Pharmacological Inhibition of Casein Kinase 2 Enhances the Effectiveness of PI3K Inhibition in Colon Cancer Cells

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Abstract. *Background/Aim:* Serine/threonine kinase B-Raf proto-oncogene (*BRAF*) mutant colon cancer has a poor prognosis and there is an absence of targeted treatments for this subtype. Here, we investigated the effects of inhibition of casein kinase 2 (CK2) on the inhibitory effects of *BRAF* and phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) inhibition in *BRAF*-mutant colon cancer cells. *Materials and Methods:* Colon cancer cell lines with mutations in components of the mitogen-activated protein kinase (MAPK) and PI3K signalling pathway were used. Cell viability was determined after exposure to single agent and combinations of erlotinib (EGFR inhibitor), dabrafenib (MEK inhibitor), GDC0941 (PI3K inhibitor) and CX4945 (CK2 inhibitor). Western blots were used to examine MAPK and AKT serine/threonine kinase (AKT) pathway activation. *Results:* Addition of CX4945 to dabrafenib did not enhance the antiproliferative effects of single-agent dabrafenib. Use of GDC0941 alone was highly effective in controlling growth of both *BRAF*-mutant and wild-type cells and this effect was enhanced by CK2 inhibition. *Conclusion:* Inhibition of the PI3K/AKT pathway is central to regulating growth of colon cancer cells and this can be enhanced by CK2 inhibition.

Mutation of the *KRAS* proto-oncogene (*KRAS*) gene is commonly associated with the development of colorectal cancer (CRC) through the adenoma–carcinoma sequence, a gradual mutational activation and inactivation of oncogenes and tumour-suppressor genes, respectively. However, approximately 10% of CRCs are associated with mutation in the serine/threonine kinase *B-Raf* proto-oncogene (*BRAF*), which leads to unrestrained signalling through the

proliferative mitogen-activated protein kinase (MAPK) pathway in the absence of mitogen ligands. Mutations in *KRAS* and *BRAF* are mutually exclusive (1). In the case of melanoma, where 40-60% of patients harbour the *BRAF* V600E mutation, specific *BRAF* drug inhibitors such as vemurafenib and dabrafenib targeting the mutant kinase have been remarkably successful in providing short-term clinical benefit before resistance mechanisms prevail (2-4). Despite this success in combating melanoma, unfortunately in the context of *BRAF*-mutant CRC, clinical testing with the potent *BRAF* V600E inhibitor vemurafenib (PLX4032) failed to elicit treatment response (5). Subsequent studies to investigate resistance mechanisms demonstrated MAPK reactivation and alternative pro-growth and survival pathways, particularly through epidermal growth factor receptor (EGFR) or phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)/AKT serine/threonine kinase (AKT) signalling pathways (6, 7). Furthermore, in preclinical models, Mao *et al.* showed that the vemurafenib analogue PLX4720 combined with the PI3K inhibitor GDC0941 or LY294002 synergistically inhibited proliferation in *BRAF*-mutant colon cancer cell lines (7), underscoring the relevance of these pathways in colon cancer.

Previous work in our laboratory demonstrated that CX4945, a specific inhibitor of casein kinase 2 (CK2), acted in strong synergy to potentiate the inhibitory effects of vemurafenib for treating *BRAF*-mutant melanoma and *BRAF*-mutant thyroid carcinoma cells. We showed that this effect was mediated, in part, through reduced activation of AKT (8). Given the greater reliance of EGFR and PI3K/AKT pathways in CRC compared to melanoma, the focus of this work was to assess whether CK2 inhibition could similarly have a potentiating effect when used in combination with inhibitors targeting these pathways.

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Materials and Methods

Cell culture. Human colon cancer cell lines SW480 (*BRAF* wild-type, *KRAS*G12V), Colo205 (*BRAF* V600E) and HT29 (*BRAF* V600E, *PIK3CA* P449T) were obtained from American Type

Figure 1. Viability of colon cancer cells following 72-h treatment with epidermal growth factor receptor (EGFR) inhibitor (erlotinib; 2 μ M), serine/threonine kinase B-Raf proto-oncogene (BRAF) inhibitor (dabrafenib; 15 nM), casein kinase 2 (CK2) inhibitor (CX4945; 4 μ M) and combinations thereof (n=5). Data are mean values $\pm$ SD. Significantly different at * p <0.005 for erlotinib versus erlotinib/dabrafenib; # p <0.01 for erlotinib versus erlotinib/CX4945; ^ p <0.005 for erlotinib/dabrafenib versus erlotinib/dabrafenib/CX4945; ns, not significantly different.

