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Targeted Sirna Directed Therapy To Increase Chemosensitivity In Drug Resistant Bladder Cancer Cells

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Targeted siRNA-directed Therapy to Increase Chemosensitivity in Drug Resistant Bladder Cancer Cells

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Chemoresistance is a common problem encountered by patients with advanced urothelial carcinoma. Recent studies have investigated the mechanisms of drug resistance in cancer, particularly agents of the DNA damage repair pathway. One major player in this pathway is Ataxia-Telangiectasia Mutated and Rad3-Related protein (ATR), a kinase that becomes activated during nuclear damage yielding a single-stranded DNA break. Due to the fact that many traditional chemotherapeutic agents induce cytotoxicity by initiating DNA damage, ATR is an attractive target for investigating the mechanism behind multidrug resistant urothelial carcinoma.

Using two bladder cancer cell lines, MMCR (a drug-resistant cell line) and RT4 (a non-resistant parental cell line) we were able to create resistance profiles using cytotoxicity assays, which further facilitated in characterizing the extent of cellular resistance to a number of chemotherapeutic agents traditionally used in the treatment of advanced urothelial carcinoma.

We hypothesized that knockdown of ATR expression via RNA interference alone would render cells unstable and induce apoptosis, in accordance with similar studies investigating the effects of downstream members of this signaling pathway. Furthermore, we also hypothesized that by blocking ATR signaling, the cell line would be unable to repair its DNA, rendering the cell line sensitive to other chemotherapeutic agents.

Contrary to the above hypotheses, ATR downregulation via siRNA appeared to increase the cell viability of the MMCR cells, and did not significantly increase the chemoresistance to mitomycin C and doxorubicin across the majority of our treatment arms. These findings, though in opposition to some very early studies regarding ATR expression and chemoresistance in a variety of cancer types, highlight the continued need for elucidation of the role that ATR plays in chemoresistance in urothelial carcinoma.

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Introduction

Urothelial Carcinoma

Urothelial carcinoma (UC) of the bladder is the fourth most common cause of cancer in men in the United States, and is estimated to account for approximately 72,570 new cases and 15,210 deaths in 2013.[1] UC is three times more common in men than in women, and the median age of diagnosis is 69 for men and 79 for women.[2]

Patients with UC usually present to their physician with asymptomatic hematuria, and cystoscopy can be used for diagnosis. Tumors seen during cystoscopy can be resected or biopsied transurethrally. For patients who are considered to be in the low-risk stratification group, and whose tumors are determined to be localized (non-muscle invasive), transurethral resection of bladder tumor (TURBT) and immediate (within 24 hours of resection) single dose chemotherapy is recommended.[3] Those patients whose tumors are considered higher grade and are placed in an intermediate-risk stratification group, can be treated with intravesical Bacillus Calmette-Guérin (BCG) immunotherapy or chemotherapeutic instillations of agents such as mitomycin C.[4]

Those patients with tumors that extend into the bladder muscle or beyond can undergo additional imaging such as CT or MRI to determine if metastasis has occurred. If distant metastasis has not occurred, a patient can chose to get cisplatin-based neoadjuvant therapy before radical cystectomy. In those patients for whom cystectomy is not an option or who have metastatic disease, systemic chemotherapy or radiation are treatment options. For patients with metastatic disease, chemotherapeutic agents can be effective in prolonging survival, but these treatment options are rarely curative.[5, 6] Furthermore, even though up to 50% of patients with metastatic UC have initial

favorable response rates to systemic chemotherapy, increased drug resistance leads to little change in overall survival in this population.[7]

Chemotherapeutic Resistance

The mechanisms for the development of chemotherapeutic resistance can be divided into two groups: those in which resistance develops due to impeded drug bioavailability (decreased transport, increased efflux, or sequestration of drug) and those that mitigate the cell's biochemical response to (the) drug-induced DNA damage and apoptosis.[8]

One group of proteins associated with multidrug resistance is the family of ATP-binding cassette transporters known as MRPs (multidrug-associated proteins).[9] This protein family functions to export a wide array of molecules (including cytotoxic ones, such as chemotherapeutics) with flexible affinity to size, composition, or conformation.[10, 11] Differences in MRP expression, however, can vary across cell lines and tissue type. Because some cancer cell lines become resistant to entire groups of chemotherapeutics when exposed to a single agent (cross-resistance), it is likely that a single transport protein might not be the principle resistance mechanism. Furthermore, in one study, cisplatin resistance was shown to be independent of the intracellular concentration of cisplatin, and likely due to changes in different signaling pathways.[12]

Another reason to shift the focus of the present chemoresistance investigation towards a biochemical signaling pathway and not necessarily a membrane transport protein is that previous research has shown that high grade urothelial carcinomas actually have lower MRP mRNA expression than low grade urothelial carcinomas.[13, 14] One study investigating mitomycin C treatment- found that, in some adenocarcinomas, the drug

actually induces a decrease in both MRP1 mRNA and protein expression.[15] Numerous studies investigating multidrug-resistant cancer cell lines also show that different mechanisms might be responsible for inducing chemotherapeutic resistance, including activated DNA repair pathways, defective apoptosis pathways, modulated growth/signaling pathways, and even epigenetic changes.[8]

Previous studies have shown that increased expression of certain anti-apoptotic genes (such as BCL and survivin) is responsible for chemoresistance in bladder cancer cells.[16] Furthermore, expression knockdown of these and other anti-apoptotic genes resulted in sensitization of two cell lines resistant to mitomycin C.[16]

Signaling Pathways and Cancer

Agents such as BCL and survivin are likely to be upregulated in resistant strains of cancer cells, particularly because anti-apoptotic agents function to prevent the cell from dying, even when signals that lead to cell death are present. There are, however, a number of different signaling pathways in the cell that make up the repertoire of cellular responses to a chemical insult (in addition to anti-apoptotic agents) that could similarly prevent cell death. One such pathway is the DNA-damage response (DDR) pathway. This pathway is activated when DNA damage is sensed and a cascade of events are triggered that activate numerous cellular responses including DNA repair via the nuclear excision repair (NER) system, checkpoint arrest (via Chk1 and Chk2), transcription of genes necessary for DNA repair, and apoptosis signaling if the DNA damage is too extensive. Two of the major players involved in the DDR pathway are the phosphoinositol-3 kinase-like serine/threonine protein kinsases ATM (ataxia telangiectasia mutated) and ATR (ATM and Rad3-related).[17, 18] ATM and ATR are

kinases that function in response to aberrant DNA structures. ATM is activated and recruited to the site of double stranded breaks (DSBs)[19] and ATR to the site of single stranded breaks[20] or arrested DNA replication.[21] ATM primarily acts to activate Chk2,[22] which then mediates signals through multiple substrates (such as p53[23, 24] and BRCA1[25]) and functions to arrest the cell cycle.[22] Conversely, ATR primarily activates Chk1,[26] which also mediates numerous signals through different substrates (such as Cdc25A[27]) to arrest the cell cycle.

A number of chemotherapeutics function by inciting nuclear and DNA damage. Mitomycin C, for example, induces DNA damage through bioreductive formation of adducts with the N^6 atoms of adenine or the N^7 or N^2 atoms of guanine[28-30] which subsequently activate the apoptosis pathway if the DNA assault cannot be repaired. Cisplatin damages DNA by forming adducts with the N^7 atoms of purine bases along the same (intra) or opposing (inter) strand of DNA.[31] In essence, the majority of traditional chemotherapeutic agents (gemcitabine, methotrexate, etc.) being used for the treatment of UC exert the majority of their therapeutic potential by inducing DNA damage, resulting in rapid cancer cell death.

