Sorafenib Enhances Pemetrexed Cytotoxicity through an Autophagy-Dependent Mechanism in Cancer Cells

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Abstract

Pemetrexed (ALIMTA, Lilly) is a folate antimetabolite that has been approved by the U.S. Food and Drug Administration for the treatment of non–small cell lung cancer and has been shown to stimulate autophagy. In the present study, we sought to further understand the role of autophagy in response to pemetrexed and to test if combination therapy could enhance the level of toxicity through altered autophagy in tumor cells. The multikinase inhibitor sorafenib (Nexavar, Bayer), used in the treatment of renal and hepatocellular carcinoma, suppresses tumor angiogenesis and promotes autophagy in tumor cells. We found that sorafenib interacted in a greater than additive fashion with pemetrexed to increase autophagy and to kill a diverse array of tumor cell types. Tumor cell types that displayed high levels of cell killing after combination treatment showed elevated levels of AKT, p70 S6K, and/or phosphorylated mTOR, in addition to class III receptor tyrosine kinases such as platelet-derived growth factor receptor beta and VEGF receptors, known in vivo targets of sorafenib. In xenograft and in syngeneic animal models of mammary carcinoma and glioblastoma, the combination of sorafenib and pemetrexed suppressed tumor growth without deleterious effects on normal tissues or animal body mass. Taken together, the data suggest that pemetrexed and sorafenib act synergistically to enhance tumor killing via the promotion of a toxic form of autophagy that leads to activation of the intrinsic apoptosis pathway, and predict that combination treatment represents a future therapeutic option in the treatment of solid tumors. Cancer Res; 71(14): 4955–67. ©2011 AACR.

Introduction

The antifolate drug pemetrexed (ALIMTA, Lilly) was developed as an inhibitor of thymidylate synthase (TS; refs. 1–4). Pemetrexed also has at least one other target that becomes apparent from a continued antiproliferative effect of drug treatment in cell cultures exposed to exogenous thymidine, which prevents the cytotoxic effects of TS inhibition (1, 2). The identity of any secondary target(s) for pemetrexed could be of considerable interest as the drug exhibits clinical responses in non–small cell lung cancers, which is an unusual activity for folate antimetabolites (5, 6). Subsequently, the secondary target was shown to be the folate-dependent enzyme, amidonimidazole-carboxamide ribonucleotide formyltransferase (AICART; refs. 1, 2). Pemetrexed inhibition of AICART elevated the levels of AICAr/R monophosphate (5-aminoimidazole-4-carboxamide-1-D-ribofuranosyl monophosphate; ZMP), a substrate of the AICART reaction. Accumulation of ZMP caused activation of AMP-activated protein kinase (AMPK) with subsequent inhibition of mTOR and the induction of autophagy (1, 2).

There are 2 main types of programmed cell death: type I, also called apoptosis, refers specifically to an ATP energy-dependent, genetically controlled process involving transcription of specific proteins and leading eventually to a cell’s demise. Propagation of a type I apoptotic signal may occur via either the extrinsic or the intrinsic pathway (7–9). In the extrinsic pathway, trimerization of a death receptor, for example, CD95, recruits and activates caspase 8 via the death-inducing signaling complex (DISC). DISC formation and activation of procaspase 8 are suppressed by c-FLIP-l and c-FLIP-s (9). In some instances, caspase 8 may cleave the proapoptotic protein BID into its active form, tBID. After activation, tBID translocates to the mitochondria, where it contributes to mitochondrial membrane permeabilization, and cytochrome c and apoptosis inducing factor release (10, 11). Cytochrome c binds to Apaf-1 that associates with
procaspe 9 and permits procaspe 9 to autocatalyze its activation. Caspe 9 cleaves to procaspe 3; after cleavage, caspe 3 translocates to the nucleus, followed by DNA fragmentation, carried out by DFF40/45. The intrinsic apoptosis pathway comprises the mitochondrial portion of the extrinsic pathway. After an intracellular insult, BCL-2 proapoptotic family members such as BAK and BAX translocate to the mitochondria, inactivating the antiapoptotic BCL-2 family proteins such as BCL-XL and MCL-1 (12, 13). This allows BAX and BAK to form multimers (pores) which lead to mitochondrial membrane permeabilization. Inhibition of the intrinsic apoptosis pathway has previously been shown to suppress the cytotoxicity of several TS inhibitors, including pemetrexed (14, 15).

Type II programmed cell death, also called autophagy, is a ubiquitous process that occurs in all eukaryotes (16, 17). Autophagy is a nonselective process in which cytoplasm and organelles are (apparently) randomly assorted into the autophagosome, where they are degraded. The process is activated by extracellular and intracellular stimuli (18, 19). Simplistically, there are 3 types of autophagy: microautophagy, macroautophagy, and chaperone-mediated autophagy (20–25). Macroautophagy is mediated by 2 ubiquitin-like conjugation systems, ATG12–ATG5 and ATG8 (microtubule-associated protein 1 light-chain 3, LC3)–phosphatidyl ethanolamine (26–30). A green fluorescent protein (GFP)-conjugated form of LC3 (ATG8) thus provides a useful tool for the study of autophagy. After autophagosome formation, this structure fuses with an acidic endosome. The proteins in this structure are degraded, leading to the formation of autolysosomes. The lytic action of autolysosomes is completed by the pexon, which is a nonselective lysosome.

