THE EFFECTS OF COMBINATORIAL CHEMISTRY AND TECHNOLOGIES ON DRUG DISCOVERY AND BIOTECHNOLOGY – A MINI REVIEW

PIERFAUSTO SENECI[1,2*], GIORGIO FASSINA[3],
VLADIMIR FRECER[4,5], STANISLAV MIERTUS[5,6*]

[1]*Dipartimento di Chimica, Università degli Studi di Milano, Viale Golgi 19, I-20133 Milan, Italy (pierfausto.seneci@unimi.it)*
[2]*CISI scrl, Via Fantoli 16/15, I-20138 Milan, Italy*
[3]*Xeptagen S.p.A., Via delle Industrie 9, Marghera (VE) Italy*
[4]*Department of Physical Chemistry of Drugs, Faculty of Pharmacy, Comenius University, Odbojarov 10, SK-83232 Bratislava, Slovakia*
[5]*International Centre for Applied Research and Sustainable Technology (ICARST), Jamnickeho 18, SK-84104 Bratislava, Slovakia*
[6]*Faculty of Natural Sciences, University of SS. Cyril & Methodius, Nam. J. Herdu 2, SK-91701 Trnava, Slovakia (stanislav.miertus@ucm.sk)*

Abstract: The review will focus on the aspects of combinatorial chemistry and technologies that are more relevant in the modern pharmaceutical process. An historical, critical introduction is followed by three chapters, dealing with the use of combinatorial chemistry/high throughput synthesis in medicinal chemistry; the rational design of combinatorial libraries using computer-assisted combinatorial drug design; and the use of combinatorial technologies in biotechnology. The impact of "combinatorial thinking" in drug discovery in general, and in the examples reported in details, is critically discussed. Finally, an expert opinion on current and future trends in combinatorial chemistry and combinatorial technologies is provided.

Key words: combinatorial chemistry, combinatorial technologies, compound libraries, computer-assisted combinatorial drug design, virtual screening, display libraries, chemical tools, chemical biology, target identification, fragment-based drug design, combinatorial proteomics

1. Introduction

The expression "combinatorial chemistry" ("combichem") became extremely popular in the late '90s-early '00s, according to its recurrence in publications. A SciFinder search in early November 2013 shows that "combinatorial chemistry" is referenced significantly (>50 citations per year) since 1995 (n=60); then, it climbs steadily through 100 (1996, n=126), 500 (1999, n=592), 750 (2000, n=787) and 1000 citations (2002 and 2003, respectively n=1199 and 1203). During 2004 the decrease starts (n=995), gently continues to 800 (2010, n=791) and more steeply to ≈500 (2012, n=529). Partial data for 2013 (n=246, Jan to Oct) confirm the tendency. This decrease mirrors the rise and the fall for several key technologies in the past: the breakthrough reports in the late '80s (the seminal – and often forgotten – papers from FURKA *et al.* (1988; 1991), the first combinatorial libraries to fulfil HTS expectations in terms of chemical diversity by GEYSEN *et al.* (1984; 1985), HOUGHTEN (HOUGHTEN, 1985; HOUGHTEN *et al.*, 1991), LAM *et al.* (1991), ELLMAN (BUNIN *et al.*, 1994) and PIRRUNG (FODOR *et al.*, 1991), its fast spreading into the pharma community

DE GRUYTER
OPEN

around mid '90s (synthesis/biosynthesis and characterization of large libraries, new automated instrumentation, new library formats), the first concerns about quality and diversity representation in the early '00s (large peptide libraries, mix-and-split formats, false positives and negatives in screening), the "extraction" of useful bits and pieces from combichem around mid-late '00s (high throughput synthesis and purification of discrete libraries, solid-supported scavengers/reagents/purifying agents, computer-assisted diversity selection, virtual HTS, display libraries). Combinatorial chemistry fell from the early breakthrough technology status to the current not-so-popular/useful tool for drug discovery.

No one denies that the stumbling progress during the early combichem years led to excessive expectations in the scientific and industrial community. No one, though, should neglect how even routine operations today are available through discoveries made and technologies developed by combi-chemists. We will provide here a sampling of examples of today's combichem usefulness and applications. This contribution is divided into three Sections, according to the main applications for combinatorial chemistry in synthetic/medicinal chemistry, computer-assisted combichem and biotechnology.

2. Combichem in synthetic/medicinal chemistry

Two recent reviews provide an articulate description of what combichem in medicinal chemistry was, is and will be. The former (KODADEK, 2011) highlights how key questions (library size and format, HTS screening technologies, "developability" of hits from combichem libraries) should be addressed to take advantage of combinatorial chemistry without improperly making it the focus of drug discovery projects. The latter (MERRITT, 2012) is a detailed account of high throughput chemistry in drug discovery that describes combichem-derived techniques, strategies and synthetic methodologies in use in many industrial drug discovery labs today. They provide a balanced view of the usefulness and the opportunities provided by combichem in pharmaceutical research.

Let's examine here two examples of important achievements attained through the use of a combinatorial chemistry-biased approach in a drug discovery project.

3. Target identification and validation

Histone deacetylases (HDACs) are zinc hydrolases that modulate gene expression through deacetylation of the N-acetyl lysine residues of histone proteins. Eleven HDAC isoforms belong to four sub-classes (GROZINGER and SCHREIBER, 2002). They are involved in many cellular pathways, and it is crucial to link their effects with specific isoforms. In 2001, naturally occurring unselective HDAC inhibitors trichostatin A (TSUJI *et al.*, 1976) and trapoxin (KIJIMA *et al.*, 1993) were known, but isoform-specific HDAC inhibitors were unavailable. The X-ray structure of the complex between TSA and an HDAC ortholog (HOUGHTEN, 1985) suggested the variation of the the dimethylaminophenyl radical in TSA to identify isoform-specific molecular interactions.

Schreiber performed the SP synthesis of a 7,392 membered pool library of zinc-binding carboxylates, hydroxamates and o-aminoanilides analogues (respectively L1-L3, Fig. 1) (STERNSON *et al.*, 2001). The three zinc-binding groups are connected to a linker region made by a C3-C6 alkyl chain ending with an amide bond. A bulky cap region, indeed capping each library individual, is composed of a disubstituted chiral 1,3-dioxane core anchored onto the linker via an o-, m- or p-phenyl group. Chemical diversity is introduced in one of the chiral 1,3-dioxane substitutions, while the other substituent is determined by final resin cleavage (STERNSON *et al.*, 2001). The library was prepared using chemical encoding on polystyrene macrobeads, taking advantage of a sophisticated library synthesis/quality control/HTS platform (BLACKWELL *et al.*, 2001; CLEMONS *et al.*, 2001).
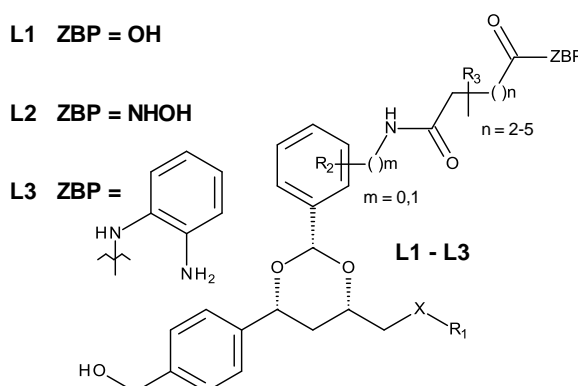

Fig. 1. Structure of carboxylate (L1), hydroxamate (L2) and o-aminoanilide (L3) libraries.

The library was conceived to identify selective inhibitors of histone deacetylases (HDACs), and to eventually validate one or more HDACs as therapeutical targets. The library was tested on HTS cellular assays (HAGGARTY *et al.*, 2003), determining a) the inhibition of histone (HDAC1-like) and α-tubulin (HDAC6-like) deacetylation; b) the selectivity of an inhibitor for either deacetylation reaction; and c) a preliminary SAR.

617 library individuals, i.e. ≈8% of the library, inhibit HDAC deacetylation (point a). 475 compounds inhibit α-tubulin and 344 inhibit histone deacetylation (202 being dual deacetylase inhibitors); according to the zinc-binding motifs (ZBMs), 18 *o*-aminoanilides, up to 80 carboxylates, and up to 519 hydroxamates (points b and c) inhibit one or both enzymatic activities. Further profiling of inhibitors identified tubacin (tubulin acetylation inducer, 1, Fig. 2), the first HDAC6-selective small molecule inhibitor (HAGGARTY *et al.*, 2003). Its use allowed the dissection of HDAC6-selective physiological and pathological events in cells, and in models of cancer (ALDANA-MASANGKAY *et al.*, 2011), neurodegeneration (D´YDEWALLE *et al.*, 2011) and inflammation (DE ZOETEN *et al.*, 2011). It also allowed the rationalization of HDAC6-selective structural features (ESTIU *et al.*, 2008), the design and structural optimization of second generation, drug-like HDAC6 inhibitors (WONG *et al.*, 2003).