Evidence suggests that enhanced DNA damage signaling can lead to chemoresistance in certain types of cancers. Cisplatin resistance, for example, has been linked to the cellular response that removes cisplatin adducts via the NER system.[32] ERCC1 (excision repair cross complementing 1) protein, a major player in the NER pathway, has been found to have high expression profiles in cancers resistant to cisplatin-based therapy in various tissues including ovarian,[33] cervical,[34] colon,[35] and bladder cancer.[36] However, recent studies suggest that inhibition of some of the other actors in

the DDR pathway (particularly ATM, ATR, and Chk1) has the effect of potentiating cytotoxicity from agents such as cisplatin and gemcitabine.[37]

Much research has been done with inhibition of Chk1 in a number of different cancer types to induce drug potentiation[37-39] and even diminish adaptive chemoresistance.[40] Until now, the ATR protein has been relatively absent from studies addressing DDR-related chemoresistance. One reason could be the relative paucity of selective ATR inhibitors, and another may be the controversy surrounding a Korean study[41] using CGK733, a molecule initially billed as a selective ATR/ATM inhibitor. This study was found to have fabricated data regarding the efficacy of CGK733, and the findings were retracted.[42] Despite this, different studies have been reported in which CGK733 has worked in inhibiting both ATR and ATM in different cancer cell lines.[43, 44]

Specific Aims/Hypotheses

The purpose of this study is multifaceted, and focuses on downregulating one of the major players in the DDR system—ATR (one of the first kinases activated during drug-induced DNA damage)—to determine the effects it has on potentiating the chemotherapeutic effect of different agents, or even inducing drug sensitivity in previously resistant bladder cancers. In order to be able to fully investigate the effect of ATR on chemosensitization, a bladder cancer cell line that was resistant to traditional chemotherapeutic agents needed to be developed. Traditional methods of inducing chemoresistance in cell lines generally involves long-term exposure to the offending agent at a low concentration.[45] Unfortunately, resistance that develops from using long-term, low dose therapies is inconsistent with the resistance that arises clinically from adjuvant chemotherapies, which tend to be limited, short-acting, high-dose

intravesical or systemic treatments. Two recent studies instead used short 1-hour exposures to mitomycin C to induce chemoresistance in bladder cancer.[46, 47] This treatment modality more closely mimics clinical intravesical adjuvant therapy (and also systemic chemotherapy), and expands on the notion that individual cells or groups of cells in a cloned line already possess resistance potential and can expand after even a single iteration of drug exposure.[47] Interestingly, the resistance profile of the cell line developed in these studies was peculiar in that, similar to some cisplatin-resistant cell lines,[32] the resistant line was not only resistant to mitomycin C, but also cross-resistant to a different agent, epirubicin,[47] further pointing to a signaling mediated pathway (likely DDR-related), and not solely a transport protein like MRP, as the mechanism of resistance.

Using the same methodology as Birare *et. al. (*2009), a multi-drug resistant cell line was previously developed in our lab (J. Liu, unpublished data) by treating a parental bladder cancer cell line (RT4, a well-differentiated papillary tumor cell line)[48] with mitomycin C. This newly created mitomycin C resistant (MMCR) cell line is the basis for the chemoresistance studies in our investigation.

The present study aims to characterize the resistance profiles of various chemotherapeutic agents in the MMCR line.

Specific Aim #1: Characterize drug resistance profiles of MMCR and RT4 lines to several different chemotherapeutic agents and calculate IC_{50} values for each drug.

Furthermore, this study also aims determine if affecting the expression of a key DNA-damage repair signal modulator, ATR, has an effect on the viability of bladder cancer cell lines, similarly to previous studies using a chemical ATR inhibitor in our lab (J. Liu, unpublished data). The reasoning behind this aim comes from the studies showing that inhibition of ATR promotes nuclear instability[18] (likely due to uncontrolled cellular division without DNA repair) and embryonic lethality from chromosome fragmentation.[49]

Specific Aim #2: Inhibit ATR expression via RNAi and determine the potential effects that ATR knockdown can have on MMCR and RT4 cells.

Hypothesis #1: ATR knockdown alone will be cytotoxic to cells.

And lastly, the overall aim is to determine if inhibition of ATR has an effect on the chemosensitivity of the resistant MMCR cell line when exposed to wide array of different chemotherapeutic agents.

Specific Aim #3: Determine if ATR knockdown can render the MMCR cell line sensitive to chemotherapeutic agents.

Hypothesis #2: ATR knockdown will render the MMCR cell line sensitive to mitomycin C and make its resistance profile similar to that of the parental RT4 line. ATR knockdown will produce similar effects in the resistance profiles for other drugs investigated in this study.

Various cytotoxicity assays were performed on the MMCR cell line, and its parental RT4 line, to make comparisons about drug resistance for various agents typically used in chemotherapy regimens for both intravesical and systemic treatment of urothelial

carcinoma. ATR kinase expression was inhibited via RNAi in both cell lines and further cytotoxicity assays were performed to determine if lowering the expression of ATR was cytotoxic to both cell lines, and could also chemosensitize the resistant (MMCR) cell line as hypothesized above.

Materials and Methods

Cell Culture & MMRC

RT4 and MMCR cells were cultured in McCoy's 5A complete media (10% fetal bovine serum, 1% penicillin, 1% streptomycin and 1% glutamine). The MMCR cell line was previously selected from the parental RT4 cell line by being incubated with 6.25 µg/mL of mitomycin C for 2 hours, and then washed with PBS 3 times (J. Liu, unpublished data). The cells that survived the mitomycin C treatment were subsequently grown and passaged at least 3 times in McCoy's complete media before being frozen and stored in liquid nitrogen.

siRNA Transfection

Oligofectamine™ reagent (Invitrogen) was used for the transfection of ATR siRNAs. The standard transfection protocol from Invitrogen was followed with scaling modifications. In brief, cells were plated (overnight) in 6-well ($1x10^5$ cells/well) or 96-well ($5x10^3$ cells/well) plates in McCoy's 5A media without antibiotics. Cells were transfected 16 hours later when cells reached 30-50% confluency. Oligofectamine™ was premixed with McCoy's 5A media (without FBS or antibiotics) in a 1:6 ratio and allowed to incubate at room temperature for 10 minutes in order to form liposomes. For 6-well plates, 15 µL of the Oligofectamine™ preparation was mixed with 185 µL of various

concentrations of siRNA diluted in McCoy's 5A media, for a final volume of 200 µL, and allowed to complex for 10 minutes. 800 µL of warm McCoy's 5A media (without FBS or antibiotics) was then added to each preparation for a final plating volume of 1 mL.The existing media from each well was aspirated, and the cells were washed once with sterile PBS. The entire 1 mL of the complexed siRNA preparation was added to each well and allowed to incubate at 37°C for 4 hours. After the incubation period, 500 mL of McCoy's 5A media with 30% FBS was added to each well to inactivate the transfection. For 96-well plates, 3 µL of the Oligofectamine™ preparation was mixed with 17µL of siRNA diluted in media, for a final volume of 20 µL, and allowed to complex for 10 minutes. 80 µL of McCoy's 5A media (without FBS or antibiotics) was added to each preparation for a final plating volume of 100 µl. Each well was washed once with PBS, and the final preparation volume was added to each well and allowed to incubate at 37°C for 4 hours. After the incubation period, 50 µL of McCoy's 5A media with 30% FBS was used to inactivate the transfection.

In the initial proof of concept experiments, different transfection vehicles other than Oligofectamine™ were used make comparisons about transfection efficiency of the ATR and control siRNAs. One agent that was subsequently used as an additional positive control was the N-TER Peptide (N-TER™ Nanoparticle siRNA Transfection System, Sigma). The standard protocol from the manufacturer's website (with scaling modifications) was used in those transfection experiments that included this positive control.

The following siRNAs (Thermoscentific) were used:

#19-ON-TARGET plus siRNA Human ATR - GAGAAAGGAUUGUAGACUA

#20-ON-TARGET plus siRNA Human ATR - GCAACUCGCCUAACAGAUA

ON-TARGET plus Non-targeting siRNA #2 - UGGUUUACAUGUUGUGUGA

Cytotoxicity/Viability Assay

Cells were grown overnight in a 96-well plate ($5x10^3$) in McCoy's 5A media (without antibiotics) and were treated with serial dilutions of chemotherapeutic agents for 1 hour with and without ATR siRNA (see transfection method above). Cell viability was assayed using WST-1 reagent (Clontech, Mountain View, CA) which is suitable for an *in vitro* model. The assay is based on the extracellular enzymatic reduction of the tetrazolium salt WST-1 to formazan due to the glycolytic production of NAD(P)H present on the surface of viable cells. Cell viability was determined on a plate reader (Molecular Devices SpectraMax 250) by measuring the absorbance at 420-480 nm after incubating with WST-1 reagent for 0.5-2 hours. Each treatment concentration had 4-6 replicates and each experiment for a given cell line was done in duplicate.

