Disruption of STAT3 by Niclosamide Reverses Radioresistance of Human Lung Cancer

Shuo You1,5, Rui Li1, Dongkyoo Park1, Maohua Xie1, Gabriel L. Sica2, Ya Cao3, Zhi-Qiang Xiao4, and Xingming Deng1

Abstract

A major challenge affecting the outcomes of patients with lung cancer is the development of acquired radioresistance. However, the mechanisms underlying the development of resistance to therapy are not fully understood. Here, we discovered that ionizing radiation induces phosphorylation of Janus-associated kinase (JAK)-2 and STAT3 in association with increased levels of Bcl2/Bcl-XL in various human lung cancer cells. To uncover new mechanism(s) of radioresistance of lung cancer, we established lung cancer cell model systems with acquired radioresistance. As compared with radiosensitive parental lung cancer cells (i.e., A549, H358, and H157), the JAK2/STAT3/Bcl2/Bcl-XL survival pathway is significantly more activated in acquired radioresistant lung cancer cells (i.e., A549-IRR, H358-IRR, and H157-IRR). Higher levels of STAT3 were found to be accumulated in the nucleus of radioresistant lung cancer cells. Niclosamide, a potent STAT3 inhibitor, can reduce STAT3 nuclear localization in radioresistant lung cancer cells. Intriguingly, either inhibition of STAT3 activity by niclosamide or depletion of STAT3 by RNA interference reverses radioresistance in vitro. Niclosamide alone or in combination with radiation overcame radioresistance in lung cancer xenografts. These findings uncover a novel mechanism of radioresistance and provide a more effective approach to overcome radioresistance by blocking the STAT3/Bcl2/Bcl-XL survival signaling pathway, which may potentially improve lung cancer outcome, especially for those patients who have resistance to radiotherapy.

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Introduction

Lung cancer is the second most common cancer diagnosed and remains the number one cancer killer among all cancers in the United States (1). The best currently available therapies for patients with lung cancer achieve overall 5-year survival rates of 16% and 6% for non–small cell lung cancer (NSCLC) and small cell lung cancer (2), respectively. A major contributor to such poor outcomes is the intrinsic or acquired resistance to radiation and/or chemotherapy. To improve the survival of patients with lung cancer, basic molecular mechanisms responsible for resistance to therapy must be carefully elucidated and such knowledge exploited for the identification of more effective therapeutic agents.

STATs, including six STAT genes and eight alternatively spliced isoforms, are a group of transcription factors that regulate cell survival, proliferation, angiogenesis, and immune response by regulation of specific gene expression (3–6). Importantly, persistent activation of STAT3 was observed in various cancers, including human lung cancer, but not in normal epithelial cells (4, 7, 8). The inactive form of STAT3, which is located in the cytoplasm, can be activated by Janus-associated kinase (JAK) or nonreceptor tyrosine kinase (Src) through phosphorylation at its Tyr705 residue (9). The Tyr705-phosphorylated STAT3 molecules interact with each other through a reciprocal SH2 domain and form dimers, which are then translocated into the cell nucleus where they bind to DNA and transcribe a broad spectrum of genes, including Bcl2, Bcl-XL, Mcl-1, etc. (7, 10). It has been reported that overexpression of STAT3 potentiates growth, survival, and radioresistance of NSCLC cells (11). The 5-year overall survival rate of patients with NSCLC with high STAT3 expression was significantly lower than that of patients with low STAT3 expression (11), indicating that STAT3 may be an attractive therapeutic target for patients with NSCLC.

