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Exploiting the co-reliance of tumours upon transport of amino acids and lactate: Gln and Tyr conjugates of MCT1 inhibitors[†]

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Abstract

Glutamine and tyrosine-based amino acid conjugates of monocarboxylate transporter types 1 and 2 inhibitors (MCT1/2) were designed, synthesized and evaluated for their potency in blocking the proliferation of a human B lymphoma cell line that expresses the transporters Asct2, LAT1 and MCT1. Appropriate placement of an amino acid transporter recognition element was shown to augment anti-tumour efficacy vs. Raji cells. Amino acid conjugation also improves the pharmacodynamic properties of experimental MCT1/2 inhibitors.

Graphical Abstract

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Introduction

Proteomics and functional analyses of many tumour types have shown a shared reliance upon active transport systems to drive rapid cancer cell growth and to sustain survival. For example, a greatly enhanced rate of uptake of amino acids, particularly glutamine and large neutral amino acids, is ubiquitous in malignant cells.^{1–5} These cells largely rely upon the Asct2 and LAT1 transporters for import of glutamine and large neutral amino acids, respectively, thus these transporters have been dubbed “partners in crime” for their role in driving cancer progression.⁵ Monocarboxylate transporters (MCTs) are also often up-regulated to transport lactic acid, a by-product of energy production via glycolysis (the Warburg effect).^{6–8} The co-reliance on MCT1, Asct2, and LAT1 is particularly manifest in tumours with *MYC* oncogene involvement.⁴

Tethered amino acids have been used to direct molecular imaging agents to tumour cells, which leads to the selective accumulation of the amino acid/imaging agent conjugates in cells that are reliant upon amino acid transporters. Examples include the imaging agents **1**⁹ and **2**¹⁰ (Figure 1), which are substrates for the Asct2 and LAT1 transporters, respectively.

The tethered glutamine residue of **1** and the tyrosine core of **2** deliver, respectively, the gadolinium-containing MRI contrast warhead and the radioactive fluorine-containing PET warhead to affected cells, permitting high sensitivity in detecting tumours. The transport of the Gd-containing complex **1**, in particular, indicates that even large complexes can be accommodated as substrates by Gln transporters.

A logical extension of this concept is to use amino acid transport to deliver not an *imaging agent* but rather an *anti-tumour agent* to cancer cells. This principle also has precedent in the mustard agent melphalan (**3**), which has enhanced brain exposure and efficacy relative to other aniline mustards because it is a LAT1 substrate (LAT1 is abundant in the bloodbrain barrier, as well as in many tumour types).¹¹ Indeed, the melphalan analogue **4** achieves ~1000-fold greater brain exposure *vs.* compound **3** due to improved LAT1 transport.¹²

A conceptually similar delivery strategy, using folates tethered to imaging agents or anti-tumour agents, has been recently reviewed.^{13,14} Here drug targeting to tumours relies upon enhanced folate receptor expression, which is manifest in ~40% of human cancers. Phase II clinical results of folate-conjugated small molecules have shown significant promise, though no agents have yet met stringent phase III clinical trial endpoints.¹³

We have begun to explore the potential of LAT1 and/or Asct2 transporters to facilitate the selective delivery of experimental therapeutic agents to tumour cells abundantly expressing these transporters. As a case study, we chose to investigate amino acid conjugation strategies to augment effectiveness of the MCT1/2 inhibitor **5** (Figure 3) that has been reported by AstraZeneca^{15–19} and that has also been studied in our labs for its anti-tumour potential.²⁰ A linker group (whether permanently attached or designed to be biodegradable) would be used to connect the MCT1/2 inhibitor to an amino acid transporter recognition element as shown schematically in compound **6** in Figure 3. The complex should direct MCT inhibitors to cells that over-express the relevant amino acid transporter. We reasoned that since most cancers are reliant upon glycolysis and many overexpress MCT1 (and are thus sensitive to this class of MCT inhibitors), their co-reliance upon lactate and the amino acid transporters LAT1 and Asct2 should result in additive or synergistic therapeutic benefits.

