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„Applying mass spectrometry to cell culture models to gain novel insight into molecular processes of cancer pathophysiology and (metallo-)drug action“

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1 Abstract

Mass spectrometry-based omics techniques detect and quantify an unprecedented number of biomolecules (e.g. lipids, proteins), which enables the comprehensive investigation of cellular systems. This combination of *in vitro* cell culture and mass spectrometry has proven to be a valuable tool to gain insight into molecular mechanisms of disease processes and drug action.

Model systems have contributed enormously to our understanding of biological processes. The possibility to study diseases or drug actions *in vitro* facilitates the investigation. But the simplification that allows the understanding in the first place sometimes exacerbates a holistic comprehension. The analysis of a melanoma brain metastasis model was able to illustrate the high flexibility of metastasis formation. The model consists of four human melanoma cell lines which were transformed in a xenograft to create a cutaneous (C) and brain metastasis (CB) variant. The CB cell lines show stable and reproducible phenotypes, namely the selective formation of brain metastasis in mice. While the observed *in vivo* behaviour is preserved over several *in vitro* passages a molecular commonality could not be observed. An in-depth proteome analysis identified known metastasis pathways for each cell pair but was not able to reveal a common mechanism for all four cell pairs. In a further study, it was possible to show a so far unappreciated influence of minor differences of eicosanoids naturally occurring in fetal calf serum, a routinely used cell culture additive. Discrepancies in nanomolar range of eicosanoids account for significant differences in the inflammatory activation of a human macrophage model.

Additional to the study of diseases the investigation of drug actions gives valuable insight for therapeutic strategies and allows optimal utilisation of the drug. Metal-based drugs are promising treatments for a variety of cancers, but their molecular targets remain elusive in most cases. However, latest studies have shown that these complexes can indeed exhibit high target specificity and interfere with specific biological mechanisms. Here, I demonstrate the possibility to unravel parts of a drug mode of action induced by the treatment with the ruthenium-based drug BOLD-100 (formerly known as KP-1339). This study illustrates how the combination of a proteomic target-response profiling, including chemical proteomics, was able to identify potential targets of the drug which could be related to earlier works. Integrating transcriptomic analysis and mass spectrometry-based imaging techniques it was possible to propose a mechanism of action accounting for its observed *in vivo* efficacy.

This thesis demonstrates how the combination of proteomics with other analytical methods like metabolomics and transcriptomics can be utilised to gain novel insight about the pathophysiology of cancer and investigate (metallo-)drug action.

2 Zusammenfassung (German abstract)

Die hohe Zahl an Biomolekülen (e.g. Lipide, Proteine) die mit Massenspektrometrie basierten Methoden detektiert und quantifiziert werden können, ermöglichen eine umfassende Untersuchung von zellulären Systemen. Vor allem die Kombination aus *in vitro* Zellkultur mit Massenspektrometrie hat sich als nützlich erwiesen, Einblicke in molekulare Mechanismen von Krankheiten und Medikamententherapien zu geben. Die Möglichkeit eben diese biologischen Prozesse an *in vitro* Modellsystemen zu untersuchen, erleichtert die Forschung enorm. Die Vereinfachung, die damit einher geht und die Untersuchung erst ermöglicht, kann aber auch ein ganzheitliches Verständnis der Mechanismen erschweren.

Die Analyse eines Modells für Gehirnmastasen, entstehend aus Melanomen, konnte die hohe Flexibilität des Mastasierungsprozesses verdeutlichen. Das Modell besteht aus vier humanen Melanomzelllinien, welche in einem Xenograft transformiert wurden, um eine kutane (C) und gehirnmastasen (CB) Variante zu erzeugen. In weiteren Untersuchungen in Mausmodellen zeigten die CB Zelllinien die selektive Bildung von Gehirnmastasen als reproduzierbaren, stabilen Phänotyp. Diese *in vivo* Beschaffenheit wurde über mehrere *in vitro* Passagen beobachtet, ihr konnte aber kein gemeinsamer molekularer Prozess zugeordnet werden. In der Proteom Analyse konnten mehrere, gut bekannte Mastasierungsmechanismen den Zellpaaren zugeordnet werden. Kein Mechanismus wurde in allen vier Paaren beobachtet. In einer weiteren Studie war es möglich, den bislang unbeachteten Einfluss von kleinen Unterschieden des Eikosanoidgehalts in fetalem Kälberserum, einem weit verbreiteten Zellkulturzusatz, aufzuzeigen. Signifikante Änderungen der Proteinexpression einer entzündlichen Stimulation konnten auf nanomolare Unterschiede der Eikosanoide zurückgeführt werden.

Die Untersuchung der molekularen Wirkung von Medikamenten kann wichtige Indizien zur korrekten Applikation einer Therapie geben. Metallbasierte Therapeutika sind vielversprechende Behandlungen für eine Vielzahl von Krebserkrankungen, ihre molekularen Ziele sind aber oftmals schwer fassbar. Neueste Studien zeigen aber, dass diese Komplexe sehr oft eine hohe Spezifität aufweisen. In dieser Arbeit demonstriere ich die Möglichkeit, Teile eines molekularen Wirkmechanismus der Ruthenium-Verbindung BOLD-100 (vormals bekannt als KP-1339) aufzudecken. Die Studie zeigt, wie mit der Kombination aus Target-Response Profiling potentielle Zielmoleküle identifiziert werden konnten. Durch die Ergänzung mit Transkriptomik und Massenspektrometrie basierten, bildgebenden Techniken war es möglich, einen molekularen Mechanismus für die Wirkung des Medikaments vorzuschlagen.

Diese Arbeit demonstriert, wie die Kombination von Proteomik mit anderen analytischen Methoden wie Metabolomik und Transkriptomik verwendet werden kann, um neue Erkenntnisse über die Pathophysiologie von Krebs und die Wirkung von neuartigen metallbasierten Therapeutika zu erhalten.

3 Introduction and theoretical background

Multi-omics aims to integrate different omics levels. The analysis of the genome, transcriptome, proteome, metabolome or any other holistic analysis of biological samples termed *-ome* are combined to create novel insights. The acquisition and especially data interpretation of omics datasets requires thorough understanding of the analytical techniques, biological pathways and the chemistry of the investigated molecules to avoid misinterpretation. Therefore, it is not surprising, that a multi-omics analysis combining proteomics, lipidomics and metabolomics is still rarely seen today. This thesis aims to investigate the implications that subsequent (omics) analysis has on proteome profiling studies and how these multi-omics combinations enhance the exploration of molecular mechanisms related to cancer pathophysiology and therapeutic approaches.

3.1 Omics

The term omics describes the analysis of the entirety of a class of biological molecules, termed with the suffix '*-ome*'. The first technique developed was genomics which, following the given description strives to analyse the complete genome of a species, tumour or cell. While the analysis of the first complete human DNA took several collaborating groups and months of research,^[1] new generation sequencing methods enable such analysis within hours and is available as commercial services.^[2] The DNA is built up by only 4 different components, the nucleobases, which have very similar chemical. Furthermore, DNA does not change its quantity very much during a cells lifetime but is always limited to one single molecule except during mitosis which doubles the amount. These biochemical properties are quite favourable when it comes to analysis and made the investigation of the genome the consequential first step in the development of omics techniques. Figure 1 illustrates the classical biological dogma where DNA is an organism's functional blueprint that gets transcribed into RNA which is subsequently translated into proteins. The proteins are the main effectors that catalyse reactions or transport metabolites. The increasing quantity and chemical complexity of the biomolecules affected the development of the respective omics techniques in a similar way as demonstrated in Figure 1.

Studying the DNA sequence will give insight into possible mutations but will only give limited information on a cell's differentiation state or its capability to react to stress stimuli. Modifications present on the DNA like methylation or acetylation on the other hand can give information on active and silenced genes. The analysis of these modifications is referred to as epigenomics and can give an idea about the cells possibility to react to certain triggers.

The analysis of transcribed RNA molecules is termed as transcriptomics and offers the information on which genes are actually transcribed in a living cell. The development of transcriptomics was much more difficult although the chemical background of RNA is similar the DNA. The biggest issue that had to be overcome was the much higher dynamic range. RNA

molecules are in quantities of up to six orders of magnitude and can change significantly within hours.^[3]

Since mRNA is the template for protein synthesis the transcriptome already gives insight into proteins that can be present in a cell. Due to the high dynamic that comes with the constant synthesis and degradation of the proteins the comparisons between mRNA and proteins show low abundance correlations of around 40%.^[4,5] The exact correlation however, strongly depends on the investigated genes, tissue and species. Furthermore, a proteins' activity is also strongly influenced by its post-translational modifications which cannot be anticipated from the RNA and therefore led to the inevitable investigation of proteomics and further post-genomic omics techniques.

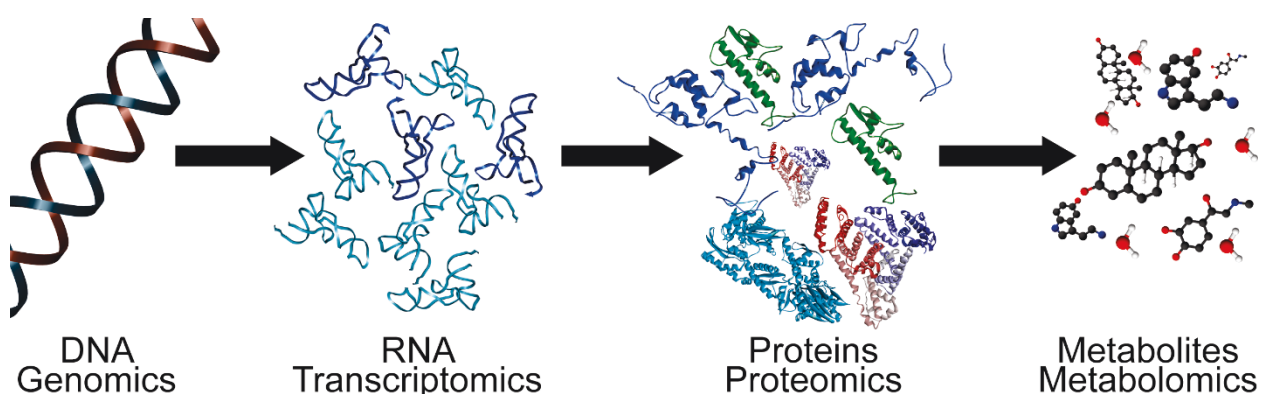


Figure 1: Typical biological dogma and the respective omics analysis methods. The DNA serves as blueprint for cell mechanisms and is transcribed into RNA. Through translation mRNA is used as template for protein synthesis which are effectors in the cell. Metabolites are imported and exported, degraded for energy generation or synthesised via enzymes. The illustrated direction is certainly not a one-way process and all molecules are influencing each other's activity. The increasing chemical complexity influenced the development of the respective omics methods.

3.1.1 Proteomics

The involvement of proteins in all biological processes made the analysis of these biomolecules of special interest. From synthesis of other proteins in the ribosomes, over transport in the cell and through membranes, to the transduction of signalling cascades proteins are involved in virtually all functional aspects of cellular life. The Edman-degradation, first released in 1949, was among the first analytical methods to investigate the amino acid sequence of proteins. In this technique the proteins are N-terminally labelled and with the help of an endopeptidase the amino acids are cleaved one by one.^[6] This method is quite laborious but set the foundation for modern analysis methods since it allowed the determination of the unique sequence of proteins. Thirty years later the invention of Western Blotting as modification from the Southern Blotting used for DNA again revolutionised the field and made protein analysis faster, easier and accessible for all laboratories.^[7-9] The Western Blot remains important for protein identification and quantification in biological samples in molecular biology approaches. The method relies on the interaction of target

proteins with a specific antibody that is either directly or indirectly fluorescently labelled. Protein mixtures are separated according to their molecular weight using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).^[10] The proteins' own charge is covered with SDS which distributes an equal negative charge around each protein with about 1.4 g of SDS per 1 g protein.^[11] The denatured and negatively charged proteins are loaded onto a polyacrylamide gel and a constant electric field forces the proteins to migrate into the gel. The proteins with lower molecular weight will move faster through the gel and separation takes place. Subsequently they are transferred to a nitrocellulose paper where they are incubated with an antibody specific for a certain protein or modification. A secondary antibody specific against the primary antibody is linked to a fluorescent dye which ultimately allows an assessment of the proteins' abundance. Since every protein requires a specific antibody the investigation of several molecules is quite laborious and expensive. Also, it has been shown that up to 50% of commercially available antibodies do not yield satisfactory results and are strongly dependent on the matrix and context they are used in.^[12]

Label-free proteomics methods

The theoretical foundation for shotgun proteomics was first described by Mann *et al.*^[13,14] in the early 1990s and has since evolved into a high throughput method. The method is based on the measurement of fragments of proteins and peptides and the comparison of their sequence to databases. To identify a protein with bottom-up proteomics the first step is the controlled fragmentation of the protein into smaller peptides using peptidases. Trypsin is an endopeptidase which hydrolyses a peptide bonding specifically after basic amino acids lysine and arginine and is the most widely used enzyme for proteomics.^[15] Additionally to the digestion of the protein it is possible to predict all peptides that will be formed. This process is called *in-silico* digestion and calculates all possible peptides and their exact masses from the sequence of the protein. This information can then be integrated to identify a protein by means of their measured mass spectra. The first usage of this principle was the so-called peptide mass fingerprinting (PMF) in which the proteins are digested and the masses of the resulting peptides measured by mass spectrometry. This method is limited to protein mixtures of relatively low complexity. An additional separation dimension is required for biological samples since their proteome tends to be more complex. Two-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (2D-SDS-PAGE) is used to separate proteins of samples and allow identification of proteins in complex mixtures such as cell lysates. The proteins are separated according to their isoelectric point before further separation according to molecular weight. They can be visualised by staining them with AgNO₃. Single spots of interest are cut out of the gel, digested and identified using the above mentioned PMF (Figure 2).^[16]

Separation according to isoelectric point (pH gradient)

Separation according to molecular weight (SDS-PAGE)



Figure 2: Two-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (2D-SDS-PAGE). The first dimension (horizontal) separates the proteins according to their isoelectric point using a pH gradient. Afterward proteins are separated according to their molecular mass. Proteins are visualised through silver staining and spots can be cut out and analysed used PMF analysis.

The identification of peptides from tryptic digests was then further enhanced by the implementation of tandem-mass spectrometry (MS/MS, MS2). Peptides are collided with neutral gas molecules with specific energies and this collision-induced dissociation (CID) shows very distinct fragmentation pattern. Figure 3 illustrates the most probable cleavage points of the protein backbone using different fragmentation techniques. The fragments with the N-terminal end are termed a, b and c while the C-terminal fragments are named x, y and z. B and y fragments are obtained by cleavage of the peptide amide bond which is normally formed in the protein biosynthesis and is mostly used for the identification in the context of proteomics. Figure 4 shows a MS2 spectrum for a unique peptide of human serum albumin recorded with a Q Exactive orbitrap. The y-fragments of the peptide are shown and correlated to the measured m/z values and almost show the complete sequence. Additional peaks represent modified b- or y-fragments which have lost H_2O (-18 Th) or NH_3 (-17 Th) during the fragmentation process. This (partial) determination of the sequence makes it possible to differentiate between two peptides with the same amino acids but different sequence. Therefore, a peptide can be exactly determined and assigned to a given protein sequence. While there are peptide sequences that occur in several proteins the so-called *unique peptides* are specific for one protein or even isoform of a protein.

This enhancement of the identification allowed the determination of proteins in complex mixtures and was the basis for high throughput shotgun proteomics. In contrast to PMF the proteins are not separated before digestion but the complex biological sample is digested and the resulting peptides are separated using liquid chromatography. Schirmer *et al.* introduced Multidimensional Protein Identification Technology (MudPIT) in 2003 using a 2-dimensional LC before MS analysis to identify up to 2.000 proteins within a single run.^[17] The high separation performance of modern HPLC systems and the speed that MS are able to create high quality MS2 spectra makes it today possible to identify and quantify up to 6.000 proteins within a single run of two hours.^[18]

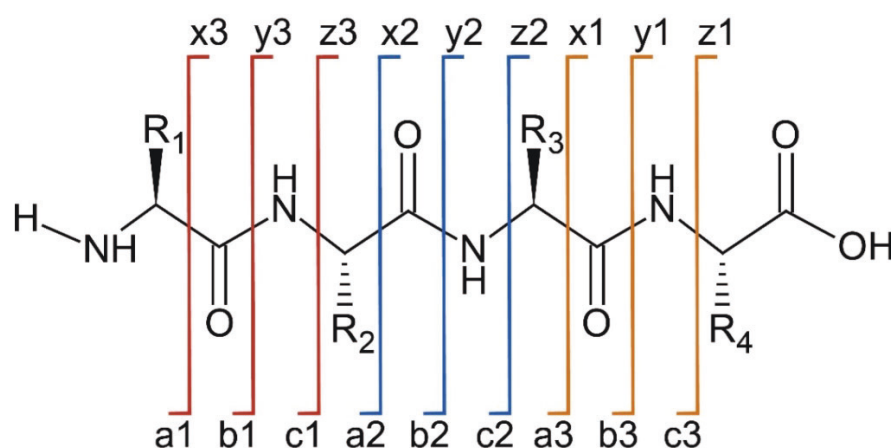


Figure 3: Possible fragmentation sites of hypothetical peptide consisting of 4 amino acids. a-x, b-y and c-z lines represent typical breaking points used in peptide fragmentation. b- and y-chains cleave the peptide amide bond and are most commonly used for peptide identification. a, b and c are the fragments named occurring at the N-terminal end of the peptide with the numbers rising with the number of included amino acids. x, y and z are the corresponding fragments on the C-terminal end of the peptide. b1 occurs together with y[n], where [n] is the number of total amino acids of the peptide. The counterpart to b2 is y[n-1] etc.

Labelling methods for proteomics analysis

Label-free quantification (LFQ) describes methods that quantify proteins without labelling them first like PMF or shotgun proteomics. These are most used for proteomics experiments since they require no additional sample preparation or high-resolution MS². Also, the number of comparable samples is basically unlimited. But the consecutive measurements are very time intensive which is challenging for very large sample cohorts or high throughput applications. Using labelling methods can strongly increase these drawbacks and further enhance the analysis.^[19]

Stable isotopes are a great way to tag analytes in mass spectrometry since the mass difference can easily be detected while keeping the chemical properties (e.g. retention time) unaltered. But using stable isotope labelled molecules in living system can change biological processes when they are dependent on quantum mechanics.^[20] For example, enzymatic reactions can be influenced by tunnelling properties which are sometimes altered by different isotopes.^[21]

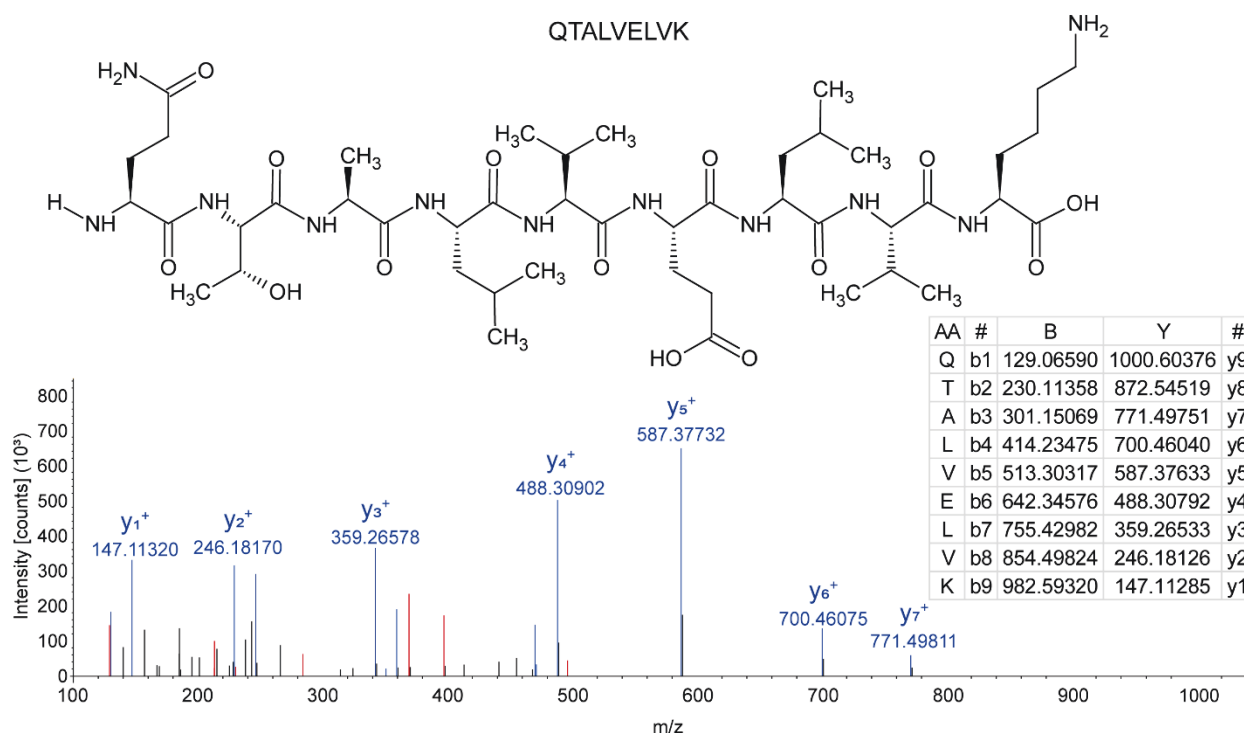


Figure 4: Fragmentation spectrum (MS2) of the 9 amino acid peptide QTALVELVK a unique peptide from human serum albumin (HSA, ALBU), recorded on a Q Exactive Hybrid Quadrupol-Orbitrap mass spectrometer. The y-fragments are correlated to the measured m/z values.

The two most widely used labelling methods in mass spectrometry today are stable isotope labelling by amino acids (SILAC) and tandem mass tags (TMT).^[22] SILAC is used to label proteins in cell culture experiments. Therefore, stable isotope labelled versions of essential amino acids are added to the cell culture medium for several passages.^[23] Deuterated leucine (Leu-D3) is often used for these setups. This assures that almost all leucine in the cell's proteins are exchanged with the stable isotope version. For the actual experiment the cells are again grown with the natural amino acid. This allows to investigate the synthesis and degradation of the proteins. TMT on the other hand is not used in living cells but applied during sample preparation. Tandem mass tags are chemical structures that form covalent bounds with peptides and therefore 'tag' them. The tags' chemical structure is similar but labelled with different stable isotopes, namely C^{13} and N^{15} .^[24,25] The different mass tags are applied to each sample after digestion of the proteins. Finally, the samples are pooled and measured. This allows for much shorter measurement times since multiple samples don't have to be measured consecutively. Since the chemical structure of the tags are similar except for the mass tag, the same peptides will co-elute and only differ in detected mass. Therefore, an accurate relative comparison of the peptides' abundance is achieved. TMT is available in different complexities. Early methods were only able to distinguish between 6 different tags since they all differed with at least 1 Th. The high resolution on MS2 level of modern mass spectrometers allow up to 16-plex systems since they have the power to resolve the small differences of C^{12} - C^{13} and N^{14} - N^{15} , corresponding to $\Delta m = 0.00632$.

Statistical testing for large datasets: FDR and q-value

The big amount of data that is created during a typical proteomics measurement makes it possible to identify thousands of proteins within analysis times. But due to variations and noise of the experimental spectra and uncertainties in the predictions of the theoretical spectra wrong assignment of peptides are possible.^[26] The high number of spectra created during a proteomics analysis (up to 10^5 - 10^6 spectra per experiment) makes a false positive identification even more likely.^[27] To overcome this problem mathematical solutions were developed that greatly decrease false positive identifications while not losing the important identifications (false negatives). Especially, the development of false discovery rate (FDR) calculations by Benjamini and Hochberg in 1995^[28] and its implementation with decoy databases^[29] greatly increased proteomics data quality. According to this concept, a recorded MS2 spectra is matched to a database entry, but also rated against a decoy database. The identification of a peptide is achieved by the comparison of a recorded spectrum with a sequence from a database and creating a so-called peptide-spectrum-match (PSM) and a score is assigned to it. Currently used search algorithms like Mascot^[30] or Andromeda^[31] employ a probability based scoring system. This means that the probability to observe a match between the experimental data and the database is calculated. The score is an arbitrary number that is dependent on the algorithm but a higher score always represents a higher confidence for the identification. Additional to the search against the database, all MS2 spectra are searched against a so-called decoy database. This is a database that consist of amino acid sequences that do not make up actual proteins. It can be created by inverting or shuffling the target database or can be simulated. Matching recorded spectra to both the target and decoy database identifies true and imaginary hits, respectively. While a higher score will be assigned to proteins from the target database, the decoy proteins will be assigned with lower scores. The scores will be distributed in a gaussian manner as illustrated in the schematic in Figures 5. But the distributions will overlap to a certain extent. The FDR gives a measure of the score that is acceptable for a certain threshold of data confidence. An FDR of 0.05 will allow 5% of matched PSMs to be wrongly assigned to a peptide. While the FDR is very important for the identification of peptides and proteins the subsequent statistical analysis of proteomics data has to be corrected for the large number of analytes as well. These statistical tests were originally developed for genomics data but also apply to other omics data sets.^[32] When testing for statistical significance each analyte is tested against a null hypothesis (truly null) and alternative hypothesis. The p-value gives a measure of significance to each of the features for the applied test. The q-value represents a p-value adjusted to fit the purpose of testing multiple variables as they occur in omics analysis. While the false positive rate and the false discovery rate (FDR) are often used synonymously they are substantially different and therefore represented by the p-value and q-value respectively. A false positive rate of 5% means that on average 5% of the truly null features are significant while an FDR of 5% means that among all

significant features on average 5% are truly null. While this difference does not seem striking at first it nevertheless makes the q-value the more appropriate measure for proteomics data since it considers that thousands of analytes are tested simultaneously.

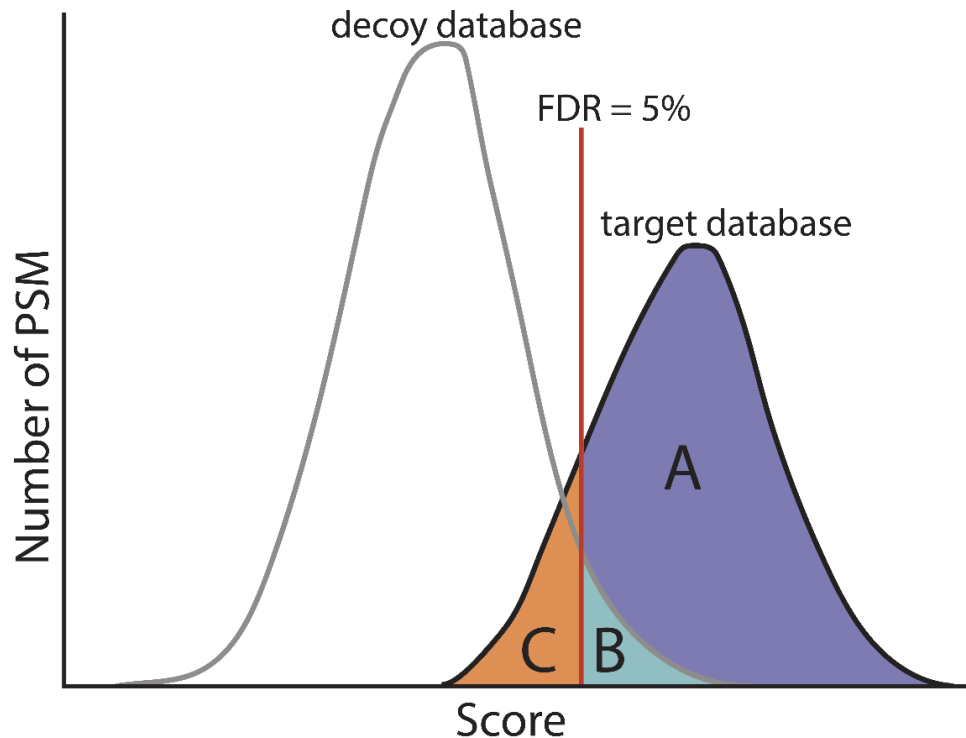


Figure 5: Schematic of peptide spectrum matches (PSMs) plotted against peptide score. The grey line shows the distribution of hits against the decoy database while the black line represents number of assigned peptides for the target database. The red threshold represents the FDR of 5% that is used for the search. The blue area (A) shows the correct positive hits, usually those with the highest scores (95%). Turquoise (B) are the false positive hits, namely the 5% accepted for a certain FDR. Orange (C) are false negative hits that are discarded for the sake of the data quality.

Database for proteins

Since the human (*homo sapiens*) is the main interest of biological sciences and the mouse (*mus musculus*) has long been known to be a very good model system these organisms have the best annotated and most accurate databases. UniProt^[33] is among the most important databases when looking for information about proteins. The entries include the sequences of all known isoforms, post-translational modifications, subcellular location and short descriptions of the functions. Reviewed entries are manually curated from information gathered from literature and many more unreviewed entries are generated automatically. To date, over half a million entries are reviewed and 20.394 human proteins are manually verified. The information is freely accessible and the amino acid sequence can easily be downloaded and used for search algorithms for shotgun proteomics experiments.

Sample preparation for mass spectrometry-based protein analysis

The sample preparation for MS based shotgun protein analysis is basically the same since the very development of the method, but the reagents and exact operating procedure have been optimised over the years. Depending on the analysing matrix it is necessary to remove other biomolecules like lipids, metabolites or DNA/RNA. The denaturation hence precipitation of proteins from a complex mixture is well suitable to enrich the protein fraction. This can be achieved through strong acids or organic solvents like acetonitrile, methanol or cold ethanol.^[34,35] The precipitated proteins are then dissolved in a suitable buffer containing chaotropes (e.g. urea) and detergents to increase the solubility of (hydrophobic) proteins for the subsequent sample preparation.^[36] The following steps represent the optimal conditions for HPLC-MS analysis and consist of reduction, alkylation and digestion. The reduction and alkylation steps ensure that the disulfide bonds naturally formed between cysteine residues are broken up and covered. The protein folding normally stabilised by these disulfide bonds is hence further disrupted and the following digestion enhanced. The reduction is achieved *via* a reducing agent like dithiothreitol (DTT) or Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) and directly followed by the alkylation with a iodoacetamide (IAA) or N-ethylmaleimide (NEM). The digestion is typically performed by trypsin although over the years many alternative enzymes have been used for specific applications.^[15]

The used chemicals for sample preparation are often incompatible with HPLC-MS analysis. Especially detergents are known to cause ion suppression and impair the performance of the chromatographic system and have to be removed.^[37-39] In the sample preparation for PMF the proteins are trapped in the poly-acrylamide gel until they are digested into peptides, which makes a removal of the detergents easy. Digestion of protein mixtures in-solution often requires subsequent clean-up. The introduction of filter-aided sample preparation (FASP) enhanced the workflow since it allowed to wash off any unwanted reagents while the proteins are trapped on a molecular weight filter.^[40] Today proteins are often trapped on functionalised beads before sample preparation which allows for even faster workflows with higher recoveries and less contamination.^[41,42]

Post-translational modifications

Protein profiling typically considers non-modified proteins only. Thus, differential expression of post-translational modifications (PTM) may bias data interpretation. There are a number of different modifications that are characteristic for biological processes. Ubiquitination as signal of degradation, glycosylation for cell-cell recognition or acetylation as regulators of protein-DNA interaction on histones are just some prominent examples which are worth investigating.^[43] Especially phosphorylation is very important when it comes to signal transduction and signalling cascades. Up to 30% of proteins in eukaryotes are phosphorylated.^[44] A phosphorylation is a

covalent binding of a phosphate group to the side chain of mostly serine, threonine or tyrosine. The enzymes catalysing these reactions are kinases and there are over 500 different kinases known in humans.^[45] The presence or absence of a known phosphorylation can give valuable insight into the present state of a biological system or the activity of a kinase. PTMs can be analysed with mass spectrometry since the addition of the modifications significantly alters the mass of the amino acid. Even in shotgun experiments PTMs can be detected if the search parameters are set accordingly. But the abundances of the modified proteins are low compared to the complete proteome which hinders the analysis. To overcome this, several enrichment strategies have been developed that allow a much deeper insight into the phosphoproteome.^[46] Although there are different methods available the same principle applies to all of them. Materials that show high affinity specifically against the phosphate modification are immobilised and bind the phosphopeptide. The negative charge of the phosphorylation shows high affinity to metals like iron (Fe^{3+}) or zirconium (Zr^{4+}) and methods employing these metals are summarised as immobilised metal affinity chromatography (IMAC).^[46] But also other materials like titanium dioxide (TiO_2) are used to enrich the phosphorylated peptides. The specificity for (phosphor-)peptides can vary depending on the employed material.^[47]

3.1.2 Metabolomics

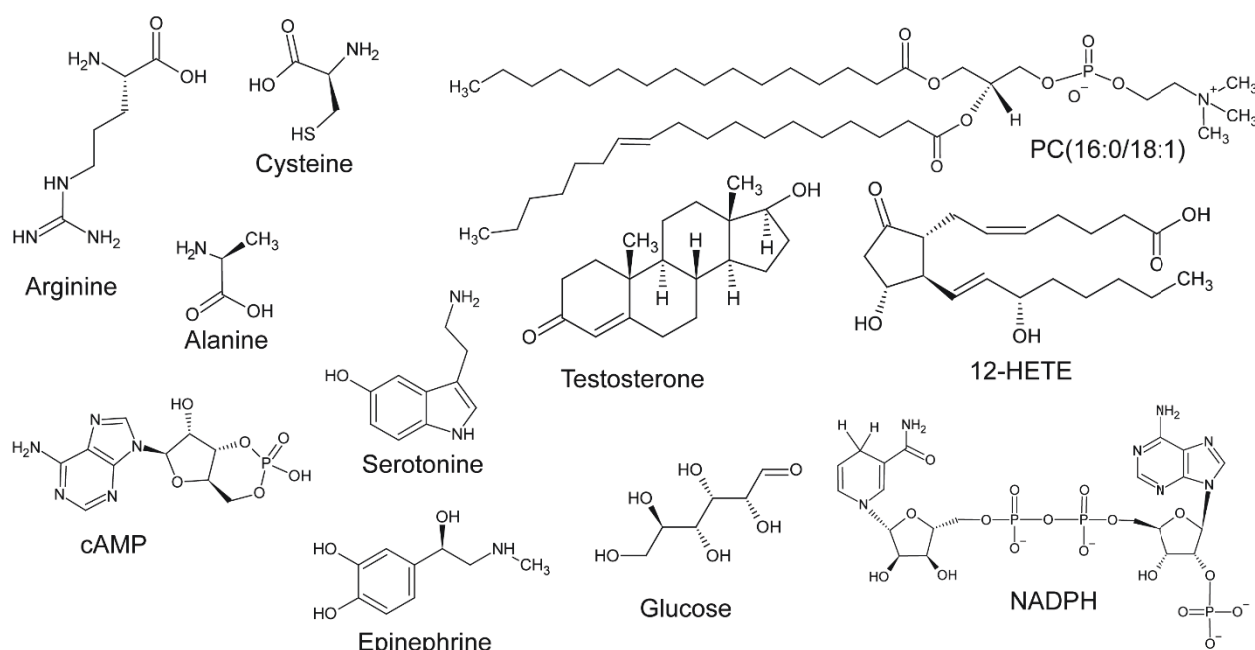


Figure 6: Collection of metabolites to visualise the high variety of different chemical properties making a complete metabolome analysis challenging. Arginine, alanine, and cysteine are shown as examples for amino acids. Cyclic adenosine monophosphate (cAMP) is an example of an intracellular second messenger. Serotonin is found outside of cells as neurotransmitter. Epinephrine and testosterone are examples for hormones. Phosphatidylcholine (PC (16:0/18:1)) is an example for a membrane lipid. Another lipid class, eicosanoids, is represented by 12-Hydroxyeicosatetraenoic acid (12-HETE). Glucose is shown as an example for sugars often used for energy production or as posttranslational modification. Nicotinamide adenine dinucleotide phosphate (NADPH, reduced form) is an important electron carrier used for catabolism, anabolism, and cell detoxification.^[44]

calculations using different computational approaches to generate possible spectra of non-natural metabolites.^[51] These investigations showed that, analogous to proteomics data, a simple score cut-off is not enough and needs these statistical approaches. Since the differentiation between actual metabolites and false positive hits is even more complex than with proteomics further advances will be necessary to ensure high data quality for high throughput untargeted metabolomics experiments.

3.2 Mass spectrometry

Mass spectrometry (MS) describes the measurement of the mass-to-charge ratio (m/z) for analytes in the gas phase. Although the basic measurement is the same since J.J. Thomson developed the first mass spectrometer around 1900^[52] this technology has evolved enormously during the recent decade. The Q Exactive mass spectrometer was predominantly used for this thesis and will be used to explain the basic measurement strategies of modern MS. Figure 8 shows the basic design of the Q Exactive Hybrid Quadrupole-orbitrap MS.

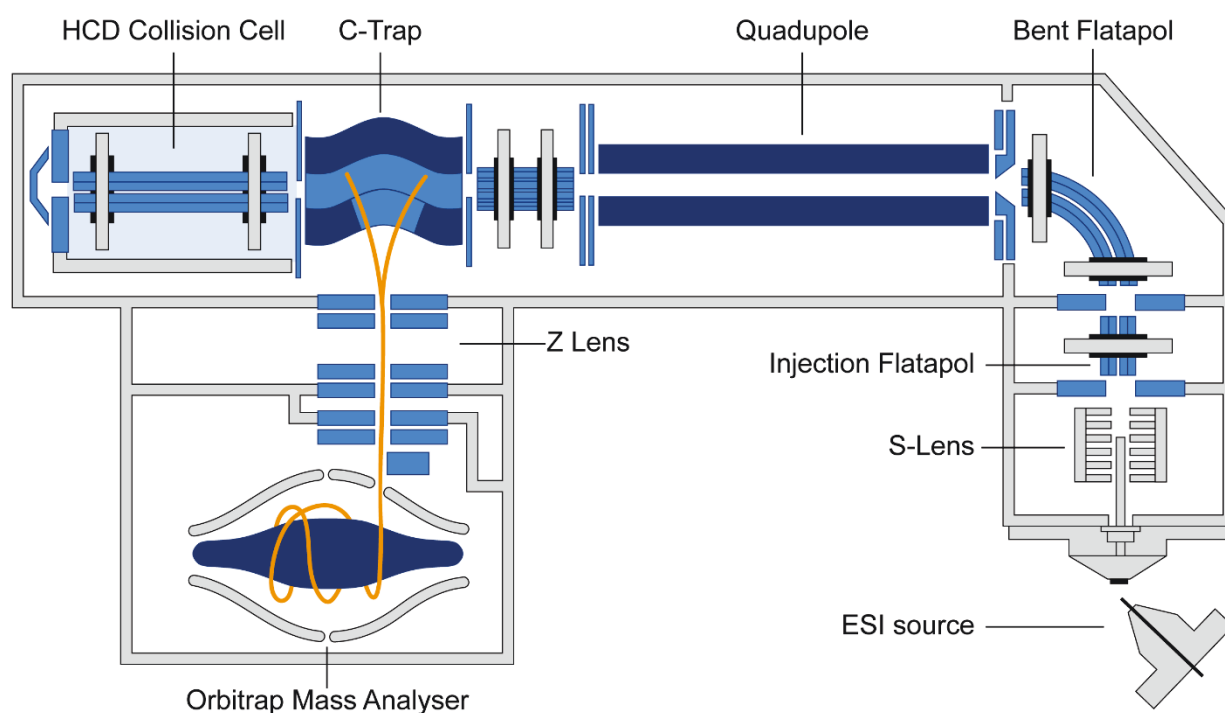


Figure 8: Schematic setup of a Q Exactive Hybrid Quadrupole-Orbitrap to highlight the most important analytical constituents. Redrawn from Michalski *et al.*^[53] (The original image was originally published in Molecular and Cellular Proteomics. ^[53] © Michalski *et al.*)

The first important step is the ionisation of the analytes in the gas phase. From the numerous different ionisation forms that have been developed over the years electrospray ionisation (ESI) is widely applied in proteomic and metabolomic analysis. Since liquid chromatography (LC) is often needed to reduce the complexity of the sample the coupling to the MS via ESI has proven to be quite advantages. In a mass spectrometric analysis using ESI, the analytes are transferred from a liquid phase into the gaseous phase and ionised. As illustrated in figure 9 the analyte

solution exits the capillary and forms a so-called Taylor cone finally emitting small droplets of analytes, solvent and charged excess ions in an aerosol. The solvent of the droplets is rapidly evaporated, which is in some case assisted by a counter-stream of heated nitrogen, resulting in smaller droplets with higher charge density. The repulsion of the same charge causes the creation of even smaller droplets until the charged analytes are completely solvent free resulting in ions which can be measured in the mass spectrometer. ESI is operated at atmospheric pressure and considered a soft ionisation technique, meaning that the analytes are rarely fragmented during the ionisation process.

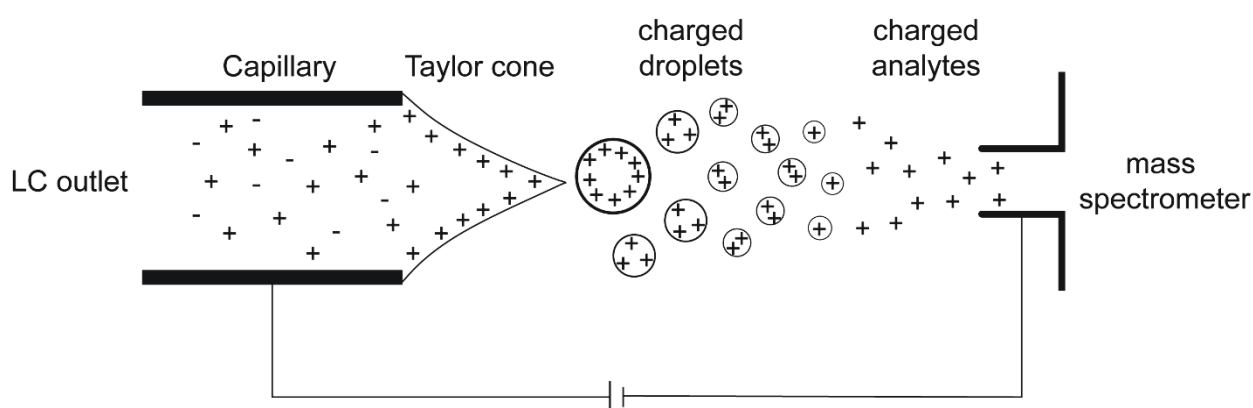


Figure 9: Schematic of an electrospray ionisation (ESI) run in positive ionisation mode coupling a liquid chromatography to a mass spectrometer.

ESI can be operated in positive and negative ionisation mode. This means that depending on the settings the created ions are either positively or negatively charged. The chosen ionisation mode is strongly dependent on the chemical properties of the analytes. While proteins/peptides are exclusively ionised in positive mode for metabolites and lipids the ionisation strongly depends on the analytes of interests. Often samples are analysed in both ionisation modes either consecutively or even in the same method by switching the polarisation of the ESI and the subsequent lenses in MS instrument.^[54] The exact identification of lipids can be achieved with the information from both polarities. Membrane lipids for example (Figure 6, PC (16:0/18:1)) are made of a polar headgroup and nonpolar fatty acid chain. While the headgroup bears the information of the lipid class, the fatty acid chain gives closer information on the length number and position of the double bond.^[55] The lipid can be charged positively and negatively but the charge will be located on different parts of the molecule which comes into account when fragmenting the analyte since only the charged part will be measured by the detector. The headgroup will be predominantly charged positively while the fatty acid chain will mainly bear the negative charge. A consecutive measurement in both ionisation modes can therefore enable an identification of unknown lipids.

The detector is the heart of the mass spectrometer and actually determines the mass-to-charge (m/z) ratio of the analytes. Years of research have developed different detector types like time-

of-flight (TOF), quadrupole, Fourier-transform ion cyclotron resonance (FT-ICR) or Orbitrap mass spectrometer. Due to the speed and high resolution the Orbitrap has proven to be a suitable tool for shotgun or untargeted analysis of proteins and metabolites. It was developed by Alexander Makarov^[56] and uses the orbital trapping of ions in an electrostatic field first described by Kingdon in 1923.^[57] The Orbitrap basically consists of two axially symmetric electrodes where the inner one looks spindle-like while the outer electrode is formed barrel-like as depicted in figure 10. The ions are injected tangentially to the outer electrode and move on stable trajectories around the inner electrode. The oscillation of the ions on the z-axis are detected *via* an image current that is induced on the outer electrode. The recorded transient is finally Fourier transformed, which leads to mass peaks of the injected ions and can be correlated to their m/z ratios.

Two or more mass analyser can be combined in one machine to create tandem mass spectrometry. Using two different types of detector (e.g. quadrupole and TOF → QTOF) This allows to isolate and fragment analytes and determine the fragmentation pattern, which is essential to identify the molecules. Different levels of fragmentation are termed as MS_n where n equals a natural number bigger 0. MS_1 spectra reflect the analysis of unfragmented ions while MS_2 spectra show the ions that have been fragmented once etc.

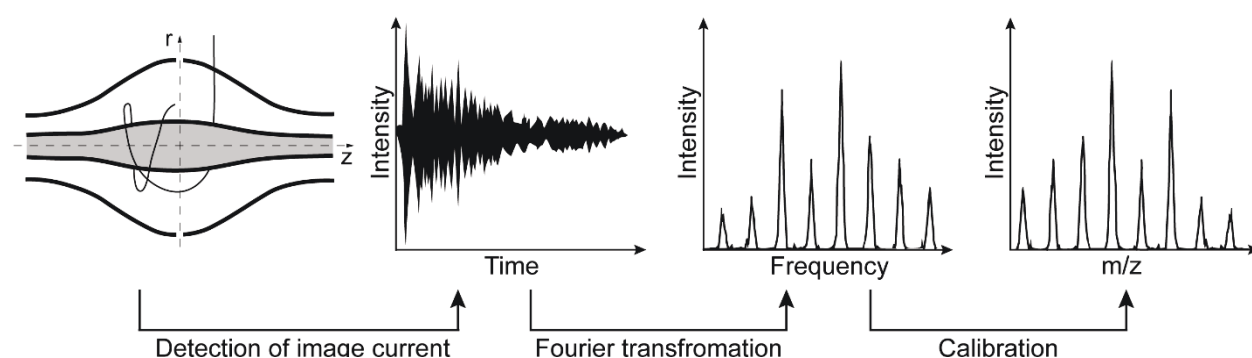


Figure 10: Signal processing after orbitrap measurement. The detected image current is Fourier transformed from time to frequency domain and subsequently correlated to the m/z values via calibration.

A typical shotgun proteomics experiment on a Q Exactive Hybrid Quadrupole-Orbitrap (Figure 8) features several consecutive measurements in order to allow a detailed investigation. The ions generated in the source will therefore make their way to the quadrupole which serves as a mass filter and will only allow a certain range of ions within distinct m/z ratios to pass to the detector. This first measurement is a full mass spectrum (MS_1) and gives an overview of all ions in the specific m/z range. Based on the MS_1 spectrum ions can be chosen for fragmentation. In data-independent acquisition (DIA) all ions within a defined m/z , which is considerably smaller than for MS_1 , are isolated and submitted to fragmentation. This is repeated with different mass-to-charge ratios until the desired range is completely covered.^[58] In contrast, for data-dependent acquisition (DDA) ions are selected based on their intensity and each ion is isolated and fragmented separately. The Quadrupole is therefore set to the exact m/z ratio that is selected and the passing

ions are submitted to the collision cell. In the Q Exactive a higher-energy collisional dissociation (HCD) is used for the fragmentation, which functions similar to CID. The ions are therefore transferred into the HCD cell, accelerated and allowed to collide with neutral gases, typically nitrogen. The exact energy that is used for the fragmentation heavily depends on the analyte but is in the range of 30 eV for peptide fragmentations.^[59] The fragments of the analyte are then again injected into the orbitrap and all fragments are analysed simultaneously, resulting in an MS2 spectrum.

3.3 Liquid chromatography:

The characteristic high complexity of biological samples makes its analysis and interpretation quite challenging. But the density of information that can be gathered within short time strongly outweighs all other methods. The today common combination of liquid chromatography with mass spectrometry has been proven to overcome many of the challenges that high complexity proposes.

