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Probing the substrate specificity of the ergothioneine transporter with methimazole, hercynine, and organic cations

Silke Grigat [a], *Stephanie Harlfinger* [a], *Sonia Pal* [a], *Ralph Striebinger* [a], *Stefan Golz* [b], *Andreas Geerts* [b], *Andreas Lazar* [a], *Edgar Schömig* [a,c], *Dirk Gründemann* [a,c,*]

[a] *Department of Pharmacology, University of Cologne, Gleueler Straße 24, 50931 Cologne, Germany*
[b] *Pharma Research—Target Research, Bayer Healthcare AG, 42096 Wuppertal, Germany*
[c] *Center for Molecular Medicine, University of Cologne (CMMC), Germany*

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ABSTRACT

Recently, we have identified the ergothioneine (ET) transporter ETT (gene symbol *SLC22A4*). Much interest in human ETT has been generated by case-control studies that suggest an association of polymorphisms in the *SLC22A4* gene with susceptibility to chronic inflammatory diseases. ETT was originally designated a multispecific novel organic cation transporter (OCTN1). Here we reinvestigated, based on stably transfected 293 cells and with ET as reference substrate, uptake of quinidine, verapamil, and pyrilamine. ETT from human robustly catalyzed transport of ET (68 μl/(min mg protein)), but no transport of organic cations was discernible. With ET as substrate, ETT was relatively resistant to inhibition by selected drugs; the most potent inhibitor was verapamil (K_i = 11 μmol/l). The natural compound hercynine and antithyroid drug methimazole are related in structure to ET. However, efficiency of ETT-mediated transport of methimazole (K_i = 7.5 mmol/l) was 130-fold lower, and transport of hercynine (K_i = 1.4 mmol/l) was 25-fold lower than transport of ET. ETT from mouse, upon expression in 293 cells, catalyzed high affinity, sodium-driven uptake of ET very similar to ETT from human. Additional real-time PCR experiments based on 16 human tissues revealed ETT mRNA levels considerably lower than in bone marrow. Our experiments establish that ETT is highly specific for its physiological substrate ergothioneine. ETT is not a cationic drug transporter, and it does not have high affinity for organic cation inhibitors. Detection of ETT mRNA or protein can therefore be utilized as a specific molecular marker of intracellular ET activity.

1. Introduction

Ergothioneine (ET) is a natural antioxidant which is biosynthesized solely by fungi and mycobacteria [1]. Humans like other mammals absorb it exclusively from food in which it is distributed very unevenly; a distinguished source of ET are mushrooms (0.1–1 mg/g dried material). ET is rapidly cleared from the circulation and then avidly retained with minimal metabolism. The content of ET varies greatly among human tissues [2]. High ET levels

* *Corresponding author*. Tel.: +49 221 478 7455; fax: +49 221 478 5022.
E-mail address: dirk.gruendemann@uni-koeln.de (D. Gründemann).

Abbreviations: ETT, ergothioneine transporter; LC, liquid chromatography; MPP^+, 1-methyl-4-phenylpyridinium; MS, mass spectrometry; TEA, tetraethylammonium

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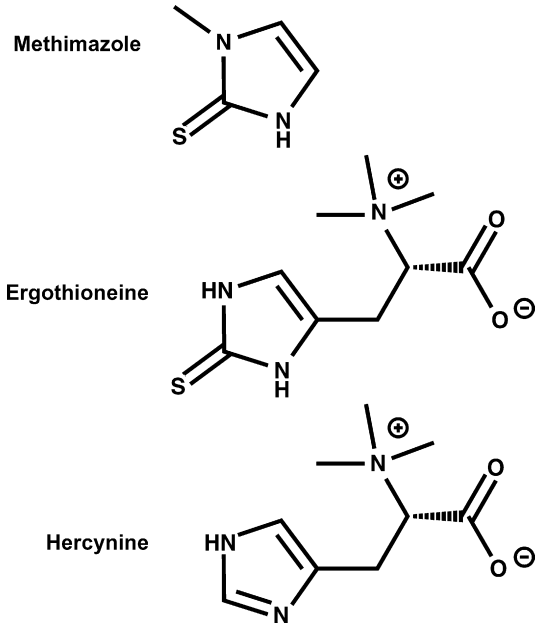

Fig. 1 – Structures of ET, hercynine, and methimazole.

have been found in erythrocytes, bone marrow, seminal fluid and eye.

