

Chemical Targeting of Membrane Transporters: Insights into Structure/Function Relationships

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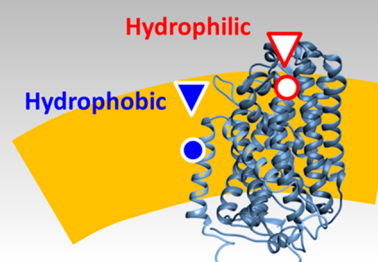
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ABSTRACT: Chemical modification of proteins is a vintage strategy that is still fashionable due to the information that can be obtained from this approach. An interesting application of chemical modification is linked with membrane transporters. These proteins have peculiar features such as the presence of hydrophobic and hydrophilic domains, which show different degree of accessibility to chemicals. The presence of reactive residues in the membrane transporters is at the basis of the chemical targeting strategy devoted to investigating structure/function relationships; in particular, information on the substrate binding site, regulatory domains, dimerization domains, and the interface between hydrophilic loops and transmembrane domains has been obtained over the years by chemical targeting. Given the difficulty in handling membrane transporters, their study experienced a great delay, particularly concerning structural information. Chemical targeting has been applied with reasonable success to some membrane transporters belonging to the families SLC1, SLC6, SLC7, and SLC22. Furthermore, some data on the potential application of chemical targeting in pharmacology are also discussed.

Amino acid (●) targeting reagents (▼)



1. INTRODUCTION

1.1. Relevance of Studying Membrane Transporters.

As in the case of the widely studied soluble enzymes, chemical targeting of membrane transport proteins can be considered a physiological mimicking strategy. Indeed, chemical modifications known as post-translational modifications (PTMs) occur in cells for regulating protein functions, driving protein localization, and accomplishing signaling phenomena. Even though in the case of membrane transporters the information on PTMs is not as large as for soluble proteins, it is well acknowledged that PTMs cause changes in function and structure of membrane transporters, as well. However, the size of such a phenomenon is unpredictable since the transporter proteome is still poorly defined. Rough data, available in databases together with some more extensive studies, indicate that threonine, serine, tyrosine, asparagine, lysine, arginine, and cysteine are the residues involved in PTMs of membrane transporters.¹ However, only some of the above listed amino acids are exploited for chemical targeting approaches. One of the reasons is that the suitability of an amino acid residue is limited by its intrinsic reactivity, while the physiological PTM process often involves the action of enzymes, thus allowing targeting of any residue even under mild conditions of pH and temperature. Moreover, the intrinsic reactivity of each residue in a protein can be influenced by the neighboring amino acids, which modulate the responsiveness to the administered reagent. Finally, the size and the hydrophilicity of a reagent

may affect its ability to interact at a specific site of the target protein. The hydrophilic/hydrophobic balance of a reagent has to be considered in particular when the target is a membrane protein in which hydrophobic and hydrophilic moieties coexist and can influence the reactivity. Therefore, by exploiting the features of reactants and their accessibility to protein residues, insights into the structure/function relationships of membrane transporters can be obtained. This issue is very important owing to the delay of the knowledge on membrane transporters with respect to that of soluble proteins.² Indeed, the interest in studying membrane transporters increased in the past decade due to their well-assessed role in cell homeostasis and potential pharmacological implications. Indeed, these proteins regulate the flux of metabolites and ions from the extracellular to the intracellular milieu and vice versa and, within a cell, among different organelles, allowing compartmentalized metabolic pathways to occur.² A great variety of membrane transporters are necessary to regulate the very intricate traffic of compounds. Then, it is not a surprise that roughly 10% of the human genome encodes for proteins related to transport function. After genome annotation,

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membrane transporters of human cells have been classified in ABC (ATP binding cassette) and SLC (solute carrier) superfamilies. In the first case, the superfamily includes seven families whose members exploit ATP hydrolysis as the driving force for transport (<https://www.genenames.org/data/genegroup/#!/group/417>). The SLC superfamily includes, to date, 65 families whose members gain energy by the concentration gradient of the transported substrate or by coupling the vectorial reaction of a substrate to the cotransport or counter-transport of another molecule or ion (<http://slc.bioparadigms.org/>). These transport mechanisms are called uniport, symport, or antiport, respectively. The crucial role of membrane transporters in maintaining cell homeostasis is demonstrated by the occurrence of pathologies, with a wide range of severity, due to inherited defects of genes encoding these proteins. Further proofs come from human diseases characterized by metabolic alterations, such as cancer and diabetes, in which the expression of some membrane transporters is changed for accomplishing the different nutritional needs of cells.

1.2. Chemical Targeting of Membrane Proteins: An Overview. Chemical targeting for function/structure relationship investigations has been widely used for membrane transporters as testified by several papers published since the beginning of transport studies.³ The main challenge in performing chemical targeting on membrane transporters resides in the difficulty of handling these hydrophobic proteins. At the same time, the advantage of chemical targeting is the possibility to explore and recognize the boundary between hydrophobic and hydrophilic protein moieties. As an example, using selective hydrophobic or hydrophilic chemical reagents, it is possible to map the substrate path or to reveal the relationships among the protein and the membrane. In summary, an accurate use of chemical targeting approaches can give a lot of information on both the protein surface and the protein interior³ (Figure 1). The purposes of using a chemical targeting strategy changed over the years.

Some alkylating, reducing, or oxidizing reagents have been used initially only for inhibiting the activity of membrane transporters in cells, in vesicles, or in isolated organelles as a control for identifying the activity of transporters. Later on, the achievement of methodologies for purifying membrane transporters from cells, tissues, or heterologous host expression enlarged the information inferable from chemical targeting such as the accessibility of water, substrates, or effectors to specific protein domains; the role of key residues for transport reaction or for transport regulation; the identification of protein–protein interactions; and the study of protein trafficking to the membrane surface. It is worth noting that few 3D structures of mammalian membrane transporters are available so far; thus, the chemical targeting approaches cannot be disregarded when investigating the structural and/or functional roles of specific residues or domains as well as for validating structures and topologies obtained by computational analyses. Interestingly, the knowledge acquired by chemical targeting very often anticipated crucial and key findings demonstrated only much later by the 3D structure of the transporters, confirming the suitability of this strategy.⁴ Nowadays, the chemical targeting is still largely used to obtain information that cannot be derived by the sole 3D structure, such as conformational changes underlying the transport cycle or the functional role of single amino acid residues.⁴ For all the mentioned reasons, a sizable number of chemical reagents,

