Examining molecular factors of inactive versus active bivalent EGFR inhibitors: A missing link in fragment-based drug design.

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Abstract: The optimization of linkers that connect fragments within drug binding sites represents an impediment in fragment-based drug discovery (FBDD). To improve our understand of the molecular factors that enable effective fragment linking, we have produced a series of compounds that bind to the ATP and allosteric sites of the EGFR kinase domain connected by two distinct linker structures. We find the linker is responsible for opposing impacts on potency against EGFR mutants, the most potent of which are active in human cancer cells. Comparison of X-ray cocrystal structures of active versus inactive molecules provide unique experimentally derived insights into linker design criteria such as how fragment flexibility and intermolecular interactions can serve compound design broadly in drug optimization.

Fragment-based drug discovery (FBDD) consists of several strategies to develop highly effective lead compounds against protein targets leveraging structure-based methods.[1-3] Initiated by the structural characterization of weakly bound low molecular weight fragments, chemical elaboration through growing, linking, and/or merging is implemented with the goal of rapidly enhancing the binding affinity of the drug lead.[4-6] For fragment linking, where two fragments are chemically coupled to form a single compound, the desired outcome is the production of a substantially higher affinity molecule, otherwise known as superadditivity.[7, 8] Despite the straightforwardness of this approach, connecting fragments with a well-optimized linker in practice has proven to be a considerable challenge.[5, 9-11] Fragment linker design criteria that maximize chances of success have been proposed previously,[4, 5] but little is known about how certain linkers can improve or impair a compound's potency.

An informative model system for studying fragment linker design in FBDD is the well-studied kinase target the epidermal growth factor receptor (EGFR).[12, 13] As a subject of decades of drug development, lung cancers harboring the EGFR activating mutations L858R (LR) and exon19del, as well as drug resistant T790M (TM) gatekeeper and C797S (CS) mutants, have led to a diverse set of small-molecule inhibitors.[14, 15] A growing number of mutant-selective ATP-site (orthosteric) inhibitors are known in addition to the more distinct allosteric (type 3) inhibitors.[16-20] Structurally, the allosteric inhibitor binding site is positioned adjacent to the ATP site allowing for co-binding of inhibitors to both sites, which are associated with positive cooperativity and synergy *in vivo*.[18, 20, 21] Additionally, there are recently emerged examples of molecules that are designed to bind simultaneously to both ATP and allosteric sites.[22-24]

Due to the proximity of these sites, we sought to explore different chemical linkers in compounds that span the ATP and allosteric EGFR sites (**1-4**, Scheme 1, Figure S1). We selected ATP-competitive inhibitors based on our structurally-characterized trisubstituted imidazole molecules (**5-7**),[25-28] and the EGFR 5,10-dihydro-11*H*-dibenzo[b,e][1,4]diazepin-11-one (benzo) allosteric inhibitors **8** and **9**.[17] We synthesized a set of bivalent ATP-allosteric inhibitors bridged by an *N*-linked methylene (**1**) and *C*-linked amide (**2**-**4**) (SI methods, Scheme 1). We utilized a Suzuki−Miyaura cross-coupling reaction strategy for the combination of the fragments of *N*-linked derivative **1**, while fragments of the *C*-linked derivatives were assembled prior to connecting them through amide coupling conditions (Schemes S1-S5).

To characterize the binding modes of the *N*- and *C*-linked scaffolds, we determined X-ray cocrystal structures through compound soaking with crystals of EGFR(T790M/V948R), which reliably crystallizes in the inactive conformation (Table S1, Figures 1 and S2). A 2.1 Å-resolution cocrystal structure shows the imidazole and benzo groups of **1** bound within the ATP and allosteric sites, respectively (Figure 1A). Analogously, a 2.2 Å-resolution cocrystal structure of **2** shows this compound bound identically at the ATP site as **1** but with a significantly different conformation within the allosteric site (Figure 1B-C). Additionally, alternative intermolecular interactions are observed such as H-bonding with T854 and D855 enabled by the *C*-linked amide linker in **2**, which are not possible in the *N*-linked methylene **1**. A "swing" of K745 toward the benzo ketone is seen for **2** opening a position on the imidazole, which now binds a solvent water (Figure 1B). Importantly, the binding mode of the parent allosteric inhibitors **8** and **9** is best matched by the *C*-linked **2** benzo (Figure 1D). These cocrystal structures indicate that these compounds bind as designed within the EGFR kinase but are unique in that changes to the linker results in different conformations in the allosteric sites as well as alternative H-bonding interactions.

