A cellular target engagement assay for the characterization of SHP2 (PTPN11) phosphatase inhibitors

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The nonreceptor protein-tyrosine phosphatase (PTP) SHP2 is encoded by the proto-oncogene PTPN11 and is a ubiquitously expressed key regulator of cell signaling, acting on a number of cellular processes and components, including the Ras/Raf/Erk, PI3K/Akt, and JAK/STAT pathways and immune checkpoint receptors. Aberrant SHP2 activity has been implicated in all phases of tumor initiation, progression, and metastasis. Gain-of-function PTPN11 mutations drive oncogenesis in several leukemias and cause developmental disorders with increased risk of malignancy such as Noonan syndrome. Until recently, small molecule-based targeting of SHP2 was hampered by the failure of orthosteric active-site inhibitors to achieve selectivity and potency within a useful therapeutic window. However, new SHP2 allosteric inhibitors with excellent potency and selectivity have sparked renewed interest in the selective targeting of SHP2 and other PTP family members. Crucially, drug discovery campaigns focusing on SHP2 would greatly benefit from the ability to validate the cellular target engagement of candidate inhibitors. Here, we report a cellular thermal shift assay that reliably detects target engagement of SHP2 inhibitors. Using this assay, based on the DiscoverX InCell Pulse enzyme complementation technology, we characterized the binding of several SHP2 allosteric inhibitors in intact cells. Moreover, we demonstrate the robustness and reliability of a 384-well miniaturized version of the assay for the screening of SHP2 inhibitors targeting either WT SHP2 or its oncogenic E76K variant. Finally, we provide an example of the assay's ability to identify and characterize novel compounds with specific cellular potency for either WT or mutant SHP2.

The dynamic regulation of tyrosine phosphorylation by protein-tyrosine kinases (PTKs) and protein-tyrosine phosphatases (PTPs) is of singular importance in the control of cell-signaling pathways, and the dysregulation of PTK or PTP function is known to play a major role in many human diseases (1, 2). The inhibition of PTKs has been heavily pursued and has led to multiple successful therapies in the clinic (3, 4). Alternatively, efforts to modulate the reciprocal PTPs have received less attention because these proteins have been historically viewed as less specific and were deemed intractable from a drug discovery standpoint (5). However, research over the past 15 years has made it clear that PTPs, similar to PTKs, are highly regulated enzymes and specific for their substrates in vivo, primarily because of the combined effects of regulatory domains, post-translational modifications, and subcellular sequestration (6). Moreover, recent reports of novel small molecule inhibitors have opened up new avenues for drug discovery, shifting the focus away from compounds that target the highly conserved PTP active site, to allosteric inhibitors with novel mechanisms of action (5, 7, 8). Currently, many of the over 100 members of the human PTP superfamily are being explored as drug targets in all major disease areas, including cancer (9–11), cardiovascular disease (12–14), metabolic disease (15, 16), neurological disorders (17, 18), and autoimmunity and immunodeficiency (19, 20).

The Src homology 2 (SH2) domain-containing phosphatase 2 (SHP2) has recently gained considerable interest because of its fundamental role in tumor initiation, progression, and metastasis in a growing number of cancers (21). SHP2 (encoded by the PTPN11 gene) is a well-known proto-oncoprotein that drives carcinogenesis through activation of the Ras/Raf/Erk, PI3K/Akt, or JAK/STAT signaling pathways (22). Germ-line mutations in SHP2 were first observed in ~50% of cases in Noonan syndrome, a developmental disorder with an increased risk of malignancy (23). Numerous somatic mutations in SHP2 have been identified in cancer, primarily in leukemias (24).

This article contains supporting text, Tables S1 and S2, and Figs. S1 and S2. To whom correspondence should be addressed: Cancer Metabolism & Signaling Networks Program, NCI-Designated Cancer Center, Sanford Burnham Prebys Medical Discovery Institute, 10901 North Torrey Pines Rd., La Jolla, CA 92037. E-mail: tautz@sbpdiscovery.org.

This work was supported by National Institutes of Health Grants 1R21CA195422 (to L. T.) and 1R21NS067502 (to E. B. P.), NCI Experimental Therapeutics (NEXT) Program Grant Leidos-16X113-SHP2 (to L. T.), Epstein Family Foundation Award (to N. D. P. C.), and NCI Cancer Center Support Grant P30CA030199. The authors declare that they have no conflicts of interest with the contents of this article. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

2 The abbreviations used are: PTK, protein-tyrosine kinase; PTP, protein-tyrosine phosphatase; SHP2, Src homology 2 domain-containing phosphatase 2; PTKs, protein thermal shift; CETS, cellular thermal shift assay; GST, glutathione S-transferase; TCEP, (tris-2-carboxyethylphosphine); DIFMuP, 6,8-difluoro-4-methylumbelliferyl phosphate; IRS-1, insulin receptor substrate 1; SH2, Src homology 2; EFC, enzyme fragment complementation; ePL, enhanced ProLabel.© 2020 Romero et al. Published under exclusive license by The American Society for Biochemistry and Molecular Biology, Inc.
Normally, SHP2 is tightly regulated by intramolecular interactions between its N-terminal SH2 and phosphatase (PTP) domains, which under resting conditions form a closed, autoinhibited inactive conformation (Fig. 1A) (25, 26). The activity of WT SHP2 in cancer is primarily enhanced because of increased levels of phosphorylated SHP2-binding proteins (Fig. 1B). In leukemias, oncogenic mutations located at the interface between the N-SH2 and PTP domains prevent SHP2 from closing, resulting in a constitutively active SHP2. D, crystal structure of the SHP2:SHP099 complex (PDB accession number 5EHR) with the N-SH2 (blue), C-SH2 (green), and phosphatase domain (orange) in the closed, autoinhibited conformation. The allosteric inhibitor SHP099 binds in a “tunnel” formed at an interface of the three domains and stabilizes SHP2 in its inactive conformation. E, allosteric SHP2 inhibitors such as SHP099 or RMC-4550 compete with SHP2 activation, as the allosteric binding site only exists in the closed conformation. The effect of SHP2 gain-of-function mutations is to destabilize the autoinhibited confirmation of SHP2. Therefore, many oncogenic SHP2 mutants are resistant to inhibition by the SHP099 class of compounds.

