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Fragment linking strategies for structure-based drug design

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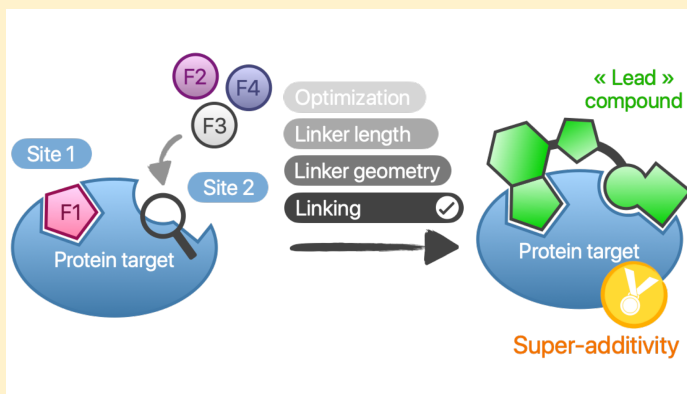
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ABSTRACT: Fragment-based drug discovery is a strategy widely used both in academia and pharmaceutical companies, to generate small-molecule protein inhibitors and drug candidates. Among the approaches



reported in the literature (growing, linking and merging), the linking approach theoretically offers the opportunity to rapidly gain in binding energy. Nevertheless, this approach is poorly represented when considering the compounds currently in clinical trials. Here, we report an exhaustive view of the cases published so far in the literature, together with the methods used to identify the two initial fragments either simultaneously or successively. We review the different types of linkers published and discuss how these linkers are designed to obtain the lead compound. Mixing merging and linking methods,

where the linker is duplicated from a known inhibitor appears as an interesting strategy. To reach super-additivity, we propose to grow one of the fragments in order to minimize the distance between the two binders, and then link the resulting compounds using flexible alkyl-derived linkers.

1. INTRODUCTION

Fragment-based drug discovery (FBDD) has been acknowledged in the past two decades as a powerful tool for rational design of drug leads.¹⁻⁶ The first step of the FBDD process consists in the identification of fragments that weakly bind the target protein, typically in the micromolar to millimolar affinity range. Fragments are defined as low molecular weight (< 300 g/mol) and highly soluble organic molecules. Jhoti and co-workers⁷ proposed a rule of three to describe the physicochemical properties of the fragments, by analogy to Lipinski's rule of five. Due to the weak affinity of the fragments for their targets, biophysical techniques sensitive towards micromolar to millimolar affinities are required to identify hits; among them Nuclear Magnetic Resonance (NMR), X-ray crystallography and Surface Plasmon Resonance (SPR) are the most widely used.^{1,2,3,5} In a second, iterative and time-consuming step, the fragments that form high quality interactions are optimized into lead compounds exhibiting higher affinity and selectivity, through the so-called fragment growing, fragment merging or fragment linking strategies. As we will see in the examples discussed below, fragment linking may include growing or merging steps and distinguish between the three strategies may be ambiguous. Vemurafenib,⁸ the first drug originating from a fragment screen, was approved in 2011 by the Food and Drug Administration (FDA), and two other drugs (venetoclax⁹ and erdafitinib¹⁰) were approved in 2016 and 2019, respectively. Vemurafenib and erdafitinib exemplify the growing approach, while venetoclax illustrates the linking method. To have an overview of the fragment-based development of therapeutic compounds, we report in Table 1 the 43 small-molecules currently in clinical trials (phase 1, 2 and 3) and the 3 FDA-approved compounds. We indicate the therapeutic target and the fragment-based strategy used. As reported in Table 1, most of the compounds in the clinic were obtained using the growing approach, where chemical groups are progressively added to the fragment hit to maximize the favorable interactions with the binding site residues. In some cases, fragment hits can be merged, as reported for

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3 the induced myeloid leukemia cell differentiation protein (MCL1) (Table 1). Here, we focus on the
4 fragment linking approach, where two fragments that bind at distinct protein pockets are linked together
5 to obtain a new compound. While this approach theoretically represents a very attractive methodology
6 to rapidly gain in binding affinity from the initial fragments, it faces two major challenges. First, the
7 observation of two fragments binding simultaneously at two distinct binding pockets of a protein is not
8 systematic. This is related to the non-equivalence of the corresponding hot-spots in the binding pockets.
9 As reported in Table 2, a second screening to identify fragments capable to bind in the second pocket
10 may be necessary. In addition, the fragments do not necessarily bind to the protein in suitable positions
11 for linking. The second challenge remains the design of a linker that will maintain the protein-fragment
12 interactions. Fesik and co-workers^{11,12} first successfully used the fragment linking methodology through
13 their Structure-Activity Relationships (SAR) by NMR approach (Figure 1), with the generation of
14 nanomolar compounds for the FK506-binding protein (FKBP), a prolyl isomerase belonging to the
15 immunophilin family. Due to its binding properties to the immunosuppressant molecule FK506, FKBP
16 is a target for neurodegenerative disorders.

17
18 In 2011, Whittaker and colleagues¹³ reviewed examples where the fragment linking approach was used
19 and focused their discussion on the super-additivity concept. The concept of super-additivity (also called
20 positive cooperativity) was defined by Jencks.¹⁴ It stipulates that the affinity of the linked compound
21 should be greater than the affinity resulting from the addition of the binding energies of the two
22 fragments. The super-additivity energetic term includes changes in translational and rotational entropy,
23 loss of binding energy due to the linker and change in binding orientation of the two fragments. When
24 the orientation of the fragments is not disturbed, changes in translational and rotational entropy
25 dominate. Whittaker and colleagues¹³ proposed that one key criterion to achieve super-additivity is to
26 maintain the binding modes of the initial fragments. To be successful, linking should therefore involve
27 one fragment that interacts with the protein through polar interactions and a second fragment that
28 interacts via van der Waals interactions, as the latter will be more tolerant to changes in binding mode.
29 This statement will be further discussed later in the perspective.

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31 Here we have reviewed, to the best of our knowledge, all cases where the fragment linking approach
32 has been used, through 2018. To our point of view, the first step that consists in the identification of two

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3 fragment hits is not straightforward. As we report below, two fragments bound in two distinct protein
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5 pockets were directly identified in 22 cases out of 39, while 17 cases required the conduction of two
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7 successive screens. Once two fragments have been identified, the main challenge is the design of the
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9 linker, in particular when the distance between the two fragments is large ($> 3\text{\AA}$). One possibility
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11 consists in reducing the distance between the two fragments. To do so, one of the two fragments is
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13 selected and a growing approach is applied until the distance is close to 3\AA . Regarding the chemical
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15 structure of the linker, simple linkers such as alkyl of different lengths are widely used. More
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17 sophisticated linkers include functional groups, which may be utilized to fit particular geometries such
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19 as 90° bends required to conserve the positions and orientations of the fragments. Such functionalized
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21 linkers also enable the addition of interactions between the designed compound and the protein.
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24 The article is organized as follows. First, we summarize the methods used for the identification of the
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26 two fragments that will be further linked. This includes successive identification as well as simultaneous
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28 identification of the two fragment hits. We also review the particular case of fragment dimerization.
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30 Then we discuss the second challenge of the linking process and analyze different types of linkers that
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32 have been designed to generate the new linked compounds.
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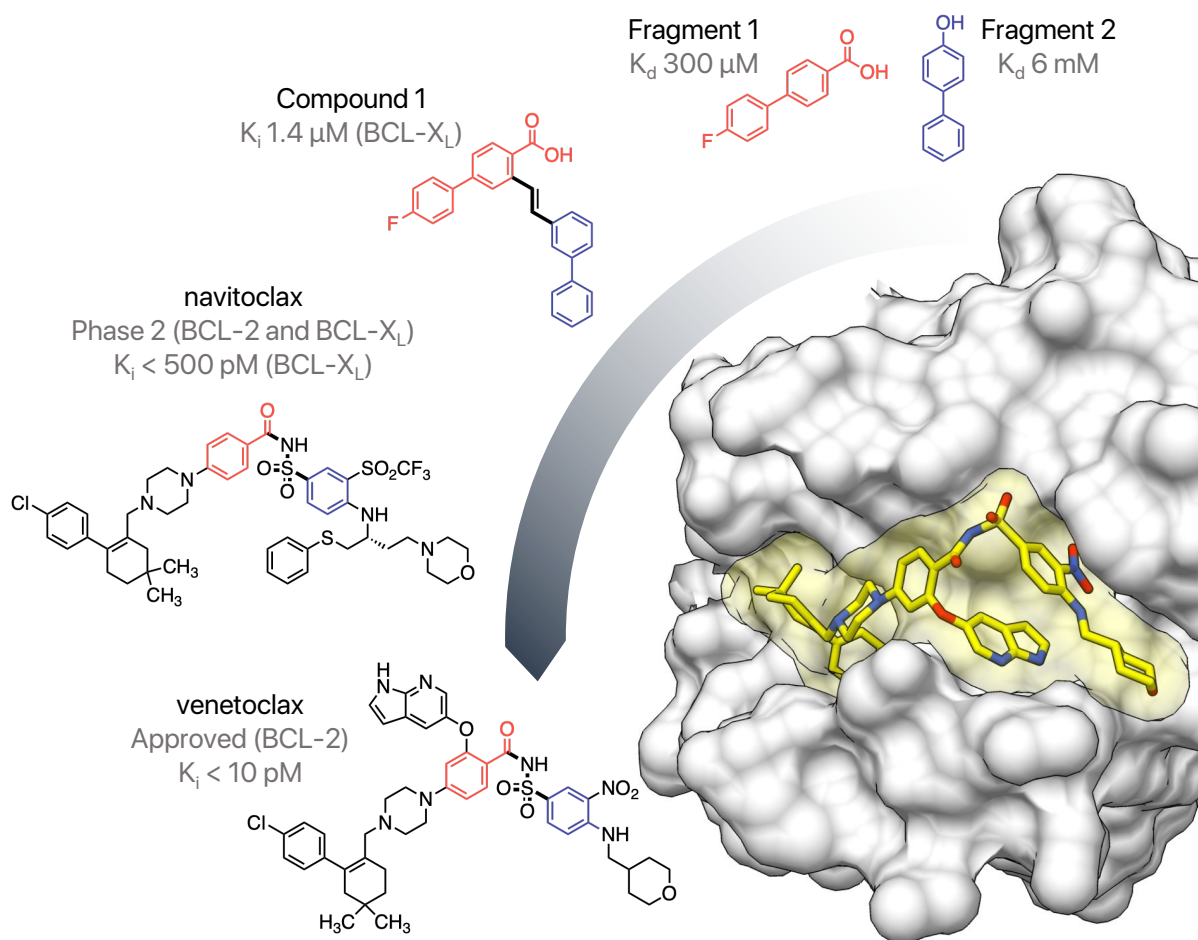


Figure 1. Fragment linking approach with venetoclax. The initial fragments discovered through “SAR by NMR” were linked with an alkene moiety. Navitoclax, exhibiting high affinity for both BCL- X_L and BCL-2, was generated and finally, medicinal chemistry led to venetoclax, a selective BCL-2 inhibitor. The drug was first approved for use in patients with chronic lymphocytic leukemia and 17p deletion.

Table 1. Fragment-based compounds in clinical trials or approved (From *clinicaltrials.gov*, 2020 January)

Drug*	Company	Status	Initial fragment strategy
Biological target			
Erdafitinib FGFR	Johnson & Johnson / Astex	Approved	Growing
Vemurafenib BRAF-V600E	Plexxikon	Approved	Growing
Venetoclax BCL-2	AbbVie / Genentech, Inc.	Approved	Linking
Asciminib BCR-ABL	Novartis	Phase 3	Growing
PLX3397 CSF1R, KIT, FLT3	Plexxikon	Phase 3	Growing
Verubecestat BACE1	Merck	Phase 3	Growing
AZD3293 BACE1	Astex / AstraZeneca / Lilly	Phase 2/3	Growing
ABT-737 BCL-2 & BCL-X _L	AbbVie	Phase 2	Linking
AT7519 CDK1, CDK2, CDK4, CDK5 & CDK9	Astex	Phase 2	Growing
AT9283 Aurora & JAK2	Astex	Phase 2	Growing
AUY922 HSP90	Novartis, Vernalis	Phase 2	Growing
AZD5363 AKT	AstraZeneca / Astex	Phase 2	Growing
CPI-0610 BET	Constellation	Phase 2	Growing
eFT508 MNK1 & MNK2	eFFECTOR	Phase 2	Growing
Indeglitazar PPAR agonist	Plexxikon	Phase 2	Growing
LY3202626	Lilly	Phase 2	Growing

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3	BACE1			
4	LY517717			
5		Lilly / Protherics	Phase 2	Growing
6	FXa			
7	Navitoclax			
8		AbbVie	Phase 2	Linking
9	BCL-2 & BCL-X _L			
10	Onalespib			
11		Astex	Phase 2	Growing
12	HSP90			
13	PF-06650833			
14		Pfizer	Phase 2	Growing
15	IRAK4			
16	PF-06835919			
17		Pfizer	Phase 2	Growing
18	KHK			
19				
20	AMG 510			
21		Amgen	Phase 1/2	Growing
22	KRAS			
23	ASTX029			
24		Astex	Phase 1/2	Growing
25	ERK1 & ERK2			
26	ASTX660			
27		Astex	Phase 1/2	Growing
28	XIAP & cIAP1			
29	LY2886721			
30		Lilly	Phase 1/2	Growing
31	BACE1			
32	MAK683			
33		Novartis	Phase 1/2	Growing
34	PRC2			
35	ABBV-075			
36		AbbVie	Phase 1	Growing
37	BRD2			
38	ABT-518			
39		AbbVie	Phase 1	Linking
40	MMP2 & MMP9			
41	AT13148			
42		Astex	Phase 1	Growing
43	AKT, S6K1 & ROCK			
44	AZD3839			
45		AstraZeneca / Astex	Phase 1	Growing
46	BACE1			
47	AZD5099			
48		AstraZeneca	Phase 1	Growing
49	Bacterial topoisomerase II			
50	AZD5991			
51		AstraZeneca	Phase 1	Merging
52	MCL1			
53	BCL201			
54		Vernalis / Servier / Novartis	Phase 1	Growing
55	BCL-2			
56	BI691751			
57		Boehringer Ingelheim	Phase 1	Growing
58	LTA4H			
59	ETC-1907206			
60		EDDC / A*STAR Research Entities	Phase 1	Growing
60	MNK1 & MNK2			

GDC-0919	Genentech, Inc.	Phase 1	Growing
IDO1			
GDC-0994	Genentech, Inc.	Phase 1	Growing
ERK2			
HTL0014242	Sosei Heptares	Phase 1	Growing
mGlu5			
IC-776	Lilly / ICOS	Phase 1	Growing
LFA1			
LP-261	Locus Pharmaceuticals	Phase 1	Growing
Tubulin			
LY2811376	Lilly	Phase 1	Growing
BACE1			
PLX5568	Plexxikon	Phase 1	Growing
RAF			
SGX-393	SGX Pharmaceuticals	Phase 1	Growing
BCR-ABL			
SGX-523	SGX Pharmaceuticals	Phase 1	Growing
MET			
SNS-314	Sunesis Pharmaceuticals	Phase 1	Growing
Aurora			
S64315	Servier / Novartis / Vernalis	Phase 1	Growing
MCL1			

* Drugs are listed alphabetically within each phase.

