NOVEL ROLE OF JAK/STAT3 INHIBITORS IN OVERCOMING CISPLATIN RESISTANCE IN SQUAMOUS CARCINOMAS

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INDEX OF FIGURES

INDEX OF TABLES

ABSTRACT

In squamous cell carcinomas (SCC) of the uterine cervix and head and neck, the success of cisplatin treatment is limited by the development of resistant cell populations. The activation of EGFR has been shown to trigger many survival pathways that result in the development of these types of resistant populations. Western blot analysis revealed that proteins in multiple pathways are overexpressed in the cisplatin resistant cell lines UM-SCC-29, UM-SCC-33 (Head and Neck) and ME-180R (Cervical) including the downstream EGFR pathway proteins P-STAT3, P-Akt, Bcl-xL and survivin. In this study, we show that treatment with EGFR siRNA did not alter P-STAT3, STAT3 or survivin expression in cisplatin resistant cell lines, but inhibition of the downstream protein survivin enhanced cisplatin-induced apoptosis in this model. This data suggests that inhibition of EGFR is not sufficient to overcome cisplatin resistance and targeting a downstream signaling molecule like STAT3 may be a more effective strategy. Because many EGFR pathways implicated in cisplatin resistance converge at activation of STAT3, we proposed that targeting STAT3 phosphorylation may be more suitable for treatment in resistant cell lines. Treatment of cisplatin resistant ME-180R cells with JSI-124, a Jak/Stat3 inhibitor, decreased growth and resulted in decreased survivin and Bcl-xL expression, but not EGFR, STAT3, or AKT expression. In addition, JSI-124 sensitized ME-180R cells to cisplatin-induced apoptosis. In contrast, AG490, a proposed Jak2/Stat inhibitor only induced additive cytotoxicity with cisplatin in ME-180R, did not induce apoptosis, and did not affect the protein expression of P-STAT3, Bcl-xL or survivin. We next investigated this mechanism in HNSCC cisplatin-resistant cell lines. In these cell lines, we show that treatment with JSI-124 sensitized resistant head and neck cell lines to cisplatin. However, the mechanism of the STAT3 inhibitors (JSI-124 and AG490) is unclear. We observed no effect of JSI-124 or AG490 on P-STAT3 at concentrations that eliminated Bcl-xL expression, reduced survivin expression and activated Caspase-3. Together, our data begin to define a targetable cisplatin resistance mechanism in squamous carcinomas. Our results suggest that the Bcl-xL–survivin–caspase-3 pathway is a novel mechanism of cisplatin resistance in head and neck and cervical squamous carcinomas and that JSI-124 counteracts that survival mechanism, sensitizing resistant cell lines to cisplatin.

INTRODUCTION

Cervical carcinomas are the second most common cancer in women and not normally diagnosed until the late stages of the cancer. Most cervical carcinomas are the result of an infection by one of the 15 HPV (Human Papilloma Virus) genotypes (3). Until recently, the links between cervical cancer and head and neck cancer seemed remote, since the latter has been associated primarily with heavy tobacco and alcohol abuse. However, there is currently an epidemic of oropharyngeal and oral cancers that are, in part, arising in patients who are not tobacco users. Furthermore, the tumors arising in this population contain the same high risk HPV virus types that are considered to be the etiologic factor in cervical cancer. Additionally, cervical carcinomas are very similar to head and neck carcinomas in that they are both mucosal diseases and therefore have many disease phenotype similarities. HNSCC is the sixth most commonly diagnosed cancer and even with the HPV epidemic, smoking remains the main risk factor for this disease (4), and many patients who have HPV positive oropharyngeal cancers also use tobacco products. Although the role of the HPV-cervical cancer risk is well known, much less is noted about the very strong independent causal effect of smoking on cervical cancer risk (5). Thus, there are many similarities between the two tumor types, including epidemiology and HPV transmission between infected partners. Generally HPV-positive tumors tend to be responsive to therapy, including surgery, radiation and chemotherapy or a combination thereof. However, some tumors that are HPV-positive and many that are HPV negative persist after initial therapy and are typically treated with regimens based on cisplatin or its analogue. Some such tumors are very resistant to treatment. Thus, there is a need to understand the cell survival mechanisms involved in tumor cell resistance and to develop new strategies to overcome resistance.

To date, advanced, Stage 3 and 4 uterine cervix SCC and HNSCC cancers are difficult to treat and less than fifty percent of patients survive longer than five years (6). Unfortunately even though advancements have been made in the treatment of these cancers, the survival rate has remained relatively unchanged in the past years (7). Therefore, development of novel treatment options must be developed that focus on more individualized treatment based on identification of biomarkers that would lead to an improvement in the quality of life and survival rates of HNSCC patients. Much work has been done to identify these biomarkers in HNSCC. For example, clinical trials have shown that high EGFR (epidermal growth factor) expression, and wild type p53 together with high Bcl-xL are associated with a poor survival rate in oropharyngeal cancer (8). In addition, others have shown that transcription factors, such as STAT3 (Signal Transducer and Activator of Transcription 3) are important in regulating anti-apoptotic genes. STAT3 has been shown to upregulate Bcl-xL (a Bcl-2 family protein) and survivin (an IAP family protein) and this regulation results in reduction in treatment induced apoptosis levels (9). This observation correlates well with the aforementioned clinical data which implicate STAT3 as an important biomarker in HNSCC and a potential therapeutic target. As expected, STAT3 has also been shown to act as an oncogene in cervical carcinomas (10). Therefore, it seems likely that STAT3 is important in the development of resistant populations in cisplatin treatment which is the standard of care for treatment of HNSCC and cervical carcinomas.

STAT3 is dormant in the cytoplasm until it is activated by a signal from either a cytokine or growth factor (or a peptide) that results in kinase activation and STAT3 phosphorylation which then moves it to the nucleus where it affects expression of response proteins. There are two different isoforms of STAT3—STAT3α and STAT3β—which are known to be expressed in mammalian cells (11). The mechanism of this activation occurs as follows: in growth factor

mediated pathways, non-receptor tyrosine kinases (such as c-Src) are among the proteins recruited to the receptor. For the cytokine pathways, there is a chain of three tyrosine phosphorylations that ultimately result in the activation of STAT3. These phosphorylations are carried out by Janus kinase (JAK) proteins that interact with specific receptors. Recruited JAK proteins dimerize and self-activate by phosphorylation (12), (13). These tyrosine phosphorylations allow for the interaction of specific residues with STAT3 proteins that bind to JAK via their Src-2 homology (SH2) domain. The STAT3 proteins are then phosphorylated by JAK at tyrosine 705 (Y705) which is necessary for subsequent translocation to the nucleus and activation of transcription (13), (14). For the most part, this interaction is transient, and acts to regulate biological functions. However, in cancer, many oncogenic pathways have been identified in which STAT3 is constitutively active and this continuous signaling supports cellular transformation and the malignant phenotype (14). Survivin and Bcl-xl are two genes which

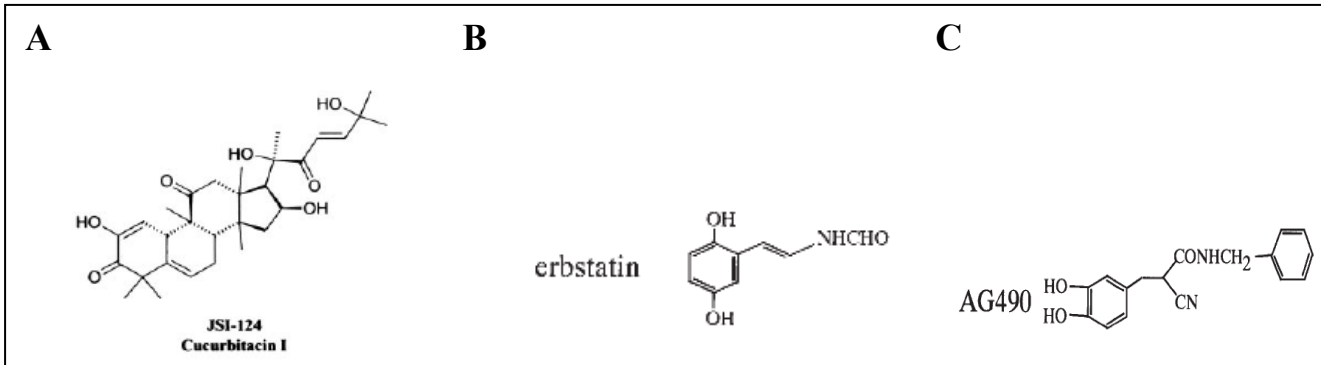


Figure 1. The chemical structure of the STAT3 inhibitors. a) structure of JSI-124 (1) b) structure of erbstatin, the compound that AG490 was synthesized from c) structure of AG490 (2)

have been shown to be activated upon expression of phosphorylated STAT3 (15),(16).

Because STAT3 has been shown to be activated in numerous cancer types, many strategies have been employed to target STAT3 signaling. One way to target the STAT3 is to target the upstream tyrosine kinases responsible for the activation of STAT3 using small

molecule inhibitors. Inhibitors have been developed that target tyrosine kinases such as JAK, SRC, BCR-ABL, FLT3 and EGFR (9). The STAT3 inhibitors JSI-124 and AG490 that block JAK mediated phosphorylation and nuclear translocation of STAT3 will be used in this thesis. There are other methods to target STAT3 more directly including oligonucleotides, decoy oligonucleotides, dominant-negative expression vectors and small interfering RNA molecules.

JSI-124 was identified in a high throughput cytoblot assay that used the NCI diversity compound set to identify any compounds that showed decreased interaction with p-STAT3 as determined by loss of interaction with a p-STAT3 (Y705) antibody. In this assay, JSI-124 was determined as a potent inhibitor of STAT3 phosphorylation (1). This drug, also known as Cucurbitacin I, (Figure 1A) is a natural compound isolated from plant species such as Cucurbitaceae (the melon family, extracted commonly from cucumbers) and Cruciferae (mustard/cabbage family). This compound is a component of folk medicines used in China and India to treat cancer and chronic hepatitis (17). JSI-124 has been shown to selectively induce apoptosis in endometrial and cervical cancer cell lines (10). AG490 was identified as a JAK2 specific inhibitor in a tyrphostin study that showed that the drug inhibited the growth of human B-precursor leukemia. AG490 was specifically identified to increase anti-proliferative effects in these cells and this activity was correlated with downregulated JAK2 but not JAK1 or JAK3. AG490 is a modified tyrphostin, (see structure in Figure 1C) that is synthesized from a natural tyrosine kinase inhibitor, erbstatin (Figure 1B), isolated from fungal extracts (2).

Resistance to cisplatin is an important factor in the management of advanced head and neck and cervical cancers. Analysis of pretreatment biopsies from completed clinical trials testing the effectiveness of organ sparing therapies for advanced laryngeal and oropharynx cancer revealed consistent biomarkers that are associated with response to cisplatin based

treatments. Analysis of biomarkers in pretreatment tissue from the chemotherapy arm of the VA larynx cancer trial revealed that failure to respond to induction chemotherapy was associated with the presence of wild type p53 (18). This was contrary to the expectation that tumors with wild type p53 would undergo p53-regulated apoptosis in response to DNA damage induced by cisplatin. To investigate this surprising finding, our group investigated the relationship between p53 status and cisplatin resistance in vitro, using the head and neck cell lines in our laboratory. Thirteen of 23 tested cell lines were shown to have mutated p53. The average IC_{50} value for the cell lines with mutant p53 was 6.8 μM as compared to the average IC_{50} value of 13.7 μM for cell lines that had wild-type p53. These results showed that cell lines with mutant p53 had increased cisplatin sensitivity as compared to the cell lines with wild-type p53 (19). This observation that mutant p53 was involved in a cisplatin sensitivity mechanism was surprising and the specifics of this mechanism were yet to be defined. In order to address this conundrum, a study in our laboratory on laryngeal pretreatment biopsies was done to identify correlations in larynx preservation and survival. Although mutations in p53 have been associated with poor response to chemotherapy (20), the results showed that high and low Bcl-xL with expression of wild-type p53 make up the worst and best survival rates, respectively (18). Mutant p53 has been found in 50% of all head and neck tumors (18). Therefore, this data is puzzling because it is expected that wild-type p53 would induce apoptosis and the cells would be expected to be much more sensitive to cisplatin treatment. Overexpression of the antiapoptotic proteins Bcl-2 and Bcl-xl are known to result in resistance to chemotherapy and radiation (21). In laryngeal tumors, overexpression of Bcl-xL has been found in 74% of tumors while overexpression of Bcl-2 has been found in 15% of tumors (22). Further investigation demonstrated that high levels of the anti-apoptotic protein Bcl-xL was associated with failure to respond to chemotherapy whereas a

good response was more likely when Bcl-xL expression was low. In the same study Bcl2 levels were associated with good response to chemotherapy (22). When Bcl-xL and p53 were analyzed together, it became clear that patients whose tumors expressed the combination of wild type p53 and high levels of Bcl-xL were at highest risk for laryngectomy due to failure of induction chemotherapy. In contrast, patients whose tumors expressed wild type p53, but had low or no detectable Bcl-xL expression had a very low risk of laryngectomy. In fact, no patient with this tumor biomarker profile of wild type p53 in the setting of low Bcl-xL, required laryngectomy, all were responders to induction chemotherapy (18). Whereas those with the wild type p53 high Bcl-xL phenotype had a 16 fold increased risk for laryngectomy due to chemo-resistance. Thus, understanding how to target tumors based on their individual biomarker profile will be important for treatment of head and neck tumors. In an oropharyngeal cancer study from our lab, other markers were identified to be important in response to patient survival. Low EGFR and high p16, high HPV copy number, and/or low p53 and low Bcl-xL were found to be markers of excellent response to an organ sparing protocol. In contrast, high EGFR expression, low p16, absence of HPV, low p53/high BcL-xL expression, and smoking correlated with poor outcome (8). Furthermore, together EGFR expression and HPV identified those tumors with the poorest survival (high EGFR, HPV-) and the best survival (low EGFR ± HPV) and an EGFR+ HPV+ subset with poor survival. Therefore, these markers further classify a phenotype for poor prognosis in head and neck cancer carcinomas.