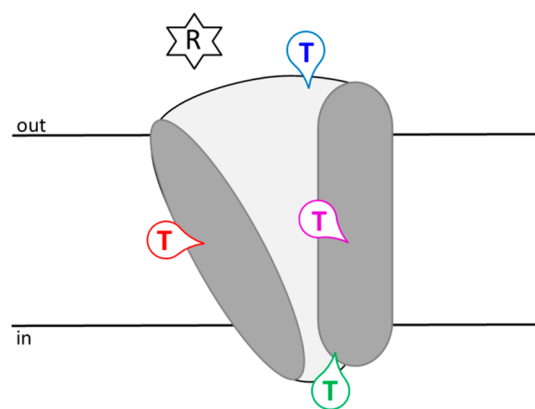


Figure 1. Chemical targeting of membrane transporters. The sketch depicts a typical asymmetric transporter that, upon conformational changes, exposes the substrate site alternatively to the external or internal side of the membrane. The reagent, depicted as a starred “R”, can have different size as well as degree of hydrophilicity/hydrophobicity. The target, indicated by T, can be any lateral group of an amino acid such as a thiol residue (SH) of cysteine as well as a NH₂ of lysine, etc. The T group exhibits four different typical exposures: (1) T facing the hydrophilic protein exterior (blue) that can be targeted by large hydrophilic reagents; (2) T facing the internal hydrophilic transport path (purple) that can be targeted by small hydrophilic reagents; (3) T facing the intracellular hydrophilic surface (green) that can be targeted by hydrophobic membrane permeant reagents; and (4) T facing the hydrophobic protein moiety in contact with the membrane (red) that can be targeted by hydrophobic small reagents. Effects observed upon targeting can then be ascribed to the targeting of a specific protein moiety based on the type of reagent.

more or less specific for an amino acid, harboring a different degree of hydrophobicity and different size, have been made available over the years [(Table 1) and see ref 5 for a comprehensive list of reagents].

The most exploited amino acid in chemical targeting of transporters is cysteine and, in a few cases, lysine or others.⁶ Indeed, cysteine abundance in proteins increased during evolution, but it is still one of the least abundant, thus being a favorable residue for specific targeting. Moreover, the side group of cysteine may give rise to different kinds of reactions at physiological pH, making this residue one of the most employed in the study of structure/function relationships of transporters [refs 7 and 8 and refs therein]. Thus, many reagents have been developed with different reaction mechanisms, such as alkylation (maleimides), the formation of mixed disulfides (methane thiosulfonates), or the formation of metal–S bonds (simple or derivatized heavy metals).³ All these classes of reagents include a lot of different compounds characterized by different properties of hydrophilic/hydrophobic balance and size. Moreover, bifunctional, fluorescent reagents and high molecular mass reagents are available for exploring protein–protein interactions and identifying exposed residues using a simple SDS-PAGE analysis.^{5,8} Differently, lysine is relatively more abundant, thus being a good candidate for extensive or unspecific targeting, as in the case of some cross-linking strategies.⁶ Currently, two major approaches are used in handling reagents: (i) the canonical chemical targeting and (ii) the chemical targeting coupled to site-directed mutagenesis (Figure 2A and B). The first mostly gives predictions on the location and exposure of protein moieties; in this respect, a parallel computational analysis may improve

Table 1. Chemical Reagents Commonly Used to Target Amino Acid Residues of Membrane Transport Proteins

reagent	type	target AA		
<i>N</i> -ethylmaleimide (NEM)	maleimides	Cys		
<i>N</i> -pyrene maleimide				
<i>N</i> -pyrroloisomaleimide				
<i>N,N'</i> -phenylene-bismaleimides				
<i>N</i> -(4-dimethylamino-3,5-dinitrophenyl)-maleimide				
<i>N,N'</i> -hexamethylene-bismaleimide				
4-chloroacetylphenylmaleimide				
4-azidophenylmaleimide				
iodoacetate	alkylating reagents	Cys		
iodoacetamide				
bromoacetate				
chloroacetate				
bromopyruvate				
ethylene oxide and -imine				
methyl iodide				
dimethylsulfate				
fluorodinitrobenzene (FDNB)	aryating reagents	Cys		
chlorodinitrobenzene (CDNB)				
Ag ⁺ , Cu ⁺ , Au ⁺ , Hg ²⁺ , Pb ²⁺ , Cu ²⁺ , Cd ²⁺ , Zn ²⁺ , Sb ³⁺	metal ions	Cys		
<i>p</i> -chloromercuribenzoate (pCMB)	organic mercurials	Cys		
<i>p</i> -chloromercuriphenylsulfonate (pCMBS)				
phenylmercuryacetate				
methylmercury iodide				
methylmercury bromide				
<i>O</i> -(3-hydroxymercuri-2-methoxypropyl)carbamyloxyacetate (mersalyl)				
chloromercurinitrophenols				
<i>S</i> -mercuric <i>N</i> -dansyl cysteine				
arsenite	arsenic reagents	Cys		
arsenoxides				
iodine	oxidizing reagents	Cys		
ferricyanide				
tetrathionate				
Na ₂ S ₄ O ₆				
H ₂ O ₂				
sulfoxides				
benzofuroxan				
dansyl chloride				
thiocyanogen				
(SCN) ₂				
tetranitromethane				
1,1'-azobis(<i>N,N</i> -dimethylformamide) (diamide)				
<i>S,S'</i> -dithio-bis(2-nitrobenzoic acid) (DTNB)			disulfides	Cys
<i>n</i> -propyl-2-pyridyl disulfide				
<i>N</i> -succinimidyl-3-(2-pyridylthio)propionate (SPDP)				
β -hydroxyethyl-2,4-dinitrophenyl disulfide				
oxidized glutathione (GSSG)				
cystamine				
cystamine monosulfoxide				
di- <i>N</i> -(2-nitro-4-azidophenyl)-cystamine- <i>S,S</i> -dioxide (DNCO)				
sodium (2-sulfonatoethyl)methanethiosulfonate (MTSES)	methanethiosulfonates	Cys		
2-aminoethyl methanethiosulfonate hydrobromide (MTSEA)				
(2-(trimethylammonium)ethyl)methanethiosulfonate bromide (MTSET)				
methyl methanethiosulfonate (MMTS)				
1-ethyl-3-(3'-dimethylaminopropyl)carbodiimide (EDC)	various	Asp/Glu		
2-hydroxy-5-nitrobenzyl bromide (Koshland's Reagent)				
sulfo-NHS-SS-biotin				
4,4'-diisothiocyanatostilbene-2,2'-disulfonate (DIDS)				
4-acetamido-4'-isothiocyanato-stilben-2,2'-disulfonate (SITS)				
pyridoxal phosphate (PLP)				
Woodward's reagent K (2-ethyl-5-phenylisoxazolium-3'-sulfonate)				

