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Perspective

Inside job: ligand-receptor pharmacology beneath the plasma membrane

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Most drugs acting on the cell surface receptors are membrane permeable and thus able to engage their target proteins in different subcellular compartments. However, these drugs' effects on cell surface receptors have historically been studied on the plasma membrane alone. Increasing evidence suggests that small molecules may also modulate their targeted receptors through membrane trafficking or organelle-localized signaling inside the cell. These additional modes of interaction have been reported for functionally diverse ligands of GPCRs, ion channels, and transporters. Such intracellular drug-target engagements affect cell surface expression. Concurrent intracellular and cell surface signaling may also increase the complexity and therapeutic opportunities of small molecule modulation. Here we discuss examples of ligand-receptor interactions that are present in both intra- and extracellular sites, and the potential therapeutic opportunities presented by this phenomenon.

Keywords: ligand-receptor interaction; phamacological chaperone; endoplasmic reticulum; GPCR; ion channel; transporter

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Introduction

Generally, the activities of most endogenous receptor ligands or exogenous small molecule modulators have been studied at the plasma membrane, where they initiate signaling cascades along intracellular pathways. However, increasing evidence suggests that such interactions may also occur while the target receptor is inside the cell, either in nascent form in the endoplasmic reticulum (ER) or as a mature component residing on intracellular organelle membranes (Table 1).

A number of studies show that compounds may modulate the processing and trafficking efficiency of transmembrane proteins to the cell surface, acting as chemical 'chaperones' or 'pharmacoperones'[1–5]. This terminology is used to describe two classes of modulators.

The first are relatively nonspecific compounds such as dimethyl sulfoxide (DMSO), 4-phenylbutyrate (4-PBA), thapsigargin, and glycerol, which modulate the trafficking of diverse targets. Such nonspecific compounds may act through a number of mechanisms. Modulators of endoplasmic reticulum calcium, such as thapsigargin, may enhance activity of endogenous chaperones and allow exit of misfolded proteins from the ER[6]. Compounds such as 4-PBA may increase expression of heat shock proteins to aid export from the ER[7]. Finally, osmolytes such as glycerol may aid protein folding by solvating hydrophobic regions and preventing aggregation of partially-folded intermediates[8].

The second class of molecules termed 'pharmacological chaperones' are selective ligands, which promote the folding and expression of specific targeted proteins. While the first class resemble interactions of native chaperone molecules such as the heat shock proteins, the second presumably engage specific binding site(s) on their target(s). In this perspective we have chosen to distinguish these two classes of small molecule chaperones as 'general' and 'specific'. Independently from these trafficking effects, receptors including dopaminergic D2/D3 or serotonin 5-HT_{2A} receptors may be endogenously targeted to organelles in addition to the cell surface, where they may have functional sub-populations and unique signaling roles on intracellular membranes[9, 10].

We speculate that these additional sites of action may help explain the complex therapeutic mechanisms of known drugs[5], or offer potential treatments for pathological defects in trafficking or processing such as long QT type 2 (LQT2) and cystic fibrosis (CF)[11–17]. In this perspective we discuss examples that highlight the intracellular activities of ligands for three major drug target classes on the plasma membrane: G-protein coupled receptors (GPCRs), ion channels, and transporters (Figure 1). Illustrative examples for each group

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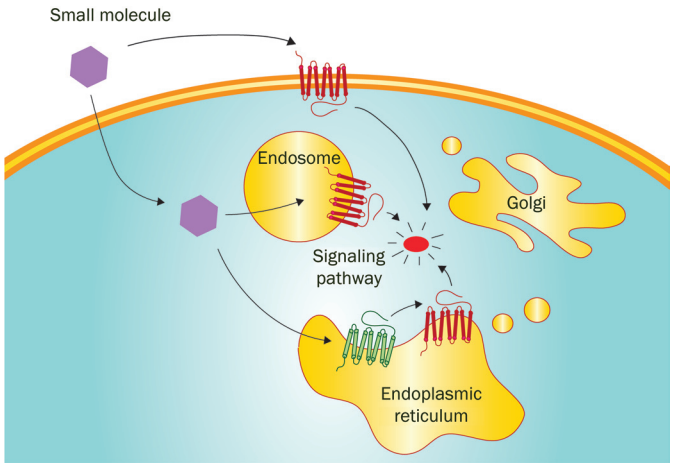