Our lab has shown that Bcl-xL also plays a role in modulating cisplatin resistance in HNSCC *in vitro*. The cisplatin resistant cell lines UM-SCC-5PT* and -10BPT* that were thought to be derived from UM-SCC-5 and UM-SCC-10B by long term selection in increasing concentrations of cisplatin have high Bcl-xL and wild-type p53 and do not respond to cisplatin

treatment. This is presumably due to the high levels of Bcl-xL in the setting of wild type p53 which allows for blockade of apoptosis by Bcl-xL and induction of arrest and repair by wild type p53 (23). However, more recent studies of the purported UM-SCC-5PT* and -10BPT* cell lines revealed that these lines, but not the parental lines contained HPV68. This suggested that a cell line mix-up had occurred at some point in the cisplatin selection and a different cell line had been selected for resistance. A search of the literature showed that there was one known HPV68 positive cell line and that line was called ME-180, a cervical carcinoma cell line (M. Longuet 1996). To test this, we genotyped the original ME-180 cell line (also HPV68 positive), that we had in our bank, and the resistant lines (so called 5PT* and 10BPT*) and found that the resistant lines had identical genotypes to one another and this genotype did not match the genotypes of the original UM-SCC-5 or UM-SCC-10B cells, but did match the genotype of ME-180 from our bank. Thus, we concluded that the cells selected for cisplatin resistance were ME-180; these were designated ME-180R.

In this thesis I investigated the role of EGFR, survivin, and STAT3 activation/inhibition on cisplatin resistance and the therapeutic potential of targeting these proteins.

MATERIALS AND METHODS

Reagents. Cis-Diammineplatinum(II) dichloride (Cisplatin) and AG 490 were purchased from Sigma (St. Louis, MO) and dissolved to a stock concentration of 3.33mM in 0.9% Sodium Chloride and 35mM in EtOH, respectively. JSI-124 was obtained from Calbiochem (San Diego, CA) and suspended at 100μM in EtOH. All agents, JSI-124, cisplatin, and AG-490, were stored in light protective containers and experiments were conducted in minimal light.

Cell Culture. The cell lines were grown in complete Dulbecco's Modified Eagle's Medium (cDMEM) containing 2 mM L-glutamine, 1% nonessential amino acids, 1% Penicillin-Streptomycin from Invitrogen (Carlsbad, CA) and 10% fetal bovine serum, in a humidified atmosphere of 5% CO_2 at 37˚C. The cell lines were tested for mycoplasma, using the MycoAlert Detection Kit from Cambrex (Rockland, ME) every 3-6 weeks to ensure that they were free of contamination. Cell line identity was confirmed by genetic mapping using nine different short tandem repeat loci (24). ME-180R was isolated from an omental metastasis of a cervical carcinoma by J.A. Sykes in 1967 (25). UM-SCC-10B was derived as a lymph node metastasis with a primary site at the larynx; the patient received salvage surgery treatment (26). UM-SCC-17B was derived as a local cartilage invasion with a primary site at the supraglottis, the patient received radiation treatment (26). UM-SCC-29 was derived as a primary lesion at the alveolus, patient received chemotherapy treatment (27). UM-SCC-33 was derived as a neck metastasis that spread from the maxillary sinus, the patient received chemotherapy treatment. UM-SCC-74B was a recurrence at an intraoral site following treatment of chemotherapy, radiation, and surgery.

Plasmid Construction and shRNA Design. RNA interference constructs were prepared using sense and anti-sense oligonucleotides designed to create short 19bp hairpins. These were then cloned into a shRNA cloning site under control of the H1 promoter in a self-inactivating lentiviral vector with a green fluorescent protein (GFP) marker under control of human ubiquitin-C promoter. Oligos were ordered from Invitrogen (Carlsbad, CA). Target sequences for survivin (GenBank NM_001168) are as follows: survivin shRNA1 5'-GGAGCTGGAAGGCTGGGAG (307-325), survivin shRNA2 5'- CCTAGCCTGCCAACAGCCA (1751-1769), survivin shRNA3 5'- CCAACTGCCATCCTGGAAA (2487-2505). A scrambled shRNA with no known target gene was designed as a control: CTGACTAGGATAGTACTAC. All vectors were forward and reverse sequence confirmed.

Transfection and Lentiviral Infection. Human embryonic kidney 293FT cells (2-5 × 10^6) were co-transfected with 4 μg each of the indicated plasmid DNA (including 3 packaging plasmids) (28) by the calcium phosphate method. Recombinant virus containing culture media was collected 24-48 hours after transfection. ME-180R cells were plated (2-4 × 10^6) for one day and then incubated in viral media for 4 hours with 4 μg/ 5 mL polybrene from Sigma (St. Louis, MO), washed with cDMEM and allowed to proliferate for another 24-72 hrs. In all experiments, 90-100% infection efficiency was achieved 48-72 hrs after the lentiviral infection. Cells were sorted for the top 50% of GFP expressing cells by flow cytometry. Viral infected ME-180R cells were used in all experiments between passages 2-10, during which western blot analysis revealed no loss in knockdown or expression efficiency.

RNA Isolation and cDNA Preparation. Exponentially growing (60-90% confluence) cells were washed in PBS and re-suspended in 500 μL TRIzol from Invitrogen (Carlsbad, Ca). RNA was

extracted according to manufacturer's protocol and quantified using a Nanodrop spectrophotometer. Prior to cDNA synthesis, RNA was DNase treated. Two μg of total RNA and a poly-T primer were used to make cDNA using Superscript III, Invitrogen (Carlsbad, Ca). All cDNA was subsequently treated with RNase A (Invitrogen, Carlsbad, CA).

qPCR. Survivin mRNA expression was quantified using the QuantiTect, Sybr-green qPCR kit available from Qiagen (Valencia, CA). Primers were designed to sit in exons and flank at least one intron as follows: survivin-F: 5'-GCCCAGTGTTTCTTCTGCTT, survivin-R: 5'-GACAGAAAGGAAAGCGCAAC, GAPDH-F: 5'-GGTCATCCATGACAACTTTGG, GAPDH-R: 5'-GAGGCAGGGATGATGTTCTG, Beta-Actin-F: 5'-ATTGGCAATGAGCGGTTC, Beta-Actin-R: 5'-AGCACTGTGTTGGCGTACAG, RPL19-F: 5'-GCTGCTCAGAAGATACCGTGA, RPL19-R: 5'-TTGTCTGCCTTCAGCTTGTG. All samples were run in triplicate. Data analysis was completed using the ΔΔCt method comparing expression to the geometric mean of the three house keeping genes (29). Each experiment was repeated at least 3 times.

Western Blot Analysis. Exponentially growing (60-90% confluence) cells were washed in PBS and lysed in a PBS buffer containing 1% NP-40 (Sigma), 1 mM PMSF (Sigma), and a cocktail of protease inhibitors (Boehringer Mannheim, Germany). Total protein from cell extracts was quantified using the Bradford Assay (Bradford Reagent; BioRad; Hercules, CA). For western blotting, protein (15-25 μg) was electrophoresed on 12% Tris-glycine SDS-polyacrylamide gels under denaturing conditions, and transferred to Hybond-P PVDF membranes (Amersham Pharmica Biotech, Buckinghamshire, England). Membranes were blocked in Tris-buffered

saline plus 0.05% Tween containing either 5% nonfat dry milk or 5% BSA at room temperature for 1 hour followed by incubation for 3 hours with primary antibody; rabbit anti-survivin polyclonal antibody, 2 μg/mL (Novus Biologicals, Littleton, CO), Bcl-xL (Cell Signaling) or mouse anti-GAPDH monoclonal antibody, 1:10,000 (Chemicon International, Temecula, CA) or an overnight incubation at 4^{o}C with rabbit anti-EGFR polyclonal antibody (Cell Signaling), rabbit anti-STAT3 (Cell Signaling), rabbit anti-Phospho-STAT3 (Tyr705) monoclonal antibody (Cell Signaling), AKT, P-AKT (Cell Signaling). Membranes were then incubated for 45 minutes with a secondary horseradish peroxidase-conjugated sheep anti-mouse antibody (Amersham), or horseradish peroxidase-conjugated donkey anti-rabbit antibody (Amersham) and analyzed using the Enhanced Chemiluminescence Plus reagent (Amersham) by exposing membranes to X-ray film (Kodak, X-Omat). All experiments were repeated in triplicate, representative images are shown. Protein expression was quantified using densitometry and fold-change was assessed using NIH ImageJ software.

Immunofluorescence. Cells were seeded at 25,000 cells/mL in 4-well chamber slide 24 hours prior to the addition drug or vehicle control. After the desired incubation period, MitoTracker Deep Red 633 was added to cells for 15 minutes. Cells were then washed once in PBS, fixed for 15 minutes in 4% formaldehyde, washed in PBS containing 0.1M glycine 3 times, permeabilized for 5 minutes in PBS containing 0.2% Triton-X, washed twice in PBS, blocked for 30 minutes in PBS containing 1% BSA, held for 30 minutes in PBS containing 1% BSA and primary antibody rabbit anti-Phospho-STAT3 (Tyr705) monoclonal antibody (Cell Signaling), washed 3 times with PBS, held for 30 minutes in PBS containing 1% BSA and Alexa Fluor 594-anti-Rabbit

(Invitrogen). Cells were then stained with DAPI for 5 minutes and slides were mounted 12 hours prior to analysis using ProLong Gold.

Chemosensitivity (MTT) Assay. Logarithmically growing cells were plated at 5,000-10,000 cells per well in five replicate wells in 96-well plates and allowed to attach and grow in cDMEM at 37˚C overnight. The next day, cisplatin (0, 3.125, 6.25, 12.5, 25, 50 μM) was added to five-replicate wells. Cells were incubated with drug or vehicle control in 300 μL of cDMEM for two hours, after which the media was removed from the wells, and replaced with DMEM. Five days later, MTT assays were performed according to the manufacturer's instructions (Roche Diagnostics, Mannheim, Germany), as previously described (19). Percent absorbance (optical density) value relative to vehicle control (y-axis) was plotted as a function of drug concentration (x-axis). The concentration of drug required for a 50% reduction in absorbance relative to control was taken as the 50% inhibitory concentration (IC_{50}). All experiments were performed in triplicate.

Trypan Blue Dye Exclusion Assay. Cells were seeded at 50,000 cells per well in a 24-well plate and allowed 24 hours to adhere. Cells were then incubated in fresh DMEM (10% FBS) containing cisplatin for 2 hours, before media was replaced with cisplatin-free DMEM (10% FBS). Cultures were then grown for 48 hours at 37˚C. Supernatant (containing some dead cells and debris) was collected and pooled with the attached cells, which were removed from the plate using trypsin and the mixture was centrifuged at 8000 x g for 5 minutes. Cell pellets were re-suspended in 40μL Trypan Blue (Invitrogen, Carlsbad, Ca) and counted in triplicate using a

hemacytometer. Percentage trypan blue stained cells are presented along the y-axis. All experiments were repeated in triplicate.

Determination of Caspase-3 Activity. Cells were seeded at 50,000 cells per well in a 24-well plate and allowed 24 hours to adhere. Cells were then incubated in fresh DMEM (10% FBS) containing cisplatin for 2 hours, after which media was replaced with cisplatin-free DMEM (10% FBS). Cells were grown for 24 hours at 37˚C, trypsinized, and held at -80C until analysis with the EnzChek Caspase-3 Assay Kit (Molecular Probes, Eugene, OR) according to the manufacture's protocol. Fluorescence of the Z-DEVD-AMC substrate was recorded 30 minutes after addition of the substrate to cell lysates.

Annexin V Staining. One day before treatment, cells were plated (100,000 cells/mL) in a 6-well plate. Cells and cell supernatants were then collected 48 hours after treatment and 100,000 cells were resuspended in 100µL of Annexin V binding buffer (10mM HEPES with 140mM NaCl and 2.5mM $CaCl_2$, pH 7.4) with 0.2µL of Annexin V-APC (Invitrogen, Carlsbad, CA) and 10ng DAPI (Invitrogen, Carlsbad, CA). After 15 minute room temperature incubation, 300µL of binding buffer (without Annexin V-APC or DAPI) was added and cells were immediately analyzed by flow cytometry. All experiments were repeated thrice.

EGFR siRNA Knockdowns. Knockdowns were accomplished by RNA interference using commercially available control or EGFR siRNA duplexes (Dharmacon, Lafayette, CO). Transfections were performed with OptiMEM (Invitrogen) and oligofectamine (Invitrogen) as previously described (30).

Chromatin immunoprecipitation assay. Chromatin immunoprecipitation assays were done using the Magna-ChIP^{TM} assay kit (Millepore). ME-180R was treated with various concentrations of JSI-124 and then incubated with formaldehyde to crosslink DNA-protein complexes. The crosslinking reaction was quenched, the cells were lysed, the chromatin extracted, and sheared by sonication (18 pulses, 15 seconds each) to 500 base pairs. The sheared chromatin was incubated with anti-STAT3 monoclonal antibody or controls consisting of anti-immunoglobulin G, or anti-RNA polymerase polyclonal antibody followed by overnight incubation with Magnetic Protein A beads. The next day the beads were trapped with a magnet, washed, and the anti-STAT3 chromatin eluted. Then the crosslinking was reversed according to manufacturer's instructions and the free DNA was extracted. Purified DNA was used for PCR reaction with primers specific for the STAT3 putative binding sites in the human survivin, XIAP, and Bcl-xL promoters. The sequences of the primers used are as follows: survivin forward primer, 5'-CAGTGAGCTGAGATCATGCC-'3, survivin reverse primer 5'-TATTAGCCCTCCAGCCCCAC-3' (15), Bcl-xL forward primer 5'-CACCCTCACCCAGTCTTTGT-3', Bcl-xL reverse primer 5'-GGCCTGTCTGTGATGTTGAA-3', XIAP forward primer 5'-TGCCTGCTTAAATATTACTTTCCTCAAAA-3', XIAP reverse primer 5'-ACTACACGACCGCTAAGAAACATTCT-3'(31), Actin forward primer 5'-TGCACTGTGCGGCGAAC-3', Actin reverse primer 5'- TCGAGCCATAAAAGGCAA-3' (32).

