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Identification of Niclosamide as a Novel Anticancer Agent for Adrenocortical Carcinoma

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Abstract

Purpose—Adrenocortical carcinoma (ACC) is a rare and aggressive cancer, and no current effective therapy is available for locally advanced and metastatic ACC. Drug repurposing is an emerging approach for identifying new indications for existing drugs, especially for rare cancers such as ACC. The objective of this study was to use quantitative high-throughput screening to identify agents with antineoplastic activity against ACC.

Experimental Design—A screening of 4,292 compounds was performed on three ACC cell lines: BD140A, SW-13, and NCI-H295R.

Results—Twenty-one active compounds were identified, with an efficacy of >80% in all three cell lines. Of these, niclosamide showed higher efficacy and lower IC_{50} than established anti-ACC drugs. We then validated niclosamide-inhibited cellular proliferation in all three ACC cell lines. Next, we investigated the mechanism by which niclosamide inhibited ACC cell proliferation, and found that it induced caspase-dependent apoptosis and G1 cell cycle arrest. Niclosamide also decreased cellular migration and reduced the level of mediators of epithelial-to-mesenchymal transition, such as N-cadherin and vimentin. Furthermore, niclosamide treatment resulted in decreased expression of β-catenin. We also evaluated the effect of niclosamide on energy metabolism in ACC cell lines and found it resulted in mitochondrial uncoupling. Niclosamide treatment inhibited ACC tumor growth with no observed toxicity in mice *in vivo*.

Conclusions—Our findings suggest that niclosamide has anti-ACC activity through its inhibition of multiple altered cellular pathways and cellular metabolism in ACC. Our results provide a preclinical rationale for evaluating niclosamide therapy in a clinical trial for ACC.

Keywords

adrenocortical carcinoma; quantitative high-throughput screening; niclosamide; β-catenin; mitochondrial uncoupler

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Introduction

Adrenocortical carcinoma (ACC) is a highly aggressive endocrine cancer, with an incidence of one to two cases per million in the general population (1, 2). Our knowledge of the molecular pathogenesis of ACC has improved, and alterations in CTNNB1, IGF-2, and TP53 are common in ACC cases and are associated with ACC prognosis (3). With a five-year overall survival rate ranging from 16% to 38%, the prognosis of patients with locally advanced and/or metastatic ACC is dismal (4–6). Surgical resection is the only available curative treatment, yet 60–80% of patients who undergo complete resection experience a recurrence (7). Mitotane and systemic chemotherapy with etoposide, doxorubicin, and cisplatin is commonly used for patients with advanced or unresectable disease. Unfortunately, the available agents provide little clinical benefit for patients, leaving them with few treatment options. Given the limited success and significantly high toxicity of current drug regimens, there is an urgent need for new therapeutic options for patients with locally advanced and unresectable ACC.

The traditional drug-development process is costly and time consuming, with a high failure rate. It is estimated that it requires approximately $1 billion and 10 years to bring a drug to market (8). Drug repositioning or repurposing is an emerging field in which new applications are found for existing drugs. Drug repositioning has an advantage over *de novo* drug discovery because many drugs already have known pharmacokinetics, pharmacodynamics, and toxicity profiles, and this knowledge hastens the evaluation of the drug in clinical trials. For rare cancers such as ACC, drug repositioning can play an essential role in a disease that would otherwise be neglected due to high costs. Furthermore, drug repurposing may uncover new molecular pathways involved in carcinogenesis or reveal new molecular targets for therapy.

In this study, we performed quantitative high-throughput screening (qHTS) of 4,292 clinically approved compounds in three ACC cell lines. We identified 21 compounds that were active in all cell lines. One of the most potent compounds was niclosamide, an antihelminthic drug approved for human use for over 50 years. We demonstrated that niclosamide inhibits ACC cellular proliferation, induces caspase-dependent apoptosis and G_1 cell cycle arrest, and decreases ACC cellular migration. More importantly, niclosamide treatment dramatically inhibited ACC tumor growth *in vivo* with no observed side effects or toxicity in the mice. Our findings suggest that niclosamide is a promising new agent for the treatment of ACC.

Materials and Methods

Cell culture

NCI-H295R and SW-13 cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 2.5% NuSerum (BD Biosciences, San Jose, CA) and 0.1% ITS premix (BD Biosciences, San Jose, CA). BD140A cells, kindly provided by Drs. Kimberly Bussey and Michael Demeure (Phoenix, AZ), were cultured in RPMI supplemented with 1% L-glutamate (Gibco, Grand Island, NY), 1% penicillin-streptomycin (Gibco, Grand Island,

NY), and 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA). The cell lines were authenticated using short tandem repeat profiling. Cells were maintained in a 5% CO_2 atmosphere at 37°C.

qHTS screening

The National Institutes of Health Chemical Genomic Center pharmaceutical library consists of 4,292 small molecules and compounds. Compounds were prepared as described previously (9). The cell viability of treated cells was measured using the luciferase-coupled ATP quantitation assay, Cell Titer-Glo (Promega, Madison, WI). Doxorubicin hydrochloride and tetraoctylammonium bromide were used as positive controls. The final concentration of compounds in the assay ranged from 0.6 nM to 46 μM. The titration-response data was plotted and modeled by a four-parameter logistic fit-to-yield half-maximal inhibitory concentration (IC_{50}) and efficacy (maximal response), as compared to tetraoctylammonium bromide values.

