L-lactate is an important metabolite, energy source, and signaling molecule in health and disease. In mammals, its transport across biological membranes is mediated by monocarboxylate transporters (MCTs) of the solute carrier 16 (SLC16) family. Malfunction, overexpression or absence of transporters of this family are associated with diseases such as cancer and type 2 diabetes. Moreover, lactate acts as a signaling molecule and virulence factor in certain bacterial infections. Here, we report the rational, structure-guided identification of potent, nanomolar affinity inhibitors acting on an L-lactate-specific SLC16 homologue from the bacterium Syntrophobacter fumaroxidans (SfMCT). High-resolution crystal structures of SfMCT with bound inhibitors uncovered their interaction mechanism on an atomic level and the role of water molecules in inhibitor binding. The presented systematic approach is a valuable procedure for the identification of L-lactate transport inhibitors. Furthermore, identified inhibitors represent potential tool compounds to interfere with monocarboxylate transport across biological membranes mediated by MCTs.
The solute carrier 16 (SLC16) family constitutes a diverse group of membrane transporters composed of 14 genes in the human genome (i.e., SLC16A1-A14). The broad range of transported substrates (e.g., L-lactate, pyruvate, ketone bodies, short-chain fatty acids, thyroid hormones, aromatic amino acids, amino acid metabolites, or drugs) reflects the diversity of the SLC16 family. Within the transporter classification (TC) system, the SLC16 family represents the monocarboxylate transporter (MCT) family (TC-ID 2.A.1.13), which is a subgroup of the major facilitator superfamily (MFS, TC-ID 2.A.1). MFS transporters share a common fold, which is characterized by 12 transmembrane helices (TMs) assembled into two interconnected six-helix bundles. These bundles are related to each other by a pseudo-twofold symmetry axis that runs perpendicular to the membrane plane and lies in the substrate translocation pathway. An arginine residue is conserved in transmembrane helix 8 of most SLC16 family members and its positively-charged guanidinium group is involved in the binding of the negatively-charged carboxylate group of substrates and ligands. Several SLC16 family members are associated with health disorders. For example, MCT1 and MCT4 are overexpressed in certain tumors and play an essential role in their metabolism. MCT11 and MCT13 are associated with type 2 diabetes risk. Mutations in the SLC16 family represent potential tool compounds to interfere with bacterial infections. In Neisseria meningitidis, it acts as a signaling molecule for microcolony dispersal. An arginine residue is conserved in transmembrane helix 8 of most SLC16 family members and its positively-charged guanidinium group is involved in the binding of the negatively-charged carboxylate group of substrates and ligands.

L-lactate is considered an important glycolytic metabolite, energy source, and signaling molecule in human health and disease. Additionally, lactate has an important role in some bacterial infections. In Neisseria meningitidis, it acts as a signaling molecule for microcolony dispersal. In Staphylococcus aureus-induced biofilms, it functions as a virulence factor, sustaining an anti-inflammatory environment that promotes bacterial persistence.

We have recently determined crystal structures of the proton-dependent, L-lactate-specific SLC16 family homologue SfMCT with bound L-lactate and thiosalicylate (TSA), which is a nontransported ligand. The structures show SfMCT in the pharmacologically-relevant outward-open conformation and they provide important mechanistic insights into the role of critical residues involved in ligand binding. The negatively-charged carboxylate groups of L-lactate and TSA interact with the positively-charged guanidinium group of R280 (TM8), which is conserved in TM8 of most SLC16 family members. Here, we use this structural information for the rational identification of potent, nanomolar (nM) affinity L-lactate transport inhibitors starting from a low-affinity compound. We have solved high-resolution crystal structures of SfMCT with bound potent inhibitors to understand the molecular mechanism underlying the binding of these molecules. Furthermore, we uncovered the role of water molecules in the interaction between inhibitors and key residues based on high-quality structural data. Identified inhibitors represent potential tool compounds to interfere with monocarboxylate transport across biological membranes.

Results

Rational identification of potent L-lactate transport inhibitors.

