Dabrafenib, a selective BRAFV600E inhibitor, is not able to reverse the CpG island methylator phenotype in RKO colorectal cell line

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Abstract

**Background:** Colorectal cancer is considered as one of the most common death causes among cancer types in the developed countries. Methylation in the promoter of genes shows specific patterns, which define the molecular pathogenesis and prognosis of the cancer. Therefore, reversal of DNA methylation constitutes a potential therapeutic target. Coexistence of B-RAF V600E mutation with hypermethylation in the promoter of specific genes and chromosomal instability characterize the serrated pathway of carcinogenesis in colorectal cancer and has been associated with poor prognosis. The purpose of this study was to investigate if inhibition of BRAF V600E mutation by the selective inhibitor Dabrafenib in the RKO cell line has any effect on the methylation phenotype of the Weisenberger’s CIMP panel genes.

**Materials and methods:** RKO cancer cell line was cultured under various conditions of Dabrafenib concentrations, time of treatment, cell passage and culture medium provision. Cells from every condition were counted and the subsequently extracted DNA was modified using sodium bisulfate. The characterization of the methylation phenotype was performed by MS-PCR analysis. Modified genomic DNA from Caco2 cancer cell line was used as a control.

**Results:** Dabrafenib treatment resulted in a 50% inhibition of cell growth rate, independent of the concentration used and has no effect on the methylation status of the genes tested under all conditions.

**Conclusions:** Inhibition of the B-RAFV600E by Dabrafenib was not able to reverse the CIMP phenotype in the RKO cell line.

**Keywords:** Colorectal cancer, Dabrafenib, CIMP phenotype, B-Raf inhibitor, RKO cell line
Introduction
Colorectal cancer (CRC) is widely recognized as one of the most frequent causes of cancer related morbidity and mortality, particularly in western world, (1). As highly heterogeneous disease, CRC has been divided into subgroups according to genetic and epigenetic aberrations, including BRAF or KRAS mutation, chromosomal instability (CIN), microsatellite instability (MSI) and CpG island methylator phenotype (CIMP) (2). BRAF mutations harbor in approximately 10% of sporadic CRCs leading to up-regulation of MAPK signal pathway and, as a consequence, dysregulation of cell differentiation, growth and apoptosis (3). Aberrant methylation of CpG islands in the promoter’s region of tumor suppressor genes leading gene silencing is a common phenomenon during the process of carcinogenesis (4,5). CRC cell lines have been used extensively to map the molecular mechanisms of carcinogenesis as they, according to integrated studies, demonstrate similar genetic and epigenetic aberrations to primary tumors (6-8). During the last years, research is focused on the serrated neoplasia pathway characterizing the progression of sessile serrated adenomas or traditional serrated adenomas into invasive adenocarcinomas (9-11). This particular molecular pathway of carcinogenesis is associated with female in older age predominance, proximal colon location, BRAF mutation, microsatellite instability (MSI), CIMP high (CIMP-H) and poor differentiation (9). Prognosis and overall survival of these tumors with MSI and CIMP-H, harboring BRAF mutation is poor making an effective targeted molecular therapy crucial (2,12,13). Until recently, the everyday clinical practice for BRAF mutated CRC consisted of chemotherapy regimens as FOLFOX (5-FU, leucovorin and oxaliplatin) plus the anti-VEGF agent bevacizumab, FOLFIRI (5-FU, leucovorin and irinotecan) plus bevacizumab or FOLFOXIRI as a combination. However, BRAF mutated CRC has been proved refractory to standard chemotherapy compared to wild type BRAF CRC. In terms of an effective treatment against BRAF mutated CRC, a variety of BRAF inhibitors like sorafenib, PLX4720, vemurafenib, encorafenib and Dabrafenib, have been tested as monotherapy by assessing MAPK signal pathway downregulation as well as cell and/or tumor growth inhibition (1, 14-16). Although, an initial downregulation of MAPK pathway is to be seen, this effect fades because of feedback reactivation of MAPK pathway through EGFR overexpression and PI3K pathway upregulation (17). Currently, a variety of pending clinical studies investigate the therapeutic effect of molecular targeted therapy by using BRAF (Dabrafenib, Vemurafenib, Encorafenib),
MEK (Trametinib) and EGFR (Panitumumab, Cetuximab, Erlotinib) inhibitors as a combination for more effective and durable downregulation of MAPK signaling ([18], [19]). These combined molecular target therapies are under investigation and they are not yet approved by Food and Drug Administration (FDA) for CRC treatment as for melanoma. According to studies, BRAF mutation, in these sessile serrated adenomas of right colon, is highly associated with CIMP-H although a causal association between them has not been proven yet. On the other side, CIMP-H has been suggested to induce MSI through hypermethylation of MLH1 promoter leading to mismatch-repair deficiency (MMR deficiency) ([14]). The purpose of this study was to investigate current implications, that BRAF mutation directly induces hypermethylation and CIMP-H ([15], [20], [21]).

