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Synthetic lethal interaction between PI3K/Akt/mTOR and Ras/MEK/ERK pathway inhibition in rhabdomyosarcoma

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ABSTRACT

Rhabdomyosarcoma (RMS) frequently exhibits concomitant activation of the PI3K/Akt/mTOR and the Ras/MEK/ERK pathways. Therefore, we investigated whether pharmacological cotargeting of these two key survival pathways suppresses RMS growth. Here, we identify a synthetic lethal interaction between PI3K/Akt/mTOR and Ras/MEK/ERK pathway inhibition in RMS. The dual PI3K/mTOR inhibitor PI103 and the MEK inhibitor UO126 synergize to trigger apoptosis in several RMS cell lines in a highly synergistic manner (combination index <0.1), whereas either agent alone induces minimal cell death. Similarly, genetic knockdown of p110α and MEK1/2 cooperates to induce apoptosis. Molecular studies reveal that cotreatment with PI103/UO126 cooperates to suppress PI3K/Akt/mTOR and Ras/MEK/ERK signaling, whereas either compound alone is not only less effective to inhibit signaling, but even cross-activates the other pathway. Accordingly, PI103 alone increases ERK phosphorylation, while UO126 enhances Akt phosphorylation, consistent with negative crosstalks between these two signaling pathways. Furthermore, PI103/UO126 cotreatment causes downregulation of several antiapoptotic proteins such as XIAP, Bcl-x_L and Mcl-1 as well as increased expression and decreased phosphorylation of the proapoptotic protein Bim_{EL}, thus shifting the balance towards apoptosis. Consistently, PI103/UO126 cotreatment cooperates to trigger Bax activation, loss of mitochondrial membrane potential, caspase activation and caspase-dependent apoptosis. This identification of a synthetic lethal interaction between PI3K/mTOR and MEK inhibitors has important implications for the development of novel treatment strategies in RMS.

1. Introduction

Rhabdomyosarcoma (RMS) is the most frequent pediatric soft tissue sarcoma and can be subdivided by histological and molecular markers into the embryonal (ERMS) and alveolar (ARMS) subtypes [1]. The prognosis for children with RMS is still poor irrespectively of aggressive multimodal treatment protocols [2], underscoring the need for innovative therapeutic approaches. Escape of programmed cell death is a frequent event in human cancers [3]. Apoptosis is one of the best characterized forms of programmed cell death and involves two key signaling pathways, i.e. the extrinsic (death receptor) and the intrinsic (mitochondrial) pathway of apoptosis [4].

The PI3K/Akt/mTOR and the Ras/MEK/ERK (MAPK) pathways represent key signaling cascades that are linked via receptor tyrosine kinases (RTKs) to extracellular survival signals emitted, for example, by the tumor microenvironment [5]. Upon binding of growth factors, transmembrane RTKs undergo phosphorylation, leading to activation of both the PI3K/Akt/mTOR pathway and the Ras/MEK/ERK pathways [6,7]. Enhanced signaling via these pathways affects various cellular functions, including stimulation of proliferation and/or inhibition of programmed cell death via apoptosis [8]. Both the PI3K/Akt/mTOR and the Ras/MEK/ERK pathway have been described to be dysregulated in RMS, e.g. by genetic lesions such as activating mutations in *RAS*, *FGFR4* or *PIK3CA* or by hyperactivation of growth factor signaling for example via insulin-like growth factor II (IGFII) [9–14]. Also, constitutive activation of the PI3K/Akt/mTOR pathway has been linked to poor outcome in RMS [15].

There is mounting evidence that the PI3K/Akt/mTOR and the Ras/MEK/ERK pathways, which are often simultaneously activated in human malignancies, can exert complementary and redundant functions when only one single pathway is inhibited [5]. In addition, PI3K/Akt/mTOR and Ras/MEK/ERK signaling is regulated by various positive and negative crosstalks and feedback loops [5,16–18]. The existence of coactivated survival cascades and their crosstalks has raised the possibility that these interdependencies can be exploited therapeutically. Therefore, simultaneous cotargeting of both cascades is currently considered as an attractive anticancer strategy [19]. Indeed, preclinical data demonstrated that

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combining pharmacological inhibition of PI3K/mTOR together with MEK/ERK results in synergistic suppression of tumor growth and may overcome resistance, for example in lung cancer [20–23]. However, the question whether this targeted combination approach can be exploited in RMS has not yet been answered. Searching for novel therapeutic strategies for RMS, in the present study we evaluated the rational combination of molecular targeted therapeutics that disrupt complementary tumor cell survival pathways, using inhibitors of the PI3K/Akt/mTOR and the Ras/MEK/ERK pathways.

