CHEMOTHERAPY-INDUCED CXC-CHEMOKINE/CXCR2 SIGNALING IN METASTATIC PROSTATE CANCER CELLS CONFERS RESISTANCE TO OXALIPLATIN THROUGH POTENTIATION OF NF-κB TRANSCRIPTION AND EVASION OF APOPTOSIS.

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a) **Running Title**: CXC-chemokine signaling attenuates platinum cytotoxicity.

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c) **Text Pages**: 42 (entire manuscript including references)
   Number of Tables: 0
   Number of Figures: 9
   Number of References: 39
   Number of Words in Abstract: 211
   Number of Words in Introduction: 721
   Number of Words in Discussion: 1418

d) **Non-Standard Abbreviations**:  
AIPC, androgen-independent prostate cancer  
CaP, prostate cancer  
EMSA, electromobility shift assay  
L-OHP, oxaliplatin;

e) **Chemotherapy, Antibiotics, and Gene Therapy**
ABSTRACT

Constitutive activation of 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 activity. Administration of oxaliplatin to PC3 and DU145 cells increased NF-κB activity promoting anti-apoptotic 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 CXCR2 that 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 anti-apoptotic genes of the Bcl-2 and IAP families. Co-administration of a CXCR2-selective antagonist, AZ10397767, attenuated oxaliplatin-induced NF-κB activation, increased oxaliplatin cytotoxicity and potentiated oxaliplatin-induced apoptosis in AIPC cells. Pharmacological inhibition of NF-κB or RNAi-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.
INTRODUCTION

Interleukin-8 (CXCL8) is a pro-inflammatory CXC-chemokine whose expression is primarily regulated by the AP-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) while 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 (Murphy et al., 2005; Huang 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 de-regulation 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 following paclitaxel-therapy (Uslu et al., 2005), while high levels of CXCL8 expression in the peritoneal fluid and serum correlate with a poor initial response to paclitaxel chemotherapy (Penson et al., 2000). Similarly, 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) while reductions in intra-tumoral 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 (Uslu et al., 2005; Collins et al., 2000).
Similarly, administration of adriamycin and 5-fluoro-2’-deoxyuridine to breast cancer cells
(DeLarco et al., 2001), the addition of 5-FU 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. Tumour necrosis factor-related apoptosis inducing ligand
(TRAIL)-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 TRAIL-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.,
2004). Since CXCL8 transcription is regulated by NF-κB (Brat et al., 2005), our study aimed to
determine whether this CXC-chemokine may contribute to the resistance of AIPC cells to
cytotoxic chemotherapeutic agents. We report our findings that the expression of CXCL8, a
related CXC-chemokine CXCL1 and their signaling competent receptors CXCR1 and CXCR2 are each increased in AIPC cells in response to administration of the platinum agent, oxaliplatin, a chemotherapy agent whose activity has been shown to be modulated by 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).
METHODS

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

Chemicals. Chemicals were sourced from Sigma Chemical Company (St. Louis, MO) unless otherwise stated. BAY 11-7082 and SC-514 were purchased from Calbiochem (La Jolla, CA). Oxaliplatin (L-OHP) (Woynarowski 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., 2007).

Immunoblotting. Protein was prepared, resolved and blotted as previously described (Murphy et al, 2005; MacManus et al., 2007). Membranes were probed with monoclonal antibodies to anti-CXCL8 (1:150 dilution) (Abcam Ltd., Cambridgeshire, UK), anti-CXCR1 (1:500 dilution) or anti-CXCR2 (1:250 dilution) (Biosource, Camirillo, CA) anti-Bcl2 (1:500 dilution), anti-Survivin (1:1000 dilution), (all from Cell Signaling Technology, Beverly, MA) and Poly(ADP-ribose) polymerase (1:500 dilution) (PARP; e-biosciences, San Diego, CA) overnight at 4°C. Following washing in TBS / 0.1% Tween, membranes were incubated with HRP-conjugated secondary antibodies (Amersham Life Sciences). Specific staining was detected using chemiluminescence (Supersignal, Pierce, Rockford, IL or ECL plus Amersham, Buckinghamshire, UK). Equal loading was assessed using a GAPDH mouse monoclonal primary antibody.
ELISA. Cells (1x10^5 cells/well) were incubated overnight at 37°C in a humidified 5% CO₂ atmosphere and replenished in serum free RPMI 1640 medium prior to treatment with oxaliplatin. Cell media was collected at indicated times, processed and subjected to specific ELISA. CXCL8 levels were measured using the Pelikine Compact™ CXCL8Elisa Kit (SanquinReagents, Amsterdam, The Netherlands) while CXCL1 levels were determined using the Quantikine® kit (R&D Systems, Abingdon, UK). The manufacturer’s instructions were employed in the application of each ELISA kit.

