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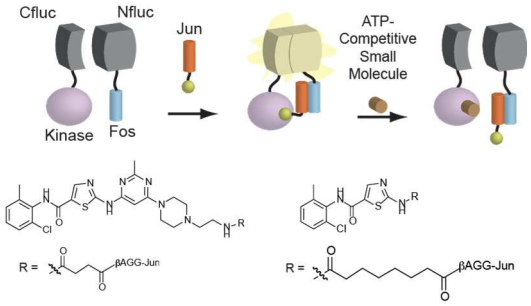

We report new CIDs based on Dasatinib and its analogues for profiling kinase inhibitors using a split-luciferase screen.

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ARTICLE TYPE

When Tight is too Tight: Dasatinib and its Lower Affinity Analogue for Profiling Kinase Inhibitors in a Three-Hybrid Split-luciferase System

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The development of methods for profiling inhibitors of protein kinases has seen tremendous progress over the last decade. We have previously reported a split-luciferase based three-hybrid approach for determining kinase inhibitor selectivity that utilized the promiscuous staurosporine warhead for designing chemical inducers of dimerization (CID). Herein we describe the extension of this methodology to target the tyrosine kinase (TK) group using a Dasatinib warhead based CID. We found that though the Dasatinib enabled CID provided a means for assembling the split-protein fragments, it had too tight an affinity in the context of the three-hybrid system for several TKs and could not be displaced with inhibitors. By tuning the affinity of Dasatinib, we were able to successfully target multiple TKs that could subsequently be assayed for inhibition by small molecules. We further demonstrated that the new CID allowed for the screening and identification of inhibitors against ABL.

Introduction

Human protein kinases catalyze the transfer of a phosphate group from ATP to a serine, threonine or tyrosine residue of a protein substrate, while protein phosphatases remove them. This reversible phosphorylation controls a vast array of signalling pathways and cellular events from apoptosis to cell division.[1, 2] The aberrant function of protein kinases has been associated with a multitude of diseases such as cancer, metabolic disorders[3], and inflammation[4,5]; hence protein kinases have emerged as important therapeutic targets. The human kinome is composed of over 500 distinct protein kinases that display very similar active site architectures. Since most inhibitors function by targeting the ATP binding cleft[6,7], it is not surprising that the selectivity of inhibitors is rarely optimal. Determining inhibitor selectivity is important for optimization of inhibitor specificity or promiscuity and potentially predicting the pharmacology of potential drugs.[8]

We have previously reported a split-protein based methodology for determining kinase inhibitor selectivity[9]. In this approach, a protein kinase is attached to the C-terminal fragment of split-firefly luciferase (Cfluc) and the coiled coil Fos is attached to the N-terminal fragment (Nfluc). With the addition of the chemical inducer of dimerization (CID) consisting of Jun, a coiled-coil peptide specific for Fos, conjugated to a small molecule kinase inhibitor, such as staurosporine, a three-hybrid complex is formed resulting in the reassembly of the split-luciferase (Figure 1a). By measuring luminescence of the split-reporter, the interaction between Jun-conjugated inhibitor based CID and the protein kinase can be detected. Upon addition of a small molecule that prevents the CID from binding to the kinase, the CID dissociates as do the luciferase fragments, resulting in loss in luminescence. This approach was successfully developed using staurosporine as the active site directed ligand. However, many kinases, in particular the tyrosine kinase (TK) group, are not as effectively targeted by staurosporine. Hence there is a need for finding new probes that can expand this three-hybrid approach to the entire kinome, particularly the TK group.

Many laboratories that target protein kinases for inhibitor profiling, proteomic studies, or the design of bivalent ligands have developed probes based on existing SAR and structural data on known inhibitors[10, 11 12-17]. This data guides the incorporation of chemical handles that do not significantly perturb binding of the probes for the target kinase. Such inhibitor based reagents have been developed for several inhibitors, including decorated Dasatinib analogues[18-22]. Herein we report the synthesis and implementation of Dasatinib based CIDs as active site targeted ligands for the interrogation of TKs and TKLs. We found that the Jun conjugated carboxy-Dasatinib could successfully recapitulate split-luciferase activity for most TKs tested. More interestingly, however, a CID based on a lower affinity analogue of carboxy-Dasatinib was found to be necessary for allowing the reversible assembly of split-luciferase. Finally, as proof of principle, we utilized the three-hybrid system to screen a library of known kinase inhibitors against ABL. Based on our data, we suggest that lower affinity analogues of known inhibitors may sometimes be necessary for displacement based methods and also be of utility for reversible and irreversible covalent inhibitors.[23-27]