The thiosalicylate (TSA)-bound structure of the proton-dependent, L-lactate-specific SLC16 homologue SfMCT in its outward-open conformation served as a structural framework for the rational identification of potent L-lactate transport inhibitors. The previously characterized ligand binding site provides space to accommodate molecules that are larger than TSA. Since TSA is a sulfanyl-derivative of the aromatic monocarboxylate benzozoate (BA), BA was selected as starting compound of a rational search for molecules that potently inhibit L-lactate transport (Supplementary Fig. 1). Only molecules containing one carboxylate group were considered (i.e., monocarboxylates) as R280 (TM8) is the only positively-charged residue in the ligand binding site region. Furthermore, previous experiments have shown that SfMCT does not have significant affinity for di- and tricarboxylates. The binding site also contains hydrophobic and aromatic residues, which can be involved in hydrophobic and interacting with bound molecules. Therefore, the focus of the screen was set on aromatic monocarboxylates, which are summarized in Supplementary Fig. 1. To evaluate the inhibitory effect of the selected molecules, an inhibition assay was set up where the transport of [14C]L-lactate through SfMCT was measured in the absence and presence of different inhibitors at decreasing concentrations. In a first step, the inhibitory effects of BA, as well as hydroxylated and methylated BA derivatives on L-lactate transport, were investigated at a concentration of 500 µM (Fig. 1A, red). Among the hydroxylated BA derivatives, a methyl group at the C2-carbon atom of BA (i.e., salicylate (SA)) leads to the strongest decrease in L-lactate transport activity. BA and SA inhibit L-lactate transport with comparable Ki values of 431 µM and 362 µM, respectively (Fig. 1B, C). In contrast to hydroxylation, the C4-carbon atom of BA is the optimal position for derivatization by a methyl-group (i.e., 4-methyl-BA (4MBA)) resulting in the strongest reduction of L-lactate transport activity followed by methylation of the C3- and C2-carbon atoms (Fig. 1A, red). 4MBA inhibits L-lactate transport with a Ki of 13.4 µM (Fig. 1D), which is ~30 times more potent than BA, the starting compound of the rational search. Inhibition experiments performed at a compound concentration of 100 µM show that combining hydroxylation and methylation of BA at the determined optimal positions (i.e., C2-carbon atom for hydroxylation, C4-carbon atom for methylation) does not lead to a synergistic improvement of L-lactate transport inhibition (Fig. 1A, blue). As observed for BA derivatives, methylation at the C4-carbon atom of SA (4-methyl-SA (4MSA)) leads to the strongest reduction of L-lactate transport among the methylated SA-derivatives with a Ki of 24.4 µM (Fig. 1E). This inhibitory potency is comparable with the value obtained for 4MBA. The hydroxyl group of 4MSA cannot be shifted from the C2- to the C3-carbon atom as shown by the strongly reduced inhibition of L-lactate transport by 3-hydroxy-4-methyl-BA (3OH4MBA; Fig. 1A, blue). The fact that adding methyl-groups to the C3- and C4-carbon atoms increased the inhibitory effect of BA and SA, suggests fusing a second benzene ring to these compounds to further increase their affinity. This results in two naphthoates (i.e., 1-naphthoate (N1C) and 2-naphthoate (N2C)), and three hydroxy-naphthoate derivatives (i.e., 1-hydroxy-2-naphthoate (1OHN2C), 3-hydroxy-2-naphthoate (3OHN2C) and 2-hydroxy-1-naphthoate (2OHN1C)). The inhibitory effect of these compounds on L-lactate transport was determined at a concentration of 25 µM (Fig. 1A, orange). N2C, where the second benzene ring is fused to BA via the C3- and C4-carbon atoms, and 1OHN2C show the strongest reduction of L-lactate transport among the tested naphthoate derivatives with Ki values of 2.4 and 16.2 µM, respectively (Fig. 1F, G). 