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Medicarpin and millepurpan, two flavonoids isolated from *Medicago sativa*, induce apoptosis and overcome multidrug resistance in leukemia P388 cells

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ABSTRACT

Background: High consumption of flavonoids has been associated with a decrease risk of cancer. Alfalfa (*Medicago sativa*) leaves have been widely used in traditional medicine and is currently used as a dietary supplement because of their high nutrient content. We previously reported the cytotoxic activity of alfalfa leaf extracts against several sensitive and multidrug resistant tumor cell lines.
Hypothesis/purpose: We aimed to determine whether medicarpin and millepurpan, two isoflavonoids isolated from alfalfa leaves, may have pro-apoptotic effects against drug-sensitive (P388) and multidrug resistant P388 leukemia cells (P388/DOX).
Study design/methods: Cells were incubated with medicarpin or millepurpan for the appropriate time. Cell viability was assessed by the MTT assay. DNA fragmentation was analyzed by agarose gel electrophoresis. Cell cycle analysis was realized by flow cytometry technics. Caspases 3 and 9 activities were measured using Promega caspACE assay kits. Proteins and genes expression were visualized respectively by western-blot using specific antibodies and RT-PCR assay.
Results: P-glycoprotein-expressing P388/DOX cells did not show resistance to medicarpin ($IC_{50} \approx 90$ μM for P388 and P388/DOX cells) and millepurpan ($IC_{50} = 54$ μM and 69 μM for P388 and P388/DOX cells, respectively). Treatment with medicarpin or millepurpan triggered apoptosis in sensitive as well as multidrug resistant P388 cells. These effects were mediated through the mitochondrial pathway by modifying the balance pro/anti-apoptotic proteins. While 3 μM doxorubicin alone could not induce cell death in P388/DOX cells, concomitant treatment with doxorubicin and subtoxic concentration of medicarpin or millepurpan restored the pro-apoptotic cascade. Each compound increased sensitivity of P388/DOX cells to doxorubicin whereas they had no effect in sensitive P388 cells. Vinblastine cytotoxicity was also enhanced in P388/DOX cells ($IC_{50} = 210$ nM to 23 and 25 nM with medicarpin and millepurpan, respectively). This improved sensitivity was mediated by an increased uptake of doxorubicin in P388/DOX cells expressing P-gp. P-gp expression was not altered by exposure to medicarpin and millepurpan.
Conclusion: These data indicate that medicarpin and millepurpan possess pro-apoptotic properties and potentiate the cytotoxicity of chemotherapy drugs in multidrug resistant P388 leukemia cells by modulating P-gp-mediated efflux of drugs. These flavonoids may be used as chemopreventive agents or as sensitizer to enhance cytotoxicity of chemotherapy drugs in multidrug resistant cancer cells.
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Introduction

Resistance to chemotherapy is a major cause of failure in cancer treatment, one of the main mechanisms being the overexpression of drug efflux pumps such as the 170-kDa P-glycoprotein (P-gp). P-glycoprotein is a member of the highly conserved superfamily of ATP-binding cassette (ABC) transporters proteins. It acts as an

Abbreviations: DOX, doxorubicin; MED, medicarpin; MIL, millepurpan; P-gp, P-glycoprotein; VBL, vinblastine; VPL, verapamil..

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ATP-dependent drug efflux pump that reduces intracellular accumulation of antineoplastic agents and thereby hampers their effectiveness in clinic (Bates et al. 2001; Borst and Elferink 2002; Gottesman et al. 2002).

A multitude of approaches to attempt reversal of multidrug resistance have been investigated but efforts to reverse drug resistance in tumor cells gave little results due to dose-limiting toxicity in clinical trials (Volm 1998). Therefore, there is a need to develop new compounds capable of inducing apoptosis despite the existence of mechanisms which limit drug accumulation in tumor cells. Apoptosis is a regulated program of cell death that occurs under a variety of physiological and pathological conditions. It is characterized by the activation of a complex intracellular pathway leading to a cascade of biochemical and morphological changes (Hengartner 2000). Induction of apoptosis depends on the balance between pro-apoptotic factors such as Bax or Bak, and anti-apoptotic factors such as Bcl-2 or Bcl-X_L (Chao and Korsmeyer 1998; Gross et al. 1999).

In the last twenty years, researches focused on naturally-occurring compounds, especially flavonoids. A lot of evidences have been accumulated, showing that the beneficial effects of plant extracts in cancer chemoprevention may be in part attributed to the presence of these polyphenolic compounds (Khan et al. 2008; Ramos 2008). Flavonoids were shown to possess a wide variety of biological effects, including apoptosis induction and multidrug resistance reversal in tumor cells (Limtrakul et al. 2005; Qian et al. 2005; Ramos 2007).

Alfalfa (*Medicago sativa*) is a plant from the *Fabaceae* family whose culture is mainly intended to cattle feeding. However alfalfa leaves are also used as dietary supplements because of their high protein and vitamin contents, and have been widely used for 1500 years to cure various ailments. It displays numerous pharmacological properties, recently reviewed by Bora and Sharma (Bora and Sharma 2011).

