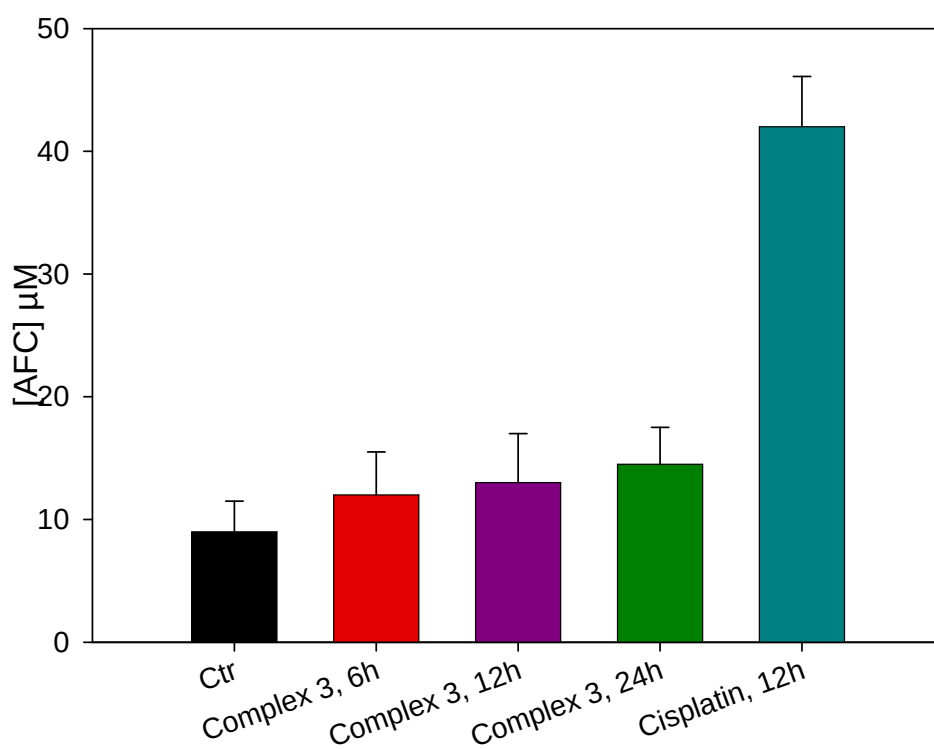


Fig. 58. Induction of caspase-3. The 2008 cells were incubated with IC_{50} concentrations of complex **3** or cisplatin. At the end of incubation time, cells were processed and Caspase-3 activation was estimated by AFC fluorescence.

RESULTS AND DISCUSSION

Cell cycle analysis. To explore complex **3** mode of action on inducing cell death, we performed a time-dependent evaluation of the cell-cycle profile of 2008 tumor cells treated with cisplatin (as a positive control) and complex **3** by fluorescence-activated cell sorting (Figure 54).

Treatment of cells with IC₅₀ concentrations of complex **3** resulted in a time-dependent increase of G2/M subpopulation within 1-48 hours (Figure 59, panels A-C). In particular, after 48 hours treatment, the percent of cells in the G2/M phase was significantly higher (about 30%) than that of control cells (Table X). However, a reduction of G1 cells fraction was observed even at 18 hours incubation (Figure 59, panel A). On the contrary, the percentage of 2008 cells with hypodiploid DNA markedly increased by treatment with cisplatin; in particular, the percentage of cells with fragmented DNA was higher (27%) compared to the control samples (Table X). These results, pointing out a scarce presence of classical signs of apoptosis, suggest that complex **3**-treated cells mainly died through a nonapoptotic cell death mechanism.

An additional insight into the cytotoxic potential of the new copper(I) complex has been offered by forward and side light scatter that are used as indices of cell size and cell granularity, respectively. After exposure to IC₅₀ concentrations of complex **3**, 2008 cells showed a marked increase of cell size and of cell granularity (Figure 60, upper right quadrant); these alterations became extremely pronounced after a 48 hours treatment (Figure 60, panel C). These remarkable morphological changes may be related to the ability of complex **3** to cause an extensive cytosolic vacuolization. Actually, it is well-known that cells with increased light scattering properties may contain structures such as vacuoles or aggregated proteins.

Table X Cell cycle phases

	18 hours			24 hours			48 hours		
	Ctr	3	<i>p</i> -value	Ctr	3	<i>p</i> -value	Ctr	3	<i>p</i> -value
SubG1	3.34±0.89	2.21±1.20	>0.05	2.31±0.22	10.43±1.90	0.03	2.44±0.17	9.23±0.46	<0.001
G1	63.88±0.57	50.02±0.99	<0.001	46.28±0.11	28.25±1.33	0.003	59.51±1.36	24.32±1.55	<0.001
G2/M	33.23±0.95	48.26±0.54	<0.001	52.27±0.14	62.43±0.55	0.002	38.65±1.24	67.09±1.66	<0.001

Percentage of cells in different cell cycle phases as function of time exposure to complex **3** vs control untreated cells. (*p*-Value was estimated by Welch test).

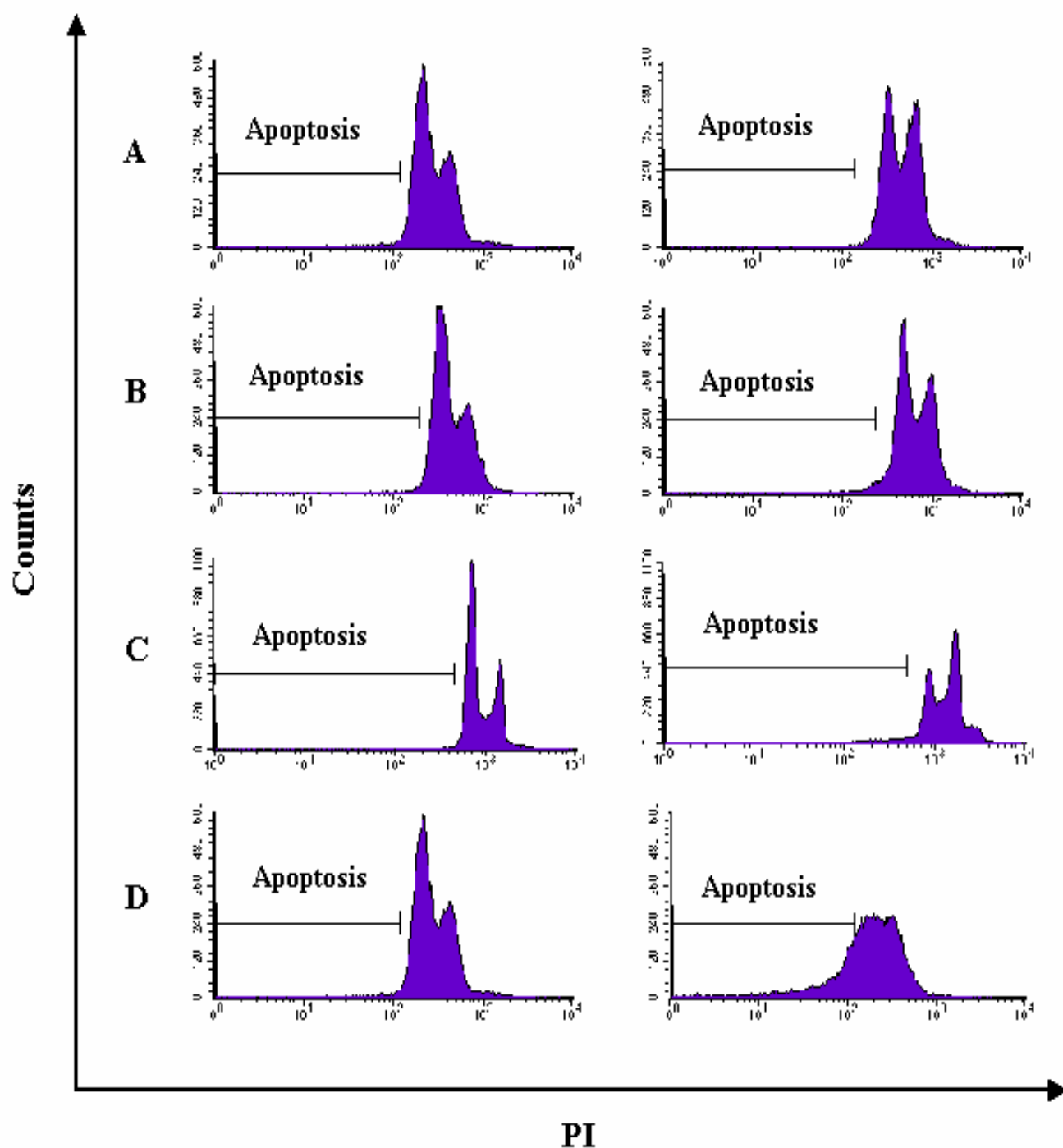


Fig. 59. Cell cycle analysis of 2008 human ovarian cancer cells. Panels A-C represent different time points (18, 24, and 48 hours, respectively) of control cells (left) and cells treated with IC_{50} concentrations of complex **3** (right). Panel D represents control cells after 18 hours in normal medium (left) and cells treated with IC_{50} concentration of cisplatin (right).

