Chemotherapy-Induced CXC-Chemokine/CXC-Chemokine Receptor Signaling in Metastatic Prostate Cancer Cells Confers Resistance to Oxaliplatin through Potentiation of Nuclear Factor-κB Transcription and Evasion of Apoptosis

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ABSTRACT

Constitutive activation of nuclear factor (NF)-κB is linked with the intrinsic resistance of androgen-independent prostate cancer (AIPC) to cytotoxic chemotherapy. Interleukin-8 (CXCL8) is a transcriptional target of NF-κB whose expression is elevated in AIPC. This study sought to determine the significance of CXCL8 signaling in regulating the response of AIPC cells to oxaliplatin, a drug whose activity is reportedly sensitive to NF-κB signaling in AIPC. Administration of oxaliplatin to PC3 and DU145 cells increased NF-κB activity, promoting antiapotptic gene transcription. In addition, oxaliplatin increased the transcription and secretion of CXCL8 and the related CXC-chemokine CXCL1 and increased the transcription and expression of CXC-chemokine receptors, especially CXC-chemokine receptor (CXCR) 2, which transduces the biological effects of CXCL8 and CXCL1. Stimulation of AIPC cells with CXCL8 potentiated NF-κB activation in AIPC cells, increasing the transcription and expression of NF-κB-regulated antiapoptotic genes of the Bcl-2 and IAP families. Co-administration of a CXCR2-selective antagonist, AZ10397767 (Bioorg Med Chem Lett 18:798–803, 2008), attenuated oxaliplatin-induced NF-κB activation, increased oxaliplatin cytotoxicity, and potentiated oxaliplatin-induced apoptosis in AIPC cells. Pharmacological inhibition of NF-κB or RNA interference-mediated suppression of Bcl-2 and survivin was also shown to sensitize AIPC cells to oxaliplatin. Our results further support NF-κB activity as an important determinant of cancer cell sensitivity to oxaliplatin and identify the induction of autocrine CXCR2 signaling as a novel mode of resistance to this drug.

Interleukin-8 (CXCL8) is a proinflammatory CXC-chemokine whose expression is primarily regulated by the activator protein-1 and NF-κB transcription factors (Brat et al., 2005). Overexpression of this chemokine has been detected in the serum of patients with metastatic prostate cancer (CaP) (Veltri et al., 1999; McCarron et al., 2002), whereas colorimetric in situ hybridization has reported elevated expression of CXCL8 in the tumor cells of androgen-independent CaP (AIPC) tissue (Uehara et al., 2005). Recently, we and others have demonstrated elevated CXCL8 expression and CXCL8 receptor expression in cancer cells of prostate biopsy tissue (Huang et al., 2005a; Murphy et al., 2005). Using immunohistochemistry, we determined that the intensity of CXCL8, CXCR1, and CXCR2 staining increased with stage of disease, reaching a maximal level in AIPC. The concurrent expression of the ligand and its receptors suggests that CaP cells are subject to a continuous autocrine CXCL8 signaling stimulus in situ. Significantly, increased expression of CXCL8 has been correlated with the angiogenesis, tumorigenicity, and incidence of lymph node metastasis arising from orthotopic or xenograft implantation of human AIPC cells in athymic nude mice (Inoue et al., 2000; Kim et al., 2001), suggesting...
that deregulation of this chemokine in patients may have functional significance with regard to disease progression.

Increasingly, monitoring CXCL8 expression in cancer patients has been used as a prognostic marker in assessing patient response to chemotherapy. In ovarian cancer, elevated serum CXCL8 levels identify those patients with a high residual tumor burden after paclitaxel therapy (Uslu et al., 2005), whereas high levels of CXCL8 expression in the peritoneal fluid and serum correlate with a poor initial response to paclitaxel chemotherapy (Penson et al., 2000). Likewise, decreased CXCL8 serum levels have been described as an indicator of response to chemotherapy in stage IV melanoma (Brennecke et al., 2005) and non-small cell lung cancer patients (Orditura et al., 2002), whereas reductions in intratumoral CXCL8 expression has been reported in esophageal adenocarcinoma patients exhibiting a complete pathological response to chemotherapy (Abdel-Latif et al., 2005). Chemotherapy agents have been shown to directly regulate CXCL8 transcription in cancer cells. Paclitaxel increases CXCL8 transcription and secretion in ovarian, breast, and lung cancer cell lines (Collins et al., 2000; Uslu et al., 2005). Likewise, administration of doxorubicin (Adriamycin) and 5-fluoro-2′-deoxyuridine to breast cancer cells (De Larco et al., 2001), the addition of fluorouracil to oral cancer cells (Tamatani et al., 2004), doxorubicin addition to small cell lung cancer cells (Shibakura et al., 2003), and dacarbazine administration to melanoma cells (Lev et al., 2003) all result in increased CXCL8 expression. However, the significance of this chemokine in modulating the response of cancer cells to chemotherapy is less well understood. Tumor necrosis factor-related apoptosis-inducing ligand-mediated increases in CXCL8 expression attenuate cell death as a consequence of decreased DR4 expression and reduced caspase-8 activation in ovarian carcinoma cells (Abdollahi et al., 2003). We have also demonstrated that CXCL8 signaling regulates the transcription of the native caspase-8-inhibitory protein, c-FLIP, to attenuate tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis in prostate cancer cells (Wilson et al., 2008). Conversely, the increased expression of CXCL8 was not shown to affect the sensitivity of osteosarcoma cells to paclitaxel (Duan et al., 2002).

