RESEARCH

Thermodynamic genome-scale metabolic modeling of metallodrug resistance in colorectal cancer

Helena A. Herrmann^{1†}, Mate Rusz^{1,2†}, Dina Baier², Michael A. Jakupiec^{2,3}, Bernhard K. Keppler^{2,3}, Walter Berger^{3,4}, Gunda Koellensperger^{1,5,6} and Jürgen Zanghellini^{1*}

Abstract

Background: Mass spectrometry-based metabolomics approaches provide an immense opportunity to enhance our understanding of the mechanisms that underpin the cellular reprogramming of cancers. Accurate comparative metabolic profiling of heterogeneous conditions, however, is still a challenge.

Methods: Measuring both intracellular and extracellular metabolite concentrations, we constrain four instances of a thermodynamic genome-scale metabolic model of the HCT116 colorectal carcinoma cell line to compare the metabolic flux profiles of cells that are either sensitive or resistant to ruthenium- or platinum-based treatments with BOLD-100/KP1339 and oxaliplatin, respectively.

Results: Normalizing according to growth rate and normalizing resistant cells according to their respective sensitive controls, we are able to dissect metabolic responses specific to the drug and to the resistance states. We find the normalization steps to be crucial in the interpretation of the metabolomics data and show that the metabolic reprogramming in resistant cells is limited to a select number of pathways.

Conclusions: Here we elucidate the key importance of normalization steps in the interpretation of metabolomics data, allowing us to uncover drug-specific metabolic reprogramming during acquired metal-drug resistance.

Keywords: omics data integration; constraint-based modeling; data normalization

Background

A reprogramming of metabolism is a hallmark of multiple diseases, including cancer [1]. Changes in glucose, amino acid, lipid, and cholesterol metabolism, for example, have all been associated with aberrant metabolic phenotypes observed in cancers [2]. Resulting differences in metabolism between healthy and cancerous cells hold the potential for selectively targeting cancerous cells through pharmacological and dietary interventions. As such, understanding the extent to which metabolic reprogramming occurs in different cancer cells is a fundamental requirement for better treatment options. However, not only malignant transformation, but also therapy response on drug resistance acquisition might be paralleled or even driven by metabolic changes in the malignant cells [3, 4]. Especially, in case of acquired therapy resistance, dissection of the respective metabolic alterations and mechanism on a larger scale are only at the beginning.

In silico methods have the potential to integrate existing experimental data and to generate new hypotheses about the underlying mechanisms associated with metabolic reprogramming. Genome-scale metabolic models (GSMMs), which capture the known biochemical reactions of a given system, have previously been applied in various cancer studies [5, 6] and have led to the discovery of new drug targets and biomarkers [7, 8, 9, 10, 11]. There are, however, areas of cancer research, where GSMMs have not yet been applied due to a lack of available experimental data. For example, GSMMs have not yet been used extensively to study acquired drug resistance against different drug classes in different cancer cell types. Acquired therapy resistance is considered a major obstacle for curative systemic cancer treatment at progressed stages and also affects the success of anticancer metal drugs. [12, 13, 14, 15].

* Correspondence: {gunda.koellensperger, juergen.zanghellini}@univie.ac.at

¹ Department of Analytical Chemistry, University of Vienna, Vienna, Austria

Full list of author information is available at the end of the article

[†]Equal contributor

¹ Metal-based drug treatments involving oxaliplatin
² are a standard therapy for colorectal cancer, the third
³ most commonly diagnosed cancer [16, 17, 18, 19]. Drug
⁴ resistance, however, has been reported to develop in
⁵ nearly all patients with colorectal cancer; even when
⁶ using modern targeted and immunotherapy options,
⁷ chemotherapy remains a major part of the colorec-
⁸ tal cancer treatment regimen [13, 14]. Platinum-based
⁹ drugs are still prescribed in different lines of sys-
¹⁰ temic cancer treatment in diverse tumor types and pa-
¹¹ tient cohorts [20]. Although platinum-based anticancer
¹² drugs like oxaliplatin are widely-used, intrinsic and ac-
¹³ quired resistances remain a crucial impediment in the
¹⁴ treatment of colorectal cancer.
¹⁵

¹⁶ Acquired resistance against platinum drugs is thought
¹⁷ to be mainly based on elevated DNA-repair mech-
¹⁸ anisms, detoxification, evading apoptosis and au-
¹⁹ tophagy [21]. However, there is an increasing amount
²⁰ of evidence that metabolic alterations might play a
²¹ pivotal role as well [22]. The clinically-investigated
²² ruthenium-based anticancer drug BOLD-100/KP1339
²³ has shown promising results with regards to colorec-
²⁴ tal cancer treatment [23]. BOLD-100/KP1339 (sodium
²⁵ trans-[tetrachloridobis (1H-indazole) ruthenate(III)])
²⁶ is a prodrug [24] displaying preferential activation by
²⁷ reduction in the hypoxic milieu of solid tumors and
²⁸ does not primarily target DNA [25] and metabolic al-
²⁹ terations are expected to be relevant. Unlike BOLD-
³⁰ 100/KP1339, which is still under investigation for
³¹ clinical applications, oxaliplatin is an already widely-
³² applied, clinical cancer treatment. As a result, the
³³ body of literature addressing oxaliplatin resistance is
³⁴ notably larger than that of BOLD-100/KP1339 resis-
³⁵ tance. Nonetheless, the extent to which metallodrug
³⁶ resistance results in an altered metabolic profile re-
³⁷ mains poorly understood for both drug treatments
³⁸ and has not yet been compared. As such, it is not
³⁹ yet known whether metabolic reprogramming during
⁴⁰ resistance development against anticancer compounds
⁴¹ with different metal centers and activity parameters
⁴² are comparable or drug-specific.

⁴³ Metabolomics aims to directly measure metabolite
⁴⁴ abundance from a global and unbiased perspective
⁴⁵ and has the potential to not only detect metabolic
⁴⁶ alterations but to discover diagnostic and prognostic
⁴⁷ markers and to generate hypotheses that can be val-
⁴⁸ idated with genetic experiments [26]. Recent progress
⁴⁹ in targeted and untargeted metabolomics approaches
⁵⁰ have resulted in a wide-ranging toolkit for study-
⁵¹ ing metabolic phenotypes in terms of cellular concen-
⁵² trations. Mass spectrometry-based metabolomics ap-
⁵³ proaches can be used for the metabolic profiling of
⁵⁴ drug-treatment responses in cancer cell lines [27, 28].
⁵⁵

¹ While metabolomics studies provide an effective in-
² terrogation window for the cellular changes that oc-
³ cur in response to a change in conditions, they do
⁴ not necessarily provide mechanistic insights into the
⁵ reprogramming of metabolism. Metabolite pools do
⁶ not inform about pathway activity, ergo correspond-
⁷ ing metabolic fluxes are sometimes measured. Measur-
⁸ ing metabolic fluxes, however, also suffers from several
⁹ practical limitations. For example, a prolonged time
¹⁰ for peripheral pathways to reach isotopic steady-state,
¹¹ the fact that simple linear pathways can only be inves-
¹² tigated with non-stationary labelling or an increased
¹³ number of samples, and the complex data analyses re-
¹⁴ quired for nonstationary labelling experiments often
¹⁵ hinder a successful and comprehensive application of
¹⁶ isotopic labelling methods[29].

¹⁷ Recent trends in metabolomics have shown, it is al-
¹⁸ ways possible to measure more metabolites at more
¹⁹ time points and to analyse the obtained results in com-
²⁰ bination with other 'omics data sets [30, 31, 32, 33].
²¹ While multi-omics have allowed for the identifica-
²² tion of numerous regulatory mechanisms in cancer
²³ [34, 35], their integration with fluxomics is required
²⁴ to gain a holistic understanding of metabolic repro-
²⁵ gramming. To understand the mechanisms that under-
²⁶ pin a potential reprogramming of metabolism during
²⁷ resistance development, observed changes in metabo-
²⁸ lite concentrations need to be placed in the context
²⁹ of changes in metabolic flux. GSMMs provide a plat-
³⁰ form for doing so [36]. Multiple techniques for integrat-
³¹ ing omics data sets into GSMMs have been developed
³² [10, 37, 38, 39, 40]. While expression data sets are of-
³³ ten used to generate system-specific models [41, 7],
³⁴ metabolomics and proteomics data sets are used to
³⁵ constrain the solution space of the generated models
³⁶ [37, 40].

³⁷ Typically, constraint-based modelling (CBM) is em-
³⁸ ployed to study GSMMs and to explore metabolic phe-
³⁹ notypes in the form of steady-state fluxes [42, 43]. Flux
⁴⁰ balance analysis (FBA), for example, uses linear opti-
⁴¹ mization techniques to model the fluxome of GSMMs
⁴² [see Orth et al [44] for a review]. FBA, however, can
⁴³ lead to the prediction of thermodynamically infeas-
⁴⁴ ible flux solutions. Thermodynamic flux analysis (TFA)
⁴⁵ imposes additional constraints on stoichiometric mod-
⁴⁶ els to ensure thermodynamically valid fluxes and pro-
⁴⁷ vides a framework for integrating metabolomics data
⁴⁸ into GSMMs [45, 46]; extracellular metabolite data are
⁴⁹ used to constrain the directionality of exchange reac-
⁵⁰ tions of the model and intracellular metabolite data
⁵¹ can be used to constrain reactions in the model. Both
⁵² intra- and extracellular metabolite data have previ-
⁵³ ously been integrated into system-specific metabolic
⁵⁴ models to draw physiological conclusions about can-
⁵⁵ cerous and healthy cells [47, 48, 49, 50].

¹ In this work, we integrate experimentally deter-
²mined absolute concentrations of intracellular metabo-
³lites and medium-based metabolites and growth rates
⁴of the colorectal cancer cell-line HCT116 into a cell-
⁵line specific, thermodynamic, genome-scale metabolic
⁶model (GSMM). We consider two different models of
⁷acquired resistance in colon cancer: oxaliplatin resis-
⁸tant (OxR) and BOLD-100/KP1339 resistant (RuR)
⁹HCT116 cells as well as their sensitive controls to gen-
¹⁰erate four model instances. To identify metabolic dif-
¹¹ferences between resistant and sensitive cells, we nor-
¹²malize the calculated flux values by their representa-
¹³tive growth rates. As oxaliplatin and BOLD-100 are
¹⁴prepared in different solvents (water versus dimethyl-
¹⁵sulfoxid (DMSO)), OxR and RuR cells were grown in
¹⁶the same media, but RuR and its respective control
¹⁷were exposed to a low DMSO background equivalent
¹⁸to the drugs' stock solution solvent. To account for
¹⁹metabolic difference that are the results of a differ-
²⁰ence in solvent, we further normalized the results ob-
²¹tained for the resistant cells by their sensitive controls.
²²Eliminating both differences in growth rate and solvent
²³background, we are able to draw drug-specific conclu-
²⁴sions about the metabolic changes that occur upon
²⁵resistance. As a result, we are able to identify specific
²⁶changes in flux that are the direct result of an acquired
²⁷resistance to either OxR or RuR treatment.

²⁸Materials and Methods

²⁹Cell Culture

³⁰HCT116 colon cancer cells were generously provided
³¹by Dr. Vogelstein from John Hopkins University,
³²Baltimore. Cells were cultured in McCoy's medium
³³(Sigma Aldrich) supplemented with 10% fetal calf
³⁴serum (FCS; PAA, Linz, Austria) and 2 mM glutamine
³⁵(Sigma Aldrich). Cells were selected for acquired
³⁶drug resistance over several months via exposure to
³⁷increasing concentrations of oxaliplatin or BOLD-
³⁸100/KP1339 followed by drug-free recovery phases.
³⁹Finally, the oxaliplatin-resistant HCT116 (OxR) cells
⁴⁰were selected with 5 μ M of oxaliplatin [51, 52] for 24 h
⁴¹and BOLD-100/KP1339-resistant (RuR) cells with
⁴²200 μ M of BOLD-100/KP1339 for 72 h in two-week-
⁴³intervals. All cultures were grown under standard cell
⁴⁴culture conditions and checked for *Mycoplasma* con-
⁴⁵tamination.

