Development of potent small-molecule inhibitors to drug the undruggable steroid receptor coactivator-3

Xianzhou Song,a,1 Jianwei Chen,a,b, Mingkun Zhao,a,b, Chengwei Zhang,a, Yang Yu,c, David M. Lonard,c,d, Dar-Chone Chow,a,b,c,d, Timothy Palzkill,a,b,c,d, Jianming Xu,e, Bert W. O’Malley,a,b,c,d, and Jin Wang,a,b,c,d

*Department of Pharmacology, Baylor College of Medicine, Houston, TX 77030; †Integrative Molecular and Biomedical Sciences Graduate Program, Baylor College of Medicine, Houston, TX 77030; ‡Department of Molecular and Cellular Biology, Baylor College of Medicine, Houston, TX 77030; and §Center for Drug Discovery, Baylor College of Medicine, Houston, TX 77030

Contributed by Bert W. O’Malley, March 15, 2016 (sent for review January 19, 2016; reviewed by Thomas Burris and Roy G. Smith)

Protein–protein interactions (PPIs) play a central role in most biological processes, and therefore represent an important class of targets for therapeutic development. However, disrupting PPIs using small-molecule inhibitors (SMIs) is challenging and often deemed as “undruggable.” We developed a cell-based functional assay for high-throughput screening to identify SMIs for steroid receptor coactivator-3 (SRC-3 or AIB1), a large and mostly unstructured nuclear protein. Without any SRC-3 structural information, we identified SI-2 as a highly promising SMI for SRC-3. SI-2 meets all of the criteria of Lipinski’s rule (Lipinski et al. (2001) Adv Drug Deliv Rev 46(1-3):3–26) for a drug-like molecule and has a half-life of 1 h in a pharmacokinetics study and a reasonable oral availability in mice. As a SRC-3 SMI, SI-2 can selectively reduce the transcriptional activities and the protein concentrations of SRC-3 in cells through direct physical interactions with SRC-3, and selectively induce breast cancer cell death with IC50 values in the low nanomolar range (3–20 nM), but not affect normal cell viability. Furthermore, SI-2 can significantly inhibit primary tumor growth and reduce SRC-3 protein levels in a breast cancer mouse model. In a toxicology study, SI-2 caused minimal acute cardiotoxicity based on a hERG channel blocking assay and an unappreciable chronic toxicity to major organs based on histological analyses. We believe that this work could significantly improve breast cancer treatment through the development of “first-in-class” drugs that target oncogenic coactivators.

Coactivators are non-DNA binding proteins that mediate transcriptional activities of nuclear receptors (NRs) and many other transcription factors (6–10). Since the O’Malley group identified the first coactivator, steroid receptor coactivator 1 (SRC-1) (11), there have been more than 400 coactivators identified and associated with a wide range of human diseases, including neurological and metabolic disorders, inflammatory diseases, and cancer (6–8). Taking estrogen receptor-positive (ER+ ) breast cancer as an example, cancer cells can use a number of mechanisms to overcome selective estrogen receptor modulators to silence the NR activity. Although breast cancer cells can become resistant to endocrine therapies, it is essential for them to recruit coactivators to survive. Earlier efforts have been focused on developing peptides and SMIs to interfere with the interactions between NRs and coactivators (12–14). A major drawback of this strategy is that overexpression of coactivators, a hallmark of endocrine resistance, often occurs regardless of the context of which NR is expressed in the cancer cell. Coactivators also partner with other transcription factors; therefore, SMIs that can directly target the overexpressed coactivators and reduce their activity or stability should be preferred for drug development.

Identification of SMIs for coactivators is challenging because coactivators are usually considered as undruggable because of their large and flexible structures (6–9). We recently developed a cell-based functional assay for high-throughput screening to identify SMIs for steroid receptor coactivator-3 (SRC-3). Without any SRC-3 structural information, we identified and improved a

Significance

Steroid receptor coactivator-3 (SRC-3) sits at the nexus of many intracellular signaling pathways critical for cancer formation and proliferation. Although the oncogenic role of SRC-3 has been well established in breast and other cancers, coactivators are usually considered as “undruggable” because of their large and flexible structures. Herein, we developed SI-2 as a new class of potent small-molecule inhibitors for SRC-3. SI-2 can selectively reduce the transcriptional activities and the protein concentrations of SRC-3 in cells and significantly inhibit primary tumor growth in a breast cancer mouse model. This work not only has the potential to improve breast cancer treatment, but also to provide a viable strategy to target often “undruggable but important” protein targets without ligand-binding sites.

Author contributions: D.M.L., J.X., B.W.O., and J.W. designed research; X.S., J.C., M.Z., C.Z., Y.Y., and D.-C.C. performed research; X.S., J.C., D.M.L., D.-C.C., T.P., J.X., B.W.O., and J.W. analyzed data; and X.S., J.C., B.W.O., and J.W. wrote the paper.

