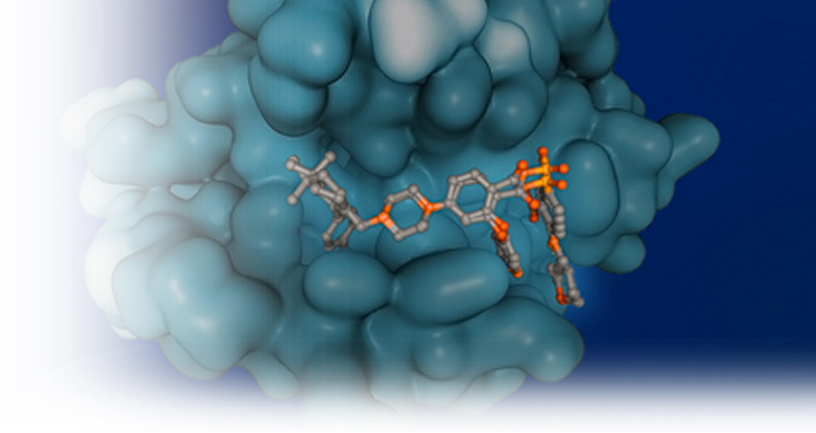

APPLICATION NOTE

InCELL Pulse Cellular Target Engagement Assays for Characterizing Novel Small Molecule Inhibitors of Oncogenic SHP2 Variants

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INTRODUCTION

SHP2 (Src-homology 2 domain-containing phosphatase 2) is a protein tyrosine phosphatase encoded by the human *PTPN11* gene. SHP2 is a key player in many signaling cascades such as the JAK/STAT, Ras-Raf-MAP kinase, and PI3 kinase pathways[1]. Mutations of *PTPN11* that cause aberrant SHP2 activity have been implicated in several cancers including leukemia and breast cancer[2]. Abnormal SHP2 function has also been well-documented in 50% of Noonan Syndrome patients[3]. Because of its role in multiple pathways and diseases, SHP2 continues to be a critical therapeutic target.

Inhibiting SHP2 with orthosteric small molecules has been challenging in the past. Drugs either exhibited low efficacy, or failed to bind selectively to SHP2 owing to its highly conserved active site[4,5,6]. Recently however, a novel allosteric SHP2 inhibitor (SHP099) by Novartis has revealed the existence of druggable allosteric sites on SHP2, opening a new avenue for SHP2 drug discovery. SHP099 displayed remarkable selectivity and efficacy for SHP2[5]. However, further research revealed that SHP099 and similar inhibitors were only effective on wild-type SHP2 (SHP2-WT) and not the oncogenic variants[7,8,9]. Such a finding has created a need for allosteric SHP2 inhibitors that selectively bind to disease-relevant SHP2 variants. Since the discovery of allosteric SHP2 inhibitors, efforts to find similar therapeutics that successfully target SHP2 variants continues.

For a more effective drug discovery process, determining compound-target binding during the early stages is critical. This compound binding determination is especially true for challenging targets such as SHP2 that lack properties that can easily be assayed. While methods such as biochemical assays are commonly used for this purpose, they do not capture the cellular environment's complexities. Therefore, cell-based assays are used to evaluate compound-target engagement like biochemical assays, but also determine compound permeability, measure EC_{50} values, and rank compounds all in the native cellular environment. A common cellular target engagement assay is the cellular thermal shift assay, but like many assays, it has its own limitations. Cellular thermal shift assays consist of low throughput methods (Western blot, mass spectrometry, etc.), require custom chemical tracers, or use target-specific antibodies. Thus, a simpler cell-based assay format is needed that is easily amenable to high throughput and does not require chemical tracers or antibodies.

