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Systems Biology of the Proteomic Analysis of Cytotoxic Gold Compounds in A2780 Ovarian Cancer Cell Line: A Network Analysis

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Abstract

Gold and ruthenium compounds hold today great promise as new metallodrugs for treatment of human ovarian carcinoma; yet, their mode of action is still largely unknown. To shed light on their molecular mechanisms in previous studies we performed proteomic analysis in the A2780 human ovarian cancer cell line, after treatment with four different gold and two ruthenium compounds. To gain a better interpretation of their cellular effects we reported here the bioinformatic analysis of proteomic data that confirmed the action of these metallodrugs on previously identified signaling pathways such as glucose metabolism, protein folding, and cell division cycle and apoptosis. In addition, this new study pointed out the involvement of novel pathways such as synthesis and degradation of purine/pyrimidine nucleotides as well mitochondrial energy metabolism. These findings strengthen the idea of energy metabolism and mitochondrial function as possible target of both gold and ruthenium compound in A2780 cell line.

Keywords: Network Analysis; Proteomics; Metallodrugs; Systems Biology; Ovarian Cancer Cells

Introduction

The protein composition represents the functional status of biological processes at a given time. Proteomics methods may provide an analysis of the alteration induced by drugs on protein expression and these alterations may be related to the mode of action of the drugs themselves. Consequently, identification of protein alterations specific to diseases by proteomic approaches has been helpful to clarify the therapeutic benefit of drugs [1]. Moreover, proteomic profiling offers the opportunity to identify proteins that mediate apoptotic pathways, in cells treated with cytotoxic agents [2]. In cancer chemotherapy, platinum coordination compounds are essential agents, with proven efficacy against a variety of tumors [3]. Ovarian cancer is the second among gynecological cancers in number of new cases and the platinum-based chemotherapy is the election treatment [4]. However, the occurrence of chemoresistence is the main cause of chemotherapy failure [5]. The use of transition metal compounds other than platinum has also attracted attention to overcome the disadvantages of platinum-based chemoresistance. Several authors evaluated various metal complexes (e.g., Pt, Au, and Ru), either clinically or experimentally, as therapeutic agents in treatment of malignant diseases [6]. Recently research has increasingly focused

on gold compounds as anticancer drug candidates, and the most important classes of cytotoxic gold compounds under study have been gold (III) porphyrins [7,8], gold (III) dithiocarbamates [9,10], cyclometalated gold(III) complexes [11], dinuclear gold complexes [12,13], gold carbenes [14,15] and 2,3,4,6-tetra-o-acetyl-l-thio-β-D-glucopyranosato-S-(triethyl-phosphine) gold(I) (manufactured as auranofin) [16]. This because gold(III) complexes typically display the same electronic configuration (d8) and similar structural and reactivity features of platinum(II) [17].

An organometallic species containing ruthenate (III), has been shown to have selectivity towards metastatic tumors. These complexes are based on a ruthenium metal center and demonstrate a low general toxicity that apparently reduces the side-effects associated with chemotherapy [18].

In previous papers, we reported lists of proteins found to be differentially expressed in the human ovarian cancer cisplatin-sensitive and resistant cell line (A2780/S and A2780/R), after treatment with four different cytotoxic gold compounds, namely: Auranofin [19,20], Auoxo6 [19,20], AuL12 [21], Au_2Phen [21] and $Aubipy_c$ [22,23]. Figure 1 shows chemical structure of these gold compounds. Auranofin is a typical gold (I) drug in clinical use as antiarthritic [16] endowed with significant cytotoxic properties in vitro being able to strong inhibit the mitochondrial thioredoxin reductase. This selenoenzyme is involved in the control of redox state of thioredoxin, a small protein directly implicated in the regulation of intracellular redox metabolism and its inhibition may result in perturbation of the mitochondrial membrane potential that lead to mitochondrial dysfunction and then to apoptosis [16]. On the contrary Auoxo6 and Au_2Phen are binuclear gold (III) complexes whose display, as reported above, the same configuration and similar reactivity of platinum complexes. AuL12 is a gold (III) dithiocarbamate compound and $Aubipy_c$ is characterized both chemically and biologically. Its structure consists of a square planar gold (III) center receiving three donors from the tridentate bipyridyl ligand while the fourth coordination position is occupied by a hydroxide group that is the preferential site for ligand replacement reaction and for protein binding. All these gold compounds result able to induce cytotoxicity toward the human ovarian cancer cell line A2780. Moreover we have used proteomics to investigate, in the same human cancer cell lines, the mode of action of two anticancer ruthenium compounds namely trans-[tetrachloro (DMSO) (imidazole) ruthenate(III)] (NAMI-A) and [Ru(η6-toluene)Cl2(PTA)] (RAPTA-T), where PTA is 1,3,5-triaza-7-hosphaadamantane [24]. For all these metallodrugs we monitored changes in the protein expression levels in A2780 sensible or resistant to cisplatin treated with them.. Regarding the alteration induced by Auranofin and Auoxo6 in cisplatin-sensitive cell line (A2780/S) we observed that the expression level of six proteins resulted modified in both the two treatments. Some of these proteins were involved in intracellular redox homeostasis and this suggest that cell damage is the consequence of oxidative stress inducing apoptosis [19]. On the contrary, the effects of both gold compounds on A2780/R (cisplatin-resistant) cancer cells showed that the two metallodrugs cause different proteomic modifications [20]. Auranofin mainly acts by altering the expression of Proteasome apparatus proteins, while Auoxo6 by an expression modification of proteins related to mRNA splicing, trafficking and stability. Moreover, after cell treatments with both compounds we found a decrease in the expression level of the thioredoxin-like protein. This protein is involved in cell oxidative stress defense and we can conclude that a possible mechanism involved in cytotoxicity of these gold compounds is the induction of a massive oxidative stress that lead to cell death. Our proteomics studies continued with the investigation of the biological effects of two more gold compounds: AuL12 and Au_2Phen in cell line sensitive to cisplatin A2780 [21]. The results suggested that the cytotoxic effects of the two gold-compounds could be related to an impairment of protein degradation pathway. We found that both treatments cause an overexpression of Ubiquilin-1 involved in inhibiting protein degradation. Moreover, by comparing the results of these two metallodrugs with the results obtained with Auranofin and Auoxo6 treatment, we found that these proteins could be correlated. In detail, we can observe that with all these treatments the expression levels of few proteins involved in RNA processing resulted modified. Moreover, we found another correlation regarding two proteins involved in connections of cytoskeletal components to membrane. In a very recent paper, we investigate the proteomic alterations associated to the cytotoxic effect of the gold compound $Aubipy_c$ on A2780/S cancer cell line [22]. The results pointed out many differentially expressed proteins. Most of these proteins belong to stress response and chaperones, protein synthesis, metabolism and cell redox homeostasis and cytoskeleton and cell structure. Bioinformatics and functional analysis of these proteins pointed out that Aubipyc affects several glycolytic enzymes leading to a possible down-regulation of glucose metabolism. We also carried out a parallel proteomic study on the A2780/R cell line in order to verify if $Aubipy_c$ treatment lead to proteomic variations similar to those highlighted in A2780/S cell line [23]. It emerged that gold treatment induced similar modifications similar in both cell lines. Indeed, $Aubipy_c$ mainly acted by affecting glycolytic pathway. This work therefore strengthens the role of glycolysis in $Aubipy_c$ cytotoxic mechanism of action. We have investigated using a proteomic analysis the mode of action of two anticancer ruthenium compounds, NAMI-A and RAPTA-T, in A2780/S human cancer cells [24]. We observed for both ruthenium compounds reduced changes in protein expression levels. It is interesting to note that NAMI-A modifies the expression level of proteins showing an important role in removing peroxides and this may suggest that NAMI-A induces ROS increase damaging cell macromolecules.

