Unlocking medicinal chemistry innovation with FBDD strategies.

Transforming fragments into candidates: small becomes big in medicinal chemistry

Gerdien E. de Kloe[1], David Bailey[2], Rob Leurs[1] and Iwan J.P. de Esch[1,2]

[1] Leiden/Amsterdam Center for Drug Research (LACDR), Division of Medicinal Chemistry, Department of Pharmacochemistry, Faculty of Exact Sciences, VU University Amsterdam, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands
[2] IOTA Pharmaceuticals Ltd, St Johns Innovation Centre, Cowley Road, Cambridge CB4 0WS, United Kingdom

Fragment-based drug discovery (FBDD) represents a logical and efficient approach to lead discovery and optimisation. It can draw on structural, biophysical and biochemical data, incorporating a wide range of inputs, from precise mode-of-binding information on specific fragments to wider ranging pharmacophoric screening surveys using traditional HTS approaches. It is truly an enabling technology for the imaginative medicinal chemist. In this review, we analyse a representative set of 23 published FBDD studies that describe how low molecular weight fragments are being identified and efficiently transformed into higher molecular weight drug candidates. FBDD is now becoming warmly endorsed by industry as well as academia and the focus on small interacting molecules is making a big scientific impact.

Introduction

The promise of more efficient lead discovery has fuelled enthusiasm for fragment-based drug discovery (FBDD), accompanied by a paradigm shift from high-throughput screening towards design intensive approaches, enabled by structural biology [1,2]. An impressive number of FBDD reviews have been published focusing on general concepts and screening methods [3–12]. In this review we focus on medicinal chemistry strategies for optimising fragments, taking examples from the published literature. By organising the data by research groups, a clearer appreciation of the FBDD strategies preferred by individual labs is evident. Together, the different studies encompass a wide variety of technologies and methodologies, illustrating the expanding utility of FBDD in lead development. Although 'fragmentology' has become a central part of modern medicinal chemistry, several issues continue to be debated with this new technology. We highlight these in the medicinal chemistry campaigns described in the next subsections.

Corresponding author: de Esch, Iwan J.P. (i.de.esch@few.vu.nl)

GERDIEN DE KLOE MSC
Gerdien de Kloe MSc studied Pharmaceutical Sciences at the VU University in Amsterdam. Currently she is a PhD student working on the design and synthesis of novel nicotinic acetylcholine receptor ligands using FBDD. Her focus is on the comparison of orthogonal screening methods and the development of potent compounds by linking and growing hit fragments.

DAVID BAILEY
David Bailey headed up the Molecular Sciences Department at Pfizer in Sandwich, UK, before becoming Vice President at Incyte Genomics. Currently, he is CEO of IOTA Pharmaceuticals, having previously founded two Cambridge-based start-ups, De Novo Pharmaceuticals Ltd and Purely Proteins Ltd. He is a Board Director of the Babraham Institute in Cambridge and consultant entrepreneur for the University of Greenwich in London. Dr Bailey holds a PhD in biochemistry from the University of Cambridge.

ROB LEURS
Rob Leurs obtained his PhD in pharmacochemistry from the VU University Amsterdam in 1991. As a postdoctoral fellow (1992–1993) at INSERM (Unite de Neurobiologie and Pharmacologie, Paris), he was involved in the cloning of genes encoding histaminergic and serotonergic receptors. Thereafter, he was awarded with a 5 year fellowship (1993–1998) of the Royal Netherlands Academy of Arts and Sciences. He was appointed as assistant and full professor in Medicinal Chemistry in 1998 and 2000.

IWAN J.P. DE ESCH
Iwan J.P. de Esch received an MSc in organic chemistry and holds a PhD in pharmacochemistry. In 1998, he joined the Drug Design Group of the University of Cambridge, UK. This group spun out of the university to form De Novo Pharmaceuticals. Iwan returned to academia in 2003 and is now associate professor at the Medicinal Chemistry Department of the VU University, Amsterdam. Next to studying G-protein-coupled receptors, the group has established a strong fragment-based drug discovery research line. Iwan is also co-founder of IOTA Pharmaceuticals, a company that provides fragment-based drug discovery services to partners in the pharmaceutical industry.

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Fragment library design

Although FBDD originated from strategies for identifying smaller starting structures for medicinal chemistry programmes, Hann *et al*. demonstrated that there is a balance between too low a complexity (which may result in not finding an unambiguous binding mode) and too high a complexity (which may not enable precise matching with the receptor). Furthermore, a degree of complexity is needed to provide enough potency to be able to measure the binding event. These considerations determine the ligand complexity needed to find unique binding modes in fragment screening [9,13].

The link between complexity and unambiguous binding mode has been investigated in detail by Babaoglu and Shoichet, who fragmented a β-lactamase inhibitor and analysed the binding modes of the resulting fragments using X-ray approaches. None of the resulting structures bound in an equivalent position to those in the complete inhibitor [14], prompting the question of how much complexity is needed to obtain a well-defined binding mode and how many alternative binding modes can usefully be explored by small fragments. The studies below include several examples of common fragments with multiple binding modes, some illustrating binding mode changes during optimisation. Fragment library design has been reviewed recently [15].

Ligand efficiency

Kuntz *et al*. showed that high molecular weight is not a necessity for high binding affinity [16]. A useful approach is to measure binding affinity per atom, that is, to monitor ligand efficiency (LE). Initially, this concept was suggested as a tool for hit selection [17]. In FBDD, LE is used to select the most promising fragment, and also to monitor the fragment growing or linking process and guide optimisation of the hit structure [18]. Several formulae have been proposed to determine LE [17,19]. In this review we have chosen the binding efficiency index (BEI) proposed by Abad-Zapatero [19], defined as BEI = (pK_i, pK_d or pIC_{50}/MW) × 1000.

Fragment growing. . .

On the basis of a retrospective study of 18 drug discovery programmes at Abbott, Hajduk found that the increase in molecular weight from a fragment towards a high-affinity lead compound was linear with respect to affinity. In other words, ligand efficiency remained fairly constant, serving as a quantitative measure of effective fragment elaboration [18]. Growing fragments has the advantage that subtle changes in binding mode, often reflected in ligand efficiency, can be taken into account during step-wise optimisation. Various examples of this are discussed below.

. . .or fragment linking?

Reduction of rotational freedom [20] predicts that efficiently linking two simultaneously binding fragments should result in the affinity of the product being higher than simply the sum of the affinities of the non-linked fragments, at the same time increasing ligand efficiency. Unfortunately, linkage of two fragments, retaining potency, is still an enormous challenge. In many cases the orientation of both fragments in the site after linkage is compromised [21], or energy is lost owing to linker strain [22]. In addition, the chemical nature of the linker often has an influence on binding [23]. Despite these issues, there are several examples of successful fragment linking [24].

Astex Therapeutics

Astex Therapeutics is a fragment-based drug discovery company with expertise in high-throughput crystal soaking and X-ray structure determination (dubbed high-throughput crystallographic screening, HTX), complemented by other screening technologies (e.g. *in silico* screening and NMR studies) [25,26]. As a leader in the field of FBDD, Astex has published a significant number of studies describing the structure-based optimisation of fragment hits [3,21,27–32], the most recent of which are discussed below.

β-*Secretase*

Exploration of co-crystal structures of β-secretase (BACE-1) with various fragments reveals significant conformational changes in the crystallised complex [33]. Astex screened a small number of fragments (347) by this method, yielding two hits (a hit rate of 0.6%), including fragment 1. Virtual analogue screens were then performed, in which fragment 2 was identified. This compound was grown into the hydrophobic S_1 pocket of the target, leading to 3. With an indoline substituent, the hydrophobic pocket was even better occupied, and an additional pyridine-containing substituent was introduced to reach out for the S_2′ region. This resulted in compound 4 with low micromolar affinity, judged as an interesting lead compound [3,30] (Figure 1).

Protein kinase B

Protein kinase B (PKB) is an important anti-cancer target. For this target, Astex deployed an initial virtual screen, followed by validation through bioassay and crystallography. Fragment 5 is an example of the hits that were found. The azaindole moiety was exchanged for a purine scaffold to facilitate synthesis, and this fragment was grown at the 9-position with several phenyl groups. Compound 6 was one of the best compounds in this series, with further extension of the vector using a 4-chlorosubstituted phenyl group, giving the nanomolar inhibitor 7. Unfortunately, the compound showed low potency. In efforts to improve this property, secondary amines were synthesised, and compound 8 (with a piperidine moiety) gave good potency while retaining acceptable ligand efficiency [34]. The purine scaffold could be replaced with a pyrazole moiety to give equal affinity and slightly better ligand efficiency [31].