Reagents

The following antibodies were used: anti-beta catenin (1:1,000) from R&D Systems (Minneapolis, MN); anti-GAPDH (1:3,000) from Santa Cruz Biotechnology (Dallas, TX); anti-N-cadherin (1:4,000) from EMD Millipore (Billerica, MA); and anti-vimentin (1:5,000) from Cell Signaling Technology (Boston, MA). Niclosamide (2′,5-dichloro-4′-nitrosalicylanilide) was purchased from Sigma-Aldrich (St. Louis, MO) and dissolved in DMSO for *in vitro* studies.

Cellular proliferation assay

3×10^3 and 6×10^3 cells were plated in 96-well plates depending on the cell line. 100 μL of fresh culture medium containing the drug or vehicle was added. Cell count was determined using the CyQuant kit (Life Technologies, Grand Island, NY), according to the manufacturer's instructions, and cell number was measured using a SpectraMax M5 microplate reader (ex485/em538; Molecular Devices, Sunnyvale, CA). Assays were performed in quadruplicate and the experiments were repeated three times.

NCI-H295R and SW-13 cells, which form multicellular aggregates (MCA) or spheroids, were plated in Ultra Low Cluster 24-well plates (Costar, Corning, NY) at 1×10^5 cells/0.5 mL or 6×10^4 cells/0.5 mL depending on the cell line. Spheroids were allowed to develop for one or two weeks at 37°C in 5% CO_2, and media was exchanged twice a week. Spheroids were treated with niclosamide or the vehicle at varying concentrations, and imaged weekly.

Caspase 3/7 activity assay

Cells were plated in 96-well plates and treated with niclosamide or the vehicle. Caspase 3/7 activity was measured using the Caspase-Glo 3/7 assay (Promega, Madison, WI), according to manufacturer's instructions.

Cell cycle analysis

Cells plated in six-well plates were treated with niclosamide or the vehicle. At 48 hours, cells were fixed for 30 minutes in 70% ethanol at 4°C, and stained with 50 μg/mL of propidium iodide containing 100 mg/mL of ribonuclease A. Flow cytometry was performed on a Canto I flow cytometer (Becton-Dickinson, Franklin Lakes, NJ) using CellQuest software (BD Biosciences, San Jose, CA). Data was generated for at least 20,000 events per sample and analyzed using Modfit software (Verity Software House, Inc., Topsham, ME).

Cellular migration assays

NCI-H295R and SW-13 cells were plated in six-well plates and treated with varying concentrations of niclosamide or the vehicle for 24 hours. Cells were trypsinized and plated in transwell chambers (BD Biosciences, San Jose, CA) at a density of 1×10^5 cells per 0.5 mL. The lower chamber was filled with DMEM supplemented with 10% FBS as a chemoattractant. Cells were allowed to migrate for 24 hours or 48 hours depending on the cell line, and were fixed and stained with Diff-Quik (Dade Behring, Newark, NJ). Cells were imaged and counted in three random fields per well, and the experiments performed in triplicate. For the wound-healing assay in BD140A cells, which do not migrate in the Boyden chamber model, cells were plated in six-well plates until confluent and treated with niclosamide or the vehicle. The cells were scratched using a sterile pipette tip and photographed at various time points.

Western blot analysis

Cell lysates were analyzed by SDS-PAGE and transferred to a PVDF membrane. The membranes were incubated with the appropriate primary antibodies overnight at 4°C, followed by horseradish peroxidase conjugated IgG (anti-rabbit 1:3,000, Cell Signaling Technology; anti-mouse 1:3,000, Santa Cruz Biotechnology; anti-goat 1:2,000, R&D Systems, Inc.). Proteins were detected by enhanced chemiluminescence (ECL; Thermoscientific, Rockford, IL).

Mitochondrial metabolism assays

The Seahorse XF96 assay was performed according to the manufacturer's instructions (Seahorse Bioscience, Billerica, MA). Briefly, cells were plated in XF96 plates and media was replaced with unbuffered DMEM supplemented with 2 mmol of glutamine and 2.5 g/L of D-Glucose. After 1 hour in a 37°C CO_2-free incubator, the oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were measured before and after injection of the drug, the vehicle, or carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) using the Seahorse XF96 extracellular flux analyzer.

Cells in 96-well plates were treated with the drug or vehicle. Cells were then stained with 200 nM of tetramethylrhodamine, ethyl ester (TMRE) for 20 minutes at 37°C and were washed with PBS; fluorescence was measured using a SpectraMax M5e 96-well fluorescence microplate reader. FCCP was used as a positive control.

In vivo mouse studies

Animal studies were approved by the National Cancer Institute Animal Care and Use Committee. Mice were maintained according to National Institutes of Health (NIH) Animal Research Advisory Committee (ARAC) guidelines. 5×10^6 NCI-H295R cells were injected into the flank of Nu^+/Nu^+ mice. Tumors were allowed to grow and mice were randomized into three treatment groups (8 mice per treatment group). Mice were treated with 100 mg/kg of niclosamide, 200 mg/kg of niclosamide, or the vehicle (PEG500) everyday by oral gavage. Tumor sizes were measured in two dimensions every week with calipers and recorded.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 6 software (GraphPad Software, La Jolla, CA). Data were analyzed using a two-tailed t-test or Mann-Whitney test. Statistical significance was defined as a *P*-value of less than 0.05. Data are presented as mean ± standard deviation (SD) or mean ± standard error of the mean (SEM).