We next sought to characterize the biochemical potencies of these molecules in HTRF-based activity assays with purified EGFR kinase domains (Table 1). Strikingly, the *N*-linked **1** is limitedly potent against WT and mutant EGFR with IC_{50} values > 1 μM while the *C*-linked inhibitors **2-3** show substantially lower IC_{50} values of 1.2-1.5 nM for LR and 51-64 pM for LRTM and LRTMCS (Figure S3). Time-dependent kinetic parameters for the covalent analogue **4** were obtained with Sox-based reagents, and k_{inact}/K_I values show that this molecule is most potent against LR (Table 2). Compared to IC_{50} values of the parent ATP site **5-6** imidazoles (≥6 μM) and allosteric **8-9** (39-59 nM), the potent **2-3** exhibit markedly improved potencies (≥10^3-fold lower) over the analogous ATP and allosteric fragments alone. These results indicate that the *C*-linked **2-3** are far better suited for binding and inhibiting EGFR compared to the *N*-linked **1**, which can be mainly attributed to the structure of the linker connecting the ATP and allosteric sites.

Table 1. Reversible Inhibitor Biochemical EGFR IC_{50} values in nanomolar against wt and mutant EGFR kinase domains.

Compound	wt	LR	LRTM	LRTMCS
1	> 10000	1300 ± 100	> 10000	> 10000
2	< 10	1.5 ± 0.1	0.059±0.005	0.064±0.004
3	< 10	1.2 ± 0.09	0.051±0.005	0.063±0.005
4	-[c]	-[c]	-[c]	1.8 ± 0.3
5	n.d.	n.d.	5800±300	6000±500
6	n.d.	n.d.	> 10000	> 10000
7	-[c]	-[c]	-[c]	130 ± 40[b]
8 DDC4002[a]	> 1000	690 ± 120	39 ± 4	59 ± 8
9 EAI002[a]	> 1000	n.d.	52	n.d.

[a] from [17] [b] from [23] [c] Left blank as these compounds are time-dependent covalent inhibitors of these enzymes. IC_{50} value is a higher-limit due to the WT EGFR enzyme concentration is 10 nM.

Table 2. Time dependent inhibition of EGFR by **4**.

4	wt	LR	LRTM
k_{inact}/K_I ($M^{-1}s^{-1}$)	2500±10	14100± 300	1070± 40
k_{inact} (min^{-1})	0.18±0.004	0.52±0.02	0.10±0.004
K_I (μM)	1.20±0.04	0.61±0.03	1.6±0.1

These results from structural and functional experiments present a unique opportunity to examine the molecular factors that enable highly potent versus practically inactive EGFR bivalent inhibitors dependent entirely on linker composition and structure. Interestingly, we do not observe significant differences in the length of the fragments, which is often a key route to linker optimization (Figure S4).[5, 8] However, we observe alternative binding conformations within the allosteric pocket for the active **2** versus inactive **1** best matches the parent allosteric inhibitors **8**-**9** (Figure 1D & S4). This

supports the notion that ideal linkers must retain the original interactions for the original protein-fragment-interactions, and indicates that the more hydrophobic fragment (allosteric site) is more tolerant to alterations in binding mode.[4, 5] Furthermore, the linker is rotationally restrictive and reinforces the "outward" conformation of the *N*-linked methylene **1** whereas the *C*-linked amide in **2** allows for rotational flexibility enabling the allosteric fragment to bind more favorably (Figure 2). Enhanced binding affinity of **2** is likely afforded through the more extensive H-bond network due to the amide functional group (Figure 1B). This side-by-side comparison of compounds with vastly different potencies demonstrates the importance of prioritizing fragment linkers that enable rotational freedom and intermolecular interactions.