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

Luciferase Reporter Assays. Cells (1 x10^5 cells/well) were seeded in 6-well plates and incubated in RPMI 1640 medium at 37 °C for 48 h. Cells were then transfected using GeneJuice (Merck Chemicals) according to manufacturer’s protocol incorporating 2μg pGL3 basic vector (Promega, Madison, WI) or 2μg of an NF-κB-LUC-pGL3 plasmid (kindly provided by Dr. James Purcell, QUB). Cells were also co-transfected with 0.2μg of a Renilla luciferase plasmid as a transfection control for pGL3 and NF-κB. Transfected cells were incubated for a further 24 h prior to drug addition. Either oxaliplatin or the CXC-chemokine was added for the desired time and the
samples were analyzed by luciferase assay using the Promega Dual Luciferase assay kit (Promega, Madison, WI) according to manufacturer’s protocol.

**Quantitative Real-Time PCR Analysis.** RNA was harvested from cultured cells using RNAStat60™ (Biogenesis Ltd) according to manufacturer’s instructions and cDNA was synthesised from 2µg total RNA by priming with random hexamers (Invitrogen™ Life Technologies, Paisley, Scotland) and reverse transcribing with MMLV reverse transcriptase (Invitrogen™ Life Technologies, Paisley, Scotland), as per the manufacturer’s protocol. Quantitative real time PCR (qPCR) analysis was performed on the DNA Engine Opticon®2 Continuous Fluorescence Detector (MJ Research, Bio-Rad Laboratories, Inc. Waltham, MA) with product amplification determined by SYBR Green 1 fluorescence detection (Finnzymes, Oy, Espo, Finland). cDNA was synthesized as described previously (Maxwell et al., 2007). The forward and reverse primers used were: CXCL8 Forward: ATg ACT TCC AAg CTg gCC gTg g; CXCL8 Reverse: CAT AAT TTC TgT gTT ggC gCA gTg Tgg (Invitrogen™ Life Technologies, Paisley, Scotland); BCl2 Forward: AAA ggA CCT gAT CAT Tgg gg; BCl2 Reverse: CAA CTC TTT TCC TCC CAC CA; Survivin Forward: TCT gCT TCA Agg AgC Tgg AA; Survivin Reverse: CgC ACT TTC TTC gCA gTT TC; CXCR1 Forward: TgC ATC AgT gTg gAC CgT TA; CXCR1 Reverse: TgT CAT TTC CCA ggA CCT CA; CXCL1 Forward: TgC ATC AgT gTg gAC CgT TA; CXCR2 Reverse: CCg CCA gTT TgC TgT ATT g; CXCL1 Forward CCC AAg AAC ATC CAA AgT gTC A; CXCL1 Reverse: gTg gCT ATg ACT TCg gTT Tg; 18S Forward: CAT TCg TAT TgC gCC gCT A; 18S Reverse: CgA Cgg TAT CTg ATC gTC. (MWG Biotech AG, Ebersberg, Germany). The qPCR reaction consisted of 1µl undiluted cDNA, 0.225µM final concentration of forward and reverse primers and 2 X SYBR Green 1 master-mix.
(Finnzymes, Oy, Espoo, Finland). Standard cycling procedures were employed with an annealing temperature of 55°C for all primer pairs tested. Specific amplicon formation with each primer pair was confirmed by melt curve analysis. Gene expression was quantified relative to an 18S housekeeping gene.

**Cell Count Analysis.** Cells (1 x 10^5 cells/well) were allowed to adhere overnight. Media was replaced with fresh RPMI 1640 medium and treated with a range of concentrations of oxaliplatin in the absence or presence of AZ10397767 (20nM) or BAY 11-7082 (1μM). 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, Miami, FL) at threshold values of Tₑ 21 and Tₗ 5. Cell numbers were normalized to control values and statistical analyses of the data performed using GraphPad PRISM 3.0 software.

