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Identification of a selective inhibitor of human monocarboxylate transporter 4

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Abstract

The human monocarboxylate transporters (hMCTs/SLC16As) mediate the uptake of various monocarboxylates. Several isoforms of hMCTs are expressed in cancerous tissue as well as in normal tissue. In cancerous tissue, hypoxia induces the expression of hMCT4, which transports the energetic metabolite L-lactate across the plasma membrane. Since hMCT4 is involved in pH regulation and the transport of L-lactate in cancer cells, an hMCT4 inhibitor could function as an anticancer agent. Although several non specific hMCT inhibitors have been developed, a selective hMCT4 inhibitor has not yet been identified. The aim of this study was therefore to identify a selective hMCT4 inhibitor for use as a pharmacological tool for studying hMCT4. The heterologous expression system of the *Xenopus* oocyte was used to assess the effects of test compounds on hMCT4, whereupon isobutyrate derivatives, fibrates, and bindarit (2-[(1-benzyl-1H-indazol-3-yl)methoxy]-2-methylpropanoic acid) were demonstrated to exhibit selective inhibitory effects against this transporter. It is suggested that the structure formed from the joining of an isobutyrate moiety and two aromatic rings by appropriate linkers is important for acquiring the selective hMCT4-inhibiting activity. These findings provide novel insights into the ligand recognition of hMCT4, and contribute to the development of novel anticancer agents.

Keywords: Monocarboxylate transporter; hMCT1; hMCT4; Lactic acid; Oocyte

1. Introduction

The human monocarboxylate transporters (hMCTs/SLC16As) belong to solute carrier family 16 and mediate the uptake of various monocarboxylates (1). Among the hMCTs, hMCT1 and hMCT4 are prominently expressed in cancerous tissue, and transport energetic metabolites, such as L-lactate and pyruvate (2–5). The cancerous tissue microenvironment is different from that of normal tissue in terms of oxygen levels (6). To adapt to hypoxia, the expression of various proteins is induced in cancerous tissue. Hypoxia-inducible factor 1 plays its central role by inducing glycolytic enzyme genes to suppress the influx of glucose-derived acetyl CoA into the tricarboxylic acid cycle and to promote the production of lactate from pyruvate. The upregulation of glycolysis leads to the expression of hMCT4, which is involved in pH regulation and the transport of L-lactate in cancer cells (7–10). Hence, an hMCT4 inhibitor could function as an anticancer agent.

In previous studies, α -cyano-4-hydroxycinnamate (CHC) and 4,4'-diisothiocyanostilbene-2,2'-disulfonate (DIDS) have been used as weak and non selective inhibitors of hMCT1 and hMCT4 (11–13). Although the antitumor agent lonidamine has been recently identified as a potent inhibitor of rat MCTs, it displays non selective inhibitory activity for the transporters, similar to CHC and DIDS (14). Hence, these compounds are not suited as a pharmacological tool for distinguishing hMCT1 and hMCT4 in native cells. Although pteridine derivatives and coumarin derivatives have been created as selective hMCT1 inhibitors, no selective hMCT4 inhibitor has yet been developed (15, 16).

In the current study, we present the identification of bindarit (2-[(1-benzyl-1H-indazol-3-yl)methoxy]-2-methylpropanoic acid) as the first potent and highly selective inhibitor of hMCT4 that works in a non competitive manner.

2. Materials and methods

2.1. Materials

Sodium L-[¹⁴C]lactate was purchased from PerkinElmer (Waltham, MA, USA). Propionic acid, L-alanine, benzoic acid, and tetrahydrofuran-2,4-dione (tetronic acid) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Sodium D-lactate, sodium L-lactate, sodium pyruvate, sodium oxamate, and bezafibrate were purchased from Sigma-Aldrich (St. Louis, MO, USA). L-Proline was purchased from Peptide Institute (Osaka, Japan). 5-Ethyl-1H-1,2,3,4-tetrazole was purchased from Chem-Impex International (Wood Dale, IL, USA). 1-Benzyl-1H-indazole-3-carboxylic acid was purchased from Fluorochem (Hadfield, Derbyshire, UK). Bindarit was purchased from Cayman Chemical Company (Ann Arbor, MI, USA). All other test compounds used were purchased from Tokyo Chemical Industry (Tokyo, Japan). *Xenopus laevis* frogs were purchased from Hokudo (Sapporo, Hokkaido, Japan). Permission for this study was obtained from the Committee on Animal Experimentation, Hokkaido University. All compounds used were of the highest purity available.