We next evaluated the biological activity of the *C*-linked bivalent compounds in human cancer cells. The covalent analogue **4** is found most effective at suppressing EGFR activity (pY1068) as well as downstream pERK and pAKT in the NSCLC cell line H1975 (Figure 3A). Antiproliferative activity experiments in Ba/F3 cells are consistent with this observation, showing ~220 nM and ~770 nM potency of **4** against L858R and L858R/T790M, respectively (Table S2). Comparably, the reversible binding **2-3** are less effective in H1975 cells (Figure 3B, S5) and Ba/F3 cells likely due to the importance of forming a covalent bond in driving cellular activity.[23] We also confirmed that **4** is similarly active in HCC827 cells driven by the del(E746-A750) exon19del mutation (Figure 3C). This observation is informative as EGFR allosteric inhibitors are limitedly effective against exon19del mutants indicating the bivalent **4** is more related to ATP-competitive inhibitors despite extensive binding within the allosteric pocket (Figure 1).[29, 30] While we were unable to obtain a cocrystal structure of **4**, in silico docking provided an analogous docking pose as seen for **2** with the correct orientation for covalent bond formation with C797 (Figure S6). We confirm **4** is selective across the kinome exhibiting a selectivity score of S(35) = 0.084 (Table S3) and metabolically stable in liver microsome assays (Figure S7). The biological activity and early-phase medicinal chemistry properties of **4** validate this linker-focused design of bivalent EGFR inhibitors and consists of a viable route to compounds for further pre-clinical evaluation.

In conclusion, we have designed and characterized a set of bivalent EGFR inhibitors that exhibit a range of biochemical potencies dependent on the chemical structure of the connecting linker bridging the ATP and allosteric sites. A direct comparison of cocrystal structures of active and inactive bivalent EGFR inhibitors showcases the importance of flexibility and interactions enabled by small changes to the linker structure. Our study shows that selecting a linker through merging the parent fragments[17, 25] did not yield an effective inhibitor **1** necessitating exploration of alternative groups and points of connection showcasing a common pitfall in this approach. Effective FBDD linking should place higher emphasis on surveying linkers with different points of connection that enable proper orientation while allowing linker-enabled intermolecular interactions. Future studies of diverse linker structures and binding modes in kinases or other receptor model systems will enable needed molecular-level understanding to access new starting points more swiftly in FBDD and drug development, and potentially open new avenues in classically undruggable targets.

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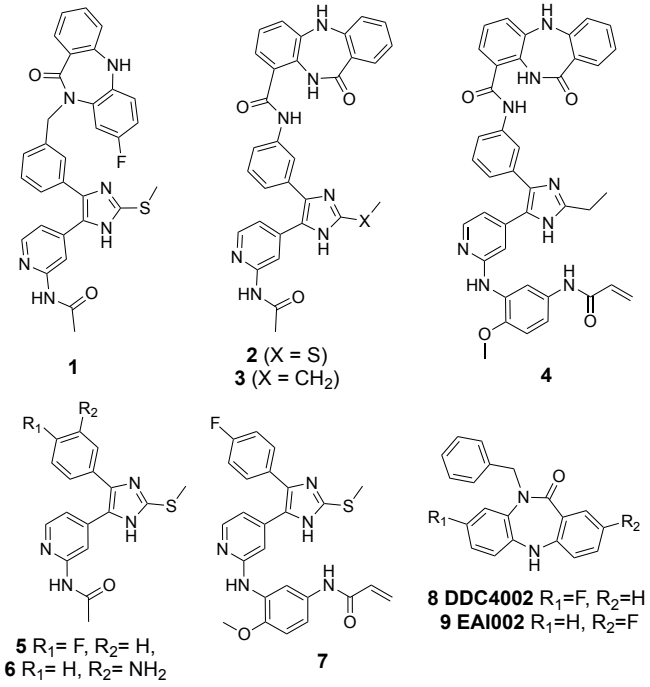

Scheme 1. Chemical structures of bivalent ATP-allosteric inhibitors consisting of *N*-linked reversible (**1**) as well as *C*-linked reversible (**2,3**) and covalent (**4**) scaffolds. Parent ATP site imidazole reversible (**5-6**) and covalent (**7**) inhibitors as well as dibenzodiazepinone allosteric inhibitors (**8-9**).

