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A simple and widely applicable hit validation strategy for protein–protein interaction inhibitors based on a quantitative ligand displacement assay

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ABSTRACT

Identification of inhibitors for protein–protein interactions (PPIs) from high-throughput screening (HTS) is challenging due to the weak affinity of primary hits. We present a hit validation strategy of PPI inhibitors using quantitative ligand displacement assay. From an HTS for Bcl-xL/Mcl-1 inhibitors, we obtained a hit candidate, **I1**, which potentially forms a reactive Michael acceptor, **I2**, inhibiting Bcl-xL/Mcl-1 through covalent modification. We confirmed rapid reversible and competitive binding of **I1** with a probe peptide, suggesting non-covalent binding. The advantages of our approach over biophysical assays include; simplicity, higher throughput, low protein consumption and universal application to PPIs including insoluble membrane proteins.

Fundamental processes in living cells, such as intracellular signal transduction and intercellular communication are largely controlled by proteins often acting together with other proteins through protein–protein interactions (PPIs). Since inappropriately promoted PPIs are observed in many diseases, including cancer[1–3] and autoimmune diseases[4–6], inhibition of PPIs is an area of growing interest to pharmaceutical science.[7–10] Currently, nearly all PPI inhibitors on the market are therapeutic antibodies limiting their application to targets on cellular surface. For disruption of intracellular PPIs, identification of small molecule inhibitors with drug-like properties is necessary.

Examples of the intercellular targets include B-cell lymphoma-extra large (Bcl-xL) and myeloid cell leukemia 1 (Mcl-1), which belong to anti-apoptotic member of the B-cell lymphoma 2 (Bcl-2) protein family and are key regulators of mitochondrially mediated apoptosis.[1,2,11] They suppress apoptosis by interacting with pro-apoptotic members of Bcl-2 protein family such as Bcl-2-associated X protein (Bax) and BH3 interacting-domain death agonist (Bid).[1,2,11] These interactions control the pathways leading to the release of cytochrome *c* from the mitochondrial membrane, activation of the caspase cascade, and execution of apoptosis. Overexpression of Bcl-xL and Mcl-1 protein or amplification of these genes is correlated with the escape of tumor cells from apoptosis.[12–16] Interestingly, simultaneous inhibition of Bcl-xL in addition to Mcl-1 is anticipated to have a stronger anticancer effect compared with inhibition of Mcl-1 alone.[12] Based on such information, inhibition of these PPIs may restore the apoptotic process in cancer cells, making a promising approach to cancer therapy.

While PPIs are attractive targets for drug discovery, identification of small molecule PPI inhibitors with drug like properties from high-throughput screening (HTS) campaigns remains challenging despite great efforts of academia and the pharmaceutical industry.[7] A major obstacle to identifying PPI inhibitors in an HTS is the lack of deep cavities for binding at the PPI interfaces.[7,10,17] The shallow protein–protein interface of these targets means initial hits will have low affinity for their targets requiring compound screening to be conducted at high concentrations (>10 μM). Screening at high inhibitor concentration leads to high false-positive rates due to factors such as nonspecific covalent adduction, denaturation of target proteins and assay interference.[7] Accordingly, the inhibitory activity of primary hit compounds must be examined with great care.

Conventionally, biophysical methods have been widely applied for the validation of PPI inhibitors.[18,19] For example, surface plasmon resonance, isothermal calorimetry, and nuclear magnetic

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resonance each have been applied to remove false positive compounds with high stoichiometry binding properties and unusual binding kinetics.[7,18–21] These biophysical methods permit the direct measurement of binding without the need for fluorescent or radioisotope labeling, thus providing strong evidence that a compound actually binds to a target. However, biophysical methods are not always available because they require expensive equipment, a large supply of proteins and specialized experimental skills. In addition, these methods generally cannot be applied to membrane proteins limiting their application to soluble purified proteins. A simple and universally applicable method of lead compound validation would be ideal to reduce false-positive results.

For the validation of hit compounds from primary HTS hits, in this Letter, we introduce an easy and high-throughput hit confirmation strategy for PPI inhibitors based on a quantitative ligand displacement assay and illustrate its use in the examination of a dual PPI inhibitor of Bcl-xL and Mcl-1.

To obtain hit compounds for lead optimization for anti-cancer drug, we performed an HTS and among approximately 800,000 compounds screened, **I1** (Fig. 1) was selected as a potential Bcl-xL and Mcl-1 inhibitor due to its unique chemical structure and low molecular weight property for PPI inhibitors. The potency of **I1** was evaluated with K_i values of 4.7 and 14 μM for Bcl-xL and Mcl-1, respectively (Table 1), by use of a ligand displacement fluorescence polarization (FP) assay.