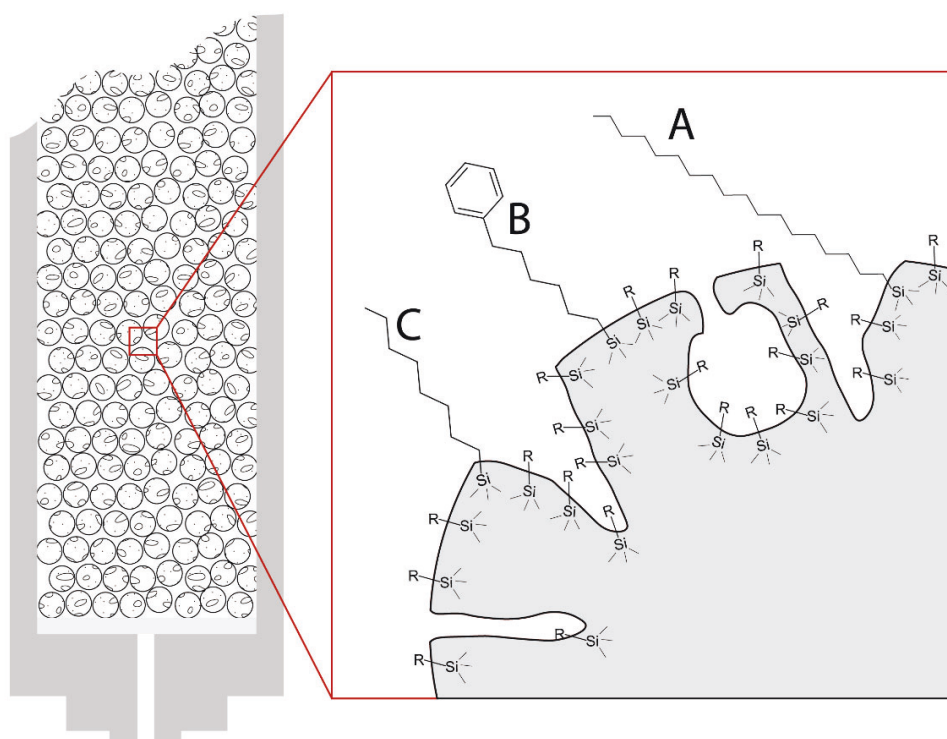


Figure 11: Schematic setup of a typical column used for liquid chromatography (left). Examples for functional groups used for reversed phase liquid chromatography. Aliphatic molecules are linked to the Si of the silica beads and give the material its characteristic hydrophobic properties. **(A)** and **(C)** show C18 and C8 residues respectively. For proteomics analysis C18 is used almost exclusively while C8 shows a little less hydrophobicity and is used for more hydrophobic molecules.^[60,61] The **(B)** Phenyl-Hexyl stationary phase can be used especially for aromatic compounds.^[62]

Chromatography is a technique developed in the early decades of the 20th century to separate molecules based on their distribution between a stationary and mobile phase.^[63] The analytes are dissolved in the mobile phase and move along the stationary phase. Strong interactions with the

stationary phase cause the molecules to move slower and lead to a separation depending on their molecular structure. While thin layer chromatography (TLC) is one of the oldest and still used methods the possibility to couple gas chromatography (GC) and liquid chromatography (LC) to detectors like mass spectrometer make them the most widely used chromatography system today. Size exclusion chromatography (SEC) separates the molecules depending on their steric properties.^[64] Affinity chromatography utilises the affinity of molecules to immobilised metals as described for phosphoproteomics enrichment or the purification of recombinant proteins with poly-histidine tags which react with copper ions.^[65] Supercritical fluid chromatography (SFC) uses high pressures and temperatures to create supercritical fluids as mobile phase which enable unique separation possibilities.^[66] There are numerous additional techniques that can be used for special applications but in the following we will take a closer look into the commonly used systems in proteomics.

GC uses inert carrier gases like N₂ or He as mobile phase and is used for clinical standard measurements like the analysis of hormones.^[67] The biggest limitation of the method is the need for the analytes to be evaporated into gas phase without decaying. The possibility to derivatise molecules and make them available in the gas phase exists but is certainly not suitable for every class of analytes.^[68] This led to the rise of LC which is the most used chromatography system for bioanalytic research today. Depending on the separation principle and used combination of stationary and mobile phase several different methods are used today. Normal phase (NP) chromatography uses a polar stationary phase like silica and elutes the analytes with non-polar organic solvents (e.g. chloroform, hexane). Today the most used technique in the analysis of biological molecules is reversed phase (RP) chromatography that uses non-polar stationary phases and polar, aqueous mobile phases.^[69] The separation hence depends on the hydrophobicity of the analyte, meaning that more hydrophobic, non-polar analytes will show more interaction with the stationary phase and retain longer on the column.

Figure 11 illustrates the composition of a chromatographic column. The column is filled with a porous material, mostly silica, that is functionalised with different molecules determining its chemical properties. The most used column type is the C18 column having an octadecyl (18) carbon chain. Other lengths of the aliphatic chain ranging from C1 to C30 are commercially available, but C8 and C18 are by far the most used.^[60,61] Also, phenyl based stationary phases are used which exploit π - π -stacking which are specially interesting for aromatic compounds.^[62] Furthermore, the developed phases can be mixed to further refine the separation. Beside the actual chemical interaction of an analyte with the stationary phase the physical properties of the support material silica strongly influence the chromatographic performance and resolution. Size, shape, and distribution of the material, surface area, pore size, diameter, volume, and distribution are critical factors that determine a column's physical properties.^[70-72] With the advances in the manufacturing process of the particles the size could be reduced steadily. Smaller particles as

well as column diameters require higher pressures and finally led to the development of high-performance liquid chromatography (HPLC) and ultra-high-performance liquid chromatography (UHPLC) which are today most used in analytical sciences.

3.4 Cell culture

The introduction of human cell culture models in the 1950s with the first extraction and cultivation of the HeLa cell line was a milestone in modern life sciences.^[73] Prior to this discovery, investigation of molecular mechanism was limited to bacterial and yeast models. Today HeLa is distributed all over the world and cell culture became an indispensable part of science. Since then innumerable cell lines of a multitude of human tissues and many disease states have been established and are used to study the basic mechanisms of human life, pathomechanisms of diseases and effects of drugs and toxins.^[74]

Despite their obvious advantages cell cultures are model systems and thus, do not entirely reflect an organism. One of the major issues is the reproducibility. Strategic analysis of published works has shown, that up 89% of the results are not reproducible by other working groups of even the same laboratory.^[75-77] While the cause for this is manifold and cannot be easily pinned down a major problem that is often discussed is the issue that comes with the use of natural additives like fetal calf serum (FCS, also fetal bovine serum FBS).^[78] FCS is the most used cell culture substitute today and considered essential for most cell lines. FCS is created as a side product of the meat industry. About 8% of the cows that are slaughtered are gravid. These unborn foetuses are used to draw their blood directly from the still pounding heart and extract the serum from it.^[79,80] The exact formulation of FCS is unknown and shows the same biological variances that are normally found in the comparison of different individuals. And although companies strive to achieve high similarity of their products, different batches of FCS may consist of varying amounts of biomolecules. In fact, it is common practice to buy large quantities of the same batch to reduce such variations in research projects that span long periods. It is also possible to buy products, that are analysed and controlled more carefully. But the analysis is never able to cover the complete formulation of the serum which means that the uncertainty of unknown factors is still present. Furthermore, a study showed, that the processing of FCS after extraction is as crucial as the biological variances. Researchers investigated the effects of different batches of charcoal stripped FCS after their assay didn't work properly with the newer batches. They revealed that the vendor changed the processing procedure to deplete the hormones and lipids from the FCS and therefore dramatically changed the composition in a completely unexpected way.^[81] To overcome the increasing issue with natural products scientists strive to grow their cells in chemically defined media (CDM). This means that every concentration of every molecule present in the growing medium is exactly known and therefore controlled. To date, there are only a handful of cells that are successfully grown in CDM.^[82-89]

3.5 Metal-based anticancer drugs and the analytical approach to investigate its mechanisms of action

Ever since the accidental discovery of cisplatin and its anti-neoplastic effect^[90] metal-based therapeutics have risen in number and priority. Metal complexes and organometallic compounds can today be found in diagnostics and for therapies of several diseases. Radioactive isotopes play an important role for diagnostic purposes, since they can be tracked inside the body by imaging techniques. But also for the therapy of solid tumours some radioactive metals are investigated for their ability to ablate cancer cells.^[91,92] Cisplatin was the first metal-based anticancer drug to be approved by the Food and Drug Administration (FDA) as cancer therapy and thus, the platinum class played a predominant role for a long time. The major mechanism of action (MOA) of cisplatin involves DNA lesions, which lead to double-strand breaks and ultimately death of the cell.^[93] The indiscriminate targeting of fast growing cells is associated with a considerable toxicity of the drug and leads to the well-known side effects associated with cancer therapy. In recent years more and more metals have been recognised as promising base for cancer therapies and science has started to investigate their properties. Beside arsenic trioxide (As_2O_3) as treatment for acute promyelocytic leukemia no other nonplatinum metallodrug is in clinical use as anticancer treatment.^[94] But many different metal-based drugs are used and explored in preclinical and clinical studies. Gold, rhenium, tungsten and ruthenium are among the most prominent metals that are currently studied intensively.^[95-98] But in order to gain approval for a (cancer) therapy the drug has to show an advantage over already existing therapies. Insight into the molecular mechanism of action is therefore quite valuable since it allows a more precise application of the therapy.

The investigation of a drug's efficacy that hopefully concludes in its approval is a long and very expensive process.^[99] Since many anticancer drugs show high cytotoxicity a test in humans is only reasonable if an advantageous effect is foreseeable. The prior test on cell lines and animal models is therefore a necessary step and scientists strive to gather as much information about the mechanism of action as possible to bring the drug to optimal use. The NCI-60 cancer cell line panel is a strategy that aims to help give a quick overview of a potential anticancer activity of drugs. The National Cancer institute (NCI) therefore tests the potential drug on 60 selected cancer cell lines representing a wide variety of different tumours and monitors the growth inhibition.^[100] Highly effective drugs are then used for further investigations. These preliminary tests on cancer cell lines are quick, cheap and represent an approach to detect potential new drug candidates. Such assays monitoring the survival of cells in the context of increasing drug concentrations are often the first steps in the investigation. As such, the colorimetric MTT assay^[101] is often used to measure the metabolic activity of cells and indirectly conclude on their death rate or growth inhibition upon treatment. The assay gives a measure for the cells ability to metabolise the added dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) into its insoluble form. While this

is indeed not dependent on the cells death, the convergence is often made for the easy utilisation and fast results that the method provides. These investigations allow the user to identify potential cell toxicity on certain cell lines and give assumption on whether further studies are worthwhile. The study of molecular mechanisms of potential anticancer agents has been greatly supported by the advances in mass spectrometry. Interaction studies of therapeutics with all sort of biomolecules can give holistic insights into its molecular mechanisms.^[102] Especially the utilisation of proteomics and other omics techniques can give insight into potential mechanisms of action.^[103]

BOLD-100/KP-1339

Sodium *trans*-[tetrachlorido-bis(1*H*-indazole)ruthenate(III)] (BOLD-100/KP-1339, Figure 14) is a ruthenium-based drug strongly investigated due to its promising anticancer effects in an autochonomous tumour model and its low side effects in human trials.^[102,104] Also the downregulation of the endoplasmic reticulum chaperone BiP (HSPA5; also 78 kDa glucose-regulated protein, GRP78) that has been recognised as major mechanism of action enhanced the interest in the drug.^[105] Since GRP78 upregulation is a known resistance mechanism of cancer treatment, the combination of BOLD-100 with established therapies seems a promising approach.^[106,107] Interestingly, the drug shows very high binding capacity to serum proteins transferrin and serum albumin.^[108,109]

3.5.1 Omics supported metal drug investigations

Studies show that about 90% of drug targets are proteins.^[110] This makes proteomics a powerful tool in the investigation of the biological effects of drug treatments. Proteome response profiling compares the proteome profiles of cells in two or more different states (e.g. control vs drug treatment) and tries to conclude the ongoing molecular mechanism. The significantly regulated proteins received from the comparison to the untreated control cells will give an indication about the global changes triggered by the drugs interaction. This method has proven to be incredibly useful in the investigation of the mechanisms of action of metal-based drugs.^[103] To create proteomic response profiles for metal-drug treatments a sensitive cell line is used and treated with subcytotoxic concentrations of the drug. The half maximal inhibitory concentration (IC₅₀ value) is the concentration that is needed to inhibit 50% of a biological process. For investigations on anticancer drugs the main interest is a growth inhibition or induction of apoptosis. The half IC₅₀ value that is typically used for proteomics analysis is chosen to assure meaningful analysis. At this concentration most of the cells have not actually induced apoptosis but already show signs of drug effect and the cells response. Although a bioinformatic analysis with gene ontology terms (GO), network analysis or comparison to other, already investigated drugs (FITeXP)^[111] can give quite a lot information, an assignment of an actual drug target can hardly be done. To actually investigate the drug-protein interactions other methods have been developed and successfully used to unravel molecular targets of metal-based anticancer drugs.

3.5.2 Target identification methods

Small molecules (e.g. metal-based drugs) are prone to multiple interactions within a biological system which makes the analysis of its direct binding partners quite challenging. Even cisplatin whose mechanism of action has been correlated with its DNA damaging effect has been shown to have specific protein binding partners.^[112] This is of special interest when it comes to resistance mechanisms and how to overcome them. Several mass spectrometry-based methods have been developed to help investigate potential binding partners for metal-based drugs. Early studies exposed cell lysates to metal drugs *in vitro* and modified the search algorithms to search for mass shifts and isotope patterns indicating metal binding to the proteins.^[113] The major disadvantage of this method lies in the unwanted selectivity. In order to measure a metal-amino acid adduct the formed complex has to be very stable. A HPLC-MS measurement is only feasible for multi-dentate complexes which unwillingly discriminates all other, weaker bindings although they might be just as important for the biological effects. The creation of covalent bindings of the metal drug to the target protein has become a well-suited alternative to investigate direct binding partners of the drug. Photo-affinity linker (PAL) are chemical structures that form covalent bonds upon activation with light.^[114] Phenylazides, phenyldiazirines and benzophenones are PALs often used for ligand-target interactions. In order to use these linkers for target investigations the drug of interest has to be modified with it to create a probe. The probe is then introduced into the biological system and upon activation with UV-light the linker creates a covalent bond to a close biological structure. In a successful experiment the metallodrug is linked to its specific binding partner. In order to identify the target the reporter tag of the probe is needed. This can be either biotin or a fluorescent structure. A biotin-modified structure can then be used to extract the ligand-protein complex from the biological sample via streptavidin beads. Fluorescent probes can be used in 2D-Gels to visualise the bound proteins and cut them out of the gel. Either way, the purified proteins can be detected by mass spectrometry using PMF or any other suitable detection method. Although these methods are quite sophisticated and widely used they have to be used with care to avoid falsifications, e.g. unspecific/hydrophobic binding.^[115,116] The modification of the drug certainly changes its biochemical properties. The linker and reporter tag have to be bound to the drug, which inevitably introduces structural changes that might affect the binding to the target. A prior knowledge of the active groups of the drug is therefore advantageous. Furthermore, the probe is considerably bigger than the original drug. To avoid a steric hinderance of the drug binding, PAL and reporter tag are often linked to the drug *via* a spacer. Polyethylene glycol (PEG) is often used due to its stability, low hydrophobicity and availability in different lengths. The modified probes are often used in competition with the original drug to see if they compete for the same binding site, minimising unspecific identifications.^[117] For some complexes a linker modification is not amendable which requires to work around the immobilisation with a covalent linker, like it is reported for BOLD-100.

There are methods available, which allow an investigation of ligand-protein binding with the original drug. Thermal protein profiling (TPP) uses the fact that a proteins denaturation process is altered when a ligand is bound to it. The method uses cell lysates with and without drugs, heats them from 37-67 °C and measures the soluble proteins. When comparing a treated with an untreated cell lysate protein binding to the drug will show different melting curves and therefore identify direct binding partners for the metal drug.^[118-120] For these investigations the drugs are used in their native form and do not need any modification with a linker or reporter. With the TMT labelling method all different heat points are analysed in the same measurement which makes the relative quantification of the different conditions even more accurate showing even minor changes.

Integrated target-response profiling provides a method to investigate the biological interactions of a (metallo-) drug and bioinformatically correlates them to a protein response profiling.^[117,121,122] For the target identification the drug needs to be immobilised in an appropriate way. The modification of the drug with biotin and binding to Streptavidin beads is a good way to do this but requires the drug to be amendable with the biotin without loss of the drug effect. The immobilised drug is then submitted to cell lysates that are either preincubated with the parent drug or not. This incubation of the native cell lysate allows the free drug to bind to its target proteins and therefore inhibiting the binding possibility of the immobilised drug. The proteins that are finally bound to the drug-loaded beads are digested and used for MS based proteomics analysis. This competitive approach shows the enrichment of the proteins that are specifically bound to the drug. The target identification is complimented with a response profiling of the same sensitive cell line, that is also used for the drug pulldown. Figure 14 shows the schematic workflow used for the target response profiling in this thesis.

3.6 The confounding problem in the context of omics data interpretation

The term confounding is not the most familiar amongst the scientific community especially in life sciences although the concept might unconsciously be hidden in many deliberations.^[123,124] While the exact definition of confounder may vary^[125] confounding factors are basically variables that influence the result of a study and introduce bias. Therefore, controlling for a confounder, which basically means knowing or measuring the exact value, will allow for a correct interpretation of obtained results. To actually understand the concept, I will paraphrase an example by Judea Pearl's *The Book of Why*.^[126] If you consider the reading capability of kids you can show that the bigger the kids shoe size is, the better they are able to read. Only regarding these two variables you would be tempted to see a direct correlation between the two variables, which is not necessarily correct. Figure 12 will give a visualisation on the following relations the variables can show. The first important differentiation that we have to make now is between correlation and causation. Correlation meaning that two observed variables (A, B) change in coherence, e.g. if A

risers B rises as well. But this correlation does not mean that there is an actual connection between the two variables, they can be completely unrelated (Figure 12.1). Causation on the other hand means that one variable actually causes the change of the other (Figure 12.2). For example, the activity of a kinase and the phosphorylation of its ligand correlate because the first one actually causes the latter. To come back to the confounding problem, there is no actual correlation between a kid's shoe size and its reading capability but there is a confounder, the age. If you control the study for age, meaning you only compare children of the same age, the correlation between shoe size and reading abilities will vanish. This is simply because the confounder age actually causes both variables to rise: Older children will on average have bigger feet and will be better at reading (Figure 12.3).

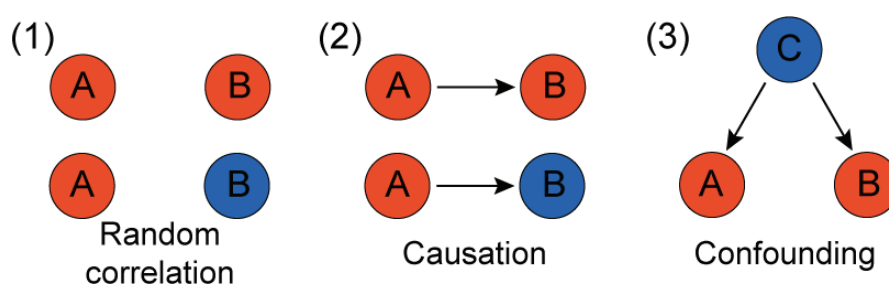


Figure 12: Graphical representation of the concept of a random unrelated correlation, causation and confounding. **(1)** Variables A and B seem to be correlated although there is no causation behind it, the data correlation is completely random *e.g.* due to the high data density. Changing A will not cause B to change. **(2)** Variable A causes the change of B. Changing A will always force B to change as well. **(3)** Variables A and B show a correlation that is caused by a confounder C. Changing A **will** force a change in B only when C is not controlled or in other words, when the confounder C is controlled changing A **will not** cause a change in B.

The interpretation of omics datasets in this context is blessing and curse in one. With the latest mass-spectrometry based proteomics methods several thousand proteins can be observed from a single experiment.^[18] With so many observed variables a random correlation is not only possible but actually quite probable.^[127] The interpretation of the data therefore requires utmost attention to avoid overinterpretation. On the other hand, the number of measured hence controlled molecules is bigger than ever. This allows the scientist to control for many different variables and evade random correlations when no causation is evident within the data. This advantage can be utilised even better when multiple complementary omics techniques are used to investigate the same scientific question.

3.7 Multi-omics: the method for future investigations

At least since the finalisation of the human genome project when all human genes were discovered and mapped on the corresponding chromosomes scientists understood that, although the genome inherits the ultimate plan of a cell that is passed on during cell division and reproduction it does not describe the function of a cell. Post-genomic analysis methods

investigating proteins and metabolites with all its subgroups are looking to achieve what genomics was not able to do. Today we do not know all proteoforms and their function neither the concentrations of all metabolites in the cells to correlate them to a cells' function nor are we yet able to comprehensively analyse it.^[51,128] Nevertheless, it is foreseeable that each method alone will not be able to absolutely predict a cell's functional state even if it was able to analyse all proteins. Analogous to the human senses, only the interplay of two or more enables the complex knowledge that we are able to withdraw from it. As an example, humans are very good at estimating the distance, shape and size of a certain object by just looking at it. But this is only possible because our brain learned to interpret the complex data that is produced by photons hitting our eyes.^[129] Babies, as soon as they open their eyes are not able to predict these parameters correctly because they do not know what it actually should be. But they will start to grab anything within their reach, or at least try to. They will look at it from different angles and will study how it changes while it moves. With this additional information the brain learns to predict the correct distance, shape and size with just the sights information. After learning how to correctly interpret an objects' properties just from its looks it is even possible to have a mere picture of it but still know what you are looking at.^[130] Coming back to the investigation of biological systems the knowledge we gain from proteomics studies is quite powerful, but often does not give us enough information to give a correct interpretation of the cells functions. The ways in which a proteins function can be constrained or enhanced are numerous from well-known post-translational modifications over cofactors to often underestimated lipid compositions.^[44] Especially, the last-mentioned point depicts a very interesting biological property that can easily be missed and would represent a perfect example of an unknown confounder that would alter the interpretation. Receptor tyrosine kinases (e.g. endothelial growth factor receptor; EGFR) are transmembrane proteins which are activated by forming a homodimer. The two single proteins need to migrate through the membrane of a cell and find each other before activating their downstream kinase activity. Their activity is therefore dependent on their migration speed which is strongly influenced by the membrane rigidity which again depends on the lipid composition of the membrane.^[131] This means that two cells with the same concentration of the receptor but different lipid composition would show significant differences on their reaction to the receptor's ligand.

So analogous to a babies' brain we need to learn on how to meaningfully combine all the different omics methods. In order to actually understand biological processes, pathomechanisms and drug mechanism of action different methods have to complement each other's findings. But to use high-end technologies like genomic, transcriptomic, proteomic and metabolomics is not trivial, nor is the combination. It is not a commercial assay that is easy to use and interpret. The sensibility of the analysis and high amount of data makes it challenging to properly control it and demands expertise. This together with proper bioinformatic tools to meaningfully interpret different omics

datasets within reasonable time will make the ultimate tool to actually overcome mere correlation and understand biological causations.

3.8 Research justification

Mutations are major causes for the development of tumours which has been shown in numerous epidemiologic studies.^[132] But years of genomic research demonstrated that gene alterations are not the single cause for the emergence of cancer. The aim of this thesis was to expand post-genomic analytical methods in cancer research, by combining disease models with mass spectrometry-based omics technologies. These approaches were applied to clarify metastasis processes in melanoma, investigate the impact of cell culture conditions on cellular responses and elucidate the mode of action of anticancer drug candidates as outlined below.

The treatment of late stage melanoma and especially the formation of metastasis in the brain is an often-described challenge.^[133] Genomic studies showed high mutation rates in these tumours and hence underlined the observed clinical outcome.^[134] In the first work the molecular profiling of a melanoma brain metastasis model was able to further confirm these observations. The model consisted of four human melanoma cell lines transformed to create cell pairs of cutaneous and brain metastasis versions of each cell line. Although several well-described pathways essential for the formation of metastasis could be detected, each cell pair showed a different specialisation that allowed the selective formation of brain metastasis. This strengthened the upcoming realisation that external factors like the tumour microenvironment strongly influences the development of cancers.

Reproducibility of cell culture experiments is a well-accepted challenge in modern life sciences and fetal calf serum (FCS) has long been identified as a major factor.^[135,136] The second work shows the severity of minimal differences exemplified by two eicosanoids. Similar works of cell cultures incubated with HETEs used higher concentrations which makes this the first study to investigate their biological effect at naturally occurring nanomolar differences.^[137,138]

The comprehensive characterisation of the mechanism of action of anticancer drug candidates represents a milestone for their correct application. Especially, metal-based anticancer drugs were long prejudiced to be unspecific and this, associated with being toxic. The third study shows the identification of the molecular protein targets of the widely investigated BOLD-100 (formerly KP-1339) and validated them in the context of the biological response to the drug *in vitro*. This is the first time a molecular target could be proposed and directly linked to the mechanism of action of BOLD-100.^[139-141] Metal-based anticancer drugs were long prejudiced to be unspecific. This work adds further evidence that metal-based anticancer agents may be specific to their mechanisms of action.^[115-121] The investigation of AuTMX₂ further strengthens the importance of an in-depth molecular profiling and validation by alternative biochemical approaches to elucidate the mechanism of metal-based drug action.

4 Methodology used in this thesis

4.1 Cell culture routine

Over the course of this thesis different cell lines were cultured under standard conditions in a humidified Heracell incubator (Thermo Fisher Scientific, Austria) at 37 °C and 5% CO₂. Cells were subcultured depending on their rate of growth every 3-4 days upon reaching 80-90% confluency. Suspension cells were removed from their cell culture flask, pipetted into a 15 mL falcon tube and centrifuged at 236 g for 5 min at room temperature. Adherent cells were washed with sterile phosphate-buffered saline (PBS) and trypsin-EDTA solution (Sigma-Aldrich, Austria) was added at 2 mL for T25 flasks and 3 mL for T75 flasks (both Sarstedt, Austria). The cells were then incubated for 3-5 min until they lost adherence. The enzyme was inhibited with 3 and 4 mL of fully supplemented medium for T25 and T75 flasks, respectively. The supernatant was moved to a 15 mL falcon tube and centrifuged at 236 g for 5 min at RT. The cell pellet was carefully resuspended in fully supplemented media and diluted in the desired ratio. DP-C, DP-CB, YDRF-C, YDFR-CB, M12-C, M12-CB, M16-C, M16-CB, U937 and A2780 were grown in RPMI-1640 (1X with L-glutamine; Gibco, Thermo Fisher Scientific) with 10% FCS (Sigma-Aldrich) and 1% penicillin/streptomycin (Sigma-Aldrich). HCT116 cell line was grown in McCoy's 5A modified medium (1X with L-glutamine; Gibco) supplemented with 10% FCS and 1% penicillin/streptomycin. All adherent cells were grown in polystyrene cell culture flasks with cell growth surface for adherent cells (Sarstedt). Suspension cells were grown in polystyrene cell culture flasks for suspension cells (Sarstedt). MOXI Z mini automated cell counter together with MOXI Z Type M Cassettes (both ORFLO Technologies, USA) were used to count cells before seeding them in the required concentration for the experiments. Mycoplasma contamination was tested regularly for all cell lines using the MycoAlert™ Mycoplasma Detection Kit (Szabo-Scandic, Austria).

4.2 Cell fractionation for protein analysis

For standard proteomics analysis three different fractions were analysed: supernatant (SN), cytoplasm (CYT) and nuclear extract (NE). To analyse the proteome of the supernatant of a cell culture experiment the fully supplemented medium was removed and the cells were washed twice with PBS to remove FCS contamination. The cells were then further incubated for 4-6 hours to allow the cells to secrete proteins. The supernatant was removed from the cells and diluted with ice cold ethanol (-20 °C) and a ratio of 4 parts ethanol for 1 part supernatant and left overnight to precipitate the proteins.

The complete cell fractionation was done on ice to minimise enzyme activity. For cell lyses the supernatant was removed from the cells and then washed thoroughly twice with PBS. Isotonic lysis buffer (IAP) consisting of 10 mM HEPES/NaOH (pH 7.4), 10 mM NaCl, 3,5 mM MgCl₂, 1 mM

EGTA, 0.25 M Sucrose and 0.5% Triton X-100 (all chemicals from Sigma-Aldrich) in H₂O was supplemented with Protease and Phosphatase Inhibitor Cocktail (1:100, Sigma-Aldrich) and PMSF (1 mM final concentration) shortly before use. The final fractionation buffer was added to the flask and the cells were scraped off using a cell scraper (Fisherbrand, Thermo Fisher Scientific). The cells were lysed by pushing them through a needle (23 G) and applying enough shear force to break up the outer cell wall but keep the nuclear membrane intact. The cells were pushed through the needle 10-15 times depending on the cell line and the progress was tracked through microscopy. (Figure 13) Afterwards, the sample was centrifuged at 2200 g for 5 min at 4 °C. The supernatant was again precipitated in ice cold ethanol (4:1) and the remaining liquid was carefully removed with a paper towel to ensure as little contamination as possible. 100 µL of TE-NaCl buffer (10 mM Tris/HCl, 1 mM EDTA, 0.5 M NaCl) was added to the remaining pellet, resuspended and incubated for 10 min on ice. If the pellet did not dissolve an ultrasonic stick was used to ensure solvation. After the incubation 900 µL of TE-NP-40 buffer (10 mM Tris/HCl, 1 mM EDTA, 0.5 % NP-40) was added. After another 15 min incubation on ice the sample was centrifuged at 2200 g for 5 min at 4 °C and the remaining supernatant was again precipitated with ice cold ethanol (4:1) overnight.

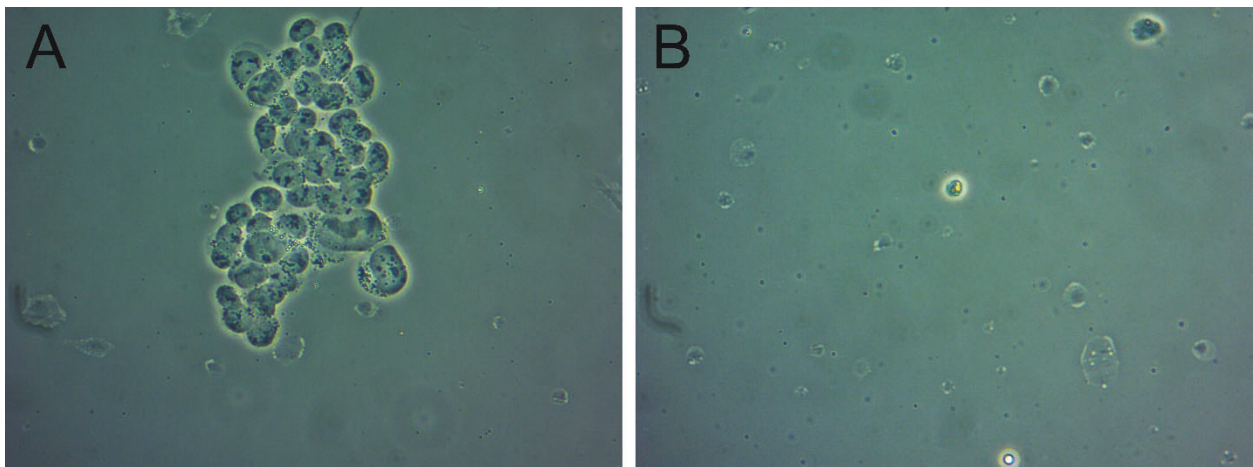


Figure 13: The different stages of cell lysis from HCT116 cell line. **(A)** Sample scraped of the fell flask and not jet pushed through the needle. Most cells are not lysed, sticking together and forming clusters. There is little debris visible. **(B)** No intact cells are visible but a significant amount of cell debris is floating around while cell nuclei are still not lysed.

4.3 Protein sample preparation

After at least overnight precipitation samples were centrifuged at 3500 g for 30 min at 4 °C. The supernatant was discarded and the protein pellet was dried with the vacuum desiccator. The dry sample was solved in about 50-100 µL of sample buffer (7.5 m urea, 1.5 m thiourea, 4% CHAPS, 0.05% SDS and 100 mM DTT) depending on the size of protein pellet. After determination of the protein concentration using Bradford assay (Bio-Rad-Laboratories, Germany) 20 µg of protein was used for the FASP workflow. The sample was loaded onto a prewetted 10 kDa cut-off filter

(Pall Austria Filter GmbH, Austria), reduced with DTT and alkylated with IAA. The proteins were then digested with trypsin/Lys-C solution (Promega, Germany) over-night at 37 °C. The peptides were eluted, dried via vacuum centrifugation and stored at -20 °C.

4.4 Protein analysis using HPLC-MS

For the HPLC-MS analysis an Ultimate 3000 RSLCnano chromatography was coupled to a Q Exactive Hybrid Quadrupol-Orbitrap mass spectrometer. The two column system consisted of an Acclaim™ PepMap™ 2 cm precolumn (C18, 5 µm particle size, 100 µm diameter; Thermo Fisher) and an Acclaim™ PepMap™ 50 cm main column (C18, 2 µm particle size, 100 Å pore size, 75 µm diameter; Thermo Fisher). Loading pump eluent C and eluent A consisted of 97.9% H₂O, 2% acetonitrile (ACN) and 0.1% formic acid (FA) while eluent B was made of 19.9% H₂O, 80% ACN and 0.1% FA.

Name	Sequence	Molecular weight	Final con (fmol*µL ⁻¹)
Glu1-Fibrinopeptide B	EGVNDNEEGFFSAR	1569,6696	1.1
M28	TTPAVLDSGSGSYFLYSK	1862,8938	2.2
HK0	VLETKSLYVR	1206,6972	1.1
HK1	VLETK(ε-AC)SLYVR	1248,7078	1.1

Table 1: Peptide standards used as internal control for proteomics measurements

The dried peptides were dissolved in 5 µL 30% FA in H₂O containing 4 internal, synthetic peptides (Table 1). The sample was further diluted with 40 µL of eluent A and transferred into a glass vial. Depending on the sample two different gradients were used for the HPLC separation. For supernatant samples, which show lower complexity than cytoplasm or nuclear fraction, 1 µL of the sample was loaded onto the precolumn. From there it was eluted onto the main column and separated in an 85 min method with a 43 min gradient from 7% to 40% eluent B. For cytoplasmic and nuclear fractions 10 µL were injected and separated with a 135 min method including a gradient from 8% to 40% eluent B over 95 min at a flow rate of 300 nL min⁻¹ for both methods. Mass spectrometry settings were similar for both methods adjusted for the HPLC method timings. MS1 was set to 400-1400 m/z at a resolution of 70,000 (at 200 m/z). The top eight most abundant masses were chosen for fragmentation at 30% normalised collision energy. MS2 fragments were analysed at a resolution of 17,500 (at 200 m/z).

4.5 Target-response profiling of BOLD-100/KP-1339

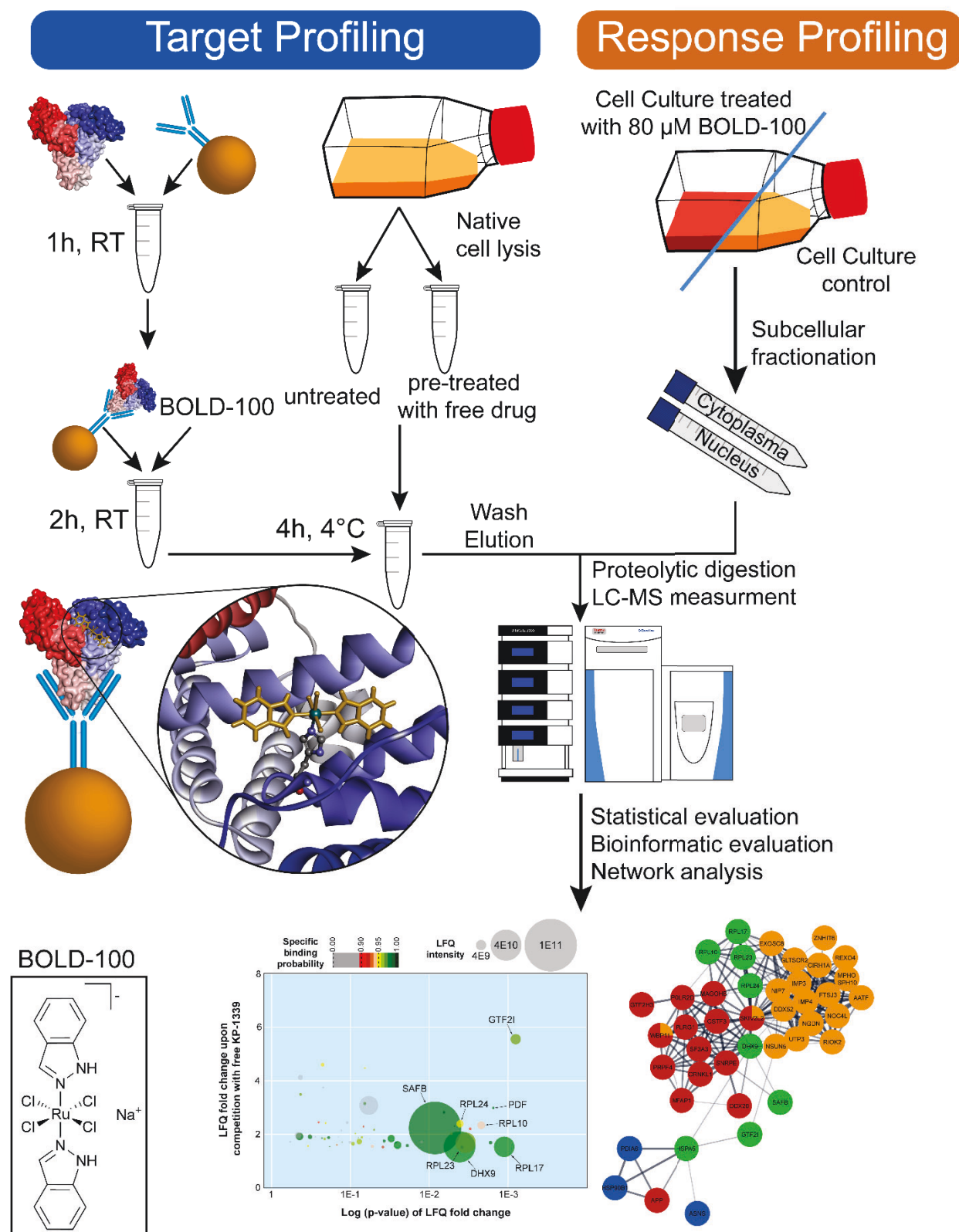


Figure 14: Schematic of the target-response profiling workflow used for the identification of BOLD-100 (formerly KP-1339) protein interaction partner

The workflow of the target response profiling for BOLD-100 is schematically shown in Figure 14. For the target profiling HCT116 cells were grown in 6 T75 polystyrene cell culture flasks with cell growth surface for adherent cells (Sarstedt) to about 95% confluency. The cells were lysed using a non-denaturing lysis buffer consisting of NaCl (100 mM), Tris HCl (50 mM), MgCl₂ (1.5 mM), NaF (25 mM), NP-40 (0.2%), glycerol (5%), TLCK (10 µg mL⁻¹), DTT (1 mM), Na₃VO₄ (1 mM), PMSF (1 mM) and pepstatin/leupeptin/aprotinin (each 1 µg mL⁻¹), at pH 7.4. The cells were scraped off the surface and homogenised by passing 20-times through a 23G needle. The samples were centrifuged (3500 rpm, 5 min, 4 °C) and the supernatant was transferred. 50% of the cell lysates were incubated with 50 µM BOLD-100 for 1 h at 4 °C.

Since it was not possible to modify BOLD-100 with biotin or a comparable method to generate an immobilisation, its natural high affinity to human serum albumin (HSA) was used.^[142] Therefore, 1 mL of PureProteome™ Albumin Magnetic Beads (Millipore, Germany) were incubated with 2 mg HSA (Albumin from human serum, Sigma-Aldrich) for 1 h at room temperature. The beads were washed with PBS and 95 µg BOLD-100 were added for 2 h at room temperature which resulted in a magnetic bead-HSA-BOLD-100 complex as depicted in Figure 14. Although this is a big complex compared to the size of the drug the immobilisation reflects the *in vivo* situation since the drug is bound to HSA very quickly. It imitates the way the cells are exposed during a treatment in cell culture through binding to bovine serum albumin contained in the FCS. Furthermore, this binding also takes place when administering the treatment to mice or humans. The suspension was split into six similar aliquots and incubated with the cell lysates previously described (3 control and 3 pre-incubated with BOLD-100) for 4 h at 4 °C while softly shaken at 300 rpm. The beads were washed extensively 5 times with lysis buffer and 3 times with HEPES buffer (HEPES-NaOH (50 mM), EDTA (0.5 mM), PMSF (1 mM), TLCK (10 µg mL⁻¹) and pepstatin/leupeptin/aprotinin (each 1 µg mL⁻¹)). The bound proteins were finally eluted using a 0.1 M citrate buffer (pH = 2,4) and used for a proteolytic digestion as described previously.

For the response profiling HCT116 cells were seeded in 6 T25 cell culture flasks at 5·10⁶ cells per flask and allowed to grow for 24 h. 3 flasks were incubated with 80 µM BOLD-100 for 24 h while the others served as controls. All 6 flasks were lysed and fractionated into CYT and NE as previously described. The samples were proteolytically digested and submitted to HPLC-MS analysis.

4.6 Data analysis and statistical evaluation

For Data analysis different versions (from 1.6.1.0 to 1.6.17.0) of MaxQuant^[143,144] software containing the Andromeda search algorithm were used. As database the Uniprot KB^[33] containing 20,316 reviewed human proteins (downloaded 03/2018) was used for the main search and reverse as decoy database to calculate FDR. In order to successfully identify a protein, at least two peptides, of which one had to be unique, had to be identified. The FDR for peptide and protein

identification was set to 0.01, based on the q-value. Trypsin/P was set as digesting enzyme alongside Carbamidomethyl as fixed modification on cysteine residues. Oxidation of methionine and acetylation on the protein N-term was further set as variable modification.

The search results were used in the MaxQuant companion software Perseus^[145] (version 1.6.6.0) for statistical evaluation of the data. The generated matrix of the LFQ intensities was therefore filtered for potential contaminants, reverse and only identified by site. The LFQ values were further logarithmised on base 2 and categorical annotation was used to assign samples to their respective group. The matrix was filtered for invalid values, only allowing proteins that showed at least in 70% valid values of samples per group. The remaining invalid values were replaced from normal distribution with a downshift of 1.8σ (standard deviation of the normal distribution) and a width of 0.3σ . These matrices were used for all statistical analysis mainly principle component analysis (PCA), volcano plots and two-sided t-tests. Significantly regulated proteins coming from volcano plots or t-tests were used for further bioinformatic analysis. DAVID^[146] bioinformatic tool was used to assign Gene ontology (GO) terms to groups of proteins. Cytoscape^[147] (version 3.8.0) was used for network analysis with plug-ins STRING^[148] and ClueGO^[149]. Origin (Pro), Version 2020 (OriginLab Corporation, Northampton, MA, USA) was used to generate radar plots, bar plots and Venn diagrams. Heatmaps were created using RStudio and a custom-made R script.

4.7 Eicosanoid analysis

Standard Mixture	Name	Abbreviation	Conc. Stock solution [nM]	Final conc. [nM]
Standards spiked before solid phase extraction	12S-Hydroxyeicosatetraenoic acid d8	12S-HETE-d8	609,2	20,3
	15S-Hydroxyeicosatetraenoic acid d8	15S-HETE-d8	609,2	20,3
	5-Oxo-Eicosatetraenoic acid d7	5-Oxo-ETE-d7	1844,6	61,5
	11,12-Dihydroxy-5,8,14-icosatrienoic acid d11	11.12-DiHETrE-d11	1122,8	37,4
	Prostaglandin E2-d4	PGE2-d4	613,0	20,4
	20-Hydroxyeicosatetraenoic acid d6	20-HETE-d6	572,5	19,1
Standards spiked after solid phase extraction	5S-Hydroxyeicosatetraenoic acid d8	5S-HETE-d8	609,2	20,3
	14,15-Dihydroxy-5,8,11-icosatrienoic acid d11	14.15-DiHETrE-d11	572,5	19,1
	8-Isoprostaglandin F2 α d4	8-iso-PGF2 α -d4	1116,5	37,2

Table 2: Eicosanoid standards used as control for eicosanoid measurements. **Before** standards are spiked before protein precipitation and serve as internal control for the sample preparation. **After** standards are added during dissolving in LC standard conditions and serve as control for the LC-MS measurement.

Eicosanoids were analysed from cell culture supernatants containing FCS. The supernatant was therefore spiked with internal standards (Table 2) and added to ice cold ethanol (ratio 4:1) and the proteins precipitated over-night. Afterwards the sample was centrifuged at 4536 g and 4 °C for 30 min and the supernatant was transferred into a new 15 mL falcon. Ethanol was evaporated

via vacuum centrifugation at 37 °C until the original volume of the supernatant was reached ensuring less the 10% ethanol in the sample. The sample was then loaded onto a 30 mg mL⁻¹ StrataX solid phase extraction (SPE) column (Phenomenex, Torrance, CA) preconditioned with 2 mL Methanol. After washing the columns with 2 mL MS grade H₂O the cleaned sample was eluted using 500 µL methanol (MeOH abs; VWR International, Vienna, Austria) containing 2% FA. Samples were dried under Nitrogen stream and resolved in reconstitution buffer (64.8% H₂O, 31.5% ACN, 3.5% MeOH, 0.2% FA) containing another set of internal standards (Table 2). The samples were submitted to HPLC-MS analysis without further storage. The system consisted of a Thermo Scientific Vanquish (UHPLC) with a 15 cm Kinetex C18 column (C18, 2.6 µm particle size, 100 Å pore size, 2.1 mm diameter). Eluent A consisted of 98.8% H₂O with 0.2 FA while Eluent B was 89.9% ACN and 9.9% MeOH with 0.2% FA. 20 µL of sample was injected onto the column and the analytes were separated via a gradient from 35% to 90% eluent B within 10 min at a flow rate of 200 µL min⁻¹ with a total method time of 20 min including washing and equilibration of the column. A Q Exactive HF Hybrid Quadrupol-Orbitrap MS was coupled to the UHPLC via a HESI source run in negative ionisation mode. MS1 were recorded at a resolution of 60,000 (at 200 *m/z*) in a range from 250 to 700 *m/z*. The two most abundant ions were selected for fragmentation at 24% normalised collision energy in the HCD to look for unexpected oxylipins alongside the eicosanoids that were confirmed with synthetic standards. MS2 was operated at a resolution of 15,000 (at 200 *m/z*). The generated raw files were submitted to TraceFinder™ software (version 4.1) and a pre-existing list with eicosanoids, containing the exact mass and retention time was used to automatically integrate the peaks. The peak piking was manually verified. The generated intensity matrices were used in Perseus, Origin and R for statistical evaluation and creation of graphs and heatmaps as described for proteomics data.

4.8 Measurement of oxidised and reduced glutathione

For glutathione measurements a modified protocol from Tomin et al was used.^[48] 5*10⁵ cells were seeded in a 6-well plate in the appropriate medium, allowed to adhere for 24 h and treated according to the experimental setup. After incubation the supernatant was removed and the cells were washed fast and thoroughly with PBS twice. The cells were then incubated with 500 µL N-ethylmaleimide (NEM, 2.5 mM in PBS, Sigma-Aldrich) for 20 min at RT. After removal and washing with PBS the cells were lysed using 200 µL of 80% methanol in water for 20 min at 4 °C in the dark. The lysed cells were scraped of and centrifuged for 10 min at 13000 g to remove precipitated proteins. The supernatant was dried under nitrogen and resuspended in 100 µL NH₄OAc (50 mM, pH 7, Sigma-Aldrich). The sample was further cleaned using 300 µL dichloromethane (DCM, Sigma-Aldrich) and thoroughly vortexing the samples. After centrifugation (5 min, 13000 g, 4 °C) 45 µL of the upper watery phase were collected and reduced for 30 min at 37 °C with 2.5 µL TCEP (5 mM, pH 7, Sigma-Aldrich). Finally, 2.5 µL of N-ethyl-D5-

Maleimide (D5-NEM, 100 mM, Merck, Germany) were added for 20 min at RT and the samples diluted with 200 μ L of NH_4OAc (50 mM, pH 7).

For each sample 5 μ L were injected and measured on a Thermo Scientific Vanquish UHPLC coupled to a Q Exactive HF. A Kinetex XB-C18 column (2.6 μ m, 100 $\times$ 2.1 mm, 100 $\text{\AA}$, Agilent) was used for separation at 300 μ L min^{-1} . During the total runtime of 12 min a gradient of 0-30% mobile phase B (Methanol with 0.2% FA) in 5 min followed by 30-70% B in 2 was used. Afterwards the column was washed with 95% B for 3 min and finally equilibrated with mobile phase A (H_2O with 0.2% FA). MS1 scans were acquired in a mass range of 100-1000 m/z and a resolution of 60.000 (at 200 m/z). The reduced NEM-GSH form was found at 433 m/z and the oxidised D5-NEM-GSH adduct was found at 438 m/z . Tracefinder Software was used to analyse the data and gather the intensities which were used in Origin for further statistical evaluation and visualisation.

