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The B-cell lymphoma 2 (BCL2)-inhibitors, ABT-737 and ABT-263, are substrates for P-glycoprotein

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ABSTRACT

Inhibition of BCL2 proteins is one of the most promising new approaches to targeted cancer therapy resulting in the induction of apoptosis. Amongst the most specific BCL2-inhibitors identified are ABT-737 and ABT-263. However, targeted therapy is often only effective for a limited amount of time because of the occurrence of drug resistance. In this study, the interaction of BCL2-inhibitors with the drug efflux transporter P-glycoprotein was investigated. Using ^{3}H labelled ABT-263, we found that cells with high P-glycoprotein activity accumulated less drug. In addition, cells with increased P-glycoprotein expression were more resistant to apoptosis induced by either ABT-737 or ABT-263. Addition of tariquidar or verapamil sensitized the cells to BCL2-inhibitor treatment, resulting in higher apoptosis. Our data suggest that the BCL2-inhibitors ABT-737 and ABT-263 are substrates for P-glycoprotein. Over-expression of P-glycoprotein may be, at least partly, responsible for resistance to these BCL2-inhibitors.

1. Introduction

Targeted therapy is becoming increasingly more important for cancer treatment. Amongst the most promising targets in apoptosis signalling are the B-cell lymphoma 2 (BCL2) proteins, which have been found to contribute both to oncogenesis and to chemoresistance [1]. The BCL2 protein family consists of both pro- and antiapoptotic members [2]. The antiapoptotic BCL2 proteins, comprising BCL2, BCL-X_L, MCL1, BCL-w and BCL2A1, inhibit apoptosis by preventing cytochrome *c* release from mitochondria into the cytosol. Thus, they block the activation of the intrinsic apoptotic pathway, which is induced upon cellular stress and DNA damage. Recently, several small molecule inhibitors of antiapoptotic BCL2 proteins have been identified, which display promising anti-tumor activity in preclinical studies [3]. Amongst the most promising compounds are ABT-737 [4] and its analogue ABT-263 (Navitoclax) [5]; the latter is currently being tested in clinical trials for multiple malignancies including leukemia, lymphoma and lung cancer. ABT-737 was initially discovered using nuclear magnetic resonance-based screening, chemical synthesis and structure based-design [4]. ABT-737 causes a rapid induction of apoptosis mainly in leukemia cells and exerts potent anti-cancer activity either alone or in combination with chemotherapeutic drugs. However, ABT-737 is rapidly metabolized, has a short half-life *in vivo* and is not orally bioavailable. Therefore, it was modified in three key positions, resulting in the synthesis of ABT-263, which is more metabolically stable and orally bioavailable [6]. Both compounds bind with very high affinities to BCL2, BCL-X_L and BCL-w but do not inhibit MCL1 or BCL2A1. To date, acquired drug resistance to ABT-737 has only been investigated in cell lines cultured over a long period of time with increasing drug concentrations and the postulated mechanism of resistance is an increased expression of MCL1 and BCL2A1 [7].

The ATP-binding cassette (ABC) transporter superfamily has been implicated in multi-drug resistance, a phenomenon by which tumor cells acquire simultaneous cross-resistance to multiple chemotherapeutic drugs, contributing to chemotherapy failure [8]. The main mediator of multi-drug resistance is P-glycoprotein (*MDR1*, *ABCB1*), a 180 kDa membrane glycoprotein that displays broad substrate specificity and is involved in efflux of hydrophobic xenobiotics [9]. By mediating chemotherapeutic drug efflux, P-glycoprotein and related members of the ABC superfamily may render chemotherapy inefficient by lowering intracellular drug concentrations. The discovery of P-glycoprotein and its function in drug-resistance led to the development of several small

Abbreviations: ATP, binding cassette (ABC); BCL2, B-cell lymphoma 2; CLL, chronic lymphocytic leukemia.

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molecule inhibitors of P-glycoprotein, which inhibit drug efflux and might potentially circumvent or reverse drug resistance [10]. P-glycoprotein inhibitors can be categorized into three groups: first generation (licensed drugs shown to be high affinity substrates; e.g. cyclosporin), second generation (e.g. PSC-833) and third generation (e.g. tariquidar). Several of these inhibitors, including tariquidar, have been shown to reverse resistance to multiple drugs like doxorubicin, paclitaxel and vincristine *in vitro*. However, in clinical trials the first and second generation inhibitors, as adjuvants for cancer therapy, have failed due to either a high incidence of toxicity or lack of clinical benefit. Consequently, no P-glycoprotein inhibitor has as yet received approval from the FDA or European regulatory agencies as an adjuvant treatment [11,12]. The situation with third generation inhibitors may be more encouraging, but they have only been tested in early phase clinical trials to date [13]. In this study, the contribution of drug efflux transporters to acquired resistance to ABT-737 and ABT-263 was investigated.

2. Materials and methods

2.1. Material

CEM and VBL_{100} cells [14] were kind gifts from Dr. R. Davey (Sydney, Australia), and MDCKII cells with sublines transfected with *MDR1* [15] were kindly provided by Prof P. Borst (Netherlands Cancer Institute, Amsterdam, The Netherlands). ABT-737 and ABT-263 were purchased from Selleck Chemicals (Houston, TX). ^{3}H-ABT-263 was custom-labelled by PerkinElmer (Boston, MA) with a specific activity of 41.6 Ci/mmol. Verapamil was acquired from Sigma–Aldrich (Gillingham, UK) and tariquidar was synthesized by Dr. O. Langer, Medical University of Vienna, Austria. For Western blotting, rabbit anti-BCL-X_L (BD Biosciences, San Diego, CA) and mouse anti-P-glycoprotein (Abcam, Cambridge, UK) were used.