1OHN2C revealed a comparable Ki value as observed for 4MBA and 4MSA. The naphthalene rings of N2C and 1OHN2C are completely planar. In order to increase conformational flexibility, the carbonate-containing benzene ring of N2C was replaced by an sp2 hybridized alkene (i.e., trans-cinnamate (CA)) or an sp hybridized alkane (i.e., 3-phenyl-propionate (3PP)) chain. CA and N2C have similar Ki values of 2.5 and 2.4 µM, respectively (Fig. 1F, H). In contrast, 3PP shows a stronger inhibitory effect compared to N2C (Fig. 1A, gray), which is highlighted by a lower Ki value of 812 nM (Fig. 1I). Accordingly, we selected aromatic monocarboxylates where the
Fig. 1 Identification of SFMCT inhibitors. A Screening for L-lactate transport inhibitors using a transport inhibition assay (final inhibitor concentrations are indicated). Full names and molecular structures of the used compounds are given in Supplementary Fig. 1. For compounds highlighted by a star, $K_i$ values were determined. Residual uptake in the presence of competitor is normalized with respect to control samples without competitor (control). B–J $K_i$ determination of selected inhibitors with displayed structures. The determined $K_i$ values and the 95% confidence intervals are indicated in the corresponding panels. Data are represented as mean ± SEM from three to five independent experiments, each in triplicate. If not visible, error bars are smaller than symbols. Individual data points are shown as open circles.
carboxylate-group and the benzene ring are connected by an aliphatic chain in the further rational screening procedure. An aromatic monocarboxylates of different length (i.e., benzoate (BA), phenylacetate (PA), 3-phenylpropionate (3PP), and 4-phenylbutyrate (4PB); Fig. 1A, black) served as molecular rulers to determine the optimal length of the aliphatic linker. Among the tested compounds, 3PP is the most potent inhibitor (Fig. 1A, black), which is ~530 times more potent than the starting compound (BA) of the rational search. In a final step, the effect on L-lactate transport of hydroxylation and methylation of the benzene ring of 3PP was systematically analyzed at an inhibitor concentration of 10 μM (Fig. 1A, magenta). Hydroxylation of the C3-carbon atom (3-(3-hydroxyphenyl)-propionate (3OH3PP)) leads to the strongest L-lactate transport reduction among the tested hydroxylated derivatives (Fig. 1A, magenta). In contrast, a methyl-group at the C2-carbon atom (3-(2-methylphenyl)-propionate (2M3PP)) reveals the strongest inhibitory effect on L-lactate transport among the tested methylated derivatives. 2M3PP inhibits L-lactate transport with a Kᵢ value of 469 nM (Fig. 1I), which is significantly lower than the inhibition induced by 3PP. In contrast to SMCT, the here identified key inhibitors (i.e., 1OHN2C, N2C, 3PP, and 2M3PP) do not affect the transport activity of E. coli L-lactate permeases (i.e., LdP and GcA), whose function depends on an intact transmembrane proton-gradient (Supplementary Fig. 2). Therefore, the reduced SMCT-mediated L-lactate transport, which was measured in the presence of these key inhibitors, is due to transport inhibition and does not result from a protonophoric effect of these molecules. We have also performed a transport assay using radiolabeled SA and 3PP, which are both key compounds of our rational search. SMCT does not transport SA or 3PP, which allows the assumption that also derivatives thereof are not transported (Supplementary Fig. 3).

