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Enhancement of 5- Fluorouracil-induced *In Vitro* and *In Vivo* Radiosensitization with MEK Inhibition

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Abstract

Purpose—Gastrointestinal cancers frequently exhibit mutational activation of the Ras/MAPK pathway, which is implicated in resistance to ionizing radiation (IR) and chemotherapy. Concurrent radiotherapy and 5-fluorouracil (5-FU) based chemotherapy is commonly used for treatment of gastrointestinal malignancies. We previously reported radiosensitization with selumetinib, an inhibitor of MEK1/2. The purpose of the current study was to evaluate if selumetinib could enhance radiosensitivity induced by 5-FU.

Experimental Design—Clonogenic survival assays were performed with the HT29 (colorectal), HCT116 (colorectal) and MiaPaca-2 (pancreatic) cell lines using pre-IR treatment with selumetinib, 5-FU and 5-FU+selumetinib. Cell proliferation was determined using a tetrazolium conversion assay. Mitotic catastrophe and DNA repair were analyzed using immunocytochemistry. Flow cytometry was used to analyze cell cycle and apoptosis. Growth delay was used to determine effects of 5-FU+selumetinib on *in vivo* tumor radiosensitivity.

Results—Pre-IR treatment with 5-FU+selumetinib significantly decreased clonogenic survival compared to either agent alone. Dose modifying factors at a surviving fraction of 0.1 for 5-FU +selumetinib was 1.78, 1.52, and 1.3 for HT29, HCT116, and MiaPaca-2, respectively. Cell proliferation was decreased by treatment with selumetinib+5-FU as compared to single agent treatment regardless of treatment sequencing. Enhancement of 5-FU cytotoxicity and 5-FU mediated radiosensitization with selumetinib treatment was accompanied by an increase in mitotic catastrophe and apoptosis, and reductions in Stat3 phosphorylation and survivin expression. *In vivo*, an additive growth delay was observed with 5-FU+selumetinib+5Gy versus 5-FU+5Gy and selumetinib alone.

Conclusion—These data suggest that selumetinib can be used with 5-FU to augment radiation response.

Keywords

selumetinib; AZD6244; 5-fluorouracil; radiosensitization; MEK1/2

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Introduction

One of the hallmarks of cancer cells is the up-regulation of cellular pathways that provide survival advantages by promoting proliferation and/or decreasing cell death [1]. One such pathway is the Ras/Raf/mitogen activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) pathway, which is involved in cell proliferation, differentiation, apoptosis, and survival. Inhibition of the Ras/MAPK pathway has been explored as an anti-cancer therapy. Selumetinib (AZD6244, ARRY-142886), a selective inhibitor of MEK1/2, has been shown to have single agent efficacy in a variety of human tumor cell lines, with greater single agent efficacy observed in *BRaf* and *KRas* mutant cell lines [2].

Activation of the Ras/MAPK pathway has been implicated in resistance to ionizing radiation [3, 4] and cytotoxic chemotherapy [5, 6]. We and others have previously shown that inhibition of signaling via the Ras/MAPK pathway enhances sensitivity to radiation [7–9]. Inhibition of the Ras/MAPK pathway has also been exploited as a means to sensitize tumors cells to cytotoxic chemotherapy [5, 6].

Gastrointestinal cancers frequently exhibit activation of the Ras/MAPK pathway via activating mutations in *Ras* [10] and/or *Raf*[11–14]. The presence of either a Ras or Raf mutation is associated with an inferior prognosis compared to non-mutated tumors [14–16]. Concurrent radiotherapy and radiation sensitizing 5-fluorouracil (5-FU) based chemotherapy is a common treatment strategy for gastrointestinal malignancies. Despite aggressive chemoradiotherapy, local failure remains a troubling clinical problem that requires development of more effective regimens.

The purpose of the current study was to evaluate if inhibition of MEK1/2 could enhance 5-FU mediated radiosensitization. We describe enhancement of radiation response with 5-FU and selumetinib compared to either agent alone. We also demonstrate an enhancement of the cytotoxic and anti-proliferative activity of 5-FU in the setting of MEK1/2 inhibition regardless of treatment sequencing. The enhancement of 5-FU cytotoxicity and 5-FU mediated radiation sensitization with selumetinib treatment was accompanied by an increase in mitotic catastrophe and apoptosis, a reduction in stat3 phosphorylation, and a reduction in survivin expression. These data suggest that concurrent treatment of selumetinib with 5-FU may be used in patients with gastrointestinal malignancies to augment radiation response.

Materials and Methods

Cells lines and Treatments

The colorectal carcinoma cells lines, HT29 (ATCC # HTB-38) and HCT116 (ATCC # CCL-247), and pancreatic carcinoma cell line MiaPaCa-2 (ATCC # CRL-1420) were maintained in RPMI 1640 medium (ATCC, Manassas, VA) containing 10% FBS at 37°C in a humidified atmosphere with 5% CO_2. All cell lines were passed for fewer than 6 months after resuscitation. Selumetinib (supplied by Astra Zeneca) was reconstituted in DMSO and stored at −20°C. 5-Fluorouracil (5-FU; Teva Parenteral Medicines, Irvine, CA) was diluted in PBS (Invitrogen, Carlsbad, CA) immediately prior to each experiment. The final concentration of 5-FU used was 15μM in all experiments.