Figure 1. Intracellular drug mechanisms. Hypothetical modes of action for a GPCR ligand at the cell surface, in the trafficking pathway, and at intracellular organelle membranes.

including disease associations and relevant ligands are summarized in Table 1.

G-protein coupled receptors

A classic example of a pharmacological chaperone capable of restoring surface expression of misfolded receptors is the vasopressin 2 receptor (V2R) antagonist SR121463A[18]. This compound was found to rescue surface expression of trafficking-deficient V2R mutants, while a membrane-impermeable antagonist lacked this effect[18]. This observation was accompanied by a decrease in ER-retained V2R and increased production of full-length glycosylated receptors that are presumably expressed on the cell surface[18]. Besides SR12463A and antagonist SR121463B, less specific compounds such as glycerol, DMSO, thapsigargin, curcumin and ionomycin can also rescue some V2R mutants[19]. However, the spectrum and magnitude of activity appears to be broader for specific compounds[19]. As ~90% of documented V2R mutations linked to diseases such

Table 1. Examples of intracellular ligand-receptor targets, molecules, and mechanisms.

Class	Target	Ligands	Disease links	Reference(s)
GPCRs	DRD2-4	Quinpirole, haloperidol, butaclamol, clozapine, domperidone	Attention-deficit hyperactivity disorder	[25]
	5-HT_{2A}	SR46349B	Schizophrenia	[31]
	A1	DCPX, IBMX	NA	[30]
	GnRHR	Indoles, quinolones, erythromycin derivatives	Congenital hypogonadotropic hypogonadism	[35, 130, 131]
	Rhodopsin	β-Ionone	Retinosa pigmentosa	[39]
	δ-opioid	Naltrexone	Analgesia/pain	[37]
	μ-opioid	Naloxone, etorphine	Analgesia/pain	[36]
	hMCHR1	NBI-A	Anxiety, feeding	[34]
	MC4R	ML00253764	Obesity	[29]
	VR	SR49059, SSR149415, SR121463A, B, thapsigargin, glycerol, DMSO, curcumin, ionomycin	Nephrogenic diabetes insipidus	[18, 19, 132–4]
	S1P1	Fingolimod	Multiple sclerosis	[49]
Ion channels	nAChR	Nicotine, cytosine, dihydro-β-erythroidine	Neuroprotection in Parkinson's	[1, 5]
	hERG	Celastrol, E4031, thapsigargin, fluconazole, fluoxetine, ketoconazole, pentamidine, probucol, cardiac glycosides, astemizole, cisapride	Long QT Syndrome 2	[11, 13, 54–6, 58–61, 70]
	KCNQ2	Retigabine	Benign neonatal familial convulsions	[69]
	CFTR	Quinazolines, thapsigargin	Cystic fibrosis	[12, 63, 76]
	K_{ATP}	Tolbutamide, glibenclamide, repaglinide,	Congential hyperinsulinism	[84]
Transporters	ABCA1,3	4-PBA, thapsigargin	Cardiovascular disease, respiratory distress syndrome, tangier disease	[64, 95, 101]
	ABCD1,2	4-PBA	X-linked adrenoleukodystrophy	[103]
	ABCB1,4	Glycerol, cyclosporin A 4-PBA	Progressive familial intrahepatic cholestasis type 3	[105]
	ABCC6		Dystrophic mineralization	[107]
	ABCG2,5,8	Mitoxantrone, tauroursodeoxycholate	Gout	[108–10]
	SERT	Ibogaine	NA	[111, 112]
	ATP7B	4-PBA, curcumin	Wilson's disease	[115]
	MNK	Copper, glycerol	Menke's disease	[114]
	hABST	Cyclosporin A	Cholesterol transport	[113]

as nephrogenic diabetes insipidus (NDI) cause ER membrane retention amenable to this manner of correction[20, 21], this specific pharmacological chaperone was proposed as a novel prospective therapeutic[18].