2. SECOND-SITE SCREENING FOR IDENTIFICATION OF A SECOND FRAGMENT

As widely reported in the literature, it can be necessary to perform a second screening to identify a fragment bound in another binding site (Table 2). To do so, it may be worth occupying the first site to block its entry. This is partly due to the fact that the fragments typically bind a preferred hot-spot on the protein surface. Moreover, the detection of second-site ligands is generally more difficult because these fragments tend to have a weaker affinity for the target than the first-site ligands. We present below six different strategies that have been used in the literature to identify second-site fragments. The strategies are illustrated in Figure 2.

2.1. Screening using protein-observed NMR or “SAR by NMR”. NMR is an established technique for fragment screening and is particularly attractive for linking strategies. In protein-detected experiments, the protein is typically ^{15}N or ^{13}C labeled and two-dimensional heteronuclear NMR experiments are used to observe the protein NMR peak modifications upon ligand addition. Most of the published cases deal with small to medium-sized proteins, such as protein domains, for which the NMR assignment is not a difficult task. Fesik and collaborators^{11,12} at Abbott established the “SAR by NMR” approach to design a potent inhibitor of the FKBP binding domain. Two fragments bound in two pockets were identified from the NMR experiments and a linker was elaborated using structural data from ^{15}N - ^{13}C -filtered protein-ligand Nuclear Overhauser Effect (NOE) (Table 2, entry 1). With the same approach, a linked compound was designed for the stromelysin protein (Table 2, entry 2).¹⁵ Navitoclax, the orally active anti-cancer drug,¹⁶⁻¹⁸ which led to venetoclax,⁹ was discovered using the SAR by NMR strategy (Figure 1). In another study, the group of Hadjuk¹⁹ designed a highly potent B-cell lymphoma 2 (BCL-2) inhibitor using a library of 17,000 compounds for a first screen and a small library of 70 compounds for a second screen (Table 2, entry 3). Another specific inhibitor of B-cell lymphoma-extra-large (BCL-X_L) was designed by Souers and colleagues.²⁰ In this case, the first site was blocked using a compound derived from High-Throughput Screening (HTS) and medicinal chemistry. 875 fragments were screened to identify a second-site ligand that could be linked to the first-site compound (Table 2, entry 4). The fragment linking approach was also successfully applied to the heat shock protein 90

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3 (HSP90) (Table 2, entry 5)²¹ and to the protein tyrosine phosphatase-1B (PTP1B) (Table 2, entries 6,
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10 **2.2. Screening using ligand-observed ¹H NMR.** Ligand-observed NMR offers different opportunities
11 for identifying second-site ligands that bind at the vicinity of a first-site ligand. By contrast to the “SAR
12 by NMR” approach, ligand-observed experiments are performed for medium-size to very large proteins.
13 One strategy is to use 2D Nuclear Overhauser Effect Spectroscopy (NOESY) experiments that allow the
14 identification of compounds that bind near (~5Å) a first-site ligand through the observation of Inter-
15 Ligand NOEs (ILOEs).²⁴⁻²⁶ It is possible to modify the first-site ligand through the addition of methyl
16 group(s), allowing the generation of a suitable NOESY probe for the observation of strong NOESY
17 cross peaks in a spectral region with little overlap. Such experiments, referred as “SAR by ILOE”, have
18 been used for the design of protein-protein interaction inhibitors for the targets BCL-X_L and MCL1
19 (Table 2, entry 8).^{27,28} In addition to the identification of second-site ligands, the ILOE experiment can
20 be used to assess the orientation of the second-site ligand relative to the first-site ligand, as shown by
21 the group of Abell for the *Mycobacterium tuberculosis* pantothenate synthetase (PS) (Table 2, entry
22 9)²⁹ and confirm previously results obtained by X-Ray crystallography.³⁰
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36 Another strategy is to use paramagnetic labeling,^{31,32} taking advantage of the paramagnetic enhancement
37 (PRE) obtained thanks to the paramagnetic labeling of a first-site ligand. Typically, a 2,2,6,6-
38 tetramethylpiperidin-1-oxyl (TEMPO) moiety is used. The transverse relaxation rate of the protons
39 located within a radius of ~10Å of the first-site ligand is drastically enhanced, which significantly
40 reduces the NMR signal intensities.^{33,34} This approach has been successfully applied for the development
41 of nanomolar E-selectin antagonists by Ernst and co-workers³⁵ (Table 2, entry 10).
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51 **2.3. Screening using ligand-observed ¹⁹F NMR.** ¹⁹F NMR spectroscopy is another tool for fragment
52 screening, with the possibility to screen large mixtures of more than 20 fragments, taken advantage of
53 the chemical shift dispersion of the ¹⁹F nucleus. ¹⁹F NMR is also used to search for second-site ligands,
54 as exemplified for inhibitors of the Alzheimer target beta-secretase 1 (BACE1) (Table 2, entry 11).^{36,37}
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3 FAXS-NMR screening (fluorine chemical shift anisotropy and exchange for screening) was used to
4 identify a novel class of HSP90 inhibitors. The principle is to use a fluorinated molecule as a spy to
5 recognize fragments that competitively bind with the spy molecule. A fragment hit selected from a 1,200
6 fragments library was further characterized by X-ray crystallography and linked to the known HSP90
7 ligand resorcinol, leading to a low nanomolar compound (Table 2, entry 12).³⁸
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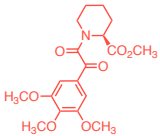
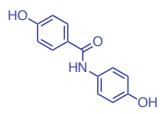
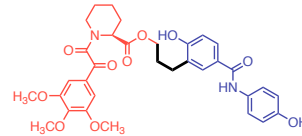
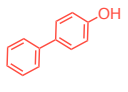
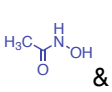
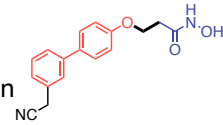
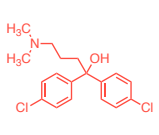
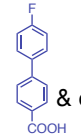
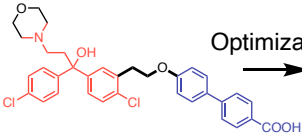
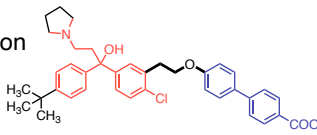
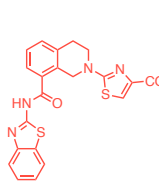
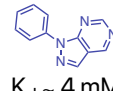
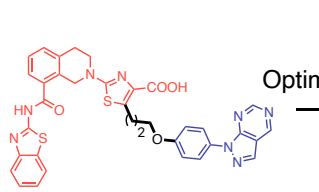
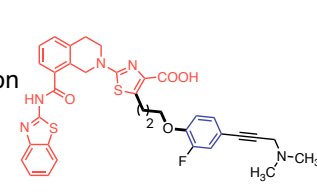
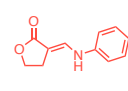
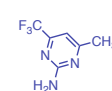
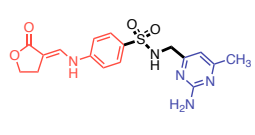
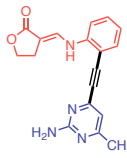
16 **2.4. Second-site ligand resulting from elaboration of a first-site ligand.** Fragment growing may also
17 generate a new ligand that bind in a pocket that differs from the one of the initial fragment hit. This is
18 exemplified by the group of Dalgarno³⁹ who screened 735 fragments against the lactate dehydrogenase
19 A (LDHA) using Saturation Transfer Difference (STD) NMR. A fragment bound in the substrate pocket
20 and part of the cofactor site was identified by X-ray crystallography. Fragment growing led to a new
21 compound that do not bind in the cofactor binding site. Therefore, the initial fragment and the optimized
22 compound were linked to generate the final structure (Table 2, entry 13).
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33 **2.5. Second-site ligand following tethering-based first-site ligand.** Wells and colleagues⁴⁰ have
34 developed an alternative strategy to identify fragments that bind to proteins, using a cysteine residue
35 that can form a disulfide bond with disulfide containing fragments. The disulfide bond of the fragments
36 that display affinity for the protein are entropically stabilized while the other disulfide bonds can be
37 easily reduced. Tethered compounds were then recognized by Mass Spectrometry (MS). The approach
38 was used to generate a novel non-peptidic inhibitor of caspase 3 (Table 2, entry 14).⁴¹ The methodology
39 was also used by Raimundo and co-workers.⁴² They developed a potent small-molecule inhibitor of
40 interleukin 2 (IL2) starting from a library of 7,000 disulphide-containing fragments (Table 2, entry 15).
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51 **2.6. Second-site ligand screening with focused libraries.** To identify second-site ligands, another
52 strategy is to screen a fragment library that is chemically oriented to bind a particular protein-binding
53 pocket. For example, the group of Hudson⁴³ screened a focused library of 450 fragments containing
54 acids to find hits able to bind to the negatively charged region of the LDHA protein. The hit fragment
55 was then linked to another fragment bound in the adenine region of the cofactor nicotinamide adenine
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3 dinucleotide (NADH) (Table 2, entry 16). Another example was published by Gaul and co-workers⁴⁴
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5 regarding disruptor of telomeric silencing 1-like (DOT1L), a potential therapeutic target involved in
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7 leukemias. The first site was blocked with a DOT1L inhibitor to search for fragments that bind in the
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9 cofactor S-adenosylmethionine (SAM) pocket using NMR (Table 2, entry 17). In parallel, a focused
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11 virtual screening followed by a biochemical screen was performed for the cofactor pocket. The NMR
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13 and biochemical hits were further studied by X-ray crystallography. Modeling studies suggested to link
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15 a fragment and a second-site binder, which led to a new structure with low nanomolar affinity.
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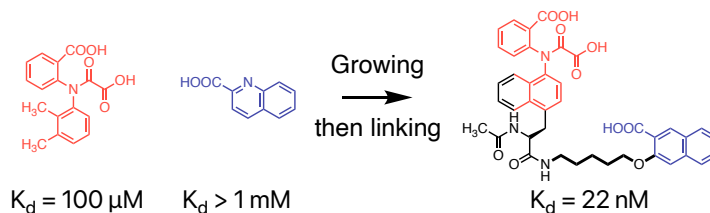
Table 2. Fragment linking by second-site screen.

Entry [Ref.] Target (Pathologies)	Fragments & leads	
Method		
1 [11,12] FKBP (Neuro-degenerative diseases) NMR	 $K_d = 2 \mu\text{M}$	 $K_d = 100 \mu\text{M}$
	Linking →  $K_d = 49 \text{ nM}$	
2 [15] MMP3 (Cardiovascular diseases) NMR	 $K_d = 280 \mu\text{M}$	 $K_d = 17 \text{ mM}$
	Linking & optimization →  $K_d = 15 \text{ nM}$	
3 [19] BCL-2 (Cancer & autoimmune diseases) NMR	 $K_d = 20 \mu\text{M}$	 $K_d = 300 \mu\text{M}$
	Linking & optimization →  $K_d = 220 \text{ nM}$	
	Optimization →  $K_d = 40 \text{ nM}$	
4 [20] BCL-X_L (Cancer & autoimmune diseases) NMR	 $\text{IC}_{50} = 91 \text{ nM}$	 $K_d \sim 4 \text{ mM}$
	Linking →  $K_i = 42 \text{ pM}$	
	Optimization →  $K_i < 10 \text{ pM}$	
5 [21] HSP90 (Cancer & neuro-degenerative diseases) NMR	 $K_d = 150 \mu\text{M}$	 $K_d = 20 \mu\text{M}$
	Linking →  $K_i = 1.9 \mu\text{M}$ Closed state	
	 $K_i = 4 \mu\text{M}$ Open state	

6 [22]

PTP1B

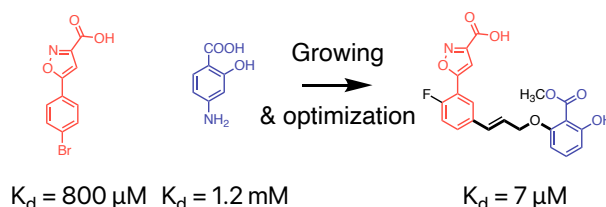
(Obesity,
diabetes and
cardiovascular
diseases)

NMR

7 [23]

PTP1B

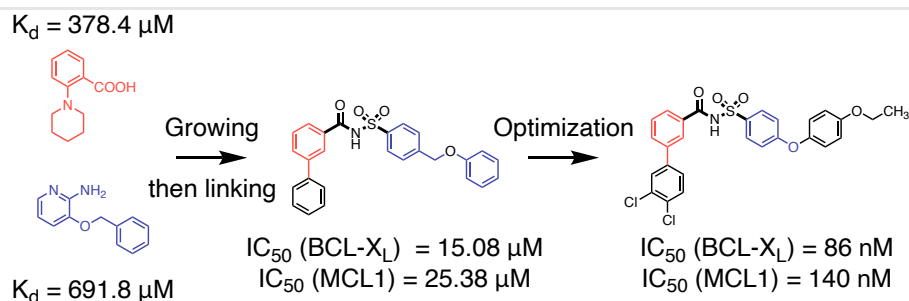
(Obesity,
diabetes and
cardiovascular
diseases)

NMR

8 [27,28]

BCL-X_L,**MCL1**

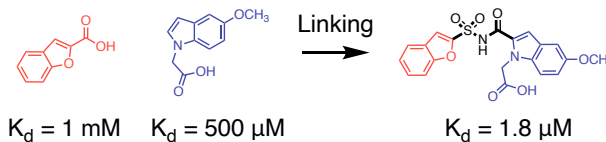
(Cancer &
autoimmune
diseases)

NMR

9 [29,30]

PS

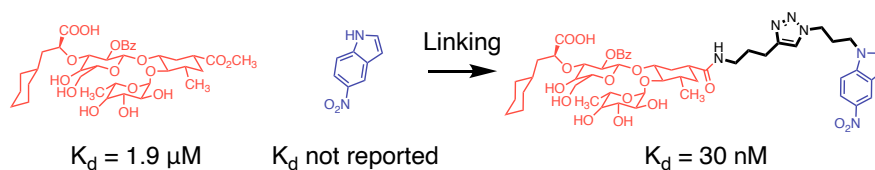
(Infectious
diseases)

