Bufalin Is a Potent Small-Molecule Inhibitor of the Steroid Receptor Coactivators SRC-3 and SRC-1

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Abstract

Virtually all transcription factors partner with coactivators that recruit chromatin remodeling factors and interact with the basal transcription machinery. Coactivators have been implicated in cancer cell proliferation, invasion, and metastasis, including the p160 steroid receptor coactivator (SRC) family composed of SRC-1 (NCOA1), SRC-2 (TIF2/GRIP1/NCOA2), and SRC-3 (AIB1/ACTR/NCOA3). Given their broad involvement in many cancers, they represent candidate molecular targets for new chemotherapeutics. Here, we report on the results of a high-throughput screening effort that identified the cardiac glycoside bufalin as a potent small-molecule inhibitor for SRC-3 and SRC-1. Bufalin strongly promoted SRC-3 protein degradation and was able to block cancer cell growth at nanomolar concentrations. When incorporated into a nanoparticle delivery system, bufalin was able to reduce tumor growth in a mouse xenograft model of breast cancer. Our work identifies bufalin as a potentially broad-spectrum small-molecule inhibitor for cancer. Cancer Res; 74(5): 1–12. ©2014 AACR.

Introduction

The steroid receptor coactivator (SRC) family comprises three members, SRC-1 (NCOA1; ref. 1), SRC-2 (NCOA2; refs. 2, 3), and SRC-3 (NCOA3; refs. 4–7). Numerous studies have established broad molecular and physiologic roles for the SRC family in activating nuclear receptor–mediated gene expression (8). Besides nuclear receptors, they also coactivate other transcriptional factors, including NF-kB (NFkB1), E2F1, and insulin-like growth factor I (IGF-I)–dependent transcriptional factors (9–11). The expansive roles that they play in mediating gene expression is consistent with a breadth of studies pointing to their regulation of diverse physiologic and pathophysiologic processes, including cancers in which they are frequently overexpressed (8).

Among the SRcs, SRC-3 has been the family member most prominently linked to a wide variety of cancers. SRC-3 overexpression in the mouse mammary gland leads to spontaneous tumor formation (12). In contrast, loss of SRC-3 suppressed oncogene- and carcinogen-induced breast cancer initiation, progression, and metastasis in mouse tumor models (13, 14). In humans, SRC-3 gene amplification has been found in 9.5% of breast cancers and the mRNA for SRC-3 is overexpressed 64% of the time (4). Clinical and preclinical studies have shown that overexpression of SRC-3 and SRC-1 is linked to resistance to endocrine therapies in breast cancers. For instance, high expression of SRC-3, especially conjunction with high levels of EGF receptor (EGFR) and HER2 (ERBB2), is associated with poor outcome and recurrence after tamoxifen treatment (15). In ERBB2-overexpressing breast cancer cells, overexpression of SRC-3 also contributes to resistance against the ERBB2-targeting drug trastuzumab (Herceptin; ref. 16). SRC-3 overexpression has also been observed in a wide range of other cancers, including ovarian (17), endometrial (18), prostate (19, 20), liver (21), pancreatic (22), colorectal (23), and lung cancers (24).

SRC-1 has also been clearly implicated in cancer progression. In a mouse mammary tumor virus polyoma middle T (MMTV-PyMT) model system, loss of SRC-1 markedly reduces tumor cell metastasis (25). Consistent with this, elevated SRC-1 expression has been reported in approximately 20% of primary human breast cancers, with higher expression positively correlating with disease recurrence and poor survival (26, 27). A positive association between high SRC-1 expression and tumor recurrence in patients with breast cancer who received tamoxifen therapy has also been reported (26, 27).

Considering the ability of SRC coactivators to activate multiple growth factor signaling pathways that drive cancer cell growth and promote resistance to endocrine therapy, SRC small-molecule inhibitors (SMI) are anticipated to be effective new agents to treat cancer. In an initial “proof-of-principle” study, we recently showed that gossypol is able to inhibit SRC-3 and SRC-1 (28), demonstrating that these oncogenic coactivators are a class of accessible targets for SMI-based chemotherapy. This work established the feasibility for engaging in a high-throughput compound library screen to identify more
effective SRC SMIs. As a result of these high-throughput screens, here we describe the characterization of the cardiac glycoside bufalin as potent SRC SMI that is able to effectively reduce SRC-3 and SRC-1 cellular protein concentration and block cancer cell growth in cell culture and animal models.