In summary, the presented rational screening approach allowed the identification of two nM-affinity inhibitors starting from a low-affinity compound (i.e., BA). The best inhibitor 2M3PP, which has a Kᵢ value of 469 nM, is almost 1000 times more potent than the starting compound of the rational search.

Crystal structures of SMCT with bound inhibitors. To understand the molecular interactions underlying the binding of the identified key inhibitors (i.e., 1OHN2C, N2C, 3PP, and 2M3PP), we established a procedure to successfully co-crystallize SMCT with these compounds. Purified and concentrated SMCT (8 mg/ml; ~0.18 mM) was supplemented with 1 mM of key inhibitors, is the most potent inhibitor (Fig. 1A, black), which is ~530 times more potent than the starting compound (BA) of the rational search. In a final step, the effect on L-lactate transport of hydroxylation and methylation of the benzene ring of 3PP was systematically analyzed at an inhibitor concentration of 10 μM (Fig. 1A, magenta). Hydroxylation of the C3-carbon atom (3-(3-hydroxyphenyl)-propionate (3OH3PP)) leads to the strongest L-lactate transport reduction among the tested hydroxylated derivatives (Fig. 1A, magenta). In contrast, a methyl-group at the C2-carbon atom (3-(2-methylphenyl)-propionate (2M3PP)) reveals the strongest inhibitory effect on L-lactate transport among the tested methylated derivatives. 2M3PP inhibits L-lactate transport with a Kᵢ value of 469 nM (Fig. 1I), which is significantly lower than the inhibition induced by 3PP. In contrast to SMCT, the here identified key inhibitors (i.e., 1OHN2C, N2C, 3PP, and 2M3PP) do not affect the transport activity of E. coli L-lactate permeases (i.e., LdP and GcA), whose function depends on an intact transmembrane proton-gradient (Supplementary Fig. 2). Therefore, the reduced SMCT-mediated L-lactate transport, which was measured in the presence of these key inhibitors, is due to transport inhibition and does not result from a protonophoric effect of these molecules. We have also performed a transport assay using radiolabeled SA and 3PP, which are both key compounds of our rational search. SMCT does not transport SA or 3PP, which allows the assumption that also derivatives thereof are not transported (Supplementary Fig. 3). In summary, the presented rational screening approach allowed the identification of two nM-affinity inhibitors starting from a low-affinity compound (i.e., BA). The best inhibitor 2M3PP, which has a Kᵢ value of 469 nM, is almost 1000 times more potent than the starting compound of the rational search.

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Role of Y331 and water molecules in ligand binding. An aromatic residue in TM10, which corresponds to Y331 in SMCT, is involved in ligand recognition in several bacterial and human L-lactate transporters. While the hydroxyl group of Y331 (TM10) is directly hydrogen-bonded to the hydroxyl group of 1OHN2C and the thiol group of TSA, it interacts indirectly via a water molecule with the carboxylate groups of N2C, 3PP, and 2M3PP (Fig. 2B–F). Therefore, the hydroxyl and thiol groups as well as the identified water molecule are important for the interaction with the functionally-relevant Y331 (TM10) and thus have a similar functional role in ligand binding. In addition to structural data, transport inhibition experiments corroborate the important role of Y331 (TM10) in ligand recognition. Replacing Y331 (TM10) by a phenylalanine, which removes the hydroxyl group of Y331 (TM10), results in a ~2-fold reduction of L-lactate transport inhibition by 1OHN2C, N2C, 3PP, and 2M3PP (Supplementary Fig. 6). This highlights the functional significance of the hydroxyl group of Y331 (TM10) for ligand recognition. A second water molecule was identified as part of an extended hydrogen-bonding relay network, which connects functionally-relevant residues from the amino- and carboxy-terminal six-helix bundle (i.e., L145 (TM1), N276 (TM8), R280 (TM8), and Y331 (TM10); Supplementary Fig. 7).