Libraries L1-L3 are a strongly biased chemical diversity effort (few ZBM-linker variations, one substituent on a position of the 1,3-dioxane ring, wide substituent sampling on the other). Nevertheless, the exploitation via combinatorial chemistry of synthetically accessible modifications of scaffolds inspired by the structure of biologically active natural products allowed the characterization of HDAC isoforms, and the subsequent identification of drug-like, selective HDAC6 inhibitors (KALIN and BERGMAN, 2013). Similar examples are available in literature; others could stem from a mixed "serendipitous/rational" approach, inspired by Schreiber's efforts.
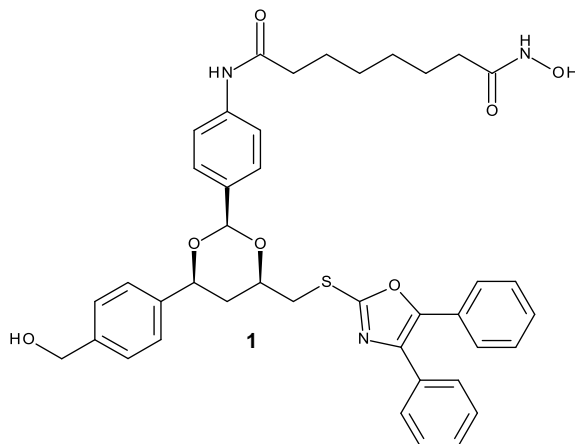

Fig. 2 Structures of HDAC6-selective tubacin (1).

4. Chemical tools

The 4,4-difluoro-4-bora-3a,4a-diaza-s-indacene nucleus (BODIPY, 2, Fig. 3) is a fluorescent probe for biological applications (LOUDET and BURGESS, 2007). It conjugates excellent photophysical properties with lack of toxicity, and the decoration of its nucleus provides specificity for single molecular targets. BODIPY-based probes were reported in the past (BOENS *et al.*, 2012), but a thorough exploration of the chemical space around 2 required a combinatorial chemistry approach.
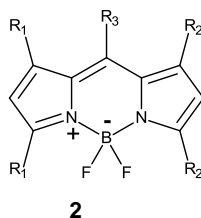

Fig. 3. The BODIPY scaffold (2).

Chang reported the library L4 of 317 mono-styryl BODIPY analogues, obtained via microwave-assisted Knoevenagel condensation on the asymmetric scaffold 3 (Fig. 4) (LEE *et al.*, 2009; LEE *et al.*, 2011).
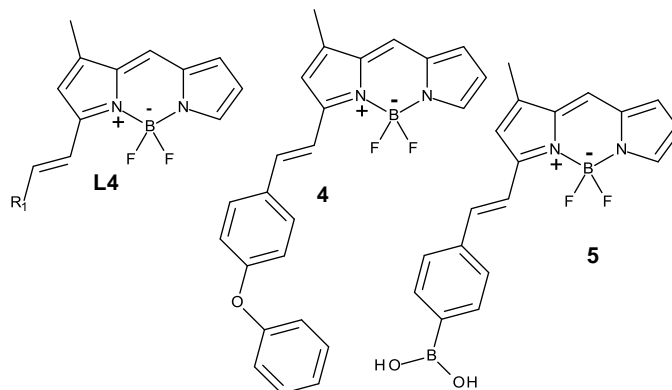
Fig. 4. Structure of the 317-membered mono-styryl BODIPY library L4, of a BSA-selective (4) and a dopamine-selective (5) BODIPY-based fluorescent probe from L4.

The library was tested on 94 biomolecules in intracellular-like conditions, observing the fluorescence variations at four concentrations (317 compounds, 94 biomolecules, 4 concentrations = 117,192 data points). A SAR map was obtained, determining the highest response for proteins; the high fluorescence changes at low pH for p-benzylamine- or heterocycle-decorated library members; and their fluorescence decrease in presence of negatively charged biomolecules (DNA, RNA) (LEE *et al*., 2011). Two compounds showed excellent turn on (4, Fig. 4, binding to bovine serum albumin/BSA with a 212-fold fluorescence increase) and turn off properties (5, Fig. 4, binding to dopamine with a 10-fold fluorescence emission decrease), with good correlations between fluorescence modulation and concentration of the binding partner (LEE *et al*., 2011).
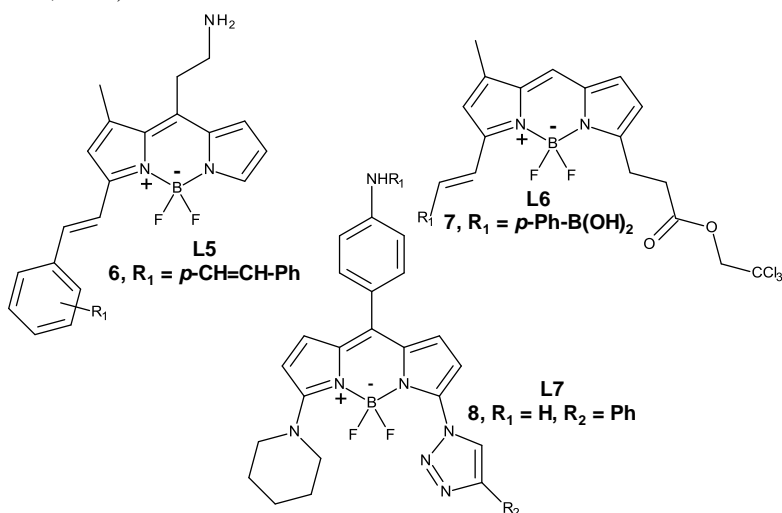

Fig. 5. Structures of the BODIPY libraries L5-L7, of a IgG-selective (6), a fructose-selective (7), and a human serum albumin-selective (8) BODIPY-based fluorescent probe.

Other library/active pairs (Fig. 5) include L5/6 (VENDRELL *et al.*, 2011) (160 library members, solid-phase synthesis, 6 showing a 75-fold fluorescence increase with immunoglobulin IgG); L6/7 (ZHAI *et al.*, 2012) (160 library members, 7 showing a 24-fold fluorescence increase with fructose); and L7/8 (ER *et al.*, 2013) (120 library members, 8 showing a 220-fold fluorescence increase with human serum albumin). BODIPY sensors 4-8 show excellent selectivity for their target biomolecule, and similar targets (i.e., albumins from animals for 4 and 8; saccharides for 7) do not cause comparable fluorescence changes.
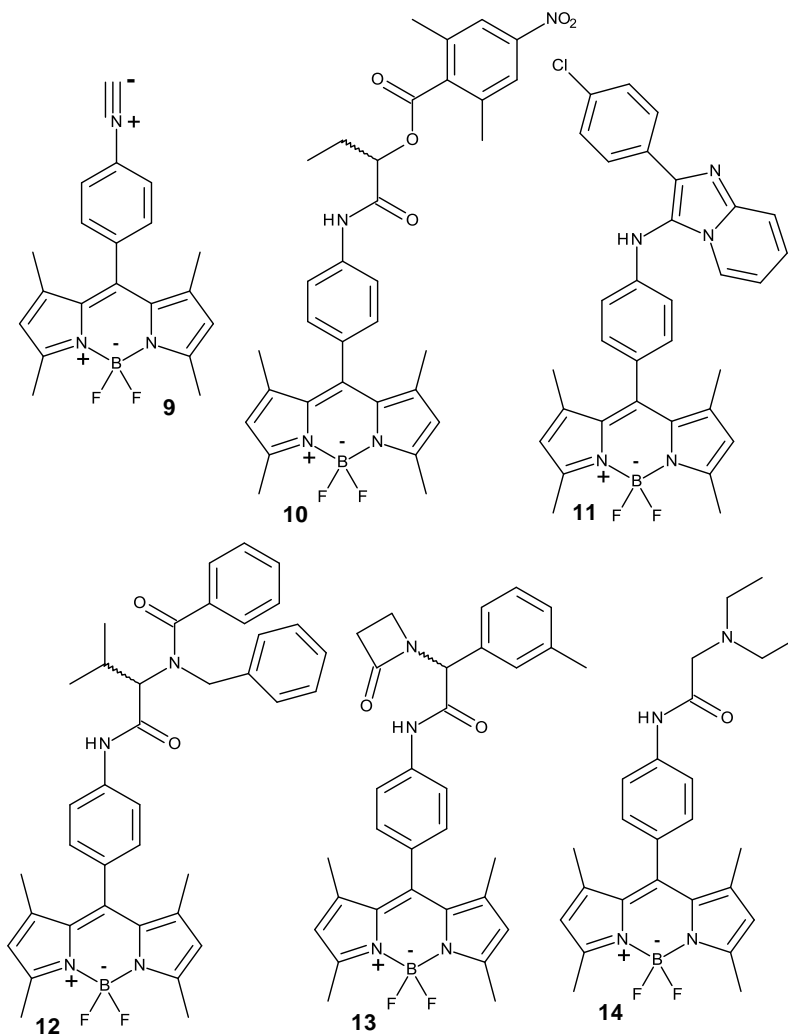
Fig. 6. Structures of the isocyanate 9, and of BODIPY-based adducts resulting from the Passerini (10), Bienaymè-Blackburn-Groebke (11), 4-MCR (12) and 3-MCR Ugi (13,14) reactions.

