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LIST OF ABBREVIATIONS

2DSEC-RP-ICP-MS	Two-dimensional size-exclusion chromatography-reversed-phase chromatography coupled to inductively coupled plasma mass spectrometry.
BEH	Ethylene bridged hybrid.
CE	Capillary electrophoresis.
DNA	Deoxyribonucleic acid.
EI	Electron ionization.
ESI	Electrospray ionization.
FCS	Fetal calf serum.
FI-ICP-MS	Flow injection inductively coupled plasma mass spectrometry.
GC	Gas chromatography.
HILIC	Hydrophilic interaction liquid chromatography.
HMF	High molar fraction.
HR-MS	High-resolution mass spectrometry.
HSA	Human serum albumin.
ICP-QMS	Quadrupole inductively coupled plasma mass spectrometry.
ICP-TOFMS	Time of flight inductively coupled plasma mass spectrometry.
LA-ICP-MS	Laser ablation inductively coupled plasma mass spectrometry.
LC	Liquid chromatography.
LMF	Low molar fraction.
LOD	Limit of detection.
MRM	Multiple reaction monitoring.
NMR	Nuclear magnetic resonance.
m/z	Mass to charge ratio.
QQQ	Triple quadrupole.
RAM	Restricted access material.
RNA	Ribonucleic acid.
RP	Reversed-phase chromatography.
SEC-IC	Size-exclusion chromatography-ion chromatography.
SPE	Solid-phase extraction.
SRM	Selected reaction monitoring.
Tf	Transferrin.
TFC	Turbulent flow chromatography.
UHPLC	Ultra-high performance liquid chromatography.

ABSTRACT

Different analytical techniques are employed in the field of metal-based anticancer drugs to investigate the metabolism and interaction with cellular targets in preclinical studies prior to clinical translation. The aim of this dissertation was to compare and discuss different analytical techniques as well as to integrate the field of metallomics with another –omics field, metabolomics. This combination will lead to an improved understanding of the drug's mode of action, and in the best scenario to understand unwanted acquired or intrinsic resistance in chemotherapy.

In the first publication, four different analytical approaches, size-exclusion chromatography (SEC), ultrahigh performance size-exclusion chromatography (UHPLC SEC), turbulent flow chromatography (TFC) and centrifugal ultrafiltration, all of them coupled to inductively coupled plasma-mass spectrometry (ICP-MS), were compared and discussed. For this end, a platinum(IV)-based drug non-reactive and a platinum(IV)-based drug with binding properties towards albumin were incubated *ex vivo* in fetal calf serum (FCS). UHPLC SEC-ICP-MS showed the best results regardless of the chemistry of the analytes.

In the second publication, the kinetics of oxaliplatin was investigated in *ex vivo* incubations in FCS and in cytosolic fractions of the colorectal cancer line HCT116 by heart-cut UHPLC SEC coupled to reversed phase (RP) chromatography followed by ICP-MS detection. This analytical methodology was established after observing in the first publication the superiority of UHPLC SEC compared to other analytical techniques. However, the resolution was still low for intact metallodrug quantification needing of an orthogonal approach.

In the third publication, metallomics and metabolomics were integrated in *in vitro* studies of oxaliplatin in sensitive and resistant cells of the colorectal cancer line HCT116. The results showed the possibility of combining both -omic approaches into one single cell extraction. In metallomics, the total platinum in the cytosolic fraction as well as in the nucleus containing fraction was quantified by flow injection coupled to (FI)-ICP-MS. In metabolomics, the cytosolic fraction was analyzed by hydrophilic interaction liquid chromatography (HILIC) coupled to high resolution mass spectrometry (HR-MS) merging targeted and untargeted analysis. A clear difference was observed between sensitive and resistant cells on both –ome levels which will be further investigated to understand the mechanism of action of the metallodrug and the resistance in chemotherapy.

ZUSAMMENFASSUNG

Im Rahmen präklinischer Untersuchungen werden verschiedene Analysentechniken eingesetzt, um Stoffwechsel und zelluläre Angriffspunkte metallbasierter zu charakterisieren, bevor diese in klinischen Studien näher untersucht werden. Das Ziel dieser Dissertation war es, verschiedene analytische Techniken zu vergleichen und zu diskutieren, sowie die Forschungsfelder metallomics und metabolomics zu verbinden. Diese Kombination kann helfen, die Wirkungsweise des Arzneimittels, aber auch unerwünschte erworbene oder intrinsische Resistenzen in der Chemotherapie besser zu verstehen.

In der ersten Veröffentlichung wurden vier verschiedene analytische Ansätze - Größenausschlusschromatographie (SEC), Ultrahochleistungs-Größenausschlusschromatographie (UHPLC SEC), Turbulent-Flow-Chromatographie (TFC) und Zentrifugal-Ultrafiltration, jeweils gekoppelt mit ICP-MS (inductively coupled plasma mass spectrometry) - verglichen und diskutiert. Zu diesem Zweck wurden ein auf Platin (IV) basierendes Arzneimittel, das nicht reaktiv ist, und ein Platin (IV)-basiertes Arzneimittel mit Bindungsaffinität zu Albumin *ex vivo* in fötalem Kälberserum (FCS) inkubiert. UHPLC SEC-ICP-MS zeigte die besten Ergebnisse unabhängig von der Chemie der Analyten.

In der zweiten Veröffentlichung wurde die Kinetik von oxaliplatin in *ex vivo* Inkubationen in FCS und in zytosolischen Fraktionen der Darmkrebslinie HCT116 mittels heart-cut UHPLC SEC verbunden mit RP-Chromatographie (RP) und anschließender ICP-MS-Detektion untersucht. Diese Strategie wurde festgelegt, weil in der ersten Veröffentlichung zwar die Überlegenheit von UHPLC SEC gegenüber anderen Analysetechniken beobachtet wurde. Allerdings war die Auflösung für die Quantifizierung des intakten Metallodrugs aber nicht ausreichend und deshalb die Einführung einer orthogonalen Trenntechnik erforderlich war.

In der dritten Veröffentlichung wurde oxaliplatin *in vitro* an sensiblen und resistenten Zellen der Darmkrebslinie HCT116 unter Kombination von metabolomics- und metallomics-Ansätzen getestet. Beide -omics-Untersuchungen konnten am selben Zellextrakt durchgeführt werden. Die Gesamtplatinkonzentration wurde für die zytosolische und die kernhaltige Extraktfraktion mittels Fließinjektion gekoppelt an (FI)-ICP-MS bestimmt. Zur Metabolitenquantifizierung wurde die zytosolische Fraktion mittels hydrophiler Interaktionsflüssigkeitschromatographie (HILIC) in Verbindung mit hochauflösender Massenspektrometrie (HR-MS) analysiert, wobei zielgerichtete und ungezielte Analysen zusammengeführt wurden. Es wurde ein deutlicher Unterschied zwischen empfindlichen und resistenten Zellen auf beiden Ebenen beobachtet, der weiter untersucht werden, um den Wirkungsmechanismus des Metallodrugs und die Resistenz in der Chemotherapie zu verstehen.

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1 INTRODUCTION

1.1 Metallomics related to preclinical metallodrug research

The term metallome appeared for the first time in 2001 to define the distribution of a metal and the metalloid species in the cells or in its different compartments¹. Two years following this definition by Williams, Haraguchi coined the term metallomics for the first time to integrate the research fields related to this term². However, since these terminologies were coined, the biological scope of metallomics has been somewhat ill-defined. Accordingly, the definition of the term itself saw many modifications, relating to the speciation of metals in cells and their compartments to cells or tissues³ and more recently being expanded to a biological organism or its parts including body fluids as well⁴⁻⁶. The latter expansion was based on the fact that the metals must first go through the body fluids being submitted to chemical reactions with proteins and metabolites which ultimately influence the distribution of the metal in the cells and tissues. All these chemical reactions either in the body fluids, in the tissues or in the cells are decisive not only in physiological processes but also in cases where metals are applied for medical purposes since these reactions govern metal delivery and metal activation/deactivation. A prime example would be the biology of iron and its homeostasis, determined by its interaction with proteins⁷.

Bioinorganic studies on metal-based anticancer drugs contributed largely to the development of metallomics. The most employed anticancer drugs are based on platinum cores⁸. It is believed that their main mechanism of apoptosis is through deoxyribonucleic acid (DNA) platination^{9,10}. However, it is clear that there are other biomolecule interactions leading to adduct formations that must be considered and investigated as well. For example, the binding to human serum albumin (HSA) in plasma is known to lead to deactivation of these platinum-based drugs^{10,11}. Once inside the cells, the adduct formation with glutathione and metallothioneins are believed to result in deactivation potentially associated with resistance¹². However, the role of this key intracellular interaction is still a matter of debate¹³. Not only the adduct formation of these platinum(II)-based drugs but also the hydrolysis products have been investigated since they can show a completely different reactivity^{10,11}. Several investigations have been carried out to improve the stability of these drugs and their targeting to the DNA. In the different investigations, the synthesis of platinum(IV)-based prodrugs has shown promising results exploiting this same binding to albumin for targeted drug delivery since this protein accumulates in tumor tissues¹⁴⁻¹⁶. Nowadays it has become clear that the mechanism of cytotoxicity of the existing metallodrugs in general and of these platinum-based drugs in particular demands for reconsideration and further investigations. Accordingly, the development

of new metallodrugs integrating studies on mode of action is a very active area of research. In this regard, studies integrating metallomics and genetic approaches have been extensively investigated. For example, Bruno et al.¹⁷ proved this way that the assumption that all the platinum-based drugs exhibit cytotoxicity through DNA platination is not always the case. Their results showed that whereas this mode of action was true for cisplatin, oxaliplatin provokes apoptosis by inducing ribosome biogenesis stress as well. This type of fundamental investigations were not restricted to platinum-based drugs but were expanded to other anti-cancer drugs with a different metal center⁸. Hence, since already a different drug's behavior is observed within the family of platinum-based drugs, it is not surprising that this is also true between metallodrugs with a different core. For example, the combination of metallomics with proteomics was reported to investigate ruthenium-based drugs showing a different mechanism of action¹⁸. A same -omics combination for ruthenium and arsenic-based drugs showed that their effect might be cell type specific¹⁹.

Our metallomics studies on metal-based anticancer drugs were method oriented. We addressed the current challenges of the analytical instrumentation in platinum-based drugs in *ex vivo* incubations. Moreover, we investigated the integration of metallomics and metabolomics in *in vitro* incubations of oxaliplatin since the platination of the DNA leads to perturbations on the proteome and on the metabolome level. These perturbations will be further investigated to understand the mechanism of action of these drugs (section 1.2). The instrumentation and sample preparation for these studies is shown and discussed in the next sections.

1.1.1 Analytical tools in the field of preclinical metallodrug research

Inductively coupled plasma-mass spectrometry (ICP-MS) has been extensively employed in speciation analysis and metallomics in general and in metallodrug research in particular due to the metal signal detection without the need for species specific standards, low matrix effects, high sensitivity and multielement and multi-isotopic information^{11,20-24}. As inconveniences, ICP-MS does not provide molecular information although this can be solved by the combination with electrospray ionization (ESI)-MS^{25,26} as explained later in section 1.3.1.3. Interferences hampering the detection of sulfur and phosphorus necessary for the monitoring of DNA, protein and metabolites can be addressed by using high-resolution ICP-MS (double focusing sector-field ICP-MS or Time of flight, ICP-(TOF)MS), and collision/reaction cells^{4,25}. When an on-line or off-line separation/fractionation method is employed prior to ICP-MS detection in *ex vivo* incubations of metallodrugs in serum²⁷ and cell extracts^{13,28}, a general in-

formation can be obtained about the hydrolysis products and the interaction with biomolecules^{11,29} prior to *in vitro* and *in vivo* studies. In this regard, capillary electrophoresis (CE) or size-exclusion chromatography (SEC) prior to ICP-MS have been applied in numerous cases to get a general screening of metallodrug-biomolecule interactions and hydrolysis in biological samples. CE has shown a higher separation power, however, the sensitivity and robustness is lower^{11,20,23,27,30} being SEC a more suitable approach for this purpose. Nevertheless, in SEC, different metalloproteins are difficult to separate in the protein rich high molar mass fraction (HMF). On the other hand, metallometabolites and/or hydrolysis products cannot be separated from the intact metallodrug in the low molar fraction (LMF) due to the lower separation power of SEC. Extraction/fractionation techniques such as turbulent flow chromatography (TFC) or centrifugal ultrafiltration have been used to extract the HMF and determine the total metal in the LMF by flow injection (FI)-ICP-MS or the different metalloid species and the intact metallodrug by liquid chromatography (LC)-ICP-MS. In **Figure 1** we show a summary of the different analytical tools that have been and can be applied for metallodrug research in *ex vivo* incubations in serum and cell lysates with the corresponding references.

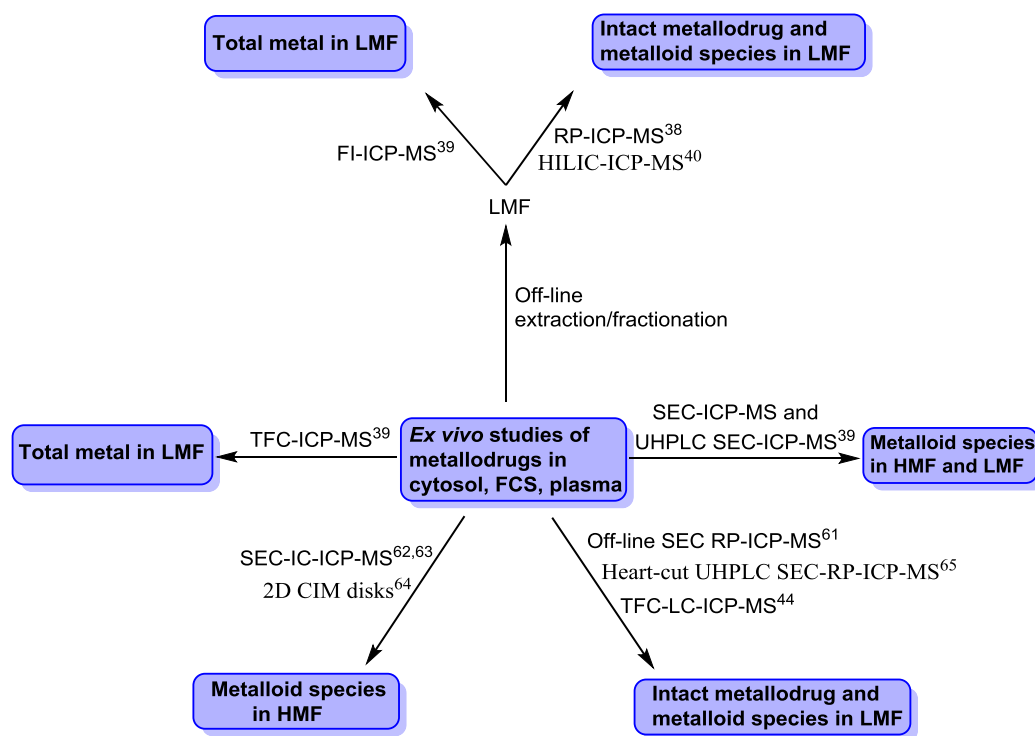


Figure 1 The different analytical techniques that have been employed in metallodrug research in *ex vivo* incubations in fetal calf serum (FCS), cytosol extracts and/or human plasma with the corresponding information obtained in the low molar fraction (LMF) and/or in the high molar fraction (HMF).

An important development regarding throughput was the introduction of UHPLC SEC, allowing high protein recoveries, excellent separation efficiency within analysis times of few minutes. This is further discussed in section 1.1.1.6. The new stationary phase enabled to couple SEC to reversed-phase (RP)-ICP-MS improving the resolution for quantification of intact metallodrug in *ex vivo* incubations (section 1.1.1.7). This way the sample preparation for the protein extraction can be avoided, circumventing low stabilities and recoveries of the investigated metallodrug derived from this process. Moreover, this methodology can be applied to any metallodrug as long as they do not have a high polarity in order to interact with the functional groups of the RP column. The different analytical techniques are discussed in detail in the next sections.

1.1.1.1 Emerging trends

In vitro studies using cancer cell models increase the complexity of the analytical task. As shown in **Figure 2**, two different powerful analytical approaches address *in vitro* studies: single-cell analysis and lysate-based assays. Whereas in lysate-based assays the metallodrug information is inferred from bulk analysis of different cell fractions, in single-cell approaches the metallodrug information is obtained separately from each cell³¹. Single-cell information can be obtained either by implementing high spatial or time resolution allowing distinguishing between single cells. In the first concept, Laser ablation (LA)-ICP-MS using low dispersion ablation cells features spatial resolution at 1-2 μm , allowing addressing elemental patterns for single cells. However, a scanning analyser like a quadrupole, ICP-(Q)MS, cannot be used as it is necessary to record multi-elemental information within microseconds. For this task, ICP-TOFMS can be employed since the isotopes are detected quasi-simultaneously^{26,31}. For the analysis of single-cells in liquid samples, a cytometry concept is combined to ICP-TOFMS. Different articles and reviews discuss this emerging field in more detail^{31,32}. Single-cell analysis was beyond the scope of this thesis, focusing on lysate-based assays.

1.1.1.2 Preparation of cells

As shown in **Figure 2**, complete mineralization of the biological samples followed by FI-ICP-MS can be carried out. While this strategy provides complete metal content only, lysate-based assays yield information on drug distribution in the different cell fractions. Different lysis procedures can be carried out³³ as observed in **Figure 2** but the method of choice must ensure the full recovery and stability of the metallodrug and the metalloid species under

study. For example, it was shown that for cisplatin investigations lysis buffers showed a good lysis efficiency but freeze-thaw cycles were beneficial with regard to cisplatin stability³⁴.

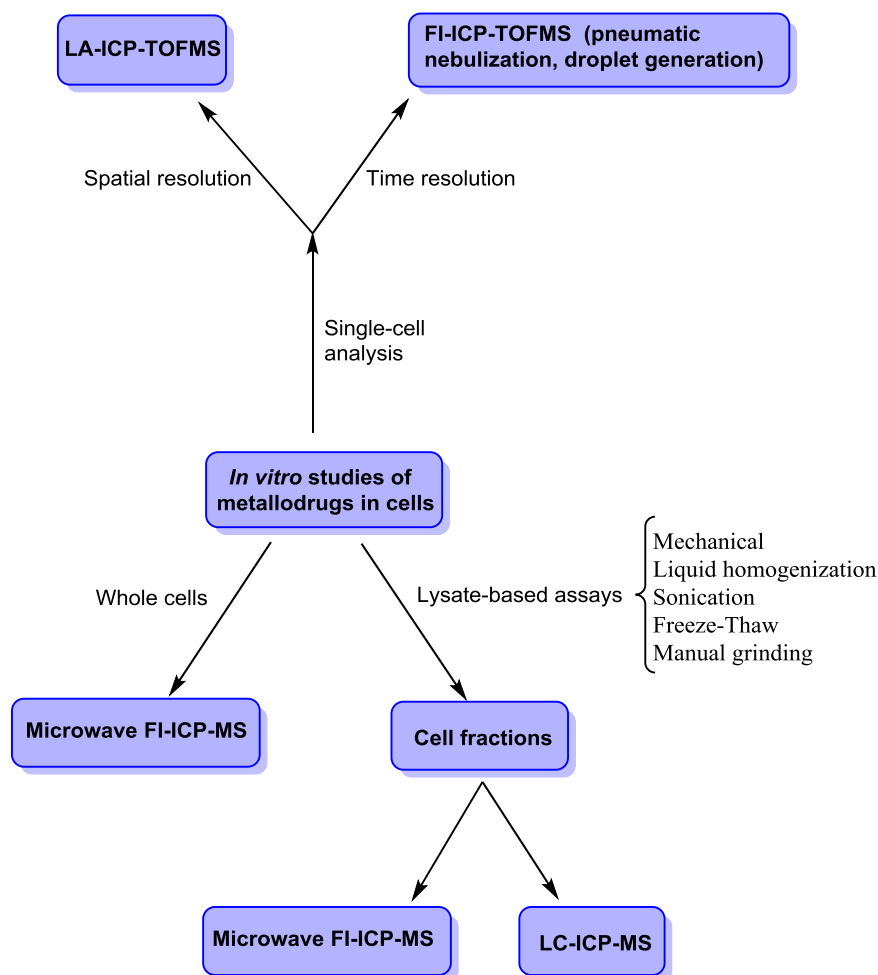


Figure 2 The different analytical techniques employed for *in vitro* studies of metallodrugs in cells.

1.1.1.3 Normalization

In *in vitro* studies the number of cells is usually different upon incubation time or between cell lines but also in the cell-wells of the same plate due to experimental conditions. In this case, a normalization to the number of cells is always necessary since a different number of cells is accompanied by a different intracellular metal content³⁵. Cell counting and protein assays can be employed for this normalization purpose although other authors showed the possibility of freeze-drying and weighing the cell precipitates³⁶. In this work, a metallomics study was combined with metabolomics in *in vitro* incubations of the cancer cell line HCT116 and hence

protein assay was the only option for such a normalization purpose since the other procedures were not suitable for metabolomics (section 1.3.1.6).

1.1.1.4 Off-line fractionation/extraction techniques

Off-line fractionation/extraction techniques are usually employed in *ex vivo* incubations of metallodrugs in order to extract the HMF and analyze either the total metal with FI-ICP-MS or the different metalloid species with LC-ICP-MS in the LMF. Centrifugal ultrafiltration and protein precipitation are two of the most employed off-line fractionation/extraction techniques.

Centrifugal ultrafiltration, developed in the late 1960s³⁷, has been extensively used for metallodrug research^{27,28,38}. Although it could seem an easy method, it is time consuming since a pre-conditioning of the filter is necessary to reduce the interactions of the metallodrug with it and to get good recoveries. It is important here to choose the suitable chemistry of the filter for the metallodrug under study as well as to choose a good pre-conditioning method to get the best possible results³⁹. Hence, these results are going to depend on these filters, their pre-conditioning and the chemistry of the researched metallodrug making this technique more difficult as expected. Protein precipitation has been also reported as an off-line extraction technique for metallodrug research due to its faster preparation compared to centrifugal ultrafiltration. Here, the HMF precipitation is usually achieved by addition of organic solvents although other protein precipitation methods such as changing temperature, pH or ionic strength of the solvent have been reported as well⁴⁰. However, co-precipitation effects must be considered. Besides, when using organic solvents, the analysis of the different species in the supernatant or LMF, after separation from the precipitate or HMF by centrifuging, must be carried out using hydrophilic interaction liquid chromatography (HILIC). The disadvantage of HILIC coupled to ICP-MS compared to RP relies in the need of additional high oxygen flow rates to make it compatible with ICP-MS and avoid carbon deposition. This increase in the oxygen flow rates is accompanied by an increase in the limits of detection (LODs), an inconvenience when studying metallodrugs at low clinical concentrations. As an alternative, the supernatant or LMF can be evaporated and re-suspended in water for later RP-ICP-MS analysis. Nevertheless, this process must be evaluated for each metallodrug under study since it might not be suitable, degrading the intact drug and its metalloid species as we observed in oxaliplatin investigations (publication II).

1.1.1.5 On-line fractionation/extraction techniques

On-line fractionation/extraction techniques are used for an automatic processing of biological samples to extract the HMF and analyze the LMF directly with ICP-MS or with LC-ICP-MS. Turbulent flow chromatography (TFC) and restricted access materials (RAM) are nowadays two of the most employed on-line fractionation/extraction techniques leaving solid-phase extraction (SPE) aside with regard to analytical throughput. On top of that, often challenges arising from poor analyte recovery and efficiency are reported⁴¹.

RAMs are sorbents such as polymers or silica-based materials with hydrophilic groups to create a chemical barrier and small pores accessible only to small molecules⁴². On the contrary, TFC, developed in the late 1990s, merges SEC and traditional stationary phases with large pores (30-80 μm). At very high flow rates on these columns, turbulent flow regimes are provoked which generate a mass transfer cut-off sending the HMF directly to the waste without any interaction with the functional groups of the stationary phase. The small molecules, including the intact metallodrug, hydrolysis products and metallometabolites, can interact with the functional groups of the stationary phase which are later eluted by backflushing with a suitable eluent⁴³⁻⁴⁵. TFC has the advantage of being faster and more stable compared to laminar-flow regimes⁴⁶. As a drawback of this technique, the use of high flow rates of ~ 1 mL/min necessary to reach these turbulent conditions would make it a difficult technique to use when a big batch of samples need to be analyzed without the presence of an operator to replace the solvents. There are different TFC columns with different chemistries and dimensions^{47,48}. It is important to choose a suitable column for the metallodrug under study although it is difficult in some cases to foresee the chemistry of the different new formed metalloid species and their retention on the TFC column.

1.1.1.6 SEC and UHPLC SEC

Size-exclusion chromatography (SEC) was originally developed in the 1950s⁴⁹. In the late 1990s, SEC-ICP-MS was reported as a screening tool for metallodrug-protein interactions⁵⁰ and since then it has been extensively employed in metallodrug research. The columns in SEC are mostly polymeric and silica-based. The separation is based only in molar size and not based on the chemistry of the molecules. The big molecules do not enter the pores showing shorter flow paths compared to small molecules⁴⁹.

In the early years, the resolution in SEC was considered low and it was a time consuming method. The chromatographic separation times ranged between 20 and 40 min⁵¹. Various

approaches were developed to reduce this analysis time⁵². For this matter, the reduction of the stationary particle size to less than 2 μm was key. This reduction in the particle size was accompanied by a reduction of the eddy dispersion and the dispersion due to mass transfer, responsible for band broadening in liquid chromatography⁵³⁻⁵⁵.

In 2000, a first generation silica-polymer hybrid was introduced, combining the higher pH range of the polymeric-based columns and the higher chemical rigidity of the silica-based columns. However, the mechanical rigidity was still not enough for UHPLC, needing a second hybrid generation, the ethylene bridged hybrid (BEH) particles. The improvement in the mechanical stability of these 1.7 μm particles was obtained by the binding of methyl groups in the silica matrix⁵⁶ (**Figure 3**). They were first introduced in RP and HILIC separations and finally in 2010 in SEC separations. A recent review discuss this relatively new technique, UHPLC SEC⁵⁷, including its application to biotherapeutic proteins, proteomics and polymers^{58,59}. The main advantage of this technique in comparison to off-line extraction techniques is the acquisition of a general screening of metallodrug-protein interaction with an easier sample preparation or even without any sample preparation (*ex vivo* experiments in FCS). However, although SEC or UHPLC SEC can be employed to quantify the low molar mass fraction of the metallodrug (publication I), it is necessary to consider one or more orthogonal methods when the aim is to accurately quantify the intact metallodrug, as the separation selectivity of SEC in general is limited. Hence, the separation of molecules with similar sizes like human serum albumin HSA and transferrin (Tf) in the HMF or metallometabolites, hydrolysis products and intact metallodrug in the LMF is challenging or not possible.

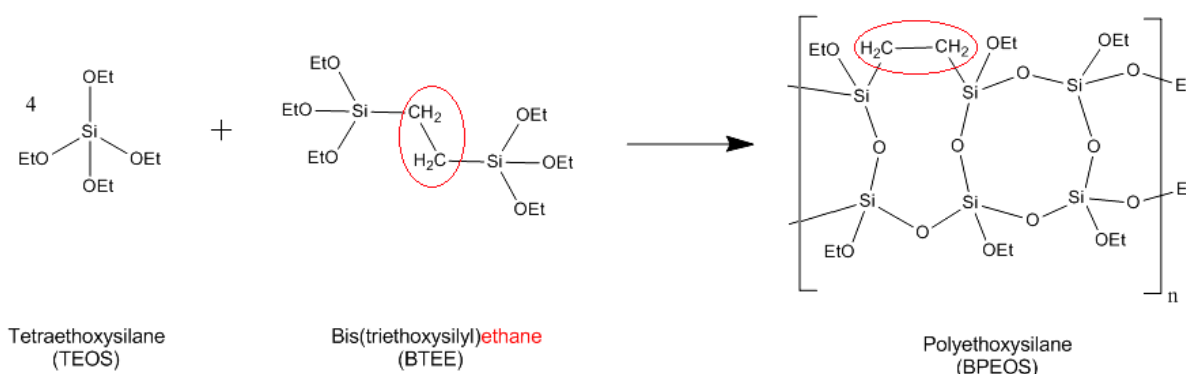


Figure 3 ethylene bridged hybrid (BEH) particles introduced on the market by Waters Corp, (Milford, MA, USA), adapted from elsewhere⁶⁰.

1.1.1.7 Two-dimensional chromatography

As mentioned in section 1.1.1.6, SEC is a straightforward analytical tool to obtain a general mapping of metallodrug-protein interactions. However, the accurate quantification of intact metallodrug, of paramount importance for kinetic studies, is hampered due to the co-elution with other metalloid species. In order to overcome this obstacle, different orthogonal approaches have been investigated. Off-line SEC and later analysis of the collected fractions with RP-ICP-MS was previously reported for Cr speciation⁶¹. The main drawback of this approach is the dilution of the collected samples and therefore it would not be compatible when the metallodrug is at low clinical concentrations. On-line two dimensional SEC-ion chromatography SEC-(IC), fast column switching SEC-IC separation coupled to ICP-MS and 2D CIM monolithic disks bearing weak anion-exchange diethylamino groups and immunoglobulin G ligands were reported for *ex vivo* incubations of metallodrugs⁶²⁻⁶⁴. However, the aim of these set-ups was the quantification of the metallodrug in the HMF and not in the LMF and they operated at low pressures.

So far, the on-line coupling of SEC to RP was not possible due to the low mechanical rigidity of the conventional SEC columns limiting the increase in the pressure⁶⁵. This, as mentioned in the previous section, was overcome with the introduction of the stationary phase utilizing BEH particles. However, the use of the analogous stationary phase (sub 2µm particles) for RP chromatography on the second dimension was not possible for two practical reasons: (1) the pressure limit of the employed pump (600 bars) and (2) the high back pressure generated by the second dimension (>500 bars) not tolerated by the first dimension. Hence, 3 µm-particle reversed phase columns were selected at low flow rates (350 bars). This approach avoided any sample preparation for protein extraction compared to off-line fractionation/extraction techniques. Moreover, we made sure that all the species present in the LMF were sent to the second dimension due to a lower interaction on the stationary phase of the UHPLC SEC column compared to TFC. In the second dimension, a 100% wettable RP was employed to increase the retention and separation of polar compounds^{66,67} (publication II).

1.2 Metallomics as connected to other –omics studies

As mentioned in section 1.1, either in physiological processes or in medical purposes, the interactions of metals with proteins and metabolites already occur in the body fluids before the metals reach the tissues and cells. Even inside the tissues and cells these interactions are still present together with interactions with ribonucleic acid (RNA) and DNA. In this re-

gand, metallomics alone can be applied to investigate these direct interactions. Metals play a key role at all different -ome levels (see **Figure 4**). Hence, a further combination with other -omics technologies is necessary to study regulations, perturbations or connectivity of the metallome with the different -ome levels. This concept of combining different -omics strategies can be also employed upon drug treatment to elucidate the mechanism of action and resistance of the drug. Up to date, the simultaneous investigation of all the -omic levels is technically challenging if not impossible and for a start one must choose the -ome to integrate with metallomics for the investigation of the metallodrug. While there are several researches of platinum-based drugs integrating transcriptomics^{11,68-72}, few studies exist pursuing proteomics^{73,74} and even less integrating metabolomics⁷⁵⁻⁷⁷, the youngest wide spread -omics discipline. The reason could be that metabolomic platforms and procedures are still not as mature as the other -omics and some challenges must be considered to adapt analytical methods⁷⁸. In this work we integrated metallomics with metabolomics in *in vitro* incubations of oxaliplatin in the colorectal cancer line HCT116. The field of metabolomics is discussed in the next sections.

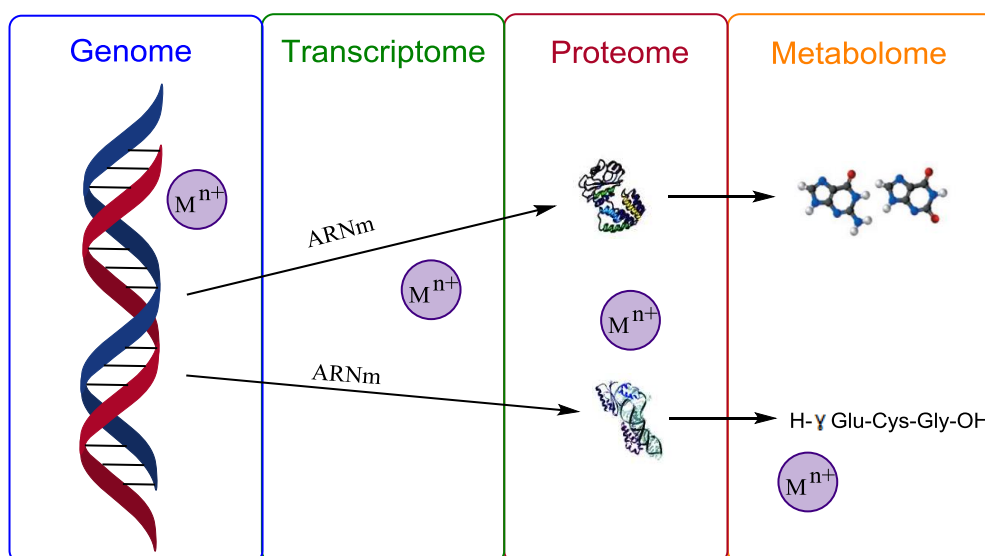


Figure 4 Metallomics in relation to the -omics cascade. Adapted from elsewhere^{4,79,80}.