Similar pharmacological rescue of trafficking deficiency has also been documented in other GPCRs, suggesting this activity may represent a potential general strategy for diverse pathologies. Indeed, an estimated 65% of the more than 600 documented GPCR mutations are missense mutations[22], of which >80% are estimated to affect receptor folding in the ER[23, 24]. Assuming equal propensity for disease mutations among GPCRs, approximately half of all GPCR mutations may cause such localization defects. As previously indicated, the activity and specificity profiles of compounds rescuing such mutants may also be diverse. This is the case for trafficking-deficient D4-class dopamine receptors, which are rescued by specific chaperones including antagonists (such as domperidone), agonists (*eg*, quinpirole), or even endogenous ligands such as dopamine itself, independent of effects on translation or transcription[25]. Unlike similar effects observed for general chaperone compounds such as glycerol and DMSO[26, 27], which rescue both D4 and CFTR trafficking mutants, these examples appear to be specific for the dopamine receptor[25]. Further, the observation that brefeldin A blockade of Golgi-ER vesicle traffic may be reversed by these ligands suggests that these compounds may play a role in facilitating proper folding of the immature receptor, rather than later steps, in which they might instead promote trafficking of the fully glycosylated, mature protein through the Golgi machinery[25]. Pharmacological chaperone activity also appears to rescue D4 receptors from proteasomal as opposed to lysosomal degradation, as suggested by the differential effects of lactacystin and chloroquine treatment on receptor levels in the ER membrane[25], results correlated with similar studies of the V2R receptor[28]. However, it is not clear whether this is a universal pattern for proteins rescued by such ligands. As variants in D4 have been linked to conditions such as attention deficit hyperactivity disorder (ADHD), modulation of receptor surface expression may offer a therapeutic pathway[25]. Intriguingly, the trafficking of the wild type dopamine receptor to the cell surface is also amplified by both specific and general pharmacological chaperones, an observation that may correlate with the relatively inefficient endogenous processing of this receptor leaving a large, latent pool of functional D4 primed in the ER membrane for export[25]. Further evidence is offered by the pro-trafficking effects of antagonist ML00253764 on both wild type and mutant melanacortin 4 receptors, which are also inefficiently processed endogenously[29]. However, in cases such as the adenosine 1 (A1) receptor, the wild-type protein is unaffected by the specific pharmacological chaperones 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) and isobutylmethylxanthine (IBMX), suggesting this effect may vary with the natural efficiency of the protein's transport to the cell surface[30] and site(s) of drug-target interaction(s).

Another common antipsychotic drug target, the 5-HT serotonin receptor, has also been shown to be up-regulated in response to treatment with antagonist SR46349B[31], though it is unclear whether this effect is due to modulation of trafficking or transcription/translation. Similarly, multiple point mutations in the melanin-concentrating hormone receptor 1 (hMCHR1) (whose signaling is associated with stress response[32] and metabolism[33]) leading to impaired trafficking may be rescued by the antagonist NBI-A[34]. The gonadotropin-releasing hormone receptor (GnRHR), for which ~90% of inactivating mutations causing hypogonadotropic hypogonadism are due to impaired trafficking, may also be functionally rescued with diverse compound classes such as indoles, quinolones, thienopyrimidinediones, and erythromycin-derived macrolides[35].

