

# BRAF mutant colorectal cancer: ErbB2 expression levels as predictive factor for the response to combined BRAFi/ErbB1 inhibitors

Evelina Miele (✉ [evelina.miele@opbg.net](mailto:evelina.miele@opbg.net))

<https://orcid.org/0000-0002-4747-1032>

**Luana Abballe**

Universita degli Studi di Roma La Sapienza

**Gian Paolo Spinelli**

Universita degli Studi di Roma La Sapienza

**Besharat Zein Mersini**

Universita degli Studi di Roma La Sapienza

**Giuseppina Catanzaro**

Universita degli Studi di Roma La Sapienza

**Martina Chiacchiarini**

Universita degli Studi di Roma La Sapienza

**Alessandra Vacca**

Universita degli Studi di Roma La Sapienza

**Agnese Po**

Universita degli Studi di Roma La Sapienza

**Carlo Capalbo**

Universita degli Studi di Roma La Sapienza

**Elisabetta Ferretti**

Universita degli Studi di Roma La Sapienza

---

## Research article

**Keywords:** BRAFV600E mutation, colon cancer, molecular therapy, afatinib, vemurafenib, ErbB2

**Posted Date:** October 11th, 2019

**DOI:** <https://doi.org/10.21203/rs.2.15911/v1>

**License:**  This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

**Version of Record:** A version of this preprint was published on February 17th, 2020. See the published version at <https://doi.org/10.1186/s12885-020-6586-0>.

# Abstract

**Background:** Colorectal cancer (CRC) is a heterogeneous disease with a complex biology and has a wide number of altered genes such as BRAF, KRAS and PIK3CA. Advances with new-targeted therapies have been achieved and available treating options have prolonged patient's survival. However, BRAF-mutated CRC patients remain unresponsive to available therapies with RAF inhibitors (RAFi) alone or combined with ErbB inhibitors (ErbBi). These unmet needs require further exploitation of oncogenic signaling in order to set up individualized treatments.

**Methods:** To this end, we tested the efficacy of single agent or combined treatments using the BRAFi, vemurafenib and two different ErbBi: panitumumab and afatinib in CRC cells characterized by different molecular phenotypes.

**Results:** Combination strategies with BRAFi and ErbBi achieved a better response in BRAF V600E mutated cells expressing high levels of ErbB2.

**Conclusions:** In conclusion our findings allow us to indicate that high ErbB2 expression levels is a positive predictor factor since those patients are responsive to BRAFi/ErbBi combination.

## Background

Colorectal carcinoma (CRC) is the third leading cause of death from cancer worldwide [1]. Recent studies have shed light on the complex biology of CRC, which reflects its heterogeneous genetic background with alterations involving a variety of genes (e.g., TP53, EGFR, RAS, PIK3CA, BRAF, and PTEN). These efforts have provided fundamental information for the development of novel treatments targeting key signaling pathways in the tumor [2]. Advances made in the diagnosis and treatment of CRC have undoubtedly prolonged patient survival. However, unfavorable outcomes are still the rule for certain CRCs that are particularly aggressive [3,4]. Up to 10% of metastatic CRCs are BRAF V600E-mutated, and display a worse prognosis [3,5]. These tumors are relatively unresponsive to currently used chemotherapy protocols [6,7], and in these cases, second-line treatment with anti-epidermal growth factor receptor (EGFR)-antibodies is rarely beneficial [8,9,10].

BRAF (along with CRAF and ARAF) belongs to a family of genes that encode serine–threonine kinases, which heterodimerize and are activated by several receptor tyrosine kinases (RTKs) such as EGFR. Together with downstream MEK and ERK proteins, RAF kinases constitute a powerful mitogen-activated protein kinase (MAPK) pathway [3,4]. The BRAF mutations found in CRC usually occur at the V600 hotspot and lead to constitutive activation of BRAF<sup>V600E</sup>, which signals as a monomer in the absence of upstream signaling through the RAS signaling pathway. This explains at least in part the resistance of BRAF<sup>V600E</sup> CRCs to EGFR pathway blockade [11,3].

Selective inhibitors of RAF kinases, such as vemurafenib, regorafenib [12], and dabrafenib [13], have recently been introduced into clinical practice, and BRAF inhibitors have proved to be highly effective for

the treatment of BRAF-mutant melanoma. In contrast, when used alone, these drugs produce only minimal benefits in BRAF-mutant colorectal cancer [14] because they cause reactivation of RAS (via a feedback mechanism) [15,16,17] or activation of signaling through the PI3K/AKT pathway [18].

This issue has been addressed in several preclinical and clinical studies, which indicate that EGFR blockade and BRAF targeting, act synergically to inhibit ERK pathway signaling in BRAF<sup>V600E</sup> mutant colon cancers [3,16,19]. However, in a recent study, the efficacy of targeted therapies with RAF inhibitors or combinations with MEK and EGFR inhibitors were found to vary widely in cohorts of patients with BRAF<sup>V600E</sup> CRCs [20]. The therapeutic failure of BRAF inhibition in these cases might be related to other mutations and/or the activation of other signaling pathways. It is becoming increasingly clear that the biology of BRAF mutant patients varies widely and this heterogeneity must be taken into account for the development of effective targeted therapies [20].

In the study described below, we tested the efficacies of BRAF and EGFR inhibitors, alone and in combination, against BRAF<sup>V600E</sup> mutant colon cancer cell lines with different mutational profiles (Table 1). Based on the results that emerged, we propose use of these drugs in the treatment of patients with BRAF-mutant CRCs, based on the tumor's expression of ErbB2.

## Materials And Methods

### Cell lines and Reagents

SW-48 (EGFR mutant), Colo-205 (BRAF mutant, TP53 mutant, APC mutant) and HT-29 (BRAF mutant, PIK3CA mutant) human colorectal cancer cells (Table 1) were obtained from the American Type Culture Collection. Colo-205 was grown in RPMI-1640 (supplemented with 10% (v/v) fetal bovine serum, 1% (v/v) penicillin (50U/ml) - streptomycin (50U/ml) and 2mM L-glutamine) and the other ones in Dulbecco's Modified Eagle's Medium - high glucose D6429 (supplemented with 10% (v/v) fetal bovine serum, 1% (v/v) penicillin (50U/ml) - streptomycin (50U/ml) and 2mM L-glutamine) confirmed free of mycoplasma contamination by regular testing with PCR Mycoplasma Detection Kit (ABM, Cat. No. G238).

