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# New Insights into the Molecular Mechanisms of Selected Anticancer Metal Compounds through Bioinformatic Analysis of Proteomic Data

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## Abstract

Research has increasingly focused on cytotoxic gold and ruthenium compounds as anticancer drug candidates. From proteomic investigations, clearly emerged that a few different cellular pathways relevant for the comprehension of the pharmacological actions are specifically modulated by them. To gain a better intepretation of their cellular effects, we decided to undertake a comprehensive bioinformatic analysis of the available proteomic results. Data obtained from prevously published treatments were grouped and mapped in the PPI Spider on the web portal Bioprofiling (http://www.BioProfiling.de/gene_list). A preliminary map of protein-protein interactions was built up, and some mechanistically relevant features highlighted. In total, 34 proteins resulted to be direct gold and ruthenium compounds interactors; we built a statistically significant interaction network that grouped together all the proteins differentially expressed. Moreover, we showed as intermediate protein cREL a Component of the NF-kappa-B. This study explores the affected protein pathways from an interactomic prospective stressing the importance of advanced bioinformatic analysis.

**Keywords:** Proteomics of metallodrugs; Protein-protein interaction; Bioinformatic analysis

## Introduction

Proteomic profiling offers the opportunity to identify proteins that mediate apoptotic pathways, when cells are treated with cytotoxic agents [1]. Proteomic results have the potential to provide new insight into the molecular mechanisms of this group of molecules at the cellular level [2]. More than 99% of currently approved clinical drugs are organic compounds. In contrast, the percentage of metal-containing drugs (metallodrugs) is very low [3]. In cancer chemotherapy, however, platinum coordination compounds are essential agents, with proven efficacy against a variety of tumors. The discovery of the anticancer properties of cisplatin (cis-dichloro diammineplatinum (II), cis-$[Pt^{II}Cl_2(NH_3)_2]$ has triggered the development of several other metal-based compounds [4]. Because of the established clinical applications of these platinum-based drugs, the number of research initiatives to identify other metallodrugs that can be used in cancer therapy has increased. The use of transition metal compounds other than platinum has also attracted attention, to improve the therapeutic effects and to overcome the disadvantages of current platinum-based drugs [5]. Various metal complexes (e.g., Pt, Au, and Ru) are currently evaluated, either clinically or experimentally, as therapeutic agents in treatment of malignant diseases, including several cancer types.

Gold coordination complexes, for instance, demonstrate outstanding cytotoxic properties, and certain ruthenium complexes possess a remarkable ability to inhibit metastases of solid invasive tumors. Previous studies showed that cytotoxic gold(III) compounds are able to induce cell death through apoptosis [6,7]; essentially triggered through a direct mitochondrial damage [8]. Within this frame, the pivotal role of thioredoxin reductase as a probable target for cytotoxic gold compounds was highlighted [9]. Among currently investigated anticancer metallodrugs, a few ruthenium- compounds hold great promise as experimental antimetastatic agents [10,11]. Interestingly, their relevant antimetastatic actions are usually associated to weak cytotoxicity, and to acceptable profiles of systemic toxicity that render them very suitable for further drug development and for clinical use [12]. For instance, imidazolium trans-[tetrachloro(DMSO)(imidazole) ruthenate(III)] NAMI-A was found to reduce dramatically the number and the size of metastases in certain *in vivo* models of cancer, e.g. the Lewis lung carcinoma, and is currently undergoing phase II clinical trials [13]. Similarly, the ruthenium(II) organometallic complex [Ru(η6-toluene)Cl2(PTA)] (where PTA=1,3,5-triaza-7-phospha-tricyclo-[3.3.1.1] decane) named RAPTA-T is weakly cytotoxic *in vitro*, while showing favorable antimetastatic effects *in vivo* [14], as well as remarkable anti-angiogenic properties [15]. The mode of action of antimetastatic ruthenium compounds remains elusive and largely unexplored. For these reasons, a proteomic approach was utilized for investigating the mode of action of these anticancer metallodrugs [16,17].

In previous papers, we reported lists of proteins found to be differentially expressed in the human ovarian cancer cisplatin-sensitive

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cell line (A2780/S), after treatment with four different cytotoxic gold compounds, namely: Auranofin [2], Auoxo6 [2], AuL12 [1] and Au2Phen [1]. In a very recent paper, we have used for the first time, 2D-DIGE to investigate the mode of action of two anticancer ruthenium compounds, namely NAMI-A and RAPTA-T in A2780/S human cancer cells [18]. Changes in the expression of intracellular proteins after the treatment at non-cytotoxic concentrations were monitored.