Clonogenic Survival Assay

Cells were trypsinized to form single cell suspensions and plated at appropriate concentrations in triplicate in 6-well plates. Six hours after plating, 5-FU or vehicle control (PBS) was added. Sixteen hours after the addition of 5-FU, selumetinib (final concentration 250nM for HCT116 and MiaPaCa-2, 100nM for HT29) or DMSO (vehicle) was added. Cells were irradiated 2 hours after the addition of selumetinib using a Pantak X-ray unit at a

dose rate of 1.55 Gy/min. 5-FU was removed from the media immediately following irradiation and incubation was continued with selumetinib. Ten to 12 days after irradiation, cells were fixed, stained with crystal violet, and the number of colonies containing at least 50 cells was determined. Surviving fractions for each treatment were determined by normalizing the average plating efficiency for each dose to the plating efficiency at 0 Gy. Assays were performed in triplicate. Dose modification factors (DMFs) were calculated by taking the ratios of the radiation dose that resulted in 10% survival (control radiation dose divided by the drug-treated radiation dose). DMF values greater than 1.0 indicate enhancement of radiosensitivity.

Western Blot

Cell lysates were prepared using RIPA buffer (Pierce, Rockford, IL) containing phosphatase (Sigma-Aldrich, St. Louis, MO) and protease (Roche, Indianapolis, IN) inhibitors and protein concentration was determined using a DC protein assay kit (Bio-Rad, Hercules, CA). Equal amounts of protein were subjected electrophoresis performed under reducing conditions and gels were blotted to nitrocellulose membranes. All primary antibodies used were purchased from Cell Signaling (Danvers, MA) with the exception of anti-phospho γH2AX and anti-actin (Millipore, Billerica, MA). All western blotting experiments were conducted in duplicate.

Immunocytochemistry for γH2AX

Cells were fixed in 2% paraformaldehyde for 15 min at room temperature and permeabilized with 1% Triton X-100 for 10 min on ice. Slides were incubated in anti-phospho γH2AX (Millipore, Billerica, MA) for 1 hr at 4°C, an Alexa Fluor-conjugated secondary antibody (Invitrogen, Carlsbad, California) for 1 hr at room temperature, and nuclei were counterstained with DAPI (Sigma-Aldrich, St. Louis, MO). Slides were viewed using a Leica microscope with fluorescence capability and images were captured using a QImaging camera. The percentage of cells containing >50 foci was determined in 150 cells for each condition. Experiments were performed in triplicate.

Evaluation of Mitotic Catastrophe

The presence of fragmented nuclei was used as the criteria for defining cells undergoing mitotic catastrophe. To visualize nuclear fragmentation cells were fixed with 2% parformadehyde for 15 minutes at room temperature, permeabilized with 0.5% Triton X-100 for 1 hour on ice, and stained with anti-α-tubulin antibody (Millipore, Billerica, MA) followed by staining with an Alexa Fluor-conjugated secondary antibody (Invitrogen, Carlsbad, California), each for 1 hour at room temperature. Nuclei were counterstained with DAPI. Cells were imaged with a Leica microscope with fluorescence capability. Nuclear fragmentation was defined as the presence of more than two distinct nuclear lobes within a single cell. The percentage of cells with nuclear fragmentation was determined in 150 randomly selected cells in each of 2 separate experiments.

Flow Cytometry

For cell cycle analysis, cells were trypsinized, washed with PBS, fixed with 100% ethanol, and stored at −20°C. Fixed cells were washed with 0.5% BSA and incubated in a solution containing 0.1% Triton X-100, 0.2mg/ml RNase A, and 20ug/ml propidium iodide. DNA content was determined from 2 individual experiments using FACSCaliber cytometry (BD Biosciences, San Jose, CA) and FlowJo software (Tree Star, Inc., Ashland, OR).

For apoptosis analysis, a modified protocol from the ApopNexin FITC Apoptosis Detection Kit (Millipore, Billerica, MA) was used. Briefly, media was aspirated and collected with

attached cells. Attached cells were trypsinized, collected and washed with ice-cold PBS. Cells were resuspended in binding buffer and incubated with ApopNexin FITC and propidium iodide at room temperature in the dark. Cells positive for Annexin V were determined from 2 individual experiments using FACSCaliber cytometry (BD Biosciences San Jose, CA) and analyzed using FlowJo software (Tree Star, Inc., Ashland, OR).

Proliferation Assay

Cells were plated in 96-well plates and treated with 5-FU and selumetinib at the time-points and durations indicated. Following the indicated treatment, media was replaced to remove 5-FU. The number of viable cells at 72 hours after plating was determined using the CellTiter 96 Non-radioactive Cell Proliferation Assay according to the manufacturer's protocol (Promega, Madison, WI). Briefly, 72 hours after plating, cells were treated with 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) for 4 hours at 37°C before addition of stop solution. After 1 hour, changes in absorbance were read at 570nm in a plate reader and expressed as a percent of control absorbance. All conditions were assayed in triplicate and experiments were repeated 3 times.

