Induction of apoptosis in human lymphoblastoid cells by kaempferol 3-O- β -isorhamninose and rhamnocitrin 3-O- β -isorhamninose from *Rhamnus alaternus* L. (Rhamnaceae)

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Abstract

Objective: Kaempferol 3-O- β -isorhamninose (K3O-ir) and rhamnocitrin 3-O- β -isorhamninose (R3O-ir) from *Rhamnus alaternus* L leaves are investigated for their ability to induce apoptosis in human lymphoblastoid cells. We have attempted to characterize apoptotic pathway activated by these two flavonoids.

Material and methods: Apoptosis of the human TK6 lymphoblastoid cell line was detected by DNA fragmentation, PARP cleavage and by evaluating caspase activity.

Results: Apoptosis was observed after 24- and 48-h incubation of the cells with the tested compounds. DNA fragmentation was observed after treatment with flavonoids; this was confirmed by demonstration of PARP cleavage. Caspase-3 and caspase-8 activities were induced by both K3O-ir and R3O-ir flavonoids showing highest activity with compound concentration of 400 μ g/ml.

Conclusion: We have demonstrated that K3O-ir and R3O-ir induce apoptosis in human lymphoblastoid cells by the extrinsic pathway of apoptosis.

Introduction

Apoptosis is genetically programmed cell death that is essential for development, maintenance of tissue homeostasis and elimination of unwanted or damaged cells from multicellular organisms (1). When apoptosis is dysregulated, it can contribute to various diseases such as

cancer, autoimmune and neurodegenerative diseases (2,3). Thus, understanding mechanisms of apoptosis is important for preventing and treating many diseases (4). Cell death by apoptosis is characterized by cell shrinkage, chromatin condensation, nuclear collapse and cellular fragmentation into apoptotic bodies.

In most cases, these morphological changes are accompanied by intranucleosomal DNA fragmentation (5). Central components of the conserved apoptotic machinery include Bcl-2, Apaf-1 (apoptotic protease activating factor 1) and caspase family members (6). Caspases compose a family of conserved cysteine proteases, which play pivotal roles in apoptosis (7).

Choi *et al.* (6) and Guo *et al.* (8) claim that caspases are synthesized as dormant proenzymes, which upon proteolytic activation, acquire the ability to cleave key intracellular substrates that result in morphological and biochemical changes associated with apoptosis.

Poly-(ADP-ribose)-polymerase (PARP) is a substrate for caspase-3; it is a 116-kDa enzyme involved in DNA repair (9). Activated caspase-3 cleaves PARP between amino acids 216 and 217, generating 89- and 24-kDa inactive fragments. Loss of PARP function precludes DNA repair, which contributes to the apoptotic phenotype (10). In apoptosis, the death receptor pathway requires membrane receptors such as Fas, which trimerize, then recruit an adaptor molecule, a Fas-associated death domain and procaspase-8, forming the death-inducing signaling complex (DISC) (11). At the DISC, procaspase-8 is processed and caspase-8 is activated, ensuring direct activation of caspase-3 (12).

Induction of apoptosis in tumour cells has been shown to be the most common anti-cancer mechanism targeted by therapy. Some researchers [for example, Bradham *et al.* (13); Friesen *et al.* (14); Kaufmann (15)] admit that compounds used in cancer chemotherapy, such as

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etoposide, cisplatin, doxorubicin and paclitaxel, have apoptosis-inducing activity (13–15). Therefore, natural agents exhibiting strong apoptosis-inducing activity would be expected to have potential utility as anticancer drugs. Regulation of caspase-3/caspase-8 activities could be a promising way to control apoptosis. Based on this idea, we have tested two triglycoside flavonoids isolated from the leaves of *Rhamnus alaternus*, kaempferol 3-O- β -isorhamnnoside and rhamnocitrin 3-O- β -isorhamnnoside.

