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Navigating Solute Carrier Transporters—A Comprehensive Review of Functionalized Small Molecule Probes for Target Identification and Characterization

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ABSTRACT

Solute carrier transporters (SLCs) are integral membrane proteins that play pivotal roles in maintaining cellular homeostasis by mediating the transport of a diverse range of substrates across cell membranes. With their involvement in fundamental physiological processes such as nutrient uptake, neurotransmitter signaling, and drug transport, SLCs have emerged as crucial players in health and disease. Dysregulation of SLC function has been implicated in a spectrum of disorders, including metabolic diseases, cancer, and neurological afflictions. Despite their significance, SLCs remain relatively understudied compared to other protein classes, resulting in a gap in understanding their molecular mechanisms of action and potential as therapeutic targets. This review aims to address this gap by providing a comprehensive overview of the diverse array of small-molecule probes utilized in the study of SLCs. Various types of functionalized probes, amongst which fluorescent probes, bivalent probes, covalent inhibitors, affinity-based probes, photoswitchable inhibitors and proteolysis targeting chimeras (PROTACs), have been designed to investigate transporter function, substrate specificity, and regulatory mechanisms. In this review, we describe the principles underlying the design and synthesis of these probes, highlights key examples of their application in elucidating transporter function and regulation, and discuss insights gained from such studies. Furthermore, we examine current challenges and future directions in the development and utilization of small-molecule probes for SLC transporter research. By shedding light on the intricate mechanisms involved in transporter

Abbreviations: 2-NBDG, 2-[*N*-(7-nitrobenz-2-oxa-1.3-diazol-4-yl)-amino]-2-deoxyglucose; 5-HT, 5-hydroxytryptamine (serotonin); AAC1, ADP/ATP carrier (old nomenclature ANT1); ABC, ATP-binding cassette; ABP, activity-based probe; ADME, absorption, distribution, metabolism, and excretion; ADP, adenosine diphosphate; AE1, anion exchanger 1; AfBP, affinity-based probe; ANT1, mitochondrial adenine nucleotide translocator 1; AOG, (2′-azi-*n*-octanol)-β-D-glucoside; APP^+, 4-(4-(dimethylamino)phenyl)-1-methylpyridin-1-ium; ASBT, apical sodium ion-dependent bile acid transporter; ASCT2, alanine/serine/cysteine transporter 2; ATP, adenosine triphosphate; BODIPY, boron-dipyrromethene; BPP^+, 1-butyl-4-(4-(dimethylamino)phenyl)pyridin-1-ium; BzG, [(4′-benzoyl)phenyl]-D-glucopyranoside; CCC9, cation-chloride cotransporter 9; CDCA, chenodeoxycholic acid; CNT, concentrative nucleoside transporter; CTL4, choline transporter-like 4; CuAAC, copper(I)-catalyzed azide-alkyne cycloaddition; Cy, cyanine; DAT, dopamine transporter; DBD, 4-*N*,*N*-dimethylaminosulfonyl-2,1,3-benzoxadiazole; DCPO, (*E*)-2-(2-(4-hydroxystyryl)-4H–chromen-4-ylidene)-malononitrile; DCSN, (*E*)-2-(3-(4-(diethylamino)styryl)-5,5-dimethylcyclohex-2-en-1-ylidene)malononitrile; dSTORM, direct stochastic optical reconstruction microscopy; dTAG, degradation tag; EAAT, excitatory amino acid transporter; ECHD, epoxycyclohexenedione; EMA, European Medicines Agency; ER, endoplasmatic reticulum; FATP3, fatty acid transport protein 3; FDA, U.S. Food and Drug Administration; FFF, fully functionalized fragment; FFN, fluorescent false neurotransmitter; FITC, fluorescein isothiocyanate; FRET, Förster (or fluorescence) resonance energy transfer; GABA, γ-aminobutyric acid; GAT, γ-aminobutyric acid transporter; GDC, Graves disease carrier; Glt_{Tk}, glutamate transporter (*Thermococcus kodakarensis*); GLUT, glucose transporter; GlyT, glycine transporter; GOLD, gating-oriented live-cell distinction; GPCR, G protein-coupled receptor; IFN, interferon; IngMeb, Ingenol mebutate; LAT1, L-type amino acid transporter 1; LYTAC, lysosome targeting chimeras; MAT, monoamine transporter; MATE, multidrug and toxin extrusion protein; MCT, monocarboxylate transporter; MPC2, mitochondrial pyruvate carrier subunit 2; MS, mass spectrometry; mTOR, mammalian target of rapamycin; NBD-MTMA, *N*,*N*,*N*-trimethyl-2-(methyl(7-nitrobenzo[*c*][1,2,5]oxadiazol-4-yl)amino)ethan-1-aminium; NBD-TMA, *N*,*N*,*N*-trimethyl-2-((7-nitrobenzo[*c*][1,2,5]oxadiazol-4-yl)amino)ethan-1-aminium; NBTI, *S*-(4-nitrobenzyl)-6-thioinosine; NET, norepinephrine transporter; NHE1, sodium-hydrogen antiporter 1; NIR, near-infrared; NPC, Niemann-Pick disease type C; NPC1, NPC intracellular cholesterol transporter 1; NT, nucleoside transporter; NTCP, sodium/bile acid and sulphated solute cotransporter 1; OAT, organic anion transporter; OATP, organic anion transporting polypeptide; OCT, organic cation transporter; PARADISO, paralog-dependent isogenic cell assay; PAT1, proton-coupled amino acid transporter 1; PHT1, peptide transporter 4; POI, protein of interest; PPP^+, 1-methyl-4-(4-(piperidin-1-yl)phenyl)pyridin-1-ium; PROTAC, proteolysis targeting chimeras; ROS, reactive oxygen species; SAEATA, 5′-*S*-(2-aminoethyl)-N^6-(4-azidobenzyl)-5′-thioadenosine; SAENTA, 5′-*S*-(2-aminoethyl)-N^6-(4-nitrobenzyl)-5′-thioadenosine; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SERT, serotonin transporter; SGLT, sodium-glucose cotransporter; SLC, solute carrier transporter; TCA, taurocholic acid; TCI, targeted covalent inhibitor; TFB-TBOA, TFB-threo-β-benzyloxyaspartic acid; THCA, 3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid; TIPDG, [2’-iodo-4′-(3″-trifluoromethyldiazirinyl)phenoxy]-D-glucopyranoside; TPD, targeted protein degradation; T-ALL, T cell acute lymphoblastic leukemia; VMAT, vesicular monoamine transporter; ZIP7, zinc transporter 7; ZmYS1, iron-phytosiderophore/yellow stripe 1 transporter (*Zea mays*); ZnT3, zinc transporter 3.

function and regulation, this review not only enhances the understanding of SLCs but also highlights their potential as therapeutic targets in drug discovery and thereby may facilitate systematic implementation of these innovative research approaches and the refinement of existing methodologies.

1 | Introduction

Cellular processes rely on complex mechanisms that ensure the proper transport of essential molecules across the cell membranes. Among these mechanisms, solute carrier transporters (SLCs) stand out as fundamental players in maintaining cellular homeostasis and facilitating multiple physiological functions [1]. With over 455 members organized into 66 subfamilies, SLCs form a diverse protein family, second in abundance only to G protein-coupled receptors (GPCRs) [2, 3]. These membrane-bound proteins mediate the translocation of a broad range of substrates, including ions, nutrients, neurotransmitters, and metabolites, across the cell as well as organelle membranes [4, 5]. While SLCs exhibit a general consistency in the types of molecules they transport within their subfamilies, individual proteins can display varying degrees of selectivity. Some SLCs transport a range of biomolecules, while others are restricted to a single type, and some remain "orphan" transporters with no known substrate [1, 6, 7]. Moreover, while SLCs encompass a wide spectrum of transport activities within various cell organelles, the majority of the identified transporters are known for their presence at the cell surface, specifically on the plasma membrane [4]. SLCs operate through facilitative transport where substrates move down their concentration gradients, or secondary active transport, in which the movement of one solute down its gradient provides energy for the transport of another solute against its respective gradient [5]. Within the latter, SLCs can function as symporters or antiporters, dynamically mediating the simultaneous or opposite movement of multiple solutes across membranes.

The transport function of SLCs, acting as the primary regulator of molecules entering and leaving cells, is intricately linked to a wide range of cellular and physiological processes. Certain SLC families control essential nutrient transport across membranes, impacting metabolism, energy production, and biosynthesis [8]. SLCs are also involved in the efflux of metabolic waste products from cells, facilitating detoxification while maintaining cellular homeostasis [4]. In addition, SLCs are integral components of the neurotransmitter system, modulating the synaptic concentrations of neurotransmitters and their precursors by means of neurotransmitter transporters such as norepinephrine transporter (NET, SLC6A2), dopamine transporter (DAT, SLC6A3), serotonin transporter (SERT, SLC6A4), and γ-aminobutyric acid transporters (GATs, SLC6 family) [9].

SLCs are also known to influence drug pharmacokinetics and pharmacodynamics, whether it is through direct or indirect mechanisms [10–12]. Drug transporters like the organic cation and anion transporters (OCTs, OATs respectively, SLC22 family), nucleoside transporters (NTs, SLC28 and SLC29 families), and multidrug and toxin extrusion proteins (MATEs, SLC47 family) facilitate the bidirectional transport of a wide array of drugs across the biological membranes in important ADME-related organs such as intestine, liver and kidney [11, 13, 14]. Upregulation or inhibition of these transporters can lead to drug-drug interactions when multiple drugs compete for the same transporter, altering therapeutic efficacy and toxicity [10, 15]. Emphasizing the crucial role of SLC proteins in drug absorption across various interfaces, recent FDA and EMA guidelines even highlight the importance of screening these proteins for potential drug-drug interactions, with analyses linking numerous pharmacological compounds to transporter activity [16, 17].

As a consequence of the significant roles that SLCs play, dysfunction of SLCs has been linked to a spectrum of pathophysiological conditions. Polymorphisms in SLC genes have been connected to complex disorders [18]. For instance, mutations in genes encoding SLCs, such as with monocarboxylate transporter 1 (MCT1, SLC16A1) and zinc transporter 3 (ZnT3, SLC30A3), can lead to inherited metabolic disorders characterized by impaired nutrient absorption or excretion [19]. Moreover, alterations in the expression or activity of SLCs, such as cation-chloride cotransporter 9 (CCC9, SLC12A8) and choline transporter-like 4 (CTL4, SLC44A4), have been implicated in the development and progression of various cancers [20, 21]. Dysregulated nutrient transport mediated by SLCs can contribute to the metabolic reprogramming observed in cancer cells, supporting their proliferation and survival [22–24]. Additionally, irregular expression of drug transporters can result in multidrug resistance in cancer cells, limiting the efficacy of chemotherapy and leading to treatment failure [25].

Despite their significance, SLCs remain significantly understudied when compared to other protein classes of similar importance, with a narrowed focus in drug discovery on specific members [26–28]. This bias does not consistently align with functional significance, with some owing their status to positive feedback loops from previous successes and experimental convenience, while other SLCs gained prominence due to their role as targets for existing drugs. Exploring the reasons behind this study gap also reveals a series of challenges, such as the lack of a universal classification system, difficulties in protein purification and crystallization, and limited availability of cell-based assays [29, 30]. Moreover, challenges in replicating physiological conditions in transport assays further hinder our ability to simultaneously explore the structural and functional aspects of human SLC proteins, highlighting the need for innovative approaches in SLC research. To overcome this, the RESOLUTE consortium aimed to advance research on solute carriers (SLCs) by systematically characterizing these transport proteins to unlock their potential as therapeutic targets [31, 32]. The public-private partnership, supported by the European Innovative Medicines Initiative, developed comprehensive tools and resources, such as in vitro assays and data sets, to enhance understanding and facilitate the discovery of novel drug targets within the SLC family [33].

Besides being studied from an assay perspective, multiple new chemical modulators for SLCs have been developed in this

regard [34]. These small molecules, which are designed to bind to and modulate the activity of specific transporters, provide a means to investigate transporter function, substrate specificity, and regulatory mechanisms both in vitro and in vivo [35]. By utilizing such chemical probes, it is not only possible to elucidate the physiological roles of individual SLCs, but also to uncover their involvement in disease processes, and identify potential therapeutic targets for drug development. Chemical probes provide flexibility by enabling studies in the native environment without extensive purification or crystallization of target proteins, thereby preserving natural conformation and function [36]. Besides the classical chemical probes, which have been reviewed previously, a range of more functionalized tool molecules emerged as an invaluable addition to the resources that are available to study SLCs. These functionalized probes are often equipped with detection or purification handles, while still being designed with high selectivity to target specific SLCs. Among other things, functionalized chemical probes can facilitate the observation of dynamic conformational changes in real-time, a challenge for static methods like crystallization or computational docking experiments.

While several comprehensive reviews of these tools are available for other protein families like GPCRs, a complete overview of what is available for SLCs is still missing [37–39]. Therefore, in this review, we aim to provide a comprehensive overview of the diverse array of small-molecule chemical probes employed as tools in the study of SLCs. While the focus will primarily be on fluorescent probes, covalent inhibitors, affinity-based probes (AfBPs), bitopic probes such as bivalent ligands and proteolysis targeting chimeras (PROTACs), and photoswitchable probes (schematically represented in Figure 1), it is also important to note the existence of other chemical probes that are as important yet too extensive to cover. We have considered newly designed or synthesized probes from 2000 up until July 2024 to ensure relevance to current research, which have been summarized in Table 1 at the end of this review. We explore the principles underlying the design and synthesis of these probes, highlight key examples of their application in elucidating transporter function and regulation, and discuss the insights gained from such studies. Furthermore, we examine the current challenges and future directions in expanding the SLC toolbox and therefore the development and utilization of small molecule probes for SLC research.

2 | Fluorescent Probes

Small molecule fluorescent probes are essential tools in drug discovery, offering high sensitivity and versatility for studying protein localization, interactions, and dynamics [114, 115]. These probes, primarily organic dyes, have significantly advanced our understanding of protein behavior across various biological contexts. Compared to traditional functionalized probes such as

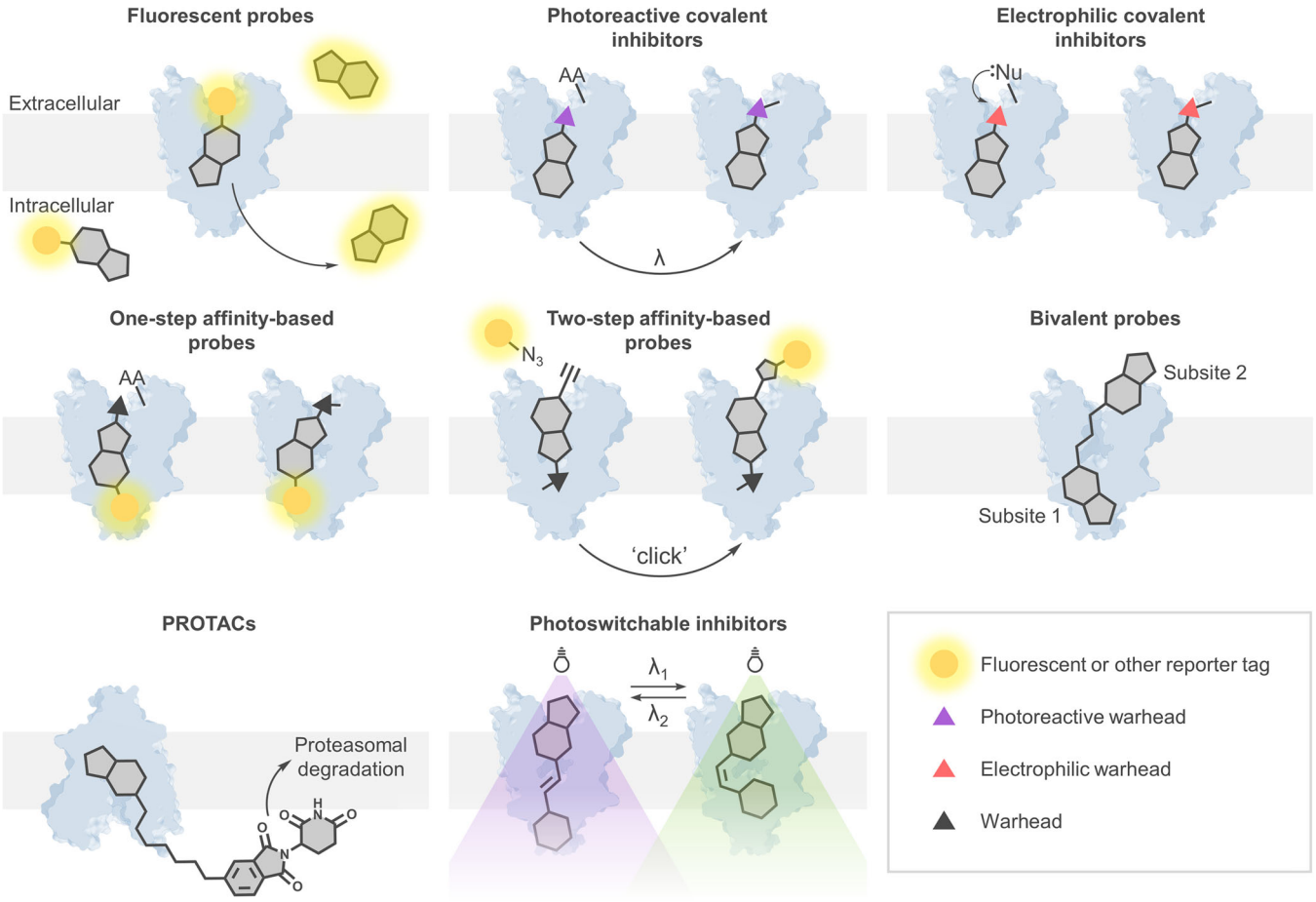


FIGURE 1 | Schematic representation of functionalized small molecule probes for SLCs discussed in this review. [Color figure can be viewed at wileyonlinelibrary.com]

TABLE 1 | Overview of all functionalized small molecule probes and their target SLCs including their functionality, activity or affinity values discussed in this review.