Results

qHTS identifies niclosamide as an active agent against ACC cell lines

qHTS was performed in order to identify compounds with anti-ACC activity in BD140A, SW-13, and NCI-H295R. A total of 21 compounds were found to be pan-active in all cell lines, with an efficacy >80% (Supplemental Table 1). One of the highly active agents identified was niclosamide, which had an IC_{50} of 0.12 μM, 0.15 μM, and 0.53 μM in BD140A, SW-13, and NCI-H295R, respectively; these values are well below the known C_{max} in humans of 18.34 μM. A comparison of niclosamide to commonly used drugs for ACC showed that niclosamide had better activity (lower IC_{50} and higher efficacy) compared to cisplatin, doxorubicin, etoposide, mitotane, and streptozocin (Fig. 1).

Niclosamide inhibits cellular proliferation and induces caspase-dependent apoptosis

The antiproliferative effects of niclosamide were validated in the three ACC cell lines. We first evaluated the effect of niclosamide in a monolayer cell culture. Niclosamide inhibited proliferation in a time- and dose-dependent manner (Fig. 2A). Also, at higher concentrations, niclosamide treatment was cytotoxic, killing pre-existing cancer cells (Fig. 2A). We then assessed the antiproliferative activity of niclosamide in the cell lines (SW-13 and NCI-H295R) that form MCAs. After three and four weeks of treatment of SW-13 and NCI-H295R, respectively, we observed growth inhibition and disintegration of the MCAs (Fig. 2B).

To further elucidate the mechanism by which niclosamide inhibited cellular proliferation and caused cell death, we determined the effect on cell cycle progression and apoptosis. Niclosamide treatment of NCI-H295R and SW-13 cell lines increased caspase 3/7 activity at 48 hours and 96 hours, respectively (Fig. 2C). However, no increase in caspase 3/7 activity was observed in BD140A cells. In contrast, niclosamide treatment induced G_1 cell cycle arrest, with an observed increase in the number of cells in the G_1 phase and a decrease in the number of cells in the S phase in all three ACC cell lines (Fig. 2D).

Niclosamide results in mitochondrial uncoupling

Niclosamide's antiparasitic mechanism of action has been reported to be due to the uncoupling of oxidative phosphorylation (10). To determine whether niclosamide has an uncoupling effect in ACC cell lines, we measured the OCR and ECAR using the Seahorse XF96 analyzer. The OCR and ECAR increased with niclosamide treatment in all three cell lines, which is consistent with an uncoupling of the electron transport chain from ATP synthesis and the subsequent metabolic shift to glycolysis for energy production (Fig. 3A). Staining of niclosamide-treated ACC cells with TMRE confirmed a decrease in mitochondrial membrane potential after 3 hours and 6 hours of niclosamide treatment (Fig. 3B).

Niclosamide reduces the expression of β-catenin, and decreases cellular migration and mediators of epithelial-to-mesenchymal transition

We next investigated whether niclosamide had any effect on β-catenin, an important pathway altered in over 30% of ACC cases. Niclosamide reduced the expression of β-catenin in all three cell lines (Fig. 4A).

Since WNT/β-catenin signaling has been well established as playing an important role in epithelial-to-mesenchymal transition (EMT), we explored whether niclosamide treatment reduced cellular migration. We found reduced cellular migration with niclosamide treatment in transwell migration assays (Fig. 4B). Niclosamide's effect on migration in BD140A cells was assessed through a wound-heal assay because BD140A cells do not migrate in the Boyden chamber model. We observed that niclosamide treatment decreased BD140A cell migration after 6 hours and 12 hours of treatment, as compared to the vehicle control (Fig. 4C). Given the observed effect of niclosamide on migration, we investigated whether niclosamide altered the level of EMT mediators. Niclosamide reduced the expression of N-cadherin and vimentin (Fig. 4D).

Niclosamide inhibits ACC tumor growth *in vivo*

To confirm our *in vitro* observations, the effect of niclosamide treatment was evaluated in ACC xenografts. Niclosamide treatments, at both doses (100 mg/kg and 200 mg/kg), were well tolerated, with no observed toxicity or side effects in the mice. There were no significant weight differences among the groups (Fig. 5). Four weeks after treatment, mice treated with niclosamide at 100 mg/kg and 200 gm/kg showed a 60% and 80% inhibition in tumor growth, respectively, as compared to the vehicle control group ($P < 0.01$ for both groups) (Fig. 5). The same treatment schedule was maintained for 8 weeks, at which time, more than 90% tumor growth inhibition was observed for the two treated groups, as compared to the control group.

Discussion

In this study, we demonstrated an effective strategy for identifying novel antineoplastic agents for ACC using qHTS. A large pharmaceutical library containing 4,292 compounds was screened for drug activity in multiple ACC cell lines. We identified 21 active compounds with an efficacy >80% in all cell lines and validated the antiproliferative activity

of niclosamide *in vitro* and *in vivo*. Mechanistically, we found that niclosamide induces caspase-dependent apoptosis and G_1 cell cycle arrest, and decreases cellular migration. Furthermore, we determined that niclosamide is a potent mitochondrial uncoupler that inhibits important cellular pathways involved in ACC. Most importantly, our *in vivo* experiment showed that niclosamide treatment greatly inhibited ACC xenograft tumor growth with no observed side effects or toxicity in mice, suggesting that niclosamide should be evaluated in the clinic in ACC patients who do not respond standard therapies.