Materials and Methods

Chemicals

For high-throughput screening, an MLSMR library was provided by Evotec through the Roadmap Molecular Libraries Initiative of the NIH. Details about compound selection for this library can be found online (29). Digoxin, bufalin, ouabain, and digitoxin were obtained from Sigma and dissolved in ethanol. Cinobufagin, cinobufotalin, cycloheximide, and MG132 were obtained from Sigma and dissolved in dimethyl sulfoxide (DMSO). Strophanthidin and resibufogenin were purchased from Santa Cruz Biotechnology and dissolved in ethanol and DMSO, respectively. MK-2206 was purchased from Selleck-Chem and dissolved in DMSO. Antibodies to SRC-1, SRC-3, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were purchased from Cell Signaling Technology. Antibodies to CARM1 and SRC-2 were obtained from Bethyl Laboratories.

Cell culture

Human cancer cell lines were obtained from the American Type Culture Collection (ATCC). Cell lines were maintained in Dulbecco’s Modified Eagle Medium (DMEM; HeLa; MCF-7) and RPMI-1640 (A549) supplemented with 10% fetal calf serum (FCS), penicillin, and streptomycin (100 U/mL), unless otherwise indicated. All cells were cultured at 37°C under 5% CO2. All cells were used within 6 months from the time when they were obtained from ATCC, expanded, and resuscitated except for MDA-MB-231-LM3.3, which were verified to be derived from MB-MBA-231 cells by short tandem repeat DNA profiling before use in xenograft animal model experiments.

Plasmids and transfections

The expression vectors for the GAL4-responsive luciferase reporter pG5-LUC and Gal4 DBD fusion proteins with SRC-1, SRC-2, and SRC-3 were described previously (30). Twenty-four
hours before transfection, HeLa cells were plated in 24-well dishes. Cells were transfected with the indicated expression vector plasmids using Lipofectamine LTX reagent (Invitrogen) according to the manufacturer’s protocol before incubation with chemicals at the indicated concentrations.

**Cell extraction and assays**

For luciferase assays, collected cell pellets after treatments were lysed and assayed for luciferase activity. Luciferase activities were normalized against *Renilla* luciferase activities according to the manufacturer’s recommendations (Promega). For Western blotting, cells were harvested and lysed in lysis buffer (50 mmol/L Tris, pH 7.5, 150 mmol/L NaCl, 0.5% NP-40) and then centrifuged for 15 minutes at 21,000 × g at 4°C. After the total cellular protein concentration was determined by Bradford analysis (Bio-Rad), protein lysates were resolved by SDS-7.5% PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad). Membranes were blocked and incubated with indicated antibodies as previously described (28). All experiments were repeated at least three times. Intensities of the bands of interest in the Western blot analysis were quantitated using ImageJ software (31).

**1,536-well plate SRC-1 and SRC-3 HTS assays**

A detailed protocol for the high throughput screening (HTS) assay can be found on the PubChem Bioassay website (32).

**Quantitative PCR analysis**

MCF-7 cell total RNAs were isolated from 12-well culture dishes using the RNeasy Mini Kit (Qiagen). The mRNAs for SRC-1, SRC-2, SRC-3, and GAPDH were quantitated by TaqMan-based reverse transcriptase PCR (RT-PCR) using the ABI Prism 7700 sequence detection system (Applied Biosystems). For SRC-1, the primer pair 5'-gcaaccagctctcatccact-3' and 5'-