X-Ray & NMR

10 [35]

E-selectin

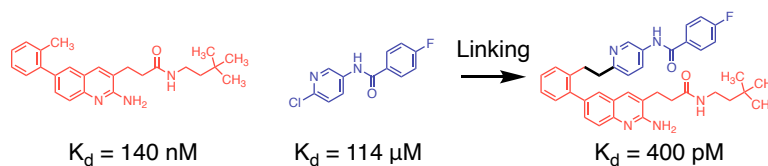
(Cardiovascular
diseases)

NMR

11 [36,37]

BACE1

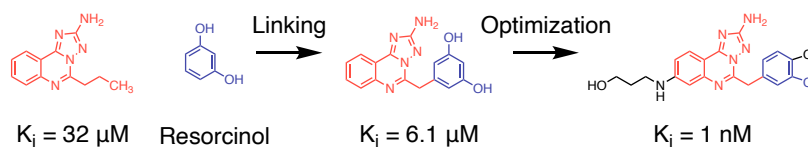
(Neuro-
degenerative
diseases)

NMR

12 [38]

HSP90

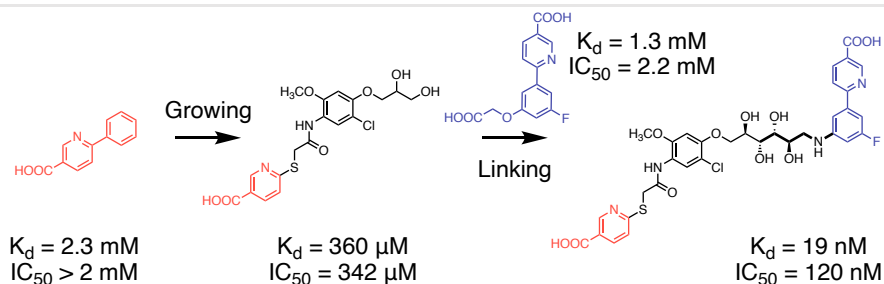
(Cancer & neuro-degenerative diseases)

NMR

13 [39]

LDHA

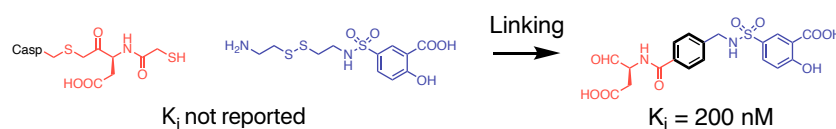
(Cancer)

X-Ray & NMR

14 [41]

CASP3

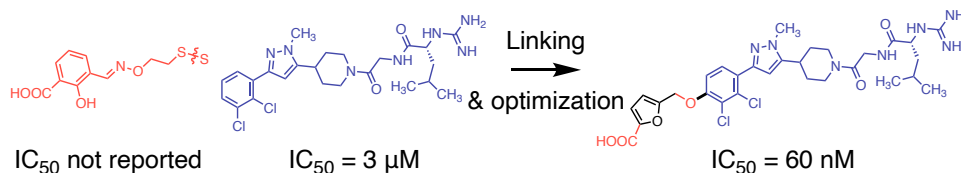
(Neuro-degenerative diseases)

Tethering

15 [42]

IL2

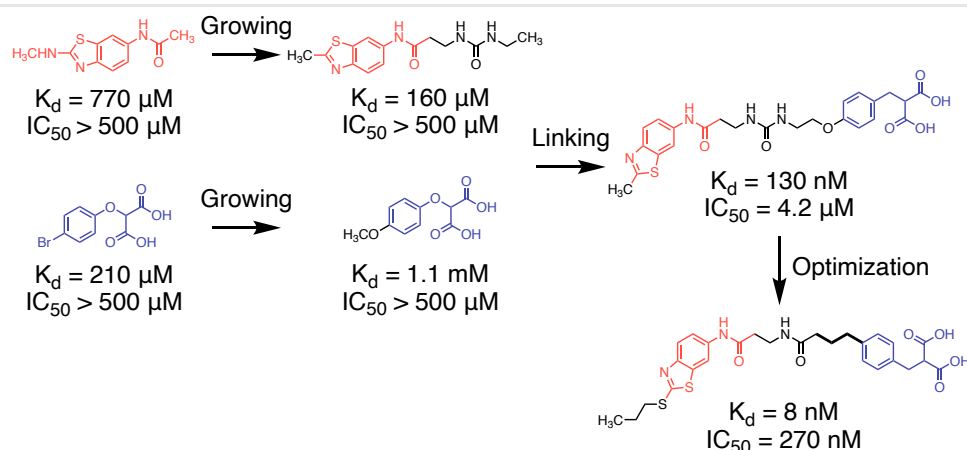
(Autoimmune diseases)

Tethering

16 [43]

LDHA

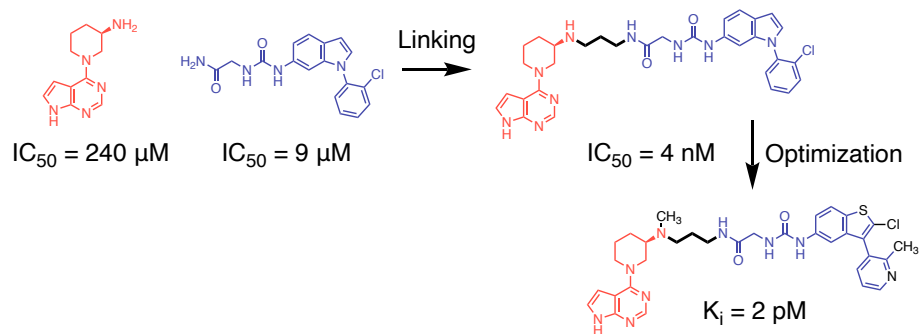
(Cancer)

NMR & SPR

17 [44]

DOT1L

(Cancer)

Bioassay, X-
Ray & NMR

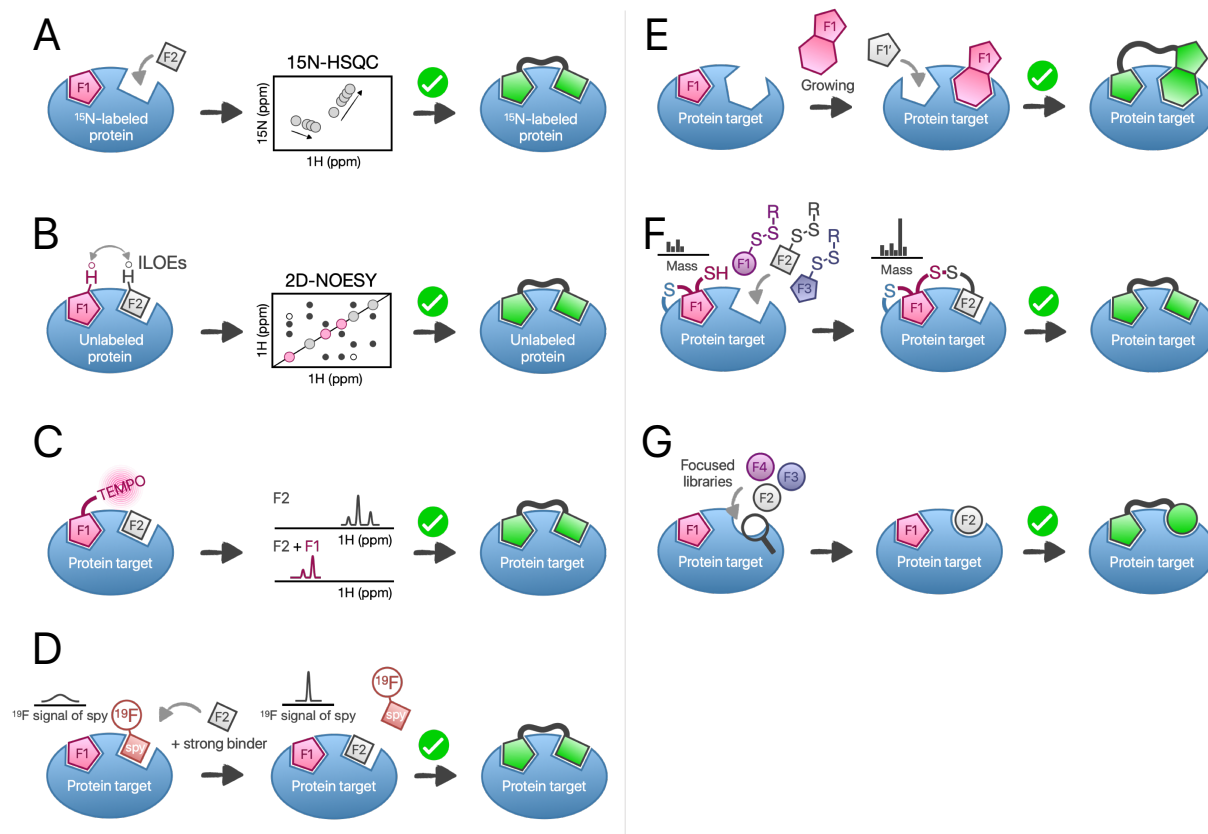


Figure 2. Schematic representation of methods for second-site screening for identification of a second fragment hit. **A)** Screening using protein-observed NMR or “SAR by NMR”. A first hit (pink) is identified through chemical shift perturbations of a 2D ^{15}N or ^{13}C labeled protein spectrum. Chemical shifts perturbations observed in a second site of the protein (grey) indicate the binding of a second fragment. **B)** Screening using ligand-observed ^1H NMR: the ILOE method. A first fragment hit is used as a spy to discover a second fragment hit that binds in the vicinity of the first binder (distances ($\sim 5\text{\AA}$), through NOESY correlations observed between the two adjacent ligands. **C)** Screening using ligand-observed ^1H NMR : the PRE method. A first fragment hit is used as a spy labeled with paramagnetic tag using a 2,2,6,6-tetramethylpiperidin-1-oxyl (TEMPO) moiety. The second fragment hit displays reduced NMR signal intensities due its proximity to the TEMPO moiety. **D)** Screening using ligand-observed ^{19}F NMR. A spy molecule is labeled with a fluorine tag to identify fragments that competitively bind with the spy molecule. **E)** Second-site ligand resulting from elaboration of a first-site ligand. Fragment growing generates a new ligand that bind in a distinct pocket. **F)** Second-site ligand

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3 following tethering-based first-site ligand. A first fragment is covalently bound to the protein through a
4 cysteine residue. A second cysteine residue is used to form a disulfide bond with disulfide containing
5 fragments. Tethered compounds are identified by Mass Spectrometry. **G) Second-site ligand screening**
6 with focused libraries. When the nature of a second pocket is known, a focused library can be used to
7 identify a second-site binder.
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3. DIRECT IDENTIFICATION OF FRAGMENTS BOUND TO TWO DISTINCT BINDING SITES

When techniques such as X-ray crystallography, NMR or virtual screening are used, the identification of two fragments that bind in two distinct sites can be a direct outcome of the screening process. As discussed below, additional methods have also been used to identify two fragments that simultaneously bind the protein. The different strategies are summarized in Figure 3.

3.1. Screening using X-ray crystallography. X-ray crystallography is a particularly attractive technique in FBDD as it directly gives the 3D structure of the protein-fragment complex. Using a focused library of 80 fragments on the protein thrombin,⁴⁵ Astex Therapeutics observed fragments bound to the S1 pocket of the enzyme and one compound that bound to the S2-S4 region. The X-ray structures then helped the design of a linked compound (Table 3, entry 18). The group of Yarnold⁴⁶ identified, using biochemical screening followed by X-ray crystallography, fragments that bound to separate sub-sites within the ATPase pocket of HSP90 (Table 3, entry 19). In another example, Guichou and colleagues⁴⁷ used computational screening of 34,409 fragments to identify fragments of the cyclophilin D (CYPD) active site as well as the gatekeeper pocket. Fourteen X-ray structures were obtained, showing four fragments bound to the catalytic site of the protein while five fragments were observed in the gatekeeper pocket (Table 3, entry 20). Using a thermal shift assay followed by X-ray crystallography, Barral and co-workers^{48,49} identified 3 compounds that bind near the S-Adenosylmethionine (AdoMet) binding pocket of the NS5 AdoMet-dependent mRNA methyltransferase (MTase) domain, highlighting a new targetable site (Table 3, entry 21). More recently, Spring *et al.*⁵⁰⁻⁵² found a fragment bound at multiple sites on the protein casein kinase 2 (CK2), including a previously unreported site adjacent to the adenosine triphosphate (ATP) site. The fragment was modified to generate a compound that specifically binds the newly identified pocket and linked with ATP-site ligands (Table 3, entry 22).