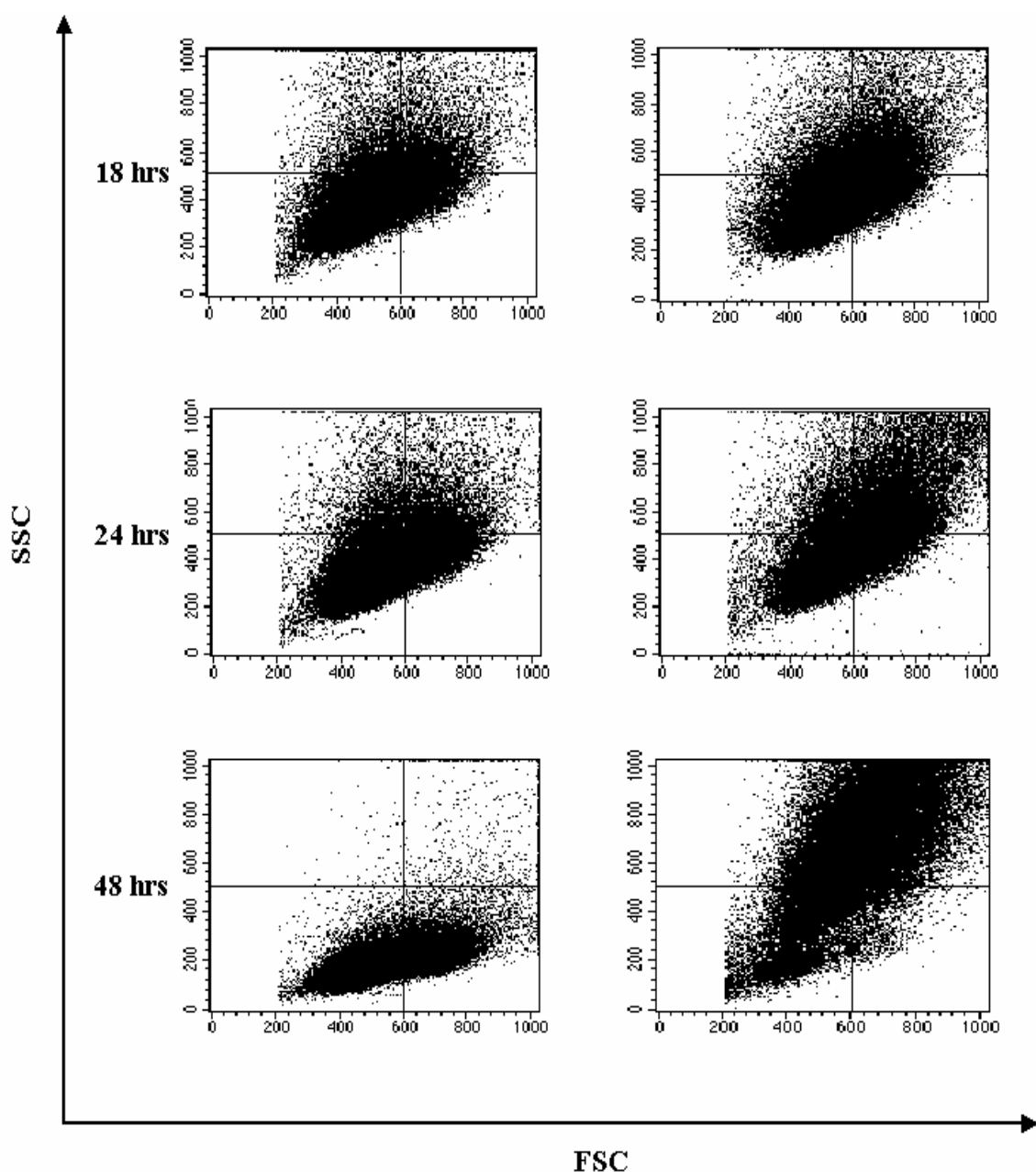


Fig. 60. Forward scattering vs side scattering as a function of time in 2008 cells untreated (left) and treated with IC_{50} concentration of complex **3** (right).

Taken together, all our data appear to be in agreement with the hypothesis that the new copper(I) complex may activate a cell death pathway distinct from apoptosis.

4.1.2 Copper(I) complexes bearing the tris(hydroxymethyl)phosphine ligand

Cytotoxicity Studies.

Complexes $[\text{Cu}(\text{thp})_4][\text{PF}_6]$ (**4**) and $[\text{Cu}(\text{bhpe})_2][\text{PF}_6]$ (**5**) and the corresponding uncoordinated ligands and counteranions were examined for their cytotoxic properties against a panel of human tumor cell lines containing examples of lung (A549), colon (CaCo2, HCT-15), cervix (Hela) and breast (MCF-7) cancer, leukaemia (HL60), lymphoma (Daudi), epatoma (HepG2) and melanoma (A375). For comparison purposes, the cytotoxicity of cisplatin was evaluated under the same experimental conditions. IC_{50} values, calculated from the dose-survival curves obtained after 48 hours of drug treatment from the MTT test, are shown in Table XI.

Complex **5** showed a relatively low cytotoxic activity against all cancer cell lines, with IC_{50} values roughly 3-fold higher than those of cisplatin. On the contrary, complex **4** showed the greatest antitumor efficacy with an average IC_{50} over 9 cell types that was over 5 times lower than that of cisplatin. In particular, in HCT-15, HepG2 and HL60 cells IC_{50} values were about 12, 17 and 26 fold lower than those calculated for cisplatin, respectively. Remarkably, against CaCo2 cells, cytotoxic potency of complex **4** exceeded over than 30-fold that of the reference drug, thus reflecting an useful selectivity for this human colon adenocarcinoma cell type.

The uncoordinated thp and bhpe ligands, and the PF_6 counteranion proved to be scarcely effective, even if on certain tumor cell types, detectable IC_{50} values were collected; in any case, their values were markedly higher than those displayed by complex **4**.

The negligible activity shown by the copper complex **5** could be associated with the high kinetic inertness of this molecule, as assessed by ESI-MS studies. This property determines a low tendency to dissociate and participate in physiologically mediated transport mechanisms which may be relevant for the cellular internalization of Cu(I) species.

RESULTS AND DISCUSSION

Table XI Cytotoxic activity

Compound	IC ₅₀ (μM) ± S.D.								
	HL60	A549	MCF-7	Daudi	HepG2	A375	CaCo2	HCT-15	HeLa
[Cu(thp) ₄][PF ₆] 4	0.60±0.02	9.11±2.71	11.08±0.52	6.94±0.18	1.26±0.10	4.58±2.41	1.08±0.12	2.00±0.03	8.21±1.50
[Cu(bhpe) ₂][PF ₆] 5	47.40±2.92	57.60±2.19	49.71±2.03	65.5±1.22	78.23±1.11	68.21±1.23	52.50±0.81	57.36±1.31	62.41±1.33
thp	68.63±2.44	72.91±2.44	64.23±4.29	>100	98.71±3.63	88.70±3.88	>100	>100	>100
bhpe	83.72±3.23	>100	>100	>100	71.71±1.64	73.21±1.22	84.11±2.22	91.71±4.01	>100
KPF ₆	>100	>100	>100	>100	>100	>100	>100	>100	>100
Cisplatin	15.91±1.51	29.21±1.92	19.04±1.51	23.97±2.51	21.50±1.41	20.33±1.33	35.42±1.40	25.34±1.31	10.50±1.51

S.D.= standard deviation

N.D.= not detectable

IC₅₀ values were calculated by probit analysis ($P < 0.05$, χ^2 test). Cells ($5-8 \cdot 10^4 \cdot \text{mL}^{-1}$) were treated for 48 h with increasing concentrations of tested compounds. Cytotoxicity was assessed by MTT test

RESULTS AND DISCUSSION

Cross-resistance profiles.

The encouraging results obtained against the in-house panel of cell lines prompted us to test the cytotoxic activity of the most potent derivative, complex **4**, onto three additional cell line pairs, two of which (2008/C13* ovarian cancer cells and A431/A431-Pt cervix carcinoma cells) selected for their resistance to cisplatin and one (LoVo/LoVoMDR) for its resistance to doxorubicin, thus retaining the MDR phenotype. Cytotoxicity of tested compounds in sensitive and resistant cells was assessed after a 48 h drug exposure by the MTT test. Data are cumulated in Table XII. Cross-resistance profiles were evaluated by means of the resistance factor (RF).

Table XII Cross-resistance profiles

Compound	Human ovarian adenocarcinoma cells		
	2008	C13	R.F.
	IC ₅₀ [μM] ± S.D.	IC ₅₀ [μM] ± S.D.	
[Cu(thp) ₄][PF ₆] 4	1.48±0.21	2.88±1.07	1.9
Cisplatin	12.69±1.72	89.18±4.50	7.02
Compound	Human cervix squamous carcinoma cells		
	A431	A431/Pt	R.F.
	IC ₅₀ [μM] ± S.D.	IC ₅₀ [μM] ± S.D.	
[Cu(thp) ₄][PF ₆] 4	14.37±1.41	13.26±0.80	0.92
Cisplatin	22.06±1.62	57.76±1.81	2.61
Compound	Human colon adenocarcinoma cells		
	LoVo	LoVo-MDR	R.F.
	IC ₅₀ [μM] ± S.D.	IC ₅₀ [μM] ± S.D.	
[Cu(thp) ₄][PF ₆] 4	1.54±0.03	2.9±0.1	1.88
Doxorubicin	1.46±2.30	44.89±0.90	30.74

S.D. = standard deviation. IC₅₀ values were calculated by probit analysis ($P < 0.05$, χ^2 test). Cells ($3\cdot 5\cdot 10^4\cdot \text{mL}^{-1}$) were treated for 48 hours with increasing concentrations of tested compounds. Cytotoxicity was assessed by MTT test. Resistant factor (R.F.) is defined as IC₅₀ resistant/parent line.

RESULTS AND DISCUSSION

Remarkably, complex **4** showed activity levels very similar on both cisplatin sensitive and resistant cell lines that indicates a different cross-resistance profile than that of cisplatin. Against the ovarian cancer, RF value was about 4 times lower than that calculated for cisplatin, while against the cervix cell line pair, it was about 3 times lower. The overcoming of cross-resistance phenomena in both cell line pairs strongly supports the hypothesis of a different pathway of action of this copper(I) complex from that of cisplatin.

Acquired multidrug resistance (MDR), whereby cells become refractory to multiple drugs, poses a most important challenge to the success of anticancer chemotherapy. Cytotoxicity assays testing complex **4** against LoVo/ LoVo MDR cell line pair allowed calculation of a RF value about 16-fold lower than that obtained with doxorubicin, thus suggesting that this copper(I) complex is not a potential MDR substrate.

Cytotoxicity in non-tumor cells

Cytotoxicity experiments were performed on murine and human non-transformed cells treated with complex **4**, in order to get an indication of the drug selectivity.

Cytotoxicity was evaluated by MTT test after 48 hours incubation with increasing amounts of complex **4** and cisplatin (reference drug). As show in Table XIII, complex **4** exhibited a a similar cytotoxicity than that of cisplatin. However, calculating the ratio between mean IC₅₀ values over 15 tumor cell lines and those arising from non-tumor cells, a higher selectivity ratio towards tumor cells occurred for complex **4** in comparison with the reference drug cisplatin. Indeed, while cisplatin required almost the same concentration in both tumor and non-tumor cells to inhibit viability of half cell population, complex **4** needed roughly 4 times lower dose.

