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Original article

Identification of competitive inhibitors of the human taurine transporter TauT in a human kidney cell line

Michelle Richter, Selina J. Moroniak, Hartmut Michel*

Department of Molecular Membrane Biology, Max Planck Institute of Biophysics, Frankfurt am Main, Germany

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ABSTRACT

Background: The osmolyte and antioxidant taurine plays an important role in regulation of cellular volume, oxidative status and Ca^{2+}-homeostasis. Taurine uptake in human cells is regulated by the Na^+- and Cl^--dependent taurine transporter TauT. In order to gain deeper structural insights about the substrate binding pocket of TauT, a HEK293 cell line producing a GFP-TauT fusion protein was generated.
Methods: Transport activity was validated using cell-based [^{3}H]-taurine transport assays. We determined the K_m and IC_{50} values of taurine, β-alanine and γ-aminobutyrate. Additionally we were able to identify structurally similar compounds as potential new substrates or inhibitors of the TauT transporter. Substrate induced cytotoxicity was analyzed using a cell viability assay.
Results: In this study we show competitive effects of the 3-pyridinesulfonate, 2-aminoethylhydrogen sulfate, 5-aminovalerate, β-aminobutyrate, piperidine-4-sulfonate, 2-aminoethylphosphate and homotaurine. We demonstrate that taurine uptake can be inhibited by a phosphate. Furthermore our studies revealed that piperidine-4-sulfonate interacts with TauT with a higher affinity than γ-aminobutyrate and imidazole-4-acetate.
Conclusion: We propose that piperidine-4-sulfonate may serve as a potential lead structure for the design of novel drug candidates required for specific modulation of the TauT transporter in therapy of neurodegenerative diseases.

Introduction

The transport and homeostasis of bioactive solutes and small compounds is of fundamental importance in human physiology. The conditionally essential β-amino acid taurine (2-aminoethanesulfonate) is one of the most highly accumulated amino acids in the brain and plays a critical role in neural development [1]. The total taurine content in mammals is derived from two sources: mainly by dietary intake and partly also by taurine biosynthesis in liver and kidney [2]. Taurine is involved in fundamental biological processes. For instance, taurine is an indirect antioxidant and plays a key role in osmoregulation, membrane stabilization and modulation of Ca^{2+}-signaling [3]. In brain and retinal tissues, the function of taurine as antioxidant and osmolyte is essential for cell volume homeostasis. In this context it was shown that the regulatory effect of taurine on cell volume leads to neuroprotection [4]. Taurine deficiency in humans causes development of retinal disorders, which in severe cases can lead to blindness [5,6]. The function of solute carriers (SLC) in drug absorption and disposition has increased their clinical importance in recent years [7].

Taurine supply is regulated by the taurine transporter TauT (SLC6A6) [8] in combination with the gamma-aminobutyric acid (GABA) transporter GAT2 (SLC6A13) [9] and the proton-coupled amino acid transporter PAT1 (SLC36A1) [10]. The transport activity of TauT is sodium- and chloride dependent with a stoichiometry of 2:1:1 (Na^+:Cl^-: taurine) [11]. As a high-affinity and low-specificity transporter TauT has the capability to transport hypotaurine, β-alanine and γ-aminobutyrate (GABA), apart from taurine [12]. In addition there are several drug candidates that interfere with

Abbreviations: 2AEP, 2-Aminoethylphosphate; 3APP, 3-Aminopropylphosphonate; 5AVA, 5-Aminovalerate; AMSA, Aminomethanesulfonate; Aza-THIP, 4,5,6,7-tetrahydropyrazolo[5,4-c]pyridin-3-ol; BABA, β-Aminobutyrate; CAHS, cis 2-Aminocyclohexanecarboxylate; Caco-2, Colorectal adenocarcinoma cells; COS, CV-1 in origin, carrying SV40 cells; DMEM, Dulbecco's Modified Eagle Medium; EOS, 2-Aminoethylhydrogen sulfate; FBS, Fetal bovine serum; GABA, γ-Aminobutyrate; GABAAR, γ-Aminobutyrate receptor type A; GAT2, GABA transporter 2; GES, Guanidinoethyl sulfonate; GFP, Green fluorescent protein; I4AA, Imidazole-4-acetate; IC50, Median inhibitory concentration; JAR, Choriocarcinoma cells; Km, Michaelis constant; LCIS, Live cell imaging solution; MESNA, 2-Mercaptoethanesulfonate; MMT, N-Methyltaurine; P4S, Piperidine-4-sulfonate; PAT1, Proton-coupled amino acid transporter 1; Pen/Strep, Penicillin/streptomycin; PYR, 3-Pyridinesulfonate; SLC, Solute carrier; TauT, Na+- and Cl–dependent taurine transporter; THIP, 4,5,6,7-Tetrahydroisoxazolo(5,4 c)pyridin-3-ol; wt, Wild type.

* Corresponding author.

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GABA transporters, but so far there is only little knowledge about the interference of structural analogs with TauT.

In 2016, Rasmussen et al. investigated the influence of basic GABA-analogs like nipecotic acid, guvacine, δ-aminolevulinic acid, vigabatrin und gaboxadol on taurine uptake [13].

The anti-epileptic drug vigabatrin increases the intracellular level of GABA by irreversible inhibition of the GABA-aminotransferase [14]. The intestinal absorption of vigabatrin is mainly regulated by PAT1 with a partial involvement of TauT [15]. Gaboxadol, also known as 4,5,6,7-tetrahydroisoxazolo(5,4-c)pyridine-3-ol (THIP), acts as a $GABA_A$ receptor type A ($GABA_AR$) agonist. $GABA_A$ receptors are Cl^--channels, which form the major inhibitory system in the central nervous system. Gaboxadol showed inhibitory properties on the $GABA_AR$ as well as on the TauT-mediated uptake of taurine in SKPT cells and ARPE-19 cells [13,16].

Valembois et al. demonstrated 2017 that various other GABA analogs (imidazole-4-acetat (I4AA), 4,5,6,7-tetrahydropyrazolo [5,4-c]pyridin-3-ol (Aza-THIP), muscimol and thiomuscimol) also act as competitive inhibitors of taurine transport [16]. In particular the histamine metabolite I4AA significantly inhibits taurine uptake in a concentration-dependent manner. Due to its additional interaction with the $GABA_AR$, they defined I4AA as a new lead structure for the development of new compounds.

