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In silico fragment-based drug design with SEED

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ABSTRACT

We report on two fragment-based drug design protocols, SEED2XR and ALTA, which start by high-throughput docking. SEED2XR is a two-stage protocol for fragment-based drug design. The first stage is *in silico* and consists of the automatic docking of 10^3-10^4 fragments using SEED, which requires about 1 s per fragment. SEED is a docking software developed specifically for fragment docking and binding energy evaluation by a force field with implicit solvent. In the second stage of SEED2XR, the 10-10^2 fragments with the most favorable predicted binding energies are validated by protein X-ray crystallography. The recent applications of SEED2XR to bromodomains demonstrate that the whole SEED2XR protocol can be carried out in about a week of working time, with hit rates ranging from 10% to 40%. Information on fragment-target interactions generated by the SEED2XR protocol or directly from SEED docking has been used for the discovery of hundreds of hits. ALTA is a computational protocol for screening which identifies candidate ligands that preserve the interactions between the optimal SEED fragments and the protein target. Medicinal chemistry optimization of ligands predicted by ALTA has resulted in pre-clinical candidates for protein kinases and bromodomains. The high-throughput, very low cost, sustainability, and high hit rate of the SEED-based protocols, unreachable by purely experimental techniques, make them perfectly suitable for both academic and industrial drug discovery research.

1. Introduction

The development of novel ligands for physiologically relevant targets can be a difficult task. Fragment-based drug design (FBDD) is a strategy for rational drug development. FBDD has shown the potential to reach higher success rates and higher efficiency (time- and resource-wise) than traditional high-throughput screening driven drug discovery, although both techniques can be complementary [1–4]. The discovery of novel chemical matter with FBDD benefits from the much larger chemical space covered by a library of fragments (small molecules with less than 20 non-hydrogen atoms) than a similar-size collection of larger molecules. The process starts from the discovery of small fragment hits, the properties of which are finely tuned to grow the molecule into a desirable lead compound. The biophysical toolkit of FBDD comprises fluorescence-based thermal shift, 1D/2D NMR (nuclear magnetic resonance), mass spectrometry, surface plasmon resonance, isothermal titration calorimetry, X-ray crystallography, and *in silico* screening [3,5]. Of particular note, X-ray crystallography brings some of the most crucial information for the progression of a FBDD project with the precise description of the binding mode of hits in the binding pockets and potential fragment-growing axis, but is low-to medium-throughput in spite of advances in automation software and robotics [6,7]. High-throughput *in silico* screening, on the other hand is an efficient tool for discovering protein binders at almost no cost, but produces false positives and needs constant experimental validation [8,9]. Molecular docking and X-ray crystallography are complementary methods for kick-starting a FBDD project and also very useful for the subsequent FBDD hits optimization campaign. Fragment docking methods seem particularly powerful for the identification of initiating hits for a FBDD optimization program: fragment based virtual screening campaigns can be performed within hours on a desktop computer and produce hits rates in crystallography validation experiments reaching 10%–40% [10–13].

We review here the high-throughput docking campaigns carried out by the software SEED (solvation energy for exhaustive docking) in the context of FBDD, starting with its integration in an efficient protocol for the identification of small and mainly rigid fragments that bind to a target protein, which we call SEED2XR (SEED docking followed by X-ray crystallography). First, the high-throughput docking of 10^3-10^4 fragments is carried out by SEED

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[14,15], a program specifically developed for fragment docking, which requires about 1 s of computational time per fragment. SEED samples exhaustively all positions of the fragment around a user-defined binding site and relies on a transferable force field with a generalized-Born description of solvation effects. Then, the 10-10^2 fragments with the most favorable predicted binding energies are tested in protein X-ray crystallography experiments [10,11]. If a reproducible protocol of production of soakable crystals has been established, the validation step is very efficient in confirming the binding pose of the hits to give insights into optimization strategies. We will also discuss other uses of SEED, potentially ignoring the crystallography step, such as the ALTA (anchor-based library tailoring approach) protocol, in which the docking information has been directly used for the discovery of potent binders [16–27]. ALTA has also guided the medicinal chemistry optimization of fragment hits into selective, nanomolar pre-clinical candidates for protein kinases [28,29] and bromodomains [30,31]. Finally, we discuss the main advantages and limitations of SEED and the SEED2XR protocol, and review other computational tools for fragment docking. It emerges that a very limited set of docking programs have been developed for the specific task of identifying fragment binders. Tools with an energy evaluation based on a transferable force field with (implicit) solvation (*e.g.,* SEED) may deliver the highest enrichment rates in fragment docking.

2. FBDD protocols: SEED2XR and ALTA

2.1. SEED2XR

In the first stage of the SEED2XR protocol (Fig. 1), the program SEED is used to dock and score the fragments in the target binding site. SEED performs an exhaustive search of all positions and orientations that result in optimal protein/fragment hydrogen bonds and/or lipophilic contacts (Fig. 2). The search space is limited to vectors generated around residues specified by the user. These vectors can be considered as a multitude of small pharmacophore points of the binding pocket with a directionality. Once the poses are generated, they are scored with a two-step approach based on a force field. First, a fast scoring scheme is applied to filter out unlikely poses. The evaluation of the binding energy of the fast scoring scheme relies on a simplified energy function, with pre-calculated potentials on a grid for van der Waals interactions and partial desolvation of the receptor and the ligand. The electrostatic interaction between the atoms of the fragment and the protein is calculated with a linear distance-dependent dielectric model. In a second step, the poses are clustered geometrically and the best binding energies in each cluster are rescored using a more complex scoring scheme. At this stage, the binding free energy is estimated as the sum of the van der Waals interaction and the electrostatic energy including desolvation penalties. The van der Waals interaction is computed between the fragment and an automatically detected neighbor list of protein atoms. The electrostatic desolvation of the protein is calculated by integrating the square of the electric displacement vector over the volume occupied by the fragment. The fragment desolvation and the screened fragment-receptor interaction are calculated with the generalized-Born formula [15]. SEED uses the CHARMM36 force field [32] for the protein and CGenFF [33,34] for the fragment, which are fully consistent as they were parametrized with the same paradigm and protocol. SEED has a very good predictive power with success ratio (*i.e.*, percentage of true binders in a set of experimentally tested molecules) that ranges between 10% and 40%, and predicted binding poses generally confirmed by X-ray structures [10–13]. Moreover, it is computationally very efficient as a library of 10^4 fragments can be screened in a few hours on a desktop computer. The computational time depends on the number of user-specified residues set as definition of the binding pocket. To give an example, the docking of a fragment in a bromodomain, with two to eight residues specified as docking anchors takes about 1 s [10,11,24].

