Development of novel MOG analogues with increased stability to explore MCT2 and α -ketoglutarate biology *in vivo*

Louise Fets^{1,4}, Patrícia M. Nunes¹, Sebastien Campos², Mariana Silva dos Santos³, Natalie Bevan¹, Emma Sherriff², James I. MacRae³, David House², Dimitrios Anastasiou^{1*}

¹Cancer Metabolism Laboratory, Francis Crick Institute, UK; ²Crick–GSK Biomedical LinkLabs, UK; ³Metabolomics Science Technology Platform, Francis Crick Institute, UK;

⁴Present address: Drug Transport and Tumour Metabolism Lab, MRC London Institute of Medical Sciences, UK

*Corresponding author

ABSTRACT

α -ketoglutarate (α KG) is a central metabolic node with far-reaching influence on cellular physiology. The α KG analogue *N*-oxalylglycine (NOG) and its membrane-permeable pro-drug derivative dimethyloxalylglycine (DMOG) have been broadly used as tool compounds both *in vitro* and *in vivo* to study α KG-dependent processes. In cell culture media, DMOG is rapidly converted to MOG, a substrate of the monocarboxylate transporter MCT2. The expression level of MCT2 determines the intracellular concentration of NOG, and, as such, influences the molecular targets NOG engages with. Here we show that DMOG and MOG are highly unstable also in mouse blood. We therefore designed and characterised a series of MOG analogues with two aims: to improve pharmacokinetic properties, and to explore the pharmacophore of MCT2, a relatively understudied member of the SLC16 family. We report MOG analogues that maintain MCT2-dependent uptake, including NOG-generating compounds that replicate the metabolic effects of MOG in a concentration-dependent manner. One such analogue, IPOG, shows significantly increased blood stability, and an improved overall pharmacokinetic profile, leading to increased NOG accumulation in MCT2-expressing tumours versus isogenic controls.

KEYWORDS

Dimethyl-oxalylglycine (DMOG), methyl-oxalylglycine (MOG), *N*-oxalylglycine (NOG), monocarboxylate transporter 2 (MCT2), *SLC16A7*, α -ketoglutarate, prolyl hydroxylases, transporter-mediated drug uptake, structure-activity relationship.

INTRODUCTION

The study of metabolism has long been aided by the use of metabolite analogues that allow the rapid and reversible inhibition of enzymes and pathways in different experimental settings¹. In the field of cancer metabolism, analogue compounds such as 2-deoxyglucose, 6-diazo-5-oxo-L-norleucine (DON) and dichloroacetate (DCA) continue to complement genetic approaches to dissect the strengths and vulnerabilities associated with oncogene-driven metabolic changes in tumours^{2–4}. Metabolite analogues are also among some of the most important clinically used chemotherapeutic compounds: from gemcitabine and 5-fluoro-uracil (5-FU), nucleoside analogues used as therapies in pancreatic and colorectal cancer; to methotrexate and pemetrexed, folate analogues administered to treat a range of malignancies^{5,6}. The development and refinement of metabolite analogues for *in vivo* studies therefore provides valuable tools to facilitate improved mechanistic understanding of both metabolism and tumorigenesis.

α -ketoglutarate (α KG) is a key metabolic node and understanding its complex biology has been significantly facilitated by the structural analogue *N*-oxalylglycine (NOG), which has been extensively used *in vitro* along with its cell-permeable derivative, dimethyl-oxalylglycine (DMOG), to inhibit a range of α KG-dependent enzymes^{7–9}. Most commonly, DMOG is utilised to elicit hypoxia signalling by inhibiting prolyl hydroxylase domain (PHD) enzymes leading to stabilisation of the transcription factor Hypoxia Inducible Factor 1 α (HIF1 α)^{8,10}. HIF1 α stabilisation is a therapeutic aim in conditions ranging from ischaemia and anaemia to inflammatory diseases^{11,12}, and previous studies using DMOG have demonstrated the potential benefits of inhibiting PHDs to achieve these goals^{13,14}.

As well as being a cofactor for α KG-dependent enzymes, α KG is also the entry point for glutamine carbons into the TCA cycle, a substrate for a large number of transaminase reactions, and has also been shown to influence mechanistic target of rapamycin (mTOR) activity¹⁵. Through regulation of ten-eleven translocation (TET) hydroxylases and Jumonji demethylases, α KG can modify epigenetic profiles during development⁹ and in pathogenic contexts¹⁶. Additionally, through an as-yet unclear mechanism, α KG can influence aging¹⁷, highlighting that there is still much to be discovered about the physiological functions of this metabolite.

DMOG is selectively toxic to some cancer cell lines, in a manner that strongly correlates to the expression level of the monocarboxylate transporter MCT2¹⁸. DMOG is unstable in cell culture media and is non-enzymatically converted to the monocarboxylate methyl-oxalylglycine (MOG) with a half-life of 10 min. MOG is a substrate for MCT2, the expression of which determines the concentration of NOG that accumulates intracellularly

72 ([NOG]_{IC}). In cells with high MCT2 expression, [NOG]_{IC} can reach millimolar levels sufficient
73 to engage low-affinity α KG-binding enzymes such as isocitrate dehydrogenase (IDH) and
74 glutamate dehydrogenase (GDH) to severely disrupt metabolism and cause cytotoxicity.

75 DMOG has been extensively used *in vivo* for pre-clinical studies where, typically, it is
76 administered at concentrations that far exceed those required to inhibit the intended
77 intracellular targets^{11,19,20}. Instability as a result of chemical or enzymatic de-esterification and
78 a subsequent loss of cell-permeability could explain the disparity in potency observed between
79 *in vitro* (purified enzyme) and *in vivo* studies, particularly in light of the high level of esterase
80 activity in blood. In support of this hypothesis, here we show that, in mouse blood, DMOG
81 degradation leads to a transient accumulation of MOG, which is rapidly converted to NOG.
82 With the aim of generating a more stable compound for *in vivo* exploration of α KG biology, we
83 designed and characterised a series of MOG analogues, identified those that recapitulate the
84 metabolic effects of MOG in cells, and then tested them in mice. The creation of a range of
85 analogues additionally enabled us to explore the pharmacophore of MCT2 ligands and thereby
86 increase understanding of this lesser-studied member of the *SLC16* transporter family.

87 88 **RESULTS**

89 *Design and synthesis of MOG analogues*

90 Given its relative instability in cell culture media¹⁸ we reasoned that DMOG, and its
91 derivative MOG, may also have a short half-life *in vivo* (Fig. 1a). To test this idea, we examined
92 DMOG stability in whole mouse blood using liquid chromatography-mass spectrometry (LC-
93 MS) and found that it was almost instantaneously converted to MOG, with a half-life of less
94 than 1 min. (Fig. 1b). The MOG formed did not accumulate and was rapidly de-esterified to
95 NOG, with a half-life of 4 min. Synthetic MOG, which only contains the glycinate methyl ester
96 group, was converted to NOG (Fig. 1c) with a half-life mirroring the conversion of DMOG-
97 derived-MOG to NOG, suggesting that the oxoacetate methyl group of DMOG is lost first and
98 more rapidly, followed by the glycinate methyl group. The half-lives of these compounds in
99 mouse blood are significantly shorter than those previously measured in aqueous solution¹⁸,
100 which we attribute to the well-documented high level of blood esterase activity.