⁴⁶Cell viability assay

⁴⁷Cells were seeded at densities of 3.5×10^4 cells/well
⁴⁸cells in 96-well microtiter plates and allowed to adhere
⁴⁹overnight. Cells were exposed to indicated concentra-
⁵⁰tions of the respective drugs for 72 h. Cell viability
⁵¹was determined using the 3-(4,5-dimethylthiazol-2-yl)-
⁵²2,5-diphenyltetrazolium bromide (MTT) assay (EZ4U,
⁵³Biomedica, Vienna, Austria) following the manufac-
⁵⁴turer's recommendations.
⁵⁵

¹Metabolomics experiment

²HCT116 cells, HCT116 cells with acquired oxaliplatin
³resistance, and HCT116 cells with acquired BOLD-
⁴100/KP1339 resistance were seeded as 2×10^5 cells/well
⁵in 12-well plate formate in 1 mL McCoy's medium
⁶(Sigma Aldrich) supplemented with 2 mM glutamine
⁷and 10% FCS. After overnight growth, wells were sup-
⁸plemented with 1 mL fresh medium each. HCT116
⁹with acquired BOLD-100/KP1339 resistance and its
¹⁰sensitive control contained the same medium with
¹¹0.5% dimethyl sulfoxide (DMSO) used as BOLD-
¹²100 solvent. 24 h after supplementing with additional
¹³medium, cells are still not confluent. At this point, the
¹⁴medium was removed and cells were washed 3-times
¹⁵with 2 mL PBS (37 °C) and snap frozen with liquid
¹⁶nitrogen.
¹⁷

¹⁸Metabolomics sample preparation

¹⁹The samples were randomized at the stages of the ex-
²⁰periment including seeding, sample preparation and
²¹extraction as well as LC-MS measurement sequence.
²²Extraction and measurement of the metabolites were
²³based on a protocol described elsewhere [53]. Shortly,
²⁴the protocol comprised cell scraping and extraction
²⁵with 180 μ L cold 80% methanol containing 5 mM
²⁶N-ethylmaleimide (dissolved in 10 mM ammonium-
²⁷formate at pH 7) with 20 μ L fully 13 C-labeled internal
²⁸standard, ISOTopic solutions (Vienna, Austria). Af-
²⁹ter a centrifugation step (14,000 rcf, 4 °C, 10 min) cell
³⁰extracts were directly measured with high-resolution
³¹OrbiTrap mass spectrometer.
³²

³³LC-MS analysis of metabolites

³⁴The quantification of metabolites was based on Schwaiger
³⁵et al. [54] and the LC-MS gradient was adapted and
³⁶shortened to suit metabolites as described elsewhere
³⁷[55]. Full mass scan data was acquired both in positive
³⁸and negative ion mode.
³⁹

⁴⁰LC-MS analysis of coenzymes

⁴¹The analysis of free coenzyme A (CoA), acetyl-
⁴²coenzyme A, palmitoyl-coenzyme A (malonyl-coenzyme
⁴³A below LOD) was carried out in an additional mea-
⁴⁴surement series of the same samples and on the same
⁴⁵instrumental setup but a with a dedicated LC-MS
⁴⁶method. The same separation was used with the
⁴⁷same gradient and eluents, but flushing of the column
⁴⁸started already at 6 min instead of 7 min min, shorten-
⁴⁹ing the total measurement time from 15 min to 14 min.
⁵⁰The OrbiTrap MS settings were changed with regards
⁵¹to the mass range to 750-1100 m/z and the capillary
⁵²temperature was lowered from 280 °C to 200 °C to re-
⁵³duce in-source fragmentation and the S-lense RF-level
⁵⁴was increased from 30 to 60.
⁵⁵

¹Determination of total protein content

²The applied extraction and centrifugation resulted in a
³pellet containing the high-molecular fraction and non-
⁴polar metabolites. This pellet was dissolved in 0.2 M
⁵NaOH overnight, diluted 1:10 in the same NaOH solu-
⁶tion and determined for total protein content with the
⁷Thermo Micro BCA kit, according to manufacturer's
⁸instructions.

¹⁰Data analysis of metabolomics measurement

¹¹Targeted analysis of the data was done with Skyline
¹²20.2 (MacCoss Lab Software) extracting the $[M-H]^-$
¹³and $[M+H]^+$ ions with 5 ppm mass tolerance. The ab-
¹⁴solute concentrations relied on the external calibration
¹⁵with internal standardization. The compounds
¹⁶were standardized compound-specifically where pos-
¹⁷sible and class-specifically when the $U^{13}C$ equivalent
¹⁸was not reliably available or by $U^{13}C$ -glutamate if nei-
¹⁹ther of the aforementioned were available.

²⁰Metabolites with technical repeatability (relative
²¹standard deviation) above 30% were removed from
²²the dataset. This was based on the repeated injection
²³and measurement of a pooled quality control sample.
²⁴Furthermore, metabolites which had mean concentra-
²⁵tion below the determined lowest limit of quantifica-
²⁶tion (LOQ) according to the validation of the LC-MS
²⁷method described in [54] were removed.

²⁸Datasets were combined by joining the metabolite
²⁹data acquired in both positive and negative mode,
³⁰as well as coenzyme data in the negative acquisition
³¹mode. A further calibration was measured in positive
³²mode for several carnitines (propionyl-carnitine, O-
³³acetyl-carnitine, propionyl-carnitine, palmitoyl-carnitine)
³⁴with the method for metabolites, since these com-
³⁵pounds were not contained in our original calibration
³⁶mixture. Also the calibration row for coenzymes was
³⁷prepared freshly in this mixture to avoid degradation
³⁸by storage. The external calibration of the different
³⁹coenzymes (coenzyme A, acetyl coenzyme A, malonyl
⁴⁰coenzyme A, palmitoyl coenzyme A) was measured in
⁴¹negative mode. For all primary thiols in the dataset
⁴²(coenzyme-A, glutathione, cysteine etc.) its N-ethyl
⁴³maleimide adduct was used for quantification after it
⁴⁴was made sure that the conversion was quantitative.

⁴⁶Measurement of extracellular metabolite concentrations

⁴⁷ 10^5 HCT116 cells as well as HCT116 cells with ac-
⁴⁸quired oxaliplatin resistance and HCT116 cells with
⁴⁹BOLD-100/KP1339 resistance were seeded ($N = 4$ for
⁵⁰each respectively) into 12-well plate (StarLab) with
⁵¹2 mL McCoy's 5A medium (Sigma-Aldrich) contain-
⁵²ing 10% FCS (BioWest) and 4 mM glutamine. Also
⁵³in the case of the sensitive HCT116 cells and the
⁵⁴BOLD-100/KP1339-resistant cells the experiment was
⁵⁵

run with and without 0.5% DMSO. 100 μ L of was col-
lected from the starting medium at beginning of the
experiment, and directly from the wells after 24 h, 48 h
and 72 h after seeding. Also, a cell free experiment was
run to determine the contribution of abiotic glutamine
decay.

Determination of dry-weight for the cell lines

Measurements were carried out as described by Szélio-
vá et al. [56]

HCT116-specific genome-scale metabolic model

Robinson et al. [57] provide the latest consensus
GSMM of human metabolism called Human1. The au-
thors used Human1 to generate cell-line specific mod-
els using gene essentiality data from previous CRISPR
knockout screens [58]. Using the tINIT algorithm [41]
and RNA-Seq data from HCT116 colorectal carci-
noma cells they select reactions from Human1 asso-
ciated with moderately and highly expressed genes
to build a cell-line specific model for HCT116. We
obtained the model from the authors, removed the
enzyme constraints and added a further seven ex-
change reactions to the model to account for the ex-
cretion or uptake of cis-aconitate, fumarate, isocitrate,
malate, sarcosine, succinate and xanthine that we ob-
served in our measured time-course of the medium
composition. We use this updated model for all our
analyses presented here. The model is available at
https://github.com/HAHerrmann/Hct116_DrugRes/blob/master/Models/Colon_Combined.xml.

Growth rates (Figure S1) and exchange rates (Fig-
ure S2) were fitted as described in Szélio-
vá et al. [59]. In short, we fitted an exponential model to estimate
the initial concentration, X_0 , and the growth rate, μ .
The fitted growth rate and the initial biomass, B_0 ,
were then used to calculate the specific exchange rates
for all of the measured medium-based metabolites. B_0
was calculated from the fitted X_0 and the experimen-
tally determined dry mass per cell (Figure S3). The fit-
ting was done in Python (Version 3.7.9) using the opti-
mize function in scipy (Version 1.5.2) with parameters
`soft_l1` for the loss function and `f_scale = 0.3` for
outlier detection. The obtained growth and exchange
rates were used to constrain the respective import and
export reactions of the model. Flux constraints were
set such that the applied upper and lower bounds ac-
counted for the relative standard error of the mea-
surement. We further constrained the directionality of
uptake and excretion rates of 50 metabolites, using
HCT116 cell line specific data obtained by Jain et al.
[60]. The "blood pool" reactions were removed from
the model because we did not consider *in vivo* condi-
tions. Instead, we allowed for an unconstrained influx

¹of stearate, palmitate, oleate, linolenate, linoleate, and
²arachidonate. These fatty acids have previously been
³shown to make up the majority of lipids present in
⁴fetal calf serum [61, 62] which was used as a growth
⁵medium supplement.

⁶
⁷Thermodynamic metabolic modeling
⁸The pyTFA package [46], <https://github.com/EPFL-LCSB/pytfa>, formulates thermodynamic flux analysis (TFA) of GSMM as a mixed-integer linear programming problem that incorporates metabolite concentrations as thermodynamic constraints into a traditional flux balance analysis (FBA) model. Masid et al. [48] have recently constructed an extensive thermodynamic database containing the thermodynamic information for compounds, reactions and compartments in human metabolism; this includes the Gibbs free energy formation of compounds and the associated error estimation, the pH, ionic strength and membrane potentials. Using Biopython (Version 1.78) we annotated the GSMM with SEED identifiers which allowed us to match the information in the GSMM to the thermodynamic database of Masid et al. [48]. This allowed us to achieve a thermodynamic coverage of 89% of the compounds and to estimate the Gibbs free energy for 20% of the reactions. We initially applied default metabolite concentrations from 10^{-12} to 0.1 mol per total protein. We then scaled our measured metabolite concentrations to fall within that same range and applied them, condition-dependent, to different instances of the GSMM. Using a parsimonious FBA (pFBA) that maximizes a linear objective while minimizing the total sum of fluxes [63], we calculated the minimum total sum of fluxes and set this as an additional constraint to our linear model prior to performing a Flux Variability Analysis (FVA) on the thermodynamic model, here referred to as TFVA. TFVA applies the same constraints as TFA but instead of returning a single feasible solution, the lowest and highest possible flux value for each reaction is returned [64]. Because pFBA does not necessarily return a unique solution when two alternative pathways with the same total sum of fluxes exist, we chose to implement a parsimonious TFVA (pTFVA) to compare different model instances to one another. Upon parallelizing the existing TFVA implementation in pyTFA for an improved run time, we ran a pTFVA for different instances of the HCT116 cell-lines specific GSMM. Flux analyses were done in Python (Version 3.7.9) using cobrapy (Version 0.19.0) [65].

⁴⁹Data processing and flux normalization

⁵⁰We constrained four different instances of the HCT116
⁵¹model: oxaliplatin-resistant cells (OxR) and their sensitive parental counterpart (HCT116) and BOLD-
⁵²100/KP1339-resistant cells (RuR) and their sensitive parental counterpart in a DMSO-based medium

(HCT116-DMSO). Model instances were constrained¹ using the condition-specific exchange fluxes (Figure S1) and growth rate (Figure S2). All blocked reactions were removed using the `find.blocked.reactions` in cobrapy (Version 0.19.0) with default parameters, resulting in a model with 4530 reactions and 4492 degrees of freedom. Upon calculating flux values for each model instance using pTFVA as described, we divided each set of flux values by the outgoing flux to biomass production of that model instance, effectively normalizing for difference in growth. We checked for reactions for which both the upper and the lower bound differed by at least 15%. Furthermore, we feature-scaled all flux values to lie between 0 and 1 and divided the flux values obtained in the drug-resistant instances by the corresponding flux values obtained for their respective controls. Having thus normalized for differences in the medium composition, we were able to compare the flux profiles of the two metallocrug resistance to another, again checking for which reactions both the upper and lower bounds differed by at least 15%.