Table 1. continued

reagent	type	target AA
phenylglyoxal		Arg
phenylmethylsulfonyl fluoride (PMSF)		Ser
diethyl pyrocarbonate (DEPC)		His
<i>N</i> -bromosuccinimide (NBS)		Trp

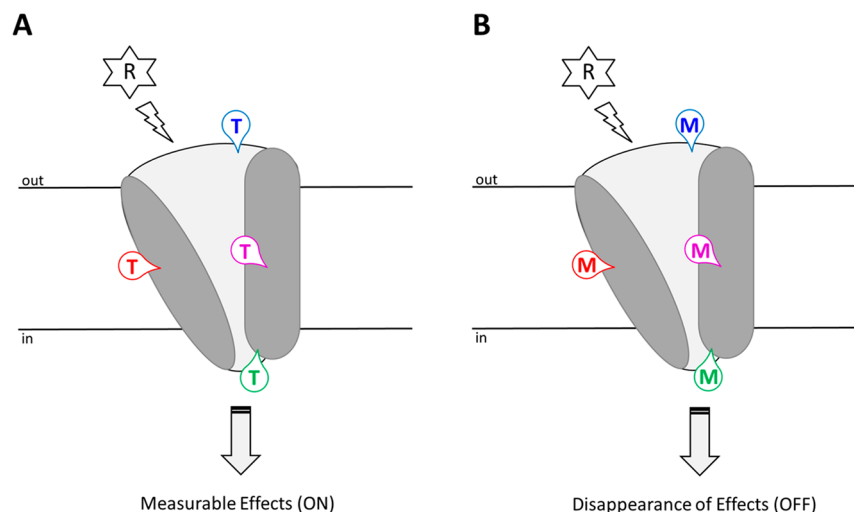


Figure 2. Chemical targeting coupled to site-directed mutagenesis. The sketch depicts a typical asymmetric transporter inserted in a cell membrane. Targetable residues (T) or their mutants (M) are exposed to the reagent (R). (A) In the wild-type transporter, after the interaction with the reagent, a measurable effect called “ON” is observed. (B) In the mutant protein, in which one of the targetable residues (T) is substituted by a nontargetable residue (M), the effect observed in (A) disappears (“OFF”), if the mutated residue is actually the target of the reagent.

the accuracy of the results. The second gives more precise information on the structure/function relationships of specific residues or more defined protein moieties. However, the site-directed mutagenesis approach is based on the availability of a recombinant transporter and on a method for assaying transport *in vitro* such as in proteoliposomes.⁹ In principle, the recent and up to date CRISPR/Cas9 technology could also allow *in vivo* site-directed mutagenesis, but this strategy does not yet have a wide application in structure/function relationship studies. A peculiar and classical example of chemical targeting coupled to site-directed mutagenesis is the methodology known as Cys-scanning mutagenesis.¹⁰ Similar chemical targeting approaches have been employed to study a huge number of different transporters from plasma and organelle membranes, with a special focus on those from an inner mitochondrial membrane.¹¹ Therefore, a systematic dissertation cannot be here afforded. In this mini-review, a compact overview of the use of chemical targeting for gaining insights into structure/function relationships of plasma membrane transporters will be given. The described strategies have been chosen because they have a general application in the field of membrane transporters. A special focus on the widely used cysteine targeting will be proposed. Besides cysteine, two examples of a peculiar targeting approach based on tryptophan and lysine will also be provided. The proteins dealt with are members of the SLC1, SLC6, and SLC7 families responsible for the transport of amino acids and the SLC22 family responsible for the transport of organic cations and carnitine. The choice of these proteins derives from their relevance in human pathologies and pharmacology for drug design, thus representing a future perspective on the use of chemical targeting also in the pharmaceutical field.⁷ Indeed, the targeted covalent inhibitors (TCIs) approach applied on a

transport protein was revealed to be already fruitful in the case of the largely used drug, omeprazole.

2. CANONICAL CHEMICAL TARGETING

As stated in the **Introduction**, chemical targeting has been employed for specifically inhibiting the transport activity of membrane transporters. This strategy allowed identifying a single transporter or a class of transporters characterized by reactivity toward a specific reagent. Indeed, the first classification of membrane transporters, in the pregenomic era, has been performed based on sensitivity to inhibitors of two different types: covalent inhibitors which form bonds with specific amino acid residues or noncovalent inhibitors which interact via weak bonds such as hydrogen bonds. The canonical chemical targeting is intended as covalent modification of an amino acid residue of a protein. Later on, information on structure/function relationships has been collected by combining chemical targeting data with topology predictions. The information on protein topology derived by hydrophathy plots or by refined 3D homology models was obtained based on template 3D structures with enough similarity with the unknown protein. In the next paragraph, examples of using canonical chemical targeting on some amino acid transporters are reported together with basic information on the function and relevance of each transporter.

2.1. Rat Amino Acid Transporters SLC1A5 and SLC6A19: Targeting Cysteine Residues by Mercurial Reagents. SLC1A5 and SLC6A19 are transporters for neutral amino acids with different specificity, transport mechanism, and structural folds. SLC1A5, known as ASCT2, shows high specificity for glutamine, alanine, serine, asparagine, and threonine and obeys a peculiar transport mechanism that is

an obligatory antiport that is strictly sodium dependent [ref 12 and refs therein]. Based on the transport mode and the substrate specificity, ASCT2 has been proposed as a harmonizer of amino acid pools in cells. The studies on murine ASCT2 began in the '90s with the annotation of the rat gene and the study of tissue distribution of the protein. Then, the development of methodologies for the transport assay in intact cells allowed the study of substrate specificity and kinetics. Later, an *in vitro* model for the transport assay of the rat ASCT2 based on the proteoliposome technology was set up. This model is suitable for the structure/function relationship studies because proteoliposomes are artificial vesicles in which a single protein, derived from homogenized tissue(s) or by recombinant production, is inserted with the same orientation as in the native cell membranes, and transport activity can be measured by reducing or abolishing the interferences derived from other transporters or metabolic pathways present in intact cells; this feature may represent also a limitation of the system that lacks potential physiological regulator(s) of the protein under investigation; indeed, the most comprehensive studies are conducted by combining transport assays in proteoliposomes with those in the intact cell system.⁹ The proteoliposome model allowed us to investigate the functional properties of ASCT2. As an example, the rat protein was revealed to be competent also for glutamate transport, besides neutral amino acids. This feature links ASCT2 to the glutamine/glutamate cycle between astrocytes and neurons that are responsible for reducing glutamate toxicity during neurotransmission.¹² SLC6A19, known as B0AT1, is a Na⁺-dependent transporter with high specificity for glutamine, leucine, cysteine, valine, isoleucine, methionine, phenylalanine, alanine, serine, and asparagine. Other amino acids are transported with lower affinity, while cationic and anionic amino acids are not substrates. The transport reaction mediated by B0AT1 is an electrogenic cotransport Na⁺: neutral amino acid. The first studies on murine B0AT1 have been performed in intact cells, and some information on the molecular mechanism of transport and stoichiometry has been collected.¹³ Later on, as for ASCT2, the proteoliposome assay model has been set up for B0AT1, as well. Owing to this experimental tool, some basic properties of B0AT1 have been confirmed, and some controversies, regarding the regulatory properties and the transport mechanism, have been solved such as the allosteric modulation by K⁺, the mechanism of transport, and the lack of involvement of ACE2 (angiotensin converting enzyme 2) and collectrin in the intrinsic transport function of B0AT1.