In the meantime, Suárez et al. reported guanidinoethyl sulfonate [17] to mimic the effects of taurine on long-term synaptic potentiation. GES, a natural guanidine-taurine analog, causes taurine depletion by acting as a competitive inhibitor of TauT, PAT1 and GAT2 as well as interfering with taurine biosynthesis [18–20]. In order to analyze the involvement of taurine transport by TauT in relation to PAT1- and GAT2-mediated taurine uptake, TauT was recombinantly overproduced in this study.

To gain a deeper knowledge about the structural requirements of ligands for interaction with the taurine transporter (TauT), we investigated the competitive inhibition by various GABA- and TauT-analogs. Therefore we selected structural similar compounds containing one acidic and one amino head group separated by a carbon chain and determined their inhibitory properties on taurine uptake (Fig. 1). The compounds analyzed in this study were chosen mostly due to their known physiological effects on either humans or other eukaryotes. Our taurine uptake assays using HEK293-cells producing TauT-GFP indicated that homotaurine, ethanolamine-O-sulfate (EOS), piperidine-4-sulfonate (P4S) and 3-pyridinesulfonate (PYR) act as taurine analogs; 5-aminovalerate (5AVA) and β-aminobutyrate (BABA) as GABA analogs and 2-aminoethylphosphate (2AEP) as competitive inhibitors for TauT with a phosphate group. Here we report a robust *in vitro* model for studying TauT and the inhibitory potencies of substrate analogs on taurine uptake.

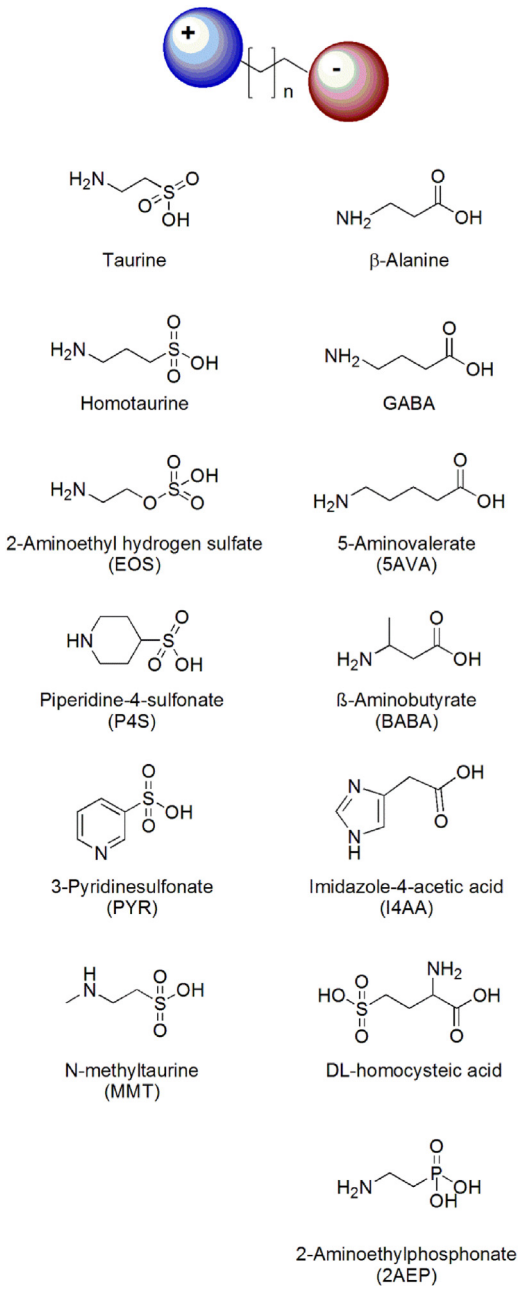

Fig. 1. Structures of substrate analogs.

Materials and methods

Chemicals

N-Methyltaurine (PubChem CID: 7882) was purchased from Santa Cruz Biotechnology (Dallas, TX, USA) and sodium dodecyl sulphate (PubChem CID: 3423265) from Carl ROTH (Karlsruhe, Germany). Taurine (PubChem CID: 1123), β-alanine (PubChem CID: 239), γ-aminobutyrate (PubChem CID: 119), hypotaurine (PubChem CID: 107812), 3-pyridinesulfonate (PubChem CID: 23677935), DL-homocysteic acid (PubChem CID: 92117), 2-Aminoethylhydrogen sulfate (PubChem CID: 70223), 5-Aminovalerate (PubChem CID: 138), β-aminobutyrate (PubChem CID: 10932), Piperidine-4-sulfonate (PubChem CID: 4838), 2-aminoethylphosphate (PubChem CID: 19985431), imidazole-4-acetate (PubChem CID: 96215) and other chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA). Assays were performed in live cell imaging solution (LCIS) provided by Thermo Fisher Scientific (Waltham, MA, USA). 2,2-^{3}H-taurine ([^{3}H]-taurine; 20 Ci/mmol, 1 mCi/mL) and β-[3-^{3}H]-alanine ([^{3}H]β-alanine; 50 Ci/mmol, 1 mCi/mL) was acquired from Biotrend (Cologne, Germany). γ-[2,3-^{3}H(N)]-aminobutyrate ([^{3}H]-GABA; 80.8 Ci/mmol, 1 mCi/mL) and pony vials were obtained from PerkinElmer (Llantrisant, UK). Rotizint Eco Plus scintillation liquid was obtained from Carl ROTH.

The following chemicals were used for cell culture: Dulbecco's Modified Eagle Medium (DMEM)/high glucose and accutase solution were purchased from Sigma–Aldrich. Fetal bovine serum (FBS), 100x penicillin/streptomycin (Pen/Strep) and doxycycline hdyrochloride were obtained from Thermo Fisher Scientific. Further antibiotics, hygromycin B and blasticidin S hydrochloride, were purchased from AppliChem (Darmstadt, Germany). Cell culture plastic ware was purchased from Greiner Bio-One

(Kremsmünster, Austria). For microscopy, millicell EZ slides were purchased from Merck (Darmstadt, Germany). Collagen-coated 12-well plates used for uptake assays were obtained from Corning (New York, USA).

Cloning of human TauT

We obtained the human SLC6A6 gene sequence [21] in a pQE-T7 vector provided by Qiagen (Venlo, The Netherlands). This sequence was isolated by the restriction enzyme digestion, using SacI/EcoRI of New England Biolabs (Ipswich, MA, USA). The SLC6A6 gene was inserted into a pOET1 vector, containing a downstream eGFP gene, form Oxford Expression Technologies (Oxford, UK). A GFP-fusion construct was chosen for a fast analysis of protein overproduction. SLC6A6-eGFP pOET1 plasmid was digested by the restriction enzyms AflII and XhoI. The SLC6A6-eGFP insert was excised an inserted into the pcDNA5/FRT/TO vector from Thermo Fisher Scientific. To confirm integrity cloning was verified by DNA sequencing.

