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1 # Rational cell culture optimization
2 # enhances experimental reproducibility in
3 # cancer cells

4

5 Marina Wright Muelas[1,2,6]*, Fernando Ortega[5], Rainer Breitling[6], Claus Bendtsen[2], Hans V. Westerhoff[1,4,6]
6
7 [1]Manchester Centre for Integrative Systems Biology and Doctoral Training Centre, Manchester Institute of Biotechnology, University of
8 Manchester, 131 Princess Street, Manchester M1 7DN, UK
9 [2]Quantitative Biology, Discovery Sciences, AstraZeneca R&D, Cambridge Science Park, Cambridge, Cambs, CB4 0WG, UK
10 [4]Netherlands Institute for Systems Biology, VU University Amsterdam and University of Amsterdam, The Netherlands
11 [5]Manchester Pharmacy School, University of Manchester, Stopford Building, Oxford Road, Manchester, M13 9PT, UK
12 [6]Manchester Institute of Biotechnology, University of Manchester, 131 Princess Street, Manchester M1 7DN, UK
13 *Corresponding author: Marina Wright Muelas (marina.wrightmuelas@manchester.ac.uk)

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14

15 **Abstract**

16

17 Optimization of experimental conditions is critical in ensuring robust experimental
18 reproducibility. Through detailed metabolomic analysis we found that cell culture conditions
19 significantly impacted on glutaminase (GLS1) sensitivity resulting in variable sensitivity and
20 irreproducibility in data.
21 Baseline metabolite profiling highlighted that untreated cells underwent significant changes
22 in metabolic status. Both the extracellular levels of glutamine and lactate and the intracellular
23 levels of multiple metabolites changed drastically during the assay. We show that these
24 changes compromise the robustness of the assay and make it difficult to reproduce.
25
26 We then devised 'metabolically rationalized standard' assay conditions, in which
27 glutaminase-1 inhibition reduced glutamine metabolism differently in both cell lines assayed,
28 and decreased the proliferation of one of them. The adoption of optimized conditions such
29 as the ones described here should lead to an improvement in reproducibility and help
30 eliminate false negatives as well as false positives in these assays.
31

32 **Keywords:** Cell culture, metabolomics, cancer cell lines, non-small cell lung cancer, drug discovery.

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1 Introduction

Reproducibility has increasingly become a topic of concern in biomedical research[1,2]. Scientists acknowledge that they fail to reproduce even their own experiments, let alone those of their colleagues around the globe[3]. When testing a potential anticancer drug, a novel and potent allosteric inhibitor specific for the glutaminase-1 enzyme (EC 3.5.1.2), we initially experienced a similar irreproducibility. Our focus on metabolomics led us to experiments that then produced an explanation for the lack of reproducibility, and employed a more comprehensive assay development approach which we believe can be of benefit for the scientific community.

One of the initial steps in the development of therapeutic agents for cancer involves testing these agents *in vitro* using human cancer cell lines as experimental models[4,5]. Using primary cell lines in culture, the effects of compounds or perturbations on cell proliferation, DNA replication or cell death is generally investigated over a period of time. These types of read-out are highly dependent on cell physiology and as such these assays need to fulfill a number of conflicting conditions. On the one hand, cells need to be kept in culture long enough to attain a steady state and for the effects of treatments to be observed. On the other hand, they should not be kept there too long because of the gradual accumulation of waste products that can be inhibitory or toxic to cells, such as lactate and ammonia[6]. The concentration of nutrients will fall over time, pH will change, and as cells grow and divide, space may become limiting. As cell density increases, effects of paracrine signaling become more pronounced and as cells reach confluence, contact inhibition may suppress proliferation. Although cancer cells are able to proliferate for some time after reaching confluence by then accumulating on top of one another, this crowding still limits individual cells' access to nutrients and growth factors[7], eventually resulting in cell cycle arrest and apoptosis, but long before then, in shifts in cell metabolism. Cell viability assays are affected by the metabolic state of the cells and therefore any shift in metabolic states during the assay, and particularly different shifts between sensitive and resistant cell lines, would confound the outcome of such assays.

Recently, Haibe-Kains *et al.* highlighted multiple inconsistencies between two large-scale pharmacogenomic studies, the Cancer Genome Project (CGP[8]) and the Cancer Cell Line Encyclopedia (CCLE[9]), *viz.* the sensitivity profiles of common cell lines and drugs[10]. It has been suggested that differences in the cell culture conditions were amongst the reasons for

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71 these discrepancies[11] and that consistency should be achievable with appropriate laboratory
72 and analysis protocols[12]. For example, for each cell line the CGP study determined the
73 seeding density that ensured that each was still in the growth phase at the end of the assay
74 (~70% confluence), whilst the seeding density was not reported for the CCLE study[8]. In
75 addition, for adherent cells, the test compound was added 'around 12–24 hours' after
76 seeding cells and studied over a further 72–84 hours in the CCLE study, whereas in the
77 CGP study this was added 1 day after seeding and assayed 72 hours after treatment. This
78 lack of standardized and well-described culture conditions is common to most literature in
79 this field (see refs. [13-19] for examples). Living cells are complex; they adjust to altering
80 environments, by quick metabolic or somewhat slower gene-expression regulation and this
81 may readily change the extent to which any target limits cell physiology and survival. This
82 can make results of drug targeting studies irreproducible unless the relevant environmental
83 conditions are well controlled at the appropriate time scale. Both academia and the
84 pharmaceutical industry recognize the necessity of much more thorough standardization to
85 improve reproducibility [20].
86
87 The metabolic performance of the cell lines during drug targeting assays is not assessed
88 routinely, or at least not reported. Metabolic changes could have strong implications for
89 therapeutic targets in, or affected by, intermediary metabolism. Metabolic enzymes involved
90 in cellular proliferation and growth have been identified as altered in cancers, either through
91 the expression of cancer-specific isoforms, through mutations, or through altered expression
92 levels[21]. And it is precisely these targets that are witnessing revived interest of late[22,23]: these
93 altered metabolic pathways are now being targeted directly, used to enhance the efficacy of
94 existing therapeutic agents or to overcome resistance to current treatment strategies for
95 cancer. In addition, anti-cancer drugs that do not target metabolism itself are often assayed
96 in survival based assays. If metabolism is so involved in cell survival, its variability and
97 during survival based assays could therefore be a prime cause of irreproducibility of the
98 outcome of the many experimental assays. We thus investigated whether variability in
99 cellular metabolic status is linked with different phenotypic responses.
100
101 Here we show how culture conditions widely used to investigate the effects of an inhibitor of
102 the glutaminase-1 enzyme on cell proliferation and metabolism, result in drastic and rapid
103 changes in the metabolic state of the cells, compromising the robustness and reproducibility
104 of the results. We then present the pipeline we engage in such cases in order to identify