4.9 Melanoma brain metastasis model

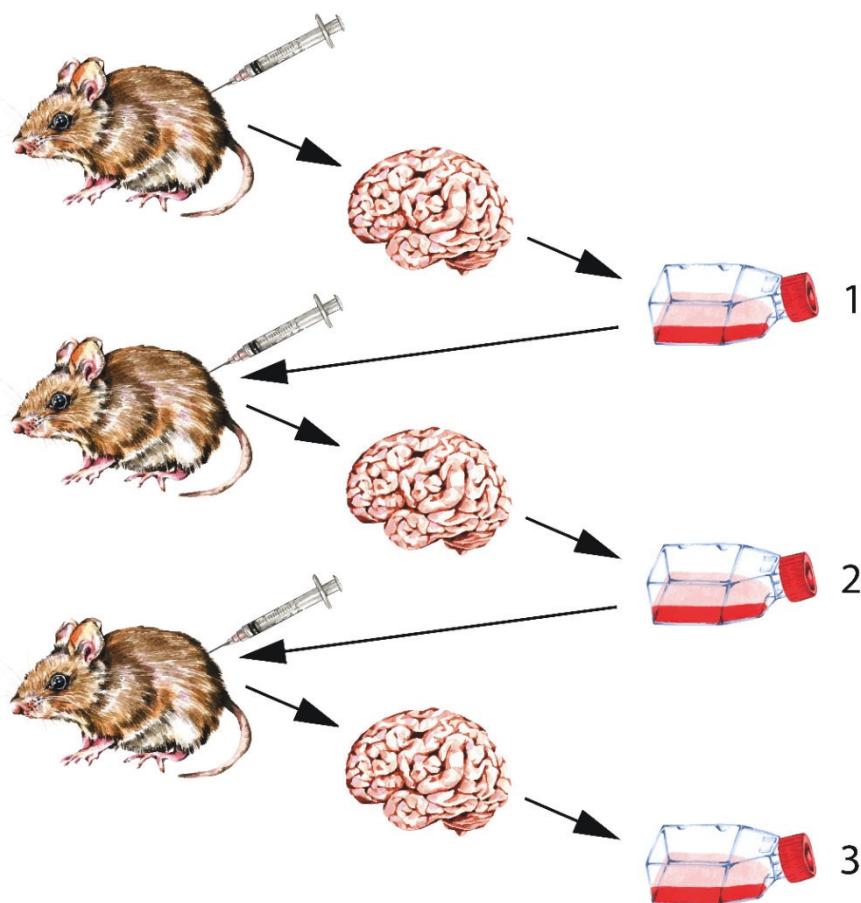


Figure 15: Schematic representation of the generation of the brain metastasis model. Cells are inoculated intracardiac into nude mice and the tumour formed in the brain is removed and again put into culture. The procedure is repeated 2-3 times as indicated by the numbers on the right until stable brain metastasis phenotype is formed.

Although the generation of the melanoma brain metastasis model (MBM) was not a direct task of this thesis the model does have a central importance within it and therefore requires further

explanation. Figure 15 depicts the workflow needed to create the brain metastasis model as described and carried out by Izraely et al.^[150] For this thesis 4 different human melanoma cell lines were used, namely YDFR, DP, M12 and M16. These four cell lines were used to generate a cutaneous and brain metastasis variant indicated with -C and -CB respectively. To create the cell lines male athymic nude mice (Balb/c background) at 7-10 weeks were housed under pathogen-free conditions. To get the subdermal C variant 10^6 cells of each cell line in 100 μ L of RPMI-1640 with 5% FCS were inoculated subdermally into the right thigh of the mice. After a solid tumour has formed the mice were sacrificed and the cells were put into culture. The creation of the CB variant was done several consecutive times to strongly associate the brain metastasis phenotype to the cell lines. Therefore, the cells were inoculated intracardiac into the anesthetised mice. After a solid tumour formed in the brain of the mice the animals were sacrificed and the tumour was removed and put into culture. This newly created cell line (CB1) was again inoculated intracardiac and the formed brain metastasis was removed, repeating 2-3 times (CB2, CB3) for each cell line.

4.10 List of abbreviations

cAMP – cyclic adenosine monophosphate, **CDM** – chemically defined medium, **CYT** – cytoplasm, **DDA** – data dependent acquisition, **DNA** – deoxyribonucleic acid, **DTT** – dithiothreitol, **EDTA** – ethylenediaminetetraacetic acid, **EGTA** – ethylene glycol-bis(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid, **ESI** – electrospray ionisation, **FA** – formic acid **FASP** – filter-assisted sample preparation, **FBS** – fetal bovine serum, **FCS** – fetal calf serum, **FDA** – food and drug administration, **FDR** – false discovery rate, **FITeXp** – functional identification of target by expression proteomics, **FT-ICR** – Fourier-transform ion cyclotron resonance, **GC** – gas chromatography, **GO** – gene ontology, **GSH** – glutathione (reduced monomer), **GSSG** – glutathione (oxidised dimer), **HCD** – higher-energy collisional dissociation, **HEPES** – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, **HETE** – hydroxyeicosatetraenoic acid, **HPLC** – high-performance liquid chromatography, **HSA** – human serum albumin, **IAA** – iodoacetamide, **IAP** – isotonic lysis buffer (isotoner Aufschlusspuffer), **IC50** – half maximal inhibitory concentration, **IMAC** – immobilised metal affinity chromatography, **kDa** – kilodalton, **LC** – liquid chromatography, **MBM** – melanoma brain metastasis, **MOA** – mechanism of action, **MS** – mass spectrometry, **MTT** – 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium, **NADPH** – nicotinamide adenine dinucleotide (reduced form, H), **NCI** – national cancer institute, **NE** – nuclear extract, **NEM** – N-ethylmaleimide, **PAL** – photo-affinity linker, **PBS** – phosphate buffered saline, **PC** – phosphatidylcholine, **PEG** – polyethylene glycol, **PMF** – peptide mass fingerprinting, **PMSF** – phenylmethylsulfonyl fluoride, **PSM** – peptide-spectrum-match, **PTM** – post-translational modification, **RNA** – ribonucleic acid, **ROS** – reactive oxygen species, **RP** – reversed phase, **RT** – room temperature, **SDS-PAGE** – sodium dodecyl sulfate polyacrylamide gel electrophoresis, **SEC** – size exclusion chromatography, **SFC** – supercritical fluid chromatography, **SILAC** – stable isotope labelling by amino acids, **SN** – supernatant, **SPE** – solid phase extraction, **TCEP** – tris(2-carboxyethyl)phosphine hydrochloride, **TLC** – thin layer chromatography, **TLCK** – Tosyl-L-lysyl-chloromethane hydrochloride, **TMT** – tandem mass tag, **TOF** – time of flight, **TPP** – thermal proteome profiling, **UHPLC** – ultra high-performance liquid chromatography

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6 Results and discussion

6.1 The Challenge of Classifying Metastatic Cell Properties by Molecular Profiling Exemplified with Cutaneous Melanoma Cells and Their Cerebral Metastasis from Patient Derived Mouse Xenografts

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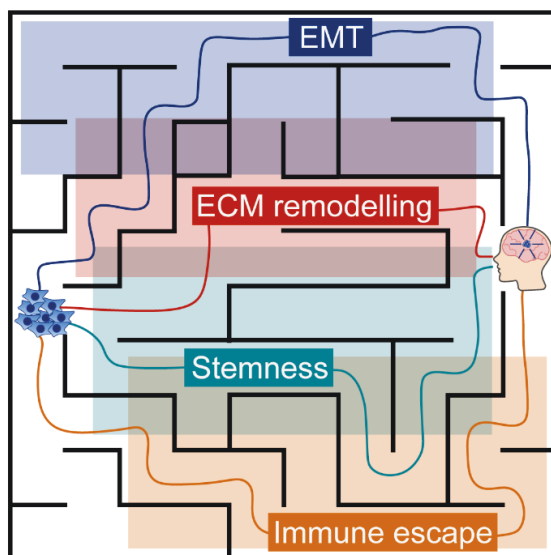
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Contributions to this publication:

- Planning and conduction of experiments and MS analysis
- Analysis and interpretation of data
- Preparation of figures and manuscript



The Challenge of Classifying Metastatic Cell Properties by Molecular Profiling Exemplified with Cutaneous Melanoma Cells and Their Cerebral Metastasis from Patient Derived Mouse Xenografts^{*§}

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The prediction of metastatic properties from molecular analyses still poses a major challenge. Here we aimed at the classification of metastasis-related cell properties by proteome profiling making use of cutaneous and brain-metastasizing variants from single melanomas sharing the same genetic ancestry. Previous experiments demonstrated that cultured cells derived from these xenografted variants maintain a stable phenotype associated with a differential metastatic behavior: The brain metastasizing variants produce more spontaneous micro-metastases than the corresponding cutaneous variants. Four corresponding pairs of cutaneous and metastatic cells were obtained from four individual patients, resulting in eight cell-lines presently investigated. Label free proteome profiling revealed significant differences between corresponding pairs of cutaneous and cerebellar metastases from the same patient. Indeed, each brain metastasizing variant expressed several apparently metastasis-associated proteomic alterations as compared with the corresponding cutaneous variant. Among the differentially expressed proteins we identified cell adhesion molecules, immune regulators, epithelial to mesenchymal transition markers, stem cell markers, redox regulators and cytokines. Similar results were observed regarding eicosanoids, considered relevant for metastasis, such as PGE2 and 12-HETE. Multiparametric morphological analysis of cells also revealed no characteristic alterations associated with the cutaneous and brain metastasis variants. However, no correct classification regarding metastatic potential was yet possible with the present data. We thus concluded that molecular profiling is able to classify cells according to known functional categories but is not

yet able to predict relevant cell properties emerging from networks consisting of many interconnected molecules. The presently observed broad diversity of molecular patterns, irrespective of restricting to one tumor type and two main classes of metastasis, highlights the important need to develop meta-analysis strategies to predict cell properties from molecular profiling data. Such base knowledge will greatly support future individualized precision medicine approaches. *Molecular & Cellular Proteomics* 19: 478–489, 2020. DOI: 10.1074/mcp.RA119.001886.

Molecular profiling of cells aims at the prediction of relevant cell properties eventually supporting the optimal choice of therapy based on individualized data related to a given patient. In fact, the prediction of cell properties directly related to the expression of a defined set of functional proteins such as e.g. matrix metalloproteinases mediating the degradation of extracellular matrix, already works in a reliable fashion (1). However, the prediction of complex cell properties rather indirectly related to an unknown number of mediators, such as malignant phenotypes of metastatic diseases, still poses major challenges. In this work we made use of metastatic melanoma cells with stable and well-described patterns of metastasis to evaluate whether and how such prediction of cell properties out of molecular profiling data might become feasible.

The incidence of brain metastasis in melanoma patients is one of the highest for all tumors and it is generally associated with poor prognosis (2). Moreover, brain metastasis generally

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relates to intrinsic drug resistance properties (3). Investigating genetic traits of such tumor cells revealed a significant genetic diversity among melanoma tumors associated with high mutation rates, eventually accounting for the present difficulties in understanding the underlying mechanisms of metastasis (4). To draw general statements on the molecular events sustaining the development of metastasis proves to be a very challenging task, sometimes associated with apparently contradicting conclusions. Remarkably, genetic traits of melanoma cells also hardly correlate with survival or with the time from primary diagnosis to the detection of brain metastasis (5). Thus, the absence or presence of certain mutations in key molecules such as BRAF, NRAS or KIT is not directly related to the capability of melanoma cells to colonize the brain (5). This was our motivation to apply post-genomic techniques searching for molecular patterns potentially associated with brain metastasis which might also support the functional understanding of accompanying drug resistance properties. We have previously applied proteome profiling to investigate melanoma drug resistance features as well as melanoma brain metastases (6–8).

To systematically investigate potential molecular patterns associated with brain metastasis, we have used stable and well-described melanoma cell models originating from four different patients. Primary melanoma cells isolated from the patients were xenografted into nude mice and repeatedly inoculated into either the hypoderm or the brain thus establishing human melanoma xenograft models encompassing four pairs of local (cutaneous - C variants) and brain metastasis variants (CB variants) (9). Stable phenotypes were obtained and subsequently characterized (10–12). Each corresponding pair of cutaneous and brain metastasis variants share an identical genetic background. Any molecular difference between C and CB cells of each pair may thus be mainly attributed to post-genomic differences between these variants originating from cellular adaptation to different microenvironments. Certain CB variants spontaneously migrate into the brain when inoculated subdermally (9) suggesting that these variants may have gained stable brain metastasizing properties.

The subsequent molecular profiling analysis of these variants was designed to support two independent strategies. First, the large number of identified proteins allowed us to specifically investigate how known molecular players are expressed in these models. Cell functions known to be related to metastasis comprising migration, intravasation, survival in circulation and extravasation through the blood brain barrier (13) were considered with priority. Second, statistical analysis was performed to search for potentially unknown molecules significantly associated with metastatic properties. The results demonstrate that indeed the applied molecular profiling methods revealed many apparently meaningful molecular alterations associated with the metastatic variants, supporting a potential classification of cells according to metastasis-related molecules. However, no molecular pattern could be

ascertained which would support an unequivocal classification regarding the known metastatic phenotypes of the cells. The data suggest that current classification strategies are not yet capable of predicting relevant cell properties in a satisfying fashion. We present evidence for the establishment of largely individual and specific strategies for metastasis on adaptation which need to be comprehended accordingly to make molecular profiling efficient for individualized precision medicine.

MATERIALS AND METHODS

Cell Culture Conditions and Sample Preparations—Cutaneous human melanoma cells (YDFR-C, DP-C, M12-C and M16-C) and brain metastatic human melanoma cells (YDFR-CB, DP-CB, M12-CB and M16-CB) were previously obtained as described previously (9). Briefly, human melanoma cells from four different melanoma patients were inoculated subdermally in nude mice to generate cutaneous melanoma tumors. Tumors were excised, minced into single cell suspension, and put in culture, until a pure melanoma culture was obtained, representing the cutaneous (C) variants. These cells were then inoculated intracardially 2–3 times into nude mice, each time resulting in the isolation and culturing of brain metastases, until a pure melanoma culture was obtained, representing the brain metastatic (CB) variants. These cells were subsequently cultured in RPMI 1640 (1X with L-Glutamine; Gibco, Thermo Fischer Scientific, Austria) with 10% (v/v) heat inactivated fetal calf serum (FCS¹, Sigma-Aldrich, Austria) and 1% (v/v) Penicillin and Streptomycin (Sigma-Aldrich) in humidified incubators at 37 °C and 5% CO₂. Cells were grown in T25 polystyrene cell culture flasks with cell growth surface for adherent cells (Sarstedt, Austria) and sub-cultured every 3–4 days. Therefor the medium was removed, the cells were washed twice with 5 ml PBS and incubated with 1.5 ml Trypsin-EDTA solution 0.25% (Sigma-Aldrich) for 3 min. The reaction was stopped by adding 4 ml fully supplemented medium and centrifuged for 5 min at 220 × g. After removing the supernatant, the cells were re-suspended in fully supplemented medium and seeded in a ratio of 1:6. Cells were regularly tested for absence of mycoplasma contamination and consistency in cell growth and morphology (i.e. size distribution and aggregation coefficient) was monitored during sub-culturing as well during the experiments with CASY TT Cell Counter and Analyzer (OMNI Life Science GmbH & Co. KG, Bremen, Germany). Cells were counted with a MOXI Z Mini Automated Cell Counter (ORFLO Technologies) using Moxi Z Type M Cassettes (ORFLO Technologies) and the number of seeded cells for the experiments calculated based of these results. For the performance of the assays, cells were used between passage 2 and 25.

Animals—Male athymic nude mice (BALB/c background) were purchased from Harlan Laboratories Limited (Israel). The mice were housed and maintained in laminar flow cabinets under specific pathogen-free conditions in the animal quarters of Tel Aviv University and in accordance with current regulations and standards of the Tel-Aviv

¹ The abbreviations used are: FCS, fetal Bovine Serum; ACN, acetonitrile; CSC, cancer stem cells; CYT, cytoplasm; ECM, extracellular matrix; EMT, epithelial–mesenchymal transition; FA, formic acid; FDR, false discovery rate; GSH, glutathione (reduced); GSSG, glutathione (oxidized); 12-HETE, 12-hydroxyeicosatetraenoic acid; NE, nuclear extract; NEM, N-ethylmaleimide; NP-40, nonidet P-40; PCA, principal component analysis; PGE2, prostaglandin E2; SIM, structured illumination microscopy; SN, supernatant; SPE, solid phase extraction; TCEP, tris(2-carboxyethyl)phosphine hydrochloride; TME, tumor microenvironment.

University Institutional Animal Care and Use Committee. The mice were used when they were 7–8 weeks old.

Orthotopic Inoculation of Tumor Cells and Metastasis Formation Assays— 1×10^6 melanoma cells in 100 μ l of 5% FCS supplemented RPMI 1640 medium were inoculated subdermally into the right thigh of male nude BALB/c mice. Brains were harvested 2 months following the inoculation and immediately frozen and stored at -70°C , until used for RNA extraction. Detection of human cells (micrometastases) in mouse brain by RT-qPCR was performed as described previously (9).

Proteomics of Cytoplasmic and Nuclear Fractions—For the proteomics analysis of the cytoplasmic and nuclear fraction 2×10^6 cells of passage 2 to 4 were seeded in a T25 cell culture flask with 3 ml of fully supplemented medium. After 24 h incubation the cells were lysed as previously described (14). In short, the medium was removed and the cells were carefully washed with 5 ml PBS twice. The lysis buffer was added onto the cells and lysed by pushing the suspension through a needle, centrifuged and the supernatant was precipitated in ice cold ethanol (EtOH, abs. 99%; AustroAlco, Austria). The remaining nuclear pellet was lysed with a Tris-EDTA, NP-40 buffer and after centrifugation the supernatant was again precipitated and kept on -20°C over night.

Supernatant Analysis of Eicosanoids and Proteomics— 2×10^6 cells of passage 4 to 6 were seeded in a T25 cell culture flask with 3 ml fully supplemented medium and kept in the incubator. After 24 h the supernatant was removed, spiked with 10–100 nM of each internal standard (supplemental Table S1), centrifuged with $726 \times g$ and 3 ml precipitated with 12 ml ethanol (EtOH, abs. 99%; AustroAlco) and stored at -20°C over night. Precipitated proteins containing fetal calf serum proteins were discarded, while the eicosanoids were isolated from the supernatant. The cells were washed twice with 5 ml PBS and 3 ml of RPMI without supplements was added. After 4 h incubation the supernatant was removed, centrifuged and precipitated in ethanol overnight to obtain the secreted proteins.

Protein Sample Preparation—The proteins were used for a filter-assisted protein digest as described previously (15). In short, the precipitated proteins were centrifuged at $4536 \times g$ for 30 min, the supernatant discarded and the protein pellet dried. After dissolving in sample buffer the protein concentration was determined and 20 μ g of total protein was used for the digestion. After reduction with dithiothreitol and alkylation with iodoacetamid (both Sigma-Aldrich) proteins were digested with Trypsin/Lys-C (MS grade; Promega Corporation, Madison, WI) and dried via vacuum centrifugation.

Eicosanoid Extraction—Samples were centrifuged (30 min, $4536 \times g$, 4°C) and the supernatant transferred to a new 15 ml Falcon tube. Ethanol was evaporated via vacuum centrifugation at 37°C until the original sample volume was restored. Samples were loaded on conditioned 30 mg/ml StrataX solid phase extraction (SPE) columns (Phenomenex, Torrance, CA). Columns were washed with 2 ml MS grade water and eicosanoids were eluted with 500 μ l methanol (MeOH abs.; VWR International, Vienna, Austria) containing 2% formic acid (FA; Sigma-Aldrich). MeOH was evaporated using N_2 stream at room temperature and reconstituted in 150 μ l reconstitution buffer ($\text{H}_2\text{O}/\text{ACN}/\text{MeOH} + 0.2\% \text{ FA} = 65:31.5:3.5$), containing a set of internal eicosanoid standards at a concentration of 10–100 nM (supplemental Table S1).

Analysis of Reduced and Oxidized Glutathione— 5×10^5 cells of passage 10–12 were seeded in a 6-well polystyrene plate with cell growth surface for adherent cells (Sarstedt) with 1 ml fully supplemented medium and allowed to grow for 24 h. Cells were washed twice with 1 ml PBS. After that, 500 μ l of 2.5 mM N-Ethylmaleimide (NEM, Sigma-Aldrich) in PBS was added to each well. Samples were kept in the dark for 20 min at room temperature. The NEM-PBS solution was removed; cells were washed twice with 1 ml PBS. Two

hundred microliters of 80% MeOH were pipetted to each well and incubated on ice for 20 min. Cells were scraped off, transferred into an Eppendorf tube and spun down for 10 min at $13,000 \times g$ and 4°C . The supernatant was moved to a 500 μ l Eppendorf Tube, dried under nitrogen stream and then re-suspended in 100 μ l 50 mM ammonium acetate (NH_4OAc ; pH = 7; Sigma-Aldrich). Three hundred microliters dichloromethane were added, and samples were vortexed. Next, samples were centrifuged for 5 min at $13,000 \times g$ and 4°C . Forty-five microliters of the upper phase were transferred to a new tube and 2.5 μ l of 5 mM Tris(2-carboxyethyl)phosphine hydrochloride (TCEP; pH = 7; Sigma-Aldrich) was added. Samples were left on a thermoshaker for 30 min at 37°C . Afterward, 2.5 μ l of 100 mM N-Ethyl-D5-Maleimide (D5-NEM, Sigma-Aldrich) were added and samples were again placed in the dark for 20 min at room temperature. Finally, samples were diluted 1:5 in 50 mM NH_4OAc and stored at 4°C until further analysis.

HPLC-MS/MS for Proteomics—For the HPLC-MS/MS analysis the peptides were resolved in 5 μ l 30% formic acid and diluted with 40 μ l of mobile phase A (97.9% H_2O , 2% acetonitrile, 0.1% formic acid). 1 μ l for the supernatant samples and 10 μ l of cytoplasmic and nuclear samples were injected into the Dionex UltiMate 3000 RSLCnano LC system coupled to the QExactive Orbitrap MS (all Thermo Fisher Scientific). Peptides were trapped on a C18 2 cm $\times$ 100 μ m precolumn and LC separation was performed on a 50 cm $\times$ 75 μ m Pepmap100 analytical column (both Thermo Fisher Scientific). For the supernatant samples 1 μ l of sample was injected. The 85 min HPLC method included a 43 min gradient from 7% to 40% mobile phase B (79.9% acetonitrile, 20% H_2O , 0.1% formic acid) at a flow rate of 300 nL/min. For the cytoplasmic and nuclear samples 10 μ l were injected. The 135 min HPLC method included a 95 min gradient from 8% to 40% mobile phase B at a flow rate of 300 nL/min. Mass spectrometric settings were the same for all fractions. The resolution on the MS1 level was set to 70,000 (at $m/z = 200$) with a scan range from 400 to 1400 m/z . The top eight abundant peptide ions were chosen for fragmentation at 30% normalized collision energy and resulting fragments analyzed in the Orbitrap at a resolution of 17,500 (at $m/z = 200$).

Proteomics Data Analysis—Raw data were subjected to the freely available software MaxQuant (version 1.6.0.1) utilizing the Andromeda search engine, followed by statistical evaluation with the Perseus software (version 1.6.0.2). For the MaxQuant search, a minimum of two peptide identifications, at least one of them being a unique peptide, was required for valid protein identification. Digestion mode was set to “Specific” choosing Trypsin/P. The peptide mass tolerance was set to 50 ppm for the first search and to 25 ppm for the main search. The false discovery rate (FDR) was set to 0.01 both on peptide and protein level, based on the q-value. The database applied for the search was the human Uniprot database (version 03/2018, reviewed entries only) with 20,316 protein entries. Further settings for the search included carbamidomethylation as fixed modification and oxidation of methionine and acetylation of the protein C terminus as variable modifications. Each peptide was allowed to have a maximum of two missed cleavages and two modifications, “Match between runs” was enabled and the alignment window set to 10 min, with the match time window of 1 min.

UHPLC-MS/MS for Eicosanoid Measurements—Analytes were separated using a Thermo Scientific Vanquish (UHPLC) system and a Kinetex[®] C18 - column (2.6 μ m C18 100 Å, LC Column 150 $\times$ 2.1 mm; Phenomenex[®]). Applying a 20 min gradient flow method, starting at 35% B steadily increasing to 90% B (1–10 min), going up to 99% B in 0.25 min. Flow rate was kept at 200 μ l/min, 20 μ l injection volume and column oven temperature was set to 40°C . Eluent A contains $\text{H}_2\text{O} + 0.2\% \text{ FA}$ and eluent B ACN:MeOH (90:10) + 0.2% FA.

Mass spectrometric analysis was performed with a Q Exactive HF Quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific), equipped with a HESI source for negative ionization. Mass spectra were recorded operating from 250 to 700 m/z at a resolution of 60 000 @ 200 m/z on MS1 level. The two most abundant precursor ions were selected for fragmentation (HCD 24 normalized collision energy), preferentially molecules from an inclusion list which contained 32 m/z values specific for eicosanoids (supplemental Table S2). MS2 was operated at a resolution of 15,000 @ 200 m/z . For negative ionization, a spray voltage of 2.2 kV and a capillary temperature of 253 °C were applied, with the sheath gas set to 46 and the auxiliary gas to 10 arbitrary units.

Raw files generated by the Q-Exactive™ Orbitrap™ were analyzed manually using Thermo Xcalibur 4.1.31.9 (Qual browser), comparing reference spectra from the Lipid Maps depository library from July 2018 (3). For peak integration the software TraceFinder™ (version 4.1 - Thermo Scientific) was used.

UHPLC-MS/MS for Targeted Glutathione Measurements—Chromatographic separation was performed on a Kinetex 2.6 μ m XB-C18 100Å (100 × 2.1 mm) column using a 1290 Infinity UHPLC System (Agilent Technologies). Mobile phase A consisted of water with 0.2% formic acid and mobile phase B of methanol with 0.2% formic acid. The following gradient profile was run: 0 to 30% B in 5 min, 30 to 70% B in 2 min, afterward washed for 3 min at 95% B and at 10.1 min the system was reequilibrated at 0% B giving a total runtime of 12 min. Flow rate was 0.3 μ l/min, the column temperature was set to 40 °C and the injection volume was 5 μ l. MS detection was performed in the positive ion mode on a triple quadrupole mass spectrometer (QqQ 6490, Agilent technologies).

ESI spray voltage was 4500V, gas flow 12 L/min, gas temperature 250 °C, sheath gas temperature 400 °C, sheath gas flow 12 L/min, and nebulizer gas pressure 35 psi.

MRM transitions and the collision energies are summarized in supplemental Table S3. As quantifier in the final analysis of GSH-NEM the transition from m/z 433 to 84.2 was used, for GSH-d5NEM the transition from m/z 438 to 84.2 was used. The other transitions were used as qualifiers to ensure identity of the analytes.

GSH-NEM is a diastereomer, therefore GSH-NEM and GSH-d5NEM elute as two peaks because they have different physical and chemical properties (16). The areas under both peaks were summarized and used for integration and quantification. The obtained raw data was processed with Skyline (MacCoss Lab Software).

Experimental Design and Statistical Rationale—Three independent cell culture experiments were carried out for every cell line. All samples were analyzed with LC-MS/MS in 2 technical replicates which resulted in 144 measurements for the proteomics, 48 measurements for eicosanoids and 48 measurements for glutathione. The proteins were identified using MaxQuant software (version 1.6.0.1), including the Andromeda search engine. For statistical evaluation, the Perseus software (version 1.6.0.2) was used. Potential contaminants, reversed sequences and proteins identified only by site were removed. The normal distribution of the data was manually checked using the histogram function. After the Label-free quantification (LFQ) values were logarithmized to base 2, the technical replicates were averaged. Invalid values were filtered with the criteria that at least 70% of the samples per group, being each individual cell line, have valid values. After application of these procedures, a final list of 5665 protein identifications regarding cytoplasmic and nuclear fractions and 1238 proteins in the supernatants was obtained. The still missing LFQ values were then replaced from a normal distribution with a down shift of 1.8 and a width of 0.3 to enable t-tests. Volcano plots were created to analyze the significantly regulated proteins ($FDR < 0.05$, $S0 = 0.1$). Identified proteins as well as statistical values of the t-tests are available in supplemental Tables S5–S8. The heatmaps were gener-

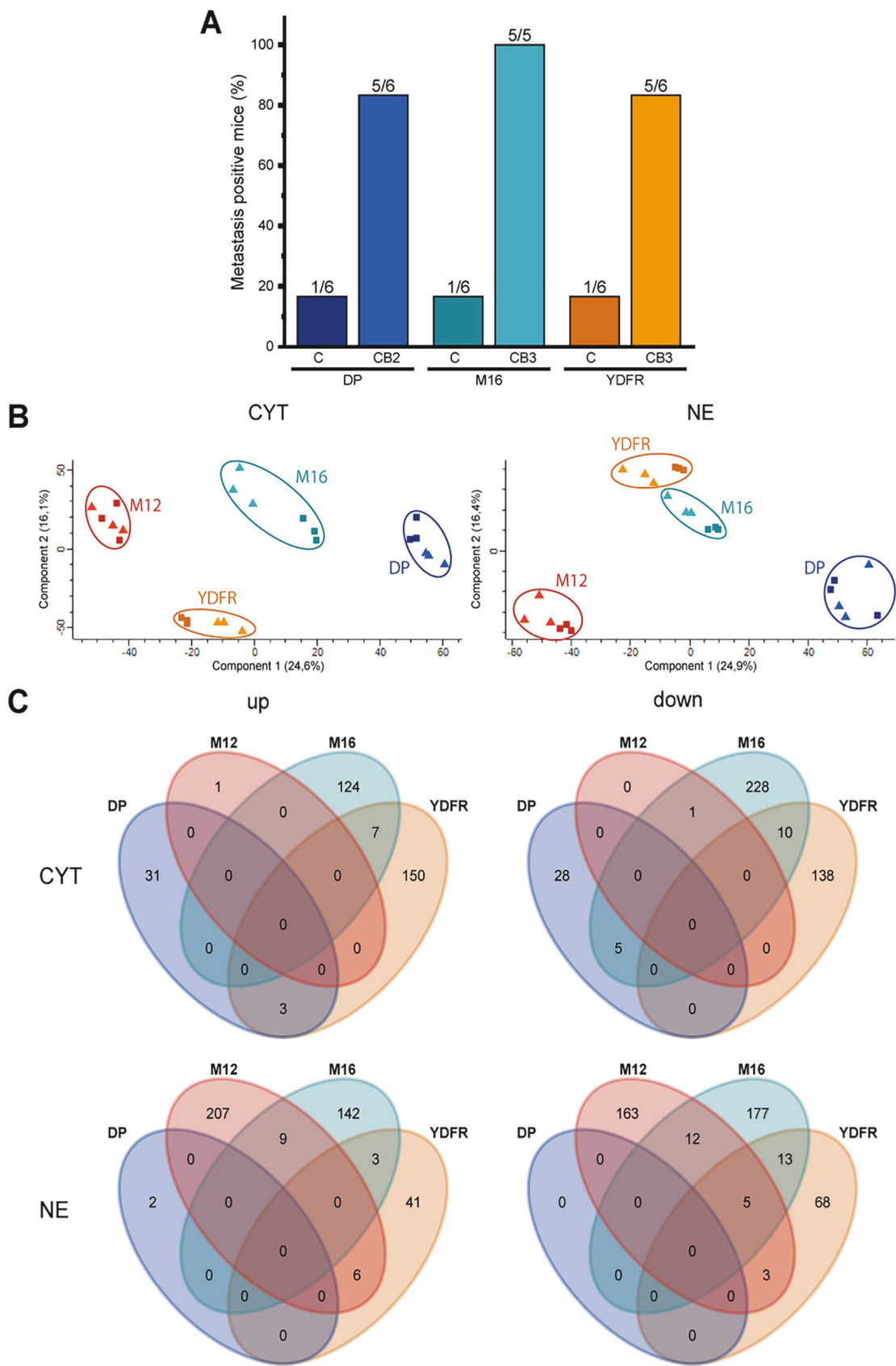
ated by a custom R (<https://www.r-project.org>) script. OriginPro 2018b was used for the generation of the spiderweb plots (Fig. 4), for the bar plots (Fig. 3) as well as for the two-sided-t-tests for the eicosanoid data ($S0 = 0.1$) which is indicated in the heatmap (Fig. 2).

Morphological Evaluation Using Bright-field Microscopy—To obtain the multiparametric evaluation of the morphology of the melanoma variants, cells were seeded in 6-wells plates (polystyrene, Cell+ growth surface for adherent cell; Sarstedt AG & Co., Nümbrecht, DE). After 72 h settling time, bright field images were taken with an Olympus CKX53 Inverted Microscope (10× magnification). Images were analyzed manually deriving area, perimeter, minimum and maximal radius of the cells with the software CellSens Entry. Circularity (4π area/perimeter) and roundness (minimum radius/maximum radius) were calculated as previously described (17). For the purpose of the study, 8–10 cells were randomly chosen from 5–6 optical fields and the experiments were repeated in 5 independent cell preparations, resulting in the quantification of more than 200 cells for every condition. To avoid selection bias, image analysis was performed after acquiring all the images from all the experiments and quantification workflow was independently verified with the software Gen5 (BioTek, Winooski, VT). Determination of cell volume and diameter was performed as previously described with the cell counter analyzer CASY-TT (18). Briefly, cells were gently detached with trypsin and after inactivation of the enzyme 100 μ l cell suspension was diluted in 10 ml CASYton solution. Measurement range threshold was optimized at the beginning of the experiments and kept constant for all the subsequent analysis. Measurements were performed in technical duplicates from 5 independent cell preparations. For the graphical representation of the results values were corrected through adjustment values as reported in supplemental Table S4 and the software Origin Pro 9.1G was used for the generation of the spiderwebs.

Immunolocalization Nrf2—For the localization of Nrf2, an immunofluorescence experiments were performed for all the melanoma variants as previously described (19, 20). Briefly, cells were seeded on μ -Slides (35000 cells/well, Ibidi treatment coating, ibidi GmbH Martinsried, Germany), fixed with pre-warmed formaldehyde (FA, 3.7%, 15 min) and permeabilized with Triton X (0.2%, 10 min). Blocking of unspecific binding sites was obtained with donkey serum (2%, 1h). For the localization of transcription factor a mouse monoclonal and Nrf2 antibody was used (ab 89443 Abcam, dilution 1:600, 2 h). Detection was obtained with a donkey anti mouse antibody (Alexa Fluor 488-conjugated, 715–545-150 Jackson ImmunoResearch, dilution 1:1000, 1.5 h). After washing, cells were post-fixed with FA (10 min.) and glycine (100 mM) was used to mask reactive sites. Slides were mounted and sealed with Roti-Mount FluoCare with DAPI to counterstain the nuclei (Roth, Graz, Austria). Images were acquired with a Confocal LSM Zeiss 710 equipped with ELYRA PS. 1 system. Structured illumination microscopy (SIM) images were acquired with a Plan Apochromat 63X/1.4 oil objective.

RESULTS

Unbiased Statistical Analysis of Proteome Profiles of Metastatic Variants—Four pairs of melanoma cells representing cutaneous (C) and cerebellar (CB) variants of metastases, here in short referred to as DP, M12, M16 and YDFR, were analyzed for this study. The distinct metastatic potential of the CB and C variants, as used for the molecular profiling experiments, was successfully verified in case of three cell pairs as depicted in Fig. 1A. Almost all animals established brain metastases on subdermal inoculation of any of the CB variants, whereas only one out of six tested animals did so in case of the C variants. Label-free proteome profiling was performed



with all four cell pairs after cell fractionation to obtain cytoplasm (CYT), nuclear extracts (NE) and cell supernatants (SN). The experimental workflow is depicted in [supplemental Fig. S1](#).

A principal component analysis (PCA) of CYT and NE fractions revealed a clear distinction of all four cell pairs but rather incomplete separation of the C and CB variants (Fig. 1B). This would indicate that the variation between donors exceeds the variation observed between C and CB variants. Only in case of M16 and YDFR cells the variants were fully separated in both fractions, whereas the DP variants were only separated in the cytosolic fraction and the M12 variants only in the nuclear extracts. PCA of cell supernatants revealed similar results ([supplemental Fig. S2A](#)). Imaginary vectors in the PCA graphs pointing from the experiments representing C variants to experiments representing corresponding CB variants would point in different directions. These observations already indicated rather few commonalities shared by the CB variants representing a distinct metastatic phenotype.

Wondering whether we would be able to identify a proteome signature associated exclusively with the metastatic subgroup, a statistical analysis comparing all CB variants to all C variants was performed. However, not a single protein regulatory event was found apparently associated with a metastatic variant. Two scenarios might explain such a result. Either the experimental variation is too high rendering the power of the analysis insufficient or, alternatively, no specific commonality between the individual differences between C and CB variants would potentially be able to account for the distinct cell properties. Actually the experimental variation seemed to be rather acceptable, as all CB-C pairs displayed significant proteome alterations ($FDR < 0.05$) when compared with each other separately (Fig. 2). However, comparing the commonalities of these deregulated proteins between all pairs revealed only very little overlap (Fig. 1C). Although the CB variants of M16 and YDFR shared at least a small number of regulated proteins compared with the corresponding C variants, the other donors showed rather different regulatory events.

Although the strategies listed above were restricted to the consideration of significant regulatory events, we wondered whether some kind of co-regulated proteome signature would emerge considering all proteome alterations regardless of significance thresholds. To this aim, we blotted ranked difference levels between CB-C pairs and projected difference levels of the corresponding proteins derived from another CB-C pair. As illustrated in [supplemental Fig. S3](#), no similarities between such regulatory patterns were becoming apparent. In addition, correlation analyses of all regulatory events

regardless of significance levels between all potential pairs of donors was performed and depicted in [supplemental Fig. S4](#). All R^2 values of these correlation analyses were as little as ranging from 0.0001 to 0.05. These observations again supported the notion of a lack of commonalities of CB-related proteome alterations.

Specific Consideration of Metastasis-related Proteins Distinguishing CB and C Variants—To test the hypothesis that the CB variants may have altered the proteome profile in a biologically meaningful fashion providing them with relevant properties, we not only performed statistical analyses in an unbiased fashion as described above, but also focused on known metastasis-related proteins. In a first step, the question was investigated whether such proteins were found altered in any of the analyzed CB-C pairs. Thus, we started to investigate the significantly altered proteins depicted in Fig. 2 and listed in [supplemental Tables S5–S8](#) whether they comprised such proteins.

Results of this analysis are visualized in heatmaps (Fig. 3). First, clinically relevant melanoma marker molecules PMEL and MLANA (21) were detected in all cells and found significantly down-regulated in the YDFR-CB variant. All detected melanoma markers varied substantially between the cells of the different donors, whereas CSPG4 (21, 22) was found uniformly increased in all four CB variants lacking significance in all of them. Proteins potentially regulating immune responses of the host such as class II histocompatibility antigens HLA-DRB1 and CD74 (23), and the innate immune response regulator ANXA1 were found specifically altered between the variants and between the donors with no consistent expression pattern. The same notion applies to cell adhesion molecules TGFBI, L1CAM, LAMC1, ITGAV, THBS1 (24), the EMT marker molecules FN1, CDH2 and VIM (25), and the cancer stem cell marker molecules NGFR (CD271), ALDH1A3, and PLEKHA5 (26) as depicted in Fig. 3A. In contrast, the cancer stem cell marker molecule SOX10 was rather uniformly expressed in all eight cells (Fig. 3A). The analysis of secretomes specifically identified inflammatory mediators such as interleukin-6 and the chemokine CXCL2, and a broad variety of extracellular matrix proteins (Fig. 3B). In addition, the lymphangiogenesis regulator INHBA (27), the fibroblast activating protein basigin/CD147/BSG (28), the multiple cell regulator CCN3/GOV (29), and the exosome marker molecule CD9 (30) also specifically altered between the variants and between the donors with no consistent expression pattern. LDHA levels in the cell supernatants served to control for necrotic cell death levels and were found to hardly vary (Fig. 3B).

Fig. 1. A, Percentage of mice with positive brain metastasis after subdermal inoculation with cultured melanoma cells as used for the present experiments. B, Principal component analysis (PCA) for cytoplasmic (CYT) and nuclear fraction (NE). The C variants are represented by the rectangles whereas the CB variants are indicated by the triangles. C, Venn diagrams comparing all significant regulations indicated in the volcano plots (Fig. 2). The diagrams show the up- and down-regulated proteins from the cytoplasm and nuclear fraction separately.

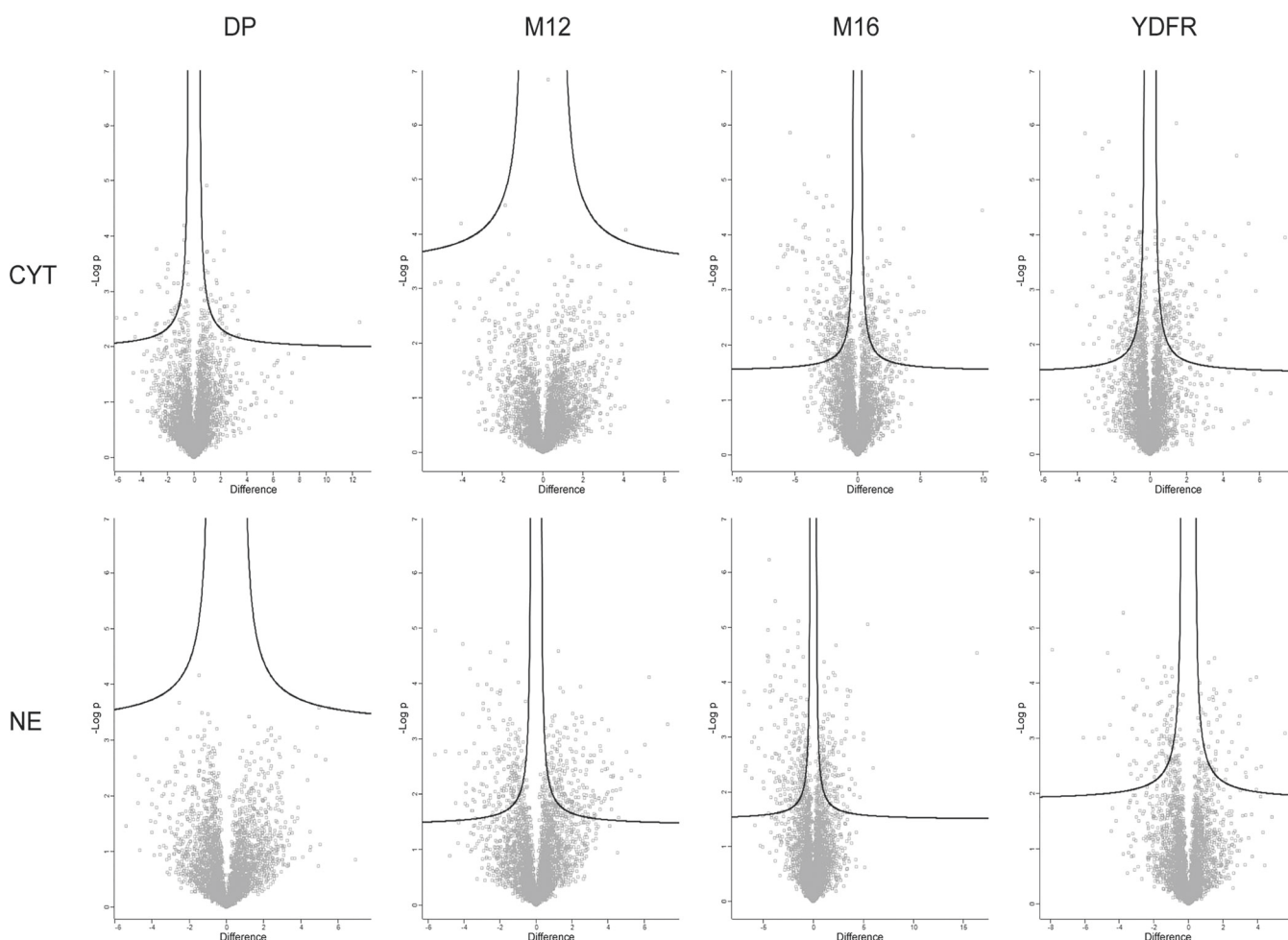


FIG. 2. Volcano plots for all 4 cell pairs comparing each C-CB pair (FDR < 0.05, S0 = 0.1). Up- or down-regulation corresponds to higher or lower LFQ intensities in the CB variant compared with the C variant, respectively.

Eicosanoid Formation in Metastatic Variants—Besides genes and proteins, lipids and especially eicosanoids with hormone-like activities were described to have the capability to affect and control metastatic processes (31). This is why the analysis of the cancer and metastasis promoting molecules PGE2 (32) and 12-HETE (33) was included in the present study. Indeed, significant alterations of the precursor molecule arachidonic acid as well as PGE2 and 12-HETE were observed between some C-CB pairs, again potentially contributing to a molecular classification system for metastatic properties. However, again none of these alterations showed uniformity regarding the metastatic variants and would thus be able to predict the given cell phenotype (Fig. 3C).

Redox Regulation in Metastatic Variants—Redox regulation is known to be highly relevant for metastasis and invasion (34). Thus, redox-regulating proteins as well as the ratio of reduced *versus* oxidized glutathione were investigated. Although no significant alterations were detected, the determined glutathione levels were found to remarkably parallel expression levels of glutathione reductase, an essential enzyme for glutathione metabolism (Fig. 4A, 4B). The transcrip-

tion factor NRF2 is known to coordinate antioxidant responses in cells and mediates MAPK inhibitor resistance in melanoma cells (20, 35). Although not successfully identified by proteome profiling, we performed immunofluorescence staining and detected positive signals in all investigated melanoma cells (Fig. 4C). In accordance with the glutathione measurements, no characteristic difference in the localization of the transcription factor (nuclear/cytoplasmic ratio) was observable in any of the c-CB cell pairs.

Cell Morphology Assessment in Metastatic Variants—On cell culture, some specific morphologic features of the cells were becoming apparent. EMT affects both cell morphology and the cells capability for metastasis (36). This motivated us to test the hypothesis that EMT-associated features might correlate with the different variants of metastasis. Eight different morphologic features of the cultured cells were determined manually. Microscopic images of the analyzed cells as well as spider webs depicting these selected morphologic characteristic are shown in Fig. 5. The spiderwebs revealed a similar tendency for all the 4 pairs (Fig. 5B) and in general a more “consistent” appearance for the cutaneous

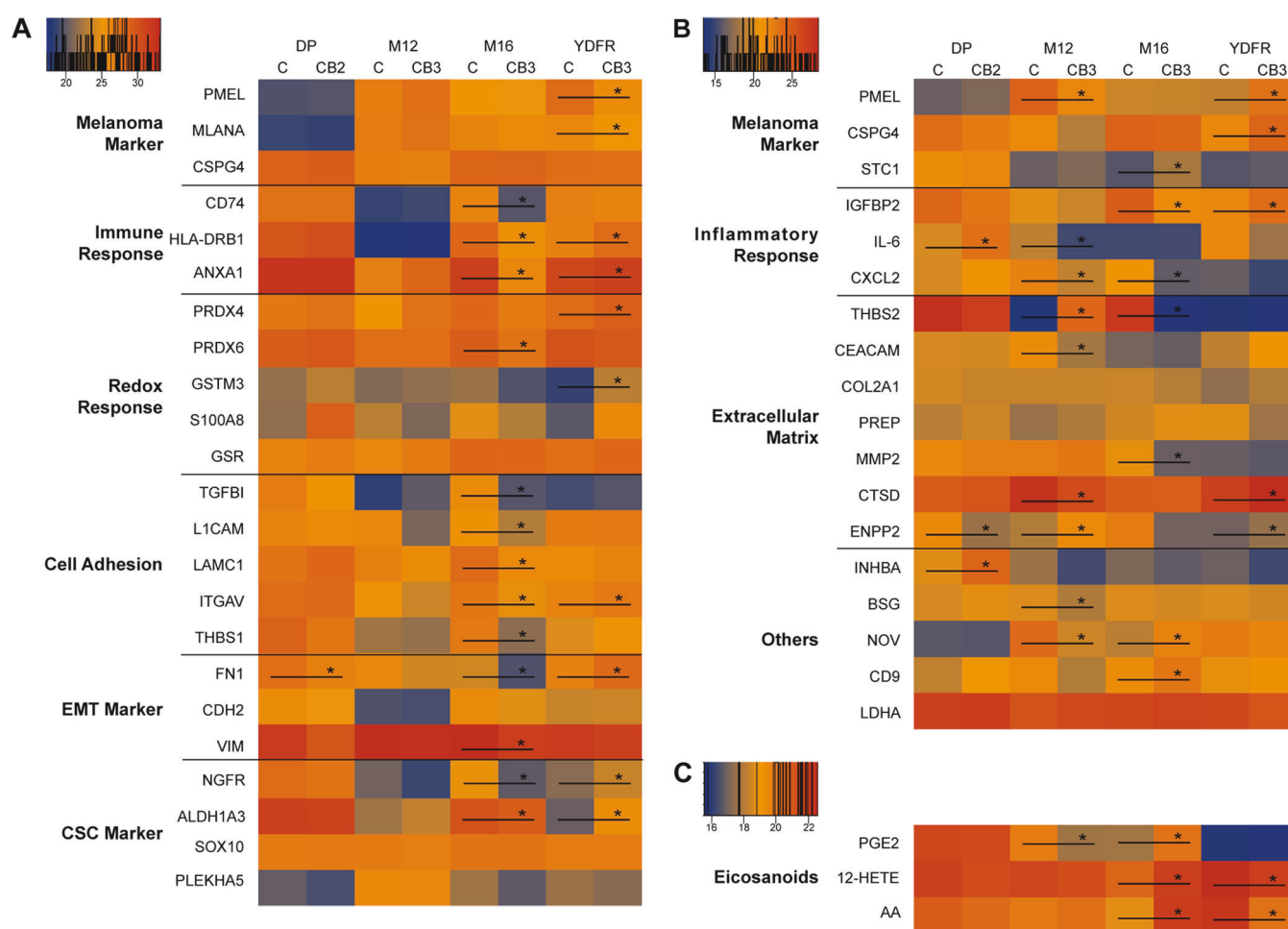


FIG. 3. **A**, Heatmap for selected proteins of the cytoplasmic fraction for all cell lines. **B**, Heatmap for selected proteins of the supernatant. Legend values are logarithmic LFQ intensities calculated by MaxQuant. **C**, Heatmap for the eicosanoids PGE2, 12-HETE and the precursor AA of the supernatant. Values correspond to logarithmic intensities normalized to the internal deuterated standards. Black bars with stars (*) indicate significant regulations (FDR < 0.05, S0 = 0.1) between C and CB variants.

variants compared with the brain metastasis. However, although characteristic features were discerned, no uniform alterations were found to successfully distinguish cutaneous from brain metastases.