In vivo Tumor Model

Six to eight week-old female nude mice (Fredrick Labs, Frederick, MD) were caged in groups of five or less, and fed a diet of animal chow and water *ad libitum*. HCT116 tumor cells (1×10^6 cells) were injected subcutaneously into the right hind leg. When tumors grew to a mean volume of 172 mm^3, the mice were randomized to treatment groups (8 mice per group). At time 0, mice receiving 5-FU were given 100 mg/kg via intraperitoneal injection while all other mice received an injection of PBS. Two hours later, mice receiving selumetinib were given a single oral dose of selumetinib at 50 mg/kg while all other mice received an oral dose of DMSO. Six hours after 5-FU and 4 hours after selumetinib, mice received a dose of 3 Gy to the tumor. Irradiation was performed using a Therapax DXT300 X-ray irradiator (Precision X-ray Inc., North Branford, CT) using 2.0 mm Al filtration (300 KVp) at a dose rate of 1.9 Gy/min with animals restrained in a custom jig that allows exposure of the tumor bearing leg with shielding of the rest of the body. To obtain a tumor growth curve, perpendicular diameter measurements of each tumor were measured every 3 days with digital calipers, and volumes were calculated using the formula $(L \times W \times W)/2$. Tumors were followed individually until they measured greater than 600 mm^3. Tumors that failed to regrow were followed for 90 days after treatment. Specific tumor growth delay was calculated for each individual animal. The mean growth delay for each treatment group was calculated as the number of days for the mean of the treated tumors to grow to 600 mm^3 minus the number of days for the mean of the control group to reach the same size. Each animal study was conducted in accordance with the principles and procedures outlined in the NIH Guide for the Care and Use of Animals.

Statistics

All data were analyzed with SAS using Proc GLM with a significance level of $P < 0.05$. Duncan's multiple range test was used to determine significant differences between means.

Results

To determine if selumetinib could enhance the radiation sensitization observed with 5-fluorouracil, we performed clonogenic survival assays with three tumor cells lines. Doses and timing of 5-FU were chosen based on published data [17, 18] and preliminary work performed in our laboratory confirming radiation sensitization. Clonogenic survival in all 3 cell lines was significantly reduced with 5-FU, selumetinib, and combined 5-FU+ selumetinib pre-IR treatment. Clonogenic survival after pre-IR treatment with 5-FU+

selumetinib was reduced beyond that observed with either agent alone with DMFs of 1.78, 1.52, and 1.3 for HT29, HCT116, and MiaPaCa-2, respectively (Figure 1). Dose-dependency of the selumetinib and 5-FU+ selumetinib combination effect was observed with a less dramatic enhancement of radiosensitization in HCT116 and MiaPaCa-2 cells treated with a combination of a lower dose of selumetinib (100μM) and 5-FU (data not shown). Toxicity was calculated by subtracting the plating efficiency for each treatment in the unirradiated condition from one. Toxicity was greatest with 5-FU+ selumetinib compared to selumetinib or 5-FU in all cell lines.

In an effort to better understand the cellular mechanisms underlying enhancement radiation sensitization with the combined treatment of 5-FU+ selumetinib, we focused on the HCT116 cell line. To confirm that target inhibition with selumetinib was present in the setting of 5-FU, IR, or the combination, phosphorylation of ERK1/2 was determined in each setting. Western blot analysis confirmed basal ERK phosphorylation, consistent with the known status as *Ras* mutants. Selumetinib treatment inhibited basal phosphorylation of ERK1/2. Treatment with selumetinib was sufficient to inhibit ERK phosphorylation after exposure to 5-FU, IR, or the combination of 5-FU and IR (Figure 2).

Antagonism of the cytotoxic and cytostatic effects of 5-FU is a major concern when combining 5-FU treatment with agents known to alter cell cycle distribution. The cytotoxic effects of 5-FU are known to be cell cycle dependent, and selumetinib is known to redistribute cells into G0 and G1 phases of the cell cycle [19, 20]. Theoretically, treatment with selumetinib could reduce the cycling fraction and result in antagonism of 5-FU. Therefore, we evaluated if sequencing of 5-FU in relation to selumetinib would alter the anti-proliferative effects of either agent. To determine the effects of altering treatment order and duration, three treatment sequences were evaluated, which altered the timing of 5-FU treatment in relation to selumetinib administration. Proliferation assays showed that the combined treatment of 5-FU+ selumetinib significantly decreased cell proliferation as compared to 5-FU single agent treatment (Supplemental figure 1). In sequence 1, 5-FU treatment was delivered 16 hours prior to the addition of selumetinib. In this condition both 5-FU and selumetinib led to a reduction in proliferation with the greatest anti-proliferative effect observed with combined 5-FU+selumetinib (Supplemental figure, sequence 1). When selumetinib treatment was delivered six or even 16 hours prior to 5-FU (Supplemental figure, sequences 2&3), the combined treatment of 5-FU+ selumetinib still significantly decreased cell proliferation as compared to 5-FU single agent treatment. Therefore, although the magnitude of anti-proliferative effects changed with the duration of exposure to each agent, altering treatment order did not eliminate the significant increase in anti-proliferative effect of the combined treatment of 5-FU+ selumetinib compared to 5-FU alone suggesting a lack of antagonism with any of the tested sequences. The need to alter pre-and post 5-FU exposure to selumetinib necessitated different total durations of exposure to 5-FU and selumetinib between the sequences; thus while these data can be used to provide evidence of lack of antagonism within a sequence, direct comparisons of efficacy between sequences are not possible.

Cells in the G2 and M phases of the cell cycle are preferentially sensitive to irradiation. Cell cycle analysis was performed to evaluate if changes in cell cycle distribution could be responsible for enhanced radiation sensitivity in cells treated with selumetinib and 5-FU compared to either agent alone or vehicle. Pre-treatment of cells with selumetinib, 5-FU, and selumetinib and 5-FU did not result in significant changes in the proportion of cells in the G2 and M phases of the cell cycle compared to vehicle treated controls (data not shown) suggesting that cell cycle distribution is not responsible for the increased sensitivity to radiation.

