Molecular recognition: the fragment approach in lead generation

Daniela Fattori

The successful practice of medicinal chemistry is crucially dependent on the principles of molecular recognition: the first and 'fundamental' requirement for a drug is to bind to its target; specificity, or at least selectivity, of binding is also a must. Subsequent optimization steps to develop a lead compound into a drug are a complex mixture of processes that are not yet fully understood or predictable. Fortunately, criteria exist to discard leads that would be intractable for optimization. The concepts of non-lead-likeness and lead-likeness, in respect to drug-likeness and non-drug-likeness, have prompted a rich discussion in the recent medicinal chemistry literature. The fragment approach is an emerging philosophy in the process of lead compound discovery. The basic interactions responsible for binding affinity are defined from the 'protein interactions world' and key structural fragments are combined according to the criteria of three-dimensional diversity to find new leads. New techniques in screening are used for the detection of the weaker interactions of fragments with their targets that might be undetectable in classical biological assays.

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'Reversible molecular interactions are at the heart of the dance of life.'
(Lubert Stryer)

Molecular recognition is at the heart of biochemical regulation, communication and transformation. The most important prerequisite for binding appears to be a good steric and electronic complementarity between ligands. But biological interactions take place in aqueous media: solvation and desolvation effects involving both ligands, protein and small molecule, must be taken into account [1]. Measured binding constants (K_i) are related to the free energy of interactions, which in turn depend on the interplay between the enthalpic and entropic contributions (as shown in the modified Gibb's equation below), each of which is strongly influenced by the nature of the solvent.

$$-RT \ln K_i = \Delta G = \Delta H - T\Delta S$$

The free energy of a hydrogen bond in the gaseous phase can be 1–10 kcal mol^{-1}. When considered in the context of a biological system, this energy depends on the microscopic environment of the protein–ligand complex: desolvation effects can completely compensate for the intermolecular hydrogen bond, so that the net contribution to binding is close to zero [2]. Electrostatic interactions buried in a hydrophobic environment can greatly enhance affinity, and at long range in aqueous media they can still facilitate the approach of ligands with complementary electrostatic potential surfaces. Alternatively, unpaired buried polar groups at the protein–ligand interface are strongly averse to binding. Regarding lipophilic interactions, it is generally accepted that the energetic gain is mainly because of the replacement and release of ordered water molecules, and therefore the recognition process is entropically driven. In addition, there is a positive enthalpic contribution coming from favourable contacts between the lipophilic groups (dispersion interactions) [3]. Aromatic rings play a key role in the field of molecular recognition [4]. Apart from lipophilic interactions (mentioned previously), specific contacts between aromatic rings are also relatively common. Because of their large polarizability, aromatic side chains can also interact with positively charged ammonium groups or act as hydrogen bond acceptors [5,6]. Finally, aromatic hydrogen atoms can be involved in C–H hydrogen bonding [7].

These non-covalent interactions regulate life at the molecular level. Evolutionary pressures have caused proteins to develop recognition systems for their targets that function with high selectivity and specificity.

The 20 natural amino acid side chains, together with backbone amides, furnish proteins

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with a complete 'toolbox' for productive molecular recognition. Secondary, tertiary and quaternary structures provide the correct placement in space of the chemical motifs, and eventual flexibility [8], as required.

An important aspect of protein–ligand interactions is that group contributions to the binding energy are generally additive [9]. For lipophilic interactions it has been shown [10] that the free enthalpy of binding is proportional to the lipophilic surface hidden from the solvent. Contributions to free energy for lipophilic surfaces being removed from the aqueous solution and becoming buried on binding, range from 80 to 200 J/(mol A^2). Even if, in general, there is not a clear relationship between the number of hydrogen bonds of a ligand to its binding site and the corresponding binding affinity, data from protein mutation studies give a value of +/–5 kJ mol^{-1} for the contribution of individual hydrogen bonds to the binding affinity or protein stability [4]. This consistent value derived from different proteins, suggests some degree of additivity in the hydrogen-bonding interactions. Analogous observations have been reported for polar interactions in host–guest complexes [11].

A simple 4.2 kcal mol^{-1} gain in binding free energy (a couple of good hydrogen bonds, a few van der Waals interactions) can account for an increase in binding affinity of three orders of magnitude. When searching for a lead, low to medium K_d are acceptable, and even welcome, if related to a hit with other desirable qualities. It is important to be aware of how these affinities might be obtained from, and depend on, the three-dimensional manipulation of polar and hydrophobic groups.