Culture Collection. Lim2405 (BRAF V600E, PTEN-null) were obtained from Professor J. Cebon (Olivia Newton-John Cancer Research Institute, Melbourne, Australia). SW480 and HT29 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% dialysed foetal bovine serum (FBS), 1% penicillin/streptomycin and 10 mM HEPES. Colo205 and Lim2405 cells were cultured in Roswell Park Memorial Institute medium (RPMI) supplemented with 10% dialysed FBS, 1% penicillin/streptomycin, 10 mM HEPES and 10 mM L-glutamine.

Viability assays. The inhibitors erlotinib (2 μ M), dabrafenib (15 nM), CX4945 (4 μ M), GDC0941 (4 μ M) were obtained from Selleckchem, TX, USA. Cells were seeded into 96-well plates at 5.0×10^3 per well and were allowed to settle for 1 h in a laminar flow-hood (to reduce edge-effect) before being transferred into an incubator for at least 12 h before use. Medium was removed and replaced with that containing single drugs or combinations thereof. Dimethyl sulfoxide (DMSO) was used as a control treatment for all drugs. Cells were incubated for 72 h before being analysed for cell viability using PrestoBlue reagent (ThermoFisher, CA, USA) and read at 570 nm.

Western blot. Cells were seeded into 6-well plates at 5.0×10^5 per well. Cells were allowed to settle for 1 h in a laminar flow-hood (to reduce edge-effect) before being transferred into an incubator for at least 12 h before use. The medium was removed and replaced with that containing single drugs or combinations thereof. Cells were incubated for 60 min before lysis using a buffer consisting of 20 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, pH 7.5. Cell lysate was collected using a cell scraper sonicated on ice for 3 s at 10% amplitude.

BCA assay was used to determine protein concentration and 10 μ g of protein from cells was prepared for 4-12% Bis-Tris gels run at 200 V for 1 h. The gel was transferred onto nitrocellulose using a TurboBlot system (BioRad, CA, USA) for 10 min at 1.5 V. Membranes were washed with Tris-buffered saline (TBS) (1 M Tris-HCl, 3 M NaCl) and blocked in 5% bovine serum albumin (BSA) in TBS-T (TBS/0.1% Tween 20). Membranes were then washed with TBS-T and incubated with primary antibody (in 5% BSA/TBS-T) overnight at 4°C. Membranes were washed in TBS-T and incubated

with secondary antibody for 30 min at room temperature. Membranes were rinsed in water and imaged using an Odyssey imager (LI-COR Biotechnology, Lincoln, NE, USA) at 700 nm or 800 nm. Primary antibodies used in this study were: mouse monoclonal anti-ERK1/2 (1:2,000 dilution, Cat #9107), rabbit polyclonal anti-phospho-ERK1/2 (T202/Y204) (1:1000 dilution, Cat #9101), rabbit monoclonal anti-panAKT (1:1,000 dilution, Cat # 4691), rabbit monoclonal anti-phospho-AKT (S473) (1:1,000 dilution, Cat# 4060), rabbit monoclonal anti-p70 S6 kinase (1:1,000 dilution, Cat # 2708), rabbit polyclonal anti-phospho-p70 S6 kinase (T389) (1:1000 dilution, Cat# 9205) (Cell Signaling Technology, Danvers, MA, USA).

Statistical analysis. Two-tailed, paired Student's *t*-test was performed to evaluate significant changes in cell viability following drug treatments. *p*-Value of less than 0.05 were considered significant.

Results

CK2 inhibition failed to potentiate the antiproliferative effects of dabrafenib in BRAF-mutant colon cancer cells. We previously observed that inhibition of constitutively active CK2 strongly potentiated the antiproliferative effects of the BRAF inhibitor vemurafenib in melanoma and thyroid cancer cells which carry the BRAF V600E/K mutation (8). Here, we set out to examine whether a similar response could be realized in BRAF-mutant colon cancer where monotherapy with BRAF kinase inhibition has been unsuccessful (5). It has been hypothesized that activating mutations within the PI3K pathway in BRAF-mutant colon cancer and amplification of EGFR provide sufficient pro-growth signalling pathway alternatives to overcome the effectiveness of BRAF blockade (9). For this study, we utilised commonly reported colon cancer cell lines with mutations affecting BRAF and PI3K signalling pathways.