On the basis of the system and the drugs/stimulus being studied, autophagy can either act to protect tumor cells from a toxic stress or can facilitate the toxicity of the stress: we have published evidence for both protective and toxic forms of autophagy based on the stimulus and cell type being examined (31–38). Apoptosis pathways have been linked with autophagy; for example, knockdown of caspase 8 can induce autophagic death (30). Beclin1 contains a BH3 domain that binds to BCL-2/BCL-XL/MCL-1, and release of Beclin1 from these proteins permits induction of autophagy (26–30). The ser/thr kinase mTOR acts as one gatekeeper in the autophagy process, exerting an inhibitory effect; mTOR acts both in a signal transduction cascade that activates autophagic transcription and translation and by inhibiting the ATG proteins directly via their phosphorylation (39, 40). The phosphoinositide 3-kinase (PI3K) class I/akt pathway is involved in downregulation of autophagy by activation of mTOR, whereas Beclin1 and the class III-type PI3K complex are positive regulators of autophagy (41).

Sorafenib is a RAF family kinase inhibitor (Nexavar, Bayer), which is a multikinase inhibitor that was originally developed as an inhibitor of RAF-1, a component of the extracellular signal-regulated kinase (ERK)1/2 pathway, but which was subsequently shown to inhibit multiple other kinases, including class III tyrosine kinase receptors such as platelet-derived growth factor (PDGF), VEGF receptors 1 and 2, c-Kit, and FLT3 (31–35, and references therein). Antitumor effects of sorafenib in renal cell carcinoma and in hepatoma have been ascribed to antiangiogenic actions of this agent through inhibition of the growth factor receptors (31–35, and references therein). However, several groups of investigators, including ours, have shown in vitro that sorafenib kills human leukemia cells at concentrations below the maximum achievable dose (Cmax) of 15–20 μmol/L through a mechanism involving downregulation of the antiapoptotic BCL-2 family member MCL-1. In these studies, sorafenib-mediated MCL-1 downregulation occurred through a translational rather than a transcriptional or post-translational process that was mediated by endoplasmic reticulum stress signaling and the regulation of autophagy. More recently, we have shown that sorafenib-mediated inhibition of PDGF receptor β (PDGFR-β) plays a key role in the ability of this agent to promote autophagy in tumor cells (35).

The present studies attempted to define whether pemetrexed toxicity was modified by the drug-induced induction of autophagy, and whether sorafenib, a drug that also is known to modulate autophagy, could interact with pemetrexed to kill tumor cells.

**Materials and Methods**

Sorafenib tosylate was purchased from Eton BioScience, Inc. Pemetrexed was purchased from LC Laboratories. Trypsin-EDTA, Dulbecco’s modified Eagle’s medium (DMEM), RPMI, and penicillin-streptomycin were purchased from GibcoBRL Life Technologies. All cells, except for human GBM cells and MCF7, were purchased from the American Type Culture Collection (ATCC) and were not further validated beyond that claimed by ATCC. Cells were repurchased every 6 months. GBM cells came from the repository at the Mayo Clinic. MCF7 cells were obtained by Dr. K.P. Nephew from their primary source (University of Michigan, Ann Arbor, MI). MCF7F cells were generated as noted in (42). Plasmids to express active p70 S6K and active mTOR were purchased from Addgene. Commercially validated small hairpin RNA molecules to knockdown RNA/protein levels were from Qiagen. Reagents and performance of experimental procedures were described in refs. 31–38.

**Culture and in vitro exposure of cells to drugs**

All cell lines were cultured at 37°C [5% (v/v) CO2] in vitro using RPMI supplemented with dialyzed 5% (v/v) fetal calf serum and 10% (v/v) nonessential amino acids. Human serum does not contain significant amounts of thymidine. If the fetal calf serum, generally used by many scientists, for in vitro studies has not been dialyzed to remove the thymidine, experiments will only obtain, at best, ~30% of the combination killing effect due to a diminution of the actions of pemetrexed (see Supplementary Data). Cells growing in complete fetal calf serum that contains thymidine were gradually weaned into dialyzed serum lacking thymidine over 2 weeks and were then used for experimental analyses for the following 3 weeks before being discarded (1, 2). Cells were re-isolated in thymidine-less media as required. For short-term cell killing assays and immuno-blotting studies, cells were plated at a density of 3 × 104 per cm2 (~2 × 105 cells per well of a 12-well plate) and 48 hours after...
plating treated with various drugs, as indicated. In vitro pemetrexed and sorafenib treatments were from 100-nmol/L stock solutions of each drug, and the maximal concentration of vehicle (DMSO) in media was 0.02% (v/v). Cells were not cultured in reduced serum media during any study described in this article.