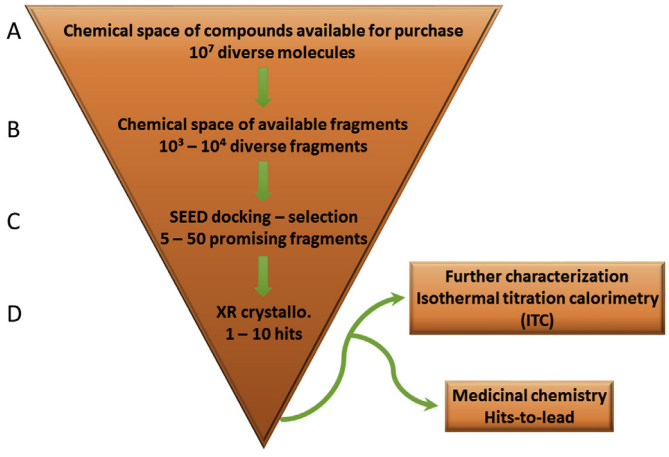

Fig. 1. SEED2XR protocol. (A) Commercially available chemical libraries usually contain up to tens of millions of molecules. (B) The size of a fragments library is much smaller, from thousands to tens of thousands. Yet, it contains most of the chemical diversity of larger molecules libraries. (C) SEED docking of the fragment library takes only few hours and leads to the selection of a small set of compounds to test. (D) X-ray crystallography experiments are carried out only on a very limited set of fragments from SEED, with reported hit rates from 10% to 40%. These hits can be further characterized by binding assay, in particular ITC experiments, which provide information on thermodynamics of binding. They serve as starting points for hits-to-lead medicinal chemistry optimization.

The fragments with the most favorable predicted binding energy are then selected and soaked into crystals of the protein if the binding pocket of the apo target is not occluded by crystal contacts (Fig. 1). Alternatively, co-crystals of the ligand-macromolecule complex can be grown. X-ray crystallography is used as a primary tool for the identification of the true binders in the set of selected compounds from docking. This technique is highly sensitive and can identify binders up to millimolar (mM) affinities. In addition, it brings important information for the follow-up of the project by confirming the binding pose, describing the contacts between the ligand and the protein, revealing possible rearrangements of the binding pocket, and giving insights into strategies for hits-to-lead optimization of the hit fragments. As an alternative to X-ray crystallography, NMR spectroscopy experiments can be considered [12,13]. Fragment hits are expected to bind with modest affinities in the range of high micromolar (μM) to mM. They serve as starting anchors for a fragment-based hit optimization medicinal chemistry program.

2.2. ALTA

In other projects, SEED has been integrated in a virtual screening platform called ALTA for high-throughput docking [24] (Fig. 3). The purpose of ALTA is to integrate the first steps of FBDD automatically in the *in silico* platform, *i.e.*, find interesting fragment hits betting on the predictive power of SEED and grow the molecule in order to improve the affinity of the fragment hits. The target is to reach the low μM range with desirable properties, such as high lipophilic efficiency (LLE) [35–37], halfway on the path of the design of selective low nanomolar (nM) ligands. Two main versions of ALTA have been developed, $ALTA_{v1}$ [23] and $ALTA_{v2}$ [24]. Both versions lay on a four-step protocol: (1) decomposition of a chemical library into predominantly rigid fragments, (2) rigid-fragment docking with SEED, (3) flexible docking of the parent compounds of the

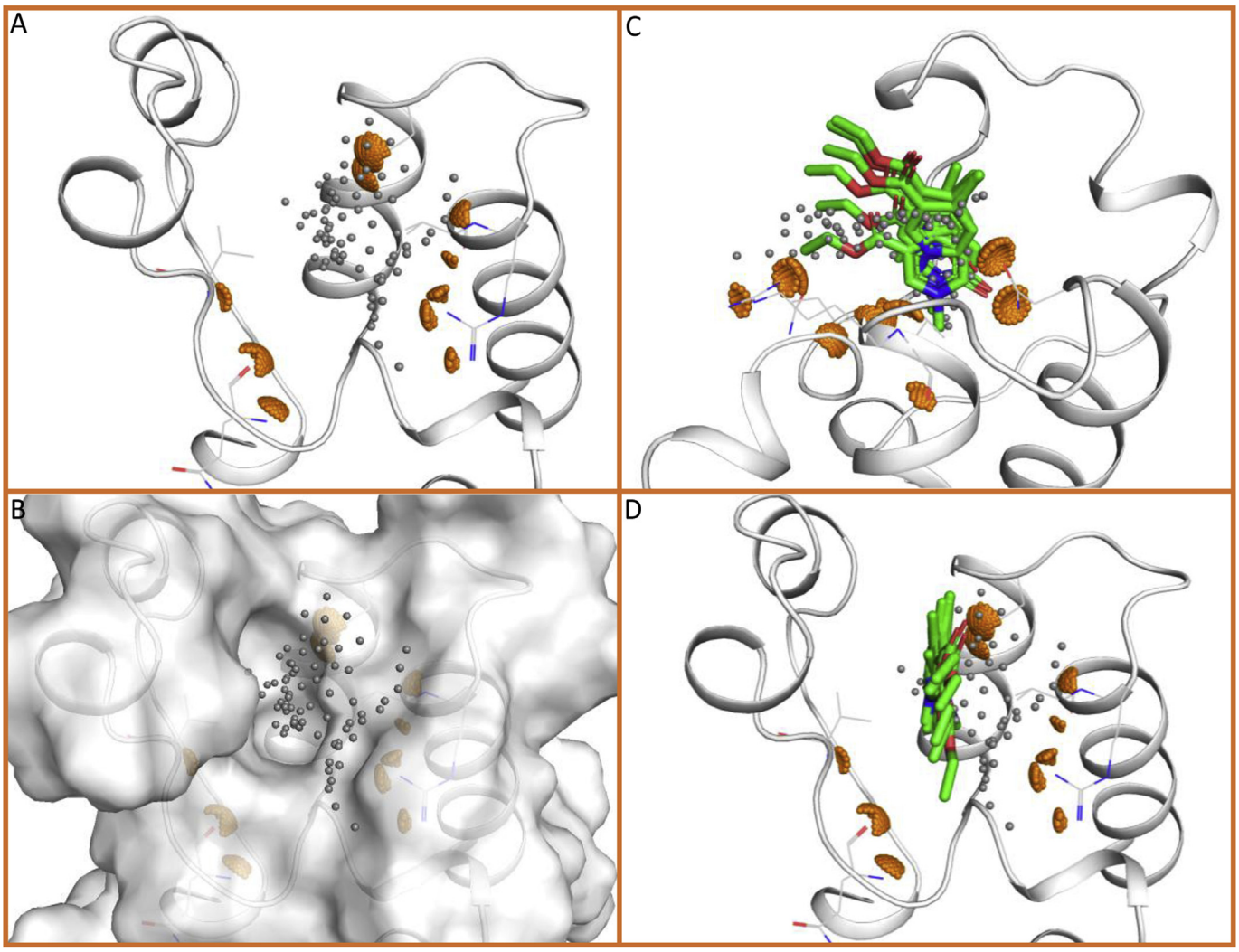

Fig. 2. SEED docking. (A) Polar (orange spheres) and apolar (grey spheres) pharmacophore points are generated around user-specified residues of the binding pocket (directionality not shown). They serve as anchor points for placing the fragment and performing the exhaustive search. (B) Same as A, with surface representation of the protein. (C) Example of a fragment docked in the pocket (carbon atoms in green). The six best poses according to the accurate energy model are displayed. (D) Same as C, top view. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