Results and Discussion

Inhibitor design

Preparing amino acid-conjugated analogues of inhibitor **5** required the identification of regions that are tolerant to substitution by large appended groups. In the published AstraZeneca synthesis, the naphthylmethyl group was attached to the pyrrole core by alkylation.¹⁹ Thus, we explored the anti-cancer cell-based activity of substituted analogues. We were encouraged to find that the bromide **7** (Figure 4) was similar in activity to MCT1/2 inhibitor **5** (EC₅₀ values 11 and 5 nM, respectively, in a standard MTT assay using a Raji human B-cell lymphoma line).²⁰

The bromine substituent²¹ provided access to alkyne-substituted analogues via Sonagashira coupling reactions.²² For example, a Pd-catalyzed coupling reaction of **7** with N-phthaloyl-protected 1-amino-4-pentyne gave alkyne **8**. Further, hydrazinolysis of **8** followed by coupling with N-Boc-Gln and Boc deprotection gave the Gln conjugate **9**. Coupling of bromide **7** with a terminal alkyne O-linked to a protected tyrosine residue gave, after deprotection, the Tyr conjugate **10**. Details of the synthetic route are discussed below; experimental conditions are provided in the supplementary material that accompanies this article.

The Raji B-cell lymphoma cell line was selected for these studies based upon expression profiling analyses, wherein we found that, relative to levels expressed in wild type (WT) B cells, *Asct2*, *Lat1* and *Mct1* mRNA levels were highly elevated in premalignant and neoplastic B cells from the Eμ-*Myc* transgenic mouse, a validated model of human Burkitt B cell lymphoma such as Raji cells that have *MYC/Immunoglobulin* gene translocations (Figure 5).²⁰ Co-elevation of all three transporters is undoubtedly not unique to Burkitt lymphoma. By qRT-PCR this cell line expresses the MCT1 and MCT2 isoforms but not the

MCT4 isoform (Figure 6). The absence of MCT4 blocks a possible pathway for the development of treatment resistance upon exposure to MCT1/2 inhibitors. *Together, these findings indicate that this human lymphoma cell line is appropriate for biological assessment of our new conjugates.* As a control we used MDA-MB-231 cells, which highly express MCT4, moderately express MCT2, and do not express MCT1 (Figure 6). Cytotoxicity (MTT assay) of compounds in Raji cells with no toxicity in MDA-MB-231 cells is thus consistent with an MCT1/2 inhibition-driven effect. Unless indicated, all compounds reported here with activity vs. Raji cells were inactive vs. MDA-MB-231 cells.

The Tyr conjugate **10** (Figure 4) was significantly more active in blocking lymphoma cell proliferation ($EC_{50} = 0.8$ nM) than was the parent MCT1/2 inhibitor **5** ($EC_{50} = 5$ nM), which was similar in potency to the Gln conjugate **9** ($EC_{50} = 4$ nM). Enhanced cell-based efficacy in Gln conjugates was, however, observed when the naphthyl group was replaced with an isoquinoline group and a diamine linker was also used instead of an alkyne-containing linker (see compound **11**, Figure 6). This compound was highly efficacious vs. Raji cells ($EC_{50} = 0.16$ nM). An attempt to reduce the lipophilicity of the parent MCT1/2 inhibitor, using a pyridine ring in place of the isoquinoline group, gave a compound (**12**) with reduced cell-based efficacy ($EC_{50} \sim 100$ nM), supporting the known preference of the MCT1 transporter for large aromatic groups in this region.¹⁹ A quinoline group was only slightly less preferred (compound **13**, $EC_{50} = 0.8$ nM) and was superior to the pyridine analogue **14** ($EC_{50} \sim 500$ nM). Glutamine conjugates **9** and **11–15** were in every case more highly efficacious vs. Raji cells than were related compounds lacking the Gln residue (compounds **5** and **15–18**), see Figure 7. The enhanced efficacy of the LAT1 conjugate **10** and the Gln conjugates **11–14** in MTT assays in Raji cells is consistent with the hypothesis that upregulated amino acid transporters in this tumour cell line recognize the conjugates as substrates and thus augment tumour exposure to these compounds. Based upon modelling studies that use a published MCT1 homology-based structure,²³ the linker and conjugated amino acid of **9–11** should occupy a solvent-exposed region and should contribute very little to their intrinsic affinity for MCT1. In support of this, an assay using ¹⁴C-labeled lactate showed no appreciable difference in effects upon lactate flux among compounds **9–11** (data not shown).