Niclosamide (C13H8Cl2N2O4, MW: 327.119) is U.S. Food and Drug Administration-approved small-molecule drug...
of the teniacide anthelmintic family that is effective against human tapeworms (12). It exerts its anthelmintic effects by uncoupling oxidative phosphorylation in the tapeworm (13). Intriguingly, niclosamide has recently been identified as a potent STAT3 inhibitor that disrupts STAT3 transcriptional activity by suppressing phosphorylation and nuclear translocation of STAT3 (14). Niclosamide is safe, well tolerated, and readily available (15, 16). Here, we discovered that the JAK2/STAT3/Bcl2/Bcl-XL survival pathway is persistently more active in lung cancer cells with acquired radioresistance than in radiosensitive lung cancer cells. Inhibition of STAT3 by niclosamide overcomes radioresistance of human lung cancer in vitro and in vivo, which may significantly improve the outcome of patients with lung cancer, especially those who have resistance to radiotherapy.

Materials and Methods

Materials
Niclosamide was purchased from Sigma-Aldrich. Phospho-JAK2 (Tyr1007/1008), phospho-STAT3 (Tyr705), STAT1, STAT3, STAT5, p-mTOR (Ser2448), p-p70S6K (Thr389), p-4EBP1 (Thr37/46), PARP, cleaved caspase-3, and β-actin antibodies were obtained from Cell Signaling Technology. Mcl-1 and K-Ras antibodies were purchased from Santa Cruz Biotechnology. Bcl2 antibody was obtained from Epitomics, Inc. Bcl2 antibody was purchased from Calbiochem. Alexa Fluor 555 Goat Anti-Rabbit immunoglobulin G (IgG; H + L) as well as ProLong Gold antifade reagent with 4', 6-diamidino-2-phenylindole (DAPI) was obtained from Invitrogen Life Technologies Inc. All other reagents used were purchased from commercial sources unless otherwise stated.

Cell lines and cell culture
A549, H358, H157, and H292 lung cancer cell lines were purchased from the American Type Culture Collection. A549 cells were maintained in DMEM/F12 medium supplemented with 10% FBS. H358, H157, and H292 cells were cultured in RPMI-1640 medium with 10% FBS. These cell lines were used for the described experiments without further authentication.

Preparation of cell lysates and Western blot analysis
Cells were washed with cold PBS and resuspended in ice-cold EBC buffer (0.5% Nonidet P-40, 50 mmol/L Tris, pH 7.6, 120 mmol/L NaCl, 1 mmol/L EDTA, and 1 mmol/L β-mercaptoethanol) containing protease inhibitor mixture set I. Following cell lysis by sonication and centrifugation at 14,000 × g for 15 minutes at 4°C, the resulting supernatant was collected as the total cell lysate. Protein expression was analyzed by Western blot analysis as previously described (17).

Establishment of irradiation-resistant cell lines
A549, H157, and H358 cell lines were used to establish ionizing radiation-resistant (IRR) lung cancer cell lines (A549-IRR, H157-IRR, and H358-IRR) as described (18). Briefly, A549, H157, and H358 cells (1 × 10⁶) were serially irradiated with 2 Gy of X-rays to a final dose of 80 Gy using X-RAD 320 (Precision X-ray, Inc.). Culture medium was renewed immediately after each dose of radiation. After growing to approximately 90% confluence, cells were trypsinized and then passaged into new culture dishes. Reirradiation of the newly passaged cells with 2 Gy of X-rays occurred at about 60% confluence, and this was repeated 40 times over a period of 5 months, for a total dose of 80 Gy. The parental cells (A549-P, H157-P, and H358-P) were trypsinized, counted, and passaged under the same conditions without ionizing irradiation as described (18).

Colony formation assay
A549/A549-IRR, H358/H358-IRR, and H157/H157-IRR cells were trypsinized and suspended into single-cell suspension and plated into 6-well plates (200 cells/well). Cells were treated with ionizing radiation or niclosamide as indicated. Cell culture medium was replaced every 3 days. After 10 days, cells were then stained and fixed with 0.1% crystal violet in 20% methanol. Surviving colonies were counted and the surviving fraction (SF) was calculated using the formula SF = treatment colony numbers/control colony numbers after at least three independent experiments as described (19, 20).