Selectivity of the inhibitors for PKB over the close homologue PKA was measured while growing the fragments. Replacing the phenyl moiety of hit 6 for a non-planar linker while also performing scaffold hopping to introduce the more hydrophilic pyrrolopyrimidine moiety, resulted in compound 9 that has an eightfold selectivity for PKB. Extension with a 4-chlorosubstituted phenyl (inspired by 7) resulted in 10, which, despite its higher affinity, lost its selectivity. The position of the primary amine is important for selectivity; compound 11 gave nanomolar affinity and 30-fold selectivity [35].

Cyclin dependent kinase 2 inhibitors

Cyclin dependent kinases (CDKs) are key regulatory proteins in cell cycle progression, and, as such, are important anti-cancer

targets. Indazole 12 was found in a crystal-soaking screen of about 500 fragments, which included both focused kinase and CDK2 fragment sets. The fragment binds to the hinge region, and structural information suggested that it could be grown at the 3-position and 5-position. The introduction of a benzamide at the 3-position (13) gave a 60-fold improvement in potency, but the crystal structure of this complex showed restricted opportunities for extension at the 5-position of 13 owing to the changed orientation of the indazole ring. Scaffold hopping to the smaller pyrazole heterocycle gave comparable ligand efficiency and allowed growth from the 4-position of the ring. Introduction of an additional benzamide gave significant increase in affinity (14). The crystal structure revealed that the phenyl moiety was twisted out of plane with the amide bond, thereby adopting an energetically unfavourable conformation. Two *ortho*-substituents were then introduced to decrease the difference between global and binding conformations, which resulted in 50 times improvement in affinity. Finally, the 4-fluorophenyl group was replaced by a more hydrophilic piperidine group to improve cell membrane permeability, producing compound 15, which has entered clinical development [32].

Urokinase-type plasminogen activator

Urokinase-type plasminogen activator (uPA) binding with its receptor is associated with multiple sclerosis and cancer. Fragment 16 was a hit from an HTX campaign against uPA. The amine functionality makes three important hydrogen bonds, but is less basic than guanidine or amidine groups present in known uPA inhibitors. The lower pK_a favours oral bioavailability. The X-ray structure of 16 shows that the hit can be expanded around the phenyl moiety and several substitution patterns were explored, amongst which 17 gave a significant increase in affinity, with 18 showing 10 times better potency. Selectivity and pharmacokinetic studies showed that compound 18 was a good lead compound with high selectivity and good oral bioavailability [27].

Plexxikon

Plexxikon, a small Bay-area biotech, was founded in 2001. The company uses bioassays for screening, followed by X-ray crystallography-based structure-guided lead optimisation.

Phosphodiesterase inhibitors

Inhibitors of phosphodiesterases (PDEs) have broad therapeutic potential, especially as anti-inflammatory and anti-asthmatic agents. Plexxikon screened a substantial library of some 20 000 fragments against a representative subset (5) of members of the PDE family using a high-throughput scintillation proximity assay. A total of 316 compounds showed >30% inhibition at 200 μM for three or more PDEs. Co-crystallisation with two subtypes (PDE4D and PDE4B) led to 269 co-crystallised compounds and ultimately to 107 solved co-crystal structures. The combination of biochemical screening of larger fragment libraries with rapid structure determination of bound fragments has proven very powerful. Fragment 19, with an IC_{50} value of 60 μM for PDE4B, was chosen for optimisation.

Four different vectors (see Figure 2) on this scaffold were probed by the synthesis of ten compounds. Only the introduction of a phenyl group at the I position was tolerated, leading to 20 with an IC_{50} of 270 nM. Importantly, the binding mode of the aromatic heterocycle did not change when compared to the original fragment hit 19 and, for this reason, was qualified as a so-called validated scaffold. Next, using structural data from hit 20 and *in silico* library enumeration (taking into account synthetic feasibility and scoring all possible compounds *in silico*), 10 further compounds were designed that differed in substituents on the phenyl group. This led to compound 21 with low nanomolar affinity. Thus, two rounds of synthesising, screening and co-crystallisation of only 20 compounds, each led to a 2000-fold improvement in affinity [36].

B-Raf kinase inhibitors

Another Plexxikon FBDD target was the oncogenic target B-Raf kinase. Of particular interest is the V600E mutant, a well-described activating missense mutation often found in tumour cells. The Plexxikon library was screened against a panel of several kinases to find a non-specific kinase binding fragment. In the strategy chosen by Plexxikon, selectivity was built into this novel lead series during fragment growth. Compounds that inhibited the activity of Pim-1, P38α and CSK by at least 30% at 200 μM concentrations (238 compounds) were co-crystallised with at least one of these kinases. More than 100 structures were obtained in this programme, including the complex of 7-azaindole (22) with Pim-1.

Interestingly, different binding modes were identified for this fragment. A subsequent analogue screen with mono-substituted 7-azaindoles yielded the 3-aminophenyl analogue 23, a compound with significantly increased affinity and only one observed binding mode. This compound, however, was still a non-specific kinase binder. Subsequently, a library of analogues with varying substituents at three different positions was synthesised and screened against the panel of kinases, including the B-Raf^{V600E} mutant. Using co-crystal structures, optimisation chemistry led to designs that bind not only to the well known and highly conserved hinge region of the kinases but also to a selectivity pocket almost unique to the activated B-Raf, with lead compound 24 (PLX4720) showing nanomolar affinity for B-Raf^{V600E}. This compound is highly selective for the activated state of B-Raf^{V600E} in a diverse panel of 70 other kinases. Studies in animal models have confirmed 24 as a promising lead compound with therapeutic potential for treating B-Raf^{V600E} driven tumours, one of the first examples of successful progression from fragment to drug candidate [37].

SGX Pharmaceuticals

SGX Pharma, recently acquired by Eli Lilly, is one of the more mature players in the FBDD field. Founded in 1998 as Structural Genomix, SGX has focused on anti-cancer discovery, although to date, only a limited number of medicinal chemistry programmes have been published in the scientific literature.

BCR-Abl inhibitors

A crystallographic screen was performed against the wild type and T315I mutant of BCR-Abl, a tyrosine kinase associated with cancer. A total of 800 compounds were screened, giving 28 hits binding at two different sites (representing a 3.5% hit rate) [38].

Fragment 25, binding in the ATP-binding pocket, was subsequently optimised at both the 3-position and 5-position. Although optimisation at the 3-position was more efficient than that at the 5-position, the combination of both optimisation