Lead-like versus drug-like

Over the past decade, an enormous effort has been expended on trying to increase the success rate of the drug discovery process and one aspect of this has been the use of technologies for synthesizing and screening large libraries of molecules in the search for leads. However, the success rate has not been as high as expected and medicinal chemists have started to debate the reasons for this outcome. Among the subjects discussed, the quality of the leads stands out.

Not every molecule can be a lead. The lead compound must be easily amenable to structure–activity relationship analysis, bind in a non-covalent way and ideally be free of known toxicophores. Papers have already been written on reactive false positives [12], promiscuous inhibitors [13] and frequent hitters [14]. Experienced medicinal chemists know just how misleading and time-consuming it can be to deal with these kinds of molecules.

Alternatively, it is becoming widely accepted that the physicochemical properties suitable for drug-like compounds are different to those required for lead-like compounds in the early stages of drug discovery programs [15]. Lipinski's Rule of Five is useful for assessing the risk profile of an oral drug candidate entering development, but does not necessarily define the properties of a good lead. As Rishton recently pointed out [16], the definitions of both drug-likeness and lead-likeness serve their own ends; 'drug-likeness serving to identify compounds suitable for drug development and product candidacy, and lead-likeness serving to identify compounds that are tractable for optimization by medicinal chemists. Lead-like compounds bind appropriately to their protein targets in biochemical assays and subsequently enable viable SAR development by medicinal chemistry'.

The fragment approach in the search for a lead

The complexity issue

Hann and collaborators demonstrated, in theory and in practice, that there is an optimal complexity for molecules that should be considered while screening collections of compounds for leads in medicinal chemistry programs (Figure 1a) [17]. Libraries containing highly complex molecules have a low chance of individual molecules binding. The reported data showed in an elegant way, that from a probabilistic and statistical point of view, when searching for new leads, 'the smaller the better'. Using the model system shown in Figure 1a, they have computed, for various levels of complexity of binding site and ligands, the probability that a randomly selected ligand of a given complexity matches a binding site. For a binding site of complexity twelve, the highest probability of single way matching, 39.9%, is found for a ligand of complexity three, whereas for a ligand of complexity seven, this probability drops to 4.6%. As the authors themselves recognize, 'the model we have used here has the simplest possible (i.e. +/–) recognition event. More sophisticated models could be developed which more realistically represent the true 3D nature of property gradients. However, the underlying principles explored with the 1D pattern model will be present in all sophisticated models'. As a molecule becomes more complex the chance of observing a useful interaction for a randomly chosen ligand falls dramatically, and the probability of mismatches increases exponentially!

The same concept had already been anticipated in 1984 by Andrews, who, when introducing the binding potential for molecular functionalities and comparing the calculated binding energy with the experimentally measured value, showed that poor binders were generally found to be large flexible molecules, rich in polar groups [18]. Often, in trying to quickly identify high-affinity matches, the industry has focused on screening relatively complex compounds

leading to, on statistical grounds, low hit rates and then eventually to leads that are not easy to modify without losing potency. In the light of past experience, screening simpler compounds initially might be a better strategy in the long run for drug discovery.

The selectivity issue

Some concern might arise regarding issues of selectivity when one considers working on particularly small molecules. In general, the multitude of energetically similar, but structurally different binding modes increases the fewer the interaction features of the ligand. When this happens, the free energy of binding landscape has been termed 'frustrated' [19]. But this is not always the case.

Structural studies on FKB12–ligand complexes, using mutant T4 lysozyme–ligand complexes and streptavidin–peptide complexes, suggest that molecular recognition might be fulfilled by a conserved, relatively rigid portion of the receptor active site interacting with an anchoring fragment of the ligand acting as its recognition core in the binding process [20]. Molecular anchors are small molecules or molecular fragments that bind within the enzyme–receptor binding site in a specific binding mode, characterized by a pronounced gap between the free energy of the favourable binding mode and that of alternative binding modes. This gap ensures the thermodynamic stability of the favourable binding mode and has been shown to be an important prerequisite for the specificity of ligand–protein recognition (Figure 1b). Molecular anchors, although responsible for specific binding, are usually not sufficient to provide tight binding unless they are incorporated into a larger ligand in which peripheral groups confer additional binding affinity. From a practical point of view it is not uncommon that a lead compound might contain a fragment that appears to be essential for binding affinity, even if it itself did not exhibit binding. Further, it is not uncommon that the remainder of the molecule, which might appear to contribute significantly to the binding affinity, might be amenable to significant modification without loss of binding.