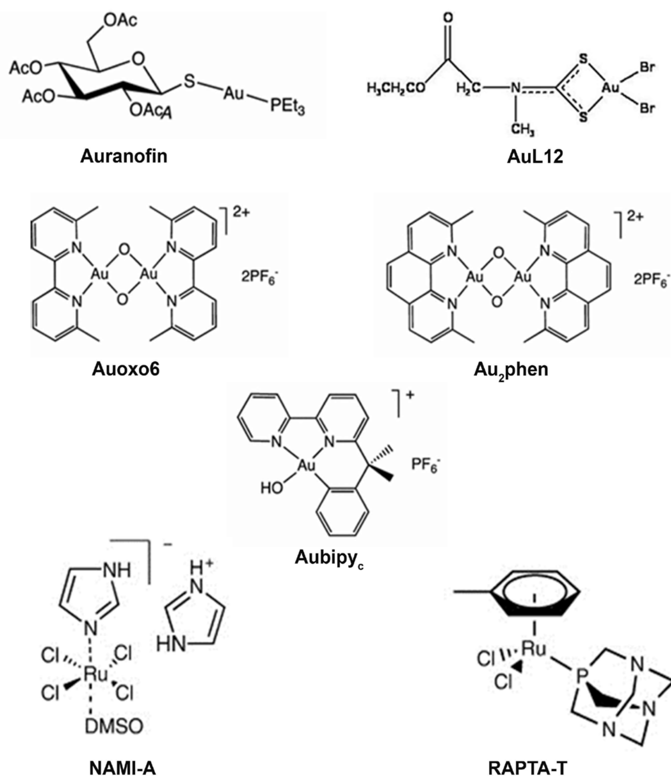

Figure 1. Chemical structure of gold and ruthenium compounds.

Proteomic methods may offer a great deal of information on the functional alterations occurring at the cellular level because of treatment. In turn, integrated bioinformatics analysis of proteomic data in terms of affected metabolic and signaling pathways may help to understand the molecular mechanisms of these drugs within a Systems Biology perspective [25,26]. Systems Biology mainly refers to the ability to obtain integrate and analyze complex sets of data from multiple experimental sources using interdisciplinary tools [25,26]. Omics sciences such as Genomics, Epigenetics, Transcriptomics, Interferomics, Proteomics, Metabolomic, etc, represent the platforms for systems biology [27]. As these disciplines have the potential to produce large amounts of new experimental data, has been developed a robust method to analyze and interpret such data. Bioinformatics is the discipline specifically deputed to this goal [27]. In recent years, Bioinformatics has been extensively applied to systems biology and to the interpretation of proteomic data. Related to this and to interpret all the results obtained we carried out a first bioinformatics analysis on proteins identified in our proteomic studies showing variation in expression level following treatment with AuL12, Au_2Phen, Auoxo6, Auranofin and with NAMI-A and RAPTA-T in a cisplatin-sensitive human ovarian cancer cell line (A2780/S) [28]. We used an analytical web portal BioProfiling (www.bioprofiling.de) [29] that employs statistical methodology for the network based interpretation of the protein list (Rspider, PPIspider) to identify a possible combination of metallodrugs and to increase the effectiveness of metallodrugs treatment [30,31]. A preliminary network map was built up and the software found two classes of proteins correlated to each other. These two classes were i) Apoptosis, cellular proliferation and DNA stability and ii) Cytoskeleton dynamics, splicing regulation and protein biosynthesis.

The study presented here deals with the application of bioinformatics to analyze all the proteins lists obtained by proteomics experiments performed on cisplatin-sensitive human ovarian cancer cell line treated with Auranofin, Auoxo6, AuL12, Au_2Phen, $Aubipy_c$, NAMI-A and RAPTA-T. We used a network strategy to generate statistically valid hypotheses about biological mechanisms involved in metallodrugs treatment, and to find possible common features in the mechanism of action of these compounds. To proceed, we carried out a detailed bioinformatics analysis (using open source software for analyses/interpretation of biological data and visualization of complex networks obtained) on proteins identified in our previous proteomic studies. The protein list was first analyzed by over- representation analysis (ORA) using the web-based tool Webgestalt (WEB-based GEne SeT AnaLysis Toolkit) (http://bioinfo.vanderbilt.edu/webgestalt/) [32,33]. Afterwards, the same protein list was submitted to the web portal BioProfiling (www.bioprofiling.de) to provide models of protein interactions [29].

Methods

The Input List

We analyzed a list of proteins showing variation in expression level in cisplatin-sensitive human ovarian cancer cell line (A2780/S) following treatment with the gold compounds Auranofin [19], Auoxo6 [19], AuL12 [21], Au_2Phen [21], $Aubipy_c$ [22], and with the ruthenium compounds NAMI-A and RAPTA-T [24] and previously identified in our experimental proteomics studies. We also added to the input list the proteins obtained as intermediate nodes by our previously bioinformatic study carried out using the web portal Bioprofiling (www.bioprofiling.de) [28]. The input list, named A2780/S, is reported in Table 1.