**In vitro cell treatments, microscopy, SDS-PAGE, and Western blot analysis**

For in vitro analyses of short-term cell death effects, cells were treated with vehicle or pemetrexed/sorafenib for the indicated times in the legends for Figs. 1–5. For apoptosis assays where indicated, cells were isolated at the indicated times, and either subjected to trypan blue cell viability assay by counting in a light microscope or fixed to slides, and stained using a commercially available Diff Quick (Geimsa) assay kit. Alternatively, the Annexin V–propidium iodide (PI) assay was carried out to determine cell viability as per the manufacturer's instructions (BD PharMingen) using a Becton Dickinson FACScan flow cytometer. Pemetrexed/sorafenib lethality, as judged by Annexin V–PI, was first evident approximately 12 hours after drug exposure (data not shown).

For SDS-PAGE and immunoblotting, cells were plated at 5 × 10^5 cells/cm² and treated with drugs at the indicated concentrations and after the indicated time of treatment, lysed in whole-cell lysis buffer (0.5 mol/L Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 1% β-mercaptoethanol, and 0.02% bromophenol blue), and the samples were boiled for 30 minutes. The boiled samples were loaded onto 10%–14% SDS-PAGE, and electrophoresis was run overnight (10–100 μg/lane based on the gel size). Proteins were electrophoretically transferred onto 0.22-μm nitrocellulose and immunoblotted with various primary antibodies against different proteins. All immunoblots were visualized using an Odyssey Infrared imager. For presentation, immunoblots were digitally assessed using the provided Odyssey Imager software [the data sets presented are the fold increase ± SEM (n = 3) in expression of the indicated protein compared with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) loading control; for phosphoproteins the fold increase ± SEM (at least n = 3) is normalized to the total protein level of the indicated kinase or substrate]. Errors are not numerically shown because of space restrictions in the figures displayed in this article; any significant differences between the expression/phosphorylation levels of proteins are indicated by an asterisk or other annotation (P < 0.05).

**Transfection of cells with siRNA or with plasmids**

For plasmids, cells were plated as described above and transfected 24 hours after plating. For mouse embryonic fibroblasts (2–5 μg) or other cell types (0.5 μg), plasmids expressing a specific mRNA (or siRNA) or appropriate vector control plasmid DNA were diluted in 50 μL serum-free and antibiotic-free medium (1 portion for each sample). Concurrently, 2 μL LipoFectamine 2000 (Invitrogen), was diluted into 50 μL of serum-free and antibiotic-free medium (1 portion for each sample). Diluted DNA was added to the diluted LipoFectamine 2000 for each sample and incubated at room temperature for 30 minutes. This mixture was added to each well/dish of cells containing 200 μL serum-free and antibiotic-free medium for a total volume of 300 μL, and the cells were incubated for 4 hours at 37°C. An equal volume of double-strength medium was then added to each well. Cells were incubated for 48 hours and then treated with pemetrexed/sorafenib.

**Transfection with siRNA**

Cells were plated in 60-mm dishes from a fresh culture growing in log phase as described above, and transfected 24 hours after plating. Prior to transfection, the medium was aspirated, and 1 mL serum-free medium was added to each plate. For transfection, 10 nmol/L of the annealed siRNA, the positive sense control double-stranded siRNA targeting GAPDH or the negative control (a scrambled sequence with no significant homology to any known gene sequences from mouse, rat, or human cell lines) was used. siRNA (10 nmol/L; scrambled or experimental) was diluted in serum-free media. HiPerfect (4 μL; Qiagen) was added to this mixture, and the solution was mixed by pipetting up and down several times. This solution was incubated at room temperature for 10 minutes and then added dropwise to each dish. The medium in each dish was swirled gently to mix and then incubated at 37°C for 2 hours. One microliter of 10% (v/v) serum-containing medium was added to each plate, and cells were incubated at 37°C for 48 hours before replating (50 × 10^3 cells) each) onto 12-well plates. Cells were allowed to attach overnight and then treated with pemetrexed/sorafenib (0–48 hours). Trypan blue exclusion/terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL)/flow cytometry assays and SDS-PAGE/immunoblotting analyses were conducted at the indicated time points.

**Recombinant adenoviral vectors; infection in vitro**

We generated and purchased previously described recombinant adenoviruses to modulate protein expression and to express constitutively activated and dominant-negative proteins, dominant-negative caspase 9, and BCL-XL (Vector Biolabs). Cells were infected with these adenoviruses at an approximate multiplicity of infection of 50. Cells were further incubated for 24 hours to ensure adequate expression of transduced gene products prior to drug exposures.

**Microscopy for LC3–GFP expression**

Cells were transfected with a plasmid to express an LC3–GFP fusion protein and then were cultured for 24 hours. Cells were then treated with drugs, as indicated. LC3–GFP transfected cells were visualized at the indicated time points on the Zeiss Axiosvert 200 microscope using the fluorescein isothiocyanate (FITC) filter.