The constitutive activity of NF-κB detected in AIPC cell lines and tissue is proposed to underpin the poor response of this disease to chemotherapy (Fradet et al., 2004; Sweeney et al., 2005). Likewise, treatment with oxaliplatin has been shown to modulate the NF-κB activity (Rakitina et al., 2003). In addition, we demonstrate that oxaliplatin-induced CXCR2 signaling potentiates NF-κB activation and potentiates anti-apoptotic gene expression. Consistent with a role for CXCL8 signaling in promoting cellular resistance to a chemical stress, we further show that inhibition of CXCR2 signaling and its downstream effectors, NF-κB, Bcl-2, and survivin, increases the sensitivity of AIPC cells to undergo oxaliplatin-induced apoptosis. These findings support our recent characterization of CXCL8 signaling in mediating the attenuated response and reduced sensitivity of hypoxic AIPC cells to etoposide (Maxwell et al., 2007).

Materials and Methods

Cell Culture. PC3 and DU145 cells were sourced and cultured as described previously (MacManus et al., 2007; Maxwell et al., 2007).

Chemicals. Chemicals were sourced from Sigma-Aldrich (St. Louis, MO) unless otherwise stated. BAY 11-7082 and SC-514 were purchased from Calbiochem (La Jolla, CA). Oxaliplatin (L-OHP) (Wyynarowski et al., 2000) was obtained from the Bridgewater Chemotherapy Suite, Belfast City Hospital. AZ10397767 (AZ767) was kindly provided by Dr. Simon T. Barry and Dr. David Blakey (AstraZeneca, Alderley Park, Cheshire, UK) (Walters et al., 2008).

Immunoblotting. Protein was prepared, resolved, and blotted as described previously (Murphy et al., 2005; MacManus et al., 2007). Membranes were probed with monoclonal antibodies to anti-CXCL8 (1:150 dilution) (Abcam plc, Cambridge, UK), anti-CXCR1 (1:500 dilution) or anti-CXCR2 (1:250 dilution) (BioSource International, Camarillo, CA), anti-Bcl-2 (1:500 dilution) or anti-survivin (1:1000 dilution) (all from Cell Signaling Technology Inc., Danvers, MA), and poly(ADP-ribose) polymerase (PARP; 1:500 dilution) (Biogenes, San Diego, CA) overnight at 4°C. After washing in Tris-buffered saline/0.1% Tween 20, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (GE Healthcare, Chalfont St. Giles, UK). Specific staining was detected using chemiluminescence (SuperSignal, Pierce Chemical, Rockford, IL; or ECL Plus; GE Healthcare). Equal loading was assessed using a GAPDH mouse monoclonal primary antibody.

ELISA. Cells (1 × 10^5 cells/well) were incubated overnight at 37°C in a humidified 5% CO2 atmosphere and replenished in serum-free RPMI 1640 medium before treatment with oxaliplatin. Cell media were collected at indicated times, processed, and subjected to specific ELISA. CXCL8 levels were measured using the Pelikine Compact CXCL8Elisa Kit (Sanquin Reagents, Amsterdam, The Netherlands), whereas CXCL1 levels were determined using the Quantikine kit (R&D Systems Europe Ltd, Abingdon, Oxfordshire, UK). The manufacturer’s instructions were employed in the application of each ELISA kit.

Electromobility Shift Assays. Nuclear extracts (8 μg of protein) were incubated with 35,000 cpm of a 22-bp oligonucleotide containing the NF-κB consensus sequence, which had been previously end-labeled with [γ-32P] ATP (10 mCi/mmol) by T4 polynucleotide kinase. Incubations were performed for 30 min at room temperature in the presence of 2 μl of poly(dI-dC) and 100 μM Tris-HCl, pH 7.5, containing 10 mM EDTA, 50 mM dithiothreitol, and 40% (v/v) glycerol. The NF-κB complexes were resolved on 5% acrylamide gels, and the migration of NF-κB complexes was determined by detection of radioactivity on the gel by autoradiography.

Luciferase Reporter Assays. Cells (1 × 10^5 cells/well) were seeded in six-well plates and incubated in RPMI 1640 medium at 37°C for 48 h. Cells were then transfected using GeneJuice (Merck, Darmstadt, Germany) according to the manufacturer’s protocol incorporating 2 μg of pGL3 basic vector (Promega, Madison, WI) and 2 μg of an NF-κB-LUC-pGL3 plasmid (kindly provided by Dr. James Purcell, Queens University Belfast). Cells were also cotransfected with 0.2 μg of a Renilla luciferase plasmid as a transfection control for pGL3 and NF-κB. Transfected cells were incubated for a further 24 h before drug addition. Either oxaliplatin or the CXCL-chemokine was added for the desired time, and the samples were analyzed by luciferase assay using the Promega Dual Luciferase assay kit (Promega) according to the manufacturer’s protocol.