1.3 Metabolomics

Metabolomics is the study of small molecules or metabolites (<1500 Da) present in cells, tissues and body fluids⁸¹. It provides a link between genotypes and phenotypes since these metabolites are the ultimate product to genetic and environmental changes⁸². Despite of the discovery in the 1920s of a different metabolism between cancer and non-cancer cells^{83,84},

the genomics and transcriptomics field dominated in cancer research^{78,85,86} until the resurgence of metabolomics in recent years⁸⁷. The explanation of this resurgence is that as mentioned before, metabolomics is directly connected to the biochemical activity (genetic and environmental changes)^{80,82}. For this same reason it can also be applied to determine the effect of the anticancer drugs in the different biochemical pathways and provide valuable information about their efficacy and mechanism. These investigations could lead ultimately to an improvement of the treatment and an understanding of the acquired or intrinsic resistance⁸⁸⁻⁹⁰. As pointed in different references, resistance to chemotherapy is a complex process where different mechanisms and alterations are involved such as changes in drug accumulation due to lower intracellular metallodrug uptake and/or higher intracellular metallodrug excretion⁹¹⁻⁹⁴. In this work, we investigated the integration of metallomics and metabolomics in *in vitro* incubations of oxaliplatin in cancer cells resistant and sensitive to this metallodrug (publication III). The sample preparation and analytical techniques in the field of metabolomics are discussed in the next sections. The metabolome information can be acquired by targeted or untargeted methods discussed in sections 1.3.1.1 and 1.3.1.2.

1.3.1 Instrumentation and sample preparation in the field of metabolomics

LC coupled to electrospray ionization mass spectrometry LC-(ESI-MS) has shown to be the analytical approach of choice in this field^{95,96} gaining nowadays ground to other approaches such as nuclear magnetic resonance (NMR)⁷⁸ and gas chromatography (GC) due to the lower sensitivity and resolution of the former and the limited application of the latter⁹⁷. However, some obstacles present in this -omics field like the great variety of metabolites with different physicochemical properties, ion suppression, matrix effects and the sample preparation^{98,99} must be considered to adapt analytical methods. These challenges observed in metabolomics are discussed in the next sections.

1.3.1.1 Targeted metabolomics

Targeted metabolomics is the measurement of characterized metabolites. With the use of external and internal standards (section 1.3.1.5) quantitative or semi-quantitative information can be obtained¹⁰⁰.

For GC a single quadrupole with a hard ionization source (electron ionization, EI) is used which leads already to the fragmentation of the metabolites to select the product ions in the quadrupole. The method in GC-MS has been standardized for decades since the same colli-

sion energy is employed (70 eV) with similar separation methods (similar columns and eluents) getting libraries based on retention time and mass spectra¹⁰¹. Moreover, the higher resolution of the GC separation reduces the co-elution of metabolites. However, as mentioned in the previous section, the application of GC is limited since the analytes under study must be volatile or they must be derivatized to make them volatile and this derivatization process is experimentally demanding^{97,102}.

On the other hand, for LC, a fragmentation is employed for metabolite selectivity and accurate quantification (LC-MS-MS or MS²). Multiple reaction monitoring (MRM) or selected reaction monitoring (SRM) with triple quadrupole (QQQ) is usually used for this approach where a mass to charge ratio (m/z) is selected in the first Q for later fragmentation in the second Q and selection of the fragments (specific for each compound) in the third Q⁹⁶. Unlike in GC, the method is not standardized (different columns and eluents)¹⁰¹ and the libraries are based only in mass spectra although nowadays there are studies to try to predict the retention time in LC-MS¹⁰³.

Although QQQ is the most employed analytical approach for targeted metabolomics, especially in routine analysis, since 2010 improvements have been done in the application of high resolution mass spectrometry (HR-MS) for quantitative analysis¹⁰⁴. In HR-MS, the selectivity is based on the mass resolution and this selectivity in full scan (MS¹) was shown to be similar compared to SRM in QQQ. The use of MS² in HR-MS did not improve this selectivity in most cases¹⁰⁴. This, together with the fact that HR-MS can be employed for untargeted metabolomics (as discussed in the next section) makes it a powerful analytical tool.

1.3.1.2 Untargeted metabolomics

Untargeted metabolomics is the analysis of as many metabolites as possible including: (1) Known unknown metabolites. Metabolites that are initially unknown for the researcher but that were previously characterized and can be found and annotated by comparison to MS¹ and/or MS² spectra libraries. (2) Unknown unknown metabolites. Metabolites that are not characterized and they cannot be found in any libraries.^{104,105}

The high resolution, the accurate mass and the short cycle time provided by HR-MS make it the analytical tool of choice for this purpose. TOF and Orbitrap are the most employed instruments in HR-MS¹⁰⁶. They work in a different way and they show some differences in the

acquisition parameters. Whereas in TOF the rate scan is higher, in Orbitrap, on average, the resolution is superior.

Here, in HR-MS, as mentioned before, the metabolite identification can be done in MS¹ since the high mass accuracy obtained reduces the number of possible sum formulas for metabolite identification. When a quadrupole is employed for fragmentation, MS² information can be also applied for metabolite identification in different libraries. In untargeted metabolomics the researchers do not know which metabolites they are looking for¹⁰⁴. For this reason, the fragmentation is carried out by selecting the most abundant ions or by selecting a list of ions that the researchers are interested in. Nevertheless, the use of standards is suggested especially for MS¹ but for MS² as well since the fragmentation might be different depending on the instrument¹⁰⁷. The different strategies for metabolite identification are discussed in more detail elsewhere¹⁰⁸⁻¹¹¹.

1.3.1.3 Targeted and untargeted metallometabolomics

The isotope pattern is also employed for metabolite identification in HR-MS reducing the number of possible sum formulas¹⁰⁸. This isotope pattern detection can be also applied to the detection of the metallodrug under study as well as its different species since the isotope pattern is characteristic for each element²⁵ (**Figure 5**).

However, this is only possible for multi-isotopic metals which are ionized in ESI. Moreover, it can be applied only to small molecules since in big molecules the isotope pattern of carbon would not allow the detection of the isotope pattern of the metal. In addition to comparably high limits of detection for metal compounds, species specific standards would be necessary for quantification^{25,26} (as opposed to LC-ICP-MS where species unspecific quantification is possible; discussed in section 1.3.1.5). Then, although LC-ESI-MS is the choice for the targeted and untargeted analysis of organic compounds, for trace analysis of metal-containing compounds LC-ICP-MS remains the method of choice. When paralleled by LC-ESI-MS, complementary molecular information can be provided.

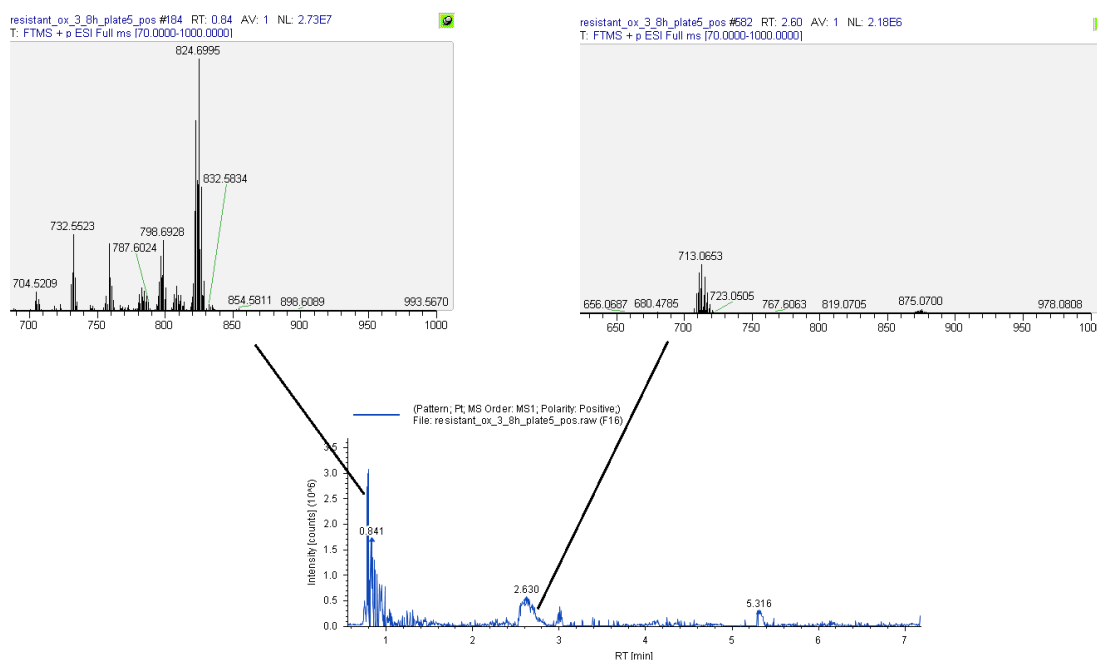


Figure 5 The Isotope pattern of platinum detected with HILIC-HR-MS in a cell extract of the colorectal resistant cancer line HCT116 after 8h of incubation with oxaliplatin 5 μ M. The column UPLC BEH Amide (2.1 x 100 mm, 1.7 μ m) was employed at pH4 in positive mode (see publication III for more detailed information about the instrumentation and the HILIC method). Different peaks containing the platinum pattern were obtained in the void volume (0.841 min) and they could not be completely separated with this method. Another peak could be observed at 2.630 min. The masses could not be identified and the formation of artefacts during the sample preparation could not be prevented. Software: Compound Discoverer 3.0 and Thermo Xcalibur 4.0.

1.3.1.4 Data treatment

Another important aspect to consider in metabolomics is the data treatment to find differences between metabolomes¹⁰², a complex task in untargeted metabolomics. In untargeted metabolomics thousands of features can be detected in one single sample. Different features, at the same time, can be generated from one single metabolite (adducts, fragments, multiple-charge states) or can be introduced during the sample preparation (artefacts) or in the data acquisition (bioinformatic errors)¹¹². Hence, a method validation is necessary to get accurate biological information and although this is common in targeted analysis, it is still in process for untargeted analysis¹⁰².

1.3.1.5 Matrix effects and ion suppression

In both ionization sources, ICP-MS and ESI-MS, ion suppression and matrix effects must be considered for accurate quantification being isotope dilution the key to overcome this obstacle. This isotope dilution is ideally carried out with standards that are chemically similar to the analyte. However, in ICP-MS the compounds are completely atomized and ionized and hence the enriched isotope of the element to be analyzed (species-unspecific standards) can be employed for quantification purposes. That is not the case in ESI-MS where hundreds of metabolites are usually quantified and the standards of them (species-specific standards) are always needed, making this task more difficult.

In this regard, the use of bioreactors to obtain isotopically labeled metabolites has shown to be the solution to this inconvenience. In this work we used internal standards obtained from a bioreactor containing yeast cells (*Pichia pastoris*) where ^{13}C labeled glucose was employed as the only nutrient obtaining metabolites labeled with ^{13}C . These labeled metabolites can be later employed for internal standardization to correct for these matrix effects and ion suppressions¹¹³⁻¹¹⁸. Nevertheless, although this validation is straightforward for targeted metabolomics, it is still difficult or not possible in untargeted metabolomics.

1.3.1.6 Cell extraction, protein normalization and LC separations

Due to the great variety of metabolites with different physicochemical properties, it is not possible to extract all of them simultaneously. Hence, the results are going to be conditioned from the first step of the sample preparation.

A fast and effective quenching is essential to stop the biochemical activity of the metabolites during the sample preparation¹¹⁷, but the sample storage, homogenization and extraction solvent must be carefully considered as well. All these features were previously reviewed by Mushtaq et al¹¹⁹. In this work, we used liquid N_2 for the quenching of adherent mammalian cells of the colorectal cancer line HCT116 which shows no cell leakage or low cell leakage¹¹⁹ followed by cold MeOH extraction which showed to be suitable for these cells¹²⁰ extracting in this case polar metabolites.

As mentioned before in *in vitro* studies for metallomics (section 1.1.1.3), the number of cells is usually different when different incubation times or cell lines are compared but also in the cell-wells of the same plate due to different experimental conditions. It is then necessary of a normalization since a different number of cells implies a different metabolite content³⁵. How-

ever, in metabolomic studies on adherent cells, the cells are extracted by scraping them from the surface of the wells (Dettmer et al.¹²¹), since trypsinization alter metabolites¹²¹⁻¹²³. This hampers cell counting for normalization. To overcome this inconvenience, kits can be employed for protein quantification which is directly correlated to the number of cells and hence the protein content can be used for normalization (publication III).

It is also important to consider the different LC techniques for the different coverage of the metabolome^{99,124,125}. In this work we employed HILIC for the retention of polar metabolites. Different reviews^{126,127} showed also the use of sub-2- μ m particle size in HILIC separations to improve efficiency at short separation times. In this work we employed the already mentioned sub-2- μ m BEH stationary phase (section 1.1.1.6) with uncharged amide groups. The HILIC separation in general and this column in particular are discussed in the next section.

1.3.1.7 HILIC

HILIC was initially employed for carbohydrate investigations in the 1970s. The primary retention mechanism in HILIC is the liquid-liquid partitioning of the analyte between the organic mobile phase and the water layer¹²⁸. It offers a complementary selectivity to RP increasing the retention of polar metabolites^{124,125,129,130}. It also offers a better sensitivity and moderate backpressures due to the use of organic solvents^{127,128}. Unlike in ICP-MS where organic solvents lead to carbon deposition and ion suppression, in ESI-MS they improve the efficiency of the spraying and desolvation increasing the sensitivity¹²⁸. However, at the beginning, the HILIC separations showed long analysis times and usually short pH ranges^{131,132}. In this work we employed the UPLC BEH Amide column based on the sub-2- μ m bridged-ethyl hybrid (BEH) technology mentioned in section 1.1.1.6 and shown in **Figure 3** with uncharged amide groups improving efficiency in a shorter analysis time. This column was previously reported in different metabolomics studies including cell extracts of adherent mammalian cancer cells^{113,123,133}. Investigations in our group confirmed the power of this separation with regard to metabolite coverage when compared to other conventional HILIC columns (publication III).

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2 RESEARCH OBJECTIVES

This thesis deals with the investigation of different analytical techniques in preclinical anti-cancer research of platinum-based drugs as well as with the combination of different analytical fields (metallomics and metabolomics) in this stage. This combinatorial study is important to get accurate information on the drug's behavior prior to the clinical stage.

- I. *Ex vivo* incubations combined with ICP-MS has been extensively employed for the investigation of metallodrugs and their behavior (adduct formation and hydrolysis). However, depending on the analytical approach, the results might vary due to stabilities and recoveries of the metallodrug during the sample preparation. Hence, we carried out a comparison study of different analytical approaches in order to quantify platinum-based drugs with opposite binding to proteins. Comparing not only the results but also different features such as time of analysis or sample preparation, the relatively new UHPLC SEC approach showed the best results even when the analytes showed opposite chemistries.
- II. After observing the superiority of UHPLC SEC in the first publication, the resolution was still low for the separation and quantification of intact metallodrug, important for kinetic studies. Thanks to this column based on BEH particles able to resist higher pressures compared to the conventional SEC columns, we developed a 2DSEC-RP-ICP-MS technique for the screening of metallodrugs in *ex vivo* incubations. This technique allows the quantification of intact metallodrug extracting the HMF in the first dimension (UHPLC SEC) and analyzing the LMF in the second dimension (RP). This automatic protein extraction avoids the use of off-line sample preparations which could lead to low stabilities and recoveries of the metallodrug.
- III. Metallomics can be combined to the other -omics to investigate the behavior of the drug and its efficacy. Whereas metallomics have been extensively combined with genetic approaches, the combination with proteomics and especially with metabolomics has been less investigated. In this work, metallomics and metabolomics were integrated in *in vitro* studies of oxaliplatin in sensitive and resistant cells of the colorectal cancer line HCT116. The differences observed in both -omic fields will be further investigated to understand the mechanism of action of this drug as well as its acquired resistance.

3 RESULTS

This cumulative PhD thesis consists of three publications which appeared and will appear in international peer-reviewed journals, listed in chronological order:

I. Critical assessment of different methods for quantitative measurement of metallodrug-protein associations

Luis Galvez, Sarah Theiner, Márkó Grabarics, Christian R. Kowol, Bernhard K. Keppler, Stephan Hann, Gunda Koellensperger. *Analytical and Bioanalytical Chemistry*, **2018**, 410, 7211-7220.

II. Heart-cut 2DSEC-RP-ICP-MS as a screening tool in metal-based anti-cancer research

Luis Galvez, Mate Rusz, Michael A. Jakupiec, Gunda Koellensperger. *Journal of Analytical Atomic Spectrometry*, **2019**.

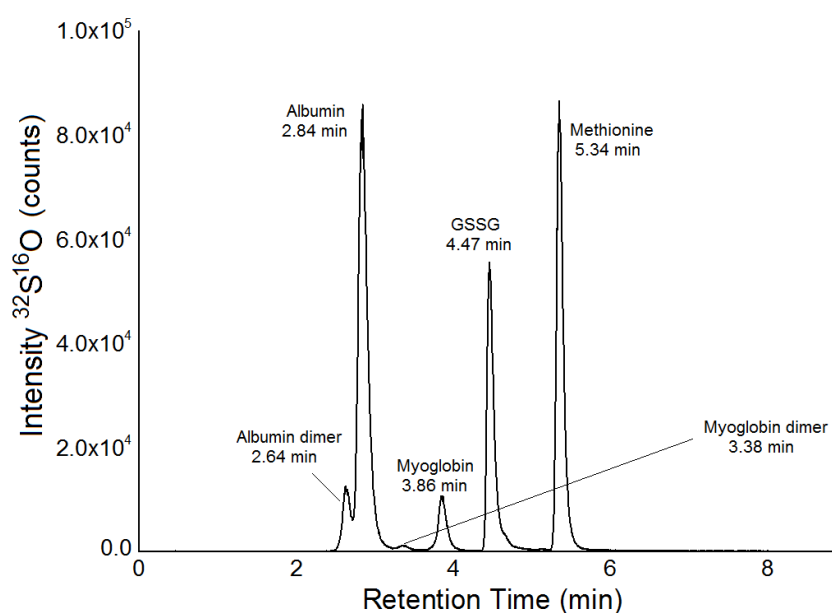
III. Preclinical studies on metal based anticancer drugs as enabled by integrated metallomics and metabolomics

Luis Galvez, Mate Rusz, Michaela Schwaiger-Haber, Yasin El Abiead, Gerrit Hermann, Ute Jungwirth, Walter Berger, Bernhard K. Keppler, Michael A. Jakupiec, Gunda Koellensperger. Submitted.

3.1 PUBLICATION I

Critical assessment of different methods for quantitative measurement of metallodrug-protein associations

Luis Galvez, Sarah Theiner, Márkó Grabarics, Christian R. Kowol, Bernhard K. Keppler, Stephan Hann, Gunda Koellensperger.



Analytical and Bioanalytical Chemistry, **2018**, 410, 7211-7220.

A quantitative study of two platinum(IV)-prodrugs with opposite binding to proteins was carried out with different analytical techniques. UHPLC SEC showed the most accurate result independently of the chemistry of the metallodrug in a short analysis time.

I carried out the experiments for TFC, SEC and UHPLC SEC. The experimental part for centrifugal ultrafiltration was carried out in collaboration with the co-authors Sarah Theiner and Márkó Grabarics.



Critical assessment of different methods for quantitative measurement of metallodrug-protein associations

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Abstract

Quantitative screening for potential drug–protein binding is an essential step in developing novel metal-based anticancer drugs. ICP–MS approaches are at the core of this task; however, many applications lack in the capability of large-scale high-throughput screenings and proper validation. In this work, we critically discuss the analytical figures of merit and the potential method-based quantitative differences applying four different ICP–MS strategies to ex vivo drug–serum incubations. Two candidate drugs, more specifically, two Pt(IV) complexes with known differences of binding affinity towards serum proteins were selected. The study integrated centrifugal ultrafiltration followed by flow injection analysis, turbulent flow chromatography (TFC), and size exclusion chromatography (SEC), all combined with inductively coupled plasma-mass spectrometry (ICP–MS). As a novelty, for the first time, UHPLC SEC–ICP–MS was implemented to enable rapid protein separation to be performed within a few minutes at > 90% column recovery for protein adducts and small molecules.

Keywords Metal-based anticancer drugs · High-throughput · ICP–MS · Elemental speciation analysis · Metal–protein interaction

Introduction

The development of metal-based anticancer drugs continues to be a topical research theme. In fact, a recent upsurge of activities regards the development of metal-based compounds and non-classical platinum complexes whose mechanism of action is distinct from known drugs such as the clinically established cisplatin [1–3]. Many metal-based compounds have been synthesized by redesigning the existing chemical structure through ligand substitution or building the entire

new compound with enhanced safety and cytotoxic profile [3, 4].

As in any drug development, studying the chemistry of the drug in solution-based ex vivo experiments is a first crucial step. In the case of metal-based anticancer drugs, in vitro protein binding studies received major attention, as it was recognized soon that protein binding occurred for many compounds already in short time scales in human blood and would be therefore decisive regarding drug delivery, drug deactivation, or vice versa drug activation. For example, whereas for platinum (II)-based drugs such as cisplatin binding to serum proteins, especially human serum albumin (HSA) leads to deactivation and lower bioavailability of the drug [5], other concepts exploit HSA for targeted drug delivery to the tumor tissue [6, 7]. HSA is known to accumulate in cancer cells, an advantage that was investigated by the preparation of functionalized platinum (IV) prodrugs that were designed to bind selectively to HSA [8–10].

Evidently, tools of trade for this type of ex vivo investigations are offered by elemental speciation approaches utilizing ICP–MS as a detection technique of choice [11–14]. ICP–MS featuring species unspecific quantification capabilities was combined with intact-protein separation- or fractionation techniques ensuring native, i.e., mild chemical conditions, being

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the latter a prerequisite ensuring that protein binding was not reversed upon chromatographic separation, as drug protein association could be based on coordinative and/or covalent binding [3, 11, 15–19]. A comprehensive review about the different approaches is given elsewhere [20, 21]. In brief, capillary electrophoresis (CE) and SEC have been the most commonly applied native separation methods [22, 23]. Already in 1999 [24], SEC combined with ICP–MS emerged as essential tool for studying the interaction of metallodrugs and serum proteins and has seen numerous applications ever since in support of new drug design concepts. Despite this success, the separation was considered a low-resolution, time-consuming method. The involved length of single experiments was regarded as major disadvantage especially when kinetics studies were addressed. Typical chromatographic separation times ranged between 20 and 30 min [25]. As an alternative to on-line separation, several studies resorted to an off-line fractionation technique, which had been developed in the late 1960s [26], namely centrifugal ultrafiltration using cut-off filters. As a key advantage, this strategy is technically very simple; however, it became very clear soon that filter material selection and preconditioning strategy was crucial regarding drug recovery [27–30], making this technique more tedious than expected. Flow injection (FI)-ICP–MS [28, 31] proved to be a valuable method addressing the metal-based drug distribution between low (LMF) and high molar mass fraction (HMF) in very small sample volumes. In some cases, off-line protein removal was followed by LC-ICP–MS analysis in order to assess potential low molar mass transformation products of the metal-based drugs [16, 27, 32]. Only recently, on-line protein removal by turbulent flow chromatography (TFC) was introduced in combination with ICP–MS for studying metal-based anticancer drugs and their protein binding. TFC was first developed in the late 1990s as an emerging, alternative approach to study biological samples by extracting on-line the HMF and analyzing the LMF. In TFC, very high mobile phase linear velocities are combined with stationary phases consisting of large porous particles (30–80 μm). This combination leads to a turbulent flow regime which generates a mass transfer cut-off allowing large molecules, e.g., proteins, to pass without any interaction. Small molecules can interact with the functional groups of the stationary phase and are then eluted subsequently by an appropriate solvent or buffer [33–36].

The aim of this work, thus, was to provide a comparison between different state-of-the-art methods focusing on the aspect of quantitative protein binding studies. The investigated approaches offered a varying degree of automation. The objective was to shed light on crucial and sometimes overlooked shortcomings that make a generalizable straightforward quantitative application without considering the chemistry of the metallodrugs difficult. Moreover, as a novelty, UHPLC SEC was combined with ICP–MS detection for studying

metallodrug–protein interaction in human serum. Various approaches have been developed to improve the speed of size-exclusion chromatography [37], with the recently introduced UHPLC SEC or sub-2 μm technique being one of the most promising ones, reducing both eddy dispersion and resistance towards mass transfer, two of the most important factors contributing to band broadening in liquid chromatography [38]. For SEC, these sub-2 μm materials have been introduced several years ago; however, their application in speciation studies is novel. A recent review reports on developments in UHPLC SEC [39] including the key application areas such as, e.g., purity monitoring of protein-related compounds in industry, the development process of biotherapeutic proteins, the field of proteomics, and the polymer industry [40, 41]. To the best of our knowledge, this technology has not been applied to speciation analysis with ICP–MS detection so far.

Despite the fact that numerous methods addressing metallodrug–biomolecule interactions are available, a comparative study on different metallodrugs (different ligand chemistry) is still lacking. Hence, next to size-exclusion-based approaches, the cross-validation study involved centrifugal filtration and turbulent flow chromatography, always in combination with ICP–MS detection. All investigated approaches were applied to test compounds in serum incubations. For this purpose, two platinum (IV)-based prodrugs (1) with selective albumin-binding properties by a maleimide moiety and (2) a negative control being non-reactive towards biomolecules with a succinimide moiety were selected. The novel drugs release oxaliplatin upon reductive activation. The compound with selective albumin binding was already tested in vivo colon carcinoma-bearing mice and treatment resulted in significantly reduced tumor growth and even disease stabilization. Additionally, for a very similar compound, a highly increased plasma half-life and very efficient tumor accumulation were observed [8].

Experimental

Reagents, chemicals, and standards

A reagent I grade water ($> 10 \text{ M}\Omega \text{ cm}^{-1}$ resistance according to ISO 3696 water specifications) purification system (Ultra Clear basic Reinstwassersystem, SG Wasseraufbereitung und Regenerierstation GmbH, Barsbüttel, Germany) was used to obtain purified water. Ammonium acetate ($\geq 99\%$), glutathione disulfide ($\geq 98\%$), albumin from human serum (96–99%), and myoglobine from equine skeletal muscle (95–100%) were purchased from Sigma Aldrich, St. Louis, MO, USA. Methionine ($\geq 99\%$) was purchased from Merck (Darmstadt, Germany). Pt standard ($1002 \pm 6 \mu\text{g/mL}$) was purchased from Inorganic Ventures, Christiansburg, VA, USA. Fetal calf serum (FCS) was kindly provided by Prof. Walter Berger from

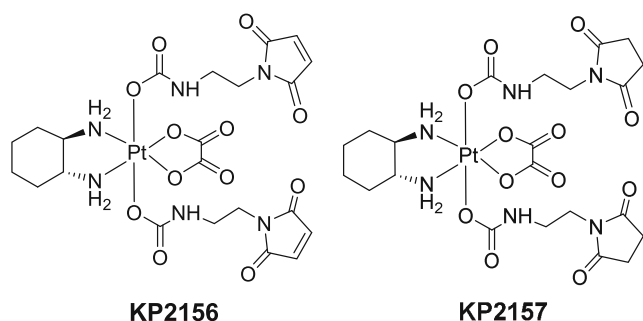


Fig. 1 Chemical structures of KP2156 containing a maleimide moiety designed to bind covalently to the cystein residues of serum proteins via maleimide-thiol coupling reaction and KP2157 containing a succinimide moiety lacking of the ability to bind to sulfhydryl groups [8]

the Institute of Cancer Research, University of Vienna, Vienna, Austria. The analytical figures of merit for metallodrug studies were assessed by the use of two platinum (IV) compounds synthesized according to literature procedures [8] at the Institute of Inorganic Chemistry, University of Vienna, Vienna, Austria. The structures of these compounds, KP2156 and KP2157, are shown in Fig. 1. More specifically, the selection comprised KP2156 (trans-bis(2-maleimideethylcarbamato)-dihydroxido(1R,2R-diamminocyclohexane)-oxalatoplatinum (IV)), a maleimide-functionalized Pt (IV) complex with oxaliplatin core and KP2157 (trans-bis(2-succinimideethylcarbamato)-dihydroxido(1R,2R-diamminocyclohexane)-oxalatoplatinum (IV)), its succinimide analog [8]. While KP2156 is designed to bind covalently to the cystein residues of serum proteins via maleimide-thiol coupling reaction, in order to utilize the enhanced permeability and retention effect for targeting, its succinimide derivative, KP2157, inherently lacks the ability to form covalent bounds with sulfhydryl groups, due to the missing double bonds in the axial linker moiety (Fig. 1). Prior kinetics studies by UHPLC SEC in our laboratory showed this difference in the affinity towards serum proteins (see Electronic Supplementary Material (ESM) Table S1 for chromatography and ICP-MS conditions and Fig. S1 for chromatograms).

Ex vivo incubations

Stock solutions of KP2156 and KP2157 were prepared in water (1 μ M) right before the incubation in FCS. 100 μ L of the stock solution was incubated in 900 μ L of FCS (corresponding to a molar ratio of drug:albumin of 1:6000 approximately) for 45 min at 37 °C. Immediately prior to ICP-MS determination, the incubations were diluted by a factor of 10 resulting in a drug concentration of 0.01 μ M. In all experiments, aqueous solutions of the drugs were employed, as the drugs are stable under these conditions [8].

ICP-MS

The ICP-MS instrument used for the quantification studies with conventional SEC and TFC was an iCAP-QMS Thermo Scientific (Bremen, Germany) with oxygen (purity 5.0, Linde Gas GmbH, Vienna, Austria) as reaction gas and Qtegra Intelligent Scientific Data Solution (version 2.4.1800.33) for the data treatment. For the quantification in the centrifugal ultrafiltration studies and for UHPLC SEC, the Agilent 8800 ICP-MS/MS (Agilent Technologies, Tokyo, Japan) was used with Agilent MassHunter software package (Work-station Software, Version C.01.03, 2016) for the data treatment. The ICP-MS parameters are summarized in Table 1.

SEC and UHPLC SEC

The Agilent 1260 infinity Bio-inert HPLC system (Agilent Technologies, Waldbronn, Germany) was employed for the UHPLC SEC measurements. For the quantification studies with conventional SEC, the Thermo Scientific Transcend HPLC system (San Jose, CA, USA) was used. The chromatographic conditions are given in Table 2.

Centrifugal ultrafiltration

Centrifugal ultrafiltration was carried out using Amicon Ultra 0.5-mL centrifugal regenerated cellulose membrane filters,

Table 1 ICP-MS operation parameters

Instrumentation ICP-MS	iCAP-QMS Thermo Scientific	ICP-MS/MS Agilent 8800
Nebulizer	PFA-ST	MicroMist
Spray chamber	Cyclonic	Scott double-pass
Nebulizer gas flow	1.01 L/min	1.05 L/min
Aux. gas	0.99 L/min	0.90 L/min
Plasma gas	14 L/min	15 L/min
Reaction gas	0.370 mL/min	0.300 mL/min
ICP RF Power	1550 W	1550 W
m/z measured	194.97, 47.97	195, 48

Table 2 Chromatographic conditions

Instrumentation HPLC	Agilent 1260 infinity Bio-inert	Thermo Scientific Transcend system
HPLC Column	Acquity UPLC Protein BEH SEC, 4.6 × 150 mm, 125Å, 1.7 µm, 1 kDa–80 kDa, Waters	BioBasic SEC-60 A, 4.6 × 250 mm, 5 µm, 0.1 kDa–10,000 kDa Thermo
Eluent	50 mM CH ₃ COONH ₄ , pH = 6.0	50 mM CH ₃ COONH ₄ , pH = 6.0
Flow rate	400 µL/min	250 µL/min
Injection volume	5 µL	10 µL
Column temperature	30 °C	Room temperature
Autosampler Temperature	4 °C	4 °C
Combined with	ICP–MS/MS Agilent 8800	ICP–QMS iCAP Thermo Scientific

with a 10-kDa molecular weight cut-off (MWCO) (Millipore Co, Carrigtwohill, Co. Cork, Ireland). The samples were ultracentrifuged with a Hermle Z 466 K device with a precooled rotor (4 °C) at a G-force of 12,500g. For preconditioning of the membrane filters and ultracentrifugation of the drug solutions, a centrifugation time of 60 min and 15 min was used, respectively. The filters used for centrifugal ultrafiltration were preconditioned using the solvents with the highest recovery for the drugs, respectively. For KP2156, 50 mM CH₃COONH₄, pH = 6.0 was employed, whereas for KP2157 FCS was used. Flow injection measurements for platinum quantification were performed using an Agilent 1260 Infinity Bio-inert HPLC system (Agilent Technologies, Waldbronn, Germany) coupled to an Agilent 8800 ICP–MS/

MS (Agilent Technologies, Tokyo, Japan). The HPLC system was operated with CH₃COONH₄ (50 mM, pH = 6) as eluent, a flow rate of 250 µL/min, and an injection volume of 10 µL. The ICP–MS operation parameters are summarized in Table 1.