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3 **3.2 Screening using protein-observed NMR.** Protein-observed NMR experiments can also be used to
4 identify two different fragments that bind simultaneously two distinct protein pockets. The group of
5 Fesik⁵³ examined 14,976 fragments of replication protein A (RPA) by NMR and found compounds that
6 bind to two distinct sites in the basic cleft of RPA70N. X-ray structures revealed the binding modes of
7 the fragments, suggesting a strategy for fragment optimization and linking (Table 3, entry 23).
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15 **3.3. Screening using MS.** Similarly to the “SAR by NMR”, a “SAR by MS” approach has been
16 proposed. Ibis Therapeutics⁵⁴ used MS to screen fragments against the 1061 region of bacterial 23S
17 ribosomal ribonucleic acid (rRNA). This process includes a first step where compounds are screened
18 against the RNA by MS. SAR is obtained by chemical modification of the hits and MS experiments.
19 Data obtained for structurally modified hits, for which competition or absence of competition in the MS
20 experiments was observed, led to distant restraints between initial fragments that were then linked (Table
21 3, entry 24).
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32 **3.4. Screening using biochemical assays.** Biochemical assays constitute straightforward screening
33 methods that require knowledge of the biochemical protein functions. A proof of concept of the linking
34 strategy was reported by Green⁵⁵ with biotin analogues binding avidin, illustrating the super-additivity
35 that can be achieved with the fragment linking approach (Table 3, entry 25). Another report⁵⁶ showed
36 that the knowledge of the 3D structure of the target was not crucial. Ellman and co-workers screened at
37 high concentration a library of oximes and a small library of di-hydroxylamine linkers against the
38 tyrosine kinase c-SRC. They could demonstrate the utility of the method with the identification of a
39 potent and subtype-selective inhibitor (Table 3, entry 26).
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51 **3.5. Fragment self-assembly.** We have also considered fragment self-assembly as examples of
52 fragment linking, even if no linker is designed to obtain the linked compound. The process called
53 dynamic combinatorial chemistry (DCC) is observed *in situ* in the presence of a target protein. As a first
54 example, Huc and Lehn⁵⁷ reported the generation of carbonic anhydrase (CA) inhibitors by combination
55 of aldehyde and amine moieties to *in situ* form imines (Table 3, entry 27). Other applications were
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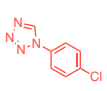
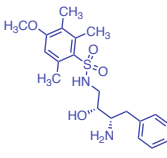
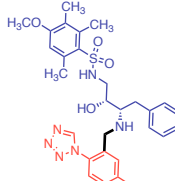
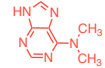
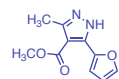
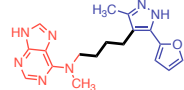


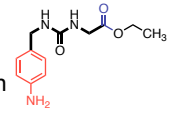
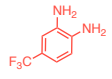

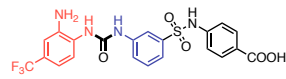
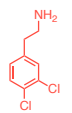
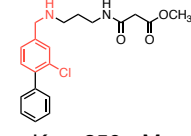
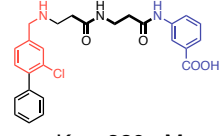
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3 published for a neuraminidase (NA) inhibitor (Table 3, entry 28)^{58,59} and an aspartic protease
4 endothiapepsin (Table 3, entry 29).⁶⁰ Dynamic combinatorial X-ray crystallography, when DCC takes
5 place inside protein crystals, was demonstrated for the cyclin-dependent kinase 2 (CDK2), using a
6 mixture of hydrazines and isatins (Table 3, entry 30).⁶¹ Another methodology called dynamic ligation
7 screening (DLS) was reported by Rademann and co-workers⁶² for the protease caspase 3 (Table 3, entry
8 31). Using a fluorescent probe attached to a fragment, competitors or fragments that bind cooperatively
9 to the fluorescent probe are detected with the fluorescence polarization assay. The authors also extended
10 the approach to form amide bonds (Table 3, entry 32).⁶³ Protein-templated click chemistry (PTCC) that
11 relies on the 1,3-dipolar cycloaddition of azides and alkynes to generate a triazole moiety was also
12 reported by the team of Sharpless⁶⁴ with the generation of a femtomolar inhibitor of acetylcholinesterase
13 (Table 3, entry 33). Additional examples concern inhibitors developed for histone deacetylases (Table
14 3, entry 34),⁶⁵ chitinase B (Table 3, entry 35)⁶⁶ and the aspartic protease endothiapepsin (Table 3, entry
15 36).⁶⁷ The approach can also lead to protein-protein interaction inhibitors, as demonstrated for BCL-X_L
16 (Table 3, entry 37).^{68,69}

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34 **3.6. Inhibitor deconstruction and reconstruction.** Deconstruction of known ligands can provide a
35 useful strategy for the reconstruction of a new compound.⁷⁰ Using HTS, two compounds were identified
36 to bind the human *N*-myristoyltransferase 1 (NMT1) protein and X-ray crystallography indicated that
37 the molecules bind in two different pockets.⁷¹ To preclude a steric clash upon compound linking, one of
38 the hits was deconstructed to remove the quinoline moiety (Table 3, entry 38).

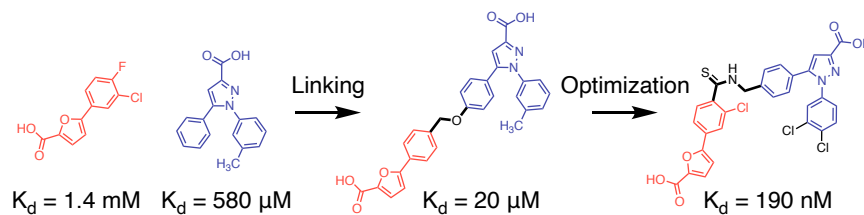
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41 The linking approach can be particularly useful to replace a part of a compound that is not suitable. For
42 example, fragments were searched to substitute a chymase inhibitor benzothiophene moiety that
43 produced reactive metabolites.⁷² Takahashi and co-workers screened one thousand fragments and
44 identified a fragment that binds in the same pocket as the benzothiophene core. The deconstruction and
45 reconstruction of the initial inhibitor led to a compound with improved metabolic properties (Table 3,
46 entry 39).

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3 **3.7. Cooperative binding.** In some cases, the second binding pocket is only observed in the presence
4 of a first ligand bound to a distinct pocket. For example, Varani and colleagues⁷³ identified six fragments
5 capable to bind the human immunodeficiency virus 1 (HIV-1) transactivation response RNA only in the
6 presence of the first hit, an arginine mimetic.
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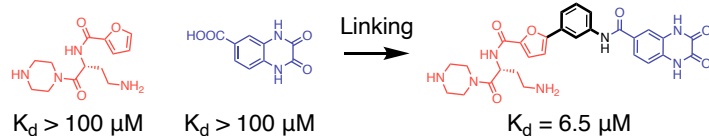
Table 3. Fragment linking by direct identification of two fragments.

Entry [Ref.] Target (Pathologies)	Fragments & leads		
Method			
18 [45] Thrombin (Neuro-degenerative diseases) X-Ray	 $IC_{50} = 330 \mu M$	 $IC_{50} = 12 \mu M$	 $IC_{50} = 1.4 nM$
19 [46] HSP90 (Cancer & neuro-degenerative diseases) Bioassay & X-Ray	 $IC_{50} = 1.5 mM$	 $IC_{50} = 1 mM$	 $IC_{50} = 1.5 \mu M$
20 [47] CYPD (Viral & neuro-degenerative diseases) X-Ray	 $IC_{50} > 5 mM$	 $IC_{50} > 5 mM$	 IC_{50} (CYPA) = 13.1 μM IC_{50} (CYPB) = 6.1 μM IC_{50} (CYPD) = 6.2 μM
21 [48,49] DENV-MTase (Viral diseases) X-Ray	 $IC_{50} = 9 mM$	 $IC_{50} = 2.8 mM$	 IC_{50} (DENV 2'O-MTase) = 91 μM IC_{50} (DENV N7-MTase) = 1.1 mM IC_{50} (WNV 2'O-MTase) = 51 μM
22 [50-52] CK2α (Cancer) X-Ray	 K_d not reported	 $K_d = 250 \mu M$	 $K_d = 320 nM$

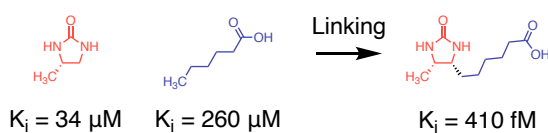
23 [53]
RPA
(Cancer)
NMR



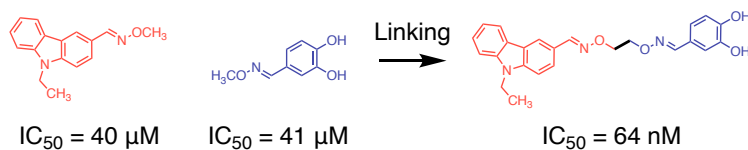
24 [54]
23S rRNA
(Viral &
bacterial
diseases)
MS



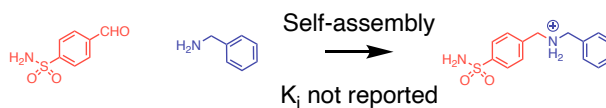
25 [55]
Avidin
-
Bioassay



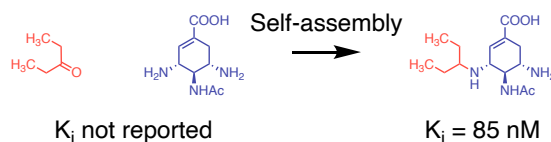
26 [56]
c-SRC
(Cancer)
Bioassay



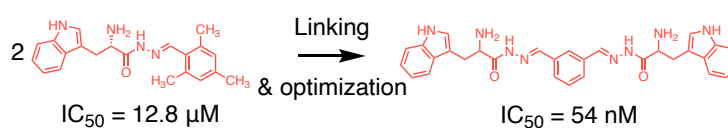
27 [57]
CA
(Osteoporosis)
DCC



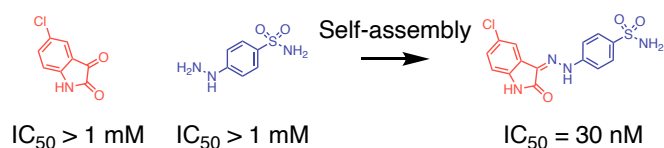
28 [58, 59]
NA
(Viral diseases)
DCC



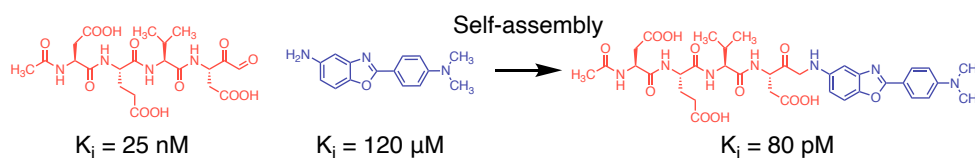
29 [60]
Endothia-
pepsin
(Infectious, viral
& neuro-
degenerative
diseases)
DCC



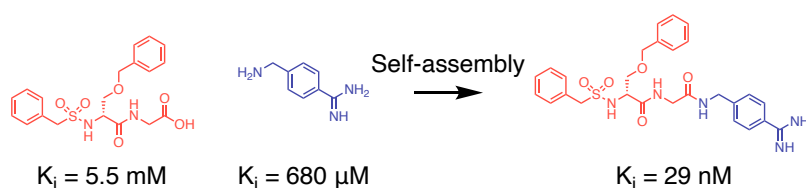
30 [61]
CDK2
 (Cancer)
DCX



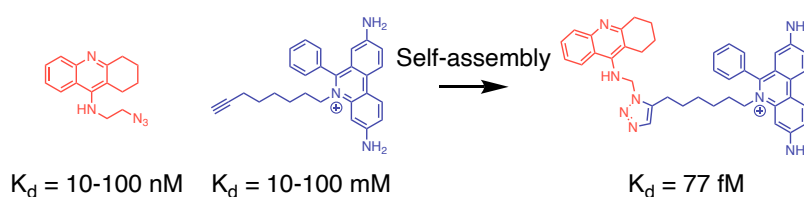
31 [62]
CASP3
 (Neuro-
 degenerative
 diseases)
DLS



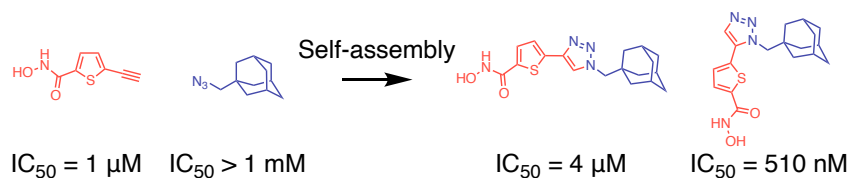
32 [63]
FXa
 (Bleeding
 disorders)
DLS



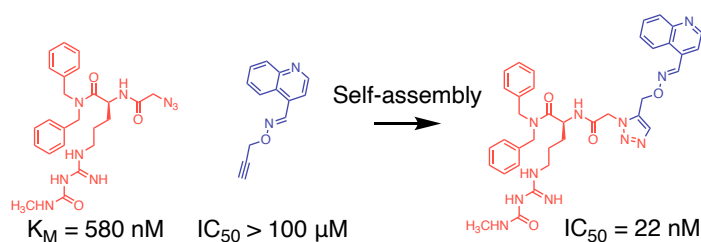
33 [64]
ACHE
 (Neuro-
 degenerative
 diseases)
PTCC



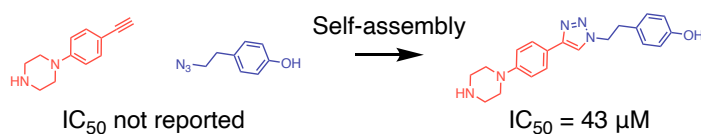
34 [65]
HDAC
 (Cancer,
 inflammatory &
 neuro-
 degenerative
 diseases)
PTCC



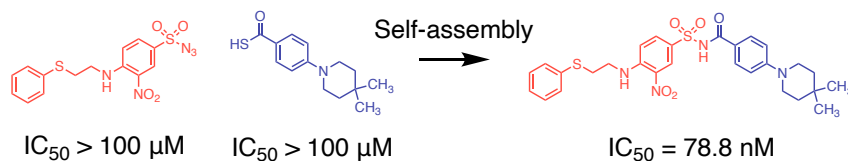
35 [66]
SmChiB
 (Infectious &
 inflammatory
 diseases)
PTCC



36 [67]
**Endothia-
 pepsin**
 (Infectious, viral
 & neuro-
 degenerative
 diseases)
PTCC



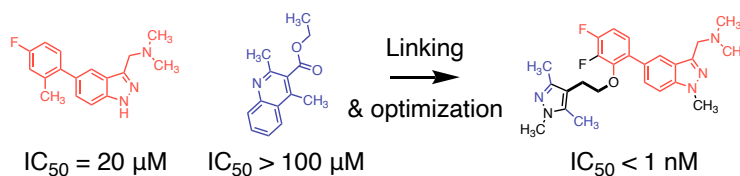
37 [68, 69]

BCL-X_L(Cancer &
autoimmune
diseases)**PTCC**

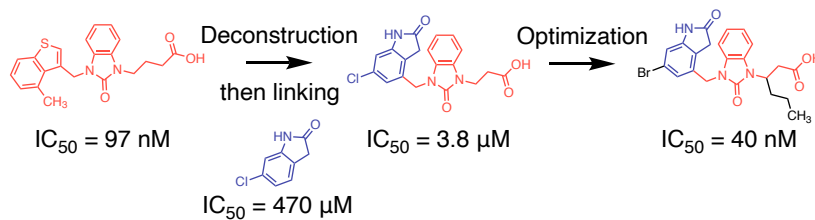
38 [71]

NMT1

(Viral diseases)

X-Ray

39 [72]