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**Figure 2.** A panel of cardiac glycosides can reduce cellular SRC-3 protein concentrations in MCF-7 breast cancer cells. Cells were treated with plant-derived cardiac glycosides (ouabain, digitoxin, digoxin, and strophanthidin) and Asiatic toad-derived cardiac glycosides (bufalin, cinobufagin, cinobufotalin, and resibufogenin) at increasing doses for 24 hours, then SRC-3 protein levels were determined by Western blot analysis.
gacctcagcaaacacctga-3' was used along with Universal Roche Probe #3 (Roche). For SRC-2, the primer pair 5'-aggcaacctgttcccaaac-3' and 5'-actggcttcagcagtgtcag-3' was used along with Universal Roche Probe #27. For SRC-3, the primer pair 5'-agctgagctgcgaggaaa-3' and 5'-gagtccaccatccagcaagt-3' was used with Universal Roche Probe #70. For GAPDH, the primer pair 5'-agccacatcgctcagacac-3' and 5'-gcccaatacgaccaaatcc-3' was used along with Universal Roche Probe #60. RT-PCR was performed using 1/C2 TaqMan Universal PCR Master Mix (Applied Biosystems). All mRNA quantities were normalized against GAPDH RNA and experiments were repeated two times.

**Fluorescence spectrometry**

The glutathione S-transferase (GST) fusion proteins of different portions of SRC-3 were expressed and purified as described previously (33). Fluorescence spectrometric measurements were performed using an SLM 48000S fluorescence spectrophotometer (SLM-Aminco) and an Agilent Cary Eclipse Fluorescence spectrophotometer (Agilent Technologies, Inc.). A total of 1.5 μmol/L of GST SRC-3 receptor-interacting domain (RID), CBP-interacting domain (CID), or basic helix-loop–helix (bHLH) was placed in a fluorescence cuvette and excited with UV light at a wavelength of 278 nm with a 2-nm bandwidth, and the emission spectra were recorded from 295 nm to more than 400 nm with a bandwidth of 4 nm. The aliquot size of test compound was maintained below 5% of the total sample volume to minimize the effects of dilution.

**Cell viability assays**

Cells were seeded in 96-well plates in medium supplemented with 10% FCS and allowed to reach 70% to 80% confluence, while relative numbers of viable cells were measured with the CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega) after compound treatment. IC50 values were determined using the Prism 4 software (GraphPad Software, Inc.).

**Bufalin PLGA nanoparticles**

A block copolymer of polyactic-co-glycolic acid (PLGA) and polyethylene glycol (PEG) was chosen as a nanocarrier material. PLGA–PEG was synthesized and bufalin-containing nanoparticles were produced using a nanoprecipitation method. Specifically, a mixture of bufalin and PLGA–PEG in trifluorothanol (TFE) was dripped into water under constant stirring. The formed bufalin nanoparticles were verified for proper formation by dynamic light scattering (DLS) and transmission electron micrography (TEM). The amount of drug encapsulated in nanoparticles was determined to be 1% by weight using high-performance liquid chromatography (HPLC) followed by detection of absorption at a wavelength of 210 nm.

**MDA-MB-231-LM3.3 xenograft tumor model**

Six- to 7-week-old severe combined immunodeficient mice (SCID) mice were obtained from Charles River Laboratories. Of note, 750,000 MDA-MB-231-LM3.3 cells were injected into one of the second mammary fat pads on day 1 with tumors becoming palpable in 6 days. At this time, animals were treated...
with either PBS, empty nanoparticles, or bufalin-loaded nanoparticles three times per week. Tumor length and width were measured daily, with volume estimated using the formula \((\text{length} \times \text{width} \times \text{height})/2\).

**Results**

**High-throughput screening of SRC-3 and SRC-1 inhibitors**

Through a high-throughput luciferase assay-based screen, a MLPCN chemical library containing 359,484 compounds (34) was screened to identify compounds capable of inhibiting the intrinsic transcriptional activities of SRC-3 (PubChem AID:588362) and SRC-1 (PubChem AID:588354; Fig. 1). Here, compounds were evaluated by measuring luciferase expression from cells transiently transfected with a GAL4 responsive luciferase reporter (pG5-LUC) and an expression vector for either a GAL4 DNA-binding (DBD) SRC-3 or GAL4 DBD SRC-1 fusion protein (30). Compounds that inhibited luciferase gene expression greater than 3σ over DMSO were counted as SMI hits. In this primary screen, the transfected HEK293 cells were treated with test compounds at a concentration of 3.6 μmol/L in 0.36% DMSO. Of note, 36 μmol/L gossypol was used as a positive control, which was able to elicit 100% inhibition. On the basis of a 3-σ cutoff, 620 (0.17%) compounds were able to inhibit SRC-3 and 428 (0.12%) compounds were able to inhibit SRC-1. These active