²²Results

²³Differences in metabolite concentrations may not
²⁴correlate to changes in flux

²⁵To investigate the metabolic changes associated with
²⁶metallo-resistance in colorectal cancer, we compared
²⁷the metabolic profiles of resistant and sensitive cells.
²⁸Using the HCT116 colorectal cancer cell line, cells
²⁹with resistance to either oxaliplatin (OxR) or BOLD-
³⁰100/KP1339 (RuR) were compared to their sensitive
³¹counterparts. The two acquired resistance models are
³²largely independent of one another: while OxR cells
³³show moderate cross-resistance for the ruthenium-
³⁴based drug, RuR cells display no cross-resistance and
³⁵remain sensitive to oxaliplatin treatment (Figure S5).
³⁶This implies a difference in the molecular basis of resistance between the two models. OxR cells and their parental sensitive counterparts were grown in a standard medium, while RuR cells and their parental sensitive counterparts were grown in the same medium but with a low solvent-background (DMSO) as outlined in the Materials and Methods. Relative differences in the cellular metabolite concentrations of sensitive versus resistant cells highlight the extent to which the acquired metallocrug resistance results in an altered metabolome (Figure 1). We observe that some responses, such as an increase in palmitoylcarnitine and a decrease in lactate upon resistance, are shared across the two metallo-resistance phenotypes. Nevertheless, many of the metabolic changes associated with resistance are drug-specific. Pyruvate and carnitine concentrations, for example, are higher in RuR cells but lower in OxR cells when compared to their sensitive counterparts. Palmitoyl-CoA, on the other hand, is lower

Figure 1 Observed changes in metabolite concentrations upon acquired resistance. Oxaliplatin (OxR) and BOLD-100/KP1339 (RuR) resistance models are compared to their sensitive counterparts. Relative differences in the measured metabolite concentrations of resistant and sensitive cells are shown. A positive relative difference (red) indicates a higher metabolite concentration in the resistant as compared to the parental sensitive model, whereas a negative relative difference (blue) indicates a lower metabolite concentration in the resistant than in the sensitive counterpart. Relative differences were calculated from using the mean values of six replicates. Only those metabolites for which we observed an absolute relative change greater than 15% between sensitive and resistant, in at least one of the two conditions, are shown. The following abbreviations were used: ADP - adenosine diphosphate, AMP - adenosine monophosphate, CMP - cytidine monophosphate, CTP - cytidine triphosphate, GMP - guanosine monophosphate, IMP - inosine monophosphate, NAD - nicotinamide adenine dinucleotide, UDP - uridine diphosphate, UMP - uridine monophosphate, UTP - uridine triphosphate, CoA - coenzyme A, dATP - deoxyadenosine triphosphate, dCMP - deoxycytidine monophosphate.

part (sensitive), a BOLD-100/KP1339-resistant (RuR)¹ and a parental sensitive counterpart for the DMSO-² containing medium (sensitive-DMSO). Measuring 110³ metabolite concentrations and 37 exchange fluxes,⁴ we constrained the solution space of a model with⁵ 6479 metabolites and 6716 reactions. Growth rates⁶ were used to constrain the biomass production of⁷ each model instance. Resistant cells grow slower than⁸ sensitive cells and OxR cells grow even slower than⁹ RuR cells (Figure S1). Exchange rates (Figure S2)¹⁰ were determined from time-course measurements of¹¹ the medium composition and were applied as flux¹² bounds on the corresponding import and export reac-¹³ tions of the model. Intracellular metabolite concen-¹⁴ trations were applied as constraints using the pyTFA¹⁵ package [46]. Using a parsimonious thermodynamic¹⁶ flux variability analysis (pTFVA), as outlined in the¹⁷ Materials and Methods, we calculated flux solutions¹⁸ for each of the four model instances, each of which¹⁹ was constrained with the corresponding experimen-²⁰ tal data. By incorporating the growth and exchange²¹ rates as well as the intracellular metabolite concentra-²² tions into a GSMM, we were able to calculate possible²³ changes in metabolic fluxes. Metabolic rates, rather²⁴ than concentrations, could then be normalized accord-²⁵ ing to the cellular growth rate observed under those²⁶ conditions. We compared the four sets of flux solutions²⁷ against one another, both before and after normaliz-²⁸ ing all flux values by the respective growth rate (Fig-²⁹ ure 2). Growth rate normalization was implemented³⁰ by dividing all of the calculated flux values by the ex-³¹ perimentally measured growth rate used to constrain³² that model instance.³³

The maximum relative standard error observed³⁴ across the metabolite measurements was less than³⁵ 15%. Thus, when integrating the data into the GSMM³⁶ and comparing flux differences between condition-³⁷ specific instances of the model, we used a cutoff of³⁸ 15% to determine whether fluxes were significantly³⁹ different across conditions. Comparing resistant cells⁴⁰ to sensitive cells, we identify pathways with the most⁴¹ prominent changes in flux upon acquired resistance⁴² (Figure 2). Differences in flux observed prior to growth⁴³ standardization directly correspond to predictions of⁴⁴ *in vivo* fluxes. Differences in flux observed post growth⁴⁵ standardization are no longer predictions of *in vivo*⁴⁶ fluxes, but are predictions of flux differences that are⁴⁷ assumed to be the direct result of a metabolic re-⁴⁸ programming upon acquired resistance rather than⁴⁹ changes in growth rate.⁵⁰

Initially, the oxidative phosphorylation pathway⁵¹ shows the highest amount of flux changes in response⁵² to OxR. Upon growth normalizing, however, it is⁵³ RuR that shows a higher number of flux changes in⁵⁴ ⁵⁵

Figure 2 Metabolic fluxes in resistant versus sensitive models before and after growth rate normalization. Both intracellular and extracellular metabolite constraints were applied to generate four instances of the HCT-specific GSMM [oxaliplatin (OxR) and BOLD-100/KP1339 (RuR) and their parental sensitive counterparts (sensitive and sensitive-DMSO, respectively)] as described in the Materials and Methods. A parsimonious thermodynamic flux variability analysis (pTFVA) was done on each model instance. Flux values of the resistant instances were compared to their respective controls. Metabolic reactions that had an absolute relative difference greater than 15% in both the highest possible and the lowest possible flux value were considered to be different. The proportion of reactions that show a difference in flux between the OxR and sensitive condition [OxR before growth rate normalization (GRN); red bars] and the RuR and sensitive conditions (RuR before GRN; orange bars) are shown for each subsystem. All flux values were then normalized according to the corresponding growth rate of that condition (Figure S1) and were again checked for a relative difference between OxR (OxR after GRN; black bars) and RuR (RuR after GRN; gray bars) and their sensitive controls. Subsystems for which no relative changes in flux between resistant and sensitive instances were observed were omitted from the figure for clarity.

however, indicate significant changes in flux with regards to detoxification. While the number of reactions that appear to be affected in starch and sugar and tricarboxylic acid (TCA) metabolism appears to be drug resistance-specific prior to growth normalization, this effect disappears upon growth normalization. The comparison of non-normalized and growth-normalized results in Figure 2 emphasizes that observed changes in metabolite concentrations are not necessarily indicative of cellular changes in flux. It further highlights that flux results must be growth normalized in order to distinguish a resistance model effect from a growth effect when comparing the metabolic profiles of resistant and sensitive cells. Changes in the pentose phosphate pathway (PPP), oxidative phosphorylation, glycolysis/gluconeogenesis, TCA, nucleotide, ROS and fatty acid pathways, for example, appear to be a direct result of acquired resistance when comparing OxR and RuR to their parental sensitive counterparts (Figure 2).

Metallo drug resistance is linked to changes in energy metabolism

Integrating metabolite measurements into GSMMs allows for growth rate normalization of the calculated fluxes which in turn allows for a direct flux comparison between resistant and sensitive cells. The reprogramming of energy metabolism to support cell growth and proliferation is a major hallmark of cancer [1] and has previously been linked to the emergence of acquired drug resistance [3]. To further investigate the role of a reprogramming of energy metabolism upon acquired metallo drug resistance, we used the four instances of the HCT116 model (OxR, sensitive, RuR, sensitive-DMSO) to specifically assess differences in flux in pathways related to energy metabolism.

In the growth-conditions considered here, glucose acts as the primary carbon source (Figure S2). Glucose is catabolized to pyruvate, generating two ATP during glycolysis. Pyruvate can then be transported into the mitochondria and converted to acetyl-CoA which then enters the TCA cycle or, in what is known as the Warburg effect in cancer cells [66], pyruvate can be converted to lactate. Acetyl-CoA can also be generated from fatty acid oxidation and sometimes amino acid catabolism (see [67] for a review). Fluxes corresponding to these three well-established energy pathways of colorectal cells along with the oxygen consumption are shown for each cell type in Figure 3. While glutaminolysis is another common means by which cancer cells support the Warburg effect [68], we did not measure high glutamine uptake rates in the considered growth conditions. In fact, our determined glucose and glutamine uptake rates are in the same orders of magnitude as previously determined for HCT116 cell lines grown in fetal bovine serum [60].

Figure 3 Comparison of fluxes through key energy metabolism reactions for ruthenium- and oxaliplatin-based resistant cells after growth rate normalization. Growth normalized flux values through (a) the oxygen uptake reaction - HMR_9048, (b) the ATP synthase reaction - HMR_6916, (c) the lactate secretion reaction - HMR_9135, and (d) the fatty acid influx - sum of m01362s_FAx, m02387s_FAx, m02389s_FAx, m02646s_FAx, m02674s_FAx, m02938s_FAx, across the four model instances (sensitive - Sen - blue bars; sensitive in a DMSO-containing medium - Sen-DMSO - gray bars; oxaliplatin-resistant - OxR - red bars; BOLD-100/KP1339 - RuR - yellow bars) are shown. Fatty acid influx is the combined influx of stearate, palmitate, oleate, linolenate, linoleate, arachidonate.

Figure 4 Simulating minimum fatty acid uptake requirement in response to various oxygen uptake constraints. Oxygen influx constraints were applied to each of the model instances (a) sensitive, (b) oxaliplatin-resistant, (c) sensitive in DMSO-containing medium, and (d) BOLD-100/KP1339-resistant. Using a parsimonious thermodynamic flux variability analysis (pTFVA; see Methods and Materials for details) the minimum possible fatty acid uptake was calculated for each oxygen constraint as shown. Fatty acid influx is the combined influx (black line) of stearate (orange), palmitate (cyan), oleate (dark purple), linolenate (yellow), linoleate (green), arachidonate (light purple).

We observe that OxR cells convert less pyruvate into lactate, but in turn consume a higher relative amount of fatty acids compared to their parental sensitive cells. RuR cells, however, show a high glycolytic flux and a high oxygen consumption as well as higher fatty acid consumption than their sensitive counterparts (Figure 3). Notably, the flux values shown in Figure 3 are growth normalized and may therefore not directly correspond to what would be observed in a traditional oxygen consumption rate (OCR) versus extracellular acidification rate (ECAR) experiment [69]. When comparing experimentally determined OCR and ECAR measurements to the non-normalized model results, we find a close agreement with regards to the differences in glycolysis and respiration between sensitive and resistant cells (Figure S4, S6); thus further validating the set model constraints.