The InCELL Pulse™ assay platform by Eurofins DiscoverX seeks to address many of the challenges posed by conventional cellular thermal shift assays. InCELL Pulse cell-based assays are designed for rapid, high-throughput experiments for the detection of shift in the thermal stability of target proteins brought about by compound binding without the need for chemical tracers or antibodies. These assays are based on the industry-validated Enzyme Fragment Complementation (EFC; discoverx.com/efc) technology (see Figure 1.) that has been utilized in screening of billions of data points over the past two decades. EFC consists of two inactive fragments of the β-galactosidase (β-gal) enzyme – a small enzyme donor fragment and a larger enzyme acceptor (EA) fragment – that when complexed together generate an enzymatically active β-gal. The small donor fragment, called ePL, is tagged to the target protein of interest and expressed in the relevant cell line. When the cell line is subjected to a heat pulse, the target protein undergoes denaturation at a specific temperature in the absence

of a compound, leading to protein aggregation. However, when a compound successfully enters the cell and binds with the target protein, the proteins stability is increased. The protein is protected from thermally induced denaturation, and a shift in denaturation temperature is observed. In the presence of EA and a chemiluminescent substrate, the ePL-tagged thermally stable protein binds EA, the EA-ePL complementation forms an active β-gal enzyme. The active enzyme then hydrolyzes the substrate to generate a chemiluminescent signal that can be read on any standard luminometer.

On a quest for discovering effective therapeutics targeting SHP2 variants, a study published by Romero *et al.* focused on evaluating an array of allosteric SHP2 inhibitors using a 384-well miniaturized, high throughput InCELL Pulse™ cellular thermal shift assay[10]. In this paper, we briefly describe how they developed this assay and demonstrate the suitability, robustness, and reliability of this assay for characterizing novel allosteric SHP2 inhibitors.

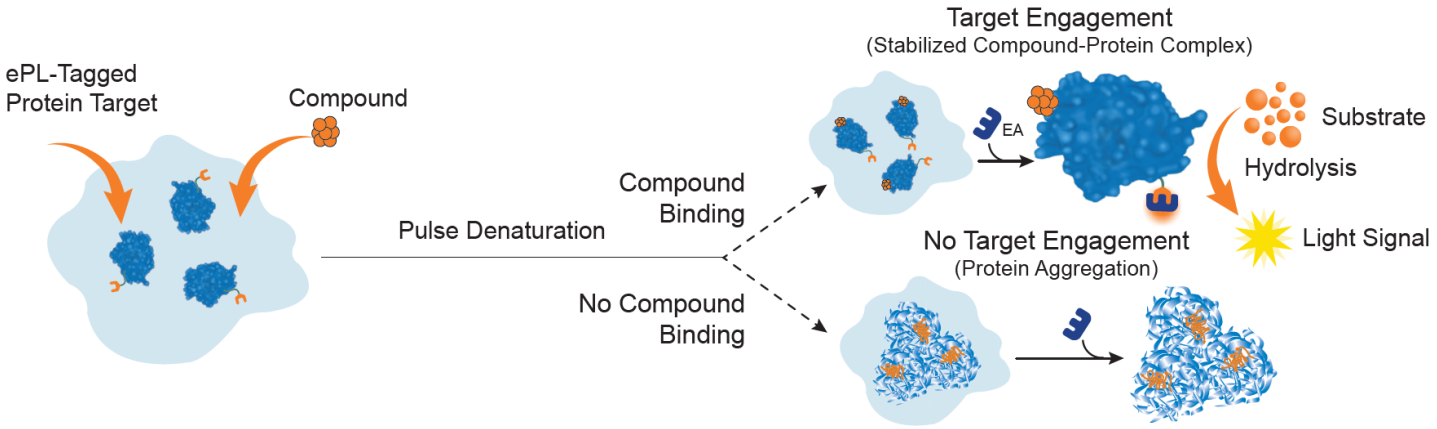

Figure 1. InCELL Pulse Assay Principle. The intracellular target protein in these assays is fused with the ePL fragment of β-gal. Upon addition of a compound that can permeate the cell membrane and bind to its intended protein target, the cells are subjected to elevated temperatures during a pulse denaturation step. Compound binding protects the target protein from thermal denaturation, which enhances the complementation between EA and ePL and increases the chemiluminescent signal measured using the EFC-based detection system. In the absence of compound binding, the target protein forms denatured aggregates that poorly complement with EA, resulting in a low chemiluminescent signal.

