Proteomics as a Tool to Disclose the Cellular and Molecular Mechanisms of Selected Anticancer Gold Compounds

Tania Gamberi[1], Alessandro Pratesi[2], Luigi Messori[3*], Lara Massai[3*]

[1] *Department of Experimental and Clinical Biomedical Sciences "Mario Serio", University of Florence, Florence, Italy;* [2] *Department of Chemistry and Industrial Chemistry, University of Pisa, Pisa, Italy;* [3] *Department of Chemistry "Ugo Schiff", University of Florence, Florence, Italy*

**Corresponding author: Lara Massai, Tel. +39 055 4573130, E-mail lara.massai@unifi.it*

**Corresponding author: Luigi Messori, Tel. +39 055 4573388, E-mail luigi.messori@unifi.it*

Keywords: Proteomics; Gold complexes; Mass Spectrometry; Metal-based drugs; Mechanism of action

Abstract

Gold compounds form an attractive class of cytotoxic metal compounds of potential application as anticancer agents. Notably, the mode of action of cytotoxic gold compounds appears to differ from that of the widely used anticancer Pt drugs -to which they were initially inspired- and to be basically DNA-independent. However, mechanistic details are still largely lacking for this class of metal-based drugs. To shed light on these issues we have developed a proteomic strategy that is capable of highlighting the perturbations in protein expression elicited by gold drugs in a selected cancer cell line with the final aim to disclose the underlying molecular mechanisms. In recent years, this type of strategy has been systematically applied, in our laboratory, to a representative panel of gold compounds including seven outstanding cytotoxic agents, i.e. six experimental gold(III) and gold(I) compounds and the clinical gold(I) drug, Auranofin. A2780 human ovarian cancer cells were used as the standard cellular model for these studies. Proteins differentially expressed upon treatment were separated by 2DE and identified by MALDI TOF and their meaning tentatively assessed through bioinformatic analysis. The occurrence of various and often overlapping molecular mechanisms was revealed. The affected proteins were found to belong -in most cases- to redox control systems and/or to the proteasome machinery implying that the severe cellular damage induced by gold compounds predominantly originates at these two distinct levels. However, for one Au(III) and one Au(I) compound, i.e. [(bipy^{dmb}-H)Au(OH)][PF_6] (bipy^{dmb}-H = deprotonated 6-(1,1-dimethylbenzyl)-2,2'-bipyridine) (Aubipyc) and the bis(1-butyl-3-methyl-imidazole-2-ylidene) gold(I) [Au(NHC)$_2$]PF_6, a substantially greater number of proteomic alterations were detected pointing out, in both cases, to glucose metabolism as an additional target process of the cytotoxic action. The results that were obtained with the seven gold complexes are discussed in the frame of the available knowledge on anticancer gold drugs and their mechanisms. In general, our studies underscore the large amount of information that proteomic measurements may provide on the mode of action of metal-based drugs at the cellular level and delineate a very effective methodology for the identification of the respective cytotoxic mechanisms. We propose that the interpretation of the proteomic data in terms of the main affected cellular processes is further supported and validated through the implementation of complementary metabolomics and metallomics experiments.

Contents

1. Introduction 2

2. A representative panel of gold compounds 5

3. The classical proteomic experiment 9

4. Systematic proteomic studies on panel gold compounds 14

4.1. Auranofin and Auoxo6 14

4.2. AuL12 and Au_2phen 16

4.3 Aubipyc 18

4.4. Au(NHC)Cl and $[Au(NHC)_2]PF_6$ 22

5. Overall interpretation of the proteomic results 26

6. Conclusions and perspectives 29

Acknowledgements 30

References 30

1. Introduction

Gold compounds are promising experimental agents for cancer treatment. Indeed, during the last two decades, several gold compounds were shown to manifest outstanding antiproliferative properties *in vitro* against selected human tumor cell lines, and some of them performed remarkably well even in a few *in vivo* tumor models [1–7].

The investigation of the cytotoxicity scores of gold complexes initially focused on Auranofin, and its analogues, which present linear mixed-ligand gold phosphane/thiolate structures [4]. More recently, a variety of other gold complexes showing a remarkable structural diversity, with gold in the oxidation states either +3 or +1, were prepared and characterized. Most of them were tested as potential antitumor agents *in vitro* with rather encouraging results [5,8–11]. The biological data gathered so far testify to the importance of gold-based compounds as a new class of prospective anticancer agents, that warrant further investigation [12]. In this context, it is worth mentioning that the established antiarthritic gold drug auranofin has recently entered three distinct clinical trials for cancer treatment according to emerging drug repurposing strategies (ClinicalTrials.gov Identifier: NCT01419691, NCT01747798, NCT01737502) [13–15]; in this perspective, several studies have been carried out in the last twenty years [16,17], also taking advantage of the clinical and the pharmacological studies previously described for Auranofin as an antiarthritic drug [18,19].

For a long time, scientists working in the field of metal-based drugs have relied on the so-called "DNA paradigm", inspired by cisplatin and its analogues, according to which DNA is the primary and nearly exclusive target of anticancer metal-based drugs [20–25].

Accordingly, the newly synthesized and developed anticancer metal complexes, in most cases, were designed in such a way to reproduce the main chemical features of cisplatin in terms of structure and reactivity, capable of conferring them -at least in principle- a specific DNA tropism. However, more recently, the implementation of powerful and advanced bioanalytical techniques has revealed that metal-based drugs, including cisplatin, in "real" biological systems, interact with a plethora of biomolecular targets, in particular proteins, thus affecting several distinct biological processes beyond DNA and its functioning. This implies that for many metallodrugs DNA may not be the primary or exclusive target as earlier postulated [26–29].

In particular, advanced metallomics approaches disclosed the fate of metal compounds in biological systems revealing that the metal is largely associated to the protein fraction. This is typically found for most gold-based anticancer agents for which DNA seems not to be a relevant target [30].

On the ground of the studies carried out so far, the tested medicinal gold complexes for cancer treatment, in relation to their behavior in biological systems, may be roughly classified as follows:

i) Gold(I) and (III) compounds that are *prodrugs* undergoing activation in *vivo* by metabolic processes and conversion into their active form through a ligand exchange or a redox reaction. Then, the activated gold species is able to coordinate tightly thiols, imidazoles, and selenols that are present in the side chains of many proteins thus impairing the functions of those proteins [12,29,31].

ii) Gold compounds that are big delocalized lipophilic cations capable of crossing membranes in their intact form and binding strongly but non-covalently to biomolecules (proteins, enzymes, DNA) [32,33] or other cellular structures (e.g. mitochondria) thus producing their biological effects.

iii) Gold(III) compounds endowed with a conspicuous oxidizing character that are able to react with biomolecules through direct redox chemistry inferring them severe oxidative damage and producing oxidative stress [34,35].

Some mechanistic insight on cytotoxic gold compounds has now been gained. Evidence is emerging that a few proteins, in particular, those presenting accessible selenocysteine or cysteine residues are preferred biomolecular targets for gold compounds. Among them, thioredoxin reductase is the generally accepted target for gold(I) compounds [11,36–38], but several other proteins including a few cysteine proteases [39] and a variety of transcription factors with zinc finger motifs are other plausible targets [40]. In addition, other selenoproteins beyond thioredoxin reductase seem to be favorite targets for gold compounds in relation to the strength of the gold(I) selenolate bond. In any case, the molecular mechanisms of currently studied anticancer gold drugs that unfold intracellularly remain largely unexplored and warrant further investigation.

Traditionally, the mechanism of action of a cytotoxic metallodrug having proteins as targets may be elucidated through the identification of the individual proteins with which the metallodrug interacts and the assessment of the precise biological consequences of each metallodrug-protein interaction. Indeed, metal binding often results in a severe impairment of protein's function which is the first step of the observed cascade of biological events ultimately leading to cancer cell death; thus, the occurrence and identification of a definite protein's loss of function with a large impact on cell homeostasis will allow to identify the pharmacologically relevant biomolecular targets [12,41,42].

On the other hand, given the inherent complexity of biological systems, an integrated approach is usually required to characterize satisfactorily the overall biological response of a cell to a metallodrug, to provide a whole description of the occurring drug-induced processes and establish a hierarchy of the relevant events according to a Systems Biology perspective [43,44].

Within this frame, proteomics techniques represent an excellent investigative tool to gain a detailed picture of the cellular processes that are affected by a certain metallodrug and of the underlying molecular mechanisms. Indeed, in recent years, proteomic strategies relying on mass spectrometry have emerged as a powerful and systematic approach for large-scale proteome-wide identification of drug-protein interactions and for the elucidation of the associated mechanisms [45,46].

Proteomics is particularly useful in monitoring signaling and metabolic events in a cell under standard conditions and their perturbations induced by any xenobiotic; this has led to the opening of several new horizons in the fields of drug discovery and drug delivery. Accordingly, the complete elucidation of the protein configuration of a human biological system enables us to identify a protein that is implicated in a specific disorder, whilst a comparison between differentially expressed proteins in treated or not treated samples indicates which proteins are responsive toward a chosen metallodrug. Comparative proteomics or protein-protein interactions studies assisted by bioinformatic analysis may turn crucial to decipher the true mechanism of a certain metal-based drug [47,48].

Based on these arguments, proteomics has become an essential tool to investigate cancer biology and anticancer drug mechanisms; in addition, proteomic studies may greatly help drug design. Proteins represent indeed a type of therapeutic target that is more functionally relevant than DNA or RNA, given their intrinsically greater druggability [49]. In the case of metal-based drugs, proteomic studies may be complemented effectively by metallomics. Notably, some elegant papers by Hongzhe Sun and his group stressed the importance of metallomics studies and described suitable methodologies to identify target proteins for bismuth metallodrugs; those studies were successful in pinpointing a few proteins that are likely targets for this class of metal drugs. Some general information on the associated investigative strategies may be found in recent review articles [48,50].

Conversely, metabolomics studies grounded on NMR or MS measurements may reveal the alterations in the metabolites' patterns induced by cell treatment with a few selected metallodrugs. These alterations may be correlated to the observed proteomic changes [51].

The present review article is specifically devoted to illustrating the proteomic studies conducted in our laboratories on the effects of panel gold compounds on A2780 cancer cells. The results are discussed in the frame of the existing knowledge on this topic.

2. A representative panel of gold compounds

Both gold(I) and gold(III) compounds manifest promising anticancer properties [52]. After the discovery of auranofin, different series of gold(I) complexes were evaluated for *in vitro* cytotoxicity and *in vivo* antitumor activity [53]; on the other hand, gold(III) complexes that are isostructural and isoelectronic to platinum(II) complexes, were presumed to manifest biological and pharmacological properties similar to those of cisplatin. So, for gold(III) complexes, it was postulated that DNA should be the main target but this hypothesis was never validated. Indeed, apart from a few Au(III) complexes for which there is clear evidence of direct DNA damage leading to apoptosis [54], the interactions of gold(III) complexes with DNA turned out, in most cases, to be weaker than those of platinum analogues [55]: it is difficult to believe that DNA is the main biomolecular target.

Thus, several studies were carried out to unveil the true targets of cytotoxic gold compounds and their specific effects at the molecular level [3]. It is now well documented that gold(I) and gold(III) compounds interact strongly and preferentially with model and target proteins, suggesting that the mechanism of action of these complexes is likely to be profoundly different from that of platinum compounds, protein-mediated, and virtually DNA-independent [55].

Among the dozens of medicinal gold compounds prepared and tested so far, only a few gold compounds were selected by us for the intended proteomics and mechanistic studies to disclose how these metal complexes exert their action at the cellular level. The selection of the gold compounds to build up the investigational panel was mainly dictated by the following criteria:

1. An acceptable stability and solubility in aqueous media.
2. The previous observation of appreciable antiproliferative properties *in vitro* against human cancer cells.
3. A remarkable structural and chemical diversity.
4. The presence of gold compounds that are representative of the two main oxidation states of the metal, *i.e.* +3 and +1.
5. A reasonably limited total number of metal compounds to form the panel (less than 10).

The seven gold compounds that were chosen to build up our panel are synoptically represented in Chart 1. The panel includes: Auranofin, Aubipyc, AuL12, Auoxo6, Au_2phen, Au(NHC)Cl and $[Au(NHC)_2]PF_6$.

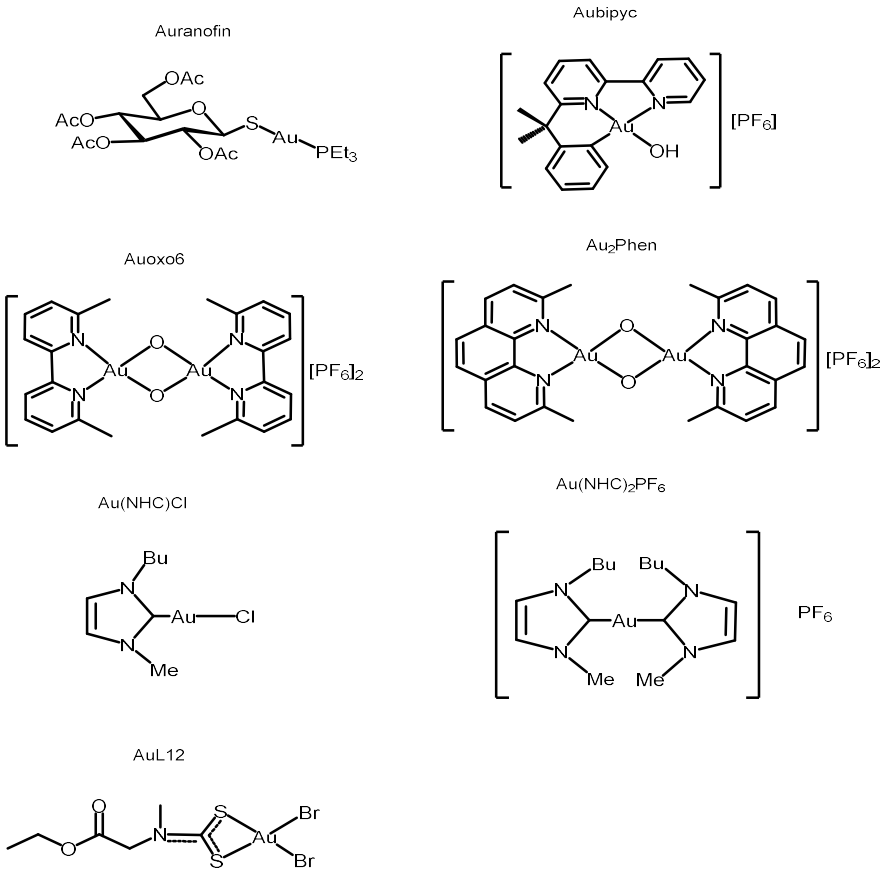

Chart 1. *The panel of the selected gold complexes showing high cytotoxicity against A2780 cells.*

Some additional information about the individual gold compounds is given below.