original library with anchored fragment from SEED data, and (4) rescoring. $ALTA_{v1}$ was introduced in 2005 and uses at least three fragments from SEED docking as binding site pharmacophores to guide the placement of the flexible molecules with FFLD (fragment-based flexible ligand docking), *i.e.,* the flexible docking affects only the conformation of the ligand since its placement in the binding site is determined by SEED [38]. The final rescoring step for the selection of molecules to test experimentally relies on a fitted scoring function called linear interaction energy with continuum electrostatics (LIECE) [39]. These two characteristics can be impractical for some targets. Three subpockets cannot be defined for all drug targets, in particular when the binding site is exposed, such as in the case of bromodomains and protein-protein interaction pockets in general. A fitted scoring function can be very performant in the case of targets for which enough true binders are known but is in general not transferable from protein to protein, in particular across families [39]. Thus, application of $ALTA_{v1}$ is not recommended for targets with less than about 10 known ligands. To circumvent those pitfalls, a modified ALTA version was designed recently, referred to here as $ALTA_{v2}$ [24]. Rigid fragments are docked with SEED and few head-groups are selected as anchoring points for a growing strategy, *i.e.,* $ALTA_{v2}$ uses only one major anchor point in the pocket, such as the mandatory interaction with the hinge of kinases or the conserved asparagine of bromodomains [10,11,24]. Once the top scoring fragments are selected, tethered flexible docking is performed keeping the anchor in place. The rest of the molecule is free to be placed anywhere around the binding pocket, without pre-assigned positions. The final scoring is based on a force field with a finite-difference Poisson Boltzmann treatment of desolvation effects, without fitting parameters for the target, which makes it transferable and applicable to targets without known binders [24]. The two versions of ALTA are somewhat complementary as they are most adequate for targets with known ligands or completely new targets. The same can be said about SEED2XR and ALTA, which are complementary as they are appropriate for mainly rigid fragments and flexible molecules, respectively.

3. Applications of SEED in FBDD

The fragment docking program SEED has been used since the early 2000s to discover fragment binders of human bromodomains, kinases, proteases; viral proteases; parasite proteases; and bacterial

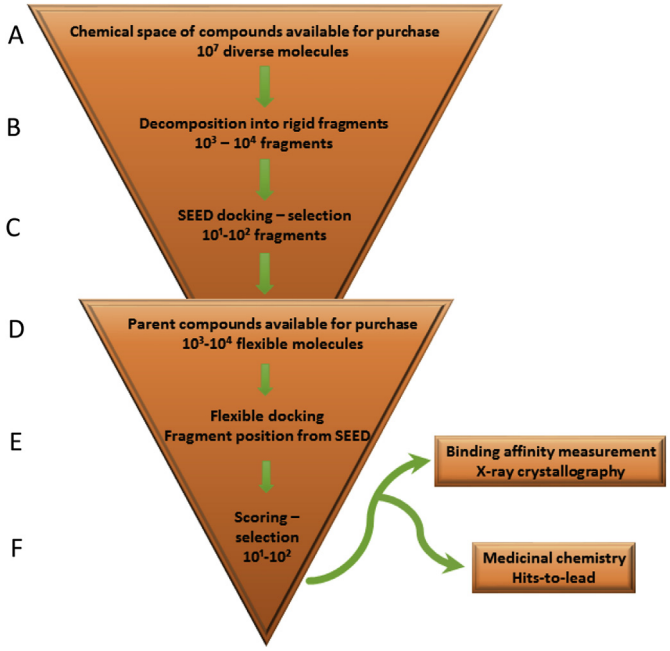

Fig. 3. ALTA protocol. (A–C) These steps are very similar to SEED2XR and consist of the preparation of a fragment library and docking with SEED. (B) In ALTA, the fragment library consists of virtual fragments from A, *i. e,* all rotatable bonds are cut and the library is deduplicated. (C) Fragments are docked with SEED and selected based on their calculated binding energies. The position of the fragments is kept as pharmacophoric restraint for the following steps. (D) The compounds from A that contain the fragments selected in C, so-called parent compounds, are retrieved. (E) Flexible docking of the parent compounds is performed with pharmacophoric constraints consisting of the position of the original fragments as docked with SEED (tethered docking). (F) Final rescoring and selection of compounds to test *in vitro*. The binding affinity is measured and eventually X-ray crystallography validates the binding poses.

dehydroquinases (Table 1). More than a hundred protein binders have been discovered in the period 2005–2017, with hit rates routinely in the double-digit points of percentage and an overall 15% average hit rate across 21 projects.

3.1. SEED2XR protocol

Recently, four reports of the straight application of the SEED2XR protocol to bromodomains were published [10–13] (Table 2). Bromodomains are relatively recent targets for drug discovery and important efforts are made in order to develop potent selective inhibitors that could be promising drugs for the treatment of diverse cancers and other pathologies in the clinics [41–44]. Interestingly, the 28 clinical trials of 17 different bromodomain inhibitors all target the same subset of bromodomains, the BET subfamily [44,45]. Significant interest lays in the development of non-BET bromodomain inhibitors [46]. The four recent applications of SEED2XR have led to the discovery of fragment inhibitors of the bromodomains of BAZ2A, BAZ2B, BRPF1, and CREBBP, respectively, all outside of the BET subfamily (Table 2). Importantly, these projects yielded successful results within only few days equivalent of active work time. This is expected to be the case for any protein for which a crystal structure is available, as well as the knowledge of a reproducible production protocol of protein-ligand crystals.