An essential aspect of hit candidate selection for lead optimization is proven target engagement not just in vitro but critically also in the cell. A testing funnel that includes such a metric can assure that resources for medicinal chemistry are efficiently deployed on compounds with promising cellular efficacy. Multiple approaches to validate small molecule engagement with their target protein in living cells have been developed, primarily for protein kinases (36). With our focus on SHP2, we chose to investigate the use of a cellular thermal shift assay (CETSA), which monitors the thermal stability of a target protein in cells (37). Similar to the in vitro protein thermal shift (PTS) assay (38), binding of a small molecule typically changes the levels of intact protein over a temperature gradient compared with a vehicle control. The CETSA as originally reported uses specific antibodies to the target protein to quantify its levels. This method has a relatively low throughput and is limited by the...
In vitro characterization of SHP2 allosteric inhibitors using biochemical inhibition and protein thermal shift assays

To develop a cellular target engagement assay for SHP2, we selected a set of known SHP2 allosteric inhibitors and characterized them first in vitro in biochemical inhibition and PTS assays (Table 1). We selected the Novartis compound SHP099 (30) as the prototypical example of a new class of SHP2 allosteric inhibitors. Further, we selected SHP836 (30), the original hit that led to the development of SHP099. SHP836 is substantially less potent than SHP099 in enzymatic phosphatase assays; the reported IC50 values for SHP2-WT are 5 μM for SHP836 and 70 nM for SHP099. We also selected Example 57 (Ex-57) from a Novartis patent (42), a more potent analog of SHP099 with a reported IC50 for SHP2-WT of 3.0 nM. Finally, we selected Revolution Medicines’ SHP2 inhibitor RMC-4550, which is ~100-fold more potent than SHP099 (reported SHP2-WT IC50 = 0.58 nM), but shares its 2,3-dichlorophenylpyrazine core scaffold (32). For in vitro assays, highly pure (>95%) full-length recombinant SHP2-WT protein (residues 1–594) was expressed as a GST-tagged fusion in Escherichia coli and purified via GSH affinity column chromatography, subsequent thrombin cleavage of the GST tag, and S200-column size-exclusion chromatography. Because SHP099-like inhibitors have a greatly reduced effect on many of the SHP2 oncogenic mutants (33–35), we also included the most common SHP2 variant in cancer, SHP2-E76K (24), into our testing regimen. Site-directed mutagenesis was used to generate the full-length SHP2-E76K construct, which was expressed and purified similarly to SHP2-WT. Experiments also included the SHP2 catalytic domain (SHP2cat; residues 248–527) as a negative control, because SHP099-like inhibitors do not have detectable activity against SHP2cat because of their unique binding mode that requires the presence of the SHP2 SH2 domains (30, 32).

First, we tested all SHP2 compounds in biochemical dose-response kinetic inhibition assays. We utilized a standard fluorescence intensity phosphatase assay in 384-well plate format using 6,8-difluoro-4-methylumbelliferyl phosphate (DiFMUP) as the substrate (43). Because WT SHP2 adopts an inactive conformation in which its active site is occluded by its N-terminal SH2 domain (Fig. 1A), SHP2-WT was activated by preincubation with a dually phosphorylated insulin receptor substrate 1 (IRS-1) peptide that served as an SH2 domain–binding protein surrogate. Titration of IRS-1 indicated maximal SHP2-WT activation at 1000 × SHP2 concentration. IRS-1 at the corresponding concentration (500 nM final concentration) was added to the full-length SHP2-WT and SHP2-E76K assays. IRS-1 was not used in experiments with SHP2cat, which does not contain the SH2 domains. Michaelis–Menten kinetic experiments were performed for each SHP2 construct to determine the Michaelis–Menten constants (Km) as described previously (44). Dose-response assays to determine IC50 values utilized DiFMUP at a concentration corresponding to the respective Km value for each SHP2 protein. Calculated IC50 values are provided in Table 1 and largely match reported values in the literature. As expected, all compounds exhibited ~3 orders of magnitude greater activity for SHP2-WT compared with SHP2-E76K. None of the four allosteric inhibitors showed any appreciable inhibitory activity toward SHP2cat.

Next, we set out to determine the effects of the allosteric inhibitors on the melting temperature of the SHP2 proteins in vitro using a ThermoFluor PTS assay (38). We established PTS assays in 384-well format for SHP2-WT, SHP2-E76K, and SHP2cat. Under optimized assay conditions, all SHP2 constructs yielded melting profiles with a single inflection point, a primary peak in the first derivative plot, and melting temperatures (Tm) with low error margins (Fig. 2). Notably, the Tm was substantially reduced in the SHP2 mutant protein (Tm = 42.1 ± 0.1 °C) compared with WT (Tm = 51.3 ± 0.1 °C), reflecting a lower thermal stability caused by the E76K mutation, which prevents SHP2 from adopting its closed and compact conformation. The melting temperature of the catalytic domain (Tm = 44.0 ± 0.1 °C) was also reduced compared with the full-length protein. To demonstrate the suitability of thermal shift for the identification of allosteric SHP2 inhibitors, we first tested SHP099 in the established PTS assays. At 50 μM concentration, SHP099 substantially increased the Tm of SHP2-WT (∆Tm = 4.8 °C; Fig. 2A), and we confirmed that the Tm shift

### Table 1

| Compound | Chemical Structure | ΔTm (°C) | Enzymatic IC50 (μM) |
|----------|--------------------|----------|---------------------|
| SHP099   | ![Structure](image1) | 1.8 ± 0.2 | 0.1 ± 0.1 |
| SHP836   | ![Structure](image2) | 0.2 ± 0.1 | 0.1 ± 0.1 |
| Ex-57    | ![Structure](image3) | 4.4 ± 0.1 | 3.3 ± 0.1 |
| RMC-4550 | ![Structure](image4) | 6.3 ± 0.1 | 3.9 ± 0.1 |
| DMSO**   | ![Structure](image5) | 5.3 ± 0.1 | 42.1 ± 0.1 |