Among the several gold(III) compounds that were prepared and characterized so far we decided to include in the panel the following four ones, *e.g.*, Aubipyc, AuL12, Auoxo6, and Au_2phen. All these compounds seem particularly promising for medicinal application as they already manifested remarkable cytotoxicity against several tumor cell lines [56]. More in detail, Aubipyc is an organogold(III) compound bearing the 6-(1,1-dimethylbenzyl)-2,2'-bipyridine ligand; the three donor atoms from the terdentate bipyridine ligand and the oxygen atom of the hydroxo ligand coordinate the gold(III) center according to a slightly distorted square planar geometry. Notably, this gold(III) complex exhibits a quite large redox stability in solution due to the presence of a direct gold-carbon bond [57].

Auoxo6 and Au_2phen are two structurally related binuclear gold(III) compounds; both of them contain the so-called "Au_2O_2 diamond core" that crystallographic studies revealed to be an approximately planar system. So, each gold(III) center is tetracoordinated with a N_2O_2 chromophore, with two bridging oxygen atoms and two nitrogen atoms belonging to a chelating bipyridyl ligand in the case of Auoxo6 and to phenanthroline in the case of Au_2phen [58,59].

AuL12 is a gold(III) dithiocarbamate complex where the metal center is bound to two bromide ligands and to the bidentate NCSS group of ethylsarcosine-dithiocarbamate (ESDT) resulting in a slightly distorted square-planar geometry [60].

The panel also includes two distinct gold(I) compounds bearing one or two identical carbene ligands, namely Au(NHC)Cl and $[Au(NHC)_2]PF_6$, with the 1-butyl-3-methyl-imidazole-2-ylidene moiety acting as the NHC ligand coordinating the gold center through a direct gold-carbon bond. The different nature of the second ligand, *i.e.* a chloride in Au(NHC)Cl or a second NHC ligand in $[Au(NHC)_2]PF_6$, makes the chemical and biological behavior of these two gold carbenes very distinct. Indeed, the monocarbene complex is neutral and less stable, with the chloride ligand acting as the leaving group, whilst the bis-carbene complex $[Au(NHC)_2PF_6]$ manifests a greater stability in solution, as previously demonstrated by us [61] and typically behaves as a delocalized lipophilic cation (DLC).

Auranofin *i.e.* (1-thio-β-D-glucopyranose-2,3,4,6-tetraacetato-S) (triethyl-phosphine) gold(I), was included in the panel as a reference compound. Auranofin (AF), in clinical use since 1985 for the treatment of rheumatoid arthritis, is a linear, two-coordinate gold(I) complex with triethylphosphane and tetracetylthioglucose as gold(I) ligands [16,62,63]. In the last years, Auranofin has been reported to exhibit favorable *in vivo* anticancer properties and was included in a few clinical trials for cancer treatment as stated above.

The cytotoxic activity of several gold(III) and gold(I) complexes of potential medicinal application toward a large variety of cancer cell lines (CCRF-CEM, HCT-8, HEC1-A, etc.) has been tested for a large number of gold(III) and gold(I) complexes [5,56]. For our proteomics studies, we chose a reference cancer cell line: the A2780 human ovarian cancer cell line. During the past years, all the panel gold compounds were evaluated for their antiproliferative properties *in vitro* against this cell line taken as a standard; all of them turned out to produce potent cytotoxic effects with IC_{50} values typically falling in the low micromolar range. In the case of $[Au(NHC)_2PF_6]$, the most potent cytotoxin, an IC_{50} around 100 nM was measured (see Table 1). Such remarkable antiproliferative effects, in some instances greater than those of cisplatin, make these gold compounds excellent candidates for further biological evaluation.

Table 1. *The cytotoxic activity of the studied gold complexes towards A2780 human ovarian carcinoma cell lines over 72 h drug treatment. The reported IC_{50} values are the classical ones used in the proteomic experiments discussed in the review.*

Gold compound	**A2780** IC_{50} **(μM) ± SD**	**Ref.**
Au(NHC)Cl	1.98 ± 0.17	[64]
$Au(NHC)_2PF_6$	0.10 ± 0.02	[64]
Aubipyc	3.30 ± 1.40	[57]
Auoxo6	1.79 ± 0.17	[58]
Au_2Phen	0.80 ± 0.10	[65]
AuL12	4.00 ± 0.10	[65]
Auranofin	0.50 ± 0.39	[66]
CDDP	2.10 ± 0.20	[58]

3. The classical proteomic experiment

In the Systems Biology era, the various Omics sciences, such as genomics, transcriptomics, proteomics, and metabolomics are aimed to identify and characterize in its entirety, respectively, the genome, the transcriptome, the proteome and the metabolome of a cell, tissue or organism and its changes. Before describing in detail the results obtained for each selected gold compound, it is worth reminding shortly the main features of a classical proteomic experiment and the kind of information that may be extracted.

The term "proteome" was first used by Marc Wilkins in 1994 [67] and refers to the total set of proteins contained in a biological organism. O'Farrell [68] and Klose [69], first described, independently, the total set of proteins of an organism by electrophoretic separation in a two-dimensional (2-D) gel. This method succeeded in resolving a complex mixture of more than 1100 different proteins of *Escherichia coli* into distinct bands of individual components on the gel. Later on, the application of mass spectrometry in conjunction with genomics allowed to carry out proteomic studies on a large scale, achieving, in this manner, better separation and more accurate identification of the proteins [70–72].

Proteomics analysis, ultimately identifying the proteins that are present in the sample and their respective amounts, provides a detailed picture of a biological system, usually a cell, at a certain time. It is possible, at least in principle, to characterize all the present proteins (but more often only the abundant ones) with their spatial distribution and their temporal dynamics and measure the response of the cell to a variety of environmental stimuli. A crucial step is the identification of the differences in protein expression (i.e. up-regulated or down-regulated proteins) between the diseased cells and the healthy cells (control), or between treated and control cells [73].

Classical proteomic techniques include gel-based separation methods, in most cases, two-dimensional gel electrophoresis (2-DE), followed by mass spectrometry (MS) analysis (figure 1). Through the 2-DE experiment, the proteins are separated by letting them run in sequence in two orthogonal directions, first according to their isoelectric point and then according to charge-to-radius ratio; the protein spots of interest are eventually cut from the 2-DE gel and digested with a suitable enzyme, *e.g.* trypsin. Afterwards, the digested gel spots are analyzed through mass spectrometry to identify the associated proteins. The most commonly used MS techniques for protein spot identification are matrix-assisted laser desorption ionization (MALDI) and electrospray ionization (ESI), in combination with time-of-flight (TOF) mass spectrometry and tandem mass spectrometry (MS/MS) detection. Briefly, ESI generates multiply charged ions while MALDI produces separated charged pseudo-molecular ions of analytes. Both ion sources are coupled with separation techniques; ESI-MS, usually, with LC to increase the sensitivity and MALDI-TOF-MS with two-dimensional (2D) gel electrophoresis [74].

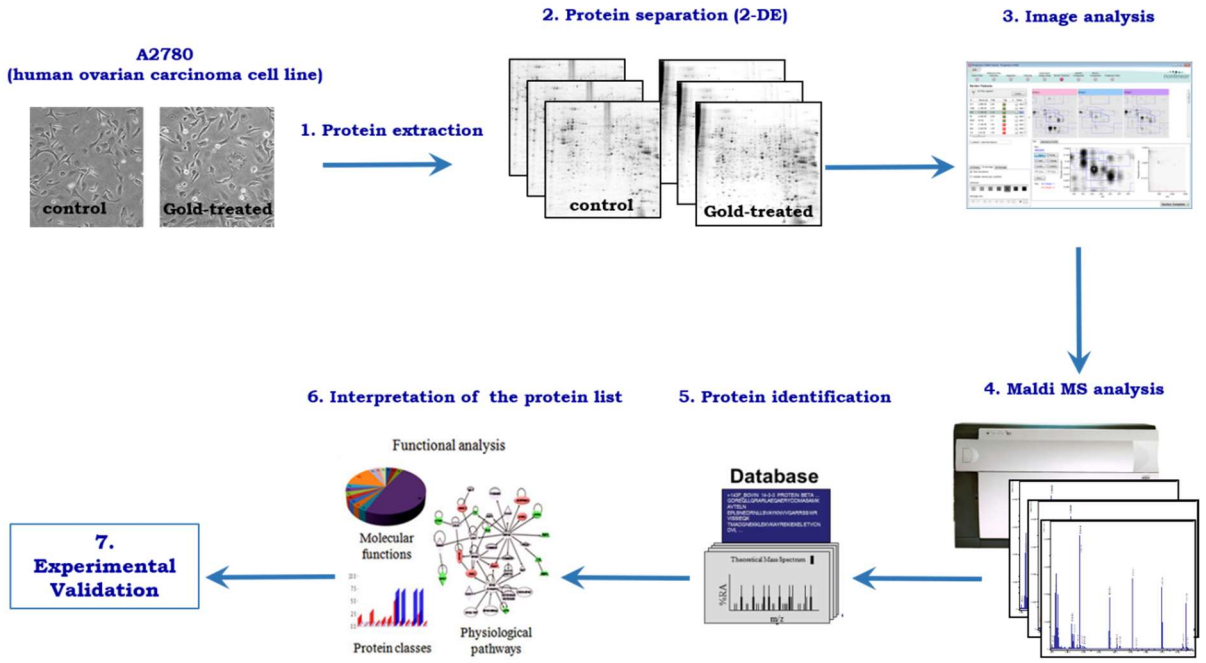

Figure 1. *The classical gel-based proteomic workflow.*

A greater reproducibility and a better protein resolution may be obtained thanks to the introduction of a relatively new technique, *i.e.* 2D-DIGE (two-dimensional difference in gel electrophoresis) [74,75]; the latter, though being more laborious than the classical approach, reduces significantly gel to gel variations and allows to examine and resolve several protein samples in a single gel. This method generates fluorescence images; indeed, proteins of different samples (*e.g.* control and treated cells) are pre-labeled with different fluorescent cyanine dyes (Cy2, Cy3, and Cy5) and identical lysate concentrations of both samples are mixed and co-separated in one 2DE gel. A comparison of generated 2D-DIGE images allows identifying the differentially abundant spots.

Two different methods can be applied to mass proteomics: the "bottom-up" and the "top-down" approach. With the top-down approach it is possible to analyze the intact proteins without enzyme digestion while bottom-up methods require enzymatic digestion of the analytes (so a more laborious sample preparation) before the MS acquisition. This latter technique is more time consuming but allows to investigate bigger proteins and protein mixtures. The advantage relies on the analysis of short peptides that are easily fractionated by both strong cation-exchange and reversed-phase chromatography, and well ionized by electrospray ionization (ESI). For these reasons, the "bottom-up" approach is thus largely used for protein analysis [74,76]. The adopted experimental procedure typically includes a comparison between the expressed proteins in cancer cells treated with a specific metallodrug and those normally expressed in untreated cancer cells. This type of analysis, in principle, allows identification of those differentially expressed proteins belonging to the main affected biological processes that are strictly associated with the cells stress response to the drug, and to the primary events of cell damage. If the cells are unable to repair themselves because the drug inferred

damage is too severe, the programmed cell death process starts, and the proteins associated to the apoptotic cascade are soon detected [43].

Proteomic studies, with the identification of the differentially expressed proteins after metallodrug administration in reference cancer cell lines, can be a crucial point in the drug discovery pipeline (figure 2), since this method may lead to the identification and validation of the targets associated to specific cancer disease and to the monitoring of their modulation. Moreover, proteomic profiles can offer significant information about drug-affected cellular signaling pathways on a global scale, and on protein-drug interactions.

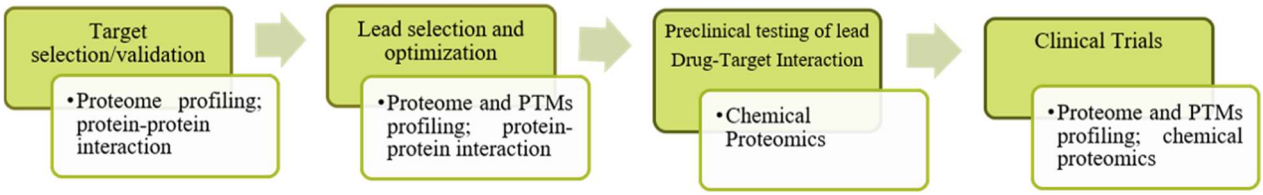

Figure 2. *Drug discovery pipeline. Proteomics contributes to the drug discovery process through protein interactions studies and proteome analysis.*

A few proteomic studies have been carried out so far to identify changes in protein expression associated with the mechanism of action of metal-based drugs [46]. Typically, the differentially expressed proteins are correlated with many other proteins and subcellular components and are involved in specific cellular pathways and networks, thus being well amenable for subsequent bioinformatic analysis (see below) [48,77,78]. In turn, bioinformatic analysis is able to pinpoint what are the main cellular processes that are affected by the various metallodrugs and offer a mechanistic explanation of their mode of action (see below).

