Available online at www.sciencedirect.com
SCIENCE DIRECT
Cancer Letters 245 (2007) 315–320
CANCER Letters
www.elsevier.com/locate/canlet

Pomolic acid may overcome multidrug resistance mediated by overexpression of anti-apoptotic Bcl-2 proteins

Janaina Fernandes [a,1], Ricardo Weinlich [b,1], Rachel Oliveira Castilho [c], Gustavo Pessini Amarante-Mendes [b], Cerli Rocha Gattass [a,*]

[a] *Instituto de Biofísica Carlos Chagas Filho, Laboratório de Imunoparasitologia, Universidade Federal do Rio de Janeiro, Centro de Ciências da Saúde, Bloco G, Cidade Universitária, 21949-900 Rio de Janeiro, RJ, Brazil*
[b] *Department of Immunology, Institute of Biomedical Sciences, University of São Paulo, and Institute for Investigation in Immunology, Millenium Institute, Av. Prof. Lineu Prestes, 1730 Cidade Universitária, 05508-900 São Paulo, SP Brazil*
[c] *Universidade Católica Dom Bosco, Av. Tamandaré 6000, 79117-900 Campo Grande, Mato Grosso do Sul, Brazil*

Received 4 October 2005; received in revised form 2 December 2005; accepted 16 January 2006

Abstract

Multidrug resistance (MDR) is multifactorial and may be mediated by overexpression of anti-apoptotic proteins. This paper investigated whether pomolic acid (PA) was able to overcome resistance mediated by overexpression of Bcl-2 or BcL-xL. The results obtained showed that overexpression of these proteins partially inhibited the PA-induced apoptosis, loss of mitochondrial membrane potential and caspase -3 and -9 activation observed in HL-60/neo. Since Bcl-2 transfected cell lines were shown to be quite resistant to a series of chemotherapeutic agents, the data presented call attention to the possible clinical significance of PA as an anti-MDR drug.

Keywords: Pomolic acid; MDR; Anti-apoptotic proteins; Bcl-2 and Bcl-xL; Leukemia; Apoptosis

1. Introduction

The expression of anti-apoptotic Bcl-2 proteins are clinically related to multidrug resistance (MDR) in several types of cancer [1–3]. MDR, the major cause of chemotherapeutic failure in cancer, is a multifactorial phenomenum. Overexpression of transporter glycoproteins such as P-gp/ABCB1, MRP1/ABCC1, and BCRP/ABCG2 are some of the most well known MDR effectors [4,5]. By actively extruding the drug, these proteins decrease their intracellular concentration and prevent cell death. However, although expression of these proteins play an important role in MDR, they do not account for all the MDRs. Other MDR mechanisms, such as resistance associated with drug entrapment [4,6] and with altered apoptotic response due to altered p53 expression and function [7] or altered expression of Bcl-2 proteins [8–10] are attracting much attention.

The role of Bcl-2 family of proteins in modulating the mitochondrial (intrinsic) pathway of apoptosis is well

Abbreviations: Apaf-1, apoptotic protease activating factor-1; $\Delta\psi_m$, mitochondrial transmembrane potential; $DiOC_6(3)$, 3,3′-dihexyloxacarbocyanide iodide; FACS, fluorescence-activated cell sorter; PI, propidium iodide; PTP, permeability transition pore.

* Corresponding author. Address: Dra Cerli Rocha Gattass, Instituto de Biofísica Carlos Chagas Filho, Laboratório de ImunologiaCelular, Universidade Federal do Rio de Janeiro, Centro de Ciências da Saúde, Bloco G, Cidade Universitária, CEP 21949-900, Rio de Janeiro, R.J, Brazil. Tel.: +55 21 2562 6564; fax: +55 21 2280 8193.
E-mail address: cerli@chagas.biof.ufrj.br (C.R. Gattass).
[1] Janaina Fernandes and Ricardo Weinlich contributed equally to this paper.

0304-3835/$ - see front matter © 2006 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.canlet.2006.01.009

known. Classically, activation of this pathway is initiated by the opening of mitochondria permeability transition pore (PTP), loss of transmembrane potential ($\Delta\psi_m$), and release of apoptogenic factors from the mitochondrial intermembrane space [11,12]. Release of cytochrome *c* into the cytosol triggers the formation of the cytochrome *c*/dATP/Apaf-1/caspase-9 containing apoptosome and the subsequent activation of effector caspases (−3, −6 and −7), the executioners of the apoptotic program [11,13]. It has been proposed that members of the Bcl-2 family may regulate the central events of the intrinsic pathway of apoptosis: opening of the PTP and loss of $\Delta\psi_m$ [14,15]. Anti-apoptotic members of the Bcl-2 family (Bcl-2, Bcl-xL), inhibit cell death by forming complexes with pro-apoptotic ones (Bax, Bad) [16–19]. It was also shown that the reduced expression of Bcl-2 sensitizes the tumor cells to apoptosis [20,21].