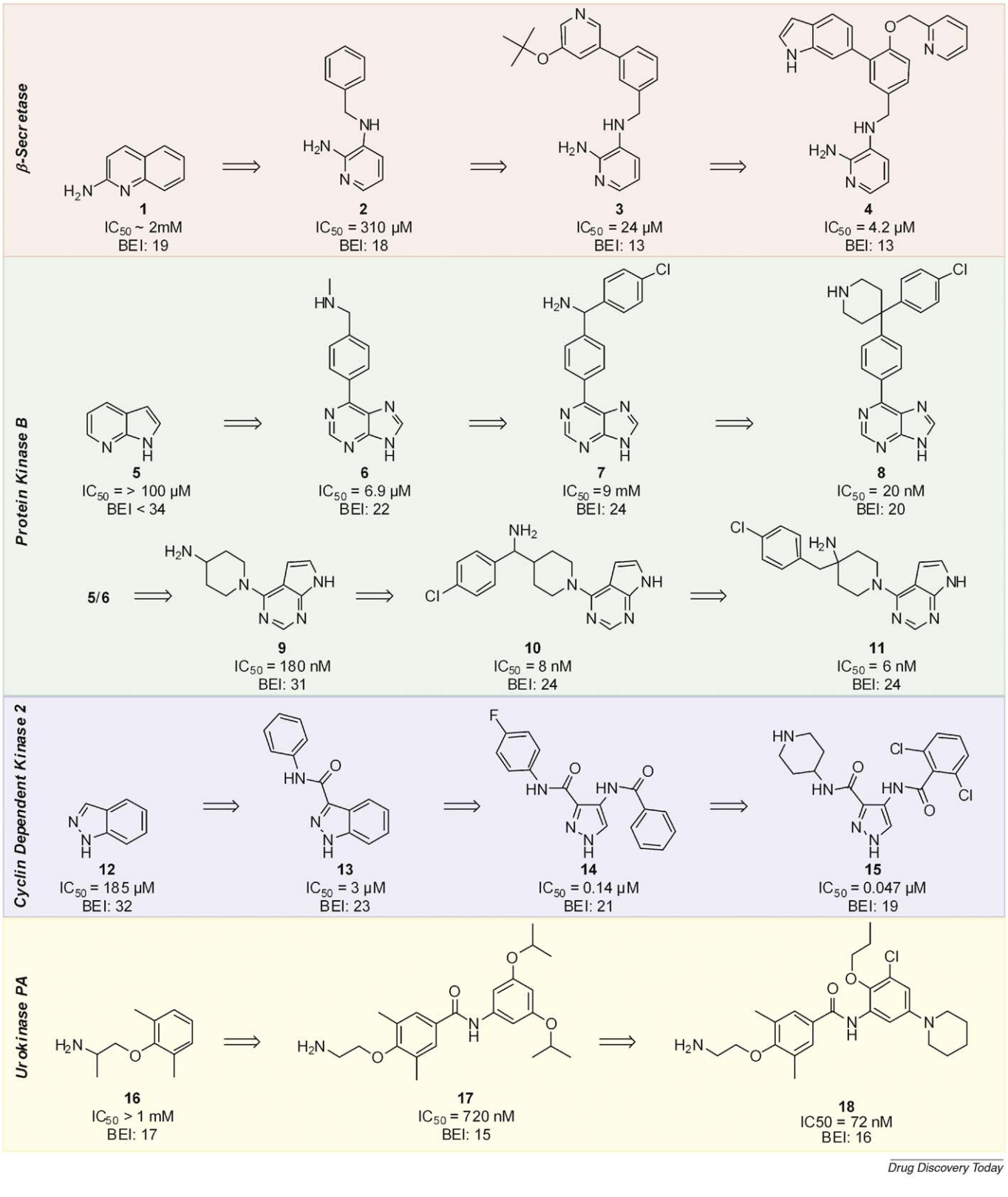

FIGURE 1

Astex's fragment-based optimisation studies.

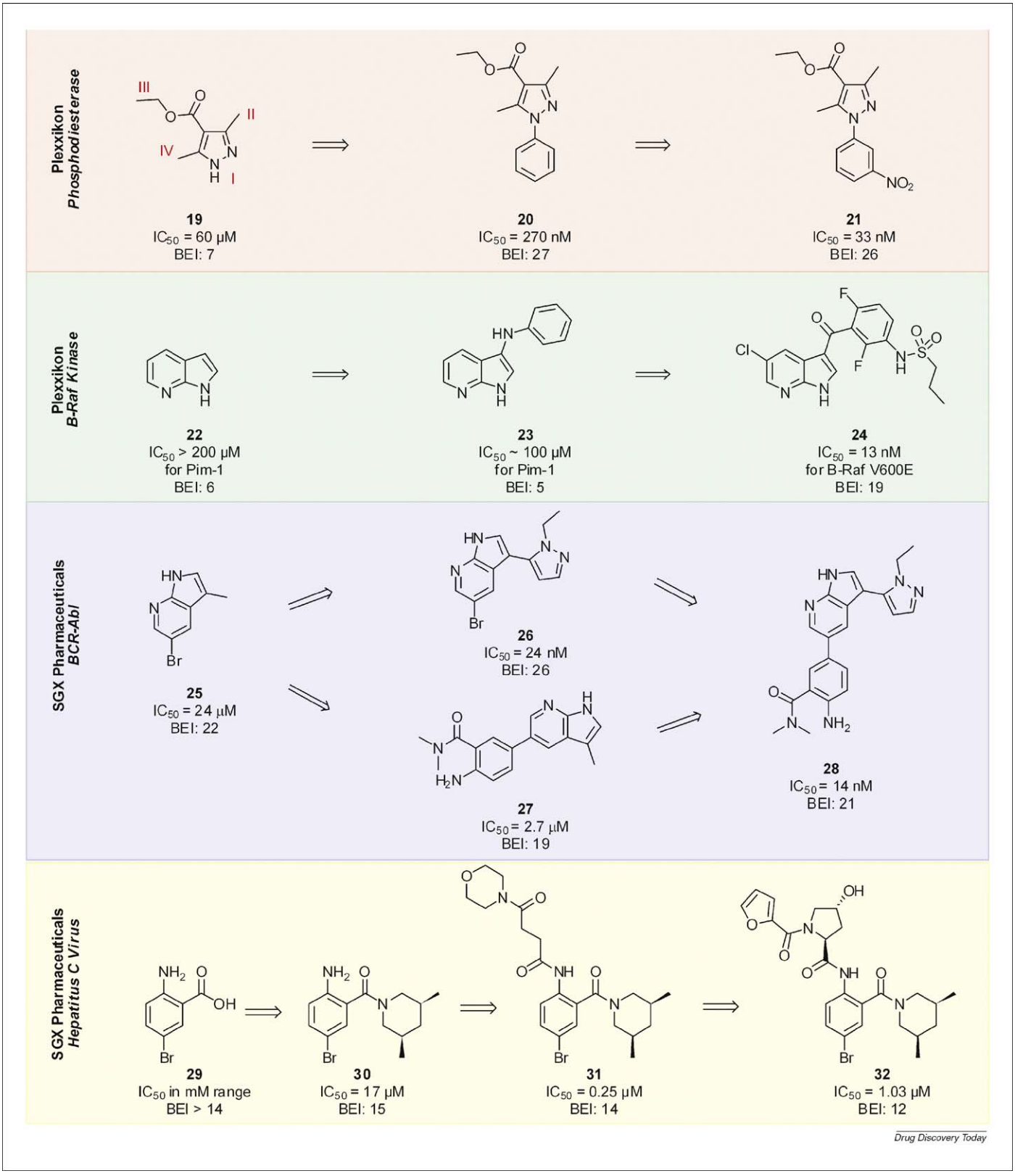

FIGURE 2

Representative fragment-based medicinal chemistry programmes at Plexxikon and SGX Pharmaceuticals.

directions yielded 28, a nanomolar lead compound (S. Reich, FBLD Conference San Diego, 2008). This compound was further optimised and submitted to the FDA (June, 2008; S. Burley *et al.*, unpublished).

Hepatitis C virus

Infection with the virus Hepatitis C (HCV) is the leading cause of chronic liver disease, and thus, HCV represents an important therapeutic target. SGX performed crystal soaking fragment

screening on the essential HCV target, NS5b RNA polymerase. A total of 960 fragments were screened, 20 hits were found, of which 17 interestingly were bound at an allosteric site. One of the hits was fragment 29, containing a bromine atom to help structural elucidation. The aromatic ring occupied a well-defined hydrophobic pocket, with the bromine atom pointing towards the floor of the pocket and the aniline and carboxylic acid moieties pointing outwards. After X-ray analysis, fragments and their derivatives were characterised, both in biochemical assays to measure their IC_{50}s and in surface plasmon resonance (SPR) technology to measure binding affinities.

Fragments were then grown by introducing several amines to the carboxylic acid, yielding a small series of compounds, including 30. Interestingly, this fragment had a different binding mode than its precursor, as the phenyl moiety was flipped by 180°, and the carbonyl amide oxygen atom interacted with very different residues at the entrance of the hydrophobic pocket than did the carboxylic acid in hit fragment 29.

Crystal structures showed that the compound could be grown further by substituting the aniline nitrogen atom. Compound 31 was the best of this series of amides, in which the attached morpholine ring stacked (but did not hydrogen-bond) with an adjacent histidine residue. Exchange of the morpholine ring with (heterocyclic) aromatic rings did not improve affinity. Finally, the ethylene spacer was rigidified with a proline moiety to give compound 32 with the highest potency in the biochemical assay, although its affinity for the polymerase was not improved [39].

Sareum

Sareum, a UK structure-based drug discovery company founded in 2003 and recently acquired by Galapagos, uses biochemical assays to prioritise active fragments, subsequently analysing hits using X-ray approaches.

Checkpoint kinase 1

Sareum screened a fragment library of 361 compounds against Chk1, an important anti-cancer target. Twenty fragments showed >25% inhibition, and were assessed by X-ray crystallography, from which 13 crystal structures were obtained. One of these compounds, 33, was grown from two vectors, leading to intermediates 34 and 35. Compound 36 was further optimised into a nanomolar inhibitor reportedly with great selectivity and equal ligand efficiency to 33, but this structure has not yet been published [40] (Figure 3).

Vernalis

Vernalis is another UK-based company, focusing on novel lead candidates for CNS diseases and cancer. Vernalis has developed several structure-based methodologies, including a FBDD platform called SeeDs. The construction of their fragment library has been described in detail by Baurin *et al*. [41].

Hsp90

Heat Shock Protein 90 (Hsp90) is an abundantly expressed and highly conserved cellular chaperone. Many Hsp90 client proteins such as erbB2/Her-2, c-raf, bcr-abl and p53, are members of well-characterised oncogenic pathways, making Hsp90 inhibitors attractive anti-cancer agents. Vernalis identified fragment 37 as a hit for Hsp90 in a competitive NMR screen of 790 fragments. This low-affinity compound was used to search for commercially available analogues, which were ranked by *in silico* docking. A number of compounds were purchased and tested, including compound 38 (IC_{50} = 0.5 μM) [42]. Ironically, this compound was found independently in a high-throughput screen of 50 000 compounds by collaborators, illustrating that FBDD and HTS are complementary approaches [43]. Further structure-based optimisation focused on the 3-position of pyrazole 38. The amido-moiety (39) was able to pick up an additional hydrogen bond with a glycine residue in Hsp90 that was already interacting with the pyrazole NH, while the carbonyl moiety made a hydrogen bond with a flexible lysine residue [44].