The lead-likeness issue

The statistical work of Hann [17] tells us that in the search for affinity, a lead must be a small molecule, and Rejito [19] demonstrated that in the search for specificity a lead can be small. What about the process of transforming a lead compound into a clinical candidate? An answer to this question is given by Teague *et al.* [15] who, after careful analysis of published and internal data found that, on average, historical leads had lower MW, lower lipophilicity, fewer aromatic rings, fewer hydrogen bond acceptors and lower Andrew's binding energy functions [18] than the corresponding final drug. During the optimization process the MW and lipophilicity of the initial leads typically increase, and the proposal is that libraries consisting of compounds with MW = 100–350 and ClogP = 1–3 are greatly superior for finding leads than those containing drug-like compounds, with higher MW and ClogP.

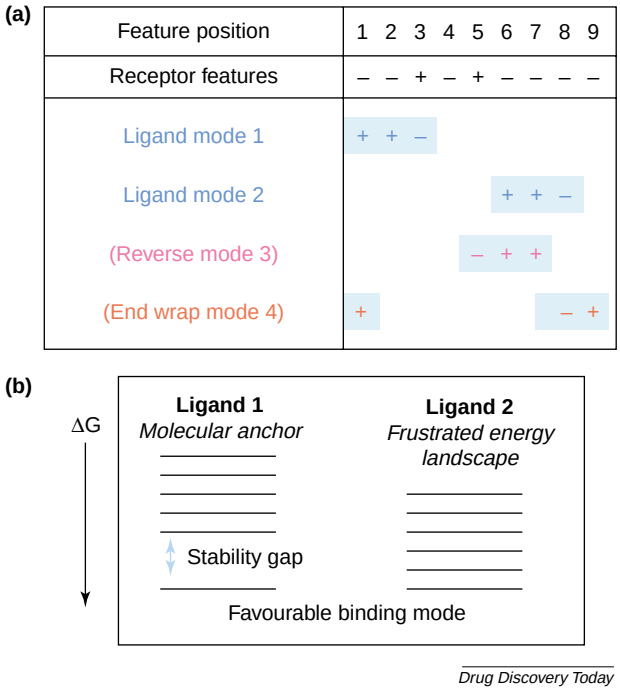


Figure 1. (a) Evaluation of ligand–receptor matching possibilities for a random receptor of complexity 9 and ligand of complexity 3 according to Hann *et al.* [17]. Localized recognition elements are represented by '+' and '–'. A successful recognition event is considered the one where for a given ligand all the + features match a binding site – feature (or visa versa). These features represent any aspect of a molecule (shape, dipolarity, hydrophobicity, among others) that need to be matched to the binding site. Possible ligands are then restricted in pattern length to be equal or less than that of the binding site. In the reported example there are two ways the ligand can match the receptor: mode 1 and mode 2. The authors decided to exclude reverse binding such as mode 3 and end wrap matches such as mode 4 from their model. **(b)** The concept of stability gap in binding energy for molecular anchors. Some molecular fragments may work as receptor-specific recognition nuclei, so-called molecular anchors. This happens when they are able to provide not only relative thermodynamic stability of a favorable binding mode, but also to ensure the kinetic accessibility of this binding mode. Rejito *et al.* [19] demonstrated that 'a basic requirement of a molecular anchor is the formation of a single binding mode that is both thermodynamically stable, compared with alternative binding modes, and kinetically accessible' (i.e. fragments having average energy and average structural consensus have little or no energy gap and are unlikely to be structurally stable molecular anchors).