In a first project, fourteen small fragment hits of the BRPF1 bromodomain belonging to five different chemical series have been identified by the SEED2XR protocol [11] (Fig. 4A). At the time of the study, only two holo crystal structures of the complex BRPF1 bromodomain–ligand were known, with both ligands from the same chemical series. The identification of new binders with novel chemotypes informs on specific bromodomain–ligand contacts for the development of potent BRPF1 inhibitors. The screening of an original library of 24 133 molecules with SEED required less than 7 h on one core of a desktop computer. Thirteen molecules were

Table 1
High-throughput docking campaigns by SEED in the group of the last author.[A] Protocols such as $ALTA_{v1}$-LIECE, $ALTA_{v2}$, and SEED2XR are explained in the FBDD PROTOCOLS section. $ALTA_{v1.5}$-SEED refers to a project that does not fit in the description of either of the two ALTA versions as flexible docking was performed without anchoring of the selected SEED fragments. Correct position of the (unique) fragment anchor was checked as post processing of the poses, and final rescoring was done with SEED energies. $ALTA_{v1}$-filt. corresponds to a procedure in which the final rescoring was done by iterative steps of filtering and ranking with different energy terms, rather than a LIECE model. $ALTA_{v1}$-cons. presents a final rescoring with a consensus score of different energy terms.[B] Numbers of hits, with the number of purchased molecules in parenthesis.[C] The binding affinity of the most potent ligand identified in the campaign. These data are difficult to compare directly as they include IC_{50} and K_d values, and they were measured with very different experimental techniques, *e.g.* AlphaScreen, ITC, enzymatic assay. XR = no binding affinity was measured and binding was validated only by X-ray crystallography.

ORGANISM	PROTEIN FAMILY	PROTEIN	PROTOCOL[A]	N HITS (PURCH.)[B]	%HIT RATE	BEST HIT μM[C]	LIBRARY SIZE	REF.	YEAR
HUMAN	Bromodomains	ATAD2	$ALTA_{v2}$	19 (142)	13	23	$8{\cdot}10^4$	[24]	2017
		BAZ2A	SEED2XR	4 (20)	20	51	$1{\cdot}10^3$	[12]	2017
		BAZ2B	SEED2XR	4 (12)	33	XR	$4{\cdot}10^2$	[10]	2016
			$ALTA_{v2}$	2 (25)	8	6	$8{\cdot}10^4$	[24]	2017
		BRD4(1)	$ALTA_{v2}$	3 (38)	8	22	$8{\cdot}10^4$	[24]	2017
			SEED	1 (1)	NA	7	1	[40]	2016
		BRPF1	SEED2XR	5 (13)	39	21	$2{\cdot}10^4$	[11]	2016
		CREBBP	$ALTA_{v1.5}$-SEED	2 (17)	12	13	$2{\cdot}10^6$	[27]	2016
			$ALTA_{v2}$	2 (25)	8	55	$8{\cdot}10^4$	[24]	2017
			SEED2XR	4 (39)	10	4	$1{\cdot}10^3$	[13]	2017
	Kinases	EphB4	$ALTA_{v1}$-LIECE	3 (43)	7	2	$4{\cdot}10^4$	[22]	2008
			$ALTA_{v1}$-LIECE	8 (43)	19	1	$2{\cdot}10^4$	[23]	2008
		CDK2	$ALTA_{v1}$-LIECE	1 (30)	3	8	$4{\cdot}10^4$	[22]	2008
	Proteases	BACE1	$ALTA_{v1}$-LIECE	12 (72)	17	58	$5{\cdot}10^5$	[21]	2005
			$ALTA_{v1}$-LIECE	10 (88)	11	7	$3{\cdot}10^5$	[20]	2006
		Cathepsin-B	$ALTA_{v1}$-filt.	1 (29)	3	5	$5{\cdot}10^5$	[26]	2008
		Caspase-3	$ALTA_{v1}$-LIECE	3 (21)	14	XR	$7{\cdot}10^2$	[19]	2011
VIRUS	Proteases	NS3	ALTA $_{v1}$-LIECE	6 (22)	27	40	$1{\cdot}10^4$	[17]	2009
			ALTA $_{v1}$-filt.	2 (5)	40	15	$2{\cdot}10^4$	[16]	2009
PARASITE	Proteases	Plasmepsin	ALTA $_{v1}$-cons.	13 (59)	22	2	$4{\cdot}10^4$	[18]	2009
BACTERIUM	Dehydroquinase	Type II	ALTA $_{v1}$-LIECE	8 (9)	89	19	$2{\cdot}10^4$	[25]	2017

Table 2
Application of SEED2XR to bromodomains.[A] Central processing unit (CPU) time in minutes estimated on one core of a desktop computer.[B] Number of protein-ligand complexes obtained by X-ray crystallography. The number in parentheses denotes the number of fragments tested.

PROTEIN	LIBRARY SIZE	CPU TIME[A]	CRYSTAL STRUCTURES[B]	HIT RATE	REF.
BAZ2A	1413	24	4 (20)	20%	[12]
BAZ2B	350	6	4 (12)	33%	[10]
BRPF1	24133	402	5 (13)	38%	[11]
CREBBP	1413	24	4 (39)	10%	[13]

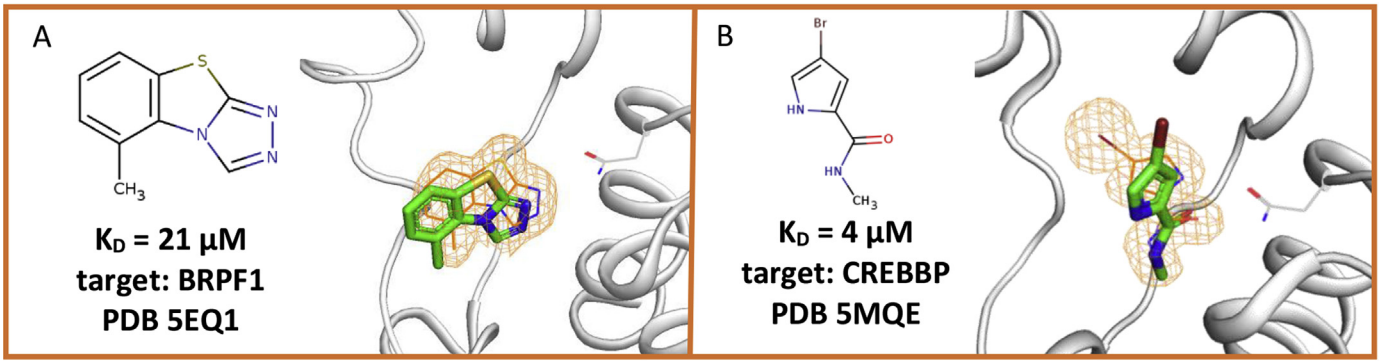

Fig. 4. Examples of fragment hits identified by SEED. (A) BRPF1 bromodomain. 2D structure and superimposition of predicted binding mode of compound **1** of reference [11] (carbon atoms in green) and binding mode in the crystal structure (orange). The $2F_0$-F_C map of the ligands is contoured at 1σ (orange mesh). (B) Same as A, with compound **1** of reference [13] in CREBBP. The docked pose mostly fits in the electron density, but the ring of the docked pose is flipped 180° compared to the crystal pose, a consequence of the rigid docking of only one conformation of a fragment that has two conformers. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

purchased from the 30 top ranking fragments and tested by soaking experiments in apo crystals of BRPF1. Five of the 13 tested molecules were found bound to the target, representing a hit rate of 38%. The five molecules belong to five different chemotypes. Nine additional molecules were further purchased based on analogy with these hits, all of them had unambiguous densities in the binding pocket of BRPF1, raising the total to fourteen BRPF1 binders originating from SEED docking (five chemical series). In addition, six further crystal structures were disclosed, with BRPF1 in complex with bromosporine (first bromodomain—bromosporine crystal structure) [47,48] and compounds analog to an acetylindole derivative published as a binder of another bromodomains [49]. Of note, the most potent binder among the hits discovered with SEED2XR had an affinity K_d of 21 μM to BRPF1 and consisted of only 13 non-hydrogen atoms. The interaction motifs between different chemical moieties and the bromodomain, as well as the analysis of the plasticity of the bromodomain has been used recently to develop selective BRPF1 inhibitors [50].