Cmpd nr.	Name	Functionality	Activity or affinity[a]	Target SLC	Reference	Remarks[b]
Fluorescent probes (Figures 2 and 3)						
1	Glu-1N-Cy3	Substrate	n.r.	GLUTs	[132]	Includes Glu-1N-Cy5 in article
2	6N-Gly-Cy3	Substrate	n.r.	GLUTs	[133]	Includes 6N-Gly-Cy5 in article
3	Glu-Alexa532	Substrate	n.r.	GLUT1 (SLC2A1)	[134]	
4	N2	Substrate	n.r.	GLUT1 (SLC2A1)	[135, 136]	Includes other probes in article
5	ASqαGl	Substrate	n.r.	GLUTs	[40]	
6	Glc-SiR-CO_2H	Substrate	n.r.	GLUT1 (SLC2A1) GLUT4 (SLC2A4)	[41]	Includes version with another fluorophore in article
7	Glu-1-O-DCSN	Substrate	n.r.	GLUTs	[42]	
8	WZB117-IR820	Inhibitor	n.r.	GLUT1 (SLC2A1)	[43]	Includes a FITC version in article
9	PPP^+	Substrate	n.r.	SERT (SLC6A4)	[44]	
10	BPP^+	Substrate	n.r.	SERT (SLC6A4)	[44]	
11	MFN103	Substrate	n.r.	DAT (SLC6A3) VMAT2 (SLC18A2)	[45]	Earlier reported as Mini103 in SI of Lee et al. [46]
12	SERTlight	Substrate	K_T ~ 100 μM (apparent Michaelis-Menten constant)	SERT (SLC6A4)	[47]	
13	HT-CA	Substrate	n.r.	SERT (SLC6A4)	[48]	
14	PA-CA	Inhibitor	n.r.	SERT (SLC6A4)	[48]	
15	GC04-38	Inhibitor	K_i: 191 ± 16.5 nM	DAT (SLC6A3)	[49]	Highly selective; includes other fluorescent probes of same chemotype in article
16	ZWZ-3	Substrate	n.r.	OATP (SLCO)	[50]	
17	CDrB	Substrate	n.r.	OATP1B3 (SLCO1B3, influx) SCaMC-3L (SLC25A41, efflux)	[51]	
18	IR-817	Substrate	n.r.	OATP (SLCO)	[52]	

(Continues)

TABLE 1 | (Continued)

Cmpd nr.	Name	Functionality	Activity or affinity[a]	Target SLC	Reference	Remarks[b]
19	rose bengal	Substrate	n.r.	OATP1B1 (SLCO1B1) OATP1B3 (SLCO1B3)	[53]	
20	SAENTA-Cy5	Inhibitor	n.r.	ENT1 (SLC29A1)	[54]	
21	UCPH-102F	Inhibitor	pIC_{50}: 4.85 ± 0.05	EAAT1 (SLC1A3)	[55]	Due to unspecific binding could not be visualized with microscopy
22	6FGA	Substrate	K_m: 20.3 μM	SGLT (SLC5)	[56]	
23	10i/SC70-p	Inhibitor	pIC_{50}: 5.11 ± 0.05 (mGAT2) pIC_{50}: 5.15 ± 0.05 (mGAT4) pK_i: 6.40 ± 0.07 (GlyT2)	mGAT (Slc6) GlyT2 (SLC6A5)	[57, 58]	
24	tauro-nor-THCA-24-DBD	substrate	K_m: 40.6 ± 14.0 μM	ASBT (SLC10A2)	[59]	Previously reported for NTCP (SLC10A1) by de Bruyn *et al.* [60]
25	NBD-TMA	Substrate	n.r.	OCT2 (SLC22A2)	[61]	
26	NBD-MTMA	Substrate	n.r.	OCT2 (SLC22A2) MATE1 (SLC47A1)	[62]	
27	CDoB	Substrate	n.r.	GDC (SLC25A16)	[63]	
28	CDg18	Substrate	n.r.	FATP3 (SLC27A3)	[64]	
29	uridine-furan	Substrate	EC_{50}: 0.5 ± 0.1 μM (CNT1) EC_{50}: 1.6 ± 0.5 μM (CNT3)	CNT1 (SLC28A1) CNT3 (SLC28A3)	[65]	
30	fluorescent nicotianamine 15	Substrate	n.r.	PAT1 (SLC36A1)	[66]	
Photoreactive covalent inhibitors (Figure 4)						
31	Compound 4	Inhibitor	K_i: 60 μM	GLUT4 (SLC2A4)	[67]	Species: rat; includes other photo-AfBP of same chemotype in article
32	TIPDG	Inhibitor	K_i: 22 ± 5 μM	SGLT1 (SLC5A1)	[68]	Species: rabbit
33	BzG	Inhibitor	K_i: 12 ± 2 μM	SGLT1 (SLC5A1)	[68]	Species: rabbit; structure wrongly depicted in original article [68]
34	AOG	Inhibitor	K_i: 43 ± 12 μM	SGLT1 (SLC5A1)	[69]	Species: hog
35	Compound 15	Inhibitor	IC_{50}: 0.11 ± 0.02 μM (EAAT1)	EAAT1 (SLC1A3)	[70]	

(Continues)

TABLE 1 | (Continued)

Cmpd nr.	Name	Functionality	Activity or affinity[a]	Target SLC	Reference	Remarks[b]
			IC_{50}: 0.40 ± 0.03 μM (EAAT2) IC_{50}: 4.03 ± 0.80 μM (EAAT3)	EAAT2 (SLC1A2) EAAT3 (SLC1A1)		
36	Compound 16	Inhibitor	IC_{50}: 1.23 ± 0.29 μM (EAAT1) IC_{50}: 0.82 ± 0.08 μM (EAAT2) IC_{50}: 2.11 ± 0.58 μM (EAAT3)	EAAT1 (SLC1A3) EAAT2 (SLC1A2) EAAT3 (SLC1A1)	[70]	
Electrophilic covalent inhibitors (Figure 5)						
37	Compound 26	Inhibitor	IC_{50}: 31.5 ± 3.1 nM	DAT (SLC6A3)	[71]	Species: rat; includes other covalent inhibitors of same chemotype in article
38	Compound 16	Inhibitor	K_i: 537 ± 37 nM	DAT (SLC6A3)	[72]	Species: rat
39	Compound 14b	Inhibitor	IC_{50}: 68.3 ± 6.6 nM	DAT (SLC6A3)	[73]	Species: rat; includes other covalent inhibitors of same chemotype in article
40	HD-205	Inhibitor	IC_{50}: 284 ± 92 nM (NET) IC_{50}: 4.07 ± 0.93 nM (DAT) IC_{50}: 14.1 ± 0.91 nM (SERT)	NET (SLC6A2) DAT (SLC6A3) SERT (SLC6A4)	[74]	Species: rat; includes HD-244 as one-step AfBP in article
41	Compound 6	Inhibitor	IC_{50}: 24 nM	DAT (SLC6A3)	[75]	
42	1,2,3-dithiazoles	Inhibitors	—	LAT1 (SLC7A5) ASCT2 (SLC1A5)	[76, 159]	Includes multiple covalent inhibitors of same chemotype in article for both SLCs
43	1,2,4-dithiazines	Inhibitors	—	LAT1 (SLC7A5)	[159]	Includes multiple covalent inhibitors of same chemotype in article
44	3β-Cl-CDCA	Inhibitor	K_i: 0.81 μM	ASBT (SLC10A2)	[77]	
45	7α-Ms-CDCA	Inhibitor	K_i: 1.19 μM	ASBT (SLC10A2)	[77]	
46	TF2A	Inhibitor	Remaining [^{3}H]-TC uptake at 100 μM: 37.4 ± 2.8%	ASBT (SLC10A2)	[78]	
47	TF2B	Inhibitor	Remaining [^{3}H]-TC uptake at 100 μM: 33.8 ± 4.0%	ASBT (SLC10A2)	[78]	

(Continues)

TABLE 1 | (Continued)

Cmpd nr.	Name	Functionality	Activity or affinity[a]	Target SLC	Reference	Remarks[b]
48	AMM-59	Inhibitor	n.r.	ANT1 (SLC25A4)	[79]	Species: bovine; includes AMM-120 as two-step AfBP in article
49	YY4	Inhibitor	n.r.	MCP2 (SLC54A2)	[80]	Includes YY4-yne as two-step AfBP in article
One-step photoaffinity-based probes (Figure 6)						
50	[^{125}I]11	Inhibitor	K_i: 15.1 ± 2.2 nM (DAT) K_i: 109 ± 14 nM (SERT) (nonradioactive iodine)	DAT (SLC6A3) SERT (SLC6A4)	[81]	Species: rat (labelling in human)
51	[^{125}I]16b	Inhibitor	IC_{50}: 163 ± 25 nM (nonradioactive iodine)	DAT (SLC6A3)	[73]	Species: rat
52	[^{125}I]-6	Inhibitor	K_i: 78 ± 18 nM (nonradioactive iodine)	DAT (SLC6A3)	[82]	
53	(±)-[^{125}I]-SADU-3-72	Inhibitor	K_i: 3071 ± 497 nM (nonradioactive iodine)	DAT (SLC6A3)	[83]	
54	[^{125}I]IAPEGlyMER	Inhibitor	IC_{50}: 200 ± 67 nM (nonradioactive iodine)	VMAT2 (SLC18A2)	[84]	Species: bovine
55	*trans*-[^{125}I] TBZ-AIPP	Inhibitor	IC_{50}: 2100 ± 450 nM (nonradioactive iodine)	VMAT2 (SLC18A2)	[84]	Species: bovine
56	^{125}I-labeled 4	Inhibitor	n.r.	MCT1 (SLC16A1)	[85]	
57	SAEATA-x14-biotin	Inhibitor	K_i: 2.69 ± 0.36 nM	ENT1 (SLC29A1)	[86]	
Two-step photoaffinity-based probes (Figure 7)						
58	Azidobupramine	Inhibitor	pK_i: 6.59 ± 0.06 (hNET) pK_i: 5.80 ± 0.07 (hDAT) pK_i: 6.99 ± 0.03 (hSERT) pK_i: 7.52 ± 0.03 (rSERT)	NET (SLC6A2) DAT (SLC6A3) SERT (SLC6A4)	[87]	Species: human and rat
59	Photoprobe 6	Inhibitor	K_i: 0.16 nM	SERT (SLC6A4)	[88]	
60	NVS-ZP7-6	Inhibitor	n.r.	ZIP7 (SLC39A7)	[89]	
61	mo56AZK	Pharmacochaperone	EC_{50}: 0.078 μM	NPC1 (SLC65A1)	[90]	
62	itraAZY	Pharmacochaperone	EC_{50}: 0.168 μM	NPC1 (SLC65A1)	[91]	
63	Photoprobe 1	Inhibitor	IC_{50}: 3 nM	MCT4 (SLC16A3)	[92]	Another photo-AfBP for MCT4 reported by Goldberg *et al.* (2023)

(Continues)

TABLE 1 | (Continued)

Cmpd nr.	Name	Functionality	Activity or affinity[a]	Target SLC	Reference	Remarks[b]
						but did not include synthesis [93]
64	sICeMM1-PAP	Inhibitor	n.r.	MCT4 (SLC16A3)	[94]	
65	AJ2-32	Inhibitor	IC_{50}: 2.5 μM	PHT1 (SLC15A4)	[95]	
66	Ing-DAyne	Inhibitor	n.r.	CAC (SLC25A20)	[96]	
One-step electrophilic affinity-based probes (Figure 8)						
67	[^{125}I]MFZ-3-37	Inhibitor	n.r.	DAT (SLC6A3)	[97]	Species: rat
68	[^{125}I]HD-244	Inhibitor	IC_{50}: 20 nM (nonradioactive iodine)	DAT (SLC6A3)	[74]	Species: rat; includes HD205 as covalent inhibitor in article
69	probe 2	Inhibitor	n.r.	AE1 (SLC4A1)	[98]	Species: sheep
Two-step electrophilic affinity-based probes (Figure 9)						
70	AMM-120	Inhibitor	n.r.	ANT1 (SLC25A4)	[79]	Species: bovine; includes AMM59 as covalent inhibitor in article
71	YY4-yne	Inhibitor	n.r.	MCP2 (SLC54A2)	[80]	Includes YY4 as covalent inhibitor in article
Bivalent ligands (Figure 10)						
72	Compound 17	Inhibitor	K_i: 180 ± 72 nM (NET) K_i: 6.6 ± 4 nM (DAT) K_i: 2600 ± 1100 nM (SERT)	NET (SLC6A2) DAT (SLC6A3) SERT (SLC6A4)	[99]	More bivalent inhibitors reported from same chemotype
73	Compound 30	Inhibitor	K_i: 8.5 ± 2 nM (NET) K_i: 1.0 ± 0.6 nM (DAT) K_i: 0.86 ± 0.79 nM (SERT)	NET (SLC6A2) DAT (SLC6A3) SERT (SLC6A4)	[99]	More bivalent inhibitors reported from same chemotype
74	Compound 10	Inhibitor	IC_{50}: 11 ± 3 nM (DAT) IC_{50}: 31 ± 11 nM (SERT)	DAT (SLC6A3) SERT (SLC6A4)	[100]	More bivalent inhibitors reported from same chemotype
75	Compound 2	Inhibitor	IC_{50}: 4.8 μM	SERT (SLC6A4)	[101]	More bivalent inhibitors reported from same chemotype
76	Compound 51	Inhibitor	[^{3}H]nisoxetine displacement at 10 μM: 65.1% (NET)	NET (SLC6A2) SERT (SLC6A4)	[102]	Species: rat

(Continues)

TABLE 1 | (Continued)