TFC

TFC combines SEC and traditional stationary phase chemistry. In this case, a reversed phase was employed. The HPLC used was a HPLC Thermo Scientific Transcend system (San Jose, CA, USA) coupled to an ICP–QMS iCAP Thermo Scientific (Bremen, Germany). The separation and elution of the HMF and LMF were carried out by a two inert six-port

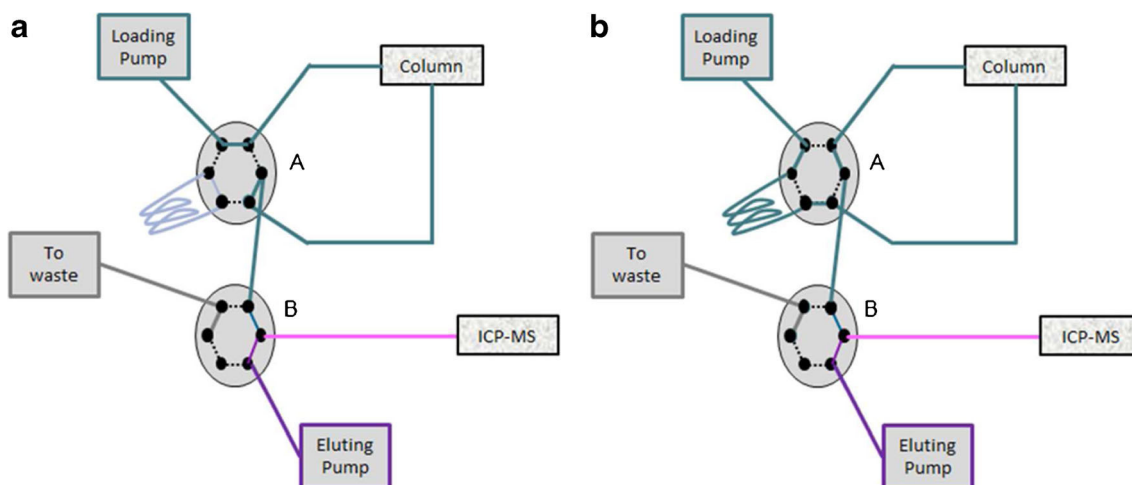


Fig. 2 **A** At turbulent flow conditions (1 mL/min, 20% MeOH, delivered by the loading pump, while the eluting pump is set to 0 mL/min flow), the HMF (bound fraction of metal/drug) shows no retention on the column and can hence be directed to the ICP–MS (depending on the valve B position). The free drug corresponding to the low molar mass fraction LMF is retained. Subsequently, the flow delivered by the loading pump is reduced to 0.2 mL/min (20% MeOH) and mixed with the flow delivered by the eluting pump (0.8 mL/min 100% buffer). In the following step, the valve A is switched (**B**) and the “loading pump”- eluent, mixed with a

solvent plug of 100% MeOH in a loop, is sent back-flush to the column. This high organic solvent conditions (0.2 mL/min) result in elution of the LMF from the column. Before reaching the ICP–MS, the latter flow is combined with the flow of the eluting pump, in order to reduce the MeOH content. Throughout all steps, the flow rate entering the introduction system of the ICP–MS is set to 1 mL/min. Finally, the last steps involve column cleaning and the loop loading with 100% MeOH. In the last step, the valve A is switched to the initial position (**A**) and the column is preconditioned for the next injection

Table 3 Chromatographic conditions and TFC method

Chromatographic conditions																
HPLC Column			Fluoro XL 0.5 × 50 mm													
Eluent			Eluent B 50 mM CH ₃ COONH ₄ , pH = 6.0, Eluent C MeOH													
Injection volume			10 μL													
Column temperature			Room temperature													
Autosampler temperature			4 °C													
TFC Method																
Step	Start	Sec	Loading pump						Tee	Loop	Eluting pump					
			Flow	Grad	A	B	C	D			Flow	Grad	A	B	C	D
1	0	40	1	Step	–	80	20	–	T	out	0	step	–	80	20	–
2	0.67	5	0.2	Step	–	80	20	–	T	out	0.8	step	–	100	–	–
3	0.75	55	0.2	Step	–	80	20	–	T	in	0.8	step	–	100	–	–
4	1.67	10	1	Step	–	–	100	–	===	in	1	step	–	80	20	–
5	1.83	200	1	Step	–	–	100	–	===	in	1	step	–	80	20	–
6	5.17	240	1	Step	–	80	20	–	===	out	1	step	–	80	20	–

valves present in this HPLC instrument (Fig. 2). The chromatographic conditions and the method are shown in Table 3.

Results and discussions

SEC and UHPLC SEC

The UHPLC SEC-ICP-MS approach established in this work relied on the most advanced columns available on the market. More specifically, the implemented SEC column was based on the use of high pore-volume ethylene-bridged hybrids (BEH) particles with diol-bonded surface which combines an increase of ~75% in pore volume with the required mechanical rigidity to maintain their integrity under high pressure

and shear conditions. This stationary phase shows significantly reduced acidity of residual silanol groups, thereby reducing the contribution of unwanted ionic interaction between separated molecules and the stationary phase. UHPLC SEC when compared to conventional SEC for methionine (see ESM Table S2), (compound selection based on comparable retention time factors on the two columns) showed significantly higher separation efficiency. In fact, the number of theoretical plates/m was increased drastically, due to smaller peak widths. Figure 3A, B shows typical UHPLC SEC-ICP-MS separations accomplished within a few minutes. The size ladder consisting of a protein/amino acid mixture (Fig. 3A) and undiluted FCS (Fig. 3B) was analyzed monitoring sulfur (*m/z* 47.97 with oxygen as reaction gas). The retention time of the most abundant protein in FCS corresponded to albumin

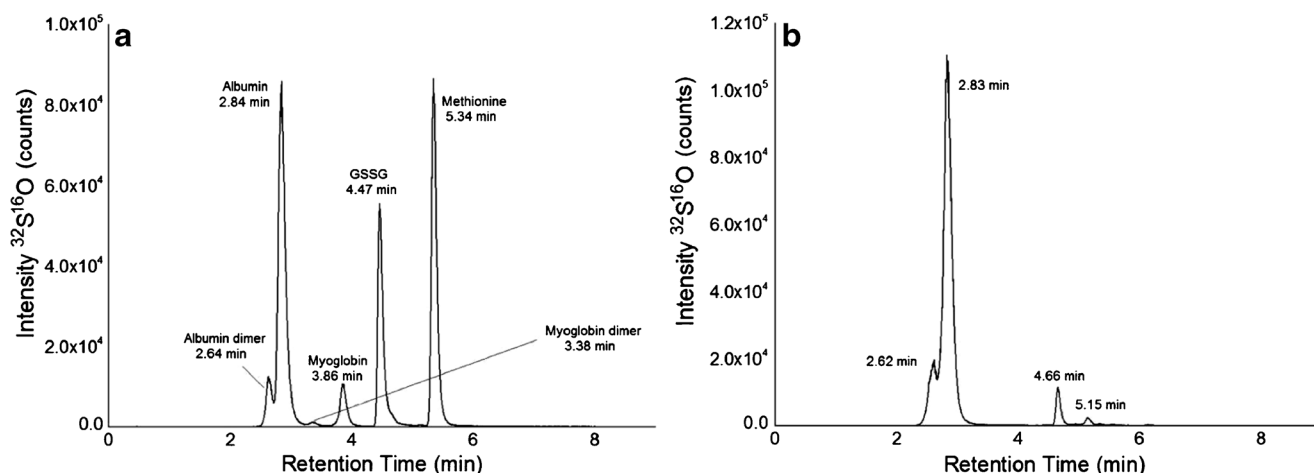


Fig. 3 UHPLC SEC chromatograms with the column Acquity UPLC Protein BEH SEC, 4.6 × 150 mm, 125A, 1.7 µm, size range 1 kDa–80 kDa, at a flow rate of 400 µL/min and an injection volume of 5 µL of (A) a protein/amino acid mixture containing standards of HSA

(66 kDa) (~9.32 µM), Myoglobin (17 kDa) (~34.6 µM), glutathione disulfide (GSSG, 0.6 kDa) (~375 µM) and methionine (0.1 kDa) (~1700 µM) and (B) undiluted FCS. Sulfur was monitored as ³²S¹⁶O at *m/z* 47.97 with oxygen as reaction gas

Table 4 Analytical figures of merit of the standards and the samples

Acquity UPLC Protein BEH SEC, 4.6 × 150 mm, 125A, 1.7 μm				
Compound	Recovery (%)	LOD (nM) 3σ criterion	LOD (μg/L Pt) 3σ criterion	Absolute LOD (fg Pt) 3σ criterion
KP2156	100 ± 5	0.50	0.10	500
KP2157	99 ± 9	0.51	0.10	500
KP2156 in serum	101 ± 4	0.71	0.14	700
KP2157 in serum	97 ± 3	0.36	0.07	350

(2.83 min). The optimal size range of the used column was 1–80 kDa, presenting thus, the dimer of albumin (132 kDa) in the exclusion limit/void volume (2.64 min).

The analytical figures of merit for metallodrug studies were assessed by the use of KP2156 and KP2157 which served as perfect structural analogs of each other, with significantly different affinity towards serum proteins as explained in the experimental part and shown in the ESM (Table S1 and Fig. S1).

As can be readily seen in Table 4, UHPLC SEC-ICP-MS resulted in excellent recoveries for Pt. Regardless whether drug serum incubations (45 min of incubation of KP2156 and KP2157 in FCS at 37 °C, resulting in a molar ratio of drug:albumin of ~1:6000) or drug only (0.01 μM) was analyzed, the mass balance for Pt was complete as the diol bonded surface of the porous hybrid organic/inorganic particles featured significantly decreased acidity of the residual silanol groups. In this way, the SEC eluent composition could be optimized with the aim of reducing salt content thereby increasing the robustness of the method without compromising protein or small molecule recovery. Moreover, excellent limits of detection could be obtained which were comparable to conventional SEC (Table 7), despite the fact that the latter approach utilized lower flow rates (by a factor of 2) and higher injection volumes (factor of 2). Excellent intermediate repeatabilities of 2% were observed for UHPLC SEC-ICP-MS (flow rate of 400 μL/min and an injection volume of 5 μL) upon $n = 18$ and $n = 15$ injections over 24 h for albumin

(monitored via sulfur) and a serum incubation of KP2156 (monitored via Pt). The chromatographic retention time repeatability ranged at 0.4 and 0.5% respectively.

Centrifugal ultrafiltration

An orthogonal approach to SEC and UHPLC SEC-ICP-MS suitable to ex vivo screening of metallodrug-protein interaction is offered by centrifugal ultrafiltration. The sample is fractionated into a protein rich high molar mass fraction (HMF) and a filtrate which constitutes the low molar mass fraction (LMF). The established procedure is to analyze the total Pt content of the sample prior to fractionation, followed by the determination of the Pt content of the filtrate. Due to practical reasons, the HMF is derived from the difference of the two obtained Pt amounts provided that the mass balance of the procedure is complete. Although it could seem a straightforward method, the often encountered poor recoveries of the metal-based drugs pose severe limitations to this otherwise simple method. As the quantitative results critically depend on filter materials [27–30] and preconditioning, we evaluated different preconditioning strategies for the two test compounds KP2156 and KP2157 (Table 5). Regenerated cellulose filters with a cut-off limit of 10 kDa were selected as these filters showed excellent recoveries for different Pt(II) compounds [27]. In this study, regardless which preconditioning method was tested, the recovery of the free drug from diluted drug standards was poor. However, the obtained LODs were lower by a factor of 10 compared to the SEC strategy. The LODs ranged at 0.03 nM (0.006 μg/L of Pt) and 0.01 nM (0.002 μg/L of Pt) for KP2156 and KP2157, respectively (3σ criterion).

Table 5 Centrifugal ultrafiltration

Standards (0.1 μM)		
Compound	Preconditioning	Recovery (%)
KP2156	–	8.7 ± 1.1 $n = 3$
KP2156	Milli-Q water	10 ± 1 $n = 3$
KP2156	50 mM CH ₃ COONH ₄ , pH = 6.0	9.6 ± 2.1 $n = 3$
KP2156	FCS	7.5 ± 1.4 $n = 3$
KP2157	–	38 ± 2 $n = 9$
KP2157	Milli-Q water	41 ± 3 $n = 9$
KP2157	50 mM CH ₃ COONH ₄ , pH = 6.0	38 ± 6 $n = 9$
KP2157	FCS	60 ± 3 $n = 9$

TFC-ICP-MS

TFC has appeared more recently as an on-line extraction technique to study metallodrug-protein interactions, where the protein rich HMF is removed on-line by utilizing TFC. In essence, the two fractions HMF and LMF can be analyzed only considering their Pt content or alternatively, in a more sophisticated approach, the LMF is analyzed on a second analytical column [33–36]. TFC columns are available in

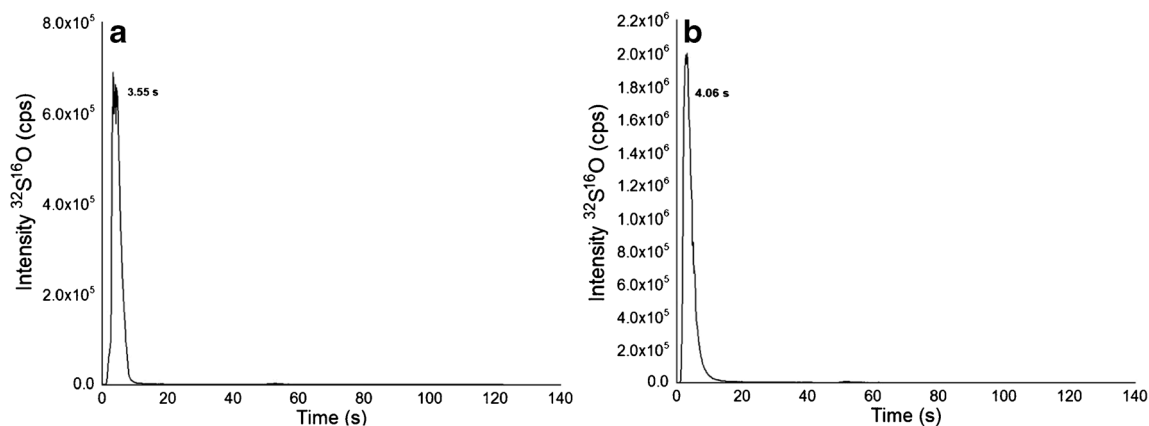


Fig. 4 TFC chromatograms of (A) a standard of albumin 3.79 nM. The LOD was 0.006 nM (3σ criterion). **B** FCS diluted 1:10 in water. Sulfur was monitored at m/z 48 with oxygen as reaction gas

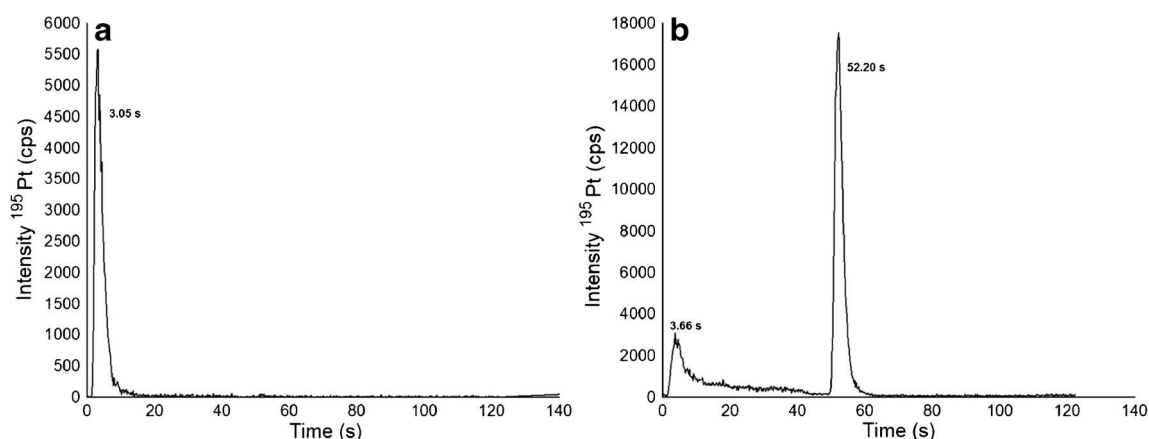


Fig. 5 TFC chromatograms of (A) an incubation of KP2156 0.1 μ M (20 μ g/L of Pt) 1:10 in FCS during 45 min at 37 $^{\circ}$ C and diluted 1:10 in water prior to the analysis (B) an incubation of KP2157 0.1 μ M (20 μ g/L

of Pt) 1:10 in FCS during 45 min at 37 $^{\circ}$ C and diluted 1:10 in water prior to the analysis. Pt was monitored at m/z 195

different chemistries and dimensions [42, 43]. In this work, a silica-based fluorinated alkyl stationary phase was employed which provided a unique selectivity compared to other reversed phase chemistries [43]. TFC is a rapid analytical process compared to SEC; however, the duty cycle between samples is 9 min due to loop filling and preconditioning of the TFC column (Fig. 2). Overall, comparable throughput to UHPLC SEC could be achieved, but at the expense of higher solvent consumption necessary to reach the turbulent conditions. Figure 4 shows the TFC-ICP-MS analysis of albumin and FCS, where the protein shows virtually no retention.

Figure 5 shows the results from the incubations of KP2156 and KP2157 with serum. In accordance with the fact that KP2156 shows pronounced protein binding, no retention for Pt was observed. Only the free KP2157 showed retention. The free drug (52.2 s) was retained on the column until the back flushing with MeOH was applied. TFC-ICP-MS analysis revealed two peaks for KP2157 which could be explained by the occurrence of KP2157 protein adducts and/or the generation of a hydrophilic hydrolysis product of KP2157 showing no reversed phase retention on the TFC stationary phase. The latter explanation is in accordance with UHPLC SEC-ICP-

Table 6 Analytical figures of merit of the standards and the samples

Compound	Recovery (%) $n = 3$	Precision (%) $n = 3$	LOD (nM) 3σ criterion	LOD (μ g/L Pt) 3σ criterion	Absolute LOD (fg Pt) 3σ criterion
KP2157	85 $\pm$ 3	2	0.04	0.008	80
KP2156	54 $\pm$ 1	5	0.05	0.01	100
KP2156 in FCS	64 $\pm$ 2	1	0.03	0.006	60
KP2157 in FCS	90 $\pm$ 5	7	0.04	0.008	80

MS measurements revealing the presence of low molar mass transformation products. As can be observed in Table 6, the TFC approach provided excellent LODs in the sub nM range. As a drawback, the drug recoveries and repeatabilities were compromised compared to the UHPLC SEC-ICP-MS analysis.

Cross-validation of SEC-ICP-MS, UHPLC SEC-ICP-MS, TFC-ICP-MS, and centrifugal ultrafiltration

Finally, the discussed methods were compared regarding their suitability of quantifying the degree of protein metallodrug-adduct formation. KP2156 and KP2157 (0.1 μ M, 20 μ g/L of t) were incubated in fetal calf serum for 45 min at 37 °C, corresponding to a molar ratio of drug:albumin of approximately 1:6000. The incubation solutions were analyzed by conventional size-exclusion (optimal size range 0.1 kDa–10,000 kDa), (see ESM Fig. S2) by UHPLC SEC-ICP-MS (optimal size range of 1–80 kDa), (see ESM Fig. S3) by centrifugal ultrafiltration combined with flow injection analysis and finally, by TFC-ICP-MS (Fig. 5). While for the SEC- and centrifugal ultrafiltration-based methods, standard dilutions of one Pt(IV) compound served for calibration of all investigated Pt(IV) drugs and protein adducts, this species-unspecific quantification concept could not be expanded to the TFC methodology without considering the fact that the high molar mass fraction and the retained hydrophobic low molar mass fraction eluted under completely different conditions from the

TFC column. Fig. S4 in the ESM shows the TFC measurements of the established calibration strategy.

Table 7 summarizes the most important findings of the cross-validation study. KP2157 was assessed in the LMF by calibration using a dilution series of the drugs. However, since Pt was < LOD in the HMF, for all methods except in TFC-ICP-MS, the bound Pt was assessed by difference to the total Pt measured by FI-ICP-MS. The opposite was true for KP2156. In this case, Pt adducts were actually quantified while the LMF was assessed as difference of the experimentally assessed Pt adduct to the total Pt. While SEC-ICP-MS and UHPLC SEC-ICP-MS were in excellent agreement for both quantification exercises, the values obtained by ultrafiltration and TFC-ICP-MS revealed inconsistencies. The former approach was compromised by poor recoveries of the free Pt(IV) compounds. Therefore, correction for recovery resulted in biased values regarding the degree of protein binding of KP2156. While with all other approaches, the LMF fraction of KP2156 was < LOD upon incubation in serum, in ultrafiltration a LMF fraction of 38% was found. TFC-ICP-MS resulted in a biased quantification in the case of KP2157. Here, a low molar mass product co-eluted with the protein fraction.

Conclusions

Different speciation methods were investigated regarding their screening capabilities towards protein binding of

Table 7 Findings of the cross-validation study

Results	SEC	UHPLC SEC	Centrifugal ultrafiltration	TFC
Sample Preparation	No	No	Yes (1.25 h)	No
Time of Analysis	30 min/sample	7 min/sample	1 min/sample	9 min/sample
Solvent consumption	Medium	Medium	Low	High
Recovery (%) KP2157	93 $\pm$ 3	97 $\pm$ 3	60 $\pm$ 3 ^a	90 $\pm$ 5
Recovery (%) KP2156	96 $\pm$ 1	101 $\pm$ 4	9.6 $\pm$ 2.1 ^a	64 $\pm$ 2
LOD (μ g/L Pt) (3 σ criterion)	0.04–0.16	0.07–0.14	0.003–0.004	0.006–0.01
Assessed by calibration				
KP2157 in the LMF (%)	96 $\pm$ 2, <i>n</i> = 3	93 $\pm$ 2, <i>n</i> = 3	95 $\pm$ 4b, <i>n</i> = 9	74 $\pm$ 4, <i>n</i> = 3
KP2157 in the HMF (%)	–	–	–	38 $\pm$ 11, <i>n</i> = 3
KP2156 in the LMF (%)	–	–	38 $\pm$ 6 ^b , <i>n</i> = 3	–
KP2156 in the HMF (%)	102 $\pm$ 1, <i>n</i> = 3	98 $\pm$ 4, <i>n</i> = 3	–	98 $\pm$ 3, <i>n</i> = 3
Assessed by difference				
KP2157 in the LMF (%)	–	–	–	–
KP2157 in the HMF (%)	3.6 $\pm$ 1.9, <i>n</i> = 3	6.9 $\pm$ 2.5, <i>n</i> = 3	4.6 $\pm$ 4.4 ^b , <i>n</i> = 9	26 $\pm$ 4, <i>n</i> = 3
KP2156 in the LMF (%)	– ^c	– ^c	–	– ^c
KP2156 in the HMF (%)	–	–	62 $\pm$ 6 ^b , <i>n</i> = 3	–

^a Corresponding to the standards of the drugs in water

^b Using the preconditioning method with the best recoveries: 50 mM CH₃COONH₄, pH = 6.0 and FCS for KP2156 and KP2157 respectively (see Table 5)

^c Below the uncertainty

metallodrugs. Among the techniques, the analytical performance of the UHPLC SEC-ICP-MS suited best to perform such studies in a high-throughput manner as required in preclinical drug development without the necessity of finding dedicated analysis conditions upon investigating drugs with very different protein-binding affinity.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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Analytical and Bioanalytical Chemistry

Electronic Supplementary Material

Critical assessment of different methods for quantitative measurement of metallodrug-protein associations

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Table S1 ICP-MS operation parameters and chromatographic conditions for the kinetic studies of KP2156 and KP2157 in FCS

ICP-QMS iCAP Thermo Scientific operation parameters	
Nebulizer	PFA-ST
Spray chamber	Cyclonic
Nebulizer gas flow	1.01 L/min
Aux. gas	0.99 L/min
Plasma gas	14 L/min
Reaction gas	0.370 mL/min
ICP RF Power	1550 W
m/z measured	194.97, 47.97
Thermo Scientific Transcend system chromatographic conditions	
HPLC Column	Acquity UPLC Protein BEH SEC, 4.6 x 150 mm, 200Å, 1.7 µm, 10kDa-500kDa, Waters
Eluent	50 mM CH ₃ COONH ₄ , pH = 6.0
Flow Rate	400 µL/min
Injection Volume	2 µL
Column Temperature	37°C

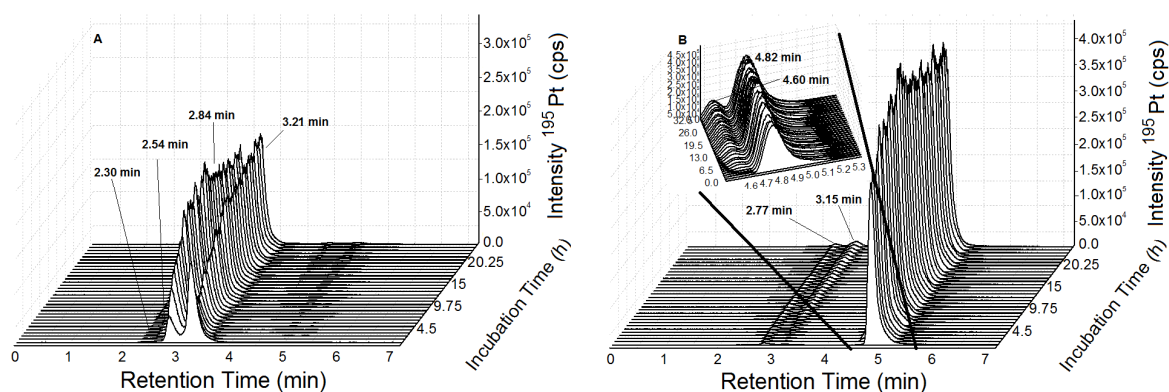


Fig. S1 Chromatographic separations with the column Acquity UPLC Protein BEH SEC, 4.6 x 150 mm, 200Å, 1.7 µm, 10kDa-500kDa, of the platinum complexes (~1 µM) upon incubation in FCS during 24h at 37°C (corresponding approximately to a molar ratio of drug:albumin of ~1:600). (A) KP2156 shows a high binding to albumin (3.21, 2.84, 2.54 and 2.30 min). The free drug could not be observed. (B) KP2157 shows a lower binding to albumin (3.15 and 2.77 min). The free drug could be observed at 4.82 min. A hydrolysis product was observed in kinetics studies of the drug in water as well as in the incubation in FCS (4.60 min)

Table S2 Analytical figures of merit of the SEC and UHPLC SEC columns

Column	Compound	Retention Time (min)	N (m ⁻¹) ^a	H (μm) ^b
BioBasic SEC-60 A, 4.6x250 mm, 5μm, Thermo.	Methionine	12.9	17072	59
Acquity UPLC Protein BEH SEC, 4.6 x 150 mm, 125A, 1.7 μm, Waters.	Methionine	5.34	61960	16

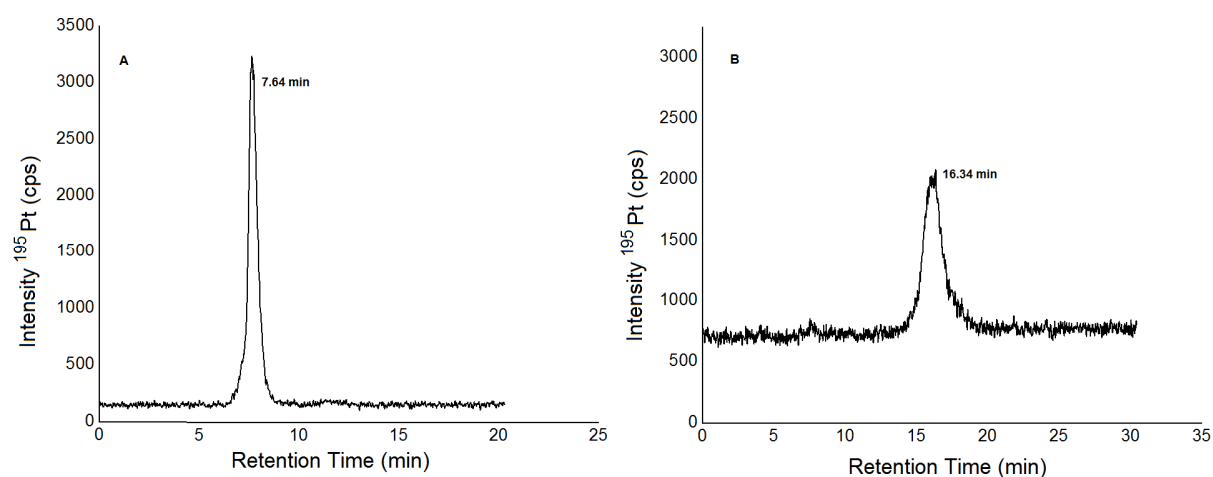
^aTheoretical Plate Number m⁻¹.^bTheoretical Plate Height μm.

Fig. S2 SEC chromatograms (BioBasic SEC-60 A, 4.6x250 mm, 5μm) of (A) an incubation of KP2156 0.1μM (20 μg/L of Pt) 1:10 in FCS during 45 min at 37°C and diluted 1:10 in water prior to the analysis. . A high binding of this compound to albumin could be observed (7.64 min). (B) An incubation of KP2157 0.1 μM (20 μg/L of Pt) 1:10 in FCS during 45 min at 37°C and diluted 1:10 in water prior to the analysis. This compound showed no binding to albumin appearing mainly as free drug (16.34 min)

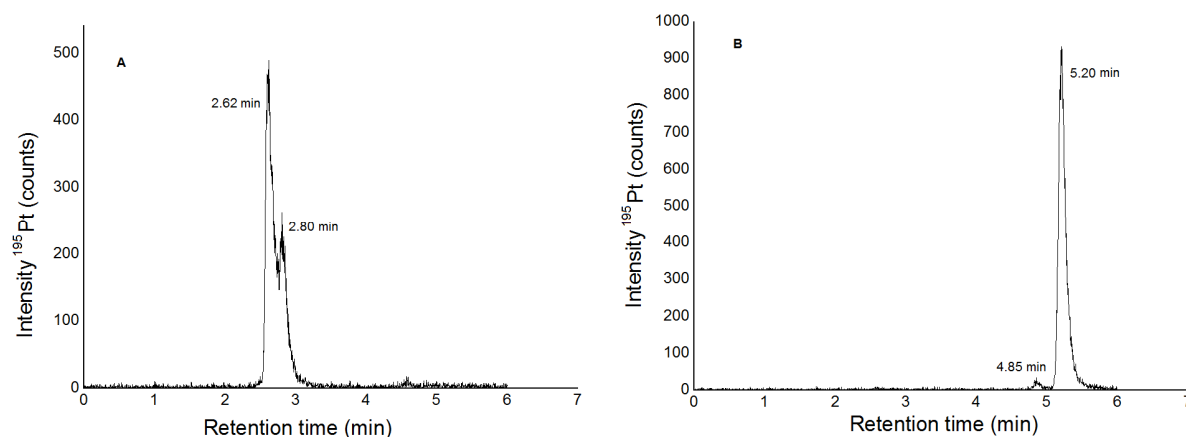


Fig. S3 UHPLC SEC chromatograms (Acquity UPLC Protein BEH SEC, 4.6 x 150 mm, 125A, 1.7 μ m) of (A) an incubation of KP2156 0.1 μ M (20 μ g/L of Pt) 1:10 in FCS during 45 min at 37°C. A high binding of this compound to the monomer and the dimer of albumin could be observed (2.62 and 2.80 min respectively) (B) an incubation of KP2157 0.1 μ M (20 μ g/L of Pt) 1:10 in FCS during 45 min at 37°C. This compound showed no binding to albumin appearing mainly as free drug (5.20 min). A hydrolysis product was observed in the standard of the drug in water as well as in the incubation in FCS (4.85 min)

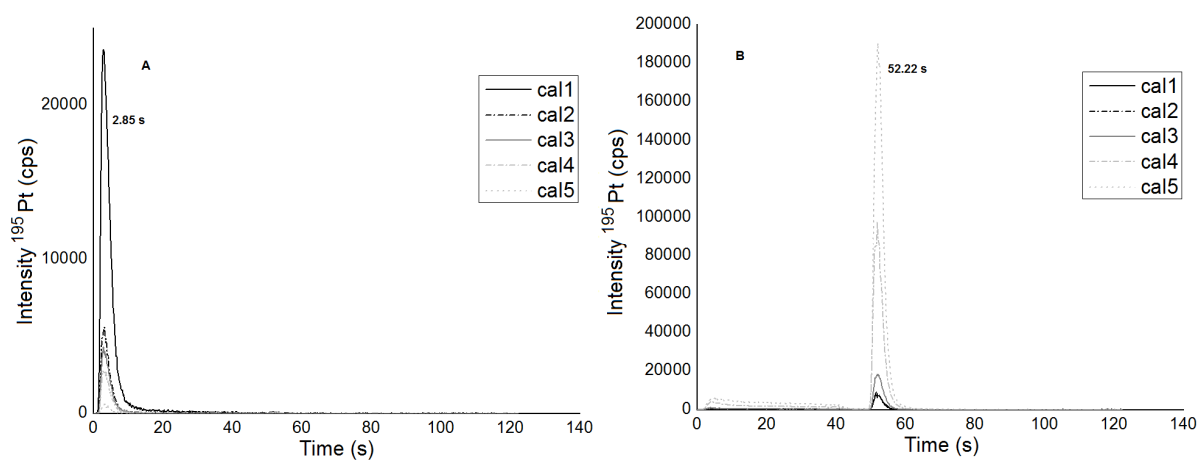
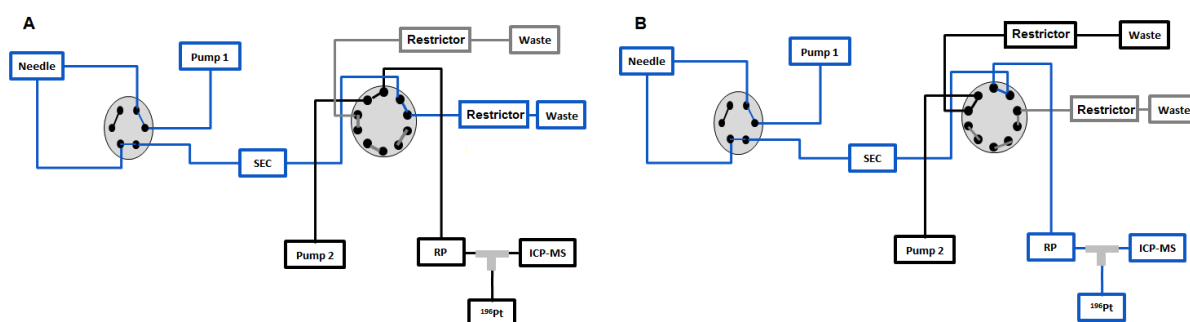


Fig. S4 TFC chromatograms (Fluoro XL 0.5x50 mm) of (A) standards of KP2156 in FCS 1:10 at different concentrations. (B) standards of KP2157 in water at different concentrations

3.2 PUBLICATION II

Heart-cut 2DSEC-RP-ICP-MS as a screening tool in metal-based anticancer research

Luis Galvez, Mate Rusz, Michael A. Jakupiec, Gunda Koellensperger..



