Journal of Molecular Recognition

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Journal of Molecular Recognition

REVIEW OPEN ACCESS

Toward Understanding the Mechanism of Client-Selective Small Molecule Inhibitors of the Sec61 Translocon

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Received: 28 June 2024 | **Revised:** 28 August 2024 | **Accepted:** 27 September 2024

Funding: This work was supported by Deutsche Forschungsgemeinschaft.

ABSTRACT

The Sec61 translocon mediates the translocation of numerous, newly synthesized precursor proteins into the lumen of the endoplasmic reticulum or their integration into its membrane. Recently, structural biology revealed conformations of idle or substrate-engaged Sec61, and likewise its interactions with the accessory membrane proteins Sec62, Sec63, and TRAP, respectively. Several natural and synthetic small molecules have been shown to block Sec61-mediated protein translocation. Since this is a key step in protein biogenesis, broad inhibition is generally cytotoxic, which may be problematic for a putative drug target. Interestingly, several compounds exhibit client-selective modes of action, such that only translocation of certain precursor proteins was affected. Here, we discuss recent advances of structural biology, molecular modelling, and molecular screening that aim to use Sec61 as feasible drug target.

1 | The Role of Sec61 in Protein Translocation

In eukaryotes, a large number of precursors of polypeptides and secretory proteins with amino-terminal signal peptides (SPs) are translocated across the endoplasmic reticulum (ER) membrane or, when instead possessing transmembrane helices (TMHs), are integrated into its membrane [1, 2]. In total, ER protein import applies to about 30% of the proteome [5, 6]. Hence, this is the first step in the biosynthesis of precursors for a large number of soluble proteins and membrane proteins in secretory pathway compartments, the plasma membrane, and outside the cell [3–5, 7]. This translocation and membrane insertion of nascent peptide chains is mostly mediated by a dynamic protein-conducting channel, namely the heterotrimeric Sec61 complex located in the eukaryotic ER membrane or SecY in the plasma membrane of prokaryotes [1, 2]. Sec61 is a hetrotrimeric complex that comprises three subunits, Sec61α (Sec61p in *Saccharomyces cerevisiae* and SecY in bacteria and archaea), Sec61β (Sbh in *S. cerevisiae*, Secβ in archaea), and Sec61γ (Sss1p in *S. cerevisiae*, SecE in bacteria and archaea). Cross-linking experiments provided evidence that Sec61 is a protein-conducting channel [8]. The first structures of the Sec channel consolidated much of the biochemical information and showed that the nascent inserting polypeptide passes through the central channel of the translocon [55]. When passaging across the membrane, the nascent polypeptide chain is surrounded by the α-subunit of Sec61 complex [8]. For less competent SPs, a driving force needs to be provided by associated protein partners for the translocation process to occur via the passive Sec61 pore [1].

There exist two different Sec-dependent translocation modes that is, co- and post-translational translocation, depending on the associated partner, respectively, see Figure 1 [1, 9]. Co-translational translocation is an intricate ribosome-dependent process applying to the integration of most transmembrane (TM) proteins into the ER membrane. This process begins with a targeting phase, where the SP emerging from the ribosome is recognised by the cytosolic signal recognition particle (SRP) [10–12] and SRP binding induces translational slowdown [14–17]. The ribosome-nascent chain complex, with the help of SRP, is then directed to the ER membrane via the binding

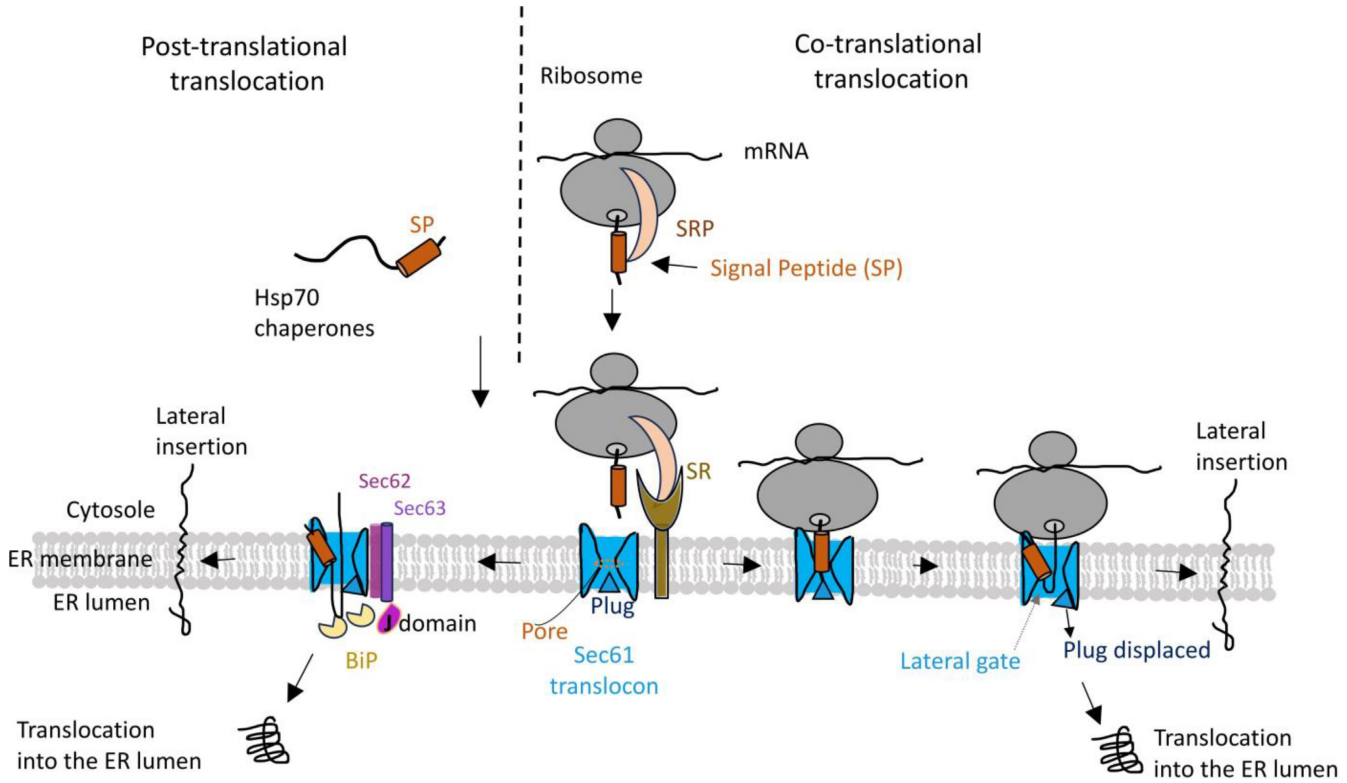

FIGURE 1 | Schematic representation of the two translocation modes of a secretory protein via the Sec61 translocon in mammals. Co-translational translocation (right) is SRP-dependent and involves the binding of the particle signal recognition protein (SRP) to the ribosome carrying a nascent chain signal sequence (SP or TMH). Subsequently, this ribosome-nascent chain-SRP complex docks to a membrane receptor named SR, and SRP dissociates from the ribosome. Then, the signal peptide can insert into Sec61 as a result of altered conformational dynamics of Sec61. Post-translational translocation (left) is an SRP independent pathway where the binding of a completely synthesized polypeptide chain with low hydrophobicity SP to the Sec61 complex along with Sec62/Sec63 membrane proteins is facilitated by the chaperones BiP/Grp78. Their binding to the polypeptide assists its translocation to happen in a net forward direction. Thereof the signal peptidase is cleaved off the signal peptide followed by folding and N-glycosylation of the translocated protein. In fungi, the cytosolic face of Sec63 is bound to two additional proteins, termed Sec71 and Sec72.

of SRP to its SRP receptor (SR) [12, 13], which is present on the ER surface. After the ribosome binds to the cytosolic face of Sec61, SRP is released and translation can proceed [18]. The recognition and then penetration of the polypeptide chain into the Sec61α pore either lead to lateral insertion into the membrane or to translocation into the ER lumen (Figure 1). SRP not only targets the nascent polypeptide to the ER, but may also favor the targeting of RNCs having high binding affinity for SRP [19, 20].

Post-translational translocation takes place after translation is completed, and usually applies to nascent chains with low hydrophobicity SPs that are not recognized by SRP. However, Lakkaraju et al. reported that in mammalian cells the length of the polypeptide chain can also affect signal recognition. Post-translational translocation also occurs for short precursor proteins comprising ≤~100 amino acid residues and strongly depends on Sec62 for efficient translocation. For example, Sec62 played an important role in promoting the post-translational translocation of preproinsulin [21]. This mechanism is thus SRP-independent [7, 9, 22]. In this case, translocation of the completely synthesized polypeptide is supported by chaperones as shown schematically in Figure 1 [7, 9, 22]. Likewise, prokaryotes commonly use post-translational translocation for soluble proteins. In eukaryotes, along with Sec61, two other essential integral membrane proteins, Sec62 and Sec63, and the lumenal chaperone immunoglobulin heavy chain binding protein (BiP), which is a member of the Hsp70 ATPase family, contribute to post-translational translocation [23]. The J domain of Sec63 activates BiP binding to the translocating polypeptide, thus preventing the peptide from sliding back into the cell cytosol (Figure 1). Therefore, translocation occurs in a net forward direction. In fact BiP acts via a molecular ratcheting mechanism that provides the driving force for this pathway. The net forward direction is also driven by folding and posttranslational modification in the ER lumen [24]. In fungi, the cytosolic side of Sec63 is coordinated to the additional nonessential proteins, Sec71 and Sec72 [1, 25, 26].