2. Materials and methods

2.1. Cell culture and chemicals

RMS cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA) and maintained in RPMI 1640 or DMEM medium (Life Technologies, Inc., Eggenstein, Germany), supplemented with 10% fetal calf serum (FCS) (Biochrom, Berlin, Germany), 1 mM glutamine (Invitrogen, Karlsruhe, Germany), 1% penicillin/streptomycin (Invitrogen) and 25 mM HEPES (Biochrom). Treatment with PI103 and/or UO126 was performed in medium containing 0.7% FCS. N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (zVAD.fmk) was purchased from Bachem (Heidelberg, Germany), and all chemicals from Sigma (Deisenhofen, Germany) unless indicated otherwise.

2.2. Determination of apoptosis

Apoptosis was determined by fluorescence-activated cell-sorting (FACSCanto II, BD Biosciences, Heidelberg, Germany) analysis of DNA fragmentation of propidium iodide-stained nuclei as described previously [24]. The percentage of specific apoptosis was calculated as follows: % specific apoptosis = 100 × (% induced apoptotic cells − % spontaneous apoptotic cells)/(100 − % spontaneous apoptotic cells).

2.3. Western blot analysis

Western blot analysis was performed as described previously [24] using the following antibodies: mouse anti-caspase-8, mouse anti-Noxa (Alexis Biochemicals, Grünberg, Germany), mouse anti-XIAP (clone 28), mouse anti-Akt, mouse anti-Bcl-2, rabbit anti-Bcl-X_L, mouse anti-Bax, rabbit anti-Bak (BD Transduction Laboratories), rabbit anti-caspase-3, mouse anti-caspase-9, rabbit anti-pAkt, rabbit anti-pERK, rabbit anti-p4E-BP1, rabbit anti-4E-BP1, rabbit anti-pS6, mouse anti-S6, rabbit anti-Bim (Cell Signaling, Beverly, MA), goat anti-cIAP1 (R&D Systems, Wiesbaden, Germany), rabbit anti-Mcl1 (Stressgene Bioreagents, Germany). Mouse anti-GAPDH (HyTest, Turku, Finland) or mouse anti-β-actin (Sigma) were used as loading controls. Goat anti-mouse IgG, donkey anti-goat IgG, goat anti-rabbit IgG conjugated to horseradish peroxidase (Santa Cruz Biotechnology) and goat anti-mouse IgG1 or goat anti-mouse IgG2b (Southern Biotech, Birmingham, AL) conjugated to horseradish peroxidase were used as secondary antibodies. Enhanced chemiluminescence was used for detection (Amersham Bioscience, Freiburg, Germany). Representative blots of at least two independent experiments are shown.

2.4. Determination of mitochondrial membrane potential and Bax activation

To determine mitochondrial transmembrane potential cells were incubated with tetramethylrhodamine methyl ester (TMRM) (1 μM; Molecular Probes) for 30 min at 37 C and immediately analyzed by flow cytometry. For detection of active Bax, cells were lysed in CHAPS lysis buffer (10 mM HEPES (pH 7.4); 150 mM NaCl; 1% CHAPS) as previously described [25]. A total of 1000 μg protein was immunoprecipitated with 2 mg mouse anti-Bax antibody (6A7, Sigma) and 5 μl Dynabeads Pan Mouse IgG (Dako, Hamburg, Germany). The precipitate was analyzed by Western blotting using the BaxNT antibody (Upstate Biotechnology).

2.5. Transient RNA interference

Cells were seeded and simultaneously transfected with 10 nM SilenderSelcet siRNA (Invitrogen) targeting p110α (s10520), MEK1 (s11168) and MEK2 (s11170) using Lipofectamine RNAi Max (Invitrogen) and OptiMEM (Life Technologies).

