Metallomics

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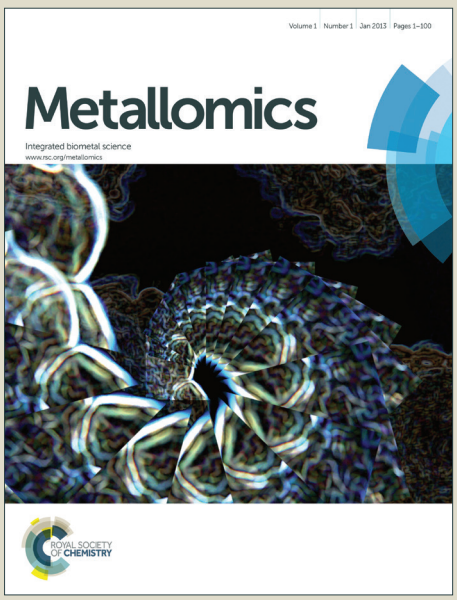

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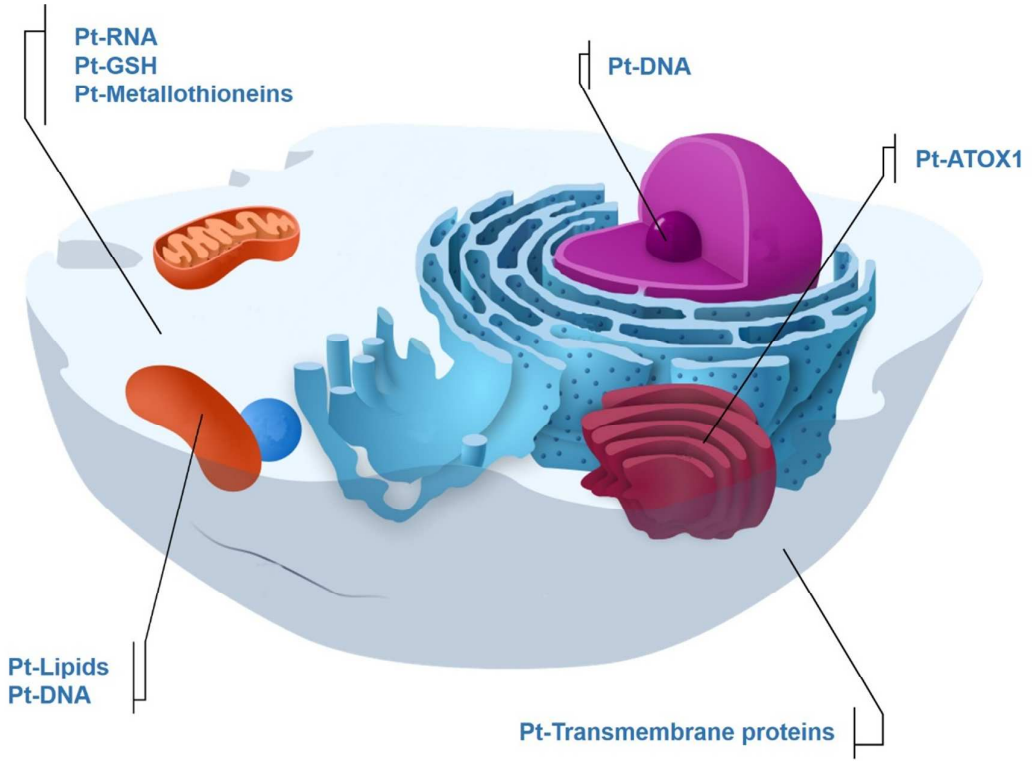

The growing importance of biomarkers in platinum-based chemotherapy schemes are forseen to play important role in medical decision-making. This mini review points out some targets for metallomics to help them to reach this goal sooner.

Biomarkers to Assess the Efficiency of Treatment with Platinum-Based Drugs: What Can Metallomics Add to it?

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Abstract

Since the approval of cisplatin as an antineoplastic drug, the medical and the scientific communities are concerned about the side effects of platinum based drugs, which have been the dose limiting factor that leads to reduced treatment efficiency. Other important issue is the intrinsic or acquired resistance of some patients to treatment. Identifying proper biomarkers is crucial to evaluate the efficiency of the treatment, assisting physicians to determine, at early stages, whether the patient presents or not resistance to the drug, minimizing severe side effects, and allowing them to redirect the established course of chemotherapy. A great effort is being made to identify biomarkers that can be used to predict the outcome of the treatment of cancer patients with platinum-based drugs. In this context, the metallomic approach is not yet being used to its full potential. Since the basis of these drugs is platinum, the monitoring of biomarkers containing this metal should be the natural approach to evaluate the treatment progress. This review intends to show where the research in this field stands and points out some gaps that can be filled by metallomics.

Introduction

Since the late 1970's, platinum based drugs are being successfully used against a wide variety of tumours and they are still some of the most important agents for cancer treatment[1]. The severe side effects concern the medical and scientific communities since the first trials and have been the dose limiting factor, which reduces treatment efficiency[2]. Another important limitation of such treatment is the intrinsic or acquired resistance to these drugs. The mechanisms of such resistance have been reviewed recently by Galluzzi *et al.*[3] and this is still a hot topic of scientific investigation[3, 4, 5]. Once the treatment is not being effective or the prognostic is not favourable, there is no point to expose patients to the severe side effects caused by platinum based drugs[6, 7].