It must be stressed that during the last few years gel-based proteomic studies are being progressively replaced by gel-free proteomics studies *e.g.* shotgun proteomics. The latter approach requires less sample and is much faster than gel proteomics [74,79]. The gel-free workflow requires the proteolysis of complex protein samples and the separation of the corresponding peptide mixtures through reverse-phase liquid chromatography, followed by ionization via nano ESI coupled to MS (LC-ESI-MS systems). The LC-ESI-MS system is a more powerful technology for the analysis of large numbers of proteins and allows the identification and quantification of low-abundance proteins such as transcription factors, protein kinases, and other regulatory proteins, and is therefore widely used today in disease research. A few shotgun proteomics studies on the mechanism of metal-based drugs have indeed appeared [80–83].

We must say that, despite the evident advantages of gel-free proteomics, the gel electrophoresis-based approach still has an important role in proteome research and nowadays several scientists suggest a new top-down functional proteomics based on an integrated gel-based and gel-free strategy. Indeed, the major advantages of gel-proteomics are the ability to separate proteoforms (all different molecular forms of a protein derived from post-translational modifications, genetic variations as well as alternatively spliced RNA transcripts) and to easily interface with many powerful biochemistry techniques (such as Western Blotting) used for the validation of proteomic data. An example of the informative results obtained through the 2DE technique is presented below (figure 3); the image shows the spots of the identified differentially expressed proteins after A2780 cells treatment with Au(NHC)Cl and $Au(NHC)_2PF_6$. In addition, using the 2DE technique instead of gel-free analysis, the possible incorrect mass spectrometry protein identifications due to the complexity of peptide digestion are avoided.[74,79].

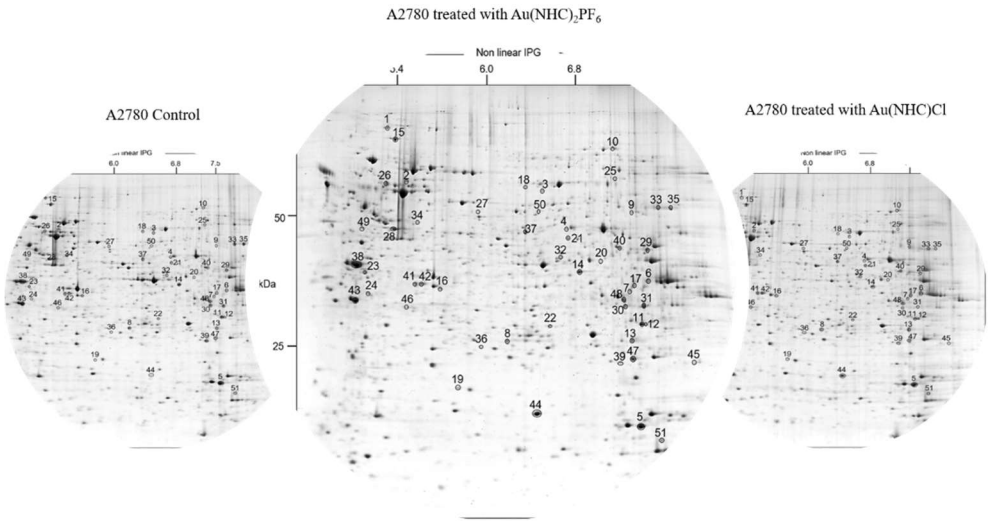

Figure 3 *An example of representative 2-DE gel images for control-A2780 cells, Au(NHC)Cl and* $Au(NHC)_2PF_6$*-treated A2780 cells for 24 h at a concentration equal to their 72-h-exposure* IC_{50} *values. The numbers indicate the 51 statistically differentially expressed protein spots.*

A major challenge in proteomics research today is the effort to develop powerful bioinformatic tools capable of analyzing the huge datasets that are generated from both gel-based and gel-free proteomics studies. Over the years, several software have been developed to carry out the so-called "functional enrichment analysis", a methodology, mostly based on the Gene Ontology (GO) classification system (http://www.geneontology.org) [84], that allows to identify genes/proteins statistically overrepresented in a dataset of interest [85–87]. The GO is a structured, controlled vocabulary for the classification of gene function at the molecular and cellular level. It includes three distinct GO terms: the biological process (e.g., signal transduction), the molecular function (e.g.,

ATPase activity) and the cellular component (e.g., ribosome), each of them hierarchically clustered and with a unique identifier [88]. The statistical tests commonly used to find significant differences in the frequency of GO terms associated with the dataset of MS identified proteins relative to their frequency in the genome, are Fisher's exact test and the hypergeometric test. The most widely used functional enrichment analysis web-accessible programs include DAVID (The Database for Annotation, Visualization and Integrated Discovery; https://david.ncifcrf.gov/) [89], WebGestalt (WEB-based Gene SeT AnaLysis Toolkit) [90], PANTHER (Protein ANalysis THrough Evolutionary Relationships) [91] and STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) [92]. These programs are not only limited to GO term enrichment analysis, but they also allow to perform pathway and protein-protein interaction networks functional enrichment analysis by using biological pathway annotations databases such as KEGG/PATHWAY [93] and Reactome [94].

Bioinformatic analysis is fundamental not only to collect and analyze proteomic datasets but also to provide new valuable information in terms of biochemical knowledge. Indeed, without a robust bioinformatic analysis, the following validation strategy is not standardized; most often, it is based on confirming changes in protein abundance detected by proteomics by using real-time quantitative polymerase chain reaction (RT-qPCR) and/or Western Blotting. At variance, the enrichment analysis allows to highlight biological processes and pathways that need to be deeper investigated by different approaches; only in this way is it possible to provide solid biological interpretations of the experimental data.

4. Systematic proteomic studies on panel gold compounds

In the course of the last ten years a number of proteomic studies have been carried out in our research group to characterize the cellular alterations produced in A2780 cancer cells by a variety of cytotoxic gold compounds, all included in the above investigational panel, according to a systematic approach. All these studies were conducted following a roughly standard experimental protocol: we will focus our attention only on studies concerning the A2780 (sensitive to cisplatin) cancer cell line, treated with the individual gold compounds for 24 h at a concentration equal to their 72-h-exposure IC_{50} values (see table 1). The results of these investigations have been published between 2010 and 2018 [64–66,95,96]. For a few gold compounds, additional studies have been carried out in the A2780 cancer cell line resistant to cisplatin (A2780R) [64,96]. Complete proteomic data have been gathered for all panel compounds.

It is worth reminding that in the case of Aubipyc and of the two gold carbene complexes the proteomic results were acquired taking advantage of slightly different methodology guidelines known as the "minimum information about a proteomics experiment" (MIAPE; http://www.psidev.info/miape). These methodology guidelines are those suggested by the Human Proteome Organization for the standardization of gel image processing and of data format.

The classification of the identified proteins was based on the Gene Ontology (GO) terms annotated in the UniProt database (http://www.uniprot.org/). Proteomic results obtained for each gold compound of the panel are described in detail below.

4.1. Auranofin and Auoxo6

Auranofin and Auoxo6 are two profoundly different gold compounds in terms of structure and reactivity. The former is a linear gold(I) complex with the metal center that binds a thiosugar ligand and a phosphane ligand; the latter is a binuclear gold(III) complex with an Au_2O_2 diamond core and a conspicuous oxidizing character [35]. Distinct reactivity patterns and biomolecular interaction profiles were reported for these two gold compounds. When both were tested in the A2780 cell line to monitor the induced changes in protein expression (in a study appeared in 2012 [66]), they showed, rather unexpectedly, a very similar pattern of proteomic alterations, also taking into account that the number of differentially expressed proteins was quite low, only 12 out of almost 1300 analyzed spots. The limited number of detected changes may be traced back, at least in part, to a less efficient statistical analysis strategy of 2-DE gel images than today. Indeed, in this period, in the scientific discussion, there were two concepts for considering differentially expressed genes/proteins. The mathematical concept of statistical significance based on the p-value and the biological concept based on a threshold value of expression change to be considered as meaningful, with 1.5/2-fold typically considered a worthwhile cut-off.

In detail, in this study the computer-aided 2D image analysis was carried out using the software ImageMaster 2D Platinum 6.0 (GE Healthcare) that allows calculating the relative spot volumes as %V (Vsingle spot/Vtotal spots, where V is the integration of the optical density over the spot area). The statistical analysis was performed using the A-NOVA test followed by Tukey's post hoc multiple comparisons. Proteins with A-NOVA pvalue<0.05 and with the additional arbitrary cut-off (1.5-fold change in abundance) were considered as statistically ''changed'' and selected for MS identification [66]. Probably, the selected arbitrary fold change cut-off may have drastically reduced the number of proteins included in our final analysis [97,98]. Very remarkably, almost half of the observed changes were in common between the two gold compounds, pointing to a similar mode of action. Specifically, the five altered proteins in common between Auranofin and Auoxo6 were: i) Triosephosphate isomerase 1 (TPI1); ii) Heterogeneous nuclear ribonucleoprotein H (HNRNPH1), whose increased cleavage induces the caspase 3 activation leading to apoptosis; iii) Histidine triad nucleotide-binding protein 1 (HINT1); iv) Peroxiredoxin-1 (PRDX1), that controls the cell redox balance and, finally, v) Ezrin (EZR), a protein related to cytoskeleton and apoptosis.

In this case, due to the very low number of affected proteins, Gene Ontology (GO) was only used to classify the identified proteins (Table 2 and 3).

Remarkably, this pioneering proteomic study pointed out that Auoxo6 and Auranofin exhibit a similar mode of action in the biological milieu regardless of the oxidation state of Au; indeed, for both of them, the cell death process is connected with caspase 3 activation and apoptosis. Moreover, it is noteworthy that some of the altered proteins are directly involved in redox homeostasis.

Table 2. *Auranofin's differential expressed proteins have been classified according to Gene Ontology (GO) terms belonging to the biological process category.*

Protein description	AC[a]	Gene Name	Subcellular localization	Auranofin *vs* control*
Metabolism				
(Glucose Metabolism)				
Triosephosphate isomerase 1	P60174	TPI1	cytoplasm	-1.9
(Lipid Metabolism)				
3-hydroxyacyl-CoA dehydrogenase type-2	Q99714	HSD17B10	mitochondrion	-1.6
Protein synthesis				
Heterogeneous nuclear ribonucleoprotein H	P31943	HNRNPH1	nucleus	-1.7; 7#
Cell Cycle and Apoptosis				
Histidine triad nucleotide-binding protein 1	P49773	HINT1	cytoplasm/nucleus	-10
Cell redox homeostasis				
Peroxiredoxin-6	P30041	PRDX6	cytoplasm	-1.5
Peroxiredoxin-1	Q06830	PRDX1	cytoplasm	13
Cytoskeleton and Cell Structure				

Ezrin	P15311	EZR	cytoskeleton	6.7

[a] *Accession number in Swiss-Prot/UniprotKB (http://www.uniprot.org/).*
** Ratio between the mean normalized 2DE spot volumes of treated and untreated A2780 cells.*
 # Protein identified in more than one spot placed in different sites on 2DE-gels and associated with a different isoelectric point (pI) or to different molecular weight (Mr).

***Table 3**. Auoxo6's differential expressed proteins have been classified according to Gene Ontology (GO) terms belonging to the biological process category.*

Protein description	**AC[a]**	**Gene Name**	**Subcellular localization**	**Auoxo6 *vs* control***
Metabolism				
(Glucose Metabolism)				
Triosephosphate isomerase 1	P60174	TPI1	cytoplasm	-1.4
Protein synthesis				
Heterogeneous nuclear ribonucleoprotein H	P31943	HNRNPH1	nucleus	-1.3; 13.5#
Cell Cycle and Apoptosis				
Histidine triad nucleotide-binding protein 1	P49773	HINT1	cytoplasm/nucleus	-8.7
Cell redox homeostasis				
Peroxiredoxin-1	Q06830	PRDX1	cytoplasm	12
Cytoskeleton and Cell Structure				
Ezrin	P15311	EZR	cytoskeleton	6

[a] *Accession number in Swiss-Prot/UniprotKB (http://www.uniprot.org/).*
** Ratio between the mean normalized 2DE spot volumes of treated and untreated A2780 cells.*
 # Protein identified in more than one spot placed in different sites on 2DE-gels and associated with a different isoelectric point (pI) or to different molecular weight (Mr).

4.2. AuL12 and Au$_2$phen

At a later time, the proteomic effects of AuL12 and Au$_2$phen, in A2780 cells, were explored using 2-DIGE experiments followed by MALDI-TOF MS and ESI-Ion trap MS/MS analysis [65]. However, the analysis process of gel images was basically the same (*i.e.* ImageMaster 2D Platinum software and the statistics based on A-NOVA p value<0.05 along with the additional arbitrary cut-off 1.5-fold change in abundance). Both these compounds possess reactive gold(III) centers; however, while Au$_2$phen is a binuclear gold(III) complex structurally similar to Auoxo6, AuL12 is a mononuclear gold(III) complex with a dithiocarbamato ligand.

The obtained proteomics results were similar to those previously obtained for Auoxo6 and Auranofin, at least formally, as only a limited number of proteomic alterations were again detected. Only one protein, *i.e.* HITN1, involved in the apoptotic cell death mediated by the nuclear transcription p53 factor, was in common to all four gold compounds.

For AuL12 and Au$_2$phen seven perturbed protein spots in common were identified.

In particular, two interesting proteins were identified as differentially expressed after the administration of both compounds, *i.e.* Ubiquilin-1 and NAP1L1 (nucleosome assembly protein 1-like 1). Ubiquilin-1 is a protein linked to the ubiquitin-proteasome system (UPS - which is involved in misfolded proteins' degradation) and overexpressed in both treatments [99], NAP1L1, a prognostic biomarker for primary tumors, is down-regulated in AuL12-treated cells in comparison with control and with Au_2phen-treated cells. These changes were validated by Western blot analysis using specific antibodies against Ubiquilin-1 and NAP1L1.

The altered proteins were classified according to GO biological function category (Table 4 and 5); notably, both compounds mainly affected proteins belonging to protein synthesis, mRNA splicing, and proteasome-mediated protein degradation GO category.

