Proteomic and Metallomic Strategies for Understanding the Mode of Action of Anti-cancer Metallodrugs

Chiara Gabbiani[a], Francesca Magherini[b], Alessandra Modesti[b] and Luigi Messori[a,*]

[a]*Department of Chemistry, University of Florence, via della Lastruccia, 3, 50019 Sesto Fiorentino, Florence, Italy;* [b]*Department of Biochemical Sciences, University of Florence, Viale G. Morgagni, 50, 50134 Florence, Italy*

Abstract: Since the discovery of cisplatin and its introduction in the clinics, metal compounds have been intensely investigated in view of their possible application in cancer therapy. In this frame, a deeper understanding of their mode of action, still rather obscure, might turn crucial for the design and the obtainment of new and better anticancer agents. Due to the extreme complexity of the biological systems, it is now widely accepted that innovative and information-rich methods are absolutely needed to afford such a goal. Recently, both proteomic and metallomic strategies were successfully implemented for the elucidation of specific mechanistic features of anticancer metallodrugs within an innovative "Systems Biology" perspective. Particular attention was paid to the following issues: i) proteomic studies of the molecular basis of platinum resistance; ii) proteomic analysis of cellular responses to cytotoxic metallodrugs; iii) metallomic studies of the transformation and fate of metallodrugs in cellular systems. Notably, those pioneering studies, that are reviewed here, allowed a significant progress in the understanding of the molecular mechanisms of metal based drugs at the cellular level. A further extension of those studies and a closer integration of proteomic and metallomic strategies and technologies might realistically lead to rapid and significant advancements in the mechanistic knowledge of anticancer metallodrugs.

Keywords: Cancer, metallodrugs, metallomics, proteomics, systems biology, 2D- gel electrophoresis.

1. ANTICANCER METALLODRUGS: SOME INTRODUCTORY REMARKS

Metal-based chemotherapy is nowadays a well established therapeutic option for cancer treatment [1]. Remarkably, cisplatin, carboplatin and oxaliplatin, three strictly related inorganic platinum(II) complexes (Fig. **1**), are among the most widely used anticancer drugs worldwide [2]. They proved to be very effective in the treatment of a variety of cancers, especially testicular cancer (for which cisplatin has a >90% cure rate), ovarian cancer, head and neck cancers and, also, for colorectal cancer (specifically responding to *oxaliplatin*); moreover, classical platinum drugs are now part of several established chemotherapeutic protocols for cancer treatment.
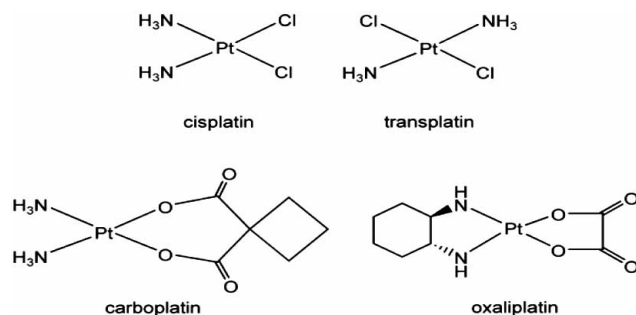

Fig. (1). Chemical formulas of classical platinum compounds.

Anticancer platinum(II) complexes are believed to exert their relevant biological effects primarily by interacting with double helix DNA, causing irreversible DNA damage and thus inducing apoptotic death of the cancer cell [2]. The mechanism of action of platinum compounds, on which there is now a rather large consensus, is summarised in Fig. (**2**); it basically relies on bidentate platinum(II) coordination to two adjacent DNA purine nucleobases leading to a relevant local distortion of the DNA double helix. This

is the clue event capable of triggering a complex cascade of down stream biochemical processes, ultimately ending with cancer cell apoptosis.

Unfortunately, similar to most established anticancer drugs, cisplatin and its analogues manifest a rather poor selectivity toward cancer cells and may also attack several other types of rapidly dividing healthy cells [3]. Therefore, nearly all patients treated with platinum drugs commonly experience severe side effects often leading to treatment suspension. The issue of drug resistance (i.e. *platinum resistance*) is another important limitation for platinum drugs; indeed, platinum chemotherapy often induces acquired tumor resistance, in most cases causing treatment failure. Accordingly, the molecular mechanisms of platinum drugs leading either to systemic toxicity or to acquired platinum resistance have been the subject of intense investigations; it is believed that a full understanding of those biochemical processes might greatly help in counteracting and overcoming these phenomena, thus resulting into important clinical progresses.

Owing to the relevant drawbacks of classical platinum(II) drugs, several additional platinum and non-platinum metal-based compounds were designed, prepared, and tested during the last three decades with the goal of identifying compounds that might lack the undesirable activities of cisplatin, particularly host toxicity, and manifest a different spectrum of anticancer actions.

Some significant success was obtained with a variety of unconventional platinum drugs. For instance, a few polynuclear platinum complexes, developed by Farrell *et al.*, manifested outstanding antiproliferative effects *in vitro* and favourable antitumor effects *in vivo* [4]; pairwise some *trans* platinum(II) compounds, developed by various research groups worldwide, showed very promising pharmacological profiles [5-7]. Platinum(IV) compounds were also shown to behave as useful *precursors* for platinum(II) drugs with a generally lower systemic toxicity than platinum(II) congeners; indeed, upon reduction within the reducing milieu of cancer cells, they may be converted into biologically active platinum(II) species [8].

In addition, other classes of metal compounds (*non platinum metallodrugs*) were developed and intensely investigated in the search of new anticancer leads (e.g. ruthenium, titanium, tin, gold, copper, etc.). Some of them displayed a promising pharmacological

*Address correspondence to this author at the Department of Chemistry, University of Florence, via della Lastruccia, 3, 50019 Sesto Fiorentino, Florence, Italy; Tel: +39 055 4573388; Fax: +39 055 4573385; E-mail: luigi.messori@unifi.it
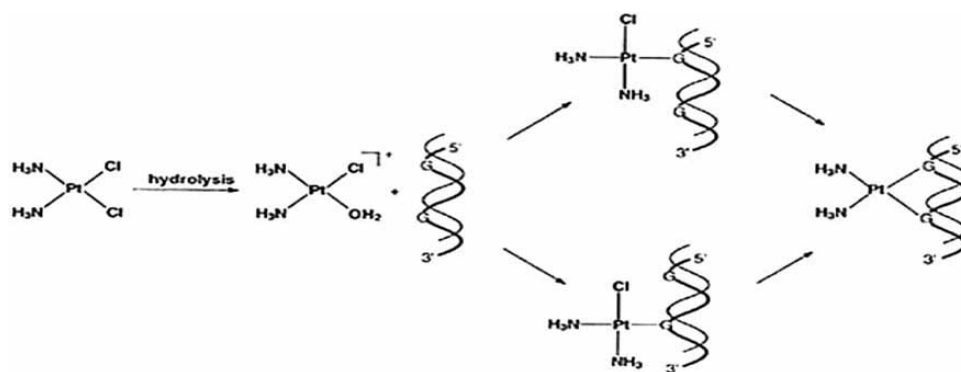
Fig. (2). The mechanism of action of platinum compounds.

profile and peculiar biological features that are profoundly different from those of classical platinum compounds.

Ruthenium compounds, in particular, were the object of much interest. Among them, NAMI A, KP1019 and a few novel ruthenium arene compounds manifested, on the whole, very attractive pharmacological properties, both *in vitro* and *in vivo*. Remarkably, NAMI A, while being poorly cytotoxic, displayed excellent antimetastatic properties and, for this reason, is currently undergoing phase II clinical trials [9, 10].

Gold compounds constitute another class of promising cytotoxic and anticancer metallodrugs. Several gold compounds, both gold(I) and gold(III), showed favourable antiproliferative properties *in vitro* against a variety of human tumor cell lines. For some of them excellent results *in vivo* were obtained as well. In particular, gold(III) dithiocarbamates [11-13] and gold(III) porphyrins [14, 15] form two classes of promising agents for further pharmacological development and are currently undergoing advanced preclinical testing [16].

In the course of the numerous investigations on anticancer metallodrugs, much interest has focused on the elucidation of the likely molecular mechanisms in the belief that a precise knowledge of their mode of action might better direct the design and the synthesis of novel anticancer metallodrugs. Indeed, the "true" molecular mechanisms of anticancer metallodrugs are still largely unknown as well as the involved biochemical pathways leading to resistance and toxicity. The understanding of those mechanisms might bring to a significant progress in this research area and allow the obtainment of more effective anticancer metallodrugs. At the same time, it is now evident that understanding to a satisfactory degree the mode of action of a drug at the molecular level requires gathering a great amount of experimental data on drug/biomolecules interactions and deciphering and interpreting, at least partially, the huge complexity that is intrinsic to biological systems, as it will be discussed below.

2. COPING WITH THE INHERENT COMPLEXITY OF BIOLOGICAL SYSTEMS: *"SYSTEMS BIOLOGY"* AND THE *"OMICS"* SCIENCES

Biological systems are characterised by a very high degree of internal *complexity*. Even "simple" isolated cells are the result of the complex interplay of thousands of genes, proteins and small molecules according to well defined -but yet not completely understood- principles of structural and functional organisation. Following the dramatic increase in the biological knowledge that has taken place during the last two decades, this concept, "the inherent complexity of biological systems", has reached its maturity and is widely accepted within the scientific community. Thus, new disciplines and new methods are now absolutely needed to tackle with the huge complexity of biological systems and try to interpret it. These new disciplines and methods may turn crucial to understand in more depth the mode of action of biologically active substances at the molecular level.

In this frame, *Systems biology* is a term now largely employed to describe current trends in bioscience research [17-19]. *Systems biology* may be defined as an inter-disciplinary study field which is mainly devoted to the analysis of the complex interactions that occur within biological systems. Basically, those interactions are investigated with the aid of new experimental methods according to an innovative conceptual perspective (*holism* instead of *reductionism*). In other words, systems biology is the study of an organism, considered as an *integrated* and *interacting network* of genes, proteins and biochemical processes that give rise to life. Instead of studying individual components or aspects of a certain organism, *systems biologists* rather focus on all its components and on their mutual interactions, each considered as a part of one single system. According to this new perspective, the action of a drug is no more interpreted in terms of the simple interaction of the drug with its primary target but, rather, on the basis of the numerous
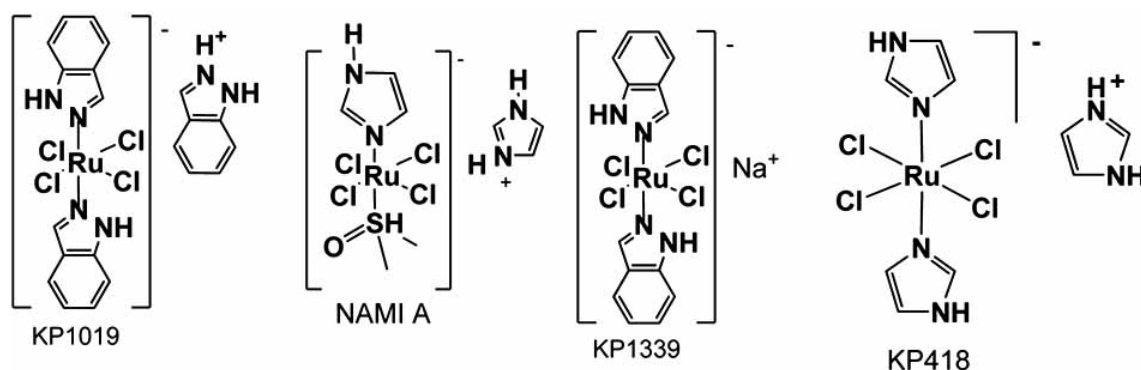