In this paper, we carry out a detailed bioinformatic analysis on proteins identified in those previous proteomic studies, including the protein-protein interaction network, in order to better understand the biological processes involved in metallodrugs treatment, and find possible common features in the mechanism of action of these compounds. To interpret all the protein lists obtained in our previous proteomics results, with the two classes of metallodrugs, we used a BioProfiling.de [19] tool: PPI spider [20] freely available on the analytical web portal (http://www.BioProfiling.de/gene_list). In one submission, the protein/gene list can be analyzed by a collection of tools. The input list is translated into a network model, according to the topology of the two web-based tools. We provide models of protein interactions that represent the most probable scenario of how proteins identified within the list are connected, in order to identify a possible combination of metallodrugs in experimental chemotherapy of cancer disease, to increase the effectiveness of metallodrugs treatment. The expression of many of these proteins resulted affected by most of the gold compounds used, and this strengthens the validity of the results.

## Methods

### Bioinformatic analysis

**BioProfiling.de**: BioProfiling.de provides a comprehensive analytical toolkit for the interpretation of protein lists. Asinput, BioProfiling.de accepts a protein list. As output, in one submission, the gene list is analyzed by a collection of tools, which employs advanced enrichment or network-based statistical frameworks. BioProfiling.de provides a common interface for the collection of recently developed tools [19]; description and details of the tools can be found in original publications [20]. The gene list is profiled with respect to the most information available regarding gene function, protein interactions, pathway relationships, *in silico* predicted microRNA to gene associations, as well as, information collected by text mining. The web portal is freely available at http://www.BioProfiling.de/gene_list.

To better understand the key regulated biological processes occurring after metallodrugs A2780/S cells treatment, bioinformatics analysis to the differentially expressed list of proteins identified by our previous quantitative proteomic studies, were carried out. The network analysis used has been freely available web-based tool for the interpretation of experimentally derived protein lists in the context of a global PPI network. A dataset containing the standard gene symbols of the identified proteins was uploaded. The significantly enriched PPI networks have been determined with the default parameter settings.

**Statistical methodology**: PPI Spider implements a statistical framework for the interpretation of protein lists in the context of a global PPI network. The statistical significance of the inferred model is estimated based on the distribution of the model size for a random protein list. Statistical significance of the model is computed by a Monte Carlo simulation procedure [19,20].

**Network visualization using Cytoscape**: Cytoscape (http://www.cytoscape.org/) is a software platform for visualizing molecular interaction networks and integrating these interactions with gene expression profiles. Cytoscape supports several algorithms for the layout of networks [21].

## Results

### Bioinformatic analysis of proteomic data

To gain a deeper insight into the mechanism of action of selected anticancer Gold and Ruthenium compounds, and establish whether these metallodrugs have common protein targets, we performed a bio-informatic analysis on the lists of proteins previously identified by proteomic studies. We have used the web-based tool BioProfiling.de, which provides a common interface for the collection of recently developed tools; description and details of the tools can be found in the original publications [20].

We used proteins previously identified, showing variation in expression level following treatment with AuL12, Au2Phen, Auoxo6, Auranofin and with NAMI-A and RAPTA-T in a cisplatin-sensitive human ovarian cancer cell line (A2780/S).

To start the analysis, we uploaded a text file with the 34 proteins, and we selected *Homo sapiens* as organism. We put in the same analysis all the proteins, listed in Table 1, whose expression resulted modified after the two different treatments. The expression of many of these proteins resulted affected by most of the gold compounds used, and this strengthens the validity of the results. The table reported in Figure 1 summarizes the results for this submission using the PPI Spider. In the table, it is also shown the models' definition, which specifies the maximal allowed distance between two input proteins connected (D0 means directly connected, D1 one intermediate is allowed, D2 two intermediate are allowed), the number of input proteins covered by the system, and the corresponding significance of the best inferred model. As it is evident the inferred network model D2 covers 22 proteins of the 34 used as input. The p-value estimated for this model is statistically significant (p-value less than 0.005). We decided to analyze this network, and in Figure 1 is reported, a graphical illustration of the protein-protein interaction network created by PPI Spider and obtained by Cytoscape: http://www.cytoscape.org/, the software platform for visualizing the results. Four proteins belong to the Ruthenium molecules treatments (circle in the Figure 1), and 18 proteins to the Gold metallodrugs. This network includes intermediate proteins not previously detected in our proteomic studies, reported in Table 2, and represented as triangles in the network of Figure 1. These intermediate proteins could be potentially interesting targets for metallodrugs, even if their expression level could not be directly influenced by the treatment.

