Synthetic membrane transporters

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An increasing number of synthetic compounds have been shown to facilitate ion and polar molecule transport across bilayer membranes. Most notably, recent advances in anion transport have yielded synthetic chloride channels and phospholipid translocases. Attention has also turned to the ability of short amino acid sequences to transport peptides and proteins across cellular membranes.

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Abbreviations

PC	phosphatidylcholine
PE	phosphatidylethanolamine
PIP_n	phosphoinositide polyphosphate
TREN	tris(aminoethyl)amine

Introduction

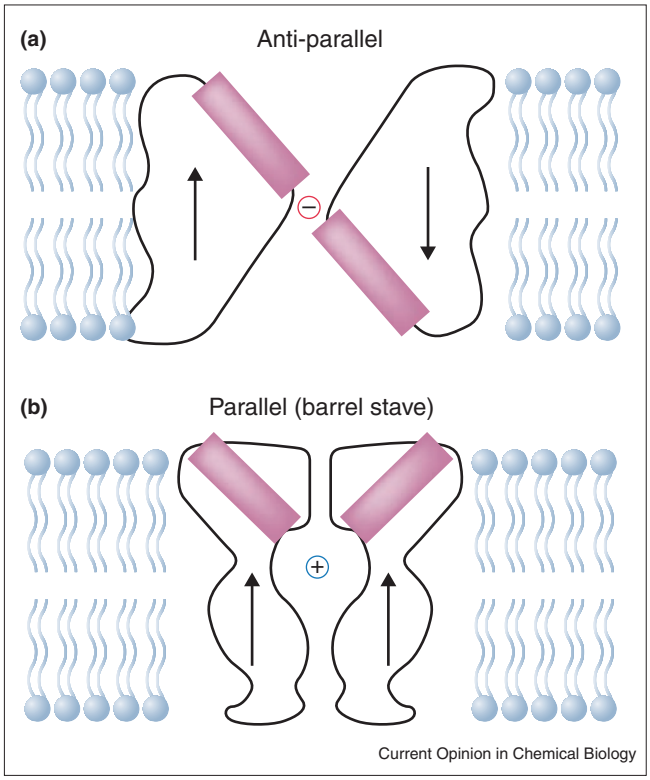
The facilitated transport of ions and polar molecules across biological membranes is essential for normal cell function and is also a major objective in the rapidly growing field of drug delivery. Researchers strive to simulate naturally occurring channel-forming proteins or carrier molecules. The resultant compounds may utilize endogenous peptide sequences or may be novel organic structures. This review summarizes the most recent synthetic transporters shown to be active in both model bilayers and cellular membranes.

Mechanism of ion transport across bilayer membranes

Pohorille and colleagues [1,2] have performed molecular dynamics simulations of unassisted Na^+ and Cl^- ion transport across a bilayer membrane. As the ion enters the outer monolayer, it generates a deep, local defect that allows solvated water molecules and polar phospholipid head-groups to also enter the membrane. When the ion advances past the midplane of the bilayer into the inner monolayer, the local defect switches to the outgoing side. Consequently, the ion remains solvated by water or phospholipid head-groups throughout the process.

The recently published crystal structures of two prokaryotic ClC Cl^- channels [3••], along with the previously solved structure of the KscA K^+ channel [4], have contributed significantly to the current understanding of assisted ion transport. A simplified view of both channel architectures is illustrated in Figure 1. Anion selectivity in the Cl^- channel is due to partial positive charges generated by main-chain amide nitrogen atoms, serine/threonine side-chain hydroxyl groups, and positive helix dipoles (N-terminal ends); whereas cation selectivity in the K^+ channel is due to partial negative charges contributed by main-chain carbonyl oxygen atoms and negative helix dipoles (C-terminal ends). Partial charges prevent the ion from being bound too tightly, enabling rapid flow through the channel. The Cl^- channel pore is shaped like an hourglass with a narrow constriction in the center; whereas the K^+ channel pore widens in the center producing a cavity. MacKinnon and colleagues [3••] offer an intriguing explanation for these structural differences. Because of the dipole layer on the plane of the water–lipid interface, a positive electrostatic potential (+300 mV) exists inside the membrane, which results in a more stable environment for anions than for cations. Consequently, K^+ ions require a water cavity in the center of the membrane in addition to helix dipoles to overcome the dielectric barrier, whereas Cl^- only require helix dipoles.

