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Dasatinib sensitizes KRAS mutant colorectal tumors to cetuximab

Emily F. Dunn, **Mari Iida**, **Rebecca A. Myers**, **Kylee A. Hintz**, **David A. Campbell**, **Eric A. Armstrong**, **Chunrong Li**, and **Deric L. Wheeler**[1]

Department of Human Oncology, University of Wisconsin School of Medicine and Public Health, Madison, Wisconsin, USA

Abstract

KRAS mutation is a predictive biomarker for resistance to cetuximab (Erbitux®) in metastatic colorectal cancer (mCRC). This study sought to determine if KRAS mutant CRC lines could be sensitized to cetuximab using dasatinib (BMS-354825, sprycel®) a potent, orally bioavailable inhibitor of several tyrosine kinases, including the Src Family Kinases. We analyzed 16 CRC lines for: 1) KRAS mutation status, 2) dependence on mutant KRAS signaling, 3) expression level of EGFR and SFKs. From these analyses, we selected three KRAS mutant (LS180, LoVo, and HCT116) cell lines, and two KRAS wild type cell lines (SW48 and CaCo2). In vitro, using Poly-D-Lysine/laminin plates, KRAS mutant cell lines were resistant to cetuximab whereas parental controls showed sensitivity to cetuximab. Treatment with cetuximab and dasatinib showed a greater anti-proliferative effect on KRAS mutant line as compared to either agent alone both *in vitro* and *in vivo*. To investigate potential mechanisms for this anti-proliferative response in the combinatorial therapy we performed Human Phospho-kinase Antibody Array analysis measuring the relative phosphorylation levels of phosphorylation of 39 intracellular proteins in untreated, cetuximab, dasatinib or the combinatorial treatment in LS180, LoVo and HCT116 cells. The results of this experiment showed a decrease in a broad spectrum of kinases centered on the β-catenin pathway, the classical MAPK pathway, AKT/mTOR pathway and the family of STAT transcription factors when compared to the untreated control or monotherapy treatments. Next we analyzed tumor growth with cetuximab, dasatinib or the combination *in vivo*. KRAS mutant xenografts showed resistance to cetuximab therapy, whereas KRAS wild type demonstrated an anti-tumor response when treated with cetuximab. KRAS mutant tumors exhibited minimal response to dasatinib monotherapy. However, as *in vitro*, KRAS mutant lines exhibited a response to the combination of cetuximab and dasatinib. Combinatorial treatment of KRAS mutant xenografts resulted in decreased cell proliferation as measured by Ki67 and higher rates of apoptosis as measured by TUNEL. The data presented herein indicate that dasatinib can sensitize KRAS mutant CRC tumors to cetuximab and may do so by altering the activity of several key-

[1]Correspondence: Deric L Wheeler PhD, Department of Human Oncology, University of Wisconsin Comprehensive Cancer Center, 1111 Highland Avenue, WIMR 3159, Madison, Wisconsin 53705. Phone: (608) 262-7837; fax: (608) 263-9947;, dlwheeler@wisc.edu.

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signaling pathways. Further, these results suggest that signaling via the EGFR and SFKs may be necessary for cell proliferation and survival of KRAS mutant CRC tumors. This data strengthen the rationale for clinical trials in this genetic setting combining cetuximab and dasatinib.

Keywords

Cetuximab; resistance; KRAS mutation; dasatinib; EGFR; SRC; colorectal cancer

INTRODUCTION

The epidermal growth factor receptor (EGFR) is a member of the HER family of receptor tyrosine kinases and consists of four members: EGFR (ErbB1/HER1), HER2/Neu (ErbB2), HER3 (ErbB3) and HER4 (ErbB4). Stimulation of the receptor through ligand binding activates the intrinsic receptor tyrosine kinase and promotes receptor homo- or heterodimerization with HER family members. EGFR activation leads to the downstream stimulation of several signaling cascades, including RAS/RAF/ERK/MAPK, phosphatidylinositol 3-kinase (PI3K/Akt) pathway and the phospholipase C-protein kinase C (PLCg/PKC) pathway. In addition, several other pathways are activated including Src family kinase (SFKs) and the Signal Transducers and Activators of Transcription (STATs). Collectively, these pathways influence several cellular responses including cell proliferation, survival, angiogenesis, migration, and metastasis (reviewed in (Abram and Courtneidge, 2000; Biscardi *et al.*, 1999b; Blume-Jensen and Hunter, 2001; Marmor *et al.*, 2004; Prenzel *et al.*, 2001; Schlessinger, 2000; Yarden and Sliwkowski, 2001)). Aberrant expression or activity of the EGFR is linked to the etiology of several human epithelial cancers including head and neck squamous cell carcinoma (HNSCC), non-small cell lung cancer (NSCLC), brain cancer and colorectal cancer (CRC). Therefore, the EGFR has emerged as one of the most promising molecular targets in oncology.

Although EGFR is activated through ligand binding and autophosphorylation of its cytoplasmic tail, it is well established that Src, or Src family kinases (SFKs), are necessary for full activation of the EGFR (Biscardi *et al.*, 1999a). Src is the prototype member of a family of non-receptor tyrosine kinases (nRTKs) including Src, Yes, Fyn, Lyn, Lck, Hck Fgr, Blk and Yrk. These cytoplasmic membrane associated nRTKs are transducers of mitogenic signaling emanating from a number of RTKs including EGFR, HER2, fibroblast growth factor receptor (FGFR), platelet derived growth factor (PDFGR), colony-stimulating factor-1 receptor (CSF-1R) and hepatocyte growth receptor (Belsches *et al.*, 1997; Bowman *et al.*, 2001; DeMali *et al.*, 1999; Mao *et al.*, 1997; Muthuswamy *et al.*, 1994; Tice *et al.*, 1999). Investigations into the molecular interactions between SFKs and EGFR have revealed that SFKs can physically associate with activated EGFR (Belsches-Jablonski *et al.*, 2001; Maa *et al.*, 1995; Muthuswamy and Muller, 1995). This interaction results in a conformational change in the SFK and leads to autophophorylation at Y419 and transient activity (Xu *et al.*, 1999). This interaction of SFKs with RTKs can result in enhanced or synergistic SFK activation and has been demonstrated in tumor types, most notably in HNSCC, NSCLC and CRC (Fu *et al.*, 2008; Koppikar *et al.*, 2008; Mao *et al.*, 1997; Zhang *et al.*, 2007).

Activation of SFKs occurs with high frequency during the development of CRC. An increase in SFK activity in CRC tumors as compared to normal adjacent mucosa has been reported (Bolen *et al.*, 1987; Cartwright *et al.*, 1989). In addition, activation of SFKs was reported at an early stage of colorectal tumor development in polyps with high malignant potential but not in small benign polyps of the colon (Cartwright *et al.*, 1990). Further, premalignant ulcerative colitis epithelium has been reported to have elevated SFK activity (Cartwright *et al.*, 1994), suggesting that SFKs activity may be a critical step in the development from non-malignant to malignant transformation in CRC. Talamonti *et al* reported increased activity and expression of SFKs in progressive stages of human colorectal cancer, suggesting that colon cancer progression may be dependent on increased SFK protein level and subsequent activity (Talamonti *et al.*, 1993). Similar studies by Termuhlen *et al* looking at colorectal metastases to either the liver or the regional lymph nodes exhibited increased SFK activity levels when compared to the primary tumor (Termuhlen *et al.*, 1993). Collectively these studies suggest a putative link between increased SFK activity and metastatic potential. Irby *et al* indicated that overexpression of normal c-Src in poorly metastatic human colon cancer cells enhances primary tumor growth but not the metastatic potential of these cancers (Irby *et al.*, 1997). Additional studies by Irby *et al* cited that activating mutations in Src, as compared to increased expression and activity of Src, in a subset of human colorectal cancers might have a role in the malignant progression of human CRC (Irby *et al.*, 1999). It has been reported that increased SFK expression occurs in approximately 80% of CRC specimens when compared to the normal adjacent colonic epithelium (Hurwitz *et al.*, 2004). Recent studies looking at 64 individual CRC cell lines found a striking diversity of SFK activity. The authors reported that all lines tested depended on SFK activity for growth and concluded from this work SFK activity is important for the growth of CRC lines (Emaduddin *et al.*, 2008). In addition to SFK activity and CRC progression, SFK activity has been reported as a marker of poor clinical prognosis (Aligayer *et al.*, 2002). Collectively these investigations provide a wide body of evidence implicating Src and its family of kinases in CRC development and progression (for further review (Summy and Gallick, 2003)). Regardless of the form of activation, activated SFKs lead to the phosphorylation of several targets including the EGFR, STATS, PLCγ, PKCs, FAK, RAS, RAF and mucin 1 (MUC1) (Biscardi *et al.*, 2000; Bivona *et al.*, 2003; Blake *et al.*, 1999; Bromberg *et al.*, 1998; Chiu *et al.*, 2002; Denning *et al.*, 1996; Fabian *et al.*, 1993; Joseloff *et al.*, 2002; Kijima *et al.*, 2002; Kronfeld *et al.*, 2000; Li *et al.*, 1994; Li and Kufe, 2001; Li *et al.*, 2001a; Li *et al.*, 2001b; McLean *et al.*, 2005; Schaller *et al.*, 1994; Yu *et al.*, 1995).

