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Development of inverse electron demand Diels–Alder ligation and TR-FRET assays for the determination of ligand–protein target occupancy in live cells†‡

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Determination of target engagement following drug administration under physiological conditions is essential for understanding clinical outcomes of therapeutic candidates. While the list of potential techniques that enable studies of target engagement is continuously expanding, identification of the best method to evaluate interactions between a ligand and its cellular binding partner(s) remains far from straightforward. We developed and compared the applicability of two label-based techniques; inverse electron demand Diels–Alder (IED-DA) ligation-based pull-down and TR-FRET assays for in-cell determination of target occupancy of c-Src kinase and p38- α kinase by the reversible inhibitor Dasatinib. Significantly, none of the assays required engineering proteins-of-interest. Moreover, cellular TR-FRET assay emerged as a very promising platform for the determination of target occupancy of specific protein in a high-throughput manner. Our studies suggest that both IED-DA assay and TR-FRET assay should be considered as methods of choice for the determination of target engagement of small molecule protein binders in live cells.

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Introduction

A detailed understanding of the mode of action of a therapeutic candidate is not possible without accurate assessment of its ability to engage target(s) and off-target(s) of interest under physiologically relevant conditions.¹ Moreover, it is increasingly recognized that evaluation of target engagement is critical to the advancement and further success of therapeutic programs.² Accurate determination of on- and off-target engagement can guide early-stage medicinal chemistry efforts towards the most promising drug candidates.³ Advanced programs also benefit from the ability to correlate the level of target engagement with observed efficacy or lack thereof.¹ If and when possible, measurement of target engagement should be an integral part of every medicinal chemistry campaign as early in the program as possible.

The growing recognition of the value of target engagement studies is paralleled by the continuous increase in available methods to analyze interactions between small molecules

and their protein partners in living cellular systems.^{4,5} Currently available methods can be divided into three categories: 1) methods requiring simultaneous engineering (through the introduction of labels/reporters) of small molecule and its protein partner; 2) methods limited to modification of small molecule, and 3) label-free methods.^{6–9} It is frequently an open question as to which of these techniques is most well suited to a specific scenario (*e.g.* dependent on known *in vitro* affinity of a small molecule ligand, cellular localization of target protein, target protein's size and structure *etc.*) to best assess drug–protein interactions. Given the substantial investment required to establish target engagement, it would be beneficial to understand the advantages and limitations of each technique.

Dasatinib (**1**) (BMS-354825, Sprycel®) is an FDA-approved, reversible inhibitor of Bcr-Abl and Src family of tyrosine kinases utilized in the treatment of chronic myelogenous leukemia and Philadelphia chromosome-positive acute lymphoblastic leukemia.¹⁰ As in the case of many other ATP-competitive inhibitors, the list of Dasatinib's cellular binding partners goes far beyond its primary targets and includes a significant number of other kinases.^{11,12} We envisioned that we could take advantage of the promiscuous nature of Dasatinib to gain an understanding of the applicability of two methods, IED-DA ligation-based pull-down assay and TR-FRET assay, for the measurement of cellular target

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engagement of multiple targets. In this context and with a goal to resemble scenario similar to the process of identification of therapeutic candidates, we set to compare the applicability of these methods against two protein targets of Dasatinib that varied in binding affinity.

Herein, we report our findings based on the application of two different Dasatinib-derived probes: *trans*-cyclooctene (TCO) (2) and fluorescent BODIPY-FL (3) (Fig. 1). We employed these probes to perform IED-DA ligation-based pull-down assay and cellular TR-FRET assay (Fig. 2) to determine the level of Dasatinib's cellular target occupancy of two kinases; c-Src and mitogen-activated protein kinase 14 (MAPK14) also known as p38- α . Our studies showed that the cellular TR-FRET assay, which did not require engineering the target protein, very closely reflected results of the biochemical binding assay however its development was limited to the higher affinity target c-SRC. In contrast, the IED-DA ligation-based pull-down assay was successfully developed for both proteins-of-interest, c-SRC and p38- α , and as such it emerged as a relatively straightforward method allowing classification of the level of cellular occupancy of two Dasatinib targets in parallel albeit in lower throughput.