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105 these changes and to optimize culture conditions accordingly. The reward is a robust study
106 of the effects of a potentially important anti-proliferative agent.
107
108

109 2 Results

110
111 To investigate the effect of an inhibitor (GLS1i) of the glutaminase-1 enzyme (GLS1,
112 EC 3.5.1.2), on cell metabolism and proliferation, we started by employing culture conditions
113 that are widely used in the scientific literature for proliferation assays and should enable the
114 application of metabolomics[13-19]. We seeded cells at a density of 8×10^5 cells/well in 1 mL of
115 culture media 24 hours prior to commencing the experiment at a time point denoted as time
116 0 by adding 1.0 µM of a GLS1 inhibitor (see Materials and Methods section 1.2). The effect
117 of this inhibitor on cell survival was determined 48-hours later. We used two cell lines, A549
118 and H358, that are dependent on glutamine for proliferation[24], but differ in sensitivity to a
119 novel and potent inhibitor of GLS1 activity developed jointly by AstraZeneca and Cancer
120 Research Technology: proliferation of A549 cells is inhibited by this ‘GLS1i’, whereas
121 proliferation of H358 cells is insensitive to GLS1 inhibition (Figure S1).

122 2.1 The problem: the inhibitor does not seem to work

123
124 We had expected that treatment with a GLS1 inhibitor would lead to a reduced consumption
125 of glutamine, a reduced production of glutamate, an increased intracellular concentration of
126 glutamine, a reduced intracellular concentration of glutamate and reduced intracellular
127 concentrations of all TCA cycle intermediates, particularly in the GLS1i sensitive A549 cell
128 lines. The initially observed effects of GLS1i treatment were very different to what we
129 expected (Table 1): GLS1i treatment did not affect cell numbers in either cell line when
130 compared to control treatment (Figure S2a). Equally unexpectedly, the amount of glutamine
131 consumed was reduced to a much greater extent in the resistant cell line than in the
132 sensitive cell line (Figure 1a). Intracellular glutamine concentrations were raised in treated
133 conditions in both cell lines (Figure 1b), particularly in the resistant H358 cell lines compared
134 to controls, in agreement with our expectations. However, intracellular glutamate
135 concentrations were reduced in the GLS1i resistant H358 cell lines only (Figure 1c). The
136 abundance of TCA cycle intermediates, such as alpha-ketoglutarate (α-KG), citrate and
137 fumarate, was unaffected by treatment of A549 cells with GLS1i. Only α-KG was reduced in
138 H358 cells (Figure S3).

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139
140 A first clue on what could be responsible for the lack of effect of the metabolic inhibitor on
141 the cell proliferation and the paradoxical effects on metabolism, was the extracellular
142 concentration of glutamine at the end of this assay: this was very close to undetectable
143 levels, suggesting that during the assay cells had been subject to a concentration of
144 glutamine varying between 2mM and 0 mM. In the absence of glutamine, an inhibitor of
145 glutaminase should perhaps not be expected to have any effect; either directly or due to
146 metabolic rewiring.

147 **2.2 Explanation: The cellular environment is uncontrolled**

148
149 To understand why GLS1i treatment in the above assay failed to show any significant effects
150 on proliferation or metabolism by A549 and H358 cells we examined the changes in cell
151 numbers and intracellular and extracellular metabolites with enhanced time resolution
152 (Figure 2 and Figure S4).
153
154 Our results suggest that, throughout the course of the assay, the cells' environment in
155 control conditions was changing in ways that would be expected to interfere with the cells'
156 internal state[25]. Firstly, the concentration of lactate in spent media 1 hour into the assay was
157 already above 10 mM in both cell lines (Figure 2a). H358 cells had already secreted nearly
158 20 mM of lactate by this point, suggesting that most of the glucose available in culture media
159 had already been consumed. In H358 cells this increase in lactate continued over the time
160 points sampled, but in A549 cells the concentration of lactate reached its maximum level of
161 around 14 mM 6 hours post dosing. Secondly, the concentration of glutamine in spent media
162 was already reduced by ≥ 70% 1 hour into the assay in both cell lines and undetectable by
163 24 hours and 48 hours post dose in A549 cells and H358 cells, respectively (Figure 2b).
164
165 The fluctuating environment that the cells were exposed to in this assay likely contributed to
166 the changes in the specific growth rate of these cells: a small increase in cell numbers was
167 observed over the first 24 hours post dosing (Figure 2c) but was much slower than the
168 expected growth kinetics of these two cell lines[26]. Moreover, between 24 and 48 hours post
169 dosing, the number of cells in control conditions seemingly *decreased*. This could be due to
170 the depletion of glutamine, glucose or other essential substrates not measured, to the
171 increases in the concentration of lactate, to the resulting decrease in pH, or to contact
172 inhibition of the cells. Deprivation of nutrients and growth factors has been shown to lead to
173 cell cycle arrest and subsequently cell death in NSCLC cell lines indicating that this is a

