Cysteine 159 delineates a hinge region of the alternating access monocarboxylate transporter 1 and is targeted by cysteine-modifying inhibitors

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Keywords
basigin; L-lactate; methanethiosulfonates; monocarboxylate transporter; pCMBS

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Monocarboxylate transporter isoforms 1–4, MCT, of the solute carrier SLC16A family facilitate proton-coupled transport of L-lactate. Growth of tumors that exhibit the Warburg effect, that is, high rates of anaerobic glycolysis despite availability of oxygen, relies on swift L-lactate export, whereas oxygenic cancer cells import circulating L-lactate as a fuel. Currently, MCTs are viewed as promising anticancer targets. Small-molecule inhibitors have been found, and, recently, high-resolution protein structures have been obtained. Key questions, however, regarding the exact binding sites of cysteine-modifying inhibitors and the substrate translocation cycle lack a conclusive experimental basis. Here, we report Cys159 of the ubiquitous human MCT1 to reside in a critical hinge region of the alternating access-type transporter. We identified Cys159 as the binding site of the organomercurial pCMBS. The inhibitory effect of pCMBS was proposed to be indirect via modification of the chaperone basigin. We provide evidence that pCMBS locks MCT1 in its outward open conformation in a wedge-like fashion. We corroborated this finding using smaller cysteine-modifying reagents that size-dependently inhibited L-lactate transport. The smallest modifiers targeted additional cysteines as shown by a C159S mutant. We found a Cys399/Cys400 pair to constitute the second hinge of the transporter that tolerated only individual replacement by serine. The hinge cysteines, in particular the selectively addressable Cys159, provide natural anchors for placing probes into MCTs to report, for instance, on the electrostatics or hydration upon binding of the transported L-lactate substrate and the proton cosubstrate.

Lactate is a key metabolite in human physiology. Most notably, it is the end product of anaerobic glycolysis of highly glycolytic cells and a fuel for oxygenic cells [1,2]. To maintain lactate homoeostasis and to allow lactate shuttling, of special importance in tumors, cells express distinct monocarboxylate transporters (MCT) of the solute carrier family SLC16A [3]. Extensive functional characterization determined MCT1–4 as bidirectional, proton-coupled transporters that mediate secondary-active transport of a diverse spectrum of monocarboxylates including lactate, pyruvate, short-chain-fatty acids and ketone bodies [3]. Substrate selectivity and transport kinetics as well as tissue distribution differ between the MCT isoforms. MCT1 (K_m 3.5 mM) is ubiquitously expressed and has been found to mediate in- and efflux of L-lactate at physiological

Abbreviations
BMTS, benzyl methanethiosulfonate; BSG, basigin; EPR, electron paramagnetic resonance; MCT, monocarboxylate transporter; pCMBS, p-chloromercuribenzen sulfonate; PfFNT, Plasmodium falciparum formate-nitrite transporter; PMTS, propyl methanethiosulfonate; SLC, solute carrier; TMDs, transmembrane domains.
conditions. MCT2 is a high-affinity isoform (K_m 0.74 mM) and mainly confers 1-lactate uptake, whereas lactate efflux from highly glycolytic cells is brought about by MCT4 (K_m 28 mM) [4]. MCTs consist of 12 transmembrane domains (TMDs), intracellular N- and C-terminal ends and a long flexible loop between TMD 6 and 7 [5,6]. The recently published structure of MCT1 confirmed the existence of two distinct transporter conformations and a two-domain architecture of six helices each (TM1–6 and TM7–12) arranged in a pseudo-twoworld symmetry. Relative movement of both domains enables an alternating access mechanism in which the transporter toggles between an outward and an inward open conformation [5]. Early on, protonation of an exposed residue in the transporter cavity was suggested to generate a positive charge suitable for substrate anion attraction and H^+ cotransport [7,8]. Subsequent domain reorganization to the inward open conformation would then release the substrate and a proton to the cytosolic side. This process is reversible, and the restoration of the original conformation was defined as the rate-limiting step of trans- 
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protein hints at a functional association and an increased basigin-mediated translocation to the plasma membrane. This is in line with previous reports on MCT plasma membrane trafficking in yeast [22]. The obtained transport activities were suitable for carrying out inhibitor studies.