**Animal studies**

Animal studies in flank and brain were carried out under approved protocols by the Virginia Commonwealth University Institutional Animal Care and Use Committee. For studies with human mammary carcinoma cells, athymic nu/nu mice (8-week-old, female) were injected, into the fourth mammary.
fat pad, with $1.0 \times 10^7$ BT474 cells. Tumors of approximately 75 mm$^3$ grew over the following month. Animals were segregated into tumor volumes of approximate equivalent mean tumor size and standard error. The animals were administered vehicle diluent (Cremophore, BASF), sorafenib (25 mg/kg), pemetrexed (50 mg/kg), or the drug combination by oral gavage once every day for 5 days. We carried out the experiment twice with a total of 10 animals per treatment group. Tumor volumes were measured every 2 to 3 days as indicated. For studies with mouse mammary tumor cells, Balb/c mice (8-week-old, female) were obtained from the Nathional Cancer Institute, and animals were injected into the fourth mammary fat pad with $1.0 \times 10^4$ 4T1 cells. Five days after implantation, the animals were administered vehicle diluent (Cremophore), sorafenib, pemetrexed, or the drug combination (as above) by oral gavage for 5 days. The volumes of the tumors in each group were calculated 14 days after the final drug treatment. We carried out the experiment twice with a total of 10 animals per treatment group.

For studies using GBM6-luciferase, athymic female were anesthetized via intraperitoneal administration of ketamine (40 mg/kg) and xylazine (3 mg/kg) and immobilized in a stereotactic frame (David Kopf Instruments). A 24-gauge needle attached to a Hamilton syringe was inserted into the right basal ganglia to a depth of 3.5 mm and withdrawn 0.5 mm to make space for tumor cell accumulation. The entry point at the skull was 2-mm lateral and 1-mm dorsal to the bregma. Intracerebral injection of 0.5 $\times 10^6$ GBM6-luc glioma cells (~40 mice per cell line per separate experiment) in 2 µL of DMEM was carried out over 10 minutes. The skull opening was enclosed with sterile bone wax, and the skin incision was closed using sterile surgical staples. The animals were administered vehicle diluent (Cremophore), sorafenib (25 mg/kg), pemetrexed (50 mg/kg), or the drug combination by oral gavage once every day for 5 days. We carried out the experiment twice with a total of 12 animals in each treatment group.

For normal tissue toxicity studies, animals were administered vehicle diluent (cremophore) or with sorafenib (25 mg/kg) and pemetrexed (50 mg/kg) in combination by oral gavage once every day for 5 days. Animals were rested for 2 days and then treated for an additional 5 days with vehicle or drugs. Two days after cessation of drug treatment, animals were humanely sacrificed and tissues (brain, lung, liver, heart, kidney, and spleen) isolated, 10 µmol/L sections taken, and hematoxylin and eosin (H&E) stained. For immunohistochemy and staining of fixed tumor sections following sacrifice, tumors were fixed in optimal cutting temperature compound (Tissue Tek) and cryostat sectioned (Leica) as 10-µm sections. Data shown are representative slides from several sections from the same tumor with multiple tumors (from multiple animals and multiple experiments) having been examined at least 3–6 animals — tumors per group.

**Data analysis**

Comparison of the effects of various treatments was carried out using ANOVA and Student t-test. Differences with $P < 0.05$ were considered statistically significant. Experiments shown are the means of multiple individual points ($\pm$ SEM). Statistical examination of in vivo animal survival data utilized log-rank statistical analyses between the different treatment groups.

**Results**

Mammary, hepatoma, and lung carcinoma cells were grown in thymidine-less serum to replicate growth conditions of an in situ tumor in a patient. Cells were treated with low clinically relevant doses of the antifolate pemetrexed and in response cells rapidly increased their levels of autophagy as judged by vesiculization of a GFP tagged form of LC3 (ATG8; LC3–GFP) and by increased processing of LC3 to LC3II (Fig. 1A; Supplementary Fig. S1; refs. 1, 2). Drug-induced autophagy was first observed at a higher pemetrexed dose within 6 hours of treatment, and autophagy was noted at all doses within 12 hours. The induction of autophagy was blocked by a small molecule inhibitor of the class III PI3K Vps34, 3 methyl-adenine (3MA), or by knockdown of Beclin1 or ATG5 (data not shown; Supplementary Fig. S2). Within 24 hours, treatment of breast cancer cells with pemetrexed caused a dose-dependent reduction in cell viability as judged by Annexin V–PI flowcytometry, an effect that was blocked by 3MA or by knockdown of Beclin1 (Fig. 1B and C).