RESULTS

UM-SCC-29, UM-SCC-33 and ME-180R are resistant to cisplatin treatment and show increased Annexin V staining following cisplatin treatment

MTT chemosensitivity growth inhibition assays were carried out to compare the relative cisplatin resistance or IC_{50} values of each cell line. The cell lines were grouped into three categories of varying cisplatin-resistance as shown in Figure 2A. Cisplatin sensitive cell lines with IC_{50} < 10 uM, included two very sensitive lines, UM-SCC-17B (4.0 μM) and UM-SCC-74B (4.8 μM), and one mildly cisplatin resistant line, UM-SCC-10B (8.0 μM). Cisplatin resistant lines included two inherently resistant cell lines, UM-SCC-29 (12 μM) and UM-SCC-33 (>50 μM) that were derived from primary tumors of patients that had no prior therapy, and one line that had been previously selected for resistance to cisplatin, ME-180R (IC50-25uM). In figure 2B a comparison between the original ME-180 and ME-180R is shown, demonstrating that ME-180R is ~5x more resistant than the parent cell line. The IC_{50} values for each cell line are listed in table 1.

The relative sensitivity of the lines was assessed for entry into apoptosis with annexin V staining and FACS analysis. Annexin V dye has been used to detect apoptotic cells by interacting specifically with the phosphatidylserine residues at the outer plasma membrane. Exposure of phosphatidylserine has been shown to be an early event in apoptosis (33). It was intriguing that two of the most resistant HNSCC lines UM-SCC-29 and UM-SCC-33, (Figure 3) and the highly resistant ME-180R (See cisplatin alone Figure 13) exhibited the highest annexin V staining. This data suggests that at least a subpopulation within the resistant group starts the apoptotic pathway, but a large proportion of the population escapes from cell death. On the other hand, the cisplatin-sensitive cells apparently do not undergo the membrane changes that are typical of the apoptotic process detected by Annexin V staining.

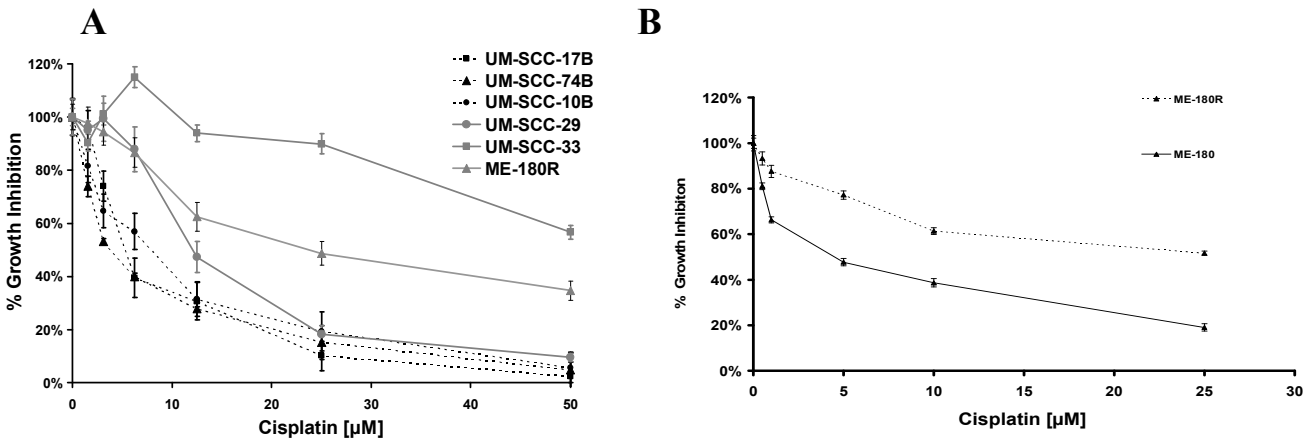


Figure 2. Relative cisplatin sensitivities of UM-SCC-29, UM-SCC-33, ME-180R, UM-SCC-10B, UM-SCC-17B and UM-SCC-74B as determined by MTT growth assays. Cell lines were exposed to cisplatin treatment continuously for 48 hours before assessing viability with the MTT dye conversion assay. a) Comparison of three sensitive lines to three resistant lines. b) comparison of ME-180 to ME-180R that was selected for cisplatin resistance. The experiments were repeated three times.

Cell Line Name	IC50 Value (μM)
UM-SCC-17B	7.2
UM-SCC-74B	4.8
UM-SCC-10B	9.8
UM-SCC-29	12.5
UM-SCC-33	>50
ME-180R	25
ME-180	4.8

Table 1. IC50 values for each of the cell lines.

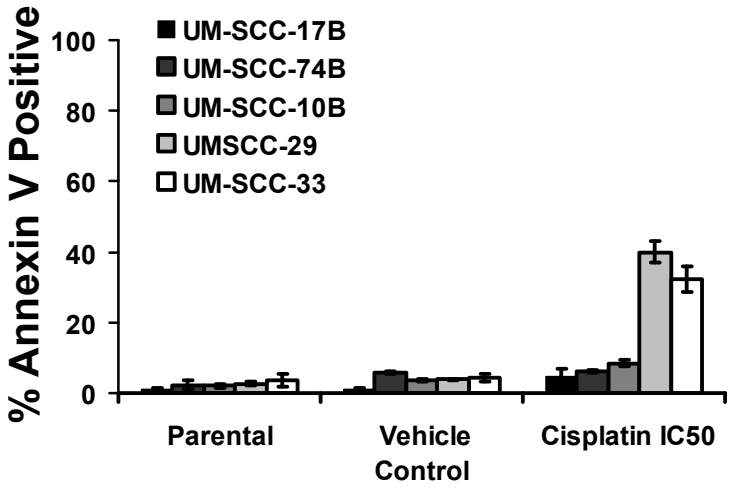


Figure 3. Flow cytometry was used to assess the percentage of Annexin V positive, Hoechst negative (dye excluding) in cells continuously exposed to their IC50 dose of cisplatin. Plot shows the average and standard deviation of three independent experiments.

Overexpression of cell survival proteins in cisplatin resistant SCC lines

The EGFR-STAT3 pathway has been frequently implicated in outcome and survival studies of patients with squamous cell carcinomas, therefore, the proteins that are downstream of EGFR (Fig 4A) were examined in cells in mid-logarithmic growth. To select a group of potentially activated signaling pathways that might trigger a cisplatin resistance mechanism, we selected three pathways that converge at the level of STAT3 activation. These include the P13K/Akt, MAPK pathway, and the direct stimulation of STAT3 by JAK. Figure 4A gives a general scheme of these activation pathways. Because of known overexpression of Bcl-xL in some cisplatin resistant cancer cell lines, we hypothesized that we would see overexpressed activated STAT3 in addition to protein products from the transcription of STAT3-regulated genes including XIAP, Bcl-xL and STAT3. In addition, Caspase 3 was examined for its implications in downstream apoptotic mechanisms. As shown in Figure 4B, EGFR, p-STAT3, and p-AKT were generally up-regulated in the resistant lines (boxed in red) as were Bcl-xL,

survivin, and caspase 3. Interestingly, XIAP was only upregulated in the ME-180R cell line, however, EGFR was expressed to a much lower degree in this cell line. Other differences between these resistant lines include strong upregulation of survivin in UM-SCC-33 and ME-180R. Importantly, even through STAT3 was upregulated in all of the cell lines in the panel, activated STAT3 was typically higher in the cisplatin resistant lines suggesting a cascade of activation of cell survival proteins in the resistant lines as indicated in the cartoon shown in Figure 4A. Although these differences were not unique to the resistant lines there seemed to be a pattern of greater activation of most proteins in the resistant lines.

Treatment of UM-SCC-29 and UM-SCC-33 with IMC-C225 did not result in EGFR downregulation *in vitro*

Based on the proposed diagram in figure 4A, EGFR stimulation results in a signaling cascade that subseqently upregulates STAT3 and other downstream targets in resistance cell lines. We hypothesized that by knocking down EGFR in the cell lines UM-SCC-29 and UM-SCC-33, which are inherently resistant to cisplatin, we would see deregulation of the STAT3 signaling pathway. We investigated the effects of the humanized anti-EGFR chimeric mouse antibody that is presently being used to treat SCC patients by competitively binding to the extracellular receptor and interfering with the binding of natural EGFR ligands including TGF-α and EGF (34). In this study, EGFR expression was not affected by IMC-C225 in UM-SCC-29 and UM-SCC-33 after continuous treatment at 48 or 72 hours (Figure 5). Possibly, the humanized form of the EGFR monoclonal antibody may not be effective in these *in vitro* experiments. However, IMC-C225 has been shown to work in other *in vitro* systems (35). It is unclear as to why this experiment did not work in the HNSCC resistant cell lines studied in this experiment. In order to further attempt to knockdown EGFR expression, siRNA was used to target EGFR as previously shown (30).

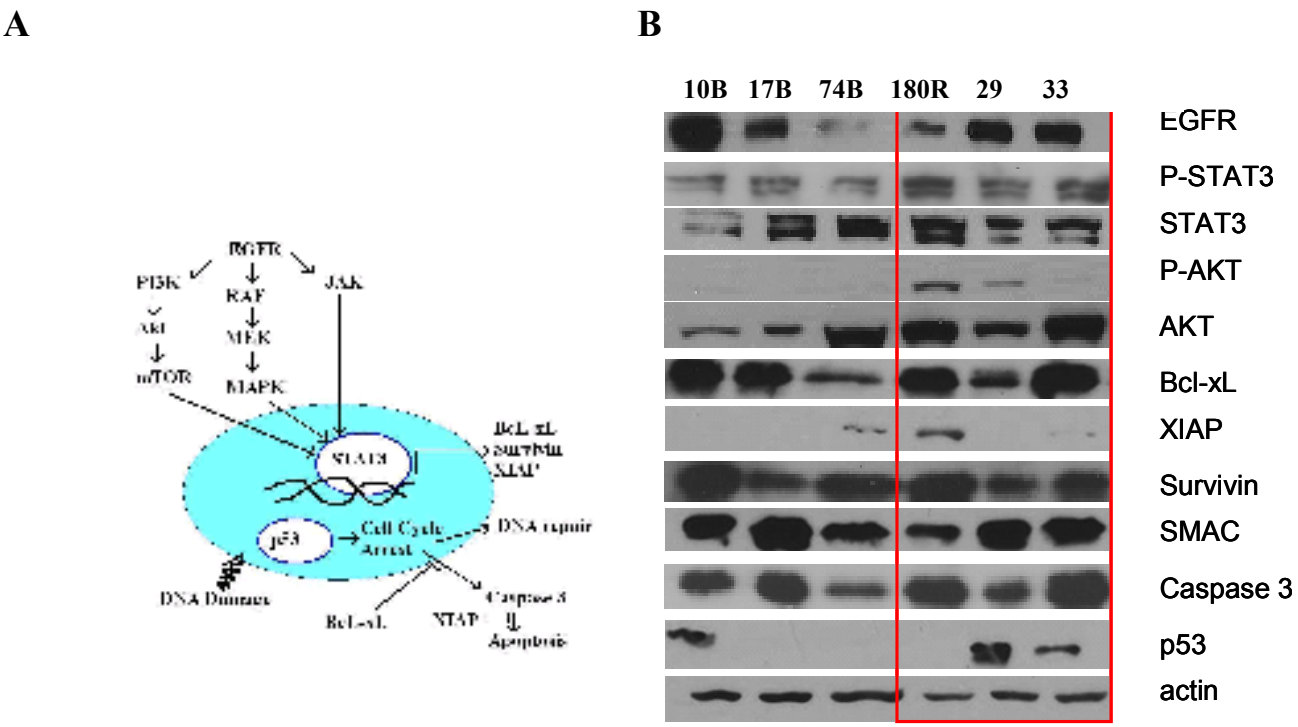


Figure 4. EGFR and apoptosis pathway protein expression in sensitive and resistant UM-SCC and cervical cell lines. a) Schematic of EGFR signaling as involved in STAT3 activation. The proteins are listed in the blot in the order that they signal. Importantly, STAT3, Bcl-xL, and Survivin are overexpressed in both of the resistant cell lines. b) Western blot analysis was used to compare the expression of several proteins involved in both the EGFR pathway and the intrinsic apoptosis pathway in the cisplatin sensitive cell lines UM-SCC-17B and UM-SCC-74B, the moderately cisplatin resistant cell line UM-SCC-10B and the cisplatin resistant cell lines UM-SCC-29, UM-SCC-33 and ME-180R. Beta Actin shows equal loading in all blots. The experiments were repeated three times.

	UM-SCC-29			UM-SCC-33		
Starved	+	-	-	+	-	-
C225-48	-	+	-	-	+	-
C225-72	-	-	+	-	-	+

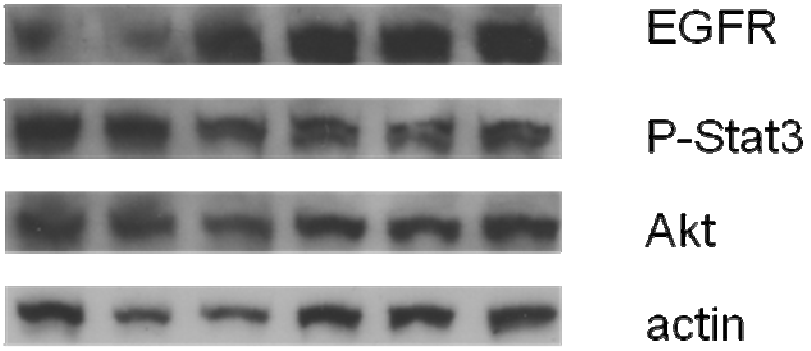


Figure 5. C225 Treatment of UM-SCC-29 and UM-SCC-33 did not result in loss of EGFR expression *in vitro*. Western blot analysis was done on starved cells, cells treated with C225 for 48 hours and 72 hours. The blots were probed with P-STAT3, AKT, and EGFR. Beta actin was used as a loading control. The experiments were repeated three times.

