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TUTORIAL REVIEW

Diversity-oriented synthesis: producing chemical tools for dissecting biology

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Small molecule modulators of biological function can be discovered by the screening of compound libraries. However, it became apparent that some human disease related targets could not be addressed by the libraries commonly used which typically are comprised of large numbers of structurally similar compounds. The last decade has seen a paradigm shift in library construction, with particular emphasis now being placed on increasing a library's structural, and thus functional diversity, rather than only its size. Diversity-oriented synthesis (DOS) aims to generate such structural diversity efficiently. This *tutorial review* has been written to introduce the subject to a broad audience and recent achievements in both the preparation and the screening of structurally diverse compound collections against so-called 'undruggable' targets are highlighted.

Introduction

The ability of small molecules to interact with macromolecules and perturb their function has emerged as a powerful tool for dissecting biological processes and indeed forms the basis of modern medicine.[1–4]

Small molecules function as critical components of signalling pathways regulating a host of cellular processes from stem cell differentiation to the molecular basis of memory.[8–12] The majority of FDA approved drugs currently available are small molecules which offer several distinct advantages over biologics.[13,14] For example, the small molecule approach provides a rapid, temporal and often reversible method for modulating biological function in a concentration dependent manner, making them not only useful as drugs but also as chemical probes to study biological systems.[1,4,15,16]

Traditionally high-throughput screening (HTS) of large libraries of compounds has been employed by the pharmaceutical industry, to identify bioactive small molecules.[17,18] Similarly, chemical genetics screens large compound collections in phenotypic assays to identify compounds which elicit a particular biological effect.[4,16] Since the beginning of the 1990s, library

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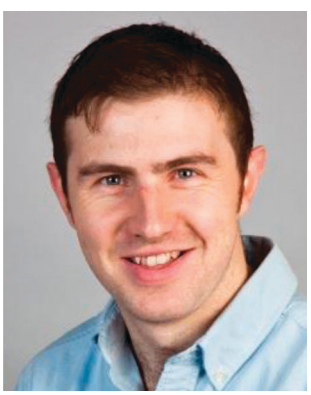


Cornelius J. O' Connor

Cornelius (Con) O' Connor grew up in Carlow, in the south east of Ireland. He obtained his B.A. (Mod.) in Chemistry in 2004 from Trinity College Dublin where he also undertook his PhD under the supervision of Dr Mike Southern, studying nucleoside mimetics and developing synthetic methodology. He subsequently joined the Connon Group at Trinity, where he worked on the development of novel catalytic systems. Con then moved to the University of Cambridge where he is currently a member of the Spring Group, researching the use of small molecules to modulate protein-protein interactions, diversity-oriented synthesis and chemical genetics.

Henning S. G. Beckmann

Henning S. G. Beckmann grew up in Soest (Germany). He studied chemistry at the University of Konstanz (Germany) interrupted by a six-month internship with Aventis Pharma (Frankfurt a. M., Germany). He earned his PhD at the University of Konstanz under the supervision of Prof. Dr V. Wittmann working on chemoselective ligations and the preparation of carbohydrate arrays. In 2010 Henning joined the Spring Group at the University of Cambridge as a DAAD postdoctoral fellow, undertaking a DOS project synthesizing a library of diverse non-peptidic macrocycles.

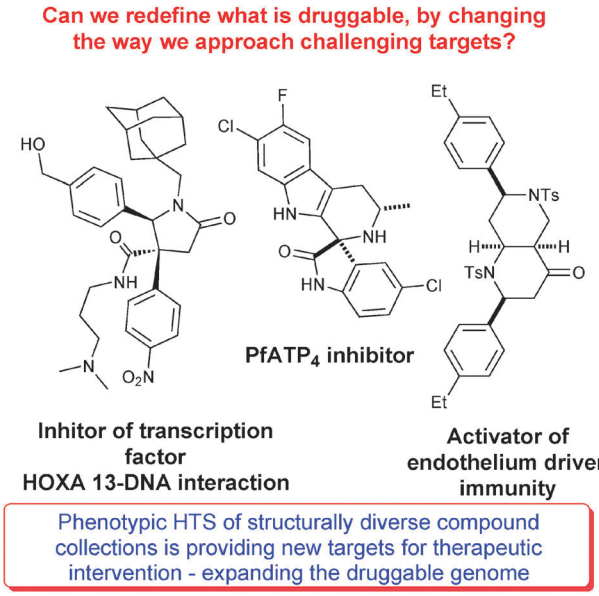


Fig. 1 Schematic illustrating some small molecule modulators of protein function discovered from screening structurally diverse libraries.[5–7]

composition has been scrutinized and it has become clear that the structural diversity of its components is a crucial determinant, when selecting an appropriate library for a particular screening campaign (see **Assessing Diversity** *vide infra*). Screening focused libraries assembled around known natural ligands or structures derived from molecular modelling has proven to be very successful at generating leads of known and evaluated targets, such as G-protein coupled receptors (GPCRs) or kinase enzymes (the current repertoire of pharmacopeia is comprised of almost 36% GPCR inhibitors and approximately 29% enzyme modulators).[19] However, many other human disease-related targets such as transcription factors, protein-protein interactions (PPIs) and protein-DNA interactions have been termed 'undruggable' as they have proved less addressable to the small molecule approach typically used in drug-screening programs. But are these targets really undruggable or have they simply been challenged with the wrong types of molecules? It is becoming increasingly evident that the latter is indeed the case[5–7] (see Fig. 1).

David R. Spring

David Spring is currently a Reader at the University of Cambridge within the Chemistry Department. He received his DPhil (1998) at Oxford University under Sir Jack Baldwin. He then worked as a Wellcome Trust Postdoctoral Fellow at Harvard University with Stuart Schreiber (1999–2001), after which he joined the faculty at the University of Cambridge. His research programme is focused on synthetic chemistry and chemical biology.

HTS of *functionally diverse* compound collections is emerging as a powerful method for identifying chemical probes of biological function and as a consequence, lead compounds for drug development.[17,22] The construction of functionally diverse compound libraries has become a venerable field of organic chemistry known as 'diversity-oriented synthesis' (DOS), which aims to synthesize, structurally complex small molecules in an efficient manner.[23–26] In this *tutorial review* we outline recent developments in the field with particular focus on the discovery of small molecule modulators of challenging biological targets from DOS screening campaigns.

Chemical space

The construction of an 'ideal', functionally diverse library of small molecules would comprise modulators for all biological processes. That is, such a library would span the total bioactive area of chemical space which is the entirety of thermodynamically stable molecules. A widely cited back-of-an-envelope calculation estimated the size of drug-like chemical space (*i.e.* compounds of mass < 500 Da) to be about 10^{63} compounds.[27] The size of the bioactive area of chemical space is considerably smaller, as it has been constrained by nature through evolution; however, its boundaries remain undefined. Advancements in genomic and proteomic technologies are likely to continue to reveal targets for therapeutic intervention, but only chemical exploration of these disease states with functionally diverse compounds, will determine whether these targets are amenable to small molecule modulation. Clearly, library composition is of paramount importance, when screening for chemical bioprobes. This poses the question: When constructing a library, how does one maximize functional diversity?