Modern combinatorial chemistry shifts its attention towards diversity, rather than numerosity. A small number of BODIPY analogues are prepared from diversity-multiplying multicomponent reactions (MCRs) on the isocyanate intermediate 9 (VAZQUEZ-ROMERO *et al.*, 2013) (Fig. 6). 3-MCR Passerini (BANFI and RIVA, 2005), 3-MCR Bienaymè-Blackburn-Groebcke (BIENAYMÉ and BOUZID, 1998), and 3- and 4-MCR Ugi (UGI *et al.*, 1996) protocols were used to prepare 10-14 (Fig. 6). Each compound could lead to a combinatorial library of analogues. Compound 14 (PhagoGreen) is a cell-permeable, non-toxic, pH-sensitive fluorescent probe to image phagosomal acidification in macrophages with sensitivity and specificity (VAZQUEZ-ROMERO *et al.*, 2013).

5. Computer-assisted combinatorial drug design

Combinatorial chemistry has revolutionized the drug discovery process in both academic and industrial settings. In the early years of the combinatorial boom drug design relied mainly on the screening of large diversity libraries. However, the initial screening results have been disappointing in terms of the achieved hit rates. In addition, the screening often produced hits with undesirable molecular properties, which were not suitable as lead compounds (GILLET, 2002; LEACH and HANN, 2000). Although the number of compounds synthesised and screened has increased by several orders of magnitude, the quantity of new chemical entities per year remained virtually unchanged. It was soon recognized that the diversity libraries are more appropriate for initial screening of compounds against a range of biological targets to identify biological activities pertinent to the portion of chemistry space covered by the library. An increased rate of identification of drug-like hits was observed when the libraries were rationally designed and focused by applying compound filtering and selection methods. Application of the Lipinski's rule-of five for the identification of analogues, to avoid insufficient oral bioavailability, was a first step in this direction. Training of neural networks with drugs and chemicals, performed in industrial research laboratories resulted in the design of combinatorial libraries with a higher content of biologically active compounds (KUBINYI, 2002). It became apparent that incorporation of relevant information on the targeted biomolecule or knowledge about active compounds affecting the target is beneficial for the library design. Thus focused (targeted) libraries were designed to cover restricted regions of the chemistry space with boundaries defined by the available information on the biological target. Additional restraints dictated by computed ligand-receptor interaction energies, structures of known active molecules and ADMET (absorption, distribution, metabolism, excretion and toxicity) properties shifted the emphasize from drug-like libraries to more biologically focused lead-like libraries (ROSE and STEVENS, 2003; OPREA, 2002). Moreover, it has turned out to be more cost effective to design and *in silico* screen virtual libraries of molecular models to identify subsets of the chemistry space that contain promising molecules, before going to the synthesis of compounds and to their high-throughput screening. Computational modelling led to an increased hit rate of drug-like molecules in combinatorial libraries, and focused the synthetic efforts onto those synthetically accessible molecules that are most likely to bind to the

target, as predicted by a computational algorithm. Later on, computer-assisted drug design methods such as generation of virtual libraries, analogue docking and *in silico* screening against a three-dimensional model of a targeted biomolecule or a pharmacophore derived from active analogues, became the standard procedure routinely used in medicinal chemistry. This blend of combinatorial chemistry and structure-based design (also referred to as combinatorial docking) (BÖHM and STAHL, 2000) has emerged as a new and promising approach to drug discovery.

Today, similarity searching, clustering, nonlinear mapping and other established virtual screening tools are used to aid in the selection of the most interesting library subsets. A new approach to generation of drug-like libraries was introduced by the CombiGen program (WOLBER and LANGER, 2001). This approach combined several hundreds of 'privileged' drug-specific fragments to produce structurally diverse virtual combinatorial libraries with an elevated content of drug-like molecules. If the 3D structure of the biological target is known, methods of flexible docking, which simultaneously consider the flexibility of the ligand and the binding site, are applied for compounds selection. Another successful strategy is the combinatorial design of ligands within the receptor binding site (BÖHM *et al.*, 1999; ERLANSON, 2006; VILLAR *et al.*, 2004; BLUNDELL and PATEL, 2004). Instead of docking thousands of individual analogues from a combinatorial library to a receptor model, this approach constructs the whole library directly within the receptor binding site.

In an attempt to increase the hit rates, the emphasis in computational and medicinal chemistry has shifted toward the rational design of small focused libraries that are biased toward one specific biological target. A greater understanding of new biochemical targets through genomics and chemical biology has also increased the number of novel drug targets for which biological screens are being developed. Libraries are now being focused, through the use of computer-assisted design strategies intended to hit a single specific therapeutic target. Results from docking and in silico screening of a virtual library and/or predictions of activity from a QSAR (quantitative structure-activity relationships) analysis or 3D pharmacophore models are now routinely used for selection of combinatorial subsets of targeted libraries. Focus on biological targets represents one aspect of a multi-objective optimisation design process, which considers also synthetic feasibility, availability and cost of reagents, diversity, drug- or lead-likeness, ADMET properties and other commercial factors (GILLET *et al.*, 2002; AGRAFIOTIS, 2002).

From a methodological point of view, structure-based combinatorial library design is an extension of traditional structure-based drug design, with the design and screening applied to virtual libraries of analogues instead of individual compounds (BEAVERS and CHEN, 2002). Consequently, the major computational strategies that have been developed for structure-based library design originate from the field of small molecule docking and scoring (BÖHM *et al.*, 1999), or alternatively from library searching for analogues that match a pharmacophore model (ROLLINGER *et al.*, 2008; GRIFFITH *et al.*, 2005). The frequently used focussing strategy is straightforward. All possible members of a virtual library are first enumerated, according to the available reagents and established synthetic scheme. Individual members are then separately docked into the binding site of a receptor and finally, a

library subset is selected for synthesis, based on the ranking of their docking scores and/or predicted ADMET properties (KITCHEN *et al*., 2004). Alternatively only the substituents (fragments) may be scored by attaching them to the common scaffold docked to the receptor binding site (BEAVERS and CHEN, 2002; ZHOU, 2008). Docking combined with the use of a scoring function is a fast method for ranking compounds in terms of binding potency, or complementarity to a biological target which can be used for relatively large libraries. During the docking, ligand poses are generated by fitting conformers of the ligand into three-dimensional model of the binding pocket of the macromolecular receptor. Then, poses are evaluated based on a scoring function, which yields estimates of binding affinity to the receptor. Docking algorithms may perform systematic or random searches for ligand conformations and then map them to the three-dimensional structure of the receptor. Many scoring functions have been developed, and they may score using a force field-based, empirically based, knowledge- based, or consensus-based algorithms. There are two key requirements of all computational algorithms for combinatorial docking: first, the ability to correctly predict the conformation of the docked ligand; and second, the ability to correctly predict the binding affinity of a putative ligand. It is clear that getting the geometry correct is a prerequisite for being able to predict binding affinities (BÖHM and STAHL, 2000; KITCHEN *et al*., 2004; ZHOU, 2008; RUPASINGHE and SPALLER, 2006; DUFFY *et al*., 2012; MCINNES, 2007).