2.2. Expression of cRNA in *Xenopus* oocytes

Xenopus oocytes were isolated as previously described (13). The plasmid constructs of hMCT1 and hMCT4 had been generated previously (13, 17). cRNA was synthesized *in vitro* and was injected into each *Xenopus* oocyte (17). Water-injected oocytes were used as negative controls.

2.3. Substrate transport assays

The transport activities of hMCT1 and hMCT4 were measured according to a previously published procedure with some modifications (18). Groups of 3–5 oocytes were incubated at 25 °C in a standard buffer containing L-[¹⁴C]lactate (0.14 µCi/mL). Uptake of the radiolabeled compound was measured over 10 min since it was within the linear portion of uptake in oocytes expressing hMCT1 and hMCT4 (13, 19). Transporter-specific uptake was calculated by subtracting the uptake in water-injected oocytes from the uptake in cRNA-injected oocytes.

2.4. Calculations

The kinetic parameters were calculated by nonlinear regression methods using SigmaPlot 13 (Systat Software, San Jose, CA, USA). All experimental data are expressed as the mean ± S.E. Calculator plugins were used for structure property prediction and calculation (MarvinSketch 17.24.0; ChemAxon [<http://www.chemaxon.com>]).

3. Results and discussion

3.1. Effects of simple monocarboxylates and their bioisosteres on L-lactate uptake via hMCT1 and hMCT4

In order to search for compounds with selective inhibitory effect on hMCT4, we investigated the effects of various simple monocarboxylates and their bioisosteres on L-lactate uptake via hMCT1 and hMCT4 expressed in *Xenopus* oocytes.

L-Lactate, which is a typical substrate of hMCT1 and hMCT4, consists of propionate substituted with a hydroxy group on the α -carbon. Initially, we confirmed the influence of differences in α -carbon functional groups on the inhibitory effect toward hMCT1 and hMCT4 (Fig. 1A).

Propionate, isobutyrate, and L-lactate inhibited the transport activity of both hMCT1 and hMCT4, suggesting that the molecular size of these compounds is acceptable at the ligand binding site of the transporters. D-Alanine and L-alanine did not have inhibitory effects on hMCT1 and hMCT4, implying that ligand recognition is attenuated when the α -carbon group is cationic. The inhibitory effect of D-lactate on hMCT4 was markedly lower than that on hMCT1. Since isobutyrate showed an inhibitory effect on hMCT4, we considered that the decrease in D-lactate recognition of the transporter was not due to the size of the hydroxy group of this monocarboxylate. Because of the high conformational flexibility of L-lactate and its bioisosteres, it is difficult to estimate the structure necessary for their interaction with hMCT1 and hMCT4. Thus, we examined the inhibitory effects on the transporters using rigid bioisosteres of L-lactate (Fig. 1B). The inhibition of both hMCT1 and

