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ORIGINAL RESEARCH ARTICLE

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Neratinib augments the lethality of [regorafenib + sildenafil]

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Abstract

Regorafenib is approved for the treatment of colorectal cancer and hepatocellular carcinoma. In the trial NCT02466802, we have discovered that regorafenib can be safely combined with the phosphodiesterase 5 inhibitor sildenafil in advanced solid tumor patients. The present studies determined whether the approved ERBB1/2/4 and RAS downregulating drug neratinib, could enhance the lethality of [regorafenib + sildenafil]. Neratinib enhanced [regorafenib + sildenafil] lethality in a greater than additive fashion in colon cancer cells. The drug combination reduced the expression of mutant K-RAS and of multiple histone deacetylase (HDAC) proteins that required autophagosome formation. It caused green fluorescent protein or red fluorescent protein–tagged forms of K-RAS V12 to localize into large intracellular vesicles. Compared with [regorafenib + sildenafil], the three-drug combination caused greater and more prolonged activation of the ATM-AMPK-ULK-1 pathway and caused a greater suppression and prolonged inactivation of mammalian target of rapamycin, AKT, and p70 S6K. Approximately 70% of enhanced lethality caused by neratinib required ataxia-telangiectasia-mutated (ATM)–AMP-dependent protein kinase (AMPK) signaling whereas knockdown of Beclin1, ATG5, FADD, and CD95 completely prevented the elevated killing effect. Exposure of cells to [regorafenib + sildenafil] reduced the expression of the checkpoint immunotherapy biomarkers programmed death-ligand 1, ornithine decarboxylase, and indoleamine 2,3-dioxygenase-1 and increased the expression of major histocompatibility complex A (MHCA), which also required autophagosome formation. Knockdown of specific HDAC proteins recapitulated the effects observed using chemical agents. In vivo, using mouse cancer models, neratinib significantly enhanced the antitumor efficacy of [regorafenib + sildenafil]. Our data support performing a new three drug Phase I trial combining regorafenib, sildenafil, and neratinib.

KEYWORDS
autophagy, DNA damage, HDAC

Abbreviations: AIF, apoptosis inducing factor; AMPK, AMP-dependent protein kinase; CMV, empty vector plasmid or virus; ER, endoplasmic reticulum; ERK, extracellular-regulated kinase; HDAC, histone deacetylase; MAPK, mitogen-activated protein kinase; mTOR, mammalian target of rapamycin; NER, neratinib; PDE5, phosphodiesterase 5; REG/REGO, regorafenib; RNS, reactive nitrogen species; STAT, signal transducers and activators of transcription; RNS, reactive nitrogen species; SCR, scrambled; SIL, sildenafil; siRNA, small interfering RNA; VEH, vehicle.

1 | INTRODUCTION

The drug regorafenib (Stivarga®), a multikinase and chaperone inhibitor, was originally approved for the treatment of colon

cancer and more recently for hepatocellular carcinoma (Carr et al., 2013; Røed Skårderud, Polk, Kjeldgaard Vistisen, Larsen, & Nielsen, 2018). In 2017 the drug neratinib (Nerlynx®), an irreversible inhibitor of ERBB1/2/4, was approved by the Food and Drug Administration as an adjuvant therapy alongside the antiERBB2 antibody herceptin in ERBB2+ breast cancers (Echavarria, López-Tarruella, Márquez-Rodas, Jerez, & Martin, 2017). Sildenafil (Viagra®) is a phosphodiesterase 5 (PDE5) inhibitor and was approved to treat erectile dysfunction (Booth et al., 2014). Several years ago, we demonstrated that sildenafil and other PDE5 inhibitors could enhance the lethality of multikinase inhibitors such as sorafenib, regorafenib, and pazopanib (Booth, Albers, et al., 2016; Booth, Shuch, et al., 2016; Tavallai et al., 2015). One component of drug-combination lethality was the inhibition of chaperone function, which resulted in enhanced endoplasmic reticulum (ER) signaling, reduced expression of protective proteins such as myeloid cell leukemia sequence 1 (MCL-1) and B-cell lymphoma-extra large (BCL-XL), and the induction of death receptor signaling in parallel with formation of toxic autophagosomes. Based on our preclinical data, a Phase I trial was initiated in collaboration with Bayer combining regorafenib and sildenafil in advanced solid tumors (NCT02466802). This trial is still recruiting patients at the highest dose level, with no DLTs at present observed.

Our work in 2017 with the ERBB1/2/4 inhibitor neratinib has shown that this agent, when compared with other ERBB receptor inhibitors for example, gefitinib, erlotinib, lapatinib, afatinib, cannot only inhibit the catalytic activity of ERBB1/2/4 but also can cause receptor internalization and degradation, requiring autolysosomes and cathepsin B (Booth, Roberts, Poklepovic, Avogadri-Connors, et al., 2017). Subsequent analyses demonstrated that other receptor tyrosine kinases, for example, c-MET, potentially associated with ERBB receptors in quaternary signaling complexes on the cell surface, could also be downregulated, although the mechanisms of downregulation of ERBB1 and c-MET were not identical. Building upon this concept, we discovered that neratinib, especially neratinib combined with an histone deacetylase (HDAC) inhibitor, caused the rapid autolysosomal-dependent degradation of mutant K-RAS and mutant N-RAS proteins (Booth, Albers, et al., 2017; Booth, Roberts, Rais, et al., 2017; Booth et al., 2018).

In vivo studies combining [regorafenib + sildenafil] revealed via multiplex assays on tumor tissue and plasma, that the levels of plasma fibroblast growth factor (FGF), and ERBB1 and AKT phosphorylation in tumor cells, were biomarkers that were associated with lower levels of cell killing by the [regorafenib + sildenafil] combination (Tavallai et al., 2015). In further studies, the ERBB1/2/4 inhibitor lapatinib significantly enhanced the antitumor effect of [regorafenib + sildenafil] in vivo; the ERBB1/2/4 inhibitor afatinib enhanced the antitumor effects of (sorafenib + sildenafil; Booth, Albers, et al., 2016). The present studies were performed to determine whether neratinib enhanced the lethality of [regorafenib + sildenafil] in gastrointestinal (GI) tumors, particularly colorectal carcinomas. Our findings reveal that neratinib significantly enhanced the antitumor efficacy of [regorafenib + sildenafil] in vitro and in vivo. Collectively our data support performing a new three drug Phase I trial in colorectal cancer patients combining regorafenib, sildenafil, and neratinib.

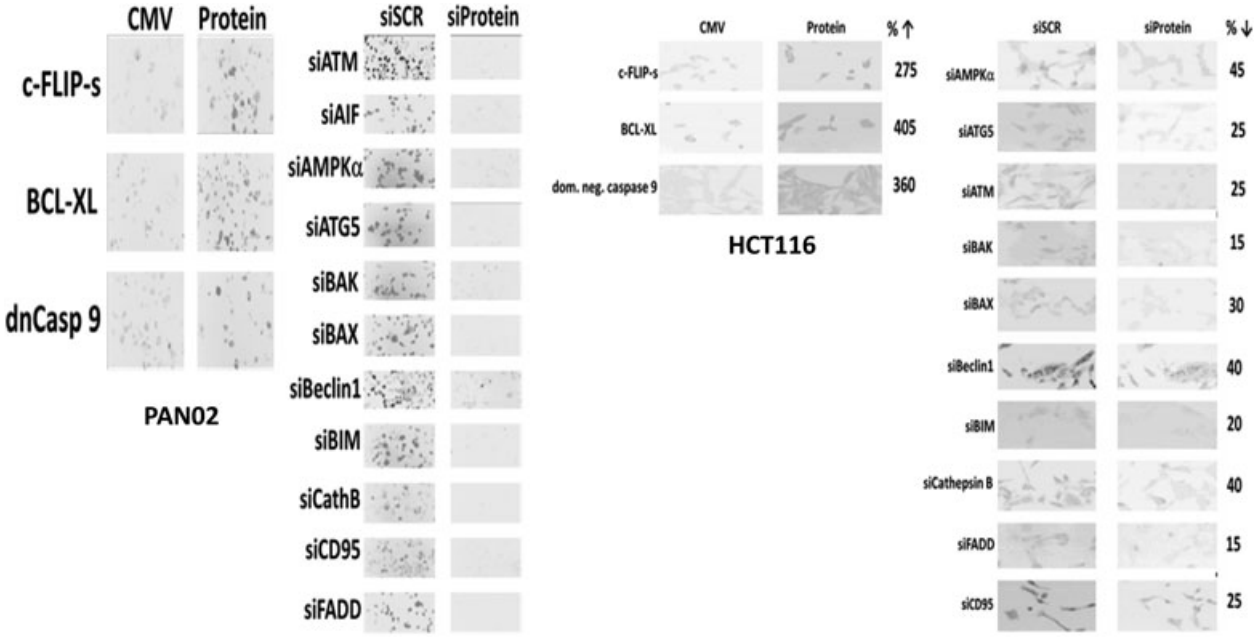

FIGURE 1 Representative images of proteins knocked down or overexpressed in the present studies. The data are from PAN02 (mouse) cells and HCT116 (human) cells. AIF: apoptosis inducing factor; AMPK: AMP-dependent protein kinase; ATM: ataxia-telangiectasia-mutated; BAX: BCL2-associated X; BAK: BCL2-antagonist/killer; BCL-XL: B-cell lymphoma-extra large; c-FLIP: cellular FLICE inhibitory protein; CMV: empty vector plasmid or virus