Niclosamide is an antihelminthic agent that has been approved by the United States Food and Drug Administration for the treatment of tapeworm infections in humans, and it has been in use for the past 50 years (10). This agent has a good safety profile and exhibits little toxicity even after long-term exposure (10). Niclosamide inhibits oxidative phosphorylation in the mitochondria of cestodes, and this mechanism, which has been exploited to alter metabolism in mice, may serve as a potential treatment for Type 2 diabetes (11). In addition, niclosamide has been found to have antineoplastic activity in various cancers by inhibiting multiple cellular pathways known to play important roles in carcinogenesis, including WNT/β-catenin, notch, mTOR, NF-kB, and STAT3 (12–20). Our screening revealed that niclosamide has potent anticancer activity against multiple ACC cell lines, with an IC_{50} that is well below the C_{max} in both mice and humans. Niclosamide has low water solubility and oral bioavailability. These factors may result in a wide range of serum concentrations of niclosamide, which can result in variable anti-cancer efficacy. When compared to current drug treatments for ACC, niclosamide was found to have better activity than cisplatin, doxorubicin, etoposide, mitotane, or streptozocin, suggesting that it may be a viable novel agent that could be translated into clinical therapy for ACC patients who do not respond standard therapeutic regimens.

Validation of the screening results confirmed that niclosamide inhibits cellular proliferation in a time- and dose-dependent manner in both monolayer cell culture and MCA, and that it induces cell death at high doses. MCA has been widely considered to better reflect *in vivo* tumor growth due to similar volume of growth kinetics, proliferation gradients, and extracellular matrix production that supports cancer cell growth (21). We further characterized the mechanism by which niclosamide inhibits cell proliferation, demonstrating that it induces G_1 cell cycle arrest and caspase-dependent apoptosis.

The WNT/β-catenin pathway has been shown to play an important role in ACC, with alteration of this pathway found in over 30% of ACC cases (22, 23). In canonical WNT/β-catenin signaling, β-catenin complexes with adenomatous polyposis coli, axin, and glycogen synthase kinase-3b (GSK3b) are subsequently phosphorylated and degraded. In the presence of WNT, GSK3b inhibition results in the stabilization of β-catenin, which is then translocated into the nucleus and targets the expression of genes that regulate cell growth, motility, and differentiation (24). Mutations in β-catenin have been associated with poor prognosis in patients with ACC, and higher-grade ACC is associated with higher β-catenin expression (25). Silencing of β-catenin in NCI-H295R has been shown to decrease proliferation, induce cell cycle arrest and apoptosis, reverse the EMT phenotype, and inhibit *in vivo* tumor development (26, 27). These findings suggest that the WNT/β-catenin pathway plays a major role in ACC tumorigenesis and may be an effective therapeutic target for ACC

treatment. Despite the key role of WNT/β-catenin signaling in ACC, current treatments do not effectively target this pathway. Thus, niclosamide's ability to inhibit β-catenin expression may prove to be an exciting and effective new strategy for ACC treatment, especially given the large subset of patients with ACC who have alterations in the WNT/β-catenin pathway in their tumors.

Because niclosamide reduces β-catenin levels and plays a key role in the induction of EMT, we investigated whether niclosamide affects cellular migration (24). ACC is a highly invasive cancer with a high rate of metastasis. Cancer progression is associated with the loss of epithelial properties and the gain of mesenchymal characteristics, and this process is regulated by multiple proteins that mediate EMT in cancer progression (28). Consistent with the reduced level of β-catenin, we observed decreased ACC cellular migration with niclosamide treatment. Furthermore, this decrease was associated with reduced expression of the mesenchymal markers N-cadherin and vimentin. Thus, the observed reversal of the EMT phenotype may have important implications for the *in vivo* effects of niclosamide on ACC progression.

We found that niclosamide acts as a mitochondrial uncoupler in ACC cell lines. The mitochondrial dysregulation of cancer cells has been well studied, beginning with the observation in the 1920s by Otto Warburg that cancer cells often rely on glycolysis even in the presence of an adequate oxygen supply (Warburg effect) (29). Cancer-specific changes in mitochondrial metabolism have been linked to malignant cell transformation, apoptosis evasion, the high proliferative capacity of cancer cells, and driver gene/pathway mutations (30, 31). Mitochondrial uncouplers exert their effect by dissipating the proton gradient formed by the electron transport chain, thus uncoupling it from ATP production. While under the Warburg hypothesis many cancers appear to decrease their dependence on oxidative phosphorylation for ATP production, studies have shown that the ATP produced by oxidative phosphorylation may still be necessary to initiate glycolysis through hexokinase II activation (32). In addition, uncoupling oxidative phosphorylation has been associated with shifts in cancer cell metabolism, from the oxidation of pyruvate to the oxidation of glutamine and fatty acids (33). Studies on the antineoplastic effects of other mitochondrial uncouplers have demonstrated that they have an effect on cell cycle arrest and apoptosis (34–37). Furthermore, in a study by Jin and colleagues on the effect of niclosamide in acute myelogenous leukemia, niclosamide was shown to cause mitochondrial damage, increase reactive oxygen species, and induce apoptosis through increased levels of cytochrome C (15). The altered mitochondrial function seen in cancer cells may also explain the low toxicity of niclosamide in normal cells. The mitochondria of cancer cells are often hyperpolarized compared to normal cells, which is why cationic compounds such as niclosamide will preferentially accumulate in cancer cells (38, 39). In addition, the acidic environment produced by the high glycolytic rate in tumor cells may promote the availability and activity of the drug (40).