Cmpd nr.	Name	Functionality	Activity or affinity[a]	Target SLC	Reference	Remarks[b]
			K_i: 30.2 ± 4.29 nM (SERT)			
77	Compound 2	Inhibitor	K_i: 0.64 ± 0.01 nM (SERT) K_i: 1676 ± 128 nM (DAT)	DAT (SLC6A3) SERT (SLC6A4)	[103]	More bivalent inhibitors reported from same chemotype
78	D-362	Inhibitor	K_i: 51.91 ± 4.54 nM	DAT (SLC6A3)	[104]	More bivalent inhibitors reported from same chemotype
79	Compound 5c	Inhibitor	IC_{50}: 14 ± 3 nM	SERT (SLC6A4)	[105]	Species: rat
80	Compound 11	Inhibitor	IC_{50}: 1.4 ± 0.1 μM (DAT) IC_{50}: 0.21 ± 0.02 μM (SERT)	DAT (SLC6A3) SERT (SLC6A4)	[106]	More bivalent inhibitors reported from same chemotype
PROTACs (Figure 11)						
81	d9A-2	Degrader	EC_{50} < 0.1 μM (in leukemia cell lines)	NHE1 (SLC9A1)	[107]	Can also partially degrade other SLC9 family members, also in other cancer cell lines
Photoswitchable inhibitors (Figure 12)						
82	(*R*)-6e	Inhibitor	pIC_{50}: 6.39 ± 0.08 (*E*) pIC_{50}: 5.78 ± 0.03 (*Z*)	GAT1 (Slc6a1)	[108]	Species: mouse
83	(*R*)-*m*-20a	Inhibitor	pIC_{50}: 4.65 ± 0.03 (*E*) pIC_{50}: 6.38 ± 0.04 (*Z*)	GAT1 (Slc6a1)	[109]	Species: mouse
84	ATT	Inhibitor	IC_{50}: 8.1 ± 1.1 nM (*E*, EAAT1) IC_{50}: 66.4 ± 4.9 nM (*Z*, EAAT1) IC_{50}: 0.9 ± 0.1 nM (*E*, EAAT2) IC_{50}: 12.7 ± 1.4 nM (*Z*, EAAT2) IC_{50}: 341.2 ± 19.5 nM (*E*, EAAT3) IC_{50}: 1132 ± 51 nM (*Z*, EAAT3)	EAAT1 (SLC1A3) EAAT2 (SLC1A2) EAAT3 (SLC1A1)	[110]	
85	*p*-MeO-azo-TBOA	Inhibitor	IC_{50}: 2.5 ± 0.4 μm (*E*) and 9.1 ± 1.5 μm (*Z*)	Glt_{Tk} (bacterial glutamate transporter)	[111]	Species: *Thermococcus kodakarensis*
86	azo-escitalopram	Inhibitor	IC_{50}: 819 nM (*E*) and 18.9 nM (*Z*)	SERT (SLC6A4)	[112]	
87	compound 3	Inhibitor	IC_{50}: 5.36 μM (*E*) and 9.94 μM (*Z*)	GlyT2 (SLC6A5)	[113]	

[a]n.r.: not reported.
[b]Probes target the corresponding human SLC unless stated otherwise.

radiolabeled ligands, fluorescent probes are more accessible, cost-effective, and simpler to use, requiring only standard fluorescent microscopy for visualization [116]. Additionally, they provide greater flexibility in experimental design, allowing multiplexed imaging studies that enable the simultaneous visualization of multiple targets within a single sample. Improvements in probe affinity and selectivity, as well as fluorophore (photo)stability [117], have broadened their applicability across various methodologies, ranging from single-cell microscopy to high-throughput binding assays [118]. These advancements have led to a significant increase in the use of fluorescent probes that extend beyond visualizing protein expression and distribution to facilitate studies on (real-time) oligomerization, internalization, downstream signaling, and binding properties [119, 120].

Two primary approaches characterize the design of fluorescent probes. The first involves creating fluorescent ligands that mimic either endogenous or exogenous ligands, typically benefiting from smaller sizes and easier transport. The second approach employs a combination of a high-affinity parent ligand, an optimized linker, and a suitable fluorescent dye. For example, organic dyes like fluorescein, rhodamine, and cyanine are renowned for their bright fluorescence, enabling the detection of low-abundance proteins [121]. Their ability to conjugate with small molecules or peptides allows specific targeting, making them invaluable for fixed-cell imaging, flow cytometry, and other applications [115]. Additionally, near-infrared (NIR) fluorescent probes, which emit light in the 650–900 nm range, have gained importance in recent years [122–124]. NIR dyes offer deeper tissue penetration and reduced background due to the distinct wavelength when compared to the autofluorescence of endogenous biomolecules, making them ideal for in vivo imaging [115]. Furthermore, they enhance image resolution and signal-to-noise ratio compared to classic organic dyes. Although many challenges surrounding these probes have been tackled over the years, some still remain, like photobleaching and toxicity, driving continuous efforts to develop more stable and selective fluorescent probes. Additionally, the addition of fluorescent groups to high-affinity ligands can have a profound effect on the selectivity profile and (intracellular) localization of the probe, which warrants careful investigation. Through these studies, fluorescent probes have advanced the research of SLCs, prompting the development of numerous ligands across various SLC families. Moreover, gating-oriented live-cell distinction (GOLD) has been defined as an important mechanism for the development of cell-selective imaging probes in which transport of these probes is ATP-binding cassette (ABC) transporter- or SLC-mediated [125–127].

Specifically in case of glucose transporters (GLUTs, SLC2 family), many fluorescent probes have been reported and previously reviewed, highlighting their importance in biochemical and biomedical research [128–131]. This paragraph focuses on new advances in the field not discussed in these reviews. While probes had been developed to investigate glucose uptake, many suffered from limitations in terms of sensitivity, specificity, or applicability in complex biological systems. Seeking to address these shortcomings the Gao group introduced several innovative cyanine-based and glycoconjugate glucose probes, demonstrating high sensitivity and specificity for GLUT-mediated processes [132, 133]. Xu et al. developed two cyanine-based glucose conjugates, among which Glu-1N-Cy3 (**1**, Figure 2), synthesized through direct amide coupling with cyanine fluorophores [132]. These probes showed higher sensitivity compared to traditional probes like 2-NBDG, making them valuable for theranostics and drug research. Expanding on this study, 6N-Gly-Cy3 (**2**, Figure 2) was designed, similar to the aforementioned probes with a cyanine substituted on the C-6 position instead of C-1 [133]. This probe demonstrated enhanced fluorescence and low cytotoxicity, rendering it suitable for live-cell imaging and as potential tracer for galactokinase metabolism. The cellular uptake of these probes was competitively inhibited by endogenous GLUT substrates, confirming their specificity for GLUTs. Additionally, 6N-Gly-Cy3 showed potential for single-molecule FRET-based studies due to its high fluorescence intensity. Wu *et al.* developed D-glucose-based fluorescent probes for super-resolution imaging of GLUTs in HeLa cells and erythrocytes [134]. These probes, such as the thio-glucose Glu-Alexa532 (**3**, Figure 2), offered high labeling density and specificity, potentially providing new opportunities to study glucose metabolism in different cell lines. In an attempt to circumvent low sensitivity and permeability of earlier reported fluorescent GLUT probes, Chen et al. synthesized N2 (**4**, Figure 2), a NIR fluorescent glucose analogue utilizing a DCPO dye [135]. N2 exhibited improved membrane permeability and high uptake in cancer cells overexpressing glucose transporter 1 (GLUT1, SLC2A1) compared to previously reported NIR-based probes, demonstrating its potential for cancer cell imaging and understanding cancer metabolism [136]. Similarly, several squaraine-based probes have been developed for selective optical imaging of cancer cells [40]. These dyes target the Warburg effect, where cancer cells exhibit increased glucose uptake via GLUTs. Conjugating squaraine dyes with glucose allows for selective absorption by cancer cells, triggering a "turn-on" fluorescence upon internalization through aggregation-mediated quenching. The unsymmetrical dye with α-glucose, ASqαGl (**5**, Figure 2), showed superior imaging properties compared to its β-glucose counterpart. The dyes are nontoxic up to 20 μM and provide bright fluorescence in cancer cells, making these probes promising for cancer imaging due to their specificity, strong NIR emission, and low toxicity. In addition, Jo et al. synthesized Glc-SiR-CO_2H (**6**, Figure 2), another NIR fluorescent tracer synthesized by conjugating glucosamine with silicon (Si) rhodamine (R) fluorochromes. This tracer effectively differentiated cancer cells from normal cells, providing a tool for measuring cytotoxicity of anticancer agents and monitoring anticancer effects [41]. Glu-1-O-DCSN (**7**, Figure 2), emitting deep-red fluorescence with a large Stokes shift, mimics natural glucose uptake and localizes in mitochondria via GLUT1-mediated transport, which are overexpressed in tumor cells [42]. In vivo studies in mouse models showed selective tumor accumulation without diffusion to other organs as well as the ability to cross the blood-brain barrier which suggests potential applications in tumor detection and brain disease imaging. Most recently, Tian et al. reported on precision detection and fluorescence image-guided surgery for oral squamous cell carcinoma using a novel probe, WZB117-IR820 (**8**, Figure 2) [43]. This fluorescent probe, conjugated with a GLUT1 inhibitor and a NIR dye, demonstrated high specificity in tumor detection and facilitated complete tumor resection in surgical mouse models. While certain limitations, like subtype specificity, quantitative detection, and in vivo toxicity remain,

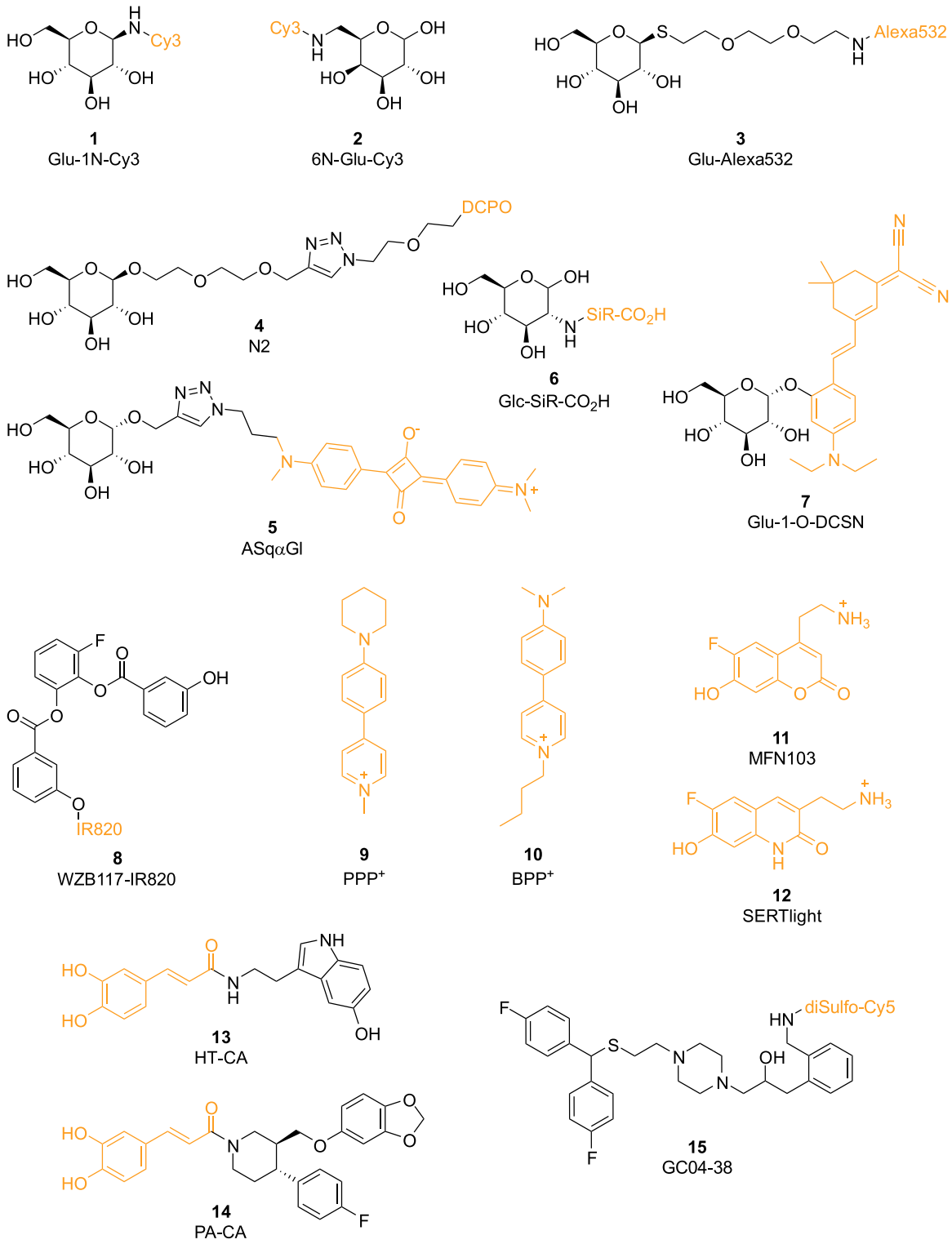


FIGURE 2 | Molecular structures of fluorescent probes 1–15 targeting GLUTs and (V)MATs. In case of a fluorescent dye attached to a substrate or inhibitor, the fluorescent dye is highlighted in orange. [Color figure can be viewed at wileyonlinelibrary.com]

these probes represent significant progress in imaging and characterizing GLUT-mediated processes in vivo, advancing diagnostic and therapeutic strategies.

Unlike GLUTs, which primarily regulate the influx of metabolic fuel, the (vesicular) monoamine transporters ((V)MATs) are primarily involved in the uptake and release of neurotransmitters. This difference in localization and substrate specificity requires unique design principles for their respective molecular probes. The (V)MATs, consisting of NET, DAT, SERT, VMAT1 and VMAT2 have been extensively studied with the use of fluorescent probes as was recently reviewed by the Newman group as well as by the Sulzer team [137, 138]. Nevertheless, since publication of these reviews, several new fluorescent probes for these SLCs have been reported, addressing some challenges left unattended by previous probes. In an attempt to improve imaging resolution,

Wilson et al. developed two new analogues of APP^+, PPP^+ and BPP^+ (**9** and **10**, respectively, Figure 2), and investigated their binding-induced fluorescence with SERT [44]. PPP^+ and BPP^+ exhibited turn-on fluorescence upon binding to SERT, demonstrating high sensitivity to their chemical environments. Molecular docking revealed that both probes access the nonpolar binding pocket of SERT, showing distinct fluorescence from intracellular accumulations. These findings provide valuable insights into SERT interactions and the potential application of these probes in differentiating serotonergic and catecholaminergic cells. Other fluorescent probes for visualizing (V)MATs stem from the development of so called fluorescent false neurotransmitters (FFNs). MFN103 (**11**, Figure 2), combines fluorescent and magnetic resonance properties to study dopamine storage and release in synaptic vesicles mediated by DAT and the vesicular monoamine transporter 2 (VMAT2, SLC18A2) [45]. SERTlight (**12**, Figure 2), designed for serotonergic neuron labeling, demonstrates high selectivity for SERT without significant binding to other receptors, providing stable, non-releasable optical signals compatible with genetically encoded sensors [47]. Furthermore, two new fluorescent probes, HT-CA and PA-CA (**13** and **14**, respectively, Figure 2), for imaging superoxide anion radicals ($O_2^{\bullet-}$) in serotonergic neurons for SERT were reported by Wang et al. [48]. These probes enable real-time detection of oxidative stress in living mouse brains, revealing increased superoxide levels in depression models, providing the ability to monitor reactive oxygen species (ROS) dynamics in vivo to elucidate mechanisms underlying depression. The Newman group reported recent advancements focusing on developing fluorescent probes tailored for rapid detection of DAT in peripheral blood mononuclear cells [49]. Fluorescent probe GC04-38 (**15**, Figure 2), had high affinity and was selective for DAT, leading to reliable high-throughput detection of DAT-expression using flow cytometry from human and mouse samples, potentially serving as a non-invasive biomarker for conditions like Parkinson's disease.

Understanding SLC-mediated drug transport with fluorescent probes, can be important for predicting and managing such interactions and has been extensively reviewed very recently in case of liver transporters by Özvegy-Laczka et al. following the previous review of drug transporters by Fardel et al. [139, 140]. Newly reported fluorescent probes for these transporters are discussed below. In addition to their involvement in drug transport, organic anion transporting polypeptides (OATPs, SLC22 family) also play a pivotal role in the uptake of NIR dyes in cancer and immunology, enabling targeted imaging and therapy. Several new advances in this field have been reported over the years as has been reviewed by Zhang et al. [141]. More recently, the novel probe ZWZ-3, developed for melanoma imaging and therapy, exemplifies this important application. ZWZ-3 (**16**, Figure 2) is a hemicyanine-based probe that targets melanoma mitochondria via an OATP-dependent mechanism, inducing apoptosis and autophagy, significantly suppressing tumor growth both in vitro and in vivo [50]. Another innovative probe, CDrB (**17**, Figure 2), discriminates between T and B lymphocytes by utilizing distinct solute carriers, OATP1B3 (SLCO1B3) and the mitochondrial transporter SCaMC-3L (SLC25A41), showcasing its potential for immune cell differentiation [51]. Additionally, IR-817 (**18**, Figure 2), a NIR fluorescent agent, targets melanoma mitochondria, inducing apoptosis and cell cycle arrest, significantly reducing tumor size in preclinical models [52]. Both ZWZ-3 (**16**) and IR-817 (**18**) highlight the advantages of mitochondrial-targeting agents, while CDrB (**17**) underscores the importance of specific transporter mechanisms, both advancing diagnostic and therapeutic strategies in cancer and immunology. Recently, OATP1B1 and OATP1B3 (SLCO1B1 and SLCO1B3, respectively) were identified to mediate transport of rose bengal, a xanthene-based dye with anticancer properties (**19**, Figure 3) [53]. This selective uptake mechanism was determined in hepatic cancer cells overexpressing these transporters, as confirmed by confocal microscopy and flow cytometry highlighting the importance of these transporters in photodynamic therapy as targeted cancer treatment.