hMCT4 by cyclopentanecarboxylate (CPC) suggested that the molecular size of this compound was acceptable at the ligand binding site. Similar to D-alanine and L-alanine, the transporters were not inhibited by D-proline and L-proline, which have the cationic α -carbon group. The inhibitory effects of (*S*)-tetrahydrofuran-2-carboxylate (D-THFC) and (*R*)-tetrahydrofuran-2-carboxylate (L-THFC) were lower in hMCT4 than in hMCT1. Given that D-lactate (with a D-isomeric hydroxy group at the α -carbon) also showed a weaker inhibitory effect on hMCT4, it is possible that the orientation of oxygen lone pairs of the hydroxy group or the orientation of an electropositive hydrogen atom bonded to the oxygen atom affects the ligand recognition by hMCT4. Our previous research identified a selective hMCT1 substrate, L-5-oxoproline (L-OPro) having a structure in which an oxo group is introduced at position 5 of L-proline, which showed no inhibitory effect on hMCT1 and hMCT4 (17; Fig. 1B). In order to investigate the influence of the oxo group on ligand recognition by hMCT1 and hMCT4, the inhibitory potency of L-OPro bioisosteres against the transporter-mediated L-lactate uptake was verified (Fig. 1C). Inhibitory effects on hMCT1 were observed with 3-oxocyclopentanecarboxylate (OCPC), L-OPro, (*R*)-5-oxotetrahydrofuran-2-carboxylate (D-OTHFC), and (*S*)-5-oxotetrahydrofuran-2-carboxylate (L-OTHFC), suggesting that the molecular size of these compounds is acceptable at the ligand binding site of the transporter. On the other hand, no inhibitory effect was observed in hMCT1 with D-OPro, indicating that the D-isomeric $-\text{NH}-$ structure markedly weakens the inhibitory effect on the transporter. In hMCT4, the inhibitory effect was observed with CPC, whereas the inhibitory effects were abrogated in any compound in which an oxo group is

introduced at position 5 of CPC. Thus, it is suggested that the molecular size of these compounds is not acceptable at the ligand binding site of hMCT4. Based on these results, the structural reasons for the selective recognition of L-OPro by hMCT1 are as follows: (1) L-isomeric form; (2) reduced basicity of the nitrogen at position 1 through the introduction of an oxo group; and (3) reduced ligand recognition by hMCT4 through the introduction of an oxo group.

Next, we examined the effect that replacement of the scaffold structure and the carboxy group would have on ligand recognition by hMCT1 and hMCT4. It has been reported that pyruvate, in which an oxo group is introduced at the α -carbon of propionate, is a selective substrate of hMCT1 (12, 20). Thus, we verified the recognition of pyruvate and its bioisosteres by hMCT1 and hMCT4 (Fig. 1D). Pyruvate showed an inhibitory effect on the transporters, whereas oxamate and methyl pyruvate exhibited selective inhibitory effects against hMCT1, and no inhibitory effect was observed in the transporters with diacetyl. The results imply the importance of the proper arrangement of the two anionic oxygen atoms of the carboxy group, and the hydrophobicity or the hydrogen atom orientation of the methyl group of the scaffold structure, for ligand recognition by hMCT1 and hMCT4. To further investigate the effect of different scaffold structures on ligand recognition by hMCT1 and hMCT4, we tested several aromatic carboxylates and verified that they all had inhibitory effects on the transporters (Fig. 1E). This result implies that the molecular size of these compounds (including benzoate, which is a substrate of rat MCT1) is acceptable at the ligand binding site of hMCT1 and hMCT4 (21). Since the inhibitory effect of 3-pyrrolinecarboxylate (3-PC) was weaker than that of the

other compounds, it is suggested that the presence of the electropositive hydrogen atom bonded to the nitrogen atom interferes with the interaction between the ligand and hMCT1 or hMCT4. To further explore the influence that replacement of the carboxy group by its bioisosteres would have on ligand recognition by hMCT1 and hMCT4, we investigated the inhibitory effects of propionate bioisosteres on the transporters (Fig. 1F). The examination using pyruvate bioisosteres had revealed that the inhibitory effects of those compounds on hMCT1 and hMCT4 were increased when the carboxy group is negatively charged (Fig. 1D). Hence, we selected to use carboxylate bioisosteres having a pK_a of <5.5 , which is the same value as or lower value than that of the transport assay condition (pH 5.5; 22–25). Since 5-Ethyl-1H-1,2,3,4-tetrazolide (5-ETZ) showed the same inhibitory potency on hMCT1 and hMCT4 as propionate, replacing the carboxy group with a tetrazole group seems to be an effective approach for the search and optimization of hMCT1 and hMCT4 inhibitors. Tetrazolates are well known as carboxylate bioisosteres, and are capable of interacting with the amidines, which are partial structures of Arg residues (26, 27). We considered that propionate and 5-ETZ interact with the Arg residue, because transmembrane domain 8 of rat MCT1 and hMCT4 contains the Arg residue required for the substrate recognition (13, 28). Other propionate bioisosteres showed no inhibitory effect on both hMCT1 and hMCT4, suggesting that carboxylates and tetrazolides having a planar structure and capability to form two-point interactions with the Arg residue are important for ligand recognition by the transporters.

