Novel Small-Molecule Inhibitor for the Oncogenic Tyrosine Phosphatase SHP2 with Anti-Breast Cancer Cell Effects

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ABSTRACT: The oncogenic property of the Src homology phosphotyrosine phosphatase 2 (SHP2) is well-known, but developing specific inhibitors has been very difficult. Based on our previous reports that showed the importance of acidic residues surrounding SHP2 substrate phosphotyrosines for specific recognition, we have rationally designed and chemically synthesized a small-molecule SHP2 inhibitor named 4,4′-(4′-carboxy)-4-nonyloxy-[1,1′-biphenyl]-3,5-diyl)dibutanoic acid (CNBDA). Molecular modeling predicted that CNBDA packs well into the SHP2 active site and makes extended interactions primarily with positively charged and polar acids surrounding the active site. In vitro PTPase assays showed that CNBDA inhibits SHP2 with an IC_{50} of 5 μM. However, the IC_{50} of CNBDA toward SHP1, the close structural homologue of SHP2, was 125 μM, suggesting an approximately 25-fold effectiveness against SHP2 than SHP1. Because SHP2 is known for its positive role in breast cancer (BC) cell biology, we tested the effect of SHP2 inhibition with CNBDA in HER2-positive BC cells. Treatment with CNBDA suppressed cell proliferation in 2D culture, anchorage-independent growth in soft agar, and mammosphere (tumorisphere) formation in suspension cultures in a concentration-dependent manner. Furthermore, CNBDA inhibited EGF-induced signaling and expression of HER2 by inhibiting the PTPase activity of SHP2 in BC cells. These findings suggest that CNBDA is a promising anti-SHP2 lead compound with anti-BC cell effects.

INTRODUCTION

Dysregulation of receptor tyrosine kinase (RTK) signaling is prevalent in many cancer types, including breast, lung, brain, ovarian, colon, gastric, liver, thyroid, pancreatic, and blood cancers.1 As such, RTKs have been useful targets for cancer therapy, transforming the standard of care for patients.2 However, development of resistance to anti-RTK drugs and disease relapse remains a challenging clinical problem.3 This is true in HER2-positive breast cancer (BC) as well, which is caused by overexpression of the HER2 protein. Several antibody and small molecule-based anti-HER2 drugs that inactivate its oncogenic property have been developed, but tumor cells often find ways to overcome the effect of the drugs. These observations underpin the need for discovering and exploiting alternative therapeutic targets and developing specific inhibitors against them.

The Src homology 2-containing protein tyrosine phosphatase 2 (SHP2) is a critical signaling node for many RTKs that are dysregulated in cancer. The importance of SHP2 in RTK signaling is so critical that effective activation of the Ras-ERK and PI3K-Akt pathways, for epithelial mesenchymal transition, for anchorage-independent growth, and for xenograft tumor growth and metastasis.4−9,12−13 These prior reports suggest that targeting SHP2 might be a useful strategy for BC treatment. SHP2 is a cytoplasmic protein tyrosine phosphatase (PTP) with two SH2 domains in the N-terminal region and a PTP domain in the C-terminal region.14,15 Although the SH2 domains mediate interaction of SHP2 with signaling complexes, the PTP domain catalyzes dephosphorylation reactions in substrate proteins.16,17 The biological role of SHP2 primarily lies on its PTPase activity because mutation of the catalytic Cys (Cys459) to Ser and expression in cells effectively abrogates its function as a mediator of RTK signaling. SHP2 is an autoregulated enzyme that closes and show that SHP2 is essential for sustained and augmented activation of the Ras-ERK and the PI3K-Akt signaling pathways, for epithelial mesenchymal transition, for anchorage-independent growth, and for xenograft tumor growth and metastasis.8,9,12−13 These prior reports suggest that targeting SHP2 might be a useful strategy for BC treatment.

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opens depending on the state of interaction with signaling complexes. When the SH2 domains are not engaged in interaction with Tyr-phosphorylated (pTyr) signaling partners, the N-terminal SH2 domain (N-SH2) binds to the PTP domain and blocks the active site, leading to what is known as the closed conformation. Engagement of the SH2 domains with pTyr induces conformational changes that open up the PTP domain for substrate recognition and catalyzing dephosphorylation reactions.\textsuperscript{16,17} It is therefore highly likely that SHP2 assumes a sustained open conformation in cancers with dysregulated RTK signaling, leading to a sustained mediation of signaling. In support of this possibility, previous reports by us and others show the importance of SHP2 in mediating augmented and sustained activation of the Ras-ERK and the PI3K-Akt signaling pathways.\textsuperscript{10,18,19}

The positive role of SHP2 in RTK signaling and cancer biology has led to numerous attempts to produce specific inhibitors with the objective to develop targeted therapies.\textsuperscript{20−24} However, active site-directed small-molecule inhibitors have faced major challenges in terms of specificity because of the conserved nature of amino acid sequences surrounding the active site cleft of many PTPs.\textsuperscript{25} As such, small-molecule inhibitors that target SHP2 tend to target other PTPs as well, particularly, SHP1 that is highly homologous to SHP2.\textsuperscript{26} Here, we report invention of a new active site-directed and specific SHP2 inhibitor termed (4,4′-(4′-carboxy)-4-nonyloxy-[1,1′-biphenyl]-3,5-diyldibutanoic acid (CNBDA) that shows promising effects in inhibiting RTK-induced and SHP2-mediated signaling and in suppressing BC cell proliferation and transformation.