Like the OATPs, the human equilibrative nucleoside transporter 1 (hENT1, SLC29A1) is involved in the cellular uptake of chemotherapeutic drugs and has been studied using fluorescent ligand techniques. In 2023, Sui et al. reported the synthesis of SAENTA-Cy5 (**20**, Figure 3), a fluorescent probe designed for imaging with nanoscale resolution using direct stochastic optical reconstruction microscopy (dSTORM) [54]. This probe offers enhanced specificity and resolution over traditional antibody-based methods, reducing steric hindrance and improving labeling accuracy. SAENTA-Cy5 (**20**) enabled detailed visualization of hENT1 clustering, facilitating advanced studies of nucleoside transporter organization and their critical role in drug metabolism.

Since 2000, each SLC with a newly developed fluorescent probe, aside from those previously discussed, has been covered in only one or two articles. For example, Huynh et al. explored the synthesis of fluorescent probes based on excitatory amino acid transporter 1 (EAAT1, SLC1A3) inhibitor UCPH-101 [55]. Derivative UCPH-102F (**21**, Figure 3) is a coumarin-based analog that selectively inhibits EAAT1 with micromolar potency. While it emits fluorescence at physiological pH, its nonspecific binding complicated EAAT1 visualization in HEK293 cells to distinguish EAAT1 expression over EAAT3 (SLC1A1). Despite these limitations, the results hold promise for further developing UCPH-101 derivatives as fluorescent probes to study EAAT1. Very recently, 6FGA (**22**, Figure 3), a novel fluorescent glucose analogue, was designed to study sodium-glucose cotransporter (SGLT, SLC5) activity [56]. Synthesized by click conjugation of azido-D-glucose to a Cy5.5 dye, 6FGA (**22**) demonstrates nontoxic behavior and effectively measures transporter activity in cells, with uptake strongly dependent on sodium ions, indicating SGLT-mediated uptake. Compared to well-known fluorescent probe 2-NBDG, 6FGA (**22**) offers superior photophysical properties and sensitivity, making it a valuable tool for investigating glucose transport mechanisms in biological research. In 2020, the Wanner group reported the synthesis and evaluation of BODIPY-labeled ligands for murine γ-aminobutyric acid (GABA) transporters 1 to 4 (mGAT1-mGAT4, Slc6 family) [58]. These ligands were tested for their inhibitory potency across the different transporter subtypes. The study reports compounds with longer linkers, while more lipophilic, sterically demanding cyclic amines demonstrated higher inhibitory potency. Specifically, compound 10i (**23**, Figure 3), with a pentyl linker and methoxy group, showed improved potency for mGAT2-4 transporters, emphasizing the role of spacer length and structural modifications in enhancing activity. The same fluorescent probe emerged in a library screen in search

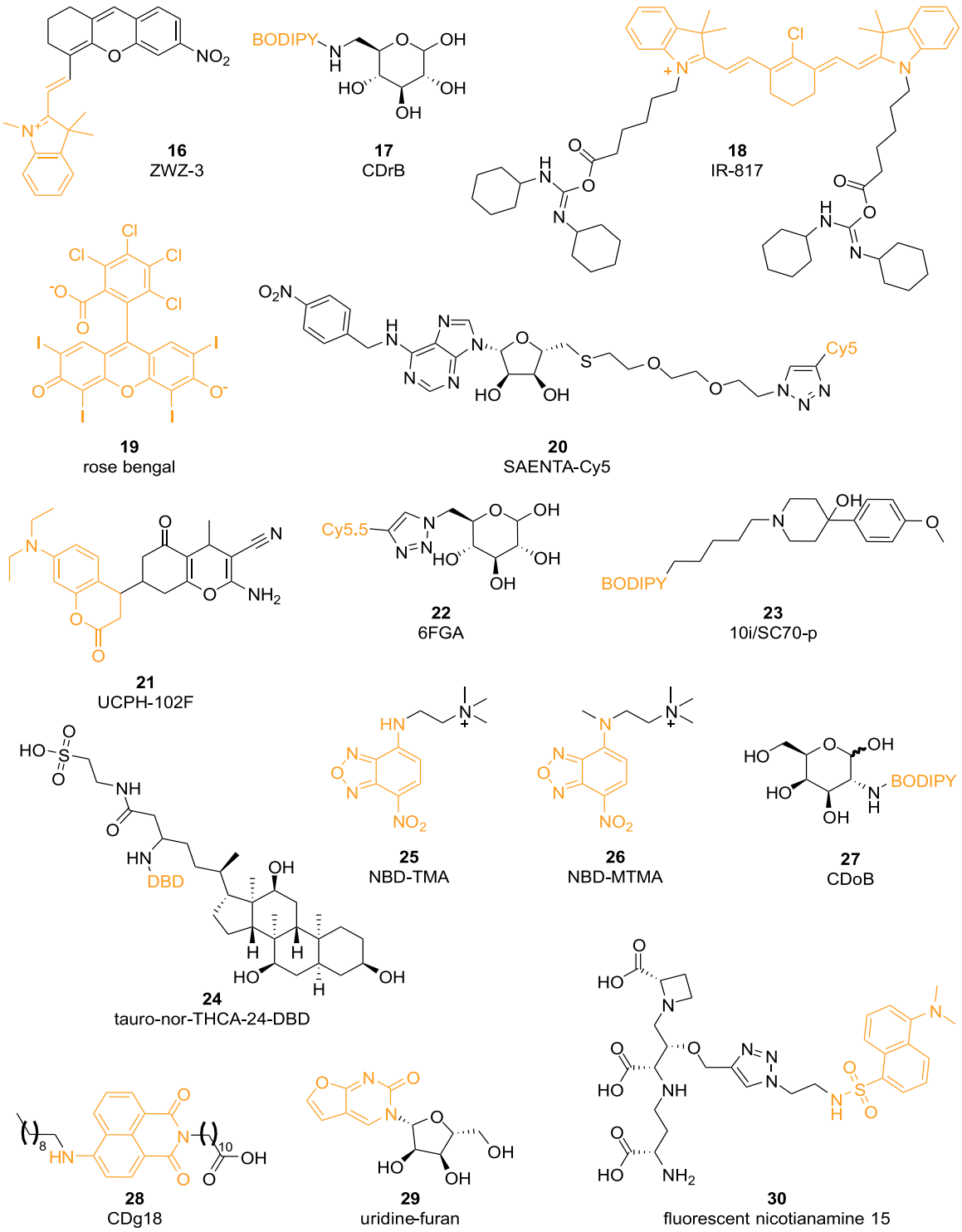


FIGURE 3 | Molecular structures of fluorescent probes 16–30 targeting SLCs. In case of a fluorescent dye attached to a high-affinity substrate or inhibitor, the fluorescent dye or moiety is highlighted in orange. [Color figure can be viewed at wileyonlinelibrary.com]

of new inhibitors for glycine transporters GlyT1 (SLC, SLC6A9) and GlyT2 (SLC6A5) [57]. SC70–p (10i in the aforementioned study, **23**, Figure 3) showed the highest binding affinity for GlyT2. The study highlights the compound's potential as tools in fluorescence-based experiments to explore GlyT2 inhibition and related pharmacological processes.

Similarly, Zhu et al. explored the potential of tauro-nor-THCA-24-DBD (**24**, Figure 3) for the apical sodium ion-dependent bile acid transporter (ASBT, SLC10A2), a fluorescent probe previously described as a substrate for several hepatic bile acid transporters [59]. ASBT-expressing Xenopus oocytes showed increased uptake of both the *R*- and *S*-isomers, indicating that tauro-nor-THCA-24-DBD (**24**) is a substrate for ASBT. The uptake was inhibited by taurocholic acid and elobixibat, an ASBT inhibitor, confirming ASBT-mediated transport. These findings support its potential as a tool for studying ASBT activity in intestinal tissues. The Wright group has advanced the

study of organic cation transporter 2 (OCT2, SLC22A2) with new fluorescent probes. Bednarczyk *et al.* reported NBD-TMA (**25**, Figure 3), a fluorescent substrate, that enables real-time measurement of cation transport in renal tubules, displaying high temporal and spatial resolution [61]. NBD-TMA (**25**) remains stable across physiological pH levels and unaffected by chloride ion concentration changes, indicating its robustness compared to traditional radiolabeled compounds. In a follow-up study, the same research group developed NBD-MTMA (**26**, Figure 3) as an improved version of NBD-TMA (**25**), which maintains stable fluorescence from pH 5.5 to 8.5, making it ideal for studying cation transport in biological systems without pH interference [62]. Both compounds are improved tools for investigating OCT2 functions and cellular transport mechanisms albeit with much less fluorescent intensity compared to fluorescein.

Regarding the mitochondrial Graves disease carrier (GDC, SLC25A16) and the fatty acid transport protein 3 (FATP3, SLC27A3) two new fluorescent probes were reported that are cell type specific via a GOLD mechanism. CDoB (**27**, Figure 3) distinguishes B cell developmental stages (progenitor, transitional, and mature) based on its fluorescence intensity [63]. CDoB localizes to mitochondria and correlates with SLC25A16 overexpression in mature B cells. The study demonstrates the ability of CDoB to selectively visualize mature B cells in spleen tissue, making it a useful tool for monitoring B cell maturation and identifying SLC25A16 as a potential biomarker for mature B lymphocytes. CDg18 (**28**, Figure 3) selectively targets M2 macrophages, which are associated with anti-inflammatory and tumor-promoting activities [64]. CDg18 distinguishes M2 cells by FATP3 transport, which is overexpressed in M2 macrophages. The probe accumulates in lipid droplets, allowing real-time visualization of macrophage polarization. Using CDg18 alongside the M1-selective probe CDr17 enables monitoring the reprogramming of M2 to M1 macrophages, which could be valuable for cancer treatment strategies targeting tumor-associated macrophages.

Aside from ENT1, the concentrative nucleoside transporters (CNTs, SLC28 family) have been the target for development of fluorescent probes as well due to their role in nucleoside homeostasis and transport of nucleoside-derived antiviral and anticancer drugs. One such probe, uridine-furan (**29**, Figure 3), was synthesized by the Pastor-Anglada group [65]. It demonstrated the ability to inhibit uridine uptake in HeLa cells expressing hCNT1 (SLC28A1) and hCNT3 (SLC28A3), showing high-affinity interaction in the low micromolar range. Confocal microscopy and flow cytometry confirmed uridine-furan's utility in detecting hCNT activity in living cells, suggesting its potential as a biomarker for transporter function in vivo.

Lastly, Kayano et al. recently reported the synthesis of fluorescent nicotianamine probe 15 (**30**, Figure 3), based on nicotianamine which is involved in the intestinal proton-coupled amino acid transporter 1 (PAT1, SLC36A1)-mediated uptake of iron ions in mammals [66]. The synthesis involved a click reaction of the protected 2′-hydroxy-nicotianamine with a dansyl group, creating the fluorescent probe. This probe effectively forms iron ion complexes that are transported via plant and mammalian transporters, ZmYS1 and PAT1, respectively. This development facilitates the study of iron ion uptake mechanisms in plants and mammals, potentially offering insights into addressing iron deficiency issues.

Collectively, advances in the design and application of fluorescent probes for SLCs have greatly contributed to our understanding of the roles SLCs play in cellular homeostasis and how they are involved in pathological states, highlighting their utility for fundamental research and the translation of SLC insights into clinical applications.

3 | Covalent Inhibitors

Covalent inhibitors represent a powerful class of molecular tools used in chemical biology and drug discovery to elucidate interactions with and functions of proteins within complex biological systems [35, 142]. Unlike more traditional reversible inhibitors, covalent inhibitors form stable bonds with their target, enabling specific and permanent labeling of proteins in their native cellular environments. From a therapeutic perspective, covalent inhibitors have revitalized interest in targets previously considered undruggable. Many proteins deemed difficult to inhibit due to the absence of well-defined binding pockets can be targeted by exploiting covalent binding [143]. Moreover, irreversible binding can result in improved efficacy, reduced off-target effects, and lower drug resistance, making covalent inhibitors particularly attractive in the treatment of diseases like cancer, where mutation-driven resistance is a major challenge [144]. While irreversible binding is not without challenges (i.e., it can lead to on- and off-target toxicity) and the inherent reactive nature of covalent inhibitors can lead to metabolic instability, the field has made strides in evaluating and overcoming these pitfalls [145]. Several covalent drugs have already been approved, such as ibrutinib for chronic lymphocytic leukemia, Osimertinib for non-small cell lung cancer and nirmatrelvir as antiviral against COVID-19, demonstrating the clinical viability and therapeutic potential of this approach [146–148]. In addition to drug discovery, the implications of covalent inhibitors for chemical biology are noteworthy. These inhibitors provide powerful tools for examining the function of proteins in a cellular context, enabling studies of the dynamics of protein activity and regulation with high precision [149]. Furthermore, covalent inhibitors can serve as chemical probes to validate the function of newly discovered proteins and to explore the structural aspects of protein-ligand interactions for instance by stabilizing a protein-ligand complex which allows crystallization, contributing to our understanding of protein function at a molecular level [37]. The strategic design of covalent inhibitors includes the incorporation of a photoreactive or electrophilic warhead on the inhibitor [150, 151]. Photoreactive warheads form highly reactive radical intermediates such as carbenes upon activation by light facilitating covalent binding, while electrophilic warheads are designed to covalently bind to nucleophilic amino acid residues of the target protein. This approach enhances the precision of this type of covalent inhibitors, also referred to as targeted covalent inhibitors (TCIs) [152], and thereby minimizes adverse effects and promotes drug safety profiles [153]. Through the application of covalent inhibitors, the molecular determinants for substrate selectivity, transport kinetics, and the impact of posttranslational

modifications on transporter function can be elucidated, thus advancing the understanding of SLC transporter biology. Subsequently, these insights provide opportunities for therapeutic intervention in various diseases through precise, selective and durable target engagement.

3.1 | Photoreactive Covalent Inhibitors

Besides serving as a template for functionalized probes such as AfBPs, which will be discussed in subchapters 3.1 and 3.2, photoreactive covalent inhibitors have been used primarily to characterize the molecular basis of the inhibitor-transporter binding. For this purpose, common photoreactive warheads such as aromatic and aliphatic diazirines, benzophenones, and aryl azides, are installed on existing SLC-targeting inhibitors [154, 155]. Below we will discuss the design and application of such inhibitors, as well as common challenges. While photoaffinity labeling of membrane fractions and the resulting identification of transport-related proteins has been attempted since the 80s [156], here we will focus on advances since 2000.

In an effort to find inhibitors for the glucose transporter type 4 (GLUT4, SLC2A4) in 2002, the Holman group reported two new bis-glucose derivatives integrating the photoactivatable trifluoromethyl diazirine warhead [67]. Introducing nitro- and iodo-substituents on the phenyl ring substituted with the warhead (**31**, Figure 4) resulted in 60 to 130-fold increased affinities for the GLUT4 compared to D-glucose. Moreover, these substitutions led to increased reactivity of the warheads upon determination of the rate of photolysis.

Another glucose transporter which was identified as a potential target for diabetes, the sodium-glucose co-transporter 1 (SGLT1, SLC5A1), has been studied with the use of several photoreactive covalent inhibitors by the Kinne group. Based on the high-affinity substrate arbutin, and the potent inhibitor phlorizin, the probes TIPDG (**32**, Figure 4) and BzG (**33**, Figure 4) were synthesized containing trifluoromethyl diazirine and benzophenone warheads, respectively [68]. These probes can be activated at 350–360 nm, avoiding protein-damaging wavelengths while exhibiting fully competitive inhibition in sodium-dependent D-glucose uptake assays, with K_i values comparable to their parent compounds. Furthermore, to illustrate the utility of this approach in exploring SGLT1 and related transporter proteins, MALDI-TOF MS analyses of TIPDG- and BzG-bound SGLT1 revealed efficient labeling of truncated loop 13, the proposed substrate and inhibitor recognition site. In addition, AOG (**34**, Figure 4), an alkyl glucoside-based probe, was introduced for SGLT1 [69]. In a similar manner, AOG was determined to bind to loop 13 indicating this part of the transporter as a binding domain for aglucone residues of inhibitors such as alkyl glucosides and phlorizin.