Journal of Analytical Atomic Spectrometry, 2019

The relatively new sub-2-µm BEH particles can resist higher pressures due to their higher mechanical rigidity in comparisons to the conventional columns. This allowed for the first time the direct coupling of UHPLC SEC to RP for the screening of *ex vivo* incubations of metal-based drugs avoiding a prior sample preparation.

I carried out the cell extraction procedures for the *ex vivo* cytosolic incubations and I established and validated this methodology.



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Heart-cut 2DSEC-RP-LC-ICP-MS as a screening tool in metal-based anticancer research†

Luis Galvez, ^a Mate Ruzs, ^b Michael A. Jakupc ^b and Gunda Koellensperger^{*a}

In this work, we introduce a new screening tool for metal-based anticancer drugs, enabled by on-line heart-cut two-dimensional liquid chromatography combined with inductively coupled plasma mass spectrometry (2DLC-ICP-MS). This method employs ultrahigh performance size-exclusion chromatography (UHPLC SEC) in the first dimension and reversed phase liquid chromatography (RP-LC) in the second dimension. In this way, the quantification of free and intact metal-based anticancer drugs was addressed in biologically relevant matrices without otherwise necessary sample preparation steps such as protein removal and extraction of the drug. In a proof of principle study the half-life of oxaliplatin upon *ex vivo* incubation in serum and cellular extracts was assessed by repeated analysis every 25 min over several hours. This method showed good column recoveries for intact oxaliplatin (80% assessed by on-line isotope dilution) and limits of detection <3 nM. Oxaliplatin exhibited a half-life of 1.6 ± 0.1 h ($N = 3$) in serum. In the cellular extract prepared from the colorectal cancer cell line HCT116 oxaliplatin showed a prolonged half-life of 3.5 ± 0.3 h and 4.2 ± 0.4 h, assuming zero order or first order kinetics, respectively.

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Introduction

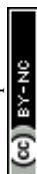
The discovery of platinum drugs, which are also on the WHO list of “essential medicines”, dates back to 1969.¹ Currently, about every second anticancer treatment scheme makes use of one of the three worldwide-approved platinum therapeutics, cisplatin, carboplatin or oxaliplatin. With regard to the mode of action, for a long time it has been assumed that their activity against cancer cells is solely based on DNA platination followed by stalling of replication and cell death. Over the last few years, however, it has become clear that metal-based anticancer drugs exert multifaceted cytotoxic activity,^{2,3} fueling the development of next generation metal-based anticancer agents. As a consequence, following chemical synthesis of a drug candidate and assessment of preliminary safety and efficacy profiles, serious efforts in analytical chemistry are required before its nomination as a clinical development candidate. Current optimization of lead compounds is accompanied by solution based experiments mimicking intracellular chemistry. *Ex vivo* studies using drug incubation in serum⁴ and cell lysates^{5,6} provided valuable information preceding *in vivo* and *in vitro* screening. Combined with mass spectrometric analysis (elemental and molecular mass spectrometry), these types of experiments emerged as

a valuable tool supporting preclinical drug development and promoting the basic understanding of drug biomolecule interaction.^{1,3} For example, *ex vivo* incubation provided information about distribution of metal-based drugs in blood cells, plasma and ultra-filtrate,⁷ which is key to determining the pharmacokinetics of the drug, which in turn is key for ascertaining the optimum dosage. The assessment of biotransformation products is another crucial aspect of drug development, as distinctly different cytotoxicity/toxicity profiles arise.^{2,3} Again, in the past, incubation experiments together with advanced techniques provided useful information prior to clinical studies showing non-enzymatic bio-transformations such as hydrolysis product and/or adduct formation.^{8,9} The fact that metal-based anticancer drugs can be determined by their metal content following diverse separation or fractionation methods carried out either on-line or off-line proved to be advantageous in many preclinical studies. Powerful methods for the quantification of metaldrug-protein association and biotransformation were developed for different matrices such as human plasma, serum and cell lysates.^{4,5,10} Recently, a systematic study evaluated different methods for quantitative screening of protein adduct formation. These methods included separation methods/fractionation methods such as centrifugal ultrafiltration, size exclusion and turbulent flow chromatography in combination with ICP-MS.¹¹ However, using these methods precluded accurately differentiating between the free intact drug, small-molecule transformation products and protein adducts. Tailored chromatographic separation for elemental speciation analysis of small molecular transformation products required sample preparation steps (*e.g.* centrifugal ultrafiltration or

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extraction) for removing the protein rich fraction of the samples.¹⁰ Only in this way was the selectivity offered by the one dimensional separation sufficient for the accurate quantification of the free intact drug *versus* transformation products. Evidently, the additional sample preparation step was time consuming (centrifugal ultrafiltration required up to 30 min) and prone to error.¹¹ The procedures could not be generalized to a wide panel of metal-based drugs, as co-precipitation, species interconversion during preparation and recovery had to be validated for each compound individually. As a drawback, extraction using polar organic solvents hampered subsequent application of reversed phase chromatographic separation. Either an additional sample preparation step for removing organic solvents was implemented or hydrophilic interaction chromatography (HILIC)⁷ was employed resulting in poor limits of detection. In both cases, drug stability needed to be considered. In this work, we present a novel elemental speciation strategy for quantifying the free and intact drugs in biological matrices omitting off-line sample preparation. More specifically, we present an on-line combination of two orthogonal separations, *i.e.* size exclusion chromatography (SEC) and reversed phase liquid chromatography (RP-LC). In the past, two dimensional separation involving size exclusion addressed heart-cut SEC-ion chromatography SEC-(IC) and comprehensive 2DLC as established by fast column switching SEC-IC (monoliths).^{12–14} Both set-ups were dedicated to the determination of metal/drug–protein interaction and operated at low backpressures (a requirement for the implemented SEC). This pressure limitation of size exclusion columns was overcome with the introduction of UHPLC SEC, facilitating the combination with reversed phase separations as established in this work. The presented approach allowed separating the low molar mass fraction (LMF) eluted from the size exclusion in the second chromatographic dimension. In this way, kinetic studies of metal-based drug degradation in biologically relevant matrices are significantly improved. In a proof of principle study the half-life of oxaliplatin was investigated in plasma and cell extracts.

Experimental

Reagents, chemicals and standards

A Pt standard ($1002 \pm 6 \mu\text{g mL}^{-1}$) was purchased from Inorganic Ventures, Christiansburg, Virginia, USA. A platinum spike enriched in ¹⁹⁶Pt (abundance ~97.25%, Science Technical Centre “Stable Isotopes” of State Scientific Centre of the Russian Federation–Institute of Physics and Power Engineering, Obninsk, Kaluga Region, Russia)¹⁵ was kindly provided by professor Stephan Hann at the Department of Chemistry, Division of Analytical Chemistry, University of Natural Resources and Life Sciences – BOKU, Vienna, Austria, and it was employed for species-unspecific quantification by on-line isotope dilution. Glucose and ammonium acetate were purchased from Sigma Aldrich (St. Louis, MO, USA). LC-MS grade water and MeOH were purchased from Honeywell (Muskegon, MI, USA). Oxaliplatin and fetal calf serum (FCS) were kindly provided by professor Christian R. Kowol at the

Department of Inorganic Chemistry, University of Vienna, Vienna, Austria. The media and the reagents for cell culture were purchased from Sigma-Aldrich Austria and all the material such as dishes, plates and flasks were from StarLab, Germany unless indicated otherwise.

Cancer cell culture

Cells were grown as adherent monolayer cultures in 160 cm² culture flasks (Iwaki, Japan) in McCoy's medium supplemented with 10% heat-inactivated fetal bovine serum (Biowest, USA) and 4 mM L-glutamine (Sigma-Aldrich, Austria) without antibiotics at 37 °C under a humidified atmosphere containing 5% CO₂ and 95% air. Cells from the human colon cancer line, HCT116, acquired from ATCC (American Type Culture Collection, USA) were harvested from the culture flasks by trypsinization. The trypsinization was stopped by addition of fresh medium and the cell suspension was washed three-times by centrifugation (300 rcf, 3 min) and addition of PBS. Subsequently, the cell number in the suspension was determined and 30×10^6 cell aliquots were distributed in 5 mL Eppendorf tubes. Following this, the aliquots were centrifuged twice and the excess PBS was aspirated. Finally, the cell pellets were snap-frozen with liquid N₂ and stored at –80 °C until analysis.

Ex vivo incubation, cell extraction and cytosolic incubation

Oxaliplatin was weighed and dissolved in glucose 5% (w/v) to reach a concentration of 1 mM. This stock solution was kept in ice and it was further diluted in glucose 5% (w/v) to reach a concentration of 10 μM and finally diluted 1 : 10 in FCS (molar ratio of drug : albumin of ~1 : 600, ~8 times higher compared to clinical infusions¹⁶) to reach a concentration of 1 μM and incubated in the autosampler of a HPLC system at 37 °C and injected every 0.83 h for 2.5 h.

A lysis procedure using freeze/thaw cycles with NaCl as described elsewhere was implemented.¹⁰ Briefly, 100 μL of 15 mM NaCl was added in a 5 mL Eppendorf tube containing 30×10^6 cells of the human colon cancer line HCT116. The Eppendorf tube was frozen with liquid N₂ and thawed at room temperature. This procedure was repeated two more times but in the last repetition 90 μL of 15 mM NaCl was added. Then it was centrifuged (Hermle Z446 K, 4350 rcf, 15 min, 4 °C) and the supernatant (cytosolic fraction) was transferred to a 1.5 mL Eppendorf tube whereas the pellet (nuclei fraction) was discarded. Since the 5 mL Eppendorf tube can be

Table 1 ICP-MS operation parameters

Nebulizer	MicroMist
Spray chamber	Scott double-pass
Nebulizer gas flow	1.05 L min ^{–1}
Aux. gas	0.90 L min ^{–1}
Plasma gas	15 L min ^{–1}
ICP RF power	1550 W
<i>m/z</i> measured	195, 196, 48
Reaction gas	0.3 L min ^{–1}



Table 2 Chromatographic conditions for the heart-cut UHPLC SEC-RP method

Chromatographic conditions	
SEC column	Acquity UPLC Protein BEH SEC, 4.6 × 150 mm, 200 Å 1.7 μm, 10–500 kDa, Waters
RP column	Atlantis T3, 2.1 × 150 mm, 3 μm, Waters
Eluent pump 1	A 20 mM CH ₃ COONH ₄ , pH = 7
Flow pump 1	250 μL min ⁻¹
Eluent pump 2	A 20 mM CH ₃ COONH ₄ , pH = 7
	B 20 mM CH ₃ COONH ₄ , pH = 7, 80% MeOH (v/v)
Flow pump 2	250 μL min ⁻¹
Injection volume	2 μL
Temperature SEC	37 °C
Temperature RP	37 °C
Autosampler temperature	37 °C

Chromatographic method

Time (min)	Position 10-port valve ^a	Pump 1	Pump 2	
		A (%)	A (%)	B (%)
0	A	100	100	0
7.5	B	100	100	0
11	A	100	100	0
17.5	A	100	80	20
21	A	100	80	20
21.1	A	100	100	0
25	A	100	100	0

^a See Fig. 1A and B for the position of the 10-port valve.

centrifuged only at a maximum speed of 4350 rcf in the Hermle Z446 K centrifuge, the 1.5 mL Eppendorf tube containing the cytosolic fraction was centrifuged again at a higher speed (Hermle Z366, 21 380 rcf, 60 min, 4 °C) in order to separate the rest of the pellet (nuclei fraction) that could not be separated in the first centrifugation, and 290 μL of the supernatant (cytosolic fraction) was finally transferred to a HPLC vial (final biomolecule dilution 1 : 100). 10 μL of a fresh stock solution of oxaliplatin in glucose 5% (w/v) was added in the vial containing the cytosolic fraction to reach a final concentration of 2 μM. This oxaliplatin concentration

was calculated assuming a cell volume of 10⁻¹² L and an intracellular concentration corresponding to typical oxaliplatin incubation in a cell culture medium of 20 μM as explained elsewhere.¹⁰ The vial containing the oxaliplatin in the cytosolic extract was placed in the autosampler of a HPLC at 37 °C to inject it at different times. After the analysis, an aliquot of 50 μL was used for protein quantification with a BCA protein assay kit obtaining a concentration of 35.2 ± 0.7 mg protein per mL (corresponding to a molar ratio of ~1 : 300 drug : biomolecule based on albumin molarity).

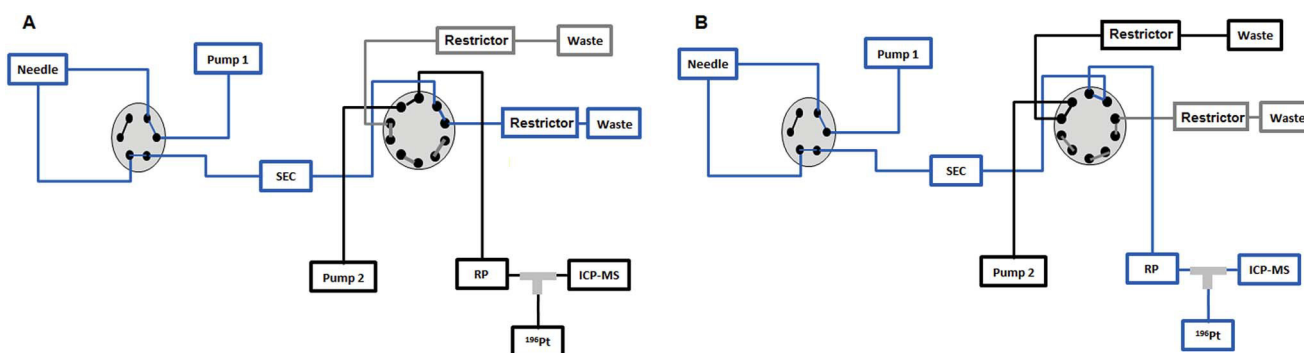


Fig. 1 (A) The sample is injected by means of the first pump from the 6-port valve of the Agilent 1260 Infinity Bio-Inert HPLC system to the UHPLC SEC column and to the external 10-port valve to send the high molar fraction (HMF) to the waste in the first 7.5 min. (B) After 7.5 min the 10-port valve is switched to send the low molar fraction (LMF) eluted from the UHPLC SEC column to the RP column. Then the valve is switched again at 11 min to position (A) and the second pump is used to separate the different species present in the LMF on the RP column.



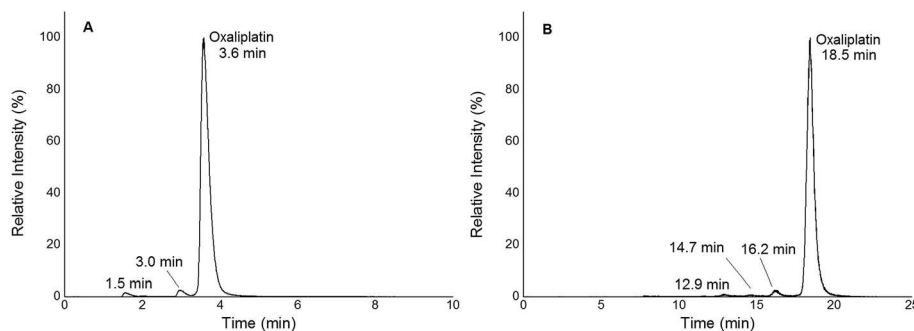


Fig. 2 (A) 1D RP-LC separation (column Accucore PFP, 2.1×150 mm, $2.6 \mu\text{m}$) of $2 \mu\text{M}$ oxaliplatin in 15 mM NaCl incubated for 2 h at 37°C ; gradient adapted from elsewhere.¹⁹ The hydrolysis products were observed at 1.5 and 3.0 min. Oxaliplatin was observed at 3.6 min. (B) 2D SEC-RP-LC separation (Acquity UPLC Protein BEH SEC, 4.6×150 mm, $200 \text{ \AA}$; Atlantis T3, 2.1×150 mm, $3 \mu\text{m}$) of $1 \mu\text{M}$ oxaliplatin in 15 mM NaCl incubated for 2 h at 37°C . The hydrolysis products were observed at 12.9, 14.7 and 16.2 min. Oxaliplatin was observed at 18.5 min.

Instrumentation

ICP-MS. An Agilent 8800 ICP-MS/MS (Agilent Technologies, Tokyo, Japan) was used with oxygen as the reaction gas for the monitoring of proteins *via* $\text{S}^{32}\text{O}^{16}$ (for efficient control of protein removal); data not shown. The Agilent MassHunter software package (Workstation Software, Version C.01.03, 2016) was employed for data treatment. The ICP-MS parameters are summarized in Table 1.

Heart-cut UHPLC SEC-RP. An Agilent 1260 Infinity Bio-Inert HPLC system (Agilent Technologies, Waldbronn, Germany) was employed for this setup. The sample was injected into a 6-port valve of the HPLC and sent to a 10-port valve (Agilent Technologies, Waldbronn, Germany) where the removal of the HMF was carried out by means of a UHPLC SEC column in the first 7.5 min and the LMF was sent to a RP-ICP-MS system by switching this 10-port valve after the 7.5 min. The chromatographic conditions and the method are shown in Table 2. The instrumental setup for the heart-cut UHPLC SEC followed by RP-ICP-MS is shown and explained in detail in Fig. 1.

Post-column isotope dilution. The peristaltic pump of the ICP-MS instrument was employed with a mass flow of 98 mg min^{-1} to introduce a platinum spike enriched in ^{196}Pt (abundance 97.25%) ($0.82 \mu\text{g Pt per L}$) after the RP column (Fig. 1) for species-unspecific quantification of oxaliplatin by on-line isotope dilution in order to calculate accurately the recovery by this heart-cut 2D-LC approach.

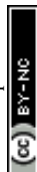
Results and discussion

In this work, quantification of intact and free metal-based anticancer drugs in protein rich samples was addressed.

More specifically, a heart-cut 2D-LC approach enabling combination with ICP-MS without off-line protein removal was validated for oxaliplatin. When analysing (pre)clinically relevant samples, 1D separation combined with ICP-MS detection required dedicated sample preparation. The 2DLC-ICP-MS method provided on-line separation of proteins by SEC. The SEC separation time could be reduced to a few minutes as a sub- $2 \mu\text{m}$ particle UHPLC SEC stationary phase was implemented.^{17,18} The size range of the column was 500 kDa to 10 kDa, (exclusion limit 500 kDa) resulting in the complete elution of the high molar mass fraction (HMF) within 6 min when employing moderate flow rates of $250 \mu\text{L min}^{-1}$. Under these conditions oxaliplatin was eluted in the low molar mass fraction (LMF) within 9 min with a LOD of 2.7 nM (3σ criterion) and a peak width of 1.7 min (see ESI Fig. S1†). The separation range of size exclusion was selected in a way such that the LMF containing intact drugs and potential transformation products (*e.g.* hydrolysis products) were eluted within a short time. With the use of a post column 10-port valve, a heart cut 2D separation was established separating the LMF by RP-LC (see Fig. 1). The reversed phase separation was carried out using either a fully wettable C18 material or pentafluorophenyl phases allowing stacked injections (high volume) of metal-based drugs showing medium to high retention on the reversed phase.^{19,20} Due to the use of quaternary pumps, $3 \mu\text{m}$ -particle RP-LC stationary phases were selected, as imposed by the pressure limit of the pump. Reversed phase separations using polar embedded groups or other functionalized C18 material had been shown in the past to be suitable for the selective separation of oxaliplatin and its hydrolysis products.^{19,20} Using 1D RP-LC-ICP-MS or 2D UHPLC

Table 3 2D SEC-RP-ICP-MS analytical figures of merit assessed for intact $1 \mu\text{M}$ oxaliplatin in glucose 5% (w/v) and in FCS for the first injection (incubation time 0 h). The recovery was assessed by on-line isotope dilution

Matrix	Recovery (%) $n = 3$	RSD retention time (%) $n = 3$	LOD (nM) 3σ criterion	LOD ($\mu\text{g L}^{-1}$ Pt) 3σ criterion
Glucose	80 ± 1	0.49	2.53	0.58
FCS	80 ± 2	0.50	2.19	0.50



SEC-RP-ICP-MS for the analysis of intact oxaliplatin in 15 mM NaCl excellent separation of oxaliplatin from hydrolysis products could be observed (Fig. 2). After 2 h incubation time at 37 °C, >80% was still intact. The observed two-phase decay is in accordance with that in other publications.²¹

Fig. 1 depicts the novel heart-cut 2D-LC separation. At 7.5 min the installed switching-valve directed the SEC flow for 3.5 min towards the second dimension, where the intact metal-based drug was retained. Backpressures of 350 bar were observed when both columns were connected in series. Restrictors established a constant backpressure upon valve switching. For this purpose a HPLC column (Atlantis T3, 2.1 × 150 mm, 3 μm, Waters) was installed in the flow path of pump 2 whereas for pump 1 a narrow bore capillary (inner diameter 0.127 mm) was introduced (Fig. 1). Table 3 summarizes the key analytical figures of merit for intact oxaliplatin quantification obtained using the novel 2DLC-ICP-MS set-up. As can be readily observed, oxaliplatin recovery and measurement repeatability was fit for purpose despite the high injection volume. The recovery was assessed by on-line isotope dilution.

The limits of detection (2.5 nM, 3σ criterion) were comparable to those for RP-LC-ICP-MS analysis only (1.3 nM, 3σ criterion) and were significantly improved as compared to typical HILIC-ICP-MS limits of detection (13 nM, 3σ criterion; see ESI Table S1 and Fig. S2†). Oxaliplatin was quantified by on-line isotope dilution compensating for signal suppression with RP-LC gradients. By using the 2D-LC set-up excellent separation of the low molar mass biotransformation products could be obtained (Fig. 3). For a proof of principle study, *ex vivo* incubations of oxaliplatin in fetal calf serum were investigated by 2DLC-ICP-MS. In this model, the drug concentration of 1 μM resembled drug concentrations found in the plasma of chemotherapy patients.¹⁶ The decay of intact oxaliplatin was monitored over 2.5 hours. For the investigated period the observed decay resembled first order kinetics (linear regression for first order $R^2 = 0.9970$). A half-life of 1.6 ± 0.1 h was calculated for three independent incubation experiments. Comparative experiments by using UHPLC SEC only (Fig. 4) revealed a half-life of 2.3 ± 0.1 h ($N = 3$) (Fig. 5). This higher value can be attributed to the poor chromatographic

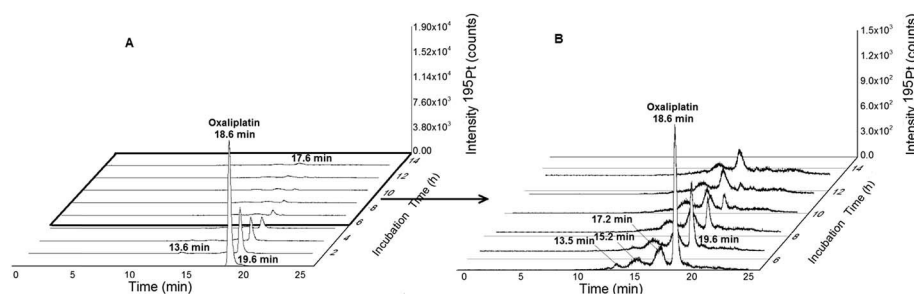


Fig. 3 (A) 2DLC-ICP-MS separation (columns Acquity UPLC Protein BEH SEC, 4.6 × 150 mm, 200 Å and Atlantis T3, 2.1 × 150 mm, 3 μm, Waters at a flow rate of 250 μL min⁻¹) of oxaliplatin (10 μM previously dissolved in glucose 5% (w/v) and finally diluted 1 : 10 in FCS to a final concentration 1 μM) incubated in serum measured at 37 °C for 14 h. (B) Incubation time 5–14 hours.

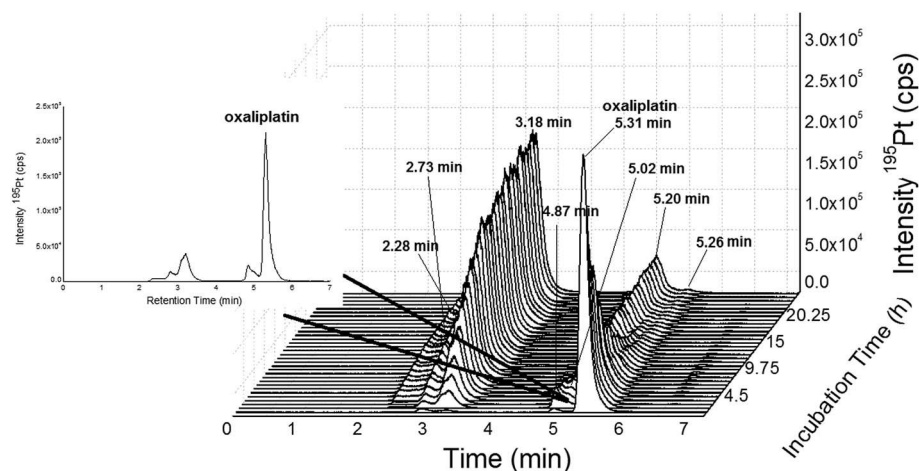
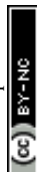


Fig. 4 SEC-ICP-MS of oxaliplatin (Acquity UPLC Protein BEH SEC, 4.6 × 150 mm, 200 Å, 1.7 μm, 10–500 kDa) at a flow rate of 400 μL min⁻¹; 50 mM CH₃COONH₄, pH = 6.8 at a temperature of 37 °C; 10 μM oxaliplatin diluted 1 : 10 in FCS (final concentration 1 μM) and incubated for 24 h at 37 °C. Oxaliplatin shows binding to albumin (3.18, 2.73 and 2.28 min). The free drug could be observed at 5.31 min. The other peaks (5.20, 5.02, 4.87 and 6.60 min) could be due to binding to other biomolecules in the LMF or degradation products of oxaliplatin. The peak at 5.20 min could not be completely separated from the peak of oxaliplatin at 5.31 min.



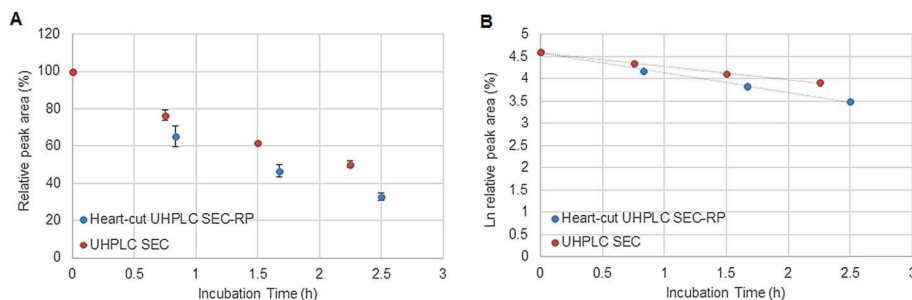


Fig. 5 (A) Stability of oxaliplatin in FCS assessed using the 2D-setup compared to UHPLC SEC in $N = 3$ biological replicates and (B) the logarithmic plot of oxaliplatin relative area versus incubation time. Linear regression analysis of the data plot showed that for the investigated period the decay showed first order kinetics. A half-life of 2.3 ± 0.1 and 1.6 ± 0.1 h was found for UHPLC SEC and heart-cut UHPLC SEC-RP, respectively.

selectivity of UHPLC SEC regarding the separation of biotransformation products in the LMF. The obtained results were in good agreement with those in previously reported studies based on ultrafiltration and metal quantification. Using comparable drug concentrations, a half-life of 2.21 ± 0.72 h was reported for oxaliplatin in human plasma.²² In clinical samples of patients undergoing chemotherapy even faster clearance was observed due to the high degree of binding to erythrocytes.¹⁶

In the field of metal-based anticancer drugs, only a few studies addressing elemental speciation analysis in cancer cell models exist.^{5,6,10} As the metal concentrations in pre-clinically relevant settings are in the low sub-nM range, these experiments were hampered by the moderate limits of detection offered by elemental speciation approaches (especially in the case of HILIC-ICP-MS²³). Accordingly, simplified model experiments such as drugs exposed to cell lysates contributed to the understanding of intracellular drug chemistry. In this study, the novel 2DLC-ICP-MS method was employed to address the kinetics of oxaliplatin degradation in the cytosolic preparation of a colon cancer model. 2 μ M oxaliplatin was incubated with a cell lysate resulting in an estimated molar drug/biomolecule ratio of 1 : 300. Assuming a volume of 1 pL for a single cell, the cytosol of 30×10^6 cells was diluted by a factor <100 in 15 mM NaCl. The estimated ratio was

calculated considering the protein concentration (expressed as molar albumin concentration) of the cell preparation. The decay of oxaliplatin was monitored in 15 mM NaCl in parallel to the cellular preparation over 1.8 hours. As a drawback, in some cases, precipitation was observed in the highly concentrated cell extract after several hours at 37 °C limiting the investigation time to 2 hours. Oxaliplatin is known to be activated by forming hydrolysis products in 15 mM NaCl.²¹ The experiments in this work show that the oxaliplatin transformation in cells is governed by interaction partners such as proteins and other biomolecules and not only by the reduced intracellular NaCl concentration. Due to the limited investigation period, no unambiguous kinetic model could be found. Assuming zero (Fig. 6A) and first (Fig. 6B) order kinetics ($R^2 = 0.9977$ and $R^2 = 0.9922$ for zero and first order kinetics, respectively) a half-life of 3.5 ± 0.3 h and 4.2 ± 0.4 h was estimated, respectively. For the oxaliplatin in NaCl stabilities $>80\%$ in 2 h of incubation at 37 °C and two-phase decay were observed as shown in other publications.²¹ The difference in half-life between serum and cell incubations can be explained by protein interactions in the protein rich fetal calf serum. In fact, UHPLC SEC-ICP-MS measurements of both sample matrices exposed to >24 hours revealed 90% of Pt in the HMF versus 85% Pt in the LMF, for serum incubation and cell incubation, respectively.

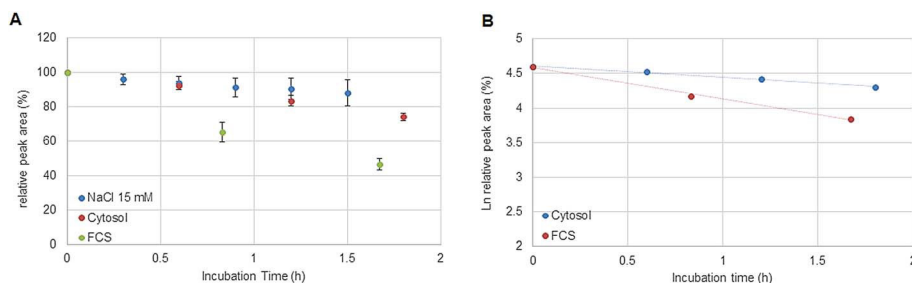


Fig. 6 (A) Stability of oxaliplatin in 15 mM NaCl assessed by RP-ICP-MS with the column Accucore PFP, 2.1×150 mm, 2.6 μ m, Thermo Scientific and stability of oxaliplatin in the cytosolic extract in $N = 3$ biological replicates assessed using the 2D-setup employing the column Accucore PFP, 2.1×150 mm, 2.6 μ m, Thermo Scientific compared to the previous investigated stability in FCS. The decay observed in the cytosol was different from the decay observed in 15 mM NaCl and in FCS showing a linear regression for zero order kinetics with a half-life of 3.5 ± 0.3 h although a (B) logarithmic plot of oxaliplatin relative area versus incubation time was also observed to be linear for the investigated period for first order kinetics, as it was observed for the incubation in FCS, obtaining a half-life of 4.2 ± 0.4 h.



Conclusions

Clinically established metal-based anticancer drugs either in their intact form or as activated transformation products bind to erythrocytes, plasma proteins, amino acids and nucleic acids. The extent of these key interactions ultimately determines the cytotoxic active fraction of the drug reaching the target DNA. In the case of candidate metal-based anticancer drugs, DNA is not always the final target. Despite this, biotransformation and interaction with cellular building blocks need to be carefully evaluated. Accordingly, the drug half-life in different (pre)clinically relevant matrices is an important parameter, which can be approximated by *ex vivo* incubation experiments. As a prerequisite, the intact and free drug has to be accurately quantified in biological complex matrices following different time points of drug exposure. In this work, we introduce a method enabling the quantification of the metal-based drug in serum samples and cell extracts without the need for *a priori* sample preparation. Samples are exposed to drugs at a fixed temperature (37 °C autosampler) and injected at defined time intervals on a heart-cut 2DLC-ICP-MS system. In a proof of principle study, the kinetics of oxaliplatin degradation in serum and cancer cell lysates was investigated. However, this approach is applicable to any metallodrug showing a significant retention on reversed phase chromatography.

Conflicts of interest

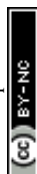
There are no conflicts to declare.

Acknowledgements

We acknowledge Professor Stephan Hann at the Department of Chemistry, Division of Analytical Chemistry, University of Natural Resources and Life Sciences – BOKU, Vienna, Austria for providing the enriched ¹⁹⁶Pt for post-column species-unspecific isotope dilution and Professor Christian R. Kowol at the Department of Inorganic Chemistry, University of Vienna, Vienna, Austria for providing oxaliplatin and FCS.

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HEART-CUT 2DSEC-RP-LC-ICP-MS AS A SCREENING TOOL IN METAL-BASED ANTICANCER RESEARCH

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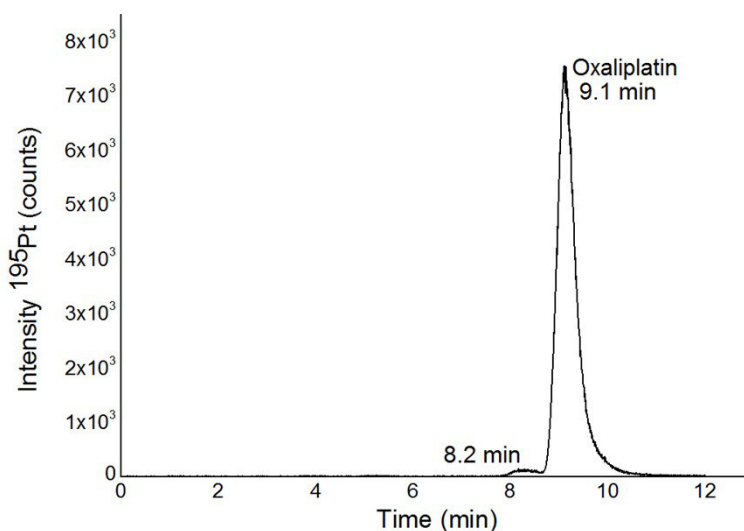


Figure S1 UHPLC SEC-ICP-MS separation of oxaliplatin 2 μ M in NaCl 15 mM at a flow rate of 250 μ L/min in isocratic mode with 20 mM $\text{CH}_3\text{COONH}_4$, pH = 7. A hydrolysis product was observed at 8.2 min. Oxaliplatin was observed at 9.1 min.