Results and discussion

Dasatinib (1) consists of four ring systems – 2-chloro-6-methyl phenyl, aminothiazole, pyrimidine and piperazine (Fig. 1). As reported previously, the hydroxyethyl chain, which decorates the piperazine ring, is responsible for the enhancement of Dasatinib's solubility and does not significantly impact protein affinity.¹³ Furthermore, as revealed by X-ray crystallography, the hydroxyethyl chain occupies a solvent exposed portion of kinase binding site, and is not responsible for any critical interactions with its protein binding partners.^{14,15} Therefore, replacement of the hydroxyethyl chain through attachment of linker-like substituents was expected, as shown previously, to have limited impact on affinities of the newly created probes for target proteins as compared to affinities exhibited by Dasatinib itself.^{11,16} Detailed description of the synthesis of TCO probe 2 and BODIPY-FL probe 3 can be found in the ESI.†

To confirm that attachment of bulky substituents in the structure of probe 2 did not attenuate its ability to bind to c-Src and p38- α , the compound was evaluated in an *in vitro* TR-FRET competitive binding assay with recombinant c-Src and p38- α . Probe 2 was observed to bind to c-Src and p38- α with a K_i of 0.11 nM and 11 nM respectively (Table 1). In comparison, in the same experiment, Dasatinib was observed to bind to c-Src with K_i = 0.6 nM, and to p38- α with K_i = 47 nM (Table 1). Therefore, as expected and reported before,^{11,16} derivatization of Dasatinib *via* the TCO attachment did not significantly impact the affinity of probe 2 for c-Src or p38- α nor their broader kinome profile (Fig. S1A†). While evaluation of fluorescent probe 3 in an *in vitro* TR-FRET binding assay was prevented by experimental constraints posed by the reporter interference, both probes were found to retain low nM activity similar to the parent compound in a K562 cell proliferation assay (Dasatinib EC_{50} = 0.4 nM (95% confidence interval (95% CI), 0.18–0.74 nM); probe 2 EC_{50} = 3 nM (95% CI, 2.3–4.3 nM); probe 3 EC_{50} = 13 nM (95% CI, 10–17 nM)) (Table 1, Fig. S1C†). Although it is possible to observe considerable differences between biochemical and cellular potency for select ligands,¹⁷ these results in combination with previous reports provided us with greater confidence in the ability of probe 2 and probe 3 to recapitulate the modality of unmodified Dasatinib.^{11,16}

Bioorthogonal reactions are defined as chemical transformations that occur between two functional groups in the context of a biological environment without compromising that environment. In recent years, a growing number of transformations that fulfill the conditions of bioorthogonality, namely selectivity and biocompatibility, have been reported.¹⁸ Introduced in 2008, the inverse-electron demand Diels–Alder (IED-DA) reaction between strained alkenes or alkynes and tetrazines has attracted considerable attention.¹⁹ IED-DA displays an extremely fast rate constant (up to 10^6 M⁻¹ s⁻¹ for the reaction between a strained TCO and tetrazines) that is comparable to enzyme catalyzed reactions, and has not been matched by any other bioorthogonal transformation. Moreover, as a reflection of its bioorthogonal character IED-DA is marked by high selectivity and stability of reactants and products in biological systems. Consequently, IED-DA-based

Fig. 1 Structure of Dasatinib (1), *trans*-cyclooctene (TCO) probe 2 and BODIPY-FL probe 3 used in the study.