Table XIII Cytotoxicity in non-tumor cells

Compound	IC ₅₀ (μM) ± S.D		
	V79	CHO	HFF-1
{Cu[P(CH ₂ OH) ₃] ₄ }PF ₆	35.72±2.03	13.00±1.81	13.00±1.81
Cisplatin	33.46±0.31	15.20±1.65	15.20±1.65

S.D. = standard deviation. IC₅₀ values were calculated by probit analysis ($P < 0.05$, χ^2 test). Cells ($5 \cdot 10^4 \cdot \text{mL}^{-1}$) were treated for 48 hours with increasing concentrations of tested compounds. Cytotoxicity was assessed by MTT test.

Cellular uptake

With the aim to correlate complex **4** cytotoxicity to its intracellular accumulation, cellular uptake experiments were performed in 2008 cells after continuous treatment, lasting 6, 12, 18, 24 and 36 hours, with 12.5 and 6.25 μM of the investigated copper(I) complex (Fig. 61). Intracellular copper amount was quantified by means of ICP analysis and expressed as ng metal/ mg of cellular proteins.

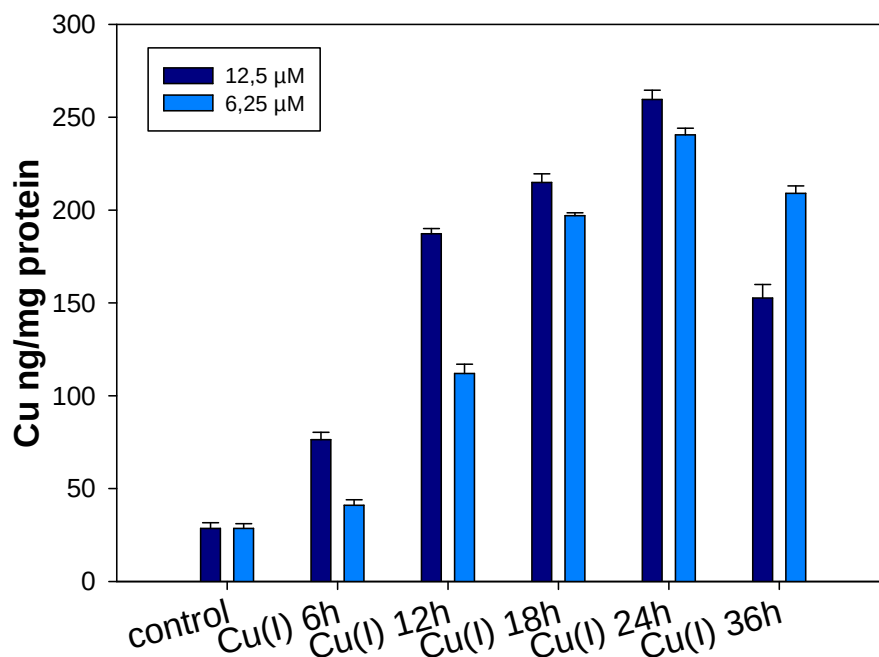


Fig. 61. Intracellular copper(I) complex accumulation. 2008 cells were incubated with complex **4** for 6, 12, 18, 24 and 36 hours. Aliquots of lysate were submitted to ICP analysis.

Intracellular accumulation was time and dose dependent within 12 hour treatment, whereas, following 18 or 24 hour exposure, the approximately linear dependence on concentration and time was missed. These data, revealing a saturability of copper complex uptake, clearly suggest the involvement of active transport mechanisms. In particular, complex **4** uptake could be mediated by hCtr1 multiprotein complex, the ATP-dependent carrier responsible for endogenous copper uptake [168]. This transmembrane protein complex is endowed with a high selectivity for monovalent cations and, in particular, for Cu^+ ions [169]. With the aim to get more informations concerning complex **4** cellular accumulation we carried out some competitive uptake experiments in 2008 cells treated with complex **4** together with a monovalent (Ag^+) or a bivalent (Zn^{2+}) ion (Fig. 62).

RESULTS AND DISCUSSION

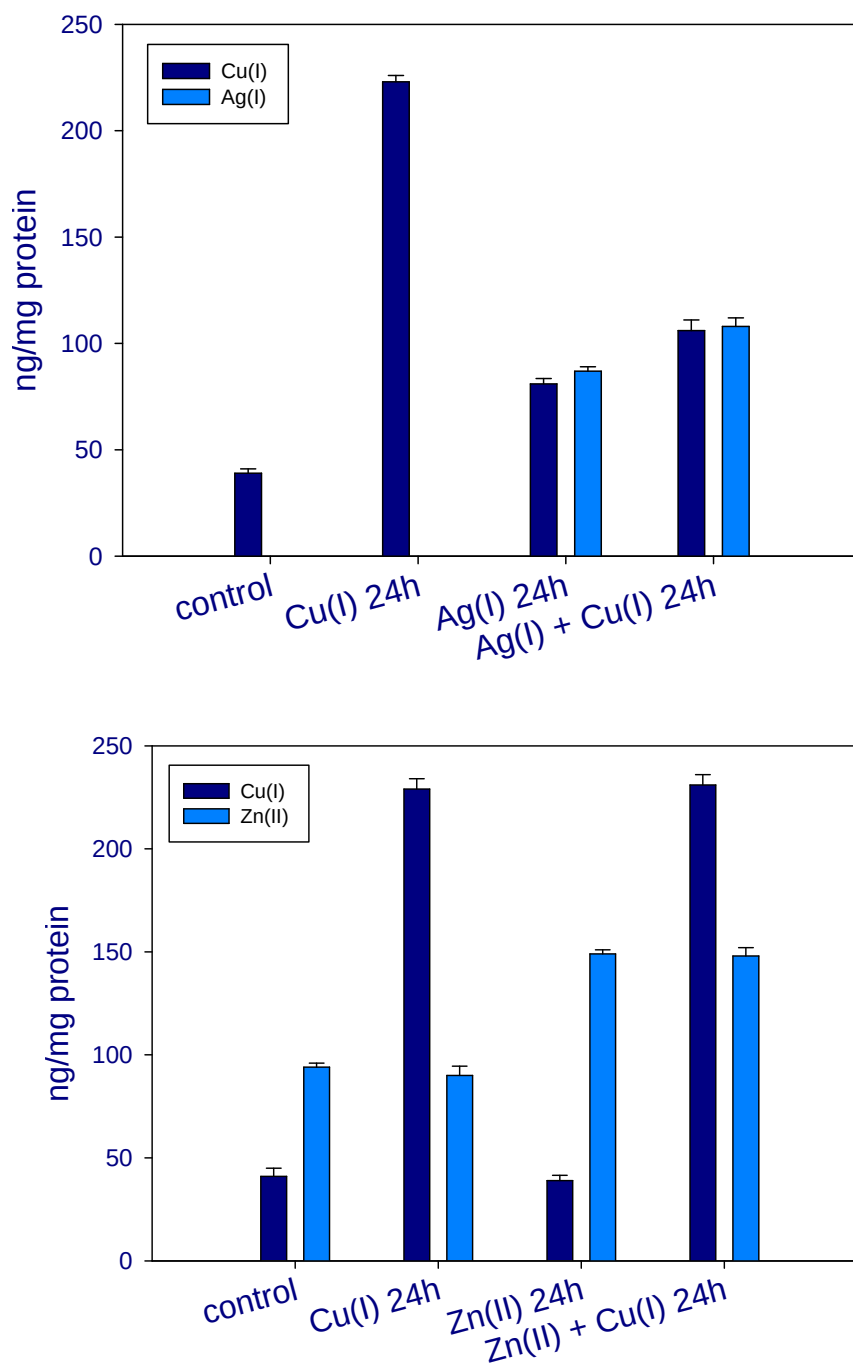


Fig. 62. Competitive uptake experiments. 2008 cells were incubated with: (A) complex **4** and/or AgNO_3 for 24 hours (B) complex **4** and/or ZnCl_2 . Aliquots of lysate were submitted to ICP analysis.

RESULTS AND DISCUSSION

As underlined in Fig. 62, a significant decrease of complex **4** intracellular accumulation was induced by the co-treatment with Ag(I) whereas any effect in copper uptake was underlined following the co-treatment with Zn(II). These findings clearly attest a certain selectivity of transporter involved in complex **4** cellular accumulation for monovalent cations and in particular, strongly support the hypothesis that complex **4** uptake is mediated by an ATP-dependent carrier, possibly identified in hCtr1.

Cytotoxicity tests to determine different subcellular organelle damages

In order to provide information on the antiproliferative effect of complex **4**, we performed a time-dependent evaluation of its cytotoxicity by using three different standard methods which, at relatively short exposure times, assess the damage to different subcellular organelles. MTT test, detecting the viable mitochondria able to reduce the MTT dye to blue formazan, mainly reflects damage to mitochondria. The NR assay, measuring the intracellular accumulation of a weak cationic dye, indicates damage to lysosomes and Golgi apparatus. Finally, TB test quantifying dead cells by means of their staining after a vital dye treatment, reveals damage to cell membrane.

Fig. 63 shows the comparison of IC₅₀ values detected by MTT, NR and TB assays in 2008 cells treated with complex **4** for different exposure times. At 3 hours, neither of the three methods revealed significant IC₅₀ values; otherwise a time dependent increase in cytotoxicity becomes apparent after longer exposure times. Anyway, the NR test revealed the strongest and earliest effects among those revealed by all three assays; in fact, following the 12 hours treatment, IC₅₀ values obtained with NR were about one half and one third than those calculated from the dose-response profiles by MTT and TB test, respectively.

These results suggest that lysosomes/Golgi apparatus are more sensitive to complex **4** treatment. On the contrary, the scarce permeability to vital dye indicates that plasma membrane function is still maintained until the late phase of cell death.