Sulforhodamine B colorimetric assay
Cells were seeded at a density of 6 × 10³ to 8 × 10³ per well in 96-well plates and allowed to grow overnight. Cells were treated with niclosamide for 72 hours. The surviving cell fraction was determined using the sulforhodamine B (SRB) assay as described (19).

Cell-cycle analysis
After treatment with niclosamide, cells were washed once with ice-cold PBS and resuspended in 100 μL of ice-cold PBS. Then 900 μL of cold methanol was added to the cells, mixed gently, and then incubated on ice or in a −20°C freezer for at least 30 minutes. Cells were washed once with PBS and resuspended in 500 μL PBS. RNase (100 μg/mL) was added and incubated at room temperature for 60 minutes. Next, 500 μL of 0.1 mg/mL propidium iodide was added to cells and incubated at room temperature for 30 minutes. Cell cycle was analyzed by flow cytometry as described (21).

Immunofluorescence staining
A total of 2 × 10⁸ cells were plated into chamber slides with cell culture medium. After treatment with niclosamide, cells were fixed with 4% formaldehyde at room temperature for 15 minutes. After fixation, cell membranes were permeabilized with 0.1% Triton-100 at room temperature for 20 minutes. Cells were then washed with 1 × PBS and blocked with 10% goat serum for 1 hour before primary antibody incubation. Rabbit anti-human STAT3 antibody was mixed at 1:100 dilution in 1 × PBS containing...
stained with DAPI, and mounted and stored at 4°C under dark conditions. Pictures were taken under an Olympus BX41 microscope (Olympus Imaging America Inc.).

**RNA interference**

Lentiviral pSIH1-puro-control short hairpin RNA (shRNA) and pSIH1-puro-STAT3 shRNA were purchased from Addgene. Control shRNA hairpin sequence: CCT AAG GTT AAG TCG CCC TCG CTC GAG CGA GGG CGA CTG AAC CTT AGT TGT TTG. Pseudovirus was produced by cotransfecting lentiviral packaging plasmid mixture (System Biosciences) with STAT3 shRNA into 293FT cells using NanoJuice transfection kit (EMD Chemical, Inc.) as described (22). After 48 hours, the virus-containing media were harvested by centrifugation at 20,000 × g. Cells were infected with the virus-containing media in the presence of polybrene (8 μg/mL) for 24 hours following which stable positive clones were selected using 1 μg/mL puromycin.

**Lung cancer xenografts and treatments**

Animal experiments were approved by the Institutional Animal Care and Use Committee of Emory University (Atlanta, GA). Six-week-old Nu/Nu nude mice were purchased from Harlan and housed under pathogen-free conditions in microisolator cages. A total of 5 × 10⁶ A549 and A549-IRR cells were injected into the subcutaneous tissue over the flank region of nude mice. Tumors were allowed to grow to an average volume of 145 mm³ before initiation of therapy as described (23). Tumor-bearing mice were randomly assigned into four groups (6 mice each group) as follows: (1) vehicle control (0.5% dimethyl sulfoxide, 100 μL/day, i.p.); (2) whole-body ionizing radiation (2 Gy, twice per week); (3) niclosamide (30 mg/kg, i.p.); (4) niclosamide (30 mg/kg/d, i.p.) + whole-body ionizing radiation (2 Gy, twice per week). Tumor volume was assessed by caliper measurements once every 2 days and calculated with the formula: \( V = \frac{L \times W^2}{2} \) (L, length; W, width) as described (24). At the end of experiments, mice were euthanized by CO₂ inhalation. Harvested tumor tissues were used for further analysis.

**Immunohistochemical staining**

Tumors were harvested, fixed in formalin, and embedded in paraffin. Representative sections from paraffin-embedded tumor tissues were analyzed by immunohistochemical (IHC) staining using an active caspase-3-specific antibody or Ki-67 antibody, respectively. Active caspase- or Ki-67–positive cells in tumor tissues were scored at ×400 magnification. The average number of positive cells per 0.0625 mm² area was determined from three separate fields in each of the three independent tumor samples as described (23).