Figure 1

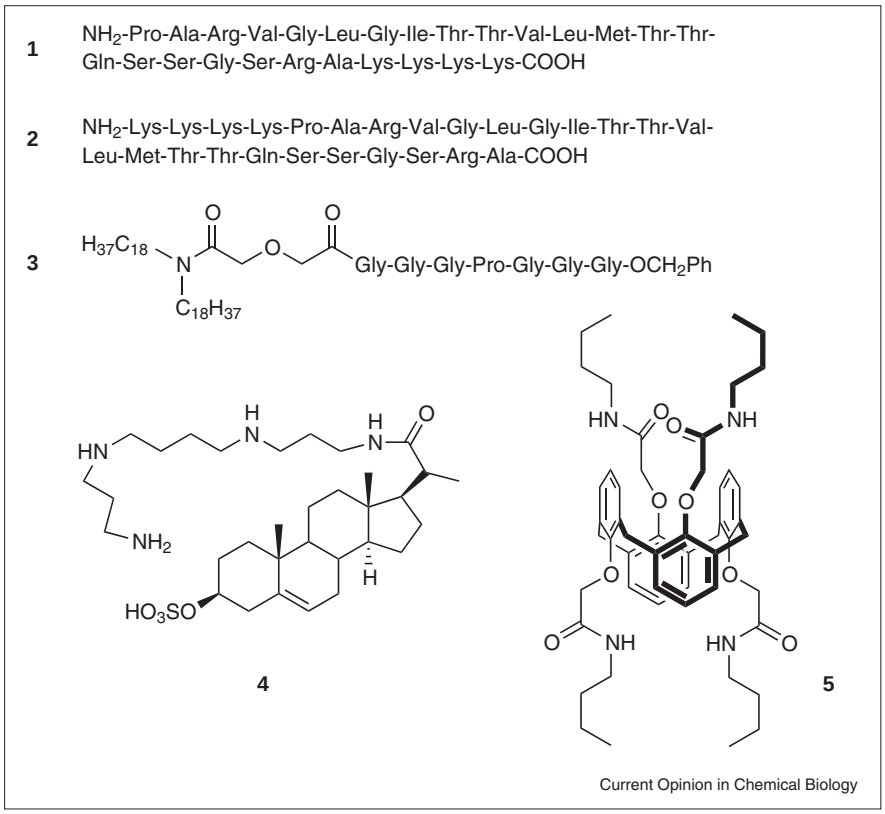


Two architectures for ion channel proteins. **(a)** Cl^- channel. **(b)** K^+ channel. Arrows indicate the direction of ion transport orientation of protein subunits. Adapted from [3••] with permission. Copyright *Nature Publishing* 2002.

Cation transport

Over the past decade, a variety of synthetic compounds have been shown to form cation-conducting channels.

Figure 2



Synthetic Cl^- channels.