DNA damage repair is an important component of 5-FU and radiation- induced cytotoxicity. To evaluate the induction and repair of DNA double-strand breaks, we counted phosphorylated histone H2AX (γH2AX) foci. The percent of cells with greater than 50 foci was calculated at 1, 6, and 24 hours after cells were treated with 5-FU and selumetinib as in the clonogenic assays and irradiated to 4Gy. In vehicle and selumetinib treated cells, irradiation induced a significant increase ($p<0.05$) in the number of γH2AX foci at 1 hr, which progressively declined to 24 hrs (Figure 3). Exposure to 5-FU, selumetinib, or 5-FU+ selumetinib followed by 4 Gy resulted in a number of γH2AX foci not significantly higher to that observed with 4Gy alone at 1 hr suggesting that these treatments do not impact immediate DNA damage after IR. At 24 hrs after radiation, the number of cells with more than 50 γH2AX foci per cell was higher in the 5-FU+selumetinib treated cells compared to 5-FU, vehicle, or selumetinib treated cells, suggesting that the combination of 5-FU and selumetinib resulted in inhibition of DNA repair (Figure 3). ($p<0.05$)

Mitotic catastrophe is a common method of cell death after IR. To determine if treatment with 5-FU and selumetinib together enhanced mitotic catastrophe after IR compared to the individual treatments, we determined the percentage of cells that contained more than 2 distinct nuclear bodies at 24, 48, and 72 hours after IR [21]. Within 48 hours after radiation, a significantly greater number of cells were scored as undergoing mitotic catastrophe in the 5-FU+selumetinib treated cells as compared to either selumetinib or 5-FU treated cells at the same time-point (Figure 4A), an effect which persisted at 72 hours. As supplemental evidence, we determined DNA content using flow cytometry. A significant increase in cells with >4N DNA content was observed in HCT116 cells (Supplemental figure 2). A significant increase in mitotic catastrophe therefore may be a mechanism by which treatment with selumetinib enhances 5-FU radiosensitization.

To determine if apoptotic cell death was also an important component of cell death after treatment with 5-FU and/or selumetinib after irradiation, we evaluated the percentage of apoptotic cells after treatment with 5-FU, selumetinib, or the combination after exposure to IR. A significant increase ($p<0.05$) in apoptotic cells was found in irradiated cells after treatment with 5-FU+ selumetinib compared to single agent or vehicle treatments (Figure 4B).

In an attempt to identify a molecular explanation for the increase in mitotic catastrophe and apoptosis observed in cells treated with 5-FU in combination with selumetinib, we determined the protein expression of survivin, a known inhibitor of apoptosis that plays a role in mitotic progression, and one of survivin's transcriptional regulators, Stat3, by western blot analysis. Survivin is a known downstream target of the MAPK/ERK pathway [22]. A decrease in both phosphorylated Stat3 and survivin protein expression and an increase in cleaved caspase 3 and cleaved PARP were found in cells treated with the combination of 5-FU+selumetinib as compared to radiation, 5-FU, or selumetinib alone (Figure 5A), suggesting that the enhancement of radiation effect observed with selumetinib and 5-FU compared with either agent individually may be related to the inhibition of downstream mediators of apoptosis and mitotic catastrophe after IR.

Survivin expression is known to be regulated by the cell cycle, with dominant expression observed in G2 and M phases [23]. To confirm that cell cycle redistribution was not responsible for the observed reduction in survivin expression with selumetinib treatment, we evaluated the cell cycle distribution of cells treated with selumetinib, 5-FU, and IR. At the time-point in which reduced survivin expression was observed with selumetinib +5-FU (24 hours after IR), there was no significant difference in the percentage of cells in the G2 and M phases of the cell cycle (Figure 5B).

To confirm that the enhancement of radiation sensitization observed *in vitro* could be translated into an *in vivo* tumor model, a tumor growth delay assay using HCT116 cells grown subcutaneously (sc) in the hind leg of mice was used. Athymic nude mice bearing sc xenografts (172 mm^3) were randomized into four groups: vehicle; selumetnib only; 3 Gy + 5-FU; and selumetinib +5-FU + 3 Gy. Selumetinib was delivered as a single 50 mg/kg dose by oral gavage 4 hours prior to IR. 5-FU was delivered as a 100 mg/kg IP dose 6 hours prior to IR. Treatment was on the day of randomization. The growth rates for the HCT 116 tumors exposed to each treatment are shown in figure 6. For each group, the time for tumors to triple in size (from volume at the time of treatment) was calculated using the tumor volumes from the individual mice in each group (mean ± SE).

For the HCT116 xenograft model, the time required for tumors to triple in volume from the initiation of treatment increased from 7.1 ± 0.5 days for vehicle treated mice to 8.8 ± 0.6 days for selumetinib (50 mg/kg) treated mice. Treatment with 3Gy + 5-FU increased the time to triple in volume to 11.7 ± 1.0 days. However, in mice that received the combination of 3Gy + 5-FU + selumetinib, the time for tumors to triple increased to 22.9 ± 10.4 days. The large SEM value for the 3Gy + 5-FU + selumetinib treatment group was due to a tumor cure (no regrowth at 90 days). The absolute growth delays (the time in days for tumors in treated mice to triple in volume minus the time in days for tumors to reach the same size in vehicle treated mice) were 1.7 days for selumentib alone, 4.6 days for 3 Gy + 5-FU, and 15.8 days for 3 Gy + 5-FU + selumetinib. Thus, the combined treatment was more than the sum of the growth delays caused by individual treatments.