Hydrophobic interactions. In addition to salt bridges and hydrogen bonds, hydrophobic interactions contribute to inhibitor binding (Fig. 3). The benzene ring of F335 (TM10), which is part of the carboxylate group-accommodating confinement, is involved in a hydrophobic interaction with the aromatic moiety of naphthoate-derivatives, as well as with the alkane chains of 3PP and 2M3PP (Fig. 3). Furthermore, there is a hydrophobic interaction between the methyl-group of 2M3PP and the aromatic side chain of F335 (TM10), which provides an additional binding interaction with SMCT compared to 3PP. The surface representation of the binding site region clearly highlights that the methyl-group of 2M3PP points towards a cavity between TM7 and TM10 (Fig. 3H). This cavity is exclusively located in the carboxy-terminal six-helix bundle and therefore dictates the
orientation of bound 2M3PP. A hydrophobic environment formed by L28 (TM1), C57 (TM2), F60 (TM2), F359 (TM11), and C362 (TM11) accommodates the second benzene ring of the naphthalene moiety of 1OHN2C and N2C as well as the benzene ring of 3PP and 2M3PP (Fig. 3). Structural analysis revealed that the carboxylate group-containing benzene ring of N2C and the benzene rings of 3PP and 2M3PP are involved in a T-shaped π-stacking interaction with the aromatic side chain of F359 (TM11) (Fig. 3)21. With the exception of L28 (TM1), residues, which are involved in inhibitor binding, adopt similar rotamer conformations in SMCT crystal structures with bound key inhibitors and bound TSA (Fig. 2). In contrast to the TSA-bound structure, L28 (TM1) is rotated away from the central cavity in the presence of the here identified inhibitors (Fig. 2F, star). This rotamer conformation of the isobutyl side-chain of L28 (TM1) allows the formation of hydrophobic interactions with the carboxylate group-containing benzene ring of the naphthoate-derivatives (Fig. 3A, B) or with the benzene ring of 3PP and 2M3PP (Fig. 3C, D). The distance between the benzene ring of 3PP and the adjacent TMs 2 and 11 is <4.5 Å, which precludes the extension of the inhibitor by another methylene-group. This structural information is in line with functional data. In a molecular ruler experiment using aromatic monocarboxylates, which differ in the length of the aliphatic chain connecting the carboxylate-group and the benzene ring, as L-lactate transport inhibitors (i.e., BA, PA, 3PP, and 4PB; Fig. 1A), 3PP was identified as the inhibitor with the optimal length among the tested molecules (Fig. 1A, black). Apparently, while longer molecules (i.e., 4PB) do not

Fig. 2 SMCT ligand binding site. A Overall structure of SMCT in the outward-open conformation viewed in the plane of the membrane with indicated binding site residues (cyan) and surface representation. B-F Binding mechanism of 1OHN2C, N2C, 3PP, 2M3PP, and TSA to SMCT. Corresponding omit maps are shown in Supplementary Fig. 4. Residues within a distance of 4 Å from the bound compounds are displayed as ball-and-stick models and highlighted in cyan. Pink spheres indicate the centers of benzene rings of aromatic residues and inhibitors, which are involved in π-π stacking interactions. Water molecules are indicated by labeled red spheres. The role of the second water molecule (2) is shown in Supplementary Fig. 7. The different rotamer conformation of L28 in the case of TSA binding is indicated by a star in (F). Distances are given in Ångström (Å).
properly fit into the binding pocket, shorter compounds (e.g., BA, PA) cannot establish sufficient interactions with SfMCT, which reduces their affinities.

Discussion

L-lactate is no longer solely regarded as glycolytic end-product, but is now considered an important energy source and signaling molecule in human health and disease\(^1\). Also, there is evidence of its role as a macromolecular guanidinium group, which was also observed for carboxylate-containing drugs bound to their targets\(^23\) and the thiol group of cysteine (Supplementary Fig. 8). Alignment of the 2M3PP-bound SfMCT structure with an outward-open cryo-EM structure of human MCT1\(^30\) suggests that the here presented SfMCT inhibitors (i.e., 1OHN2C, N2C, 3PP, 2M3PP) might be differently oriented in human MCT1 since the distance between the \(\eta\) atoms of the guanidinium group of R313 and the oxygen atoms of the carboxylate group of 2M3PP are 4.7 and 4.9 Å (Supplementary Fig. 8C), which is significantly larger than observed for the inhibitor-bound SfMCT structure (Fig. 2). Furthermore, there would be clashes between the inhibitors and MCT1 side chains (Supplementary Fig. 8). Nevertheless, it can be hypothesized that SfMCT inhibitors might indicate transport-modulating effects on certain human MCTs as all compounds contain a carboxylate group.

Water molecules are involved in ligand binding in several membrane transporters where they bridge the distance between transporter residues and the bound ligands\(^32-38\). Removing the hydroxyl group of Y331 (TM10) (i.e., Y331F mutation) reduces the transporter affinity for the identified inhibitors.

Fig. 3 Hydrophobic interactions involved in ligand binding. Ligands A 1OHN2C, B N2C, C 3PP, and D 2M3PP as determined by structural analysis\(^21\). Hydrophobic residues within a distance of 4 Å from the bound compounds are displayed as ball-and-stick models and highlighted in cyan. The centers of benzene rings of aromatic residues and inhibitors, which are involved in \(\pi-\pi\) stacking interactions, are indicated by pink spheres. Distances are given in Ångström (Å).