**siRNA strategy.** Cells were seeded at 5 x 10^5 per P90 plate in Optimem 1 (Invitrogen)-10% (v/v) FCS medium and allowed to grow to 50% confluency. SiRNA oligonucleotide sequences were designed as follows – Bcl2 Targeted CAg CUg CAC CUg ACg CCC UUC; Bcl2 Scrambled CCU UCA gCC gCA UgC UgC CCA; Survivin Targeted AAC gAg CCA gAC UUg gCC CA; Survivin Scrambled ACU ggC AAU CCA gCA gCC. SiRNA transfections were conducted by incubating cells in unsupplemented Optimem 1 medium using oligofectamine reagent (Invitrogen) for 4 h at 37°C, following which cells were replenished in 20% (v/v) FCS-enriched medium. The concentration of oligonucleotides employed and the period of incubation for each experiment is described in the relevant textual description of results and the corresponding figure legend.
**FACS Analysis.** Cells (5 x 10^4 cells) were seeded in a 6-well tissue culture plate, allowed to adhere overnight, then replenished in fresh RPMI 1640 medium. Cells were treated with increasing concentrations of oxaliplatin and incubated for a further 72 h prior to fixation in 100% ethanol. Cellular DNA profile was evaluated by propidium iodide staining of cells using an EPICS XL Flow Cytometer (Beckman Coulter). In some experiments, oxaliplatin was administered in the presence of 20nM AZ10397767 or 1µM BAY 11-7082.

**Statistical Analysis.** The nature of the interaction between oxaliplatin and AZ10397767 was determined by calculating the R index (RI), using the methods recently described (Longley et al., 2004). Differences observed in comparing apoptosis levels observed in control and drug-treated groups were analyzed for statistical significance using a two-tailed students-t-test comparison (Prism 3.0 software).
RESULTS

Oxaliplatin promotes NF-κB activation in AIPC cells.

Oxaliplatin induces cytotoxicity through formation of adducts with cellular DNA (Woynarowski et al., 2000). Previously published studies suggest that the sensitivity of cancer cell lines to oxaliplatin is modulated by the level of constitutive NF-κB activity (Rakitina et al., 2003). An electromobility shift assay (EMSA) and luciferase reporter assays were each conducted to determine whether oxaliplatin promoted NF-κB activation in AIPC cells. Initial studies conducted in the PC3 cell line confirmed that administration of 1 µM oxaliplatin resulted in a rapid increase in the DNA binding activity of NF-κB (Fig. 1a). Subsequently, luciferase reporter assays confirmed an increased level of NF-κB transcriptional activity in two AIPC cell lines, PC3 and DU145 cells (Fig. 1b). Furthermore, using real-time PCR analysis, we observed increased mRNA transcript levels for two representative NF-κB target genes, Bcl-2 and survivin following exposure of PC3 and DU145 cells to 1µM oxaliplatin (Fig. 1c, left and right panels, respectively). In addition, oxaliplatin was also shown to increase Bcl-2 and survivin protein expression in PC3 (Fig. 1c, left panel) and DU145 cells (Fig. 1c, right panel).

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 increase in the CXCL8 mRNA transcript level in PC3 and DU145 cells (Fig. 2a, left panel). Furthermore, oxaliplatin treatment induced the expression of the related CXC-chemokine, CXCL1 in each of these CaP cell lines (Fig. 2a, right panel). This induction of CXCL8 and CXCL1 by oxaliplatin was attenuated 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 regulation of these CXC-chemokines. Immunoblotting also confirmed a time-dependent increase in the intracellular CXCL8 protein level in response to 1 μM oxaliplatin (Fig. 2c) while 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 following exposure to 1 μM oxaliplatin for 3h (Fig. 2d).

Significantly, the administration of oxaliplatin also increased the expression of CXCR1 and CXCR2, the receptors 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 following addition of oxaliplatin, especially that of the CXCR2 receptor (Fig. 2f). Consequently, the potentiation of CXCL8 and CXCL1 secretion and the increase in CXCR2 expression suggests that exposure to oxaliplatin increases the magnitude of the autocrine CXC-chemokine signaling stimulus that AIPC cells are subject to and furthermore, promotes a selective increase in CXCR2-mediated signaling.

**CXC-chemokine signaling potentiates NF-κB transcription in AIPC cells.**

Further experiments were conducted 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 3nM recombinant human-CXCL8 (rCXCL8) and an EMSA 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 panel). 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 post-addition of rCXCL8 (Fig. 3a, right panel). 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 (20nM) 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 3nM CXCL8 (Fig. 3c, left panel) or 3nM CXCL1 (Fig. 3c, right panel), 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 co-administration of the CXCR2 antagonist, AZ10397767.