In conclusion, the qHTS of 4,292 compounds identified niclosamide as a novel antineoplastic agent for ACC. We established that niclosamide has antiproliferative and proapoptotic activities, and induces G_1 cell cycle arrest. In addition, niclosamide reduces β-catenin expression and acts as a potent mitochondrial uncoupler. Importantly, niclosamide

greatly inhibited xenograft tumor growth *in vivo* with no toxicity in mice. Our findings suggest that niclosamide has anticancer activity through the inhibition of multiple altered cellular pathways and cellular metabolism, and, thus, is a promising new agent for the treatment of patients with ACC who do not respond to standard therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Translational Relevance

Adrenocortical carcinoma is a rare but lethal malignancy. There are no standard or effective therapies for locally advanced and metastatic adrenocortical carcinoma, and most patients with localized adrenocortical carcinoma develop recurrence even after complete surgical resection. We performed high-throughput drug screening in adrenocortical carcinoma cell lines using a library of 4,292 compounds. We identified and show niclosamide induces durable anticancer activity in adrenocortical carcinoma *in vitro* and *in vivo*. Niclosamide induced caspase-dependent apoptosis and G1 arrest. Moreover, niclosamide treatment reduced β-catenin and mediators of epithelial-to-mesenchymal transition protein levels, and resulted in mitochondrial uncoupling, features omnipresent in human adrenocortical carcinoma samples. Our findings provide a preclinical basis upon which to evaluate niclosamide therapy for locally advanced and metastatic adrenocortical carcinoma in humans.

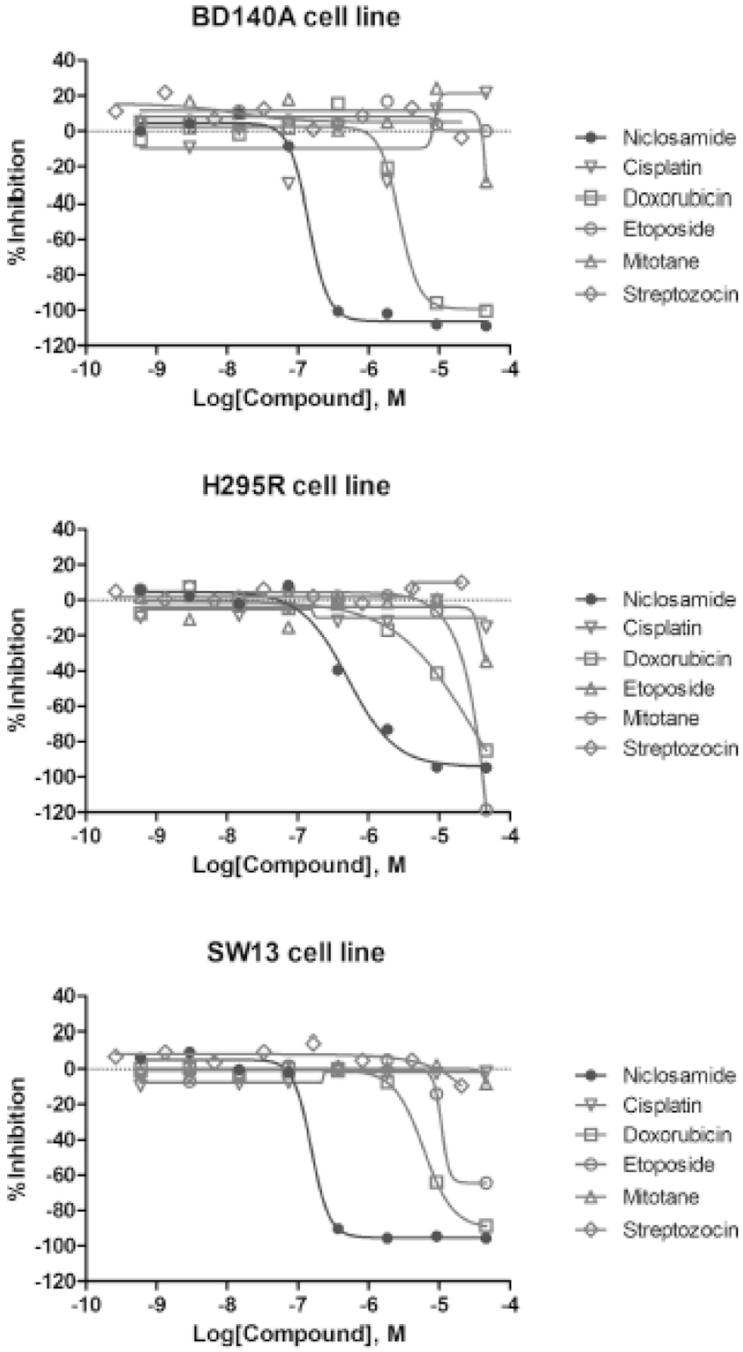

Figure 1.

Dose-response curves comparing niclosamide to cisplatin, doxorubicin, etoposide, mitotane, and streptozocin from qHTS.