Another project focused on the bromodomain of BAZ2B, one of the least druggable member of the bromodomain family [10]. The same SEED2XR approach was used, although the initial chemical library for screening was much smaller than in the BRPF1 campaign as it consisted of only 350 fragments. As a consequence, the docking with SEED took only 6 min. Twelve fragments were selected for testing and four were found to bind BAZ2B, a success ratio of 33%. Again, apo crystals of the bromodomain could be produced reliably with a non-occluded binding pocket, and thus, soaking into apo crystal was used for X-ray crystallography. The four binders belong to four different scaffolds and informed on the potential displacement of conserved water molecules by fragment hits inside the binding pocket of the bromodomain of BAZ2B, a rarely occurring event [51]. The same procedure was applied to the bromodomain of BAZ2A with a library of 1413 fragments and led to the identification of four binders of this bromodomain, cognate to the one of BAZ2B [12]. The data on fragment hits for BAZ2A and BAZ2B was used for hit expansion [12,24,52]. The bromodomains of BAZ2A and BAZ2B are almost identical in terms of sequence. Their crystal structures in complex with diverse small molecules inhibitors discovered with the SEED2XR protocol and follow-up projects show minor differences that influence the binding mode of ligands. These differences are being investigated currently for the development of BAZ2B inhibitors that are selective against BAZ2A, or vice versa [53].

Finally, an application of the SEED2XR protocol to the bromodomain of CREBBP led to the discovery of four binders in a set of 39 molecules tested [13] (Fig. 4B). The success ratio of this project is the lowest of the cases presented here, 10%, but an interesting observation can be made. The strongest binder discovered with SEED2XR has a K_d of 4 μM to the protein and consists of only 10 non-hydrogen atoms.

3.2. ALTA protocol

SEED was frequently involved in ALTA virtual screening campaigns. ALTA is a purely *in silico* FBDD protocol based on SEED, which has led to the discovery of many low μM ligands, summarized in Table 1 [16–27]. Ligands discovered by ALTA are larger than those discovered by SEED2XR, making them harder to soak into apo crystals. On the other hand, they reach in general a higher affinity than fragments, making them easier to measure in high-throughput assays, such as FRET (Förster resonance energy transfer), AlphaScreen or thermal shift assays. Moreover, ALTA can lead to the proposition of several probable binders deriving from the same head group, which can give starting insights into a SAR (structure-activity relationship) analysis. When dealing with small rigid fragments, the energy function of SEED has a strong ability to identify binders and predict their binding poses [10–15]. The ALTA protocol uses the prediction of small rigid anchors from SEED to grow the fragments in the chemical space of commercial compounds [24]. The larger molecules are placed with a flexible docking approach, retaining the positions of the SEED anchors (Fig. 5).

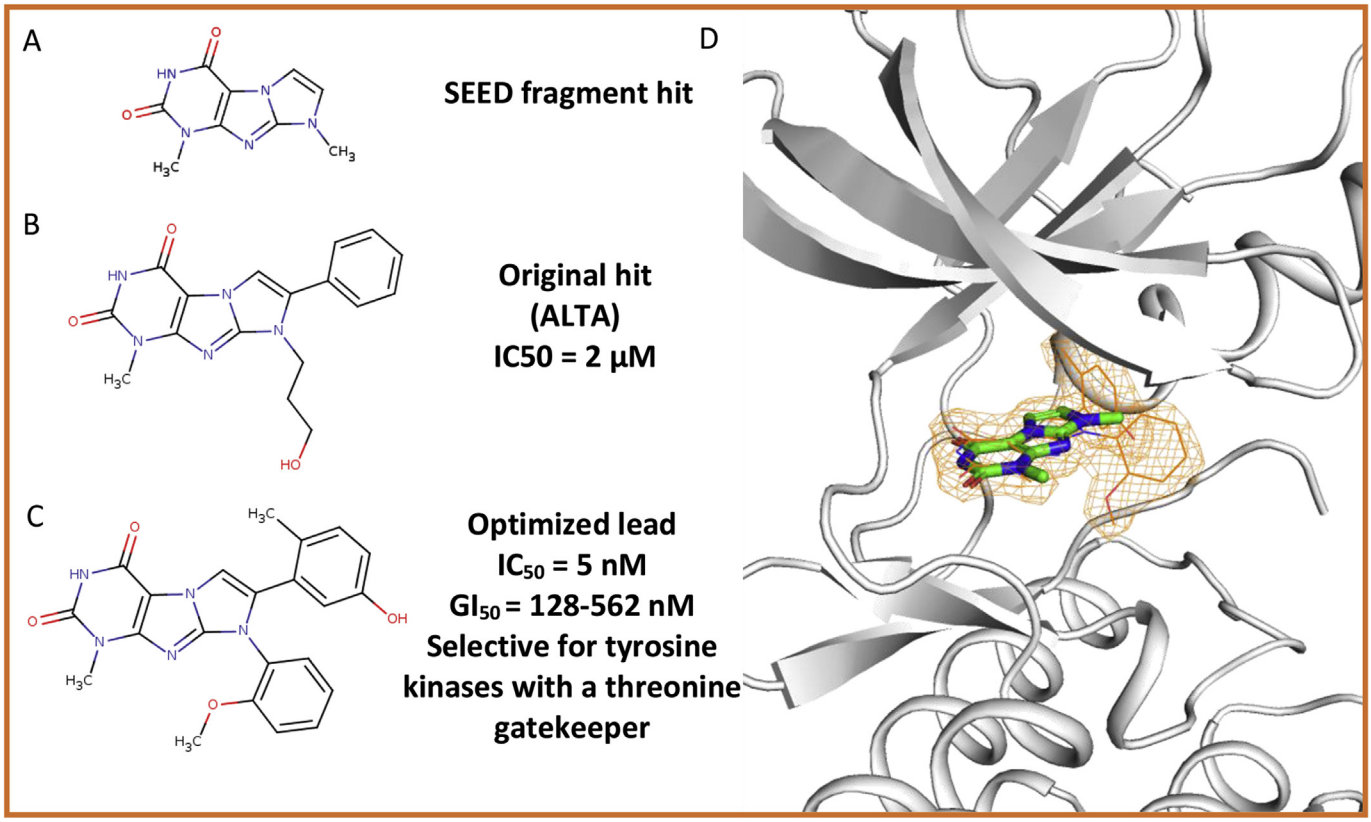

Fig. 5. Evolution of the fragment hit discovered with SEED for EphB4 [54]. (A) Original fragment hit positioned by SEED in the binding pocket in an ALTA protocol. (B) Hit discovered at the end of the ALTA virtual screening. IC_{50} measured in FRET-based enzymatic assays. (C) Lead issued from A, B, and a medicinal chemistry optimization program based on the structural analysis of the docking poses. The low nanomolar affinity was reached after the synthesis of 34 compounds (28 tested in binding experiments). (D) Comparison of the original fragment hit pose in SEED (carbon atoms in green) from A and the crystal structure of the optimized lead in C (orange, PDB code 4GK2). The $2F_0$-F_C map of the ligand is contoured at 1σ (orange mesh). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

