Bcl-xl and Mcl-1 are the major determinants of the apoptotic response to dual PI3K and MEK blockage

ELINA JOKINEN and JUSSI P. KOIVUNEN

Department of Medical Oncology and Radiotherapy and Medical Research Center Oulu, Oulu University Hospital and University of Oulu, Oulu, PB22 90029 OYS, Finland

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Abstract. The dual targeting of PI3K-AKT-mTOR and Ras-Raf-MEK-ERK pathways is a potential anticancer therapy, but unfortunately, the response rate has been low in early phase clinical trials. Pre-clinical models have suggested that an apoptotic response to dual PI3K and MEK targeting is relatively rare and understanding apoptotic avoidance could lead to increased clinical efficiency. This study investigated solid cancer cell lines, which are known to be sensitive to dual PI3K and MEK inhibition but to have a limited apoptotic response. The cells were exposed to dual PI3K and MEK blockage in combination with a panel of additional pharmacological agents and cytotoxicity and apoptosis were analyzed. Our results indicated that the BH3 mimetic ABT-263, the HDAC inhibitor entinostat and the multikinase inhibitor dasatinib increased the cytotoxicity and apoptotic response of dual PI3K and MEK targeting. Furthermore, ABT-263 and entinostat was able to induce apoptosis in combination with single agent PI3K and MEK inhibitors. Protein expression, immunoprecipitation and siRNA knockdown models suggested that Bcl-xl and Mcl-1 were the most important factors circumventing PI3K and/or MEK inhibition-mediated apoptosis. The results suggest that the cytotoxicity of PI3K and/or MEK inhibitor treatments can be augmented by combinatory approaches targeting anti-apoptotic mediators Bcl-xl and Mcl-1.

Introduction

In solid malignancies, the PI3K-AKT-mTOR and Ras-Raf-MEK-ERK pathways have been identified as the most important oncogenic pathways. Considering the central role of the pathways in transmitting upstream oncogenic signals, their inhibition could be an effective therapy as regards various cancer genotypes (1).

The clinical efficiency of single pathway inhibitors of PI3K-AKT-mTOR and Ras-Raf-MEK-ERK has generally been disappointing, with some exceptions such as their use in cases of B-Raf mutant melanoma. Cancers can be de novo-dependent concurrently on these parallel pathways and cross-signaling of the pathways is also evident (2,3). Many in vivo and in vitro studies have shown that the PI3K-AKT-mTOR and Ras-Raf-MEK-ERK pathways regulate each other's activity through feedback mechanisms and have shared downstream targets (4,5). Interaction of the PI3K-AKT-mTOR and Ras-Raf-MEK-ERK signaling pathways is thought to explain the inefficiency of single agent treatments and provide a rationale for the concurrent targeting of both pathways.

Preclinical studies have shown that dual targeting with PI3K and MEK inhibitors has antitumor activity in various cancer models and genotypes (6-8). Many preclinical studies have concerned predictive factors as regards dual PI3K and MEK inhibitor therapy, but so far no clear factors have been identified (8,9). Numerous early-phase clinical studies concerning dual PI3K and MEK targeting are ongoing and some results have recently been presented. Generally, combined PI3K and MEK inhibitor therapy seems to be feasible but, unfortunately, the rate of response is low (10-12). In preclinical models, the vast majority of cancer cell lines do not show apoptosis in response to dual PI3K and MEK targeting, which could be a major factor behind the limited clinical activity of the approach (8,9). Some recent investigations have revealed that drugs affecting the apoptotic pathways, such as BH3 mimetics, could dramatically increase the rate of apoptosis and the efficiency of MEK and/or PI3K/mTOR inhibitors (13,14). Bcl-2 family members have been suggested to be important determinants of cell fate in targeted cancer therapies. Induction of pro-apoptotic proteins such as BIM is often linked to apoptosis, while anti-apoptotic proteins such as Mcl-1 and Bcl-xl promote cell survival. The general balance between anti- and pro-apoptotic mediators is crucial for the determination of cell survival or apoptosis (15).