Protein translocation and membrane insertion depend on the nature of the targeting SPs or alternatively, the first TM helix of the TM proteins. SPs possess a three-domain structure, a positively charged N-terminal domain (or "N-region"), a central hydrophobic region ("H-region"), and a short polar C-terminal region ("C-region") [27]. SPs are typically 12–30 amino acid residues long and usually show very variable sequence similarity to each other [28]. The signal sequences of soluble proteins are cleaved off once translocated across the membrane by either one of two ER-membrane embedded enzymes termed signal peptidases [29–31].

Subsequent to ribosome priming of Sec61 that is by binding of ribosome to its cytosolic loops (between TMH 6/7 and 8/9), SPs of nascent presecretory polypeptides and TMHs both with "strong" amino-termini can gate the Sec61 channel by themselves without need for any further assistance. As will be explained below, the targeting peptide should be hydrophobic enough to displace the plug and/or intercalate between the gating TMHs of Sec61α to open the lateral gate. Polypeptide precursors with "weak" SPs and/or TMHs are incapable of translocating on their own and need extra help of auxiliary factors, such as the heterotetrameric translocon-associated protein (or TRAP) complex or the translocating chain-associated membrane protein (TRAM), or of the Sec62/Sec63 complex with BiP (Figure 2). The notion of being "strong" or "weak" mostly refers to the hydrophobic strength of the targeting signal that is crucial for their translocation via the channel, or for lateral membrane insertion. Experimental data for TRAP-dependent clients from human suggested that weak α-helical propensity for example due to a rather high glycine and proline content may be another property of "weak" SPs [32]. The oligosaccharyl-transferase (OST) complex is located in proximity of Sec61 and is responsible for the catalysis of co-translational N-glycosylation of substrates [33]. These accessory factors are Sec61 interaction partners and are thought to assist or facilitate the biosynthesis of different subsets of substrates at the ER [30, 34, 35]. After a nascent peptide chain is transferred to Sec61, its SP or TMH engages with the Sec61 core via its N-terminal head that is in a "head-on" or loop insertion. The orientation of SP and TMH in the Sec61 channel follows the positive inside rule according to which positively charged amino acid residues in the N-region favor loop insertion (N_{in}-C_{out}) whereas positively charged side chains downstream of the SP or TMH support "head-on" insertion [36–40, 152]. To allow the nascent chain to enter the ER lumen while the SP remains in the membrane segment, a subsequent inversion of the SP orientation is required ("flip-turn") [40]. "Strong" SPs with very hydrophobic H-regions are capable of re-orienting themselves to open/gate the channel pore. In contrast, SPs with lower hydrophobicity require the help of auxiliary components to facilitate inversion and Sec61 channel gating [36]. These allosteric effectors are believed to affect the energetics of the Sec61 channel gating by reducing the energy barrier for full opening of the channel [7]. In vitro experiments suggested that TRAP and BiP facilitate the channel opening in a substrate-specific way by aiding translocation of weakly gating precursors [41–43]. Schorr and co-workers revealed the role of human Sec62/63 together with BiP in facilitating the co-translational translocation of proteins with long and weak SPs [44]. Recently, a single particle cryo-EM study revealed the interaction of mammalian TRAP with the translating ribosome and the associated Sec61 complex [45]. The full aspects of how TRAP functions in protein translocation and ER stress still need to be determined [46, 72, 108, 166, 167].

The closed or inactive translocon complex is primed by ribosome binding to cytosolic loops 6 and 8 of Sec61α. The N-terminus of Sec61γ then presents a hydrophobic patch of residues in the cytosolic funnel, which is the interaction site for the hydrophobic H-region of the SP. Multiple side chains of Sec61 residues form a pore constriction zone surrounding the translocating chain [47]. Photo-crosslinking experiments showed that the SP recognition and insertion site is located at the interface of the lateral gate helices towards the cytosolic face of the translocon [48, 49]. When the translocation process is completed, the Sec61 channel becomes leaky for Ca^{2+} ions, what has been linked to pathological situations [43, 50, 131].

Additional components such as the ER membrane protein complex (EMC), PAT, and TMCO1 complexes have been discovered, which are thought to play important roles in the

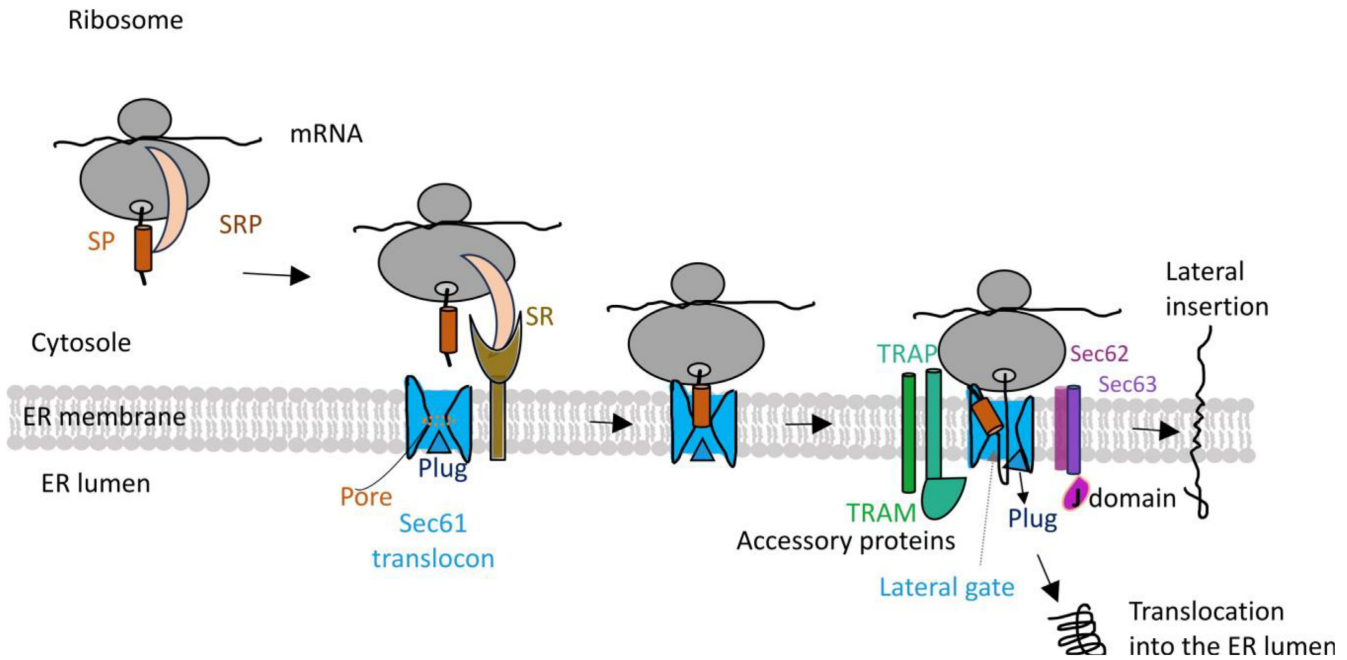

FIGURE 2 | Schematic diagram showing the co-translational translocation of a "weak" signal peptide, which needs extra help from accessory proteins (residing in the local membrane environment) such as heterotetrameric translocon-associated protein (TRAP) complex, translocating chain-associated membrane protein (TRAM), Sec62/Sec63 complex with or without BiP. The presence of these proteins alters the conformational dynamics of Sec61 to facilitate protein translocation or membrane integration.

biogenesis of less hydrophobic multi-pass membrane proteins [51–53]. They all contain multiple subunits and are conserved TM chaperones.

Recently, Hegde and co-workers suggested a new framework for the biogenesis of multipass membrane proteins. Based on biochemical and structural analysis, they described an interesting mechanism related to the insertion of a series of early intermediates of a few TMHs of rhodopsin, a 7-TMH G-protein-coupled receptor (GPCR). They showed that the nascent chain of rhodopsin is not inserted via Sec61 but along its membrane-surface facing a nearby PAT complex [54], that itself is composed of CCDC47 and Asterix. The presence of CCDC47 between the ribosome exit tunnel and the cytosolic vestibule of Sec61 apparently restricts the Sec61 in a closed conformation. It directs the nascent chain to the TMCO1-PAT complex associated with the Sec61 complex for insertion. The authors suggested that the client TMHs are held in a large central cavity formed between these three complexes (Sec61, TMCO1, and PAT) in the TM region [54].

2 | Sec61 Structure and Dynamics

In this section, we summarize evidence from structural biology about the overall Sec61 conformation and conformational substates. This is followed by a summary of a series of computational studies that aimed at characterizing conformational characteristics of Sec61.

The first structure in atomic detail of an ortholog of human Sec61 was determined by van den Berg et al. who presented an x-ray crystallographic analysis of a closed conformation of the SecY complex from the archaea *Methanocaldococcus jannaschii* [55]. SecY and Sec61 subunits possess high sequence conservation suggesting an evolutionarily conserved architecture and conformational dynamics, which was confirmed later by cryo-EM studies of eukaryotic Sec61s [48, 56–58]. The α-subunit of Sec61 is the central subunit forming the polypeptide-conducting channel. Its 10 TMHs may be divided into two covalently linked N- and C-terminal halves (TMH1-5 and TMH6-10). The 10 TMHs are arranged around a central constriction or "pore ring" that is sealed by six hydrophobic conserved residues from TMHs 2b, 5, 7, and 10, and a short helical (TMH2a) "plug" halfway across the membrane. The pore ring forming residues are usually amino acids having bulky side chains. The TMHs are connected by a total of nine loops (four cytosolic and five ER lumenal) clamped together by the γ subunit as shown in fig. 1 in Reference [55]. As mentioned, polypeptides either translocate along the Sec61 pore into the ER lumen or laterally insert into the membrane via a so-called "lateral gate" formed by TMHs 2 and 7 of Sec61α. As depicted in Figure 3, these two mechanisms are coupled to relative motions of the N- and rather static C-terminal pseudo-symmetric halves of Sec61α. The location of the plug domain just below the pore ring in the idle or inactive state of Sec61 was confirmed by several cryo-EM studies [56, 57]. In the SP engaged state, the plug moves away from the pore and SPs are inserted into the lipid phase via the lateral gate [48, 58] or translocated across the ER lumen as shown in Figure 3.