Chemically, ET is the betaine of histidine with a sulfur atom attached to position 2 of the imidazole ring (Fig. 1). It should not be considered a thiol compound, but rather a thione, a derivative of thiourea. As a consequence of the prevailing thione tautomer, ET is a very stable antioxidant with unique properties; e.g. it does not auto-oxidize at physiological pH and does not promote the generation of hydroxyl radical from H_2O_2 and Fe^{2+} ions (=Fenton reaction) [2]. The precise physiological role of ET is still unclear. Most authors consider it an intracellular antioxidant.

Recently, we have discovered an ET transporter (ETT; gene symbol *SLC22A4*) [3]. ETT from human (ETTh) has high affinity for ET (K_m = 21 μmol/l) and catalyzes cotransport of ET with Na^+. Cells lacking ETT do not accumulate ET, since their plasma membrane is virtually impermeable for this compound. By contrast, cells with expression of ETT accumulate ET to high levels. Based on the expression profile, we judge ETT to be necessary for the supply of ET primarily to erythrocyte progenitor cells and to monocytes.

Much interest in ETT has been generated by case-control studies that suggest an association of polymorphisms in the *SLC22A4* gene with susceptibility to chronic inflammatory diseases. The association with Crohn's disease [4] has been largely confirmed [5–8]. Moreover, associations with ulcerative colitis [9] and Type I diabetes [10] have been reported. There was association with rheumatoid arthritis in a Japanese cohort [11], but this could not be replicated in British [12] and Spanish cohorts [13]. Clearly, in order to elucidate the role of ETT in the genesis of chronic inflammatory diseases, it will be necessary to fully understand substrate specificity and localization of the carrier.

Our previous results suggest that expression of ETT in specific cells indicates intracellular ergothioneine activity. However, the gene product of *SLC22A4* was originally designated a multispecific novel organic cation transporter (OCTN1), because it was reported to transport tetraethylammonium [14], and quinidine, verapamil, and pyrilamine [15]. If ETT also functions as an organic cation transporter, then it cannot serve as a specific molecular marker of ET activity. Thus, it was one of our aims here to clarify, with ET as reference substrate, whether ETT transports organic cations. In addition, we tested organic cations as inhibitiors to see if ETT displays high affinity towards organic cations with ET as substrate.

The widely used antithyroid drug methimazole (=1-methyl-imidazole-2-thione) and the side chain of ET have, except for the methyl moiety, identical structures (cf. Fig. 1). In view of the possible involvement of ETT in chronic inflammatory diseases, it is very interesting that methimazole has immunosuppressive [16–18] and powerful anti-inflammatory [19] activity. In order to evaluate whether ETT could provide a specific route of entry of methimazole into those cells that express this carrier, we tested methimazole as a substrate. The substrate specificity of ETT was probed further with the natural precursor of ET, hercynine, which lacks the sulfur atom but is otherwise identical to ET (Fig. 1).

Finally, we expressed and analyzed ETT from mouse in 293 cells to see if key functional properties are conserved over species.

2. Materials and methods

2.1. *Plasmid constructs*

The construction of pEBTetD/ETTh has been described previously [20]. The cDNA of ETTm was inserted into the polylinker of a plasmid related to pEBTet but without the cassette for expression of the Tet repressor; the latter was supplied by a second plasmid [20]. The cDNA sequence of ETTm corresponds to GenBank entry AB016257 except for a single base deviation at position 66 downstream of the stop codon (C > T). The 5′-interface between cDNA and vector is **GTTTAAACTT AAGCTT** CGCGCCGAAT (polylinker in bold, cDNA underlined); the 3′-interface is TCAAAAGCCT **GGATCC ACTA**. The construct was assembled by standard cloning methods; the whole insert was verified by DNA sequencing.

2.2. *Cell culture*

293 cells (ATCC CRL-1573), a transformed cell line derived from human embryonic kidney, were grown at 37 °C in a humidified atmosphere (5% CO_2) in plastic culture flasks (Falcon 3112, Becton Dickinson, Heidelberg, Germany). The growth medium was Dulbecco's Modified Eagle Medium (Life Technologies 31885-023, Invitrogen, Karlsruhe, Germany) supplemented with 10% fetal calf serum (PAA Laboratories, Cölbe, Germany). Medium was changed every 2–3 days and the culture was split every 5 days.

Stably transfected cell lines were generated as reported previously for the pEBTetD vector [20]; cell culture medium always contained 3 μg/ml puromycin (PAA Laboratories) to ascertain plasmid maintenance. To turn on protein expression, cells were cultivated for at least 20 h in regular growth