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Dual phosphatidylinositol 3-kinase/mammalian target of rapamycin inhibitor NVP-BEZ235 synergizes with chloroquine to induce apoptosis in embryonal rhabdomyosarcoma

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ABSTRACT

Aberrant activation of the phosphatidylinositol 3-kinase (PI3K)/mammalian target of rapamycin (mTOR) pathway has been reported for rhabdomyosarcoma (RMS) and is implicated in survival of tumor cells as well as therapeutic resistance. In the present study, we searched for combination therapies with the dual PI3K/mTOR inhibitor NVP-BEZ235 (BEZ235) in RMS. Here, we identify a synthetic lethal interaction of BEZ235 together with the lysosomotropic agent chloroquine (CQ), which is effective against embryonal rhabdomyosarcoma (ERMS). BEZ235 and CQ at subtoxic concentrations synergize to induce apoptosis in ERMS cells, as confirmed by calculation of combination index (CI). BEZ235 and CQ cooperate to activate caspase-9, -3 and -8, which is crucial for apoptosis induction given that the broad-range caspase inhibitor *N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (zVAD.fmk) blocks BEZ235/CQ-induced apoptosis. Additionally, pharmacological inhibition of lysosomal enzymes significantly reduces BEZ235/CQ-induced apoptosis, indicating concomitant activation of the lysosomal compartment. Importantly, BEZ235/CQ-induced apoptosis is significantly inhibited by antioxidants, implying that increased oxidative stress contributes to BEZ235/CQ-induced cell death. Importantly, our molecular studies reveal that BEZ235/CQ-induced apoptosis is mediated by cooperative downregulation of the antiapoptotic BCL-2 family protein MCL-1, since stabilization of MCL-1 by expression of a non-degradable MCL-1 phosphodefective mutant significantly decreases BEZ235/CQ-induced apoptosis. Also, overexpression of antiapoptotic BCL-2 leads to a significant reduction of BEZ235/CQ-induced apoptosis, emphasizing that an intact mitochondrial pathway of apoptosis is required for BEZ235/CQ-induced cell death. This identification of a synthetic lethality of BEZ235 and CQ has important implications for the development of molecular targeted therapies for RMS.

Introduction

RMS, the most frequent pediatric soft tissue sarcoma, is characterized by typical histological and genetic features and classified into two major entities, i.e. alveolar RMS (ARMS) and ERMS [1]. Current therapeutic strategies against RMS in general include surgery, chemotherapy and radiation [2]. However, a complete tumor response is still rare for patients with high-risk or relapsed disease [2], highlighting the need for novel, more efficient treatment approaches.

The PI3K/mTOR pathway is often aberrantly activated in RMS [3] and integrates survival signals provided by extracellular and intracellular stimuli to promote cell growth and to inhibit cell death in cancer cells [4,5]. Chemotherapeutic drugs usually exert their anticancer activity by activation of cell death signaling pathways, which are inhibited by aberrant PI3K/mTOR signaling [6].

The most extensively studied form of cell death is apoptosis, a programmed cell death pathway [7]. Activation of the extrinsic or intrinsic apoptosis pathway eventually leads to activation of caspases as effector molecules for the execution of cell death [7]. Evasion of apoptosis is a characteristic feature of cancer cells promoting treatment resistance and can, among others, be mediated by overexpression of antiapoptotic proteins. High expression levels of the antiapoptotic BCL-2 family proteins BCL-2, BCL-X_L and MCL-1 have previously been reported for RMS [8–10].

BEZ235, a dual PI3K/mTOR inhibitor [11], has recently been evaluated as a therapeutic option for sarcoma including RMS, especially for combination strategies [12,13]. CQ, a lysosomotropic agent which was originally used as an anti-malarial or immune-suppressive drug,

Abbreviations: ARMS, alveolar RMS; ATTC, American Type Culture Collection; CI, combination index; CQ, chloroquine; ERMS, embryonal rhabdomyosarcoma; FCS, fetal calf serum; GSK3, glycogen synthase kinase 3; mTOR, mammalian target of rapamycin; NAC, N-acetylcysteine; PI, propidium iodide; PI3K, phosphatidylinositol 3-kinase; RMS, rhabdomyosarcoma; ROS, reactive oxygen species; Trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; UPS, ubiquitin–proteasome system; zVAD.fmk, *N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone.

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was lately found to additionally display promising anticancer activity [14]. Searching for new synergistic combinations with inhibitors of the PI3K/mTOR pathway, we investigated the potential of BEZ235 in combination with CQ in the present study.

Materials and methods

Cell culture and chemicals

RMS cell lines were obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA). ERMS cells were maintained in DMEM GlutaMAX™-I medium, ARMS in RPMI 1640 GlutaMAX™-I (Life Technologies, Inc., Carlsbad, CA, USA), both supplemented with 10% fetal calf serum (FCS) (Life Technologies, Inc.), 1% penicillin/streptomycin (Life Technologies, Inc.), 1 mM sodium pyruvate (Life Technologies, Inc.). BEZ235 was kindly provided by Novartis Institute for BioMedical Research (Oncology Basel, Novartis Pharma AG, Basel, Switzerland). CQ, N-acetylcysteine (NAC), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), CA-074me and E64d were obtained from Sigma (Deisenhofen, Germany), zVAD.fmk from Bachem (Heidelberg, Germany). If not indicated differently, chemicals were purchased from Carl Roth (Karlsruhe, Germany).

