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Brefeldin A triggers apoptosis associated with mitochondrial breach and enhances HA14-1- and anti-Fas-mediated cell killing in follicular lymphoma cells

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Abstract

Follicular lymphoma (FL) remains a fatal disease of increasing worldwide incidence. Since patients with FL eventually develop resistance to conventional anticancer agents, and due to BCL-2 overexpression present with profoundly compromised execution of mitochondrial pathway of apoptosis, targeting alternative pathways of cell demise may appear therapeutically beneficial. Herein we report for the first time the effects of an ER-Golgi transport inhibitor, Brefeldin A (BFA), alone and in combination with a small molecule Bcl-2 inhibitor HA14-1 or agonistic anti-Fas mAb, in the recently established human FL cell lines. All cell lines tested were sensitive to BFA-induced cytotoxicity and apoptosis. Moreover BFA-induced cell death was associated with profound ER stress, mitochondrial breach and subsequent caspase cascade activation, including caspase 2 activation. Interestingly, BFA-induced ER stress did not result in appearance of autophagic morphology in FL cells. Of importance, small molecule Bcl-2 antagonist, HA14-1 and agonistic anti-Fas mAb significantly enhanced BFA-mediated cytotoxicity and apoptosis, revealing novel and previously unexplored means to enhance ER stress-mediated cell killing in follicular lymphoma cells.

Keywords: Brefeldin A; ER stress; HA14-1; Apoptosis; Follicular lymphoma; Multiparameter flow cytometry

1. Introduction

Follicular lymphoma (FL), a mostly indolent and still incurable non-Hodgkin's lymphoma, is usually characterized by overexpression of anti-apoptotic Bcl-2 and/or Bcl-X_L proteins [1–4]. Although survival-favouring misbalance within the Bcl-2 family of proteins is reportedly associated with alterations in sensitivity to anticancer agents, follicular lymphoma remains responsive to first-line treatments, and it is the relapse disease that develops drug resistant phenotype [4–8]. We and others have previously shown that small molecule Bcl-2 inhibitors effectively induce apoptosis in a single agent scenario and enhance efficacy of classical chemotherapeutic drugs in combinatorial treatments [9–16]. Since in FL tumour cells eventually develop resistance to conventional anticancer agents and may present with profoundly compromised execution of mitochondrial (intrinsic) apoptotic pathway, targeting additional, failsafe pathways of cell demise may appear therapeutically beneficial [17–19].

Although the burden of data shows that the elimination of malignant cells of hematopoietic origin depends heavily

Abbreviations: ARF, ADP ribosylation factor; Bcl-2, B-cell lymphoma 2 protein; BFA, Brefeldin A; CLL, chronic lymphocytic leukaemia; DMSO, dimethyl sulfoxide; ER, endoplasmic reticulum; FL, follicular lymphoma; FLICA, fluorescently labelled inhibitors of caspases; HA14-1, ethyl 2-amino-6-bromo-4-(1-cyano-2-ethoxy-2-oxoethyl)-4H-chromene-3-carboxylate; MMP, mitochondrial membrane permeabilization; PI, propidium iodide; 7-AAD, 7-amino actinomycin D; SERCA, sarcoplasmic-endoplasmic calcium ATPase; TEM, transmission electron microscopy; TMRM, tetramethylrhodamine methyl ester; UPR, unfolded protein response

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on classical apoptotic pathways, the evidence is mounting that alternative apoptotic and non-apoptotic mechanisms may effectively contribute to tumour suppression and deliver novel anti-cancer targets [18–22]. A growing number of reports recognize endoplasmic reticulum (ER) and Golgi apparatus as key players in the sensing and execution of apoptotic signals [22–26]. It is plausible that stress responses from ER-Golgi network involve: transcriptional signalling, intracellular Ca^{2+} mobilization, activation of apical caspases like caspase 12, 4 or 2, induction of ganglioside GD3 synthesis and surge release of latent death receptors [22,23,26]. Interestingly, it has been reported that cell demise programs launched from ER-Golgi network may proceed with or without contribution of mitochondrial pathway of apoptosis [22,25]. Because of constant trafficking between the ER and Golgi apparatus it is conceivable that this network stands at the nexus of sensing and processing a plethora of incoming traumatic signals [24,26]. As the interest in the role of ER and Golgi during induction/execution of apoptosis has been gaining momentum, they also attract growing attention in the development of novel targets for selective anti-cancer therapies [22,25].

