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Review

Biased and unbiased strategies to identify biologically active small molecules

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ABSTRACT

Small molecules are central players in chemical biology studies. They promote the perturbation of cellular processes underlying diseases and enable the identification of biological targets that can be validated for therapeutic intervention. Small molecules have been shown to accurately tune a single function of pluripotent proteins in a reversible manner with exceptional temporal resolution. The identification of molecular probes and drugs remains a worthy challenge that can be addressed by the use of biased and unbiased strategies. Hypothesis-driven methodologies employs a known biological target to synthesize complementary hits while discovery-driven strategies offer the additional means of identifying previously unanticipated biological targets. This review article provides a general overview of recent synthetic frameworks that gave rise to an impressive arsenal of biologically active small molecules with unprecedented cellular mechanisms.

Contents

1. Introduction

Synthetic small molecules and natural products are key players in molecular medicine programs. Drug substances can perturb cellular processes underlying diseases, providing the means to reveal biological targets suitable for therapeutic intervention. Small molecules have been shown to accurately tune a single function of pluripotent proteins in a reversible and dose-dependent manner with temporal resolution that cannot be achieved with RNA silencing strategies. As such, forward chemical genetic approaches offer the additional means of identifying associated chemical hits suitable for drug development.[1]

The discovery of potent and selective agents remains a worthy challenge that can be addressed by the establishment of novel synthetic and screening methodologies. Combinatorial chemistry, a process designed to produce large libraries of closely related structural analogues, has emerged as a result of technological advancement associated to solid-phase synthesis.[2] The use of a solid support can increase reaction yields, facilitate purifications

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and enable split-pool synthesis to mix and match reagents in a strategic manner, thereby generating a high number of compounds readily available for biological evaluation. Based on this, combinatorial chemistry has long been considered a powerful process in drug discovery programs.

Compounds from combinatorial libraries, however, remain structurally closely related with a common central core harboring appendages with a high degree of variability. While combinatorial chemistry can provide a useful starting point, the fairly limited chemical space covered by combinatorial libraries may not be sufficient to discover new biologically active structures.[3] Over the past two decades, several approaches have been introduced with the aim of covering biologically-relevant chemical space. This includes dynamic combinatorial chemistry (DCC),[4] in situ click chemistry,[5] fragment-based drug discovery (FBDD)[6] and diversity-oriented synthesis (DOS).[7] While these strategies rely on conceptually distinct principles, recent examples from the literature demonstrate that these methods display complementary features and can be strategically used to fulfill different purposes.

This article describes recent strategies implemented to accelerate the process of drug discovery and the production of small molecule probes to study biology. The development of such diverse approaches reflects the inherent difficulties chemical biologists and medicinal chemists are facing to identify new biologically active compounds. No method has proven to be a 'one size fits all' route. This review outlines some of the advantages and pitfalls of each methodology, illustrating relevant examples that helped identify compounds with unprecedented properties.

2. Target guided synthesis (TGS)

Target guided synthesis (TGS) represents a subset of combinatorial approaches designed to produce biologically active small molecules. These nature-inspired strategies rely on adaptive libraries in which the biological target itself is able to select the best small molecule binder, thus avoiding the difficult task of drug design, cost of individual synthesis, characterization and screening of each library component. In contrast to traditional combinatorial chemistry, TGS methods are inherently biased towards a pre-defined biological target that is used to select for small molecule binders. Therefore, the impact of such an approach highly depends on the choice of the biological target and whether its targeting leads to a phenotype that can be exploited for therapeutic benefits. TGS may be illustrated by two types of processes: the 'thermodynamic' approach, named *dynamic combinatorial chemistry* (DCC) and the 'kinetic' approach, among which *in situ click chemistry* has received considerable attention.[8]

2.1. Dynamic combinatorial chemistry (DCC)

Dynamic combinatorial chemistry (DCC), independently conceptualized in the mid-90s by Lehn and Sanders,[9] can be described as a chemical process taking advantage of the reversible nature of chemical bonds to drive the composition of a mixture of building blocks at steady-state to a different composition upon introduction of a bias to the mix (i.e. protein or nucleic acid targets).

To do so, a dynamic combinatorial library (DCL) can be obtained by mixing building blocks capable of undergoing reversible bond formation, thereby producing adducts in variable proportion. The distribution of each adduct relies on the initial composition of the mixture and intrinsic reactivity of each building block. The constituents present at any moment are just a subset of all those that are potentially accessible, hence defining a virtual library.[10] By subjecting the mixture to an external pressure, it becomes possible to drive the equilibrium and influence the product distribution. In particular, when the *stimuli* is an external template able to engage supramolecular interactions with specific members of the library, the change in product distribution can lead to the amplification, and thus to the identification of the best binder. This concept has been mainly exploited in two different settings: (i) 'substrate casting', where a biomolecule acts as a host for the assembly of the fittest ligand and (ii) 'receptor molding', where a small molecule acts as a guest for the optimal assembly of a synthetic receptor.[9b]

Huc and Lehn reported the first example of this concept in 1997, using carbonic anhydrase (CAII) as target.[9b] The library generated by DCC purposely included products with structural features close to known inhibitors; the positive outcome validated the method and outlined the basis of subsequent research in the field.

The design of a dynamic combinatorial systems depends on a set of basic principles, that can be divided in few key steps: (i) selection of building blocks, (ii) choice of the reversible chemistry for the generation of dynamic diversity and (iii) the external template that can 'trap' and amplify the best binder at the expense of the other members of the library.

To efficiently produce a DCL, building blocks must fit several requirements. Firstly, they should contain functional groups that can be engaged in reversible covalent or non-covalent interactions. Secondly, to avoid the bias imposed by the competition, it is important that all members of the library display a comparable reactivity. Finally, library members should be designed to interact with the target in the most diverse geometrical and functional ways. It is noteworthy that dynamic combinatorial selections must be carried out at physiological conditions, which limits the choice of reversible reactions at use. A desirable feature to implement in the system is a 'switch off' mechanism to freeze the exchange process and analyze the composition of the mixture after selection. This includes pH, temperature, solvent composition or the use of quenching reagents. As a result, organic reactions most frequently associated with DCC involves condensation reactions (e.g. imine and hydrazone exchange) and disulfide chemistry.

DCC relies on the generation of dynamic libraries in the presence of a template; if one of the products better interacts with the template, it will be subtracted from the equilibrating pool causing a redistribution of the mixture according to Le Chatelier's principle (Fig. 1, pathway A). The preparation of such libraries, known as 'adaptive libraries', is restricted by several factors such as the need to use mild equilibrating reaction conditions and stoichiometric amounts of template to achieve high amplification turn over. Alternatively, 'pre-equilibrated libraries' (Fig. 1, pathway B) can be generated and frozen in the absence of the template, then screened with standard assays. In this case, no amplification takes place. Here, identification of the active components may be achieved through dynamic deconvolution protocols, where sub-libraries are generated in the absence of one of the building blocks

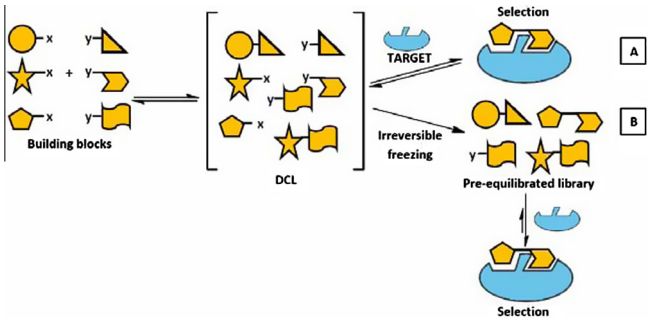
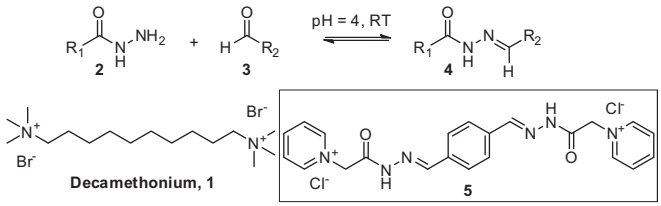


Figure 1. Dynamic combinatorial chemistry with (A) adaptative libraries and (B) pre-equilibrated libraries.



Scheme 1. Reversible acylhydrazone formation and AChE inhibitors.

and evaluated. A loss of activity indicates which of the fragments depleted from the pool is an active component of the best binder.[11]

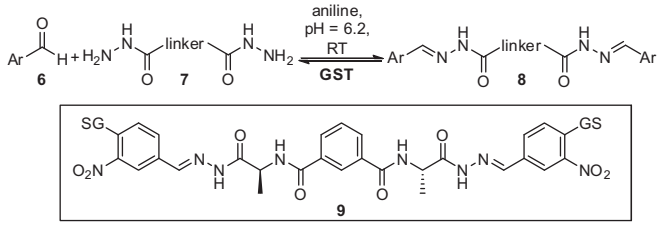
DCC relies on prior knowledge of the target. As such, libraries are frequently designed to improve molecules that already exhibit a detectable affinity for the substrate of interest. DCC can be seen as a biased method to refine the properties and selectivity of known small molecule partners. The biological substrate is strategically chosen for its therapeutic relevance and may include proteins and nucleic acids.[7]

Lehn and co-workers used DCC to identify acetylcholinesterase (AChE) inhibitors.[12] This enzyme has been linked to myasthenia gravis, glaucoma, Alzheimer's and Parkinson's diseases. It displays two distinct binding sites that can be the target of bridging ligands, such as the clinically approved Decamethonium (**1**, Scheme 1). Acylhydrazines (**2**) and aldehydes (**3**) containing library were used to identify acylhydrazone inhibitors (**4**) through reversible exchange, varying the molecular nature of the spacer between building blocks (Scheme 1). Due to the limited stability of the target, a pre-equilibration strategy was employed with the identification by dynamic deconvolution of compound **5** as the best binder out of a mixture of 66 possible adducts. Additional work revealed an inhibitor with an IC_{50} value of 2.3 nM, significantly more potent than **1** (IC_{50} = 1.0 μM) and other commercial drugs.

Greaney and co-workers established a DCC protocol to identify isoform specific inhibitors of the GST (glutathione-*S*-transferase) enzyme family known to catalyze the conjugation of glutathione (GSH) with a variety of substrates.[13]

The relevance of modulating GST activity has been recognized in a range of therapeutic areas, from oncology to tropical diseases including malaria. These enzymes generally exist as homodimers containing a GSH binding site and a hydrophobic region that varies between isoforms, for which the purpose is to trap electrophilic substrates. Using aniline-catalyzed acylhydrazone exchange,[14] libraries of bivalent inhibitors were generated from a series of aldehydes (**6**) and acylhydrazones (**7**) in the presence of four distinct GST isozymes (Scheme 2). The experiments led to the identification of acylhydrazones (**8**) including the most potent inhibitor to date (**9**, IC_{50} = 50 nM) that is selective for the mGSTM1-1 isoform.