Chymase(Cardiovascular
diseases)**X-Ray**

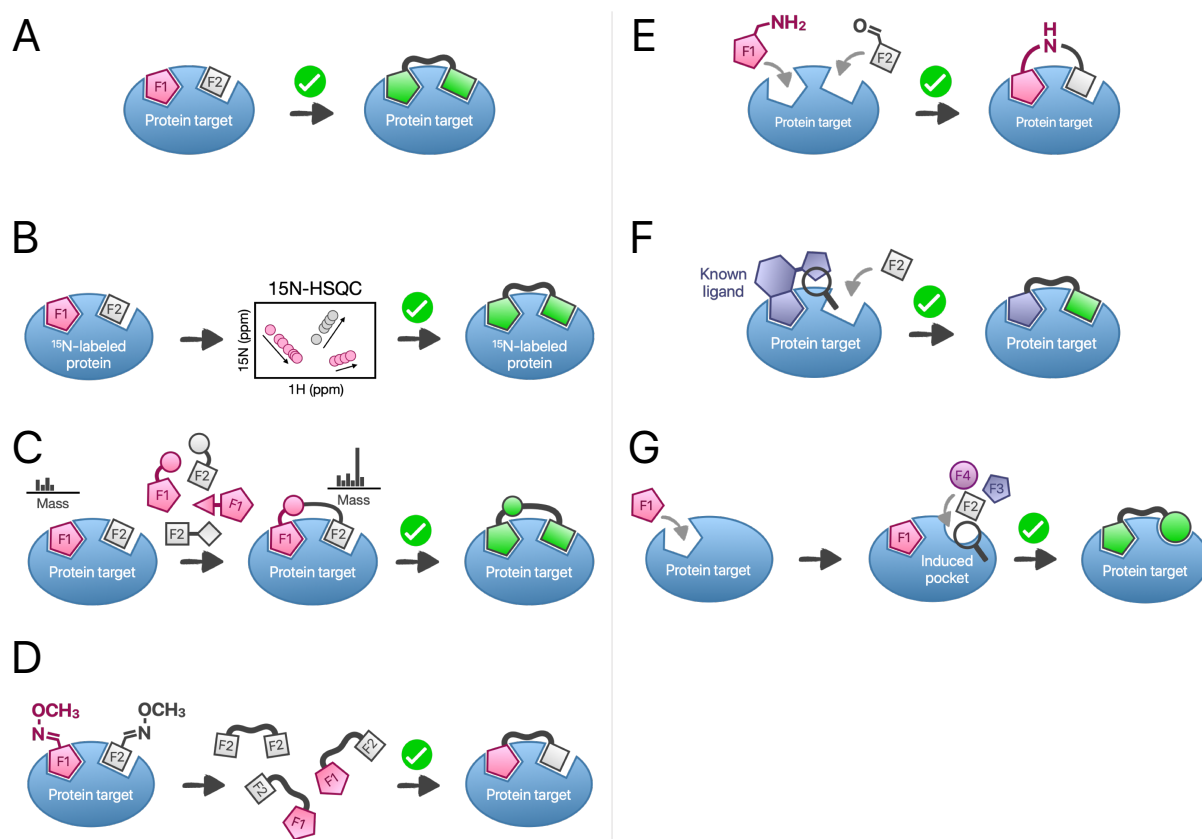


Figure 3. Schematic representation of methods for direct identification of fragment binds to two distinct binding sites. **A)** Screening using X-ray crystallography. When fragments are screened in cocktails, fragments bound to distinct sites can be observed. **B)** Screening using protein-observed NMR. Two fragments that bind simultaneously two distinct protein pockets can be identified on 2D NMR protein spectra. **C)** Screening using MS. Fragment hits identified by MS are modified and competition experiments are performed to derive structural restraints. **D)** Screening using biochemical assays. The example shows the use of the oxime function together with a small library of di-hydroxylamine linkers. **E)** Fragment self-assembly in the presence of a target protein: dynamic combinatorial chemistry (DCC). The example is illustrated with aldehyde and amine moieties that *in situ* form imines. **F)** Inhibitor deconstruction and reconstruction. A known ligand can be improved by replacing a moiety by a fragment hit identified in the presence of the deconstructed fragment. **G)** Cooperative binding. A first ligand induces a new pocket for a second fragment hit.

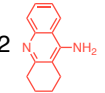
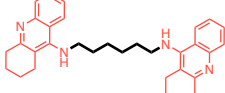
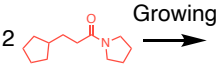
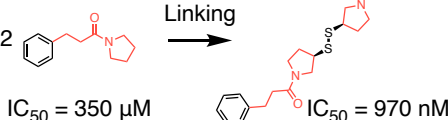
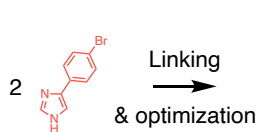
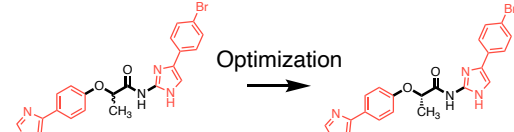
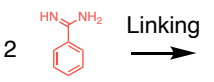
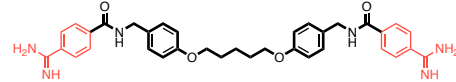
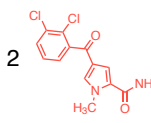
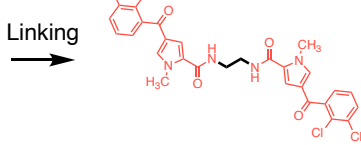
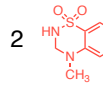
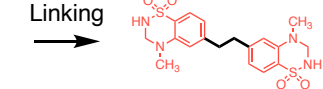
4. DIMERIZATION OF A FRAGMENT HIT

A special application of fragment linking is dimerization. This approach allows one to link together two copies of the same fragment binding to either adjacent sites or in the same pocket of a protein.

4.1. The fragment binds twice in the protein binding pocket. The first example was reported by Brimijoin and co-workers⁷⁴ for the acetylcholine esterase. The observation that two copies of an acridine analogue could be modelled in the active site led to the design of dimeric compounds with different linker lengths. An inhibitor that was 1,000-fold more potent than the original molecule was synthesized (Table 4, entry 40). Another case was reported by Blundell and co-workers⁷⁵ for the transcription factor from *Mycobacterium tuberculosis* (EthR), involved in antibiotic resistance. Thermal shift assay followed by crystallography identified two copies of a fragment hit in the allosteric pocket of the protein, starting from 1,250 fragments (Table 4, entry 41). Similarly, biochemical assay (enzyme inhibition by monitoring NADH absorbance) followed by crystallography identified two copies of a fragment hit in the active site of the protein inosine-5'-monophosphate dehydrogenase (IMPDH) (Table 4, entry 42).⁷⁶ They synthesized an inhibitor that was 2,500-fold more potent than the original molecule.

4.2. The fragment binds in the same pocket in two protein sub-units. Human mast cell tryptase, a trypsin-like serine protease, exists as a tetramer. The ligand benzamidine was modified to contain two benzamidine moieties with a flexible linker region spanning the space between two monomers (Table 4, entry 43).⁷⁷ With a similar approach, an anti-cancer pyruvate kinase M2 (PKM2) activator was designed (Table 4, entry 44)⁷⁸ as well as an inhibitor of the α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors (Table 4, entry 45).⁷⁹

Table 4. Fragment linking by fragment dimerization

Entry [Ref.] Target (Pathologies)	Fragments & leads	
Method		
40 [74] ACHE (Neuro-degenerative diseases) Bioassay	 $K_i = 600 \text{ nM}$	 $K_i = 400 \text{ pM}$
41 [75] EthR (Infectious diseases) Bioassay & X-Ray	 $IC_{50} = 280 \text{ }\mu\text{M}$	 $IC_{50} = 350 \text{ }\mu\text{M}$ $IC_{50} = 970 \text{ nM}$
42 [76] IMPDH (Infectious diseases) Bioassay & X-Ray	 $IC_{50} = 674 \text{ }\mu\text{M}$	 $IC_{50} = 520 \text{ nM}$ $IC_{50} = 270 \text{ nM}$
43 [77] Tryptase (Asthma) Bioassay	 $K_i = 22 \text{ }\mu\text{M}$	 $K_i < 10 \text{ pM}$
44 [78] PKM2 (Cancer) HTS	 $AC_{50} = 17 \text{ }\mu\text{M}$	 $AC_{50} = 14 \text{ nM}$
45 [79] AMPA receptor (Neuro-degenerative diseases) X-Ray	 $EC_{50} = 176 \text{ }\mu\text{M}$	 $EC_{50} = 13 \text{ nM}$

5. LINKERS

Once two fragments have been identified, a linker is designed, which can be done using various strategies. From a chemical point of view, we have considered two types of linkers, the alkyl-derived linkers, and the functionalized linkers. We also report two promising approaches, a first one that we have called “linkers inspired by known ligands” and the other one “growing before linking”.

5.1. Alkyl linkers and derivatives. Alkyl linkers of variable lengths are largely used in the literature to link two fragments bound in two pockets (Table 5). In some studies, the linkers also include more rigid structures such as phenyl core, alkyne function (Table 5 entry 11) and cyclohexane (Table 5 entry 26). Typically, the likely binding mode and structural constraints for the lengths are obtained from NMR experiments (Table 5 entries 1, 2 & 3), crystal structure of fragments (Table 5 entries 4, 6, 18, 38 & 39) or a combination of crystallography and molecular modelling (Table 5 entries 7, 12, 16, 17, 19, 40, 44 & 45). One publication showed that mass spectroscopy competition experiments can also generate distance restraints (Table 5 entry 24). Linking strategy with an alkyl chain has also successfully generated compounds in the absence of structural information, using biochemical data (Table 5 entries 25 & 43).

In a majority of cases, a small set of compounds with varying linker lengths and attachment points are synthesized to explore the hypotheses. In addition, the linking can start from an ether or an amine moiety, making chemical synthesis more straightforward.

5.2. Linkers with functional groups. Alkyl functions are more problematic when the linking of the two molecules requires a non-linear geometry. For example, the team of Barral used a urea function as suggested by *in silico* studies (Table 5 entry 21). Hadjuk and co-workers reported a case where a sulfonamide was chosen as it could bend by 180°. In the same study, in order to position correctly a furanone moiety included in the fragment with a 90° twist, an acetylene linker was used (Table 5 entry 5). Sulfonamide and acyl sulfonamide were also used by the Abell group to replace ester and amide moieties in the case of the pantothenate synthase (Table 5 entry 9).

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3 In some cases, the linker has been carefully optimized in order to add molecular interactions between
4 the atoms of the linker and the protein residues. For example, Fesik's team chose to add rigidity and to
5 introduce interactions between the linker and the protein by introducing a thioamide linker for a BCL-
6 2 inhibitor (Table 5 entry 23). In the Dalgarno group, the initial ether-based linker was modified with
7 the addition of functional groups including ester, amide, amine and hydroxyl functions. The eight- atom
8 linker containing four hydroxyl groups interacting with protein atoms provided the most active
9 compound (Table 5 entry 13). A further strategy reported in the literature consisted in selecting the
10 linkers by *in silico* studies. For example, for the design of a BCL-X_L inhibitor (Table 5 entry 8), several
11 types of linkers were proposed and led to more active compounds.
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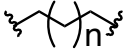
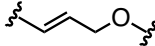
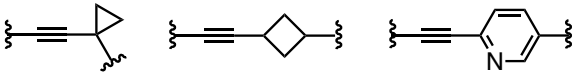
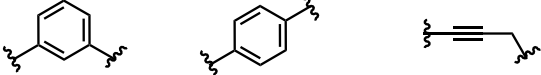

24 **5.3. Structural data for the linker design.** As illustrated in Table 5, in the large majority of the cases
25 (26 out of 31 publications), structural information is used to design the linker. X-ray crystallography
26 appears as the method of choice, alone or in combination with modeling, while NMR is much rarely
27 utilized. Structural data can also result from the merging strategy, where a known ligand offers an
28 opportunity to design the linker (see below, 5.4). Also, the growing strategy may be necessary to first
29 reduce the distance between the fragments before linking, which necessitates structural characterization
30 of the protein-fragment complexes (see 5.5).
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41 **5.4. Linkers inspired by known ligands (mixing merging and linking strategies).** Another strategy
42 for the linker design is to take advantage of the knowledge of natural substrates or other known
43 inhibitors. For example, Guichou et *al.* chose to use a urea moiety, after superimposition of fragments
44 with the known structures of sangliferin A and cyclosporine A, two cyclophilin inhibitors. Two
45 molecules with two different linkers were obtained and one of them directly showed micromolar activity
46 (Table 5 entry 20). Abell group similarly merged a previously known inhibitor with two copies of a
47 fragment hit (Table 5 entry 42), which has led to a successful linker.
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58 **5.5. Growing before linking.** We want to particularly emphasize one strategy that consists in growing
59 one of the fragments before linking, when the distance between the two fragments is particularly large.
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3 This strategy has been used with both alkyl and functionalized linkers. In the report by AstraZeneca
4 (Table 5 entry 16) on the LDHA protein, growing of one of the fragments was performed until the
5 distance between the two fragments was reduced to 3Å. Then, a molecular study was used to select
6 suitable linkers. In the case of the protein kinase CK2, due to the long distance between the initial
7 fragments, Spring and colleagues choose to modify one of the fragments by addition of chemical groups
8 with different lengths. The synthesized molecules were then evaluated and X-ray data were solved to
9 measure the resulting distance between the two fragments (Table 5 entry 22), before linking.
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Table 5. Linker chemical structures and structural methods.

Entry from tables 2, 3, 4 [Ref.]	Structural methods	Linker structure
Alkyl linkers and derivatives		
1 [11,12] 2 [15] 3 [19]	NMR	
4 [20] 6 [22] 18 [45] 38 [71] 39 [72]	X-Ray	
12 [38] 16 [43] 17 [44] 19 [46] 40 [74] 44 [78] 45 [79]	X-ray & molecular modeling	Variable lengths
25 [55] 43 [77]	-	
7 [23]	X-ray & molecular modeling	
11 [36, 37]	NMR, X-ray & molecular modeling	
24 [54]	MS	
26 [56]	-	

Linkers with functional groups

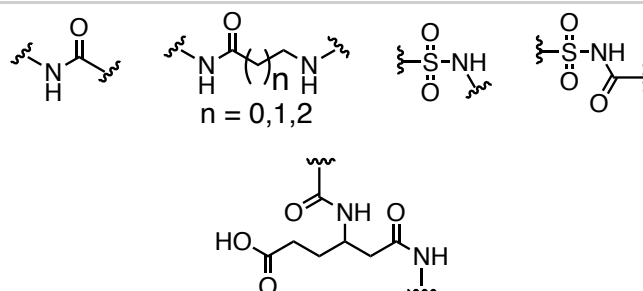
5 [21]

NMR & X-ray

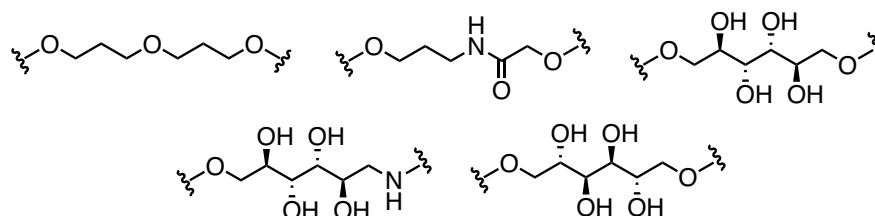


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Molecular modeling

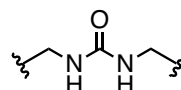


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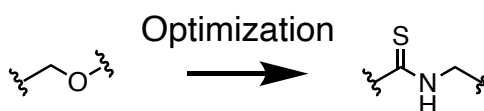


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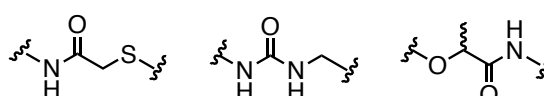
X-ray



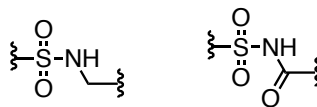
23 [53]



42 [76]



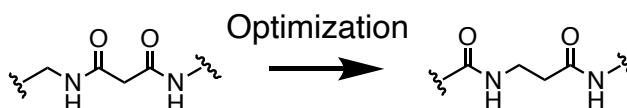
9 [29, 30]



21[48, 49]

X-ray &
molecular
modeling

22 [50-52]



41 [75]



* Ranking by calculating the internal-strain energies

** Molecular dynamics simulation

6. FUTURE PERSPECTIVES AND CONCLUSION

FBDD is a powerful method for the generation of new drug candidates, which so far has led to three FDA-approved drugs and to clinical trials for nearly fifty molecules. While strategies for developing an active molecule from a fragment are typically growing, linking and merging approaches, some examples reported here show that these strategies may be used one after another to increase the chances of success.