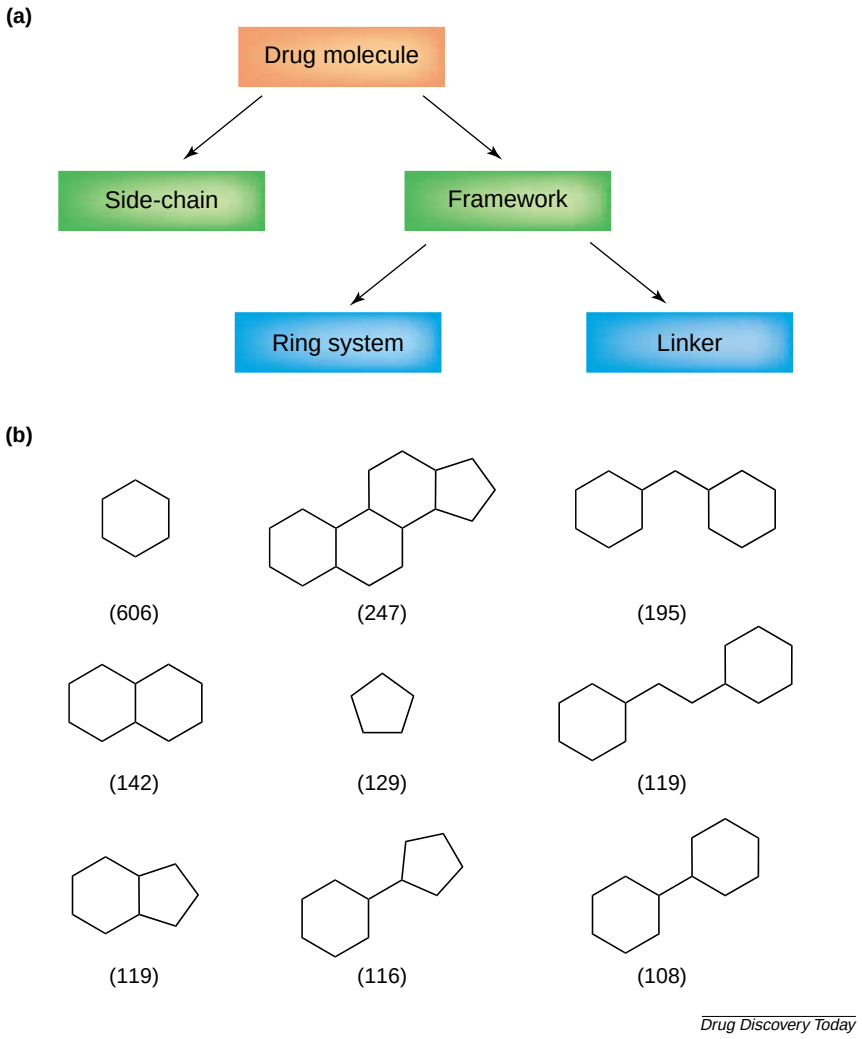


Figure 2. Database mining for molecular frameworks [21]. **(a)** The structures of molecules are dissected into four units: ring system, linker, framework and side-chain. Ring systems are defined to be cycles within the graph representation of molecules and cycles sharing an edge; linker atoms are atoms that are on the direct path connecting two ring systems; side-chain are any non-ring, non-linker atoms; the framework is defined as the union of ring systems and linkers in a molecule. **(b)** When this hierarchical dissection was applied to the Comprehensive Medicinal Chemistry (CMC) database components [22], well-represented frameworks could be identified. Here are reported the first nine (frequency of occurrence).

The choice of fragments

It is clear that fragment choice is an important issue. The first consideration is size. A fragment has to be large enough to act as a molecular anchor, but at the same time small enough to have good potential lead characteristics. The presence of opportune functional groups is important for the subsequent application of expansion and optimization chemical techniques. Reactive functional groups have to be avoided; covalent acting agents are useless in binding assays because they are not 'hit-like' [12]. Finally, there is diversity and space coverage. In the search for optimal binding, groups with diverse binding capacities (shape and polarity) have to be placed in complementary positions within the structural space.

Common archetypes and building blocks

Stimulated by the desire to find some guiding rules for the process of drug design – up to now largely driven by intuition, experience and serendipity – many groups have focused on drug database mining in the search for common drug properties. Some have tried to extract the basic fragments, 'the archetypes' according to Bemis and Murcko's definition [21], setting up a hierarchical analysis in which drug molecules' component parts have been categorized into ring systems, linker atoms and side chains (Figure 2). The union of ring systems and linkers defines a framework, the archetype. Through the analysis of an opportune set of molecules from the Comprehensive Medicinal Chemistry (CMC) database [22] it was found that a group of 32 common shapes or frameworks accounted for 50% of the 5120 drug molecules considered. Whether these fragments had intrinsic characteristics that gave them drug-like properties or that their presence was as a result of chemists' habits, familiarities or synthetic versatility was an issue that was recognized, but not addressed.

RECAP (Retrosynthetic Combinatorial Analysis Procedure), a database mining system published in 1998 by GlaxoSmithKline researchers, addresses the fragment choice for biased libraries. The procedure starts by collecting a set of structures with activity at a common target or target class. These structures are fragmented according to 11 chemical bond types easy to form in a synthetic sequence. The goal is to identify fragments occurring most frequently and to use them as building blocks for libraries on the same target [23].

Both these methods analyse databases of drugs or drug-like compounds; the starting molecules contain the features responsible for affinity, along with those important for metabolic stability and good physicochemical properties.