*SHP2 inhibitor concentration tested.
**Vehicle.
***Tm (°C).

availability of highly specific antibodies. Alternatively, we chose to investigate a recently developed variant of CETSA that utilizes the InCell Pulse DiscoverX platform (39). Based on this technology, we developed cellular target engagement assays for several forms of SHP2, including WT SHP2 (SHP2-WT), a frequent SHP2 oncogenic variant (SHP2-E76K) (24), and the SHP2 catalytic domain (SHP2cat). We showed the power of this miniaturized assay to reliably detect target engagement of SHP2 allosteric inhibitors in cells and demonstrate a high degree of correlation among inhibitor in vitro potency, in vitro thermal shift, and cellular thermal shift data. Finally, we provide an example of the assay’s ability to assess and prioritize novel SHP2 inhibitors from our laboratory. Given the relatively simple assay protocol and instrumentation, we propose that this cellular target engagement assay can be valuable for small molecule inhibitor screening campaigns against SHP2 and other phosphatases.

### Results

**In vitro characterization of SHP2 allosteric inhibitors using biochemical inhibition and protein thermal shift assays**

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induced by this compound was dose-dependent (Fig. 2B). Thermal shift of the SHP2-E76K mutant by SHP099 ($\Delta T_m = 1.2^\circ C$; Fig. 2C) was attenuated compared with SHP2-WT, which was in agreement with the reduced inhibition of SHP2-E76K by SHP099. Stabilization of SHP2cat by SHP099 did not occur (Fig. 2D), reflecting the lack of potency of SHP099 and similar compounds toward the catalytic domain alone. Next, we tested SHP836, Ex-57, and RMC-4550 in similar experiments, and the results, demonstrating the utility of the PTS assay for benchmarking SHP2 allosteric inhibitors, are shown in Table 1. The more potent SHP099 analogs Ex-57 and RMC-4550 produce a greater thermal shift than SHP099, and this is true for both SHP2-WT and SHP2-E76K. Similar to SHP099, the shift caused by the analogs is reduced for E76K compared with SHP2-WT. As expected, the least potent inhibitor, SHP836, produces the smallest thermal shift of the four compounds, which again is greater for SHP2-WT than for the E76K mutant. Finally, none of the SHP2 allosteric inhibitors caused a thermal shift of the SHP2 catalytic domain. Thus, the PTS data and relative thermal shift potencies track with the biochemical IC$_{50}$ measurements. Moreover, the significant thermal shifts observed with these compounds provided the necessary confidence that target engagement of SHP2 allosteric inhibitors in a complex cellular environment could be assessed and interpreted using a cellular thermal shift assay.

Development of SHP2 cellular thermal shift assays

A cellular thermal shift assay is based on the same core concept as the in vitro PTS assay (Fig. 3A). In each technique, an elevated temperature is applied to a protein of interest that causes the protein to unfold. With the PTS assay, measurement of this thermal transition is achieved by performing the assay in the presence of a fluorescent dye that binds and detects increasing concentrations of the exposed hydrophobic interior residues of a target protein (Fig. 3B). With CETSA, the heat-induced thermal transition that causes the target protein to unfold and aggregate is monitored by the measurement of thermal-induced depletion of the signal from the nonaggregated protein (Fig. 3C). In contrast to PTS, each data point used to generate a thermal profile in CETSA is obtained from an individually assayed well. The originally described CETSA technique relied on a relatively low-throughput immunoblot assay for quantification of the target protein that remained in solution. An innovative effort by McNulty et al. (39) recently addressed multiple challenges to improve the original detection method by application of the InCell Pulse$^\text{TM}$ (DiscoverX) platform for efficient cellular target engagement measurement. InCell Pulse uses a $\beta$-gal–based enzyme fragment complementation (EFC) assay to determine levels of intact protein using chemiluminescence as a readout (Fig. 3D). The protein of interest is expressed in cells with an enhanced ProLabel$^\text{®}$ tag (ePL, 42-amino acid fragment of $\beta$-gal) fused either to the N or C terminus of the target protein. The cells are incubated with compounds of interest and subjected to a brief heat treatment using a temperature gradient delivered by a thermocycler. Based on their thermal stability, proteins denature and become aggregated with the temperature increase. However, in the presence of bound ligand, ePL-tagged proteins exhibit an elevated thermal stability and are able to complement the reporter enzyme acceptor at higher temperatures, resulting in detectable $\beta$-gal activity using a luminescence substrate (Fig. 3A).

Based on the pICP–ePL plasmid designed for the InCell Pulse technology, we engineered transient expression constructs for N-terminally ePL-tagged SHP2-WT, SHP2-E76K,
and SHP2cat. We tested the levels of transient expression of these constructs in HEK293T cells by immunoblotting using anti-ePL antibodies (Fig. S1). As a control, we included a construct encoding the MTH1 protein, which is provided with the InCell Pulse kit. Comparable levels of expression were observed for ePL-tagged MTH1, SHP2-WT, and SHP2-E76K, whereas SHP2cat was expressed at a slightly reduced level. Analysis of cellular viability after transfection did not indicate a difference between cells transfected with MTH1, SHP2-WT, SHP2cat, or SHP2-E76K. Next, we demonstrated the utility of the InCell Pulse assay format under miniaturized conditions using the MTH1 plasmid and control compound TH588, an MTH1 inhibitor. HEK293T cells were plated and transiently transfected with pICP-ePL-MTH1. A 384-well PCR plate was prepared by spotting TH588 or vehicle control (DMSO) across an entire row (24 wells) in duplicate using an acoustic dispenser for a final TH588 concentration of 10 µM and a corresponding final DMSO concentration of 0.1% (v/v). Transfected cells were diluted to a density of ~125 cells/µl, and a 5-µl volume of cells was added to compound and control wells, respectively. The
cells were incubated with compound or vehicle for 1 h at 37 °C, before a pulse temperature gradient of 38–68 °C was applied across the 24 wells for 3 min using a thermocycler. The heat pulse denaturation and subsequent recovery period was immediately followed by the addition of 5 μl of InCell Pulse detection reagent containing lysis buffer, the β-gal enzyme acceptor, and the luminescence substrate. After an incubation for 1 h, chemiluminescence was detected using a microplate reader. Thermal profiles were generated by analyzing the normalized data using nonlinear regression (Fig. 4). The melting temperature of MTH1 was determined to $T_m = 45.7 \pm 0.1$ °C (Fig. 4A). In the presence of 10 μM TH588, MTH1 was dramatically stabilized ($T_m = 14.4 \pm 0.2$ °C) in agreement with previous results. Importantly, the well-constrained data points provided confidence not only of target engagement by the MTH1 inhibitor but also of a reliable miniaturized InCell Pulse protocol.