Fig. 2 General representation of label-based target engagement methods evaluated in the study. **A.** IED-DA ligation-based pull-down assay combined with immunoblot-based readout. Treatment of intact cells for 1 h with descending concentrations of a ligand-of-interest is followed by the addition of a TCO probe for 2 h. Subsequent exposure of cell lysate to tetrazine-decorated beads leads to IED-DA reaction-mediated protein enrichment that is followed by the immunoblot analysis. Densitometry-based quantification of the level of protein-of-interest captured by TCO probe that was not outcompeted by a ligand-of-interest allows for determination of cellular target occupancy. **B.** *In cellulo* TR-FRET assay combined with plate-based readout. Treatment of intact cells with descending concentrations of a ligand-of-interest for 1 h is followed by the addition of a fluorescent probe. Subsequent exposure of cell lysate to a protein-of-interest specific antibody and Tb-labeled secondary antibody enables detection of TR-FRET signal. Quantification of TR-FRET signal leads to generation of a dose response curve and determination of target occupancy.

transformations have been utilized in a variety of applications spanning from affinity pull-down-driven target validation efforts to genetic code expansion.^{20–24}

Recently, Rutkowska *et al.* described an effort to identify a modular single probe-based strategy that allows for a combination of applications including identification of drug localization, target identification, and target occupancy.²⁵ IED-DA-based ligation between TCO-derived small molecule probe and tetrazine-conjugated fluorescent reporter was determined to be the most suitable amongst available bioorthogonal transformations for this convergent approach. By merging

IED-DA ligation with fluorescent microscopy or FACS, the authors demonstrated the ability to determine *in cellulo* target occupancy of reversible PARP1 inhibitors without the need to isolate the protein-of-interest. In this report, we set out to establish if IED-DA ligation approach could enable evaluation of target occupancy in cases when the ligand is suspected to have many cellular binding partners, and the application of imaging techniques would be challenging. This approach would involve merging IED-DA ligation-based pull-down, with immunoblot analysis as a readout method to quantify target occupancy of multiple targets in parallel (Fig. 2A). In more detail, TCO probe 2 would be utilized to engage proteins-of-interest that are not out-competed by Dasatinib in intact cells. Subsequent cell lysis, followed by exposure to tetrazine-modified beads would lead to enrichment of proteins-of-interest and their detection by immunoblot allowing for assessment of target occupancy. Analogous affinity capture methods utilizing probe-conjugated beads were described previously.²⁶ However, their application was limited to cell homogenates as a source of probe–target protein interactions.

To establish if cellular target occupancy, defined as ligand concentration in cells that provides 50% occupancy of

Table 1 Inhibition constants (K_i) and EC_{50} values determined for Dasatinib (1), probe 2 and probe 3. *In vitro* competitive TR-FRET binding assay utilizing recombinant c-SRC and p38- α was applied to determine K_i of Dasatinib and probe 2. CellTiter-Glo luminescent assay conducted with a use of K562 cells was applied to determine EC_{50} of Dasatinib, probe 2 and probe 3

	c-Src K_i [nM]	p38- α K_i [nM]	K562 EC_{50} [nM]
Dasatinib (1)	0.6	47	0.4
TCO probe (2)	0.11	11	3
BODIPY-FL probe (3)	NA	NA	13

proteins-of-interest (OC_{50}), could be determined using IED-DA ligation-based pull-down combined with immunoblot analysis, we investigated the ability of TCO probe 2 to capture c-Src and p38- α in intact cells. Live K562 cells were treated with probe 2 at concentrations 0.5 μ M, 1 μ M, 2 μ M, 5 μ M or 10 μ M for two hours. Subsequently, cells were harvested and lysates were mixed with tetrazine-conjugated beads to allow for IED-DA reaction to occur. After 45 min incubation, followed by elution in LDS buffer and SDS-PAGE electrophoresis, immunoblot analysis was conducted which showed that probe 2 captured from 0.2% to 0.5% of cellular c-Src, and from 5% to 27% of cellular p38- α (Fig. S2 †). We concluded that capture efficiency achieved with the use of probe 2 at a concentration of 2 μ M should be sufficient for the competition experiments. Consequently, we began an effort to determine the level of cellular occupancy of c-Src and p38- α by Dasatinib. Live K562 cells were subjected to treatment with increasing concentrations of Dasatinib. Based on the results of biochemical assays, Dasatinib concentrations of 0.1–1000 nM for c-Src and 100 nM–100 μ M for p38- α in 10-fold increments were applied. After one hour incubation, probe 2 (2 μ M) was added and the aforementioned set of steps performed to isolate both proteins-of-interest. Densitometry-based quantification of immunoblots revealed an average OC_{50} = 31 nM (95% CI, 18–41 nM) for c-Src, and OC_{50} = 220