METHODS

Refer to Romero *et al.* JBC 2020 for complete details of materials and methods.

Reagents

The InCELL Pulse™ Target Engagement Starter and InCELL Pulse Detection kits were purchased from Eurofins DiscoverX (Fremont, CA).

Molecular Cloning

For cellular thermal shift experiments, N-terminal, ePL-tagged SHP2-WT (wild type) and SHP2cat (catalytic subunit) were obtained by insertion of SHP2-WT or SHP2cat DNA between the Eco RI and Xba I sites of pICP-ePL-N vector (Eurofins DiscoverX). Site-directed mutagenesis was used to generate pICP-ePL-SHP2-E76K (oncogenic mutant variant) from SHP2-WT.

InCELL Pulse SHP2 Cellular Target Engagement Assays

HEK 293T or HEK 293 cells expressing ePL-tagged SHP2 were maintained in tissue culture. Transfection of pICP derived plasmid constructs was performed and stable cell lines were obtained by antibiotic selection. For use in assays, cells were detached from cell culture plates, added to round bottom PCR assay plates and immediately incubated with the indicated concentration(s) of compounds at 37°C and 5% CO_2 for 1 hour.

After incubation with compounds or vehicles (DMSO control sample), the assay plates were subject to thermal denaturation using a gradient thermocycler. For thermal gradient experiments, a 3-minute thermal pulse was applied using a temperature gradient. For example, a typical temperature gradient from 38 to 68°C was applied across 24 wells yielded temperature points with increments of 1.25°C. For isothermal experiments, plates were subjected to a 3-minute heat pulse at a fixed temperature. For both gradient and isothermal experiments, after the thermal pulse, a 4-minute recovery step at 20°C was applied. InCELL Pulse lysis and detection reagent (including EA and substrate) were then immediately added to the assay plates. Chemiluminescence was read using a multimode luminescent microplate reader after a 30-minute incubation at room temperature. Additional details are provided in the Material and Methods, and the Supporting Information sections of Romero *et al.* JBC 2020.

Isothermal SHP2 Cellular Target Engagement Screen

Cellular target engagement compound screening assays of fixed, single concentration isothermal (at a fixed temperature) were performed using the same cell lines and cell handling procedures as described above for thermal profiling experiments. Assay plates were prepared with SHP2 inhibitors or vehicle control spotted for a final compound concentration of 30 µM in a 5 µL assay volume. HEK 293T cells transfected with either SHP2-WT or SHP2-E76K ePL-tagged plasmid constructs were applied to the assay plates containing the compounds, centrifuged, and incubated at 37°C and 5% CO_2 for 1 hour. After incubation, the plates were subjected to a 3-minute heat pulse at 54°C (SHP2-WT) or 50°C (SHP2-E76K) followed by a 4-minute recovery at 20°C. The addition of detection reagent and reading of luminescence were performed as described above.

RESULTS

Biochemical assays were first conducted to ascertain the potencies of four SHP2 allosteric inhibitors. The compounds tested were SHP099, SHP836, Example 57 (Ex-57), and RMC-4550. Each of these compounds were tested with three variants of SHP2 [wild-type (SHP2-WT), oncogenic variant (SHP2-E76K), and the catalytic domain (SHP2cat)]. Results from the biochemical assays are listed in Table 1. Out of the four compounds tested, RMC-4550 was found to be the most potent (IC_{50}=0.0082 µM), while SHP836 showed the lowest potency (IC_{50}=23.5 µM), when acting on SHP2-WT. Furthermore, reports suggest that allosteric SHP099-like inhibitors do not act on oncogenic SHP2 variants, and the same was reflected for SHP2-E76K in this biochemical assay. As expected for allosteric inhibitors, none of the four compounds showed any activity towards the catalytic domain SHP2cat.