Biomarkers that can securely indicate the resistance of tumours or help to determine a prognostic to patients under treatment may play an important role, improving treatment efficiency through the adjustment of the chemotherapy strategy, and minimizing patients exposure to the drug side effects[6, 8]. According to the National Cancer Institute, on its Dictionary of Cancer Terms[9], a biomarker is defined as a "*biological molecule found in blood, other body fluids, or tissues that is a sign of a normal or abnormal process, or of a condition or disease. A biomarker may be used to see how well the body responds to a treatment for a disease or condition*". However, the distance between the first attempts to identify a biomarker and its acceptance and use in the clinic is huge, and despite that, their scientific, social and economic potentials makes such endeavour worth[8].

The importance of biomarkers can be inferred from the number of publications about the topic and by the growing number of specific databases condensing information about them, such as GOBIOM, BiomarkersBase, CancerDrive among others. A simple

search for "cisplatin" at GVK BIO's Online Biomarker Database (GOBIOM)[10] returns around nine hundred molecules, however, the United States Food and Drug Administration (FDA) has approved only ten of them. Within this database, the biomarkers are classified as biochemical, genomic, cellular, scoring scale, imaging, or physiological. These classes are related to the molecule characteristics and/or to the method applied to measure them. The biomarkers classified as the biochemical ones accounts for 59.7% of the outcome of the search, while the genomic ones represent 29.3% of the total. Other classes of biomarkers are also represented: cellular (4.9%) and imaging (1.7%). Scoring scale and physiological types of biomarkers to access the effectiveness of platinum-based chemotherapy were not considered due to the scope of this review. The number of "under investigation" biomarkers reflects Drucker *et al.* recent statement, that after the development of the "omics" the search for molecular indicators increased substantially[8]. Most biochemical and genomic biomarkers were conceived using genomic or proteomic techniques. Metallomics, as the newest *omic* approach, could be useful to identify biomarkers, especially to diagnose the action of metal-containing drugs, but, so far, the authors were not able to find any reported biomarker in clinical use identified through this approach.

Many biochemical and genomic biomarkers have been suggested for the detection of different diseases, however, most of them is not specific and does not allow the identification of diseases in their earlier stages. Most of potential biomarkers did not jump from the laboratory stage to the clinical studies[8]. Despite that difficulty, the research effort put on the identification of molecular predictors is huge. By understanding the biochemistry of platinum-based drugs in the cellular environment, their mechanisms of action or tumour resistance towards them, potential biomarkers can be foreseen and lead to reliable indications of the efficacy of treatment. In a near future,

as the platinum pathway in human organism will be determined, other biological processes can be understood and reliable predicted. Platinum-containing biomarkers are post treatment indicators, which can bring information about individual's metabolism of the drug, making possible to evaluate and predict the outcome of the treatment. Platinum-based antineoplastic drugs allow the search for specific biomarkers to go into this direction, as platinum is a very rare element and can be detected in a sensitive way. In this work, selected biomarkers being used as clinical assistants in cancer prognostic, in the evaluation of the effectiveness of platinum chemotherapy or in the determination of the tumour resistance towards platinum-based drugs are reviewed. In addition, brief descriptions of the mechanism of action of these drugs, tumour resistance and the technologies used to investigate the biomarkers are presented, aiming to indicate the potential of metallomics in identifying reliable Pt-containing biomarkers.

Mechanisms of Platinum Based Drugs Activity

A complete comprehension of the mechanisms of action of platinum-based drugs has not yet been established, and conflicting data is observed in the literature[11]. What seems as consensus is that the main cytotoxic mechanism of platinum-based drugs is the formation of covalent bond with the DNA, preventing cell division and inducing cell apoptosis. However, several other factors corroborate to their efficiency. Undoubtedly, cisplatin is the more studied drug of this group and serves as a reference in terms of activity and pharmacological principle to other drugs, thus its pharmacokinetics is briefly presented, and main differences among the drugs are highlighted.

After application of cisplatin intravenously, the molecule undergoes hydrolysis due to the chloride concentration in the blood plasma and is carried by the blood in its original form. About 90% of cisplatin physically interacts with plasma proteins such as albumin,

and the remainder is solubilized. Carboplatin is more stable than cisplatin in the blood; on the other hand oxaliplatin is hydrolysed even at high chloride concentrations. In the blood stream oxaliplatin interacts strongly with erythrocytes and blood proteins and only 12 % is available to enter other cells[12]. Cisplatin is rapidly distributed in organs and tissues and is found mainly in the liver and kidney[13]. The drug concentrations in plasma rapidly decreases, with a half-life of ultrafilterable platinum in plasma ranging from 20 to 45 min. Approximately 25% of the drug is excreted in the urine over the first 24 h, up to 90% is eliminated by up to five days[14].