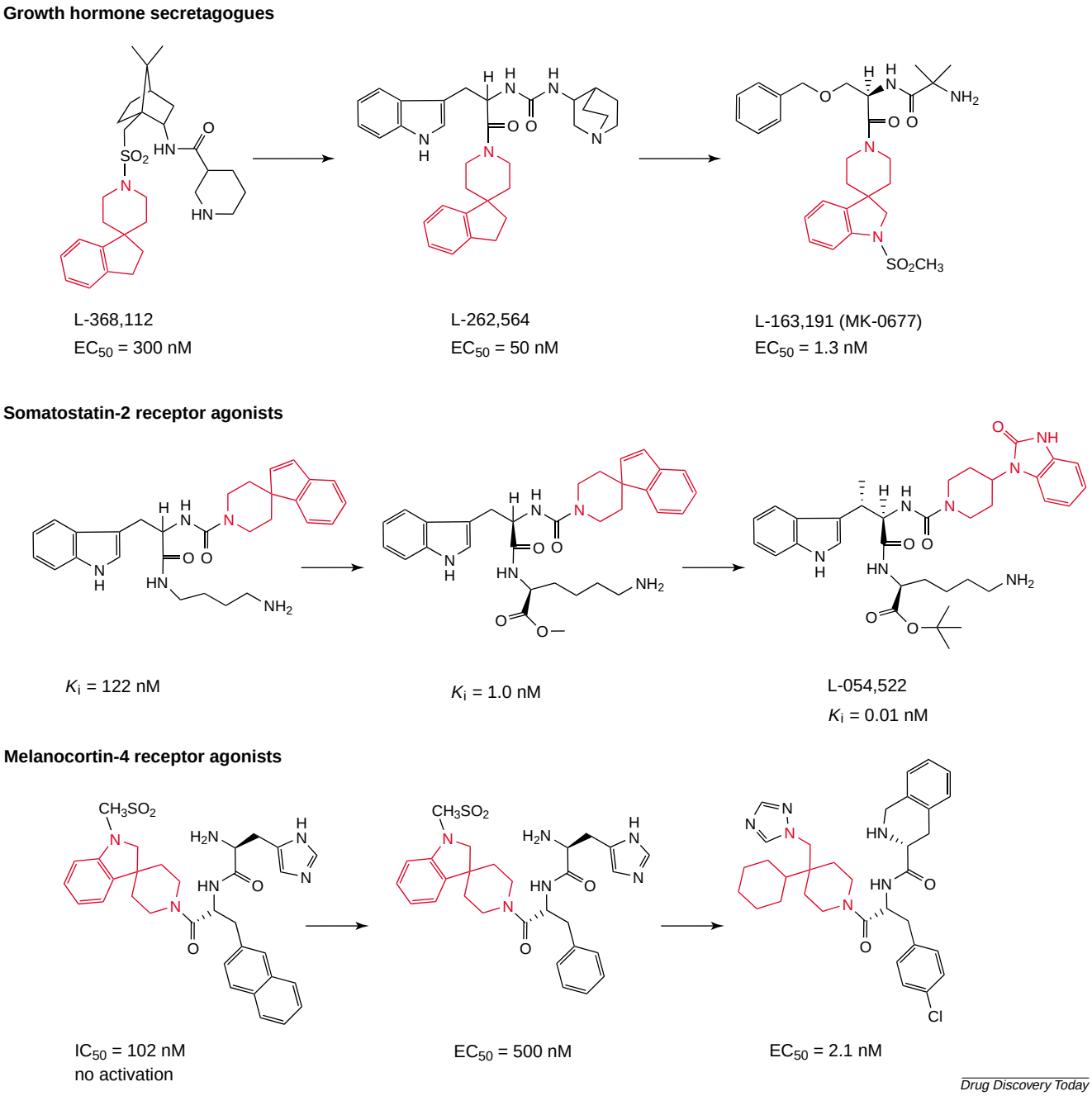


Figure 3. Growth hormone secretagogues, somatostatin-2 agonists and melanocortin-4 agonists obtained in Merck through derivatization of one among the privileged fragments, the spiropiperidine unit (red fragment) [25]. Evolution from the lead (left side) to the corresponding pre-clinical or clinical candidates [26–28].

The privileged structures

In attempts to rationalize the drug discovery process, others have taken an apparently similar path, but with a different underlying philosophy.

In 1988 Evans *et al.* [24], describing their development of the benzodiazepine-based CCK-A antagonists, had proved that certain substructural motifs, called privileged structures, were capable of providing useful ligands for more than one receptor and concluded that 'these structures appear to contain common features which facilitate binding to various proteinaceous receptor surfaces, perhaps through binding elements different from those employed for binding of the natural ligands'. The selection and derivatization of these 'privileged structures' has become a

working strategy in Merck, coinciding with the rapid growth of combinatorial chemistry in the mid-1990s, and has produced several interesting leads (Figure 3) [25].