Generation of the TauT-GFP-HEK293 cell line

The recombinant human cell line Flp-In™ T-REx™293 TauT-GFP was created by using the commercially available Flp-In™ T-REx™ host cell line according to the manufacturer's instructions from Thermo Fisher Scientific. The Flp-In™ T-REx™293 cells contain a single, stably integrated Flp recombinase target (FRT) site at a transcriptionally active genomic locus. This ensures high level gene expression of integrated target under the control of a cytomegalovirus promoter and the tetracylcline operator sequence. In order to integrate the TauT-GFP sequence into the genome of the Flp-In™ T-REx™ host cell line, the SLC6A6-eGFP pcDNA5/FRT/TO vector was co-transfected with the Flp recombinase encoding expression vector pOG44 by Thermo Fisher Scientific in a 1:13 ratio. Transfection was performed with Lipofectamine 2000 reagent according to the manufacturer's instructions of Thermo Fisher Scientific. Stable clones were selected by cultivation in growth medium containing 100 μg/ml hygromycin B. After 14 days the TauT production was induced with 2 μg/mL doxycycline hydrochloride for 48 h. Thereafter cells were analyzed by fluorescence microscopy and uptake studies.

Cell culture

For the experiments in this study the stable transfected cell line containing the TauT fusion construct gene with an N-terminal Strep- and green fluorescent protein (GFP)-tag as well as a C-terminal 6xHis tag was utilized. The cells were cultured in DMEM containing 10% (v/v) FBS, 2x Pen/Strep, 100 μg/ml hygromycin B and 10 μg/ml blasticidin S hydrochloride. Cells (5×10^4 cells/well) were seeded in collagen-coated 12-well plates and protein production was induced with 2 μg/mL doxycycline hydrochloride for 48 h. Cells were grown in an atmosphere of 5% CO_2 and at 37 °C.

Fluorescence microscopy

Cells were cultured in collagen coated millicell EZ slides and protein production was induced with 2 μg/mL doxycycline hydrochloride for 48 h. Cells were grown in an atmosphere of 5% CO_2 at 37 °C. For cell imaging, the medium was removed and subsequently the cell layer was rinsed and covered in pre-warmed LCIS. Cells were examined using the EVOS FL cell imaging system with a light cube for GFP (Ex(nm) 470/22, Em(nm) 510/42) of Thermo Fisher Scientific.

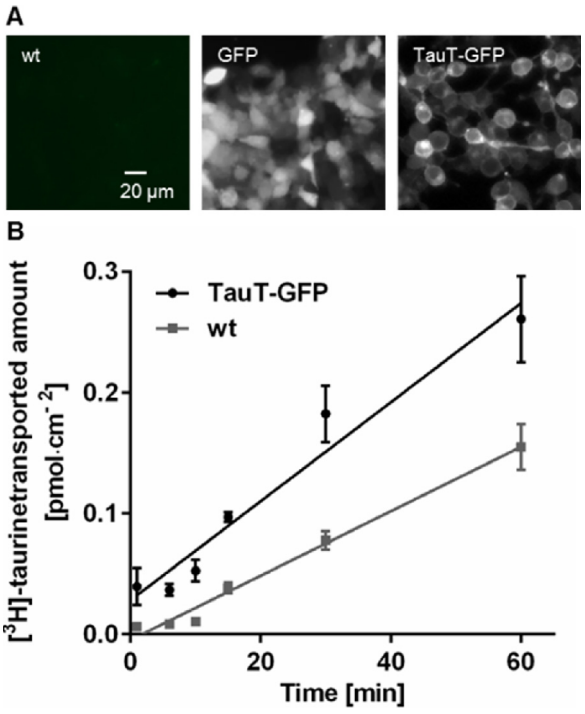

Fig. 2. (A) Fluorescence micrograph of Flp-In™ T-REx™ 293 cells and cells producing GFP and TauT-GFP at 40× magnification, (B) time dependence of [^{3}H]-taurine uptake into wild type (wt) and TauT-GFP producing Flp-In™ T-REx™ 293 cells in LCIS at pH 7.4 and 37 °C. The uptake of taurine was measured after 1, 3, 6, 10, 30 and 60 min. The values are reported as means ± SD and based on experiments performed in triplicates. The reported linear regression corresponds to the equations of $y = 0.0102x - 0.01081$ for wt and $y = 0.01596x + 0.09931$ for TauT-GFP.

Cellular transport assays

The experimental setup and data analysis for cell based transport assays was adapted from Rasmussen et al. 2016 [13]. Modifications of the latter approach are presented in the following. The cellular uptake studies with [^{3}H]-taurine, [^{3}H]-β-alanine and [^{3}H]-GABA were performed in LCIS (140 mM NaCl, 2.5 mM KCl, 1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$ buffered with 20 mM HEPES, pH 7.4, 300 mOsm). Before performing the uptake experiments, cells were equilibrated in pre-warmed LCIS for 5 min. Uptake experiments were initiated by incubating the cells at 37 °C in 0.5 mL LCIS containing indicated ratios of radioligand labeled substrates and non-radioligand compounds of interest for 45 min (see results). Experiments were terminated by removing the donor solutions on ice and gently rinsing the cell layer twice with 500 μL ice cold LCIS. The cell layer was subsequently detached using 400 μL accutase solution. Cell suspension was transferred into pony vials containing 400 μL 2% (w/v) SDS and lysed for 5 min at RT. Finally 4.2 ml Rotizint Eco Plus scintillation liquid was added and the samples were then counted *via* liquid scintillation. The intrinsic taurine uptake is not subtracted from the presented data in this work.

