

1 Novel MOG analogues to explore the MCT2 pharmacophore,  $\alpha$ -  
2 ketoglutarate biology and cellular effects of *N*-oxalylglycine

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18 **ABSTRACT**

19  $\alpha$ -ketoglutarate ( $\alpha$ KG) is a central metabolic node with a broad influence on cellular  
20 physiology. The  $\alpha$ KG analogue *N*-oxalylglycine (NOG) and its membrane-permeable pro-drug  
21 derivative dimethyl-oxalylglycine (DMOG) have been extensively used as tools to study prolyl  
22 hydroxylases (PHDs) and other  $\alpha$ KG-dependent processes. In cell culture media, DMOG is  
23 rapidly converted to MOG, which enters cells through monocarboxylate transporter MCT2,  
24 leading to intracellular NOG concentrations that are sufficiently high to inhibit glutaminolysis  
25 enzymes and cause cytotoxicity. Therefore, the degree of (D)MOG instability together with  
26 MCT2 expression levels determine the intracellular targets NOG engages with and, ultimately,  
27 its effects on cell viability. Here we designed and characterised a series of MOG analogues  
28 with the aims of improving stability and exploring the functional requirements for interaction  
29 with MCT2, a relatively understudied member of the SLC16 family. We report MOG analogues  
30 that maintain ability to enter cells via MCT2, and identify compounds that do not inhibit  
31 glutaminolysis or cause cytotoxicity but can still inhibit PHDs. We use these analogues to show  
32 that glutaminolysis-induced activation of mTORC1 can be uncoupled from PHD activity.  
33 Therefore, these new compounds can help deconvolute cellular effects that result from the  
34 polypharmacological action of NOG.

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37 **KEYWORDS**

38 Dimethyl-oxalylglycine (DMOG), methyl-oxalylglycine (MOG), *N*-oxalylglycine (NOG),  
39 monocarboxylate transporter 2 (MCT2), SLC16A7,  $\alpha$ -ketoglutarate, prolyl hydroxylases,  
40 transporter-mediated drug uptake, structure-activity relationship.

41 **INTRODUCTION**

42 The study of metabolism has long been aided by the use of metabolite analogues that  
43 allow the rapid and reversible inhibition of enzymes and pathways in different experimental  
44 settings<sup>1</sup>. In the field of cancer metabolism, analogue compounds such as 2-deoxyglucose, 6-  
45 diazo-5-oxo-L-norleucine (DON) and dichloroacetate (DCA) continue to complement genetic  
46 approaches to dissect the strengths and vulnerabilities associated with oncogene-driven  
47 metabolic changes in tumours<sup>2–4</sup>. Metabolite analogues are also among some of the most  
48 important clinically used chemotherapeutic compounds: from gemcitabine and 5-fluoro-uracil  
49 (5-FU), nucleoside analogues used as therapies in pancreatic and colorectal cancer; to  
50 methotrexate and pemetrexed, folate analogues administered to treat a range of  
51 malignancies<sup>5,6</sup>. The development and refinement of metabolite analogues can therefore  
52 provide valuable tools for mechanistic studies of both metabolism and tumorigenesis.

53  $\alpha$ -ketoglutarate ( $\alpha$ KG) is a key metabolic node and understanding its complex biology  
54 has been significantly facilitated by the structural analogue *N*-oxalylglycine (NOG), which has  
55 been extensively used *in vitro* along with its cell-permeable derivative, dimethyl-oxalylglycine  
56 (DMOG)<sup>7–9</sup> (Fig. 1a). Most commonly, DMOG is utilised to elicit hypoxia signalling by inhibiting  
57 prolyl hydroxylase domain (PHD) enzymes leading to stabilisation of the transcription factor  
58 Hypoxia Inducible Factor 1 $\alpha$  (HIF1 $\alpha$ )<sup>8,10</sup>. HIF1 $\alpha$  stabilisation is a therapeutic aim in conditions  
59 ranging from ischaemia and anaemia to inflammatory diseases<sup>11,12</sup>, and, in these settings,  
60 previous studies have used DMOG to demonstrate the potential therapeutic benefits of  
61 inhibiting PHDs<sup>13,14</sup>.

62 As well as being a cofactor for  $\alpha$ KG-dependent enzymes,  $\alpha$ KG is also the entry point  
63 for glutamine carbons into the TCA cycle, a substrate for a large number of transaminase  
64 reactions, and has also been shown to mediate the activation of the mechanistic target of  
65 rapamycin complex 1 (mTORC1) by glutamine<sup>15</sup>.  $\alpha$ KG can modify epigenetic profiles during  
66 development<sup>9</sup> and in pathogenic contexts<sup>16</sup> by regulating ten-eleven translocation (TET)  
67 hydroxylases and Jumonji demethylases. Additionally,  $\alpha$ KG can influence aging<sup>17</sup>, through an  
68 as-yet unclear mechanism, highlighting that there is still much to be discovered about the  
69 physiological functions of this metabolite.

70 Though DMOG is able to inhibit PHDs and thereby stabilise HIF1 $\alpha$  in a broad range of  
71 cell lines, it is selectively toxic to some, in a manner that strongly correlates with the expression  
72 level of the monocarboxylate transporter MCT2<sup>18</sup>. DMOG is unstable in cell culture media and  
73 is non-enzymatically converted to the monocarboxylate methyl-oxalylglycine (MOG) with a  
74 half-life of 10 min. MOG is a substrate for MCT2, the expression of which determines the  
75 concentration of NOG that accumulates intracellularly ( $[NOG]_{ic}$ ). In cells with high MCT2

76 expression,  $[NOG]_{IC}$  can reach millimolar levels, which are sufficiently high to additionally  
77 engage low-affinity  $\alpha$ KG-binding enzymes such as isocitrate dehydrogenase (IDH) and  
78 glutamate dehydrogenase (GDH), leading to severely disrupted metabolism and cytotoxicity.  
79 Such a polypharmacological mode of action makes it challenging to disentangle the exact  
80 mechanism(s) that account for the effects of NOG on cellular physiology.

81 In addition to MOG, MCT2 transports endogenous monocarboxylates ranging from  
82 pyruvate and lactate to larger ketone bodies such as  $\beta$ -hydroxybutyrate, acetoacetate,  $\alpha$ -  
83 ketoisovalerate and  $\alpha$ -ketoisocaproate<sup>19</sup> (Supplementary Fig. 1a) with a higher affinity than  
84 the other SLC16 family members<sup>9</sup>. MCT2 plays important physiological roles including the  
85 uptake of astrocyte-secreted lactate into neurons within the brain<sup>20,21</sup>. MCT2 is highly  
86 expressed in some human cancers (Supplementary Fig. 1b) and has been proposed as a  
87 biomarker for prostate cancer<sup>22</sup>, as well as having pro-tumorigenic<sup>23</sup> and pro-metastatic roles<sup>24</sup>  
88 in breast cancer. Therefore, there is an emerging need to develop chemical probes to study  
89 MCT2 functions.

90 Here, we report the design and synthesis of MOG-based analogues and use them to  
91 explore the MCT2 pharmacophore, and  $[NOG]_{IC}$ -dependent interference with intracellular  
92 targets in the context of their effects on cellular proliferation and survival.

93

## 94 RESULTS

### 95 Design and synthesis of MOG analogues

96 Based on our previous findings<sup>18</sup>, we reasoned that MOG could be used as a scaffold  
97 to explore both the chemical space accommodated by MCT2 and the cellular roles of  $\alpha$ KG-  
98 binding proteins. The conversion of DMOG to MOG and subsequently NOG generates  
99 compounds with progressively decreased capacity to transverse the plasma membrane (Fig.  
100 1a), so, the stability of MOG and, by extension, that of its analogues, could influence their  
101 mode of entry into cells and subsequently the degree of intracellular target engagement.  
102 Therefore, with the outlook of generating compounds that could also be used in the future for  
103 studies *in vivo*, we first determined the stability of MOG in whole mouse blood using liquid  
104 chromatography-mass spectrometry (LC-MS).

105 Synthetic MOG was converted to NOG with a half-life comparable that was short and  
106 comparable to that of MOG that was transiently generated from DMOG (Fig. 1b,c). Notably,  
107 the degradation of DMOG to MOG was even more rapid, with a half-life of just 0.61 minutes.  
108 The half-lives of DMOG and MOG in mouse blood are significantly shorter than those  
109 previously measured in aqueous solution<sup>18</sup>, which we attribute to the well-documented high  
110 level of blood esterase activity. These data suggested that increasing stability would be a  
111 desirable attribute of MOG analogues.

112 We designed and synthesised compounds using MOG as the chemical scaffold (**1**,  
113 Fig. 1d, e). Based on our previous findings and the fact that esters are typically used as pro-  
114 drugs for poorly absorbed carboxylic acid drugs<sup>20–23</sup>, we focused on the methyl ester of MOG  
115 as the primary cause of compound instability in plasma. Furthermore, as the pharmacophore  
116 required for MCT2-driven transport is unknown, substitutions were kept relatively  
117 conservative. Therefore, we replaced the glycinate ester on MOG with i) bulkier alkyl esters  
118 such as ethyl or isopropyl (compounds **2–3**), ii) esters possessing methyl substituents at the  $\alpha$   
119 position (compounds **4–6**), iii) a ketone (compound **7**), or iv) 5-membered aromatic  
120 heterocycles (compounds **8–10**). Importantly, compounds **1–6** are predicted to be de-esterified  
121 to form NOG or methyl-substituted NOG, and therefore likely also able to engage intracellular  
122 targets.

123 Esters **2** and **3** were designed to minimally increase the steric hindrance of the ester  
124 substituent, which would be expected to decrease both chemical and enzyme-mediated  
125 instability<sup>24–28</sup>. We also explored branched esters (**4–6**), as substitutions on the  $\alpha$ -carbon can  
126 increase chemical stability<sup>29</sup> and cellular esterases can exhibit surprising selectivity toward  
127 complex esters<sup>30–32</sup>. Ketones and amides (**7**) are classical ester isosteres<sup>33</sup>, with the amide  
128 typically used to improve the stability of drugs<sup>34</sup>. Notably, such small changes can have a  
129 significant effect on the binding affinity of the compounds to their targets<sup>35</sup>. Finally, 5-  
130 membered ring heterocycles, such as oxadiazoles (**10**) or oxazoles (**8**), have also been  
131 successfully used as ester bioisosteres<sup>36–39</sup>.