## RESULTS

### Rational Design and Synthesis of the Small-Molecule SHP2 Inhibitor.

We have previously reported that SHP2 selectively dephosphorylates target phosphotyrosine substrates in proteins based on the primary amino acid sequence N-terminal to the phosphorylation site.\textsuperscript{4,9,11} Particularly, enrichment in acidic residues in this region of substrates is critical for specific binding and dephosphorylation. A tyrosine-phosphorylated peptide derived from this region of substrates is able to inhibit the PTPase activity in vitro and SHP2-mediated signaling in cells.\textsuperscript{27} Based on this information, we have rationally designed and chemically synthesized a small-molecule SHP2 inhibitor whose structure is shown in Figure 1A. The chemical name of this compound is CNBDA with a formula weight of 512. The biphenyl ring forms the core of the compound to which the 4′-carboxylate group that mimics a phosphate and two butanoic acids that mimic carboxylic side chains of acidic amino acids in natural SHP2 substrates are attached. The aliphatic group was added to promote cellular permeability.

To predict how CNBDA might bind to SHP2, in silico molecular modeling and interaction studies were performed. CNBDA was docked into the active site of SHP2 (PDB: 4DGP)\textsuperscript{28} using the molecular modeling program Glide (Schrodinger) followed by an induced-fit docking and binding energy calculations with Prime MM-GB/SA.\textsuperscript{29} The docking results showed that CNBDA packs well into the active site of SHP2 with a $\Delta G$ of $\textasciitilde 54.55$ kcal per mole (Figure 1B). To show the details of the interaction, a two-dimensional flattened structure was drawn. In this diagram, the stick structure represents CNBDA, while the balloon-like structures represent amino acid residues surrounding the SHP2 active site (Figure 1C).
Flattened two-dimensional diagram of CNBDA interaction with SHP2, drawn using the MAESTRO 2D sketcher, shows the details of the interaction. The oval red outline shows the 9-carbon aliphatic group of CNBDA. As indicated by the color code in the key to the interaction map, the 9-carbon aliphatic group remains on the surface exposed to the solvent.

As shown, the carboxyl moiety of the biphenyl core group interacts with R465 and the catalytic nucleophile C459 in a fashion similar to phosphotyrosyl substrates. In addition, the backbones of I463 and G464 participate in positioning the carboxyl group deep into the active site. This mode of binding seemed to permit further interaction by the carboxylate groups of the butanoic acid arms with side chains of positively charged and polar residues surrounding the active site, including Q281, R362, K364, and K366 (Figure 1C). The 9-carbon aliphatic group that is added for mediating cellular permeability stayed exposed to the surface (red oval outline), suggesting that it does not interfere with the binding of CNBDA to the active site.

**CNBDA is More Effective in Inhibiting SHP2 than SHP1 in In Vitro PTPase Assays.** As predicted by the molecular modeling studies described in Figure 1B,C, CNBDA packs well into the SHP2 active site and makes extended interactions. To experimentally test an inhibitory effect, we conducted an in vitro phosphotyrosine phosphatase (PTPase) assay, using a purified PTP domain of SHP2 as an enzyme, CNBDA as a test compound, and DiFMUP (6,8-diﬂuoro-4-methylumbelliferyl phosphate) as an artiﬁcial substrate. We also used a puriﬁed PTP domain of SHP1, the close structural homologue of SHP2, as a speciﬁcity control for the PTPase assay. CNBDA concentrations ranging from 61 nM to 4 mM and puriﬁed PTP domains of SHP2 and SHP1 at 1 μM concentration were used in the reactions. The reactions were performed in triplicate at 30 °C in 100 μL volume of PTPase buffer for 10 min. Production of DiFMU (ﬂuorescent) was followed in a plate-reading visible spectrophotometer at 455 nm as described previously.30 The effect of CNBDA on the PTPase activity was presented as percent inhibition by transforming averages of absorbance values at each CNBDA concentration; absorbance values at zero CNBDA were used as 100 percent activity or no inhibition. The results showed inhibition of both SHP2 and SHP1 by CNBDA in a concentration-dependent manner. However, CNBDA inhibited SHP2 at an IC_{50} of approximately 5 μM, but the IC_{50} for SHP1 inhibition was approximately 125 μM (Figure 2A,B), suggesting an approximately 25-fold effectiveness against SHP2 than SHP1.

