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An emerging combinatorial technology harnesses the ribosome to access unnatural peptide chemical space for the rapid discovery of novel macrocycles.

mRNA display: from basic principles to macrocycle drug discovery

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We describe a new discovery technology that uses mRNA-display to rapidly synthesize and screen macrocyclic peptide libraries to explore a valuable region of chemical space typified by natural products. This technology allows high-affinity peptidic macrocycles containing modified backbones and unnatural side chains to be readily selected based on target binding. Success stories covering the first examples of these libraries suggest that they could be used for the discovery of intracellular protein–protein interaction inhibitors, highly selective enzyme inhibitors or synthetic replacements for monoclonal antibodies. The review concludes with a look to the future regarding how this technology might be improved with respect to library design for cell permeability and bioavailability.

Introduction

Drug developers continue to explore new approaches and molecular modalities in their continued efforts to identify modulators of the extremely well validated targets that have proven 'undruggable' with small molecules. These targets are often difficult to address with small molecules because they are not enzymes with suitable small molecule binding sites, and their function in various signaling pathways is based on their interaction with other proteins. It is believed that drugging these targets will require accessing new chemical space where larger yet cell permeable molecules reside. In the past few years there has been an increased interest in exploring larger (700–1900 Da) macrocyclic compounds as a new modality for inhibiting intracellular protein–protein interactions. To date, the best representatives of this region of chemical space are macrocyclic natural products and their analogs, which include rings of polyketides, peptides, depsipeptides, peptoids, peptidomimetics, lipopeptides and backbone heterocycle-containing peptide oligomers.

Polyketide synthases (PKSs) and nonribosomal peptide synthases (NRPSs) are complex biosynthetic machines that synthesize the majority of known macrocyclic natural products [1,2]. PKSs and NRPSs use thioester-activated building blocks to synthesize linear co-polymers that are cyclized via an enzyme-catalyzed intramolecular reaction. PKSs link residues with a carbon–carbon bond via a reaction known as a Claisen condensation, whereas NRPSs mediate the attack of an amine or hydroxyl functional group on a thioester-activated residue to form

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1359-6446/06/$ - see front matter http://dx.doi.org/10.1016/j.drudis.2013.10.011

amide (N–C) and ester (C–O) bonds. Interestingly, the peptidyl transfer center (PTC) of the ribosome has the ability to catalyze the formation of the latter two types of inter-residue bond using a different mechanism, and this activity can be harnessed *in vitro* to construct peptide macrocycles with heterogeneous natural-product-like structures [3].

Despite the chemical distinction between PKSs and NRPSs, at the biological level these two biosynthetic pathways share enough similarities to be able to 'crosstalk', allowing organisms to expand the diversity and chemical space achievable through the synthesis of hybrid polyketide or polypeptide secondary metabolites. Polyketides, in general, contain fewer H-bond donors (HBDs) compared with nonribosomal peptides, a crucial parameter for the ability of a macrocycle to partition into a membrane, potentially leading to increased cell permeability.

Several groups are using their detailed understanding of PKSs and NRPSs to engineer these biosynthetic machines for the production of novel hybrid macrocycles [4,5]. However, it is also conceivable that novel hybrid molecules could be produced ribosomally by incorporating polyketide-like residues as amino acid side chains or as N-terminal extensions on the initiating aminoacyl-tRNA [6]. Following translation, the hybrid molecules could then be cyclized using the various chemistries discussed later in this review to generate hybrid macrocycles.

Although the majority of macrocyclic natural products are synthesized on PKSs and NRPSs, some organisms use the ribosome to synthesize similar compounds by post-translational enzymatic processing of linear peptides to generate peptide macrocycles that can contain multiple nonproteinogenic residues [7]. Regardless of synthetic route, the macrocyclic natural products have yielded several useful drugs, despite the fact that their physicochemical properties would have predicted poor bioavailability. The structural characteristics of natural macrocyclic molecules that lead to their unique abilities, inhibition of protein–protein interactions combined with cell permeability, are described below.

Features of natural product macrocycles

Cyclization

Cyclization restricts the conformational flexibility of a molecule to a subset of the structures sampled by the acyclic form, and can effectively pre-organize a larger compound for target binding. As a result of this pre-organization, the entropic penalty for target binding is decreased, and in some cases cyclization can therefore lead to an increase in target affinity. Because macrocycles have fewer conformations that can be compatible with the binding off-targets, they can also achieve improved target specificity. Achieving specificity among closely related enzymes or target isoforms can be challenging with small molecules, and this is another area in which macrocycles have garnered interest.

Depending on the method of cyclization, the C and/or N terminus can be sequestered, which increases the stability against proteolytic degradation by carboxyl, amino and dipeptidyl peptidases [i.e. dipeptidyl peptidase 4 (DPP-4)]. The enhanced proteolytic stability of macrocycles can also derive from the conformational distortion of endopeptidase recognition sites.

Several cyclic topologies are observed in natural product macrocycles, including head-to-tail, side-chain-to-side-chain, branched-chain cyclization (to either the N or C terminus) and bi-cyclization. Not all natural product macrocycles are 'true cycles' because they can contain an acyclic extension (lariat) or multiple cycles linked by short acyclic regions. In addition to the rigidity afforded by cyclization, natural product macrocycles also utilize proline (and its derivatives), N-methyl amino acids, oxazoles, thiazoles and alpha-alpha di-substituted residues to restrict dihedral angles locally.

Chemical diversity

Although the properties of natural product macrocycles derive in part from their reduced conformational flexibility, this class of molecules also displays remarkable chemical diversity with over 500 nonproteinogenic amino acids identified [8] (http://bioinfo.lifl.fr/norine/). This set of nonproteinogenic amino acids is rich and diverse, however their presence does not necessarily directly translate to cell permeability and suitable bioavailability. The important features of these natural product building blocks and their role in macrocycles are described below.

Backbone modifications

N-methylation is a common modification present in natural macrocycles found on the nitrogen of the peptide bond or nitrogen-containing side chains. N-methylation can impact the *cis–trans* equilibrium at the local amide bond as a result of the increased steric hindrance caused by the methyl group. Backbone N-methylation greatly enhances proteolytic stability and reduces the number of HBDs. The patterns of N-methylation in macrocycles can also influence their conformation by favoring a particular set of intramolecular H-bonds that stabilizes one structure to the exclusion of others [9]. The incorporation of D-amino acids into a polypeptide chain leads to conformational changes as a result of the directionality of the side chain. D-residues are also known to be involved in the induction of beta and gamma turns [10], and the nonproteogenic D-configuration is also a poor substrate for proteases.

The replacement of a backbone amide bond by an ester bond, as seen in depsipeptides, is an effective way of removing a HBD from a macrocycle and can have a positive impact on its membrane permeability. Potential drawbacks of this modification are that it decreases resistance to chemical hydrolysis and proteases relative to amide bonds, while increasing conformational flexibility because of the lower rotational barrier of the C–O–C bond compared with the C–NH–C bond [11]. The stability of an ester bond in a macrocycle can be context-dependent and more studies are required to establish ways to modulate this parameter by the introduction of neighboring groups that slow down ester hydrolysis. Despite the reduced proteolytic and chemical stability of the ester bond, the natural ester-containing macrocycle FK506 has been recently approved for the treatment of cutaneous T cell lymphoma.

Lipid moieties

One of the functionalities most commonly observed in natural product macrocycles is the presence of lipophilic residues. Saturated and unsaturated (mono and poly) hydrocarbon chains (C5 to C16) are common, either as amino acid side chains or as attach-

ments to amine-, hydroxyl- and/or sulfur-containing side chains. These lipophilic moieties can be as simple as a prenyl group or can be relatively complex with branched or linear chains that contain additional chemical functionalities such as hydroxyl, epoxides, aromatic rings or halogens.

The function of lipidation in natural product macrocycles is generally associated with the ability of hydrophobic moieties to cause membrane targeting and disruption, but lipidation can also co-localize macrocycles with membrane-associated protein targets. Lipidation is also known to effect the conformational dynamics of peptides by providing a hydrophobic surface that can interact in an intramolecular fashion with other side chains and the backbone. Lipidation can also facilitate endocytotic cell uptake by localizing the macrocycle in the membrane. Finally, lipid moieties can increase plasma protein binding, which can decrease the rate of clearance and increase overall bioavailability [12].

An emerging and important area in the study of macrocycles relates to the identification of mechanisms that facilitate permeation across lipid bilayers. These mechanisms can be organized into two general categories: passive spontaneous permeability; and active transport [13]. From a medicinal chemistry perspective, the passive mechanism is more desirable for achieving oral bioavailability, but only a few examples of larger passively permeable natural product peptidic macrocycles are known (Fig. 1).