Table 4. *AuL12's differential expressed proteins have been classified according to Gene Ontology (GO) terms belonging to the biological process category.*

Protein description	AC[a]	Gene Name	Subcellular localization	AuL12 *vs* control*
Stress Response and Chaperones				
Peptidyl-prolyl cis-trans isomerase A	P62937	PPIB	cytoplasm	1.49
(Amino-acid and Protein Metabolism)				
D-3-phosphoglycerate dehydrogenase	O43175	PHGDH	cytoplasm	
Cytosol aminopeptidase	P28838	LAP3	cytoplasm	-1.35
(Nucleotide Metabolism)				
Thymidylate kinase	P23919	DTYMK	cytoplasm	1.41
Protein synthesis				
Elongation factor 1-beta	P24534	EEF1B2	cytoplasm	1.45
Serine-threonine kinase receptor-associated protein	Q9Y3F4	STRAP	cytoplasm/nucleus	-1.35
Cell Cycle and Apoptosis				
Histidine triad nucleotide-binding protein 1	P49773	HINT1	cytoplasm/nucleus	1.33
Ubiquilin-1	Q9UMX0	UBQLN1	membrane/proteasome	1.37
Signal Transduction				
Macrophage migration inhibitory factor	P14174	MIF	cytoplasm, secreted	1.39
Cytoskeleton and Cell Structure				
Actin, cytoplasmic 1	P60709	ACTB	cytoskeleton	1.61
Tubulin beta chain	P07437	TUBB	cytoskeleton	-1.40
DNA replication and Repair				
Nucleosome assembly protein 1-like 1	P55209	NAP1L1	nucleus	-1.54
Proliferating cell nuclear antigen	P12004	PCNA	nucleus	-1.45

[a] *Accession number in Swiss-Prot/UniprotKB (http://www.uniprot.org/).*

** Ratio between the mean normalized 2DE spot volumes of treated and untreated A2780 cells.*

Table 5. *Au_2phen differential expressed proteins have been classified according to Gene Ontology (GO) terms belonging to the biological process category.*

Protein description	AC[a]	Gene Name	Subcellular localization	Au_2phen *vs* control*
Stress Response and Chaperones				
Peptidyl-prolyl cis-trans isomerase A	P62937	PPIB	cytoplasm	1.49
(Amino-acid and Protein Metabolism)				
Cytosol aminopeptidase	P28838	LAP3	cytoplasm	-1.35
Omega-amidase NIT2	Q9NQR4	NIT2	cytoplasm	1.42
(Nucleotide Metabolism)				
Thymidylate kinase	P23919	DTYMK	cytoplasm	1.41
Protein synthesis				
Heterogeneous nuclear ribonucleoprotein K	P61978	HNRNPKH	nucleus	1.63
Cell Cycle and Apoptosis				
Histidine triad nucleotide-binding protein 1	P49773	HINT1	cytoplasm/nucleus	1.33
Ubiquilin-1	Q9UMX0	UBQLN1	membrane/proteasome	1.37
Signal Transduction				
Macrophage migration inhibitory factor	P14174	MIF	cytoplasm, secreted	1.39
Cytoskeleton and Cell Structure				
Actin, cytoplasmic 1	P60709	ACTB	cytoskeleton	1.61
Unknown				
Reticulocalbin-2	Q14257	RCN2		1.68

[a] *Accession number in Swiss-Prot/UniprotKB (http://www.uniprot.org/).*

** Ratio between the mean normalized 2DE spot volumes of treated and untreated A2780 cells.*

4.3 Aubipyc

The proteomic analysis of Aubipyc treated A2780 cells was carried out by the classical 2-DE approach coupled to MALDI-TOF mass spectrometry [95]. However, unlike the previous compounds, the analysis of the two-dimensional gels was carried out taking advantage of a different statistical approach based on the HUPO (Human Proteome Organization) guidelines (MIAPE; http://www.psidev.info/miape). These guidelines rely on parameters such as a false discovery rate p-value ≤ 0.05 and a power ≥ 0.8 to select differentially expressed proteins. Conversely, the fold-change cut-off is not applied since it does not take variability into account or ensure reproducibility, and besides, decrease the number of differentially expressed proteins increasing the risk of false negatives. Moreover, it has become increasingly evident that the biological significance of an arbitrary fold-change is likely to depend on the gene/protein and on the experimental context [97]. Moreover, in this study the statistical analysis of 2-DE gel images was improved by using a different software, i.e. Progenesis SameSpots (NonLinear Dynamics).

This software allowed to perform a statistical analysis similar to that well established for DNA microarray experiments [100]. In detail, we carried out a univariate analysis on the 2D protein spots using ANOVA test along with multivariate analyses based on false discovery rate called q-value, PCA and power analysis by using Progenesis SameSpots software. The statistically different protein spots were selected using the following parameters: a q-value ≤ 0.05 and a power ≥ 0.8. Thus, we decided for the first time, to exclude the arbitrary fold-change criterion i.e. the popular "minimum 1.5 fold change" to increase the number of proteins included in the analysis and reduce the risk of creating false negatives.

This methodology allowed us to find out a greater number of protein changes; this made possible to apply bioinformatic analysis based on functional enrichment analysis approaches, by the web-accessible program DAVID (https://david.ncifcrf.gov/) version 6.7 using BIOCARTA and KEGG/PATWHAYS databases.

These innovations certainly contributed to pinpoint for Aubipyc a far greater number of affected proteins in A2780 cells compared to Auranofin, Auoxo6, AuL12 and Au_2phen. Indeed, 95 differentially expressed protein spots were disclosed following Aubipyc treatment; 87 of those were identified and, among them, 29 resulted up-regulated and 58 down-regulated.

Notably, all the identified proteins are involved in specific biological pathways that modulate a variety of cellular processes. The GO functional classification of up-regulated and down-regulated proteins is depicted in Tables 6 and 7.

Table 6. *– Aubipyc's differential expressed up-regulated proteins have been classified according to Gene Ontology (GO) terms belonging to the biological process category.*

Protein description	**AC[a]**	**Gene Name**	**Subcellular localization**	**Aubipyc *vs* control***
Stress Response and Chaperones				
Stress-70 protein, mitochondrial	P38646	HSPA9	mitochondrion	1.7; 1.9#
T-complex protein 1 subunit gamma	P49368	CCT3	cytoplasm	1.6
T-complex protein 1 subunit epsilon	P48643	CCT5	cytoplasm	1.3; 1.8#
T-complex protein 1 subunit alpha	P17987	TCP1	cytoplasm	1.8; -2.4#
T-complex protein 1 subunit zeta	P40227	CCT6A	cytoplasm	1.7
T-complex protein 1 subunit eta	Q99832	CCT7	cytoplasm	1.6
60 kDa heat shock protein, mitochondrial	P10809	HSPD1	cytoplasm	2
(Cellular respiration and ATP metabolism)				
NADH-ubiquinone oxidoreductase 75 kDa subunit, mitochondrial	P28331	NDUFS1	mitochondrion	1.7
Dihydrolipoyl dehydrogenase, mitochondrial	P09622	DLD	mitochondrion	1.5
(Nucleotide Metabolism)				

C-1-tetrahydrofolate synthase, cytoplasmic	P11586	MTHFD1	cytoplasm	2.3
GMP synthase [glutamine-hydrolyzing]	P49915	GMPS	cytoplasm	-1.6; 1.8#
Protein synthesis				
Elongation factor 2	P13639	EEF2	cytoplasm	2.9; -2; -2.3#
Elongation factor Tu, mitochondrial	P49411	TUFM	mitochondrion	1.6
Pre-mRNA-processing factor 19	Q9UMS4	PRPF19	nucleus	1.7
Heterogeneous nuclear ribonucleoprotein Q	O60506	SYNCRIP	nucleus	2.2
Heterogeneous nuclear ribonucleoprotein K	P61978	HNRNPKH	nucleus	1.8
Cell Cycle and Apoptosis				
Programmed cell death 6-interacting protein	Q8WUM4	PDCD6IP	cytoplasm	2.3
Cell redox homeostasis				
Glutathione synthetase	P48637	GSS	cytoplasm	1.4
Cytoskeleton and Cell Structure				
Vinculin	P18206	VCL	cytoskeleton	1.8; -3.9#
Actin-related protein 3	P61158	ACTR3	cytoskeleton	1.6
Ezrin	P15311	EZR	cytoskeleton	2
DNA replication and Repair				
RuvB-like 1	Q9Y265	RUVBL1	nucleus	1.7
Single-stranded DNA-binding protein, mitochondrial	Q04837	SSBP1	mitochondrion	1.2

[a] *Accession number in Swiss-Prot/UniprotKB (http://www.uniprot.org/).*

** Ratio between the mean normalized 2DE spot volumes of treated and untreated A2780 cells.*

 # Protein identified in more than one spot placed in different sites on 2DE-gels and associated with a different isoelectric point (pI) or to different molecular weight (Mr).

Table 7. *Aubipyc's differential expressed down-regulated proteins have been classified according to Gene Ontology (GO) terms belonging to the biological process category.*

Protein description	**AC[a]**	**Gene Name**	**Subcellular localization**	**Aubipyc *vs* control***
Stress Response and Chaperones				
Heat shock 70 kDa protein 4L	O95757	HSPA4L	cytoplasm	-1.6; -1.8; 2; 1.8#
Heat shock cognate 71 kDa protein	P11142	HSPA8	cytoplasm	-2.9; 2.2; -2#
T-complex protein 1 subunit alpha	P17987	TCP1	cytoplasm	1.8; -2.4#
T-complex protein 1 subunit beta	P78371	CCT2	cytoplasm	-2; -2.2#
LDLR chaperone MESD	Q14696	MESDC2	cytoplasm	-1.5
Stress-induced-phosphoprotein 1	P31948	STIP1	cytoplasm/nucleus	-1.9; -7.6#
Peptidyl-prolyl cis-trans isomerase A	P62937	PPIB	cytoplasm	-1.4
Metabolism				
(Glucose Metabolism)				
Pyruvate kinase isozymes M1/M2	P14618	PKM	cytoplasm	-2; -2.3; -2.4#
Glyceraldehyde-3-phosphate dehydrogenase	P04406	GAPDH	cytoplasm	-1.6; -1.7; -6.2#
Alpha-enolase	P06733	ENO1	cytoplasm	-2.3
Phosphoglycerate kinase 1	P00558	PGK1	cytoplasm	-1.5

(Galactose Metabolism)				
Galactokinase	P51570	GALK1	cytosol	-1.7
(Retinol Metabolism)				
Retinal dehydrogenase 1	P00352	ALDH1A1	cytosol	-1.5
(Cellular respiration and ATP metabolism)				
ATP synthase subunit alpha, mitochondrial	P25705	ATP5A1	mitochondrion	-3.6
(Lipid Metabolism)				
Isocitrate dehydrogenase	O75874	IDH1	cytosol/peroxis	-2.5
Enoyl-CoA hydratase, mitochondrial	P30084	ECHS1	mitochondrion	-1.8
(Ketone metabolism)				
Succinyl-CoA:3-ketoacid coenzyme A transferase 1, mitochondrial	P55809	OXCT1	mitochondrion	-5.9
(Amino-acid and Protein Metabolism)				
D-3-phosphoglycerate dehydrogenase	O43175	PHGDH	cytoplasm	-3.9
Cytosol aminopeptidase	P28838	LAP3	cytoplasm	-4.4
(Nucleotide Metabolism)				
Bifunctional purine biosynthesis protein PURH	P31939	ATIC	mitochondrion	-2.6
GMP synthase [glutamine-hydrolyzing]	P49915	GMPS	cytoplasm	-1.6; 1.8#
Protein synthesis				
Elongation factor 2	P13639	EEF2	cytoplasm	2.9; -2; -2.3#
Cleavage stimulation factor subunit 2	P33240	CSTF2	nucleus	-1.4
Far upstream element-binding protein 2	Q92945	KHSRP	nucleus	-2.8
Far upstream element-binding protein 3	Q96I24	FUBP3	nucleus	-1.8; -1.6#
Mitochondrial-processing peptidase subunit alpha	Q10713	PMPCA	mitochondrion	-1.4
Serine/arginine-rich splicing factor 1	Q07955	SRSF1	nucleus	-2.3
Heterogeneous nuclear ribonucleoprotein A/B	Q99729	HNRNPAB	nucleus	-2.1
Heterogeneous nuclear ribonucleoprotein H	P31943	HNRNPH1	nucleus	-1.9; 2.1#
Calreticulin	P27797	CALR	Endo. reticulum	-1.7
Eukaryotic translation initiation factor 4H	Q15056	EIF4H	cytosol	-1.9
Cell Cycle and Apoptosis				
Anamorsin	Q6FI81	CIAPIN1	cytoplasm/nucleus	-1.7
Proliferation-associated protein 2G4	Q9UQ80	PA2G4	cytoplasm/nucleus	-2.7
Prohibitin	P35232	PHB	mitochondrion	-2
Cell redox homeostasis				
Thioredoxin reductase 1, cytoplasmic	Q16881	TXNRD1	cytoplasm	-1.2
Peroxiredoxin-6	P30041	PRDX6	cytoplasm	-4.6; -1.7#
3-mercaptopyruvate sulfurtransferase	P25325	MPST	mitochondrion	-1.6
Protein disulfide-isomerase A3	P30101	PDIA3	Endoplasm.	-2.8; -1.6#
Signal Transduction				
Heme-binding protein 1	Q9NRV9	HEBP1	mitochondrion	-2.2

Ankyrin repeat domain-containing	Q8N9B4	ANKRD42	nucleus	-3.5
Cytoskeleton and Cell Structure				
Vinculin	P18206	VCL	cytoskeleton	1.8; -3.9#
Protein enabled homolog	Q8N8S7	ENAH	cytoskeleton	-2.7
Transport				
Mitochondrial inner membrane protein	Q16891	IMMT	mitochondrion	-1.4; -1.5#
Protein SCO2 homolog, mitochondrial	O43819	SCO2	mitochondrion	-1.7
Voltage-dependent anion-selective channel protein 2	P45880	VDAC2	mitochondrion	-1.4

[a] *Accession number in Swiss-Prot/UniprotKB (http://www.uniprot.org/).*
** Ratio between the mean normalized 2DE spot volumes of treated and untreated A2780 cells.*
 # Protein identified in more than one spot placed in different sites on 2DE-gels and associated with a different isoelectric point (pI) or to different molecular weight (Mr).