nM (95% CI, 155–320 nM) for p38- α (Fig. 3A and B). While the latter value was found to be in good agreement with biochemical assay results, a 30-fold difference was observed between K_i and OC_{50} determined against c-Src, presumably due to inefficient release of c-Src from the beads after capture with tightly bound probe 2.^{26,27} However, we were pleased to see that IED-DA assay clearly distinguished and ranked both proteins in correlation with their *in vitro* binding affinities against Dasatinib.

The process of Förster resonance energy transfer (FRET) involves donation of energy between two neighboring fluorophores. The donor fluorophore absorbs light energy to subsequently transfer it in a non-radiative way to an acceptor fluorophore. The result of this sequence is emission of longer-wavelength light by the acceptor. For the process to be successful, certain conditions must be met. First, the donor and acceptor must be characterized by overlapping emission and excitation spectra. Second, both fluorophores must be located within 10 nm of each other. FRET can be utilized to detect interactions between molecules, and has been applied in the development of numerous biochemical and cellular assays.^{28,29} In the last decade, one key contribution to the advancement of FRET technique was the introduction of rare-earth lanthanides in the role of donors.³⁰ Owing to their relatively long emission half-lives, lanthanides can transmit

Fig. 3 Cellular Dasatinib occupancy of c-Src and p38- α determined using TCO probe 2. A. Immunoblot-based depiction of c-Src (left panel) or p38- α (right panel) captured by TCO probe 2 (2 μ M) that was not out-competed by Dasatinib. B. Densitometry-based quantification of immunoblots shown in A. Results are representative of three independent experiments. C. Dasatinib target occupancy plot for c-Src (left panel) and p38- α (right panel) generated based on quantification shown in B. Results are representative of three independent experiments.

energy in a time-resolved manner, thus contributing to a reduction of background fluorescence and an overall improvement in the signal-to-noise ratio. Consequently, biochemical time-resolved FRET (TR-FRET) has become the most popular format behind development of homogenous high-throughput assays (HTRF) that do not require additional separation steps.³¹ Furthermore, numerous HTRF-type assays, that monitor protein-of-interest in the context of cellular settings, have been developed.³² However, previously reported HTRF assays require engineering the protein-of-interest through the introduction of a reporter tag that could be recognized by a lanthanide-conjugated antibody. We decided to investigate if an alternative approach could be developed which did not require engineered protein to evaluate interaction of Dasatinib with its targets. After successful development of a live-cell TR-FRET drug-target engagement assay for covalent kinase inhibitors (unpublished results), our goal was to identify conditions for TR-FRET-based detection of endogenous c-Src and p38- α that could determine the level of occupancy of those targets by non-covalent binders (Fig. 2B).

First, with the use of recombinant c-Src or p38- α , we set out to identify primary antibodies whose application would produce the highest TR-FRET signal-to-noise ratio in an *in vitro* setting (Fig. S3†). As a source of lanthanide donor, we

used commercial Tb-labeled secondary antibodies (Lantha Screen). By conducting the competition assay with Dasatinib and probe 3 (20 nM), we identified the anti-Src antibody Ab16885 (at the initial concentration 2 nM) to be most suitable for the desired task. Its application produced a TR-FRET signal with 20-fold difference over background (in comparison Ab109381 gave only a 3-fold difference in TR-FRET signal-to-noise ratio). In contrast, we were unable to identify an antibody, that in combination with probe 3 would produce TR-FRET signal-to-noise ratio significant enough for detection of p38- α , presumably due to low probe affinity to the target and need for higher probe concentration (>100 nM). Next, several parameters of the TR-FRET assay were optimized including lysis buffer (Fig. S4A†), number of cells used per treatment (Fig. S4B†), and antibody and probe concentrations (Fig. S5 and S6† respectively). It was established that the highest TR-FRET signal-to-noise ratio could be detected with 62 500 cells per well in NP40 buffer (0.1% NP40, 5 mM EDTA, 50 mM MgCl₂) in the presence of Ab16885 (0.15 nM) and probe 3 (3 nM). Finally, Dasatinib occupancy of c-Src was measured (Fig. 4). In the first approach, K562 cells pre-incubated with Dasatinib for one hour were harvested, and probe 3 (3 nM), Ab16885 (0.15 nM) and Tb-labelled secondary antibody (0.1 μ g) were added to the lysates. In the second