The current study builds upon our earlier work (9) where we identified three cell lines from solid tumors, which showed sensitivity to dual PI3K and MEK blockage but with limited apoptotic response. In this study, we investigated in vitro with
these cell lines if an additional agent could increase efficiency and apoptotic response to dual PI3K and MEK blockage. We identified some pharmacological agents that could enhance the cytotoxicity of dual PI3K and MEK blockage, and more importantly, induce marked apoptosis. Furthermore, Bcl-xl (Bcl2L1) and Mcl-1 were identified as being important determinants of cell fate.

Materials and methods

Cell lines. The cell lines used in the current study included the triple-negative NSCLC (non-small cell lung cancer) cell line H1437, the basal-like breast cancer line MDA-MB231 and the K-Ras mutant colorectal cell line HCT116. The NSCLC cell line H1437 was a kind gift from Dr Pasi Jänne (Dana-Farber Cancer Institute, Boston, MA, USA) and the breast and colorectal lines (MDA-MB231 and HCT116) were from Dr Peppi Koivunen (Oulu University, Oulu, Finland). The cell lines were cultured in RPMI-1640 supplemented with fetal bovine serum (10%) plus penicillin and streptomycin (100 IU/ml). All the cell culture reagents were purchased from HyClone (Logan, UT, USA).

Inhibitors. Pharmacological agents used in the study and their final concentrations are listed in Table I. The drugs were dissolved in DMSO to a concentration of 10 mmol/l, except for cisplatin, which was diluted in distilled water. All the agents were stored in aliquots at -20°C. Further dilutions were made in the cell culture medium.

MTS cell viability/cytotoxicity analysis. Cells were plated onto 96-well plates with three or six parallel wells for each treatment, the experiments being replicated at least twice. The following day the cells were drug treated for 72 h, after which the plates were developed using an MTS reagent mix ((3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt), Promega, Madison, WI, USA) and treated with the drugs 24 h later for the desired time, after which the cells were plated on 96- (for MTS) or 6-well (for western blotting) plates and drug treated, the experiments being replicated at least twice.

Western blot analysis. The cells were plated onto 6-well plates and treated with the drugs 24 h later for the desired time, after which they were lysed in RIPA buffer [1% Igepal CA-630, 20 mM Tris-HCl pH 8.0, 137 mM NaCl, 10% glycerol, 2 mM EDTA, 1 mM sodium orthovanadate, aprotinin (10 µg/ml), leupeptin (10 µg/ml) and pepstatin (10 µg/ml)]. Protein concentrations were measured by Bio-Rad Protein Assay. Protein (200 µg) at a concentration of 1 mg/ml was incubated overnight at 4°C with primary antibody at a 1:1000 concentration in 5% BSA. Anti-mouse (β-actin) or anti-rabbit (others) IgG HRP-linked antibody was used as the secondary antibody. All antibodies were from Cell Signaling Technology (Danvers, MA, USA).

Immunoprecipitation. For immunoprecipitation, cells were plated on 6-well or 10-cm diameter plates and drug treated the following day for the desired time, after which the cells were lysed in RIPA buffer and protein concentrations were determined by Bio-Rad Protein Assay. Protein (200 µg) at a concentration of 1 mg/ml was incubated overnight at 4°C with primary antibody at a 1:100 concentration. The next day protein A/G agarose beads (Santa Cruz Biotechnology, Dallas, TX, USA) were added to each sample and incubated for 1-3 h at 4°C, after which the samples were pelleted by centrifugation. The pellets were washed with cell lysis buffer, resuspended in Laemmli buffer and analyzed with western blotting.

siRNA knockdown. For the knockdown studies, BCL-XL (BCL2L1) and MCL-1 (MCL1) specific siRNAs (Dharmacon smart pools) were used at 25 nmol/l and transfected with DharmaFECT transfection reagent (both from Dharmacon, Lafayette, CO, USA). The experiments were performed according to the manufacturer's protocol. In short, the cells were plated on 96- (for MTS) or 6-well (for western blotting) plates in non-antibiotic media. After 24 h the medium was changed...