Quantitative Real-Time PCR Analysis. RNA was harvested from cultured cells using RNAsafe (Biogene, Poole, Dorset, UK) according to the manufacturer’s instructions, and cDNA was synthesized from 2 μg of total RNA by priming with random hexamers.
were normalized to control values, and statistical analyses of the
Cells were incubated at 37°C in a humidified 5% CO2 atmosphere for
absence or presence of AZ10397767 (20 nM) or BAY 11-7082 (1
1 gTC (MWG Biotech, High Point, NC). The qPCR reaction consisted of
gCT CAg Agg TAg TAg; survivin reverse, CgC ACT TTC TTC TTC TCA;
CXCR2 reverse, gCT TCA Agg AgC Tgg AA; survivin targeted, AAC gAg CCA gAC UUg gCC CA; and survivin
CCC UUC; Bcl2 scrambled, CCU UCA gCC gCA UgC UgC CCA;
CXCL8 reverse, CAT AAT TTC TgT gTT ggC gCA gTg Tgg
primers used were: CXCL8 forward, ATg ACT TCC AAg CTg gCC
described previously (Maxwell et al., 2007). The forward and reverse
series particle count and size analyzer (Beckman Coulter, Fullerton,
72 h, then trypsinized and counted in triplicate using a Coulter Z
count analysis. Cells (1 × 10^5 cells/well) were allowed to
Cell Count Analysis. Cells were replaced with fresh RPMI 1640
medium and treated with a range of concentrations of oxaliplatin in the
absence or presence of AZ10397767 (20 nM) or BAY 11-7082 (1 μM).
Cells were incubated at 37°C in a humidified 5% CO2 atmosphere for
72 h, then trypsinized and counted in triplicate using a Coulter Z
Series particle count and size analyzer (Beckman Coulter, Fullerton,
CA) at threshold values of TUPPER 21 and TLOWER 5. Cell numbers
were normalized to control values, and statistical analyses of the
data were performed using GraphPad Prism 3.0 software (GraphPad
Software Inc., San Diego, CA).

siRNA Strategy. Cells were seeded at 5 × 10^5 per 90 plate in
Opti-MEM I (Invitrogen)-10% (v/v) fetal calf serum medium and
allowed to grow to 50% confluence. siRNA oligonucleotide sequences
were designed as follows: Bel2 targeted, CAg C Ug CAC C Ug ACg
CCC UUC; Bel2 scrambled, CUC UCA gCC gCA UgC UgC CCA;
survivin targeted, AAC gAg CCA gAC UgU gCC CCA; and survivin
scrambled, ACU gCc AAU CCA gCa gCc. siRNA transfections were
conducted in fresh RPMI 1640 medium. Cells were treated with increas-
ing concentrations of oxaliplatin and incubated for a further 72 h
before fixation in 100% ethanol. Cellular DNA profile was evaluated by
propidium iodide staining of cells using an EPICS XL Flow Cy-
tometer (Beckman Coulter). In some experiments, oxaliplatin was
administered in the presence of 20 nM AZ10397767 or 1 μM oxaliplatin (Fig. 1c, left and right, respectively). In addition,
oxaliplatin was also shown to increase Bcl-2 and survivin
protein expression in PC3 (Fig. 1c, left) and DU145 (Fig. 1c, right) cells.

Oxaliplatin Potentiates CXCL8 and CXC-Chemokine
Receptor Expression in PC3 Cells. Transcription of the
CXCL8 gene is regulated by NF-κB. Using a qPCR protocol,
oxaliplatin was shown to induce a rapid and sustained in-
crease in the CXCL8 mRNA transcript level in PC3 and
DU145 cells (Fig. 2a, left). Furthermore, oxaliplatin treat-
ment induced the expression of the related CXC-chemokine,
CXCL1, in each of these CaP cell lines (Fig. 2a, left). Furthermore, oxaliplatin was at-
tested by addition of the NF-κB inhibitor, BAY 11-7082 (Fig.
2b), suggesting that NF-κB activation is the predominant
factor underpinning oxaliplatin-induced transcriptional reg-
ulation of these CXC-chemokines. Immunoblotting also con-
firmed a time-dependent increase in the intracellular CXCL8
protein level in response to 1 μM oxaliplatin (Fig. 2c),
whereas ELISA-based analysis of PC3 or DU145 cell culture
medium revealed an increased level of CXCL8 and CXCL1
secretion from each of these CaP cell lines after exposure to
1 μM oxaliplatin for 3 h (Fig. 2d).

It is significant that the administration of oxaliplatin also
increased the expression of CXCR1 and CXCR2, the recep-
tors that mediate the biological activity of both CXCL8 and
CXCL1. The increase in the mRNA transcript level detected for
CXCR2 was more sustained and greater in magnitude than
that detected for CXCR1 in both the PC3 and DU145
cells in response to 1 μM oxaliplatin, respectively (Fig. 2e).
Immunoblotting also confirmed increased CXCR1 and CXCR2
receptor expression in the PC3 and DU145 cells after addition of
oxaliplatin, especially that of the CXCR2 receptor (Fig. 2f). As
a consequence, the potentiation of CXCL8 and CXCL1 secretion and
the increase in CXCR2 expression suggest that exposure to
oxaliplatin increases the magnitude of the autocrine CXC-che-
monine signaling stimulus that AIPC cells are subject to and,
furthermore, promotes a selective increase in CXCR2-mediated
signaling.