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3 Nevertheless, the literature clearly shows that the growing strategy is by far the most widely used
4 strategy, even if the linking strategy raised great expectations due to theoretical considerations.¹⁴ Are
5 there some experimental, technical difficulties that might explain the moderate success of the linking
6 strategy? The first step in this process is the identification of two fragments that bind the protein in two
7 distinct sub-sites. Screening a fragment library with a structural method offers a clear advantage in this
8 context. While both NMR and X-Ray crystallography techniques theoretically offer the opportunity to
9 directly identify two compounds that simultaneously bind the protein target at two different sites,
10 published examples highlight X-Ray crystallography as the most successful (or used) method. By
11 contrast, NMR is a method of choice for the iterative identification of a second-site binder.⁸⁰ However,
12 when using NMR as a screening technique, two screens are generally essential. Second-site screening
13 also offers the opportunity to identify new binding pockets, which may open the route for the design of
14 highly selective compound such as allosteric molecules. The optimization of the second-site ligands
15 might be necessary to increase the affinity of those ligands that are typically weaker binders than the
16 first-site ligands. Once the screening has been performed, the most appropriate method to guide the
17 linking process will depend on the distance between the two identified binders. NMR can be an
18 interesting approach for short distances, typically when the two fragments bind in adjacent pockets,
19 using ILOE 2D experiments that do not require protein labeling. We believe this strategy is particularly
20 suitable for catalytic sites of enzymes (Table 2, entries 8 & 9). For larger distances, which might be
21 observed for protein-protein interaction inhibitors or for bivalent compounds targeting two sites (for
22 example active site and allosteric site), modelisation and X-Ray crystallography are more efficient
23 techniques than NMR, as the linker design will necessitate the resolution of the complex 3D structure.
24 NMR would require labeled protein samples.

25
26 For the majority of the fragment-linking published examples, super-additivity is not observed. This
27 might explain why the growing approach remains the preferred strategy. Out of the forty-five examples
28 reported here, nine only fulfill the super-additivity criterium (Entries 2, 4, 18, 20, 25, 30, 32, 33 & 43),
29 including the well-known avidin case, one dimerization case and three self-assembly examples. This
30 shows that achieving super-additivity is challenging. In particular for non-catalytic sites such as protein-
31 protein interaction sites, protein conformational rearrangement upon compound binding might induce

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3 changes in the fragment binding modes. Translational and rotational entropy changes due to the linker
4 also need to be counteracted. It is not straightforward to understand what criteria (regarding the fragment
5 or the linker) lead to super-additivity. Previously, Whittaker and colleagues¹³ suggested that successful
6 linking could be achieved by carefully selecting the pair of fragments to be linked, with one forming
7 polar interactions with the protein while the second fragment should interact through van der Waals
8 interactions. The idea is that the binding mode of the fragment that does bind through nonpolar
9 interactions can be changed without penalty upon linking. There are examples in the literature that
10 contradict this assumption and successful cases here showed polar interactions for both fragments. To
11 our point of view, the linking strategy in FBDD essentially depends on the optimization of the two initial
12 fragments, the geometry required to link the fragments and the length of the linker. As observed for the
13 compounds for which the super-additivity was reached, the linkers are rather short and flexible, mainly
14 alkyl-derived linkers. Therefore, these findings tend to suggest that mixing the growing and linking
15 approaches could be a promising method to achieve the super-additivity. Growing one fragment helps
16 reduce the length of the linker required. Also, optimization of the fragments before linking may help
17 adapt the linker in case of protein conformational changes. Another promising approach consists in
18 mixing merging and linking strategies, where the linker is designed thanks to the imitation of chemical
19 groups observed in known ligands.
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Notes

The authors declare no competing financial interest.

Biographies

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Claire Raingeval received her Ph.D. from the Chemistry Department at the University of Lyon, France, in 2019, under the supervision of Dr Isabelle Krimm at the The Very High field NMR Center in Lyon. Her research was focused on fragment screening against the G protein-coupled receptors.

Thierry Lomberget obtained his Ph.D. in Organometallic Chemistry from University of Lyon in 2002 under the supervision of Dr. Geneviève Balme. After a two years' post-doctoral stay at the University of Geneva in the research group of Prof. E. Peter Kündig, he came back to Lyon as a temporary teacher/researcher and then was appointed as a lecturer at ISPB - Faculty of Pharmacy of Lyon in 2005. He was promoted as a full professor of medicinal chemistry in 2016 at the same institute. His actual main research interests are the design and optimization of anticancer agents, such as microtubule-targeting agents and kinases inhibitors, multidrug resistance modulators and anti-infective compounds.

Marc Le Borgne has his Ph.D. in medicinal chemistry under the supervision of Prof. Guillaume Le Baut at the University of Nantes (France) in 1997. He began his research and academic career in the School of Pharmacy at the University of Nantes. In 2008, he moved to the University of Lyon as a full professor in medicinal chemistry. He is currently the Head of the research Team Bioactive Molecules and Medicinal Chemistry (B2MC). His research interests focus on the design and synthesis of bioactive small molecules as anti-infective compounds or kinase-targeted anticancer therapeutic agents.

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9 He has been working on various therapeutic targets, using SBDD or FBDD approaches.
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20 **Isabelle Krimm** received her Ph.D. from the Chemistry Department in 2000 in the University of Lyon,
21 France. She served as a postdoctoral fellow at the CEA in Paris for 2 years in order to resolve the 3D
22 structure of the N-terminal part of the lamin protein, using NMR. She then joined a Chemistry laboratory
23 at the University of Lyon, and started a new group dedicated to fragment-based approaches for drug
24 design in 2013. In 2019, she joined as Principal investigator both the The Very High field NMR Center
25 in Lyon and the Center for Research in Cancer in Lyon.
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36 **ACKNOWLEDGMENTS**

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38 We thank the CNRS, the Ligue contre le cancer and ANR (Convergence PLAScan) for financial support.
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42 **ABBREVIATIONS USED**

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44 AMPA, α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; ACHE, acetylcholinesterase;
45 AdoMet, adenosylmethionine; AKT, protein kinase B; ATP, adenosine triphosphate; BACE1, beta-
46 secretase 1; BCL-2, B-cell lymphoma 2; BCL-X_L, B-cell lymphoma-extra-large; BET, bromo- and
47 extra-terminal domain family; BRAF, v-raf murine sarcoma viral oncogene homolog B; BRD2,
48 bromodomain-containing protein 2; c-SRC, proto-oncogene tyrosine-protein kinase SRC; CA, carbonic
49 anhydrase; CASP3, caspase 3; CDK1/2/4/5/9, cyclin-dependent kinases 1/2/4/5/9; cIAP1, cellular
50 inhibitor of apoptosis protein 1; CK2, casein kinase 2; CSF1R, colony stimulating factor 1 receptor;
51 CYPD, cyclophilin D; DCC, dynamic combinatorial chemistry; DENV, dengue virus; DLS, dynamic
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3 ligation screening; DOT1L, disruptor of telomeric silencing 1-like; ERK1/2, mitogen-activated protein
4 kinase 3/1; EthR, *Mycobacterium tuberculosis* transcriptional repressor; FAXS, fluorine chemical shift
5 anisotropy and exchange for screening; FBDD, Fragment-based drug discovery; FDA, Food and Drug
6 Administration; FGFR, fibroblast growth factor receptors; FKBP, FK506-binding protein; FLT3, fms-
7 like tyrosine kinase 3; FXa, factor X; HDAC, histone deacetylases; HIV-1, human immunodeficiency
8 virus 1; HSP90, heat shock protein 90; HTS, high-throughput screening; IDO1, indoleamine-pyrrole
9 2,3-dioxygenase; IL-2, interleukin 2; ILOEs, inter-ligand NOEs; IMPDH, inosine-5'-monophosphate
10 dehydrogenase; IRAK4, interleukin-1 receptor-associated kinase 4; JAK2, Janus kinase 2; KHK,
11 ketohexokinase; KIT, tyrosine-protein kinase KIT; KRAS, Kirsten rat sarcoma viral oncogene; LDHA,
12 lactate dehydrogenase A; LFA1, lymphocyte function-associated antigen 1; LTA4H, leukotriene A4
13 hydrolase; MCL1, induced myeloid leukemia cell differentiation protein; MET, tyrosine-protein kinase
14 MET; mGlu5, metabotropic glutamate receptor 5; MNK1/2, MAP kinase-interacting serine/threonine-
15 protein kinase 1/2; MMP2/9, matrix metalloproteinase 2/9; mRNA, messenger ribonucleic acid; MS,
16 Mass Spectrometry; MTase, methyltransferase; NA, neuraminidase; NADH, nicotinamide adenine
17 dinucleotide; NMR, Nuclear Magnetic Resonance; NMT1, *N*-myristoyltransferase 1; NOE, nuclear
18 overhauser effect; NOESY, nuclear overhauser effect spectroscopy; PKM2, pyruvate kinase M2; PPAR,
19 peroxisome proliferator-activated receptor; PRC2, polycomb repressive complex 2; PRE, paramagnetic
20 enhancement; PS, pantothenate synthetase; PTCC, protein-templated click chemistry; PTP1B, protein
21 tyrosine phosphatase-1B; RAF, rapidly accelerated fibrosarcoma; ROCK, rho-associated protein kinase;
22 RPA, replication protein A; rRNA, ribosomal ribonucleic acid; S6K1, ribosomal protein S6 kinase beta-
23 1; SAM, S-adenosylmethionine; SAR, structure-activity relationships; SmChiB, *Serratia marcescens*
24 chitinase B; SPR, surface plasmon resonance; STD, Saturation Transfer Difference; TEMPO, 2,2,6,6-
25 tetramethylpiperidin-1-oxyl; VRE, vancomycin-resistant *enterococci*; XIAP, x-linked inhibitor of
26 apoptosis protein
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54
55
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57
58
59
60

REFERENCES

- (1) Rees, D. C.; Congreve, M.; Murray, C. W.; Carr, C. Fragment-based lead discovery. *Nature*. **2004**, *3*, 660-672.
- (2) Hajduk, P. J.; Greer, J. A decade of fragment-based drug design: strategic advances and lessons learned. *Nat. Rev. Drug Discov.* **2007**, *6*, 211-219.
- (3) Chessari, G.; Woodhead, A. J. From fragment to clinical candidate - a historical perspective. *Drug Discov. Today*. **2009**, *14*, 668-675.
- (4) Murray, C. W.; Blundell, T. L. Structural biology in fragment-based drug design. *Curr. Opin. Struct. Biol.* **2010**, *20*, 497-507.
- (5) Erlanson, D. A.; Fesik, S. W.; Hubbard, R. E.; Jahnke, W.; Jhoti, H. Twenty years on: the impact of fragments on drug discovery. *Nat. Rev. Drug Discov.* **2016**, *15*, 605-619.
- (6) Mortenson, P. N.; Erlanson, D. A.; de Esch, I. J. P.; Jahnke, W.; and Johnson, C. N. Fragment-to-lead medicinal chemistry publications in 2017. *J. Med. Chem.* **2019**, *62*, 3857-3872.
- (7) Congreve, M.; Carr, R.; Murray, C.; Jhoti, H. A "Rule of three" for fragment-based lead discovery. *Drug Discov. Today*. **2003**, *8*, 876-877.
- (8) Bollag, G.; Hirth, P.; Tsai, J.; Zhang, J.; Ibrahim, P. N.; Cho, H.; Spevak, W.; Zhang, C.; Zhang, Y.; Habets, G.; Burton, E. A.; Wong, B.; Tsang, G.; West, B. L.; Powell, B.; Shellooe, R.; Marimuthu, A.; Nguyen, H.; Zhang, K. Y.; Artis, D. R.; Schlessinger, J.; Su, F.; Higgins, B.; Iyer, R.; D'Andrea, K.; Koehler, A.; Stumm, M.; Lin, P. S.; Lee, R. J.; Grippo, J.; Puzanov, I.; Kim, K. B.; Ribas, A.; McArthur, G. A.; Sosman, J. A.; Chapman, P. B.; Flaherty, K. T.; Xu, X.; Nathanson, K. L.; Nolop, K. Clinical efficacy of a RAF inhibitor needs broad target blockade in BRAF-mutant melanoma. *Nature*. **2010**, *467*, 596-599.
- (9) Souers, A. J.; Levenson, J. D.; Boghaert, E. R.; Ackler, S. L.; Catron, N. D.; Chen, J.; Dayton, B. D.; Ding, H.; Enschede, S. H.; Fairbrother, W. J.; Huang, D. C. S.; Hymowitz, S. G.; Jin, S.; Khaw, S. L.; Kovar, P. J.; Lam, L. T.; Lee, J.; Maecker, H. L.; Marsh, K. C.; Mason, K. D.; Mitten, M. J.; Nimmer, P. M.; Oleksijew, A.; Park, C. H.; Park, C.; Phillips, D. C.; Roberts, A. W.; Sampath, D.; Seymour, J. F.; Smith, M. L.; Sullivan, G. M.; Tahir, S. K.; Tse, C.; Wendt, M. D.; Xiao, Y.; Xue, J. C.; Zhang, H.; Humerickhouse, R. A.; Rosenberg, S. H.; Elmore, S. W. ABT-199, a potent and selective BCL-2 inhibitor, achieves antitumor activity while sparing platelets. *Nat. Med.* **2013**, *19*, 202-208.
- (10) Murray, C. W.; Newell, D. R.; Angibaud, P. A successful collaboration between academia, biotech and pharma led to discovery of erdafitinib, a selective FGFR inhibitor recently approved by the FDA. *MedChemComm.* **2019**, *10*, 1509-1511.
- (11) Shuker, S. B.; Hajduk, P. J.; Meadows, R. P.; Fesik, S. W. Discovering high-affinity ligands for proteins : SAR by NMR. *Science*. **1996**, *274*, 1531-1534.
- (12) Hajduk, P. J.; Meadows, R. P.; Fesik, S. W. Discovering high-affinity ligands for proteins. *Science*. **1997**, *278*, 497-499.