Western Blot & Quantification

Cells were lysed with a lysis buffer which contained protease and phosphatase inhibitors (25 mM Tris-HCl, 12.5 mM EDTA, 12.5 mM NaF, 1.25 mM EGTA, 1.25 mM Na_3PO_4, 12.5 nM Na-orthovenadate, 1.25% Triton X-100, with added cOmplete Mini EDTA-free protease inhibitor tablet from Roche). Lysates were mixed with an equal amount of 2X sample buffer (125 mM Tris-HCL, 25 mM EDTA, 20%glycerol, 10% ß-mercaptoethanol, 4% SDS, 0.004% bromophenol blue), scraped off the culture dish, collected into screw-cap microcentrifuge tubes, and boiled for 5 minutes at 95°C. 20 µL of extract was subsequently loaded onto a 4-20% polyacrylamide gel (Bio-Rad) and electrophoresed at 160 Volts for 1 hour in running buffer (25 mM Tris base, 192 mM

glycine, 0.1%SDS). The protein in the gel was transferred to a PVDF membrane in transfer buffer (25 mM Tris base, 192 mM glycine, 20% methanol) at 100 Volts for 90 minutes. The membrane was then blocked with 5% non-fat milk (Krasdale®) in TBST (Tris buffered saline containing 0.1% Tween 20) for 2 hours, incubated overnight at 4°C with one of 4 different antibodies: rabbit anti-ATR polyclonal antibody (1:5,000 dilution) (Millipore, Temecula, CA); rabbit anti-p-ATR polyclonal antibody (1:5,000 dilution) (Sigma, St. Louis, MO); rabbit-anti-p-STAT3 polyclonal antibody (1:2,000 dilution) (Sigma, St. Louis, MO); and goat anti-beta actin monoclonal antibody (1:2,000 dilution) (Santa Cruz Biotechnology, Santa Cruz, CA). After washing with TBST (3X for 5 minutes), the membranes were incubated with secondary antibody using 5% non-fat milk TBST for 1 hour at room temperature using donkey anti-goat antibody (1:5,000 dilution) (Santa Cruz Biotechnology, Santa Cruz, CA) or donkey anti-rabbit antibody (1:2,000 dilution) (Jackson ImmunoResearch Laboratories). After washing with TBST (5X for 5 minutes), samples were visualized by an enhanced chemiluminescence detection system (Chemiluminescent Substrate, Rockford, IL, USA) on Kodak film. The films were scanned and densitometry of protein bands was quantified with ImageJ using the standard protocol outlined on the NIH ImageJ website.[50]

Relative protein expression for each cell line was obtained by normalizing the densitometry data relative to an internal loading control, β-actin. Briefly, areas for each band in the treatment wells (all three bands present: p-ATR, p-STAT3 and β-actin) were first normalized to the corresponding bands the “Untreated” control lane across all scanned images. A second normalization step was then performed on the data, creating a ratio from the either the p-ATR or p-STAT3 bands to the internal loading

control, β-actin. Normalized data from experiments run in duplicate or triplicate (if any) could then be averaged and depicted on a graph using Prism (GraphPad).

Quantitative PCR

Total RNA was extracted from bladder cancer cells using Trizol® reagent (Life Technologies, Carlsbad, CA), following the manufacturer's standard protocol. cDNA was subsequently synthesized from the RNA via reverse transcription using the iScript cDNA synthesis kit (Bio Rad). qRT-PCR was then performed using iQ™ SYBR® Green Supermix (Bio Rad), 100 nM of forward and reverse primers, and equivalent amounts of cDNA, The following primers were used:

ATR Forward: ATTCACCACAGGCACAATCA

ATR Reverse: TAGCCCGGATTACTTCATGG

GAPDH Forward: CCACTAGGCGCTCACTGTTCT

GAPDH Reverse: GCGAACTCACCCGTTGACT

MyIQ™ Single Color Detection RT-PCR system was used for the reactions. Each reaction was run in triplicate, and data was exported to Excel (Microsoft) and relative ATR gene expression was calculated using the $2^{-\Delta\Delta C_T}$ as outlined by Livak and Schmittgen.[51]

Statistical Analysis

All data points (consisting of 4-6 replicates, in cytotoxicity assays) are expressed as mean ± SEM for each treatment group. For cytotoxicity assays, dose-response curves were plotted using Prism (GraphPad) for data points within each cell line. IC_{50} was calculated using non-linear regression (best-fit curves have not been juxtaposed in the

cytotoxicity profiles for simplicity), using a variable slope model. R^2 was greater than .90 for a majority of the RT4 line cytotoxicity assays except where noted. R^2 was less than .90 for the majority of the MMCR cytotoxicity assays.

Results

Drug Resistance Profiles for RT4 (Parental) and MMCR (Resistant) Cell Lines

To compare the differences in resistance profiles between the two cell lines, cytotoxicity assays were performed on both cell lines using seven different drugs: mitomycin C, doxorubicin, cisplatin, methotrexate, vinblastine, gemcitabine, and 5-fluorouracil. Cell viability for each of the treatments was measured using a WST-1 absorbance assay.

High doses of 5-fluorouracil (300 μM) and cisplatin (324 μM) were insufficient to completely eliminate the MMCR cell line, although these concentrations did produce a significantly stronger response in the parental RT4 cell line (Fig. 1A & 1B). The IC_{50} value of 5-fluorouracil in the MMCR line was indeterminate because a best-fit line could not be interpolated. The IC_{50} for 5-fluorouracil in the RT4 line was determined to be 49.4 μM (R^2 = .91). The IC_{50} values for cisplatin in the MMCR and RT4 lines were determined to be 804 μM (R^2 = .62) and 114 μM (R^2 = .90), respectively.

Methotrexate and doxorubicin produced toxicity profiles that were more adequately measured in the drug concentration ranges tested (< 300 μM), but (like with 5-fluorouracil and cisplatin) were unable to produce a complete cytotoxic response in the MMCR line. The IC_{50} values for methotrexate in the MMCR and RT4 lines were 43.4 μM

(R^2 = .60) and 0.59 μM (R^2 = .67), respectively (Fig. 1C). The IC_{50} values for doxorubicin in the MMRC and RT4 lines were 2.29 μM (R^2= .90) and 0.051 μM (R^2 = .98), respectively (Fig. 1D).

Vinblastine, unlike the other drugs being investigated, did not produce a significantly different drug resistance profile (Fig. 1E). The IC_{50} values for the two cell lines were almost identical at 0.06 μM (R^2 of .78) for MMRC and 0.05 (R^2 of .96) μM for RT4.

Traditional systemic treatment of metastatic urothelial carcinoma involves a combination treatment with methrotrexate (30 mg/m^2), vinblastine (30 mg/m^2), doxorubicin (3 mg/m^2), and cisplatin (70mg/m^2). This drug combination, termed MVAC was investigated in both cell lines at a dose ratio that parallels standard adjuvant therapy. The initial maximum drug concentration chosen for this assay was one in which at least 2 out of the 4 drugs had greater than 50% toxicity (when assessed individually) so as to be able to accurately regress a best-fit line and calculate the IC_{50}. The IC_{50} values for MVAC in the MMCR and RT4 lines were calculated to be 3.69 μM (R^2 = .77) and 0.04 μM (R^2 = .96), respectively (Fig. 1F).

Doses of mitomycin C, and gemcitabine above 50 μM were cytotoxic to both cells lines. Both cell lines were reincubated with lower starting concentrations and subsequent serial dilutions of these two agents. The IC_{50} for each cell line was calculated using regression curve-fitting, as previously. The IC_{50} value for mitomycin C in the MMCR cell line was determined to be 4.63 μM (R^2 = .90) and 0.15 μM (R^2 = .93) for the RT4 line (Fig. 2A). The IC_{50} values for gemcitabine in the MMCR cell and RT4 cell lines were determined to be 12.7 μM,(R^2 = .82) and 1.7 μM (R^2 = .91), respectively (Fig. 2B).