Targeting EGFR has been intensely pursued in the last decade and has resulted in the FDA approval of five new molecular targeting agents since 2003 in four distinct solid tumors including metastatic, NSCLC, HNSCC, breast cancer and colorectal cancer (mCRC). One molecular strategy of EGFR inhibition has been the development of monoclonal antibodies (mAb, cetuximab and panitumumab) directed against the extracellular domain of the EGFR. This approach results in 1) blockade of endogenous ligand binding to the receptor, 2) inhibition of dimerization with other HER family members and 3) receptor internalization and degradation. Cetuximab and panitumumab have been approved for the treatment of mCRC when used alone or in combination with irinotecan in patients with irinotecan-

refractory mCRC (cetuximab) or as a single agent in patients who do not respond to all available chemotherapies (panitumumab).

Despite the approval of this promising biological therapeutics many individuals do not respond to this class of drug. Intensive clinical trials have evaluated the outcomes of patients with mCRC in relation to their KRAS mutational status. The conclusions of this analysis demonstrated a strong correlation between mutated KRAS and a lack of response to cetuximab therapy indicating that KRAS status as a predictive factor (Amado *et al.*, 2008; Bokemeyer *et al.*, 2008; De Roock *et al.*, 2008; Di Fiore *et al.*, 2007; Karapetis *et al.*, 2008; Khambata-Ford *et al.*, 2007; Lievre *et al.*, 2008; Punt *et al.*, 2008; Van Cutsem *et al.*, 2008). One the basis of these clinical trials ASCO published guidelines that strongly support the use of anti-EGFR antibodies in mCRC patients with wild type KRAS status (Allegra *et al.*, 2009; Bardelli and Siena, 2010). These guidelines leave very few therapeutic options for mCRC patients harboring a KRAS mutation.

In this report we investigated whether or not targeting the EGFR, using cetuximab, and SFKs, using the broad spectrum inhibitor dasatinib, in the KRAS mutant colorectal setting would lead to anti-proliferative effects on colon tumor growth. We found that dasatinib treatment could sensitize KRAS mutant, cetuximab resistant cells to cetuximab therapy *in vitro* and *in vivo*. This combinatorial therapy led to altered signaling in 1) components of the MAPK pathway, 2) the β-catenin pathway and 3) the activation of several members of the STAT family of transcription factors. Taken together this suggests that the EGFR and SFKs play a role in the KRAS mutant CRC setting and that dual targeting the EGFR and SFKs with dasatinib and cetuximab may be a beneficial approach in this genetic subset of mCRC patients.

RESULTS

Characterization and selection of KRAS mutant colorectal tumor lines

We screened 16 CRC lines for the expression of EGFR and SFKs (Figure 1A). Fourteen of the 16 lines expressed EGFR and all lines expressed SFKs. Relative EGFR and SFK expression was quantitated using ImageJ and normalized to Colo320DM and SW620 for EGFR and SW48 for total SFK. Next we screened each line for *KRAS* mutations at codon 12 and 13 and for *BRAF* mutations at codon 600 by pyrosequencing (Figure 1B). Nine of 16 lines had a *KRAS* mutation. Four cell lines (LS123, LS180, SW480, and SW620) had a mutation at codon 12, whereas five lines (DLD1, HCT115, HCT116, LoVo, and SW1417) had a mutation at codon 13. Two of the 16 lines (HT29 and WiDR) demonstrated *BRAF* mutations. *BRAF* mutations were analyzed to ensure that selected lines were mutated for *KRAS* only. To further analyze these tumor cells, we performed *in vivo* tumor growth analysis to determine ability of each CRC cell line to grow in a xenograft model. For this analysis $1.0 \text{ X } 10^6$ were inoculated into the dorsal flank of athymic nude mice and allowed to grow for 4 weeks. Tumors that reached a minimum size of 500 mm^3 were considered xenograftable. The results of this study showed that 12 of 16 lines were able to form tumors *in vivo* (**annotated with an asterisk in** Figure 1A). From these results we selected three lines LS180, LoVo and HCT116 for further studies. To determine their dependence on KRAS we performed proliferation assays using siRNAs targeting KRAS (Figure 1C).

Results from this study showed that each line had dependence on mutated KRAS for proliferation. Significant reductions of KRAS protein levels were demonstrated by Western blot analysis for KRAS knockdown in these experiments (Figure 1C inset). In addition, these lines were also screened for other known dasatinib targets such as EphA2, c-KIT and PDGFR. However, Western blot analysis did not detect expression of these proteins in the three KRAS mutant lines (data not shown). Collectively, this analysis of CRC lines led to the selection of three KRAS mutant, EGFR- and SFK-expressing lines (LS180, LoVo, HCT116), two KRAS wild type lines expressing EGFR and SFKs (CaCo2, SW48), and one non-EGFR expressing KRAS wild type control line (Colo320DM).

Dasatinib sensitizes KRAS mutant colorectal tumors to cetuximab *in vitro*

We performed a series of *in vitro* experiments using two KRAS wild type (CaCo2 and SW48) and three KRAS mutant lines (LS180, LoVo and HCT116) to investigate the mechanisms of sensitization of KRAS mutant CRC lines to cetuximab using dasatinib. To determine if KRAS mutant lines were resistant to cetuximab therapy *in vitro* we performed a series of proliferation assays using plastic plates, fibronectin, laminin, fibronectin/laminin coated plates or Poly D-lysine/laminin (PDL/laminin) coated plates. KRAS mutant CRC cell lines were sensitive to cetuximab on plastic and fibronectin plates (data not shown), however, when plated on PDL/laminin plates, KRAS mutant lines showed decreased response to cetuximab whereas KRAS wild type lines showed increased sensitivity to cetuximab (Figure 2A). These results mimic clinical and *in vivo* findings. Therefore we used PDL/laminin plates for all *in vitro* studies. Next we examined if dasatinib could sensitize KRAS mutant CRC lines to cetuximab therapy. We performed proliferation assays on PDL/laminin plates using DMSO control, 100 nM cetuximab, 50 nM dasatinib or the combination on LS180, LoVo and HCT116 cell lines. The results of these experiments indicated the **1)** KRAS mutant lines were resistant to cetuximab **2)** dasatinib induced mild growth inhibition on KRAS mutant lines and **3)** but the combination of the two drugs exhibited abrogation of cell proliferation (Figure 2B). Figure 2C shows the effects of cetuximab, dasatinib and the combination on their respective kinase targets in KRAS mutant CRC cell lines. These results suggest that signaling via the EGFR and SFKs may be necessary for cell proliferation and survival of KRAS mutant CRC tumors. This data strengthen the rationale for clinical trials in this genetic setting combining cetuximab and dasatinib.

Treatment of KRAS mutant CRC lines with dasatinib plus cetuximab results in distinct alterations of phospho-kinase activity

The development of CRC is characterized by a number of events that lead the normal mucosa through a transformation to dysplastic lesions, adenoma, adenocarcinoma *in situ* and finally to invasive adenocarcinoma. Some of the events lead to deregulated expression and ultimate over activation in the EGFR, KRAS and SFK signaling pathways. Many other alterations have been well documented cell signaling pathways that lead to CRC (Fearon and Vogelstein, 1990). Given the beneficial results seen by the combination of dasatinib and cetuximab in each of the three KRAS mutant lines (Figure 2C) we were curious about potential mechanistic underpinnings that may have resulted in this beneficial effect. Given the complexity and cross-talk of each of these pathways we elected to perform Human Phospho-kinase array analysis on each KRAS mutant line (LS180, LoVo and HCT116)

treated with vehicle, cetuximab (500 nM), dasatinib (50 nM), or the combination to gain an aerial view. This Human Phospho-kinase array analyzed 39 individual proteins involved in cellular proliferation and survival. Each cell line was plated on PDL/laminin plates and allowed to adhere overnight. Vehicle, cetuximab, dasatinib or the combination were placed onto the cells and allowed to incubate for 24 hours. Protein lysates were collected and Human Phospho-kinase Arrays were analyzed for each treatment group in all three cell lines. The results of this series of experimentations were quantitated for each line and summarized in Figure 3. Interestingly the results of this study showed a very unique kinase signature for each cell line treated with the cetuximab, dasatinib or the combination.