3.2. Effects of isobutyrate derivatives on L-lactate uptake via hMCT1 and hMCT4

Although a basic structure showing selective hMCT4 inhibition could not be found by replacement of the α -carbon functional group, the carboxy group, and the scaffold structure, we identified a number of basic structures showing an inhibitory effect on both hMCT1 and hMCT4. In the search for compounds showing a selective inhibitory effect against hMCT4, we decided to use preexisting pharmaceutical agents having the basic structures, because the agents often contain “privileged structures” that are capable of providing high-affinity ligands for more than one type of target molecule (29, 30). We focused on lipid-lowering agents, fibrates, which have an isobutyrate moiety as the basic structure, and examined their inhibitory effect on hMCT1 and hMCT4 (31; Fig. 2A). Bezafibrate, fenofibrate anion (the active metabolite of fenofibrate), and clinofibrate selectively inhibited the L-lactate transport activity of hMCT4, whereas clofibrate anion (the active metabolite of clofibrate) showed no inhibitory effect on hMCT1 and hMCT4. By comparing the structures of these compounds, it is suggested that the structure in which an isobutyrate moiety and two aromatic rings are joined by appropriate linkers is important for acquiring the selective hMCT4-inhibiting activity. In addition, the inhibitory potency of clinofibrate against hMCT4 was similar to that of bezafibrate and fenofibrate anion, whereas its potency against hMCT1 was slightly higher than that of the other fibrates, suggesting that the two carboxy groups and/or two 2-methylbutyrate moieties of clinofibrate enhanced the ligand recognition by hMCT1.

It has been reported that lonidamine, which has a benzylindazole scaffold, shows potent and

non selective inhibitory effects on rat MCTs ($K_{0.5} \approx 40 \mu\text{M}$; 14). To search for a more potent selective hMCT4 inhibitor, we examined the inhibitory effect of benzylindazole derivatives on hMCT1 and hMCT4 (Fig. 2B). 1-Benzyl-1H-indazole-3-carboxylate (BIC), which is the dechlorinated form of lonidamine, inhibited both hMCT1 and hMCT4 only slightly. Comparison of the lonidamine structure with that of BIC revealed that the introduction of chlorine atoms into the *ortho* and *para* positions of the benzyl group could potentiate the inhibitory activity. Similar to BIC, the non steroidal anti-inflammatory drug bendazac, which has a 2-atom linker between the benzylindazole scaffold and the carboxy group, showed slight inhibitory effects on hMCT1 and hMCT4 (32). As shown in Fig. 2A, an appropriate positional relationship between the aromatic rings and the isobutyrate scaffold is important for selective inhibitory potency against hMCT4. Hence, we also examined the inhibitory effect of bindarit (an anti-inflammatory agent that inhibits the production of inflammatory cytokines), which has a 2-atom linker between the benzylindazole scaffold and isobutyrate group (33). We found that bindarit exhibited much greater hMCT4 selectivity and a more potent inhibitory effect than fibrates and the other benzylindazole derivatives.

3.3. Inhibitory property of bindarit on L-lactate uptake via hMCT4

In order to investigate the inhibitory property of bindarit on hMCT4, we performed a dose-response experiment using different concentrations of the drug (0–500 μM ; Fig. 3A). Bindarit inhibited hMCT4 with a K_i value of $30.2 \pm 1.4 \mu\text{M}$; this is 100-fold lower than the reported L-lactate

K_m for hMCT4 (2.8–3.4 mM; 13, 18). On the other hand, the L-lactate transport activity of hMCT1 remained >50% even in the presence of 500 μ M bindarit, indicating that this drug is at least 15 times more selective for hMCT4 than for hMCT1. To determine the mode of inhibition by bindarit, kinetic analyses were performed (Fig. 3B, C). Bindarit inhibited the L-lactate uptake via hMCT4 by reducing the apparent V_{max} value from 388.7 ± 34.2 pmol/min/oocyte to 80.4 ± 26.2 pmol/min/oocyte without affecting the apparent K_m value (4.5 ± 0.8 mM to 2.7 ± 1.3 mM). These results suggest that bindarit is a highly selective and non-competitive inhibitor of hMCT4.