Fig. (3). Schematic drawings of some representative ruthenium compounds.
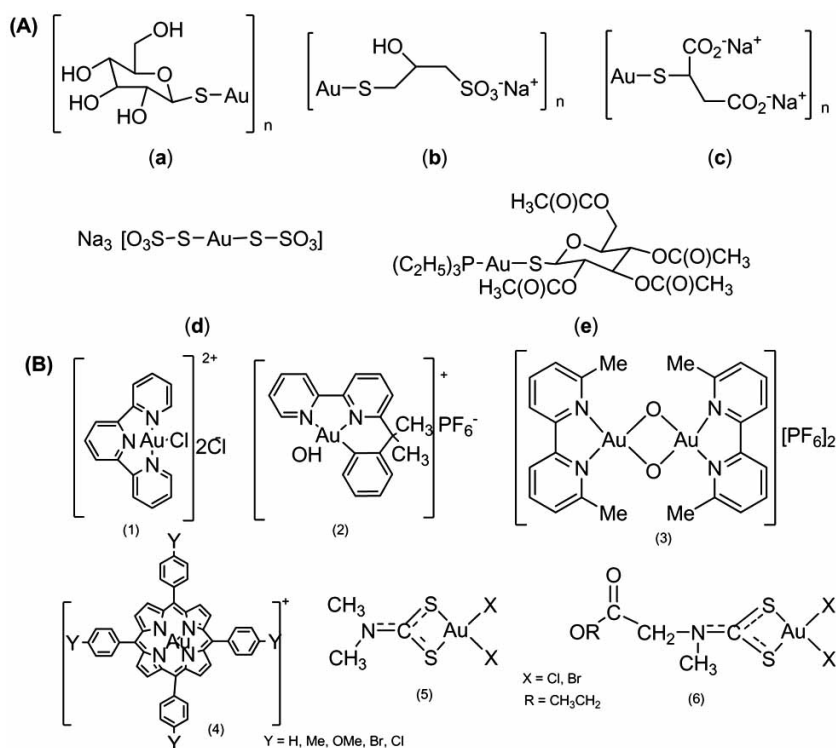
Fig. (4). (A) Established gold(I) compounds in clinical use: solganol (a), allocrysin (b), myocrysin (c), sanocrysin (d) and auranofin (e). **(B)** Some representative gold(III) compounds: [Au(terpy)Cl]Cl$_2$ (1), [Au(bipy^c-H)(OH)][PF$_6$] (2), the dinuclear AuOXO6 complex (3), gold (III) *meso*-tetraarylporphyrins complexes(4) and the gold(III) dithiocarbamate complexes containing N,N-dimethyldithiocarbamate (5) and ethylsarcosinedithiocarbamate (6) ligands.

interactions that the drug itself establishes with a large variety of biomolecules. These several interactions will determine not only drug's activity but also its toxicity, bioavailability, biodistribution, metabolism and so on, in other words its overall pharmacological profile.

Thus, *Systems Biology* mainly refers to the ability to obtain, integrate and analyze complex sets of data from multiple experimental sources using interdisciplinary tools. *Omics sciences* such as Genomics, Epigenetics, Transcriptomics, Interferomics, Proteomics, Metabolomic, etc, represent the typical technological platforms for systems biology. As these disciplines have the potential to produce huge amounts of new experimental data, a major goal for systems biologists will be to develop smart and robust methods, to mine, analyse and interpret such data. *Bioinformatics* is the discipline specifically deputed to this goal [20-23].

The bioinformatic analysis often involves the development of mechanistic models, such as the reconstruction of dynamic systems starting from the quantitative properties of their elementary building blocks [24, 25]. For example, a cellular network can be modelled using methods derived from chemical kinetics and from control theory. Due to the great number of parameters, variables and constraints, that are typically present in a cellular network, advanced numerical and computational techniques are often employed for bioinformatic analysis.

In recent years *Bioinformatics* has been extensively applied to systems biology and to the interpretation of proteomic data [26, 27]. The main goal of these studies consists in referring the observed alterations of the proteomic profiles –recorded under well defined experimental conditions- to specific modifications in selected metabolic or signalling pathways. This type of analysis requires extensive identification and functional annotation of proteins.

Owing to the intrinsic complexity of biological systems it is evident that determining the exact mode of action of a drug represents today a formidable and very ambitious task. Moreover, as most metallodrugs are *prodrugs,* characterised by a high propensity

to react with very many biomolecules and to give rise to several active metabolites, a full understading of their mechanism of action turns out to be a goal even more difficult and more ambitious than in the case of classical organic drugs.

Within this frame the *Omics sciences* offer valuable tools to gain mechanistic information on metallodrugs. Indeed, large collections of data can be straightforwardly obtained from their application that, need, afterward, to be analysed and organised systematically. The interpretative efforts based on novel bioinformatics tools have the potential to refer the observed alterations to specific modifications of metabolic and signalling pathways of the cell. This kind of analysis may be extremely valuable in the process of target discovery as nicely illustrated in a recent paper [28].

The focus of our review article will be on both Proteomics and Metallomics. While Proteomics is now an established branch of the new omics sciences, Metallomics is a younger discipline still in the search of a definitive conceptual and methodological organisation as it will be discussed below in more detail. In particular, we will report here on the exploitation of novel proteomic and metallomic methodologies to gain insight into the mode of action, and also the mechanisms of toxicity and resistance, of a few representative anticancer metallodrugs. An interesting review on the application of proteomics methods to investigate the mechanism of anticancer metallodrugs has appeared a couple of years ago [29]. Moreover, a very comprehensive review on the studies concerning the interactions of metallodrugs with serum proteins has appeared somehow earlier [30].

3. PROTEOMICS: GENERAL REMARKS

Proteomics is the "*large-scale study of proteins*", with a particular emphasis on their structures and functions [31, 32]. Proteins form a crucial part of the living organisms, as they are the main components of the metabolic and signalling pathways of cells while playing, concomitantly, very important structural roles. The term *proteomics* was first introduced in 1997 [33] in analogy with
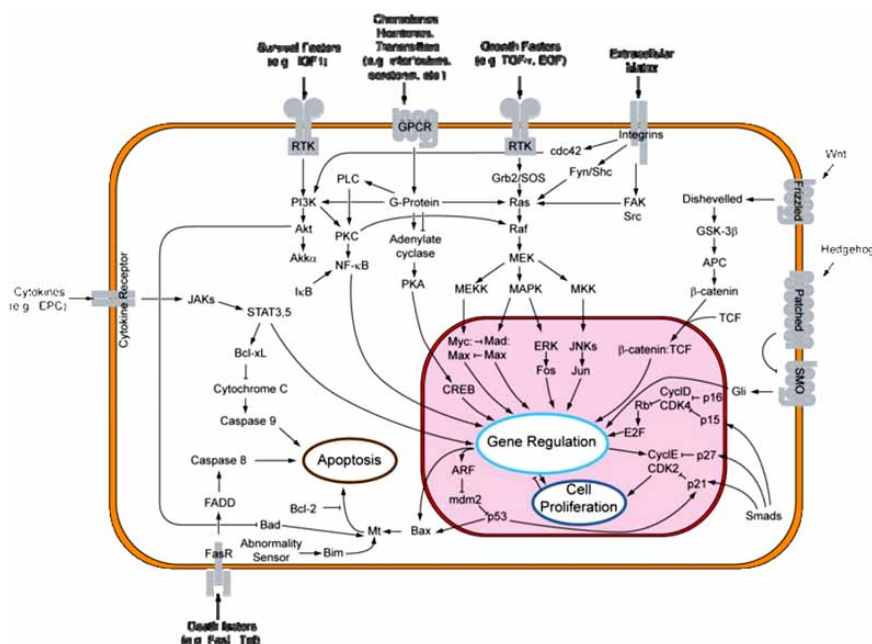
Fig. (5). Graphical presentation of some relevant signal transduction pathways.

genomics, the "*study of the genes*". In turn, the word *"proteome*", coined by Marc Wilkins in 1994, is a blend of "protein" and "genome". It defines the entire complement of proteins [34], produced by an organism or system. Thus, the proteome may greatly vary with time in dependence of the requirements, or stresses, to which a cell or organism may be exposed and these alterations may be the object of specific investigation.

The *proteomic technology*, is aimed to separate, identify, and characterize a global set of proteins in such a way to provide information on protein abundance, location, modification, and protein-protein interaction in the proteome of a given biological system. This technology offers a direct measurement of the presence and of the relative abundance of proteins, and reveals the consequence of protein functioning and networking in determining the biological phenotype of organisms under various conditions. By studying changes in protein expression, in health and disease or upon drug treatment, proteomics may provide important insights into the molecular basis of disease, may help in the validation of drug targets, and may offer accurate descriptions of drug action, toxicity, and side effects.

In the field of proteomics, several well-established methods are now available to resolve and analyze complex mixtures of proteins derived from cells and tissues. Currently, the most commonly used proteomic platform includes a method for proteins separation, such as two-dimensional gel electrophoresis (2D-GE) or Liquid chromatography (LC) (Table **1**), most often coupled with a variety of mass spectrometry methods (matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) [35], electrospray ionization mass spectrometry (ESI-MS) [36], and/or tandem mass spectrometry (MS/MS). In addition, inductively coupled plasma (ICP) mass spectrometry and surface enhanced laser desorption ionization time of flight (SELDI-TOF) [37] were also applied with success in the proteomic research.

In general, several different technological platforms can be used in proteomic studies and each technique has its own advantages and limitations (see Table **1**).

Protein separation with two dimensional electrophoresis (2D-GE), still the standard approach, involves initial separation of proteins based on their isoelectric point followed by subsequent separation based on size. This strategy is a robust method that separates

intact proteins, and allows evidencing the presence of isoforms and post-translational modifications as well as determining their respective expression levels. Unfortunately, this method is not applicable to polypeptides smaller than 10 kDa; in addition, 2D-GE usually shows severe problems in the separation of highly hydrophobic proteins and is time consuming. The introduction of Differential In Gel Electrophoresis (DIGE) by the group of Jonathan Minden in 1997 [38] has been one of the major hallmarks in the application of 2D electrophoresis method to proteomics in the last years. This method is based on the fluorescently tagging of proteins in different samples with different dyes and allows a more rapid and sensitive comparison of samples. In combination with MALDI-TOF MS, 2D-GE constitutes the standard method in expression proteomics.

Hydrophobic membrane proteins are hardly detectable after 2D-GE. Therefore, any global and comprehensive analysis of the membrane proteomes has not been reported yet. Blue native polyacryamide gel electrophoresis represents an alternative strategy to separating membrane proteins with high resolution while conserving their enzymatic function. The method is powerful between 10 and 10 000 kDa. Also, membrane protein complexes are separated well after solubilization of complexes with mild neutral detergents. The separation principle relies on binding of Coomassie blue G250 which provides negative charges to the surface of the protein [39].