Knockdown of EGFR Expression Did Not Abrogate STAT3 Activation in Inherently Cisplatin Resistant Cell Lines

To successfully knockdown EGFR and to determine its importance in downstream signaling pathways, siRNA was used for infection into UM-SCC-29 and UM-SCC-33 cells. As shown by Western blot analysis in Figure 6, EGFR expression was significantly reduced upon infection with siRNA. To determine if proteins were affected downstream of EGFR, the blots were stripped and probed with antibodies for P-STAT3 and survivin. Activation of STAT3 was not affected by decreased EGFR expression. Survivin, one of the downstream targets of STAT3 was also unaffected. These results suggest that STAT3 is constitutively activated in the inherently resistant cell lines UM-SCC-29 and UM-SCC-33, and therefore, are activated independent of EGFR stimulation. Disrupting EGFR stimulation will not be effective in disregulating the potential cisplatin resistance pathways. The pathway will need to be targeted further downstream to interfere with STAT3 regulated genes.

Survivin shRNA Sensitizes ME-180R to Cisplatin

To investigate the effects of reducing the expression of a protein product regulated by STAT3, ME-180R shRNA constructs were developed to target survivin. Lentiviral shRNA vectors for survivin containing GFP on an independent ribosomal entry site were developed and used to infect ME-180R cells, a resistant cell line that was selected for cisplatin resistance. These cells were then sorted for the highest integration of the vector by FACS sorting for green cells. Western blot analysis (data not shown) confirmed a greater than 80% knockdown in survivin shRNA transduced lines as compared to scrambled control transduced or parental ME-180R. MTT chemosensitivity assays (figure 7A) revealed that the parental and scrambled control shRNA cell lines had IC_{50} values of 20.8 to 22.1μM cisplatin, while knockdown cell lines were partially sensitized to cisplatin and had IC_{50} values of 5.9 to 6.1μM cisplatin. With

increasing concentrations of cisplatin, caspase 3 activity increased (figure 7B) in comparison to the parental and scrambled control. Survivin shRNA sensitized ME-180R to cisplatin-mediated cell death, as determined by Trypan blue staining (figure 7C). The trypan blue exclusion assay employs the use of a dye that can only stain the cells if the cellular membranes have broken down, which is characteristic of dying cells. Due to the fact that cellular death is observed in addition to caspase activation 3, it is most likely that these cells are undergoing apoptosis. Since survivin is directly regulated by activated STAT3, it seems likely that targeting STAT3 could potentially sensitize ME-180R cells to cisplatin treatment.

Survivin Overexpression in UM-SCC-17B Results in Increased Survivin Cisplatin Resistance

To further address the importance of survivin in cisplatin-mediated cellular resistance, UM-SCC-17B (a cell line with relatively low endogenous expression of survivin) was infected with 5 different lentiviral survivin expression constructs, one of these was highly effective in transducing survivin expression and was used in subsequent experiments (data not shown). We hypothesized that introducing survivin into a cisplatin sensitive cell line, would result in increased resistance to cisplatin treatment. Figure 8A shows that survivin was highly expressed in the cells transfected with the survivin expression vector but remained low or undetectable in the cells without infection or infection with a vector control. In figure 8B, a MTT growth chemosensitivity assay was performed with continuous exposure to cisplatin for 48 hours. Based on the IC_{50} concentration determined in prior experiments (Table 1), the expected cisplatin concentration at which fifty percent of the wild-type UM-SCC-17B cells were viable was 4.8 μM. Indeed, the calculated IC_{50} values of the parental UM-SCC-17B and UM-SCC-17B infected with the vector control was 5 μM, a difference which is statistically insignificant. However, with overexpression of survivin, UM-SCC-17B resistance dramatically increased to an IC_{50}

concentration of 15 μM, above the arbitrary 10 μM level we used to assess sensitive and resistant lines. Trypan blue exclusion assays (Figure 8C) with UM-SCC-17B cell lines revealed that about sixty percent of the cells were unable to extrude the die after treatment with 10 μM cisplatin. UM-SCC-17B cells expressing survivin were able to evade cell death as demonstrated by Trypan blue staining of only 15% percent of the cell lines. Even at a very high concentration of cisplatin (25 μM), survivin continued to show some protective effect such that the proportion of dead cells was 15% lower than that in the uninfected cells. Therefore, expression of survivin in a cell line with limited endogenous expression allowed for the reversal of cisplatin sensitivity.

	UM- SCC- 29			UM- SCC- 33		
Parental	+	-	-	+	-	-
Scrambled	-	+	-	-	+	-
EGFR siRNA	-	-	+	-	-	+

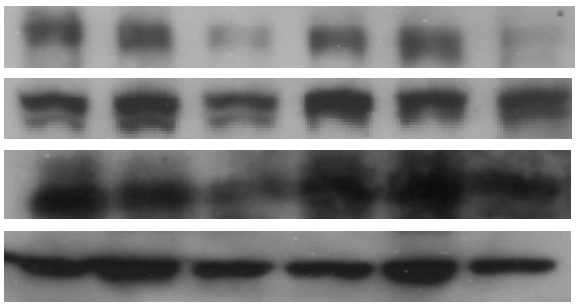


Figure 6. Knockdown of EGFR expression with siRNA did not abrogate STAT3 activation in UM-SCC-29 and UM-SCC-33. A) Western blot analysis of resistant cells either without treatment, treatment with a scrambled vector or with EGFR siRNA contstructs. The blots were then probed with EGFR, P-STAT3, and survivin. Beta Actin was used as a loading control. The experiments were replicated three times.

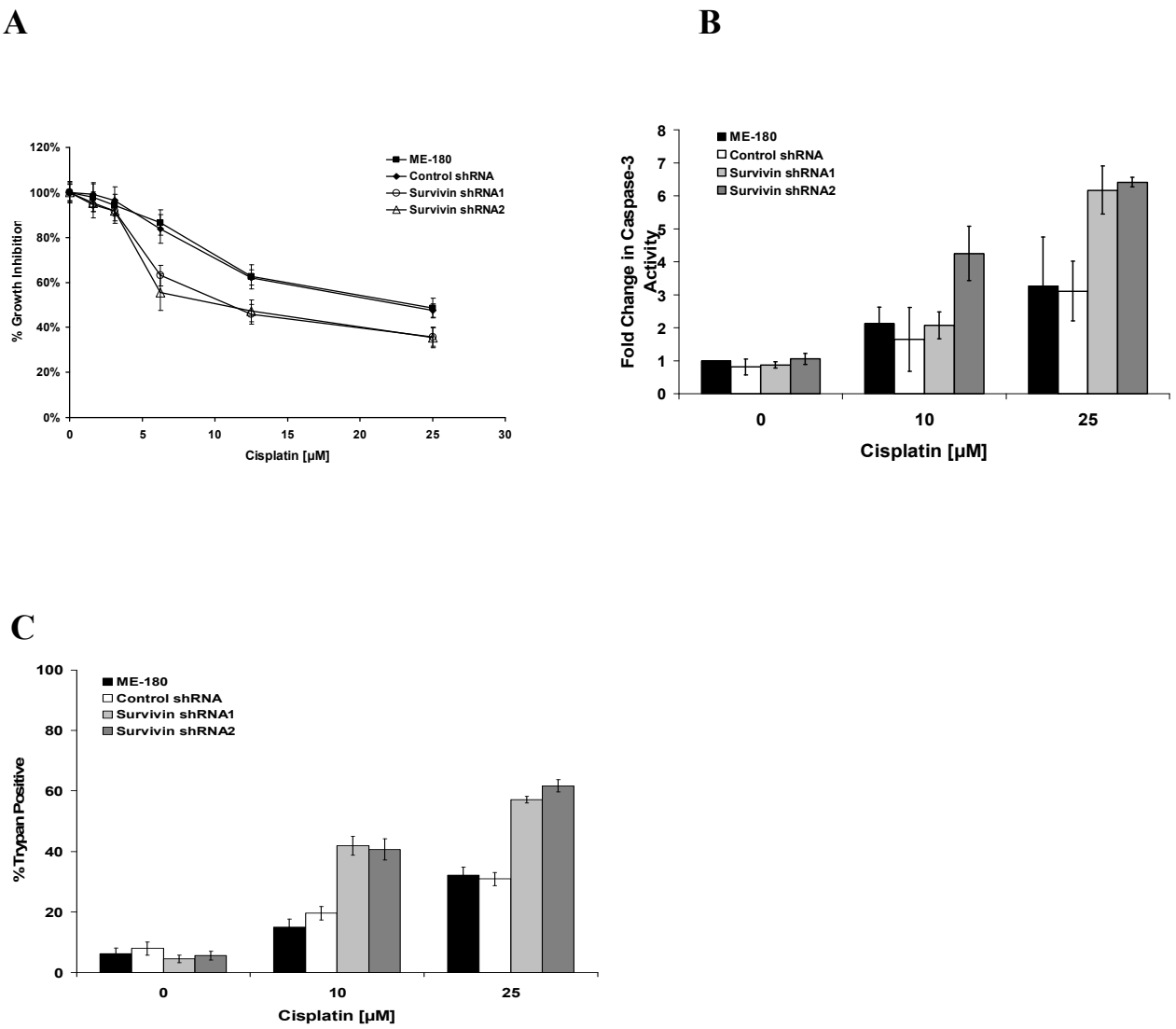


Figure 7**.** Survivin shRNA sensitizes ME-180 cells to cisplatin-induced apoptosis. A) Western blot of protein lysates from cells treated with either control or survivin shRNA was probed for total survivin expression. Glyceraldehyde-3-phosphate dehydrogenase shows equal loading in all blots. B) MTT chemosensitivity assays were used to obtain IC_{50} values for each cell line. Cells were treated continuously with cisplatin for 48hours prior to analysis of (C) caspase-3 activity and (D) trypan blue dye exclusion.

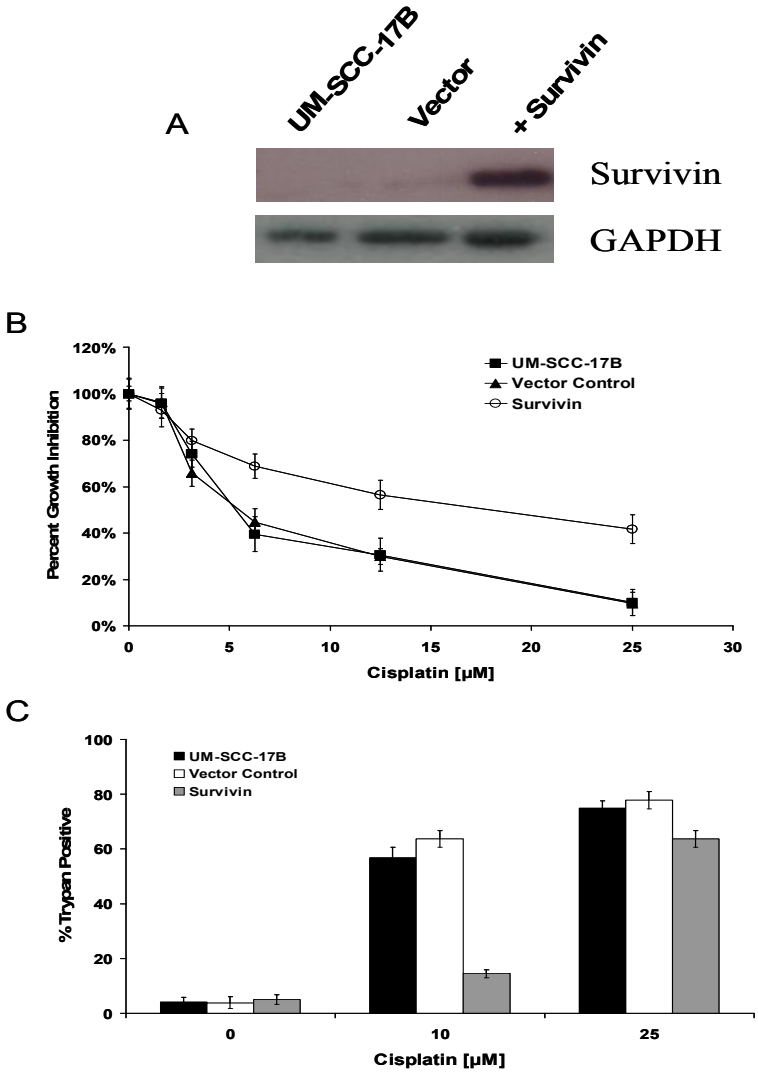


Figure 8. Survivin overexpression in UM-SCC-17B results in increased survivin cisplatin resistance. a) Western blot analysis of survivin expression in UM-SCC-17B with and without infection of survivin expression vectors. GAPDH was used as a loading control. b) MTT chemosensitivity assays and c) trypan blue experiments were performed to test for cisplatin resistance in UM-SCC-17B cells expressing survivin.