Only a brief update will be provided here as extensive reviews have been published [5•,6,7]. In the past year, Gokel and co-workers [8] explored a modified version of their original hydraphile channel by substituting two 12-carbon sidearms in the place of the single sidearm. The resulting compound was more organized within the bilayer and able to transport Na^+ more efficiently. Regen and colleagues [9] attached four molecules of cholic acid to a spermine backbone to generate a series of facial amphiphiles. These amphiphiles form membrane-spanning dimers that transport Na^+ via water-filled pores. Kobuke's laboratory [10,11] has also manipulated the structure of cholic acid and designed synthetic cation channels with rectification properties. Novel alkyl resorcin[4]arenes produce current patterns that are indicative of selective potassium channels in planar lipid bilayers [12]. The aliphatic chains of two resorcin[4]arenes originating from opposite sides of the bilayer may meet to form a water-filled pore. Similar tail-to-tail structures may produce the ion channel activity seen with 5-(12-tricosanoxy)-isophthalic acid monomers, which are the lowest molecular weight channel-forming compounds to date [13]. Fyles and colleagues [14] have also synthesized macrocyclic tetraesters from 5-substituted isophthalic acids and 1,8-octane diol. The majority of these bolaamphiphiles were extremely insoluble but one derivative was successfully incorporated into bilayers and found to form active ion channels.