The bioinformatic analysis allowed us to propose an innovative mode of action for this gold compound, that is correlated to its action on the glycolysis pathway; indeed, several identified down-regulated proteins are glycolytic enzymes, *i.e.* glyceraldehyde-3-phosphate dehydrogenase (GAPDH), alpha-enolase (ENO1), phosphoglycerate kinase 1 (PGK1) and pyruvate kinase isozymes M1/M2 (PKM). The proteomics results were then validated by western blot analysis and metabolic investigations pointing out that the impairment of glycolytic enzymes produces a decrease of glucose metabolism that activates, in turn, the cellular response to stress stimuli.

4.4. Au(NHC)Cl and [Au(NHC)$_2$]PF$_6$

The study of the proteomic alterations induced by Au(NHC)Cl and [Au(NHC)$_2$]PF$_6$ in A2780 cells was carried out in line with the study performed for Aubipyc, with a similar statistic methodology [64]. Notably, [Au(NHC)$_2$]PF$_6$ shows superior cytotoxic properties with respect to Au(NHC)Cl and this results in a greater number of altered proteins in the case of [Au(NHC)$_2$]PF$_6$.; however, the proteomic alterations elicited by both gold carbenes roughly exhibit a similar trend. Concerning the bioinformatic analysis of GO terms, it was found that most of the identified proteins belong to a few GO biological process categories and functional classes (Table 8 and 9). Also, for these two complexes, an overrepresentation enrichment analysis (ORA) of pathways and GO terms could be performed. This bioinformatic analysis was carried out by the web tool WebGestalt (WEB-based GEne SeT AnaLysis Toolkit; http://www.webgestalt.org/) using two different databases: the KEGG database (www.kegg.jp), that highlights an enrichment of carbon metabolism and amino acid biosynthesis pathways, and the Panther database (www.pantherdb.org) that discloses the involvement of glycolysis pathways [64]. Indeed, several proteins belonging to these specific pathways were identified. For instance, six proteins of the hnRNP (heterogeneous nuclear ribonucleoproteins) family were found, a family that comprises multifunctional proteins participating in a variety of cellular

functions related to different steps of mRNA processing. The altered expression of the individual hnRNPs plays a key role in cancer progression connected to inhibition of apoptosis and promotion of cell invasion. Another important protein affected by the treatment is the phosphoprotein nucleophosmin (NPM1 – chaperone family) often over-expressed in cancer cells [101] and involved in several pathways such as mRNA transport, apoptosis, and regulation of tumor suppressors p53/TP53 and AR.

In addition, both gold carbenes downregulated some enzymes implicated in cellular respiration and ATP production *e.g*. ACO2, AK2, ATP5C1. Treatment with $[Au(NHC)_2]PF_6$ also led to the decrease of other enzymes such as CYP4A22 and VCP involved in those pathways. Moreover, $[Au(NHC)_2]PF_6$ treatment, similarly to Aubipyc treatment, affected the glycolytic pathway; specifically, the enzymes GAPDH, ALDOA and TPI1 were altered.

Owing to the greater number of altered proteins and subsequent bioinformatic analysis, these results allowed us to propose a detailed mechanism of action for the bis-carbene gold complex, as previously done for Aubipyc complex. The potent cytotoxic properties of the $[Au(NHC)_2]PF_6$ depend, most likely, on the inhibition of an important redox enzyme, namely thioredoxin reductase, that plays a crucial role in the mitochondrial functions; the drastic decrease of the activity of this enzyme causes, in turn, a mitochondrial metabolism impairment that leads to cell death through apoptosis. Interference with the glycolytic pathway may also play an important though accessory role.

To further validate this hypothesis, a variety of biochemical determinations were carried out: the study of glucose transport inside the cell, the measure of intracellular ATP, the measure of oxygen consumption, the determination of lactate production, the determination of the levels of citrate synthase expression; on the whole, the obtained results are consistent with the above mechanistic hypothesis.

Table 8. *Au(NHC)Cl's differential expressed proteins have been classified according to Gene Ontology (GO) terms belonging to the biological process category.*

Protein description	**AC[a]**	**Gene Name**	**Subcellular localization**	**Au(NHC)Cl *vs* control***
Stress Response and Chaperones				
Stress-70 protein, mitochondrial	P38646	HSPA9	mitochondrion	-1.6
Peptidyl-prolyl cis-trans isomerase A	P62937	PPIB	cytoplasm	-1.2
(Cellular respiration and ATP metabolism)				
ATP synthase subunit gamma, mitochondrial	P36542	ATP5C1	mitochondrion	-1.2
Aconitate hydratase, mitochondrial	Q99798	ACO2	mitochondrion	-1.4
Adenylate kinase 2, mitochondrial	P54819	AK2	mitochondrion	-1.3; -1.4#
(Nucleotide Metabolism)				

Deoxyuridine 5'-triphosphate nucleotidohydrolase, mitochondrial	P33316	DUT	mitochondrion	-1.7
Protein synthesis				
Elongation factor 1-delta	P29692	EEF1D	nucleus	-1.3
Heterogeneous nuclear ribonucleoprotein A1	P09651	HNRNPA1	nucleus/spliceosome	2.2
Heterogeneous nuclear ribonucleoprotein K	P61978	HNRNPKH	nucleus	-1.4
Heterogeneous nuclear ribonucleoprotein D0	Q14103	HNRNPD	cytoplasm/nucleus	1.6
Heterogeneous nuclear ribonucleoproteins A2/B1	P22626	HNRNPA2B1	nucleus	1.5
RNA-binding protein 4	Q9BWF3	RBM4	cytoplasm/nucleus	-1.3
Cell redox homeostasis				
Peroxiredoxin-1	Q06830	PRDX1	cytoplasm	-1.3
Cytoskeleton and Cell Structure				
Nucleophosmin	P06748	NPM1	cytoskeleton/nucle	-1.4
Tubulin beta chain	P07437	TUBB	cytoskeleton	-1.4
Cofilin-1	P23528	COF1	cytoskeleton/membrane	-3.1
Cytoskeleton-associated protein 2	Q8WWK9	CKAP2	cytoskeleton	1.5
Transport				
Voltage-dependent anion-selective channel protein 2	P45880	VDAC2	mitochondrion	1.4

[a] *Accession number in Swiss-Prot/UniprotKB (http://www.uniprot.org/).*

** Ratio between the mean normalized 2DE spot volumes of treated and untreated A2780 cells.*

 # Protein identified in different 2DE spots.

Table 9. *$Au(NHC)_2PF_6$'s differential expressed proteins have been classified according to Gene Ontology (GO) terms belonging to the biological process category.*

Protein description	**AC[a]**	**Gene Name**	**Subcellular localization**	**$Au(NHC)_2PF_6$ *vs* control***
Stress Response and Chaperones				
Heat shock 70 kDa protein 4L	O95757	HSPA4L	cytoplasm	1.4
Stress-70 protein, mitochondrial	P38646	HSPA9	mitochondrion	-1.4
T-complex protein 1 subunit zeta	P40227	CCT6A	cytoplasm	1.5
Stress-induced-phosphoprotein 1	P31948	STIP1	cytoplasm/nucleus	-1.5
Peptidyl-prolyl cis-trans isomerase A	P62937	PPIB	cytoplasm	-1.3
Metabolism				
(Glucose Metabolism)				
Glyceraldehyde-3-phosphate dehydrogenase	P04406	GAPDH	cytoplasm	1.5
Fructose-bisphosphate aldolase A	P04075	ALDOA	cytoplasm	1.6
UTP--glucose-1-phosphate uridylyltransferase	Q16851	UGP2	cytoplasm/ER	-1.5
Triosephosphate isomerase 1	P60174	TPI1	cytoplasm	1.5

(Cellular respiration and ATP metabolism)				
ATP synthase subunit gamma, mitochondrial	P36542	ATP5C1	mitochondrion	-1.4
Aconitate hydratase, mitochondrial	Q99798	ACO2	mitochondrion	-1.6
Adenylate kinase 2, mitochondrial	P54819	AK2	mitochondrion	-1.5; -1.6#
Cytochrome P450 4A22	Q5TCH4	CYP4A22	mitochondrion	-1.2
(Ketone metabolism)				
Succinyl-CoA:3-ketoacid coenzyme A transferase 1, mitochondrial	P55809	OXCT1	mitochondrion	1.6
(Amino-acid and Protein Metabolism)				
3-hydroxyisobutyryl-CoA hydrolase, mitochondrial	Q6NVY1	HIBCH	mitochondrion	-1.4
(Nucleotide Metabolism)				
Bifunctional ethylenetetrahydrofolate dehydrogenase/cyclohydrolase	P13995	MTHFD2	mitochondrion	-1.5
Deoxyuridine 5'-triphosphate nucleotidohydrolase, mitochondrial	P33316	DUT	mitochondrion	-2.1
Protein synthesis				
Elongation factor 2	P13639	EEF2	cytoplasm	1.8
Elongation factor Tu, mitochondrial	P49411	TUFM	mitochondrion	1.5
Elongation factor 1-delta	P29692	EEF1D	nucleus	-1.4
Heterogeneous nuclear ribonucleoprotein A1	P09651	HNRNPA1	nucleus/spliceosome	2.4
Heterogeneous nuclear ribonucleoprotein Q	O60506	SYNCRIP	nucleus	1.8
Heterogeneous nuclear ribonucleoprotein K	P61978	HNRNPKH	nucleus	-1.6; 1.4#
Heterogeneous nuclear ribonucleoprotein F	P52597	HNRNPF	nucleus/spliceosome	-1.6
Heterogeneous nuclear ribonucleoprotein D0	Q14103	HNRNPD	cytoplasm/nucleus	1.7
Heterogeneous nuclear ribonucleoproteins A2/B1	P22626	HNRNPA2B1	nucleus	1.8
RNA-binding protein 4	Q9BWF3	RBM4	cytoplasm/nucleus	-1.4
Zinc finger protein 18	P17022	ZNF18	nucleus	1.6
Zinc finger protein 486	Q96H40	ZNF486	nucleus	2.2
Plasminogen activator inhibitor 1 RNA-binding protein	Q8NC51	SERBP1	cytoplasm/nucleus	2.1
WD repeat-containing protein 61	Q9GZS3	WDR61	cytoplasm/nucleus	-1.4
N-myc (and STAT) interactor	Q13287	NMI	cytoplasm	-1.4
Cell Cycle and Apoptosis				
Proliferation-associated protein 2G4	Q9UQ80	PA2G4	cytoplasm/nucleus	-1.5
26S protease regulatory subunit 10B	P62333	PSMC6	proteasome	-1.2
Cell redox homeostasis				
Peroxiredoxin-1	Q06830	PRDX1	cytoplasm	-1.4
Cytoskeleton and Cell Structure				
Nucleophosmin	P06748	NPM1	cytoskeleton/nucleus	-2.5
Mitogen-activated protein kinase kinase kinase MLT	Q9NYL2	ZAK	cytoplasm/nucleus	1.4
Tubulin beta chain	P07437	TUBB	cytoskeleton	-1.6
F-actin-capping protein subunit alpha-1	P52907	CAPZA1	cytoskeleton	-1.2
Tropomyosin alpha-3 chain	P06753	TPM3	cytoskeleton	-1.3

Cofilin-1	P23528	COF1	cytoskeleton/membrane	-4.3
Cytoskeleton-associated protein 2	Q8WWK9	CKAP2	cytoskeleton	1.6
Vascular cell adhesion protein 1	P19320	VCAM1	membrane	1.7
Transport				
Voltage-dependent anion-selective channel protein 2	P45880	VDAC2	mitochondrion	1.4
Transitional endoplasmic reticulum ATPase	P55072	VCP	ER/cytoplasm	-1.4; 1.8#
Oxysterol-binding protein-related protein 1	Q9BXW6	OSBPL1A	endosome	2
DNA replication and Repair				
RuvB-like 1	Q9Y265	RUVBL1	nucleus	-1.4
Single-stranded DNA-binding protein, mitochondrial	Q04837	SSBP1	mitochondrion	1.5

[a] *Accession number in Swiss-Prot/UniprotKB (http://www.uniprot.org/).*

** Ratio between the mean normalized 2DE spot volumes of treated and untreated A2780 cells.*

 # Protein identified in different 2DE spots.

5. Overall interpretation of the proteomic results

Thanks to a series of systematic proteomic investigations carried out in our laboratories during the last ten years, a large amount of data was gathered concerning the proteomic alterations induced by a panel of selected gold-based drugs in A2780 human ovarian cancer cells. To the best of our knowledge, this is one of the largest experimental efforts in the field of proteomics of metal-based drugs and of the inherent mechanistic studies. Herein, we will try to recapitulate the main alterations that were observed in our experiments, perform a comparative analysis and identify a few general trends of mechanistic relevance.

The classification of the identified proteins, deriving from the analysis of the proteomic alterations induced by the seven gold compounds, was carried out through UniProtKB lists that selected terms correlated to the GO annotation. Remarkably, the identified proteins, that are affected by the treatment with the seven gold compounds, are involved in a variety of biological processes as shown in Figure 4.