101 Low stability of DMOG or MOG in blood prevented further well-controlled studies with
102 either compound *in vivo*. In order to overcome these issues, we designed a number of MOG
103 analogues, with two main objectives. Our first and major aim was to improve compound
104 stability while maintaining the ability to produce NOG intracellularly, in order to improve the *in*
105 *vivo* utility of this compound. Secondly, since MOG is a substrate for MCT2, by generating a
106 chemically diverse range of analogues, we aimed to broaden understanding of the chemical
107 space accommodated by this transporter.

We initiated our compound design using MOG as a chemical scaffold (**1**, Fig. 1a). Based on our previous findings and the fact that esters are typically used as pro-drugs for poorly absorbed carboxylic acid drugs,^{21–24} we focused on the methyl ester of MOG as the primary cause of compound instability in plasma. Furthermore, as the pharmacophore required for MCT2-driven transport is unknown, substitutions were kept relatively conservative. Therefore, we replaced the glycinate ester on MOG with i) bulkier alkyl esters such as ethyl or isopropyl (compounds **2-3**), ii) esters possessing methyl substituents at the α position (compounds **4-6**), iii) a ketone (compound **7**), or iv) 5-membered aromatic heterocycles (compounds **8-10**). Importantly, compounds **1-6** are predicted to be de-esterified to form NOG or methyl-substituted NOG, and therefore able to engage intracellular targets for functional studies.

Esters **2** and **3** were designed to minimally increase the steric hindrance of the ester substituent, which, would be expected to increase both chemical and metabolic stability^{25–29}. We also explored branched esters (**4-6**), as substitutions on the α -carbon can increase chemical stability³⁰ and cellular esterases can exhibit surprising selectivity toward complex esters^{31–33}. Ketones and amides (**7**) are classical ester isosteres³⁴, with the amide typically used to improve the stability of drugs³⁵. Notably, such small changes can have a significant effect on the binding affinity of the compounds to their targets³⁶. Finally, 5-membered ring heterocycles, such as oxadiazoles (**10**) or oxazoles (**8**), have also been successfully used as ester bioisosteres^{37–40}.

MCT2-dependent uptake is maintained by a subset of MOG analogues

To determine the MCT2-dependence of MOG analogue uptake into cells, we utilised HCC1569 cells, a human breast cancer line that expresses very low levels of MCT2 and is naturally resistant to MOG-induced toxicity¹⁸. We compared compound uptake in these cells transduced with a control ‘empty vector’ plasmid (HCC1569-EV) to an isogenic line expressing exogenous MCT2 (HCC1569-MCT2) (Fig. 2a, b). After incubation of both cell lines with each analogue for 4 h, all tested compounds or derivatives thereof could be detected intracellularly. In the case of compounds **7-10**, we detected the intact parent compound, however, as expected, compounds **1-6** were all de-esterified intracellularly and therefore in these cases we quantified NOG, or the NOG analogues that were formed. Intracellular accumulation of these compounds occurred to varying extents, with the bulkier alkyl esters (compounds **2** and **3**) and α -methyl substituents (compounds **4-6**) reaching concentrations in the millimolar range (Fig. 2c). The de-esterification of these compounds within cells further decreases their membrane permeability, which could effectively trap them inside cells and may explain the

higher concentrations observed. We defined MCT2-dependent uptake as a two-fold increase in compound accumulation in HCC1569-MCT2 cells compared with HCC1569-EV cells⁴¹. The MCT2-dependence of uptake varied between groups, but was maintained in both of the bulkier alkyl esters (with the fold-change in uptake of compound **2** equivalent to MOG), as well as in all 3 of the 5-membered aromatic heterocycles (compounds **8-10**, Fig. 2d). Although there was a modest increase in the uptake of the α -methyl and ketone analogues in MCT2-expressing cells compared to controls, these compounds did not meet the two-fold cut off criterion and so were not further studied.

MCT2-dependent analogues replicate the effects of MOG in a NOG-concentration dependent manner

MOG inhibits cell proliferation and leads to apoptosis in MCT2-expressing cells by disrupting metabolism¹⁸. To test the relative efficacy of the bulkier alkyl esters and 5-membered aromatic heterocycles in eliciting metabolic changes and death in cells, we first assayed their effect on cell proliferation at varying concentrations, utilising our HCC1569±MCT2 cell model. Over 96 h, MOG inhibited cell mass accumulation in a concentration-dependent manner. This inhibition was markedly higher in MCT2-expressing cells and was associated with increased [NOG]_{IC} and increased apoptosis (Fig. 3a). The bulkier alkyl esters (**2,3**) were also capable of slowing cell proliferation in a manner proportional to the observed [NOG]_{IC} achieved after dosing with these compounds (Fig. 3b). Aligned with this observation, only the highest concentrations of compounds **2** and **3** were able to increase apoptosis in MCT2-expressing cells. The 5-membered aromatic heterocycles (**8-10**) did not affect proliferation of cells, either in the presence or absence of MCT2, except in the case of compound **9**, where the highest dose led to a small decrease in proliferation and a concomitant increase in apoptosis.

The most common use of DMOG as a tool compound is to inhibit PHDs and thereby promote the accumulation of the transcription factor HIF1 α . Similar to the effects observed on cell growth and apoptosis, after 4 h of treatment with 1 mM of each analogue (the concentration commonly used for treatment of cells with DMOG) either of the alkyl esters (**2, 3**), which produce NOG intracellularly, but not the aromatic heterocycles (**8-10**), induced HIF1 α stabilisation (Fig. 3c) in a [NOG]_{IC}-dependent manner.

A subset of metabolic effects of MOG are maintained by the bulkier alkyl ester analogues

As well as inhibiting prolyl hydroxylases, upon MCT2-driven accumulation to high concentrations, NOG is able to engage other, lower-affinity α KG-binding enzymes. The effects of MOG treatment on metabolism are rapid and extensive, and at high [NOG]_{IC}, targets include

α KG-binding enzymes GDH and IDH¹⁸. Inhibition of these targets can be determined by monitoring levels of the citrate m+4 and m+5 isotopologues derived from glutamine uniformly labelled with ¹³C ([U-¹³C]-glutamine). Citrate m+4 is formed from the oxidative use of glutamine carbons in the canonical TCA cycle (Fig. 3d), and decreases as a result of GDH inhibition by NOG¹⁸. Citrate m+5 is produced by the reductive carboxylation of α KG by IDH (Fig. 3d). Reductive carboxylation is important in a range of physiological and pathological settings, including cancer, to enable the use of glutamine-derived carbons for the production of fatty acids, and to maintain redox balance^{42–45}.

We analysed the metabolism of HCC1569±MCT2 cells treated for 4 h with MOG analogues in the presence of [U-¹³C]-glutamine. The depletion of TCA metabolites that had previously been observed after MOG treatment was partially recapitulated by compounds **2** and **3**, again in a [NOG]_{IC}-dependent manner (Fig. 3e). Citrate m+5 was significantly decreased after treatment with either of the bulkier alkyl esters (**2**, **3**), whereas citrate m+4 was not (Fig. 3f), in line with the fact that IDH is inhibited by lower [NOG]_{IC} than GDH¹⁸. These data therefore demonstrate that bulky alkyl ester MOG analogues are able to disrupt reductive carboxylation independently of effects on the oxidative TCA cycle. This differential inhibition of IDH and GDH relative to the [NOG]_{IC} is reflective of their relative *in vitro* K_d values^{18,46} and is consistent with our previous findings¹⁸.