**CNBDA Suppresses Cell Proliferation or Induces Cell Death in a Concentration-Dependent Manner.** After demonstrating the effect of CNBDA on the PTPase activity of SHP2, we sought to determine the effect on cell growth and viability. We chose the BT474 and JIMT-1 BC cells for these studies because they have dysregulated HER2 expression for which SHP2 is an essential mediator of signaling, transformation, and tumorigenesis.9,13,31 The nontransformed MCF-10A breast epithelial cells were used as normal controls. Cells were thinly seeded in 2D culture and then treated with varying concentrations of CNBDA every 24 h for a total of 72 h by replacing both the growth medium and CNBDA doses. Cell proliferation was monitored by observation under a microscope and pictures were collected every 24 h for a total of 72 h. Normally, the BT474 cells grow as patchy cellular aggregates in 2D culture. Treatment with CNBDA suppressed the expansion of these cellular aggregates at 0.25 μM and induced cell death at 0.5 μM concentrations (Figure 3A). On the other hand, the JIMT-1 cells exhibit spindle-shaped and elongated morphology when sparsely growing and a monolayer sheet when confluent. Treatment with CNBDA suppressed cell growth and induced cell death in a concentration-dependent manner (Figure 3B). However, the nontumorogenic MCF-10A cells were relatively resistant to CNBDA even at the higher concentration used (Figure 3C). These findings suggest that HER2+ BC cells are highly sensitive to CNBDA.

The PTPase assay data in Figure 2 suggest that the IC_{50} of CNBDA against the purified SHP2 enzyme domain is approximately 5 μM, but the cell treatment data in Figure 3A,B suggest that CNBDA is more effective in cells than in **in vivo**.
vivo PTPase assays. To obtain additional insight on this point, we conducted cell viability studies using the Promega protocol that measures cell growth and viability based on ATP levels. As mentioned above, the MCF-10A cells that showed relative resistance were used as controls. Cells were treated with CNBDA concentrations ranging from 100 nM to 1.6 μM in a 2x serial dilution for 24 h. The results showed a drastic reduction in cell viability in both the BT474 and the JIMT-1 cells with an IC_{50} of 300 and 400 nM, respectively (Figure 3D,E). In the case of the control MCF-10A cells, the cell viability was reduced by only 20 percent even at the highest concentration used, which is 1.6 μM (Figure 3F). These results suggest that CNBDA might be more effective in inhibiting SHP2 in cells than in in vivo PTPase assays.

**CNBDA Suppresses Anchorage-Independent Growth and Cancer Stem Cell Properties of BC Cells.** We have previously shown that inhibiting SHP2 by shRNA silencing or dominant-negative expression in BC cells blocks colony formation in soft agar and mammosphere formation in suspension culture, respectively. Although colony formation in soft agar is an assay for cell transformation, mammosphere formation is commonly used for determining the presence of cells with cancer stem cell (CSC) properties. We used these assays as readouts for the effect of CNBDA on cell transformation and CSC properties. Approximately, 10^5 BT474 or JIMT-1 cells were seeded in soft agar in 6 cm plates and then treated with a vehicle or three different concentrations of CNBDA. Although vehicle-treated cells
formed larger colonies, CNBDA-treated cells formed smaller and fewer colonies at 0.25 and 0.5 μM and not at all at 1 μM (Figure 4A,B). These findings suggest that CNBDA suppresses the transformation phenotype of HER2-positive BC cells.

Figure 4. CNBDA abrogates anchorage-independent growth in soft agar and mammosphere formation in suspension culture in a concentration-dependent manner. (A) Effect of CNBDA on colony formation in soft agar by the BT474 cells. (B) Effect of CNBDA on colony formation in soft agar by the JIMT-1 cells. (C) Effect of CNBDA on mammosphere formation in suspension culture by the BT474 cells. (D) Effect of CNBDA on mammosphere formation in suspension culture by the JIMT-1 cells. P: primary culture; S: secondary (passaged) culture.

CNBDA Blocks EGF-Induced Signaling in BC Cells. Because SHP2 is an essential mediator of mitogenic and cell survival signaling induced by RTKs and other signaling pathways,32−34 we asked whether CNBDA affects cellular phenotypes through inhibition of SHP2-mediated signaling. To test this possibility, we treated cells with a sublethal concentration of CNBDA, 200 nM, which is lower than the minimum concentration used in colony and mammosphere formation assays. The JIMT-1 and the BT474 BC cells used in the abovementioned cellular assays were seeded in 6 cm plates, grown to approximately 80% confluency, serum-starved overnight in the presence of 200 nM CNBDA or vehicle only, and then stimulated with 20 ng/mL EGF in a time course fashion, ranging from 10 min to 4 h. Total protein extracts from these cells were separated by SDS-PAGE and analyzed by immunoblotting for the effect on activation of ERK1/2 and Akt as readouts for activation of the Ras-ERK and the PI3K-AKT signaling pathways. Akt and ERK1/2 activation in the vehicle-treated cells was augmented and sustained for at least 4 h, but it was suboptimal and short-lived in the CNBDA-treated cells (Figure 5A,B). Reblotting for total ERK2 and Akt proteins showed comparable protein levels in all lanes. These findings are consistent with CNBDA inhibiting SHP2-mediated signaling. Because silencing SHP2 with shRNA in HER2-positive BC cells or genetic knockout in the mammary glands of the MMTV-HER2/Neu mice leads to downregulation of the HER2 oncogene,35 we asked whether pharmacological inhibition of SHP2 also leads to similar downregulation of the HER2 protein in these cells. Consistent with these prior observations, CNBDA treatment led to downregulation of HER2 expression in both cell lines (Figure 5A,B, top panel).