2 | MATERIALS AND METHODS

2.1 | Materials

Regorafenib and sildenafil were from Selleckchem (Houston, TX). Neratinib was supplied by Puma Biotechnology Inc. (Los Angeles, CA). Cell culture materials were purchased from GIBCOBRL Life Technologies (Grand Island, NY). Established cell lines were purchased from the American Tissue Type Culture Collection (Bethesda, MD). The adenosine diphosphate (ATP) assay kit (MAK190-1KT) was from Sigma-Aldrich (St. Louis, MO). The green fluorescent protein (GFP) and red fluorescent protein (RFP)-tagged K-RAS V12 plasmids were provided by Dr. Hancock. Commercially available validated short hairpin RNA molecules to knockdown RNA and protein levels were from Qiagen (Valencia, CA; Figure 1). Reagents and the performance

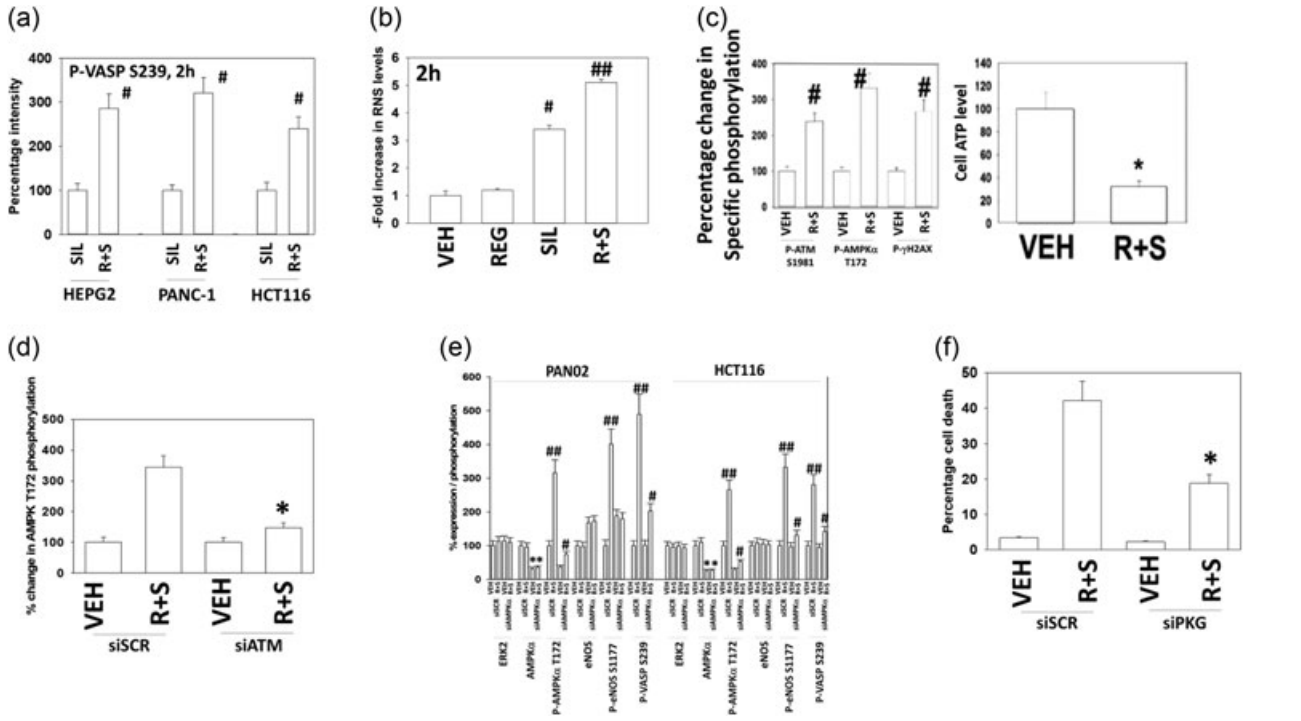

FIGURE 2 [Regorafenib + sildenafil] causes concerted signaling to activate toxic PKG signaling. (a) GI tumor cells were treated with vehicle control, regorafenib (0.5 μM), sildenafil (2.0 μM), or the drugs in combination for 2 hr. Cells were fixed in place and immunostaining performed to determine the staining intensity for VASP-1 S239. At least 40 cells per condition were imaged in independent triplicate and plotted as a percentage of sildenafil treatment; no staining for VASP-1 S239 was observed in control treated or regorafenib treated cells ($n = 3$; mean ± *SD*). $^{\#}p < 0.05$, greater than sildenafil value. (b) HEPG2 cells were loaded with DAF-FM DA and were then treated with vehicle control, regorafenib (0.5 μM), sildenafil (2.0 μM), or the drugs in combination for 2 hr. Data are expressed as a fold increase in RNS levels compared with those in vehicle control treated cells ($n = 3$; mean ± *SEM*). $^{\#}p < 0.05$, greater than vehicle control value; $^{\#\#}p < 0.05$, greater than sildenafil value. (c) Left: HEPG2 cells were treated with vehicle control or with [regorafenib (0.5 μM) + sildenafil (2.0 μM)] in combination for 2 hr. Cells were fixed in place and immunostaining performed to determine the staining intensity for total ATM, P-ATM S1981, total AMPKα, P-AMPKα T172, total γH2AX, and P-γH2AX. At least 40 cells per condition were imaged in independent triplicate and plotted as a percentage of vehicle treatment ($n = 3$; mean ± *SD*). $^{\#}p < 0.05$, greater than vehicle control value; right: HEPG2 cells were treated with vehicle control or with [regorafenib (0.5 μM) + sildenafil (2.0 μM)] in combination for 2 hr. The levels of ATP in treated cells were determined according to manufacturer's instructions ($n = 3$; mean ± *SD*). $^{*}p < 0.05$, less than vehicle control value. (d) PANC1 cells were transfected with a scrambled siRNA (siSCR) or an siRNA to knockdown expression of ATM. Twenty-four hours later, cells were treated with vehicle control or with [regorafenib (0.5 μM) + sildenafil (2.0 μM)] in combination for 2 hr. Cells were fixed in place and immunostaining performed to determine the staining intensity for total AMPKα and P-AMPKα T172. At least 40 cells per condition were imaged in independent triplicate and plotted as a percentage of vehicle treatment ($n = 3$; mean ± *SD*). $^{*}p < 0.05$, less than corresponding value in siSCR.(e) HCT116 (colorectal) and PAN02 (pancreatic cancer cells were transfected with a scrambled siRNA (siSCR) or an siRNA to knockdown expression of AMPKα. Twenty-four hours later, cells were treated with vehicle control or with [regorafenib (0.5 μM) + sildenafil (2.0 μM)] in combination for 2 hr. Cells were fixed in place and immunostaining performed to determine the staining intensity for P-eNOS S1177 and P-VASP-1 S239. At least 40 cells per condition were imaged in independent triplicate and plotted as a percentage of vehicle treatment ($n = 3$; mean ± *SD*). $^{\#}p < 0.05$, greater than vehicle control value; $^{\#\#}p < 0.05$, greater than corresponding value in siAMPKα cells. (f) Cells were transfected with a scrambled siRNA (siSCR) or an siRNA to knockdown expression of PKGI and PKGII. Twenty-four hours later, cells were treated with vehicle control or with [regorafenib (0.5 μM) + sildenafil (2.0 μM)] in combination for 24 hr. Cells were isolated and viability determined by trypan blue exclusion ($n = 3$; mean ± *SD*). $^{*}p < 0.05$, less than corresponding siSCR value. AMPK: AMP-dependent protein kinase; ATM: ataxia-telangiectasia-mutated; eNOS: endothelial nitric oxide synthase; ERK: extracellular regulated kinase; GI: gastrointestinal; PKG: protein kinase G; RNS: reactive nitrogen species; siRNA: small interfering RNA

of experimental procedures were all as described in Booth and Albers, et al. (2016); Booth and Shuch, et al. (2016); Booth, Roberts, Poklepovic and Avogadri-Connors, et al. (2017); Booth, Roberts and Rais, et al. (2017); Booth et al. (2018); Tavallai et al., (2015).

2.2 | Methods

2.2.1 | Culture, transfection, and in vitro exposure of cells to drugs

All cell lines were cultured at 37°C (5% (vol/vol) CO_2) in vitro using RPMI supplemented with 5% (v/v) fetal calf serum and 10% (v/v) nonessential amino acids (Booth, Albers, et al., 2016; Booth, Shuch, et al., 2016; Booth, Roberts, Poklepovic, & Avogadri-Connors et al., 2017; Booth, Roberts, & Rais et al., 2017; Booth et al., 2018; Tavallai et al., 2015). Cells were transfected with small interfering RNA (siRNA) molecules or plasmids as described in prior manuscripts (Booth, Albers, et al., 2016; Booth, Shuch, et al., 2016; Booth, Roberts, Poklepovic, & Avogadri-Connors, etal., 2017; Booth, Roberts, & Rais, et al., 2017; Booth et al., 2018; Tavallai et al., 2015).

Detection of cell viability, protein expression, and protein phosphorylation by immunofluorescence using a Hermes WiScan machine (Idea Biotechnologies, Revohot, Israel) (http://www.idea-bio.com/; Booth, Albers, et al., 2016; Booth, Shuch, et al., 2016; Booth, Roberts, Poklepovic, & Avogadri-Connors, et al., 2017; Booth, Roberts, & Rais, et al., 2017; Booth et al., 2018; Tavallai et al., 2015). Live and dead assays used a live–dead reagent (Thermo Fisher Scientific, Waltham, MA) and we visualized cells at ×10 magnification. Green cells = viable; yellow/red cells = dying/dead. For immunofluorescence studies, cells were visualized at either ×10 or ×60. All immunofluorescent images for each individual protein and phosphoprotein were taken using the identical machine settings. Images were processed at 9999 dpi using Adobe Photoshop (Adobe Systems Inc., San Jose, CA), and figures generated in Microsoft PowerPoint (Redmond, WA) (Booth, Albers, et al., 2016; Booth, Shuch, et al., 2016; Booth, Roberts, Poklepovic, & Avogadri-Connors, al., 2017; Booth, Roberts, & Rais, et al., 2017; Booth et al., 2018; Tavallai et al., 2015).