Assessing diversity

Biological macromolecules interact with each other in a three-dimensional environment. Functional diversity is directly related to the three-dimensional chemical information that the surface of a small molecule presents to a macromolecule. Thus, the functional diversity is directly associated with the structural diversity of a compound collection (see Fig. 2). Structural diversity is typically divided into four principal components:[24]

(1) Appendage diversity—variation in structural moieties around a common skeleton

(2) Functional group diversity—variation in the functional groups present

(3) Stereochemical diversity—variation in the orientation of potential macromolecule-interacting elements

(4) Skeletal (scaffold) diversity—presence of many distinct molecular skeletons

The advent of combinatorial chemistry enabled the synthesis of huge compound collections, and improvements in robotics enabled HTS of these vast libraries against diverse protein targets. However, a commensurate increase in marketed drugs was not observed.[28] Structural analyses revealed that the combinatorial compound collections had high appendage diversity but very limited skeletal diversity. Indeed, it was shown that the overall three-dimensional shape diversity of a library is primarily dependent on the diversity of the central

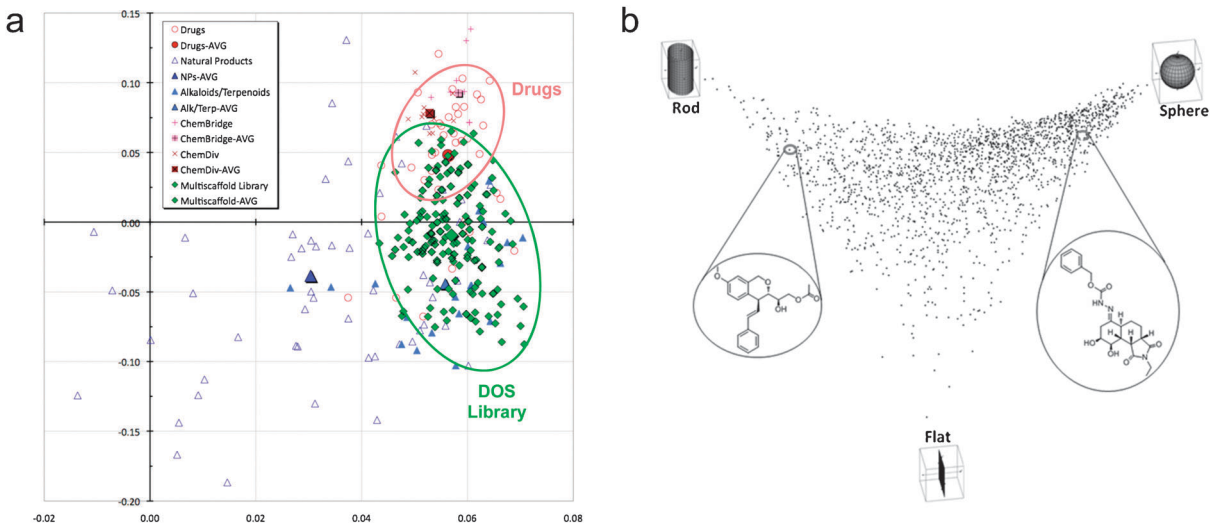


Fig. 2 Diversity assessment of compound collections. (a) Principal component analysis (PCA) of the 40 top-selling drugs (red circles), 60 diverse natural products (open blue triangles), 20 polycyclic alkaloids and terpenoids (filled blue triangles), 20 ChemBridge and ChemDiv library members (crosses), and 190 members of a DOS library synthesized by Tan and coworkers (green diamonds).[20] The DOS library spans a large area of chemical space distinct from the area populated by approved drugs and commercial compound collections. Adapted with permission from ref. 20. (b) Principle moments of inertia (PMI) analysis of the 2070 membered diversity compound collection of the Center for Chemical Methodology and Library Development at Boston University (CMLD-BU).[21] The analysis reveals a significant predominance of spherically shaped compounds over flat compounds typically found in commercial collections. Adapted with permission from ref. 21.

scaffolds, with the peripheral substituents being of minor importance.[29] Thus, a high degree of skeletal diversity is essential to maximize the functional diversity of a compound collection.

In a recent study, Schreiber and co-workers assessed the importance of stereochemical complexity in library composition when screening for bioactivity. They obtained binding profiles of 15,000 compounds comprising commercially available compounds; compounds created in academic labs (*e.g. via* DOS); and natural products against 100 sequence unrelated proteins using microarrays.[30] The stereochemical complexity of each compound was assessed based on the ratio of stereogenic centers to the total number of carbons (restricted to the scaffold) and was matched with the binding profile of the compound. Compounds of simple stereocomplexity ($C_{stereo}/C_{total} = 0$) exhibited the highest promiscuity in protein binding. Generally, binding selectivity increased with enhanced stereocomplexity. However, for compounds of high stereochemical complexity ($C_{stereo}/C_{total} > 0.25$) the lowest overall hit rate was found. Thus, the highest proportion of selective binders was observed for compounds with an intermediate level of stereochemical complexity ($0 < C_{stereo}/C_{total} < 0.25$). These findings coincide with the observation that the average level of stereocomplexity of drug candidates increases from discovery through clinical trials to approval presumably due to the enhanced binding selectivity of more complex candidates.[31] Thus, libraries used for the identification of new small molecule modulators should exhibit a high degree of structural diversity and an enhanced level of stereochemical complexity.

The structural diversity of a library can be computationally assessed, by completing a comparative statistical analysis of a defined set of molecular descriptors for a given collection of molecules. Two statistical approaches are typically employed to this end: principal component analysis (PCA)[32] and principal moments of inertia (PMI) analysis.[29] PCA utilizes a defined number of descriptors (usually physicochemical or biological properties), such as, molecular weight, logP values, or the number of hydrogen bond donors, to represent each molecule as a vector in *n*-dimensional space. *n*-Dimensional vectors can then be reduced to 2-dimensional vectors which can be re-plotted, giving an illustrative representation of the library diversity. PMI analysis employs shape based descriptors: the minimum energy conformation of each library member is determined, PMI ratios are calculated and normalized, and a subsequent triangular graph plot depicts the shape diversity of the library. Examples of both PCA and PMI analysis are shown in Fig. 2.

Sources of small molecules for use in biological screens

Natural products. Traditionally, natural products have been a valuable source of leads in drug development, especially in the areas of cancer therapeutics and anti-infective agents.[28,33,34] Although natural products represent a vast pool of structural diversity, there are several problems associated with using natural products in biological screens and drug-development (*e.g.* access and supply, purification, identification, chemical modification, and concerns about intellectual property rights).[33,34]

Commercial compound collections. Alternative sources for small molecules are commercially available libraries and pharmaceutical proprietary compound collections. These collections tend to be highly biased towards traditional drug targets such as GPCRs, ion channels and kinases – areas in which they have proven to be quite successful.[22] However, these compound collections turned out to be less successful in screening campaigns

against non-traditional targets such as PPIs. A large fraction of commercial compound libraries are prepared in combinatorial chemistry programs by attaching different appendages to a limited number of common skeletons. Consequently, the scaffold diversity of such libraries is relatively limited. For instance, 83% of the core ring scaffolds found in natural products are absent among commercially available compounds.[35] Additionally, commercially available compounds are typically of low stereochemical complexity, containing few stereogenic centers and a high proportion of sp^2-hybridized carbon atoms.[30] Given these limitations of structural, and thus functional diversity, there is reason to doubt that libraries based on commercially available compounds are appropriate to tackle so-called 'undruggable' targets.