Reviewers: T.B., St. Louis University; and R.G.S., The Scripps Research Institute.

Conflict of interest statement: J.C., T.P., J.X., D.M.L., B.W.O., and J.W. are coinventors of a patent application related to this work. T.P., J.X., D.M.L., B.W.O., and J.W. are cofounders and hold stock in Corgenx, Inc., which is developing steroid receptor coactivator inhibitors for clinical use.

1X.S. and J.C. contributed equally to this work.
2To whom correspondence may be addressed. Email: berto@bcm.edu or wangj@bcm.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1604274113/-/DCSupplemental.
series of SMIs that can target SRC-3 (15–17). We initially reported gossypol as our first "proof-of-concept" SRC-3 SMI (17). Despite the encouraging success of gossypol as the first selective SRC-3 SMI, the IC50 values of gossypol are in the micromolar range, which is suboptimal for drug development and may cause off-target toxicity (17). Subsequently, we reported bufalin, a cardiac glycoside, as a potent SRC-3 SMI (16). Bufalin is an active component in the Chinese medicine Huachansu, which is prepared from the skin and parotid venom glands of the Asian toad. Bufalin directly binds to SRC-3 in its receptor interacting domain (RID) and selectively reduces the concentration of SRC-3 in breast, lung, and pancreatic cancer cell lines without perturbing overall protein expression patterns (16). Additionally, bufalin is selectively toxic to cancer cells with IC50 values in the low nanomolar range, and normal cell viability is not affected (16, 18, 19).

Importantly and most excitingly, bufalin sensitizes breast and lung cancer cells to the inhibitory effects of other chemotherapeutics and inhibits primary tumor growth in vivo. Other groups also reported that bufalin and other cardiac glycosides can inhibit transcription factors and induce synergistic immune responses (19–28). Unfortunately, cardiac glycosides are well known for their cardiotoxicity. Although we have developed bufalin nanoparticles (16) and a phosho-bufalin produg (29) to reduce cardiotoxicity, the potential of cardiac glycosides to cause cardiac arrest dampens the enthusiasm for their clinical use.

In this report, we describe a new class of SRC-3 SMI: SRC-3 inhibitor-2 (SI-2). Unlike gossypol and bufalin, SI-2 is an unnatural compound and identified through a combination of high-throughput screening and medicinal chemistry optimization. SI-2 meets all of the criteria for drug-like compounds. Because of the well-established roles of SRC-3 in endocrine resistance and tumor metastasis in breast cancer, we tested the therapeutic efficacy of SI-2 in a series of breast cancer cell lines and an orthotopic triple-negative breast cancer (TNBC) mouse model. Similar to bufalin, SI-2 can selectively reduce the protein concentrations and transcriptional activities of SRC-3, and selectively kill breast cancer cells with IC50 values in the low nanomolar range but not affecting normal cell viability. Different from bufalin, SI-2 has a much improved toxicity and pharmacokinetic profile. In addition, an animal study showed that SI-2 can significantly inhibit breast tumor growth in vivo without any observable toxicity. Based on our strong data, we envision that SI-2 is a promising drug candidate as an SRC-3 SMI that could potentially expand breast cancer treatment. In addition, our efforts to identify coactivator SMIs will build confidence for other researchers to develop approaches to target relatively unstructured, regulatory proteins that are designated as "important but difficult" targets in the future.

**Results**

**Identification of SI-2 as a Potent SRC-3 SMI.** Through a high-throughput compatible luciferase assay, we screened with the Molecular Libraries Probe Production Centers (MLPCN) library of the NIH to identify compounds targeting the intrinsic transcriptional activities of SRC-3 (PubChem AID 588352). In these assays, we evaluated the effects of compounds by measuring the output of a GAL4-responsive luciferase reporter (pGL-LUC) in the presence of GAL4 DNA binding (DBD) SRC coactivator fusion proteins (pBIND-SRC) (30). Compounds that inhibit the intrinsic ability of SRC coactivators to activate transcription will lead to a decrease in expression of the luciferase gene, resulting in reduced luminescence. Additionally, active compounds also were tested in a counter screen using cells transfected with a DBD-VP16 fusion protein to exclude those perturbing pGL/pBIND systems (AID 588794). Only those compounds that reduce SRC-3 activity greater than they do toward the VP16 control were defined as potential SRC-3 inhibitors. Active compounds retrieved from the primary screens were clustered according to structure similarities in PubChem. Through dose-dependent toxicity studies, we identified SI-1 (Fig. S1) as a promising candidate for further development. SI-1 has a submicromolar IC50 value in a MDA-MB-468 cell line, a TNBC cell line used as a model in this study.