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174 possible explanation for the changes seen in cell numbers in this type of assay[27-29]. The
175 drastic reductions in glutamine could influence normal cell metabolism and physiology, as a
176 result of forcing cells to switch to alternative fuel sources and to deal with the problem of
177 ammonium toxicity[6]. Indeed, the intracellular concentration of glutamine fell drastically
178 throughout the assay, as did the abundance of other metabolites, albeit to a smaller extent
179 (Figure S4). The high concentration of secreted lactate in spent media is likely to be
180 accompanied by drastic acidification of culture media and cellular damage[30,31]; the culture
181 media had a buffering capacity of around 20 mM/pH unit, whilst some 20 mM of lactic acid
182 may have been produced, a large proportion of which was likely derived from glucose.
183
184 Our results suggest that these commonly used assay conditions are unsuitable for
185 comparing inhibitors of molecular targets with each other.
186

187 **2.3 Assay optimization: Reducing the seeding density and increasing culture**
188 **volume stabilizes cellular state**

189
190
191 Increasing the volume of culture media alone from 1 to 3 mL was not sufficient to avoid
192 these problems of variations in metabolic state (Figure S5): Whilst this reduced the
193 magnitude of changes in the extracellular concentrations of glucose and glutamine, these
194 key nutrients were still close to depletion 72 hours after seeding. pH changes remained
195 within acceptable ranges in A549 cells, but not in H358 cells where pH changed by > 1 pH
196 unit. A slight improvement in the proliferation of these two cell lines was observed but this
197 was still much slower than expected. Confluence was reached early into the assay (24–36
198 hours after seeding) when the cell lines were seeded at a density of 8×10^5 cells/well
199 (Figure 3, upper purple line). This, together with the drastic reductions in nutrient
200 concentrations through the assay, may account for the reduced rate of proliferation observed
201 in our previous assays as a result of the induction of cell cycle arrest and apoptosis[7,27-29,32].
202
203 Indeed, we observed steadier metabolic conditions and cell proliferation when the initial
204 seeding density of cells was reduced *and* the volume of culture media increased from 1 to 3
205 mL (Figure 3 and Figure S5). The period of time during which cells were able to grow
206 exponentially was also increased (Figure 3). Ensuring that confluence remained below ~ 80
207 % throughout the assay window (24–72 hours post seeding), or that this level of confluence
208 was reached as late as possible in the assay, required a significant reduction in the initial

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209 seeding density of cells, and this was cell-line specific. The time required to recover from
210 reseeding also differed between cell lines and was affected by the initial seeding density.
211 This initial lag phase was very short in duration for A549 cells (< 6 hours) compared to the
212 approximately 24 hours required by H358 cells (Figure 3), which extended beyond 24 hours
213 when H358 cells were seeded at 2×10^5 cells/well. These differences in growth kinetics
214 could well compromise inhibitor assays.
215
216 Lowering the initial seeding density of cells also reduced the magnitude of changes in the
217 concentrations of key nutrients such as glucose and glutamine (Figure S5b and c), and in pH
218 (Figure S5a) throughout the assay window (24–72 hours post seeding). In the case of the
219 H358 cell line, using these conditions, assays beyond 48 hours after seeding may not be
220 suitable since these cells displayed a high rate of glucose consumption (Figure S5b) and the
221 corresponding lactate secretion would lead to significant reductions in pH (Figure S5a).
222 When H358 cells were seeded at 3×10^5 cells/well, the concentration of glucose reached
223 limiting levels (~2 mM) 72 hours after seeding, which would constitute 48 hours post dosing
224 in an assay where treatment was applied 24 hours after seeding (Figure S5b).
225
226 We conclude that, in order to ensure that (1) cells are in exponential growth from 24 hours
227 after seeding, (2) confluence is reached as late as possible, and (3) changes in glucose,
228 glutamine and pH are kept to a minimum, the volume of culture media should be increased
229 up to 3 mL and seeding density reduced according to individual cell line growth kinetics. In
230 our case, seeding A549 cells at a density of 2×10^5 cells/well or less, and H358 cells at
231 around 3×10^5 cells/well in 3 mL of culture media, fulfills these criteria.
232

233 2.4 Optimized in vitro culture conditions enable successful hypothesis validation 234 and discovery

235
236 To validate the expected improvement in assay performance we then seeded A549 and
237 H358 cells at a density of 1.5×10^5 and 3×10^5 cells/well respectively in 3 mL of media in a
238 6 well plate format. Cells were growing exponentially at rates comparable to those reported
239 in the literature[26] (Figure S6) throughout the assay in control conditions. From plates
240 prepared in parallel, the levels of various metabolites in cell and spent media extracts as well
241 as cell numbers were measured for 24 hours after treatment with 1 µM of the GLS1 inhibitor.
242 In agreement with our expectations (Table 1), treatment with the GLS1 inhibitor over 24
243 hours led to a reduction in cell numbers of around 20% in A549 cells but not in H358 cell

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244 lines (Figure 4a). Throughout the assay, the changes in the cells' environment were now
245 minimal in both cell lines regardless of treatment conditions (Figure 4b-f): the concentrations
246 of glucose and glutamine were reduced by less than 50% over the assay and the lactate
247 secreted caused a pH drop < 1 unit under these improved assay conditions. The amount of
248 glutamine consumed appeared reduced in both cell lines by treatment with the GLS1
249 inhibitor although these changes were small and only statistically significant in A549 cells:
250 the achieved stability of culture conditions had the consequence that differences in cellular
251 metabolism were no longer strongly reflected in the changes of the exometabolome, such
252 that assay conditions were now under control and steady.
253
254 We therefore assessed intracellular metabolism to investigate whether, under the optimized
255 conditions, the predicted effects of GLS1i on intracellular metabolites were observed that
256 had not been observed under the previous unstable conditions (Table 1). Our results confirm
257 that the glutaminase inhibitor engaged with the intended target: large reductions ($p < 0.01$) in
258 glutamate were observed in both cell lines (Figure 5a). Only minor increases in the
259 concentration of glutamine were seen, probably as a result of rapid equilibration with the
260 external medium via the glutamine transporter (Figure 5b). The intracellular abundance of
261 TCA cycle intermediates was also affected by GLS1i treatment in both cell lines (Figure 5c-
262 e).
263
264 Our results confirm that the optimized culture conditions devised here provide a robust and
265 stable environment in which to reproducibly assay the effects of a GLS1 inhibitor on cell
266 metabolism and proliferation.
267