2.2. Cell culture

MDCKII wild type or *MDR1*-over-expressing cells were maintained in DMEM plus 10% FCS and 2 mM L-glutamine. CEM and VBL_{100} cells were maintained in RPMI 1640 plus 10% FCS and 2 mM L-glutamine (all from Life Technologies, Inc, Paisley, UK). VBL_{100} cells constitute a multidrug-resistant subline that over-expresses *MDR1* through exposure to vinblastine. All cell lines were negative when tested for mycoplasma infection. Peripheral blood samples from patients with chronic lymphocytic leukemia (CLL) were obtained with patient consent and local ethical committee approval (REC 06/Q2501/124). Lymphocytes were purified using histopaque1077 separation and cultured in RPMI 1640 medium supplemented with 10% FCS and 2 mM L-glutamine.

2.3. Uptake of radioactively labelled ABT-263

Cells were incubated for 30 min in transport buffer (Hank's Buffered Salt Solution with 25 mM HEPES at pH 7.4 with 0.1% BSA) at 37 °C or 4 °C with 1 μM of ^{3}H-ABT-263 (radioactivity concentration of 0.2 μCi/ml with sufficient non-radiolabelled compound added to give a final concentration of 1 μM) in the presence or absence of transport inhibitors. After incubation, ice-cold phosphate-buffered saline (PBS) was added to the cells and centrifuged at 250*g* at 4 °C for 5 min. The resultant cell pellet was washed twice with ice-cold PBS and centrifuged again. Distilled water (100 μl) was added to the cells and vortexed to break up the pellet. Cells were solubilized by incubation with 200 μl 0.2 M NaOH and 1% sodium dodecyl sulphate (SDS) for 1 h. The resultant solution was mixed with 4 ml scintillation fluid, and radioactivity was counted using a β-counter (1500 Tri Carb LS Counter; PerkinElmer).

2.4. Determination of apoptosis

Cells were incubated for the indicated times with different concentrations of ABT-737 or ABT-263 with or without transport inhibitors. To assess apoptosis, cells were stained for 10 min with annexinV-FITC and propidium iodide in annexin buffer (10 mM HEPES, 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, pH 7.4). Externalization of phosphatidylserine during apoptosis was measured by binding of annexinV-FITC and membrane permeability assessed by uptake of propidium iodide using flow cytometry.

2.5. Rhodamine123 efflux studies

P-glycoprotein activity was assessed as described previously [16]. Purified CLL cells were stained for 45 min at room temperature with 150 ng/ml rhodamine123. After two washes in ice-cold medium, CLL cells were resuspended in rhodamine123-free medium with or without a transport inhibitor (10 μM verapamil or 1 μM tariquidar) and incubated at 37 °C. The rhodamine123 efflux or retention was determined at 2 or 3 h after staining by flow cytometry.

2.6. Statistical analysis

To assess statistical significance for drug accumulation data in Fig. 3B, Prism 5 was used with the samples compared by one-way ANOVA followed by Tukey–Kramer test for multiple comparisons. For cell death data shown in Fig. 1, statistical analysis was done using student's *t*-test in Microsoft Excel.

3. Results

3.1. ABT-263 is a substrate for P-glycoprotein

In order to investigate whether the BCL2-inhibitor ABT-263 was a substrate for P-glycoprotein, we assessed the cellular accumulation of ^{3}H-ABT-263 in P-glycoprotein expressing cells. To this end, we used MDCKII cells transfected with *MDR1*, the gene encoding P-glycoprotein [17]. Cells were exposed to ^{3}H-ABT-263 for 30 min before cellular drug accumulation was measured. When exposed to ^{3}H-ABT-263, *MDR1* expressing MDCKII cells accumulated less ABT-263 than wild type MDCKII cells, and the reduced ABT-263 accumulation was reversed in the presence of the P-glycoprotein inhibitor, tariquidar (Fig. 1A). Expression of P-glycoprotein in MDCKII-MDR1 cells was confirmed by Western blotting, while BCL-X_L, the target of ABT-263, was not differentially expressed in wild type or MDR1 MDCKII cells (Fig. 1B). Taken together, these data indicate that P-glycoprotein is involved in ABT-263 efflux and that ABT-263 is a substrate for P-glycoprotein.

3.2. P-glycoprotein expression confers resistance to BCL2-inhibitors

Since drug efflux is directly linked to drug resistance, we next asked whether expression of P-glycoprotein might result in reduced drug efficacy. To this end, we investigated apoptosis induced by ABT-263 and its analogue, ABT-737, in both wild type cells and cells expressing P-glycoprotein. After 24 h of exposure, MDCKII cells transfected with *MDR1* showed significantly less apoptosis induced by ABT-737 (Fig. 1C) and ABT-263 (Fig. 1D) than wild type cells, indicating that expression of P-glycoprotein may confer resistance to both these BCL2-inhibitors.