132

### 133 *MCT2-dependent uptake by cells is maintained in a subset of MOG analogues*

134 To determine the MCT2-dependence of MOG analogue uptake into cells, we utilised  
135 HCC1569 cells, a human breast cancer line that expresses very low levels of MCT2 and is  
136 naturally resistant to MOG-induced toxicity<sup>18</sup>. We compared compound uptake in these cells  
137 transduced with a control 'empty vector' plasmid (HCC1569-EV) to an isogenic line that  
138 expresses exogenous human MCT2 (HCC1569-MCT2) (Fig. 2a, b). After incubation of both  
139 cell lines with each analogue for 4 h, we tested whether compounds or derivatives thereof  
140 could be detected intracellularly. In the case of compounds **7–10**, we detected the intact parent  
141 compound, however, as expected, compounds **1–6** were all de-esterified intracellularly and  
142 therefore in these cases we quantified NOG, or the methyl-substituted NOG that were formed.  
143 Intact MOG analogues or their products accumulated intracellularly to varying extents, with  
144 those derived from the bulkier alkyl esters (**2** and **3**) and  $\alpha$ -methyl substituents (**4–6**) reaching  
145 concentrations in the millimolar range (Fig. 2c). The de-esterification of compounds **1–6** within  
146 cells is expected to further decrease their membrane permeability, which could effectively trap

147 them inside cells and may explain the higher concentrations observed. We defined MCT2-  
148 dependent uptake as a two-fold increase in compound accumulation in HCC1569-MCT2 cells  
149 compared with HCC1569-EV cells<sup>40</sup>. The dependence of uptake on MCT2 varied between  
150 compound groups but was maintained in both of the bulkier alkyl esters (with the fold-change  
151 in uptake of compound **2** being comparable to that of MOG), as well as in all 3 of the 5-  
152 membered aromatic heterocycles (compounds **8-10**, Fig. 2d).

153 Although we found a modest increase in the uptake of the  $\alpha$ -methyl and ketone  
154 analogues in MCT2-expressing cells compared to controls, these compounds did not meet  
155 the two-fold cut off criterion. Since these compounds (**4-7**) harbour very minor modifications  
156 of the MOG scaffold, we considered whether they might interact with MCT2 in an inhibitory  
157 manner. To test this idea, we assessed the ability of **4-7** to prevent MOG-induced, MCT2-  
158 dependent inhibition of respiration<sup>18</sup> in INS1 cells (a rat pancreatic  $\beta$ -cell line with low  
159 expression of all endogenous MCT isoforms<sup>41</sup>) that expressed exogenous human MCT2  
160 (INS1-MCT2)(Fig. 2e and *Methods*). Upon treatment with MOG, basal respiration of INS1-  
161 MCT2 (but not EV control) cells decreased by 60%. AR-C155858, a previously described  
162 inhibitor of MCT2<sup>42</sup> almost completely prevented inhibition of respiration by MOG. Addition of  
163 the  $\alpha$ -methyl substituents had no effect on MOG-induced decrease of respiration, however,  
164 co-incubation with compound **7** decreased the inhibitory effect of MOG by half. This finding  
165 suggests that the replacement of the glycinate ester of MOG with a ketone group switches the  
166 nature of the interaction with MCT2 from substrate to inhibitor.

167 To test whether the difference in  $[NOG]_{IC}$  in cells treated with analogues **2** and **3** can  
168 be accounted for by altered stability and thereby compound availability to cells, we measured  
169 the conversion of these compounds to NOG in cell culture media. Both compounds  
170 demonstrated similar, improved stability compared to MOG (Fig. 2f), suggesting that increased  
171  $[NOG]_{IC}$  in cells treated with compound **2** compared to compound **3** is likely due to differences  
172 in transport or intracellular de-esterification rates rather than differences in their stability in  
173 media. Interestingly, the half-life of compound **3** in blood was significantly longer than that of  
174 either MOG or compound **2**, also mirrored by the greater persistence of **3** compared to MOG  
175 in the blood of animals administered with these compounds (Supplementary Fig. 2b).

176 In summary, we generated MOG analogues with differential dependence on MCT2 for  
177 cell entry and with the ability to generate a range of  $[NOG]_{IC}$ . Further investigations were  
178 focused on compounds that showed MCT2-dependent uptake.

179

180 *MCT2-dependent cellular effects of MOG analogues are proportional to the  $[NOG]_{IC}$  they elicit*

181 MOG inhibits cell proliferation and leads to apoptosis in MCT2-expressing cells in a  
182 manner that depends on  $[NOG]_{IC}$ <sup>18</sup>. We therefore assessed whether the bulkier alkyl esters

183 and 5-membered aromatic heterocycles maintain the ability to elicit cytotoxicity in our  
184 HCC1569±MCT2 cell model. Over 96 h, MOG inhibited cell mass accumulation in a  
185 concentration-dependent manner (Fig. 3a). This inhibition was markedly higher in MCT2-  
186 expressing cells and was associated with increased apoptosis. In contrast, although the  
187 bulkier alkyl esters (**2,3**) also slowed cell mass accumulation to a larger extent in HCC1569-  
188 MCT2 than HCC1569-EV cells, they did not elicit significant apoptosis except at the highest  
189 concentration. The cytostatic effects of analogues **2** and **3** in HCC1569-MCT2 cells were  
190 proportional to the observed  $[NOG]_{ic}$  achieved after treatment with these compounds,  
191 respectively (Fig. 3b). The 5-membered aromatic heterocycles (**8-10**) did not affect cell mass  
192 accumulation, either in the presence or absence of MCT2, except compound **9**, which, at the  
193 highest dose, led to a small increase in apoptosis.

194 We also tested cellular effects and uptake of our analogues in LN229 (glioblastoma),  
195 MCF7 (breast cancer) and SN12C (kidney cancer) cells that express endogenous MCT2<sup>18</sup>.  
196 MOG attenuated cell mass accumulation in all three cell lines (Fig. 3c). Compounds **2** and **3**  
197 caused either a small or no decrease in cell mass accumulation and led to much lower  $[NOG]_{ic}$   
198 relative to that achieved with MOG (Fig. 3d). Notably, **2** and **3** elicited  $[NOG]_{ic}$  in these cells  
199 that was almost 10-fold lower than the  $[NOG]_{ic}$  elicited in HCC1569-MCT2 cells (Fig. 3b), likely  
200 explaining the attenuated cytostatic effects of these compounds in cells with endogenous  
201 MCT2 expression levels relative to MCT2-overexpressing cells.

202 Together, these findings demonstrated that substitution of the methyl-ester of MOG  
203 with bulkier alkyl esters created compounds that, under equivalent treatment conditions, yield  
204 lower intracellular NOG levels and lower or no cytotoxicity compared to MOG.

205

206 *Bulkier alkyl-ester MOG analogues have attenuated effects on metabolism compared to MOG*

207 MCT2 expression promotes MOG-induced cytotoxicity by eliciting metabolic changes  
208 due to high  $[NOG]_{ic}$ <sup>18</sup>. We hypothesised that the lack of cytotoxic effects by MCT2-dependent  
209 MOG analogues (**2, 3, 8-10**, Fig. 2d) was linked to a decreased ability to perturb metabolism.  
210 To test this idea, we treated cells for 4 h with MOG analogues and analysed their metabolism  
211 by gas chromatography-mass spectrometry (GC-MS). As previously described<sup>18</sup>, MOG  
212 caused a characteristic MCT2-dependent decrease in TCA cycle intermediates and increase  
213 in amino acid concentrations (Fig. 4a and Supplementary Fig. 3a-b). Consistent with our  
214 hypothesis, this metabolic signature was significantly dampened in cells treated with any of  
215 the analogues tested (Supplementary Fig. 3a, b). Similarly, cells with endogenous levels of  
216 MCT2 (MCF7, SN12C and LN229) showed an attenuated metabolic response to analogues **2**  
217 and **3** compared to MOG (Fig. 4a).

218 Metabolic changes induced by high  $[NOG]_{IC}$  are, in part, driven by inhibition of GDH  
219 and IDH. In cells labelled with  $[U^{13}C]$ -glutamine, the extent of GDH or IDH inhibition can be  
220 determined by monitoring, respectively, levels of the citrate m+4 isotopologue formed from the  
221 oxidative use of glutamine carbons in the canonical TCA cycle and the citrate m+5  
222 isotopologue produced by the reductive carboxylation of  $\alpha$ KG (Fig. 4b)<sup>18</sup>.

223 Treatment of cells with **2** or **3** did not affect citrate m+4 labelling and caused a modest  
224 MCT2-dependent decrease in citrate m+5, which was, however, significantly less pronounced  
225 than that caused by MOG (Supplementary Fig. 3c). The analogues demonstrated similarly  
226 attenuated effects on  $[U^{13}C]$ -glutamine-derived citrate labelling in SN12C, MCF7 and LN229  
227 cells (Fig. 4c). Notably, the modest decrease in citrate m+5 was more pronounced with **2** than  
228 with **3**, reflecting the higher  $[NOG]_{IC}$  in cells treated with the former (Fig. 3d).

229 Together, these metabolic analyses support the idea that lower  $[NOG]_{IC}$  elicited by  
230 bulkier alkyl-MOG analogues does not suffice to fully inhibit glutamine metabolism, and,  
231 together with previous observations<sup>18</sup>, explain their decreased ability to induce cytotoxicity.