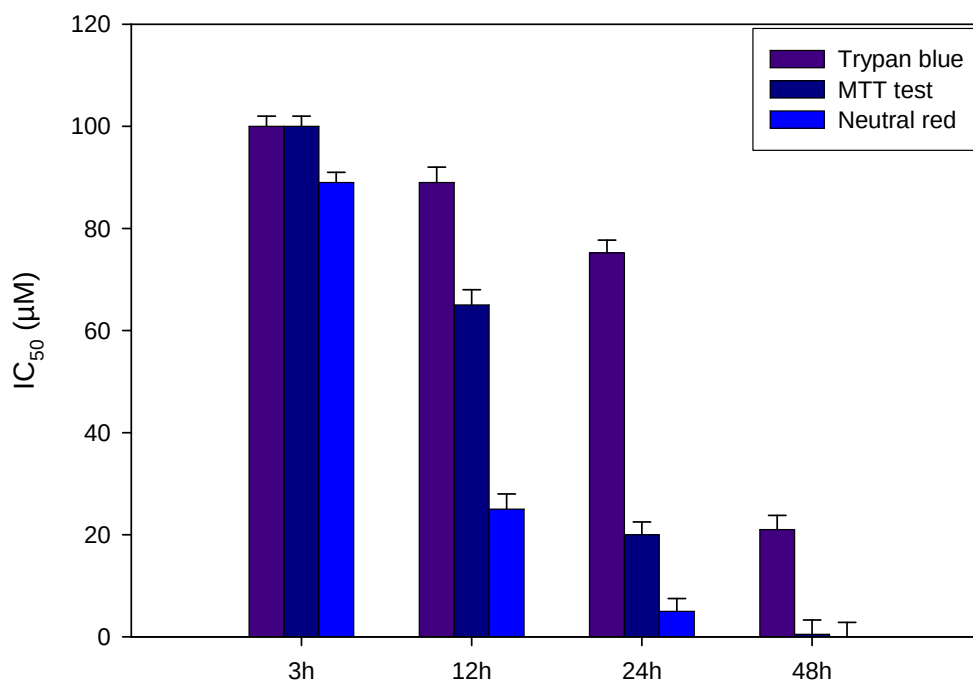


Fig. 63. Comparison of IC₅₀ values detected by MTT, NR and TB test after incubation of 2008 cells with complex **4** for 3, 12, 24 and 48 hours.

Cytotoxicity with ROS scavengers

It is well-known that copper represents an excellent catalyst of redox cycles and therefore it can stimulate cellular ROS formation [201]. To evaluate the contribution of oxidative stress in the execution of tumor cell death, complex **4**-mediated cytotoxicity against 2008 cells was investigated in the presence of various ROS scavengers. Mannitol (1 mM) and DABCO (1.2 mM) were respectively used as scavengers of hydroxyl radical and singlet oxygen, the main damaging oxidative species. Ascorbic acid plus α -tocopherol (1 mM), working in synergism, act as direct antioxidants towards singlet oxygen and superoxide anion and indirect antioxidants breaking the chain reaction of lipid peroxidation [201]. Furthermore, selenium, as cofactor of various selenoproteins, has been found to protect at low concentrations tumor ovarian cells from oxidative damage induced by hydrogen peroxide [201]. Chemicals were added to 2008 cell growth medium in concomitance with complex **4** treatment which was extended to 48 hours. The results presented in Fig. 64 clearly attest the cytoprotective properties of all ROS scavengers; in particular, the co-presence of α -tocopherol and ascorbic acid totally prevent the cytotoxic

activity of complex **4**. Taken together, these data suggest the involvement of ROS production in copper(I) complex mechanism.

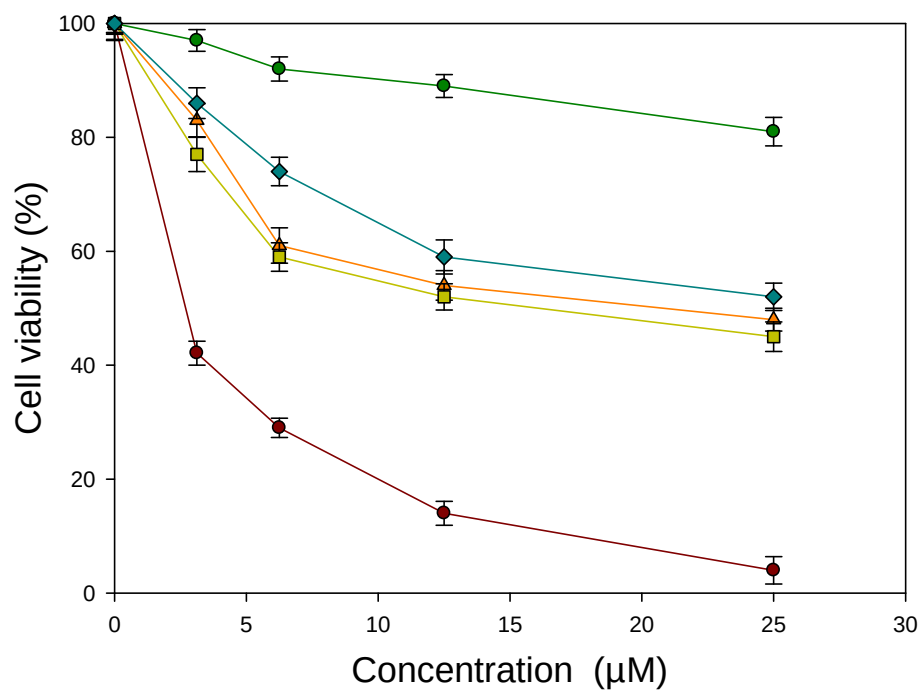


Fig. 64. Effects of ROS scavengers on complex **4** cytotoxicity against 2008 cells. Mannitol (1mM, ▲), DABCO (1.2 mM, ■), tocopherol + ascorbic acid (1mM, ●) and sodium selenite (0.1 μM, ◆) were added to cell cultures for 48 hours in concomitance with complex **4**. Complex **4** alone (●). MTT test was used to measure cell viability. Results are expressed as percentage viability of control cells.

ROS production

Cellular production of ROS upon treatment with complex **4** was measured in 2008 cells by the fluorogenic probes CM-H₂DCFDA and DHR. 2008 cells, pre-incubated with CM-H₂DCFDA or DHR, were treated with IC₅₀ concentrations of complex **4** for increasing exposure times.

Figure 65 shows that no significant levels of reactive species production with respect to the basal one of control cells was detected after either exposure times with both H₂DCFDA (panel A) and DHR (panel B) probe.

Similar negative results have been already obtained by other authors investigating the cellular effects induced by a thioxo-1,2,4-triazole copper(II) complex [34]. Most probably, as stated in that context, the intracellular ROS production associated with copper complexes needs to be analyzed by other methods. In our case, the potential presence of free phosphines, a well known

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reducing agent in chemical reactions, released partially from the metal centre may interfere in ROS determination.

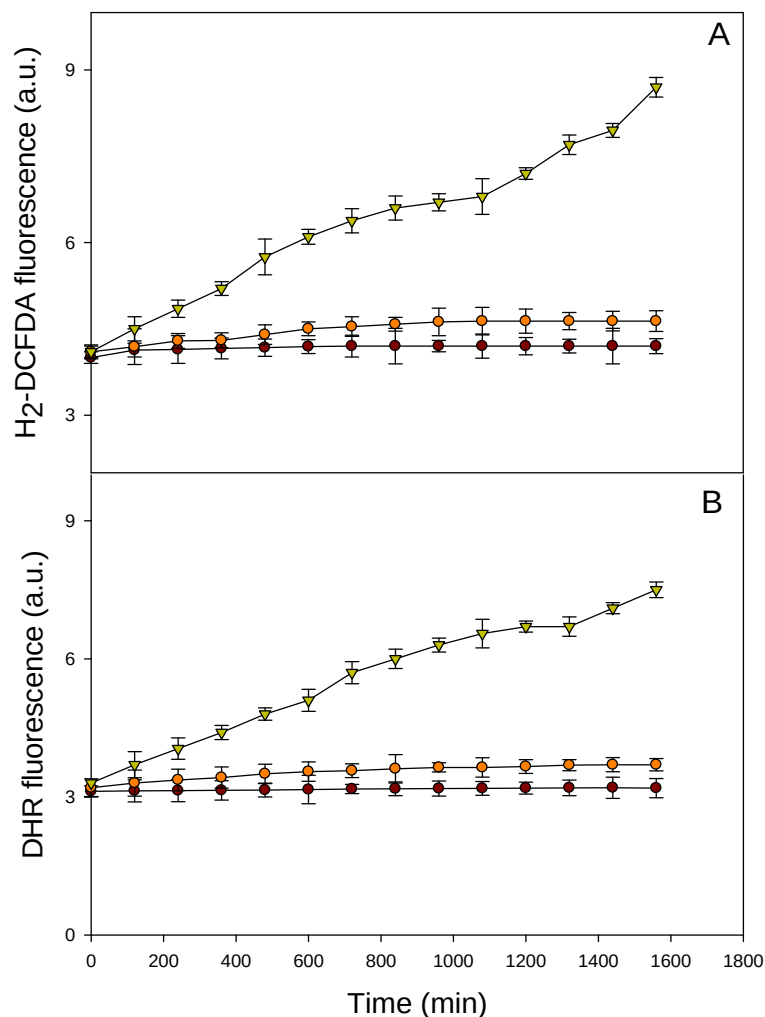


Fig. 65. Effect of IC_{50} concentrations of complex **4** on ROS production in 2008 cells. Cells were loaded with the fluorescent probes CM-H₂DCFDA (10 μ M, panel A) and DHR (50 μ M, panel B) and increase in fluorescence was monitored. Symbols: $\bullet$, control; $\circ$ complex **4**; ∇ antimycin (3 μ M).

Apoptosis studies.

Aiming at the understanding of a possible mechanism of action, we have subsequently studied the cellular effects induced by the representative complex **4**, in terms of apoptosis induction.

Caspase-3. This protease involved in both intrinsic and extrinsic apoptosis pathways was found activated by different copper(II) complexes. Furthermore, cisplatin may cause apoptosis

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associated with increased Caspase-3 activity. Complex **4** was tested for its ability to induce Caspase-3 activation in 2008 cells treated for 18 hours (Fig. 66). Treatment with IC_{50} concentration of complex **4** did not result in a significant enhancement of enzyme activity with comparison to untreated cells.

Differently, cisplatin determined a Caspase-3 activity increase, being AMC fluorescence roughly from 5 to 7 times higher in treated cells with respect to the control cells.