Several evidences indicate that the overexpression of the anti-apoptotic members of the Bcl-2 family impairs apoptosis induced by several chemotherapeutic drugs actually in clinical use, thus leading to chemoresistance. In addition to impairing apoptosis induced by several chemotherapeutic drugs [1,22,23], the involvement of the anti-apoptotic Bcl-2 protein in resistance to radiotherapy [24] and disease progression in prostate and breast cancer [25,26] has also been demonstrated. Similarly, the over expression of Bcl-xL, was also related to chemoresistance and tumor progression in several types of tumors [3,27].

The search for new drugs able to overcome resistance mechanisms is of great interest for cancer therapy. In a previous study, our group [28] showed that pomolic acid (PA), a pentacyclic triterpene isolated from *Chrysobalanus icaco*, was highly effective in inhibiting the growth of leukemia cell lines, including a MDR-resistant leukemia line that overexpresses P-gp [29]. We also showed that the apoptotic activity of this triterpene is mediated through a direct effect on the mitochondria [30]. Considering the regulatory role played by Bcl-2 proteins on the mitochondria pathway of apoptosis, the aim of this work was to evaluate if PA was able to overcome resistance mediated by the overexpression of Bcl-2 and Bcl-xL.

2. Materials and methods

2.1. Chemicals and cell culture

Pomolic acid, isolated from *Chrysobalanus icaco* L. as described previously [28], was dissolved in dimethyl sulfoxide (DMSO, Sigma, St Louis) and diluted in RPMI 1640 for use. $DiOC_6(3)$ was from Molecular Probes, Inc. (Eugene, OR), mAbs for caspases-3 and -9 were from Pharmingen (San Diego, CA, USA), anti-actin mAb was from ICN (Costa Mesa, USA), and anti-mouse IgG-HRP from Amersham Biosciences (Arlington, IL). The human promyelocytic leukemia cell line HL-60 and its stably transfected clones HL-60-Bcl-2, HL-60-Bcl-xL [31], were cultured at 37 °C and 5% CO_2) in RPMI 1640 (Life Technologies, Inc., USA) supplemented with heat inactivated 10% fetal calf serum (FCS; Life Technologies, Inc., USA), 2 mM L-glutamine, 100 U/ml of penicillin and 100 μg/ml of streptomycin (Life Technologies, Inc., USA). The transfected cell lines were previously shown to overexpress Bcl-2 or Bcl-xL and be resistant to a series of chemotherapeutic drugs [31].

2.2. Detection of apoptosis

Apoptosis was evaluated by cell cycle analysis [32]. Platted cells (2×10^5/well) were treated with medium or 50 μg/mL of PA. This concentration was established from dose response curves done previously [30]. After 18 h incubation, cells were harvested and resuspended in 300 μL of Hypotonic Fluorescent Solution (50 μg/mL propidium iodide (PI) and 0.1% Triton X-100 in 0.1% Na Citrate buffer). After 1 h incubation in the dark at 4 °C, the DNA content was measured by flow cytometry (FL-2) (FACSCalibur, Becton Dickinson, San Jose, CA). Data acquisition and analysis were controlled by Cellquest software version 3.1f. Subdiploid populations were considered apoptotic. The results represent the average ± SD of three experiments.

2.3. Measurement of mitochondrial transmembrane potential ($\Delta\psi_m$)

Variations of mitochondrial membrane potential (MMP) was assessed with the fluorochrome $DiOC_6(3)$ (40 nM) as previously described [33]. Cells were plated and treated with PA under the same conditions described for detection of apoptosis. Stained cells were analyzed by flow cytometry (FL-1). The results represent the average ± SD of three experiments.

2.4. Western blot analysis

SDS-PAGE and western-blot analysis were performed as described elsewhere [34]. Briefly, plated cells (2×10^5/well) were treated with medium or 25 μg/ml PA, for 24 h. After this time, they were collected by centrifugation, washed once in ice-cold PBS, lysed directly in SDS-sample buffer (50 mM Tris–HCl, pH 6.8, 2% SDS, 10% glycerol, 2.5% 2-ME), and boiled for 5 min. Equal amounts of protein were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes at 150 mA for 5 h. Blots were blocked for 2 h in TBST (10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.05% Tween) containing 5% nonfat dried milk, probed with anti-inactive caspases-3 or -9 for 1 h at RT and then with anti-mouse IgG-HRP. Blots were developed using the enhanced chemiluminescence system (ECL, Amersham, Arlington, IL).