After the functional characterization, chemical targeting has been applied to rat ASCT2¹² and rat B0AT1.⁹ This kind of study has been designed in the absence of 3D structures of ASCT2 and B0AT1 for which only homology models have been built based on bacterial homologues. Both ASCT2 and B0AT1 were revealed to be sensitive to the cysteine targeting by metal–S bonds using mercurial reagents and some other heavy metals. Starting from the assumption that the binding of a metal to the protein impairs its function, the interactions have been evaluated as the extent of transport inhibition in proteoliposomes harboring ASCT2 or B0AT1 extracted from a rat kidney. Inhibition by HgCl₂, methyl-Hg, and mersalyl unveiled structure/function relationships of both B0AT1 and ASCT2. Indeed, the mercurial targeting is linked to the presence, in both proteins, of a metal-binding domain, namely, CXXX, that is accessible to the hydrophilic reagents. Rat

B0AT1 contains an additional CXXXC motif that is also acknowledged as a metal target. The more hydrophilic and larger compound mersalyl was revealed to be an inhibitor of ASCT2 and B0AT1 with the same mechanism of HgCl₂ and methyl-Hg but with lower affinity explained by a different degree of accessibility of the compound to the reactive domain of the proteins (Figure 1). In line with the specificity of the metal–transporter interaction (metal–S bond), some differences in the ability of chemical or physiological antioxidants to reverse the effect of metals have been highlighted between ASCT2 and B0AT1. In the first case, the inhibition is fully reversed, while in the second case, the reversal of B0AT1 inhibition is revealed to be less efficient, indicating that some additional mechanism rather than merely a covalent one should take place in the interaction with metals. As a proof of this hypothesis, in the case of B0AT1, the inhibition by the hydrophilic mersalyl is prevented to some extent by the presence of the natural substrate of the transporter, i.e., glutamine. This indicates that the binding site for mersalyl is close to, or is part of, the substrate binding site that forms an aqueous cavity exposed toward the extracellular milieu as predicted by a homology model. Taken together, the data of cysteine targeting allowed gaining insights into the structure/function relationships of ASCT2 and B0AT1, probing the exposure of some hydrophilic domains of these proteins. The collected results represent typical examples of the canonical chemical targeting coupled to structure predictions (homology modeling) to investigate the role of protein moieties in the transport function. Such an application of the canonical chemical targeting can be employed with relatively simple procedures for a large variety of membrane transporters thanks to the availability of a huge number of SH reagents with very different features.^{5,8} In the case of ASCT2 and B0AT1, the data obtained with the mercury compounds HgCl₂ and methyl–Hg also had relevance to toxicology: the two compounds are, indeed, well-known pollutants often spread in the environment by industry activities, and the measured IC₅₀ values are in the micromolar range, i.e., that are normally present in the environment. Concerning the rat ASCT2, besides mercury, Cu²⁺ also showed inhibitory effect. Other divalent cations such as Zn²⁺ and Cd²⁺ are weaker inhibitors, indicating that the mere presence of a binding motif does not guarantee an efficient interaction, but the surrounding amino acid environment influences the binding and inhibition.

2.2. Human Amino Acid Transporter SLC1A5: A Peculiar Targeting Tryptophan Residue by the Koshland's Reagent. The chemical targeting of tryptophan residues has been rarely used for membrane transporters (Table 1). In the case of human ASCT2, this strategy allowed us to probe the sites of the interaction of cholesterol with the transporter. A brief history of the human ASCT2 will be given for introducing its relevance to human health. The human gene coding for ASCT2 has been annotated after the initial studies on the murine isoforms, and then the protein has been identified [ref 12 and refs therein]. The relevance of ASCT2 to human health emerged after the discovery of its strong overexpression in virtually all human cancers, boosting the efforts for deepening the knowledge of this protein [ref 12 and refs therein]. One of the major aspects dealt with is the biological significance of the overexpression and the structure/function relationships that could help to improve the design of new potential drugs able to specifically target the protein. Regarding the first aspect, it is now well accepted that the

overexpression of ASCT2 in cancers is linked to the glutamine addiction of cancer cells. This amino acid is used for energy production thanks to a modification of the terminal energy metabolism consisting of a truncation of the tricarboxylic acid cycle. Thanks to this metabolic rewiring, the carbon skeleton of glutamine can be converted, by one carbon atom catabolism, into ATP using a low amount of oxygen. This allows cancer cells to sustain their high rate of proliferation by furnishing metabolites for anaplerotic reactions.¹⁴ The ASCT2 has been studied in intact cell systems and proteoliposomes, harboring the recombinant protein obtained by overexpression in the methylotrophic yeast *P. pastoris*. The functional properties described in cell systems overlapped those obtained with the recombinant protein, while some differences have been highlighted in terms of substrate specificity and the molecular mechanism of transport with respect to the rat protein: one distinctive difference is that human ASCT2 does not recognize cysteine as a substrate, and this amino acid, possibly by binding to an allosteric site of the protein, can modulate the human ASCT2 transport function.¹² Very importantly, a relevant difference between the human and the rat isoforms exists in a stretch of 31 amino acids in which the local identity is lower than 15%. This is concurrent to the significant difference in the abundance of cysteine residues between the two orthologues; rat ASCT2 harbors 16 cysteine residues and human ASCT2 only 8. These findings indicate that the results collected with a protein from a model organism cannot always be considered fully valid also for the human orthologue protein. An accurate evaluation of the similarity of the proteins between the human and model organism must be performed before drawing any conclusions. Recently, using the protein produced in *P. pastoris*,¹² the 3D structure of the human ASCT2 has been solved by CryoEM; interestingly, it has been demonstrated that the protein is organized in a trimeric structure as hypothesized by previous functional and kinetics data.¹² Moreover, allosteric sites for cholesterol have been predicted, based on patches of densities found in the solved trimer that we have confirmed by computational docking analysis [ref 15 and references therein]. In this respect, chemical targeting has been employed to validate such predicted areas of interaction and to obtain novel structure/function information. A peculiar strategy based on targeting tryptophan residues in the vicinity of a cholesterol molecule has been used. In this approach, the Koshland's reagent that is specific for tryptophan residues at pH 7.0 has been employed to treat the functionally active human ASCT2 (Figure 1). Upon the treatment, the protein was revealed to be less sensitive to cholesterol modulation, validating the predicted interaction of cholesterol with tryptophan residues. The same strategy has been applied using SH reagents able to target cysteine residues lying on the interface with cholesterol;¹⁵ in that case, additional information has been obtained owing to the availability of some specific cysteine mutants as described in the next paragraph. This peculiar type of chemical targeting strategy to probe the site of interaction of cholesterol assumes great importance due to the emerging role of cholesterol for membrane transporter function and structure. These aspects find a biological explanation also in the localization of membrane transporters in lipid rafts of the membrane where other lipids, besides cholesterol, play a regulatory function as recently demonstrated for ASCT2. The ganglioside GD2 is responsible for the malignant phenotypes of several human cancers through the interaction with ASCT2.¹⁶