Experiments with the rat μ-opioid receptor have demonstrated pharmacological rescue of deletion mutants of transmembrane and carboxyl-terminal motifs using the specific agonist etorphine and antagonist naloxone[36]. Likewise, pain and analgesia associated δ-opioid receptors have demonstrated similar enhanced trafficking through interaction with naltrexone[37]. Because these receptors form heterodimers, mutants can function as 'dominant negatives' to retain wild type receptors in the ER through protein-protein interactions[37], allowing small molecule chaperones to rescue both wild type and mutant trafficking. In related studies, higher order-oligomerization of α_{1b}-adenoreceptors appears to be conserved in some trafficking-deficient mutants of transmembrane segment I, suggesting that such mutations can alter surface expression without effecting intermolecular interactions between receptors retained in the ER/Golgi[38]. The potential for systematic discovery of specific small molecule chaperones has been demonstrated for rhodopsin, for which high-throughput *in silico* docking was combined with *in vitro* competitive binding studies to discover potential therapeutic compounds for retinosa pigementosa (RP) such as β-ionone[39].

Intriguingly, dopamine, serotonin, adrenergic, and thrombin receptors have also been identified in the ER membrane[40–45], with D2 receptors appearing to be capable of activating G-protein signaling while retained in the ER and this expression correlating with increased vacuolization[46]. In related studies, dopamine receptors have been identified in endosomes through immunohistochemical analysis of the rat cerebral cortex and hippocampus[47]. As organelle membranes such as endosomes have been proposed as alternative signaling 'platforms' distinct from the plasma membrane[48], these observations are suggestive of additional opportunities for tuning the activity of these receptors beyond biogenesis. For instance, the immunomodulator drug fingolimod (FTY720), a therapeutic for multiple sclerosis, promotes internalization of the sphingosine-1-phosphate receptor 1 (S1P1) as well as persistent signaling via extracellular-signal-regulated kinase(ERK) and adenylyl cyclase from endosomes[49]. Similarly, experiments with chemical blockers of endocytosis have revealed that thyroid stimulating hormone (TSH) receptor signaling appears to require internalization, suggesting that non-endogenous ligands of the TSH receptor might also promote intracellular activity[50].

Ion channels

Therapeutic modulation of intracellular receptor activity via ligands active at the cell surface pertains not just to disease targets, but also to anti-targets linked to drug side effects. An illustrative example is provided by the human ether-a-go-go related (hERG) potassium channel, a frequent target of promiscuous inhibition by small molecules and drug-induced cardiac arrhythmias, for which genetic mutations are linked to long QT syndrome type 2 (LQT2)[51]. Like the GPCRs described above, diverse LQT2 mutations have been documented, which have detrimental effects on surface expression of hERG[11, 13–16, 52, 53]. Intriguingly, these studies also determined that hERG inhibitors such as E4031, astemizole, and cisapride, while potential causes of drug-induced LQT2 through blockade of channel current across the plasma membrane, could also rescue surface expression of LQT2 mutant channels by potentiating biogenesis of the mature, glycosylated protein[11, 13]. In addition to these pharmacological chaperones, many hERG blockers also appear to inhibit trafficking. These effects, like chemical inhibition of hERG channel conductance, appears to be promiscuous, with evidence that acute blockers such as the antifungal fluconazole[54], the Chinese thunder god vine component celastrol[55], as well as fluoxetine[56, 57], and ketoconazole[58], also inhibit trafficking of the wild-type channel. However other molecules, such as pentamidine[59], and probucol[60] and cardiac glycosides including digitoxin[61] inhibit trafficking without acute effects on current density. Similarly, the sarcoplasmic/endoplasmic recticulum calcium ATPase (SERCA) inhibitor thapsigargin can rescue trafficking without effects on current[62], though the mechanism may be not be specific to hERG as this compound also rescues the trafficking of CFTR channels[63] and the transporter ABCA1[64]. Intriguingly, other SERCA inhibitors cannot rescue these hERG mutants, suggesting thapsigargin might have broad activity to modulate trafficking for particular protein classes[62]. While the inhibitory activity of the above ligands on current or trafficking limits their therapeutic application, these observations raise the possibility that activators or 'silent', functionally inactive ligands might find value as chemical rescuers of hERG trafficking. Computational studies of estrogen receptor ligands and modulators of translation initiated at the internal ribosome entry site (IRES) of the encephalomyocarditis virus (EMCV) have identified inactive molecular series that are rendered active through small changes in chemical functionalization[65, 66]. While these studies did not biochemically confirm whether the inactive series bind their target, such results provide support for identification of 'silent' modulators.