**CXCL8 signaling potentiates anti-apoptotic gene transcription in AIPC cells.**

qPCR analysis was conducted to determine whether CXCL8 signaling increased the transcriptional of established, NF-κB-regulated anti-apoptotic genes. Bcl-2 mRNA transcript levels were shown to increase by a factor in excess of 3-fold and 2.5-fold within 1 h of adding
3nM rCXCL8 to PC3 and DU145 cells, respectively (Fig. 4a, left panel). A sustained increase in the transcription of the Bcl-2 gene was evident in both AIPC cells at further timepoints out to 12 h post-stimulation. CXCL8 signaling also induced statistically-significant increases in survivin mRNA transcript levels in PC3 and DU145 cells (Fig. 4a, right panel). Immunoblotting further confirmed that CXCL8 signaling increased the expression of these representative NF-κB-regulated, anti-apoptotic genes in both PC3 (Fig. 4b, left panel) and DU145 cells (Fig. 4b, right panel). Expression of Bcl-2 was observed to increase in both cell lines at time points out to 4 h post-stimulation, with a secondary increase in expression detected 10 h post-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 following stimulation with rCXCL8. In contrast, CXCL8 induced a sustained increase in the expression of survivin out to 6 h post-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 anti-apoptotic 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 anti-apoptotic gene expression, PC3 and DU145 cells were stimulated with 3nM 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). Similarly, 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 following inhibition of
CXCL8-signaling using AZ10397767 or BAY-11-7082 (data not shown). Accordingly, our data indicates that the CXCR2 receptor primarily couples CXCL8 to NF-κB activation and that CXCR2/NF-κB signaling underpins CXC-chemokine-promoted regulation of anti-apoptotic protein expression in these cells.

Inhibition of CXCR2 signaling attenuates oxaliplatin-induced activation of NF-κB.

Since 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 co-administration of AZ10397767 attenuated oxaliplatin-induced complexing of NF-κB with the radiolabelled oligonucleotide, with marked decreases observed 1 h and 2 h post-addition of the two drugs (Fig. 5a). Co-administration 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 anti-apoptotic genes (Bcl-2 and survivin) in the PC3 (Fig. 5c, left panel) and DU145 cells (Fig. 5c, right panel). Co-administration 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 co-administration 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 to 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 nM – 50 nM range, but not at higher concentrations of oxaliplatin (ie. greater than 10 μM). The interaction between oxaliplatin and AZ10397767 was synergistic in the PC3 and DU145 cell line respectively at oxaliplatin concentrations ranging from 0.1 μM to 1 μM (RI>1.5 and RI>1.3, respectively). In addition, co-administration of AZ10397767 corresponded to calculated increases of 10-and 19-fold in the potency of oxaliplatin in PC3 cells (oxaliplatin and AZ10397767 IC_{30} = 0.2μM +/- 0.005 versus oxaliplatin alone IC_{30} = 1.9μM +/- 0.01; n=6, p<0.01) and DU145 cells (IC_{25} = 0.021μM +/- 0.02 versus 0.4μM +/- 0.09; n=3), respectively.

Addition of AZ10397767 by itself failed to induce apoptosis in either PC3 or DU145 cells. However, co-administration of AZ10397767 with 0.1 or 1μM oxaliplatin resulted in a marked increase in the sub G0/G1 cell population in either cell line (Fig. 6b, left and right panels). In PC3 and DU145 cells respectively, the level of apoptosis induced by oxaliplatin increased from 5.48 ± 0.69% to 10.55 ± 2.5% (p<0.05; n=4) and 4.99 ± 0.49% to 9.13 ± 1.74%
(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 panels). While treatment with 1µM oxaliplatin reduced full-length PARP expression in PC3 cells relative to untreated cells, the concurrent administration of AZ10397767 with 1µM oxaliplatin effected a more significant reduction in PARP expression. Similarly, in the DU145 cells AZ10397767 had the ability to enhance oxaliplatin-induced cleavage of PARP. Accordingly, 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 1µM 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, non-iterative non-linear regression analysis of the cell count data predicted a two-site model to be more appropriate in modeling the data resulting from the co-administration of oxaliplatin and BAY-11-7082. The combination of oxaliplatin with BAY-11-7082 increased the calculated IC_{25} value from 0.73±0.63 to 0.008±0.011 (P<0.05; n=3) in PC3 cells. In DU145 cells, inhibition of NF-κB activity using BAY-11-7082 increased the calculated IC_{30} from 0.287±0.456 to 0.015±0.04 (P<0.05; n=3). Although oxaliplatin was ineffective in
inducing apoptosis in either cell line, treatment with 1µM BAY-11-7082 did induce apoptosis in PC3 (Fig. 7b, *left panel*) and DU145 cells (Fig. 7b, *right panel*). 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 µM and/or 1µM oxaliplatin (Fig. 7b). These results were verified by use of a second inhibitor of NF-κB, the IκK-2 inhibitor, SC-514 which was administered to PC3 cells at a final concentration of 2µM. SC-514 inhibited oxaliplatin (1µM)-induced NF-κB binding in EMSA analysis, sensitized PC3 cells to sub-µM concentrations of oxaliplatin in cell count analysis and when co-administered with 0.1 µM oxaliplatin, SC-514 increased the accumulation of apoptotic-cells detected by FACS analysis in the sub Go/G1 peak relative to that observed when treated with 0.1 µM oxaliplatin alone (data not shown).