232

233 *MOG analogues retain ability to inhibit PHDs and help uncouple their activity from regulation  
234 of mTORC1 by glutaminolysis*

235 DMOG inhibits PHDs and thereby promotes stabilisation of HIF1 $\alpha$  at lower  $[NOG]_{IC}$   
236 than those required to inhibit glutaminolysis due to the higher affinity of NOG for PHDs than  
237 for metabolic targets<sup>18</sup>. To test whether the low  $[NOG]_{IC}$  we found with MOG analogues suffice  
238 to stabilise HIF1 $\alpha$ , we treated cells for 4 h with each analogue at 1 mM (a typical concentration  
239 at which DMOG is used to stabilise HIF1 $\alpha$  in cell culture studies) and monitored HIF1 $\alpha$  levels  
240 by western blot. In HCC-1569±MCT2 cells, either of the alkyl esters (**2**, **3**), which produce  
241 NOG intracellularly, induced HIF1 $\alpha$  stabilisation (Fig. 4d) in an MCT2- and  $[NOG]_{IC}$ -dependent  
242 manner. Conversely, the aromatic heterocycles (**8–10**) did not induce HIF1 $\alpha$  stabilisation,  
243 suggesting that despite the conservation of the oxoacetate moiety of NOG, the addition of an  
244 aromatic group on the glycinate site is incompatible with inhibition of PHDs at the compound  
245 concentrations we used. In MCF7 cells, compounds **2** or **3** stabilised HIF1 $\alpha$  with kinetics  
246 similar to those of MOG (Fig. 4e). Importantly, the protein levels of lactate dehydrogenase A  
247 (LDHA) and pyruvate kinase M2 (PKM2), two prototypical HIF1 $\alpha$  gene targets, were equally  
248 upregulated in response to treatment with compounds **2** and **3**, and with comparable kinetics.  
249 These data showed that, even though analogues **2** and **3** lead to lower  $[NOG]_{IC}$  than MOG,  
250 they stabilise HIF1 $\alpha$  to the same extent as MOG in cells with endogenous expression of MCT2.

251 In addition to regulation of HIF1 $\alpha$ , PHDs have also been reported to mediate  
252 glutaminolysis-fuelled mTORC1 activation<sup>43</sup>. Given that MOG analogues fail to inhibit  
253 glutaminolysis but can still inhibit PHDs, we compared their effects on ribosomal protein S6

254 kinase (S6K) phosphorylation (a typical readout of mTORC1 activity) to those of MOG, which  
255 can inhibit both glutaminolysis and PHDs. Even though **2** and **3** could inhibit PHDs (as shown  
256 by HIF1 $\alpha$  stabilisation) they failed to inhibit mTORC1 signalling after 4 or 8 h of treatment (Fig.  
257 4e). All three compounds suppressed S6K phosphorylation after 24 h suggesting this latent  
258 mTORC1 inhibition is likely secondary to HIF1 $\alpha$  activation rather than a direct effect of the  
259 analogues. Therefore, comparison of the effects of analogues to MOG revealed that the  
260 inhibitory effects of (D)MOG on mTORC1 signalling are likely due to attenuated glutaminolysis  
261 rather than inhibition of PHDs.

262 In summary, our data showed that compounds **2** and **3** led to inhibition of PHDs but  
263 caused minimal metabolic effects, cytotoxicity and mTORC1 inhibition compared to MOG,  
264 thus enabling us to uncouple the cellular effects of NOG elicited by metabolic targets from  
265 those that occur due to PHDs.

266

## 267 DISCUSSION

268 Metabolism has far-reaching effects on cellular physiology that extend beyond  
269 biomass accumulation, energy production and redox balance. A prototypical example of this  
270 concept is  $\alpha$ -KG, a central metabolic node that is not only the entry point of glutamine-derived  
271 carbons into the TCA cycle, but also has important regulatory roles for key signalling proteins  
272 such as mTOR and HIF1 $\alpha$ <sup>8,15</sup>. Furthermore,  $\alpha$ KG acts as a cofactor for DNA and chromatin  
273 modifying enzymes such as TET hydroxylases<sup>9</sup> and Jumonji demethylases<sup>7</sup>; consequently,  
274 fluctuations in the concentration of  $\alpha$ KG can also influence epigenetic processes, leading to  
275 long lasting effects within the cell. NOG is a structural analogue of  $\alpha$ KG that has been used to  
276 help understand many of the established roles of this important metabolite. DMOG is a  
277 membrane-permeable NOG ester that is rapidly de-esterified in cell culture media to the  
278 monocarboxylate MOG, a substrate of the transporter MCT2. The expression level of MCT2  
279 determines the  $[NOG]_{IC}$ , which, at high levels, inhibits a number of low affinity metabolic  
280 targets such as GDH and IDH, leading to toxicity in MCT2-expressing cancer cells<sup>18</sup>.

281 In addition to its *in vitro* use, DMOG has been extensively used, primarily as a  
282 pharmacological stabiliser of HIF1 $\alpha$ , *in vivo* for pre-clinical studies<sup>46,47</sup> where, typically, it is  
283 administered at concentrations that far exceed those required to inhibit the intended  
284 intracellular targets<sup>11,48,49</sup>. DMOG instability as a result of chemical or enzymatic de-  
285 esterification and a subsequent loss of cell-permeability could explain the disparity in potency  
286 observed between *in vitro* (purified enzyme) and *in vivo* studies, particularly in light of the high  
287 level of esterase activity in blood. In support of this hypothesis, here we show that DMOG and  
288 MOG are both rapidly converted to NOG in blood, each with a half-life of less than 5 minutes.

289 This poor stability in blood should therefore be a key consideration when using DMOG as a  
290 tool compound *in vivo*.

291

292 *Insights into the MCT2 pharmacophore*

293 MCT2 has a number of established physiological as well as pathological roles yet is  
294 one of the lesser-studied members of the monocarboxylate transporter family. A more detailed  
295 mechanistic understanding of this transporter could therefore open up new therapeutic  
296 opportunities and provide the basis for further studies to generate *in vivo* imaging tools. The  
297 new MOG analogues we report here helped us to further explore the structure-activity  
298 relationship (SAR) between MCT2 and its ligands, beyond what has been established based  
299 on endogenous substrates<sup>19</sup>.

300 Interestingly, we observed very little tolerance for the  $\alpha$ -methyl substitutions (**4-6**), all  
301 of which failed to meet our 2-fold threshold for MCT2-dependent uptake. These three  
302 analogues bare some structural similarity to  $\alpha$ -ketoisocaproate (Fig. 1d, Supplementary Fig.  
303 1a), for which MCT2 has a  $K_m$  of 100  $\mu$ M<sup>19</sup>. Their lack of transport therefore suggests that the  
304 combination of an  $\alpha$ -methyl substitution with a carboxyl-ester group cannot be accommodated  
305 by MCT2 (Fig. 2c, d). Unexpectedly, while we also observed no MCT2-dependent transport  
306 of the ketone analogue (**7**), this compound prevented a MOG-induced decrease in cellular  
307 respiration, suggesting it can inhibit MCT2 transport activity (Fig. 2e). This finding could  
308 potentially indicate that though **7** can still bind to MCT2, the oxygen within the MOG ester  
309 participates in an interaction within the substrate binding pocket in that is required for transport.

310 MCT2-dependent transport was maintained in the bulkier alkyl esters. We found that  
311 replacement of the methyl-ester leaving group with an ethyl-ester (**2**) was well-tolerated by  
312 MCT2, with an almost eight-fold increase in uptake by MCT2-expressing cells compared to  
313 the control cell line. MCT2-dependent transport was maintained with an isopropyl-ester  
314 substitution (**3**), however, it was lower compared to **2** indicating that the increased size of the  
315 substitution led to some steric hindrance within the transporter.

316 The 5-membered aromatic heterocycles (**8-10**) were also all transported in an MCT2-  
317 dependent manner with between a 2- and 4-fold enrichment in MCT2-expressing cells. Given  
318 the increasing interest in the role of MCT2 in cancer<sup>50-52</sup>, this finding provides a useful set of  
319 scaffolds for the development of ligands to image MCT2 activity *in vivo*.

320

321 *Transporter-dictated intracellular concentration of compound determines target engagement*

322 Even in the case of very selective drugs, intracellular concentrations higher than those  
323 required to engage the intended target could lead to off-target effects and toxicity. Here, we  
324 demonstrate that transporter-mediated uptake determines intracellular concentration of

325 compounds and thereby dictates their efficacy and toxicity. Although both the bulkier alkyl  
326 esters (**2**, **3**) are converted to NOG intracellularly, the [NOG]<sub>IC</sub> achieved in MCT2-expressing  
327 cells varied widely (48.1, 16.4 and 4.65 mM for MOG, **2** and **3**, respectively), despite each  
328 analogue being dosed at the same concentration. The [NOG]<sub>IC</sub> achieved by each compound  
329 determined their effects within cells, as reflected by their relative impact on cell mass  
330 accumulation and apoptosis (Fig. 3a, b). PHD engagement by **2** and **3** was maintained across  
331 a range of cell lines with both over-expressed and endogenous levels of MCT2, based on the  
332 observed stabilisation of HIF1 $\alpha$  (Fig. 4d, e). Similarly, compounds **2** and **3** only partially  
333 inhibited reductive carboxylation and did not significantly suppress the oxidative production of  
334 citrate (Fig. 4c, Supplementary Fig. 3c) via inhibition of GDH<sup>18</sup>; as such these analogues only  
335 minimally depleted TCA intermediates relative to that seen when dosing with MOG (Fig. 4a,  
336 Supplementary Fig. 3a). Together, our observations suggest that the lack of cytotoxicity in  
337 cells treated with **2** and **3** is because these compounds result in a [NOG]<sub>IC</sub> that is not sufficient  
338 to engage all the NOG targets that are collectively required for the cellular effects seen with  
339 MOG.