Another voltage-gated potassium channel, KCNQ2, has been linked to conductive disorders such as neonatal seizures, which might be corrected with chemical openers[67, 68]. While it had been assumed to act only by shifting the voltage activation curve of the channel, recent evidence has also suggested the antiepileptic retigabine (RTG) may function as a specific pharmacological chaperone[69]. Incubation with RTG has been shown to correct processing of a folding-defective mutant linked to neonatal seizures, independent of effects on the open-probability of the channel, as demonstrated by increased current density even following RTG washout[69]. While molecular determinants of RTG have been linked to the channel pore[70–74], direct evidence of compound-ligand interaction is unavailable. Given the availability of many functionally distinct KCNQ2 activators[75], there exists the possibility that other pharmacological chaperones remain to be characterized for this channel.

A similar example of this phenomenon among channel proteins is the cystic fibrosis transmembrane conductance regulator (CFTR), for which ~90% of North American cases result from an in-frame deletion (ΔF508) that causes defects in trafficking, membrane half-life, and gating[12, 17]. Thus, correction of CFTR processing for this high-frequency mutation has therapeutic potential for a large patient population. High-throughput screening has been utilized to discover small molecule modulators using the response of membrane potential or halide-sensitive fluorescence dyes as a proxy for activity of the channel[12, 76]. Among other chemical families, these studies discovered a class of quinazolinones that increase the population of core-glycosylated CFTR mutant proteins and surface expression, without modulating either ubiquitin-proteasome activity or transcription levels, suggesting these specific chaperones might increase the efficiency of ER processing[12, 76]. Later studies utilized crosslinking assays and *in vitro* reconstitution of the protein's ATPase activity to verify that these 'corrector' compound activities are indeed due to direct intracellular interactions[77, 78]. Additional evidence suggests these compounds also stabilize CFTR on the cell surface, rather than purely acting as modulators of ER processing[76, 79]. Supportive for the direct chemical-CFTR interaction, the identified quinazoline series promote surface expression and at high concentrations they also appear to inhibit CFTR conductance[80]. Conversely, other series can enhance the chloride current[76]. While the quinazoline series described above and the corrector phenol VRT-532 remain the only chemicals with evidence of direct physical intracellular association with CFTR, other compound classes with similar functional phenotypes await more detailed biochemical characterization[78, 81]. Further, the recent FDA approval of a CFTR potentiator (ivacaftor) as a treatment for conductance mutants comprising 5% of CFTR cases suggests the possibility of combination therapy promoting conductance and proper trafficking of the channel[82].

Like the V2R receptor, five aquaporin-2 (AQP2) mutants associated with X-linked diabetes insipidus may be rescued by treatment with general chemical chaperones such as glycerol, trimethylamine N-oxide (TMAO), or DMSO when expressed in *Xenopus* oocytes or Chinese Hamster Ovary (CHO) cells[27]. However, the lack of selective aquaporin modulators[83] places limits on potential therapeutic interventions to date. An example of endogenous intracellular ligand-channel interaction is presented by the nicotinic acetylcholine receptor (nAChR). Experimental evidence suggests that nicotine interactions with the receptor inside the cell promote trafficking to the cell surface[2–4] and reduce ER stress, as judged by attenuated acti-