Our aim was to inhibit the mutant BRAF V600E in the CRC cell line RKO using the potent BRAF inhibitor Dabrafenib in order to study the impact on CIMP phenotype.

**Material and Methods**

**Cell lines and culture conditions**

RKO colorectal cancer cell line characterized by the presence of two mutant alleles of BRAF V600E mutation, CIMP-H and MSI, and Caco2 cell line characterized by with type BRAF, CIMP negative and microsatellite stability, MSS were used. The CRC cell lines RKO and Caco-2 were kindly provided by A. Pinztas (National Hellenic Research Foundation, Greece).

Cells were grown at 37°C in a water-saturated atmosphere containing 5% CO₂ in DMEM supplemented with supplemented with 100 units/mL penicillin, 100 mg/L streptomycin and 10% FBS (Gibco, Thermofisher Scientific).

**Treatment with BRAF inhibitor Dabrafenib**

Dabrafenib, a selective inhibitor of mutant BRAF (Dabrafenib, GSK2118436, Selleckchem, Houston, Texas, USA), was dissolved in dimethylsulfoxide (DMSO). All experiments have been performed in duplicates. RKO cells (250.000) were seeded on 6-wells and four hours later the medium was replaced with fresh media, supplemented with 1 μM or 10 μM Dabrafenib. The final concentration of DMSO, in
medium didn’t exceed 0.1% (v/v). There were four different conditions of treatment. In the first condition cells were treated with solvent, 1µM Dabrafenib or 10µM Dabrafenib for three days. In the second condition cells were treated with solvent, 1µM Dabrafenib or 10µM Dabrafenib for 7 days by adding fresh agent every 72 hours. In the third condition after 72 hours cells passaged 1:4 for DMSO, 1:2 for 1µM Dabrafenib and 1:2 for 10µM Dabrafenib in order to reduce growth inhibitory effect of cell confluency and treated with agent for total seven days. Finally, in the fourth condition, cells were treated with DMSO, 1µM Dabrafenib and 10µM Dabrafenib for 7 days.

Genomic DNA isolation, DNA conversion and Methylation-Specific PCR for CIMP status characterization

Genomic DNA extraction was performed using DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) according to manufacturer’s protocol. Genomic DNA samples (1µg each) were treated with bisulfite using the Zymo EZ DNAmethylation kit (Zymo Research, Irvine, California). To characterize the CIMP status of treated RKO cells Weisenberger’s CIMP panel containing the following genes; CACNA1G, IGF2, NEUROG1, RUNX3, and SOCS1, was utilized (22). Primers and Methylation-Specific PCR (MS-PCR) protocol for these genes were used according to protocol described by Lee et al (23). Polymerase chain reaction products were electrophoresed in 2% agarose gels and visualized under ultraviolet illumination after ethidium bromide staining.

Results

Cell growth inhibition of RKO cell line

Concerning cell growth inhibition, Dabrafenib, in both concentrations 1µM and 10µM, showed efficient inhibitory effect as it reduced cell growth rate by approximately 50% in both concentrations compared to DMSO. The same effect was observed in both 3- and 7-day treatment without medium change (Figure1). However, no significant difference between the two concentrations of the drug, regarding
growth inhibition rate, was ascertained. After 3-day treatment 1,925,000 cells for DMSO, 1,075,000 cells for 1μM Dabrafenib and 1,110,000 cells for 10μM Dabrafenib were counted on average. After 7-day treatment 2.100.000 cells for DMSO, 920.000 cells for 1μM Dabrafenib and 1,105,000 cells for 10μM Dabrafenib were counted on average. Dabrafenib had no effect on Caco-2 growth/proliferation (data not shown).

**Characterization of CIMP after BRAF inhibition in RKO cell line**

In order to define CIMP phenotype after Dabrafenib induced BRAF inhibition in RKO cells, Weisenberger’s 5-marker CIMP panel was used. Caco-2 cell line was used as a negative control in all promoter methylation analysis experiments.

For the characterization of the CIMP phenotype, the Weisenberger criteria were used, according to which ≥3 of the 5 genes (CACNA1G, IGF2, NEUROG1, SOCS1 and RUNX3) positive for methylation constitute the CIMP + phenotype whereas ≤2 of the 5 genes constitute the CIMP- phenotype. After the intervention with Dabrafenib, no interventional condition reversed the CIMP phenotype. After 3-day treatment with DMSO, 1μM or 10μM Dabrafenib (samples 1-3), there is no change in the methylation status of CACNA1G, NEUROG1, RUNX3 and IGF2 gene promoters, all tested sequences are methylated, while the SOCS1 gene remains unmethylated (4/5, CIMP +). In the 7-day treatment with no change of nutritional medium, addition of DMSO, 1μM or 10μM Dabrafenib every 3 days (samples 4-6), methylation is also observed in the genes CACNA1G, NEUROG1, RUNX3 and IGF2, while the SOCS1 gene remains unmethylated (4 / 5, CIMP +). At 7 days, after dissection of the plate and change of nutrient, DMSO and Dabrafenib every 3 days (samples 7-9), the CACNA1G, NEUROG1, RUNX3 and IGF2 genes remained methylated, while the SOCS1 gene was unmethylated (4/5, CIMP +). Finally, in the cells, which were maintained in the original dish with change of nutritive material, DMSO and Dabrafenib every 3 days (samples 10-12), methylation was also found in the genes CACNA1G, NEUROG1, RUNX3 and IGF2, while the SOCS1 gene remained unmethylated (4/5, CIMP +). In Caco-2 cells, the genes tested were unmethylated (0/5, CIMP-). The results of MS-PCR are set forth in Figure 2 (A-E).