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Target/Class</th>
<th>Concentration in µM</th>
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<tr>
<td>ABT-263</td>
<td>BH3</td>
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<tr>
<td>Afinib</td>
<td>HER2</td>
<td>1</td>
</tr>
<tr>
<td>AG-1024</td>
<td>IGF1R1</td>
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<td>AZD-2281</td>
<td>PARP</td>
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<tr>
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<tr>
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<td>ALK</td>
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<td>Multi-TKI</td>
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<td>γ-Secretase</td>
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Table I. Agents studied in combination with dual PI3K and MEK inhibition.
to medium containing siRNA+lipid, or scramble siRNA+lipid (controls), prediluted in Opti-MEM (Gibco/Invitrogen, Carlsbad, CA, USA). The following day, the medium was changed to normal medium. Incubation was continued for the desired time (total of 24–96 h) before assays.

**Statistical analysis.** Student's two-tailed t-test was used for the statistical analysis. For the analysis, percentage change compared to PI3Ki+MEKi treated (Fig. 1) or scramble siRNA treated cells (knockdown studies) was used. p-values <0.001 and <0.01 are indicated.

**Results**

**ABT-263, entinostat and dasatinib increase the cytotoxicity of dual PI3K and MEK inhibition.** The effect of combining dual PI3K and MEK inhibition with other pharmacological agents was studied in HCT116 (colon cancer), MDA-MB231 (breast cancer) and H1437 (NSCLC) cell lines, which are known to be sensitive to dual PI3K and MEK inhibition but to show limited apoptotic responses to the treatments (9). The cells were exposed to dual PI3K and MEK inhibition (ZSTK474 and CI-1040) in combination with a panel of other small-molecule inhibitors and cell viability was analyzed by using 72-h MTS cytotoxicity assays (Table I). The concentrations chosen for each drug were based on our preliminary experiments, where we used MTS assay to determine the lowest concentration causing the maximal cytotoxic effect (not shown). Of the 21 tested drugs, the BH3 mimetic ABT-263, the HDAC inhibitor entinostat and the multikinase inhibitor dasatinib were observed to decrease cell viability compared with dual blockage alone, excluding the H1437 line and ABT-263 treatment combination. Of these three drugs, entinostat and dasatinib had single agent cytotoxic activity, while ABT-263 was active only in the MDA-MB231 line. ABT-263, entinostat and dasatinib were chosen for further studies.

We next assessed whether concurrent dual blockage is needed for cytotoxicity of the other agent or if single PI3K or
MEK inhibition is sufficient, using MTS cytotoxicity assays. In the HCT116 line, ABT-263 was seen to decrease viability in combination with PI3K inhibition (PI3Ki) but not with MEK inhibition (MEKi). Conversely, when HCT116 cells were treated with entinostat or dasatinib, concurrent administration of either PI3Ki or MEKi decreased cell viability (Fig. 1A). In the MDA-MB231 line, a marked increase in cytotoxicity was seen with concurrent administration of ABT-263 or dasatinib with ZSTK474 or CI-1040. Synergistic cytotoxicity was also seen with entinostat co-administration with PI3Ki but not with MEKi (Fig. 1B). In the H1437 line, entinostat was the only agent noted to increase cytotoxicity in combination with either PI3Ki or MEKi, while no marked change was seen with ABT-263 or dasatinib combinations (Fig. 1C).

ABT-263, entinostat and dasatinib increase apoptotic responses to dual PI3K and MEK inhibition. Next we investigated whether the cytotoxic responses seen in MTS assays are accompanied by apoptosis. Western blot analysis of PARP (cPARP) in response to treatment with ZSTK474 (3.3 µM), CI-1040 (1 µM), ABT-263 (1 µM), entinostat (1 µM) or dasatinib (0.1 µM) or their combinations for 6 h (MDA-MB231 cells), 48 h (HCT116 cells) or 72 h (H1437 cells). (A) ABT-263-containing treatments. (B) Entinostat-containing treatments. (C) Dasatinib-containing treatments.