CXC-Chemokine Signaling Potentiates NF-κB Trans-
scription in AIPC Cells. Further experiments were con-
ducted to characterize the effect of CXCL8 signaling upon
established cell survival pathways, focusing on whether
NF-κB was a downstream effector of CXCL8 signaling in these AIPC cells. PC3 cells were stimulated with 3 nM recombinant human-CXCL8 (rCXCL8), and an EMSA was used to determine whether this stimulus increased the DNA binding activity of NF-κB. Increased complexing of NF-κB with DNA was detected within 20 min of adding rCXCL8, reaching a peak level of interaction at 40 min (Fig. 3a, left). Analysis over a longer time course also confirmed that NF-κB complexing with DNA was detected within 1 h of stimulation with rCXCL8 and identified a secondary increase in the DNA binding of this transcription factor 4 h after addition of rCXCL8 (Fig. 3a, right). The secondary increase in NF-κB complexing was lower in intensity than the initial response to exogenous rCXCL8. We also examined the receptor selectivity underpinning the CXCL8-induced activation of NF-κB and the CXCL8-induced potentiation of Bcl-2 expression. Blockade of CXCR2 receptor signaling was effected using a small-molecule CXCR2 receptor antagonist, AZ10397767, which when administered at a concentration (20 nM) selectively antagonizes CXCR2 activation but has no activity on the CXCR1 receptor in vitro (Walters et al., 2008). In EMSA analysis, CXCL8-promoted DNA binding of NF-κB was attenuated in the presence of AZ10397767 (Fig. 3b). Luciferase reporter assays were also conducted to demonstrate that CXC-chemokine signaling not only potentiated nuclear translocation and DNA binding of NF-κB but also induced the transcriptional activity of this factor in AIPC cells. Stimulation with either 3 nM CXCL8 (Fig. 3c, left) or 3 nM CXCL1 (Fig. 3c, right) was observed to increase NF-κB-promoted luciferase activity in both PC3 and DU145 cells. Consistent with the EMSA analysis, the CXC-chemokine-promoted NF-κB transcriptional activation was attenuated by coadministration of the CXCR2 antagonist, AZ10397767.

**CXCL8 Signaling Potentiates Antiapoptotic Gene Transcription in AIPC Cells.** qPCR analysis was conducted to determine whether CXCL8 signaling increased the transcriptional activity of established, NF-κB-regulated antiapoptotic genes. Bcl-2 mRNA transcript levels were shown to increase by a factor in excess of 3- and 2.5-fold within 1 h of adding 3 nM rCXCL8 to PC3 and DU145 cells, respectively (Fig. 3d, left). A sustained increase in the transcription of the Bcl-2 gene was evident in both AIPC cells at further time points out to 12 h after stimulation. CXCL8 signaling also induced statistically significant increases in survivin mRNA transcript levels in PC3 and DU145 cells (Fig. 3d, right). Immunoblotting further confirmed that CXCL8 signaling increased the expression of these representative NF-κB-regulated, antiapoptotic genes in both PC3 (Fig. 4b, left) and
DU145 (Fig. 4b, right) cells. Expression of Bcl-2 was observed to increase in both cell lines at time points out to 4 h after stimulation, with a secondary increase in expression detected 10 h after stimulation. This may reflect the secondary increases in NF-κB/DNA binding and the secondary rise in transcript levels detected for this gene in each of the AIPC cells after stimulation with rCXCL8. In contrast, CXCL8 induced a sustained increase in the expression of survivin out to 6 h after stimulation in both AIPC cells (Fig. 4b). Similar to the effect of CXCL8, CXCL1 also induced increases in the expression of Bcl-2 and survivin in these AIPC cells, although the induction of antiapoptotic protein expression in
response to this chemokine was more transient in the DU145 cells than PC3 cells (Fig. 4c).

To confirm the mechanism regulating CXC-chemokine-induced antiapoptotic gene expression, PC3 and DU145 cells were stimulated with 3 nM rCXCL8 in the absence and presence of the CXCR2 antagonist, AZ10397767, or the NF-κB inhibitor, BAY 11-7082. AZ10397767 was shown to reverse the CXCL8-promoted increase in Bcl-2 and survivin mRNA transcript levels to basal or sub-basal levels in both the PC3 and DU145 cells (Fig. 4d). Likewise, administration of BAY 11-7082 attenuated the CXCL8-promoted transcription of the Bcl-2 and survivin genes in each of these AIPC cell lines (Fig. 4d). Reduced expression of Bcl-2 and survivin protein was also observed in immunoblotting experiments after inhibition of CXCL8-signaling using AZ10397767 or BAY 11-7082 (data not shown). In accordance, our data indicate that the CXCR2 receptor primarily couples CXCL8 to NF-κB activation and that CXCR2/NF-κB signaling underpins CXC-chemokine-promoted regulation of antiapoptotic protein expression in these cells.

**Inhibition of CXCR2 Signaling Attenuates Oxaliplatin-Induced Activation of NF-κB.** Because CXCR2-mediated signaling promotes NF-κB activation in PC3 cells, we determined whether oxaliplatin-induced CXC-chemokine signaling may reinforce and prolong oxaliplatin-induced NF-κB activation. Oxaliplatin was added to PC3 cells in the absence or presence of the CXCR2 antagonist, AZ10397767. EMSA analysis confirmed that coadministration of AZ10397767 attenuated oxaliplatin-induced complexing of NF-κB with the radiolabeled oligonucleotide, with marked decreases observed 1 and 2 h after addition of the two drugs (Fig. 5a). Coadministration of AZ10397767 was also shown to inhibit oxaliplatin-induced NF-κB transcriptional activity, using a further luciferase reporter assay (Fig. 5b). Furthermore, qPCR analysis confirmed that the presence of AZ10397767 attenuated the oxaliplatin-induced increases in mRNA transcript levels for each of the CXC-chemokines (CXCL8 and CXCL1) and antiapoptotic genes (Bcl-2 and survivin) in the PC3 (Fig. 5c, left) and DU145 (Fig. 5c, right) cells. Coadministration of AZ10397767 also attenuated oxaliplatin-induced increases in Bcl-2 and survivin protein expression in AIPC cells (data not shown).