268 3 Discussion

269
270 The assay with which we started this study failed to demonstrate any consistent effects of
271 glutaminase inhibition on either glutamine metabolism or proliferation in these two cancer
272 cell lines: addition of the inhibitor to cells (A549) known to be sensitive to the inhibitor, had
273 no apparent effect on their proliferation (Figure S2a). Conversely, the glutamine metabolism
274 by cells (H358) that are *in*sensitive to the same inhibitor was reduced to a much greater
275 extent than that by sensitive (A549) cells (Figure 1). We then demonstrated that these
276 inconsistencies were artifacts, for one, because most glutamine had been depleted in the
277 pre-incubation period (Figure 1b and 2b) leaving too little glutamine for effects of the inhibitor
278 to become statistically noteworthy.

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279
280 With our improved assay conditions we were able to show that the glutaminase inhibitor
281 (GLS1i) does have an effect on glutamine metabolism of both cell lines, but that only the
282 proliferation of the A549 cells is reduced (Figure 4). GLS1 inhibition was also apparent from
283 the changes in levels of intracellular metabolites in both cell lines, but with distinct
284 differences between sensitive (A549) and resistant (H358) cell lines (Figure 5).
285
286 Our findings highlight the importance of *in vitro* assay optimization for the assessment of the
287 potential of metabolic, and probably also other, inhibitors as anti-cancer drugs that impact on
288 cellular metabolism. Variability in the metabolic state during the assay may well create false
289 positives and false negatives because intermediary and energy metabolism is full of
290 pleiotropic implications. Importantly perhaps, the implications of our findings are unlikely to
291 be limited to studies of metabolic inhibitors. Other inhibitors, such as those of cell signaling
292 or transcription require even longer cell incubations, and may therefore be compromised
293 even more by changes in the levels of metabolites such as ATP, NADH, acetyl-CoA and
294 glutamate that cross-talk widely. Even though metabolism may not be the drug target in
295 these cases, its perturbation due to inappropriate culture conditions, might produce a false
296 response. And since the drug may well affect metabolism indirectly, the impact of metabolic
297 status could be overlooked in both control and treated conditions.
298
299 Perhaps even more so than this, our results should warn against the straightforward
300 implementation of historically-fixed sets of conditions for drug assays in cell lines. Living
301 cells are complex enough to engage in all sorts of metabolic changes, these changes may
302 well differ between individual cell lines, and the metabolome is sensitive to such changes
303 well before the metabolic fluxes produced by the cells are[33]. We therefore advocate that
304 reports on drug assays are accompanied by a thorough description of the experimental
305 procedure used as well as by a metabolic characterization of the cells during the assay,
306 such as in the workflow we demonstrated here. After all, such characterization has become
307 possible over recent years.
308
309 Indeed, reports in the literature regarding the characteristics of cell lines under basal and
310 perturbed conditions may have overlooked changes in the metabolic environment of cells or
311 high cell density. Such aspects are typically not reported or measured and may contribute to
312 the irreproducibility of the results when repeating the assay in a different laboratory. Such
313 irreproducibility is fueling the reproducibility debate[1,2].

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314
315 Not only do our results highlight the need for reporting experimental 'details' concerning
316 culture conditions, they also show a way towards rationalizing and standardizing these.
317 Required details would include, but not be limited to, information on the source of cell lines
318 and passage number (or at least whether all cells used were below a certain passage
319 number), number of cells per well at the time of seeding and throughout the assay,
320 density/confluence throughout the assay, volume of culture media used, details of cell
321 culture flasks used, length of assay (from time of seeding), choice of cell culture medium
322 (including concentrations of all components) and sera (concentration used and source), and
323 how concentrations of key nutrients (e.g. glucose and glutamine) and pH change throughout
324 the assay. This would complement existing efforts for standardization across biomedical
325 research[34-36] and improve reproducibility, transparency and evaluation of the experimental
326 data, points that are of critical concern[37].
327
328 A number of reporting guidelines for the results of biological assays have been in existence
329 for some time, e.g. the Minimum Information About a Microarray Experiment (MIAME)
330 standard. MIAME is now a reporting requirement for a number of funding agencies and
331 journals[38]. Similarly, there are now minimum reporting standards in use for metabolomics [35],
332 proteomics[36] and systems biology models[39]. Since 2008, the Minimum Information for
333 Biological and Biomedical Investigations (MIBBI) project has acted as a repository for the
334 many minimum reporting guidelines that have since been created; there are now over 40 for
335 the biological and biomedical sciences (https://biosharing.org/standards/, accessed 04 July
336 2016).
337
338 The assay developed and discussed here, as well as the contention that it should be
339 accompanied by metabolic analyses of the assay cell lines, should contribute to improved
340 assay reproducibility in cell biology and drug discovery.
341

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Figures and Tables

Table 1: Predicted versus observed effects of treatment with the GLS1i inhibitor using the prevalent culture conditions compared with the optimized culture conditions devised in this study.

Predicted effect of GLS1i treatment…		**Prevalent culture conditions**	**Optimized culture conditions**
…in sensitive A549 cells			
Cell numbers ↓		✗	✓
Glutamine consumption ↓		✓	✓
Glutamate production ↓		✗	✓
Glutamine (intracellular) ↑		✓	✓
Glutamate (intracellular) ↓		✗	✓
TCA cycle intermediates (intracellular):	α-KG ↓	✗	✗
	Citrate ↓	✗	✓
	Fumarate ↓	✗	✓
….in resistant H358cells			
Cell numbers unchanged		✓	✓
Glutamine consumption ↓		✓	✓
Glutamate production ↓		✗	✓
Glutamine (intracellular) ↑		✓	✓
Glutamate (intracellular) ↓		✓	✓
TCA cycle intermediates (intracellular)	α-KG ↓	✓	✓
	Citrate ↓	✗	✗
	Fumarate ↓	✗	✓

✓: significantly as predicted at $p < 0.05$

✗ : not significantly as predicted, i.e. $p > 0.05$.