Similar to diazirines, benzophenones have an excitation range of 350 to 360 nm [157]. Despite being easier to incorporate, their bulkier nature tends to hinder proper interactions between the photoprobe and its biological targets, posing potential drawbacks [155]. This may explain why benzophenone is less utilized compared to diazirines when it comes to studying SLCs. Nevertheless, two TFB-threo-β-benzyloxyaspartic acid (TFB-TBOA)-based covalent inhibitors were reported bearing either a diazirine (compound 15, **35**, Figure 4) or benzophenone (compound 16, **36**,

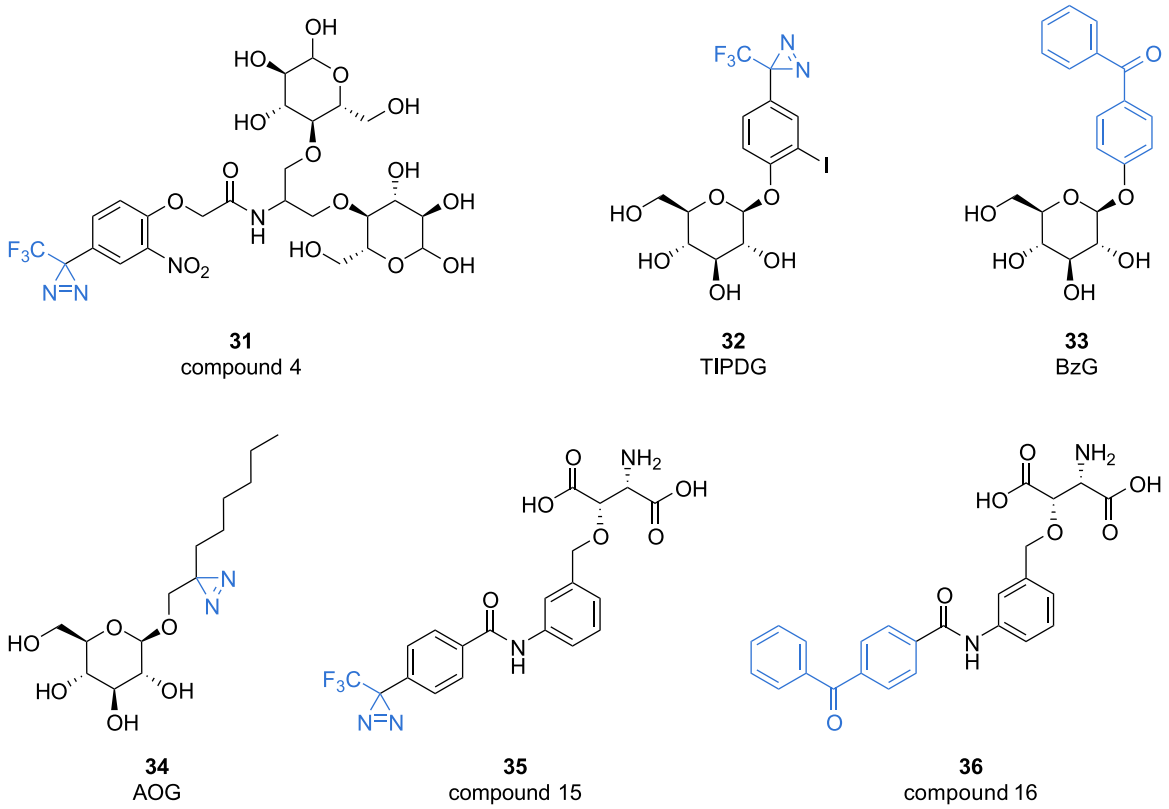


FIGURE 4 | Molecular structures of SLC-targeting photoreactive covalent inhibitors 31–36. Photoreactive warhead is highlighted in blue. [Color figure can be viewed at wileyonlinelibrary.com]

Figure 4) warhead targeting the EAATs (SLC1 family) [70]. The probes displayed submicromolar to micromolar inhibitory potency enabling the development of new TFB-TBOA-based probes for EAATs to understand binding mechanisms and for further therapeutic applications.

3.2 | Electrophilic Covalent Inhibitors

As mentioned above, where photoreactive inhibitors utilize light-activated chemistry, electrophilic covalent inhibitors rely on the inherent reactivity of an electrophilic warhead to form a covalent bond with a nucleophilic amino acid residue. For many years the Newman group has focused on the development of new inhibitors and probes for MATs to elucidate the molecular mechanisms of these transporters and develop new therapeutics for a variety of neuropsychiatric conditions as well as drug abuse disorders. Based on previously reported one-step photoaffinity-based probes (photo-AfBPs), multiple other *N*-substituted tropane-based dopamine reuptake inhibitors were synthesized containing an electrophilic isothiocyanate warhead to initiate covalent binding [158]. Compound 26 (**37**, Figure 5) showed submicromolar potency and successfully binds to DAT in a wash-resistant manner indicating covalent attachment to an amino acid residue in the proximal binding site [71]. To further characterize potentially different MAT binding sites for tropane-based ligands, compound 16 (**38**, Figure 5) was designed. However, substitution with the aryl isothiocyanate on the 2-position of the

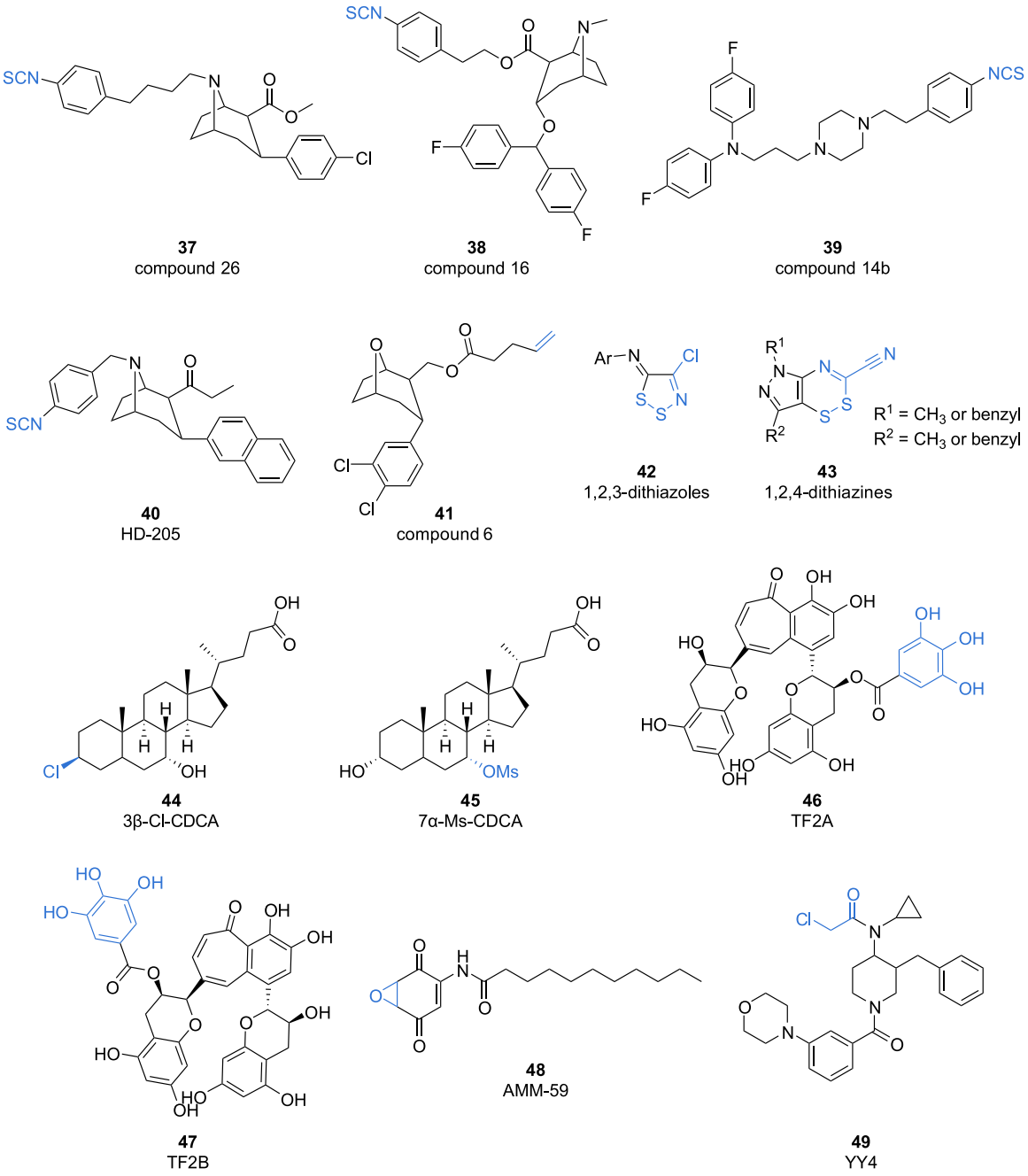


FIGURE 5 | Molecular structures of SLC-targeting electrophilic covalent inhibitors 37–49. Electrophilic warhead is highlighted in blue. [Color figure can be viewed at wileyonlinelibrary.com]

benztropine scaffold resulted in significantly decreased inhibitory potency compared to the 2-substituted 3-phenyltropane counterparts, further suggesting different binding sites and limited tolerance of DAT for steric bulk at the 2-position of benztropines [72]. The different binding profiles of 3-phenyltropanes, benztropines and GBR-12909-based DAT inhibitors inspired the synthesis of rimcazole analogues as potential covalent inhibitors as the parent molecule showed distinct pharmacological and behavioral activity profiles in cocaine abuse animal models [73]. In this study, Cao et al. included synthesis of isothiocyanate derivatives with submicromolar inhibitory potency such as compound 14b (**39**, Figure 5). Based on the increased potency compared to corresponding the iodo-azido analogues, it was thought that during the binding experiments covalent interactions between the DAT and isothiocyanate analogues were formed leading to higher apparent IC_{50} values, however further characterization has not been reported.

Another phenyltropane-derived isothiocyanate covalent inhibitor was reported by Murthy et al. [74]. HD-205 (**40**, Figure 5), derived from the potent 2-napthyl tropane WF-23, showed high affinity for all MATs in radioligand binding studies. The research highlights HD-205's potential to irreversibly label DAT, which was confirmed through the use of an iodinated analog, HD-244, as further discussed in subchapter 3.2. In the pursuit of dopamine-sparing cocaine antagonists crucial for mitigating the addictive properties of cocaine, Meltzer et al. proposed a novel strategy [75]. They designed irreversible inhibitor 6 (**41**, Figure 5) that covalently binds to DAT and leaves behind a small fragment that obstructs cocaine access while allowing dopamine transport. This innovative approach targets a reactive cysteine residue in DAT, aiming to selectively inhibit cocaine binding while preserving dopamine transport. Moreover, their compounds exhibited promising selectivity for DAT and demonstrated considerable inhibition of cocaine binding while allowing substantial dopamine uptake, offering a potential avenue for developing effective pharmacotherapeutics to combat cocaine abuse.

Besides the MATs, extensive research has also been done on the covalent inhibition of amino acid transporters, focusing on the alanine/serine/cysteine transporter 2 (ASCT2, SLC1A5) and L-type amino acid transporter 1 (LAT1, SLC7A5), both crucial in cancer metabolism. Oppedisano *et al*. explored the inhibition of ASCT2 using 1,2,3-dithiazoles (**42**, Figure 5) [76]. Their findings revealed that several derivatives inhibited over 50% of glutamine transport at 20 μM, with inhibition reversible by 1,4-dithioerythritol, suggesting mixed sulfide formation with cysteine residues on the transporter. Kinetic studies indicated noncompetitive inhibition, likely through covalent binding to cysteines outside the substrate site, with Cys207 or Cys210 identified as potential targets. Complementing this, Napolitano et al. targeted LAT1, overexpressed in many cancers. Herein, several dithiazole and dithiazine compounds (**42** and **43**, respectively, Figure 5) were screened, discovering multiple ligands with significant inhibitory effects on LAT1 [159]. The inhibition mechanism involved disulfide bond formation with LAT1's cysteine residues, particularly Cys407, confirmed by docking simulations and mutant studies. Effective inhibitors induced cell death in cancer cells with high LAT1 expression, highlighting their potential as anticancer agents. These studies collectively advanced the understanding of targeting amino acid transporters in cancer therapy, offering promising opportunities for the development of novel anticancer drugs by inhibiting crucial transport mechanisms in tumor cells.

Inspired by photoaffinity labeling experiments previously reported for ASBT (SLC10A2) [160] González et al. reported the synthesis of electrophilic chenodeoxycholic acid (CDCA) derivatives with the purpose to identify the bile acid binding sites of ASBT in a more specific manner [77]. 3β-Cl-CDCA and 7α-Ms-CDCA (**44** and **45**, respectively, Figure 5) were found to be potent and selective irreversible inhibitors of ASBT that bind to the transmembrane domain 7 (TM7) cleft of the transporter as was reported for rabbit ASBT with taurocholic acid (TCA) derivatives [160]. However, no mechanistic evidence was found for a covalent nature of irreversible binding. Another chemotype of inhibitors was found to covalently modify ASBT and significantly reduce TCA uptake in Caco-2 cells [78]. Two theaflavins, theaflavin-3-gallate (TF2A, **46**, Figure 5) and theaflavin-3′-gallate (TF2B, **47**, Figure 5) competitively inhibited ASBT activity without affecting cell viability. It was proposed that these compounds exert their inhibitory effect through the oxidation of their benzotropolone rings, leading to covalent binding with ASBT's cysteine residues. Hence, these theaflavins could be potential therapeutic agents for lowering plasma cholesterol levels by modulating bile acid transport and might help in designing more effective dietary strategies for preventing hypercholesterolemia.

Based on an extensive chemical library screen, epoxycyclohexenedione (ECHD)-type of compounds were identified to be potent inhibitors consisting of a new chemotype for the mitochondrial adenine nucleotide translocator 1 (ANT1 or AAC1, SLC25A4) [79]. Compound AMM-59 (**48**, Figure 5), was found to inhibit ADP uptake in bovine heart submitochondrial particles. In addition, these type of compounds were hypothesized to form covalent bonds because of the electrophilic epoxide and α,β-unsaturated carbonyl within the structure. By testing the reactivity against ethanethiol in thin-layer chromatography experiments, potential reactivity against cysteines was confirmed and further explored by converting AMM-59 into a two-step AfBP (further discussed in subchapter 3.2). Similarly, a broad proteomic screen using a fragment-based approach identified YY4 (**49**, Figure 5), with an α-chloroacetamide warhead, as a covalent inhibitor targeting Cys54 of the mitochondrial pyruvate carrier subunit 2 (MPC2, SLC54A2) [80]. These findings also prompted the development of a YY4-based two-step AfBP as a new tool to study MPC2 as a cellular engagement probe in Western blot and mass-spectrometry-based proteomics, which are highlighted in subchapter 3.2. Besides serving as a stepping stone towards further research, covalent inhibitors have yielded a wealth of information on SLC structure, function and therapeutic potential.

4 | Affinity-Based Probes (AfBPs)

AfBPs are essentially covalent ligands that are able to not only bind the target irreversibly, but also tag it with a specific purification or visualization moiety. AfBPs have evolved from the principles underlying activity-based probes (ABPs), which were initially created to target enzymes [161]. Serving as invaluable

tools for navigating the complex pathways of cellular transport, AfBPs typically entail a sophisticated design comprising a ligand with high-affinity binding to a target protein and a reactive group facilitating covalent binding between the ligand and the target as the previously discussed covalent probes [162, 163]. However, they are differentiated through the inclusion of a reporter group which enables visualization, quantification, and/or purification of the labeled target protein in biochemical assays.

Similar to covalent inhibitors, AfBPs utilize two types of reactive groups: electrophilic or photoreactive warheads. Depending on the labeling strategy of the reporter groups, AfBPs are categorized into one-step or two-step probes. In the one-step approach, the reporter group is directly attached to the AfBP. Common reporter groups used in one-step probes include radioactive isotopes, fluorophores, and biotin tags. While radioactive isotopes offer benefits such as small size, high sensitivity, and ease of detection, they also pose environmental risks, sometimes short half-lives, and the need for specialized handling. Biotin and fluorophores aid in the enrichment, isolation, and detection of target proteins but can be bulky and potentially interfere with interactions between the probe and target protein.