- 1
2
3 (13) Ichihara, O.; Barker, J.; Law, R. J.; Whittaker, M. Compound design by fragment-linking. *Mol. Inform.* **2011**, *30*,
4 298-306.
5
6 (14) Jencks, W. On the attribution and additivity of binding energies. *Proc. Natl. Acad. Sci. U.S.A.* **1981**, *78* (7), 4046-
7 4050.
8
9 (15) Hajduk, P. J.; Sheppard, G.; Nettlesheim, D. G.; Olejniczak, E. T.; Shuker, S. B.; Meadows, R. P.; Steinman, D. H.;
10 Carrera, G. M.; Marcotte, P. A.; Severin, J.; Walter, K.; Smith, H.; Gubbins, E.; Simmer, R.; Holzman, T. F.; Morgan,
11 D. W.; Davidsen, S. K.; Summers, J. B.; Fesik, S. W. Discovery of potent nonpeptide inhibitors of stromelysin using
12 SAR by NMR. *J. Am. Chem. Soc.* **1997**, *119*, 5818-5827.
13
14 (16) Oltersdorf, T.; Elmore, S. W.; Shoemaker, A. R.; Armstrong, R. C.; Augeri, D. J.; Belli, B. A.; Bruncko, M.;
15 Deckwerth, T. L.; Dinges, J.; Hajduk, P. J.; Joseph, M. K.; Kitada, S.; Korsmeyer, S. J.; Kunzer, A. R.; Letai, A.; Li,
16 C.; Mitten, M. J.; Nettlesheim, D. G.; Ng, S.; Nimmer, P. M.; O'Connor, J. M.; Oleksijew, A.; Petros, A. M.; Reed, J.
17 C.; Shen, W.; Tahir, S. K.; Thompson, C. B.; Tomaselli, K. J.; Wang, B.; Wendt, M. D.; Zhang, H.; Fesik, S. W.;
18 Rosenberg, S. H. An inhibitor of BCL-2 family proteins induces regression of solid tumours. *Nature.* **2005**, *435*, 677-
19 681.
20
21 (17) Petros, A. M.; Dinges, J.; Augeri, D. J.; Baumeister, S. A.; Betebenner, D. A.; Bures, M. G.; Elmore, S. W.; Hajduk,
22 P. J.; Joseph, M. K.; Landis, S. K.; Nettlesheim, D. G.; Rosenberg, S. H.; Shen, W.; Thomas, S.; Wang, X.; Zanze, I.;
23 Zhang, H.; Fesik, S. W. Discovery of a potent inhibitor of the antiapoptotic protein Bcl-X_L from NMR and parallel
24 synthesis. *J. Med. Chem.* **2006**, *49*, 656-663.
25
26 (18) Tse, C.; Shoemaker, A. R.; Adickes, J.; Anderson, M. G.; Chen, J.; Jin, S.; Johnson, E. F.; Marsh, K. C.; Mitten, M.
27 J.; Nimmer, P.; Roberts, L.; Tahir, S. K.; Xiao, Y.; Yang, X.; Zhang, H.; Fesik, S.; Rosenberg, S. H.; Elmore, S. W.
28 ABT-263: A potent and orally bioavailable Bcl-2 family inhibitor. *Cancer Res.* **2008**, *68*, 3421-3428.
29
30 (19) Petros, A. M.; Huth, J. R.; Oost, T.; Park, C. M.; Ding, H.; Wang, X.; Zhang, H.; Nimmer, P.; Mendoza, R.; Sun, C.;
31 Mack, J.; Walter, K.; Dorwin, S.; Gramling, E.; Lador, U.; Rosenberg, S. H.; Elmore, S. W.; Fesik, S. W.; Hajduk,
32 P. J. Discovery of a potent and selective Bcl-2 inhibitor using SAR by NMR. *Bioorg. Med. Chem. Lett.* **2010**, *20*,
33 6587-6591.
34
35 (20) Tao, Z. F.; Hasvold, L.; Wang, L.; Wang, X.; Petros, A. M.; Park, C. H.; Boghaert, E. R.; Catron, N. D.; Chen, J.;
36 Colman, P. M.; Czabotar, P. E.; Deshayes, K.; Fairbrother, W. J.; Flygare, J. A.; Hymowitz, S. G.; Jin, S.; Judge, R.
37 A.; Koehler, M. F.; Kovar, P. J.; Lessene, G.; Mitten, M. J.; Ndubaku, C. O.; Nimmer, P.; Purkey, H. E.; Oleksijew
38 A.; Phillips, D. C.; Sleebs, B. E.; Smith, B. J.; Smith, M. L.; Tahir, S. K.; Watson, K. G.; Xiao, Y.; Xue, J.; Zhang,
39 H.; Zobel, K.; Rosenberg, S. H.; Tse, C.; Levenson, J. D.; Elmore, S. W.; Souers, A. J. Discovery of a potent and
40 selective Bcl-X_L inhibitor with in vivo activity. *ACS Med. Chem. Lett.* **2014**, *5*, 1088-1093.
41
42 (21) Huth, J. R.; Park, C.; Petros, A. M.; Kunzer, A. R.; Wendt, M. D.; Wang, X.; Lynch, C. L.; Mack, J. C.; Swift, K.
43 M.; Judge, R. A.; Chen, J.; Richardson, P. L.; Jin, S.; Tahir, S. K.; Matayoshi, E. D.; Dorwin, S. A.; Lador, U. S.;
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

- 1
2
3 Severin, J. M.; Walter, K. A.; Bartley, D. M.; Fesik, S. W.; Elmore, S. W.; Hajduk, P. J. Discovery and Design of
4 Novel HSP90 inhibitors using multiple fragment-based design strategies. *Chem. Biol. Drug. Des.* **2007**, *70*, 1-12.
5
6 (22) Szczepankiewicz, B. G.; Liu, G.; Hajduk, P. J.; Abad-Zapatero, C.; Pei, Z.; Xin, Z.; Lubben, T. H.; Trevillyan, J. M.;
7 Stashko, M. A.; Ballaron, S. J.; Liang, H.; Huang, F.; Hutchins, C. W.; Fesik, S. W.; Jirousek, M. R. Discovery of a
8 potent, selective protein tyrosine phosphatase 1B inhibitor using a linked-fragment strategy. *J. Am. Chem. Soc.* **2003**,
9 *125*, 4087-4096.
10
11 (23) Liu, G.; Xin, Z.; Pei, Z.; Hajduk, P. J.; Abad-Zapatero, C.; Hutchins, C. W.; Zhao, H.; Lubben, T. H.; Ballaron, S. J.;
12 Haasch, D. L.; Kaszubska, W.; Rondinone, C. M.; Trevillyan, J. M.; Jirousek, M. R. Fragment screening and
13 assembly: a highly efficient approach to a selective and cell active protein tyrosine phosphatase 1B inhibitor. *J. Med.*
14 *Chem.* **2003**, *46*, 4232-4235.
15
16 (24) Krimm, I. INPHARMA-based identification of ligand binding site in fragment-based drug design. *MedChemComm.*
17 **2012**, *3*, 605-610.
18
19 (25) Li, D.; DeRose, E. F.; London R. E. The inter-ligand Overhauser effect: a powerful new NMR approach for mapping
20 structural relationships of macromolecular ligands. *J. Biomol. NMR.* **1999**, *15*, 71-76.
21
22 (26) Leone, M.; Freeze, H. H.; Chan, C. S.; Pellechia, M. The Nuclear Overhauser Effect in the lead identification process.
23 *Curr. Drug Discov. Technol.* **2006**, *3*, 91-100.
24
25 (27) Becattini, B.; Culmsee, C.; Leone, M.; Zhai, D.; Zhang, X.; Crowell, K. J.; Rega, M. F.; Landshamer S.; Reed, J. C.;
26 Plesnila, N.; Pellecchia, M. Structure-activity relationships by interligand NOE-based design and synthesis of
27 antiapoptotic compounds targeting Bid. *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103*, 12602-12606.
28
29 (28) Rega, M. F.; Wu, B.; Wei, J.; Zhang, Z.; Cellitti, J. F.; Pellecchia, M. SAR by interligand nuclear overhauser effects
30 (ILOEs) based discovery of acylsulfonamide compounds active against Bcl-X_L and Mcl-1. *J. Med. Chem.* **2011**, *54*,
31 6000-6013.
32
33 (29) Sledz, P.; Silvestre H. L.; Hung, A. W.; Ciulli, A.; Blundell, T. L.; Abell, C. Optimization of the interligand
34 Overhauser effect for fragment linking: application to inhibitor discovery against Mycobacterium tuberculosis
35 pantothenate synthetase. *J. Am. Chem. Soc.* **2010**, *132*, 4544-4545.
36
37 (30) Hung, A. W.; Sivistre, H. L.; Wens, S.; Ciulli, A.; Blundell, T. L.; Abell, C. Application of fragment growing and
38 fragment linking to the discovery of inhibitors of Mycobacterium tuberculosis pantothenate synthetase. *Angew.*
39 *Chem. Int. Ed. Engl.* **2009**, *48*, 8452-8456.
40
41 (31) Jahnke, W. Spin labels as a tool to identify and characterize protein-ligand interactions by NMR spectroscopy.
42 *ChemBioChem.* **2002**, *3*, 167-173.
43
44 (32) Jahnke, W.; Florsheimer, A.; Blommers, M. J.; Paris, C. G.; Heim, J.; Nalin, C.M.; Perez, L. B. Second-site NMR
45 screening and linker design. *Curr. Top. Med. Chem.* **2003**, *3*, 69-80.
46
47 (33) Vazquez, J.; Tautz, L.; Ryan, J. J.; Vuori, K.; Mustelin, T.; Pellecchia, M. Development of molecular probes for
48 second-site screening and design of protein tyrosine phosphatase inhibitors. *J. Med. Chem.* **2007**, *50*, 2137-2143.
49
50
51
52
53
54
55
56
57
58
59
60

- 1
2
3 (34) Vazquez, J.; De, S. K.; Chen, L. H.; Riel-Mehan, M.; Emdadi, A.; Cellitti, J.; Stebbins, J. L.; Rega, M. F.; Pellecchia,
4 M. Development of paramagnetic probes for molecular recognition studies in protein kinases. *J. Med. Chem.* **2008**,
5 *51*, 3460-3465.
6
7
8 (35) Egger, J.; Weckerle, C.; Cutting, B.; Schwardt, O.; Rabbani, S.; Lemme, K.; Ernst, B. Nanomolar E-selectin
9 antagonists with prolonged half-lives by a fragment-based approach. *J. Am. Chem. Soc.* **2013**, *135*, 9820-9828.
10
11 (36) Cheng, Y.; Judd, T. C.; Bartberger, M. D.; Brown, J.; Chen, K.; Fremeau, R. T. Jr.; Hickman, D.; Hitchcock, S. A.;
12 Jordan, B.; Li, V.; Lopez, P.; Louie, S. W.; Luo, Y.; Michelsen, K.; Nixey, T.; Powers, T. S.; Rattan, C.; Sickmier,
13 E. A.; St Jean, D. J. Jr.; Wahl, R. C.; Wen, P. H.; Wood, S. From fragment screening to in vivo efficacy: optimization
14 of a series of 2-aminoquinolines as potent inhibitors of beta-site amyloid precursor protein cleaving enzyme 1
15 (BACE1). *J. Med. Chem.* **2011**, *54*, 5836-5857.
16
17 (37) Jordan, J. B.; Whittington, D. A.; Bartberger, M. D.; Sickmier, E. A.; Chen, K.; Cheng, Y.; Judd, T. A fragment-
18 linking approach using 19F NMR spectroscopy to obtain highly potent and selective inhibitors of β -secretase. *J. Med.*
19 *Chem.* **2016**, *59*, 3732-3749.
20
21 (38) Casale, E.; Amboldi, N.; Brasca, M. G.; Caronni, D.; Colombo, N.; Dalvit, C.; Felder, E. R.; Fogliatto, G.; Galvani,
22 A.; Isacchi, A.; Polucci, P.; Riceputi, L.; Sola, F.; Visco, C.; Zuccotto, F.; Casuscelli, F. Fragment-based hit discovery
23 and structure-based optimization of aminotriazoloquinazolines as novel HSP90 inhibitors. *Bioorg. Med. Chem.* **2014**,
24 *22*, 4135-4150.
25
26 (39) Kohlmann, A.; Zech, S. G.; Li, F.; Zhou, T.; Squillace, R. M.; Commodore, L.; Greenfield, M. T.; Lu, X.; Miller, D.
27 P.; Huang, W. S.; Qi, J.; Thomas, R. M.; Wang, Y.; Zhang, S.; Dodd, R.; Liu, S.; Xu, R.; Xu, Y.; Miret, J. J.; Rivera,
28 V.; Clackson, T.; Shakespeare, W. C.; Zhu, X.; Dalgarno, D. C. Fragment growing and linking lead to novel
29 nanomolar lactate dehydrogenase inhibitors. *J. Med. Chem.* **2013**, *56*, 1023-1040.
30
31 (40) Erlanson, D. A.; Braisted, A. C.; Raphael, D. R.; Randal, M.; Stroud, R. M.; Gordon, E. M.; Wells, J. A. Site-directed
32 ligand discovery. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 9367-9372.
33
34 (41) Erlanson, D. A.; Lam, J. W.; Wiesmann, C.; Luong, T. N.; Simmons, R. L.; DeLano, W. L.; Choong, I. C.; Burdett,
35 M. T.; Flanagan, W. M.; Lee, D.; Gordon, E. M.; O'Brien T. In situ assembly of enzyme inhibitors using extended
36 tethering. *Nat. Biotechnol.* **2003**, *21*, 308-314.
37
38 (42) Braisted, A. C.; Oslob, J. D.; Delano, W. L.; Hyde, J.; McDowell, R. S.; Waal, N.; Yu, C.; Arkin, M. R.; Raimundo,
39 B. C. Discovery of a potent small molecule IL-2 inhibitor through fragment assembly. *J. Am. Chem. Soc.* **2003**, *125*,
40 3714-3715.
41
42 (43) Ward, R. A.; Brassington, C.; Breeze, A. L.; Caputo, A.; Critchlow, S.; Davies, G.; Goodwin, L.; Hassall, G.;
43 Greenwood, R.; Holdgate, G. A.; Mrosek, M.; Norman, R. A.; Pearson, S.; Tart, J.; Tucker, J. A.; Vogtherr, M.;
44 Whittaker, D.; Wingfield, J.; Winter, J.; Hudson, K. Design and Synthesis of novel lactate dehydrogenase A inhibitors
45 by fragment-based lead generation. *J. Med. Chem.* **2012**, *55*, 3285-3306.
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