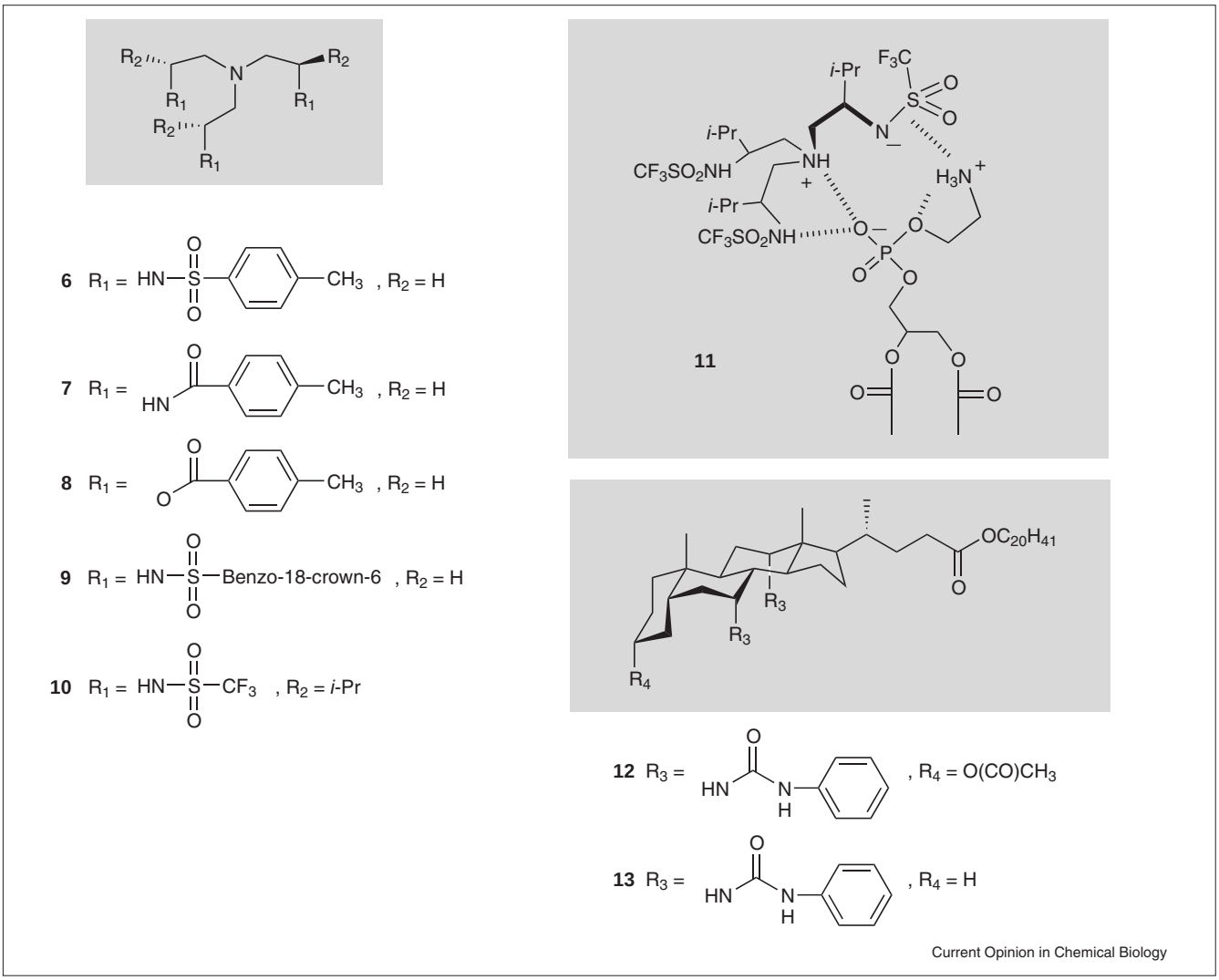
Anion transport

In comparison to cation transport, much less attention has been devoted to the development of artificial anion channels or carriers. However, the past few years have witnessed the first examples of synthetic Cl^- channels. The need for a synthetic Cl^- channel is great because of the number of diseases affected by insufficient Cl^- transport, most notably cystic fibrosis [15].

In the early 1990s the α-helical peptide M2GlyR was reported to aggregate in lipid bilayers and form a chloride-selective channel [16]. The peptide sequence corresponds to the second transmembrane domain of the glycine receptor protein and forms the lining of its Cl^- channel. Solubility of the synthetic peptide was improved by adding four lysine residues to the C-terminal region [17]. This modified peptide, C-K_4-M2GlyR, (Figure 2, **1**) induces both Cl^- and fluid secretion when added to the apical surface of epithelial cell monolayers [18,19].

More recently, the same laboratory has explored an N-terminally modified peptide, N-K_4-M2GlyR (**2**), which generates an enhanced Cl^- current [20]. Potential explanations for the increased activity include a more stable multimeric form, a decreased dissociation rate of the multimers, and the possibility that an endogenous Cl^- secretion pathway is activated in addition to the formation of a new synthetic channel. The peptide has recently been

Figure 3



Synthetic phospholipid translocases.