On the other hand, two-step probes often employ a click handle, such as a terminal alkyne or aliphatic azide moiety, which can be functionalized after the initial covalent binding of the probe to the target protein, typically through bio-orthogonal ligation reactions [155, 164]. This approach is notable for the small size and easy incorporation of click handles, minimizing potential disruptions to crucial ligand-target interactions and preserving biological activity [165]. Additionally, these functional groups are compatible with biological systems, allowing for in vivo applications. However, this method has the disadvantage of added complexity and time requirements. Furthermore, the use of potentially cytotoxic reagents in bio-orthogonal conjugation reactions necessitates careful handling to mitigate risks. Therefore, AfBPs are versatile tools for investigating cellular transport pathways, employing either electrophilic or photoreactive groups for covalent binding to SLCs and using one-step or two-step strategies for reporter group labeling.

4.1 | Photoaffinity-Based Probes

One-step photo-AfBPs gained importance in the 1970s and 1980s for mapping receptor-ligand interactions. With regard to studying SLCs, most one-step photo-AfBPs that were developed, target MATs (including NET, DAT, SERT and VMATs) and GLUTs, both of which have been extensively reviewed elsewhere [166–171]. The probes that have not been discussed in these reviews as well as their applications are discussed below.

Looking to expand the tropane-based toolbox for MATs the Newman and Vaughan group incorporated a photoreactive aryl azide on the 3β-position to give [^{125}I]11 (**50**, Figure 6) [81]. Using SDS-PAGE experiments it was shown that both hDAT and hSERT were photolabeled, indicating a shared binding site with cocaine for these SLCs. This also highlighted the importance of positioning the photoreactive warhead away from the tropane pharmacophore to achieve effective labeling. Additionally, another class of dopamine reuptake inhibitors was investigated by the Newman group, based on rimcazole, an antipsychotic agent which was in clinical trials for treatment of acute schizophrenia [73]. Establishing a small structure–activity relationship led to the synthesis of photoaffinity probe [^{125}I]16b (**51**, Figure 6). The probe was shown to effectively and covalently label hDAT in western blot experiments and was used in further studies of the hDAT binding site.

More recently, Lapinsky et al. synthesized pyrovalerone-based ligands, substituting the aromatic ring with a photoreactive azido group to create [^{125}I]-6 (**52**, Figure 6), which labeled and bound hDAT with nanomolar affinity [82]. In contrast to the tropane-based one-step photo-AfBPs, the photoreactive group could be incorporated directly on the pharmacophore of (±)-[^{125}I]-SADU-3-72 (**53**, Figure 6), a probe based on the antidepressant bupropion, which was synthesized to investigate its interactions with DAT and nicotinic acetylcholine receptors [172].

In addition to the previously reviewed inhibitors, substrates and photoaffinity labeling agents for VMATs, two additional probes have been reported based on the nanomolar-affinity inhibitors reserpine and tetrabenazine [168]. [^{125}I]IAPEGlyMER (**54**, Figure 6) and *trans*-[^{125}I]TBZ-AIPP (**55**, Figure 6) both labeled VMAT2 in bovine chromaffin granules, providing tools to further study the binding site of these inhibitors [84]. Moreover, decreased labeling with *trans*-[^{125}I]TBZ-AIPP (**55**) in the presence of ATP provided further indications of the transporters' conformational change upon generation of a proton gradient in the vesicles.

In 2005, AstraZeneca published the discovery of MCT1 (SLC16A1) as the target of immunomodulating quinazolinediones with the use of ^{125}I-labeled 4 (**56**, Figure 6) in SDS-PAGE and proteomics experiments [85]. Upon identification of MCT1 as a target for inhibiting T-lymphocyte proliferation, many studies have followed to map the SLC's role in the tumor microenvironment and to design novel inhibitors [173].

Before the crystal structure of hENT1 was elucidated in 2019, the Buolamwini group made multiple efforts to explore the inhibitor binding interactions of ENT1. For this purpose, the well-characterized ENT1 inhibitor NBTI was converted into photo-AfBP SAEATA-x14-biotin (**57**, Figure 6) by substituting the aromatic nitro group with an azide and addition of a linker attached to a biotin reporter group on the 5′-OH of the original ribose ring similar to the fluorescent derivative 5-(SAENTA)-x8-fluorescein [86, 174]. SAEATA-x14-biotin (**57**) was able to cross-link ENT1 in a wash-resistant manner and could be visualized using streptavidin detection after SDS-PAGE.

The first two-step photo-AfBP for MATs, azidobupramine (**58**, Figure 7), was reported in 2016 [87]. As an analogue of the tricyclic antidepressant imipramine, azidobupramine was designed to covalently target hMATs using an aryl azide. Moreover, the addition of an alkyne tag at the terminal amine allowed for copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC) to attach reporter moieties, such as fluorophores. This novel probe maintained binding affinity comparable to various tricyclic antidepressants while enabling the visualization of azidobupramine-SERT complexes in membrane fractions with induced SERT expression as well as in living cells. The Lapinsky

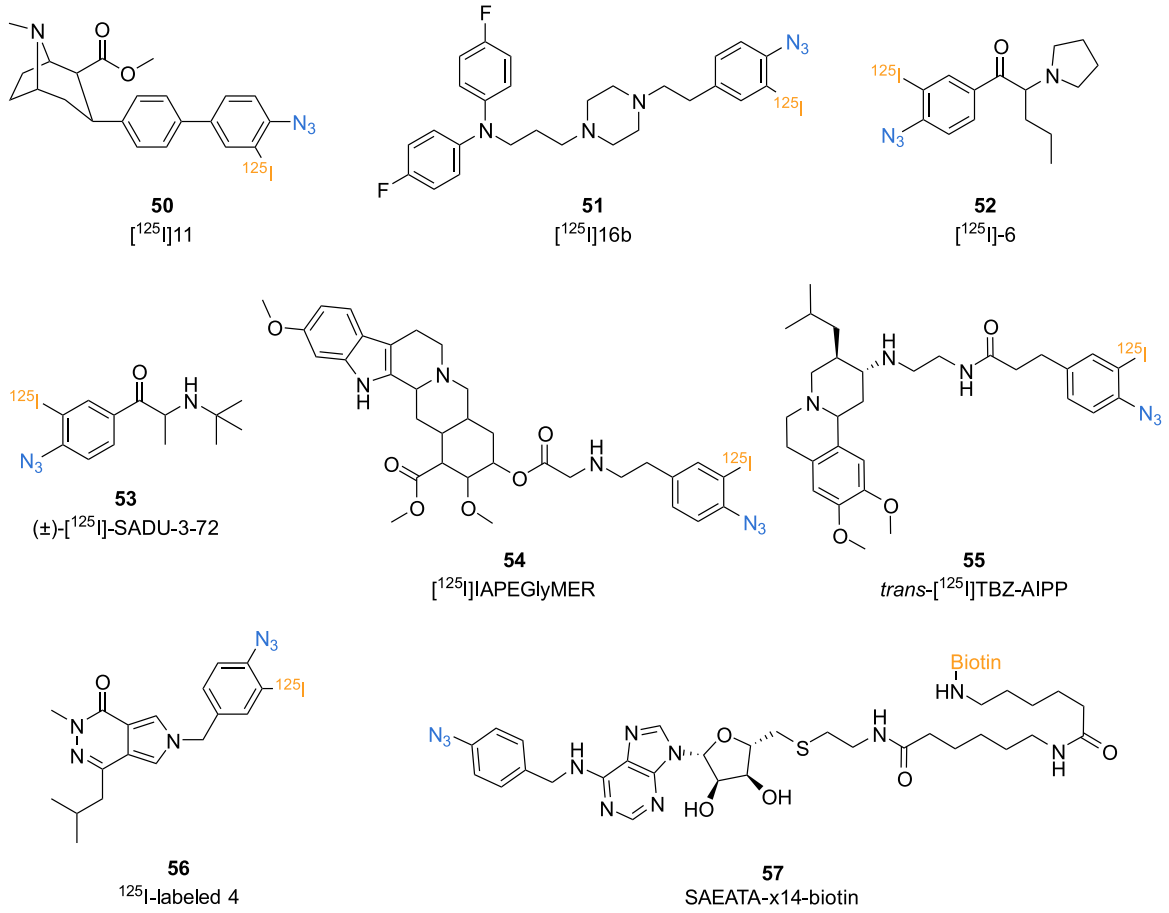


FIGURE 6 | Molecular structures of SLC-targeting one-step photoaffinity-based probes 50–57. Photoreactive warhead and reporter group are highlighted in blue and orange, respectively. [Color figure can be viewed at wileyonlinelibrary.com]

group built upon this pioneering work to further design multifunctional tools derived from clinically approved antidepressants. Leveraging escitalopram's unique pharmacological properties, such as its high affinity for both the S1 and S2 hSERT binding sites, they synthesized a series of clickable photoprobes [88]. Photoprobe 6 (**59**, Figure 7), featuring a benzophenone moiety and a ligation handle at the C-5 position of escitalopram, demonstrated a remarkable 11-fold improvement in hSERT binding affinity compared to the parent compound and was clickable to IRDye 800CW. Western blot analysis revealed that the probe labeled purified hSERT, facilitating structure-function studies and imaging applications involving hSERT. These advancements in the field of neuropsychopharmacology offer a promising approach for identifying antidepressant target proteins without using radioactive isotopes, marking a significant step forward in drug discovery and understanding antidepressant mechanisms.

In search of biological targets in the Notch signaling pathway, a study by Nolin et al. employed a photoaffinity labeling approach [89]. As a result of siRNA knockdown experiments and equipping a newly identified inhibitor of Notch signaling with a diazirine and terminal alkyne (NVS-ZP7-6, **60**, Figure 7), zinc transporter 7 (ZIP7, SLC39A7) was validated as target to disrupt Notch protein trafficking, induce ER stress, and trigger apoptosis in T cell acute lymphoblastic leukemia (T-ALL) cells. These findings show ZIP7's critical role and open avenues for novel cancer therapies by modulating Notch signaling, particularly in cancers with dysregulated Notch pathways.

The Ohgane group investigated NPC intracellular cholesterol transporter 1 (NPC1, SLC65A1), another SLC highly implicated in disease [90]. Inherited mutations in the protein can lead to the rare Niemann-Pick disease type C by causing folding defects ultimately leading to lysosomal and late endosomal cholesterol accumulation [175]. This study on pharmacological chaperones for NPC1 focused on oxysterol derivatives, which demonstrated significant potential in repairing NPC1-I1061T protein misfolding. Photocrosslinking followed by SDS-PAGE experiments with the oxysterol-derived two-step photo-AfBP mo56AZK (**61**, Figure 7) and N-terminal domain-deleted NPC1 revealed that these molecules interact with a non-N-terminal domain sterol-binding site on NPC1, suggesting the presence of a second sterol-binding site. This interaction aids in the proper folding and maturation of the mutant protein, improving its localization and function within cells. Additionally, in a follow-up study an image-based screening method was used to identify potential new chaperones for NPC1 from a drug-repurposing point of view [91]. Itraconazole, a known antifungal, emerged as a potential pharmacochaperone to the NPC1-I1061T mutant protein amongst other azole antifungal compounds. This suggested that the *N*,*N*-diaryl piperazine moiety in these molecules may be important for the misfolding rescue properties. By converting itraconazole into the photo-crosslinking compound itraAZY (**62**, Figure 7) and the use

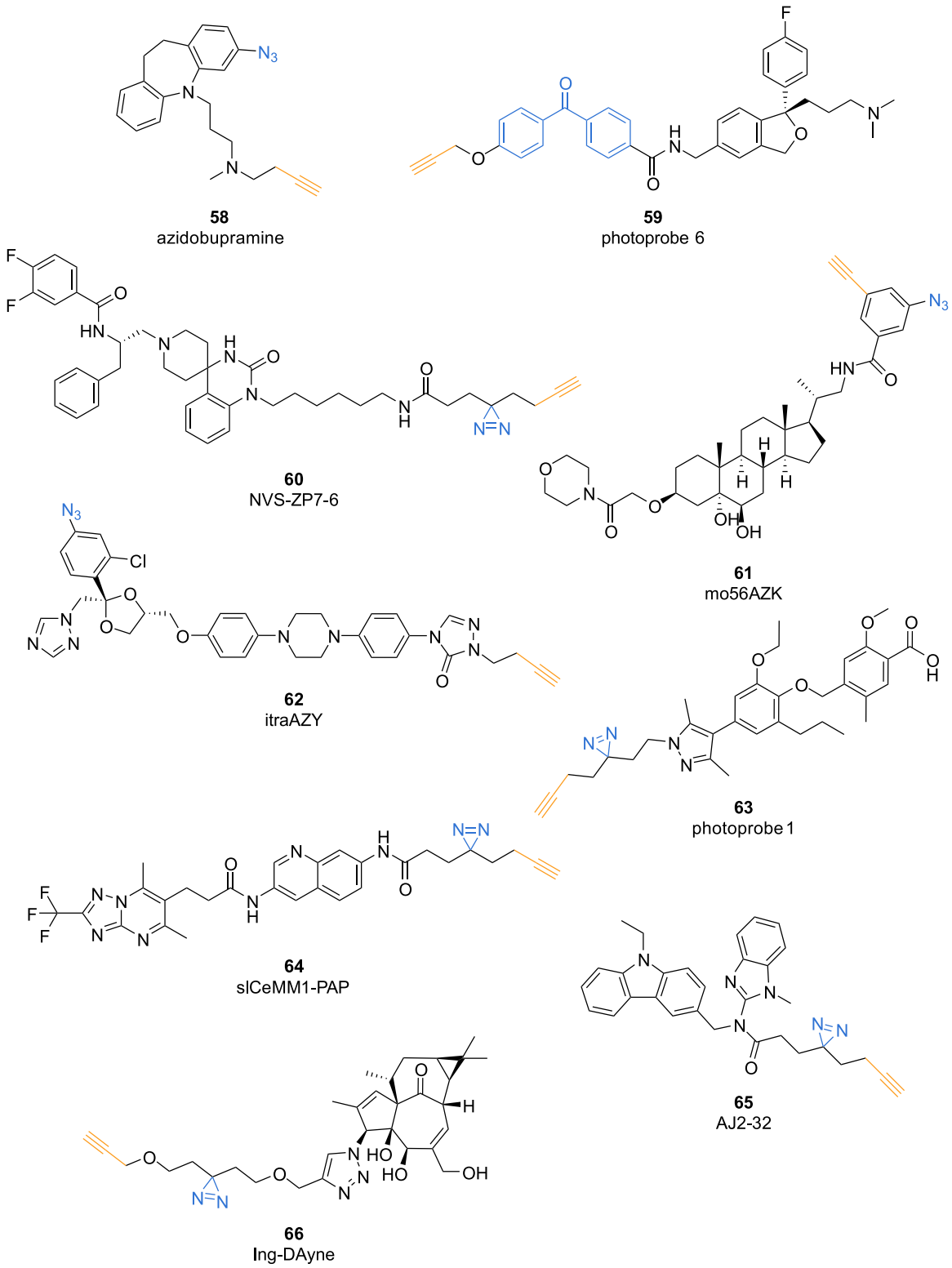


FIGURE 7 | Molecular structures of SLC-targeting two-step photoaffinity-based probes 58-66. Photoreactive warhead and ligation handle are highlighted in blue and orange, respectively. [Color figure can be viewed at wileyonlinelibrary.com]

of competitive binding studies, it was confirmed that this antifungal binds directly to NPC1-I1061T and shares binding sites with oxysterol-based chaperones near the sterol-sensing domain. Although less effective at reducing cholesterol accumulation than oxysterol-derived chaperones or the histone deacetylase inhibitor LBH-589, itraAZY provides a promising starting point for developing pharmacological chaperones to treat NPC1-related diseases through targeted medicinal chemistry efforts.

In search of new selective inhibitors for the monocarboxylate transporter 4 (MCT4, SLC16A3), AstraZeneca developed photoprobe 1 (**63**, Figure 7), a clickable photo-AfBP that retains

cellular biological activity in the MCT4-driven lactate efflux assay [92]. Photoprobe 1 (**63**) was employed in reverse competition assays with AZ1422, demonstrating its utility in visualizing probe localization through fluorescence imaging. In live NCI-H358 cells, photoprobe 1 enabled the confirmation of cellular localization of AZ1422. This study shows the importance of photoprobe 1 in validating target engagement and elucidating the mechanism of action for therapeutic compounds targeting MCT4 in cancer. In another study, by the Superti-Furga group, the photoaffinity probe slCeMM1-PAP (**64**, Figure 7) was developed to study SLC16A3 [94]. This probe, derived from the selective SLC16A3 inhibitor slCeMM1, incorporates a diazirine moiety for covalent crosslinking and an alkyne handle for reporter tag conjugation. slCeMM1-PAP demonstrated biological activity similar to slCeMM1 but with increased toxicity at higher concentrations. Chemoproteomic analyses using slCeMM1-PAP revealed the probe's high selectivity for SLC16A3, confirming its effectiveness in distinguishing target proteins from off-targets within the proteome, thereby validating the used paralog-dependent isogenic cell assay (PARADISO) as a cell-based assay approach in drug discovery screening for SLCs.