Figure 4. Development of a cellular target engagement assay for WT and oncogenic mutant (E76K) SHP2 proteins. Transiently transfected HEK293T cells were used to investigate the utility of a cellular thermal shift assay based on the InCell Pulse technology. A, thermal profiles of the control protein MTH1 in the presence (blue) or absence (vehicle, red) of the MTH1 inhibitor TH588 (10 μM). As expected, TH588 does not engage with MTH1. B, thermal profiles of the SHP2 catalytic domain in the presence (blue) or absence (vehicle, red) of the SHP2 allosteric inhibitor SHP099 (10 μM). The SHP099-like allosteric inhibitors RMC-4550 (10 μM, green), Ex-57 (10 μM, blue), or SHP836 (50 μM, violet) exhibit a greater stabilization of SHP2-WT than SHP099, in agreement with the greater potency of these compounds compared with SHP099 in both the in vitro PTS and biochemical inhibition assays. Similarly, the muted effect of SHP836 on SHP2-WT in cells corresponds with the lower potency of this compound in the in vitro assays. F, thermal profiles of SHP2-E76K in the absence (vehicle, red) or presence of SHP099-like allosteric inhibitors RMC-4550 (10 μM, green), Ex-57 (10 μM, blue), or SHP836 (50 μM, violet). All compounds exhibit an attenuated effect on the SHP2-E76K mutant in cells, which is also in agreement with the in vitro PTS binding and enzymatic inhibition data. The data points and error bars (± S.D.) represent duplicate measurements.
Next, using the established assay conditions, we set up similar experiments for SHP2cat, SHP2-WT, and SHP2-E76K in the presence or absence of SHP099 (Fig. 4, B–D). All SHP2 constructs produced thermal melt profiles with low error margins and good curve fits ($r^2$ for all conditions $> 0.97$). Interestingly, the observed melting temperatures of SHP2 proteins in the absence of inhibitor were comparable with those measured in the in vitro PTS assay (Table S1). The destabilizing effect of the E76K point mutation on the SHP2 protein was also evident in cells. In the presence of 10 μM SHP099, SHP2-WT was substantially stabilized ($\Delta T_m = 3.7°C$). This shift was comparable with the measured shift in vitro using the PTS assay ($\Delta T_m = 1.8°C$ at 12 μM SHP099). SHP099 at 10 μM did not affect the thermal stability of SHP2-E76K or SHP2cat, which again mirrored the results observed in the PTS assay. Next, we tested SHP836, Ex-57, and RMC-4550 in similar experiments against both SHP2-WT and SHP2-E76K (Fig. 4, E and F). Evident in the thermal profiles was the increased stabilization of SHP2-WT afforded by Ex-57 ($\Delta T_m = 7.0°C$) and RMC-4550 ($\Delta T_m = 7.0°C$) compared with SHP099 ($\Delta T_m = 3.7°C$). Thus, the cellular thermal shift magnitude of SHP2 inhibitors tracks with both the biochemical inhibition and PTS potency. When cells expressing the SHP2-E76K mutant were subjected to treatment with Ex-57 or RMC-4550, an attenuated thermal shift was observed (Ex-57, $\Delta T_m = 2.3°C$; RMC-4550, $\Delta T_m = 2.3°C$). The greater scatter in points at the inflection point somewhat restricted interpretation of the E76K curves. However, the data did suggest that these compounds are able to bind and stabilize the SHP2-E76K mutant to some degree in cells. Again, these results are in agreement with the PTS and biochemical inhibition data. Finally, the weaker allosteric inhibitor SHP836 did not affect the thermal stability of either SHP2-WT or SHP2-E76K at 10 μM ligand concentration (data not shown). When we tested SHP836 at an increased concentration of 50 μM, we observed a modest thermal shift for SHP2-WT ($\Delta T_m = 1.9°C$) and a lesser shift for SHP2-E76K ($\Delta T_m = 0.9°C$) (Fig. 4, E and F). Collectively, our data demonstrate the effectiveness of the InCell Pulse assay for evaluating target engagement of SHP2 allosteric inhibitors in cells. Moreover, our results suggest substantial consistency among biochemical inhibition, in vitro PTS, and cellular thermal shift data, providing assurance that the observed cellular efficacy of inhibitors is a result of direct interactions with their target. In summary, we have developed cellular target engagement assays for SHP2-WT and SHP2-E76K that provide both an indication of a compound’s quantitative capacity to stabilize its cellular target and a means to benchmark SHP2 inhibitors.

**SHP2 CETSA isothermal dose response for the SHP099 allosteric inhibitor**

Next, we established CETSA isothermal conditions to evaluate the dose-dependent target engagement of SHP2 inhibitors. Isothermal titration measurements at the melt curve transition temperature provide a useful comparative cellular dose response for individual compounds (37, 39). It was evident in the thermal profile for the binding of SHP099 to SHP2-WT (Fig. 4C) that the temperature-induced aggregation occurs in a relatively narrow temperature range (−6°C). To obtain the best results for isothermal measurements, we determined the optimal temperature experimentally. To optimize this assay, we took advantage of the ability to invert the axis of the applied temperature gradient on a 384-well plate (Fig. S2). We performed 5-point dose-response measurements for SHP2-WT with titration of SHP099 on the “long axis” and a temperature gradient on the “short axis” (Fig. 5A). When analyzed, it was evident that the best results were obtained in a narrow temperature window (54−57°C). Isothermal 10-point titration measurements of SHP099 with SHP2-WT probed at 55°C followed by nonlinear regression analysis yielded an EC50 value for SHP099 of 4.2 μM (Fig. 5B).