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this cavity and dictates the orientation of bound molecules on an atomic level. These crystal structures revealed an asymmetric binding site region with a cavity whose length is mainly restricted by TMs 2, 8, and 11 (Fig. 3G). The methyl group of 2M3PP points toward this cavity and dictates the orientation of bound 2M3PP, since no cavity has been identified in the amino-terminal six-helix bundle. The compound 3PP represents an inhibitor scaffold, whose length is mainly restricted by TMs 2, 8, and 11 (Fig. 3G). The 3PP- and 2M3PP-bound, outward-open SfMCT crystal structures suggest that the inhibitor size can be vertically increased in the direction of the substrate translocation pathway by attachments at the Cβ carbon atom (Supplementary Fig. 1).

In summary, we have identified n-affinity transport inhibitors of the L-lactate-specific SCL6 homologue SfMCT using a rational, structure-guided screening approach, which started with a low-affinity compound. Accordingly, the inhibitory potency could be increased by almost three orders of magnitude. We have solved high-resolution crystal structures of SfMCT with bound inhibitors, which provided important insights into the binding mechanism of these molecules on an atomic level. These crystal structures clearly explain the molecular reasons, which underlie the improvement of the inhibitory potency of the identified inhibitors. This highlights the impact of high-resolution, inhibitor-bound crystal structures on the interpretation of inhibition data and the design of potent inhibitors. The here identified L-lactate transport inhibitors represent valuable compounds that might potentially also interfere with substrate transport across biological membranes mediated by SCL6 family members. In contrast to human MCTs, only a very low number of the innumerable bacterial homologues have been studied. Considering the presence of SCL6 family homologues in human pathogenic bacteria, application of monocarboxylate transport inhibitors to compromise pathogen’s survival might turn out to be a valid antibacterial approach in the near future.

**Methods**

**Cloning of SfMCT.** Cloning of the bacterial SfMCT (UniProt ID code A0LNN5) gene into the pZUF21-rbs-3C-10His plasmid was done as described previously.39,40 The gene, which was codon-optimized for expression in E. coli (GenScript), was ligated into the pZUF21-rbs-3C-10His plasmid using 5′ HindIII and 3′-Xhol restriction sites for overexpression, or into the pET20 plasmid using 5′ EcoRI and 3′-Xhol restriction sites for functional studies.41 The resulting constructs (pZUF21-rbs-3SMCT-3C-10His and pET20-SfMCT-3C-10His) contained recombinant SfMCT followed by a C-terminal human rhinovirus 3C (HRV3C) protease cleavage site and a decahistidine tag (His-tag). The Y331F mutant was generated by QuikChange site-directed mutagenesis (Agilent Technologies) using the primer set (5′-3′) GATATGACGAGCGTGATGCTG and 5′-3′ CGTATCGAGCGGCGCCTG, respectively.