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**Figure 4.** Digoxin and bufalin selectively reduce cellular protein levels of SRC-3 and SRC-1, but not that of SRC-2. A, MCF-7 cells were treated with digoxin (0, 0.5, 1, and 2 μmol/L) for 24 hours. Cell extracts were then blotted and probed using antibodies against SRC-1, SRC-2, SRC-3, CARM1, actin, and GAPDH. B and C, A549 cells were treated with bufalin (0, 1, 2, and 5 nmol/L; B) and MCF-7 cells (C) were treated with bufalin (0, 1, 2, 3, 4, and 5 nmol/L), and analyzed as described in A. Relative intensities of bands were normalized by either GAPDH or by actin as shown.
compounds were then tested in the confirmatory screen in the same manner as the primary screen in quadruplicate and in a counter screen using cells transfected with an expression vector for a GAL4 DBD VP16 fusion protein instead of an expression vector for either GAL4 DBD SRC-3 or SRC-1 to exclude general inhibitors of transcription and/or luciferase activity.
 Bufalin promotes proteasome-mediated degradation of the SRC-3 protein. A, effects of bufalin on the mRNA levels of SRC-1, SRC-2, and SRC-3. MCF-7 cells were incubated with bufalin (0, 1, 2, and 5 nmol/L) for 24 hours and total RNA was extracted and analyzed by qPCR. Differences between the control and the treated samples were analyzed for significance using a Student t test. Only P values less than 0.05 are shown in the chart above its corresponding column. B, effects of bufalin on SRC-3 protein turnover rates. (Continued on the following page.)
Structure–activity cluster analyses identify cardiac glycosides as a functional group of SRC-3 and SRC-1 SMIs

Active compounds retrieved from the primary screens were clustered according to the structural similarities in PubChem (data not shown). The biggest cluster contains 25 compounds, sharing a common steroid nucleus, the majority of which contain a lactone moiety characteristic of cardiac glycosides (35). Cardiac glycosides are known to inhibit the Na+–K+ ATPase in cardiac myocytes, leading to an increase in intracellular Ca2+ and stronger myocardial contraction (36). Interestingly, numerous epidemiologic studies revealed that patients taking cardiac glycosides have either better outcome or lower risk for various cancers, including breast cancers (37, 38), leukemia/lymphoma (39), and prostate cancer (40), although the mechanism has hitherto been unknown.

Evaluation of cardiac glycoside family members for their efficacy as SRC SMIs

Because a cluster of cardiac glycoside series was identified as active compounds in our high-throughput screens, we sought to evaluate a panel of cardiac glycosides to identify which were most potent as SRC SMIs. We examined SRC-3 protein concentrations in MCF-7 cells treated for 24 hours with cardiac glycosides, including ouabain, digitoxin, strophanthinid, cinuebufagin, cinobufotalin, and resibufogenin. All tested compounds downregulated cellular SRC-3 protein levels, but at varying doses (Fig. 2). Ouabain and digitoxin seemed to be more effective than digoxin, while bufalin was found to be the most potent of all. In comparison, strophanthinid also reduced SRC-3 protein, but at a higher dose than digoxin. Bufalin was the most potent bufadienolide, followed (in order) by cinobufagin, cinobufotalin, and resibufogenin.