Phospho-array analysis of LS180 identified several pathways, which were downregulated by the combination of dasatinib and cetuximab. These pathways included the AKT/mTOR/p70 S6 kinase pathway (AKT and p70 S6 Kinase), MAPK/RSK (RSK1/2/3) and components of the β-catenin pathway (GSK α/β and β-catenin). In addition to signaling pathways several key transcription factors were down regulated including, STAT1, STAT3, STAT4, STAT5A/B, STAT6 and p53. Other signaling molecules that were down regulated in the combination group include: eNOS, and p27. Figure 3A presents in histogram form the notable changes.

In LoVo the members of the MAPK signaling pathway appeared to be down regulated with the combination of dasatinib and cetuximab including MEK 1/2 and MSK 1/2. In regards to transcription factor activity, the combination of dasatinib and cetuximab resulted modulation of phosphorylation of several STAT family members including STAT2, STAT3, STAT5A, STAT5B and STAT6. Other signaling molecules that were down regulated with the combination treatment were; AMPKα1, HSP27 and most notably FAK. Figure 3B presents in histogram form the notable changes.

Phospho-array analysis of HCT116 identified similar pathways as in LS180, These pathways included the AKT/mTOR/p70 S6 kinase pathway (AKT and p70 S6 Kinase), MAPK/RSK (RSK1/2/3) and components of the β-catenin pathway (GSK α/β and β-catenin). However, like LS180 and LoVo the combination seemed to have effects on the STAT transcription factor family including STAT1, STAT2, STAT4, STAT5A, STAT5B, and STAT6. Other signaling molecules that were down regulated in the combination group include: p27, Paxillin, and AMPKa1. Figure 3C presents in histogram form the notable changes.

Collectively these results suggest that three independent KRAS mutant, cetuximab resistant CRC tumor lines, have several shared cell signaling pathways effected by the combination of cetuximab and dasatinib with the most notable similarities in the MAPK pathway, AKT/mTOR pathway, β-catenin pathway and the activation of the STAT family of transcription factors.

Dasatinib sensitizes KRAS mutant colorectal tumors to cetuximab *in vivo*

Next we performed a series of mouse xenograft studies to confirm that KRAS wild type CRC lines are sensitive to cetuximab therapy *in vivo*. To test the non-specific effects of cetuximab, we utilized a non-EGFR, KRAS wild type line, Colo320DM (Figure 4A). A total

of 40 mice (20 per group) were analyzed with bilateral flank tumors. Established tumors (>100 mm^3) were randomized and treated twice weekly with 0.3 mg of cetuximab or 0.3 mg of immunoglobulin G (IgG) for 3 weeks. Next, we utilized a known EGFR-expressing, cetuximab-sensitive NSCLC line, H226, for a positive control (Figure 4B). A total of 20 mice (10 per group) were analyzed with bilateral flank tumors. Similarly, mice were randomized to cetuximab or IgG and treated twice weekly once tumors were established with 0.3 mg of cetuximab or 0.3 mg IgG for 4.5 weeks. The data in Figure 4A and 4B indicate that the EGFR negative line showed no off-target effects of cetuximab whereas H226 showed a similar response to cetuximab as has been previously reported (Wheeler *et al.*, 2008).

Next we tested the KRAS wild type lines, SW48 and CaCo2, for response to cetuximab *in vivo* (Figure 4C and 4D). For both SW48 and CaCo2, 20 mice per cell line were analyzed (10 per group) with bilateral flank tumors. Mice were randomized to IgG or cetuximab and treated twice weekly with 0.3 mg of cetuximab or IgG. SW48 mice were treated for 3.5 weeks whereas the CaCo2 mice were treated for 5.5 weeks based on relative tumor growth rates. This set of experiments confirmed that these KRAS wild type CRC lines are sensitive to cetuximab and manifested a response after the first treatment.

In Figure 5 we performed a series of experiments using three KRAS mutant CRC lines (LS180, LoVo, and HCT116) to test cetuximab and dasatinib as single agents, given sequentially, or in combination. Athymic nude mice were injected with cells (2×10^6) and established tumors from KRAS mutant cell lines were randomized to treatment or control groups. Each line was treated with cetuximab or dasatinib alone (Figure 5A, 5B, 5C, **cetuximab; upper left panels, dasatinib; upper right panels**). For LS180, 37 mice established tumors and were analyzed with bilateral flank tumors (19 in the vehicle group, 18 in the dasatinib treatment group). For LoVo, 42 mice were analyzed (21 per treatment group) with bilateral flank tumors. For HCT116, 40 mice (20 per treatment group) were analyzed with bilateral flank tumors. The results confirmed the clinical finding that these tested KRAS mutant lines were resistant to cetuximab. Dasatinib monotherapy in HCT116 and LS180 showed minimal tumor growth delay and was not shown to be statistically significant, whereas treatment of LoVo with dasatinib appeared to have a slight proliferative effect. These results indicated that dasatinib monotherapy is not effective in these KRAS mutant CRC cell lines.

Next we performed both sequential (cetuximab followed by dasatinib) and combinatorial treatment regimens (Figure 5A, 5B, 5C, **sequential; lower left panels, combination; lower right panels**). In the sequential experiments, mice were randomized to treatment (cetuximab followed by dasatinib) or control (IgG followed by vehicle) groups. For each line (LS180, LoVo, and HCT116), 20 mice were analyzed (10 per treatment group) with bilateral flank tumors. Mice were given cetuximab or IgG (0.3 mg) twice weekly by intraperitoneal injection until tumors demonstrated a resistant phenotype - defined as growth without deviation from the IgG controls. At this time, cetuximab and IgG were ceased and dasatinib or vehicle was started the next day for five days a week by oral gavage (70 mg/kg). Treatment with dasatinib or vehicle was continued for the specified times. The results of these experiments indicated that sequential treatment could lead to an anti-tumor growth

effect (Figure 5A, 5B, 5C, **lower left panels**). The most pronounced effect was in seen in the LS180 and LoVo sequential experiments.

In the combinatorial experiments, mice were randomized to treatment or control groups. For each line (LS180, LoVo, and HCT116), 30 mice from each line (15 per treatment group) were analyzed with bilateral flank tumors. Established tumors were treated with either the combination of IgG and vehicle or cetuximab (0.3mg, twice weekly) and dasatinib (70mg/kg, 5 times weekly) for the time indicated (Figure 5A, 5B, 5C, **lower right panel**). These experiments demonstrated statistically significant tumor growth inhibition in the combinatorial treatment regimen compared to vehicle controls that was distinguishable after the first treatment in LS180 and LoVo cell lines ($P \leq 0.001$). HCT116 demonstrated a statistically significant response at the beginning ($P < 0.05$) and by the end of treatment ($P < 0.001$); although response was modest compared to the other two KRAS mutated cell lines. Collectively, this series of mice xenograft experiments suggests sequential or combinatorial treatment regimens of cetuximab and dasatinib may be effective in KRAS mutant CRC tumors. In addition the combination of cetuximab and dasatinib appears to be more efficacious than the sequential experiments.

Combinatorial dasatinib and cetuximab treatment decreases proliferation and enhances apoptosis

To determine the impact of the combination of dasatinib plus cetuximab we examined rates of cell proliferation and apoptosis in tumor samples from each line. Cell proliferation was analyzed by immunohistochemistry for Ki67. Each tumor shown Figure 6A was collected 3 hours after the last dasatinib or vehicle treatment and 24 hours after the last cetuximab or IgG treatment. In each respective line, Ki67 expression is decreased in the treatment samples (+) compared to vehicle controls (−) (Figure 6A, **Ki67 panels**). To further analyze the effects of cetuximab and dasatinib on tumor growth, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay was completed on tumor samples. Figure 6A **TUNEL assay panels** demonstrate apoptosis in each KRAS mutant cell line was increased by the combination treatment (+) compared to vehicle controls (−). Quantification of the immunohistochemistry staining for Ki67 and TUNEL is shown in Figure 6B. Concomitant treatment samples were obtained from mice euthanized at 3, 12, and 24 hour time points after the last dasatinib or vehicle treatment and 24, 27, 36, and 48 hours after the last cetuximab or IgG treatment. Five random fields, 4 sections for each sample were analyzed at 400× and compared to control slides for false positive DAB staining. The rates of Ki67 expression in LoVo and HCT116 demonstrated statistically significant differences in proliferation between treatment and control groups (66% and 68% decrease in proliferation, respectively). LS180 demonstrated decrease in proliferation of 27%. All lines demonstrated a statistically significant increase in percentage of apoptosis compared to vehicle controls based on TUNEL assays. LS180 demonstrated a 97% increase in apoptosis, LoVo demonstrated a 93% increase in apoptosis, and HCT116 demonstrated a 71% increase in apoptosis. Collectively, this series of experiments suggests that the combination of cetuximab and dasatinib may lead to decreased tumor growth by increasing cell death (apoptosis) and decreasing cell proliferation (Ki67) in KRAS mutant CRC lines.