Discussion
It is generally acceptable that CRC, with its raising incidence in general population, demonstrates a high heterogeneity concerning its deregulated molecular pathways and epigenetic and/or genetic aberrations (22). A particular subtype of CRC, characterized by BRAF mutation, MSI and CIMP-H, progresses highly aggressive with poor prognosis and shows resistance to current chemotherapy regimens such as FOLFOX and FOLFIRI (24). Treatment of BRAF mutated CRC remains a challenge as no current monotherapy has a sustainable effect on tumor inhibition. Recent research and clinical trials focus on combined molecular targeted therapy of CRC with BRAF, MEK and EGFR inhibitors, which shows a more effective downregulation of MAPK pathway (18, 19). RKO cancer cell line, selected for our experiment, assembles the same molecular features with hypermethylated BRAF mutated CRC (2). In a recent systematic review of literature, Jia et al. cited that variety of existing CIMP panels and cutoffs is responsible for discrepancies among the published results rendering the definition of CIMP problematic (25). Although Ogino et al. suggested that the 8-marker CIMP panel shows the highest sensitivity and specificity for CIMP-H detection, Kaneda et al. showed through a comprehensive analysis that Weisenberger’s 5-marker CIMP panel can efficiently detect CIMP-H tumors, and that is the reason we used the 5-marker CIMP panel (26, 27). Inhibition of BRAF V600E mediated with agent Dabrafenib has been shown to efficiently inhibit phosphorylation of the B-Raf downstream targets Mek1/2 and Erk 1/2 (1). In cell lines encoding BRAFV600E mutation, Dabrafenib inhibited pERK and pMEK in a concentration-dependent manner with IC50 values of 3nM and 6nM, respectively. RKO cell line seems to require higher concentrations of this RAF inhibitor due to additional mutations of PI3K signal pathway in order to overcome tumor cell’s resistance to growth inhibition (16, 28). Most studies so far have focused either on cell growth inhibitory effect of BRAF inhibitors or their tumor suppressive efficiency without investigating their impact on CIMP status. Our experiment hints that BRAF inhibitor Dabrafenib is not able to reverse CIMP status in the CRC RKO cell line. Future perspectives of these MAPK signal pathway inhibitors require more studies, where a comparative analysis of multiple RAF inhibitors in different concentrations and durations of treatment will take place in order to clarify the association of BRAF mutation with CIMP status in CRC. Our future goal is to trace a potential reversal of CIMP status through BRAF inhibition by assessing more or combinations of BRAF inhibitors and a wider range of CIMP genes as well as different treatment conditions.
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Conflict of interest
The authors declare no conflicts of interest.
References


Figures and Tables

**Figure 1.** Cell growth inhibition after 3-day and 7-day treatment with DMSO (blue), 1μM Dabrafenib (pink), or 10μM Dabrafenib (yellow).
**Figure 2.** Agarose gel electrophoresis of MS-PCR amplicons from: (A) CACNA1G (the expected sizes of methylated (m) and unmethylated (u) products are 104 and 100 bp, respectively), (B) SOCS1 (expected sizes of methylated and unmethylated products are 106 and 116 bp, respectively), (C) RUNX3 (expected sizes of methylated and unmethylated products are 104 and 120 bp, respectively), (D) NEUROG1 (expected sizes of methylated and unmethylated products are 118 and 119 bp, respectively), (E) IGF2 (expected sizes of methylated and unmethylated products are 94 and 120 bp, respectively).

(1-3: 3-day treatment with solvent, 1μM Dabrafenib and 10μM Dabrafenib respectively, 4-6: 7-day treatment with DMSO, 1μM Dabrafenib and 10μM Dabrafenib respectively without refreshing culture medium, 7-9: 7-day treatment with DMSO, 1μM Dabrafenib and 10μM Dabrafenib respectively, 10-12: 7-day treatment with DMSO, 1μM Dabrafenib and 10μM Dabrafenib respectively with refreshing of agent and culture medium, 13: Caco-2 cells without treatment, L: Ladder 100 bp, m:methylated, u:unmethylated)