**Inhibition of CXCR2 Signaling Potentiates the Cytotoxicity and Induction of Apoptosis by Oxaliplatin in AIPC Cells.** Cell count analysis was used to determine the sensitivity of AIPC cells to a 72-h continuous exposure to oxaliplatin. PC3 and DU145 cells were largely insensitive to oxaliplatin at concentrations up to 1 μM. However, reduced cell viability was detected above this concentration. Interestingly, the coadministration of AZ10397767 rendered both PC3 and DU145 cells more sensitive to oxaliplatin-induced cytotoxicity, with marked decreases in cell number detected in cell populations treated with lower concentrations of this platinum drug (Fig. 6a). Analysis of cell count data conducted on either PC3 or DU145 cells determined that a two-site curve fit was statistically superior for oxaliplatin-induced cytotoxicity in the presence of AZ10397767 as compared with the one-site model, which was preferential for modeling the effects of oxaliplatin alone. The potentiating effect of AZ10397767 on oxaliplatin-induced cytotoxicity in PC3 cells was evident at concentrations in the 10 to 50 nM range but not at higher concentrations of oxaliplatin (i.e., greater than 10 μM). The interaction between oxaliplatin and AZ10397767 was synergistic in the PC3 and DU145 cell lines, respectively, at oxaliplatin concentrations ranging from 0.1 to 1 μM (RI > 1.5 and RI > 1.3, respectively). In addition, coadministration of AZ10397767 corresponded to calculated increases of 10- and 19-fold in the potency of oxaliplatin in PC3 cells (oxaliplatin and AZ10397767, IC50 = 0.2 ± 0.005 μM versus oxaliplatin alone, IC50 = 1.9 ± 0.01 μM; n = 6, p < 0.01) and DU145 cells (IC25 = 0.021 ± 0.02 versus 0.4 ± 0.09 μM; n = 3, respectively).

Addition of AZ10397767 by itself failed to induce apoptosis in either PC3 or DU145 cells. However, coadministration of
Fig. 4. Characterization of CXC-chemokine-induced antiapoptotic protein expression in AIPC cells. a, bar graphs illustrating the effect of 3 nM rCXCL8-induced signaling upon the mRNA transcript levels detected for Bcl-2 (left) and survivin (right) in PC3 and DU145 cells. All values were normalized to the levels detected in unstimulated cells. Data shown are the mean ± S.E.M. values, calculated from three independent experiments.

b, immunoblots demonstrating the time-dependent increases in Bcl-2 and survivin expression in PC3 (left) and DU145 (right) cells after stimulation with 3 nM rCXCL8. c, immunoblots demonstrating the time-dependent increases in Bcl-2 and survivin expression in PC3 (left) and DU145 (right) cells after stimulation with 3 nM rCXCL1. All immunoblots shown in the figure are representative of two to three experiments.

d, bar graph illustrating the effect of the CXCR1 antagonist, AZ10397767 (AZ767), or the NF-κB antagonist BAY 11-7082 on rCXCL8-induced increases in the mRNA transcript levels detected for Bcl-2 (left) and survivin (right) in PC3 and DU145 cells. Data shown are the mean ± S.E.M. values, calculated from five independent experiments. Statistically significant differences in mRNA transcript level were determined using Student’s t test: *, p < 0.05; **, p < 0.01; and ***, p < 0.001.
AZ10397767 with 0.1 or 1 μM oxaliplatin resulted in a marked increase in the sub-G0/G1 cell population in either cell line (Fig. 6b, left and right). In PC3 and DU145 cells, respectively, the level of apoptosis induced by oxaliplatin increased from 5.48 ± 0.69 to 10.55 ± 1.74% (p < 0.05; n = 4) and 4.99 ± 0.49 to 9.13 ± 2.5% (p < 0.05; n = 3) in the presence of the CXCR2 antagonist. AZ10397767-mediated potentiation of oxaliplatin-induced apoptosis was also studied by immunoblotting for cleavage of the caspase-3 and caspase-7 substrate PARP (Fig. 6c, left and right). Although treatment with 1 μM oxaliplatin reduced full-length PARP expression in PC3 cells relative to untreated cells, the concurrent administration of AZ10397767 with 1 μM oxaliplatin effected a more significant reduction in PARP expression. Likewise, in the DU145 cells, AZ10397767 had the ability to enhance oxaliplatin-induced cleavage of PARP. In accordance, these cell-based and molecular experiments indicate that inhibition of CXCR2 receptor signaling potentiates oxaliplatin-induced apoptosis in AIPC cells.