Initially, we assayed the effect of AZD3965 on MCT1 and found an IC₅₀ value of $96 \pm 2 \text{nM}$ (Fig. 2A). The inhibitor was equally effective on the BSG-MCT1 fusion construct yielding an IC₅₀ of $118 \pm 15 \text{nM}$ (Fig. 2B). This establishes a direct and basigin-independent inhibitory effect of AZD3965 on MCT1 that is in accordance with the very recent structural data [5].

**pCMBS is a direct inhibitor of MCT1 acting via covalent Cys159 modification**

The cysteine-modifying organomercurial pCMBS was suggested to exert indirect effects on MCT by covalently binding to basigin. This assumption was in part based on several cysteine point mutations of MCT1 of which none reversed inhibition by pCMBS [15].

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**Fig. 1.** Expression of functional MCT1 alone and as fusion with basigin in yeast. (A) Western blot showing expression of MCT1 wild-type and C159S mutants alone and in fusion with basigin (BSG). Five micrograms of total protein was loaded per lane. Protein was detected via a Penta-His antibody. Nonexpressing cells served as control (—). The arrowhead indicates the full-length fusion proteins. (B) Transport rates for the uptake of $^{14}$C-labeled L-lactate of cells expressing MCT1 alone and the BSG-MCT1 fusion protein. Minimal background of nonexpressing cells was subtracted. Data represent mean $\pm$ SEM of three biological replicates.

**Fig. 2.** Inhibition of MCT1-mediated L-lactate transport by AZD3965 and pCMBS. A, B. IC₅₀ curves showing transport activity of MCT1 alone (A) and fused to basigin (BSG, B) after treatment with AZD3965 (15 min). The data points represent mean $\pm$ SEM of 3–9 replicates. (C, D) Time-dependent inhibition of MCT1 alone (C) and in fusion with BSG (D) by the covalent cysteine-modifier pCMBS (30 µM). Data depict mean $\pm$ SEM of three biological replicates. Minimal background of nonexpressing cells was subtracted.
yeast system allowed us to address this question more specifically. When expressing functional MCT1 alone, that is, in the absence of basigin, incubation with 30 µM pCMBS blocked L-lactate transport in a time-dependent manner (Fig. 2C). Fifteen minutes of incubation resulted in nearly complete inhibition of MCT1 transport. The extent and time profile were unaltered in the BSG-MCT1 fusion construct (Fig. 2D). This clearly indicates that MCT1 is the direct target of covalent cysteine modification by pCMBS.

In order to identify the cysteine in MCT1 that is targeted by pCMBS, we excluded the previously mutated cysteines [15] and focused on cysteines that are conserved in the pCMBS-inhibitable MCT1 and MCT4 isoforms but are absent in the insensitive MCT2 and MCT3. This drastically decreased the number of candidates and unveiled MCT1-Cys159 (Cys161 in MCT4) as the most-likely candidate (Fig. 3A,B).

According to the recently published structure of MCT1 [5], Cys159 is located close to the substrate binding site at the bottom of a deep cleft. Cys159 appears solvent-accessible in the outward open conformation yet buried in the inward open state (Fig. 3A). Its position can be described as a hinge region in the MCT structure because movement between the outward and inward open conformations pivots around Cys159.

We mutated Cys159 of MCT1 to serine and expressed the mutant protein alone (MCT1-C159S) or fused with basigin (BSG-MCT1-C159S). The mutants exhibited indistinguishable expression levels (Fig. 1A) and comparable (MCT1) or slightly decreased (BSG-MCT1) transport rates compared with the respective wild-type proteins (Fig. 3C). Strikingly, the Cys159 to serine exchange rendered MCT1 insensitive to treatment by pCMBS (Fig. 3D). Even prolonged incubation for 15 min did not affect MCT1 transport activity. Further, fusion with basigin did not rescue inhibitor sensitivity, indicating that the inhibitory action of pCMBS is solely due to binding to Cys159 of the MCT1 protein (Fig. 3D).