Subsequently, we performed a series of medicinal chemistry optimizations to enhance the potency of SI-1. We discovered that SI-2 (Fig. 1A), which is formed by introduction of N-methylation to the benzimidazole ring in SI-1, can decrease the IC50 value by ~60-fold in the same TNBC cell line. In addition, we also substituted the pyridine ring in SI-2 with a series of hetero-aromatic groups (Fig. S1). Unfortunately, this substitution significantly reduced the potency of these compounds in MDA-MB-468 cells. In this study, we chose SI-2 as a leading SRC-3 SMI candidate in the following in vitro and in vivo tests.

![Image](https://example.com/image.png)

**Fig. 1.** SI-2 as a potent SRC-3 SMI. (A) Structure of SI-2. (B) Physical characterization of SI-2 drug-likeliness properties. (C) SI-2 can selectively reduce the SRC-3 intrinsic transcriptional activities. Luciferase assays were performed in HeLa cells transiently transfected with the reporter vector pG5-LUC in combination with expression vectors for pBIND and pBIND-SRC-3 before incubation with SI-2 for 24 h. (D) Selective inhibition of SRC-3 by 24 h of SI-2 treatment in MDA-MB-468 cells. SRC-3, CARM-1, and actin were detected using Western blotting. Relative band intensities were normalized by actin. (E) Endocrine sensitive MCF-7 and T47D, and endocrine resistant BT474 cells were treated with SI-2 (100 nM) for 24 h, followed by Western blotting. Relative band intensities were normalized by actin. (F) SI-2 treatment does not decrease the SRC-3 mRNA level. The relative mRNA levels vs. RPS6 were quantified using qPCR in MDA-MB-468 cells treated with SI-2 for 24 h. (G) The viabilities of HeLa and HeLa SRC-3 KO cells treated with SI-2 for 72 h. (H) SI-2 directly binds to the RID of SRC-3. Intrinsic tryptophan fluorescence emission spectra of SRC-3 RID (ex = 278 nm) were quenched with increasing concentrations of SI-2. All of the data represent mean ± SD.

Song et al.
Drug-Like Properties of SI-2. SI-2 is a drug-like molecule and meets all of the criteria for Lipinski’s rule (31), Veber’s rule (32), and Oprea’s rule (33) of drug-likeness (Fig. 1B). SI-2 has a molecular weight of 265 g·mol⁻¹ and an experimental LogP value of 0.44. In addition, SI-2 has five rotatable bonds and its numbers of hydrogen bond (H-bond) donor and acceptor are 1 and 4, respectively. The molecular polar surface area of SI-2 is calculated to be 52 Å² based on a topological method (34), which is well below 140 Å² and suggests good oral availability based on Veber et al. (32) rules. The thermodynamic and kinetic solubilities of SI-2 in PBS are 168 and 327 μg/mL, respectively.

SI-2 Inhibits Intrinsic Transcriptional Activity of SRC-3. We investigated the effects of SI-2 on the intrinsic transcriptional activities of SRC-3. HeLa cells were transiently transfected with a pGL5-LUC reporter and expression vectors for pBIND and pBIND-SRC-3, followed by 24 h of treatment with different concentrations of SI-2. As shown in Fig. 1C, SI-2 significantly reduced the luciferase reporter activities in cells transfected in pBIND-SRC-3 in a dose-dependent manner, but only minimally affected the activity of pBIND. This result suggests that SI-2 can selectively inhibit the intrinsic transcriptional activities of SRC-3. Similar tobufalin, SI-2 also can inhibit the transcriptional activities of SRC-1 and SRC-2 (Fig. S2A), the other two members of the p160 family of steroid-receptor coactivators.

SI-2 Selectively Inhibits SRC-3 Protein Levels Posttranscriptionally. We showed that the steady-state levels of coactivator proteins correlate with their transcriptional activities and with cancer progression (35). Therefore, we studied the effects of SI-2 on SRC-3 protein levels in MDA-MB-468 breast cancer cells after 24 h of incubation. As shown in Fig. 1D, SI-2 selectively reduces cellular protein levels of SRC-3, but not that of coactivator-associated arginine methyltransferase 1 (CARM-1), which is part of a multiprotein coactivator complex with SRC-3. Furthermore, inhibition of SRC-3 levels also was observed in other breast cancer cell lines, including endocrine sensitive MCF-7, T47D cells, and endocrine-resistant BT-474 cells (Fig. 1E). In addition, SI-2 can inhibit the protein levels of SRC-1 and SRC-2, but to a lesser extent than for SRC-3, especially at lower concentrations (Fig. S2 B and C).