Among them, the software found few proteins correlated with anti-proliferation, and/or apoptosis. To group the proteins present in the PPI network, we divided them in two classes: Class A including proteins involved in apoptosis, cellular proliferation and DNA stability, and Class B that includes proteins responsible of cytoskeleton dynamics, splicing regulation and protein biosynthesis.

The two classes are reported in Figure 1:

a) Class A–Apoptosis, cellular proliferation and DNA stability: Important nodes of this Group are represented by two intermediate proteins: the TNF receptor-associated factor 1 (TRAF1–Q13077), an adapter molecule that plays a role in the regulation of cell survival and

| Accession number[a] | Protein name | Top Function | Gold | Ruthenium |
|---|---|---|---|---|
| P07437 | Tubulin beta chain | constituent of microtubules. | Guidi et al. [1] | |
| Q9BWD1 | Acetyl-CoA acetyltransferase, cytosolic | 2 acetyl-CoA = CoA + acetoacetyl-CoA. | | Guidi et al. [18] |
| P60174 | Triosephosphate isomerase | associated with cardiomyopathy and increased susceptibility to infection. | Magherini et al. [2] | |
| P55209 | Nucleosome assembly protein 1-like 1 | chromatin formation and regulation of proliferation. | Guidi et al. [1] | |
| P29692 | Elongation factor 1-delta | Protein synthesis | | Guidi et al. [18] |
| P23919 | Thymidylate kinase | Pyrimidine metabolism | Guidi et al. [1] | Guidi et al. [18] |
| P49773 | Histidine triad nucleotide-binding prot 1 | Hydrolyzes adenosine 5'-monophosphoramidate substrates | Guidi et al. [1] Magherini et al. [2] | Guidi et al. [18] |
| Q9Y3F4 | Ser-thr kinase receptor-associated protein | spliceosomal snRNP assembly | Guidi et al. [1] | |
| Q9NQR4 | Omega-amidase NIT2 | omega-amidase activity | Guidi et al. [1] | Guidi et al. [18] |
| P30041 | Peroxiredoxin-6 | redox regulation of the cell. | Magherini et al. [2] | |
| P62253 | Ubiquitin-conjugation enzyme E2 G1 | Protein modification | | Guidi et al. [18] |
| P31943 | Heterog nuclear ribonucleoprot H | ribonucleoprotein complexes. | Magherini et al. [2] | |
| P09429 | High mobility group protein B1 | DNA binding proteins that associates with chromatin and bend DNA. | Magherini et al. [2] | |
| Q9NRF9 | DNA pol epsilon sub 3 | nucleosome-remodeling activity | | Guidi et al. [18] |
| P62258 | 14-3-3 protein epsilon | Adapter protein binds to a large number of partners. | Guidi et al. [1] | |
| P63104 | 14-3-3 protein zeta/delta | Adapter protein implicated in the regulation of signaling pathways. | Guidi et al. [1] | |
| P12004 | Proliferating cell nuclear antigen CICLIN | This protein is an auxiliary protein of DNA polymerase delta | Guidi et al. [1] | |
| P07339 | Cathepsin D | active in intracellular protein breakdown. | | Guidi et al. [18] |
| P62937 | Peptidyl-prolyl cis-trans isomerase A Cyclophilin A | accelerate the folding of proteins. | Guidi et al. [1] | |
| P61978 | Heterog nuclear ribonucleoprot K | pre-mRNA-binding proteins. | Guidi et al. [1] | |
| P15311 | EZRINA | connections of major cytoskeletal structures. | Magherini et al. [2] | |
| Q06830 | Peroxiredoxin 1 | redox regulation of the cell | Magherini et al. [2] | Guidi et al. [18] |
| Q9UMX0 | Ubiquilin 1 | Interacts with the proteasome 19S subunit | Guidi et al. [1] | |
| P14174 | Macrophage migration inhibitory factor | Pro-inflammatory cytokine | Guidi et al. [1] | |
| P60709 | Actin, cytoplasmic 1 | involved in various types of cell motility. | Guidi et al. [1] | |
| Q14257 | Reticulocalbin-2 | Binds calcium. | Guidi et al. [1] | |
| P24534 | Elongation factor 1-beta | Protein synthesis | Guidi et al. [1] | |
| P61758 | Prefoldin subunit 3 | Binds specifically to cytosolic chaperonin | | Guidi et al. [18] |
| P26447 | Protein S100-A4 | calcium-binding protein | | Guidi et al. [18] |
| P28838 | Cytosol aminopeptidase | involved in the processing turnover of intracellular proteins | Guidi et al. [1] | |
| Q99714 | 3-hydroxyacyl-CoA dehydrogenase type-2 | Functions in mitochondrial tRNA maturation. | Magherini et al. [2] | |
| P33316 | Deoxiuridine 5' triphosphate nucleotidohydrolase, mit | involved in nucleotide metabolism | | Guidi et al. [18] |
| Q969Q6 | Serine/Treonine-protein phosphatase 2A | play a role in the activation-induced cell death | | Guidi et al. [18] |
| Q08752 | Peptidyl-prolyl cis-trans isomerase D | accelerates the folding of proteins | | Guidi et al. [18] |