Nicolaou and colleagues have developed a target-accelerated combinatorial approach to produce antibiotics effective against Vancomycin-resistant bacteria.[15] Vancomycin (**10**, Scheme 3) analogues exhibit antibacterial activity due to their ability to



Scheme 2. DCL generation by aniline-catalyzed acylhydrazone exchange and selected compound **9** (GS = *S*-glutathionyl).

inhibit peptidoglycan biosynthesis required for the bacterial cell wall. Vancomycin is known to exert its activity through its binding to the terminal Lys-D-Ala-D-Ala fragment of the growing precursor. The library was based on the homo- and hetero-dimerization of eight Vancomycin analogues (**11**) bearing spacers of variable lengths, using olefin metathesis as a dynamic reversible process (Scheme 3). Equilibration led to the amplification of dimers **12**. Analogue **13,** selected in the presence of the target (Ac_2-L-Lys-D-Ala-D-Ala), exhibited a higher cytotoxic potential compared to the commercial drug against both Vancomycin-sensitive (antibacterial activity: Minimum Inhibitory Concentration (MIC) = 0.03 μg/ml) and resistant strains (MIC = 2 μg/ml).

It is noteworthy that the size of DCLs are significantly smaller compared to those typically observed in combinatorial and high throughput-screening studies. This difference may be seen as a limitation of the methodology and is mostly dominated by the analysis of DCLs, the smaller being easier to resolve. In principle, DCLs should evolve towards the production of a small set of major products.[10,16] In practice, this is hardly ever the case, which considerably limits library size and the overall scope of DCC.

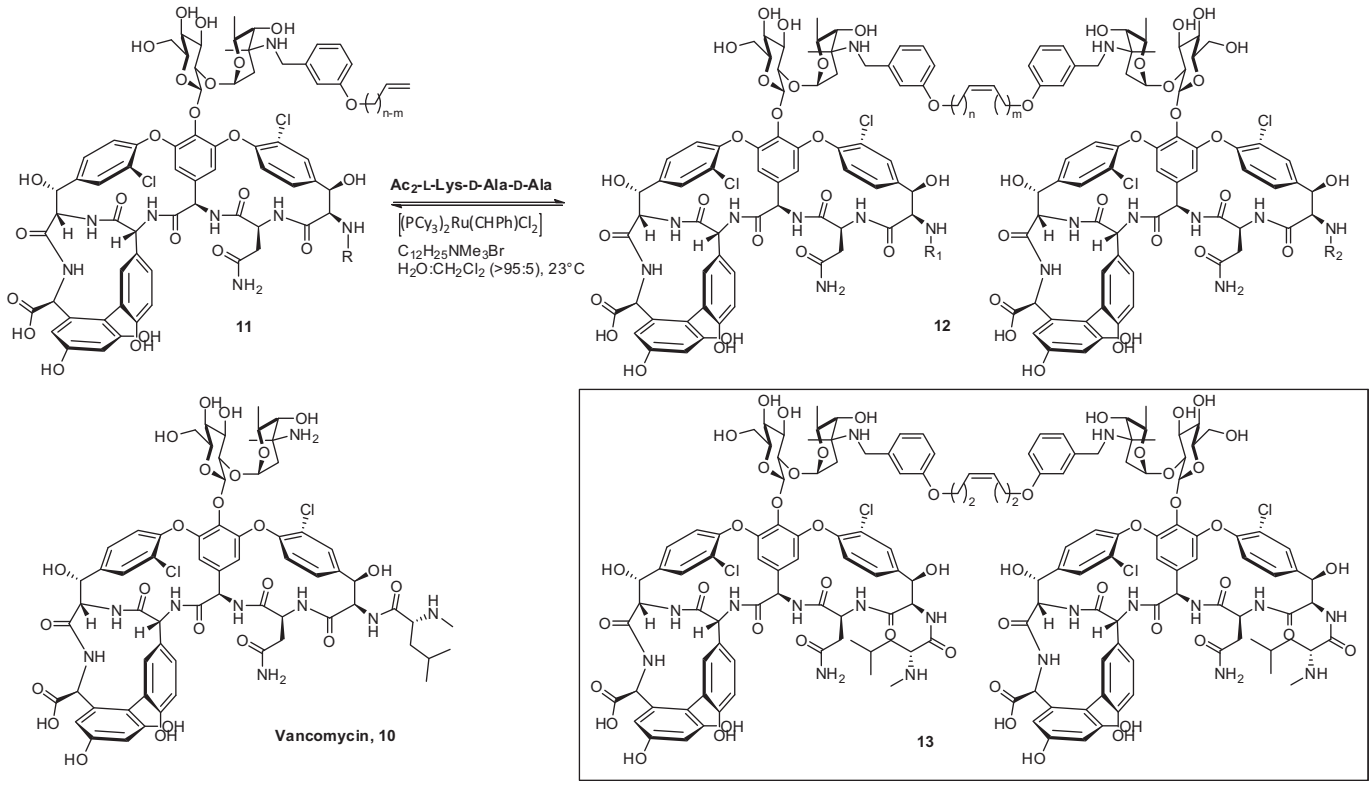
To challenge this notion, Miller developed an approach to identify molecules capable of selectively inhibiting the interaction between RNA containing CUG repeats and RNA binding proteins such as MBNLs (Muscleblind-like proteins). MBNL are a family of splicing regulators essential for muscular functions. In a genetic disease, CUG repeats are expanded on the mRNA of the DMPK (Dystrophia Myotonica Protein Kinase) gene leading to a depletion in active MBNL responsible of myotonic dystrophy.[17] To target RNA, a resin-bound dynamic combinatorial library (RBDCL) of 11,325 compounds was generated using 150 resin-attached cysteine-containing building blocks (compounds **14**–**16**, Scheme 4) and an identical set of solution-phase building blocks, allowed to undergo disulfide exchange in the presence of a labeled RNA target. This approach consisted of the fast identification of high-affinity binders (**17**) by physical removal of beads showing retention of the fluorescent RNA, followed by MS (Mass Spectrometry) analysis of the hit compounds.[18] The experiments provided a set of ligands with good affinity and selectivity for CUG motifs, representing the first example of compounds able to inhibit the (CUG)-RNA/MBLN1 interaction. A selected compound (**18**, K_i = 3.6 μM) was then chemically improved by replacing the disulfide moiety with a more stable olefin bridge suitable for biological evaluation. This example demonstrates the high value of dynamic combinatorial approaches to identify hit compounds exhibiting an activity in vivo.[19]

These examples represent only a subset of the DCC literature. Frequently, compounds arising from dynamic libraries exhibit biological properties at concentration ranges comparable to that of corresponding commercial drugs. It is worth mentioning that the use of reversible chemistry produce unstable compounds in some cases and late stage replacement of these linkers is amenable to a loss of potency, challenging the very purpose of the method.

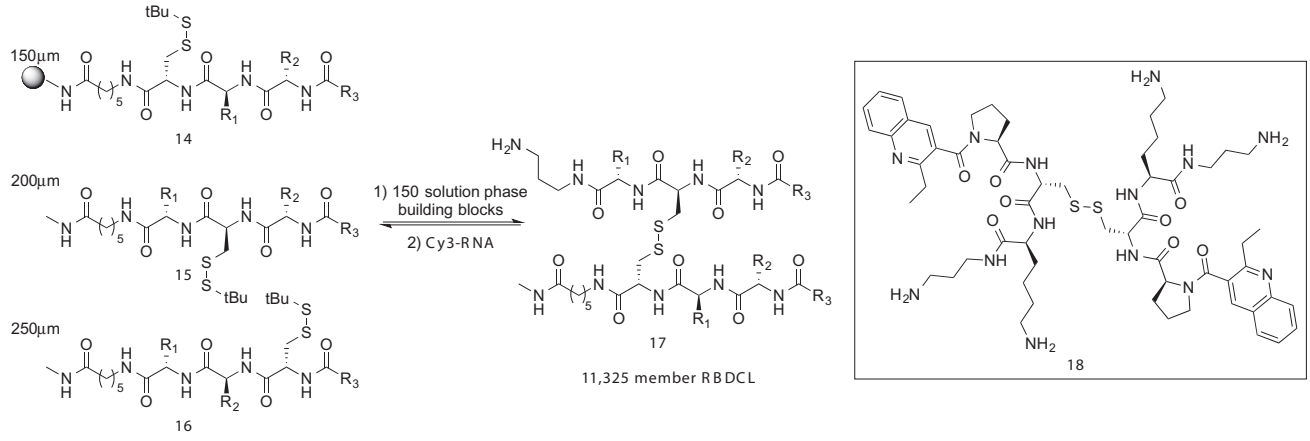
2.2. In situ click chemistry

In situ click chemistry is similar in that a biological template is used as the reaction vessel to produce the best binder in a kinetically driven manner based on irreversible covalent bond formation. The reactive building blocks come in close proximity within the binding pocket, thereby accelerating the rate of ligation in proximity within the cavity, producing a stable adduct due to the irreversible nature of the chemistry at work (Fig. 2).[20] This process is made possible by the fact that chemical ligation is slower than the selection of fragments but faster than its non-templated counterpart.

The concept of click chemistry was first introduced by Kolb, Finn and Sharpless. It describes a set of reactions that must be modular, broad in scope, high-yielding, generate only inoffensive



Scheme 3. Vancomycin homo- and hetero-dimerization in the presence of the target and active compound 13 (Cy = cyclohexyl).



Scheme 4. RBDCL (for clarity only one of the possible combinations is represented) and active compound **18**.