As expected, the 5-membered aromatic heterocycles (**8-10**, which do not form NOG) had no effect on the concentrations of TCA intermediates. One of the other key metabolites affected by MOG is α -glycerophosphate (α GP). Interestingly, even in DMSO-treated cells, a modest increase in α GP was observed in the MCT2-expressing cells suggesting that this transporter may lead to some alteration of cellular metabolism in HCC1569 cells (Fig. 3d). However, even relative to the MCT2-dependent increase, α GP levels were more than doubled after MOG treatment, with modest potentiation also seen with compounds **2**, **3** and **9** (Supplementary Fig. 1a).

Together, our data showed that compounds **2** and **3** entered cells and recapitulated the metabolic effects of MOG, in a manner that was proportional to the [NOG]_{IC}. We therefore chose to examine the stability of these two compounds in mice to enable more in-depth *in vivo* studies.

Higher alkyl ester analogues of MOG have improved pharmacokinetic profile

To test whether the alkyl ester substitutions changed stability for *in vivo* use, we measured the conversion of compounds **2** and **3** to NOG in mouse whole blood. In blood, the half-life of compound **2** was marginally lower than that of MOG (95% confidence intervals (CI) of 2.27-2.96 min and 3.01-3.69 min, respectively), however, compound **3** had a half-life of 14.4

min (95% CI = 10.18-21.24 min), a 4-fold improvement in blood stability compared with MOG (Fig. 4a). As predicted, the three aromatic heterocycles were all stable in blood (Supplementary Fig. 2a).

Based on its improved stability in blood, we decided to further characterise compound **3** to better understand its pharmacokinetic and distribution profile. After intra-peritoneal (i.p.) injection at 100 mg/kg into *Nod* SCID gamma (NSG) mice, the measured blood concentration of the intact analogue was higher in compound **3**-dosed animals at both 15 and 60 min post-injection than in MOG-dosed animals, with a corresponding increase in exposure (Fig. 4b). In a small number of animals examined after 2 hours post-injection with MOG or (**3**) most NOG was found in the liver and kidney, the two major sites of drug metabolism and excretion, with lower levels observed in the spleen and at detectable, albeit very low, levels in heart, skeletal muscle and brain (Supplementary Fig. 2b).

Since compound **3** demonstrated an improved pharmacokinetic profile relative to MOG, it was taken forward into *in vivo* studies, and will henceforth be referred to as isopropyl-oxalylglycine (IPOG).

MCT2-dependent transport of IPOG in vivo

To explore the utility of IPOG for *in vivo* experiments, we tested whether it would selectively accumulate in MCT2-expressing tumours. To this end, we established an orthotopic xenograft assay in which HCC1569 cells±MCT2, were injected into mammary fat pads on opposing flanks of female NSG mice (Fig. 5a). Tumours grew slowly, as previously reported⁴⁷, and there was no significant difference between the size of tumours from HCC1569-EV and HCC1569-MCT2 cells (Fig. 5b).

Once tumours had reached 5 mm in diameter, mice were acutely dosed i.p. with either MOG or IPOG at 100 mg/kg. At 2 hours post injection, mice were sacrificed and tumours were collected. Analysis by LC-MS showed that the overall concentration of NOG in tumours from mice dosed with IPOG was higher than in those dosed with MOG (Fig. 5c). Importantly, tumours expressing MCT2 accumulated significantly more NOG when dosed with IPOG than paired control tumours, whereas this was not the case in MOG-dosed animals.

Since there was no MCT2-specific accumulation of NOG in MOG-dosed animals, and MOG is so rapidly converted to NOG in blood, we considered whether the observed NOG in these tumours could be the result of direct NOG uptake. Indeed, it has been shown that NOG is a substrate for the organic anion transporter OAT1⁴⁸, so to control for this possibility, we also dosed animals with NOG directly. The concentrations of NOG in both EV and MCT2 tumours were similar to those dosed with MOG (Supplementary Fig. 2c, Fig. 5c), suggesting that direct NOG uptake is likely the main source of NOG accumulation in this case, rather than

uptake of MOG itself. We can therefore infer that the increased stability of IPOG compared to MOG drives the increased NOG concentrations observed in MCT2-expressing tumours.

In addition to LC-MS analysis of NOG levels, we extracted metabolites from the paired, treated tumours and analysed them using gas chromatography mass spectrometry (GC-MS) metabolomics. Within the time-frame tested, there were minimal metabolic effects seen within the tumours after dosing with either compound, compared to the metabolic effects of MOG in cells¹⁸. Although there was a relative decrease in α KG and increase in α GP in MCT2-expressing cells upon IPOG dosing (which was not observed with MOG or NOG treatment) these changes were not statistically significant (Fig. 5d, Supplementary Fig. 3d).

Together, these data demonstrate that as a more stable pro-drug, IPOG enabled increased accumulation of NOG in MCT2-expressing tissues and could be used as a scaffold for further chemical iterations to develop additional prodrugs with improved utility as *in vivo* tool compounds.

DISCUSSION

Metabolism has far reaching effects on cellular physiology which extend beyond biomass accumulation, energy production and redox balance. A prototypical metabolite that illustrates this concept is α -KG; a central metabolic node which is not only the entry point of glutamine-derived carbons into the TCA cycle, but also a metabolite with important regulatory roles for key signalling proteins such as mTOR and HIF1 α ^{8,15}. The effects of fluctuations in its concentration can be long lasting, with α KG also acting as a cofactor for DNA and chromatin modifying enzymes such as TET hydroxylases⁹ and Jumonji demethylases⁷, thereby influencing epigenetic processes within the cell. The α KG mimic NOG has been used to help understand many of the established roles of this important molecule. DMOG is a membrane-permeable, esterified analogue of NOG, which is rapidly de-esterified in cell culture media to the monocarboxylate MOG, a substrate of MCT2. The expression level of MCT2 determines the [NOG]_{ic}, which at high levels, inhibits a number of low affinity metabolic targets such as GDH and IDH, leading to toxicity in MCT2-expressing cancer cells¹⁸.

Although DMOG continues to be used *in vivo*^{49,50}, it is dosed at very high concentrations, significantly beyond those required to inhibit intended targets *in vitro*¹¹. Here, we show that DMOG and MOG are both rapidly converted to NOG in blood, each with a half-life of less than 5 minutes. We generated a series of analogues to improve the stability of MOG, allowing us to further increase its potential utility *in vivo*, while also exploring the structure-activity relationship of MCT2-dependent transport.

Insights into the MCT2 pharmacophore

As well as MOG, MCT2 transports endogenous monocarboxylates ranging from pyruvate and lactate to larger ketone bodies such as β -hydroxybutyrate, acetoacetate, α -ketoisovalerate and α -ketoisocaproate⁵¹ (Supplementary Fig. 3) with a higher affinity than the other SLC16 family members⁹. Although less well studied, MCT2 plays important physiological roles including the uptake of astrocyte-secreted lactate into neurons within the brain^{20,21}. MCT2 is highly expressed in some human cancers (Supplementary Fig. 4) and has been proposed as a biomarker for prostate cancer²², as well as having pro-tumourigenic²³ and pro-metastatic roles²⁴ in breast cancer. A more detailed mechanistic understanding of this transporter could therefore open up new therapeutic opportunities, and provide the basis for further studies to generate *in vivo* imaging tools. By generating a series of MOG analogues, we therefore also aimed to further explore the structure-activity relationship (SAR) between MCT2 and its ligands, beyond what has been established based on endogenous substrates⁵¹.