As shown in Figure 1, *in vitro* blocking of EGFR signalling with erlotinib alone was ineffective in reducing

Figure 2. Western blot of extracellular signal-regulated kinase 1/2 (ERK1/2) activation following 1-h treatment with casein kinase 2 (CK2) inhibitor (CX4945; 4 μ M), serine/threonine kinase B-Raf proto-oncogene (BRAF) inhibitor (dabrafenib; 15 nM), epidermal growth factor receptor (EGFR) inhibitor (erlotinib; 2 μ M) and combinations thereof ($n=3$).

cell growth below 80% of the control in the four colon cancer cell lines after 72-h drug exposure. Treatment with the clinically approved second-generation BRAF inhibitor dabrafenib resulted in reduced cell viability in Colo205 (BRAF V600E) (46%), HT29 (BRAF V600E and PIK3CA P449T) (49%) and Lim2405 cells (BRAF V600E/PTEN-null) (10) (63%), but not BRAF wild-type SW480 cells. The combination of erlotinib and dabrafenib further improved antiproliferative activity in Colo205 and Lim2405 by an additional ~15% and ~27% respectively, compared to dabrafenib alone. Paradoxical activation of wild-type BRAF in SW480 cells (KRAS G12V) was observed when erlotinib

was combined with dabrafenib, while a similar response was seen in HT29 cells where this dual combination treatment was less effective than dabrafenib alone. Sensitivity to single-agent CK2 inhibition using the drug CX4945 was modest, reducing growth by 66-85% of controls, similarly to our previous experience for monotherapy in BRAF-mutant melanoma and BRAF-mutant thyroid cancer cell lines. However, in those cancer types, CK2 inhibition with CX4945 or siRNA knockdown was strongly synergistic in potentiating the antiproliferative effects of BRAF or MEK inhibition (8), but this was not observed here with colon cancer cell lines. Interestingly, use of CK2 inhibitor in

combination with erlotinib was a useful approach to inhibit cell growth compared to erlotinib monotherapy and in fact was the most successful regimen for slowing proliferation of *KRAS*-mutant SW480 cells. Triple treatment of erlotinib, dabrafenib and CX4945 added further modest reduction in proliferation to each of the tested models.

We used western blotting of phospho-ERK to monitor MAPK pathway activity after short-term incubation (1 h) with some of these drugs used as monotherapy or in combination (Figure 2). Interestingly, in many cases, but especially with dabrafenib, pERK was reduced with drug treatments, indicating effective pharmacodynamic target inhibition. However, as shown in Figure 1, this did not always translate into effective control of cell proliferation, underscoring the importance of MAPK-independent growth-stimulating pathways in colon cancer cells. For example, dabrafenib and CX4945 strongly inhibited ERK phosphorylation in Colo205 and Lim2405 cells, but not in HT29 cells, while the corresponding viability data showed growth reduction to 50%, 57% and 53% of controls, respectively.

Controlling activity of PI3K/AKT pathway is effective for BRAF-mutant colon cancer and is enhanced by CK2 inhibition. To investigate the contribution of other pathways in driving cell proliferation, we focused on the PI3K/AKT pathway, where components are known to be commonly mutated in CRC. Furthermore, we previously showed that inhibition of CK2 effected reduced priming of AKT through restricting phosphorylation of Ser129 as a likely contributor for its synergistic activity with BRAF inhibition in melanoma and thyroid cells (8). As shown in Figure 2, 1-h treatment with the potent ATP-competitive PI3K α/δ inhibitor GDC0941 had almost no effect on MAPK activity in these cells, however, we observed potent inhibition of the AKT Ser 473 activation site and subsequent ablation of phosphorylation of the downstream effector p70S6 kinase in each of the four cell lines, consistent with arresting AKT signalling (Figure 3A). We next examined cell proliferation control following 72-h treatment with GDC0941 and this showed cell viability was significantly reduced in all four cell lines (Figure 3B). Interestingly, treatment with 4 μ M GDC0941 was more effective than 15 nM dabrafenib alone in *BRAF*-mutant cells and was most effective against HT29 cells, which have a constitutively active mutant PI3K enzyme. Furthermore, GDC0941 was the most potent monotherapy trialled for regulating growth in the *PTEN*-null Lim2405 cell line, reducing growth to 45% of the control. These observations are consistent with a previous report (7) and confirm the importance of the PI3K pathway in promoting colon cancer cell proliferation. Combination of GDC0941 with dabrafenib predictably reduced growth of HT-29 and Colo205 cells even further, while addition of CX4945 to GDC0941 appeared to be synergistic and produced the surprising effect of greatly reducing growth of *BRAF* wild-type SW480 cells to less than 40% of controls.