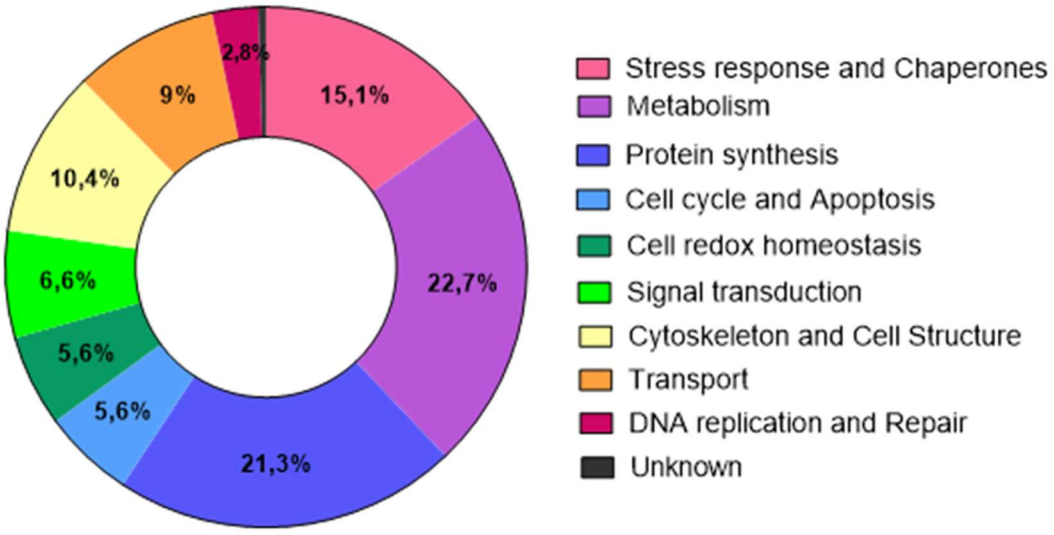

Figure 4. *The percentage of the involved proteins classified into functional categories based on the Gene Ontology (GO) terms related to their major biological functions using UniprotKB database (http://www.uniprot.org/).*

The main outcomes of the above proteomics studies and their tentative mechanistic interpretation for A2780 cancer cells are summarized below.

The mode of action of Auoxo6 appears to be strictly related to that of Auranofin: five of the altered proteins were the same in the two cases. These two gold compounds affected proteins involved in metabolism, in the cell redox homeostasis and stress response (peroxiredoxins 1 and 6), and in triggering caspase 3 activation and apoptosis (Ezrin, Heterogeneous nuclear ribonucleoprotein H). Notably, both metallodrugs caused relatively few and modest changes in protein expression in comparison with controls.

In the proteomic analysis of the changes induced by AuL12 and Au_2Phen, only a few altered proteins were identified, in line with the case of Auoxo6 and Auranofin. In any case, treatment with AuL12 and Au_2Phen led to significant quantitative alterations of proteins belonging to protein synthesis, mRNA splicing and proteasome pathway of protein degradation.

For Aubipyc, [Au(NHC)Cl] and $[Au(NHC)_2PF_6]$, thanks to the greater number of identified altered proteins (compared to the other gold compounds), a bioinformatic and functional analysis could be carried out and some likely mechanisms of action could be suggested that were later supported by independent biochemical assays.

In the Aubipyc treated cells the impairment of glycolysis has been indicated as one of the main effects leading to cell death; indeed, several glycolytic enzymes turned out to be largely downregulated.

Conversely, proteomic analysis on A2780 cells treated with the two related gold carbenes pointed out that $[Au(NHC)_2]PF_6$ is the most effective compound in inducing cell apoptosis *via* strong

inhibition of thioredoxin reductase [102,103] and associated mitochondrial dysfunction (lower oxygen consumption and lower membrane potential), besides a depletion of ATP production.

From the comparative analysis of the obtained results, it is evident that proteins belonging to selected functional classes are more prone to the alterations caused by gold compounds. It is also evident that various independent functional classes are affected simultaneously in line with the concept that gold complexes may use a variety of independent molecular mechanisms to induce cellular damage and possess a multifactorial mode of action. This fact was nicely highlighted in a pioneering work of ours where COMPARE analysis conducted at Oncotest revealed the occurrence of a variety of modes of action for a few gold compounds [56].

As far as the individual components of the cell machinery are concerned, redox metabolism and the proteasome emerge as the most affected cellular processes by treatment with four gold compounds (*i.e.* Auranofin, AuL12, Aubipyc and Auoxo6), according to the identification of a few proteins related to these pathways. In agreement with some recent studies [104,105], we can state that redox metabolism probably remains the main cellular process that is impaired by gold compounds with pharmacological and cytotoxic activity [82].

Another important observation arising from our studies is that at least two of the considered gold compounds (*i.e.* Aubipyc and $[Au(NHC)_2PF_6]$) can strongly affect glucose metabolism. This is a new type of molecular mechanism for cytotoxic gold compounds. This latter observation has now been strongly supported by independent cellular determinations carried out in cells treated with the bis-carbene gold complex [64].

Upon analyzing the results obtained by the treatment of A2780 cancer cells with the seven gold compounds, it is evident that the number of altered proteins is highly variable in the different cases. Though this may be traced back, at least in part, to the technological and methodological advancements recorded in the meantime (the first study was published in 2012, the last one in 2019), there are no doubts that some gold compounds are able to impact a greater number of metabolic and signaling pathways than the others, thus producing a greater number of proteomic changes. Also, it must be pointed out that our approach mainly monitors the abundant proteins (typically about 1,000-1,300 proteins are monitored as a maximum in 2DE experiments) in such a way that the metabolic pathways will be privileged over signaling pathways, the latter typically including a smaller number of protein copies.

In conclusion, we can state that we have carried out a very detailed and systematic proteomic investigation on the effects of seven structurally different gold compounds in A2780 human ovarian cancer cells. This analysis has been supported by a robust bioinformatics analysis performed on all the affected proteins.

From these studies, three main modes of action emerge for the investigated cytotoxic gold compounds:

1. Alteration of the redox state of the cell, increased oxidative stress-mediated by thioredoxin reductase inhibition, and ultimately induction of intrinsic apoptosis (Auranofin and Auoxo6).

2. Alterations of Protein synthesis, of mRNA splicing and of proteins involved in proteasome-mediated protein degradation (AuL12 and Au_2phen).

3. Impairment of glucose metabolism (glycolysis or mitochondrial respiration) (Aubipyc, [Au(NHC)Cl] and [$Au(NHC)_2PF_6$]).

A final point concerns the nature of the target proteins for gold compounds, *i.e.* the proteins to which the gold center will directly bind. Several targets for gold compounds were suggested in previous studies, *e.g.* inhibition of the mitochondrial function and inhibition of Thioredoxin Reductase, referred to the first and third proposed modes of action [12,82]; inhibition of proteasome catalytic activity referred to the second mode [106]. Up to date, only thioredoxin reductase inhibition by gold complexes is well documented and proved in the very recent literature; other potential targets and other different modes of action remain to be explored and validated. We are confident that new emerging metallomics and metalloproteomics methods may quickly offer some independent clues on these aspects. In addition, it will be possible to challenge individually selected target proteins with the panel gold compounds and monitor adduct formation by direct ESI MS analysis. An investigation of this kind was recently performed in our laboratory for Auranofin challenged with a variety of representative proteins [31].

6. Conclusions and Perspectives

Gold-based metallodrugs represent a very promising class of cytotoxic compounds, complementary to platinum drugs, that exert their pharmacological actions mainly by targeting a variety of cellular proteins rather than DNA or RNA. Yet, their molecular mechanisms are still debated and largely unexplored.

In this review article, we have shown how a small but representative panel of gold compounds could be investigated in depth through a classical proteomic approach, to better elucidate the respective mechanisms of action and identify the main cellular functions that are impaired upon treatment.

In parallel with the technological progress and the availability of more advanced, more sensitive and more performing instrumentation, we can state that proteomic analysis emerges as one of the most powerful tools to offer a comprehensive description of the cellular effects caused by metal-based drugs. The chance offered by proteomics to investigate the differential expression of proteins

has turned out of paramount importance in modern oncological research to understand the altered regulation of cancer cells compared to healthy ones, opening the way for a rational design of new and more effective metallodrugs. Thanks to the proteomics approaches here described and to related bioinformatic analysis, a significant body of data on A2780 cancer cells treated with seven distinct gold complexes was gathered; these results allowed us to gain important information on their respective modes of action. In particular, the proteomic evidences allowed us to disclose a few distinct modes of action (that might coexist) in relation to the chemical nature of the tested complexes (different ligands, different oxidation states etc.) and to identify at least some of the main protein targets.

Although proteomics may be considered nowadays as an optimal tool for the detection of a relatively large number of biological alterations induced in the cell by a certain metallodrug, the picture may be further expanded and completed through the implementation of additional strategies. Indeed, thanks to emerging metallomics approaches and technologies, new information can be gained on the metal's fate [43,107,108]. We are confident that Metallomics studies in the near future will be able to provide a satisfactory description of metal uptake and localization, of its biomolecular interactions and its biological targets. Conversely, Metabolomics studies revealing the alterations in the metabolite patterns and their time-dependent evolution may effectively enrich the description of the metabolic alterations at the cellular level induced by treatment offering independent and complementary information with respect to proteomic studies.

Acknowledgements

The authors thank AIRC and Fondazione Cassa Risparmio Firenze for funding the projects "Advanced mass spectrometry tools for cancer research: novel applications in proteomics, metabolomics and nanomedicine" (Multi-user Equipment Program 2016, grant number 19650) and AIRC (grant numbers IG 12085, IG-16049). Beneficentia Stiftung (Vaduz, Liechtenstein), CIRCMSB and University of Pisa (Rating Ateneo 2019-2020) are also acknowledged for financial support.

References

[1] B. Bertrand, M.R.M. Williams, M. Bochmann, Gold(III) Complexes for Antitumor Applications: An Overview, Chem. – A Eur. J. 24 (2018) 11840–11851. https://doi.org/10.1002/chem.201800981.

[2] D. Aldinucci, L. Ronconi, D. Fregona, Groundbreaking gold(III) anticancer agents, Drug Discov. Today. 14 (2009) 1075–1076. https://doi.org/https://doi.org/10.1016/j.drudis.2009.07.011.

[3] T. Zou, C.T. Lum, C.N. Lok, J.J. Zhang, C.M. Che, Chemical biology of anticancer gold(III) and gold(i) complexes, Chem. Soc. Rev. 44 (2015) 8786–8801. https://doi.org/10.1039/c5cs00132c.

[4] E.R.T. Tiekink, Phosphinegold(I) thiolates - Pharmacological use and potential, Bioinorg. Chem. Appl. 1 (2003) 53–67. https://doi.org/10.1155/S1565363303000050.

[5] S. Nobili, E. Mini, I. Landini, C. Gabbiani, A. Casini, L. Messori, Gold compounds as anticancer agents: Chemistry, cellular pharmacology, and preclinical studies, Med. Res. Rev. 30 (2010) 550–580. https://doi.org/10.1002/med.20168.

[6] A. Casini, L. Messori, Molecular Mechanisms and Proposed Targets for Selected Anticancer Gold

Compounds, Curr. Top. Med. Chem. 11 (2011) 2647–2660. https://doi.org/10.2174/156802611798040732.
[7] C. Gabbiani, L. Messori, Protein Targets for Anticancer Gold Compounds: Mechanistic Inferences, Anticancer. Agents Med. Chem. 11 (2012) 929–939. https://doi.org/10.2174/187152011797927607.
[8] L. Ronconi, D. Fregona, The Midas touch in cancer chemotherapy: from platinum- to gold-dithiocarbamato complexes, Dalt. Trans. (2009) 10670. https://doi.org/10.1039/b913597a.
[9] R.W.-Y. Sun, C.-M. Che, The anti-cancer properties of gold(III) compounds with dianionic porphyrin and tetradentate ligands, Coord. Chem. Rev. 253 (2009) 1682–1691. https://doi.org/https://doi.org/10.1016/j.ccr.2009.02.017.
[10] I. Ott, On the medicinal chemistry of gold complexes as anticancer drugs, Coord. Chem. Rev. 253 (2009) 1670–1681. https://doi.org/10.1016/j.ccr.2009.02.019.
[11] A. Bindoli, M.P. Rigobello, G. Scutari, C. Gabbiani, A. Casini, L. Messori, Thioredoxin reductase: A target for gold compounds acting as potential anticancer drugs, Coord. Chem. Rev. 253 (2009) 1692–1707. https://doi.org/https://doi.org/10.1016/j.ccr.2009.02.026.
[12] C. Yeo, K. Ooi, E. Tiekink, Gold-Based Medicine: A Paradigm Shift in Anti-Cancer Therapy?, Molecules. 23 (2018) 1410. https://doi.org/10.3390/molecules23061410.
[13] Auranofin and Sirolimus in Treating Participants With Ovarian Cancer - Full Text View - ClinicalTrials.gov, (n.d.). https://clinicaltrials.gov/ct2/show/NCT03456700 (accessed November 9, 2020).
[14] Sirolimus and Auranofin in Treating Patients With Advanced or Recurrent Non-Small Cell Lung Cancer or Small Cell Lung Cancer - Full Text View - ClinicalTrials.gov, (n.d.). https://clinicaltrials.gov/ct2/show/NCT01737502 (accessed November 9, 2020).
[15] Phase I and II Study of Auranofin in Chronic Lymphocytic Leukemia (CLL) - Full Text View - ClinicalTrials.gov, (n.d.). https://clinicaltrials.gov/ct2/show/NCT01419691 (accessed November 9, 2020).
[16] T. Onodera, I. Momose, M. Kawada, Potential Anticancer Activity of Auranofin, Chem. Pharm. Bull. 67 (2019) 186–191. https://doi.org/10.1248/cpb.c18-00767.
[17] C. Roder, M.J. Thomson, Auranofin: Repurposing an Old Drug for a Golden New Age, Drugs R D. 15 (2015) 13–20. https://doi.org/10.1007/s40268-015-0083-y.
[18] N.L. Gottlieb, Pharmacology of auranofin: overview and update., Scand. J. Rheumatol. Suppl. 63 (1986) 19–28.
[19] M. Chaffman, R.N. Brogden, R.C. Heel, T.M. Speight, G.S. Avery, Auranofin, Drugs. 27 (1984) 378–424. https://doi.org/10.2165/00003495-198427050-00002.
[20] J. Reedijk, Fast and slow versus strong and weak metal-DNA binding: Consequences for anti-cancer activity, in: Metallomics, Royal Society of Chemistry, 2012: pp. 628–632. https://doi.org/10.1039/c2mt20032e.
[21] J. Reedijk, New clues for platinum antitumor chemistry: Kinetically controlled metal binding to DNA, Proc. Natl. Acad. Sci. 100 (2003) 3611 LP – 3616. https://doi.org/10.1073/pnas.0737293100.
[22] S.M. Cohen, S.J. Lippard, Cisplatin: From DNA damage to cancer chemotherapy, in: Prog. Nucleic Acid Res. Mol. Biol., 2001: pp. 93–130. https://doi.org/10.1016/S0079-6603(01)67026-0.
[23] C.X. Zhang, S.J. Lippard, New metal complexes as potential therapeutics, Curr. Opin. Chem. Biol. 7 (2003) 481–489. https://doi.org/10.1016/S1367-5931(03)00081-4.
[24] K.R. Barnes, S.J. Lippard, Cisplatin and related anticancer drugs: recent advances and insights., Met. Ions Biol. Syst. 42 (2004) 143–77.
[25] D. Wang, S.J. Lippard, Cellular processing of platinum anticancer drugs, Nat. Rev. Drug Discov. 4 (2005) 307–320. https://doi.org/10.1038/nrd1691.
[26] A. Casini, J. Reedijk, Interactions of anticancer Pt compounds with proteins: An overlooked topic in medicinal inorganic chemistry?, Chem. Sci. 3 (2012) 3135–3144. https://doi.org/10.1039/c2sc20627g.
[27] P.C. Bruijnincx, P.J. Sadler, New trends for metal complexes with anticancer activity, Curr. Opin. Chem. Biol. 12 (2008) 197–206. https://doi.org/10.1016/j.cbpa.2007.11.013.
[28] L. Massai, C. Zoppi, D. Cirri, A. Pratesi, L. Messori, Reactions of medicinal gold(III) compounds with proteins and peptides explored by electrospray ionization mass spectrometry and complementary biophysical methods, Front. Chem. 8 (2020) 581648. https://doi.org/10.3389/fchem.2020.581648.
[29] I. Tolbatov, D. Cirri, L. Marchetti, A. Marrone, C. Coletti, N. Re, D. La Mendola, L. Messori, T. Marzo, C. Gabbiani, A. Pratesi, Mechanistic Insights Into the Anticancer Properties of the Auranofin Analog Au(PEt3)I: A Theoretical and Experimental Study, Front. Chem. 8 (2020) 812.