Inhibitor	Enzymatic IC_{50} (µM) on Different SHP2 Variants		
	SHP2-WT	SHP2-E76K	SHP2cat
Ex-57	0.0079	7.6	>99
RMC-4550	0.0082	5.6	>99
SHP099	0.15	>99	>99
SHP836	23.5	>99	>99

Table 1. Biochemical assay experiments. IC_{50} values of four inhibitors tested on SHP2-WT, SHP2-E76K, and SHP2cat.

As the goal was to develop a high-throughput cellular thermal shift assay, it was necessary to confirm that measuring protein thermal shift is an appropriate method to determine target protein engagement of allosteric inhibitors. Protein thermal shift (PTS) assays were deployed for this purpose using the same three SHP2 variants and the same four inhibitors from the biochemical assay

experiments. The highest thermal shift values were recorded for RMC-4550 and Ex-57 acting on SHP2-WT (Table 2.). SHP836 again ranked the lowest, bringing about a negligible thermal shift of 0.2°C. All four inhibitors caused significantly lower thermal shifts on SHP-E76K, and no effects were observed on the SHP2cat. These results correlate well with those from the biochemical assay, indicating the suitability of measuring cellular protein thermal shift for compound target engagement.

Inhibitor	Thermal Shift (ΔTm) (°C)		
	SHP2-WT (12 µM of inhibitor)	SHP2-E76K (12 µM of inhibitor)	SHP2cat (50 µM of inhibitor)
Ex-57	4.4 ± 0.1	3.3 ± 0.1	-0.2 ± 0.1
RMC-4550	6.3 ± 0.1	3.9 ± 0.1	0.0 ± 0.1
SHP099	1.8 ± 0.1	0.81 ± 0.1	-0.1 ± 0.1
SHP836	0.2 ± 0.1	0.1 ± 0.1	-0.1 ± 0.1

Table 2. Protein thermal shift assay experiments. Thermal shifts (ΔT_m) of SHP2-WT, SHP2-E76K, and SHP2cat treated with four inhibitors.

The InCELL Pulse™ assay was then used to develop a 384-well miniaturized, high-throughput cellular thermal shift assay. After transfecting all three SHP2 variants tagged with ePL, levels of ePL-tagged SHP2 expression were measured in cells after 24 hours. It was determined that the addition of the ePL tag did not alter SHP2 protein expression, and the transfection of ePL-SHP2 did not alter cell viability. Therefore, the assay was deemed suitable to measure all variants' thermal shifts mediated by the four previously mentioned inhibitors. In this experiment, the 10 µM SHP099 inhibitor altered the thermal stability of SHP2-WT (ΔT_m = 3.7°C), as shown in Figure 2. A. However, it did not significantly affect the thermal stability of either SHP2-E76K or SHP2cat variants (see Romero *et al.* JBC 2020). When SHP2-WT was treated with Ex-57 and RMC-4550, significantly larger thermal shifts were observed (ΔT_m = 7°C, Figure 2. B.). When these inhibitors were added to the SHP2-E76K variant, Ex-57 and RMC-4550 brought about smaller thermal shifts of 2.3°C (Figure 2. C.). Again, SHP836 did not cause significant thermal shifts of SHP2-WT and SHP-E76K (Figure 2. B. and C.). All these results were in agreement with both the biochemical and PTS assays conclusions, thus confirming the reliability of this assay.