With the four instances of the HCT116-specific GSMMs, further conditions encountered in the tumor environment can be simulated. Simulating the effect of hypoxic growth conditions, we first set the oxygen influx for each model instance to the minimum possible value and then observe the minimum required fatty acid influx as we iteratively increase the oxygen influx, thus plotting the growth normalized production

envelope of oxygen versus minimum fatty acid influx for each of the four conditions (Figure 4). While RuR cells appear to have a lower tolerance for hypoxic conditions, they also have a higher fatty acid requirement under those conditions when compared to the sensitive simulations (Figure 4c,d). While the same differences can be observed between OxR and sensitive simulations, it is less pronounced (Figure 4a,b).

We then set a minimum possible fatty acid influx and iteratively increased the total fatty acid influx to the model while calculating the minimum required oxygen influx (Figure 4). We repeated this calculation for various biomass constraints and note that there is an optimal fatty acid influx for minimizing the total oxygen required. In fact, this optimal value corresponds directly to the fatty acid uptake rates observed in Figure 3d and is in accordance with the parsimonious thermodynamic flux variability analysis which minimizes the total sum of fluxes (see Method and Materials for details).

Crucially, while a direct comparison between resistant and sensitive cells for each drug respectively can be made, we cannot make a direct comparison between the two drug resistance models (Figure 1-5). Because RuR cells were grown in a DMSO-containing medium whereas OxR cells were not, we cannot, at this stage,

Figure 5 Simulating minimum oxygen requirement in response to various fatty acid uptake and biomass constraints. Fatty acid influx constraints were applied to each of the model instances (a) sensitive - blue lines, (b) oxaliplatin-resistant - red lines, (c) sensitive in DMSO-containing medium - black lines, and (d) BOLD-100/KP1339-resistant - yellow lines. Using a parsimonious flux variability analysis (pTFVA; see Methods and Materials for details) the minimum possible oxygen uptake was calculated for each fatty acid constraint. The calculations were performed for various growth rate constraints ranging from 5 to 15 h⁻¹, as indicated by the fading lines. Fatty acid influx constraints were applied as the combined influx of stearate, palmitate, oleate, linolenate, linoleate, arachidonate.

sensitive and resistant cells are the result of differences¹ caused by DMSO. As such, this normalization step al-² lows us to directly compare the two acquired resis-³ tances, OxR and RuR, to one another even though⁴ RuR, unlike OxR, was grown in a medium with low⁵ solvent (DMSO) background. The comparison of OxR⁶ and RuR (Figure 6) cells highlights an upregulation⁷ of fluxes associated with amino acid and fatty acid⁸ metabolism in RuR. OxR cells, on the other hand,⁹ show an upregulation in glycolysis and starch and¹⁰ sugar metabolism when compared to RuR cells (Fig-¹¹ ure 6b).¹²

Notably, when comparing the OxR and RuR model¹³ instances to their respective parental HCT116 drug-¹⁴ sensitive counterparts, prior to growth normalization,¹⁵ we identified 1039 (OxR) and 1180 (RuR) fluxes¹⁶ that were significantly different. Upon growth nor-¹⁷ malization, these numbers reduced to 743 (OxR) and¹⁸ 883 (RuR), highlighting that hundreds of differences¹⁹ observed in the non-normalized results are simply²⁰ the result of a difference in growth rate. The OxR²¹ versus RuR comparison upon growth-media DMSO-²² background normalization highlighted 670 different re-²³ actions, suggesting that another 73 of reactions were²⁴ initially observed as significantly different because of²⁵ presence of 0.5% DMSO.²⁶

Discussion

Genome-scale metabolic models (GSMMs) provide²⁹ a platform for integrating omics data sets and for³⁰ analysing them in the context of metabolic fluxes.³¹ As we have shown, GSMMs can be constrained using³² both extracellular and intracellular metabolite con-³³ centrations to study metalloid drug resistance in colon³⁴ cancer. Approximately one-hundred metabolite con-³⁵ straints were applied to study the effect of changes³⁶ in their concentrations in thousands of reactions.³⁷ Colorectal-specific GSMMs have previously been con-³⁸ structed [47, 57, 70, 11] but have not yet been applied³⁹ to study metallo-drug resistance specifically. Here, we⁴⁰ compared metabolic flux alterations in HCT116 cell⁴¹ models with acquired oxaliplatin- (OxR) vs BOLD-⁴² 100/KP1339 resistance (RuR) relative to parental,⁴³ drug-sensitive HCT116 cells grown in the respective⁴⁴ growth media without or with 0.5% DMSO.⁴⁵

In this study, we investigated various pathways in⁴⁶ silico including glycolysis, the tricarboxylic acid cycle,⁴⁷ fatty acid and amino acid metabolism, beta-oxidation,⁴⁸ the pentose phosphate pathway. A comprehensive sta-⁴⁹ ble isotope resolved metabolic flux analysis consider-⁵⁰ ing such a diverse set of pathways would require the⁵¹ application of multiple different positionally labelled⁵² isotopic tracers [71, 26]. In addition to economic fac-⁵³ tors, practical challenges may also play a role in exper-⁵⁴ imental design. The application of palmitate to study⁵⁵

distinguish a resistance model-specific effect from a sol-
vent background-induced effect.

Growth rate and medium normalization allows for a
direct comparison of fluxes of cells grown across
heterogeneous conditions

In order to be able to compare the metabolic profiles
of the two metalloid drug resistance phenotypes directly,
we finally normalized the flux results obtained from the
metallo-resistant model instances against their respec-
tive parental sensitive counterparts (see Materials and
Methods for further details). By dividing growth rate
normalized and feature-scaled flux values calculated
for the resistant models by those calculated for the re-
spective sensitive models, we add a further normaliza-
tion step. This normalization step eliminates observed
differences in flux values that are the result of differ-
ences due to the presence of DMSO-background. Be-
cause the parental sensitive counterparts were grown
in the same medium as their resistant counterparts,
we can assume that shared differences in flux between

Figure 6 Predicted differences in the metabolic fluxes of ruthenium- and oxaliplatin-based resistances after growth rate and medium normalization. Both intracellular and extracellular metabolite constraints were applied to generate four instances of the HCT-specific GSMMs (oxaliplatin (OxR) and BOLD-100/KP1339 (RuR) and their sensitive parental counterparts (WT and WT-DMSO, respectively)) as described in the Materials and Methods. A parsimonious thermodynamic flux variability analysis (pTFVA) was done on each model instance. Each set of flux values was divided by the corresponding flux through biomass (Figure S1), thus normalizing for differences in growth. Flux values were then feature-scaled to lie between 0 and 1 and the flux values obtained in the drug-resistant instances were divided by the flux values of the corresponding control instances, thus normalizing for difference in medium composition. (a) The relative changes in flux between OxR and RuR instances were calculated and the total number of reactions that showed an absolute relative difference greater than 15% in relative upper and lower flux values were counted for each subsystem. The proportion of reactions that are significantly different in each subsystem is shown as black bars. Subsystems for which no relative changes in flux between the two resistant instances were observed were omitted from the figure for clarity. (b) Percentage of reactions in a subsystem which were identified as significantly different out of all reactions that were identified as significantly different between the two conditions are shown as a pie chart. Subsystems for which the total flux values were higher in OxR are shown in red. Subsystems for which total flux values were higher in the RuR are shown in orange. The subsystems transport reactions were omitted from this analysis as together they make up over 95% of the significantly different reactions.

beta-oxidation, for example, requires the conjugation of fatty acid free bovine serum albumin [72]. Moreover, it usually has high background contamination from plastic materials [73]. Finally, stable isotope labeling in living organisms is even more complex from a data evaluation perspective [74]. Thus, a purely experimental study that provides a holistic analysis of metabolic reprogramming in cancer is currently infeasible.

There is no simple relationship between changes in metabolite concentrations and changes in flux [75]. This notion also applies to acquired resistance in the HCT116 colorectal cancer cell line. We have shown that observed differences in metabolite concentrations between resistant and sensitive conditions may not necessarily reflect a drug resistance-specific response but may instead arise as a result of differences in

growth rate or solvent conditions. If we want to compare changes in metabolic flux of cells grown in heterogeneous conditions, data needs to be normalized in order for valid comparisons to be made. Here we have outlined a procedure for this kind of normalization based on thermodynamic genome-scale metabolic modelling of the HCT116 cell line.

Accurate comparative profiling of metabolic changes observed across heterogeneous conditions remains a challenge. Differences in growth rates and impact of solvent necessities will result in observed differences in metabolite concentrations but are not causal to a reprogramming of metabolism [76]. Considering cellular fluxes as the metabolic phenotype through the use of GSMMs has the advantage that fluxes, unlike concentrations, can easily be normalized with regards to

other rate measurements, such as growth rate or exchange rates (e.g. [77, 78, 79]).

As a prerequisite to produce accurate, quantitative metabolomics data [76], we normalized the metabolite amounts to total protein content, calculating absolute concentrations based on internal standardization. Even though this approach is superior to relative quantification and is able to compensate for technical variation of the sample preparation and differences in extracted biomass, it does not account for biological processes like growth or environmental factors such as differences in medium composition.

Integrating the metabolite data as part of a thermodynamic flux analysis allows us to normalize the calculated reaction rates by the growth rate observed under the corresponding conditions. We showed that growth-normalization reduces the number of reactions that are different between resistant and sensitive model instances and changes some of the conclusions about altered metabolic pathways entirely. Growth normalization is therefore a critical step when looking for drug-specific metabolic phenotypes. A second limitation to studying non-normalized metabolite concentrations is that data obtained from heterogeneous conditions cannot be directly compared. Using the growth-normalized flux results we further normalized each resistant model against its sensitive counterpart which was grown in an identical medium and solvent composition. Hence, we were able to do a direct comparison between the two metallo-resistances and to identify drug resistance-specific responses.

A limitation to our approach is that we first normalized our flux results to differences in growth rate and then normalized each resistant model against its respective counterpart. This means that we are unable to capture emergent properties that result from differences in growth rate and solvent impact; we assume that a combined effect of the two is minimal. Furthermore our flux analyses assume metabolism to be in steady-state, such that intracellular concentrations are constant. Nonetheless, we have clearly demonstrated that a growth and medium/solvent normalization is non-trivial as it allows for comparisons across heterogeneous conditions. We expect this method to be of wider applicability in studies where the effects of medium compositions, such as the availability of carbon sources to a cell, are of interest.

Time-dependent changes of metabolite profiles have previously been considered [80, 81], but are not typically integrated at a genome-scale level. Measuring metabolite concentrations alongside cell counts at various time points and quantifying the relative metabolite abundance per cell using linear regression Dubuis et al. [80] accounts for deviations from steady-state. The

method was then further developed, using intermediates of fatty acid metabolism and other metabolites to account for differences in cell size [81]. While the difference in cell size can be interpreted as a proxy of growth rate it cannot be assumed that the observed changes in metabolite concentrations directly translate to differences in metabolic activity, i.e. fluxes. Metabolic responses associated with an acquired metalloidrug resistance in cancer have not yet been studied extensively using constraint-based flux analyses [82, 83, 38]. The use of GSMMs to integrate metabolomics data to study cellular fluxes, however, provides multiple new opportunities in this field.

Defense mechanisms and acquired resistance are well known phenomena when applying metal-based drugs, as anticancer agents. Reduced efficacy due to acquired resistance remains a major challenge in systemic anticancer therapy. The complexity is increasingly recognized, as the contribution of epigenetic and metabolic effects will be uncovered. Drug-specific and tumor tissue specific mechanisms have been described, and more recently the tumor microenvironment has come into focus [84]. Accordingly, response profiling with metabolomics analysis can be a powerful tool to investigate drugs and drug candidates [28, 27] and dissecting emerging resistance [85]. Currently, only a handful of studies consider metalloidrugs applied to cancers with metabolomics [86, 87, 88], and even fewer investigate acquired metalloidrug resistance [52].

In this work, we consider an *in vitro* study of colon cancer. Gastrointestinal cancer cell lines, including colorectal cancer cell line HCT116 activate beta-oxidation as response to oxaliplatin treatment and conversely become more sensitive to oxaliplatin upon inhibition of fatty acid catabolism [22]. A seminal study in the field integrates both metabolomics and transcriptomics and finds that, within 59 NCI60 cell lines, the metabolic basis of platinum-sensitivity can largely be attributed to energy metabolism (TCA cycle, glutaminolysis, pyruvate metabolism), lipoprotein uptake, and nucleotide synthesis [89]. The results from our *in silico* analysis are in line with these findings, also highlighting the importance of energy metabolism (OXPHOS, glycolysis, TCA).

