TETHERING: A NOVEL TOOL IN DRUG DISCOVERY

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ABSTRACT

The concept of tethering has become enormously popular in the past few years which facilitated binding of protein targets with smaller molecules or fragments in order to achieve successful leads and their optimization. The concept behind tethering is additive binding to yield a high affinity lead molecule which involves binding to protein, chemical modification and then its optimization to get a lead structure. Three types of tethering studies are Cooperative tethering, Extended tethering and Breakaway tethering which are studied with reference to various enzymes like Caspase3, Interlukin-2, Protein Tyrosine Phosphatase 1B etc. The need for rapid target identification and evaluation is resulting in an increase in pressure on an already strained pharmaceutical industry. As a first step toward target validation, tool compounds are invaluable in assessing the potential of drug targets. This review attempts to summarize how Tethering can rapidly identify tool compounds for a difficult target and provide effective hits. Although Tethering was initially developed to provide high-quality starting points for well-validated, highly characterized targets, the use of IL-2 as a model system proves its potential in early target assessment.

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INTRODUCTION

The expensive drug discovery procedures and time consuming development requires that the validity and druggability of targets are assessed as early as possible. Protein–protein interactions are clinically important but are usually high-risk targets when dealing with small-molecule approaches. Discovering drugs in pieces can reduce the dimensionality of the search and dramatically improve the chances of finding good starting points for the drug discovery process against novel drug targets. Hence researchers are putting their efforts to search new drug designing techniques which are precise and less time consuming. This led to the emergence of Tethering which is a disulphide-based drug discovery. Tethering is one of the unique method which uses covalent, reversible bond to stabilize the interaction between a fragment and a target protein. Search of smaller molecules remains a challenge for researchers for years. Tethering being a powerful new technology helps to find a good small-molecule starting point in diverse chemical space. Tethering can also find highly polar compounds i.e. fragments often missed in high-throughput screens. Tethering provides a much more efficient means to discover nanomolar inhibitors than traditional medicinal chemistry. Background of this technique comes from the example that shows weak binding affinity that can be linked to form higher affinity molecules was first shown by ligand binding to metal ion, which is known as “Chelate effect”. Jencks applied this concept to small molecule-protein interactions [1]. Conceptually tethering performs series of functions as shown in the following Figure 1 to generate a potential lead compound.

![Figure 1: Lead generation by tethering.](image)

The lead compound generated is within the fewer time frames as compared to traditional way by medicinal chemistry and provides efficient lead development. The general process includes the use of a cysteine near the targeted area which is either found naturally or introduced via site directed mutagenesis as shown in Figure 2. The protein is reacted with a library of disulphide containing fragments in a partially reducing environment and identification of the dominating species using spectrometry.

![Figure 2: Binding of cysteine residues [2].](image)

In this review, we discuss on method, theory and uses of tethering. Consideration of this technique to identify fragments along with their linking of these fragments to rapidly identify starting points for drug discovery [3]. Basic overview comprises of tethering technologies which contributes in finding active sites of enzyme and accordingly identify fragments (potent inhibitors).

Concept behind Tethering

Concept behind tethering is additive binding to yield a high affinity lead molecule which involves identification of a small molecule that binds to the protein pocket, chemical modification of starting fragment to generate a binder of higher affinity and optimization to get a lead structure [4]. The concept has become very popular for two main reasons; firstly, there are much fewer fragments than drug-sized molecules. Rough estimates indicate that approximately $10^7$ fragments with up to 12 heavy atoms do exist (excluding 3- and 4-ring-containing structures), whereas there are $10^{63}$ possible small drug like molecules with up to 30 heavy atoms [5]. For comparison, only approximately $10^8$ molecules have been synthesized to date [6]. Thus, initial screening of fragment libraries is expected to sample the chemical space much more efficiently than traditional conventional methods. The second reason is that
fragment-derived lead structures have significantly higher ligand efficiency (free binding energy per non-hydrogen atom of the ligand) than molecules discovered by screening of large compound libraries.