- 1
2
3 (44) Möbitz, H.; Machauer, R.; Holzer, P.; Vaupel, A.; Stauffer, F.; Ragot, C.; Caravatti, G.; Scheufler, C.; Fernandez,
4 C.; Hommel, U.; Tiedt, R.; Beyer, K. S.; Chen, C.; Zhu, H.; Gaul, C. The discovery of potent, selective and
5 structurally novel DOT1L inhibitors by a fragment linking approach. *ACS Med. Chem. Lett.* **2017**, *8*, 338-343.
6
7
8 (45) Howard, N.; Abell, C.; Blakemore, W.; Chessari, G.; Congreve, M.; Howard, S.; Jhoti, H.; Murray, C. W.; Seavers,
9 L. C.; van Montfort, R. L. Application of fragment screening and fragment linking to the discovery of novel thrombin
10 inhibitors. *J. Med. Chem.* **2006**, *49*, 1346-1355.
11
12
13 (46) Barker, J. J.; Barker, O.; Courtney, S. M.; Gardiner, M.; Hesterkamp, T.; Ichihara, O.; Mather, O.; Montalbetti, C.
14 A.; Müller, A.; Varasi, M.; Whittaker, M.; Yarnold, C. J. Discovery of a novel HSP90 inhibitor by fragment linking.
15 *ChemMedChem.* **2010**, *5*, 1697-1700.
16
17
18 (47) Ahmed-Belkacem, A.; Colliandre, L.; Ahnou, N.; Nevers, Q.; Gelin, M.; Bessin, Y.; Brillet, R.; Cala, O.; Douguet,
19 D.; Bourguet, W.; Krimm, I.; Pawlotsky, J. M.; Guichou, J. F. Fragment-based discovery of a new family of non-
20 peptidic small-molecule cyclophilin inhibitors with potent antiviral activities. *Nat. Commun.* **2016**, *7*, 12777.
21
22
23 (48) Benmansour, F.; Trist, I.; Coutard, B.; Decroly, E.; Querat, G.; Brancale, A.; Barral, K. Discovery of novel dengue
24 virus NS5 methyltransferase non-nucleoside inhibitors by fragment-based drug design. *Eur. J. Med. Chem.* **2017**,
25 *125*, 865-880.
26
27
28 (49) Hernandez, J.; Hoffer, L.; Coutard, B.; Querat, G.; Roche, P.; Morelli, X.; Decroly, E.; Barral, K. Optimization of a
29 fragment linking hit toward Dengue and Zika virus NS5 methyltransferases inhibitors. *Eur. J. Med. Chem.* **2019**,
30 *161*, 323-333.
31
32
33 (50) Brear, P.; De Fusco, C.; Hadje Georgiou, K.; Francis-Newton, N. J.; Stubbs, C. J.; Sore, H. F.; Venkitaraman, A. R.;
34 Abell, C.; Spring, D. R.; Hyvönen, M. Specific inhibition of CK2 α from an anchor outside the active site. *Chem. Sci.*
35 **2016**, *7*, 6839-6845.
36
37
38 (51) De Fusco, C.; Brear, P.; Iegre, J.; Georgiou, K. H.; Sore, H. F.; Hyvönen, M.; Spring, D. R. A fragment-based
39 approach leading to the discovery of a novel binding site and the selective CK2 inhibitor CAM4066. *Bioorg. Med.*
40 *Chem.* **2017**, *25*, 3471-3482.
41
42
43 (52) Iegre, J.; Brear, P.; De Fusco, C.; Yoshida, M.; Mitchell, S. L.; Rossmann, M.; Carro, L.; Sore, H. F.; Hyvönen, M.;
44 Spring, D. R. Second-generation CK2 α inhibitors targeting the α D pocket. *Chem. Sci.* **2018**, *9*, 3041-3049.
45
46
47 (53) Frank, A. O.; Feldkamp, M. D.; Kennedy, J. P.; Waterson, A. G.; Pelz, N. F.; Patrone, J. D.; Vangamudi, B.; Camper,
48 D. V.; Rossanese, O. W.; Chazin, W. J.; Fesik, S. W. Discovery of a potent inhibitor of replication protein A protein-
49 protein interactions using a fragment-linking approach. *J. Med. Chem.* **2013**, *56*, 9242-9250.
50
51
52 (54) Swayze, E. E.; Jefferson, E. A.; Sannes-Lowery, K. A.; Blyn, L. B.; Risen, L. M.; Arakawa, S.; Osgood, S. A.;
53 Hofstadler, S. A.; Griffey, R. H. SAR by MS: a ligand based technique for drug lead discovery against structured
54 RNA targets. *J. Med. Chem.* **2002**, *45*, 3816-3819.
55
56
57 (55) Green, N. M. Avidin. *Adv. Protein Chem.* **1975**, *29*, 85-133.
58
59
60

- 1
2
3 (56) Maly, D. J.; Choong, I. C.; Ellman, J. A. Combinatorial target-guided ligand assembly: identification of potent
4 subtype-selective c-SRC inhibitors. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 2419-2424.
5
6 (57) Huc, I.; Lehn, J. M. Virtual combinatorial libraries: Dynamic generation of molecular and supramolecular
7 diversity by self-assembly. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 2106-2110.
8
9 (58) Hochgürtel, M.; Kroth, H.; Piecha, D.; Hofmann, M. W.; Nicolau, C.; Krause, S.; Schaaf, O.; Sonnenmoser, G.;
10 Eliseev, A. V. Target-induced formation of neuraminidase inhibitors from in vitro virtual combinatorial libraries.
11 *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 3382-3387.
12
13 (59) Hochgürtel, M.; Biesinger, R.; Kroth, H.; Piecha, D.; Hofmann, M. W.; Krause, S.; Schaaf, O.; Nicolau, C.; Eliseev,
14 A. V. Ketones as building blocks for dynamic combinatorial libraries: highly active neuraminidase inhibitors
15 generated via selection pressure of the biological target. *J. Med. Chem.* **2003**, *46*, 356-358.
16
17 (60) Mondal, M.; Radeva, N.; Köster, H.; Park, A.; Potamitis, C.; Zervou, M.; Klebe, G.; Hirsch, A. K. Structure-based
18 design of inhibitors of the aspartic protease endothiapepsin by exploiting dynamic combinatorial chemistry. *Angew.*
19 *Chem. Int. Ed. Engl.* **2014**, *53*, 3259-3263.
20
21 (61) Congreve, M. S.; Davis, D. J.; Devine, L.; Granata, C.; O'Reilly, M.; Wyatt, P. G.; Jhoti, H. Detection of ligands
22 from a dynamic combinatorial library by X-ray crystallography. *Angew. Chem. Int. Ed. Engl.* **2003**, *42*, 4479-4482.
23
24 (62) Schmidt, M. F.; El-Dahshan, A.; Keller, S.; Rademann, J. Selective identification of cooperatively binding fragments
25 in a high-throughput ligation assay enables development of a picomolar caspase-3 inhibitor. *Angew. Chem. Int. Ed.*
26 *Engl.* **2009**, *48*, 6346-6349.
27
28 (63) Jaegle, M.; Steinmetzer, T.; Rademann, J. Protein-templated formation of an inhibitor of the blood coagulation factor
29 Xa through a background-free amidation reaction. *Angew. Chem. Int. Ed. Engl.* **2017**, *56*, 3718-3722.
30
31 (64) Lewis, W. G.; Green, L. G.; Grynspan, F.; Radić, Z.; Carlier, P. R.; Taylor, P.; Finn, M. G.; Sharpless, K. B. Click
32 chemistry in situ: acetylcholinesterase as a reaction vessel for the selective assembly of a femtomolar inhibitor from
33 an array of building blocks. *Angew. Chem. Int. Ed. Engl.* **2002**, *41*, 1053-1057.
34
35 (65) Suzuki, T.; Ota, Y.; Kasuya, Y.; Mutsuga, M.; Kawamura, Y.; Tsumoto, H.; Nakagawa, H.; Finn, M. G.; Miyata, N.
36 An unexpected example of protein-templated click chemistry. *Angew. Chem. Int. Ed. Engl.* **2010**, *49*, 6817-6820.
37
38 (66) Hirose, T.; Maita, N.; Gouda, H.; Koseki, J.; Yamamoto, T.; Sugawara, A.; Nakano, H.; Hirono, S.; Shiomi, K.;
39 Watanabe, T.; Taniguchi, H.; Sharpless, K. B.; Omura, S.; Sunazuka, T. Observation of the controlled assembly of
40 preclick components in the in situ click chemistry generation of a chitinase inhibitor. *Proc. Natl. Acad. Sci. U.S.A.*
41 **2013**, *110*, 15892-15897.
42
43 (67) Mondal, M.; Unver, M. Y.; Pal, A.; Bakker, M.; Berrier, S. P.; Hirsch, A. K. H. Fragment-based drug design
44 facilitated by protein-templated click chemistry: fragment linking and optimization of inhibitors of the aspartic
45 protease endothiapepsin. *Chem. Eur. J.* **2016**, *22*, 14826-14830.
46
47 (68) Hu, X.; Sun, J.; Wang, H. G.; Manetsch, R. Bcl-X_L-templated assembly of its own protein-protein interaction
48 modulator from fragments decorated with thio acids and sulfonyl azides. *J. Am. Chem. Soc.* **2008**, *130*, 13820-13821.
49
50
51
52
53
54
55
56
57
58
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2
3 (69) Kulkarni, S. S.; Hu, X.; Doi, K.; Wang, H. G.; Manetsch, R. Screening of protein-protein interaction modulators via
4 sulfo-click kinetic target-guided synthesis. *ACS Chem. Biol.* **2011**, *6*, 724-723.
- 5
6 (70) Chen, H.; Zhou, X.; Wang, A.; Zheng, Y.; Gao, Y.; Zhou, J. Evolutions in fragment-based drug design : the
7 deconstruction-reconstruction approach. *Drug Discov. Today.* **2015**, *20*, 105-113.
- 8
9 (71) Mousnier, A.; Bell, A. S.; Swieboda, D. P.; Morales-Sanfrutos, J.; Pérez-Dorado, I.; Brannigan, J. A.; Newman, J.;
10 Ritzefeld, M.; Hutton, J. A.; Guedán, A.; Asfor, A. S.; Robinson, S. W.; Hopkins-Navratilova, I.; Wilkinson, A. J.;
11 Johnston, S. L.; Leatherbarrow, R. J.; Tuthill, T. J.; Solari, R.; Tate, E. W. Fragment-derived inhibitors of human N-
12 myristoyltransferase block capsid assembly and replication of the common cold virus. *Nat. Chem.* **2018**, *10*, 599-
13 606.
- 14
15 (72) Taylor, S. J.; Padyana, A. K.; Abeywardane, A.; Liang, S.; Hao, M. H.; De Lombaert, S.; Proudfoot, J.; Farmer, B.
16 S. 3rd.; Li, X.; Collins, B.; Martin, L.; Albaugh, D. R.; Hill-Drzewi, M.; Pullen, S. S.; Takahashi, H. Discovery of
17 potent, selective chymase inhibitors via fragment linking strategies. *J. Med. Chem.* **2013**, *56*, 4465-4481.
- 18
19 (73) Davidson, A.; Begley, D. W.; Lau, C.; Varani, G. A small molecule probe induces a conformation in HIV TAR RNA
20 capable of binding drug-like fragments. *J. Mol. Biol.* **2011**, *410*, 984-996.
- 21
22 (74) Pang, Y. P.; Quiram, P.; Jelacic, T.; Hong, F.; Brimijoin, S. Highly potent, selective, and low cost bis-
23 tetrahydroaminacrine inhibitors of acetylcholinesterase. Steps toward novel drugs for treating Alzheimer's disease.
24 *J. Biol. Chem.* **1996**, *271*, 23646-23649.
- 25
26 (75) Surade, S.; Nancy, T. Y.; Hengrung, N.; Lechartier, B.; Cole, S. T.; Abell, C.; Blundell, T. L. A structure-guided
27 fragment-based approach for the discovery of allosteric inhibitors targeting the lipophilic binding site of transcription
28 factor EthR. *Biochem. J.* **2014**, *458*, 387-394.
- 29
30 (76) Trapero, A.; Pacitto, A.; Singh, V.; Sabbah, M.; Coyne, A. G.; Mizrahi, V.; Blundell, T. L.; Ascher, D. B.; Abell, C.
31 A fragment-based approach to targeting inosine-5'-monophosphate dehydrogenase (IMPDH) from *Mycobacterium*
32 *tuberculosis*. *J. Med. Chem.* **2018**, *61*, 2806-2822.
- 33
34 (77) Burgess, L. E.; Newhouse, B. J.; Ibrahim, P.; Rizzi, J.; Kashem, M. A.; Hartman, A.; Brandhuber, B. J.; Wright, C.
35 D.; Thomson, D. S.; Vigers, G. P.; Koch, K. Potent selective nonpeptidic inhibitors of human lung tryptase. *Proc.*
36 *Natl. Acad. Sci. U.S.A.* **1999**, *96*, 8348-8352.
- 37
38 (78) Matsui, Y.; Yasumatsu, I.; Asahi, T.; Kitamura, T.; Kanai, K.; Ubukata, O.; Hayasaka, H.; Takaishi, S.; Hanzawa,
39 H.; Katakura, S. Discovery and structure-guided fragment-linking of 4-(2,3-dichlorobenzoyl)-1-methyl-pyrrole-2-
40 carboxamide as a pyruvate kinase M2 activator. *Bioorg. Med. Chem.* **2017**, *25*, 3540-3546.
- 41
42 (79) Drapier, T.; Geubelle, P.; Bouckaert, C.; Nielsen, L.; Laulumaa, S.; Goffin, E.; Dilly, S.; Francotte, P.; Hanson, J.;
43 Pochet, L.; Kastrop, J.; Pirotte, B. Enhancing action of positive allosteric modulators through the design of dimeric
44 compounds. *J. Med. Chem.* **2018**, *61*, 5279-5291.
- 45
46 (80) Sun, Q.; Phan, J.; Friberg, A. R.; Camper, D. V.; Olejniczak, E. T.; Fesik, S. W. A method for the second-site
47 screening of K-Ras in the presence of a covalently attached first-site ligand. *J. Biomol. NMR.* **2014**, *60*, 11-14.
- 48
49
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