### Inhibitors

The selective BRAF inhibitor vemurafenib and the dual TKI inhibitor afatinib (BIBW2992) were both purchased from SelleckChem (Cat. No. S1267-10MM/1ML and Cat. No. S1011, respectively). Each was dissolved in DMSO to obtain a 10-mM stock solution. Panitumumab (Vectibix®, Amgen, Thousand Oaks, CA) was kindly provided by GPS in a stock solution of 20 mg/ml.

### Cell viability and Cell death assays

Cell viability was evaluated after 48 hours of treatment by MTS assay (CellTiter 96® Aqueous One Solution Reagent assay, Promega) and cell count by Trypan blue exclusion. Each sample was measured

in triplicate and repeated at least three times.

The dye trypan blue exclusion test was used to determine the number of viable or death cells present in a cell suspension (Warren Strober APPENDIX 3B Trypan Blue Exclusion Test of Cell Viability, Current Protocols in Immunology, 2001).

## Western blot assay

Protein lysates were prepared using RIPA buffer (50 mM Tris-HCl, pH 7.6, 0.5% deoxycholic acid sodium salt, 140 mM NaCl, 1% NP-40, 5 mM EDTA, 100 mM NaF, 2 mM Na pyrophosphate) with fresh protease inhibitors (Roche). Lysates were separated on 6% SDS polyacrylamide gels. Proteins were transferred to nitrocellulose membranes (0.45 micron) (PerkinElmer). Membranes were blocked for 1 h at room temperature in 5% nonfat dry milk and incubated overnight at 4 °C with the following antibodies: rabbit anti-human ErbB2 (HER2-Neu) (clone A0485, Dako), rabbit anti-HSP70 (sc-33575; Santa Cruz Biotechnology). HRP-conjugated secondary antibodies (Santa Cruz Biotechnology) were used in combination with enhanced chemiluminescence (ECL Amersham). Densitometry calculations were made with Image-J software ([rsb.info.nih.gov](http://rsb.info.nih.gov)), after verification of non-saturation and background subtraction. Values are expressed as the integrals of each band normalized to weakest band and a ratio between target protein and calibrator proteins (housekeeping-HK: HSP70) is shown.

## Statistical Analysis

Results are expressed as means  $\pm$  s.d. from an appropriate number of experiments.

Statistical analysis was performed using GraphPad Prism software Version 6.0 (La Jolla, CA, USA). Two-way ANOVA test for multiple comparison was used unless otherwise specified.

## Results

### **BRAF<sup>V600E</sup> CRC cell lines are sensitive to the selective BRAF inhibitor vemurafenib and insensitive to the EGFR-inhibitor panitumumab**

For our investigations, we selected colorectal carcinoma cell lines according to their mutational status (Table 1). In particular, mutations of the EGFR signaling pathway were considered, in the order of the following molecules: EGFR, RAS (including K-RAS, H-RAS, N-RAS), BRAF, TP53, and PI3K.

Two (Colo205, HT-29) harbored the BRAF<sup>V600E</sup> mutation with wild-type EGFR; the third (SW-48) was BRAF wild-type (wt) with an EGFR mutation.

## **Both BRAF<sup>V600E</sup> CRC cell lines are sensitive to the selective BRAF inhibitor vemurafenib and insensitive to the EGFR-inhibitor panitumumab in vitro**

To explore their in vitro sensitivity to BRAF blockade, we first treated the three CRC cell lines with the selective BRAF<sup>V600E</sup> inhibitor, vemurafenib (VEM). As expected, the BRAF wt SW-48 cells were unaffected by the drug (Fig. 1A and 1B). In contrast, the BRAF<sup>V600E</sup> lines, Colo205 and HT-29, both displayed significantly reduced viability and increased death rates after exposure to VEM, which is consistent with previous reports [18]. To investigate the in vitro sensitivity to EGFR blockade, the cell lines were then exposed to panitumumab (PAN), a fully human recombinant monoclonal IgG2 antibody highly specific against EGFR/ErbB-1/HER1. PAN is indicated for the treatment of metastatic CRC with wild-type KRAS [21]. As shown in Figures 1C and D, however, PAN had no significant growth-limiting effects in any of the cell lines tested, regardless of their EGFR status.

## **Co-exposure to panitumumab may enhance the efficacy of vemurafenib in some BRAF<sup>V600E</sup> CRC cells**

Since a synergy between EGFR and BRAF<sup>V600E</sup> inhibition was previously demonstrated [3,11], we investigated the possible combined action of vemurafenib and panitumumab in cell models tested.

As shown in Figure 2A and B, vemurafenib and panitumumab, administered singly or combined, had no effects on cell viability or cell death rates in the BRAF-wt/EGFR-mutant SW-48 line. As for the BRAF<sup>V600E</sup> CRC cells, the two drugs exerted synergic growth-limiting effects in the Colo205 line (Fig. 2C and D). In contrast, in HT-29 cells, the efficacy of the combination was not significantly different from that of vemurafenib alone (Figures 2E and F).

Our results from data presented in Figure 1 and 2 show that the combined targeting of EGFR and BRAF<sup>V600E</sup> can be beneficial in BRAF<sup>V600E</sup> cells.

## **BRAF<sup>V600E</sup> CRC cells respond to different doses of the pan-ErbB-family inhibitor afatinib**

We then proceeded to explore whether a molecule with a broader action on ErbB family receptors could achieve better results with respect to the highly anti-EGFR specific panitumumab.

We evaluated the responses of the CRC cell lines to afatinib (AFA), a small-molecule receptor tyrosine kinase inhibitor (TKI), which irreversibly blocks signaling activity from all ErbB-family homo- and heterodimeric receptors [22]. Afatinib is currently approved for first-line treatment of patients with EGFR-mutation-positive lung cancer [23].

Viability and death rates were assessed in all three CRC cell lines after exposure to AFA at concentrations ranging from 0.01  $\mu$ M to 10  $\mu$ M (log<sub>10</sub> escalations). As expected, SW-48 cells, which harbor an EGFR mutation targeted by afatinib (i.e., p. G719S), were sensitive to low doses of the drug (0.1 and 1  $\mu$ M) (Figures 3A-B). The responses of the BRAF<sup>V600E</sup> cell lines—both of which are EGFR-wildtype—differed: in Colo205 cells, significant changes in both viability and death rates were already evident after exposure to the lowest concentration tested (0.01  $\mu$ M) (Figures 3C and D), whereas HT-29 cells were significantly affected only by the highest concentration used (10  $\mu$ M) (Figures 3E and F).