To gain insights into the mechanism of SI-2-mediated SRC-3 protein down-regulation, we assessed whether 24 h of SI-2 treatment affected the production of mRNAs for each SRC family member in MDA-MB-468 cells. Quantitative PCR (qPCR) revealed that mRNA levels for SRC-1 and SRC-2 were not significantly altered (Fig. S2D), whereas the mRNA levels for SRC-3 were actually increased upon SI-2 incubation (Fig. 1F). This result is consistent with the mRNA level changes for the SRC family in bufalin-treated cells (16) and suggests that SI-2 reduces SRC protein levels posttranscriptionally.

SRC-3 Is Required for SI-2 Activity. It is well established that targeting SRC-3 with siRNAs inhibits cell growth in many cancer types (7, 36). Considering the fact that the decreased cell viability induced by SI-2 is accompanied by reduced SRC-3 protein levels, we sought to investigate the specific role of the SRC-3 protein in blocking cancer cell proliferation. Previously, we used a zinc finger nuclease to knockout both SRC-3 alleles in the HeLa cell line and developed HeLa SRC-3 KO cells (16). Compared with parental SRC-3⁺⁺ cells, the response of HeLa SRC-3 KO cells to SI-2 treatment is reduced by ~fivefold (Fig. 1G). This finding supports the idea that SRC-3 protein is involved in mediating the cell response to SI-2 treatment. However, the remaining response of HeLa SRC-3 KO cells to SI-2 is likely because of the SRC-1 and SRC-2 that continue to be expressed in these cells and that also respond to SI-2 at a similar dose as SRC-3, in addition to any unknown off-target actions of the compound.

Fig. 2. SI-2 selectively kills and inhibits migration of breast cancer cells, but spares normal cells. (A) SI-2 is selectively toxic to MDA-MB-468 cells with an IC₅₀ value of 3.4 nM (Left axis), and normal cell viability (i.e., primary hepatocytes) is not affected. The toxicities were determined by MTT assays. (B) SI-2 treatment kills MCF-7 and BT-474 cells with IC₅₀ values in the low nanomolar range, measured using MTT assays. (C) SI-2 treatment induces apoptosis in cancer cells. MDA-MB-468 cells were treated with SI-2 for 24 h and stained with Annexin V. Flow cytometry was performed to quantify the percentages of cancer cells in the early apoptosis (EA) and late apoptosis (LA) phases. (D) SI-2 treatment in cancer cells causes PARP cleavage, an apoptosis marker. MDA-MB-468 cells were treated with SI-2 for 24 h and PARP cleavage (using Western blotting). (E) SI-2 inhibits migration of triple negative breast cancer cells. Cell migration assay of MDA-MB-468 cells was performed using a Cellomics cell motility kit. The bright area of 50 images for each sample were analyzed using ImageJ. (Magnification: 100×). (F) Treatment of MDA-MB-468 cells with SI-2 (100 nM) for 12 h showed minimal toxicity but significant motility reduction. All of the data represent mean ± SD.
SI-2 Inhibits SRC-3 Through Direct Physical Interactions. We used a fluorescence assay to demonstrate that down-regulation of SRC-3 by SI-2 proceeds through direct physical interaction with SRC-3. We found that the intrinsic tryptophan fluorescence emission spectra of the SRC-3 RID ($\lambda_{em} = 278$ nm) were quenched with increasing concentrations of SI-2, indicating that SI-2 binds directly to the RID of SRC-3 (Fig. 1H). In contrast, there were no changes of the intrinsic fluorescence observed for a KPC-2 β-lactamase, a negative control, upon addition of SI-2 (Fig. S3), which negates the possibility that the fluorescence changes of RID are a result of nonspecific interactions with SI-2.

SI-2 Selectively Kills Cancer Cells but Spares Normal Cells. We then evaluated the cytotoxic effect of SI-2 on cancer cells. First, MTT assays were performed on MDA-MB-468 cells treated with SI-2 at different concentrations for 72 h. SI-2 can block MDA-MB-468 cell growth with an IC$_{50}$ value of 3.4 nM (Fig. 2A), in line with the dose of SI-2 required to reduce SRC-3 protein levels in the cell (Fig. 2A). In addition, similar low nanomolar IC$_{50}$ values also were observed for SI-2 in many other cell lines, including endocrine-sensitive, endocrine-resistant, and TNBC cells (Fig. 2B).

We performed apoptosis assays to examine the cause of the cytotoxic effect of SI-2 on cancer cells. Annexin V and propidium iodide were used to stain MDA-MB-468 cells treated with SI-2 at different concentrations for 24 h. Flow cytometry revealed that the percentages of cancer cells in the early and late apoptosis phases increase in a SI-2 dose-dependent manner (Fig. 2C), which is similar to etoposide, a positive control, caused apoptosis (Fig. S4). In addition, we confirmed the apoptotic effect of SI-2 on cancer cells by measuring the poly(ADP ribose) polymerase (PARP) cleavage using Western blotting (Fig. 2D).