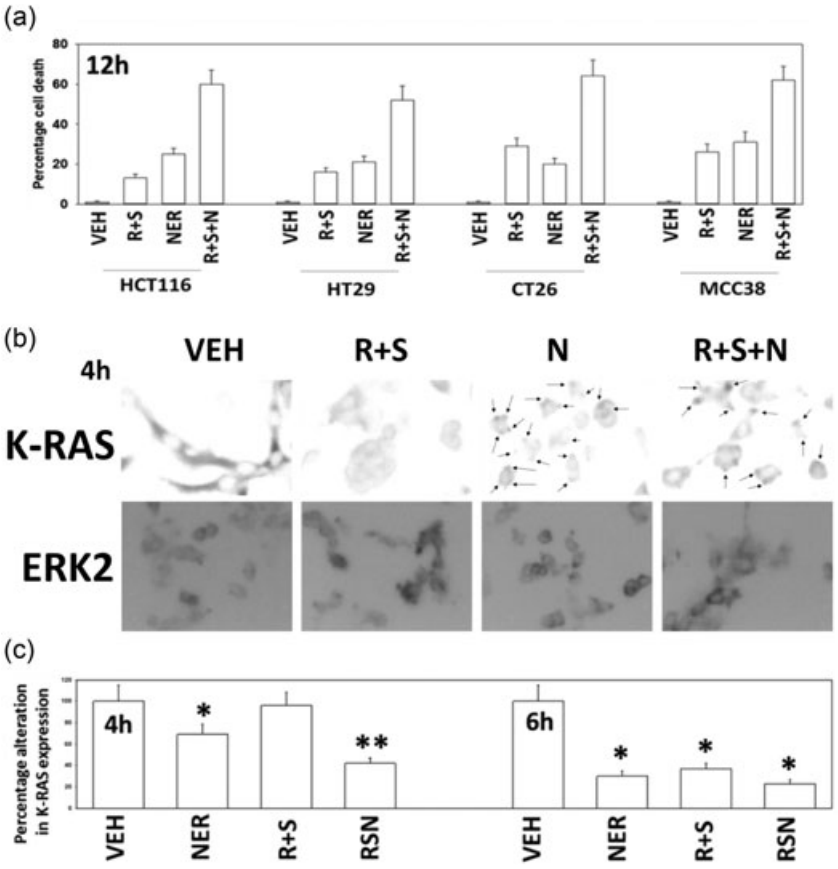

FIGURE 3 Neratinib enhances [regorafenib + sildenafil] lethality, which correlates with enhanced K-RAS degradation and inactivation of AKT, mTOR, p70 S6K, and ERK1/2. (a) Tumor cells (HCT116, HT29: human colorectal cancer; CT26, MCC38: mouse colorectal cancer) were treated with vehicle control, [regorafenib (0.5 μM) + sildenafil (2.0 μM)], neratinib (50 nM), or the drugs in combination for 12 hr. Cells were then isolated, and viability determined by trypan blue exclusion assay ($n = 3 \pm$ SD). (b) CT26 cells were treated with vehicle control, [regorafenib (0.5 μM) + sildenafil (2.0 μM)], neratinib (50 nM), or the drugs in combination for 4 hr. Cells were fixed in place at each time point, and immunofluorescent staining performed to determine the total expression of K-RAS and total expression of ERK2. At least 40 cells per condition were imaged in independent triplicate and the intensity ratio of K-RAS levels to total ERK2 expression plotted as a percentage of control treatment ($n = 3 \pm$ SD). *$p < 0.05$, less than vehicle control; **$p < 0.05$, less than neratinib alone value. Upper images: K -RAS protein become localized in punctate bodies 4 hr after exposure to neratinib (teal arrows). ERK: extracellular regulated kinase; mTOR: mammalian target of rapamycin

2.2.2 | Analysis of reactive nitrogen species (RNS) levels

RNS levels were determined in a vector 3 plate reader (PerkinElmer Life, Waltham, MA and Analytical Sciences). Cancer cells were loaded for 30 min with 3-amino,4-aminomethyl-2′,7′-difluorescein (DAF-FM DA, 4 μM) which is sensitive to oxidation by nitric oxide (NO). Cells were treated with vehicle control, regorafenib, and/or sildenafil and fluorescence measured after 2 hr. Data are presented corrected for basal fluorescence of vehicle-treated cells.

2.2.3 | Assessment of autophagy

Cells were transfected with a plasmid to express a GFP and RFP tagged form of LC3 (ATG8). GFP punctae detect autophagosome formation and RFP punctae detect autolysosome formation (Booth, Albers, et al., 2016; Booth, Shuch, et al., 2016; Booth, Roberts, Poklepovic, & Avogadri-Connors, et al., 2017; Booth, Roberts, & Rais, et al., 2017; Booth et al., 2018; Tavallai et al., 2015).

2.4 | Animal studies

Studies were performed per USDA regulations under VCU IACUC protocol AD20008. Female CT26 mouse colorectal and male PAN02 mouse pancreatic carcinoma cells (2×10^4) were implanted into rear flanks of female BALB/c and male C57/BL6 mice, respectively. Tumors were permitted to form until the mean tumor volume was ~40 mm^3. Animals were then segregated into groups with near identical mean volumes and the animals then treated for 3 days with the indicated therapeutic agents: Vehicle control (cremophore); regorafenib (20 mg/kg q.d. Days 1–3); regorafenib (20 mg/kg q.d. Days 1–3 and sildenafil (5 mg/kg q.d. Days 1–3); neratinib (15 mg/kg q.d. Days 1–3); or the three drugs in combination. Tumor volumes were measured before drug administration and every 3 days after the initiation of therapeutic interventions ($n = 10$ mice per group; mean ± *SEM*). Before, during and after drug treatment tumors are calipered as indicated in the Figure and tumor volume was assessed up to 24 days later. When the volume of the tumor reached >1,500 mm^3, animals were humanely killed, and the normal tissues removed for further hematoxylin and eosin (H&E) staining studies.

2.5 | Transfection

Cells were transfected with plasmids to express GFP-K-RAS V12 and RFP-K-RAS V12 (0.1 μg) using Lipofectamine 2000 (Thermo-Fisher, Waltham, MA). Twenty-four hours after transfection, cells were used in assays examining their staining for GFP and RFP.

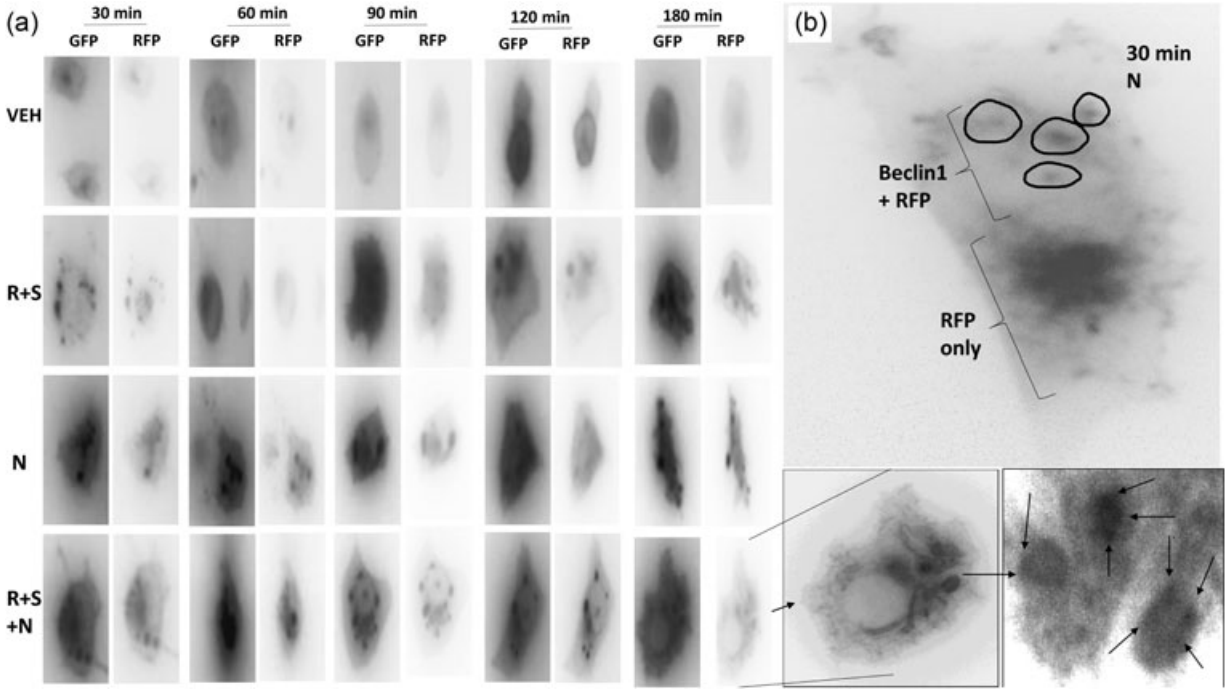

FIGURE 4 Neratinib and [regorafenib + sildenafil] both cause the rapid intracellular vesicularization of GFP-K-RAS V12 and RFP-K-RAS V12. (a) PANC1 cells were cotransfected with plasmids to express GFP-K-RAS V12 and RFP-K-RAS V12. Twenty-four hours later, cells were treated with vehicle control, [regorafenib (0.5 μM) + sildenafil (2.0 μM)], neratinib (50 nM), or the drugs in combination for 30, 60, 90, 120, and 180 min. Live cells were imaged at each time-point at ×60 magnification. The images presented are representative of three independent experiments. (b) In parallel to the studies in (a), cells were fixed in paraformaldehyde after 30 min, that preserves the GFP and RFP fluorescence. Cells were immunostained to detect the expression of Beclin1 and images taken at ×60 magnification. The image presented is representative of three independent experiments. GFP: green fluorescent protein; RFP: red fluorescent protein

2.6 | Data analysis

Comparison of the effects of various treatments (in triplicate three times) was using one-way analysis of variance and a two-tailed Student's *t* test. Statistical examination of in vivo animal survival data utilized a two-tailed Student's *t* test and log rank statistical analyses between the different treatment groups. Differences with a $p < 0.05$ were considered statistically significant. Experiments are the means ± *SEM* of multiple individual points from multiple experiments.