Figure 6 highlights the relevance of fatty acid metabolism, as fluxes from this subsystem contribute to 12.5% of all observed differences (excluding all transport reactions) between the RuR and OxR, showing elevated fluxes in RuR. This supports existing evidence of beta-oxidation activation in response to metalloidrug treatment [22]. Interestingly, for the majority of observed flux differences between OxR and RuR, flux values are higher in the RuR, implying

¹a higher metabolic activity in this phenotype, inde-
²pendently of differences in growth rate. OxR, how-
³ever, does exhibit elevated activity in the Glycoly-
⁴sis/Glyconeogenesis and pentose phosphate pathways.
⁵Thus, our comparison of the growth rate- and medium-
⁶normalized reaction rates supports the notion that an
⁷acquired resistance to the two metallodrugs is marked
⁸by differences in their metabolic phenotype with an
⁹overall higher metabolic activity in the RuR system.

¹⁰The high metabolic plasticity of cancer cells enables
¹¹efficient detoxification and protection strategies [84].
¹²Normalization of the flux values by growth rate sub-
¹³stantially reduces the observed differences (Figure 2) in
¹⁴most of the investigated pathways. In contrast, both
¹⁵the pentose phosphate pathway and ROS detoxifica-
¹⁶tion subsystem, which includes glutathione-synthesis,
¹⁷were emphasized to the same extent in both the OxR
¹⁸and RuR resistance models upon growth standardiza-
¹⁹tion. This supports the notion that metallodrugs in-
²⁰terfere with cellular redox homeostasis and stimulate
²¹a readiness to counter reactive oxygen species (also by
²²synthesizing NADPH via the pentose phosphate path-
²³way) which has previously been described to conjugate
²⁴glutathione to platinum complexes with glutathione-S-
²⁵transferase [90].

²⁶Despite shared commonalities like the production of
²⁷ROS, it is expected that RuR and OxR models dis-
²⁸play different metabolic phenotypes, because of known
²⁹differences in their modes of action [91, 92, 25]. Oxali-
³⁰platin, for example, is primarily a DNA targeting drug,
³¹whereas BOLD-100/KP1339 has recently been found
³²to have a prodrug nature and is capable of causing ER-
³³stress and the downregulation of GRP78, encoding a
³⁴endoplasmic reticulum chaperone protein, which has
³⁵been linked to malignancy [93]. It is widely accepted
³⁶that DNA repair mechanisms play a crucial role in re-
³⁷sistance to oxaliplatin [94]. It is important to note that,
³⁸using GSMM, we have here focused solely on metabolic
³⁹changes to compare metabolic reprogramming of the
⁴⁰two acquired resistances but cannot exclude further
⁴¹regulatory events.

⁴²The comparison of fluxes through key energy me-
⁴³tabolism reactions (Figure 3) shows that both acquired
⁴⁴resistances are defined by lowered glycolytic flux than
⁴⁵their sensitive parental cells, although this is less pro-
⁴⁶nounced with RuR. Growth normalization does not af-
⁴⁷fect this observation (Figure S4). The same cannot be
⁴⁸said about the fatty acid beta-oxidation, where upon
⁴⁹growth standardization the acquired resistance models
⁵⁰both show a higher fatty acid requirement than their
⁵¹sensitive controls (Figure 3d; Figure S4d). Addition-
⁵²ally, upon growth normalization OxR has lower and
⁵³RuR higher respiration rates than corresponding sensi-
⁵⁴tive counterparts (Figure 3a). The calculated rates cor-
⁵⁵respond well to the experimentally determined results

with a Seahorse assay (Figure S6). As expected the ex-
perimentally determined and non-normalized *in vitro*
results align more closely with the non-normalized flux
values modelled *in silico*.

Drastic changes in oxygen and fatty acid availability
are known stress conditions in a tumor microenviron-
ment, and are assumed to be managed with metabolic
adaptations [4]. Lipid dependency, for example, is
more pronounced under hypoxic conditions and relies
on the uptake of extracellular fatty acids [95, 96, 72].
We thus used the condition-specific instances of our
constrained GSMM to further inspect the relationship
between hypoxia and fatty acid uptake. We found that
the composition of fatty acids taken up changes in
response to oxygen limitation (Figure 4). Under nor-
moxic conditions linolenate can act as the sole fatty
acid source. As oxygen limitation becomes more pro-
nounced, linoleate, arachidonate, oleate, stearate and
finally palmitate are also required. RuR cells requires
less fatty acids under oxygen limitation compared to
its sensitive counterpart (Figure 4c,d); while the same
is true for OxR the observed difference is notably less
pronounced (Figure 4a,b).

Additionally, the investigation of minimum oxygen
requirement at various fatty acid influxes (Figure 5) re-
vealed that the optimal fatty acid composition, which
has the lowest oxygen demand, is the same across
growth rates. Overall, OxR has the lowest oxygen re-
quirement, which suggests that if sufficient fatty acids
are available, OxR will be the most resilient of the
investigated model against hypoxia (Figure 5).

Conclusion

There are different ways to capture the metabolic phe-
notype of a cell. Metabolic profiling via metabolomics
provides an interrogation window of the intracellular
concentrations at a given point in time. Extracellu-
lar concentrations measured over time provide insight
to the cellular uptake and excretion rates of cells. To-
gether they can be integrated to constrain the solution
space of a genome-scale metabolic model. The calcu-
lated flux values can then be normalized according to
growth rates and environmental conditions, allowing
for drug resistance specific metabolic responses to be
identified across heterogenous conditions. We find the
outlined normalization steps to be crucial in the in-
terpretation of the results and show that metabolic re-
programming is more extensive in BOLD-100/KP1339
resistant cells than in oxaliplatin resistant cells. We
identify pathways, such as fatty acid and amino acid
metabolism, to be upregulated in response to a resis-
tance acquired to a ruthenium-based drug when com-
pared to a platinum-based drug. All in all, genome-
scale metabolic modelling provides a valuable platform

¹for putting observed changes in metabolite concentra-
²tions in the context of metabolic fluxes.

⁴Availability of data and materials

⁵All data and code used to conduct the analyses presented in this
⁶manuscript are available on GitHub
⁷(https://github.com/HAHerrmann/Hct116_DrugRes) and Zenodo (DOI:
⁸[10.5281/zenodo.4633725](https://doi.org/10.5281/zenodo.4633725)). Metabolomics data (LC high-resolution mass
⁹spectrometry-based metabolomics dataset in rawdata and total protein
¹⁰contents corresponding to the samples) have been deposited to the
¹¹EMBL-EBI MetaboLights database [97] with the identifier MTBLS2665 for
¹²the OXr-batch and MTBLS2681 for the RuR-batch. The complete dataset
¹³can be accessed at <https://www.ebi.ac.uk/metabolights/MTBLS2665>
¹⁴and <https://www.ebi.ac.uk/metabolights/MTBLS2681> for the OXr- and
¹⁵RuR-batch, respectively.

¹⁴Competing interests

¹⁵The authors declare that they have no competing interests.

¹⁶Author's contributions

Author contributions	HAH [†]	MR [‡]	DB	MAJ	BKK	WB	GK	JZ
Conceptualization								
Funding acquisition								
Methodology								
Software								
Formal analysis								
Investigation								
Resources								
Validation								
Supervision								
Visualization								
Writing – original draft								
Writing – review & editing								

²⁴Author details

²⁵¹Department of Analytical Chemistry, University of Vienna, Vienna,
²⁶Austria. ²Institute of Inorganic Chemistry, University of Vienna, Vienna,
²⁷Austria. ³Research Cluster Translational Cancer Therapy Research,
²⁸University of Vienna and Medical University of Vienna, Vienna, Austria.
²⁹⁴Institute of Cancer Research and Comprehensive Cancer Center, Medical
³⁰University of Vienna, Vienna, Austria. ⁵Vienna Metabolomics Center
³¹(VIME), University of Vienna, Vienna, Austria. ⁶Research Network
³²Chemistry Meets Microbiology, University of Vienna, Vienna, Austria.

³¹References

- ³²1. Hanahan, D., Weinberg, R.A.: Hallmarks of Cancer: The Next
³³Generation. *Cell* **144**(5), 646–674 (2011).
³⁴doi:[10.1016/j.cell.2011.02.013](https://doi.org/10.1016/j.cell.2011.02.013)
- ³⁵2. Vernieri, C., Casola, S., Foiani, M., Pietrantonio, F., Braud, F.d.,
³⁶Longo, V.: Targeting Cancer Metabolism: Dietary and Pharmacologic
³⁷Interventions. *Cancer Discovery* **6**(12), 1315–1333 (2016).
³⁸doi:[10.1158/2159-8290.CD-16-0615](https://doi.org/10.1158/2159-8290.CD-16-0615)
- ³⁹3. Zaal, E.A., Berkens, C.R.: The Influence of Metabolism on Drug
⁴⁰Response in Cancer. *Frontiers in Oncology* **8** (2018).
⁴¹doi:[10.3389/fonc.2018.00500](https://doi.org/10.3389/fonc.2018.00500)
- ⁴²4. Faubert, B., Solmonson, A., DeBerardinis, R.J.: Metabolic
⁴³reprogramming and cancer progression. *Science* **368**(6487) (2020).
⁴⁴doi:[10.1126/science.aaw5473](https://doi.org/10.1126/science.aaw5473)
- ⁴⁵5. Agren, R., Bordel, S., Mardinoglu, A., Pornputtapong, N., Nookaew,
⁴⁶I., Nielsen, J.: Reconstruction of Genome-Scale Active Metabolic
⁴⁷Networks for 69 Human Cell Types and 16 Cancer Types Using INIT.
⁴⁸*PLOS Computational Biology* **8**(5), 1002518 (2012).
⁴⁹doi:[10.1371/journal.pcbi.1002518](https://doi.org/10.1371/journal.pcbi.1002518)
- ⁵⁰6. Nilsson, A., Nielsen, J.: Genome scale metabolic modeling of cancer.
⁵¹*Metabolic Engineering* **43**, 103–112 (2017).
⁵²doi:[10.1016/j.ymben.2016.10.022](https://doi.org/10.1016/j.ymben.2016.10.022)
- ⁵³7. Folger, O., Jerby, L., Frezza, C., Gottlieb, E., Ruppin, E., Shlomi, T.:
⁵⁴Predicting selective drug targets in cancer through metabolic networks.
⁵⁵*Molecular Systems Biology* **7**(1), 501 (2011). doi:[10.1038/msb.2011.35](https://doi.org/10.1038/msb.2011.35)