Downregulation of Bcl-xl and Mcl-1 correlates with apoptosis in cells treated with PI3K and MEK dual blockage. We further analyzed the effects of the inhibitor treatments on the pro-apoptotic protein BIM and the anti-apoptotic proteins Mcl-1 and Bcl-xl. HCT116 and H1437 lines were selected for the analysis since these cell lines respond to treatment within days and are more reliably assessable as regards expression. Conversely, the MDA-MB231 line undergoes very rapid apoptosis and on the basis of the results of our preliminary experiments, protein levels remain unaltered in this line and therefore we excluded it from these experiments. In HCT116 cells, BIM upregulation was detected in response to all treatments except for co-targeting with ABT-263 or entinostat plus dual PI3K and MEK blockage. Furthermore, there was a tendency for BIM upregulation to occur with single agent MEK inhibition.

In H1437 cells, BIM upregulation correlated with MEK inhibition, but, surprisingly, this was absent when PI3Ki was co-administered. In the HCT116 line, Mcl-1 upregulation was noted in connection with ABT-263 and its combinations and downregulation was seen when dual PI3K and MEK blockage was combined with ABT-263, entinostat, or dasatinib. In the H1437 line, Mcl-1 downregulation correlated with PI3K inhibition. In HCT116 cells, Bcl-xl downregulation was noted in entinostat combinations, and in ABT-263 and dual PI3K and MEK blockage treatments. In the H1437 line, Bcl-xl downregulation was seen with ABT-263 combinations, and with entinostat+PI3Ki and dasatinib+PI3Ki+MEKi. In general, Bcl-xl downregulation does not indicate apoptosis but is required in most cases. As an exception, we did not see Bcl-xl downregulation when the HCT116 line was treated with apoptosis-inducing ABT-263 combinations, but since ABT-263 is an indirect inhibitor of the protein, this is not surprising (Fig. 3A-C).

**BIM heterodimerization with Bcl-xl and Mcl-1 in response to dual PI3K and MEK blockage.** Next, we assessed whether there is a difference in Bcl-2 family member dimers in cells treated with PI3K and MEK inhibitor combination. After drug treatments, the cell lysate were immunoprecipitated with BIM antibody and detected with Mcl-1 or Bcl-xl antibodies. In HCT116, dual PI3K and MEK targeting increased incorporation of Bcl-xl to BIM while Mcl-1 levels remained low. In H1437, we saw an increase in Bcl-xl and Mcl-1 incorporation to BIM, but some increase in the Mcl-1 attachment. In MDA-MB231, we could not detect any increase in Bcl-xl or Mcl-1 attachment to BIM in response to dual targeting. This could be related to the short period of drug treatment and quick
apoptotic response seen with pharmacological Bcl-2/Bcl-xl blockage in this specific cell line (Fig. 3D).

**Knockdown of Bcl-xl expression increases the cytotoxicity of dual PI3K and MEK blockage.** HCT116, MDA-MB231 and H1437 cells were subjected to BCL-XL-specific siRNA knockdown. The cells were first analyzed by western blotting for Bcl-xl expression. In MDA-MB231 cells we saw some downregulation of Bcl-xl after 24- and 48-h treatment, while almost complete absence of the protein was noted following 72- and 96-h treatments. In the HCT116 and H1437 lines, 72- or 96-h treatment with BCL-XL-specific siRNA induced downregulation of Bcl-xl expression, but this, however, was lower in the H1437 line when compared with the MDA-MB231 line (Fig. 4A).