Uptake studies

Prior to competition experiments uptake of [^{3}H]-taurine (20 Ci/mmol, 1 mCi/mL, 60 nM) in LCIS buffer was measured as a function of time and in dependancy of ions and pH value. The collected data was resolved in a time frame of 60 min. Samples were collected after an incubation time of 1, 3, 6, 10, 15, 30 and 60 min of cells treated with [^{3}H]-taurine. Uptake dependancy on the presence of sodium- and chloride-ions was studied using sodium- and chloride-free LCIS, respectively. Selective ion-free LCIS was prepared by substituting sodium with choline

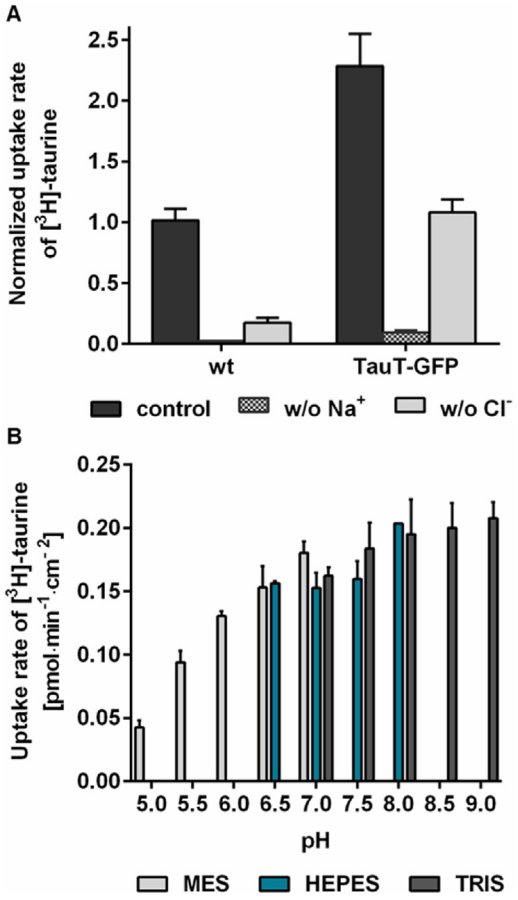

Fig. 3. (A) The effect of Na^+ or Cl^--ions on [^{3}H]-taurine uptake in Flp-In™ T-REx 293™ cells without (wt) and with induced TauT-GFP production at pH 7.4 for 45 min at 37 °C. (B) Rate of [^{3}H]-taurine influx in TauT-GFP producing Flp-In™ T-REx™ 293 cells at pH values ranging from 5.0 to 9.0 in 20 mM MES, HEPES or TRIS buffered LCIS. Mean ± SD. Experiments performed twice in triplicates.

(in mM: $C_5H_{14}ClNO$, 140; KCl, 2.5; $CaCl_2$, 1.8; $MgCl_2$, 1.0; HEPES, 20 pH 7.40) and chloride with gluconate (in mM: sodium D-gluconate, 140; potassium D-gluconate, 2.5; calcium D-gluconate, 1.8; magnesium D-gluconate, 1.0; HEPES, 20; pH 7.40). In addition, the impact of pH on [^{3}H]-taurine uptake was examined by changing HEPES to TRIS or MES buffers in some experiments and adjusting to various pHs between 5.0 and 9.0.

Inhibition of [^{3}H]-taurine (20 Ci/mmol, 1 mCi/mL, 60 nM), [^{3}H]-β-alanine (50 Ci/mmol, 1 mCi/mL, 15 nM) or [^{3}H]-GABA (80.8 Ci/mmol, 1 mCi/mL, 2.5 nM) uptake in Flp-In™ T-REx™ 293 cells was measured with logarithmical increasing concentrations of unlabeled taurine, β-alanine or GABA. For determination of Michaelis–Menten constants cells were incubated with logarithmical increasing concentrations of radiolabeled taurine, β-alanine and GABA.

[^{3}H]-taurine uptake in the presence of substrate-analogs

Inhibition of [^{3}H]-taurine uptake (20 Ci/mmol, 1 mCi/mL, 60 nM) by taurine, β-alanine, GABA, homotaurine, 2-aminoethylhydrogen sulfate, 5-aminovalerate, piperidine-4-sulfonate, β-aminobutyrate, 3-pyridinesulfonate, imidazole-4-acetate, N-methyltaurine, DL-homocysteic acid and 2-aminoethylphosphate in Flp-In™ T-REx™ 293 cells was measured in LCIS. The compounds were first tested at concentrations of 100 mM, which was expected to clearly exceed an inhibitory concentration based on IC_{50} values in the mM range from the previous known inhibitors, e.g. vigabatrin [14]. The obtained uptake values were compared to uptake values in the absence of the tested compounds. Taurine uptake reducing compounds were used in logarithmically increasing concentrations to investigate the concentration-dependent inhibition of [^{3}H]-taurine influx within an incubation time of 45 min.

Cell viability assay

The trypan blue exclusion test of cell viability was used to determine the number of viable cells. The protocol used here is based on the protocol of Strober [22]. Cells were grown in the presence of 2 μg/mL doxycycline hydrochloride in collagen-coated 12-well plates for two days. Subsequently the cell layer was washed with pre-warmed LCIS and afterwards incubated at 5% CO_2 and at 37 °C in LCIS containing 100 mM of each tested compound for 45 min, respectively. Cells were gently detached using 500 μL accutase solution. A volume of 10 μL of 0.4% trypan blue was mixed with 10 μL of cell suspension and incubated at room temperature for 3 min. Subsequently cells were counted immediately, to avoid an influence of trypan blue on cell viability. Unstained (viable) and stained (nonviable) cells were counted with the TC20 automated cell counter from Bio-Rad (Hercules, CA, USA).

Data processing and statistics

The amount of imported [^{3}H]-taurine, [^{3}H]β-alanine or [^{3}H]-GABA (Δc, μmol/h) per defined time period (Δt, min) and per surface (A, cm^2) area across the cellular membrane of Flp-In™ T-REx™ 293 cells was calculated from the experimental uptake value (dpm) prior to the statistical analyses.

$$\Delta c = \frac{\text{dmp} \cdot 4.504 \cdot 10^{-13}}{\Delta t \cdot A}$$

IC_{50} values of the tested compounds were analyzed using non-linear regression (one site)–Fit logIC50 and Michaelis–Menten kinetics, respectively. Our data is presented as mean ± standard deviation [23] values. The kinetic parameter estimates (IC_{50} and K_m) are reported as best estimates ± SD. For data processing and visualization the GraphPad Prism software version 6.04 (GraphPad Software, Inc., La Jolla, CA, USA) was used.

Results

Impact of incubation time on taurine uptake

In order to increase the TauT-mediated uptake signals, TauT-GFP was produced in a Flp-In™ T-REx™ 293 cell line. SLC6A6-GFP gene expression was induced with doxycycline hydrochloride, instead of up regulation of SLC6A6 gene expression by hyperosmotic conditions [24], was chosen to avoid effects by altered osmolality. GFP-Fusion was chosen for a rapid evaluation of protein production in stably transfected cell line.