Discussion

The absence of effective targeted therapies to treat *BRAF*-mutant colon cancer greatly contrasts the situation observed clinically in *BRAF*-mutant melanoma (5). We previously demonstrated that inhibition of the ubiquitous CK2 strongly potentiates the effect of BRAF and MEK inhibition in *BRAF*-mutant melanoma and *BRAF*-mutant thyroid cancer cells (8) and set out here to evaluate such an approach for *BRAF*-mutant colon cancer. In contrast to our previous findings, we observed no additional benefit of CK2 inhibition when used in combination with dabrafenib, although proliferation was significantly reduced when used in combination with the EGFR blocking drug erlotinib. Furthermore, triple treatment of adding CK2 to erlotinib and dabrafenib was effective for some cell lines and could be further investigated given encouraging outcomes of pilot clinical testing using combinations of EGFR and BRAF inhibition in patients with metastatic *BRAF* V600E CRC (11).

Colon cancer has a strong reliance on exploiting the pro-growth PI3K/AKT signalling pathway and several pre-clinical studies have investigated the use of dual PI3K/mechanistic target of rapamycin (mTOR) and mitogen-activated protein kinase kinase 1/2 (MEK1/2) inhibitors as single agents and in combination therapy in models of human cancer (12-16), although only limited reports have focused specifically on *BRAF*-mutant colon cancer (6, 7). Here, it was of interest to determine whether CK2 inhibition could potentiate the effects of PI3K inhibition given we had previously elucidated that CK2 exerts its effects independently on AKT to regulate its priming (8, 17), which facilitates subsequent phosphorylation by pyruvate dehydrogenase kinase 1 (PDK1) and mTOR complex-2. Cell viability assays following 3-day exposure to the ATP-competitive PI3K α/δ inhibitor GDC0941 was highly effective in reducing proliferation in all colon cancer models tested and indeed was more effective than single-agent dabrafenib for -mutant colon cancers. Subsequent addition of CX4945 with GDC0941 successfully potentiated the response in *KRAS*-mutant, *BRAF*-wild-type SW480 cells and *BRAF*-mutant HT29 and Colo205 cells, but not *BRAF*-mutant/*PTEN*-null Lim2405 cells. Similar observations were seen when GDC0941 was combined with dabrafenib, except in the *BRAF* wild-type SW480 cells, where this approach activated MAPK signalling through established mechanisms (18).

Clinical testing of controlling the PI3K/AKT signalling axis in *BRAF* CRC is very limited and our findings further support deeper evaluation of therapeutic targets within this pathway as is being carried out for numerous cancer types (19). van Geel and colleagues recently reported a phase Ib/II study evaluating the RAF inhibitor encorafenib in combination with cetuximab for EGFR blocking, with and without the PI3K α inhibitor alpelisib in *BRAF*-mutant metastatic CRC (20). The overall response rates were 19%

Figure 3. A: Western blot for AKT serine/threonine kinase (AKT) pathway activity following 30-min treatment with phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) inhibitor GDC0941 (4 μ M). B: Cell viability following 72-h exposure to GDC0941, serine/threonine kinase B-Raf proto-oncogene (BRAF) inhibitor (dabrafenib; 15 nM), casein kinase 2 (CK2) inhibitor (CX4945; 4 μ M) and combinations thereof (n=8). Data are mean values $\pm$ SD. Significantly different at * p <0.001 for GDC0941 versus GDC0941/dabrafenib and GDC0941 versus GDC0941/CX4945; ns, not significantly different.

and 18% in the two-and three-drug arms, with a median progression-free survival of 3.7 and 4.2 months, respectively. Unfortunately, the maximum tolerated dose was not reached in either arm in this heavily pre-treated cohort and the addition of PI3K inhibitor did not improve overall response rates compared to the doublet combination.

Our investigations showed a role for CK2 inhibition in potentiating the inhibitory effects of GDC0941 in *BRAF*-mutant and wild-type colon cancer cell lines. We found this combination to be superior to targeting MAPK activity using either BRAF or EGFR inhibition, combinations of

which have produced modest clinical responses in metastatic *BRAF* V600E colorectal cancer (21). *In vivo* studies are now required to further determine efficacy and toxicity associated with PI3K/CK2 combinations for treating colorectal cancer.

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