Based on the MTS cytotoxicity assay, siRNA knockdown of BCL-XL was not cytotoxic by itself in any of the tested lines. To evaluated whether BCL-XL knockdown would sensitize the cells to dual PI3K and MEK blockage, the cells were pretreated with or without BCL-XL siRNA for 24-48 h, after which they were exposed to PI3Ki, MEKi, or their combination for an additional 72 h and analyzed by using MTS cytotoxicity assays. Increased cytotoxicity was seen in the MDA-MB231 line with PI3Ki and/or MEKi treatment after BCL-XL knockdown. This was not observed in the other two cell lines tested except for single agent MEKi in the HCT116 cell line (Fig. 4B). We then investigated whether BCL-XL knockdown would increase apoptosis in response to PI3Ki and/or MEKi. In HCT116 cells, PARP cleavage was evident in PI3Ki and more notably, in MEKi treated cells after BCL-XL knockdown, but not in control cells. Furthermore, a marked increase in the cleaved PARP signal was seen after dual PI3K and MEK targeting when BCL-XL was suppressed. In the MDA-MB231 line, PARP cleavage was evident in cells treated with PI3Ki and/or MEKi combination in the cells with BCL-XL knockdown, but not in controls (Fig. 4C). In H1437 cells, we did not see a marked change in the PARP cleavage profile in cells with BCL-XL knockdown as expected (not shown).

Knockdown of MCl-1 expression increases the cytotoxicity of dual PI3K and MEK blockage. Since H1437 line showed increased attachment of Mcl-1 to BIM in response to dual PI3K and MEK targeting and was less sensitive to pharmacological or siRNA blockage of BCL-XL, we assessed whether knockdown of MCl-1 on would increase the cytotoxic and apoptotic response in this cell line. HCT116, MDA-MB231, and H1437 lines were exposed to siRNA-mediated knockdown of MCl-1. Marked decrease of Mcl-1 expression was noted in cells treated with MCl-1-specific siRNA but not with scramble siRNA (Fig. 5A). Based on MTS cytotoxicity assay, siRNA knockdown of MCl-1 was not cytotoxic by itself in any of the tested cell lines. In the HCT116 line, knockdown of MCl-1 had no synergistic cytotoxic effect with the drugs tested. In the MDA-MB231 cell line, MCl-1 knockdown was able to provoke increased cytotoxicity with all the tested agents. The most dramatic difference was seen in MDA-MB231 cells treated with ABT-263 as a single agent where control cells showed some cytotoxicity, which was highly promoted by MCl-1 knockdown. In the H1437 cell line, siRNA knockdown of MCl-1 was able to induce significant cytotoxicity in treatment regimens containing PI3Ki and with MEKi+ABT-263 combination (Fig. 5B).

Next, we analyzed whether MCl-1 knockdown would lead to apoptotic response in H1437 and MDA-MB231 lines. In
MDA-MB231 cells apoptosis was evident in the cells treated with ABT-263 containing treatment regimens but expression of cleaved PARP was increased after MCl-1 knockdown. In the H1437 cell line, a trace of PARP cleavage was evident only in cells treated with PI3Ki+MEKi+ABT-263 after scramble siRNA treatment. Conversely, when MCl-1 was knocked down, marked apoptosis was evident in cells treated with PI3Ki+MEKi, PI3Ki+ABT-263, and PI3Ki+MEKi+ABT-263 (Fig. 5C).

Discussion

The PI3K-AKT-mTOR and Ras-Raf-MEK-ERK pathways are central transmitters of oncogenic signals in solid malignancies. Considering the central role of the pathways, their inhibition could be an effective therapy in various cancer genotypes. Even though PI3K-AKT-mTOR and Ras-Raf-MEK-ERK are the most commonly altered signaling pathways in solid malignancies, the clinical efficiency of single pathway inhibitors has generally been disappointing and combinational approaches have been applied. Preclinical models have shown that dual targeting with PI3K and MEK inhibitors has antitumor activity in various cancer models (3,8,16,17). Numerous early-phase clinical studies concerning dual PI3K and MEK targeting are ongoing and some results have already been presented. Generally, combined PI3K and MEK inhibitor therapy seems to be feasible, but, unfortunately, the rate of response seems to be low (10-12). In preclinical models, the vast majority of cancer cell lines do not show apoptosis in response to dual PI3K and MEK blockage, which could be the major factor behind the limited clinical activity of the approach (8,9).