340

#### 341 *Building new tools to probe $\alpha$ KG-dependent processes*

342 Given the extensive roles of  $\alpha$ KG in cells, it is widely appreciated that NOG, as an  $\alpha$ KG  
343 mimic, is a promiscuous compound. Significant efforts have been made to generate tool  
344 compounds and potential clinical leads to inhibit a number of its targets more selectively, in  
345 particular the prolyl hydroxylases<sup>11</sup>. The differential transport of our analogues and  
346 subsequent differences in [NOG]<sub>IC</sub> have enabled us to better understand the mechanism of  
347 action of (D)MOG. Previous studies implicated PHDs in the regulation of the mTORC1  
348 pathway, in part by demonstrating that DMOG inhibits mTORC1 activity<sup>43</sup>. However, since  
349 glutaminolysis is also known to activate mTORC1<sup>15</sup>, the simultaneous actions of (D)MOG on  
350 both glutaminolysis and PHD activity complicate these conclusions. We demonstrate here that  
351 while treatment of cells with MOG leads to rapid inhibition of mTORC1 signalling, compounds  
352 **2** and **3**, which inhibit PHD activity but do not recapitulate the metabolic effects of MOG, are  
353 unable to inhibit mTORC1 signalling. These findings therefore suggest that PHD inhibition,  
354 alone, is insufficient to impact mTORC1 activity.

355 For  $\alpha$ KG-dependent dioxygenases beyond PHDs, there are far fewer specific chemical  
356 inhibitors available. The TET enzymes are of particular interest, given their well-established  
357 roles in regulating DNA methylation during early embryonic development. More recently, it has  
358 become clear that these enzymes also mediate the effects of cellular metabolic state upon  
359 epigenetic regulation<sup>53,54</sup>, which, in turn, can influence differentiation in cancer<sup>16</sup>. Isoform-  
360 specific TET inhibitors have yet to be developed, and so ‘bump and hole’-based approaches<sup>55</sup>

361 have been employed to allow individual isoform targeting via engineered enzyme isoforms  
362 with expanded active sites which can accommodate bulkier NOG analogues<sup>56</sup>. Our work could  
363 aid in the creation of cell-permeable derivatives of these NOG analogues, enabling the study  
364 of specific TET enzymes both in cells and potentially also *in vivo*.

365 Finally, to enable the study of MCT2-specific interactions our analogue series were  
366 designed to mimic MOG. However, our findings could also be of use, more generally, for the  
367 many labs that use DMOG as a tool. Further development of compound **3** with analogues that  
368 also feature bulkier oxoacetate carboxyl ester groups will likely enhance blood stability while  
369 maintaining general membrane permeability, thereby further improving the pharmacokinetic  
370 profile of this tool compound.

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386

## 387 **AUTHOR CONTRIBUTIONS**

388 S.C. and D.H. designed (with input from L.F. and D.A.) and synthesised MOG analogues, and  
389 advised on mouse dosing experimental design; N.B. assisted with cell line work and related  
390 western blots and performed cellular respiration experiments together with P.N.; P.N. also  
391 assisted with compound dosing in mice; E.S. assisted with and advised on compound stability  
392 measurements; M.S.d.S. and J.I.M. assisted with and advised on metabolomics experiments;  
393 L.F. designed and performed all other experiments, analysed and interpreted data. D.A.  
394 supervised the study, designed experiments and interpreted data. L.F. wrote the first draft of  
395 the manuscript and developed it with support from D.A. and input from S.C. and D.H. All  
396 authors reviewed and commented on the manuscript.

397 **MATERIALS AND METHODS**

398

399 *Chemical Synthesis*

400 Please see Supplementary Methods.

401

402 *Cell lines and cell culture*

403 HCC1569, MCF7, LN229 and SN12C cells were obtained from the American Type Culture  
404 Collection. Cells were cultured in RPMI 1640 medium (Gibco, 31840) containing 10% fetal calf  
405 serum (FCS), 2 mM glutamine and 100 U/ml penicillin/streptomycin, and were incubated in a  
406 humidified incubator at 37 °C and 5% CO<sub>2</sub>. All cell lines tested mycoplasma-free, and identity  
407 was confirmed by short-tandem-repeat profiling (Francis Crick Institute Cell Services Science  
408 Technology Platform). Generation of HCC1569-MCT2 over-expressing cells was achieved  
409 using retroviral transduction of HCC1569 cells with a pBabe-puro vector containing the  
410 SLC16A7 cDNA sequence, as described previously<sup>18</sup>. HCC1569-EV cells transduced with an  
411 empty pBabe-puro vector were used as controls. in MCF7 cells. MCT2 expression was  
412 knocked-down using pLKO-vector-based shRNAs obtained from Dharmacon  
413 (TRCN0000038504, sequence: GCAGGTAAATTGGTGGATTAA).

414

415 *Western blotting*

416 Cells on cell culture dishes were washed twice with PBS, before scraping in SDS sample  
417 buffer (without beta-mercaptoethanol or bromophenol blue) and boiled for 5 min at 95 °C.  
418 Protein was quantified using a BCA assay before adding beta-mercaptoethanol and  
419 bromophenol blue and resolving by SDS-PAGE. Proteins were transferred to nitrocellulose  
420 membranes by electroblotting, before blocking with 5% milk in Tris-buffered saline (50 mM  
421 Tris-HCl, pH 7.5, and 150 mM NaCl) containing 0.05% Tween 20 (TBS-T). Membranes were  
422 then incubated with the primary antibody overnight at 4 °C, washed with TBS-T and incubated  
423 with horseradish peroxidase-conjugated secondary antibody for 1 h at RT in 5% milk TBS-T.  
424 Antibodies were visualized by chemiluminescence and imaged using an Amersham  
425 Imagequant 600 RGB.

426

427 Primary antibodies used were obtained from Cell Signalling Technology: P-S6 kinase #9234  
428 1:1000, S6 kinase #2708 1:2000, LDHA: #2012 1:1000, PKM2 #3198 1:1000; Sigma: β-actin  
429 A5316 1:1000; BD Biosciences: HIF1α #610959 1:250; MCT2 1:500 (generated by the  
430 Anastasiou lab). Secondary antibodies were goat anti-rabbit or anti-mouse IgG from Millipore  
431 (#AP132P, #AP127P respectively).

432

433

434 *Cell confluence and apoptosis measurements*

435 Cell proliferation and apoptosis were measured in real time using an IncuCyteZoom (Essen  
436 Bioscience). Cell lines were seeded in 96-well plates at between 4,000 and 9,000 cells per  
437 well (depending on growth rate), in the presence of Incucyte Caspase 3/7 Green Apoptosis  
438 Assay Reagent (Essen Bioscience, used according to manufacturer's instructions). MOG  
439 analogues were added at the indicated doses 16–20 h after seeding. The IncuCyteZoom was  
440 programmed to image cells (phase and fluorescence) at 3 h intervals, and automated image  
441 analysis was used to determine confluence and number of apoptotic cells.

442

443 *Assessing ability of analogues to inhibit MCT2-mediated MOG-induced cellular respiration*

444 Oxygen consumption was measured in intact INS1 cells that stably expressed human MCT2  
445 or an empty vector control using an Orobos Oxygraph-2K oxygen electrode system  
446 (Orobos Instruments) at 37°C. Cells from one confluent 10 cm cell culture dish were used  
447 per replicate, per condition. After trypsinisation, cells were resuspended in Hank's buffered  
448 saline solution (HBSS) and incubated with 0.1% DMSO or 1 mM of the indicated analogue for  
449 30 mins before the start of oximetry. Under each treatment condition, following an initial  
450 measurement of basal oxygen consumption, 0.25 mM MOG were added to the cell suspension  
451 in the oximeter chamber. Oxygen consumption was normalised for cell number. Inhibition of  
452 MCT2 was determined by the ability of analogues to prevent a MOG-induced decrease in  
453 cellular respiration.

454

455 *Stable isotope labelling and metabolite extraction for metabolomics*

456 Cells were seeded 1 day prior to the experiment in 6-cm dishes in RPMI medium (as described  
457 above), containing dialysed FCS (3,500-Da MWCO). Medium was replaced with fresh at t = –  
458 1 h. At t = 0, medium was replaced again to medium containing [ $U-^{13}C$ ]-glutamine (2 mM) and  
459 the MOG analogue of interest (1 mM) or 0.1% DMSO (vehicle control). Treatment with  
460 compounds and labelling was carried out for 4 h unless otherwise stated. Four or five  
461 technical-replicate plates were used per condition and two or three additional plates of each  
462 cell line were counted to use for normalisation of metabolite measurements. Cell diameter was  
463 also recorded for calculation of cell volumes in order to determine intracellular concentrations.  
464 Cell diameter and number were measured using a Nexcelcom Bioscience Cellometer Auto  
465 T4. At the end of the experiment, plates were washed twice with ice-cold PBS, before  
466 quenching cells with the addition of 725  $\mu$ l dry-ice-cold methanol. Each plate was then scraped  
467 on ice, and samples were transferred to a microcentrifuge tube containing 160  $\mu$ l  $CHCl_3$  and  
468 180  $\mu$ l  $H_2O$  (containing 2 nmol of scyllo-inositol as an internal standard). Plates were scraped  
469 once more with an additional 725  $\mu$ l of cold MeOH, which was then added to the  
470 microcentrifuge tube containing the rest of the sample. Samples were sonicated for 3  $\times$  8 min

471 in a water bath, and metabolites were extracted overnight at 4 °C. Precipitated material was  
472 removed by centrifugation and samples were subsequently dried and resuspended in 3:3:1  
473 (vol/vol/vol) MeOH/H<sub>2</sub>O/CHCl<sub>3</sub> (350 µl total), to separate polar and apolar metabolites into an  
474 upper aqueous phase and lower organic phase, respectively.

475

476 *Analogue uptake assays*

477 Cells were incubated with MOG or MOG analogues (1 mM) for 4 h. Cells were then washed  
478 with ice-cold PBS, and extracted as described for GC–MS above. Samples of the polar phases  
479 were diluted 50-fold in 1:1 (vol/vol) MeOH/H<sub>2</sub>O (containing 5 µM [U-<sup>13</sup>C,<sup>15</sup>N]-valine as an  
480 internal standard) and analysed by LC–MS as described below.