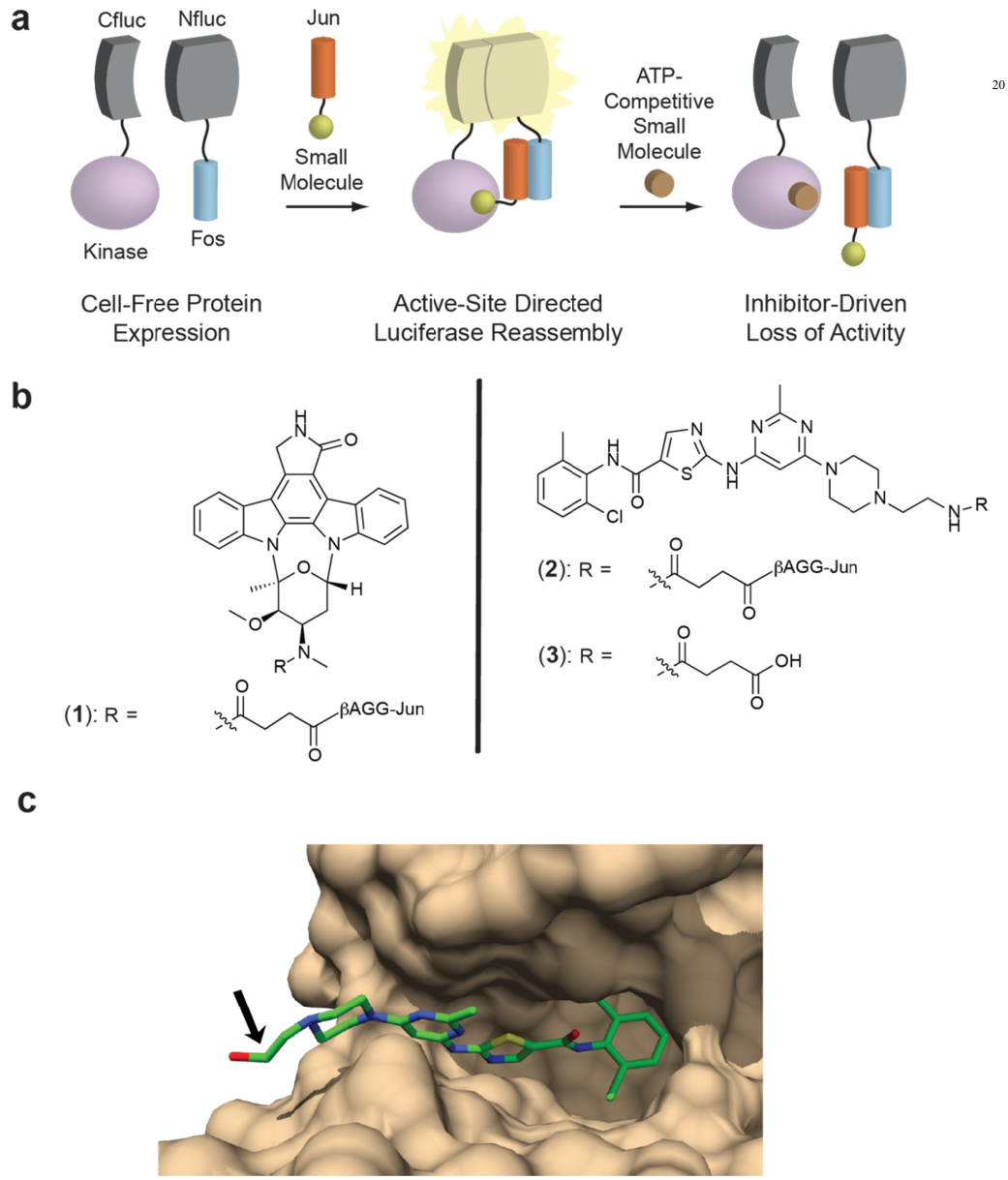

Fig.1 (a) Split-Luciferase reassembly driven by the Fos-Jun and kinase-small molecule interaction. Displacement of the CID occurs when a small molecule competitive ligand is introduced, causing loss in luminescence signal, (b) Structures of staurosporine (left) and Dasatinib analogues (right). (c) Crystal structure of SRC bound to Dasatinib (PDB ID: 3G5D), arrow shows site of modification for attachment to Jun.