**Optimization and robustness of the SHP2 cellular target engagement assay**

For the routine use of the SHP2 target engagement assay, we evaluated the performance of the miniaturized assay in multiple tests in a 384-well format. Importantly, we compared the transiently transfected SHP2/HEK293T cells to HEK293 cells stably expressing SHP2. Given the reduced expression level of SHP2 in the stable cell line (Fig. S1) and potential variability caused by day-to-day variation in the transfection protocol, we determined the reliability of using cells with stable integration. Using an optimized assay window (chemiluminescence signal to background value greater than 100), the conditions tested were: 1) optimized 5.0 μl of transfected cells (140 cells/μl); 2)
optimized 5.0 μl of 2× stable cells (350 cells/μl); and 3) optimized 12.5 μl of stable cells (140 cells/μl). The assays were performed at a fixed DMSO concentration of 0.5% (v/v), using SHP099 at 10 μM. Temperatures spanned 38.0–68.0 °C with seven replicate wells per point for the DMSO control and the corresponding SHP099 conditions. Assays were performed on four or five separate days. The chemiluminescent data were normalized using the minimum (highest temperature) and maximum (lowest temperature) value, and the temperature profile was fit using a nonlinear Boltzman-Sigmoidal equation to generate \( T_m \) values. The tabulated \( Z' \) values are shown in Table S2 and were calculated as described under “Materials and methods.” The results indicate that the stably integrated SHP2 cell line at 12.5 μl is as reliable as the transiently transfected cells (average \( Z' \) stable = 0.8; average \( Z' \) transient = 0.79). For the stable cell line, the 12.5-μl volume performs better than a lower volume with higher cell concentration (average \( Z' \) stable = 0.8; Average \( Z' \) 2× stable = 0.74). The combined results suggest that stable integration of ePL-tagged SHP2 allows the assay to be performed with high reproducibility at reduced effort.

**Application of the SHP2 cellular target engagement assay in ligand discovery**

As part of a program to identify small molecule compounds that modulate SHP2 activity, we have introduced the SHP2 cellular target engagement assay into our screening and testing funnel. Here, we demonstrate the value of this assay for two series of SHP2 inhibitors, which we initially discovered using a PTS screen of an in-house small molecule library (to be published elsewhere). PTS identified two chemical scaffolds, biphenylpyrimidines and benzothiophenones, as SHP2 ligands exhibiting low micromolar IC\(_{50}\) values (<10 μM; determined in subsequent biochemical inhibition assays against SHP2-WT and/or SHP2-E76K). To assess membrane permeability and target engagement of these inhibitors in cells, we subjected a panel of these compounds to an isothermal CETSA screen against SHP2-WT and SHP2-E76K (Fig. 6A). Among the biphenylpyrimidines tested, seven compounds contained a carboxylic acid moiety in the R\(^1\) position. In four additional compounds of this series, the hydrophilic carboxylic acid group was replaced by the more lipophilic carboxylic acid bioisostere tetrazole. Interestingly, at 30 μM concentration, none of the carboxylic acid derivatives produced a substantial response, compared with the DMSO control, whereas all of the tested tetrazoles exhibited a stabilizing effect on SHP2-WT, indicating target engagement in cells. Compared with carboxylic acid, tetrazole is known to greatly increase the membrane permeability of compounds (46). Thus, the CETSA results for the biphenylpyrimidine series likely reflects the drug likeness of the compounds and specifically their rate of passive permeation across cell membranes, which is a fundamental measure in drug discovery. We also tested the top five compounds from the benzothiophenone series of SHP2 inhibitors and found that all of them destabilize both SHP2-WT and the E76K mutant to various degrees, indicating membrane permeability and target engagement. Notably, the decrease in SHP2 cellular thermal stability reflects the decrease in the melting temperature of SHP2 caused by these compounds in the *in vitro* PTS assay (data not shown). As expected, SHP099 (added as a control) stabilized SHP2-WT while having a negligible effect on SHP2-E76K (Fig. 6A).

Next, we further characterized a representative inhibitor from each series, SBI-221 (Fig. 6B) and SBI-668 (Fig. 6C), by establishing thermal profiles using CETSA. At 30 μM, SBI-221 showed a modest capacity to stabilize the SHP2-WT protein in cells (\( \Delta T_m = 0.71 \) °C; Fig. 6D). Compound SBI-668, which showed a selective preference for destabilizing SHP2-E76K in the isothermal experiment (Fig. 6A), substantially shifted the thermal melting curve of SHP2-E76K (\( \Delta T_m = -2.79 \) °C; Fig. 6E). Interestingly, the preference of SBI-668 to destabilize SHP2-E76K over SHP2-WT was also reflected in the compound’s biochemical IC\(_{50}\) values (E76K: IC\(_{50}\) = 2.1 μM; WT: IC\(_{50}\) = 15 μM).

We further corroborated the effects of SBI-221 and SBI-668 in cells by performing isothermal CETSA dose-response measurements for both SHP2-WT (Fig. 6F) and SHP2-E76K (Fig. 6G). A dose-dependent capacity of SBI-221 to stabilize SHP2-WT was apparent, whereas SBI-668 only had a minimal effect on the SHP2-WT protein (Fig. 6F). Isothermal titration using SHP2-E76K yielded a reversed profile: SBI-668 destabilized the SHP2 mutant protein dose-dependently, whereas SBI-221 had no effect on the mutant (Fig. 6G), confirming the observations from the single concentration experiment. In both cases saturation of the cellular target was not achieved, consistent with the low/submicromolar biochemical inhibitory potency of these compounds. However, the value of the cellular target engagement assay for compound prioritization within a SHP2 inhibitor testing funnel is evident.