Before investigation of taurine uptake, TauT-GFP production in Flp-In™ T-REx™ 293 cells was verified by fluorescence microscopy. A distinct GFP fluorescence was identified after 48 h of induction in proximity of the cell membrane.

For optimization of the experimental setup, the dependence of the taurine uptake on the incubation time was determined for native TauT and recombinant TauT in Flp-In™ T-REx™ 293 cells. The uptake of taurine was therefore measured after incubation times ranging from one to 60 min (Fig. 2). In our study taurine uptake by Flp-In™ T-REx™ 293 cells producing TauT-GFP was doubled in comparison to natively produced TauT (wt) in the control experiments. We chose to work in the linear range between 30 and 60 min incubation time for obtaining significant uptake

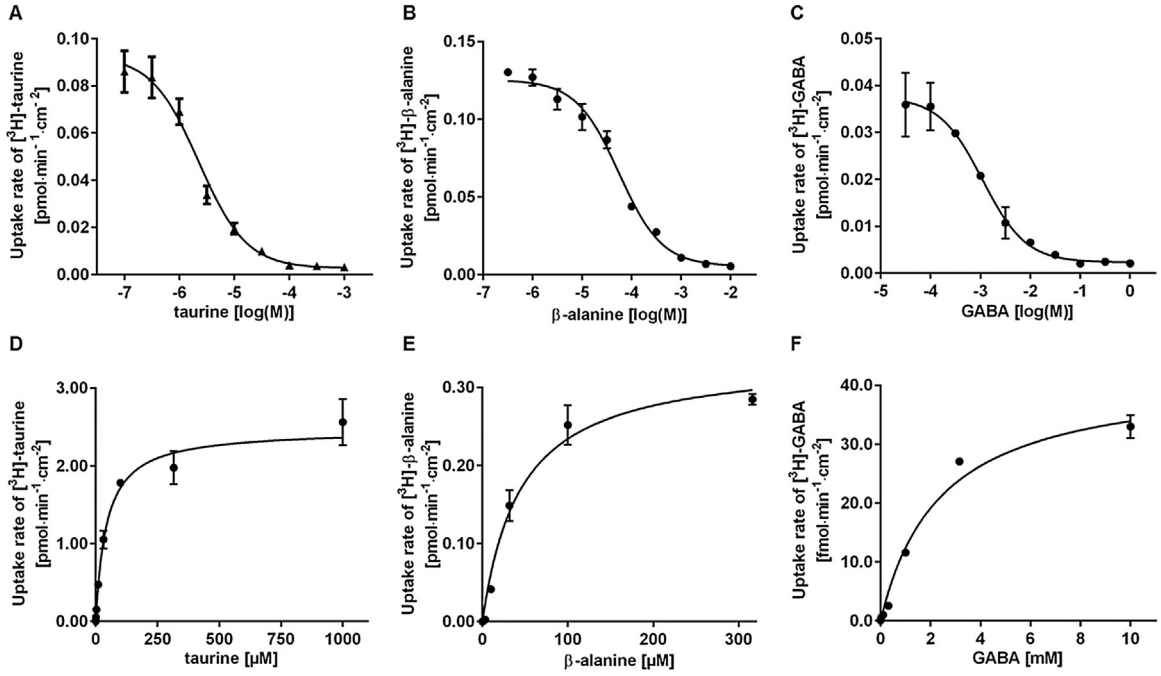

Fig. 4. (A-C) Dependence of the uptake of taurine, β-alanine and GABA; (D-F) Substrate concentration dependence of [^{3}H]-taurine, [^{3}H]-alanine and [^{3}H]-GABA uptake rate in Flp-In™ T-REx™ 293 cells producing TauT-GFP. Values are reported as means ± SD and based on experiments performed in triplicates. Taurine: IC_{50} = 2.9 ± 0.75 µM, K_m = 37.3 ± 6.4 µM and V_{max} = 2.46 ± 0.15 pmol·min^{-1}·cm^{-2}. β-Alanine: IC_{50} = 55.5 ± 6 µM, K_m = 44.5 ± 5.3 µM and V_{max} = 0.34 ± 0.03 pmol·min^{-1}·cm^{-2}. GABA: IC_{50} = 1.0 ± 0.004 mM, K_m = 2.4 ± 0.6 mM and V_{max} = 42.1 ± 3.4 fmol·min^{-1}·cm^{-2}.

Table 1
Kinetic data for the effect of selected compounds on taurine, β-alanine or GABA influx in a TauT-GFP producing Flp-In™ T-REx™ 293 cell line.

Compound	IC_{50} value [µM]	K_m value [µM]	V_{max} value [pmol·min^{-1}·cm^{-2}]
Taurine	2.9 ± 0.75	37.3 ± 6.4	2.46 ± 0.15
β-Alanine	55.5 ± 6	44.5 ± 5.3	0.34 ± 0.03
GABA	1014 ± 4	2400 ± 582	0.042 ± 0.003
P4S	582 ± 9	–	–
I4AA	785 ± 14	–	–
MMT	992 ± 16	–	–
2AEP	1228 ± 24	–	–
5AVA	1420 ± 16	–	–
EOS	2714 ± 71	–	–
Homotaurine	3433 ± 54	–	–
DL-Homocysteic acid	3553 ± 83	–	–
BABA	4393 ± 92	–	–
PYR	5378 ± 60	–	–

increase between TauT-GFP and wild type and for values above the range of systematic errors.

Dependence on Na$^+$- and Cl$^-$-ions and influence of pH on taurine uptake

TauT belongs to the family of secondary active membrane transporters. Thus, taurine transport by TauT is dependent on electrochemical transmembrane gradients formed by of Na$^+$- and Cl$^-$-ions. To validate that the measured uptake into Flp-In™ T-REx™ 293 cells was mediated by TauT, the rate of taurine uptake in the absence of either Na$^+$- or Cl$^-$-ions, respectively, was investigated (Fig. 3). The absence of Na$^+$-ions led to a 20 fold lower taurine uptake rate. Whereas the uptake rate of taurine in Cl$^-$-ion absence was decreased by half. In summary our Flp-In™ T-REx™ 293 system is able to produce and integrate the functionally active TauT transporter which can be modulated in transport activity by Na$^+$- and Cl$^-$-Ions.