To obtain semiquantitative data, band densities of pAkt and pERK1/2 levels from three independent experiments were determined. The raw band density data were transformed into fold activation, using the mean of non-EGF-treated data from the vehicle-treated cells as a reference point. EGF-induced ERK1/2 and Akt activation in vehicle-treated cells was 14−15-fold at the 10 min time point and was sustained for up to 4 h with minimal decline. On the other hand, the pAkt levels in CNBDA-treated cells was approximately 7-fold at the 10 min time point and was sustained for at least 4 h, but it was suboptimal and short-lived in the CNBDA-treated cells (Figure 5B,E). Similar patterns were observed in the case of pERK1/2 except that the overall intensity of activation was higher. For instance, the pERK1/2 level in vehicle-treated cells was 18−20-fold at 10 min and declined to 5-fold in 2 h, and to baseline in 4 h (Figure 5B,E). Overall, these data show that CNBDA suppresses EGF-induced signaling and expression of the HER2 oncogene in BC cells.

CNBDA Inhibits SHP2 PTPase Activity in Cells. An important question that followed the observed effects of CNBDA on cell viability and signaling was whether it directly engages SHP2 in cells. Previous reports have shown that SHP2 becomes Tyr-phosphorylated following growth factor stimulation of cells and is capable of rapidly auto-dephosphorylating itself.35 This property of SHP2 is demonstrated by the inability of the PTPase-dead C459S-SHP2 mutant to auto-dephosphorylate itself. To verify this point under our experimental conditions, we determined the state of Tyr phosphorylation of wild-type SHP2 (WT-SHP2) and phosphatase-dead C459S-SHP2 expressed as FLAG-tagged proteins showed comparable protein levels in all lanes. These observations, CNBDA treatment led to downregulation of HER2 expression in both cell lines (Figure 5A,B, top panel).

For determining the effect of CNBDA on CSC properties, approximately, 10^5 cells were seeded in nonadherent 6 cm plates in suspension cultures, in which only cells with stem-like properties can grow. Because the proportion of CSCs increases upon passaging from primary to secondary cultures, we used this strategy to test the efficacy of CNBDA. Although the control cells formed larger and numerous mammospheres that became exhausted upon passaging from primary to secondary cultures, the CNBDA-treated cells formed fewer and smaller ones that became exhausted upon passaging in a concentration-dependent manner (Figure 4C,D). Hence, inhibition of SHP2 with CNBDA blocks the CSC properties of the HER2-positive BT474 and JIMT-1 cells.
proteins in BC cells as described previously. Cells expressing the vector alone, WT-SHP2, and C459S-SHP2 were grown to about 80% density in 2D culture, serum-starved them overnight, and then stimulated with EGF for 10 min or left unstimulated. Total cell lysates were cleared by centrifugation, subjected to immunoprecipitation with the anti-FLAG antibody, and analyzed by immunoblotting with the anti-pTyr-SHP2 (pTyr542-SHP2) antibody. Consistent with previous reports, the PTPase-dead C459-SHP2 was highly Tyr-phosphorylated and this event was enhanced by EGF stimulation, but the WT-SHP2 was not regardless of the presence of comparable amounts of both proteins (Figure 6A). We used this biological event as a surrogate readout for pharmacological inhibition of SHP2 in cells.

The BT474 and the JIMT-1 cells used in the above-mentioned cellular and signaling experiments were seeded in 6 cm plates, grown to approximately 80% confluency, serum-starved overnight in the presence of 200 nM CNBDA or vehicle only, and then stimulated with 20 ng/mL EGF for 10 min. Total cell lysates were cleared by centrifugation, subjected to immunoprecipitation with the anti-SHP2 antibody, and analyzed by immunoblotting with the anti-pTyr-SHP2 anti-
body. Consistent with inhibition of the PTPase activity, SHP2 in CNBDA-treated cells was highly phosphorylated on Tyr4542, which was enhanced by EGF stimulation. Reblotting for SHP2 showed the presence of comparable SHP2 proteins in all lanes although the bands look fatter because of the increase in size caused by phosphorylation. These findings show that CNBDA engages the SHP2 protein in cells and inhibits its PTPase activity.

■ DISCUSSION AND CONCLUSIONS

Since 2006, several attempts have been made to develop selective inhibitors against SHP2. Particularly, active site-directed small-molecule inhibitors have faced significant challenges because of the similarity of the active sites of many PTPs. For instance, designing inhibitors that specifically target SHP2 without impacting SHP1 that has significant sequence and structural homology, but plays opposite biological roles, has been very difficult. The major challenge is the incomplete knowledge on how SHP2 selectively binds target pTyr substrates in proteins, which could help guide the design of small-molecule inhibitors. We have previously reported that SHP2 selectively dephosphorylates substrates in proteins based on primary amino acid sequences surrounding the target pTyr. In our recent report, we have further shown that acidic residues at the -2 and the -1 position (N-terminal) of the target phosphotyrosine are critical for specific substrate recognition. Based on this information, we were able to design and successfully synthesize a small-molecule SHP2 inhibitor with an abbreviated name of CNBDA (Figure 1A). As discussed below, CNBDA is effective in inhibiting SHP2 and in suppressing cell growth, transformation, and mitogenic and cell survival signaling.