Materials and methods

Chemicals

Dimethylsulphoxide (DMSO), monoclonal antibody, anti-poly-(ADP-ribose) polymerase (anti-PARP) and goat anti-mouse alkaline phosphatase-conjugated antibody, plus caspase-3 and caspase-8 colorimetric assay kits were purchased from Sigma RBI, (St. Louis, MO, USA). RPMI-1640 Glutamax, foetal bovine serum and gentamicin were purchased from GIBCO BRL Life technologies (Grand Island, NY, USA). Proteinase K, ethylene diamine tetra-acetic acid (EDTA), sodium dodecyl sulphate (SDS) and RNase A were obtained from Sigma Aldrich Co (St. Louis, MO, USA). Acrylamide and bisacrylamide, 5-bromo-4-chloro-3-indolyl phosphate (BCIP)/nitro blue tetrazolium (NBT) and Tween 20 were purchased from Promega (Madison, WI, USA). Ethidium bromide (EtBr) and bromophenol blue were purchased from Merck (Darmstadt, FR, Germany) and agarose and polyvinylidene difluoride (PVDF) membranes were obtained from Invitrogen Life Technologies, Paisley, UK.

Method of extraction

Dried and powdered leaves (100 g) of *R. alaternus* were first defatted using petroleum ether (1 l), then extracted with chloroform (1 l), ethyl acetate (1 l) and methanol (1 l) using Soxhlet apparatus (6 h). Four different extracts were obtained. These were concentrated to dryness and kept at 4 °C in the absence of light. Amongst these extracts, only the Soxhlet methanolic extract was fractionated and purified here. In addition, in order to obtain a total oligomer flavonoid (TOF)-enriched extract, the powdered leaves were macerated in water: acetone mixture (1:2) for 24 h, with continuous stirring. The extract was filtered and acetone was evaporated under low pressure, to obtain an aqueous phase. Phlobaphenes were removed by precipitation with excess of NaCl at 5 °C for 24 h. Supernatant was extracted with ethyl acetate, concentrated and precipitated in an excess of chloroform. The precipitate was then separated and TOF extract yielded.

Fractionation and isolation methods

K3O-ir was directly obtained by fractionation of TOF extract, on a silica gel column with EtOAc:MeOH:H₂O (100:15:13) solvent system as eluent.

The methanolic extract (6 g) was fractionated by vacuum liquid chromatography on a silica gel column eluted with CH₂Cl₂:MeOH with gradual increasing of MeOH content and eight fractions were collected. Fractions 5, 6 and 7 were rechromatographed over a silica gel column using an EtOAc:MeOH:H₂O (100:15:13) solvent system, to give seven subfractions. Subfraction 5 was rechromatographed on a C18 gel column using a H₂O:MeOH (70:30 to 0:100) gradient solvent system to afford R3O-ir.

K3O-ir and R3O-ir were identified by analysis of negative fast atom bombardment mass spectra (FAB-MS), ¹H NMR (nuclear magnetic resonance) spectroscopy and ¹³C NMR spectroscopy (Fig. 1) (16).

Cell lines, cell culture and chemicals

Human lymphoblastoid cell line TK6 (Kindly provided by Pr. Pierre Biscoff, Centre Paul Strauss, Strasbourg, France) expresses wild-type p53, and is thus p53 proficient. Cells were cultured in RPMI-1640 glutamax supplemented with 10% (v/v) foetal bovine serum, 1 mM sodium pyruvate, 1 mM non-essential amino acids, 50 µg/ml gentamicin at 37 °C in humid atmosphere of 5% CO₂. Experiments were performed after approximately two passages to limit chromosome instability due to cell maintenance in culture.

Alamar blue proliferation test

This assay was performed according to the manufacturer's instructions (Alamar, Sacramento, CA, USA). Briefly, after treatment either with 0.5% DMSO (control cells) or with the tested compounds dissolved in 0.5% DMSO,

Figure 1. Chemical structures of compounds.

cells were seeded at 5000 cells/well (200 µl) in 96-well microplates. Each experimental assay was performed in triplicate, then 20 µl of Alamar blue working solution was added to each well. After incubation at 37 °C in a humidified atmosphere containing 5% CO₂ for 4 h, plates were read at 590 nm emission wavelength (excitation: 560 nm) on a microplate Flow Multiscan reader (Dynex Technologies Issy-Les-Moulineaux, France). Absorbance was compared with that of control cells treated with 0.5% DMSO.