Table S1 HILIC-ICP-MS operation parameters and chromatographic conditions for the stability study of oxaliplatin.

Agilent 1260 infinity Bio-inert			
Chromatographic conditions			
Eluent	A 50 mM HCOONH ₄ , pH = 4.0, B 80% ACN 50 mM HCOONH ₄ , pH = 5.0		
HILIC Column	Acquity UPLC BEH Amide (2.1 x 100 mm, 1.7 μm)		
Flow Rate	250 μL/min		
Injection Volume	5 μL		
Autosampler Temperature	4°C		
Gradient Elution			
	Time (min)	A (%)	B (%)
	0	0	100
	2	0	100
	8	50	50
	10	50	50
	10	0	100
	15	0	100
ICP-MS Triple Quadrupole Agilent 8800			
Nebulizer	MicroMist		
Spray chamber	Scott double-pass		
Nebulizer gas flow	1.05 L/min		
Plasma gas	15 L/min		
ICP RF Power	1550 W		
Carrier Ar gas	0.70 L/min		
Option O ₂ gas	0.50 L/min		
m/z measured	195		

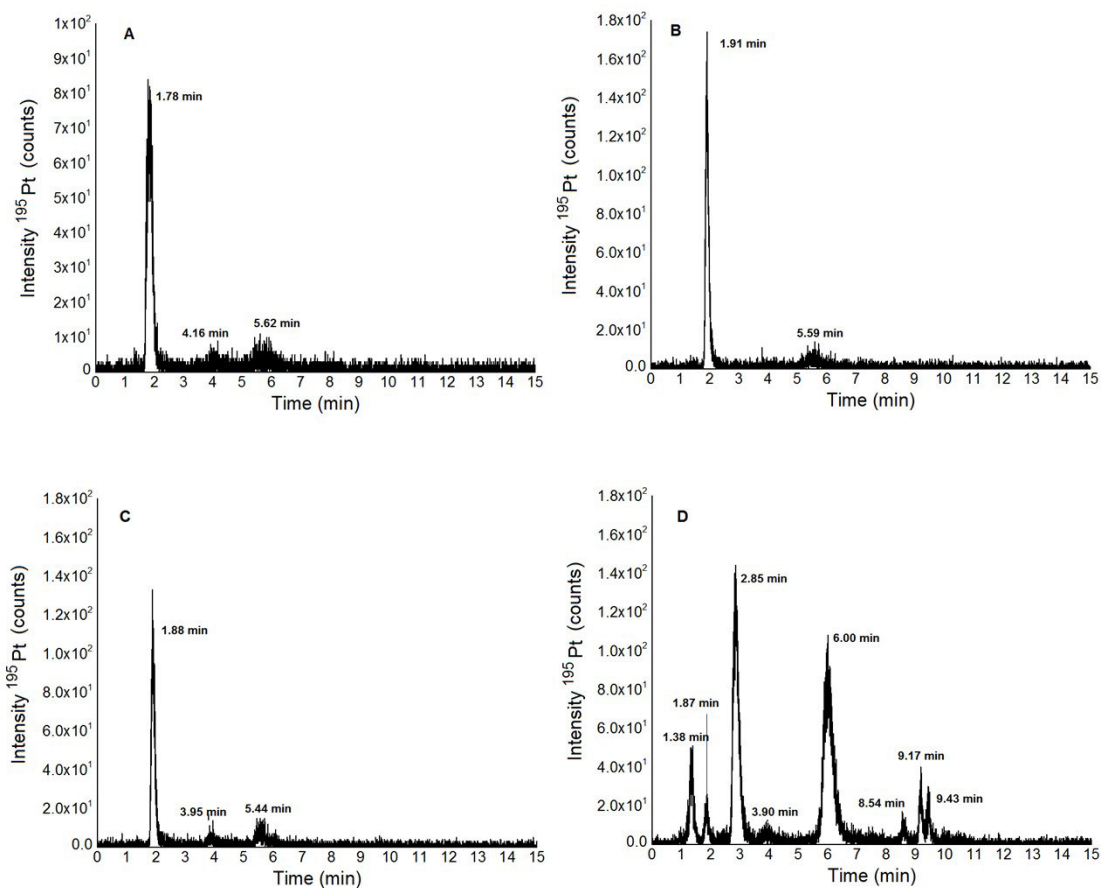
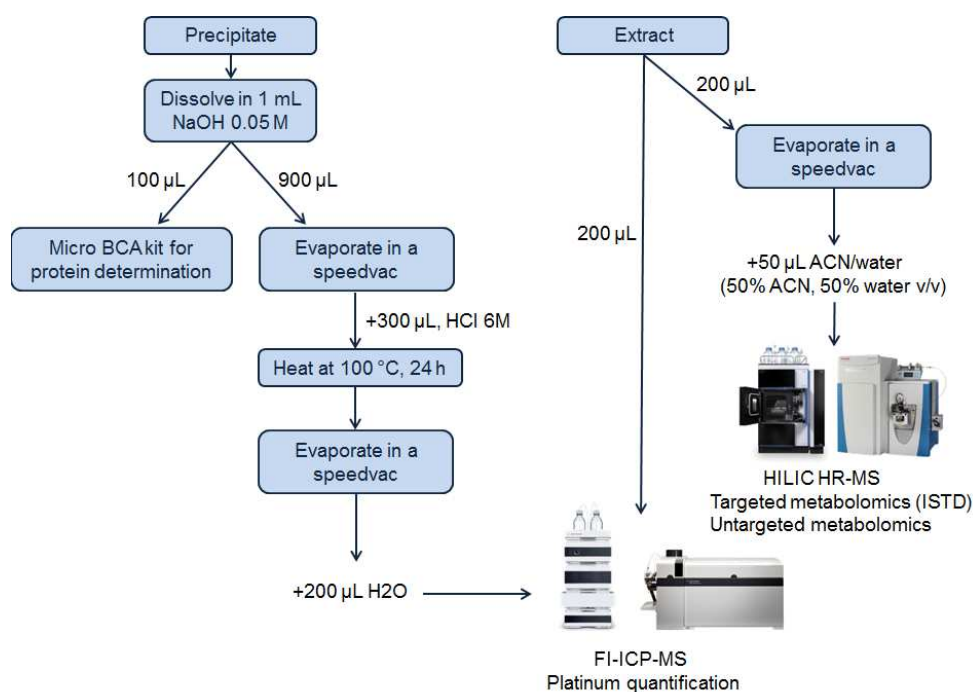


Figure S2 Chromatographic separations with the HILIC-ICP-MS method of (A) oxaliplatin 0.5 μM in glucose 5% (w/v) (B) oxaliplatin 0.5 μM in MeOH (80% methanol, 20% water, v/v) stored at -80°C for 96h (C) oxaliplatin 0.5 μM in ACN (50% ACN, 50% water, v/v) (D) 200 μL oxaliplatin 0.5 μM in MeOH (80% methanol, 20% water, v/v) evaporated in a speedvac and reconstituted in 50 μL ACN (50% ACN, 50% water, v/v).

3.3 PUBLICATION III

Preclinical studies on metal based anticancer drugs as enabled by integrated metallomics and metabolomics

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da Koellensperger.



Submitted manuscript

Metallomics and metabolomics were integrated in *in vitro* studies of oxaliplatin in sensitive and resistant cells of the colorectal cancer line HCT116. The differences observed in both –
ome levels will be further investigated to understand the behavior of this drug as well as the
acquired resistance.

After the seeding of the cell culture, incubation with the drug and quenching in collaboration
with the co-author Mate Rusz, I carried out the cell extraction followed by the HILIC-HR-MS
and FI-ICP-MS measurements and data evaluation.

Preclinical studies on metal based anticancer drugs as enabled by integrated metallomics and metabolomics

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Abstract

Resistance development is a major obstacle for platinum-based chemotherapy, with the anticancer drug oxaliplatin being no exception. Acquired resistance is often associated with altered drug accumulation. In this work we introduce a novel –omics workflow enabling the parallel study of platinum drug uptake, its distribution between nucleus and cytosol along with metabolic changes after different treatment time points. This integrated metallomics/metabolomics approach is facilitated by a tailored sample preparation workflow suitable for preclinical studies on adherent cancer cell models. Inductively coupled plasma mass spectrometry monitors the platinum drug, while the metabolomics tool-set is provided by hydrophilic interaction liquid chromatography combined with high-resolution Orbitrap mass spectrometry. The implemented method covers biochemical key pathways of the cancer cell metabolism as shown by a panel of >130 metabolite standards. Furthermore, the addition of yeast-based ¹³C-enriched internal standards upon extraction enabled a novel targeted/untargeted analysis strategy. In this study we used our method to compare an oxaliplatin sensitive human colon cancer cell line (HCT116) and its corresponding resistant model. In the acquired oxaliplatin resistant cells distinct differences in oxaliplatin

accumulation correlated with differences in metabolomic rearrangements. Using this multi-omics approach for platinum-treated samples facilitates the generation of novel hypothesis regarding the susceptibility and resistance towards oxaliplatin.

Introduction

Already in the 1930s Warburg and his contemporaries reported the concept of aerobic glycolysis in cancer. They highlighted the fundamental differences in the central metabolic pathways between healthy and malignant tissues¹. While for many years cancer research focused on genomic changes and oncogenes^{2,3,4} links between metabolism and cancer only recently re-emerged as an exciting area of investigation⁵. Indeed, dynamic changes of biological systems can be tracked by measuring the metabolome. Furthermore, molecular signatures are extremely powerful for hypothesis generation, offering novel insights, e.g. in the crosstalk of tumor cells with the tumor microenvironment⁶ or in the drug mode of action^{7,8}. Metabolome profiling of drug-perturbed cell lines *in vitro* offers the unique opportunity to identify potential weaknesses in cellular drug response, allowing correlations of biochemical pathways and/or genetic polymorphisms with drug susceptibility^{3,9}.

Until now only few metabolomic studies exist in the field of metal-based anticancer agents, despite the fact that platinum-based therapies are among the most frequently clinically applied anticancer therapies¹⁰. The amount of registered clinical studies involving metal-based anticancer drugs (novel compounds and combination therapies) is constantly high with increasing numbers over the last years^{11,12}, such as successful combinations with immunotherapies in the recent years¹³. As for many drugs, including the clinically approved platinum compounds it is not fully defined how the drug is taken up by the malignant tissues^{14,15}. A clear-cut mechanism remains to be elucidated even after years of clinical practice. Intrinsic and acquired platinum resistance are a major obstacle jeopardizing successful therapy¹⁶⁻¹⁹. For the clinically approved platinum drugs, DNA is the established molecular target^{11,15}. However, the fact that only 1% of the platinum is reaching the nucleus strongly suggests additional important cellular interactions²⁰. Recently, oxaliplatin was found to kill cancer cells by inducing ribosome biogenesis stress rather than by engaging a DNA damage response²¹.

The few metabolomic studies investigating platinum drugs (1) address the role of metabolism in chemosensitivity of tumor cells towards platinum compounds²², (2) investigate metabolomics as predictive tool^{23,24}, (3) used metabolomics to discriminate isogenic sensitive and resistant cell types^{19,25,26} and (4) proposed metabolomics as tool-set for early detection of nephrotoxicity²⁷. By correlating biochemical pathways (measured by transcriptomics and metabolomics) from the NCI60 cell line panel with tumor cell chemosensitivity, it was found that pathways including the tricarboxylic acid (TCA) cycle, pyruvate metabolism, lipoprotein uptake and nucleotide synthesis (salvage pathways and de novo synthesis) were specific for sensitivity towards platinum compounds^{28,29}.

Overall metabolomic platforms and procedures are still not as mature as other –omic technologies⁴. There is a need for new methods and technologies as analytical protocols have often to be adapted to the specific applications to try to cope with the intrinsic challenges of analysis such as metabolome coverage, analytical throughput and accuracy³⁰. In this work we propose the combination of metabolomics and metallomics as a novel powerful tool-set in preclinical metallodrug research to address metal uptake and cellular drug distribution within the same sample. This unique combination ultimately allows assessing metabolome perturbations exerted by the metallodrugs and correlating the corresponding biochemical pathways with drug accumulation and/or cytotoxicity. Complementary use of elemental inductively coupled plasma mass spectrometry (ICP-MS) and high-resolution molecular mass spectrometry (Orbitrap-MS) together with the developed sample preparation enabled the study of drug uptake, drug distribution between nucleus and cytosol and metabolic signatures upon drug exposure from one cell culture sample. Sample preparation in metabolomics is highly demanding^{25,31} and absolutely key when acquiring a snap-shot of the highly dynamic metabolome, particularly with regards to the handling time. Several preparation protocols have been established to simultaneously measure proteomics and metabolomics³²⁻³⁴, but the combination of metal analysis and metabolomics has not been addressed so far. In the past, it has been shown that choice of chromatography was key for the selectivity, throughput and metabolome coverage of the analysis.^{35,36} In this work we implemented hydrophilic interaction liquid chromatography (HILIC) for the retention of polar metabolites³⁷⁻⁴⁴. Finally, ¹³C internal standardization based on yeast standards^{25,35,45,46} was employed at the extraction stage.

Together with high-resolution mass spectrometry this strategy allows (1) improving the identification task in untargeted metabolomics, an approach coined as credentialing⁴⁷, and (2) merging targeted quantification and untargeted analysis in one workflow²⁵. With this method targeted relative quantification between different sample groups was facilitated without compromising untargeted data evaluation²⁵. To test the novel metabolomics/metallomics strategy in a preclinical setting we investigated metabolic changes and drug accumulation in an oxaliplatin sensitive and resistant cell model over time.

Experimental

Reagents, chemicals and standards

The metabolite standards were purchased from Sigma-Aldrich or Fluka (Vienna, Austria) except for malic acid which was purchased from Merck (Vienna, Austria). A multistandard stock solution of 100 µM was prepared in water and used as a quality control (QC) of 10 µM. A fully ¹³C labeled yeast extract of *Pichia pastoris* (2 billion cells) from ISOTopic solutions e.U., (Vienna, Austria) was reconstituted in 2 mL of water and added in same amounts to the the samples. The final dilution of the internal standard for the measurement was 1:10. The platinum standard (1002±6 µg/mL) was purchased from Inorganic Ventures (Christiansburg, Virginia, USA). Media and reagents for cell culture were purchased from Sigma-Aldrich (Vienna, Austria) and all material such as dishes, plates and flasks from StarLab (Hamburg, Germany) unless indicated otherwise.

Cell culture and cytotoxicity tests

The human colon cancer line HCT116, acquired from ATCC (American Type Culture Collection, USA), and the oxaliplatin resistant subline (established as described elsewhere⁴⁸) were grown as adherent monolayer cultures in McCoy's 5A medium (Sigma-Aldrich) supplemented with 10% heat-inactivated fetal bovine serum (BioWest) and 4 mM L-glutamine (Sigma-Aldrich) without antibiotics at 37 °C (StarLab) under a humidified atmosphere containing 5% CO₂. HCT116 cells were seeded at a density of 5×10^3 viable cells per well into 96-well microculture plates (StarLab) 24 h prior to the treatment. Stock solutions of oxaliplatin were prepared in McCoy's 5A medium and diluted stepwise. Cells were incubated for 24 h at the indicated drug concentrations. Cell viability was measured using resazurin sodium salt (Sigma-Aldrich) (440 μ M in PBS (Sigma-Aldrich)). After 4 h at 37 °C for 4 h the fluorimetric measurement of the formed resorufin was performed with a Synergy HT reader (BioTek) (excitation wavelength 530 nm, emission wavelength 590 nm). The concentration–effect curves from three independent experiments were averaged. An average IC₅₀ value of 35 ± 6 μ M after 24 h of incubation ensured that a concentration of 5 μ M oxaliplatin was adequate (sub-cytotoxic) for the following preparations (see supplementary information **Figure S1** for the concentration-effect curves).

Preparation of cell extracts

5×10^5 cells/well were seeded of each cell line in a 6-well plate in 3 mL/well (N=3 for 4 h and 8 h and N=6 for 24 h experiments). After 24 h, 1 mL of the control medium or oxaliplatin-containing (final concentration 5 μ M) medium was added. Plates were incubated for 4 h, 8 h or 24 h. After incubation, the medium was removed, and the wells were washed once with 4 mL of PBS. Then, liquid nitrogen was added to quench the metabolism in the cells and samples were stored at -80 °C until analysis. Upon extraction, 50 μ L internal standard at room temperature and 1 mL ice-cold methanol (80% methanol, 20% water, v/v) were added. The cells were scraped into the extraction solvent and transferred to an Eppendorf tube. The cell scraper and the wells were washed two times with 475 μ L extraction solvent to reach a final volume of 2 mL extract. After thorough mixing and centrifugation (20,000 RCF, 5 min, 4 °C), the supernatant (extract) was separated from the precipitate and transferred into a fresh Eppendorf tube. From the extract, an aliquot of 200 μ L was transferred into a HPLC vial with an insert for evaporation and reconstitution in 50 μ L ACN (50% ACN, 50% water, v/v) leading to a pre-concentration factor of 4. Additionally, a pooled sample was prepared by mixing aliquots (10 μ L) of all the pre-concentrated samples. Another aliquot (200 μ L) of the extract was used for platinum quantification by flow injection inductively (FI)-ICP-MS. The precipitate was dissolved in 1 mL of 0.05 M NaOH and mixed thoroughly to ensure proper dissolving. For metallomics, 900 μ L of the dissolved precipitate was evaporated in a SpeedVac and 300 μ L HCl 6 M were added, thoroughly vortexed, transferred to reaction vessels and heated at 100 °C for 24 h for the hydrolysis of the proteins. After the hydrolysis of the proteins, the HCl was removed by evaporation. The dry samples were finally re-dissolved in 200 μ L H₂O for platinum quantification with FI-ICP-MS. A scheme summarizing the sample preparation is shown in **Figure 1**.

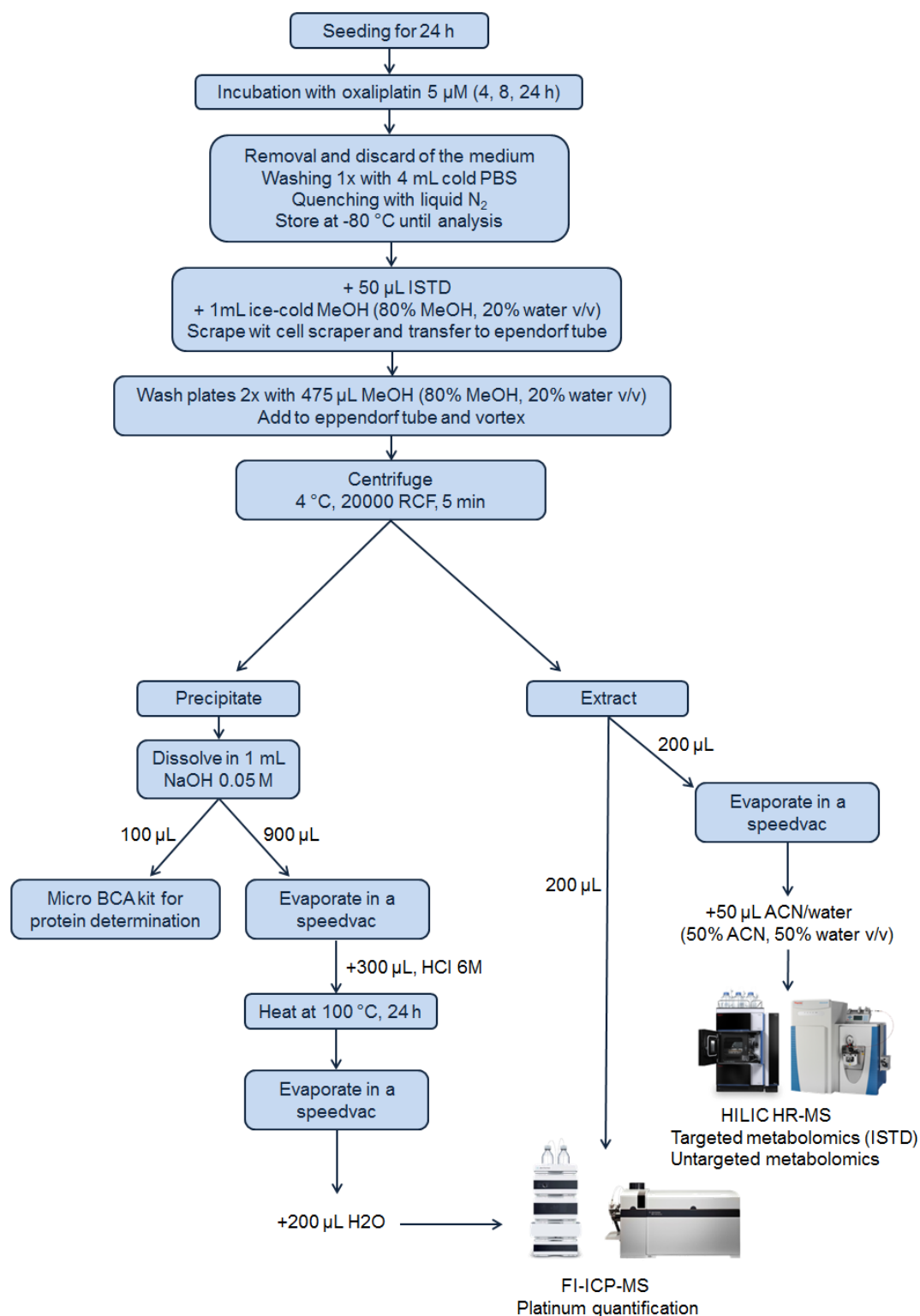


Figure 1 Scheme of integrated metallomics/metabolomics - from sample preparation to analysis. The precipitate was employed for the protein determination and the platinum quantification, the latter requiring protein hydrolysis before injection on the FI-ICP-MS system. In parallel to the analysis in the precipitate, two aliquots of the extract were analyzed: one was pre-concentrated by evaporation, re-suspended in ACN/water (1:1, v/v), and injected on the HILIC HR-MS system for metabolomic analysis whereas the other one was directly injected on the FI-ICP-MS system for platinum analysis.

Determination of protein content for normalization of adherent cell cultures

The protein concentration was assessed from the remaining precipitate dissolved in NaOH 0.05 M (100 μ L). For that purpose, the commercially available micro BCA protein assay kit (Thermo Fisher Scientific, Pierce Biotechnology, Rockford, USA) was employed. In order to validate this approach for normalization purposes, different number of cells were seeded in 6-well plates and extracted as described before, prior to protein quantification. A standard curve with BSA was used for calibration (0.5-20 μ g/mL).

FI-ICP-MS

Platinum uptake was quantified by FI-ICP-MS. Briefly, HPLC Agilent 1260 infinity Bio-inert HPLC (Waldbronn, Germany) was coupled to a ICP-MS Triple Quadrupole ICP-MS Agilent 8800 (Agilent Technologies, Tokyo, Japan) with Agilent MassHunter software package (Work-station Software, Version C.01.03, 2016) for the data treatment. The chromatographic and ICP-MS conditions are summarized in **Table S1**.

HILIC-HR-MS

HILIC-HR-MS was employed for metabolomic analysis. Briefly, a Vanquish UHPLC (Thermo Scientific) was coupled to a high field Thermo Scientific™ Q Exactive HF™ quadrupole-Orbitrap mass spectrometer equipped with an ESI source. The separation was conducted on an ACQUITY® UPLC BEH Amide column 2.1 x 100 mm, 1.7 μ m (Waters Corp, Milford, MA) with trifunctional amide groups bonded to ethylene bridged hybrid substrate as stationary phase and a sub-2- μ m particle. The column was operated at 250 μ L/min and 40°C. The injection volume was 5 μ L. Mobile phase A consisted of 50 mM HCOONH₄ in water, pH = 4.0. Mobile phase B consisted of 80% ACN and 20% water with 50 mM HCOONH₄. The chromatographic run started at 100% B (2 min) followed by a linear gradient to 50% B (6 min). Then, it was held at 50% B for 2 min and re-equilibrated at 100% B for 5 min. The parameters for the full mass scan (Full-MS), ESI-source (ESI) and untargeted fragmentation approach (ddMS2) are summarized in **Table S2**. The targeted data evaluation was performed in TraceFinder™ 4.1 from Thermo Scientific™ with internal standardization. Thermo Scientific™ Compound Discoverer™ 3.0 was employed for the untargeted data processing. This software combines feature detection with statistical data evaluation. For the detection of unknown features at MS1 level a minimum peak intensity of 10000 with a mass tolerance of 3 ppm and a minimum number of 2 isotopes were used. For identification, mzCloud search was performed with 5 ppm mass tolerance and a threshold for compound annotation of 70.

Results and discussions

To achieve an integrated metallomics/metabolomics workflow (**Figure 1**) several key steps required optimization and/or novel analytical developments. In the following, the methodological aspects of this study will be described.

Sample preparation

A sample preparation method based on simultaneous direct scraping and extraction adopted from metabolomic workflows (Dettmer et al.³¹ and Schwaiger et al.²⁵) was implemented in this work. As internal standard ¹³C-enriched *Pichia pastoris* was added upon extraction. The yeast extract served as a standard for both, the metabolome and the metal-based anticancer drug. After the centrifugation step the supernatant (extract) contains the low molar mass fraction of the cytosol and the remaining precipitate includes of high molecular weight proteins and the nucleus fraction.

Normalization strategy

The application of normalization strategies is an established approach to ensure comparability and improved biological repeatability of quantitative studies addressing either different cell types or different cultivation time points. A typical metabolomics experiment relates either relative or absolute concentration changes to cell number, protein amount or dry/wet weight^{49,50}. The same holds true for quantitative drug uptake studies. In platinum accumulation studies of adherent cell cultures, cells are often trypsinized to consequently normalize the platinum content to total cell number. As trypsinization introduces significant alterations to the metabolome^{31,42,51}, protein content was used for normalization in this study. For this purpose, the protocol of a commercially available protein assay was adapted to adherent cell cultures omitting the precipitation step of the kit by directly dissolving the precipitate for protein quantification. An independent validation experiment assessing cell number and protein content showed the validity of the normalization method. As can be observed in **Figure S2** and **Table S3** a linear calibration of cell number versus protein content could be achieved. The working range was at low µg/mL level resulting in biological repeatability of typically 10%.

Metabolomics enabled by HR-MS

A standard panel consisting of >130 metabolites, including amino acids, sugars, sugar phosphates, organic acids, nucleotides, cofactors and coenzymes served for selection of chromatographic separation. The selection covered pathways key in cancer metabolism⁵², such as glycolysis, TCA cycle, energy metabolism, amino acid synthesis, fatty acid synthesis, purine and pyrimidine pathways. In this work, a HILIC separation based on a sub-2µm stationary phase with trifunctional amide groups bonded to ethylene bridged hybrid substrate was implemented. Compared to chromatographic separation on a mixed mode stationary phase in targeted metabolomics, the selected HILIC phase provided higher

metabolite coverage (see **Figure 2** bar chart and pie chart of recovered metabolite mix). Based on retention time distribution (see violin plots **Figure S3**) and ionization coverage, pH 4 was selected as optimum separation condition. Detailed information on separated metabolites, retention times, ionization mode and separation of critical isomers are summarized in the supplementary data (**Figure S4-S5** and **Tables S4-S6**). Repeated injections of a quality control 10 μ M multi-metabolite standard over >25 hours revealed good technical repeatability (see **Table S7**). Using the sub-2 μ m HILIC phase, most standard peaks revealed a full width at half maximum (FWHM) <10 s (see **Table S8**). ^{13}C enriched standards derived from labeled yeast extract were added to the cancer cells upon sample preparation with the aim of improving biological repeatability of sample preparation and subsequent HILIC analysis. This isotopically enriched biomass has been successfully used in numerous metabolomic studies addressing targeted absolute quantification^{35,43,45,46} and novel workflows merging targeted and untargeted analysis^{25,53}. It was shown that the addition of internal standard did not hamper the untargeted data evaluation, but enabled simultaneous absolute quantification of a targeted panel²⁵. In this work, we implemented the internal standardization strategy for the first time with the aim of improving biological repeatability of relative quantification based on high-resolution mass spectrometry. **Figure 3a** summarizes metabolites according to compound classes and numbers as found up-to-date in ^{13}C -enriched *Pichia pastoris*^{25,35,43,45,46,53}, comparing these figures to metabolites recovered in cancer cell extract versus yeast cell extracts (**Figure 3b and 3c**) by targeted analysis using the here established protocols and available standards. Overall >60 metabolites could be internally standardized upon sample extraction improving technical and biological repeatability (see **Figure 4a and b** respectively).

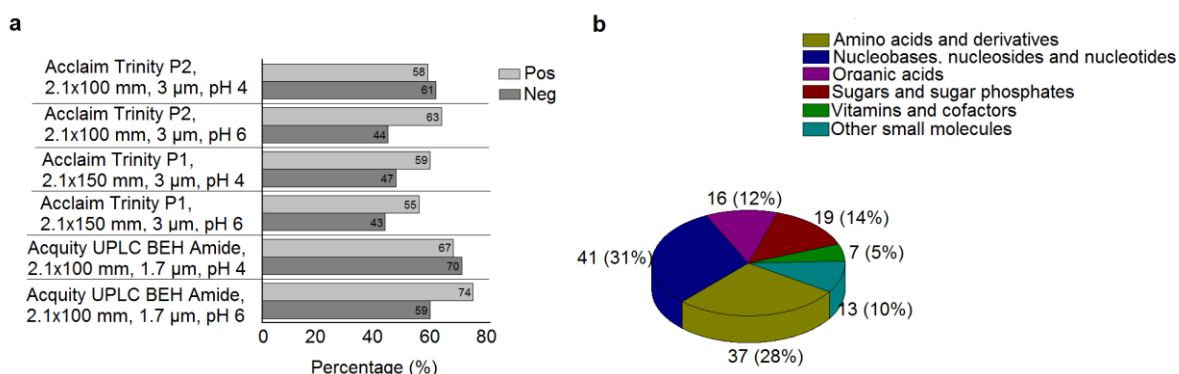


Figure 2 (a) Percentage of metabolites found in a 10 μ M multicomponent standard with the different HILIC columns at pH4 and pH6 in positive and negative mode. **(b)** Number of metabolites and their classes (combining positive and negative mode) as found in a 10 μ M multicomponent standard of >130 metabolites with the UPLC BEH Amide column at pH 4.

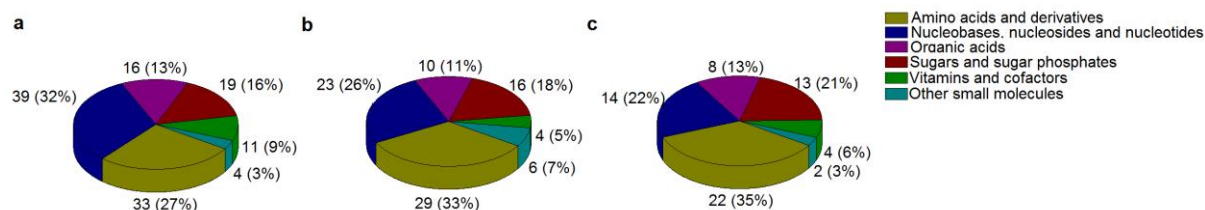


Figure 3 Number of metabolites and their classes (combining positive and negative mode) as found **(a)** up to date in ^{13}C -enriched *Pichia pastoris*^{25,35,43,45,46,53} **(b)** in a sensitive control HCT116 cell extract measured with the UPLC BEH Amide column at pH 4 **(c)** in ^{13}C -enriched *Pichia pastoris* added in sensitive control HCT116 cell extract and measured with the UPLC BEH Amide column at pH 4.

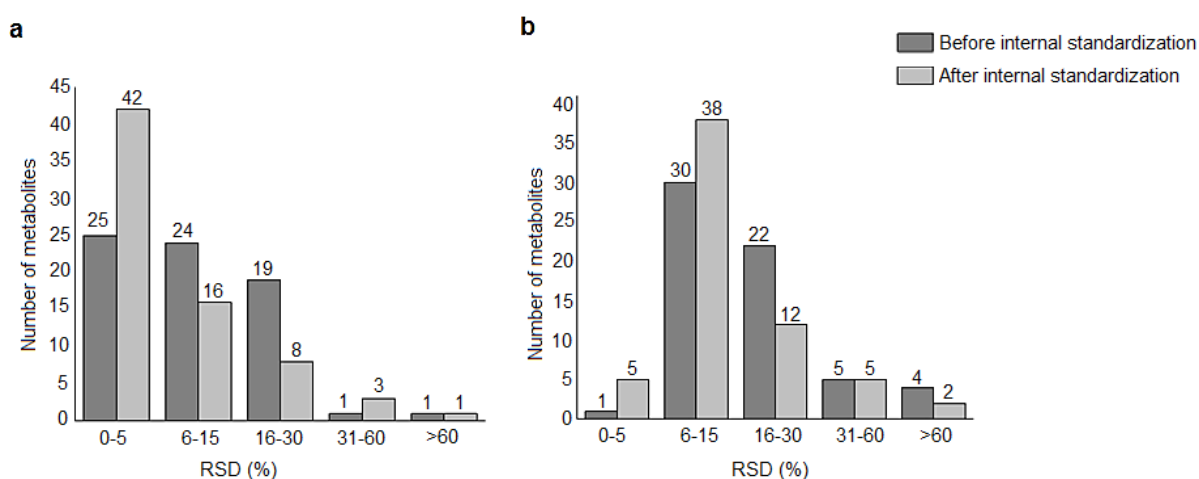


Figure 4 Repeatability of the HILIC-HR-MS approach combining positive and negative mode regarding **(a)** a pooled cell extract (n=3 biological replicates) with and without internal standardization and **(b)** a sensitive line control cell extract (n=6 biological replicates) with and without internal standardization. The internal standard was added prior to the extraction.