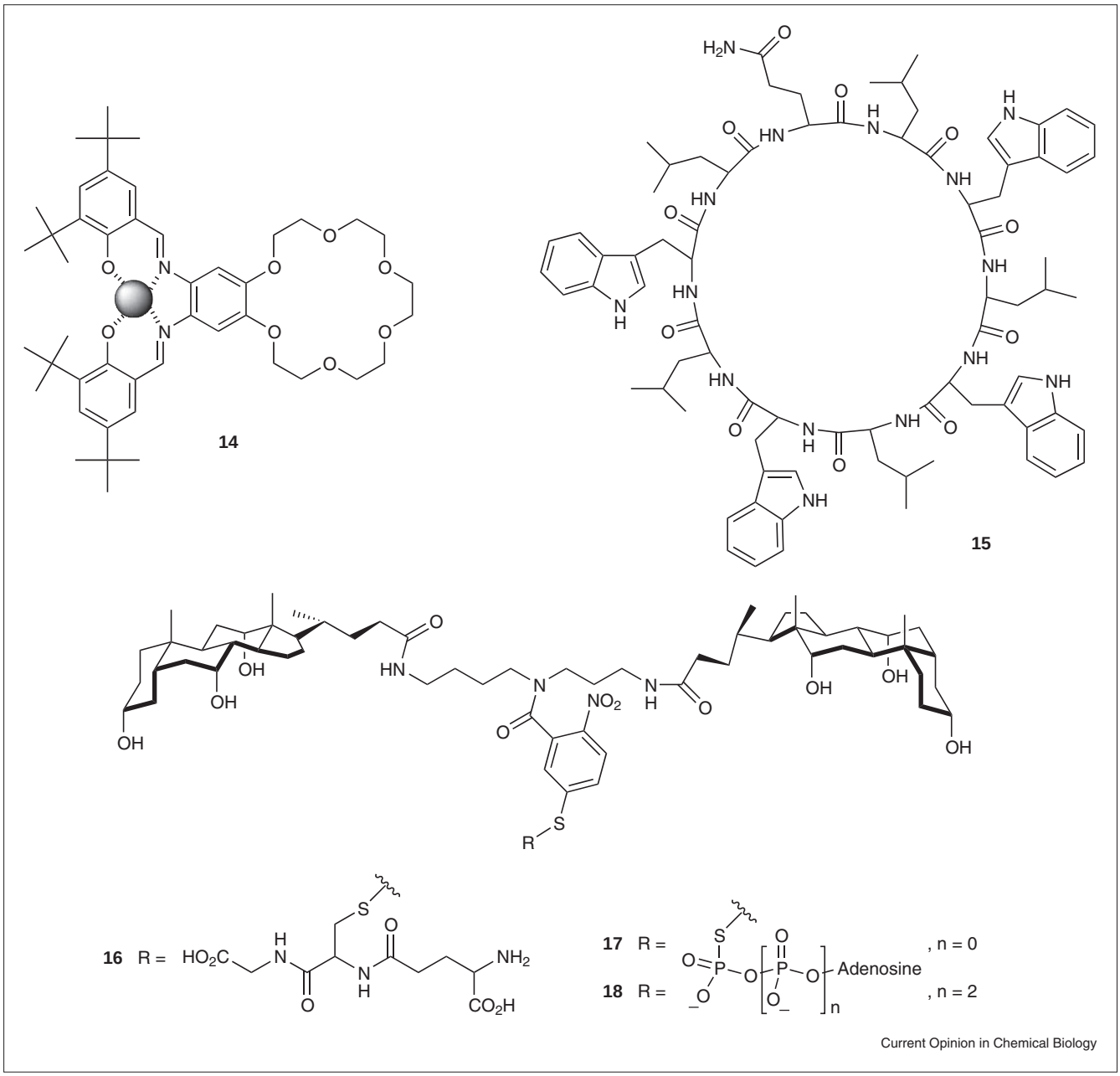
shown to increase glutathione efflux in epithelial cell monolayers derived from a cystic fibrosis patient [21]. The simultaneous demonstration of enhanced Cl^- and glutathione secretion is a new lead for cystic fibrosis treatment.

Aware of the prominence of proline residues in naturally occurring Cl^- transporters, Gokel, Schlesinger and co-workers [22•,23] have designed a heptapeptide that consists of proline flanked on either side by three glycines. Diglycolic acid links the hydrophilic peptide to the hydrocarbon chains that anchor in the membrane. The overall structure of the derivative (**3**) was designed to mimic a phospholipid monomer. Addition of **3** to unilamellar vesicles resulted in a rapid, concentration-dependent release of Cl^-. The importance of the proline-induced 'kink' is revealed by the failure of a leucine-substituted derivative to induce activity. Voltage clamp methods determined that **3** forms a voltage-gated channel with >10:1 Cl^-/K^+ selectivity. The authors hypothesize that two molecules of **3** associate in the external monolayer to generate a 6–7 Å pore that spans the bilayer as a result of the penetration of the anchor group's tails into the internal monolayer.

Early examples of non-peptidic Cl^- channels are a unimolecular, rigid rod-shaped polyol that operates by an OH^-/Cl^- antiport mechanism (transported ions move in opposite directions) [24] and a cholate–spermidine conjugate **4** that operates by a H^+/Cl^- symport (transported ions move in the same direction) mechanism [25]. Recently, compound **4** was used to partially correct defective Cl^- secretion in cystic fibrosis epithelial cells [26]. The synthetic channels restore 30% of the endogenous Cl^- conductance.