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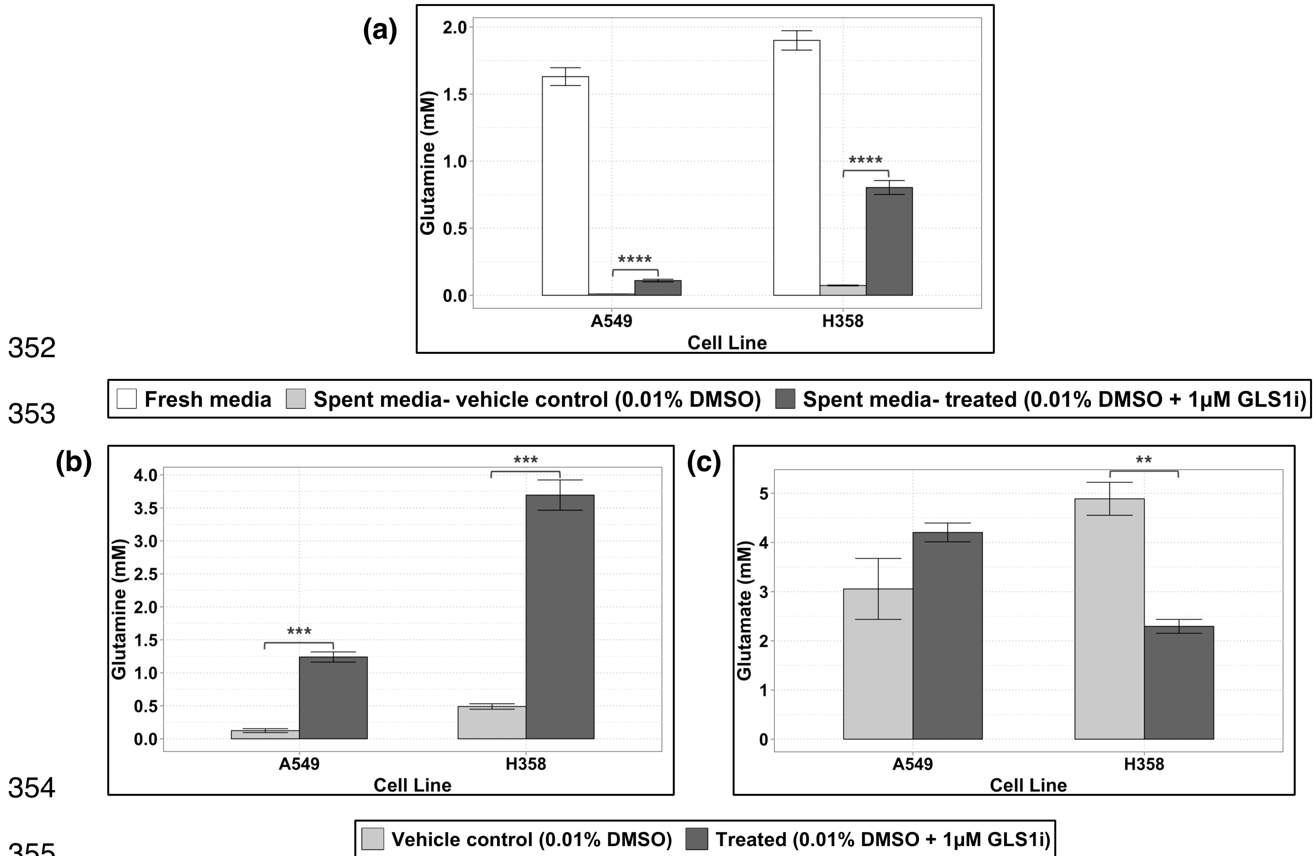

356 **Figure 1: Glutamine and glutamate concentrations 48 hours after treatment with 0.01% DMSO ± 1 µM**
357 **GLS1i using a prevalent assay method.** A549 and H358 are known as sensitive and resistant cell lines,
358 respectively, vis-à-vis glutaminase 1 inhibitors. Concentrations of **(a)** extracellular glutamine **(b)** intracellular
359 glutamine and **(c)** intracellular glutamate measured by LC-UV after 24 h of treatment with 0.010 % DMSO ± 1.0
360 µM (final concentrations) GLS1i. For this single experiment, measurements were performed in triplicate for
361 control and treated conditions. Cells had been seeded at a density of $8x10^5$ cells/well in 1 ml of culture media 24
362 h prior to commencing the experiment. Shown are the mean ± SEM for the 3 technical replicates per cell line
363 and treatment condition. Unadjusted p-values of the differences between control and treated samples obtained
364 using a two-tailed Student's t-test are denoted with asterisks: **: $p \leq 0.01$; ***: $p \leq 0.001$; ****: $p \leq 0.0001$.