**Mouse blood analysis**

Whole blood (250 μL) was collected in EDTA-coated tubes via cardiac puncture of anesthetized mice for hematology studies. Specimens were analyzed for white blood cells (WBC), red blood cells (RBC), hemoglobin (Hb), platelets (PLT), alanine aminotransferase (ALT), aspartate aminotransferase (AST), and blood urea nitrogen (BUN) in the Clinical Pathology Laboratory at the University of Georgia (Athens, GA).

**Statistical analysis**

Significant differences between two groups were analyzed using two-sided unpaired Student t test or two-way ANOVA. The \( P < 0.05 \) was considered statistically significant. Statistical analysis was performed with GraphPad Prism 5 software (25).

**Results**

**Exposure of human lung cancer cells to ionizing radiation results in the activation of JAK2/STAT3 and elevated levels of Bcl2/Bcl-XL**

To test whether ionizing radiation induces activation of the JAK2/STAT3/Bcl2/Bcl-XL survival signaling pathway, various human lung cancer cells (i.e., A549, H588, H157, and H292) were treated with ionizing radiation as indicated. Results revealed that ionizing radiation enhanced phosphorylation of JAK2 at Tyr1007/1008 and STAT3 at Tyr 705 in dose- and time-dependent manners (Fig. 1 and Supplementary Fig. S1). STAT3 functions as a physiologic transcriptional factor of Bcl2 and Bcl-XL that can directly bind to Bcl2 or Bcl-XL promoter to regulate Bcl2 and Bcl-XL at transcriptional levels (26–29). This helps explain how radiation also increased the expression levels of Bcl-XL and/or Bcl2 in various lung cancer cells (Fig. 1 and Supplementary Fig. S1). These findings suggest that radiation activated the JAK2/STAT3/Bcl2/Bcl-XL survival signaling pathway in lung cancer cells, which may negatively affect the efficacy of radiotherapy. Because similar effects of ionizing radiation on JAK2, STAT3, Bcl2, and Bcl-XL were observed in H292 cells (i.e., a non-K-Ras–mutated lung cancer cell line) as compared with cell lines harboring K-Ras mutation (i.e., A549, H588, and H157; Fig. 1), this suggests that K-Ras mutation does not affect ionizing radiation-induced activation of the STAT3/Bcl2/Bcl-XL survival pathway.

**The JAK2/STAT3/Bcl2/Bcl-XL survival pathway is highly and persistently activated in human lung cancer cells with acquired radioresistance**

To uncover the mechanism(s) of acquired radioresistance, we established three lung cancer cell lines with acquired radiation resistance (i.e., A549-IRR, H588-IRR, and H157-IRR).
and H157-IRR) as described (18). Increased levels of 
pJAK2 (Tyr1007/1008), pSTAT3 (Tyr705), Bcl2, and Bcl-
XL were observed in A549-IRR, H358-IRR, and H157-IRR
cells as compared with A549, H358, and H157 parental
cells (Fig. 2A). A549, H358, and H157 parental cells
remained sensitive but A549-IRR, H358-IRR, and H157-
IRR cells became insensitive to ionizing radiation (Fig. 2B).
These results provide strong evidence that ionizing
radiation activation of the JAK2/STAT3/Bcl2/Bcl-XL
survival pathway may contribute to acquired radioresis-
tance. Similar levels of K-Ras protein were observed in
parental and radioresistant cell lines (Fig. 2A), Sugges-
ting that K-Ras is not increased in radioresistant cells
compared with parental cells. It is known that the active
form of STAT3 is localized in the nucleus (30). As
compared with parental cells, significantly higher levels
of nuclear STAT3 were observed in A549-IRR, H358-
IRR, and H157-IRR cells (Fig. 3), indicating that more
activated STAT3 molecules accumulate in the nucleus in
radioresistant lung cancer cells.

