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BMX Acts Downstream of PI3K to Promote Colorectal Cancer Cell Survival and Pathway Inhibition Sensitizes to the BH3 Mimetic ABT-737[1,2]

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Abstract

Evasion of apoptosis is a hallmark of cancer, and reversing this process by inhibition of survival signaling pathways is a potential therapeutic strategy. Phosphoinositide 3-kinase (PI3K) signaling can promote cell survival and is upregulated in solid tumor types, including colorectal cancer (CRC), although these effects are context dependent. The role of PI3K in tumorigenesis combined with their amenability to specific inhibition makes them attractive drug targets. However, we observed that inhibition of PI3K in HCT116, DLD-1, and SW620 CRC cells did not induce apoptotic cell death. Moreover, these cells were relatively resistant to the Bcl-2 homology domain 3 (BH3) mimetic ABT-737, which directly targets the Bcl-2 family of apoptosis regulators. To test the hypothesis that PI3K inhibition lowers the apoptotic threshold without causing apoptosis *per se*, PI3K inhibitors were combined with ABT-737. PI3K inhibition enhanced ABT-737–induced apoptosis by 2.3- to 4.5-fold and reduced expression levels of MCL-1, the resistance biomarker for ABT-737. PI3K inhibition enhanced ABT-737–induced apoptosis a further 1.4- to 2.4-fold in CRC cells with small interfering RNA–depleted MCL-1, indicative of additional sensitizing mechanisms. The observation that ABT-737–induced apoptosis was unaffected by inhibition of PI3K downstream effectors AKT and mTOR, implicated a novel PI3K-dependant pathway. To elucidate this, an RNA interference (RNAi) screen of potential downstream effectors of PI3K signaling was conducted, which demonstrated that knockdown of the TEC kinase BMX sensitized to ABT-737. This suggests that BMX is an antiapoptotic downstream effector of PI3K, independent of AKT.

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Introduction

Single-agent treatments rarely prove sufficient for cancer cure. This is in part due to a variety of innate or acquired, drug-specific, or pleiotropic drug resistance mechanism(s), one of which is suppression of drug-induced cell death. Consequently, there is considerable motivation to overcome drug resistance mechanisms by identifying rational combinations of molecular targeted drugs. To aid this, the US Food and Drug Administration (FDA) is considering early-phase drug combination trials without the necessity for prior single-agent approval [1]. Historically, choice of drug combinations is predicated

Abbreviations: CRC, colorectal cancer; PI3K, phosphoinositide 3-kinase; PtdIns(3,4,5)P_3, phosphatidylinositol-3,4,5-triphosphate; PH, pleckstrin homology; siRNA, small interfering RNA; SRB, sulforhodamine B

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on nonoverlapping drug toxicities; however, the concept of targeting multiple different hallmarks of cancer is an emerging approach [2]. Two such hallmarks are sustained inappropriate proliferative signaling and suppression of apoptotic cell death [2]. Molecular features that contribute to these hallmarks in several human tumors, including colorectal cancer (CRC), are aberrations in the phosphoinositide 3-kinase (PI3K) signaling pathway and up-regulation of antiapoptotic Bcl-2 family proteins. This study examines the combined effect of inhibiting PI3K signaling and interrupting the protein-protein interactions between proapoptotic and antiapoptotic members of the Bcl-2 family in CRC cells.

PI3K phosphorylates the 3-hydroxy group of phosphatidylinositol lipid rings to generate a secondary messenger that is implicated in many intracellular signaling pathways. The most studied are the class I PI3Ks, which phosphorylate phosphatidylinositol-4,5-bisphosphate generating phosphatidylinositol-3,4,5-triphosphate [PtdIns(3,4,5)P_3] [3]. PtdIns(3,4,5)P_3 is a docking site for a number of proteins that contain PtdIns(3,4,5)P_3-binding motifs such as pleckstrin homology (PH) domains, frequently leading to activation of the docked protein. The best characterized effectors of the pathway are phosphoinositide-dependent kinase 1 (PDK1) and AKT (AKA protein kinase B). PDK1 and AKT bind to PtdIns(3,4,5)P_3, allowing PDK1 to phosphorylate and activate AKT [4]. Activation of AKT has multiple cell fate outcomes including increased cell survival, sustained cell proliferation, and enhanced cell migration, all of which have potential to promote oncogenesis [5]. Aberrant PI3K signaling is implicated in many cancer types. For example, loss of the PI3K antagonistic phosphatase PTEN and activating mutations in *PIK3CA*, the gene encoding the catalytic PI3K subunit p110α, are among the most common genetic aberrations in cancer [6]. Consequently, the PI3K signaling pathway is a major focus of drug discovery programs, with multiple small-molecule inhibitors targeting PI3K, AKT, and other PI3K pathway components undergoing clinical trials [6]. Multiple lines of preclinical evidence suggest that PI3K signaling acts to suppress apoptosis through mechanisms including the modulation of Bcl-2 family proteins that control the release of potent apoptogens from mitochondria [5]. However, despite this body of evidence, apoptosis is not induced in many cancer cell lines after specific inhibition of PI3K pathway signaling [7,8]. Furthermore, emerging evidence shows that, whereas in some cell types, combining PI3K inhibition with conventional chemotherapeutic agents induces apoptosis [9,10], this is not the case with CRC cells [7], a disease where aberrant PI3K activation is common.