The essential idea of ALTA is that a small-molecule ligand contains at least one fragment that perfectly fits in a sub-pocket of the binding site. About a hundred hits were discovered by the ALTA protocol in the period 2005–2017 (Table 1), targeting the binding pockets of the human proteases of β-secretase/BACE1 [20,21], cathepsin B [26], and caspase-3 [19], the West Nile Virus NS3 protease [16,17], the *P. falciparum* plasmepsin family proteases [18], the human kinases EphB4 [22,23] and CDK2 [22], the human bromodomains of CREBBP [24,27], BAZ2B [24], BRD4(1) [24], and ATAD2 [24], and the *Helicobacter pylori* type II dehydroquinase [25].

3.3. Medicinal chemistry optimization of SEED hits into preclinical candidates

Two medicinal chemistry projects based on initial hits from SEED led to potent and selective inhibitors of the kinase domain of EphB4 [28,29,49,54,55], which we will present here, and the bromodomain of CREBBP [30,31]. EphB4 is a receptor of the erythropoietin-producing human hepatocellular carcinoma pathway and has been linked to tumor-related angiogenesis [56]. The inhibition of EphB4 has been shown to reduce tumor growth in murine tumor xenograft models [57,58]. Starting from 11 µM inhibitors of EphB4 discovered in two ALTA campaign [22,23], a low nanomolar inhibitor of the Eph kinase family was developed [28] (Fig. 5). The best binder derived from a xanthine scaffold and reached an IC_{50} of 5 nM for EphB4. The optimization process was rationally designed with the help of structural data from SEED, and consisted of the synthesis of only 34 molecules (28 tested *in vitro*). Modifications of the original scaffold were suggested solely on the base of molecular docking and molecular modeling analysis. SAR analysis confirmed the *in silico* hypothesis in the absence of a crystal structure, which were only resolved later, giving a definitive validation of the binding mode [29]. The 28 derivatives of xanthine tested span a variety of affinities to EphB4, from low nanomolar to mid micromolar and few non binders. Eighteen of the inhibitors are type I kinase inhibitors, as the original hit. Type I inhibitors are defined such as they bind to the activated state of the kinase, in competition with ATP. The 10 remaining inhibitors are type $I_{½}$ inhibitors, which also bind to an active state of the kinase, but interact also with a hydrophobic back pocket, typical of type II inhibitors. Type II inhibitors bind to an inactive conformation of the kinase. The most potent inhibitors is a type $I_{½}$ inhibitor, with an IC_{50} of 5 nM for EphB4 in a FRET enzymatic assay and a cellular IC_{50} of 130 nM. The compound suffers from significant efflux by *P*-glycoprotein transporters, as observed on Caco-2 monolayer cell permeability experiments. The selectivity was assessed with a panel of 124 kinases. The compound was shown to have a good selectivity profile with a strong inhibition of a relatively small portion of the human kinome, and is less promiscuous than the approved drug Dasatinib [59]. Antiproliferative activity was assessed in the NCI-60 cancer cell line. Nanomolar growth inhibition (GI_{50}) values were described for central nervous system, leukemia and breast cancer cell lines [54], and very significant inhibition of angiogenesis in an *in vitro* matrigel angiogenesis assay [29]. Another series of inhibitors of EphB4 was synthetized, benefiting from the information of the xanthine scaffold SAR study [55]. Out of 24 derivatives of the new quinoxaline scaffold, three expressed favorable properties similar to the best xanthine lead.

Preclinical testing was extended to metabolic stability in human liver microsomes, with half-lives of the quinoxaline derivatives longer than 60 min, which compares favorably to approved human drugs. Ultimately, *in vivo* experiments were conducted on tumor xenograft mouse models, derived from an aggressive breast cancer cell line. Results showed inhibition of tumor growth by 80% over 21 days compared to the control mice, with a tolerable decrease of mean body weight [55].

4. Discussion

4.1. Limitations and workarounds

On the computational side, the main limitation of SEED-based FBDD protocols is the availability of a crystal structure of the target protein or a close homologous of it for the docking campaign. If a structure is not available at the beginning of the project and docking has to be performed concomitantly to the optimization of the crystallization protocol, a homology model can be used for docking, a strategy that already proved useful [23,60]. The best-case scenario is the availability of a holo structure of the protein. The structure of the target in complex with a small organic compound gives insights into potentially crucial protein/ligand contacts. It also guarantees a conformation of the binding pocket to be used for docking that can accommodate a ligand. If only an apo structure is available, the preparation of the target for docking is critical. A strategy to generate holo-like conformations of the apo protein is to run molecular dynamics with a small fragment in the binding pocket, such as the cosolvent DMSO [61]. In addition, empirical information of binding hotspots is crucial for improving the success of high-throughput docking campaign and in particular to define an adequate binding site for SEED [23,24]. Several test docking runs can be performed with a sample library of small size, in order to identify the most favorable binding subpockets of the target. Optionally, the stability of test fragments in their docked conformation can be investigated with molecular dynamics simulations [62,63]. The definition of the anchoring points for fragments in the binding pocket can be optimized upon results from crystallography. Another type of information that may be critical for certain systems is the presence of discrete structural water molecules in the binding pocket [64–66]. Bromodomains, presented as test cases in this review, contain a stable network of five to six structural water molecules that directly interact with most ligands [41]. SEED permits the use of explicit discrete water molecules, *e.g.*, the TIP3P water model [10–13,24,52]. An additional possible drawback of SEED is the rigid approximation of ligand docking. Both the ligand and the receptor are treated as rigid. This is consistent with the strategy of soaking in a crystal a small fragment: the protein is assumed to not rearrange massively and the ligand should be small enough so that it can enter the binding pocket. In the cases for which the fragments contain a limited amount of rotatable bonds, one can generate an ensemble of conformers for the studied fragment and run SEED on all the conformers, or run an ALTA screening campaign. Finally, the calculation of the desolvation of the protein is computed with a method that is valid under the assumption that the electric displacement of the protein does not change significantly upon fragment docking [14]. This assumption is not valid for large molecules (more than about 20 heavy atoms), and/or if the ligand interacts with a cluster of charges. Scoring of charged fragments requires special care and we have proposed to rank them separately [24] or using more accurate models, *e.g.*, semiempirical quantum mechanics calculations [67]. Free energies of solvation of charged species are typically an order of magnitude larger than the ones of equivalent neutral compounds [68]. Therefore, scoring charged compounds with implicit solvent models is challenging. The energy values involved are on a different scale than neutral compounds, potentially ending up with an incorrect ranking of charged molecules among a set of neutral compounds. Ranking separately compounds according to their charge status is an easy workaround, since the energies are comparable among a charge group. Limitations and advantages of the ALTA protocol were discussed in Ref. [24].