Crystal structures of this series of compounds showed that *meta*-substituents and *para*-substituents at the 4-phenyl moiety pointed towards the solvent. A solubilising functionality was, therefore, introduced at this position, with a morpholine moiety giving the highest functional activity. Substitution of the chlorine atom by a hydrogen atom gave a 20-fold loss in affinity. This vector points towards a flexible part of the binding pocket and will tolerate several large groups. The moderately sized isopropyl group gave the highest activity (GI_{50}, inhibition of cell proliferation).

Finally, the exchange of the pyrazole ring for an isoxazole moiety gave similar affinity but higher activity. Kinetic analysis of these compounds by SPR showed that, while the k_{on} was very similar, the k_{off} of the isoxazole compound was 10-fold slower. According to crystallographic data, no extra interactions were made by the isoxazole moiety, but the orientation must lead to a better complementarity with the binding pocket [45], illustrating the importance of obtaining such kinetic data.

The best compound of this series, with respect to both affinity and activity, was compound 40 with an IC_{50} of 21 nM and almost equal functional activity. This compound is currently in Phase I clinical studies.

PDK1

The Ser-Thr kinase PDK1 is an important regulator of other kinases and, as such, an important target against tumour growth. Again using NMR-based screening, Vernalis identified fragments 41 and 42 by screening only 80 fragments. These hits, in combination with the structure of a known CDK2 inhibitor, enabled an analogue search for commercially available derivatives to be performed. Candidates were selected on the basis of their docking poses, a process yielding the micromolar compound 43. Subsequent optimisation by introduction of a benzimidazole ring system, a substructure also present in ligands targeting the homologous kinase Chk1, resulted in the nanomolar lead compound 44 [42].

Uppsala University

Surface plasmon resonance (SPR) is a very useful and informative biophysical method for analysis of molecular interactions. Having previously confirmed the value of SPR with respect to kinetic and thermodynamic parameters by defining these properties of candidate HIV-1 reverse transcriptase inhibitors [46–48], the group at Uppsala recently published the first SPR fragment screening study [49]. A company, Beactica, was spun out of Uppsala University after the study and is now using the approach to develop leads against a number of different classes of targets.

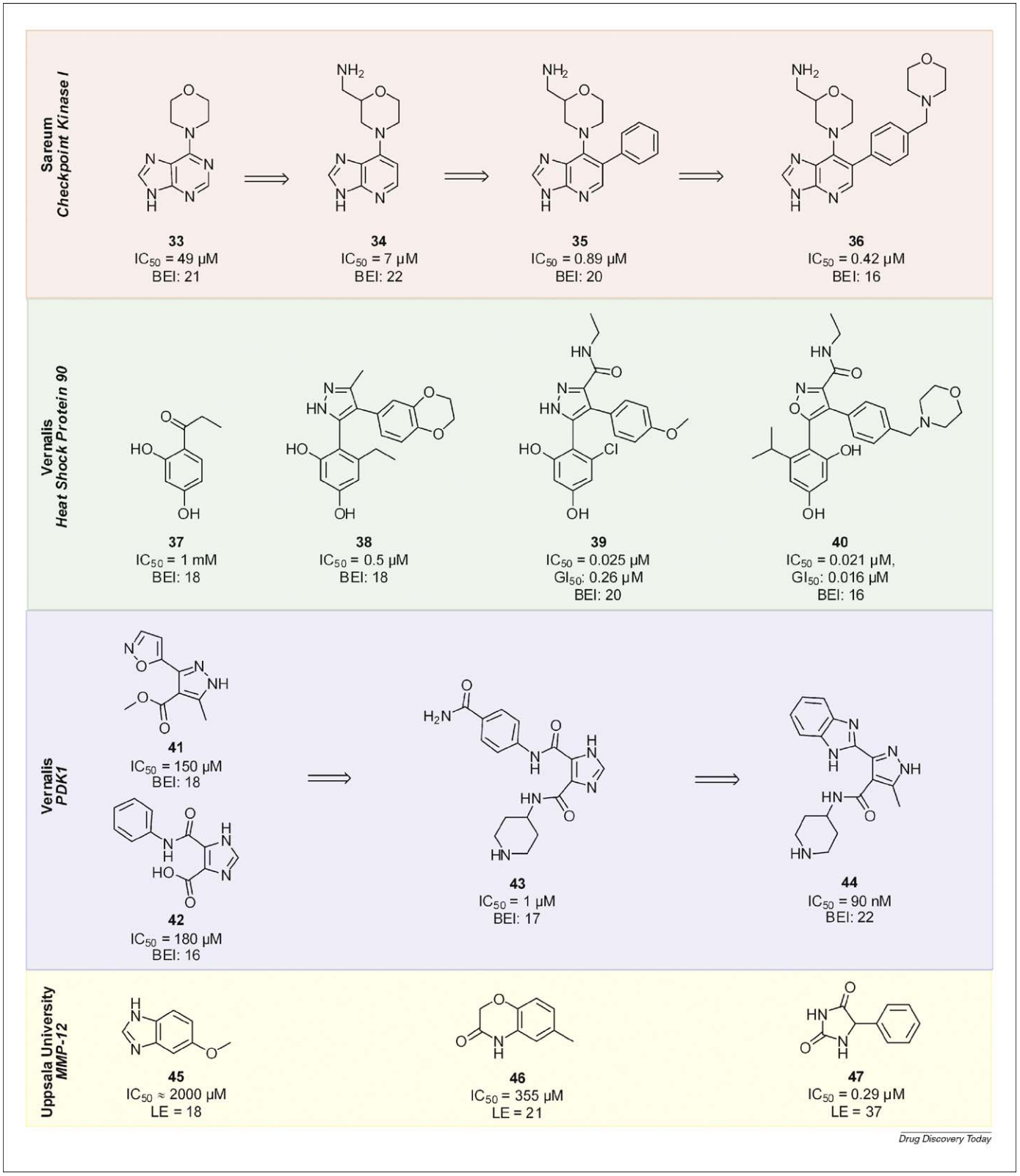

FIGURE 3

Representative fragment-based medicinal chemistry programmes at Sareum, Vernalis and Uppsala University.

Matrix metalloproteinase 12

Matrix metalloproteinase 12 (MMP-12) is a potential drug target for emphysema and chronic obstructive pulmonary disease (COPD). A major problem with the known MMP-12 inhibitors is their non-selectivity. Uppsala University used SPR to identify and characterise selective fragments. Fragment affinity was simultaneously measured for native MMP-12, MMP-12 in the presence of a known inhibitor and a homologous zinc-containing protease.

Using this screening procedure, fragments 45–47 were found to be selective, competitive inhibitors of MMP-12 with good ligand efficiencies, representing good starting points for lead optimisation [49].

Abbott Laboratories

Scientists at Abbott are pioneers in the FBDD field. More than a decade ago, Steven Fesik described the classic 'SAR-by-NMR' approach [50]. Since then, their ~10 000 fragment library has been screened against many targets [22,24,50–58]. In recent years, NMR fragment screening has been combined with an X-ray screening protocol called CrystalLEAD [59]. Crystals are soaked in carefully assembled mixtures of 100 fragments with different shapes. Abbott scientists have noted that a disadvantage of X-ray analysis as a fragment screening technology is that the outcome is highly dependent on the conformation of the protein in the crystal [60]. A small, but illustrative, number of publications are discussed below (Figure 4).

PTP1B inhibitors

Protein tyrosine phosphatase 1B, or PTP1B, is responsible for downregulation of the insulin receptor, a well-validated target in diabetes and obesity. Abbott discovered the diaryloxamic acid 50 in a 2D NMR-based fragment screen using their 10 000 compound library. Chemical shift changes were similar to those for phosphotyrosine, indicating an interaction with the positively charged catalytic site that is highly conserved amongst the tyrosine phosphatases. Naphthyloxamic acid 51 was synthesised to fill the space within the active site, leading to a 7.5-fold improvement in affinity. An X-ray structure verified that the hit fragment had tight interaction with the active site. While the crystal structure suggested the naphthyl 5-position as an appropriate vector for growing out of the active site, substituents at both the 5-position and 6-position of the naphthalene resulted in lost affinity. By contrast, substitution at the 4-position, leading to compound 52, resulted in a 40-fold affinity gain. X-ray analysis revealed that the hydrogen-bonding contacts of the oxamic acid were preserved but that the nahpthyl group had flipped 180°, thereby allowing the pentyl chain to point out towards a second, non-catalytic site, which was not conserved in the target class and a good site to address selectivity. Selective amino-acid protein labeling and NMR screening enabled fragment screening to the non-catalytic site. A further screen of 10 000 compounds indicated that fused-ring aromatic acids could be used to fill this pocket. This led ultimately to the enantiomerically pure compound 53 that showed an affinity of 22 nM [53,61].