Sorafenib is a multi kinase inhibitor with biologic actions that have often been tied to inhibition of class III receptor tyrosine kinases (RTK), for example, VEGFRs and PDGFR-$\beta$ (35). We and others have also noted that sorafenib can stimulate autophagy through inhibition of class III RTKs, and that in a dose-dependent effect this response can either be a protective form of autophagy or a toxic form of autophagy (e.g., 35, 36). Hence, we next determined whether sorafenib enhanced, or suppressed, pemetrexed toxicity. Sorafenib and pemetrexed interacted in a greater than additive fashion to increase the number of autophagic vesicles in tumor cells that correlated with increased processing of LC3 to LC3II and that was inhibited by knockdown of Beclin1 (Fig. 2A). Sorafenib enhanced the toxicity of pemetrexed in a dose-dependent fashion in short-term 24-hour cell viability assays using multiple tumor cells from a diverse range of tissue types (Fig. 2B and C; Supplementary Fig. S3–S7). Knockdown of Beclin1 blocked the pemetrexed + sorafenib drug combination–stimulated induction of autophagy and suppressed the cytotoxic interaction between sorafenib and pemetrexed (Fig. 2A and D; Supplementary Fig. S8). Incubation of cells with 3MA also suppressed pemetrexed + sorafenib–induced toxicity (data not shown).

It has been noted by Racanelli and colleagues and Rothbart and colleagues that pemetrexed treatment increases the intracellular concentration of the chemical ZMP resulting in ZMP-induced activation of the AMPK; AMPK activation in turn acts to suppress mTOR activity (1, 2). Reduced mTOR activity has been associated with elevated levels of autophagy. Because of these findings, we investigated whether suppression of mTOR function by use of rapamycin-altered pemetrexed lethality (1, 2, 39, 40). Rapamycin (Rap) enhanced pemetrexed toxicity in multiple tumor cell types (Supplementary Fig. S9). Thus
The development estrogen independence in recurrent mammary tumors; that is, those tumors initially diagnosed as being estrogen receptor–positive (ER+), suppression of the PI3K–mTOR pathway at the level of a growth factor receptor or at the level of mTOR could enhance pemetrexed lethality.
Figure 2. Pemetrexed interacts with sorafenib in a dose-dependent fashion to increase autophagy and tumor cell killing that is suppressed by knockdown of Beclin1. A, BT474, 4T1, and HuH7 cells as indicated were transfected with siRNAs (si-scramble, siSCR; siBeclin1; 20 nmol/L) and with a plasmid to express LC3–GFP. Twenty-four hours after transfection cells were treated with vehicle (PBS) or pemetrexed (0.03–3.0 μmol/L) and/or vehicle (DMSO) or sorafenib. Twelve hours after drug exposure cells were examined under a fluorescent microscope ×40 at the indicated times after drug exposure, and the mean number of vesicles in 40 random cells in triplicate calculated per experiment (2 studies; ± SEM; *, P < 0.05 less than corresponding siSCR value). B, HuH7 and H460 cells were treated as indicated with vehicle (PBS) or pemetrexed and/or vehicle (DMSO) or sorafenib. Viability was determined in triplicate 24 hours later by trypan blue exclusion (HuH7, H460) or Annexin V–PI staining, as noted in the panel (%; ± SEM; *, P < 0.05 greater than vehicle control value). C, parental MCF7 and fluvestrant-resistant MCF7 cells (MCF7F) were treated as indicated with vehicle (PBS) or pemetrexed and/or vehicle (DMSO) or sorafenib. Viability was determined in triplicate 24 hours later by trypan blue exclusion (%; ± SEM; *, P < 0.05 greater than vehicle control value; †, P < 0.05 greater than corresponding value in parental MCF7 cells). D, BT474 and HuH7 cells as indicated are transfected with siRNAs (si-scramble, siSCR; siBeclin1; 20 nmol/L). Twenty-four hours after transfection in triplicate, cells were treated with vehicle (PBS) or pemetrexed (0.03–3.0 μmol/L) and/or vehicle (DMSO) or sorafenib. Viability was determined in triplicate 24 hours later by trypan blue exclusion (%; ± SEM; *, P < 0.05 less than corresponding siSCR value).
chronically treated with tamoxifen, has frequently been observed. For ER⁺ postmenopausal breast cancer patients, the development of other "purer" antiestrogen therapeutics such as fulvestrant has also been beneficial though even these purer antiestrogen modalities have potential to fail with the delayed outgrowth of estrogen-independent breast cancer cells (43). We next determined the impact of fulvestrant resistance on the lethality of the pemetrexed + sorafenib drug combination in a well-characterized ER⁺ breast cancer cell line that had been isolated from a pleural effusion, MCF7.