## **Vemurafenib and afatinib produce additive growth-limiting effects in CRC BRAF<sup>V600E</sup> cell lines**

We then wanted to investigate if the combination of vemurafenib with afatinib, which is able to target ErbB2 and ErbB4 receptors in addition to EGFR, could achieve better results than the combination of vemurafenib and panitumumab.

We then exposed the cells to vemurafenib and afatinib—the latter at doses of 1  $\mu$ M or 10  $\mu$ M (VEM + AFA1 and VEM + AFA10, respectively).

As shown in Fig. 4A and B, the addition of vemurafenib did not enhance the effect produced in SW-48 cells by exposure to AFA1 or AFA10 alone, which was consistent with these cells' unresponsiveness to the BRAF inhibitor alone (Fig. 1). In the BRAF<sup>V600E</sup> lines (Colo205 and HT-29), the two drugs produced additive growth-limiting effects that were afatinib dose-dependent (Fig. 4C-F). In the Colo205 cells, which responded to both VEM and AFA when used alone (in terms of both decreased viability and increased death rates), the response to the combination VEM+AFA was stronger than those achieved with VEM+PAN (Fig. 2C and D), and these effects were even more evident when the higher dose of AFA was used (VEM+AFA10). As for the HT-29 cells, which were not significantly affected by VEM-PAN, the VEM-AFA1 combination was also effective, but the level of efficacy observed in Colo205 cells could be achieved only with the higher dose of AFA (VEM+AFA10).

## **ErbB2/HER2/Neu is differentially expressed in untreated BRAF<sup>V600E</sup> CRC cell lines**

To explore possible mechanisms underlying the differential responses to the inhibition of the ErbB receptor family members of the BRAF<sup>V600E</sup> cell lines, we analyzed their expression levels of ErbB2/HER2/Neu, which is targeted by AFA but not by PAN. Unlike EGFR, ErbB2 is activated not by ligand binding, but as a result of its heterodimerization with other ErbB family members. Owing to ErbB2's constitutive kinase activity, EGFR/ErbB2 heterodimers are more active than EGFR homodimers, but this interaction is limited by the relatively low-level of ErbB2 expression in normal cells [24]. Mutations or amplifications in one of the four ERBB family genes are present in 22 out of 165 (13%) non-hypermutated

and 16 out of 30 (53%) hypermutated cases [25]. Interestingly, activating mutations and amplifications of ErbB2 account for 7% of cases (Cancer Genome Atlas Network) and patients with HER2-amplified metastatic CRC are less likely to respond to anti-EGFR therapy [26].

As shown in Figure 5A and B, ErbB2 expression in Colo205 cells was significantly higher than that found in HT-29 (or in the BRAF<sup>wt</sup> SW-48 cells). This molecular feature could conceivably explain the high sensitivity of Colo205 cells to afatinib shown in Figures 3 and 4. These findings suggest that ErbB2 expression levels in BRAF<sup>V600E</sup> CRC cells are an important predictor of their responsiveness to ErbB blockade, alone or with BRAF inhibition. Patients whose tumors express high levels of ErbB2 are likely to be sensitive to afatinib monotherapy or combined treatment with afatinib and vemurafenib.

Indeed, in clinical settings, vemurafenib alone proves to be insufficient in CRC tumors owing in many cases to feedback-mediated reactivation of RAS. ErbB2 (HER2-Neu) has been identified as a possible mediator of this feedback. Our experiments showed that the small molecule pan-ErbB family inhibitor afatinib effectively reduces the viability of CRC cells. Moreover, the responses to afatinib treatment were heterogeneous. Based on the ErbB2 protein levels we demonstrated in the cell lines we used, it seems reasonable to speculate that high-level expression of ErbB2 might predict an effective response to AFA at lower doses. We therefore propose that combined treatment with vemurafenib and afatinib be used for BRAF<sup>V600E</sup> CRCs. Assessment of ErbB2 (HER2-Neu) expression levels in these tumors should also be used to inform treatment decision-making.

## Discussion

CRC includes different categories of tumors based on their specific mutational spectra and molecular phenotype, driving distinct oncogenic signaling pathways. The main goal of molecular oncology is the development of personalized medicine, and this is particularly urgent for BRAF mutant CRC patients since effective therapies are still lacking indeed the inhibition of the BRAF<sup>V600E</sup> oncoprotein by the small-molecule drug vemurafenib (VEM), which is highly effective in melanoma [27], showed a very limited response in these patients [28]. In BRAF mutant CRC, some studies have indicated that EGFR reactivation contributes to insensitivity to vemurafenib. In this context, in vivo studies showed that BRAF mutant patients could be responsive to association of BRAFi and EGFRi [3].

Our study aimed to explore the effectiveness of different targeted drugs either alone or in combination in BRAF<sup>V600E</sup> cell lines, with the aim to shed light on the possible mechanisms driving resistance to biological therapy in BRAF mutant patients. We found that the BRAF<sup>V600E</sup> cell lines we tested differed markedly in their sensitivity to short-term exposure to the BRAF inhibitor VEM, as reflected by assays of cell viability and cell death. All cell lines were unresponsive to the anti-EGFR blockade with panitumumab, whereas all displayed sensitivity to afatinib, which covalently binds to and blocks HER2 and HER4, in addition to EGFR, a dual receptor tyrosine kinase inhibitor for the treatment of solid tumors [29,30]. Notably, combination strategies VEM+AFA at low dose achieved good response in Colo205 BRAF<sup>V600E</sup> mutated cell lines. Looking for molecular aspects that could explain the different responsiveness of the

BRAF mut cells analyzed, we showed that Colo205 cells, that express higher levels of ErbB2 (HER2-Neu) in respect to the other cells, were the cells that presented the best impairment after afatinib treatment, even at low dose. We decided to consider the HER2-Neu molecular aspect in our cells since it has been described that human CRC are positive for HER2 staining [31] and that activating mutations and amplifications of ErbB2 account for 7% of cases (Cancer Genome Atlas Network). Moreover, a very recent paper proposed the use of ErbB protein levels as biomarker for selection and stratifications of CRC patients [32]. Our data support the importance of ErbB2 evaluation, by the ICH staining of tumor samples after surgery, as a new molecule aspect in CRC mutated patients for personalized therapy selection.

We suggest screening tumors for the ErbB2 (HER2-Neu) expression, where its high levels could be considered as positive predictive factor of treatment response using afatinib or using afatinib+vemurafenib.

## Conclusion

Our work presents new molecular aspects of BRAF mutated CRC cells which can occur in resistant patients and support the notion that, besides the specific BRAF<sup>V600E</sup> mutation, other signaling pathway activations could be responsible for therapy failure. Therefore, BRAF mutant patients should not be considered as having a unique underlying biology but heterogeneous paths [20] that may be identified and exploited for effective personalized targeted therapies [33].