DISCUSSION

The process of metastasis into distant organs such as the brain is complex, encompassing several steps including detachment of cells associated with ECM degradation, invasion through basement membranes into neighboring tissues, intravasation into the blood stream or lymphatic vessels and arrest and extravasation at a distal hospitable site to form the metastatic lesion (13). It is still not trivial to identify specific proteins or other molecules employed by the tumor cells to cope with these different kinds of challenges *in vivo* (37). Thus, it may be expected that it is hard to find a uniform molecular pattern associated with a given metastatic phenotype. The challenge is greatly aggravated by the known inter-tumor heterogeneity (38) as well as the plasticity of tumor cells

in general (5) and of melanoma cells (16, 39). The challenge of inter-tumor heterogeneity underlying divergent clinical manifestations of different tumors belonging to the same histological type as well as distinct responses of such tumors to therapy is addressed by current precision medicine approaches (35, 40). The need for improved prediction capabilities out of molecular profiling data is thus evident.

The functional plasticity of tumor cells independent of genetic alterations is well recognized, rendering it conceivable that selection processes exerted by given *in vivo* conditions such as hypoxia, metabolic stress, biophysical stress or other tumor microenvironment (TME-) mediated effects may influence the properties of a developing tumor. We have previously aimed at the characterization of such kind of selection processes in case of chronic lymphocytic leukemia exerted via aging-related cell stress (15) and in case of multiple myeloma exerted via hypoxia (41).

The present model for brain metastasis, though associated with limitations as any other model, has major advantages to

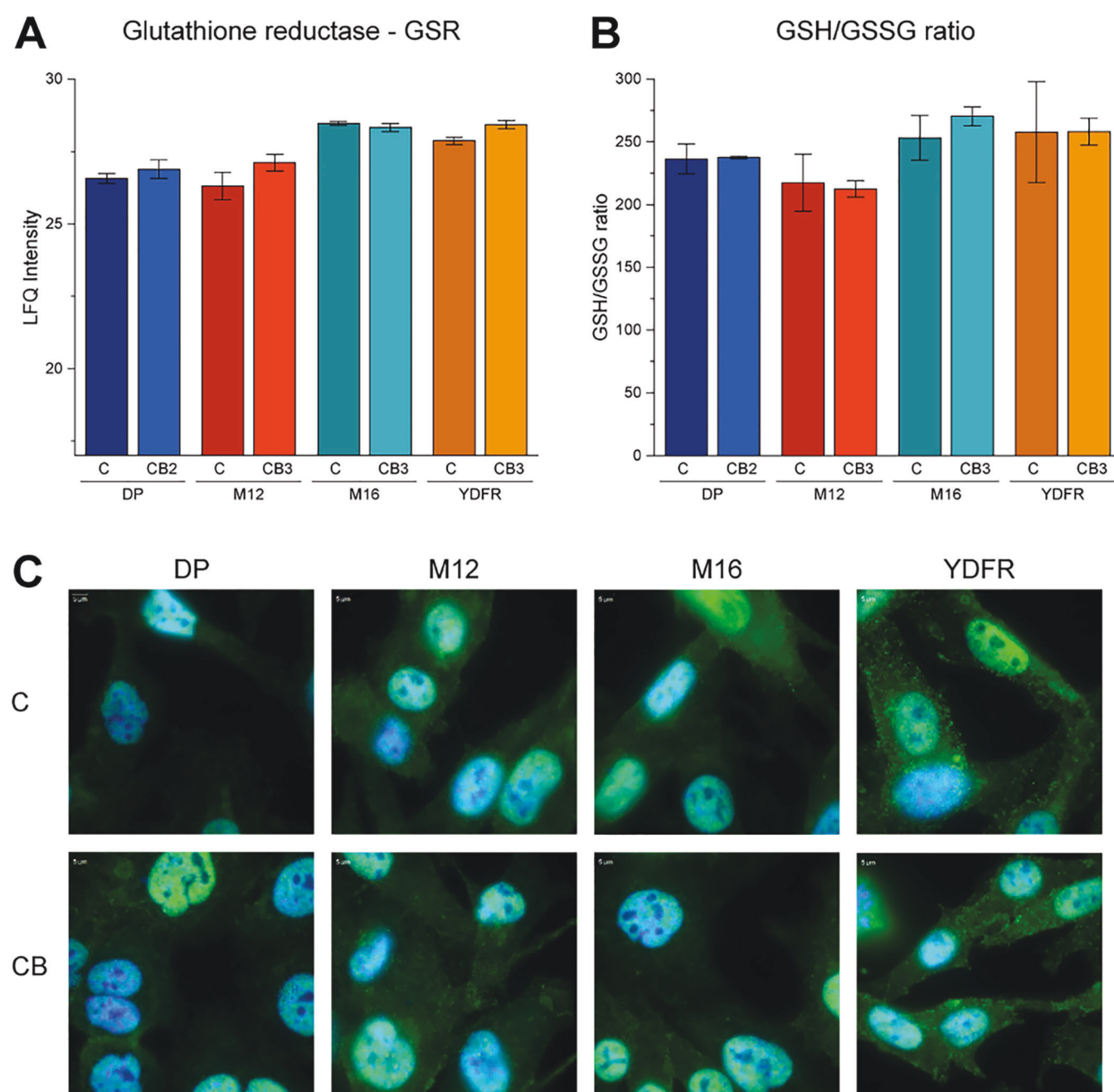


FIG. 4. **A**, LFQ intensities of glutathione reductase (GSR) of cytoplasmic fraction. **B**, Ratio of reduced (GSH) and oxidized (GSSG) form of glutathione (GSH/GSSG) of internal cellular intensities. **C**, Fluorescent microscopy shows the cellular distribution of NRF2 (green), co-stained with DAPI (blue) to indicate the nuclei.

address these challenges. The cells derived from a common genetic ancestry were exposed to selection processes exerted by two different kinds of microenvironment via successive xenotransplantation homing into the sub-dermis or the cerebellum, respectively. The cells were subsequently grown *in vitro* at the same standard cell culture conditions and demonstrated to stably maintain different properties obviously related to their previous adaptation to the different kinds of microenvironment (Fig. 1A) (9). Thus the presently described differences between

the local and metastatic (C and CB) variants represent intrinsic properties determined by their differential somatic genetic or epigenetic profile. These differences apparently remain independent of their actual microenvironment, as these two types of variants were maintained under standard cell culture conditions devoid of influences exerted by the original microenvironment. However, the original exposure to organ-specific microenvironments seems to have imprinted in the cells stable properties determining their metastatic phenotype.

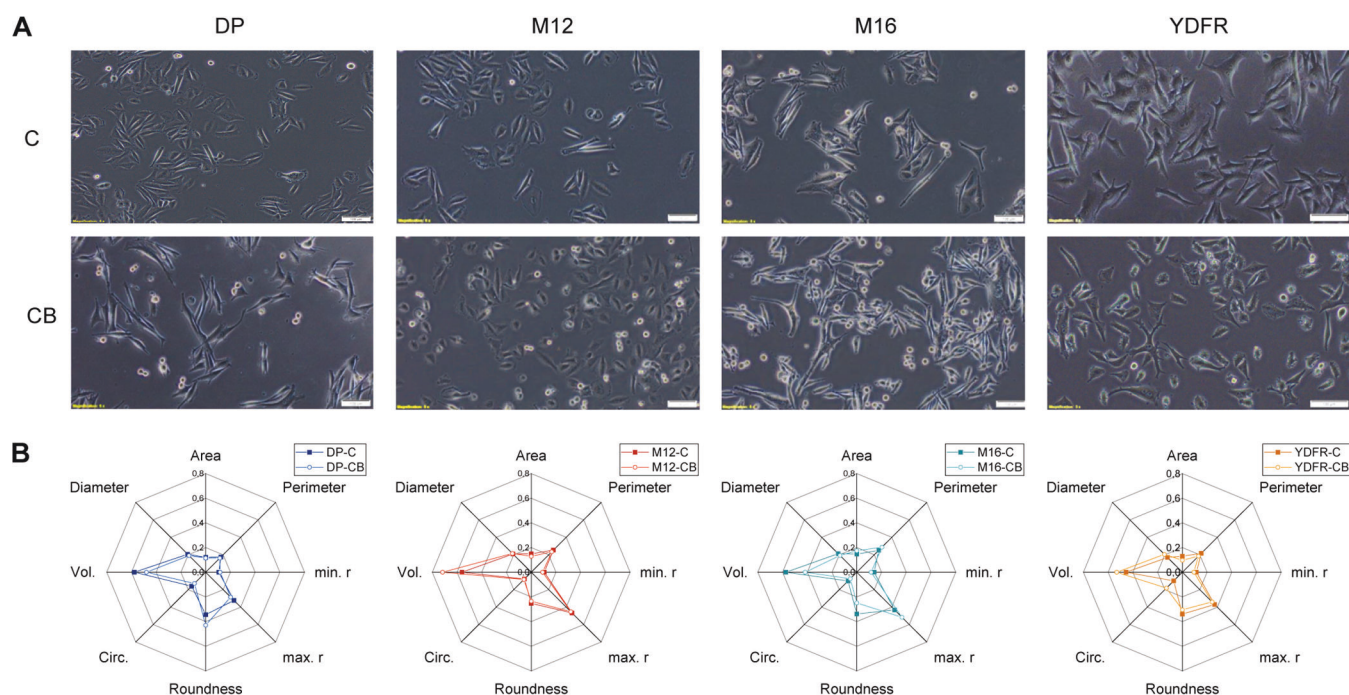


FIG. 5. **A, Morphological appearance of the melanoma variants; scale bars 100 μm .** **B, Multiparametric morphological evaluation of melanoma cells: area [μm^2], perimeter [μm], max. radius [μm], min. radius [μm], circularity, roundness, volume [fL] and diameter [μm].**

Genetic traits, usually accounting for differences in stable phenotypes, do not always account for the potential to form metastasis in general and in the brain (42–44). If brain-metastasis drivers do indeed exist, we searched for molecular patterns that would differentiate local melanomas from corresponding brain metastasis. For this purpose we employed post-genomic techniques such as proteomics and eicosadomics as applied previously (22, 45).

Rather subtle alterations in the proteome profile were detected when comparing CB-C variants in case of two patients and more pronounced in case of the other two. However, indeed these alterations comprised many proteins known to be critical for mechanisms of metastasis.

This is the reason why we consider the present findings representative and relevant to the understanding of metastasis. Although a large variety of proteins including secreted proteins and lipid mediators, *i.e.* eicosanoids, were differentially expressed in local and brain metastatic variants, no characteristic pattern for metastatic properties emerged. If there was a common mechanism for brain metastasis, it seems that the presently employed analysis strategies were not yet able to discern it.

We suggest this observation is characteristic for a common but hardly recognized limitation of current molecular profiling analysis strategies. Functional cell properties based on rather linear molecule-to-function relations may be reliably classified, *i.e.* the expression of metalloproteinases directly mediates the capability of cell invasion. In contrast, complex cellular properties such as metastatic features potentially emerging from networks consisting of many interconnected

molecules are hard to discern. Similarly, a photograph is composed of many pixels, associated with defined colors and signal intensity values. If the same pixels are stochastically scattered regarding their position, we lose the capability to interpret such a photograph correctly. We cannot interpret a photograph by the consecutive assessment of single data points, the relevant information can only be retrieved as meta-data emerging from the positional information of each pixel. In general, a molecular profiling data set contains quantitative information regarding a large number of distinct molecules. Although gene ontology classification allows us to relate most proteins to cellular properties, we are hardly capable of organizing the data points supporting improved retrieval of relevant meta-information as we can do in case of a photograph.

The present data strongly suggest there is no single regulatory event associated to a molecule which can determine metastatic properties of cancer cells. Future development of data interpretation strategies will demonstrate whether all the information contained in the present data would theoretically be enough to correctly classify the tumor cells.

Conclusions and Outlook —To search for a molecular classification strategy discerning two different variants of metastasis we made use of a molecular profiling approach combined with morphological characterization of eight cell lines paired as cutaneous and brain metastasis. Focusing on the protein expression levels we were able to observe several significant proteomic profiles related to biological processes that are known to be essential for metastasis. Every cell line displayed specific molecular profiles that may contribute to

the cell properties observed *in vivo*, but no classification strategy appeared yet suitable to classify the cells correctly. These observations are in line with the known plasticity and heterogeneity of tumor cells. We suggest that a common effort of the scientific community focusing on the development of improved data analysis strategies will be required to eventually qualify molecular profiling as a prediction tool supporting individualized precision medicine.

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DATA AVAILABILITY

The Proteomics data have been deposited to the ProteomeXchange Consortium (<http://proteomecentral.proteomexchange.org>) via the PRIDE repository and is available via the project accession PXD013765.

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§ This article contains [supplemental material](#).

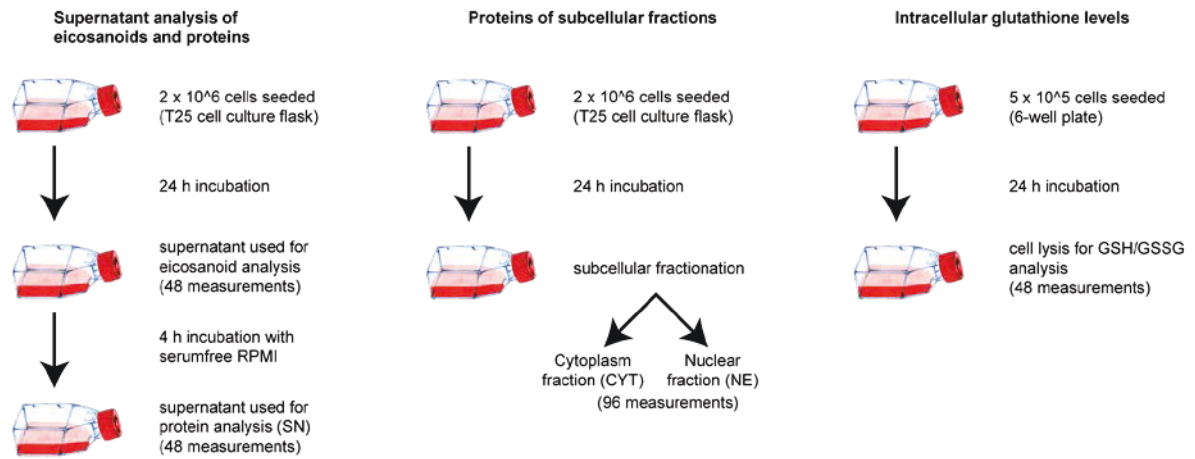
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Author contributions: B.N., L.J., L.N., J.B., K.K., and G.D.F. performed research; B.N., G.D.F., and C.G. analyzed data; B.N., I.P.W., and C.G. wrote the paper; S.I., O.S.-A., T.M., B.K., I.P.W., and C.G. contributed new reagents/analytic tools; I.P.W. and C.G. designed research.

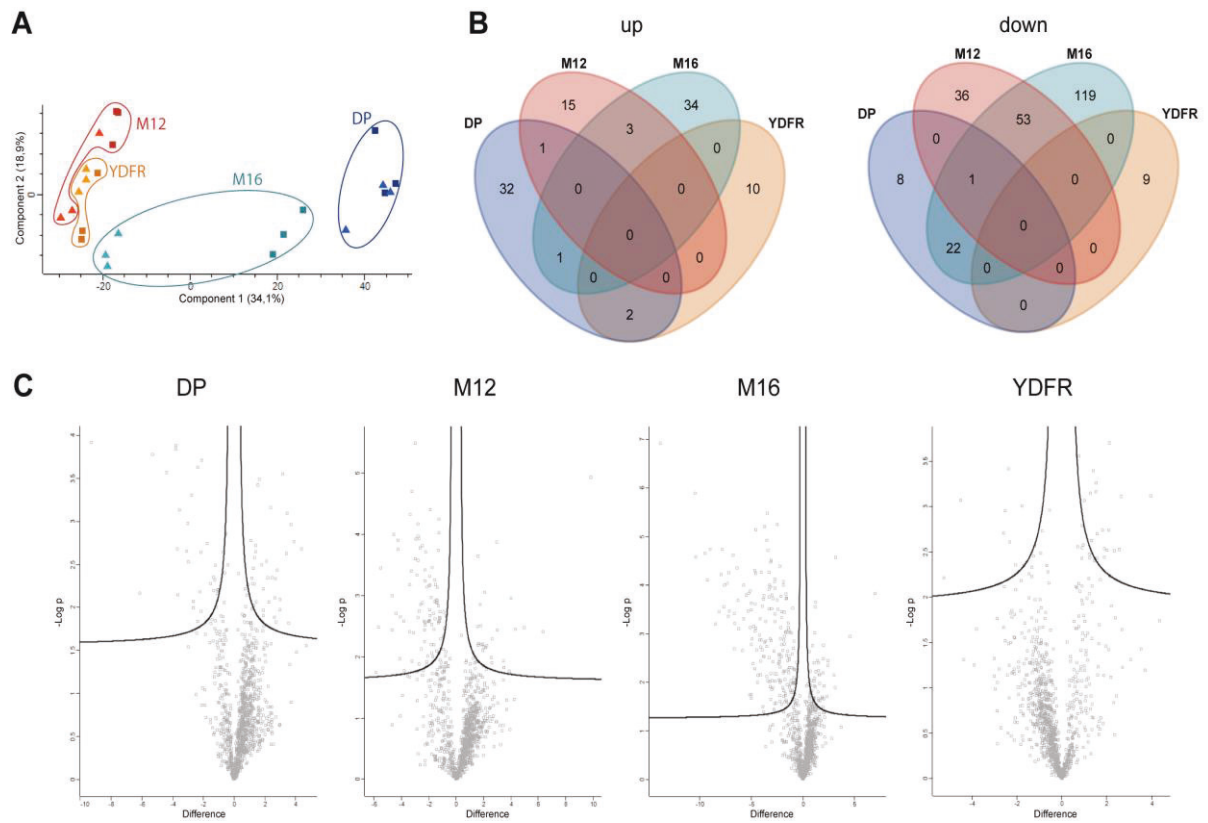
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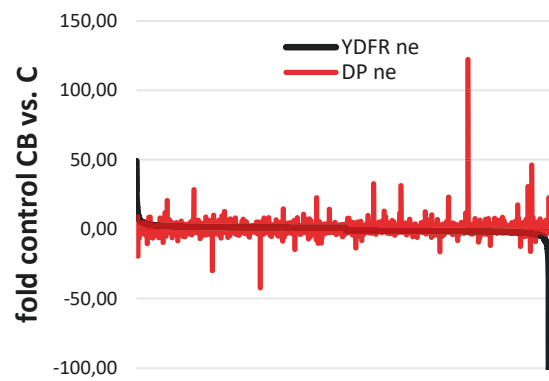
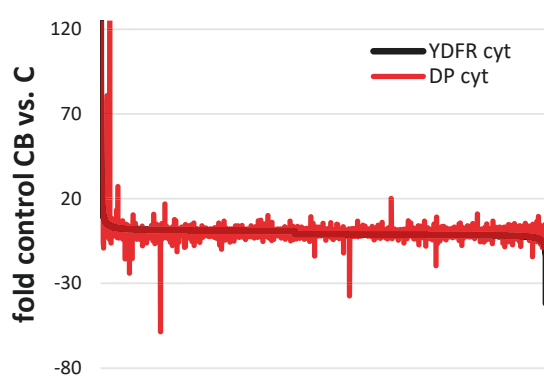
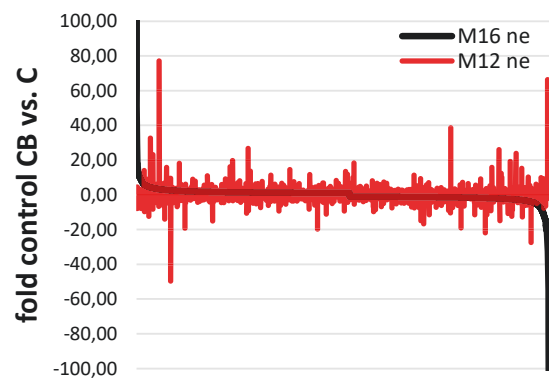
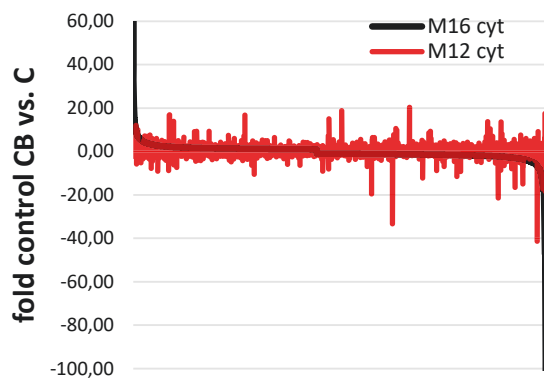
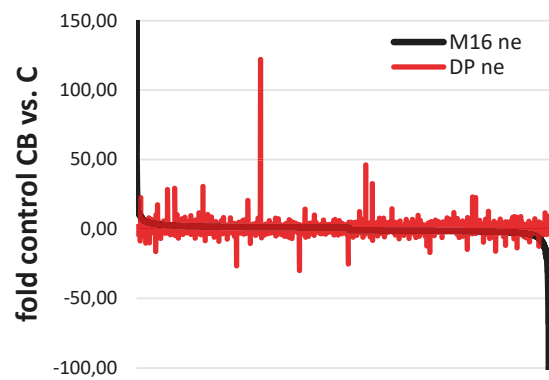
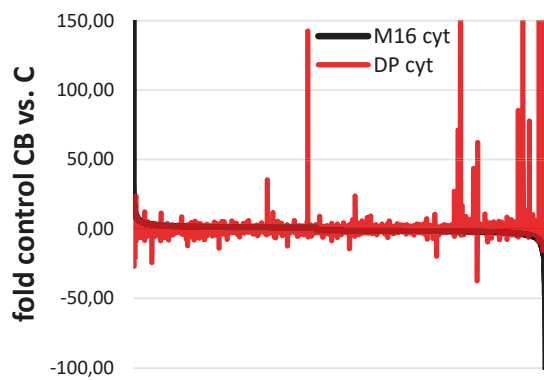
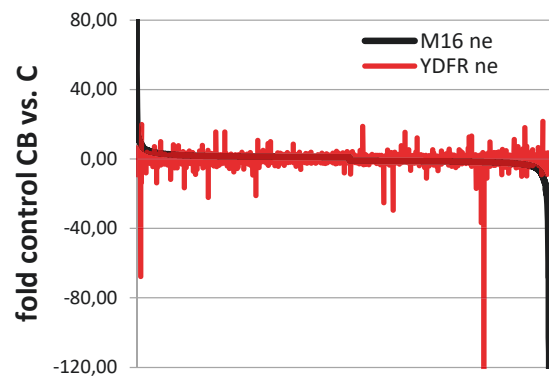
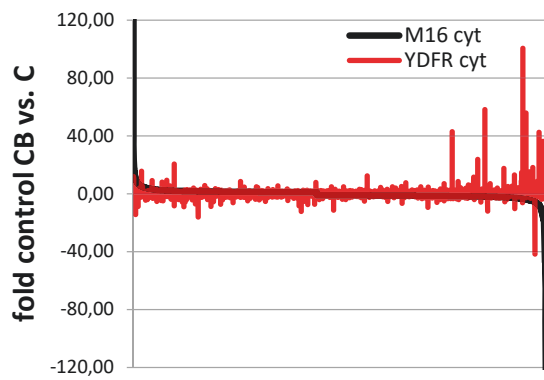
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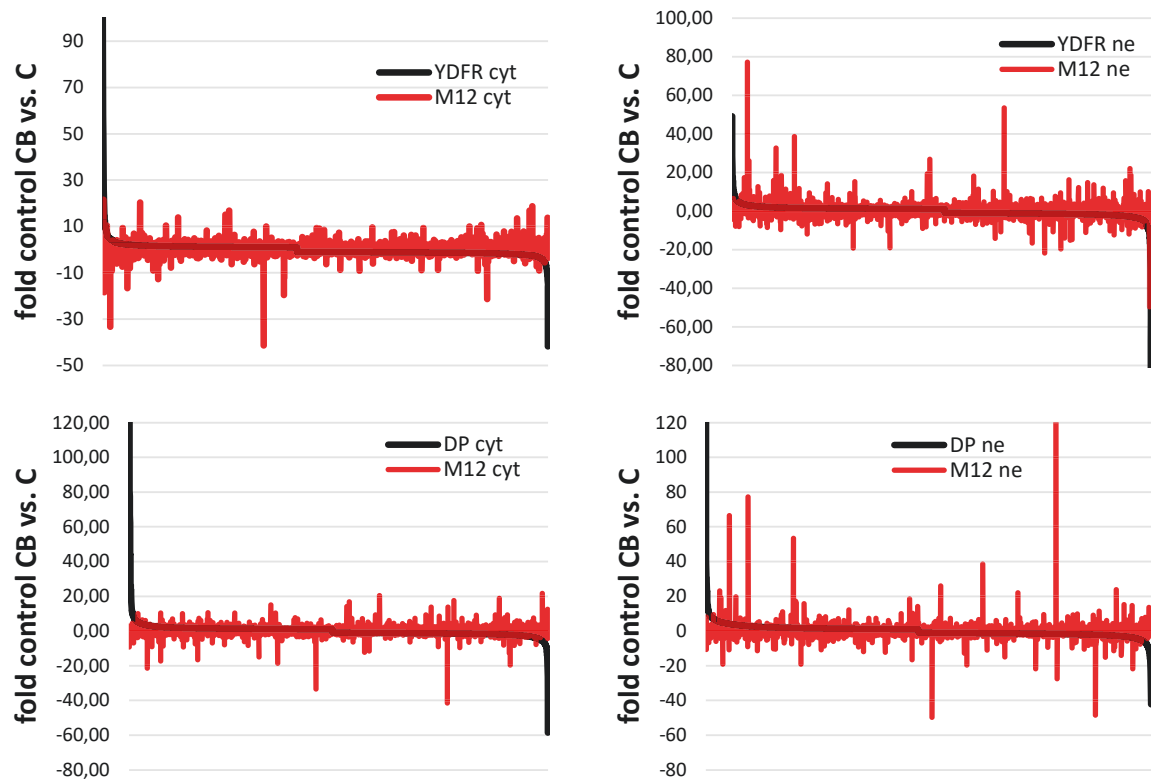


Supplementary Figure 1: Schematic experimental workflow for the molecular analysis of proteins, eicosanoids and glutathione.

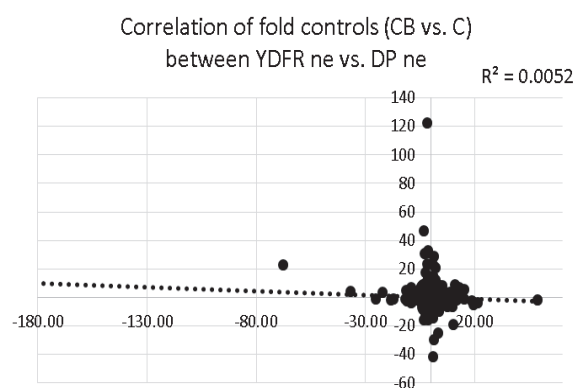
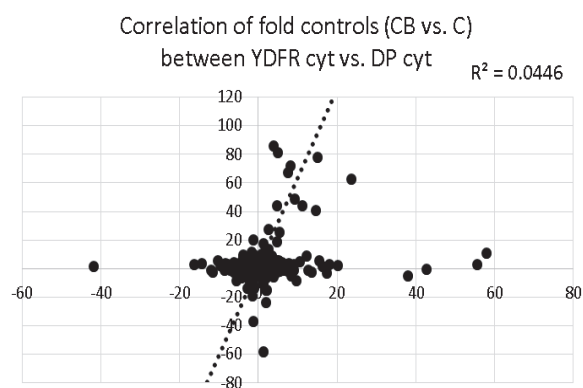
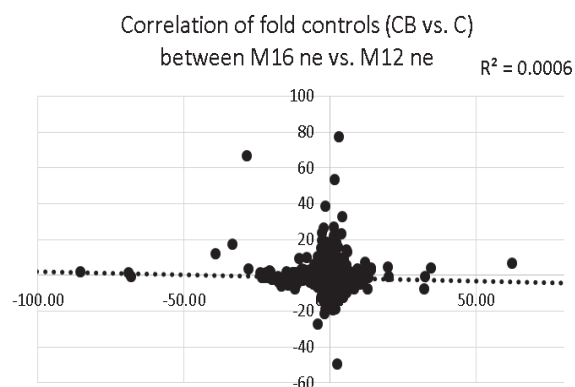
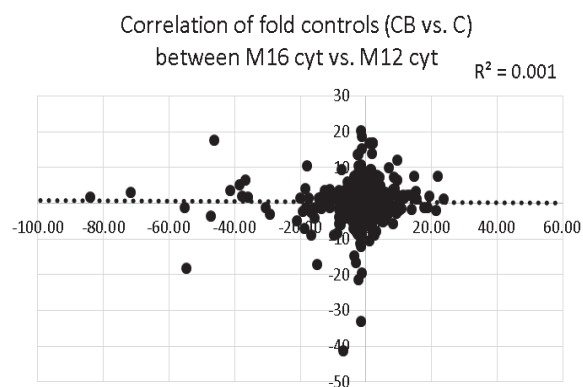
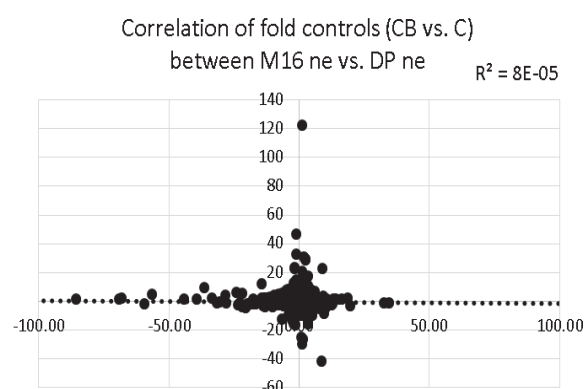
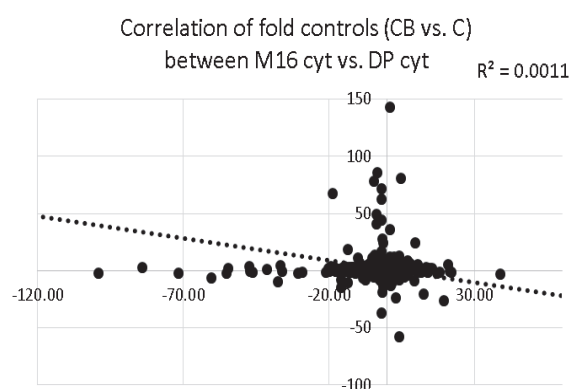
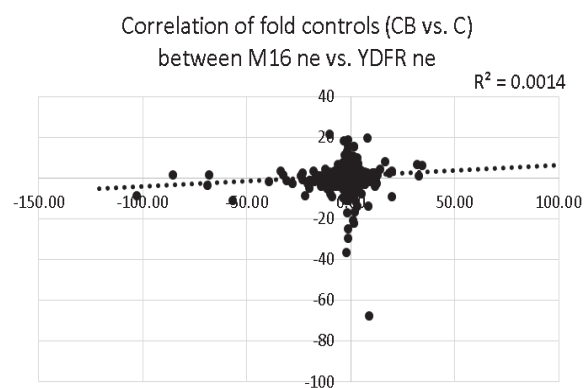
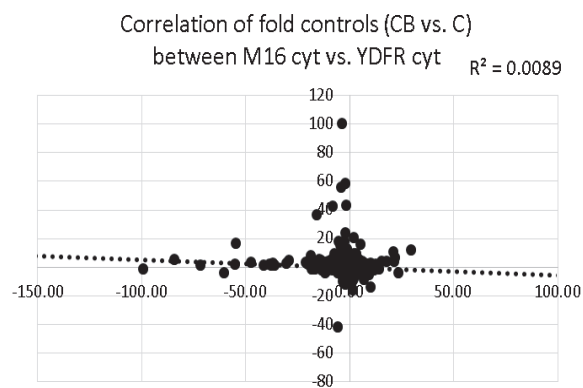


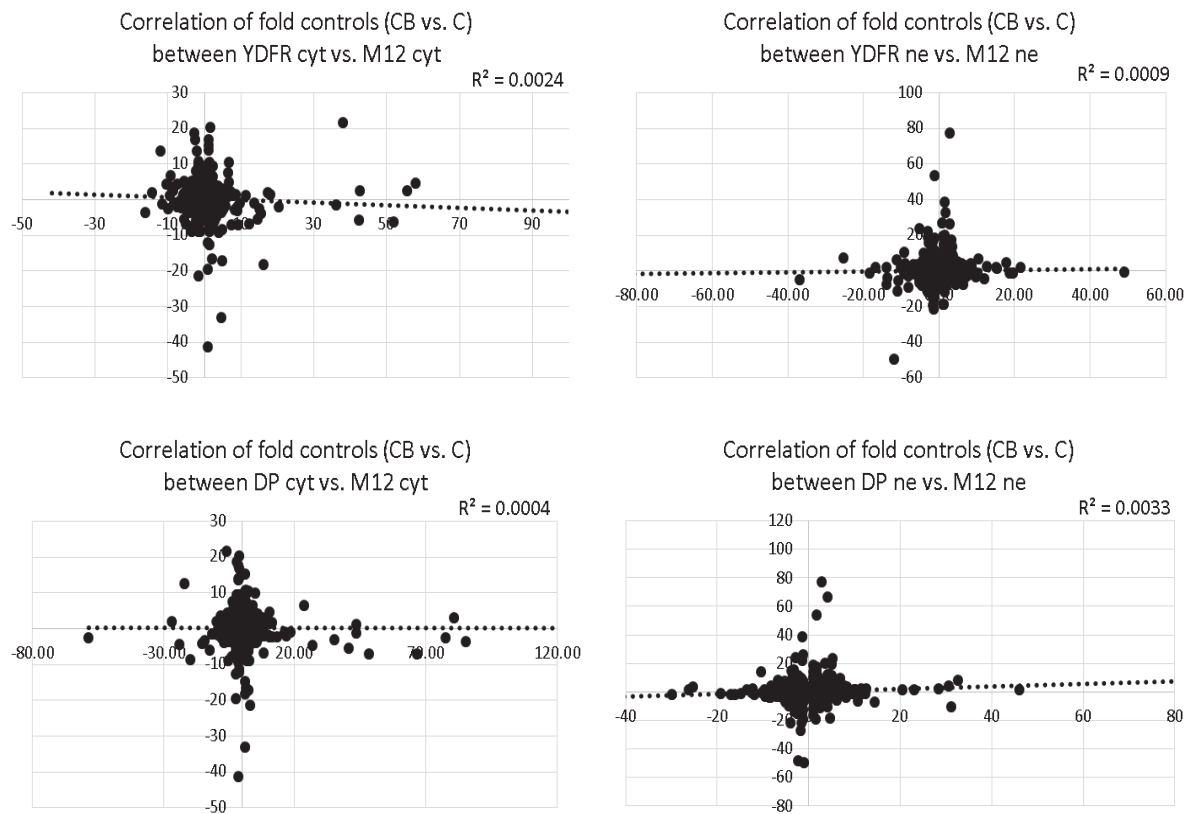
Supplementary Figure 2: (A) Principal component analysis (PCA) of the supernatant (SN). The C variants are represented by the rectangles while the CB variants are indicated by the triangles. **(B)** Venn diagrams comparing all significant regulations indicated in the volcano plots. The diagrams show the up- and down-regulated proteins from the supernatant separately. **(C)** Volcano plots for all 4 cell pairs comparing each C-CB pair (FDR = 0.05, SO = 0.1). Up- or downregulation corresponds to higher or lower LFQ intensities in the CB variant compared to the C variant, respectively.





Supplementary Figure 3: Differences in protein regulation of cell pairs. Fold-change values between cerebellar (CB) and cutaneous (C) variants of a cell pair were sorted in a descending fashion, separately for cytoplasmic (cyt) and nuclear (ne) proteins. The corresponding fold-change values of another cell pair were blotted in red. A positive correlation of deregulated proteins would be visible by alignments between black and red lines especially at the edges. Figures were created using Excel 2010.





Supplementary Figure 4: Correlation analysis of differential expression values of proteins. Correlation analysis of fold-change values between cerebellar (CB) and cutaneous (C) variants was performed for cell pairs as indicated, separately for cytoplasmic (cyt) and nuclear (ne) proteins. Graphs were created using Excel 2010.

6.2 Interaction with ribosomal proteins accompanies ER stress-induction of the anticancer metallodrug BOLD-100/KP1339

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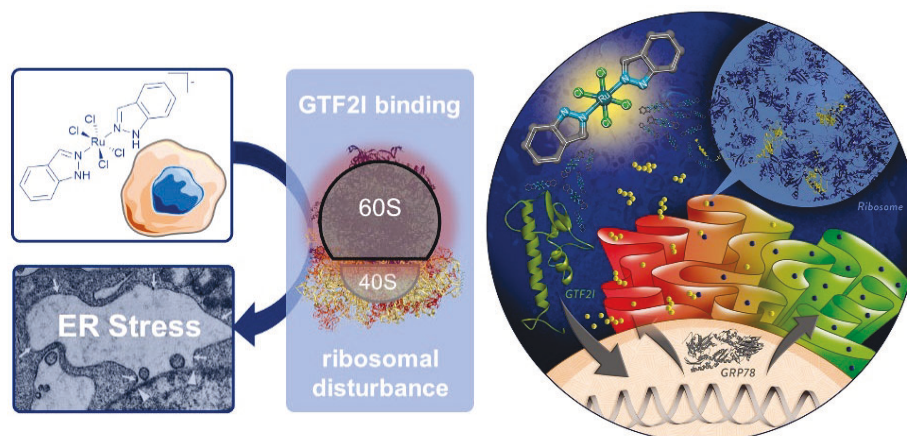
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- Planning and conduction of experiments and MS analysis
- Analysis and interpretation of data
- Preparation of figures and manuscript

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Interaction with Ribosomal Proteins Accompanies Stress Induction of the Anticancer Metallodrug BOLD-100/KP1339 in the Endoplasmic Reticulum

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Abstract: The ruthenium-based anticancer agent BOLD-100/KP1339 has shown promising results in several in vitro and in vivo tumour models as well as in early clinical trials. However, its mode of action remains to be fully elucidated. Recent evidence identified stress induction in the endoplasmic reticulum (ER) and concomitant down-modulation of HSPA5 (GRP78) as key drug effects. By exploiting the naturally formed adduct between BOLD-100 and human serum albumin as an immobilization strategy, we were able to perform target-profiling experiments that revealed the ribosomal proteins RPL10, RPL24, and the transcription factor GTF2I as potential interactors of this ruthenium(III) anticancer agent. Integrating these findings with proteomic profiling and transcriptomic experiments supported ribosomal disturbance and concomitant induction of ER stress. The formation of polyribosomes and ER swelling of treated cancer cells revealed by TEM validated this finding. Thus, the direct interaction of BOLD-100 with ribosomal proteins seems to accompany ER stress-induction and modulation of GRP78 in cancer cells.

The ruthenium complex sodium *trans*-[tetrachlorido-bis(1*H*-indazole)ruthenate(III)] (BOLD-100/KP1339, Figure 1) is

among the most widely investigated non-platinum metal-based anticancer drugs.^[1] It has shown promising anticancer effects in an autochthonous tumour model^[2] and its safety has been proven in clinical trials^[3] with clinical phase 1b trials under way (NCT04421820). In contrast to the clinically widely administered platinum-based anticancer agents that target DNA, this ruthenium(III) anticancer drug candidate acts on other biomolecules.^[4] Recently, the down-modulation of the glucose-regulated protein of 78 kDa (GRP78, HSPA5) combined with endoplasmic reticulum (ER) stress, as well as the induction of reactive oxygen species (ROS) emerged as accepted drug effects in several 2D and 3D tumour models.^[5] However, the mechanism of ER-stress induction and possible targets of BOLD-100 remained rather elusive despite intense research efforts.

Metalloproteomics is an emerging tool to elucidate potential targets and mechanisms of action for metallodrugs, and also in conjunction with other omics techniques.^[1b,6] Recently, solutions were found to identify and validate protein targets of largely substitution-inert metal-based anticancer agents based on gold^[7] and platinum,^[8] but also of labile organoruthenium prodrugs.^[9] Moreover, metallopro-

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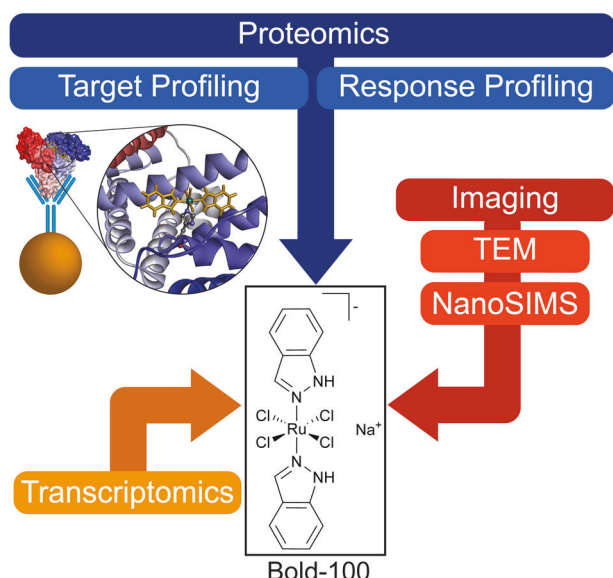


Figure 1. Workflow of the multi-omics approach followed in this study. Immobilisation of BOLD-100 with human serum albumin (HSA) and anti-HSA antibody on magnetic beads for target profiling. This is integrated with findings from proteome profiling and global transcriptome profiling. Correlative TEM/NanoSIMS imaging was applied for ultrastructure characterization and visualization of the sub-cellular drug distribution in HCT116 cancer cells.

teomes were characterized following a similar strategy in living cells.^[10] Furthermore, integrating metalloproteomics with other omics layers (e.g. transcriptomics) promises to reveal unprecedented details regarding the mode of action of metal-based anticancer agents.^[6]

BOLD-100 is a double-prodrug that undergoes hydrolysis via ligand exchange of chlorido ligands and subsequent reduction to ruthenium(II).^[1a,4] The oxidation state + III was found to be relatively stable even in tissues over prolonged time periods.^[11] The drug features a rapid binding to human serum albumin (HSA) via non-covalent bonding, which is transformed into a stable Ru-HSA adduct, characterized by coordinative bonding.^[12] X-ray diffraction studies further showed that the *trans*-indazole ligands are retained in some cases,^[13] but may be exchanged in others.^[14] The natural reactivity of BOLD-100 towards HSA is used as a tumour targeting mechanism.^[1a] The formation of a stable BOLD-100-HSA adduct was exploited here as an immobilization strategy to enable proteomic target profile experiments since this compound was not amenable for suitable derivatization so far. Here, we show that BOLD-100 may directly interact with ribosomal proteins

and provide evidence of the proposed mechanism by a multi-omics approach in conjunction with imaging techniques based on ultrastructure-resolving microscopy and nano-probe secondary ion mass spectrometry (NanoSIMS).^[15]

The study of the potential molecular targets of BOLD-100 was carried out by adapting a previously described shotgun proteomics approach based on label-free quantification (LFQ).^[16] This target-response profiling combines the investigation of potential interacting proteins of an immobilized drug with a comprehensive perturbation study. First, HSA was immobilized on magnetic beads with anti-HSA antibodies and then reacted with BOLD-100 to generate the BOLD-100-HSA adducts (s. experimental section, Figure 1). This immobilization reflects the *in vivo* situation of the anticancer drug candidate, which rapidly binds to serum proteins within a short period of time, as shown in mouse experiments amongst others.^[17] Target profiling was performed using native whole cell lysates of the colon carcinoma cell line HCT116. The cell lysates were exposed to the beads for 2 h and after thorough washing the bound proteins were eluted under mild conditions with citrate buffer and then processed for analysis by shotgun proteomics. The pull-downs were performed in a differential approach to remove non-selective binding events. Next to a normal pull-down experiment, a competitive pull-down was performed by pre-treating the cell lysate with free BOLD-100 before exposure to the immobilized drug on the beads. This latter strategy saturates selective binding sites so that the resulting target profile includes only non-selective binding partners. Subtracting these from the target profile obtained by normal pull-down cancels the non-selective interactors and provides a list of the selective binding partners.

The enrichment plot highlights the protein target profile of BOLD-100 (Figure 2A). The selectivity of binding is expressed as the fold-change of enrichment between normal and competitive pull-down experiments on the y-axis. The x-axis features the concomitant significance of enrichment and

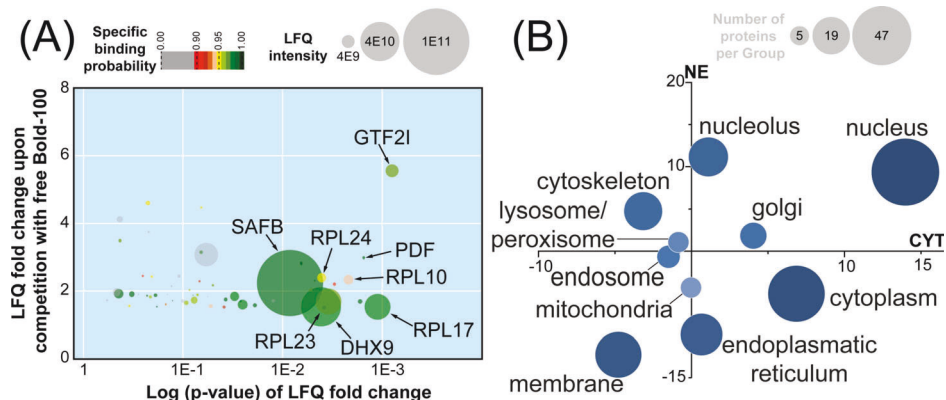


Figure 2. (A) Enrichment plot displaying potential interactors of BOLD-100 immobilized via HSA-adduct formation from HCT116 cell lysates. The y-axis represents the LFQ fold-change between normal and competitive pull-downs and the associated p-values are given on the x-axis. The colour indicates the specific binding probability and the size correlates to the abundance of each protein. (B) Significantly regulated proteins upon treating HCT116 cancer cells with BOLD-100 (80 μ M, 24 h) were grouped according to cellular compartments and displayed according to the summed fold-change in cytoplasmic (CYT, x-axis) and nuclear fraction (NE, y-axis). Circle size is determined by number of proteins in the respective group.

is expressed as a p-value. The pull-down experiment employing BOLD-100-HSA adducts provided 57 proteins as probable interaction partners, which are shown as circles in the enrichment plot, whose size is dependent on their LFQ intensity (Figure 2A). This number is smaller compared to previously investigated metallodrugs, which may be caused by the employed HSA-drug adducts in this study. Interestingly, proteins of the large ribosomal subunit (RPL10, RPL17, RPL23, RPL24) represent a major cluster of the target profile and suggest a direct interaction of BOLD-100 with ribosomal constituents (Figure S1). Moreover, the protein with the highest enrichment factor corresponded to the general transcription factor II-I (GTF2I), a transcription factor regulating the response to ER stress, for example through binding to the promoter region of HSPA5 (GRP78).^[18] The disruption of the promotion function of GTF2I through interaction with BOLD-100 may therefore directly account for the previously observed down-modulation of GRP78. Thus, the target spectrum of BOLD-100 clearly shows potential interactors that are involved in ER homeostasis.