On the experimental side, the protein production and crystallization are important bottlenecks, particularly for proteins that are recalcitrant to expression in *E. coli* and proteins for which crystallization is difficult or leads to non-soakable crystals. These points depend heavily on the protein of interest and their discussion falls beyond the scope of this review. In short, robust, stable crystals under soaking experiments need to diffract below 2.5 Å in order to place unambiguously fragments in the electron density [6,69]. Modifications of the protein target can help to optimize crystal growth. These modifications include the removal of flexible loops, terminal regions or post-translational modifications to decrease the number of regions that frustrate the formation of crystal contacts [70,71]; protein engineering, *e.g.* mutations of hydrophobic residues to increase solubility or of cysteine residues to reduce reactivity [72,73]. More details can be found in the literature [6,7,74–77]. On the fragment side, some pitfalls have to be avoided. As an example, soaking cocktails are not appropriate for fragments that would compete for binding, in particular in the case of highly enriched libraries such as those originating from SEED. The solubility of the fragments is important as the concentration can be very high in crystal soak buffers (*e.g.,* 10 mM). Another potential issue is the use of cosolvents, *e.g.* DMSO or MPD, which may interact directly with the target [10,78]. In addition, some molecules are refractive to soaking and need to be co-crystallized with the protein, which triggers additional work [11]. In non-optimal conditions, the throughput of the experimental validation by X-ray crystallography can be low. However, this point highlights again the usefulness of a protocol such as SEED2XR, in which the computational screening and selection of only few molecules (*e.g.*, 5 to 20 fragments) reduces the time required for X-ray crystallography experiments. Orthogonal experimental assays are useful to rule out false positives/negatives.

4.2. Advantages

Fragment-based lead discovery is usually more successful than high-throughput screening of large libraries of compounds, particularly for protein targets that crystallize easily [3]. The key strength of the SEED2XR protocol is the initial filtering of the chemical library by docking which, for fragments, is very rapid and accurate. SEED is a docking tool developed for screening fragments and has been now successfully used for almost 20 years [14]. Many improvements have been added since the original release and will be the subject of a technical paper in the next months. The advantages of SEED for docking fragments over other docking software are numerous. First, SEED performs exhaustive search of all the possible fragment positions around predefined residues in the binding pocket. Compared to stochastic search algorithm implemented in most docking software, the exhaustive search avoids missing binding poses because they were not sampled (provided that the binding pocket is defined accordingly). Second, SEED relies on a continuum description of solvation effects, based on a generalized-Born approximation. The accurate treatment of solvation is crucial to decrease the number of false positive in a docking run [22]. Third, docking fragments with a few functional groups is a much less complex task than docking a large flexible molecule with a high number of pharmacophoric characteristics. This latter point is not restricted to SEED, but the combination of the exhaustive

search, the placement of fragments' features on complementary pharmacophores of the protein, and the evaluation of the binding energy with a continuum treatment of electrostatics make SEED perfectly adequate and efficient for this task.

SEED is a FBDD *in silico* tool which was originally published in 1999 [14,15]. SEED contributed to the discovery of inhibitors of multiple protein targets, *viz*., the human proteases of β-secretase/BACE1 [20,21], cathepsin B [26], and caspase-3 [19], the West Nile Virus NS3 protease [16,17], the *P. falciparum* plasmepsin family proteases [18], the human kinases EphB4 [22,23,28,29] and CDK2 [22], the human bromodomains of CREBBP [13,24,27,30], BAZ2B [10,24], BRPF1 [11], BRD4(1) [24,40], BAZ2A [12], and ATAD2 [24], and the *Helicobacter pylori* type II dehydroquinase [25] (Table 1). This listing presents two notable properties. First, SEED has been applied to targets of diverse organisms, *i.e.,* viral, protozoan parasite, bacterial, and human proteins. Then, SEED produced results for a wide range of protein folds, such as for the large substrate binding-site of proteases, the mainly hydrophobic ATP binding-pockets of kinases, and the exposed binding pockets of bromodomains or a dehydroquinase. Moreover, docking involving SEED was performed on X-Ray structures, NMR structures, and homology models. This highlights the versatility of the docking software and its scoring function across protein families and structures.

4.3. Other fragment docking methods

Surprisingly very few programs have been developed for the specific task of fragment docking as a primary screening technique, when compared to the dozens of ligand docking tools [79,80]. In general, fragment docking projects rely on the use of conventional ligand docking tools, such as FlexX [81], Glide [82–84], S4MPLE [85], RosettaLigand [86], or GOLD [87,88], applied to chemical libraries of fragments [89]. While this strategy worked in some cases, one can question the general transferability of scoring functions trained on datasets containing mostly large lead-like ligands [80,90–93]. The SAMPL3 blind prediction docking challenge showed the difficulties in predicting binding affinities of small molecular fragments [94]. The use of a force field-based evaluation of the binding energy with an approximation of desolvation effects upon binding, as in the program SEED [14,15], was shown to frequently outperform other types of scoring functions for fragment docking [90,95–98], albeit higher predictive ability may be system and force field parameters dependent [82].