- ¹9. Gatto, F., Miess, H., Schulze, A., Nielsen, J.: Flux balance analysis
²predicts essential genes in clear cell renal cell carcinoma metabolism.
³*Scientific Reports* **5**(1), 10738 (2015). doi:[10.1038/srep10738](https://doi.org/10.1038/srep10738)
- ⁴10. Jerby, L., Ruppin, E.: Predicting Drug Targets and Biomarkers of
⁵Cancer via Genome-Scale Metabolic Modeling. *Clinical Cancer*
⁶*Research* **18**(20), 5572–5584 (2012).
⁷doi:[10.1158/1078-0432.CCR-12-1856](https://doi.org/10.1158/1078-0432.CCR-12-1856)
- ⁸11. Zhang, C., Aldrees, M., Arif, M., Li, X., Mardinoglu, A., Aziz, M.A.:
⁹Elucidating the Reprogramming of Colorectal Cancer Metabolism Using
¹⁰Genome-Scale Metabolic Modeling. *Frontiers in Oncology* **9** (2019).
¹¹doi:[10.3389/fonc.2019.00681](https://doi.org/10.3389/fonc.2019.00681)
- ¹²12. Dallas, N.A., Xia, L., Fan, F., Gray, M.J., Gaur, P., Buren, G.v.,
¹³Samuel, S., Kim, M.P., Lim, S.J., Ellis, L.M.: Chemoresistant
¹⁴Colorectal Cancer Cells, the Cancer Stem Cell Phenotype, and
¹⁵Increased Sensitivity to Insulin-like Growth Factor-I Receptor
¹⁶Inhibition. *Cancer Research* **69**(5), 1951–1957 (2009).
¹⁷doi:[10.1158/0008-5472.CAN-08-2023](https://doi.org/10.1158/0008-5472.CAN-08-2023)
- ¹⁸13. Hu, T., Li, Z., Gao, C.-Y., Cho, C.H.: Mechanisms of drug resistance
¹⁹in colon cancer and its therapeutic strategies. *World Journal of*
²⁰*Gastroenterology* **22**(30), 6876–6889 (2016).
²¹doi:[10.3748/wjg.v22.i30.6876](https://doi.org/10.3748/wjg.v22.i30.6876)
- ²²14. Stein, A., Atanackovic, D., Bokemeyer, C.: Current standards and new
²³trends in the primary treatment of colorectal cancer. *European Journal*
²⁴*of Cancer* **47**, 312–314 (2011). doi:[10.1016/S0959-8049\(11\)70183-6](https://doi.org/10.1016/S0959-8049(11)70183-6)
- ²⁵15. Vasan, N., Baselga, J., Hyman, D.M.: A view on drug resistance in
²⁶cancer. *Nature* **575**(7782), 299–309 (2019).
²⁷doi:[10.1038/s41586-019-1730-1](https://doi.org/10.1038/s41586-019-1730-1)
- ²⁸16. Gill, S., Thomas, R.R., Goldberg, R.M.: Colorectal cancer
²⁹chemotherapy. *Alimentary Pharmacology & Therapeutics* **18**(7),
³⁰683–692 (2003). doi:[10.1046/j.1365-2036.2003.01735.x](https://doi.org/10.1046/j.1365-2036.2003.01735.x)
- ³¹17. Anthony, E.J., Bolitho, E.M., Bridgewater, H.E., Carter, O.W.L.,
³²Donnelly, J.M., Imberti, C., Lant, E.C., Lemyte, F., Needham, R.J.,
³³Palau, M., Sadler, P.J., Shi, H., Wang, F.-X., Zhang, W.-Y., Zhang,
³⁴Z.: Metalloids are unique: opportunities and challenges of discovery
³⁵and development. *Chemical Science* **11**(48), 12888–12917 (2020).
³⁶doi:[10.1039/D0SC04082G](https://doi.org/10.1039/D0SC04082G)
- ³⁷18. Savvas, P., Efrosini, K., Athanasios, S.: Metalloids in Targeted
³⁸Cancer Therapeutics: Aiming at Chemoresistance- related Patterns and
³⁹Immunosuppressive Tumor Networks. *Current Medicinal Chemistry*
⁴⁰**26**(4), 607–623 (2019)
- ⁴¹19. Sung, H., Ferlay, J., Siegel, R.L., Laversanne, M., Soerjomataram, I.,
⁴²Jemal, A., Bray, F.: Global cancer statistics 2020: GLOBOCAN
⁴³estimates of incidence and mortality worldwide for 36 cancers in 185
⁴⁴countries. *CA: A Cancer Journal for Clinicians* (2020).
⁴⁵doi:[10.3322/caac.21660](https://doi.org/10.3322/caac.21660)
- ⁴⁶20. Johnstone, T.C., Park, G.Y., Lippard, S.J.: Understanding and
⁴⁷Improving Platinum Anticancer Drugs – Phenanthriplatin. *Anticancer*
⁴⁸*research* **34**(1), 471–476 (2014)
- ⁴⁹21. Zhou, J., Kang, Y., Chen, L., Wang, H., Liu, J., Zeng, S., Yu, L.: The
⁵⁰Drug-Resistance Mechanisms of Five Platinum-Based Antitumor
⁵¹Agents. *Frontiers in Pharmacology* **11** (2020).
⁵²doi:[10.3389/fphar.2020.00343](https://doi.org/10.3389/fphar.2020.00343)
- ⁵³22. Wang, Y., Lu, J.-H., Wang, F., Wang, Y.-N., He, M.-M., Wu, Q.-N.,
⁵⁴Lu, Y.-X., Yu, H.-E., Chen, Z.-H., Zhao, Q., Liu, J., Chen, Y.-X.,
⁵⁵Wang, D.-S., Sheng, H., Liu, Z.-X., Zeng, Z.-L., Xu, R.-H., Ju, H.-Q.:
⁵⁶Inhibition of fatty acid catabolism augments the efficacy of
⁵⁷oxaliplatin-based chemotherapy in gastrointestinal cancers. *Cancer*
⁵⁸*Letters* **473**, 74–89 (2020). doi:[10.1016/j.canlet.2019.12.036](https://doi.org/10.1016/j.canlet.2019.12.036)
- ⁵⁹23. Burris, H.A., Bakewell, S., Bendell, J.C., Infante, J., Jones, S.F.,
⁶⁰Spigel, D.R., Weiss, G.J., Ramanathan, R.K., Ogden, A., Hoff, D.V.:
⁶¹Safety and activity of IT-139, a ruthenium-based compound, in
⁶²patients with advanced solid tumours: a first-in-human, open-label,
⁶³dose-escalation phase I study with expansion cohort. *ESMO Open* **1**(6)
⁶⁴(2016). doi:[10.1136/esmoopen-2016-000154](https://doi.org/10.1136/esmoopen-2016-000154)
- ⁶⁵24. Trondl, R., Heffeter, P., Kowol, C.R., Jakupec, M.A., Berger, W.,
⁶⁶Keppler, B.K.: NKP-1339, the first ruthenium-based anticancer drug
⁶⁷on the edge to clinical application. *Chemical Science* **5**(8), 2925–2932
⁶⁸(2014). doi:[10.1039/C3SC53243G](https://doi.org/10.1039/C3SC53243G)
- ⁶⁹25. Meier-Menches, S.M., Gerner, C., Berger, W., Hartinger, C.G.,
⁷⁰Keppler, B.K.: Structure–activity relationships for ruthenium and
⁷¹osmium anticancer agents – towards clinical development. *Chemical*