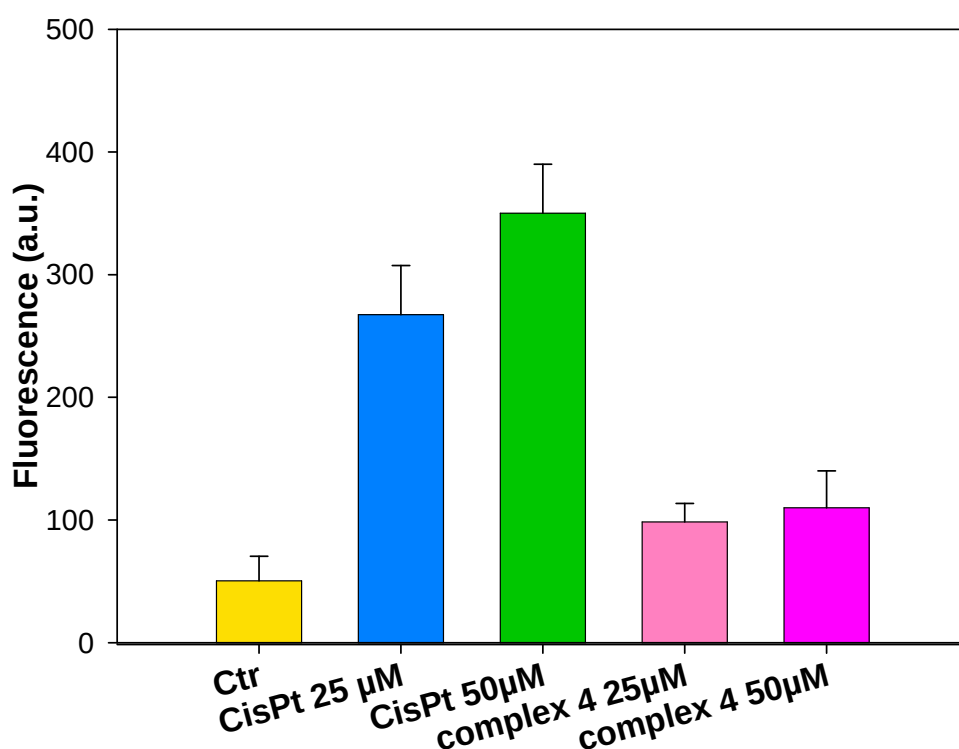


Fig. 66. 2008 cells were treated with complex **4** or cisplatin for 18 hours. Cell were processes and Caspase-3 activation was estimated by AMC fluorescence

Nuclear DNA fragmentation. The apoptosome complex formation represent a critical step in the apoptotic process. Complex **4** was studied for its ability to induce nucleosome formation in 2008 cells treated for 24 hours (Fig. 67).

While cisplatin treated-cells presented about a 20-fold increase in nuclear DNA fragmentation, IC_{50} concentrations of complex **4** did not enhanced mono- and oligo-nucleosome formation.

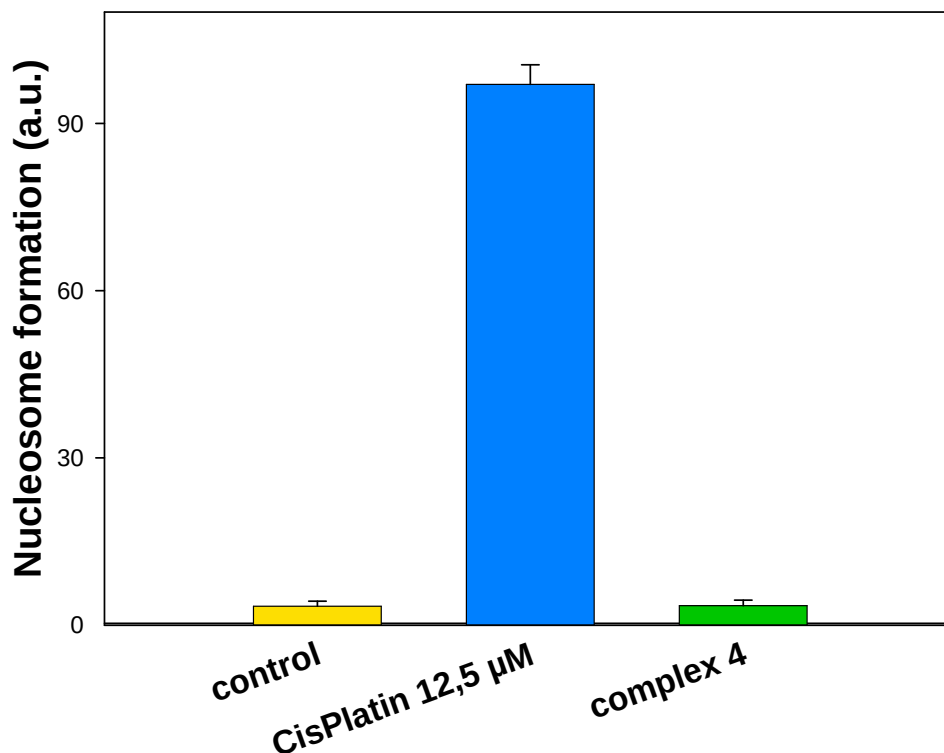


Fig. 67. Complex **4** effect on nuclear DNA fragmentation. 2008 cells were treated for 24 hours with IC₅₀ concentration of complex **4** or 12.5 µM of cisplatin. Cell were processed and the quantitative estimation of DNA fragmentation was obtained with an ELISA test

Citocrome c release. Release of pro-apoptotic factor citocrome c from the mitochondria to cytosole represents a central event in apoptosis induction. As seen in Fig. 68, complex **4**, tested for its ability to induce citocrome c release in 2008 treated cells, was found quite ineffective even at much higher concentration than IC₅₀.

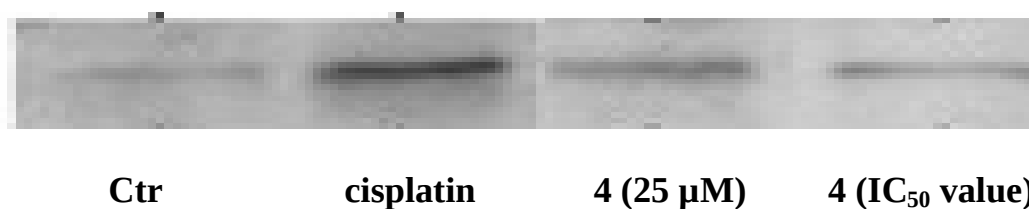


Fig. 68. Complex **4** effect on cytochrome c release. 2008 cells were treated with complex **4** or 12.5 µM cisplatin for 24 hours. Cytochrome c was estimated in the cytosolic fraction by Western blotting

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PI and Hoechst staining. Healthy, apoptotic and necrotic cells could be distinguished through their ability to exclude/include specific cationic dyes as PI and Hoechst 33342. Complex **4** ability to induce cell death through activation of apoptotic and/necrotic pathway was analyzed by means of a double staining with PI and Hoechst 33342 probes. As seen in Fig. 69, 24 hours cisplatin-treated cells showed, according with thousand of literature results, an extensive Hoechst 33342 positivity, compared with control cells, confirming cisplatin ability to determine apoptotic cell death. Conversely, 24 hour treatment with complex **4** did not lead to an increase of percentage of Hoechst 33342 and PI positive cells. Only prolonging exposure time to 48 hours and utilizing concentration of copper(I) complex exceeding its IC_{50} value, a slight number of PI positive cells was detected.

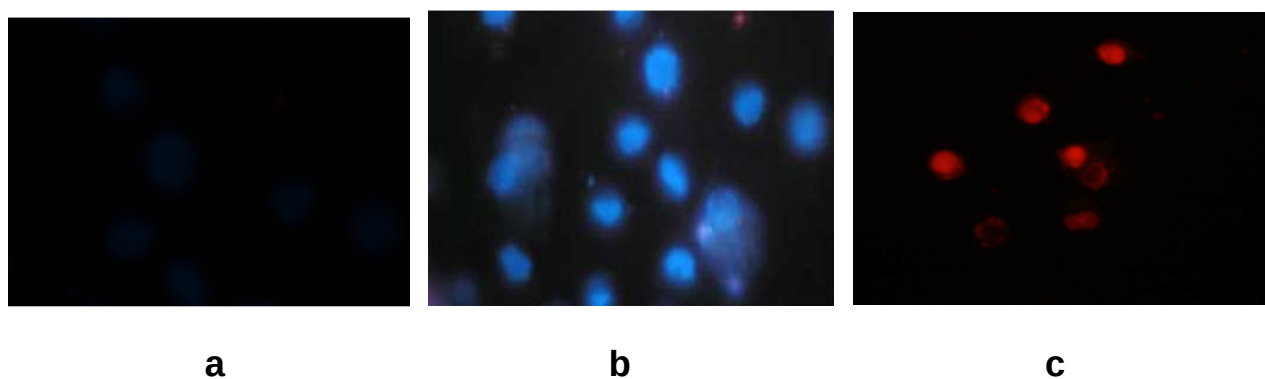


Fig. 69. .PI and Hoechst 33342 double staining of 2008 cells. **a:** 2008 control cells, **b:**2008 cells treated with IC_{50} concentration of cisplatin for 24 hours, **c:** 2008 cells treated with IC_{50} concentration of complex **4** for 48 hours.

Cell cycle analysis and mitotic index

With the aim to deeply investigate the cellular effect induced by complex **4** in terms of cell cycle modifications and induction of apoptosis, a time-dependent evaluation of cell cycle profile was performed by FACS in 2008 human ovarian cancer cells treated with IC_{50} concentrations of complex **4** (Fig. 70). Cell cycle analysis revealed a significant time-dependent decrease of G1 phase. After 48 h treatment, with respect to control cells, about 1.8 fold reduction of G1 phase as well as about 1.5 fold increase of S, G2 and M phases, were observed (Fig. 70, panels G, H; Table XIV).

PI cytofluorimetric analysis is not able to discriminate the S and M phase from the G2 phase because of the equal DNA cellular content. To better evaluate the influence of complex **4** on M

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phase, the mitotic index in 2008 cells treated with IC₅₀ concentrations of copper(I) complex for 12 and 24 hours was calculated (Table XIV). Mean percentages of mitotic index attests a significant time-dependent decrease of the number of treated cells able to complete the mitotic division.

Moreover, by the examination of histograms, no DNA fragmentation was detected following 3, 12 and 24 hour exposure (Fig. 70, panels A-F). However, cells treated with complex **4** for 48 hours showed a slightly appreciable DNA fragmentation signal compared to the control samples (15.4%) (Fig. 70, panels G-H, Table XIV).