Bioinformatics Analysis

To identify the potential functional associations between the protein set obtained in our studies we performed over-representation analysis (ORA) of the input list A2780/S using the Webgestalt online tool ("WEB-based GEne SeT AnaLysis Toolkit") (http://bioinfo.vanderbilt.edu/webgestalt/) against Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG), WikiPathway and Pathway Commons databases [32,33] (Figure 2). This method assesses the statistical

Table 1. The input list A2780/S

Uniprot Accession Number[1]	Gene Name	Protein name	Reference
Q969Q6	PPP2R3C	Serine/threonine-protein phosphatase 2A regulatory subunit B	[23]
Q08752	PPID	Peptidyl-prolyl cis-trans isomerase D	[23]
P29692	EEF1D	Elongation factor 1-delta	[23]
P07339	CTSD	Cathepsin D	[23]
P26447	S100A4	Protein S100-A4	[23]
Q06830	PRDX1	Peroxiredoxin-1	[23, 18]
Q9BWD1	ACAT2	Acetyl-CoA acetyltransferase, cytosolic	[23]
P33316	DUT	Deoxyuridine 5'-triphosphate nucleotidohydrolase, mitochondrial	[23]
P62253	UBE2G1	Ubiquitin-conjugating enzyme E2 G1	[23]
Q9NQR4	NIT2	Omega-amidase NIT2	[19, 23]
P23919	DTYMK	Thymidylate kinase	[19, 23]
P49773	HINT1	Histidine triad nucleotide-binding protein 1	[19, 23, 18]
Q9NRF9	POLE3	DNA polymerase epsilon subunit 3	[23]
P61758	VBP1	Prefoldin subunit 3	[23]
O95757	HSPA4L	Heat shock 70 kDa protein 4L	[21]
P38646	HSPA9	Stress-70 protein, mitochondrial	[21]
P11142	HSPA8	Heat shock cognate 71 kDa protein	[21]
P49368	CCT3	T-complex protein 1 subunit gamma	[21]
P48643	CCT5	T-complex protein 1 subunit epsilon	[21]
P17987	TCP1	T-complex protein 1 subunit alpha	[21]
P40227	CCT6A	T-complex protein 1 subunit zeta	[21]
P78371	CCT2	T-complex protein 1 subunit beta	[21]
Q99832	CCT7	T-complex protein 1 subunit eta	[21]
Q14696	MESDC2	LDLR chaperone MESD	[21]
P10809	HSPD1	60 kDa heat shock protein, mitochondrial	[21]
P31948	STIP1	Stress-induced-phosphoprotein 1	[21]
P23284	PPIB	Peptidyl-prolyl cis-trans isomerase B	[21]
P14618	PKM	Pyruvate kinase PKM	[21]
P04406	GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	[21]
P06733	ENO1	Alpha-enolase	[21]
P00558	PGK1	Phosphoglycerate kinase 1	[21]
P51570	GALK1	Galactokinase	[21]
P00352	ALDH1A1	Retinal dehydrogenase 1	[21]
P28331	NDUFS1	NADH-ubiquinone oxidoreductase 75 kDa subunit, mitochondrial	[21]
P25705	ATP5A1	ATP synthase subunit alpha, mitochondrial	[21]
P09622	DLD	Dihydrolipoyl dehydrogenase, mitochondrial	[21]
O75874	IDH1	Isocitrate dehydrogenase [NADP] cytoplasmic	[21]
P30084	ECHS1	Enoyl-CoA hydratase, mitochondrial	[21]
P55809	OXCT1	Succinyl-CoA:3-ketoacid coenzyme A transferase 1, mitochondrial	[21]
O43175	PHGDH	D-3-phosphoglycerate dehydrogenase	[21]
P28838	LAP3	Cytosol aminopeptidase	[21, 20]
P31939	ATIC	Bifunctional purine biosynthesis protein PURH	[21]
P11586	MTHFD1	C-1-tetrahydrofolate synthase, cytoplasmic	[21]
P49915	GMPS	GMP synthase [glutamine-hydrolyzing]	[21]
Q16891	IMMT	Mitochondrial inner membrane protein	[21]
O43819	SCO2	Protein SCO2 homolog, mitochondrial	[21]
P45880	VDAC2	Voltage-dependent anion-selective channel protein 2	[21]
P13639	EEF2	Elongation factor 2	[21]
P49411	TUFM	Elongation factor Tu, mitochondrial	[21]
P33240	CSTF2	Cleavage stimulation factor subunit 2	[21]
Q92945	KHSRP	Far upstream element-binding protein 2	[21]