Spatial distribution of platinum in lymphocytes of patients treated with cisplatin and in cultured cell lines are shown to be nearly the same: about 20% remain in the cell membrane; about 60% are found in the cytosolic fraction; 10% at the cytoskeleton and 10% in the nucleus[15]. After crossing the cell membrane by passive or active transport[16], cisplatin is in a medium with a chloride concentration of about 3 to 20 mmol L^{-1} and undergoes hydrolysis converting into the most active forms of the drug[3]. Upon entering the cell, cisplatin interacts with the plasma membrane disrupting lipid-lipid and lipid-protein interactions[17]. It is believed that this interaction may lead to recruitment and activation of caspase 8 and, consequently, in the other caspase cascade leading to caspase-dependent cell apoptosis. Cisplatin in the cytoplasmic fraction interacts with a number of nucleophilic species such as endogenous reduced glutathione (GSH), methionine, metallothionein, and other cytoplasmic proteins[3,18,19,20]. The action of cisplatin in the cytoplasm has a depletive character over reduced species, providing a favourable environment to oxidative stress, which enhance the action of the drug in the cell nucleus[3], at the endoplasmic reticulum and at the mitochondria[17,21,22]. In the cytoplasmic fraction of the cell, cisplatin interacts with RNA, pledging to cell signalling and gene expression[23]. Considering what is known today, these are the major cytotoxic

mechanisms of cisplatin. Most of other drug interactions in the cytoplasm are considered to be sources of resistance.

In the cell nucleus, cisplatin binds to the DNA modifying its structure and preventing cell replication. About 1% of the total cisplatin absorbed by the cell is connected to the nuclear DNA[24]. Cisplatin interacts with DNA to form a series of adducts, preferably linking the N7 position of the purine bases and can form double adducts with connections to a single strand or between the DNA strands. Cisplatin may also form adducts with DNA bases or DNA-protein adducts. If DNA damage is too extensive the cell progresses to apoptosis, the most important signalling pathway linking the DNA damage caused by cisplatin to apoptotic cell death involves the activation, in sequence, of a series of proteins in the nucleus and cytoplasm. The process starts by activating damage checkpoint 1 kinase (CHEK 1) proteins, and in turn phosphorylates the tumour suppressor protein p53[25,26,27]. Once activated p53 triggers a number of lethal functions in the nucleus and cytoplasm, which lead to apoptosis of the cell[28,22.29].

Current biomarker investigation tools

In the last 20 years, the possibility of biomarkers investigation has immensely grown due to the advent of new technologies and tools made available to researchers. A myriad of approaches making use of these tools and the possible combinations of them are described on the literature. The advances in data treatment are also very important once scientists need to combine numerous results emerging from different sources in order to extract the information about the responses to cancer treatment. These advances represent the basis for the implementation of new reliable indicators that will be used by physicians in a near future. Some of the main advances present in the literature are briefly discussed.

At genomic field, the Next-Generation Sequencing (NGS) has been extensively used to identify somatic mutations and to determine the expression of many genes involved in crucial pathways such as EGFR and BRAF[30]. It has also been used to determine the expression of KRAS gene, an relevant potential biomarker[31]. NGS platforms are based on sequencing-by-synthesis technology, with a DNA polymerase or ligase as the key component. Roche 454, Illumina, Helicos, and PacBio (Pacific Biosciences) use a DNA polymerase to drive their sequencing reaction, while SOLiD (Life Technologies) and Complete Genomics use a DNA ligase. The sequencing platforms can be further categorized as either single molecule-based (sequencing a single molecule) or ensemble-based (sequencing of multiple identical copies of a DNA molecule)[32]. Due to the huge amount of data produced with one single sequencing experiment (up to 200 million 100-nucleotides reads), careful experimental delimitation and a clear definition of aimed data are mandatory. Data treatment for identification of biomarkers through differential expression, for example, must be sought with the aid of specialized and validated software[32].

Besides NGS, other powerful tools to investigate biomarkers are DNA microarrays. They enable high-throughput gene expression profiling though specific reaction of complementary DNA (cDNA) fixed on a rigid support with target RNA present in the sample[33]. Through the overall RNA determination, the genic expression can be inferred. Many examples of the use of DNA microarray platform to study differences in genetic expressions, that could be used as biomarkers, are found in the literature for different malignancies such as lung adenocarcinoma[34], prostate cancer[35], among others , the approach was also used to evaluate DNA methylation changes[36].

Proteomic approach makes use a group of techniques that enable large-scale identification, characterization, and quantification of proteins in complex biological

samples[37]. Classical techniques making use immunoassays such Enzyme-Linked Immunosorbent Assay (ELISA) on research and are the most common approach in clinical exams already prescribed by clinicians[38]. Although ELISA is reliable and well established, the need for high throughput and great specificity bring other techniques to the spotlight. The analysis of proteome using 2D electrophoresis followed by mass spectrometry (MS) is currently one of the most used approaches in comparative proteomics. Samples are prepared in parallel and proteins are separated by 2D high resolution electrophoresis, the different spots on the gel are usually characterised by Electrospray ionization MS (ESI-MS) or matrix-assisted laser desorption/ionisation (MALDI) time-of-flight (TOF) MS (MALDI-TOF) either in top-down or in bottom-up approach. MALDI-MS is the most common mass analyser employed due to its ability to acquire peptide mass fingerprinting (PMF) with high throughput[39]. PMF is a very useful approach for the rapid identification of a well-separated isolated protein. Tandem mass spectrometry (MS/MS) analysis exploits the fragmentation of selected precursor peptide ions, showing greater confidence in protein identification[37].