Interestingly, we observed very little tolerance for the α -methyl substitutions (**4-6**), all of which failed to meet our 2-fold threshold for MCT2-dependent uptake. These three analogues bare some structural similarity to α -ketoisocaproate (Fig. 1d, Supplementary Fig. 3), for which MCT2 has a K_m of 100 μ M⁵¹. Their lack of transport therefore suggests that the combination of an α -methyl substitution with a carboxyl-ester group cannot be accommodated (Fig. 2c, d). Unexpectedly, we also observed no MCT2-dependent transport of the ketone analogue (**7**), potentially indicating that the oxygen in the MOG ester participates in an interaction with the substrate recognition pocket of MCT2 that is required for substrate binding and transport.

MCT2-dependent transport was maintained in the bulkier alkyl esters. We found that replacement of the methyl-ester leaving group with an ethyl-ester (**2**) was well tolerated by MCT2, with an almost eight-fold increase in uptake by MCT2-expressing cells compared to the control line. While transport was maintained with an isopropyl-ester substitution (**3**, IPOG), it occurred at a decreased level (a 2-fold increase in MCT2-expressing cells, Fig. 2d) indicating that the increased size led to some steric hindrance within the transporter.

The 5-membered aromatic heterocycles (**8-10**) were stable in blood (Supplementary Fig. 2a) and were also all transported in an MCT2-dependent manner with between a 2- and 4-fold enrichment in MCT2-expressing cells. Given the increasing interest in the role of MCT2 in cancer⁵²⁻⁵⁴, this finding provides a useful set of scaffolds that could be built upon for the design of PET ligands to image MCT2 activity *in vivo*.

Transporter-dictated effective concentration of compound determines target engagement

Even in the case of very selective drugs, intracellular concentrations significantly beyond those required to engage the intended target could lead to off-target effects and toxicity. Here, we were able to demonstrate that transporter-mediated uptake heavily influences compound efficacy and toxicity by determining intracellular concentration. Although both the bulkier alkyl esters (**2,3**) are converted to NOG intracellularly, the [NOG]_{IC} achieved in MCT2-expressing cells varied widely (48.1, 16.4 and 4.65 mM for MOG, **2** and **3** respectively), despite each analogue being dosed at the same concentration. The [NOG]_{IC} achieved by each compound determined their effects within cells, reflected by their relative impact on cell growth and apoptosis (Fig. 3a, b). PHD engagement by **2** and **3** was lower than that by MOG based on the observed stabilisation of HIF1 α (Fig. 3b). Similarly, compounds **2** and **3** only partially inhibited reductive carboxylation, but they did not significantly suppress the oxidative production of citrate (Fig. 3e) via inhibition of GDH¹⁸; as such only minimally depleted TCA intermediates relative to that seen when dosing with MOG (Fig. 3c). Together, our observations suggest that the decrease in proliferation that is observed with **2** and **3** is modest because these compounds result in a [NOG]_{IC} only sufficient to engage a subset of the NOG targets that are collectively required for the maximal effects seen with MOG.

Trade-off between blood stability and MCT2-dependent uptake

The uptake of compound **2** into cells was higher than IPOG, however the ethyl-ester substitution failed to improve its stability in blood relative to MOG. IPOG, on the other hand, had a blood half-life more than four-fold higher than MOG. As a proof of principle, we were able to show that its increased metabolic stability was sufficient to promote a significant increase in NOG accumulation in MCT2-expressing tumours compared to control tumours within the same animal (Fig. 5c). Therefore, while the MCT2-mediated uptake of IPOG analogues would ideally be improved with further design iterations, IPOG, exemplifies a fine balance between compound metabolic stability and MCT2 transporter-mediated uptake towards future *in vivo* applications.

Building new tools to probe α KG-dependent processes

Given the extensive roles of α KG in cells, it is widely appreciated that NOG, as an α KG mimic, is a promiscuous compound. Significant efforts have been made to generate tool compounds and potential clinical leads to inhibit a number of its targets more selectively, in particular the prolyl hydroxylases¹¹. For other groups of α KG-dependent dioxygenases, there are far fewer specific chemical inhibitors available. The TET enzymes are of particular interest, given their well-established roles in regulating DNA methylation during early embryonic

development. More recently, it has become clear that they also enable cellular metabolic state to impact upon epigenetic regulation^{55,56} which can influence differentiation in cancer¹⁶. Isoform-specific TET inhibitors have yet to be developed, and so ‘bump and hole’-based approaches⁵⁷ have been employed to allow individual isoform targeting via engineered enzyme isoforms with expanded active sites which can accommodate bulkier NOG analogues⁵⁸. The studies presented here could aid in the creation of cell-permeable derivatives of these NOG analogues, enabling the study of specific TET enzymes both in cells and potentially also *in vivo*.

Finally, although our analogue series were designed to mimic MOG to enable the study of its MCT2-specific interactions *in vivo*, our findings could also be of use for the many labs using DMOG as an *in vivo* tool more generally. Further development of IPOG with analogues that also feature bulkier oxoacetate carboxyl ester groups will likely enhance blood stability while maintaining general membrane permeability, improving the pharmacokinetic profile of this tool compound.

ACKNOWLEDGEMENTS

We thank all members of the D.A. laboratory for valuable discussions and input throughout this work, as well as members of the laboratory of L.F. for critical reading of the manuscript. L.F.’s laboratory is funded by the MRC (MC-A654-5QC70). This work was funded by the MRC (MC_UP_1202/1) and by the Francis Crick Institute which receives its core funding from Cancer Research UK (FC001033), the UK Medical Research Council (FC001033) and the Wellcome Trust (FC001033) to D.A. For the purpose of Open Access, the authors have applied a CC BY public copyright licence to any Author Accepted Manuscript version arising from this submission.

AUTHOR CONTRIBUTIONS

S.C. and D.H. designed (with input from L.F. and D.A.) and synthesised MOG analogues, and advised on mouse dosing experimental design; P.N. performed orthotopic cell injections and assisted with compound dosing in mice; N.B. assisted with cell line work; E.S. assisted with and advised on compound stability measurements; M.S.d.S. and J.I.M. assisted with and advised on metabolomics experiments; L.F. designed and performed all other experiments, analysed and interpreted data. D.A. supervised the study, designed experiments and interpreted data. L.F. wrote the first draft of the manuscript and developed it with support from D.A. and input from S.C. and D.H. All authors reviewed and commented on the manuscript.

MATERIALS AND METHODS

Chemical Synthesis

Please see Supplementary Methods.

Cell lines and cell culture

HCC1569 cells were obtained from the American Type Culture Collection. Cells were cultured in RPMI 1640 medium (Gibco, 31840) containing 10% fetal calf serum (FCS), 2 mM glutamine and 100 U/ml penicillin/streptomycin, and were incubated in a humidified incubator at 37 °C and 5% CO₂. All cell lines tested mycoplasma-free, and identity was confirmed by short-tandem-repeat profiling (Francis Crick Institute Cell Services Science Technology Platform). Generation of ‘HCC1569-MCT2’ over-expressing cells was achieved using retroviral transduction of HCC1569 cells with a pBabe-puro vector containing the *SLC16A7* cDNA sequence, as described previously¹⁸. ‘HCC1569-EV’ cells transduced with an empty pBabe-puro vector were used as controls.

Western blotting

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

Primary antibodies: HIF1 α 1:500 (BD Biosciences 610958), MCT2 1:500 (generated by Anastasiou lab). The secondary antibodies were as follows: goat anti–rabbit IgG conjugated to HRP.