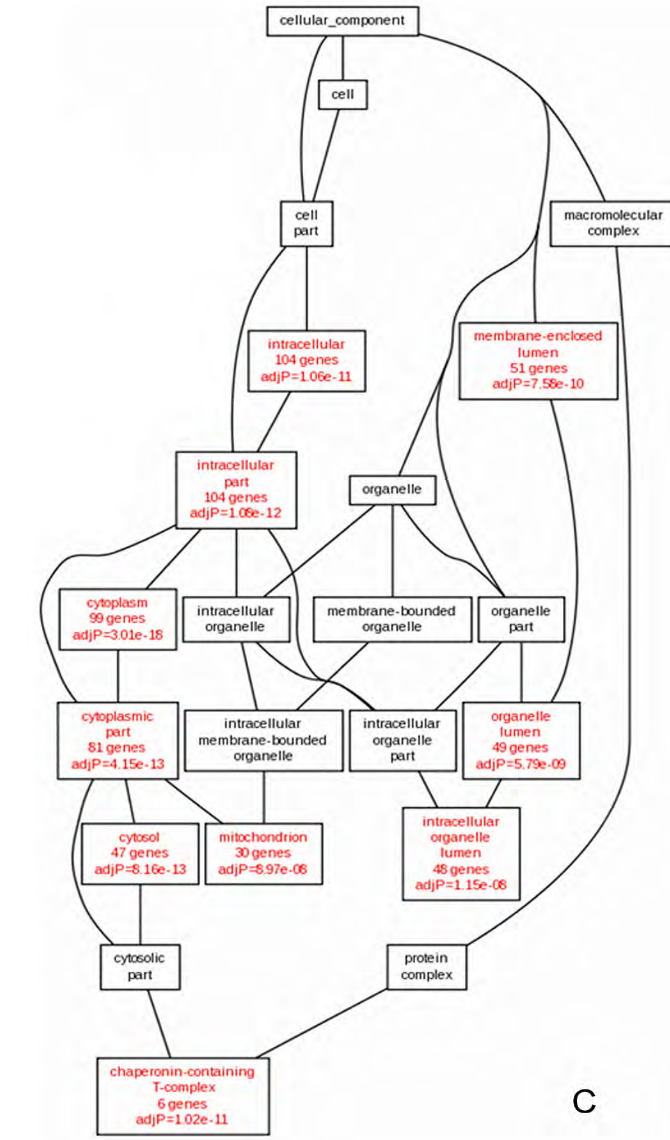

Figure 2. Gene Ontology over-representation analysis of the input list performed by Webgestalt online tool ((http://bioinfo.vanderbilt.edu/webgestalt/). Statistically significantly, over-represented categories are highlighted in red. Panel **A**: GO Biological Process (BP). Panel **B**: GO Molecular Function (MF). Panel **C**: Cellular Component (CC).

over-representation of a user-defined, pre-selected gene/protein list of interest in a reference list of known gene/protein sets using a statistical test, e.g. the one-sided Fisher's exact test or the hypergeometric distribution followed by multiple test adjustment using Benjamini-Hochberg correction [32,33]. The significant enrichment, using a cut-off of $p < 0.001$, were further considered for proteomics data interpretation and discussion. Afterwards the web portal BioProfiling.de (http://www.bioprofiling.de/) was used to build enriched networks. This portal accepts a directory of Uniprot (http://www.uniprot.org/) Accesion Number (AC) as input and provides a list of genes as output [29]. It covers most of the available infor-

mation regarding signaling and metabolic pathways (database: Reactome) and physical protein interactions (database: IntAct) [29] (Figure 2). Accordingly, the input list A2780/S was analyzed by Rspider [29] and by PPIspider [30]. In all cases, the molecular networks inferred by spider tools are profiled according to three different models, named D1, D2, and D3. In model D1 are considered only the direct interactions between proteins list, while in models D2 and D3 are allowed one and two intermediate nodes, respectively. We analysed the input list A2780/S allowing the insertion of one intermediate node (model D2). Moreover, both tools (Rspider and PPIspider) employ advanced enrichment or network-based statistical frameworks [30,31]. The p-value provided was computed by the Monte Carlo simulation (http://www.bioprofiling.de/statistical_frameworks.html) and referred to the probability of obtaining a model of the same quality for a random gene list of the same size [29,30]. The significant networks, using a cut-off of $p < 0.01$, were further considered for proteomics data interpretation and discussion. The enriched network was exported as .xgmml file, visualized, and modified by Cytoscape 3.1.0 (http://www.cytoscape.org/) [34].

Statistical Analysis

The statistics performed in the over-representation analysis (ORA) dealt with the p value obtained from the Fisher exact test followed by the p value adjusted for multiple testing by Benjamini- Hochberg correction. The accepted p value adjusted was $p < 0.001$ after Benjamini-Hochberg correction.

The statistics performed in the enriched network analysis is based on p-value computed provided by the Monte Carlo procedure (http://www.bioprofiling.de/statistical_frameworks.html) and referred to the probability of obtaining a model of the same quality for a random gene list of the same size [29,30]. The networks were considered statistically significant using a cut-off of $p < 0.01$.

Results

Over-representation analysis of the input list A2780/S

The input list A2780/S was uploaded to the Webgestalt portal and over-representation analysis (ORA) was performed [32,33]. Figure 2 shows the significantly over-represented GO Biological Processes (BP, Panel A), Molecular Functions (MF, Panel B) and Cellular Components (CC, Panel C) in the input protein set (for details, see Supplementary Table 1). We found enriched Gene Ontology Biological Process (GO BP) terms involved in metabolic process (GO:0008152 – 89 genes), including primary metabolic process (GO:0044238 – 85 genes), cellular metabolic process (GO:0044237 – 84 genes) and single-organism metabolic process (GO:0044710 – 88 genes). Other BP terms appear to be more closely related to the protein folding (GO:0006457 – 17 genes),'de novo' protein folding (GO:0006458 – 10 genes) and 'de novo' posttranslational protein folding (GO:0051084 – 9 genes). Of note, some of the BP terms corresponded to processes involved in cellular component organization including macromolecular complex subunit organization (GO:0034621–16 genes) and macromolecular complex assembly (GO:0034622 – 14 genes). Molecular function terms associated with binding were over-represented in the list. The enriched GO MF terms contain ontologies involved in binding, including small molecule binding (GO:0036094 – 43 genes), organic cyclic compound binding (GO:0097159 – 53 genes) and heterocyclic compound binding (GO:1901363 – 52 genes). The molecular function terms associated with protein binding results enriched (GO:0005515 – 78 genes) including the unfolded protein binding (GO:0051082 – 13 genes). There were also enriched GO MF terms involved in translation elongation factor activity (GO:0003746 – 5 genes). The enriched GO cellular components terms pointed out two major over-represented classes. The first were associated with the intracellular part and included cytoplasm (GO:0005737 – 47 genes) and intracellular organelle including mitochondrion (GO:0005739 – 30 genes). The other class was those components on the membrane-enclosed **lumen (GO:0031974 – 51 genes) or in the organelle lumen (GO:0043233 – 49 genes)**

The set was then analyzed with respect to: KEGG (Table 2), PathwayCommons (Table 3) and WikiPathways (Table 4) databases. A significant over-representation of pathways related to metabolic pathways like glycolysis and proteasomal system was found (11 genes). KEGG pathways indicated that proteins in the list were linked with either with energetic metabolism (Glycolysis / Gluconeogenesis 6 genes) anddegradation of essential amino acids (5 genes) and protein processing in endoplasmic reticulum (5 genes). Results achieved from the PathwayCommons ORA also supported the link of the proteins set with both glucose metabolism and protein degradation (glycolysis and gluconeogenesis - 6 genes; Parkin-Ubiquitin proteasomal system pathway - 5 genes). In addition, we found enriched pathways associated with protein translation and maturation of mRNA (translation factors – 5 genes; mRNA processing – 5 genes). Moreover, WikiPathways ORA indicated a possible connection between the input list and glucose metabolism and protein biosynthesis and degradation.