https://doi.org/10.3389/fchem.2020.00812.
[30] Z. Adhireksan, G. Palermo, T. Riedel, Z. Ma, R. Muhammad, U. Rothlisberger, P.J. Dyson, C.A. Davey, Allosteric cross-talk in chromatin can mediate drug-drug synergy, Nat. Commun. 8 (2017) 14860. https://doi.org/10.1038/ncomms14860.
[31] C. Zoppi, L. Messori, A. Pratesi, ESI MS studies highlight the selective interaction of Auranofin with protein free thiols, Dalt. Trans. 49 (2020) 5906–5913. https://doi.org/10.1039/d0dt00283f.
[32] J.L. Hickey, R.A. Ruhayel, P.J. Barnard, M. V. Baker, S.J. Berners-Price, A. Filipovska, Mitochondria-targeted chemotherapeutics: The rational design of gold(I) N-heterocyclic carbene complexes that are selectively toxic to cancer cells and target protein selenols in preference to thiols, J. Am. Chem. Soc. 130 (2008) 12570–12571. https://doi.org/10.1021/ja804027j.
[33] P.J. Barnard, M. V. Baker, S.J. Berners-Price, D.A. Day, Mitochondrial permeability transition induced by dinuclear gold(I)-carbene complexes: Potential new antimitochondrial antitumour agents, J. Inorg. Biochem. 98 (2004) 1642–1647. https://doi.org/10.1016/j.jinorgbio.2004.05.011.
[34] M. Altaf, M. Monim-ul-Mehboob, A.N. Kawde, G. Corona, R. Larcher, M. Ogasawara, N. Casagrande, M. Celegato, C. Borghese, Z.H. Siddik, D. Aldinucci, A.A. Isab, New bipyridine gold(III) dithiocarbamate-containing complexes exerted a potent anticancer activity against cisplatin-resistant cancer cells independent of p53 status, Oncotarget. 8 (2017) 490–505. https://doi.org/10.18632/oncotarget.13448.
[35] C. Gabbiani, L. Massai, F. Scaletti, E. Michelucci, L. Maiore, M.A. Cinellu, L. Messori, Protein metalation by metal-based drugs: Reactions of cytotoxic gold compounds with cytochrome c and lysozyme, J. Biol. Inorg. Chem. 17 (2012) 1293–1302. https://doi.org/10.1007/s00775-012-0952-6.
[36] J.H. Sze, P. V Raninga, K. Nakamura, M. Casey, K.K. Khanna, S.J. Berners-Price, G. Di Trapani, K.F. Tonissen, Anticancer activity of a Gold(I) phosphine thioredoxin reductase inhibitor in multiple myeloma, Redox Biol. 28 (2020) 101310. https://doi.org/https://doi.org/10.1016/j.redox.2019.101310.
[37] A. Pratesi, C. Gabbiani, M. Ginanneschi, L. Messori, Reactions of medicinally relevant gold compounds with the C-terminal motif of thioredoxin reductase elucidated by MS analysis, Chem. Commun. 46 (2010) 7001–7003. https://doi.org/10.1039/c0cc01465f.
[38] A. Pratesi, C. Gabbiani, E. Michelucci, M. Ginanneschi, A.M. Papini, R. Rubbiani, I. Ott, L. Messori, Insights on the mechanism of thioredoxin reductase inhibition by Gold N-heterocyclic carbene compounds using the synthetic linear Selenocysteine containing C-terminal peptide hTrxR(488-499): An ESI-MS investigation, J. Inorg. Biochem. 136 (2014) 161–169. https://doi.org/10.1016/j.jinorgbio.2014.01.009.
[39] S.P. Fricker, Cysteine proteases as targets for metal-based drugs, Metallomics. 2 (2010) 366–377. https://doi.org/10.1039/b924677k.
[40] A. Jacques, C. Lebrun, A. Casini, I. Kieffer, O. Proux, J.-M. Latour, O. Sénèque, Reactivity of Cys4 Zinc Finger Domains with Gold(III) Complexes: Insights into the Formation of "Gold Fingers," Inorg. Chem. 54 (2015) 4104–4113. https://doi.org/10.1021/acs.inorgchem.5b00360.
[41] E. Boros, P.J. Dyson, G. Gasser, Classification of Metal-Based Drugs according to Their Mechanisms of Action, Chem. 6 (2020) 41–60. https://doi.org/10.1016/j.chempr.2019.10.013.
[42] T. Gianferrara, I. Bratsos, E. Alessio, A categorization of metal anticancer compounds based on their mode of action, Dalt. Trans. (2009) 7588–7598. https://doi.org/10.1039/b905798f.
[43] C. Gabbiani, F. Magherini, A. Modesti, L. Messori, Proteomic and Metallomic Strategies for Understanding the Mode of Action of Anticancer Metallodrugs, Anticancer. Agents Med. Chem. 10 (2012) 324–337. https://doi.org/10.2174/187152010791162315.
[44] R. Haas, A. Zelezniak, J. Iacovacci, S. Kamrad, S.J. Townsend, M. Ralser, Designing and interpreting "multi-omic" experiments that may change our understanding of biology, Curr. Opin. Syst. Biol. 6 (2017) 37–45. https://doi.org/10.1016/j.coisb.2017.08.009.
[45] J. Reinders, ed., Proteomics in Systems Biology, Springer New York, New York, NY, 2016. https://doi.org/10.1007/978-1-4939-3341-9.
[46] S. Jia, R. Wang, K. Wu, H. Jiang, Z. Du, Elucidation of the Mechanism of Action for Metal Based Anticancer Drugs by Mass Spectrometry-Based Quantitative Proteomics, Molecules. 24 (2019) 581. https://doi.org/10.3390/molecules24030581.
[47] L. Severi, L. Losi, S. Fonda, L. Taddia, G. Gozzi, G. Marverti, F. Magni, C. Chinello, M. Stella, J. Sheouli, E.I. Braicu, F. Genovese, A. Lauriola, C. Marraccini, A. Gualandi, D. D'Arca, S. Ferrari, M.P. Costi, Proteomic and Bioinformatic Studies for the Characterization of Response to Pemetrexed in Platinum Drug Resistant Ovarian Cancer, Front. Pharmacol. 9 (2018) 454. https://doi.org/10.3389/fphar.2018.00454.

[48] Y. Wang, H. Li, H. Sun, Metalloproteomics for Unveiling the Mechanism of Action of Metallodrugs, Inorg. Chem. 58 (2019) 13673–13685. https://doi.org/10.1021/acs.inorgchem.9b01199.
[49] U. Rix, G. Superti-Furga, Target profiling of small molecules by chemical proteomics, Nat. Chem. Biol. 5 (2009) 616–624. https://doi.org/10.1038/nchembio.216.
[50] H. Wang, Y. Zhou, X. Xu, H. Li, H. Sun, Metalloproteomics in conjunction with other omics for uncovering the mechanism of action of metallodrugs: Mechanism-driven new therapy development, Curr. Opin. Chem. Biol. 55 (2020) 171–179. https://doi.org/10.1016/j.cbpa.2020.02.006.
[51] F. De Castro, M. Benedetti, L. Del Coco, F.P. Fanizzi, NMR-Based Metabolomics in Metal-Based Drug Research, Molecules. 24 (2019) 2240. https://doi.org/10.3390/molecules24122240.
[52] C. Gabbiani, A. Casini, L. Messori, Gold(III) compounds as anticancer drugs, Gold Bull. 40 (2007) 73–81. https://doi.org/10.1007/BF03215296.
[53] E.R.T. Tiekink, Gold derivatives for the treatment of cancer, Crit. Rev. Oncol. Hematol. 42 (2002) 225–248. https://doi.org/10.1016/S1040-8428(01)00216-5.
[54] C.M. Che, R.W.Y. Sun, W.Y. Yu, C.B. Ko, N. Zhu, H. Sun, Gold(III) porphyrins as a new class of anticancer drugs: Cytotoxicity, DNA binding and induction of apoptosis in human cervix epitheloid cancer cells, Chem. Commun. 3 (2003) 1718–1719. https://doi.org/10.1039/b303294a.
[55] L. Messori, P. Orioli, C. Tempi, G. Marcon, Interactions of selected gold(III) complexes with calf thymus DNA, Biochem. Biophys. Res. Commun. 281 (2001) 352–360. https://doi.org/10.1006/bbrc.2001.4358.
[56] A. Casini, G. Kelter, C. Gabbiani, M.A. Cinellu, G. Minghetti, D. Fregona, H.H. Fiebig, L. Messori, Chemistry, antiproliferative properties, tumor selectivity, and molecular mechanisms of novel gold(III) compounds for cancer treatment: A systematic study, J. Biol. Inorg. Chem. 14 (2009) 1139–1149. https://doi.org/10.1007/s00775-009-0558-9.
[57] G. Marcon, S. Carotti, M. Coronnello, L. Messori, E. Mini, P. Orioli, T. Mazzei, M.A. Cinellu, G. Minghetti, Gold(III) complexes with bipyridyl ligands: Solution chemistry, cytotoxicity, and DNA binding properties, J. Med. Chem. 45 (2002) 1672–1677. https://doi.org/10.1021/jm010997w.
[58] A. Casini, M.A. Cinellu, G. Minghetti, C. Gabbiani, M. Coronnello, E. Mini, L. Messori, Structural and solution chemistry, antiproliferative effects, and DNA and protein binding properties of a series of dinuclear gold(III) compounds with bipyridyl ligands, J. Med. Chem. 49 (2006) 5524–5531. https://doi.org/10.1021/jm060436a.
[59] M. A. Cinellu, L. Maiore, M. Manassero, A. Casini, M. Arca, H.-H.H. Fiebig, G. Kelter, E. Michelucci, G. Pieraccini, C. Gabbiani, L. Messori, M.A. Cinellu, L. Maiore, M. Manassero, A. Casini, M. Arca, H.-H.H. Fiebig, G. Kelter, E. Michelucci, G. Pieraccini, C. Gabbiani, L. Messori, [Au2(phen2Me)2(μ-O)2] (PF6)2, a novel dinuclear gold(III) complex showing excellent antiproliferative properties, ACS Med. Chem. Lett. 1 (2010) 336–339. https://doi.org/10.1021/ml100097f.
[60] L. Ronconi, L. Giovagnini, C. Marzano, F. Bettìo, R. Graziani, G. Pilloni, D. Fregona, Gold Dithiocarbamate Derivatives as Potential Antineoplastic Agents: Design, Spectroscopic Properties, and in Vitro Antitumor Activity, Inorg. Chem. 44 (2005) 1867–1881. https://doi.org/10.1021/ic048260v.
[61] L. Messori, L. Marchetti, L. Massai, F. Scaletti, A. Guerri, I. Landini, S. Nobili, G. Perrone, E. Mini, P. Leoni, M. Pasquali, C. Gabbiani, Chemistry and biology of two novel gold(I) carbene complexes as prospective anticancer agents, Inorg. Chem. 53 (2014) 2396–2403. https://doi.org/10.1021/ic401731a.
[62] C.K. Mirabelli, R.K. Johnson, C.M. Sung, L. Faucette, K. Muirhead, S.T. Crooke, Evaluation of the in vivo antitumor activity and in vitro cytotoxic properties of auranofin, a coordinated gold compound, in murine tumor models., Cancer Res. 45 (1985) 32–9.
[63] L. Li, M.A. Fath, P.M. Scarbrough, W.H. Watson, D.R. Spitz, Combined inhibition of glycolysis, the pentose cycle, and thioredoxin metabolism selectively increases cytotoxicity and oxidative stress in human breast and prostate cancer, Redox Biol. 4 (2015) 127–135. https://doi.org/10.1016/j.redox.2014.12.001.
[64] F. Magherini, T. Fiaschi, E. Valocchia, M. Becatti, A. Pratesi, T. Marzo, L. Massai, C. Gabbiani, I. Landini, S. Nobili, E. Mini, L. Messori, A. Modesti, T. Gamberi, Antiproliferative effects of two gold(I)-N-heterocyclic carbene complexes in A2780 human ovarian cancer cells: A comparative proteomic study, Oncotarget. 9 (2018) 28042–28068. https://doi.org/10.18632/oncotarget.25556.
[65] F. Guidi, M. Puglia, C. Gabbiani, I. Landini, T. Gamberi, D. Fregona, M.A. Cinellu, S. Nobili, E. Mini, L. Bini, P.A. Modesti, A. Modesti, L. Messori, 2D-DIGE analysis of ovarian cancer cell