DISCUSSION

Colon cancer continues to be the second most common cancer-related death in the United States (Jemal *et al.*, 2008). The etiology of mCRC is a complex series of genetic events that are characterized by several alterations including p53, EGFR and SFK expression and mutations in KRAS (Fearon and Vogelstein, 1990; Summy and Gallick, 2003). The EGFR protein is expressed in ~ 85% of mCRC as measured by the specific binding of ^{125}I-EGFR to tumor plasma membrane preparations, Western blotting and immunohistochemistry (Normanno *et al.*, 2003). In addition, It is estimated that 30–40% of patients with CRC have a KRAS mutation (Bardelli and Siena, 2010; Normanno *et al.*, 2009). Further, it has been demonstrated in several clinical trials that patients with mCRC and a KRAS mutation do not respond to cetuximab therapy (Benvenuti *et al.*, 2007; Cappuzzo *et al.*, 2008; De Roock *et al.*, 2008; Di Fiore *et al.*, 2007; Karapetis *et al.*, 2008; Khambata-Ford *et al.*, 2007; Lievre *et al.*, 2008). These trial results leave a large population of patients with mCRC that cannot benefit from cetuximab therapy. The data presented herein indicate that dasatinib can sensitize cetuximab resistant, KRAS mutant CRC tumors to cetuximab. Further, this combinatorial treatment was marked by downregulation of components of the MAPK, AKT/mTOR, β-catenin and STAT pathways.

We screened 16 CRC lines for EGFR and SFK expression, and KRAS or BRAF mutations and dependency on KRAS signaling (Figure 1). Next we determined if these model systems mimic clinical findings in that KRAS mutant CRC lines would be resistant to cetuximab therapy. To test this hypothesis we treated all KRAS mutant lines *in vitro* and challenged them with increasing concentrations of cetuximab (data not shown). The results of this indicated that KRAS mutant CRC lines showed a robust response to cetuximab on plastic plates and did not mimic what is seen *in vivo* and the clinic. Therefore we performed a series of cell culture experiments using plastic plates, fibronectin, laminin, fibronectin/laminin or PDL/laminin coated plates. These experiments indicated that PDL/laminin plates could most closely mimic clinical findings showing that KRAS mutant CRC lines were resistant to cetuximab (Figure 2A). This finding suggests that the interaction between the extracellular matrix *in vitro*, and most likely *in vivo*, plays a critical role in KRAS mutant CRC response to EGFR targeting agents. Viloria-Petit and colleagues reported that cetuximab resistant lines established *in vivo*, were sensitive to cetuximab *in vitro* (with plastic plates) after establishment of cell lines taken from mouse xenografts (Viloria-Petit *et al.*, 2001). Collectively these findings underscore the importance of the experimental approach to study therapeutic targeting KRAS mutant CRC lines and indicate that factors in the cell's environment are critical in the treatment of KRAS mutant CRC.

In figure 2B and 2C three KRAS mutant lines were tested for their response to cetuximab, dasatinib or the combination. Each line was resistant to cetuximab and semi-responsive to dasatinib. However, the combination of the two molecular targeting agents led to decreased proliferative potential as compared to either agent alone (Figure 2B). We verified that the cetuximab and dasatinib could reduce the activity of their respective targets (Figure 2C). Although, the EGFR couples growth factor signaling to the RAS/RAF/MEK/ERK pathway, and mutations in KRAS uncouple this pathway from the receptor, the EGFR still plays a role in the activation of other key pathways such as the PI3K/AKT pathway, STATs pathway

and the PLCγ/PKC pathways (Marmor *et al.*, 2004). These pathways may still be activated by the EGFR, even in the KRAS mutant setting. To determine the effects of co-inhibition of SFKs and the EGFR we used phospho-array analysis on the three KRAS mutant CRC lines treated with vehicle, dasatinib, cetuximab or the combination. The results of these experiments revealed common pathways inhibited by the combination of these two agents in mutant KRAS CRC lines. Firstly, in LS180 and HCT116 the β-catenin pathway appeared to be downregulated (Figure 3A and 3C). This was evident by the decrease in phosphorylation of GSK3α and GSK3β. Decreased activity in this enzyme results in decreased β-catenin phosphorylation (also noted in the phospho-array), thus allowing it to translocate to the nucleus and where it binds the Lef/Tcf transcription factors and activating target genes involved in cancer progression. Secondly, in LS180 and HCT116, downregulation of the AKT/mTOR/p70S6 Kinase pathway was noted. In both lines activating phosphorylation events on AKT were decreased. AKT, through a series of complex signal transduction cascades, leads to the activation of the mTOR1 complex (Engelman, 2009). This serine-threonine kinase then phosphorylates p70 S6 kinase which leads to the increased translation of mRNAs that encode proteins for cell cycle regulators (MYC and cyclin D1) as well as ribosomal proteins and elongation factors involved in translation (reviewed in (Rini, 2008)). Finally, in all three lines tested, the combination of dasatinib and cetuximab resulted in the downregulation two pathways involved in tumor proliferation: **1)** members of the STAT family and **2)** members of the MAPK signaling cascade. The STAT family is comprised of seven members, STAT1-4, STAT5a, STAT5b and STAT6. Binding of growth factors or cytokines to their receptors results in intrinsic kinase activity or recruitment of receptor-associated kinases (janus kinase (JAK) and SFKs). These phosphorylated receptors in turn phosphorylates STATs on key residues leading to their dimerization and translocation to the nucleus where they regulate genes involved in cell proliferation, apoptosis, and angiogenesis and tumor growth. In terms of the MAPK signaling pathway the combination of dasatinib and cetuximab impacted proteins within this cascade albeit at different levels of the pathway. At the terminal end of the classical RAS/RAF/MEK/ERK cascade sits two proteins the 90 kDa ribosomal S6 kinase (RSK1) and MSK1/2. RSKs are phosphorylated at the end of the classical where ERK phosphorylates RSK1 in the kinase activation loop (Richards *et al.*, 1999). Activation of RSK1 can lead to the phosphorylation of the pro-apoptotic protein BAD that, when phosphorylated, abrogate BAD's pro-apoptotic function (Shimamura *et al.*, 2000). In addition, RSK1 can phosphorylates IkBa, the inhibitor of NF-kB, inducing its degradation and allowing its translocation and function in the nucleus (Ghoda *et al.*, 1997). Decreased RSK1 phosphorylation was noted in LS180 and HCT116. MSK1/2 are believed to play a pivotal role in the activation of the CREB transcription factor by phosphorylation of serine 133 (Wiggin *et al.*, 2002). This molecule along with MEK1/2 was down regulated in LoVo. Collectively these data suggest that therapeutic treatment with dasatinib and cetuximab results in the downregulation of several critical pathways involved in the progression of cancer.

Both *in vitro* and *in vivo* (Figures 2B and 5C) the HCT116 data demonstrate a statistically significant response to the combination of cetuximab and dasatinib, but not as robust as compared to LS180 or LoVo. This may be explained due to the reported PI3 kinase mutation in HCT116 (Jhawer *et al.*, 2008; Wee *et al.*, 2009), which would lead to enhanced signaling

through the AKT pathway, independent of cetuximab treatment. However, AKT activity, as measured by phospho-array analysis (Figure 3C) did show decreased AKT activity as compared to either agent alone. This suggests that other, yet to be identified mechanisms exist for the decreased response to the combination in the HCT116 cell line.

Dasatinib is an orally bioavailable and promising therapeutic agent for the treatment of several human malignancies including chronic myelogenous leukemia, non-small cell lung cancer, small cell lung cancer, advanced breast cancer (including triple negative), pancreatic cancer, prostate cancer and head and neck squamous cell carcinoma (reviewed in (Kim *et al.*, 2009). Dasatinib was discovered through the synthesis and testing of a series of thiazole-based compounds with activity against SRC and ABL kinases to target imatinib-resistant BCR-ABL mutants (Kantarjian *et al.*, 2006). Dasatinib, although relatively specific for ABL, BCR-ABL and the SFKs, it possesses a broad-spectrum of inhibition of kinases including Kit, PDGFR, EphA receptors and several others (Hantschel *et al.*, 2008). Non-specific effects must always be considered when developing a mechanism but regardless, the effect of cetuximab and dasatinib on anti-tumor growth is evident and dasatinib's broad spectrum of kinase inhibition may, in part, be linked to its clinical success thus far as well as in combination with cetuximab in the KRAS mutant CRC setting. The combination of cetuximab and dasatinib has shown to be effective in other circumstances these include in the situation of overcoming acquired resistance to cetuximab in NSCLC (Li *et al.*, 2009; Wheeler *et al.*, 2009). In addition, clinical trials looking at this combination are currently in recruitment in HNSCC, mCRC and other solid tumors (clinical trials.gov).

