Biochemical Pharmacology 85 (2013) 927–936

Contents lists available at SciVerse ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

ABT-737 resistance in B-cells isolated from chronic lymphocytic leukemia patients and leukemia cell lines is overcome by the pleiotropic kinase inhibitor quercetin through Mcl-1 down-regulation

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ARTICLE INFO

Article history:
Received 30 October 2012
Accepted 15 January 2013
Available online 24 January 2013

Keywords:
ABT-737
Quercetin
Mcl-1
Chronic lymphocytic leukemia
Apoptosis

ABSTRACT

Chronic lymphocytic leukemia (CLL) is the most frequent form of leukemia in adult population and despite numerous studies, it is considered an incurable disease. Since CLL is characterized by overexpression of pro-survival Bcl-2 family members, treatments with their antagonists, such as ABT-737, represent a promising new therapeutic strategy. ABT-737 is a BH3 mimetic agent which binds Bcl-2, Bcl-X_L and Bcl-w with high affinity, while weakly interacts with Mcl-1 and Bfl-1. Previous studies demonstrated that quercetin, a flavonoid naturally present in food and beverages, was able to sensitize B-cells isolated from CLL patients to apoptosis when associated with death ligands or fludarabine, through a mechanism involving Mcl-1 down-regulation. Here, we report that the association between ABT-737 and quercetin synergistically induces apoptosis in B-cells and in five leukemic cell lines (Combination Index <1). Peripheral blood mononuclear cell from healthy donors were not affected by quercetin treatment. The molecular pathways triggered by quercetin have been investigated in HPB-ALL cells, characterized by the highest resistance to both ABT-737 and quercetin when applied as single molecules, but highly sensitivity to the co-treatment. In this cell line, quercetin down-regulated Mcl-1 through the inhibition of PI_3K/Akt signaling pathway, leading to Mcl-1 instability. The same mechanism was confirmed in B-cells. These results may open new clinical perspectives based on a translational approach in CLL therapy.

1. Introduction

Chronic lymphocytic leukemia (CLL) is the most frequent form of leukemia in adults in the Western world with an incidence of 3.5 and 6.15 per 100,000 per year in the United States and UK, respectively [1]. In 2008, 15,110 new cases of CLL were diagnosed in the United States, and 4390 patients died as a result of this disease [2]. Some patients with CLL survive for many years or decades without any treatment because of the relatively slow progression rate of the disease. Other patients experience a rapid and fatal disease despite therapy. Treatment depends on the clinical staging, Rai or Binet, which classify patients according to tumor burden and hematopoietic impairment. Front-line therapy for CLL for several decades has been represented by treatment with alkylating agents and purine analogues, such as chlorambucil, cyclophosphamide, bendamustine, fludarabine, pentostatin and cladribine [3]. However, despite the improved efficacy of CLL treatment, resistance to treatments and relapse are frequent events. As an example, up to 37% of previously untreated and 76% of previously treated patients with CLL do not respond to fludarabine monotherapy [4]. More recently, significant clinical outcomes have been achieved using chemo-immunotherapy with rituximab, a chimeric monoclonal antibody against CD20, and alemtuzumab, a recombinant, fully humanized, monoclonal antibody against the CD52 antigen, when administrated in monotherapy or in combination with alkylating agents and purine analogues [1,3–5]. Nevertheless, as relapse remains problematic, particularly in older patients, the identification of innovative and specific agents against CLL remains of high interest, including new monoclonal antibodies, protein kinase inhibitors, Bcl-2 antagonists [2,6].

Abbreviations: Bcl-2, B-cell lymphoma; BH3, Bcl-2 homology domain 3; CI, combination index; CLL, chronic lymphocytic leukemia; CR, complete remission; Mcl-1, myeloid cell leukemia 1; MEK, mitogen-activated protein kinase kinase; PI_3K, phosphatidylinositol-3-kinase; PBMC, peripheral blood mononuclear cell; PS, phosphatidylserine; qPCR, quantitative PCR.

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http://dx.doi.org/10.1016/j.bcp.2013.01.011

In the late ninety, independent research group demonstrated that over-expression of the anti-apoptotic protein B-cell lymphoma (Bcl-2) can be considered a hallmark of CLL [7]. In this contest, the so-called BH3 mimetic agents represent novel promising agents for the treatment of CLL. One of such agents is ABT-737. Such small molecule binds with high affinity (<1 nM) to Bcl-2, Bcl-X_L and Bcl-w and has proven effective as a single agent in animal models of cancer and in killing various tumor cell lines and primary cells derived from patients, including CLL [8,9]. However, the therapeutic application of ABT-737 is hampered by its very low oral bioavailability and water solubility. Recently a second generation of orally bioavailable BH3 mimetic, ABT-263, became available opening new clinical perspectives for the use of the molecule as a single agent and in combination regimens. In fact, ABT-263 exhibits similar selective cytotoxicity (K_i <1 nM for Bcl-2, Bcl-X_L, and Bcl-w) than ABT-737 in cell lines and preclinical animal models [10]. A phase I study in CLL patients with relapsed or refractory disease demonstrated that ABT-263 (Navitoclax) showed encouraging results in terms of patient susceptibility and establishment of doses for phase II clinical trials [11].