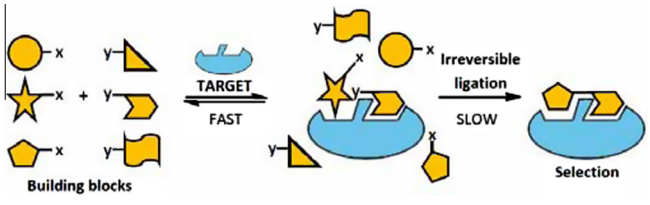
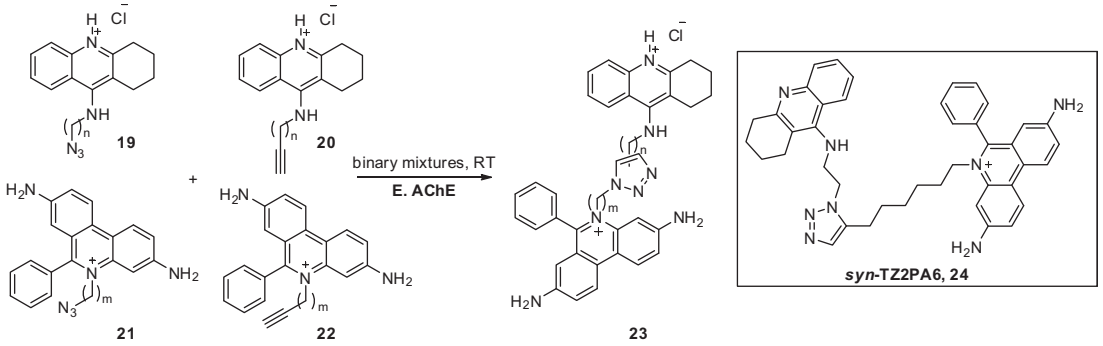


Figure 2. In situ click chemistry strategy for drug discovery.

byproducts that can be removed by non-chromatographic methods and be stereospecific.[21] Among the reactions that satisfy these requirements, 1,3-dipolar cycloadditions of azides and alkynes[22] has emerged as a powerful ligation method suitable for target-guided synthesis. The slow reaction rate of the uncatalyzed reaction and the remarkable acceleration observed by Moch and co-workers due to trapping of the reagents in close proximity inside a host structure[23] prompted Sharpless and colleagues to use this methodology in the presence of a biological target,[24] which gave rise to the concept of click chemistry performed in situ. Furthermore the reaction is bio-orthogonal,[25] can be easily performed under physiological conditions of pH and temperature, and the resulting triazole is an artificial moiety that can be involved in a network of hydrogen bonds with the target molecule.[20a] It is worth noting that, in contrast to DCC, the high energy barrier of the uncatalyzed reaction minimizes the generation of false positive, representing a valuable asset in the discovery of biologically active small molecules. As is the case for DCC, in situ click chemistry relies on the use of analytical techniques, which imposes significant limitations regarding library sizes.[26]



Scheme 5. In situ synthesis of AChE inhibitors from binary mixtures (for clarity only one of the possible combinations is represented) and active compound **24**.

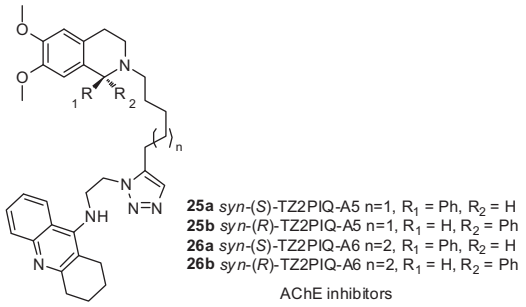
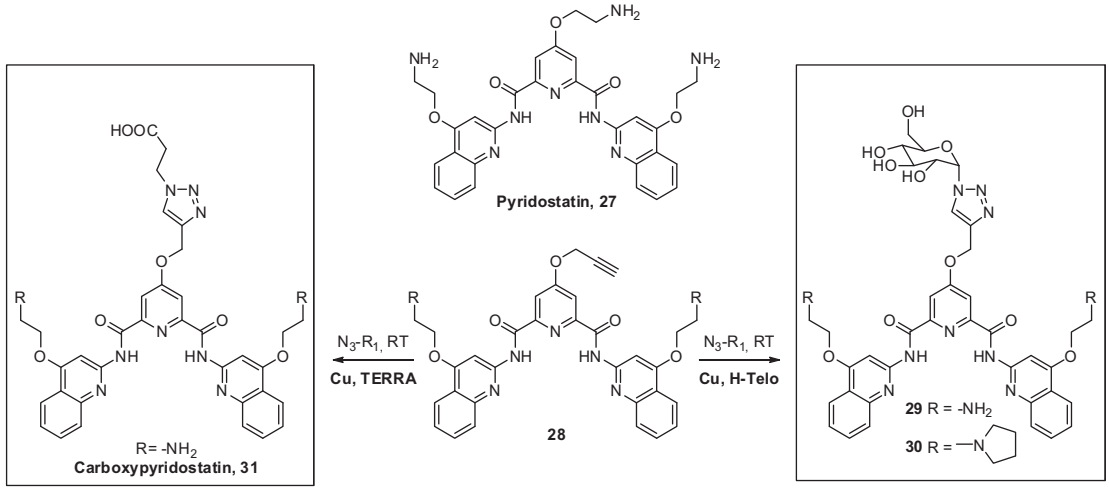


Figure 3. Selected hits from multicomponent in situ screening of AChE inhibitors.

The first example of in situ click chemistry was designed to identify AChE inhibitors.[24] Two proximal binding sites can be targeted either by mono- or bivalent ligands. In this model, the enzyme was used to select triazole-linked bivalent inhibitors based on known site-specific binders. Tancrine and phenanthridinium building blocks (**19**–**22**), functionalized with azides and alkynes, were reacted in 49 binary mixtures in the presence of *Electrophorus electricus* (*electric eel*) AChE (Scheme 5). Remarkably, 1,5-triazole isomers (*syn*-TZ2PA6, **24**) were predominantly produced at the expense of 1,4-triazoles, exhibiting unprecedented dissociation constants (K_d = 77–410 fM) against AChE from various species.

The success of this method, further validated by the X-ray structure of an inhibitor–enzyme complex,[27] led Sharpless and Kolb to improve the design of the inhibitor.[28] A range of building blocks not previously known to interact with the target including chiral components was used. The reaction was performed in a multicomponent manner in the presence of eel or mouse AChE. Among 10 possible combinations, only two products were amplified as compared to the reaction carried out in thermal conditions. All products exhibited low dissociation constants, especially against the eel enzyme, with the two enantiomers *syn*-(*S*)-TZ2PIQ-A5 and *syn*-(*R*)-TZ2PIQ-A5 (**25a** and **25b** respectively, Fig. 3) the most potent non-covalent AChE inhibitors known to date (K_d = 36 and 33 fM respectively).

Balasubramanian and co-workers used in situ click chemistry to identify potent and selective G-quadruplex (G4) binding small molecules. G-quadruplexes are non-Watson–Crick secondary nucleic acid structures that fold within G-rich regions including telomeres and whose targeting has potential implication in cancer biology.[29] Two alkynes-containing building blocks based on the potent G4 binding molecule pyridostatin (**27**) (compounds **28** Scheme 6), were reacted with a set of 6 azides in the presence of copper and sub-stoichiometric amounts of telomeric DNA G4 (H-Telo). The presence of both catalysts led to the amplification of the *anti* 1,4-sugar-containing adducts **29** and **30**, showing a strong preference for G4 DNA over double stranded DNA (ds-DNA) as monitored by Förster Resonance Energy Transfer (FRET)-melting experiments (ΔT_m (G4-DNA) = 30 K and 28 K, ΔT_m (ds-DNA) = 0.0 and 0.1 for **29** and **30** respectively). Compound **29** was subsequently evaluated by fluorescence microscopy for its ability to displace TRF1 (Telomeric Repeat-Binding Factor 1), a protein that binds to and protects the end of chromosomes from the DNA damage-response machinery. Treatment of human



Scheme 6. In situ click chemistry for the synthesis of DNA/RNA G4 ligands and selected compounds **29**, **30** and **31**.

MRC5-SV40 fibroblasts with the in situ generated lead resulted in a dose-dependent decrease in TRF1 *foci* per cell, with an IC_{50} value of 1.3 μM. It is noteworthy that the amplified compounds were also obtained in the presence of DNA and absence of copper, yet with a slower turn over but an higher selectivity. In the same report, a second in situ click experiment was performed in the presence of copper and the Telomeric Repeat-containing RNA (TERRA), an oligonucleotide known to fold into stable G4 structures in vitro. Remarkably, the reaction mixture evolved towards the production of the negatively charged adduct carboxypyridostatin (**31**, Scheme 6) for which the proportion was significantly decreased when the selection was carried out in the presence of DNA catalyst. This study led to the identification of the first G4 RNA selective ligand with a ΔT_m value of 20.7 K. Carboxypyridostatin was then employed to stabilize and visualize G4 RNA in cells providing evidence for the formation of RNA G-quadruplexes within the human transcriptome.[30]

Another promising application of in situ strategies includes the iterative peptide in situ click chemistry (IPISC), a technique first proposed by Heath et al. in 2009 to produce protein capture agents.[31,32] Protein capture agents are a class of ligands designed to replace expensive and relatively unstable antibodies and are used either as therapeutics or as molecular tools for protein recognition. Several technologies have been proposed for the production of 'antibody-like' reagents. IPISC exploits the target protein as a scaffold for the in situ assembly of high affinity probes. The experiment involved the selection of a ligand from a one-bead-one-compound library (OBOC) that served as an 'anchor ligand' for in situ click chemistry. This led to the identification of a second ligand, which in combination with the first was converted into a new anchor for the selection of the third ligand and so forth and so on (Fig. 4). In each case, beads were used to isolate compounds of interest that were identified by Edmann sequencing.

Using this approach, triligand capture agents were identified against bovine and human carbonic anhydrase II (**32**, K_d (bCAII) = 65 nM, K_d (hCAII) = 45 nM, Fig. 5) and protein kinase B (**33**, K_d (Akt1) = 200 nM) demonstrating their efficacy as surrogate antibodies in protein-detection assays.[32,33]

3. Fragment based drug discovery (FBDD)

Until recently, most lead compounds within the pharmaceutical industry have been discovered using high throughput screening (HTS) techniques, where large libraries of compounds were screened against specific targets or cell lines. To achieve the widest possible range, libraries tend to be libraries of natural products as well as compounds synthesized in-house, in collaboration and bought in, restricting the use of HTS to industry or well-funded academic laboratories. Inevitably not all of these molecules have particularly good drug-like properties, resulting in a potential waste of resources due to the screening of molecules that will fail and lead compounds that exhibit unsuitable pharmacokinetics and thus will not move further along the development process. It is arguable that HTS is a decidedly inefficient process given that screening of libraries of upwards of one million compounds is not easy, particularly in the scattergun approach required. HTS leads have high binding affinities resulting from making multiple interactions with the target. However, as it is not necessarily obvious which parts of the molecule contribute to the high binding affinity, and so subsequent modification of the lead compound can prove difficult.