Inhibition of STAT3 by niclosamide reverses
acquired radioresistance of human lung cancer cells
Niclosamide has been recently identified as a potent
STAT3 inhibitor that can abolish STAT3 transcriptional
activity (14). To test whether niclosamide blocks ion-
izing radiation-induced activation of the STAT3/Bcl2/
Bcl-XL survival pathway, A549, H358, and H157
parental cells were treated with ionizing radiation in
the absence or presence of niclosamide (1 μmol/L)
for 24 hours. Results indicated that niclosamide not
only blocked ionizing radiation-induced STAT3 phos-
phorylation at Tyr 705, but also inhibited ionizing
radiation-enhanced Bcl2 or Bcl-XL expression leading
to activation of caspase-3 (Fig. 4A). Niclosamide had no
inhibitory effect on JAK2 phosphorylation (Fig. 4A),
indicating its specificity for STAT3. Our findings sug-
gest that persistent activation of the JAK2/STAT3/
Bcl2/Bcl-XL pathway contributes to acquired radioresis-
tance (Fig. 2). To further test whether inhibition
of STAT3 can reverse radioresistance, A549-IRR and
H157-IRR cells were treated with increasing concentra-
tions of niclosamide (i.e., 0.2–2 μmol/L) for 24 hours.
Results indicated that niclosamide reduced levels of
pSTAT3, Bcl2, and Bcl-XL in association with activation of
caspase-3 in radioresistant lung cancer cells (Fig. 4B).
Mechanistically, treatment of radioresistant A549-IRR
cells with niclosamide resulted in decreased nuclear
localization of STAT3 (Supplementary Fig. S2). In addi-
tion, niclosamide not only blocked ionizing radiation-
stimulated phosphorylation of mTOR, p70S6K, and 4-EBP1 in A549, H358, and H157 parental cells (Fig. 4A), but also inhibited their phosphorylation in radioresistant lung cancer cells (Fig. 4B). These findings indicate that, in addition to STAT3, niclosamide may also inhibit mTOR activity. Colony formation analysis shows that niclosamide not only sensitized A549 cells to radiation, but also reversed acquired radioresistance of A549-IRR cells (Fig. 4C). Niclosamide also reduced Mcl-1 expression levels in both lung cancer parental and radioresistant cells (Fig. 4A and B). It has been reported that STAT3 also functions as a Mcl-1 transcriptional factor that can positively regulate Mcl-1 expression (29, 31). This may explain how inhibition of STAT3 by niclosamide downregulated Mcl-1 (Fig. 4A and B). Niclosamide-reduced Mcl-1 expression may also contribute to the sensitization of lung cancer cells to radiotherapy. To test the effect of niclosamide on cell cycle, A549 and A549-IRR cells were treated with niclosamide (0.8 μmol/L) for 24 hours. Results show that niclosamide increased the proportion of the cell population in Go–G1 phase in both A549 (i.e., from 60%→77.9%) and A549-IRR cells (from 51.4%→61.7%; Supplementary Fig. S3).

Specific knockdown of STAT3 using shRNA restores sensitivity of lung cancer cells to ionizing radiation

To determine whether the STAT3-mediated survival pathway is required for acquired radioresistance, STAT3 was knocked down from A549 and A549-IRR cells using STAT3 shRNA. Transfection of STAT3 shRNA but not control shRNA significantly depleted endogenous STAT3 levels by more than 99% but did not affect the expression of STAT1 or STAT5 (Fig. 5A), indicating that the effect of STAT3 shRNA on STAT3 expression is specific. Importantly, silencing of STAT3 also significantly reduced its downstream survival effectors, such as Bcl2 and Bcl-XL (Fig. 5A). Colony formation assay shows that depletion of STAT3 by RNA interference (RNAi) restored the sensitivity of A549-IRR cells to radiation (Fig. 5B and C). These findings indicate that depletion of STAT3 can reverse radioresistance of human lung cancer cells. To examine whether knockdown of STAT3 can block the ability of cells to attain radiation resistance, A549-IRR cells were transfected with control shRNA or STAT3 shRNA. Then, cells were treated with 2 Gy ionizing radiation twice, followed by a colony formation assay. Radiation did not significantly affect the growth of A549-IRR cells after transfection with control shRNA. However, no cell growth was
observed after treatment with radiation when STAT3 was depleted by STAT3 shRNA (Fig. 5D). These findings suggest that specific knockdown of STAT3 blocks the ability of cells to attain radioresistance.

