Disruption of STAT3 by niclosamide reverses radioresistance of human lung cancer

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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 (IR) induces phosphorylation of JAK2 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 to 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.
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 lung cancer patients achieve overall 5-year survival rates of 16% and 6% for non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC) (2), respectively. A major contributor to such poor outcomes is the intrinsic or acquired resistance to radiation and/or chemotherapy. In order to improve the survival of lung cancer patients, basic molecular mechanisms responsible for resistance to therapy must be carefully elucidated and such knowledge exploited for the identification of more effective therapeutic agents.

Signal transducers and activators of transcription (STATs), including 6 STAT genes and 8 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 non-receptor 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 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 NSCLC patients 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 NSCLC patients.
Niclosamide (C₁₃H₈Cl₂N₂O₄, MW: 327.119) is an FDA-approved small molecule drug of the
teniacide anthelmintic family that is effective against human tapeworms (12). It exerts its
anthelminthic 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 (St. Louis, MO, USA). Phospho-JAK2
(Tyr1007/1008), phospho-STAT3 (Tyr705), STAT1, STAT3, STAT5, p-mTOR (Ser2448), p-
P70S6K (Thr389), p-4EBP1 (Thr37/46), PARP, cleaved caspase 3, β-actin antibodies were
obtained from Cell Signaling Technology (Beverly, MA). Mcl-1 and K-Ras antibodies were
purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Bcl-XL and Ki67 antibody were
obtained from Epitomics, Inc (Burlingame, CA). Bcl2 antibody was purchased from Calbiochem
(Darmstadt, Germany). Alexa Fluor® 555 Goat Anti-Rabbit IgG (H+L) as well as ProLong Gold
antifade reagent with 4', 6-diamidino-2-phenylindole (DAPI) were obtained from Invitrogen Life
Technologies Inc (Carlsbad, CA). 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 (ATCC, Manassas, VA). A549 cells were maintained in DMEM/F12 medium supplemented with 10% fetal bovine serum. H358, H157 and H292 cells were cultured in RPMI 1640 medium with 10% fetal bovine serum. These cell lines were employed for the described experiments without further authentication.

**Preparation of cell lysates and Western blot**

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

**Establishment of irradiation resistant (IRR) cell lines**

A549, H157 and H358 cell lines were used to establish ionizing radiation resistant lung cancer cell lines (A549-IRR, H157-IRR and H358-IRR) as described (18). Briefly, A549, H157, and H358 cells (1x10^6) were serially irradiated with 2Gy of X-rays to a final dose of 80Gy using X-RAD 320 (Precision X-ray, Inc., North Branford, CT). 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. Re-irradiation of the newly passaged cells with 2Gy 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 80Gy. 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, 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 IR 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 (SRB) colorimetric assay**
Cells were seeded at a density of 6 x 10^3 - 8 x 10^3 per well in 96-well plates and allowed to grow overnight. Cells were treated with niclosamide for 72h. 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 phosphate-buffered saline (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 (PI) was added to cells and incubated at room temperature for 30 minutes. Cell cycle was analyzed by flow cytometry as described (21).

**Immunofluorescence staining**
2 x 10^4 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 min. After fixation, cell membranes were permeabilized with 0.1% Triton X-100 at room temperature for 20 min. 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 10% goat serum, added into the chambers and incubated overnight at 4°C. After washing with 1×PBS three times, Alexa Fluor® 555 Goat Anti-Rabbit IgG was added to the chambers at 1:1000 dilution in 10% goat serum for 1 hour. The slides were washed three times with 1×PBS, counterstained with DAPI, mounted and stored at 4°C under dark conditions. Pictures were taken under an Olympus BX41 microscope (Olympus Imaging America Inc., PA).

RNA Interference

Lentiviral pSIH1-puro-control shRNA and pSIH1-puro-STAT3 shRNA were purchased from Addgene (Cambridge, MA). Control shRNA hairpin sequence: CCT AAG GTT AAG TCG CCC TCG CTC GAG CGA GGG CGA CTT AAC CTT AGG. STAT3 shRNA hairpin sequence: GAT CCG CAT CTG CCT AGA TCG GCT ATT CAA GAG ATA GCC GAT CTA GGC AGA TGT TTT TTG. Pseudovirus was produced by cotransfecting lentiviral packaging plasmid mixture (System Biosciences, CA) with STAT3 shRNA into 293FT cells using NanoJuice transfection kit (EMD Chemical, Inc.) as described (22). After 48h, 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 (8ug/ml) for 24h following which stable positive clones were selected using 1ug/ml puromycin.