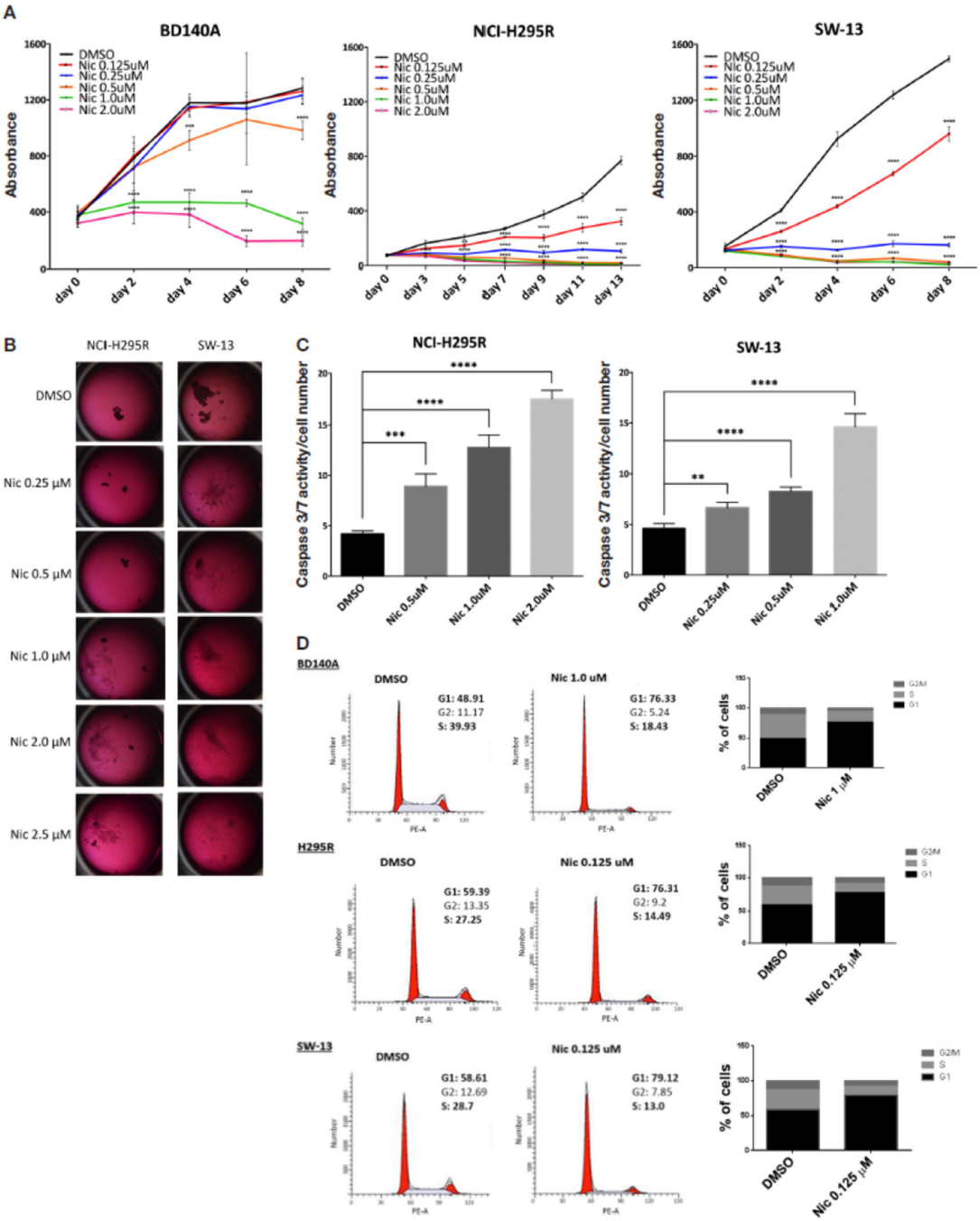

Figure 2.

The effect of niclosamide on cellular proliferation, three-dimensional multicellular aggregates, and apoptosis. A, Niclosamide inhibits cellular proliferation in a time- and dose-dependent manner in ACC cell lines. Error bars represent ± SD; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$. B, Three-dimensional multicellular aggregates (MCA) treated with niclosamide. C, Niclosamide treatment results in increased caspase 3/7 activity at 48 hours (SW-13) and 96 hours (NCI-H295R); **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$. D,

Niclosamide induces G_1 cell cycle arrest after 48 hours of treatment. Right panel shows percent of cells in each cell cycle phase.