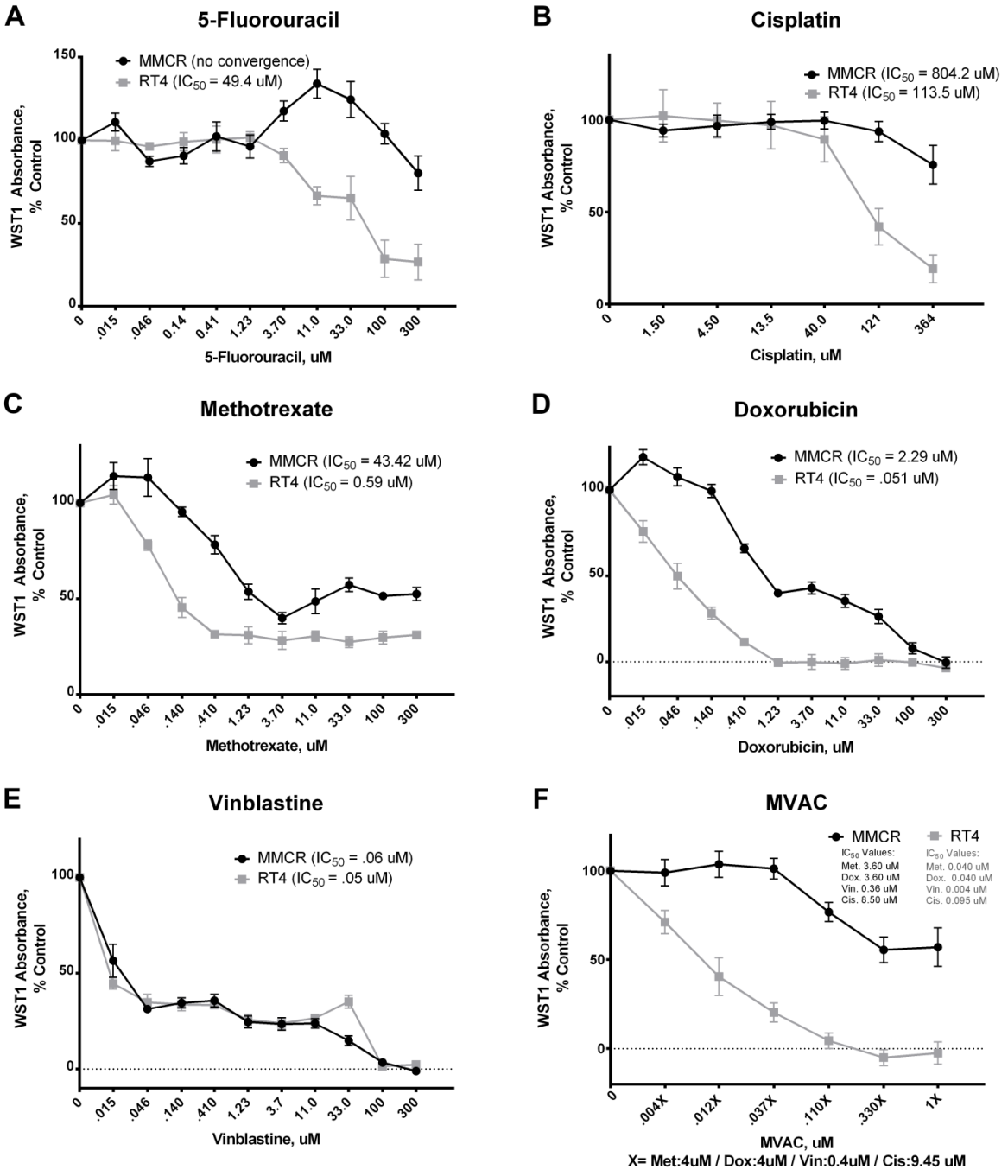

Fig. 1. Drug Treatment Cytotoxicity Profiles for 5-Fluorouracil, Cisplatin, Methotrexate, Doxorubicin, Vinblastine and MVAC

Representative dose-response curves for chemotherapeutic treatments (not including juxtaposed best-fit curves), normalized to a control (no drug). Vertical bars represent SEM. IC_{50} values represent population IC_{50} (n=44) **A.** 5-fluorouracil was ineffective at producing cytotoxicity in the range being investigated for the MMCR line. The IC_{50} for the RT4 line was determined to be 49.4 μM (R^2 = 0.91). **B.** The IC_{50} values for cisplatin in the MMCR and RT4 lines were determined to be, 804 μM (R^2 = 0.62) and 114 μM (R^2 = 0.90), respectively. **C.** At concentrations approximating 300 uM, methotrexate was unable to completely produce a cytotoxic response in

either cell line. The IC_{50} values were 43.4μM (R^2 = 0.60) and 0.59 μM (R^2 = 0.67), respectively. **D.** Doxorubicin was able to produce complete cytotoxicity in both cell lines. IC_{50} for MMCR was 2.29 μM (R^2 = .90). IC_{50} for RT4 was 0.051 μM (R^2 = .98). **E.** Vinblastine did not produce significant difference in cytotoxicity across the two cell lines. IC_{50} values for MMCR and RT4 were 0.06 μM (R^2 = .78) and 0.05 μM (R^2 = .96) respectively. **F.** The IC_{50} values for MVAC in the MMCR and RT4 lines were calculated to be 3.69 μM (R^2 = .77) and 0.04 μM (R^2 = .96), respectively.

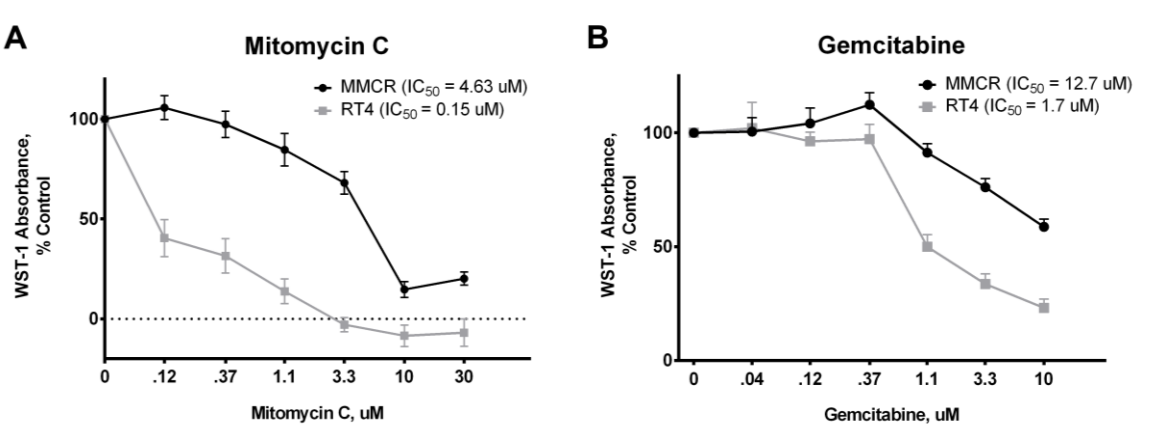

Fig. 2. Drug Treatment Cytotoxicity Profiles for Mitomycin C and Gemcitabine
Representative dose-response curves for chemotherapeutic agent treatments, normalized to a control (no drug). Vertical bars represent SEM. IC_{50} values represent population IC_{50} (n=44). **A.** Cytotixicity profile for mitomycin C in MMCR and RT4 cells. Mitomycin C was cytotoxic to RT4 cells at very low concentrations. IC_{50} for MMCR was 4.63 μM (R^2 = .90) and 0.15 μM (R^2 = .93) for RT4. **B.** Cytotoxoicty profile for gemcitabine in MMCR and RT4 cells. IC_{50} for MMCR was 12.7 μM (R^2 = .82) and RT4 was 1.7 μM (R^2 = .91).

CGK733 inhibitor effect on RT4 and MMCR Cell Viability

Previously, CGK733 had been shown to be cytotoxic to both MMCR and RT4 cell lines at relatively low concentrations. (J. Liu, unpublished data). The cytotoxicity assay (Fig. 3) shows that CGK733 inhibitor was toxic to both cell lines at increasing concentrations. 100% cytotoxicity occurred between 6.67 μM and 20 μM for both cell lines. The IC_{50} values for the MMCR and RT4 cell lines was calculated to be 5.72 μM (R^2 =.99) and 6.66 μM (R^2 = .93) for the RT4 line.