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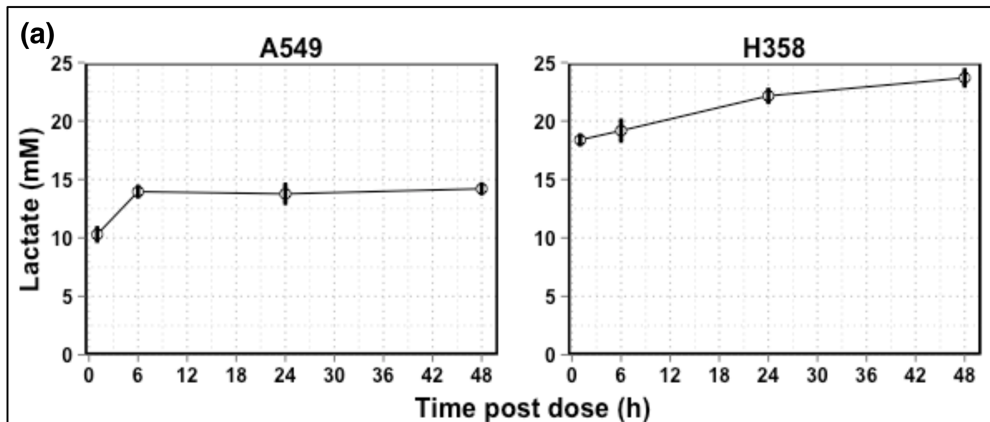

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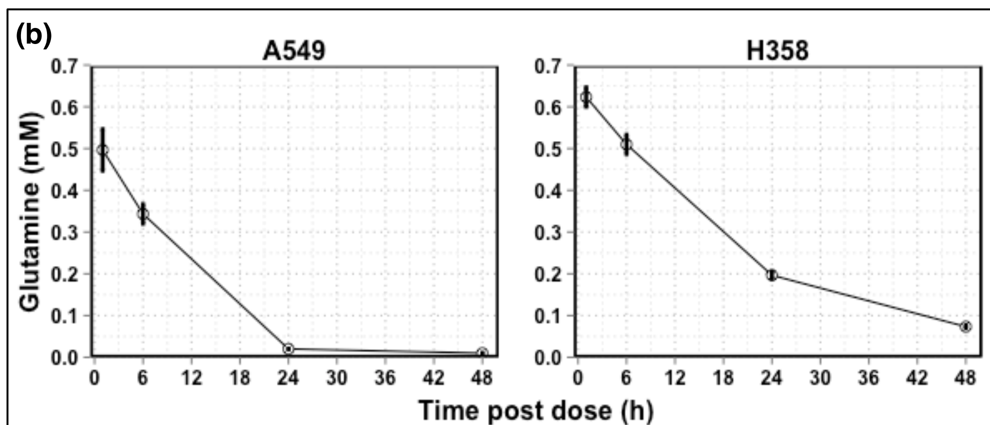

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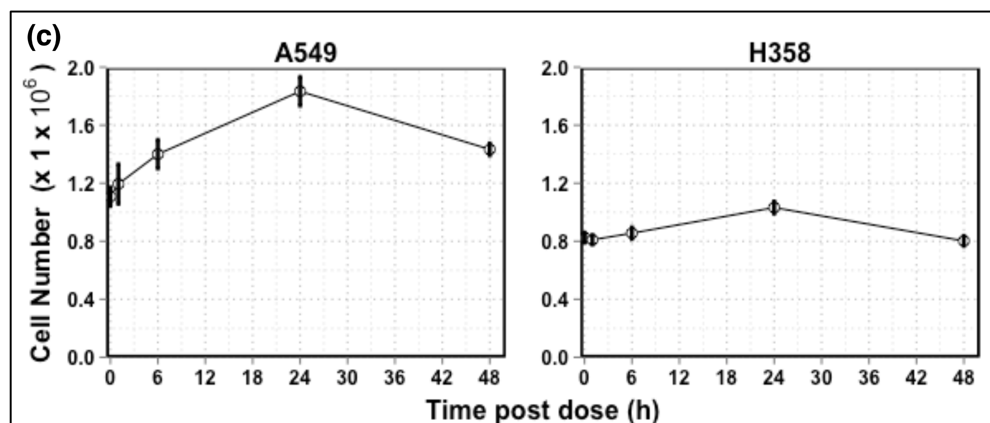

367
368

369 **Figure 2: Changes with time after seeding of the state of a cell culture in a traditional assay in vehicle**
370 **control (0.01% DMSO) and treatment conditions**. **(a)** Lactate (measured by LC-MS) and **(b)** glutamine
371 (measured by LC-UV) in spent medium. **(c)** Number of live cells per well as measured using the Trypan blue
372 exclusion technique using a Countess automated cell counter (Thermo Scientific, Loughborough, UK). Zero time
373 corresponds to 24 hours after seeding of cells into a medium containing 10 mM of glucose, 2 mM of glutamine, in
374 addition to dialyzed fetal calf serum, vitamins and both essential and non-essential amino acids at concentrations
375 well below 1mM except for arginine (0.95 mM) and glutamine (2 mM), as shown in Table M1 in Materials and
376 Methods. The cell lines were: A549 (left) and H358 (right). For this single experiment, measurements were
377 performed in triplicate. Shown are the mean ± SEM for the 3 technical replicates per cell line in control
378 conditions.

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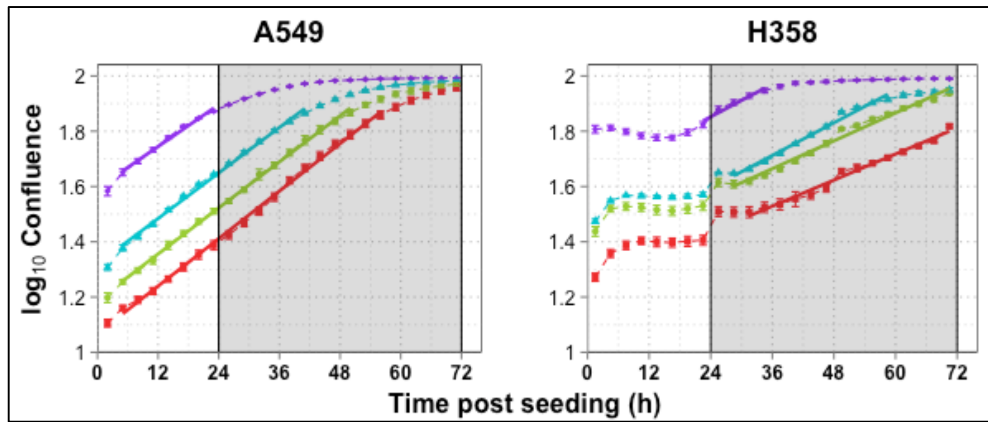