Membrane permeability

Macrocycles that spontaneously cross cell membranes are characterized by their predominantly hydrophobic side chains, ester backbone linkages, specific patterns of multiple N-methylation and their nonproteinogenic amino acids (Fig. 1) [14–18]. Desolvation of any passively permeable molecule is a crucial step in the partitioning between the aqueous environment and a membrane [19], and hydrophobic side chains facilitate this process. However, the desolvation of the HBDs and H-bond acceptors (HBAs) present in amide, thioester and ester backbone linkages is also crucial. Nature has found an elegant solution to this problem: it employs low-energy macrocycle conformations that engage HBDs and HBAs in intramolecular H-bond networks that effectively replace all of the water interactions. In cyclosporine, the best example of a compound that employs this mechanism, the fully H-bonded conformation also minimizes its 3D polar surface area (Fig. 2). In fact, the understanding of how cyclosporine, a molecule with a molecular weight of ~1200 Da, achieves passive membrane permeability has served as a guide for the design of molecules that can assume similar H-bond networks to achieve intrinsic membrane permeability [20,21].

The detailed characterization of natural product and synthetic macrocycles has allowed the identification of common features that correlate with the ability of these large molecules to permeate cells. If these features could be combined in screenable combinatorial libraries, then compounds from this special region of

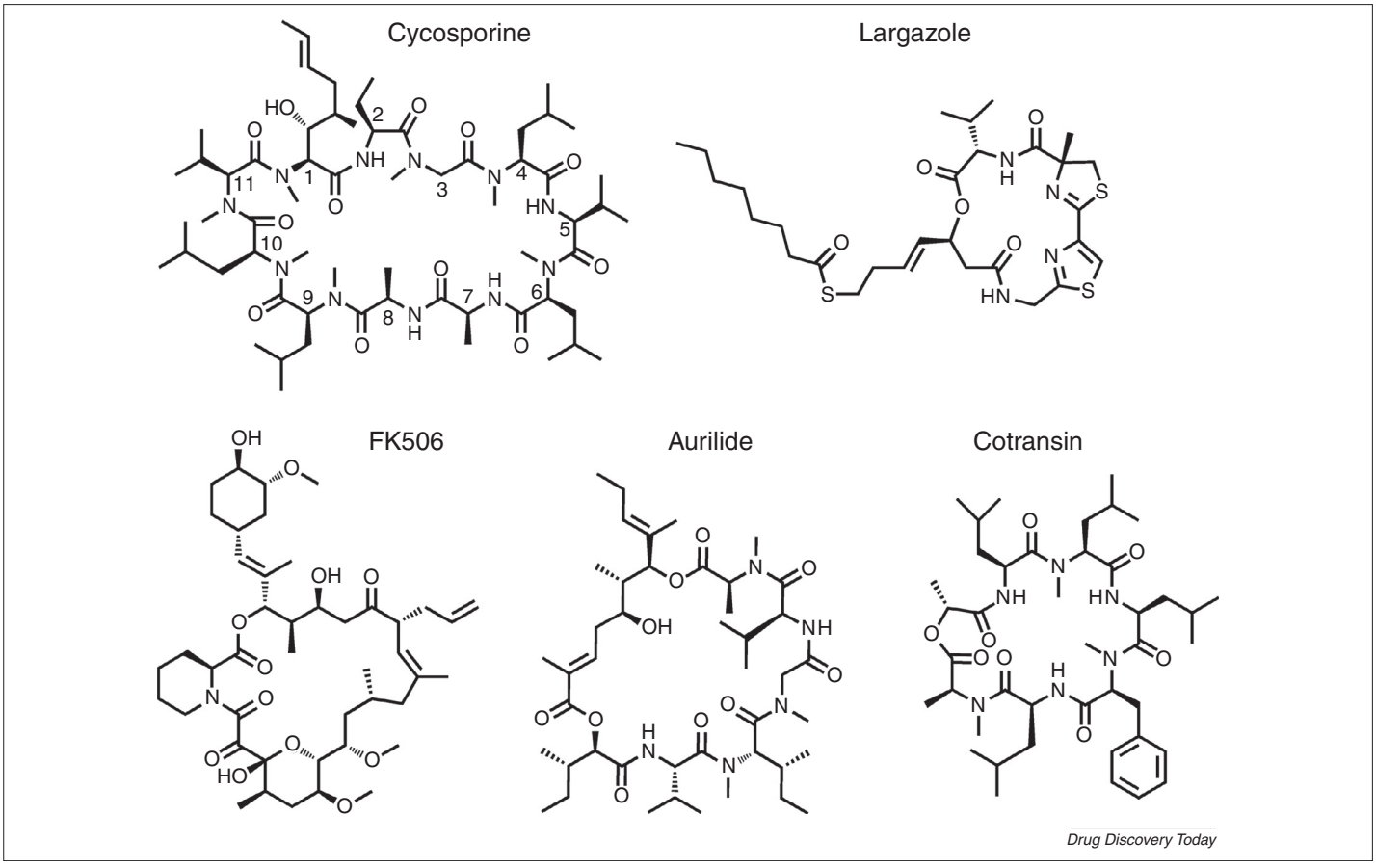

FIGURE 1

Examples of natural product macrocycles that are reported to be cell permeable. Chemical structures of aurilide [52], largazole [53], cotransin [54], cyclosporine [55] and FK506 [55] are shown, exemplifying important features discussed in this review.

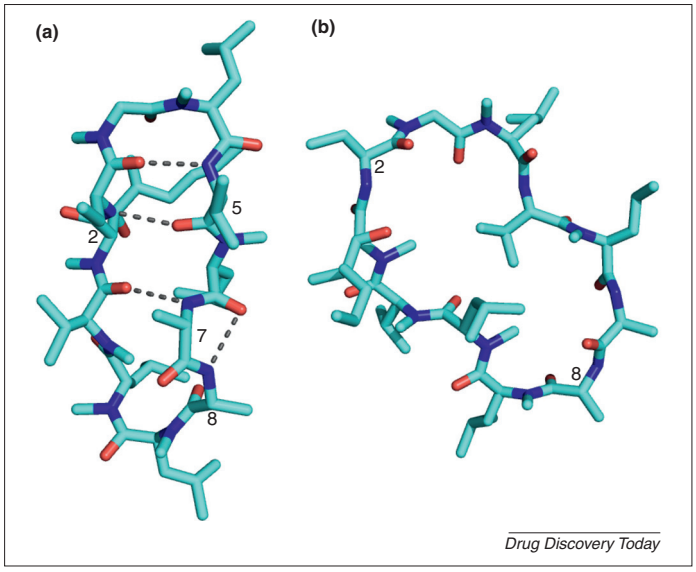

FIGURE 2

Comparison of cyclosporine conformations. **(a)** Crystal structure (CCDB code: DEKSAN.cif) of cyclosporine in chloroform, a low polarity solvent. In this compact structure the H-bond donors (HBDs) and H-bond acceptors (HBAs) are engaged in intramolecular H-bonds, which disfavors interactions with water and promotes membrane permeability. This conformation is thought to be in equilibrium with conformations that interact more extensively with water. **(b)** Cyclosporine structure (PDB code: 1IKF) extracted from a complex with a Fab in water in which the HBDs and HBAs are interacting with water or the Fab.

chemical space with novel functions might be identified. Over the past decade several groups have turned to mRNA-display to create and screen macrocyclic peptide libraries with some of the features considered important for cell permeability. The remainder of this review summarizes this technology and describes the current state-of-the-art and the challenges going forward.

mRNA-display and the PURE system

Discovering macrocycles by mRNA-display

mRNA-display is a peptide *in vitro* selection technology based on the physical linkage of a peptide to a nucleic acid tag (the mRNA that encoded it) that can be amplified by PCR and read by DNA sequencing [22]. The key to this technology is the antimicrobial natural product puromycin, which inhibits translation by mimicking the substrate of the ribosome – the 3′ end of an aminoacyl-tRNA (Fig. 3a). The structure of puromycin resembles the amino acid tyrosine linked via a stable amide bond to the 3′ carbon of a modified adenine nucleoside.