DNA fragmentation analysis

DNA fragmentation was analysed by agarose gel electrophoresis as described by Wang *et al.* (17), with slight modifications. TK6 cells (1.5×10^6 cells/ml) were exposed to tested compounds at concentrations of 400, 600 and 800 µg/ml, for 24 and 48 h, and then harvested by centrifugation. Control cells were also treated, with DMSO 0.5%. Cell pellets were resuspended in 200 µl of lysis buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 0.5% *N*-Lauroyl Sarcosine Sodium Salt) at room temperature for 1 h, then centrifuged at 12 000 *g* for 20 min at 4 °C. Supernatants were incubated overnight at 56 °C with 250 µg/ml proteinase K. Cell lysates were then treated with 2 mg/ml RNase A and incubated at 56 °C for 2 h. DNA was extracted with chloroform/phenol/isoamyl alcohol (24/25/1, v/v/v) and precipitated from the aqueous phase by centrifugation at 14 000 *g* for 30 min at 0 °C. Solutions recuperated were transferred to a 1.5% agarose gel and electrophoresis was carried out at 67 V for 3/4 h with TAE buffer (Tris 2 M, sodium acetate 1 M, EDTA 50 mM) as the running buffer. DNA in gels was visualized with EtBr (0.5 µg/ml) under UV light (17).

Western blot analysis

Cells treated with different concentrations of each reagent (400, 600 and 800 µg/ml) for 6, 24 and 48 h, as well as control cells treated with 0.5% DMSO, were lysed in lysis buffer (62.5 mM Tris-HCl and 6 M urea, pH = 6.8). Protein concentrations were determined in cell lysates using the Bradford method (18). Equal amounts of proteins were loaded on to polyacrylamide gel and separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). They were then transferred to PVDF membrane, which was blocked with 5% non-fat milk in 0.1% Tween 20-phosphate buffer saline overnight at 4 °C. Membranes were then incubated with primary antibody, anti-PARP, at 1:100 dilution for 2 h at room temperature. They were then washed and incubated in goat anti-mouse alkaline phosphatase-conjugated antibody at 1:7500 dilution for 1 h. Next, they were washed and chromogenic substrate BCIP/NBT was added to localise antibody binding proteins.

Caspase-3 and caspase-8 activities

Cells were cultured (10^6 cells/ml) in 25 cm² flasks for 24 h in absence or in presence of 400, 600 and 800 µg/ml of K3O-ir or R3O-ir at 37 °C. Controls were performed at the same time with 0.5% DMSO. Cells were harvested and centrifuged at 600 *g* and pellets were washed in PBS, incubated in ice cold lysis buffer for 15 min, then centrifuged at 16 000 *g* for 20 min. Supernatants (cell extracts eventually containing caspase-3 and caspase-8) were retrieved. Aliquots corresponding to 50 µg total protein were incubated along with acetylated tetrapeptide (Ac-DEVD) substrate labelled with chromophore *p*-nitroaniline (pNA), in presence of caspase buffer in a 96-well flat-bottomed microplate. Cleavage and release of pNA from substrates occur when caspase-3 and caspase-8 are active. Free pNA produces a yellow final reaction product, detected spectrophotometrically at 405 nm. Absorbance at 405 nm was performed against a blank, simultaneously, and containing assay buffer (200 mM HEPES, 1% CHAPS, 50 mM DTT, 200 mM EDTA). A standard curve allows determination of correspondence between absorbance and pNA concentration. Results were calculated according to manufacturers instructions then expressed versus caspase-3 and caspase-8 specific activities (µmol pNA per min/ml protein).

Statistical analysis

All tests were carried out in triplicate and results presented as means ± SD. Data were tested for statistical differences by one-way ANOVA followed by Dunnett's test using STATISTICA (Version 6.0; Tulsa, OK, USA, Statsoft Inc.) to compare results achieved for control (untreated cells) to those of cells treated with the different extracts. Statistical differences were determined at $P < 0.05$.

Results

Evaluation of cytotoxic activity

Human lymphoblastoid TK6 cells were treated with tested compounds, for 48 h at 37 °C. Cytotoxic activity of these compounds was evaluated using the Alamar blue assay. Results, summarized in Fig. 2, indicate that neither K3O-ir nor R3O-ir reached 50% inhibition of TK6 cell proliferation.