Diversity-oriented synthesis. DOS was developed over the last decade, in order to address the need for functional diverse small molecule collections and in consideration of the problems associated with the use of natural products and commercially available compounds.[23,24,26,36] DOS has been defined as the deliberate, simultaneous and efficient synthesis of more than one target compound in a diversity-driven approach.[25] DOS programs aim to prepare structurally, and thus functionally, diverse libraries that interrogate large areas of chemical space including known and previously 'un-tapped' regions of bioactive chemical space.

In order to create such a library, all four principal types of structural complexity mentioned previously (see **Assessing Diversity**) have to be addressed. However, the synthetically most challenging facet of DOS, and of central importance to its success, is the efficient generation of scaffold diversity within a library.[24,29] Different strategies to achieve this requirement efficiently are discussed below. Additionally, DOS libraries usually have an enhanced level of stereochemical complexity compared to commercially available compounds which should be beneficial in terms of binding selectivity.[30] Another important feature of DOS libraries is that complex molecules are prepared in an efficient and modular manner, typically in no more than five synthetic steps. If hits have been identified in a screening campaign, interesting compounds can be obtained easily in larger amounts and focused libraries around the hit structures can be generated conveniently. Thus, DOS compounds overcome the disadvantages associated with the availability and modification of natural products and as a consequence DOS holds the promise of significantly accelerating the drug discovery process.[37]

Generating diverse libraries using DOS

In general, the different strategies used to generate scaffold diversity can be categorized into two principal approaches, the reagent-based approach and the substrate-based approach.[24]

The reagent-based approach comprises methods based on pluripotent functional groups and methods that use densely functionalized molecules. A somewhat complementary classification is the build/couple/pair concept that can be recognized in many DOS strategies. In this section, recent outstanding examples for these approaches are highlighted.

Reagent-based DOS using densely functionalized molecules

In this approach different functional groups of a densely functionalized molecule are transformed, or paired, intramolecularly by different reagents to create scaffold diversity. Crucial to the success of such a strategy is the choice of a densely functionalized molecule that enables many transformations or pairing reactions.

In a recent example, Tan and co-workers identified *tert*-butylsulfinimide tethered enynes and diynes as promising densely functionalized molecules.[20] Solid supported enynes **1a** and diynes **1b** were subjected to a number of different reaction conditions that stereoselectively paired the unsaturated functionalities leading to different scaffolds (Scheme 1(a)).

In total, a library of 190 compounds comprising 10 distinct polycyclic, alkaloid/terpenoid-like scaffolds was synthesized on solid support.

In another outstanding example, Waldvogel and co-workers[38] used readily accessible racemic polycyclic scaffold **2** as a densely functionalized molecule (Scheme 1(b)). Compound **2** was subjected to various reaction conditions under which the scaffold underwent remarkable stereoselective transformations and rearrangements. In total, 13 compounds based on 9 complex polycyclic scaffolds were prepared in only 13 reactions.

Further notable examples of this strategy are the synthesis of 11 different scaffolds starting from a common keto-dienoate precursor reported by Robbins *et al.*,[39] and the combinatorial screening for diversity generating cascade reactions resulting in 52 compounds comprising 12 distinct scaffolds reported by Kumar and co-workers.[40]

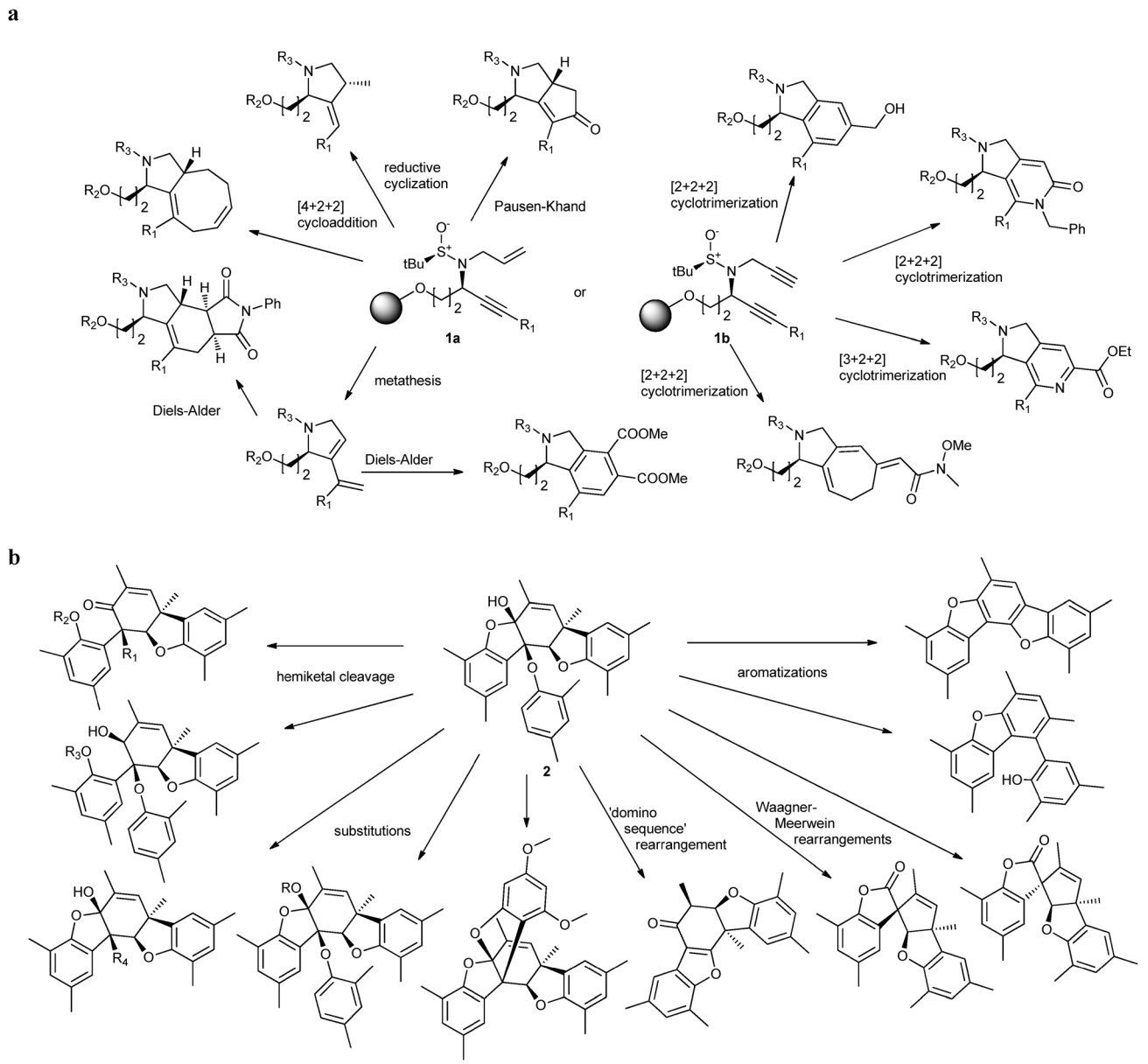
Reagent-based DOS using a pluripotent functional group strategy

In this approach a pluripotent functional group is transformed into different products by different reagents (Scheme 2). Ideally, each product of this first reaction sequence enables a new round of diversity generating reactions. As a result, a branching pathway leads to a collection of diverse scaffolds. For instance, Thomas *et al.*,[41] starting from α,β-unsaturated acylimidazolidinones, (pluripotent functional group), followed a branching pathway, from which they prepared a library of 242 compounds based on 18 molecular frameworks. Further illustrative examples for this approach are the use of nucleophilic phosphine catalysis starting from allenes or alkynes reported by Cruz *et al.*[7] and the construction of a library of 223 compounds comprising 30 distinct scaffolds reported by Spring and co-workers.[42]

The substrate-based approach

In contrast to the reagent-based approaches discussed above, the substrate-based approach applies common reaction conditions to a collection of substrates and a folding process transforms the 'pre-encoded' substrates into different molecular skeletons.