To synthesize and select macrocyclic peptides by mRNA-display an mRNA pool is modified with puromycin on its 3′ end, and then translated in an *in vitro* translation reaction (Fig. 3b). As ribosomes complete the translation of individual mRNAs to the corresponding peptides they encounter the 3′ puromycin. Because puromycin is chemically similar to the 3′ end of aminoacyl-tRNA, it is recognized by the peptidyl transfer center of the ribosome, which catalyzes the transfer of the nascent peptide to the modified tyrosine of puromycin. The mRNA is now covalently attached to the corresponding translated peptide via the puromycin, and the ribosomes are stalled. The initial pool or library mRNAs have

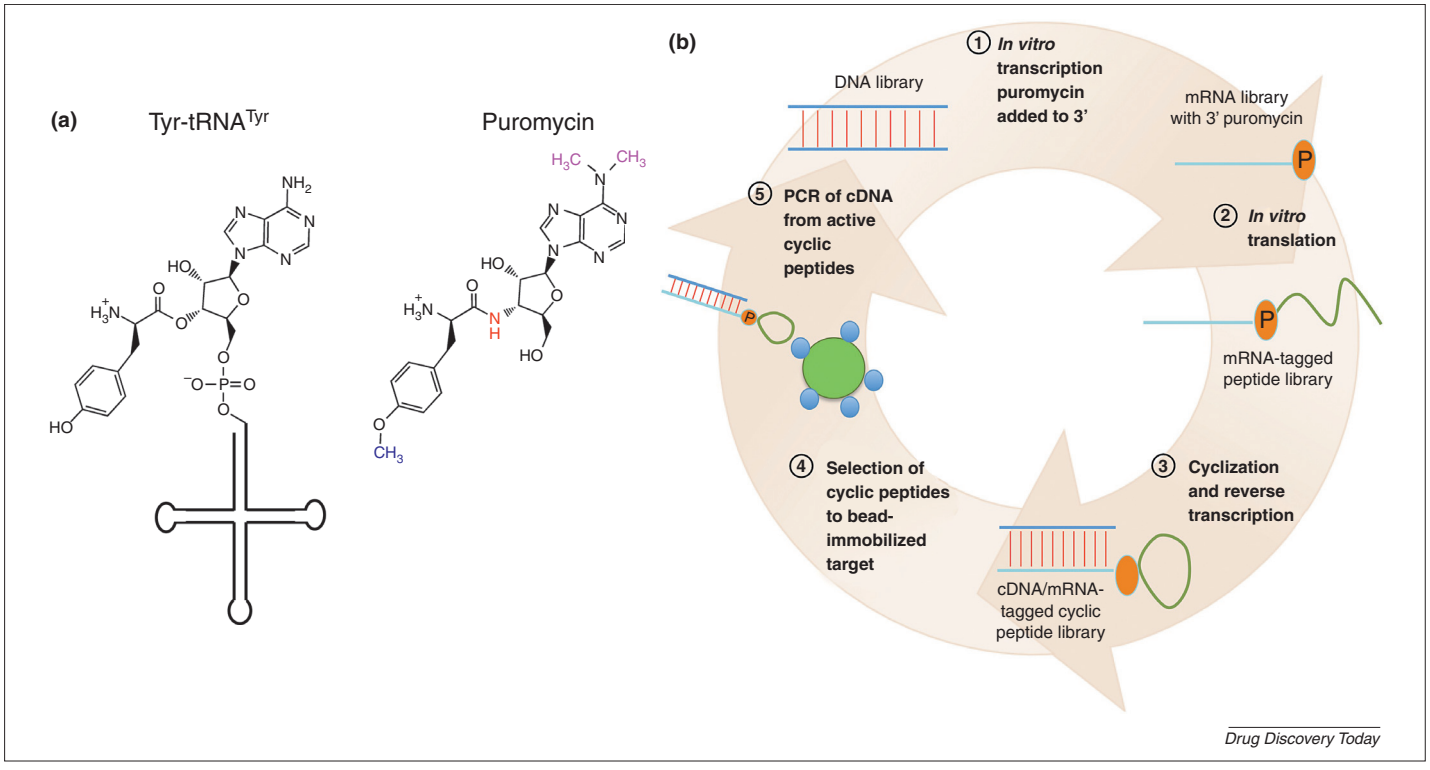

FIGURE 3

Overview of mRNA-display. **(a)** The chemical structure of the 3′ end of Tyr-tRNA^{Tyr} (left) is compared with puromycin (right). Highlighted differences include a *para*-methoxy group on the tyrosine side chain (blue), a nonhydrolyzable amide bond between the tyrosine and the 3′ hydroxyl of ribose (red) and dimethylation of the exocyclic amine of adenine (pink). **(b)** The steps required to perform a single round of *in vitro* peptide-macrocycle selection are described.

now been translated and linked via puromycin to the peptides that they encode in a stable molecular conjugate referred to as an mRNA–peptide fusion. In two separate steps, the peptide and the mRNA component of these fusion molecules are further modified before being subject to *in vitro* selection. The peptide component is cyclized using various chemistries (described below) and the mRNA is reverse transcribed to cDNA for amplification by PCR during *in vitro* selection.

To select cyclic peptides that bind a target of interest, the target (typically a purified biotinylated protein) is immobilized and incubated with the library of cyclized peptide–mRNA:cDNA fusions. The inactive molecules that do not bind the target are washed away, and the cDNAs of the peptides retained by the target are amplified by PCR. The amplified DNA, which has been enriched in sequences that at the peptide level bind the target, can then be used as the template for transcription to regenerate mRNAs. This cycle, from mRNA transcription, through translation, peptide cyclization and reverse transcription, *in vitro* selection against an immobilized target, to PCR, is commonly referred to as round of selection. Because there are practical limitations on the enrichment achievable in each round, even a relatively stringent selection might require between five and seven rounds to converge sufficiently on the tightest binders from libraries of this size.

As a peptide *in vitro* selection technology, mRNA-display is conceptually similar to phage display, which works by linking the peptide on the surface of the phage capsid (phenotype) with the amplifiable and readable DNA (genotype) that encoded it contained within the phage DNA [23]. However, there are three main differences that are important for macrocycle discovery. The first is that all of the steps of mRNA-display are entirely *in vitro* and, therefore, library size is not limited by the need to transform bacteria. Transformation steps impose an upper limit on the diversity of phage display libraries of $\sim 10^9$. By contrast, peptide mRNA-display libraries can be as large as $\sim 10^{13}$, which enables complete coverage of macrocylic peptide libraries ($\sim 10^{12}$) with approximately ten copies of each variant. The oversampling afforded by the sequence capacity of mRNA-display ensures that essentially every sequence has sufficient copies to be selected. A second advantage of mRNA-display is that it is monovalent, with only one copy of the peptide displayed on a given mRNA. The monovalent nature of peptide–mRNA fusions allows the displayed peptides to be enriched based solely on their intrinsic target affinity. By contrast, phage particles display several copies of the same peptide, which produces an avidity effect that allows peptides with modest target affinities to enrich during the course of the selection [23]. Finally, the synthesis of the peptide–mRNA fusion molecules can be done in a modified *in vitro* translation reaction that has been reconfigured with a more drug-like set of amino acids [24–26].

PURE *in vitro* translation system

Historically, mRNA-display has relied on *in vitro* translation systems based on various cellular lysates. These lysate systems are perfectly suited for the selection of proteins and peptides comprising the 20 proteinogenic amino acids, but cannot easily be reconfigured for selection with unnatural amino acids. The most flexible and therefore powerful *in vitro* translation system for mRNA-display is the reconstituted *Escherichia coli* PURE system. PURE is the acronym for 'protein synthesis using recombinant elements'. The PURE system is built from highly purified ribosomes, translation factors, tRNA-synthetases, total *E. coli* tRNA (commercially available), amino acids and a completely defined buffer system. Because the components of the PURE system are entirely defined, it is tremendously flexible, allowing natural amino acids to be readily replaced by more-drug-like unnatural amino acids.

The simplest way to reprogram this system for the selection of unnatural peptides is to omit natural amino acids from the PURE reaction and replace them with chemically similar analogs that are recognized by tRNA-synthetases [26,27]. In fact, over the years an extensive collection of analogs that are efficiently charged by these enzymes, or mutants with altered or relaxed substrate specificity, has accumulated. This approach is straightforward because the unnatural aminoacyl-tRNAs are synthesized *in situ* on their respective natural fully modified tRNAs by the components of the PURE system. However, the overall drug-likeness of the building blocks that are efficient tRNA-synthetase substrates is somewhat limited, and for some amino acids suitable drug-like analogs have not been reported.

To circumvent the selectivity of tRNA-synthetases, peptide libraries have also been prepared using versions of the PURE system in which selected tRNA-synthetases are omitted and the system is supplemented with synthetic tRNAs pre-charged with the desired unnatural amino acids [25,28]. There are three ways to produce these synthetic unnatural aminoacyl-tRNAs that have been used to prepare peptide macrocycle libraries for selection by mRNA-display. The first approach is commonly referred to as 'chemical charging'. This is, in fact, a chemoenzymatic approach, where the tRNA body lacking the terminal two nucleotides is produced by *in vitro* transcription, and is enzymatically ligated to an unnatural aminoacyl dinucleotide that is prepared chemically [29]. The second approach uses chemical transformation to modify a synthetase-charged amino acid while it is attached to a tRNA [30]. This approach is most commonly used to produce N-methyl versions of the natural amino acids by reductive amination. The final approach was developed by Suga and colleagues, who evolved a ribozyme that is capable of esterifying activated amino acids on full-length *in vitro* transcribed tRNAs [31]. Fittingly, this ribozyme has been named 'flexizyme', because it does not recognize the side chain of the activated amino acid, and therefore can be used as a generic tRNA-synthetase to charge a wide range of amino acids and amino acid analogs.