**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 RNAi-based approaches to selectively suppress the expression of these genes in AIPC cells. PC3 cells were initially transfected using 50nM 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 post-transfection. In contrast, transfection with Sur-T oligonucleotide was shown to be markedly more effective in depleting survivin expression in the PC3 cells (Figure 8a, *top and bottom panels*).

The effect of transiently reducing anti-apoptotic gene expression upon the induction of apoptosis in PC3 cells in the absence and presence of oxaliplatin was determined using FACS
analysis. Initially, PC3 cells were treated with either 50nM of Bcl2-T oligo, Sur-T oligo or Sc oligo for 48 h, following which cells were exposed to increasing concentrations of oxaliplatin for a further 72 h. In control experiments, transfection of PC3 cells using concentrations up to 200nM of the Sc oligo did not increase the level of apoptosis over that observed in control PC3 cells (data not shown).

Transfection with 50nM Bcl2-T alone increased the magnitude of the sub G0/G1 fraction of the cell population (8.53 ± 0.57%, p<0.01, n=4) relative to both mock-transfected control (4.68 ± 0.18%) and scrambled-oligo transfected cells (4.75 ± 0.18%) (Fig. 7c), suggesting that even sub-optimal reductions in the expression of this protein initiates 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 50nM Bcl2-T-transfected PC3 cells (p<0.001; n=4) and 50nM Sur-T oligo-transfected PC3 cells (p<0.05, n=4), respectively (Fig. 8b). The relative importance of these anti-apoptotic 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 1μM oxaliplatin resulted in a modest decrease in PARP expression. In contrast, transfection of PC3 cells with 50nM 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 co-treated 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 while 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 200nM of this 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 co-incide 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 following the addition of 1μM oxaliplatin to 200nM Bcl2-T-transfected PC3 cells. However, due to 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 200nM Bcl2-T was combined with 1μM 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 while 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 co-transfected cells to undergo apoptosis was compared to 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 50nM, given our observation of high levels of spontaneous apoptosis when anti-apoptotic gene oligos were used at increased concentrations. Co-transfection with 50nM Bcl2-T and 50nM Sur-T oligos was shown to induce a more pronounced accumulation of cells in the sub Go/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 Go/G1 fraction of 18.77 ± 0.89%) in Bcl2-T/Sur-T co-transfected cells relative to that observed in Bcl2-T (10.58 ± 0.72 %, p<0.05, n=4) or Sur-T transfected cells (8.3 ± 0.39 %, p<0.01, n=4).
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 anti-apoptotic 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 anti-apoptotic 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). Consequently, 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 anti-apoptotic genes, Bcl-2 and survivin in AIPC cells. These responses were attenuated by co-administration 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 anti-apoptotic genes in AIPC cells.

The selective increase in CXCR2 expression suggests that signaling through this receptor may pre-dominate over CXCR1 in dictating the response of AIPC cells to the administration of oxaliplatin. While 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). Consequently, 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 anti-apoptotic 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. Co-
administration 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 while RNAi-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 anti-apoptotic 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 sustaining NF-κB transcription and reinforcing the expression of anti-apoptotic proteins in AIPC cells (Fig. 8).

Prior studies have shown that the combination of oxaliplatin with the heat shock protein-90 inhibitor 17-AAG results in a more effective suppression of cellular NF-κB activation and promotion of anti-cancer activity (Rakitina et al., 2003). Therefore, the ability of AZ10397767 to also suppress NF-κB activity and reduce anti-apoptotic 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 co-administration of either the CXCR2 antagonist or the NF-κB inhibitor BAY11-7082 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) suggests that CXCL8 signaling may attenuate the capacity of oxaliplatin to initiate mitochondrial-driven apoptosis in these cells by altering the ratio of pro-apoptotic 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 following the administration of oxaliplatin.