In an alternative approach, called *shotgun proteomics*, [40] proteins are digested and peptides are then separated by liquid-based separation in one or several dimensions prior to detection by tandem mass spectrometry (MS/MS). This is a fast method that requires little sample handling and manual work. The peptide masses are then interpreted using bioinformatic tools. This is a critical point in *shotgun proteomics*, where reliable statistical methods are needed in order to distinguish true protein hits from false positives. One limit of shotgun approaches is that information on the intact proteins and isoforms is lost during the digestion step. Recently, a big effort was made in order to develop "*gel-free*" quantitative methods. Briefly, these approaches can be divided into two main families: "*stable isotope based*" such as SILAC (stable isotope labelling in cell culture), GIST (global internal standard technology), iTRAQ,(isotope tags relative and absolute quantification),or ICAT (isotope coded affinity tag) and "*label-free methods*". The first kind of approach uses stable isotopes to label peptides, introducing a mass difference between the labeled and unla-
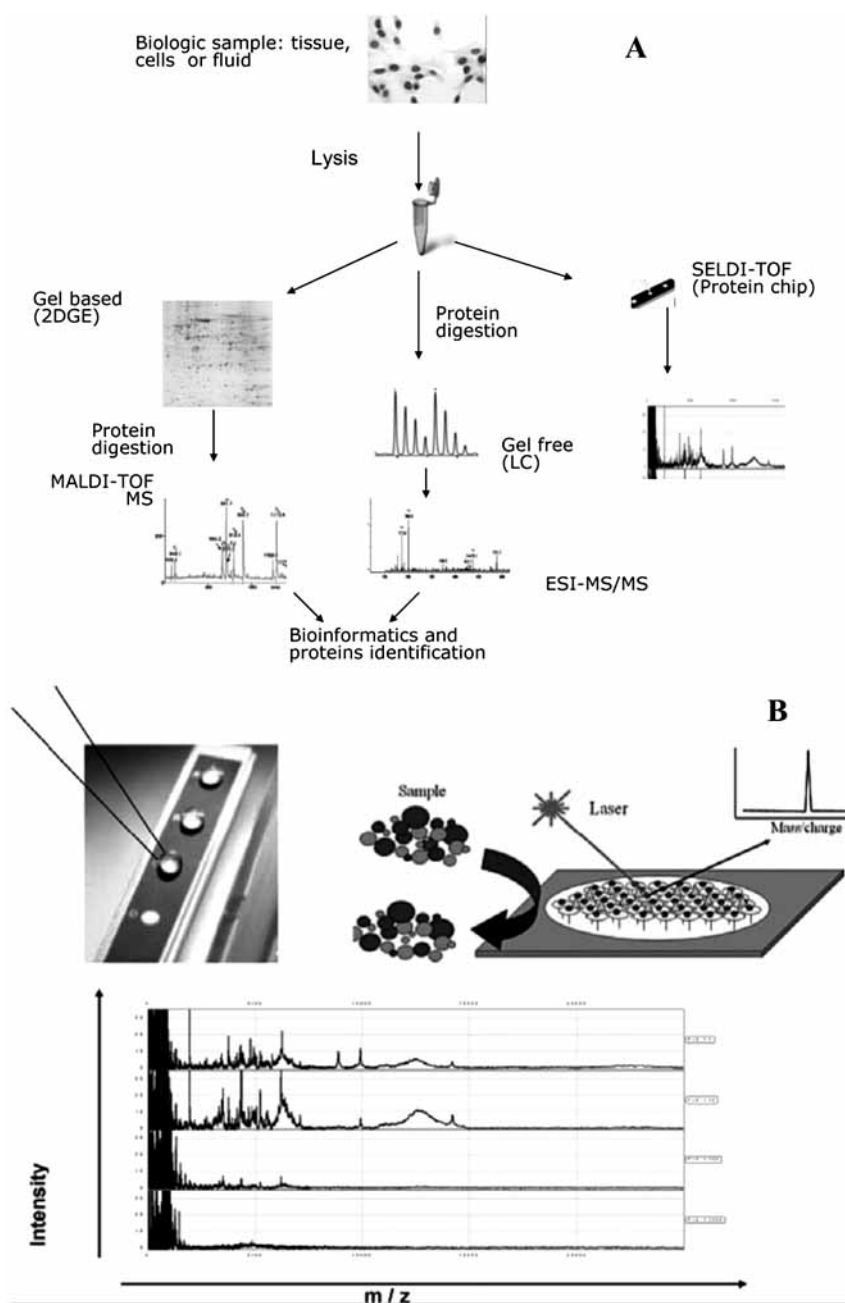
Fig. (6). (A) Schematic overview of current approaches for mass spectrometry-based proteomic studies. Proteins are extracted from biological sample, separated by 2DGE or LC and identified by MS. **(B)** In SELDI-MS approach the sample is deposited on the active chip surface. After several washing steps, only a few proteins stay bound to the surface; these are subsequently analyzed using low-resolution MS.

Table 1.   A Summary of the Main Advantages and Limitations of Different Proteomic Methodologies

	Advantages	Limitations
2DGE	2-DE Available in most proteomics labs, simple to perform, quantitative, low cost.	Time-consuming, no automation, not applicable for proteins/polypeptides with molecular masses < 10 kDa, limited use for hydrophobic proteins.
LC-MS	Automation, multidimensional, high sensitivity.	Quantitative analysis is not simple, sensitive toward interfering compounds, digestion destroy information on isoforms, high cost.
ICAT	Quantitative.	Only cysteine residues are labelled, digestion destroy information on isoforms, high cost.
SILAC	Quantitative, all proteins are labeled.	Digestion destroys information on isoforms.
SELDI-TOF MS Restricted to selected proteins,	Easy-to-use system, high-throughput, automation, low sample volume required.	low-resolution MS, low information content, reproducibility is still problematic.

beled peptides in the process. The intensity signals obtained from the unlabeled and labeled peptide will then provide quantitative information in the MS spectrum [for review see 41,42]. The second family of methods does not introduce any label, and usually relies on chromatographic and mass spectrometry data to quantify peptides in the samples. This last method is not easy to perform, requires high resolution of peptides and an accurate process for data normalization.

In the last decade we also assisted to an increasing utilization of SELDI-MS technology in clinical proteomics. The process involves binding of the sample to a Protein Chip array which is subsequently washed several times to remove unbound proteins and buffers. The Protein Chip is then directly subjected to mass spectrometry by MALDI-TOF. The mass-to-charge ratios for desorbed molecules are analysed as they fly down the TOF tube and an individual protein spectrum is generated for each sample tested. Differentially expressed proteins are determined from the protein profiles by comparing the peak intensities of spectra [for review see 43].

While the analysis of large biopolymers is successfully performed by electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI), analysis of low molecular weight molecules such as amino acids, pharmaceuticals, hormones, etc. is predominantly performed by ESI mass spectrometry with quadrupole mass analyzers. The major reasons for not using MALDI are the abundance of matrix ions in the low mass region and the lack of reasonable precursor selection in a MALDI tandem instrument. Also, until recently, MALDI was not considered to be a technique capable of providing robust quantitative data.

However, a new matrix-assisted laser desorption/ionization (MALDI) time-of-flight/time-of-flight (TOF/TOF) high-resolution tandem mass spectrometer technique is now available that combines the advantages of high sensitivity for peptide analysis associated with MALDI and comprehensive fragmentation information provided by high-energy collision-induced dissociation (CID) [44].

Proteomics thus holds great promise as a powerful technique for analysing in detail drug induced proteomic changes and to identify pathways and protein targets that are specifically affected by a certain substance. It must be reminded, however, that numerous drug-targeted proteins are membrane-bound proteins, for example, receptors and ion channels. These proteins may not be suitable for classical proteomic investigations due to their poor solubility and low abundance, and accordingly may be severely underrepresented or even completely lost in the proteome profiles.

4. PROTEOMIC STUDIES OF ANTICANCER METALLO-DRUGS

During the last few years a number of studies have appeared where classical proteomics methodology was applied to the mechanistic investigation of anticancer metallodrugs. Roughly, those studies may be referred to two main applications.

The *first type* of application deals with the elucidation of the *molecular basis of resistance* to platinum drugs. Resistance to platinum most likely relies on the overexpression of specific proteins, endowed with a variety of functions that make the cell far less sensitive to the insult caused by the platinum compounds. The rationale for these studies is thus rather straightforward: the proteomes of platinum sensitive cancer cell lines and of their platinum resistant counterparts are comparatively assayed in order to highlight differentially expressed proteins among which are *most likely* those responsible for platinum resistance.

The *second type* of application concerns the analysis of proteomic alterations induced by cells exposure to a cytotoxic metallodrug. Experimentally, the proteomic profiles of cancer cell lines treated with a specific metallodrug are analysed in comparison to those of untreated cells, and differentially expressed proteins are highlighted and identified. This type of analysis, in principle, may

allow identification of proteins belonging to the main affected biochemical pathways that are strictly related to early events of cell damage and to the molecular mechanisms of stress response. When the insult is too severe and cannot be repaired the apoptotic process is started and the corresponding proteomic signatures may be detected.

a) Analysis of the Molecular Basis of Platinum Resistance Through Comparative Proteomic Analysis of Pt Sensitive vs Pt Resistant Cell Lines

The studies on the molecular basis of drug resistance are abundant in the field of platinum drugs. Indeed, acquired resistance is often the reason for treatment failure and attracts therefore great interest. Understanding the molecular mechanism of resistance might hopefully lead to new methods to circumvent it. Thus, a conspicuous number of Proteomic studies were specifically devoted to this issue.

An interesting study on platinum resistance was reported a few years ago by Castagna *et al.* [45]. In this paper, cisplatin resistance and drug response in human tumor cervix squamous carcinoma cell lines A431 were investigated. The experimental set-up involved not just a two-way comparison of the control vs. the drug-resistant cell line, but also an acute cisplatin treatment of both cell lines, leading to a four-way comparison. An appreciable modulation of protein expression was thus revealed, that could be classified under various functional classes, such as molecular chaperones (e.g. heat-shock proteins HSP60, HSP90, HSC71, heat-shock cognate 71 kDa protein), calcium binding proteins (e.g. calmodulin, calumenin), proteins involved in drug detoxification and redox metabolism (such as peroxiredoxins PRX 2 and PRX 6, and glutathione-S-transferase, GST), anti-apoptotic proteins (such as 14-3-3 switched on in cisplatin-exposed cells) and ion channels (such as VDAC-1, voltage-dependent anion-selective channel).

In particular, the basal levels of HSC71 and HSP60 were greatly increased in A431/Pt cells compared to A431 cells. Moreover, cisplatin exposure up-regulated the anti-apoptotic 14-3-3 protein in both cell lines, GST in the sensitive cells and PRX6 in A431/Pt cells. These findings are consistent with a constitutive expression of defence factors by resistant cells and with activation by cisplatin of mechanisms capable of protecting cells from drug-induced damage. This pattern of response, also observed in parental cells, could reflect an intrinsic resistance of this tumor type.

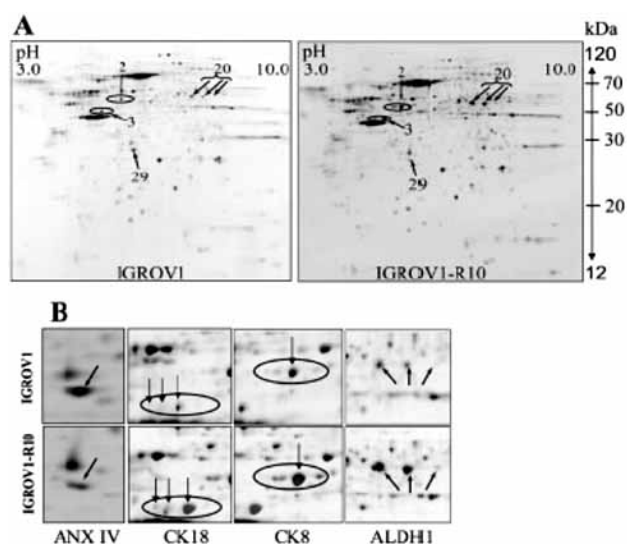Another important study was communicated by Le Moguen *et al.* in 2006 [46].

Fig. (7). Proteomic comparison between IGROV1 and IGROV1-R10 cells using 2-DE and MALDI-TOF-MS (from ref. 46).