There are advantages and disadvantages to each approach that impact the selection of unnatural amino acids used to reconfigure the PURE system for the synthesis of macrocyclic peptide libraries. The *in situ* approach in which unnatural amino acids are charged onto tRNAs by tRNA-synthetases is the easiest to implement because it does not require the production of individual tRNAs, and only requires changing the amino acid mixture used in the assembly of the PURE system. The translation reaction is also generally more productive because the aminoacyl-tRNAs are continuously synthesized during the translation reaction, and therefore tRNAs are turned over multiple times. However, these systems require careful optimization, are difficult to change and are limited to 20 amino acids because each synthetase and the corresponding codons must be dedicated to a single unnatural amino acid. The

pre-charging approaches, chemical charging or flexizyme, are single turnover and are much more labor intensive, requiring chemical synthesis to prepare the amino acid for attachment to individual *in vitro* transcribed tRNAs. These approaches, however, are necessary for using many of the most interesting unnatural building blocks that are not efficient tRNA-synthetase substrates and breaking the degeneracy of the genetic code to construct libraries with more than 20 amino acids.

Building blocks available for macrocycle libraries

The approaches described above for preparing aminoacyl-tRNAs have enabled several groups to validate the translatability of a chemically diverse collection of unnatural amino acids. This set of PURE-compatible unnatural amino acids represents a virtual menu of building blocks from which macrocyclic peptide libraries can be built. Amino acid analogs with modified backbones are of particular interest in the design of macrocyclic peptide libraries and, although the translational machinery has evolved with L-amino acids, a remarkable set of D-amino, α-hydroxy, N-methyl and N-substituted glycines have been validated for use in the PURE system (Fig. 4a) [6,28,32,33]. D-amino acids are commonly found in natural products and are resistant to cleavage by proteases. The inclusion of a limited number of D-amino acids also allows a macrocyclic peptide library to access unique conformations not available to the corresponding L-amino acid library. When incorporated into a peptide, α-hydroxy acids produce a chemically labile ester bond, which makes them less desirable, but they are the defining feature of depsipeptides, an important class of natural products. N-methyl amino acids are perhaps the most attractive modification of the amino acid backbone. Like D-amino acids, N-methyls are resistant to cleavage by proteases, but they also remove the HBD of the peptide bond, a crucially important feature for intrinsic membrane permeability. Finally, the N-alkyl glycines allow the PURE system to be co-opted for the discovery of peptoids by mRNA-display. Peptoids are inherently more flexible than peptides, and must pay a larger entropic penalty in target binding. However, the inclusion of a limited number of N-substituted glycines might be of interest to explore mixed peptide and/or peptoid macrocyclic libraries.

Among the PURE-validated unnatural side chains there are many types of heterocycles of the type favored by medicinal chemists that are based on modifications of histidine, tryptophan and phenylalanine (Fig. 4b) [27]. In addition to the stacking and HBD and/or HBA opportunities of these side chains, fluorinated derivatives are of particular interest because fluorine is often used in small molecule drugs to increase hydrophobicity, reduce metabolism and to make novel interactions with protein targets [34]. The available heterocyclic ring systems can be complemented with several straight and branched aliphatic side chains to enable libraries to sample side-chain chemical space thoroughly (Fig. 4b) [27,35]. In addition, several pairs of amino acids with reactive side chains for library cyclization have been identified (discussed in detail below).

Library cyclization

Decades of peptide chemistry have established the challenges of cyclizing linear peptides while maintaining their activity. One of the advantages of the mRNA-display approach described here is that the peptides are cyclized before selection against the target. This is crucial to the identification of very potent macrocycles, because it allows the ring size to be optimized concurrently with the peptide sequence. In fact, it is fairly common in selections from macrocyclic peptide libraries with variable ring sizes that each family of related sequences will have only one ring size, suggesting that target-binding motifs and cycle topology are interdependent. Moreover, the precise ring size and conformation of high-affinity macrocylces is typically so crucial for maintaining target affinities that even a single stereochemical inversion (the change of one residue from the L to D configuration) will abrogate target binding.

Peptide libraries screened by mRNA-display are not compatible with head-to-tail cyclization because puromycin and the mRNA block the C termini of the displayed peptides. Therefore, these libraries must be cyclized between the N terminus and a side chain or between two side chains. The components of the cyclization reaction must be compatible with the mRNA tag and the chemistry must be compatible with aqueous solvents. It is also crucial that the cyclization reaction is highly efficient, even with larger rings of up to 12–15 residues, to avoid the selection of linear peptides. The most convenient cyclization chemistries are those that occur spontaneously based on the proximity of the reactive groups in the macrocycle. Peptide libraries cyclized by disulfide bonds between two cysteine side chains are the most straightforward to produce, because the disulfide forms spontaneously by air oxidation in the absence of reducing agents (Fig. 5). However, because disulfide bonds are readily reduced inside cells, these libraries must be reserved for extracellular targets.

Suga and colleagues have developed, and used successfully in numerous projects, a stable thioether cyclization that occurs spontaneously immediately after the peptide is translated [36]. The reaction occurs between a fixed N-terminal chloroacetyl group, introduced via a synthetic aminoacylated initiator tRNA, and a variable cysteine residue in head-to-side-chain fashion. The reaction is entirely dependent on the very high effective concentration of these groups when they are within the same peptide, and goes to completion with macrocyclic ring systems of at least 17 residues.

It is possible to produce a similar side-chain-to-side-chain thioether cross-link between dehydroalanine and cysteine [35]. Dehydroalanine can be generated fairly easily by oxidative elimination of selenolysine, which is an efficient substrate for the lysyl-tRNA-synthetase. However, an issue with this approach is that the reaction is not stereoselective, so the resulting library will be a mixture of D and L alanine. Both of these approaches produce a thioether linkage that, although stable, is prone to oxidation of the sulfur. The sensitivity of thioethers to oxidation is somewhat context dependent, and when it is a problem it can typically be addressed during lead optimization.

Dibromoxylene has been used to cyclize peptide libraries by cross-linking two cysteine side chains. This chemistry is attractive because it is very efficient, and the reaction is compatible with mRNA and aqueous solvents [37]. However, it does not occur spontaneously and the resulting cross-linker contains two thioethers, which potentially doubles the issue of thioether oxidation. It is also possible to cross-link the N-terminal amine of the peptide library to an internal lysine side chain using disuccinimidyl