To test whether niclosamide kills lung cancer cells through inhibition of STAT3, we tested the effects of niclosamide on A549 cells with knocked down STAT3. A549 cells were transfected with control shRNA or STAT3 shRNA and then treated with niclosamide (i.e., 0.8 μmol/L) for 72 hours. Cell growth was measured by SRB analysis. Results showed that niclosamide-induced growth inhibition was diminished in A549 cells transfected with STAT3 shRNA but not control shRNA, suggesting that the killing effect of niclosamide in lung cancer occurs, at least in most part, through inhibition of STAT3 (Supplementary Fig. S4).

Niclosamide overcomes radioresistance in lung cancer xenografts

To test whether niclosamide can overcome acquired radioresistance of lung cancer in vivo, Nu/Nu nude mice with A549 or A549-IRR xenografts were treated with ionizing radiation (2 Gy, twice per week), niclosamide (30 mg/kg/d), or in combination for 21 days as described in "Materials and Methods." Lung cancer xenografts from A549 parental cells were sensitive to ionizing radiation with significant tumor shrinkage, whereas A549-IRR xenografts were resistant to radiotherapy but sensitive to niclosamide treatment (Fig. 6A). These findings reveal that niclosamide is able to overcome acquired radioresistance of lung cancer in vivo. IHC analysis shows that ionizing radiation enhances the numbers of active caspase-3–positive cells only in A549 tumor tissues but not in A549-IRR tumor tissues (Fig. 6B). In contrast, treatment of mice with niclosamide alone or in combination with ionizing radiation resulted in an increased number of active caspase-3–positive cells and a decreased number of Ki-67–positive cells in both A549 and A549-IRR tumor tissues (Fig. 6B and Supplementary Fig. S5). Western blot analysis further confirmed that treatment of A549 and A549-IRR xenografts with niclosamide blocked the STAT3/Bcl2/Bcl-XL pathway in tumor tissues (Fig. 6C). PARP is a 116 kDa nuclear protein that is a death substrate and can be specifically cleaved by caspase-3 or caspase-6 into a signature 85 kDa apoptotic fragment (32). Thus, PARP cleavage can be used as a marker for activation of caspase-3 or -6 during apoptosis. As shown in Fig. 6C, PARP cleavage and caspase-3 activation were observed in radiosensitive A549 xenografts but not in radioresistant A549-IRR xenografts following radiotherapy. In contrast, niclosamide alone or in combination with ionizing radiation could induce PARP cleavage and caspase-3 activation in both A549 and A549-IRR xenografts (Fig. 6C). These findings demonstrate that niclosamide suppression of tumor growth and/or ability to overcome radioresistance may occur through induction of apoptosis and suppression of proliferation in tumor tissues.