As mentioned above, the lateral gate is lined by TMH2 and TMH7. Compared with the crystal structure [55] from *M. jannaschii*, TMH2 showed a small displacement (less than 5 Å) in the structure of a eukaryotic ribosome-bound Sec61 complex determined by Becker et al. [57]. Also, a slight shift of the lumenal part of TMH7 toward the N-terminal half was observed in the structure of a ribosome-bound idle Sec61 complex from *Canis lupus familiaris* by Gogala et al. [48] Subsequent structural determination established the Sec61 conformation in various functional states upon nascent chain arrival [56, 58]. According to the structure of a solubilized ribosome-Sec61 complex by Voorhees et al. [56], ribosome binding appears to trigger conformational changes that result in translocon priming to accept an incoming polypeptide. Precisely, the loops between TMHs 6/7 and 8/9 are displaced in comparison to the crystal structure of the archaeal SecY, while the conformation of pore ring residues and of the lumenal and membrane sides of the lateral gate remained largely unchanged.

These structural studies mostly characterized conformational states of Sec61 in a detergent-solubilized condition without taking into account the presence of further translocon associated components. Instead, Pfeffer et al. reported a cryo-tomography structure of a laterally open ribosome-bound Sec61 in a non-inserting state under native conditions that had around 9 Å resolution. Based on this structure, the authors argued that ribosome binding alone is sufficient to open the Sec61 lateral gate without the presence of a nascent polypeptide chain [59].

Later, Voorhees and Hegde determined an active SP engaged structure of the canine ribosome-Sec61 translocon complex [58]. The obtained electron density showed the nascent chain to be in a looped conformation and to be intercalated between the lateral-gate helices, similar to the structure by Gogala et al. In comparison to their "primed" structure, Voorhees and Hegde observed asymmetric lateral gate opening together with rigid rotation by ca. 22 deg. of TMHs 1, 2, 3, 4, 5, and 10 of Sec61α along with TMH of Sec61β, relative to the membrane plane [56]. Also, several important hydrogen bonding interactions between lateral gate helices 2 and 7, termed "polar cluster" [60] and hydrophobic patch residues (present in the lateral gate and the pore ring), were disrupted in the primed state relative to the "quiescent" state. The position of SP in the SP-engaged state closely matched to that of TMH2 in the "quiescent" state. This suggested that the hydrophobic patch could be the interaction site for the SP, which then displaces TMH2 and opens the Sec61 channel. Alternatively, the open lateral gate conformation of Sec61 suggested that the channel could be in an open form during translocation and even after the termination of protein synthesis.

Weng and co-workers reported a cryo-EM structure of the yeast Sec complex in a post-translational mode with a bound SP [61]. As mentioned earlier, the Sec complex of yeast consists of seven subunits (Sec61α, Sbh1, Sss1, Sec62/63, Sec71/72). In the translocating state, the SP was observed to bind to a similar location at the lateral gate as observed by Voorhees et al. [58].

As evidenced by the structural characterizations of Sec61 in various substates, the conformational dynamics of the translocon are essential for the protein translocation process. Hence, various molecular dynamics (MD) simulation studies were performed for partial models of the Sec61 channel embedded

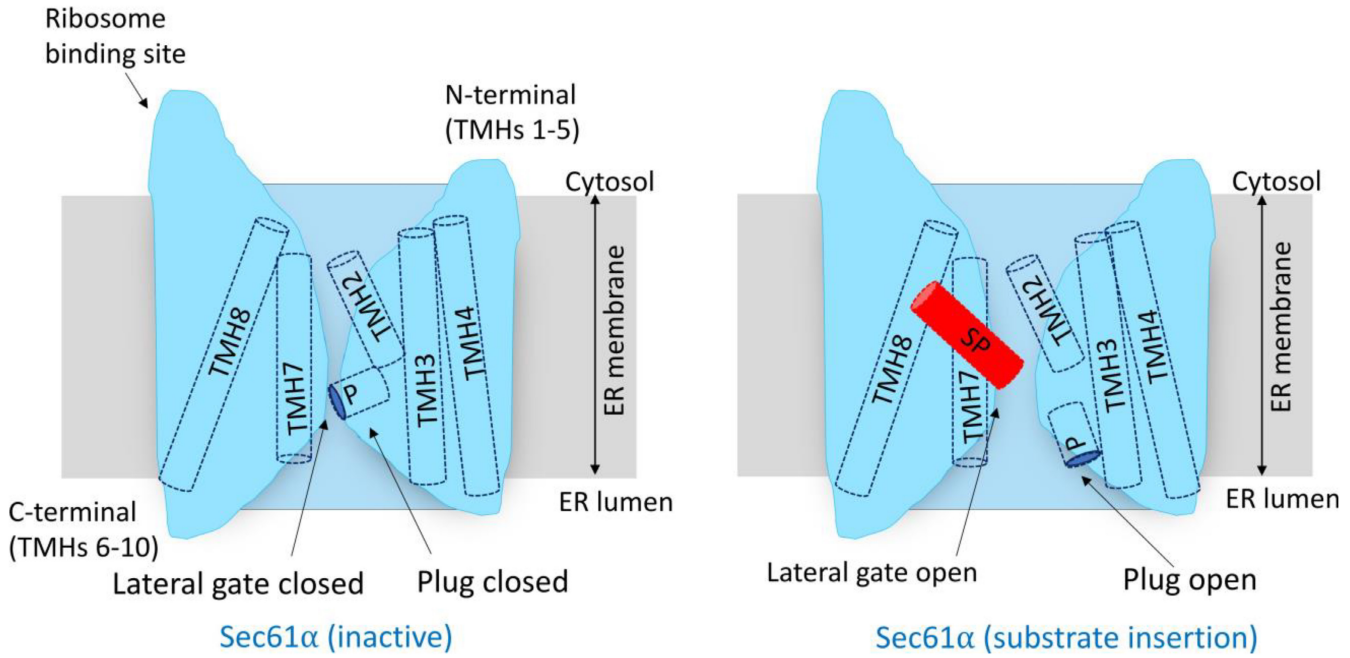

FIGURE 3 | Schematic illustration of Sec61α conformations in the resting state and during translocation. View onto the lateral gate side, where TMH2, TMH3, TMH4, TMH7, TMH8, and plug, respectively, are shown as tubes. A relative motion of the N- and rather static C-terminal halves of Sec61α and a movement of the plug moiety lead to channel opening. The picture is based on the structures described in References [59, 166].

in a planar bilayer membrane to derive detailed insight into the translocon-assisted mechanism of protein translocation and membrane integration. To characterize its intrinsic flexibility, Haider et al. [62] performed MD simulations of the pore (α-subunit) of SecY alone, and confirmed a "clamshell-like" conformational change of SecY during nascent peptide chain transport, as it was suggested by the x-ray structure of the SecY complex [63]. Haider et al. also compared their results to steered MD simulations of SecYE by Schulten and Gumbart [64]. Although, in vivo, the transport of polypeptides via the SecY pore was reported to take about 1 s [65], in their steered MD simulations, Schulten and colleagues pulled the polypeptide through the pore at a pulling time scale of ca. 1–5 ns in total (10^9 times faster than in vivo). They suggested a displacement of the plug along with the hydrophobic polypeptide used in their study [64].

A similar computational study of SecY gating using coarse-grained and atomistic MD simulations employed an expanding sphere to enforce the opening of the SecY lateral gate needed for membrane insertion [66]. These simulations suggested that the SecY pore ring is able to expand considerably and can accommodate the incoming nascent chain. Gumbart et al. separately addressed the nature of the pore ring and plug domain, because these are the necessary units to seal the channel, and studied the effect of ribosome binding on the channel. The loops between TMHs 6/7 and 8/9 were found to be more rigid in the ribosome-bound state of the channel than in its free state [67, 68]. An enhanced flexibility of the free state was also reported by Haider et al. as mentioned above [62].

Recently, using atomistic MD simulations and molecular docking, Bhadra et al. studied how the accessory proteins Sec63 and Sec62 individually affect the conformational dynamics of the yeast Sec61 translocon in the post-translational mode. First, via multiple independent simulations, they studied how the presence/absence of Sec63 affected the conformations of the gating elements that is lateral gate, pore ring and plug domain. For this, they monitored diagonal distances between the N-termini of the pore ring (V82, I86, I181, T185, M294, and M450) forming TMHs and their angular shifts along the MD trajectories. Pore opening was suggested to be due to a reorientation of TMH4 of the Sec61 channel and due to the interaction between TMH1 of Sec61 and TMH3 of Sec63 [69]. Peptide docking studies indicated that the hydrophobic cores of signal sequences and anchors interact with the TMHs lining the lateral gate (C-terminus of TMH2 and N-terminus of TMH7) and occupy the volume between them, what is supported by experiments [70]. Subsequently, Bhadra et al. also investigated how Sec62 affects the conformation of the Sec61 channel. Here, they observed that interactions between TMH2 of Sec62 and TMH7 of Sec61 mediated a widening of the lateral gate towards the lumenal side of the Sec61 pore. In the presence of Sec62, the distance between the C-terminal helical turns of TMH7 and TMH3 was found to be larger compared with the starting cryo-EM structure (about 0.8 nm) than in the absence of Sec62 (about 0.4 nm) [71]. The authors concluded that Sec63 mainly affects the orientation of TMH2 of Sec61 whereas Sec62 affects that of TMH7 of Sec61 [69, 71]. According to these simulation studies, the pore can be widened by movements of the pore forming TMHs up to a diameter between 15 and 20 Å. In this rather narrow pore the polypeptide may be either in an extended or in an alpha helical conformation, respectively.