Cell proliferation and apoptosis measurements

Cell proliferation and apoptosis were measured in real time using an IncuCyteZoom (Essen Bioscience). HCC1569 EV or MCT2 cells were seeded in 96-well plates at 9,000 cells per well, in the presence of Incucyte Caspase 3/7 Green Apoptosis Assay Reagent (Essen Bioscience). MOG analogues were added at doses indicated 16–20 h after seeding. Cells

were imaged (phase and fluorescence) at 3 h intervals, and automated image analysis was used to determine confluence and number of apoptotic cells.

Stable Isotope labelling and metabolite extraction for metabolomics

Cells were seeded 1 day prior to the experiment in 6-cm dishes in RPMI medium (as described above), containing dialysed FCS (3,500-Da MWCO). Medium was replaced with fresh at $t = -1$ h. At $t = 0$, medium was replaced again to medium containing [U- ^{13}C]-glutamine (2 mM) and the MOG analogue of interest (1 mM) or 0.1% DMSO (vehicle control). Treatment/Labeling was carried out for 4 h unless otherwise stated. Four or five technical-replicate plates were used per condition and two or three additional plates of each cell line were counted to use for normalisation of metabolite measurements. Cell diameter was also recorded for calculation of cell volumes in order to determine intracellular concentrations. Cell diameter and number were measured using a Nexcelcom Bioscience Cellometer Auto T4. At the end of the experiment, plates were washed twice with ice-cold PBS, before quenching cells with the addition of 725 μl dry-ice-cold methanol. Each plate was then scraped on ice, and samples were transferred to a microcentrifuge tube containing 160 μl CHCl_3 and 180 μl H_2O (containing 2 nmol of scyllo-inositol as an internal standard). Plates were scraped once more with an additional 725 μl of cold MeOH, which was then added to the microcentrifuge tube containing the rest of the sample. Samples were sonicated for 3×8 min in a water bath, and metabolites were extracted overnight at 4 °C. Precipitated material was removed by centrifugation and samples were subsequently dried and resuspended in 3:3:1 (vol/vol/vol) MeOH/ H_2O / CHCl_3 (350 μl total), to separate polar and apolar metabolites into an upper aqueous phase and lower organic phase, respectively.

Analogue uptake assays

Cells were incubated with MOG or MOG analogues (1 mM) for 4 hours. Cells were then washed with ice-cold PBS, and extracted as described for GC–MS above. Samples of the polar phases were diluted 50-fold in 1:1 (vol/vol) MeOH/ H_2O (containing 5 μM [U- ^{13}C , ^{15}N]-valine as an internal standard) and analysed by LC–MS as described below.

Gas Chromatography-Mass Spectrometry

For GC–MS analysis, 150 μl of the aqueous phase was dried down in a vial insert, before washing twice with 40 μl MeOH and drying again. Samples were methoximated (20 μl of 20 mg/ml methoxyamine in pyridine, RT overnight) before derivatising with 20 μl of N,O-bis(trimethylsilyl)trifluoroacetamide + 1% trimethylchlorosilane (Sigma, 33148) for ≥ 1 h. An Agilent 7890B-5977A GC-MS system was used to perform metabolite analysis. Splitless injection (injection temperature 270 °C) onto a 30 m + 10 m $\times$ 0.25 mm DB-5MS + DG column

(Agilent J&W) was used, using helium carrier gas, in electron-impact ionization (EI) mode. Initial oven temperature was 70 °C (2 min) with a subsequent increase to 295 °C at 12.5 °C/min, then to 320 °C at 25 °C/min (before holding for 3 min). Metabolite identification and quantification was performed using MassHunter Workstation software (B.06.00 SP01, Agilent Technologies) by comparison to the retention times, mass spectra and responses of known amounts of authentic standards. Fractional labelling of individual metabolites is reported after correction for natural abundance.

Liquid Chromatography-Mass Spectrometry

The LC–MS method was adapted from ref⁵⁹. Samples were injected into a Dionex UltiMate LC system (Thermo Scientific) using a ZIC-pHILIC (150 mm × 4.6 mm, 5-µm particle) column (Merck Sequant). A 15-min elution gradient was used (80% solvent A to 20% solvent B), followed by a 5-min wash (95:5 solvent A to solvent B) and 5-min re-equilibration; solvent A was 20 mM ammonium carbonate in water (Optima HPLC grade, Sigma Aldrich) and solvent B was acetonitrile (Optima HPLC grade, Sigma Aldrich). Flow rate, 300 µl/min; column temperature, 25 °C; injection volume, 10 µl; and autosampler temperature, 4 °C. MS was performed with positive/negative polarity switching using a Q Exactive Orbitrap (Thermo Scientific) with a HESI II (heated electrospray ionization) probe. MS parameters: spray voltage, 3.5 kV and 3.2 kV for positive and negative modes, respectively; probe temperature, 320 °C; sheath and auxiliary gases, 30 and 5 arbitrary units, respectively; full scan range, 70 to 1,050 m/z with settings of AGC target and resolution as ‘balanced’ and ‘high’ (3 × 10⁶ and 70,000), respectively. Xcalibur 3.0.63 software (Thermo Scientific) was used to record data. Prior to analysis, mass calibration was performed for both ESI polarities using the standard Thermo Scientific Calmix solution. Calibration stability was enhanced by application of lock-mass correction to each analytical run using ubiquitous low-mass contaminants. Parallel reaction monitoring acquisition parameters: resolution, 17,500; auto gain control target, 2 × 10⁵; maximum isolation time, 100 ms; isolation window, m/z 0.4; and collision energies, set individually in high-energy collisional-dissociation mode. Equal volumes of each sample were pooled to provide quality-control samples and were analysed throughout the run, thereby providing a measurement of the stability and performance of the system. Xcalibur Qual Browser and Tracefinder 4.1 software (Thermo Scientific) were used to perform qualitative and quantitative analysis respectively, according to the manufacturer’s workflows.

Blood stability assay

Blood was collected from euthanised NSG female mice into heparinised tubes, and used immediately for experiments. To test stability, compounds were incubated at a final concentration of 100 µM in blood (600 µL total volume), pre-warmed to 37 °C. Samples were

collected in triplicate at 0 (compound added in to sample after extraction), 5, 15, 30 and 60 minutes. At the indicated time, 30 µl was taken and added to a tube on ice containing 100 µl chloroform, 300 µl MeOH and 270 µl H₂O. [¹³C-¹⁵N] valine was present in the aqueous phase at a final concentration of 5 µM. Immediately after collection, samples were vortexed and placed on ice. After all samples had been collected, they were sonicated for 3 x 8 mins and incubated for 1h at 4 °C to allow extraction to proceed. Samples were then centrifuged (10 min, 4°C, full speed) and the aqueous phase transferred to a new tube, and stored at -80 until they were ready to run on the LC-MS (Q Exactive) system, as described above. Samples were quantitated against a 7-point standard curve of compound in mouse blood extract to minimise matrix effects.

Short term analogue dosing in vivo

Adult female NSG mice were dosed by intra-peritoneal injection with MOG or MOG analogues at 100 mg/kg. Blood samples were collected from the saphenous vein onto a Mitra micro-sampling device (10 µl capacity, 100601-A, neoteryx) prior to injection (t=0) and at 15 minutes, 1h and 2h post injection. After two hours, dosed animals were euthanised by cervical dislocation and tissues were harvested immediately and snap-frozen in liquid nitrogen before storing at -80 °C. For blood samples, the micro-sampler 'sponge' tip was transferred into a microfuge tube containing 33.3 µl CHCl₃, 100 µl MeOH and vortexed thoroughly. [¹³C-¹⁵N]-valine was included to a final concentration of 5 µM in aqueous phase. Samples were sonicated 3 x 8 mins at 4 °C in sonicating water bath and stored at -80 until required. Phases were split by addition of 90 µl H₂O before spinning at 4 °C full speed for 5 mins (since MOG analogues are susceptible to aqueous hydrolysis, H₂O was added immediately prior to running). Aqueous phase was run on LC-MS as described above.