481

482 *Gas Chromatography-Mass Spectrometry*

483 For GC–MS analysis, 150 µl of the aqueous phase was dried down in a vial insert, before  
484 washing twice with 40 µl MeOH and drying again. Samples were methoximated (20 µl of  
485 20 mg/ml methoxyamine in pyridine, RT overnight) before derivatising with 20 µl of N,O-  
486 bis(trimethylsilyl)trifluoroacetamide + 1% trimethylchlorosilane (Sigma, 33148) for ≥1 h. An  
487 Agilent 7890B-5977A GC-MS system was used to perform metabolite analysis. Splitless  
488 injection (injection temperature 270 °C) onto a 30 m + 10 m × 0.25 mm DB-5MS + DG column  
489 (Agilent J&W) was used, using helium carrier gas, in electron-impact ionization (EI) mode.  
490 Initial oven temperature was 70 °C (2 min) with a subsequent increase to 295 °C at 12.5  
491 °C/min, then to 320 °C at 25 °C/min (before holding for 3 min). Metabolite identification and  
492 quantification was performed using MassHunter Workstation software (B.06.00 SP01, Agilent  
493 Technologies) by comparison to the retention times, mass spectra and responses of known  
494 amounts of authentic standards. Fractional labelling of individual metabolites is reported after  
495 correction for natural abundance.

496

497 *Liquid Chromatography-Mass Spectrometry*

498 The LC–MS method was adapted from ref<sup>57</sup>. Samples were injected into a Dionex UltiMate  
499 LC system (Thermo Scientific) using a ZIC-pHILIC (150 mm × 4.6 mm, 5-µm particle) column  
500 (Merck Sequant). A 15-min elution gradient was used (80% solvent A to 20% solvent B),  
501 followed by a 5-min wash (95:5 solvent A to solvent B) and 5-min re-equilibration; solvent A  
502 was 20 mM ammonium carbonate in water (Optima HPLC grade, Sigma Aldrich) and solvent  
503 B was acetonitrile (Optima HPLC grade, Sigma Aldrich). Flow rate, 300 µl/min; column  
504 temperature, 25 °C; injection volume, 10 µl; and autosampler temperature, 4 °C. MS was  
505 performed with positive/negative polarity switching using a Q Exactive Orbitrap (Thermo  
506 Scientific) with a HESI II (heated electrospray ionization) probe. MS parameters: spray  
507 voltage, 3.5 kV and 3.2 kV for positive and negative modes, respectively; probe temperature,

508 320 °C; sheath and auxiliary gases, 30 and 5 arbitrary units, respectively; full scan range, 70  
509 to 1,050 m/z with settings of AGC target and resolution as 'balanced' and 'high' (3 × 106 and  
510 70,000), respectively. Xcalibur 3.0.63 software (Thermo Scientific) was used to record data.  
511 Prior to analysis, mass calibration was performed for both ESI polarities using the standard  
512 Thermo Scientific Calmix solution. Calibration stability was enhanced by application of lock-  
513 mass correction to each analytical run using ubiquitous low-mass contaminants. Parallel  
514 reaction monitoring acquisition parameters: resolution, 17,500; auto gain control target,  
515 2 × 105; maximum isolation time, 100 ms; isolation window, m/z 0.4; and collision energies,  
516 set individually in high-energy collisional-dissociation mode. Equal volumes of each sample  
517 were pooled to provide quality-control samples and were analysed throughout the run, thereby  
518 providing a measurement of the stability and performance of the system. Xcalibur Qual  
519 Browser and Tracefinder 4.1 software (Thermo Scientific) were used to perform qualitative  
520 and quantitative analysis respectively, according to the manufacturer's workflows.

521

522 *Blood stability assay*

523 Blood was collected from euthanised NSG female mice into heparinised tubes and used  
524 immediately for experiments. To test stability, compounds were incubated at a final  
525 concentration of 100 µM in blood (600 µL total volume), pre-warmed to 37 °C. Samples were  
526 collected in triplicate at 0 (compound added in to sample after extraction), 5, 15, 30 and 60  
527 minutes. At the indicated time, 30 µL was taken and added to a tube on ice containing 100 µL  
528 chloroform, 300 µL MeOH and 270 µL H<sub>2</sub>O. [U<sup>13</sup>C-<sup>15</sup>N] valine was present in the aqueous phase  
529 at a final concentration of 5 µM. Immediately after collection, samples were vortexed and  
530 placed on ice. After all samples had been collected, they were sonicated for 3 × 8 mins and  
531 incubated for 1h at 4 °C to allow extraction to proceed. Samples were then centrifuged (10  
532 min, 4°C, full speed) and the aqueous phase transferred to a new tube, and stored at -80 until  
533 they were ready to run on the LC-MS (Q Exactive) system, as described above. Samples were  
534 quantitated against a 7-point standard curve of compound in mouse blood extract to minimise  
535 matrix effects.

536

537

538

539 **FIGURE LEGENDS**

540

541 **Figure 1. Design and synthesis of MOG analogues**

542 a) Schematic depicting chemical structures for DMOG, MOG and NOG, their relative cell  
543 permeability and cellular targets depending on the intracellular NOG concentrations  
544 ( $[NOG]_{IC}$ ) they elicit. DMOG is converted to MOG and subsequently to the active  $\alpha$ KG  
545 analogue NOG. DMOG is cell-permeable whereas MOG is transported via MCT2  
546 leading to higher  $[NOG]_{IC}$  compared to that elicited by DMOG. High  $[NOG]_{IC}$  inhibits  
547 metabolic enzymes in addition to PHDs. NOG cannot pass through the plasma  
548 membrane.

549 b) Analysis of synthetic MOG stability over time in whole mouse blood by LC-MS.

550 c) LC-MS analysis of DMOG stability in whole mouse blood over time. DMOG is very  
551 rapidly converted to MOG, which is also unstable and subsequently forms NOG with  
552 similar kinetics to those of synthetic MOG measured in (b). Table shows calculated  
553 half-lives of DMOG conversion to MOG and subsequently NOG, or of synthetic MOG  
554 conversion to NOG from the data shown in panels b and c.

555 d) Structures of MOG glycinate methyl ester replacement analogues designed,  
556 synthesised and reported in this work. i) bulkier alkyl esters (2,3), ii)  $\alpha$ -methyl  
557 substituents (4-6), iii) ketone analogue (7), iv) 5-membered aromatic heterocycles (8-  
558 10).

559 e) Synthetic route for the preparation of MOG analogues 2-10 shown in panel (d).

560

561 **Figure 2. MCT2-dependent entry into cells is maintained by alkyl ester and aromatic**  
562 **heterocycle MOG analogues**

563 a) Schematic to illustrate the cell system used to assess dependence of MOG analogue  
564 cellular uptake on MCT2. HCC1569 human breast cancer cells were transduced with  
565 either an empty pBabePuro vector control (EV) or pBabePuro-MCT2 to stably express  
566 exogenous MCT2.

567 b) Western blot demonstrating expression of exogenous MCT2 in HCC1569-EV or  
568 HCC1569-MCT2 cells generated as described in (a).

569 c) Concentration of each of the analogues, or the indicated compounds they produce in  
570 cells, in HCC1569-EV or HCC1569-MCT2 cells incubated for 4 h with 1 mM of each of  
571 the indicated analogues (n=4 independent wells, mean  $\pm$  SD).

572 d) Fold-difference in intracellular concentration of each analogue, or the indicated  
573 compounds they produce in cells, in HCC1569-MCT2 cells relative to HCC1569-EV  
574 cells. Analogues with a >2-fold (dashed line) increase were considered to be taken up  
575 in an MCT2-dependent manner (n=4, mean  $\pm$  SD).

576 e) Left: Schematic illustrating the strategy for testing analogues **4-7** as putative MCT2  
577 inhibitors. MCT2 inhibits metabolic enzymes and thereby leads to decreased cellular  
578 respiration. Putative MCT2 inhibitors prevent MOG entry and are expected to attenuate  
579 MOG-induced inhibition of respiration. Right: Mean  $\pm$  SD change in basal cellular  
580 respiration after treatment of INS1-EV or INS1-MCT2 cells with MOG in the presence  
581 or absence of the indicated MOG analogues. MOG does not inhibit respiration in the  
582 absence of exogenous MCT2 expression illustrating the specificity of the assay. AR-  
583 C155858 was used as a positive control for MCT2 inhibition. The ketone analogue **7**  
584 attenuates MOG-induced inhibition of respiration consistent with this compound being  
585 an MCT2 inhibitor. Significance tested using a one-way ANOVA with Dunnett's test for  
586 multiple comparisons ( $n=2-5$  independent measurements).

587 f) LC-MS analysis to assess stability of MOG or the bulkier alkyl MOG analogues **2** and  
588 **3** in cell culture media over time.

589

590 **Figure 3. Analogues elicit lower  $[NOG]_{IC}$  and decreased cytotoxicity compared to MOG**

591 a) Confluence and apoptosis measurements, over time, of HCC1569-EV or HCC1569-  
592 MCT2 cells in the presence of MOG analogues added to cells at the indicated  
593 concentrations at 20 h (dotted line)( $n=3$  independent wells, mean  $\pm$  SD).

594 b) Degree of inhibition of cell mass accumulation after treatment with 1 mM of the  
595 indicated analogues (data from panel a) is proportional to the corresponding  $[NOG]_{IC}$   
596 elicited by each analogue. Error bars represent  $\pm$  SD.

597 c) Confluence, over time, of MCF7, SN12C or LN229 cells in the presence of the  
598 indicated compounds added to cells at 16 h ( $n=3$  independent wells, mean  $\pm$  SD)

599 d)  $[NOG]_{IC}$  in MCF7, SN12C or LN229 cells treated with 1 mM of each of the indicated  
600 for 4 h ( $n=4$  independent wells, mean  $\pm$  SD).

601

602 **Figure 4. MOG analogues help deconvolute cellular effects of NOG elicited by inhibition**  
603 **of metabolic targets from those due to inhibition of PHDs**

604 a) Heat map showing  $\log_2$  fold-changes in the abundance of the indicated metabolites in  
605 MCF7, LN229 and SN12C cells treated for 4 h with the indicated compounds relative  
606 to DMSO (vehicle)-treated controls. Metabolites are ordered from the highest to the  
607 lowest fold-change values in the MCF7 MOG-treated condition.