Most recently, the Davis laboratory [27••] described a calix[4]arene tetrabutylamide (**5**) that binds HCl in solution and self-assembles in both vesicle and cell membranes to

Figure 4



Amino-acid and other small-molecule transporters.

form long-lasting, stable Cl^- selective channels. Rapid Cl^- transport from unilamellar vesicles occurred at a calix[4]arene:lipid ratio of 1:100. Experiments performed with control compounds highlighted the importance of the hydrophobic butyl chains, secondary amide NHs, and the calix[4]arene framework.

Phospholipid translocation

The asymmetric distribution of phospholipids across mammalian plasma membranes controls important cellular processes such as signaling, fusion, blood coagulation and clearance [28••]. Endogenous translocase enzymes expend energy to maintain this arrangement by enhancing the rate of phospholipid translocation (or flip-flop) between the two monolayers. The main barrier to translocation is passage of the polar phospholipid head-group through the hydrophobic interior of the membrane. The head-group consists of an anionic phosphate group coupled to a charged or zwitterionic moiety such as choline, ethanolamine, or serine. Our laboratory has recently explored two series of compounds that complex the anionic phosphate residue and facilitate phospholipid membrane translocation.

Our first-generation tris(aminoethyl)amine (TREN)-derived translocases were reported in 1999 (Figure 3, **6**–**8**) [29]. The sulfonamide derivative **6** enhances the inward translocation of a fluorescent phosphatidylcholine (PC) probe across surface differentiated vesicles. Amide **7** has only a minor effect, whereas control ester **8** fails to facilitate transport because it lacks available hydrogen bond donors. A structure/activity study of various TREN derivatives revealed mechanistic details [30•]. The increased activity of sulfonamide **6** compared with that of amide **7** is due to two structural factors: stronger association with the PC head-group because of the increased acidity of the sulfonamide NH groups, and a molecular geometry that is able to form a tridentate complex with one of the phosphate oxygens on the head-group. Both sulfonamide **6** and amide **7** facilitate PC transport across human red blood cell membranes [31].

The TREN sulfonamide crown ether **9** possesses sulfonamide NH groups and benzo-18-crown-6 moieties to bind the phosphate and ammonium residues that make up the phosphatidylethanolamine (PE) head-group [32]. Translocase **9** has no effect on the transport of a fluorescent PC probe, whereas it facilitates the translocation of a PE probe. Sulfonamide **10** is significantly more effective as a PE translocase. This may be due to a zwitterionic form of **10** that is complementary with the zwitterionic PE head-group (illustrated as complex **11**).

More recently, we have reported a second-generation of PC translocases based on the cholate scaffold (**12**,**13**) [33••]. These bis(phenylurea) derivatives are approximately an order of magnitude more active than sulfonamide **6**. Concentration and binding studies performed with **12** suggest that a 1:1 supramolecular complex is formed with the PC head-group. A more hydrophilic methyl ester version of **12** was shown to facilitate PC transport across human red blood cell membranes.

Small-molecule transport

A handful of reports regarding small-molecule transport across phospholipid bilayers has appeared in the past year. Transport of the hydrophobic amino acid tryptophan and the neurotransmitter serotonin across bulk liquid membranes was facilitated by crown-ether-functionalized Mn^{III} salicylaldimine complexes (Figure 4, **14**) [34]. These ditopic carriers employ electron-pair-accepting sites (salicylaldimine-bound metal) and hydrogen-bond-accepting sites (crown ether cavity) to bind the polar molecules. They have also been incorporated into phospholipid vesicle membranes and shown to release encapsulated amino acids and neurotransmitters [35]. Ghadiri and co-workers [36•] have used their elegant self-assembling peptide channel to transport L-glutamic acid across unilamellar vesicle membranes. The cyclic decapeptide consists of hydrophobic amino acids with alternating D and L configurations (**15**). Hydrogen bonding dictates the formation of tubular antiparallel β-sheet structures in lipid bilayers.