In conclusion, we have presented the first-identified potent and highly selective hMCT4 inhibitor, bindarit, which can be used as a pharmacological tool for future exploration of the physiological role of hMCT4. Further improvement of the inhibitory activity and bioavailability of bindarit is expected through the introduction of chlorine atoms into its aromatic rings and replacement of its carboxy group by a tetrazole group.

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Conflict of interest

The authors declare that they have no conflicts of interest with the contents of this article.

Author contributions

YF designed and conducted the experiments, analyzed the results, and wrote the first draft of the manuscript. MK, KN, AF, and KI contributed to the writing of the manuscript. All authors reviewed the results and approved the final version of the manuscript.

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Figure legends

Fig. 1. Effects of simple monocarboxylates and their bioisosteres on L-lactate uptake via hMCT1 and hMCT4.

The uptake of L-[¹⁴C]lactate was measured in the presence of the following compounds (10 mM): L-lactate bioisosteres (A), rigid L-lactate bioisosteres (B), L-OPro bioisosteres (C), pyruvate bioisosteres (D), aromatic carboxylates (E), and propionate bioisosteres (F). Oocytes were incubated for 10 min at 25 °C in a standard buffer of pH 5.5, containing 1 μM L-[¹⁴C]lactate with monocarboxylates and their bioisostere compounds. Transporter-specific uptake was calculated by subtracting the uptake in water-injected oocytes from the uptake in cRNA-injected oocytes. Values represent the means ± S.E. from 3–9 independent experiments, each performed in 3–5 replicates. CPC, cyclopentanecarboxylate; THFC, tetrahydrofuran-2-carboxylate; OCPC, 3-oxocyclopentanecarboxylate; OTHFC, 5-oxotetrahydrofuran-2-carboxylate; OPro, 5-oxoproline; FC, furancarboxylate; PC, pyrrolecarboxylate; 5-ETZ, 5-Ethyl-1H-1,2,3,4-tetrazolide; ES, ethanesulfonate; EP, ethylphosphonate; 3-OCP, 3-oxo-1-cyclopenten-1-olate.

Fig. 2. Effects of isobutyrate derivative on L-lactate uptake via hMCT1 and hMCT4.

cis-Inhibition assays were performed using 500 μM fibrates (A) and 100 μM 1-benzyl-1H-indazole derivatives (B), as described in Fig. 1. Transporter-specific uptake was

calculated by subtracting the uptake in water-injected oocytes from the uptake in cRNA-injected oocytes. Values represent the means $\pm$ S.E. from 3 independent experiments, each performed in 3–5 replicates. BIC, 1-benzyl-1H-indazole-3-carboxylate.

Fig. 3. Inhibitory property of bindarit on L-lactate uptake via hMCT4.

(A) The uptake of L-[14 C]lactate was monitored for 10 min at 25 °C in the presence of increasing doses of bindarit (0–500 μ M). The K_i value was determined using non linear fitting of the equation $R = \text{Max} \times K_i / (K_i + [I])$, where R is the fractional transport activity, Max is the maximum transport activity, and [I] is the concentration of bindarit. (B, C) The kinetics of L-[14 C]lactate uptake in the absence and presence of bindarit (30 μ M) were monitored for 10 min at 25 °C. Data are presented in Michaelis–Menten plot (B) and Eadie–Hofstee plot (C). The apparent K_m and apparent V_{max} values were determined using non linear fitting of the Michaelis–Menten equation. Transporter-specific uptake was calculated by subtracting the uptake in water-injected oocytes from the uptake in cRNA-injected oocytes. Values represent the means $\pm$ S.E. from 3 independent experiments, each performed in 3–5 replicates.

A

Clofibrate anion

Fenofibrate anion

Bezafibrate

Clinofibrate

Fig. 2**B** Lonidamine
(Potent, Non-selective)

Bendazac

1-Benzyl-1H-indazole-3-carboxylate
(BIC)

Bindarit

A **B** **C** **Fig.3**