STAT3 Inhibitors Sensitize Resistant Cell Lines to Cisplatin-Induced Growth Inhibition

Because survivin expression is regulated by activated STAT3 (9,15), and we have shown the implications of survivin in cisplatin resistance (Figure 8), we postulated that targeting STAT3 with a small molecule inhibitor might sensitize both the inherently resistant cell lines UM-SCC-29 and UM-SCC-33 and the cell line selected for resistance, ME-180R. To test this, we used JSI-124 to determine the growth inhibitory effects on the entire panel of head and neck cell lines. JSI-124 was quite potent in the HNSCC panel with IC_{50} values in the nanomolar range (Figure 9A). Next, we hypothesized that by targeting pathways affected by cisplatin in addition to targeting the proposed STAT3 resistance pathway, we could sensitize to cisplatin treatment. Because all of the cell lines had similar JSI-124 IC_{25} values, clustering around 50 nM, we used this value for our drug treatments. Each cell line in the HNSCC panel was treated with 50 nM JSI-124 in combination with logarithmic concentrations of cisplatin. Importantly, UM-SCC-29 showed an increased cisplatin resistance to combination treatment, with a shift in cisplatin IC_{50} concentrations from 12 μM to 0.4 μM (Figure 9B and C). To further address this shift in chemotherapeutic resistance, a smaller cisplatin dosage range was used for the HNSCC panel (Figure 9C). This increase is much greater than what is expected from the effects of JSI-124 and cisplatin individually, implicating drug synergy and reduced cisplatin sensitivity in the nanomolar range (Figure 9B and C). However, JSI-124/cisplatin combinational treatment was unable to sensitize UM-SCC-33 to cisplatin. UM-SCC-33 did not show this same effect. Because UM-SCC-17B, UM-SCC-74, and UM-SCC-10B were already quite sensitive to cisplatin, these combinational effects were not seen in the MTT assays.

Because STAT3 inhibitors were shown to be effective in cell lines inherently resistant, we investigated whether the same effects would be seen in a cell line artificially selected for

chemotherapeutic resistance. Once again, we used MTT chemosensitivity assays to analyze the growth inhibitory effects of the small molecule STAT3 inhibitors JSI-124 and AG490. JSI-124 and AG 490 treatment revealed IC_{50} values of 203.3 nM and 22.5 μM, respectively for treated ME-180R (figures 10A and 10B). The drugs were then studied for their ability to increase cisplatin sensitivity in ME-180 cells. Continuous treatment with 50nM JSI-124 or 35μM AG 490 in combination with cisplatin treatment resulted in increased cisplatin-induced growth inhibition and reduced the IC_{50}s to less than 5 μM cisplatin (figure 10C). Trypan blue dye exclusion assays demonstrated a 27% and 29% loss of cell viability 48 hours after treatment with either 10 μM cisplatin or 50 nM JSI-124, respectively, while combination of the two drugs resulted in >50% reduction in trypan blue dye exclusion (figure 10D). Significantly, ME-180R can also be sensitized to cisplatin treatment when complimented with JSI-124 treatment.

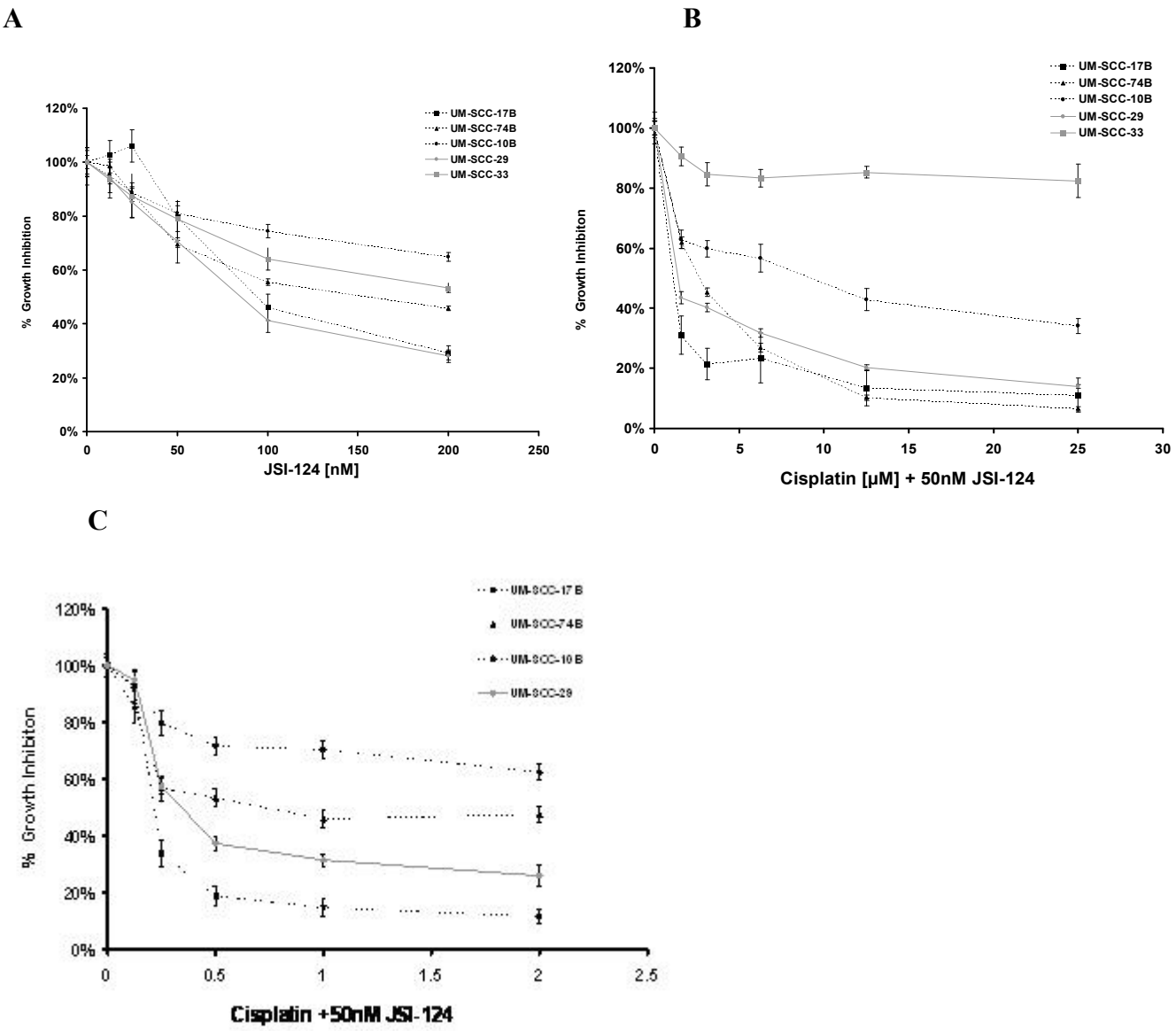


Figure 9. The small molecule inhibitor JSI-124 inhibits UM-SCC cell line growth and sensitizes cisplatin resistant, mutant p53 cell lines. A, MTT chemosensitivity assays were used to calculate the JSI-124 IC50 (50% growth inhibition) and IC25 values for each cell line. B, The effect of combination 50nM JSI-124/cisplatin treatment is quantified using the MTT chemosensitivity assay to assess changes in the cisplatin dose response. Standard deviation error bars are shown. C, Lower window of combinational JSI-124 and using cisplatin dosages in the low micromolar range. UM-SCC-33 was eliminated due to its resistance to cisplatin. All experiments were done in triplicate.

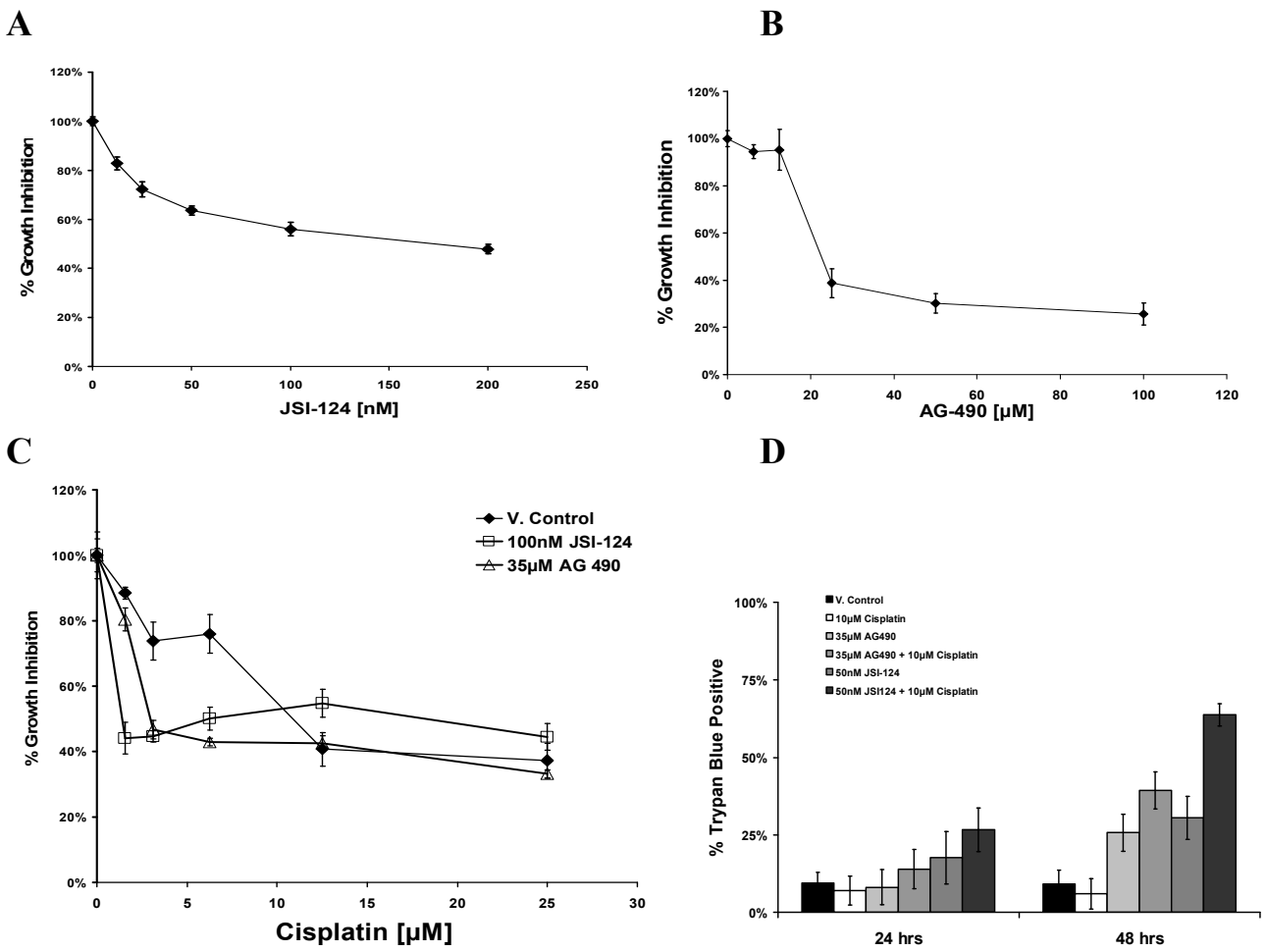


Figure 10. STAT3 inhibitors sensitize ME-180R to cisplatin. ME-180R cells were treated with different doses of either (A) JSI-124 or (B) AG-490 to obtain IC50 values. C) ME-180R cells were treated with vehicle control, 100nM JSI-124, or 35µM AG490 and then subjected to a cisplatin MTT chemosensitivity assay. D) Results of trypan blue dye exclusion assays of cells treated either with cisplatin alone or in combination with a STAT3 inhibitor for either 24 or 48 hours. All experiments were run at least three times.

JSI-124 Blocks p-Stat3 and Bcl-xL Expression in ME-180R

Because loss of survivin and JSI-124 both increased sensitivity to cisplatin treatment in ME-180R, we hypothesized that combinational treatment JSI-124/cisplatin treatment would result in downregulation of survivin and other proteins in the STAT3 signaling pathway. ME-180R cell lysates were collected after 48 hours of continuous exposure to either 10 μM cisplatin, 100 nM JSI-124, 35 μM AG490, or combinations of these three treatments. Subsequently, Western blot analysis was performed and blots were probed with the same antibodies initially used to determine a pre-treatment protein profile. Treatment with10 μM cisplatin showed reduction of total EGFR protein expression, loss of p-STAT3, p-AKT and total AKT, but only a slight reduction in STAT3 and surviving (Figure 11). Treatment with 100 nM JSI-124 led to a change in expressed p-STAT3 (loss of isoform STAT3β) but had no significant effect on expression of other EGFR pathway proteins, while 35 μM AG-490 treatment also resulted in loss of the lower STAT3β band but had no effect on total EGFR, p-AKT or AKT protein expression. It is important to note that at 100 nM, JSI-124 did not inhibit activated STAT3 expression as expected. Interestingly, treatment with either cisplatin or JSI-124 led to an induction of p53, in this wild type p53 containing cell line, however, p53 was not induced the combination of cisplatin and JSI-124. Induction of p53 was unexpected because the cervical carcinoma cell line ME-180R expresses E6/E7 which is expected to result in degradation of p53. Importantly, treatment with cisplatin in combination with JSI-124 results in loss of both phosphorylated p-STAT3, loss of Bcl-xL expression, and slight downregulation of STAT3. Importantly, treatment with cisplatin in combination with JSI-124 results in loss of phosphorylated p-STAT3, XIAP, and Bcl-xL expression. Also, there was a slight reduction in the expression of survivin. These

effects were not seen when using AG490 for unknown reasons. Apparently, AG490 is ineffective at disrupting STAT3 signaling in these cell lines.