**Table 1:** Experimentally identified proteins list used as input in PPI Spider analysis.

| Model Defenition | The Number of input genes (covered by the model) | p-value |
|---|---|---|
| Model D1 | 1 | < 0.99 |
| Model D2 | 22 | < 0.005 |
| Model D3 | 29 | < 0.395 |

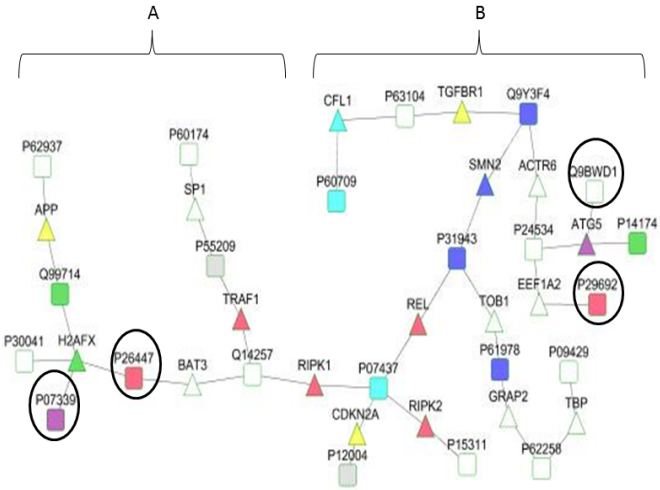


**Figure 1:** (Upper side) Table of results obtained by PPI Spider analysis after the submission of the previously identified proteins list and (lower side) network inference model D2 visualization obtained by Cytoscape. See text for details.

apoptosis [22], and the Transcription factor Sp1 (SP1–P08047). This protein can activate or repress transcription in response to physiological and pathological stimuli, and regulates the expression of a large number of genes involved in apoptosis, differentiation and immune responses [23]; moreover, it plays a role in the cellular response to DNA damage. Among the proteins of Class A, we found the previously identified Reticulocalbin-2 (Q14257) [1], belonging to the CREC protein family, including promising biomarkers in a variety of diseases [24]. We found other proteins strictly correlated with the DNA damage and DNA assembly, and identified in previous proteomics experiments. Among them, the apoptotic marker NALP1 (Nucleosome assembly protein 1 P55209) [1], connected with the two previously discussed intermediate nodes: the adapter molecule TRAF1 and the transcription factor Sp1. An intermediate node mapped in this network and involved in DNA repair mechanisms [25] is the variant histone H2A (H2AFX–P16104), which replaces conventional H2A histon in a subset of nucleosomes [26].