## List Of Abbreviations

ErbB2: Receptor tyrosine-protein kinase erbB-2

CRC: Colo-rectal Cancer

KRAS: Kirsten RAt Sarcoma

PIK3CA: Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic unit

TP53: Tumor Protein p53

EGFR: Epidermal Growth Factor Receptor

PTEN: phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase protein

RTKs: Receptor Tyrosine Kinsae

ERK: extracellular signal-regulated kinases

MAPK: mitogen-activated protein kinase

APC: Adenomatous polyposis coli

PCR: Polymerase chain reaction

TKI: Tyrosine Kinase Inhibitor

HSP70: heat shock protein 70

VEM: Vemurafenib

PAN: Panitumumab

AFA: Afatinib

WT: Wild type

## Declarations

- *Ethical Approval and Consent to participate*: Not applicable

- *Consent for publication*: Not applicable

- *Availability of data and material*: Not applicable

- *Competing interests*: The authors declare no competing interests.

- *Funding*: This work was supported by Associazione Italiana Ricerca Cancro (AIRC 5XMILLE), Ministry of University and Research (FIRB, PRIN and PON), Sapienza University of Rome (Ateneo), Italian Institute of Technology (IIT) projects.

- *Authors' contributions*: EM and EF conceived the laboratory experiments and wrote the manuscript, LA, ZMB, GC, MC, AP performed laboratory experiments, GPS and CC contributed to clinical hints, manuscript writing and review; AV and EF supervised testing and data interpretation.

- *Acknowledgements*: Not applicable

## References

1. De Rosa M, Pace U, Rega D, Costabile V, Duraturo F, Izzo P, Delrio P. Genetics, diagnosis and management of colorectal cancer. *Oncol Rep.* 2015; 34(3):1087–96. doi: 10.3892/or.2015.4108.

2. Cancer Genome Atlas Network. Comprehensive molecular characterization of human colon and rectal cancer.

*Nature.* 2012; 18;487(7407):330–7. doi: 10.1038/nature11252.

3. Capalbo C, Marchetti P, Coppa A, Calogero A, Anastasi E, Buffone A, et al. Vemurafenib and panitumumab combination tailored therapy in BRAF-mutated metastatic colorectal cancer: a case report. *Cancer Biol Ther*. 2014; 15(7):826–31. doi: 10.4161/cbt.28878.
4. Sartore-Bianchi A, Loupakis F, Argilés G, Prager GW. Challenging chemoresistant metastatic colorectal cancer: therapeutic strategies from the clinic and from the laboratory. *Ann Oncol*. 2016; 27(8):1456–66. doi: 10.1093/annonc/mdw191.
5. Van Cutsem E, Köhne CH, Láng I, Folprecht G, Nowacki MP, Cascinu S, et al. Cetuximab plus irinotecan, fluorouracil, and leucovorin as first-line treatment for metastatic colorectal cancer: updated analysis of overall survival according to tumor KRAS and BRAF mutation status. *J Clin Oncol*. 2011; 29(15):2011–9. doi: 10.1200/JCO.2010.33.5091
6. Yokota T, Ura T, Shibata N, Takahari D, Shitara K, Nomura M, et al. BRAF mutation is a powerful prognostic factor in advanced and recurrent colorectal cancer. *Br J Cancer*. 2011; 104(5):856–62. doi: 10.1038/bjc.2011.19
7. Tol J, Nagtegaal ID, Punt CJ. BRAF mutation in metastatic colorectal cancer. *N Engl J Med*. 2009; 361(1):98–9. doi: 10.1056/NEJMc0904160.
8. De Roock W, Claes B, Bernasconi D, De Schutter J, Biesmans B, Fountzilas G, et al. Effects of KRAS, BRAF, NRAS, and PIK3CA mutations on the efficacy of cetuximab plus chemotherapy in chemotherapy-refractory metastatic colorectal cancer: a retrospective consortium analysis. *Lancet Oncol*. 2010; 11(8):753–62. doi:10.1016/S1470–2045(10)70130–3.
9. Di Nicolantonio F, Martini M, Molinari F, Sartore-Bianchi A, Arena S, et al. Wild-type BRAF is required for response to panitumumab or cetuximab in metastatic colorectal cancer. *J Clin Oncol*. 2008; 26(35):5705–12. doi: 10.1200/JCO.2008.18.0786
10. Laurent-Puig P, Cayre A, Manceau G, Buc E, Bachet JB, Lecomte T, et al. Analysis of PTEN, BRAF, and EGFR status in determining benefit from cetuximab therapy in wild-type KRAS metastatic colon cancer. *J Clin Oncol*. 2009; 27(35):5924–30. doi: 10.1200/JCO.2008.21.6796.
11. Pietrantonio F, Petrelli F, Coinu A, Di Bartolomeo M, Borgonovo K, Maggi C, et al. Predictive role of BRAF mutations in patients with advanced colorectal cancer receiving cetuximab and panitumumab: a meta-analysis. *Eur J Cancer*. 2015; 51(5):587–94. doi: 10.1016/j.ejca.2015.01.054.
12. Grothey A, Van Cutsem E, Sobrero A, Siena S, Falcone A, Ychou M, et al. Regorafenib monotherapy for previously treated metastatic colorectal cancer (CORRECT): an international, multicentre, randomised, placebo-controlled, phase 3 trial. *Lancet*. 2013; 381(9863):303–12. doi: 10.1016/S0140–6736(12)61900-X.

