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Cross-resistance and synergy with bendamustine in chronic lymphocytic leukemia

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ABSTRACT

Bendamustine (BEN) has structural similarities to an alkylating agent and a nucleoside analog, and effective against tumor cells that are resistant to standard therapy. In this study we compared the activities of BEN against that of the alkylating agent, chlorambucil (CLB), and the nucleoside analogs, fludarabine (FLU) and deoxyadenosine/pentostatin (dADO/PEN), in primary chronic lymphocytic leukemia (CLL) cells *in vitro*. Cross-resistance was observed between BEN, CLB and FLU, with previously treated patients or those with a deletion 17p being most resistant. In contrast, some resistant CLL cells retained moderate sensitivity to dADO/PEN. Like FLU and CLB, BEN induced apoptosis through both the mitochondrial and death receptor pathways. There was a greater increase in DNA double-strand breaks (DSB) following FLU, as compared to BEN and CLB. Synergistic cytotoxicity was seen on combining BEN or CLB with FLU or dADO/PEN, but not when combining BEN with CLB. These results demonstrate that BEN acts as an alkylating agent, demonstrates cross-resistance to CLB and FLU and resistance to cells with a del 17p. Synergistic cytotoxic activity was seen between BEN and dADO/PEN suggesting that the combination of BEN and PEN should be evaluated in the clinic.

1. Introduction

The traditional therapy for chronic lymphocytic leukemia (CLL) was with the alkylating agents, chlorambucil (CLB) or cyclophosphamide, while the nucleoside analogs, pentostatin (PEN) and fludarabine (FLU), came into use in the past 20 years [1]. Nowadays, combinations of these agents with a monoclonal antibody are commonly used in CLL [2]. The combination of FLU, cyclophosphamide and rituximab (FCR) is the standard regimen for fit patients with CLL, although its use is limited by myelosuppression and the subsequent risk of myelodysplasia and acute myeloid leukemia [3,4]. While PEN is more likely than FLU to cause nausea, it is less myelosuppressive [5]. As a result, there has been renewed interest in combining this agent with alkylating agents, particularly for the elderly [6,7].

Bendamustine (BEN) was originally developed as a bifunctional alkylating agent with a benzimidizole ring, thus having the features of both an alkylating agent and a nucleoside analog (Fig. 1) [8–10]. More recently, this agent has become a standard treatment for CLL and the indolent lymphomas, and can safely be administered with mild renal insufficiency [9]. When compared to CLB as the first-line treatment for CLL, BEN produced a two-fold increased response rate and prolongation of progression-free survival [11,12]. As second line therapy after CLB, BEN was shown to have at least equivalent activity to FLU [13]. The major toxicity with this agent remains myelosuppression. However, although BEN has been used clini-

Abbreviations: BEN, bendamustine; CI, combination index; CLB, chlorambucil; CLL, chronic lymphocytic leukemia; Cyclo, cyclophosphamide; dADO, deoxyadenosine; Del, deletion; DHE, dihydroethidium; DiOC6, 3,3′-dihexyloxacarbocyanine iodide; DMSO, dimethyl sulfoxide; DR, death receptor; DSBs, double strand breaks; FCR, fludarabine cyclophosphamide and rituximab; FDC, fraction of dead cells; FDR, fold-dose reduction; FISH, fluorescent *in situ* hybridization; FLU, fludarabine; FR, fludarabine rituximab; *IGVH*, heavy chain immunoglobulin; LD_{50}, lethal dose to kill 50% of cells; LDT, lymphocyte doubling time; M, mutated; Mito mem, mitochondrial membrane; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; NCI, national cancer institute; PBMC, peripheral blood mononuclear cells; PEN, pentostatin; RCD, rituximab cyclophosphamide dexamethasone; ROS, reactive oxygen species; SFM, serum-free hybridoma media; SSB, single strand breaks; U, unmutated; UNK, unknown; WBC, white blood cell count; x2, double deletion; y, years; 7AAD, 7-aminoactinomycin D.

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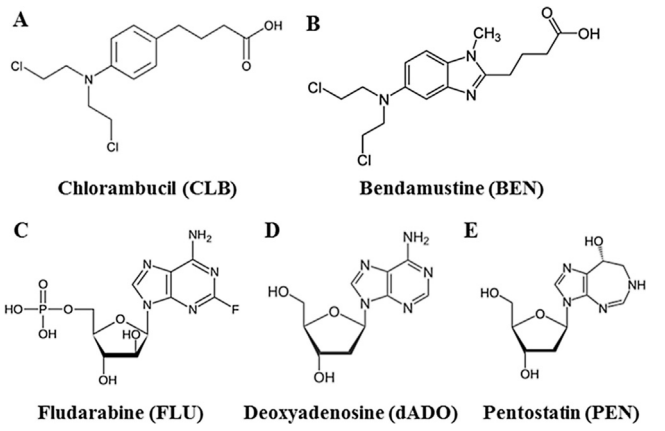

Fig. 1. Bendamustine has a similar structure to alkylating agents and nucleoside analogs.

cally for many years, primarily for lymphoid malignancies, there have been relatively few studies evaluating its mechanism of action. Despite its similarity to alkylating agents and nucleoside analogs, BEN was found to be active against CLL cells taken from patients who had become resistant to FLU or CLB, perhaps related to differences in DNA repair [8,14]. Moreover, BEN was active against cells with a p53 mutation, suggesting a unique mechanism of action [15].