Results and Discussion

In order to create general tyrosine kinase (TK) selective three-hybrid systems, we chose to conjugate Dasatinib to Jun at a position not involved in binding to the protein active site (Figure 1c). We also selected six TKs for testing, PTK6, EPHB3, RIPK2, ABL, SRC and EPHA2 that have weak reported affinities for staurosporine[11] but are potently bound by Dasatinib. Due to the potentially low affinity interactions with staurosporine, we anticipated that the Jun-staurosporine CID (**1**) would be unable to bind to any of these kinases, necessitating the use of Jun-carboxy-Dasatinib (**2**). When tested we found that the Cfluc-kinase and Fos-Nfluc proteins, alone or in the presence of **1,** did not show significant luminescence. However, upon addition of Jun-carboxy-Dasatinib, **2,** a marked increase in luminescence was observed for all the tested kinases (Figure 2a). We then set out to test whether this three-hybrid system could be used as a platform for competitive inhibition assays for the identification of kinase inhibitors. The addition of a Dasatinib-competitive small molecule inhibitor was expected to result in the displacement of the Jun-carboxy-Dasatinib, causing the dissociation of the luciferase fragments and a loss of luminescence. For this purpose Cfluc-kinase and Fos-Nfluc mRNA were co-translated and compound **2** was added to induce the assembly of luciferase. A

competitive inhibitor of Dasatinib, carboxy-Dasatinib, **3**, at 10 μM concentration was added to the three-hybrid system and incubated for 1 h, while no inhibitor was added to a negative control reaction (Figure 2b). Inhibition was determined as loss of luminescence signal relative to the signal obtained from the negative control. Three of the six tested kinases (PTK6, EPHB3, and RIPK2), showed a significant knock down of the signal with a percentage of inhibition of > 80% upon addition of the small molecule inhibitor. We were very surprised to observe that addition of free carboxy-Dasatinib to the three-hybrid systems for ABL, SRC, and EPHA2 exhibited little to no inhibition with compound **3**.

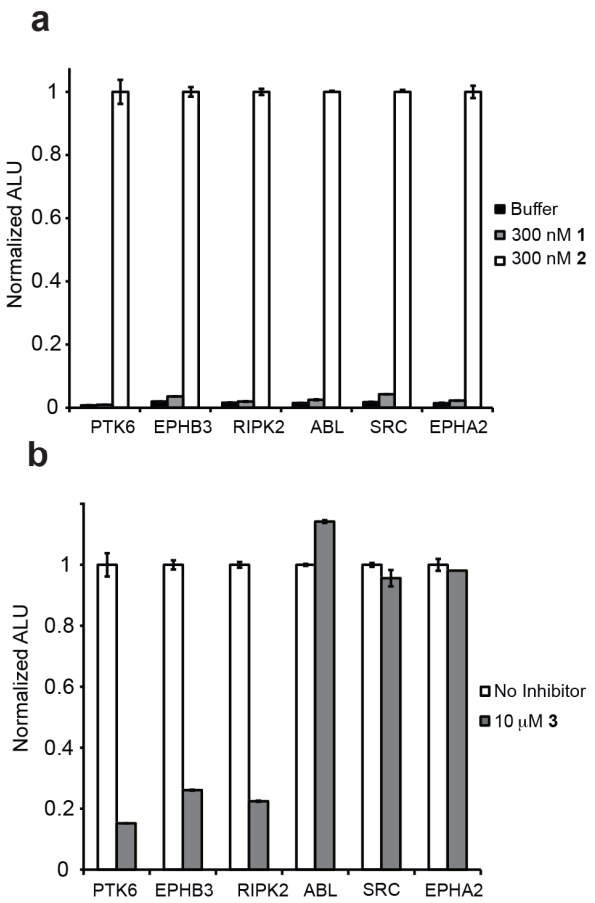

Fig.2 A) Split Luciferase Reassembly using staurosporine and Dasatinib as the active site directing ligand on six tyrosine kinases (PTK6, EPHB3, RIPK2, ABL, SRC, EPHA2). (B) Inhibition assay using Carboxy-Dasatinib, **3**, as the competitive ligand.

We reasoned that since these three kinases have reported apparent K_d values < 1 nM, their binding to the Jun-carboxy-Dasatinib conjugate may be too tight for the displacement of the three-hybrid complex by a free inhibitor in 1 h. Time dependent inhibition results support slow off-rates for Jun-carboxy-Dasatinib when tested against PTK6, EPHB3, and DDR2 (supporting information, Fig S1). DDR2, with a reported apparent K_d value of 3.2 nM,[11] only shows inhibition if added simultaneously as Jun-carboxy-Dasatinib, clearly a kinetic phenomenon. Thus, it would appear that inhibitors with reported apparent K_d values of less that < 7.0 nM as reported by Ambit,[11] have slow off-rates that preclude thermodynamic measurements.