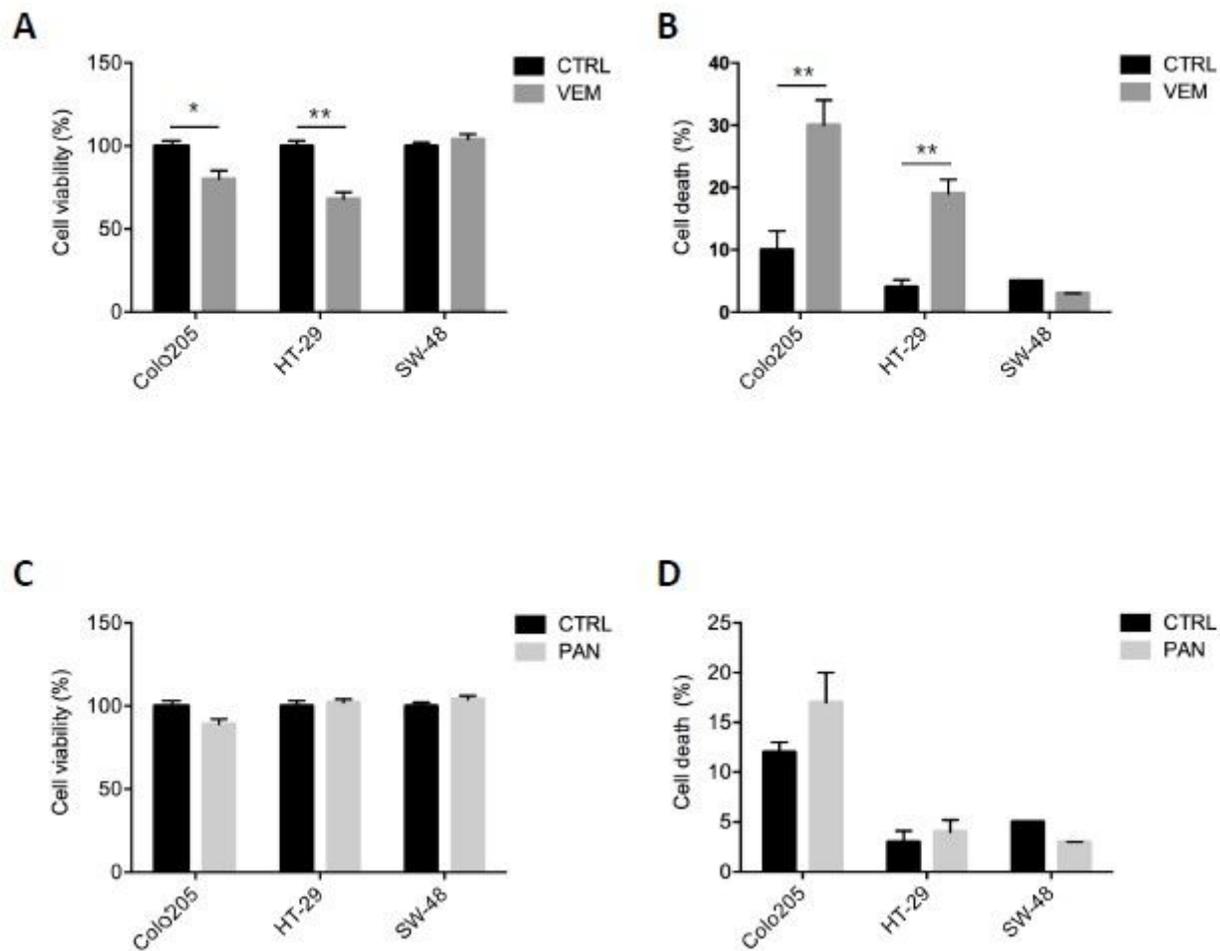
13. Corcoran RB, Atreya CE, Falchook GS, Kwak EL, Ryan DP, Bendell JC, et al. Combined BRAF and MEK Inhibition With Dabrafenib and Trametinib in BRAF V600-Mutant Colorectal Cancer. *J Clin Oncol*. 2015;33(34):4023–31. doi: 10.1200/JCO.2015.63.2471.
14. Kopetz S, Desai J, Chan E, Hecht JR, O'Dwyer PJ, Maru D, et al. Phase II Pilot Study of Vemurafenib in Patients With Metastatic BRAF-Mutated Colorectal Cancer. *J Clin Oncol*. 2015;33(34):4032–8. doi: 10.1200/JCO.2015.63.2497.
15. Lito P, Pratilas CA, Joseph EW, Tadi M, Halilovic E, Zubrowski M N, et al. Relief of profound feedback inhibition of mitogenic signaling by RAF inhibitors attenuates their activity in BRAFV600E melanomas. *Cancer Cell*. 2012;22(5):668–82. doi: 10.1016/j.ccr.2012.10.009.
16. Yaeger R, Cercek A, O'Reilly EM, Reidy DL, Kemeny N, Wolinsky T, et al. Pilot trial of combined BRAF and EGFR inhibition in BRAF-mutant metastatic colorectal cancer patients. *Clin Cancer Res*. 2015;21(6):1313–20. doi: 10.1158/1078-0432.CCR-14-2779.
17. Corcoran RB, Ebi H, Turke AB, Coffee EM, Nishino M, Cogdill AP, et al. EGFR-mediated re-activation of MAPK signaling contributes to insensitivity of BRAF mutant colorectal cancers to RAF inhibition with vemurafenib. *Cancer Discov*. 2012; (3):227–35. doi: 10.1158/2159-8290.CD-11-0341.
18. Mao M, Tian F, Mariadason JM, Tsao CC, Lemos R Jr, Dayyani F, et al. Resistance to BRAF inhibition in BRAF-mutant colon cancer can be overcome with PI3K inhibition or demethylating agents. *Clin Cancer Res*. 2013;19(3):657–67. doi: 10.1158/1078-0432.CCR-11-1446.
19. Price TJ, Hardingham JE, Lee CK, Weickhardt A, Townsend AR, Wrin JW, et al. Impact of KRAS and BRAF Gene Mutation Status on Outcomes From the Phase III AGITG MAX Trial of Capecitabine Alone or in Combination With Bevacizumab and Mitomycin in Advanced Colorectal Cancer. *J Clin Oncol*. 2011;29(19):2675–82. doi: 10.1200/JCO.2010.34.5520.
20. Barras D, Missiaglia E, Wirapati P, Sieber OM, Jorissen RN, Love C, et al. BRAF V600E Mutant Colorectal Cancer Subtypes Based on Gene Expression. *Clin Cancer Res*. 2017;23(1):104–115. doi: 10.1158/1078-0432.CCR-16-0140.
21. Dubois EA, Cohen AF. Panitumumab. *Br J Clin Pharmacol*. 2009;68(4):482–3. doi: 10.1111/j.1365-2125.2009.03492.x.
22. ref EMA/491185/2013 Committee for Medicinal Products for Human Use (CHMP) Giotrif - CHMP assessment report International non-proprietary name: Afatinib Procedure No. EMEA/H/C/002280).
23. Sequist LV1, Yang JC, Yamamoto N, O'Byrne K, Hirsh V, Mok T, et al. Phase III study of afatinib or cisplatin plus pemetrexed in patients with metastatic lung adenocarcinoma with EGFR mutations. *J Clin Oncol*. 2013;31(27):3327–34. doi: 10.1200/JCO.2012.44.2806.