Vehicle Control	-	+	-	-	-	-	-
10 µM Cisplatin	-	-	+	-	-	+	+
100 nM JSI-124	-	-	-	+	-	+	-
35 µM AG-490	-	-	-	-	+	-	+

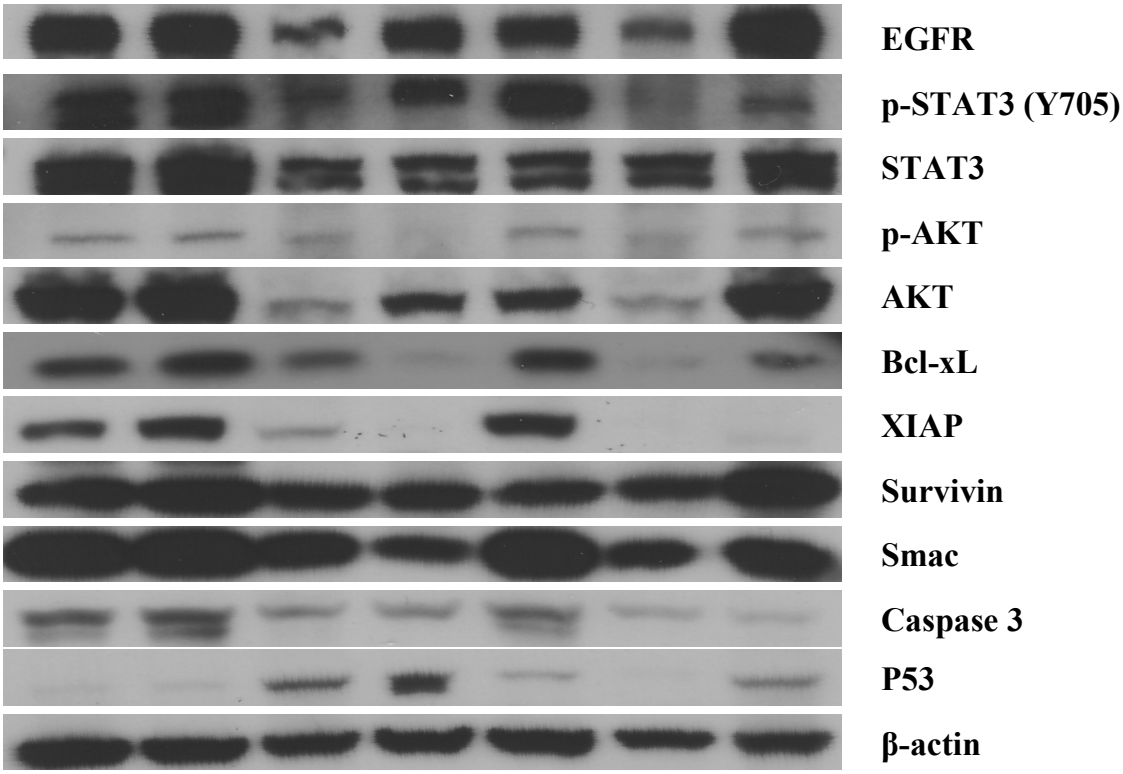


Figure 11. Alterations in the EGFR signaling axis following treatment. A) Western blot analysis of whole cell protein lysates from ME-180R cells treated for 48 hours with the annotated conditions. Blots were stripped and re-probed with multiple antibodies as indicated in the figure. Beta Actin was used to demonstrate equal loading in all lanes. This experiment was run in triplicate.

Phosphorylation of STAT3 is Completely Inhibited at 1 μM JSI-124

Treatment with JSI-124 alone did not result in deactivation of STAT3 at the concentrations where JSI-124/cisplatin drug synergy was observed. Therefore, we performed a dosage response assay in which ME-180R was treated with concentrations of JSI-124, determined logarithmically. The JSI-124 drug treatments ranged from 3 nM to 1 uM as shown in Figure 12 and cell lysates were collected after 48 hours of continuous exposure. The cell lysates were analyzed with Western blots using the same antibodies in the previous blot (Figure 11). As shown in figure 12, both p-STAT3 bands disappeared at JSI-124 concentrations of 1 μM suggesting the disruption of the interaction between STAT3 and JAK kinase. Interestingly, at a concentration that completely STAT3 activation, the protein expression profile looks strikingly similar to that of the combination JSI-124/cisplatin treatment shown in figure 11. With increasing concentrations of JSI-124, EGFR expression is slightly reduced. The levels of STAT3 and SMAC stay relatively the same while p-AKT, AKT, XIAP, survivin, and Bcl-xL expression are lost. In addition, p53 expression is induced at 100 nm but then begins to decline at higher concentrations of JSI-124. Interestingly, the molecular weight of caspase 3 changes slightly at higher concentrations of JSI-124. Overall, the protein expression levels are quite similar to those seen in the combinational treatment implying that treating with smaller doses of both cisplatin and JSI-124 can have the same effect as treating with one or the other at more toxic levels. Importantly, expression of Bcl-xL, XIAP, and survivin are abolished at high concentrations of JSI-124. When STAT3 expression is eliminated in ME-180R, the hypothesized downstream targets also appear to be effected. This data suggests that a STAT3 pathway is implicated in STAT3 mediated resistance.

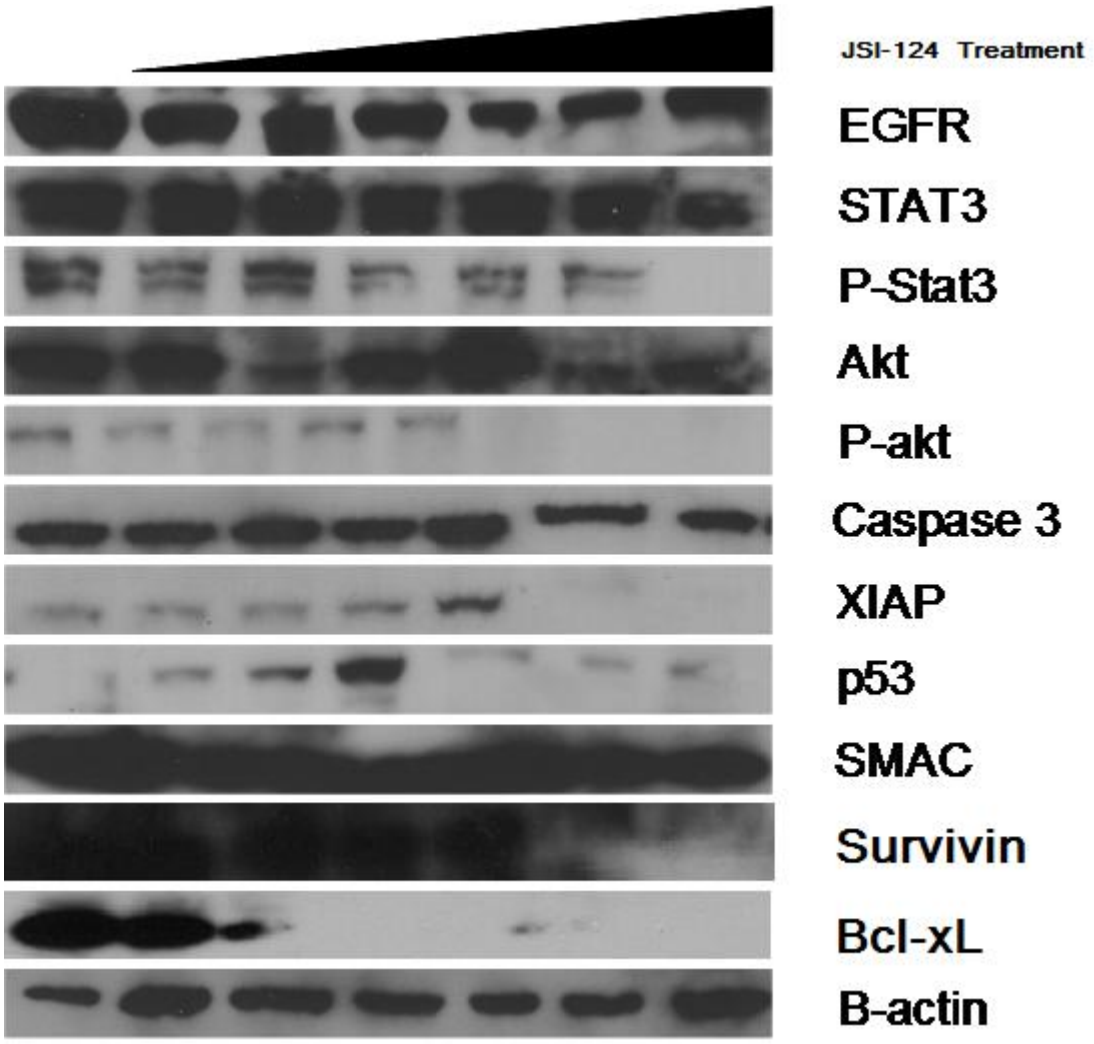


Figure 12. Treatment of ME-180R with 1 μM JSI-124 eliminates expression of activated STAT3. Cell lysates were collected after treatment with logarithmic concentrations of JSI-124. Western blot analysis was completed and the blots were probed with antibodies shown in the figures. The experiments were done in triplicate.

STAT3 Inhibitors Sensitize ME-180R to Cisplatin-Induced Apoptosis

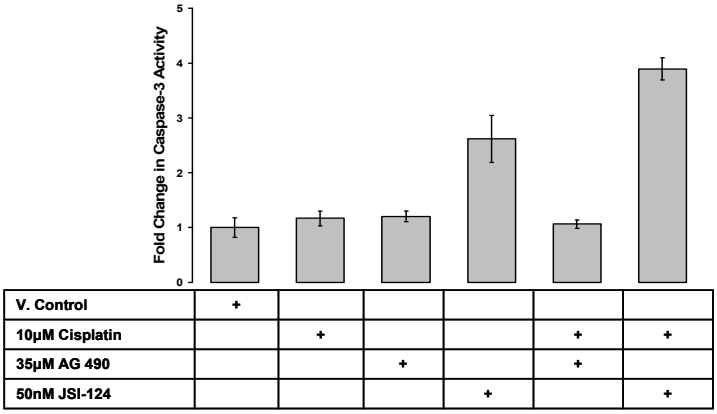
Now that we have identified that Bcl-xL, XIAP, and survivin may be implicated in a STAT3-mediated resistance mechanism, we explored whether treatment with STAT3 inhibitors would result in increased sensitivity to cisplatin-induced apoptosis. To do this, we performed both caspase 3 activity and annexin V staining assays. Treatment of ME-180R with 50 nM JSI-124 produced a 2-fold increase in caspase 3 activity in comparison to the caspase 3 activity in the parental ME-180R cell line. Combinational treatment with JSI-124/Cisplatin, resulted in even higher caspase 3 activity (Figure 13A). It was interesting that AG490 and AG490/Cisplatin treatment did not induce caspase 3 activation. Caspase 3 fold activity was determined as compared to fluorescence blank. Subsequently, Annexin V staining was completed after continuous drug treatment for 48 hours. Treatment with JSI-124 produced greater than 20% annexin V staining as compared to the vehicle control. Combinational treatment with JSI-124 and Cisplatin resulted in about an 80% increase in annexin V staining (Figure 13 B). These drastic increases in Annexin V staining were not seen in treatments with AG490 and with cisplatin alone. Taken together, these results suggest that JSI-124 and cisplatin combinational treatment target the cell for apoptosis.

Treatment with 50 nM JSI-124 Resulted in Membrane Changes Characteristic of Apoptosis in ME-180R

Because both JSI-124 and AG490 produced contrasting data in both protein expression and apoptosis assays, confocal microscopy was used to simultaneously assess changes in STAT3 phosphorylation (Tyr705) and cell morphology. As shown in figure 14, treatment of ME-180R cells with 50nM JSI-124, but not AG 490, led to decreased phospho-STAT3 staining. Based on the results from the dose response Western blot analysis, p-STAT3 expression was not completely eliminated. Significant membrane blebbing, a hallmark of apoptosis, was observed in

JSI-124 treated cells. Interestingly, following exposure of ME-180R cells to AG 490, large vacuoles more characteristic of autophagy appeared in cells (figure 14). Therefore, this data suggests that AG490 and JSI-124 target STAT3 by different mechanisms.

A



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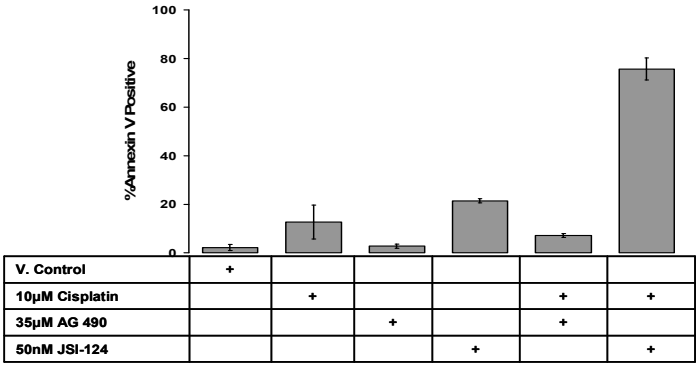

Figure 13. JSI-124 enhances cisplatin-induced apoptosis. A) Caspase 3 activity was measured in whole cell lysates 48 hours after treatment with either cisplatin, AG490, JSI-124 or in combination. Graph represents the average of three independent experiments run in quadruplicates. B) ME-180R cells were treated for 48 hours as indicated and stained with Annexin V antibody and PI (live/dead). %Apoptosis represents the percent of all size gated, PI excluding cells with high Annexin V staining. This experiment was run in triplicate and the graph represents compiled data from these experiments.