Inhibition of NF-κB Signaling Increases Sensitivity of AIPC Cells to Oxaliplatin. Further cell count assays were conducted to determine whether the suppression of drug-induced NF-κB activity by AZ10397767 may explain its ability to enhance oxaliplatin cytotoxicity in AIPC cells. In these experiments, the activity of NF-κB was perturbed using well characterized pharmacological inhibitors of this transcription factor. Administration of BAY 11-7082 at a final concentration of 1 μM to PC3 and DU145 cells was shown to be effective in enhancing the cytotoxicity of oxaliplatin in each of these AIPC cells (Fig. 7a). As observed with AZ10397767, noniterative nonlinear regression analysis of the cell count data predicted a two-site model to be more appropriate in modeling the data resulting from the coadministration of oxaliplatin with BAY 11-7082. The combination of oxaliplatin with BAY 11-7082 increased the calculated IC25 value from 0.73 ± 0.63 to 0.008 ± 0.01 μM (p < 0.05; n = 3) in PC3 cells. In DU145 cells, inhibition of NF-κB activity using BAY 11-7082 increased the calculated IC30 from 0.287 ± 0.456 to 0.015 ± 0.04 μM (p < 0.05; n = 3). Although oxaliplatin was ineffective in inducing apoptosis in either cell line, treatment with 1 μM BAY 11-7082 did induce apoptosis in PC3 (Fig. 7b, left) and DU145 (Fig. 7b, right) cells. Furthermore, the inhibition of NF-κB activity using BAY 11-7082 further enhanced the apoptosis induced by exposure of PC3 or DU145 cells to 0.1 and/or 1 μM oxaliplatin (Fig. 7b). These results were verified by use of a second inhibitor of NF-κB, the IκB kinase inhibitor, SC-514, which was administered to PC3 cells at a
Suppression of Bcl-2 and Survivin Sensitizes PC3 Cells to Oxaliplatin. The functional significance of the downstream transcriptional targets of NF-κB, Bcl-2 and survivin, in underpinning the resistance of AIPC cells to oxaliplatin was studied using gene-targeted RNA interference-based approaches to selectively suppress the expression of these genes in AIPC cells. PC3 cells were initially transfected using 50 nM of a single gene-specific oligonucleotide (oligo) against either Bcl-2 (Bcl2-T) or survivin (Sur-T) or with a scrambled oligonucleotide (Sc), used at an identical concentration. At this concentration, transfection of PC3 cells with the Bcl2-T oligo was shown to decrease but not abrogate Bcl-2 expression in these cells 72 h after transfection. In contrast, transfection with Sur-T oligonucleotide was shown to be markedly more effective in depleting survivin expression in the PC3 cells (Fig. 8a, top and bottom).

The effect of transiently reducing antiapoptotic gene expression upon the induction of apoptosis in PC3 cells in the absence and presence of oxaliplatin was determined using FACS analysis. c, immunoblots illustrating loss of full-length PARP expression in PC3 cells (left) and PARP cleavage in DU145 cells (right) in response to addition of 1 μM oxaliplatin in the absence and presence of AZ767. Equal protein loading was confirmed by reprobing the membrane for GAPDH expression. Statistically significant differences in cell viability or apoptotic cell populations were determined using Student’s t-test: *, p < 0.05; and **, p < 0.01.
spontaneous apoptosis in PC3 cells. In contrast, abrogation of survivin expression using the Sur-T oligo only effected a modest increase in the apoptotic fraction (7.55 ± 0.4% of the cell population; p < 0.01), indicating that PC3 cells are not critically dependent upon survivin for viability. Although treatment with 1 μM oxaliplatin alone was largely ineffective in increasing the level of apoptosis in Sc oligo-transfected PC3 cells, oxaliplatin did increase the apoptotic cell population to 10.58 ± 0.72 and 8.3 ± 0.4% in 50 nM Bcl2-T-transfected PC3 cells (p < 0.001; n = 4) and 50 nM Sur-T oligo-transfected PC3 cells (p < 0.05, n = 4), respectively (Fig. 8b). The relative importance of these antiapoptotic proteins in modulating PC3 cell viability and the sensitivity of these cells to oxaliplatin was reaffirmed using PARP cleavage as a marker of apoptosis induction. In Sc oligo-transfected cells, treatment with 1 μM oxaliplatin resulted in a modest decrease in PARP expression. In contrast, transfection of PC3 cells with 50 nM Bcl2-T had a pronounced effect in promoting PARP degradation in both the absence and presence of 1 μM oxaliplatin, shown by the reduced expression of the full-length protein. Suppression of survivin alone had no effect on PARP expression but did potentiate the degradation of PARP observed when cells were cotreated with 1 μM oxaliplatin (Fig. 8c). Therefore, decreasing Bcl-2 expression in PC3 cells appears to be of major importance in sensitizing cells to oxaliplatin, whereas suppressing survivin expression contributes to an enhanced response.

To determine the effect of increasing the effectiveness of targeting Bcl-2 expression, PC3 cells were treated with increasing concentrations of the Bcl2-T oligo. Transfection with 200 nM Bcl2-T oligo was shown to deplete endogenous Bcl-2 expression in these cells in immunoblots conducted on protein lysates extracted from transfected cells (Fig. 8d). Suppression of Bcl-2 expression alone was observed to coincide with a spontaneous loss of full-length PARP expression and the emergence of PARP cleavage, indicative of apoptosis. Furthermore, use of this Bcl2-T oligo at this higher concentration induced a more marked increase in the apoptotic cell fraction detected by FACS analysis, increasing the level to 20.4 ± 2.9% (p < 0.001) (Fig. 8e). A further increase in the apoptotic cell fraction was observed after the addition of 1 μM oxaliplatin to 200 nM Bcl2-T-transfected PC3 cells. However, because of the high level of spontaneous apoptosis resulting from treatment with the Bcl2-T oligo at this higher concentration, there was no statistically significant enhancement when 200 nM Bcl2-T was combined with 1 μM oxaliplatin.