**Discussion**

Recognized more than a decade ago as the “first proto-oncogene that encodes a tyrosine phosphatase” (24), SHP2 has become an attractive target for cancer therapy. The spark that truly ignited the recent surge in SHP2 discoveries was the report of SHP099, a potent and highly selective SHP2 inhibitor with antitumor efficacy *in vivo* (30, 31). Based on the unique allosteric mechanism by which SHP099 acts as a “molecular glue” (21) that stabilizes the compact SHP2 autoinhibited conformation, two SHP2 inhibitors (TNO155 and RMC-4630 from Novartis and Revolution Medicines, respectively) are currently in phase I clinical trials for the treatment of solid tumors with enhanced signaling through the Ras/Raf/Erk pathway. A paramount requirement for any drug discovery testing funnel is the proof of specific target engagement of a small molecule lead compound in intact cells. Here, we report the development of a cellular target engagement assay for SHP2 WT and mutant proteins utilizing the InCell Pulse assay platform. This assay is based on the cellular thermal shift paradigm and can be integrated into an early stage of a SHP2 inhibitor testing funnel. Playing a complementary role to cellular readouts of SHP2 activity, such as Erk activation, the thermal shift technique yields mechanistic insights in a cellular context that are difficult to obtain using other methods. Importantly, the assay evaluates physiologically relevant full-length SHP2 and is therefore able to provide information on the intricacies of SHP2 allostery. As
SHP2 inhibitor cellular target engagement assay

A

Biphenylpyrimidine Scaffold

B

IC_{50} WT (µM)

IC_{50} E76K (µM)

SBI-221

SBI-668

7.4

2.1

15.0

2.1

C

D

SHP2 Wild-Type

DMSO

SBI-221

ΔT_m = 0.71

E

SHP2 E76K

DMSO

SBI-668

ΔT_m = -2.79

F

SHP2 Wild-Type

G

SHP2 E76K

Log [Compound] (M)

Luminescence ALU

Log [Compound] (M)

Luminescence ALU

* p<0.05

** p<0.001
described, this assay is both an important validation and benchmark method, because it has the ability to discriminate between low and high affinity SHP2 allosteric inhibitors. Here, we present essential instructions for the successful establishment, optimization, and implementation of the SHP2 cellular thermal shift assay. This protocol is compatible with other high-throughput components of a typical drug discovery workflow. Assay volumes as low as 5 μl in 384-well format allow efficient use of InCell Pulse reagents and enable the screening of large numbers of compounds for cellular target engagement. A liquid dispenser to deliver precise nanoliter quantities of compounds of interest greatly simplifies the miniaturized assay protocol. Although we had ready access to an Echo acoustic liquid handler, other, less expensive options do exist that are capable of delivering similar performance for the described workflow. This is important, because the assay cannot tolerate DMSO concentrations above 1%. However, the observed effects on cells expressing SHP2 should be scalable, and laboratories that are reliant on manual methods of compound application should be able to adapt the protocol accordingly. A relatively inexpensive thermocycler is needed to apply temperature gradients to either plate direction or to provide isothermal treatments. Overall, we recommend this assay as a useful component of a diverse drug discovery strategy for the development of novel SHP2 inhibitors targeting WT SHP2 or one of its many oncogenic mutants.

Materials and methods

Reagents and compounds

Inhibitors of the SHP2 phosphatase (SHP099, SHP836, and Ex-57) were resynthesized according to previously described synthetic protocols (11, 30, 32, 35). RMC-4550 was purchased from ProbeChem (Shanghai, China). The InCell Pulse Target engagement starter kit was purchased from DiscoverX (Eurofins DiscoverX, Fremont, CA). DifMUP was purchased from Invitrogen/Thermo Fisher Scientific, and TH588 was obtained from Sigma–Aldrich. The IRS-1 peptide was synthesized by PepMic (Suzhou, China). Reagents and buffer components were purchased from Thermo Fisher Scientific unless noted.

Molecular cloning

For recombinant expression of the full-length SHP2 (amino acid residues 1–594), a GST fusion construct in pGEX-4T1 was used to produce a thrombin-cleavable construct (Addgene plasmid 8322). Site-directed mutagenesis was used to introduce the E76K gain-of-function mutation into the primary sequence of the bacterial expression construct. For recombinant expression of the catalytic domain of SHP2 (SHP2cat), the SHP2 sequence (amino acid residues 248–527) was inserted into a pETNK1-His3C-LiC-KAN (Netherlands Cancer Institute) by ligation-independent cloning. For cellular thermal shift experiments, fusion ePL-tagged SHP2-WT was obtained by directional insertion into the pICP-ePL-N vector (Eurofins Discover
erX) between the EcoRI and XbaI sites according to the vendor’s user manual. The ePL tag was added to the N terminus of SHP2 (pICP-ePL-N-SHP2-WT). The plasmid vector also includes a cytomegalovirus promoter for expression in mammalian cells and a neomycin/kanamycin resistance gene for selection in mammalian cells for stable cell line generation. Site-directed mutagenesis was performed on the SHP2-WT expression construct to introduce the E76K mutation, generating pICP-ePL-N-SHP2-E76K. The SHP2cat cDNA was similarly inserted to create pICP-ePL-N-SHP2cat.