3 | RESULTS

Sildenafil, via inhibition of PDE5, enhances cyclic guanosine monophosphate (cGMP) levels and thus protein kinase G (PKG) activity. Sildenafil rapidly promoted the phosphorylation of a well-recognized PKG substrate, VASP-1 S239 (Figure 2a). Regorafenib as a single agent did not alter VASP-1 S239 phosphorylation, however, [regorafenib + sildenafil] caused a greater amount of VASP-1 S239 phosphorylation than sildenafil alone, implying that regorafenib further enhanced PKG activation in the presence of cGMP. In parallel to enhancing PKG activity, the addition of regorafenib to sildenafil also enhanced the production of NO in tumor cells (Figure 2b). Exposure of tumor cells to [regorafenib + sildenafil] increased the phosphorylation of γH2AX, ATM S1981, and AMPK T172 and reduced cellular ATP levels, which collectively imply that the AMP-dependent protein kinase (AMPK) was being activated via phosphorylation and allosteric mechanisms (Figure 2c). Knockdown of ataxia-telangiectasia-mutated (ATM) significantly reduced [regorafenib + sildenafil]-induced phosphorylation of AMPKα T172 (Figure 2d). The AMPK has been shown by other groups to phosphorylate endothelial nitric oxide synthase (eNOS) at serine 1177; elevated S1177 phosphorylation was associated with higher eNOS catalytic activity and the production of NO (Zhang et al., 2009). In an AMPK-dependent fashion, [regorafenib + sildenafil] enhanced eNOS S1177 phosphorylation (Figure 2e). In cells with AMPKα knockdown, regorafenib was significantly less capable at promoting sildenafil-dependent phosphorylation of VASP-1 S239 (Figure 2e). Combined knockdown of PKGI and PKGII abolished the ability of regorafenib and sildenafil to cause enhanced tumor cell killing (Figure 2f). Collectively the data in Figure 2 demonstrates that regorafenib and sildenafil interact to generate NO, which activates cytosolic guanylate cyclase that enhances cGMP levels, and in parallel activates PKG by blocking PDE5 function, thereby preventing cGMP degradation.

In prior studies, based on our data showing that [regorafenib + sildenafil] treated tumors had stable high ERBB1 phosphorylation, expressed more FGF and had higher AKT activity we determined that inhibition of ERBB1/2 signaling using the first-generation drug lapatinib enhanced [regorafenib + sildenafil] lethality in vitro and in vivo (Tavallai et al., 2015). Low clinically relevant concentrations of regorafenib, sildenafil, and the novel irreversible ERBB1/2/4 inhibitor neratinib interacted in a greater than additive fashion to kill human and mouse colorectal cancer cells (Figure 3a). The three-drug combination enhanced the rate of mutant K-RAS downregulation in CT26 cells when compared with neratinib alone (Figure 3b). Note the arrows showing the mutant K-RAS in neratinib treated CT26 cells is localizing in punctate intensely staining bodies. PANC1 cells were cotransfected with two plasmids, to express K-RAS V12-GFP and the other to express K-RAS V12-RFP; with the expectation that both tagged forms of K-RAS V12 would, at least at early time points, colocalize within the cells (Cho et al., 2016). Over a 3-hr time-course, [regorafenib + sildenafil] or neratinib alone caused the formation of small and large intracellular vesicles, respectively (Figure 4a). The GFP and RFP tagged forms of K-RAS V12 colocalized arguing that the tag was not responsible for the vesicularization effect. Note that by 120–180 min, in cells treated with [regorafenib + sildenafil + neratinib], that cells had flattened outward, and that not only were large vesicles present that contained GFP-K-RAS V12 and RFP-K-RAS V12, but that within those vesicles intense punctate staining was present

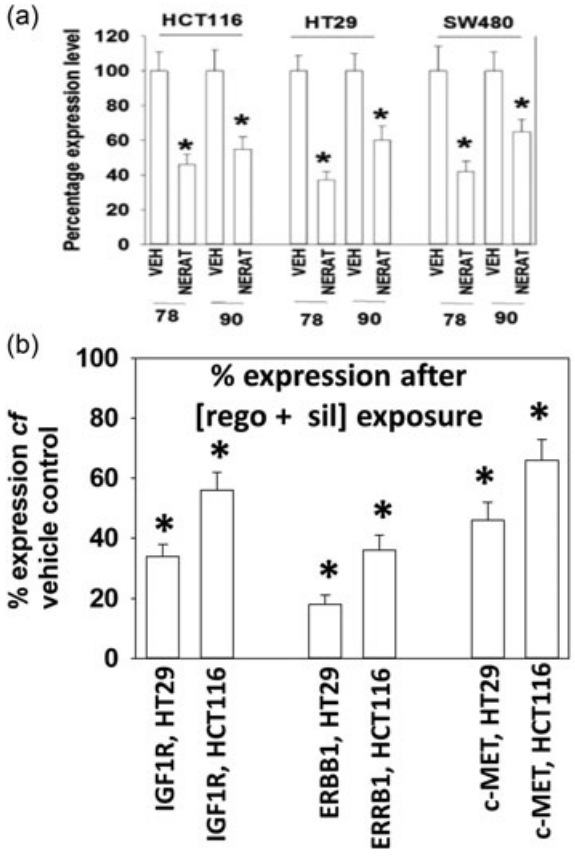

FIGURE 5 Neratinib downregulates the expression of chaperone proteins. (a) HCT116, SW480 and HT29 cells were treated with vehicle control or with neratinib (50 nM). Cells were fixed in place after 6 hr and immunostaining against the chaperones COOH-termini performed to determine the total expression of GRP78 and HSP90. ($n = 3$). *$p < 0.05$, less than vehicle control value; mean ± *SEM*. (b) HCT116 colon cancer cells were treated with vehicle control or with [regorafenib (0.5 μM) + sildenafil (2.0 μM)] for 6 hr. Cells were fixed in place and immunostaining performed to determine the expression of the indicated growth factor receptors. ($n = 3$). *$p < 0.05$, less than vehicle control value; mean ± *SEM*

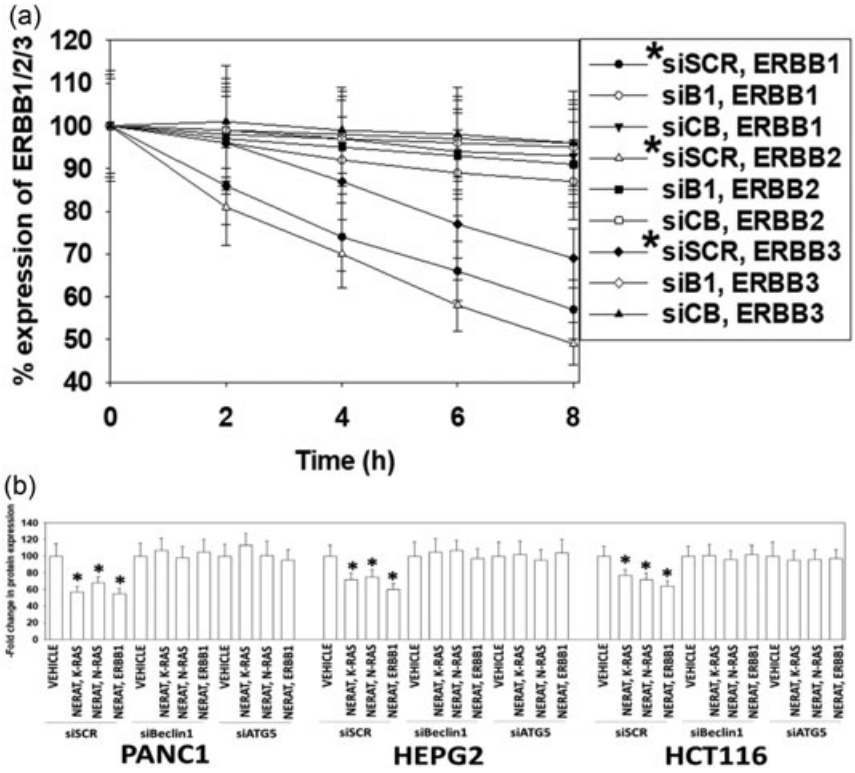

FIGURE 6 Neratinib downregulates the expression of ERBB1, K-RAS, and N-RAS via autophagy. (a) HCT116 colorectal tumor cells were transfected with a scrambled siRNA, with an siRNA to knockdown the autophagy regulatory protein Beclin1, or with an siRNA to knockdown the lysosomal protease cathepsin B. After 24 hr, the expression of Beclin1 or of cathepsin B had been reduced by >75% compared with scrambled control. Twenty-four hours after transfection, cells were treated with vehicle control or with neratinib (50 nM) for 0–8 hr. Vehicle control exposure did not alter receptor basal expression. Cells were fixed in place at the indicated time points and immunofluorescence staining performed to determine the total expression of ERBB1, ERBB2, ERBB3. Cells were visualized in the Hermes WiScan instrument at ×10 magnification. The intensity of 40 cells per condition was determined using the machine's own software combined with data from another two wells of separately treated cells, and with background fluorescence subtracted. Secondary antibody alone staining did not generate any signal. $^*p < 0.05$, less than vehicle control; the data are the mean fluorescence from 120 data points from three separate drug exposures; mean ± *SEM*). (b) PANC1 (mut. K-RAS), HEPG2 (mut. N-RAS), and HCT116 (mut. K-RAS) cells were transfected with a scrambled siRNA control molecule or siRNA molecules to knockdown the expression of Beclin1 or ATG5. Twenty-four hours after transfection cells were treated with vehicle control or neratinib (50 nM) for 6 hr. Cells were fixed in place at the indicated time points and immunofluorescence staining performed to determine the total expression of ERBB1, K-RAS, and N-RAS. Cells were visualized in the Hermes WiScan instrument at ×10 magnification. The staining intensity of 40 cells per condition was determined using the machine's own software combined with data from another two wells of separately treated cells, and with background fluorescence subtracted. Secondary antibody alone staining did not generate any signal. $^*p < 0.05$, less than vehicle control; the data are the mean fluorescence from 120 data points from three separate drug exposures; mean ± *SEM*). siRNA: small interfering RNA

for GFP/RFP suggestive that K-RAS even within a vesicle was being concentrated at specific sites. In fixed cells, without quenching endogenous GFP/RFP staining, GFP/RFP staining largely did not colocalize with the autophagosome regulatory protein Beclin1, though some vesicles, circled, were double positive staining (Figure 4b).