Several final issues warrant mention. Firstly, 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 anti-apoptotic 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 ie. 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. Accordingly, 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 anti-apoptotic 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.
References.


Footnotes.

a) This work was supported by the Ulster Cancer Foundation (PGJ and DJJW), the Prostate Research Campaign UK (DJJW), the Northern Ireland HPSS R&D Office (Grants RRG9.14 (DJJW) and EAT/2546/03 (PS, RHW and DJJW)), Cancer Research UK (C212/A5720 to PGJ, RHW and DJJW) and the Association for International Cancer Research (AICR-04-516).

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c) The authors gratefully acknowledge Drs. Simon T. Barry and David Blakey of AstraZeneca (Alderley Park, Cheshire) for provision of AZ10397767. We extend our thanks to Stephen McNiece and the Dispensing Pharmacy of the Bridgewater Suite, Belfast City Hospital for consultation and for provision of the chemotherapy drugs used in this study.
Legends to Figures

Figure 1. Oxaliplatin potentiates NF-κB-promoted transcription of anti-apoptotic genes in AIPC cells. (a) EMSA blot demonstrating the promotion of NF-κB binding to its consensus binding site in PC3 cells in response to the addition of 1 μM oxaliplatin (L-OHP). (b) Bar graph illustrating the magnitude and time-dependent induction of NF-κB-induced luciferase activity in PC3 and DU145 cells following stimulation with 1 μM L-OHP. Data shown is the mean ± S.E.M. values, calculated from four independent experiments. (c) Bar graphs illustrating the time-dependent increase in the mRNA transcript levels detected for Bcl-2 (left panel) and survivin (right panel) in PC3 cells in response to addition of 1 μM L-OHP. All values were normalized to the levels detected in unstimulated cells. Data shown is the mean ± S.E.M. values, calculated from four and three independent experiments, respectively. (d) Immunoblots demonstrating an increase in Bcl-2 and survivin expression in PC3 cells (left panel) and DU145 cells (right panel) in response to the addition of 1 μM L-OHP. Equal protein loading was confirmed by reprobing the membrane for GAPDH expression.

Figure 2. Oxaliplatin potentiates CXC-chemokine expression in AIPC cells. (a) Bar graphs illustrating the time-dependent increase in mRNA transcript levels detected for CXCL8 (left) and CXCL1 (right) in PC3 and DU145 cells over a 12 h timecourse, in response to stimulation with 1 μM L-OHP. Data shown is the mean ± S.E.M. values, calculated from five independent experiments. (b) Bar graph illustrating the effect of the NF-κB inhibitor, BAY-11-7082 on L-OHP-induced CXCL8 mRNA levels in PC3 and DU145 cells, measured 24 h post-treatment with 1 μM L-OHP. Data shown is the mean ± S.E.M. values, calculated from four independent experiments.
experiments. (c) Immunoblot illustrating the time-dependent increase in intracellular CXCL8 expression following the addition of 1μM L-OHP to PC3 cells. (d) Bar graph illustrating the time-dependent increase in the secretion of CXCL8 (left panel) and CXCL1 (right panel) from PC3 and DU145 cells following stimulation with 1μM L-OHP. Data shown is the mean ± S.E.M. values, calculated from three independent experiments. (e) Bar graph illustrating the time-dependent increase in mRNA transcript levels detected for CXCR1 (left panel) and CXCR2 (right panel) in PC3 and DU145 cells in response to stimulation with 1 μM L-OHP. Data shown is the mean ± S.E.M. values, calculated from four independent experiments. (f) Immunoblots illustrating the effect of L-OHP-stimulation upon the expression of CXCR1 and CXCR2 receptors in PC3 (left panel) and DU145 cells (right panel). All immunoblots shown are representative of three independent experiments and equal protein loading was confirmed by re-probing the membranes for GAPDH expression. All values shown for QPCR analysis were normalized to the respective levels detected in unstimulated cells. Statistically significant differences were determined using Students t-test; *, p<0.05; **, p<0.01; ***p<0.001.