In general most approaches focus upon developing high affinity probes. Interestingly, carboxy-Dasatinib proved to be too potent to be implemented in our assay. In order to render our split luciferase assay applicable to TKs with reported dissociation constants lower than 1 nM for Dasatinib, such as ABL and the EPHA/B family, we sought to modulate the binding of these kinases to the Jun-ligand conjugate by replacing carboxy-Dasatinib with an analogue that had similar specificity for TKs but an overall lower affinity. Compound **4** (Figure 3a), previously reported by Das and co-workers on the way to the discovery of Dasatinib, had been shown to bind ABL with lower affinity [28-32]. Therefore we sought to derivatize the previously described Dasatinib analogue, **4**, and couple it to Jun (compound **5**) for binding to TKs.

The luminescence signal obtained upon reassembly of the three-hybrid system with compound **5** was between 6- and 63- fold over background when tested against the six kinases (Figure 3b). To further test the utility of derivative **5**, each of the six TKs was allowed to first assemble with CID, followed by subsequent addition of the carboxy-Dasatinib (10 μM). All six kinases showed a loss in luminescence with an inhibition > 80%, demonstrating that this new lower affinity three-hybrid complex can be disrupted, in contrast to what was observed with the Jun-carboxy-Dasatinib-complex (Figure 3b). Thus, we had successfully optimized a lower affinity Dasatinib derivative with the potential for being amenable to displacement in the three hybrid assay (Figure 3b).

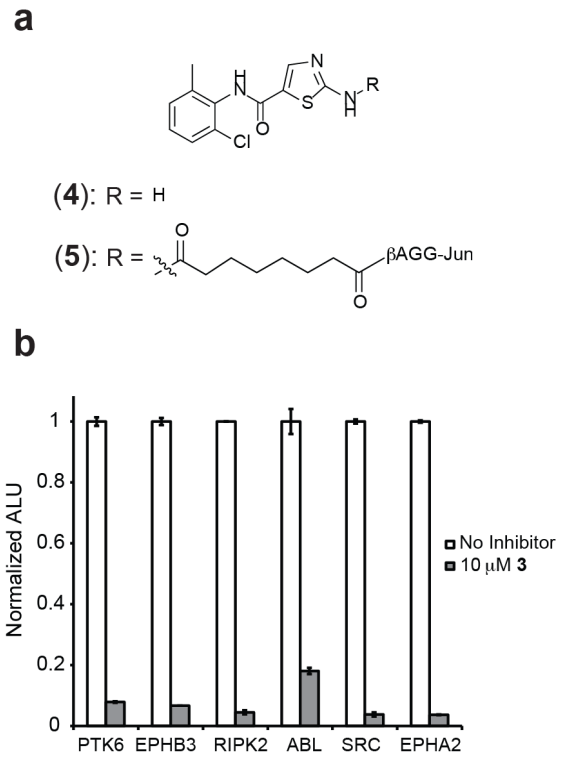

Fig.3 Inhibition assay using the Jun-Dasatinib analogue, **5**, as the active site directing ligand and **3** as the competitive inhibitor.

Finally, we wanted to verify whether we could apply this methodology, using compound **5**, to test TKs against a larger panel of kinase inhibitors. ABL was chosen as an initial test case with its relevance as an anticancer target. ABL was tested against 72 known kinase inhibitors that do not appreciably inhibit luciferase, from a commercial kinase panel (Tocriscreen Kinase Inhibitor Toolbox) supplemented with several addition compounds. The assay was conducted in a 96-well plate, with each well containing 10 μM of a specific inhibitor (Figure 4a). Several interesting inhibitors were identified from this screen, showing inhibition values ranging from 27% to 92%. Ki 8751, a VEGFR and PDGFR inhibitor with an IC_{50} of 0.9 nM and 67 nM respectively, was the most potent inhibitor identified. Ki 8751 was able to outcompete **5** with an inhibition of 92% against ABL. IKK16, reported previously as an IKK inhibitor (IC_{50} of 40 nM for IKK2, 200 nM for IKK-1), showed an inhibition of 53%; ZM 336372 known to inhibit c-Raf kinase (IC_{50} of 70 nM) and SAPK2/p38 (IC_{50} of 2 μM), showed an inhibition of 40%; ZM 306416 reported as a KDR and FLT kinase inhibitor (IC_{50} of 100 nM and 2 μM respectively), was found to inhibit at 35%; and PHA 665757 reported previously as a MET kinase inhibitor (ABL IC_{50} of 1.4 μM), showed an inhibition of 27 % (Figure 4b). Future experiments will establish the relative apparent binding constant of these compounds. The inhibition data reported herein as well as the new probes developed may be useful for designing either specific or promiscuous inhibitors or their analogues.