A classical proteomic approach was applied by these authors to identify proteins associated with platinum resistance in IGROV1 ovarian carcinoma cells. First, the proteomic pattern of the cisplatin-sensitive ovarian cell line IGROV1 was established using MALDI-TOF-MS and PMF. Then, the resulting 2-D pattern was compared with that of the cisplatin-resistant counterpart IGROV1-R10. Among the 40 identified proteins, cytokeratins 8 and 18 and aldehyde dehydrogenase 1 were greatly overexpressed in IGROV1-R10, whereas annexin IV was down-regulated. These observations were subsequently confirmed by Western blotting.

This kind of investigation was deepened and extended shortly after by the same research group [47]. A kinetic analysis of IGROV1 cells following treatment with cisplatin and subsequent statistical analysis revealed time and/or concentration-dependent modifications in protein expression. This study evidenced events such as a decreased amino-acid and nucleotide synthesis possibly associated with cell cycle blockade, and variations that might be related to resistance acquisition, such as enhanced glycolysis and increased proliferating potential. Overexpression of aldehyde dehydrogenase 1 and of both cytokeratins 8 and 18 were confirmed in consistency with the previous study. The expression of these proteins was increased in cisplatin-resistant IGROV1-R10 compared to IGROV1 parental cells. Identification of such differentially expressed proteins could allow an improved understanding of the mechanisms leading to cell death or survival and, thus, to the acquisition of chemoresistance. In a way, this study established some meaningful correlations between the cellular response to metallodrugs and the mechanisms of acquired resistance.

In turn, Pan *et al.* [48], established a few platinum-resistant human ovarian cancer cell lines and identified differentially expressed proteins related to platinum resistance. The total proteins of two sensitive (SKOV3 and A2780) and four resistant (SKOV3/CDDP, SKOV3/CBP, A2780/CDDP, and A2780/CBP) human ovarian cancer cell lines were isolated by two-dimensional gel electrophoresis (2-DE). The differentially expressed proteins were identified using MALDI-TOF MS. In total, 57 differential protein spots were detected. Five proteins, including annexin A3, destrin, cofilin 1, Glutathione-S-transferase omega 1 (GSTO1-1), and cytosolic NADP+-dependent isocitrate dehydrogenase (IDHc), were specifically affected compared with their parental platinum sensitive cells. The expression of the above mentioned five proteins was validated by quantitative PCR and Western blot; notably, the Western blot results showed nearly complete consistency with proteomic results. The above five proteins are likely to be good candidates for platinum resistance. These may be useful for further study of resistance mechanisms and for the screening of resistance biomarkers.

A very comprehensive study was reported by Stewart JJ *et al.* [49]. Indeed, ICAT coupled with tandem MS is a quantitative proteomic technique for high throughput protein expression profiling of complex protein mixtures. Using ICAT/MS/MS approach, the nuclear, cytosolic, and microsomal fractions obtained from IGROV-1 (cisplatin-sensitive) and IGROV-1/CP (cisplatin-resistant) ovarian cancer cell lines were comparatively profiled. The proteomes of cisplatin-sensitive and -resistant ovarian cancer cells were compared , and protein expression was correlated with mRNA expression profiles. A total of 1117 proteins were identified and quantified. The relative expression of 121 of these varied significantly between the two cell lines. Sixty-three proteins were overexpressed in cisplatin-sensitive, and 58 were over expressed in cisplatin-resistant cells. Examples of proteins at least 5-fold overexpressed in resistant cells and with biological relevance to cancer include cell recognition molecule CASPR3 (13.3-fold), S100 protein family members (8.7-fold), junction adhesion molecule Claudin 4 (7.2-fold), and CDC42-binding protein kinase beta (5.4-fold). Examples of cancer-related proteins at least 5-fold overexpressed in sensitive cells include hepatocyte growth factor inhibitor 1B (13.3-

fold) and programmed cell death 6-interacting protein (12.7-fold). The direction of changes in expression levels between proteins and mRNAs were not always the same, possibly reflecting posttranscriptional control of protein expression. Proteins whose expression profiles correlate with cisplatin resistance in ovarian cancer cells were identified. These proteins may be involved in modulating response to cisplatin and may have potential as markers of treatment response or treatment targets.

A further proteomic study by Smith L *et al.* [50] analysed cisplatin resistance in breast cancer cells. The MCF-7 breast cancer cell line and a novel derivative displaying significant resistance to cisplatin were analyzed using two-dimensional gel electrophoresis. The protein profiles were compared and 15 differentially expressed proteins identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Downregulation of beta-tubulin type 3, cytokeratin 17, tropomyosin 1-alpha, peroxiredoxin 4, heat shock 27-kDa protein 1, glutathione-S-transferase mu 3, ribosomal protein P0, isocitrate dehydrogenase 3, and peptidyl-prolyl isomerase A isoform 1 was detected in cisplatin-resistant cells. In contrast, the expression of hydroxyprostaglandin dehydrogenase 15-(NAD), matrix metalloproteinase 9, heterogeneous nuclear ribonucleoprotein A3, proteasome beta 1 subunit, electron transfer flavoprotein beta-polypeptide isoform 1, and peptidyl-propyl isomerase B precursor was enhanced in cisplatin-resistant cells. The downregulation (at least twofold) of glutathione-S-transferase mu 3, cytokeratin 17, and peroxiredoxin 4 was confirmed by Western blotting.

Very recently Martinez-Balibrea E *et al.* [51] published an interesting proteomic study on the molecular mechanisms involved in oxaliplatin resistance in colorectal cancer. A 5-fold oxaliplatin-resistant cell line, HTOXAR3, was compared with its parental cell line, HT29, using two-dimensional PAGE. Mass spectrometry, Western blot, and real-time quantitative PCR confirmed the downregulation of pyruvate kinase M2 (PK-M2) in HTOXAR3 cells. In a panel of eight colorectal cancer cell lines, a negative correlation was found between oxaliplatin resistance and PK-M2 mRNA levels. Oxaliplatin exposure in both HT29 and HTOXAR3 led to PK-M2 mRNA up-regulation. PK-M2 mRNA levels were measured by real-time quantitative PCR in 41 tumors treated with oxaliplatin/5-fluorouracil. Tumors with the lowest PK-M2 levels attained the lowest response rates (20% versus 64.5%). High PK-M2 levels were associated with high p53 levels. In conclusion, the data presented by these authors provided a clear link between PK-M2 expression and oxaliplatin resistance mechanisms and further implicated PK-M2 as a predictive marker of response in patients with oxaliplatin-treated colorectal cancer.

Overall, the above mentioned studies, although not conclusive, have delineated some common investigative strategies and also highlighted some general trends in the platinum resistance mechanisms. In several cases, a few specific proteins have emerged as a signature of platinum resistance being the likely candidates for sustaining the associated biochemical processes. A further understanding of these processes at the molecular level might hopefully lead to specific therapeutic strategies aimed to circumvent Pt resistance.

b) Proteomic Analysis of Cellular Responses to Metallodrugs

There is much interest in monitoring, in *"real time",* the proteomic responses of cells to cytotoxic metallodrugs as such responses might provide valuable information on the mechanism of action of the drug itself and might highlight which metabolic or signalling pathways of the cell are primarily affected and/or activated. If the damage is too intense and cannot be repaired, specific biochemical pathways will be triggered ultimately leading to cell apoptosis. A number of studies illustrating this kind of strategy have appeared in the recent literature.

For instance, *Yim et al.* [52] examined differential protein expression in cisplatin-treated HeLa cervical carcinoma cells. In total, 21 protein spots were found to be differentially expressed following cisplatin treatment, of which 12 were upregulated (e.g. regulator of G-protein signaling, TRAF:TNF (tumor necrosis factor) receptor-associated factor-interacting protein [I-TRAF], and cyclin-dependent kinase inhibitor p27 [p27(kip1)]) and 9 were downregulated (*eg,* myc proto-oncoprotein [c-myc] and proliferating cell nuclear antigen). On the basis of proteomic data, these authors revealed that cisplatin induced TRAF2-mediated NF-kappaB downregulation; in addition, they demonstrated that cisplatin triggered both membrane death receptor-mediated and mitochondria-mediated apoptosis pathways.

In turn, *Yao et al.* [53] carried out comparative proteomic studies of colon cancer cells in response to oxaliplatin treatment. Two-dimensional gel electrophoresis coupled with MALDI-TOF/TOF mass spectrometry revealed 57, 48, and 53 differentially expressed proteins in three distinct cell lines (HT29, SW620 and LoVo, respectively) after Oxaliplatin treatment.
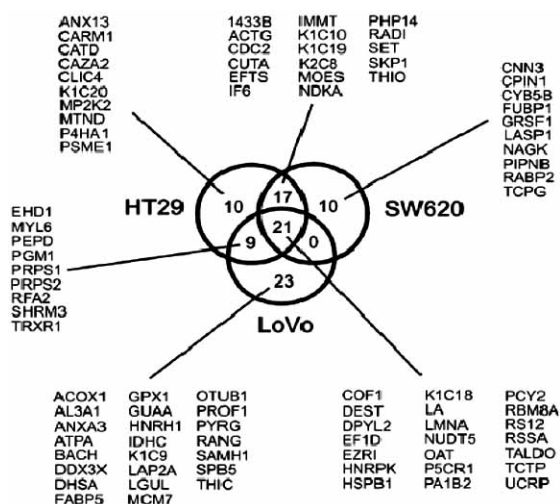

Fig. (8). Venn diagram of differentially expressed protein that overlapped between the cell lines, as identified by MALDI_TOF/TOF analysis. The identified proteins are indicated by their HUGO gene name (from ref. 53).

Of these proteins, 21 overlapped among all three cell lines. These overlapping proteins participate in many cellular processes, such as apoptosis, signal transduction, transcription and translation, cell structural organization, and metabolism. Additionally, the expression levels of ezrin (EZRI), heat-shock protein beta-1 (HSPB1), translationally controlled tumor protein (TCTP), and cell division control protein 2 homolog (CDC2) were confirmed by immunoblotting. Several interesting proteins that were identified in this study warrant further investigation owing to their potential role in the antitumor effect of Oxaliplatin.

Remarkably, a few detailed studies concerning proteomic analysis of cellular response to treatment with gold-based metallodrugs were reported by the group of Prof. Chi Ming Che, Hong Kong. [54] A 2DE-based proteomic technology was used by these authors to investigate protein expression profiles in human nasopharyngeal carcinoma SUNE1 cells treated with gold (III) porphyrin 1a. [38]. Relevant changes in the expression of a few proteins involved in redox metabolism, in the mitochondrial functions and in apoptosis were highlighted. In particular, the voltage-dependent anion channel 1 (VDAC 1) was found to be greatly upregulated in consistency with previous observations. VDAC1 is a mitochondrial outer membrane channel protein that constitutes main pathway for the transfer of various substances in and out of the mitochondria [55]. It is considered to be part of the permeability transition pore oligoprotein complex. Such large alterations of VDAC expression

point out that mitochondria could be a primary target for gold(III) porphyrin 1a. Overall, the above mentioned papers documented the feasibility and the effectiveness of this kind of strategy. However, several aspects of the mechanism of gold(III) porphyrins still remain to be clarified.