Amino acid conjugation also affects both the rate of compound uptake and the tissue distribution of compounds over time. In a head-to-head study, the tyrosine conjugate **10** and the parent compound **5** were each dosed in mice at 20 mg/kg IP using a 1 mg/ml formulation in 10:10:80 DMSO:Tween80: water. Compound **10** was found to be present at higher levels in nearly all tissues at the 6h and 24h time points (Table 1). Further, unlike parent compound **5**, Compound **10** was detectable in all tissues a full *seven days* after IP administration. Amino acid conjugation thus apparently facilitates uptake following IP dosing and also increases compound residence time in all tissues. While this experiment was not done in tumour-bearing mice, a similar increase in compound residence time within tumour cells is consistent with the observation of increased efficacy for compound **10** vs. compound **5** in the four day MTT assays used to assess effects upon tumour cell growth. Also in support of this proposed mechanism of efficacy enhancement for the conjugates, a single point study of six tumour-bearing *NOD/SCID* mice showed that, at 24h, the concentration of conjugate **10** in

tumours ranged from 210–460 nM, with a mean of 300 nM. Thus, tumour drug levels significantly exceed the drug levels in plasma and blood. Increased accumulation in the liver, spleen and kidney may be lipophilicity-driven. Accordingly, more hydrophilic MCT1 inhibitors should localize to tumours with higher specificity.

Synthesis Procedures

The preparation of test MCT1/2 inhibitors in this series relied upon a scalable synthesis of the core scaffold with all of the required substituents except for the aryl-CH₂- or heteroaryl-CH₂ chain. Compound **27** (Scheme 1) was prepared in a 7-step route that featured an olefin metathesis reaction²⁴ and one-pot directed lithiation-alkylation strategy, as we previously reported.^{25,26} Alkylation with 1-(bromomethyl) naphthalene and desilylation of **27** is used to prepare the MCT1/2 inhibitor **5**.¹⁹

Using other electrophiles to alkylate the pyrrole nucleus gave other parent MCT1/2 inhibitors and amino acid conjugates. For example (Scheme 2), alkylation with bromide **29** gave access to compound **7** by deprotection, compound **8** by Sonogashira coupling, compound **9** by phthalimide deprotection, Gln residue coupling, and Boc deprotection, and compound **10** by Sonagashira coupling using alkyne **31**, prepared from Boc-Tyr methyl ester in two steps.²⁷ For the full structures of compounds **7**, **9**, and **10**, please see Figure 4.

The alkylation using electrophiles containing quinoline, isoquinoline, 4-pyridyl, and 3-pyridyl-containing heterocycles is shown in Scheme 3. The chloro groups of alkylation products **32–35** were present in test MCT1 inhibitors **36–39**. Buchwald-Hartwig reactions²⁸ of the chloroaryl compounds with 1,5-diaminopentane gave the diamine-containing analogues **40–43**, and after desilylation gave the test MCT1 inhibitors **15–18**. Diamines **40–43** were then used to access the glutamine conjugates **11–14** by amide coupling reactions followed, in each case, by TBDMS deprotection.

Structure-Activity Relationships

Results from a 96h MTT assay using Raji Burkitt B-cell lymphoma²⁰ are shown in Table 2. Larger aromatic groups were preferred (**36** vs. **38**, **37** vs. **39**, **17** vs. **18**, **15** vs. **16**, **13** vs. **14**, **11** vs. **12**). Isoquinolines, in the most potent compounds, were slightly preferred over quinolines (**11** vs. **13**). Amino acid-containing compounds were among the most potent compounds studied in this cell-based assay (compounds **9** and **11–14**). As discussed earlier, in six separate instances the amino acid-containing MCT1 inhibitor was more cytotoxic to Raji cells than were related compounds lacking the tethered amino acid: compare the tyrosine (LAT1-transported) conjugate **10** to the parent MCT1 inhibitor **5**, and also compare the glutamine (Asct2-transported) conjugates **9** and **11–14** to compounds **5** and **15–19**.