Induction of apoptotic DNA fragmentation by K3O-ir and R3O-ir

Fragmentation of TK6 cell DNA was detected by 1.5% agarose gel electrophoresis after exposing 1.5×10^6 cells to 0, 400, 600 and 800 µg/ml of each compound for 24

Figure 2. Inhibitory effect, of kaempferol 3-O- β -isorhamnoside (K3O-ir) and rhamnocitrin 3-O- β -isorhamnoside (R3O-ir), on viability of TK6 cells. Results are represented by the means $\pm$ SD of $n = 3$. * $P < 0.05$ means a significant difference between untreated and treated cells.

and 48 h. Examination of DNA electrophoresis profiles revealed ladder formation, which is characteristic of apoptosis (Figs 3 and 4). At exposure to 800 $\mu\text{g/ml}$ of K3O-ir for 24 h and at exposure to 400, 600 and 800 $\mu\text{g/ml}$ K3O-ir for 48 h, ladder DNA profiles were clearly observed. In the same way, we observed ladder DNA profiles at exposure to 400 and 600 $\mu\text{g/ml}$ of R3O-ir for 24 h and at exposure to 400, 600 and 800 $\mu\text{g/ml}$ of R3O-ir for 48 h, whereas control cells did not exhibit DNA ladder profiles. We deduced that K3O-ir and R3O-ir compounds from *R. alaternus* induce apoptosis of TK6 cells.

Effect of *Rhamnus alaternus* compounds on proteolysis of PARP

DNA fragmentation is often associated with activation of a family of cysteine proteases, the caspases. Caspase 3, in particular, seems to play an important role in several models of apoptosis (19,20). To confirm the apoptotic process of our observed DNA fragmentation, we investigated enzymatic activation of apoptotic proteins by measuring cleavage of PARP, which is a caspase-3 substrate. As shown in Fig. 5a,b and in Fig. 6a,b when cells were treated with *R. alaternus* flavonoids, increase in formation of 85 kDa fragment and decrease or total disappearance of the 116 kDa band, were observed. Addition of K3O-ir or R3O-ir induced cleavage of 116 kDa PARP into fragments of 85 and 31 kDa in inverse concentration-dependent manner, at the tested times.

Caspase-3 and caspase-8 activation assay

Cell pathways of K3O-ir and R3O-ir induced cell death were examined by assessing caspase-3 and caspase-8 activities, which play a critical role in apoptosis. Following

Figure 3. Effect of kaempferol 3-O- β -isorhamnoside on DNA fragmentation of TK6 cells and ladders, were detected by 1.5% agarose gel electrophoresis. A: untreated cells; M: shows the molecular weight markers (bp).

Figure 4. Effect of rhamnocitrin 3-O- β -isorhamnoside on DNA fragmentation of TK6 cells and ladders were detected by 1.5% agarose gel electrophoresis. A: untreated cells; M: shows the molecular weight markers (bp).

Figure 5. Changes in expression of apoptotic-related protein in response to treatment with *Rhamnus alaternus* compound. TK6 cells were treated with 400, 600 and 800 µg/ml of Rhamnocitrin 3-O-β-isorhamminoside for 6, 24 and 48 h. Protein extracts were subjected to western blotting to determine immunoreactivity levels of PARP, as described in methods section. PARP 116 and 85 kDa bands are shown. T: Control cells were treated by the vehicle only. (a) Quantification by scanning densitometry of PARP bands intensity (b).

this, 24 and 48 h treatments of TK6 cells with various concentrations of each of K3O-ir and R3O-ir, caspase-3 and caspase-8 activities were measured and compared with those of control cells (treated with 0.5% DMSO). As shown in Figs 7 and 8, cells treated with K3O-ir and R3O-ir for both 24 and 48 h, showed significant inverse concentration-dependent increase of both caspase-3 and caspase-8 activities.

Highest values of caspase-3 activity obtained after 24 h treatment with K3O-ir and R3O-ir were respectively 15 and 72 µmol pNA/min/ml. At this concentration (400 µg/ml), the compounds showed significant induction of caspase-3 activity compared to untreated cells. In the same way, K3O-ir and R3O-ir showed highest caspase-8 activity at the same concentration (400 µg/ml) after 24 h treatment. Values obtained were respectively 22 and 30 µmol pNA/min/ml. These results suggest that apoptosis induced by the tested flavonoids may occur through activation of common executors of apoptosis, such as caspase-3 through activation of caspase-8.