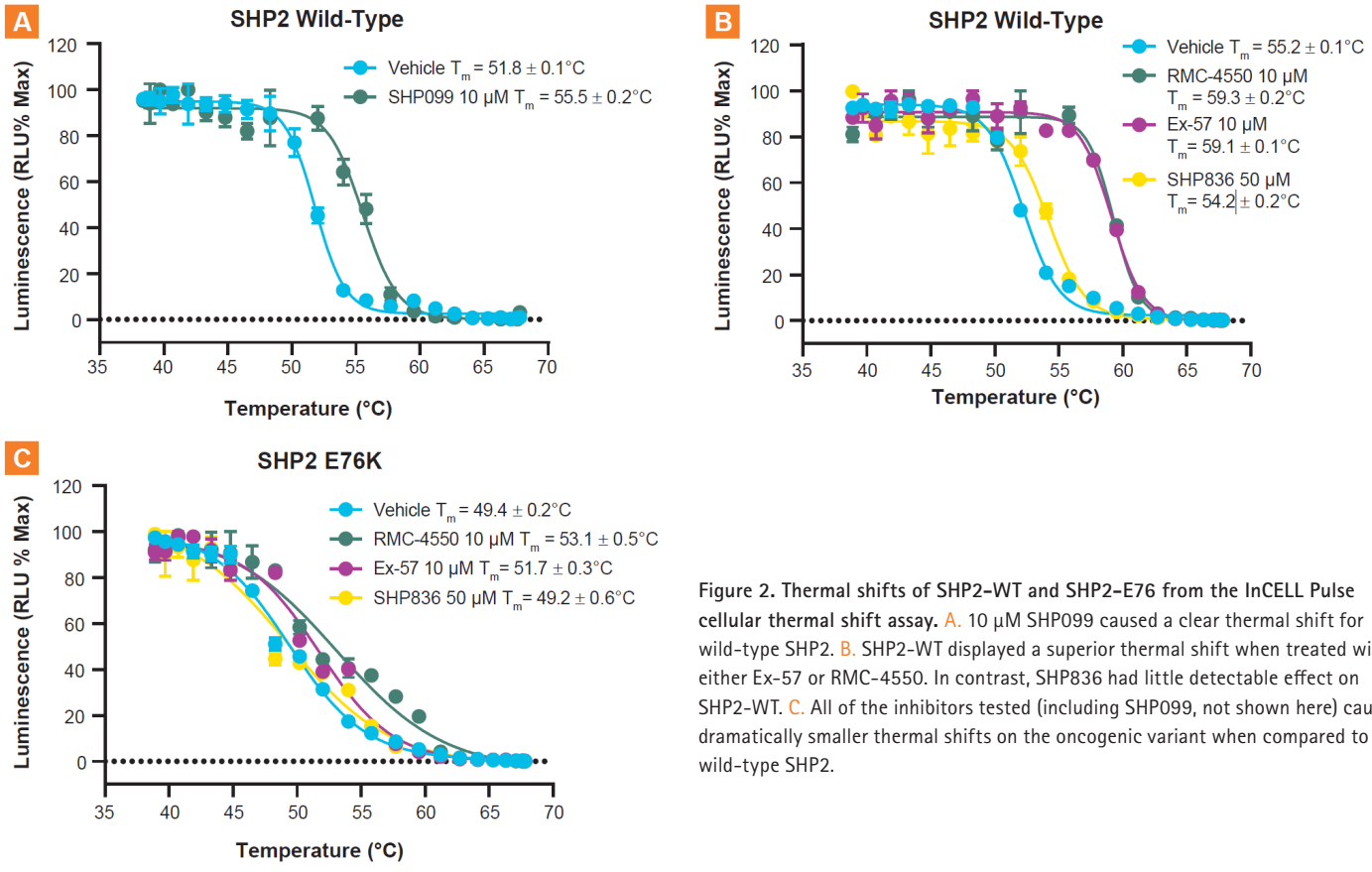

Figure 2. Thermal shifts of SHP2-WT and SHP2-E76 from the InCELL Pulse cellular thermal shift assay. A. 10 µM SHP099 caused a clear thermal shift for wild-type SHP2. B. SHP2-WT displayed a superior thermal shift when treated with either Ex-57 or RMC-4550. In contrast, SHP836 had little detectable effect on SHP2-WT. C. All of the inhibitors tested (including SHP099, not shown here) caused dramatically smaller thermal shifts on the oncogenic variant when compared to wild-type SHP2.