Rat erythrocyte MCT1 was shown before to be non-reactive toward pCMBS [15]. The species specificity was attributed to the observation that the rat MCT1 variant preferably associates with embigin rather than basigin. Aligning the rat and human MCT1 protein sequences revealed that rat MCT1 naturally carries a serine residue instead of a cysteine at position 159 (Fig. 4A). We successfully expressed functional rat MCT1 before in yeast in the absence of embigin or basigin [25]. Here, we assayed the effect of pCMBS on

Fig. 3. Position of Cys159 in human MCT1 and effect of mutation to serine. (A) Side and top views of the 3D structure of MCT1 in the inward (left, PDB #7CKO) and outward open conformations (right, PDB #6LZ0). All cysteines are highlighted in red/yellow, and cysteines at hinge positions are labeled; the image was generated using the Chimera software [40]. (B) Section of a sequence alignment around MCT1-Cys159 of human MCT1--4. (C) Transport rates for the uptake of 14C-labeled L-lactate of cells expressing MCT1-C159S alone and in fusion with basigin (BSG). (D) Transport activity of MCT1-C159S treated with pCMBS (30 µM, 15 min). For (C and D), background of nonexpressing cells was subtracted, and data represent mean ± SEM of three biological replicates.
Indeed, treatment with pCMBS did not alter transport activity (100% /C6 18%). Together, these experiments identify Cys159 as the target site for direct inhibition of human MCT1 by organomercurials.

Carrying a heavy-metal atom and a phenyl ring, pCMBS is a bulky reagent. Considering the Cys159-bound pCMBS to act as a wedge fixing the outward open conformation of MCT1, we asked whether smaller cysteine modifiers would lead to partial, more differentiated inhibition of MCT1-mediated transport. Therefore, we used a set of established cysteine-modifying methanethiosulfonate (MTS) reagents carrying differently sized transfer groups (Fig. 5A).

Reagents transferring a neutral thiomethyl (MMTS), thiopropyl (PMTS), or thioethyl moiety (BMTS) decreased MCT1 transport in a time- and concentration-dependent manner (Fig. 5B). MTSES carrying a negatively charged thioethylsulfonate moiety did not affect MCT1 lactate transport to a residual 34% ± 5% and 57% ± 8%, respectively. To confirm covalent modification of MCT1 by the compounds, we washed the cells to remove excess MTS reagent prior to the transport assay, yet inhibition remained (Fig. 5C). To evaluate whether the MTS incubation procedure is specific for MCT1 cysteine modification and does not interfere with the yeast cells or the transport assay system in an unspecific way, we expressed an MCT-unrelated microbial lactate transporter, PfFNT [26]. PfFNT lacks solvent-accessible cysteine residues and is not inhibitable by pCMBS [26]. Incubation of PfFNT with MMTS did not affect t-lactate transport (Fig. 5D) confirming an MCT1- and cysteine-specific mode of action of the MTS reagents. Strikingly, the achievable degree of MCT1 inhibition with MTS reagents correlated with the molecular volume of the moiety transferred by the cysteine modifier (Fig. 5E).

The residual activity of MCT1 after modification by MTS allowed us to test for Cys159 site-specificity by subsequently measuring pCMBS inhibition. Indeed, pretreatment of MCT1-expressing cells with MMTS prevented further inhibition by pCMBS (Fig. 5F). Preincubation with MTSES that failed to inhibit MCT1 previously, in turn maintained pCMBS reactivity and MCT1 transport, was fully inhibited (Fig. 5F).

Size-dependent inhibition in connection with the localization in the transporter structure points to Cys159 as a pivot point in the translocation cycle.

Cysteine-modifier size determines site-specificity

We next examined the MTS reagents regarding their specificity for modification of Cys159 using the MCT1-C159S mutant. Other than with pCMBS before, the mutant was not fully resistant to MTS treatment (Fig. 6A). Although none of the compounds gave rise to a complete block of MCT1-C159S transport activity, we observed substantial inhibition. This indicated that other cysteines were additionally targeted.