To investigate the effect of pH on taurine uptake in Flp-In™ T-REx™ 293 cells, taurine uptake was measured in the presence of MES, HEPES or TRIS buffered solutions enabling to carry out experiments at different pH values ranging from 5.0 to 9.0 (Fig. 3, Supplementary Fig. 1). In our studies we found that taurine uptake positively correlates to increasing pH values and approaches a plateau above pH 8. Furthermore, we could experimentally exclude a pH independent influence of different buffer systems on the measured uptake rates. Based on these results, all following experiments were performed at physiological pH using a HEPES buffer system.

Competitive uptake of taurine, β-alanine and GABA

The uptake of taurine, β-alanine and GABA into TauT-GFP producing Flp-In™ T-REx™ 293 cells was measured at increasing concentrations of these analogs in order to determine IC_{50}-, K_m- and V_{max}-values (Fig. 4). Our data strongly suggest that taurine, β-alanine and GABA uptake rates into Flp-In™ T-REx™ 293 cells are concentration dependent with an apparent IC_{50} value of 2.9 ± 0.75 µM for taurine, 44.5 ± 5.3 µM for β-alanine and 1.0 ± 0.004 mM for GABA. This study demonstrates a K_m and V_{max} of 37.3 ± 6.4 µM and 2.46 ± 0.15 pmol·min^{-1}·cm^{-2} for taurine; 44.5 ± 5.3 µM and 0.34 ± 0.03 pmol·min^{-1}·cm^{-2} for β-alanine as well as 2.4 ± 0.6 mM and 42.1 ± 3.4 fmol·min^{-1}·cm^{-2} for GABA (Table 1). These results include the intrinsic TauT uptake, which is shown in (Supplementary Fig. 2).

Interaction of substrate analogs with TauT

In our next experimental approach we investigated the potential inhibitory effect on taurine uptake of compounds which show structural similarities to taurine and GABA (Fig. 5). At a working concentration of 100 mM taurine, β-alanine, GABA, hypotaurine, PYR, DL-homocysteic acid, EOS, 5AVA, BABA, P4S, 2AEP, MMT, I4AA and homotaurine we observed a significant

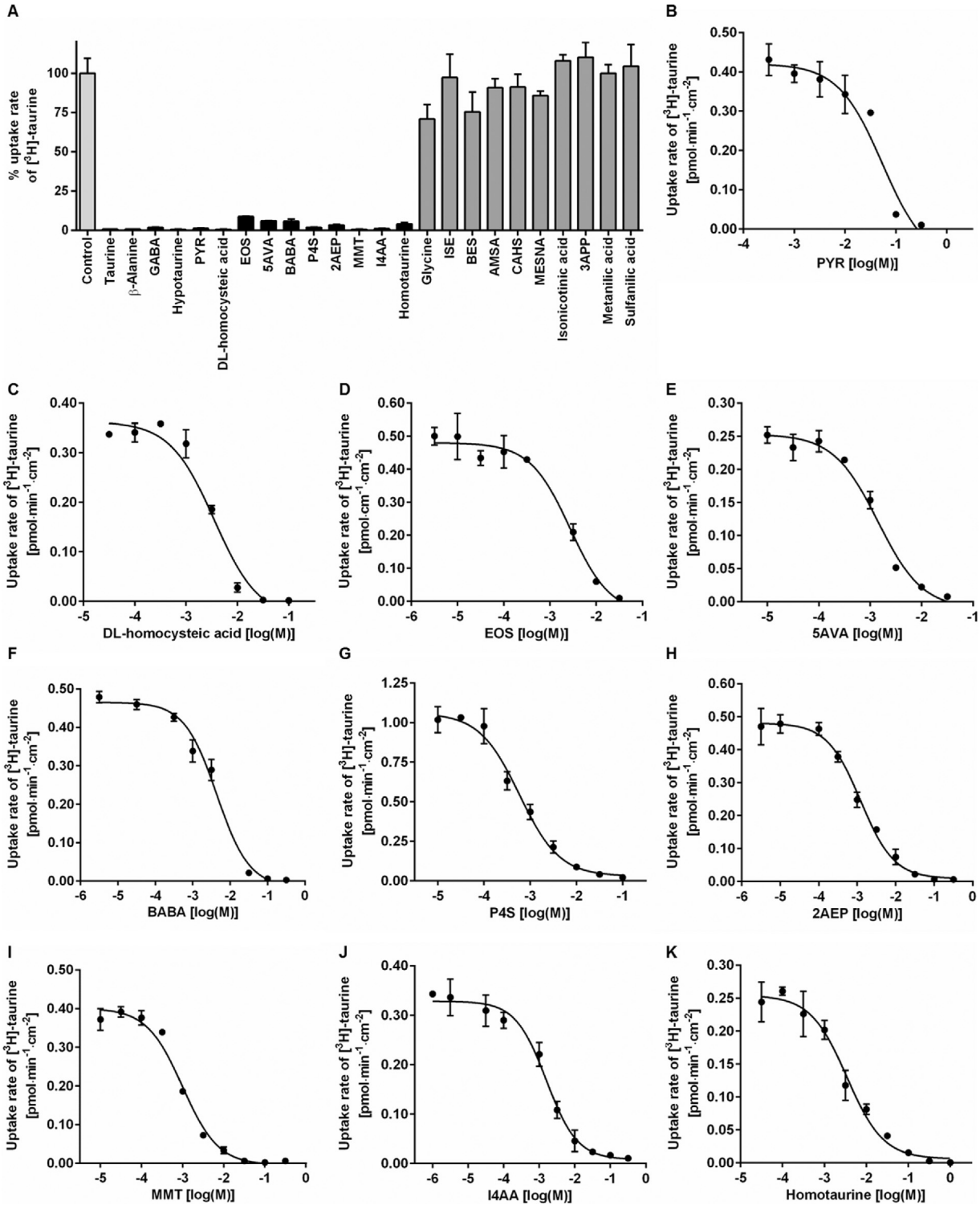

Fig. 5. Influence of taurine analogs on the [^{3}H]-taurine uptake rates by TauT-GFP producing Flp-In^{TM} T-REx^{TM} 293 cells (A) Uptake rates at concentrations of 100 mM of compounds structurally related to taurine and GABA; concentration-dependence of inhibition of the taurine uptake rate by (B) 3-pyridinesulfonate (PYR), (C) DL-homocysteic acid, (D) 2-aminoethylhydrogen sulfate (EOS), (E) 5-aminovalerate (5AVA), (F) β-aminobutyrate (BABA), (G) piperidine-4-sulfonate (P4S), (H) 2-aminoethylphosphate (2AEP), (I) N-methyltaurine (MMT), (J) imidazole-4-acetate (I4AA) and (K) homotaurine. Values are reported as means ± SD and based on experiments performed in technical triplicates and biological dublicates.