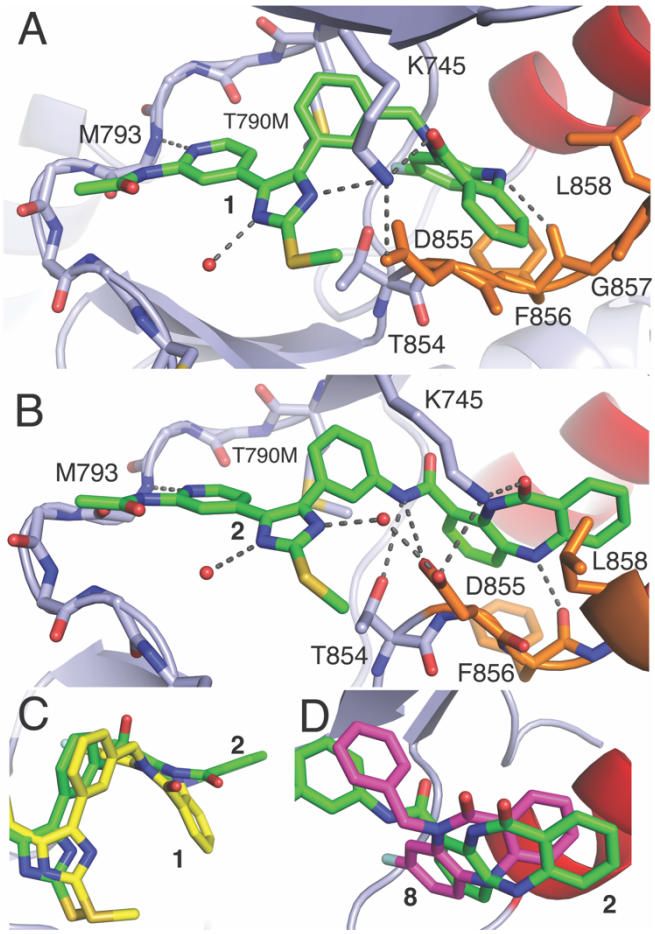

Figure 1. Binding modes of A) **1** (PDB ID 8FV3) and B) **2** (PDB ID 8FV4) in complex with EGFR(T790M/V948R). C) Top view overlay of **1** and **2** cocrystal structures within the allosteric pocket. D) Overlay of **2** and **8** (PDB ID 6P1D).

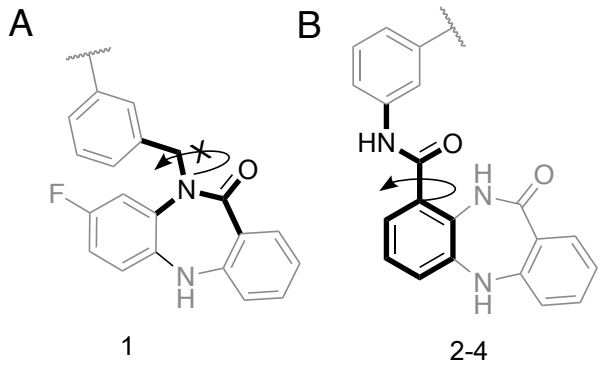

Figure 2. The *N*-linked methylene structure of A) **1** is restrictive compared to the more flexible *C*-linked amide of B) **2-4** allowing for more effective binding of the allosteric site fragment.

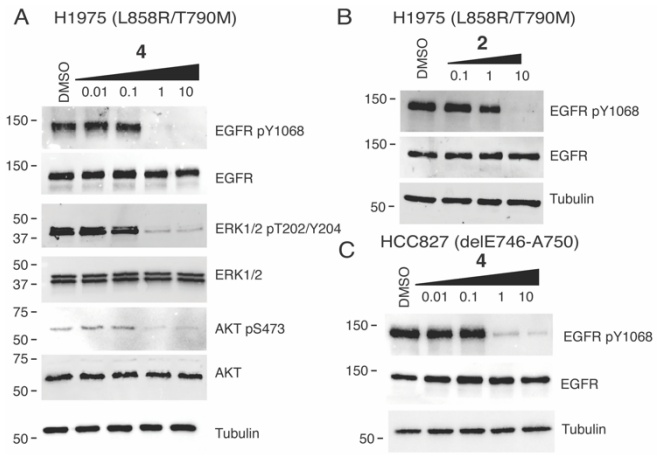

Figure 3. A) The covalent bivalent EGFR inhibitor **4** reduces EGFR(L858R/T790M), ERK1/2, and AKT phosphorylation in H1975 lung cancer cells. B) The reversible binding **2** suppresses EGFR phosphorylation in H1975 cells. C) **4** effectively ablates EGFR(delE746-A750) phosphorylation in HCC827 cells. All experiments performed after 6 hours treatments and western blots are representative of three independent experiments.

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