Neratinib as a single agent rapidly reduced the protein levels of HSP90 and GRP78 (Figure 5a). Growth factor receptors, chaperoned by HSP90, also had their protein levels rapidly reduced after [regorafenib + sildenafil] exposure (Figure 5b; Tavallai et al., 2015). Others have previously noted that sildenafil can cause the translocation of mutant K-RAS from the plasma membrane into intracellular vesicles, where it is primed for degradation (Cho et al., 2016). Within 8 hr of exposure, neratinib was capable of significantly reducing the expression of ERBB1, ERBB2, and ERBB3 in colon cancer tumor cells (Figure 6a). These effects were significantly reduced by knockdown of either Beclin1 or of cathepsin B. Reduced expression of ERBB1, as well as K-RAS and N-RAS, in these cells was blocked by knockdown of the autophagy regulatory proteins Beclin1 or ATG5 (Figure 6b).

We then determined the impact of neratinib exposure on changes in protein phosphorylation caused by [regorafenib + sildenafil]. Neratinib interacted with [regorafenib + sildenafil] to promote additional phosphorylation of eIF2α, ATM, AMPK, ULK-1, S317 and ATG13 (Figure 7a). The drugs interacted to further reduce phosphorylation of mammalian target of rapamycin (mTOR), AKT, p70 S6K, and extracellular regulated kinase (ERK)1/2. The drugs combined to promote a more prolonged inactivation of AKT, p70

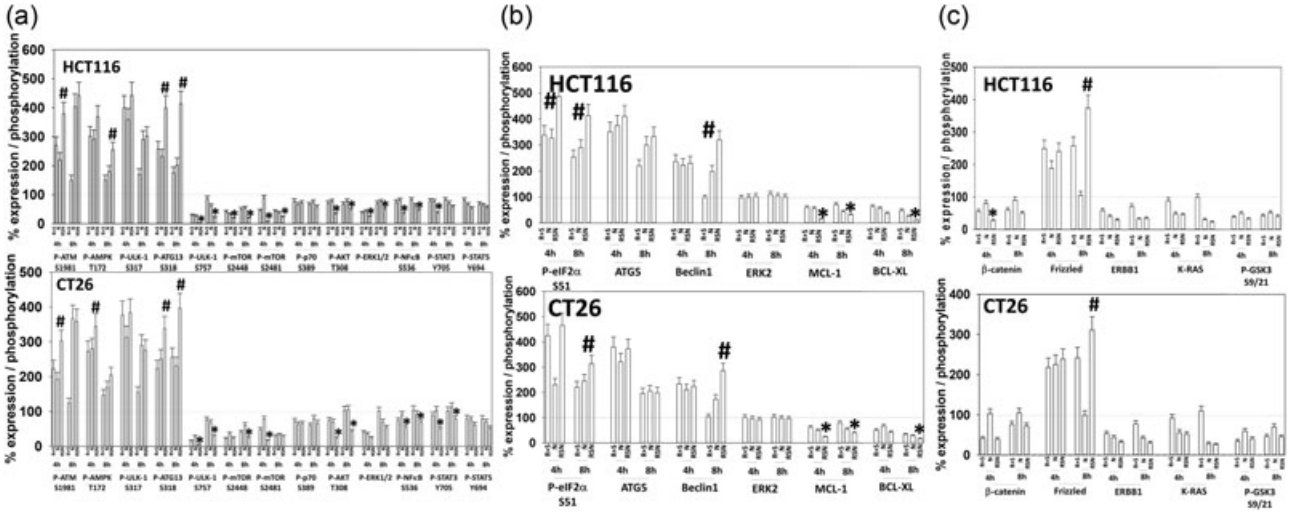

FIGURE 7 Neratinib prolongs and enhances the [regorafenib + sildenafil]-induced inactivation of mTOR that is associated with increased expression of ATG5 and Beclin1, and decreased expression of MCL-1 and BCL-XL. (a–c) CT26 (mouse) and HCT116 (human) colon cancer cells were treated with vehicle control, [regorafenib (0.5 μM) + sildenafil (2.0 μM)], neratinib (50 nM), or the drugs in combination for 4 and 8 hr. Cells were fixed in place at each time point, and immunofluorescent staining performed to determine the total expression of ERK2, and the changes in protein phosphorylation and protein expression of the indicated proteins. At least 40 cells per condition were imaged in independent triplicate and the intensity ratio of phosphorylation/expression levels to total ERK2 expression determined ($n = 3$; mean ± *SD*). $^{*}p < 0.05$, less than values in either individual treatment; $^{\#}p < 0.05$, than values in either individual treatment. AMPK: AMP-dependent protein kinase; ATM: ataxia-telangiectasia-mutated; BCL-XL: B-cell lymphoma-extra large; ERK: extracellular regulated kinase; MCL-1: myeloid cell leukemia sequence 1; mTOR: mammalian target of rapamycin; NF-κB: nuclear factor κB

S6K, mTOR, and ERK1/2. Our prior studies with [regorafenib + sildenafil], as well as with neratinib had linked their abilities to cause ER stress signaling via eIF2α to enhance Beclin1 and ATG5 expression and to reduce MCL-1 and BCL-XL levels. In agreement with our eIF2α phosphorylation data, the three-drug combination significantly enhanced ATG5 and Beclin1 expression and decreased the levels of MCL-1 and BCL-XL (Figure 7b). Increased levels of ATG13 phosphorylation and increased expression imply autophagosome formation was occurring, which was confirmed using an LC3-GFP-RFP construct (not shown; Booth, Albers, et al., 2016; Booth, Shuch, et al., 2016; Booth, Roberts, Poklepovic and Avogadri-Connors, et al., 2017; Booth, Roberts and Rais, et al., 2017; Booth et al., 2018).

The protein β-catenin has been shown to facilitate colon cancer growth, and is a target of the adenomatous polyposis coli (APC) protein; a gene frequently mutated inactive in colon cancer (Fiedler, Mendoza-Topaz, Rutherford, Mieszczanek, & Bienz, 2011; Gottardi & Peifer, 2008; Kimelman & Xu, 2006; Liu, Xing, Hinds, Zheng, & Xu, 2006; MacDonald, Tamai, & He, 2009). APC plays an essential role in the formation of the β-catenin destruction complex in which β-catenin is eventually ubiquitinated and directed toward degradation via the proteasome (Markowitz & Bertagnolli, 2009). To undergo degradation, β-catenin must also be phosphorylated by glycogen synthase kinase (GSK3). GSK3 is phosphorylated by AKT, which results in GSK3 inactivation that in the instance of insulin signaling, leads to increased activity of glycogen synthase (Cross, Alessi, Cohen, Andjelkovich, & Hemmings, 1995). We have previously noted that insulin can reduce GSK3 activity in rabbit skeletal muscle in vivo, following animal starvation and administration of insulin and propranolol (unpublished data; Dent et al., 1990). Treatment of colon cancer cells with [regorafenib + sildenafil] reduced the expression of β-catenin, ERBB1, and K-RAS, and alone or in combination with neratinib reduced the phosphorylation of AKT T308 and GSK3 S9/S21 (Figure 7c). The levels of Frizzled were increased. The dephosphorylation and activation of GSK3 correlated with the reduced expression of β-catenin. Lower levels of β-catenin, ERBB1, and K-RAS would predict for reduced tumor growth and viability.

We next determined the molecular mechanisms by which neratinib enhanced [regorafenib + sildenafil] lethality. Knockdown of ATM or AMPKα reduced the ability of neratinib to enhance [regorafenib + sildenafil] lethality by ~70% (Figure 8a). However, knockdown of CD95, FADD, ATG5, Beclin1, or the lysosomal protease cathepsin B abolished the ability of neratinib to enhance killing by [regorafenib + sildenafil]. Knockdown of the toxic BH3 domain proteins BAX or BAK or knockdown of the apoptosis inducing factor (AIF), also largely abolished the neratinib potentiating effect. Thus, the three-drug combination promotes greater signaling from CD95 and greater toxic autophagosome formation that results in greater lysosomal dysfunction and release of cathepsin B, in parallel with increased mitochondrial dysfunction and release of AIF. Additional studies were then performed to assess the effects of expressing either a caspase 8/10 inhibitor, cellular FLICE inhibitory protein (c-FLIP)-s, the mitochondrial protective protein BCL-XL, or a dominant negative form of caspase 9. Overexpression of c-FLIP-s or of dominant negative caspase 9 significantly reduced the ability of neratinib to enhance [regorafenib + sildenafil] killing (Figure 8b). BCL-XL abolished the ability of neratinib to enhance