Conclusion

We have demonstrated that the split-luciferase based three hybrid system is general and extendable to the TK family using Dasatinib based CIDs. We have also shown that the assay can be used to identify several potential starting points for designing ABL inhibitors. Most notably, we find that kinase inhibitors that bind too tightly may need to be rendered less potent for small molecule displacement based methods that operate under thermodynamic conditions.

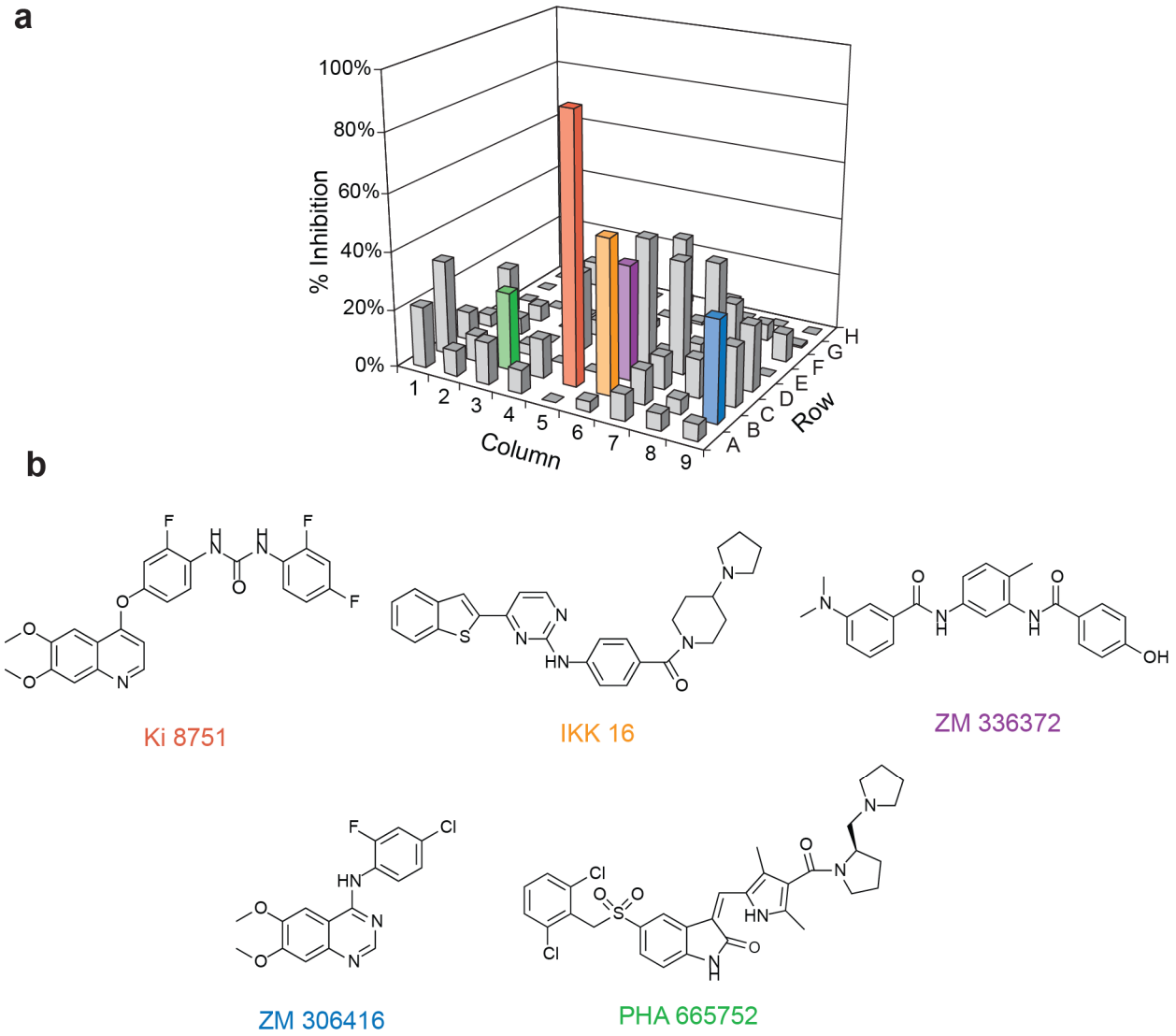

Fig.4 a) Results of a kinase inhibitor panel screened against ABL using an attenuated Jun-CID. b) Structures of five highlighted inhibitors with percentage inhibition ranging from 27 to 92%. Ki 8751 (red), IKK16 (orange), ZM 336372 (purple), ZM 306416 (blue), PHA 665757 (green).

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