This can be clarified by a DOS project conducted by Nelson and co-workers.[43] They prepared a collection of linear metathesis substrates by the combinatorial attachment of two building blocks (the so-called 'propagating' and 'capping' blocks) to a fluorous tagged linker that facilitated convenient purification



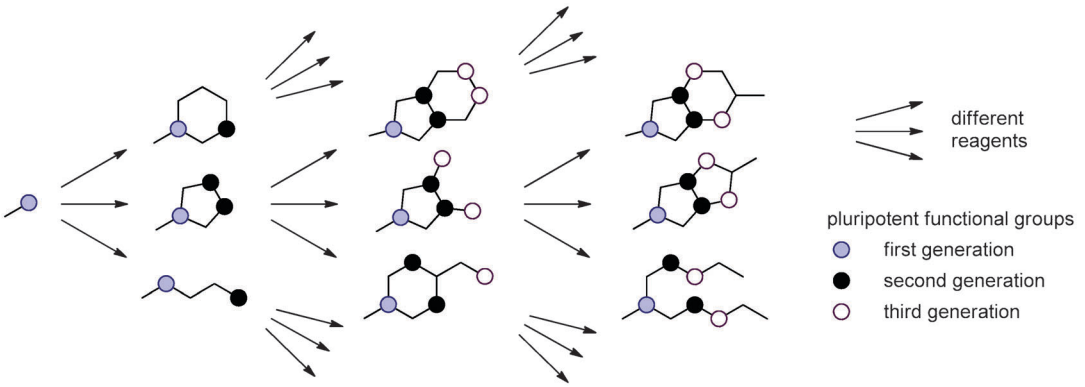
Scheme 1 Reagent based approaches using densely functionalized molecules. (a) Tan and co-workers used a various set of pairing reactions to create a library of 190 compounds based on 10 distinct scaffolds.[20] (b) Waldvogel and co-workers used a set of reagent-induced rearrangements and transformations to create 13 compounds based on 9 complex polycyclic skeletons.[38]

by fluorous solid-phase extraction. These substrates contained several unsaturated moieties. When subjected to metathesis conditions, they underwent intramolecular cyclization cascades in terms of a folding process. Depending on the 'pre-encoded' location of unsaturated moieties, different molecular scaffolds were formed. Additionally, only cyclized products were released from the fluorous tag due to the elegant design of the linker. Using this substrate-based approach Morton *et al.* synthesized 96 compounds comprising 84 distinct scaffolds (Scheme 3).

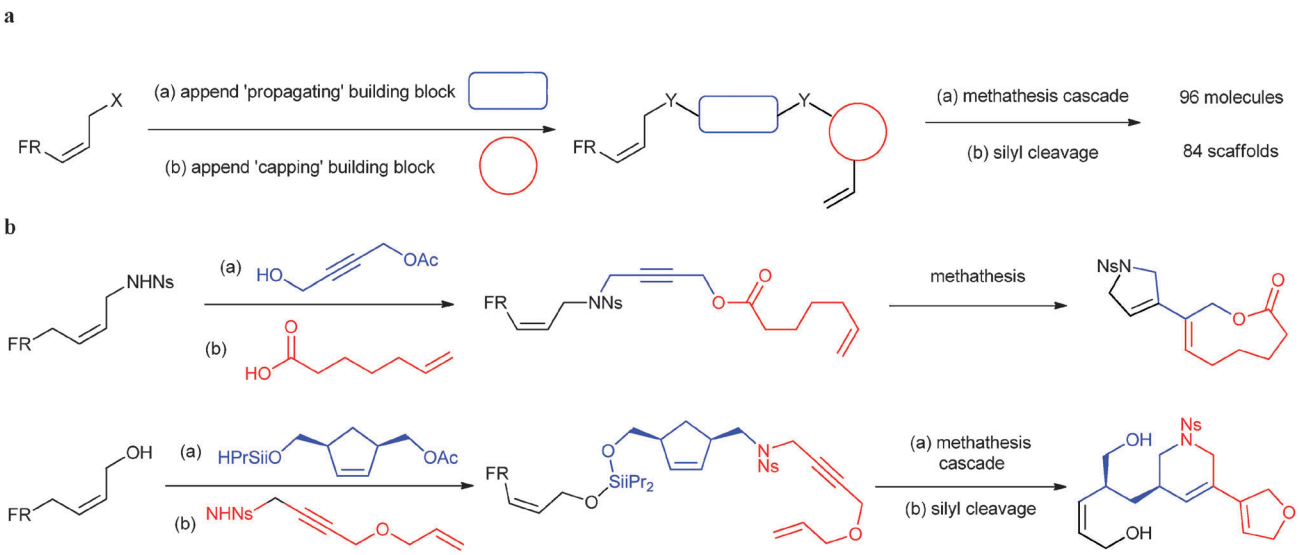
Not all DOS programs can be clearly classified into the distinct categories discussed above. For instance, Cui *et al.*[44] recently reported the construction of a DOS library using aspects of both reagent- and substrate-based DOS approaches.

The build/couple/pair strategy

The build/couple/pair (B/C/P) algorithm which was introduced by Nielsen and Schreiber is a strategic feature that can be found in both reagent based and substrate-based approaches.[37] For instance, the DOS program depicted in Scheme 3 can be described as a B/C/P strategy. In the initial 'build' phase the 'propagating' and 'capping' building blocks were synthesized. The 'couple' phase involves the attachment of the different building blocks to the linker. The metathesis cascade is performed in the final 'pair' phase. Other recent DOS approaches that can be analysed in terms of a B/C/P strategy were reported by Pizzirani *et al.*,[45] Uchida *et al.*[46] and Spring and colleagues.[47]



Scheme 2 The reagent based approach using pluripotent functional groups which are transformed into different products by different reagents in a branching pathway.



Scheme 3 The reagent based approach using pluripotent functional groups which are transformed into different products by different reagents in a branching pathway.[43]

Screening small molecules for biological activity

In recent years, DOS has revolutionized the construction of diverse compound libraries; however, the synthesis of an ideal library covering total bioactive space still remains utopian. Additionally, most synthetic academic labs have limited access to screening centers to assess the bioactivity of compounds they synthesize. Thus, collections of high bioactivity diversity are often subjected to screening against a small number of biological targets and their full potential remains under-explored. National and international compound repositories and screening initiatives try to overcome both problems. By clustering diverse libraries, a large compound collection of superior bioactivity diversity is obtained that can be screened in a large number of drug discovery and chemical genetics programs.