A response profiling experiment^[19] was then conducted to complement the data obtained from the pull-down. HCT116 cancer cells were treated with BOLD-100 (80 μ M) for 24 h. The cytoplasmic (CYT) and nuclear (NE) fractions of the cells were isolated and proteolytically digested for analysis by LFQ shotgun proteomics. At this dose, the treatment featured relatively minor proteome alterations. Of a total of 4193 proteins detected applying a false-discovery rate (FDR) of 0.01 on the peptide and protein level, 116 and 86 proteins featured significant abundance changes in CYT and NE, respectively (FDR = 0.05 and S_0 = 0.1). Thus, BOLD-100 does not seem to affect the proteome to the extent of other ruthenium drug candidates.^[16,20] The cellular response profile is visualized as global protein expression changes to the perturbation with BOLD-100. This is constructed by grouping the significantly regulated proteins according to their cellular compartments and subsequently plotting the summed protein fold-changes along the CYT and NE axes (Figure 2B). The circle size corresponds to the number of proteins in each group. A pronounced down-regulation of ER-associated proteins was observed in the NE fraction, while proteins associated with the nucleus and nucleolus were upregulated. In contrast to other ruthenium drug candidates, BOLD-100 treatment did only induce minor regulations in mitochondrial proteins.^[20]

The selection of potential interactors from the target profile and the significantly regulated proteins from the response profiling experiments were then integrated in a network using STRING^[21] analysis in Cytoscape^[22] (Figure 3A, Figure S2). This network reveals whether potential targets are mechanistically connected to drug-induced perturbations in the cancer cells. The obtained network showed proteins involved in three major biological processes, namely the endoplasmic reticulum unfolded protein response (blue), ribosome biogenesis (orange) and mRNA processing (red). The potential interactors are shown in green. Interestingly, the network displayed central ribosomal proteins that connect to protein regulations in ribosome biogenesis and mRNA processing. Furthermore, the analysis also showed the men-

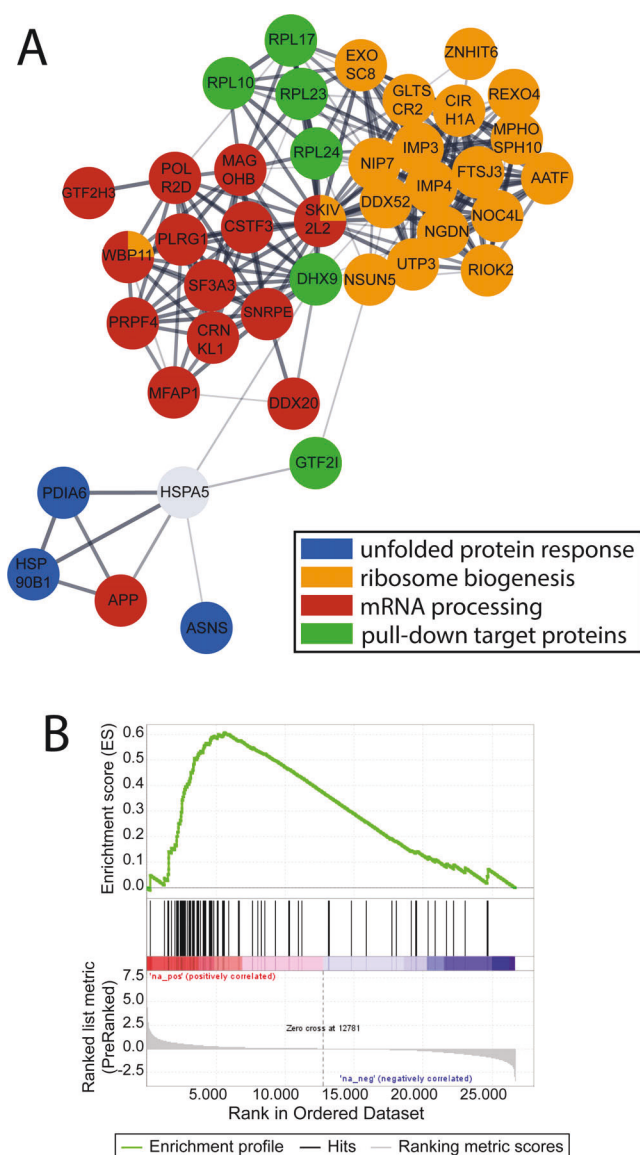


Figure 3. (A) Excerpt of the STRING analysis from the significantly regulated proteins (FDR = 0.05, S_0 = 0.1) created in Cytoscape. Colours indicate the affiliation to Gene ontology terms assigned by the STRING analysis. Blue: endoplasmic reticulum unfolded protein response (GO.0030968); Orange: ribosome biogenesis (GO.0042254); Red: mRNA processing (GO.0006397); Green: Proteins obtained in the pull-down experiment, additional HSPA5 as known response of the drug and link to the UPR. (B) Gene set enrichment analysis according to the KEGG database demonstrating significant enrichment of the term “ribosome”.

tioned connection between GTF2I and HSPA5, which extended to the cluster of ER stress. Thus, the target-response network indicated a direct interaction of BOLD-100 with ribosomal proteins and potentially GTF2I that mediate the observed alterations in proteins of ribosome biogenesis and ER homeostasis in treated HCT116 cells.

The involvement of ribosomal proteins was further investigated on the mRNA level by a global transcriptome profiling. For this purpose, HCT116 cells were treated with BOLD-100 (100 μ M) for 6 h and total mRNA was isolated

and analysed. Importantly, the term “ribosome”, containing multiple mRNA for ribosomal proteins, was generally up-regulated and showed an overall enrichment score of 0.6, representing one of the most enriched gene sets ranked top 16 (Figure 3B). mRNA of PRL10, RPL17, RPL23 and RPL24 was detected, as identified in the protein target profile, but only mRNA of RPL10 and RPL24 was upregulated.

Further support of a direct interaction of BOLD-100 with ribosomes was previously reported by analysing Ru in treated HCT116 cell lysates using size-exclusion chromatography coupled to inductively-coupled plasma MS. In these studies, ruthenium was predominantly detected in the very high molecular weight fraction (> 700 kDa) suggesting that this compound interacts with very large protein complexes such as ribosomes.^[23]

Then, NanoSIMS was employed to reveal the subcellular distribution of ruthenium in HCT116 cells after treatment with BOLD-100 for 24 h (Figure 4). The cell pellets obtained from mono-layer grown cancer cells were embedded in a low viscosity resin and cut in slices of 100 nm thickness. Ruthenium was found homogenously distributed between major cellular compartments and no significant difference in accumulation between cytoplasm, nucleus or nucleolus was observed. The cytoplasm displayed distinct organellar hotspots with a higher tendency of ruthenium accumulation. Consequently, the largely homogenous distribution of ruthenium in the cellular interior provided further support of the direct interaction of BOLD-100 with cytoplasmic ribosomes (Figure 4A).

The consecutive 100 nm section of BOLD-100-treated HCT116 cells embedded in resin was analysed by transmission electron microscopy (TEM) after post-staining with gadolinium acetate and lead citrate (Figure 5). The images revealed swelling of the ER, resulting in perinuclear space (PS) exaggeration (Figure 5A). These effects were absent in untreated controls (Figure S3). Furthermore, ribosomes were prone to detach from the ER and formed clusters of polyribosomes, which were observed as string-like formations in the cytoplasm (Figure 5D, asterisks). BOLD-100 was previously found to be able to cross-link proteins, which might underline this finding.^[24] Rounded single-membrane cytoplasmic organelles with lamellar and vesicular content were observed. These vesicles overlapped with the NanoSIMS visualized cytoplasmic ruthenium hotspots and might constitute a cellular attempt of metal detoxification (Figure S4).

In summary, using a multi-omics approach complemented by NanoSIMS and TEM as imaging techniques, this work highlights molecular events that could lead to the known ER stress response induced by BOLD-100. ER stress was previously identified as a rather unique mechanism of action of this ruthenium(III) anticancer agent.^[1a,c] BOLD-100 seems to directly interact with ribosomal proteins, most probably with RPL10 and/or RPL24, not only resulting in the inhibition of proliferation, but also in a functional disturbance of the ER. This was further evidenced by the formation of polyribosomes, concomitant ER swelling and vesicle formation in cancer cells upon treatment with BOLD-100 at higher concentrations.

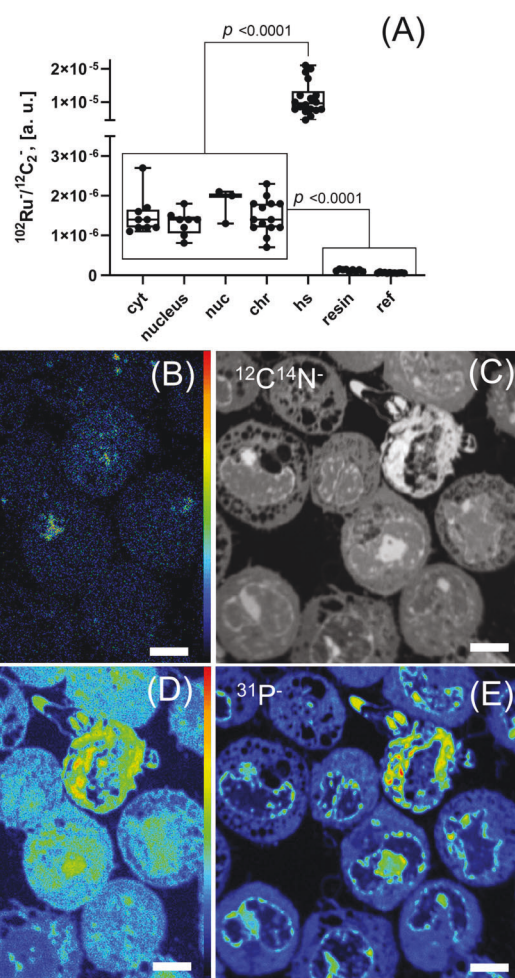


Figure 4. Visualization of the ruthenium distribution in BOLD-100 treated colon carcinoma cells by means of NanoSIMS. (A) Evaluation of the relative ruthenium content in subcellular compartments of HCT116 cells upon exposure to BOLD-100 (400 μ M, 24 h) via region of interest (ROI) specific evaluation of the $^{12}\text{C}_2^-$ normalized $^{102}\text{Ru}^-$ signal intensities. $^{102}\text{Ru}^-$ (B), $^{12}\text{C}^{14}\text{N}^-$ (C), $^{34}\text{S}^-$ (D) and $^{31}\text{P}^-$ (E) secondary ion maps revealing sites of drug accumulation. Abbreviations: cyt—cytoplasm, nuc—nucleolus, chr—heterochromatin structures, hs—cytoplasmic hotspots of Ru accumulation, resin—extracellular regions (epoxy resin), ref—values from an untreated control sample. Scale bars: 5 μ m.

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Conflict of interest

The authors declare no conflict of interest.

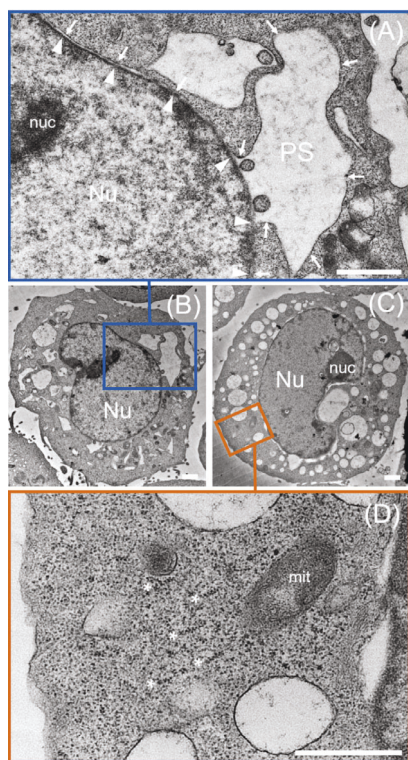


Figure 5. Endoplasmic reticulum stress morphology upon exposure of HCT116 cancer cells to BOLD-100 (400 μ M, 24 h). Transmission electron micrographs showing endoplasmic reticulum (ER) swelling, which results in perinuclear space (PS) exaggeration and ER vacuolization (A–D). In (A, a close-up of B) the outer nuclear membrane continuous with ER is marked with arrows, while the inner nuclear membrane is marked with arrowheads. The ribosomes are forming clusters of polyribosomes (marked with asterisks) visible in cytoplasm (D, a close-up of C). Abbreviations: Nu—nucleus, nuc—nucleolus, mit—mitochondria, PS—perinuclear space. Scale bars: 1 μ m.

Keywords: bioinorganic chemistry · metals in medicine · multi-omics · ribosome · ruthenium

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Materials and Methods

Compound

BOLD-100/KP1339 was synthesized as a brown powder according to published procedures.^[1] BOLD-100 was always freshly dissolved in a dimethylsulfoxide (20 mM) stock solution, which was further diluted to the appropriate concentration with complete medium.

Cell culture

HCT116 cells were kindly provided by M. Jakupc (University of Vienna). They were cultured in McCoy's 5A modified medium (Gibco, Thermo Fischer Scientific, Life Technologies, with L-glutamine) supplemented with 1% penicillin/streptomycin (Sigma-Aldrich, Austria) and 10% heat-inactivated fetal calf serum (FCS, Sigma, Austria) at 37 °C and 5% CO₂.

Proteomics

Target profiling. HCT116 cells were grown in T75 polystyrene cell culture flasks with cell growth surface for adherent cells (Sarstedt, Austria) in three biological replicates per condition to approximately 95% confluence. The cells were washed, lysed and homogenised by passing through a gauge syringe 20-times as described elsewhere.^[2] In brief, a non-denaturing lysis buffer was used for pull-down experiments consisting of NaCl (100 mM), Tris HCl (50 mM), MgCl₂ (1.5 mM), NaF (25 mM), NP-40 (0.2%), glycerol (5%), TLCK (10 µg mL⁻¹), DTT (1 mM), Na₃VO₄ (1 mM), PMSF (1 mM) and pepstatin/leupeptin/aprotinin (each 1 µg mL⁻¹), at pH 7.4. The samples were centrifuged (3500 rpm, 5 min, 4 °C) and the supernatant was used for further processing. For the competitive pull-down experiments the cell lysates were incubated with BOLD-100 (50 µM) for 1 h at 4 °C prior to exposure to the immobilised drug.

To immobilise BOLD-100 a complex with human serum albumin (HSA) was formed. Therefore, anti-HSA beads (1 mL) used for depletion of human serum (PureProteome™ Albumin Magnetic Beads, Millipore, Germany) were incubated with HSA (2 mg, 30,3 nmol, Albumin from human serum, Sigma-Aldrich, Austria) for 1 h at room temperature. After washing with PBS, BOLD-100 (95 µg, 189 nmol)

was added and incubated for 2 h at room temperature. The formed BOLD-100–HSA adduct consisting of magnetic beads, HSA, and BOLD-100 (depicted in Figure 1) was then washed and split into six aliquots used for the pulldown experiment. The cell lysates (with and without pre-incubated drug) were added to the magnetic beads and shaken for 4 h at 4 °C. Afterwards, the supernatant was removed and the beads were extensively washed with lysis buffer and HEPES buffer (HEPES-NaOH (50 mM), EDTA (0.5 mM), PMSF (1 mM), TLCK (10 µg mL⁻¹) and pepstatin/leupeptin/aprotinin (each 1 µg mL⁻¹) at pH 8. The proteins were eluted with a citrate buffer (0.1 M, pH 2.4) and proteolytically digested using trypsin/Lys-C for LC-MS/MS analysis.

Response profiling. HCT116 cells (5·10⁶ cells) were seeded in T25 polystyrene cell culture flasks with cell growth surface for adherent cells (Sarstedt, Austria) in three biological replicates per condition and allowed to grow for 24 h. Cells of three T25 flasks were treated with BOLD-100 (80 µM) for 24 hours, while the others served as controls (including solvent control). The cells were lysed and separated into cytoplasmic and nuclear fraction as previously described.^[2] In short, the cells were washed with PBS and 1 ml of isotonic lysis buffer containing protease inhibitors was added. The cells were scraped and homogenised using a gauge syringe. After centrifugation the supernatant was precipitated in ethanol overnight and the remaining nuclear pellet was swelled in hypertonic buffer and ultimately lysed with NP-40 containing buffer. The samples were again centrifuged and the supernatant precipitated in ethanol.

Protein sample preparation. The proteins were proteolytically digested in-solution using a filter-assisted protein digestion (FASP) protocol as described previously.^[3] In short, the precipitated proteins were centrifuged at 4536 g for 30 min, the supernatant was decanted and the protein pellet dried under vacuum. After dissolving in sample buffer (7.5 M Urea, 7.5 M Thiourea, 0.1 M DTT, 4% CHAPS, 0.25% SDS) the protein concentration was determined and 20 µg total protein per samples was used for further processing. After reduction with DTT and alkylation with iodacetamide (both Sigma-Aldrich, Austria) the proteins were digested with trypsin/Lys-C (MS grade; Promega Corporation, Madison, WI, USA) and dried in a SpeedVac.

Proteome analysis by LC-MS/MS. For the HPLC-MS/MS analysis the peptides were resolved in 5 μ L formic acid (aq. 30%) and diluted with 40 μ L mobile phase A (97.9% H₂O, 2% acetonitrile, 0.1% formic acid). The injection volumes were 5 μ L for the pull-down samples and 10 μ L for samples from cytoplasmic and nuclear fractions. Chromatographic separation was carried out with a Dionex UltiMate 3000 RSLCnano LC system, which was coupled to the QExactive Orbitrap mass spectrometer (all Thermo Fisher Scientific, Austria). Peptides were trapped on a C18 2 cm $\times$ 100 μ m pre-column and LC separation was performed on a 50 cm $\times$ 75 μ m Pepmap100 analytical column (both Thermo Fisher Scientific, Austria). The total run time of the HPLC method for analyzing pull-down samples was 85 min and included a 43 min gradient from 7% to 40% mobile phase B (79.9% acetonitrile, 20% H₂O, 0.1% formic acid). For samples of the cytoplasmic and nuclear fractions were separated using a HPLC method of 135 min total run time, including a 95 min gradient from 8% to 40% mobile phase B. The flow rate was always 300 nL min⁻¹. Mass spectrometric settings were the same for all fractions. The resolution on the MS1 level was set to 70,000 (at m/z = 200) with a scan range from m/z 400–1,400. The top eight abundant peptide ions were chosen for fragmentation at 30% normalized collision energy and resulting fragments analyzed in the Orbitrap at a resolution of 17,500 (at m/z = 200).

Proteomics Data Analysis. Raw data were subjected to the freely available software MaxQuant (version 1.6.0.1) utilizing the Andromeda search engine, followed by statistical evaluation with the Perseus software (version 1.6.0.2). For the MaxQuant search, a minimum of two peptide identifications, at least one of them being a unique peptide, was required for valid protein identification. Digestion mode was set to “Specific” choosing Trypsin/P. The peptide mass tolerance was set to 50 ppm for the first search and to 25 ppm for the main search. The false discovery rate (FDR) was set to 0.01 both on peptide and protein level, based on the q-value. The database applied for the search was the human Uniprot database (version 03/2018, reviewed entries only) with 20,316 protein entries. Further settings for the search included carbamidomethylation as fixed modification and oxidation of methionine and acetylation of the protein C terminus as variable modifications. Each peptide was allowed to have a maximum of two missed cleavages and two modifications, “Match between runs” was enabled and the

alignment window set to 10 min, with the match time window of 1 min. Gene ontology (GO) term analysis was performed with DAVID^[4] against the human gene background. The confidence of enrichment of a term is expressed by a Q-value, which is a multiple testing-corrected P-value according to Benjamini-Hochberg. The enrichment plot contained four dimensions. The y-axis represents the enrichment of a protein and is calculated as the ratio of the LFQ-areas of a given protein (three biological and two technical replicates) in the normal pull-down with respect to the competitive pull-down. The x-axis denotes the confidence of the enrichment and is obtained by a p-value of the mentioned LFQ-areas. The size of the bubbles represents the intensity in the mass spectrometric analysis. Finally, the colour represents the specific binding probability (P) and is a measure of specificity of a given protein to bind to the small molecule probe. It is obtained via the CRAPome database^[5] and is calculated as follows: $P = 1 - (\text{CRAPome N}^\circ \text{ of samples containing the protein}) / (\text{CRAPome total N}^\circ \text{ of samples}) \cdot (\text{CRAPome average SC}) / (\text{Average SC of Pull Down})$, where SC denotes spectralcounts. The total number (N^o) of samples in the CRAPome database was 411.

Cytoscape^[6] (v 3.8.0) was used for the STRING^[7] analysis with a confidence score cutoff of 0,4 and 0 additional interactors. The significantly regulated proteins (FDR = 0.05, S0 = 0.1) from cytoplasm (CYT) and nuclear extract (NE) were used for the analysis. To study the enriched gene ontology terms and for the assignment of the proteins to GO terms of biological processes the ClueGO app^[8] (v2.5.6) was used. The mass spectrometry proteomics data has been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD022449.

Total-RNA isolation and whole genome gene expression array

Whole genome gene expression array analysis was performed as described elsewhere^[9]. In brief, mRNA was isolated using the RNeasy Mini Kit (QIAGEN GmbH, Hilden, Germany) from HCT116 cells that were either untreated or treated with 100 µM of BOLD-100 for 6 h. Whole genome gene expression analyses were performed on 4 × 44K oligonucleotide microarrays (G4845A, Agilent Technologies, Santa Clara, CA, USA) according to manufacturer's recommendation. Feature extraction was carried out using the Feature Extraction software (version 11.5.1.1.). Differentially expressed genes in BOLD-

100-treated vs. untreated HCT116 cells were analysed using the GeneSpring software (version 13.0) and ranked according to their significance level in pre-defined gene sets using the Gene Set Enrichment Analysis software^[10].

Cell culture and growth conditions for TEM and NanoSIMS analysis

The human cancer cell line HCT116 (colon carcinoma) was grown in 75 cm² culture flasks (Starlab) as adherent monolayer cultures in complete medium [i.e., RPMI 1640 supplemented with 10% fetal bovine (Biowest) and 4 mM L-glutamine (Merck)]. The cell line was officially authenticated and confirmed to be mycoplasma and cross-contamination free (Multiplexion, Germany, 2014). Cell cultures were incubated at 37 °C in a moist atmosphere containing 5% CO₂.

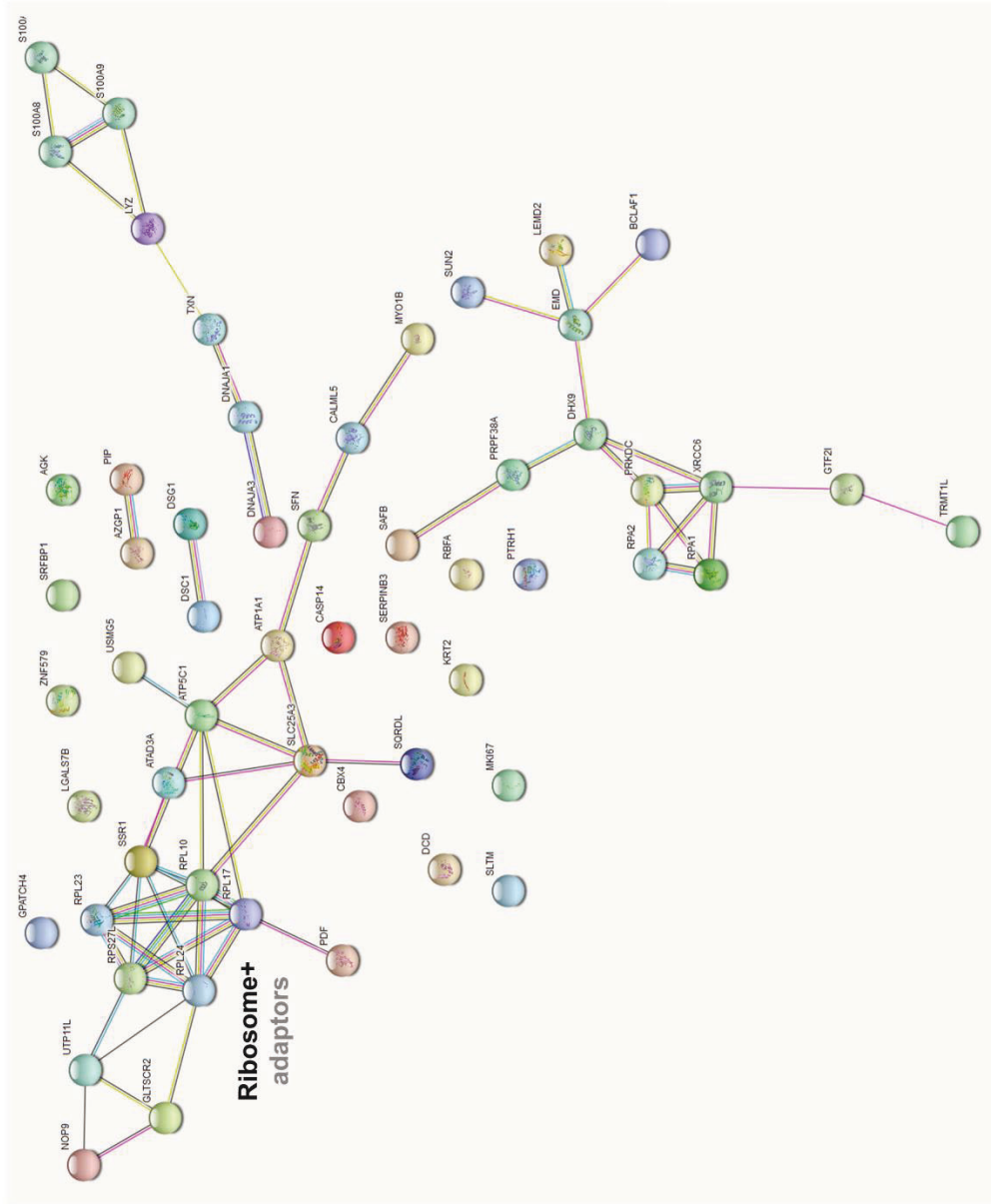
TEM analysis and sample preparation

HCT116 cells were harvested from culture flasks by trypsinization and seeded in 5 mm dishes (CytoOne, Starlab) in densities of $2 \cdot 10^6$ cells per well. Cells were allowed to grow for 72 h to reach 80-90% confluence. After growing the cells were treated with BOLD-100 solution in complete RPMI medium prepared from fresh DMSO stock (100 mM). The cells were incubated with compound for 24 h. After the treatment the cells were detached by trypsinization, washed with PBS (2×), subdivided into 1 ml aliquots (each comprising around $2.5 \cdot 10^6$ cells), pelleted by centrifugation (300 g), and fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4, RT). After chemical fixation, cell cultures were washed with cacodylate buffer (3×), and stained with 1% OsO₄ (dissolved in 0.1 M sodium cacodylate buffer overnight) for 1 h. After washing the pellets with cacodylate buffer (3×) the samples were dehydrated in an ascending ethanol series (30%, 50%, 70%, 90% and 100% ethanol, 10 min each). Ethanol (100%) was then replaced with propylen oxide (2× for 5 min, 1× for 10 min). The samples were infiltrated and embedded in low viscosity resin (Agar Scientific). The resin blocks were polymerized in an oven at 60 °C for 32 h. To obtain two consecutive thin sections (100 nm in thickness, 1× for TEM, 1× for NanoSIMS) the resin-embedded cell monolayers were cut by using an ultramicrotome (Ultracut S; Leica) with a diamond knife (Diatome). The first section was placed on a copper grid, counterstained with gadolinium acetate and lead citrate, and imaged in a TEM Libra 120 (Zeiss) at 120 kV. Images

were acquired by using a bottom stage digital camera, TRS (4 MP), and iTEM software (Soft Imaging System GmbH). The second consecutive section was deposited onto antimony-doped silicon wafer platelets ($7.1 \times 7.1 \times 0.75$ mm; Active Business Company, GmbH, Germany) for spatially correlated metal distribution analysis by NanoSIMS.

NanoSIMS analysis, image processing and numerical data evaluation

NanoSIMS measurements were carried out on a Cameca NS 50L instrument (France), as described previously.^[11] Briefly, the detectors of the multicollection assembly were positioned to enable parallel detection of $^{12}\text{C}_2^-$, $^{12}\text{C}^{14}\text{N}^-$, $^{31}\text{P}^-$, $^{34}\text{S}^-$ and $^{102}\text{Ru}^-$ secondary ions for drug distribution measurements. Prior to data acquisition, analysis areas were pre-conditioned *in situ* by rastering of a high intensity, defocused Cs^+ ion beam in the following sequence of high and extreme low ion impact energies (HE / 16 keV and EXLIE / 50 eV, respectively): HE at 100 pA beam current to a fluence of 5.0×10^{14} ions/cm²; EXLIE at 400 pA beam current to a fluence of 5.0×10^{16} ions/cm²; HE to a fluence of 3.1×10^{14} ions/cm². An area of $40 \times 40 \mu\text{m}^2$ was scanned with 512×512 pixel image resolution and approx. 80 nm physical resolution (probe size). The primary ion beam was scanned with a per-pixel dwell time of 10 ms per cycle. In total, 30 cycles were acquired in each measurement. NanoSIMS image data were evaluated by using the WinImage software package (version 2.0.8) provided by Cameca. Prior to stack accumulation, the individual images were drift corrected manually. Secondary ion signal intensities were corrected for detector dead time on a per-pixel basis and quasi-simultaneous arrival (QSA) of C_2^- and CN^- secondary ions on a per-region of interest (ROI) basis.^[12] ROI specific drug accumulation patterns were inferred from signal intensity ratios associated with Ru and the carbon matrix ($^{102}\text{Ru}^-$ and $^{12}\text{C}_2^-$, respectively) displayed in arbitrary units (a. u.). ROI data were analyzed for normal distribution (Kolmogorov-Smirnov test, $p < 0.05$). Normally distributed data were tested for significant differences in the arithmetic means by application of Welch's t-test. Statistical calculations were conducted with the GraphPad Prism software. TEM/NanoSIMS correlation images were generated utilizing the GIMP software (version 2.10).



Protein IDs	p-value	fold-change	Intensity	selective binding probability
P78347	7.94E-04	5.6	4.06E+09	0.97
P18621	1.11E-03	1.5	1.65E+10	0.98
O9HBH1	1.53E-03	3.0	1.84E+08	1.00
P43307	1.67E-03	3.0	1.84E+08	1.00
P27635	2.17E-03	2.3	2.34E+09	0.94
Q9UHQ9	3.01E-03	2.2	2.40E+08	0.92
P62829	3.44E-03	1.7	1.60E+10	0.98
Q96EV1	3.82E-03	1.5	3.90E+08	0.99
P83731	4.05E-03	2.4	2.03E+09	0.96
Q08211	4.12E-03	1.5	3.81E+10	0.99
Q8NC56	4.75E-03	2.3	6.24E+07	1.00
Q9NWH9	5.24E-03	2.0	2.41E+08	0.98
Q9Y6N5	6.50E-03	2.8	1.53E+08	1.00
Q53H12	6.51E-03	2.8	1.28E+08	0.99
P78527	7.76E-03	1.8	6.25E+07	0.956
Q15424	8.45E-03	2.2	1.07E+11	0.99
P05023	1.24E-02	1.7	2.30E+08	0.87
Q9Y3A2	1.90E-02	1.6	4.05E+08	1.00
Q71UM5	2.51E-02	1.6	2.38E+09	0.98
Q96IX5	2.64E-02	1.7	1.23E+08	1.00
Q9NV17	3.03E-02	1.8	2.00E+09	0.99
Q86U38	3.73E-02	1.7	3.25E+08	1.00
P31689	3.87E-02	1.5	2.21E+08	0.92
O5T300	5.00E-02	2.2	1.03E+08	0.97
Q00335	5.27E-02	1.6	3.78E+08	0.94
P50402	5.69E-02	1.8	3.28E+07	0.82
P35908	5.85E-02	3.1	1.40E+10	0.82
P31944	6.56E-02	4.5	1.45E+08	0.96
Q9NZM5	6.62E-02	3.1	3.23E+08	0.99
Q08554	6.85E-02	2.3	1.17E+08	0.92
P15927	7.15E-02	1.9	1.67E+08	0.96
P12956	7.73E-02	1.7	1.21E+09	0.99
Q8NEF9	7.94E-02	1.9	2.42E+07	0.99
P25311	8.65E-02	2.0	8.64E+07	0.97
P10599	9.72E-02	1.7	4.16E+08	0.97
O43795	1.01E-01	1.7	2.94E+08	0.90
P81605	1.10E-01	2.4	2.93E+08	0.68
Q8NDV3	1.28E-01	1.7	2.54E+07	1.00
Q02413	1.30E-01	2.1	1.02E+08	0.71
Q9NYF8	1.44E-01	1.9	3.51E+07	-2.25
Q8NAV1	1.49E-01	1.6	8.42E+07	0.93
O00257	1.84E-01	1.5	5.96E+07	1.00
Q86V79	1.89E-01	1.9	2.95E+08	1.00
P12273	2.06E-01	2.4	1.53E+08	0.96
P27694	2.13E-01	1.8	2.76E+08	0.97
P25908	2.23E-01	3.7	1.19E+08	0.85
P05109	2.25E-01	4.6	5.02E+08	0.96
Q8NAF0	2.29E-01	1.9	8.42E+07	1.00
P31947	2.54E-01	2.4	1.37E+08	0.92
P61626	2.80E-01	1.9	9.01E+07	0.86
P31151	3.28E-01	1.9	7.28E+08	0.99
Q9NZT1	3.47E-01	1.6	1.38E+08	0.97
P47929	4.30E-01	3.5	2.55E+08	0.96
P46013	4.32E-01	4.1	9.94E+08	-0.42
P06702	4.44E-01	1.9	2.21E+09	0.99
Q72275	4.66E-01	2.0	1.50E+09	0.88
P36542	5.84E-01	1.7	1.20E+08	0.82

Figure S1: STRING analysis of all 57 proteins from the target profiling experiment, including a table with the p-value, fold-change, intensity and selective binding probability of all proteins (FDR = 0.05, S0 = 0.1).

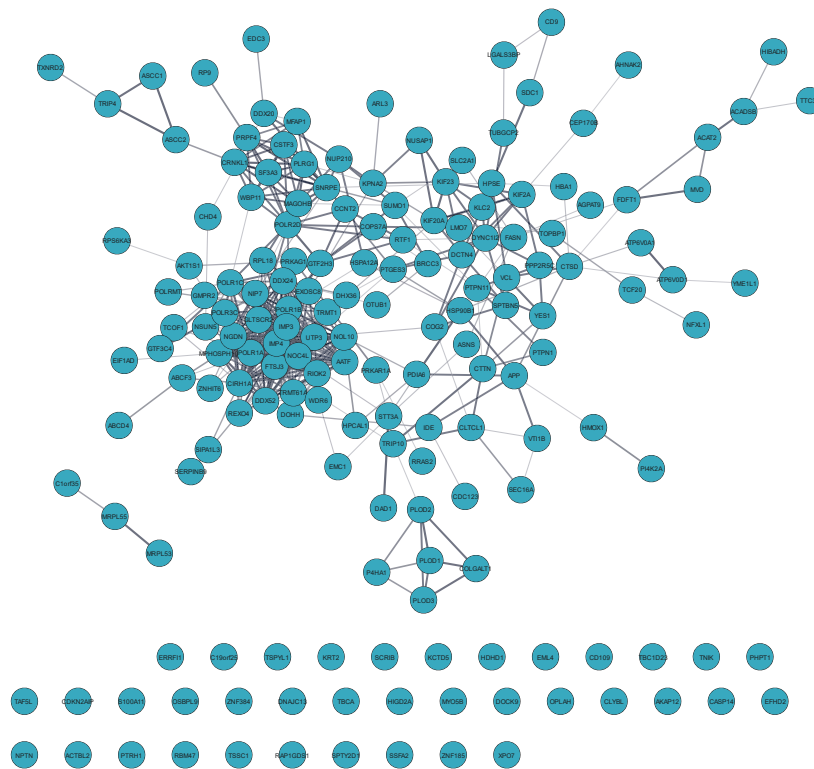


Figure S2: Complete STRING analysis of all significantly regulated proteins from cytoplasmic (CYT) and Nuclear extraction (NE) (FDR = 0.05, S0 = 0.1) created in Cytoscape. Several proteins from the target profiling are included in green. The confidence score was set to cutoff 0.4 with 0 additional interactors.

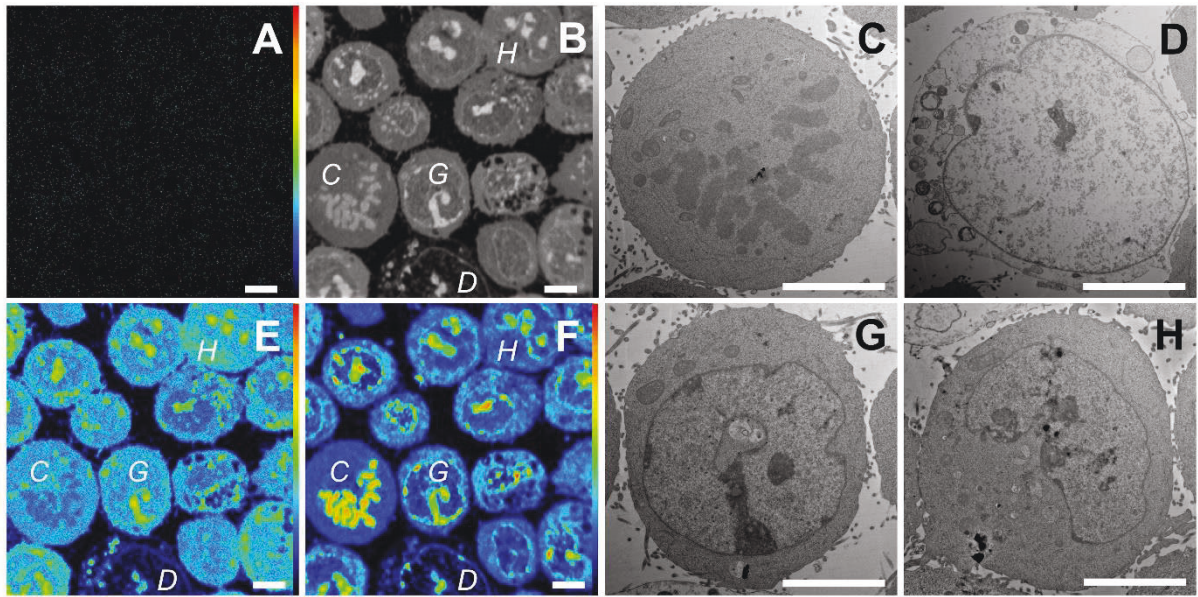


Figure S3. Untreated colon carcinoma cells. $^{102}\text{Ru}^-$ (A), $^{12}\text{C}^{14}\text{N}^-$ (B), $^{34}\text{S}^-$ (E) and $^{31}\text{P}^-$ (F) secondary ion maps (NanoSIMS) and electron micrographs (C, D, G, H) reveal the elemental distribution and general morphology of HCT116 cells (negative control). In untreated cells no signatures of severe stress induction can be observed. A cell undergoing mitosis with undisturbed endoplasmic reticulum is present (C). (D) – an example of a necrotic cell in a negative control. (G, H) are additional examples of cells with unaffected ultrastructure. Scale bars: 5 μm .

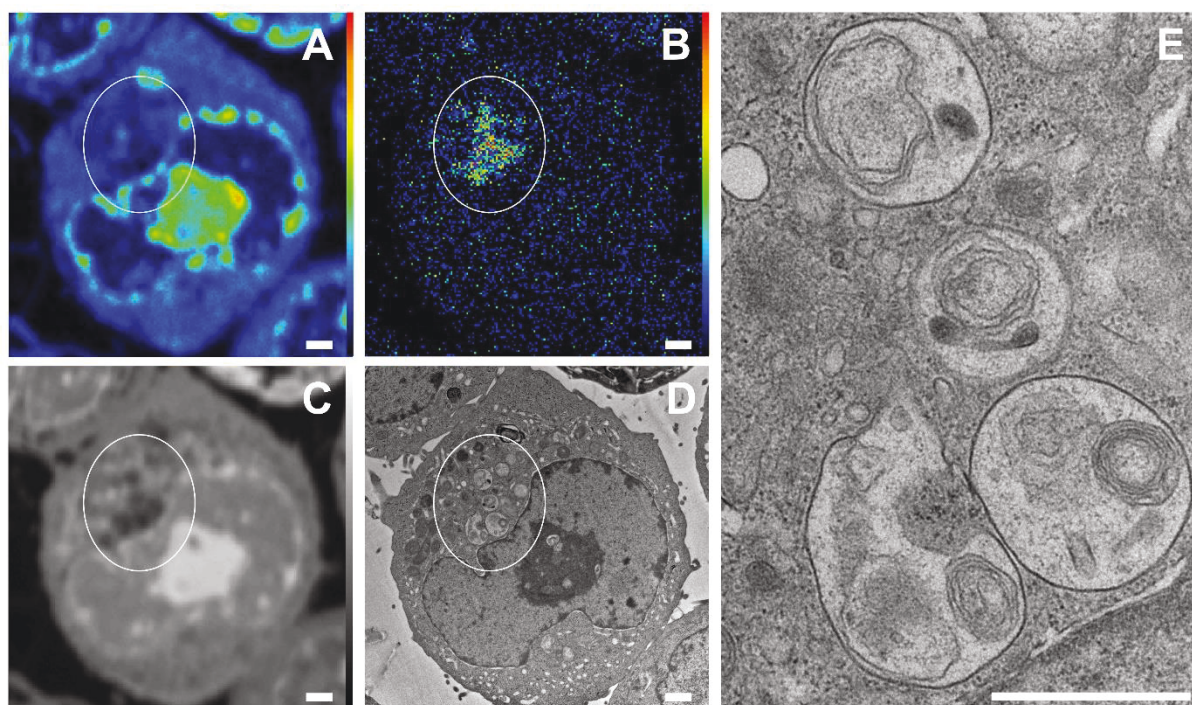


Figure S4. Cytoplasmic organelles responsible for ruthenium accumulation. Subcellular $^{102}\text{Ru}^-$ distribution (B) in HCT116 cells exposed to BOLD-100 (400 μM , 24 h). $^{31}\text{P}^-$ (A) and $^{12}\text{C}^{14}\text{N}^-$ (C) secondary ion maps (NanoSIMS) reveal no specific affinity of the compound to P- or N-rich molecules in the loci of $^{102}\text{Ru}^-$ (B) accumulation. Transmission electron micrographs (D, E) show the corresponding cellular ultrastructure. Cytoplasmic organelles that accumulate ruthenium (E, area labeled with a white oval in A-D) are featuring some major endosomal signatures, namely: rounded single membrane cytoplasmic organelles with lamellar and vesicular content. Scale bars: 1 μm ,

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6.3 Eicosanoid content in fetal calf serum accounts for reproducibility challenges in cell culture

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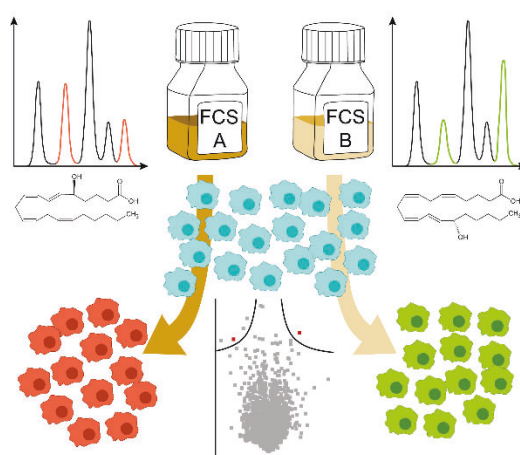
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Contributions to this publication:

- Planning and conduction of cell culture experiments
- MS measurements for protein analysis
- Analysis and interpretation of proteomics data, integration with eicosanoid data
- Preparation of figures and manuscript

Article

Eicosanoid Content in Fetal Calf Serum Accounts for Reproducibility Challenges in Cell Culture

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Abstract: Reproducibility issues regarding in vitro cell culture experiments are related to genetic fluctuations and batch-wise variations of biological materials such as fetal calf serum (FCS). Genome sequencing may control the former, while the latter may remain unrecognized. Using a U937 macrophage model for cell differentiation and inflammation, we investigated whether the formation of effector molecules was dependent on the FCS batch used for cultivation. High resolution mass spectrometry (HRMS) was used to identify FCS constituents and to explore their effects on cultured cells evaluating secreted cytokines, eicosanoids, and other inflammatory mediators. Remarkably, the FCS eicosanoid composition showed more batch-dependent variations than the protein composition. Efficient uptake of fatty acids from the medium by U937 macrophages and inflammation-induced release thereof was evidenced using C13-labelled arachidonic acid, highlighting rapid lipid metabolism. For functional testing, FCS batch-dependent nanomolar concentration differences of two selected eicosanoids, 5-HETE and 15-HETE, were balanced out by spiking. Culturing U937 cells at these defined conditions indeed resulted in significant proteome alterations indicating HETE-induced PPAR γ activation, independently corroborated by HETE-induced formation of peroxisomes observed by high-resolution microscopy. In conclusion, the present data demonstrate that FCS-contained eicosanoids, subject to substantial batch-wise variation, may modulate cellular effector functions in cell culture experiments.

Keywords: batch variations; eicosanoids; fetal calf serum; mass spectrometry; peroxisomes; proteomics

1. Introduction

Problems with the inter-laboratory reproducibility of results obtained with in vitro cell culture models are increasingly being recognized [1,2]. The need to reduce the use of animal models for research purposes relies also on the use of accurate in vitro test models [3,4]. Important decisions such as the choice of drug candidates to be evaluated in clinical studies may be based on such experiments [5]. Thus, the identification of influencing factors potentially modulating such in vitro data is mandatory. Biological materials and reference materials have been recognized as the main contributors for irreproducibility, resulting in a current focus on the investigation of genetic heterogeneity and genetic instability of cell culture models [6,7]. Here we present Fetal Bovine Serum (FBS; also fetal calf

serum, FCS) as another relevant contributor to reproducibility issues. FCS is commonly used as cell culture supplement sustaining the growth and duplication of mammalian cells *in vitro*. Since its introduction in the 1950s its use has been established world-wide, irrespective of evident limitations regarding scientific as well as ethical points of view [8]. Fetal serum is basically a by-product of meat production collected from the still beating hearts of living fetuses. While efforts are made to reduce the use of FCS, they have shown rather limited successes [9].

As for other supplements of natural origin, the main variability source associated to FBS can be traced back to largely uncharacterized bioactive components. Due to low concentrations or lack of experimental standard measurements, they may remain poorly controlled, but may still influence the outcome of cell-based experiments. While some effort has been spent to define the composition of FCS, the main bioactive constituents subjected to meaningful variation are hardly known [8]. Batch-dependent variations have been described to affect biological outcomes but such considerations remain limited to rather specialized topics such as hormone regulation [10]. Chemically defined media (CDM) represent a general and consequent solution for these problems, but have only been established and available for a limited number of cell model systems [9].

The focus of the present study was to investigate whether it was possible to identify bioactive compounds in FCS accounting for relevant batch-specific effects and correlate this information with proven and biochemically evident readouts on cell functions. Variations of amino acid and metabolite composition of FCS may be considered as less likely as they should be subjected to the homeostatic control of the organism and, further limited by the dilution of FCS in the accurately produced cell culture media also containing these molecules (typically 5–10% FCS is used). Thus proteins as well as eicosanoids and other polyunsaturated fatty acids (PUFAs) are profiled as the most relevant bioactive candidate molecules to account for inter-batch variation. Indeed, they are responsible for regulating biological processes associated with inflammation [11] and inflammation-associated pathomechanisms [12–14]. The monocyte cell line U937, a well-established cell model for macrophages [15–17], was chosen for these investigations. Overall, the data demonstrated significant effects of FCS-contained eicosanoids with batch-dependent variations on relevant cell functions, proving that bioactive lipid content in serum contributes to reproducibility issues in cell culture experiments.