608 b) Schematic to demonstrate different routes of citrate synthesis and subsequent  
609 labelling patterns from  $[U-^{13}C]$ -Glutamine.

610 c) Labelling of citrate from  $[U-^{13}C]$ -Glutamine in MCF7, LN229 or SN12C cells treated  
611 with 1 mM of each of the indicated analogues for 4 h.  $n=4$  independent wells;

612 significance tested by multiple t-tests with Holm-Sidak correction for multiple  
613 comparisons. (\* = p< 0.05, \*\* = p< 0.01, \*\*\* = p< 0.001).

614 d) Western blot showing HIF1 $\alpha$  protein expression in HCC1569-EV or HCC1569-MCT2  
615 cells treated with 0.1 or 1.0 mM of the indicated analogues, or with DMSO for 4 h.

616 e) Western blot showing protein levels of HIF1 $\alpha$ , the HIF1 $\alpha$  target gene protein products  
617 LDHA and PKM2, and the mTORC1 kinase substrate S6K (total and phosphorylated  
618 at Thr389) in lysates of MCF7 cells treated with 1mM of the indicated compounds for  
619 4, 8 or 24 h.

620

## 621 **SUPPLEMENTARY FIGURE LEGENDS**

622

### 623 **Supplementary Figure 1**

624 a) Chemical structures of endogenous MCT2 substrates.  
625 b) Ridge plots showing the range of expression levels of *SLC16A1* (MCT1), *SLC16A7*  
626 (MCT2) and *SLC16A3* (MCT4) in different human cancer types. Data source: The  
627 Cancer Genome Atlas, SKCM – Skin cutaneous melanoma; PAAD – Pancreatic  
628 Adenocarcinoma; LUSC – Lung squamous cell carcinoma; LUAD – Lung  
629 adenocarcinoma; LGG – Low grade glioma; GBM – Glioblastoma; ESCA –  
630 Oesophageal carcinoma; BRCA – Breast Invasive Carcinoma.

631

### 632 **Supplementary Figure 2**

633 a) Stability of MOG, **2** and **3** in blood over time assessed by LC-MS measurements of the  
634 levels of parent compound and NOG produced from their degradation (n=3 animals,  
635 each sampled once at each of the indicated time points, mean  $\pm$  SD).  
636 b) MOG or compound **3** were administered intraperitoneally to mice (MOG: n=6 mice, **3**:  
637 n=7 mice) at 100 mg/kg and concentration of the compounds in blood samples  
638 collected at different time points were quantified by LC-MS. The bar graph shows the  
639 means ( $\pm$ SEM) of areas under the curve values for each compound.

640

### 641 **Supplementary Figure 3**

642 a) Heat map showing log<sub>2</sub> fold-changes in the abundance of the indicated metabolites in  
643 HCC1569-MCT2 cells relative to HCC1569-EV cells treated with DMSO (vehicle  
644 control) or 1mM of the indicated compounds for 4 h. Metabolites are ordered from the  
645 highest to the lowest fold-change values in the MOG condition.  
646 b) Amounts of the TCA intermediates fumarate and malate in HCC1569-EV or HCC1569-  
647 MCT2 cells treated with 1mM of the indicated compounds for 4 h. Significance tested

648 by multiple t-tests with Holm-Sidak correction for multiple comparisons. (\* = p< 0.05,  
649 \*\* = p< 0.01, \*\*\* = p< 0.001, n=4 independent wells).  
650 c) Labelling from [ $^{13}\text{C}$ ]-Glutamine in HCC1569-EV or HCC1569-MCT2 cells after 4 h  
651 of incubation with 1 mM of the indicated analogues. M+4 citrate is derived from  
652 oxidative use of glutamine carbons through the TCA cycle, whereas m+5 is formed as  
653 a result of reductive carboxylation. Significance tested by multiple t-tests with Holm-  
654 Sidak correction for multiple comparisons. (\* = p< 0.05, \*\* = p< 0.01, \*\*\* = p< 0.001,  
655 n=4 independent wells).

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663 **Supplementary Table 1.**

664 Mass spectrometry parameters used throughout this study for detection of MOG and MOG  
665 analogues by LC-MS.

Compound Name/Number	m/z (negative mode)	Fragments	HCD
DMOG	174.0408	-	
NOG	146.0095	74.0245	35
<b>1 (MOG)</b>	160.0254	84.009, 59.013, 72.993, 100.004	35
<b>2</b>	174.0405	74.024, 84.009, 100.004	30
<b>3 (IPOG)</b>	188.0567	74.024, 84.009, 100.004, 85.029	30
<b>4</b>	174.0405	98.025, 114.020, 72.993, 59.013	30
<b>5</b>	174.0405	98.025, 114.020, 72.993, 59.013	30
<b>6</b>	188.0559	102.0559, 128.0354	30
<b>7</b>	144.0302	99.926	35
<b>8</b>	169.0255	68.014, 82.029	30
<b>9</b>	236.0171	135.0058, 114.9996, 164.0323	30
<b>10</b>	184.0358	89.0429, 68.9955	30

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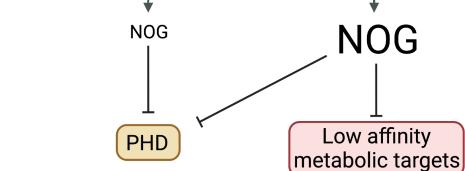
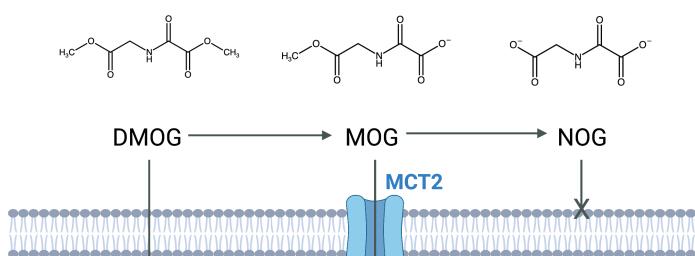
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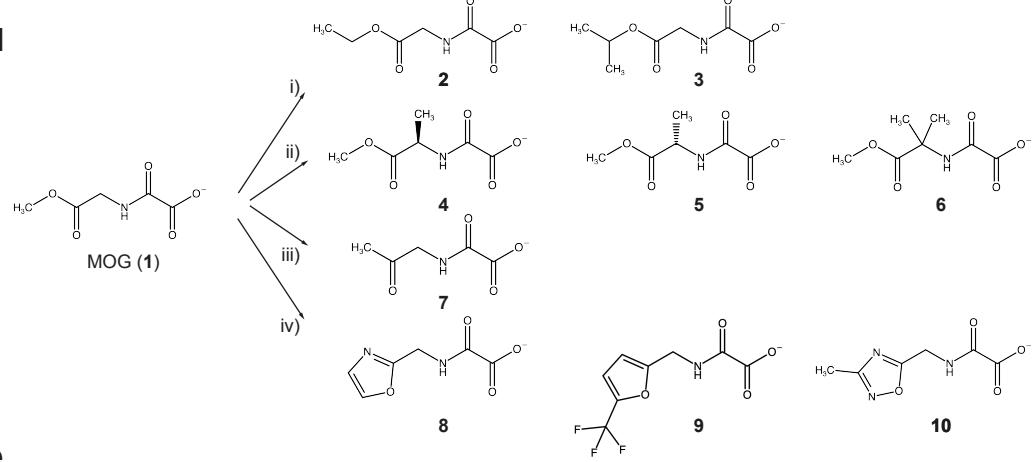
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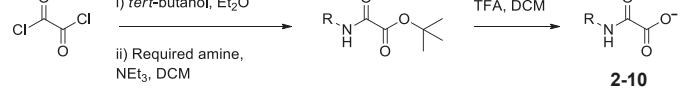
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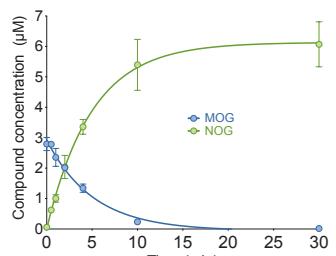
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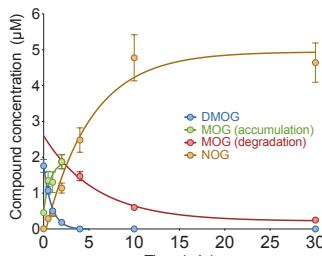
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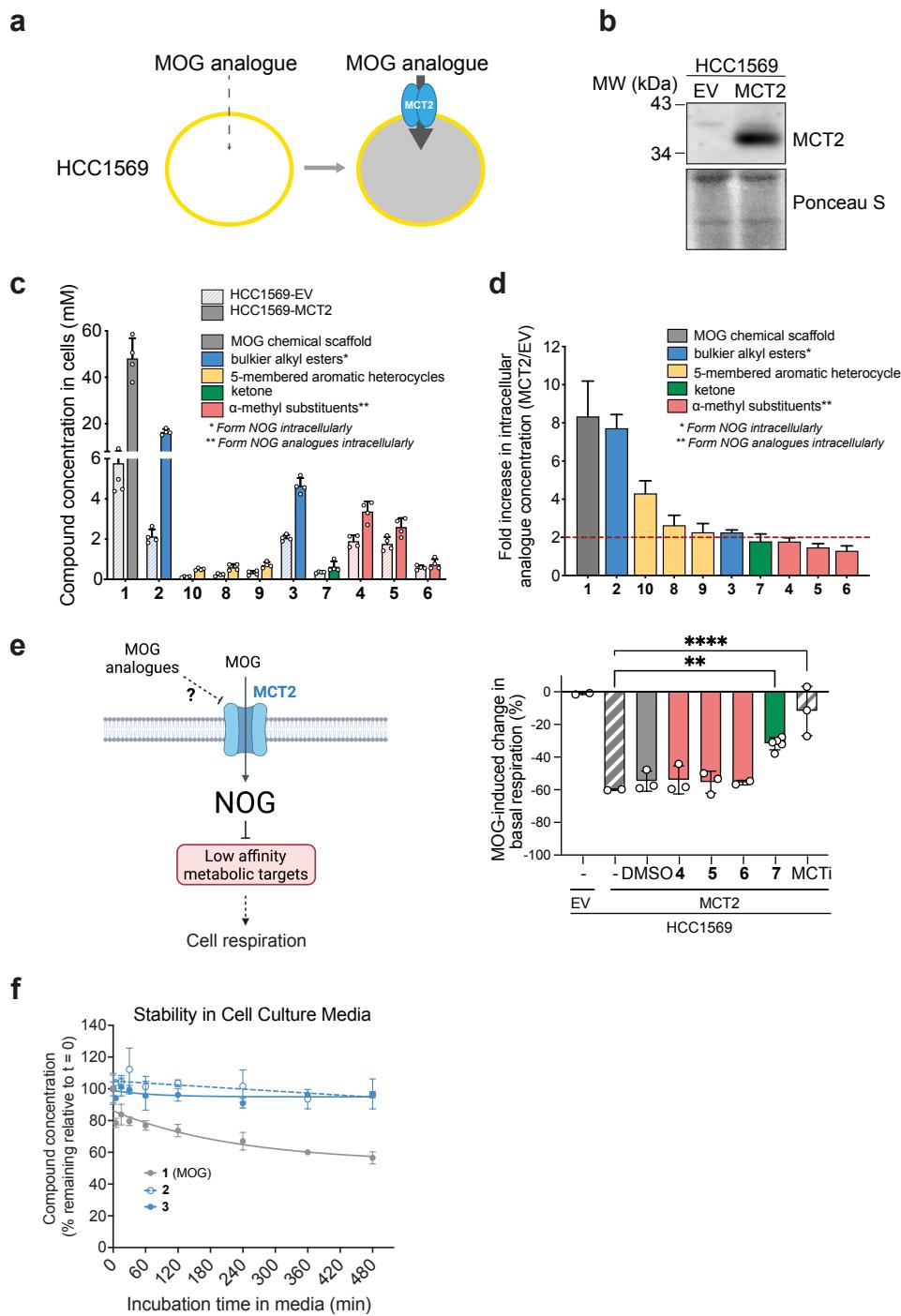
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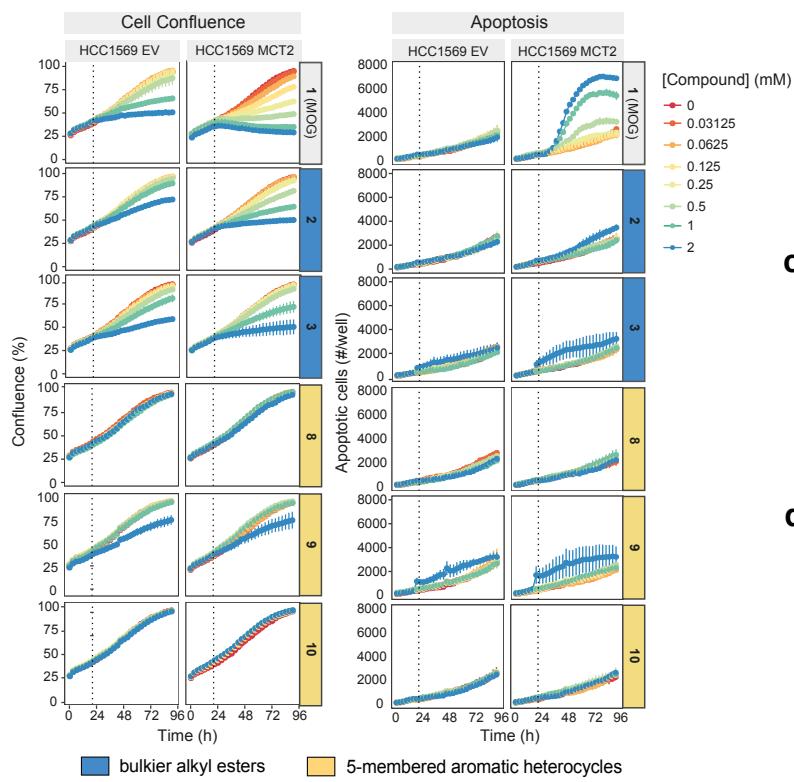
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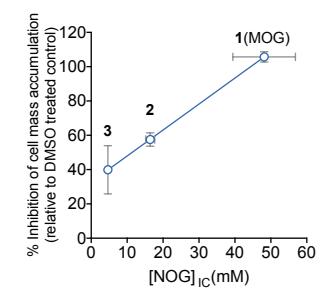
Parent compound	Species	Half Life (min)	R <sup>2</sup>
DMOG	DMOG	0.61	0.99
	MOG (accumulation)	0.54	0.82
	MOG (degradation)	4.01	0.97
MOG	NOG	3.49	0.95
	MOG	3.54	0.98
	NOG	3.46	0.97