Table 2. Web-based gene/protein set analysis tools

Web portal	URL	Database	Functional annotation	Reference
Over-representation analysis (ORA)				
WebGestalt (WEB-based Gene SeT AnaLysis Toolkit)	http://bioinfo.vanderbilt.edu/webgestalt/	GO KEGG Wikipathway Pathway Commons	Pathway analysis Gene ontology	[31, 32]
Network analysis				
BioProfiling	http://www.bioprofiling.de/	IntAct KEGG Reactome GO	Interaction networks Pathway analysis Gene ontology	[28, 29, 30]
Graphical representation tools				
Cytoscape	http://www.cytoscape.org/	Apps for connecting with databases	Graphical representation of protein network	[33]

Table 3. Over-representation of KEGG pathways in the input list A2780/S. p < 0.001 after Benjamini-Hochberg correction.

Pathway Name	Gene	Uniprot Accession Number	Statistics
Metabolic pathways	24	P61978; P28331; P04406; P23919; P30041; P28838; P30084; Q99714; P14618; O75874; P48637; O43175; P00558; P33316; P31939; P49915; P25705; Q9NRF9; P06733; P00352; P09622; P60174; P11586	C=1130;O=24;E=2.78;R=8.64;rawP=4.34e-16;adjP=5.64e-15
Glycolysis / Gluconeogenesis	6	P04406; P14618; P00558; P06733; P09622; P60174	C=65;O=6;E=0.16;R=37.56;rawP=1.40e-08;adjP=9.10e-08
Valine, leucine and isoleucine degradation	5	Q9BWD1; P55809; P09622; P30084; Q99714	C=44;O=5;E=0.11;R=46.23;rawP=8.21e-08;adjP=3.56e-07
Pathogenic Escherichia coli infection	4	P15311; P62258; P60709; P07437	C=56;O=4;E=0.14;R=29.06;rawP=1.15e-05;adjP=3.74e-05
Parkinson's disease	5	P62253; P25705; P28331; Q08752; P45880	C=130;O=5;E=0.32;R=15.65;rawP=1.83e-05;adjP=3.97e-05
Shigellosis	4	P60709; A9UGY9; O43353; P18206	C=61;O=4;E=0.15;R=26.68;rawP=1.61e-05;adjP=3.97e-05
Protein processing in endoplasmic reticulum	5	P27797; P62253; O95757; Q9UMX0; P11142	C=165;O=5;E=0.41;R=12.33;rawP=5.72e-05;adjP=9.85e-05
Alzheimer's disease	5	P25705; P28331; P04406; P05067; Q99714	C=167;O=5;E=0.41;R=12.18;rawP=6.06e-05;adjP=9.85e-05
Huntington's disease	5	P25705; P28331; Q08752; P08047; P45880	C=183;O=5;E=0.45;R=11.12;rawP=9.33e-05;adjP=0.0001
Pyrimidine metabolism	4	Q16881; 1P23919; Q9NRF9; P33316	C=99;O=4;E=0.24;R=16.44;rawP=0.0001;adjP=0.0001

The statistics column lists: C: the number of reference genes in the category; O: the number of genes in the gene set and also in the category; E: the expected number in the category; R: ratio of enrichment; rawP: p value from the Fisher exact test; adjP: p value adjusted for multiple testing by Benjamini-Hochberg correction.

Table 4. Over-representation of PathwayCommons pathways in the input list A2780/S. p < 0.001 after Benjamini-Hochberg correction.

Pathway Name	Gene	Uniprot Accession Number	Statistics
Glycolysis and Gluconeogenesis	6	P04406; P14618; P06733; P02622; P00558; P60174	C=50;O=6;E=0.12;R=48.82;rawP=2.78e-09;adjP=3.89e-08
Translation Factors	5	P13639; Q15056; Q05639; P29692; P24534	C=51;O=5;E=0.13;R=39.89;rawP=1.75e-07;adjP=1.22e-06
Parkin-Ubiquitin Proteasomal System pathway	5	P62253; P38646; P17980; P11142; P07437	C=71;O=5;E=0.17;R=28.65;rawP=9.33e-07;adjP=4.35e-06
Pathogenic Escherichia coli infection	4	P15311; P 60709; P63104 ; P07437	C=66;O=4;E=0.16;R=24.66;rawP=2.21e-05;adjP=6.19e-05
mRNA processing	5	P33240; P61978; P31943; Q99729; Q07955	C=132;O=5;E=0.32;R=15.41;rawP=1.97e-05;adjP=6.19e-05
Regulation of Actin Cytoskeleton	5	P15311; Q8N8S7; P60709; P18206; P23528	C=157;O=5;E=0.39;R=12.96;rawP=4.52e-05;adjP=0.0001
Prolactin Signaling Pathway	4	P23284; P63104; P07339; P62937	C=92;O=4;E=0.23;R=17.69;rawP=8.16e-05;adjP=0.0002
SIDS Susceptibility Pathways	5	P63104; P04406; P62258; P10809; P08047	C=214;O=5;E=0.53;R=9.51;rawP=0.0002;adjP=0.0003
Leukocyte TarBase	4	Q16881; P23284; P14618; O75874	C=160;O=4;E=0.39;R=10.17;rawP=0.0007;adjP=0.0010

The statistics column lists: C: the number of reference genes in the category; O: the number of genes in the gene set and also in the category; E: the expected number in the category; R: ratio of enrichment; rawP: p value from the Fisher exact test; adjP: p value adjusted for multiple testing by Benjamini-Hochberg correction.