As predicted by computational molecular modeling, CNBDA packs well into the SHP2 active-site cleft by making extended interactions with a ΔG of −54.78 kcal per mole (Figure 1B,C). The modeling data suggest that CNBDA inserts itself deep into the active site of SHP2 in a manner that resembles interaction of phosphotyrosyl substrates. As such, the molecule is predicted to interact with R465 that is known to coordinate the phosphate moiety in biological substrates, the catalytic nucleophile C459, the three positively charged residues that exist in the context of R360GK362SK366 motif, but reside far away from the signature motif (VHC459SAGIGR465T), and other residues that surround the active site, including backbones of I463 and G464 and side chain of Q281 (Figure 1C). Future studies that use X-ray crystallography are needed to confirm the computational data.

Using in vitro PTPase assays, we have shown that CNBDA inhibits SHP2 at an IC50 of 5 μM, but the IC50 for SHP1 inhibition was 125 μM (Figure 2), suggesting an approximately 25-fold difference in effectiveness. Although we have not exhaustively analyzed the effect of CNBDA toward other PTPs, the observed 25-fold selectivity for SHP2 over that of SHP1 that has significant structural homology is very promising. Given the extensive interactions CNBDA make with the SHP2 active site that mediates specific interactions, it is likely that CNBDA is more specific to SHP2. Future studies shall address the specificity questions by testing against the PTPase activity of other PTPs.

Accumulating evidence suggests that SHP2 is a bona fide oncogene. As such, there is an increased interest to develop specific inhibitors of SHP2 for cancer treatment. In line with this interest, we have tested the effect of SHP2 targeting with CNBDA in two HER2-positive BC cells and found suppression of cell proliferation in 2D culture (Figure 3), anchorage-independent growth in soft agar (Figure 4A,B), and mammosphere formation in suspension cultures (Figure 4C,D), which is consistent with inhibition of SHP2 by shRNA silencing, dominant-negative expression, or genetic knockout. Another important observation was differences in efficacy of CNBDA in vitro and in cells. Although the IC50 in PTPase assays toward SHP2 was 5 μM, the IC50 in cell viability assays was 300–400 nM (Figure 3D,E). It is possible that the compound binds better to full-length SHP2 overexpressed in cancer cells than to the isolated PTP domain used in enzyme assays. Overall, data presented in Figures 3 and 4 suggest that CNBDA has promising anti-BC activity that needs to be further investigated under in vivo conditions in future studies.

One of the well-characterized biological roles of SHP2 is mediating RTK signaling. Consistent with previous reports in which inhibition of SHP2 by dominant-negative expression, shRNA silencing or conditional genetic knockout in the mammary glands of BC model mice, treating the HER2-positive BC cells with CNBDA inhibited EGF-induced signaling and also downregulated the expression of HER2 (Figure 5). These findings suggest that the anti-BC cell effect of CNBDA is through inhibiting mitogenic and cell survival signaling and inhibiting receptor expression.

Finally, we determined whether or not CNBDA engages SHP2 in cells. We used the self-dephosphorylation (auto-dephosphorylation) property of SHP2 to verify this point and found that treating cells with CNBDA leads to accumulation of Tyr-phosphorylated SHP2 (Figure 6). Although this is an indirect way to show interaction of CNBDA with SHP2 in cells, it clearly suggests that the compound permeates into cells and disables the PTPase activity. However, our data cannot rule out the possibility of CNBDA inhibiting other PTPs as...
well. Future studies are needed to address some of these questions.

**CONCLUSIONS**

We have used a nontraditional approach to rationally design and chemically synthesize a unique active-site SHP2 inhibitor, which shows promising results in inhibiting the PTpase activity in vitro and cancer cell phenotypes and signaling in culture. Our previous reports on the SHP2 substrate provided key structural information for designing CNBDA. The compound showed a substantial inhibitor effect against SHP2 than the close structural homologue SHP1, but the effect on other PTps is currently unknown. Given the extensive interactions CNBDA make with side chains and backbones of the SHP2 active site, it is likely that CNBDA is more specific to SHP2. However, future studies are needed to verify this point. Our recent report using peptide inhibitors of SHP2 can inform active site, it is likely that CNBDA is more specific to SHP2. Overall, we conclude that CNBDA is a unique and promising lead compound for future development of anti-SHP2 drugs.

**EXPERIMENTAL SECTION**

**Synthesis of CNBDA.** Synthesis of Compound 2—Methyl 4′-hydroxy-[1,1′-biphenyl]-4-carboxylate. Concentrated sulfuric acid (~0.5 mL) was added to a suspension of 4′-hydroxy-[1,1′-biphenyl]-4-carboxylic acid (5.00 g) in methanol (100 mL). After 15 h of reflux, the yellow-brown suspension was obtained. This suspension was cooled to room temperature and the solid was filtered, washed with cold methanol (2 × 25 mL), and dried to give compound 2 (4.84 g) as a tan solid.