Protein microarrays are technical platforms for target proteomics based on quantitative protein expression focused on high throughput analysis. Similar to DNA microarrays these techniques count on a solid surface where hundreds to thousands of probing molecules are immobilized by robotic printing and the liquid sample is incubated on its surface[40]. The application of protein microarray to identify new biomarkers are enormous and examples present in the literature account for the assessment of breast cancer recurrence, the evaluation of risk of developing bone metastasis from breast cancer[41], as well as the evaluation of expression profile of colon cancer[42], NSCLC[43], pancreatic cancer[44] and acute myelogenous leukemia[45].

Regarding metallomics, the main characteristic of its approach is to be able to detect the elements associated with biomolecules. This characteristic is, in general fulfilled by inductively coupled plasma mass spectrometry (ICP-MS) hyphenated to a separating technique such as HPLC. The separation techniques are employed to determine the metallome because the detectors used are selective only for the differential elements, (like Pt in the case of Pt based drugs). The target molecules must be separated prior to the detection event. Electrophoresis has been used along with laser ablation (LA) in the hyphenated technique LA-ICP-MS for the identification of metal containing molecules. Most recently, LA-ICP-MS has been reported to be used in conjunction with protein microarrays making possible to probe metal containing proteins from tissue lysates[46], representing a huge step in metallomic development. The ability to probe a great number of species with known activity is very promising for the investigation of biomarkers and of biological processes in general.

The association of metallomic techniques with proteomic allow information about the general protein expression and which of them contain a specific element. In the imaging field, Bianga *et al* used LA-ICP-MS and MALDI imaging to study the penetration and distribution of two Pt-based metallodrugs (cisplatin and oxaliplatin) in human tumour samples removed from patients diagnosed with colorectal or ovarian peritoneal carcinomatosis[47].

The extensive databases used both by genomics and proteomics require powerful algorithms to assemble the pieces of the produced information, organise and manage them in a systematic way so that useful information can be obtained. Bioinformatics is fundamental for treating the huge amount of data produced by the techniques mentioned above and to extract from them valid and reliable information. Great efforts are being made on this field and databases such as Protein Data Base (PDB), Database of metal-

binding sites in protein structures (MDB), Database of information on the catalytic mechanisms of metal-dependent enzymes (Metal-MACiE), and Database of literature-based annotation of metalloproteins (PROMISE)[48] are available. Software for data treatment such as MITICS[49], OmniSpect[50], BioMap, MATLAB and Origin among others are making possible to organize the data in a suitable way.

It is important to point out that all the mentioned technologies are analytical tools, thus standardization, validation and quality control are mandatory for good quality of results[51].

Gene expression based biomarkers

The gene expression control is of fundamental importance in the cell behaviour. This becomes very clear if one considers that the whole genome is present in every cell, and the differentiation among these cells is due to the genic expression. Which, when and how much of a molecule will be expressed is dependent on a series of proteins and enzymes that control DNA transcription[52]. All cancerous processes are related to changes in cell genic expression. Many of the typical cancer cells behaviour, for instance increased cell proliferation, insufficient apoptosis and cell differentiation, are related to the expression of proteins and RNA that regulate these processes[53]. The genic expression biomarkers are the most common clinically in use and are being structured to become a standardized practice[54, 55, 56]. These biomarkers are the first to be used in the development of the personalized medicine. Sentence commenting techniques employed removed.

TPMT gene encodes some enzymes involved in cisplatin metabolism, it presents some single nucleotide polymorphism (SNP) that have been associated to a predisposition to ototoxicity during or after cisplatin treatment[57]. There still is some discussion on the

literature whether this genetic marker is reliable[58], but this is the only biomarker cited in cisplatin label with recommendation and approval of FDA. The gene mutations are screened with *Illumina GoldenGate* assay and quantified by RT-PCR[57]. Other genes are used in platinum-based therapy, but they are not specific for the patient response to treatment with this drug. Some important selected examples are shown below.

A germline mutation in let-7 complementary site 6 (LCS6) within the untranslated region of the KRAS gene is known to be associated with poor outcome and drug resistance in various cancers compared to the wild type allele59. KRAS gene is a proto-oncogene and a single nucleotide substitution is responsible for activating mutation. The translated protein that results is implicated in various malignancies, including lung, mucinous adenoma, adenocarcinoma, ductal carcinoma of the pancreas and colorectal carcinoma60. Besides, KRAS-variant is a potentially promising biomarker of poor prognosis and a predictive biomarker of cisplatin resistance in head and neck squamous cell carcinoma (HNSCC)59, for Non-Small Cell Lung Cancer (NSCLC)61, among others. The genevariation is determined with a PCR-based assay, and validation is being pursued[59].