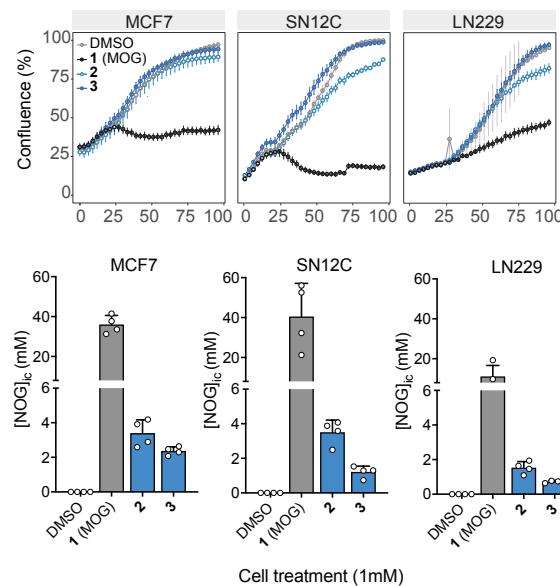
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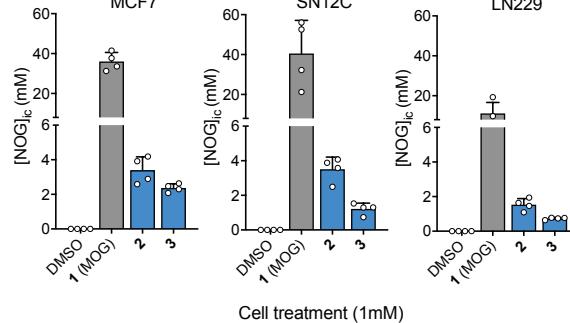
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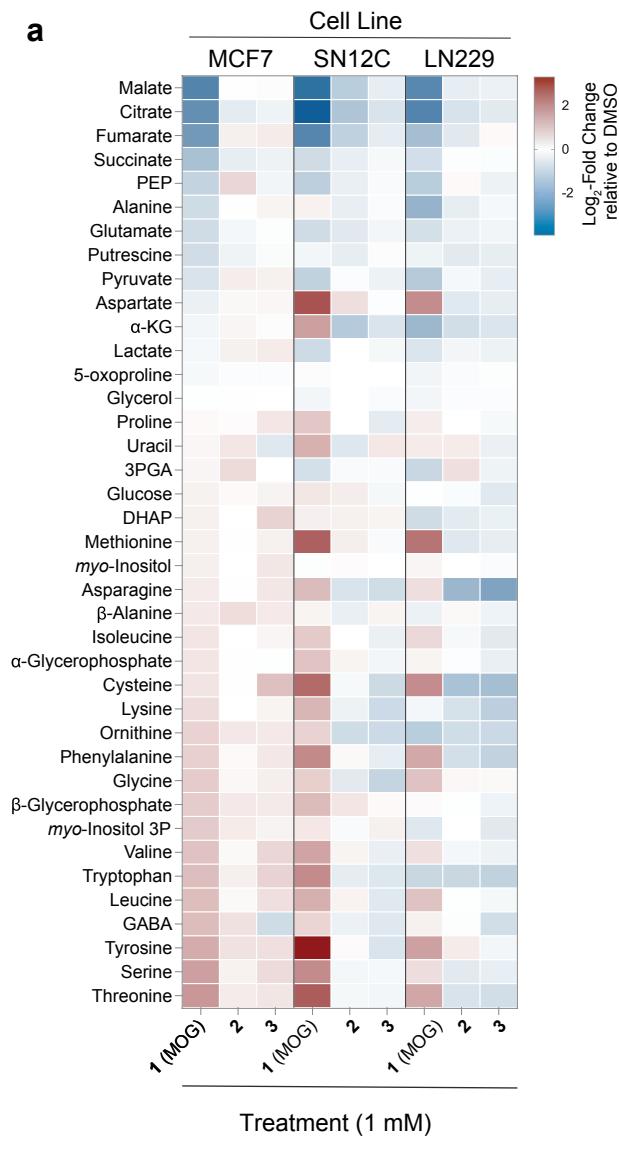
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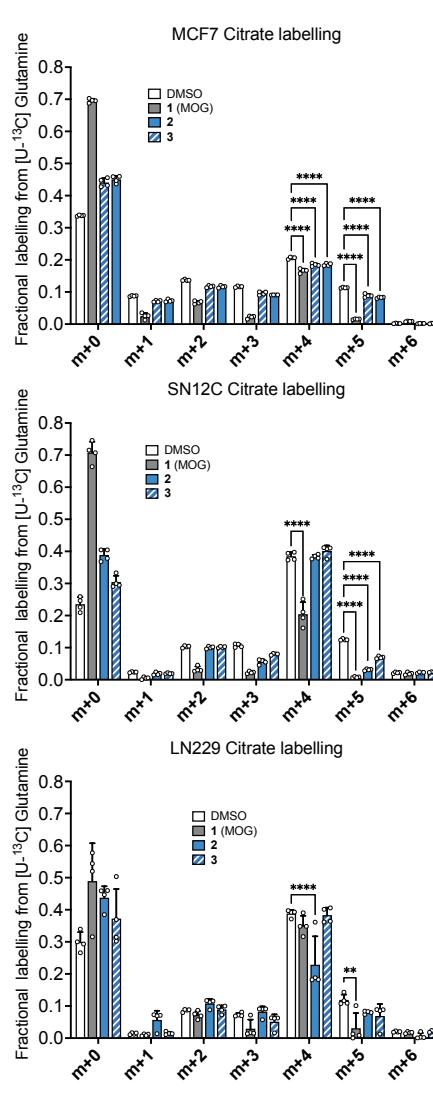
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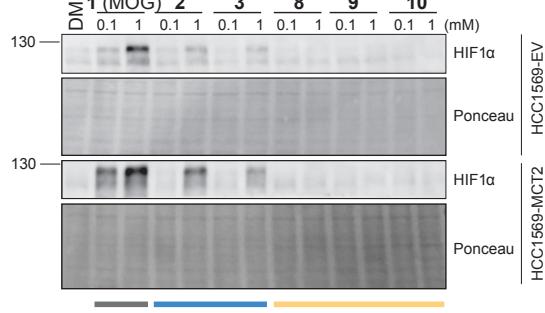
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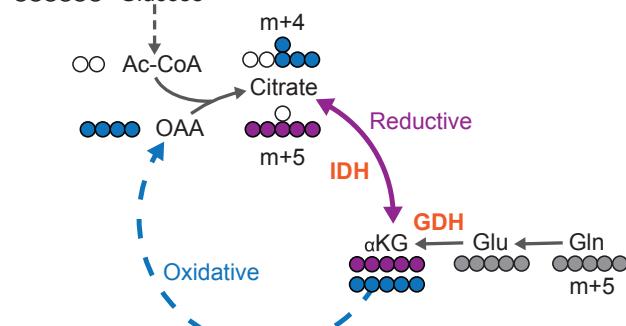
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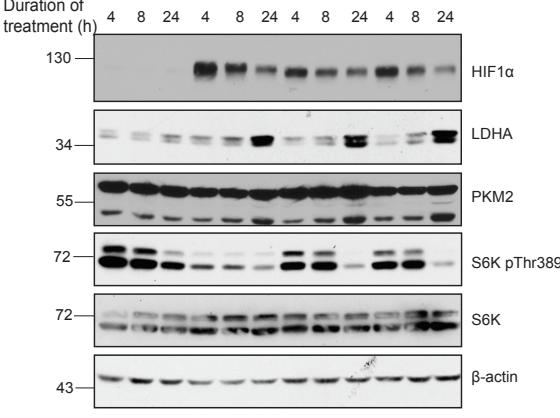
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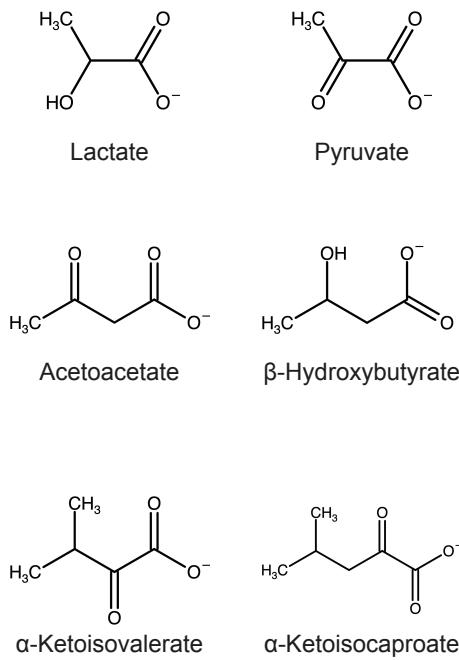
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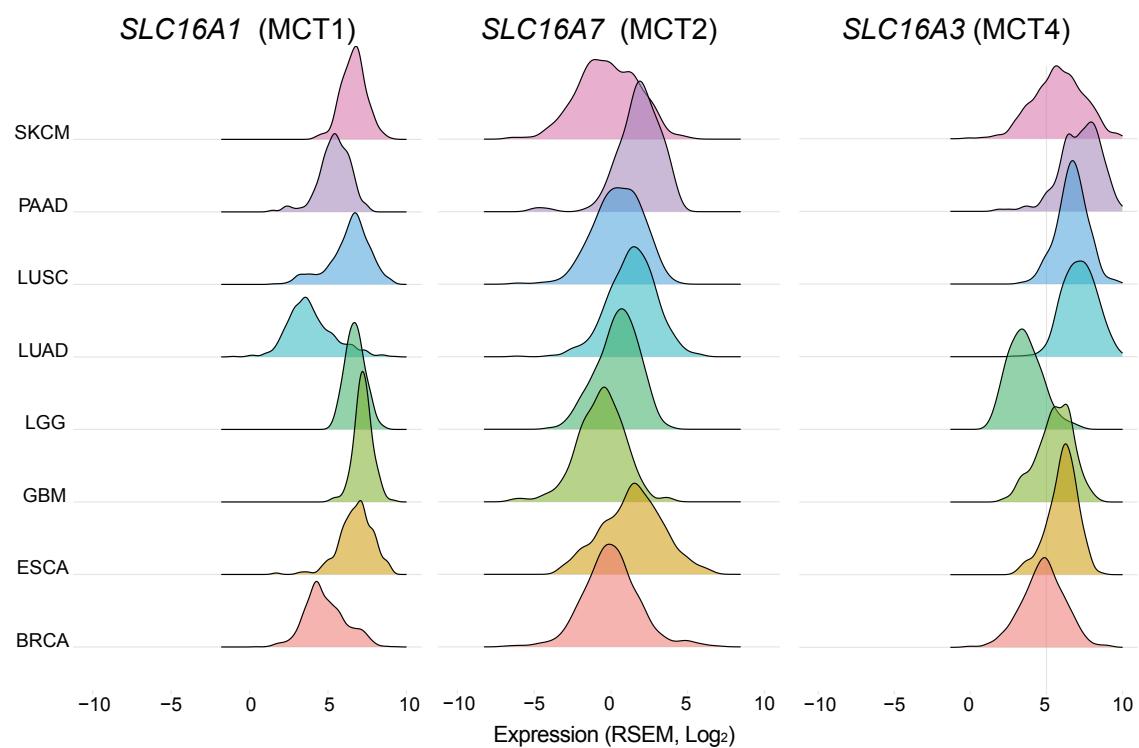
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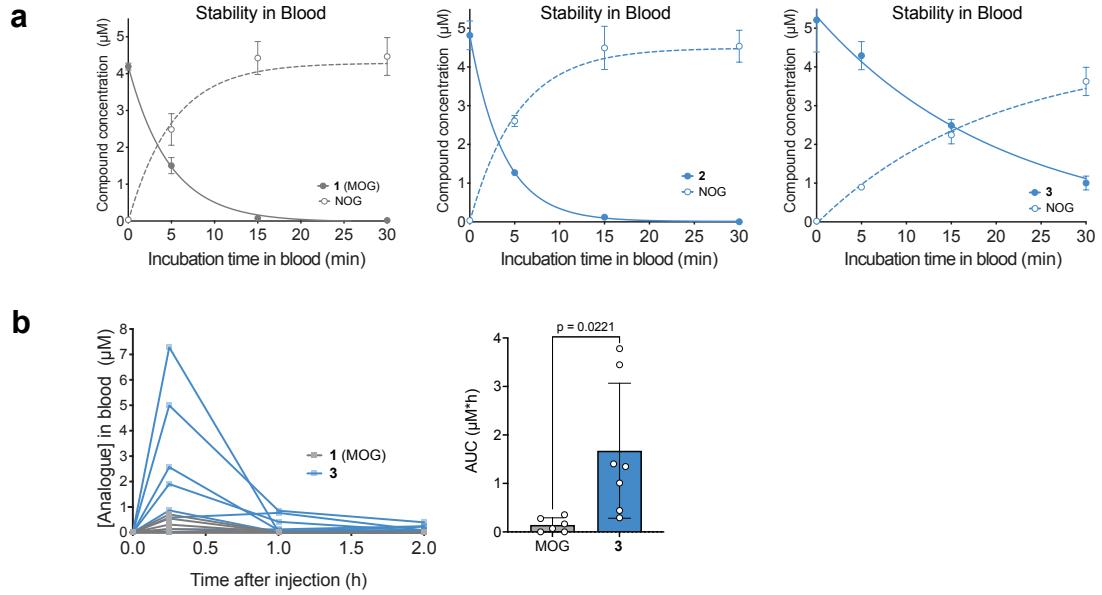