Moving away from amino acid transport, Regen and colleagues [37] have facilitated the transport of glutathione across phospholipid vesicles. Glutathione is covalently attached to a chlolate–spermidine–Ellman's reagent derivative (**16**), and these monomers are transported across bilayers via an umbrella mechanism. The facial amphiphilicity of the cholate derivatives allows the glutathione moiety to be shielded from the hydrophobic core as it traverses the membrane. More recently, the same group successfully transported two molecular umbrella-nucleoside conjugates across vesicle membranes (**17**,**18**) [38]. Upon reduction of the disulfide bond, thiolated AMP and ATP were released into the inner aqueous compartment with half-lives of 20 min and 120 min, respectively. Walde and co-workers [39] have demonstrated that sodium cholate facilitates nucleotide (ATP, ADP, UTP, UDP) transport through vesicle membranes at non-solubilizing concentrations. It is hypothesized that Mg^{2+} complexes with both cholate and nucleotide to aid in transport.

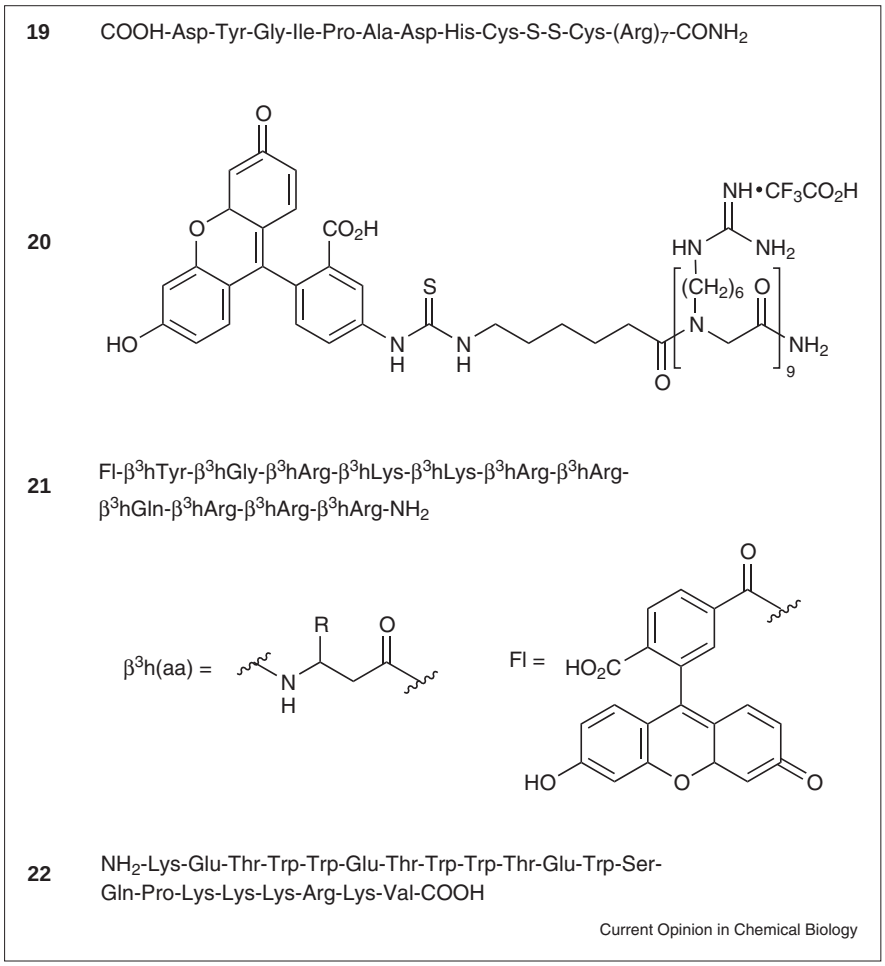
Phosphoinositide polyphosphates (PIP_ns) and inositol polyphosphates (IP_ns) have also been transported across bilayer membranes [40]. Preformed complexes with cationic polyamine carriers ('charge neutralizers') resulted in efficient passive movement across mammalian cells. The efficacy of transport depended upon the carrier molecule (aminoglycoside antibiotic, dendrimeric polyamine, or polybasic protein histone), the specific polyphosphate, and the cell type. Interestingly, PIP_ns could potentially be used in the future to specifically deliver aminoglycosides to eukaryotic cells in an effort to overcome antibiotic resistance.