**Tethering in Fragment based drug discovery**

Broadly there are two methods in this approach, which are classified as Non-tethering methods involving non-covalent interactions between target proteins and small molecule ligands. This includes High throughput screening (HTS), Nuclear Magnetic Resonance (NMR), Mass spectroscopy (MS) and X-ray Crystallography [7-9]. The second approach is the Tethering. The preceding methods of fragment discovery rely on non-covalent interactions between the target protein and small-molecule ligands; hence their binding to targets is unstable requiring high ligand concentrations for detectable occupancy on the protein. In contrast, tethering relies on reversible covalent bond formation between the fragment and the protein of interest so they form stable binding which amplifies affinity of the fragment for the target molecule, enabling detection at lower concentrations. Tethering requires relatively little protein (10–50 mg to screen more than 10,000 fragments) compared to other fragment-based approaches and is not limited by protein size or crystallization properties. Tethering also controls the region where selected fragments bind on the protein; hence it also provides information on site-directed binding of fragments on protein target. The only essential requirements for Tethering are a coarse three-dimensional model of the protein target and the ability to analyse the protein by MS. Steps involved in tethering are as follows:

**Selection of desired property of a molecule**

The system of tethering is generally set up to thermodynamically select molecules that have a desirable property (for example, high affinity to a receptor) through bond-forming and bond-breaking reactions among a group of fragments. Cysteine residue is naturally occurring into proteins so it’s easy for a molecule to undergo disulphide exchange. This work emphatically demonstrates that cooperative Tethering is a useful way to develop potent inhibitors, especially on dynamic regions of a protein surface previous research has shown that the stability of intermolecular [10,11] and intramolecular [12] disulphide bonds can be coupled to the binding energies of the interacting molecules.

**Requirements necessary for tethering**

To be useful for drug discovery, fragments should be small (molecular weight preferably lower than 250 Da), heavily functionalized, and contain no “toxicophores” or other malignant functionalities. Several researchers have written about the design of compounds for fragment-based discovery methods [13]. Fragments for Tethering must also contain a thiol or disulfide bond.

**Requirements necessary for target proteins**

If the target protein contains a cysteine residue within or near the targeted site, it can be used directly. Otherwise, site-directed mutagenesis can introduce a cysteine residue.

**Screening of molecules by tethering**

Once it is confirmed that cysteine is in place, the protein is reacted with a library of disulphide-containing fragments under partially reducing conditions. In theory, the cysteine should form a disulphide with each of the fragments, and the exchange should occur rapidly.

**Types of Tethering**

There are three types of tethering are co-operative tethering, extended tethering and breakaway tethering.

**Co-operative tethering**

Tethering utilizes engineered cysteine residues (mutated cysteine for targeted binding) on proximal regions to interrogate binding fragments that interact cooperatively with one another or to interrogate cooperative interactions between known binding elements and newly discovered disulphide-containing monophores. Many binding sites are very adaptive, which, stated differently, means that the energy barriers between various conformations are very small so a relatively small change in the system (i.e. binding of a Tethering monophore) can favour another conformational state. Interleukin-2 is one of the best examples by which Co-operative tethering can be explained. This example demonstrates discovery of small molecules and their binding to protein interface. Co-operative tethering has been most successfully applied in the case of IL-2 (interleukin 2). Use of Co-operative tethering led to the development of a series of compounds that bound to IL-2 and strongly antagonized IL-2 receptor binding. First binding site is identified. This binding site was in an adaptive region, the conformation of which changed upon binding to non-peptidic small molecules. Hyde and co-workers observed that the adaptive region extended beyond the residues that were occupied by early compounds, and selected several adjacent residues for cysteine substitutions to probe a larger portion of the adaptive region with tethering. These residues were selected from the crystal structure and none had any effect on the structure or function of IL-2 [14].

**Extended tethering**

The idea of tethering with extenders (Extended tethering) is that a disulphide-containing small-molecule anchor can be used in the active site or binding site to probe adjacent regions for chemical moieties that bind a little further on protein. This anchor is often an irreversible alkylator that reacts specifically with a single cysteine (native or engineered) on the protein of interest. Extended tethering has most widely been used against the Caspases where the active-site cysteine is suitable for alkylation by an “extender” molecule. From all members of the caspase family, caspase-3 has a linear peptide binding site that neatly accommodates four amino acids.
acids residues; tetrapeptides have been used as starting points for small-molecule inhibitors. The most critical recognition element for all caspases is an aspartyl group immediately preceding the substrate amide bond. The aspartyl residue binds in a highly conserved pocket in the enzyme. Irreversible inhibitors have been designed by starting with aspartyl-containing peptides and replacing the C-terminal amide with a ketone functionalized with a good leaving group alpha to the carbonyl. This extender contains the essential aspartyl group with a reactive aryl acyloxy methyl ketone and a cleavable thioester. One of the strengths of Extended tethering is that it suggests a defined way that the extender plus the hit can be converted to a useful inhibitor.