The pharmaceutical industry and biotechnology companies have employed HTS in drug discovery programs for decades.[17,48] However, since the turn of the century, this approach for hit finding has become increasingly popular in academic settings. Many large academic institutions now have the capability to carry out medium- to high-throughput screening programs and this can largely be attributed to the availability of commercial compound collections and affordable automated instrumentation and associated systems software.[18]

The Society for Laboratory Automation and Screening (SLAS) currently lists 85 academic screening facilities (Fig. 3) over half of which are located in the US (http://www.slas.org). Many of the listed facilities work co-operatively in screening networks, increasing their screening capabilities by pooling resources. For instance, the US National Institutes of Health (NIH) installed the Molecular Libraries Small Molecules Repository (MLSMR) which collected more than 400 000 compounds from various sources, including academic groups (http://mlsmr.glpg.com). This compound collection is the basis for a comprehensive screening program run by the Molecular Libraries Probe Production Centers Network (MLPCN). The MLPCN is a network of nine US institutions, each of which has different screening capabilities (http://mli.nih.gov/mli/mlpcn/mlpcn). In Europe, ChemBioNet (http://www.chembionet.info/) and the UK Drug Discovery Consortium (http://www.ukddc.org/) are networks with screening facilities and associated support

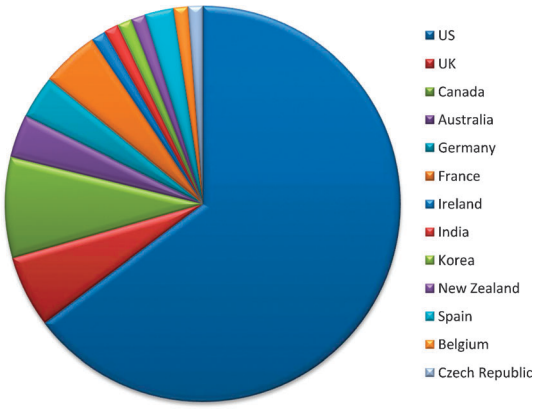


Fig. 3 Geographical distribution of academic screening centres.

services, designed to support chemical biology programs for the development of bioactive small molecules. The EU-OPENSCREEN initiative which will enter its operational phase this year, is a pan-European network integrating high-throughput screening centers, chemical libraries, medicinal chemistry facilities for hit optimization, informatics support and a central database (http://www.eu-openscreen.eu/).

Despite the increase in academic screening networks, their success in generating new leads for drug discovery will hinge on continued diversification of the compound collections and increasing the variety and robustness of biological assays available for screening.

Discovering bioactive small molecules using DOS

Most drug discovery initiatives (both academic and industrial) rely on screening large compound collections for activity against biological targets. However, despite the huge advancements in genomics and proteomics, in the present-day druggable genome only 500 genes out of approximately 20 000 genes are amenable to small molecule modulation.[49] Improvements in assay development and library construction will increase the identification of small molecule modulators of what are now considered challenging therapeutic targets and gradually redefine what is druggable. The following examples highlight the potential of DOS as a tool for discovering bioactive small molecules, focusing on three challenging areas in drug discovery: disrupting protein-protein interactions (PPIs), discovering new antibiotics and inhibiting histone deacetylase (HDAC) enzymes.

Protein-protein interactions (PPIs)

PPIs regulate a host of critical cellular functions, from DNA replication and repair, to intracellular communication and programmed cell death (apoptosis). Aberrant or malfunctioning PPIs can cause a plethora of diseases, and as such they are a very attractive target for therapeutic intervention.[50] Despite the considerable difficulties associated with PPIs as a drug-target class, several approaches have been adopted to discover highly potent small molecule modulators.[50–53] In the following section recent key examples of small molecule modulators of PPIs discovered through the screening of DOS libraries are highlighted.

Inhibition of the sonic hedgehog signaling pathway

The Schreiber Group recently reported the discovery of robotnikinin (see Fig. 4), a small molecule inhibitor of a PPI involved in the hedgehog signal transduction pathway.[54]

The hedgehog signaling pathway plays an essential role in embryonic development by regulating cell proliferation and differentiation. Sonic hedgehog (Shh) initiates the hedgehog signaling cascade when it binds to a 12-pass transmembrane receptor Patched (Ptch1).[55] When Shh binds to Ptch1, its inhibitory activity on Smoothened (Smo), a 7-pass transmembrane receptor which resembles a GPCR, is removed. Activation of Smo results in the release of Glioma (Gli) family transcription factors, which translocate to the nucleus and regulate the transcription of target genes including *Gli1* and *Ptch1*.[54–56] Mutation of genes in the hedgehog signaling pathway, reactivation of the pathway in adults and/or aberrant Shh signaling are associated with tumor initiation and maintenance in basal cell carcinoma (BCC), meduloblastoma, panctreatic and prostate cancers.[55]

Several small molecule agonists and antagonists of the hedgehog signaling pathway have been identified using cell based phenotypic screening, which act on targets downstream of the Shh-Ptch1 interaction.[55] Schreiber and co-workers screened a DOS library of 2070 small molecules arrayed onto microscope slides, for binding affinity to a bacterially expressed N-terminal construct of the sonic hedgehog protein, ShhN.[54,57] Lead optimization on initial hits produced robotnikinin (Fig. 4), which showed low micromolar affinity for ShhN (K_d of 3.1 μM).[54] In a Gli-luciferase reporter gene assay, robotnikinin was found to inhibit Gli transcription by targeting a protein upstream of Smo in the Shh signal transduction pathway. Gli transcription was also inhibited by robotnikinin, in a dose dependent manner, when tested in human derived keratinocytes and in a synthetic model for human skin.

No inhibitory activity of the Shh pathway was observed in a cell line lacking the Ptch1 receptor, and no competition was observed with agonist- or antagonist-Smo interactions.[54] These results support a novel mechanism of action involving the inhibition of the Shh pathway upstream of Ptch1 whereby robotnikinin directly targets the ShhN protein complex.

Inhibiting members of Bcl-2 family

The Bcl-2 family of proteins is composed of both pro- and anti-apoptotic members, which regulate cell proliferation and

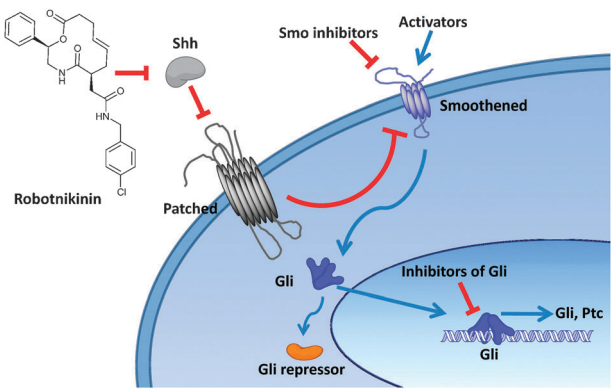


Fig. 4 Robotnikinin inhibits the induction of the Shh pathway.[54]

programmed cell suicide (apoptosis).[58–60] Up regulation of anti-apoptotic proteins is observed in many cancers. Inhibiting the interactions of proteins in the anti-apoptotic signaling cascade, results in cell death. Small molecule modulators of the Bcl-2 family of proteins therefore offer huge therapeutic potential for the treatment of angiogenic disorders. DOS, through its exploration of unchartered chemical space, is providing promising lead compounds with this chemotype.