Using a fully functionalized fragment (FFF) approach, the Parker group reported the development of the first inhibitors that disrupt all functions of the peptide transporter 4 (PHT1, SLC15A4) [95]. Based on the candidate hits several inhibitors and a two-step photo-AfBP AJ2-32 (**65**, Figure 7) were developed to perform target engagement experiments. The inhibitors were validated as having significant potential for treating inflammatory and autoimmune conditions. The inhibitors, particularly AJ2-30, were shown to reduce the production of interferons (IFN-α, IFN-β, and IFN-γ) and suppress early mTOR signaling, which mimics the functional loss of SLC15A4. This suggests that targeting SLC15A4 could be effective in managing lupus and other autoimmune diseases. Another photoreactive and clickable probe by Parker *et al.* was used to determine the involvement of the molecular targets of Ingenol mebutate (IngMeb), a drug for actinic keratosis [96]. Ing-Dayne (**66**, Figure 7) was designed with a diazirine group for crosslinking and despite its reduced potency, retained sufficient biological activity for proteomic studies. Using mass spectrometry, the probe identified multiple protein targets, notably carnitine/acylcarnitine carrier (CAC, SLC25A20), a mitochondrial transporter. Ing-DAyne revealed that IngMeb blocks SLC25A20, impairing fatty acid oxidation and causing mitochondrial dysfunction, suggesting a new mechanism for IngMeb's therapeutic effects.

4.2 | Electrophilic Affinity-Based Probes

Electrophilic AfBPs for SLCs have also provided valuable insights into transporter ligand-binding sites, though to a lesser degree compared to photoaffinity AfBPs. For this type of probes isothiocyanate is the most frequently used reactive electrophilic warhead. In addition to the aforementioned one-step photo-AfBPs developed for the MATs, two tropane-based probes were synthesized to covalently bind the transporters in rat striatal membranes in a covalent manner [74, 97]. These probes, [^{125}I]MFZ-3-37 (**67**, Figure 8) and [^{125}I]HD-244 (**68**, Figure 8), both bound rDAT albeit with off-target labeling of multiple other proteins. The off-target labeling, which was not reported for the aryl azide analogue probes, was attributed to the reactivity of the isothiocyanate warhead.

To enable the identification of a putative binding pocket of cholesterol in the anion exchanger 1 (AE1, SLC4A1), Moriyama *et al.* demonstrated a novel chemical tagging approach in erythrocyte membranes using the thiourea of probe 2 (**69**, Figure 8) [98]. The study highlighted the possibility of analyzing lipid-protein interactions within complex membrane protein systems, paving the way for comprehensive proteomic analyses and potential therapeutic developments.

The exploration of two-step electrophilic affinity-based probes for SLCs was pioneered by Aoyama et al. in 2018 [79]. In this study, ECHD-like compounds, specifically the AMM-59 above

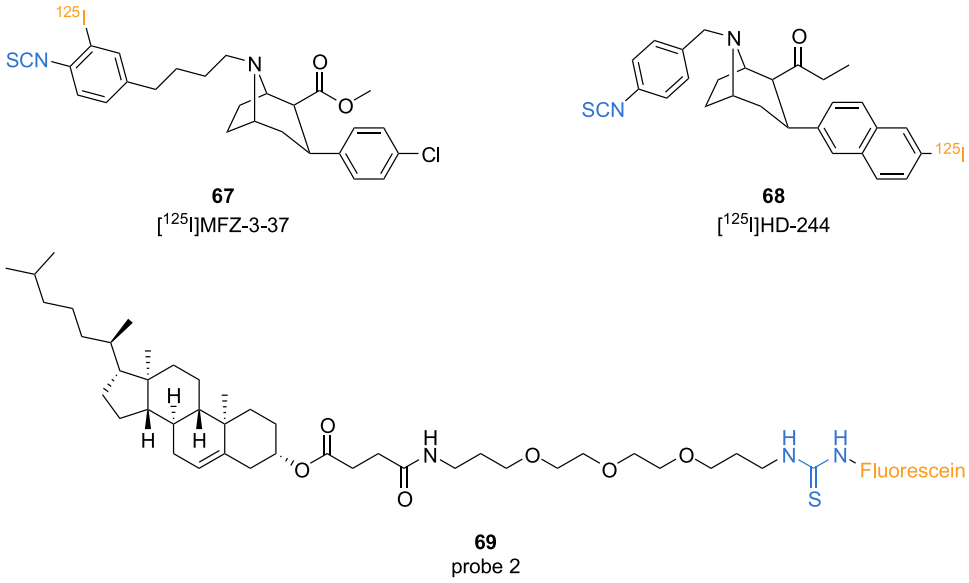
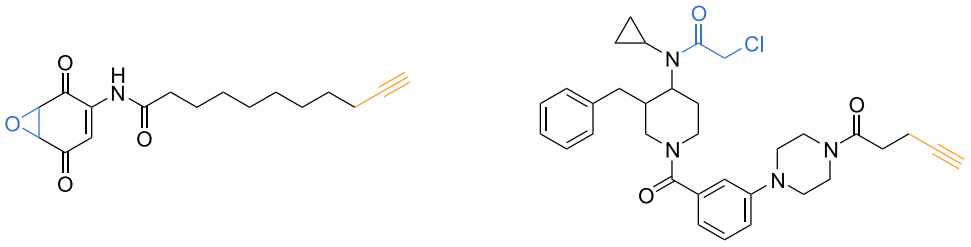


FIGURE 8 | Molecular structures of SLC-targeting one-step electrophilic affinity-based probes 67–69. Electrophilic warhead and reporter group are highlighted in blue and orange, respectively. [Color figure can be viewed at wileyonlinelibrary.com]



70
AMM-120

71
YY4-yne

FIGURE 9 | Molecular structures of SLC-targeting two-step electrophilic affinity-based probes 70 and 71. Electrophilic warhead and ligation handle are highlighted in blue and orange, respectively. [Color figure can be viewed at wileyonlinelibrary.com]

(**48**, Figure 5) and AMM-120 (**70**, Figure 9), were investigated as potent inhibitors of bovine ANT1 (SLC25A4, old nomenclature of AAC1 used in original article) [79]. Analysis by SDS-page and proteomics experiments of AMM-120-bound ANT1 revealed that these compounds inhibit ANT1 function primarily by forming covalent bonds with amino acid residue Cys57 and secondarily with Cys160 of the protein. Moreover, prolonged exposure to high concentrations of ECHDs induced aggregation of ANT1 suggesting a significant structural alteration. Importantly, this aggregation was not attributed to intermolecular disulfide linkages but rather to hydrophobic stacking mediated by probes bound to Cys57 and possibly Cys160. This phenomenon represents a unique characteristic of ECHDs, presenting a novel avenue for studying ANT1 transporters and offering insights into their structural dynamics and potential regulatory mechanisms.

Recently, Yamashita et al., also presented a two-step AfBP approach utilizing the aforementioned α-chloroacetamide (αCA) inhibitor YY4 (**49**, Figure 5) to selectively modify amino acid residue Cys54 within MPC2 (SLC54A2) [80]. YY4-yne (**71**, Figure 9), facilitated the study of MPC2 but not MPC1 in living cells using Western blot analysis as well as proteomics techniques showing the selective nature of the probe. Moreover, their findings shed light on the mechanism of action of UK-5099, a MPC2 inhibitor with hypothesized covalent reversible mechanism of action, suggesting this inhibitor may also interact with Cys54.

5 | Bitopic Probes

Bitopic probes such as bivalent ligands, molecular glues and PROTACs are molecules designed to establish dual engagement within one protein or between two proteins. The probes, bearing two high-affinity binding moieties for their respective protein, offer a wide arrange of strategies to study proteins. Such probes may target combined orthosteric and allosteric binding sites on one protein, which may enhance selectivity, induce protein-protein interactions or cause targeted protein degradation [176–179].

Bivalent strategies have been employed to enhance inhibitor-protein interactions on SLCs. By simultaneously engaging with multiple substrate recognition sites on transporters, bivalent probes may induce interactions with increased affinity compared to individual monovalent molecules [180]. In addition, achieving selectivity can be crucial, as multiple SLCs within one family often exert various physiological effects. Since for many SLCs there are multiple binding sites, the length of the linker connecting the two binding components in bivalent ligands could offer a way to fine-tune the selectivity of these ligands for specific transporter types as previously described for GPCRs [181]. Theoretically, their specificity can be attained through two potential bridging mechanisms [182]. In the first case, the bivalent ligand is linked with a spacer of suitable length, facilitating both ligands to attach to similar or identical adjacent recognition sites. With the correct linker configuration, the bivalent binding is favored, while inadequate spacer length permits only monovalent binding. The second bridging approach occurs when the second ligand binds to an accessory site of a neighboring protein. However, this is specific to receptors and has not yet been shown for SLCs [183].

Targeted protein degradation (TPD) has emerged as one of the most significant developments in chemical biology and drug discovery over the past years [178, 184, 185]. A prominent class within TPD are PROTACs, which have emerged as a novel method that deviates from conventional therapeutic strategies based on optimizing ligand-protein occupancy [186]. PROTACs are heterobifunctional small molecules, comprising two ligands interconnected by a linker. One ligand moiety participates in the recruitment and binding of the protein of interest (POI), while the other engages with an E3 ubiquitin ligase. The simultaneous binding of the POI and E3 ligase leads to the ubiquitination of the POI, initiating its subsequent proteasomal degradation and recycling of the PROTAC for reuse. Suitable protein candidates for TPD typically exhibit characteristics that promote disease-causing gain of function [187]. Structurally, effective PROTAC targets require a small molecule binding site accessible to an E3 ligase [188]. Additionally, it is beneficial to have an unstructured section that makes engagement with the proteasome easier [189]. Despite these common requirements, proteins with scaffolding roles, mutations inducing resistance to targeted treatments, or those traditionally deemed "undruggable" can also be excellent candidates for PROTAC intervention [190, 191]. As a result, the catalytic nature of PROTACs and the resulting depletion of a POI could circumvent challenges such as drug-resistance and off-target effects observed with small-molecule inhibitors.

5.1 | Bivalent Ligands

Where in previous paragraphs examples from various SLC's were presented, the application of bivalent ligands in SLC

research focused solely on MATs. In the early 2000s, the Kozikowski and the Cashman groups designed multiple novel dimeric MAT inhibitors based on 3,4-disubstituted piperidines and phenyl tropanes as reviewed previously [192–196]. Nielsen et al. extended the work on bivalent phenyltropanes by designing a number of probes linked with amide-connected aromatic spacers (**72**, Figure 10) or ester connected triazole linkers (**73**, Figure 10), which displayed higher affinity for MATs compared to parent monovalent compound RTI-31 [99]. This supported the hypothesis reported by Fandrick et al. that incorporating longer linkers in bivalent phenyltropanes can reverse the low DAT inhibitory potency observed in monovalent compounds suggesting the existence of a secondary tropane binding site (S2 binding site) in addition to the primary binding site (S1 binding site) [195]. Subsequent work by Meltzer *et al.* however challenged the hypothesis, reporting tropane-based bivalent ligands (**74**, Figure 10) that were less effective than monovalent counterparts despite maintaining high potency, suggesting the absence of proximal binding sites [100].

The same effect is also observed for bivalent ligands based on imipramine, an antidepressant with potential anticancer effects (**75**, Figure 10). Although the bivalent ligands had higher potential anticancer effects compared to the monomeric imipramine,

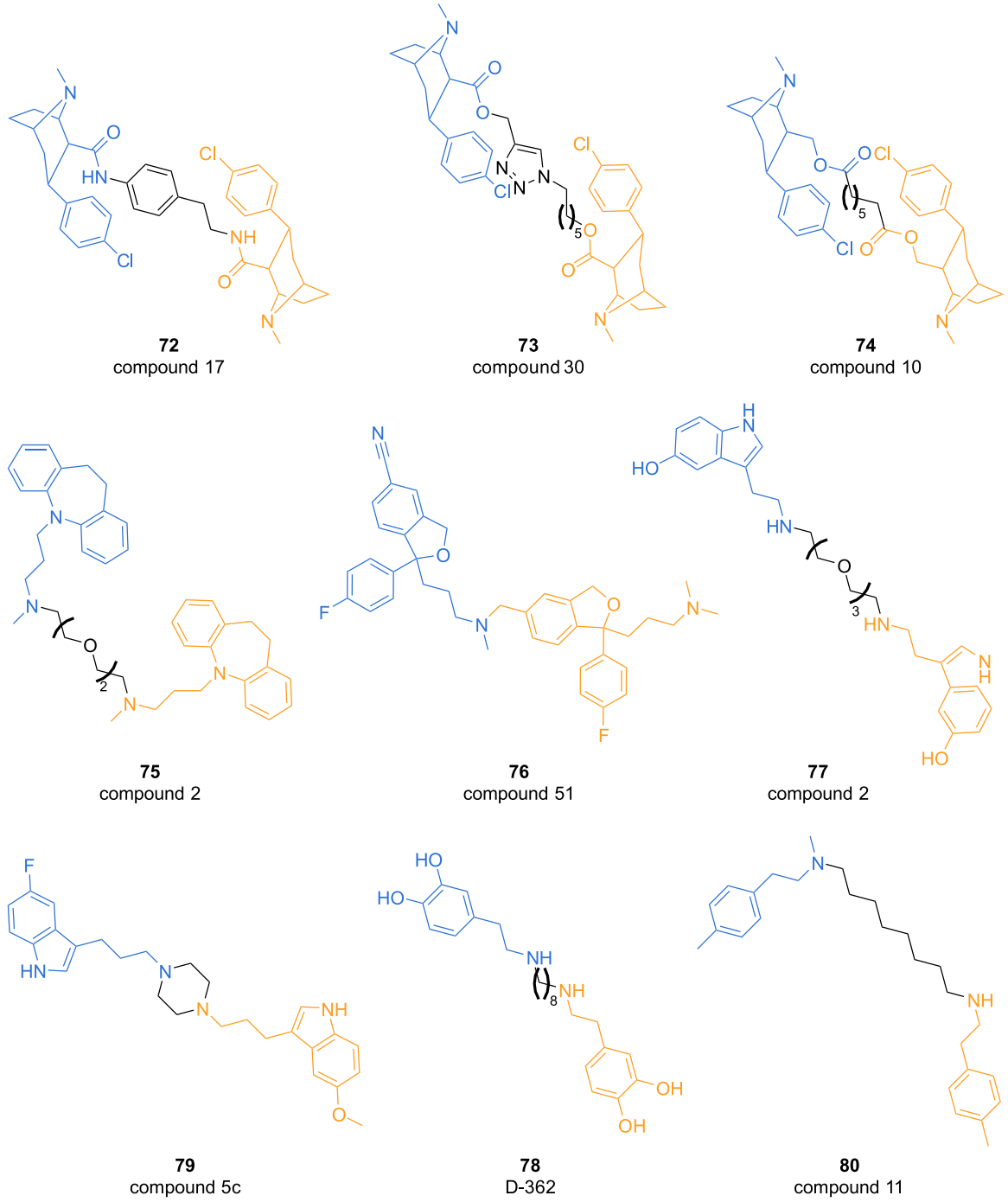


FIGURE 10 | Molecular structures of exemplary SLC-targeting bivalent probes 72–80. Monovalent ligand moieties (connected by a linker) are highlighted in blue and orange. [Color figure can be viewed at wileyonlinelibrary.com]

a two-fold decreased affinity was found against SERT [101]. This indicates that bivalent ligands should express specific structural characteristics to bind to MATs with high affinity. In addition, a follow-up computational study predicted the S2 site approximately 10-12 Å above S1, after which imipramine-based bivalent probes were optimized accordingly to facilitate binding to both sites simultaneously [197]. As opposed to previous studies, the dimers with the shortest chain length had the highest potency, albeit all with significantly lower affinity compared to monovalent imipramine. Importantly, it was proposed that S2 facilitates a secondary, low-affinity allosteric site for inhibitors and interaction of inhibitors with this site influences the rate at which molecules detach from their primary binding sites. This is thought to occur due to a mechanism involving the physical obstruction of the exit pathway [198]. The study supported this concept by showing that expanding the S2 site through mutations led to an increased potency of inhibitors, implying that S2 may indeed negatively contribute to inhibitor potency.