To determine the optimal temperature for cellular thermal shift isothermal measurements with SHP2-WT, 14 isothermal 5-point SHP099 dose-response curves were generated at different temperatures spanning 50.5 to 65.2°C in the SHP2-WT InCELL Pulse™ assay (Figure 3. A.). Of the 14 isothermal curves, SHP099 can be seen to stabilize completely (at high concentrations) at 49.9 and 56.6°C. The ideal temperature for isothermal testing was determined to be 55°C. A 10-point isothermal dose-response curve at 55°C was also generated for SHP099 acting in the SHP2-WT InCELL Pulse assay, and allowed determination of an EC_{50} value of 4.2 µM (Figure 3. B.).

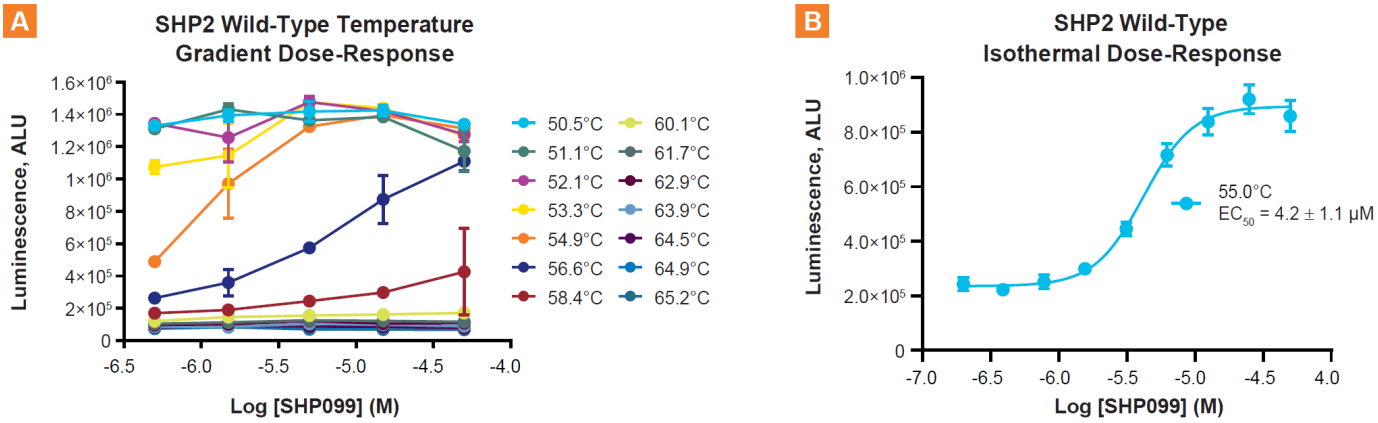

Figure 3. Cellular thermal shift isothermal dose-response experiments with the SHP099 using the SHP2-WT InCELL Pulse assay. A. Isothermal 5-point dose-response curves were produced at each of the 14 listed temperatures. The ideal temperature for isothermal experiments was found to be 55°C. B. A 10-point isothermal dose-response curve at 55°C revealed an EC_{50} value of 4.2 µM of SHP099 binding to SHP2WT.

The miniaturized (384-well format) InCELL Pulse cellular thermal shift assay was then used to test two series of SHP2 inhibitors with SHP2-WT and SHP2-E76K to assess membrane permeability and target engagement of these inhibitors in cells. These compounds were grouped according to their chemical scaffold. One group has a biphenylpyrimidine scaffold, while the other has a benzothiophenone scaffold (see Figure 4. A.). From the biphenylpyrimidines group, four compounds out of eleven tested were able to stabilize or engage in the SHP2-WT cells compared to the oncogenic variant. Interestingly, these four compounds had tetrazole moieties, which are known to greatly increase membrane permeability of compounds[11]. This correlation demonstrates the utility of being able to simultaneously interrogate target engagement and permeability in one assay. From the benzothiophenone group, five inhibitors were tested on both SHP2 variants and demonstrated destabilized, negative thermal shifts. Notably, these five compounds also destabilized SHP2 in PTS assays. This correlation demonstrates the accuracy and predictiveness of InCELL Pulse cellular thermal shift assay with respect to compound mechanism of action.