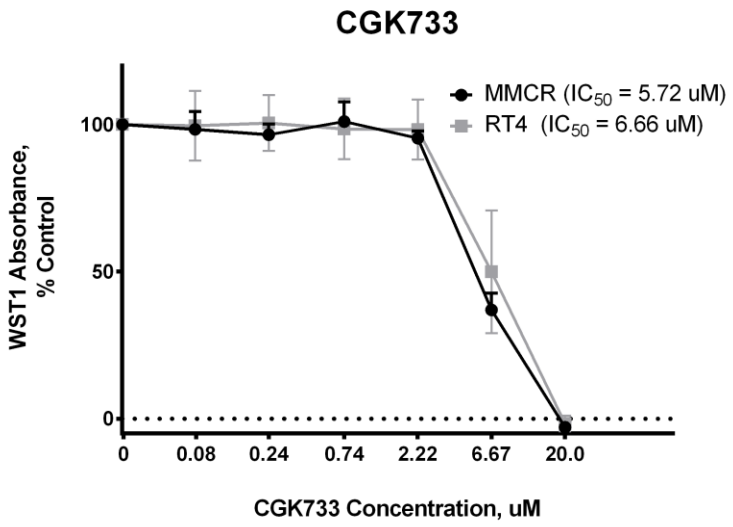

Fig. 3. CGK733 Inhibitor Treatment
Both the MMCR and RT4 cell lines were treated with the chemical inhibitor, CGK733. The inhibitor was quickly cytotoxic to both cell lines at very similar concentrations. IC_{50} was 5.72 μM (R^2 = .99) for the MMCR line, and 6.66 μM (R^2 = .93) for the RT4 cell line.

CGK733 Inhibitor Effect on ATR Expression in MMCR Cell Line

Incubation of cells with the chemical inhibitor CGK733, at two different concentrations, 5 μM and 6 μM, knocked down ATR protein expression in the MMCR cell line (Fig.4). The relative expression of phosphorylated-ATR (p-ATR) protein was 0% at 6 μM CGK733 + mitomycin C, though a considerable drop in this activated form of the protein was also noted in the mitomycin C-only lane.

siRNA Effect on Activated p-ATR Protein Expression

In order to test the ATR pathway specifically, different siRNAs were used to determine knockdown of ATR protein and mRNA expression in both cell lines. ATR siRNA #20 (100nM) produced approximately 50% relative p-ATR knockdown in RT4 cells, and 76% p-ATR knockdown in MMCR cells (data not shown). ATR siRNA#19 (at a concentration of 100 nM) produced a 66% relative protein knockdown of p-ATR in RT4

cells (circle, Fig. 5A) and a 91% relative protein knockdown in MMCR cells (circle, Fig. 5C). STAT3 is a transcription factor in a signaling pathway unrelated to the ATR/Chk1 pathway. Phosphorylated-STAT3 was examined in these blots to control for 1) adequate phosphorylated protein collection in each sample and 2) specificity of ATR/Chk1 pathway inhibition. P-STAT3 expression was, interestingly, elevated at higher concentrations of ATR siRNA in the RT4 cells, but relatively unchanged in the MMCR cell line (Fig. 5B and 5D).

Expression levels of p-ATR mRNA for both cell lines were measured via qPCR (Fig. 6). In parallel to the western blot data, the relative expression of ATR mRNA was less profound in the RT4 cells, with 78% knockdown as compared to the MMCR cell line, which had an impressive 97% knockdown.

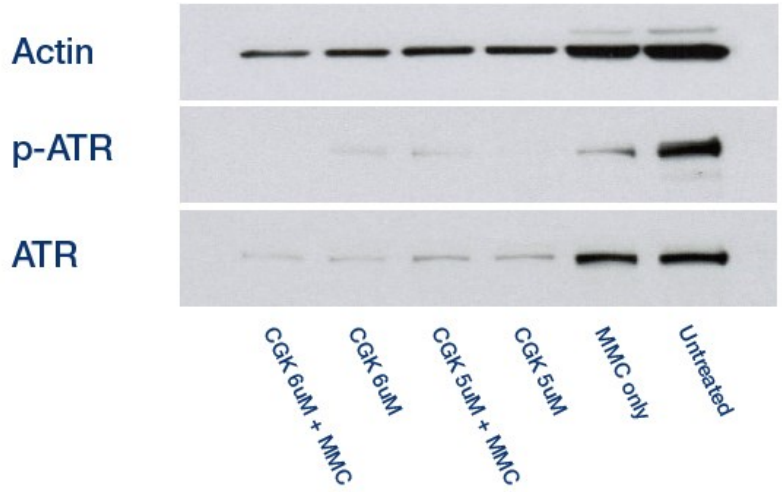

Fig. 4. Western Blot. ATR and Activated p-ATR Protein Expression with CGK733
Expression of ATR and phosphorylated-ATR in the MMCR cell line after treatment with the ATR inhibitor, CGK733. P-ATR was undetectable (via ImageJ) in the lane with 6μM CGK733 + mitomycin C. The decrease in intensity of actin (with higher concentrations of CGK733) can be attributed to lower amounts of cells recovered in the same volume of protein lysate due to the cytoxicity of the inhibitor agent.

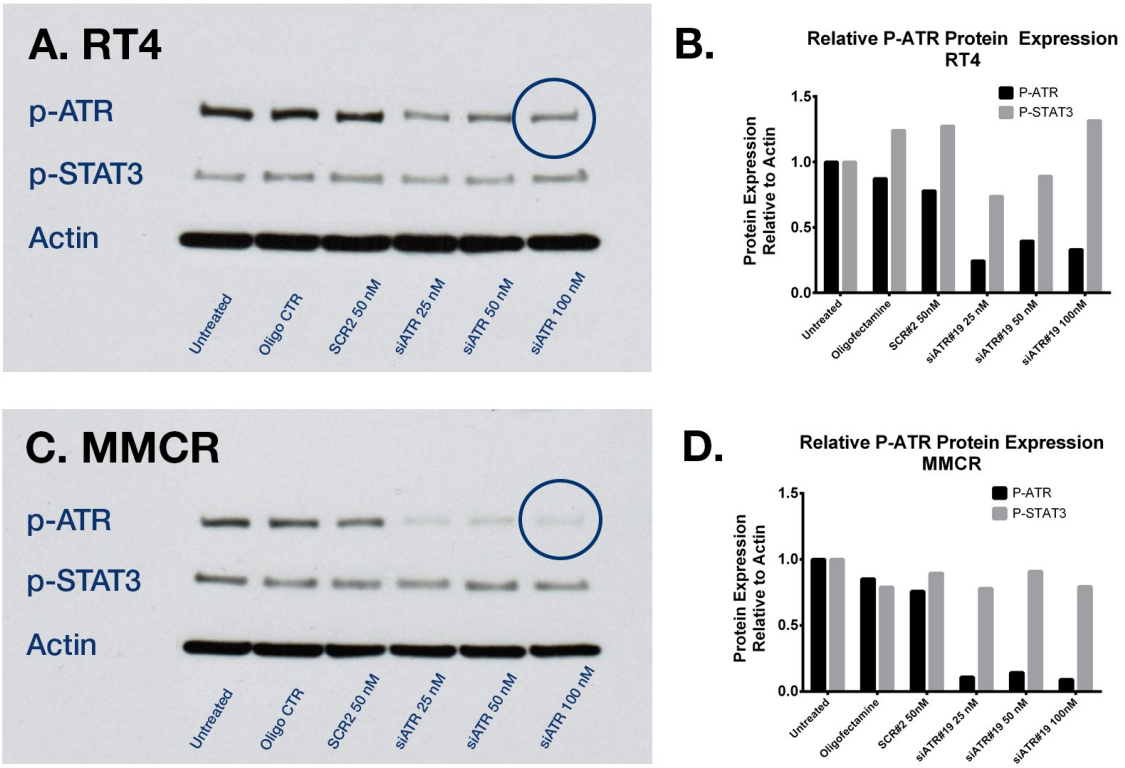

Fig. 5. Western Blot, Activated ATR (p-ATR) Protein Expression with siRNA Treatment
Phosphorylated-ATR (p-ATR) and phosphorylated-STAT3 (p-STAT3) protein expression in RT4 and MMCR cells. Oligo CTR is a positive control using only the Oligofectamine™ transfection vehicle (no siRNA). SCR#2 is the Non-targeting siRNA #2 positive control. **A.** RT4 western blot image. **B.** Graph depicting expression of p-ATR and p-STAT3 relative to B-actin (loading control) protein expression in RT4 cells. **C.** MMCR western blot image and (**D**) representative graph of relative protein expression.