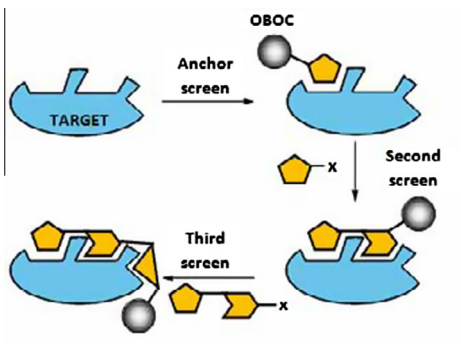


Figure 4. IPISC strategy for protein capture agent design.

One suggested alternative methodology is fragment based drug discovery (FBDD). This process screens small molecules (<300 Da) for weak binding to a preselected target in vitro. While small fragments bind weakly, high micromolar to low millimolar concentrations, due to the loss of entropy upon binding, Jencks argued that these interactions have to be 'high quality'.[34] This can be illustrated by the difference in binding free energy between a 100 μM and a 3 nM binder. While the affinity of the former is 33,000 times the latter, its free energy is only roughly half that of the latter.[35] As a consequence, fragments are considered to have high ligand efficiency, as defined by the free energy of binding per heavy atom, thus constituting an excellent basis for lead compounds. Ligand efficiency can be measured quantitatively by finding the ratio of the Gibbs free energy to the number of non-hydrogen atoms.[36]

The choice of library compounds screened is very important to the success of this process. A library that is too small will not cover enough chemical diversity, while a library that is too large loses the efficiency of the FBDD process. Typically FBDD libraries contain between 1000 and 2000 molecules, although libraries of 140 and 16,000 compounds have been used.[37,38] The design of the fragment library is a key step in the FBDD process. Generally, molecules included in a library are 'rule of three' compliant and contain moieties that are suitable for future elaboration.[39] Depending on the type of screening and how much is known about the target, libraries can attempt to cover as much chemical space as possible or be highly tailored. For example, hits against protein–protein interactions tend to be more hydrophobic and heavier than hits for other drug targets.[40] The relatively small size of these libraries allows FBDD to be performed in academic settings, resulting in its use for less commercial targets as well as the development of molecular probes for particular targets.[41,42]

Once a library of compounds has been synthesized, it can be screened against the desired target making this approach inherently biased. There is a selection of biophysical techniques that can be used to screen the fragments in vitro. The most commonly used are NMR spectroscopy, fluorescence based thermal shift, mass spectroscopy, surface plasmon resonance and X-ray crystallography. It is also possible to virtually screen fragments using in silico molecule docking programs.

Once the library has been screened and a number of hits have been found, the next step is fragment elaboration or development, in order to prepare a viable lead compound. The simplest approach is to link together two fragment hits that bind to proximal sites. Howard et al. found that linking two fragments that bind in adjacent pockets of thrombin resulted in a 100,000 reduction in IC_{50}.[43] Whilst fragment linking is conceptually simple, the choice of the appropriate linker is not trivial. The ideal linker must be flexible enough to allow both fragments to adopt the optimal orientation to maximize binding, whilst avoiding unfavorable interactions with the target. Another approach to developing a fragment hit is to incorporate the structural and chemical features of several fragments into one molecule. Fragment merging is particularly useful for overlapping fragments, which would be unsuitable for linking. Brough et al. merged fragments from in silico screening with those discovered using more traditional methods and identified an orally

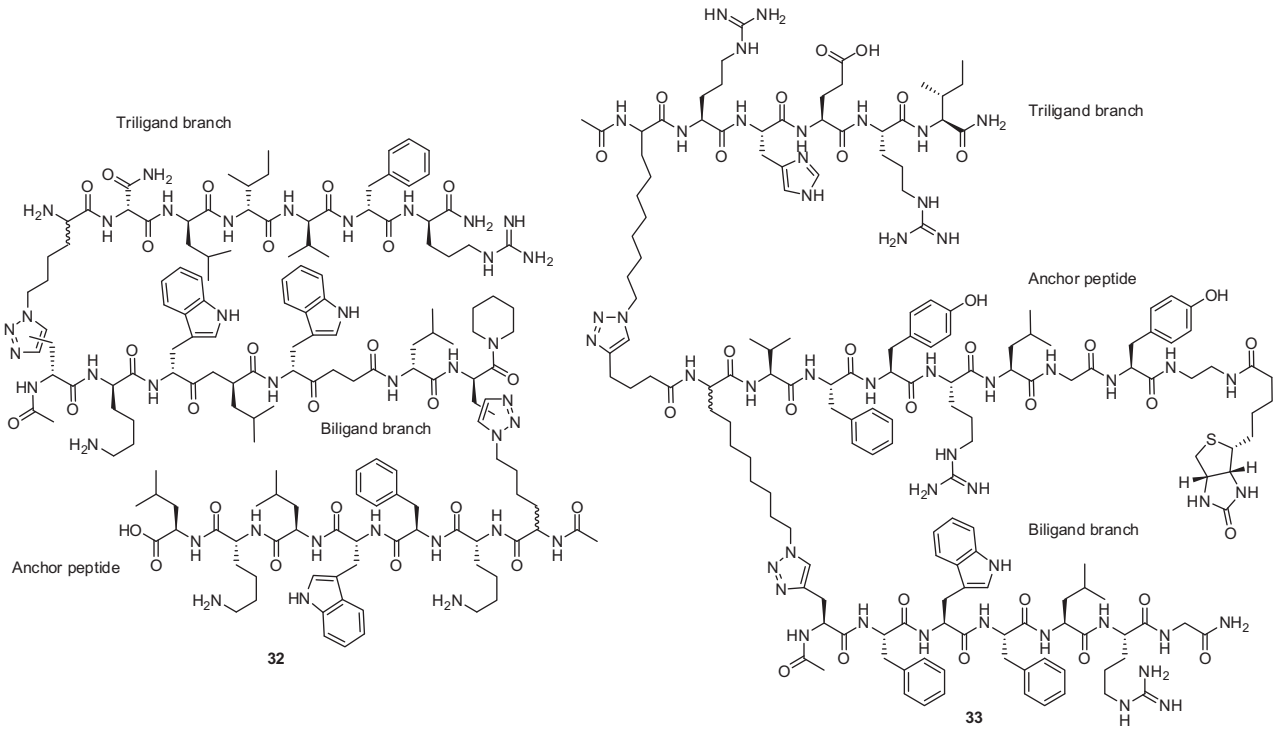


Figure 5. Triligand capture agents **32** and **33** identified by IPISC methodology.

available HSP90 (Heat Shock Protein) inhibitor which showed significant efficacy in a subcutaneous human breast tumor model.[44] A more popular method of fragment elaboration is fragment growth. Here, using structural information gathered during fragment screening, groups are added to the fragment to increase the number of interactions with the target and increase potency. After every round of group addition, the compound should be evaluated, not just for potency, but also for metrics, such as ligand efficiency to ensure that the added mass is effective. Frederickson et al. found that, after three rounds of fragment growth, their starting fragment with an IC_{50} of greater than 1 mM, now had an IC_{50} of 72 nM against urokinase-type plasminogen activator.[45]

A different methodology that, to some extent, combines both fragment discovery and fragment elaboration is tethering. Developed by Sunesis Pharmaceuticals, this strategy involves the use of a protein with a free cysteine next to the targeted pocket, or the mutation of the target protein so as to have an appropriately exposed cysteine residue.[46] A fragment library of disulfide compounds is then screened against the protein under conditions that promote rapid thiol exchange, resembling DCC. Under these conditions, molecules with low affinity for the target will not be retained by the protein, whereas high affinity binders will form stable disulfide bonds and so the complex will become detectable by mass spectrometry. If a protein has two adjacent pockets, which can be targeted, this methodology can be extended to find molecules that bind in both pockets simultaneously. Firstly, a fragment that binds within the pocket in close proximity to the cysteine residue can be identified either from the literature or from screening. This fragment is then modified by the introduction of two disulfide linkers and the resulting protein–fragment complex is screened against a range of disulfide containing fragments in a similar way to the basic tethering protocol.[38]

Zelboraf (**34**, Fig. 6) was the first FDA approved drug developed using a fragment based method.[47] It is a kinase inhibitor specifically targeting the B-Raf^{V600E} mutation, which is one of the most frequent oncogenic protein kinase mutation known.[48] The search for inhibitors began by screening a library of 20,000 compounds at a concentration of 200 μM against multiple structurally characterized kinases. While more than 100 compounds were found to successfully bind, 7-azaindole was found to interact with the ATP binding site. Further investigation of this structure led to a 3-aminophenyl analog **35** (Fig. 6), which appeared to have the potential to be a generic structure suitable for kinase inhibition capable of hydrogen bonding interactions with the kinase hinge region and multiple sites for substitution. Libraries of substituted 7-azaindoles

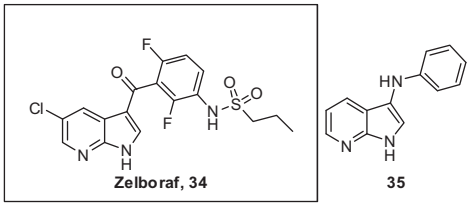


Figure 6. Drug molecule Zelboraf and initial fragment hit.