Calculation of a selectivity index, that is, the difference in inhibition of MCT1 wild-type and C159S mutant related to the inhibition of the wild-type protein, \( \frac{I_{\text{wt}} - I_{\text{C159S}}}{I_{\text{wt}}} \), provided a quantitative measure for the extent to which Cys159 contributes to the inhibition. Here, a value of 1 indicates that Cys159 is...
specifically modified, whereas a value of 0 is obtained for Cys159-independent effects. Accordingly, the Cys159-specific pCMBS gave a value of 1.08 ± 0.06 (Fig. 6B). PMTS and BMTS yielded an equal selectivity index of 0.17 ± 0.20, whereas MMTS action appeared independent of Cys159 modification (Fig. 6B). This points to other cysteines that are size-dependently targeted by the MTS reagents but are out of reach for the bulky pCMBS.

We identified a Cys399/Cys400 pair residing in a similar hinge position as Cys159, yet across the substrate binding region at the opposite side of the protein. Considering the thermal motion of the Cys399/Cys400 sidechains in particular in the outward open conformation of MCT1 (b-factors of 67 vs. 37 in the inward open structure), they might be accessible for small modifiers from within the transport path (Fig. 3A). Strikingly, an MCT1-C399S/C400S double mutant was expressed by the yeast cells but the transporter was not functional hinting at a critical position in the protein. Individual mutations of C399S and C400S were tolerated and functional. The single C399S and C400S mutants and combinations with the C159S mutation exhibited the same degree of MMTS sensitivity throughout (Fig. 6C). We conclude that alteration of cysteines in the hinge region interferes with protein motion during substrate translocation. Selectivity for Cys159 increases with the size of the cysteine modifier, and the organomercurial pCMBS being fully Cys159-specific (Fig. 6D).

**Discussion**

Using yeast expression to produce functional human MCT1 in the absence of its chaperone basigin was key in finding direct MCT1 inhibition by pCMBS. The cysteine modifier is a long-known inhibitor of MCT1 and MCT4 functionality [27,28]. The proposal that pCMBS targets basigin rather than the transporter itself derived from cysteine mutations based on structure models of MCT4 using crystal data of the *Escherichia coli* glycerol-3-phosphate transporter and lactose permease [15]. Since none of the mutations in the MCT conferred resistance to pCMBS, interference
with structure-relevant disulfide bridges of the ancillary protein basigin was assumed. The cysteines at positions 41 and 87 of the predominant basigin variant 2 assure proper folding of the extracellular immunoglobulin-like C2 domain. Covalent modification and misfolding were assumed to be transmitted to the MCT and block transport [15]. Nonetheless, given that a misfolded basigin prevents trafficking of the MCT to the plasma membrane, direct evidence for transport modulation is lacking. Noteworthy, Cys159 of human MCT1 has not been under investigation.

Today, three high-resolution structures of MCT family members are available. These are the bacterial SfMCT [29], human MCT2 [6], and, just recently, human MCT1 [5]. Our data show that pCMBS binds covalently to Cys159 in MCT1, and we postulate that this modification obstructs movement locking MCT1 in its outward open conformation.

A small number of previous studies addressed effects of certain cysteine residues on the MCT transport properties. Cys362 of the bacterial SfMCT was shown to be relevant for lactate transport [29]. The crystal structure revealed that its side chain contributes to the substrate binding site and mutation to alanine greatly reduced transport activity. Reported mutations of Cys336 in rat MCT1 to alanine (corresponding to Cys343 in human MCT1) [30], as well as Cys399 of human MCT1 to alanine, did not change transport properties [31]. We confirmed the latter in this study and also found the neighboring Cys400 not to affect transport if mutated alone. Strikingly, though, the C399S/C400S double mutation was not tolerated. The new structure data show that Cys159 at the one hand side and Cys399/Cys400 at the other are positioned at the two hinges around which the MCT pivots during the translocation cycle.

Covalent modification of the hinge Cys159 resulted in decreased functionality that correlated with the size of the introduced group (Fig. 5E). The spacious aromatic moieties of pCMBS (Fig. 6D) and BMTS fully blocked the transporter, whereas with the smallest modifier MMTS a maximal decrease in transport activity by about half was achievable. The molecular size of the reagent further determines which cysteines are accessible for modification. As a membrane-impermeable reagent, pCMBS can only reach cysteines that are well exposed to the extracellular solvent. Cys159 fulfills this property. MMTS is membrane-permeable; therefore, it is thinkable that in addition to Cys159, and possibly Cys399/Cys400, even more cysteines might be modified. Clearly, smaller reagents tend to elicit more unspecific effects due to their enhanced mobility.