- 1 Society Reviews **47**(3), 909–928 (2018). doi:[10.1039/C7CS00332C](https://doi.org/10.1039/C7CS00332C)
26. Jang, C., Chen, L., Rabinowitz, J.D.: Metabolomics and Isotope
2 Tracing. *Cell* **173**(4), 822–837 (2018). doi:[10.1016/j.cell.2018.03.055](https://doi.org/10.1016/j.cell.2018.03.055)
- 3 27. Armitage, E.G., Godzien, J., Peña, I., López-González, Á., Angulo,
4 S., Gradillas, A., Alonso-Herranz, V., Martín, J., Fiandor, J.M.,
5 Barrett, M.P., Gabarro, R., Barbas, C.: Metabolic Clustering Analysis
6 as a Strategy for Compound Selection in the Drug Discovery Pipeline
7 for Leishmaniasis. *ACS Chemical Biology* **13**(5), 1361–1369 (2018).
8 doi:[10.1021/acschembio.8b00204](https://doi.org/10.1021/acschembio.8b00204)
- 9 28. Armitage, E.G., Southam, A.D.: Monitoring cancer prognosis,
10 diagnosis and treatment efficacy using metabolomics and lipidomics.
11 *Metabolomics* **12**(9), 146 (2016). doi:[10.1007/s11306-016-1093-7](https://doi.org/10.1007/s11306-016-1093-7)
- 12 29. Zamboni, N., Saghatelian, A., Patti, G.J.: Defining the Metabolome:
13 Size, Flux, and Regulation. *Molecular Cell* **58**(4), 699–706 (2015).
14 doi:[10.1016/j.molcel.2015.04.021](https://doi.org/10.1016/j.molcel.2015.04.021)
- 15 30. Buescher, J.M., Antoniewicz, M.R., Boros, L.G., Burgess, S.C.,
16 Brunengraber, H., Clish, C.B., DeBerardinis, R.J., Feron, O., Frezza,
17 C., Ghesquiere, B., Gottlieb, E., Hiller, K., Jones, R.G., Kamphorst,
18 J.J., Kibbey, R.G., Kimmelman, A.C., Locasale, J.W., Lunt, S.Y.,
19 Maddocks, O.D., Malloy, C., Metallo, C.M., Meillet, E.J., Munger, J.,
20 Nöh, K., Rabinowitz, J.D., Ralser, M., Sauer, U., Stephanopoulos, G.,
21 St-Pierre, J., Tennant, D.A., Wittmann, C., Vander Heiden, M.G.,
22 Vazquez, A., Voudsen, K., Young, J.D., Zamboni, N., Fendt, S.-M.: A
23 roadmap for interpreting 13C metabolite labeling patterns from cells.
24 *Current Opinion in Biotechnology* **34**, 189–201 (2015).
25 doi:[10.1016/j.copbio.2015.02.003](https://doi.org/10.1016/j.copbio.2015.02.003)
- 26 31. Eicher, T., Kinnebrew, G., Patt, A., Spencer, K., Ying, K., Ma, Q.,
27 Machiraju, R., Mathé, E.A.: Metabolomics and Multi-Omics
28 Integration: A Survey of Computational Methods and Resources.
29 *Metabolites* **10**(5), 202 (2020). doi:[10.3390/metabo10050202](https://doi.org/10.3390/metabo10050202)
- 30 32. Meng, C., Kuster, B., Culhane, A.C., Gholami, A.M.: A multivariate
31 approach to the integration of multi-omics datasets. *BMC*
32 *Bioinformatics* **15**(1), 162 (2014). doi:[10.1186/1471-2105-15-162](https://doi.org/10.1186/1471-2105-15-162)
- 33 33. Rampler, E., Abiead, Y.E., Schoeny, H., Ruzs, M., Hildebrand, F.,
34 Fitz, V., Koellensperger, G.: Recurrent Topics in Mass
35 Spectrometry-Based Metabolomics and Lipidomics—Standardization,
36 Coverage, and Throughput. *Analytical Chemistry* **93**(1), 519–545
37 (2021). doi:[10.1021/acs.analchem.0c04698](https://doi.org/10.1021/acs.analchem.0c04698)
- 38 34. Chen, Y.-X., Chen, H., Rong, Y., Jiang, F., Chen, J.-B., Duan, Y.-Y.,
39 Zhu, D.-L., Yang, T.-L., Dai, Z., Dong, S.-S., Guo, Y.: An integrative
40 multi-omics network-based approach identifies key regulators for breast
41 cancer. *Computational and Structural Biotechnology Journal* **18**,
42 2826–2835 (2020). doi:[10.1016/j.csbj.2020.10.001](https://doi.org/10.1016/j.csbj.2020.10.001)
- 43 35. Ghaffari, S., Hanson, C., Schmidt, R.E., Bouchonville, K.J., Offer,
44 S.M., Sinha, S.: An integrated multi-omics approach to identify
45 regulatory mechanisms in cancer metastatic processes. *Genome*
46 *Biology* **22**(1), 19 (2021). doi:[10.1186/s13059-020-02213-x](https://doi.org/10.1186/s13059-020-02213-x)
- 47 36. Bordbar, A., Monk, J.M., King, Z.A., Palsson, B.O.: Constraint-based
48 models predict metabolic and associated cellular functions. *Nature*
49 *Reviews Genetics* **15**(2), 107–120 (2014). doi:[10.1038/nrg3643](https://doi.org/10.1038/nrg3643)
- 50 37. Pandey, V., Hadadi, N., Hatzimanikatis, V.: Enhanced flux prediction
51 by integrating relative expression and relative metabolite abundance
52 into thermodynamically consistent metabolic models. *PLOS*
53 *Computational Biology* **15**(5), 1007036 (2019).
54 doi:[10.1371/journal.pcbi.1007036](https://doi.org/10.1371/journal.pcbi.1007036)
- 55 38. Yizhak, K., Chaneton, B., Gottlieb, E., Rupp, E.: Modeling cancer
metabolism on a genome scale. *Molecular Systems Biology* **11**(6), 817
(2015). doi:[10.15252/msb.20145307](https://doi.org/10.15252/msb.20145307)
39. Yizhak, K., Benyamini, T., Liebermeister, W., Rupp, E., Shlomi, T.:
Integrating quantitative proteomics and metabolomics with a
genome-scale metabolic network model. *Bioinformatics* **26**(12),
255–260 (2010). doi:[10.1093/bioinformatics/btq183](https://doi.org/10.1093/bioinformatics/btq183)
40. Zur, H., Rupp, E., Shlomi, T.: iMAT: an integrative metabolic
analysis tool. *Bioinformatics* **26**(24), 3140–3142 (2010).
doi:[10.1093/bioinformatics/btq602](https://doi.org/10.1093/bioinformatics/btq602)
41. Agren, R., Mardinoglu, A., Asplund, A., Kampf, C., Uhlen, M., Nielsen,
J.: Identification of anticancer drugs for hepatocellular carcinoma
through personalized genome-scale metabolic modeling. *Molecular*
Systems Biology **10**(3), 721 (2014). doi:[10.1002/msb.145122](https://doi.org/10.1002/msb.145122)
42. Lewis, N.E., Nagarajan, H., Palsson, B.O.: Constraining the metabolic
genotype–phenotype relationship using a phylogeny of in silico
methods. *Nature Reviews Microbiology* **10**(4), 291–305 (2012).
doi:[10.1038/nrmicro2737](https://doi.org/10.1038/nrmicro2737)
43. O'Brien, E.J., Lerman, J.A., Chang, R.L., Hyduke, D.R., Palsson, B.:
Genome-scale models of metabolism and gene expression extend and
refine growth phenotype prediction. *Molecular Systems Biology* **9**(1),
693 (2013). doi:[10.1038/msb.2013.52](https://doi.org/10.1038/msb.2013.52)
44. Orth, J.D., Thiele, I., Palsson, B.Ø.: What is flux balance analysis?
Nature Biotechnology **28**(3), 245–248 (2010). doi:[10.1038/nbt.1614](https://doi.org/10.1038/nbt.1614)
45. Henry, C.S., Broadbelt, L.J., Hatzimanikatis, V.:
Thermodynamics-Based Metabolic Flux Analysis. *Biophysical Journal*
92(5), 1792–1805 (2007). doi:[10.1529/biophysj.106.093138](https://doi.org/10.1529/biophysj.106.093138)
46. Salvy, P., Fengos, G., Ataman, M., Pathier, T., Soh, K.C.,
Hatzimanikatis, V.: pyTFA and matTFA: A Python package and a
Matlab toolbox for Thermodynamics-based Flux Analysis.
Bioinformatics **35**(1), 167–169 (2019).
doi:[10.1093/bioinformatics/bty499](https://doi.org/10.1093/bioinformatics/bty499)
47. Aurich, M.K., Paglia, G., Rolfsson, Ó., Hrafnisdóttir, S., Magnúsdóttir,
M., Stefaniak, M.M., Palsson, B.Ø., Fleming, R.M.T., Thiele, I.:
Prediction of intracellular metabolic states from extracellular
metabolomic data. *Metabolomics* **11**(3), 603–619 (2015).
doi:[10.1007/s11306-014-0721-3](https://doi.org/10.1007/s11306-014-0721-3)
48. Masid, M., Ataman, M., Hatzimanikatis, V.: Analysis of human
metabolism by reducing the complexity of the genome-scale models
using redHUMAN. *Nature Communications* **11**(1), 2821 (2020).
doi:[10.1038/s41467-020-16549-2](https://doi.org/10.1038/s41467-020-16549-2)
49. Volkova, S., Matos, M.R.A., Mattanovich, M., Marín de Mas, I.:
Metabolic Modelling as a Framework for Metabolomics Data
Integration and Analysis. *Metabolites* **10**(8), 303 (2020).
doi:[10.3390/metabo10080303](https://doi.org/10.3390/metabo10080303)
50. Zielinski, D.C., Jamshidi, N., Corbett, A.J., Bordbar, A., Thomas, A.,
Palsson, B.Ø.: Systems biology analysis of drivers underlying hallmarks
of cancer cell metabolism. *Scientific Reports* **7**(1), 41241 (2017).
doi:[10.1038/srep41241](https://doi.org/10.1038/srep41241)
51. Jungwirth, U., Xanthos, D.N., Gojo, J., Bytze, A.K., Körner, W.,
Heffeter, P., Abramkin, S.A., Jakupc, M.A., Hartinger, C.G.,
Windberger, U., *et al.*: Anticancer activity of methyl-substituted
oxalipatin analogs. *Molecular pharmacology* **81**(5), 719–728 (2012)
52. Galvez, L., Ruzs, M., Schwaiger-Haber, M., Abiead, Y.E., Hermann,
G., Jungwirth, U., Berger, W., Keppler, B.K., Jakupc, M.A.,
Koellensperger, G.: Preclinical studies on metal based anticancer drugs
as enabled by integrated metallomics and metabolomics. *Metallomics*
11(10), 1716–1728 (2019). doi:[10.1039/C9MT00141G](https://doi.org/10.1039/C9MT00141G)
53. Ruzs, M., Del Favero, G., El Abiead, Y., Gerner, C., Keppler, B.K.,
Jakupc, M.A., Koellensperger, G.: Morpho-metabotyping the
oxidative stress response. *Scientific Reports*, (2021)
54. Schwaiger, M., Schoeny, H., Abiead, Y.E., Hermann, G., Rampler, E.,
Koellensperger, G.: Merging metabolomics and lipidomics into one
analytical run. *Analyst* **144**(1), 220–229 (2018).
doi:[10.1039/C8AN01219A](https://doi.org/10.1039/C8AN01219A)
55. Oberhuber, M., Pecoraro, M., Ruzs, M., Oberhuber, G., Wieselberg,
M., Haslinger, P., Gurnhofer, E., Schleider, M., Limberger, T.,
Lagger, S., Pencik, J., Kodajova, P., Högl, S., Stockmaier, G.,
Grund-Gröschke, S., Aberger, F., Bolis, M., Theurillat, J.-P.,
Wiebringhaus, R., Weiss, T., Haitel, A., Brehme, M., Wadsak, W.,
Griss, J., Mohr, T., Hofer, A., Jäger, A., Pollheimer, J., Egger, G.,
Koellensperger, G., Mann, M., Hantusch, B., Kenner, L.:
STAT3-dependent analysis reveals PDK4 as independent predictor of
recurrence in prostate cancer. *Molecular Systems Biology* **16**(4), 9247
(2020). doi:[10.15252/msb.20199247](https://doi.org/10.15252/msb.20199247)
56. Szélliová, D., Schoeny, H., Knez, v., Troyer, C., Coman, C., Rampler,
E., Koellensperger, G., Ahrends, R., Hann, S., Borth, N., Zanghellini,
J., Ruckerbauer, D.E.: Robust Analytical Methods for the Accurate
Quantification of the Total Biomass Composition of Mammalian Cells.
In: Nagrath, D. (ed.) *Metabolic Flux Analysis in Eukaryotic Cells:*
Methods And Protocols. Methods in Molecular Biology, pp. 119–160.
Springer, New York, NY (2020). doi:[10.1007/978-1-0716-0159-4_7](https://doi.org/10.1007/978-1-0716-0159-4_7)
57. Robinson, J.L., Kocabaş, P., Wang, H., Cholley, P.-E., Cook, D.,
Nilsson, A., Anton, M., Ferreira, R., Domenzain, I., Billa, V., Limeta,
A., Hedin, A., Gustafsson, J., Kerkhoven, E.J., Svensson, L.T.,
Palsson, B.Ø., Mardinoglu, A., Hansson, L., Uhlén, M., Nielsen, J.: An
atlas of human metabolism. *Science Signaling* **13**(624) (2020).