Selectivity over other phosphatases was addressed while growing the fragments. For example, when second-site screen hit 48 was combined with a close analogue of 52, the more selective compound 54 was obtained [53]. Having identified fragment 48 as selective, this structure was coupled with another hit fragment, 49, to form 55, which showed more than 30-fold selectivity over any other phosphatase studied [54].

Hsp90 inhibitors

Abbott screened their fragment library using NMR experiments to study Hsp90. Chemical shift changes of selected residues of the protein were detected in $^{1}H/^{13}C$ 2D HSQC NMR experiments [62]. Two structurally related hits were found (56 and 57). X-ray structures showed key interactions between the aminopyrimidine and triazine moieties with an aspartic acid residue of the protein and two bound water molecules. The naphthyl moiety of 56 induced a conformational change that opened up a larger binding site. In order to improve interactions at this new binding site, the two hits were combined and 128 analogues were synthesised and tested, resulting in a high nanomolar lead compound (58). Replacement of the 4-methyl with a 4-chloro substituent resulted in a threefold increase in affinity (59). Although an example of successful FBDD optimisation, further development of this series was halted owing to IP considerations.

In a second-site screening approach, fragment 60 was shown to bind co-operatively with hit 57. Structural information about the binding of these two fragments was obtained from X-ray and NOE experiments. Interestingly, the X-ray structure showed face-to-face π-stacking between the aminopyrimidine ring of 57 and the phenyl moiety of 60, while the NMR data indicated that 60 occupied a more distant new binding site. Different linkers were designed on the basis of these two binding modes. Inspired by the X-ray structure, compound 61 was designed to make an internal π−π interaction, while compound 62 was devised to occupy the two distinct binding sites as guided by the NMR data. Both strategies resulted in low-micromolar inhibitors, although there was a significant drop in ligand efficiencies. [22].

AstraZeneca

AstraZeneca has applied FBDD extensively across their discovery portfolio, against targets such as bacterial enzymes, GPCRs (including melanocortin 4 receptor), prostaglandin D2 synthase, phosphatases such as protein tyrosine phosphatase 1B, and proteases such as β-secretase. Screening by NMR is favoured, often in combination with a range of secondary screening approaches [63]. It has been reported that FBDD is becoming the preferred line of approach for new drug targets at AstraZeneca (J. Albert, at EFMC, Vienna 2008).

β-Secretase inhibitors

β-Secretase is an important target for the treatment of Alzheimer's disease. HTS attempts to find small molecule inhibitors proved unsuccessful and so a ligand-based, WaterLOGSY NMR fragment screen was performed, both in direct and in competitive experiments with a known binder, to detect non-specific binding. SPR was used as a secondary screen. This screening cascade yielded isocytosine 63, amongst other hits. Despite its low affinity, its high ligand efficiency made it a good starting point for optimisation. Close analogue screening led to compound 64, with an extra phenyl substituent that increased affinity. The binding mode of 64 was determined with the related, and more readily available, aspartyl protease endothiapepsin by X-ray analysis. The two catalytic aspartic acid residues interacted strongly with the scaffold, the first interacting with the two hydrogen atoms of the 2-amino group, the second interacting with the N^1 atom and the 2-amino group. The phenyl group pointed towards the S_1 and S_3 pockets, which were not filled optimally. Replacement of the phenyl moiety with an indole group (65) led to an extra hydrogen bond in the S_1/S_3 pocket, and low micromolar affinity. Methylation of N^3 of the isocytosine doubled this affinity (66). In collaboration with Astex, a crystal structure was obtained that indicated that the S_1/S_3

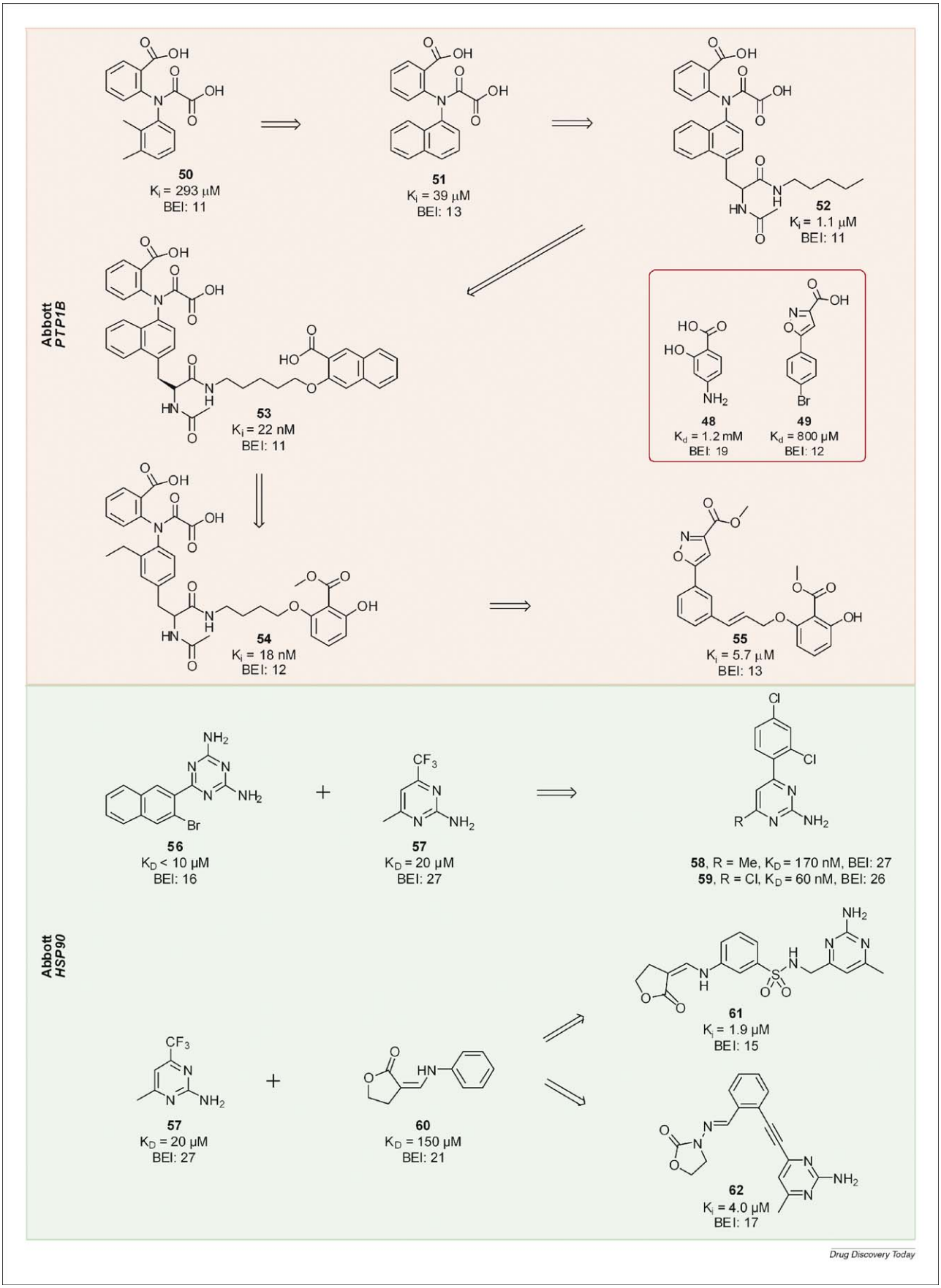

FIGURE 4

Two of Abbott's fragment-based medicinal chemistry programmes.

pocket was still not fully occupied, whereupon several biphenyl moieties were synthesised. Compound 67 had the highest affinity in this series.

Meanwhile, other in-house 2-amino heterocycles were screened, and compound 68 was found. This close analogue of the isocytosine compounds was also co-crystallised with β-secretase. The structure indicated that the 6-phenyl group adopted a pseudo-axial conformation, which is significantly higher in energy than the pseudo-equatorial conformation (calculated $\Delta E = 1.4$ kcal/mol). In order to cancel out the difference between the global minimum energy and the binding conformation energy of the ligand, a 6-methyl substituent was added to give compound 69 (calculated $\Delta E = 0.1$ kcal/mol). As in the isocytosine series, the introduction of a N^3-methyl substituent improved affinity; a better occupation of the S_1/S_3 pocket with compound 70 led to submicromolar affinity, with the active isomer (71) having an IC_{50} of 80 nM [64,65] (Figure 5).