Recently, Karki et al. determined a cyro-EM structure of mammalian Sec61 bound to the TRAP complex and the ribosome [72]. To study the dynamics and molecular interactions of the Sec61-TRAP-ribosome complex, the authors also performed atomistic and coarse-grained MD simulations of Sec61 bound to

TRAP and ribosome, and of Sec61 alone, embedded in a lipid bilayer. TRAP binding was observed to cause Sec61-associated local membrane perturbations, which in turn was speculated to affect the dynamics of Sec61 and ER protein import. Also, binding of TRAP was shown to maintain and/or stabilize the initial open lateral gate conformation of Sec61 in particular the TMH2-TMH7 distance, as compared with unbound Sec61 where the lateral gate is usually closed [72]. These findings are in line with the literature on the putative role of the accessory protein TRAP, that is believed to assist inefficient SPs and TMHs in the lateral gate engagement [32, 41].

Genetic studies in *Escherichia coli* showed that certain mutations in the SecY complex can cause the transportation of crippled or even severely damaged secretory protein signal sequences [55]. These mutations are located in important domains of SecY/Sec61, either at the lateral gate, in the plug helix or in the pore ring residues in the hydrophobic patch and polar cluster. For example, Phe64Cys and Asn65Tyr in the α subunit of *E. coli* SecY and Ile408Asn at the pore ring are thought to stabilize the channel in an open state [55]. Voorhees and Hegde speculated that Sec61 mutations may also increase the conformational dynamics of the translocating channel, so that it can open/initiate even without SP insertion to the lateral gate and this allows promiscuous translocation across Sec61 bypassing the so-called signal recognition step [58]. In mammalian cells, various diseases are associated with the mutations Sec61α1V67G, Sec61α1T185A, Sec61α1V85D, Sec61α1Q92R, and Sec61α1Y344H near to the plug helix, the pore ring, and the hydrophobic patch residues [7, 73]. The Sec61α mutation V85D was also reported to enhance Ca^{2+} leakage from the ER [73].

In summary, cryo-EM structures provide the picture of Sec61 undergoing a PacMan-like opening/closing transition where the 5 TMHs of the N-terminal half slide relative to the 5 TMHs of the C-terminal half. This motion opens up the lateral gate between TMHs 2 and 7, so that the SP of the nascent chain may dock to the hydrophobic patch formed by these lateral gate helices. When active nascent chain translocation takes place, the plug helix swings sideways toward the C-terminal of Sec61γ to clear the Sec61 pore full-length [55, 166]. Binding of accessory proteins Sec62, Sec63, and TRAP to Sec61 induces a partial opening of Sec61 and may lower the energy barrier for full opening.

Similar to mutations, small molecule inhibitors are known to affect Sec61 channel gating and ER import. Some of these Sec61 inhibitors provide important insights into the different routes taken by substrate proteins during translocation in a SP selective as well as nonselective way [74, 75]. Therefore, it is highly warranted to determine the precise interaction sites of these inhibitors inside Sec61 and their exact modes of action. We will now discuss some of the known Sec61 inhibitors and suggest why and how some of them show a SP-selective inhibition mechanism.

3 | Classes of Sec61 Inhibiting Small Molecules

Sec61 inhibitors are of interest as putative anticancer and immunosuppressive, analgesic, antiviral, anti-malaria, anti-anthrax, anti-coagulant, anti-anxiety agents, and as drugs for treatment of osteoporosis, Alzheimer's, rheumatoid arthritis, muscle loss, autism, neurodegeneration, and for stroke recovery [74–79]. The main idea behind targeting and/or blocking Sec61 is to suppress the production of harmful proteins, such as cytokines. Examples of Sec61-targeted translocon inhibitors are mycolactone, ipomoeassin F (Ipo-F), cotransin CP2, KZR-8445, decatransin, apratoxin F, eeyarestatin (ES1), and CK147. The chemical structures of these compounds are shown in Figure 4. Most of them are macrocyclic natural products. Some of them are produced by fungi, others were derived from bacteria and plant species. In the following, we will discuss them one-by-one.

Mycolactone is a polyketide-derived macrolide produced by *Mycobacterium ulcerans*. It possesses cytotoxic and analgesic properties and is the causative toxin of Buruli ulcers, necrotizing lesions in the skin [80–84]. Untreated painless Buruli ulcers can lead to severe secondary infections [85]. Initially, the two-dimensional chemical structure of this toxin was characterized by Small and co-workers [86]. They showed that mycolactone consists of three units, a 12-membered lactone core/ring and two polyketide side chains in north ("Northern" chain) and in south ("Southern" chain) positions. A mycolactone soluble fraction separated by thin layer chromatography (TLC) (silica gel, chloroform/methanol/water [90:10:1]) and ^{1}H NMR data recorded in deuterated acetone solution revealed that the molecule exists in a 3:2 equilibrated mixture of two *cis*/*trans* isomers, termed mycolactone.

A (Z-isomer) and B (E-isomer) differ in their conformation regarding the C4′–C5′ double bond in the oxygen linked southern fatty acid side chain [86–88]. The northern carbon-linked side chain is invariant and known as "core extension." The unsaturated acyl side chains play an important role in its activity as reported by Guenin-Mace et al. [89]. Variants lacking either the southern side chain or both chains showed no competitive activity [113]. Variant B has been reported to be more cytotoxic than A and was identified as the primary virulence factor for Buruli ulcer [90].

The cytotoxic macrocyclic compound ipomoeassin F (Ipo-F), derived from the leaves of *Ipomoea squamosa*, is a member of the ipomoeassin family and was reported to be a potent cytotoxic resin glycoside against human ovarian cancer and many other cancer cell lines [91, 92]. Three independent total syntheses of its unique natural macrocyclic carbohydrate-based architecture have been reported [93–95]. Its chemical structure contains two α, β-unsaturated esters (cinnamate and tiglate) and a disaccharide core (Figure 4).

Cotransin and its variants KZR-8445 and KZR-9508 form a group of cyclic heptadepsipeptides. Decatransin (30-atom membered ring) is a large fungal cyclodecadepsipeptide that is highly N-methylated (Figure 4) [96]. These compounds will be discussed in detail further below.

Apratoxins form another series of cyclic depsipeptides that were isolated from the marine cyanobacteria *Lyngbya majuscula*, *Lyngbya* sp., and *Lyngbya bouillonii* and were shown to possess cytotoxic activities [97, 98]. Their macrocyclic structures comprise peptide and polyketide units connected to a thiazoline moiety (Figure 4). Apratoxin A from *L. majuscula* was found to