Using a split pool solid phase DOS approach Marcaurelle *et al.*[61] designed and synthesized a diverse library of approximately 15 000 compounds. The compounds were tested for binding affinity against Bcl-2 and Bcl-xL using a fluorescence polarization (FP) assay modified for HTS with a fluorescently labeled BH3 peptide (peptide segment from natural binding partner). Bridged bicyclic pyridone compounds **3** (Fig. 5) displayed low micromolar activity against both Bcl-2 and Bcl-xL, whereas compounds of the type **4** (Fig. 5) selectively bound Bcl-2. In a related project, Castro *et al.*[62] screened a second DOS library for binding affinity to Bcl-2 from which several nanomolar inhibitors of Bcl-2 were discovered. Promising candidates from the initial FP assay were tested *in vitro* for cytotoxicity in RL cells (a human follicular lymphoma cell line), and a cell death assay with human pancreatic cancer cell line, Panc1, which led to the identification of compounds with IC_{50} of < 2 μM and < 1.3 μM respectively. Additionally, an *in vivo* study where the effects of treating a mouse xenograft model of human follicular lymphoma with **5** indicated a substantial decrease (> 2 fold reduction depending on route of administration) in relative tumor volume (RTV) when compared to untreated vehicle mice biopsies.

In 2009, in a multidisciplinary initiative to discover small molecule antagonists of Bcl-xL, Bifulco and colleagues combined *in silico* screening with the DOS approach.[63] They designed a virtual DOS library based on elaboration of 6 core scaffolds. Their approach involved iterative docking studies, followed by chemical synthesis of promising compounds and subsequent determination of binding affinities using *in vitro* assays. The discovery of several binders of Bcl-xL, validates this novel approach to discover small molecule modulators of PPIs, and demonstrates the utility of DOS to this end.

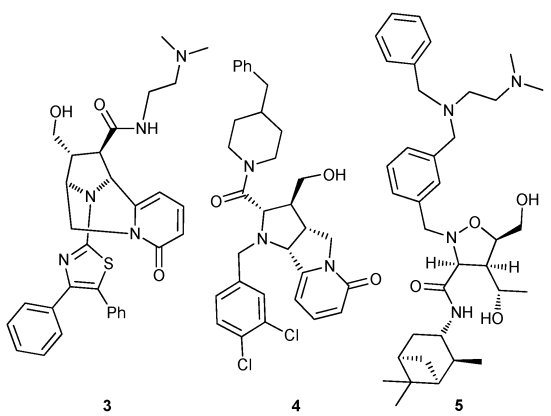


Fig. 5 Small molecule modulators of Bcl-2 family proteins.[61,62]

DNA damage checkpoint inhibitors

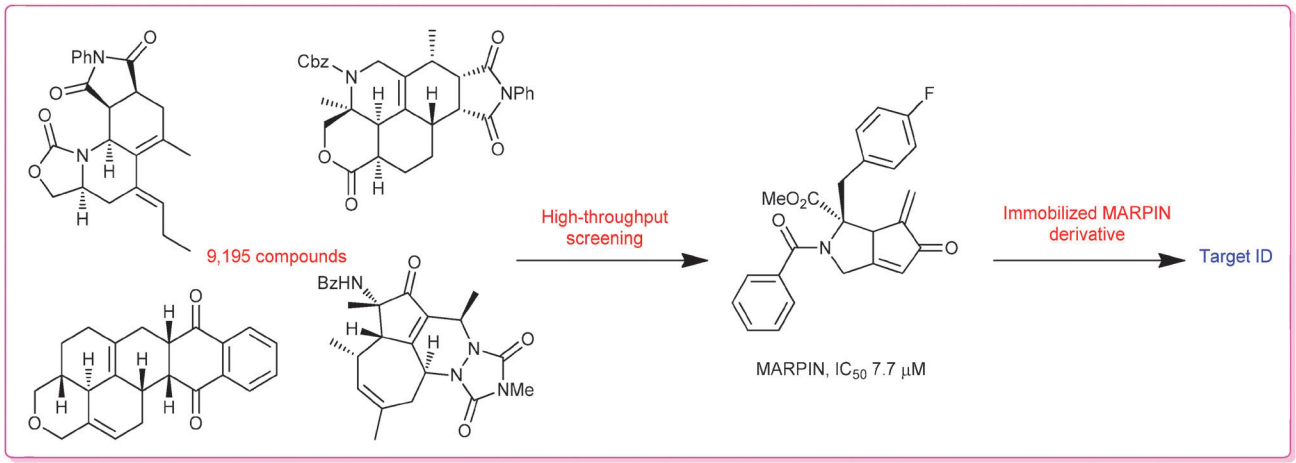
In order to maintain genomic integrity, cells activate DNA damage checkpoints which elicit cell cycle arrest and DNA repair, after exposure to genotoxic agents.[64] Members of the phosphatidylinositol 3-kinase superfamily, ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and Rad3 related (ATR) proteins, play critical roles in regulating DNA damage checkpoints. Defects in checkpoint genes and/or aberrant checkpoint PPIs can result in sensitivity to DNA damaging agents and increased genomic instability. If the ATM/ATR pathways are inhibited and DNA integrity is compromised, excessive genomic instability leads to the induction of apoptosis. Consequently the administration of DNA damage checkpoint inhibitors as adjuvant therapeutics could potentiate the efficacy and selectivity of DNA damaging agents.[65]

In order to discover novel small molecule inhibitors of ATM/ATR pathways, Huryn *et al.*[66] screened a DOS library of 9195 compounds in a cell-based assay for inhibition of Chk1 phosphorylation (a downstream protein kinase) after activation of ATR by induction of replication stress. The authors identified several active compounds, which inhibited ATM/ATR pathways and sensitized p53-deficient cells to DNA damaging agents, without suppressing ATR's catalytic activity. The authors suggest that this might indicate the inhibition of mediator proteins involved in the ATM/ATR pathways. Structure activity relationship (SAR) studies were carried out on the lead structure (Scheme 4), named MARPIN (ATM and ATR pathway inhibitor) and an immobilized derivative of MARPIN was developed. Pull-down assay experiments indicated binding to several protein targets. Further chemical genetics studies using this bioprobe, and the identification of these protein targets may illuminate previously unknown intricacies of the DNA damage checkpoint response.

Antibiotics

The discovery of antibiotics over 70 years ago revolutionized modern medicine. The accelerating emergence of resistant microbes, was considered a modern phenomenon, brought about through misuse and over prescription, but has since been verified as an inherent and ancient microbial defense mechanism.[67] The disturbing rise in resistance has transformed the panacea of penicillin into an interminable sprint, where our very survival is incumbent on the development of new antibiotics with novel modes of action, at a rate greater than the emergence of resistance.[68–70] Screening of existing combinatorial libraries has had limited success in identifying novel antibiotics, owed to the narrow focus of bioactive space being interrogated, and the inherent bias toward known protein targets.[71] DOS provides a means of populating diverse areas of bioactive space and is becoming increasingly popular as a drug discovery tool in the search for novel antibacterial therapeutics.[72]

In 2008, Spring and co-workers[41] screened a DOS library of 242 compounds, composed of 18 natural-product-like scaffolds, for antibacterial activity against three strains of *Staphylococcus aureus*: a methicillin susceptible *S. aureus* (MSSA) and two UK epidemic methicillin-resistant strains (EMRSA 15 and EMRSA 16), which are the predominant class of MRSA