Prostaglandin inhibitors

In the search for small molecule inhibitors of the hematopoietic form of Prostaglandin D_2 Synthase (H-PGDS), AstraZeneca screened a library of 2500 fragments, again with NMR, observing protein signals because the protein could easily be expressed and isotope-labeled. No assignment of chemical shifts was undertaken and, as a consequence, no confirmation of amino acids affected by fragment binding was obtained, although experiments with reference compounds indicated that all compounds and fragments occupied the same binding pocket. A total of 24 primary hits were found (1% hit rate) in the range of K_d ~50–500 μM. These included fragment 72.

Making use of X-ray structures to determine the docking modes of the hits, a pharmacophore model was constructed. Simple 2D substructure searching was performed for analogue selection, and these were evaluated in pharmacophore modelling and docking studies. On the basis of the predicted poses, 10–20 compounds per initial hit were selected and screened. Fragment 73 is an example of a low-micromolar analogue resulting from these studies. Again, pharmacophore features were combined and analogue screening was performed. Several efficient inhibitors for PGDS were found, for example, compound 74 with an affinity of 21 nM and excellent ligand efficiency. During the study, several structurally different lead compounds were identified after screening 300 hit analogues and six X-ray structures, without any synthesis [66].

Burnham Institute

The non-profit Burnham Medical Research Institute has developed several NMR-based fragment screening methods, including Inter-Ligand nuclear Overhauser Effect (ILOE) and ^{19}F-NMR measurement [67–70].

Anthrax lethal factor inhibitors

Lethal factor protease (LF) is an important enzyme excreted by Bacillus anthracis, the causative agent of anthrax. A fragment screen was set up by monitoring the changes in ^{19}F-NMR signal upon cleavage of a fluorinated peptide substrate of the protease. Screening 300 fragments using this assay led to the hit fragment 75. Analogues of this fragment with different substituents at the 5-position of the furan and 1-position of the 4-oxo-2-thioxothiazolidin-3-yl were screened, leading to compound 76 as the best compound. The SAR information from this series indicated that substitutions on the phenyl group could increase affinity, and that a methylene spacer between thiazolidin-3-yl and acid was optimal. This led to compound 77 with an affinity of 32 nM and promising *in vivo* activities [68].

FKBP12 inhibitors

FK506-binding proteins (FKBPs) catalyse peptidyl-prolyl *cis-trans* isomerisation, which is important in protein folding. Inhibitors of this activity confer neuroprotection, making this an interesting therapeutic target in neurodegenerative diseases such as Parkinson's and Alzheimer's disease.

Using 1D NMR-based screening of about 300 mixtures of 10 fragments identified several hit fragments, including 78. Dissociation constants were measured by evaluating protein chemical shift changes upon titration of the hits. Complementary ITC measurements were also performed. A set of 51 commercially available analogues were purchased and, on the basis of the SAR data obtained, new derivatives were synthesised in which the methyl group of 78 was replaced by an isopropyl group, leading to a 75-fold improvement in affinity. Anticipating a similar binding mode as reference compound 81, especially with respect to the aliphatic rings, substituents at the morpholine ring of 79 were introduced, yielding compounds such as 80. This did not, however, improve affinity illustrating subtle differences in binding mode. Docking studies with the help of NMR chemical shift mapping data indeed indicate that the amide carbonyl of 79, makes a similar H-bonding interaction with a tyrosine residue of the receptor as that of the α-keto-amido group of 81, implying that the positioning of the morpholine ring must be somewhat changed [70].

P38α MAP kinase inhibitors

In the search for inhibitors of P38α MAP kinase, Burnham devised the elegant Interligand Nuclear Overhauser Effects (ILOE) technique. This NMR approach is able to identify fragments that simultaneously bind to the protein site. Mixtures of 2–96 fragments were screened in the presence of protein and 82 and 83 were identified as hits.

The strongest NOE signals were observed between the protons of the piperidine ring of 82 and the phenyl ring of 83. The data proved ideal for a fragment linking approach, in this case, resulting in compound 85. Alternatively, ILOE data can be used to construct a pharmacophore based on the binding fragments. A query of commercially available compounds for the developed pharmacophore resulted in compound 84. This compound was further characterised and was shown to have micromolar affinity and an interesting selectivity profile [67].

F. Hoffmann-La Roche

Originators of the 'needles' approach to fragment-based screening, reviewed in [70], Hoffmann-La Roche have devised several novel FBDD screening methods, including computational methods such as *in silico* screening and *de novo* structure generation, as well as experimental applications such as SPR.

DNA gyrase inhibitors

At Roche, the FBDD approach was first used to find new classes of DNA gyrase inhibitors as antibacterial agents. On the basis of a

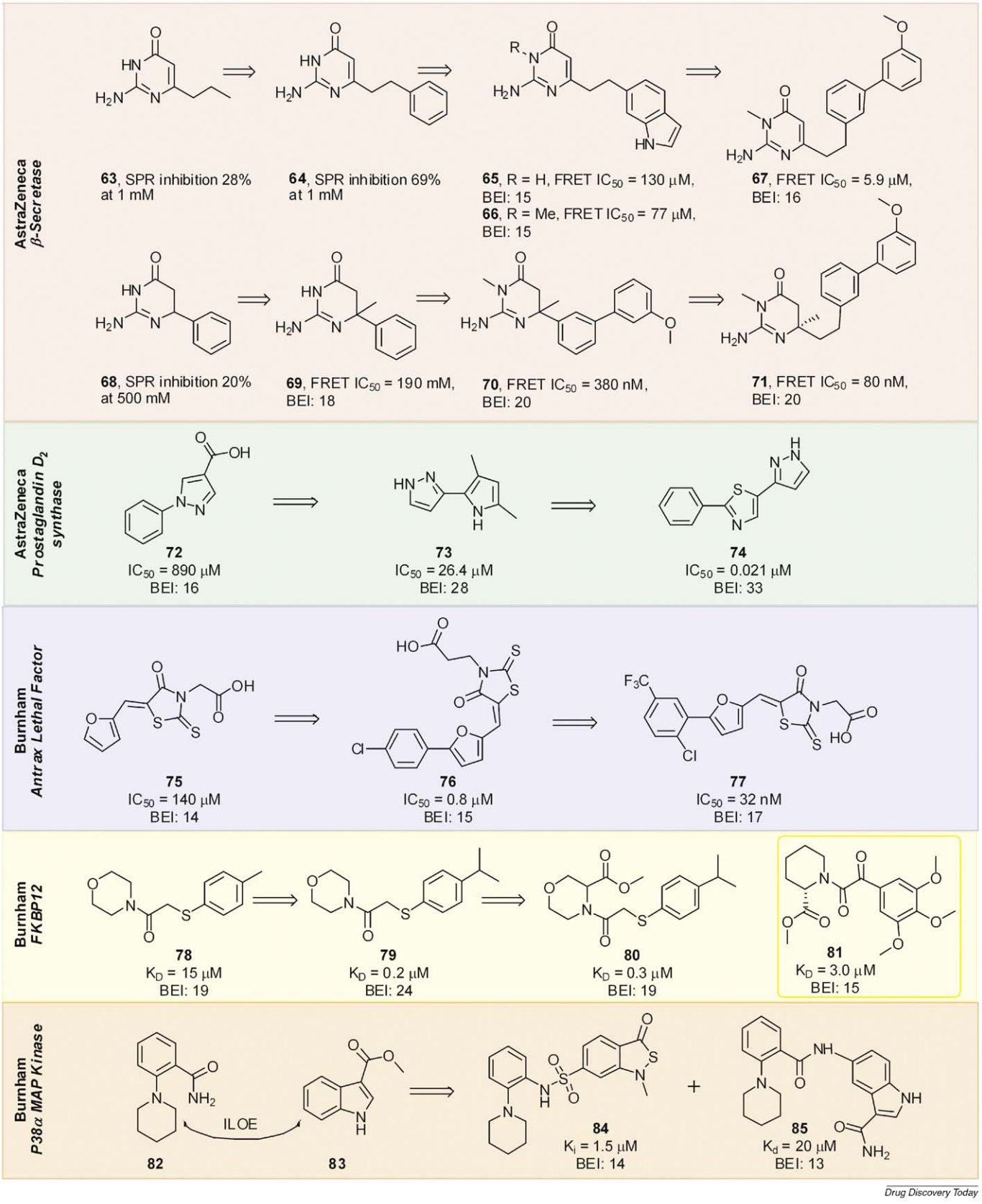

FIGURE 5

Representative fragment-based medicinal chemistry programmes at AstraZeneca and Burnham.

pharmacophore model of the enzyme, virtual screening was performed with 350 000 compounds, and 600 initial hits were followed up in a robust and sensitive-coupled ATPase assay. Then 2400 close analogues of the initial hits were screened, which resulted into a total of 150 hits (5% hit rate) divided into 14 structurally diverse classes. These classes were validated using various assays including a functional assay (supercoiling), as well as biophysical assays such as SPR and NMR. Specific binding could be confirmed in seven compound classes [71]. The optimisation of the hit fragments, such as compound 86, has been described in a more recent publication. Ring closure gave 87. Different substituents were introduced at the 3-position of this 2,3-dihydroisoindol-1-one, including a series of small aromatic heterocycles, but only very small substituents, such as in compound 88, were tolerated. Again ring closure improved affinity. Compound 89 was the most promising from this series [72] (Figure 6).