To obtain a dose enhancement factor comparing the tumor radiation response in the setting of 5-FU in mice with and without selumetnib treatment, the normalized tumor growth delays were calculated, which accounts for the contribution of selumentib to tumor growth delay induced by the combination treatment. Normalized tumor growth delay was defined as the time in days for tumors to triple in volume in mice exposed to the combined modality minus the time in days for tumors to triple in size in mice treated with selumetinib only. The dose enhancement factor, obtained by dividing the normalized tumor growth delay in mice treated with 3Gy + 5-FU + selumetinib by the absolute growth delay in mice treated with 3Gy + 5-FU, was 3.4 for the addition of selumetinib.

Collectively, these data confirm a lack of antagonism with the addition of selumetinib to 5-FU and IR and indicate that selumetinib may provide at least an additive enhancement of radiation response when combined with 5-FU and IR. These effects correlate to an increase in DNA double strand breaks and an increase in mitotic catastrophe after irradiation in selumetinib treated cells compared cells treated with irradiation alone.

Discussion

Selumetinib is a selective MEK1/2 inhibitor that is currently being tested in combination with a variety of other agents in Phase I and II trials. Selumetinib has broad preclinical activity with increased single agent efficacy in cancer cell lines with *BRaf* or *KRas* mutations [24]. Selumetinib has previously been shown, by our lab and others, to enhance the radiation response of tumor cell lines both *in vitro* and *in vivo* [7, 8].

In the present study, we evaluated the radiosensitizing effects of selumetinib when combined with 5-FU and irradiation in three gastrointestinal cell lines. This evaluation is critical to the clinical translation of selumetinib as a radiation sensitizer in the setting of combined therapy with 5-FU and IR, the current standard therapy for a wide range of gastrointestinal malignancies. In mammalian cells, 5-FU is converted into metabolites that either damage DNA directly by incorporation into nucleic acids or indirectly by inhibition of thymidylate

synthase, thus causing uridine incorporation into DNA due to a nucleotide pool imbalance (reviewed in [25, 26]).

We found enhanced radiosensitization when cells were treated with 5-FU + selumetinib as compared either agent alone with IR. Evaluation of ERK phosphorylation in each setting confirmed target inhibition in the presence of 5-FU and irradiation with selumetinib treatment. These findings were confirmed in HCT116 xenografts in which the addition of selumetinib to 5-FU and IR led to augmentation of radiation response.

An increase in mitotic catastrophe and apoptosis were identified as two mechanisms of cell death correlating with enhanced radiosensitization. We identified a greater persistence of DNA double strand breaks in the setting of treatment with 5-FU and selumetinib with IR compared to IR alone. Consistent with the reduction in DNA repair, we found an increase in the proportion of cells undergoing mitotic catastrophe after exposure to 5-FU and selumetinib with IR compared to either agent alone with IR.

Both 5-FU [27] and selumetinib [7] have been shown separately to induce mitotic catastrophe. We found a synergistic induction in mitotic catastrophe when 5-FU, selumetinib, and IR were combined that was preceded by a decrease in survivin expression. Decreased survivin expression was accompanied by decreased expression of one of its transcriptional regulators, phosphorylated Stat3. As Stat3 is a known to function downstream of phosphorylated ERK [28], this provides one potential mechanism to explain the increase in apoptosis and mitotic catastrophe observed in irradiated cells after exposure to 5-FU and selumentib.

Survivin is known to play an important role in several aspects of cell division such as sister chromatid segregation, microtubule assembly/stabilization, and regulation of mitotic progression [23, 29]. Importantly, mitosis but not cytokinesis occurs in survivin- depleted cells [29]. Combined with the finding that survivin expression affects DNA repair [30–33], these results suggest that decreased survivin expression may lead to disordered mitosis and mitotic catastrophe through a number of mechanisms.

Interesting in light of the current results, previous studies have indicated that survivin levels affect the response of some cells, including colorectal and pancreatic cancer cells, to radiation therapy [30, 31, 34] and treatment with 5-FU [35–38]. Survivin was previously identified as an inhibitor of apoptosis and it has been shown that over-expression of survivin leads to an inhibition of apoptotic cell death (reviewed in [39]). The reverse, that down-regulation of survivin leads to an increase in apoptosis, has also been shown [30, 33, 35]. Therefore, the increase in apoptotic cell death observed after treatment with selumetinib+5-FU+IR could also be attributed to survivin depletion.

Our current results indicate that in the absence of radiation, the combination of 5-FU and selumetinib resulted in a decrease in tumor cell proliferation. Because treatment with selumetinib may have effects on cell cycle [19, 20] and the cytotoxicity of 5-FU depends on progression through S-phase ([40], reviewed in [41]), we wanted to ensure that coupling treatment with 5-FU and selumetinib did not impair the efficacy of 5-FU in the absence of radiation. This information is critical when considering that subclinical distant disease will not be targeted by irradiation. Our results indicate that the addition of selumetinib to 5-FU results in enhanced anti-proliferative and cytotoxic effects compared to that observed with selumetinib or 5-FU alone, regardless of treatment sequencing. Even in the setting of prolonged exposure to selumetinib prior to treatment with 5-FU, there was no evidence of antagonism between the two agents. The data from proliferation experiments correlate to the observed reduction in plating efficiency observed with the combination of 5-FU and selumetinib compared to either agent alone in clonogenic assays. Our data suggesting an

additive effect of 5-FU and selumetinib are consistent with prior studies suggesting that ERK phosphorylation is a resistance factor to 5-FU mediated cytotoxicity [5].