Rich has provided a theoretical explanation for these findings based on molecular interactions in water [29]. A large amount of binding energy for interactions in aqueous media comes from hydrophobic interactions. Proteins have evolved in a way that enables them to generate stable hydrophobic pockets at the surface of the enzyme/receptor, pockets that are used by the enzyme/receptor itself to bind its natural ligand. The proteins have evolved in such a way that these pockets do not undergo hydrophobic collapse in the absence of the ligand. The same is also true for some ligands. In some natural products (cyclosporin) and in some flexible synthetic molecules (Argatroban), it is hydrophobic collapse that generates the bioactive conformation. However, this is not always the case. In general, small peptides and organic molecules are not rigid enough to prevent neighbouring hydrophobic chains from interacting once in aqueous media, generating conformations far from those calculated *in vacuo* and far from the bioactive one. There are structural motifs containing multiple hydrophobic groups that are conformationally constrained from hydrophobic stacking. By examining the structures of known drugs, many templates containing two or more aromatic or hydrophobic groups can be found that, because of constraint, cannot stack (Figure 4).

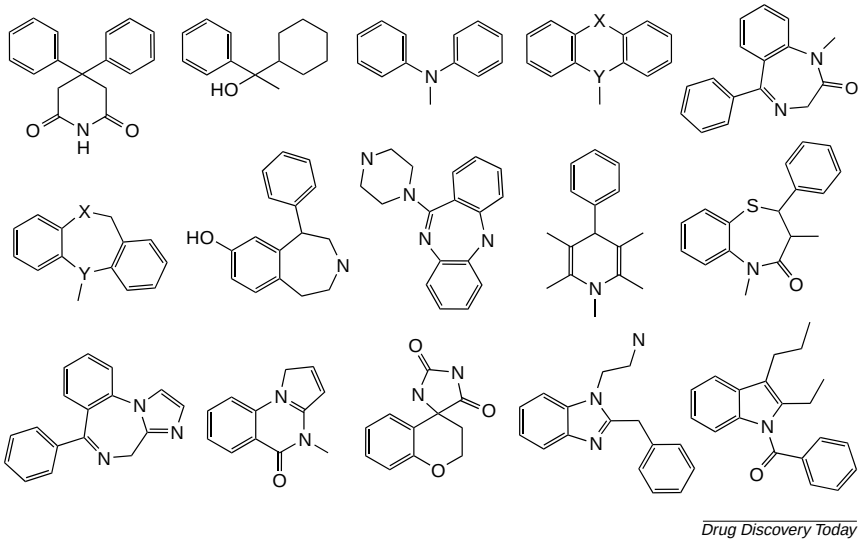


Figure 4. Examples of structural units that will not stack aromatic groups intramolecularly in aqueous media according to Ref.[29]. All reported units are portions of clinically used drugs.

It is noteworthy that the archetypes found by Bemis and Murcko [21] through the analysis of drug databases (except for the steroid skeleton and the cytotoxic fused polycyclic systems) describe many of Rich's 'privileged structures' [29], and the latter share many of the qualities required by 'molecular anchors' once decorated with opportune functionalities.

Modification of these core structures has produced a large number of highly potent agonists and antagonists in the field of GPCRs [30], and many of the privileged fragments are also found in protease inhibitors [31], kinase inhibitors [32], and even in protein–protein interaction inhibitors [33].

Building blocks inspired by the privileged structure concept are now commercially available under the name of 'optimers' (http://www.arraybiopharma.com).

The target family-directed masterkeys

Recently Mueller pointed out that another kind of fragment is used for focused libraries [34]. In fact, given that target families often recognize common small chemical entities, target family-directed masterkeys, such as hydroxyethylene dipeptide mimics for aspartic proteases, hydroxamates for metalloproteases, and so on. These might be referred to as privileged recognition fragments, or according to Rejito's definition [19,20] 'molecular anchors' specific for protein functional classes. The HIV protease inhibitors already on the market, or the advanced renin inhibitor from Pharmacia (Figure 5), which share the hydroxyethylene fragment, are a good example of the power of masterkeys as anchoring fragments.

The experimental approach

The strengths of the fragment approach are the potential to use less complex and therefore more 'druggable' starting points, and the possibility to easily enter into areas of chemistry and biology not previously explored. One drawback is the expected low target affinity that is not always compatible with classical screening assays. Indeed, because higher compound concentrations have to be employed to detect binding over the usual micromolar cut-off, serious problems of protein stability can arise because of the high DMSO concentration necessary to solubilize the tested molecules and/or high compound concentrations [35]. Several innovative assay technologies have appeared in recent years to obviate this problem.

Fesik and his colleagues at Abbott pioneered the use of NMR for the *de novo* design of small drug molecules: the

approach is termed 'NMR-based screening' [36]. Individual small fragments, selected using a modification of the RECAP algorithm, are tested against a ^{15}N-labelled protein. The affinity and binding site location are determined by watching how the ^{15}N/^{1}H-HSQC (heteronuclear single quantum coherence) spectra of the protein change.