**Concurrent Suppression of Bcl-2 and Survivin Potentiates L-OHP Cytotoxicity in PC3 Cells.** Bcl-2 primarily antagonizes Bax/Bak-induced mitochondrial membrane permeabilization, whereas the survivin/IAP gene family directly inhibits the activation of downstream caspases, including caspase-9 (Reed, 2001). To determine whether oxaliplatin and CXCL8-promoted increases in Bcl-2 and survivin expression describe two complementary mechanisms of resistance to oxaliplatin, we determined the effect of concurrently suppressing Bcl-2 and survivin expression on oxaliplatin-induced apoptosis. The sensitivity of Bcl2-T/Sur-T cotransfected cells to undergo apoptosis was compared with the effect of transfecting PC3 cells with equivalent concentrations of either the Bcl2-T or Sur-T oligos alone or with a Sc oligo (Fig. 8b). All transfections were conducted using a final oligonucleotide concentration of 50 nM, given our observation of high levels of spontaneous apoptosis when antiapoptotic
gene oligos were used at increased concentrations. Cotransfection with 50 nM Bcl2-T and 50 nM Sur-T oligos was shown to induce a more pronounced accumulation of cells in the sub-G0/G1 peak (15.0 ± 1.3%; n = 4) relative to transfection with Bcl2-T (8.53 ± 0.58%, p < 0.05) or Sur-T (7.55 ± 0.41%) alone. In the context of sensitizing PC3 cells to oxaliplatin, the combined suppression of Bcl-2 and survivin potentiated the level of apoptosis detected in PC3 cells in response to oxaliplatin (sub-G0/G1 fraction of 18.77 ± 0.89%) in Bcl2-T/Sur-T cotransfected cells relative to that observed in Bcl2-T (10.58 ± 0.72%, p < 0.05, n = 4) or Sur-T transfected (8.3 ± 0.39%, p < 0.01, n = 4) cells.

**Discussion**

Deregulation of NF-κB has been proposed as a major factor contributing to the resistance of AIPC to chemotherapy. Nuclear localization of this transcription factor, indicative of constitutive activity, has been detected in biopsy tissues of AIPC (Fradet et al., 2004; Sweeney et al., 2004). Further
studies conducted in cell lines have shown that inhibition of NF-κB renders CaP cells more susceptible to apoptosis, mediated in part by the ability of this transcription factor to regulate antiapoptotic gene expression (Huang et al., 2005; Li et al., 2005). The sensitivity of colorectal cancer cells to oxaliplatin-induced death is adversely affected by elevated NF-κB activity (Rakitina et al., 2003), suggesting that the dysregulation of this transcription factor in AIPC may underpin the low sensitivity of this disease to oxaliplatin (Droz et al., 2003).

We have shown that administration of oxaliplatin further potentiates NF-κB activity in AIPC cells, increasing transcriptional regulation and expression of antiapoptotic genes. Furthermore, we have shown that oxaliplatin induced a dynamic process of increased CXCL8 gene transcription, intracellular protein expression, and ultimately secretion from PC3 cells that is predominantly mediated downstream of NF-κB activation. Oxaliplatin was also shown to increase the transcription and expression of the CXCL8 receptors, CXCR1 and CXCR2, in AIPC cells. To our knowledge, this is the first report demonstrating an effect of chemotherapy drugs upon the expression of these CXC-chemokine receptors in cancer cells. Furthermore, these observations are consistent with our recent demonstration that hypoxia-induced transcription of the CXCR1 and CXCR2 receptors is mediated in part by increased NF-κB transcriptional activity (Maxwell et al., 2007). As a consequence, our demonstration of increased ligand and receptor expression suggests that exposure of AIPC cells to oxaliplatin increases the magnitude of the autocrine CXCL8 signaling stimulus received by these cells.

To determine whether drug-induced or constitutive CXCL8 signaling may alter the sensitivity of the cells to the chemotherapy drug, we characterized the effect of stimulating AIPC cells with rCXCL8, focusing on the NF-κB cell survival pathway. Administration of pharmacologically relevant concentrations of rCXCL8 induced NF-κB activation, enhancing the transcription and expression of the antiapoptotic genes, Bcl-2 and survivin, in AIPC cells. These responses were attenuated by coadministration of the CXCR2 receptor antagonist, AZ10397767, at a concentration that selectively blocks CXCR2 activation. Furthermore, pharmacological inhibition of NF-κB attenuated the rCXCL8-induced increase in Bcl-2 expression, suggesting that the CXCR2 receptor primarily, but not exclusively, couples CXCL8 to the activation of NF-κB and transcriptional regulation of these antiapoptotic genes in AIPC cells.

The selective increase in CXCR2 expression suggests that signaling through this receptor may predominate over CXCR1 in dictating the response of AIPC cells to the administration of oxaliplatin. Although CXCR2 binds and is activated by CXCL8, this receptor also binds several related CXC-chemokines, including the growth-related oncogenes (CXCL1, CXCL2, CXCL3), whose expression is also regulated at the level of transcription by NF-κB (Brat et al., 2005). As a consequence, we have shown that oxaliplatin treatment also induces expression of CXCL1 in AIPC cells. As observed for CXCL8, stimulation of these AIPC cells with CXCL1 potentiates a CXCR2-dependent activation of NF-κB and increases antiapoptotic protein expression. Irrespective of the ligand activating the receptor, the significance of CXCR2-induced signaling in modulating the sensitivity of AIPC cells to oxaliplatin was illustrated by the ability of the CXCR2 antagonist, AZ10397767, to attenuate oxaliplatin-induced NF-κB activation and reduce oxaliplatin-induced transcription of the CXCL8, CXCL1, Bcl-2, and survivin genes. Coadministration of AZ10397767 also markedly increased the sensitivity of AIPC cells to oxaliplatin and enhanced oxaliplatin-induced apoptosis.