Toxicity analysis in vivo

To evaluate the in vivo toxicity of ionizing radiation and niclosamide, the weight of each mouse was monitored every other day. Results indicated that whole-body ionizing radiation (2 Gy × 6) resulted in significant weight loss in both A549 and A549-IRR xenograft mice, whereas treatment with 30 mg/kg/d of niclosamide was well tolerated without weight loss (Fig. 7A and Supplementary Fig. S6A). Interestingly, niclosamide may have some protective effect from ionizing radiation because the combination of niclosamide and ionizing radiation did not result in significant weight loss (Fig. 7A and Supplementary Fig. S6A). Blood analysis showed that A549 and A549-IRR mice treated with radiation had reversible reduction in WBC and platelet counts (Fig. 7B and Supplementary Fig. S6B). Niclosamide had no significant toxicity to vital organ functions as reflected by the results of liver, kidney, and bone marrow function tests (ALT, AST and BUN, WBC, RBC, Hb, and PLT; Fig. 7B and Supplementary Fig. S6B). Histopathology of harvested normal tissues (heart, liver, lung, brain, spleen, kidney, intestine, etc.) revealed
no evidence of normal tissue toxicities after treatment with ionizing radiation or niclosamide alone or in combination (Fig. 7C and Supplementary Fig. S6C).

Discussion

Radiotherapy is a major therapeutic intervention for patients with lung cancer and is administered to up to 75% of patients with lung cancer during the course of their disease (33). Prognosis for patients with lung cancer remains poor, in part, due to resistance to radiation or chemotherapy. However, the mechanism(s) underlying this resistance are only partially defined. It has been reported that multiple signal transduction pathways, including the PI3K/AKT, MAPK/ERK, ATM, and EGF receptor pathways, can reduce radiation efficacy by promoting DNA repair in tumor cells (34, 35). Overexpression of Bcl2 and Bcl-XL resulted in resistance of tumor cells to apoptosis induced by radiation (36–39). Here, we discovered that radiation induces activation of the JAK2/STAT3 survival signaling pathway, leading to upregulation of its downstream transcriptional effectors, Bcl2/Bcl-XL, in various human lung cancer cells (Fig. 1 and Supplementary Fig. S1). As compared with radiosensitive parental lung cancer cells,

Figure 4. Niclosamide (Niclo) blocks ionizing radiation (IR)-induced activation of the STAT3/Bcl2/Bcl-XL pathway and reverses acquired radioresistance of human lung cancer cells. A, A549, H358, and H157 cells were treated with IR (2 Gy) in the absence or presence of Niclo (1 μmol/L) for 24 hours. Levels of pJAK2 (Tyr1007/1008), pSTAT3 (Tyr705), Bcl2, Bcl-XL, Mcl-1, p-mTOR, p-P70S6K, p-4EBP1, and active caspase-3 were analyzed by Western blot analysis. B, radioresistant human lung cancer A549-IRR and H157-IRR cells were treated with increasing concentrations of Niclo for 24 hours. Levels of pJAK2 (Tyr1007/1008), pSTAT3 (Tyr705), Bcl2, Bcl-XL, Mcl-1, p-mTOR, p-P70S6K, p-4EBP1, and active caspase-3 were analyzed by Western blot analysis. C, A549 and A549-IRR cells were treated with IR (5 Gy), Niclo (0.1 μmol/L), or in combination. Cell growth was analyzed by colony formation assay. Results represent the mean ± SD for three separate determinations.
significantly increased levels of pJAK2, pSTAT3, Bcl2, and Bcl-XL were observed in acquired radioresistant cells (Fig. 2), indicating that the JAK2/STAT3/Bcl2/Bcl-XL survival pathway is constitutively more active in radioresistant human lung cancer cell lines than in radiosensitive lung cancer cell lines. Immunostaining analysis further confirmed that STAT3 accumulated in the nucleus of radioresistant lung cancer cells (Fig. 3). Our findings indicate that the acquired radioresistance resulted from persistent activation of the JAK2/STAT3/Bcl2/Bcl-XL pathway in human lung cancer cells. Interestingly, radiation did not seem to affect Mcl-1 expression (Fig. 1 and Supplementary Fig. S1). Inversely, even lower levels of Mcl-1 were observed in radioresistant lung cancer cells than in parental cells (Fig. 2A). It is currently unclear why ionizing radiation-activated STAT3 only upregulated Bcl2/Bcl-XL but not Mcl-1 expression. It is possible that, in addition to STAT3 activation, radiation may also activate Mcl-1 E3 ligase (i.e., Mule, FBW7, etc.) to promote its degradation. Further work may be required to uncover the exact mechanism(s).