Table XIV Cell cycle phases

	3h			12h		
	Ctr	4	<i>p-Value</i>	Ctr	4	<i>p-Value</i>
Su-G1	0.83±0.76	0.82±0.51	>0.05	1.1±0.81	1.04±0.97	0.03
G1	77.81±1.22	80.51±2.53	<0.001	68.46±1.77	63.23±2.46	0.003
S,G2/M	21.72±0.98	18.99±1.43	<0.001	29.74±1.31	34.15±1.39	0.002
	24h			48h		
	Ctr	4	<i>p-Value</i>	Ctr	4	<i>p-Value</i>
Su-G1	4.24±0.71	2.43±0.66	<0.001	1.21±0.73	15.39±0.96	<0.001
G1	70.92±1.82	60.9±1.35	<0.001	65.57±1.21	41.03±1.39	<0.001
S,G2/M	20.29±1.11	33.5±1.28	<0.001	32.62±1.46	37.03±1.12	<0.001

Percentage of cells in different cell cycle phases as function of time exposure to complex **4** vs control untreated cells. (*p-Value* was estimated by Welch test).

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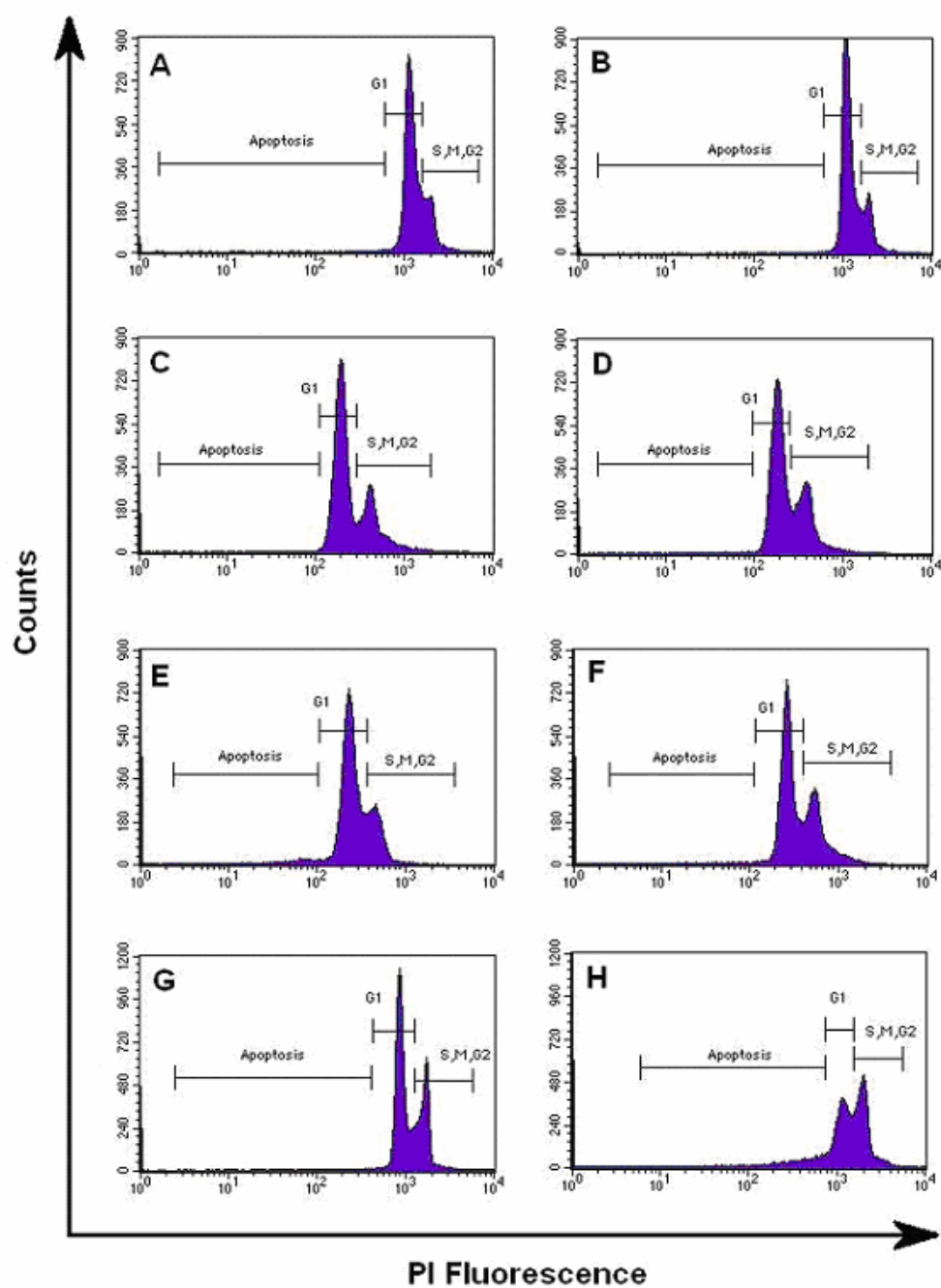


Fig. 70. Cell cycle analysis of 2008 cells. Panels A-H represent different time points (3, 12, 24, and 48 hours, respectively) of control cells (left) and cells treated with IC_{50} concentrations of complex 4 (right).

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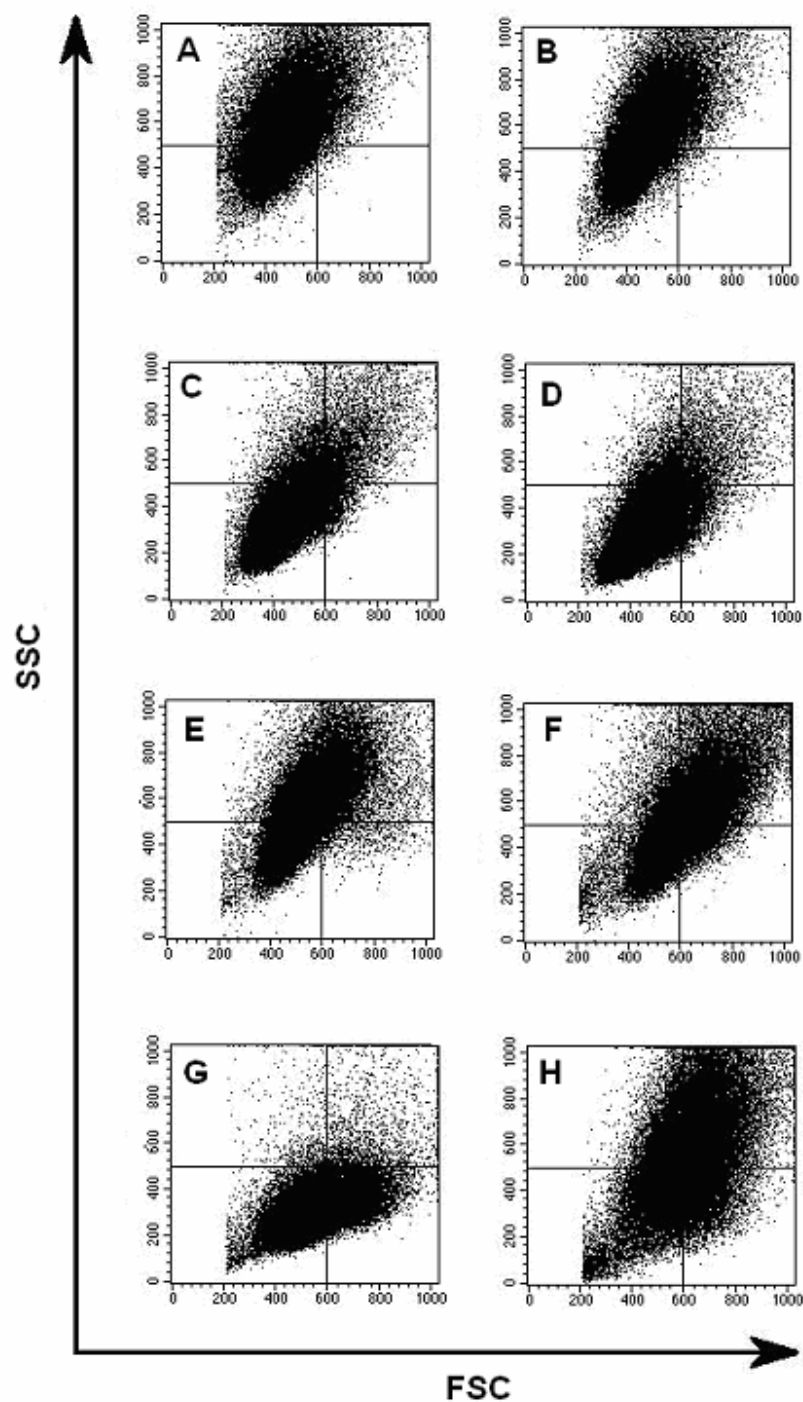


Fig. 71. Forward scattering vs side scattering as a function of time in 2008 cells untreated (left) and treated with IC_{50} concentrations of complex 4 (right). Panels A-H represent different time points (3, 12, 24, and 48 hours, respectively).

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Side scattering and forward scattering based analysis showed in complex **4** treated cells a time dependent increase of cell complexity and size (Fig. 71, panels B, D, F, H). Following 48 hours treatment, it can be noted that 36 % of cytofluorimetric counts shifted in the high complexity and size fraction of the cytogram (Fig. 67, panel H, upper right quadrant). These remarkable morphological changes may be related to the ability of complex **4** to cause an extensive cytosolic vacuolization.

Mitochondrial membrane potential ($\Delta\Psi$)

Considering that mitochondria play a key role in the pathways to cell death, we investigated the mitochondrial energization of treated tumor cells as the retention of a mitochondrial selective cationic fluorescent probe, tetramethyl rhodamine methyl ester (TMRM). Fig. 72 shows the flow cytometric profiles of 2008 cells treated with 6.25 and 12.5 μM of complex **4** for 24 hours and stained with 100 nM of TMRM.