Inhibition of SRC-3 and SRC-1 by digoxin and bufalin

Next, we chose to investigate the effects of cardiac glycosides on the intrinsic transcriptional activities of SRC coactivators. HeLa cells were transiently transfected with a pGL5-LUC reporter and expression vectors for pBIND, pBIND-SRC-1, -SRC-2, or -SRC-3, followed by 24 hours of treatment with the plant-based cardiac glycoside digoxin or the toad cardiac glycoside bufalin. Digoxin reduced luciferase reporter activities in cells transfected with pBIND–SRC-1 and pBIND–SRC-3 (Fig. 3A). In contrast, reporter activities driven by the GAL4 DBD alone were only slightly affected (pBIND, inset to the right). Similarly, administration of bufalin led to a significant decrease in pBIND–SRC-1 and pBIND–SRC-3 activities in a dose-dependent manner, while only minimally affecting the activity of pBIND (GAL4 DBD alone) and influencing pBIND–SRC-2 less strongly (Fig. 3B). This result suggests that both digoxin and bufalin preferentialy inhibit the intrinsic transcriptional activities of SRC-3 and SRC-1, while inhibiting SRC-2 to a lesser extent.

Effects of digoxin and bufalin on cellular protein concentrations of SRCs and other coactivators

Because the steady-state levels of coactivator proteins have been shown to correlate with their transcriptional activities and with cancer progression (33), we sought to examine the effects of digoxin and bufalin on SRC protein levels in MCF-7 breast and A549 lung cancer cells after 24 hours of incubation. As shown in Fig. 4A–C, SRC-3 and SRC-1 protein levels were significantly reduced by both digoxin and bufalin in a dose-dependent manner, suggesting that the loss in SRC-3 and SRC-1 intrinsic activities seen above is due to the reduced cellular concentration of either coactivator. In agreement with the finding that SRC-2 activities are less strongly affected, both digoxin and bufalin have more modest effects on the cellular level of the SRC-2 protein. We then investigated the steady-state level of CARM1 that has been shown to exist in a multi-protein coactivator complex along with SRC-3 (41). In contrast to the noticeable decrease in SRC-3 protein level, CARM1 protein level is not altered after treatment in A549 cells, but was reduced moderately in MCF-7 cells.

Digoxin and bufalin inhibit cancer cell proliferation

Next, we assessed the effects of digoxin and bufalin on cancer cell proliferation. First, MTS assays were performed on MCF-7 cells treated with digoxin at different doses for 24 hours. This revealed that digoxin can block MCF-7 cell growth with IC50 of about 500 nmol/L (Fig. 5A, left), in line with the dose of digoxin required to reduce SRC-3 protein levels in the cell (Fig. 5A, right). In contrast, the IC50 of bufalin in MCF-7 and A549 cells were below 5 nmol/L in either cell lines (Fig. 5B and C, left). Again, the dose of bufalin required to block cancer cell proliferation corresponds with the concentration required to cause downregulation of the SRC-3 protein (Fig. 5B and C, right). Differential dependence of distinct cell types toward each of the three SRCs could account for the differences in bufalin effect on cell viability and SRC-3 cellular protein concentration. Importantly, the concentrations of bufalin required to kill cancer cells tested here are less than the reported human maximum-tolerated dose (MTD) of 8.75 nmol/L (42), pointing to its potential clinical use as an anti-cancer agent. For this reason, we chose to focus on bufalin for further characterization as an SRC SMI.

Accumulating evidence has shown that targeting SRC-3 expression impairs cancer cell growth in multiple cancer types (8). Given the fact that the decreased cell viability induced by bufalin 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. To do this, we used a zinc finger nuclease (ZFN) to knockout both SRC-3 alleles in the HeLa cell line (SRC-3KO cells). It was difficult to obtain viable SRC-3KO cells initially; however, we noted that after 2 months,
they proliferated at a rate comparable with wild-type (WT) cells, likely after adapting genetically to the loss of SRC-3. As shown in Fig. 5D, SRC-3 protein is abolished in these SRC-3KO knockout cells. Compared with parental SRC-3+/+ cells, the response of SRC-3KO cells to bufalin administration is blunted. This finding supports the idea that SRC-3 protein is involved in mediating the cell response to bufalin treatment. However, the remaining response of SRC-3KO cells to bufalin, including the lack of an increase in the effective concentration required for these cells to respond to treatment, is likely due to the SRC-1 and SRC-2 that continues to be expressed in these cells and that also responds to bufalin at a similar dose as SRC-3, in addition to any unknown off-target actions of the compound.