Protein expression and purification

Full-length SHP2 (1–594) WT and the E76K mutant constructs were expressed as fusion proteins containing a GST tag at the N termini. Pilot cultures of transformed E. coli BL21(DE3) were used to inoculate 1 liter of LB medium. When they reached an A600 of 0.8, they were split into 7 liters and further grown to an A600 of 1.2. SHP2 expression was induced with 0.7 mM isopropyl β-D-thiogalactopyranoside at 25 °C overnight. The cells were collected by centrifugation the following day and resuspended in 1 × PBS with 100 mg/liter RNaseA. The cells were lysed by two passages through a microfluidizer at 124 MPa. The lysate was clarified by centrifugation at 15,000 × g for 50 min and applied to GST resin. The resin was washed, and the adhered SHP2 fusion protein was treated with high-purity bovine thrombin (catalog no. 154163; MP Biomedicals LLC; 1KU/40 ml GST resin) and allowed to cleave overnight at 4 °C. Released SHP2-WT or SHP2-E76K protein was further purified by size-exclusion chromatography using an S200 column. The purified proteins were eluted in 50 mM Tris, pH 7.5, 50 mM NaCl, supplemented with tris(2-carboxyethyl)phosphine (TCEP) to 10 mM, and concentrated by ultrafiltration before storage at −80 °C. The SHP2 catalytic domain (248–527) was expressed as a N-His–tagged fusion protein. For expression, BL21(DE3) cells were grown and induced as described above. Collected cells were resuspended in lysis buffer (25 mM Tris, pH 7.5, 300 mM NaCl, 50 mM imidazole, 10% glycerol) with 100 mg/liter RNaseA and were lysed with two passages using a microfluidizer. The lysate was clarified by centrifugation at 15,000 × g for 50 min and applied to HiTrap nickel–nitritolriacetic acid resin. The column resin was washed, and the SHP2cat protein was eluted in lysis buffer at 300 mM imidazole. The SHP2cat protein was further purified by S75 size-exclusion chromatography in 50 mM Tris, pH 7.5, 50 mM NaCl. The eluted peak fractions were used in a thermal shift assay. This protocol is compatible with other high-throughput components of a typical drug discovery workflow.
supplemented with TCEP to 10 mM, concentrated by ultrafiltration, and stored at −80 °C.

**Michaelis–Menten kinetic assays**

SHP2 activity was measured at room temperature in a 384-well plate format standard phosphatase fluorescence intensity assay using DiFMUP as a substrate and a total reaction volume of 25 μL. SHP2 working solutions were prepared at a 0.625 nM concentration (for a final concentration of 0.5 nM) in buffer containing 50 mM Bis-Tris, pH 6.0, 50 mM NaCl, 5 mM DTT, and 0.01% Tween® 20. Prior to the assay, a SHP2-binding protein surrogate (625 nM final concentration) of a dually phosphorylated IRS-1 peptide (30) was added to full-length WT and E76K SHP2 working solutions and incubated for 20 min. DiFMUP working solutions at 5× final concentration were prepared in 50 mM Bis-Tris, pH 6.0, 50 mM NaCl, and 0.01% Tween 20. 20 μL of SHP2 working solution was dispensed into a black Greiner FLUOTRAC™ 200 384-well microplate (catalog no. 781076; Greiner, Frickenhausen, Germany) in triplicate using a Multidrop™ Combi reagent dispenser (Thermo Fisher Scientific). The reaction was initiated by addition of 5 μL of DiFMUP working solutions for final DiFMUP concentrations of 400, 200, 100, 50, 25, 12.5, 6.25, 3.12, 1.56, and 0.781 μM. Fluorescence intensity was measured in kinetic mode (every minute for 10 min) using a Tecan Spark® multimode microplate reader (Tecan, Groedig, Austria) with an excitation wavelength of 360 nm and an emission wavelength of 460 nm. The initial rates were determined from the linear progression curves of the SHP2 reaction. The nonenzymatic hydrolysis of the substrate was corrected by using a control without the addition of enzyme. Michaelis–Menten plots were generated for each SHP2 construct, and Michaelis–Menten constants (Kₘ) were calculated using the program GraphPad Prism (GraphPad Software, Inc., San Diego, CA), version 8.

**Biochemical inhibition assays**

SHP2 inhibitors were tested using a similar assay format as described above. SHP2 inhibitors or vehicle (DMSO) were spotted in triplicate into a black Greiner FLUOTRAC™ 200 384-well microplate (catalog no. 781076, Greiner) for a 10-point dose-response assay using an Echo® 555 Liquid Handler (Labcyte, Inc., San Jose, CA). SHP2 working solutions were prepared and dispensed as described above and incubated with inhibitor for 20 min at room temperature. 5× DiFMUP working solutions were prepared for final concentrations corresponding to the respective Kₘ value for each SHP2 protein (SHP2-WT, 60 μM; SHP2-E76K, 20 μM; SHP2cat, 20 μM). The reaction was initiated, fluorescence intensity was measured, and initial rates were determined as described above. IC₅₀ values were determined using the nonlinear regression (sigmoidal dose-response with variable slope) function of the GraphPad Prism software.

**PTS assay**

Differential scanning fluorimetry (also known as protein thermal shift) measurements of SHP2-WT, SHP2-E76K, and SHP2cat were performed using optimized methods and conditions in accordance with those previously described (40). The reactions were prepared in a 384-well plate format by combining SHP2 proteins with known binding ligands with thermal shift dye and buffer to a final assay volume of 10 μL. Test compounds were spotted at different concentrations into MicroAmp® 384-well real-time PCR plates (catalog no. 4483285; Applied Biosystems) using an Echo® 555 liquid handler. 5 μL of SHP2 working solution (2.5 μM SHP2-WT, 1.5 μM SHP2-E76K, or 1.5 μM SHP2cat in 50 mM Tris-HCl, pH 7.5, 50 mM NaCl, and 5 mM DTT) were added to each well using a Multidrop™ Combi reagent dispenser (Thermo Fisher Scientific). 5 μL of 5× SYPRO Orange (Invitrogen/Thermo Fisher Scientific) dissolved in molecular grade water was equally dispensed into the PCR plates diluting the enzyme solution 1:2. The plates were then sealed with MicroAmp optical adhesive film (Applied Biosystems) and spun to collect the reaction mix at the bottom of the plate. The plates were analyzed using a ViiA 7 real-time PCR instrument (Applied Biosystems) and a 12-min temperature gradient with a temperature increase of 0.075 °C/s. The melting temperature and thermal profiles were determined as described previously using Protein Thermal Shift™ software, version 1.2 (Applied Biosystems) (40).