vating transcription factor 6(ATF6) translocation during the unfolded protein response (UPR)[1]. This effect may underlie the protective effect of smoking in neurodegenerative conditions such as Parkinson's, which are characterized by a loss of nAChR activity in the central nervous system[1]. Thus, synthetic ligands may also be useful probes of this intracellular nAChR activity. An additional level of complexity is revealed by studies of pharmacological chaperoning of the K_{ATP} complex of Kir6.2 and sulfonylurea receptor 1 (SUR1)[84], which is expressed as a 4:4 receptor:channel octamer in pancreatic β cells[85–87]. Mutations in the transmembrane domains of SUR1 reduces trafficking of the receptor from the ER to the cell surface, but may be rescued by both sulfonylurea and glinide compounds[84]. The observation that pharmacological rescue of trafficking-deficient SUR1 or Kir6.2 mutants requires co-expression of both components of the K_{ATP} complex suggests that the entire channel complex, rather than either individual subunit, is the target of these small molecules[84].

Like GPCRs, channels may also have functional populations at intracellular sites that complicate potential therapeutics. For example, both wild type and ΔF508-CFTR have been shown to conduct chloride currents across ER membranes as measured by patch clamp of isolated nuclear membranes from CFTR-expressing CHO cells[88]. CFTR intracellular localization is also associated with endosome fusion[89] and regulation of lysosomal pH[90], and thus intracellular modulators may have specialized functional effects within inside the cell. Concurrently, these alternative functional roles may indicate that chemical therapeutics for CFTR, like other receptors with cell surface and intracellular roles, may ameliorate defects across multiple pathways within the cell. Recent evidence has further identified ion channels expressed both on the cell surface and functionally in intracellular compartments such as endosomes. For example, TRPML3 and TRPML2 channels may be expressed at both the plasma membrane as well as membranes of intracellular compartments[91]. Recently identified small molecule modulators for these channels may be useful to dissect their functional roles at these sites[91]. The identification of increased lysosomal lipid inclusions from knocking down these channels further suggests that chemical modulators may influence not only the receptor itself, but downstream functionality of the organelle in which they are found[92]. Similarly, the 2-pore domain channel KCNK9 has been reported in mitochondrial membranes[93] as well as the cell surface functions, for which reported small molecule inhibitors[94] may be useful probes.

Transporters

Like GPCRs and ion channels, transporters regulate important physiological processes at both intracellular sites and the plasma membrane, functions which may be disrupted by mutations causing improper folding or erroneous trafficking. In many cases, however, the primary sites of localization for transporters are on intracellular membranes, with trafficking to organelles being the target of chemical modulation. In the case of the ATP-binding cassette transporter A1 (ABCA1), the transporter is an important regulator of high-density lipoprotein cholesterol (HDL-C) formation by exporting lipid to cell-surface apoA-I lipoproteins during the biogenesis of HDL particles[95]. Over 150 mutations in ABCA1 have been documented[96], many leading to mutants correlated with the cholesterol transport and cardiovascular disorder Tangier Disease[97–99], through reduced protein expression and retention in the ER[100]. Experiments employing both heterologous expression systems and fibroblasts derived from HDL-deficient patients demonstrate the general chemical chaperone 4-PBA may correct intracellular retention of ABCA1 mutants, but boost expression of the protein in the heterologous system only[95]. In the same gene family, the ABCA3 transporter is localized not to the plasma membrane, but to lysosomes and lamellar bodies in lung cells where it mediates secretion of surfactant lipids such as phosphatidylcholine, sphingomyelin, and cholesterol. Mutations are associated with respiratory distress syndrome (RDS), and cellular pathologies include lack of conversion of lysosomes to lamellar-like bodies that store lipids[101]. This failure in lamellar body formation may be rescued with 4-PBA in cultured lung and HEK293 cells[101]. A similar deficiency in trafficking to predominantly intracellular sites is observed for the lipid transporter ABCD1, for which mutations in the ATPase domain[102] block peroxisome proliferation and are associated with X-linked adrenoleukodystrophy (X-ALD)[103]. These deficiencies may be rescued by 4-PBA induction of ABCD2 expression in X-ALD patient-derived fibroblasts, though it is unclear whether this results from increased trafficking to the peroxisome or other pleiotropic effects via gene expression[103].