RRM1 gene encodes one subunit, which constitutes ribonucleoside-diphosphate reductase, an essential enzyme for the production of deoxyribonucleotides prior to DNA synthesis in S phase of dividing cells. Lower expression levels of this gene, and SNP mutations are associated to a better response to cisplatin treatment in NSCLC[62, 63]. The gene is screened and quantified by multiplex RT-qPCR. Significance of this gene expression as biomarker is usually associated with ERCC1 gene expression. Down regulation of the last is also associated with better treatment outcome and prognostic. Overexpression of ERCC1 is directly related to poorer prognostics and treatment outcome[64], expression of this is being measured with different approaches like in

peripheral blood[65], tumour tissue, among other matrices, which makes its clinical use easier. Although there are problems with the expression measurement[66] this appears to be the most promising biomarker to enter clinical use[64].

MiRNA are small non-protein-coding RNA molecules that play an important hole in different biological processes, such as proliferation, differentiation and apoptosis by regulating gene expression. MiRNA-21 transcription is correlated with resistance to cisplatin chemotherapy regimen: the lower the expression the better the outcomes. The expression level of miR-21 in tumour tissue and plasma was indicated by Gao *et al.*[67] as a biomarker to predict adjuvant platinum based chemotherapy response and disease free survival in patients with NSCLC and also with oesophageal cancer[68]. The miRNAs are measured with specific microarray and RT-qPCR, and It has also been proposed as a circulating biomarker able to provide prognostic, diagnosis and therapy progress [69].

Besides the evaluation of individual genes, the genetic signatures (which make use of statistics-based analytical tools) have been proposed as indicators of treatment outcome. Zhu *et al.* propose a 15-gene expression signature that is considered independent prognostic marker, which can predict patients most likely to benefit from adjuvant chemotherapy with cisplatin/vinorelbine[70]. Kratz and co-workers established a quantitative-PCR-based assay based on 14 genes that, according to their findings, is able to identify patients with early-stage non-squamous NSCLC at high risk for mortality after surgical resection[71].

Protein expression Biomarker

The genetic expression of a cell can be inferred by the pool of proteins found in it. Changes in the protein expression pattern are often observed when the cell is exposed to some level of stress. Based on these principles, a *proteomic* approach can evaluate the

behaviour of a tumour based on the comparative protein expression pattern of healthy/cancerous cells or treated/non-treated cell (using, for instance, patient tissue or blood). The protein expression is believed to be the next hot spot of research to clarify the metabolism of platinum based drugs[72]. (Coments about techniques removed)

Kuang *et al.* presented a proteomic approach making use of Western blot and real-time PCR to identify possible biomarkers of efficiency for cisplatin-based treatment. The group has identified eight differentially expressed proteins in one pair of cisplatin sensitive/cisplatin resistant NSCLC cell lines. Special attention is given to DDH2 protein. This protein was investigated in serum of patients with NSCLC being treated with cisplatin. They observed significantly different levels of the protein in the blood of patients who presented disease progression, stability or amelioration[73].

Fitzpatrick *et al.* used LC-MS to identify and quantify over 2000 proteins from two pairs of cisplatin sensitive/cisplatin resistant cell lines. Among these, 760 proteins showed significant expression changes. Based on the results, several potential pathways that may be involved in cisplatin resistance in human ovarian cancer can be suggested. This study provides a proteomic platform for large-scale quantitative protein analysis, besides important information for investigation of new biomarkers of cisplatin resistance in ovarian cancer[74].

The efficacy of cisplatin treatment of tumour tissue from osteosarcoma has been evaluated through protein expression profiling. A 2-D difference gel electrophoresis was applied and 33 spots were found to differ significantly, allowing the classification of patients in good or poor responders groups. These spots were later identified by ESI-MS. Identification of the higher expression of peroxiredoxin 2 (PRDX2) in poor responders was confirmed using Western blotting[75].

The transmembrane 205 (TMEM205, previously known as MBC3205), a predicted transmembrane protein, has shown expression profiles in normal human tissues indicating a differential expression pattern with higher expression levels in the liver, pancreas, and adrenal glands. Overexpression of TMEM205 in cells may be valuable as a biomarker in cancer chemotherapy. Stable transfection of the TMEM205 gene confers resistance to cisplatin by approximately 2.5 fold[76].

Secreted proteome has been also evaluated as a biomarker. Such approach focus on the possibility of collecting samples in the microenvironment of the tumour and/or around the tumour. Sixteen proteins were shown to be differentially expressed by three different epithelial ovarian carcinoma (EOC) lines, and the protein Collagen, type XI, alpha 1 (COL11A1) was proposed as a biomarker for bad response to cisplatin treatment. The authors evaluated the proteome of these cell lines by numerous techniques[77].

Proposed biomarkers based on resistance mechanisms

The molecular basis of platinum-based drugs resistance can be described following their molecular mode of action that involves several steps until the final response of the tumour cell towards the drug. Problems to achieve DNA-damage response and mitochondrial apoptosis[78, 79] are the main factors impairing these drugs efficiency, leading to poor treatment response. Herein, the goal is discuss, in the molecular level, some points of the resistance mechanisms known so far in order to identify possible biomarkers that may allow clinicians to predict and monitor clinical response to platinum-based drugs chemotherapy. A better understanding of the mechanism by which those molecules are regulated, as well as their roles in drug sensitivity and

resistance, may indicate crucial prognostic markers and therapeutic targets for cancer treatment.