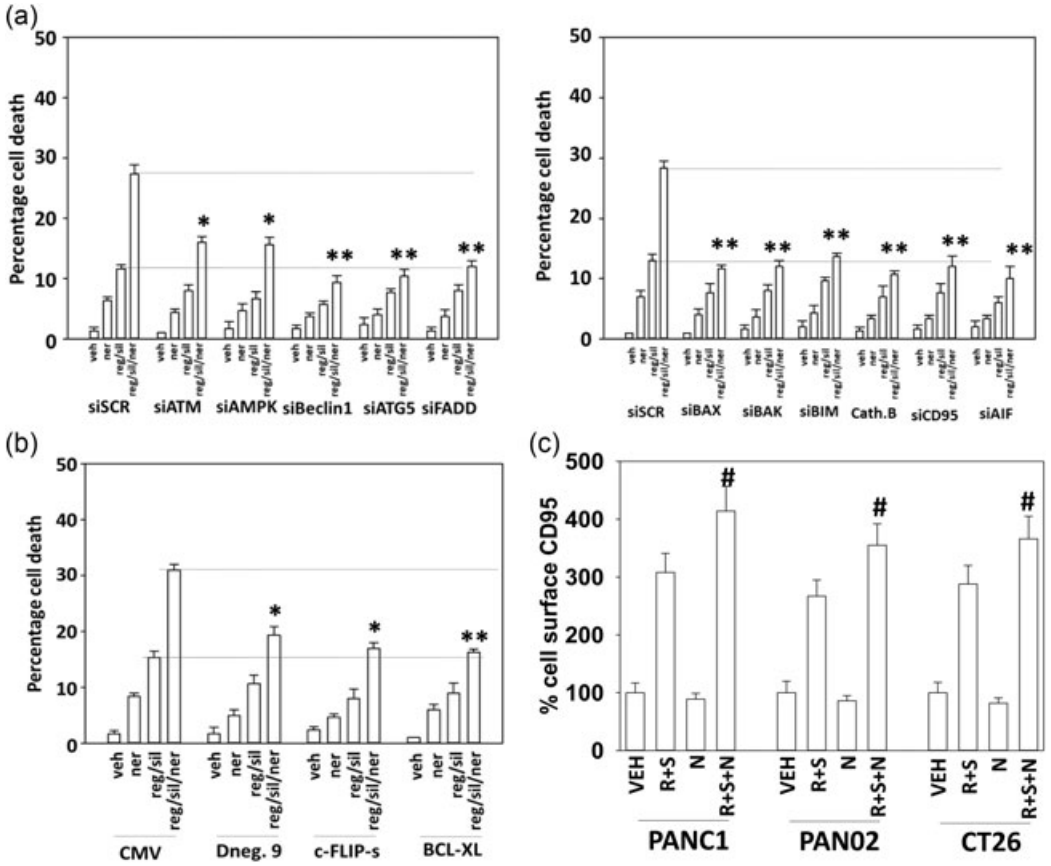

FIGURE 8 Death receptor signaling and autophagosome formation are essential for neratinib to enhance [regorafenib + sildenafil] lethality. (a) CT26 cells were transfected with a scrambled control siRNA (siSCR) or were transfected to knockdown the expression of the indicated proteins (Figure 7 for knockdown images). Twenty-four hours after transfection cells were treated with vehicle control, [regorafenib (0.5 μM) + sildenafil (2.0 μM)], neratinib [50 nM], or the drugs in combination for 12 hr. Cells were then isolated, and viability determined by trypan blue exclusion assay ($n = 3$; mean ± *SD*). $^{*}p < 0.05$, greater than reg/sil value in siSCR cells; $^{**}p > 0.05$, greater than [regorafenib + sildenafil] value in siSCR cells. (b) CT26 cells were transfected with an empty vector control plasmid (CMV) or were transfected with plasmids to express the indicated proteins: c-FLIP-s; BCL-XL; dominant negative caspase 9 (Figure 7 for expression images). Twenty-four hours after transfection cells were treated with vehicle control, [regorafenib (0.5 μM) + sildenafil (2.0 μM)], neratinib (50 nM), or the drugs in combination for 12 hr. Cells were then isolated, and viability determined by trypan blue exclusion assay ($n = 3$; mean ± *SD*). $^{*}p < 0.05$, greater than reg/sil value in siSCR cells; $^{**}p > 0.05$, greater than reg/sil value in siSCR cells. (c) PANC1, PAN02, and CT26 cells were treated with vehicle control, [regorafenib (0.5 μM) + sildenafil (2.0 μM)], neratinib (50 nM), or the drugs in combination for 3 hr. Cells were fixed in place without membrane permeabilization. Immunofluorescent staining performed to determine the total plasma membrane levels of CD95. At least 40 cells per condition were imaged in independent triplicate ($n = 3$; mean ± *SD*). $^{\#}p < 0.05$, greater than values in [regorafenib + sildenafil] treatment. AIF: apoptosis inducing factor; AMPK: AMP-dependent protein kinase; ATM: ataxia-telangiectasia-mutated; BAX: BCL2-associated X; BAK: BCL2-antagonist/killer; BCL-XL: B-cell lymphoma-extra large; c-FLIP: cellular FLICE inhibitory protein; c-FLIP: cellular FLICE inhibitory protein

killing. We have previously reported that [regorafenib + sildenafil] can increase the plasma membrane levels of the death receptor CD95, which is a biomarker for CD95 activation. Neratinib in a nonsignificant fashion modestly reduced cell surface levels of CD95 (Figure 8c). However, neratinib significantly enhanced the ability of [regorafenib + sildenafil] to increase CD95 plasma membrane levels. This data reaffirms that death receptor signaling, and mitochondrial dysfunction, are key mechanistic elements in three-drug combination lethality. The data also support the hypothesis that tumor cell killing proceeds predominantly through necroptotic mechanisms rather than apoptotic pathways.

In prior studies we have demonstrated that high levels of autophagosome formation can act to reduce the protein levels of multiple HDAC proteins (Booth, Roberts, Poklepovic, & Dent, 2017; Booth, Roberts, Poklepovic, Gordon, & Dent, 2017). Exposure of CT26 cells to [regorafenib + sildenafil + neratinib] reduced the expression of HDAC1 and HDACs4-11 but did not change the levels of HDAC2 and HDAC3 (Figure 9A). In PAN02 cells, a mouse pancreatic tumor cell line that also expresses a mutant K-RAS protein, [regorafenib + sildenafil] and more so [regorafenib + sildenafil + neratinib] reduced the expression of HDACs1-11 (Figure 9b). Knockdown of the autophagy regulatory proteins Beclin1 or ATG5 prevented any of the drug treatments of reducing HDAC levels. Very similar data were obtained using PANC1 human pancreatic cancer cells (Figure 9c). Thus, our three-drug combination not only kills tumor cells, but also rapidly alters

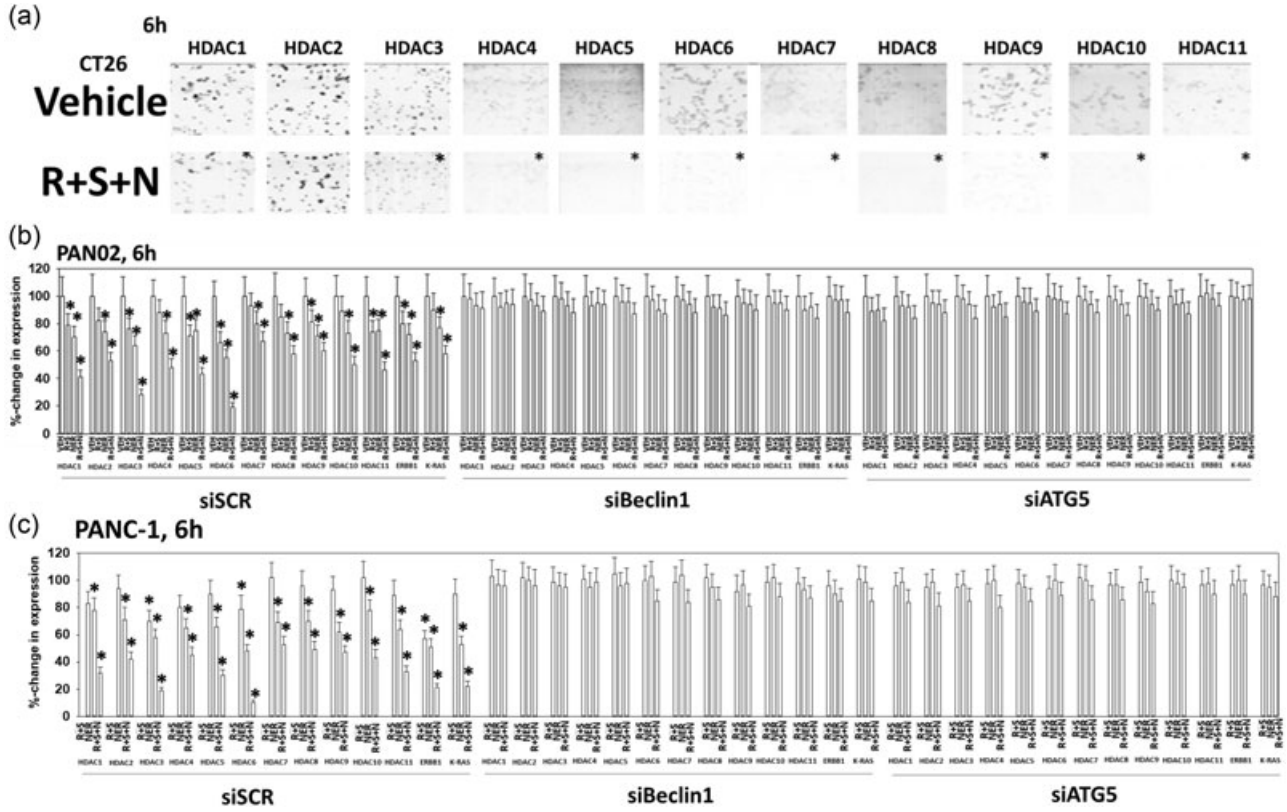

FIGURE 9 Exposure of GI tumor cells to [regorafenib + sildenafil + neratinib] reduces the expression of multiple HDAC proteins, that requires autophagosome formation. (a) CT26 cells were treated with vehicle control or with [regorafenib (0.5 μM) + sildenafil (2.0 μM) + neratinib (50 nM)] for 6 hr. Cells were fixed in place at each time point, and immunofluorescent staining performed to determine the total expression of HDACs1–11. At least 40 cells per condition were imaged in independent triplicate ($n = 3$; mean ± *SD*). $^*p < 0.05$, less than vehicle control. (b,c) PAN02 mouse and PANC1 human pancreatic cancer cells were transfected with a scrambled control siRNA (siSCR) or with siRNA molecules to knockdown the expression of ATG5 or Beclin1. Twenty-four hours after transfection cells were treated with vehicle control, [regorafenib (0.5 μM) + sildenafil (2.0 μM)], neratinib (50 nM), or the drugs in combination for 6 hr. Cells were fixed in place and immunofluorescent staining performed to determine the changes in HDAC protein expression of the indicated HDAC proteins. At least 40 cells per condition were imaged in independent triplicate ($n = 3$; mean ± *SD*). $^*p < 0.05$, less than vehicle control. GI: gastrointestinal; HDAC: histone deacetylase; siRNA: small interfering RNA

the expression of HDAC proteins, which will impact on gene function and protein stability.