b) Class B–Cytoskeleton dynamics, splicing regulation and protein biosynthesis: In this class are included many proteins present as intermediate nodes and involved in cytoskeleton organization, like the protein Cofilin1 (CFL–P23528), important for the regulation of actin cytoskeleton dynamics, and for the progression through mitosis [27]. It is connected to the cytoplasmic βactin (P60709), identified by proteomics experiments [1], that is a component of the cytoskeleton and a mediator of internal cell motility. Among this group, we found another intermediate node, an actin-related protein involved in cytoskeleton organization [28] (ACTR6–Q9GZN1). The protein SMN2 (Q16637) is a node between two input proteins involved in splicing: the Heterogeneous nuclear ribonucleoprotein H (P31943) [2], and the Serine-threonine kinase receptor-associated protein (Q9Y3F4) [1]. The SMN2 protein plays an essential role in spliceosomal assembly, and is required for pre-mRNA splicing [29]. Moreover, an intermediate node which connects the two elongation factors, previously identified by proteome analysis (Elongation factor 1-beta - P24534 [1] and Elongation factor 1-delta - P29692 [18], is the Elongation factor 1-alpha 2 (EEF1A2–Q05639). These components of the eEF1 complex have the canonical functions in translation elongation [30], and many non-canonical functions, including roles in nuclear export events, turnover of misfolded proteins, binding and bundling of the actin cytoskeleton and apoptosis. Moreover, the cellular senescence has been found associated with changes of eEF1 levels in colon and lung cancer cell lines [31]. The A and B classes of proteins are connected together by an intermediate node: Receptor-like protein kinase1 (RIPK1–Q13546) a Serine-threonine kinase, which transduces inflammatory and cell-death signals (necroptosis) and DNA damage [32].

## Enriched network analysis

The new list of proteins, obtained by the PPI Spider analysis described above, and including: the proteins previously identified by proteomics experiments and reported in Table 1, and the nodes corresponding to new intermediate proteins and inferred by the PPI spider analysis, has been used as input list for an enriched second PPI analysis. This new list of proteins was used, in order to identify a new network model to provide new insight into possible biological mechanisms of metallodrugs. The table of Figure 2 shows the results obtained with this new submission: the inferred network model D1 covers 40 proteins from the 52 used as input, and with the model D2 are covered 42 proteins. Both the models show a statistical significance with a $p<0.005$. Since the proteins connected in the model D1 belong to the input list, and there are not missing proteins in this model, we decided to analyze the model D2 to find new information about new intermediate proteins (nodes), correlated with the input list. In Figure 2 is represented the graphical illustration of the D2 model obtained by Cytoscape, and modified by the authors. In this sub-network are present two intermediate proteins (triangles in the Figure 2): the Glyceraldehyde-3-phosphate dehydrogenase (GAPDH-P04406), and the Cleavage stimulation factor subunit 2 (CSTF2-P33240). In our network, we found GAPDH connected with the Peroxiredoxin-1 (Q 06830) [2,18], identified by previous proteomic analysis, which is involved in redox regulation of the cell. Recently, Maller et al. [33], found that GAPDH's oxidation state controls the protein biosynthesis, and its nitrosylation state is important to induce apoptosis. Moreover,

| Model Defenition | The Number of input genes (covered by the model) | p-value |
|---|---|---|
| Model D1 | 40 | < 0.005 |
| Model D2 | 42 | < 0.005 |
| Model D3 | 49 | < 0.29 |

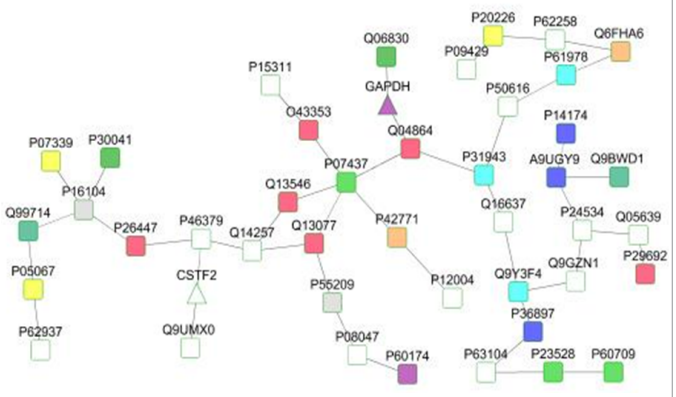


**Figure 2:** (Upper side) Table of results of the network obtained by PPI Spider analysis after the submission of the enriched proteins list and (lower side) network inference model D2 visualization obtained by Cytoscape. See text for details.