Niclosamide has recently been identified as a new small-molecule STAT3 inhibitor that inhibits Tyr705 site phosphorylation as well as transcriptional activity of STAT3, but has no obvious inhibitory effect on upstream proteins JAK2 and Src (14, 40). Here, we found that niclosamide not only selectively blocked ionizing radiation-induced activation of STAT3 (but not JAK2) but also suppressed the downstream effectors, Bcl2 and Bcl-XL, in both radiosensitive and radioresistant human lung cancer cells (Fig. 4A and B), suggesting that niclosamide functions as a specific STAT3 inhibitor to block ionizing radiation activation of the STAT3/Bcl2/Bcl-XL survival pathway in human lung cancer cells. Intriguingly, niclosamide reversed radioresistance and restored the sensitivity of radioresistant (A549-IRR) cells to ionizing radiation (Fig. 4C). Because specific depletion of STAT3 using RNAi sensitized A549 cells to radiation and also reversed radioresistance of A549-IRR cells through downregulation of Bcl2/Bcl-XL (Fig. 5), this indicates that the STAT3/Bcl2/Bcl-XL survival pathway in human lung cancer cells is not only essential for the development of acquired radioresistance, but is also a therapeutic target for overcoming radioresistance.

In vivo studies using radiosensitive (i.e., A549) and radioresistant (i.e., A549-IRR) xenografts revealed that niclosamide alone or in combination with radiation effectively overcame radioresistance in animal models (Fig. 6).
Niclosamide and ionizing radiation did not show a significant benefit in the reduction of tumor burden (P > 0.05; Fig. 6A). It is possible that the dose of 30 mg/kg/d used for niclosamide may be too high to observe a synergistic effect of the combination treatment because niclosamide alone in this dose was already very effective in repressing lung cancer growth in vivo. If a lower dose of niclosamide (i.e., 20 mg/kg or less) is used, a synergistic or additive effect between niclosamide and ionizing radiation may be observed. Consistently, niclosamide also blocked ionizing radiation-induced activation of the JAK2/STAT3/Bcl2/Bcl-XL pathway leading to enhanced apoptosis in tumor tissues (Fig. 6C). Mice tolerated the combined treatment with niclosamide and ionizing radiation well without significant normal tissue toxicities except for reversible weight loss and decrease in WBCs and platelets that resulted from radiotherapy (Fig. 7 and Supplementary Fig. S6).

In summary, we have identified a new mechanism of acquired radioresistance in lung cancer cell lines. Radiation-induced activation of JAK2/STAT3 upregulates the downstream survival effectors Bcl2 and Bcl-XL. The JAK2/STAT3/Bcl2/Bcl-XL survival pathway is persistently and highly activated in lung cancer model systems with acquired radioresistance. Inhibition of the STAT3/Bcl2/Bcl-XL pathway by niclosamide effectively overcomes acquired radioresistance in vitro and in vivo. On the basis of our findings, niclosamide alone or in combination with radiotherapy may represent a novel and more effective approach for lung cancer treatment, especially for those patients who have resistance to radiotherapy.
Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors' Contributions
Conception and design: S. You, Y. Cao, Z.-Q. Xiao, X. Deng
Development of methodology: S. You, R. Li, D. Park, X. Deng
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S. You, R. Li, G.L. Sica, X. Deng
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. You, X. Deng
Writing, review, and/or revision of the manuscript: S. You, Y. Cao, Z.-Q. Xiao, X. Deng
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S. You, D. Park, M. Xie, X. Deng
Study supervision: Y. Cao, Z.-Q. Xiao, X. Deng

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Shuo You, Rui Li, Dongkyoo Park, et al.


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