Conclusions

Appending Gln and Tyr residues to an MCT1 inhibitor, similar to their use in contrast imaging agents, consistently enhanced toxicity vs. Raji cells, which express MCT1 and the amino acid transporters LAT1 and Asct2. The enhanced cytotoxicity likely facilitated transport into Raji cells. Future work includes linker and transporter recognition element

optimization, MCT inhibitor optimization and the application of such conjugation strategies to other classes of anti-tumour agents.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Molecular imaging agents using amino acid transport

Figure 2.
Mustard agents transported by LAT1

Figure 3.
MCT1 inhibitor and its amino acid conjugates

Figure 4.
Tyr and Gln conjugates of MCT1 inhibitor 5.

Figure 5.

Expression of the Gln transporter *Asct2* (left), the essential neutral amino acid transporter *Lat1* (middle) and *Mct1* (right) in WT vs. premalignant Eμ-Myc splenic B cells and in Eμ-Myc lymphomas. Each symbol represents one mouse.

Figure 6.
Expression profiling for MCT isoforms by qRT-PCR

Figure 7.
Gln conjugates and comparator compounds

Scheme 1.

a. vinyl magnesium chloride, Et₂O, 70%; b. methyl acrylate (5 eq), Grubbs 2nd gen. metathesis catalyst²⁴ (3%), CuI (6%), Et₂O, 35°C, 81%; c. activated MnO₂ (10 eq), 99%; d. NaH (2 eq), TOSMIC acid (1.5 eq), DMF, 0–25°C, 53%; e. methyl hydrazine (3 eq), EtOH, reflux, 16h, 90%; f. Boc anhydride (2 eq), DMAP (0.1 eq), CH₃CN, RT, 95%; g. LDA (2.0 eq), THF, dithiane **26** (2 eq), 68%.

Scheme 2.

a. NBS, CCl_4 , 62%; b. NaH, KI, DMF, 80%; c. TBAF, THF, 88%; d. N-phthaloyl 1-amino-4-pentyne,²⁹ $\text{Pd}(\text{PPh}_3)_4$, pyrrolidine, 42%; e. $\text{H}_2\text{NNH}_2\text{-H}_2\text{O}$, EtOH,; then N-Boc-Gln, HATU, DIPEA, DMF, 21%; then 4M HCl in dioxane, 97%; f) $\text{Pd}(\text{PPh}_3)_4$, pyrrolidine, 80°C, 42%; then 4N HCl in dioxane, 51%; g) 5-chloro-1-pentyne, K_2CO_3 , DMF, 97%; then LiOH- H_2O , MeOH-THF, 99%.

Table 1

Tissue distribution studies for compounds **5** and **10**, each dosed at 20mg/kg IP, using solutions of 1 mg/mL in a vehicle of 10:10:80 DMSO:Tween80:water

tissue	[5] at 6h (nM)	[5] at 24h (nM)	[5] at 7 d (nM)	[10] at 6h (nM)	[10] at 24h (nM)	[10] at 7 d (nM)
plasma	27	4	0	3270	12	4
blood	140	63	0	4330	76	41
brain	190	270	0	110	62	43
liver	63	60	0	2500	940	98
spleen	740	420	43	2260	1060	190
kidney	120	160	0	1620	960	210
lung	61	74	0	1540	520	110

Table 2

Results from 96h MTT assay using Raji Burkitt B-cell lymphoma. All compounds were nontoxic over 96h at 10 μ M to the control MCT1-deficient breast cancer cell line MDA-MB-231 using an MTT assay, indicating that selective cytotoxicity is driven by MCT1 inhibition.

 structure X =	EC ₅₀ (nM)	 structure X =	EC ₅₀ (nM)
 5	60	 7	1000
 36	60	 38	1000
 37	55	 39	690
 17	24	 18	520
 15	0.8	 16	420
 13	0.8	 14	500
 11	0.16	 12	100
 9	4	 10	0.8