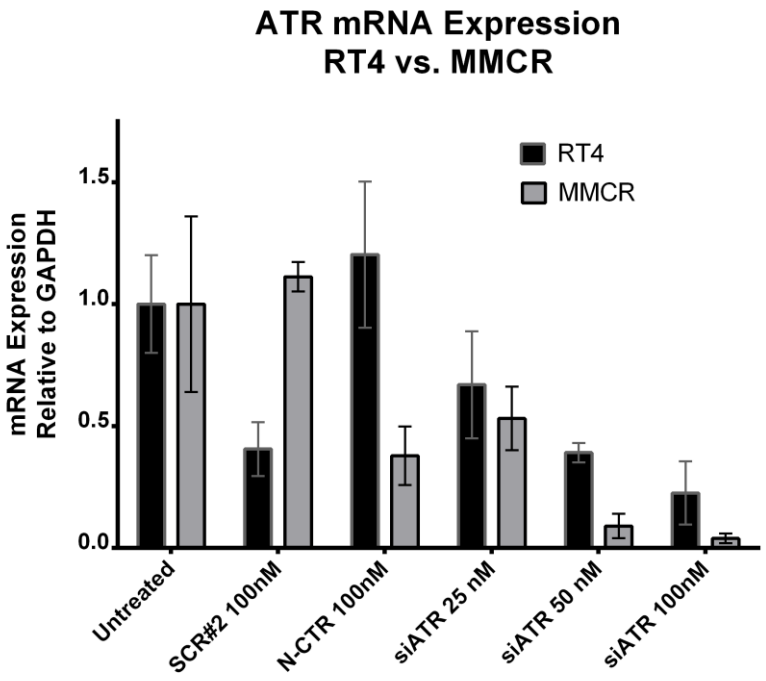

Fig. 6. Quantitative PCR: ATR mRNA Expression After siRNA Treatment
Relative mRNA expression of ATR normalized to internal control (GAPDH) via $2^{-\Delta\Delta C_T}$ method. Both SCR#2 and N-CTR have Non-targeting siRNA #2 as the transfected peptide, but the N-CTR sample used a different transfection vehicle (see methods above). Each treatment condition was run in triplicate. 97% relative knockdown of ATR was seen at a concentration of siRNA #19 100 nM in the MMCR cell line, and 78% relative knockdown in the RT4 cell line.

ATR siRNA Effect on Cell Viability

One observation that was made early during the experiments was that siRNA treatment had little to no effect on RT4 (parental) proliferation, but had a positive (slight) effect on MMCR cell viability after transfection. The cytotoxicity assay was performed using only siRNA treatment (no drug) and it showed that increasing concentrations of siATR#19 produced higher rates of proliferation (Fig. 7). Although a T- test showed that there was no significant difference among the different treatment arms *within* the RT4 line, there was a statistically significant difference between the untreated and siATR-treated data points (including the Oligofectamine™ only control) within the MMCR line.

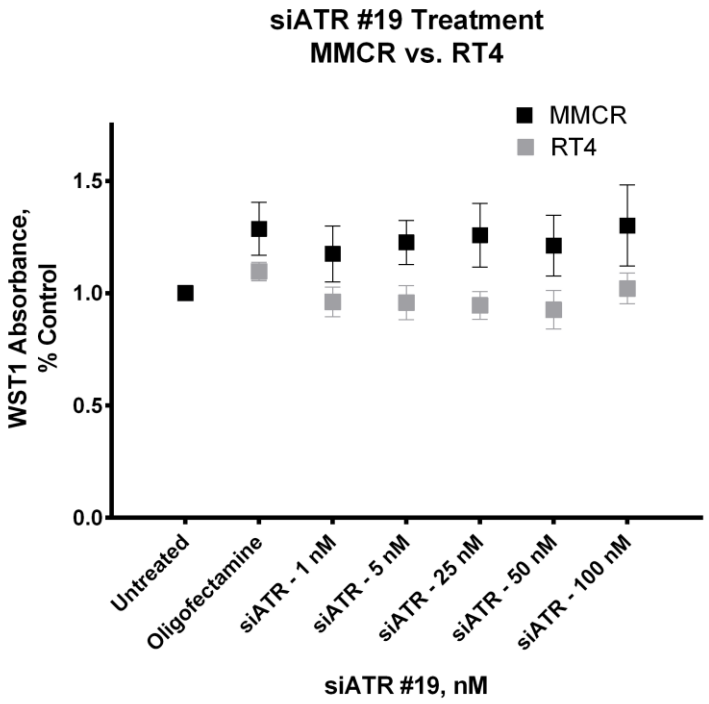

Fig. 7. Cellular Viability After siATR Treatment
Graph showing the difference in cellular proliferation between the MMCR line and the RT4 line after transfection of siRNA#19. Differences in data points were not significant within RT4 cell line across cell treatments. There was a moderate, but significant (P<.001) difference when comparing the untreated cells against the siATR-treated cells.

ATR siRNA Effect on MMC and Doxorubicin Resistance

Mitomycin C and doxorubicin resistance were investigated in the MMCR line after transfection of 100 nM siATR #19. Cells were transfected (see methods above) and subsequently treated with mitomycin C (at 48 hours post-transfection) for one hour with, a) no drug, b) high drug concentration, 15 μM (final well volume), or c) low drug concentration, 1.5 μM. The experiment was repeated under the same conditions for doxorubicin. Statistical analysis of the WST-1 assay showed that after siRNA transfection, drug treatment with mitomycin C, produced no significant difference in cell viability when compared to no drug treatment (control), except in the siATR treatment arm with a low dose of mitomycin C (Fig. 8A), where cell viability increased 18% (P<0.01). T-test showed no statistical difference among the remaining treatment arms

were for mitomycin C. In the doxorubicin treated group, no significant difference was observed in the cells transfected with siRNA among any of the treatment groups.