Network Enrichment

To gain a deeper insight into the mechanism of action of selected anticancer gold and ruthenium compounds, and to establish whether these metallodrugs have common protein targets, the input list A2780/S was enriched in terms of a network-based statistical framework. As a first approach, a pathway-based network was built by allowing the insertion of no more than one intermediate node using the Reactome database in the Bioprofiling platform (www. bioprofiling.de) as a reference [29,30]. Allowing the insertion of one intermediate node (model D2), an interconnected network were obtained (Figure 3A). In this network, 16 nodes overlapped with the input list and 11 intermediate nodes were allowed. The 11 intermediate proteins, not previously detected in our proteomic studies, were represented as triangles in the network of Figure 3A and were listed in Table 6. Furthermore, a physical-interaction-based network was built by limiting the insertion of enriched nodes to one or two by the IntAct database as a reference and using the same web platform [29,31]. A statistically significant enriched network was obtained allowing the insertion of one intermediate node (model D2). In the resulting network, 83 nodes (Figure 3B) overlapped with the input list and 27 intermediate nodes were allowed (represented as triangles). The list of the intermediate nodes was reported in Table 7. Among the intermediate nodes, associated with the proteins of the input list A2780/S in terms of pathway-based network (Figure 3A and Table 6), we found proteins involved in mitochondrial metabolism and in synthesis and degradation of purine/pyrimidine nucleotides. The nodes, involved in mitochondrial metabolism, are represented by four intermediate proteins. ACAT1 a mitochondrial enzyme involved in both amino acid degradation and ketone body metabolism. During ketolysis, it converts an acetoacetyl-CoA to two molecules of acetyl CoA. ACAD8 is an isobutyryl-CoA dehydrogenase that functions in valine catabolism. AMT is a mitochondrial aminomethyltransferase and it is one of four components of the glycine cleavage system. HADHA is the alpha subunit of the mitochondrial trifunctional protein (TFP) (HADHA / HADHB) involved in mitochondrial fatty acid beta-oxidation pathway.

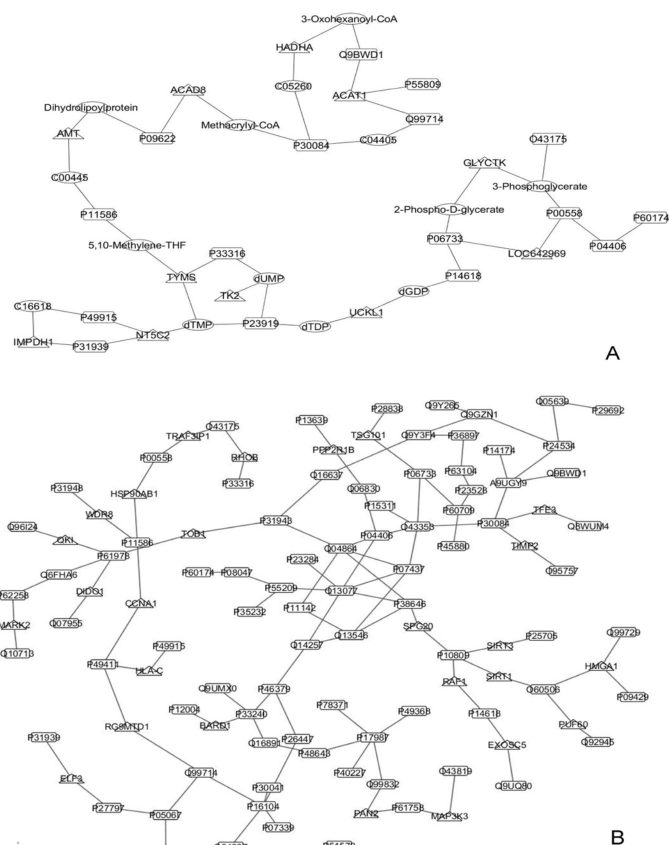

Figure 3. Enriched networks obtained by Bioprofiling (http://www.bioprofiling.de/) online tool. Results obtained by (**A**) Rspider and (**B**) PPIspider analysis after the submission of the imput list A2780/S. A maximum of one intermediate node was allowed to be added. Network visualization was obtained using Cytoscape (http://www.cytoscape.org/). Nodes arising from the "input list" are shown as white squares, whereas enriched nodes are represented by white triangles. p < 0.01. See text for details.

The intermediate nodes mapped in synthesis of purine/pyrimidine nucleotides are: IMPDH1(P20839), that catalyzes the first committed and rate-limiting step in the *de novo* synthesis of guanine nucleotides; TYMS a thymidylate synthase that contributes to the *de novo* mitochondrial thymidylate biosynthesis pathway; UCKL1 involved in UMP and CTP biosynthesis and

TK2 that specifically phosphorylates thymidine, deoxycytidine, and deoxyuridine. This enzyme localizes to the mitochondria and is required for mitochondrial DNA synthesis. The node involved in degradation of purine/pyrimidine nucleotides is NT5C2 a cytosolic purine 5'-nucleotidase that hydrolyzes inosine 5'-monophosphate (IMP) and other purine nucleotides. It may have a critical role in the maintenance of a constant composition of intracellular purine/pyrimidine nucleotides.

Table 5. Over-representation of WikiPathways pathways in the input list A2780/S. $p < 0.001$ after Benjamini-Hochberg correction.

PathwayName	Gene	Uniprot Accession Number	Statistics
Glycolysis and Gluconeogenesis	6	P04406; P14618; P06733; P09622; P00558; P60174	C=50;O=6;E=0.12;R=48.82;rawP=2.78e-09;adjP=3.89e-08
Translation Factors	5	P13639; Q15056; Q05639; P29692; P24534	C=51;O=5;E=0.13;R=39.89;rawP=1.75e-07;adjP=1.22e-06
Parkin-Ubiquitin Proteasomal System pathway	5	P62253; P38646; PP17980; P11142; P07437	C=71;O=5;E=0.17;R=28.65;rawP=9.33e-07;adjP=4.35e-06
Pathogenic Escherichia coli infection	4	P15311; P60709; P63104; P07437	C=66;O=4;E=0.16;R=24.66;rawP=2.21e-05;adjP=6.19e-05
mRNA processing	5	P33240; P61978; P31943; Q99729; Q07955	C=132;O=5;E=0.32;R=15.41;rawP=1.97e-05;adjP=6.19e-05

The statistics column lists: C: the number of reference genes in the category; O: the number of genes in the gene set and also in the category; E: the expected number in the category; R: ratio of enrichment; rawP: p value from the Fisher exact test; adjP: p value adjusted for multiple testing by Benjamini-Hochberg correction.