Figure 3: Confluence of A549 and H358 cells over 72 hours after seeding at different initial densities when the volume of culture media was increased to 3 mL. Shown are the changes in $\log_{10}$ confluence over time measured by live content cell imaging Incucyte HD system (Essen Bioscience) when A549 and H358 cells were seeded at a density of 8×10^5, 4×10^5, 3×10^5 and 2×10^5 cells/well. For this single experiment, measurements were performed in triplicate for control and treated conditions. Shown are the mean ± SEM for the 3 technical replicates per cell line and treatment condition. Shaded area denotes the assay window in a prevalent assay where samples would be taken over 48 hours from the time of dosing (24 hours after seeding). Solid lines are a fitted linear model for the exponential growth phase of cells.

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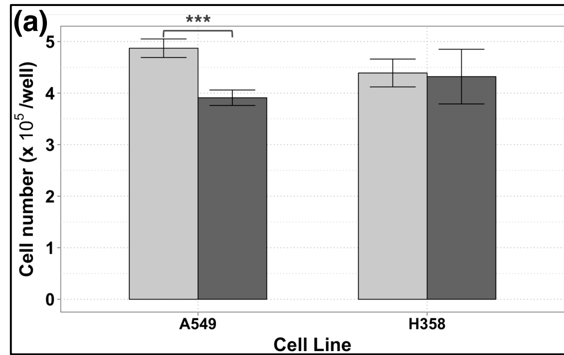
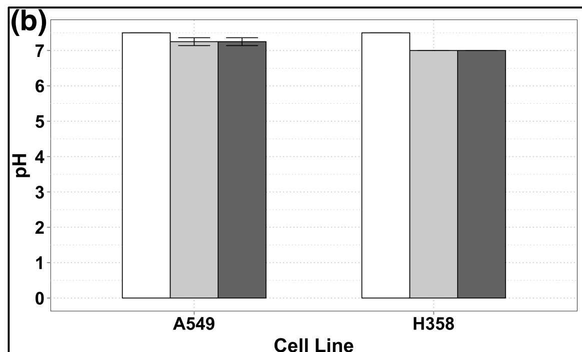
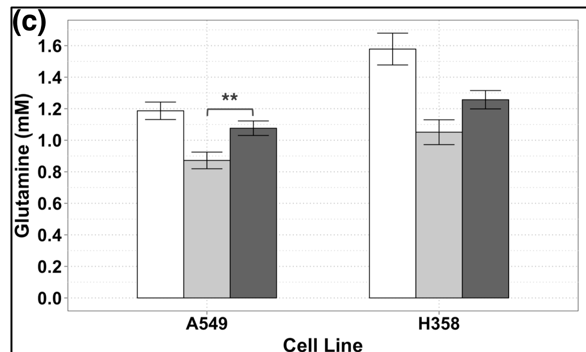


389

390 Vehicle control (0.01% DMSO) Treated (0.01% DMSO + 1μM GLS1i)





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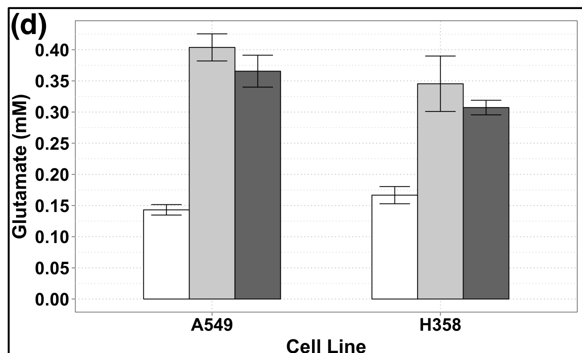

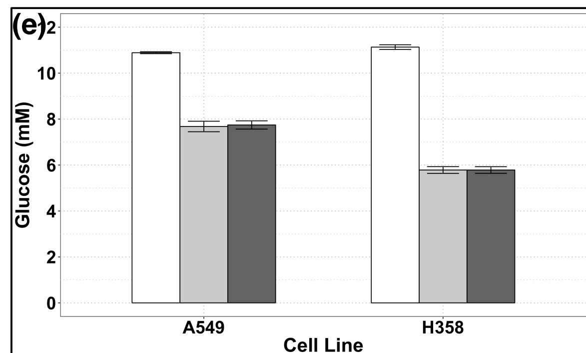

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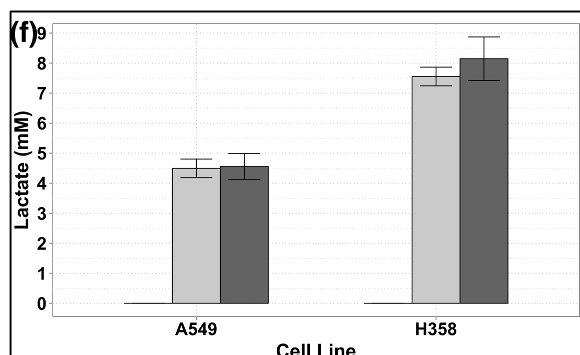

393

394 Fresh media Spent media- vehicle control (0.01% DMSO) Spent media- treated (0.01% DMSO + 1μM GLS1i)

395 **Figure 4: Live cell numbers, concentrations of various metabolites, and pH in fresh and spent media**
396 **extracts 24 hours after treatment with 0.010 % DMSO ± 1.0 μM GLS1i using the improved culture**
397 **conditions devised here. (a)** Live cell numbers as measured by automated microscopy following Hoechst
398 staining and fixation (See Materials and Methods 1.6). **(b)** pH of fresh and spent media samples measured using
399 MColorpHast indicator strips. Concentration of **(c)** glutamine, **(d)** glutamate, **(e)** glucose and **(f)** lactate in fresh

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400 and spent media samples after treatment with or without 1.0 µM GLS1i measured by LC-UV (glutamine and
401 glutamate), Accu-Chek Aviva Blood Glucose Meter System (glucose) and LC-MS (lactate). For each experiment,
402 measurements were performed in triplicate for control and treated conditions. Shown are the mean ± SEM of 3
403 (A549 cell line; 9 data points per condition) or 2 (H358 cell line; 6 data points per condition) independent
404 experiments. Note that glutamine concentrations in fresh media used for A549 and H358 cells fell by an average
405 of ~27 % and ~5 % respectively over the duration of the assay. Unadjusted p-values of the differences between
406 control and treated conditions obtained using a two-tailed Student's t-test are denoted with asterisks: **: $p \leq 0.01$;
407 ***: $p \leq 0.001$.