Several molecules have been implicated in resistance to cisplatin, Methyl-CpG binding domain protein 1 (MBD1), which plays an important role in disease progression, is one of these. It is recruited to DNA damage sites under DNA damage conditions induced by cisplatin. Silencing of MBD1 significantly impaired activation of the DNA damage checkpoint response and inhibited DNA repair capacity[80]. MBD1 binds mediator of DNA damage checkpoint protein 1 (MDC1), which is induced by radiation and regulates NBS1 activation in the presence of DNA damage repair[80].

Another modulator of cisplatin activity in tumour recently described is Jab1 (a c-Jun coactivator), a multifunctional protein that participates in controlling cell proliferation and the stability of multiple proteins, plays an important role in the cellular response to cisplatin and irradiation by regulating DNA damage and repair pathways[81]. Jab1 positively regulated Rad51 through p53-dependent pathway, and increased ectopic expression of Rad51 conferred cellular resistance to cisplatin, infrared light (IR) and UV radiation in Jab1-deficient cells. They showed that Jab1 is overexpressed in two relatively cisplatin-resistant, IR-resistant and UV-resistant nasopharyngeal carcinoma cells (NPC) cell lines.

Furthermore, cisplatin activity can be modulate by pathways that, depending of the stimulus and cell type, enhance or reduce cisplatin efficiency. Activation of autophagy in the early stages of apoptosis, by BO-1051 (an N-mustard linked with a DNA-affinity molecule) acted as a defense system against cell death[82]. Inhibition of autophagy in its early or late stages resulted in an increase in the number of annexin V-positive cells. BO-1051-induced autophagy has a cytoprotective role and is connected to the ATM

signaling pathway. This study revealed autophagy as a cytoprotective response against DNA damage-inducing chemotherapeutic agents, including BO-1051, cisplatin, and doxorubicin, in hepatocellular carcinoma cell lines[82]. On the other hand, autophagy is reported to enhance apoptosis induced by cisplatin. In lung cancer, treatment with cisplatin and radiation induced overexpression of autophagy-related genes, so as for the apoptosis signaling genes and a marked up-regulation of p21 expression, offering evidence that autophagy may enhance cisplatin efficiency[83].

FOXO transcription factors, functioning downstream of the PI3K-PTEN-AKT (PKB) signaling cascade, are essential for cell proliferation, differentiation, DNA damage repair and apoptosis[84]. Recent research indicates that the related transcription factor FOXM1 is a direct target of repression by FOXO proteins. Inactivation of FOXO or overexpression of FOXM1 is associated with tumourigenesis and cancer progression. In addition, the cytostatic and cytotoxic effects of a diverse spectrum of anti-cancer drugs, such as paclitaxel, doxorubicin, lapatinib, gefitinib, imatinib and cisplatin, are mediated through the activation of FOXO3a and/or the inhibition of its target FOXM1. Paradoxically, FOXO proteins also contribute to drug resistance by driving the expression of important genes for drug efflux as well as DNA repair and cell survival pathways in drug resistant cancers[84].

One mechanism of cisplatin-resistant cells is through reduced intracellular platinum accumulation. This may result from reduced uptake, increased drug export or intracellular sequestration. While cisplatin uptake is mediated through the copper transporter protein Ctr1, efflux is performed by two other copper transporting p-type adenosine triphosphatases (ATP7A and ATP7B). Samimi *et al.* described that changes in the expression of these proteins implicated in cisplatin resistance and poor patient survival in some types of cancer, most notably ovarian cancer[85]. The ATOX1 chaperone

has been recently described as the transporter that brings Pt from CTR1 to ATP7B, the interaction of Pt with this protein appears to be γ-L-glutamyl-L-cysteinyl-glycine (reduced glutathione or GSH) competitive and stable ATOX1-Pt adducts have been observed[86].

The cMOAT/MRP2, which is another important efflux system, have its expression increased in tumour cells and several works have shown that this membrane transporter may contribute to cisplatin resistance[87]. MRP2 requires GSH as a cofactor and its role in protection from the cytotoxic effects of cisplatin may be a result of its ability to transport GSH/cisplatin conjugates across the cell membrane[88]. GSH can work in concert with cMOAT/MRP2 to pump GSH-cisplatin conjugates out of cells in an ATP-dependent manner. Resistance to cisplatin through this mechanism is entirely GSH-dependent[89]. Increased levels of intracellular GSH are frequently observed in cisplatin-resistant tumours[90].

The capacity of cisplatin and other drugs to form bonds with thiol groups, make it suitable to form bonds with metallothioneins (MTs), which are intracellular proteins containing the highest amount of thiol groups within the cytoplasm. These thiol groups are able to bind several cytotoxic agents, such as platinum compounds. The increase of the level of MT is one mechanism of resistance to these anticancer drugs, once intracytoplasmic binding of MT prevents the active molecules from reaching their target, the intranuclear DNA of tumour cells[91, 92].