- 24.Hopper-Borge EA1, Nasto RE, Ratushny V, Weiner LM, Golemis EA, Astsaturov I. Mechanisms of tumor resistance to EGFR-targeted therapies. *Expert Opin Ther Targets*. 2009; (3):339–62. doi: 10.1517/14712590902735795
- 25.Cancer Genome Atlas Network. Comprehensive molecular characterization of human colon and rectal cancer. *Nature*. 2012;487(7407):330–7. doi: 10.1038/nature11252.
26. Sartore-Bianchi A, Amatu A, Porcu L, Ghezzi S, Lonardi S, Leone F5, et al. HER2 Positivity Predicts Unresponsiveness to EGFR-Targeted Treatment in Metastatic Colorectal Cancer. *Oncologist*. 2019; 5. doi: 10.1634/theoncologist.2018–0785
- 27.Chapman PB, Hauschild A, Robert C, Haanen JB, Ascierto P, Larkin J, et al. Improved survival with vemurafenib in melanoma with BRAF V600E mutation. *N Engl J Med*. 2011;364(26):2507–16. doi: 10.1056/NEJMoa1103782.
- 28.Kopetz S1, Hoff PM, Morris JS, Wolff RA, Eng C, Glover KY, et al. Phase II trial of infusional fluorouracil, irinotecan, and bevacizumab for metastatic colorectal cancer: efficacy and circulating angiogenic biomarkers associated with therapeutic resistance. *J Clin Oncol*. 2010;28(3):453–9. doi: 10.1200/JCO.2009.24.8252.
- 29.Solca F, Dahl G, Zoepfel A, Bader G, Sanderson M, Klein C, et al. Target binding properties and cellular activity of afatinib (BIBW 2992), an irreversible ErbB family blocker. *Pharmacol Exp Ther*. 2012;343(2):342–50. doi: 10.1124/jpet.112.197756.
- 30.Minkovsky N, Berezov A. BIBW–2992, a dual receptor tyrosine kinase inhibitor for the treatment of solid tumors. *Curr Opin Investig Drugs*. 2008;9(12):1336–46.
- 31.Seo AN, Kwak Y, Kim DW, Kang SB, Choe G, Kim WH, Lee HS. HER2 status in colorectal cancer: its clinical significance and the relationship between HER2 gene amplification and expression. *PLoS One*. 2014;9(5):e98528. doi: 10.1371/journal.pone.0098528.
- 32.Plano D, Alcolea V, Sanmartín C, Sharma AK. Methods of selecting combination therapy for colorectal cancer patients: a patent evaluation of US20160025730A1. *Expert Opin Ther Pat*. 2017;27(5):527–538. doi: 10.1080/13543776.2017.1315103.
33. La Salvia A, Lopez-Gomez V, Garcia-Carbonero R. HER2-targeted therapy: an emerging strategy in advanced colorectal cancer. *Expert Opin Investig Drugs*. 2019 Jan;28(1):29–38. doi: 10.1080/13543784.2019.1555583

## Tables

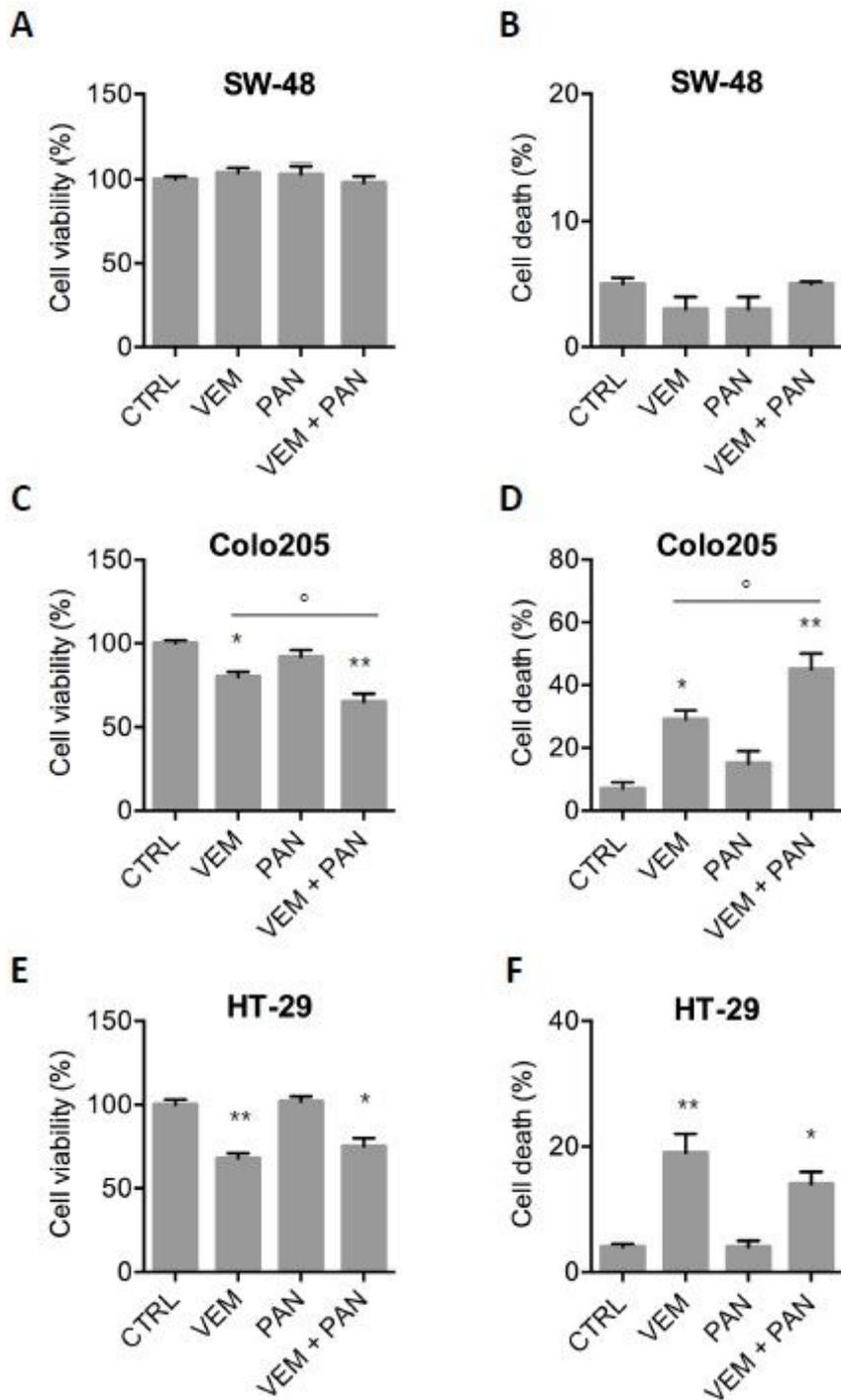
Due to technical limitations, all Table(s) are only available as a download in the supplemental files section.