Figure 3. Characterization of CXC-chemokine-induced NF-κB activation in AIPC cells. (a) EMSA blots demonstrating the promotion of NF-κB binding to its consensus binding site in PC3 cells in response to short-term (left panel) or long-term [right panel] stimulation with 3nM rCXCL8. (b) EMSA blot illustrating the effect of co-administration of 20nM AZ10397767 (AZ767), a CXCR2 receptor antagonist, upon the time-dependent DNA binding of NF-κB induced by stimulation of PC3 cells with 3nM CXCL8. (c) Bar graph illustrating the magnitude of NF-κB-induced luciferase activity in PC3 and DU145 cells, 24 h post-stimulation with 3nM CXCL8 (left panel) or 3nM CXCL1 (right panel), in the absence and presence of the CXCR2
antagonist AZ10397767 (AZ767; 20nM). Data shown is the mean ± S.E.M. values, calculated from five and three independent experiments, respectively. Statistically significant differences were determined using Students t-test; *, p<0.05; **, p<0.01.

Figure 4. Characterization of CXC-chemokine-induced anti-apoptotic protein expression in AIPC cells. (a) Bar graphs illustrating the effect of 3nM rCXCL8-induced signaling upon the mRNA transcript levels detected for Bcl-2 (left panel) and survivin (right panel) in PC3 and DU145 cells. All values were normalized to the levels detected in unstimulated cells. Data shown is 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 cells (left panel) and DU145 cells (right panel) following stimulation with 3nM rCXCL8. (c) Immunoblots demonstrating the time-dependent increases in Bcl-2 and survivin expression in PC3 cells (left panel) and DU145 cells (right panel) following stimulation with 3nM 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 (AR767) or the NF-κB antagonist BAY-11-7082 on rCXCL8-induced increases in the mRNA transcript levels detected for Bcl-2 (left panel) and survivin (right panel) in PC3 and DU145 cells. Data shown is the mean ± S.E.M. values, calculated from five independent experiments. Statistically significant differences in mRNA transcript level were determined using Students t-test; *, p<0.05; **, p<0.01; ***p<0.001.

Figure 5. Inhibition of CXCR2 signaling attenuates oxaliplatin-induced NF-κB activation. (a) EMSA blot illustrating the oxaliplatin (L-OHP)-stimulated binding of NF-κB to an oligonucleotide containing the consensus nucleotide sequence for this transcription factor, in the
absence and presence of the CXCR2 antagonist, AZ10397767 (AR767). In these experiments cells were treated with 1µM L-OHP and AZ10397767 was co-administered where indicated at a concentration of 20 nM. (b) Bar graph illustrating the magnitude and time-dependent induction of NF-κB-induced luciferase activity in PC3 and DU145 cells following a 24 h stimulation with 1µM L-OHP, in the absence and presence of the CXCR2 antagonist, AZ10397767 (AZ767). Data shown is the mean ± S.E.M. values, calculated from four independent experiments. (c) Bar graph illustrating the time-dependent alterations in mRNA transcript levels detected for CXCL8, CXCL1, Bcl-2 and survivin in PC3 cells (left panel) and DU145 cells (right panel) following a 24 h stimulation with 1µM oxaliplatin in the absence or presence of AZ10397767 (AZ767). Data shown is the mean ± S.E.M. values, calculated from five and four independent experiments, respectively. Statistically significant differences in mRNA transcript level were determined using Students t-test; *, p<0.05; **, p<0.01; ***p<0.001.

Figure 6. Inhibition of CXCR2 signaling potentiates oxaliplatin cytotoxicity and oxaliplatin-induced apoptosis in PC3 cells. (a) Graph illustrating the effect of increasing concentrations of oxaliplatin (L-OHP) upon the viability of PC3 cell populations (left panel) and DU145 (right panel) cell populations in the absence or presence of 20 nM AZ10397767 (AZ767), calculated over the course of 72h. (b) Bar graph illustrating the percentage of apoptotic cells detected in PC3 cell populations (left panel) or DU145 cell populations (right panel) following treatment with 1µM L-OHP in the absence or presence of co-administering AZ10397767 (AZ767; 20 nM). Values shown represent the percentage of cells detected in the sub G0/G1 fraction as determined by FACS analysis. (c) Immunoblots illustrating loss of full-length PARP expression in PC3 cells (left panel) and PARP cleavage in DU145 cells (right panel) in response to addition of 1µM
oxaliplatin in the absence and presence of AZ10397767 (AZ767). Equal protein loading was confirmed by re-probing the membrane for GAPDH expression. Statistically significant differences in cell viability or apoptotic cell populations were determined using Students t-test; *, p<0.05; **, p<0.01.