It is important to note that inhibition of SRC-3 is selectively toxic to cancer cells while sparing normal cells (16, 17), and prior data showed that knockout of the SRC-3 gene does not influence adult mouse life span (37). Consistent with our previously reported SRC-3 SMIs, we did not observe any toxicity of SI-2 up to 500 nM (the highest concentration used) in primary hepatocytes (Fig. 2A), demonstrating the potential of SI-2 as a targeted therapy.

SI-2 Inhibits Migration of Breast Cancer Cells. Down-regulation of SRC-3 can decrease cell motility, invasion, and tumor metastasis (38). We performed a cell migration assay of MDA-MB-468 cells in the presence and absence of SI-2 using a Cellomics cell motility kit. The bright areas of 50 images for each sample were analyzed using ImageJ. We found that SI-2 treatment can significantly reduce the motility of cancer cells (Fig. 2E and F). It should be noted that under the same conditions, SI-2 caused minimal toxicity in MDA-MB-468 cells (Fig. 2F), indicating that the decrease of cell motility is not because of decrease of cell numbers.

SI-2 Is Nontoxic to Heart and Other Major Organs. Minimizing cardioxicity is an important consideration in drug development. hERG (the human ether-à-go-go-related gene) is a gene that codes for a cardiac potassium ion channel (39). Blockage of hERG can cause cardiac arrhythmia and even sudden death. Therefore, hERG screening is commonly used in drug discovery (40). We compared the hERG blocking ability of bufalin and SI-2 using a well-established in vitro method (41). HEK293 cells that stably express hERG channels (HEK293-hERG) were used as the in vitro system. HEK293-hERG cells have a more negative membrane potential than do wild-type cells, as a result of the potassium channel activity, which can be monitored using a membrane potential-sensitive fluorescent dye DiBAC$_4$(3). Blocking of hERG can increase the membrane potential and thus the fluorescence of DiBAC$_4$(3). HEK293-hERG cells were treated with DiBAC$_4$(3) and bufalin or SI-2 at different concentrations (0.1, 1.0, and 5.0 μM). The fluorescence of DiBAC$_4$(3) was monitored using a plate reader. To offset the nonspecific interactions between the drug and DiBAC$_4$(3), a similar experiment was performed in parental HEK293 cells. After correcting for the background, we found a dramatic fluorescence increase if cells were treated with bufalin, indicating strong cardiotoxicity (Fig. 3A). In contrast, SI-2 did not cause appreciable changes in DiBAC$_4$(3) fluorescence, suggesting that SI-2 cannot block hERG even at 5 μM (Fig. 3A).

SI-2 does not cause observable acute or chronic toxicity in vivo. Intraperitoneal or oral administration of SI-2 up to 20 mg/kg (the highest dose tested) did not cause appreciable stress in mice. In addition, continuous treatment of mice with SI-2 (2 mg/kg, twice per day) for 5 wk did not cause any loss of body weight (Fig. 4C) or observable damages to the major organs, including heart, liver, spleen, kidney, lung, and stomach based on histological analyses (Fig. 3B). Based on both the in vitro and in vivo studies of the toxicity profiles, SI-2 appears to be a relatively safe and promising candidate for anticancer drug development.

SI-2 Has an Acceptable Pharmacokinetic Profile and Is Orally Available. We measured the pharmacokinetic (PK) profile of SI-2 (20 mg/kg) in CD1 mice ($n = 3$) via intraperitoneal administration. Twenty microliters of blood were collected at each time point by tail-nicking. The plasma concentration of SI-2 was quantified using liquid chromatography mass spectrometry (LC-MS). The PK data were fitted into an extravascular non-compartment model (Fig. 4A), affording half-life $t_{1/2}$ of 1 h, the maximum plasma concentration $C_{max}$ of 3.0 μM, and the time to reach the maximum plasma concentration $t_{max}$ of 0.25 h.

As a drug candidate, it is important for SI-2 to achieve a reasonable oral availability. SI-2 contains a hydrazone group (Fig. L4), which is potentially sensitive to acid-catalyzed hydrolysis. We tested the stability of SI-2 in different simulated gastric fluids (SGFs) with different pH values to mimic fasted state conditions.
(pH 1.6) SGF, and early (pH 6.4), middle (pH 5), and late (pH 3) fed-state SGFs (42). A phosphate buffer with the physiological pH 7.4 was also used as a control. We found that SI-2 only degrades slightly (less than 5%) at pH 1.6 and 3.0 within 6 h, and is stable in buffers with pH ≥ 5 (Fig. S5). Next, we measured the PK profile of SI-2 (20 mg/kg) via oral gavage in CD1 mice (n = 3). The PK profile is biphasic (Fig. 4B). We could still detect SI-2 at a 24-h time point, which approaches the lower limit of detection 10 nM. The area under the curve, an indicator of drug exposure, for oral administration is ~30% of that for intraperitoneal administration. Based on these measurements, SI-2 showed acceptable oral availability and holds promise for drug development.