In a previous study, we described the antiproliferative and apoptosis-inducing effects of alfalfa leaf extracts in several tumor cells lines and the isolation of flavonoids, including medicarpin (MED) and millepurpan (MIL), with potential antitumor activities (Gatouillat et al. 2014).

In this study, we report the *in vitro* pro-apoptotic effects of medicarpin and millepurpan, which were mediated through the mitochondrial pathway by modifying the balance between pro- and anti-apoptotic proteins. Then, we investigated whether they could sensitize P388 cells overexpressing P-glycoprotein to doxorubicin and vinblastine treatment and, thereby, overcome multidrug resistance.

Materials and methods

Antibodies and reagents

Cell culture reagents and propidium iodide were purchased from InVitrogen (Cergy-Pontoise, France). Doxorubicin (DOX), vinblastine (VBL), verapamil (VPL) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma (Saint Quentin Fallavier, France). Anti-β-actin, PARP-1, caspase-3 antibodies were from Santa Cruz Biotechnology. Anti-Bcl-2, Bcl-X_L, Bax antibodies were from Beckman Coulter. Anti-P-gp antibody was purchased from Proteogenix. Medicarpin and millepurpan were extracted and purified from *Medicago sativa* leaf extracts as previously described (Gatouillat et al. 2014). Briefly, the dried plant was supplied by Prolivim (Reims, FRANCE). After several steps of extraction, fractions eluted with cyclohexane-$CHCl_3$ (9:1) were purified by column chromatography (CC) over RP-18, eluted with MeOH–H_2O to give millepurpan. Fractions eluted with $CHCl_3$ were purified by prep. TLC in CH_2Cl_2-Methanol (98:2) to give medicarpin.

Cell culture

The murine P388 leukemia cell line was supplied by Dr. G. Atassi (Servier Lab, France) and maintained in our laboratory. A doxorubicin-resistant subline (P388/DOX) was established by culturing them with increasing concentrations of the drug. Cells were cultured in RPMI medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C in a humidified atmosphere of 5% CO_2. The resistant sublines were cultured in the presence of 1 μM doxorubicin. Before experiments, they were cultured in a drug-free medium for at least 7 days.

Cell viability assay

Cell viability was assessed by the MTT assay. Cells growing in suspension were seeded in a 96-well plate at a density of 5×10^3 cells per well and incubated with increasing concentrations of the appropriate compound for 72 h. In every control experiment, an equal volume of DMSO which is used to dissolve extracts or compounds was added in each well. After the period of incubation, 20 μl MTT (2.5 mg/ml) were added to each well for 3 h. Then, the medium was removed and formazan crystals were dissolved in 200 μl DMSO. Absorbance was measured at 540 nm using a microplate reader (Multiskan Ascent, Labsystems). Triplicate experiments were conducted in each test. Percent viability was calculated as (Absorbance of treated sample/Absorbance of non-treated sample) x 100.

RT-PCR assay

Total RNA was extracted from cultured cells using the Qiagen RNeasy Kit procedure (Qiagen; Courtaboeuf, France). One microgram of mRNA was used as a template for each RT-PCR. The primer sets were 5'-TGCTTATGGATCCCAGAGTGAC-3′ and 5′-TTGGTGAGGATCTCTCCGGCT-3′ for mdr1; 5′-GAAAGAT GGTGAACTATGCC-3′ and 5′-TTACCAAAAGTGGCCCACTA-3′ for mdr3; 5′-GAAAGATGGTGAACTATGCC-3′ and 5′-TTACCAAAAGTGGCCCACTA-3′ for 18S rRNA.

Western blotting and densitometry

Cells (3×10^6) were lysed on ice for 15 min in RIPA lysis buffer (50 mM Tris–HCl, 150 mM EDTA, 1% Igepal CA-630, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with 0.5 mM PMSF and 0.1% protease inhibitor mixed. After centrifugation (15,000 g, 20 min, 4 °C), the supernatant was collected and total protein concentration was determined using the Bradford method. Equal amounts of proteins were separated on a 4–12% polyacrylamide gel and electrotransferred on nitrocellulose membranes. Immunodetection was performed using the Western Breeze chemiluminescence detection system (InVitrogen) according to the manufacturer's instructions with the appropriate monoclonal antibodies. Densitometric measurement of immunoblots was performed using the ImageJ software. Each specific protein expression was normalized compared to β-actin expression.

DNA fragmentation analysis

After treatment, cells were washed in PBS and lysed on ice for 15 min in a lysis buffer (10 mM Tris–HCl, 1 mM EDTA, 0.5% Triton X-100). The lysate was successively treated with RNase A (100 μg/ml) and proteinase K (1 mg/ml) for 1 h at 37 °C. After centrifugation (15,000 g, 15 min, 4 °C), DNA was precipitated overnight with absolute ethanol and 3 M sodium acetate at –20 °C and electrophoresed on 1.2% agarose gel containing ethidium bromide. DNA fragmentation was observed under a UV transilluminator.

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