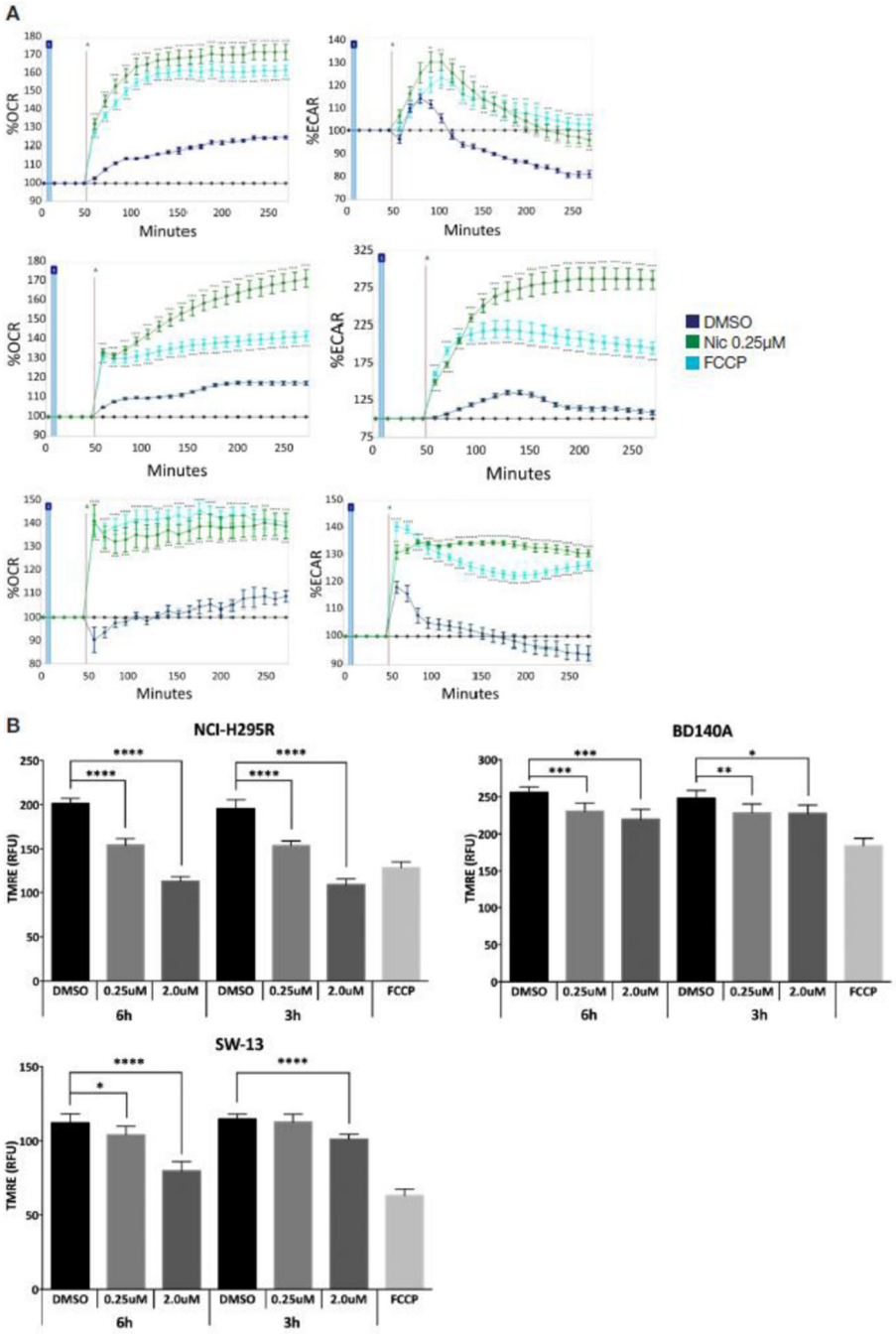

Figure 3.

Niclosamide treatment results in mitochondrial uncoupling in ACC cells. A, Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were measured using the Seahorse XF96 analyzer after niclosamide treatment (see materials and methods). Point A (red vertical lines) indicates injection of DMSO, niclosamide, or the positive control; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$. B, The effect of niclosamide on tetramethylrhodamine, ethyl ester (TMRE) in ACC cells. Cells treated for 3 hours or 6 hours with niclosamide were stained with TMRE to measure mitochondrial membrane potential.

FCCP was used as a positive control; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