3. CHEMICAL TARGETING COUPLED WITH SITE-DIRECTED MUTAGENESIS

The availability of site-directed mutants of membrane transporters enlarges a lot the range of structure/function relationship information that can be derived from chemical targeting. Even though some pitfalls may arise, such as the difficulty in the production of recombinant membrane transporters and their mutants, a sizable number of papers deal with the combination of chemical targeting with site-directed mutagenesis. Indeed, the on–off switch of the effect of a chemical reagent observable between the WT protein and a site-directed mutant (see Figure 2) is a straightforward proof of concept to demonstrate the role of a specific protein residue. An important step in this experimental strategy is the right choice of the substituting residue. Taking into account that the most used site-directed mutagenesis tool is that of cysteine, this residue can be substituted by at least two amino acids that are alanine and serine. The first substitution results in the simple removal of the thiol group from the amino acid side chain. Since the role of a residue also depends on the amino acid surroundings, the use of alanine can be deleterious for the activity of the protein when the cysteine residue is involved in some hydrogen bonds or other hydrophilic interactions. In this case, serine is a better substitution since it harbors a hydrophilic –OH residue. Generally, it is not trivial to consult a PAM (or Blosum) matrix before deciding which amino acid has to be used for changes.¹⁷ Some examples will follow for highlighting how a specific targeting approach coupled to mutagenesis and to mass spectrometry gave detailed information on the structure/function relationships.

3.1. Human Amino Acid Transporter SLC1A5: Targeting Cysteine Mutants by Thiol Reagents. As described above, the interest around SLC1A5 (ASCT2) increased due to the relevance to human health. Then, the identification of critical residues in terms of function and reactivity to reagents has been a primary objective in the last 10 years. These strategies are still in progress, even after the ASCT2 3D structure resolution, to identify moieties of the protein responsible for allosteric modulation and conformational changes occurring during the transport cycle. A big piece of this work has been performed employing proteoliposome transport assays.^{12,18} Given the presence of 8 cysteine residues in the ASCT2 structure, the targeting of SH groups has been one of the first attempts of investigation. The induction of S–S/SH (or vice versa) conversion revealed that human ASCT2 is fully active in its thiol-reduced state, while it is inactive in the oxidized state. This phenomenon has been demonstrated by using some chemical and physiological reducing or oxidizing reagents with the capacity to convert disulfides into thiols or thiols into disulfides, respectively. Among these reagents, the most commonly used are the reducing agents DTE (DiThioErythritol) and β -mercaptoethanol (chemical reagents), GSH (reduced glutathione), and H₂S (physiological reagents); the oxidizing are Cu²⁺-phenanthroline and diamide (chemical reagents), GSSG (oxidized glutathione), and H₂O₂ (physiological reagents). Moving from this premise, further aspects of cysteine reactivity have been dealt with, and chemical reagents with different degrees of hydrophobicity, such as methyl-Hg (hydrophobic) and MTSES (hydrophilic membrane impermeant and 2-sulfonatoethylmethanethiosulfonate sodium salt) have been used. Interestingly, the compounds can block the transport activity of ASCT2 with

different potency, methyl-Hg being more effective than MTSES. The different extent of inhibition suggested that the cysteine residues underlying this phenomenon are easily accessible to methyl-Hg and less accessible to MTSES. The identification of the cysteine residue responsible for the effect has been straightforward using the site-directed mutagenesis approach. Each of the eight cysteine residues has been replaced by alanine constructing eight single cysteine mutants. The substituting residue alanine has been chosen because it is the simplest one in which only the thiol group is missing. The identification strategy is based on the lack (on–off switch) or reduction of the effects of the targeting reagents in a specific cysteine mutant (Figure 2). This residue is the C467, thus identified as the main target of the SH reagents. With a more sophisticated approach of kinetic analysis, the mapping of the substrate-binding site has been completed, revealing that C467 is crucial also for the recognition of the substrate glutamine. Noteworthy, soon after, the 3D structure of human ASCT2 was solved in complex with the substrate that mapped exactly in the same area identified by the targeting/mutagenesis approach.¹⁸ The chemical targeting coupled to site-directed mutagenesis revealed to be useful to describe the mechanism of redox regulation of the protein. Two cysteine residues, namely, C308 and C309, can form a disulfide with the C467 explaining the inhibition of transport activity under oxidizing conditions. The chemical targeting coupled to site-directed mutagenesis has also been applied to validate the interaction between cholesterol and a specific moiety of ASCT2. As stated above, cholesterol can stimulate transport activity mediated by ASCT2 by physical interaction with the protein; interestingly, in the presence of cholesterol the well-known inhibitor HgCl₂ was revealed to be less efficient in blocking transport activity; in other words, cholesterol can protect from the inhibition exerted by HgCl₂. This finding is in line with the possible presence of a cholesterol molecule in a domain of the protein surrounded by the residues C308 and C309, according to molecular docking predictions. Then, two cysteine-alanine mutants, namely C308A and C309A, have been constructed, and their sensitivity to HgCl₂ in the presence of cholesterol has been tested. In good agreement with the mentioned hypothesis, the addition of cholesterol did not exert the protection phenomenon observed in the wild-type protein when treated with HgCl₂. This strategy definitively allowed us to validate the presence of other binding sites for cholesterol close to the mentioned cysteine residues as predicted by computational analysis and by the 3D structure of ASCT2.¹⁵

3.2. Human Amino Acid Transporter SLC1A5: A Peculiar Targeting Strategy of Lysine Residues. Besides cysteine, lysine is another residue employed in the chemical targeting of human ASCT2 for a more complex structure/function relationship study. Indeed, lysine targeting by biotinylation has been employed in cell systems to obtain information on the role of N-glycosylation moieties in the trafficking and stabilization of ASCT2 to the plasma membrane. Therefore, a peculiar approach involving site-directed mutagenesis and transfection into human cells has been used in which the mutated amino acid is different from the targeted one. In particular, the substituted residues are those responsible for N-glycosylation, i.e., asparagine, while the targeted ones are lysine residues according to the biotinylation protocol. This approach allowed us to definitively assess the role of N-glycosyl moieties in stabilizing the protein at the plasma membrane [ref 12 and refs therein].