Very recently, we have exploited a classical proteomic approach to gain a deeper insight into the mechanism of two cytotoxic gold compounds, namely *Auoxo6*, a binuclear gold(III) complex showing a very favourable cytotoxic profile, and *auranofin*, a clinically established gold(I) antiarthritic drug, causing relevant tumor cell growth inhibition *in vitro* [56]. First, the 72h cytotoxicity profiles of Auoxo6 and auranofin were carefully recorded against A2780 human ovarian carcinoma cells, either sensitive or resistant to cisplatin. Subsequently, protein extraction from gold-treated A2780/S cells (after 24 hours drug exposure) and 2D Gel electrophoresis protein separation were carried out according to established procedures and differentially expressed proteins were searched for. Notably, both metallodrugs turned out to induce relatively modest changes in the overall protein expression pattern in comparison to controls as only 11 out of ~1300 monitored proteins revealed appreciable quantitative changes. Very remarkably, six altered proteins were in common between the two gold drug treatments suggesting a strong similarity in their respective mode of action. Eight altered proteins were identified by MALDI TOF analysis: among them, notably, ezrin, a protein associated to the cytoskeleton and involved in apoptosis. Interestingly, two of the altered proteins, peroxiredoxins 1 and 6, play a crucial role in the cell redox metabolism suggesting that the intracellular redox balance is significantly perturbed by these gold drugs. Increased cleavage of heterogenous ribonucleoprotein H was also evidenced consistent with caspase 3 activation. Thus, the results of this proteomic study highlight that the mode of action of *Auoxo6* is strictly related to that of auranofin; that the induced alterations of protein expression are limited and selective; that both gold compounds trigger caspase 3 activation; that some of the affected proteins are primarily involved in the redox homeostasis suggesting that cell damage probably arises from oxidative stress.

Some interesting results were recently communicated by Koncarevic *et al.* [57] concerning the proteomic response of glioblastoma cells to terpyridineplatinum(II) complexes. Terpyridineplatinum(II) complexes (TPCs) efficiently inhibit the proliferation of glioblastoma cells *in vitro* and have been tested successfully in a rodent model of brain glioblastoma. Apart from intercalation with DNA, the major mechanism of action of TPCs seems to be a very potent and specific interaction with the human selenoprotein thioredoxin reductase (TrxR). TrxR plays a crucial role in the cellular redox homeostasis and protection against oxidative damage. In many malignant cells the thioredoxin system is upregulated, promoting tumor growth and progression. Accordingly, the thioredoxin system has been proposed to be an attractive target for cancer therapy. The study by Koncarevic *et al.* gives the first comprehensive overview of the effects of TPCs on the transcriptome and proteome of glioblastoma cells. Under TPC treatment, mechanisms contrasting TrxR inhibition are activated in parallel to DNA-damage-responsive pathways. TPC pressure results into long-term compensatory upregulation of TrxR expression. In parallel, p53 is activated, leading to a range of regulations typical for cell-cycle-arrested cells such as upregulation of CDKN1A, induction of GADD45, inhibition of eIF5A maturation, and reduced phosphorylation of stathmin. It was also shown that TPCs induce endoplasmic reticulum stress, as they activate the unfolded protein response. Thus, this profiling study provides a thorough insight into the spectrum of cellular events resulting from specific TrxR inhibition and characterizes the TPC mode of action.

Overall, the studies mentioned in this paragraph highlight the complex cellular responses to stress caused by metallodrugs. Obviously, more data need to be gathered to carry out well grounded
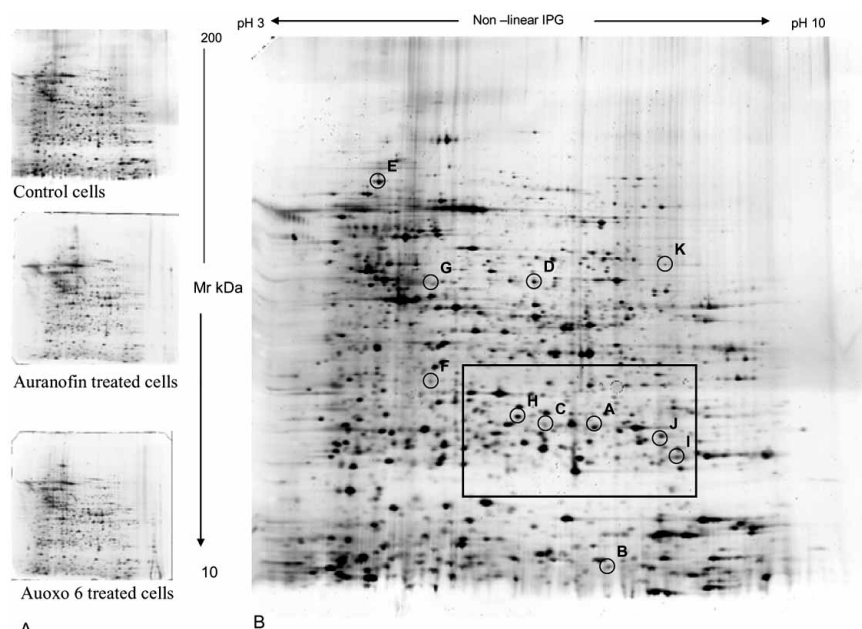
Fig. (9). A: Representative 2D gel images for control cells, auranofin and auoxo 6 treated cells. B: Representative gel image of A2780 control cells. The box highlight a major area where significant and consistent alterations of protein expression were identified Circles and letters indicate differentially expressed proteins (from ref. 56).

comparisons and establish meaningful correlations between the nature of the metallodrugs and the type of observed proteomic response. Moreover, there is the need to deepen the analysis of the proteomic data through the implementation of strong and reliable bioinformatic tools. Such efforts will facilitate the interpretation of the observed proteomic changes in term of affected biochemical pathways and processes.

However, some indications already emerge from the above described studies. In most cases, only a few categories of proteins emerged that are primarily concerned with the cell response to metallodrugs such as proteins of the redox metabolism and mitochondrial proteins. Of course, much attention must be paid now to distinguish whether the observed proteomic alterations are just a part of the overall cell defence response or represent early signs of the apoptotic process.

5. METALLOMICS: A TENTATIVE DEFINITION

The term *"metallome"* was first introduced by R.J.P. Williams in 2001 [58] to define the distribution of free metal ions in every cellular compartment. Subsequently, the term *Metallomics* was proposed to define the *"study of the metallome."* Szpunar (2005) [59] defined *"metallomics"* as the "comprehensive analysis of the entirety of metal and metalloid species within a cell or tissue type". Therefore, metallomics may be considered as a specific branch of metabolomics specifically devoted to the study of metal-containing species, even though metals are not typically considered as metabolites. There were other attempts to define *Metallomics*. According to Hiroki Haraguchi [60], metalloproteins, metalloenzymes and other metal- containing biomolecules are defined as *"metallomes"*, in a similar manner to genomes in genomics and proteomes in proteomics. Since the identification of metallomes and the elucidation of their biological or physiological functions in the biological systems is the main research target of metallomics, chemical speciation for specific identification of bioactive metallomes is one of the most important analytical technologies to establish metallomics as the integrated bio-metal science. Thus *"Metallomics"* may be viewed as an "integrated biometal science".

In his review Haraguchi also presented a classification of the major areas of metallomics and of the respective analytical tech-

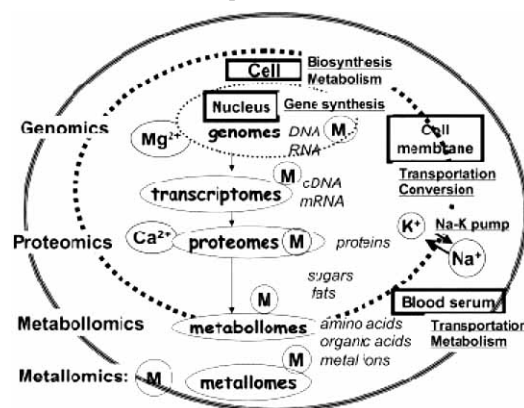niques. According to this interpretation, metallomics covers several important research areas as reported in Table **2**.

Fig. (10). A schematic model of a biological system, showing the relationships among genomics, proteomics, metabolomics and metallomics, where metallic ions are shown as M (from ref. 60).

In turn, Shi *et al.* [61] CMLS provided a clear distinction between metalloproteomics and metallomics. Metalloprotoemics is the study of metalloproteins where metallomics is the study of all metal containing species.

The object and the methods of metallomics were defined in greater detail by Szpunar and Lobisnki in a comprehensive review appeared in 2009 [62].

According to these authors, the emerging field of metallomics refers to the entirety of research activities aimed at the understanding of the molecular mechanisms of metal-dependent life processes. It follows that Metallomics is a new discipline greatly concerned with the implementation of all analytical techniques and methods that are required for probing the interactions between metal ions and the organism's genome, proteome and metabolome. Particular attention has to be paid to the *in vivo* screening for the native metal-protein and metal-metabolite complexes by hyphenated techniques that combine a high-resolution separation technique (gel electrophoresis, chromatography or capillary electrophoresis) with sensi-

Table 2.   **Research Subjects in Metallomics and the Main Respective Analytical Techniques Researches (from ref. 60)**

Research Subject	Analytical Technique
1. Distributions of the elements in the biological fluids, cell, organs, etc.	Ultratrace analysis, all-elements analysis, one atom detection, one molecule detection.
2. Chemical speciation of the elements in the biological samples and systems.	Hyphenated methods (LC-ICP-MS, GC-ICP-MS, MALDI-MS, ES-MS)
3. Structural analysis of metallomes (metal-binding molecules).	X-ray diffraction analysis, EXAFS
4. Elucidation of reaction mechanisms of metallomes using model complexes (bioinorganic chemistry).	NMR, XPS, laser-Raman spectroscopy, DNA sequencer, amino acids sequencer, time-resolution and spatial-resolution fluorescence detection.
5. Identification of metalloproteins and metalloenzymes.	LC-ES-MS, LC-Maldi-MS, LC-ICP-MS
6. Metabolisms of biological molecules and metals (metabolomes, metabolites).	LC, GC, LC-MS, GC-MS, ES-MS, API-MS[a], biosensors
7. Medical diagnosis of health and disease related to trace metals on a multielement basis.	ICP-AES, ICP-MS, graphite-furnace AAS, autoanalyser, spectrophotometry
8. Design of inorganic drugs for chemotherapy.	LC-MS, LC-ICP-MS, stable isotope tracers
9. Chemical evolution of the living systems and organisms on the earth.	Isotope ratio measurement (chronological techniques), DNA sequencer.
10. Other metal-assisted function biosciences in medicine, enviromental science, food science, agriculture, toxicology, biogeochemistry, etc.	*In-situ* analysis, immunoassay, food analysis, clinical analysis.

[a] Atmospheric pressure chemical ionization mass spectrometry.

tive elemental (inductively coupled plasma, ICP) or molecular (electrospray or MALDI) mass spectrometric detection. The contribution of bioinformatics for the prediction of metal-binding sequences in proteins may result very important. In turn, molecular biology approaches may be of great help for the detection of metal-dependent genes, proteins and metabolites.

As specified above, metallomics includes a great variety of investigative tools capable of providing information on metal distribution and speciation within organisms or cells. As this discipline is still in its infancy, standard investigation protocols are not well defined yet. Rather, a group of heterogeneous biophysical ad bioanalytical tools now exist, essentially centered on the metal, that need further refinement and require integration into standard and validated experimental procedures. In any case, ICP MS appears to constitute the election method for this new discipline as ICP MS is capable to monitor a multitude of diverse metals simultaneously and with a high sensitivity. Thus, according to Shy, the two main techniques of metallomics are ICP MS and ESI MS. From the former method detailed quantitative information may be derived on metal containing species whereas from the latter structural information is typically achieved. Remarkably, ICP MS may be coupled to other biophysical or bioseparation tools. Alternatively metals in Metallomic studies may be monitored by XAS methods as described by Ascone *et al.* [63] and by Hambley *et al.* [64]. Remarkably, despite the lack of canonical or systematic investigation procedures, a number of studies have appeared in recent years principally aimed at analysing metallodrugs in the biological systems according to a metallomic point of view. Selected studies will be described below.