responses to cytotoxic gold compounds, Mol. Biosyst. 8 (2012) 985–993. https://doi.org/10.1039/c1mb05386h.
[66] F. Magherini, A. Modesti, L. Bini, M. Puglia, I. Landini, S. Nobili, E. Mini, M.A. Cinellu, C. Gabbiani, L. Messori, Exploring the biochemical mechanisms of cytotoxic gold compounds: A proteomic study, J. Biol. Inorg. Chem. 15 (2010) 573–582. https://doi.org/10.1007/s00775-010-0624-3.
[67] M.R. Wilkins, C. Pasquali, R.D. Appel, K. Ou, O. Golaz, J.-C. Sanchez, J.X. Yan, A.A. Gooley, G. Hughes, I. Humphery-Smith, K.L. Williams, D.F. Hochstrasser, From Proteins to Proteomes: Large Scale Protein Identification by Two-Dimensional Electrophoresis and Amino Acid Analysis, Nat. Biotechnol. 14 (1996) 61–65. https://doi.org/10.1038/nbt0196-61.
[68] P.H. O'Farrell, High resolution two-dimensional electrophoresis of proteins, J. Biol. Chem. 250 (1975) 4007–4021.
[69] J. Klose, Protein mapping by combined isoelectric focusing and electrophoresis of mouse tissues - A novel approach to testing for induced point mutations in mammals, Hum. Genet. 26 (1975) 231–243. https://doi.org/10.1007/BF00281458.
[70] Y. Ahmad, A.I. Lamond, A perspective on proteomics in cell biology, Trends Cell Biol. 24 (2014) 257–264. https://doi.org/10.1016/j.tcb.2013.10.010.
[71] R. Aebersold, M. Mann, Mass spectrometry-based proteomics, Nature. 422 (2003) 198–207. https://doi.org/10.1038/nature01511.
[72] W.J. Henzel, C. Watanabe, J.T. Stults, Protein identification: The origins of peptide mass fingerprinting, J. Am. Soc. Mass Spectrom. 14 (2003) 931–942. https://doi.org/10.1016/S1044-0305(03)00214-9.
[73] B. Wen, W.F. Zeng, Y. Liao, Z. Shi, S.R. Savage, W. Jiang, B. Zhang, Deep Learning in Proteomics, Proteomics. 20 (2020) 1900335. https://doi.org/10.1002/pmic.201900335.
[74] K. Marcus, C. Lelong, T. Rabilloud, What Room for Two-Dimensional Gel-Based Proteomics in a Shotgun Proteomics World?, Proteomes. 8 (2020) 17. https://doi.org/10.3390/proteomes8030017.
[75] W.-C. Chao, J.-F. Lu, J.-S. Wang, T.-H. Chiang, L.-J. Lin, Y.-L. Lee, P.-T. Chou, Unveiling the structural features of nonnative trimers of human superoxide dismutase 1, Biochim. Biophys. Acta - Gen. Subj. 1864 (2020) 129483. https://doi.org/10.1016/j.bbagen.2019.129483.
[76] Y. Zhang, B. R. Fonslow, B. Shan, M.-C. Baek, J. R. Yates, Protein Analysis by Shotgun/Bottom-up Proteomics, Chem. Rev. 113 (2013) 2343–2394. https://doi.org/10.1021/cr3003533.
[77] T. Gamberi, New Insights into the Molecular Mechanisms of Selected Anticancer Metal Compounds through Bioinformatic Analysis of Proteomic Data, J. Proteomics Bioinform. 01 (2013) S6. https://doi.org/10.4172/jpb.S6-006.
[78] D.A. Wolters, M. Stefanopoulou, P.J. Dyson, M. Groessl, Combination of metallomics and proteomics to study the effects of the metallodrug RAPTA-T on human cancer cells, Metallomics. 4 (2012) 1185–1196. https://doi.org/10.1039/c2mt20070h.
[79] Y.-I. Kim, J.-Y. Cho, Gel-based proteomics in disease research: Is it still valuable?, Biochim. Biophys. Acta - Proteins Proteomics. 1867 (2019) 9–16. https://doi.org/https://doi.org/10.1016/j.bbapap.2018.08.001.
[80] A. Chernobrovkin, C. Marin-Vicente, N. Visa, R.A. Zubarev, Functional Identification of Target by Expression Proteomics (FITExP) reveals protein targets and highlights mechanisms of action of small molecule drugs, Sci. Rep. 5 (2015) 11176. https://doi.org/10.1038/srep11176.
[81] D. Kreutz, A. Bileck, K. Plessl, D. Wolrab, M. Groessl, B.K. Keppler, S.M. Meier, C. Gerner, Response Profiling Using Shotgun Proteomics Enables Global Metallodrug Mechanisms of Action To Be Established, Chem. - A Eur. J. 23 (2017) 1881–1890. https://doi.org/10.1002/chem.201604516.
[82] X. Zhang, K. Selvaraju, A.A. Saei, P. D'Arcy, R.A. Zubarev, E.S. Arnér, S. Linder, Repurposing of auranofin: Thioredoxin reductase remains a primary target of the drug, Biochimie. 162 (2019) 46–54. https://doi.org/10.1016/j.biochi.2019.03.015.
[83] A. Sau, G. Filomeni, S. Pezzola, S. D'Aguanno, F.P. Tregno, A. Urbani, M. Serra, M. Pasello, P. Picci, G. Federici, A.M. Caccuri, Targeting GSTP1-1 induces JNK activation and leads to apoptosis in cisplatin-sensitive and -resistant human osteosarcoma cell lines, Mol. Biosyst. 8 (2012) 994–1006. https://doi.org/10.1039/c1mb05295k.
[84] M. Ashburner, C.A. Ball, J.A. Blake, D. Botstein, H. Butler, J.M. Cherry, A.P. Davis, K. Dolinski, S.S. Dwight, J.T. Eppig, M.A. Harris, D.P. Hill, L. Issel-Tarver, A. Kasarskis, S. Lewis, J.C. Matese, J.E. Richardson, M. Ringwald, G.M. Rubin, G. Sherlock, Gene Ontology: tool for the unification of biology, Nat. Genet. 25 (2000) 25–29. https://doi.org/10.1038/75556.

[85] A. Schmidt, I. Forne, A. Imhof, Bioinformatic analysis of proteomics data, BMC Syst. Biol. 8 (2014) S3. https://doi.org/10.1186/1752-0509-8-S2-S3.
[86] T. Villavicencio-Diaz, A. Rodriguez-Ulloa, O. Guirola-Cruz, Y. Perez-Riverol, Bioinformatics Tools for the Functional Interpretation of Quantitative Proteomics Results, Curr. Top. Med. Chem. 14 (2014) 435–449. https://doi.org/10.2174/1568026613666131204105110.
[87] C. Chen, J. Hou, J.J. Tanner, J. Cheng, Bioinformatics Methods for Mass Spectrometry-Based Proteomics Data Analysis, Int. J. Mol. Sci. 21 (2020) 2873. https://doi.org/10.3390/ijms21082873.
[88] G.O. Consortium, The Gene Ontology (GO) database and informatics resource, Nucleic Acids Res. 32 (2004) D258–D261. https://doi.org/10.1093/nar/gkh036.
[89] D.W. Huang, B.T. Sherman, R.A. Lempicki, Bioinformatics enrichment tools: Paths toward the comprehensive functional analysis of large gene lists, Nucleic Acids Res. 37 (2009) 1–13. https://doi.org/10.1093/nar/gkn923.
[90] Y. Liao, J. Wang, E.J. Jaehnig, Z. Shi, B. Zhang, WebGestalt 2019: gene set analysis toolkit with revamped UIs and APIs, Nucleic Acids Res. 47 (2019) W199–W205. https://doi.org/10.1093/nar/gkz401.
[91] H. Mi, A. Muruganujan, P.D. Thomas, PANTHER in 2013: modeling the evolution of gene function, and other gene attributes, in the context of phylogenetic trees, Nucleic Acids Res. 41 (2012) D377–D386. https://doi.org/10.1093/nar/gks1118.
[92] D. Szklarczyk, A.L. Gable, D. Lyon, A. Junge, S. Wyder, J. Huerta-Cepas, M. Simonovic, N.T. Doncheva, J.H. Morris, P. Bork, L.J. Jensen, C. von Mering, STRING v11: protein–protein association networks with increased coverage, supporting functional discovery in genome-wide experimental datasets, Nucleic Acids Res. 47 (2019) D607–D613. https://doi.org/10.1093/nar/gky1131.
[93] M. Kanehisa, S. Goto, KEGG: Kyoto Encyclopedia of Genes and Genomes, Nucleic Acids Res. 28 (2000) 27–30. https://doi.org/10.1093/nar/28.1.27.
[94] D. Croft, G. O'Kelly, G. Wu, R. Haw, M. Gillespie, L. Matthews, M. Caudy, P. Garapati, G. Gopinath, B. Jassal, S. Jupe, I. Kalatskaya, S. Mahajan, B. May, N. Ndegwa, E. Schmidt, V. Shamovsky, C. Yung, E. Birney, H. Hermjakob, P. D'Eustachio, L. Stein, Reactome: a database of reactions, pathways and biological processes, Nucleic Acids Res. 39 (2011) D691–D697. https://doi.org/10.1093/nar/gkq1018.
[95] T. Gamberi, L. Massai, F. Magherini, I. Landini, T. Fiaschi, F. Scaletti, C. Gabbiani, L. Bianchi, L. Bini, S. Nobili, G. Perrone, E. Mini, L. Messori, A. Modesti, Proteomic analysis of A2780/S ovarian cancer cell response to the cytotoxic organogold(III) compound Aubipyc, J. Proteomics. 103 (2014) 103–120. https://doi.org/10.1016/j.jprot.2014.03.032.
[96] T. Gamberi, F. Magherini, T. Fiaschi, I. Landini, L. Massai, E. Valocchia, L. Bianchi, L. Bini, C. Gabbiani, S. Nobili, E. Mini, L. Messori, A. Modesti, Proteomic analysis of the cytotoxic effects induced by the organogold(iii) complex Aubipyc in cisplatin-resistant A2780 ovarian cancer cells: further evidence for the glycolytic pathway implication, Mol. Biosyst. 11 (2015) 1653–1667. https://doi.org/10.1039/c5mb00008d.
[97] A.G. Herrmann, J.L. Searcy, T. Le Bihan, J. Mcculloch, R.F. Deighton, Total variance should drive data handling strategies in third generation proteomic studies, Proteomics. 13 (2013) 3251–3255. https://doi.org/10.1002/pmic.201300056.
[98] M. Berth, F.M. Moser, M. Kolbe, J. Bernhardt, The state of the art in the analysis of two-dimensional gel electrophoresis images, Appl. Microbiol. Biotechnol. 76 (2007) 1223–1243. https://doi.org/10.1007/s00253-007-1128-0.
[99] C. Zhang, A.J. Saunders, An emerging role for Ubiquilin 1 in regulating protein quality control system and in disease pathogenesis., Discov. Med. 8 (2009) 18–22.
[100] M. Berth, F.M. Moser, M. Kolbe, J. Bernhardt, The state of the art in the analysis of two-dimensional gel electrophoresis images, Appl. Microbiol. Biotechnol. 76 (2007) 1223–1243. https://doi.org/10.1007/s00253-007-1128-0.
[101] J.K. Box, N. Paquet, M.N. Adams, D. Boucher, E. Bolderson, K.J. O'Byrne, D.J. Richard, Nucleophosmin: from structure and function to disease development, BMC Mol. Biol. 17 (2016) 19. https://doi.org/10.1186/s12867-016-0073-9.
[102] J. Zhang, X. Li, X. Han, R. Liu, J. Fang, Targeting the Thioredoxin System for Cancer Therapy, Trends Pharmacol. Sci. 38 (2017) 794–808. https://doi.org/10.1016/j.tips.2017.06.001.
[103] D.A. Mitchell, M.A. Marletta, Thioredoxin catalyzes the s-nitrosation of the caspase-3 active site cysteine, Nat. Chem. Biol. 1 (2005) 154–158. https://doi.org/10.1038/nchembio720.

[104] M. Altaf, N. Casagrande, E. Mariotto, N. Baig, A.-N.N. Kawde, G. Corona, R. Larcher, C. Borghese, C. Pavan, A.A. Seliman, D. Aldinucci, A.A. Isab, Potent in vitro and in vivo anticancer activity of new bipyridine and bipyrimidine gold (III) dithiocarbamate derivatives, Cancers (Basel). 11 (2019) 474. https://doi.org/10.3390/cancers11040474.
[105] J.H. Kim, E. Reeder, S. Parkin, S.G. Awuah, Gold(I/III)-Phosphine Complexes as Potent Antiproliferative Agents, Sci. Rep. 9 (2019) 12335. https://doi.org/10.1038/s41598-019-48584-5.
[106] N. Micale, T. Schirmeister, R. Ettari, M.A. Cinellu, L. Maiore, M. Serratrice, C. Gabbiani, L. Massai, L. Messori, Selected cytotoxic gold compounds cause significant inhibition of 20S proteasome catalytic activities, J. Inorg. Biochem. 141 (2014) 79–82. https://doi.org/10.1016/j.jinorgbio.2014.08.001.
[107] L. Galvez, M. Rusz, M. Schwaiger-Haber, Y. El Abiead, G. Hermann, U. Jungwirth, W. Berger, B.K. Keppler, M.A. Jakupec, G. Koellensperger, Preclinical studies on metal based anticancer drugs as enabled by integrated metallomics and metabolomics, Metallomics. 11 (2019) 1716–1728. https://doi.org/10.1039/C9MT00141G.
[108] Y. Wang, H. Wang, H. Li, H. Sun, Metallomic and metalloproteomic strategies in elucidating the molecular mechanisms of metallodrugs, Dalt. Trans. 44 (2015) 437–447. https://doi.org/10.1039/C4DT02814G.