3.3. Human Histidine/Essential Amino Acid Transporter SLC7A5: Targeting Substrate Site Cysteine by Thiol Reagents. SLC7A5, known as LAT1, is a membrane transporter responsible for the exchange across the cell membrane of neutral amino acids by a Na⁺- and pH-independent mechanism. The protein is mainly localized in basolateral membranes of polarized epithelia and peculiar body districts such as the blood–brain barrier and the placental barrier. LAT1 is one of the few examples of a heterodimeric transporter constituted by two subunits linked via a covalent bond between two conserved cysteine residues of each subunit, i.e., LAT1 and CD98 (SLC3A2). The interest in LAT1 increased over the years due to its well-documented overexpression in human cancers [ref 19 and refs therein]. As in the case of ASCT2, the phenomenon of the overexpression has been linked to the metabolic rewiring typical of cancer cells which become addicted to some nutrients for both energy production and signaling. Very recently, a link of LAT1 with a familiar form of autism spectrum disorder has been proposed, confirming the importance of LAT1 for brain homeostasis [ref 19 and refs therein]. In this frame, understanding the molecular mechanism of LAT1 is fundamental for both basic and applied research in terms of designing new drugs. The first studies on human LAT1 have been conducted in cell systems, and the specificity toward neutral amino acids has been assessed. A further contribution to the field has been provided by employing the proteoliposomes harboring the recombinant protein produced in *E. coli*. In this experimental setup, it has been demonstrated that LAT1 is the sole competent transport unit of the heterodimer and that histidine is one of the favorite substrates, besides almost all the essential amino acids. It has been demonstrated that the companion CD98 does not play any role in the intrinsic transport function of the heterodimer, but it should be involved in routing LAT1 to the definitive location into the plasma membrane [ref 19 and refs therein].

LAT1 harbors 12 cysteine residues that must be in a reduced state for full transport function. This feature correlates well with the sensitivity toward mercury compounds, which is also responsible for the toxicity exerted by these pollutants in the brain and placenta where LAT1 is expressed. As in the case of ASCT2, the cysteine reactivity has been exploited for chemical targeting approaches devoted to gain information on the substrate binding site and residues critical for transport function. At first, by combining computational analysis and site-directed mutagenesis, the presence of four critical residues for histidine binding has been revealed; in particular, the residue F252, analogue of the tryptophan residue in the arginine/agmatine transporter AdiC of *E. coli*, was revealed to be crucial for substrate gating. Interestingly, also two cysteine residues are required for substrate recognition. The presence of these two exposed thiol groups allowed us to investigate their role in the reactivity toward small, large, hydrophobic, or hydrophilic SH reagents. Therefore, cysteine to alanine site-directed mutants have been generated, namely, C335A and C407A: proteins lacking one or both residues did not lose their reactivity toward the tested SH reagents. This indicated that the substrate binding site of LAT1 is not easily accessible to molecules very different from the natural substrate. This information is in line with data on the minimal structural requirement for a molecule to be considered a substrate of LAT1 and with the recently solved 3D structure of human LAT1 in complex with hCD98.²⁰

3.4. Human Organic Cation Transporter SLC22A4: Targeting Cysteine Mutants by Thiol Reagents.

SLC22A4, known as OCTN1, is a plasma membrane transporter that, together with OCTN2 (SLC22A5) and OCTN3, constitutes the OCTN subfamily, a small group of proteins responsible for the traffic of organic cations and carnitine in cells. Surprisingly, OCTN3 has been lost in humans, thus no human SLC classification is currently present for this protein [ref 15 and refs therein]. While OCTN2 and OCTN3 are unequivocally identified as carnitine transporters, OCTN1 has low affinity for carnitine and is responsible for the Na^+ -independent transport of organic cations, experimentally represented by the prototype TEA (tetraethylammonium). This suggested that, physiologically, OCTN1 might be involved in the traffic of organic cations but not of carnitine. The transporters belonging to the SLC22 family are very interesting for their involvement in drug disposition and interaction. Indeed, these proteins have been listed by the ITC (International Transporter Consortium) as those to be considered when designing new drugs.¹⁵ The human isoform of OCTN1 has been studied in intact cells and in proteoliposomes harboring the recombinant protein overexpressed in *E. coli*. In proteoliposomes, as well as HeLa cells, it has been demonstrated that OCTN1 can mediate the transport of acetylcholine.¹⁵ This molecule, known as a neurotransmitter, has a relevant, even though less famous, role also in non-nervous districts. Despite this lack of knowledge, the presence of acetylcholine in non-neuronal tissues is an evolutionary old function that remained also in higher organisms where it can play the autocrine role of regulating cell growth, cell proliferation, and also the regulation of inflammatory responses [ref 15 and refs therein]. Concerning this last aspect, a natural variant of human OCTN1 has been related to inflammatory bowel disease, Crohn's disease, and is less efficient in mediating the efflux of acetylcholine from proteoliposomes. The 3D structure of this protein is not available; only incomplete homology models have been built, in which the large cytosolic loop, a typical feature of the OCTN subfamily, is not solved (Figure 3). Therefore, the chemical targeting approach coupled to site-directed mutagenesis was revealed to be very helpful to define structure/function relationships of this protein and to validate the model. Indeed, four cysteine residues are in the extracellular hydrophilic loop, and three cysteine residues are in the hydrophobic transmembrane domain of this protein (Figure 3) [ref 15 and refs therein]. This is a typical case of employing chemical targeting for obtaining information on two different regions of the protein. Then, single cysteine to alanine mutants have been generated, and their sensitivity toward both hydrophilic and hydrophobic SH reagents has been tested. The strategy has been based on the on-off switch of chemical reagent effects for identifying those responsible for targeting a single cysteine residue and identifying the hydrophilic/hydrophobic environment in which the residues are located (Figure 2). In this experimental setup, it has been shown that cysteine residues present in the large extracellular loop are the most relevant for the interaction with mercurial reagents HgCl_2 , methyl-Hg, and ethyl-Hg. The same information has been derived by using MTSEA (2-aminoethylmethanethiosulfonate hydrobromide), a prototype thiol reagent that confirmed the results obtained with mercurial reagents. From the results collected with site-directed mutants, information on the degree of exposure of the studied cysteine residues and,