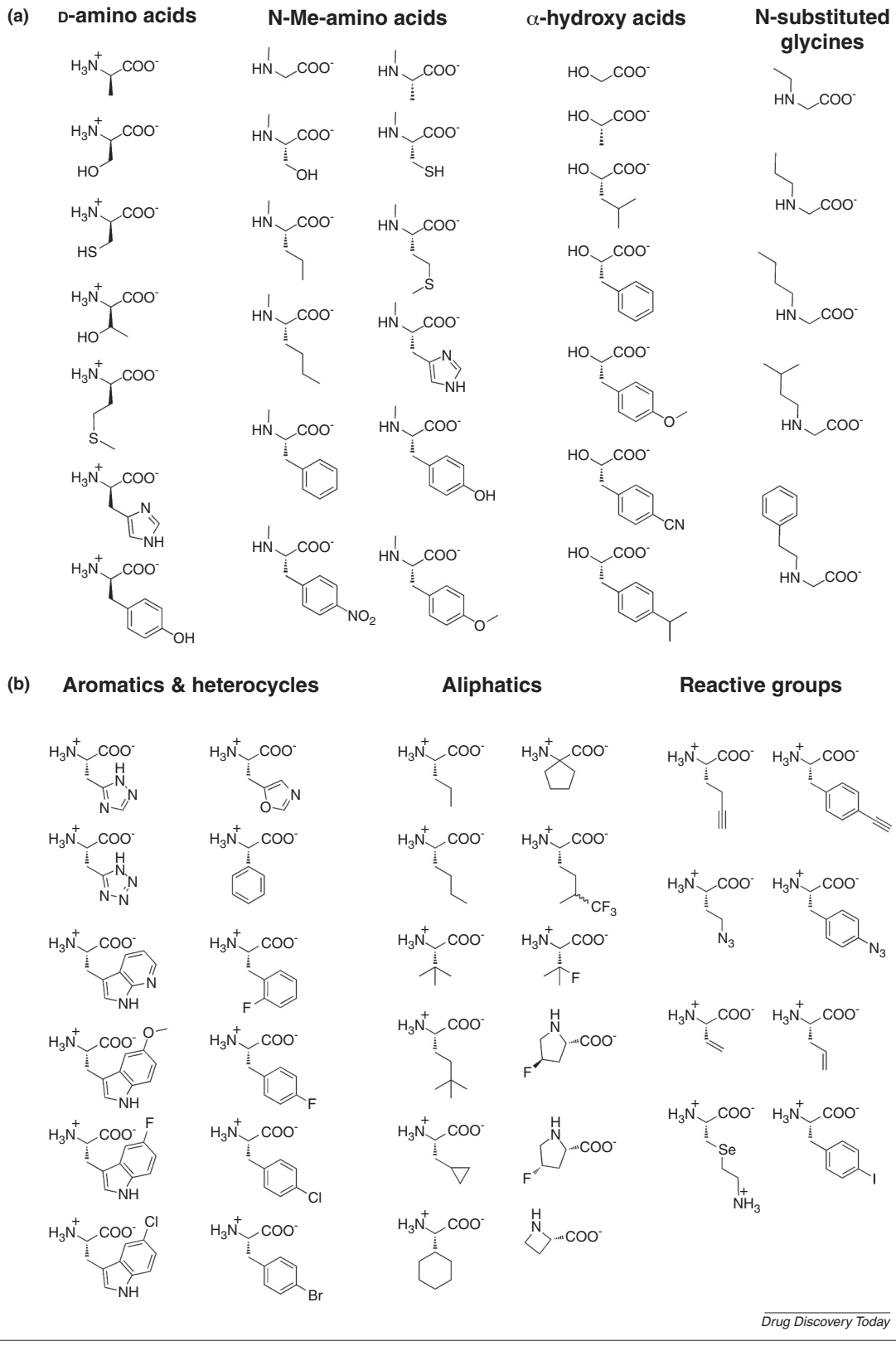

FIGURE 4

Translatable unnatural amino acids validated for use in macrocyclic peptide libraries. **(a)** The chemical structures of unnatural amino acid analogs are organized based on type of main chain modification. **(b)** The chemical structures of unnatural amino acid analogs are organized based on the structure of the side chain.

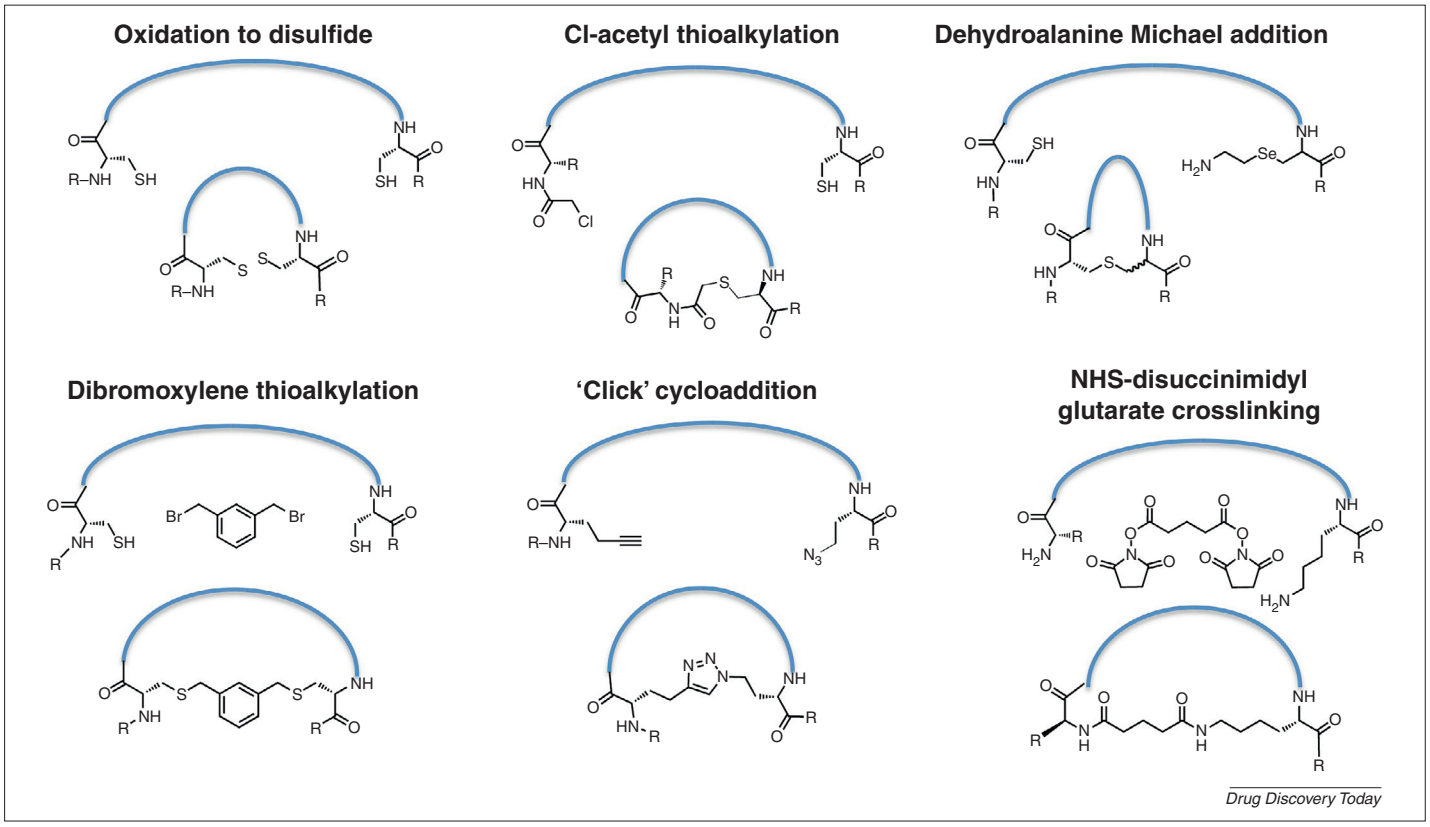

FIGURE 5

Peptide cyclization strategies compatible with mRNA-display. Six macrocycle cyclization chemistries are illustrated, which differ in their ease of use with mRNA-displayed peptide libraries and in the drug-likeness and stability of the ring-closing bond.

glutarate in a head-to-side-chain fashion [38]. This reaction occurs in aqueous solvents, is compatible with mRNA-display and produces a stable cross-linker with the formation of two amide bonds. It is also attractive because it does not require incorporating unnatural amino acids, however the resulting linker is flexible, increasing the entropy term for target binding.

The copper-catalyzed 3 + 2 cycloaddition 'click' reaction is also considered an option for cyclizing peptide libraries for selection by mRNA-display [39]. The reaction can be used to cyclize peptides in side-chain-to-side-chain fashion between azide and alkyne functional groups. The resulting triazole linkage is attractive because it is stable and relatively rigid. However, a successful macrocycle selection using mRNA-display employing click cyclization has not yet been reported.

Library design

There are several strategies for selecting building blocks for constructing macrocyclic peptide libraries depending on the target and the properties desired. For example, if membrane permeability or oral bioavailability is required, then the overall length of the library should be seven-to-nine residues to ensure that hits are less than or equal to ~1000 Da (based on the larger cell permeable natural products). In addition, building blocks probably need to be hydrophobic with minimal HBDs and HBAs, and the number of N-methyl residues should be maximized to limit HBDs further. In effect this approach will produce libraries of macrocyclic peptides with a size and composition similar to the orally available natural product cyclosporine. By contrast, a library for an extracellular target for which parenteral dosing is acceptable only needs to yield macrocyclic peptides with good plasma stability. Presumably, these libraries can be considerably longer, and the monomers could be chosen to maximize chemical diversity for improved target binding, irrespective of polarity.

The number of macrocyclic peptides discovered by mRNA-display from libraries constructed with unnatural amino acids continues to grow. However, these libraries can also be customized for individual targets by incorporating small molecule warheads that direct or enhance the interaction of the library with a desirable site on the target. The resulting macrocyclic peptides, selected in the presence of the conjugated small molecule, effectively extend and enhance the existing interaction with the target, and offer a relatively straightforward approach for rapidly improving the potency or specificity of the parent compound. Li and Roberts first demonstrated this concept in 2003 using mRNA-display to select for a linear natural-peptide–penicillin conjugate that improved the potency of the penicillin parent by 100-fold [40]. In the decade since then, mRNA-display peptide libraries have improved considerably with the advent of the PURE system, which enables the incorporation of multiple unnatural amino acids carrying side chains with orthogonal reactivity for library cyclization and small molecule conjugation.

A potentially very powerful approach could also be envisioned by combining the use of mRNA-display for the creation and screening of macrocyclic peptide libraries with very small drug

fragments identified by fragment-based screening approaches [41]. As fragment libraries and methods have matured, it has become increasingly easier to identify these small compounds, which typically have molecular weights less than 300 Da and micromolar or even low millimolar target affinities. Once these fragments are identified it can be very challenging to improve them rationally, either by 'growing' or 'linking', using structure-based design. However, because they are relatively small, they could be employed as amino acid side chains in target-directed macrocyclic peptide libraries and then subjected to *in vitro* selection by mRNA-display. The combination of these two technologies could provide a way to use small fragments rapidly and more easily in much larger fragment-containing peptide macrocycles. These molecules are likely to have much higher affinities than the free fragments and could be considered either as drug leads themselves or as templates for peptidomimetic design. A somewhat similar approach was taken by Suga and colleagues, who incorporated the ε–N-trifluoroacetyl-Lys analog of ε–N-acetyl-Lys for the selection of potent selective inhibitors of a lysine deacetylase (discussed below).