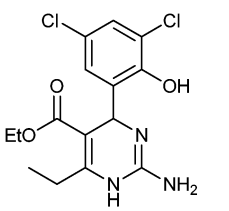


Scheme 4 Screening of DOS library identified a novel DNA checkpoint inhibitor, MARPIN.[66]

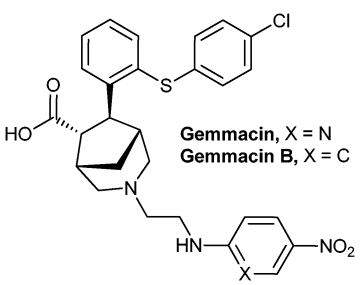
infections within the UK. A compound which they named gemmacin, exhibited broad spectrum activity against gram-positive bacteria, and demonstrated low antifungal activity and low toxicity in human epithelial cells. Gemmacin was subjected to a variety of assays to elucidate its mechanism for growth inhibition (for example, dihydrofolate reductase (DHFR) inhibition, protein synthesis and ATP synthesis uncoupling) and was identified as a cell-membrane disrupter. Spring and co-workers subsequently performed SAR studies on gemmacin,[73] which led to the identification of gemmacin B, with similar activity against EMRSA 15 and increased activity against EMRSA 16 (see Table 1).

Spring and colleagues also screened a second DOS library of 223 compounds containing 30 distinct molecular scaffolds, for antibacterial activity.[42] They identified a compound (emmacin) which inhibited the growth of EMRSA 15 and EMRSA 16 in cellular assays (Table 1). Further assays were performed which demonstrated that emmacin displayed low toxicity in mammalian cells and its mode of action was determined to be the prokaryotic-selective uncompetitive reversible inhibition of EMRSA 16 DHFR ($DfrB_{EMRSA16}$).[74] The high hit return ratio demonstrated in the antibacterial screening of these two libraries by Spring and co-workers, exemplifies the awesome power of DOS as a means for the efficient interrogation of bioactive space.

Table 1 Antibiotic activity of DOS compounds



Emmacin



	MIC_{50} (μg ml^{-1})		
	MSSA	EMRSA 15	EMRSA 16
Emmacin	2	9	9
(−)-Gemmacin	2	8	16
Gemmacin B	—	8	8
Erythromycin	0.5	>64	>64
Oxacillin	0.5	>32	>32

HDAC inhibitors

Histone deacetylase (HDAC) enzymes regulate a host of cellular processes by governing the acetylation state of histones and other non-histone proteins.[75] Aberrant HDAC activity has been implicated in a number of disease states including cancer, sickle cell anemia, rheumatoid arthritis and cardiac hypertrophy.[76] As such, the inhibition of HDAC catalysis has emerged as an important area for therapeutic intervention in a variety of diseases.

Several small molecule HDAC inhibitors have been developed, indeed several are currently in clinical trials, but to date, only two have been approved for use in humans, the hydroxamic acid vorinostat and the disulfide isotodax.[75,77] To date eighteen HDAC isoforms have been identified but in general the current armamentarium of HDAC inhibitors lack isoform-selectivity and consequently are likely to have undesired off-target effects. The discovery of new, isoform-selective, small molecule HDAC inhibitors will facilitate complete biological profiling of the functions of HDAC enzymes and may provide starting points for the development of novel therapeutics. To this end, Marcaurelle *et al.* employed an aldol-based build/couple/pair strategy to synthesize a DOS library of 14 400 macrocyclic compounds[78] which was screened for HDAC2 inhibitory activity using a high through-put fluorescence based assay.

Stereo-structure/activity relationship studies on promising compounds revealed marked differences in the inhibitory activity of individual stereoisomers, with differences in selectivity also being observed, thereby indicating the importance of stereodiversity in library composition. The inhibitory activity of the lead compound class BRD-4805 (Fig. 6) was determined to be less potent than the FDA approved vorinostat, but markedly more selective, displaying isoform-selectivity for HDACs 1–3 without inhibiting HDACs 4–8, which were inhibited by vorinostat. A subsequent focused SAR study identified BRD-8172 which exhibited marginally improved inhibitory activity *in vitro* and