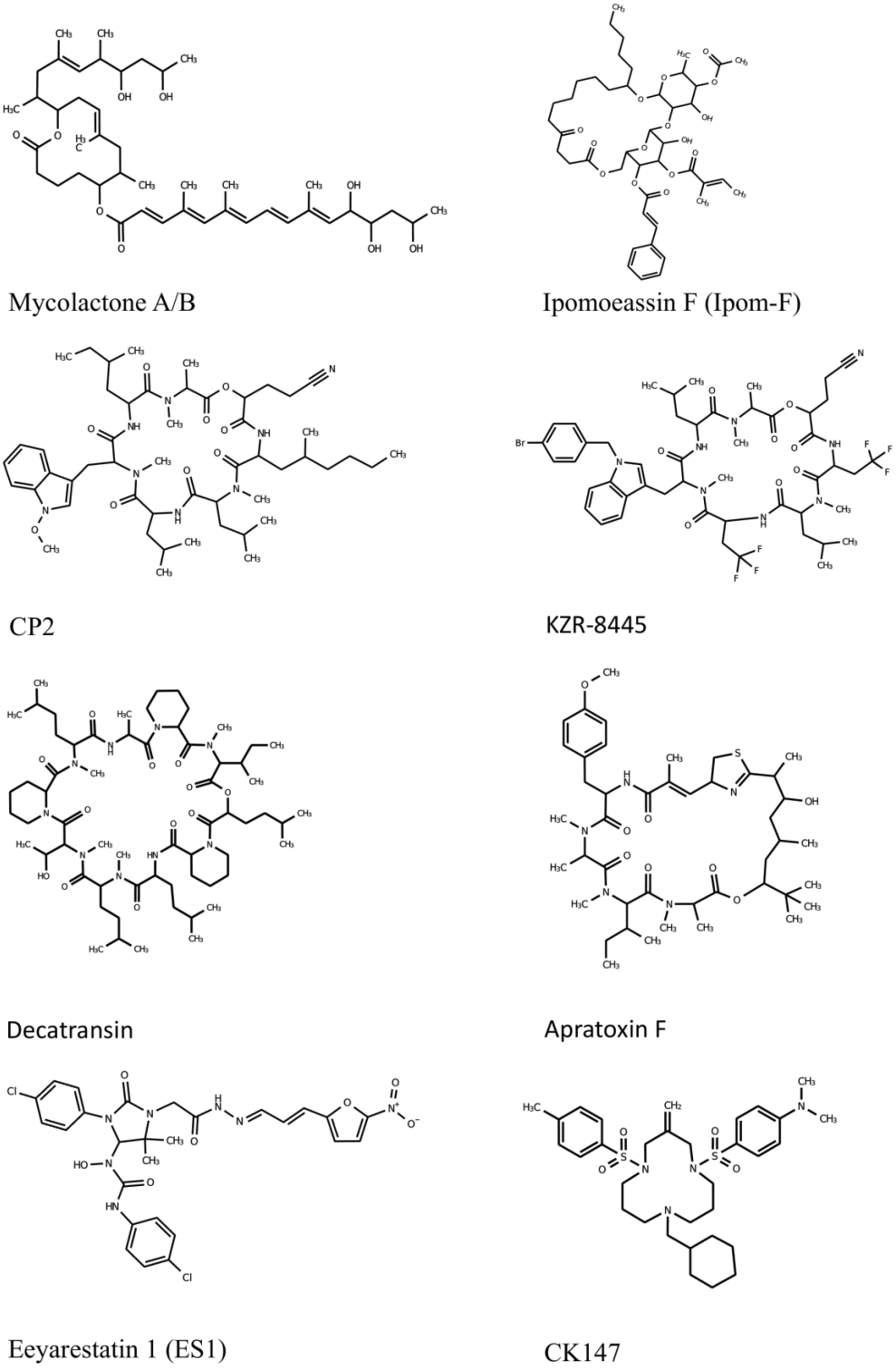

FIGURE 4 | Chemical structures of Sec61 inhibitors.

prevent growth of a variety of cancer cells [99, 100]. As identified by NMR analysis, it contains three methylated amino acids, a proline unit, an α, β-unsaturated modified cysteine residue, and a hydroxylated fatty acid moiety [99, 101]. The analogue Apratoxin F shown in Figure 4 is a cytotoxic cyclic depsipeptide with a minor structural difference, namely an N-methyl alanine unit in place of the proline unit present in the other compounds of this family.

Eeyarestatin 1 (ES1) and CK147 are synthetic compounds. ES1 was identified as an inhibitor of the ER-associated degradation (ERAD) pathway but also affects many other cellular pathways [102]. Its chemical structure includes aromatic and nitrofuran containing domains. The structure of CK147 contains a cyclohexylmethyl tail, a 4-dimethylaminobenzenesulfonyl side arm, and a tosyl side arm as shown in Figure 4. In the x-ray structures of various CADA compounds, their 12-membered

macrolide rings were found to adopt highly similar conformations [103, 104].

3.1 | Inhibitor-Bound Sec61 Structures and Docking Studies

Only recently, the first cryo-EM structures of mammalian Sec61 channels bound to small molecule inhibitors became available [105–108]. Prior to this, van Puyenbrocken and Vermeire already discussed several so-called Sec61 gating inhibitors, which were proposed to stabilize the translocon in a sealed plug conformation [109]. The modes of action of some Sec61 dependent translocon inhibitors were also discussed in [75, 169]. We focused our manuscript on those compounds where structural evidence by x-ray or cryo-EM has been established. Here, we are now able to discuss some of these Sec61 translocon inhibitors in the light of the novel structural evidence. Figure 5 shows the various interaction sites between inhibitors and Sec61α.

For quite some time, mycolactone was believed to stabilize the translocon in a closed conformation until Gérard and co-workers released the first mycolactone-bound inhibited cryo-EM structure of the canine ribosome-Sec61 complex [105]. When comparing the conformations of mycolactone-bound and unbound ribosome-translocon complexes, the authors observed no major structural differences except an elongated density at the cytosolic side of the lateral gate and certain structural changes in the N-terminal half of the translocon. Identification of the binding pose of mycolactone was assisted by MD simulations [105]. The authors found that mycolactone wedges open the cytosolic side of the lateral gate of Sec61α while keeping the translocon in a substrate-engaged state (Figure 5a), a conformation which is very similar to the one stabilized in yeast by the allosteric activators Sec62/63 [120]. In its bound conformation (in the docked model), the southern chain of mycolactone protruded into the ER membrane, and the northern chain was buried inside of Sec61α and made contacts with the pore and hydrophobic patch residues V85, L89, and I179, which are thought to be essential for SP binding. According to the Gérard et al. structure, most known resistance mutations are positioned near the plug region far from the hydrophobic patch residues. The authors suggested that these mutations indirectly reduce mycolactone binding by modulating the conformational dynamics of Sec61 [105].

Prior to the structural work of Gérard et al., mycolactone was reported to form a stable complex with the pore-forming unit Sec61α [112, 114]. At nanomolar concentrations, mycolactone inhibits the translocation to the ER of a broad range of secretory and membrane proteins such as cytokines, chemokines, and the inflammatory mediators TNF and Cox2 [84, 110–114]. Also, in vitro translocation assays and whole cell proteome analysis are consistent with mycolactone selectivity toward secreted, Type I/II single pass, and some multipass membrane proteins [113–117]. This indicates a selective effect of mycolactone on ER protein import unlike the structurally unrelated compound apratoxin A that was shown to have a nonselective effect on ER protein import [7, 111, 113, 140, 141]. The first biochemical evidence that mycolactone induces a conformational change in the Sec61 channel was provided by McKenna and co-workers [114].

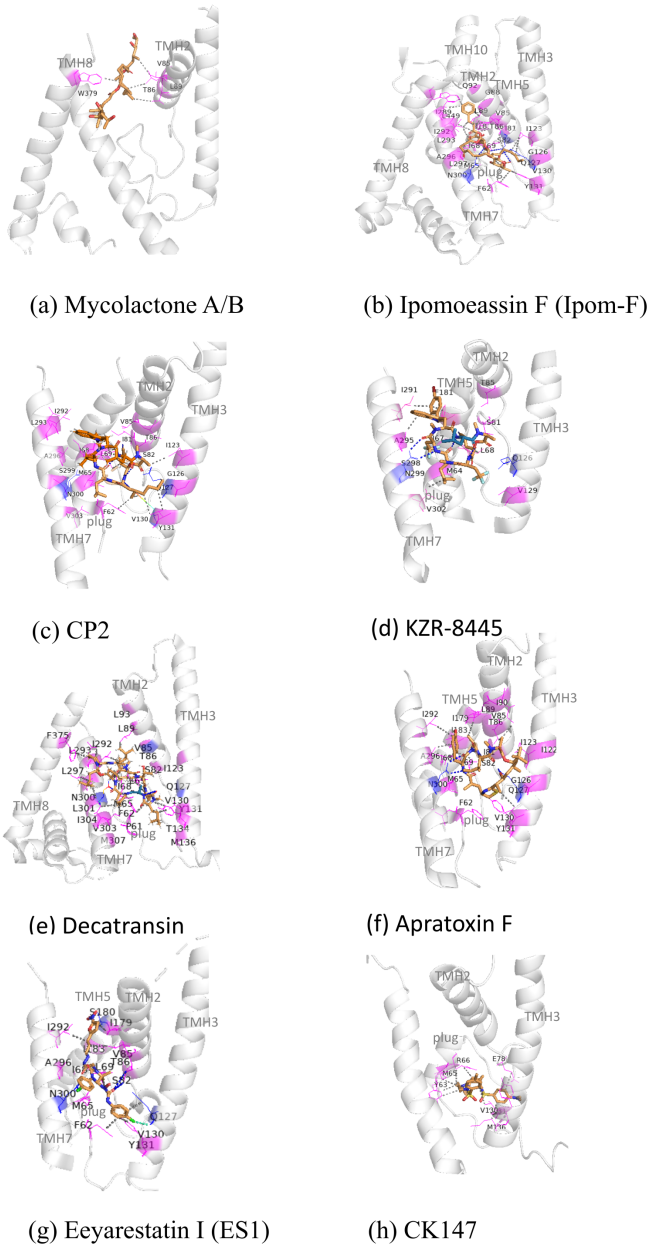

FIGURE 5 | Interaction sites between chemical inhibitors and Sec61α. All structures show the same view into the lateral gate or inhibitor binding site. Shown in atomic detail are the inhibitors and the interacting amino acids of adjacent TMHs and plug domain of Sec61α. Polar (blue) and hydrophobic (pink) residues are highlighted. The inhibitors are shown in brown with atom specific coloring. The used PDB structures are from Gérard et al. [105] (a), Rehan et al. [106] (d), Itskanov et al. [107] (b, c, e–g), and Pauwels et al. [108] (h), respectively. The below hyperlinks for these structures (a–h) enable users to inspect these 3D structures with the NCBI molecular graphics viewer [168]. (a) https://structure.ncbi.nlm.nih.gov/icn3d/share.html?HDgD1srwnK3UG4wg6; (b) https://structure.ncbi.nlm.nih.gov/icn3d/share.html?89rVzevvmgrD9Xsv6; (c) https://structure.ncbi.nlm.nih.gov/icn3d/share.html?XHDaSRChNCMRdJXe9; (d) https://structure.ncbi.nlm.nih.gov/icn3d/share.html?CMkPWKhBaoWKhw9L8; (e) https://structure.ncbi.nlm.nih.gov/icn3d/share.html?Z82M7M5ozaUiTJCt7; (f) https://structure.ncbi.nlm.nih.gov/icn3d/share.html?SSH1AAsZbeCbUrPe6; (g) https://structure.ncbi.nlm.nih.gov/icn3d/share.html?skRmobkGCgkrg2cR8; (h) https://structure.ncbi.nlm.nih.gov/icn3d/share.html?Ub1jjjozhZ2HeUgD7.

