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Nimesulide binding site in the B0AT1 (SLC6A19) amino acid transporter. Mechanism of inhibition revealed by proteoliposome transport assay and molecular modelling

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ABSTRACT

The effect of pharmaceutical compounds on the rat kidney B0AT1 transporter in proteoliposomes has been screened. To this aim, inhibition of the transport activity by the different compounds was measured on Na^+-[^{3}H]glutamine co-transport in the presence of membrane potential positive outside. Most of the tested drugs had no effect on the transport activity. Some compounds exhibited inhibitory effects from 5 to 88% at concentration of 300 μM. Among the tested compounds, only the anti-inflammatory drug nimesulide exerted potent inhibition on B0AT1. From dose response analysis, an IC_{50} value of 23 μM was found. Inhibition kinetic analysis was performed: noncompetitive inhibition of the glutamine transport was observed while competitive behaviour was found when the inhibition was analyzed with respect to the Na^+ concentration. Several molecules harbouring functional groups of nimesulide (analogues) were tested as inhibitors. None among the tested molecules has the capacity to inhibit the transport with the exception of the compound NS-398, whose chemical structure is very close to that of whole nimesulide. The IC_{50} for this compound was 131 μM. Inhibition kinetics showed behaviour of NS-398 identical to that of nimesulide, i.e., noncompetitive inhibition respect to glutamine and competitive inhibition respect to Na^+. Molecular docking of nimesulide suggested that this drug is able to bind B0AT1 in an external dedicated binding site and that its binding produces a steric hindrance effect of the protein translocation path abolishing the transporter activity.

1. Introduction

B0AT1 is a transporter for glutamine and other neutral amino acids. It is mainly involved in the absorption of these nutrients in intestine and reabsorption in kidney. B0AT1 is also expressed, even though at lower level, in other tissues, such as, skin, pancreas, prostate, stomach and liver, regulating neutral amino acids trafficking [1,2]. Since glutamine is one of the more efficiently transported substrates, B0AT1 is indirectly involved in the cell pathways underlined by this special amino acid, such as nucleotides and amino sugar synthesis, redox homeostasis through glutathione synthesis and shuttling of ammonia. The pivotal role of B0AT1 in the metabolism is demonstrated by the occurrence of the autosomal recessive Hartnup disease (OMIM: 234500) caused by defects of the

Abbreviations: $C_{12}E_8$, octaethylene glycol monododecyl ether; NS-398, N-[2-(Cyclohexyloxy)-4-nitrophenyl]methanesulfonamide.

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human gene coding for this protein, identified and annotated in the chromosome 5 [1–5]. This syndrome is characterized by metabolic disorders with serious symptoms such as a pellagra-like light-sensitive rash, cerebellar ataxia, emotional instability, and strong amino aciduria linked to loss of activity of B0AT1 in kidney. Several functional studies have been conducted on mouse and rat B0AT1 both in cell systems and in proteoliposomes [4,6–12]. From the experimental data it emerged that the transporter catalyses an electrogenic Na^+-amino acids co-transport and that Na^+ cannot be substituted by other cations. The specificity towards neutral amino acids such as glutamine, phenylalanine, leucine and several others, among which also histidine, has been well assessed [5,11]. In particular, the recent studies in proteoliposomes, clarified that internal K^+ stimulates the glutamine uptake by an allosteric positive effect. Moreover, the mechanism of Na^+-glutamine co-transport has been defined to be simultaneous [6,12]. This data represents the basis for further structure/function relationships analysis and studies of interaction with xenobiotics. It was previously demonstrated that mercurial environmental pollutants abolish the transport activity mediated by B0AT1 [12]. These results opened perspectives of screening of interactions with drugs in line with a novel aspect in pharmacology, i.e. the importance of identifying potential off-site targets of drugs, responsible of side effects [13]. Membrane transporters are likely to be included in the list of off-site targets of pharmaceutical compounds, given their localization in cell plasma membranes in epithelia. Therefore, in view of its crucial role in amino acid absorption/reabsorption, B0AT1 represents a potential first-level off-site interactor of xenobiotics. However, very few information exist on this aspect of the transporter; indeed it is still not included in the list of Package Inserts of drugs [14]. A further observation in favour of the potential involvement of B0AT1 in the interaction with drugs, comes from the fact that several therapeutic compounds interact with sodium-coupled transporters such as the GABA transporter and SERT whose structures harbour LeuT fold, like B0AT1 [15,16]. Moving from these bases, a screening of the effect of different pharmaceutical compounds, largely used in human therapy, was performed on the B0AT1 transporter in proteoliposomes. The study was conducted on the available rat isoform of B0AT1, which shares more than 87% identity with the human orthologous. Among the compounds which showed some effects, the anti inflammatory nimesulide resulted the most potent inhibitor and hence the study of the inhibition mechanism was investigated at the molecular level. To this aim, the homology model of B0AT1 was built on the basis of the structure of LeuT [17] and molecular docking of nimesulide was assessed substantiating the experimental data.