Fig. 4 Cellular Dasatinib occupancy of c-Src determined by TR-FRET assay with use of BODIPY-FL probe 3. Two orders of addition of probe 3, before and after cell lysis, as well as assay equilibration time, were investigated. A. TR-FRET signal detected using probe 3 that was not out-competed by Dasatinib. K562 cells were treated with increasing concentrations of Dasatinib (10 pM–0.1 μ M). After 1 h, cells were harvested, and probe 3 (3 nM) and c-Src-specific antibody Ab16885 (0.15 nM) were added to the lysate. TR-FRET signal was detected after 180 min incubation. Results are representative of six independent experiments. B. TR-FRET signal detected using probe 3 that was not out-competed by Dasatinib. K562 cells were treated with increasing concentrations of Dasatinib (10 pM–0.1 μ M). After 1 h, probe 3 (3 nM) was added. After 1 hour incubation, cells were harvested, and c-Src-specific antibody Ab16885 was added to the lysate. TR-FRET signal was detected after 180 min incubation. Results are representative of six independent experiments. C. Dasatinib OC₅₀ for c-Src determined based on measurements conducted after 30 min, 90 min, 180 min, 300 min or 24 h incubation using conditions described in A and B.

approach, pre-incubation with Dasatinib (1 h) was followed by addition of probe 3 (3 nM) for two hours at which point cells were harvested and mixture of Ab16885 (0.15 nM) and Tb-labelled secondary antibody (0.1 µg) was added to the lysates. As shown in Fig. 4, both approaches produced nearly identical results with $OC_{50} = 0.4$ nM (95% CI, 0.3–0.6) and 0.2 nM (95% CI, 0.1–0.3 nM), respectively. The results were found to be in good agreement with the biochemical binding assay (Table 1).

Conclusions

We developed and evaluated two approaches to measure occupancy of two protein targets c-Src and p38- α by Dasatinib in live cells. We surveyed IED-DA ligation-based pull-down assay and cellular TR-FRET assay that relied on the application of a fluorescent probe and commercially available antibody. Our study indicates that IED-DA assay can be applied to determine target occupancy for both high and low affinity binders of Dasatinib, showing high utility and relative ease of probe and assay development. The ability to rank target occupancy of multiple targets in parallel is a key strength of the IED-DA assay. Concurrently, cellular TR-FRET, due to its plate format, constitutes a very promising platform for determination of target occupancy of specific protein in a high-throughput manner. Unlike other reported methods for high-throughput target occupancy determination, cellular TR-FRET assay does not require engineering the protein-of-interest, and therefore introduces minimum perturbations to its native state and environment.⁷ The identification of a potent fluorescent probe, and the identification of a high-affinity antibody that does not impede small molecule binding, are critical to the development of a cellular TR-FRET assay. As a result, antibody screening should be performed prior to the assay optimization. However, the availability of terbium-labelled antibodies for a range of IgGs from different species obviates the need to label each primary antibody with a terbium chelate, thus significantly facilitating the identification of a suitable antibody.

The characterization of small molecule protein binders through determination of target engagement in *in vivo* setting has been recognized as one of the key pursuits to ensure success of medicinal chemistry campaigns.^{2,3} Therefore, the continuation of efforts to expand the available toolbox of target engagement techniques as well as to better understand their strengths and limitations remains critical to further advancement of the field and its ability to contribute to development of new therapeutics.

Disclosures

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