Peptide/protein transport

In addition to the rapidly progressing field of DNA transfection [41–43], attention has recently turned to the transport of peptides and small proteins across biological membranes. Several naturally occurring proteins possess a specific highly basic domain that imparts an ability to cross biological membranes. These proteins include Tat from HIV-1 virus, Antennapedia from *Drosophila melanogaster*, and VP22 from herpes simplex virus. Several research groups have manipulated these short amino acid sequences to facilitate peptide/protein transport. However, little mechanistic detail is known.

Wender, Rothbard and colleagues have contributed tremendously to this new area of peptide transfection. They established that polymers of at least six arginine residues were more effective than lysine, ornithine or histidine homopolymers at transporting a conjugated fluorescein molecule across cellular membranes [44]. The guanidine head-group was determined to be the critical structural feature. A peptide composed of seven arginine residues (R_7) was covalently attached to a cell-impermeable peptide (ψεRACK) via a cysteine–cysteine disulfide bond (Figure 5, **19**) and was successfully delivered to rat myocytes and intact hearts [45•]. Delivery resulted in increased resistance to damage induced by prolonged

Figure 5



Peptide/protein transporters.

ischemia. The Tat and Antennapedia conjugate peptides were half as effective. Wender and co-workers [46] have also developed peptoid transporter **20**, which is superior to polyarginine. The structure of **20** suggests that the peptide backbone is not a crucial element in the transport process, but the length of the alkyl chain between the arginine head-group and the backbone is important. Peptoid **20** has the added advantage of being protease resistant.

Raines, Gellman and colleagues [47] have discovered another solution to protease degradation by using a β-peptide version of $Tat_{47–57}$ (**21**). β-peptides are composed of β-substituted β-amino acid residues and are protease-resistant. As visualized by confocal microscopy, a β-$Tat_{47–57}$-fluorescein derivative is transported equally as well as an α-peptide derivative. Because the secondary structure of β-peptides is more easily controlled than α-peptides or peptoids, the authors speculate that these peptides will be crucial in elucidating the transport mechanism.

In the above examples, intracellular delivery required covalent attachment of the cargo to the arginine-based transporters. An alternative peptide carrier, Pep-1 (**22**), delivers peptides and proteins via noncovalent interactions [48••]. The 21-residue, amphiphathic peptide consists of three domains: firstly, a hydrophobic domain to efficiently target membranes and to form hydrophobic interactions with the cargo; secondly, a hydrophilic domain derived from the nuclear localization sequence of simian virus 40; and thirdly, a proline-containing spacer domain to separate the hydrophobic and hydrophilic domains. Peptides up to 50 residues in length were rapidly delivered to a variety of cell lines at a Pep-1/peptide ratio of 20:1, whereas green fluorescence protein and β-galactosidase were delivered at a ratio of 40:1. It is hypothesized that Pep-1 forms a stable noncovalent complex with the peptide cargo before crossing the membrane.

Conclusion

Significant progress has been made in the development of small molecules to transport ions, phospholipids and proteins across membrane barriers. Much work remains to be done before all of the transport mechanisms are understood, but the synthetic compounds themselves may already have valuable applications as pharmaceuticals or tools for biological membrane research. For example, synthetic $Cl^–$

channels **2** and **4** have been demonstrated to improve anomalous Cl^- transport in cystic fibrosis cell lines [21,26], whereas phospholipid translocase **6** [29] was recently used as a biological tool to study the peroxisome proliferator-activated receptor γ [49].

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