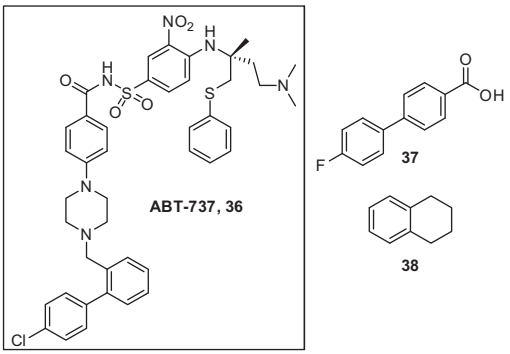


Figure 7. Drug molecule ABT-737 and initial fragment hits.

were then screened against wild type and oncogenic B-Raf in vitro and Zelboraf was found to inhibit wild type B-Raf at concentrations 10-fold higher than oncogenic B-Raf. Zelboraf also showed excellent selectivity when screened against other kinases, with at least a 100-fold difference in IC_{50} for almost all the kinases studied. Cell lines with the B-Raf^{V600E} mutation were found to be more sensitive to zelboraf inhibition with selectivity exceeding 100-fold. It also showed this selectivity in both animal and human trials.[49]

Abbott Laboratories developed ABT-737 (**36**, Fig. 7), an anticancer drug that targets the anti-apoptotic Bcl-2 (B-cell lymphoma 2) protein family, which is overexpressed in many cancers and has been implicated in tumor initiation, progression and therapy resistance.[50] NMR based screening methods called SAR (structure–activity relationship) by NMR were used to screen for fragments that would bind to the hydrophobic B-cell lymphoma 2 (Bcl-2) homologous 3 binding groove of B-cell lymphoma-extra large (Bcl-X_L). Two fragments were found to bind to different but neighboring sites within this groove, 4′-fluoro-biphenyl-4-carboxylic acid **37**, and 5,6,7,8-tetrahydro-naphthalen **38** (Fig. 7). Linking of these two fragments and subsequent synthetic development based on the binding information from the NMR structure resulted in the discovery of ABT-737. In vitro, ABT-737 showed high affinity ($K_i \leqslant 1$ nM) for Bcl-X_L, Bcl-2 and Bcl-w, but lower affinity for the less homologous proteins Bcl-B, induced myeloid leukemia cell differentiation protein 1 and β-casein A1 ($K_i = 0.46 \pm 0.11$ μM, >1 μM and >1 μM respectively). When tested in cancer cell lines in combination with existing cancer therapies, it was found that the median effective concentration value was reduced 2–20 fold depending on therapy and cell lines. This is because ABT-737 does not directly initiate apoptosis itself but enhances the effects of death signals by binding and sequestering the anti-apoptotic Bcl-2 protein family. ABT-737 also showed potent single agent activity against cell lines involved in lymphoid malignancies and small cell lung cancer. In both mouse models and clinical trials it has been found to be an effective treatment for leukemia, multiple myeloma and small cell lung cancer.[51]

Astex Therapeutics has developed AT7519 (**39**, Fig. 8) as a cyclin dependent kinase 2 (CDK2) inhibitor.[52] CDKs are key to the regulation of the cell cycle and, since loss of cell cycle control is a key characteristic of cancer, it is anticipated that inhibition of CDKs would control tumor growth and provide an effective anticancer agent. Members of the CDK family have also been implicated in DNA damage response, gene regulation, transcription and neuronal and secretory cell function, thus affecting cell growth and survival via several possible mechanisms. A library of around 500 compounds was assembled from a focused kinase collection, a drug fragment collection and compounds identified by virtual screening. More than 30 compounds were identified, all of which were hydrogen bonded to residues in the hinge region of the ATP binding site of CDK2. Several parallel synthetic developments of fragment hits were followed up, however it was fragment **40** that led, in seven steps, to AT7519 (**39**, Fig. 8). The in vitro IC_{50} dropped from 185 to 0.047 μM while the ligand efficiency for the two molecules was in the same range, 0.57 and 0.42 respectively. In HCT-116 cells, AT7519 exhibited an IC_{50} of 0.082 μM. In mouse models and

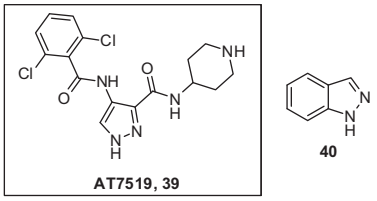


Figure 8. Developed drug molecule AT7519 and initial fragment hit.

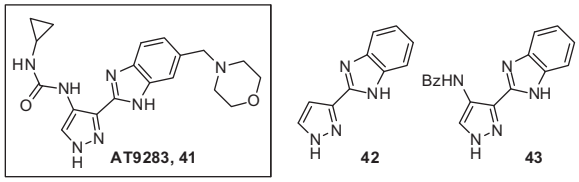


Figure 9. Developed drug molecule AT9283 and initial fragment hits.

clinical trials, it has proved effective in multiple myeloma, B-cell leukemia and refractory solid tumors.[53]

AT9283 (**41**, Fig. 9) is another anticancer drug targeting kinases, specifically Aurora A and B, serine/threonine kinases which are regulators of mitosis and are overexpressed in solid tumors and leukemias.[54] AT9283 was developed from a fragment found in the same screen as the fragments used for the basis of AT7519.[53] Fragment **42** and the benzamide analogue **43** were found to have better activities and ligand efficiencies against Aurora A than CDK2 (**42**, IC_{50} 0.91 μM vs 28 μM, **43**, IC_{50} 0.0059 μM vs 0.052 μM). Substitution on the six-membered ring of the benzoimidazole and replacement of the benzamide motif with a urea was found to increase the potency of the compound to the point where the IC_{50} of AT9283 could not be measured accurately using the available assay ($IC_{50} \approx 3$ nM). HCT-116 cells treated with the compound at 0.03 μM exhibited a clear polyploidy phenotype and showed a favorable pharmacokinetics. Interestingly, when the X-ray structures of **42** and AT9283 were compared, key hydrogen bonding interactions with the protein were identical, showing that the fragment-protein interactions were maintained throughout the optimization process. In addition, AT9283 displayed activity against proteins with structurally similar regions, thus showing that it could be used as a multitarget anticancer drug. AT9283 has proved effective in both mouse models and clinical trials.[55]

The Abell and Leeper groups have used FBDD to develop a compound that binds to riboswitches, thus modulating gene expression.[56] Riboswitches are secondary structures found on messenger RNAs that modulates their transcription, stability, splicing or translation upon binding by a natural ligand. An equilibrium dialysis method was used for initial screening of fragments against *Escherichia coli thiM* riboswitch in which the amount of [^{3}H]thiamine that is displaced can be used as a measure of a fragment's binding ability. From this screen, 20 fragments were taken through to waterLOGSY (Ligand Observed via Gradient Spectroscopy),[57] an NMR spectroscopic technique, to confirm their status as binding hits. Cross screening with a second riboswitch to remove generic RNA binders resulted in the elimination of 7 fragments (ten remaining fragments displayed in Fig. 10). These ten structures exhibit a modest range of structural diversity, but interestingly, every fragment displays at least one position for further elaboration. However, in vitro translation studies did not identify fragments that showed a significant effect on gene expression.[58]

FBDD has not only helped develop new drugs for existing targets but has also been used to identify new druggable sites on known protein targets. For example, inhibition of farnesyl pyrophosphate synthase (FPPS) has been shown to be a viable anticancer strategy.[59] All previous potent inhibitors of FPPS are biophosphate derivatives and so are rapidly absorbed into bone tissues or excreted by the kidneys, thus resulting in very low soft tissue exposure. In an attempt to find new non-biophosphate based inhibitors of FPPS, a library of 400 compounds was screened against FPPS using NMR spectroscopy. Further characterization of these hits was carried out using a competition experiment with zoledronic acid, which binds to the active site. Interestingly, most fragments were not competitive with zoledronic acid and actually showed binding independent of the competitor ligand, indicating

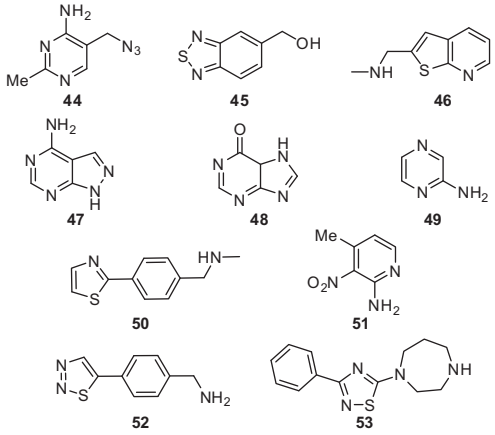


Figure 10. Fragment hits found in riboswitch binder screen.

that they were binding in a different position on FPPS. The four hits shown in Figure 11 (**54**–**57**) were all crystalized with FPPS and in all four cases it was observed that the fragment was bound in an allosteric pocket close to the C-terminus and the isopentenyl pyrophosphate binding pocket. Optimization led to compounds **58** and **59** that are very effective non-biophosphate inhibitors of FPPS. These findings raised the possibility that FPPS inhibition may take place outside of bone tissues.

4. Diversity-oriented synthesis (DOS)

Commercially available libraries of compounds remain the principle source of small molecules, but the molecular diversity represented in such collections can lack chirality and structural complexity. In addition, small molecules are generally based on a common structure, diversified by the introduction of various appendages onto the core. Even if these collections have led to the discovery of many drug substances, they hardly contain lead structure suitable for 'uncommon' targets.[60] Genome biology is uncovering new processes underlying human diseases, involving previously unknown protein–protein interactions that could be targeted and modulated by drugs. Some of these targets are considered 'undruggable', and bear little resemblance to those that are already known. Broader diversity of molecular shape increases the possibility of discovering hits with distinct biological activity or that are capable of binding in a different manner. To achieve the preparation of such collections of small molecules, harboring the complexity of natural products and the advantage of high-purity and ease of characterization of combinatorial libraries, Schreiber introduced the concept of diversity-oriented synthesis (DOS).[61]

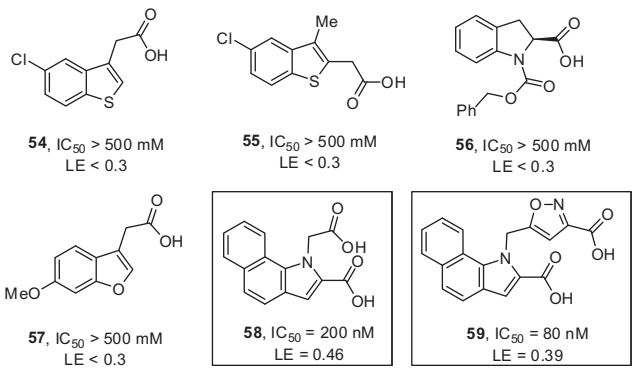


Figure 11. Initial fragment hits and optimized lead molecules in FPPS screen (LE = Ligand Efficiency).

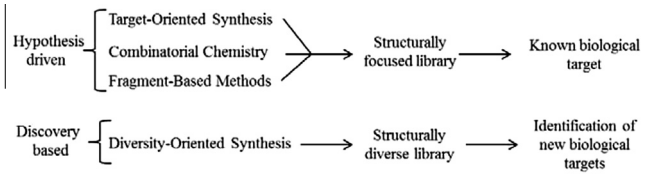


Figure 12. Scope of biased and unbiased approaches.

DOS was conceived as a method to afford in an efficient and straightforward manner a collection of complex and diverse molecules, where diversity is related not only to the appendage elements, but also to the molecular skeletons itself and the stereochemistry to cover a wide area of the chemical space relevant to biological processes. In contrast to the classical retrosynthetic approaches, which tends to convert complex molecules to very simple starting materials, DOS is a forward approach, thinking about the products of a reaction as suitable substrates for the next synthetic step. Increasing the structural diversity of a chemical collection can be a useful strategy to identifying lead compounds and to reveal unanticipated biological targets. Indeed, while target-oriented libraries are suitable for screens involving a preselected biological target, DOS libraries offer unprecedented opportunities in phenotypic-based assays (Fig. 12).