- doi:[10.1126/scisignal.aaz1482](https://doi.org/10.1126/scisignal.aaz1482)
58. Hart, T., Chandrashekar, M., Aregger, M., Steinhart, Z., Brown, K.R., MacLeod, G., Mis, M., Zimmermann, M., Fradet-Turcotte, A., Sun, S., Mero, P., Dirks, P., Sidhu, S., Roth, F.P., Rissland, O.S., Durocher, D., Angers, S., Moffat, J.: High-Resolution CRISPR Screens Reveal Fitness Genes and Genotype-Specific Cancer Liabilities. *Cell* **163**(6), 1515–1526 (2015). doi:[10.1016/j.cell.2015.11.015](https://doi.org/10.1016/j.cell.2015.11.015)
59. Széllővá, D., Ruckerbauer, D.E., Galleguillos, S.N., Petersen, L.B., Natter, K., Hanscho, M., Troyer, C., Causon, T., Schoeny, H., Christensen, H.B., Lee, D.-Y., Lewis, N.E., Koellensperger, G., Hann, S., Nielsen, L.K., Borth, N., Zanghellini, J.: What CHO is made of: Variations in the biomass composition of Chinese hamster ovary cell lines. *Metabolic Engineering* **61**, 288–300 (2020). doi:[10.1016/j.ymben.2020.06.002](https://doi.org/10.1016/j.ymben.2020.06.002)
60. Jain, M., Nilsson, R., Sharma, S., Madhusudhan, N., Kitami, T., Souza, A.L., Kafri, R., Kirschner, M.W., Clish, C.B., Mootha, V.K.: Metabolite Profiling Identifies a Key Role for Glycine in Rapid Cancer Cell Proliferation. *Science* **336**(6084), 1040–1044 (2012). doi:[10.1126/science.1218595](https://doi.org/10.1126/science.1218595)
61. Else, P.L.: The highly unnatural fatty acid profile of cells in culture. *Progress in Lipid Research* **77**, 101017 (2020). doi:[10.1016/j.plipres.2019.101017](https://doi.org/10.1016/j.plipres.2019.101017)
62. Gregory, M.K., King, H.W., Bain, P.A., Gibson, R.A., Tocher, D.R., Schuller, K.A.: Development of a Fish Cell Culture Model to Investigate the Impact of Fish Oil Replacement on Lipid Peroxidation. *Lipids* **46**(8), 753–764 (2011). doi:[10.1007/s11745-011-3558-9](https://doi.org/10.1007/s11745-011-3558-9)
63. Lewis, N.E., Hixson, K.K., Conrad, T.M., Lerman, J.A., Charusanti, P., Polpitiya, A.D., Adkins, J.N., Schramm, G., Purvine, S.O., Lopez-Ferrer, D., Weitz, K.K., Eils, R., König, R., Smith, R.D., Palsson, B.Ø.: Omic data from evolved *E. coli* are consistent with computed optimal growth from genome-scale models. *Molecular Systems Biology* **6**(1), 390 (2010). doi:[10.1038/msb.2010.47](https://doi.org/10.1038/msb.2010.47)
64. Gudmundsson, S., Thiele, I.: Computationally efficient flux variability analysis. *BMC Bioinformatics* **11**(1), 489 (2010). doi:[10.1186/1471-2105-11-489](https://doi.org/10.1186/1471-2105-11-489)
65. Ebrahim, A., Lerman, J.A., Palsson, B.O., Hyduke, D.R.: Cobrapy: constraints-based reconstruction and analysis for python. *BMC systems biology* **7**(1), 1–6 (2013)
66. Warburg, O.: Über den Stoffwechsel der Carcinomzelle. *Naturwissenschaften* **12**(50), 1131–1137 (1924). doi:[10.1007/BF01504608](https://doi.org/10.1007/BF01504608)
67. Brown, R.E., Short, S.P., Williams, C.S.: Colorectal Cancer and Metabolism. *Current Colorectal Cancer Reports* **14**(6), 226–241 (2018). doi:[10.1007/s11888-018-0420-y](https://doi.org/10.1007/s11888-018-0420-y)
68. Li, T., Le, A.: Glutamine Metabolism in Cancer. In: Le, A. (ed.) *The Heterogeneity of Cancer Metabolism*. *Advances in Experimental Medicine and Biology*, pp. 13–32. Springer, ??? (2018). doi:[10.1007/978-3-319-77736-8_2](https://doi.org/10.1007/978-3-319-77736-8_2)
69. Wu, M., Neilson, A., Swift, A.L., Moran, R., Tamagnine, J., Parslow, D., Armistead, S., Lemire, K., Orrell, J., Teich, J., Chomicz, S., Ferrick, D.A.: Multiparameter metabolic analysis reveals a close link between attenuated mitochondrial bioenergetic function and enhanced glycolysis dependency in human tumor cells. *American Journal of Physiology. Cell Physiology* **292**(1), 125–136 (2007). doi:[10.1152/ajpcell.00247.2006](https://doi.org/10.1152/ajpcell.00247.2006)
70. Uhlen, M., Zhang, C., Lee, S., Sjöstedt, E., Fagerberg, L., Bidkhori, G., Benfeitas, R., Arif, M., Liu, Z., Edfors, F., Sanli, K., Feilitzén, K.v., Oksvold, P., Lundberg, E., Hober, S., Nilsson, P., Mattsson, J., Schwenk, J.M., Brunnström, H., Glimelius, B., Sjöblom, T., Edqvist, P.-H., Djureinovic, D., Micke, P., Lindskog, C., Mardinoglu, A., Ponten, F.: A pathology atlas of the human cancer transcriptome. *Science* **357**(6352) (2017). doi:[10.1126/science.aan2507](https://doi.org/10.1126/science.aan2507)
71. Balcells, C., Foguet, C., Tarragó-Celada, J., de Atauri, P., Marin, S., Cascante, M.: Tracing metabolic fluxes using mass spectrometry: Stable isotope-resolved metabolomics in health and disease. *TrAC Trends in Analytical Chemistry* **120**, 115371 (2019). doi:[10.1016/j.trac.2018.12.025](https://doi.org/10.1016/j.trac.2018.12.025)
72. Yao, C.-H., Fowle-Grider, R., Mahieu, N.G., Liu, G.-Y., Chen, Y.-J., Wang, R., Singh, M., Potter, G.S., Gross, R.W., Schaefer, J., Johnson, S.L., Patti, G.J.: Exogenous Fatty Acids Are the Preferred Source of Membrane Lipids in Proliferating Fibroblasts. *Cell Chemical Biology* **23**(4), 483–493 (2016). doi:[10.1016/j.chembiol.2016.03.007](https://doi.org/10.1016/j.chembiol.2016.03.007)
73. Yao, C.-H., Liu, G.-Y., Yang, K., Gross, R.W., Patti, G.J.: Inaccurate quantitation of palmitate in metabolomics and isotope tracer studies due to plastics. *Metabolomics* **12**(9), 1–7 (2016)
74. Hasenour, C.M., Rahim, M., Young, J.D.: In Vivo Estimates of Liver Metabolic Flux Assessed by ¹³C-Propionate and ¹³C-Lactate Are Impacted by Tracer Recycling and Equilibrium Assumptions. *Cell Reports* **32**(5) (2020). doi:[10.1016/j.celrep.2020.107986](https://doi.org/10.1016/j.celrep.2020.107986)
75. Williams, T.C.R., Miguet, L., Masakapalli, S.K., Kruger, N.J., Sweetlove, L.J., Ratcliffe, R.G.: Metabolic Network Fluxes in Heterotrophic Arabidopsis Cells: Stability of the Flux Distribution under Different Oxygenation Conditions. *Plant Physiology* **148**(2), 704–718 (2008). doi:[10.1104/pp.108.125195](https://doi.org/10.1104/pp.108.125195)
76. Wu, Y., Li, L.: Sample normalization methods in quantitative metabolomics. *Journal of Chromatography A* **1430**, 80–95 (2016). doi:[10.1016/j.chroma.2015.12.007](https://doi.org/10.1016/j.chroma.2015.12.007)
77. Chan, S., Cai, J., Wang, L., Simons-Senftle, M., Maranas, C.: Standardizing biomass reactions and ensuring complete mass balance in genome-scale metabolic models. *Bioinformatics* **33**(22), 3603–3609 (2017). doi:[10.1093/bioinformatics/btx453](https://doi.org/10.1093/bioinformatics/btx453)
78. Pereira, R., Nielsen, J., Rocha, I.: Improving the flux distributions simulated with genome-scale metabolic models of *saccharomyces cerevisiae*. *Metabolic Engineering Communications* **3**(1), 153–163 (2016). doi:[10.1016/j.meten.2016.05.002](https://doi.org/10.1016/j.meten.2016.05.002)
79. Yuan, H., Cheung, M., Hilbers, P., van Riel, N.: Flux balance analysis of plant metabolism: The effect of biomass composition and model structure on model predictions. *Frontiers in Plant Sciences* **7**(1), 537 (2016). doi:[10.3389/fpls.2016.00537](https://doi.org/10.3389/fpls.2016.00537)
80. Dubuis, S., Ortmayr, K., Zampieri, M.: A framework for large-scale metabolome drug profiling links coenzyme a metabolism to the toxicity of anti-cancer drug dichloroacetate. *Communications biology* **1**(1), 1–11 (2018)
81. Ortmayr, K., Dubuis, S., Zampieri, M.: Metabolic profiling of cancer cells reveals genome-wide crosstalk between transcriptional regulators and metabolism. *Nature communications* **10**(1), 1–13 (2019)
82. Nam, H., Campodonico, M., Bordbar, A., Hyduke, D., Kim, S., Zielinski, D., Palsson, B.: A systems approach to predict oncometabolites via context-specific genome-scale metabolic networks. *PLOS Computational Biology* **10**(9), 1003837 (2014). doi:[10.1371/journal.pcbi.1003837](https://doi.org/10.1371/journal.pcbi.1003837)
83. Turanli, B., Zhang, C., Kim, W., Benfeitas, R., Uhlen, M., Arga, K., Mardinoglu, A.: Discovery of therapeutic agents for prostate cancer using genome-scale metabolic modeling and drug repositioning. *EBioMedicine* **42**, 386–396 (2019). doi:[10.1016/j.ebiom.2019.03.009](https://doi.org/10.1016/j.ebiom.2019.03.009)
84. McGuirk, S., Audet-Delage, Y., St-Pierre, J.: Metabolic Fitness and Plasticity in Cancer Progression. *Trends in Cancer* **6**(1), 49–61 (2020). doi:[10.1016/j.trecan.2019.11.009](https://doi.org/10.1016/j.trecan.2019.11.009)
85. Lee, S., Jang, W.-J., Choi, B., Joo, S.H., Jeong, C.-H.: Comparative metabolomic analysis of HPAC cells following the acquisition of erlotinib resistance. *Oncology Letters* **13**(5), 3437–3444 (2017). doi:[10.3892/ol.2017.5940](https://doi.org/10.3892/ol.2017.5940)
86. Fan, T.W.-M., El-Amouri, S.S., Macedo, J.K.A., Wang, Q.J., Song, H., Cassel, T., Lane, A.N.: Stable Isotope-Resolved Metabolomics Shows Metabolic Resistance to Anti-Cancer Selenite in 3D Spheroids versus 2D Cell Cultures. *Metabolites* **8**(3), 40 (2018). doi:[10.3390/metabo8030040](https://doi.org/10.3390/metabo8030040)
87. Ricci, F., Brunelli, L., Affatato, R., Chilà, R., Verza, M., Indraccolo, S., Falcetta, F., Fratelli, M., Fruscio, R., Pastorelli, R., Damia, G.: Overcoming platinum-acquired resistance in ovarian cancer patient-derived xenografts. *Therapeutic Advances in Medical Oncology* **11**, 1758835919839543 (2019). doi:[10.1177/1758835919839543](https://doi.org/10.1177/1758835919839543)
88. Rusz, M., Rampler, E., Keppler, B.K., Jakupc, M.A., Koellensperger, G.: Single Spheroid Metabolomics: Optimizing Sample Preparation of Three-Dimensional Multicellular Tumor Spheroids. *Metabolites* **9**(12), 304 (2019). doi:[10.3390/metabo9120304](https://doi.org/10.3390/metabo9120304)
89. Cavill, R., Kamburov, A., Ellis, J.K., Athersuch, T.J., Blagrove, M.S.C., Herwig, R., Ebbels, T.M.D., Keun, H.C.: Consensus-Phenotype Integration of Transcriptomic and Metabolomic Data Implies a Role for Metabolism in the Chemosensitivity of Tumour Cells. *PLOS Computational Biology* **7**(3), 1001113 (2011). doi:[10.1371/journal.pcbi.1001113](https://doi.org/10.1371/journal.pcbi.1001113)

- 1 90. Jungwirth, U., Kowol, C.R., Keppler, B.K., Hartinger, C.G., Berger,
2 W., Heffeter, P.: Anticancer Activity of Metal Complexes: Involvement
3 of Redox Processes. *Antioxidants & Redox Signaling* **15**(4), 1085–1127
4 (2011). doi:[10.1089/ars.2010.3663](https://doi.org/10.1089/ars.2010.3663)
- 5 91. Gibson, D.: The mechanism of action of platinum anticancer
6 agents—what do we really know about it? *Dalton Transactions* (48),
7 10681–10689 (2009). doi:[10.1039/B918871C](https://doi.org/10.1039/B918871C)
- 8 92. Kelland, L.: The resurgence of platinum-based cancer chemotherapy.
9 *Nature Reviews Cancer* **7**(8), 573–584 (2007). doi:[10.1038/nrc2167](https://doi.org/10.1038/nrc2167)
- 10 93. Lizardo, M.M., Morrow, J.J., Miller, T.E., Hong, E.S., Ren, L.,
11 Mendoza, A., Halsey, C.H., Scacheri, P.C., Helman, L.J., Khanna, C.:
12 Upregulation of Glucose-Regulated Protein 78 in Metastatic Cancer
13 Cells Is Necessary for Lung Metastasis Progression. *Neoplasia* **18**(11),
14 699–710 (2016). doi:[10.1016/j.neo.2016.09.001](https://doi.org/10.1016/j.neo.2016.09.001)
- 15 94. Gottesman, M.M., Fojo, T., Bates, S.E.: Multidrug resistance in
16 cancer: role of atp-dependent transporters. *Nature Reviews Cancer*
17 **2**(1), 48–58 (2002)
- 18 95. Drury, J., Rychahou, P.G., He, D., Jafari, N., Wang, C., Lee, E.Y.,
19 Weiss, H.L., Evers, B.M., Zaytseva, Y.Y.: Inhibition of Fatty Acid
20 Synthase Upregulates Expression of CD36 to Sustain Proliferation of
21 Colorectal Cancer Cells. *Frontiers in Oncology* **10** (2020).
22 doi:[10.3389/fonc.2020.01185](https://doi.org/10.3389/fonc.2020.01185)
- 23 96. Valli, A., Rodríguez, M., Moutsianas, L., Fischer, R., Fedele, V.,
24 Huang, H.-L., Stiphout, R.V., Jones, D., McCarthy, M., Vinaxia, M.,
25 Igarashi, K., Sato, M., Soga, T., Buffa, F., Mccullagh, J., Yanes, O.,
26 Harris, A., Kessler, B.: Hypoxia induces a lipogenic cancer cell
27 phenotype via HIF1 α -dependent and -independent pathways.
28 *Oncotarget* **6**, 1920–1941 (2014). doi:[10.18632/oncotarget.3058](https://doi.org/10.18632/oncotarget.3058)
- 29 97. Haug, K., Cochrane, K., Nainala, V.C., Williams, M., Chang, J.,
30 Jayaseelan, K.V., O'Donovan, C.: MetaboLights: a resource evolving in
31 response to the needs of its scientific community. *Nucleic Acids*
32 *Research* **48**(D1), 440–444 (2020). doi:[10.1093/nar/gkz1019](https://doi.org/10.1093/nar/gkz1019)

Additional Files

- 33 Additional file 1 — supplementary material
- 34 Pdf of the supplementary material containing Figure [S1](#) to [S6](#).