Moreover, it was reported that the binding of mycolactone can be prevented by a point mutation (R66G) in the pore-region of Sec61α without affecting the functionality of the channel [113]. Recently, Domenger et al. found that mycolactone is effective in inducing apoptosis in multiple laboratory-derived myeloma cell lines [118]. Already in 2005, Garrison et al. discovered that the fungal product derivative cotransin blocks the Sec61 channel in a substrate-selective manner [119]. The binding affinities of mycolactone and cotransin CT8 were measured by relative competitive binding assays and resistance mutant studies of Sec61, which showed that both molecules possess similar binding sites [113]. Other Sec61 inhibitors, namely decatransin and apratoxin, also possess partially coinciding binding sites inside Sec61α but mycolactone was found to be more potent and less specific [113]. The mechanistic linkage between mycolactone inhibition of Sec61 and apoptotic cell death still needs to be clarified.

Itskanov and co-workers recently reported inhibitor-bound cryo-EM structures of a post-translational chimeric Sec translocon complex, where the human TM domain (Sec61 from human except cytosolic loops 6 and 8) was fused to the cytosolic region from yeast. These structures had an improved resolution compared with that of human Sec complex lacking Sec62 (fig. 1c of Reference [107]). Their study reported cryo-EM structures of Sec either bound to mycolactone, Ipo-F, apratoxin F, cotransin CP2, decatransin, CADA, or ES1, respectively. Notably, almost all the inhibitors bound to Sec61 in a common overlapping binding pocket near the partially open lateral gate and a closed plug, irrespective of their different chemical structures. As this binding pocket faces towards the lipid membrane, all these compounds are thought to interact with hydrophobic lipid tails. The intrinsic conformational flexibility of Sec61 apparently enabled the differently-shaped inhibitors to tightly fit into this pocket (Figure 5b,c,e–g).

In the study of Gérard et al., the SP mediated opening of the translocon was prevented by mycolactone, whereas it is positioned at the lateral gate near to the plug in the Itskanov model [107]. There, its long southern chain is buried deeply into the channel and reaches toward the cytosolic region between the pore forming residues (fig. 3d in Reference [107]). We speculate that the different experimental protocols could have resulted in different binding locations and orientations of mycolactone in these studies.

Motivated by the studies of Gérard et al. and of Itskanov et al., Nguyen et al. performed MD simulations combined with enhanced sampling methods to investigate the binding trends of mycolactone A/B to the Sec61 translocon considering either the Gérard or Itskanov models as basis [121]. By comparing the inhibition pattern in both complexes, isomer B was found to interact more strongly with the hydrophobic patch residues and plug region of Sec61 by adopting a more open conformation as compared to isomer A, which supports its stronger inhibition efficiency [121]. Earlier, the same authors also compared the binding of mycolactone B to different model membranes to inspect its membrane selectivity using multi-scale simulation techniques [122].

The Sec61 complex also plays an important role for Ca^{2+} leakage from the ER [43]. The Sec61 mediated handling of Ca^{2+} homeostasis has been linked to pathological conditions such as cancers, neurodegenerative disorders, neutropenia, Buruli ulcer, and so on [123]. Recently, Bhadra et al. suggested that mycolactone induced Ca^{2+} leakage through the Sec61 translocon should not occur in its idle conformation when the translocon binds to the ribosome with a sealed plug and lateral gate. Instead, they argued that Ca^{2+} leakage should involve a "primed" Sec61 conformation that is ready for accommodating the SP [124]. Such an intermediate conformation that is for example stabilized by binding of mycolactone with a partially opened lateral gate is thought to enhance the Ca^{2+} leak (fig. 9 of Reference [124]). They suggested that the SP translocation process keeps the channel transiently in an open conformation, which triggers Ca^{2+} permeability via the aqueous pore until the plug closes, and ribosome rebinds and the translocon again converts into its idle state. Various Sec61α mutants resistant to mycolactone binding showed no Ca^{2+} depletion from the ER. This confirmed a direct effect of the inhibitor on the channel. Putative mycolactone binding sites suggested by docking studies of Bhadra et al. compared well with Gérard's reported mycolactone-inhibited structure, where mycolactone prefers to interact with the cytosolic entrance of the pore forming subunit. It would be interesting to study the effect of accessory proteins on the mycolactone stabilized Ca^{2+} permeable state of the channel.

Eeyarestatin 1 (ES1) was initially characterized to be an ERAD inhibitor that stabilizes the substrate MHC class I heavy chain [102]. As the ERAD factor p97 is essential for ERAD substrate release from the ER membrane, binding of ES1 to this complex blocks ERAD, which confirmed its inhibitory action [102, 125]. A subsequent study showed that micromolar concentrations of ES1 in the treated cells inhibited the ER insertion of many co-translationally imported proteins [126]. It was also shown that ES1 interferes with SP transfer to the Sec61 complex [126], what enhances the amount of cytosolic polyubiquitinated proteins and triggers unfolded protein response (UPR) [127]. In fact, ES1 also acts as an anti-tumor agent [109, 128, 129]. ES1 inhibits both ER protein insertion via Sec61, as well as the ERAD pathway through p97. Its nitrofuran moiety (Figure 4) was associated with its biological activity on protein translocation [130]. Two ES1 analogues, ES2 and ES24, possess chemical modifications mainly in the aromatic domain. Both ES1 and ES24 were shown to be potential inhibitors of bacterial protein secretion, but ES24, unlike ES1 showed broad-spectrum antibacterial activity and was less toxic to HEK293 cells. In fact, ES24 was reported to inhibit SecYEG-dependent protein translocation and membrane insertion of Gram-negative *E. coli* [132, 133]. Also, using transcriptomatic stress response profiling and phenotypic assays, Schäfer et al. recently investigated the mechanism of dual action of ES24 in Gram-positive *Bacillus subtilis*. They reported that ES24 inhibits SecYEG-dependent protein secretion in *B. subtilis* along with evidence for DNA damage [134].

As Sec61 is the target of many inhibitors and was reported to mediate Ca^{2+} efflux from the ER [131], Gamayun et al. characterized the effect of ES1 on ER Ca^{2+} homeostasis by measuring the cytosolic, as well as ER Ca^{2+} concentrations in the treated cells [132]. Treatment with ES1 led to a reduced Ca^{2+} concentration in the ER and an enhanced Ca^{2+} leak from the ER lumen in a dose-dependent manner. All of this presumably resulted from

the binding of ES1 to the Sec61 channel and prevented protein translocation. Docking studies suggested that Sec61 alpha may be stabilized by ES1 in a partially open ion-permeable conformation. After comparison of all analogues, the 5-nitrofuran unit was suggested to play a critical role on Sec61. ES24, a truncated version with a similar 5-nitrofuran moiety, was shown to strongly enhance the Ca^{2+} leak, which can be thought of as an important contributor to cytotoxicity.

To characterize putative binding cavities for ES1 derivatives, Gamayun et al. modelled canine Sec61α in an open conformation (fig. 6 of Gamayun et al. [132]) and performed a docking analysis of ES1 and ES24. The docked positions of both ES analogues with the highest predicted affinity were found in similar locations near to the cytosolic end of the lateral gate forming TMHs. Occupying this site was suggested to disturb channel reorientation required for protein translocation. ES1 was postulated to trap the translocon in an open ion-conducting stage and prevent lateral gate closure. A similar type of "foot in the door" binding was suggested for mycolactone, as was also discussed above in the mycolactone section. In the ES1-inhibited structure of Sec61 by Itskanov et al., the extended side chain of ES1 is deeply buried in the channel cavity like mycolactone where it interacts with several pore ring residues (Ile81, Val85, Ile179, Ile183, Ile292, Leu449). ES1 is pointing to the cytosolic region, while its aromatic domains face toward the lipid membrane (Figure 5g). This type of long penetration into the channel could be a reason for the broad-spectrum activity of these molecules. When residing in the pocket, they interact with pore, plug, lateral gate, cytosolic funnel, and also with lipid tails and prevent substrate insertion into the channel. Notably, Itskanov and co-workers were unable to capture the reported aspects of calcium leakage through Sec61 in presence of mycolactone and ES1 in their study.

Ipomoeassin F (Ipo-F) is another cytotoxic macrocyclic compound. It is obtained from the leaves of *Ipomoea squamosa*. By chemical proteomics studies, Zong et al. discovered Sec61α to be the binding-partner/molecular-target of Ipo-F in human cell lines [135]. Ipo-F was found to strongly inhibit (IC_{50} value of ~50 nM) Sec61 dependent protein secretion in in vitro translocation assays [135]. The known resistance mutations of Sec61α toward cotransin, mycolactone, and apratoxin A also conferred strong resistance to Ipo-F, which again explains its cytotoxicity in ER import inhibition. The size of the macrocyclic ring of Ipo-F is related to its biological activity and/or cytotoxicity, which matches structure activity-relationships [136].

In a cell-free assay, several ring-opened analogues of Ipo-F also showed Sec61 inhibition [135]. This motivated O'Keefe and co-workers to study the effect of ring-modified Ipo-F analogues (fig. 1 of Reference [137]) in vitro and/or in cellular assays and via molecular docking studies.

Based on the measured cytotoxicity of nine newly synthesized open-chain Ipo-F analogues they concluded that the macrocyclic unit is not essential for Ipo-F biological activity. They suggested that both groups of analogues (open or closed chain) share a similar cytotoxic mechanism of action. Whereas Ipo-F and open-chain analogues mediated cytotoxicity and cell death of HCT-116 cells having WT Sec61α was observed even at reduced concentrations of these molecules, cell viability of treated cells with point mutated G80W Sec61α was shown to be unaffected by these compounds. This again confirms Sec61α as their main molecular target.