**SI-2 Significantly Inhibits Breast Tumor Growth and Reduces SRC-3 Levels in Vivo.** Next, we assessed the antitumor activity of SI-2 in an orthotopic MDA-MB-468 breast cancer mouse model. We determined the dosing schedule based on the PK profile of SI-2. In the PK study (20 mg/kg, i.p.) the plasma concentration of SI-2 is 23 nM at 8 h. Therefore, if a therapeutic dose 2 mg/kg were used, the plasma concentration of SI-2 should be ~2.3 nM at 8 h, which is close to the IC_{50} value of SI-2 in MDA-MB-468 cells (3.4 nM). To maintain the plasma concentration of SI-2 above the IC_{50} value in cancer cells, we decided to treat the mice twice per day. To establish orthotropic breast tumors, 2 × 10^6 MDA-MB-468 cells were injected into one of the second mammary fat pads of SCID mice (female, 5–6 wk). When tumors became palpable, 14 d after injection, mice were randomized into two groups (n = 6 for the control group; n = 8 for the treatment group). The treatment group was injected with SI-2 (intraperitoneally, 2 mg/kg per dose, two doses per day), whereas the control group received PBS. Tumor lengths and widths and mouse weights were measured once per week. Tumor sizes were calculated by (length × width^2)/2. As shown in Fig. 4 D and E, SI-2 treatment can significantly inhibit tumor growth. After 5 wk of treatment, mice in each group were killed and tumors were harvested. To test whether SI-2 can cause SRC-3 down-regulation in vivo, the SRC-3 levels in tumor tissues were measured by immunohistochemistry. As shown in Fig. 4F, the SRC-3 levels (brown color) in SI-2–treated tumor tissues were significantly lower than the PBS treated control group. K_{i67}, a cell proliferation marker, also was immunochemically analyzed. Accordingly, the SI-2–treated tumor tissues have dramatically fewer K_{i67}^+ cells (cells with brown staining, Fig. 4F). Based on these data, we conclude that SI-2 can significantly inhibit breast tumor growth through inhibition of SRC-3.

**Discussion**

SRC-3 is a critical coactivator that regulates many transcriptional signaling pathways for cancer formation and proliferation. Although selective estrogen receptor modulators, such as tamoxifen, are first-line treatments for ER^+ breast cancer (43, 44), high expression of SRC-3 is associated with recurrence and poor overall survival in both ER^+ and TNBC (29). Inhibition of SRC-3 can circumvent endocrine therapy resistance in ER^+ breast cancer. Additionally, downregulating SRC-3 can reduce proliferation and migration in TNBC cells (29). Unlike gossypol and bufalin, SI-2 is a drug-like molecule and meets all of the criteria of Lipinski’s rule (31), Veber’s rule (32), and Oprea’s rule (33) for drug-likeness. As a SRC-3 SMI, SI-2 can selectively reduce the transcriptional activities and the protein concentrations of SRC-3 in cells through direct physical interactions with SRC-3, and can selectively induce breast cancer cell death with IC_{50} values in the low nanomolar range (3–20 nM) but not affect normal cell viability. It should be noted although SI-2 treatment in cancer cells can inhibit the transcriptional activities and protein levels of all three members of the p160 family of steroid receptor coactivators, this inhibitory effect against the SRC family should be advantageous because these three SRC proteins share many redundant functions (45). Furthermore, our in vivo study demonstrated that SI-2 can significantly inhibit primary tumor growth and reduce SRC-3 protein levels in a breast cancer mouse model. In addition, in a toxicology study, we showed that SI-2 caused minimal acute cardiotoxicity based a HERT channel blocking assay and unappreciable chronic toxicity to major organs based on histological analyses.

Our study should not only pave the way to development of “first-in-class” drugs that target oncogenic coactivators and make a potential impact on breast cancer treatment, but also provide
an example and a viable strategy to target often “undruggable but important” protein targets without ligand-binding sites. Furthermore, SRC-3 is also an important oncogene in many other types of cancer, including prostate, ovarian, and lung cancer (36). It should be evaluated whether SI-2 can provide survival benefits in these cancers. In addition, deregulation of coactivators is associated with a wide range of human diseases. We will take advantage of our experience in developing SI-2 to develop SMIs for other coactivators to improve human health.