In the current work, we employed cell lines identified in our earlier study (9) to evaluate whether the apoptotic response to dual PI3K and MEK inhibition could be augmented by pharmacological means in vitro. In the screen, we found that the Bcl-2/Bcl-xl inhibitor ABT-263, the HDAC inhibitor entinostat and the multikinase inhibitor dasatinib increased the cytotoxic response and apoptosis in combination with PI3K and MEK dual blockage. Analogously to our results, some recent publications have described how Bcl-2/Bcl-xl targeting can enhance the cytotoxicity of MEK and mTOR inhibitors, and dual PI3K and MEK blockage (13,14). Furthermore, HDAC inhibition has earlier been shown to increase the efficiency of both MEK and PI3K-AKT-mTOR targeting agents (16,18). Moreover, earlier research has proposed HDAC inhibitors to act through Mcl-1 (19). To our knowledge, however, previous work provided only limited evidence on the use of dasatinib in combination with PI3K and MEK inhibitors.

It is challenging to include three investigational agents concurrently in a clinical trial, and therefore we were also inter-
tested to see if either PI3K or MEK inhibitor efficiency could be enhanced by combining ABT-263, entinostat or dasatinib to them. All three of these agents were found to increase the cytotoxicity of either the PI3K inhibitor or the MEK inhibitor, excluding ABT-263 in the H1437 line. Apoptotic response was also seen with dual ABT-263 and PI3K or MEK therapy in the HCT116 and MDA-MB231 lines, with entinostat and PI3K or MEK therapy in the HCT116 line, and with entinostat and PI3K therapy in H1437 cells. Based on the results of our preclinical models, it would be appealing to test Bcl-xl/Bcl-2 or HDAC inhibitors in combination with PI3K or MEK inhibitors in animal models and clinical trials. Some of these, such as Bcl-2/Bcl-xl and MEK (NCT02079740), and HDAC and mTor (NCT01087554, NCT01174199) inhibitor combinations, are currently tested in ongoing early phase clinical trials.

Previous studies have identified the apoptotic proteins BIM, Puma, Mcl-1, Bcl-2 and Bcl-xl as major determinants of cell fate in response to PI3K-AKT-mTOR and/or MEK inhibitors. Bcl-xl and Mcl-1 have been suggested to be the most important anti-apoptotic mediators in solid malignancies (13,14). Our results provide similar proof, since drug treatments enabling downregulation of Bcl-xl expression, blockage of its function by the BH3 mimetic or siRNA gene knockdown resulted in apoptosis in most cases. However, downregulation or blockage of Bcl-xl did not indicate apoptosis but is required in most cases. One of the tested lines (H1437) showed more dependency on both anti-apoptotic proteins Bcl-xl and Mcl-1, since downregulation, blockage, or knockdown of BCL-XL itself was insufficient to cause apoptosis if not accompanied by Mcl-1 downregulation. Furthermore, MCL-1 knockdown in this cell line was able to produce prominent increase in cytotoxicity and apoptosis after treatment with PI3K inhibitor or its combinations. It is likely that many cancer cells are able to circumvent PI3K and/or MEK inhibition-mediated apoptosis. Drug treatments inhibiting Bcl-xl and/or Mcl-1 would, therefore, increase the apoptotic response to PI3K and/or MEK
inhibitors. In our study, BIM expression showed no correlation to apoptosis.

In the current study, we found that combining Bcl-2/Bcl-xl, HDAC and multikinase inhibitors to PI3K and MEK dual blockage can increase cytotoxicity and apoptosis in vitro. Furthermore, these agents were also able to enhance cytotoxicity and apoptosis of single-agent PI3K and MEK drugs. More importantly, we found Bcl-xl and Mcl-1 to be major determinants of cell fate in connection with PI3K and/or MEK inhibitor treatment (Fig. 6). We conclude that understanding the molecular mechanism of the anti-apoptotic response to dual PI3K and MEK treatment could provide new and smarter pharmacological approaches and lead to more efficient combinations to be tested in clinical trials.

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