It is noteworthy that MTSES failed to affect MCT1 transport activity. The transferred group is of an intermediate volume between BMTS and PMTS, yet carries a negative charge due to the sulfonic acid moiety. Another negatively charged cysteine modifier, 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), was recently tested with MCT1 and equally failed to inhibit transport [11]. From this, the authors concluded that cysteine residues in general were irrelevant for MCT transport activity. It appears likely that DTNB and MTSES, due to their negative charge, do not reach Cys159 in the hinge region of the transporter. Neither do the charged compounds gain access to the additional target cysteines of MMTS which are probably more buried in the protein structure.

The established accessibility of MCT1-Cys159 and its central role in the transduction cycle provide
mechanistic insight into MCT functionality. Importantly, the cysteine is naturally present in the protein and selectively addressable with modifiers. This opens up possibilities for labeling applications using the native MCT1 or MCT4 proteins. Various probes might be introduced to report on the local electrostatics or hydration during transport [32], and conformational changes using fluorescence [33,34], or electron paramagnetic resonance (EPR) [35]. Exploitation of Cys159 may therefore serve as a tool for further biophysical characterization of the MCT substrate recognition, attraction, and translocation cycle.

Materials and Methods

Plasmids, cloning, and mutations

Human MCT1 (GenBank NM_001166496) and basigin variant 1 (GenBank NM_001728) were obtained as ORF clones in pcDNA3.1(+)/C-KDYK (GenScript). The MCT1 ORF was cloned into the yeast expression plasmid pDR196 (Addgene #36029) [36] using PflMI and BspEI restriction sites introduced via a pair of oligonucleotides. Basigin splice variant 2 (lacking the N-terminal signal peptide) was produced via PCR with additional SpeI and PstI restriction sites and inserted upstream of the coding region for MCT1. All constructs contained an N-terminal hemagglutinin epitope tag and a C-terminal His10 affinity tag. Site-directed point mutations were introduced using oligonucleotides with respective nucleotide exchanges according to the QuikChange protocol (Agilent, Santa Clara, CA, USA). All constructs were sequenced for verification.

Saccharomyces cerevisiae W303-1A jen1A ady2Δ (MATa, can1-100, ade2-loc, his3-11-15, leu2-3,-12, trpl-1-1, ura3-1, jen1::kanMX4, ady2::hphMX4) yeast cells [37] lacking endogenous monocarboxylate transporters were kindly provided by M. Casal (Braga, Portugal). Cells were transformed using the lithium acetate/single-stranded carrier DNA/polyethylene glycol method [38]. Cells were grown at 29 °C in selective SD medium supplemented with adenine, histidine, leucine and tryptophan, and 2% (w/v) glucose but lacking uracil.

Isolation of microsomal proteins, SDS/PAGE, and Western blot

40–50 mL of yeast culture was grown to an optical density (OD600) of 1.0 ± 0.1, collected (4000 g, 5 min, 4 °C), and washed with 50 mL of ice-cold water and 10 mL of ice-cold TE buffer (25 mM Tris/HCl, 5 mM EDTA, pH 7.5). Pellets were frozen and stored for at least 30 min at –80 °C. For disruption, the cell pellets were resuspended in 0.5 mL TE buffer and vortexed with 0.5 g of acid-washed glass beads (Ø 425–600 μm, Sigma-Aldrich, Taufkirchen, Germany) in twelve cycles of 30 s each. Suspensions were cleared by centrifugation at 1000 g (5 min, 4 °C). Microsomal proteins were obtained by removal of high-density cell components at 10 000 g (5 min, 4 °C) and ultracentrifugation at 100 000 g (45 min, 4 °C). Membrane pellets were resuspended in 0.1 mL phosphate buffer (100 mM sodium phosphate, 50 mM NaCl, pH 8.0) for determination of the total protein content using the Bradford Protein Assay (Bio-Rad, Feldkirchen, Germany) and for separation by SDS/PAGE. Five micrograms of total protein was loaded per lane. For western blots, the proteins were blotted on PVDF membranes (Hybond P 0.45, GE Healthcare, Solingen, Germany) and detected via a monoclonal mouse penta-His antibody (Cat. Nr. 34660, Qiagen, Hilden, Germany) and a horseradish peroxidase-conjugated secondary goat-anti-mouse antibody (Cat. Nr. 115-035-174, Jackson ImmunoResearch, Eching, Germany) with the Clarity ECL substrate detection system (Bio-Rad). Visualization was done with a ChemiStar Touch ECL & Fluorescence Imager (Intas Science Imaging Instruments, Göttingen, Germany).