KRAS is clearly a marker of resistance to cetuximab in monotherapy for CRC and patient screening is still essential. However, our results suggest KRAS mutant CRC lines are dependent on both signals from the EGFR and SFKs. Thus, the relationship between EGFR and SFK signaling in the presence of KRAS mutations will be an area of intense investigation. The concomitant treatment of dasatinib and cetuximab may be a viable option for KRAS mutant CRC patients without PI3K, or further downstream mutations. In addition, future directions may include investigations of this combination in the KRAS wild type setting. In summary, this study combines two FDA-approved agents, dasatinib and cetuximab, in the KRAS mutant CRC setting. From the data provided it appears that dasatinib can sensitize KRAS mutant tumors to cetuximab. This work may provide rationale for further investigative clinical trials using dasatinib plus cetuximab in patients with KRAS-mutant, cetuximab-resistant mCRC.

MATERIALS AND METHODS

Compounds

Cetuximab (C225, Erbitux™) was purchased from the University of Wisconsin Pharmacy. Dasatinib (BMS-354825, Sprycel™) was generously provided by Bristol-Myers Squibb (New York, NY).

Cell culture and transfection

The human CRC cell lines CaCo2, Colo320DM, DLD1, HCT15, HCT116, HT29, LoVo, LS123, LS180, SK-CO-1, SW48, SW480, SW620, SW948, SW1417, and WiDr were purchased from ATCC (Manassas, VA). All cell lines were maintained in their respective media with 10% fetal bovine serum with 1% penicillin and streptomycin, except for CaCo2, which was maintained in 20% FBS and 1% penicillin and streptomycin. Colo320DM, DLD1, and HCT15 were maintained in RPMI 1640; HCT116 and HT29 were maintained in McCoy's media; LoVo was maintained in F12 media; CaCo2, LS123, LS180, SK-CO-1, and WiDr were maintained in minimum essential medium eagle; SW48, SW480, SW620, SW948, and SW1417 were maintained in L15 media (Life Technologies, Inc., Gaithersburg, MD). LS180, LoVo and HCT116 cells were seeded in 96-well plates Poly D-lysine/laminin plates (BD Biosciences, San Jose, CA) and transiently transfected with small interfering RNAs (SiRNA; siKRAS (Dharmacon, Lafayette, CO)) using LipofectAMINE RNAiMAX according to the manufacture's instructions (Invitrogen). The Non-targeting siRNA pool was obtained from Dharmacon. Cells were then lysed for analysis of protein knockdown by Western blot or use in cell proliferation assays 72 hour after siRNA transfection.

Cell proliferation assay

Exponentially grown cells were seeded in 96-well plates Poly D-lysine/laminin plates (BD Biosciences). Following 72 hours treatment, 10ul of tetrazolium salt from cell counting kit (Dojindo Molecular Technologies, Japan) was added to each well. After two to four hours, the percentage cell growth was calculated by comparison of the A540 reading from treated versus control wells.

Pyrosequencing

Genomic DNA was isolated from cell lines using a standard proteinase K-phenol-chloroform extraction method. For polymerase chain reaction amplification of the relevant fragments, we used PyroMark KRAS and BRAF kits (Qiagen, Valencia, CA) according to the manufacturer's protocols. The resulting PCR products were electrophoresed in 1.5% agarose gel to confirm successful amplification and 40 uL of each sample was sequenced using a Pyrosequensing PSQ96HS System (Biotage, Uppsala, Sweden) according to the manufacturer's protocol.

Immunoblotting analysis

Whole cell protein lysate was obtained with lysis buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 0.1% Tween-20, 10% glycerol, 2.5 mM EGTA, 1 mM EDTA, 1 mM DTT, 1 mM PMSF and 10 μg/ml of leupeptin and aprotinin), sonicated, fractionated and quantified. Cellular fractionation was performed as described previously (Wheeler *et al.*, 2008). Protein was quantitated using the Bradford method (Bio-Rad Laboratories, Hercules, CA). Western blotting was performed as described previously (Wheeler *et al.*, 2008). Briefly, equal amounts of protein were fractionated by SDS–PAGE. Thereafter, proteins were transferred to PVDF membrane (Millipore, Billerica, MA) and analyzed by incubation with the appropriate primary antibody. Proteins were detected via incubation with HRP-conjugated secondary antibodies and ECL chemiluminescence detection system (GE Healthcare,

Piscataway, NJ). The antibodies used in this study were as follows: EGFR, HRP-conjugated goat-anti-rabbit IgG, and goat-anti-mouse IgG were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). pEGFR 1173, SFK, pSFK and β-actin were obtained from Cell Signaling Technology (Beverly, MA). Ki67 antibody was purchased from AbCam (Cambridge, MA) and α-tubulin was obtained from Calbiochem (San Diego, CA). Image J was used to evaluate densitometry of EGFR and SFK western blots.

Phospho- kinase Array

LS180, LoVo and HCT116 cell lines were analyzed the panel of phosphorylation profiles of kinases after treatment with cetuximab, dasatinib and combination of these compounds (Human Phospho-Kinase Array, ARY003, R&D Systems, Minneapolis, MN). This array specifically screens for relative levels of phosphorylation of 39 individual proteins involved in cellular proliferation and survival. After treatment with cetuximab, dasatinib and combination of cetuximab and dasatinib, cell lysates were incubated with the membrane. Thereafter, a cocktail of biotinylated detection antibodies, streptavidin-HRP and chemiluminescent detection reagents were used to detect the phosphorylated protein. The relative expression of specific photophorylated protein was determined following quantification of scanned images by ImageJ compared to cetuximab, dasatinib, combination of cetuximab plus dasatinib and untreated control.

Mouse xenograft model

Athymic nude mice (4 to 6-week-old males) were obtained from the Harlan laboratories (Indianapolis, IN). All animal procedures and maintenance were conducted in accordance with the institutional guidelines of the University of Wisconsin. Mice were randomized into treatment or control groups. Mice were injected in bilaterally in the dorsal flank of the mouse at respective day 0 (2×10^6 cells). Once tumors reached 100–200 mm^3, mice were started on their respective treatments (cetuximab, IgG, dasatinib, vehicle, the combination of cetuximab and dasatinib, or the combination of IgG and vehicle). Cetuximab dose for all experiments was 0.3 mg intraperitoneally twice weekly. The dose for all experiments was 70 mg/kg of dasatinib five days a week by oral gavage. Tumor volume measurements were evaluated by digital calipers and calculated by the formula $(\pi)/6 \times (\text{large diameter}) \times (\text{small diameter})^2$.

Mouse tumor collection and protein isolation

Tumors were collected at 0, 3, 12, and 24 hours post-treatment. Mice were sedated using isofluorane mixed with oxygen until unconscious. Mice were euthanized by cervical dislocation and tumors were promptly collected, washed in PBS, and frozen on dry ice or fixed in formaldehyde. Tumors were crushed using a mortar and pestle until the tumor was the consistency of a powder. Whole cell protein lysate was obtained with lysis buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 0.1% Tween-20, 10% glycerol, 2.5 mM EGTA, 1 mM EDTA, 1 mM DTT, 1 mM PMSF and 10 μg/ml of leupeptin and aprotinin), sonicated, and quantified. Cellular fractionation and protein quantitation were performed as stated above.

Ki67 and TUNEL Assays

The ApopTag Plus Peroxidase *in situ* apoptosis detection kit was purchased from Millipore. Samples were prepared according to manufacturer's recommended protocol with the modification of antigen retrieval instead of proteinase K. Antigen retrieval was performed in citrate buffer (pH=6.0) with 0.05% tween 20. For immuohistochemistry, tumor samples were fixed in paraformaldehyde for 24 hours, paraffin embedded, and serially cut onto slides. Samples were deparaffinized and antigen retrieval was performed in citrate buffer (pH=6.0) with 0.05% tween 20. Samples were then incubated with Ki67 primary antibody (AbCam, Cambridge, MA). Samples were washed and incubated in secondary antibody one hour followed by with Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA). DAB staining was done using Ultravision Plus Detection System (Lab Vision Corporation, Fremont, CA). Images were captured using Biospot Advanced program software. ImageJ was used to obtain total number of cells (via thresholding that was maintained across all samples). Color deconvolution was used to identify the positive staining and was thresholded across all image samples. All images for treatment (cetuximab plus dasatinib) and control (IgG plus vehicle) were averaged and standard error mean was calculated. Ki67 samples were normalized to the vehicle images and TUNEL samples were normalized to the treatment (cetuximab plus dasatinib) images.