**Uptake of radiolabeled L-lactate into SfMCT-expressing E. coli.** Uptake experiments were done as described previously.40 Overnight precultures of E. coli JA202 (MC4100 CmAc:tac::FliB-lacZ) were transformed with a plasmid carrying SfMCT (pET20-SfMCT-3C-10His), which were diluted 1:200 into LB-medium supplemented with 100 µg/ml ampicillin. Cultures were grown at 37 °C and 180 r.p.m. in an incubator shaker (Multitron, Infors HT). At OD590 = 0.1, 5 mM 3-phenylpropionic acid spiked with [14C]salicylic acid (NG, Glycon Biochemicals GmbH) for 2 h followed by ultra-centrifugation (200,000 × g, 90 min, 4 °C). The pellet was resuspended in lysis buffer, homogenized and subjected to ultracentrifugation (200,000 × g, 90 min, 4 °C). The supernatant was diluted 1:1 with detergent-free solubilization buffer supplemented with 5 mM L-histidine. The solubilized material was then incubated with nickel-NTA beads (2.4 × 108 bacteria), 10 µl substrate master mix (67 µM sodium L-lactate spiked with [14C]L-lactate sodium salt ([14C]L-lactate, American Radiolabeled Chemicals), or 67 µM 3-phenylpropionic acid spiked with [1-14C] 3-phenyl propionic acid (14C]-3-phenyl propionic acid, American Radiolabeled Chemicals) to an activity per reaction of 0.167 µCi, resulting in a final substrate concentration of 40 mM and a final activity per reaction of 0.1 µCi. All monocarboxylate inhibitors were purchased from Merck (i.e., SigmaAldrich) with the exception of 6-methyl-salicylate (6MSA) that was purchased from Fluorescence. Uptake experiments were performed in 2 ml reaction tubes (Eppendorf) at 30 °C under agitation (1000 rpm), Thermomixer compact, Eppendorf. After 30 min, reactions were stopped by adding 900 µl stop buffer (20 mM HEPES-NaOH (pH 7.5), 150 mM NaCl) followed by centrifugation (21,000 × g, 4 min, room temperature). The supernatant was then washed two times with 900 µl stop buffer to remove free radioligand. Finally, bacteria were lysed using 50 µl 5% (w/v) sodium dodecylsulfate and transferred into a 96-well plate (Optiplate, PerkinElmer). 150 µl scintillation cocktail (MicroScint 40, PerkinElmer) were added to each well before measuring each well for 2 min with a scintillation counter (TopCount NXT, PerkinElmer). For data analysis Primet (GraphPad Software) was used.

**Expression of SfMCT in E. coli and membrane preparation.** For crystallization experiments, SfMCT was expressed in E. coli BL21 (DE3) pLysS grown at 37 °C in Luria Bertani (LB) medium supplemented with antibiotics (100 µg/ml ampicillin and 36 µg/ml chloramphenicol) at 180 r.p.m. in an incubator shaker (Multitron, Infors HT). At OD590 = 0.9, expression of SfMCT was induced by adding isopropyl-β-D-thiogalactopyranoside to a concentration of 0.1 M. After four hours, cells were harvested by centrifugation (5200 × g, 10 min, 4 °C), resuspended in lysis buffer (45 mM Tris-HCl (pH 8), 450 mM NaCl, 4 °C) and pelleted again (10,000 × g, 25 min, 4 °C). The final cell pellet was resuspended in lysis buffer and the bacteria were lysed using an M-110P Microfluidizer (Microfluidics) operated at 1,500 bar. Low-speed centrifugation (10,000 × g, 10 min, 4 °C) was done to remove unlysed bacteria. Bacterial membranes were isolated by subjecting the supernatant of the low-speed centrifugation step to ultracentrifugation (200,000 × g, 90 min, 4 °C). The pellet was resuspended in lysis buffer, homogenized and subjected to ultracentrifugation (200,000 × g, 90 min, 4 °C). Finally, membranes were resuspended and homogenized in buffer (20 mM Tris-HCl (pH 8), 150 mM NaCl, 10% (v/v) glycerol) at 100 µg/ml and stored at −80 °C. Homogenization of the membranes was done using a glass Auflosen homogenizer (Satorius). Purification of SfMCT. Purification of SfMCT was done at 4 °C. Resuspended membranes were solubilized by gentle stirring in solubilization buffer (20 mM Tris-HCl (pH 8), 150 mM NaCl, 10% (v/v) glycerol, 4% (w/v) n-nonyl β-D-glucopyranoside (NG, Glycon Biochemicals GmbH)) for 2 h followed by ultra-centrifugation (200,000 × g, 30 min, 4 °C) to remove unsolubilized material. The supernatant was diluted 1:1 with detergent-free solubilization buffer supplemented with 5 mM L-histidine. The solubilized material was then incubated with nickel-nitrilotriacetic acid resin (Ni-NTA; ProteinA) (1 ml resin bed volume for solubilized membranes from one-liter expression culture) under gentle stirring for 2 h. The resin was then transferred into a column and washed with 25 column volumes of Ni-NTA wash buffer (120 mM Tris-HCl (pH 8), 150 mM NaCl, 5 mM L-histidine, 0.4% (w/v) NG and 25 column volumes of washing buffer 2 (20 mM Tris-HCl (pH 7), 100 mM NaCl, 0.4% (w/v) NG). SfMCT was proteolytically eluted from the column by incubating the resin with His-tagged HRV3C on a rotational shaker (~16 h). Eluted, undigested SfMCT and co-eluted HRV3C were removed by an additional Ni-NTA purification step.