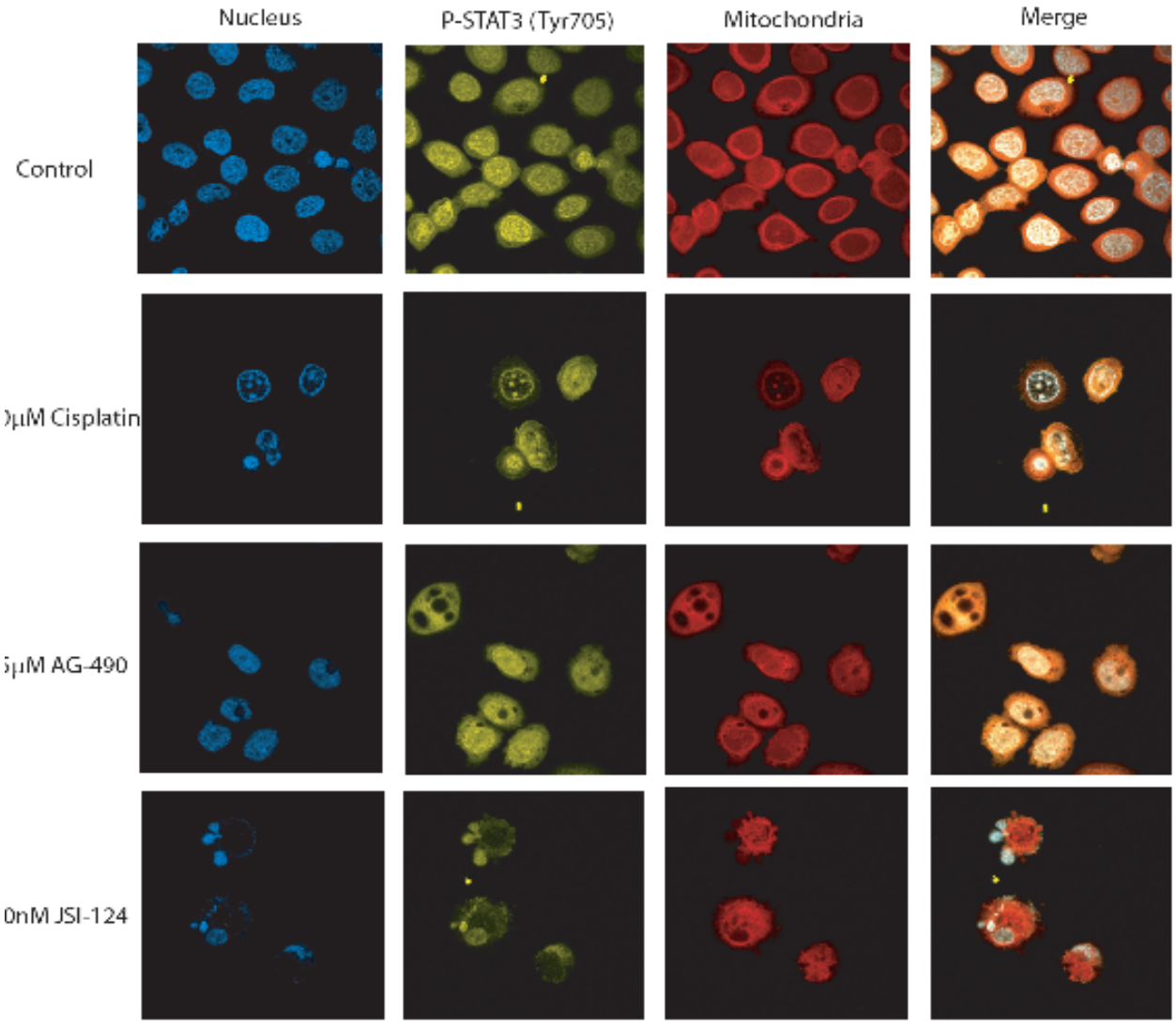

Figure 14. JSI-124 induces apoptosis at concentrations below those necessary for inhibition of STAT3 phosphorylation. ME-180R cells treated with either vehicle control, cisplatin, JSI-124 or AG490 for 48 hours were stained with DAPI (nucleus), p-STAT3 (Yellow) and MitoTracker (Red). Split images and the merge are shown for all four conditions. Note the presence of DAPI staining in the membrane blebs of cells treated with JSI-124. The experiment was run in triplicate and multiple images were recorded each time. Representative images are shown.

Preliminary ChIP Data Suggests that Bcl-xL, XIAP, and Survivin Promoters Interact with STAT3

To provide more evidence for the hypothesis that Bcl-xL, XIAP and survivin promoters are regulated by STAT3, a ChIP experiment was completed to test for these protein-DNA interactions. A simplified diagram of the experiment has been included in figure 14. First protein-DNA complexes are crosslinked by incubation with formaldehyde, forming one carbon linkers between the protein and DNA that spatially allow for the chemical linkage reaction to occur. The cells and nuclei are lysed and the chromatin is extracted. Then, the chromatin is sheared to 500 base pairs with sonication. The chromatin is incubated with either anti-STAT3, anti-immunoglobulin, or anti-RNA polymerase antibodies along with beads that react with the antibody. Subsequently, the chromatin is immunoprecipitated with the attached magnetic beads and then a reverse crosslinking interaction is done to free the chromatin from the DNA. The immunoprecipitation step serves to enrich for DNA sequences that interact with the antibody due to its specificity for a protein which is crosslinked to chromatin. Using qPCR primer sets that target the STAT3 binding site of Bcl-xL, XIAP, and survivin (which overlaps with the NF-κB binding site) (36), amplification of the DNA is determined relative to the negative immunoglobulin control. DNA from input controls along with an RNA-Pol II positive control are also amplified in this experiment. We hypothesized that treating ME-180R cells with increasing concentrations of JSI-124 would result in disruption of STAT3/DNA interactions and hence would not allow for the enrichment of DNA fragments containing the STAT3 binding site (such as Bcl-xL, XIAP, and survivin). In wild-type ME-180R cells, we hypothesized that we would see enrichment of Bcl-xL, XIAP and survivin promoter sequences at the STAT3 binding site. Currently, we have preliminary data that suggests that our hypothesis was correct. In our initial experiments, we have seen greater amplification of the Bcl-xL, XIAP, and survivin

promoters in wild-type ME-180R cells and less amplification of these DNA sequences in a JSI-124 dose dependant fashion. However, at this point, the experiment has not been done in triplicate so the results have not been verified. Further replicates of this experiment will allow for the determination of whether STAT3 interacts with the anticipated promoter regions in ME-180R. This biochemical data will be important for determination of a cisplatin resistance signal cascade implicated in STAT3 signaling.

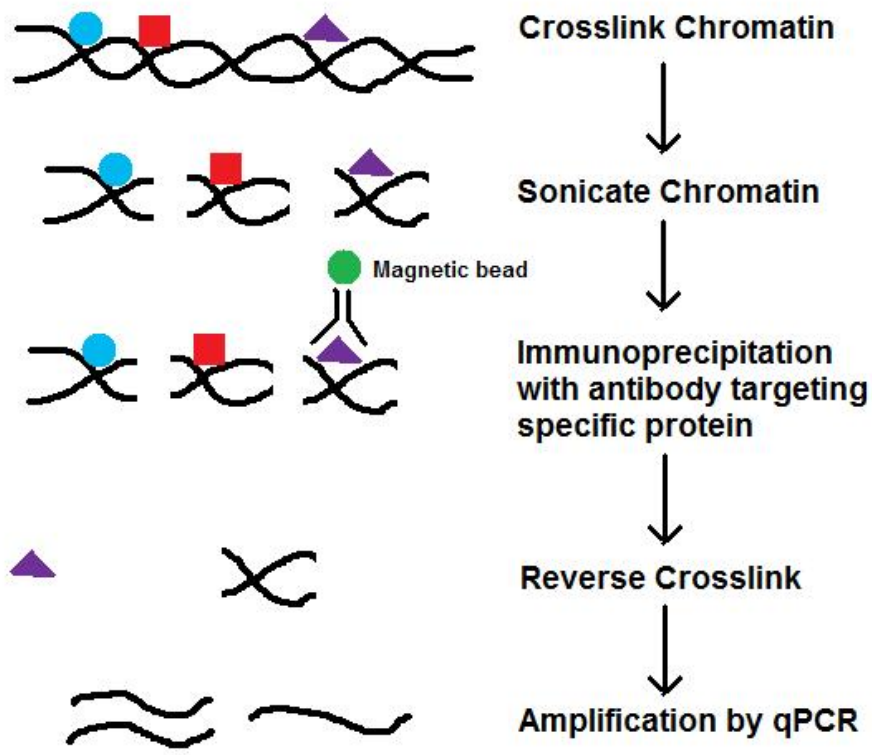

Figure 15. Basic schematic of ChIP assay.

DISCUSSION

To date, cisplatin is the most effective agent in treating squamous cell carcinomas, including the HNSCC and cervical carcinomas studied in this thesis (37). Even though cisplatin has proven to be successful in disrupting growth of squamous cell carcinomas, some tumors are inherently resistant to this therapy. Clinical trials have shown that combining cisplatin treatment with other compounds such as the mitomycin-C, vincristine, and bleomycin (MVB) regimen (38) and 5-fluorouracil (39) result in much improved patient responses. Even with the development of these chemotherapeutic cocktails, resistant cell populations develop that do not respond to current therapies. The goal of this thesis is to define a mechanism by which these resistant tumors avoid cell death. Identification of these survival pathways will be important in developing more targeted cisplatin combinational therapies for resistant cell types.

Correlation Between Biomarkers in SCC Tumors and Chemotherapeutic Resistance

Analysis of pretreatment biopsies from SCC patients and results from clinical trials are beginning to reveal biomarkers that categorize individual tumors into populations that respond to cisplatin-based treatment protocols and those that do not. In a previous study, our lab demonstrated that high Bcl-xL and wild-type p53 are indicative of poor patient prognosis, most likely due to the loss of p53 arrest and DNA repair mechanisms (18). A study by Kim et al. revealed that HPV and p16 expression are inversely related to the levels of EGFR in a tumor (40). p16, a cyclin-dependent kinase inhibitor that blocks cell cycle progression at the G1 to S checkpoint, is overexpressed when pRb is inactivated by HPV E7 protein. Notably, HPV E6 can inactivate p53, resulting in less HPV positive tumors expressing mutant p53 (8). Further studies identified p16, EGFR, and HPV as independent predictive biomarkers of poor survival. Poorest prognosis was correlated with high levels of EGFR, and within this group high levels of p16 and

HPV improved patient survival, whereas low levels of p16 and HPV did not (8,41). In this thesis, cell lines were analyzed with chemotherapeutic sensitivities and with different protein expression profiles. Explanations for cisplatin sensitivity of these cell lines are not well-developed, however, we can hypothesize why certain cells are more sensitive than others based on the identification of the aforementioned biomarkers from pretreatment biopsies. The cell lines UM-SCC-17B and UM-SCC-74B exhibit wild-type p53 and high Bcl-xL, however, these cell lines are highly sensitive to cisplatin. Western blot analysis of untreated cell lines in figure 4B demonstrated that UM-SCC-17B and UM-SCC-74B have decreased levels of EGFR expression as compared to the other resistant cell lines. As shown in analysis of pretreatment biopsies, low EGFR is correlated with increased patient survival even in the presence of other biomarkers (8). In the case of the cell lines UM-SCC-17B and UM-SCC-74B, relative HPV and EGFR expression could explain their increased sensitivities to cisplatin. UM-SCC-29 and UM-SCC-33 are cisplatin-resistant cell lines with high Bcl-xL and mutant p53. Because mutant p53 is not associated with the presence of the HPV 16 protein, it can be hypothesized that UM-SCC-29 and UM-SCC-33 do not express HPV. Therefore, low levels of HPV and high levels of EGFR in these cell lines would position them in the subgroup with the worst projected survival rates (8). Unlike these resistant cell lines, ME-180R expresses both high levels of EGFR and wild-type p53. Therefore, it is possible that ME-180R has high levels of HPV expression because p53 expression is low. These biomarkers would place ME-180R in the subgroup with poor prognosis, but in a group with better survival expectations than that of UM-SCC-29 and UM-SCC-33. Understanding the HPV and EGFR story may help explain why certain cell lines are more resistant than others and why certain survival pathways are expressed in the cisplatin-resistant cell lines.

The IC_{50} concentrations for each cell line tested in this thesis were analyzed by MTT chemosensitivity assays. For the most part, the calculated cisplatin IC_{50}s were found to be as expected based on previous studies in the laboratory. We defined a sensitive cell line as one that had an IC_{50} concentration of lower than 10μM. This designation created a group of three sensitive (UM-SCC-17B, UM-SCC-74B, UM-SCC-10B) and three resistant cell lines (UM-SCC-29, UM-SCC-33, ME-180R). At this point, it is important to note that the cisplatin sensitivities of the HNSCC derived cell lines are all inherent. In contrast, the cervical cell line ME-180R was artificially selected for chemoresistance by isolating cisplatin resistant populations by constantly treating the cells with high concentrations of cisplatin. Cells that were resistant to cisplatin died off and the remaining population consisted solely of ME-180 resistant cells. Interestingly, in this study UM-SCC-33 was found to be extremely cisplatin resistant (IC_{50} greater than 50 μM). This calculated IC_{50} is greater than the IC_{50} of ME-180R. The IC_{50} concentration of ME-180R would be expected to be larger because it is a subpopulation of cells specifically selected for chemoresistance. There are two potential reasons for the high levels of chemoresistance in UM-SCC-33. First, UM-SCC-33 may be multi-drug resistant and therefore the IC_{50} concentration that was calculated here is a real observation. This hypothesis can be further tested with particularly potent drug treatments, to determine if UM-SCC-33 can be sensitized with other treatment regimens. Second, the high resistance of UM-SCC-33 may be an artifact of a slower growth rate of the cell line. The MTT growth assay is used to determine the growth rate of the treatment wells in comparison to that of control wells. If UM-SCC-33 grows more slowly than other cell lines, the cisplatin-resistance seen in Figure 2A could be due to less detectable growth of UM-SCC-33 cells (even if some of the cells were dying) in the wells to take

up the dye. In any case, the relative cisplatin sensitivities were important in comparing protein expression in cisplatin sensitive and cisplatin resistant cell lines.