Targets and selections

Not surprisingly, the potential power of *in vitro* selection by mRNA-display to construct and screen macrocyclic peptide libraries rapidly has attracted significant investment and attention. However, there are only a handful of publications describing the application of different versions of this technology from academic labs, because most of the work is proprietary. In 2011, Hiro Suga's group at the University of Tokyo described the selection of a macrocyclic peptide inhibitor of the serine/threonine protein kinase Akt2 (Fig. 6) [42]. This study actually utilized two libraries with 4–12 random positions that enabled the 20 natural amino acids, flanked by either an N-terminal chloroacetyl-L-tyrosine or a chloroacetyl-D-tyrosine and a C-terminal cysteine, for cyclization. The most potent peptide macrocycles all had the same number of residues (14 including the flanking tyrosine and cysteine) and the N-terminal tyrosine was of the L-configuration, consistent with the concept that macrocycle ring size and conformation are crucial parameters that are readily optimized by selection for high-affinity target binding. These peptides were reasonably potent inhibitors of Akt2 with IC_{50} values of ~100 nM, and demonstrated 10–25-fold selectivity over the other Akt isoforms: Akt1 and Akt3. Because these compounds were selected against an inactive (nonphosphorylated) full-length form of Akt2 but inhibit the activity of the isolated activated kinase domain, it is speculated that their isoform selectivity is a result of an allosteric mechanism.

Around the same time Suga's group also published the selection of a macrocyclic peptide inhibitor of the ubiquitin ligase E6AP [43]. This peptide was selected from a slightly longer variable

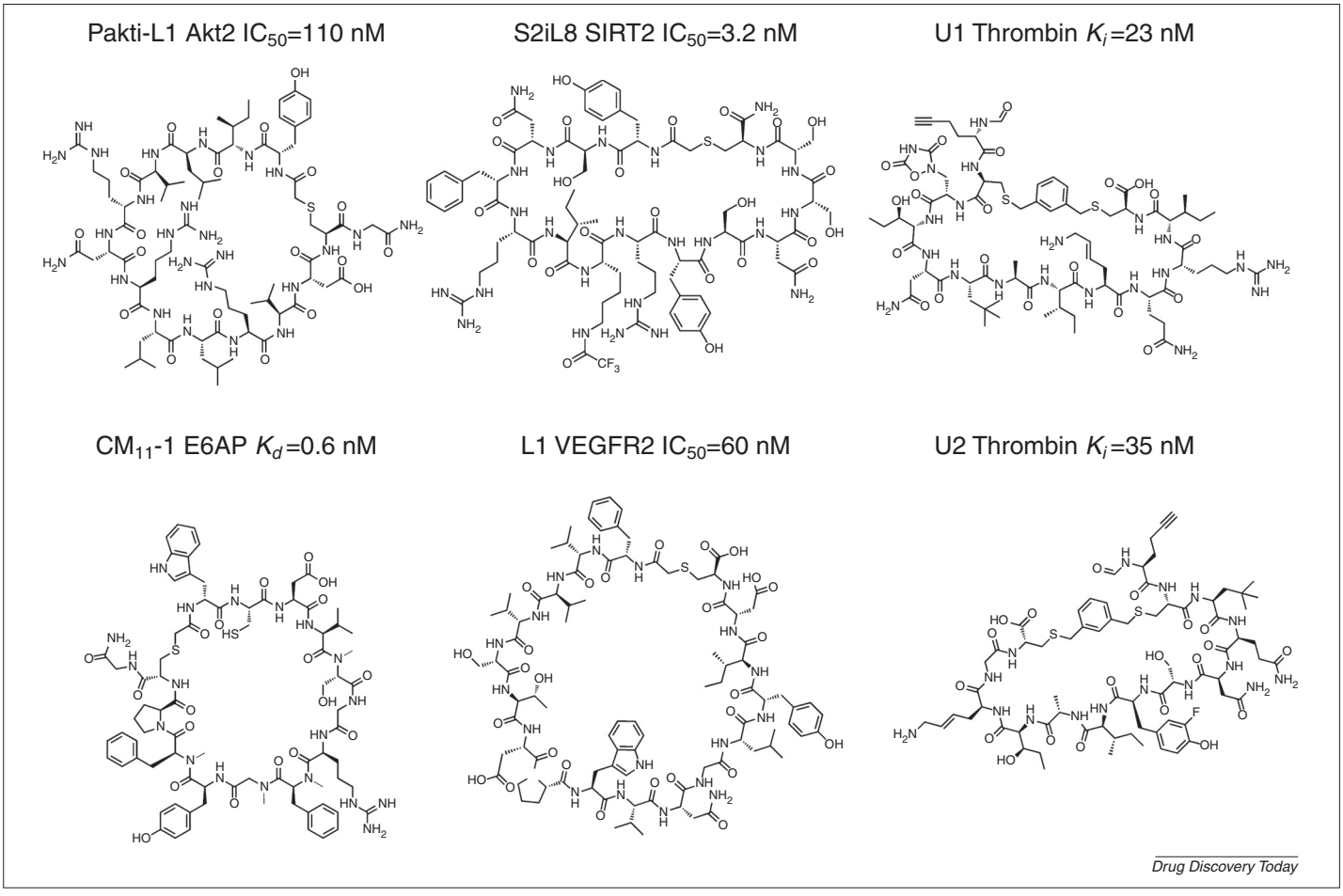

FIGURE 6

Novel macrocycles discovered by mRNA-display.

length library of 7–14 random positions cyclized between an N-terminal chloroacetyl-D-tryptophan and a C-terminal cysteine. However, the translation reaction used for this selection was configured such that each random position allowed for 12 natural amino acids plus N-methyl-phenylalanine, N-methyl-serine, N-methyl-alanine and N-methyl-glycine. The selected macrocycle contained 13 residues with four N-methyl amino acids plus the N-terminal-D-tryptophan. The peptide bound E6AP with a K_d of 0.6 nM and inhibited polyubiquitination in cell lysates at 1 μM. It is proposed that this compound inhibits polyubiquitination by blocking the interaction between E6AP and E2. It is notable that the anti-Akt2 and anti-E6AP macrocyclic peptides inhibited function despite being selected simply for their ability to bind the immobilized target. This is actually fairly common for macrocycles selected by mRNA-display, and it suggests that the ligands selected from these libraries are 'finding' relevant sites that influence target function. In fact, very little *a priori* knowledge about the target structure and function is required to set up and conduct a macrocycle selection.

Demonstrating the warhead approach, the Suga laboratory prepared a targeted library with trifluoroacetyl-lysine for selection against the lysine deacetylase, sirtuin2 (SIRT2) [44]. This residue was centered in two macrocycle libraries of 10–14 residues with either an N-terminal L or D chloroacetyl-tyrosine and a C-terminal cysteine, which allowed for 15 natural amino acids at each random position. All of the selected macrocycles shared a conserved motif around the trifluoroacetyl-lysine residue and contained 13 or 14 residues depending on the stereochemistry of the N-terminal tyrosine. These compounds bound and inhibited SIRT2 with a K_d or IC_{50} ~4 nM with 10–100-fold selectivity over SIRT1 and SIRT3.

In all three examples from the Suga laboratory, potent compounds with nanomolar affinities and selectivity over closely related isoforms were directly selected from these libraries based solely on their ability to bind the target protein without counter screening. The initial libraries that yielded these compounds were designed to sample macrocycle ring size in combination with different stereochemistries for the N-terminal residue used in cyclization. For the SIRT2 and Akt2 selections, natural proteinogenic amino acids were allowed at randomized positions. For E6AP a similar set of natural amino acids was used, except glycine, alanine, serine and phenylalanine were replaced with their N-methyl analogs.

Suga's group also recently selected inhibitors of vascular endothelial growth factor receptor 2 (VEGFR2) from libraries of 10–17 residues produced in a PURE translation system configured with 16 natural amino acids and four backbone-modified unnatural amino acids (cycloleucine, N-methyl-phenylalanine, N-methyl-histidine, D-tyrosine and D-phenylalanine) [45]. The most potent compound consisted entirely of natural L-amino acids, and blocked VEGF-induced human umbilical vein endothelial cell (HUVEC) proliferation with an IC_{50} of 60 nM and inhibited angiogenesis as measured by the HUVEC tube formation assay.

In an effort to demonstrate that a majority of the 20 proteinogenic amino acids can be simultaneously substituted with translatable unnatural analogs, Guillen-Schlippe *et al.* exploited the substrate promiscuity of tRNA-synthetases to construct a library that allowed 12 unnatural and eight natural amino acids at each of ten random positions [37]. This random region was flanked by cysteine residues for cyclization with dibromoxylene to construct a fixed-ring library. The selection yielded thrombin inhibitors with K_is of 23 and 35 nM. Although many of the unnatural amino acids used in this study did not improve drug-likeness relative to the natural amino acids they replaced, this system served as a proof-of-concept that wholesale changes could be made to the PURE translation reaction with an efficiency and fidelity suitable for *in vitro* selection by mRNA-display.