Drug development efforts to disrupt interactions between proapoptotic and antiapoptotic proteins of the Bcl-2 family yielded the Bcl-2 homology domain 3 (BH3) mimetic class of drugs [11]. The "poster-child" BH3 mimetic ABT-737 and its related clinical candidate navitoclax readily induce apoptosis in small cell lung cancer *in vitro* and *in vivo* as a single agent [12] and kill lymphoma cell lines and primary lymphoma cells *ex vivo* [12,13], and navitoclax has demonstrated promising results in a phase I clinical trial in patients with chronic lymphocytic leukemia [14]. However, in several cancer cell types, including CRC, ABT-737 treatment alone does not induce apoptosis at clinically relevant concentrations [15]. In a broad range of cancer cell types, ABT-737 acts synergistically with a variety of conventional and novel chemotherapeutic agents [16], including agents that target the PI3K pathway [17,18]. This suggests that a lowering of the apoptotic threshold by ABT-737 facilitates the coupling of drug-induced damage and/or the interruption of survival signaling events to the commitment to apoptotic cell death. Therefore, the hypothesis tested in this study was that PI3K pathway ablation using small-molecule inhibitors could "prime" CRC cells for apoptosis but that cell death would only be realized if the actions of antiapoptotic Bcl-2 family proteins were negated by a BH3 mimetic.

Materials and Methods

Cell Culture and Drugs

HCT116, DLD-1 [American Type Culture Collection (ATCC), Manassas, VA], and isogenic pairs of HCT116 and DLD-1 expressing only wild-type or mutant PIK3CA (a kind gift from B. Vogelstein) were cultured in McCoy's 5A media (Life Technologies, Inc, Paisley, United Kingdom) supplemented with 10% FBS (BioWest, Nuaillé, France). SW620 (ATCC) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FBS and glutamine (Life Technologies, Inc). All cells were maintained in a humidified atmosphere at 37°C and 5% CO_2. Cell lines were authenticated using the AmpFlSTR system (Applied Biosystems, Paisley, United Kingdom) during the study. ABT-737 (a kind gift from AbbVie, Chicago, IL), PI-103, rapamycin, Akti1/2, KU-0063794 (Merck, Nottingham, United Kingdom), GDC-0941, MK-2206, and PCI-32765 (Selleck Chemicals, Houston, TX) were all dissolved to 10 mM in DMSO (Sigma, Dorset, United Kingdom) and stored as single use aliquots at −20°C/−80°C (Figure W1).

Concentration Response

Cells were seeded into 96-well plates. After 24 hours, cells were treated with the indicated concentration of drug(s) and cultured for a further 72 hours in the presence of drug(s). Plates were stained with sulforhodamine B (SRB) and processed as previously described [7] to give an indication of cellular biomass. To determine logGI_{50}, log drug concentration was plotted against raw absorbance, and nonlinear curve fit analysis was performed (GraphPad Prism; GraphPad Software, La Jolla, CA). Statistical analysis was carried out on three independent logGI_{50} readings and transformed to growth inhibition 50 (GI_{50}) for presentation. For display purposes only, drug concentration (log scale) has been plotted against normalized absorbance.

Western Blot Analysis

Cell lysis and Western blot analysis were carried out as previously described [7]. The following primary antibodies were used: rabbit anti-pS473AKT (No. 4058), rabbit anti-AKT (No. 9297), rabbit anti-pT246 40-kDa proline-rich AKT substrate (PRAS40) (No. 2997), rabbit anti-PRAS40 (No. 2691), pS240/244S6 (No. 4838), rabbit anti-S6 (No. 2217), rabbit anti–cleaved caspase 3 (No. 9661), rabbit anti-PARP (No. 9542), rabbit anti-Bax (No. 2774; all from Cell Signaling Technology, Danvers, MA), mouse anti–Bcl-2 (M0887; Dako, Glostrup, Denmark), rabbit anti–BCL-XL (No. 610211; Becton Dickinson, Oxford, United Kingdom), mouse anti-human MCL-1 (No. 559027; Becton Dickenson), rabbit anti–MCL-1 (sc819; Santa Cruz Biotechnology, Inc, Dallas, TX), rabbit anti-Bad (AF819; R&D Systems, Minneapolis, MN), rabbit anti-Bim (No. 202000; Merck), mouse anti-Bak (AM03; Merck), mouse anti–α-tubulin (CP06; Merck), and mouse anti-GAPDH (G9545; Sigma).

Measurement of Apoptosis

Annexin V/7-aminoactinomycin D (7AAD) flow cytometry was performed as previously described [7]. For assessment of Bak conformational change, cells were cultured in a 96-well plate and