Metallomics enabled by FI-ICP-MS

Flow injection combined with ICP-MS detection enabled absolute quantification of metal based anticancer drugs in the extract omitting mineralization steps. The metal in the precipitate, resembling the protein and nucleus associated fraction, was quantified after mineralization. In this study, we focus on the third generation platinum compound oxaliplatin knowing that this compound shows *in vitro* and *in vivo* antitumor activity against colorectal cancer⁵⁵. The recovery of intact oxaliplatin in the extraction solvent was excellent ($100 \pm 7\%$, n=3). However, the preclinical application of sub-IC-50 concentrations of oxaliplatin resulted in cytosolic concentration levels ranging below 1.5 $\mu\text{g/L}$ (as assessed by total platinum concentration) which hampered elemental speciation approaches (typical LOD of HILIC-ICP-MS ideally compatible with the extraction solvent is 3 $\mu\text{g/L}$ platinum), while reversed phase approaches required removal of the organic solvent, a step which altered the elemental speciation of the metallodrug⁵⁶.

The study case – Addressing metabolic perturbation exerted by oxaliplatin in sensitive versus resistant cancer cell models of human colon cancer

More than a decade ago, oxaliplatin was approved as a standard treatment of advanced metastatic colorectal carcinoma in a combination with 5-fluorouracil and leucovorin (FOLFOX)⁵⁷. Despite being in the clinic since 2004, several key aspects regarding its mode of action are still open and are distinctly different from cisplatin. The ultimate targets of both drugs is DNA^{16,17}. Despite forming the same types of inter- and intrastrand DNA cross-links, the drug's reactivity towards DNA is different with oxaliplatin being less reactive than cisplatin. It is believed that the DACH ligand of oxaliplatin, in contrast to the amono groups of cisplatin, is responsible for its differential recognition by the mismatch repair machinery and therefore leads to different activity spectrum. The promise that oxaliplatin would have low-cross resistance to cisplatin and attack cisplatin resistant tumors was found to be only partially true and was limited to special cases such as in combination therapy against colon cancer^{58,59}.

In this work, the novel integrated metallomics/metabolomics workflow was applied in a preclinical study on oxaliplatin resistance. More specifically, we investigated the human colon cancer HCT-116 cell model with a sub-line of acquired resistance. The resistant cell model was generated by pulsed-exposure of HCT-116 cells to increasing concentrations of oxaliplatin. As compared to the parental cell model, the resistant cell models displayed more than 10-fold resistance against oxaliplatin⁶⁰. The workflow enabled addressing drug uptake and distribution between nucleus/protein fraction and cytosolic low molar mass fraction together with metabolic perturbation upon different exposure times.

Metallomics

Figure 5 summarizes the drug accumulation data obtained by ICP-MS based metallomics. While in the sensitive HCT116 cell line the extract concentration of platinum increased over time (~30% of total platinum), the cytosolic low molecular mass fraction remained constant (~70 % of total platinum). Within the first 4 h the platinum accumulation data were comparable for the sensitive and resistant cell models. Drug accumulation was reduced after 8 h of incubation, as previously shown⁶⁰. In contrast, to the parental cell line, the resistant model showed constant low nucleus/protein concentration, while the cytosol fraction was decreasing. Of note, until now there is no clear-cut uptake mechanism for oxaliplatin established. Passive diffusion and transporter-mediated uptake mechanisms are discussed^{61,62}. The comparative drug accumulation data of **Figure 4**, point towards predominant passive diffusion uptake for the first 4 hours. Differential active efflux transport mechanisms might observed after 8 h of exposure between the parental and the resistant cell line. In fact, several studies linked overexpression of transporters to therapy outcome or resistance, however knowledge is still fragmentary^{61,62}. The influx transporters involve copper transporter (CTR) proteins and organic cation transporters (OCTs). Export of platinum drugs has been linked to efflux pumps such as ATP binding cassette (ABC) multidrug transporters, copper-transporting P-type adenosine triphosphatases (ATPases), and multidrug and toxin extrusion (MATE) proteins⁶³.

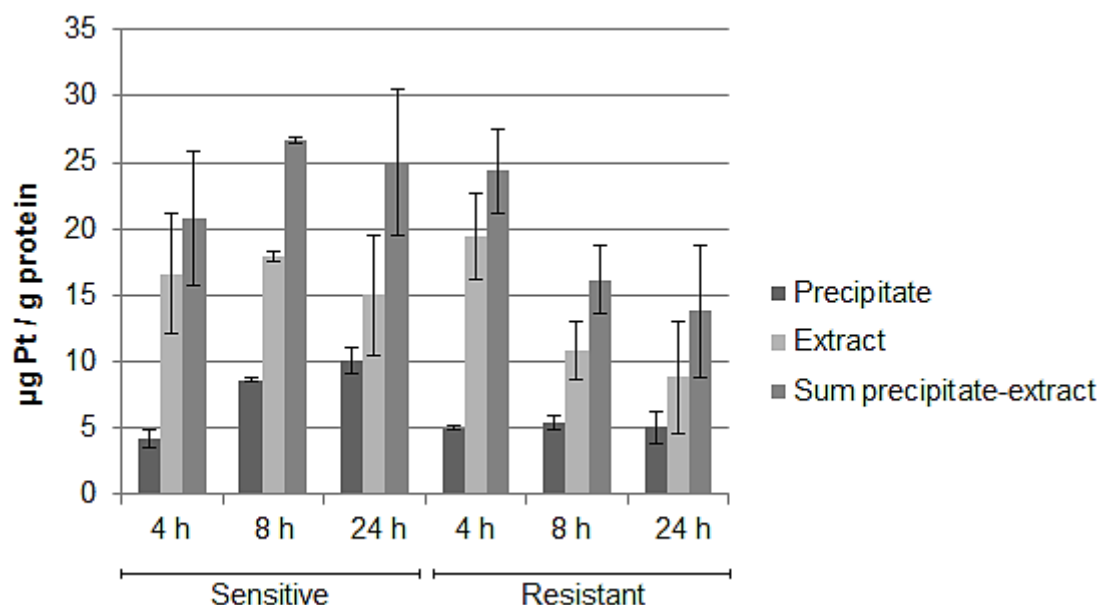


Figure 5 Total platinum concentration in the precipitate, in the extract and in the sum of both normalized to the protein content of the sensitive and resistant cancer line HCT116 upon incubation with oxaliplatin 5 μ M at 4, 8 and 24 h (n=3 biological replicates for 4 and 8 h, n=6 biological replicates for 24 h).

Metabolomics

The metabolomics experiments revealed that parental and resistant HCT-116 cell lines exhibited a distinctly different metabolite profile from the start as evidenced by multivariate statistics (see heat-map and principal component analysis **Figure S6** and **S7**). **Table 1** shows a list of significantly different metabolites after 24 hours of incubation. The list covers metabolites which were determined by targeted relative quantification (using ^{13}C internal standardization) together with metabolites discovered by untargeted data evaluation. Accordingly, their identity confirmation is based on accurate mass, isotopologue pattern and spectral library match (MS2; mzCloud database), which is considered a level 2 structure confirmation⁶⁴. Upon drug exposure, the sensitive cell models revealed a pronounced metabolic perturbation, while the resistant cells showed only subtle changes (see multivariate statistical evaluation **Figure S8-S11** and **Table 1**).

The classic hallmark of tumor cell metabolism is the Warburg effect, which was found to be frequently accompanied by altered Krebs cycle, glutaminolysis, fatty acid and lipid metabolism and nucleotide synthesis^{65,66}. In a seminal study, metabolomics profiles as obtained across the NCI-60 panel pointed towards a positive correlation between all these “Warburg-like” phenotypes and platinum chemosensitivity²⁹.

Table 1 List of Metabolites which showed significant differences ($p < 0.05$) in a HCT116 cell extract after incubation with oxaliplatin 5 μM for 24h in targeted (internal standardization) and untargeted metabolomics (without internal standardization). Column: UPLC BEH Amide pH4 in positive and negative mode.

Targeted									
Compound	Polarity	RT (min)	Resistant control vs sensitive control		Sensitive control vs Sensitive Oxaliplatin		Resistant Control vs Resistant Oxaliplatin		
			-log10 (p)	log2 (FC)	-log10 (p)	log2 (FC)	-log10 (p)	log2 (FC)	
Alanine*	Pos	4.64	2.86	-0.38	3.26	0.44	-	-	
Alpha-ketoglutarate	Neg	3.13	-	-	4.97	0.85	-	-	
AMP*	Pos/Neg	5.62	-	-	3.66	1.34	-	-	
Argininosuccinic acid	Pos	6.83	6.12	-1.21	3.80	0.73	-	-	
Asparagine	Neg	5.47	4.16	-0.48	3.40	0.46	-	-	
Aspartate	Neg	5.99	4.53	-0.48	-	-	-	-	
Carnitine	Pos	6.64	2.69	-0.29	2.96	0.35	-	-	
Choline	Pos	2.01	3.58	-0.73	-	-	-	-	
Cystathionine	Pos	6.64	6.81	0.73	-	-	-	-	
Fructose-1,6-bisphosphate	Pos/Neg	7.35	-	-	3.15	1.31	2.78	1.11	
Fumarate	Neg	3.15	6.56	-0.79	7.86	1.32	-	-	
GDP	Pos	7.62	-	-	2.31	0.45	-	-	
Glutamate	Pos/Neg	5.55	-	-	4.82	0.53	-	-	
Glutamine	Pos/Neg	5.35	4.53	-0.53	3.03	0.42	-	-	
Glutathione Oxidized	Pos	7.55	-	-	3.37	4.84	-	-	
Hexose Phospate*	Pos/Neg	6.51	-	-	3.25	0.96	-	-	
Untargeted									
Compound	Polarity	RT min	mZCloud results (match)	Resistant control vs Sensitive control		Sensitive control vs Sensitive Oxaliplatin		Resistant Control vs Resistant Oxaliplatin	
				-log10 (p)	log2 (FC)	-log10 (p)	log2 (FC)	-log10 (p)	log2 (FC)
Arginine	Pos	6.03	4 (81)	2.28	-0.53	-	-	-	-
Acetyl-carnitine	Pos	2.47	4 (99)	5.01	-0.67	-	-	-	-
Acetyl-β-methylcholine	Pos	2.12	4 (92)	2.75	-0.32	-	-	-	-
N8-Acetylspermidine	Pos	5.16	3 (81)	-	-	6.09	-1.29	-	-
Acetylcholine	Pos	2.68	2 (98)	3.2	-0.48	-	-	-	-
Palmitoylcarnitine	Pos	0.95	1 (96)	**	-1.32	6.02	-1.11	-	-
4-Acetamidobutanoic acid	Pos	1.37	1 (99)	4.07	-0.78	-	-	-	-
Nicotinic acid adenine dinucleotide	Pos	7.03	1 (93)	3.71	0.52	4.74	0.75	-	-
Glutathione (reduced)	Neg	5.97	3 (79)	2.57	-0.44	2.62	0.49	-	-

*The separation of the different isomers was not possible with this chromatographic method. If different peaks were partially separated, they were integrated as one single peak.

**Only one biological replicate was detected in the resistant line.

Parental HCT116 cells differ in glycolysis compared to the resistant HCT116 cells. Pyruvate levels are higher in sensitive cells (**Figure S6**), and are strongly influenced upon drug incubation in this cell model (**Figure S8** and **Table S9**). Additionally, the metabotype of the oxaliplatin sensitive HCT116 compared to the resistant model revealed differences regarding the intermediates of the Krebs cycle (i.e. fumarate, succinate and malate) confirming altered activity (**Table 1**, **Figure S6**). All three metabolites are higher in sensitive cells compared to resistant cells. Glutamate involved in glutaminolysis and purine synthesis via the folate cycle shows lower abundance in sensitive than resistant cells. The low glutamate levels in sensitive cells are accompanied by low alpha-ketoglutarate (AKG) levels, the metabolite linking glutamate to the Krebs cycle. The biochemical basis for this increase of AKG in resistant cells might be explained by altered generation of carbon derived from amino acid catabolism and amino acid synthesis or by altered glutamine uptake from the media. The low glutamate level in sensitive cells is accompanied by low cystathionine levels linked to glutathione biosynthesis. Glutathione decreases significantly during oxaliplatin incubation which can be inferred from the Pearson correlation data treatment addressing the differential impact of exposure time (see **Table S9-S12**) on metabolites and pathways. The involvement of glutathione in platinum drug efflux and its association to drug resistance has been discussed controversially in the past⁶⁷. Whereas Mohn et al.⁶⁸ did not observe any glutathione alteration upon oxaliplatin incubation in *in vitro* studies of the colorectal cancer line HCT-8, Zhang et al.⁶⁹ observed the opposite in chronic lymphocytic leukemia (CLL) cells. Finally, **Figure 6** gives the comparative metabolomic data on palmitoyl-carnitine, N1,N12-diacetylspermine and N8-acetylspermidine discovered by non-targeted analysis (level 2 identification, based on spectral library match). The long chain acyl-carnitine is a key metabolite of the fatty acid catabolism, more specifically of fatty acid oxidation⁷⁰. This pathway is an essential source of NADH, FADH₂, NADPH and ATP. Fatty acids are conjugated with carnitine for translocation into the mitochondria. Palmitoyl-carnitine has been reported to change activity of several enzymes and transporters localized in the mitochondrial membrane and has been shown to stimulate caspase activity^{71,72}. Resistant cells showed consistently lower levels of palmitoyl-carnitine over 24 hours of incubation time, which were not affected by drug uptake. In contrast, in sensitive cells, the levels increased with time in the control samples while drug uptake resulted in the opposite trend. Already a decade ago, Reidel et al.⁷³ observed that fatty acid metabolism played a role in platinum-resistance. More recently, a positive correlation between fatty acid oxidation and malignant phenotypes including metastasis, therapeutic resistance and relapse was found⁷⁴. Moreover, increasing levels of fatty acid oxidation have been linked to apoptosis. Interestingly, it has been shown that organic cation/carnitine transporter (OCTN1)-mediated uptake of oxaliplatin might contribute to its neuronal accumulation and treatment-limiting neurotoxicity^{61,62}. Carnitine has shown to have an impact on caspase activity⁷². Carnitine showed reduced levels in sensitive cells as compared to platinum resistant cells, which were not increasing upon drug uptake. N8-Acetylspermidine and N1,N12-diacetylspermine are metabolites of the polyamine catabolism. Interestingly, polyamine metabolites were discussed as potential biomarkers for colon cancer and oxidative damage (as ROS is produced by polyamine catabolism). Recently, it has become increasingly clear that polyamine catabolism can play a dominant role in drug response, apoptosis and response to stressful stimuli⁷⁵. In the case of platinum drugs, polyamine catabolism was associated with renal toxicity.

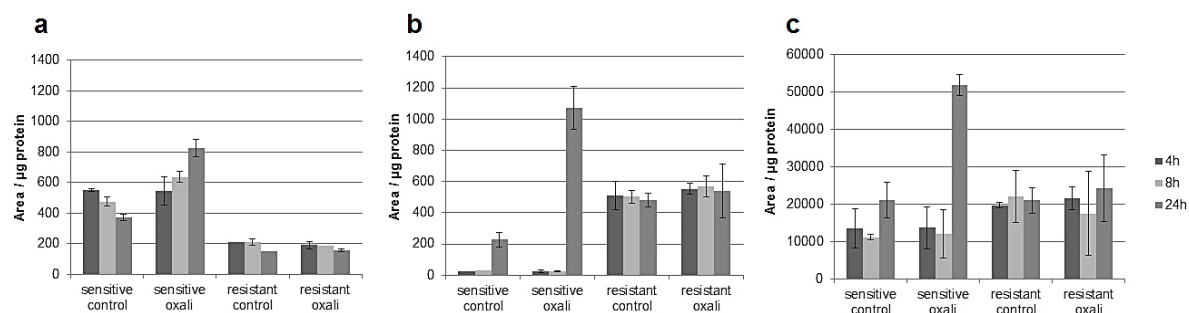


Figure 6 Relative area normalized to the protein content over the incubation time with oxaliplatin 5 μ M in untargeted metabolomics in positive mode (column: UPLC BEH Amide at pH4) of **(a)** palmitoylcarnitine. Low values were observed for the resistant line whereas higher values were observed in the sensitive line which increased upon incubation time. **(b)** N1,N12-diacetylspermine. No differences could be observed in the resistant line whereas high values were observed in the sensitive line upon incubation time. **(c)** N8-acetylspermidine. No differences could be observed in the resistant line whereas high values were observed in the sensitive line upon incubation time.

Conclusion

In this preclinical study, colon cancer resistance towards oxaliplatin was studied for the first time by an integrated metallomics/metabolomics approach. The oxaliplatin sensitive HCT116 cell line displayed a metabolic phenotype which differed most significantly in hall marks of the energy metabolism, the sulfur metabolism, the fatty acid metabolism and polyamine metabolites. From both metallomics and metabolomics data, it could be inferred that resistance in the HCT116 model was accompanied by different drug transport mechanism. It could be speculated that sensitive cells were less protected against oxaliplatin, since glutathione levels and other intermediates of the sulfur metabolism were lower than in the resistant cells. Furthermore, polyamine catabolism was significantly dysregulated upon exposure to oxaliplatin which is known to increase the ROS burden to the cell. In this way, the introduced methods revealed novel insights and provided valid tools for generating new hypothesis regarding resistance towards oxaliplatin.

In summary, we have developed a workflow suitable for preclinical metallomic and metabolomic studies on adherent cancer cell models to investigate resistance to chemotherapy.

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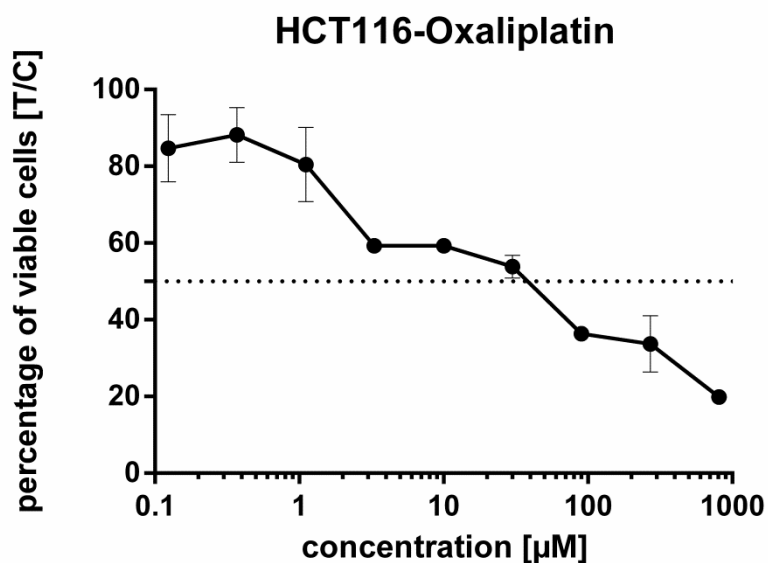


Figure S1 Concentration–effect curves for oxaliplatin in the colorectal cancer line HCT116. An average IC_{50} value of $35 \pm 6 \mu M$ ensured that a concentration of $5 \mu M$ oxaliplatin was adequate (sub-cytotoxic) for the following preparations.

Table S1 Chromatographic conditions and ICP-MS operation parameters for the quantification of the platinum uptake with FI-ICP-MS.

Agilent 1260 Infinity Bio-inert chromatographic conditions	
Eluent	H ₂ O
Flow rate	400 $\mu L/min$
Injection volume	10 μL
Autosampler temperature	2°C
ICP-MS operation parameters	
Nebulizer	MicroMist
Spray chamber	Scott double-pass
Nebulizer gas flow	1.05 L/min
Plasma gas	15 L/min
ICP RF Power	1550 W
m/z measured	195

Table S2 Parameters of the full mass scan (Full-MS), ESI-source (ESI) and untargeted approach (ddMS2) for the metabolomic studies with HILIC-HR-MS.

ESI	
Sheath gas	38
Auxiliary gas	3
Sweep gas	0
Spray voltage	3.5 kV (pos), 2.8 kV (neg)
Capillary temperature	280°C
S-Lens RF	30
Auxiliary gas heater	320°C
Full-MS	
Scan	70-1000 m/z
Polarity	Positive and Negative
Resolution	60000
Automatic Gain Control (AGC) target	1×10^6
Maximum Injection Time (IT)	200 ms
ddMS2	
TopN	5
Polarity	Positive and Negative
AGC target	1×10^5
IT	50 ms
Isolation	1 m/z
Fragmentation with HCD energy	NCE 30
Resolution	30000

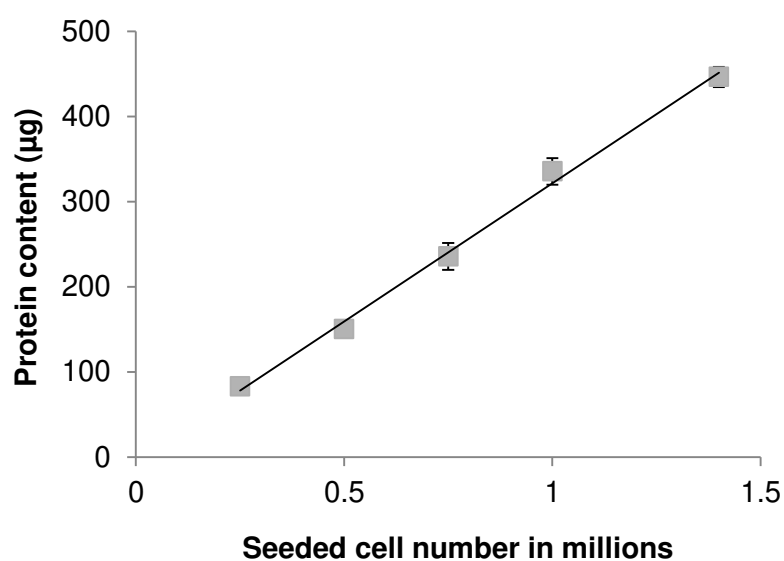


Figure S2 Protein content of different numbers of seeded cells (melanoma cell line FTSLA) after 24h of incubation (n=3 biological replicates) measured with the 2D-Quant Kit.

Table S3 Analytical figures of merit for the protein content determination with the micro BCA protein kit.

Sensitivity ($\mu\text{g/mL}$)	Linear working range ($\mu\text{g/mL}$)	Biological Repeatability (%)	Instrumental Repeatability (%)
0.5	0.5 - 20	< 11 (n=6)	< 7 (n=3)

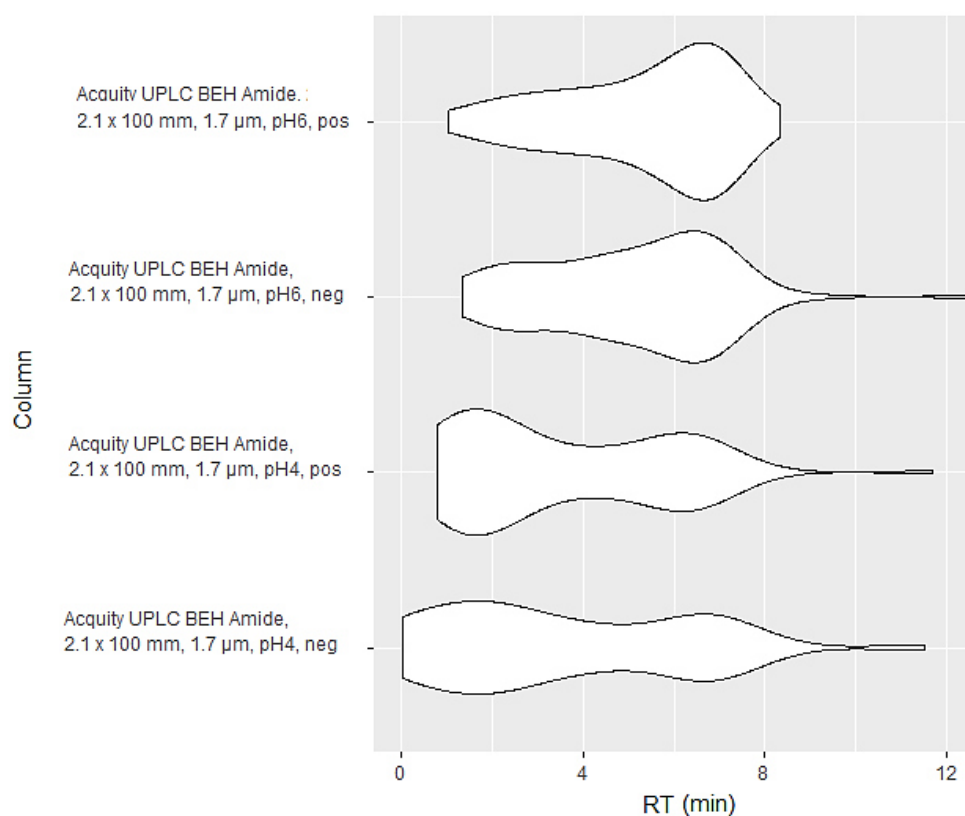


Figure S3 Retention time distribution of a 10 μM multicomponent standard measured on the UPLC BEH Amide column at pH 4 and pH 6 in positive and negative mode. A better separation was observed at pH 4.

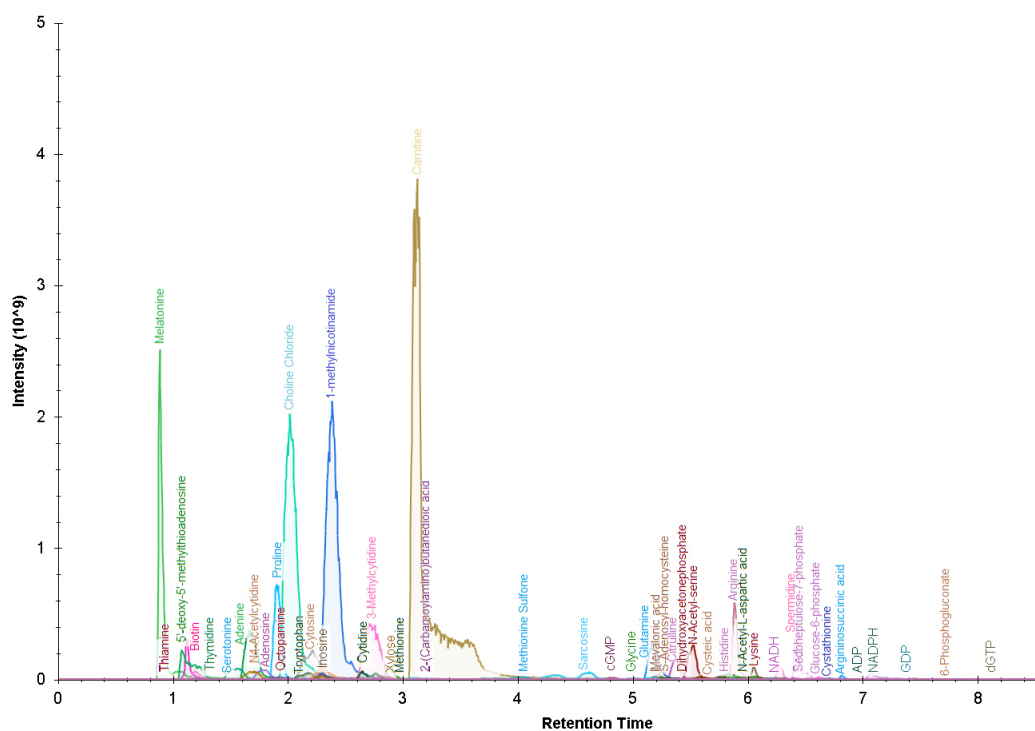


Figure S4 Chromatogram of a 10 μ M multicomponent standard measured on the UPLC BEH Amide column at pH 4 in positive mode.

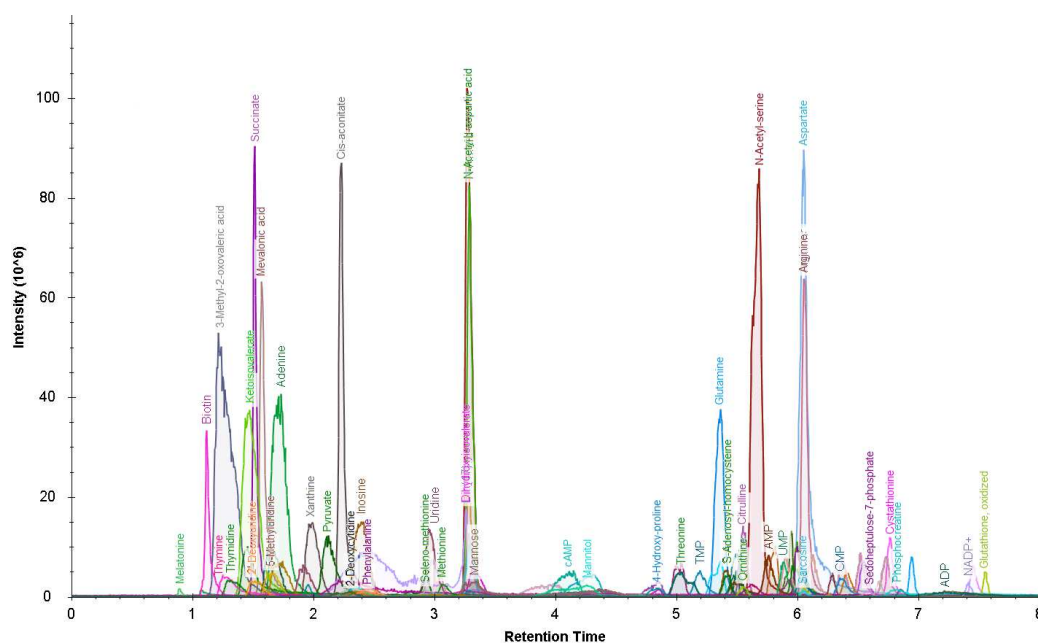


Figure S5 Chromatogram of a 10 μ M multicomponent standard measured on the UPLC BEH Amide column at pH 4 in negative mode.

Table S4 Multicomponent standard 10 μM in positive mode and retention times measured on the UPLC BEH Amide column at pH 4. The uniformly ^{13}C labeled metabolites present in the samples was used for internal standardization.