6. METALLOMIC STUDIES OF ANTICANCER METALLODRUGS

In the course of the last 10-15 years several research papers have appeared dealing with anticancer metallodrugs that may be roughly referred to a metallomic approach i.e. the study of the distribution and the speciation of a metal within a certain biological system... Most of these studies were devoted to platinum anticancer metallodrugs while only a few other concern non platinum metallodrugs. Some selected studies will be considered below in greater detail

a. Metallomic Studies of Platinum Drugs

A rather complete and exhaustive description of metallomic studies carried out so far on platinum based drugs has been provided in a very recent review [65]. This review highlights the major

role of Analytical Chemistry, and in particular of ICP MS methods, in elucidating the interactions that are established between Pt-based and bio molecules. Notably, this review offers a summary of the *main analytical techniques* and of the most common *sample treatment procedures* currently used in metallomics studies of anticancer platinum . Both are of paramount importance to investigate such complex samples in the effort of preserving the existing – biomolecule interactions. In particular, separation and detection techniques must be carefully selected to achieve the intended goals. The use of multidimensional hyphenated techniques is usually necessary for understanding Pt-based interactions in the organism. Typically, samples analysed in the studies of platinum drugs are blood, urine, cell , as well as the drugs themselves and their derivatives. However, most of the works that were carried out on platinum drugs are based on *in vitro* experiments or incubations of standards, leading in some cases to contradictory results depending on the experimental conditions used. Though *in vivo* experiments represent a great challenge due to their intrinsic high complexity and to the low concentrations of the Pt-adducts in real samples, these studies must be necessarily undertaken to gain a deeper understanding of the real interactions concerning Platinum drugs.

Among the several papers appeared on the above issue there are a few ones that seem to us of particular interest and merit further discussion.

In a pioneering study, published in 2001, Paul Dyson and co-workers [66] examined the cellular distribution of platinum in a bacterial model, following cisplatin treatment, in the attempt to identify possible metal targets. Accordingly, proteins from cisplatin treated bacterial cells were partially separated by 1D polyacrylamide gel electrophoresis and analysed by laser ablation inductively coupled plasma mass spectrometry; using peptide fingerprinting methods (PFM). Very remarkably, the band containing the highest levels of platinum was found to contain the outer membrane protein A, ompA, which might be involved in cisplatin uptake. Thus, this study already pointed out the possible way for the subsequent investigations aimed at target identification.

More recently, Sheldrick *et al.* expanded the approach originally proposed by Dyson. [67] They used multidimensional liquid chromatography and electrospray ionisation tandem mass spectrometry in combination to analyse platinated tryptic peptides from *Escherichia coli* cells treated with cisplatin at pH 7.0. Prerequisites for the LC/LC/MS/MS analysis of protein targets, that are nicely fulfilled by cisplatin, are: i) that the original protein binding sites have a high kinetic stability over a wide pH range(2.3 < pH < 8.5),

and ii) that the metal fragment remains coordinated to a significant number of b+ and y+ peptide ions under MS/MS fragmentation conditions. Matching the MS/MS spectra of the platinated tryptic peptides to sequences of proteins in the *E. coli* database allowed the identification of 31 protein targets for cisplatin. Whereas six of them were high-abundance enzymes and ribosomal proteins, five low-abundance DNA-binding proteins could be identified as specific platinum targets. These included the DNA mismatch repair protein mutS, the DNA helicase II (uvrD) and topoisomerase I (top1). Two efflux proteins (acrD, mdtA), the redox regulator thioredoxin 1 (thiO) and the external filament-like type-1 fimbrial protein A chain (fimA1) were also characterised as specific cisplatin-binding proteins. Kinetically favoured carboxylate (D, E) and hydroxy (S, T, Y) O atoms were identified as the Pt coordination sites in 18 proteins and methionyl S atoms in 9 proteins. Again, such an approach is susceptible of further extension to cisplatin treated human cancer cells.

In an interesting paper appeared in 2003, Timerbaev, Keppler *et al.* [68], using cisplatin as a reference compound, revealed that capillary electrophoresis (CE) may be exploited to reliably assess drug/HSA interactions. Since cisplatin is small compared to the size of the protein, the binding response could only be recognized when applying CE coupled to a (platinum) -specific mode of detection, namely inductively coupled plasma-mass spectrometry (ICP-MS). This coupling allowed revealing the specific affinity of cisplatin and of novel Pt complexes for HSA, the measurement of the kinetics of binding, and the determination of the number of drug molecules attached to the protein. As the cisplatin/HSA molar ratio increased, the reaction rate became faster with a maximum on the kinetic curve appearing at about 50 h of incubation at 20 times excess of cisplatin. The reaction was characterized as a pseudo-first order reaction. When incubated with a 20-fold excess of cisplatin, HSA bound up to 10 mol of Pt per mol of the protein. This is indicative for a strong metal-protein coordination occurring at several HSA sites other than the only protein cysteine residue. Structural analogs of cisplatin, bearing aminoalcohol ligands, showed comparable protein binding reactivity and stoichiometry but a common equilibrium was not reached even after one week of incubation.. Overall these results demonstrated the suitability of CE-ICP-MS as a rapid assay for high-throughput studying of drug/protein interactions.

Trevor Hambley *and coworkers* [69], in a number of papers now summarised in a comprehensive *Chem Rev.* article [70] developed some elegant strategies aimed to monitor platinum distribution inside cancer cells and also to perform detailed speciation studies. In particular XAS and fluorescence methods turned out very informative for this purpose. The use of XAS methods was specifically exploited to monitor reduction of Pt(IV) complexes inside the reducing environment of cancer cells.

In turn, Dan Gibson *and coworkers* [71] developed an ingenious method directed to discriminate between low molecular weight, intermediate molecular weight and high molecular weight ligands for cisplatin. These studies were grounded on NMR measurements coupled to simple, low resolution, separation methods. It is known that the interactions of cisplatin with a variety of biologically occurring nucleophiles can potentially enhance the efficacy of the drug by mediating its delivery to nuclear DNA or inactivate the drug by binding it irreversibly or by labilizing the NH$_3$ ligands. Despite the potential importance of trans-labilization reactions in the mechanism of action of cisplatin, few detailed studies on trans labilization of the ammines had been conducted before. Gibson et al used 2D NMR to show that some trans labilization occurs in proliferating cells and that aqueous extracts of cancer cells were able to labilize ca 20% of the amine ligands of cis-[PtCl$_2$(^{13}CH$_3$NH$_2$)$_2$] after 12-h incubation. Both low molecular mass nucleophiles (less than 3 kDa) and high molecular mass nucleophiles (more than 3 kDa) were found to labilize the amines with similar efficiency.

Studies with model compounds showed that thiols and thioethers bind to platinum(II) at similar rates, but thioethers are significantly more efficient at labilizing the am(m)ine at lower pH. Quantification of the platinum adducts obtained from incubation of cisplatin with cell extracts indicated that two thirds of the platinum is bound to cellular components with molecular mass greater than 3 kDa. In turn, Pt(IV) complexes must be reduced to kill cancer cells. The reduction of cis,trans,cis- [PtCl$_2$(OCOCH$_3$)$_2$(NH$_3$)$_2$] by extracts of three cell lines was measured, and the rates follow the order A2780cisR > A2780 > HT-29. It emerged from these measurements that reduction is not carried out by the low molecular weight (MW) antioxidants but primarily by cellular components with MW > 3000. These studies documented the suitability and the value of NMR studies coupled to simple separation procedures.

b) Metallomics Studies of Non-Platinum Metallodrugs

In addition, a few metallomic papers were devoted to the mechanistic investigation of non-platinum metallodrugs. Some of them are described below in more detail.

In analogy with the above mentioned paper on platinum compounds, Sheldrick *et al.* employed an automated multidimensional protein identification technology, (*MudPIT*) [72], which combines biphasic liquid chromatography and electrospray ionisation tandem mass spectrometry (MS/MS), to analyse tryptic peptides from *Escherichia coli* treated with the antiproliferative agent [(eta(6)-p-cymene)RuCl(2)(DMSO)]. MS/MS spectra were recorded for molecular ions generated by neutral loss of p-cymene from peptide ions coordinated by the (η p-cymene)Ru(II) fragment. Matching of the MS/MS spectra of the ruthenated peptides to spectra of proteins in the *E. coli* database allowed identification of five protein targets for this ruthenium arene. One of these proteins is the constitutive cold-shock protein cspC, which regulates the expression of genes encoding stress-response proteins, while three of the other targets, ppiD, osmY and sucC, are proteins of the latter type. The DNA damage-inducible helicase dinG was likewise established as a protein target. Aspartate carboxylate functions were identified as the probable ruthenium binding sites in cspC, ppiD and dinG, and threonine and lysine side chains in osmY and sucC, respectively. This investigation confirmed the general validity of the applied methodology.

In an interesting paper appeared in 2007 Sadler *et al.* [73] reported on the use of a radioactive label for metallodrug distribution studies. More in detail, the organometallic half-sandwich Ru^{II} arene anticancer complex [(η^6-fluorene)Ru(en)Cl]PF$_6$ (1) was synthesized in high yield and purity on a micromole scale with incorporation of the β-emitting radioisotope ^{106}Ru (half-life = 1.01 y) using a refined procedure involving conversion of RuCl$_3$ into [(η^6-fluorene) RuCl$_2$]$_2$, and then [(η^6-fluorene)Ru(CH$_3$CN)$_2$Cl]PF$_6$ as intermediates. Distribution studies 0.25 h post i.v. injection of ^{106}Ru-1 at a dose of 25 mg 1 kg^{-1} showed that ^{106}Ru is well distributed throughout the tissues of a rat. This was the first report of the radiolabelling of a potential ruthenium antitumour agent for distribution/biological studies. Despite the problems and the difficulties intrinsic to the use of radioactive isotope, radiolabeling seems still to be an attractive option due to selectivity and sensitivity.

Again, Hagen *et al.* [74] reported very recently on the use of radioisotopes for metalloproteomics studies. A combination of techniques was developed to separate and quantify the native proteins associated with a particular transition metal ion from a cellular system developed. The procedure basically involves four steps: (1) labeling of the target proteins with a suitable short-lived radioisotope (suitable isotopes are (64)Cu, (67)Cu, (187)W, (99)Mo, (69)Zn, (56)Mn, (65)Ni); (2) separation of intact soluble holoproteins using native isoelectric focusing combined with blue native polyacrylamide gel electrophoresis into native-native 2D gel electrophoresis; (3) spot visualization and quantification using autoradiography; and (4) protein identification with tandem mass spec-

Table 3. Proteins Identified as "Responsive" to Metallodrugs (from ref. 75)

Function	Proteins	Metallodrug
Chaperone	Hsp70 Hsp27 HSPgp96 HspA	As As Au, Pt Bi
Metabolic enzyme	TPI1 GAPDH Fumarase	Au, Pt As Bi
Anti oxidative stress	Peroxiredoxin 1 Peroxiredoxin 6 Thioredoxin AR TsaA	Au, Pt Au, Pt Au, Pt, Bi As Bi
Translation factor	Splicing factor 17 PDI Ef-Tu	Au, Pt Au, Pt Bi
Signal transduction	Annexin IV Cyclophilin A	Pt Au, Pt
Cellular structure	Cytokeratin 8 Cytokeratin 18 Destrin	Pt Pt Pt

trometry. The method was specifically applied to the identification of copper proteins from a soluble protein extract of wild-type Escherichia coli K12 using the radioisotope (64)Cu. The E. coli protein CueO, which has previously been only identified as a multi-copper oxidase following homologous overexpression, was now directly detected as a copper protein. The retention of the radioisotope by the copper proteins throughout the separation process corroborates the method to be genuinely native. The procedure developed here can be applied to cells of any origin, and to any metal having suitable radioisotopes. This kind of methodology, although troublesome, might be successfully applied to metallodrugs and to the identification of their protein targets.