2. Results

Formation of bioactive pro-inflammatory mediators by macrophages may be influenced by FCS batch effects

In order to systematically investigate cell culture reproducibility issues resulting from FCS batch effects, a proteome profiling experiment using a U937 macrophage differentiation and activation model was performed. A single batch of U937 cells was seeded into 24 identical aliquots, forming four groups subsequently sub-cultured with four different FCS batches (Table 1, see Materials and Methods). All cells were differentiated using phorbol 12-myristate 13-acetate (PMA) to induce macrophage formation as verified by FACS (fluorescence activated cell sorting) analysis (Supplementary Figure S1), while three aliquots of each group (FCS batch) were subsequently treated with lipopolysaccharides (LPS) to induce inflammatory stimulation, the other three per group serving as untreated controls. The formation of inflammatory mediators was investigated by comparative secretome analysis resulting in the identification of 488 proteins (Supplementary Table S3) and 54 eicosanoids and fatty acid precursor molecules (Supplementary Table S4). Whereas most molecules such as the chemokine CCL3 and CXCL5 showed rather little variation between the groups, reproducibility issues of differentiated U937 macrophages were evidenced by FCS batch-dependent significant (FDR (false discovery rate) < 0.05) differences in the formation of the chemokine CCL5, the cell growth regulator IGFBP2, and the cell migration and fibrinolysis regulator SERPINE1 (PAI1) and MMP1 (Figure 1A). In line, the amount of bioactive eicosanoids comprising the hydroxyeicosatetraenoic

acids 11-, 12-, and 15-HETE, hydroxydocosaheptaenoic acid 17-HDoHE, the prostaglandin PGJ2 and others were found to differ significantly ($FDR < 0.05$) depending on the FCS batch used for cell culture (Figure 1B).

Table 1. Tested FCS batches stating Vendor, Lot number, expiration date as well as letter used in this work.

Nomenclature	Vendor	Lot Number	Expiration Date	Origin	Processed
A	Sigma	BCBT4187	07.2021		-
B	Gibco	42Q5650K	06.2020	Brazil	-
C	Gibco	42G8378K	11.2022	Brazil	-
D	Gibco	08Q8082K	02.2023	Brazil	Heat inactivated

Inflammatory stimulation with LPS induced the secretion of a total of 67 proteins ($FDR < 0.05$, Supplementary Table S3), including tumor necrosis factors TNF and TNFSF15, chemokines such as CCL3, CXCL5, CXCL10, metalloproteinases including MMP1 and MMP10 and other promoters and mediators of inflammation (Figure 1A and Supplementary Table S3). Only 22 of those 67 proteins were found uniformly regulated independent of the FCS batch (Figure 1C), whereas other bioactive molecules such as IGFBP2 and TNFSF15 again showed FCS batch-dependent expression patterns (Figure 1C). Similarly, LPS treatment induced the formation of lipid mediators of inflammation such as 15-HETE, PGE2, PGJ2 and others (Figure 1B, Supplementary Table S4). The formation of eicosanoids varied rather strongly dependent on the FCS batch used, four out of seven LPS-induced eicosanoids showing significant batch-dependent alterations ($FDR < 0.05$, Figure 1D).

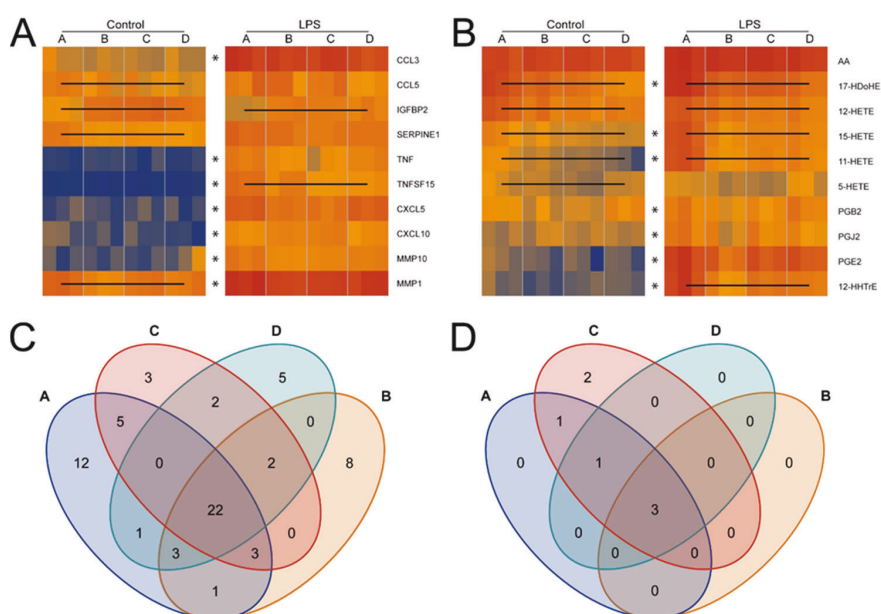


Figure 1. Heatmaps of selected proteins (A) and eicosanoids (B) determined in secretomes of control and LPS-treated U937 cells cultured with the indicated batch of FCS, A, B, C or D. Lines within a heatmap indicate a significant difference of the given molecule within at least two batches. Asterisks (*) indicate that LPS-treatment induced a significant increase. Venn diagrams of significantly up- and downregulated (C) proteins ($S0 = 2$, $FDR = 0.01$) and (D) eicosanoids comparing LPS activation with control samples for all four FCS batches (A–D).

2.1. The Eicosanoid Content of FCS Varies in a Batch-Dependent Fashion

The induction of inflammatory activities of cells may be subject to modulation by a delicate balance of pro- and anti-inflammatory molecules. Thus we investigated whether the above-described batch effects may be caused by differences of the protein and eicosanoid content of the FCS batches used for the cell culture experiments. Remarkably, the protein profile comprising 289 identified proteins (FDR < 0.01) of the four different FCS batches was rather consistent (Figure 2A, Supplementary Table S5). Several significant abundance differences between batches were observed (Supplementary Table S5), and a principle component analysis showed fairly good clustering of the FCS samples according to batches (Figure 2B). The analysis of FCS eicosanoid contents revealed even stronger batch-dependent differences, comprising mainly COX and LOX-products (Figure 2C, Supplementary Table S6). Here, an unbiased PCA clustered the FCS batches with clear distances between batch clusters (Figure 2D), and demonstrated that batch dependent differences of eicosanoid content exceeded the differences of protein content.

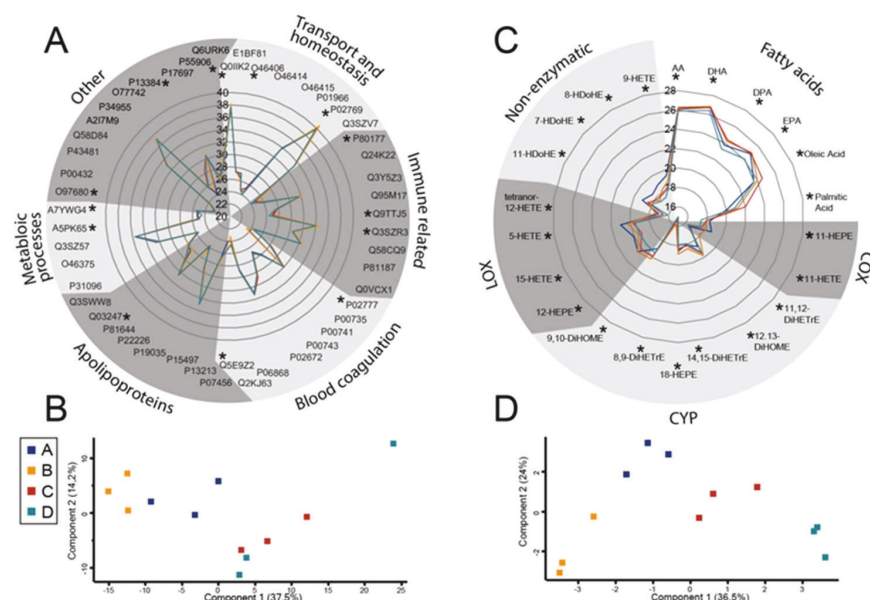


Figure 2. Radar plot for selected proteins (A) and fatty acids (C) identified in 4 different FCS batches without incubation with cells (baseline levels). Principal component analysis of protein (B) and eicosanoid (D) measurements of the same FCS batches, as indicated by different colors, demonstrates superior clustering in the case of eicosanoids.

2.2. Cell Culture Subjects Fatty Acids to a High Turnover

Growing cells require medium supply of fatty acids and fetal calf serum is rich in polyunsaturated fatty acids. In order to mediate biological effects as assumed for the FCS-contained fatty acids described above (Figure 2B), cells are supposed to take up fatty acids from the medium. In order to estimate to what extent cultured U937 cells may be able to take up fatty acids from the medium and release fatty acids back into the medium upon stimulation, we investigated the intracellular to extracellular exchange dynamics of the eicosanoid precursor molecule arachidonic acid (AA). For this purpose, cultured U937 cells were spiked with stable isotope labelled AA at a concentration double that of the endogenous AA (1.6 μ M). Stable isotope labelled AA can be clearly distinguished from endogenous AA by mass spectrometry. As demonstrated in Figure 3, upon differentiation to macrophages, U937 cells apparently picked up more than 99% of the labelled and spiked AA within 72 h and less than 1% C13-AA remained detectable in the medium after that period. As expected, subsequent LPS treatment triggered a significant increase of the

amount of C13-AA in the supernatant (Figure 3). This demonstrated that phospholipase activity was capable of releasing previously incorporated AA back into the medium. When supplementation with C13-AA was performed after PMA differentiation but before LPS treatment, the outcome was similar. Around 95% of the available AA was incorporated, but still a significant release of C13-AA was observed upon LPS treatment, clearly indicating a high turnover rate of AA. Concomitant measurement of endogenous C12-AA confirmed that AA was consumed substantially during cell culture and released back in the medium again in a smaller proportion upon LPS treatment.

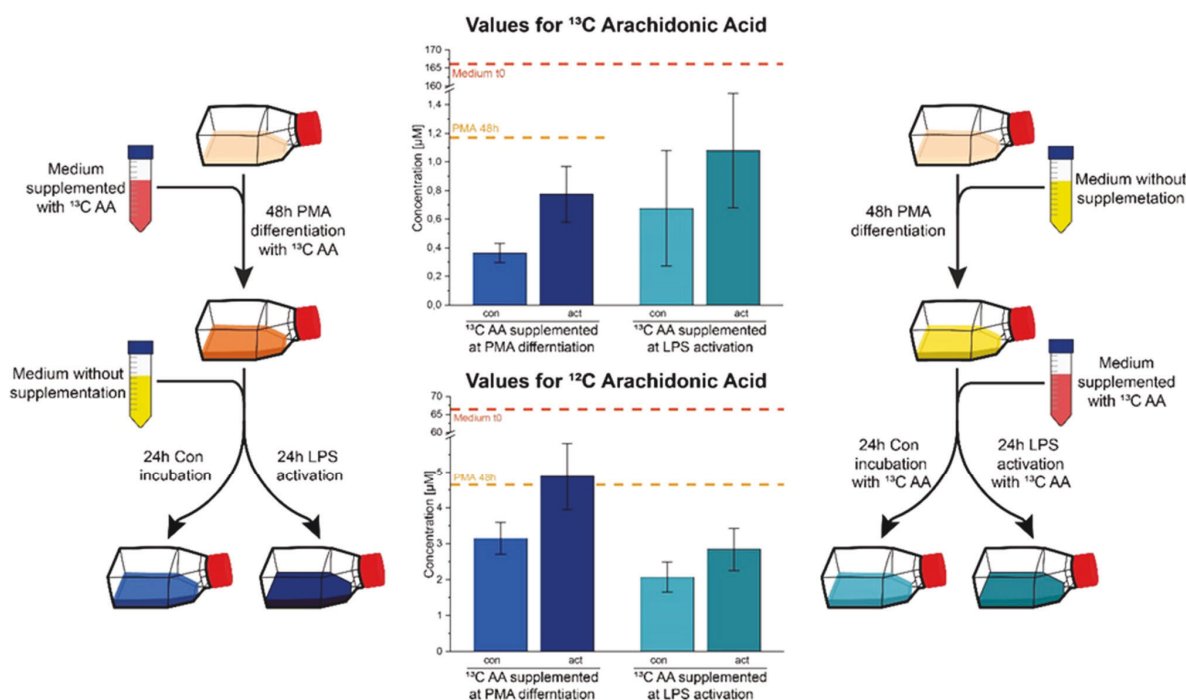


Figure 3. Experimental setup and results from AA spiking experiments. Medium was supplemented with ^{13}C AA either before differentiation (left hand side) or before LPS treatment (right hand side). AA determination of cell supernatants by LC-MS/MS revealed AUC values as indicated. Medium levels at the beginning of cell culture are indicated by lines. Error bars are derived from three independent experiments. Con, untreated cells; act, LPS-treated cells. Note that AA concentration values strongly decrease upon cell cultivation but increase again upon LPS treatment.

2.3. Supplementation of 5-HETE and 15-HETE in the Nanomolar Concentration Range Induces the Formation of Peroxisomes in U937 Macrophages

In order to demonstrate that the detected differences in eicosanoid content of FCS could originate from the observed batch effects, we performed an additional proteome profiling experiment with U937 cells at conditions only differing with regard to two selected eicosanoids, 5-HETE and 15-HETE. To this aim, we supplemented the FCS batch found to have the lowest levels of these two eicosanoids, here designated FCS-B (containing 5 nM 5-HETE and 12 nM 15-HETE), with the pure chemicals to levels close to those observed in case of FCS batch A (FCS-A, containing 42 nM 5-HETE and 49 nM 15-HETE, Figure 4A). Thus, U937 cells were grown and differentiated as before, using either FCS-A, supplemented FCS-A, or FCS-B, and subsequently subjected to proteome profiling of cytoplasmic proteins (Figure 4B). Indeed, spiking in of the two HETEs was associated with distinct proteome alterations (Figure 4B) including down-regulation of PKM and up-regulation of PEX 16, a peroxisomal membrane biogenesis protein [18]. To independently verify with a complementary method that this was a relevant observation, peroxisome formation was analyzed using immunofluorescence with an anti-PMP70 antibody. Nuclei

and mitochondria were additionally stained to demonstrate uniform appearance of these organelles serving as background control. Indeed, treatment of U937 cells with increasing concentration of HETEs induced the formation of peroxisomes in an apparently concentration dependent manner (Figure 4C).

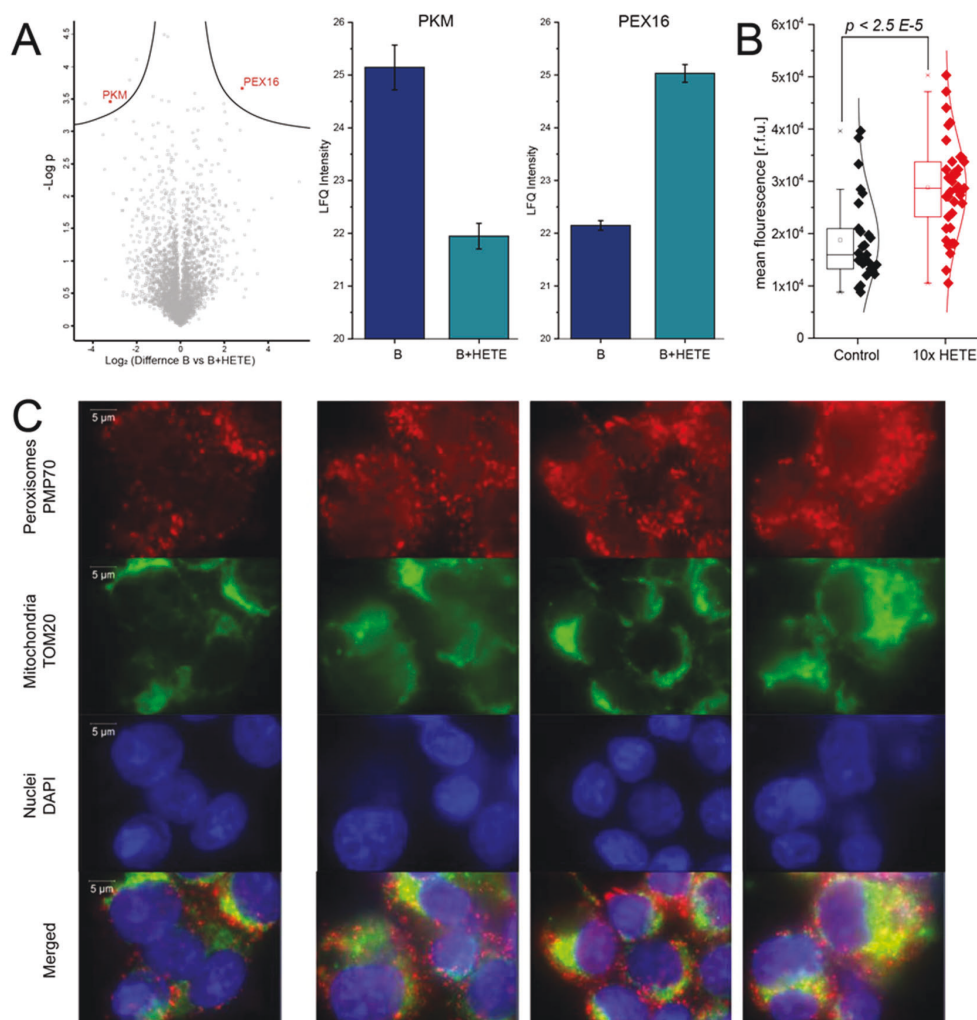


Figure 4. (A) Eicosanoid levels of 5-HETE and 15-HETE for FCS-B (before spiking), FCS-A and FCS-B after spiking with 5-HETE and 15-HETE. (B) Volcano plot for cytoplasmic proteins obtained from U937 cells after PMA differentiation when cultured in either FCS-B or FCS-B supplemented HETEs. Bar plots exemplify the significantly regulated proteins PKM and PEX16. (C) Immunofluorescence detection of peroxisomes (red, PMP70 antibody), mitochondria (green, TOM20 antibody) and nuclei (blue, DAPI) shown for control and increasing concentrations of supplemented HETEs (addition of 1, 3, or 10 times of the spiked HETE mix).

3. Discussion

The present data demonstrates that variations in the eicosanoid content of FCS may account for substantial batch effects with regard to functional readouts of a cell culture model reporting inflammatory mediators. This finding may be of great relevance for a large number of laboratories working with cell culture and using FCS, as FCS-contained eicosanoids have hardly been considered to have major implications for cell culture experiments and have thus, to the best of our knowledge, not yet been subjected to rigorous control. There are reasons, why relevant effects of eicosanoids contained in FCS were

hardly expected. First, these molecules are generally considered to be short-lived and to act mainly *in situ* [19]. Second, eicosanoids were detected in FCS in the lower nanomolar concentration range, this is much less than the concentration range applied for functional assays *in vitro*, which is typically around 1 μM [20,21]. Furthermore, fatty acids including eicosanoids contained in serum are bound to albumin and only about 0.1% is actually free from associated molecules [22–24]. This free pool has a high turnover rate of about 2 min accounting for the redistribution of albumin-bound fatty acids *in vivo* to distant organs such as muscles or the liver.

When investigating FCS batch effects, we initially expected proteins to represent the most plausible candidates contributing to reproducibility issues. Proteins as well as metabolites are strictly regulated *in vivo* to ensure homeostasis and consequently stable viability of the organism. While proteins may be rather stable in biological environments, metabolites such as fatty acids are much more vulnerable to chemical reactions such as oxidation, which may occur also during processing of FCS and are hard to control. Thus it was somewhat unexpected to see that FCS eicosanoid profiles were stable and clustered the FCS samples according to batches (Figure 2). This finding, supported by older and current literature reporting remarkable biological effects of eicosanoids [25–27], motivated us to focus on this class of molecules. Functional analyses were based on spiking experiments with the U937 cells. As a first step, the efficient and fast uptake of albumin bound arachidonic acid (AA) was verified in the present cell model system using stable isotope labelled AA. The subsequent release of labelled AA upon LPS stimulation of the cells strongly indicated the previous uptake and incorporation of AA into more complex lipids, from where AA was apparently released by the action of LPS-induced phospholipase A2 [28]. In order to test potential biological effects of eicosanoids on U937 cells, a decision was made in favor of commercially available eicosanoids, 5-HETE and 15-HETE, which were found to show remarkable concentration differences among the FCS batches. Hydroxyeicosatetraenoic acids (HETEs) are formed with AA by the action of lipoxygenases ALOX5 and ALOX15, expressed typically by epithelial cells as well as phagocytes such as neutrophils and macrophages [29,30]. Beside their effects on cell proliferation and differentiation, they are known activators of PPAR γ [31]. Actually, peroxisome proliferator-activated receptors are known to induce the uptake and metabolism of fatty acids and to strongly modulate immune functions [32]. As fatty acid metabolism takes place in peroxisomes [33], the 5-HETE/15-HETE induced up-regulation of PEX16 (Figure 4), a peroxisome biogenesis protein indicative for peroxisome proliferation [18], indicated that this treatment caused an increased demand for these organelles. The concomitant down-regulation of PKM (Figure 4), a key enzyme for glycolysis [34], may suggest that HETE-treatment of U937 cells induced a metabolic shift increasing beta oxidation and attenuating glycolysis. This interpretation was independently supported by the concentration-dependent HETE-induced formation of peroxisomes (Figure 4) observed by immunofluorescence staining using a PMP70 antibody [35].

4. Conclusions and Outlook

The present data demonstrate that batch-dependent differences of eicosanoids contained in FCS may have a profound effect on cellular functions as observed with the U937 *in vitro* cell culture model for differentiation and inflammatory stimulation. Eicosanoids affect many relevant cellular events far beyond that, suggesting that they may represent the main contributors for reproducibility issues in cell culture. The establishment of a strict quality control regime controlling eicosanoid content in FCS may alleviate this challenging problem.

5. Materials and Methods

5.1. Cell Culture

U937 cell line was cultured in RPMI medium (1X with L-Glutamine; Gibco, Thermo Fischer Scientific, Vienna, Austria) supplemented with 1% Penicillin/Streptomycin (Sigma-Aldrich, Austria) and 10% Fetal Calf Serum (FCS, Sigma-Aldrich, Vienna, Austria) in T25 polystyrene cell culture flasks for suspension cells (Sarstedt, Wiener Neudorf, Austria) at 37 °C and 5% CO₂. Cells were counted with a MOXI Z Mini Automated Cell Counter (ORFLO Technologies, Ketchum, ID, USA) using Moxi Z Type M Cassettes (ORFLO Technologies, Ketchum, ID, USA) and the number of seeded cells for the experiments calculated based of these results. For all experiments the cells were used in passages 22–26.

5.2. Differentiation with Phorbol 12-Myristate 13-Acetate (PMA) and Inflammatory Activation with Lipopolysaccharides (LPS)

All experiments were carried out in triplicates of LPS activation and control. For the proteomics and eicosadomics measurements 2×10^6 cells were seeded in T25 polystyrene cell culture flasks with cell growth surface for adherent cells (Sarstedt, Wiener Neudorf, Austria) with 5 mL fully supplemented media and 100 ng/mL PMA (Phorbol 12-myristate 13-acetate $\geq 99\%$, Sigma-Aldrich, Vienna, Austria) to induce differentiation. After 48 h incubation the medium was withdrawn and used for eicosanoid measurements. Three ml of new fully supplemented media was added either with 1 $\mu\text{g/mL}$ LPS (Lipopolysaccharides from *Escherichia coli* 055:B5, γ -irradiated, BioXtra, Sigma-Aldrich, Vienna, Austria) or 1 μL PBS per 1 mL medium as control. After 24 h activation the medium was withdrawn again and used for eicosanoid measurements. The cells were gently washed twice with 5 mL phosphate buffered saline (PBS) and 3 mL new medium without FCS was added and incubated. After 4 h the supernatant was withdrawn and used for proteomics measurements. The cells were used for a subcellular fractionation as described before and cytoplasm and nuclear fraction were used for proteomics analysis [36].

5.3. Test of Different FCS Batches

Throughout the experiments different suppliers and batches of FCS were used. Additional details concerning the FCS batches are listed in Table 1. FCS batches A-C were heat inactivated at 56 °C for 30 min, batch D was already bought heat inactivated. Also different concentrations of HETEs and labelled arachidonic acid were supplemented and the respective controls treated with the same amount of LC-MS grade methanol (5 $\mu\text{L}/3\text{mL}$ medium). The experimental workflow of PMA differentiation and LPS activation was done for every condition similarly, only exchanging the FCS batch, supplier or eicosanoid. Additionally, for every condition 3 aliquots ($3 \times 3\text{ mL}$) of the fully supplemented media were used for eicosanoid measurements to determine the default levels of eicosanoids present.

5.4. C13 Labelled Arachidonic Acid

For the investigation of the uptake and release of PUFAs an experiment was carried out with the supplementation of 1,2,3,4,5-¹³C arachidonic acid (C13 AA, Cayman chemicals, Ann Arbor, Michigan, USA). Whenever C13 AA was added, a control experiment was supplemented with the same concentration of unlabeled arachidonic acid. For the first experiment the C13 AA was added at a concentration of 1.6 μM (used for all AA supplementations) to the fully supplemented medium during the 48 h PMA differentiation step. This concentration is around double that of the endogenous arachidonic acid, thus the supplementation tripled the concentration of biologically active arachidonic acid. Afterwards, the now adherent cells were washed three times with PBS and medium without supplemented AA was added together with or without LPS for 24 h. For the second experiment the cells were differentiated with PMA in standard medium, washed three times with PBS and medium supplemented with C13 AA was added together with and

without LPS for 24 h. The eicosanoids were collected and measured in the supplemented medium without incubation (t0), after 48 h PMA differentiation and after 24 h LPS activation. The experimental setup is illustrated in Figure 3.

5.5. Proteomics of Supernatant (SN), Cytoplasm (CYT), and Nuclear Extract (NE)

For the proteomics sample preparation, the s-trap system (Protifi, Huntington, NY) was employed following the manufacturers protocol with slight modifications. The precipitated proteins were dissolved in lysis buffer (8 M Urea, 0.05 M triethylammonium bicarbonate (TEAB) and 5% sodium dodecyl sulfate SDS) and diluted to obtain a protein concentration of about 1 µg/µL. Twenty µg of protein was used for each digestion. First, the sample was reduced with 20 µL dithiothreitol DTT (Sigma-Aldrich) at a final concentration of 32 mM for 10 min at 95 °C. Afterwards, 5 µL iodoacetamide IAA (Sigma-Aldrich) was added to a final concentration of 54 mM and incubated for 30 min at 30 °C in the dark. After adding 4.5 µL 12% ortho-phosphoric acid (Sigma-Aldrich) and 297 µL S-Trap buffer (90% Methanol (v/v) in H₂O and 0.1 M TEAB) the sample was loaded onto the S-Trap Filter. The S-trap filters were centrifuged at 4000 xg for 1 min to pass through all the sample and trap the proteins onto the resin and afterwards washed four times with 150 µL S-Trap buffer. Twenty µg aliquots of Trypsin/Lys-C (MS grade; Promega Corporation, Madison, WI, USA) were dissolved in 400 µL 50 mM TEAB and 20 µg of this solution was added directly onto the resin of the filter (corresponding to 1 µg Trypsin/Lys-C per sample) and incubated for 1 h at 47 °C. After finishing the digestion, the peptides were eluted with 40 µL of 50 mM TEAB followed by 40 µL of 0.2% formic acid (FA) in H₂O and 35 µL of 50% (v/v) acetonitrile (ACN) with 0.2% FA in H₂O. The peptides were dried for about 2 h with vacuum centrifugation and stored at −20 °C until LC-MS/MS measurement.

5.6. HPLC-MS/MS for Proteomics

For the HPLC-MS/MS analysis the peptides were resolved in 5 µL 30% formic acid and diluted with 40 µL of mobile phase A (97.9% H₂O, 2% acetonitrile, 0.1% formic acid). One µL for the supernatant samples and 5 µL of cytoplasmic and nuclear samples were injected into the Dionex UltiMate 3000 RSLCnano liquid chromatography (LC) system coupled to the QExactive Orbitrap MS (all Thermo Fisher Scientific, Austria). Peptides were trapped on a C18 2 cm × 100 µm precolumn and LC separation was performed on a 50 cm × 75 µm Pepmap100 analytical column (both Thermo Fisher Scientific, Austria). Separation was achieved applying a 43 min gradient from 7% to 40% mobile phase B (79.9% acetonitrile, 20% H₂O, 0.1% formic acid) for supernatant samples and 95 min gradients from 8% to 40% mobile phase B for cytoplasmic and nuclear samples, both at a flow rate of 300 nL/min, resulting in a total run time of 85 min and 135 min, respectively. Mass spectrometric settings were the same for all fractions. The resolution on the MS1 level was set to 70,000 (at $m/z = 200$) with a scan range from 400 to 1400 m/z . The top eight abundant peptide ions were chosen for fragmentation at 30% normalized collision energy and resulting fragments analyzed in the Orbitrap at a resolution of 17,500 (at $m/z = 200$).

5.7. Proteomics Data Analysis

Raw data were subjected to the freely available software MaxQuant (version 1.6.0.1) [37] utilizing the Andromeda search engine, which returns label free quantification (LFQ) values for each identified protein as subsequently used for further data evaluation. For the MaxQuant search, a minimum of two peptide identifications, at least one of them being a unique peptide, was required for valid protein identification. Digestion mode was set to “Specific” choosing Trypsin/P. The peptide mass tolerance was set to 50 ppm for the first search and to 25 ppm for the main search. The false discovery rate (FDR) was set to 0.01 both on peptide and protein level. The database applied for the search was the human Uniprot database (version 03/2018, reviewed entries only) with 20,316 protein entries. Further settings for the search included carbamidomethylation as fixed modification and

oxidation of methionine and acetylation of the protein C terminus as variable modifications. Each peptide was allowed to have a maximum of two missed cleavages and two modifications, “Match between runs” was enabled and the alignment window set to 10 min, with the match time window of 1 min. Statistical evaluation was performed with Perseus software (version 1.6.0.2) [38] using LFQ intensities of the MaxQuant result file. After filtering potential contaminants, the LFQ values were Log(2)-transformed. Technical duplicates were averaged. Only proteins detected in three of three biological replicates in either control and/or treatment groups were considered for data evaluation. Permutation-based FDR was set to 0.05 for *t*-tests and provided significant protein expression changes corrected for multi-parameters ($S_0 = 0.1$). The mass spectrometry proteomics data were deposited in the ProteomeXchange Consortium (<http://proteomecentral.proteomexchange.org>) via the PRIDE partner repository [39] with the dataset identifier PXD020617 and 10.6019/PXD020617.

5.8. Eicosanoid Sample Preparation

Cell supernatants were spiked with 5 μ L of internal standards (Supplementary Table S1) and centrifuged at 726 g for 5 min to remove cells and debris. Three ml of the supernatant was mixed with 12 mL of ice cold ethanol and left at -20°C overnight to precipitate the contained proteins. The samples were centrifuged for 30 min with 4536 xg at 4°C and the supernatant transferred into a new 15 mL Falcon tube. Ethanol was evaporated via vacuum centrifugation at 37°C until the original sample volume was restored. Samples were loaded on conditioned 30 mg/mL StrataX solid phase extraction (SPE) columns (Phenomenex, Torrance, CA, USA). Columns were washed with 2 mL MS grade water and eicosanoids were eluted with 500 μ L methanol (MeOH abs.; VWR International, Vienna, Austria) containing 2% formic acid (FA; Sigma-Aldrich). MeOH was evaporated using N_2 stream at room temperature and reconstituted in 150 μ L reconstitution buffer ($\text{H}_2\text{O}/\text{ACN}/\text{MeOH} + 0.2\% \text{FA} - 65:31,5:3,5$), containing a second set of internal eicosanoid standards at a concentration of 10–100 nM (Supplementary Table S1).

5.9. UHPLC-MS/MS for Eicosanoid Measurements

Analytes were separated using a Thermo Scientific Vanquish (UHPLC) system and a Kinetex[®] C18-column (2.6 μm C18 100 $\text{\AA}$, LC Column 150 $\times$ 2.1 mm; Phenomenex[®]). Applying a 20 min gradient flow method, starting at 35% B steadily increasing to 90% B (1–10 min), going up to 99% B in 0.25 min. Flow rate was kept at 200 $\mu\text{L}/\text{min}$, 20 μL injection volume and column oven temperature was set to 40°C . Eluent A contains $\text{H}_2\text{O} + 0.2\% \text{FA}$ and eluent B $\text{ACN}:\text{MeOH} (90:10) + 0.2\% \text{FA}$.

Mass Spectrometric analysis was performed with a Q Exactive HF Quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific, Austria), equipped with a HESI source for negative ionization. Mass spectra were recorded operating from 250 to 700 m/z at a resolution of 60,000 @ 200 m/z on MS1 level. The two most abundant precursor ions were selected for fragmentation (HCD 24 normalized collision energy), preferentially molecules from an inclusion list which contained 32 m/z values specific for eicosanoids (Supplementary Table S2). MS2 was operated at a resolution of 15,000 @ 200 m/z . For negative ionization, a spray voltage of 2.2 kV and a capillary temperature of 253°C were applied, with the sheath gas set to 46 and the auxiliary gas to 10 arbitrary units.

Generated raw files were analyzed manually using Thermo Xcalibur 4.1.31.9 (Qual browser), comparing reference spectra from the Lipid Maps depository library from July 2018 [40]. For peak integration and quantitative data analysis the software TraceFinderTM (version 4.1-Thermo Scientific, Austria) was used. For the quantification of arachidonic acid (Figure 3), a calibration curve was generated (Supplementary Figure S4).

5.10. Immunofluorescence

For fluorescence microscopy 8×10^4 cells in 400 μL were seeded in a $\mu\text{-Slide}$ 8 well (Ibidi coating, ibidi GmbH Martinsried, Germany). Differentiation of the cells was induced with 100 ng/mL PMA for 48 h. Afterwards the cell supernatant was exchanged with fully supplemented medium without PMA for an additional 24 h. Sample preparation was performed as previously described with minor modifications [41]. Cells were fixed with pre-warmed formaldehyde (3.7%) for 15 min and permeabilized with Triton-X 100 (0.2%) for 10 min. Blocking was performed with Donkey serum (2% in PBS-A) for 1 h, room temperature (RT). Primary antibodies were incubated 2 h at RT at dilution 1:500. After washing, specie-specific fluorescent-labelled secondary antibodies were added and slides incubated in a dark humidified chamber for 1.5 h. For our study, Anti PMP70 Antibody (Rabbit polyclonal, PA1-650) and Anti TOM20 (F-10, Mouse Monoclonal Sc-17764), Alexa Fluor 488 Donkey Anti Mouse (A21202_LOT2090565) and Alexa Fluor 568 Donkey Anti-Rabbit (A10042_LOT2136776) were used. The slides were washed and post-fixed with 3.7% formaldehyde (10 min, RT); at the end of the post-fixation, 100 mM glycine was used to mask reactive sites and slides were mounted and sealed with Roti-Mount FluoCare (Roth, Graz, Austria) with DAPI. SIM Images were acquired with a Confocal LSM Zeiss 710 equipped with ELYRA PS. 1 system. Structured Illumination Microscopy (SIM) images were obtained with (Plan Apochromat 63X/1.4 oil objective) grid 5 rotation. For the quantification of fluorescence intensities (Figure 4B), 30 optical fields/region of interest (ROI) were quantified for every experimental condition from at least 3 independent experiments.

5.11. Differentiation Status by Flow Cytometry

In order to confirm the differentiation status obtained via PMA treatment the cells were tested for the differentiation marker CD11b (ITGAM) using FACS analysis. Therefore, U937 cells were treated with 100 ng/mL PMA for 48 h using 2×10^5 cells per well in 6-well plates. After the incubation time, the cells were washed three times with PBS and put on ice. The differentiation status was assessed by labelling with an anti-CD11b antibody (APC clone D12, BD Bioscience) and subsequent evaluation of the CD11b⁺ population. Three biological replicates were analyzed per condition on an FACS Canto II cytometer (BD Bioscience).

Supplementary Materials: The following are available online at www.mdpi.com/2218-273X/11/1/113/s1, Figure S1: FACS analysis of PMA-treated U937 cells, Table S1: Eicosanoid Standards, Table S2: Inclusion list of eicosanoids as used for the MS/MS analysis, Table S3: Results from proteomic analyses of U937 cells, Table S4: Results from eicosanoid analyses of U937 cells, Table S5: Results from proteomic analyses of FCS batches, Table S6: Results from eicosanoid analyses of FCS batches.

Author Contributions: L.N. performed research, analyzed and interpreted data, B.N. performed research, analyzed and interpreted data, J.B. performed research and analyzed data, L.J. performed research and analyzed data, A.B. interpreted data and wrote manuscript, G.D.F. performed research and analyzed data, C.G. conceptualized the project, interpreted data and wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The mass spectrometry proteomics data were deposited in the ProteomeXchange Consortium (<http://proteomecentral.proteomexchange.org>) via the PRIDE partner repository [39] with the dataset identifier PXD020617 and 10.6019/PXD020617.

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Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

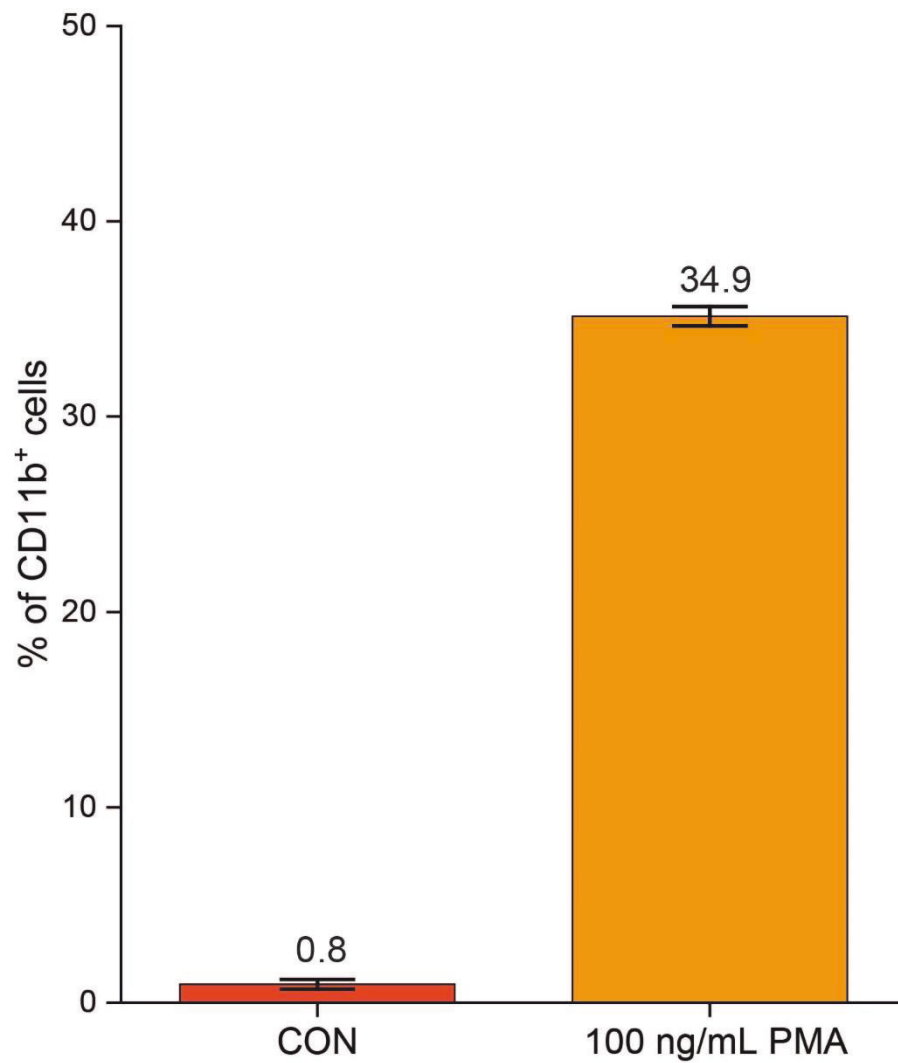
AA	Arachidonic acid
FCS	Fetal calf serum
FFA	Free fatty acids
CDM	Chemically defined medium
FBS	Fetal bovine serum
HETE	Hydroxyeicosatetraenoic Acid
LC	Liquid chromatography
LPS	Lipopolysaccharides
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
PMA	Phorbol 12-myristate 13-acetate
PUFA	Polyunsaturated fatty acids

References

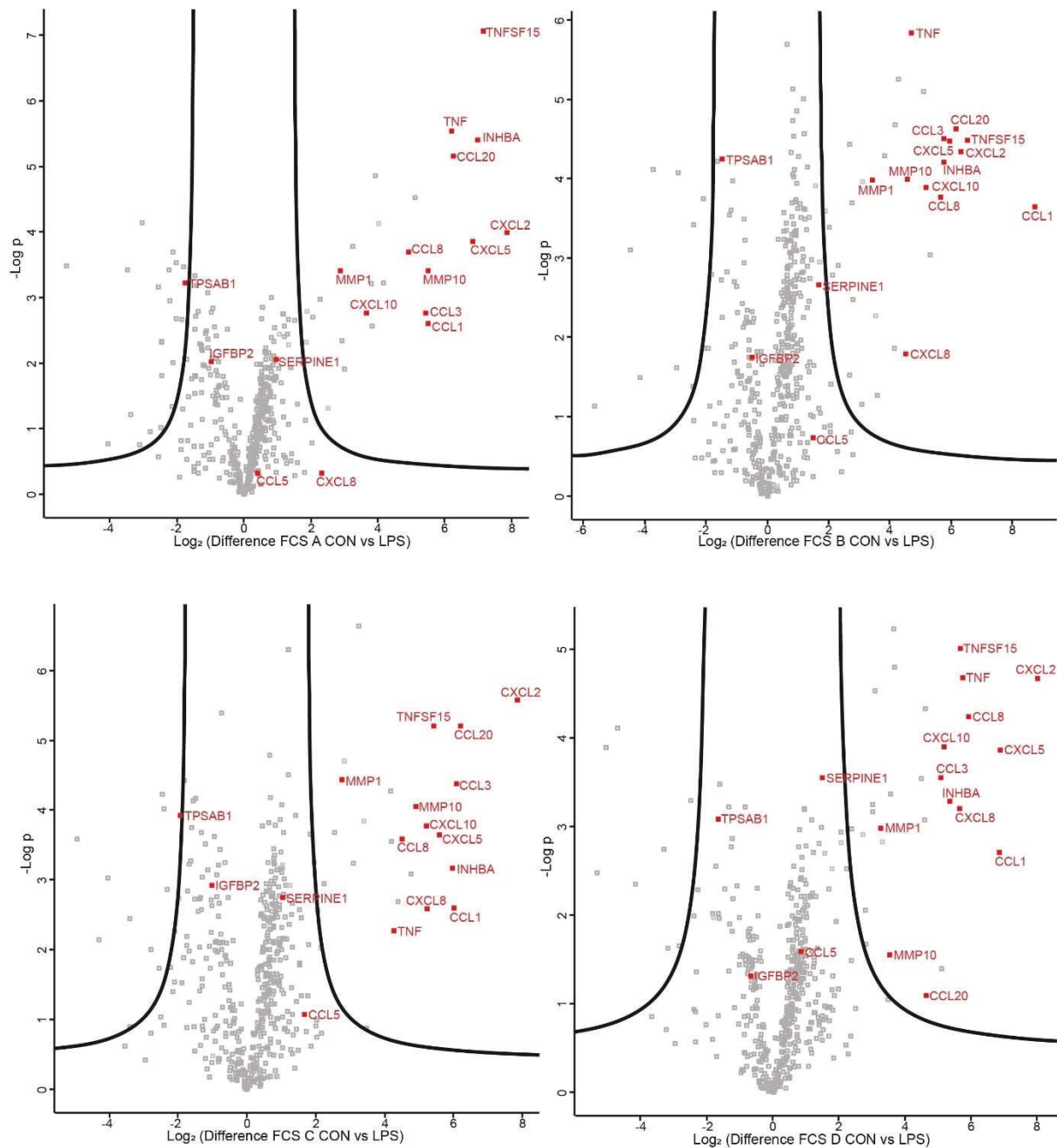
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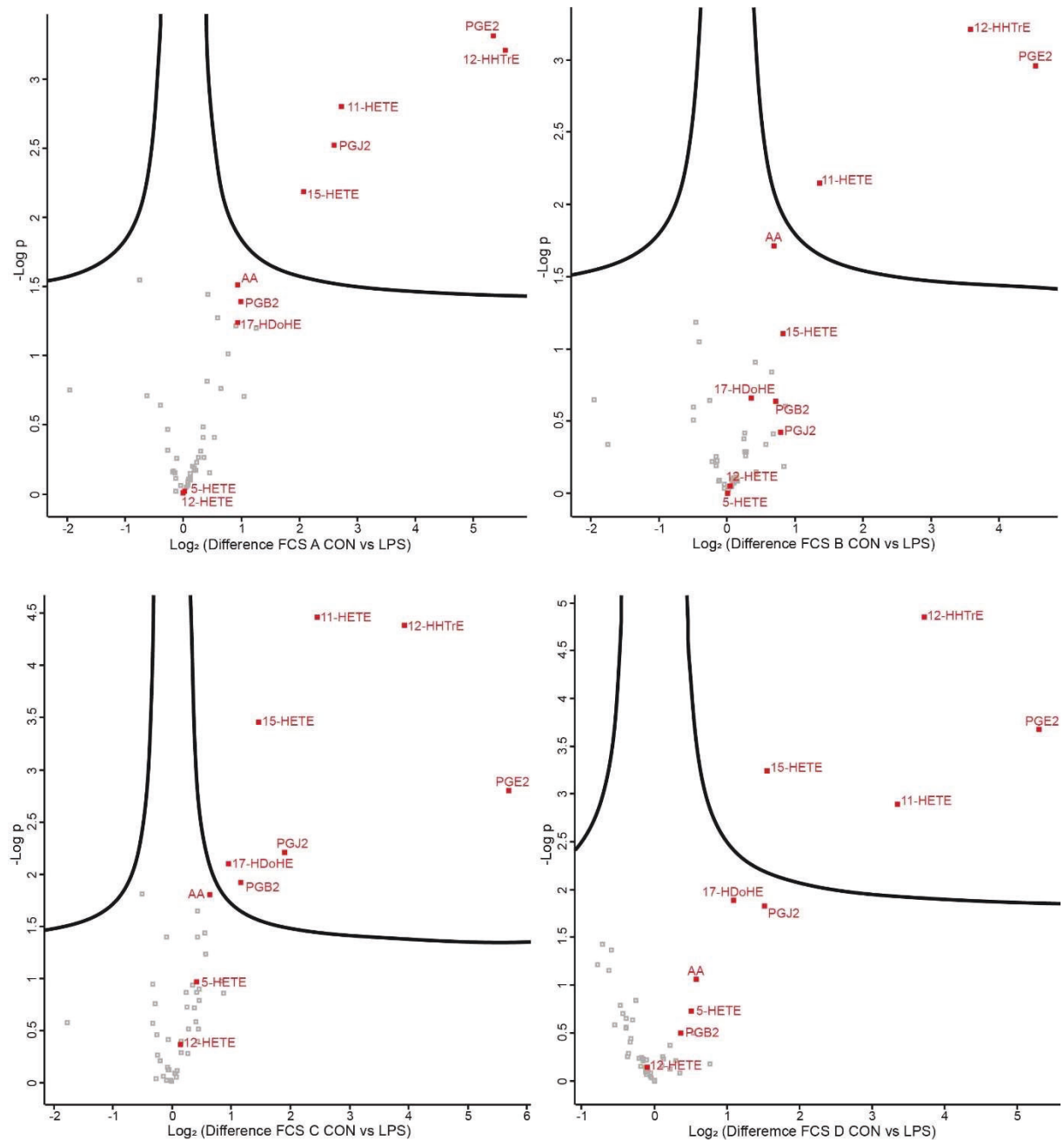
Supplementary Information



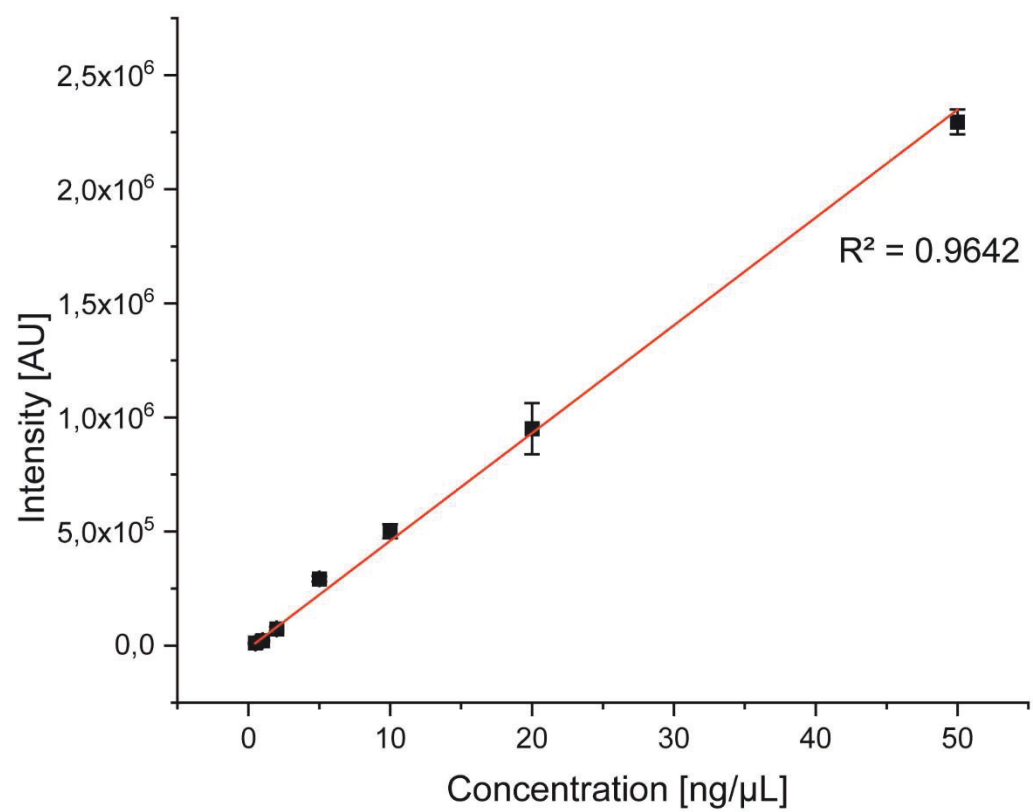
Supplementary Figure S1: Percentage of CD11b (ITGAM) positive U937 cells after 48 h treatment with 100 ng/mL PMA. The analysis shows a significant ($p\text{-value} < 10^{-8}$) increase in CD11b⁺ cells upon treatment compared to untreated cells.