4.1. Generating diverse libraries using DOS

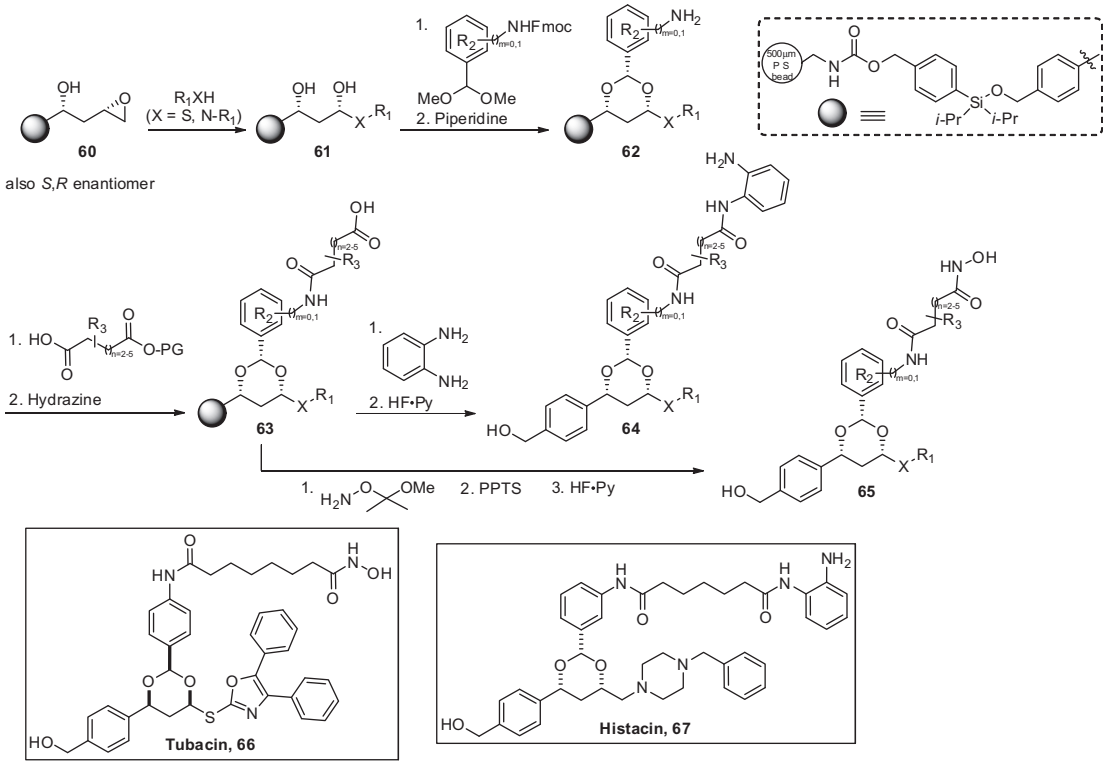
Structural diversity of small molecules is associated with one or more of these three features:

- Appendage diversity, or variation of the appending groups around a common-core unit.
- Stereochemical diversity, or variation in the three-dimensional structure of the molecule.
- Skeletal diversity, or variation in the molecular scaffold itself.

The simplest diversity-generating process can be achieved by coupling different building blocks (appendages) to a common complex scaffold. If the molecular skeleton has several reactive sites with potential for orthogonal functionalization, then it becomes feasible to generate all possible combinations of appendages. By using the technique of split-pool synthesis, Schreiber and co-workers have demonstrated the high efficiency of such an approach by preparing millions of diverse small molecules in three to five steps.[62]

The presence of various functional substituents on an active molecule is crucial to engage interactions with various polar, apolar or charged groups of the biological substrate. In this context, small molecules with functional groups differently orientated in space, which can be accomplished with different stereocenters, may exhibit distinct interactions. It is then important to implement enantio- or diastereoselective reactions to increase the number of relative orientations of potential target-interacting elements in the molecule, thus attaining stereochemical diversity.

Libraries including a broad number of diverse molecular scaffolds have the potential to cover the widest range of biological activities. Skeletal diversity can be achieved by using two strategies. In the first one, the 'reagent-based approach', a discrete molecular entity can yield a certain number of structurally distinct compounds, by employing different reagents, which impose the nature of the chemical transformation. Alternatively, in the 'substrate-based approach', structurally distinct starting materials containing suitably pre-encoded skeletal information can be converted into a library of diverse molecular scaffolds when subjected to a common set of reaction conditions.



Scheme 7. Divergent synthesis of 7200 1,3-dioxane-derivatives, as HDAC 6 inhibitors.

4.1.1. Appendage diversity

Using split-pool techniques, Schreiber and co-workers synthesized 7200 1,3-dioxanes with the aim of identifying selective small molecule inhibitors of histone deacetylases (HDACs).[63] The synthesis started by attaching γ,δ-epoxy alcohols to a silane-derivatized polystyrene resin. The functionalized resin **60** was pooled, split and reacted with 50 nucleophiles to generate 100 different 1,3-diols (**61**, Scheme 7). These solid-supported diols were pooled, split and reacted with amino dimethylacetal building blocks, yielding 600 diverse 1,3-dioxanes. The amino derivatives **62** were then transformed into carboxyamides by reacting intermediate products with diacid building blocks and were split in three portions. The first one was used for screening. The second portion was reacted to generate 2400 *o*-aminoanilides **64**, while the last one was used to prepare 2400 hydroxamic acids **65**. A multidimensional chemical genetic screening of these molecules led to the discovery of tubacin, which selectively inhibits the histone deacetylase 6 (HDAC 6), thus increasing α-tubulin acetylation in mammalian cells, and histacin, which inhibits histone deacetylase activity but HDAC 6 (**66** and **67**, respectively).[64] These selective inhibitors are extremely powerful probes, allowing the discrimination between the HDAC family-mediated effects caused by promiscuous HDAC family inhibitors. As a result, HDAC 6 has been identified as a new potential antimetastatic and antiangiogenic therapeutic target.

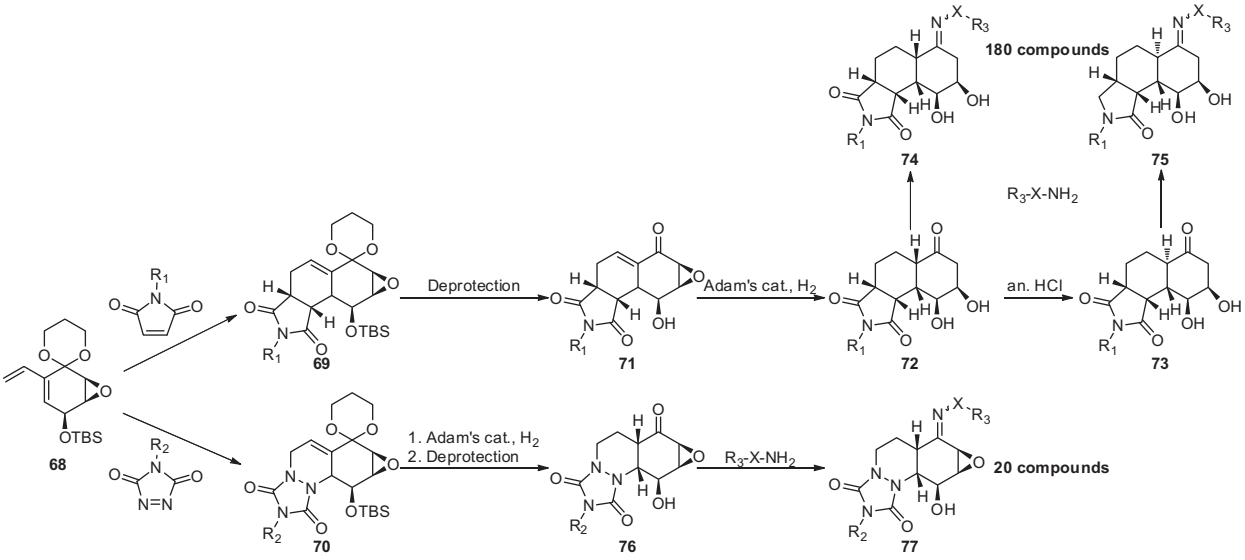
4.1.2. Stereochemical diversity

Stereochemical diversity increases the number of possible orientations of a small molecule's functional groups that are potentially involved in the interaction with the target macromolecule. It can be accomplished by employing chemical reactions that proceed with enantio- or diastereoselectivity.[65] Reactions such as Diels–Alder cycloadditions, that lead to the formation of several stereogenic centres in a single step, are widely used in DOS since they allow access to a high level of structural complexity and stereochemical diversity.

