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 different cellular pathways relevant for the comprehension of the pharmacological actions are specifically modulated by them. To gain a better interpretation of their cellular effects, we decided to undertake a comprehensive bioinformatic analysis of the available proteomic data. Results obtained from previously 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-[PtCl\(_2\)(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, to overcome the disadvantages of current platinum-based drugs [5]. For these reasons, a proteomic approach was utilized for investigating the mode of action of these anticancer metallodrugs [6,7]; essentially triggered by observation 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...
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 metallo-drugs 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 metallo-drugs, 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 metallo-drugs in experimental chemotherapy of cancer disease, to increase the effectiveness of metallo-drugs 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. As input, 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](http://www.BioProfiling.de/gene_list).

To better understand the key regulated biological processes occurring after metallo-drugs 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/](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 metallo-drugs 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/](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 metallo-drugs. 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 metallo-drugs, 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:

1. 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
Table 1: Experimentally identified proteins list used as input in PPI Spider analysis.
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 histone 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–Q09GZ1). 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 kinasel (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 metalloids. 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 (Q06830) [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,
they propose its potential peroxidase activity and the decomposition of H₂O₂ 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.

**Acknowledgements**

We thank AIRC IG 12085 for financial support.

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This article was originally published in a special issue, Microarray Proteomics handled by Editor(s), Dr. Qiangwei Xia, University of Wisconsin-Madison, USA

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