Substrate transport assays

The uptake of L-lactate was assayed as described before [26]. Briefly, yeast cells were harvested at an OD600 of 1.0 ± 0.1, washed once with ice-cold water, and resuspended in uptake buffer (50 mM HEPES/50 mM Tris, pH 6.8) to a final OD600 of 50 ± 5. Inhibitors were purchased from Toronto Research Chemicals Inc. (p-chloromercuribenzenesulfonate, pCMBS), Interchim (propyl methanethiosulfonate, PMTS; benzyl methanethiosulfonate, BMTS), Biotrend ((2-sulfonatoethyl)pyl methanethiosulfonate, PMTS, BMTS), Sigma-Aldrich (methyl methanethiosulfonate, MTSES), and Hycultec (AZD3965). Inhibitor stock solutions were prepared in water (pCMBS, MTSES, MTSES) or DMSO (PMTS, BMTS, AZD3965). The following steps were conducted in 1.5-mL tubes at 19 ± 1 °C. Transport was initiated by mixing 80 μL of cell suspension (± pretreatment with 1 μL inhibitor solution) containing 5.6 mg yeast cells with 20 μL of substrate solution (1 mM L-lactate spiked with 0.04 μCi 14C-L-lactate; Hartmann Analytics). Transport was stopped by abrupt addition of 1 mL of ice-cold water, rapid transfer of the sample to a GF/C glass microfiber filter (GE Healthcare), vacuum filtration, and washing with 7 mL of ice-cold water to remove excess substrate. Filters were analyzed in 3 mL of scintillation cocktail (QuickSafe A, Zinsser Analytic, Eschborn, Germany) and scintillation counting (Packard TriCarb 2900TR, Perkin Elmer, Rodgau, Germany). L-lactate uptake rates (± inhibitor/pretreatment) result from single-time-point measurements in the initial, linear phase.
of transport (2 min; rat MCT1, 4 min). Nonexpressing cells underwent the same treatment (cells were treated with solvent only) for background subtraction.

Structure models, analyses, and alignments

Protein structure data for human MCT1 (PDB #7CKO and #6LZ0) [5] were gathered from the RCSB Protein Data Bank [39] and visualized with UCSF Chimera [40]. Protein sequences for human MCT2 (NM_001270622), MCT3 (NM_013356), and MCT4 (NM_001206950) as well as rat MCT1 (NM_012716) were obtained from the NCBI GenBank. Van der Waals volumes of the transferred groups of the inhibitors were calculated using chemicalize.org by ChemAxon.

Statistical analysis

Graphs were visualized using SIGMAPlot (version 11.0, Systat Software, Erkrath, Germany). L-lactate uptake rates and IC₅₀ values were determined from at least three biological replicates each with two to three technical repetitions. Data represent mean ± SEM. Inhibitor efficiency was calculated after completion of cysteine modification (pCMBS: 0.03 mM, 15 min; BMTS and PMTS: 5 mM, 15 min; MMTS: 1 mM, 5 min).

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

ALK generated, expressed, and assayed the constructs and AZD3965, pCMBS, MMTS, MTSES, PMTS, BMTS modifications, analyzed data, and wrote the manuscript; KG assayed PMTS and BMTS modifications, analyzed data, and wrote the manuscript; EB conceived and designed the study, analyzed the data, and wrote the paper.

Peer Review

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