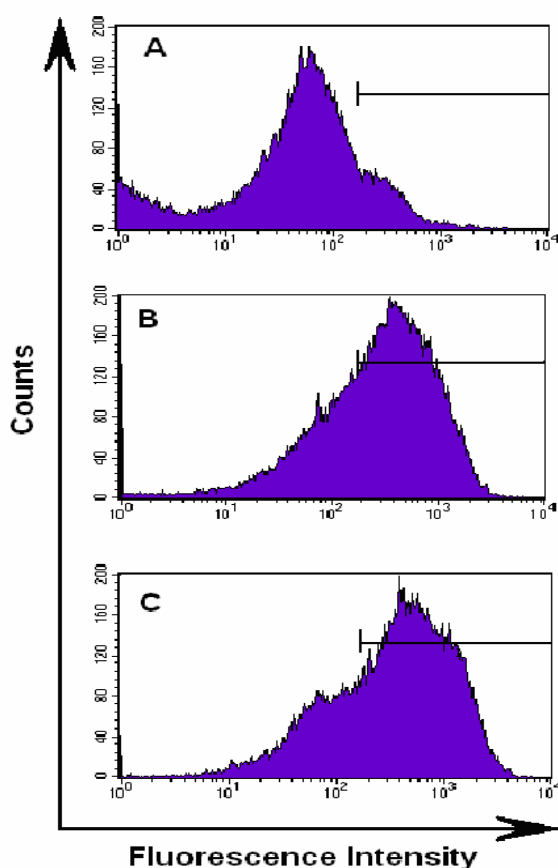


Fig. 72. Flow cytometric profiles of 2008 cells untreated (panel A) and treated with 3.125 (panel B) or 6.25 (panel C) μM of complex **4** for 24 hours and stained with TMRM (100 nM).

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The results attest that copper(I) complex induced a massive increase of the TMRM fluorescence reflecting a dramatic alteration of mitochondrial membrane potential. The marked increase of $\Delta\Psi_{mt}$ (hyperpolarisation) can be attributed to a variety of mechanisms such as increased mitochondrial mass and number per cell, or unregulated mitochondrial matrix volume [37]. In particular, mitochondrial membrane hyperpolarization induced by complex **4** might be correlated with the induction of a G2/M phase cell cycle arrest, as previously reported for other anticancer agents [38].

Morphological changes induction.

Heamatoxylin/eosin staining. To further explore morphological changes induced by investigated copper(I) complex, classical haematoxylin/eosin staining were performed in 2008 cells treated with IC_{50} concentrations of complex **4**. Following 24 hours treatment a striking cytoplasmatic vacuolization was revealed (Fig. 73) which does not appear if cells were pre-incubated with the autophagy inhibitor monensin.

Monodansylcadaverine staining. Furthermore, in 2008 cells treated for 24 hours with IC_{50} concentrations of complex **4**, vacuoles were negative to monodansylcadaverine, a fluorescent dye able to permeate only autophagic vacuoles (Fig. 74).

These results correlate well with those previously mentioned obtained from forward scattering and side scattering cytofluorimetric analyses.

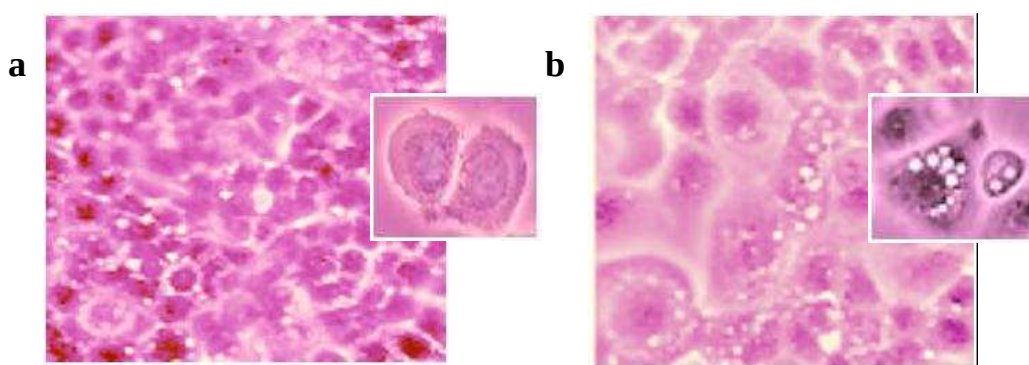


Fig. 73 Determination of morphological changes by means of heamatoxylin/eosin staining. a: 2008 control cells, b: 2008 cells treated with IC_{50} concentration of complex **4** for 24 hours (40x, inserts 100x).

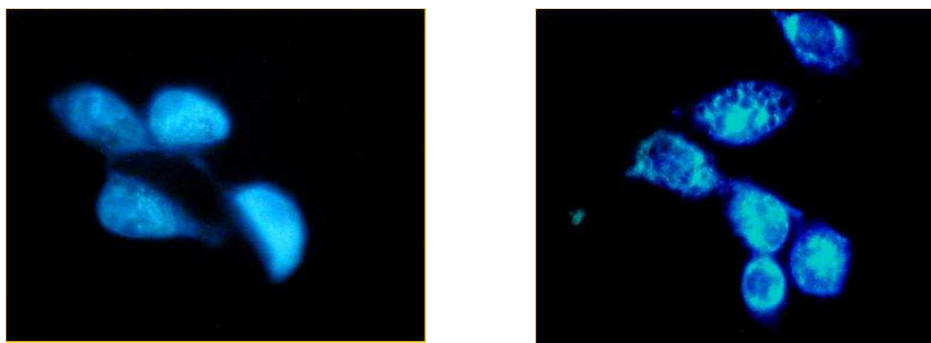


Fig. 74. Vacuoles analysis by means of monodansylcadaverine staining. a: 2008 control cells, b: 2008 cells treated with IC_{50} concentration of complex **4** for 24 hours

Taken together, these results suggest that complex **4** induced a cell death consistent with the features of type 3B cell death described by Clarke [256], characterized by dilatation of the endoplasmic reticulum, nuclear envelope, or other organelles. The morphological features of these cells remind us of a distinctive, nonapoptotic PCD, sometimes named paraptosis [256], in which the enlargement of reticular compartments, due to an uncharacterized intracellular osmotic disequilibrium, forms apparently empty spaces, sparing the plasma membrane until the late phases of the death process, eventually accompanied by cells rounding and detachment [256].

Proteasome inhibition and poly-ubiquitinated protein accumulation

Proteasome inhibition. Chen et al. have previously reported that certain copper complexes act as proteasome inhibitors in cancer cells [202]. To examine whether complex **4** is capable of inhibiting the proteasome activity, 2008 cells were treated with IC_{50} concentrations of the investigated copper(I) complex. After 48 hours exposure, cell lysates were stained with fluorescent proteasome substrates, N-Suc-Leu-Leu-Val-Tyr-AMC or Z-Leu-Leu-Glu-AMC, specific for proteasomal chymotryptic and peptidylglutamyl-hydrolyzing activity, respectively.

Following 48 hour treatment with complex **4**, the proteasomal chymotrypsin-like and the peptidylglutamyl-hydrolyzing activities of 2008 cell lysates were decreased by almost 65 and 74 %, respectively, compared with control cells (Fig. 75).

Poly-ubiquitinated protein accumulation. Consistent with the inhibition of the proteasomal chymotrypsin-like and peptidylglutamyl-hydrolyzing activity, significantly increased levels of

poly-ubiquitinated proteins were detected in the lysates prepared from cells treated with complex **4**, compared with those obtained from control cells (Fig. 76 A). Moreover, poly-biquitinated protein aggregosomes were confirmed in complex **4**-treated cells by means of immunofluorescence tests. (Fig. 76 B).

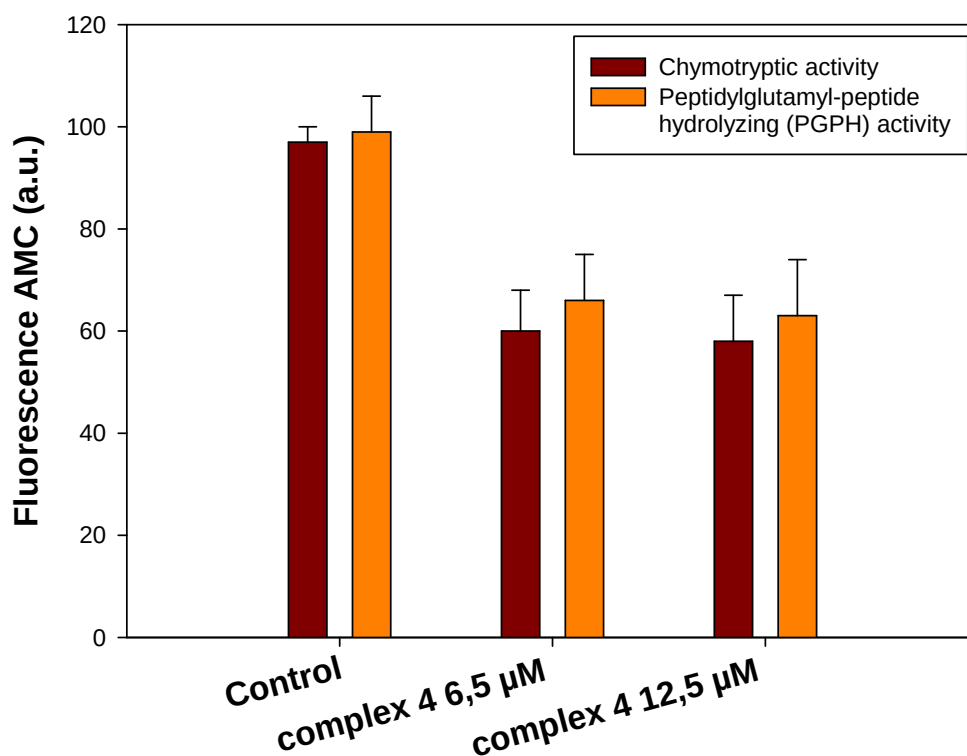


Fig. 75. Effect of complex **4** on proteasome inhibition. 2008 cells treated for 24 hours with IC_{50} concentration of complex **4**. Proteasome inhibition estimated using the fluorogenic substrates N-Suc-Leu-Leu-Val-Tyr-AMC (chymotryptic activity) or Z-Leu-Leu-Glu-AMC (peptidylgluthamyl-hydrolyzing activity).