Unlike the ABCA transporters, the multidrug resistance (MDR)/ABCB family facilitates the passage of drugs in addition to lipid substrates[104]. Despite this functional distinction, trafficking-deficient ABCB1 mutants may also be pharmacologically rescued[105]. The efficacy of rescue varies for specific and general small molecule chaperones, with cyclosporin A demonstrating greater effect than glycerol[105]. Conversely, neither heat shock protein 70 (Hsp70) overexpression nor thapsigargin treatment affects mutant ABCB1 trafficking[105]. As identical mutations in closely related ABCB4 have been identified in progressive familial intrahepatic cholestasis type 3, data from ABCB1 indicates a possible therapeutic strategy for this disorder[105]. ABCC6, another liver-localized transporter[106], has unknown substrates but its mutation is associated pathologically with deposition and accumulation of minerals within tissues, with several mutants being improperly retained in the ER and restored by 4-PBA treatment[107]. Like ABCB transporters, ABCG2 plays an important role in excretion, including regulating intracellular drug concentration[108]. The transporter substrate mitoxantrone (MX) has also been shown to serve as a pharmacological chaperone for dimerization-deficient mutants which are normally impaired for trafficking to the cell surface[108]. Trafficking-deficient mutants have been further identified in regions of the protein not associated with dimerization, alterations which are genetically linked to gout[109]. Two additional transporters in the same gene

family, ABCG5/8, form heterodimers involved in sterol secretion which have been reported to be downregulated at the cell surface in mouse models of leptin deficiency, a defect rescued by treatment with tauroursodeoxycholate[110].

In the case of the serotonin transporter (SERT) [a member of the solute carrier (SLC) family], mutants in the carboxyl-terminal binding motif are impaired for ligand binding as quantified by a radiolabeled-imipramine displacement assay, and are partially rescued by both DMSO and ibogaine, but not other substrates of the protein[111]. The binding of ibogaine to the cytoplasmic face of the transporter[112], unlike other agonists, suggests that specific binding sites of receptors may be more amenable than others to trafficking modulation and rescue. Additionally, these data highlight the role that C-terminal motifs may play in proper protein folding. Indeed, C-terminal mutations of SERT that disrupt folding also interact with vesicular component Sec24, indicating potential pleiotropic effects of trafficking-deficient mutants[111]. Trafficking-deficient mutations in the first transmembrane segment of another SLC family member, the human apical sodium-dependent bile transporter (hABST, SLC10A2), may also be pharmacologically rescued with MG132 or cyclosporin[113].

Two diseases of copper transport (Wilson's and Menkes disease) are associated with trafficking-deficient mutations in copper transporter proteins[114, 115]. In the absence of its substrate, the copper-transporting P-type ATPase MNK is localized to the *trans*-Golgi complex; sharp increases in copper concentration promote its trafficking to the plasma membrane[114]. A conditional mutant in the large cytoplasmic loop of MNK is associated with Menkes disease, and causes the transporter to be retained in the ER in the absence of copper[114]. However, this mislocalization may be corrected by glycerol or copper itself, suggesting copper supplementation as a possible treatment for Menkes[114]. Similarly, mutations in multiple structural domains of the copper transporter, ATP7B, are implicated in deficient copper extrusion by the liver and basal ganglia in Wilson's disease[115]. Many of these mutations impair proper folding and localization of the protein to the *trans*-Golgi complex, but their improper retention in the ER may be corrected with either 4-PBA or curcumin treatment[115].

Besides trafficking, transporters, like GPCRs and channels, may also be dually localized to both the plasma membrane and intracellular compartments where they have distinct signaling roles. For instance, treatment of CHO cells with the vacuolar ATPase inhibitor bafilomycin A reduces transferrin receptor trafficking[116] by interfering with the function of the vacuolar transporter in the endosomal compartment[117]. Similarly, both concanamycin and bafilomycin have been found to inhibit influenza virus entry into cells by impairing acidification of vacuoles through transporter inhibition[118]. In osteoclasts, however, cell surface activity of the vacuolar ATPase may be implicated in bone reabsorption conditional on N-terminal interactions between subunits, as this reabsorption is blocked by a benzohydrazide derivative which antagonizes this interaction[119].