decrease of [^{3}H]-taurine uptake into Flp-In^{TM} T-REx^{TM} 293 cells compared to the control value (without compound). No significant difference or only a slightly reduced uptake of taurine was caused by 100 mM glycine, isethionic acid [25], N,N-bis(2-hydroxyethyl)taurine, aminomethanesulfonate (AMSA), cis-2-aminocyclohexanecarboxylate (CAHS), 2-mercaptoethanesulfonate (MESNA), isonicotinic acid, 3-aminopropylphosphonate (3APP), metanilic acid, and sulfanilic acid. TauT inhibiting compounds were selected for measuring the concentration-dependent inhibition on taurine uptake. The inhibitory effect

decreased in the order P4S > I4AA > MMT > 2AEP > 5AVA > EOS > homotaurine > DL-homocysteic acid > BABA > PYR with the respective IC_{50} values of 582 ± 9 μM, 785 ± 14 μM, 992 ± 16 μM, 1.23 ± 0.02 mM, 1.42 ± 0.02 mM, 2.71 ± 0.01 mM, 3.43 ± 0.05 mM, 3.55 ± 0.08 mM, 5.38 ± 0.06 mM and 4.39 ± 0.09 mM (Table 1).

Impact of TauT substrates and inhibitors on cell viability

To test the physiological importance of kinetic parameters determined in this work, we assessed the impact of substrate analogs on cell viability of the Flp-In™ T-REx™ 293 cells by using the trypan blue exclusion test. According to the basic principle, unharmed cells do not take up the trypan dye, whereas cells damaged by a cytotoxic effect of the analyzed compounds do take up trypan blue. Negligible effects on cell viability were observed for PYR, EOS, 5AVA, BABA, P4S, 2AEP and homotaurine when compared to untreated (without compound) and taurine treated cells as controls (Supplementary Fig. 3). Hence, we can exclude that a decreased uptake of taurine is due to cytotoxicity of the latter compounds. DL-homocysteic acid, I4AA and MMT significantly reduce cell viability and might not directly interact with TauT as alternative substrates or inhibitors. Furthermore Flp-In™ T-REx™ 293 cells seem to be a useful model for measuring taurine uptake in a short-term presence of high concentrations of substrate analogs and for the evaluation of cytotoxic effects of small compounds.

Discussion

In the present work we investigated the effect of GABA- and taurine-analogs on ^{3}H-taurine uptake by the taurine transporter TauT, produced in the mammalian kidney cell line Flp-In™ T-REx™ 293. Our key findings reveal that several substrate analogs inhibit taurine transport to a large extent without affecting cell viability.

TauT-GFP production in Flp-In™ T-REx™ 293 cells

The first objective of this study was to develop a robust cell based model system to investigate alternative substrate molecules for the taurine transporter TauT. For ensuring homogenous TauT production, the SLC6A6 gen was stably integrated into Flp-In™ T-REx™ 293 cells. Expression of the SLC6A6-GFP fusion gene was controlled by a tetracycline-inducible promoter. In previous publications TauT production was stimulated by cultivation under hyperosmotic conditions [24]. A hyperosmolar environment affects the expression pattern of transporter genes differently in cell lines of diverse species and origins. Independent studies show that the TauT mRNA level is enhanced and taurine influx is increased in SKPT, MDCK and HepG2 cells under hyperosmotic conditions, whereas the TauT mRNA level is lowered and taurine influx is decreased in NIH3T3 cells [26,27]. In our preliminary studies, under hyperosmotic conditions Flp-In™ T-REx™ 293 cells do not indicate an alteration of [^{3}H]-taurine influx.

In the present work effects by altered osmolality were avoided by using inducible TauT production. The produced GFP-TauT fusion-protein was tracked by fluorescence microscopy. A distinct GFP-signal in proximity of the plasma membrane indicated a correctly located and properly integrated membrane protein.

In our radio ligand uptake assays, we observed a 2-fold increase of taurine uptake in comparison to wild type cells. Within the physiological pH range 6.5–8.0 of buffered solutions the taurine uptake was comparably high. Furthermore, we could confirm the Na^+- and Cl^--dependence of taurine import. Our results suggest that the taurine uptake in wild type cells was more dependent on chloride compared to the uptake by TauT-GFP producing cells. This difference in chloride-dependent taurine uptake might be the result of a higher percentage of taurine transport mediated by chloride-dependent GAT2 [9] in wild type cells.

In conclusion, our data demonstrate that TauT production in Flp-In™ T-REx™ 293 cells is a robust technique to study competitive transport kinetics.

Analysis of substrate affinities of TauT

Prior to further substrate studies we determined basic transport parameters to evaluate our system in comparison to previous studies. The determined IC_{50} values are in a close range to the calculated K_m values of 37.3 μM, 44.5 μM and 2.4 mM for taurine, β-alanine und GABA, respectively (Table 1).

In previous publications the determined K_m-values of taurine have a broad scope ranging from 2.3 μM in the human placental choriocarcinoma cell line [28,29] up to 30 μM in glioma cells (LRM55) or 43 μM in monkey kidney fibroblast cells (COS) [30,31]. Ramamoorthy et al. determined a K_m-value of 5.9 μM for human TauT over produced in HeLa cells [11]. In SKPT cells or in oocytes producing a pig taurine transporter [13,32] a similar affinity of 25 μM was reported. A variation of K_m value is known even within the same experimental system, for instance the K_m value for taurine of human epithelial colorectal adenocarcinoma cells (Caco-2) was reported to be 5.27 μM and 17.1 μM [33,34].

In addition, the transport kinetics of TauT has been investigated in several studies. K_m values for β-alanine und GABA of 79 μM and 790 μM in LRM55 cells, 40 μM and 1070 μM in SKPT cells, and 100 μM and 1 mM in MDCK cells have been previously reported [13,30,31]. In case of GABA higher K_m values up to 1.5 mM in HeLa cells and 3.5 mM in rat retinal capillary endothelial cell line (TR-iBRB2) have been published [12,35].

Considering these differences of reported K_m values, our results for the affinities of taurine, β-alanine and GABA are similar to other model systems.