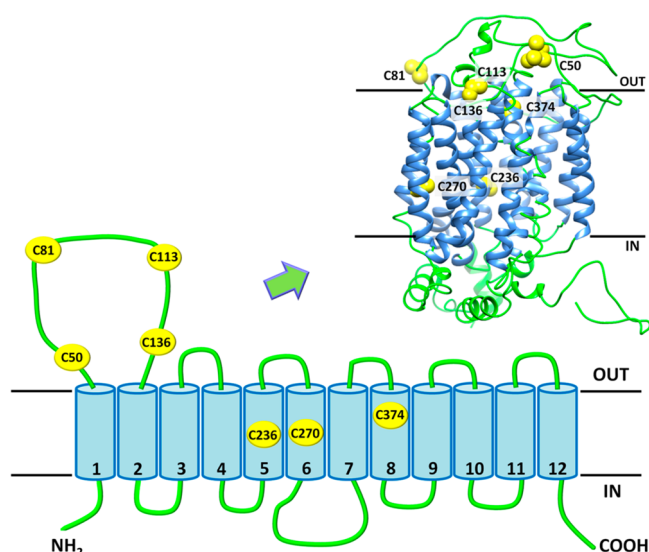


Figure 3. Prediction of the hOCTN1 structure. Topology 2D model obtained using the Kyte–Doolittle algorithm for hydropathy analysis of the hOCTN1 protein. Light blue barrels indicate 12 hydrophobic transmembrane spanning domains. Hydrophilic loops connecting transmembrane domains are depicted in green. The seven cysteine residues are numbered and depicted as yellow ovals. The N- and C-termini of the protein face toward the intracellular side. From the 2D model, the 3D homology model of the hOCTN1 protein is built. The transmembrane domains are represented as a light blue ribbon, while the loops connecting the domains are in green. The 3D model has been built using a Phyre2 Server on the structure of the eukaryotic phosphate transporter from *P. indica* (4J05) as a template. The side chains of the 7 Cys residues are highlighted by numbered balls and sticks.

hence, on the exposure of a specific protein domain has been derived. The main residue responsible for the inhibition exerted by mercury compounds was revealed to be the residue C50. Another residue, namely, C136, can also react even though with different sensitivity when comparing the HgCl_2 , methyl-Hg, and ethyl-Hg. In particular, the reactivity decreased while increasing the size and the hydrophobicity of the reagent, suggesting that this residue may be in a small hydrophilic pocket that cannot be reached by the three reagents with the same feasibility. The data have been further confirmed by using MTSEA toward which C50 and C136 mutants behaved as in the case of mercury compounds. A double mutant C50A/C136A has been also produced, and the complete lack of reactivity toward mercury compounds proved that these two residues are the most relevant for the described interaction. The higher hydrophilicity of MTSEA allowed us to conclude that another cysteine residue, namely, C81, might be close to a hydrophilic core accessible to MTSEA but not to the mercury compounds, explaining the different reactivity of the C81A mutant toward these molecules. It could be speculated that such a residue may come close to the substrate path and that the interaction with MTSEA, bigger than HgCl_2 and methyl-Hg, will affect substrate translocation. Therefore, the site-directed mutagenesis approach allowed us to validate the homology model built for human OCTN1. In line with this, the cysteine residues embedded in the transmembrane residues are not accessible to hydrophilic compounds added in the external compartment during transport assay. This strategy also allowed us to validate a homology structural model of OCTN1.¹⁵ According to the relevance of the SLC22 family

in drug disposition, other studies on members of this family have been performed. A strategy similar to that described for OCTN1 has been indeed used for the OCT2 (SLC22A2) to define the topology of the protein.²¹

3.5. Cys-Scanning Mutagenesis Approach, a Summary. A peculiar type of chemical targeting approach that has been widely used in membrane protein study is that of cysteine-scanning mutagenesis coupled to chemical targeting. This strategy consists of inserting a cysteine residue in each position of the tested protein starting from the cysteineless, that is, a protein in which all cysteine residues have been substituted by alanine or serine (or other residues). Then, the sensitivity of each mutant containing a single cysteine residue toward SH reagents with different degree of hydrophilicity is tested. The main advantage of this strategy is that of solving the topology of extensive domains of the proteins; the disadvantages are being time-consuming due to performing many mutants (scanning mutagenesis), the high costs, and the pitfalls caused by some unaccepted substitution of key residues that cause protein inactivation. A major problem that may arise at the beginning of the procedure is an inactive cysteineless protein that cannot be used as a control. This approach has been deeply employed for: mapping the substrate binding site; studying the exposure of extracellular loops; defining the relationship of the hydrophobic domains with the membrane; and determining the secondary structure of a protein domain or an allosteric region. Eminent examples of the employment of such a strategy are at the forefront of studies on lactose permease of *E. coli* and the more recent ones on glutamate transporter EAAT1.^{20,22} Noteworthy, many other papers have been published in which the approach has been applied to other membrane transporters.

3.6. Site-Directed Mutagenesis Coupled to Spectroscopy, a Summary. An innovative strategy for studying protein dynamics or protein oligomerization is the double electron–electron resonance (DEER) method. This is a spectroscopic technique based on nitroxide spin labeling at a specific cysteine residue that can also be introduced by site-directed mutagenesis. Fine structural transition could be measured in a range of 10 Å; for this reason, such an approach has been employed in the study of membrane transporters to evaluate transition between facing monomers and, within a monomer, between different structural domains. An interesting example of application of this strategy is that of the aspartate transporter of *P. horikoshii* known as GltPh. This protein represents the bacterial counterpart of the SLC1 family of proteins. It has, indeed, a homotrimeric structure, and the introduction of cysteine residues at predicted sites allowed understanding that the binding of the substrate to one monomer does not have a cooperative effect on the binding of another substrate to the adjacent monomer(s). This kinetic feature has been conserved during evolution, and the eukaryotic counterpart ASCT2 (SLC1A5), that has a trimeric assembly, does not show cooperativity phenomena as well.¹² The DEER approach has been employed also in the case of other bacterial membrane transporters and channels.²³ Mass spectrometry measurements are also very useful for unveiling structural aspects of membrane transporters, which are difficult to properly study with NMR and X-ray strategies. In this respect, covalent reagents able to specifically target amino acid residues are widely used to modify proteins, which undergo mass spectrometry analyses as previously reviewed.²⁴