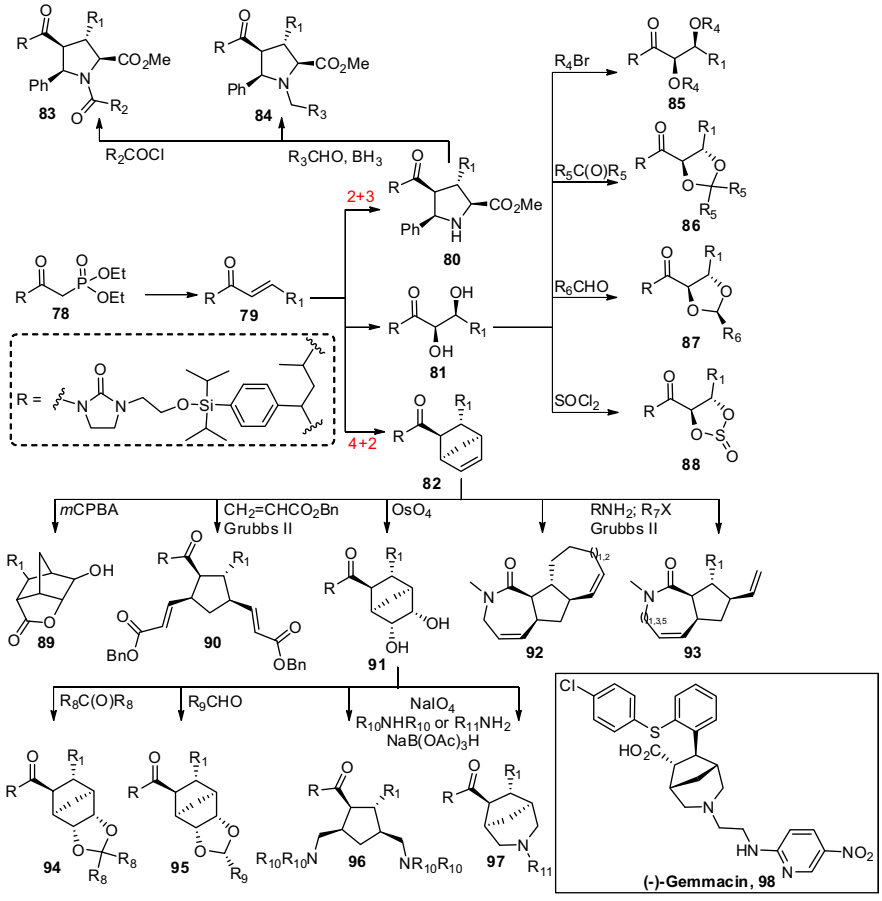
Porco and co-workers developed an efficient approach to produce a library of 200 highly functionalized and complex angular epoxyquinol scaffolds,[66] where the key step is a highly stereocontrolled [4+2] Diels–Alder cycloaddition of a chiral nonracemic epoxyquinol diene with reactive dienophiles. By using parallel solution-phase synthesis, diene **68** (Scheme 8) was reacted with ten diverse maleimides and four different triazolinediones, thus affording the corresponding *endo*-selective cycloadducts **69** and **70**. Deprotection of compounds **69** led to ten angular epoxyquinol scaffolds in good yield and purity. Subsequent hydrogenation with 5 wt% Adam's catalyst generated the *cis* isomers **72**, that were also epimerized by employing a slight excess of anhydrous HCl, in order to increase the number of stereodiverse skeletons in the library. Urazole-containing scaffolds **70** were first hydrogenated and then deprotected, yielding four epoxyquinol derivatives **76**. Final carbonyl diversification was performed through reactions with 9 nucleophiles, such as alkoxyamines and carbazates, thus obtaining a library of 200 complex compounds, where ten different frameworks possessing different stereochemistry and functional groups could be identified. The library was screened for the inhibition of the induction of heat shock proteins (HSPs). Hyperthermia, which is used in the treatment of some cancers in combination with radiation and other therapies, can rapidly induce the expression of some of HSPs,[67] resulting in antitumor activity. Biological evaluation revealed six compounds from the library that showed significant inhibition of the induction of HSP 72, a protein that is overproduced as a mechanism of resistance to hyperthermia,[68] with IC_{50}'s in the low micromolar range. These preliminary results indicated that the angular epoxyquinol scaffolds should provide access to novel molecule probes for biological research.

4.1.3. Skeletal diversity

4.1.3.1. Reagent-based approach. The reagent-based strategy, also known as the 'branching' reaction pathway, takes advantage of: (a) the use of pluripotent chemical functionalities, where the same part of the substrate can be differentially transformed by varying



Scheme 8. Synthesis of a library of 200 angular epoxyquinol scaffolds.



Scheme 9. Divergent synthesis of 242 diverse compounds based on 18 distinct molecular frameworks.

the reaction conditions and (b) the use of a densely functionalized molecule where each functionality can be transformed using different reagents. This strategy can be used either at the early stage of synthetic planning to introduce diverse functional groups or at the end stage to transform a pre-functionalized molecule into distinct molecular scaffolds. The method offers the advantage of being applicable to a collection of structurally diverse compounds exhibiting a common reactive element.

Addressing the need for the development of new antibiotics due to the emergence of drug resistance, Spring and co-workers prepared a 242 small molecule-containing library based on 18 natural-product-like scaffolds in only two to five steps.[69] The solid

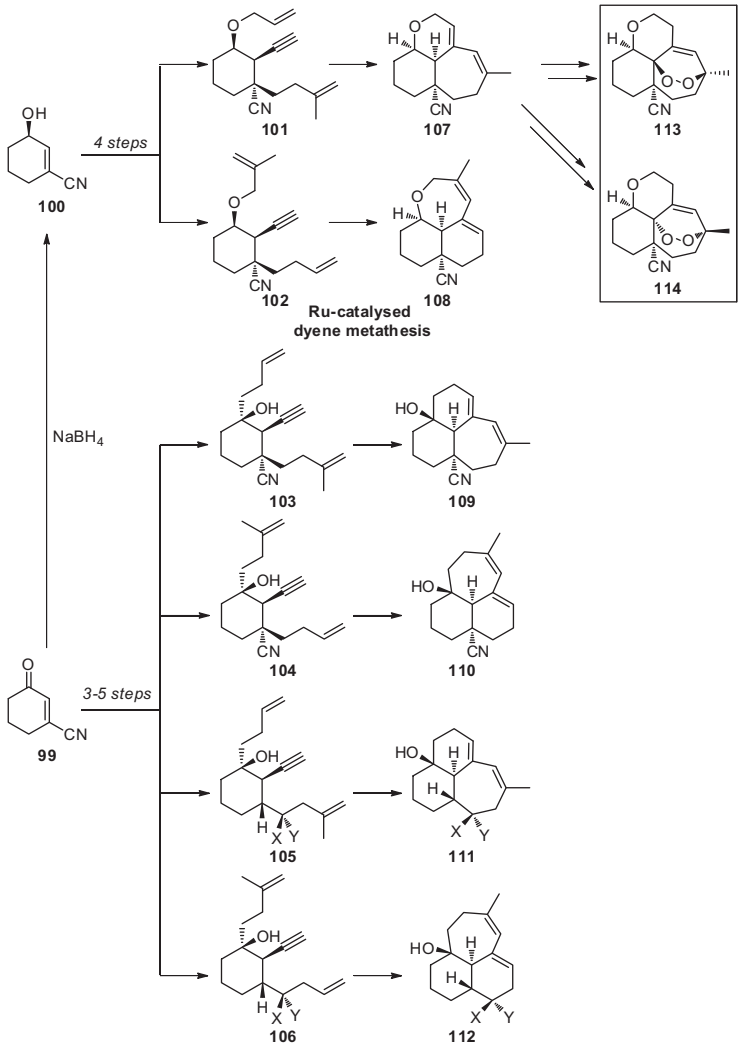
supported phosphonate **78** (Scheme 9) that was reacted with aldehyde building blocks to yield 12 α,β-unsaturated acyl imidazolidinones **79**. These compounds were processed through three catalytic, divergent synthetic pathways—a [2+3] cycloaddition, a dihydroxylation and a [4+2] cycloaddition—to deliver products **80**–**82** with good enantio- and diastereoselectivities. Using complexity-generating reactions and capitalizing on the functionalities obtained in the first set of reactions, the authors obtained 18 different scaffolds which are either rare or have no known representation in nature.

The diversity-oriented synthetic planning was designed to populate new areas of chemical space, in order to maximize the chances of discovering a new antibacterial agent with a distinct mode of action. The libraries were screened for their effects on penicillin- and erythromycin-resistant strains of *Staphylococcus aureus*. (−)-Gemmacin (**98**) was shown to prevent growth of Gram-positive bacteria, acting as a selective bacterial membrane disrupter. These results demonstrate the utility of DOS for the discovery of new potent antibiotics, through the exploration of uncharted biologically relevant regions of chemical space.[70]

4.1.3.2. Substrate-based approach. In contrast to the reagent-based approach, the substrate-based approach rely on a collection of substrates with different appendages encoding the chemical information that can be transformed into a collection of products having distinct molecular skeletons, by applying common reaction conditions. Therefore, these substrate-based transformations are referred to as folding pathways.[65]

Oguri and co-workers developed a divergent synthetic process to produce a collection of cyclic scaffolds to screen for human African trypanosomiasis, an important yet neglected disease caused by a parasitic protozoa.[71] Inspired by the structural feature of artemisinin, a natural compound active against the parasite, the authors designed six types of scaffolds with systematic structural variations, consisting of three types of stereochemical relationships on the sp^3 ring-junctions and two distinct arrays of tricyclic frameworks.

The starting cyclohexenone **99** was transformed by the introduction of three different chains harboring different relative configurations, thus delivering a series of cyclization precursors (Scheme 10). Enynene metathesis then yielded six tricyclic scaffolds incorporating a diene group (**107**–**112**). A first screening of these tricyclic stereoisomers for trypanosomal activity led to the identification of the three-dimensional shape of the pharmacophore and scaffolds **107** and **108** were selected as lead compounds for potent anti-trypanosomal agents. By introducing a peroxide bridge on the scaffold, Oguri generated artemisinin analogues with potent in vitro anti-trypanosomal activities comparable or even



Scheme 10. Divergent synthetic pathways to six types of tricyclic dienes, leading to the discovery of a potent anti-trypanosomal agent.

superior to those of artemisinin and the approved drugs, suramin and eflornithine.

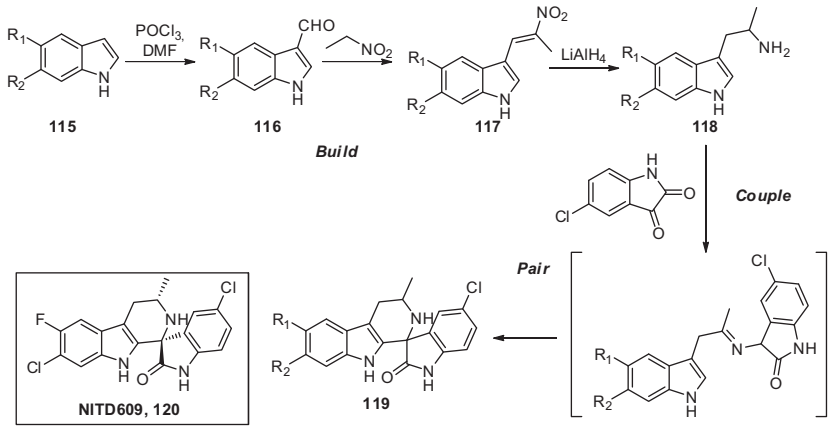
4.1.3.3. The build/couple/pair approach. Some of the ideas discussed above were further refined by Nielsen and Schreiber, who proposed the *build*/*couple*/*pair* strategy for the practical achievement of optimal synthesis of small molecules collections for biological screenings.[72] In the first phase, or *build* phase, the required building blocks, containing orthogonal reactive elements and stereogenic centers, are prepared either by asymmetric synthesis or from the chiral pool. In the second phase, or *couple* phase, these starting materials are assembled together to produce molecules with a dense array of functional groups; multicomponent reactions are generally used at this stage to couple three or more building blocks. Finally, the *pair* phase involves the intramolecular reaction of complementary functional groups strategically placed in the build phase to generate compounds with diverse molecular scaffolds. The outcome of the process is then dictated by the nature of functional groups and by their position within the starting materials, so their selection and placement are critical for a successful pathway. The new functionalities that result from pairing reactions can further participate in pairing-reactions or in appending processes, thus increasing molecular diversity.