Identification of Potential Biomarkers in Cisplatin-Resistant Cell Lines

Poor patient survival has been associated with upregulation of EGFR (8), so we investigated signaling pathways known to be activated by stimulation of EGFR. These included the JAK2/STAT3 (42), PI3K/AKT (43), and MAPK signaling pathways (44). Further analysis of these pathways revealed that they all converge at a single regulatory point, STAT3. Importantly, activated STAT3 has been shown to regulate downstream proteins implicated in survival. Survivin has been shown to be a downstream target of STAT3 in breast (15), head and neck (45), skin (46) and cervical (10) carcinomas. Bcl-xL is also known to be regulated by STAT3 (16). Further, we analyzed XIAP because of its anti-apoptotic activity and association with survivin (47). Therefore, we analyzed the expression of activated STAT3 along with genes it is known to regulate including survivin, Bcl-xL, and XIAP in conjunction with other proteins upstream of STAT3 signaling. Western blot analysis determined individualized protein expression profiles for both the cisplatin sensitive (UM-SCC-17B, UM-SCC-74B, UM-SCC-10B) and cisplatin resistant (UM-SCC-29, UM-SCC-33, ME-180R) cell lines. Some proteins appeared to be upregulated or downregulated consistently among cell lines within the same chemosensitivity group. The resistant cell lines showed high expression of EGFR, STAT3, p-STAT3, AKT, p-AKT, Survivin, Bcl-xL, SMAC, Caspase 3, and XIAP. The results were consistent with protein expression associated with EGFR-activated signaling pathways. These proteins are important in the PI3K/AKT and MAPK signaling pathways, yet, there were protein expression differences in the cell lines grouped for resistance. For example, ME-180R was the only cell line to have upregulated XIAP and UM-SCC-33 did not have increased expression of p-

AKT. Although these cell lines show many commonalities in protein expression, the fact that there are differences between each cell line is quite apparent. These variances should be addressed with diverse chemotherapeutic treatments based on a tumor's genomic "fingerprint" (48). The necessity of individualized treatment is demonstrated by the varied protein expression profiles in Figure 4B.

Targeting EGFR is Unsuccessful in Sensitizing SCC Resistant Lines to Cisplatin Treatment

Because we found EGFR to be overexpressed in the resistant cell lines UM-SCC-29 and UM-SCC-33, we hypothesized that targeting EGFR with a monoclonal antibody would sensitize the cell lines to cisplatin treatment. We postulated that deactivating a protein highly upstream the pathways shown to be upregulated by Western blot analysis in Figure 4B would be inhibited. To test this, cells were treated with Cetuximab (C225) and then analyzed for protein expression. Cetuximab is a chimeric monoclonal antibody that specifically targets the EGFR external receptor and competitively inhibits the natural ligands that bind the EGFR receptor. In clinical trials, Cetuximab has been shown to increase survival in patients with advanced head and neck cancer (49),(50) and with colorectal cancer (51). Cetuximab is a relatively new treatment and there is not significant data yet on whether or not Cetuximab treatment is improving overall survival rates of SCC patients. When treating the resistant SCC cell lines UM-SCC-29 and UM-SCC-33 with Cetuximab, we were unable to knock down expression of EGFR. There are many potential reasons for why this experiment did not work. First, the humanized monoclonal antibody may not have similar effects *in vitro* as it does *in vivo*, therefore, treating our cell lines with C225 may not have been appropriate. Recent studies have shown that treatment with Cetuximab resulted in hyperphosphorylation of tyrosine 1173. Instead of the expected deactivation of EGFR, increased activation of EGFR was observed in HNSCC cell lines. Also,

there seemed to be no effect on cell proliferation (52). Second, we cannot rule out that the treatment failed because of errors in technique; however, the protocol was followed exactly as specified by a collaborating lab. Third, other ligands may have out-competed the EGFR antibody in our *in vitro* systems. Whatever the reason, we were unable to use C225 to knockdown expression of EGFR and, alternatively, we employed the use of siRNA to reduce EGFR activation. EGFR siRNA was successful in knocking down EGFR expression, however, downstream targets such as p-STAT3 and survivin were not affected. The cumulative results of the C225 monoclonal antibody experiment and the siRNA experiment brings up the question of whether or not it is relevant to target EGFR in cell lines that are resistant to cisplatin. Quite possibly, other signaling pathways may activate STAT3 by a pathway *independent* of EGFR. For example, an integrin pathway could be implicated in STAT3 activation such as in prostate cancer signaling (53). Another possibility is that STAT3 is constitutively active and does not need to be activated by EGFR signaling. Therefore, treatment of these resistant cell lines with monoclonal antibodies may not be clinically useful for certain cell types. Defining more relevant targets will be important in sensitizing SCC resistant populations to cisplatin treatment.

Survivin Induces Cisplatin Resistance and Regulates STAT3

Knowing that EGFR was not a suitable target for downregulating STAT3 survival pathways in the cisplatin resistant cell lines, we investigated the hypothesis that the survival pathways we were studying must be deactivated for cells to be resistant to cisplatin. In SCC carcinomas, survivin has been shown to be an important survival signal that allow for cells to avoid death and has been shown to be regulated by the pathways we are studying (54). To study if survivin is implicated in increased chemosensitivity, we examined the cell line that was selected for resistance, ME-180R, because of its extremely high IC_{50} concentrations and high

expression of survivin. Here, we show that knocking down survivin in ME-180R, a cell line that contains high levels of survivin, results in increased cisplatin sensitivity (Figure 7). In contrast, when we then overexpressed survivin in UM-SCC-17B, we were able to induce cisplatin resistance (Figure 8). Importantly, when knocking down the most downstream target, we could sensitize a highly resistant cell line to cisplatin. Therefore, these results told us that we were indeed targeting the appropriate survival pathway. Based on the data from these gain and loss-of-function experiments, the most logical treatment would be to target survivin with some therapeutic approach. However, there is currently no therapeutic option for targeting survivin. There are a number of anticancer drugs that have demonstrated the ability to suppress survivin in methods that target other locations in the signaling pathway including doxorubicin, hedamycin, and lapatinib (55). In addition, the small molecule compound YM155 was shown to inhibit survivin in a survivin promoter activity assay (55). These compounds may be relevant to this study, however, they were not readily available for use. Therefore, we examined a more upstream target, STAT3, which is still a common target to all of the pathways we are studying. There are many well-established STAT3 inhibitors that could be implemented in this study. Most inhibitors do not target STAT3 directly but either inhibit the JAK/STAT3 interaction or JAK kinase activity (56). Pathways can be regulating at many different points, it is important to find the optimal protein to target for the best patient response.

STAT3 as an Optimal Target for Sensitizing Cells to Cisplatin Treatment Using JAK Small Molecule Inhibitors

It has been well-established that JAK tyrosine kinase inhibitors (such as JSI-124 and AG490) can inhibit the activation of STAT3 and the data from this thesis has provided further evidence for this activity in HNSCC and cervical carcinomas. Western blot analysis showed that in a panel of UM-SCC cell lines each cell line overexpressed activated STAT3 to some degree.

An MTT chemosensitivity assay revealed that JSI-124 treatment resulted in cell death, with very potent IC_{50} concentrations in the nanomolar range. The IC_{25} concentration for each cell line was near 50 nM so this concentration was selected as a constant (Figures 9 and 10). The cell lines were then treated with logarithmic dosages of cisplatin along with 50 nM JSI-124. The sensitive lines UM-SCC-17B, UM-SCC-74B, and UM-SCC-10B showed decreased sensitivity to cisplatin, however, there was not much of an effect because they were already highly sensitive to cisplatin treatment. The resistant cell lines UM-SCC-29 and ME-180R showed increased sensitivity to cisplatin, much greater than the simple cumulative effects anticipated from combinational cisplatin and JSI-124 treatment. Therefore, this sensitivity is a result of cisplain/JSI-124 drug synergy. When ME-180R was treated with AG490, it was also sensitized to cisplatin. This data is novel in terms of SCC cancers and suggests that targeting STAT3 with small molecule inhibitors in combination with cisplatin may be a beneficial therapeutic regimen. However, there are extreme limitations to using this approach in the clinic. STAT3 is important for the survival and proliferation of normal cells (57). AG490 and JSI-124 cannot specifically target aberrant signaling in cancer cells, and therefore, cannot be translated to treatments in the clinic.

JSI-124 Inhibits Cellular Proliferation at Concentrations that Do Not Affect STAT3 Phosphorylation

To further address the mechanism by which JSI-124 inhibited cellular growth in the MTT chemosensitivity assays, we did a western blot analysis of cell lysates that were treated with either cisplatin, JSI-124, AG490 or a combination of these treatments. Surprisingly, p-STAT3 was not deactivated in cell lines treated only with JSI-124. However, the literature does not report that JSI-124 inhibits STAT3 at concentrations in the nanomolar range. The western blot analysis does show a loss of one of STAT3 isoforms, and this could have some importance on

downstream regulation. Combinational JSI-124/cisplatin treatment results in complete downregulation of STAT3 which is most likely essential for drug synergy. We hypothesized that JSI-124 would inhibit STAT3 at the concentrations reported in the literature (1). Western blot analysis was done on cell lysates that were treated with logarithmic concentration of JSI-124. As expected, JSI-124 inhibited p-STAT3 expression at 1 μM. In addition, the protein expression profile observed at 1 μM was very consistent with that seen with combinational treatment of JSI-124/cisplatin. These protein expression trends induced by JSI-124 include loss of Bcl-xL, survivin, P-STAT3, AKT, and XIAP, induction of p53, and changes in the molecular weight of caspase 3. Therefore, the observed effect of the novel combinational treatment of JSI-124/cisplatin was comparable to that of JSI-124 treatment alone, though the former occurred at drug concentrations much lower than what would be expected to be toxic to the cell. Because synergistic potency was seen at concentrations that did not inhibit STAT3 phosphorylation, it is possible that off-target effects are the result of the cisplatin sensitivity.

JSI-124 Causes Cisplatin Sensitivity by Induction of Apoptosis

Our studies have demonstrated that STAT3 is the optimal signaling molecule to target based on current limitations in the development of small molecule inhibitors targeting downstream regulatory proteins and the specific survival pathways upregulated in the cisplatin resistant cell lines tested in this thesis. Now that we had determined that STAT3 inhibitors are capable of sensitizing the resistant cell lines to cisplatin, we were interested in the mechanism of STAT3 regulation. When JSI-124 was first identified, Blascovich et al. determined that human and murine cells underwent apoptosis when treated with micromolar concentration of JSI-124 (1). We hypothesized that the STAT3 inhibitors JSI-124 and AG490 sensitized ME-180R to cisplatin by inducing apoptosis. Trypan blue assays suggested that ME-180R underwent

increased cell death when treated with JSI-124. Greater levels of cell death were observed when the cells were treated with both JSI-124 and cisplatin. Similar effects were seen with AG490 treatment; however, more cell death was seen with the JSI-124 treatments. Therefore, the anti-proliferative effect observed in the MTT chemosensitivity assays is not the result of cellular growth arrest. Subsequent caspase 3 activity and annexin V experiments (Figure 13) suggested that ME-180R cells treated with JSI-124 or JSI-124/cisplatin in combination underwent cell death—most likely by apoptosis. However, increased caspase 3 activity and annexin V staining was not observed in treatments including AG490. This data suggests that JSI-124 treatment initiates the caspase 3 apoptotic pathway resulting in subsequent sensitivity to cisplatin. Because neither caspase 3 activation nor annexin V staining was observed in ME-180R cells treated with AG490, we further investigated the mechanism of cellular death by AG490. To do this, confocal imaging experiments were done to determine if cells treated with AG490 exhibited the hallmarks of apoptosis including membrane blebbing (58). Instead, cells treated with AG490 seemed to present with vacuole formation which is characteristic of autophagy as shown in Figure 14 (59). This is a novel mechanism for AG4900-induced cell death in SCC cells. Previously, AG490 has been shown to stimulate apoptotic pathways in other systems including a gastric cancer cell line (60) and has only been shown to induce autophagy in combination with an mTOR inhibitor (61). Interestingly, western blot analysis of combination treatment of cisplatin with either AG490 or JSI-124 showed decreased activated STAT3 only for the cells treated with JSI-124. Therefore, the mechanism by which AG490 disrupts the JAK2/STAT3 interaction may not be relevant for inducing apoptosis in SCC cell lines.

The Potential of STAT3 Inhibitors and Current Limitations

One of the major problems with developing STAT3 inhibitors is the requirement of STAT3 in normal tissues. This has been shown to be important because mice without STAT3 have an embryonic lethal phenotype which is not unexpected because STAT3 has been shown to be important in growth (62). Currently, many small molecule inhibitors have been developed that target tyrosine kinases including JAK, the kinase emphasized in this thesis. Granted, these inhibitors have worked well in downregulating STAT3 signaling; however, they have many potential targets which are problematic for patient treatment. However, it seems that the development of a more direct STAT3 inhibitor would allow for much more specific and effective targeting of the STAT3 pathway. Much work has been done in the area of developing these specific STAT3 inhibitors, and some progress is being made. STAT3 peptide inhibitors have been designed that have been shown to have little effect on non-tumor cells (63). These inhibitors disrupt the phosphotyrosine-SH2 interactions which does not allow for subsequent dimerization and activation of STAT3 (9). Another problem with STAT3 inhibitors is their potency, some STAT3 inhibitors require a high concentration to be effective but at those concentrations the treatment is toxic to normal cells (9).

Defining a Biochemical Mechanism of STAT3 Interaction with Downstream Regulators

Currently, we are doing ChIP assays in the laboratory to further confirm the interaction between STAT3 and its downstream targets including Bcl-xL, XIAP, and survivin. It is expected that the Bcl-xL, XIAP, and survivin binding sequences will be enriched in the immunoprecipitation experiment in ME-180R cells. Survivin has been previously shown to interact directly with STAT3 in breast cancer cells (15) providing further evidence for our hypothesis. We postulate that treatment with increasing concentrations of JSI-124 will abrogate

this interaction. We expect this because we have shown that JSI-124 reduces p-STAT3 expression. Loss of activated STAT3 will not allow for STAT3 translocation to the nucleus, where STAT3 normally binds to the putative binding sites of targets such as survivin. Quantitative PCR will be used to measure the amplification of the promoter regions of Bcl-xL, XIAP, and survivin using primers targeting 150-200 base pair regions. Preliminary results have shown that Bcl-xL and survivin promoter regions were amplified as compared to the IgG control in parental ME-180R cells. Amplification with primers flanking the promoter regions was much faster in the parental ME-180R cell line than in ME-180R treated with JSI-124. However, the XIAP promoter region was not amplified by qPCR. This could be due to inaccurate primer design, in which the amplification site did not include the actual STAT3 binding site. In contrast, there may not be an interaction between STAT3 and XIAP in SCC cells. These results will need to be confirmed before analysis can be completed. Overall, this ChIP data will serve to define an SCC survival pathway that may or may not require transcriptional regulation.

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