Figure 7. Inhibition of the NF-κB signaling pathway sensitizes AIPC cells to oxaliplatin. (a) Graphs illustrating the effect of co-administering the NF-κB inhibitor BAY-11-7082 at a concentration of 1 μM upon the cytotoxicity of oxaliplatin (L-OHP) in PC3 (left panel) and DU145 cells (right panel). Cells were exposed to both agents for 72 h. Data shown is the mean ± S.E.M. of four independent experiments. (b) Bar graphs presenting the level of apoptosis detected by FACS analysis in untreated, L-OHP treated, BAY-11-702 treated and L-OHP/BAY-11-7082 treated PC3 cells (left panel) and DU145 cells (right panel). Data shown is the mean ± S.E.M. of four independent experiments. Statistically significant differences in the apoptotic cell populations were determined using Students t-test; *, p<0.05; **, p<0.01.

Figure 8. Attenuation of anti-apoptotic gene expression induces spontaneous apoptosis and increases oxaliplatin-induced apoptosis in PC3 cells. (a) Immunoblots demonstrating the relative suppression of Bcl-2 or survivin in PC3 cells 72 h-post transfection with either the Bcl2-T oligonucleotide or Sur-T oligonucleotide, respectively, at a final concentration of 50 nM. The effect of the gene-targeted oligonucleotides is shown relative to that observed following transfection of PC3 cells with a scrambled oligonucleotide (Sc) at 50nM. (b) Graph illustrating the percentage of apoptotic cells detected as the sub G0/G1 fraction by FACS analysis in PC3 cell populations following transfection with the Bcl2-T or Sur-T oligonucleotides at a
concentration of 50nM, singly or in combination, in the absence or presence of 1μM L-OHP. The effect of transfection cells with the scrambled oligonucleotide at a similar concentration is shown for comparison. Values shown represent the mean ± SEM percentage, determined in four independent experiments. (c) Immunoblots illustrating the effect of suppressing Bcl-2 or survivin expression using 50nM of Bcl2-T or Sur-T respectively upon PARP cleavage in PC3 cells in the absence and presence of 1μM L-OHP. The response is shown relative to the effect of transfecting PC3 cells with 50nM of the Sc-oligo. Equal protein loading was confirmed by re-probing the membrane for GAPDH expression. (d) Immunoblots reporting the expression of full-length PARP, cleaved PARP and Bcl-2 protein expression following the transfection of PC3 cells with 200nM Bcl2-T oligo, in the absence and presence of 1μM oxaliplatin. Equal protein loading was confirmed by re-probing the membrane for GAPDH expression. (e) Bar graph illustrating the percentage of apoptotic cells detected as the sub G0/G1 fraction by FACS analysis in PC3 cell populations following transfection with the Bcl2-T oligonucleotide at a concentration of 200nM, in the absence or presence of 1μM L-OHP. The effect of transfection cells with the scrambled oligonucleotide at a similar concentration (200nM) is shown for comparison. Values shown represent the mean ± SEM percentage, determined in four independent experiments. Statistical analysis of data presented in (b) or (e) was performed using a Students t-test, comparing the effect of the combined oligonucleotide transfection (Combo) against that of Sc-oligo treated cells (directly above column), or against individual gene-targeted transfections as indicated. *, p<0.05; **, p<0.01; ***, p<0.001.

Figure 9. Schematic representation depicting the importance of NF-κB in oxaliplatin resistance. (a) Oxaliplatin promotes activation of NF-κB in AIPC cells resulting in increased
transcription/expression of anti-apoptotic protein expression, CXCL8, CXCL1 and CXCR2. The resultant autocrine/paracrine signaling reinforces NF-κB activation, promoting a survival phenotype in the cells. (b) Inhibition of CXCR2 signaling, NF-κB activation, Bcl-2 and/or survivin expression attenuates the survival phenotype and potentiates oxaliplatin-induced apoptosis in AIPC cells.
FIGURE 1

(a) L-OHP 1μM Time (h)

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(c) Bcl-2 mRNA Expression Fold change

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FIGURE 3

(a) Time (min)
0 5 10 20 40 50 60 120 rCXCL8 NF-κB

(b) Time (min)
30 60 rCXCL8 AZ767 NF-κB
- - + - +

(c) NF-KB Luciferase Activity Percentage Control
- - + - + AZ767
rCXCL8 rCXCL8

PC3 DU145

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