**Cryocrystallization.** Purified SfMCT was concentrated to 24 mg/ml using a 50,000 Da molecular weight cut-off concentration device (SARTORIUS Stedim Biotech, Vivaspin 2). Aggregated protein was removed by ultra-centrifugation (150,000 × g, 30 min, 4 °C). Concentrated SfMCT was diluted to 8 mg/ml (~180 µM) using washing buffer 2 and supplemented with 1 mM monochorboxylate inhibitor from a 10 mM stock solution (10 mM monochorboxylate, 20 mM Tris-HCl (pH 7), 100 mM NaCl) and supplemented buffer solution kept on ice for 1 h. The protein was crystallized in the sitting-drop vapor-diffusion method by mixing concentrated protein with reservoir solution (50 mM Tris-HCl (pH 7), 150 mM NaCl) in a 1:1 ratio.

**X-ray data collection and structure determination.** Data collection was done at 100 K using a Mar345 imaging plate detector synchrotron radiation (Elettra, Trieste, Italy, European Synchrotron Radiation Facility) at the beamline ID29 (1.5 Å resolution). Intensity data were processed and scaled using MOSFLM and DENZO/SCALEPACK. The structure was solved by molecular replacement using the program PHASER. The initial rigid body refinement was done with CNS. The structure was refined to R-factor of 0.221 (R-free of 0.248) using the program REFMAC5. The final model was validated using WHATIF and MolProbity.
5.0 mM ZnBr₂, 32% (v/v) PEG-1000) using a Mosquito Robot (TTP Labtech). Crystals appeared after one day of incubation at 18 °C and reached maximal size after one week. Crystals were then collected and flash frozen in liquid ethane.8 Before X-ray analysis, crystals were stored in liquid nitrogen.

Data collection and structure determination. All datasets were collected on frozen crystals at the X06SA (PIL) beamline of the Swiss Light Source (SLS; Paul Scherrer Institute, Villigen, Switzerland) using an EIGER 16M detector (Dectris). The datasets were indexed and integrated with XDS50 and then merged using BLENDS48 of the CCP4 program suite47 without truncation of the resolution. Scaling and averaging of symmetry-related intensities for all datasets were performed by aP_scale46 with truncation of the data at the best high-resolution along h, k, or l axis determined by AIMLESS51. Due to the anisotropic nature of the diffraction data the STARANISO software (http://staraniso.globalphasing.org/) was applied. This program performs an anisotropic cut-off of merged intensity data to perform Bayesian estimation of structure amplitudes and to apply an anisotropic correction to the data. The structures were obtained by molecular replacement with Phaser52 using the SMCT structure (PDB ID 6G9X) or the N2C structure (i.e., 2-naphtohydroxide SMCT structure) as search model. The final structures were obtained after multiple rounds of model building with Coot51 and refinement with phenix.refine52. The following settings were used for all refinement runs: XYZ coordinates, individual B-factors, occupancies, and TLS strategies. Full data collection, processing, and refinement statistics can be found in Supplementary Table 1. Figures involving structures were prepared using PyMol (The PyMol Molecular Graphics System; Schrodinger).

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
Relevant data are available from the corresponding author on reasonable request. The atomic coordinates of inhibitor-bound SMCT structures were deposited in the Protein Data Bank under the accession numbers: 6ZGR (10HN2C), 6ZGS (3PP), 6ZGT (N2C), and 6ZGU (2M3PP).

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Author contributions
P.D.B. and D.F. conceived and designed the experiments. P.D.B., D.K. and S.B. performed the experiments, collected and analyzed the data. P.D.B. and D.F. wrote the paper and all authors contributed to manuscript revision and approved the final version.

Competing interests
The authors declare no competing interests.

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