Often only a single rigid receptor conformation is used to dock and score a ligand for the sake of computational efficiency. However, there are ways in which docking programs can account for the receptor flexibility. Protein flexibility namely plays a major role in biomolecular recognition. Structural changes associated with ligand binding can be significant, with deviations in overall backbone structure of the receptor, or they can be more subtle such as side-chain rotations. Either way, the algorithms that predict the affinity of biomolecular binding require relatively accurate predictions of the bound structure to give an accurate assessment of the energy involved in ligand-receptor association. More accurate algorithms were subsequently developed, that accommodate the receptor flexibility during ligand docking algorithms and also explore enhanced sampling techniques. The understanding and allowance for receptor flexibility are helping to make the predictions of ligand protein binding more accurate (SINKO *et al*., 2013; HEIKAMP and BAJORATH, 2013; DAWIS *et al*., 2009). Two theories have been proposed to describe the receptor configurational change upon ligand interaction. The first is conformational selection, in which all conformations are present in the unbound receptor, but the populations of each configuration change in the bound form (MA *et al*., 1999). The second theory is the induced fit, in which a single receptor configuration is forced into another one by the ligand binding event (SHERMAN *et al*., 2006).

Besides receptor flexibility, other tools are available to improve the estimates of ligand binding affinities of virtual libraries. In our laboratories, we tested the performance of implicit solvent effect (FRECER *et al*., 1998) and implementation of target-specific scoring functions. These are generic scoring functions adjusted to predict binding free energies to a specific protein receptor, by assuming a linear relationship between the computed score and predicted inhibition constants, with

coefficients of this linear dependence derived by linear regression from a QSAR analysis. The QSAR model is typically obtained for a training set of homologous molecules, for which binding affinities to the targeted macromolecule were experimentally determined. This approach was used to design peptidomimetic and cyclic urea inhibitors of HIV-1 protease, non-peptidic inhibitors of enoyl-acyl carrier protein of *P. falciparum*, of neuraminidase from influenza H5N1 virus, as well as peptidomimetic inhibitors of NS2B-NS3 protease from dengue virus (FRECER *et al*., 2005; FRECER *et al*., 2009; RUNGROTMONGKOL *et al*., 2009; FRECER and MIERTUS, 2010; FRECER *et al*., 2011).

Another popular and powerful tool for lead discovery and optimization is the fragment-based drug design (ERLANSON, 2006; VILLAR *et al*., 2004; BLUNDELL and PATEL, 2004; ZHOU, 2008; HAJDUK and GREER, 2007; HUBBARD *et al*., 2007). In this approach, libraries of small fragments are screened experimentally by either NMR spectroscopy, crystallography or other biophysical techniques to find low affinity hits (small molecules, fragments). When the 3D structure of the target is known, screening of libraries for active fragment hits can also be done computationally. In the next step, for the hits that occupy different subpockets of the receptor binding, site proper linkers connecting these hits while maintaining their relative positions in the subpockets are designed. High affinity leads can be found by linking two to three suitable fragments or alternatively, individual fragment hits can be grown into leads by step-by-step functionalization (MORTIER *et al*., 2012; SANCINETO *et al*., 2013).

6. Combinatorial approaches in biotechnology

Combinatorial chemistry significantly accelerated the development of a whole set of combinatorial tools comprising efficient synthetic methods, reagents for solid phase synthesis, linkers, addressing strategies, screening methods, etc. This is a clear indication of the importance of the combinatorial approach in chemistry - especially medicinal chemistry -, but "combinatorial thinking" quickly spread to many other branches of pure and applied science. For example, combinatorial technologies are now primary tools in very different and apparently unrelated fields of research such as catalyst development (WOO, 2007; DOMINGUEZ, 2005) and material science (MAIER *et al*., 2007). The growing interest for combinatorial technologies and their efficacy in producing valuable results, especially when coupled to molecular modelling, makes it difficult to foresee the real number of their possible applications. Biotechnology is among the main impacted areas, as we will see below.

7. Biological libraries

The generation of molecular diversity can take advantage of the biochemical mechanisms evolved by biological systems. The immune system is an outstanding example of combinatorial chemistry embodied in living organisms, and antibody libraries can be considered naturally evolved combinatorial libraries. The libraries of antibodies generated as the result of antigenic challenges to the immune system

invariably contain high affinity binders or receptors. This feature of the immune response was also exploited to evolve antibodies against transition state analogues, thus generating antibodies endowed with catalytic activity (TANAKA, 2002; KOCHETKOV, 1998). Another way to exploit the biology of living organisms to generate combinatorial libraries is the biological display technology, which allows the preparation of random peptides fused to proteins normally expressed on the surface of microorganisms. Biological display of peptides requires the introduction of the genetic information (DNA) that codes for the peptides into a microorganism. Typically, chosen organisms are bacteriophages (SMITH and PETRENKO, 1997; SMITH, 1985; STEMBERG and HOESS, 1995) or bacteria (JOSE, 2006; LEVIN and WEISS, 2006). Once inside the microorganism, the DNA is transcribed and translated into proteins, according to the genetic code. The DNA fragment coding for the peptides is inserted into specific DNA molecules called vectors. In phage display the vector is constituted by the viral genome, while in bacterial display an extra-chromosomal circular DNA, called plasmid, is used. Vectors are usually genetically modified to allow the insertion of DNA fragments at specific sites and, sometimes, genes or regulatory regions are deleted or inserted. Phage display is nowadays a mature technology, and pre-made libraries and cloning vectors are available from standard suppliers. Peptides of different length and topology, spanning between linear 7-12mers and cyclic peptides comprising a disulfide bond, can be readily expressed. Antibodies can also be expressed on the surface of filamentous bacteriophages. In peptide phage display, the randomized peptide sequences are expressed at the N-terminus of the minor coat protein pIII which is located on the top of the filamentous phage. From a general point of view, a peptide displaying phage could be considered a "bio-bead" with peptides bound to its surface and carrying a tag, the DNA, coding for them.

The success of this approach is related to the efficacy of the method used for the selection of phages displaying the peptide with the highest affinity for the target molecules (panning). The pool of phages displaying the random peptide library is allowed to interact with a solid support coated with the target molecules. The unbound phages are removed by washing the support, and the specifically bound phages are eluted in more stringent conditions. The eluted phages are amplified by propagation in E.coli, and the new pool is allowed to interact again with the target molecule immobilised on solid support. After each cycle of binding/amplification, the pool of phages is enriched with clones displaying peptides with highest affinity for the target molecule. Usually after 3-4 rounds, individual clones are characterized by DNA sequencing and immunochemical assays. Of course, this approach allows the preparation of peptides made of proteinogenic aminoacids, even though the introduction of unnatural amino acids was reported (TIAM *et al*., 2004). Phage display libraries have been used for many applications, such as epitope mapping or identification of peptide ligands for several receptors. Usually both purified target molecules or intact cells expressing the target molecule on the cell surface can be used in the panning procedure (TINOCO *et al*., 2002; STRATMANN *et al*., 2002; GAZOULI *et al*., 2002). Rasmussen and co-workers reported a successful phage display approach for tumour cell-targeting, in particular, the authors identified phage clones able to selectively bind to colorectal WiDr tumour cell lines (RASMUSSEN *et al*., 2002).

One of most exciting potential of phage displayed combinatorial peptide libraries is to obtain small peptide molecules that mimic an antigen, at least with respect to a particular epitope. In addition to their interest as research tools, such mimotopes could in principle be useful as diagnostic tools or for eliciting antibodies to a predefined epitope. However, the reduction of the phage insert sequence to a short peptide that can compete with the antigenic and in particular with the immunogenic properties of the natural antigen faces considerable difficulties. The difficulties to use mimotopes to induce antibodies that bind to the natural antigen (crossreactive immunogenicity) and the considerable discrepancy between antigenicity and immunogenicity of phage-derived peptides have been bypassed by peptides selected with antibodies from phage displayed random peptide libraries. Such peptides may represent low molecular weight substitutes of the natural antigen, not only for proteins, but also carbohydrates and nucleic acids, and thus are now being developed as vaccines for some pathological situations including cancer (ASHOK *et al.*, 2003).

Combinatorial phage peptide libraries are also useful for identification of the specific substrates of various proteases. A substrate phage library has a random peptide sequence at the N-terminus of the phage coat protein and an additional tag sequence that enables attachment of the phage to an immobile phase. When these libraries are incubated with a specific enzyme, such as a protease, the uncleaved phage is excluded from the solution with tag-binding macromolecules. This provides a novel approach to define substrate specificity (NIXON, 2002). The delineation of the substrate specificity of proteases will help to elucidate the enzymatic properties and the physiological roles of these enzymes. Comprehensive screening of very large numbers of potential substrate sequences is possible with substrate phage libraries. Thus, this approach allows novel substrate sequences and previously unknown target molecules to be defined.