Representative inhibitors from each of the two SHP2 inhibitor groups were then chosen for a more detailed study using wild-type and oncogenic mutant variants (see Figure 4. B. and C.). Compound SBI-221, from the biphenylpyrimidine group carrying the tetrazole moiety, yielded a stabilizing thermal shift of 0.71°C for SHP2-WT and minimal effect with SHP2-E76K. In contrast, SBI-668, from the benzothiophenone group, had a minimal effect on SHP2-WT, but selectively destabilized SHP2-E76K (ΔT_m = -2.27°C). The observed destabilization of SHP2-E76K corroborated with results from biochemical experiments using SBI-668 (IC_{50} on oncogenic variant: 2.1 µM; IC_{50} on wild-type variant: 15 µM).

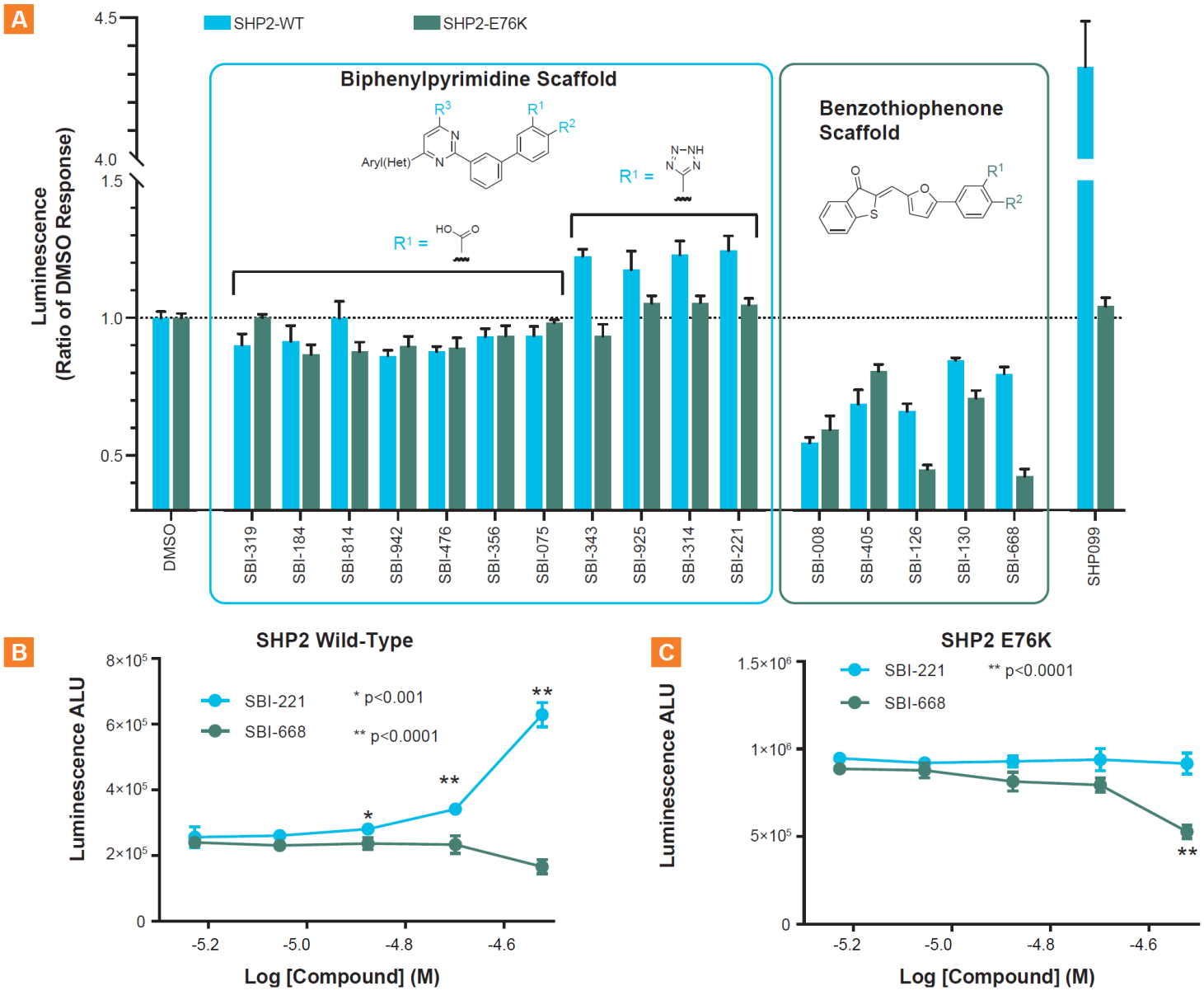

Figure 4. InCELL Pulse™-mediated high-throughput screening of a series of novel SHP2 allosteric inhibitors. A. The compounds were grouped according to their chemical scaffolds. A representative inhibitor was chosen from each group to study their effects on SHP2 and its variant. B. The dose-response of both representative inhibitors—SBI-221 and SBI-668—on SHP2-WT was measured at 55°C. SBI-221 showed stabilizing effects on SHP2-WT, while SBI-668 showed minimal effects. C. Dose-dependent activity of both inhibitors on SHP2-E76K was evaluated at 50°C. SBI-668 demonstrated preferential destabilization of SHP2-E76K, while SBI-221 showed minimal effects.

In this study, multiple experiments with the InCELL Pulse assay yielded results for compounds that acted as SHP2 inhibitors and were consistent with previously conducted biochemical and PTS assays. This demonstrated the accuracy and relevance of this cell-based assay platform. The assay enabled the use of full-length SHP2 to identify and study cell permeable and binding of allosteric inhibitors. Not only was the assay found to reliably yield unique information about the compounds (such as compounds whose increased cellular activity appears to be driven by increased cell permeability), but it was also capable of being used in a high-throughput format, thus enabling easy rank-ordering of many compounds quickly and efficiently.