2.6. Quantitative RT-PCR

Total RNA was extracted using peqGOLD Total RNA kit from Peqlab Biotechnologie GmbH (Erlangen, Germany) according to the manufacturer's instructions. cDNA was synthetized using RevertAid H Minus First Strand cDNA Synthesis Kit (MBI Fermentas GmbH, St. Leon-Rot, Germany). To quantify gene expression levels, SYBR Green based qRT-PCR was performed using the 7900HT fast RT-PCR system from Applied Biosystems (Darmstadt, Germany). Data were normalized on 28S-rRNA expression as reference gene. The relative expression of the target gene transcript was calculated as $\Delta\Delta c_t$. Primer sequences are listed in Suppl. Table 1.

2.7. Statistical analysis

Statistical significance was assessed by Student's *t*-Test (two-tailed distribution, two-sample, unequal variance). Interaction between PI103 and UO126 was analyzed by the Combination index (CI) method based on that described by Chou [26] using CalcuSyn software (Biosoft, Cambridge, UK). CI < 0.9 indicates synergism, 0.9–1.1 additivity and >1.1 antagonism.

3. Results

3.1. PI103 and UO126 synergize to induce caspase-dependent apoptosis

To explore the role of PI3K/Akt/mTOR and Ras/MEK/ERK signaling in the regulation of cell death and survival in RMS, we assessed the effect of pharmacological inhibitors of these pathways either alone or in combination using the dual PI3K/mTOR inhibitor PI103 and the MEK inhibitor UO126. We selected several RMS cell lines to represent the two major histological subtypes of RMS, i.e. ERMS (RD, TE671) and ARMS (RMS13, Rh30). To investigate the question whether pathway inhibition has an effect on the induction of apoptosis we determined DNA fragmentation as a characteristic feature of apoptotic cell death. Importantly, PI103 and UO126 cooperated to trigger DNA fragmentation in all RMS cell lines, whereas treatment with either compound alone exerted little cytotoxicity (Fig. 1). Calculation of CI revealed that this drug combination of PI103 and UO126 is highly synergistic (Table 1). Furthermore, we investigated the question whether the combination of PI103 and UO126 is cytotoxic to normal, non-malignant cells. PI103 and UO126 did not cooperate to induce apoptosis in fibroblasts at equimolar drug concentrations that synergized to trigger apoptosis in RMS cells (Suppl. Fig. 1 and Fig. 1).

To examine whether the induction of apoptosis requires activation of caspases, one of the hallmarks of apoptosis [27], we analyzed the effect of the broad-range caspase inhibitor zVAD.fmk. Notably, the addition of zVAD.fmk significantly reduced PI103/UO126-induced apoptosis in all RMS cell lines (Fig. 2). This shows that caspases are required for the induction of apoptosis upon combined treatment with PI103 and UO126.

3.2. PI103 and UO126 cooperate to block PI3K/Akt/mTOR and Ras/MEK/ERK signaling

To gain insights into the molecular mechanisms that are responsible for the synergism of PI103/UO126, we assessed the way in which PI103 and UO126 either alone or in combination affect the activation status of the PI3K/Akt/mTOR and the Ras/MEK/ERK pathways. To this end, we used phosphorylation of Akt as surrogate readout for PI3K activity, phosphorylation of ERK as surrogate readout for MEK activity and phosphorylation of 4E-BP1 and S6 ribosomal protein as surrogate readouts for mTOR activity. All RMS cell lines displayed constitutive phosphorylation of Akt, ERK, 4E-BP1 and S6 ribosomal protein (Fig. 3). Importantly, combined treatment with both PI103 and UO126 proved to be more potent than either single agent alone to inhibit phosphorylation of Akt, ERK, 4E-BP1 and S6 ribosomal protein in all RMS cell lines (Fig. 3). Single agent treatment with PI103 reduced phosphorylation of Akt, 4E-BP1 and S6 ribosomal protein in three of four RMS cell lines, i.e. RD, Rh30 and RMS13 cells (Fig. 3). In TE671 cells, PI103 failed to inhibit Akt phosphorylation and at 24 h only transiently reduced phosphorylation of 4E-BP1 and S6 ribosomal protein (Fig. 3).

Of note, we also observed that treatment with PI103 alone even increased rather than decreased phosphorylation of ERK in RD and