Tomorrow's macrocycles

To date, two types of macrocyclic peptide libraries for selection by mRNA-display have been described. The first, described by Suga and colleagues, is configured with mostly natural amino acids and a handful of N-methyl or D-amino acids [42,43,44,45]. The second type of library, described by Guillen-Schlippe *et al.*, was constructed with 12 unnatural and eight natural amino acids to demonstrate the feasibility of accessing unnatural macrocycle chemical space [37].

The macrocycles selected from these libraries have demonstrated the power of this approach to discover potent, selective and stable inhibitors against several targets of interest. Because the hits from these libraries are cyclic and highly potent, it is believed that they could be optimized for cell permeability using the principles of small molecule drugs (reduction of molecular weight, polarity, HBDs and HBAs, and rotatable bonds). However, with continued improvement in library design it should be possible to use this technology for the direct selection of cell permeable macrocyclic peptides.

The ability of macrocycle libraries to deliver cell permeable hits will be increased as the amino acid sets are further optimized to eliminate charged side chains and by the inclusion of residues with fewer HBDs and HBAs (i.e. more like cyclosporine). As such, it will be important for the next generation of amino acid sets to strike the right balance between hydrophobicity for cell permeability and carefully chosen functionality for target binding. Unlike the current libraries that have delivered inhibitors to a diverse set of targets and surfaces, the use of cyclosporine-like libraries might need to be initially restricted to the targeting of more hydrophobic binding sites. However, as library design rules for cell permeable macrocycles emerge so too will strategies for adding back the chemical diversity required for binding more polar binding sites.

Another parameter that is likely to be crucial for striking the right balance between cell permeability and activity is the overall size of the molecules in these libraries. On the one hand, shorter macrocyclic libraries of seven-to-nine residues and molecular weights in the 700–1000 Da range are most likely to yield cell permeable compounds. On the other hand, the longer libraries of 10–15 residues and molecular weights ranging from 1000–1500 Da are of considerably higher diversity (with each randomized position the diversity increases ~16–20-fold, depending on the number of amino acids).

In principle, it is possible to reconstruct the genetic code completely by co-opting the stop codons and some of the redundant sense codons to generate a translation system with approximately 35 orthogonal tRNA–codon pairs. This increased building block diversity would increase the diversity of smaller macrocycle libraries, potentially allowing the isolation of high-affinity

compounds with lower molecular weights that are more likely to be cell permeable. Building such a system would be a synthetic biology tour de force, requiring the production and optimization of an *in vitro* translation system with 35 individual synthetic tRNAs plus the synthesis of library DNA from the corresponding 35 codon trimer phosphoramidites (only 20 are currently commercially available). However, as a step toward such a system, Ra Pharmaceuticals has generated libraries from an 18 amino acid set of individual synthetic tRNAs, and continues to develop methods for increasing this number.

As the ability to construct libraries from increasing numbers of synthetic tRNAs develops, it will also be important to maximize the chemical diversity of the unnatural amino acids these tRNAs carry. Whereas the chemical space accessed by the current set of translation-validated amino acids is certainly sufficient to generate macrocylic peptide ligands to drug targets of interest, approaches to engineering the translation machinery might enable these libraries to be constructed from amino acid sets with even more diverse modifications and improved bioavailability.

EF-Tu is the translation factor that binds and delivers aminoacyl-tRNAs to the A-site of the ribosome. The affinity of EF-Tu for a specific aminoacyl-tRNA is dependent on the tRNA and the esterified amino acid, and it is believed that the poor translation of certain modified amino acids is a result of reduced EF-Tu binding [46,47]. It should be possible to use structure-based and/or *in vitro* selection approaches to generate mutant forms of EF-Tu that are customized for the translation of particular classes of desirable unnatural amino acids that are not efficiently translated with the natural translation system. Uhlenbeck and colleagues identified a key histidine side chain in EF-Tu that interacts with the side chains of aromatic amino acids charged on tRNAs, and demonstrated that mutation of this residue could modulate the interaction of EF-Tu with aminoacyl-tRNAs [48]. Lee *et al.* went even further, by developing an *in vitro* evolution strategy to select a variant of EF-Tu with six point mutations, including the key histidine residue, which improves the translation of phosphoserine [49]. Conversely, it might also be possible to compensate for the suboptimal interaction of unnatural amino acids with EF-Tu by engineering tRNAs. To this end, Uhlenbeck and colleagues have shown that three consecutive base pairs in the T-stem can be tuned to improve EF-Tu binding by >3 kcal/mol [50].

Finally, Chin and colleagues recently developed an orthogonal ribosome-mRNA system that enables the *in vivo* evolution of a duplicated *E. coli* ribosomal RNA gene without affecting protein synthesis and viability [51]. This technology was used to identify Ribi-Q1, a 16s rRNA variant with two point mutations in the A-site decoding center that efficiently translates tRNAs with four-base anticodons. This engineered ribosome could be used to expand well beyond the 35 tRNA–codon pairs currently available for reassignment to drug-like amino acids and macrocyle discovery. At this time the number of truly orthogonal codon–anticodon pairs in a four-base genetic code is unknown, but it is likely that these libraries could be generated from sets of over 100 amino acids. In principle, this orthogonal ribosome system could also be used to evolve the peptidyl transferase center of the ribosome to enable or improve the incorporation of monomers other than amino acids.

It should be noted that for cyclosporine, the best understood intrinsically membrane permeable cyclic peptide, cell permeability is not only dependent on the amino acid content but also on the ability of this molecule to access conformations in which all HBDs are engaged in intramolecular H-bonds. If it becomes evident that the cell permeability of smaller more-drug-like compounds from future libraries limited to seven-to-nine residues and improved amino acid sets is not sufficient, then it could be necessary to design conformationally templated libraries that favor conformations with extensive intramolecular H-bond networks. For example, libraries that have the potential to adopt the cyclosporine conformation could be generated by restricting the library to the ring size of cyclosporine and mimicking its pattern of N-methyl and amino acids.

Historically the sequences of peptides selected by mRNA-display have been identified simply by their abundance in 20–100 clones from the final round. However, next-generation sequencing technologies, typically used for whole-genome or transcriptome sequencing, can obtain 10–150 million sequences, and offer a considerably more powerful approach for the analysis of mRNA-display selections. The ability to analyze millions of sequences enables active sequences to be identified by abundance with statistical significance much earlier in the selection, saving time and minimizing the accumulation of biases that are inherent to each round of mRNA-display (transcription, PCR and translation). Moreover, this approach not only allows for the identification of the most abundant sequences but of whole families of related sequences, providing valuable SAR data that can be used to inform the hit optimization process. Lastly, the DNA from each round of the selection can be barcoded and multiplexed, and a global analysis of the entire dataset can be applied to track the emergence of each sequence during the selection. This global analysis can identify less-abundant sequences that might not amplify well from round to round but are very tight binders that can be identified based on their strong enrichment.

Concluding remarks

mRNA-display has been combined with customized *in vitro* translation systems and convenient cyclization chemistries to discover peptide macrocycles with unnatural side chains and modified backbone residues from libraries of $\sim 10^{12}$ variants. Early success building and applying this technology with first-generation libraries has identified novel potent molecules that have many of the features of the macrocyclic natural products that exemplify the 'large small molecule' region of drug space that has been difficult to access with combinatorial approaches. The tools and strategies are in place for improving the cell permeability of these libraries by reconfiguring them with complete sets of drug-like amino acid analogs in smaller macrocyclic rings, which will enable this technology to deliver on the promise of rapidly producing high-affinity bioavailable hits against any drug target.

Conflicts of interest

J.W.S. is a founder and a shareholder of Ra Pharmaceuticals. K.J. and A.R. are employees and shareholders of Ra Pharmaceuticals, which is commercializing a version of the technology covered in this review.