This is the first study, to our knowledge, that examines the effects of combining MEK1/2 inhibition with 5-FU and IR. In the absence of IR, we found that selumetinib enhanced in the cytotoxic and anti-proliferative effects of 5-FU, regardless of sequencing. When combined with IR, treatment with 5-FU+selumetinib enhanced radiosensitization. The enhancement of 5-FU mediated radiosensitization with selumetinib was accompanied by increases in apoptosis and mitotic catastrophe. We identified persistence in DNA double strand breaks and decrease in survivin expression as a potential mechanism to explain the increase in apoptosis and mitotic catastrophe. These data add to the growing evidence showing that targeting the Ras/MAPK pathway enhances efficacy of chemotherapeutic agents and indicate that inhibition of MEK1/2 may provide a means to enhance 5-FU mediated radiation sensitization.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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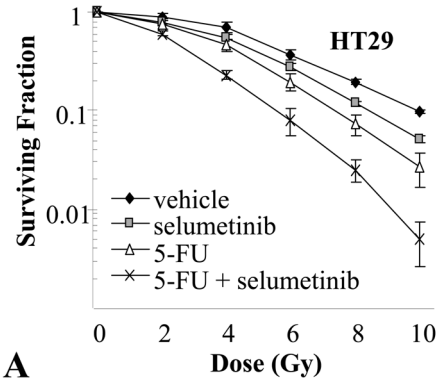
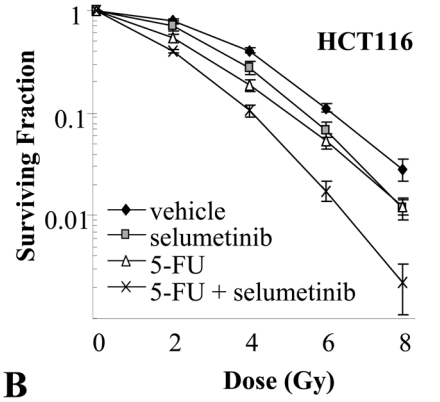
Statement of Translational Relevance

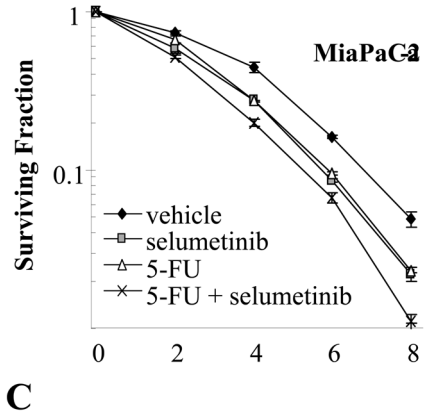
This work reports the use of a clinically relevant molecule, selumetinib, as a radiation modifier in combination with 5-fluorouracil. Selumetinib inhibits MEK1/2 and has been successfully tested in Phase I and Phase II trials. In pre-clinical models, tumors with *BRAF* and *RAS* mutations appear to be more sensitive to MEK inhibition. Gastrointestinal malignancies are commonly treated with 5-flurouracil and radiation and are known to frequently have activation of the Ras-Raf-MEK-ERK pathway via activating *Ras/Braf* mutations or EGFR pathway activation. Despite aggressive chemoradiation, local failure remains a troubling clinical problem that requires development of more effective regimens. The results of the current study indicate that the addition of selumetinib to standard 5-fluorouracil-based chemoradiotherapy for patients with gastrointestinal cancers may enhance response and are the basis for recently initiated Phase I trials.



Treatment	DMF	Toxicity
100nM selumetinib	1.18	0.21
15μM 5-FU	1.36	0.30
5-FU + selumetinib	1.78	0.60



Treatment	DMF	Toxicity
250nM selumetinib	1.13	0.10
15μM 5-FU	1.23	0.31
5-FU + selumetinib	1.52	0.38



Treatment	DMF	Toxicity
250nM selumetinib	1.19	0.37
15μM 5-FU	1.16	0.42
5-FU + selumetinib	1.30	0.51

Figure 1. The effects of selumetinib and 5-FU on tumor cell radiosensitivity

Cell lines HT29 (A), HCT116 (B), and MiaPaCa-2 (C) were exposed to 15μM 5-FU (or vehicle) for 18 hours and selumetinib (or vehicle) for 2 hours and irradiated with graded doses of X-rays. Colony forming efficiency was determined 10–12 days later and survival curves generated after normalizing for cell killing by selumetinib, 5-FU or selumetinib + 5FU in the absence of IR. The data represent the mean of three independent experiments. *Points*, mean; *bars*, standard error; DMF, dose modifying factor; toxicity, mean plating efficiency at 0 Gy.

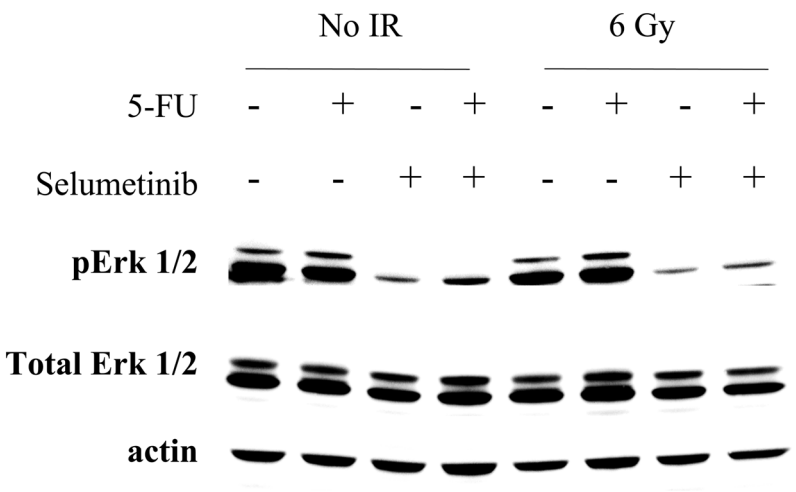


Figure 2. The effects of Selumetinib on ERK1/2 phosphorylation after exposure to IR and 5-FU

HCT166 cells were treated with 15 μM 5-FU (or vehicle) for 18 hours and 250 nM selumetinib (or vehicle) for 2 hours prior to irradiation with 4 Gy. Lysates were collected 2 hours after cells were irradiated. Blots are representative of at least 2 individual experiments.