Last but not least, combinatorial approaches, based on many different biological methods, have been used to increase enzyme stability or to refine enzyme specificity (COBB *et al.,* 2013) but the most challenging application in biotechnology is the selection of enzymes with novel catalytic properties (ACEVEDO-ROCHA *et al.,* 2014).

8. Combinatorial ProteomicTM

The naturally generated diversity of antibody libraries has been used to develop an innovative platform technology named Combinatorial ProteomicTM, which allows to profile the expression and function of protein families in complex proteomes. The use of antibodies allows the detection of iper and ipo-expressed proteins even at picomolar concentrations, overcoming one of the main limitations of proteomics: the difficulty in detecting low abundance proteins (CORTHALS *et al.*, 2000; LOPEZ, 2000). Combinatorial ProteomicTM shares with ''classical'' proteomics the goals of developing and applying technologies for the global analysis of protein expression and function. Its key advantage lies in the comparison of cells from normal tissue with those representing a disease state. Such comparisons enable the identification of disease-specific biomarkers that could be used for diagnostic tests, or to target proteins that have the potential for drug intervention. The striking feature of Combinatorial

ProteomicTM is to exploit the diversity of antibody libraries in order to select specific antibodies with high avidity for selected protein families. A single individual may produce a population of antibody specificities, an antibody repertoire, which is a reflection of all the B cell clones (lymphocyte repertoire) capable of immunoglobulin (Ig) synthesis and secretion in response to antigenic stimulation, but also in absence of exposure to environmental pathogens (CASALI and SCHETTINO, 1996). Natural antibodies, in fact, are germ line-encoded molecules produced by a distinct population of peritoneal B cells bearing the cell surface marker CD5 and are present in the sera and interstitial fluids of healthy individuals (KASAIAN *et al*., 1992; GREENBERG, 1985; KASAIAN and CASALI, 1993). The majority of natural antibodies are polyvalent immunoglobulin M (IgM) isotypes with varying – usually low – affinities. They nevertheless bind very tightly to multivalent antigens, because many low-affinity interactions can produce a single high-avidity interaction. Such a feature of IgM makes it the most suitable molecule to be used in the Combinatorial ProteomicTM technology.

Combinatorial ProteomicTM comprises the following steps:

1. *in silico* analysis of human proteome using the pattern matcher PatScan and Prosite database;
2. combinatorial synthesis of selected signature libraries by standard Fmoc chemistry and mix-split methods;
3. immobilization on solid support of selected signature libraries;
4. purification of signature-specific IgM antibodies by affinity chromatography experiments;
5. differential analysis of cellular protein expression levels.

Comparative analysis of protein and peptide levels can be conducted by a differential ELISA assay on biological samples from affected and healthy individuals. Theoretically, any disease could be studied by using molecular profiling. Specific clinical goals derived from such studies include screening tests for early disease detection, improved diagnostic markers, improved prognostic markers, new therapeutic targets, markers to evaluate therapeutic efficacy and new approaches and technologies for less invasive screening and diagnosis.

9. Ligands for bio-macromolecules

Monoclonal antibodies for therapeutic use are one of the main products deriving from biotechnology. In the quest for a synthetic ligand able to bind to monoclonal antibodies, and suitable for affinity-chromatographic applications at the industrial level, the synthesis and screening of combinatorial libraries of peptide dendrimers (FASSINA *et al*., 1996) has been exploited. Previous studies have shown that peptide ligand multimerisation enhances retention of recognition properties after immobilization on solid supports for the preparation of affinity columns (FASSINA, 1992; FOURNIER *et al*., 1992; BUTZ *et al*., 1994). Based on these considerations, a tetrameric peptide library has been designed, where four identical peptide chains are assembled starting from a tetradentate lysine core. The process is similar to that used for the production of multimeric antigenic peptides (TAM, 1988). The multimeric

library, composed of 5832 (18³) randomized tripeptide tetramers, has been realized by solid-phase peptide synthesis, following a simple manual procedure (RUVO *et al.*, 1994). Screening of the activity of the multimeric library in terms of antibody recognition has been carried out by measuring the ability to interfere with the interaction between Protein A and biotinylated immunoglobulins, monitored on a solid phase by ELISA.

The screening cycles allowed the final identification of the most active multimer as (Arg–Thr–Tyr)4–K2–K–G, named PAM (Protein A Mimetic ligand). PAM can be synthesized by solution or solid-phase methods in high yield and at limited costs. Its affinity constant for IgG, as determined by optical biosensor determinations, is close to 0.3 μM. The tetrameric ligand PAM can be easily immobilized on pre-activated solid supports, since the presence of the symmetric central core and the four peptide chains allow an oriented immobilization and the support-bound chain acts as a built-in spacer. The peptide was found to interact with other immunoglobulins such as IgE (PALOMBO *et al.*, 1998) and IgY (VERDOLIVA *et al.*, 2000). A partial inverso analogue of the PAM ligand was found to impair the interaction between IgG and the Fcγ receptor (MARINO *et al.*, 2000). Following a similar approach, a cyclic peptide able to bind to immunoglobulins was discovered as well. Linear peptides derived from the screening of combinatorial libraries, often do not display enough structural rigidity to provide recognition surfaces sufficiently selective for biotechnological or pharmaceutical applications. Cyclic peptides, on the other hand, show increased resistance to enzymatic degradation and constrained flexibility compared with the linear form. By screening dimeric tripeptide libraries, produced by starting from a bifunctional lysine residue at the C-terminus, and structurally constrained by the presence of a disulfide bond formed by two cysteine residues at the N-terminus (FASSINA *et al.*, 1995), a ligand for mouse IgG purification has been identified. The screening assay has been performed by immobilizing the library, as a set of sublibraries on microtiter plates for ELISA determination by noncovalent adsorption and then treating the microtiter plate wells with a fixed concentration of mouse monoclonal antibody. The presence of bound antibody was detected by a subsequent treatment with a goat anti-mouse IgG, labeled with peroxidase, followed by chromogenic reaction with ABTS. This screening strategy led to the identification of Peptide H, a cyclic dimeric peptide of formula (C–F–H–H)2K-G, where the two cysteine residues at the N-terminus are covalently linked by a disulfide bridge. When tested in affinity chromatography experiments, this ligand proved useful for mouse and rat IgG purification (MARINO *et al.*, 1999). The use of combinatorial libraries to identify synthetic ligands to be used in affinity chromatography for the purification of antibodies (NAIK *et al.*, 2011; MENEGATTI *et al.*, 2013) or proteins (NOPPE *et al.*, 2006; JACOBSEN *et al.*, 2007) remains today one of the most promising applications in biotechnology.

9. Conclusions

How many drugs, or at least clinical candidates, have benefited from combichem? That's usually the question causing endless debates. If you try to find hard evidence from the literature, you won't find any: no one is compelled to say if at any point of a

drug discovery project combichem was employed. If one thinks, though, of how many papers now mention that a first hit series originated either from "wet" or virtual HTS, there's your answer: compound libraries usually are made with a heavy combichem contribution, while computer-assisted computational drug design is of a great assistance to assemble meaningful virtual collections for *in silico* HTS.

Several small-medium enterprises (SMEs) have today a strong commitment in combinatorial technologies applied to catalysis, biotechnology, material sciences, biomedical devices and pharmaceutical research. The robustness and relative affordability of combinatorial methodologies that survived the disposal of less-than-perfect approaches is now widely accepted, although rarely recognized. Combinatorial libraries composed of inorganics, metallo-organics, peptides, oligonucleotides, or small molecules represent different tools for innovative research, all of them either as the products of combinatorial technologies or as part of a proprietary technology. Some SME devise, and will continue to devise their own combinatorial technologies as platforms for research and product development.

One should not see "combinatorial" and "rational" approaches as being juxtaposed concepts. For example, pharmaceutical companies now often work on small, target-focused solution-phase combinatorial libraries, where good computational design has significant impact to design the "best" library members, and is essential to increase the chances of a successful outcome – hits to be progressed to leads and candidates.

"Combinatorial thinking" represents a tool and a mindset, which – when applied to a project where rational design drives the planning and execution of activities – allows to increase productivity, to explore the meaningful diversity space, and consequently to maximize the probability to extract useful information (and industrially applicable products) from the project itself.