Automatic procedures for mapping a protein binding site with fragments or pseudo-fragments were pioneered by P.J. Goodford. GRID, originally published in 1985 [99–101], aims at placing non-atomic interactions spheres (pseudo fragments) on a grid representing the host. The interaction spheres are not molecular fragments *per se* but pseudo-groups of atoms that serve at finding the most favorable pharmacophoric interactions. This information can be used to place molecular fragments in the binding pocket with an external software, such as LUDI [102]. Of note, LUDI can be used as a standalone software and generate its own interaction map with a library of fragments [102]. MCSS [103] was the first force field-based computational protocol for determining optimal positions and orientations of small atomic groups such as acetonitrile, methanol, acetate, methane in the binding site of the target protein. Thousands of positions of the groups are placed randomly in the binding pocket, minimized, and the CHARMM energy is evaluated [103]. The poses of the most favorable groups are retained for *de novo* ligand design or optimization, with the help of other software, such as HOOK [104] or DLD [105]. Similarly, protein subunits, such as *N*-methylacetamide (for the backbone) or ethyl guanidinium (for the arginine side chain) can be used for peptide design [106]. Generally, these tools are also used to identify and characterize binding pocket hotspots. A new generation of software explicitly directed towards binding site characterization and based on the same principles was developed, *e.g.* FTSite [107] and SITEHOUND [108,109]. Following on the original ideas from MCSS and GRID, the flexible ligand docking algorithm implemented in 2001 in UCSF DOCK is an “anchor-and-grow” procedure [110]. The largest rigid substructure of the ligand is automatically identified and placed in the binding pocket. The remaining flexible portions of the ligand is built onto the best anchor orientations. This procedure is similar to the ALTA protocol [24]. Additional techniques that aim to place lead-like compounds with a preplacement of its fragments were published, *e.g.*, CAVEAT [111], LEGO [112], GroupBuild [113], SPROUT [114], SuperStar [115], CONFIRM [116], Recore [117], FTMap [118], Pingui [119], GANDI [120] (non exhaustive list). Interestingly, all of these fragment-based docking tools aim at discovering large lead-like molecules by the automatic placement of fragments and either a growing approach (from an anchor), a linking approach (between anchors), or a merging approach (fusion of anchors) [116,119,121]. Fragments are considered pharmacophoric features. These software are most likely directly applicable to the task of primary fragment screening, although such reports are scarce.

Specific challenges of fragment docking compared to lead-like ligand docking are (1) the relatively small energy difference between fragment binders and decoys [122], (2) the possible lack of transferability of scoring functions trained on lead-like compounds to small fragments [80,90–93], and (3) since fragments are simpler, a great wealth of diverse poses in a binding pocket are sterically accessible, leading to many false poses and possibly exhausting stochastic search algorithms (like those of most docking algorithm) before generating valid poses. Point 2 may be overcome with force field-based scoring functions and implicit solvent treatment of desolvation costs [90,95–98]. Importantly, transferable force fields are not fitted towards certain classes of ligands. Point 3 can be solved with exhaustive search algorithms, under the assumption that the scoring function is able to identify the correct poses and discard decoy poses. The only software encompassing these two characteristics, to our knowledge, are SEED [14,15] and UCSF DOCK [123]. In addition, eHiTS [124] is a fragment docking tool that possesses an exhaustive search algorithm, but relies on a knowledge based scoring function. A different approach is implemented in CrystalDock [125], which is a fragment docking tool that relies solely on the analysis of the PDB. Protein-ligands complexes from experimental sources are parsed to characterize molecular fragments and their protein microenvironment (up to 5 residues, 4 Å of distance). This information is stored in a database and used to identify and place fragments that are known to bind a query binding (sub-) pocket, *i.e.* a small set of residues in a certain orientation. The assumption is that this combination is present in the database. FragFEATURE [126] is another tool essentially similar to CrystalDock. The advantages of fragment docking over lead-like molecule docking are (1) since fragments are mostly rigid, the (quasi) absence of ligand entropy to take into consideration for the scoring function, (2) rigid docking approaches are possible and combinatorically less complex than flexible docking, and (3) fragments are less likely to modify the conformation of the binding pocket (induced fit).

In conclusion, the implication of computational techniques in FBDD has been mostly limited to chemical library compilation (not presented here), identification of binding pocket hotspots, and identification of lead-like molecules with automatic fragment anchor docking [127]. To our knowledge, the only tools that are explicitly directed towards fragment docking with the aim of discovering fragment binders (but not restricted to it) are UCSF DOCK [123], eHiTS [124], CrystalDock [125], FragFEATURE [126], and SEED [14,15]. More generally, fragment identification relies on

the use of lead-like ligand docking tools, of which the scoring function may not be well trained for this task [80,90–93]. The use of force field-based scoring function with estimation of desolvation costs seem to be the most appropriate way to obtain the highest enrichment rates in fragment docking [10,11,90,95–98]. Traditional ligand docking tools not based on transferable force fields may need to be extended with new scoring functions trained on sets of fragment-like small molecules. Extra care should be taken for the choice of the docking software and scoring function when running fragment docking campaigns.

5. Conclusions

We have reviewed SEED2XR and ALTA, two FBDD protocols that take advantage of the efficiency of SEED in screening fragments by high-throughput docking. These protocols for the protein structure-based identification of (fragment) hits are not only more efficient in terms of time invested and hit rate, but also more sustainable than purely *in vitro* fragment-based screening cascades in which the first filtering stage consists of differential scanning fluorimetry or the AlphaScreen assay [128]. Wet-lab consumables (compounds, buffers, etc.) are needed only for a fraction of the screened library, *i.e.*, about 10-10^2 fragments instead of 10^4-10^5 molecules. The examples provided show that the SEED2XR and ALTA protocols are robust irrespective of the protein target and initial size and composition of the chemical library. It is important to note that applications of SEED2XR and ALTA have resulted in about 200 crystal structures of ligand/protein complexes which have been deposited in the PDB since 2013 (more precisely about 150, 10, and 40 holo structures of bromodomains, tyrosine kinases, and other enzymes, respectively).

The fragment hits obtained by SEED2XR can readily serve as anchor head-groups for a medicinal chemistry project of hits optimization, in particular in combination with *in silico* fragment growing methods, *e.g.* ALTA virtual screening [24]. The potential users of the SEED2XR protocol are (computational) structural biologists, biochemists, and medicinal chemists. Of note, the recent applications of SEED2XR to bromodomains demonstrate that a single researcher can obtain structural information on novel chemical matter (*i.e.*, fragment hits and crystal structures of their complex with the target protein) in less than a week of working time [10,11]. The high-throughput, very low cost and high success ratios of the SEED2XR protocol, unreachable by purely experimental techniques, make it perfectly suitable for both academic and industrial drug discovery research. SEED is an open source software. The SEED source code, executable, test cases and documentation are freely available on gitlab (https://gitlab.com/CaflischLab/SEED).

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Abbreviations

FBDD	fragment-based drug design
NMR	nuclear magnetic resonance
SEED	solvation energy for exhaustive docking
SEED2XR	SEED to X-ray crystallography
ALTA	anchor-based library tailoring approach
FFLD	fragment-based flexible ligand docking
LIECE	linear interaction energy with continuum electrostatics
BET	bromodomain and extraterminal domain
BAZ2A	bromodomain adjacent to zinc finger domain 2A
BAZ2B	bromodomain adjacent to zinc finger domain 2B
BRFP1	bromodomain and PHD finger-containing protein 1
CREBBP	CREB-binding protein
EphB4	ephrin B4
SAR	structure activity relationship
FRET	Förster resonance energy transfer
DMSO	dimethyl sulfoxide
MPD	2-methyl-2,4-pentanediol
ITC	isothermal titration calorimetry

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