BACE-1

Pre-screening using computational chemistry and SPR measurements yielded 48 active fragments for BACE-1, which were analysed by X-ray crystal soaking. Several crystal structures were obtained, one of which was fragment 90, a metabolite of tyrosine.

This small compound has a well-defined binding mode; its protonated amine forms three hydrogen bonds, with two water molecules and a catalytic site aspartate. The phenol forms edge-to-face $\pi-\pi$ interactions with a phenylalanine and a hydrogen bond with the backbone carbonyl of this same residue. Close analogues were screened by crystal soaking. This led to the discovery of compound 91, with comparable ligand efficiency and 30 times higher affinity, a good starting point for further optimisation [73].

Ibis Therapeutics, a division of Isis Pharmaceuticals

Ibis Therapeutics have described an interesting FBDD study in which mass spectrometry (MS) was used as a sensitive screening technology [74].

Bacterial rRNA inhibitors

Fragments 92, 93 and close analogues were discovered in a screen against bacterial 23S rRNA. Using MS, hits were evaluated for simultaneous co-operative binding to the RNA, studies that indicated that compounds 92 and 93 were bound simultaneously. The RNA complex with compound 93 alone was never found. Screening close analogues revealed that the furan moiety of 92 and the allyl site chain of 93 were both needed for this simultaneous binding, indicating possible interactions between the furan and allyl side chains. Six different linked compounds were then synthesised, of which compound 94 showed low micromolar affinity [74].

Schering-Plough

Following on from their interest in SBDD [75], Schering-Plough have recently described the use of FBDD in proprietary programmes targeting viral proteases.

Hepatitis C virus protease inhibitors

Seeking low-molecular weight inhibitors for Hepatitis C Virus protease (restricted within the viral NS3/NS4A complex), a fragment library containing 3639 compounds was screened against ^{15}N-labeled protein using 2D NMR. Sixteen hits were found, binding in different regions of the protease, and these were optimised by focused SAR studies. For fragment 95, it was found that removal of the phenyl group gave highest affinity, although the acidic phenol and both iodide atoms appeared to be essential for affinity. Screening 47 analogues of compound 97 gave little improvement in affinity; methoxy group replacement of the phenyl group produced a slightly enhanced affinity, while all other modifications led to reduced affinity. Further analysis of the binding modes of these compounds using chemical shift perturbation suggested linking optimised fragments 96 and 98, giving compound 99 with an affinity of 0.8 μM [76].

Sunesis

Sunesis have developed an alternative strategy for fragment-based screening, called 'tethering', based on *in situ* ligand assembly [5]. The method relies on the formation of a disulfide bond between bound fragments and a cysteine residue of the protein (present either in the wild type or introduced by site-specific mutagenesis). The resulting complex is identified by mass spectrometry [4,73]. The tethering approach has been applied to several enzymes [77–80] and even to GPCRs [81]. An illustrative study is discussed below.

Thymidylate synthase inhibitors

This strategy was initially applied to thymidylate synthase (TS), an anti-cancer and anti-infective drug target, which naturally contains an active site cysteine. A library of 1200 thiol-containing fragments was screened, and fragment 100 and close analogues were found in complex with TS by MS. The affinity of compound 100 without linker, 101, was determined and found to be 1.1 mM. Crystal structures of both 100 and 101 in complex with TS were solved. Both structures overlapped quite well, showing that the fragment, and not the linker, determined binding. These crystal structures also showed that the tosyl group is bound in the same position as methylenetetrahydrofolate, the natural cofactor for TS. The tosyl moiety was then substituted like the cofactor, which improved affinity almost 50 times (102). Several substitutions were tried at the proline carboxyl group, of which compound 103 gave the highest affinity with a further 70-fold improvement [79].

Vertex

Vertex was one of the first companies to perform fragment screening *in silico*. More recently, computation has been supplemented by their 'SHAPES' strategy, in which a very small fragment library, based on the most commonly occurring shapes of existing drugs, has been created. This library consists of approximately 120 small fragments [82].

P38α MAP kinase inhibitors

For p38α MAP kinase, 13 confirmed hits were found by screening the SHAPES library with STD NMR. Fragments 104 and 105 are examples of these hits, which are in the millimolar range. Analogues of these fragments were selected on the basis of substructure-matching and virtual screening. In total, 300 follow-up compounds were screened, yielding eight inhibitors with potencies higher than 20 μM. Then several hundred compounds were synthesised, amongst which was compound 106, a combination of scaffolds 104 and 105, with an affinity of 13 μM [83].

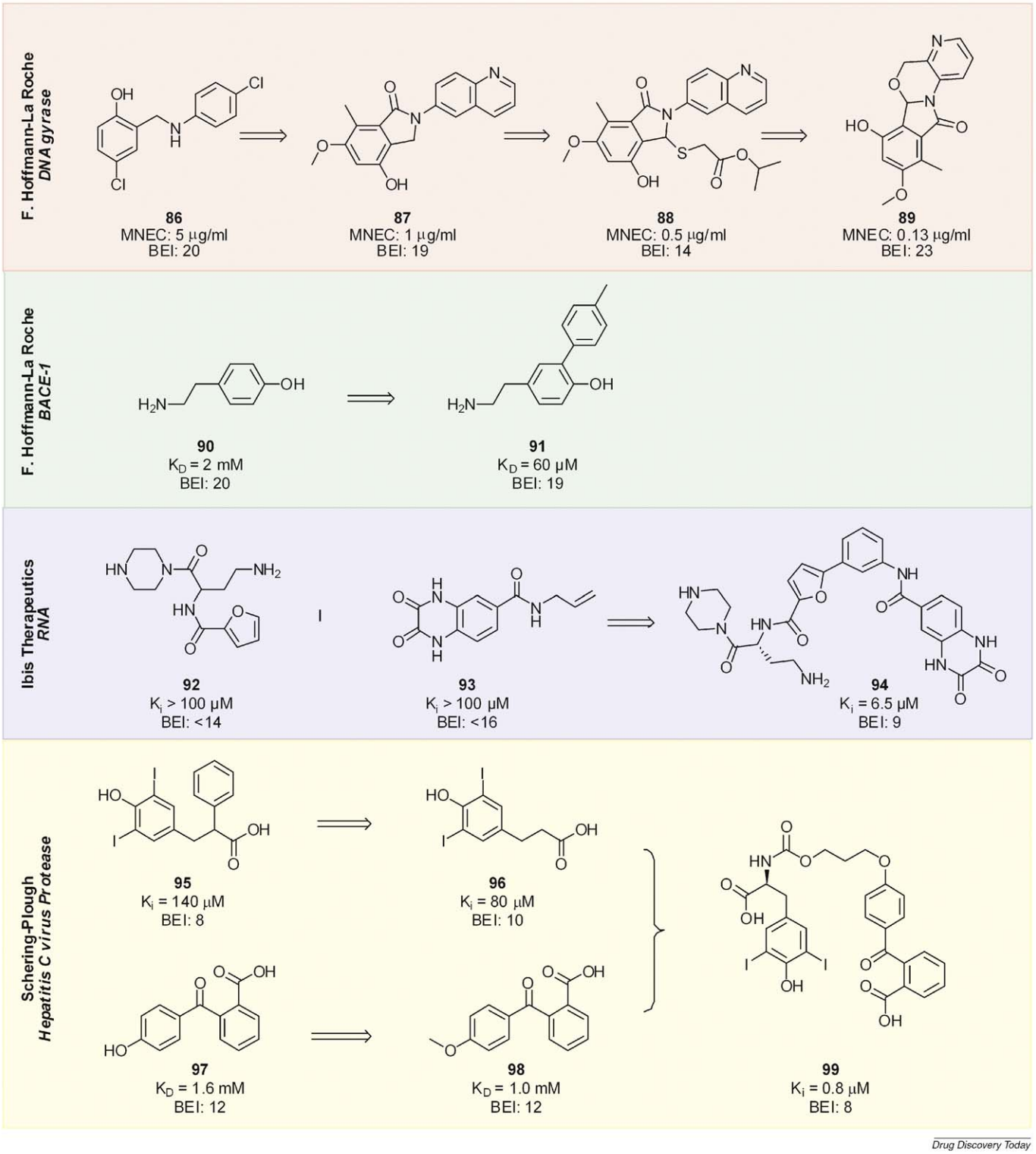

FIGURE 6

Representative fragment-based medicinal chemistry programmes at F. Hoffmann-La Roche, Ibis Therapeutics and Schering-Plough.