References

ACEVEDO-ROCHA, CG., HOEBENREICH, S., REETZ, MT.: Iterative saturation mutagenesis: a powerful approach to engineer proteins by systematically simulating Darwinian evolution. Methods Mol. Biol., 1179,2014, 103-28.

AGRAFIOTIS, D. K.: Multiobjective optimization of combinatorial libraries. J. Comput. Aided. Mol. Des., 16, 2002, 335-356.

ALDANA-MASANGKAY, G. I., RODRIGUEZ-GONZALEZ, A., LIN, T., IKEDA, A.K., HSIEH, Y.T., KIM, Y.M., LOMENICK, B., OKEMOTO, K., LANDAW, E.M., WANG, D., MAZITSCHEK, R., BRADNER, J.E., SAKAMOTO, K.M.: Tubacin suppresses proliferation and induces apoptosis of acute lymphoblastic leukemia cells. Leuk. Lymphoma, 52, 2011, 1544−1555.

ASHOK, B.T., DAVID, L., CHEN, Y.G., GARIKAPATY, V.P., CHANDER, B., KANDUC, D., MITTELMAN, A., TIWARI, R.K.: Peptide mimotopes of oncoproteins as therapeutic agents in breast cancer. Int. J. Mol. Med., 11, 2003, 465-71.

BANFI, L., RIVA, R.: The Passerini reaction. Org. React., 65, 2005, 1-140.

BEAVERS, M.P., CHEN, X.: Structure-based combinatorial library design: methodologies and applications. J. Mol. Graph. Model., 20, 2002, 463–468.

BIENAYMÉ, H., BOUZID, K.: A new heterocyclic multicomponent reaction for the combinatorial synthesis of fused 3-aminoimidazoles. Angew. Chem. Int. Ed., 37, 1998, 2234-2237.

BLACKWELL, H. E., PEREZ, L., STAVENGER, R. A., STAVENGER, R.A., TALLARICO, J.A., COPE EATOUGH, E., FOLEY, M.A., SCHREIBER, S.L.: A one bead, one-stock solution approach to chemical genetics: part 1. Chem. Biol., 8, 2001, 1167–1182.

BLUNDELL, T. L., PATEL, S.: High-throughput X-ray crystallography for drug discovery. Curr. Opin. Pharmacol., 4, 2004, 490–496.

BOENS, N., LEEN, V., DEHAEN, W.: Fluorescent indicators based on BODIPY. Chem. Soc. Rev., 41, 2012, 1130-1172.

BÖHM, H. J., BANNER, D. W., WEBER, L.: Combinatorial docking and combinatorial chemistry: design of non-peptide thrombin inhibitors. J. Comput.-Aided Mol. Design, 13, 1999, 51–56.

BÖHM, H. J., STAHL, M.: Structure-based library design: molecular modelling merges with combinatorial chemistry. Curr. Opin. Chem. Biol., 4, 2000, 283-286.

BUNIN, B. A., PLUNKETT, M. J., ELLMAN, J. A.: The combinatorial synthesis and chemical and biological evaluation of a 1,4-benzodiazepine library. Proc. Natl. Acad. Sci. U.S.A., 91, 1994, 4708-4712.

BUTZ, S., RAWER, S., RAPP, W., BIRSNER, U.: Immunization and affinity purification of antibodies using resin-immobilized lysine-branched synthetic peptides. Pept. Res., 7, 1994, 20–23.

CASALI, P., SCHETTINO, E. W. Structure and function of natural antibodies. Curr. Top. Microbiol. Immunol. 1996, 210, 167–179.

CLEMONS, P. A., KOEHLER, A. N., WAGNER, B. K., SPRIGINGS, T.G., SPRING, D.R., KING, R.W., SCHREIBER, S.L., FOLEY, M.A.: A one-bead, one-stock solution approach to chemical genetics: part 2. Chem. Biol., 8, 2001, 1183–1195.

COBB, R. E., CHAO, R., ZHAO, H.: Directed evolution: Past, present, and future. AIChE J., 59, 2013, 1432–1440.

CORTHALS, G. L., WASINGER, V. C., HOCHSTRASSER, D. F., SANCHEZ, J. C.: The dynamic range of protein expression: A challenge for proteomic research. Electrophoresis, 21, 2000, 1104–1115.

D'YDEWALLE, C., KRISHNAN, J., CHIHEB, D. M., Van DAMME, P., IROBI, J., KOZIKOWSKI, A.P., VANDEN BERGHE, P., TIMMERMAN, V., ROBBERECHT, W., Van DEN BOSCH, L.: HDAC6 inhibitors reverse axonal loss in a mouse model of mutant HSPB1–induced Charcot-Marie-Tooth disease. Nat. Med., 17, 2011, 968–974.

DAWIS, I. W., BAKER, D.: RosettaLigand docking with full ligand and receptor flexibility. J. Mol. Biol., 385, 2009, 381-392.

DE ZOETEN, E. F., WANG, L., BUTLER, K., BEIER, U.H., AKIMOVA, T., SAI, H., BRADNER, J.E., MAZITSCHEK, R., KOZIKOWSKI, A.P., MATTHIAS, P., HANCOCK, W.W.: Histone deacetylase 6 and heat shock protein 90 control the functions of Foxp3+ T-regulatory cells. Mol. Cell. Biol., 31, 2011, 2066–2078.

DOMINGUEZ, J. M.: High throughput combinatorial methods for heterogeneous catalysts design and development. In: Fassina G, Miertus S, Eds. Combinatorial

Chemistry and Technologies, 2nd Edition. CLC Press LCC, Boca Raton, 2005, 369-388.

DUFFY, B. C., ZHU, L., DECORNEZ, H., KITCHEN, D. B.: Early phase drug discovery: Cheminformatics and computational techniques in identifying lead series. Bioorg. Med. Chem., 20, 2012, 5324–5342.

ER, J. C., TANG, M. K., CHIA, C. G., LIEW, H., VENDRELL, M., CHANG, Y-T.: MegaStokes BODIPY-triazoles as environmentally sensitive turn-on fluorescent dyes. Chem. Sci., 4, 2013, 2168-2176.

ERLANSON, D. A.: Fragment-based lead discovery: a chemical update. Curr. Opin. Biotech., 17, 2006, 643–652.

ESTIU, G., GREENBERG, E., HARRISON, C. B., KWIATKOWSKI, N.P., MAZITSCHEK, R., BRADNER, J.E., WIEST, O.: Structural origin of selectivity in class II-selective histone deacetylase inhibitors. J. Med. Chem., 51, 2008, 2898-2906.

FASSINA, G., SCARDINO, P., RUVO, M., FUCILE, P., AMODEO, P., CASSANI, G.: Synthesis of conformationally constrained dimeric peptide libraries. In: Maia H, Ed. Peptides 1994. Leiden, ESCOM, 1995, 489–490.

FASSINA, G., VERDOLIVA, A., ODIERNA, M. R., RUVO, M., CASSINI, G.: Protein A mimetic peptide ligand for affinity purification of antibodies. J. Mol. Recognit., 9, 1996, 564–569.

FASSINA, G.: Oriented immobilization of peptide ligands on solid supports. J. Chromatogr., 591, 1992, 99–106.

FODOR, S. P. A., READ, J. L., PIRRUNG, M. C., STRYER, L., LIU, A. T., SOLAS, D.: Light-directed, spatially addressable parallel chemical synthesis. Science, 251,1991, 767.

FOURNIER, A., COUVINEAU, A., LABURTHE, M.: Synthesis of a hydrophilic affinity matrix for the purification of the vasoactive intestinal peptide receptor. Anal. Biochem., 211,1992, 305–310.

FRECER, V., BURELLO, E., MIERTUS, S.: Combinatorial design of nonsymmetrical cyclic urea inhibitors of aspartic protease of HIV-1. Bioorg. Med. Chem., 13, 2005, 5492-5501.

FRECER, V., MEGNASSAN, E., MIERTUS, S.: Design and *in silico* screening of combinatorial library of antimalarial analogs of triclosan inhibiting Plasmodium falciparum enoyl-acyl carrier protein reductase. Eur. J. Med. Chem., 44, 2009, 3009-3019.

FRECER, V., MIERTUS, S., TOSSI, A., ROMEO, D.: Rational design of inhibitors for drug-resistant HIV-1 aspartic protease mutants. Drug Des. Disc., 15, 1998, 211-231.

FRECER, V., MIERTUS, S.: Design, structure-based focusing and *in silico* screening of combinatorial library of peptidomimetic inhibitors of Dengue virus NS2B-NS3 protease. J. Comp.-Aided Mol. Des., 24, 2010, 195-212.