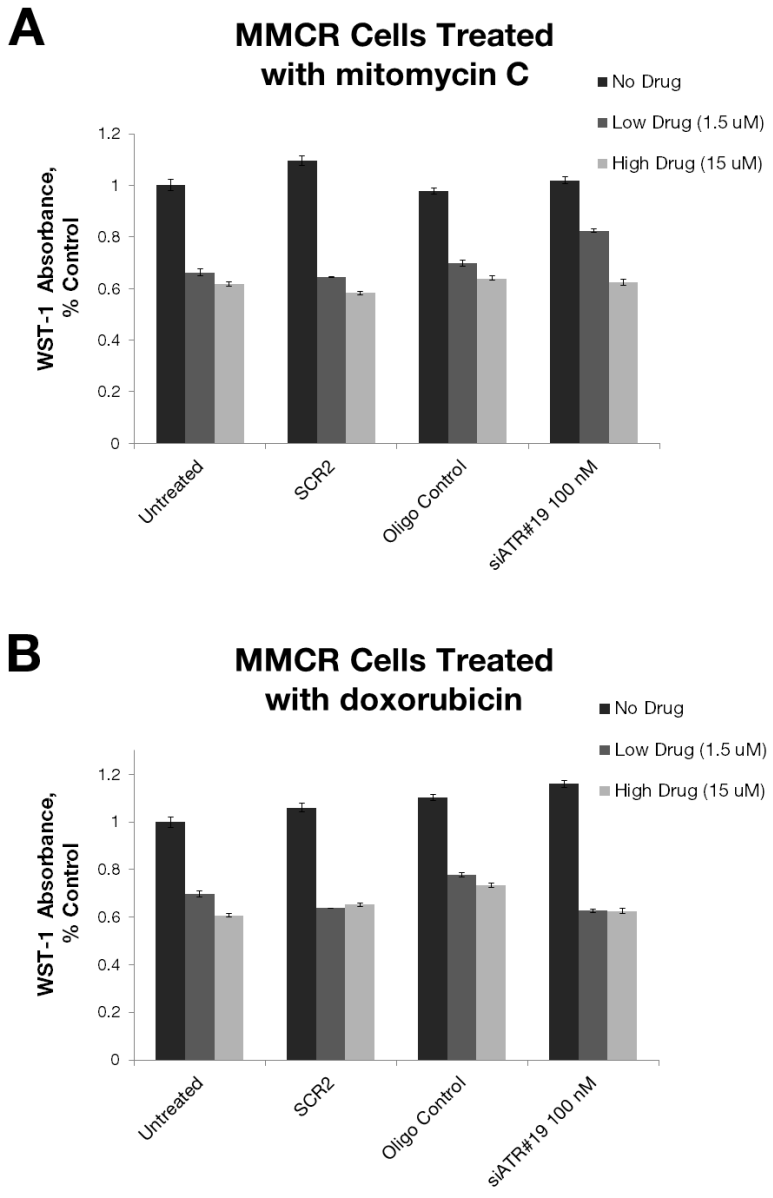

Fig. 8. Cytotoxicity Graphs for Cells Treated with Mitomycin C and Doxorubicin
MMCR cells were transfected with 100 nM siATR#19 and treated 48 hours post-transfection with mitomycin C and doxorubicin. A. No significant difference was observed between the any of the ATR treatment arms at any drug concentration with the exception of the 100 nM siATR treatment with low dose mitomycin C (18% increase, P< .01) B. No significant difference was observed between any of the ATR treatment arms at any concentration. Although there was a slight difference (can be appreciated on graph) in the no drug group between the untreated and siATR 100 nM arms (this 9% difference, however, did not reach significance, p = .12) .

Discussion

Drug Profiles

The stark differences in between the two cell lines (MMCR and RT4) can be seen both physically (Fig. 9) and can be observed from the drug resistance cytotoxicity profiles (Fig. 1 & 2). As opposed to previous research studies where mitomycin-exposed drug resistant cells were slower growing,[46, 47] the MMCR cells in this investigation grew faster on average, by reaching 80% confluency approximately 24 hours before the parental RT4 cells (J. Liu, unpublished data). These cells lines were extensively characterized previously in our laboratory, and the MMCR cell line was shown to have marked differences in size, volume (when trypsinized and measured via cell counter (Bio Rad) in comparison to the RT4 cell line), and also had higher invasion potential when grown in an extracellular matrix assay (J. Liu, unpublished data).

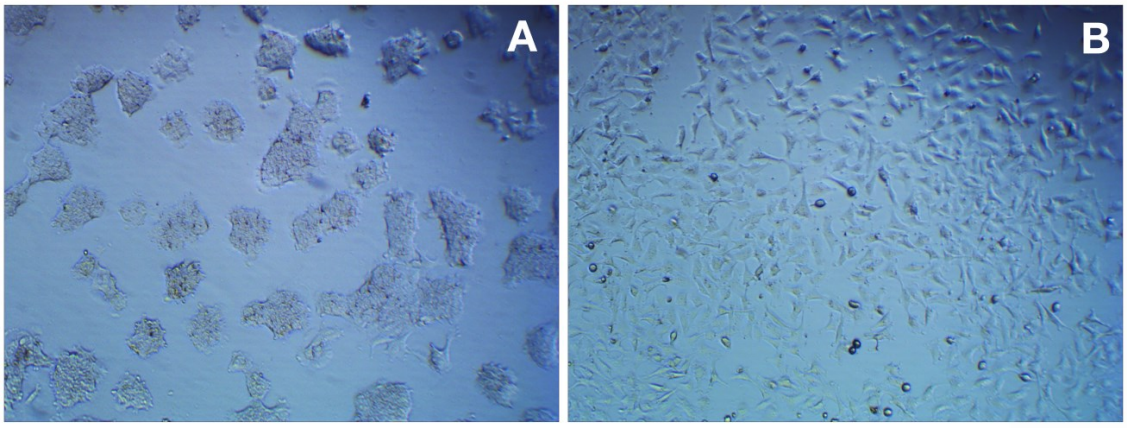

Fig. 9. Visual Differences in RT4 and MMCR Cells
Panels A (RT4) & B(MMCR) show the different cellular morphology and appearance of the different cell types. Both fields are at the same microscopic amplification at 400X.

Although high concentrations of different drug treatments (~300 μM) produced a near complete cytotoxic response the RT4 cell line, drug resistance to six of the seven agents being tested was clearly present in the MMCR line. Furthermore, the R^2 values for most of the best-fit curves in the MMCR line were <.90, likely due to the fact that the drug failed to produce greater than 50% cytotoxicity in the data gathered for the drug ranges being investigated. Neither 5-fluorouracil nor cisplatin, for example, were able to produce greater than 50% toxicity in the concentration range being investigated (~300 uM). In fact, the IC_{50} value could not be calculated for MMCR cells treated with 5-fluorouracil because a best-fit line could not be interpolated from the data gathered. For cisplatin, line interpolation and regression produced an IC_{50} of 804 μM, which is significantly higher than the range of drug concentrations investigated for other drugs in this particular study. Although an IC_{50} can be calculated using linear interpolation, without having physically subjected the cells to a concentration of 804 μM, the result obtained is inconclusive, and direct comparison of IC_{50} values between the two cells lines becomes more difficult to make. Single-agent cisplatin therapy has been shown to produce low response and high resistance rates in UC.[8] It is possible that the mechanism driving cisplatin cross-resistance after single-agent mitomycin C exposure in the MMCR line is very similar to the mechanism caused by single-agent cisplatin exposure (given the strong resistance to cisplatin observed). Regardless of the mechanism behind the resistance, however, the IC_{50} comparisons show that the MMCR cell line is very strongly resistant to 5-flurouracil and cisplatin, whereas the parental RT4 cell line is not.

Methotrexate, doxorubicin, gemcitabine, and mitomycin C also produced significant cytotoxicity differences in the two cell lines, but the IC_{50} values that were calculated did

fall within the concentration range being investigated (and similarly likely to fall within a concentration range that could be reproducible physiologically during a chemotherapy regimen). With an IC_{50} of 42.4 μm for methotrexate, the approximate 70-fold difference in concentration required to kill the MMCR cells (versus RT4 cells), shows that whichever mechanism is inducing multi-drug resistance after mitomycin C exposure is giving the cancer cells a huge advantage in escaping cell death. The MMCR cell line had an IC_{50} of 4.6 μM for mitomycin C and an IC_{50} of 12.7 μM for gemcitabine, and the apparent difference in concentrations required to kill 50% of the MMCR vs RT4 lines were under 30-fold for both of these drugs. Although the IC_{50} values for all four drugs fell within the range being tested, the less than ideal R^2 values for the MMCR best-fit lines make direct comparisons inconclusive. Similarly to 5-fluorouracil and cisplatin, however, the MMCR cell line is, again, clearly resistant to these four chemotherapeutic agents.

Vinblastine was the only agent whose drug resistance profile had no significant difference between the two cell lines (Fig. 1E). Vinblastine is a vinca alkaloid and structurally different than the remainder of the drugs being investigated. Perhaps, vinblastine resistance in cancer cells[52] might be due to a different mechanism than the cross-resistant DDR-based mechanism being investigated in the present study.

First-line therapy for metastatic urothelial carcinoma generally involves cisplatin-based multi-agent therapy, MVAC. MVAC has been shown to be more effective in in response and overall survival than single-agent cisplatin [6] therapy or CISCA therapy (cisplatin, cyclophosphamide, and doxorubicin).[53]

Similarly to the majority of other chemotherapeutic agents investigated in this study, MVAC combination failed to produce complete cytotoxicity in the MMCR line, although the starting maximum concentration for the assay was in the range where two of the individual drugs (doxorubicin and vinblastine) were shown to have at least 50% cytotoxicity in the MMCR line. Why the relatively high concentrations of doxorubicin and vinblastine failed to produce greater than 50% cytotoxicity when added together as compared to when added individually, is difficult to explain. One would have expected MVAC treatment to have a good cytotoxicity profile in both cell lines due to additive or synergistic effects of drug-induced DNA damage. Perhaps, the addition of multiple drugs in the medium affects drug transport into the cell, or perhaps metabolism/modification of one drug affects the way the other drugs are handled by the cell. It is also possible that the major mechanism of drug resistance in the MMCR line involves cisplatin and not necessarily the other three drugs. Given that the maximum starting concentration of cisplatin for this assay in the MVAC regimen was nowhere near the predicted IC_{50} of 804 μM, it becomes increasingly likely that as an agent, cisplatin concentration is not high enough in the MVAC regimen to provide any cytotoxic effect to the MMCR cells. Although the combination MVAC treatment in this study was administered in the same ratio as systemic chemotherapy MVAC, experimenting with different ratios and concentrations of the individual drugs (based on the results from the resistance profiles) could potentially lead to a stronger cytotoxic response in the MMCR cells in future experiments. Doubling the concentrations of cisplatin and methotrexate (neither of which achieved complete cytotoxicity in our profiles), while keeping the vinblastine and doxorubicin concentrations the same, might induce more cytotoxicity in both the MMCR and RT4 lines than what was observed in this study.