In agreement with a role for autophagy in the regulation of mammary tumor cell survival following sorafenib + pemetrexed, in parental MCF7, and fulvestrant-resistant MCF7 (MCF7F), sorafenib or the drug combination increased LC3II processing and decreased p62 levels (Fig. 3A). Parental MCF7 cells are known to be haplotype for expression of the autophagy regulatory gene Beclin1, and it has been postulated in clinical samples from breast cancer and lung cancer patients that loss of Beclin1 facilitates mammary/lung tumorigenesis (e.g., 43). Basal Beclin1 levels were significantly elevated in MCF7F compared with parental MCF7 cells;
Figure 4. Knockdown of PDGFR-β, mTOR, or p70 S6K enhances pemetrexed toxicity and inhibition of ERK1/2 suppresses drug combination toxicity. A, tumor cells, as indicated, growing in log phase were isolated 24 hours after plating and subjected to SDS-PAGE and immunoblotting against the indicated proteins as described in the Materials and Methods section. The intensity of immunostaining was normalized to either GAPDH for proteins or for phosphoproteins to the equivalent dephosphorylated protein; these values were then normalized with the intensity value of each protein/phospho-protein in BT474 cells defined as 1.00 (± SEM; n = 3; #, P < 0.05 greater than value in BT474; @, P < 0.05 greater than value in parental MCF7 and BT474; *, P < 0.05.
similar findings were also noted for ATG5–ATG12 levels. In parental MCF7 cells, drug combination treatment increased Beclin1 and ATG5–ATG12 expression to approximately the same extent as the basal protein levels in MCF7F cells (Fig. 3A). Beclin1 and ATG5–ATG12 levels were not further enhanced by the drug treatment in MCF7F cells.

In addition to altering expression of autophagy regulatory proteins, drug combination exposure of MCF7 and MCF7F cells also reduced expression of the mitochondrial protective proteins MCL-1 and BCL-XL (Fig. 3B). These are proteins we have previously implicated in breast cancer cells to sequester Beclin1 and reduce the ability of tumor cells to induce autophagy, as well as to suppress apoptosis. Overexpression of BCL-XL or expression of dominant-negative caspase 9, but not the expression of c-FLIP, significantly suppressed drug combination cytotoxicity, implying that autophagy was feeding into the intrinsic apoptosis pathway at the level of the mitochondrion and that the extrinsic pathway was not involved in killing (Fig. 3B).

On the basis of our in vitro cell survival data and expression data for autophagy regulatory proteins, we next examined the relative phosphorylation and expression levels of signal transduction proteins after pemetrexed + sorafenib treatment; expression and activity of these proteins have been associated by others and ourselves in a variety of systems to correlate to altered levels of autophagy and apoptosis. Tumor cell types that displayed high levels of cell killing after pemetrexed + sorafenib exposure, such as MCF7F, H460, and HuH7, tended to exhibit significantly elevated basal levels of Akt, p70 S6K, and/or mTOR phosphorylation (Fig. 4A; Supplementary Fig. S10). Cells that were more sensitive to the pemetrexed + sorafenib drug combination, such as MCF7F and HuH7, also tended to display elevated expression levels of class III RTKs such as PDGFR-β and VEGFR1, known in vivo targets of sorafenib. Thus, based on these findings, and those in Fig. 2C with estrogen-dependent and fulvestrant-resistant MCF7 cells, we further explored the signaling responses and viability changes of tumor cells after pemetrexed + sorafenib treatment.

We found that fulvestrant-resistant MCF7 breast cancer cells (MCF7F) overexpressed the class III RTK PDGFR-β, had elevated levels of ERK1/2, p70 S6K, and mTOR activity, and were more sensitive to drug combination toxicity compared with their estrogen-dependent counterparts (Figs. 2C, 4A and B). Treatment of parental MCF7 cells with pemetrexed and sorafenib, but not the individual drugs, modestly suppressed T421/S424 and mTOR S2448 phosphorylation and strongly increased ERK1/2 phosphorylation (Fig. 4B). Treatment of MCF7F cells with pemetrexed more effectively reduced p70 S6K T421/S424 phosphorylation than sorafenib, and sorafenib more effectively reduced p70 S6K T389 and mTOR S2448 phosphorylation than pemetrexed. Knockdown of PDGFR-β, or of p70 S6K or mTOR enhanced pemetrexed and pemetrexed + sorafenib lethality (Fig. 4C and D). Thus, for PDGFRβ, the observed elevated receptor expression and elevated drug toxicity in MCF7F cells define this protein as one molecular marker for a tumor cell response to the sorafenib and pemetrexed drug combination. Because sorafenib and pemetrexed treatment reduced p70 S6K and mTOR activity, we hypothesized that expression of constitutively activated forms of p70 S6K and mTOR would reduce drug cytotoxicity. Expression of a constitutively active form of p70 S6K and of mTOR significantly reduced the toxic effects of pemetrexed and sorafenib treatment (Fig. 4E).

All the prior studies cited in this article have been using 2-dimensional in vitro cultures of tumor cells. Because our ultimate goal is to translate the pemetrexed + sorafenib drug combination into the clinic, we determined whether sorafenib and pemetrexed interacted in vivo to suppress tumor cell growth in various tumor model systems. In orthotopic established HER2-positive BT474 human mammary carcinoma tumors growing in the fourth mammary fat pad, sorafenib significantly reduced tumor growth whereas pemetrexed had little impact on tumor mass (Fig. 5A). Combined exposure to sorafenib and pemetrexed significantly reduced tumor growth below that of sorafenib treatment alone and almost abolished tumor growth. The alterations in tumor growth data correlated in sections of the respective tumors with increased cleavage of procaspase 3 and TUNEL staining, a reduction in proliferation (Ki67), and a manifest disruption of tumor cytoarchitecture (Fig. 5B).