Lung cancer xenografts and treatments

Animal experiments were approved by the Institutional Animal Care and Use Committee of
Emory University. Six-week-old Nu/Nu nude mice were purchased from Harlan and housed under pathogen-free conditions in microisolator cages. 5 × 10^6 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 mm^3 prior to 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%DMSO, 100 μl/d, i.p.); (2) whole body ionizing radiation (2Gy, twice per week); (3) Niclosamide (30mg/kg/d, i.p.); (4) Niclosamide (30mg/kg/d, i.p.) + whole body ionizing radiation (2Gy, twice per week). Tumor volume was assessed by caliper measurements once every two 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 CO\(_2\) inhalation. Harvested tumor tissues were used for further analysis.

**Immunohistochemistry (IHC) staining**

Tumors were harvested, fixed in formalin and embedded in paraffin. Representative sections from paraffin-embedded tumor tissues were analyzed by IHC staining using an active caspase 3-specific antibody or Ki67 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 mm\(^2\) area was determined from three separate fields in each of three independent tumor samples as described (23).

**Mouse blood analysis**

Whole blood (250μL) 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), 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's t-test or 2-way ANOVA. The p value < 0.05 was considered statistically significant. Statistical analysis was performed with GraphPad Prism 5 software (San Diego, CA) (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 (IR) induces activation of the STAT3/Bcl2/Bcl-XL survival signaling pathway, various human lung cancer cells (*i.e.* A549, H358, H157 and H292) were treated with IR as indicated. Results revealed that IR enhanced phosphorylation of JAK2 at Tyr1007/1008 and STAT3 at Tyr 705 in dose- and time-dependent manners (Figs. 1 and S1). STAT3 functions as a physiological 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 (Figs. 1 and 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. Since similar effects of IR 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 to cell lines harboring K-Ras mutation (*i.e.* A549, H358 and H157) (Fig. 1), this suggests that K-Ras mutation does not affect IR-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, H358-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 to 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 IR (Fig. 2B). These results provide strong evidence that IR activation of the JAK2/STAT3/Bcl2/Bcl-XL survival pathway may contribute to acquired radioresistance. Similar levels of K-Ras protein were observed in parental and radioresistant cell lines (Fig. 2A), suggesting that K-Ras is not increased in radioresistant cells compared to parental cells. It is known that the active form of STAT3 is localized in the nucleus (30). As compared to 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 IR-induced activation of the STAT3/Bcl2/Bcl-XL survival pathway, A549, H358 and H157 parental cells were treated with IR in the absence or presence of niclosamide (1μM) for 24h. Results indicated that niclosamide not only blocked IR-induced STAT3 phosphorylation at Tyr 705 but also inhibited IR-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 suggest that persistent activation of the JAK2/STAT3/Bcl2/Bcl-XL pathway contributes to acquired radioresistance (Fig. 2). To further test whether inhibition of STAT3 can reverse radioresistance, A549-IRR and H157-IRR cells were treated with increasing concentrations of niclosamide (i.e. 0.2~2 μM) for 24h. 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 (Fig. S2). Additionally, niclosamide not only blocked IR-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 μM) for 24h. Results show that niclosamide increased the proportion of the cell population in G0/G1 phase in both A549 (i.e. from 60% to 77.9%) and A549-IRR cells (from 51.4% to 61.7%) (Fig S3).
Specific knockdown of STAT3 using shRNA restores sensitivity of lung cancer cells to IR

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 show that depletion of STAT3 by RNAi restored the sensitivity of A549-IRR cells to radiation (Fig. 5B, 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 2Gy IR 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 μM) for 72h. 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 (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-IR xenografts were treated with IR (2Gy, twice per week), niclosamide (30mg/kg/d) or in combination for 21 days as described in "Methods". Lung cancer xenografts from A549 parental cells were sensitive to IR with significant tumor shrinkage, while 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 IR 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 IR 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 (Figs. 6B and 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). Poly (ADP-ribose) polymerase (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 IR 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 (2Gy × 6) resulted in significant weight loss in both A549 and A549-IRR xenograft mice while treatment with 30mg/kg/d of niclosamide was well tolerated without weight loss (Figs. 7A and S6A). Interestingly, niclosamide may have some protective effect from ionizing radiation since the combination of niclosamide and IR did not result in significant weight loss (Figs. 7A and S6A). Blood analysis showed that A549 and A549-IRR mice treated with radiation had reversible reduction in WBC and platelet counts (Figs. 7B and 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 platelets; Figs. 7B and S6B). Histopathology of harvested normal tissues (heart, liver, lung, brain, spleen, kidney, intestine, etc.) revealed no evidence of normal tissue toxicities after treatment with IR or niclosamide alone or in combination (Figs. 7C and S6C).