Statistical analysis

Student T-test was used to determine the significance of the cell proliferation or tumor growth volumes between treatment and control groups for each *in vitro* and *in vivo* experiment respectively. Statistical analysis to compare treatment and control groups in positive immunohistochemistry staining was also done with a t-test. Differences between clones were considered statistically significant if $P \leq 0.05$.

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Abbreviations

ABL	v-abl Abelson murin leukemia viral oncogene homolog 1
ASCO	American Society of Clinical Oncology
BCR	breakpoint cluster region
CRC	colorectal cancer
CSF-1R	colony stimulating factor 1 receptor
DMSO	dimethyl sulfoxide
EGFR	epidermal growth factor receptor
FBS	fetal bovine serum
eNOS	endothelial nitric oxide synthase

FAK	Focal adhesion kinase-1
FDA	food and drug administration
FGFR	fibroblast growth factor receptor
GAPs	GTPase activating proteins, GSK α/β, glycogen synthase kinase α/β
HNSCC	head and neck squamous cell carcinoma
HSP27	heat shock 27kDa protein 1
IgG	immunoglobulin G, IHC, immunohistochemistry
mAb	monoclonal antibody
mCRC	metastatic colorectal cancer
MAPK	mitogen-activated protein kinase
MEK	MAPK kinase
MSK	mitogen and stress-activated protein kinase
MUC1	mucin 1
nRTK	non-receptor tyrosine kinase
NSCLC	non-small cell lung cancer
PI3K	phosphatidylinositol 3-kinase
PDGFR	platelet derived growth factor receptor
PLCγ	phospholipase C-gamma
PKC	protein kinase C
RSK	ribosomal s6 kinase
RTK	receptor tyrosine kinase
SFKs	Src-family kinases
STAT	signal transducer and activator of transcription
TUNEL	terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling

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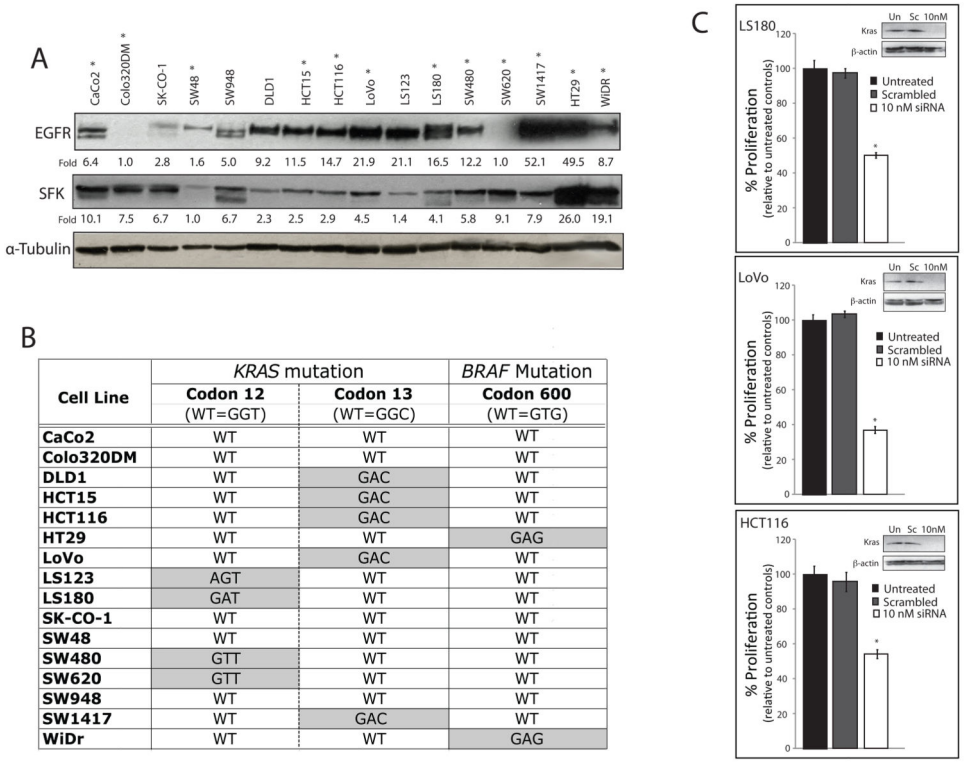

B

Cell Line	*KRAS* mutation		*BRAF* Mutation
	Codon 12 (WT=GGT)	**Codon 13** (WT=GGC)	**Codon 600** (WT=GTG)
CaCo2	WT	WT	WT
Colo320DM	WT	WT	WT
DLD1	WT	GAC	WT
HCT15	WT	GAC	WT
HCT116	WT	GAC	WT
HT29	WT	WT	GAG
LoVo	WT	GAC	WT
LS123	AGT	WT	WT
LS180	GAT	WT	WT
SK-CO-1	WT	WT	WT
SW48	WT	WT	WT
SW480	GTT	WT	WT
SW620	GTT	WT	WT
SW948	WT	WT	WT
SW1417	WT	GAC	WT
WiDr	WT	WT	GAG

Figure 1. Characterization of colorectal tumor lines

A) Analysis of EGFR and SFK expression in colon cancer lines. CRC tumor lines were grown and whole cell lysates were obtained, fractionated by SDS-PAGE and immunoblotted for the indicated proteins. α-tubulin was used as a loading control. All sixteen-tumor lines were tested for in vivo tumor growth using mouse xenografts. Tumor lines that grew greater than 500mm^3 in vivo are denoted by *. Densitometry measurements of EGFR and SFK relative to Colo320DM (1.0) for EGFR and SW48 (1.0) for SFK are shown. **B**) KRAS and BRAF mutational status was determined via pyrosequencing. **C)** KRAS mutant lines LS180, LoVo and HCT116 are dependent on KRAS. KRAS mutant lines LS180, LoVo and HCT116 were treated with transfection reagent only, scramble siRNA (10nM), or KRAS siRNA (10nM). Proliferation was measured at 72 hours after treatment using the proliferation assay as described in the experimental procedures and plotted as a percentage of growth relative to the untreated control cells. Data points are represented as mean ± SEM (n = 4). *$p < 0.05$. Inset denotes confirmation of KRAS knockdown.