Thioredoxin reductase (TrxR) is the major cellular protein disulfide reductase containing selenium. TrxR catalyzes NADPH-dependent reduction of the redox-active disulfide in thioredoxin (Trx), the so-called thioredoxin reductase/thioredoxin system, which serves a wide range of functions in cell proliferation and redox homeostasis[93].

TrxR is overexpressed in many cancer cells and TrxR and Trx can enhance tumour development and drug resistance, thus been validated as therapeutical target in several studies[94], furthermore, TrxR/Trx system has a strong impact on tumour resistance to cisplatin[95]. As shown several reports, the cisplatin-resistant variants exhibited an increased expression and activity of TRXR as well as TRX compared with the parental cells, additionally, the inhibition of the TrxR/Trx system restored cell sensitivity to cisplatin[96, 97].

The excision repair cross-complementation group 1 (ERCC1) is a key component of the platinum-DNA repair machinery responsible for nucleotide excision repair (NER). Its expression protein were markedly higher in cisplatin-resistant derivatives of several tumour cell lines[98, 99, 100, 101, 102]. The action of ERCC1 generate excised single-stranded DNA of approximately 30 nucleotides containing the Pt adduct and attached NER proteins. DNA polymerases and ligases fill in the gap using the normal strand as a template[103].

Altogether, both overexpression of proteins that regulates DNA damage response and apoptosis, so as the over activation of antioxidant mechanisms will converge to the reduced efficiency of cisplatin. Thus, an important issue for metallomics is how to efficiently monitor the outcome of metal-based treatments, so that this approach can positively improve patient survival rate.

Platinum containing biomarkers

The determination of biomolecules through the platinum atom bind to them is a unique characteristic of the metallomic approach in cancer research. This characteristic is fundamental for the investigation of a new generation of cancer biomarkers, once it allows the targeting of chemical species containing Pt that are products of the

metabolism of the drug itself. Those species might provide information about processes that each patient goes through during treatment, enabling physicians the ability to perform personalized medicine. Although metallomics present such a potential, up to now there are no biomarkers containing platinum approved by FDA or with clinical use reported. Many studies making use of metallomics have been conducted to clarify the pharmacokinetics of platinum-based drugs but none of the identified species have been reported as biomarkers so far. The information acquired through metallomics, together with information about protein content, drugs metabolites and genetic expression obtained through the different approaches may provide comprehensive information on the fate of the drug (target, metabolism and resistance).

Once the interaction of Pt with DNA is believed to be responsible for the cytotoxicity Pt based drugs, great effort have been put on the determination of Pt-DNA adducts. These studies showed also that there are between 1 to 5 Pt atoms per 10^6 nucleotides. Zayed and coworkers developed a very sensitive method for the determination of Pt-DNA adducts by liquid chromatography (LC) coupled to sector field (SF) ICP-MS (LC-SF-ICP-MS) that could be used for *in vivo* tests with a detection limit of 0,14 ng mL^{-1} of Pt [104]. Nevertheless, so far, these molecules have not been reported as biomarker for resistance, effectiveness or prognostic of treatment outcome.

Wexselblatt and coworkers has reviewed the action of platinum compounds in the cell and the DNA adducts generation. They emphasize not only the complexity and dynamic nature of cells that prevent us from monitoring the fate of platinum complexes in cells, but also they showed the inability of the current analytical techniques to provide non-invasively and in real time direct information on the speciation of the platinum complexes in cells. (Part about techniques Removed)

As a modulator of gene expression at multiple levels, RNA is an important potential biomarker. Pt-RNA adducts have the potential to impact cell fate by disrupting RNA regulatory pathways. Hostetter *et al.* used *Saccharomyces cerevisiae* for in-cell analysis of Pt adduct formation on mRNA, rRNA, and total RNA and DNA platinum adducs[23]. The estimated in-cell Pt concentrations and Pt accumulation on mRNA, rRNA, total RNA, and DNA were determined using ICP-MS. It was described that similar Pt accumulation was observed on rRNA and total RNA, but significantly less Pt accumulated on mRNA. By using the mapping by reverse transcription, they demonstrated specific Pt adduct formation on rRNA sequences conserved between yeast and humans. Taken together, these data highlight important differences in the relative accumulation of Pt on different RNA species and provide insight into the accessibility of cellular RNA to small, cationic molecules[23].

Due to a thiol group presence, numerous electron rich sites and, especially, due to its high abundance in every cell compartments GSH have been associated with resistance to Pt based drugs since the early 1990's. There are many reports associating the superexpression of GSH with resistance to platinum based drugs, Shoeib and Sharp showed many possible isomers for Pt-GSH structures[5]. Although there is controversial data about the binding of Pt to GSH many techniques to determine this biomolecule are present in the literature. In Table 1, a sample of biomarkers currently approved by FDA, in use or under investigation is listed.