In 2010, a screening campaign of 12,000 compounds focused on the discovery of antimalarial agents with a potentially new mechanism of action led to the identification of a synthetic molecule related to the spiroazepineindole class as a starting point for medicinal chemistry optimization efforts.[73] The synthesis of the library prepared using the build/couple/pair strategy involved the preparation of the building block **118** which was then coupled to an isatine-derivative (Scheme 11). Sequential in situ diastereoselective pairing employing the Pictet–Spengler cyclization afforded the corresponding spiroazepineindole compounds.[74] The four stereoisomers were resolved by chiral chromatography and tested for *Plasmodium falciparum* inhibition growth. The results showed that the 1*R*,3*S* stereoisomer was 250-fold more potent than the 1*S*,3*R* isomer, the former possessing the required stereochemical configuration for activity. Further studies demonstrated that the spiroindolones suppresses protein synthesis in the parasite, a mechanism of action distinct from that of existing antimalarial drugs. The lead compound NITD609 exhibits potency in the low nanomolar range, displays good drug-like properties and has physicochemical properties compatible with oral administration. It is currently in Phase IIa clinical trials and represents the first antimalarial drug with a novel mechanism of action to progress to this stage in the past 20 years.

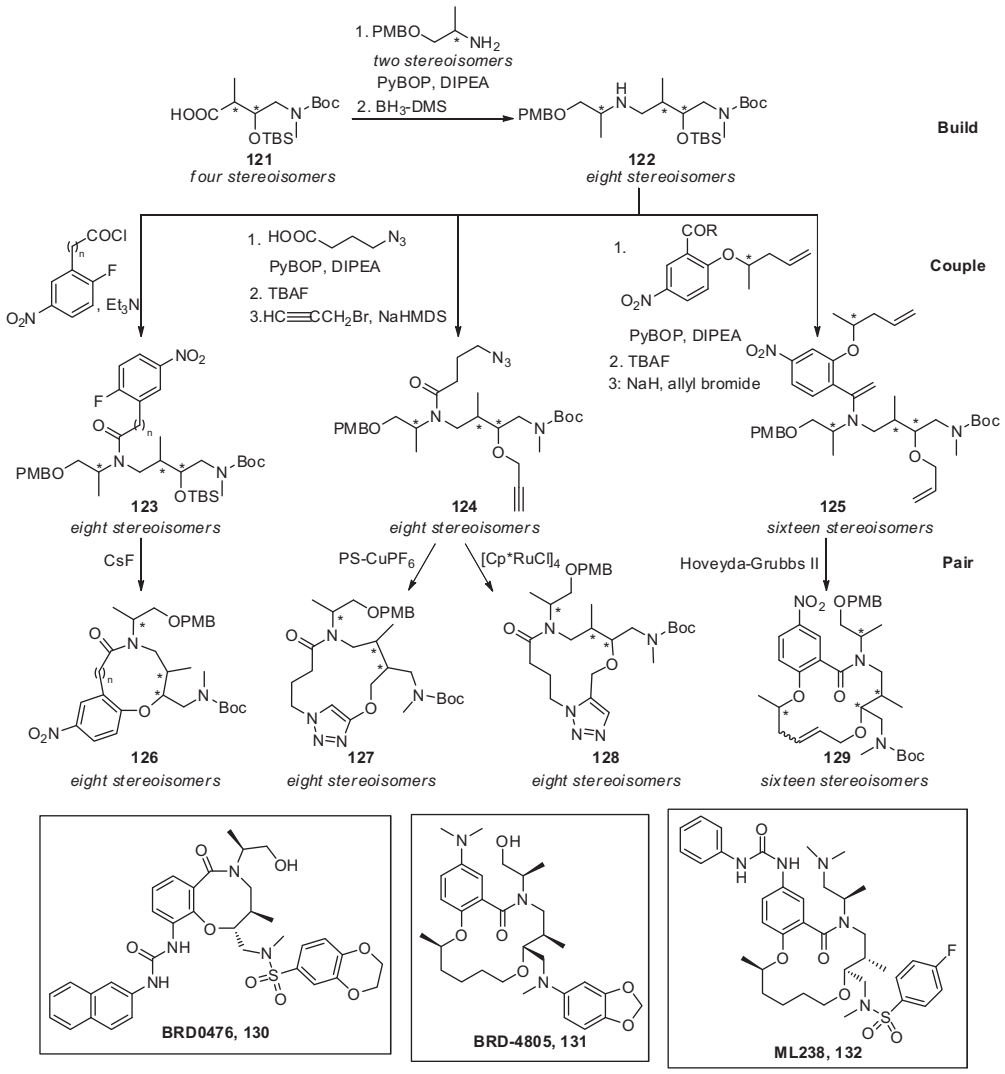
Macrocyclic compounds generally often display high affinity and selectivity for targets due to the conformationally pre-organized ring structures as exemplified by several drugs in preclinical and clinical development.[75] Marcaurelle and co-workers reported the synthesis of a collection of stereochemically and skeletally diverse medium- and large-sized ring systems through an aldol-based build/couple/pair strategy.[76] In the *build* phase, the authors prepared four stereoisomers of a chiral γ-amino acid building blocks **121** (Scheme 12), which were then coupled to two enantiomers of PMB-protected alaninol, thus generating the corresponding eight stereoisomeric secondary amines **122**, following reduction of the carbonyl. These compounds were differently functionalized depending on the type of cyclization mode used in the *pair* phase. The 8- and 9-membered rings **126** were obtained through nucleophilic aromatic substitution (S_NAr) using a one-pot TBS deprotection/cyclization sequence. The 12- and 13-membered rings **127** and **128** were synthesized through a Huisgen [3+2] cycloaddition, employing both copper and ruthenium catalysts in order to form 1,4- and 1,5-triazoles, respectively. Finally, 14-membered rings **129** were prepared through ring-closing metathesis (RCM). These 48 macrocycles were subjected to protecting group manipulations to load them onto solid support, and then derivatized, producing over 30,000 compounds, using Syn-Phase Lantern Technology.

The S_NAr-based library was screened with other DOS libraries in a phenotypic assay to identify small molecules with the ability to prevent cytokine-induced B-cell apoptosis.[77] Apoptosis of insulin-secreting B-cells in the pancreas is induced by cytokines in type 1 diabetes,[78] hence compounds that can protect B-cells from such a process can lead to novel drugs against this disease. After several rounds of biological evaluation and chemical alterations, BRD0476 (**130**, Scheme 12), a potent suppressor of B-cells death exhibiting an EC_{50} of 0.78 μM was identified. The data collected provided compelling evidence that the use of this class of compounds may represent a suitable strategy to protect pancreatic B-cells from pro-inflammatory cytokines in the context of type-1 diabetes.

The RCM-derived library was tested for inhibitory activity on histone deacetylases (HDACs). The selected hit from a pilot screen for HDAC 2 inhibitors was subjected to SAR and stereochemical structure–activity relationship (SSAR) studies, revealing marked differences in the inhibitory activity and in selectivity among all stereoisomers, thereby highlighting the importance of stereodiversity in library composition.[76] The lead compound BRD4805 (**131**, Scheme 12) displayed isoform selectivity for HDACs 1–3, without inhibiting HDACs 4–8, thus representing a starting point for a new class of HDAC selective inhibitors.



Scheme 11. Synthesis of spiroindolones, a potent class of small molecules for the treatment of malaria.



Scheme 12. Build/couple/pair strategy for generating macrocycles and medium-sized rings.

The same library was also screened in a phenotypic blood-stage malaria assay.[79] The most active hit was then analyzed by SSAR and only one of the 16 stereoisomers showed biological activity. Further SAR studies reveal ML238 (**132**, Scheme 12), a compound that showed subnanomolar activity in two *Plasmodium falciparum* strains, which was found to be more potent than chloroquine or artesunate and exhibited a similar potency compared to that of atovoquone (all three are known anti-malarial agents used as controls). ML238, which is soluble in water and nontoxic to erythrocytes and HepG2 cells is thus a promising antimalarial with a unique chemotype. Studies to discover the mechanism of action for this novel class of antimalarial agents are currently underway.

5. Conclusions

Over the past two decades TGS, FBDD and DOS have become effective new strategies employed in medicinal chemistry, lead discovery and biological target identification. Their use in both industrial and academic settings and the range of targets investigated has revealed the complementary nature of these methodologies. So far a large number of compounds discovered by DCC have not had better activity than the corresponding commercially available drugs. Also compounds discovered using this methodology have limited stability due to the reversible nature of their synthesis and attempts to replace the dynamic interconnections had frequently deleterious consequences on activity. In contrast, in situ click chemistry has proven to be an efficient tool for probing biomacromolecular chemical space and it is conceivable that molecular fragments can be used in vivo for an in situ synthesis of drug substances directly in the host.[80] FBDD on the other hand has proven that relatively small libraries can cover relevant chemical space, resulting in hits with diverse structures and chemical motifs. It represents an effective strategy to produce potent compounds, which can move successfully into in vivo and clinical trials, since they already have optimal physico-chemical properties. While these methods are inherently biased towards a target of interest, DOS strategies have made possible the efficient preparation of new types of small molecules that are currently absent in most collections, thus enhancing the chances of finding hits against the so-called 'undruggable' or non-traditional targets. DOS provides the chemical means to perform unbiased phenotypic based screen designed to discover unanticipated druggable biological partners. This represents a distinct feature of DOS compared to the other methodologies described above, TGS and FBDD, for which drug discovery requires prior knowledge of structural and biological information about the biological target of interest. Screening of TGS and FBDD libraries generally requires water-soluble proteins and tends to entail a large amount of both the target and the

compounds of interest. In contrast, the more common whole cell assays can be used when screening DOS libraries. Whilst FBDD generally provides flat molecules, DOS produces more complex structures, which more closely resemble nature's collection. While these concepts have been discussed separately in this review, examples of the combination of two or more of these approaches are appearing in the literature. For example, Young and co-workers have described the application of DOS to synthesize a library containing highly sp^3-rich scaffolds for fragment-based screening.[81] FBDD has also been combined with DCC in the screening of fragment-based dynamic combinatorial libraries for a range of targets.[82] Although these concepts have been developed relatively recently, the impact of this chemistry is undeniable. What is certain is that the dialogue between chemists and biologists is crucial to identifying new biologically active structures and elucidating mechanisms at the molecular level.

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