Generation of stable BCL-2 overexpression cell lines

Stable overexpression of murine BCL-2 was performed by lentiviral vectors according to protocols previously described [15]. Shortly, Phoenix cells were transfected with 20 μg of pMSCV plasmid (empty vector; BCL-2) using calcium phosphate transfection. The virus-containing supernatant was collected, sterile-filtered and used for spin transduction at 37 °C in the presence of 8 μg/ml polybrene. Transduced cells were selected with 10 μg/ml blasticidin (Sigma).

Transient overexpression

For transient overexpression, cells were transfected with 4 μg of pCMV Tag3B plasmid (empty vector; MCL-1 '4A' (S64A/S121A/S159A/T163A), kindly provided by Genentech, South San Francisco, CA, USA), supplied with Lipofectamine 2000 (Life Technologies, Inc.) and selected with 500 μg/ml G418.

Determination of cell death

Cell death was determined by flow cytometric analysis (FACSCanto II, BD Biosciences, Heidelberg, Germany) of DNA fragmentation of propidium iodide (PI)-stained nuclei as described previously [16].

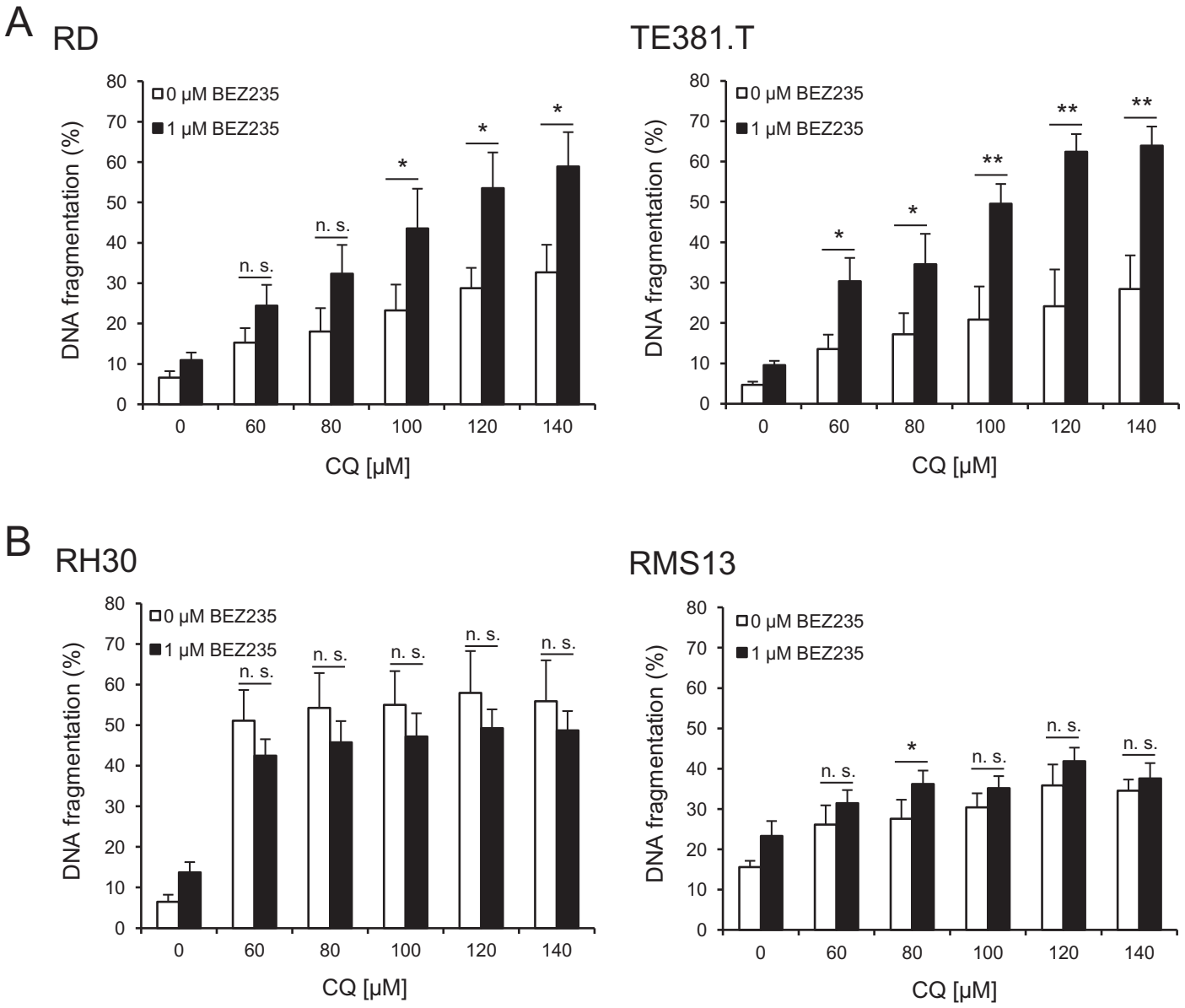

Fig. 1. BEZ235 synergizes with CQ to induce apoptosis in ERMS. ERMS (A) and ARMS (B) cell lines were treated with indicated concentrations of BEZ235 and/or CQ. Apoptosis was determined at 24 hours by analysis of DNA fragmentation of PI-stained nuclei using flow cytometry. Mean + S.D. of three independent experiments performed in triplicate are shown. Student's t-Test was used to calculate two-sided *P* values. $^{*}P < 0.05$; $^{**}P < 0.01$; *n.s.* not significant.