Table 6. Intermediate nodes inferred by Rspider network (model D2)

Uniprot Accession number[1]	Gene name	Protein name	Top Function
P49902	NT5C2	Cytosolic purine 5'-nucleotidase	It hydrolyzes inosine 5'-monophosphate (IMP) and other purine nucleotides.
Q9NWZ5	UCKL1	Uridine-cytidine kinase-like 1	May contribute to UTP accumulation needed for blast transformation and proliferation.
P40939	HADHA	Trifunctional enzyme subunit alpha, mitochondrial	Alpha subunit of the mitochondrial trifunctional protein, which catalyzes the last three steps of mitochondrial beta-oxidation of long chain fatty acids.
O00142	TK2	Thymidine kinase 2, mitochondrial	Deoxyribonucleoside kinase that phosphorylates thymidine, deoxycytidine, and deoxyuridine.
P20839	IMPDH1	Inosine-5'-monophosphate dehydrogenase 1	Catalyzes the conversion of inosine 5'-phosphate (IMP) to xanthosine 5'-phosphate (XMP).
P24752	ACAT1	Acetyl-CoA acetyltransferase, mitochondrial	Plays a major role in ketone body metabolism.
Q9UKU7	ACAD8	Isobutyryl-CoA dehydrogenase, mitochondrial	Is an isobutyryl-CoA dehydrogenase that functions in valine catabolism.
P48728	AMT	Aminomethyltransferase, mitochondrial	The glycine cleavage system catalyzes the degradation of glycine.
Q8IVS8	GLYCTK	Glycerate kinase	ATP + D-glycerate = ADP + 3-phospho-D-glycerate.
P04818	TYMS	Thymidylate synthase	Contributes to the de novo mitochondrial thymidylate biosynthesis pathway.
none	LOC642969	Phosphoglycerate mutase 1 (brain) pseudogene	This gene has characteristics of a retrotransposed pseudogene derived from the PGAM1 gene.

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

Among the 27 intermediate nodes pointed out by the physical-interaction-based network (Figure 3B and Table 7) we found that 22 proteins were previously identified by bioinformatics analysis of the Aubipy$_c$ proteomic data [22]. In particular, the PPI Spider analysis confirmed that HSP60 mitochondrial (P10809, identified by proteomic studies) represents a central node interacting directly with intermediate nodes involved in the cell division cycle, apoptosis, cell differentiation, and cell migration (RAF1, SIRT1, SIRT3, SPG20) and indirectly with proteins associated with transcription regulation and mRNA splicing (PUF60, HMGA1, EIF2C3, ELF3). Associated with this last functional group we identified two new intermediate nodes: RG9MTD1, a component of mitochondrial ribonuclease P that functions in mitochondrial tRNA maturation and, also involved in the beta-oxidation; PAN2 involved in mRNA turnover. Moreover, we confirmed as intermediate nodes proteins related to cell growth, survival and apoptosis such as TOB1, DIDO1, CCNA1, WDR8, PPP2R1B and TSG101 [22]. Besides these previously identified nodes, the PPI Spider analysis pointed out two new intermediates associated to this functional group: MARK2 involved in microtubule dynamics regulation and RHOB a member of small GTPases belonging to the Ras protein superfamily. We also identified a new intermediate node, BARD1, associated with diverse range of cellular pathways such as DNA damage repair, ubiquitination and transcriptional regulation to maintain genomic stability. BARD1 is a protein that interacts with the breast cancer gene product BRCA1. The BRCA1-BARD1 heterodimer specifically mediates the formation of 'Lys-6'-linked polyubiquitin chains and coordinates several cellular pathways such ubiquitination and cell cycle in response to DNA damage.

Table 7. Intermediate nodes inferred by PPIspider network (model D2)

Uniprot Accession number[1]	Gene name	Protein name	Top Function
Q9P2S5	WDR8	WD repeat-containing protein WRAP73	Involved in a variety of cellular processes, including cell cycle progression, signal transduction, apoptosis, and gene regulation.
P19532	TFE3	Transcription factor E3	Transcription factor that specifically recognizes and binds E-box sequences (5'-CANNTG-3').
P17096	HMGA1	High mobility group protein HMG-I/HMG-Y	Involved in the transcription regulation of genes containing, or in close proximity to A+T-rich regions.
P04049	RAF1	RAF proto-oncogene serine/threonine-protein kinase	Serine/threonine-protein kinase that acts as a regulatory link between the membrane-associated Ras GTPases and the MAPK/ERK cascade.
Q7KZI7	MARK2	Serine/threonine-protein kinase MARK2	Serine/threonine-protein kinase involved in cell polarity and microtubule dynamics regulation
O99728	BARD1	BRCA1-associated RING domain protein 1	Probable E3 ubiquitin-protein ligase.
Q504Q3	PAN2	PAB-dependent poly(A)-specific ribonuclease subunit PAN2	Catalytic subunit of the poly(A)-nuclease (PAN) deadenylation complex, one of two cytoplasmic mRNA deadenylases involved in general and miRNA-mediated mRNA turnover.
P30154	PPP2R1B	Serine/threonine-protein phosphatase 2A 65 kDa regulatory subunit A beta isoform	The PR65 subunit of protein phosphatase 2A serves as a scaffolding molecule to coordinate the assembly of the catalytic subunit and a variable regulatory B subunit.
Q99759	MAP3K3	Mitogen-activated protein kinase kinase kinase 3	Component of a protein kinase signal transduction cascade. Mediates activation of the NF-kappa-B, AP1 and DDIT3 transcriptional regulators.
Q9NQT4	EXOSC5	Exosome complex component RRP46	Non-catalytic component of the RNA exosome complex which has 3'->5' exoribonuclease activity and participates in a multitude of cellular RNA processing and degradation events.
P62745	RHOB	Rho-related GTP-binding protein RhoB	Mediates apoptosis in neoplastically transformed cells after DNA damage.
Q7L0Y3	RG9MTD1	Mitochondrial ribonuclease P protein 1	Functions in mitochondrial tRNA maturation.
Q9H9G7	EIF2C3	Protein argonaute-3	Required for RNA-mediated gene silencing (RNAi).
Q99816	TSG101	Tumor susceptibility gene 101 protein	Component of the ESCRT-I complex, a regulator of vesicular trafficking process.
Q96EB6	SIRT1	NAD-dependent protein deacetylase sirtuin-1	NAD-dependent protein deacetylase that links transcriptional regulation

Discussion

In this study we carried out a detailed bioinformatic analysis on all the protein identified by proteomics experiments performed on cisplatin-sensitive human ovarian cancer cell line (A2780/S) treated with four gold compounds (Auranofin, Auoxo6, AuL12, Au2phen, Aubipy$_c$) and two ruthenium compounds (NAMI-A and RAPTA-T). The goal was to better understand the biological processes involved in metallodrug treatment, and to find possible common features in the mechanism of action of these compounds.