One group of proteins we have studied over the past year are those that regulate the immunogenicity of tumor cells and that could potentially alter the sensitivity of tumor cells to checkpoint inhibitory antibodies. Exposure of PAN02 cells or HCT116 and HT29 cells to [regorafenib + sildenafil] or to [regorafenib + sildenafil + neratinib] reduced the expression of programmed death-ligand 1 (PD-L1), PD-L2, and indoleamine-pyrrole 2,3-dioxygenase (IDO-1; Figure 10A). The drug combinations enhanced the expression of major histocompatibility complex A (MHCA). The ability of the drug combinations to modulate protein expression required the formation of autophagosomes, as judged by the dependence of protein expression changes on the presence of Beclin1 or ATG5 (Figure 10b). Knockdown of HDAC proteins, alone or in combination, were able to recapitulate the effects of drug exposure on the levels of PD-L1, PD-L2, MHCA, IDO-1, and ornithine decarboxylase (ODC). Collectively, the data in Figures 8-10 argue that the [regorafenib + sildenafil + neratinib] drug combination, via autophagy-dependent regulation of HDAC levels, leads to altered transcription and protein expression of proteins that regulate tumor cell immunogenicity.

We wished to determine whether neratinib enhanced [regorafenib + sildenafil] lethality in tumor model systems. CT26 colorectal and PAN02 pancreatic cancer cells, that both express a mutated oncogenic K-RAS protein, were implanted into the rear flanks of syngeneic female BALB/c and male C57 black mice, respectively. Tumors formed and were then treated for 3 days with vehicle control or the drugs alone or in combination (Figures 11a,b). Neratinib significantly enhanced the ability of [regorafenib + sildenafil] to reduce tumor growth. In CT26 tumors, exposure to regorafenib reduced tumor growth by 4%; exposure to [regorafenib + sildenafil] by 12%; and neratinib alone by 10%. Combined exposure to all three drugs reduced tumor growth by 66%. This is significantly greater than the effects of neratinib and [regorafenib + sildenafil] combined. In PAN02 tumors, exposure to [regorafenib + sildenafil] reduced tumor growth by 34%; and

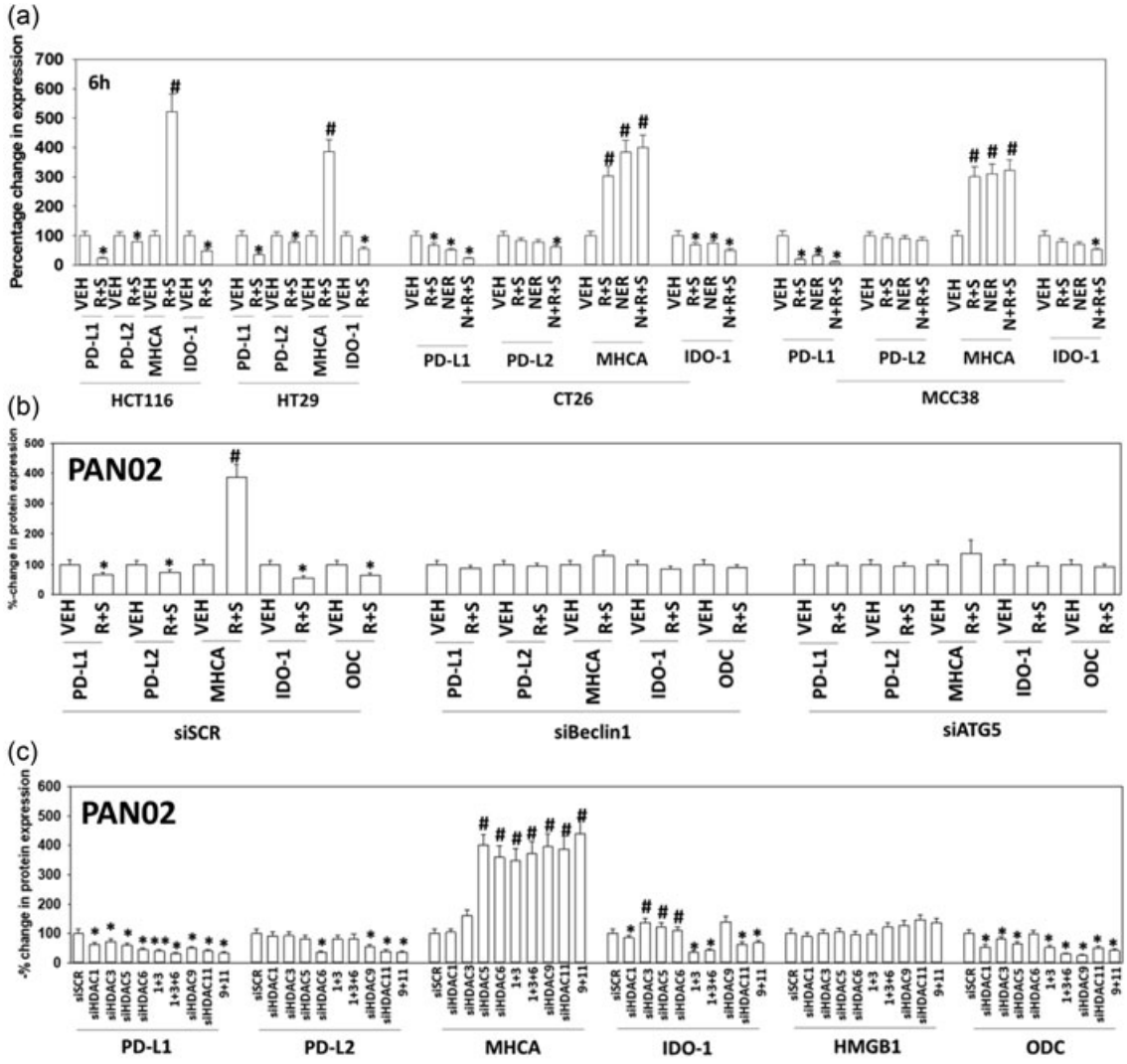

FIGURE 10 Altered expression of HDAC proteins is mechanistically linked to altered expression of immunotherapy biomarker proteins. (a) Colon cancer cells were treated with vehicle control or, as indicated, [regorafenib (0.5 μM) + sildenafil (2.0 μM)], neratinib (50 nM), or [regorafenib + sildenafil + neratinib] for 6 hr. Cells were fixed in place and immunofluorescence staining performed to determine the expression of PD-L1, PD-L2, MHCA, and IDO-1. At least 40 cells per condition were imaged in independent triplicate ($n = 3$; mean ± *SD*). $^{*}p < 0.05$, less than vehicle control; $^{\#}p < 0.05$, greater than vehicle control. (b) PAN02 cells were transfected with a scrambled control siRNA (siSCR) or with siRNA molecules to knockdown the expression of ATG5 or Beclin1. Twenty-four hours after transfection cells were treated with vehicle control, [regorafenib (0.5 μM) + sildenafil (2.0 μM)], neratinib (50 nM), or the drugs in combination for 6 hr. Cells were fixed in place and immunofluorescent staining performed to determine the changes in PD-L1, PD-L2, MHCA, IDO-1, and ODC expression. At least 40 cells per condition were imaged in independent triplicate ($n = 3$; mean ± *SD*). $^{*}p < 0.05$, less than vehicle control; $^{\#}p < 0.05$, greater than vehicle control. (c) PAN02 cells were transfected with a scrambled control siRNA (siSCR) or with siRNA molecules to knockdown the expression of HDACs 1, 3, 5, 6, 9, and 11, alone or in combination. Twenty-four hours after transfection cells were fixed in place and immunofluorescent staining performed to determine the changes in PD-L1, PD-L2, MHCA, IDO-1, and ODC expression. At least 40 cells per condition were imaged in independent triplicate ($n = 3$; mean ± *SD*). $^{*}p < 0.05$, less than vehicle control; $^{\#}p < 0.05$, greater than vehicle control. HDAC: histone deacetylase; IDO-1: indoleamine 2,3-dioxygenase-1; MHCA: major histocompatibility complex A; PD-L1: programmed death-ligand 1

neratinib alone by 52%. Combined exposure to all three drugs reduced tumor growth by 81%. This is significantly greater than the effects of neratinib and [regorafenib + sildenafil] combined. The 3-day exposure of animals to [regorafenib + sildenafil + neratinib] did not alter animal body mass or cause any changes in normal mouse behavior. No obvious normal tissue toxicities were observed in the drug-exposed mice, as judged by H&E staining (Figure 11c).