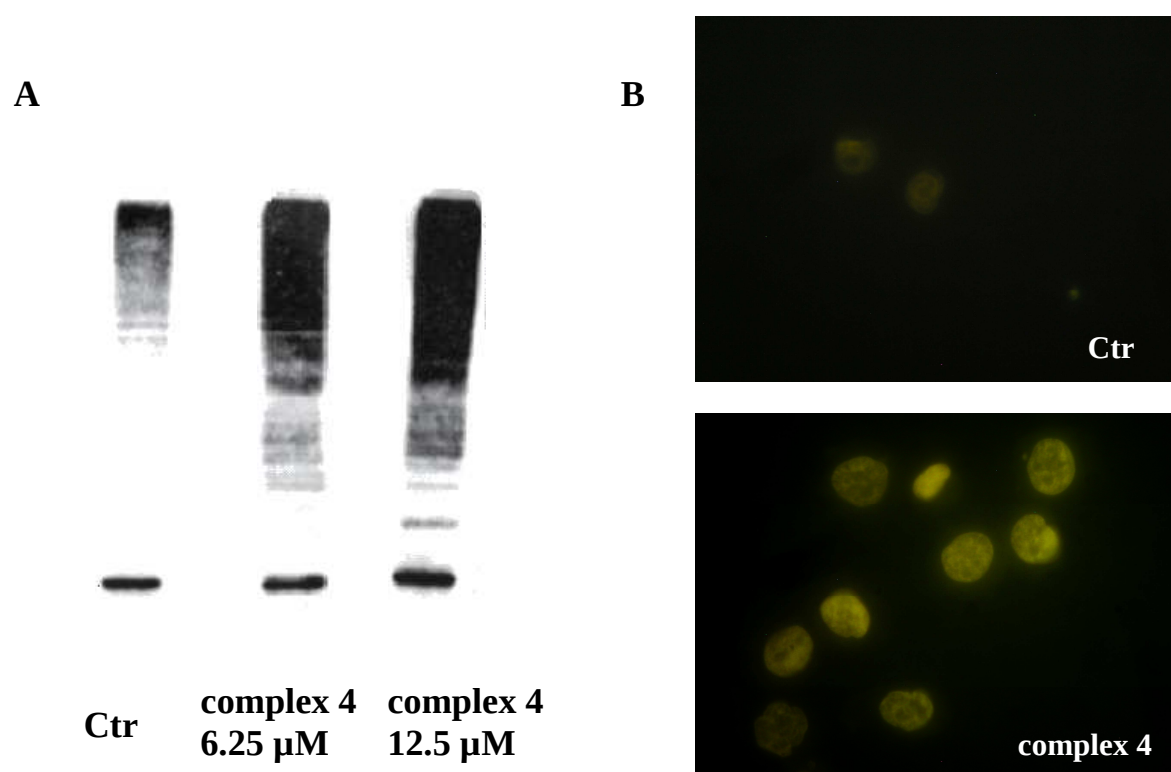
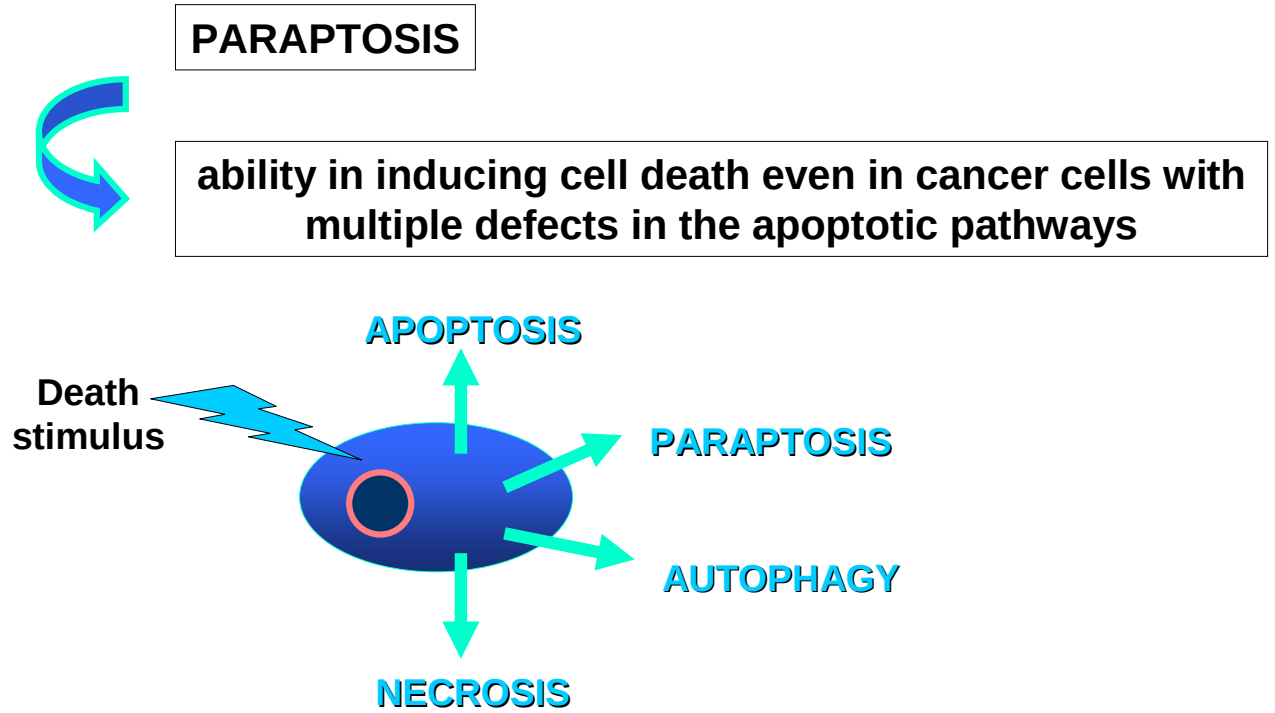


Fig. 76. Detection of poly-ubiquitinated proteins. 2008 cells treated for 24 hours with complex 4. Poly-ubiquitinated protein estimated by Western blotting (A) and immunofluorescence (B).

Taken together, these findings clearly attest that complex 4, acting as proteasome inhibitor induced intracellular accumulation of polyubiquitinated protein.

Further investigations are needed in order to elucidate complex 4 ability in inhibiting proteasome. These findings could allow to shed light on the pathways involved in paraptotic cell death triggering.

As this complex 4 activates a paraptotic pathway, it would represent the first example of an anticancer drug able to trigger this particular PCD. Many tumor cells are resistant to classical chemotherapeutic agents since they are endowed with mutations that inactivate apoptotic machinery or activate anti-apoptotic pathways. Therefore, the characterization of novel, apoptosis-independent anticancer drugs appears to be significantly relevant for possible therapeutic applications.



4. Conclusions

In the present work we have compared the cellular effects induced by auranofin on human ovarian cancer cells sensitive (2008) and resistant (C13*) to cisplatin and we have observed that this gold compound is extremely effective in decreasing the viability of both cell lines, overcoming cisplatin resistance. In particular, auranofin, acting as a potent inhibitor of thioredoxin reductase, determines an alteration of the redox state of the cell leading to an increased production of hydrogen peroxide and oxidation of the components of the thioredoxin system, thus creating the conditions for augmented apoptosis. Noticeably, cisplatin resistant ovarian cancer cells easier undergo auranofin mediated cell death, as the auranofin primary target, TrxR, is overexpressed in the resistant phenotype.

Moreover, combined treatment of 2008 or C13* cells with selenite and auranofin dramatically increased auranofin cytotoxicity. Conversely, tellurium presence did not enhance auranofin cell viability inhibitory potency. However, studies concerning thioredoxin redox state in ovarian cancer cells treated with selenite or tellurite, led to the conclusion that both chalcogens markedly increase oxidised Trx and this effect was magnified in auranofin co-presence. Indeed, combining chalcogen metalloids with auranofin, thioredoxin could be directly oxidised by chalcogens and could not be reduced back by TrxR, which in turn was inhibited by gold(I) complex. This results in an severe oxidative stress condition that leads to a massive cellular apoptosis induction. Though the role of tellurite in combined treatment with auranofin need to be further investigated, the combination of auranofin with selenite can open interesting perspectives for treatment of aggressive malignancies.

Subsequently, attempting to gain more understanding of SAR concerning gold(I) complexes as TrxR inhibitors, we have studied different newly synthesized gold(I) complexes containing phosphine ligands. All complexes showed a cytotoxic potency higher than that of cisplatin, and comparable or even higher than that of auranofin. All complexes were able to overcome either cisplatin and MDR resistance. The inhibitory effects of gold(I) complexes toward cytosolic or mitochondrial forms of rat TrxR as well as tumor cell TrxR were comparable or even higher than those obtained with auranofin. Furthermore, all gold(I) compounds were able, although to a different extent, to activate the apoptotic cell death pathway. The most effective compounds, however, were $\text{Au}(\text{xant})\text{PEt}_3$ and $\text{Au}(\text{SCN})\text{PEt}_3$ whereas less effective was $\text{Au}(\text{Tu})\text{PEt}_3$.

CONCLUSIONS

Currently, we are attempting to elucidate the fundamental issues of these enzyme-inhibitor interactions at a molecular level (through application of relativistic density-functional methods) in collaboration with Prof A.Bagno of the Chemistry Dept. of Padova University. A deeper understanding of the molecular mechanism of TrxR inhibition could lay down some important structure/activity relationships necessary for a rational design of new potent and selective TrxR inhibitors.

Concerning copper complexes, we have studied some mixed copper(I) complexes containing the tris(hydroxymethyl) phosphine coligand.

Among these complexes, $[\text{HC}(\text{CO}_2)(\text{pz}^{\text{Me}_2})_2]\text{Cu}[\text{P}(\text{CH}_2\text{OH})_3]_2$ showed IC_{50} values markedly lower than cisplatin against all tumor cell lines. Flow cytometric analysis on 2008 human ovarian carcinoma cells revealed that this complex induced a marked enlargement of both cell size and granularity and a significant increase in the fraction of G2/M cells that was not accompanied by the appearance of a relevant sub-G1 fraction.

More hydrophilic copper(I) complex $[\text{Cu}(\text{thp})_4][\text{PF}_6]$ has been shown to be about one order of magnitude more potent than cisplatin, also in cisplatin and multidrug resistance cells. Cytological stains and flow cytometric analyses indicated that the phosphine copper(I) complex is able to inhibit the growth of tumor cells via G2/M cell cycle arrest accompanied with the loss of mitochondrial transmembrane potential. In particular, it was found to activate a specific programmed cell death mechanism, namely paraptosis. As many tumor cells are resistant to apoptosis induced by classical chemotherapeutic agents, the characterization of novel, apoptotic-independent anticancer drugs appears to be significantly appealing for possible therapeutic applications.

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