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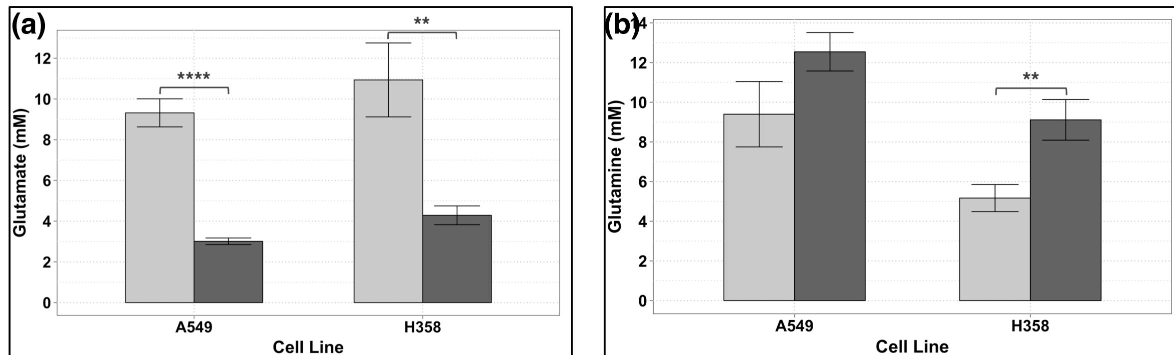

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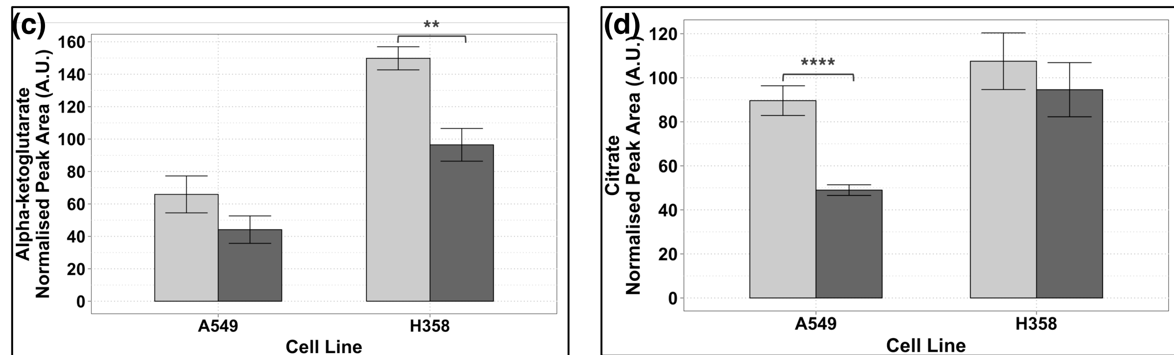

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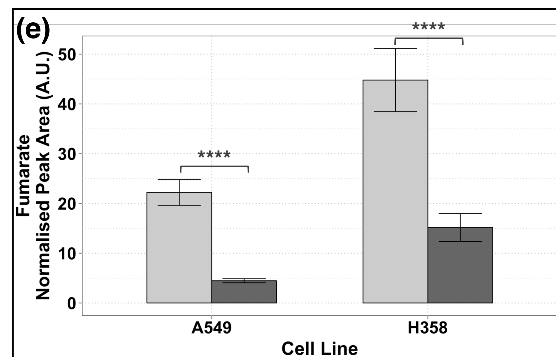

410

411 Vehicle control (0.01% DMSO) Treated (0.01% DMSO + 1μM GLS1i)

412

413 **Figure 5: Levels of various intracellular metabolites 24 hours after treatment with 0.01% DMSO ± 1 μM**
414 **GLS1i using the improved culture conditions devised here.** A549 and H358 are known as sensitive and
415 resistant cell lines, respectively. Concentration of **(a)** glutamate and **(b)** glutamine measured by LC-UV, and
416 relative abundance of the TCA cycle intermediates (measured by LC-MS) **(c)** alpha-ketoglutarate, **(d)** citrate and
417 **(e)** fumarate in cells after treatment with or without 1μM GLS1i. For each experiment, measurements were
418 performed in triplicate for control and treated conditions. Shown are the mean ± SEM of 3 (A549 cell line; 9 data
419 points per condition) or 2 (H358 cell line; 6 data points per condition) independent experiments. Unadjusted p-
420 values of the differences between control and treated conditions obtained using a two-tailed Student's t-test, the
421 results of which are denoted with asterisks: **: $p \leq 0.01$; ***: $p \leq 0.001$; ****: $p \leq 0.0001$.

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422 ## 4 Acknowledgements

423

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430

431 ## 5 Author Contributions

432 MWM and HVW established the aim and strategy of the study and
433 designed the experiments, which MWM performed. FO, RB, and CB provided advice and
434 expertise on the design of the experiments, data acquisition and analysis. SEC, BP and SP
435 (see acknowledgements) proposed the experimental system of glutaminase inhibition in
436 human non-small cell lung cancer cell lines. MWM, HVW and FO wrote the paper with
437 advice and guidance provided by RB and CB.

438

439 ## 6 Competing financial interests

440 The authors declare that they have no competing financial interests.

441

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