References

1 Grünewald, J. and Mahariel, M.A. (2006) Chemoenzymatic and template-directed synthesis of bioactive macrocyclic peptides. *Microbiol. Mol. Biol. Rev.* 70, 121–146
2 Hertweck, C. (2009) The biosynthetic logic of polyketide diversity. *Angew. Chem. Int. Ed.* 48, 4688–4716
3 Fahnestock, S. and Rich, A. (1971) Ribosome-catalyzed polyester formation. *Science* 23, 340–343
4 Du, L. *et al.* (2001) Hybrid peptide-polyketide natural products: biosynthesis and prospects toward engineering novel molecules. *Metab. Eng.* 3, 78–95
5 Bowers, A.A. (2012) Biochemical and biosynthetic preparation of natural product-like cyclic peptide libraries. *Med. Chem. Commun.* 3, 905–915
6 Kawakami, T. (2008) Ribosomal synthesis of polypeptoids and peptoid-peptide hybrids. *J. Am. Chem. Soc.* 130, 16861–16863
7 McIntosh, J.A. *et al.* (2009) Ribosomal peptide natural products: bridging the ribosomal and nonribosomal worlds. *Nat. Prod. Rep.* 26, 537–559
8 Caboche, S. *et al.* (2010) Diversity of monomers in nonribosomal peptides: towards the prediction of origin and biological activity. *J. Bacteriol.* 192, 5143–5150
9 Chatterjee, J. *et al.* (2013) *N*-methylation of peptides and proteins: an important element for modulating biological functions. *Angew. Chem. Int. Ed.* 52, 254–269
10 Mahalakshmi, R. and Balaram, P. (2007) The use of D-amino acids in peptide design. In *In D-Amino Acids: A New Frontier in Amino Acid and Protein Research* (1st edn) (Konno, R. *et al.* eds), pp. 415–430, Nova Science Publishers
11 Bionda, N. *et al.* (2012) Effects of cyclic lipodepsipeptide structural modulation on stability, antibacterial activity and human cell toxicity. *ChemMedChem* 7, 871–882
12 Zhang, L. and Bulaj, G. (2012) Converting peptides into drug leads by lipidation. *Curr. Med. Chem.* 19, 1602–1618
13 Bock, J.E. *et al.* (2013) Getting in shape: controlling peptide bioactivity and bioavailability using conformational constrains. *ACS Chem. Biol.* 8, 488–499
14 Chatterjee, J. *et al.* (2006) *N*-Methylated cyclic pentaalanine peptides as template structures. *J. Am. Chem. Soc.* 128, 15164–15172
15 White, T.R. *et al.* (2011) On-resin *N*-methylation of cyclic peptides for discovery of orally bioavailable scaffolds. *Nat. Chem. Biol.* 7, 810–817
16 Chatterjee, J. *et al.* (2008) *N*-methylation of peptides: a new perspective in medicinal chemistry. *Acc. Chem. Res.* 41, 1331–1342
17 Rand, A.C. (2012) Optimizing PK properties of cyclic peptides: the effect of side chain substitutions on permeability and clearance. *Med. Chem. Commun.* 3, 1282–1289
18 Beck, J.G. (2012) Intestinal permeability of cyclic peptides: common key backbone motifs identified. *J. Am. Chem. Soc.* 134, 12125–12133
19 Leung, S.S.F. (2012) Testing physical models of passive membrane permeability. *J. Chem. Inf. Model.* 52, 1621–1636
20 Rezai, T. *et al.* (2006) Testing the conformational hypothesis of passive membrane permeability using synthetic cyclic peptide diastereomers. *J. Am. Chem. Soc.* 128, 2510–2511
21 Loosli, H. *et al.* (1985) Peptide conformations. Part 31. The conformation of cyclosporine A in the crystal and in solution. *Helv. Chim. Acta* 68, 682–704
22 Roberts, R.W. and Szostak, J.W. (1997) RNA-peptide fusions for the *in vitro* selection of peptides and proteins. *Proc. Natl. Acad. Sci. U. S. A.* 94, 12297–12302
23 Sidhu, S.S. *et al.* (2000) Phage display for selection of novel binding peptides. *Methods Enzymol.* 328, 333–363
24 Shimizu, Y. *et al.* (2001) Cell-free translation reconstituted with purified components. *Nat. Biotechnol.* 19, 751–755
25 Forster, A.C. *et al.* (2001) A simplified reconstitution of mRNA-directed peptide synthesis: activity of the epsilon enhancer and an unnatural amino acid. *Anal. Biochem.* 297, 60–70
26 Josephson, K. *et al.* (2005) Ribosomal synthesis of unnatural peptides. *J. Am. Chem. Soc.* 127, 11727–11735
27 Hartman, M.C.T. *et al.* (2007) An expanded set of amino acid analogs for the ribosomal translation of unnatural peptides. *PLoS ONE* 10, e972
28 Ohta, A. *et al.* (2008) Polymerization of α-hydroxy acids by ribosomes. *ChemBioChem* 9, 2773–2778
29 Ellman, J. *et al.* (1991) Biosynthetic method for introducing unnatural amino acids site specifically into proteins. *Methods Enzymol.* 202, 301–336
30 Merryman, C. and Green, R. (2004) Transformation of aminoacyl tRNAs for the *in vitro* selection of "drug like" molecules. *Chem. Biol.* 11, 575–582
31 Goto, Y. *et al.* (2011) Flexizymes for genetic code reprogramming. *Nat. Protoc.* 6, 779–790
32 Fujino, T. *et al.* (2013) Reevaluation of the D-amino acid compatibility with the elongation event in translation. *J. Am. Chem. Soc.* 135, 1830–1837
33 Kawakami, T. *et al.* (2008) Messenger RNA-programmed incorporation of multiple *N*-methyl-amino-acids into linear and cyclic peptides. *Chem. Biol.* 15, 32–42
34 Muller, K. *et al.* (2007) Fluorine in pharmaceuticals looking beyond intuition. *Science* 317, 1881–1886
35 Seebeck, F.P. and Szostak, J.W. (2006) Ribosomal synthesis of dehydroalanine-containing peptides. *J. Am. Chem. Soc.* 128, 7150–7151
36 Goto, Y. *et al.* (2008) Reprogramming the translation initiation for the synthesis of physiologically stable cyclic peptides. *ACS Chem. Biol.* 3, 120–129
37 Guillen-Schlippe, Y.V. *et al.* (2012) *In vitro* selection of highly modified cyclic peptides that act as tight inhibitors. *J. Am. Chem. Soc.* 134, 10469–10477
38 Millward, S.W. *et al.* (2005) A general route for post-translational cyclization of mRNA display libraries. *J. Am. Chem. Soc.* 127, 14142–14143
39 Wang, Q. *et al.* (2003) Bioconjugation by copper(I)-catalyzed azide-alkyne [3+2] cycloaddition. *J. Am. Chem. Soc.* 125, 3192–3193
40 Li, S. and Roberts, R.W. (2003) A novel strategy for *in vitro* selection of peptide–drug conjugates. *Chem. Biol.* 10, 233–239
41 Scott, D.E. *et al.* (2012) Fragment-based approaches in drug discovery and chemical biology. *Biochemistry* 51, 4990–5003
42 Hayashi, Y. *et al.* (2012) *In vitro* selection of anti-Akt2 thioether-macrocyclic peptides leading to isoform-selective inhibitors. *ACS Chem. Biol.* 7, 607–613
43 Yamagishi, Y. *et al.* (2011) Natural product-like macrocyclic *N*-methyl-peptide inhibitors against a ubiquitin ligase uncovered from a ribosome-expressed *de novo* library. *Chem. Biol.* 18, 1562–1570
44 Morimoto, J. *et al.* (2012) Discovery of macrocyclic peptides armed with a mechanism-based war head: isoform-selective inhibition of human deacetylase SIRT2. *Angew. Chem.* 51, 3423–3427
45 Kawakami, T. *et al.* (2013) *In vitro* selection of multiple libraries created by genetic code reprogramming to discover macrocyclic peptides that antagonize VEGFR2 activity in living cells. *ACS Chem. Biol.* [Epub ahead of print]
46 LaRiviere, F.J. *et al.* (2001) Uniform binding of aminoacyl-tRNAs to elongation factor Tu by thermodynamic compensation. *Science* 294, 165–168
47 Ieong, K. *et al.* (2012) Inefficient delivery but fast peptide bond formation of unnatural L-aminoacyl-tRNAs in translation. *J. Am. Chem. Soc.* 134, 17955–17962
48 Chapman, S.J. *et al.* (2012) Histidine 66 in *Escherichia coli* elongation factor Tu selectively stabilizes aminoacyl-tRNAs. *J. Biol. Chem.* 287, 1229–1234
49 Lee, S. *et al.* (2013) A facile strategy for selective incorporation of phosphoserine into histones. *Angew. Chem.* 52, 5771–5775
50 Schrader, J.M. *et al.* (2009) Understanding the sequence specificity of tRNA binding to elongation factor Tu using tRNA mutagenesis. *J. Mol. Biol.* 386, 1255–1264
51 Neumann, H. *et al.* (2010) Encoding multiple unnatural amino acids via evolution of a quadruplet-decoding ribosome. *Nature* 464, 441–444
52 Sato, S. *et al.* (2011) Marine natural product aurilide activates the OPA1-mediated apoptosis by binding to prohibitin. *Chem. Biol.* 18, 131–139
53 Liu, Y. *et al.* (2013) A histone deacetylase inhibitor, largazole, decreases liver fibrosis and angiogenesis by inhibiting transforming growth factor-β and vascular endothelial growth factor signaling. *Liver Int.* 33, 504–515
54 Garrison, J.L. *et al.* (2005) A substrate-specific inhibitor of protein translocation into the endoplasmatic reticulum. *Nature* 436, 285–289
55 Venkataramanan, R. *et al.* (1991) Pharmacokinetics of FK506 following oral administration: a comparison of FK506 and cyclosporine. *Transplant. Proc.* 23, 931–933