Finally, we like to cite an interesting review paper published by Hong Zhe Sun *et al.* in *Metallomics.* 2009 [75].This review beautifully summarises recent developments in the identification of either binding or "responsive" proteins to metallodrugs and of their target sites for a variety of platinum-, ruthenium-, gold-, arsenic- and bismuth-containing agents, based on proteomics and metalloproteomics studies. Moreover, an interesting table is reported where a number of proteins that are "responsive" to metallodrugs are compiled (Table **3**). This kind of information may offer a rationale for understanding metal induced biochemical damage and the corresponding cellular response. Moreover, in this review, particular attention is devoted to the detailed description of the relevant analytical methodologies.

7. CONCLUSIONS AND PERSPECTIVES

The great value of proteomics in metal-based drug discovery and development, especially in elucidating and mapping drug action mechanisms, has been clearly demonstrated through many successful examples from the recent literature. Proteomic methods were recognized as a promising and powerful investigative strategy that can allow the systematic characterization of cellular responses to metal based drugs and help identifying the respective targets and pathways. However, there are still some relevant limitations to this kind of approach. Several membrane proteins are typically lost or degraded in the course of the proteomic studies; in addition low molecular weight proteins are similarly lost. Moreover, it is also possible that damage caused by metallodrugs is not immediately evident as damaged proteins are not *inducible*. In addition, discrimination of cell defence mechanisms from damage mechanisms

may be not so straightforward. All these arguments imply that a simple proteomic approach *per se* may not be sufficient to provide conclusive mechanistic information on anticancer metallodrugs.

In turn, we have shown that several analytical and physico-chemical methods, centered on the metal and recently developed and organised into the new field of *Metallomics*, are now available that allow identification of the metal in complex biological mixtures with a relatively high sensitivity. These methods permit tracking the uptake, the distribution, the transformation and the fate of metallodrugs within complex biological systems. In other words, a direct and continuous monitoring of the metal "in real time" is typically achieved.

Based on these considerations and on the incredible rapidity of technological progress, it is possible to predict the likely evolution of this research area. We believe that a tight coupling of proteomics strategies with sensitive analytical methods for metal detection (metallomics) will allow in the close future a straightforward and precise identification of metal distribution within the hundreds of proteins that are normally separated within 2D gels. Realistically, an increase in sensitivity and in spatial resolution of LA ICP MS methods will allow the rapid scanning of 2D gels to determine the quantitative distribution of the metal. This kind of strategy will permit a strong progress in this research area as in principle all proteins capable of binding tightly the metal will be identified. It is very likely that among these proteins are those that are the effective targets for the various metallodrugs: in addition proteins may be identified that are responsible for toxicity and for resistance. Obviously, such technological progresses must be accompanied and supported by a parallel progress in the interpretative tools; bioinformatics is the discipline specifically deputed to perform data mining and translate the observed metal-dependent proteomic changes into specific information on the affected cellular pathways and on the putative biomolecular targets. In conclusion we believe that a further and tight integration of the two described approaches (proteomics and metallomics) will turn crucial for a better understanding of the underlying mechanisms and for the eventual elucidation of the mode of action of anticancer metallodrugs.

REFERENCES

[1]   Zhang, C.X.; Lippard, S.J. New metal complexes as potential therapeutics. *Curr. Opin. Chem. Biol.,* **2003,** *7,* 481-489.

[2] Alderden, R.A.; Hall, M.D.; Hambley T.W. The discovery and development of cisplatin. *J. Chem. Educ.*, **2006**, *83*, 728-734.

[3] Jamieson, E.R.; Lippard, S.J. Structure, recognition, and processing of cisplatin-DNA adducts. *Chem. Rev.,* **1999**, *99*, 2467-2498.

[4] Farrell, N. Polynuclear platinum drugs. In: *Met Ions Biol Syst.* Siegel, A.; Siegel, H., Eds.: Marcel Dekker, Inc. New York, **2004**, *42*, 251-296.

[5] Coluccia, M.; Nassi, A.; Roseto, F.; Boccarelli, A.; Mariggiò, M.A.; Giordano, D.; Intini, F.P.,; Caputo, P.; Natile, G. A transplatinum complex showing higher antitumor activity than the cis congeners. *J. Med. Chem.*, **1993**, *36*, 510-512.

[6] Leng, M.; Locker, D.; Giraud-Panis, M.J.; Schwartz, A.; Intini, F.P.; Natile, G.; Pisano, C.; Boccarelli, A.; Giordano, D.; Coluccia, M. Replacement of an NH₃ by an iminoether in transplatin makes an antitumor drug from an inactive compound. *Mol. Pharmacol.*, **2000**, *58*, 1525-1535.

[7] Montero, E.I.; Díaz, S.; González-Vadillo, A.M.; Pérez, J.M.; Alonso, C.; Navarro-Ranninger, C. Preparation and characterization of novel trans-[Pt(II)Cl₂(amine)(isopropylamine)] compounds. Cytotoxic activity and apoptosis induction in ras-transformed cells. *J. Med. Chem.*, **1999**, *42*, 4264−4268.

[8] Hall, M.D.; Mellor, H.R.; Callaghan, R.; Hambley, T.W. Basis for design and development of platinum(IV) anticancer complexes. *J. Med. Chem.*, **2007**, *50*, 3403-3411.

[9] Gianferrara, T.; Bratsos, I.; Alessio, E. A categorization of metal anticancer compounds based on their mode of action. *Dalton Trans.*, **2009**, *37*, 7588-7598.

[10] Hartinger, C.G.; Jakupec, M.A.; Zorbas-Seifried, S.; Groessl, M.; Egger, A.; Berger, W.; Zorbas, H.; Dyson, P.J.; Keppler, B.K. KP1019, a new redox-active anticancer agent--preclinical development and results of a clinical phase I study in tumor patients. *Chem. Biodivers.*, **2008**, *10*, 2140-2155.

[11] Ronconi, L., Fregona, D. The Midas touch in cancer chemotherapy: From platinum- to gold-dithiocarbamato complexes *Dalton Trans.,* **2009**, *48*, 10670-10680.

[12] Aldinucci, D., Ronconi, L., Fregona, D. Groundbreaking gold(III) anticancer agents. *Drug Discov. Today,* **2009**, *14*, 1075-1076.

[13] Saggioro, D., Rigobello, M.P., Paloschi, L., Folda, A., Moggach, S.A., Parsons, S., Ronconi, L., Fregona, D., Bindoli, A. Gold(III)-Dithiocarbamato complexes induce cancer cell death triggered by thioredoxin redox system inhibition and activation of ERK pathway. *Chem. Biol.,* **2007**, *14*, 1128-1139.

[14] Sun, R.W.Y.; Che, C.M. The anti-cancer properties of gold(III) compounds with dianionic porphyrin and tetradentate ligands. *Coord. Chem. Rev.,* **2009**, *253,* 1682-1691

[15] Y. Wang; He, Q.Y.; Sun, R.W.; Che, C.M.; Chiu, J.F. GoldIII porphyrin 1a induced apoptosis by mitochondrial death pathways related to reactive oxygen species. *Cancer Res.,* **2005**, *65*, 11553-11564.

[16] Nobili, S.; Mini, E.; Landini, I.; Gabbiani, C.; Casini, A.; Messori, L. Gold compounds as anticancer agents: chemistry, cellular pharmacology, and preclinical studies. *Med. Res. Rev.,* **2009**, *in press.*

[17] Butcher, E.C.; Berg, E.L.; Kunkel, E.J. Systems biology in drug discovery. *Nat. Biotechnol.,* **2004**, *22*, 1253-1259.

[18] Snoep J.L.; Westerhoff H.V.; Alberghina L.; Westerhoff H.V. Eds. From isolation to integration, a systems biology approach for building the Silicon Cell. *Systems Biology: Definitions and Perspectives.* Springer-Verlag: USA, **2005**, p. 7.

[19] Sauer, U.; Heinemann, M.; Zamboni, N. Getting closer to the whole picture. *Science*, **2007**, *316*, 550-551

[20] Baldi, P.; Brunak, S. Bioinformatics: The Machine Learning Approach. 2nd ed. MIT Press: USA, **2001**.

[21] Barnes, M.R.; Gray, I.C.; Eds., *Bioinformatics for Geneticists,* 1ˢᵗ ed. Wiley: USA, **2003**.

[22] Baxevanis, A.D.; Ouellette, B.F.F.; Eds., *Bioinformatics: A Practical Guide to the Analysis of Genes and Proteins,* 3ʳᵈ ed. Wiley: USA, **2005**.

[23] Baxevanis, A.D., Petsko, G.A., Stein, L.D., Stormo, G.D., Eds. *Current Protocols in Bioinformatics.* Wiley: USA, **2007**.

[24] Gardner, T.S.; Di Bernardo, D.; Lorenz, D.; Collins J.J. Inferring genetic networks and identifying compound of action via expression profiling. *Science,* **2003**, *301*, 102-105.

[25] Di Bernardo, D.; Thompson, M.J.; Gardner, T.S.; Chobot, S.E.; Eastwood, E.L.; Wojtovich, A.P.; Elliot, S.J.; Schaus, S.E.; Collins,

J.J. Chemogenomic profiling on a genome-wide scale using reverse-engineered gene networks. *Nat. Biotechnol.,* **2005**, *23*, 377-383.

[26] Stefan, G.; Miguel, J.; García-Gómez, Terol, J.; Tim, D.; Williams, MJN; Robles, M.; Talón, M.; Dopazo, J.; Conesa, A. High-throughput functional annotation and data mining with the Blast2GO suite. *Nucleic Acids Res.*, **2008**, *36*, 3420-3435.

[27] Pandey, J.; Koyuturk, M.; Kim, Y.; Szpankowski, W.; Subramaniam, S.; Grama, A. Functional annotation of regulatory pathways, *Bioinformatics*, **2007**, *23*, 377-386.

[28] Yang, Y.; Adelstein, S.J.; Kassis, A.I. Target discovery from data mining approaches. *Drug Discov. Today,* **2009**, *14*, 147-154.

[29] Wang, Y.; Chiu, J.F. Proteomic approaches in understanding action mechanisms of metal-based anticancer drugs. *Met. Based Drugs,* **2008**, *2008,* 716329.

[30] Timerbaev, A.R.; Hartinger, C.G.; Aleksenko, S.S.; Keppler, B.K. Interactions of antitumor metallodrugs with serum proteins: advances in characterization using modern analytical methodology. *Chem. Rev.,* **2006**,*106*, 2224-2248.

[31] Anderson, N.L.; Anderson, N.G. Proteome and proteomics: new technologies, new concepts, and new words. *Electrophoresis,* **1998**, *19*, 1853-1861.

[32] Blackstock, W.P.; Weir, M.P. Proteomics: quantitative and physical mapping of cellular proteins. *Trends Biotechnol.,* **1999**, *17*, 121-127.

[33] James, P. Protein identification in the post-genome era: the rapid rise of proteomics. *Q. Rev. Biophys.,* **1997**, *30*, 279-331.