Synthesis of Compound 3—Methyl 3′,5′-dibromo-4′-hydroxy-[1,1′-biphenyl]-4-carboxylate. Bromine (1.45 mL) was added dropwise to a suspension of compound 2 (2.95 g) in acetic acid (75 mL). The reaction temperature slowly increased from 17 to 22 °C and the solid was dissolved to give an orange-brown solution. The mixture was stirred overnight at room temperature. The resulting yellow-orange suspension was slowly diluted with cold water (300 mL) and stirred for 30 min, which led to the development of a solid suspension. The suspension was then filtered, washed with water (3 × 100 mL), and dried on the filter for 30 min. Next, the solid was dissolved in ethyl acetate (250 mL) and the solution was washed with a mixture of saturated sodium bicarbonate (75 mL) and saturated sodium thiosulfate (75 mL), followed by saturated brine (100 mL). The organic solution was dried over sodium sulfate and filtered, and the filtrate was concentrated to near dryness. The solid was triturated with heptanes (50 mL), filtered, washed with heptanes, and dried to give compound 3 (4.76 g) as a tan solid.

Synthesis of Compound 4—Methyl 3′,5′-dibromo-4′-methoxy-[1,1′-biphenyl]-4-carboxylate. A suspension of compound 3 (4.76 g) in ethanol (40 mL) and the mixture was heated at reflux for 10 h. The solution was cooled to room temperature and stirred overnight at room temperature. The mixture was concentrated under reduced pressure to give a yellow-brown, gummy foam. The residue was purified on an AnaLogix automated chromatography system (SF15-24 g column, dry-loaded) and eluted with a gradient of 0–100% ethyl acetate in heptanes to give compound 4 (1.29 g) as a white solid.

**Synthesis of Compound 5—Diethyl (4,4′-[4′-methoxy-carbonyl]-4-methoxy-[1,1′-biphenyl]-3,5-diyl)-dibutanoate and Diethyl (4,4′-[4′-ethoxy-carbonyl]-4-methoxy-[1,1′-biphenyl]-3,5-diyl)dibutanoate.** A suspension of compound 4 (1.29 g) and [1,1′-bis(diphenylphosphino)ferrocene]-dichloropalladium(II) complexed with dichloromethane (0.24 g) in tetrahydrofuran was degassed with a stream of nitrogen for 5 min. A 0.5 M solution of 4-ethoxy-4-oxobutylzinc bromide in tetrahydrofuran (26 mL) was added via a syringe. The resulting brown solution was refluxed for 18 h, cooled to room temperature, and quenched with saturated ammonium chloride (20 mL). The biphase mixture was diluted with water (20 mL) and extracted with a 1:1 mixture of ethyl acetate and heptanes. The organic phase was washed with water (50 mL) and saturated brine (2 × 50 mL), dried over sodium sulfate, and filtered, and the filtrate was concentrated under reduced pressure. The resulting red-brown oil was purified on an AnaLogix automated chromatography system (SF15-24 g column, dry-loaded) and eluted with a gradient of 0–30% ethyl acetate in heptanes. Fractions containing the lower Rf closely running components were concentrated to give compound 5 (0.89 g) as a yellow-brown oil. Note: Compound 5 (mixture of triethyl, diethyl, and monomethyl esters) was obtained because of an unexplained partial transesterification that occurred during the Negishi coupling reaction.

**Synthesis of Compound 6—Diethyl (4,4′-[4′-ethoxy-carbonyl]-4-methoxy-[1,1′-biphenyl]-3,5-diyl)dibutanoate.** Concentrated sulfuric acid (4 drops) was added to a solution of compound 5 (0.89 g) in ethanol (40 mL) and the mixture was heated at reflux for 10 h. The solution was cooled to room temperature and stirred overnight at room temperature. The mixture was concentrated under reduced pressure to remove ethanol. The residual oil was dissolved in ethyl acetate and the solution was washed with saturated sodium bicarbonate (25 mL) and saturated brine (25 mL), dried over sodium sulfate, and filtered, and the filtrate was concentrated under reduced pressure. The crude brown oil was purified on an AnaLogix automated chromatography system (SF15-24 g column, dry-loaded) and eluted with a gradient of 0–25% ethyl acetate in heptanes to give compound 6 (0.64 g) as a pale-yellow oil.