Extraction from tissues is described below.

Orthotopic Xenograft assays

6-8 week old female NSG mice were anaesthetised with 1.5-2% isoflurane (with oxygen), and 1 million cells resuspended in Matrigel were injected into the mammary fat pad, underneath the nipple. Two injections were carried out into mammary fat pads on opposing flanks, using HCC1569 cells expressing an empty pBabe-puro vector ('HCC1569-EV') on one side and pBabe-puro-MCT2 ('HCC1659-MCT2') on the other. Cells were tested for mycoplasma and murine norovirus prior to injection. Tumours were monitored by palpation and animal weight was measured twice per week. Length and width of tumours were measured using callipers and volume calculated as 0.5*(length x width²). Short-term dosing experiments were carried out when tumours reached approximately 0.5 cm diameter.

All animal studies were ethically reviewed and carried out in accordance with Animals (Scientific Procedures) Act 1986 and the GSK Policy on the Care, Welfare and Treatment of Animals.

Metabolite/drug extraction from tissue

Snap frozen tissues were lyophilised and when dry (approx. 24h) they were ground in a pestle and mortar. Powdered tissue was weighed out, and weight noted (~7 mg), so that after the final biphasic extraction, samples can be diluted to ensure there are 2 mg/ 100 µl aqueous phase. 600 µl 2:1 CHCl₃/MeOH was added to weighed tissue and vortexed thoroughly. Samples were then sonicated in a sonicating water bath for 3 x 8 mins with 8 mins rest between. Samples were spun full speed in microfuge for 5 mins to pellet insoluble material and supernatant transferred to a new tube and dry down in a vacuum concentrator (with cold trap). 600 µl 2:1 MeOH/H₂O was added to the remaining pellets and vortexed again. Sonication (3 x 8 mins) was repeated before leaving to extract for at 4 for at least an hour. Samples were then spun full speed in a microfuge for 5 mins to pellet insoluble material and supernatant transferred to the tube containing the previous (apolar) supernatant, before drying completely in a vacuum concentrator. Dried tubes can be stored at -80. A biphasic extraction was then carried out on each sample (1:3:3 CHCl₃/MeOH/H₂O, 350 µl total) with aqueous phase containing 5 µM [U-¹³C-¹⁵N]-valine as an internal standard, before vortexing thoroughly and centrifuging at full speed, 4 °C for 5 mins to separate phases. For LC-MS analysis, the volume of aqueous phase corresponding to 2 mg of dried tissue was added into a vial insert, and made up to 100 µl with 1:1 MeOH/H₂O (with aqueous phase containing 5 µM [U-¹³C-¹⁵N]-valine as an internal standard) so that equivalent tissue amounts are analysed. For GC-MS analysis, *scyllo*-inositol was used as an internal standard, and samples were dried down after biphasic before continuing with derivatisation as described for cell samples above.

FIGURE LEGENDS

Figure 1 – Creating MOG analogues to improve blood stability and explore MCT2 pharmacophore

- a) Schematic illustrating the conversion of DMOG to MOG, and subsequently NOG, which is the active α KG analogue.
- b) LC-MS analysis of DMOG stability in whole mouse blood over time. DMOG is very rapidly converted to MOG, which is also unstable and subsequently forms NOG.
- c) LC-MS analysis of synthetic MOG stability in whole mouse blood, table showing half-lives of DMOG conversion to MOG and subsequently NOG.
- d) MOG methyl ester replacement analogues. i) bulkier alkyl esters, ii) α -methyl substituents, iii) ketone analogue, iv) 5-membered aromatic heterocycles.
- e) Synthetic route for the preparation of MOG analogues 2-10.

Figure 2 – MCT2-dependent uptake is maintained by alkyl ester and aromatic heterocycle analogues

- a) Schematic to illustrate the cell system used to measure relative uptake of MOG analogues: HCC1569 breast cancer cells transduced with either an empty pBabe vector control (EV) or pBabe-MCT2.
- b) Western blot demonstrating expression level of MCT2 in HCC1569-EV or HCC1569-MCT2 cells.
- c) Concentration of each of the analogues in EV or MCT2 expressing HCC1569 cells after 4 hours of incubation with each analogue at 1 mM (mean $\pm$ SD).
- d) Fold-change of analogue concentration in MCT2 expressing cells relative to EV cells. A 2-fold increase (dashed line) was considered the threshold for MCT2-dependent uptake (mean $\pm$ SD).

Figure 3 – Analogues replicate MOG effects in cells in a NOG-dose-dependent manner

- a) Growth curves illustrating the dose-dependent effects of MOG analogues on cell proliferation and apoptosis at different compound concentrations in either EV or MCT2-expressing HCC1569 cells (mean $\pm$ SD).
- b) Linear relationship between the inhibition of cell proliferation after treatment with 1 mM of the indicated analogue (defined as the % increase in confluence at 96 h after treatment, relative to untreated control cells, data from panel A) and the intracellular NOG accumulated by that analogue. Error bars represent $\pm$ standard deviation.

- c) Western blot showing the stabilisation of HIF1 α after 4 hours of incubation with MOG analogues in either EV or MCT2-expressing HCC1569 cells.
- d) Schematic to demonstrate different routes of citrate synthesis and subsequent labelling patterns from [U-¹³C]-Glutamine.
- e) Metabolic changes in EV or MCT2-expressing HCC1569 cells after 4 hours of incubation with MOG analogues at 1 mM. Significance tested by multiple t-tests with Holm-Sidak correction for multiple comparisons. (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$).
- f) Labelling from [U-¹³C]-Glutamine in EV or MCT2-expressing cells after 4 h of incubation with analogues at 1 mM. M+4 citrate is derived from oxidative use of glutamine carbons through the TCA cycle, whereas m+5 is formed as a result of reductive carboxylation. Significance tested by multiple t-tests with Holm-Sidak correction for multiple comparisons. (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$).

Figure 4 – Increasing size of the alkyl ester substituent improves pharmacokinetics

- a) LC-MS analysis of MOG analogue stability in whole mouse blood over time (and accumulation of NOG). Data points represent mean $\pm$ SD.
- b) Concentration of MOG or compound **3** (IPOG) in blood at different time points after IP injection at 100 mg/kg, and mean area under the curve ($\pm$ SEM).

Figure 5 – Compound 3 (IPOG) has improved MCT2-dependent uptake *in vivo*

- a) Schematic illustrating orthotopic xenograft approach, in which HCC1569 cells expressing either EV or MCT2 were injected into mammary fat pads on either flank of the mouse to allow for paired analysis of uptake.
- b) Growth rates of HCC1569 orthotopic xenograft tumours expressing either EV or MCT2 (no significant difference in growth, as determined by multiple t-tests at different time points, with holm-sidak correction for multiple comparisons).
- c) NOG accumulation in EV and MCT2 tumours 2 hours after 100 mg/kg IP injection with either MOG or IPOG. Significance tested by paired t-tests. (* = $p < 0.05$)
- d) Levels of α -ketoglutarate and α -glycerophosphate in EV and MCT2 tumours 2 hours after 100 mg/kg IP injection with either MOG or IPOG. Significance tested by paired t-tests.