Graffinity (http://www.graffinity.com) has developed a new technology based on surface plasmon resonance [37]. Small molecules are immobilized on a carrier surface and provided in a screening ready, high quality, standardized format. Incubation of the immobilized compounds with the purified and solubilized target protein yield comprehensive affinity fingerprints in a label and assay-free procedure. One such chip contains as many as 9216 microarrays of various fragments that are carefully organized in pre-determined spatial locations.

For Astex (http://www.astex-technology.com) the technology of choice for weak interaction detection is X-ray crystallography [38]. HTS methods are used to study an array of protein–fragment complexes that bind specific binding pockets. Appropriate fragments binding adjacent binding pockets are then tethered together to design and optimize drug molecules.

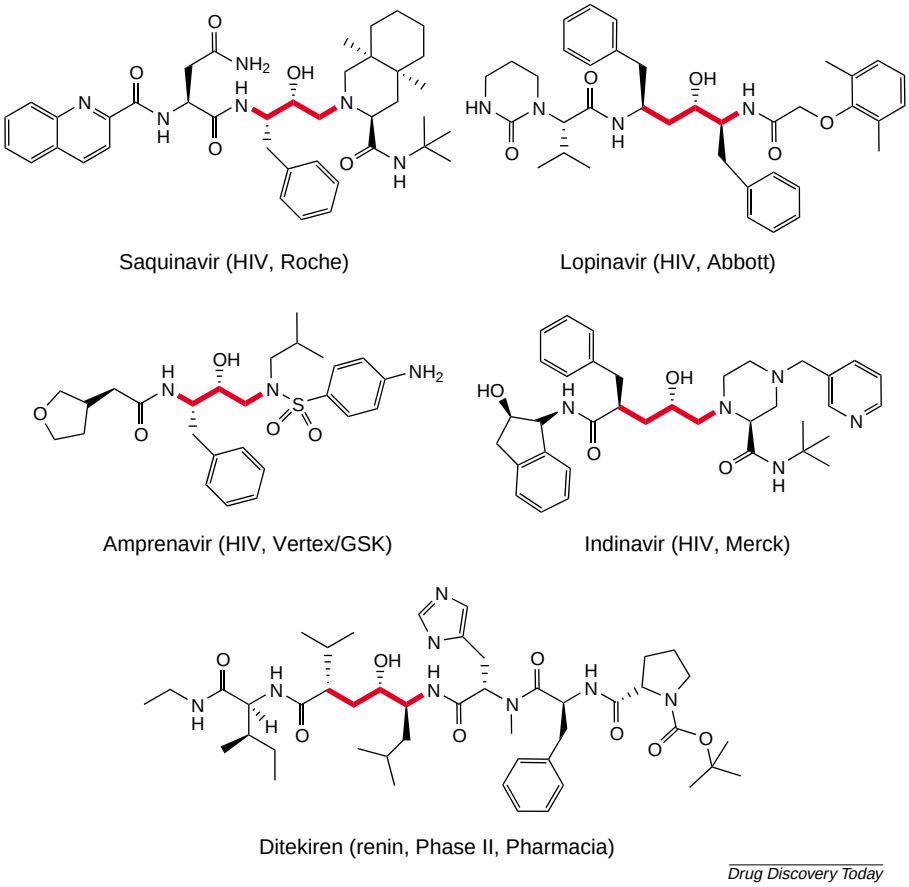


Figure 5. Protein families often recognize common small chemical entities, the target family-directed masterkeys [34]. These fragments can be used as molecular anchors to build drug molecules. Examples are the HIV protease and renin inhibitors on the market or in advanced clinical trial (Source: Prous Science Integrity®, http://integrity.prous.com), all containing the hydroxyethylene unit, a fragment specifically recognized by aspartyl proteases.

These three new techniques are applicable for a considerable number of targets, but do not, as yet, cover GPCRs, nor other membrane proteins.