**Bufalin promotes proteasome-mediated degradation of SRC-3 and binds directly to the coactivator**

To gain insights into the mechanism of bufalin-mediated SRC-3 protein downregulation, we assessed whether 24 hours of bufalin treatment affected the production of mRNAs for each SRC family member in MCF-7 cells. Quantitative PCR (qPCR) revealed that mRNA levels for SRC-1 and SRC-2 were not significantly altered, whereas the mRNA levels for SRC-3 were actually increased upon bufalin incubation (Fig. 6A). This result suggested that bufalin reduces SRC-3 and SRC-1 protein levels posttranscriptionally.

To further investigate the underlying mechanism responsible for bufalin-mediated SRC-3 protein downregulation, we compared the turnover rates of the SRC-3 protein in MCF-7 cells between vehicle and bufalin treatment in the presence of the protein synthesis inhibitor cycloheximide. Cells were treated with cycloheximide and bufalin, individually or simultaneously, and then harvested at the indicated time points for Western blot analysis. In line with a previous report (43), SRC-3 decays with a 4-hour half-life (Fig. 6B). The addition of bufalin accelerated the rate of SRC-3 turnover, indicating that bufalin promotes degradation of the SRC-3 protein.

Our laboratory has previously shown that all SRC coactivators are targets of the proteasome, as evidenced by the observation that treatment with the proteasome inhibitor MG132 increases their protein levels (30). When we examined the effects of concomitant treatment with MG132 and bufalin ranging from 1 to 5 nmol/L on SRC-3 protein levels in MCF-7 cells (Fig. 6C), MG132 treatment alone was able to strongly elevate the SRC-3 protein levels, consistent with that previously reported. The reduction in SRC-3 protein level induced by bufalin treatment was blocked by MG132, leading to protein levels up to a point comparable with cells treated with MG132 alone.

Next, we sought to determine whether bufalin promoted SRC-3 protein degradation through physical interaction with the SRC-3 protein. Through fluorescence shift analysis of SRC-3 protein fragments, bufalin was found to quench the intrinsic fluorescence and shift the emission maximum of the RID of SRC-3 (Fig. 6D), indicating that bufalin binds directly to the RID of SRC-3. In contrast, there are no changes in the intrinsic fluorescence observed for the CID or the bHLH domain of SRC-3 upon the addition of bufalin (data not shown), which mitigates the possibility that the fluorescence changes seen for the RID fragment are due to nonspecific interactions with bufalin. Because of the high affinity of bufalin binding to the SRC-3 RID, it is difficult to precisely determine its affinity; however, binding was detected even with 5 nmol/L of bufalin, consistent with its potency in cell culture experiments.

**Bufalin sensitizes cancer cells to other targeted therapeutics**

Accumulating evidence shows that SRC coactivators sit at a nexus linking diverse growth signaling cascades (see Introduction). This role for SRCs supports the notion that an SRC SMI such as bufalin should break cross-talk between different growth factor pathways, attenuating their ability to signal to downstream pathway components responsible for enacting cellular transcriptional programs that drive cell proliferation, invasion, and metastasis. SRC-3 has been shown to activate PI3K/AKT/mTOR signaling (44), and we wanted to ask whether the inhibitory effects of an AKT inhibitor on cell viability could be enhanced by cotreatment with bufalin. To test this, we treated A549 cells with a low dose of bufalin in combination with the AKT inhibitor MK-2206. As shown in Fig. 7A, combined treatment with bufalin and MK-2206 exhibited a markedly higher ability to block A549 cell proliferation than either with agent alone.