[34] Wilkins, M.R.; Pasquali; C.; Appel, R.D.; Ou, K.; Golaz, O.; Sanchez, J.-C.; Yan, J.X.; Gooley, A.A.; Hughes, G.; Humphery-Smith, I.; Williams, K.L.; Hochstrasser, D.F. From proteins to proteomes: large scale protein identification by two-dimensional electrophoresis and arnino acid analysis. *Nat. Biotechnol.,* **1996**, *14*, 61-65.

[35] Harvey, D.J. Analysis of carbohydrates and glycoconjugates by matrix-assisted laser desorption/ionization mass spectrometry: an update covering the period 2001-2002. *Mass Spectrom. Rev.,* **2008**, *27*, 125-201.

[36] Oberacher, H.; Parson, W. Forensic DNA fingerprinting by liquid chromatography-electrospray ionization mass spectrometry. *BioTechniques,* **2007**, *43*, VII-XIII.

[37] Jiang, X.; Ye, M.; Zou, H. Technologies and methods for sample pretreatment in efficient proteome and peptidome analysis. *Proteomics*, **2008**, *8*, 686-705.

[38] Minden, J.S.; Dowd, S.R.; Meyer, H.E.; Stühler, K. Difference gel electrophoresis. *Electrophoresis,* **2009**, *30*, 156-161.

[39] Reisinger, V.; Eichacker, L.A. Analysis of membrane protein complexes by blue native PAGE. *Proteomics,* **2006**, *2*, 6-15.

[40] MacCoss; M.J.; McDonald, W.H.; Saraf. A.; Sadygov, R.; Clark, J.M.; Tasto, J.J.; Gould, K.L.; Wolters, D.; Washburn, M.; Weiss, A.; Clark, J.I.; Yates, JR 3rd. Shotgun identification of protein modifications from protein complexes and lens tissue. *Proc. Natl. Acad. Sci. USA,* **2002**, *99*, 7900-7905.

[41] Elliott, M.H.; Smith, D.S.; Parker, C.E.; Borchers, C. Current trends in quantitative proteomics. *J. Mass Spectrom.*, **2009**, *44*, 1637-1660.

[42] Vaudel, M.; Sickmann, A.; Martens, L. Peptide and protein quantification: a map of the minefield. *Proteomics,* **2009,** *in press***.**

[43] Whelan, L.C.; Power, K.A.; McDowell, D.T.; Kennedy, J.; Gallagher, W.M. Applications of SELDI-MS technology in oncology. *J. Cell Mol. Med.*, **2008,** *12*, 1535-1547.

[44] Gogichaeva, N.V.; Williams, T.; Alterman, M.A. MALDI TOF/TOF tandem mass spectrometry as a new tool for amino acid analysis. *J. Am. Soc. Mass Spectrom.*, **2007**, *18*, 279-284.

[45] Castagna, A.; Antonioli, P.; Aster, H.; Hamdan, M.; Righetti, S.C.; Perego, P.; Zunino, F.; Rigetti, P.G. A proteomic approach to cisplatin resistance in the cervix squamous cell carcinoma cell line A431. *Proteomics*, **2004**, *4*, 3246-3267.

[46] Le Moguen, K.; Lincet, H.; Deslandes, E.; Hubert-Roux, M.; Lange, C.; Poulain, L.; Gauduchon, P.; Baudin, B. Comparative proteomic analysis of cisplatin sensitive IGROV1 ovarian carcinoma cell line and its resistant counterpart IGROV1-R10. *Proteomics,* **2006**, 6, 5183-5192.

[47] Le Moguen, K.; Lincet, H.; Marcelo, P.; Lemoisson, E.; Heutte, N.; Duval, M.; Poulain, L.; Vinh, J.; Gauduchon, P.; Baudin, B. A pro-

teomic kinetic analysis of IGROV1 ovarian carcinoma cell line response to cisplatin treatment. *Proteomics*, **2007**, *7*, 4090-4101.

[48]   Yan, X.D.; Pan, L.Y.; Yuan, Y.; Lang, J.H.; Mao, N. Identification of platinum-resistance associated proteins through proteomic analysis of human ovarian cancer cells and their platinum-resistant sublines. *J. Proteome Res.*, **2007**, *6*, 772-780.

[49]   Stewar,t J.J.; White, J.T.; Yan, X.; Collins, S.; Drescher, C.W.; Urban, N.D.; Hood, L.; Lin, B. Proteins associated with Cisplatin resistance in ovarian cancer cells identified by quantitative proteomic technology and integrated with mRNA expression levels. *Mol. Cell Proteomics,* **2006**, *5*, 1171.

[50]   Smith; L.; Welham, K.J.; Watson, M.B.; Drew, P.J.; Lind, M.J.; Cawkwell, L. The proteomic analysis of cisplatin resistance in breast cancer cells. *Oncol. Res.,* **2007**,*16*, 497-506.

[51]   Martinez-Balibrea, E.; Plasencia, C.; Ginés, A.; Martinez-Cardús, A.; Musulén, E.; Aguilera, R.; Manzano, J.L.; Neamati, N.; Abad, A. A proteomic approach links decreased pyruvate kinase M2 expression to oxaliplatin resistance in patients with colorectal cancer and in human cell lines. *Mol. Cancer Ther.*, **2009**, *8*, 771-778.

[52]   Yim, E.K.; Lee, K.H.; Kim, C.J.; Park, J.S. Analysis of differential protein expression by cisplatin treatment in cervical carcinoma cells. *Int. J. Gynecol. Cancer,* **2006**, *16*, 690-697.

[53]   Yao, Y.; Jia, X.Y.; Tian, H.Y.; Jiang, Y.X.; Xu, G.J.; Qian, Q.J.; Zhao, FK. Comparative proteomic analysis of colon cancer cells in response to oxaliplatin treatment. *Biochim. Biophys. Acta,* **2009**, *1794*, 1433-1440.

[54]   Wang, Y.; He, Q.Y.; Che, C.M.; Chiu, J.F. Proteomic characterization of the cytotoxic mechanism of gold (III) porphyrin 1a, a potential anticancer drug. *Proteomics*, **2006**, *6*, 131-142.

[55]   Xu, X.; Decker, W.; Sampson, M.J.; Craigen, W.J.; Colombini, M. Mouse VDAC isoforms expressed in yeast: channel properties and their roles in mitochondrial outer membrane permeability. *J. Membr. Biol.,* **1999**, *170,* 89-102

[56]   Magherini, F.; Modesti, A.; Bini, L.; Puglia, M.; Landini, I.; Nobili, S.; Mini, E.; Cinellu, M.A.; Gabbiani, C.; Messori, L. Exploring the biochemical mechanisms of cytotoxic gold compounds: a proteomic study. *J. Biol. Inorg. Chem.,* **2010**, *in press*.

[57]   Koncarevic, S.; Urig, S.; Steiner, K.; Rahlfs, S.; Herold-Mende, C.; Sueltmann, H.; Becker, K. Differential genomic and proteomic profiling of glioblastoma cells exposed to terpyridineplatinum(II) complexes. *Free Radic. Biol. Med.*, **2009**, *46*, 1096-1108.

[58]   Williams, R.J.P.; Fraústo da Silva, J.J.R. *The Biological Chemistry of the Elements: the Inorganic Chemistry of Life,* 2nd ed. Oxford University Press: Oxford and New York. **2001**

[59]   Szpunar, J. Advances in analytical methodology for bioinorganic speciation analysis: metallomics, metalloproteomics and heteroatom-tagged proteomics and metabolomics. *Analyst*, **2005**, *130*, 442-465.

[60]   Haraguchi, H. Metallomics as integrated biometal science. *J. Anal. Atomic Spectrum.,* **2004**, *19*, 5-14.

[61]   Shi, W.; Chance, M.R. Metallomics and metalloproteomics. *Cell Mol. Life Sci.*, **2008**, *65*, 3040-3048.

[62]   Mounicou, S.; Szpunar, J.; Lobinski, R. Metallomics: the concept and methodology. *Chem. Soc. Rev.,* **2009**, *38*, 1119-1138.

[63]   Ascone, I.; Strange, R. Biological X-ray absorption spectroscopy and metalloproteomics. *J. Synchrotron Radiat.*, **2009**, *16*, 413-421.

[64]   Klein, A.V.; Hambley, T.W. Platinum drug distribution in cancer cells and tumors. *Chem. Rev.*, **2009**,*109*, 4911-4920.

[65]   Esteban-Fernández, D.; Moreno-Gordaliza, E.; Cañas, B.; Palacios M.A., Gómez-Gómez M.M. Analytical methodologies for metallomics studies of antitumor Pt-containing drugs. *Metallomics*, **2010**, *2*, 19.

[66]   Khalaila, I.; Bergamo, A.; Bussy, F.; Sava, G.; Dyson, P.J. The role of cisplatin and NAMI-A plasma-protein interactions in relation to combination therapy. *Int. J. Oncol.,* **2006**, *29*, 261-268.

[67]   Will, J.; Sheldrick, W.S.; Wolters, D.J. Characterisation of cisplatin coordination sites in cellular Escherichia coli DNA-binding proteins by combined biphasic liquid chromatography and ESI tandem mass spectrometry. *J. Biol Inorg. Chem.*, **2008**, 13, 421-434.

[68]   Aleksenko, S.S.; Polec-Pawlak, K.; Ruzik, R.; Semenova, O.; Hartinger, C.G.; Oszwaldowski, S.; Galanski, M.; Jarosz, M.; Keppler, B.K. Platinum metallodrug-protein binding studies by capillary electrophoresis-inductively coupled plasma-mass spectrometry: characterization of interactions between Pt(II) complexes and human serum albumin. *Electrophoresis,* **2004**, *25*, 1988-1995.

[69]   Rademaker-Lakhai, J.M.; Terret, C.; Howell, S.B.; Baud, C. M.; de Hall, M.D.; Alderden, R. A.; Zhang, M.; Beale, P.J.; Cai, Z.; Lai, B.; Stampfl, A.P.J.; Hambley, T.W. The fate of platinum(II) and platinum(IV) anti-cancer agents in cancer cells and tumours. *J. Struct. Biol.*, **2006**, *155*, 38-44.

[70]   Klein, A.V.; Hambley, T.W. Platinum drug distribution in cancer cells and tumors. *Chem. Rev.*, **2009**, *109*, 4911-4920.

[71]   Kasherman, Y.; Sturup, S.; Gibson, D. Trans labilization of am(m)ine ligands from platinum(II) complexes by cancer cell extracts. *J. Biol. Inorg. Chem.*, **2009**, *14*, 387-399.

[72]   Will, J.; Kyas, A.; Sheldrick, W.S.; Wolters, D. Identification of (η^6-arene)ruthenium(II) protein binding sites in E. coli cells by combined multidimensional liquid chromatography and ESI tandem mass spectrometry: specific binding of [(η^6-p-cymene)RuCl$_2$ (DMSO)] to stress-regulated proteins and to helicases. *J. Biol. Inorg. Chem.*, **2007**, *12*, 883-894.

[73]   Hoeschele, J.D.; Habtemariam, A.; Muir, J.; Sadler, P.J. [106]Ru radiolabelling of the antitumour complex [(η^6-fluorene)Ru(en) Cl]PF$_6$. *Dalton Trans.,* **2007**, *43*, 4974-4979.

[74]   Sevcenco, A.M.; Krijger, G.C.; Pinkse, M.W.; Verhaert, P.D.; Hagen, W.R.; Hagedoorn, P.L. Development of a generic approach to native metalloproteomics: application to the quantitative identification of soluble copper proteins in *Escherichia coli. J. Biol. Inorg. Chem.*, **2009**, *14*, 631-640.

[75]   Sun, X.; Tsang, C.-N.; Sun, H. Identification and characterization of metallodrug binding proteins by (metallo)proteomics. *Metallomics*, **2009**, *1*, 25-31.