Materials and Methods

The synthetic procedures and characterizations of SI-1 to SI-8 and experimental procedures for SI-2 pharmacokinetics, cell culture assays, immunohistochemistry, and in vivo breast cancer models are described in Supporting Information. See Figs. S6 and S7 for the synthetic procedures of SI-1 and SI-2, respectively. All of the animal studies were conducted with the approval of the Institutional Animal Care and Use Committee at Baylor College of Medicine.

ACKNOWLEDGMENTS

This work is supported in part by National Institutes of Health Grants R01GM115622 (to J.W.), HD076596 (to D.M.L.), DK059820 (to B.W.O.), and R01CA112403 (to J.X.), Cancer Prevention and Research Institute of Texas Grants R1104 (to J.W.), RP100348 and RP101251 (to B.W.O.), and RP1027032-PS (to J.X.); Welch Foundation Grant Q-1179 (to J.W.); Susan G. Komen Foundation Grant PG12221410 (to B.W.O.); the Clayton Foundation and the Dunn Foundation (B.W.O.); the Integrative Molecular and Biomedical Sciences (IMBS) Program (NIIH S1 T32 GM008231 to M.Z.); the Center for Comparative Medicine, the Cytometry and Cell Sorting Core, the Center for Drug Discovery, and the Dan L. Duncan Cancer Center at Baylor College of Medicine; and the Texas Medical Center Digestive Disease Institute.

1. Wells JA, McClendon CL (2007) Reaching for high-hanging fruit in drug discovery at protein-protein interfaces. Nature 450(7172):1001–1009. 2. Arkin MR, Wells JA (2004) Small-molecule inhibitors of protein-protein interactions: Progressing towards the dream. Nat Rev Drug Discov 3(4):301–317. 3. Lo Conte L, Chothia C, Janin J (1999) The atomic structure of protein-protein recognition sites. J Mol Biol 285(5):2177–2198. 4. Sheng C, Dong G, Miao Z, Zhang W, Wang W (2015) State-of-the-art strategies for targeting protein-protein interactions by small-molecule inhibitors. Chem Soc Rev 44(22):8238–8259. 5. Clarkson T, Wells JA (1995) A hot spot of binding energy in a hormone-receptor interface. Science 267(5193):383–386. 6. Johnson AB, O’Malley BW (2012) Nuclear receptor coactivators: Modulators of pathology and therapeutic targets. Nat Rev Endocrinol 8(10):588–604. 7. Lonard DM, O’Malley BW (2008) SRC-3 transcription-coupled activation, degradation, and the ubiquitin clock: is there enough coactivator to go around in cells? Mol Cell 1(13):pa16. 8. Lonard DM, O’Malley BW (2006) The expanding cosmos of nuclear receptor coactivators. Cell 125(3):411–414. 9. Pardoll DG, Mani S (2013) Pregnancy xenobiotic receptor in cancer pathogenesis and therapeutic response. Cancer Lett 328(1):1–9. 10. Ohtate SA, Tsai SY, Tsai MJ, O’Malley BW (1995) Sequence and characterization of a coactivator for the steroid hormone receptor superfamily. Science 270(5240):1354–1357. 11. Chang CY, et al. (1999) Dissection of the LXXLL nuclear receptor-coactivator interaction motif using combinatorial peptide libraries: Discovery of peptide antagonists of estrogen receptors alpha and beta. Mol Cell Biol 19(12):8226–8239. 12. Norris JD, et al. (1999) Peptide antagonists of the human estrogen receptor. Science 285(5428):744–746. 13. Arnold LA, et al. (2006) Discovery of small molecule inhibitors of the interaction of the thyroid hormone receptor with transcriptional coregulators. J Biol Chem 280(52):43048–43055. 14. Yan F, et al. (2014) Identification of verrucarin as a potent and selective steroid receptor coactivator 3 small molecule inhibitor. PLoS One 9(4):e95243. 15. Wang Y, et al. (2014) Bufalin is a potent small-molecule inhibitor of the steroid receptor coactivator 3- SRC-3 and SRC-1. J Biol Chem 289(52):35797–35802. 16. Neuwman RA, Yang P, Pavlus AD, Block KI (2008) Cardiac glycosides as novel cancer therapeutic agents. Mol Interv 8(1):36–49. 17. Prassas I, Diamandis EP (2008) Novel therapeutic applications of cardiac glycosides. Nat Rev Drug Discov 7(11):926–935. 18. Mijatovic T, et al. (2006) The cardenolide UNBS1450 is able to deactivate nuclear potassium (K+)-ATPase) with bound potassium and ouabain. Proc Natl Acad Sci USA 103(33):13742–13747.