While **I1** is an interesting lead compound, it contains an α-arylthio-γ-keto-4-arylbutanoic acid group, with a potential decomposition pathway by way of a retro-Michael reaction that could lead to **I2** which contains a Michael acceptor group (Fig. 1). Similar decomposition of inhibitors and subsequent covalent modification by the decomposed products has been reported for aldehyde dehydrogenase inhibitors, where the parent inhibitors undergo enzyme-mediated retro-Michael reaction.[22] Likewise, inhibition of Bcl-xL and Mcl-1 could be through covalent modification. In fact, since a covalent Mcl-1 inhibitor has been reported[23], confirming the non-covalent binding of **I1** was a priority before starting chemical optimization. Although covalent inhibitors have many desirable features, including greater *in vivo* potency and longer duration of action[24,25], they also have the potential risk of non-selective target inhibition through the modification of reactive residues and as a downstream consequence immunogenicity from protein adducts formed by covalent inhibition, leading to allergic or drug hypersensitivity reactions.[25,26] Thus, compounds with a potential for covalent modification are widely accepted to be avoided for a lead compound for chemical optimization.

To characterize the mechanism of inhibition by **I1**, we developed a ligand displacement assay using time-resolved fluorescence resonance energy transfer (TR-FRET) technology between Bcl-xL or Mcl-1 and fluorescent Bid peptide (F-Bid) which binds to both proteins. We chose this approach because a fluorescently labeled peptide is a readily observable probe ligand, enabling a robust ligand displacement assay under wide range of ligand concentrations unlike FP assay. In the presence of K_d concentrations of an F-bid probe (10 nM for Bcl-xL and 35 nM for Mcl-1), **I1** displaced F-bid binding to Bcl-xL and Mcl-1 with equal avidity (K_i = 13 and 12 μM, respectively) (Table 1), indicating the similar potencies with those obtained by the FP assay and valid development of the TR-FRET ligand displacement assays.

Table 1
K_i values of Bcl-xL/Mcl-1 inhibitors

	Bcl-xL (μM)		Mcl-1(μM)	
	FP	TR-FRET	FP	TR-FRET
I1	4.7 (3.8–5.8)	13 (12–13)	14 (12–15)	12 (12–13)
I3		9.0 (8.6–9.5)		20 (19–21)

Numbers in parentheses represent 95% confidence interval.

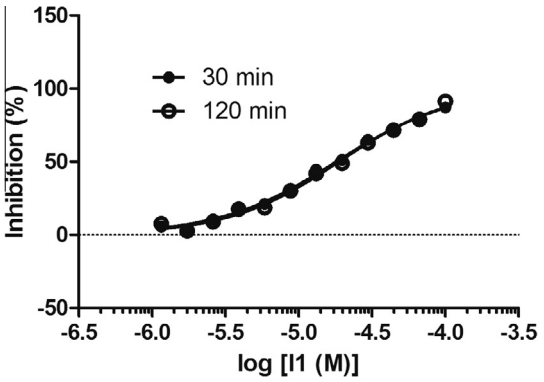

Figure 2. Effect of incubation time on Bcl-xL inhibition by **I1**. Inhibitory activity of **I1** on Bcl-xL measured at 30 (●) and 120 min (○) after addition of GST-Bcl-xL. The FRET ratio was measured at various concentrations of **I1** in the presence of 10 nM F-Bid, 1 nM Tb-anti GST and 2 nM GST-Bcl-xL. The IC_{50} value was determined by fitting the data to a sigmoidal curve. Data represent the mean ± SEM (n = 4).

A hallmark of irreversible inhibitors is that they gain potency with increasing incubation time against their target proteins. To investigate the possibility of inhibition through chemical reactivity, the time dependence of Bcl-xL inhibition by **I1** was examined using our TR-FRET ligand displacement assay. The IC_{50} values of **I1** against Bcl-xL at 30 min and 120 min incubation were identical within experimental uncertainty (18 μM and 19 μM, respectively) (Fig. 2). This lack of time-dependent inhibition supports reversible binding of **I1**.

As we have established **I1** displayed no time-dependent inhibition, we next investigated if its binding to Bcl-xL was competitive with F-Bid to further support non-covalent modification.[27–29] Using the TR-FRET binding assay, the saturation binding of F-Bid to Bcl-xL was measured in the presence of various concentrations of **I1** (Fig. 3A). The apparent K_d value of F-Bid increased linearly with the concentration of **I1** (R^2 = 0.99, Fig. 3B), while B_{max} was almost unchanged (Fig. 3C). These results are consistent with the competitive inhibition mechanism of **I1** against F-Bid peptide and illustrate the mutually exclusive binding of both compounds, suggesting non-covalent binding of **I1**. From global fitting of the binding curves, the K_i value of **I1** was determined to be 12 ± 1.0 μM, which shows good agreement with the results described so far.

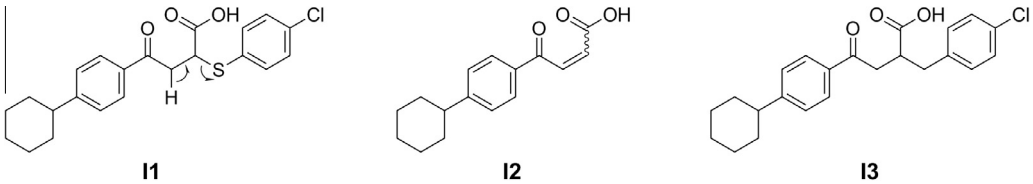

Figure 1. Chemical structures of the Bcl-xL/Mcl-1 inhibitor discussed within this study. The allows in **I1** indicate a potential decomposition pathway by way of a retro-Michael reaction.