FRECER, V., SENECI, P., MIERTUS, S.: Computer-assisted combinatorial design of bicyclic thymidine analogs as inhibitors of mycobacterium tuberculosis thymidine monophosphate kinase. J. Comp.-Aided Mol. Des., 25,2011, 31-49.

FURKA, A., SEBESTYEN, F., ASGEDOM, M., DIBO, G.: Cornucopia of peptide synthesis. Highlights of Modern Biochemistry, Proceedings of the 14th

International Congress of Biochemistry, VSP. Utrecht, The Netherland, 1988, Vol. 5, p. 47.

FURKA, A., SEBESTYEN, F., ASGEDOM, M., DIBO, G.: General method for rapid synthesis of multicomponent peptide mixtures. Int. J. Peptide Protein Res., 37, 1991, 487-493.

GAZOULI, M., HAN, Z., PAPADOPOULOS, V.: Identification of a peptide antagonist to the peripheral type benzodiazepine receptor (PBR) that inhibits hormone stimulated Leydig cell steroid formation. J. Pharmacol. Exp. Ther., 303, 2002, 627-632.

GEYSEN, H. M., BARTELING, S. J., MELOEN, R. H.: Small peptides induce antibodies with a sequence and structural requirement for binding antigen comparable to antibodies raised against the native protein. Proc. Natl. Acad. Sci. USA, 82, 1985, 178-182.

GEYSEN, H. M., MELOEN, R. H., BARTELING, S. J.: Use of peptide synthesis to probe viral antigens for epitopes to a resolution of a single amino acid. Proc. Natl. Acad. Sci. USA., 81, 1984, 3998-4002.

GILLET, V., KHATIB, W., WILLETT, P., FLEMING, P.J, GREEN, D.V.: Combinatorial library design using a multiobjective genetic algorithm. J. Chem. Inf. Comput. Sci., 42, 2002, 375-385.

GILLET, V. J.: Reactant- and product-based approaches to the design of combinatorial libraries. J. Comp. Aided Mol. Des., 16, 2002, 371-380.

GREENBERG, A. H.: Antibodies and natural immunity. Biomed. Pharmacother., 39, 1985, 4–6.

GRIFFITH, R., LUU, T. T., GARNER, J., KELLER, P. A.: Combining structure-based drug design and pharmacophores. J. Mol. Graph. Model., 23, 2005, 439-446.

GROZINGER, C. M., SCHREIBER, S. L.: Deacetylase enzymes: biological functions and the use of small-molecule inhibitors. Chem. Biol., 9, 2002, 3–16.

HAGGARTY, S. J., KOELLER, K. M., WONG, J. C., GROZINGER, C.M., SCHREIBER, S.L.: Domain-selective small molecule inhibitor of HDAC6-mediated tubulin deacetylation. Proc. Natl. Acad. Sci. USA, 100, 2003, 4389–4394.

HAGGARTY, S. J., KOELLER, K. M., WONG, J. C., BUTCHER, R.A., SCHREIBER, S.L.: Multidimensional chemical genetic analysis of diversity-oriented synthesis-derived deacetylase inhibitors using cell-based assays. Chem. Biol., 10, 2003, 383–396.

HAJDUK, P. J., GREER, J. A.: decade of fragment-based drug design: strategic advances and lesions learned. Nat. Rev. Drug Discov., 6, 2007, 211-219.

HEIKAMP, K., BAJORATH, J.: The future of virtual compound screening. Chem. Biol. Drug Des., 81, 2013, 33-40.

HOUGHTEN, R. A., PINILLA, C., BLONDELLE, S. E., APPEL, J. R., DOOLEY, C. T., CUERVO, J. H.: Generation and use of synthetic peptide combinatorial libraries for basic research and drug discovery. Nature, 354, 1991, 84-86.

HOUGHTEN, R. A.: General method for the rapid solid-phase synthesis of large numbers of peptides: specificity of antigen-antibody interaction at the level of individual amino acids. Proc. Natl. Acad. Sci. USA, 82, 1985, 5131-5135.

HUBBARD, R. E., CHEN, I., DAVIS, B.: Informatics and modeling challenges in fragment-based drug discovery. Curr. Opin. Drug. Discov. Devel., 10, 2007, 289-297.

JACOBSEN, B., GÅRDSVOLL, H., FUNCH, G. J., ØSTERGAARD, S., BARKHOLT, V., PLOUG, M.: One-step affinity purification of recombinant urokinase-type plasminogen activator receptor using a synthetic peptide developed by combinatorial chemistry. Prot. Expr. Purific., 52, 2007, 286–296.

JOSE, J.: Autodisplay: efficient bacterial surface display of recombinant proteins. Appl. Microbiol. Biot., 69, 2006, 607-617.

KALIN, J. H., BERGMAN, J. A.: Development and therapeutic implications of selective histone deacetylase 6 inhibitors. J. Med. Chem., 56, 2013, 6297-6313.

KASAIAN, M. T., CASALI, P.: Autoimmunity-prone B-1 (CD5 B) cells, natural antibodies and self recognition. Autoimmunity, 15, 1993, 315–329.

KASAIAN, M. T., IKEMATSU, H., CASALI, P.: Identification and analysis of a novel human surface CD5- B lymphocyte subset producing natural antibodies. J. Immunol., 148, 1992, 2690–2702.

KIJIMA, M., YOSHIDA, M., SUGITA, K., HORINOUCHI, S., BEPPU, T.: Trapoxin, an antitumor cyclic tetrapeptide, is an irreversible inhibitor of mammalian histone deacetylase. J. Biol. Chem., 268, 1993, 22429-22435.

KITCHEN, D. B., DECORNEZ, H., FURR, J. R., BAJORATH, J.: Docking and scoring in virtual screening for drug discovery: methods and applications. Nat. Rev. Drug Discov., 3, 2004, 935-949.

KOCHETKOV, N. K.: Catalytic antibodies: prospects for the use in organic synthesis. Russ. Chem. Rev., 67, 1998, 999-1029.

KODADEK, T.: The rise, fall and reinvention of combinatorial chemistry. Chem. Commun., 47, 2011, 9757-9763.

KUBINYI, H.: The design of combinatorial libraries. Drug Discov. Today, 7, 2002, 503-504.

LAM, K. S., SALMON, S. E., HERSH, E. M., HRUBY, V. J., KAZMIERSKI, W. M., KNAPP, R. J.: A new type of synthetic peptide library for identifying ligand-binding activity. Nature, 354, 1991, 82-84.

LEACH, A. R., HANN, M. M. The *in silico* world of virtual libraries. Drug Discov. Today 2000, 5, 326-336.

LEE, J. S., KANG, N. Y., KIM, Y. K., SAMANTA, A., FENG, S., KIM, H.K., VENDRELL, M., PARK, J.H., CHANG, Y.T.: Synthesis of a BODIPY library and its application to the development of live cell glucagon imaging probe. J. Am. Chem. Soc., 131, 2009, 10077-10082.

LEE, J. S., KIM, H. K., FENG, S., VENDRELL, M., CHANG, Y-T.: Accelerating fluorescent sensor discovery: unbiased screening of a diversity-oriented BODIPY library. Chem. Commun., 47, 2011, 2339−2341.

LEVIN, A. M., WEISS, G. A.: Optimizing the affinity and specificity of proteins with molecular display. Mol. Bio. Syst., 2, 2006, 49-57.

LOPEZ, M. F.: Better approaches to finding the needle in a haystack: Optimizing proteome analysis through automation. Electrophoresis, 21, 2000, 1082–1093.

LOUDET, A., BURGESS, K.: BODIPY dyes and their derivatives: Syntheses and spectroscopic properties. Chem. Rev., 107, 2007, 4891-4932.

MA, B., KUMAR, S., TSAI, C. J., NUSSINOV, R.: Folding funnels and binding mechanisms. Protein Eng., 12, 1999, 713-720.

MAIER, W., STOEWE, K., SIEG, S.: Combinatorial and high-throughput materials science. Angew. Chem., Int. Ed., 46, 2007, 6016-6067.

MARINO, M., CAMPANILE, M. N., IPPOLITO, A., SCARALLO, A., RUVO, M., FASSINA, G.: Structurally constrained selective ligands for mouse immunoglobulins. In: S. BAJUSZ and F. HUDECZ, Eds. Peptides 98, Academia Kiado, Budapest, 1999, 776-777.