**Immunoblotting**

The cells were lysed and scraped in radioimmunoprecipitation assay buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1.0% Igepal® CA-630, 0.5% sodium deoxycholate, and 0.1% SDS). The cells were sonicated at 20% power for 10 s on and 0 s off and centrifuged at 12,000 × g, and the supernatant was recovered. Protein concentration was quantified using a BCA assay (Pierce/Thermo Scientific). The proteins were separated on Novex™ X WedgeWell™ 4–20% Tris-glycine gels (Invitrogen) and transferred to polyvinylidene difluoride membranes (Trans-Blot® Turbo™ transfer pack) membranes. The membranes were blocked in TBS Tween 20 (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.05% Tween 20; TBST) containing 5% BSA for 1 h at room temperature and incubated at 4 °C overnight in primary antibody (PathHunter® anti-PK/PL; Eurofins DiscoverX). The blots were rinsed three times the following day with TBST. The blots were incubated in 5% nonfat milk with secondary antibody (1:5000) (anti-mouse horseradish peroxidase; Cell Signaling) for 1 h and washed three times with TBST. The blots were developed using Pierce® ECL Western blotting and imaged by chemiluminescence.

**InCell Pulse SHP2 cellular target engagement assay**

HEK293T or HEK293 cells expressing ePL-tagged SHP2 were cultured using Dulbecco’s modified Eagle’s growth medium (GlutaMAX, 10% FBS, antibiotic/antimycotic, 20 mM HEPES, sodium pyruvate; Gibco/Thermo Fisher Scientific). Transfection of plCP-derived plasmid constructs was performed using jetPRIME buffer and reagent (Polyplus-transfection® SA, Illkirch, France). For the generation of stable cell lines, transfected HEK293 cells were placed under neomycin/kanamycin selection, and after propagation, the presence of stable ePL-tagged SHP2-WT, SHP2-E76K, or SHP2cat insertion was established by immunoblot analysis.

Vehicle (DMSO) or inhibitors were spotted using an Echo® 555 liquid handler into Twin.tec® 384 real-time PCR plates.
SHP2 inhibitor cellular target engagement assay

(catalog no. 0030132734; Eppendorf, Hauppauge, NY) for a final DMSO concentration of 0.5% or less (v/v). Stable or transiently transfected cells were detached from culture plates (AssayComplete cell detachment reagent; Eurofins DiscoverX), and cell count was measured. The detached cells were diluted to 1.25 × 10⁵ cells/ml in growth medium and applied to the assay plate (transiently transfected cells, 5 μl; stable cell lines, 12.5 μl) using a multichannel pipette. The plate was centrifuged at 42 × g for 30 s to mix, and a seal was applied. The assay plate was then incubated with compounds at 37 °C and 5% CO₂ for 1 h. After incubation, the sealed plates were subjected to the following protocol using a gradient thermocycler (X500h Mastercycler; Eppendorf). For thermal gradient experiments, a 3-min heat pulse was applied using a vertical or horizontal gradient (± 15 °C). For example, a typical gradient of 38–68 °C spanned across 24 wells yielded temperature points with increments of 1.25 °C. For isothermal experiments, the plates were subjected to a 3-min heat pulse at a fixed temperature. After the heat pulse, a 4-min recovery step at 20 °C was applied. The assay plates were then immediately supplemented with the EFC lysis and detection reagent. After a 30-min incubation at room temperature, chemiluminescence was read using a TECAN Spark® Multimode Microplate Reader. A detailed step-by-step protocol is provided supporting information.

Isothermal SHP2 cellular target engagement screen

Fixed concentration isothermal cellular target engagement assays were performed using the cellular preparation procedures for thermal profile experiments described above. Detached HEK293T cells transfected with the SHP2-WT or SHP2-E76K ePL-tagged plasmid constructs were diluted to 1.25 × 10⁵ cells/ml in growth medium. Prepared assay plates with SHP2 inhibitors of interest or vehicle (DMSO) were spotted using the Echo® 555 liquid handler into Twin.tec® 384 real-time PCR plates for a final compound concentration of 30 μM in a 5-μl assay; the DMSO concentration was 0.3% (v/v). Compound target engagement was probed in quadruplicate using a plate format that separated replicate wells spatially. Detached cells were applied to the assay plate, which was centrifuged at 42 × g for 30 s and sealed. The cells were then incubated with compounds at 37 °C and 5% CO₂ for 1 h. After incubation, the plates were subjected to a 3-min heat pulse at 54 °C (SHP2-WT) or 50 °C (SHP2-E76K) followed by a 4-min recovery step at 20 °C. Assay plates were immediately supplemented with the EFC lysis and detection reagent, and chemiluminescence was measured using a TECAN Spark® multimode microplate reader.

Statistical analysis and determination of Z’ factor

All of the data are depicted as means ± S.E. from two or three experiments, p values were calculated using a t test with p < 0.05. A small number of data points were excluded by application of >3 × σ. All data were analyzed using GraphPad Prism software version 8.

The Z’ factor was calculated according to the following equation (41).

\[ Z’ = 1 - \frac{3σ_1 + 3σ_2}{(μ_1 - μ_2)} \]

(Eq. 1)

The denoted variables are: σ1, standard deviation of the bound \( T_m \) state; σ2, standard deviation of the unbound \( T_m \) state; μ1, mean bound \( T_m \); and μ2, mean unbound \( T_m \).

Author contributions—C. R., L. J. L., S. G., E. S., N. D. P. C., and L. T. conceptualization; C. R., L. J. L., D. J. S., L. J. S. D. B., S. G., R. J. H., E. S., and L. T. data curation; C. R., L. J. L., D. J. S., L. J. S. D. B., D. R.-P., M. C., S. G., S. R., J. H., E. S., and L. T. formal analysis; C. R., L. J. L., D. J. S., S. G., and L. T. validation; C. R., L. J. L., D. J. S., L. J. S. D. B., D. R.-P., M. C., S. G., S. R., J. H., E. S., and L. T. methodology; C. R., L. J. L., D. J. S., S. G., J. H., N. D. P. C., and L. T. writing-original draft; L. J. L., S. G., E. S., N. D. P. C., and L. T. supervision; L. J. L., D. J. S., and L. T. visualization; S. G., N. D. P. C., and L. T. project administration; E. B. P., N. D. P. C., and L. T. funding acquisition; E. B. P. writing-review and editing.

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