In the spontaneous mouse mammary tumor cell line 4T1, which is HER1 dependent for growth, we noted in tumors growing in the fourth mammary fat pad that sorafenib and...
pemetrexed also interacted to suppress tumor growth (Fig. 5C). Our in vivo data using 4T1 cells were in agreement with in vitro apoptosis data shown in Supplementary Fig. S5.

In an orthotopic model of human GBM, in a primary GBM tumor cell isolate that displays invasive capabilities compared with commercially available established cell lines, that is, GBM6-luc that expresses EGFR VIII, we noted that treatment with pemetrexed + sorafenib significantly suppressed tumor cell growth during and shortly after drug exposure (Supplementary Fig. S11; \( P < 0.05 \)). Many days after drug exposure, tumors still exhibited a high level of apoptosis (Supplementary Fig. S12). Finally, because we wish to move our drug combination into the clinic, consideration of normal tissue toxicity effects needed to be made. Thus, we determined whether combined sorafenib and pemetrexed treatment had any deleterious effects on normal mouse tissues. Pemetrexed + sorafenib treatment did not reduce the body mass of animals carrying GBM6-luc, BT474 tumors or in animals lacking tumors (Supplementary Figs. S13–S15).

Two weeks of pemetrexed + sorafenib treatment did not result in any obvious normal tissue toxicity as judged using H&E staining of sectioned organs and examination of nuclear morphology (apoptosis) or of tissue integrity (Supplementary Figs. S16–S18).

Discussion

The antifolate pemetrexed was recently shown to elevate ZMP levels, thereby activating AMPK, which in turn caused inactivation of mTOR and increased levels of autophagy within a tumor cell (1, 2). The precise role of autophagy in survival or death within these studies was not investigated. The mTOR pathway is, in part, responsible for regulating energy metabolism, and a number of tumor suppressor proteins play a central role in this activity of the pathway, including PI3K, PTEN, LKB1, AMPK, and mTORC1. The present studies sought to understand the role of autophagy in response of tumor cells to pemetrexed and to understand how the levels of autophagy and ER stress caused by pemetrexed could be manipulated to cause additional tumor cell killing, that is, by combined treatment with the multi-RTK inhibitor sorafenib.

In a genetically diverse group of tumor cells from a wide range of malignancies, we found that pemetrexed caused a toxic form of autophagy, as judged using 3MA and Beclin1 knockdown. The multikinase inhibitor sorafenib inhibited PDGFR-\( b \) and knockdown of PDGFR-\( b \) or sorafenib treatment of cells further promoted pemetrexed-induced autophagy that was involved in the additional levels of tumor cell killing caused by the drug combination. The drug combination exhibited elevated antitumor effects in vivo compared with the individual agents in several animal orthotopic model systems of breast cancer and also in an orthotopic model of invasive primary human glioblastoma.

As previously published by this laboratory and other groups, autophagy can be shown either to protect cells from a toxic stress or to facilitate the toxicity of the stress, all of which seems to be based on the stimulus and the tumor cell type...
being examined (31–38). In the majority of cells, the single agent toxicity of pemetrexed was dependent on autophagy. More significantly, however, for the future translational development of this drug combination, in at least 1 tumor cell type, the HCT116 and DLD1 colorectal carcinoma lines, we noted that pemetrexed-induced autophagy was a weakly protective response to single agent lethality; nonetheless, sorafenib also potentiated pemetrexed-induced autophagy and cell killing in these tumor cells in a manner consistent with the other cell lines/types examined in the majority of our studies (Park, Bareford, Moran, and Dent; unpublished data).

Pemetrexed and sorafenib treatment caused inactivation of the p70 S6K and mTOR protein kinases, as judged by their dephosphorylation at multiple regulatory sites. Of note, though, was that the protein kinases believed to phosphorylate these particular sites in p70 S6K and mTOR either exhibited little change in their phosphorylation/activity (i.e., AKT) or paradoxically displayed elevated activity (i.e., ERK1/2). In this regard, it has been shown that p70 S6K and mTOR inactivation and ERK1/2 activation have all been linked to enhanced autophagy levels (45–50). Expression of activated forms of p70 S6K and mTOR significantly reduced the toxicity of sorafenib and pemetrexed exposure. Collectively our data lead us to conclude that sustained inhibition of the p70 S6K/mTOR signaling module following sorafenib + pemetrexed exposure plays a central role in drug combination lethality. Whether the altered p70 S6K activity in our system also results in differential translation of terminal oligopyrimidine tracts in 5′ untranslated region (5′TOP) mRNAs, molecules which have recently been linked to increased autophagy, will require additional detailed experimentation (51).