| Entry name | ID | Top Function |
|---|---|---|
| SP1-human | P08047 | Transcription factor Sp1 |
| TRAF1_human | Q13077 | TNF receptor-associated factor 1 |
| BAT3 - BAG6_HUMAN | P46379 | Large proline-rich protein BAG6 |
| H2AFX | P16104 | Histone H2A.x |
| APP | P05067 | Amyloid beta A4 protein |
| RIPK1 | Q13546 | Receptor-interacting serine/threonine-protein kinase 1 **(EC 2.7.11.1)** |
| CDKN2A | Q9NXV6 | CDKN2A-interacting protein (Collaborator of ARF) |
| RIPK2 | O43353 | Receptor-interacting serine/threonine-protein kinase 2 **(EC 2.7.11.1)** |
| REL | Q04864 | Component of the NF-kappa-B p65-c-Rel complex |
| TOB1 | P50616 | Protein Tob1 |
| GRAP2 | Q6FHA6 | GRAP2-related adaptor protein 2 |
| TBP | P17980 | 26S protease regulatory subunit 6A |
| SMN2 | Q16637 | Survival motor neuron protein |
| TGFBR1 | P36897 | TGF-beta receptor type-1 **(TGFR-1) (EC 2.7.11.30)** |
| CFL | P23528 | Cofilin-1 regulation of cell morphology and cytoskeletal organization |
| ACTR6 | Q9GZN1 | Actin-related protein 6 (hArp6) (Harpx) |
| ATG5 | A9UGY9 | ATG5 autophagy related 5 homolog (S. cerevisiae), isoform CRA_b (ATG5 autophagy related 5-like) |
| EEF1A2 | Q05639 | Elongation factor 1-alpha 2 (EF-1-alpha-2) (Eukaryotic elongation factor 1 A-2) (eEF1A-2) (Statin-S1) |

**Table 2:** Intermediate nodes inferred by PPI network.

they propose its potential peroxidase activity and the decomposition of $H_2O_2$ by GAPDH is already acknowledged. In this network, GAPDH is also connected with REL (Q04864) component of the NF-kappa-B p65-c-Rel complex, which plays a role in the regulation of apoptosis [34]. CSTF2 is one of the multiple factors required for polyadenylation and 3'-end cleavage of mammalian pre-mRNAs. This subunit is directly involved in the binding to pre-mRNAs. Aragaki et al. [35], found that there is a significant correlation of CSTF2 expression, with poor prognosis for patients with lung cancers. CSTF2 could play important roles in cell proliferation and invasion.

## Discussion

The goal of this work was to elucidate whether the list of proteins previously identified by proteomics analysis, could perform biological interactions. Many biological functions are carried out by the integrated activity of interacting proteins. We aimed to build a single, representative network that possibly grouped together all the proteins differentially expressed, and reported in all previously published papers from our laboratory. It is hypothesized that proteins interact through specific interactions and a web tool, which employs a network-based statistical framework for the interpretation of protein lists has been used in this work. We used the global PPI Spider network, using all the previously identified proteins as input. In order to reduce the probability of false positives, most of the proteins used as input, have been previously validated by western blot analyses. Moreover, the expression of many of these proteins resulted affected by most of the gold compounds used, and this strengthens the validity of the results. After this first analysis, we obtained a new list of proteins, including the intermediate nodes, new proteins representing common interactors, as predicted by PPI Spider. We reload this "enriched" new list for a further PPI analysis. Comparing all the results, we noticed that the functions of some proteins could be correlated. The first correlation is between two proteins that are involved in RNA processing. The second correlation we found was between two proteins involved in connections of cytoskeletal components to membrane: the protein Ubiquilin-1 and the protein Ezrin. In our recent paper, we reported that two ruthenium compounds caused proteomic alterations, and we found that 9 proteins are in common between RAPTA-T and NAMI-A, implying that their respective mechanisms are highly correlated [18]. Analysis of all these proteins by PPI Spider resulted a significant network model, indicating two classes of proteins correlated to each other and belonging to: i) Apoptosis, cellular proliferation and DNA stability, and ii) Cytoskeleton dynamics, splicing regulation and protein biosynthesis. Among these two groups, we found some intermediate proteins able to connect most of the proteins in the input list. A new protein-protein interaction analysis, using the enriched new input list resulted in a second network model, showing as intermediate nodes two proteins involved in cellular redox regulation and dynamics RNA stability, suggesting a common mechanism of action for the metallodrugs studied. These proteins may be further investigated as possible drug targets, and more extensive bioinformatics analyses will be carried out to better identify and characterize the cellular perturbations induced by these substances.

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