Oxaliplatin cytotoxicity in AIPC cells was also increased by direct targeting of NF-κB activation or its downstream transcriptional targets. Pharmacological inhibition of NF-κB using the BAY 11-7082 or SC-514 compounds sensitized AIPC cells to low but not high concentrations of oxaliplatin, whereas RNA interference-mediated suppression of Bcl-2 and survivin was shown to increase the level of oxaliplatin-induced apoptosis detected in PC3 cells. Interestingly, reduction of Bcl-2 and survivin expression alone was shown to induce apoptosis in PC3 cells, suggesting that this AIPC cell line may have a marked dependence on the expression of these antiapoptotic proteins for maintenance of cell viability. The concurrent suppression of both Bcl-2 and survivin was also shown to result in a significantly greater induction of apoptosis in PC3 cells in response to oxaliplatin. Therefore, we propose that the potentiation of CXCL8- and CXCL1-induced CXCR2 signaling underpins a novel and contributing mode of resistance to oxaliplatin that is mediated, at least in part, by sustained NF-κB transcription and reinforcing the expression of antiapoptotic proteins in AIPC cells (Fig. 8).

Prior studies have shown that the combination of oxaliplatin with the heat shock protein-90 inhibitor 17-N-allylamino-17-demethoxygeldanamycin results in a more effective suppression of cellular NF-κB activation and promotion of anticancer activity (Rakitina et al., 2003). Therefore, the ability of AZ10397767 to also suppress NF-κB activity and reduce antiapoptotic protein expression in the presence of oxaliplatin is a likely explanation of its capacity to increase the cytotoxicity of this drug in AIPC cells. Our observations that coadministration of either the CXCR2 antagonist or the NF-κB inhibitor BAY 11-7082 sensitizes the AIPC cells to low rather than high concentrations of oxaliplatin suggests that CXCL8/CXCL1-induced NF-κB-signaling in AIPC cells confers a surmountable resistance to a specific mode of oxaliplatin-induced cytotoxicity. Further detailed studies will be required to establish the precise mechanism (mitochondrial or death receptor mediated) by which CXCR2 and NF-κB signaling impact on oxaliplatin-induced apoptosis in AIPC cells. Prior studies have shown that oxaliplatin induces apoptosis in colorectal cancer cells through the activation of caspase-8 and Bid cleavage, suggesting a role for the extrinsic apoptosis pathway (Griffiths et al., 2004; Longley et al., 2006). Our current observations that CXCL8 signaling regulates Bcl-2 gene family expression together with our recently published data demonstrating that this chemokine promotes a NF-κB-driven expression of the endogenous caspase-8 inhibitor c-FLIP in CaP cells (Wilson et al., 2008) suggest that CXCL8 signaling may attenuate the capacity of oxaliplatin to initiate mitochondrial-driven apoptosis in these cells by altering the ratio of proapoptotic cleaved Bid to the mitochondrial stabilizing effect of increased Bcl-2 expression. Furthermore, our observation that CXCL8/CXCL1 signaling increases survivin expression in AIPC cells suggests a further mechanism downstream of the mitochondria by which AIPC cells may withstand the apoptosis-inducing stress experienced after the administration of oxaliplatin.
Several final issues warrant mention. First, oxaliplatin is currently employed in the treatment of metastatic colorectal cancer. Experiments conducted in our laboratory have characterized a similar induction of CXCL8 signaling in colorectal cancer cells and demonstrated that the inhibition of chemokine signaling also has a similar effect in sensitizing colorectal cancer cells to oxaliplatin (C. Purcell and D. Waugh, unpublished observations). Therefore, these ongoing studies point to a clinical relevance of CXC-chemokine signaling in determining the response of malignant cells to oxaliplatin in this disease setting. Furthermore, in conducting experiments in the parental p53 wild-type HCT116 and the matched p53-deficient HCT116 cells, we will be able to undertake a direct investigation of how p53 status influences the magnitude of the CXC-chemokine-promoted antiapoptotic response in cancer cells. This has not been possible in the current studies given the p53-deficient and p53 mutant status in the PC3 and DU145 cell lines, respectively. Finally, we have also conducted experiments to determine whether other platinum agents induce a similar response in prostate cancer cells. Administration of cisplatin was also shown to induce NF-κB activity and increase CXC-chemokine expression in AIPC cells. However, inhibition of CXCR2 signaling did not increase the sensitivity of these cells to cisplatin (C. Wilson and D. Waugh, unpublished observations). We suspect that this reflects the inability of this pharmacological intervention to overcome the primary mode of resistance, i.e., that of mismatch repair enzyme deficiency in AIPC cells (Chen et al., 2001) that drives resistance to cisplatin but not oxaliplatin adducts (Zdraveski et al., 2002; Chaney et al., 2004).

In summary, we indicate a role for NF-κB signaling and two of its transcriptional targets, Bcl-2 and survivin, in attenuating oxaliplatin cytotoxicity in AIPC cells. Furthermore, we report the significance of oxaliplatin-induced CXC-chemokine signaling in reinforcing NF-κB transcriptional activity and, consequently, potentiating the resistance of these cells to this platinum drug. In accordance, direct targeting of NF-κB, its transcriptional targets, or inactivating CXCR2-mediated signaling may be appropriate strategies to increase the effectiveness of using oxaliplatin to treat cancer (Fig. 9). The demonstration that CXCL8 signaling potentiates antiapoptotic protein expression and attenuates chemotherapy-induced apoptosis further links CXCL8 signaling to another described “hallmark” of cancer cells (Hanahan and Weinberg, 2000), that of evading apoptosis, in addition to its proposed roles in promoting angiogenesis, invasion and metastasis.

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