MARINO, M., RUVO, M., DE FALCO, S., FASSINA, G.: Prevention of systemic lupus erythematosus in MRL/lpr mice by administration of an immunoglobulin-binding peptide. Nat. Biotechnol., 18, 2000, 735-739.

MCINNES, C.: Virtual screening strategies in drug discovery. Curr. Opin. Chem. Biol., 11, 2007, 494–502.

MENEGATTI, S., WARD, K. L., NAIK, A. D., KISH, W. S., BLACKBURN, R. K., CARBONELL, R. G.: Reversible cyclic peptide libraries for the discovery of affinity ligands. Anal. Chem., 85, 2013, 9229–9237.

MERRITT A.: High throughput chemistry in drug discovery. RSC Drug Discovery Series, 2012, 11 (New synthetic technologies in medicinal chemistry), 6-41.

MORTIER, J., RAKERS, C., FREDERICK, R., WOLBER, G.: Computational tools for *in silico* fragment-based drug design. Curr. Top. Med. Chem., 12, 2012, 1935-1943.

NAIK, A. D., MENEGATTI, S., GURGEL, P. V., CARBONELL, R. G.: Performance of hexamer peptide ligands for affinity purification of immunoglobulin G from commercial cell culture media. J. Chromatogr. A, 1218, 2011, 1691-700.

NIXON, A.E.: Phage Display as a Tool for Protease Ligand Discovery. Curr. Pharm. Biotechnol., 3, 2002, 1-12.

NOPPE, W., PLIEVA, F. M., GALAEV, I. Y., VANHOORELBEKE, K., MATTIASSON, B., DECKMYN, H.: Immobilised peptide displaying phages as affinity ligands: Purification of lactoferrin from defatted milk. J. Chromatogr. A, 1101, 2006, 79–85.

OPREA, T. I.: Current trends in lead discovery: are we looking forthe appropriate properties? J. Comput. Aided Mol. Des., 16, 2002, 371-380.

PALOMBO, G., ROSSI, M., CASSANI, G., FASSINA, G.: Affinity purification of mouse monoclonal IgE using a protein A mimetic ligand (TG19318) immobilized on solid supports. J. Mol. Recognit., 11, 1998, 247-249.

RASMUSSEN, U. B., SCHREIBER, V., SCHULTZ, H., MISCHLER, F., SCHUGHART, K.:Tumor cell-targeting by phage-displayed peptides. Cancer Gene Ther., 9, 2002, 606-612.

ROLLINGER, J. M., STUPPNER, H., LANGER, T.: Virtual screening for the discovery of bioactive natural products. Prog. Drug Res., 65, 2008, 213-249.

ROSE, S., STEVENS, A.: Computational design strategies for combinatorial libraries. Curr. Opin. Chem. Biol., 7, 2003, 331-339.

RUNGROTMONGKOL, T., FRECER, V., DE-EKNAMKUL, W., HANNONGBUA, S., MIERTUS, S.: Design of oseltamivir analogs inhibiting neuraminidase of avian influenza virus H5N1. Antivir. Res., 82, 2009, 51-58.

RUPASINGHE, C. N., SPALLER, M. R.: The interplay between structure-based design and combinatorial chemistry. Curr. Opin. Chem. Biol., 10, 2006, 188–193.

RUVO, M., SCARDINO, P., CASSANI, G., FASSINA, G.: Facile manual synthesis of *peptide* libraries. Protein Pept. Lett., 1, 1994, 187–192.

SANCINETO, L., MASSARI, S., IRACI, N., TABARRINI, O.: From small to powerful: the fragments universe and its "chem-appeal". Curr. Med. Chem., 20, 2013, 1355-1381.

SHERMAN, W., BEARD, H. S., FARID, R.: Use of an induced fit receptor structure in virtual screening. Chem. Biol. Drug Des., 67, 2006, 83-84.

SINKO, W., LINDERT, S., MCCAMMON, A. J.: Accounting for receptor flexibility and enhanced sampling methods in computer-aided drug design. Chem. Biol. Drug. Des., 81, 2013, 41-49.

SMITH, G. P., PETRENKO, V. A.: Phage display. Chem. Rev., 97, 1997, 391-410.

SMITH, G. P.: Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface. Science, 228, 1985, 1315-1317.

STEMBERG, N., HOESS, R. H.: Display of peptides and proteins on the surface of bacteriophage lambda. Proc. Nat. Acad. Sci. USA, 92, 1995, 1609-1613.

STERNSON, S. M., WONG, J. C., GROZINGER, C. M., SCHREIBER, S. L.: Synthesis of 7200 small molecules based on a substructural analysis of the histone deacetylase inhibitors trichostatin and trapoxin. Org. Lett., 3, 2001, 4239–4242.

STRATMANN, J., STROMMENGER, B., STEVENSON, K., GERLACH, G. F.: Development of a peptide-mediated capture PCR for detection of *Mycobacterium avium* subsp. *paratuberculosis* in milk. J. Clin Microb., 40, 2002, 4244-4250.

TAM, J. P.: Synthetic peptide vaccine design: synthesis and properties of a high-density multiple antigenic peptide system. Proc. Natl. Acad. Sci. USA, 85, 1988, 5409–5413.

TANAKA, F.: Catalytic antibodies as designer proteases and esterases. Chem. Rev., 102, 2002, 4885-4905.

TIAM, F., TSAO, M. L., SCHULTZ, P. G.: A phage display system with unnatural amino acids. J. Am. Chem. Soc., 126, 2004, 15962-15963.

TINOCO, L. W., DA SILVA, J. R. A., LEITE, A., VALENTE, A.P., ALMEIDA, F.C.: NMR structure of PW2 bound to SDS micelles. A tryptophan-rich anticoccidial peptide selected from phage display libraries. J. Biol Chem., 277, 2002, 36351-36356.

TSUJI, N., KOBAYASHI, M., NAGASHIMA, K., WAKISAKA, Y., KOIZUMI, K.: A new antifungal antibiotic, trichostatin. J. Antibiot., 29, 1976, 1-6.

UGI, I., GOEBEL, M., GRUBER, B., HEILINGBRUNNER, M., HEIß, C., HÖRL, W., KERN, O., STARNECKER, M., DÖMLING A.: Molecular libraries in liquid phase via Ugi-MCR. Res. Chem. Interm., 22, 1996, 625-644.

VAZQUEZ-ROMERO, A., KIELLAND, N., ARÉVALO, M. J., PRECIADO, S., MELLANBY, R.J., FENG, Y., LAVILLA, R., VENDRELL, M.: Multicomponent reactions for de novo synthesis of BODIPY probes: In vivo imaging of phagocytic macrophages. J. Am. Chem. Soc., 135, 2013, 16018−16021.

VENDRELL, M., KRISHNA, G. G., GHOSH, K. K., ZHAI, D., LEE, J.S., ZHU, Q., YAU, Y.H., SHOCHAT, S.G., KIM, H., CHUNG, J., CHANG, Y.T.: Solid-phase

synthesis of BODIPY dyes and development of an immunoglobulin fluorescent sensor. Chem. Commun., 47, 2011, 8424−8426.

VERDOLIVA, A., BASILE, G., FASSINA, G.: Affinity purification of immunoglobulins from chicken egg yolk using a new synthetic ligand. J. Chromat. B, 749, 2000, 233-242.

VILLAR, H. O., YAN, J., HANSEN, M. R.: Using NMR for ligand discovery and optimization. Curr. Opin. Chem. Biol., 8, 2004, 387–391.

WOLBER, G., LANGER, T.: CombiGen: A novel software package for the rapid generation of virtual combinatorial libraries. In: HÖLTJE, H. D., SIPPL, W., Eds., Rational Approaches to Drug Design, Prous Science, Barcelona, Spain, 2001, pp. 390-399.

WONG, J. C., HONG, R., SCHREIBER, S. L.: Structural biasing elements for in-cell histone deacetylase paralog selectivity. J. Am. Chem. Soc., 125, 2003, 5586-5587.

WOO, L. K.: Combinatorial approaches and molecular evolution of homogeneous catalysts . In: NARASIMHAN, B., MALLAPRAGADA, S. K., PORTER, M. D., Eds. Combinatorial materials science. John Wiley and Sons, Hoboken, 2007, 121–162.

ZHAI, D., LEE, S. C., VENDRELL, M., LEONG, L.P., CHANG, Y-T.: Synthesis of a novel BODIPY library and its application in the discovery of a fructose sensor. ACS Combi. Science, 14, 2012, 81-84.

ZHOU, J. Z.: Structure-directed combinatorial library design. Curr. Opin. Chem. Biol., 12, 2008, 379-385.