**Breakaway tethering**

Tethering with extenders has been useful for caspases, which contain active-site cysteine, largely because the available chemistries for modifying the active-site cysteine are robust and specificity elements for substrate binding are near the catalytic residue. But if the engineering cysteine residues in active sites or binding sites is that residue that would ideally be mutated for fragment discovery may be important for binding or catalysis. To eliminate these complications, Erlanson and co-workers developed a strategy termed as Breakaway tethering [15], which is useful for probing sites that are narrow, deep, fragile or significantly and negatively impacted by the introduction of a cysteine residue for tethering. In short Breakaway tethering is ideal for any protein where modification within the active site is not desirable. PTP-1B is the best example for breakaway tethering (finding fragments in a fragile, narrow site). PTP-1B, a negative regulator of insulin-receptor phosphorylation and signaling, a pharmaceutical target for type-2 diabetes. For many enzymes the active site is smaller and more easily disrupted. A case in point is the class of protein tyrosine phosphatases, or PTPs. These enzymes, which remove the phosphate group from phosphotyrosine (pTyr) residues in peptides and proteins, are increasingly recognized as an important but difficult class of drug targets [16, 17]. One of the primary challenges associated with these enzymes is the fact that the active site has evolved to recognize the highly charged and therefore nondrug-like pTyr residue with great specificity. Several research groups have worked to find replacements for the pTyr residue [18] but few compounds have succeeded to reach clinical trials. PTPs contain a conserved cysteine residue in the active site that is highly reactive toward electrophiles, and may in fact be regulated in vivo through disulfide bond formation with small endogenous thiols. However, the active site is deep and narrow having room for little other than the pTyr residue itself. Therefore, it was that tethering from the native active-site cysteine would not be useful for discovering pTyr replacements, as any identified fragments would likely bind outside of the active site. At the same time, it was not possible to introduce mutations too close to the active site for fear of disrupting the fragile and highly structured catalytic machinery. To access the active site for tethering without disrupting it, Tethering with breakaway extenders were developed as illustrated in Figure 4.

![Image](https://via.placeholder.com/150)

**Figure 3: Breakaway extender [2].**

In this approach, scientists first introduce a cysteine residue well outside of the active site. It was then modified to residue with a cleavable “breakaway extender,” which, when cleaved, positions a thiol close to the active site. The newly introduced thiol is then accessible for Tethering. Different extenders were prepared like prototype extender and Breakaway extender. Also this extenders were validated for there specific binding. Finally the screening is done against disulfide containing fragments. The design was confirmed by X-ray crystallography, which demonstrated that the particular extender binds as expected. Also various binding site were studied which gave sharp SAR.

**Incorporation with Medicinal chemistry**

Incorporating knowledge of medicinal chemistry is essential for tethering drug library design to ensure the tractability of the leads generated by the exercise. By incorporating functional groups of drugs early in the design phase, the quality of the leads generated by this technique is greatly enhanced. The experience of synthetic organic chemistry is equally important for the design of the library and its rapid preparation. Combined, these disciplines can shorten the overall project time and increase the chances for success.

**CONCLUSION**

Tethering offers great promise for providing new starting points for drug discovery as well as for facilitating lead optimization. Tethering provides a site-directed basis for discovery. This offers the possibility of selectively targeting and even identifying allosteric sites in proteins that may be missed in a functional screen. With the success observed it is believed that there are many untapped opportunities for Tethering. Fragment-based drug discovery offers great promise for providing new starting points for drug discovery as well as for facilitating lead optimization. The chemical information that Tethering provides can be applied to existing hits to generate higher-affinity compounds, as was shown for IL-2. Several new avenues promise to further expand the utility of Tethering. Because Tethering is a binding assay and does not rely on protein function, it is more versatile than many functional...
assays. Tethering provides a site-directed basis for discovery so that one can choose the sites to be explored. This offers the possibility of selectively targeting and even identifying allosteric sites in proteins that may be not detected in a functional screen.

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