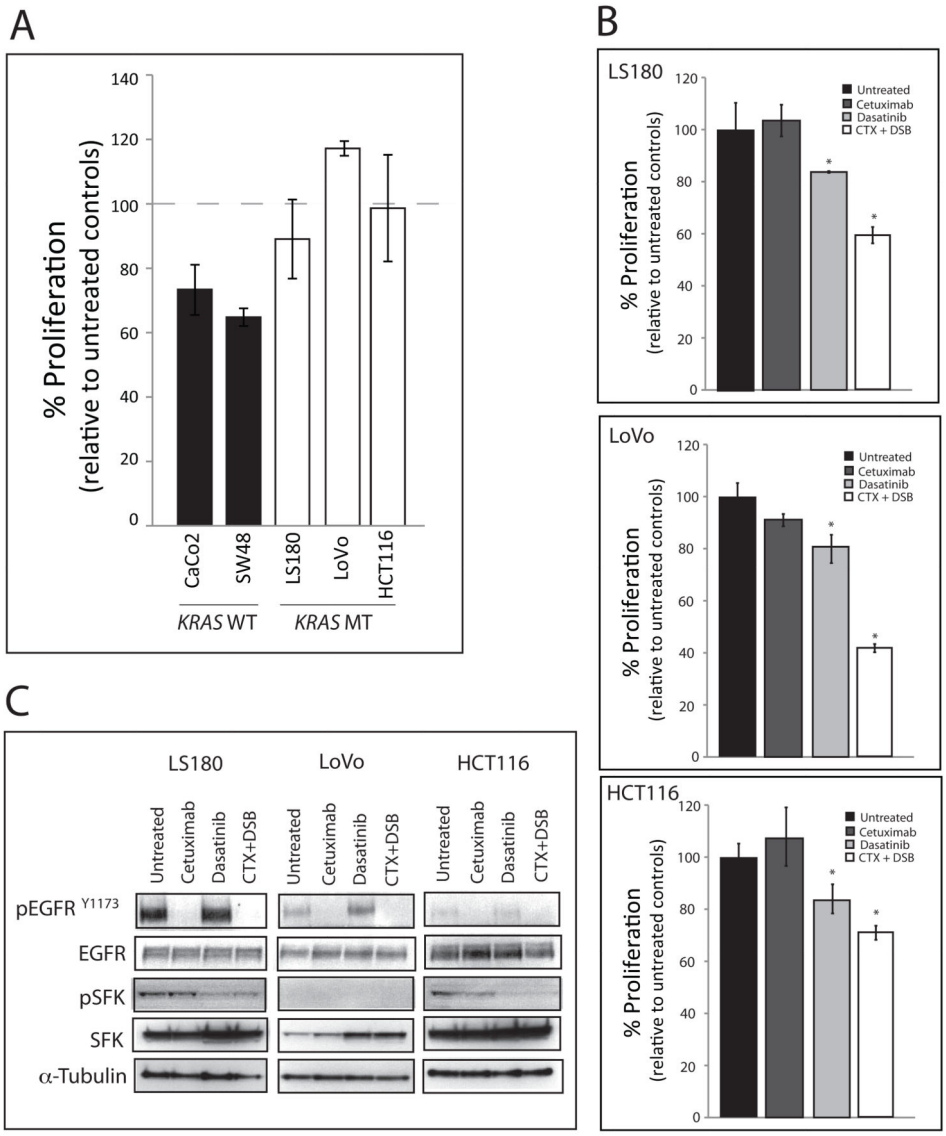

Figure 2. Dasatinib sensitizes KRAS mutant colorectal tumors to cetuximab *in vitro*

(A) KRAS mutant colorectal lines are resistant to cetuximab when compared to KRAS wild type lines. Two KRAS wild type lines (CaCo2 and SW48) and three KRAS mutant lines (LS180, LoVo and HCT116) were tested for response to cetuximab. Cells were plated on Poly D-lysine/laminin 96 well plates, allowed to adhere overnight and treated with vehicle (PBS) or 160 nM of cetuximab for 72 hours. **B)** Dasatinib sensitizes KRAS mutant lines LS180, LoVo and HCT116 to cetuximab. Cells were plated on Poly D-lysine/laminin 96 well plates and allowed to adhere overnight. Cells were treated with vehicle (PBS), 500 nM of cetuximab, 50 nM dasatinib or the combination (CTX + DSB) for 72 hours. Proliferation was measured at 72 hours after drug treatment using the proliferation assay as described in the experimental procedures and plotted as a percentage of growth relative to the untreated control cells. Data points are represented as mean ± SEM (n = 7). *$p < 0.05$.

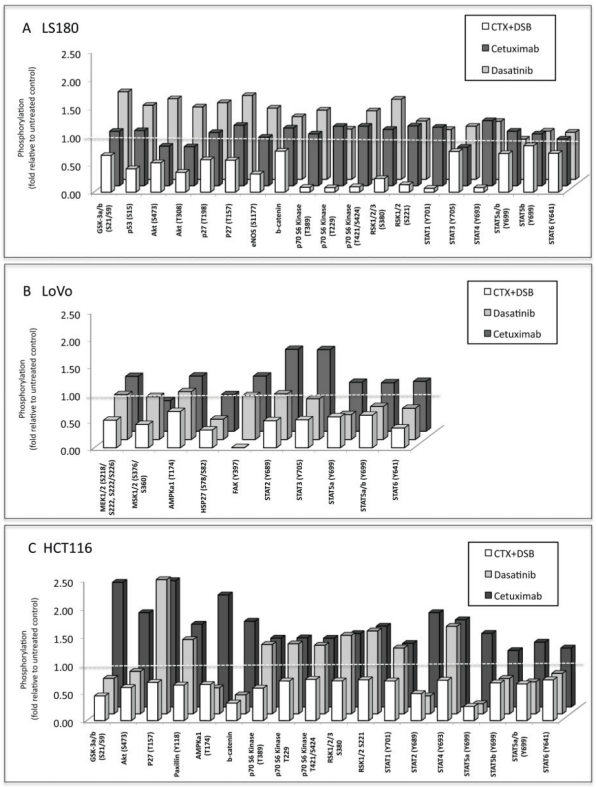

Figure 3. Human Phospho-Kinase array in KRAS mutant LS180, LoVo and HCT116 cells lines

A) AKT/mTOR/p70 S6 kinase pathway (AKT and p70 S6 Kinase), MAPK/RSK (RSK1/2/3), components of the β-catenin pathway (GSK α/β and β-catenin) and STAT family members were downregulated by the combination of dasatinib and cetuximab in LS180 cells. **B)** Members of the MAPK signaling pathway were downregulated with the combination of dasatinib and cetuximab in LoVo cells. **C)** AKT/mTOR/p70 S6 kinase pathway (AKT and p70 S6 Kinase), MAPK/RSK (RSK1/2/3), components of the β-catenin pathway (GSK α/β and β-catenin) and STAT family members were downregulated by the combination of dasatinib and cetuximab in HCT116 cells. After treatment with cetuximab, dasatinib and combination of cetuximab and dasatinib, cells were collected and cell extracts were incubated with membrane containing antibodies to 39 individual proteins. The membranes were washed and incubated with a cocktail of biotinylated detection antibodies, streptavidin-HRP and chemiluminescent detection reagents to measure the levels of phosphorylated protein. Quantitation of phosphorylated protein was completed using scanned images from ImageJ software. Data points are represented as mean of duplicate.

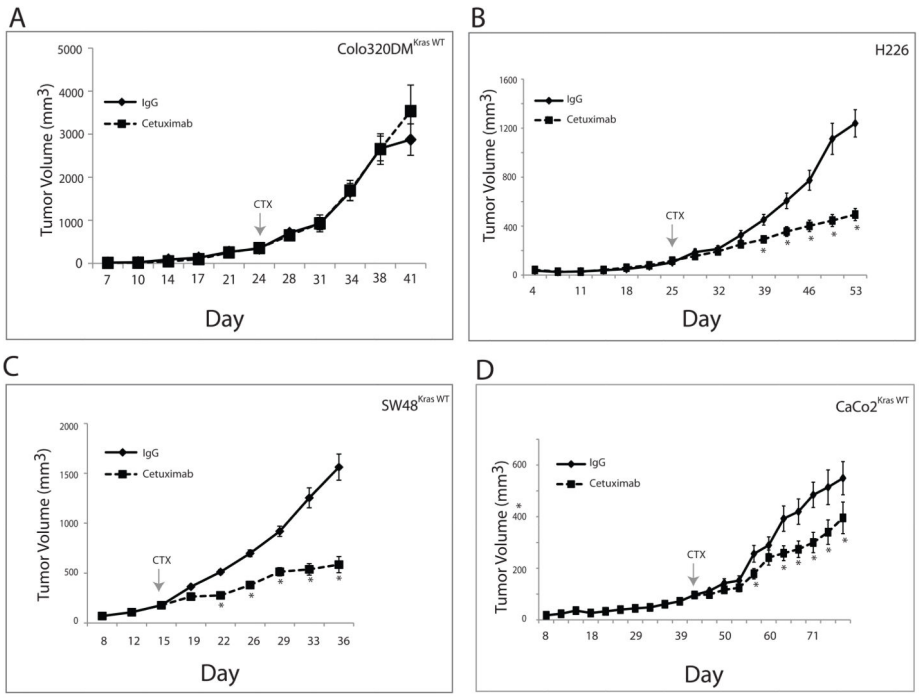

Figure 4. KRAS wild type colorectal tumors are sensitive to cetuximab *in vivo*

For the following series, all mice were randomized to cetuximab or IgG treatments. All mice received 0.3 mg of their respective treatment intraperitoneally twice weekly. **A)** A non-EGFR expressing line (Colo320DM) was used as a negative control randomizing 20 mice to cetuximab and 20 mice to IgG treatment. Mice received 3 weeks of treatment. **B)** A known-sensitive non-small cell lung cancer line (H226) was utilized as a positive control randomizing 10 mice to cetuximab and 10 mice to IgG treatment. Mice received 4.5 weeks of treatment**. C and D)** Mice (n=20) were inoculated with a KRAS wild type line (SW48 and CaCo2, respectively) and randomized to cetuximab or IgG treatment. SW48 mice received 3.5 weeks of treatment. CaCo2 mice received 5.5 weeks of treatment. Statistical significance is denoted by * ($P \leq 0.001$).