Structural features of TauT interacting compounds

Among other previously studied compounds, nipecotic acid, gaboxadol and I4AA have been shown to be potent inhibitors of taurine uptake mediated by TauT with IC_{50} values of 2.02 mM, 40 mM and 658 μM [13,16,36]. At physiological pH these compounds are of a zwitterionic nature (Fig. 1), possessing a protonated amine moiety and an acidic group separated by a carbon linker (C2-C3). In 2016, Rasmussen et al. demonstrated a competitive inhibition of TauT by cyclic analogs of β-alanine and GABA. Even nipecotic acid and guvacine, which show relatively high affinities among the set of tested compounds, possess significant lower affinities compared to their linear analogs [13]. Based on their results, the authors concluded that a β-alanine like carbon linker between the acidic and amino group is optimal for TauT transport. However, comparing the carbonated- and sulfonated-substrates reported in our study, the K_m values for taurine and β-alanine containing a C2-carbon linker are much lower than those for homotaurine and GABA with a C3 linker.

Furthermore, Valembois et al. postulated that cyclic carbon linker structures lead to restrictions of essential substrate flexibility and thus these cyclic compounds would not fit into the substrate binding pocket of TauT. In their study they used a set of modified GABA analogs to identify high affinity substrates. They reported I4AA as an equipotent inhibitor of taurine influx compared to GABA [16]. I4AA contains an acetate residue bound to an imidazole ring, which presumably imitates the amino group in GABA.

In this present study, we have investigated additional structural analogs of taurine and GABA to gain additional knowledge about the substrate specificity of TauT. No influence on TauT-mediated transport of taurine was observed for glycine, ISE, BES, AMSA, CAHS, MESNA, isonicotinic acid, 3APP, metanilic acid, and sulfanilic acid.

Of the tested taurine analogs; P4S, I4AA and MMT revealed inhibitory effects on TauT-mediated uptake of taurine, showing higher affinities than GABA. Furthermore, the taurine analogs, homotaurine, EOS and PYR, as well as the GABA analogs, 5AVA, BABA and DL-homocysteic acid inhibited the taurine influx with affinities in comparable range to GABA. In addition a competitive inhibition of taurine uptake was revealed for the phosphate analog 2AEP of taurine and β-alanine. To our knowledge this is the first demonstration of a compound inhibiting TauT with a phosphate as the negatively charged group.

The PYR inhibits taurine uptake even despite the presence of the pyridine ring. In our study PYR inhibits taurine influx with a 5 fold higher IC_{50} value than the cyclic structure I4AA postulated by Valembois et al. [16]. We therefore assume that the aromatic structure of the pyridine moiety confers rigidity to the nitrogen group leading to reduced degrees of steric freedom, perturbing structural adaptation to the binding pocket. Consequently, the affinity of PYR is significantly lower than the affinity of I4AA. However, the non-aromatic, but cyclic P4S showed a 6 fold smaller IC_{50} value in comparison to homotaurine, which is also a C3 linked compound. The more flexible piperidine ring might form a boat conformation which is more favorable to fit into the cavity of the binding site than homotaurine. The IC_{50} for P4S with 582 μM is even smaller than for I4AA, which was determined by Valembois et al. as a leading structure with 659 μM.

In case of MMT, methylation leads to a lowered compound affinity for TauT than taurine, yet higher than homotaurine. Methylation of β-alanine at the C2 position decreases the inhibitory potential by 100 fold. These decreased affinities might be caused by missing positive charges and steric interactions with the protein environment.

Structurally, EOS is a sulfonic acid ester of taurine. We observed no differences in competitive inhibition by EOS and homotaurine, as reflected in nearly identical IC_{50} values (Table 1). Hence, we can exclude that presumable hydrogen bond formation by the additional oxygen atom of the ester bond is leading to an increase of the inhibitory effect by EOS.

Surprisingly we observed a comparably high affinity for 5AVA, which contains a C4 linker between the ionic groups, as for GABA with a C3-linker. Hence, we postulate that in contrast to C3 linkers, C4 linkers could adopt a bent conformation leading to improved interactions with the protein environment [13].

Here we analyzed the competitive inhibition of taurine uptake by substrate analogs. Whether these competitive compounds are interacting with the substrate binding pocket and are consequently also transported as alternative substrates of the TauT transporter, requires to be evaluated in future studies. Further, if the TauT transporter is inhibited by the tested analogs this might also affect the potential therapeutic use of P4S [37], I4AA [38] and 5AVA [39] on $GABA_AR$ and EOS [40] on GABA transaminase modulation.

In conclusion, we investigated the kinetic properties of well-studied taurine, β-alanine and GABA on TauT transport by competitive inhibition- and substrate saturation-assays with isotopic substrates in Flp-In™ T-REx™ 293 cells, to prove the comparability of our model system [13,30,31]. In all tests Flp-In™ T-REx™ 293 cells appear to be a useful model for investigating the impact of varying concentrations of compounds on TauT-mediated uptake of taurine. We could identify taurine-, β-alanine- and GABA-analogs as potential substrates with a higher affinity for TauT than GABA.

Consideration of cytotoxic effects

Considering possible cytotoxic effects by the extremely high and non-physiological compound concentrations in the interaction studies, we investigated the impact of these compounds on cell viability (Supplementary Fig. 3). In several experiments the cytotoxicity of the tested compounds was already optically visible after treatment. In particular alcohol derivatives led to fixation of the cell layer. Our experiments revealed cytotoxic effects of DL-homocysteic acid, MMT and I4AA at concentrations of 100 mM. These results concur with studies on C6 cells; here β-alanine and GABA did not show cytotoxic effects whereas DL-homocysteic acid led to observation of impaired viability [41]. In summary, it can be concluded that inhibitory effects of DL-homocysteic acid, MMT and I4AA might be caused by cytotoxic effects. In contrast, the inhibitory effects of P4S, 2AEP, 5AVA, EOS, homotaurine, PYR and BABA are not side effects of toxicity.

Conclusion

P4S, 2AEP, 5AVA, EOS, homotaurine, PYR and BABA show affinities for TauT below or in the same range as the affinity of GABA. However, additional experiments are necessary to determine the mechanism of interaction of compounds with TauT as inhibitor or alternative substrates. Improved inhibitors are required for treatment of TauT mediated dysregulation, which is directly connected to the development of retinal blindness [1,6] and renal diseases [3]. Furthermore, TauT mediated anti-oxidation and osmoregulation is involved in other degenerative-neurological diseases, e.g. Parkinson's disease, autism, schizophrenia and Alzheimer's disease [42]. Further investigation of the substrate binding pocket of TauT is required for the design of selective and specific inhibitors. Our newly identified compounds may represent promising lead structures for further ligand based drug design.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.pharep.2018.10.005.

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