**Synthesis of Compound 7—(4,4′-[4′-Carbonyl]-4-hydroxy-[1,1′-biphenyl]-3,5-diyl)dibutanoic Acid, Mixture of Methyl and Ethyl Esters.** A solution of compound 6 (1.10 g) in dichloromethane (40 mL) was cooled in an ice bath and 1.0 M boron tribromide in dichloromethane (7.0 mL) was added dropwise. The mixture was stirred in the ice bath for 4.5 h and quenched by a slow and dropwise addition of methanol. The mixture was allowed to warm to room temperature, stirred overnight, concentrated under reduced pressure, and the yellow-brown oil was dissolved in ethyl acetate (75 mL). The solution was washed sequentially with water (2 × 50 mL), saturated sodium bicarbonate (50 mL), and saturated brine (50 mL). The organic phase was dried over sodium sulfate and filtered, and the filtrate was concentrated under reduced pressure to give crude compound 7 (1.0 g) as a yellow-brown oil.
Synthesis of Compound 8-CNBDa, Mixture of Methyl and Ethyl Esters. A mixture of potassium carbonate (0.47 g), crude compound 7 (1.00 g), and acetonitrile (25 mL) was stirred for 5 min, and 1-bromonanone (0.59 g, 0.54 mL) was added to the mixture and refluxed for 5.5 h. After stirring overnight at room temperature, the suspension was filtered and the solid was washed with ethyl acetate (25 mL). The filtrate was concentrated under reduced pressure. The resulting oil was partitioned between ethyl acetate (75 mL) and water (25 mL)—a slow phase separation. The organic phase was washed with saturated brine (25 mL), dried over sodium sulfate, and filtered, and the filtrate was concentrated to give a light-brown oil. The crude product was purified on an AnaLogix automated chromatography system (SF15-24 g column, dry-loaded) and eluted with a gradient of 0–25% ethyl acetate in heptanes to give compound 8 (0.97 g) as a colorless oil.

Synthesis of CNBDa. A solution of lithium hydroxide monohydrate (1.02 g) in water (25 mL) was added to a solution of compound 8 (0.97 g) in tetrahydrofuran (25 mL). On addition, the resulting biphasic mixture warmed slightly. The mixture was stirred at room temperature for 19.5 h, and when TLC (50% ethyl acetate/heptanes) and LCMS showed that the reaction was completed, the mixture was concentrated under reduced pressure to remove tetrahydrofuran. The resulting aqueous solution was cooled in an ice bath and made acidic (pH 1) with 1 N hydrochloric acid to give a fine white precipitate. The aqueous suspension was extracted with a 10:1 mixture of ethyl acetate and tetrahydrofuran (275 mL).

[Note]: The solid was only partially soluble in ethyl acetate.

The organic phase was washed with saturated brine (100 mL), dried over sodium sulfate, and filtered, and the filtrate was concentrated under reduced pressure to give a white solid. The solid was dried overnight in a vacuum oven at 50 °C to give CNBDa as a white solid. NMR of CNBDa (DMSO-d6): δ 0.88 (t, 3H), 1.28 (m, 10H), 1.48 (m, 2H), 1.75 (m, 2H), 1.84 (m, 4H), 2.27 (t, 4H), 2.65 (t, 4H), 3.75 (t, 2H), 7.41 (s, 2H), 7.68 (d, 2H), 8.00 (d, 2H), 12.25 (br s, 3H).

Molecular Modeling. We have used the molecular docking program Glide (by Schrodinger) that includes a receptor preprocessing and optimization program, a ligand preparation program, a flexible induced-fit docking program, and an interaction scoring system known as Prime MM-GBSA for docking CNBDa into the SHP2 active sites. We chose the SHP2 (PDB: 4DGP) that was solved with both of the SH2 domains bound to the active site for these studies. First, the 4DGP structure was loaded, preprocessed, and optimized using the Epik program in the Glide software package. Because the SHP2 structure PDB: 4DGP was solved with both of the SH2 domains, the preprocessing included removal of the first 104 amino acids from the structure because the N-SH2 domain of SHP2 blocks the active site. Because CNBDa is a presumed active-site inhibitor, a 20 × 20 × 20 Å grid box was placed around the point defined by the sulfur atom of the catalytic cysteine (C459 for SHP2 and C455 for SHP1). Next, CNBDa built in Chemdraw was saved as a mol file and docked into the active site defined by the grid box using the Glide program. The best possible docking conformation data were further refined using Prime MM-GBSA to obtain the best possible induced-fit interaction between CNBDa and the SHP2 active site. The electrostatic map of the best predicted binding structure was generated, and the best binding pose was selected and presented to predict the binding modality. A two-dimensional ligand interaction diagram was prepared from the PDB file of the docked pose, using the MAESTRO 2-D sketcher of Glide.

PTPase Assay. The inhibitory effect of CNBDa on the enzyme activity of SHP2 and SHP1 was determined by the PTPase assay as described previously44,45. Construction and expression of the GST fusions of the PTP domains of SHP2 and SHP1 were reported by us recently.27 These proteins were expressed in bacteria using a standard protocol, purified using a glutathione-conjugated sepharose column, and quantified by spectroscopic measurement of absorbance at 280 nm wavelength. The stock solutions were prepared in phosphatase reaction buffer (50 mM HEPES, 100 mM NaCl, and 2 mM EDTA, pH 7.2). For the PTPase reactions, the proteins were diluted to a final concentration of 1 μM in the same buffer and used for determining the effect of CNBDa on dephosphorylation of the artificial substrate difluoromethylbenzylidene phosphate (DiFMUP). Because the reported Km of SHP2 and SHP1 toward DiFMUP is 20 and 35 μM, respectively,46 we used these concentrations in the respective PTPase reactions. Concentrations of CNBDa ranging from 61 nM to 4 mM in 2× serial concentration increase was used to assess the approximate concentration for 50% inhibition. Graphpad Prism was used to calculate the IC50 value.