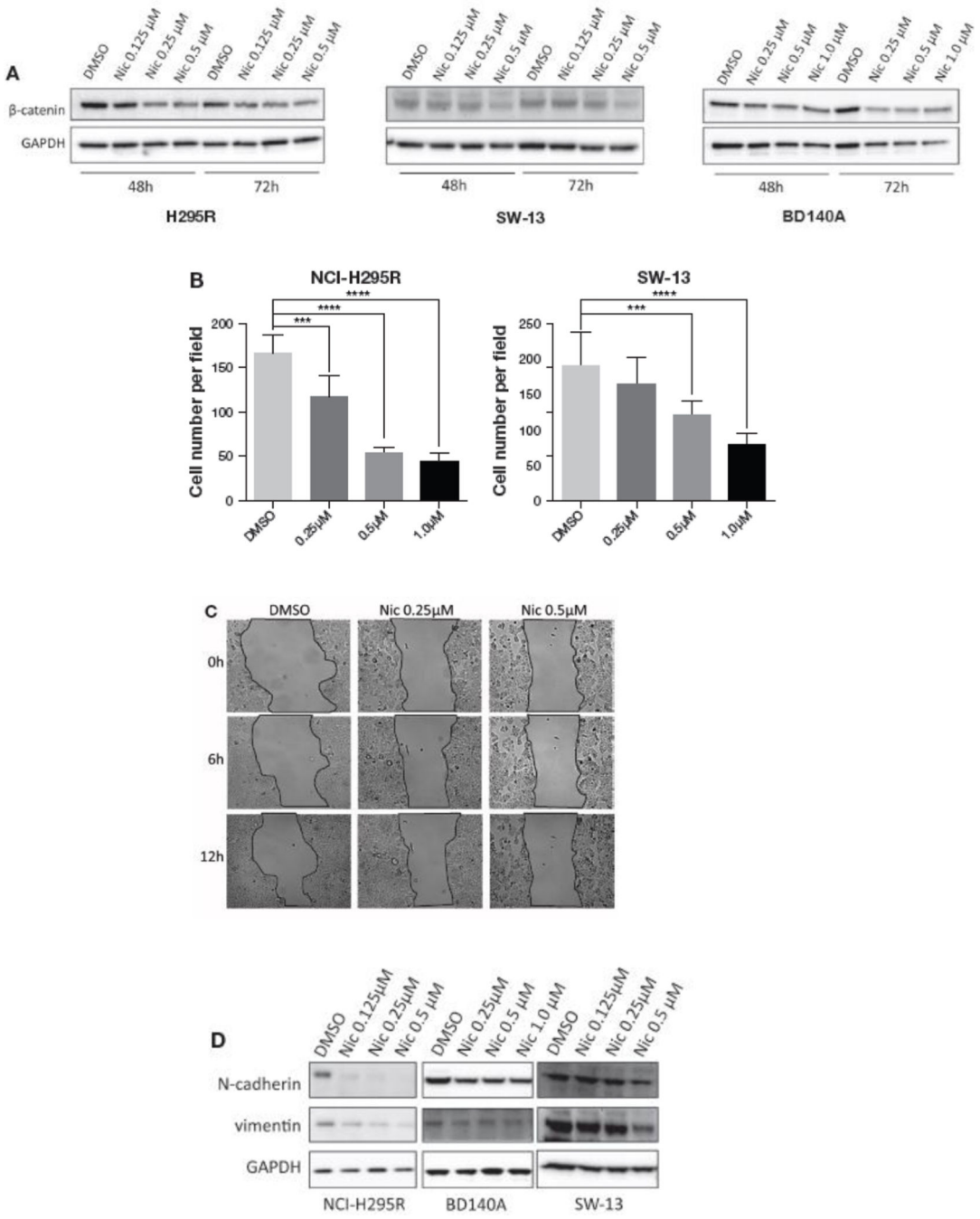

Figure 4.

The effect of niclosamide on β-catenin and cellular migration in ACC cells. A, Niclosamide reduces β-catenin in ACC cells. Western blot analysis after 48 hours and 72 hours of treatment with niclosamide. There was a dose-dependent decrease in β-catenin expression level. B, Cellular migration of NCI-H295R and SW-13 was assessed using a Boyden chamber assay. Cells were treated for 24 hours, trypsinized, and seeded in Boyden chambers, and allowed to migrate for 24 or 48 hours before fixation. Cells were counted in three random fields, and the experiment was performed in triplicate; ***, $P < 0.001$; ****, P

< 0.0001. C, Reduced cellular migration of BD-140A as measured by the wound healing assay. D, Niclosamide reduces the expression levels of N-cadherin and vimentin. Representative Western blot analysis showing reduced expression of EMT markers, N-cadherin, and vimentin after 48 hours of treatment.

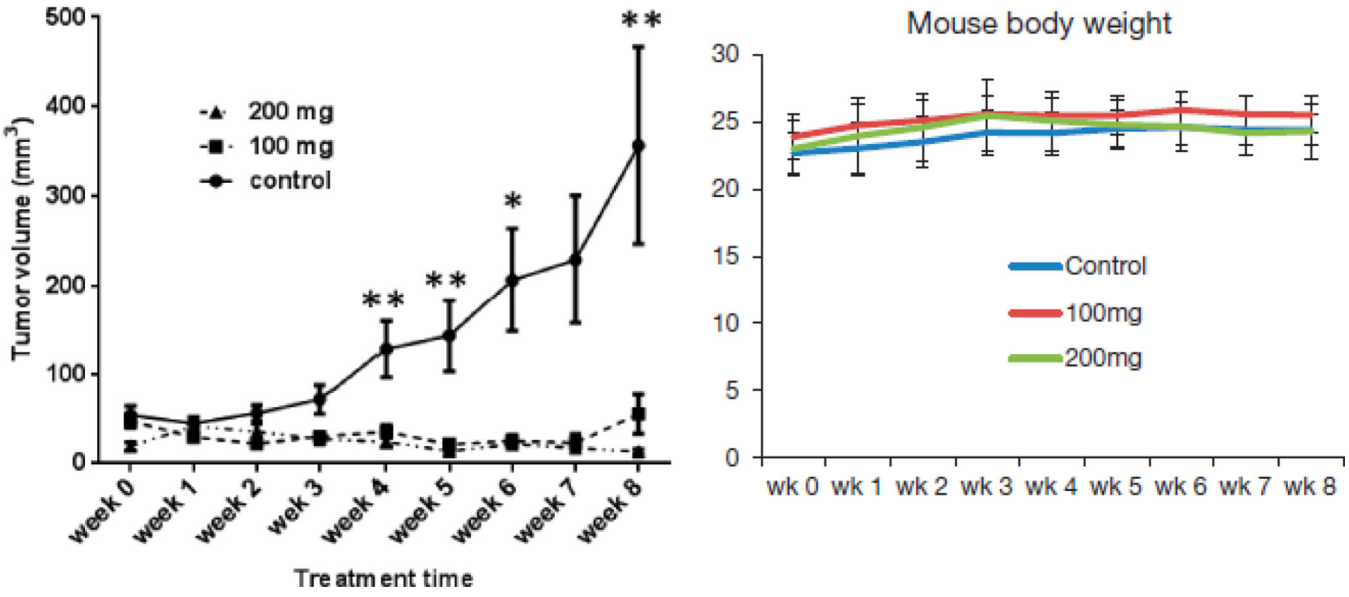

Figure 5.

Niclosamide treatment reduced ACC tumor growth *in vivo*. 5×10^6 NCI-H295R cells were injected into the flank of Nu^+/Nu^+ mice. Tumors were allowed to grow, and mice were randomized into three groups and treated as indicated. Tumor sizes (left panel) and mouse body weight (right panel) were measured weekly. Error bars are mean ± SEM for tumor volume, and mean ± STDEV for mouse body weight; *, $P < 0.05$; **, $P < 0.01$.