Apoptosis and autophagy pathways are intimately intertwined processes. On the basis of a literature search, the primary pathway of pemetrexed-induced cell killing has been postulated to occur via the intrinsic pathway, although there is relatively little information in the literature, and tested with molecular tools, to completely prove this assertion (1, 2, 52). There are several mechanisms by which endoplasmic reticulum stress, autophagy, and apoptosis can interact to alter cell viability, most notably through PKR-like endoplasmic reticulum (PERK)-dependent inhibition of translation, thereby reducing levels of protective proteins with short half-lives such as MCL-1 through altering the sequestration of Beclin1 via its BH3 domain with several protective BCL-2 family proteins (BCL-2, BCL-XL, and MCL-1) and by cleavage and activation of BID with lysosomal proteases such as cathepsins and calpains (53, 54). Sorafenib + pemetrexed treatment reduced both MCL-1 and BCL-XL protein levels, which would lead to both reduced levels of protective proteins permitting activation of toxic BH3 domain proteins such as BAX and BAK, as well as causing greater levels of unsequestrated Beclin1. In other studies, we have recently shown that drug-induced release of Beclin1 from MCL-1 using the MCL-1 inhibitor Obatoclax (Cephalon) facilitates the binding of Beclin1 to the class III PI3K vps34, thereby promoting autophagy, an effect that has been shown to either preserve or diminish cell viability based on the stimulus and cell type being examined (31–38). Increased formation of acidic lysosomes, as the final end product of autophagic flux, have potential to lead to release of active cathepsin and calpain proteases into the cytosol, where proapoptotic proteins such as BID can then be cleaved/activated (37). Other groups using a diverse variety of stimuli have observed similar lysosomal protease-dependent events (53–55). Beclin1 and ATG5 have potential to be cleaved by cysteine proteases, resulting in metabolized proteins that no longer promote autophagy but instead facilitate activation of other toxic BH3 domain proteins leading to pore formation in the outer mitochondrial membrane, causing release of cytochrome c with resultant activation of procaspase 9 and induction of apoptosis (56). At present, we do not precisely know, following pemetrexed + sorafenib treatment, how high levels of autophagy at the molecular level facilitate mitochondria-dependent apoptosis. These questions await detailed examination in a subsequent study.

It is well known that MCF7 cells, and many primary breast cancers, have a haplotype insufficiency in Beclin1 and thus are less effective at inducing autophagy than are nontransformed mammary epithelial cells (57). These findings would suggest that loss of autophagy in breast cancer facilitates tumor formation. We noted that MCF7F cells expressed higher basal levels of Beclin1, as well as of ATG5–ATG12, compared with parental MCF7 cells arguing that a portion of the process by which MCF7 cells maintain their viability in the face of estrogen deprivation/loss of ER signaling is because of an increased ability to induce a protective form of autophagy. In agreement with this finding, agents that act to suppress the later lysosomal fusion stage of autophagy, for example, chloroquine, can facilitate the antitumor effects of the antitumor tamoxifen (58). We noted that MCF7F cells were more susceptible to pemetrexed + sorafenib lethality—a lethality that was dependent on a toxic form of autophagy. At least 1 key molecular marker for the enhanced response in MCF7F cells to sorafenib was because of their having overexpression of PDGFR-β compared with parental MCF7 cells. Collectively, our data argue that the initial autophagic survival response of breast cancer cells following antiestrogen therapy, which selects for tumor cells that overexpress Beclin1, may subsequently facilitate tumor cell killing by drug combinations that utilize toxic forms of autophagy to achieve their therapeutic effects (59).

In several orthotopic models of breast cancer, we showed that pemetrexed and sorafenib interacted to suppress tumor growth that correlated with increased levels of apoptosis within the tumor. Using invasive primary human GBM cells that express an oncogene associated with shorter patient survival, EGFR VIII, we also observed a significant reduction in tumor growth and prolongation of survival. In the clinic, sorafenib is approved for treatment of renal and hepatocellular carcinomas and is believed to act by suppressing tumor angiogenesis, that is, through inhibition of VEGFR family receptors. Pemetrexed is approved for treatment and maintenance therapy of non–small cell lung cancer and mesothelioma. On the basis of the data presented in this article, we are preparing to develop and implement a protocol for an open-label phase I trial combining sorafenib and pemetrexed in patients with recurrent solid tumors. That study should open at Virginia Commonwealth University Massey Cancer Center by fall of 2011.
Disclosure of Potential Conflicts of Interest

P. Dent is The Universal Inc. Professor in Signal Transduction Research. No other potential conflicts of interest were disclosed by other authors.

Grant Support

Support for the present study was provided to P. Dent from PHS grants (R01-DK52825, P01-CA104177, R01-CA108325, R01-CA141703, and R01-CA150214).

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## Sorafenib Enhances Pemetrexed Cytotoxicity through an Autophagy-Dependent Mechanism in Cancer Cells

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*Cancer Res* 2011;71:4955-4967. Published OnlineFirst May 27, 2011.

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