# Figures



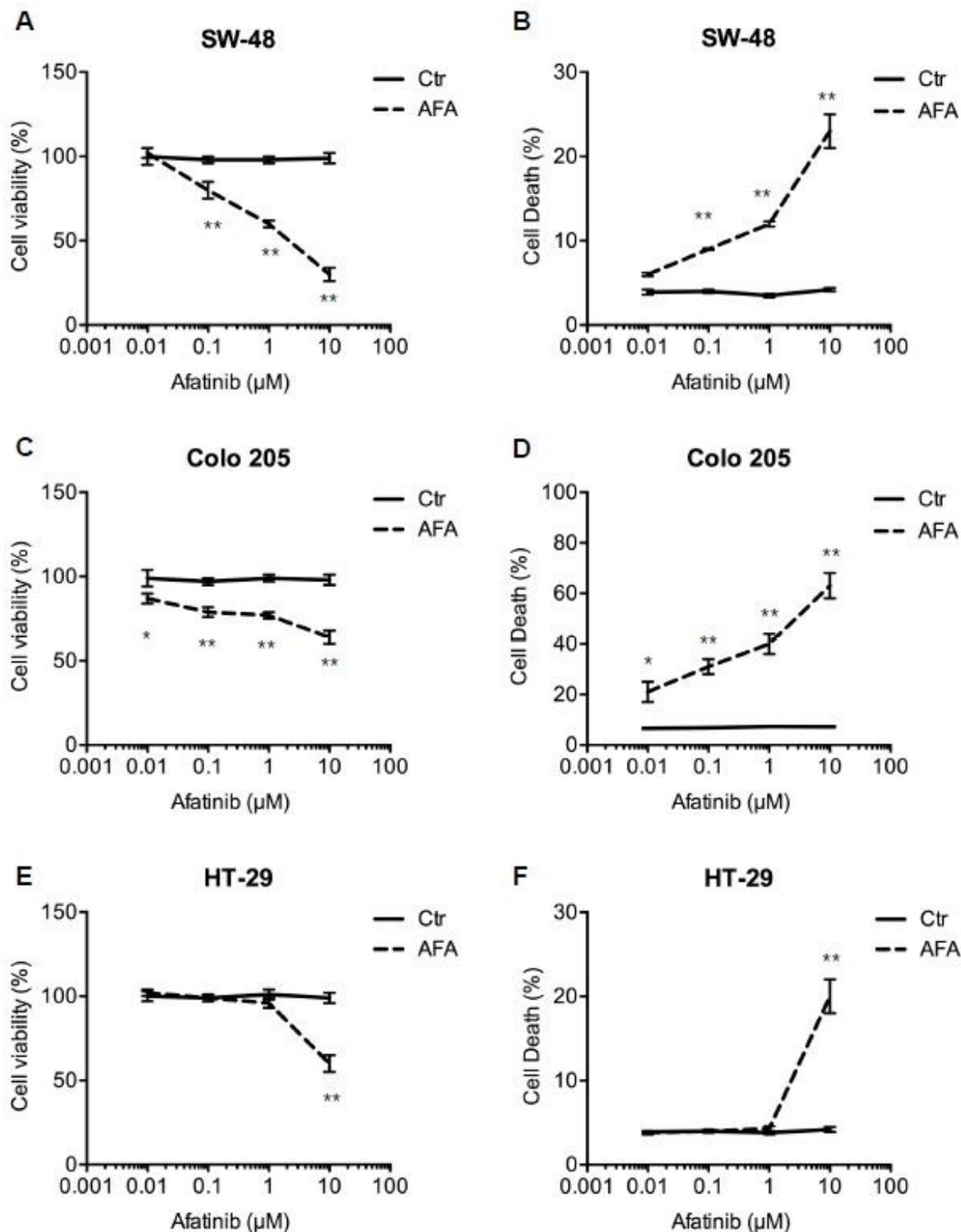
**Figure 1**

BRAFV600E CRC cell lines are sensitive to VEM but not to PAN. BRAF-mutant and wildtype cells were exposed to (A,B) vemurafenib (VEM – final concentration 3  $\mu$ M) or (C,D) panitumumab (PAN – final concentration 8  $\mu$ g/mL) for 48 hours, harvested, and assayed for cell viability (MTS assay) and cell death (trypan blue staining). Results shown are means  $\pm$  SD of three independent experiments. Statistics (unpaired t-test with equal SD): \* $p$ <0.05, \*\* $p$ <0.01 vs. CTRL (untreated cells).