**Discussion**

Radiotherapy is a major therapeutic intervention for patients with lung cancer and is administered to up to 75% of lung cancer patients during the course of their disease (33). Prognosis for lung cancer patients 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 EGFR 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 (Figs. 1 and S1). As compare to radiosensitive parental lung cancer cells, 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 (Figs. 1 and 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 IR-activated STAT3 only upregulated Bcl-2/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 IR-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, B), suggesting that niclosamide functions as a specific STAT3 inhibitor to block IR 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 IR (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). Compared to niclosamide alone, the combination of niclosamide and IR did not show a significant benefit in the reduction of tumor burden (p >0.05) (Fig. 6A). It is possible that the dose of 30mg/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. 20mg/kg or less) is used, a synergistic or additive effect between niclosamide and IR may be observed. Consistently, niclosamide also blocked IR-induced activation of the STAT3/Bcl2/Bcl-XL pathway leading to enhanced apoptosis in tumor tissues (Fig. 6C). Mice tolerated the combined treatment with niclosamide and IR well without significant normal tissue toxicities except for reversible weight loss and decrease in white blood cells and platelets that resulted from radiotherapy (Figs. 7 and 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. Based on 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.

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References


**Figure Legends**

**Figure 1.** Ionizing radiation (IR) induces activation of the JAK2/STAT3/Bcl2/Bcl-XL survival signaling pathway in various human lung cancer cells. A549, H358, H157 and H292 cells were treated with increasing doses of IR. After 2h, levels of pJAK2 (Tyr1007/1008), pSTAT3 (Tyr705), Bcl2, Bcl-XL and Mcl-1 were analyzed by Western blot.

**Figure 2.** Persistent activation of the JAK2/STAT3/Bcl2/Bcl-XL pathway in human lung cancer cells is associated with acquired radioresistance. A, levels of pJAK2 (Tyr1007/1008), pSTAT3 (Tyr705), Bcl2, Bcl-XL, Mcl-1 and K-Ras were compared in various parental (A549, H358 and H157) and acquired radioresistant (A549-IRR, H358-IRR and H157-IRR) human lung cancer cells. B, various parental and acquired radioresistant human lung cancer cells were treated with IR (5Gy). Cell growth was analyzed by colony formation assay as described in "Methods". Reported values are the mean ± SD for 3 separate experiments.

**Figure 3.** STAT3 is accumulated in the nucleus of acquired radioresistant human lung cancer cells. STAT3 was analyzed by immunofluorescence staining using STAT3 antibody in A549, A549-IRR, H358, H358-IRR, H157 and H157-IRR cells.
Figure 4. Niclosamide (Niclo) blocks 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 (2Gy) in the absence or presence of Niclo (1 μM) for 24 h. 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. B, radioresistant human lung cancer A549-IRR and H157-IRR cells were treated with increasing concentrations of Niclo for 24h. 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. C, A549 and A549-IRR cells were treated with IR (5Gy), Niclo (0.1 μM) or in combination. Cell growth was analyzed by colony formation assay. Results represent the mean ± SD for 3 separate determinations.

Figure 5. Specific knockdown of STAT3 reverses radioresistance of human lung cancer cells. A, STAT3 shRNA or control shRNA was transfected into A549 or A549-IRR cells. Expression levels of STAT3, STAT1, STAT5, Bcl-XL and Bcl2 were analyzed by Western blot. B and C, A549 or A549-IRR cells expressing STAT3 shRNA or control shRNA were treated with 5Gy of IR. Cell growth was analyzed by colony formation assay. Results represent the mean ± SD for 3 separate determinations. D, A549-IRR cells were transfected with control shRNA or STAT3 shRNA. After 48h, cells were then treated with IR (2Gy) twice, followed by colony formation assay.