Supplementary Figure S2: Volcano plots comparing the identified proteins of the control and the LPS activated U937 cells for each medium A-D. The significant regulated proteins (FDR=0.05, S0=2) were used for the Venn diagram shown in Figure 1C. The labelled proteins are described in the context of inflammatory activation and are mentioned in the main text. All identified proteins and the significant regulations with respective p-value and differences can be found in supplementary table 3.



Supplementary Figure S3: Volcano plots comparing the identified eicosanoids of the control and the LPS activated U937 cells for each medium A-D. The significant regulated oxylipins (FDR=0.05, S0=0.1) were used for the Venn diagram shown in Figure 1D. All identified oxylipins and the significant regulations with respective p-value and differences can be found in supplementary table 4.



Supplementary Figure S4: Calibration curve for arachidonic acid used for quantification. Three replicates were analysed per concentration.

6.4 An Organometallic Gold(I) Bis-N-Heterocyclic Carbene Complex with Multimodal Activity in Ovarian Cancer Cells

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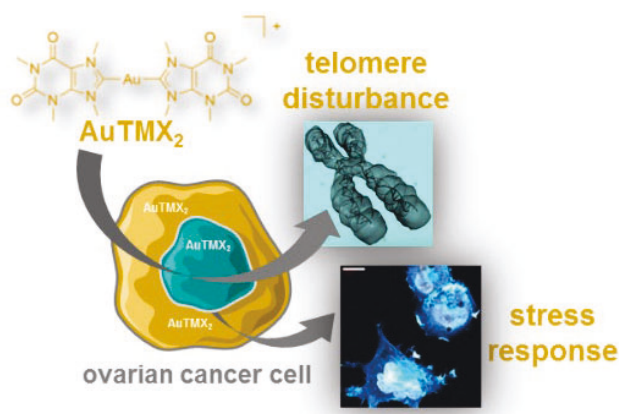
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Contributions to this publication:

- Conduction of experiments and MS analysis
- Analysis of data
- Proofreading of the manuscript

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An Organometallic Gold(I) Bis-N-Heterocyclic Carbene Complex with Multimodal Activity in Ovarian Cancer Cells

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Abstract: The organometallic Au^I bis-N-heterocyclic carbene complex [Au(9-methylcaffeine-8-ylidene)₂]⁺ (**AuTMX₂**) was previously shown to selectively and potently stabilise telomeric DNA G-quadruplex (G4) structures. This study sheds light on the molecular reactivity and mode of action of **AuTMX₂** in the cellular context using mass spectrometry-based methods, including shotgun proteomics in A2780 ovarian cancer cells. In contrast to other metal-based anti-cancer agents, this organogold compound is less prone to

form coordinative bonds with biological nucleophiles and is expected to exert its drug effects mainly by non-covalent interactions. Global protein expression changes of treated cancer cells revealed a multimodal mode of action of **AuTMX₂** by alterations in the nucleolus, telomeres, actin stress-fibres and stress-responses, which were further supported by pharmacological assays, fluorescence microscopy and cellular accumulation experiments. Proteomic data are available via ProteomeXchange with identifier PXD020560.

Introduction

During the last decades, metal complexes have occupied a pivotal role in medicinal chemistry, either as imaging or as therapeutic agents.^[1] Within the large pool of metal-based compounds, gold complexes have been extensively investigated and are now recognized as promising experimental drugs for cancer treatment.^[2] Among the various families of cytotoxic Au^I compounds, those featuring N-heterocyclic carbene

(NHC) ligands can be tuned for their steric and electronic properties, which enable modulation of the compounds' reactivity, solubility, lipophilicity and targeting properties.^[3] Concerning possible mechanisms of anticancer action, Au^I NHC complexes can affect numerous biological targets and metabolic pathways, also depending on their propensity to exchange some of their ligands to bind to intracellular nucleophiles, including sulfur and selenium-containing amino acids in proteins.^[4] For example, several homo- and heteroleptic

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Au^I NHCs were developed as potent inhibitors of seleno-enzymes.^[5]

In addition, we have recently observed that cationic Au^I bis-NHC compounds, endowed with high stability in aqueous environment, can target and stabilize DNA secondary structures of pharmacological relevance, named G-quadruplexes (G4s).^[6] In detail, the Au^I complex [Au(9-methylcaffeine-8-ylidene)]⁺ (**AuTMX₂**, Figure 1) was shown to selectively stabilize telomeric G4s using fluorescence resonance energy transfer (FRET) DNA melting assays.^[7] G4 structures may also form in promoter regions of oncogenes where they seem to control their transcription and expression.^[8] Moreover, stabilizing the folding of G4s in telomeres indirectly inhibits telomerase activity, thus, affecting cancer cell mortality.^[9] An interesting feature of **AuTMX₂** is its selective antiproliferative effect against ovarian cancer cell lines with respect to other human cancerous cells, as well as its scarce toxicity in non-tumorigenic cells and tissues.^[7] X-ray diffraction analysis of the adduct formed by **AuTMX₂** and a model of telomeric G4 (Tel23) showed that the compound binds non-covalently between neighbouring quadruplexes.^[10] Further characterization of the binding modes of **AuTMX₂** with different G4 structures was achieved by metadynamics simulations,^[11] highlighting the importance of π - π stacking and possibly electrostatic interactions in stabilizing the gold complex/G4 adducts. Of note, the presence of two NHC ligands is essential to achieve the highest possible stabilization of the G4 structure.^[12]

In parallel to their role in cancer biology, G4s are emerging and promising targets for anticancer drug discovery. Numerous studies described the efficient G4 stabilization by small molecules with associated anticancer potential,^[13] including metal complexes.^[6a] Since, to the best of our knowledge, **AuTMX₂** is the first organometallic complex reported so far for its selective G4 stabilization properties, we investigate here its cellular mode of action and pharmacological effects by an integrated approach, including mass spectrometry-based shotgun proteomics.

Proteomic approaches are being successfully employed to study drug-activity profiles and modes of action, including in vitro investigations of a variety of metal-based pharmaceuticals,^[14] as well as the effects of metal restriction on microbial physiology.^[15] Using these techniques, different classes of metal-based anticancer^[16] or antimicrobial agents^[17] were shown to trigger specific effects in target cells and evidenced that small changes in metallodrug's structure would affect dramatically its target spectrum.^[18] A few Au^I and Au^{III} anticancer compounds were investigated by gel-based^[19] and gel-free^[20]

proteomic analyses to characterize their molecular mechanisms of action, and in a few cases to identify their intracellular targets.

For example, the Au^I bis-NHC complex **Au(BMI)₂** (BMI = 1-butyl-3-methyl-imidazole-2-ylidene, Figure 1) is structurally similar to **AuTMX₂**, and was investigated using gel-based proteomics in the ovarian cancer cell line A2780.^[19a] The compound was shown to regulate a larger number of proteins compared to its mono-carbene analogue, and resulted in an impairment of metabolic activity, while being devoid of nuclear protein alterations. Here, we show that **AuTMX₂** features a multimodal mode of action by affecting nuclear and telomeric proteins in addition to inducing a stress response related to redox stress. The latter includes reactive oxygen species (ROS) and stress-sensitive proteome alterations in addition to actin stress-fibres.

Results and Discussion

The binding of the cationic Au^I bis-NHC complex **AuTMX₂** has stabilizing effects on G4 secondary DNA-structures,^[10,12] which should entail observable protein alterations due to the involvement of G4s in telomeres and promoter regions of many (onco-)genes. Hence, mass spectrometry-based shotgun proteomics was employed to comprehensively investigate the effects of **AuTMX₂** in cancer cells at the molecular level. The overall experimental workflow of this study is depicted in Figure S1 in the Supporting Information.

First, the reactivity of **AuTMX₂** was studied by electrospray ionization mass spectrometry (ESI-MS) in order to evaluate potential coordinative interactions with biomolecules that may cause off-target effects, in a similar setup as reported previously.^[21] **AuTMX₂** was stable in water and in the presence of ascorbic acid over the entire incubation period (Figure S2A). In the presence of the amino acid mixture containing L-histidine (His), L-cysteine (Cys), L-methionine (Met), L-glutamate (Glu) and L-methylselenocysteine (^{Me}Se-Cys), **AuTMX₂** remained intact even after 24 h, forming minor adducts only with His and Cys via the release of one TMX ligand (Figure S2B). Adducts corresponding to Au^I coordinated to ^{Me}Se-Cys, 9-ethyl-guanine or nucleotides were not observed, and neither were adducts with model proteins ubiquitin or cytochrome C (Figure S3).^[22] Thus, **AuTMX₂** appeared stable and largely unreactive towards biological nucleophiles, supporting the idea that its mode of action may reply primarily on non-covalent interactions. This stands in contrast to benzimidazole-based bis-NHC Au^I complexes, which appear to be less stable and tend to exchange their ligands.^[7,23]

In cells, **AuTMX₂** features selectivity for cancerous over non-cancerous cells and against the ovarian cancer cell line A2780 with respect to other human cancer cell lines.^[7] We determined the antiproliferative effect of **AuTMX₂** against A2780 cancer cells and found a half maximal effective concentration (EC₅₀) of $13.3 \pm 1.1 \mu\text{M}$ after 24 h treatment. This was similar to the published EC₅₀ in A2780 cancer cells after a 72 h treatment.^[7] Further experiments in this study were conducted by incubating A2780 cancer cells either at $8 \mu\text{M}$ ($2/3 \text{ EC}_{50}$), $13 \mu\text{M}$ (EC₅₀) or $26 \mu\text{M}$ ($2 \times \text{EC}_{50}$) concentrations.

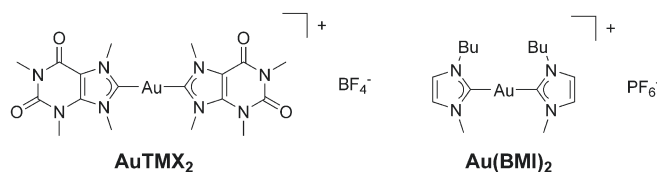


Figure 1. Chemical structures of **AuTMX₂** and **Au(BMI)₂**, BMI = 1-butyl-3-methyl-imidazole-2-ylidene. The latter was previously investigated using gel-based proteomic methods in ref. [19a].

The accumulation of **AuTMX₂** in A2780 cancer cells was studied by analysing ¹⁹⁷Au using inductively-coupled plasma mass spectrometry (ICP-MS). The total cellular accumulation was evaluated after treating the cells for 3, 6 and 24 h and at concentrations corresponding to 8 μ M and 26 μ M (Figure 2A). A significant accumulation of gold was detected only after 24 h when treating with 8 μ M **AuTMX₂** and the amount of gold corresponded to 25.0 ± 0.6 fg cell⁻¹. A steady increase in

gold levels was observed over time, when treating the cells with 26 μ M, culminating in 47 ± 2 fg cell⁻¹ after 24 h. Subsequently, A2780 cells were treated with 8 μ M of **AuTMX₂** to obtain information about its distribution between cytoplasmic and nuclear fractions. It turned out that the gold distributed to approximately one third to the nucleus and two thirds to the cytoplasmic fraction irrespective of the incubation time (Figure 2B).

Since **AuTMX₂** was shown to be a potent and selective G4-inhibitor,^[10] it was of interest to determine the percentage of Au bound to DNA. DNA was isolated from A2780 cells treated with 8 μ M and 26 μ M of **AuTMX₂** using silica-based spin columns. DNA-bound Au was assumed to co-elute with DNA, while free Au should be in the flow-through and wash fractions. The ratio of Au to DNA was found to be constant in eluate 1 and 2 (Figure S4), indicating that Au detected in these samples was actually bound to DNA. The percentage of Au bound to DNA was $25 \pm 6\%$ and $41 \pm 3\%$ for 8 μ M and 26 μ M concentrations, respectively (Figure 2C). A total of 0.6 ± 0.3 pg Au and 16 ± 3 pg Au were found per ng DNA, respectively (Figure 2D). Thus, the cellular accumulation experiments confirmed an efficient uptake of **AuTMX₂** into the nucleus of A2780 cancer cells.

Mass spectrometry-based shotgun proteomics was applied to study the response of A2780 cancer cells to the **AuTMX₂** treatment. Global changes in protein expression upon treatment were obtained using a label-free quantification (LFQ) approach.^[16,18] The compound was administered at sub-cytotoxic concentrations corresponding to 8 μ M for 24 h. Six biological replicates were analysed in each condition and after treatment, the cells were fractionated into cytoplasmic (CYT) and nuclear (NE) fractions, similar to the uptake experiments (see Supporting Information). A total of 4202 proteins were detected with a false-discovery rate (FDR) of 0.01 at the peptide and protein levels. In the cytoplasmic fraction, 36 proteins were significantly regulated, while 115 proteins were significantly regulated in the nuclear fraction (FDR=0.05, S0=0.1). The workflow spanning cell culture, nucleocytoplasmic fractionation, proteolytic digestion and LC-MS analysis was robust with correlations between replicates typically around $R^2=0.9$ (Figure 3A–B).

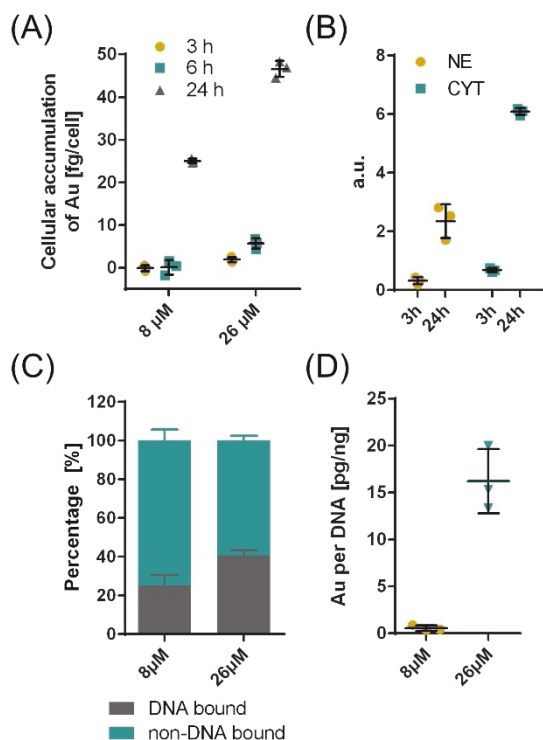


Figure 2. Cellular accumulation and distribution of **AuTMX₂** in A2780 cancer cells by analysing ¹⁹⁷Au using ICP-MS. (A) **AuTMX₂** accumulates in cells more efficiently with increasing dose or longer incubation time. (B) **AuTMX₂** distributes to one third in the nucleus (NE) and two thirds in the cytoplasmic (CYT) fraction irrespective of time when applied at 8 μ M for 24 h. (C) Relative and (D) absolute amount of Au-bound to DNA increases with applied dose after 24 h.

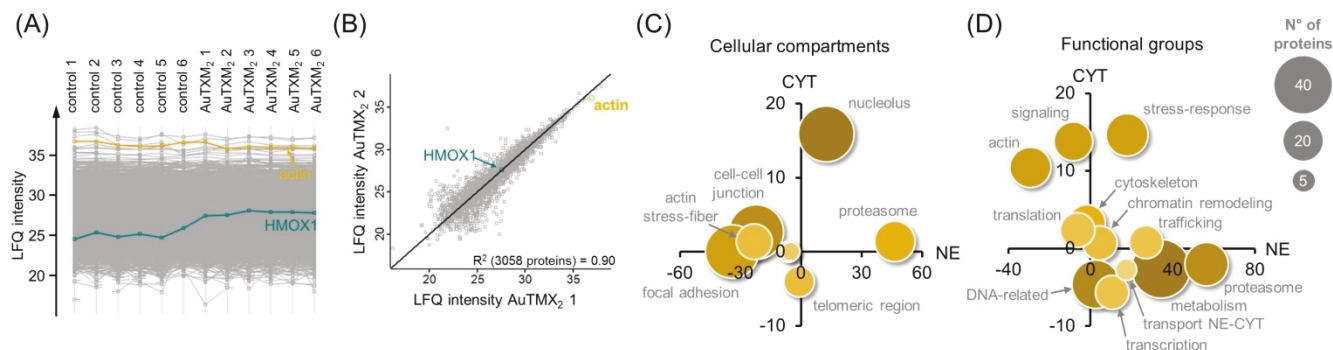


Figure 3. Response profiling by shotgun proteomics. The profile (A) and scatter (B) plots display the stability of the proteomic workflow, which contained six biological replicates per condition of **AuTMX₂**-treated A2780 cancer cells. The scatter plot highlights the global LFQ-correlation between replicate 1 and 2. Global protein expression changes were visualized by grouping significantly regulated proteins according to cellular compartment (C) and functional groups (D). The position of the protein group reflects the summed regulation in the cytoplasmic (CYT) and nuclear (NE) fractions.

Global protein expression changes of the **AuTMX₂** treatment were visualized by grouping significantly regulated proteins according to their gene ontology terms of cellular compartments and molecular functions (Figure 3C–D). The treatment with **AuTMX₂** induced pronounced changes in the nuclear compartment, especially regulating proteins belonging to the nucleolus and the telomeric region. In addition, structural elements like the actin cytoskeleton or the stress-response were regulated (e.g., the latter including heat shock proteins, Nrf2 target genes and the proteasome). Although metabolism-related proteins were regulated, it turned out that this was a very heterogeneous group largely lacking proteins of the same pathways. Interestingly, another gold(I) bis-NHC compound **Au(BMI)₂** was previously reported to induce regulations in protein synthesis, metabolism, cytoskeleton and stress-response as the most striking protein expression changes.^[19a]

Upon treatment with **AuTMX₂**, the most affected protein cluster in the nucleolus referred to stress-induced transcriptional activation and was significantly up-regulated (Table S1). This cluster is probably initiated by the up-regulated protein heme oxygenase 1 (HMOX1), which led to the regulation of the transcriptional activator BRG1 (SMARCA4), lysine-specific demethylase 2A (KDM2A) and signal transducer and activator of transcription 1- α /beta (STAT1). A total of 14 out of 20 known telomere-associated proteins were significantly regulated (Table S2).^[24] Interestingly, this protein group was generally up-regulated in the nuclear fraction, while down-regulated in the cytoplasmic fraction. For example, protein PML acts as a negative regulator of human telomerase (hTERT) and was found up-regulated upon treatment.^[25] Three components of telomere maintenance were up-regulated, including T-complex protein 1 subunits alpha (TCP1), zeta (CCT6A) and theta (CCT8). This mirrors what seems to be a common behaviour for gold-based chemotherapeutics since the Au^I complex auranofin and the dinuclear Au^{III} compound Auoxo6 were previously observed to up-regulate TCP1 and CCT8, respectively,^[19b,d] while the structurally similar **Au(BMI)₂** was found to up-regulate CCT6A.^[19a]

All significantly up-regulated proteins were searched for common transcription factor activity using oPOSSUM (Figure S5).^[26] This indicated the involvement of transcription factors responsible for mediating growth (Klf4, RREB1, ESR1), selenium metabolism (ZNF143) and oxidative stress (SP1). ZNF143 can activate the gene for selenocysteine tRNA to up-regulate selenoproteins in redox defence.^[27] Interestingly, the general transcription factor SP1 seems also to be responsible for regulating telomerase activity in cancer cells.^[28]

The observation of regulated proteins within the telomeric region (Figure 3C) together with the over-expressed transcription factor SP1 regulating telomerase activity indicates that the mode of action of **AuTMX₂** may indeed involve targeting telomeric regions in A2780 cancer cells. As **AuTMX₂** stabilizes telomeric G4 structures, we hypothesized that this interaction would reduce the efficiency of hTERT during replication. The latter is the enzyme responsible for maintaining appropriate telomere lengths required for replicative immortality^[9] and is often overexpressed in cancer cells. This stabilising effect may

lead to a reduced rate of proliferation and possibly a reduced telomere length in treated A2780 cancer cells, which is expected to be observable after several rounds of duplication. Thus, a 14 d exposure study was planned and suitable concentrations of **AuTMX₂** had to be selected to avoid unspecific effects like cell cycle arrest or apoptosis induction. A single dose of **AuTMX₂** at 13 μ M for 24 h had only a marginal effect on the cell cycle with a slight increase of cells in the G2/M fraction from 13 $\pm$ 1% to 22.0 $\pm$ 0.3% at the expense of a reduction in G0/G1 cell population (Figure 4A). **AuTMX₂** also showed a minor capacity to induce apoptosis at 8 μ M and 13 μ M concentrations, with a small dose-dependent increase of late apoptotic/necrotic cells after 72 h treatment: from 10 $\pm$ 1% in control cells to 22 $\pm$ 3% at 8 μ M and 29 $\pm$ 6% at 13 μ M concentrations (Figure 4B). This contrasts **Au(BMI)₂**, which was found to be a significant apoptosis inducer (>60%) and also induced the G0/G1 phase of the cell cycle.^[19a] Consequently, A2780 cells were treated with **AuTMX₂** at sub-cytotoxic concentrations of 0.2, 1 and 5 μ M for the long-term exposure experiments.

A biologically relevant stabilizing effect of **AuTMX₂** on telomeric G4 in A2780 cancer cells is expected to lead to shortened telomeres after a number of duplication steps due to perturbed hTERT function. Additionally, this interaction is also expected to reduce the proliferation rate of these cancer cells. Therefore, the effect of **AuTMX₂** on the telomere lengths in A2780 cancer cells was analysed after 14 d. In parallel, the effect on proliferation was evaluated by determining duplication times, number of duplications and cell cycle distributions after 7 d and 14 d. The cellular accumulation of Au was also assessed. The duplication time of the control cells was found to be 23 h throughout the experiment, resulting in 14.4 $\pm$ 0.2 duplications in total (Figure 5A). A gradual dose-dependent decrease in the number of duplications to 8.6 $\pm$ 0.2 at 5 μ M **AuTMX₂** was observed. While 5 μ M **AuTMX₂**, which roughly corresponds to 1/3 EC₅₀ (24 h), reduced the duplication time already during the first week, this effect was only observable in the second week for the lower concentrations, indicating gradual accumulation of **AuTMX₂**.

At 7 and 14 days treatment, we additionally determined the cell cycle distribution of A2780 cells by flow cytometry reasoning that the potential effects on telomeres would manifest in an altered cell cycle profile. The effect of **AuTMX₂** at low doses was also exclusively observable during the second week, while controls remained similarly distributed (Figure 5B).

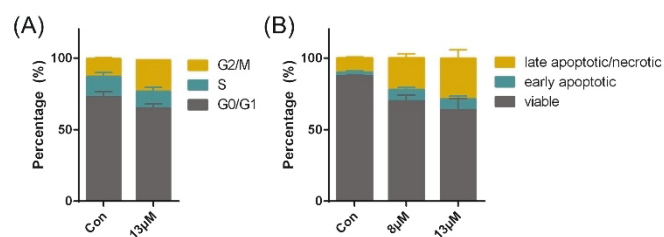


Figure 4. Effect of **AuTMX₂** on the cell cycle (A) of A2780 cancer cells after 24 h treatment and on their apoptosis induction (B) after 72 h treatment at the indicated concentrations measured by flow cytometry.

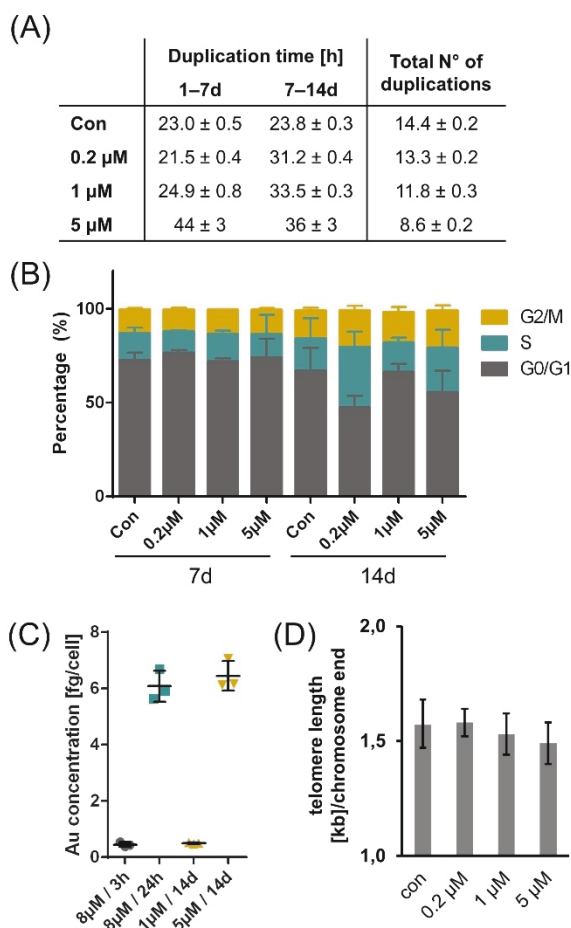


Figure 5. Effect of low dose treatments of **AuTMX₂** on A2780 cancer cells after 7 d and 14 d treatment: (A) Duplication times and number of duplications, (B) cell cycle distribution assessed by flow cytometry after the end of week 1 (7 d) and week 2 (14 d), (C) cellular accumulation of ¹⁹⁷Au in trypsinized cells analysed by ICP-MS and (D) absolute telomere lengths after 14 d. All experiments were performed in triplicate.

This result parallels the cellular accumulation of **AuTMX₂** assessed by quantifying Au in trypsinized A2780 cells by ICP-MS (Figure 5C). **AuTMX₂** seems to accumulate to a similar extent after 14 d treatment using 5 μM corresponding to 6.5 ± 0.5 fg cell⁻¹ Au, compared to the single dose of 8 μM over 24 h corresponding to 6.1 ± 0.5 fg cell⁻¹ Au. The 14 d treatment using 1 μM corresponded to 0.50 ± 0.03 fg cell⁻¹ Au, which was on the same order as an 8 μM treatment over 3 h corresponding to 0.45 ± 0.08 fg cell⁻¹ Au.

Finally, the absolute telomere length was assessed after the 14 d treatment period. A2780 cancer cells feature tightly controlled telomeres,^[29] which was evidenced in our experiments by a low variation of the telomeric length even upon drug exposure (Figure 5D). Although we were able to observe a slight reduction in telomere lengths in a dose-dependent manner, this effect was not significant. This suggests that telomere stabilizing-effects in A2780 cancer cells may contribute to the mode of action of **AuTMX₂**, but may not be the sole determinants of their anticancer activity. For example, higher concentrations of **AuTMX₂** may achieve a significant reduction in telo-

mere length, but may additionally increase stress-responses that exert a stronger influence on the proliferative capacity of cancer cells. Thus, other mechanisms seem to contribute to the mode of action of **AuTMX₂**.

The response profiling indicated a proteomic signature related to actin stress-fibres and stress-response upon treatment with **AuTMX₂** (Figure 3). The actin stress-fibres were characterized by down-regulation of their protein components (Table S3), suggesting a reduced pool of free building blocks and an increase in polymeric (insoluble) actin stress-fibres. For example, actinin-1 (ACTN1) and actinin-4 (ACTN4) were down-regulated, as well as cofilin-2 (CFL2), septin components (SEP02, 06, 07, 09 and 11) and filamin A (FLNA). The interpretation that **AuTMX₂** could induce the formation of actin stress-fibres was supported by the morphological appearance of actin filaments in A2780 cancer cells after treatment with either 13 μM or 26 μM drug concentrations for 24 h (Figure 6). Evidently, **AuTMX₂** induced a re-organization of actin toward the cell periphery, resembling the formation of stress-anchoring points.

Importantly, beside the regulation of several heat shock proteins (Table S4), the stress response profile revealed a number of Nrf2-induced genes, including glutathione related glutamate-cysteine ligase catalytic subunit (GCLC) and regulatory subunit (GCLM), glutathione reductase (GSR), glutathione S-transferase Mu 5 (GSTM5), but also thioredoxin reductase 1 (TXNRD1), peroxiredoxin 2 (PRDX2) and thioredoxin-like protein 1 (TXNL1), besides the mentioned HMOX1. Nrf2 forms a cytoplasmic complex with its inhibitor Keap1. The Nrf2-Keap1 complex is anchored to the actin cytoskeleton and mechanical stimulation may activate the Nrf2 transcription factor.^[30] Thus, rearrangement of the actin network as observed by **AuTMX₂** treatment can *per se* activate Nrf2 and induce transcription of Nrf2-induced genes.^[31] We did not directly observe Nrf2 to be

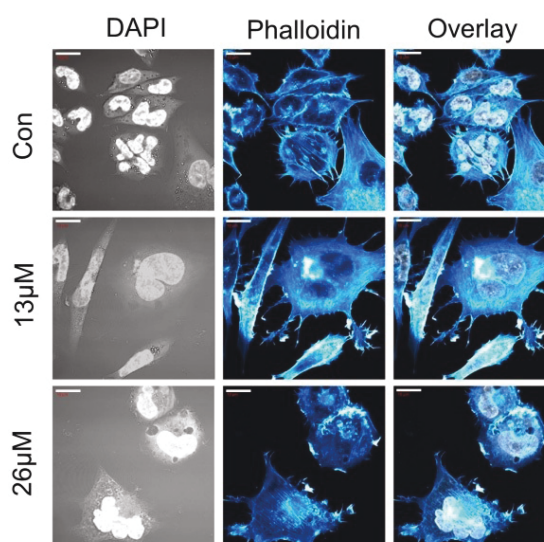


Figure 6. Fluorescence microscopy of nuclei (DAPI staining) and actin cytoskeleton (Alexa Fluor 488 phalloidin staining) of **AuTMX₂**-treated A2780 cancer cells at different concentrations for 24 h vs. untreated cells (Con). Scalebars = 20 μm.

regulated in the proteome profiling experiment, but it is the known starting point of the upregulated glutathione-related proteins. The induction of these redox-active proteins is accompanied by a significant nearly 2-fold increase in reactive oxygen species (ROS) after treating A2780 cancer cells with **AuTMX₂** for 24 h with 8 μM and 13 μM concentrations, respectively (one-way ANOVA, p -value = 0.0004) (Figure S6A). This result contrasts again **Au(BMI)₂**, which did not induce cellular ROS in A2780 cancer cells.^[19a] Furthermore, the induction of proteins involved in the glutathione pathway was paralleled by an increased abundance of glutathione (GSH) in **AuTMX₂** treated cancer cells (Figure S6B). This treatment also led to a significant 2-fold reduction of the ratio of reduced vs. oxidized glutathione indicating that GSH is actively used in the cellular defence against ROS formation (Figure S6C). It may be noted that a heterodimetallc titanium-gold(I) drug was also found to induce HMOX1 and other Nrf2 target genes in a clear cell renal carcinoma cell line.^[32]

Finally, it is known that the proteasome is affected by redox alterations.^[33] Indeed, an up-regulation of many proteasomal components, especially in the nuclear fraction, was observed during the proteomic response profiling experiment (Table S5).

Conclusions

Here, we report on the in-depth investigation of the mechanisms of reactivity and anticancer action of the cationic organometallic Au^I complex **AuTMX₂** via an integrated approach, including a combination of mass spectrometry-based methods, such as shotgun proteomics, and pharmacological assays. The obtained results suggest a multimodal mode of action involving metallodrug binding to nuclear and cytoplasmic components in human ovarian cancer cells A2780. Proteomic studies revealed that treatment with **AuTMX₂** at sub-cytotoxic concentrations induced the regulation of proteins involved in stress-induced transcriptional activation, as well as in telomere function. In parallel, in the nuclear fraction, down-regulation of proteins related to actin stress-fibres was observed.

Further experiments have been conducted to gain insight into some of these mechanisms, including effects on telomere length, actin stress-fibres and stress response signatures. Overall, while our study is not yet conclusive in validating the effects of the compound on telomeric DNA via stabilization of G4 structures, indications of stress response signatures via heat shock proteins and Nrf2-target genes were obtained and suggest the concurrence of several mechanisms that contribute to the anticancer activity of **AuTMX₂**. Future studies will be directed towards the elucidation of the proteasomal changes in the nuclear fraction of treated A2780 cancer cells.

Experimental Section

Materials: The gold-complex [Au(9-methylcaffeine-8-ylidene)]₂BF₄ (**AuTMX₂**) was synthesized as previously described.^[7,12] *N*-ethylmaleimide, NH₄OAc, tris(2-carboxyethyl)phosphine hydrochloride, *N*-ethyl-D5-maleimide and L-methionine were purchased from Sigma-Aldrich. Dimethylsulfoxide (DMSO), cytochrome C (horse

heart) and ubiquitin (from bovine erythrocytes) were obtained from Sigma. Formic acid (99%), L-glutamic acid and L-cysteine were obtained from Fluka and L-histidine from Merck. Ascorbic acid was purchased from Acros. MTT reagent powder was purchased from Cayman and dissolved in a 5 mg mL⁻¹ stock solution in PBS and stored in the dark at -20 °C. All reagents were used as received.

Interaction studies: Stock solutions (10 mM) of **AuTMX₂** were prepared in DMSO and stored at -20 °C in the dark. Aliquots were diluted to 400 μM with water for the preparation of the reaction mixtures prior to incubation. Additional stock solutions in water were prepared of ascorbic acid (400 μM), ubiquitin and cytochrome C (each 200 μM), as well as a mixture of the amino acids His, Met, Cys, Glu and ¹³⁵Se-Cys (each 100 μM). The compounds were reacted with the proteins in a 2:1 metal-to-protein ratio and with the mixture of the amino acids in an equimolar ratio. Furthermore, reaction mixtures were prepared at a 1:4 molar ratio with ascorbic acid. Reaction mixtures contained a final metal concentration of 50 μM . They were incubated at 37 °C at constant shaking (400 rpm) in the dark and aliquots were taken after 10 min, 3, 6 and 24 h.

All samples were diluted to a final concentration of 1–5 μM . Small molecule samples were diluted with H₂O and protein samples with H₂O : MeOH : formic acid (50:50:0.2). Mass spectra were recorded over 0.5 min in the positive and negative ion mode and averaged. They were processed using Compass 1.3 and DataAnalysis 4.0 software (both Bruker Daltonics, Bremen, Germany). Mass spectra of the proteins were deconvoluted by the maximum entropy deconvolution algorithm using automatic data-point spacing and a peak width of 0.2 (ion trap MS) or 30000 instrument resolving power (time-of-flight MS).

The mass spectra were recorded on an AmaZon SL electrospray ionization ion trap mass spectrometer (ESI-IT MS, Bruker Daltonics GmbH, Bremen, Germany) by direct infusion at a flow rate of 4 $\mu\text{L min}^{-1}$. Positive and negative ionization modes were recorded. Typical instrument parameters were as follows: ± 4.5 kV capillary voltage, -0.5 kV end plate offset, 6 L min⁻¹ dry gas, 180–250 °C dry temperature, 8 psi nebulizer, 89% RF level, 74.3 trap drive. Experimental mass signals include a standard deviation of $m/z \pm 0.05$.

High resolution mass spectra were recorded on a Maxis qTOF mass spectrometer (ESI-TOF MS, Bruker Daltonics GmbH, Bremen, Germany) equipped with a Triversa nanomate (Advion Biosystems Inc., Ithaca, New York, USA). ChipSoft 8.3 (Advion Biosystems Inc.) was used to control the nanomate. Typical instrument parameters were as follows: -1.8 kV capillary voltage, 0.1 psi gas flow, 4 L min⁻¹ dry gas, 250 °C dry heater, 3 eV quadrupole energy, 8 eV collision energy, 150 μs ion cooler transfer time, 10 μL sample volume and 15 °C nanomate sample plate temperature. Only the 24 h incubation samples were measured on the high resolution nESI-TOF MS. Additional mass spectra were recorded by direct infusion (3 $\mu\text{L min}^{-1}$) without the Triversa nanomate using the following parameters: -4.5 kV capillary voltage, 0.4 bar nebulizer, 4 L min⁻¹ dry gas, 180 °C dry heater, 4 eV quadrupole energy, 8 eV collision energy, 80 μs ion cooler transfer time.

Cell culture: The ovarian cancer cell line (A2780) was kindly provided by Thomas Grunt (Medicinal University of Vienna, Austria). A2780 cells were cultured in RPMI-1640 medium including L-glutamine (Gibco, Life Technologies, UK). All media contained 10% foetal calf serum (FCS, ATCC, USA) and 1% penicillin/streptomycin (ATCC, USA). Cells were grown in a humidified atmosphere containing 5% CO₂ and 95% air at 37 °C (HeraCell 150i, CO₂ incubator, ThermoFisher Scientific). Before reaching confluency, the cells were detached using Trypsin/EDTA solution (0.25%, Sigma) and split.

Cells were counted using an automated cell counter (Moxi Z, Orflo, USA).

Viability assay: The MTT assay was used to investigate the cytotoxicity of **AuTMX₂** against A2780 cancer cells. Cells were seeded in densities of 3000–50 000 cells/well in a flat-bottom 96-well plate (Corning) in 100–200 μL of the respective medium. The medium was replaced after 24 h with control medium or medium containing the appropriate concentration of **AuTMX₂** and was incubated for 24 h or 72 h. **AuTMX₂** was first dissolved as a 10 mM stock solution in DMSO and was further diluted with medium directly prior to treatment. The DMSO concentration did not exceed 1%. After the incubation time, MTT reagent (5 mg mL^{-1} in PBS) was added to each well in 10% v/v and was further incubated for 4 h. Then, the medium was removed and the formazan crystals were dissolved in 50–100 μL of DMSO. The metabolic activity was measured photometrically using a UV/Vis photometer (Multiscan GO, ThermoFisher Scientific). Each assay was blank-corrected performed in triplicates of triplicates and the half maximal effective concentration (EC_{50}) necessary to achieve 50% inhibition of cell growth was calculated using a sigmoidal fitting.

Cellular accumulation: The A2780 ovarian cancer cells were maintained in T25 or T75 culture flasks (Sarstedt, Germany) in RPMI-1640 containing 10% FCS and penicillin/streptomycin as indicated above. For accumulation studies upon **AuTMX₂** treatment, the cells were seeded at 3×10^5 cells per well in 1.5–2 mL complete culture medium in flat-bottom 6-well plates (Becton Dickinson Co.). The cells were allowed to adhere for 24 h. Then, the medium was exchanged with medium containing freshly dissolved **AuTMX₂** (at 8 or 13 μM from a 10 mM stock solution) in 1.5 mL. The cells were treated for 3, 6 and 24 h, respectively, at the respective concentrations. Appropriate absorption blanks were included, and additional wells were used to determine the cell number.

Total cellular accumulation: To determine the total cellular accumulation, the medium was removed after the treatment time and the wells were washed three times with PBS (1 $\times$, 1 mL). PBS was removed and the remaining volume was completely removed with a Gilson pipette. The cells were lysed in ultrapure HNO_3 (conc., 0.5 mL) for 3 h after which, aliquots of 400 μL were transferred into a Falcon tube (15 mL) and diluted to a total volume of 8 mL using H_2O (Millipore Advantage A10, 18.2 Ω , Merck). Alternatively, the cells were washed once with PBS (1 $\times$) and detached using Trypsin/EDTA. After transferring into Falcon tubes (15 mL), the cells were washed three times with PBS (1 $\times$). PBS was removed and the remaining PBS completely removed with a Gilson pipette. The cells were lysed in ultrapure HNO_3 (conc., 0.5 mL) for 3 h after which, aliquots of 400 μL were transferred into a Falcon tube (15 mL) and diluted to a total volume of 8 mL using H_2O (Millipore Advantage A10, 18.2 Ω , Merck).

Subcellular fractionation: The treated cells were washed three times with PBS (1 $\times$) after the incubation time. PBS was completely removed. Then, isotonic lysis buffer (1 mL, s. fractionation protocol in proteomics) was added and the cells were scraped and collected into a Falcon tube (15 mL). The cells were lysed by shear stress by pressing through a syringe (15 $\times$). The nuclei were pelleted by centrifugation at 3000 g for 5 mins. The cytoplasm was precipitated in ice-cold ethanol (abs., 99.9%, Australco, Austria) over night, while the nuclear pellets were lysed by adding ultrapure HNO_3 (conc., 0.5 mL) for 3 h. The cytoplasm was pelleted by centrifugation at 3'000 g for 5 min. The supernatant was completely removed and the lysed with ultrapure HNO_3 (conc., 0.5 mL) for 3 h. An aliquot of each sample (400 μL) was transferred into a Falcon tube (15 mL) and diluted to a total volume of 8 mL.

DNA-based experiments

Treatment for Au-DNA binding study: A total of 2×10^6 cells were seeded in T25 flasks and left to adhere for 24 h. Then, the medium was replaced with fresh complete medium or fresh complete medium containing **AuTMX₂** at doses of 8 μM ($2/3 \text{ EC}_{50}$) and 26 μM ($2 \times \text{EC}_{50}$), respectively, and was incubated for another 24 h. After trypsinization and washing with PBS, the cells were counted. The cells were pelleted, isotonic fractionation buffer was added, and the cellular membrane was lysed by shear stress (8 $\times$ through a 1 mL syringe with canula). After centrifugation (3500 rpm, 5 min, 4 $^\circ\text{C}$), the supernatant was discarded and the pellet containing the nuclei was washed with PBS. After removal of PBS, the nuclear pellets were stored at -80°C until DNA extraction. Each condition was performed in triplicates.

Treatment for telomere length quantification: A2780 cells were seeded at 3×10^5 cells per well in 6-wells, which were coated with poly-L-lysine (0.01%, Sigma). The cells were continuously treated with **AuTMX₂** at concentrations of 0.2, 1 and 5 μM (from a 10 mM DMSO stock solution) for 14 d. Cells treated with an equivalent DMSO served as controls and each condition was performed in triplicates. The cell number was followed over time. After 14 d, each sample was analysed for cell cycle distribution by flow cytometry and DNA was extracted for determination of telomere length. Additionally, the cellular accumulation of gold was determined in the samples of 1 and 5 μM **AuTMX₂** treatment after 14 d using ICP-MS.

DNA extraction: Genomic DNA was isolated with silica-based spin columns from nuclear pellets using the QIAamp DNA Mini Kit (Qiagen, Germany) according to manufacturer's instructions for cell lines with the following modifications. After incubation at 56°C for 10 min, the lysate was additionally homogenized by passing it 5 $\times$ through a 1 mL syringe with 27G canula. The filtrates after loading the lysate to the spin column (flow-through) and after the first washing step (wash 1) were collected in 2 mL Eppendorf tubes (Eppendorf, Austria). DNA was eluted using 100 μL EB buffer containing 10 mM Tris-Cl, pH 8.5 (Qiagen, Germany) and elution was repeated once. DNA concentration of eluates was determined fluorometrically with the Qubit dsDNA BR/HS Assay Kits and the Qubit 4 instrument (Thermo Scientific, Austria). All filtrates and eluates were stored at -20°C until qPCR or digestion as described below.

Telomere length quantification: Real-time quantitative polymerase chain reaction (qPCR) was performed in 20 μL reactions containing 2.5 ng genomic DNA template, 2 μL telomere or single copy reference primer and FastStart Essential DNA Green Master Mix (Roche, Austria) using the absolute human telomere length quantification qPCR assay Kit (ScienCell, USA) according to the manufacturer's protocol. Nuclease-free water served as no template control. The reference sample contained in the kit with a telomere length of 695 ± 16 kb per diploid cell was used for absolute quantification. The Rotor-Gene Q instrument (Qiagen, Germany) was used with the following temperature program: initial activation at 95°C for 10 min followed by 32 cycles of denaturation at 95°C for 20 s, annealing at 52°C for 20 s and elongation at 72°C for 45 s. Specificity of PCR was checked by subsequent melting curve analysis. Each sample was analysed in technical triplicates and in two independent runs. Absolute telomere length was calculated by the comparative $\Delta\Delta\text{CT}$ method following the manufacturer's instructions.

Inductively coupled plasma mass spectrometry: An ICP-MS 7800 (Agilent) was used to quantify the cellular and subcellular accumulation of **AuTMX₂** and was tuned on a daily basis. The instrument was controlled with MassHunter (C.01.04, Agilent) together with

the SPS 4 autosampler (Agilent). The following parameters were used: Cone material was nickel, RF power was 1550, Carrier gas 1.08 L min⁻¹, Plasma gas 15 L min⁻¹, Integration time 0.1 s, number of replicates 10, number of sweeps 100. ¹⁸⁵Re was employed as internal standard and gold was assessed using ¹⁹⁷Au. The tubing was washed with HCl (aq.) to remove carry-over of gold for 3 mins after every sample. The instrumental limit of quantification was 1.6 pg mL⁻¹.

Proteomics

Treatment: A2780 ovarian cancer cells were seeded in T25 flasks at 5 × 10⁶ cells and left to adhere for 24 h in complete medium. Then, the medium was exchanged for fresh medium (controls) or fresh medium containing freshly dissolved **AuTMX₂** (treatments, 8 μM from a 10 mM stock solution in DMSO) and incubated for another 24 h. Six biological replicates were analysed per condition.

Fractionation: After the 24 h treatment, cells were fractionated into cytoplasmic and nuclear extracts as previously described.^[16,18,34] All steps were performed on ice. Briefly, the cells were extensively washed with PBS (1 ×). Isotonic lysis buffer (10 mM HEPES, 10 mM NaCl, 3.5 mM MgCl₂, 1 mM EGTA, 0.25 M Sucrose, 0.5% Triton X-100) containing protease inhibitors (1% PMSF and 1% protease and phosphatase inhibitor cocktail from Roche) was added, the cells were scraped off and transferred into labelled 15 mL Falcon tubes (17 × 120 mm, Corning). The cellular membrane was ruptured using shear stress by pressing the cell suspension through a syringe multiple times. After centrifugation (3500 rpm, 5 min), the supernatant was transferred into ice-cold ethanol (1:5) and precipitated over night at -20 °C. The pellet containing the nuclei was incubated with a hypertonic solution (10 mM Tris-HCl, 1 mM EDTA and 0.5 M NaCl) and subsequently with NP-40 buffer (10 mM Tris-HCl, 1 mM EDTA and 0.5% NP-40) containing protease inhibitors (1% PMSF from Sigma and 1% protease and phosphatase inhibitor cocktail from Roche). After centrifugation (3500 rpm, 5 min), the soluble nuclear proteins were also transferred into ice-cold ethanol (1:5) and precipitated over night at -20 °C. The precipitated proteins were pelleted, dried under vacuum and dissolved in sample buffer (100 mM dithiothreitol (DTT), 7.5 M urea, 1.5 M thiourea, 4% CHAPS, 0.05% sodium dodecyl sulphate) and the protein concentration was determined using a Bradford assay.