ABT-737/263 antagonizes several members of Bcl-2 protein family, but has low affinity for Mcl-1 or Bfl-1, determining resistance to ABT-737 in CLL [12] and other malignant cell lines [13,14]. The (Mcl-1 + Bfl-1)/Bcl-2 ratio has been validated in a panel of leukemic cell lines as an index to predict the response of CLL to ABT-737 in view of a therapeutic application [15]. Therefore, a therapeutic strategy leading to down-regulation of Mcl-1 expression could enhance ABT-737 efficacy in CLL. To this respect, it is worthwhile to note that low expression of Mcl-1 mRNA is correlated to prolonged survival in B-CLL [16] and Mcl-1 down-regulation induces apoptosis in a number of leukemia derived cell lines and enhances rituximab-mediated apoptosis [17–19]. In primary B-cells isolated from patients with CLL, Mcl-1 protein expression has been shown to correlate with an adverse prognosis if combined to other prognostic markers, such as the stage of the disease, IgV_H mutation status, ZAP-70 positivity and CD38 expression [20]. In primary B-CLL cells, PI_3K/Akt seems to play a major role in regulating Mcl-1 depending cell survival [21,22].

In the attempt to bypass Mcl-1 mediated resistance in CLL, we recently reported that quercetin, (3,3′,4′,5,7-pentahydroxyflavone), a flavonoid widely present in fruits and beverages [23], down-regulates Mcl-1 acting directly or indirectly on its mRNA stability and protein degradation, suggesting that the same mechanism may overcome resistance to apoptosis in leukemic cells isolated from CLL patients [24]. Quercetin belongs to the wide group of naturally occurring molecules proposed as potential candidates in adjuvant chemotherapy when associated with other drugs ([25] and reviewed in Ref. [26]). Previous works from our group demonstrated that quercetin is able to re-establish sensitivity to apoptosis induction in leukemic cell lines resistant to CD95- and TRAIL-induced cell death [27–29]. More recently, we demonstrated that quercetin sensitizes leukemic cells isolated from CLL patients when associated to death ligands (e.g., TRAIL and anti-CD95) or fludarabine [30] through a mechanism involving Mcl-1 down-regulation [24].

In the present work, we demonstrate that ABT-737 in combination with quercetin synergistically induce apoptosis in B-cells isolated from CLL patients and in five leukemic cell lines (HPB-ALL, Jurkat, U-937, K562, HL-60). This sensitizing effect is associated to a decrease in Mcl-1 mRNA and protein expression upon quercetin treatment which involves the inhibition of PI_3K/Akt and/or MEK/MAPK signaling pathways, both related to Mcl-1 stability.

2. Materials and methods

2.1. Reagents

Roswell Park Medium Institute (RPMI) medium, L-glutamine 200 mM, penicillin 5000 IU/ml/streptomycin 5000 μg/ml and PBS (phosphate buffer saline) tablets were purchased from Life Technologies (Monza, Italy); fetal bovine serum from Cambrex (Milano, Italy). Neutral red solution (0.33%, v/v), trypan blue solution (0.4%, v/v), propidium iodide, quercetin, catechin, myricetin, PD98059, wortmannin, MG-132 and dimetylsulfoxide (DMSO) were from Sigma–Aldrich (Milano, Italy). Z-VAD-FMK was from Bio-Rad Laboratories (Segrate, Milano, Italy). LY294002 was a gift of Dr. Paola Ungaro (Istituto di Endocrinologia ed Oncologia Sperimentale, CNR, Naples, Italy). ABT-737 and its enantiomer were kindly provided by Abbott Laboratories (Abbott Park, IL, USA) [9], dissolved in DMSO and stored in aliquots at −80 °C.

2.2. Cell culture, isolation of lymphocyte from CLL patients, cell viability assay

Human tymoma-derived cells HPB-ALL, the human T lymphoblastoid cell line Jurkat, K562 chronic myeloid leukemia cell line, the HL-60 promyelocytic cell line, the human myelomonocytic cell line U937, were cultured in RPMI medium supplemented with 10%, fetal bovine serum and 1% penicillin/streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2.

Peripheral blood samples from anonymous CLL patients and healthy donors were provided by the Onco-Hematology Department of Moscati Hospital (Avellino, Italy) with informed consent. Patients were either untreated or had not received therapeutic treatment for a period of at least 6 months. Mononuclear cells (leukemic cells > 90%) were isolated following density gradient centrifugation (Ficoll-Paque Plus, GE Healthcare, Milano Italy). Cells were washed three times in PBS, counted with trypan blue dye to assess their viability (cell viability > 95%) and immediately cultured in RPMI supplemented with 1% penicillin/streptomycin, 2 mM L-glutamine and 10% heat inactivated autologous serum [31], at 37 °C in a humidified atmosphere containing 5% CO_2. Cells were cultured at density of 1×10^6/ml in 48 multi-well plates and incubated (24–48 h) in a medium containing specified treatments. Cell viability assay was performed using neutral red viability test as described [28].

2.3. Combination index

To evaluate the interaction between quercetin and ABT-737, combination analysis was performed. Combination index (CI) values were calculated according to the Chou and Talalay mathematical model for drug interactions [32]. Dose–response curves, dose–effect analysis and CI for the combination treatment groups were generated using the equations reported by Chou and Talalay using the CompuSyn software (freely available at: www.combosyn.com). Chou and Talalay method can be successfully be used to analyze data retrieved from experiments of drug combination designed at "constant ratio" and "non-constant ratio". We used the latter method in order to keep concentration of quercetin and ABT-737 below their respective EC_{50} (see text for discussion). A CI of >1 implies antagonism, CI = 1 is additivity and CI < 1 is synergy. All experiments were done in triplicate.

2.4. Caspase assays

To determine caspase-3 and -9 enzymatic activities, 2×10^6/ml of cells were incubated with relative treatment for 6 or 16 h. Cells were collected and centrifuged at 400 × *g* for 5 min, washed twice