Wyeth Pharmaceuticals

Wyeth, a globally operating pharmaceutical company, has used FBDD to approach the inhibition of protein–protein interactions.

ZipA/FtsZ complex

Inhibitors of the binding of FtsZ to ZipA, a membrane anchored protein in *E. coli*, have potential therapeutical value as antibacterial agents. At Wyeth, the C-terminal domain of ZipA, responsible

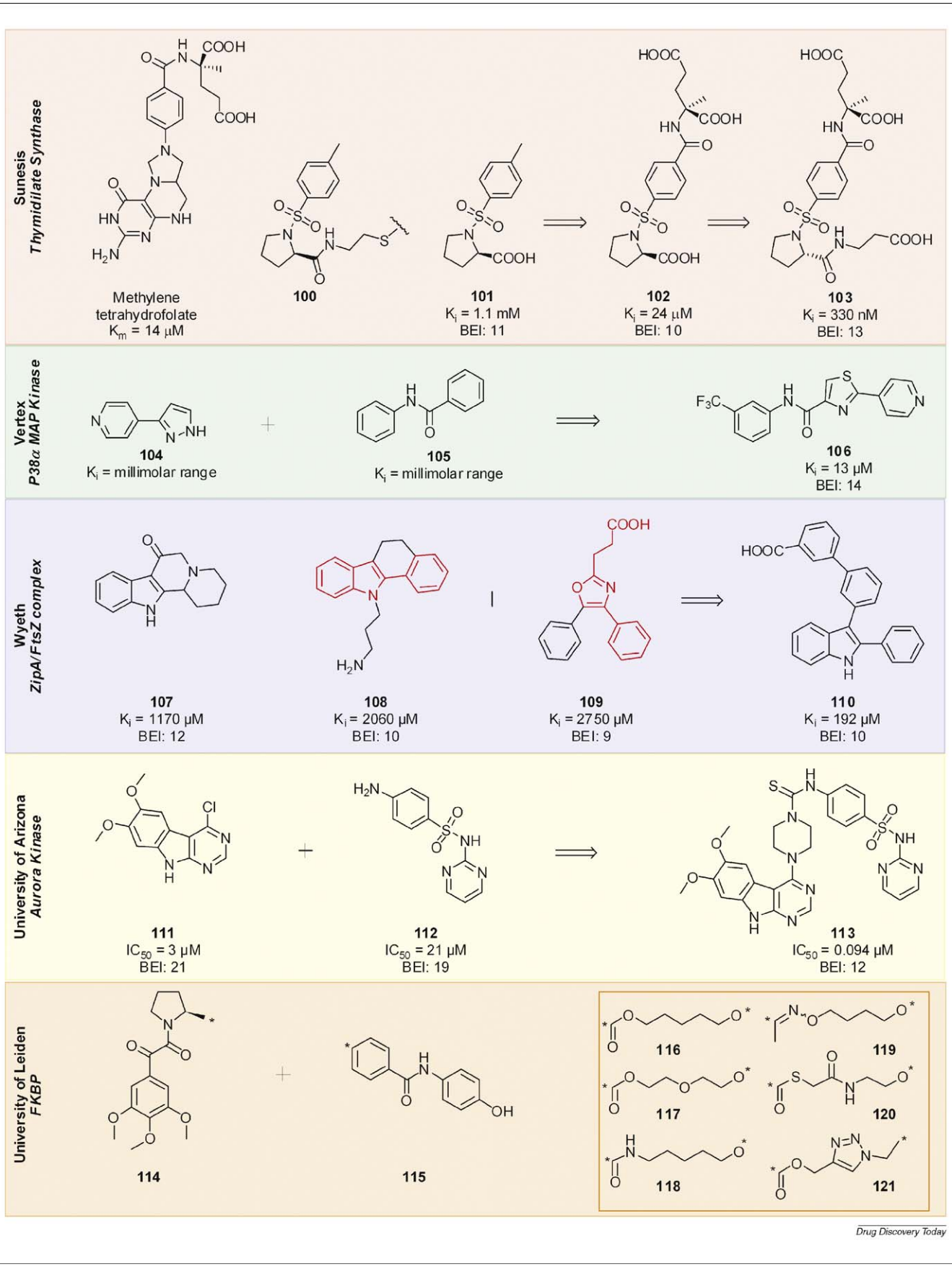

FIGURE 7

Representative fragment-based medicinal chemistry programmes at Sunesis, Vertex, Wyeth, Universities of Arizona and Leiden.

for the binding with FtsZ, was used in a 2D ^{1}H-^{15}N-NMR screening of 825 fragments. Seven structurally different hits were found, confirmed by fluorescence polarisation competition assays. For one of the hits, 107, a crystal structure with ZipA was obtained. Interactions were mainly hydrophobic, although a hydrogen bond with a conserved water molecule was also observed. About 40 analogues were screened in NMR and fluorescence assays. Six further hits were found, from which five crystal structures were obtained. One of these, fragment 108, was overlaid with the crystal structure of a known ZipA inhibitor (109). Figure 7 shows in red the parts of the inhibitor that were used in the design of compound 110, the best inhibitor of this series [84,85].

University of Arizona

Since FBDD is design-intensive rather than resource-intensive when compared to high-throughput screening, it is an ideal technique for application in academic medicinal chemistry groups, although few academic groups have so far published work on FBDD. One academic publication is described in the next subsection.

Aurora kinase inhibitors

In the search for inhibitors of Aurora kinase A and B, the *de novo* design algorithm LUDI was used to generate suitable fragments *in silico* that were subsequently docked into protein models using Glide. Fragment 111 and three others were predicted to bind at the ATP binding site, while fragment 112 and two others were found for the phosphate-binding region. In total six fragments were synthesised and tested. Fragments 111 and 112 were linked with a piperazine moiety, a known tyrosine kinase inhibitor substructure. This resulted in compound 113 with low micromolar affinity as a starting point for lead optimisation efforts [86]. This study shows how efficient FBDD can be—only seven compounds were screened.

Leiden University

At Leiden University, an NMR-based fragment screening method has been developed in which the target protein is immobilised in a flow cell inside the NMR spectrometer. This technology is being commercialised by the university spin-out ZoBio.

FK506-binding protein

Two compounds (114 and 115), which were known in the literature to bind in distinct binding sites of FK506-binding protein (FKBP), were used to explore the effect of the linkers on the binding of the linked fragments. For this, linkers 116–121 were used. The affinity of the resulting products was compared with the theoretical value of linking two fragments: the multiplication of the binding affinities of the non-linked fragments. Clear differences in binding affinities were found. Linker 116 gave the highest affinity, but still 170 times less than theoretically possible; the drop in affinity of 117 indicates that a more hydrophobic character is needed. Linker 118 has a hydrogen bond donating atom, while theoretically an acceptor atom is needed. The linkers 119 and 121 gave bad results, owing to the incorrect positioning of both fragments and, in the case of 121, a sterically too demanding linker. Surprisingly, linker 120, while constrained, gave comparable results to linker 116. Docking studies revealed that the amide of 120 may form an extra hydrogen bond.

Beyond describing the importance of the chemical nature of a linker, chemistries for rapid introduction of various linkers was also described [23].

Conclusion

The first medicinal chemistry study acknowledging FBDD as an approach was published as long ago as 1997 [24]. It has taken a decade for FBDD strategies to impact medicinal chemistry and the drug discovery process. The wide variety of contexts in which FBDD is now being used (SAR-by-NMR, HTX, scaffold-hopping, selectivity mapping) illustrates its practical utility in main stream medicinal chemistry. FBDD's recent successes, some of which are described in this review, indicate that use of this design intensive drug discovery approach is bearing fruit, promising to become the major driver for future medchem-based discovery approaches.

On a lab-by-lab basis, it is interesting to note that the adoption of FBDD now encompasses the largest of pharma and the smallest of biotech companies. This universality has been catalysed by the low cost and widespread availability of fragment collections, with many researchers developing their own tools in the area. New tools, such as SPR, are beginning to impact the FBDD process. Indeed, the great attraction of FBDD lies in its flexibility—it can incorporate the simplest of biochemical approaches, as well as the most sophisticated NMR-based and X-ray-based platforms.

One trend that can be observed is the use of orthogonal screening approaches. Companies originally specialising in one particular screening technology (e.g. Astex for X-ray-screening and Abbott for NMR-screening) now routinely implement additional primary and secondary screens using a variety of biochemical or biophysical techniques. An interesting and useful toolbox is now evolving, available to scientists in both industry and in academia, giving direction to medicinal chemisty efforts.

The impact of FBDD has been greatly accelerated by the deployment of rapid iterative structure-determination: the synergies between FBDD, protein structure determination and rapid chemical synthesis accounts for FBDD's efficiency and productivity, positioning fragment-based approaches at the very heart of today's medicinal chemistry programmes.

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