2. Materials and methods

2.1. Materials

Amberlite XAD-4, egg yolk phospholipids (3-sn-phosphatidylcoline from egg yolk) $C_{12}E_8$ were purchased from Fluka; L-[^{3}H]glutamine from Perkin Elmer; Sephadex G-75, from Sigma–Aldrich. Nimesulide, NS-398, Sulpiride, Quinidine, Scopolamine, Tetracycline, Lidocaine, Bupivacaine, Coumaric acid, Cloramphenicol, Quercetine, Omeprazole, Desipramine Hydrochloride, Verapamil, Berberine Hydrochloride, N-(3-Aminophenyl) methanesulfonamide, Acetylsalicilyc acid, Methanesulfonamide, 4-Aminobenzoic acid, Sulphamethoxazole, 4-Nitrobenzene sulphonamide, were from Sigma–Aldrich. All other reagents were of analytical grade.

2.2. Solubilization of the B0AT1 transporter

Brush-border membranes were prepared from rat kidney and stored as previously described [18,19]. The transporter was solubilized by treating the membrane preparation (50 μl, about 0.2 mg protein) with 1.3% $C_{12}E_8$, 20 mM Hepes/Tris pH 7.0 in a final volume of 150 μl and centrifuged at 13,000 × *g* for 4 min at 4 °C. The supernatant (extract) was used for the reconstitution.

2.3. Reconstitution of the B0AT1 transporter in liposomes

The B0AT1 transporter was reconstituted by the cyclic detergent removal procedure [20,21] with appropriate modifications. In brief, mixed micelles containing detergent, protein and phospholipids were repeatedly passed through the same Amberlite XAD-4 column. The composition of the initial mixture used for reconstitution was: 15 μl of the solubilized protein (about 10 μg protein in 1.3% $C_{12}E_8$), 80 μl of 10% $C_{12}E_8$, 100 μl of 10% egg yolk phospholipids in the form of sonicated liposomes prepared as described in Ref. [20], 50 mM K-gluconate and 20 mM Hepes/Tris pH 7.0 in a final volume of 700 μl. After vortexing, this mixture was passed 20 times through the same Amberlite XAD-4 column (0.5 cm diameter × 2.5 cm height) preequilibrated with a buffer of the same composition as the initial mixture but without the protein and the detergent. Functional proteoliposomes were obtained by this procedure. All operations were performed at 4 °C, except the passages through Amberlite XAD-4, which were carried out at room temperature. As previously reported [12], after reconstitution proteoliposomes contained few protein bands, the most abundant of which was B0AT1 identified by a specific antibody indicating that the transporter was substantially purified after the reconstitution procedure.

2.4. Transport measurements

To remove the external buffer, 550 μl of proteoliposomes were passed through a Sephadex G-75 column (0.7 cm diameter × 15 cm height) preequilibrated with 20 mM Hepes/Tris pH 7.0 and sucrose at an appropriate concentration to balance the internal osmolarity. Transport was started by adding 0.1 mM [^{3}H]glutamine and 50 mM Na-gluconate to the proteoliposomes, and stopped by adding 20 μM mersalyl at the desired time interval. In control samples the inhibitor was added at time zero according to the inhibitor stop method [22]. The control samples represent proteoliposome samples in which the unspecific radioactivity, incorporated by diffusion or binding to the membrane, is evaluated. The diffusion is not mediated by the transporter, thus, can be measured when the transporter is inactivated by mersalyl. This fraction of radioactive substrate also corresponds to that found in liposomes without protein. The unspecifically incorporated radioactivity is less than 5% of the [^{3}H]glutamine specifically taken up by proteoliposomes, i.e., by transporter mediated process. The assay temperature was 25 °C. Finally, each sample of proteoliposomes (100 μl) was passed through a Sephadex G-75 column (0.6 cm diameter × 8 cm height) in order to separate the external from the internal radioactivity. Liposomes were eluted with 1 ml 50 mM NaCl and collected in 4 ml of scintillation mixture, vortexed and counted. For the determination of the [^{3}H]glutamine uptake, the experimental values were corrected by subtracting the respective controls (samples inhibited at time zero). Transport rate was measured by stopping the reaction after 10 min, which is within the initial linear range of labelled substrate uptake into the proteoliposomes.

To generate a K^+ diffusion potential, 50 mM K-gluconate was added to the reconstitution mixture; K-gluconate was more efficient than other potassium salts, in activating the electrogenic process, as previously described [11]. After removal of the external salts and buffers by Sephadex G-75 chromatography, as described above, the positive outside membrane potential was generated by adding valinomycin (1 μg/mg phospholipid) in 2 μl ethanol/water 1:1 before starting transport measurement [23]. As previously