CONCLUSION

SHP2, a well-known protein tyrosine phosphatase implicated in many cancers, was previously deemed an "undruggable" target. However, since the discovery of its inhibition through allosteric mechanisms, SHP2 is once again being actively studied. Though the 1st generation allosteric inhibitors were inactive for oncogenic variants, there are now several drug discovery projects that aim to target the allosteric sites of oncogenic SHP2 mutant variants. Confirming the cell permeability and binding of a therapeutic candidate to SHP2 is an essential step in this process. Cellular thermal shift experiments are reliable for simultaneously evaluating compound-target binding and permeability in a cellular environment, which cannot be achieved in biochemical assays. In this study, the InCELL Pulse™ platform enabled a high throughput cell-based assay for quantifying SHP2 allosteric binding. The miniaturized, easy-to-use, high throughput assay was able to generate consistent results and was used successfully to characterize an array of novel allosteric SHP2 inhibitors.

The simultaneous evaluation of cellular permeability and compound binding is made possible with the InCELL Pulse assay platform. In early stages of drug discovery, not only is InCELL Pulse suitable for screening compounds, but it can be used to analyze targets whose function is not known. Thus, a broad range of targets, full-length or otherwise, can be easily studied in a cellular context. With continuous efforts to help you accelerate your drug discovery program, Eurofins DiscoverX's has developed different types of cell-based assays for your research needs. Learn more at discoverx.com/application/target-engagement/.

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