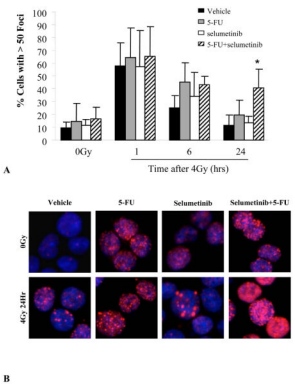


Figure 3. The effects of 5-FU and selumetinib on DNA double strand break repair

HCT116 cells growing in chamber slides were treated with 15 μM 5-FU (or vehicle) for 18 hours and 250 nM selumetinib (or vehicle) for 2 hours prior to irradiation with 4 Gy. Following irradiation, cells were washed with PBS, and media containing 250 nM selumetinib (or vehicle) was added. A) At the indicated time-points, cells were fixed and subjected to immunocytochemistry for γH2AX. The percentage of cells with greater than 50 foci per cell were determined for each treatment condition. The percentage of cells with >50 foci per cell was significantly increased at 24 hours after 4 Gy in cells treated with 5-FU +selumetinib compared to vehicle treated cells. B) Representative images of γH2AX foci (γH2AX labeled with red, nuclei with blue). *Columns*, mean from 3 independent experiments; *bars*, standard deviation; *, $p<0.05$ (selumetinib + 5-FU + 4 Gy vs. vehicle + 4 Gy and selumetinib + 4 Gy).

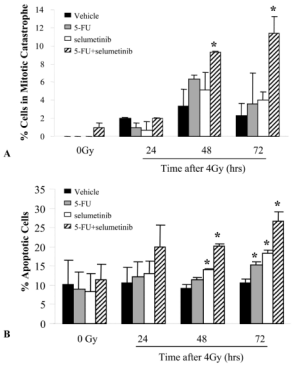


Figure 4. The effects of selumetinib on the mechanism of cell death after exposure to 5-FU and IR

A) HCT116 cells growing in chamber slides were exposed to 15 μM 5-FU (or vehicle) for 18 hours and 250 nM selumetinib (or vehicle) for 2 hours prior to irradiation with 4 Gy. Following irradiation, cells were washed with PBS, and media containing 250 nM selumetinib (or vehicle) was added. Cells were fixed at the time points indicated for immunocytochemical analysis of mitotic catastrophe. Nuclear fragmentation was evaluated in 150 cells per experiment. B) HCT 116 cells were treated with 5-FU (or vehicle) for 18 hours and selumetinib (or vehicle) for 2 hours prior to irradiation (4 Gy) and harvested at the specified times. Treated cell samples were added to a 150 μL staining solution (Guava Nexin Assay) containing 135 μL 1x apoptosis buffer, 10 μL Annexin V-PE, and 5 μL of 7-AAD. Samples (2,000 cells per sample) were evaluated by flow cytometry. *Columns*, mean; *bars*, standard deviation; *, $p<0.05$ as compared to other treatments at the specified time-point.