Using the structure of the mycolactone-bound Sec61 channel determined by Gérard et al. for molecular docking studies, O'Keefe et al. observed that Ipo-F and all its analogues (open or closed chain) were docked most favorably to the same binding pocket near the cytosolic side of the lateral gate as mycolactone. In addition, some analogues were also docked to different positions in the lateral gate where they interacted with the plug region of Sec61α pore. These different binding sites may influence the substrate engagement to the channel in various ways. The entropy change and hence the proper binding affinities of open versus closed chain Ipo-F compounds could not be accounted for due to limitations of the docking method, which we will discuss later. Furthermore, in silico-aided studies guided O'Keefe et al. to design a simpler and synthetically better accessible compound with increased lipophilicity and reduced chirality at the 11S chiral center (Figure 4). To circumvent limitations of the molecular docking tool regarding the maximum possible number of rotational bonds of the ligand, they docked two closely related compounds that differ in the chirality at the 11S position of the macrocyclic ring. Again, they were found to occupy the same binding sites as other analogues and the newly discovered achiral analogue was found to possess more interactions than its chiral counterpart. Similar to the docking models presented in O'Keefe et al. [137], Ipo-F in the inhibited translocon structure of Itskanov and co-workers was shown to interact with the plug and lateral gate residues, and bound near the lateral gate when this was almost closed. Its rigid disaccharide unit pointed into the channel interior (Figure 5b). The resistance mutations for Ipo-F are found in the vicinity of the inhibitor-binding site.

Another cyclic depsipeptide, apratoxin F, also showed potent cytotoxicity against H-460 cancer cells [138], which was similar to that of apratoxin A. Both molecules were reported to possess similar IC_{50} values in mammalian cell lines. Due to the absence of the proline moiety, apratoxin F shows high structural flexibility [101, 138]. Several related compounds (apratoxins B through E), were discovered and isolated from various marine cyanobacteria and showed similar anticancer activities [98, 99, 101, 138, 139].

It was reported that the expression of many cell surface receptors and ER proteins is downregulated in Apratoxin A treated cells. These likely results from preventing their co-translational translocation into the ER in vitro [140]. Mutagenesis and competitive photo-crosslinking studies by Paatero et al. identified Sec61 as molecular target of Apratoxin A with broad-spectrum inhibition activities. Two residues, T86 and Y131 near to the plug domain, were found to be crucial for apratoxin A activity. Altering them to T86M and Y131H conferred resistance to apratoxin and cross-resistance to cotransin. In comparison with the substrate selective cotransin CT8, apratoxin A was found to form distinct interactions near to the lumenal plug of Sec61 despite of overlapping binding sites [141]. Unlike cotransin (discussed below), which captured the SP in the cytosolic vestibule, this study showed that apratoxin inhibits the translocation at an

earlier stage that is it prevented the tumor necrosis factor-alpha (TNFα) TMH from inserting into the cytosolic vestibule and/or docking to the Sec61 lateral gate [141]. On the other hand, in the inhibited Sec61 structure of Itskanov and co-workers (overall resolution of 2.5 Å), the polypeptide unit of apratoxin F was found to interact mostly with pore, plug, and lateral gate residues, respectively, whereas the polyketide moiety was exposed to the lipid (Figure 5f).

Although the general usefulness of computational tools in drug design and discovery is out of question, many challenges still need to be addressed. A major limitation of many molecular docking tools is their inability to account for conformational flexibility of the receptor (Sec61 in the above studies). Instead of considering many possible conformations that are accessible to a flexible protein molecule, one is often limited to consider a single receptor conformation [142, 143]. An exception are a few matching algorithms that are able to consider molecular flexibility in parts of the receptor molecule [144]. A second major limitation concerns the computation of accurate binding energies by empirical scoring functions that neglect or approximate important energetic factors [143, 145, 146]. In many empirical docking scoring functions, the entropy loss of the ligand during protein-ligand binding is approximated based on the number of rotatable bonds of the ligand [137, 148]. Most docking programs are also unable to predict the flexibilities of cyclic and macro-cyclic ligands, and have limitations regarding the number of torsions/rotatable bonds [149, 150]. Up to date it is also a tricky task to deal with cavity waters in the binding pocket during the process of molecular docking [151]. MD simulations combined with molecular docking could overcome some of these limitations, because MD simulations may account for the conformational dynamics of protein and ligand on sub-millisecond timescales and are able to capture the effects of explicit water and lipid molecules [147].

3.2 | SP-Selective Sec61 Inhibitors

Interestingly, cyclotriazadisulfonamide (CADA) and the cotransin family of inhibitors were reported to mediate SP specific inhibition of the translocon. For example, CADA was reported to selectively inhibit cotranslational translocation of the human CD4 glycoprotein in vitro [152]. CD4 is very crucial in immune response, and is also known as the main entry receptor for immunodeficiency virus (HIV) [152–154]. CADA was also shown to specifically inhibit the ER translocation of five other proteins (SORT, DNAJC3, ERLEC1, PTK7, and 4-1BB) [155–157]. DNAJC3, ERLEC1, and PTK7 were identified in a proteomic study on T-cells out of 3007 quantified proteins [156]. 4-1BB was identified in Reference [157] According to Vermeire and co-workers, CADA was the first compound shown to be involved in SP inversion inside the translocon, and to prevent translocation by redirecting the SP to the cell cytosol [152]. The synthetic molecule CK147 is a more potent but also more toxic CADA derivative. It was discovered in structure-activity relationship studies, and characterized as the most potent CADA analogue having anti-HIV activities [103]. Pauwels et al. identified CK147 in a cell-free assay as a putative cytotoxic Sec61 inhibitor of huCD4 and characterized its unique binding site below the plug domain at the lumenal end of Sec61α (Figure 5h) by cryo-EM [108]. Like most of the aforementioned inhibitors, CK147 was shown to keep the translocon in a partially opened conformation [108]. Compared with the lead compound CADA, CK147 was found to strongly downregulate huCD4 in transfected HEK 293T cells with an IC_{50} of 0.04 μm [108]. Reflecting its selectivity, CK147 also reduced the expression of other CADA sensitive proteins (namely SORT, PTK7, ERLEC1, and DNAJC3) and also of some CADA resistant proteins (CD40, murine CD4), whereas it had no obvious effect on CD58 and CD86 surface receptors of T-lymphoid MT-4 cells. The proliferation rate of CK147 resistant HCT116 clones was shown to be unaffected even at high inhibitor concentrations. The *sec61* resistance mutations D60G R66G, P83H, V102I, and Q127K were shown to be located in the plug region and in the N-terminal half of lateral gate TMHs [108]. As discussed earlier, the known mycolactone resistance mutations are located far from the binding pocket of mycolactone in the Gérard et al. structure. Yet, these mutations are observed to surround the CK147 binding position. Pauwels et al. speculated that this site could also be a second binding site for mycolactone [108]. R66G showed strong resistance to CK147 binding what matches the close proximity in the structure where R66 makes extensive atom contacts to CK147. Earlier, a R66G containing strain also showed resistance to almost all known Sec61 inhibitors [109]. The V102I mutation is distal from the other resistance mutations and/or the inhibitor binding sites. Its indirect significance needs to be clarified. Binding of CK147 was shown to cause a structural rearrangement of Sec61α and the accessory protein TRAP had a less well-ordered structure when bound to Sec61:CK147. The direct interaction between the SP and CK147 still remains to be analysed. The final density map of CK147-bound complex by Pauwels et al. was calculated at 2.70 Å resolution. At the CK147-binding site, the local resolution was mentioned as ~5 Å, which was not enough for locating the binding pose of CK147. Instead, the authors used molecular docking to position CK147 in their cryoEM structure. On the other hand, in the 2.95 Å resolution structure by Itskanov et al. [107], CADA was positioned near the lateral gate of Sec61. There, its benzyl moiety was exposed to lipids while the p-toluenesulfonyl group points to the channel facing side, see fig. 3f of Reference [107]. The inhibited expression of huCD4 by CADA (IC_{50} = 0.6 μM) was reported to be similar by Itskanov et al. [107] to that of Reference [152]. We conclude by stating that the position and conformation of CK147 in the Pauwels et al. structure is different to that of CADA in the Itskanov et al. structure.

Next, cotransins are a group of cyclic heptadepsipeptides produced from HUN-7293, a fungal macrocyclic heptadepsipeptide identified in a screening program for inhibiting cell adhesion. Its chemical structure was determined by x-ray crystallography and NMR [158]. HUN-7293 was shown to selectively inhibit the expression of vascular cell adhesion molecule 1, intercellular adhesion molecule 1, and E-selection cell adhesion molecules. These molecules play an important role in immune response and a variety of inflammatory diseases [158, 159]. Garrison et al. showed that a HUN-7293 derivative, cotransin, prevented the co-translational translocation of a number of precursor proteins in a SP-selective way with the Sec61 complex as its main target [119].

Cotransin analogues with modified side chains, CT08, CT09, and compound 2 (CP2), were shown to affect Sec61-mediated translocation of substrate proteins in a variety of ways. CT08, CT09, and PS3061 inhibit the secretion/membrane integration of inflammatory cytokines such as TNFα and prevent viral replication [160–162]. Resistance mutations in Sec61 near the plug

and C-terminal of TMH3 suggest similar binding locations of CT09 and CT08 in the Sec61 vestibule with a partially open lateral gate conformation [163].