Cells, Cell Culture, and Reagents. MCF-10A (immortalized mammary epithelial cells) and BT474 BC cells were purchased from the American Tissue Culture Collection (ATCC), whereas the JIMT-1 cells were purchased from DSMZ, Germany. The BT474 and the JIMT-1 cells were grown in RPMI 1640 and Dulbecco’s modified Eagle’s medium (DMEM), respectively, and supplemented with 10% fetal bovine serum. On the other hand, the MCF-10A cells were cultured in DMEM supplemented with 10 μg/mL recombinant human insulin, 20 ng/mL EGF (PeproTech), 0.5 μg/mL hydrocortisone, 100 ng/mL cholera toxin (Sigma), and 5% horse serum. The other reagents used included DiFMUP (Invitrogen), glutathione-sepharose beads (GE Healthcare), anti-HER2, anti-SHP2, and anti-panERK2 antibodies (BD biosciences), anti-β-actin antibody (Sigma-Aldrich), and anti-phospho-ERK1/2, anti-phospho-Akt, and anti-panAkt antibodies (Cell Signaling, Inc).

Immunoblotting Analyses. For determining the effect of CNBDa on SHP2-mediated and EGF-induced signaling, cells were grown to approximately 80% density, serum-starved overnight in the presence of 250 μM CNBDa, and then stimulated with 20 ng/mL EGF for varying time points, ranging from 10 min to 4 h. They were then lysed in a buffer containing 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Triton-X-100, 10% glycerol, and 50 mM NaF supplemented with 10 μg/mL each of aprotinin, leupeptin, and phenylmethylsulfonylfluoride for inhibition of proteases and 10 mM sodium orthovanadate for inhibition of phosphatases. Lysates containing comparable protein levels were denatured by adding equal volume of 2× LaemmlI sample buffer and boiling at 100 °C for 5 min. The proteins were separated by polyacrylamide (8%) gel electrophoresis (PAGE), immobilized onto a nitrocellulose membrane, blocked with 3% bovine serum albumin (BSA) in tris-buffered saline containing 1% Tween 20 (TBST), and stained with primary antibodies for 2 h at room temperature or overnight at 4 °C as desired. Next, membranes were washed three times with TBST, incubated with hors eradish peroxidase-conjugated secondary antibodies in 5% milk, washed three times with TBST, and visualized by chemiluminescence (Pierce Inc.).
**Cell Viability Assay.** The effect of CNBDA on cell viability was determined using a luminescence cell viability assay (Promega) that measures growth based on ATP levels. We followed the manufacturer’s protocol in growing cells, preparation of the reagents, and measurement of luminescence. Cells were treated with a vehicle or CBDA concentrations ranging from 100 nM to 1.6 μM in a 2X serial dilution for 24 h. Cell viability was measured in a Synergy H3 (Biotec) plate reader and the data were analyzed with the IGEN-5 software.

**Anchorage-Independent Growth Assay.** The effect of CNBDA on anchorage-independent growth of the BT474 and the JIMT-1 cells was determined by the soft agar assay as described previously. Briefly, 6 cm cell culture plates were overlaid with 0.3% agar in a corresponding growth medium and allowed to solidify. Next, cells suspended in 3 mL of growth medium were mixed with melted agar to a final concentration of 0.3% and immediately poured onto the agar overlay. CNBDA was added to cells prior to mixing with the soft agar. After 5 min of incubation at room temperature, the plates were transferred to a 37 °C incubator with 5% CO2 supply for 10 days. During this time, the cells were fed two times with soft agar medium with or without CNBDA. Colony formation was visualized under a microscope and phase contrast pictures were taken using an Olympus IX71 microscope equipped with an Olympus DP30BW digital camera. For estimating the colony number, pictures were collected from 10 random fields per plate using the 4X objective at the same quadrants. Colonies in each image were then counted visually and the values were used to determine the effect of CNBDA on cell transformation.

**Mammosphere Formation Assay.** The mammosphere or tumorsphere assay was used to determine the effect of CNBDA on the CSC properties of the BT474 and the JIMT-1 BC cells. This assay was conducted as described by us and others previously. Briefly, approximately, 10³ cells were cultured in serum-free DMEM containing 1 μg/mL hydrocortisone, 10 μg/mL insulin, 10 ng/mL EGF, 10 ng/mL FGF, 5 ng/mL heparin, and B27 (Invitrogen) in 6 cm ultralow-adherence culture plates. For passaging, the primary spheres were collected by centrifugation, dissociated to single cells by a standard trypsin treatment method and pipetting, and seeded in new ultralow attachment plates. Both primary and secondary mammospheres were pictured after 10 days of incubation in each case.

**Author Contributions**

Z.H. carried out the PTPase assay and the molecular modeling studies. W.G. was responsible for molecular modeling and interpretation of data. Y.M.A. was responsible for designing CNBDA, for conducting cellular and signaling studies, for overseeing and directing the whole project, and for preparation of the manuscript.

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**Notes**

The authors declare no competing financial interest.

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