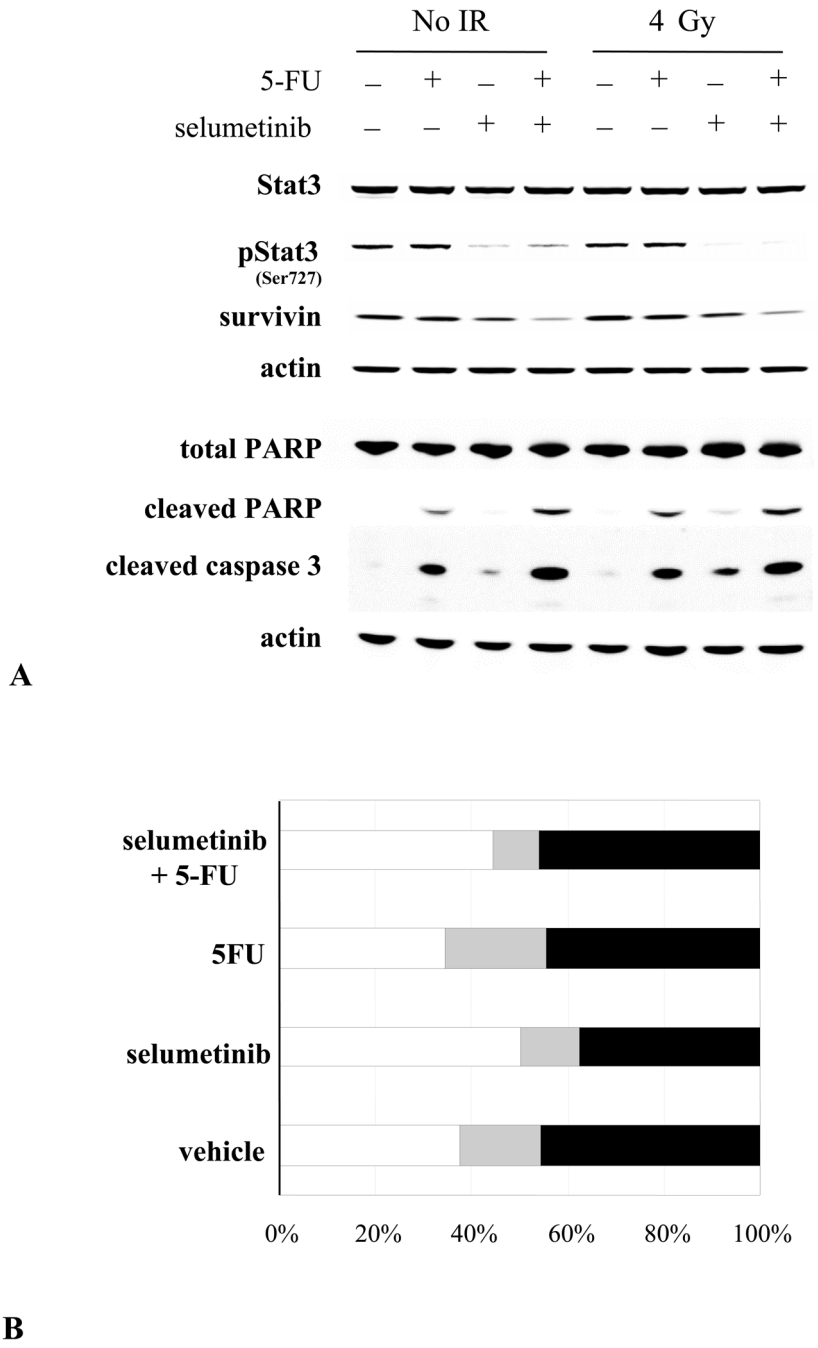


Figure 5. Effects of selumetnib on the expression of survivin, Stat3, and markers of cell death

A) HCT116 cells were exposed to 15 μM 5-FU, 250 nM selumetinib, or the combination prior to irradiation with 4 Gy. Lysates were collected at 24 hours (survivin and stat3) and 48 hours (PARP and caspase-3) after irradiation. Blots are representative of at least 2 independent experiments. B) HCT116 cells were treated as above with 5-FU, selumetinib, the combination, or vehicle prior to irradiation to 4 Gy and collected at 24 hours after IR for cell cycle analysis. No significant difference was observed between the percentage of cells in G2 and M phases of the cell cycle in any group.

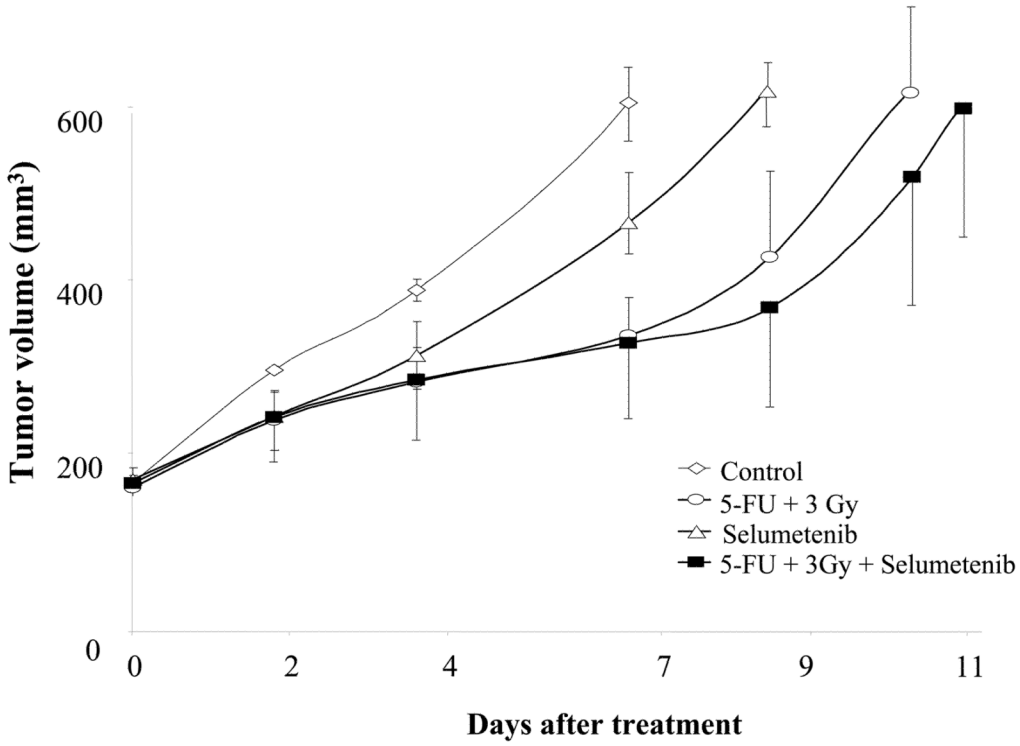


Figure 6. The effects of selumetinib on 5-FU mediated radiosensitization in HCT116 xenograft tumors

When HCT116 tumors reached 172 mm^3 in size, mice were randomized into four groups: vehicle, selumetinib, radiation + 5-FU, or selumetinib + radiation + 5-FU. Selumetinib was given by mouth (oral gavage) in a single dose of 50 mg/kg. 5-FU was delivered as a single IP injection 6 (100mg/kg) hours prior to irradiation. Radiation (3 Gy) was delivered 4 hours after selumetinib treatment. Each treated group contained at least 8 mice. A single tumor cure in the 5-FU + 3Gy + Selumetinib group is censored for clarity. Time 0 on the x axis reflects the day of randomization and treatment. Points, mean; bars, SE.