Decatransin (30-atom membered ring) is a fungal cyclodecadepsipeptide with a non-selective broad-spectrum activity on polypeptide translocation into the ER [164]. Recently, Ohsawa and co-workers reported the total synthesis and structural determination of decatransin, which turned out to be a highly N-methylated cyclodepsipeptide [96]. Decatransin was found to prevent the growth of *S. cerevisiae* and of mammalian cells by inhibiting Sec61-dependent protein translocation into the ER in both co- and post-translational modes, independently of the sequence of the targeting SP [164]. Based on mutagenesis data, the mode of action of decatransin on Sec61 was found to be similar, but not identical, to the substrate-specific cotransin [164]. The resistance mutations for decatransin identified in Sec61α1 are mostly localized in the plug domain [164]. Most of them were also resistant to cotransin CT08. Deletion of the plug and TMH2 and mutations in the constriction ring of Sec61α resulted in strong decatransin resistance [164]. In the Itskanov et al. structure, decatransin in the inhibited Sec61 channel was shown to bind to the lateral gate, pore, and plug residues (Figure 5e) [107]. There, its hexanoic acid moiety faced toward the lipid membrane. A Q129L mutant in yeast Sec61 showed strong resistance to decatransin whereas mild resistance was found for the equivalent mutation Q127L in human Sec61α [107].

Rehan and co-workers recently designed a fluorinated cotransin PS3061 variant, KZR-8445 (Figure 4), and tested a panel of 89 disease-related Sec61 clients with varying SP-inhibitor sensitivity [106]. KZR-8445 was shown to prevent the secretion of pro-inflammatory cytokines, interleukin-2 (IL-2), and TNFα, but was less effective on *sec61* (R66I) mutated cells. Also, in a mouse model of rheumatoid arthritis, this inhibitor decreased the disease-related symptoms in a dose-dependent manner without any significant toxicity. A cryo-EM structure of KZR-8445-bound Sec61 was reported with an overall resolution of 3.2 Å, with reduced resolution in the N-terminal half [106]. Here, KZR-8445 was found to form direct contacts to the Sec61α plug domain with an open lateral gate, and was trapped in the cytosolic vestibule (similar to CT08), where it blocked the entry to both ER lumen and membrane. In this SP-binding cleft, the inhibitor formed strong hydrogen bonds with N300 and polar interactions with other nearby residues (S82, T86, and Q127) that are otherwise known to stabilize the lateral gate in a closed conformation (Figure 5d).

In another recent study, the relative orientation of cotransin CP2 was found to be different from KZR-8445, but it was reported to bind to a similar location with more pronounced lateral gate opening as other Sec61 inhibitors (Figure 5c) [107]. Mutation N300 conferred resistance to KZR-8445 and to cotransin CP2, which confirms its critical role in inhibitor binding. R66I also conferred resistance to KZR-8445 and CP2, even without making direct contacts to the inhibitor what explains its indirect effect on the Sec61α plug conformation. The potential hydrogen bonding interactions between N300 and inhibitor, and unbinding with a N300A mutant agreed with MD simulation results [106].

KZR-9508 is a R5-side chain modified version of KZR-8445, where the R5 bulky bromobenzyl-tryptophan side chain was replaced by a smaller ethyl-tryptophan. The cryo-EM structure shows that the R5 bromobenzyl-tryptophan side chain in KZR-8445 reaches into the cytosolic cavity in the Sec61α interior, which explains why it blocked many SPs from accessing the lateral gate [106]. Although, the original authors speculated that KZR-9508 may bind less tightly than KZR-8455, its smaller R5 side chain enabled most of the SPs to pass through, which highlights its greater selectivity in comparison to KZR-8445. Competitive binding of substrate proteins with strong, hydrophobic SPs could cause the inhibitor to dissociate, as was also reported earlier for SP selective inhibitors [108]. Hence, variations in substrate selectivity could be achieved by tuning the inhibitor's sidechain groups as was also the case with CK147 and CADA [108]. Chemical modifications of the side chain groups in substrate-selective Sec61 inhibitors (cotransin and CADA-related) brought improved client selectivity (CADA to CK147 and KZR-8445 to KZR-9508) and less pharmaceutical toxicity [106, 108].

Recently, Wenzell et al. [165] performed SP profiling on a global scale by screening thousands of unique SPs to get mechanistic insights into the process of SP-selective inhibition of Sec61 clients. The SP-specific inhibitors KZR-8445 and its variant KZR-9873 from the cotransin family were used for this global profiling. Using a newly developed screening platform, the structural difference at 3 side chain positions of the two cotransins showed a remarkable difference in SP-sensitivity. Out of 3666 unique SPs from their synthesized nucleotide library, almost 637 (ca. 17%) were found to be sensitive to KZR-8445 and 152 (ca. 4%) to KZR-9873 at 100 nM concentration, which shows the broad effect of KZR-8445 on SPs. Wenzell et al. also showed that KZR-9873 uniquely inhibits 22 SPs out of total 152 (the remaining 130 SPs are also sensitive to KZR-8445) and KZR-9873 was also found to inhibit the cell proliferation of several tested cancer cell lines. To provide mechanistic understanding of KZR-8445 and KZR-9873 sensitivity, they specifically investigated the presence of hydrophobic and polar amino acids near the SP cleavage site. Interestingly, the hydrophobic amino acid Leu was depleted in all KZR-8445 and KZR-9873 sensitive SPs. Weak hydrophobicity of SPs, that is the presence of polar amino acids and proline residues, apparently leads to sensitivity to these drugs. Furthermore, they specifically discussed the effect of residue position on SP-selectivity for KZR-9873 by choosing the hSDC1 (human) and mSdc1 (mouse) ortholog pair because of their single amino acid sequence difference (Arg16 in mSdc1 and Ser16 in hSDC1) at the C-terminal end of the hydrophobic H-region. Here, mSdc1 was found to be more sensitive to KZR-9873 due to the presence of the positively charged Arg16. Mutation to Lys (R16K) also maintained its sensitivity. Mutations to polar and negatively charged amino acids at this position led to resistance against KZR-9873. Similar effects were also seen for human hEPHA7 (Arg20), which was more sensitive than its mouse ortholog mEpha7 (Gly20), both differing by three amino acids positions. Based on these observations, the authors speculated about the existence of a ternary complex formed by the SP, Sec61, and KZR-9873 having co-operative interactions between them, which may be stabilized by Arg or Lys. They tested this hypothesis on mSdc1 and hEPHA7, which contains an Arg residue in the H-region near

to the C-terminal end. Shifting the position of this Arg by three amino acids towards the center of the H-region compromised its sensitivity to KZR-9873 and simultaneously increased its sensitivity to KZR-8445. Hence, the two cotransins differently affected SP sensitivity, which may be due to either co-operative interactions (in case of KZR-9873) and competitive inhibition (in case of KZR-8445) or due to an allosteric effect on the inhibitor binding guided by the interactions of specific amino acids (Arg/Lys) near the C-terminus of the H-region with Sec61.

Human epidermal growth factor receptor 3 (hHER3), which has a critical role in various cancer types, was uniquely sensitive to KZR-9873 only. The authors showed that KZR-9873 mediated reduction of HER3 levels, which indicates its selective putative anti-proliferative effects on CAL27 cancer cells. This study did not consider Sec61 clients without an Arg or Lys near the C-terminal end of H-region, and also did not consider Sec61 clients without an SP, for example type-II single and multi-spanning proteins.

Selectivity appears to be governed by an interplay between the nature of the signal sequence, for example whether it contains a strongly hydrophobic and/or alpha-helical core or not, and the inhibitor binding affinities to the channel, for example whether its dissociation from Sec61 may be induced by the incoming SP or not. Resistance mutations (at the binding cavity or distal from it) presumably destabilize the inhibitor-bound translocon, likely by conferring more conformational flexibility to it. Dynamical assistance to Sec61-mediated ER translocation by allosteric accessory proteins (TRAM, TRAP, Sec62/63, OST, and BiP) could play a crucial role in inhibitor-sensitivity as well. Until now only few compounds were identified that affect Sec61-mediated translocation. More screening efforts are certainly warranted. CK147 and KZR-8445 were both observed to compete with SPs for access to the Sec61 lateral gate. Hence their binding affinities to the binding cavity inside the Sec61 channel are a contributing factor to the substrate selectivity mechanism. Furthermore, there is a major difference between substrate proteins and inhibitors, in that the inhibitors could also strongly bind to the sealed plug moiety. Based on the reported inhibitor-bound structures we can conclude that the binding of an inhibitor to the Sec61 complex largely depends on its shape, size (side groups), initial model preparation, techniques used, and of course on the experimental conditions.

4 | Concluding Remarks

Molecular screening and tracing the mode of action of natural compounds have led to discoveries of small molecules that either broadly block Sec61-mediated protein translocation into the ER or selectively block the passage of precursor proteins with specific types of SPs. Spectacular progress in structural biology allowed characterization of the binding modes for some of these compounds in the Sec61α pore. Ongoing efforts, both experimentally and using molecular simulations, attempt to relate their binding characteristics to their client-spectra of blocked substrate peptides. It appears as if Sec61 may become the focus of drug design efforts that exploit the ability of small molecules to selectively block the ER translocation of certain protein families.

Author Contributions

N.S. and V.H. planned the writing of the manuscript. N.S. then wrote the draft of the text and prepared figures. V.H. reviewed and edited the manuscript.

Acknowledgments

This work was supported by Deutsche Forschungsgemeinschaft through He3875/15-1. The authors thank their colleagues Profs. Karin Roemisch and Richard Zimmermann and the two anonymous reviewers for constructive comments on the text. Open Access funding is enabled and organized by Projekt DEAL.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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