Conclusions

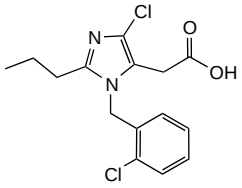
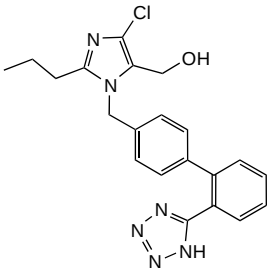
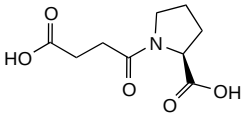
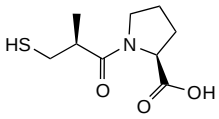
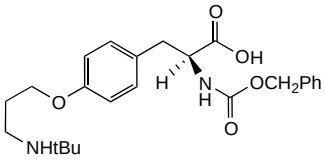
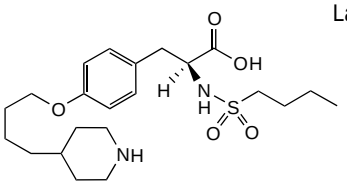
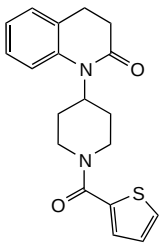
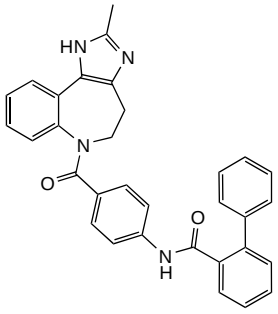
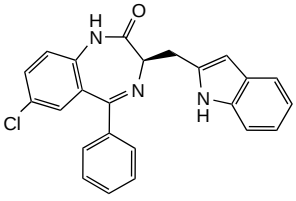
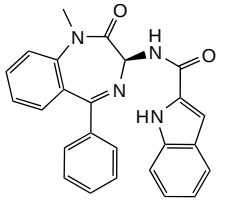
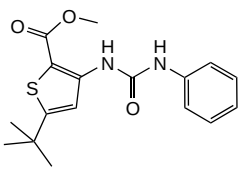
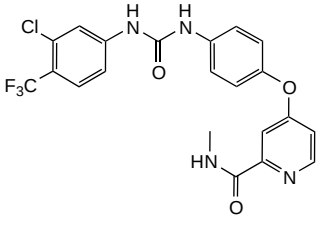
Peptides represent Nature's solution for the 1D coding of the 3D structure of molecules. With this system it is possible to build productive interactions with whatever target, given the appropriate amino acid sequence. Part of this sequence is directly responsible for productive interactions, a larger part for the positioning in space of the binding moieties. Unfortunately peptides do not possess drug-like characteristics.

The accumulated knowledge in the fields of organic synthesis and molecular recognition, enable chemists to create the desired 3D geometries with non-peptidic frameworks, with well-documented advantages [29]. There is no need for rigid scaffolds decorated with more or less functionalized fragments; the direct connection of fragments with covalent bonds is sufficient to attain the goal. In fact, the relative geometries of the binding groups can be modulated by playing on the conformational preferences of chemical bonds along with intramolecular hydrogen bonds and hydrophobic effects.

Scanning the multidimensional molecular diversity space systematically and exhaustively is still a winning strategy, provided that the working fragments have been carefully selected to produce tractable lead structures. Indeed, once the key interactions important for binding are defined, one can further modify the molecule to optimize its physicochemical and pharmacological parameters. Having a clear idea of which functionalities are important for gaining binding energy and which are important for physicochemical

and pharmacological properties, even if both must have drug-like characteristics and can often coincide, can be extremely helpful. High-throughput techniques and calculation programs are powerful instruments in the hands of drug discovery scientists, but human intelligence, intuition and experience are also important components for a successful

Table 1. Examples of drugs deriving from micromolar leads

Mechanism of action	Lead	Affinity	Drug	Phase[a]	Ref.
Angiotensin II receptor antagonist		43 μM	Losartan 	Launched	[27]
Angiotensin Converting Enzyme (ACE) inhibitor		330 μM	Captopril 	Launched	[37]
Fibrinogen receptor antagonist		22 μM	Aggrastat 	Launched	[38]
Vasopressin receptor antagonist		2.5 μM	Conivaptan 	Phase III	[39]
Cholecystokinin A receptor antagonist		3.4 μM	Devazepide 	Phase II	[40]
Raf Kinase inhibitor		17 μM	Sorafenib 	Phase II	[41]

[a]Source: Prous Science Integrity® (http://integrity.prous.com).

drug discovery group. A basic knowledge of the complex game of molecular interactions that determine the behaviour and fate of a drug molecule from the moment it enters a living organism can be considered a part of this human component.

The idea of starting a project from micromolar leads might sometimes not be welcome, but by analysing the history of some classical marketed drugs (Captopril, Losartan, Aggrastat) as well as some drug candidates currently undergoing clinical trial, several good examples can be found (Table 1). This is even more interesting if we consider that these leads were discovered using classical bioassays! As has already been pointed out, a recurring question with database mining is whether the common frameworks result from intrinsic drug-like properties or from the chemists' desire to work with known, easy to manipulate structures. We strongly believe that both aspects are important and equally relevant; chemical versatility of leads is a key point. The fragment approach is becoming a hot topic. Whether these methods might represent a major improvement in our lead compound discovery and drug development processes has yet to be demonstrated, although the prospects for the future are promising.

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