Fets *et al.* – MOG analogues

Fets et al. – MOG analogues

Fets et al; Figure 3

Fets *et al.* – MOG analogues

Fets *et al.*; Figure 4

Fets *et al.* – MOG analogues

Fets *et al.*; Figure 5

SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure 1

- a) Levels of α GP in MCT2-expressing HCC1569 cells after 4 hours of incubation with MOG analogues at 1 mM. Significance tested by ANOVA with Dunnet's test for multiple comparisons, relative to DMSO control sample. (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$).

Supplementary Figure 2

- a) LC-MS analysis of MOG analogue stability in whole mouse blood over time (mean $\pm$ SD).
- b) Small scale analysis of NOG tissue distribution after IP injection of MOG or compound 3 (2h incubation, 100 mg/kg dose).
- c) NOG accumulation in EV and MCT2 tumours 2 hours after 100 mg/kg IP injection with NOG. Significance tested by paired t-tests. (* = $p < 0.05$)
- d) Levels of α -ketoglutarate and α -glycerophosphate in EV and MCT2 tumours 2 hours after 100 mg/kg IP injection with NOG. Significance tested by paired t-tests.

Supplementary Figure 3

Chemical structures of endogenous MCT2 substrates

Supplementary Figure 4

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

Fets *et al.* – MOG analogues

Fets *et al.* – MOG analogues

Fets *et al.*; Suppl. Fig 2

Fets *et al.* – MOG analogues

Fets *et al.* – MOG analogues

Fets *et al.*; Suppl. Fig 4

Supplementary Table 1.

Mass spectrometry parameters for detection of MOG and MOG analogues

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

REFERENCES

1. Woolley, D. W. The Revolution in Pharmacology. *Perspect Biol Med* **1**, 174–197 (1958).
2. Méndez-Lucas, A. *et al.* Identifying strategies to target the metabolic flexibility of tumours. *Nat Metabolism* **2**, 335–350 (2020).
3. Liu, X. *et al.* Cystine transporter regulation of pentose phosphate pathway dependency and disulfide stress exposes a targetable metabolic vulnerability in cancer. *Nat Cell Biol* **1–11** (2020) doi:10.1038/s41556-020-0496-x.
4. Lu, H. *et al.* Rational combination with PDK1 inhibition overcomes cetuximab resistance in head and neck squamous cell carcinoma. *Jci Insight* **4**, e131106 (2019).
5. Luengo, A., Gui, D. Y. & Heiden, M. G. V. Targeting Metabolism for Cancer Therapy. *Cell Chem Biol* **24**, 1161–1180 (2017).
6. Bobrovnikova-Marjon, E. & Hurov, J. B. Targeting Metabolic Changes in Cancer: Novel Therapeutic Approaches. *Medicine* **65**, 157–170 (2014).
7. Hamada, S. *et al.* Synthesis and activity of N-oxalylglycine and its derivatives as Jumonji C-domain-containing histone lysine demethylase inhibitors. *Bioorg Med Chem Lett* **19**, 2852–5 (2009).
8. Jaakkola, P. *et al.* Targeting of HIF- α to the von Hippel-Lindau Ubiquitylation Complex by O₂-Regulated Prolyl Hydroxylation. *Science* **292**, 468–472 (2001).
9. Amouroux, R. *et al.* De novo DNA methylation drives 5hmC accumulation in mouse zygotes. *Nat Cell Biol* **18**, ncb3296 (2016).
10. Ivan, M. *et al.* HIF α Targeted for VHL-Mediated Destruction by Proline Hydroxylation: Implications for O₂ Sensing. *Science* **292**, 464–468 (2001).
11. Chan, M. C., Holt-Martyn, J. P., Schofield, C. J. & Ratcliffe, P. J. Pharmacological targeting of the HIF hydroxylases – A new field in medicine development. *Mol Aspects Med* **47–48**, 54–75 (2016).
12. Eltzschig, H. K., Bratton, D. L. & Colgan, S. P. Targeting hypoxia signalling for the treatment of ischaemic and inflammatory diseases. *Nat Rev Drug Discov* **13**, 852–869 (2014).
13. Cummins, E. P. *et al.* The Hydroxylase Inhibitor Dimethyloxalylglycine Is Protective in a Murine Model of Colitis. *Gastroenterology* **134**, 156–165.e1 (2008).
14. Milkiewicz, M., Pugh, C. W. & Egginton, S. Inhibition of endogenous HIF inactivation induces angiogenesis in ischaemic skeletal muscles of mice. *J Physiology* **560**, 21–26 (2004).
15. Duran, R. V. *et al.* Glutaminolysis Activates Rag-mTORC1 Signaling. *Mol Cell* **47**, 349–358 (2012).
16. Morris, J. P. *et al.* α -Ketoglutarate links p53 to cell fate during tumour suppression. *Nature* **1–5** (2019) doi:10.1038/s41586-019-1577-5.

Fets *et al.* – MOG analogues

- 738 17. Shahmirzadi, A. A. *et al.* Alpha-Ketoglutarate, an Endogenous Metabolite, Extends
739 Lifespan and Compresses Morbidity in Aging Mice. *Cell Metab* **32**, 447-456.e6 (2020).
- 740 18. Fets, L. *et al.* MCT2 mediates concentration-dependent inhibition of glutamine metabolism
741 by MOG. *Nat Chem Biol* **14**, 1 18 (2018).
- 742 19. Ogle, M. E., Gu, X., Espinera, A. R. & Wei, L. Inhibition of prolyl hydroxylases by
743 dimethyloxaloylglycine after stroke reduces ischemic brain injury and requires hypoxia
744 inducible factor-1 α . *Neurobiol Dis* **45**, 733–742 (2012).
- 745 20. Nagamine, Y. *et al.* Inhibition of Prolyl Hydroxylase Attenuates Fas Ligand–Induced
746 Apoptosis and Lung Injury in Mice. *Am J Resp Cell Mol* **55**, 878 888 (2016).
- 747 21. Rautio, J. *et al.* Prodrugs: design and clinical applications. *Nat Rev Drug Discov* **7**, 255–
748 70 (2008).
- 749 22. Huttunen, K. M., Raunio, H. & Rautio, J. Prodrugs--from serendipity to rational design.
750 *Pharmacol Rev* **63**, 750–71 (2011).
- 751 23. Zawilska, J. B., Wojcieszak, J. & Olejniczak, A. B. Prodrugs: A challenge for the drug
752 development. *Pharmacol Rep* **65**, 1–14 (2013).
- 753 24. Jornada, D. *et al.* The Prodrug Approach: A Successful Tool for Improving Drug Solubility.
754 *Molecules* **21**, 42 (2015).
- 755 25. Stoeckel, K. *et al.* Stability of cephalosporin prodrug esters in human intestinal juice:
756 implications for oral bioavailability. *Antimicrob Agents Ch* **42**, 2602–6 (1998).
- 757 26. Barton, P., Laws, A. P. & Page, M. I. Structure–activity relationships in the esterase-
758 catalysed hydrolysis and transesterification of esters and lactones. *J Chem Soc Perkin*
759 *Trans 2* **0**, 2021–2029 (1994).
- 760 27. Talath, S., Shirote, P., Lough, W. & Gadad, A. Stability Studies of Some Glycolamide Ester
761 Prodrugs of Niflumic Acid in Aqueous Buffers and Human Plasma by HPLC with UV
762 Detection. *Arzneimittelforschung* **56**, 631–639 (2011).
- 763 28. JOHANSEN, M. & LARSEN, C. A comparison of the chemical stability and the enzymatic
764 hydrolysis of a series of aliphatic and aromatic ester derivatives of metronidazole. *Int J*
765 *Pharmaceut* **26**, 227–241 (1985).
- 766 29. Seki, H., Kawaguchi, T. & Higuchi, T. Specificity of Esterases and Structure of Prodrug
767 Esters: Reactivity of Various Acylated Acetaminophen Compounds and
768 Acetylaminobenzoated Compounds. *J Pharm Sci* **77**, 855–860 (1988).
- 769 30. Bender, D. M. *et al.* Cyclopropanecarboxylic Acid Esters as Potential Prodrugs with
770 Enhanced Hydrolytic Stability. *Org Lett* **10**, 509–511 (2008).
- 771 31. Tian, L. *et al.* Selective esterase-ester pair for targeting small molecules with cellular
772 specificity. *Proc National Acad Sci* **109**, 4756–4761 (2012).
- 773 32. Lavis, L. D. Ester bonds in prodrugs. *Acs Chem Biol* **3**, 203–6 (2008).