4 | DISCUSSION

The present studies were performed to determine whether neratinib enhanced the ability of [regorafenib + sildenafil] to kill tumor cells. Our data demonstrated that neratinib, in a greater than additive fashion, enhanced [regorafenib + sildenafil] lethality. The elevated levels of killing caused by neratinib were mediated through both increased toxic autophagosome formation and increased death

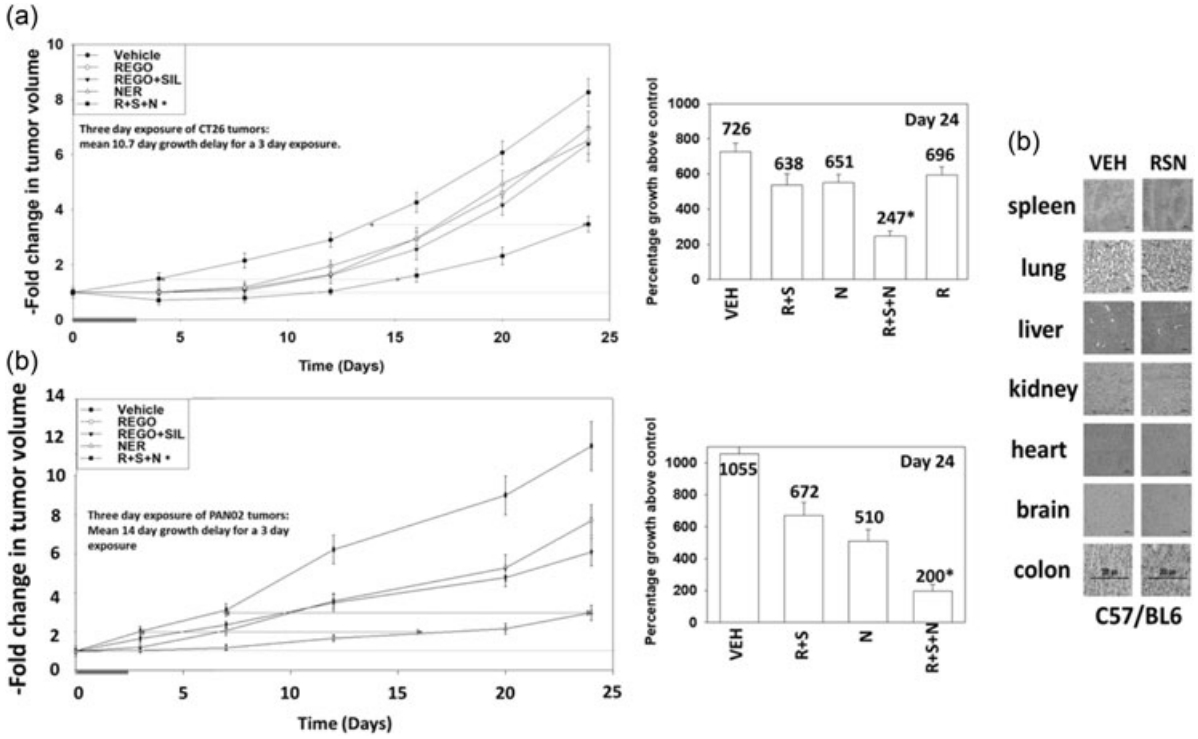

FIGURE 11 Neratinib enhances the ability of [regorafenib + sildenafil] to suppress the growth of colorectal tumors. (a,b) Female-derived CT26 mouse colorectal and male-derived PAN02 mouse pancreatic carcinoma cells (2×10^4) were implanted into rear flanks of female BALB/c and male C57/BL6 mice, respectively. Tumors were permitted to form until the mean tumor volume was ~40 mm^3. Animals were then segregated into groups with near identical mean volumes and the animals then treated for 3 days with the indicated therapeutic agents: Vehicle control (cremophore); regorafenib (20 mg/kg q.d. Days 1–3); regorafenib (20 mg/kg q.d. Days 1–3 and sildenafil (5 mg/kg q.d. Days 1–3); neratinib (15 mg/kg q.d. Days 1–3); or the three drugs in combination. Tumor volumes were measured before drug administration and every 3 days after the initiation of therapeutic interventions (n = 10 mice per group; mean ± *SEM*). Before, during and after drug treatment tumors are calipered as indicated in the Figure and tumor volume was assessed up to 24 days later. When the volume of the tumor reached >1,500 mm^3, animals were humanely killed. *$p < 0.05$, less growth than tumors treated with [regorafenib + sildenafil]. (c) Normal tissues were fixed in formalin on Day 24. Tissues were embedded in paraffin wax and five-micron sections taken. Sections were deparaffinized and H&E stained. Images of the normal tissues were taken at ×40 magnification. H&E: hematoxylin and eosin

receptor signaling. This resulted in lysosomal and mitochondrial dysfunction which in turn resulted in tumor cell execution by cathepsin B, AIF, and caspase 3. In vivo, neratinib enhanced [regorafenib + sildenafil] lethality against mouse CT26 and PAN02 tumors. Thus, our molecular and descriptive data support proposing a new Phase I trial in GI cancer patients combining the standard of care drug regorafenib with sildenafil and neratinib.

The CT26 and PAN02 cell lines, as is often found in colorectal and pancreatic tumors, expresses a mutated K-RAS protein. One "holy grail" of oncology medicine has been to develop drugs that could block signaling by mutated RAS proteins. And, at present, there is a major program at the National Cancer Institute (NCI) to develop RAS inhibitors. One limitation of directly attacking the RAS protein, however, is that all three major isoforms of RAS (K–, N–, H–) that can be mutated in cancer would each require subtly different agents to block their signaling processes. In several recent studies we have presented data arguing that neratinib, and particularly when neratinib was combined with HDAC inhibitors, could cause the proteolytic degradation of growth factor receptors and associated membrane signaling proteins, including mutated forms of K-RAS and of N-RAS (Booth, Roberts, Poklepovic and Avogadri-Connors, et al., 2017; Booth, Roberts and Rais, et al., 2017; Booth et al., 2018). Others have observed sildenafil downregulating K-RAS via PKG (Cho et al., 2016). In vitro data demonstrating neratinib's ability to downregulate K-RAS and N-RAS were recapitulated in vivo using a breast cancer model system. This indirect approach of RAS downregulation, that also lowers expression of cytoprotective growth factor receptors, will shortly move into Phase I evaluation at Massey Cancer Center in all qualifying solid tumor patients.

In the present manuscript, we discovered that neratinib and [regorafenib + sildenafil] interacted to reduce K-RAS expression, and that a 6 hr incubation of CT26 cells with [regorafenib + sildenafil] also caused the protein levels of K-RAS to decline. Confirmation that we were causing K-RAS V12 vesicularization was made using GFP and RFP tagged forms of the protein (Cho et al., 2016). Neratinib is an irreversible inhibitor, originally developed to block ERBB2 activity, but that was also shown to inactivate ERBB1 and ERBB4. That the related irreversible inhibitor afatinib, developed to block ERBB1, does not cause receptor degradation suggests that the chemical

modification of ERBB2, also the only family member not to bind a ligand, must play an elevated mechanistic role in the downregulation process. More unexpected was the observation that in a delayed fashion [regorafenib + sildenafil] exposure also reduced the levels of K-RAS. Regorafenib is an inhibitor of RAF family kinases as well as class III receptor tyrosine kinases such as PDGFRα/β. Several years ago, we presented evidence that when combined with PDE5 inhibitors such as sildenafil, regorafenib became a low-affinity inhibitor of HSP90 and HSP70 family chaperones, that correlated in GI tumor cells with reduced expression of multiple receptor tyrosine kinases (Booth, Shuch, et al., 2016). ERBB family receptor stabilities are in part controlled by HSP90 and HSP70 family chaperones, and some studies have suggested that RAS proteins are also chaperone clients (Qi et al., 2014). Thus, chaperone dysregulation could explain how [regorafenib + sildenafil] reduces RAS expression.

In prior work, including studies with neratinib, we have demonstrated a link between drug-induced autophagosome formation, the lysosomal downregulation of HDAC expression, and alterations in the protein levels of PD-L1, MHCA, ODC, and IDO-1. We were also cognascent of earlier data showing [regorafenib + sildenafil] also caused autophagosome formation. Exposure of tumor cells to [regorafenib + sildenafil + neratinib] through autophagic and HDAC-dependent mechanisms reduced the expression of PD-L1, PD-L2, ODC, and IDO-1, and increased the expression of MHCA. These changes in biomarker expression would predict for tumor cells to be more responsive to checkpoint inhibitory antibody immunotherapies that is we are turning colorectal tumors from being "cold" into being "hot." Studies beyond the scope of this manuscript will need to be performed to determine using mouse pancreatic and colorectal tumor models whether antibodies to inhibit PD-1 function or of CTLA4 function, in conjunction with [regorafenib + sildenafil + neratinib], can increase the antitumor actions of the immune system.

From our in vivo studies we demonstrated that neratinib enhanced the antitumor efficacy of [regorafenib + sildenafil] against mutant K-RAS expressing mouse CT26 colorectal tumor cells. The mice were exposed to drugs for 3 days, and the delay of tumor growth compared to vehicle control at Day 4 was 11.2 days, and at Day 24 was 10.2 days (mean 10.7). In animals exposed to the three-drug combination, tumor volumes decreased below baseline and did not return to baseline until 9 days after cessation of drug exposure. Further studies in other K-RAS mutant GI tumor cell lines, for example, PAN02, recapitulated our initial data. CT26 cells are female derived whereas PAN02 cells are male derived suggesting that our drug combination has antitumor effects regardless of sex. Based on H&E staining, no frank obvious damage was observed in normal tissues following drug combination exposure arguing that we can combine these agents for at least 3 days to achieve a nontoxic antitumor specific killing effect. Studies beyond the scope of this manuscript, using PDX colorectal and pancreatic tumor models will be required to fully validate our hypothesis.

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CONFLICTS OF INTEREST

The authors declare that there are conflicts of interest.

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