CGK733

In a previous investigation in our lab, the chemical inhibitor, CGK733 was shown to significantly lower ATR and p-ATR protein expression in both the RT4 parental line (data not shown) and the MMCR line (Fig. 3). The near complete levels of cytotoxicity over a short concentration range for both cell lines (Fig. 4), however, made this chemical inhibitor an unfavorable candidate for ATR knockdow in the present study. Furthermore, controversy regarding the authenticity of CGK733 as an ATR/ATM inhibitor, make this agent even less appealing for this study. The aim of this study was to characterize the role ATR plays in chemoresistance, and although CGK733 has been shown to work as an ATR inhibitor in numerous other studies, the need for specificity in targeting the pathway drove the focus of this study towards using RNA interference as the major method to inhibit ATR expression in the MMCR cell line.

ATR Knockdown

Similar to CGK733, siATR #19, was able to produce a significant knockdown in expression of ATR and p-ATR (activated) in both of the cell lines being investigated at both the mRNA and protein levels. The level of protein knockdown was more prominent in the MMCR cell line than RT4. The reason for this prominence in the MMCR line is unknown. Previous work in our lab showed that when quantitatively compared to RT4, several different signaling proteins, including ATR and STAT3 were more highly expressed in the MMCR line (J. Liu, data not shown). The fact that in the present study the knockdown of ATR was greater in the MMCR line, indicates that the siATR transfection was particularly efficient, but there are a number of reasons why transfection is more efficient in some cells types than others.

Because the IC_{50} values from the initial characterization of the MMCR cell line for 5-fluorouracil, cisplatin, methotrexate, and MVAC required higher than ideal concentrations of drug for the cytotoxicity experiments, they were excluded as drugs during the ATR knockdown experiments. Furthermore, as was already discussed, several of the MMCR best-fit curves had less than ideal R^2 values and making comparisons of cytotoxicity between a curve with an R^2 of .93 and one with an R^2 of .60 could lead to bias and misinterpretation of data.

Vinblastine produced no significant resistance difference between the two lines; hence it became excluded from further assays in this study. Of the three remaining drugs, the two drugs with IC_{50} values that fell within a similar concentration range were mitomycin C and doxorubicin; and these were the two drugs that were investigated during the ATR knockdown experiments. Interestingly, in the previous experiments with resistant cells resulting from mitomycin C treatment, Birare *et al.* found that the resistant cells were cross-resistant to epirubicin (an anthracycline antibiotic, very structurally similar to doxorubicin). This finding made doxorubicin an ideal candidate for further ATR knockdown studies. Gemcitabine cytotoxicity also fell within a workable range, but experiments using this drug in combination with ATR knockdown were deferred in the present study.

ATR Expression and Mitomycin C / Doxorubicin Resistance

One of the results obtained in this investigation, contrary to the both of the hypotheses of the present study, was that targeted ATR knockdown (via siRNA), appeared to actually improve cellular viability (Fig. 6) *and* (at low concentrations) might have even

increased chemoresistance to mitomycin C (Fig. 8) in the MMCR cell line. The experiment was repeated various times and under different conditions (even with different transfection vehicles, such as N-TER peptide from Sigma), and all experiments had the same final result— the transfection process (even without added siRNA) seemed to produce as slight but significant increase in MMCR cell viability, but no significant change in chemosensitivity to mitomycin C or doxorubicin. Whether cell viability changed after this time frame or not, is outside of the scope of these *in vitro* experiments.

One possible explanation for these results is that despite ATR knockdown, the numerous redundant DDR signaling pathways (including the very similar ATM-dependent pathway) might be compensating for lower expression of ATR and providing a means for the cell to survive despite treatment. One explanation for the apparent increased proliferation observed in MMCR cells that were transfected with siRNA, is that activated ATR (which is normally involved in signaling for cell-cycle arrest and DNA repair), allowed the MMCR cells to proliferate normally, despite acquiring DNA damage that would normally arrest cell growth and division.

Although previous studies have shown that inhibition of ATR can lead to genomic instability and increased chromosomal fragility, one interesting finding is that cancer cells with Chk1 (which lies downstream of ATR) knockdown, actually had increased levels of phosphorylated ATR targets. This finding is presumed to result from inhibition of Chk1, which likely functions to provide negative feedback regulation to some of the other ATR targets in the pathway. The effect these phosphorylated ATR targets have on cellular viability is unknown because ATR inhibition has not been studied extensively.

Although some studies have shown that inhibition of ATR can sensitize cancer cells to gemcitabine treatment,[37] the only major study that has examined the effects of ATR inhibition on cancer cell viability, did so in conjunction with oncogenic Ras expression. Whether the cells in the present study were more viable because of the absence of phosphorylated ATR targets is an interesting question that warrants further investigation.

Given the molecular differences between chemical inhibition of ATR and siRNA modulation of ATR, perhaps the effect of siRNA knockdown takes too long for an observable cytotoxic change in the MMCR cells. Fig. 5A shows that despite large amounts of p-ATR knockdown in RT4 cells, residual protein is visible even at high siRNA concentrations. Perhaps the reason why siATR#19 did not produce a significant increase in cell viability in RT4 cells like it did in MMCR cells, is because the residual p-ATR protein compensated for the knockdown and signaled for cell-cycle arrest. The initial hypothesis for this study was based on the notion that continuation of cell division without allowing treated cells sufficient time to repair damaged DNA and recover, would lead to compounded instability and rapid cell death. It is very possible, however, that the time frame investigated was not sufficient.

Interestingly, some recent research involving the DDR pathway in oral squamous cell carcinoma, showed that over expression or Wip1 (wild-type p53-induced phosphatase, a potent negative regulator of the ATM pathway) has been associated with increased resistance to cisplatin.[54] Whether ATR played a role in the development of resistance in the MMCR line is unlikely, but given the importance that ATR has in modulating DNA damage repair signals, it still remains a likely candidate in the search for pathways that

potentiate chemotherapeutic agent toxicity in cancer cell lines regardless of whether resistance is present or not.

Future Directions

There are several different paths that could be undertaken in an effort to further characterize the chemoresistance observed in the MMCR line. Given that the results obtained during this study are somewhat in opposition to the stated hypotheses, one possible next step for this study would be to investigate the effects of ATR *overexpression* in the MMCR and RT4 cell lines, to determine if the opposite of the hypothesis yields the expectant result. Perhaps, unlike with other carcinomas, ATR expression is not the major player in the development of chemoresistance in UC.

Given that numerous studies are underway examining ATR as a target for chemotherapeutic resistance (with hypotheses similar to the ones in the present study), and the discovery of new potent and selective ATR inhibitors such as VE-821[55, 56] and AZ20,[57] a different approach would be to repeat the experiment, perhaps using a chemical inhibitor instead of using RNAi. Because of some of the subtle, yet detectable differences in the viability and proliferation for cells treated with transfection vehicle alone, it is possible that siRNA-directed therapy might not be the ideal method to address chemoresistance *in vitro* at this time. Furthermore, while the delivery mechanism of siRNA directed-cancer therapy is a topic for a completely different discussion, it nonetheless remains a barrier that exists in getting *in vitro* treatments from the lab to the bedside of UC patients. There are many current studies investigating the effects of ATR expression in cancer cell lines. For these reasons, continued

elucidation of the role ATR plays in cancer chemoresistance is warranted for future studies.

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