Several studies have debated the roles of S1 and S2 subsites in MATs with respect to the pharmacological effects of inhibitors. While S1 has been extensively studied, the specific role of S2 remains less clear [199–203]. Notably, the S2 site has been described as being more tolerant than the S1 site, indicating that it can accommodate structural modifications to ligands with less impact on binding affinity [198, 204, 205]. Based on this observation, Banala et al. synthesized escitalopram derivatives, identifying compounds such as compound 51 (**76**, Figure 10) with selective affinity for S2 over S1 [102]. This study stresses the possibilities of bulky modifications and optimization of already known potent inhibitors that may have selectivity towards S2, and may be a starting point for further S2 studies.

Bivalent probes have also been used to elucidate substrate recognition mechanisms in MATs using homo- or heterobivalent probes based on dopamine and serotonin [103, 104]. The study by the Strømgaard group demonstrated an up to 3800-fold increased affinity for bivalent probes such as compound 2 (**77**, Figure 10) when compared to their monovalent parent substrate from which subsequent docking studies also suggested simultaneous binding to both S1 and S2 [103]. In addition, they demonstrated by using the bivalent probes together with mutational analysis that non-conserved residues in binding sites do not aid in substrate selection during binding. Additionally, Schmitt et al. synthesized MAT substrate-based bivalent probes like D-362 (**78**, Figure 10) that when subjected to binding assays with mutated transporters revealed a higher affinity towards inward-facing transporters [104]. Most recently, Ruchala et al. explored the functional characterization of various variants of *N*-octyl 4-methylamphetamine and related bivalent compounds at DAT and SERT. The use of psychostimulant-derived bivalent probes in addition to the aforementioned inhibitor and substrate-based probes revealed that certain bivalent compounds (**80**, Figure 10) exhibited potent inhibitory activity at DAT and SERT, offering insights into their potential therapeutic applications, including managing cocaine addiction [106].

Besides targeting only SLCs, bivalent ligands with a different mechanism of action to target two different proteins including a transporter and a GPCR were reported. Several bis-indole derivatives based on serotonin using propylpiperazine linkers (**79**, Figure 10) were synthesized to achieve a dual inhibitory activity at both SERT and 5-HT_{1A} receptor [105]. With the combined use of radioligand binding assays and molecular docking multiple bivalent probes with nanomolar affinity for both targets were found creating a starting point for new inhibitors with this dual transporter-receptor binding mechanism.

5.2 | PROTACs

While many advances in the field of TPD have been made, with the first examples entering the clinic [206], targeting multi-pass membrane proteins has remained challenging. Reported studies in the field of TPD are predominantly focused on targeting nuclear, cytoplasmic, and single-pass transmembrane proteins, while SLCs and other transmembrane protein families have been underexplored as targets for PROTACs. This is mainly due to their complex transmembrane topology and diverse subcellular localizations, which both can be challenging for efficient engagement with the E3 ligase and hence the proteasome. However, in 2020, this notion was challenged, demonstrating the successful degradation of multi-pass transmembrane proteins, particularly those from the SLC family, using the genetically encoded dTAG degradation system [107]. In addition, this led to the design and synthesis of d9A-2 (**81**, Figure 11), a PROTAC targeting the sodium-hydrogen antiporter 1 (NHE1, SLC9A1), and to a lesser degree, other members within the SLC9 family. The PROTAC demonstrated efficacy in initiating proteasomal degradation by the $CRL4^{CRBN}$ E3 ligase complex. Moreover, it was shown that d9A-2 (**81**) is able to induce cytotoxicity in various cancer cell lines via disturbing the intracellular pH homeostasis in which the PROTAC was most potent in leukemic cancer cells.

6 | Photoswitchable Inhibitors

The development of photoswitchable inhibitors, also known as photochromic inhibitors, represents a significant advancement

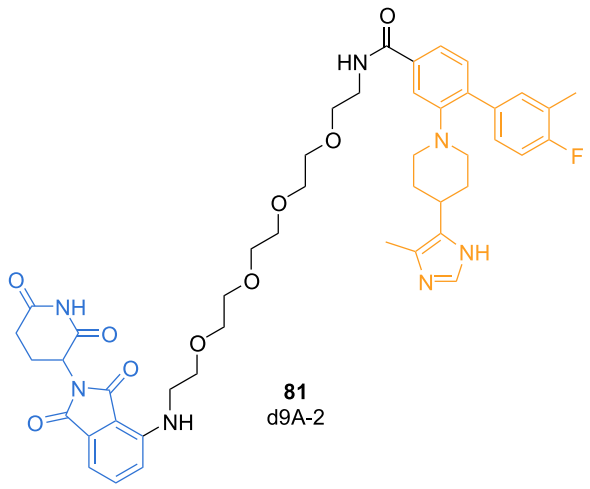


FIGURE 11 | Molecular structures of SLC-targeting PROTAC d9A-2 (81). Ligand moiety binding to the POI (sodium-hydrogen antiporter 1, NHE1, SLC9A1) and E3 ligase are highlighted in orange and blue, respectively. [Color figure can be viewed at wileyonlinelibrary.com]

in molecular pharmacology. Photoswitchable compounds undergo structural changes upon irradiation with light of a certain wavelength, leading to significant alterations in their affinity or intrinsic activity towards the target [207]. This enables precise spatiotemporal control over their activity, allowing reversible activation and deactivation of inhibitors at specific protein targets at any time using light. The use of external stimuli to control ligand activity provides powerful tools for monitoring and altering cellular processes in living cells and organisms non-invasively and therefore may enhance our understanding of transporter functions and offer valuable insights into transporter-ligand interactions [208].

Two primary strategies are employed to manipulate the activity of bioactive molecules using light, which can be either irreversible or reversible. The first involves employing photoreactive components, such as *o*-nitrobenzyl or coumarin, to temporarily deactivate a physiologically active small molecule, producing a "caged" compound that is inactive on the target [209]. Exposure to light of a specific wavelength irreversibly releases the ligand by breaking the bond and initiates a biological response in a rapid and localized manner [210]. Another approach is through the incorporation of molecular photoswitches, such as diarylethenes [211] and azobenzenes [212]. Upon irradiation, the photoswitch-incorporated molecules are able to undergo *cis-trans* isomerization, leading to changes in their 3D conformation and properties [213]. While these structural alterations can result in variations in the biological activity of the compounds, this is not a trivial matter. Azobenzenes remain the predominant choice among photoswitches, mainly because they demonstrate favorable characteristics such as accessible synthesis, small size, metabolic stability, as well as rapid isomerization with high quantum yields and little photobleaching [212]. These characteristics make them highly effective for investigating various transmembrane proteins, such as GPCRs [214] and ion channels [215]. For azobenzenes, the linear and flat *trans* (*E*)-form tends to display higher thermodynamic stability than the *cis* (*Z*)-form, which is also more polar and soluble in aqueous environment [216, 217]. Typically, the ligand is converted to the less stable isomer by one wavelength of light. Subsequently, it can either thermally revert to its stable form in the dark or be switched back to the more thermodynamically stable isomer using a second wavelength of light.

While the field of photopharmacology is still in its infancy for SLCs, the Wanner group has pioneered it with the development of the first photochromic inhibitor targeting the murine GAT1 (mGAT1, Slc6a1) [108]. A series of photoswitchable derivatives based on a nipecotic acid scaffold was reported, incorporating azobenzene moieties to confer reversible photoisomerization properties. Biological evaluation revealed that these compounds showed a subtype-selective, moderate to potent inhibitory activity against GAT1, with their efficacy being moderately reversible upon light irradiation. In addition, electrophysiological experiments further demonstrated the ability of the most promising photoswitchable inhibitor, (*R*)-6e (**82**, Figure 12), to modulate neuronal activity in mouse brain slices in an irradiation-dependent manner. As next step, Wanner *et al.* incorporated various azobenzene moieties as *N*-substituents of (*R*)-nipecotic acid [109]. New inhibitors were designed with the aim to achieve potent inhibition in the (*Z*)-form after irradiation, compared to the (*E*)-form, in contrast to the aforementioned (*R*)-6e (**82**), which was more active before irradiation [108]. This may result in a more pronounced difference in activity postirradiation due to the constrained extent to which azobenzenes can transition from the more stable (*E*)- to the less stable (*Z*)-form. This led to the discovery of a novel, potent photoswitchable inhibitor, (*R*)-*m*-20a (**83**, Figure 12), which, unlike previous inhibitors, can be "activated" by photoirradiation, showing a significantly higher pIC_{50} value in the (*Z*)-form compared to its (*E*)-form.

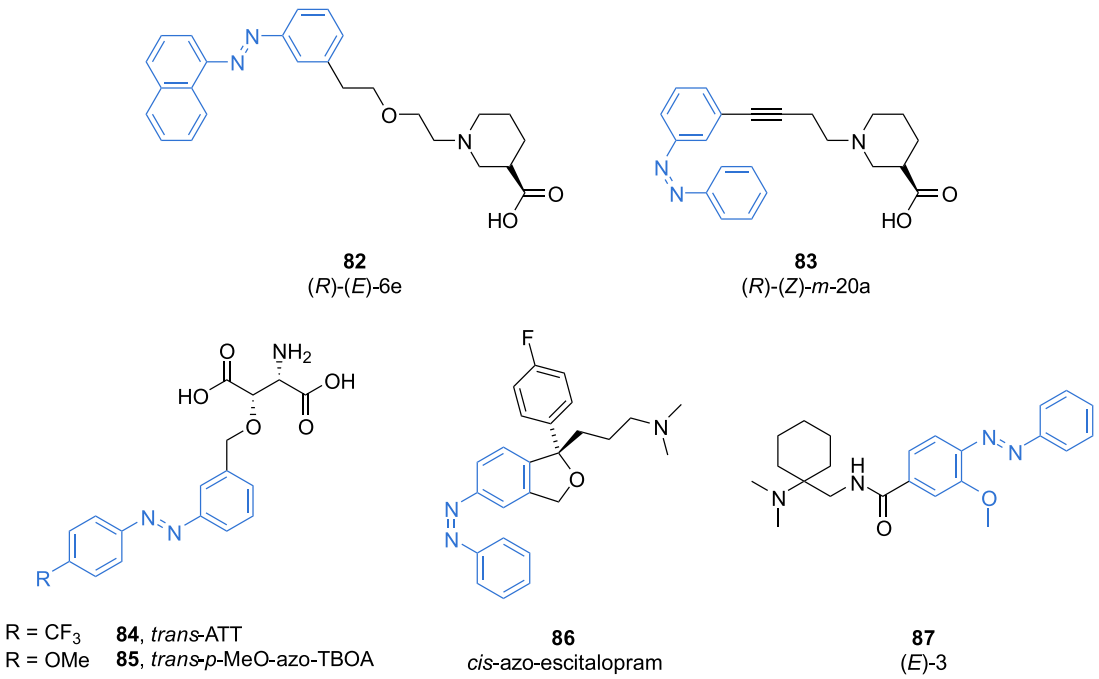


FIGURE 12 | Molecular structures of photoswitchable inhibitors 82–87 targeting SLCs. Photoreactive azobenzene moieties are highlighted in blue. Original compound names and structures are given in their most active isomer. [Color figure can be viewed at wileyonlinelibrary.com]

In addition to research focused on GABA transporters of the SLC6 family, multiple efforts in photopharmacology of the glutamate transporters have been made. In 2017 the Trauner group successfully developed the first photoswitchable glutamate transporter inhibitor targeting EAAT1-3 (SLC1A1-3) [110]. The compound azo-TFB-TBOA, or ATT (**84**, Figure 12), was synthesized by incorporating an azobenzene moiety into TFB-TBOA. TFB-TBOA is a highly potent inhibitor of glutamate uptake and is widely used for studying glutamate transporter functions in the CNS [218]. ATT displays high activity in the nonirradiated form which was reduced by 14-fold upon reversible photoswitching to the *cis*-form. In addition, another study reported several photochromic azobenzene derivatives of TBOA and TFB-TBOA targeting the archaeal homologue (*Thermococcus kodakarensis*) of glutamate transporters, Glt_{Tk} [111]. Upon biological characterization, *p*-MeO-azo-TBOA (**85**, Figure 12) was identified as the most promising photoswitch with a high inhibitory activity in the *trans*-form that drops 3.6-fold when reversibly irradiated.

While the previous photoswitches only gave minor to moderate changes in activity upon photoswitching, more recently, the Trauner group has expanded the focus of their work and developed the first photopharmacological tool for SERT (SLC6A4) by studying the structure–activity relationships of SERT and derivatives of the potent selective inhibitor, escitalopram [112]. Using rational design, an "azostere" was designed and synthesized resulting in azo-escitalopram (**86**, Figure 12), from which the stable *trans*-isomer exhibits minimal activity, serving as an inactive inhibitor. However, upon photoisomerization to the *cis*-isomer, azo-escitalopram demonstrates a striking 43-fold increase in inhibitory potency. This notable distinction between the two isomeric states underlines the potential of azo-escitalopram.

GlyT2 (SLC6A5), another member of the SLC6 family, plays a crucial role in regulating chronic pain, where inhibition of the transporter results in analgesic effects [219, 220]. Reversible inhibition is suggested to prevent toxicity while preserving function; however, the mechanisms and effects of reversible inhibition on glycine neurotransmission remain largely unknown [221]. To further investigate this transporter, Vandenberg et al. developed compound 3 (**87**, Figure 12), a photoswitchable azobenzene derivative of ORG25543 [113]. The parent GlyT2 inhibitor 2, another derivative of ORG25543 with improved toxicity profiles, is a potent inhibitor known to alleviate neuropathic pain in rodent models, featuring a benzyl phenyl ether structure that serves as a starting point for incorporation of an azobenzene [221]. Upon incorporation of the photoswitchable moiety, both isomers of compound 3 act as noncompetitive binders with similar binding activities, though the (*E*)-isomer exhibits twofold lower inhibitory potency value. The activity of both (*Z*)-3 and (*E*)-3 isomers suggests that the GlyT2 binding pocket has conformational flexibility, which should be considered in inhibitor design strategies. Besides optimizing inhibitor design, compound 3 could be valuable for investigating optimal inhibitor levels for maximal analgesic effects and for understanding the mechanisms underlying adverse effects.

Despite the advances in the field of photoswitchable ligands for SLCs, in particular the ability to precisely differentiate and control active versus inactive conformational state is still in its infancy. In contrast, in GPCR pharmacology, photoswitchable molecules enable the study of receptor signaling pathways by reversibly toggling their active states [222]. Porting these sophisticated strategies to SLCs could unlock unprecedented capabilities for studying their dynamic conformational changes in real-time.

7 | Conclusions and Outlook

This review provides a comprehensive overview of the diverse array of small molecule probes employed in the study of SLCs since 2000, with a particular focus on fluorescent probes, covalent inhibitors, affinity-based probes, bivalent probes, PROTACs and photoswitchable inhibitors. Despite the progress made, there are still considerable challenges that need to be addressed. Future research should focus on expanding the SLC molecular toolbox, enhancing the selectivity and efficacy of these probes, and especially developing chemical probes for those SLCs without any probes reported so far. Among these, PROTACs and photoswitchable probes stand out as particularly compelling avenues for future research. It is worth noting that these approaches are relatively new in the context of SLC studies. Looking ahead, researchers may also consider investigating other chemical probes that have not been traditionally used in the context of SLCs, such as molecular glues for other means of targeted protein degradation such as lysosome targeting chimeras (LYTACs). All future probe developments would also stand to benefit from targeting the less explored SLC families instead of focusing solely on those that are already extensively investigated, such as the SLC6 family. Besides this, the use of structural information (either experimental through X-Ray or CryoEM, or AI-driven through AlphFold) could greatly enhance the capabilities of probe design [223]. Expanding probe diversity and enhancing specificity and efficacy will provide deeper insights into SLC biology and advance therapeutic innovations.

Author Contributions

Conceptualization: Majlen A. Dilweg, Adriaan P. IJzerman, and Daan van der Es. Investigation: Majlen A. Dilweg and Tracie Widjaja. Funding resources and supervision: Adriaan P. IJzerman and Daan van der Es. Writing – original draft: Majlen A. Dilweg and Tracie Widjaja. Writing – reviewing and editing: Majlen A. Dilweg, Adriaan P. IJzerman, and Daan van der Es.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The authors have nothing to report.

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