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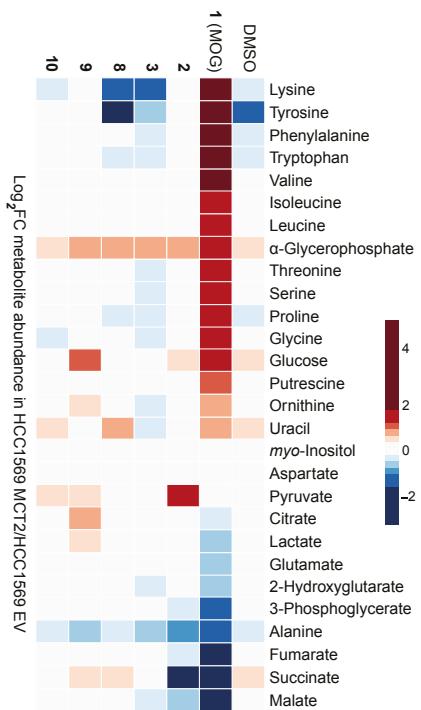


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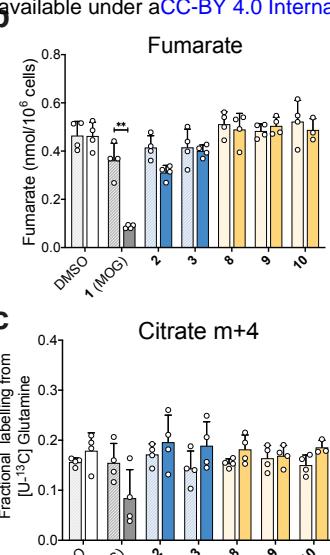




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