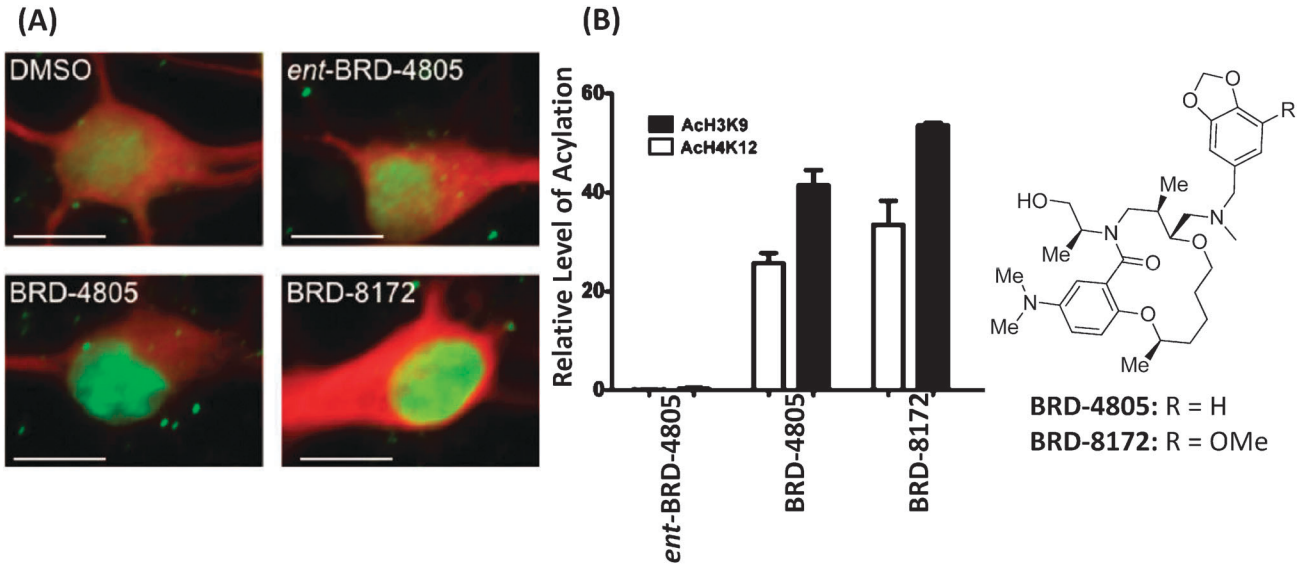


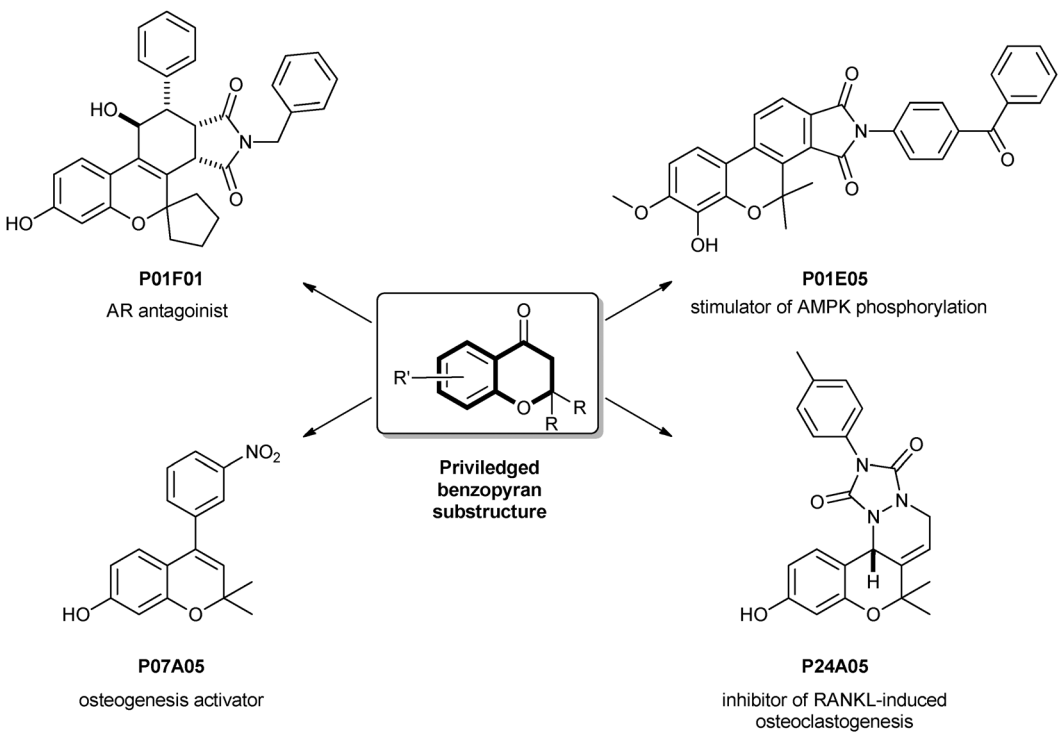
Fig. 6 (a) Imaging of histone acetylation in primary mouse forebrain neurons.[78] Compound treated cells were stained for acetylation of histone H3 lysine 9 (AcH3K9, green) and neuronal marker MAP2B (red). Immunofluorescence imaging relates the degree of acetylation to the intensity of the fluorescence signal. (b) Quantitative analysis of the relative level of acetylation as determined from immunofluorescence imaging. Adapted from ref. 78 with permission.

both compounds inhibited HDACs leading to increased levels of histone acetylation in cultured mouse neuronal cells (see Fig. 6).[78]

pDOS and BIOS

Over the last 5 years, two complementary DOS approaches have emerged for library construction, privileged-substructure-based DOS (pDOS)[79] and biology-oriented synthesis (BIOS).[80,81] Both ethea concentrate on reducing and prioritizing the areas of chemical space which should be explored to biologically pre-validated regions.[79,80]

pDOS is based on the concept that there are privileged structures or individual molecular frameworks capable of efficiently binding more than one biomolecular receptor.[82] Nature has curtailed the tracts of chemical space occupied by biomolecules through evolution, and biosynthetic natural



Scheme 5 Construction of diverse polycyclic skeletons embedded with the benzopyran motif by Park and colleagues displaying a wide variety of biological activity.

products as a consequence intimate the domains of chemical space likely to prove more fruitful when prospecting for exogenous ligands of biomolecular function. The pDOS approach correlates the omnipresence of core molecular scaffolds in (usually) clinically relevant bioactive natural products. Once a scaffold is identified, a library of compounds is created through the use of complexity generating reaction sequences, increasing scaffold diversity about the privileged core.

Park and co-workers constructed a natural-product-like library of $\sim$2500 compounds,[79] embedded with the privileged benzopyran motif, which they subsequently screened in a wide variety of biological assays (Scheme 5). They identified several small-molecule modulators of biological function including: a non-steroidal antagonist of the androgenic receptor,[83] an indirect activator of the adenosine 5′-monophosphate (AMP) activated protein kinase (AMPK),[84] an osteogenic activator,[85] and an inhibitor of the receptor activator of Nf-κB ligand (RANKL) induced osteoclastogenesis.[86] These results emphasize the importance of unbiased screening of small molecule libraries against a wide variety of biological targets. In addition, Oguri *et al*. recently demonstrated the power of the pDOS approach for generating biologically active small molecules when they constructed a library based on structural diversification of sesquiterpene analogues from which they identified several promising anti-trypanosomal agents.[87]

Biology-oriented synthesis (BIOS) identifies the evolutionary driven complementarity between bioactive natural products and their protein binding partners. Structural classification of natural products (SCONP) compartmentalizes and simplifies core (or privileged) scaffolds in a reductionist manner.[80] Protein structure similarity clustering (PSSC) recognizes likeness of protein folding sub-domains around ligand binding sites in different proteins. BIOS combines the SCONP and PSSC paradigms by classifying core scaffolds with known biological activity against a defined protein target, as likely candidates to display bioaffinity against sub-structurally related proteins.[88] Once a scaffold has been selected, a focused library is constructed with the introduction of molecular diversity through unbiased appendage variation. The Waldmann group formulated, refined and formalized this approach and has had tremendous success, developing novel bioactive small molecules through biology-inspired library syntheses.[80] The group recently reported the discovery of a small-molecule activator of the Wnt-pathway,[89] and of new classes of 5-lipoxygenase inhibitors and estrogen receptor ERα antagonists.[90]

The above examples highlight the efficacy of pDOS and BIOS for generating small molecules modulators of biological function in known bioactive space.

Conclusions and future perspectives

Over the past decade there has been a paradigm shift in how compound collections are constructed, with particular emphasis now being placed on increasing molecular diversity within libraries. This was borne out of a need to address challenging biological targets such as PPIs, which proved intractable to small molecule modulation in screens of traditional combinatorial libraries. DOS has emerged as a powerful approach for generating novel compounds with unique biological profiles, occupying previously unchartered tracts of chemical space. Through the exploration of distinct areas of chemical space, discrete areas of bioactive space can be discovered and, as a consequence, new compounds with unique modes of action against macromolecular targets may be identified. Thus, the generation and screening of DOS libraries holds the promise of expanding the druggable genome.

Furthermore, DOS has also contributed greatly to the development of novel synthetic chemistries,[91–94] and has been utilized as an approach for discovering new chemical reactions.[95] DOS has also been employed to provide diverse and complex fragment libraries for fragment-based drug discovery.[96]

In order for the full potential of the diversity-approach to be realized, significant improvements in library synthesis and screening techniques are required. The efficient generation of scaffold diversity is crucial for every DOS program. Despite recent progress made in the generation of scaffold diversity—the actual landmark number is 84 distinct scaffolds in a library of 96 compounds—the construction of large libraries with hundreds of different scaffolds remains a challenging goal for DOS. This can only be achieved by the continued development of new diversity generating synthetic methodologies. In addition, advancements in solid phase techniques, which render their applicability more general to a greater variety of chemistries, will address this shortfall in current practices.

An increase in diversity in screening is also required; by increasing the number of biological targets against which a library is screened, the likelihood of discovering a bioactive small molecule increases. The emergence of international screening initiatives is a significant development in this area which should encourage academic research groups to submit their compounds to small molecule repositories, thereby increasing library diversity and greatly improving the chances of identifying bioactive molecules.

Increased interdisciplinary collaboration between chemists and biologists in order to create and screen diverse compound collections in drug discovery and chemical genetics programs, will inevitably lead to the discovery of new small molecule modulators of novel biological targets. Although the creation of an ideal, functionally diverse library might remain utopian, continued augmentation of diversity-based synthetic approaches represents a step in the right direction.

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