We performed Gene Ontology over-representation analysis (ORA) of the protein list using the web-tool Webgestalt. We found statistically enriched BP terms involved in metabolic process and protein folding. In line with these data, the pathway ORA performed on the input list, using both KEGG, PathwayCommons and Wikipathways databases, pointed out an enrichment in glycolysis/gluconeogenesis pathway providing further evidence for the glycolytic implication in the cytotoxic effects of these compounds as previously experimentally demonstrated for Aubipy$_c$ [22,28]. Moreover, we found statistically enriched pathways associated with protein biosynthesis and degradation in response to gold and ruthenium compounds strengthening the idea the idea that protein degradation via the ubiquitin-proteasome pathway is involved in the cytotoxicity of these metallodrug. In particular, the gold (III) compound AuL12 was reported to cause proteasome inhibition, and this was interpreted as one of the main reasons for its pronounced antiproliferative effect [35]. The enriched GO cellular components terms pointed out two major over-repre-

sented classes. The first was associated with the intracellular part and included cytoplasm and intracellular organelle such as mitochondrion (GO:0005739 – 30 genes). The other class comprised components on the membrane-enclosed lumen or the lumen of these organelle.

In order to discriminate the number of proteins that could be associated to gold and ruthenium treatment, we performed network enrichment analysis. As first approach, a pathway-based network was built using the Reactome database. This analysis pointed out that the intermediate proteins were involved in mitochondrial metabolism and in synthesis and degradation of purine/pyrimidine nucleotides. Concerning the mitochondrial metabolism, we found intermediate nodes associated with valine (ACAD8) and glycine (AMT) catabolism, ketone body metabolism (ACAT1) and in mitochondrial fatty acid beta-oxidation pathway (HADHA). Unchecked proliferation is a hallmark of cancer, and several oncogenic mutations reprogram cellular metabolism to support these processes. As a central metabolic organelle, mitochondria carry out critical biochemical functions for the synthesis of fundamental cellular components, including fatty acids, amino acids, and nucleotides [36]. Despite the extensive interest in the glycolytic phenotype of many cancer cells, tumors contain fully functional mitochondria that support proliferation and survival. Furthermore, tumor cells commonly increase flux through one or more mitochondrial pathways, and pharmacological inhibition of mitochondrial metabolism is emerging as a potential therapeutic strategy in some cancer. It has been previously demonstrated that ketone body production and re-utilization drive tumor progression and metastasis [37]. Thus, ketone bodies behave as onco-metabolites, and the enzymes HMGCS2, ACAT1/2 and OXCT1/2 are bona fide metabolic oncogenes. As such, the enzymes associated with ketone body production and re-utilization should be considered as new “druggable” targets for anticancer therapy. Moreover, experimental evidences support the hypothesis that there may be a compromised metabolism of long chain fatty acids in breast cancer due to a relative deficiency of HADHA, the four alpha chains in the 8-meric mitochondrial trifunctional protein (TFP) [38] involved in the b-oxidation of long chain fatty acids.

Among the intermediate nodes involved in synthesis and degradation of purine/pyrimidine nucleotides are noteworthy IMPDH1 and UCKL1. Studies have demonstrated that IMPDH is a rate-limiting step in the de novo synthesis of guanylates, including GTP and dGTP. The importance of IMPDH is central because dGTP is required for the DNA synthesis and GTP plays a major role not only for the cellular activity but also for cellular regulation. Two isoforms of IMPDH have been demonstrated. IMPDH Type I is ubiquitous and predominately present in normal cells, whereas the IMPDH Type II enzyme is predominant in malignant cells. IMPDH plays an important role in the expression of cellular genes, such as p53, c-myc and Ki-ras [39]. Concerning the uridine-cytidine kinase like-1 (UCKL-1), It has been demonstrated that its\levels\influence the proliferation and apoptosis of K562 cell line, suggesting a role in tumor cell’s progression through a normal cell cycle and in its susceptibility to apoptosis [40]. Furthermore, a physical-interaction-based network was built using IntAct database. Analysis of the input list by PPIspider resulted a significant network model, indicating two classes of proteins correlated to each other and belonging to: i) cell division cycle, apoptosis, cell differentiation, and cell migration; ii) transcription regulation and mRNA splicing. Among the 27 intermediate nodes pointed out by this physical-interaction-based network we found that 22 proteins were previously identified by bioinformatics analysis of the Aubipy$_c$ proteomic data [22]. The five new nodes are: RG9MTD1, BARD1, MARK2, PAN2 and RHOB. RG9MTD1, BARD1 and PAN2 are associated to RNA processing. In particular, BARD1 (BRCA1-associated RING domain protein) is a very interesting node. It is a probable E3 ubiquitin-protein ligase that acts with BRCA1 in DNA double-strand break repair and also in apoptosis initiation. Studies demonstrated that deleterious mutation in BARD1 may be responsible for a certain proportion of familial breast and/or ovarian cancer [41]. Among the intermediate nodes involved in cell division cycle, apoptosis, cell differentiation, and cell migration it is noteworthy RhoB. It is a small GTPase that are receiving increasing attention from cancer researchers owing to evidence that they modulate the proliferation, survival, invasion and angiogenic capacity of cancer cells [42]. Alterations in RhoB expression have been detected in a number of human cancers and several experimental evidences support the concept that RhoB functions as a tumor suppressor or negative modifier gene in cancer [43].

Conclusion

We can conclude that our bioinformatic analysis confirmed the action of gold and ruthenium compounds previously identified signaling pathways such as glucose metabolism, protein folding, protein biosynthesis and degradation as well as cell division cycle, apoptosis, cell differentiation, and cell migration. In addition, this study pointed out the involvement of new pathways such as synthesis and degradation of purine/pyrimidine nucleotides as well mitochondrial metabolism (amino acid metabolism, ketone body metabolism and fatty acid beta-oxidation pathway). These findings strengthen the idea of energy metabolism and mitochondrial function as possible target of both gold and ruthenium compound in A2780/ cell line.

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