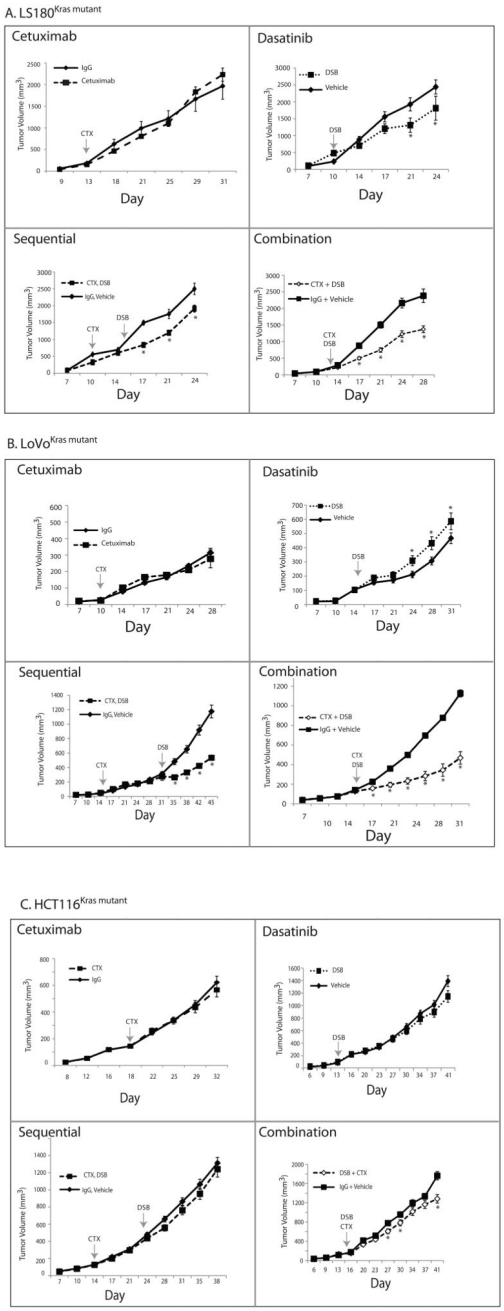

Figure 5. Dasatinib sensitizes KRAS mutant colorectal tumors to cetuximab

For the following series of experiments, all mice were randomized to treatment or control groups and treated with the following doses: cetuximab or IgG – 0.3 mg/kg intraperitoneally twice per week; dasatinib – 70 mg/kg by oral gavage five days a week. The same doses were used for sequential and combinatorial experiments. Statistical significance is denoted by * (P≤0.001). (**A, B, C upper left panel**) Cetuximab response was tested by inoculating mice with a KRAS mutant line (LS180, LoVo, and HCT116, respectively) and randomizing to cetuximab or IgG. LS180 mice received 3 weeks of treatment. LoVo mice received 3 weeks of treatment. HCT116 received 2.5 weeks of treatment. **(A, B, C upper right panel)**

Dasatinib response was tested by inoculating mice with a KRAS mutant line and randomizing to dasatinib or vehicle treatment. LS180 mice received 2.5 weeks of treatment. LoVo mice received 3 weeks of treatment. HCT116 mice received 4.5 weeks of treatment. **(A, B, C lower left panel)** Sequential treatment response of cetuximab then dasatinib was tested by inoculating mice with a KRAS mutant line and randomizing to cetuximab followed by dasatinib or IgG followed by vehicle. Cetuximab was ceased and dasatinib was started the next day once tumors displayed resistance. LS180 mice received one week of cetuximab/IgG and 2.5 weeks of dasatinib/vehicle. LoVo mice received 3 weeks of cetuximab/IgG and 2.5 weeks of dasatinib/vehicle. HCT116 mice received 2 weeks of cetuximab/IgG and 2.5 weeks of dasatinib/vehicle. **(A, B, C lower right panel)** Concomitant treatment response of cetuximab and dasatinib was tested by inoculating mice with a KRAS mutant line and randomizing to cetuximab plus dasatinib or IgG plus vehicle. LS180 mice received 2.5 weeks of concomitant treatment. LoVo mice received 3.5 weeks of concomitant treatment. HCT116 mice received 4 weeks of concomitant treatment.

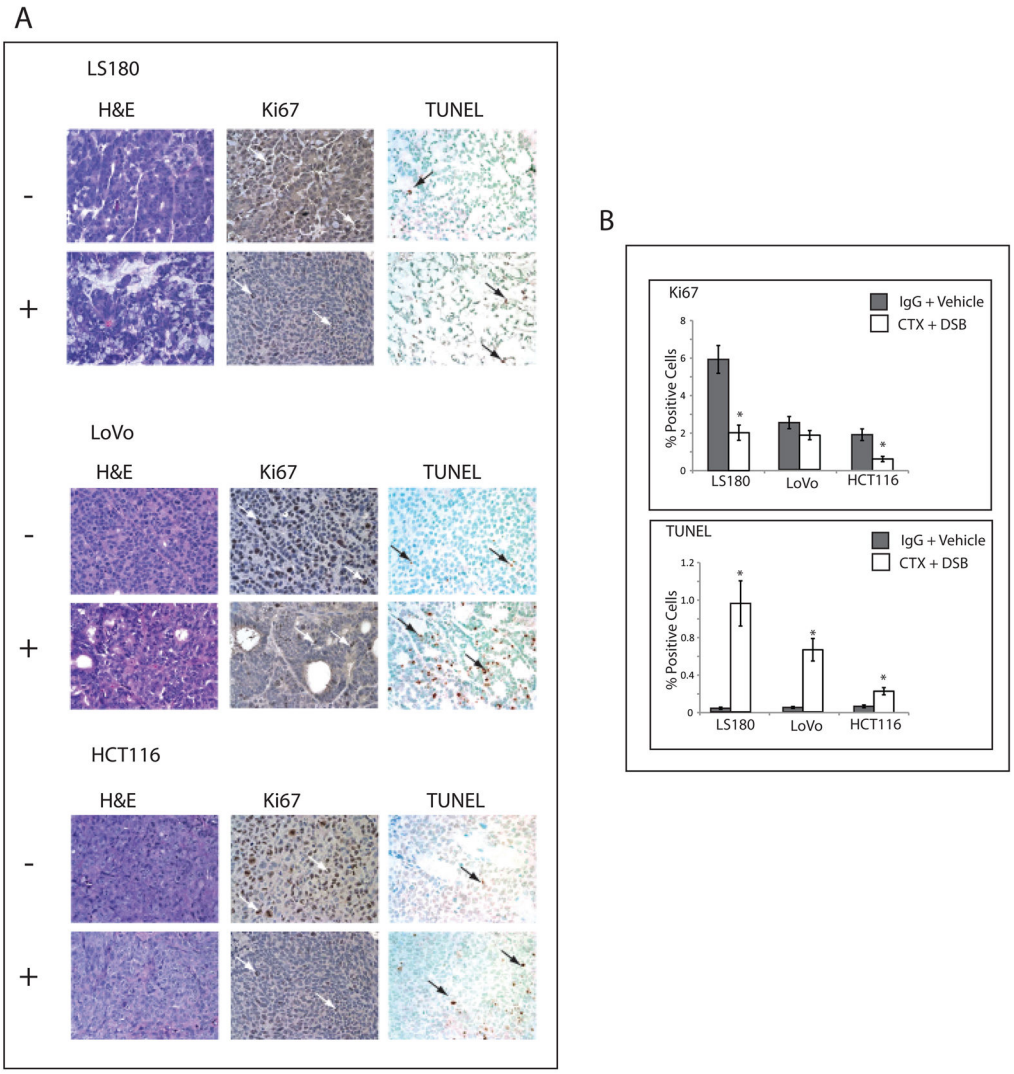

Figure 6. Combinatorial cetuximab and dasatinib treatments decrease proliferation and enhance apoptosis

A) Tumor samples from LS180, LoVo and HCT116 *in vivo* experiments were prepared and analyzed for proliferation (Ki67) and apoptosis (TUNEL). (−) denotes combinatorial IgG and vehicle control and (+) denotes combinatorial cetuximab plus dasatinib treatment. All representative samples are tumors collected three hours after the final dasatinib or vehicle treatment and 24 hours after the final cetuximab or IgG treatment. White arrows denote positive staining nuclei in Ki67 samples. Black arrows denote positive staining on TUNEL assay samples. Hematoxylin eosin stained section (magnification 400×). **B)** Quantitation of immunohistochemistry positive staining for Ki67 and TUNEL in combinatorial treatments. Graph of the Ki67 (upper) and TUNEL (lower) expression as percent of positive cells (5 random fields, 4 sections for each sample, * P≤0.05). Concomitant treatment samples were obtained from mice euthanized at 3, 12, and 24 hour time points after the last dasatinib or vehicle treatment and 24, 27, 36, and 48 hours after the last cetuximab or IgG treatment.

ImageJ was used to quantify the positive staining. CTX, cetuximab; DSB, dasatinib; IgG, immunoglobulin G,