In this context, recent studies indicated that, in comparison to normal B cells, malignant B cells feature not only increased mitochondrial content, but also more elaborate endoplasmic reticulum network [27,28]. Therefore, it has been postulated that ER-Golgi system may be imperative for endurance of malignant clones in a chronic lymphocytic leukaemia (B-CLL), and such a reliance may point out unique anti-cancer targets [28]. Pertinent to the therapy of B-CLL, the successful targeting of the ER-Golgi pathway with Brefeldin A (BFA) in fludarabine refractory CLL cells has recently provided novel insights on how to eradicate cancerous B-cells independently of their p53 status and pathological overexpression of Bcl-2, Bcl-X_L, Mcl-1 and XIAP proteins [28]. Brefeldin A, a fungal 16-membered macrolide isolated from *Penicillium brefeldianum*, exerts ER and Golgi stress via inhibition of ADP ribosylation factor (ARF) leading to a decreased coatamer proteins assembly and disruption of ER-Golgi vesicular transport [29,30]. This subsequently contributes to unfolded protein response (UPR), activation of caspase 2 and Golgi apparatus collapse [28,31]. Apart from B-CLL cells, BFA reportedly triggers apoptosis in multiple myeloma (U266, NCI-H929), Jurkat, HeLa, leukaemia (HL60, K562, BJAB), colon (HT-29), prostate and adenoid cystic sarcoma cells [28,32–36]. Importantly, a water soluble prodrug form of BFA, Breflate (7-*N*,*N*-dimethylglycinate ester of Brefeldin A; NSC656202), is currently under development as a novel antineoplastic agent [37].

Here we report for the first time that Brefeldin A potently inhibits growth and effectively induces apoptosis in follicular lymphoma cells at nanomolar concentrations. BFA-induced cell death is associated with profound ER stress, mitochondrial breach and subsequent caspase cascade activation with clear predominance of apoptosing cells at a G1 phase of the cell cycle. Moreover we provide evidence that BFA-induced ER stress is not associated with autophagic morphology in FL cells. Additionally, combinatorial targeting of apoptotic pathways with Brefeldin A and a small Bcl-2 inhibitor HA14-1 or anti-Fas mAb, a trigger of death-receptor pathway, reveals novel and previously unexplored means to enhance ER stress-mediated cell killing that may assist in the development of future regimens to combat follicular lymphoma.

2. Materials and methods

2.1. Cell lines, regents and treatments

The origin and culturing of human follicular lymphoma cell lines HF1A3, HF4.9 and HF28RA were as previously described [9,10]. Multi drug resistance efflux pump status was evaluated using cyclosporin A (Sigma–Aldrich Corp., St. Louis, MO, USA), verapamil (Alexis Biochemicals, Lausen, Switzerland), probenecid (Alexis Biochemicals) together with SYTO 16 probe (Molecular Probes, Eugene, OR, USA) as described earlier [38]. P-gp-attributable activity was not detected in any of the cell lines. Brefeldin A (Sigma) was dissolved in DNA grade ethanol and aliquots were stored at −20 °C. A pan-caspase inhibitor zVAD-fmk was procured from Calbiochem (Cambridge, MA, USA). Propidium iodide (PI) was purchased from Sigma. Cathepsin and calpain inhibitors (Pepstatin A, MDL28170, PD150606, CA-074-Me, zFA-fmk, and ALLN) were generously provided by Dr. M. Courtney (A.I. Virtanen Institute, Kuopio, Finland). All compounds were diluted in cell culture medium to working stocks immediately before use. The final concentration of ethanol/DMSO in culture media did not exceed 0.1% (v/v), and no alterations in growth variables were observed in vehicle controls.

BFA was added to cell cultures for the time indicated either alone, or following 2 h pre-treatment with caspase, cathepsin, calpain inhibitors. HA14-1, a small molecule Bcl-2 inhibitor, was from Alexis Biochemicals, whereas CD95 cross-linking mAb (clone CH11) was from Upstate (NY, USA). For combinatorial treatments, both simultaneous and sequential treatment approaches were tested.

2.2. Cell viability, proliferation and growth assays

Cell viability upon the treatments was tested using double staining of cells with YO-PRO 1/PI and SYTO16/PI probes (Molecular Probes), as described below. To access cell proliferation, cells were treated with 0–100 ng/ml BFA in complete medium for 20 h before adding 1 μCi/ml [methyl-^{3}H]-thymidine for additional 4 h at 37 °C. The incorporated radioactive thymidine was quantified by scintillation counting with Microbeta counter (Perkin Elmer, Wellesley, MA, USA). To examine long-term effects of BFA treatment, cells were seeded at initial concentration 100,000 cells/ml and treated with 0–75 ng/ml BFA for up to 5 days. At the time indicated, a sample of cells was removed and viable cell number