Fludarabine is a phosphorylated deoxyadenosine (dADO) analog, which is resistant to degradation by adenosine deaminase and is preferentially phosphorylated in lymphocytes, F-ara-AMP, F-ara-ADP and F-ara-ATP. F-ara-ATP may be incorporated into DNA as a fraudulent base and also inhibits DNA repair, features that explain its potent activity in CLL and its ability to synergize with alkylating agents, including BEN [16]. Moreover, a synergistic antitumor activity was seen with BEN even in cases which were resistant to treatment with the agents given alone and in those with a non-functioning p53 [8]. However, whether this combination will be useful in the clinic appears unlikely, as both agents produce significant marrow suppression. In contrast to FLU, PEN is a potent inhibitor of adenosine deaminase resulting in the accumulation of deoxyadenosine in plasma, which is subsequently phosphorylated in CLL cells by deoxycytidine kinase, the phosphorylation process causing the inhibition of DNA repair and leading to cell death [17]. This effect is responsible for the antitumor activity of dADO and synergy may be seen between dADO/PEN and irradiation or chlorambucil in CLL cells, but not in marrow stem cells [18,19]. However, whether dADO/PEN is synergistic with BEN has not been evaluated.

In the present study we evaluated the activity of BEN in comparison to CLB, FLU and PEN in primary CLL cells from both treated and untreated patients, to determine if there is cross-resistance between these agents and to cells with a p53 mutation. We have also compared the mechanism of action of these agents and determined whether synergy occurs between BEN and dADO/PEN in CLL.

2. Materials and methods

2.1. Patient samples and drugs

Peripheral blood mononuclear cells (PBMC) were isolated from 62 patients with a diagnosis of CLL [20]. CLL cells and clinical information were obtained from the Manitoba CLL Tumor Bank and samples processed, as previously described [21]. Freshly isolated cells were used for most experiments. Drug exposures were carried out in serum-free hybridoma media (SFM, Life Technologies) at 37 °C and 5% CO_2 in a humidified atmosphere. Informed consent was obtained from all patients and the study authorized by the human research ethics board at the University of Manitoba. The clinical information regarding the patients is summarized in Supplementary Table 1.

BEN, CLB, FLU, PEN and dADO were purchased from Sigma-Aldrich. All agents were reconstituted to 100 mM in dimethyl sulfoxide (DMSO) (Sigma-Aldrich), aliquoted and stored at −80 °C. Aliquots were then diluted in SFM (Life Technologies). For all treatments using dADO, 5–10 μM PEN was added to inhibit adenosine deaminase.

2.2. MTT assay

Cytotoxicity was assessed using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay [22]. CLL cells from 16 patients were incubated *in vitro* at 6×10^5 cell/well in a 96-well plate for 96 h with 9 increasing doses of drug (range, 0–48.8 μM for BEN and CLB, 0–18.5 μM for FLU for the majority of samples). Concentration ranges were selected and optimized to obtain the exponential phase of the dose-response curve. Each condition was performed in triplicate. DMSO treated cells were used as a negative control and for normalization. Plates were read at 570 nm using a SpectraMax M5 plate reader (Molecular Devices).

2.3. Flow cytometry and drug synergy

Cell death was determined by flow cytometry by negativity for both annexin-V and 7AAD (7-aminoactinomycin D; BD Pharmagen) [22]. For synergy and dose-response determination, CLL cells from 23 patients, representing the clinical disease spectrum (Supplementary Table 1) were treated in a 96-well plate with 8 increasing concentrations of drugs for 72 h. As for the MTT assay, drug concentration ranges were selected and optimized to ensure that the exponential phase of the dose-response curve was obtained. For the first 6 patients the concentration ranges were 0–48.8 μM for BEN, CLB, and dADO, 0–30.0 μM for FLU, and the dose of PEN maintained at 10 μM. The concentrations were then adjusted for the remaining 17 patients to 0–79.2 μM for BEN and CLB. When calculating the lethal dose for 50% of cells (LD_{50}) for BEN and CLB for the first 6 patients, the values were extrapolated to match the 0–79.2 μM dose range.

For drug combinations, a 1:1 ratio between the drugs was selected. The adjusted concentration ranges for the drug combinations were 0–48.8 μM for BEN/CLB, 0–30.0 μM for BEN/FLU, and 0–18.5 μM for CLB/FLU, 0–79.2 μM for BEN/dADO, and 0–30 μM for CLB/dADO or FLU/dADO. The concentration of DMSO was constant between samples and DMSO alone was used as a negative control and for normalization. After 72 h incubation, cells were stained with annexin-V-FITC and 7AAD (BD Pharmagen) and analyzed by flow cytometry using a NovoCyte flow cytometer (ACEA Biosciences) with a 96-well plate adapter or a BD FACSCalibur (BD Biosciences).

2.4. DNA damage analysis

DNA double strand breaks (DSBs) were measured by γH2A.X, after cells were treated for 18 h with 10 μM drug or the median LD_{50}, and DMSO or untreated cells were used as a negative control and for normalization. As a positive control, untreated cells were irradiated (20 Gy) at 16 h, using a RS-2000 Rad Source (Rad Source Technologies, Inc.). 30 min post-irradiation, samples were stained using the H2A.X phosphorylation assay kit (Millipore) as per the manufacturer's instructions, but 2 μl of anti-γH2A.X FITC-conjugated antibody was used. Samples were analyzed by flow cytometry.