**A bufalin nanoparticle formation can block tumor cell growth in a xenograft model**

Bufalin is an excellent candidate for incorporation into a nanoparticle-based delivery system because it can specifically kill cancer cells (IC50, 3–5 nmol/L) without observable toxicity in nontransformed TM4 Sertoli cells at doses up to 10 μmol/L (Fig. 7B) or primary hepatocytes even at doses as high as 300 nmol/L (data not shown), but it possesses significant cardio-toxicity—a fact we substantiated in our own animal studies with free bufalin (data not shown), and it has a short in vivo half-life. With “free bufalin,” we observed a response in metastatic tumor lesions using an in vivo model described below, but we were unable to achieve a satisfactory response in primary tumors, possibly due to dose-limiting toxicities or due to inadequate drug penetration into larger tumors (data not shown). Already, a wheat germ agglutinin–grafted lipid bufalin nanoparticle has been developed to allow for its oral delivery and improve its stability (45). Another formulation consists of a methoxy PEG, PLGA, poly-l-lysine, and cyclic arginine-glycine-aspartic acid carrier loaded with bufalin that has been shown to possess a favorable biodistribution profile and has antitumor activity in vivo (45). Here, we chose to test a similar and established PEG-PLGA–based nanoparticle delivery system (see Materials and Methods; ref. 46) that has already been successfully used to produce doxorubicin PEG–PLGA nanoparticles that have been designed to avoid distribution to cardiac tissue.

Fox Chase SCID Beige mice were injected with 750,000 MDA-MB-231−derived LM3.3 cells into the second mammary gland (cleared) with two sites per mouse. Six days after tumor cell injection, mice were separated into three groups and given the following treatments: (i) PBS vehicle control–treated (n = 5); (ii) bufalin nanoparticle (1.5 mg/kg)–treated (n = 10); and (iii)
blank nanoparticle-treated (n = 5). Mice were treated three times per week via intravenous injection. After this, primary tumor volume was measured to assess the ability of the bufalin nanoparticle to inhibit tumor growth (Fig. 7C). Aggressive tumor growth was observed in the PBS- and blank nanoparticle-treated control mice, whereas the bufalin nanoparticle-treated mice showed a significant inhibition in the rate of tumor growth 4 days after initial treatment that was sustained throughout the experiment.

Discussion

The SRC family has been widely implicated in carcinogenesis, providing a strong impetus to develop SRC SMIs as novel and effective therapeutic agents. Using a luminescence-based assay to assess coactivator intrinsic transcriptional activities, we conducted a high-throughput screen of an MLPCN compound library (34) to identify SRC-3 and SRC-1 SMI hits. Because of the large size of this library, we were able to gain insight into the comparative activities of many structurally related compounds, revealing cardiac glycosides as the largest class of compounds with SRC SMI activities. Our data demonstrate that both digoxin and bufalin selectively reduced intrinsic activities of SRC-3 and SRC-1, consistent with that observed in the primary screen. Marked differences in the potencies of cardiac glycosides were observed, with most inhibiting SRCs at concentrations higher than their MTD. However, bufalin was found to have better potency and inhibited SRCs at concentrations below its MTD of 8.75 nmol/L, leading to our focus on it as a potentially clinically useful SRC SMI.

For centuries, cardiac glycosides have been used to treat patients with edematous states, irregular heartbeats, or chronic heart failure, and epidemiologic evidence has shown that patients who take cardiac glycosides are at lower risk for various cancers, pointing to their potential as cancer therapeutic agents (47). Interestingly, cardiac glycosides have also been shown to inhibit the androgen receptor, but were found not to bind to the receptor itself (48).

Despite their anticancer properties, the effectiveness of cardiac glycosides is limited by their narrow therapeutic window. For example, the nontoxic plasma concentration of digoxin for cardiac disease patients is 2.6 nmol/L or less (49). In our study, the concentration of digoxin required to inhibit SRC-3/SRC-1 is greater than 200 nmol/L. In contrast, we show here that bufalin is effective at a low nanomolar range (∼3–5 nmol/L), which is within the concentration range observed in patient plasma in which no cardiac toxicity was observed (∼9 nmol/L; ref. 42). Given the enhanced effectiveness when combined with the AKT inhibitor MK-2206, bufalin's ability to block tumor growth might be achievable at even lower doses. Another approach we pursued to avoid the dose-limiting toxicity associated with bufalin toward the heart was to deliver bufalin in a PEG–PLGA delivery particle, in which we were able to effectively block breast tumor growth in vivo. Bufalin has already been tested in several small clinical trials (42) and the data presented here that characterize it as an SRC SMI provide new insight into its mechanism of action. Results presented here demonstrating that SRC-3 and SRC-1 are targets of bufalin promise to more rationally guide its future use as a novel therapeutic agent.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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