Compound	RT [min]	^{12}C metabolite		^{13}C labeled internal standard	
		Formula	$[\text{M}+\text{H}]^+$	ISTD formula	ISTD $[\text{M}+\text{H}]^+$
melatonin	0.88	$\text{C}_{13}\text{H}_{16}\text{N}_2\text{O}_2$	233.1285		
5'-Deoxy-5'-Methylthioadenosine	1.08	$\text{C}_{11}\text{H}_{15}\text{N}_5\text{O}_3\text{S}$	298.0968	$^{13}\text{C}_{11}\text{H}_{15}\text{N}_5\text{O}_3\text{S}$	309.1337
Biotin	1.13	$\text{C}_{10}\text{H}_{16}\text{N}_2\text{O}_3\text{S}$	245.0954		
thymidine	1.3	$\text{C}_{10}\text{H}_{14}\text{N}_2\text{O}_5$	243.0976		
2'-Deoxyuridine	1.47	$\text{C}_9\text{H}_{12}\text{N}_2\text{O}_5$	229.0819		
Uracil	1.48	$\text{C}_4\text{H}_4\text{N}_2\text{O}_2$	113.0346		
Mevalonic acid	1.55	$\text{C}_6\text{H}_{12}\text{O}_4$	149.0808		
serotonin	1.58	$\text{C}_{10}\text{H}_{12}\text{N}_2\text{O}$	177.1022		
5-methyluridine	1.59	$\text{C}_{10}\text{H}_{14}\text{N}_2\text{O}_6$	259.0925		
Adenine	1.69	$\text{C}_5\text{H}_5\text{N}_5$	136.0618	$^{13}\text{C}_5\text{H}_5\text{N}_5$	141.0785
N4-acetylcytidine	1.71	$\text{C}_{11}\text{H}_{15}\text{N}_3\text{O}_6$	286.1034		
Adenosine	1.81	$\text{C}_{10}\text{H}_{13}\text{N}_5\text{O}_4$	268.104	$^{13}\text{C}_{10}\text{H}_{13}\text{N}_5\text{O}_4$	278.1376
propionyl-L-carnitine	1.94	$\text{C}_{10}\text{H}_{19}\text{NO}_4$	218.1387		
Octopamine	1.97	$\text{C}_8\text{H}_{11}\text{NO}_2$	154.0863		
Choline chloride	2.05	$\text{C}_5\text{H}_{13}\text{NO}$	104.107	$^{13}\text{C}_5\text{H}_{13}\text{NO}$	109.1238
2-deoxycytidine	2.21	$\text{C}_9\text{H}_{13}\text{N}_3\text{O}_4$	228.0979		
Tryptophan	2.22	$\text{C}_{11}\text{H}_{12}\text{N}_2\text{O}_2$	205.0972	$^{13}\text{C}_{11}\text{H}_{12}\text{N}_2\text{O}_2$	216.1341
cytosine	2.23	$\text{C}_4\text{H}_5\text{N}_3\text{O}$	112.0505		
Phenylalanine	2.29	$\text{C}_9\text{H}_{11}\text{NO}_2$	166.0863	$^{13}\text{C}_9\text{H}_{11}\text{NO}_2$	175.1164
Inosine	2.32	$\text{C}_{10}\text{H}_{12}\text{N}_4\text{O}_5$	269.088	$^{13}\text{C}_{10}\text{H}_{12}\text{N}_4\text{O}_5$	279.1216
Kynurenine	2.32	$\text{C}_{10}\text{H}_{12}\text{N}_2\text{O}_3$	209.0921	$^{13}\text{C}_{10}\text{H}_{12}\text{N}_2\text{O}_3$	219.1256
Guanine	2.34	$\text{C}_5\text{H}_5\text{N}_5\text{O}$	152.0567	$^{13}\text{C}_5\text{H}_5\text{N}_5\text{O}$	157.0735
1-Methylnicotinamide	2.39	$\text{C}_7\text{H}_8\text{N}_2\text{O}$	137.0709	$^{13}\text{C}_7\text{H}_8\text{N}_2\text{O}$	144.0944
3-methylcytidine	2.71	$\text{C}_{10}\text{H}_{15}\text{N}_3\text{O}_5$	258.1085		
Pseudouridine	1.90/2.85*	$\text{C}_9\text{H}_{12}\text{N}_2\text{O}_6$	245.0768		
Cytidine	2.84	$\text{C}_9\text{H}_{13}\text{N}_3\text{O}_5$	244.0928	$^{13}\text{C}_9\text{H}_{13}\text{N}_3\text{O}_5$	253.123
Uridine	1.90/2.85*	$\text{C}_9\text{H}_{12}\text{N}_2\text{O}_6$	245.0768		
seleno-methionine	2.86	$\text{C}_5\text{H}_{11}\text{NO}_2\text{Se}$	198.0028		
Methionine	2.89	$\text{C}_5\text{H}_{11}\text{NO}_2\text{S}$	150.0583	$^{13}\text{C}_5\text{H}_{11}\text{NO}_2\text{S}$	155.0751
Guanosine	2.91/3.79*	$\text{C}_{10}\text{H}_{13}\text{N}_5\text{O}_5$	284.0989	$^{13}\text{C}_{10}\text{H}_{13}\text{N}_5\text{O}_5$	294.1325
Isoguanosine	2.91/3.79*	$\text{C}_{10}\text{H}_{13}\text{N}_5\text{O}_5$	284.0989	$^{13}\text{C}_{10}\text{H}_{13}\text{N}_5\text{O}_5$	294.1325
Homocysteine	3.14	$\text{C}_4\text{H}_9\text{NO}_2\text{S}$	136.0427		
N-acetyl-L-aspartic acid	3.15	$\text{C}_6\text{H}_9\text{NO}_5$	176.0554		
Tyrosine	3.15	$\text{C}_9\text{H}_{11}\text{NO}_3$	182.0812	$^{13}\text{C}_9\text{H}_{11}\text{NO}_3$	191.1114
Carnitine	3.2	$\text{C}_7\text{H}_{15}\text{NO}_3$	162.1125	$^{13}\text{C}_7\text{H}_{15}\text{NO}_3$	169.136
2-(carbamoylamino)butanedioic acid	3.25	$\text{C}_5\text{H}_8\text{N}_2\text{O}_5$	177.0506		
cAMP	3.83	$\text{C}_{10}\text{H}_{12}\text{N}_5\text{O}_6\text{P}$	330.0598		
Mannitol	3.99	$\text{C}_6\text{H}_{14}\text{O}_6$	183.0863		
methionine sulfone	4.11	$\text{C}_5\text{H}_{11}\text{NO}_4\text{S}$	182.0482		
sarcosine	4.66	$\text{C}_3\text{H}_7\text{NO}_2$	90.055	$^{13}\text{C}_3\text{H}_7\text{NO}_2$	93.065

Alanine	4.70	C ₃ H ₇ NO ₂	90.055	¹³ C ₃ H ₇ NO ₂	93.065
cGMP	4.80	C ₁₀ H ₁₂ N ₅ O ₇ P	346.0547	¹³ C ₁₀ H ₁₂ N ₅ O ₇ P	356.0883
Threonine	5.00	C ₄ H ₉ NO ₃	120.0655	¹³ C ₄ H ₉ NO ₃	122.0789
Homoserine	5.00	C ₄ H ₉ NO ₃	120.0655	¹³ C ₄ H ₉ NO ₃	122.0789
Glycine	5.01	C ₂ H ₅ NO ₂	76.0393	¹³ C ₂ H ₅ NO ₂	78.046
TMP	5.10	C ₁₀ H ₁₅ N ₂ O ₈ P	323.0639		
alpha-aminoadipic acid	5.20	C ₆ H ₁₁ NO ₄	162.0761	¹³ C ₆ H ₁₁ NO ₄	168.0962
Glutamine	5.22	C ₅ H ₁₀ N ₂ O ₃	147.0764	¹³ C ₅ H ₁₀ N ₂ O ₃	152.0932
S-Adenosyl-homocysteine	5.30	C ₁₄ H ₂₀ N ₆ O ₅ S	385.1289		
Serine	5.30	C ₃ H ₇ NO ₃	106.0499	¹³ C ₃ H ₇ NO ₃	109.0599
dAMP	5.34	C ₁₀ H ₁₄ N ₅ O ₆ P	332.0755		
Asparagine	5.42	C ₄ H ₈ N ₂ O ₃	133.0608	¹³ C ₄ H ₈ N ₂ O ₃	137.0742
Dihydroxyacetonephosphate	5.43	C ₃ H ₇ O ₆ P	171.0053		
Citrulline	5.45	C ₆ H ₁₃ N ₃ O ₃	176.103	¹³ C ₆ H ₁₃ N ₃ O ₃	182.1231
Ornithine	5.53	C ₅ H ₁₂ N ₂ O ₂	133.0972	¹³ C ₅ H ₁₂ N ₂ O ₂	138.1139
Glutamate	5.56	C ₅ H ₉ NO ₄	148.0604	¹³ C ₅ H ₉ NO ₄	153.0772
5AMP	5.62	C ₁₀ H ₁₄ N ₅ O ₇ P	348.0704	¹³ C ₁₀ H ₁₄ N ₅ O ₇ P	358.1039
3AMP	5.62	C ₁₀ H ₁₄ N ₅ O ₇ P	348.0704	¹³ C ₁₀ H ₁₄ N ₅ O ₇ P	358.1039
Cysteic acid	5.73	C ₃ H ₇ NO ₅ S	170.0178		
UMP	5.80	C ₉ H ₁₃ N ₂ O ₉ P	325.0431	¹³ C ₉ H ₁₃ N ₂ O ₉ P	334.0733
Ribose-5-phosphate	5.81	C ₅ H ₁₁ O ₈ P	231.0264	¹³ C ₅ H ₁₁ O ₈ P	236.0432
Ribulose-5-phosphate	5.81	C ₅ H ₁₁ O ₈ P	231.0264	¹³ C ₅ H ₁₁ O ₈ P	236.0432
Cysteinyl-glycine	5.84	C ₅ H ₁₀ N ₂ O ₃ S	179.0485		
Glutamyl-cysteine	5.85	C ₈ H ₁₄ N ₂ O ₅ S	251.0696		
Histidine	5.86	C ₆ H ₉ N ₃ O ₂	156.0768	¹³ C ₆ H ₉ N ₃ O ₂	162.0969
Glutathione reduced	5.87	C ₁₀ H ₁₇ N ₃ O ₆ S	308.0911	¹³ C ₁₀ H ₁₇ N ₃ O ₆ S	318.1246
Aspartic acid	5.90	C ₄ H ₇ NO ₄	134.0448	¹³ C ₄ H ₇ NO ₄	138.0582
dCMP	5.90	C ₉ H ₁₄ N ₃ O ₇ P	308.0642		
IMP	5.92	C ₁₀ H ₁₃ N ₄ O ₈ P	349.0544	¹³ C ₁₀ H ₁₃ N ₄ O ₈ P	359.0879
Arginine	5.94	C ₆ H ₁₄ N ₄ O ₂	175.119	¹³ C ₆ H ₁₄ N ₄ O ₂	181.1391
erythrose-4-phosphate	6.02	C ₄ H ₉ O ₇ P	201.0159		
Lysine	6.10	C ₆ H ₁₄ N ₂ O ₂	147.1128	¹³ C ₆ H ₁₄ N ₂ O ₂	153.1329
Flavinadenin dinucleotide	6.14	C ₂₇ H ₃₃ N ₉ O ₁₅ P ₂	786.1644	¹³ C ₂₇ H ₃₃ N ₉ O ₁₅ P ₂	813.255
CMP	6.24	C ₉ H ₁₄ N ₃ O ₈ P	324.0591	¹³ C ₉ H ₁₄ N ₃ O ₈ P	333.0893
GMP	6.30	C ₁₀ H ₁₄ N ₅ O ₈ P	364.0653	¹³ C ₁₀ H ₁₄ N ₅ O ₈ P	374.0988
NADH	6.31	C ₂₁ H ₂₉ N ₇ O ₁₄ P ₂	666.1321	¹³ C ₂₁ H ₂₉ N ₇ O ₁₄ P ₂	687.2025
S-(adenosyl)-methionine	6.35	C ₁₅ H ₂₂ N ₆ O ₅ S	399.1445		
Fructose-6-Phosphate	6.42	C ₆ H ₁₃ O ₉ P	261.037	¹³ C ₆ H ₁₃ O ₉ P	267.0571
Glucose-6-Phosphate	6.42	C ₆ H ₁₃ O ₉ P	261.037	¹³ C ₆ H ₁₃ O ₉ P	267.0571
Glucose-1-Phosphate	6.42	C ₆ H ₁₃ O ₉ P	261.037	¹³ C ₆ H ₁₃ O ₉ P	267.0571
Mannitol-1-phosphate	6.44	C ₆ H ₁₅ O ₉ P	263.0526		
spermidine	6.50	C ₇ H ₁₉ N ₃	146.1652		
Sedoheptulose-7-phosphate	6.50	C ₇ H ₁₅ O ₁₀ P	291.0476		
NAD+	6.55	C ₂₁ H ₂₇ N ₇ O ₁₄ P ₂	663.1091	¹³ C ₂₁ H ₂₇ N ₇ O ₁₄ P ₂	684.1796
Cystathionine	6.64	C ₇ H ₁₄ N ₂ O ₄ S	223.0747	¹³ C ₇ H ₁₄ N ₂ O ₄ S	230.0982
Cystine	6.70	C ₆ H ₁₂ N ₂ O ₄ S ₂	241.0311		
phosphocreatine	6.75	C ₄ H ₁₀ N ₃ O ₅ P	212.0431		
Argininosuccinic acid	6.81	C ₁₀ H ₁₈ N ₄ O ₆	291.1299	¹³ C ₁₀ H ₁₈ N ₄ O ₆	301.1635
ADP	7.08	C ₁₀ H ₁₅ N ₅ O ₁₀ P ₂	428.0367	¹³ C ₁₀ H ₁₅ N ₅ O ₁₀ P ₂	438.0702

UDP	7.12	C ₉ H ₁₄ N ₂ O ₁₂ P ₂	405.0095		
NADPH	7.15	C ₂₁ H ₃₀ N ₇ O ₁₇ P ₃	746.0984	¹³ C ₂₁ H ₃₀ N ₇ O ₁₇ P ₃	767.1688
spermine	7.15	C ₁₀ H ₂₆ N ₄	203.223		
NADP+	7.31	C ₂₁ H ₂₉ N ₇ O ₁₇ P ₃	744.0833	¹³ C ₂₁ H ₂₉ N ₇ O ₁₇ P ₃	765.1537
Fructose-1,6-bisphosphate	7.36	C ₆ H ₁₄ O ₁₂ P	341.0033	¹³ C ₆ H ₁₄ O ₁₂ P	347.0235
GDP	7.42	C ₁₀ H ₁₅ N ₅ O ₁₁ P ₂	444.0316	¹³ C ₁₀ H ₁₅ N ₅ O ₁₁ P ₂	454.0652
Glutathione oxidized	7.50	C ₂₀ H ₃₂ N ₆ O ₁₂ S ₂	613.1592	¹³ C ₂₀ H ₃₂ N ₆ O ₁₂ S ₂	633.2263
3-Phosphoglycerate	7.64	C ₃ H ₇ O ₇ P	187.0002	¹³ C ₃ H ₇ O ₇ P	190.0103
6-Phosphogluconate	7.78	C ₆ H ₁₃ O ₁₀ P	277.0319	¹³ C ₆ H ₁₃ O ₁₀ P	283.052
TTP	7.81	C ₁₀ H ₁₈ N ₂ O ₁₄ P ₃	482.9965		
dGTP	8.02	C ₁₀ H ₁₆ N ₅ O ₁₃ P ₃	508.003	¹³ C ₁₀ H ₁₆ N ₅ O ₁₃ P ₃	518.0366
dATP	8.06	C ₁₀ H ₁₇ N ₅ O ₁₂ P ₃	492.0081		
ATP	8.11	C ₁₀ H ₁₆ N ₅ O ₁₃ P ₃	508.003	¹³ C ₁₀ H ₁₆ N ₅ O ₁₃ P ₃	518.0366
GTP	8.14	C ₁₀ H ₁₆ N ₅ O ₁₄ P ₃	523.9979		
UTP	8.21	C ₉ H ₁₆ N ₂ O ₁₅ P ₃	484.9758		
CTP	8.43	C ₉ H ₁₆ N ₃ O ₁₄ P ₃	483.9918		
dCTP	8.46	C ₉ H ₁₇ N ₃ O ₁₃ P ₃	467.9969		

*Isomers which could be separated on the UPLC BEH Amide column at pH 4 in positive mode. For further identification an analysis of the single standard is necessary.

Table S5 Multicomponent standard 10 µM in negative mode and retention times measured on the UPLC BEH Amide column at pH 4. The uniformly ¹³C labeled metabolites present in the samples was used for internal standardization.

Compound	RT [min]	¹² C metabolite		¹³ C labeled internal standard	
		Formula	[M-H] ⁻	ISTD formula	ISTD [M-H] ⁻
melatonin	0.90	C ₁₃ H ₁₆ N ₂ O ₂	231.1139		
5'-Deoxy-5'-Methylthioadenosine	1.08	C ₁₁ H ₁₅ N ₅ O ₃ S	296.0823		
biotin	1.12	C ₁₀ H ₁₆ N ₂ O ₃ S	243.0809		
3-Methyl-2-oxovaleric acid	1.22	C ₆ H ₁₀ O ₃	129.0557	¹³ C ₆ H ₁₀ O ₃	135.0759
thymine	1.26	C ₅ H ₆ N ₂ O ₂	125.0357		
thymidine	1.32	C ₁₀ H ₁₄ N ₂ O ₅	241.0830		
Uracil	1.45	C ₄ H ₄ N ₂ O ₂	111.0200		
Ketoisovalerate	1.47	C ₅ H ₈ O ₃	115.0401	¹³ C ₅ H ₈ O ₃	120.0568
2'-deoxyuridine	1.50	C ₉ H ₁₂ N ₂ O ₅	227.0673		
Succinate	1.52	C ₄ H ₆ O ₄	117.0193	¹³ C ₄ H ₆ O ₄	121.0328
Mevalonic acid	1.57	C ₆ H ₁₂ O ₄	147.0663	¹³ C ₆ H ₁₂ O ₄	153.0864
5-methyluridine	1.65	C ₁₀ H ₁₄ N ₂ O ₆	257.0779		
Adenine	1.72	C ₅ H ₅ N ₅	134.0472	¹³ C ₅ H ₅ N ₅	139.0640
N4-acetylcytidine	1.75	C ₁₁ H ₁₅ N ₃ O ₆	284.0888		
Adenosine	1.84	C ₁₀ H ₁₃ N ₅ O ₄	266.0895		
Pseudouridine	1.92/2.91*	C ₉ H ₁₂ N ₂ O ₆	243.0623		
Uridine	1.92/2.91*	C ₉ H ₁₂ N ₂ O ₆	243.0623		
Xanthine	1.99	C ₅ H ₄ N ₄ O ₂	151.0262		
Lactate	2.23	C ₃ H ₆ O ₃	89.0244	¹³ C ₃ H ₆ O ₃	92.0345
cis-Aconitate	2.24	C ₆ H ₆ O ₆	173.0092	¹³ C ₆ H ₆ O ₆	179.0293
2-deoxycytidine	2.30	C ₉ H ₁₃ N ₃ O ₄	226.0833		
Tryptophan	2.33	C ₁₁ H ₁₂ N ₂ O ₂	203.0826		
gluconate	2.35	C ₆ H ₁₂ O ₇	195.0510		

Ribose	2.35/3.02*	C ₅ H ₁₀ O ₅	149.0456		
Xylose	2.35/3.02*	C ₅ H ₁₀ O ₅	149.0456		
Guanine	2.39	C ₅ H ₅ N ₅ O	150.0421		
Pyruvate	2.40	C ₃ H ₄ O ₃	87.0088	¹³ C ₃ H ₄ O ₃	90.0188
Inosine	2.42	C ₁₀ H ₁₂ N ₄ O ₅	267.0735		
Kynurenine	2.43	C ₁₀ H ₁₂ N ₂ O ₃	207.0775		
Phenylalanine	2.44	C ₉ H ₁₁ NO ₂	164.0717		
seleno-methionine	2.82	C ₅ H ₁₁ NO ₂ Se	195.9882		
Cytidine	2.90	C ₉ H ₁₃ N ₃ O ₅	242.0782		
Methionine	2.94	C ₅ H ₁₁ NO ₂ S	148.0438		
Guanosine	2.98/3.89*	C ₁₀ H ₁₃ N ₅ O ₅	282.0844		
Isoguanosine	2.98/3.89*	C ₁₀ H ₁₃ N ₅ O ₅	282.0844		
Tyrosine	3.23	C ₉ H ₁₁ NO ₃	180.0666		
Dihydroxyisovalerate	3.23	C ₅ H ₁₀ O ₄	133.0506	¹³ C ₅ H ₁₀ O ₄	138.0674
Fructose	3.26	C ₆ H ₁₂ O ₆	179.0561	¹³ C ₁₀ H ₁₃ N ₅ O ₄	185.0762
Galactose	3.26	C ₆ H ₁₂ O ₆	179.0561	¹³ C ₁₀ H ₁₃ N ₅ O ₄	185.0762
Glucose	3.26	C ₆ H ₁₂ O ₆	179.0561	¹³ C ₁₀ H ₁₃ N ₅ O ₄	185.0762
Inositol	3.26	C ₆ H ₁₂ O ₆	179.0561	¹³ C ₁₀ H ₁₃ N ₅ O ₄	185.0762
Mannose	3.26	C ₆ H ₁₂ O ₆	179.0561	¹³ C ₁₀ H ₁₃ N ₅ O ₄	185.0762
N-Acetyl-serine	3.26	C ₅ H ₉ NO ₄	146.0459		
N-acetyl-L-aspartic acid	3.29	C ₆ H ₉ NO ₅	174.0408		
alpha-Ketoglutarate	3.29	C ₅ H ₆ O ₅	145.0142	¹³ C ₅ H ₆ O ₅	150.0310
Fumarate	3.29	C ₄ H ₄ O ₄	115.0037	¹³ C ₄ H ₄ O ₄	119.0171
Hydroxyglutaric acid	3.29	C ₅ H ₈ O ₅	147.0299	¹³ C ₅ H ₈ O ₅	152.0467
2-(carbamoylamino)butanedioic acid	3.32	C ₅ H ₈ N ₂ O ₅	175.0360		
cAMP	4.15	C ₁₀ H ₁₂ N ₅ O ₆ P	328.0452		
Mannitol	4.24	C ₆ H ₁₄ O ₆	181.0718		
methionine sulfone	4.31	C ₅ H ₁₁ NO ₄ S	180.0336		
Alanine	4.66	C ₃ H ₇ NO ₂	88.0404		
sarcosine	4.66	C ₃ H ₇ NO ₂	88.0404		
guanidineacetic acid	4.83	C ₃ H ₇ N ₃ O ₂	116.0466		
Glycine	4.85	C ₂ H ₅ NO ₂	74.0248		
cGMP	5.00	C ₁₀ H ₁₂ N ₅ O ₇ P	344.0402		
Threonine	5.05	C ₄ H ₉ NO ₃	118.0510	¹³ C ₄ H ₉ NO ₃	122.0642
Homoserine	5.05	C ₄ H ₉ NO ₃	118.0510	¹³ C ₄ H ₉ NO ₃	122.0642
TMP	5.19	C ₁₀ H ₁₅ N ₂ O ₈ P	321.0493		
alpha-aminoadipic acid	5.34	C ₆ H ₁₁ NO ₄	160.0615	¹³ C ₆ H ₁₁ NO ₄	166.0817
Glutamine	5.36	C ₅ H ₁₀ N ₂ O ₃	145.0619	¹³ C ₅ H ₁₀ N ₂ O ₃	150.0786
S-Adenosyl-homocysteine	5.42	C ₁₄ H ₂₀ N ₆ O ₅ S	383.1143		
Serine	5.42	C ₃ H ₇ NO ₃	104.0353	¹³ C ₃ H ₇ NO ₃	107.0454
Asparagine	5.42	C ₄ H ₈ N ₂ O ₃	131.0462	¹³ C ₄ H ₈ N ₂ O ₃	135.0596
dAMP	5.45	C ₁₀ H ₁₄ N ₅ O ₆ P	330.0609		
Citrulline	5.49	C ₆ H ₁₃ N ₃ O ₃	174.0884	¹³ C ₆ H ₁₃ N ₃ O ₃	180.1085
Ornithine	5.53	C ₅ H ₁₂ N ₂ O ₂	131.0826	¹³ C ₅ H ₁₂ N ₂ O ₂	136.0994
Dihydroxyacetonephosphate	5.62	C ₃ H ₇ O ₆ P	168.9908	¹³ C ₃ H ₇ O ₆ P	172.0008
3AMP	5.62	C ₁₀ H ₁₄ N ₅ O ₇ P	346.0558	¹³ C ₁₀ H ₁₄ N ₅ O ₇ P	356.0894
Glutamate	5.64	C ₅ H ₉ NO ₄	146.0459	¹³ C ₅ H ₉ NO ₄	151.0627
5AMP	5.72	C ₁₀ H ₁₄ N ₅ O ₇ P	346.0558	¹³ C ₁₀ H ₁₄ N ₅ O ₇ P	356.0894
Cysteic acid	5.78	C ₃ H ₇ NO ₅ S	167.9972	¹³ C ₃ H ₇ NO ₅ S	171.0073
UMP	5.88	C ₉ H ₁₃ N ₂ O ₉ P	323.0286	¹³ C ₉ H ₁₃ N ₂ O ₉ P	332.0588
Glutamyl-cysteine	5.94	C ₈ H ₁₄ N ₂ O ₅ S	249.0551		
Glutathione reduced	5.95	C ₁₀ H ₁₇ N ₃ O ₆ S	306.0765	¹³ C ₁₀ H ₁₇ N ₃ O ₆ S	316.1101
Histidine	5.96	C ₆ H ₉ N ₃ O ₂	154.0622	¹³ C ₆ H ₉ N ₃ O ₂	160.0823
dCMP	5.99	C ₉ H ₁₄ N ₃ O ₇ P	306.0497		
Arginine	6.00	C ₆ H ₁₄ N ₄ O ₂	173.1044	¹³ C ₆ H ₁₄ N ₄ O ₂	179.1245
IMP	6.04	C ₁₀ H ₁₃ N ₄ O ₈ P	347.0398		
Aspartate	6.05	C ₄ H ₇ NO ₄	132.0302	¹³ C ₄ H ₇ NO ₄	136.0437
erythrose-4-phosphate	6.09	C ₄ H ₉ O ₇ P	199.0013		
Ribose-5-phosphate	6.13	C ₅ H ₁₁ O ₈ P	229.0119	¹³ C ₅ H ₁₁ O ₈ P	234.0287

Ribulose-5-phosphate	6.13	C ₅ H ₁₁ O ₈ P	229.0119	¹³ C ₅ H ₁₁ O ₈ P	234.0287
Lysine	6.18	C ₆ H ₁₄ N ₂ O ₂	145.0983		
Flavinadenin dinucleotide	6.29	C ₂₇ H ₃₃ N ₉ O ₁₅ P ₂	784.1499		
CMP	6.35	C ₉ H ₁₄ N ₃ O ₈ P	322.0446		
NADH	6.40	C ₂₁ H ₂₉ N ₇ O ₁₄ P ₂	664.1175		
GMP	6.41	C ₁₀ H ₁₄ N ₅ O ₈ P	362.0507	¹³ C ₁₀ H ₁₄ N ₅ O ₈ P	372.0843
Mannitol-1-phosphate	6.55	C ₆ H ₁₅ O ₉ P	261.0381		
Fructose-6-Phosphate	6.52	C ₆ H ₁₃ O ₉ P	259.0224	¹³ C ₆ H ₁₃ O ₉ P	265.0426
Glucose-6-Phosphate	6.52	C ₆ H ₁₃ O ₉ P	259.0224	¹³ C ₆ H ₁₃ O ₉ P	265.0426
Glucose-1-Phosphate	6.52	C ₆ H ₁₃ O ₉ P	259.0224	¹³ C ₆ H ₁₃ O ₉ P	265.0426
Sedoheptulose-7-phosphate	6.52	C ₇ H ₁₅ O ₁₀ P	289.033		
NAD+	6.68	C ₂₁ H ₂₇ N ₇ O ₁₄ P ₂	662.1018	¹³ C ₂₁ H ₂₇ N ₇ O ₁₄ P ₂	683.1723
Cystathionine	6.72	C ₇ H ₁₄ N ₂ O ₄ S	221.0602	¹³ C ₇ H ₁₄ N ₂ O ₄ S	228.0836
Cystine	6.80	C ₆ H ₁₂ N ₂ O ₄	239.0166		
phosphocreatine	6.82	C ₄ H ₁₀ N ₃ O ₅ P	210.0285		
Argininosuccinic acid	6.89	C ₁₀ H ₁₈ N ₄ O ₆	289.1154	¹³ C ₁₀ H ₁₈ N ₄ O ₆	299.1489
ADP	7.13	C ₁₀ H ₁₅ N ₅ O ₁₀ P ₂	426.0221	¹³ C ₁₀ H ₁₅ N ₅ O ₁₀ P ₂	436.0557
NADPH	7.21	C ₂₁ H ₃₀ N ₇ O ₁₇ P ₃	744.0838		
UDP	7.28	C ₉ H ₁₄ N ₂ O ₁₂ P ₂	402.9949		
NADP+	7.41	C ₂₁ H ₂₉ N ₇ O ₁₇ P ₃	742.0682		
Fructose-1,6-bisphosphate	7.46	C ₆ H ₁₄ O ₁₂ P	338.9888	¹³ C ₆ H ₁₄ O ₁₂ P	345.0089
GDP	7.51	C ₁₀ H ₁₅ N ₅ O ₁₁ P ₂	442.0171		
Citric acid	7.53	C ₆ H ₈ O ₇	193.0343		
Isocitric acid	7.53	C ₆ H ₈ O ₇	193.0343		
Glutathione oxidized	7.55	C ₂₀ H ₃₂ N ₆ O ₁₂ S ₂	611.1447		
Malate	7.55	C ₄ H ₆ O ₅	133.0142	¹³ C ₄ H ₆ O ₅	137.0277
6-Phosphogluconate	7.83	C ₆ H ₁₃ O ₁₀ P	275.0174		
3-Phosphoglycerate	7.87	C ₃ H ₇ O ₇ P	184.9857		
ATP	8.13	C ₁₀ H ₁₆ N ₅ O ₁₃ P ₃	505.9885	¹³ C ₁₀ H ₁₆ N ₅ O ₁₃ P ₃	516.022
dATP	8.21	C ₁₀ H ₁₇ N ₅ O ₁₂ P ₃	491.0013		
dGTP	8.25	C ₁₀ H ₁₆ N ₅ O ₁₃ P ₃	505.9885	¹³ C ₁₀ H ₁₆ N ₅ O ₁₃ P ₃	516.022
UTP	8.27	C ₉ H ₁₆ N ₂ O ₁₅ P ₃	483.9691		
GTP	8.36	C ₁₀ H ₁₆ N ₅ O ₁₄ P ₃	521.9834		
dCTP	8.66	C ₉ H ₁₇ N ₃ O ₁₃ P ₃	466.9901		
TTP	8.78	C ₁₀ H ₁₈ N ₂ O ₁₄ P ₃	481.9898		
CTP	9.14	C ₉ H ₁₆ N ₃ O ₁₄ P ₃	481.9772		

*Isomers which could be separated on the UPLC BEH Amide column at pH 4 in negative mode. For further identification an analysis of the single standard is necessary.

Table S6 Isomers present in the 10 µM multicomponent standard and measured on the UPLC BEH Amide column at pH 4 in positive and negative mode. The isomers Pseudouridine/Uridine, Gultamate/N-acetyl-serine, Ribose/Xylose, Guanosine/Isoguanosine, could be separated with this method. The rest of the isomers were partially separated and integrated separately with the exception of Homoserine/Threonine, 2-phosphoglycerate/3-phosphoglycerate, Citrate/isocitrate, 3-methylcytidine/4-methylcytidine and ATP/dGTP which could not be separated.

Isomers	Separation (positive/negative)
Hexose phosphates	partial/partial
Glutamate/N-Acetyl-serine	not separated*/separated
3AMP/5AMP	partial/partial

Hexoses	partial/partial
Ribose-5-phosphate/Ribulose-5-phosphate	partial/partial
Alanine/sarcosine	partial/partial
Pseudouridine/Uridine	separated/separated
Guanosine/isoguanosine	separated/separated
Homoserine/Threonine	not separated/not separated
2-phosphoglycerate/3-phosphoglycerate	not separated/not separated
Citrate/isocitrate	-**/not separated
3-methylcytidine/4-methylcytidine	not separated/-**
Ribose/xylose	-**/separated
ATP/dGTP	not separated/not separated

*Only one peak detected.

**Not detected in this polarity.

Table S7 Repeatability over >25 h of the UPLC BEH Amide column at pH 4 in positive and negative mode regarding a 10 μ M multicomponent standard (n=3 technical replicates).

positive		negative	
# of metabol.	RSD (%)	# of metabol.	RSD (%)
19	0-5	98	0-5
39	6-15	18	6-15
31	16-30	2	16-30
16	31-60	0	31-60
1*	>60	0	>60

*corresponding to UTP.

Table S8 Full width at half maximum (FWHM) observed with the UPLC BEH Amide column at pH 4 in positive and negative mode regarding a multicomponent standard 10 μ M.

positive		negative	
# of metabol.	FWHM	# of metabol.	FWHM
16	1.0-3.0	18	1.0-3.0
34	3.1-5.0	34	3.1-5.0
40	5.1-10.0	44	5.1-10.0
4	10.1-20.0	3	10.1-20.0
3	20.0-40.0	7	20.0-40.0
1	40.1-60.0	3	40.1-60.0
8*	>60	9*	>60

*corresponding mainly to nucleotides. Their peak width increased with the number of phosphates.

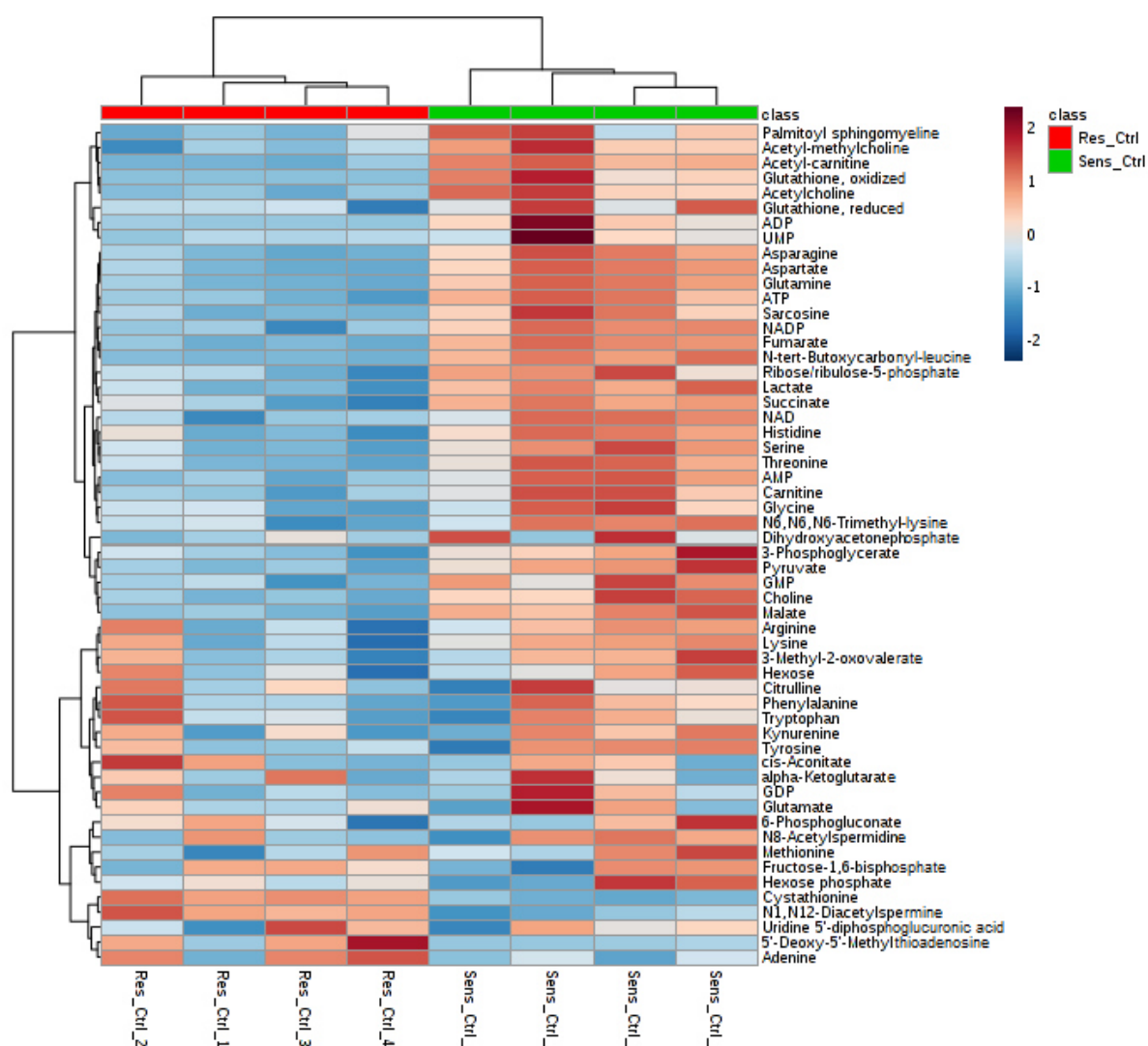


Figure S6 Heatmap of the different compounds found in sensitive HCT116 cell control and in resistant HCT116 cell control. Method: UPLC BEH Amide at pH 4 in positive and negative mode combining targeted (internal standardization) and untargeted metabolomics.