- 774 33. Yamazaki, Y., Kageyama, Y. & Okuno, H. Direct Evaluation of Stereoselectivity of Cancer
775 Esterases by Polyacrylamide Gel Electrophoresis Coupled with Activity Staining with Chiral
776 Naphthyl Esters. *Anal Biochem* **231**, 295–300 (1995).
- 777 34. Meanwell, N. A. Synopsis of Some Recent Tactical Application of Bioisosteres in Drug
778 Design. *J Med Chem* **54**, 2529–2591 (2011).
- 779 35. Nassar, A.-E. F., Kamel, A. M. & Clarimont, C. Improving the decision-making process in
780 the structural modification of drug candidates: enhancing metabolic stability. *Drug Discov*
781 *Today* **9**, 1020–1028 (2004).
- 782 36. BARLOW, R. B., BREMNER, J. B. & SOH, K. S. THE EFFECTS OF REPLACING ESTER
783 BY AMIDE ON THE BIOLOGICAL PROPERTIES OF COMPOUNDS RELATED TO
784 ACETYLCHOLINE. *Brit J Pharmacol* **62**, 39–50 (1978).
- 785 37. Patani, G. A. & LaVoie, E. J. Bioisosterism: A Rational Approach in Drug Design. *Chem*
786 *Rev* **96**, 3147–3176 (1996).
- 787 38. Diana, G. D. *et al.* Oxadiazoles as Ester Bioisosteric Replacements in Compounds Related
788 to Disoxaril. Antirhinovirus Activity. *J Med Chem* **37**, 2421–2436 (1994).
- 789 39. Street, L. J. *et al.* Synthesis and biological activity of 1,2,4-oxadiazole derivatives: highly
790 potent and efficacious agonists for cortical muscarinic receptors. *J Med Chem* **33**, 2690–
791 2697 (1990).
- 792 40. Bach, P. *et al.* 5-alkyl-1,3-oxazole derivatives of 6-amino-nicotinic acids as alkyl ester
793 bioisosteres are antagonists of the P2Y₁₂ receptor. *Future Med Chem* **5**, 2037–56 (2013).
- 794 41. Brouwer, K. L. R. *et al.* In Vitro Methods to Support Transporter Evaluation in Drug
795 Discovery and Development. *Clin Pharmacol Ther* **94**, 95–112 (2013).
- 796 42. Yoo, H., Antoniewicz, M. R., Stephanopoulos, G. & Kelleher, J. K. Quantifying Reductive
797 Carboxylation Flux of Glutamine to Lipid in a Brown Adipocyte Cell Line. *J Biol Chem* **283**,
798 20621–20627 (2008).
- 799 43. Metallo, C. M. *et al.* Reductive glutamine metabolism by IDH1 mediates lipogenesis under
800 hypoxia. *Nature* **481**, 380–384 (2012).
- 801 44. Mullen, A. R. *et al.* Reductive carboxylation supports growth in tumour cells with defective
802 mitochondria. *Nature* **481**, 385–388 (2012).
- 803 45. Jiang, L. *et al.* Reductive carboxylation supports redox homeostasis during anchorage-
804 independent growth. *Nature* **532**, 255–258 (2016).
- 805 46. Rendina, A. R. *et al.* Mutant IDH1 Enhances the Production of 2-Hydroxyglutarate Due to
806 Its Kinetic Mechanism. *Biochemistry-us* **52**, 4563–4577 (2013).
- 807 47. Torres, C. M. *et al.* The linker histone H1.0 generates epigenetic and functional intratumor
808 heterogeneity. *Science* **353**, aaf1644 (2016).
- 809 48. Hagos, Y. *et al.* α -Ketoglutarate-related inhibitors of HIF prolyl hydroxylases are substrates
810 of renal organic anion transporters 1 (OAT1) and 4 (OAT4). *Pflügers Archiv - European J*
811 *Physiology* **464**, 367–374 (2012).

- 812 49. Kato, S., Takahashi, T., Miyata, N. & Roman, R. J. DMOG, a Prolyl Hydroxylase Inhibitor,
813 Increases Hemoglobin Levels without Exacerbating Hypertension and Renal Injury in Salt-
814 Sensitive Hypertensive Rats. *J Pharmacol Exp Ther* **372**, 166–174 (2019).
- 815 50. Dirscherl, K. *et al.* Hypoxia sensing by hepatic stellate cells leads to VEGF-dependent
816 angiogenesis and may contribute to accelerated liver regeneration. *Sci Rep-uk* **10**, 4392
817 (2020).
- 818 51. BRÖER, S. *et al.* Characterization of the high-affinity monocarboxylate transporter MCT2
819 in *Xenopus laevis* oocytes. *Biochem J* **341**, 529 (1999).
- 820 52. Pérttega-Gomes, N. *et al.* Monocarboxylate transporter 2 (MCT2) as putative biomarker in
821 prostate cancer. *Prostate* **73**, 763 769 (2012).
- 822 53. Elia, I. *et al.* Breast cancer cells rely on environmental pyruvate to shape the metastatic
823 niche. *Nature* **568**, 1–5 (2019).
- 824 54. Huang, C.-K. *et al.* Adipocytes promote malignant growth of breast tumours with
825 monocarboxylate transporter 2 expression via β -hydroxybutyrate. *Nat Commun* **8**, 1
826 13 (2017).
- 827 55. Carey, B. W., Finley, L. W. S., Cross, J. R., Allis, C. D. & Thompson, C. B. Intracellular α -
828 ketoglutarate maintains the pluripotency of embryonic stem cells. *Nature* **518**, 413 (2015).
- 829 56. Schvartzman, J. M., Thompson, C. B. & Finley, L. W. S. Metabolic regulation of chromatin
830 modifications and gene expression. *J Cell Biol* **217**, jcb.201803061 (2018).
- 831 57. Islam, K. The Bump-and-Hole Tactic: Expanding the Scope of Chemical Genetics. *Cell*
832 *Chem Biol* **25**, 1171–1184 (2018).
- 833 58. Sudhamalla, B. *et al.* Complementary Steric Engineering at the Protein-Ligand Interface
834 for Analogue-Sensitive TET Oxygenases. *J Am Chem Soc* **140**, 1 7 (2018).
- 835 59. Zhang, T., Creek, D. J., Barrett, M. P., Blackburn, G. & Watson, D. G. Evaluation of
836 coupling reversed phase, aqueous normal phase, and hydrophilic interaction liquid
837 chromatography with Orbitrap mass spectrometry for metabolomic studies of human urine.
838 *Anal Chem* **84**, 1994–2001 (2012).