Cisplatin and oxaliplatin interact to a high extent with blood biomolecules, a small fraction of these drugs remains free 24 h after administration and the levels of Pt in the blood of patients treated with these drugs remain high for decades[105,106]. On the other hand, most of infused carboplatin is excreted intact, most of the drug incubated with rat

ultra-filtrated plasma is recovered intact[107], adducts with proteins having molecular weights similar to human serum albumin (HAS) and g-globulin were reported[108,109].

Conclusions

Molecular biomarkers are the most mature and are already in use to evaluate patients' prognostic, treatment outcome and overall survival rate. These biomarkers have solid and validated sample preparation and analysis kits are available in the market, which makes its application easier, less expensive and widespread among clinical analysis laboratories.

The proteomic approaches appear as a very promising field to be explored. The complexity of the interactions of platinum-based drugs with proteins (in the blood stream, entering the cell, being distributed and actually exerting its cytotoxic effect) is large and many authors recognize the lack of complete information about it [72, 86, 111].

A new biomarker generation might be identified based on the metallomic approach. The ability of atomic spectrometric methods to detect the Pt atoms bind to a diversity of biomolecules, previously separated by chromatography, electrophoresis end even with microarrays, can help to fill some of the gaps in the comprehension of the mechanisms of action of platinum-based drugs. This ability can reveal molecular markers that can help physicians to take decisions and foresee the treatment outcome and patients prognostics. Platinum containing molecules known for a long time such as Pt-GSH and metallothioneins can bring information about availability of the active compound inside the cell. However, the specific studies for them to become clinical biomarkers are still to be done. Pt-DNA adducts can bring information about activity of ERCC1 and NER

system, consequently about resistance to Pt treatment. In contrast, in all works revised in this text, the digestion of the DNA is performed prior to platinum adduct determination. In such experimental design, the biological information contained in the specific fragments generated by the NER is mixed with the other digested nucleotides, losing the biological signature of the origin of adducts. Pt-ATOX1 and Pt-RNA adducts apparently can clarify the fate of platinum-based drugs and indicate treatment outcome. Besides Pt-containing molecules, it was shown that sulphur containing ones, such as TrxR can play important role as biomarkers of resistance.

Other metallo-biomarkers, not included in the present revision, can be sought by applying cutting edge metallomics tools shown above to investigate specific cellular processes like drug efflux; repair of DNA damage; secreted Pt-containing molecules; mitochondrial induced apoptosis; outside the cell apoptosis signaling (membrane protein).

Important barriers must be overcome to reduce the apparent distance between the metallomic tools and the medical community. Once the increasing interaction of analytical scientists, biologists and medical doctors matures, the advances in the field will certainly occur. In addition, evolution in sample treatment, simplification of analytical process and instrumentation, proper analytical validation, dissemination of the approach with consequent cost reduction are also important steps to be taken to bring metallomics to examination prescription.

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Table 1: Examples of biomarkers related to Pt-based drugs.

Biomarker	Determination	Use/drug	Cancer type	Reference
TPMT*	Enzyme assay and RT-PCR	Predictive (Ear injury)/cisplatin	Solid pediatric tumours.	55-58
ATP7A and ATP7B	Microarray	Resistance/Multidrug	Diverse Malignancies	85, 110
BO-1051	qRT-PCR, immunoassay	Resitance/cisplatin	Lung	82-83
cMOAT/MRP2	HPLC-ICP-MS, qRT-PCR	Resistance/ cisplatin	Nasopharyngeal, HNSCC	87-90
COL11A1	LC-MS/MS	Resistance/ cisplatin	Ovarian carcinoma	77
ERCC1	Microarray, qRT-PCR	Predictive (treatment outcome)/Multidrug	Lung, Bladder, HNSCC, Ovarian.	6, 63-64, 98-103
FOXO	ECl; RT-PCR	Resistance/Cisplatin	Breast	84
Jab1	RPPA, qRT-PCR, immunoassay.	Resistance/cisplatin	Nasopharyngeal carcinoma	81
KRAS	RT-PCR	Treatment outcome/multidrug	Gastric, HNSCC, Lung, Pancreas	30-31, 59-61
MBD1	Immunoblot analysis	Resistence/multidrug	Pancreas	80
MiRNA-21	Specific microarray and qRT-PCR	Predict resistance/multidrug	Oesophageal, Lung	67-69
RRM1	qRT-PCR	Treatment outcome/cisplatin	bladder, lung	62-63
TMEM205	RT-PCR/ qRT-PCR	Resistance/cisplatin	Liver, pancreas, adrenal gland	76
TrxR	2D GE,MS HPLC-ICP-MS	Prognostic and Resistence/Cisplatin	Diverse malignancies	93-97
Possible Metallomic target biomarkers				
Metallothionein	2D PAGE; SEC-ICP-MS	Response indicator/multidrug	Diverse Malignancies	91-92
Pt-DNA Adduct	HPLC-ICP-MS	Many studies about these molecules/multidrug	Diverse Malignancies	15, 104
Pt-GSH	HPLC-ICP-MS	Resistence/multidrug	Diverse malignancies	5, 88-90
Pt-RNA Adduct	Microarray, GE-ICP-MS, HPLC-ICP-MS	Resitance/cisplatin	Diverse malignancies	23, 32

* This is the only biomarker approved by FDA specifically to cisplatin.

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