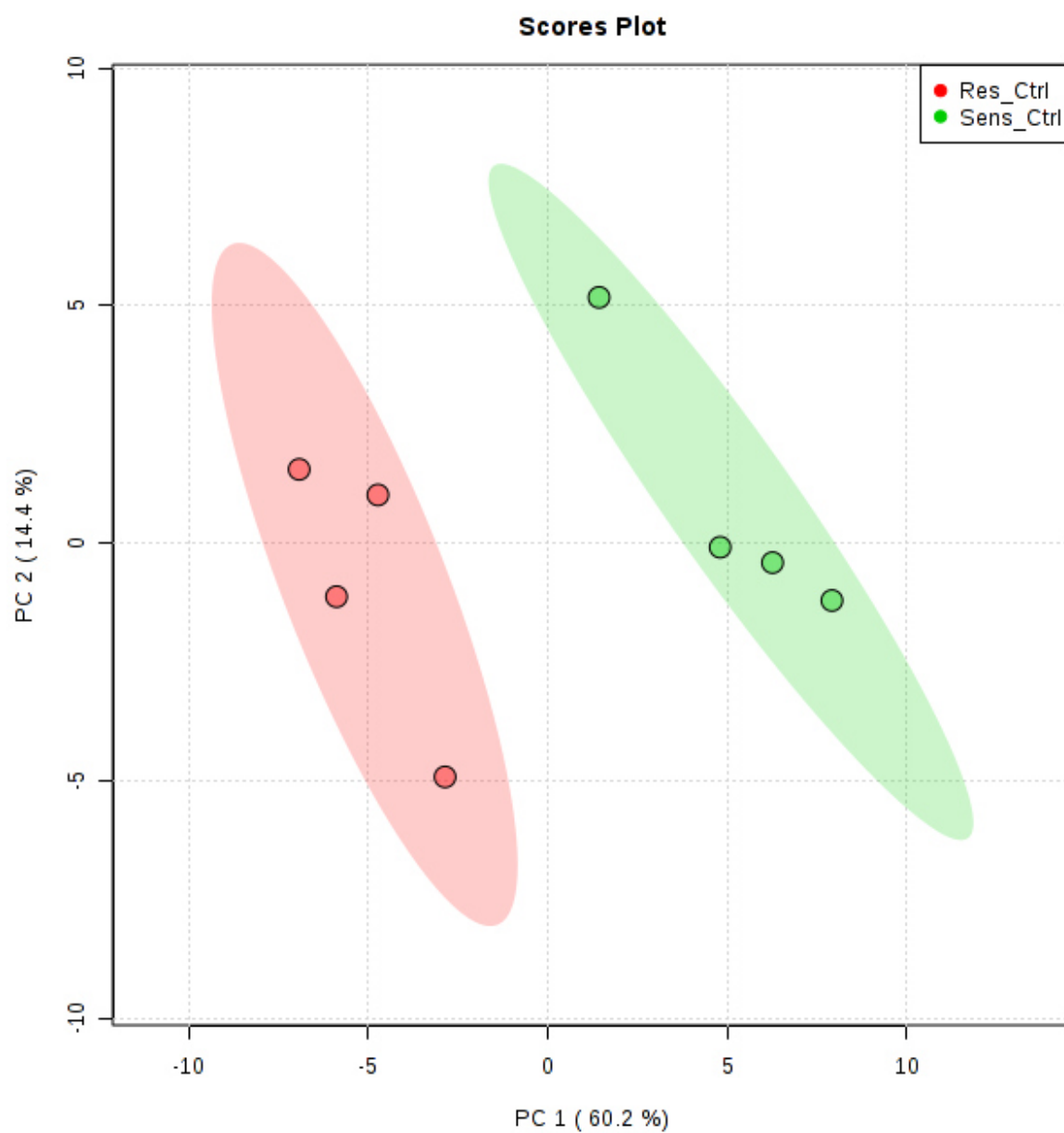


Figure S7 Principal component analysis of sensitive HCT116 cell control vs resistant HCT116 cell control. Method: UPLC BEH Amide at pH 4 in positive and negative mode combining targeted (internal standardization) and untargeted metabolomics.

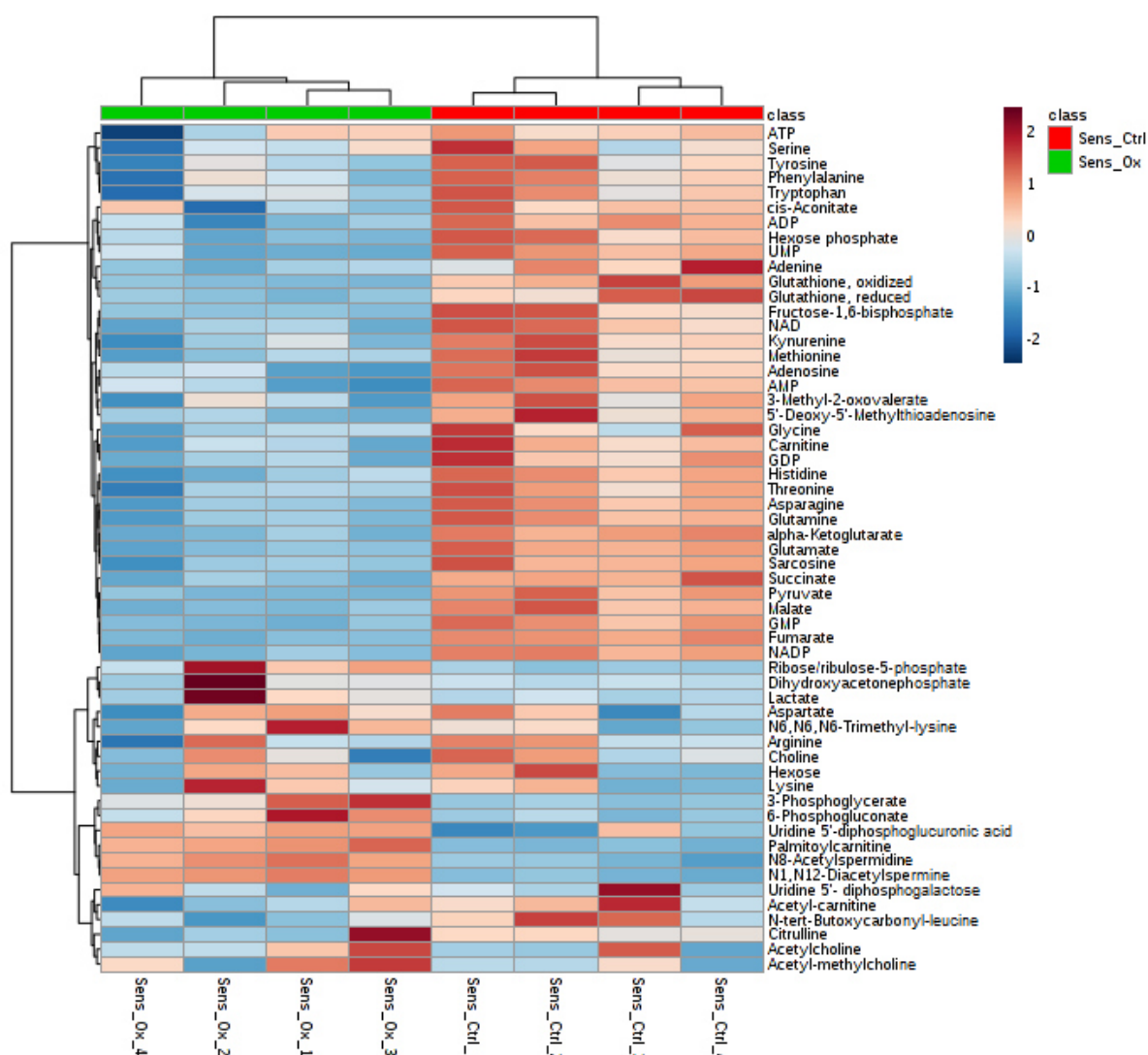


Figure S8 Heatmap of the different compounds found in sensitive HCT116 cell control and in sensitive HCT116 cell incubated with oxaliplatin 5 μ M for 24 h. Method: UPLC BEH Amide at pH 4 in positive and negative mode combining targeted (internal standardization) and untargeted metabolomics.

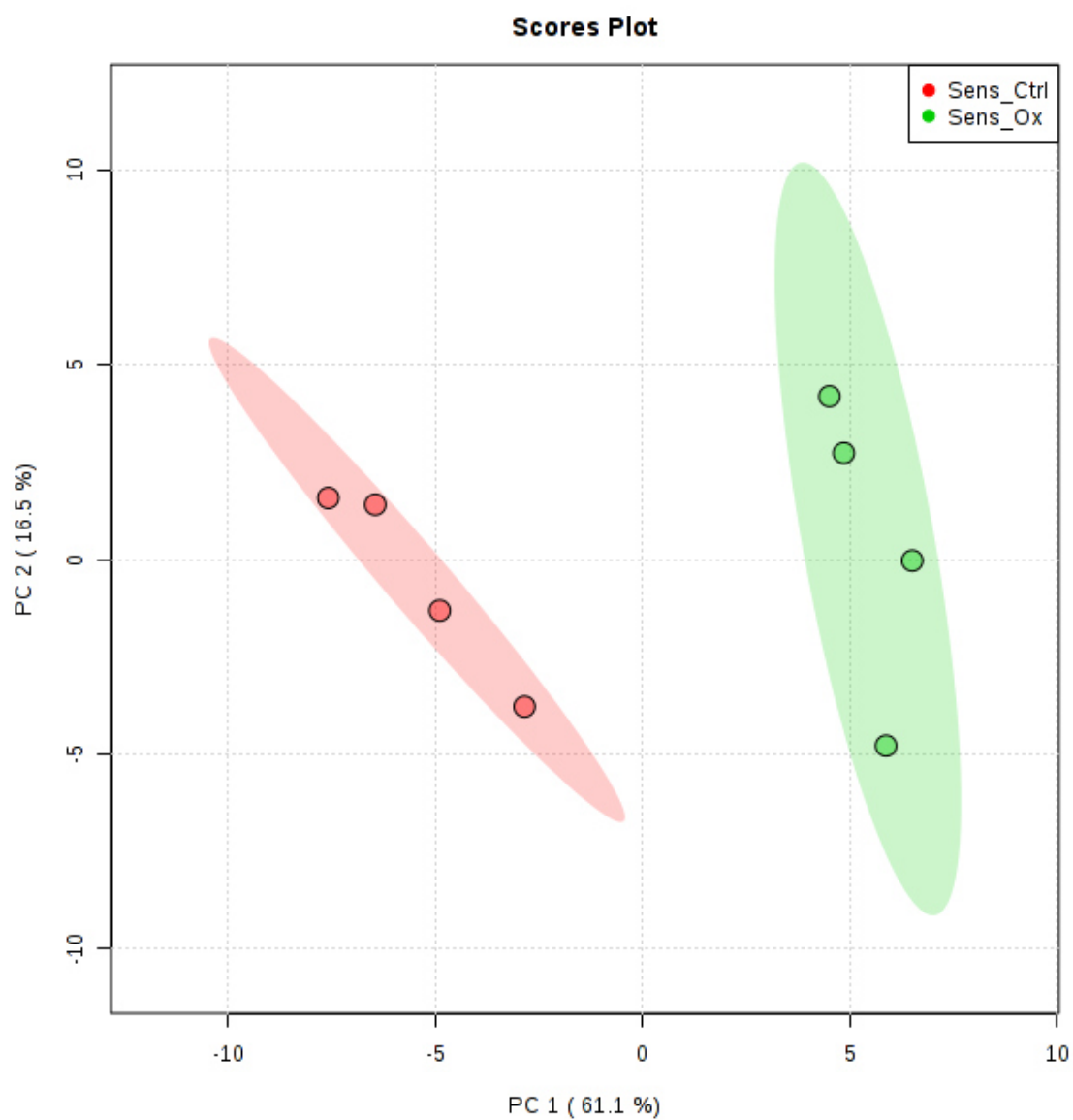


Figure S9 Principal component analysis of sensitive HCT116 cell control vs sensitive HCT116 incubated with oxaliplatin 5 μ M for 24 h. Method: UPLC BEH Amide at pH 4 in positive and negative mode combining targeted (internal standardization) and untargeted metabolomics.

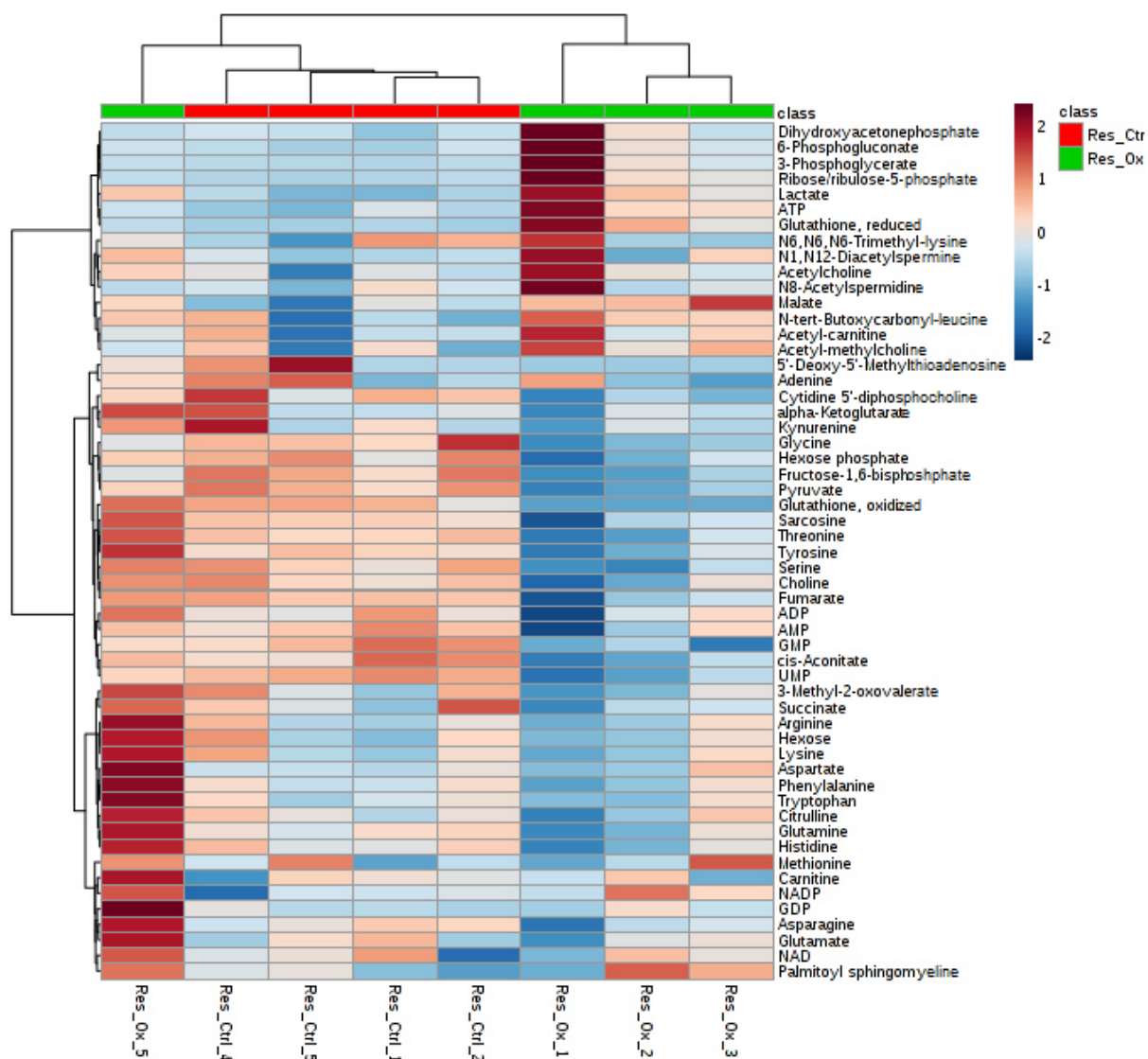


Figure S10 Heatmap of the different compounds found in resistant HCT116 cell control and in resistant HCT116 cell incubated with oxaliplatin 5 μ M for 24 h. Method: UPLC BEH Amide at pH 4 in positive and negative mode combining targeted (internal standardization) and untargeted metabolomics.

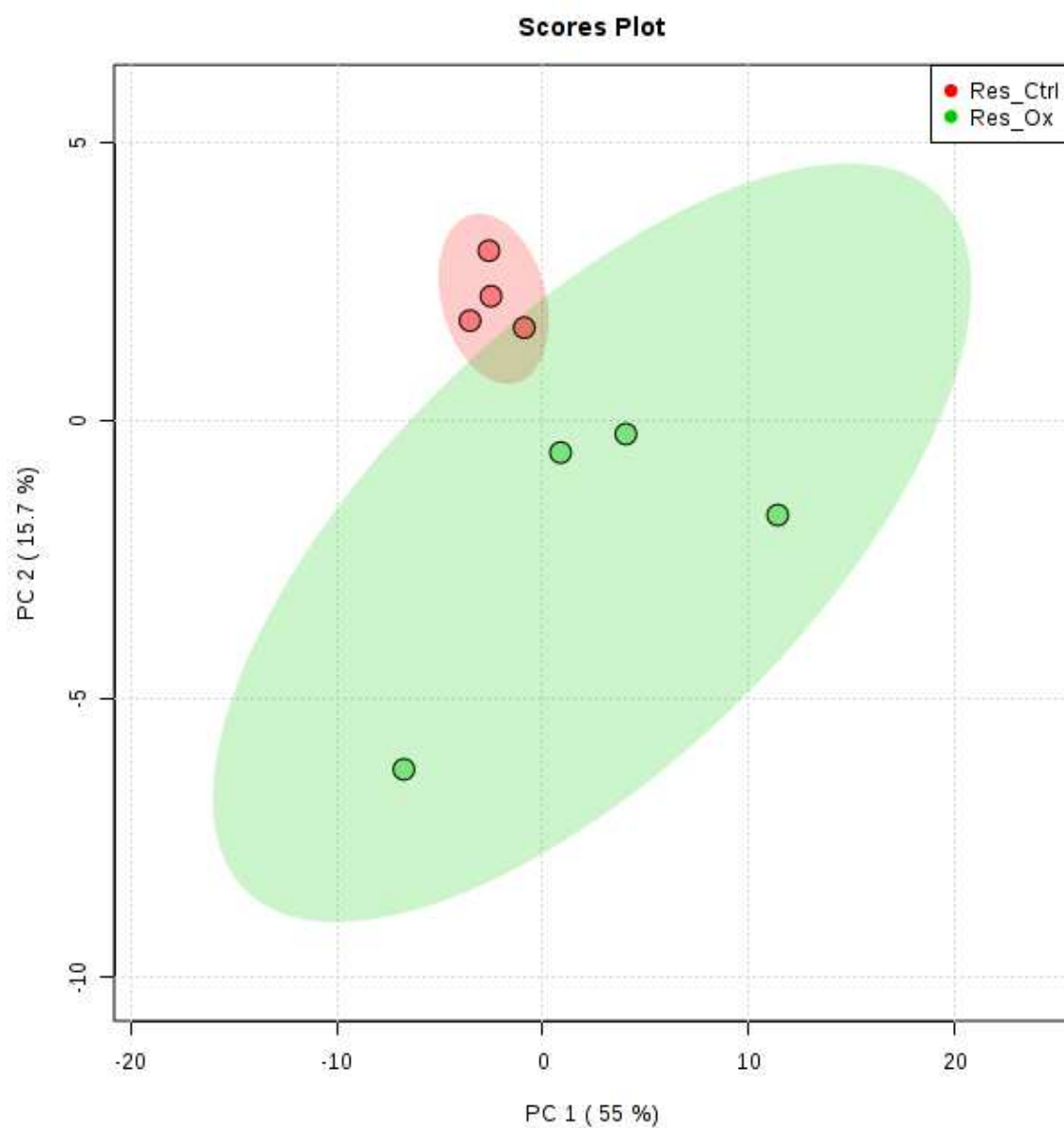


Figure S11 Principal component analysis of resistant HCT116 cell control vs resistant HCT116 incubated with oxaliplatin 5 μ M for 24 h. Method: UPLC BEH Amide at pH 4 in positive and negative mode combining targeted (internal standardization) and untargeted metabolomics.

Table S9 Pearson correlation of the different metabolites and their associated pathways with the incubation time (4, 8, 24 h) in targeted metabolomics. Samples: sensitive HCT116 cell control and sensitive HCT116 cell incubated with 5 μ M oxaliplatin. Method: UPLC BEH Amide at pH 4 in positive and negative mode with internal standardization and protein normalization.

Metabolite	Sens Ctrl	Sens Oxali	Pathway
NADP+	0.912	0.953	Glutathione metabolism. Nicotinate and nicotinamide metabolism
Glutathione, oxidized	0.810	0.778	Glutathione metabolism
Glutathione, reduced	-0.457	-0.921	Cysteine and methionine metabolism. Glutathione metabolism.
Methionine	-0.971	-0.935	Cysteine and methionine metabolism
Hexose phosphate	0.466	0.438	Glycolysis / Gluconeogenesis. Pentose phosphate pathway
Hexose	-0.900	-0.395	Glycolysis / Gluconeogenesis. Pentose phosphate pathway
Lactate	-0.819	-0.829	Glycolysis / Gluconeogenesis
Dihydroxyacetonephosphate	0.554	-0.502	Glycolysis / Gluconeogenesis
2/3-Phosphoglycerate	-0.026	-0.985	glycolysis and calvin cycle
Glutamate	0.957	0.971	Arginine biosynthesis. Alanine, aspartate and glutamate metabolism. Arginine and proline metabolism.
Glutamine	0.218	-0.230	Arginine biosynthesis. Alanine, aspartate and glutamate metabolism. Purine metabolism. Pyrimidine metabolism. D-Glutamine and D-glutamate metabolism.
Alanine	0.752	0.872	Alanine, aspartate and glutamate metabolism
Citrulline	0.926	0.344	Arginine biosynthesis
Arginine	-0.849	-0.518	Arginine and proline metabolism
Fructose-1,6-bisphosphate	0.778	-0.743	Phenylalanine, tyrosine and tryptophan biosynthesis
Phenylalanine	-0.869	-0.733	Phenylalanine, tyrosine and tryptophan biosynthesis. Phenylalanine metabolism
Tyrosine	-0.868	-0.485	Phenylalanine, tyrosine and tryptophan biosynthesis. Tyrosine metabolism
Threonine	0.921	0.973	Glycine, serine and threonine metabolism. Valine, leucine and isoleucine biosynthesis
Serine	-0.019	-0.440	Glycine, serine and threonine metabolism. Cysteine and methionine metabolism
Tryptophan	-0.829	-0.748	Glycine, serine and threonine metabolism. Tryptophan metabolism
NAD+	0.975	0.900	Oxidative phosphorylation
Carnitine	0.935	0.788	Thermogenesis
Choline	-0.962	-0.982	lycine, serine and threonine metabolism
Lysine	-0.889	-0.538	Lysine biosynthesis
Histidine	-0.785	-0.254	Histidine metabolism
Ribose-/ribose-5-phosphate	-0.916	-0.968	Pentose phosphate pathway. Purine metabolism
Glycine	0.966	0.948	Purine and pyrimidine metabolism
Adenine	0.677	-0.971	Purine and pyrimidine metabolism
GMP	0.243	-0.998	Purine and pyrimidine metabolism
GDP	0.922	0.999	Purine and pyrimidine metabolism
ADP	0.989	1.000	Purine and pyrimidine metabolism
AMP	0.845	0.982	Purine and pyrimidine metabolism

ATP	0.996	1.000	Purine and pyrimidine metabolism
alpha-Ketoglutarate	0.711	1.000	TCA cycle
Fumarate	0.864	0.790	TCA cycle
Malate	0.735	0.990	TCA cycle
Pyruvate	0.826	-0.226	TCA cycle. Glycolysis / Gluconeogenesis.
Succinate	-0.558	-0.998	TCA cycle. Oxidative phosphorylation
3-Methyl-2-oxovaleric acid	0.310	0.990	Valine, leucine and isoleucine biosynthesis/degradation
Asparagine	0.711	0.896	Alanine, aspartate and glutamate metabolism
Aspartate	0.992	0.995	Alanine, aspartate and glutamate metabolism. Glycine, serine and threonine metabolism

Table S10 Pearson correlation of the different metabolites and their associated pathways with the incubation time (4, 8, 24h) in untargeted metabolomics. Samples: sensitive HCT116 cell control and sensitive HCT116 cell incubated with 5 μ M oxaliplatin. Method: UPLC BEH Amide at pH 4 in positive and negative mode with protein normalization.

Metabolite	Sens Ctrl	Sens Oxali	Pathway
N-(tert-Butoxycarbonyl)-L-leucine	0.662	0.811	N/A
N1,N12-Diacetylspermine	0.692	0.675	N/A
N8-Acetylspermidine	0.704	0.741	N/A
Palmitoylcarnitine	-0.949	0.820	Fatty acid degradation. Fatty acid metabolism

Table S11 Pearson correlation of the different metabolites and their associated pathways with the incubation time (4, 8, 24 h) in targeted metabolomics. Samples: resistant HCT116 cell control and resistant HCT116 cell incubated with 5 μ M oxaliplatin. Method: UPLC BEH Amide at pH 4 in positive and negative mode with internal standardization and protein normalization.

Metabolite	Res Ctrl	Res Oxali	Pathway
NADP+	0.999	0.990	Glutathione metabolism. Nicotinate and nicotinamide metabolism
Glutathione, oxidized	0.983	0.976	Glutathione metabolism
Glutathione, reduced	-0.983	0.924	Cysteine and methionine metabolism. Glutathione metabolism.
Methionine	-0.116	-0.612	Cysteine and methionine metabolism
Hexose phosphate	-0.742	0.062	Glycolysis / Gluconeogenesis
Hexose	-0.900	-0.395	Glycolysis / Gluconeogenesis. Pentose phosphate pathway
Lactate	-0.874	-0.934	Glycolysis / Gluconeogenesis
Dihydroxyacetonephosphate	0.614	-0.057	Glycolysis / Gluconeogenesis
2/3-Phosphoglycerate	-0.427	-0.935	glycolysis and calvin cycle

Glutamate	0.997	0.999	Arginine biosynthesis. Alanine, aspartate and glutamate metabolism. Arginine and proline metabolism.
Glutamine	-0.107	-0.142	Arginine biosynthesis. Alanine, aspartate and glutamate metabolism. Purine metabolism. Pyrimidine metabolism. D-Glutamine and D-glutamate metabolism.
Alanine	0.981	0.983	Alanine, aspartate and glutamate metabolism
Citrulline	-0.535	-0.310	Arginine biosynthesis
Arginine	-0.820	-0.760	Arginine and proline metabolism
Fructose-1,6-bisphosphate	0.867	0.988	Phenylalanine, tyrosine and tryptophan biosynthesis
Phenylalanine	-0.497	-0.320	Phenylalanine, tyrosine and tryptophan biosynthesis. Phenylalanine metabolism
Tyrosine	0.031	-0.228	Phenylalanine, tyrosine and tryptophan biosynthesis. Tyrosine metabolism
Threonine	0.013	0.089	Glycine, serine and threonine metabolism. Valine, leucine and isoleucine biosynthesis
Serine	-0.681	-0.681	Glycine, serine and threonine metabolism. Cysteine and methionine metabolism
Tryptophan	-0.544	-0.368	Glycine, serine and threonine metabolism. Tryptophan metabolism
NAD+	0.996	1.000	Oxidative phosphorylation
Carnitine	0.991	0.989	Thermogenesis
Choline	0.979	-0.829	lycine, serine and threonine metabolism
Lysine	-0.825	-0.776	Lysine biosynthesis
Histidine	-0.433	-0.287	Histidine metabolism
Ribose-/ribulose-5-phosphate	-0.544	-0.754	Pentose phosphate pathway. Purine metabolism
Glycine	0.989	1.000	Purine and pyrimidine metabolism
Adenine	-0.629	0.450	Purine and pyrimidine metabolism
GMP	0.803	0.628	Purine and pyrimidine metabolism
GDP	1.000	0.993	Purine and pyrimidine metabolism
ADP	1.000	1.000	Purine and pyrimidine metabolism
AMP	0.985	0.966	Purine and pyrimidine metabolism
ATP	0.997	0.984	Purine and pyrimidine metabolism
alpha-Ketoglutarate	0.999	0.945	TCA cycle
Fumarate	0.999	0.993	TCA cycle
Malate	0.997	0.997	TCA cycle
Pyruvate	-0.041	0.934	TCA cycle. Glycolysis / Gluconeogenesis.
Succinate	-0.748	-0.949	TCA cycle. Oxidative phosphorylation
3-Methyl-2-oxovaleric acid	-0.654	0.777	Valine, leucine and isoleucine biosynthesis/degradation
Asparagine	0.726	0.615	Alanine, aspartate and glutamate metabolism
Aspartate	0.548	0.883	Alanine, aspartate and glutamate metabolism. Glycine, serine and threonine metabolism

Table S12 Pearson correlation of the different metabolites and their associated pathways with the incubation time (4, 8, 24 h) in untargeted metabolomics. Samples: resistant HCT116 cell control and resistant HCT116 cell incubated with 5 μ M oxaliplatin. Method: UPLC BEH Amide at pH 4 in positive and negative mode with protein normalization.

Metabolite	Res Ctrl	Res Oxali	Pathway
N-(tert-Butoxycarbonyl)-L-leucine	0.969	0.990	N/A
N1,N12-Diacetylspermine	-0.999	-0.690	N/A
N8-Acetylspermidine	0.208	0.665	N/A
Palmitoylcarnitine	-0.996	-0.920	Fatty acid degradation. Fatty acid metabolism

4 CONCLUSIONS AND OUTLOOK

Nowadays different analytical techniques are applied to preclinical metallodrug research. However, depending on the employed technique, the results may differ. Especially, the off-line fractionation/extraction techniques are prone to error needing of more automatic techniques to minimize the sample preparation. The relatively new UHPLC SEC technique with sub-2- μm BEH particles have shown to be the best approach for this purpose. Nevertheless, there is still space for improvement or for development of new approaches since the resolution in UHPLC SEC is still low for the quantification of intact metallodrug.

The combination of metallomics with other -omics has shown to be important in preclinical metallodrug research to get a better understanding of the mechanism of action of the metallodrug. Whereas the combination with proteomics and especially with transcriptomics has been extensively investigated, the combinatorial studies with metabolomics are scarce. This could be explained by the numerous challenges encountered in metabolomics to adapt an analytical method (quenching, extraction, LC separation...) due to the high diversity of the metabolome. However, the interest in the combination of metallomics with metabolomics is currently growing since metabolomics is directly connected to the biochemical activity (genetic and environmental changes) allowing the investigation of the effect of the metallodrug in these different biochemical pathways. This ultimately can provide valuable information about the efficacy and mechanism of action of the metallodrug as well as its acquired and intrinsic resistance.

Nevertheless, as mentioned before, numerous challenges are observed in metabolomics due to the high diversity in the metabolome. One of these challenges is the specific ionization of each metabolite in ESI-MS needing of specific standards. Due to the impossibility of synthesizing and acquiring hundreds of specific standards for quantitative and semi-quantitative metabolomics, the use of bioreactors to obtain isotopically labeled metabolites has shown to be the solution to overcome this inconvenience. Yet, the application of these internal standards and method validation is still in development for untargeted metabolomics. In untargeted metabolomics as many metabolites as possible are analyzed getting thousands of features from the different metabolites, from the formation of artefacts during sample preparation and from bioinformatics errors making this method validation still difficult. It was recently shown that the application of these internal standards can help in untargeted metabolomics (credentialing).

Further contributions to peer-reviewed publications

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Contributions to conferences, forums and symposiums

Poster: Galvez L., Hermann G., Kowol C.R., Heffeter P., Berger W., Keppler B.K., Hann S., Koellensperger G. Quantitative assays of oxaliplatin in preclinical cancer research. EWCPs 2015, February 22-26, Muenster, Germany.

Talk: Galvez L., Theiner S., Kowol C.R., Keppler B.K., Hann S. Koellensperger G. Platinum speciation analysis addressing metallodrug-protein interaction in preclinical anticancer research. 12th ASAC-JunganalytikerInnen Forum, June 10-11, 2016, Graz, Austria.

Poster: Galvez L., Hermann G., Kowol C.R., Heffeter P., Berger W., Mayr J., Galanski M., Keppler B.K., Hann S., Koellensperger G. Measuring metallodrug protein association on a quantitative basis - a comparative study on different techniques. EuroBic 13, August 28-September 21, 2016, Budapest, Hungary.

Poster: Galvez L., Hermann G., Kowol C.R., Heffeter P., Berger W., Mayr J., Galanski M., Keppler B.K., Hann S., Koellensperger G. In vitro investigations on oxaliplatin – a comparison study of resistant and sensitive cells. EWCPs 2017, February 19-24, Sankt Anton, Austria.

Talk: Galvez L., Rusz M., Schwaiger M., Jakupec M. and Koellensperger G. In vitro investigations on oxaliplatin – a comparison study of resistant and sensitive cells. 6th International Symposium on Metallomics August 14-17, 2017, Vienna, Austria.

Talk: Galvez L., Rusz M., Schwaiger M., Jakupec M. and Koellensperger G. In vitro investigations on oxaliplatin – a comparison study of resistant and sensitive cells. 2018 Winter conference on plasma spectrochemistry, January 8-13, Amelia Island, Florida, USA.

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