Digestion protocol: The samples were digested in-solution as previously described according to the FASP protocol.^[16,18,34] Equal amounts of 20 μg protein per sample were reduced with DTT at 37 °C and then loaded on 10 kDa centrifugal filters (Microcon-10, Merck, Millipore) and pre-concentrated. The samples were carbamidomethylated with iodacetamide. The samples were then digested over night with trypsin/lys-C (Promega, Germany) at 37 °C. Filters were washed with 0.5% TFA and the eluates were dried with a miVac duo concentrator (GeneVac Ltd, UK) and stored at -20 °C until analysis.

LC-MS/MS analysis: The data was acquired on a QExactive orbitrap mass spectrometer (Thermo Scientific, Germany), which was coupled with a nanoLC-system (Dionex Ultimate 3000, Thermo Scientific, Germany), equipped with a C-18 separation column (Dionex, Acclaim PepMap RSCL, 75 μm × 50 cm) and C-18 trapping column (2 cm × 100 μm). Dried samples were dissolved in formic acid (30%, 5 μL) containing four synthetic peptides (Glu1-fibrinopeptide B, EGVNDNEEGFFSAR; M28, TTPAVLSDSGSYFLYSK; HK0, VLETKSLYVR and HK1, VLETK(ε-AC)SLYVR) and diluted with mobile phase A (40 μL), which consisted of 98% water, 2% acetonitrile, 0.1% formic acid. Mobile phase B consisted of 20% water, 80% acetonitrile and 0.1% formic acid. Each biological sample was recorded

once by injecting 2 μL per analysis. Samples were analysed over a 135 min chromatographic run containing a 90 min gradient from 8–40% mobile phase B. MS1 resolution was 70k with 50 ms injection time and MS2 resolution was 17.5k with 75 ms injection time. A top 8 method was used in the mass range of *m/z* 400–1400. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD020560 and 10.6019/PXD020560.

Data analysis: MaxQuant (Version 1.6.3.4), including the in-built Andromeda search engine, was used for label-free quantification. For identification, we used only non-redundant Swissprot entries with at least two peptides (of which one needed to be unique). The first and main search peptide tolerance was 50 and 25 ppm, respectively. The false discovery rate (FDR) was fixed to 0.01 on the peptide and protein level. Proteins were considered if they showed at least one unique peptide. The statistical evaluation was performed with Perseus software (Version 1.6.6.0) using LFQ intensities of the MaxQuant result file. After filtering potential contaminants the LFQ values were Log(2)-transformed. Only proteins, which were found at least five times in either control and/or treatment groups were considered for data evaluation. Permutation-based FDR set to 0.05 for truncation was used for t-tests and gave multi-parameter significant protein expression changes. The final data set was further analysed using the web-based applications DAVID bioinformatics Resources 6.8, STRING 10.0 and oPOSSUM.

Flow cytometry

Three biological replicates were analysed per condition in A2780 cells (around 10⁵ cells) on a FACS Canto II cytometer (BD Bioscience).

Assessment of reactive oxygen species (ROS): The DCFDA cellular ROS detection assay kit (Abcam) was used according to the manufacturer protocol. 2',7'-dichlorofluorescein diacetate (DCFDA) is deacetylated intracellularly by esterases and then oxidized by ROS to the fluorescent 2',7'-dichlorofluorescein (DCF), which can be detected by flow cytometry. In addition to controls, A2780 cancer cells were treated for 24 h with **AuTMX₂** at concentrations of 8 and 13 μM (from 10 mM stock solution in DMSO) using 2 × 10⁵ cells per well in 6-wells. They were detached and washed three times with PBS. Cells were resuspended in PBS + DCFDA (20 μM) and incubated for 30 min at 37 °C and were then immediately transferred on ice. Without further washing steps, ROS levels were quantified by flow cytometry in the FITC channel. Three biological replicates were analysed per condition.

Assessment of apoptosis induction: A2780 cancer cells were treated for 72 h with **AuTMX₂** at concentrations of 8 and 13 μM (from 10 mM stock solution in DMSO) using 2 × 10⁵ cells per well in 6-wells. The supernatant and the detached cells were combined and washed three times with PBS and once in Annexin binding buffer (eBioscience). Samples were resuspended in Annexin binding buffer (100 μL) and incubated with 5 μL Annexin V-APC (eBioscience) for 10 min at room temperature, followed by washing once in Annexin binding buffer. Cells were then resuspended in Annexin binding buffer (200 μL) and propidium iodide was added (PI; Sigma Aldrich; final concentration 100 ng mL⁻¹). Without further washing steps, cells were analysed by flow cytometry in the PE-channel (PI) and APC-channel (Annexin-V). Annexin-V⁻/PI⁻ cells were identified as viable cells, Annexin-V⁺/PI⁻ as early apoptotic cells and Annexin-V⁺/PI⁺ and Annexin-V⁻/PI⁺ cells as late apoptotic/necrotic cells. Three biological replicates were analysed per condition.

Cell cycle analysis: For a short pulse analysis, A2780 cancer cells were treated for 24 h with **AuTMX₂** at 13 μM (from 10 mM stock solution in DMSO) using 3×10^5 cells per well in 6-wells. They were detached and washed three times with PBS, resuspended and put on ice. Three biological replicates were analysed per condition. The cell cycle distributions of control and treatment groups were analysed with the BD cycletest Plus DNA kit according to the manufacturer's protocol. In brief, cells were washed three times and permeabilized. The amount of interfering RNA was reduced using RNase after which propidium iodide was added as a DNA stain. Propidium iodide was measured by flow cytometry in the PE-channel and cells in G0/G1, S and G2/M-phase were identified according to the manufacturer's protocol.

Confocal microscopy

A2780 cancer cells were seeded in chamber slides (μ -Slide 8 well, ibidi GmbH, Germany), pre-coated with poly-L-lysine (0.01 %, Sigma) at 4×10^4 cells well⁻¹ in complete RPMI-1640 medium and were left to adhere for 24 h. For evaluation of actin morphology, the medium was exchanged, and the cells were treated at EC₅₀ (13 μM) and $2 \times \text{EC}_{50}$ (26 μM) doses (from 10 mM stock solution in DMSO), respectively, for 24 h. Thereafter, the cells were fixed (3.7 % formaldehyde, 15 min), washed with PBS and permeabilized (0.2 % triton-X, 10 min). Blocking was performed with Donkey serum (10 % in PBS-A, 1 h). Primary antibodies were incubated overnight at 4 °C. Actin was stained alexa fluor 488 phalloidin (Molecular Probes, Life Technologies, ThermoFisher Scientific). The slides were washed with PBS and post-fixed with 3.7 % formaldehyde (10 min). Finally, glycine (100 mM) was used to mask reactive sites and slides were mounted and sealed with Roti-Mount FluoCare (Roth, Graz, Austria) with DAPI. All images were acquired on a confocal LSM Zeiss 710 equipped with ELYRA PS.1 system and a plan apochromat 63x/1.4 oil objective.

Glutathione determination

Sample preparation: The ratio of reduced versus oxidized glutathione (GSH) was measured according to a modified protocol from Tomin et al.^[35] Therefore, 5×10^5 A2780 cancer cells were seeded in 6-wells in triplicates per condition. The cells were left to adhere for 24 h. Then, the medium was replaced, and the treated cells were incubated with **AuTMX₂** (13 μM) for 24 h. After removing the supernatant and washing twice with PBS the cells were incubated with 500 μL *N*-ethylmaleimide (NEM, 2.5 mM) for 20 min at RT. The NEM solution was removed and the cells were again washed twice with PBS. Then, 200 μL of 80 % methanol in water was added and incubated for 20 min at 4 °C. The cells were scraped off, transferred into an Eppendorf tube and centrifuged (10 min, 13'000 g, 4 °C). The supernatant was collected and dried under a nitrogen stream before resuspending in 100 μL NH₄OAc (50 mM, pH 7). After adding 300 μL DCM and thoroughly vortexing, the samples were centrifuged (5 min, 13'000 g, 4 °C). An aliquot of the upper phase (45 μL) containing NEM-GSH and GSSG was taken and reduced with 2.5 μL Tris(2-carboxyethyl)phosphine hydrochloride (TCEP, 5 mM, pH 7) for 30 min at 37 °C. Afterwards, 2.5 μL of *N*-ethyl-D5-Maleimide (D5-NEM, 100 mM) was added and again incubated for 20 min at RT in the dark. The samples were finally diluted with 200 μL of NH₄OAc (50 mM) for processing by HPLC-MS.

LC-MS analysis: Chromatographic separation was performed on a Kinetex XB-C18 column (2.6 μm , 100 $\times$ 2.1 mm, 100 Å, Agilent) using a Vanquish UHPLC System (Thermo Fisher Scientific). Mobile Phase A consisted of water with 0.2 % formic acid and mobile phase B of methanol with 0.2 % formic acid. The following gradient

profile was run: 0–30 % B in 5 min, 30–70 % B in 2 min, wash at 95 % B for 3 min and at 10.1 min the system was re-equilibrated at 0 % B to give a total runtime of 12 min. The flow rate was 300 $\mu\text{L min}^{-1}$, the column chamber and the preheater temperature were set to 40 °C and the injection volume was 5 μL . MS analysis was performed in the positive ion mode on a QExactive HF (Thermo Fisher Scientific). All samples were analysed in technical replicates. MS scans were acquired in the mass range of m/z 100–1000 and the resolution was set to 60'000 (at m/z 200). The four most abundant ions in the full scan were selected for further fragmentation in the HCD collision cell applying 30 eV dynamic collision energy. MS² fragments were analysed at a resolution of 15'000 (at m/z 200). Dynamic exclusion was applied for 6 s. NEM-GSH was found at m/z 433 and D5-NEM-GSH was found at m/z 438. The former corresponds to reduced GSH and the latter to oxidized GSH. The instrument was controlled using Xcalibur software (Thermo Fisher Scientific). Raw files generated by the Q Exactive HF were further analysed with Tracefinder Software 4.1 (Thermo Fisher Scientific).

Statistical analysis

Data was analysed using GraphPad Prism Version 6.

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Conflict of interest

The authors declare no conflict of interest.

Keywords: cancer • gold complexes • G-quadruplexes • N-heterocyclic carbenes • proteomics • telomeres

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Supporting Information

An Organometallic Gold(I) Bis-N-Heterocyclic Carbene Complex with Multimodal Activity in Ovarian Cancer Cells

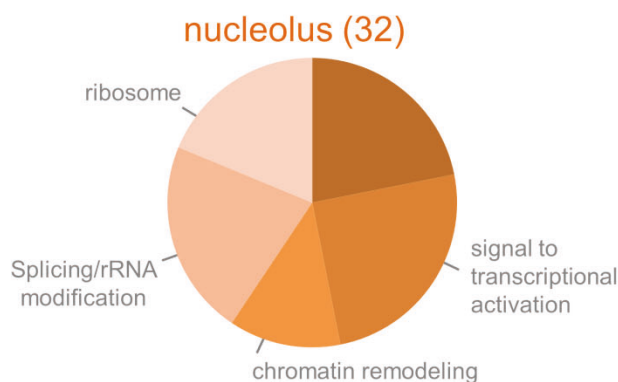
Samuel M. Meier-Menches,^{*,[a]} Benjamin Neuditschko,^[a, b] Katja Zappe,^[a] Martin Schaier,^[a] Marlene C. Gerner,^[c] Klaus G. Schmetterer,^[c] Giorgia Del Favero,^[d, e] Riccardo Bonsignore,^[f] Margit Cichna-Markl,^[a] Gunda Koellensperger,^[a] Angela Casini,^{*,[f]} and Christopher Gerner^{*,[a, e, g]}

p.S2–S6 Supplementary Tables S1–S6

p.S7–S12 Supplementary Figures S1–S6

Supplementary Tables

Table S1. Groups of significantly regulated proteins associated with the nucleolus obtained from the proteomic response profiling experiments. The LFQ intensity values of a selection of regulated proteins are reported in cytoplasmic (CYT) and nuclear (NE) fraction. # = multi-parameter significant protein regulation (FDR = 0.05, S0 = 0.1).



Group	Fraction	FC	Gene	Accession
Stress-signal to transcriptional activation	CYT	5,26	HMOX1	P09601
	CYT	5,07	KDM2A	Q9Y2K7
	CYT	1,91	TXNRD1	Q16881
	CYT	1,20	STAT1	P42224
	CYT	-2,47	SMARCA4	P51532
	NE	6,67	HMOX1	P09601
	NE	1,49	PML	P29590
	NE	2,80	STAT1	P42224
chromatin remodeling	CYT	-1,25	FEN1	P39748
	NE	-1,22	TOP2A	P11388
	NE	1,12	XRCC5	P13010
	NE	1,32	CDCA8	Q53HL2
splicing/rRNA processing	CYT	-1,26	DDX5	P17844
	NE	1,27	DDX18	Q9NVP1
	NE	-1,24	DDX6	P26196
	NE	1,21	DIMT1	Q9UNQ2
	NE	1,66	IMP4	Q96G21
	NE	1,48	NSUN2	Q08J23
	NE	1,30	RNMT	O43148
ribosome	CYT	1,16	EIF6	P56537
	NE	-1,21	RPL13A	P40429
	NE	-1,19	RPL4	P36578
	NE	-1,33	RPS19	P39019
	NE	-1,22	SBDS	Q9Y3A5

stress-signal to transcriptional activation

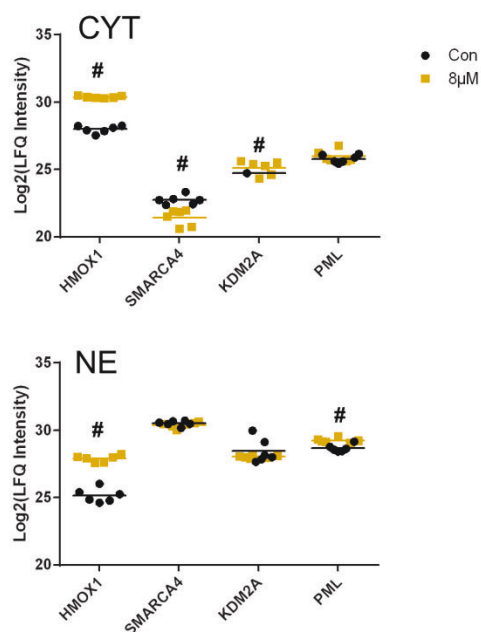


Table S2. Groups of significantly regulated proteins associated with telomeres obtained from the proteomic response profiling experiments. The LFQ intensity values of a selection of regulated proteins are reported in cytoplasmic (CYT) and nuclear (NE) fraction. # = multi-parameter significant protein regulation (FDR = 0.05, S0 = 0.1).

Telomere-associated proteins (14)

Group	Fraction	FC	Gene	Accession
telomere region	CYT	-1,25	FEN1	P39748
	CYT	-1,15	MCM6	Q14566
	CYT	-1,30	MSH2	P43246
	CYT	-1,26	POLD1	P28340
	NE	-1,44	CBX1	P83916
	NE	-1,42	CBX3	Q13185
	NE	2,49	HAT1	O14929
	NE	1,49	PML	P29590
	NE	1,30	RPA1	P27694
	NE	1,29	RPA2	P15927
	NE	1,12	XRCC5	P13010
telomere maintenance	NE	1,40	CCT6A	P40227
	NE	1,51	CCT8	P50990
	NE	1,48	TCP1	P17987

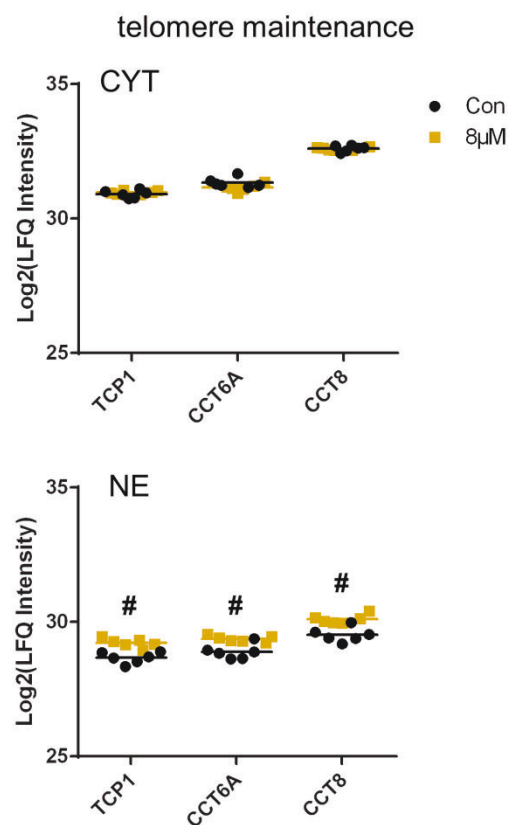


Table S3. Groups of significantly regulated proteins associated with actin stress fibers obtained from the proteomic response profiling experiments. The LFQ intensity values of a selection of regulated proteins are reported in cytoplasmic (CYT) and nuclear (NE) fraction. # = multi-parameter significant protein regulation (FDR = 0.05, S0 = 0.1).

Actin stress fibers (18)				
Group	Fraction	FC	Gene	Accession
Actin stress fiber	CYT	1,31	FLNB	O75369
	NE	3,44	RRAS	P10301
	NE	-2,07	ACTN1	P12814
	NE	-2,04	ACTN4	O43707
	NE	-2,02	CFL2	Q9Y281
	NE	-1,38	CORO1C	Q9ULV4
	NE	-1,55	FSCN1	Q16658
	NE	-1,68	FLNA	P21333
	NE	-1,49	LIMA1	Q9UHB6
	NE	-1,88	PDLIM5	Q96HC4
	NE	-1,77	PDLIM7	Q9NR12
	NE	-1,70	ENAH	Q8N8S7
	NE	-1,60	RDX	P35241
	NE	-1,45	Sep 02	Q15019
	NE	-1,51	Sep 06	Q14141
	NE	-1,47	Sep 07	Q16181
	NE	-1,56	Sep 09	Q9UHD8
	NE	-1,56	Sep 11	Q9NVA2

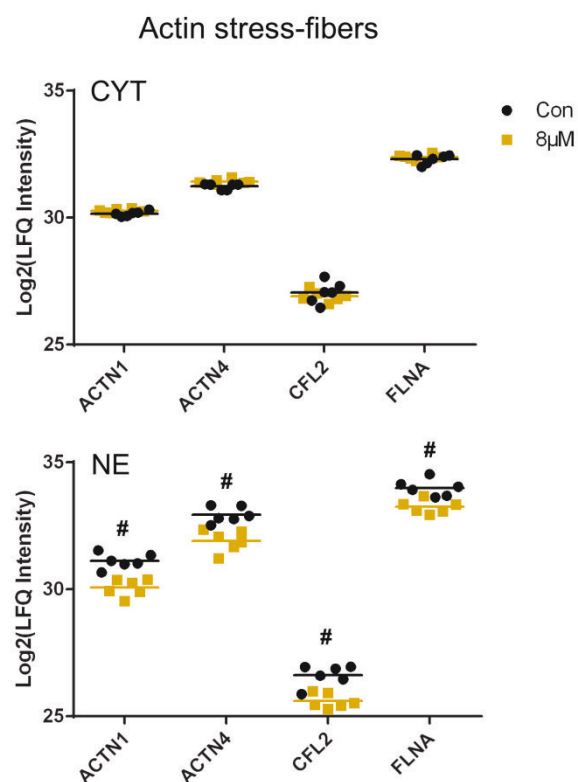


Table S4. Groups of significantly regulated proteins associated with stress-response obtained from the proteomic response profiling experiments. The LFQ intensity values of a selection of regulated proteins are reported in cytoplasmic (CYT) and nuclear (NE) fraction. # = multi-parameter significant protein regulation (FDR = 0.05, S0 = 0.1).

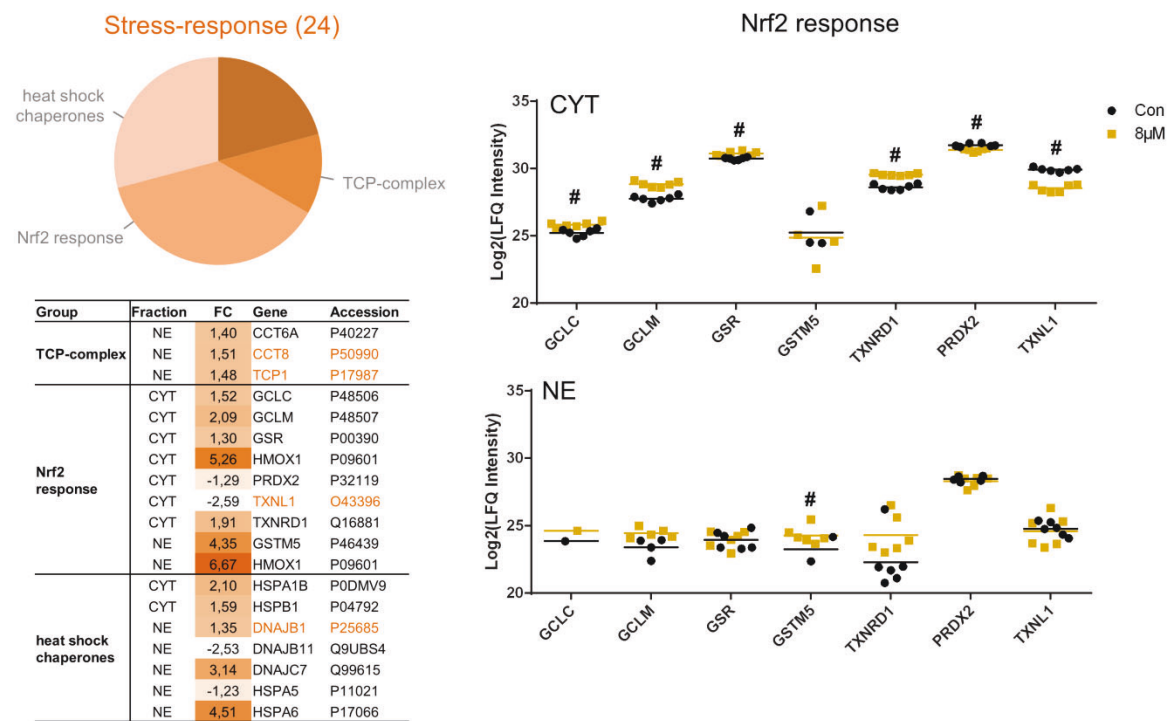
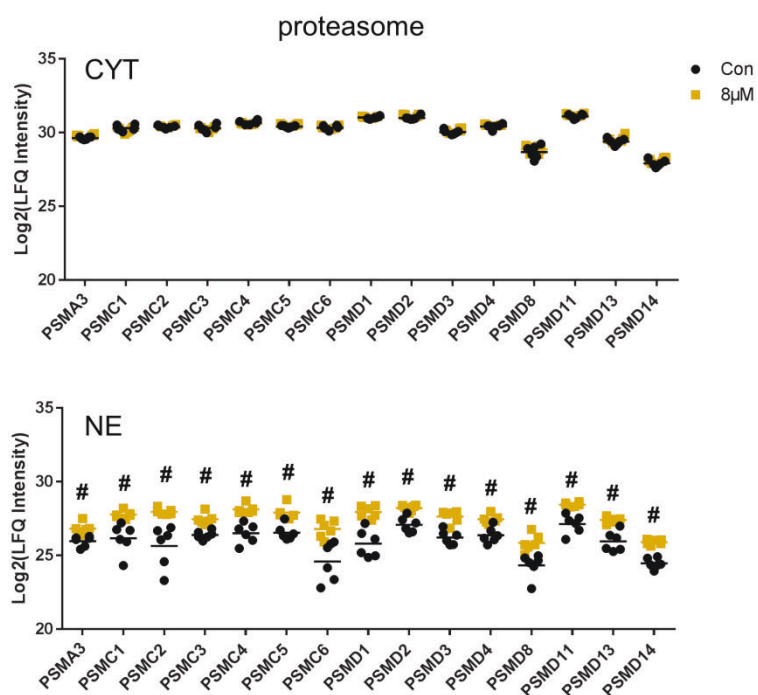


Table S5. Groups of significantly regulated proteins associated with the proteasome obtained from the proteomic response profiling experiments. The LFQ intensity values of a selection of regulated proteins are reported in cytoplasmic (CYT) and nuclear (NE) fraction. # = multi-parameter significant protein regulation (FDR = 0.05, S0 = 0.1).

Proteasome (19)

Group	Fraction	FC	Gene	Accession
proteasome	CYT	2.36	PSMB4	P28070
	CYT	-2.59	TXNL1	O43396
	NE	2.22	ADRM1	Q16186
	NE	1.80	PSMA3	P25788
	NE	3.03	PSMC1	P62191
	NE	4.97	PSMC2	P35998
	NE	2.09	PSMC3	P17980
	NE	3.08	PSMC4	P43686
	NE	2.56	PSMC5	P62195
	NE	4.58	PSMC6	P62333
	NE	4.42	PSMD1	Q99460
	NE	2.20	PSMD2	Q13200
	NE	2.69	PSMD3	O43242
	NE	2.12	PSMD4	P55036
	NE	2.86	PSMD8	P48556
	NE	2.50	PSMD11	O00231
	NE	2.73	PSMD13	Q9UNM6
	NE	2.71	PSMD14	O00487



Supplementary Figures

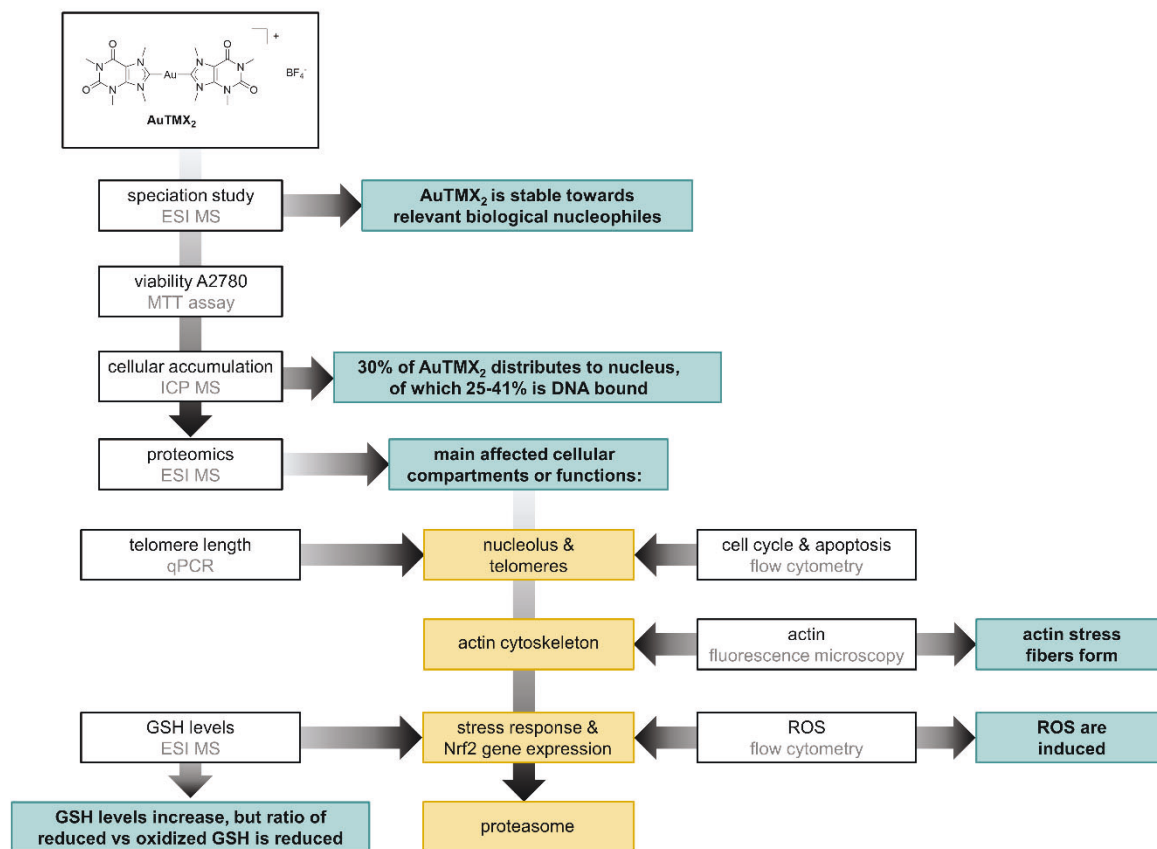


Figure S1. The figure summarizes the experimental workflow of this study and highlights the main results.

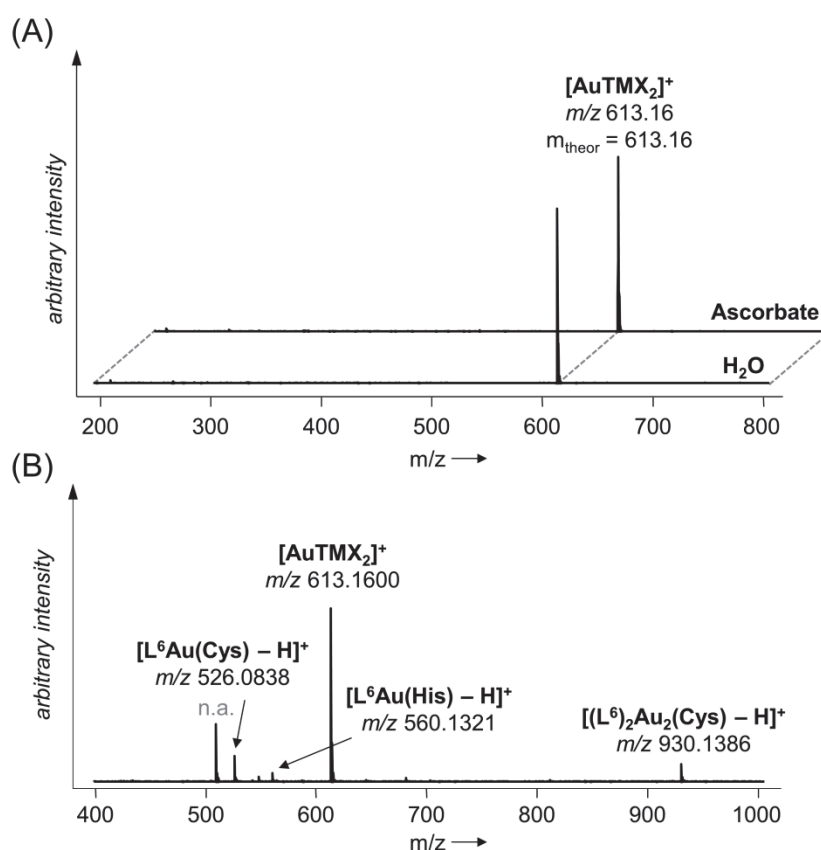


Figure S2. (A) ESI-ion trap mass spectrum of AuTMX_2 dissolved in aqueous solution or in the presence of ascorbate for 24 h. AuTMX_2 remains stable over the entire incubation period. (B) ESI-TOF mass spectrum of AuTMX_2 in the presence of His, Cys, Met, Glu and $^{\text{Me}}\text{Se-Cys}$ after 24 h. Adduct formation occurs exclusively with Cys and His after release of one TMX ligand.

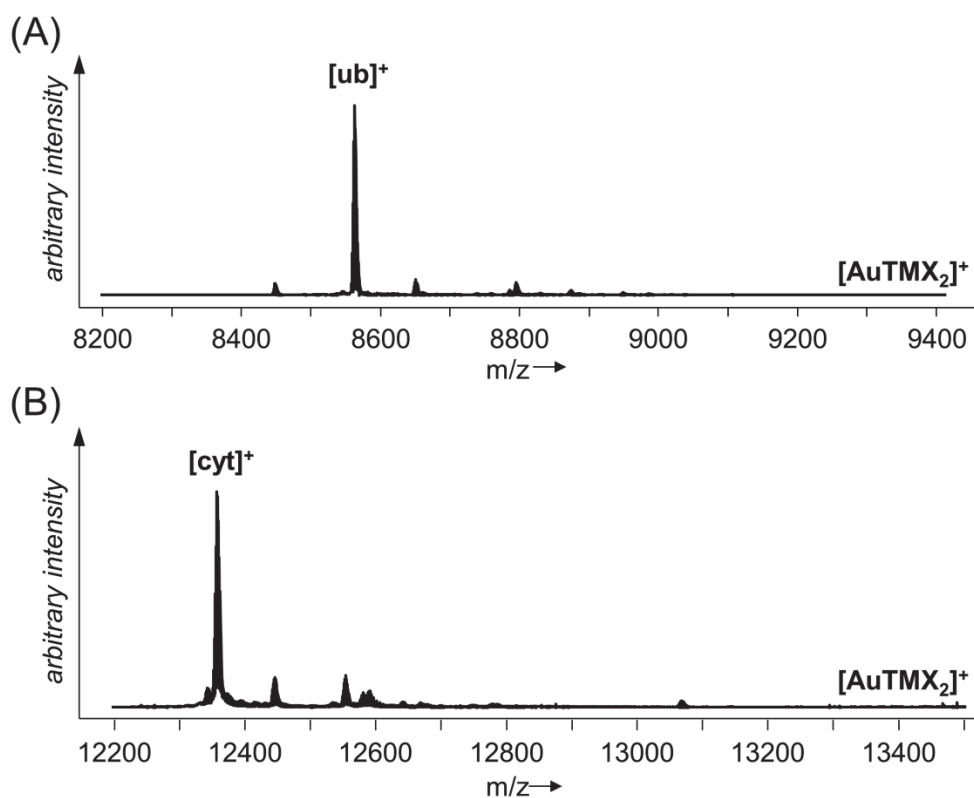


Figure S3. (A) Deconvoluted ESI-TOF mass spectra of ubiquitin (A) and cytochrome C (B) exposed to $AuTMX_2$ for 24 h. No adducts were observed.

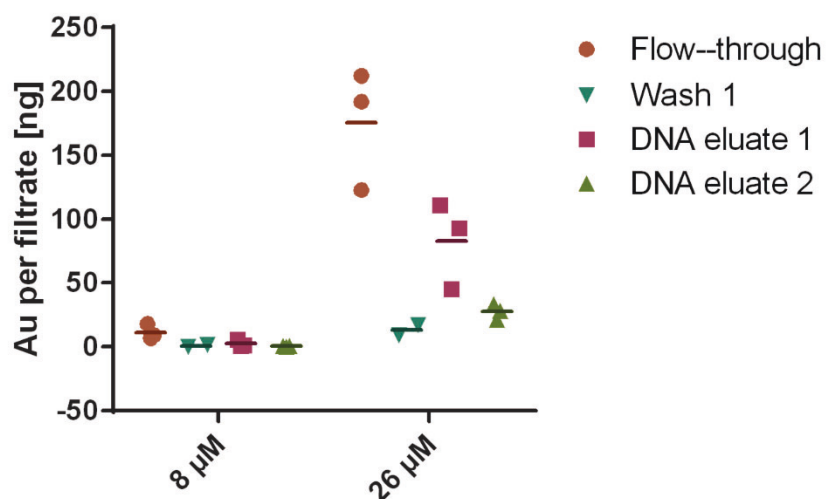


Figure S4. Amount of Au found during the DNA isolation process of A2780 (cancer) cells in filtrates after loading the lysate to the spin column (flow-through), after the first washing step (wash 1) and in both eluates. In samples treated for 24 h with 8 μM ($2/3 \text{ EC}_{50}$) **AuTMX₂**, DNA concentration of the eluate 1 and 2 were $105.6 \pm 18.9 \text{ ng } \mu\text{L}^{-1}$ and $55.0 \pm 8.6 \text{ ng } \mu\text{L}^{-1}$, respectively. After 26 μM ($2 \times \text{EC}_{50}$) treatment, DNA concentration of the eluate 1 and 2 were $110.7 \pm 17.9 \text{ ng } \mu\text{L}^{-1}$ and $51.4 \pm 12.6 \text{ ng } \mu\text{L}^{-1}$, respectively.

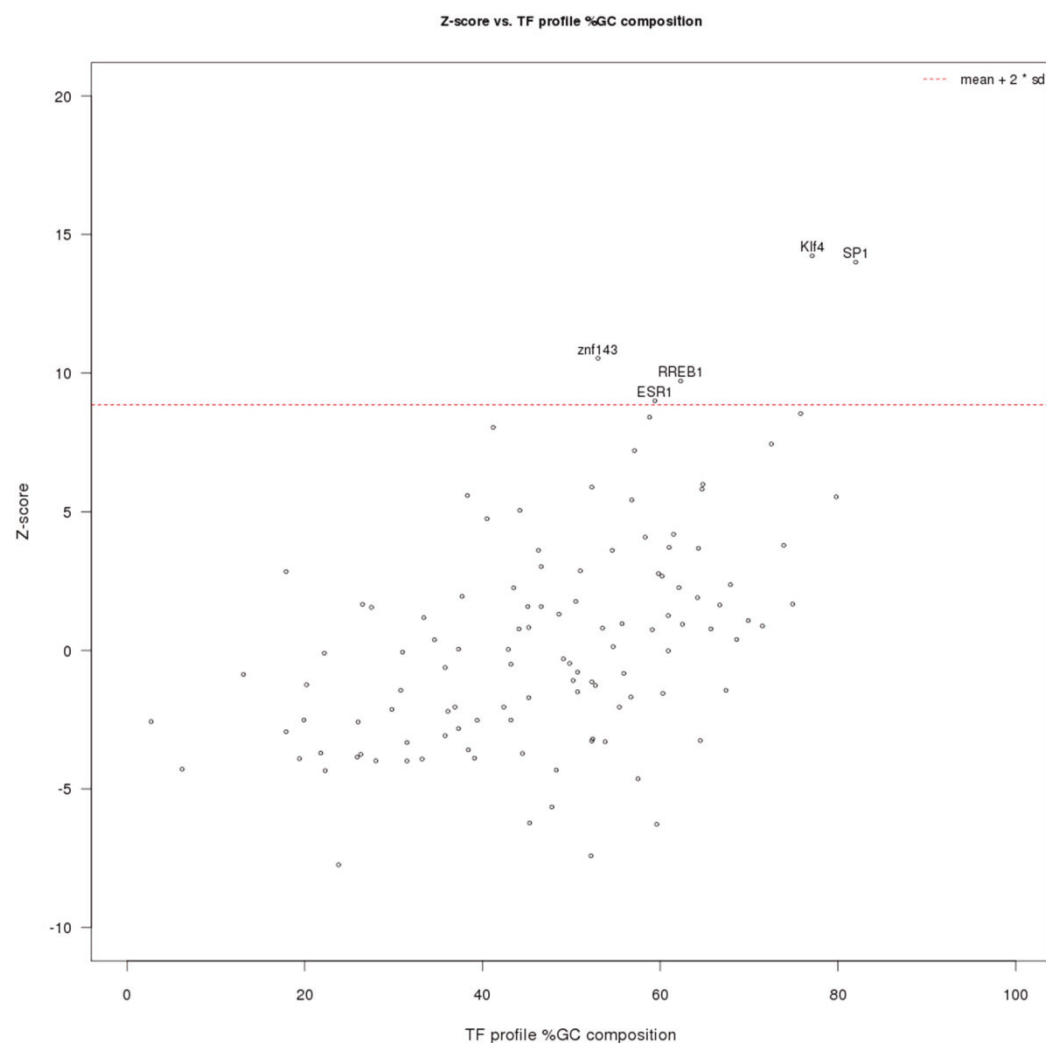


Figure S5. oPOSSUM search of enriched transcription factor activity from the set of significantly up-regulated proteins of **AuTMX₂**-treated A2780 cancer cells.

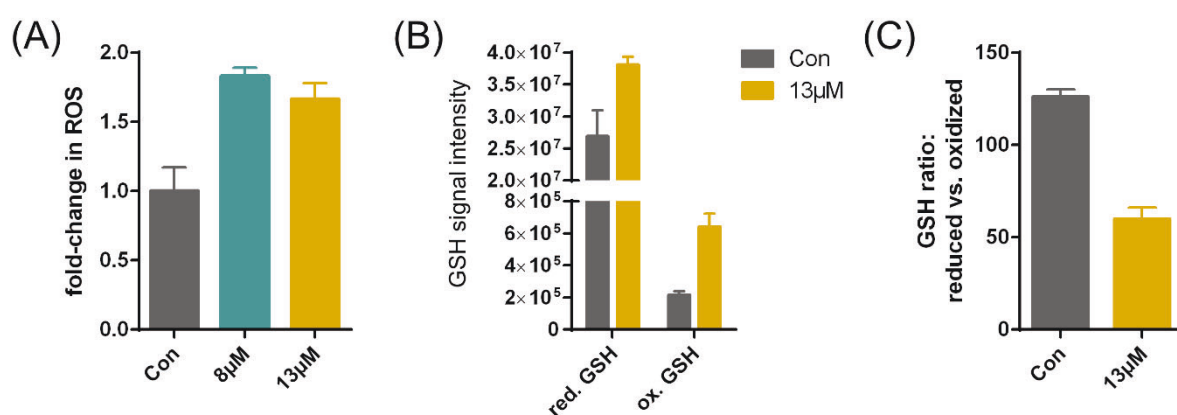


Figure S6. (A) Induction of reactive oxygen species (ROS) in A2780 cancer cells as measured by an increase in mean fluorescence intensity of the 2',7'-dichlorofluorescein (DCF) fluorophore upon treatment with **AuTMX₂** for 24 h. Data were set relative to control cells. p-value (one-way ANOVA) = 0.0004. (B) Signal intensity of GSH stemming from reduced and oxidized glutathione (GSH) after treating A2780 cancer cells with **AuTMX₂** at the indicated concentration for 24 h. (C) The ratio of reduced vs oxidized GSH of the signals in (B) as measured in untreated A2780 cancer cells and treated cells with **AuTMX₂** (13 μM) for 24 h. p-value (unpaired, two-tailed) = 0.0001.

7 Conclusion

Modern mass spectrometry-based proteomics is able to detect several thousand proteins within a short time. It crucially helps to understand the biological processes that the detected proteins are involved in on a molecular level. However, it remains challenging to connect model systems and mass spectrometry-based proteomics in a robust workflow. Thus, this thesis aims to combine the analysis of model systems used in life sciences with complementary omics methods, including eicosadomics, to elucidate the pathophysiology of disease and to gain insight into complex mechanisms such as anticancer drug treatment.

The melanoma brain metastasis model (MBM) used in this thesis consists of four pairs of human cell lines. Each cell line was transformed in a mouse xenograft to yield a cutaneous (C) and brain metastasis (CB) variant. These cell lines were shown to have a stable *in vivo* phenotype, namely the selective metastasis into the brain of the CB variants after several *in vitro* passages. A proteomic profiling revealed distinct molecular pathways characteristic for metastasis formation (e.g. immune evasion and epithelial–mesenchymal transition) in each of the four cell lines. Crucially, no common pathway was found significantly regulated in all four cell lines underlining the heterogeneity which is observed in clinical studies of melanoma brain metastasis. The study further emphasises the necessity to combine complementary omics techniques to meaningfully describe the complex, multi-step formation of metastasis.

Then, the effect of different fetal calf serum (FCS) batches on the inflammatory activation was investigated in a human macrophage model. FCS is a widely used natural additive in mammalian cell culture. In this study U937 cells were treated with phorbol 12-myristate 13-acetate (PMA) and lipopolysaccharide (LPS) using four different lots of FCS added to the cell culture medium. The subsequent proteomics and eicosadomics analysis revealed significant differences in the extent of inflammatory activation. Especially, FCS batches showed different levels of several eicosanoids. Adjusting the levels of 5-HETE and 15-HETE for two FCS batches, which differed in nanomolar range, resulted in significant protein alterations. The involvement of peroxisomes indicated by upregulated PEX16 was verified using immunofluorescence.

Combining chemical proteomics and proteome profiling with transcriptomics profiling allowed to reveal the protein-specific mechanism of action of the ruthenium-based anticancer drug BOLD-100 (formerly known as KP-1339). First, target-response profiling on the protein level revealed potential molecular targets of the drug in the HCT116 colon carcinoma cell line. The transcription factor GTF2I, which directly regulates GRP78 expression and therefore influences the unfolded protein response was identified as one of the potential interaction partners of the drug. Furthermore, a range of ribosomal proteins was identified as potential interactors of BOLD-100. Transcriptomics analysis confirmed a strong influence on ribosomal proteins and TEM showed a disturbance of the ribosomes by detecting polyribosomes in treated cells. This illustrates the

importance of an appropriate combination of comprehensive analysis methods to reveal drug mode of actions. The possibility to reveal metal-based drug mode of actions with proteomics analysis was additionally shown for the organometallic gold complex AuTMX₂. G-quadruplexes (G4s) have earlier been shown to be a major target of the drug and a strong proteomic alteration of treated A2780 ovarian cancer cells could be observed. Especially, the strong upregulation of proteins related to oxidative stress response was shown. ROS formation was also confirmed with metabolic measurements determining the glutathione levels.

Biochemical processes are characterised by their interplay of different biomolecules making multi-omics an important tool in their investigation. Inflammation for example, is mediated by both cytokines and eicosanoids hence analysis on protein and metabolite level allow a more accurate insight. Omics methods like proteomics and metabolomics are technically demanding and require high expertise in the data acquisition and interpretation which makes a combination even more complex. But the insight that is gained through multi-omics analysis is necessary to comprehensively understand the complex biological process with respect to disease and therapies.

8 Scientific contributions

8.1 List of publications

1. Neuditschko B, Niederstaetter L, Brunmair J, Janker L, Bileck A, Del Favero G, Gerner C. Eicosanoid Content in Fetal Calf Serum Accounts for Reproducibility Challenges in Cell Culture. *Biomolecules*. **2021** Jan 15;11(1):113. doi: 10.3390/biom11010113
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4. Reichl B, Niederstaetter L, Boegl T, Neuditschko B, Bileck A, Gojo J, Buchberger W, Peyrl A, Gerner C. Determination of a Tumor-Promoting Microenvironment in Recurrent Medulloblastoma: A Multi-Omics Study of Cerebrospinal Fluid. *Cancers (Basel)*. **2020** May 26;12(6):1350. doi: 10.3390/cancers12061350.
5. Neuditschko B, Janker L, Niederstaetter L, Brunmair J, Krivanek K, Izraely S, Sagi-Assif O, Meshel T, Keppler BK, Del Favero G, Witz IP, Gerner C. The Challenge of Classifying Metastatic Cell Properties by Molecular Profiling Exemplified with Cutaneous Melanoma Cells and Their Cerebral Metastasis from Patient Derived Mouse Xenografts. *Mol Cell Proteomics*. **2020** Mar;19(3):478-489. doi: 10.1074/mcp.RA119.001886.
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7. Del Favero G, Woelflingseder L, Janker L, Neuditschko B, Seriani S, Gallina P, Sbaizero O, Gerner C, Marko D. Deoxynivalenol induces structural alterations in epidermoid carcinoma cells A431 and impairs the response to biomechanical stimulation. *Sci Rep*. **2018** Jul 27;8(1):11351. doi: 10.1038/s41598-018-29728-5.
8. Kral M, Klimek C, Kutay B, Timelthaler G, Lendl T, Neuditschko B, Gerner C, Sibilia M, Csiszar A. Covalent dimerization of interleukin-like epithelial-to-mesenchymal transition (EMT) inducer (ILEI) facilitates EMT, invasion, and late aspects of metastasis. *FEBS J*. **2017** Oct;284(20):3484-3505. doi: 10.1111/febs.14207.

8.2 List of oral presentations

“Response Profiling of the anti-cancer drug arsenic trioxide”, ASAC Junganalytikerforum, Vienna, Austria, **05/05/2018**

“Molecular profiling of cutaneous melanoma cells and their cerebral metastasis from patient derived mouse xenografts suggest individually distinct adaptation strategies”, ASAC Junganalytikerforum, Linz, Austria, **23/09/2019**

“The challenge of classifying metastatic cell properties by molecular profiling of a brain metastasis model”, 31st MassSpec Forum, Vienna, Austria, **26/02/2020**

8.3 List of poster presentations

“Response profiling shows synergistic anticancer effects of the clinically successful combination therapy all-trans retinoic acid and arsenic trioxide”, Austrian Proteomics and Metabolomics Research Symposium, Vienna, Austria, **29-31/08/2018**

“Multi omics evaluation of cutaneous melanoma cells and their cerebral metastasis reveal individually distinct molecular mechanisms”, EMBL–Wellcome Genome Campus Conference: Proteomics in Cell Biology and Disease Mechanisms, Heidelberg, Germany, **7-9/03/2019**

„Ich habe mich bemüht, sämtliche Inhaber der Bildrechte ausfindig zu machen und ihre Zustimmung zur Verwendung der Bilder in dieser Arbeit eingeholt. Sollte dennoch eine Urheberrechtsverletzung bekannt werden, ersuche ich um Meldung bei mir.“

B.N. Februar 2021