4. APPLICATION OF THE CHEMICAL TARGETING TO DRUG DESIGN

A specific application of the chemical targeting in the study of membrane transporters is that of covalent drug design. This approach is not the most common in pharmacology; in fact, the majority of drugs is designed as noncovalent interactors, so far. On the contrary, covalent drugs are much fewer and are normally inhibitors of target proteins; eminent examples of this strategy are acetylsalicylate and penicillin, two blockbuster drugs which act by reacting with serine residues of the cyclooxygenases and DD-transpeptidase, respectively. Interestingly, membrane proteins have also been selected as targets of covalent drugs; in this case, cysteine residues of the purinoceptor P2Y12 and the gastric proton pump have been used as a target of other commonly used drugs, i.e., clopidogrel and omeprazole (and derivatives), respectively. Later on, this covalent approach has been upgraded to the currently known as targeted covalent inhibitors (TCIs). In such a strategy, a noncovalent interaction occurs between the drug and a target protein, followed by the covalent binding of an electrophilic group (warheads) of the drug to a specific amino acid residue of the target [refs 6 and 7 and refs therein]. In this respect, the SH group of cysteine is a very good candidate due to the sizable number of possible reactions occurring under physiological conditions.⁸ Moreover, the localization of membrane proteins at the boundary between the exterior and interior of a cell makes the covalent approach an attractive possibility for improving the efficacy/potency of pharmacological treatments. The drug design is a high-cost process in terms of both laboratory and animal tests with several challenges due to the detection of compound toxicity sometimes after completion of the costly phases of the experimentation. In this scenario, the possibility of *in silico* predictions by molecular docking analysis, before experimental testing, is one of the best approaches for reducing the number of molecules to be assayed experimentally with a drastic reduction of the costs. This issue becomes particularly important for transporters considered a “hot spot” in drug development; this is the case of ASCT2 and LAT1 dealt with in this mini-review, due to their wide overexpression in virtually all human cancers. These proteins are, indeed, eminent druggable targets, and plenty of studies arose with the scope of finding specific, potent, and efficient inhibitors able to reduce cancer cell growth and proliferation.^{12,19} The majority of the studies has been focused, for both ASCT2 and LAT1, on identifying substrate analogues able to compete with the natural substrates in a noncovalent fashion. One of the drawbacks of noncovalent inhibitors is the possibility of being displaced by the natural substrate(s) whose concentration may rise over the affinity of the transporter(s) toward the used inhibitors. This is the reason behind the choice of studying covalent inhibitors able to chemically knock out the transport activity of both ASCT2 and LAT1, exploiting the presence of reactive cysteine residues [ref 7 and refs therein]. In both cases, a library of 100 compounds with a dithiazole moiety has been tested on the transport activity mediated by ASCT2 and LAT1 [refs 12 and 19 and references herein]. The mechanism of inhibition is that of forming mixed disulfide with cysteine residues of the protein(s). At first, proteoliposomes harboring the rat isoform of ASCT2 have been used for measuring the inhibition exerted by dithiazole-based molecules differing in electronic, lipophilic, and steric properties of substituents.

From this study, six compounds with high inhibitory potency have been proposed, characterized by the presence of halogen substituents of the dithiazole scaffold (Figure 4A). Computa-

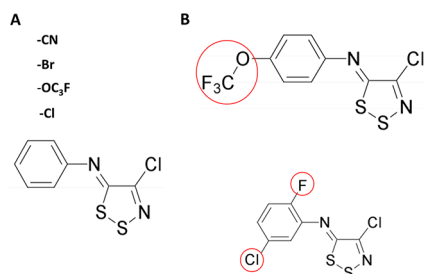


Figure 4. Dithiazole molecules. (A) The scaffold molecule responsible for interaction with rat ASCT2 and a list of substituents used for the most potent inhibitors. (B) Structures of the two most potent inhibitors able to block human LAT1 with highlighted substituents (in red) of the scaffold dithiazole.

tional analysis revealed that these molecules could be able to interact with the CXXC motif present in the rat sequence. Noteworthy, as highlighted above, this portion of the protein is the one with the lowest sequence identity with the human ASCT2. This feature makes the collected results not transferable in a straightforward fashion to the human ASCT2; indeed, these data can give only suggestions on a prototype scaffold molecule able to trigger covalent binding to ASCT2 that need, however, refinements to be adapted to the human isoform. Later on, a more advanced work has been performed using proteoliposomes harboring the human isoform of LAT1 for testing the inhibitory potency of dithiazole- and dithiazine-based molecules with different electrophilic and electron-donor substituents. In this case, eight compounds have been identified as a good inhibitor with IC_{50} lower than $1 \mu M$ *in vitro*. Importantly, information on the SAR trend could be obtained by the analyses of these results: in fact, electron withdrawal substituents increase the interaction with the protein, while electron donors and bulky substituents are revealed to be less effective. Therefore, in this case, a scaffold compound of the best inhibitor could be designed and hypothesized. Interestingly, kinetic analysis has been conducted on the two most potent inhibitors (Figure 4B) revealing that the type of inhibition is mixed, i.e., competitive and noncompetitive at the same time. This suggested that, besides a covalent type of inhibition due to the disulfide formation between molecule and cysteine residues of LAT1, there is the possibility of forming also a noncovalent interaction. Intriguingly, given the structure of the dithiazole moiety, i.e., similar to that of histidine, it could be argued that these molecules interact with the substrate binding site by noncovalent binding. Interestingly, as described above, the substrate binding site of LAT1 includes two cysteine residues; therefore, cysteine to alanine mutants have been generated. In this condition, it has been shown that the two most effective inhibitors lost the efficacy to block the transport activity of LAT1 when one of the two cysteine residues, namely, C407, was missing. These results confirmed that dithiazole-based drugs can target LAT1 at the binding site via interaction with the thiol group of an exposed cysteine residue. Interestingly, the best inhibitors can also reduce the growth of cancer cells harboring high expression of LAT1. Noteworthy, very recently a study showing the effect of triazoles on LAT1 has been

performed, confirming the reactivity of this protein toward this class of molecules.²⁵

5. CONCLUSIONS

After decades of studies with different methodologies, the pivotal role played by membrane transporters in human cells is definitively assessed. This important achievement is grounded on plenty of *in silico*, *in vitro*, *in vivo*, and clinical pieces of evidence. In this respect, the approach of amino acid chemical targeting gave a big contribution to all the steps of membrane protein studies from the identification to the regulatory studies and the application in the pharmacology of a membrane transporter. Therefore, given the versatility of this approach, a lot of work will still be done using new-generation reagents on orphan or poorly known membrane transporters, to enlarge the information about these proteins and their fundamental role in cell homeostasis.

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Notes

The authors declare no competing financial interest.

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