Therapeutic implications

Ligand-receptor interactions at both the plasma membrane and intracellular sites suggest a number of intriguing therapeutic possibilities. For example, drug synergies (achieved by combined treatment with trafficking and activity modulators) might offer complementary efficacy or effect targets inaccessible with single compound treatments. In fact this synergy may have already been achieved and unappreciated for current medications, since most therapeutic treatments involve administration of drugs for days, not the seconds or minutes in which most cell-based assays are quantified. The timespans of typical drug administration allow both the acute effects typically measured by functional assays of cell surface targets as well as chronic modulation of intracellularly retained or localized proteins. Additionally, the rescue of trafficking-deficient mutants by ligands which impair the functionality of their target presents a conundrum, as increased surface expression may be offset by functional antagonism. Thus, 'silent' modulators that bind their target without functional effect may find utility as modulators of trafficking, and suggest high-throughput screening for such chemicals. Intriguingly, chronic application of such compounds may have medicinal benefits, despite their seeming inertness in acute activity measurements. Alternatively, if the potency for acute and chronic compound effects is sufficiently different, an appropriate dosage window might be defined to avoid functional antagonism while preserving beneficial effects on trafficking. This window may be influenced by the intracellular accumulation of drugs, such as through inhibition of P-glycoprotein transporters[120–122], which may lead to chronic doses that are far higher concentrations than acute levels to which the cell is initially exposed.

In cases where receptors are functionally present at both the plasma membrane and intracellular sites, optimization of subcellular localization profiles of small molecule modulators, perhaps through conjugation with polymers[123], toxins[124], or lipids[124] directed to particular organelles, may allow fine tuning of their therapeutic phenotype through a balance of extra- and intracellular actions. Finally, the existence of these intracellular chemical activities argues for more systematic exploration of such effects among existing drugs, for possible therapeutic repurposing or to discover mechanistic explanations of chronic physiological side effects.

Perspective

While the concept of pharmacological chaperones and intracellular ligand-receptor activities has been well-established for many of the therapeutic targets and target classes described above, in other cases these activities are just beginning to be appreciated. The field will benefit from more systematic evaluation of these activities, using platforms that concurrently evaluate chronic (such as trafficking and intracellular effects) as well as acute actions of molecules in a comprehensive fashion. High-content imaging assays, allowing visualization of both receptor localization and downstream readouts such as

ion flux may offer opportunities for such multiplexed analysis[125]. Another is offered by approaches such as chemobleaching, which functionally inactivates surface receptors to allow chemical effects on trafficking to be quantified independently of acute effects[126]. Further, improved understanding of the biophysical interactions by which ligands stabilize their target receptors during biogenesis may also open new doors for rational drug design in this field. As discussed, it is clear that different drug binding sites could lead to differential rescue efficacy. Some clues have emerged from studies in which specific pharmacological chaperones have been co-crystallized with phenylalanine hydroxylase and β-glucosidase[127, 128]. These data suggest that the ligands may stabilize a particular conformation of the target protein that is optimal for export, and thus improve their processing through the ER. The observation that ligand binding sites are often located at subdomain interfaces may indicate that chemical interactions in these regions are generally conducive to stabilization of macromolecular structure[129].

More broadly, the realization that ligands may interact with receptors not just at the cell surface, but at many points along their maturation process and at distinct intra- and extracellular sites extends the concept of polypharmacology and 'network medicine' to not just interactions among diverse proteins, but complex processes involving a single target. Many drugs are being taken by patients for an extended period of time, thus making more likely to induce intracellular pharmacology in addition to the cell surface pharmacology. Further knowledge of all sites of contact between receptors and their corresponding ligands will help to expand both the functional nuance and potential impact of future medicines.

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