27. Manna SK, Sah NK, Newman RA, Cisneros A, Aggarwal BB (2000) Oleandrin suppresses activation of nuclear transcription factor-kappaB, activator protein-1, and c-Jun NH2-terminal kinase. Cancer Res 60(14):3838–3847.

28. Song S, et al. (2000) Cardiac glycosides induce Apo2L/TRAIL-induced apoptosis in non-small cell lung cancer cells by up-regulation of death receptors 4 and 5. Cancer Res 66(11):5867–5874.

29. Song X, et al. (2015) Steroid receptor coactivator-3 (SRC-3/AIB1) as a novel therapeutic target in triple negative breast cancer and its inhibition with a phospho-bufalin prodrug. Proc Natl Acad Sci U S A 108(16):10400–104011.

30. Lonard DM, Nawaz Z, Smith CL, O’Malley BW (2000) The 26S proteasome is required for estrogen receptor-alpha and coactivator turnover and for efficient estrogen receptor-alpha transactivation. Mol Cell 5(6):939–948.

31. Lipinski CA, Lombardo F, Dominy BW, Feeney P (2001) Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. Adv Drug Deliv Rev 46(1):3–26.

32. Veber DF, et al. (2002) Molecular properties that influence the oral bioavailability of drug candidates. J Med Chem 45(12):2615–2623.

33. Hann MM, Operea TI (2004) Pursuing the leadlikeness concept in pharmaceutical research. Curr Opin Chem Biol 8(3):255–263.

34. Ertl P, Rohde B, Selzer P (2000) Fast calculation of molecular polar surface area as a sum of fragment-based contributions and its application to the prediction of drug transport properties. J Med Chem 43(20):3714–3717.

35. Wu RC, Feng G, Lonard DM, O’Malley BW (2007) SRC-3 coactivator functional lifetime is regulated by a phospho-dependent ubiquitin time clock. Cell 129(6):1125–1140.

36. Xu J, Wu RC, O’Malley BW (2009) Normal and cancer-related functions of the p160 steroid receptor co-activator (SRC) family. Nat Rev Cancer 9(9):615–630.

37. Tien JCY, Xu J (2012) Steroid receptor coactivator-3 as a potential molecular target for cancer therapy. Expert Opin Ther Targets 16(11):1085–1096.

38. Qin L, et al. (2008) The AI18 oncoregome promotes breast cancer metastasis by activation of PEA3-mediated matrix metalloproteinase 2 (MMP2) and MMP9 expression. J Biol Chem 283(19):12537–12542.

39. Zhou PZ, Babcock J, Liu Q, Li M, Gao ZB (2011) Activation of human ether-a-go-go related gene (HERG) potassium channels by small molecules. Acta Pharmacol Sin 32(6):781–788.

40. Bovill BY, Perri R, Zhang H, Dunlop J (2008) HERG (KCNH2 or Kv11.1) K+ channels: Therapeutic approaches for cardiac arrhythmias. Prog Drug Metab 29(9):965–970.

41. Kerns E, Di L (2010) Drug-Like Properties: Concepts, Structure Design and Methods: From ADME to Toxicity Optimization (Academic, Burlington, MA).

42. Marques MR, Loebenberg R, Almukainzi M (2011) Simulated biological fluids with possible application in dissolution testing. Dissolut Technol 18(3):15–28.

43. Jordan VC (2003) Tamosifen: A most unlikely pioneering medicine. Nat Rev Drug Discov 2(3):205–213.

44. Jordan VC (2004) Selective estrogen receptor modulation: Concept and consequences in cancer. Cancer Cell 5(3):207–213.

45. Xu J, Li Q (2003) Review of the in vivo functions of the p160 steroid receptor co-activator family. Mol Endocrinol 17(9):1681–1692.

46. Hofmann J, Heinisch G, Easmon J, Purstinger G, Fiebig H-h (2003) Heterocyclic hydrazones for use as anti-cancer agents. US Patent 20,030,166,658.

47. Zhang Y, Huo M, Zhou J, Xie S (2010) PKSolver: An add-in program for pharmaco Kinetic and pharmacodynamic data analysis in Microsoft Excel. Comput Methods Programs Biomed 99(3):306–314.

48. Minn AJ, et al. (2005) Genes that mediate breast cancer metastasis to lung. Nature 436(7050):518–524.

49. Fu T, et al. (2012) Aberrantly elevated microRNA-34a in obesity attenuates hepatic responses to FGFl9 by targeting a membrane co-receptor β-Klotho. Proc Natl Acad Sci USA 109(40):16137–16142.

50. Dorn A, et al. (2005) Evaluation of a high-throughput fluorescence assay method for HERG potassium channel inhibition. J Biomat Screen 10(4):339–347.

Song et al.