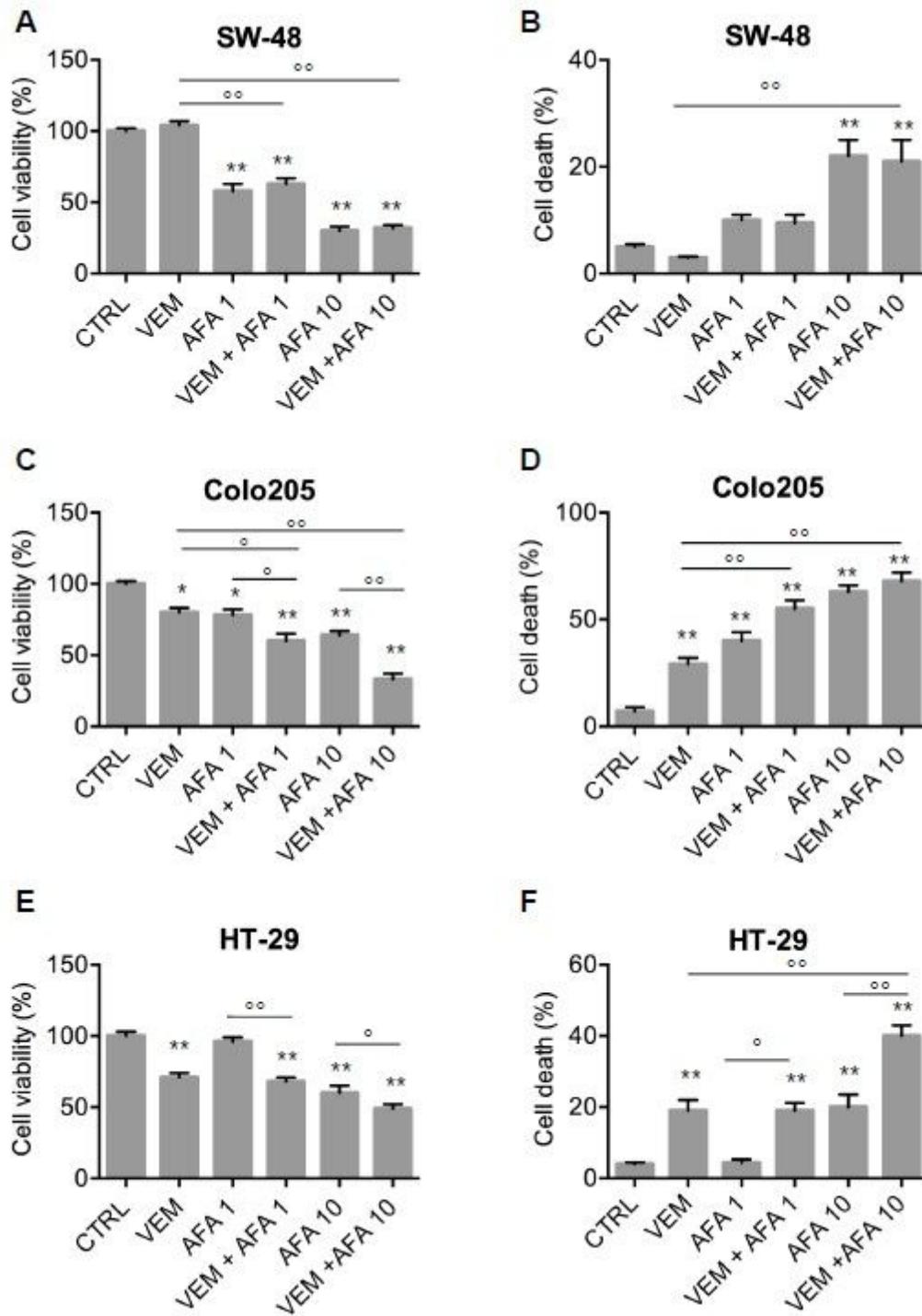
**Figure 2**

Co-exposure to panitumumab may enhance the efficacy of vemurafenib in some BRAFV600E CRC cells. A-F. Cell viability (A,C,E) and cell death (B,D,F) assessment in BRAFV600E mutant cells Colo205 and HT-29 and in SW-48 BRAFWt (EGFRmut) cell line, 48 hours after the administration of 3 $\mu$ M vemurafenib (VEM), or 8 $\mu$ g/mL panitumumab (PAN) or the combination of the two (Vem + Pan). Histograms represent mean  $\pm$  SD of three independent experiments \* $p$ <0.05, \*\* $p$ <0.01 vs CTRL; °  $p$ <0.05 vs VEM



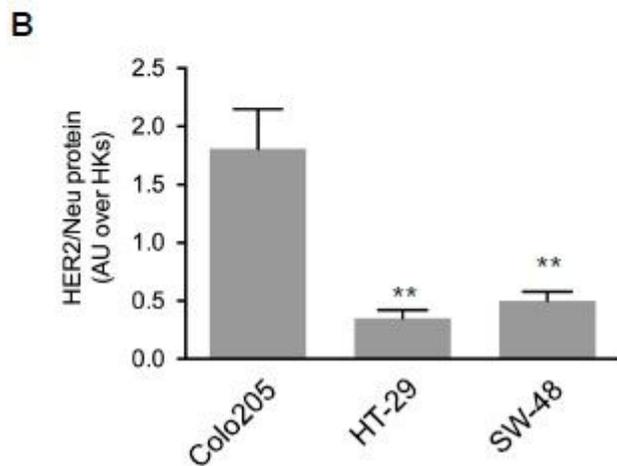
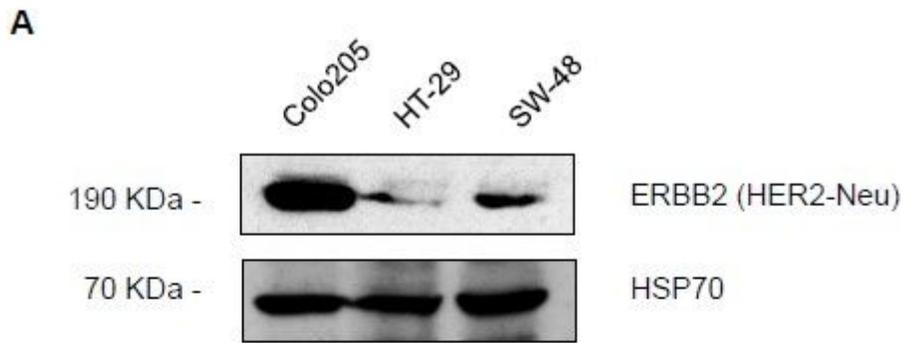
**Figure 3**

BRAFV600E CRC cells respond to different doses of the pan-ErbB-family inhibitor afatinib. Cells were exposed to log<sub>10</sub>-escalating doses of afatinib (AFA-final concentration range: 0.01 μM to 10μM) for 48 hours, harvested, and assayed for (A, C, E) cell viability and (B, D, F) cell death, as described in Figure 1. Results shown are means ± SD of three independent experiments. Statistics (two-way ANOVA for multiple comparisons): \*p<0.05, \*\*p<0.01 vs. CTRL (untreated cells).



**Figure 4**

Vemurafenib and afatinib produce additive growth-limiting effects in CRC BRAFV600E cell lines. Cells were exposed to VEM (3 $\mu$ M), AFA (1 $\mu$ M or 10  $\mu$ M), or both (VEM + AFA1, VEM+AFA10, respectively) for 48 hours, harvested, and assayed for (A, C, E) cell viability and (B, D, F) cell death. Results are means  $\pm$  SD of three independent experiments. Statistics (two-way ANOVA for multiple comparisons): \* $p$ <0.05, \*\* $p$ <0.001 vs. CTRL (untreated cells); ° $p$ <0.05, °° $p$ <0.01 vs. cells treated with the indicated drug or drug combo (horizontal bars).



**Figure 5**

ErbB2/HER2/Neu is differentially expressed in untreated BRAF V600E CRC cell lines. (A) Representative immunoblots with (B) densitometric quantification of band intensities show ErbB2/HER2/Neu protein levels in the three cell lines. (Loading control: HSP70) Bars in (B) represent means  $\pm$  SD of three independent experiments. Statistics (test used): \*\*  $p < 0.01$  vs. Colo205 cells.

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Table1.xls](#)
- [Table2.xls](#)