Figure 6. Niclosamide overcomes acquired radioresistance in lung cancer xenografts. A, mice bearing lung cancer A549 or radioresistant A549-IRR xenografts were treated with vehicle control, IR (2Gy, twice per week), Niclo (30mg/kg/d), or in combination for 21 days. Tumor volume was measured every other day. B and C, tumor tissues were removed at end of treatments. Active caspase 3 was analyzed by IHC staining using anti-active caspase 3.
antibody (B). Protein expression levels of pSTAT3 (Tyr705), STAT3, Bcl2, Bcl-XL, Mcl-1, PARP and active caspase 3 in tumor tissues were analyzed by Western blot (C).

**Figure 7.** Toxicity analysis for treatments with IR and niclosamide in mice bearing A549 xenografts. A, body weight of mice with A549 xenografts was measured once every other day during treatment with vehicle control, IR (2Gy, twice per week), Niclo (30mg/kg/d), or in combination. B, blood analysis of mice after various treatments for 21 days. C, H&E histology of various organs after various treatments for 21 days.
Fig. 1

A549

0  0.5  1  2  5  10  IR (Gy)

- p-JAK2
- p-STAT3
- STAT3
- Bcl2
- Bcl-XL
- Mcl-1
- β-Actin

H358

0  0.5  1  2  5  10  IR (Gy)

- p-JAK2
- p-STAT3
- STAT3
- Bcl2
- Bcl-XL
- Mcl-1
- β-Actin

H157

0  0.5  1  2  5  10  IR (Gy)

- p-JAK2
- p-STAT3
- STAT3
- Bcl2
- Bcl-XL
- Mcl-1
- β-Actin

H292

0  0.5  1  2  5  10  IR (Gy)

- p-JAK2
- p-STAT3
- STAT3
- Bcl2
- Bcl-XL
- Mcl-1
- β-Actin
Fig. 2

A

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B

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![Graphs showing % of control colony formation](#)
Fig. 3

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Fig. 4

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<tr>
<td>IR (2Gy)</td>
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<td>Niclo (1μM)</td>
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<td>p-JAK2</td>
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<td>Bcl2</td>
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<td>Mcl-1</td>
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<td>Active caspase 3</td>
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<tr>
<td>β-Actin</td>
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B

0 0.2 0.4 0.8 1 2 Niclo (μM)

A549-IRR

H157-IRR

C

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<tr>
<th></th>
<th>Ctrl</th>
<th>IR 5Gy</th>
<th>Niclo</th>
<th>Niclo + IR</th>
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A549

A549-IRR

% of control colony formation

IR Niclo Niclo + IR

*p < 0.001
Fig. 5

A

A549

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<tr>
<th>Ctrl shRNA</th>
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<td>STAT5</td>
<td>Bcl2</td>
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<td>Bcl-XL</td>
<td>β-Actin</td>
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A549-IRR

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<td>STAT1</td>
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<tr>
<td>STAT5</td>
<td>Bcl2</td>
</tr>
<tr>
<td>Bcl-XL</td>
<td>β-Actin</td>
</tr>
</tbody>
</table>

B

IR

0  5 Gy  0  5 Gy

Ctrl shRNA  STAT3 shRNA  Ctrl shRNA  STAT3 shRNA

A549

A549-IRR

C

% of control colony formation

IR  STAT3 shRNA  STAT3 shRNA + IR

D

IR

0  2 Gy x 2  0  2 Gy x 2

Ctrl shRNA  STAT3 shRNA

A549-IRR
Fig. 6

A

B

Active Caspase 3 IHC

C

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Fig. 7

A549 Xenografts

Mice Weight (g)

- Ctrl
- IR
- Niclo
- Niclo + IR

* p > 0.05 (vs Ctrl)
** p > 0.05 (vs Ctrl)
*** p < 0.001 (vs Ctrl)

B

<table>
<thead>
<tr>
<th>Parameter</th>
<th>1: Ctrl</th>
<th>2: IR</th>
<th>3: Niclo</th>
<th>4: Niclo + IR</th>
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<td>RBC (x10^6/μL)</td>
<td>6, 5, 4, 3</td>
<td>7, 6, 5, 4</td>
<td>8, 7, 6, 5</td>
<td>9, 8, 7, 6</td>
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<td>Hb (g/dL)</td>
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<td>ALT (IU/L)</td>
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<td>BUN (mg/dL)</td>
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C

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Molecular Cancer Therapeutics

Disruption of STAT3 by niclosamide reverses radioresistance of human lung cancer
Shuo You, Rui Li, Dongkyoo Park, et al.

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