Discussion

Cancer develops when balance between cell proliferation and cell death is disturbed, and aberrant cell proliferation leads to tumour growth. Apoptosis is a form of physiological cell death essential for normal tissue development and

Figure 6. Changes in expression of apoptotic-related protein in response to treatment with *Rhamnus alaternus* compound. TK6 cells were treated with 400, 600 and 800 µg/ml of kaempferol 3-O-β-isorhamminoside for 6, 24 and 48 h. Protein extracts were subjected to western blotting to determine immunoreactivity levels of PARP, as described in methods section. PARP 116 and 85 kDa bands are shown. T: Control cells were treated by the vehicle only. (a) Quantification by scanning densitometry of PARP bands intensity (b).

Figure 7. Effect of kaempferol 3-O-β-isorhamminoside (K3O-ir) and rhamnocitrin 3-O-β-isorhamminoside (R3O-ir) on caspase-3 activity in TK6 cells. Lysates prepared from cells treated with the kaempferol 3-O-β-isorhamminoside and rhamnocitrin 3-O-β-isorhamminoside for 24 and 48 h were assayed for *in vitro* caspase-3 activity. The rate of cleavage of the caspase substrate DEVD-pNA was measured at 405 nm. The results are presented as the mean ± SD. The experiments were done in triplicate. * $P < 0.05$ means a significant difference between the untreated and treated cells. Control: cells were treated by the vehicle only.

homeostasis (21). After receiving an apoptotic death stimulus, cells first enter a signalling phase followed by final degradation phase, in which apoptosis is identifiable microscopically by chromatin condensation, cell

Figure 8. Effect of kaempferol 3-O- β -isorhamnnoside (K3O-ir) and rhamnocitrin 3-O- β -isorhamnnoside (R3O-ir) on caspase-8 activity in TK6 cells. Lysates obtained from cells treated with the kaempferol 3-O- β -isorhamnnoside and rhamnocitrin 3-O- β -isorhamnnoside for 24 and 48 h were assayed for *in vitro* caspase-8 activity. The rate of cleavage of the caspase substrate DEVD-pNA measured at 405 nm. The results are presented as the mean $\pm$ SD. The experiments were done in triplicate. * $P < 0.05$ means a significant difference between the untreated and treated cells. Control: cells were treated by the vehicle only.

shrinkage, caspase activation, membrane lipid rearrangement, DNA fragmentation and cell fragmentation, through formation of apoptotic bodies (22).

Apoptosis and its related signaling pathways have a profound effect on progression of cancer development (23). Induction of apoptosis is, therefore, a highly desirable goal for preventative strategies for cancer control (24). Flavonoids are a group of common phenolic plant pigments. They are widely distributed in the plant kingdom and occur naturally in a broad range of fruit and vegetables. They have been suggested to be able to inhibit replication of tumour cells (25,26) and to be anticarcinogenic (27). Previous studies have shown that flavonoids induce apoptosis in various tumour cells including K562, Molt-4, Raji and MCAS. The effect has also been observed in other tumour cell lines from gastric, colon and lung carcinomas (28). In addition, flavonoids also inhibited tumour growth through cell cycle arrest and induced apoptosis through a p53-dependent mechanism (29). In the present study, we showed that our flavonoids isolated from *R. alaternus*, can induce apoptosis in TK6 cells; however, to date, the mechanism of apoptosis is unclear. Thus, we investigated the role of the caspase cascade in flavonoid-induced human lymphoblastoid cell apoptosis. Results of the present study clearly demonstrate that K3O-ir and R3O-ir suppressed TK6 cell viability through inducing apoptosis. After treatment with flavonoids, DNA fragmentation was clearly observed. These results

suggested that TK6 cells treated with K3O-ir and R3O-ir undergo typical apoptosis and treatment with the tested flavonoids caused induction of caspase-3 activity and degradation of PARP, which precedes onset of apoptosis.

Caspase-3 is one of the key proteases responsible for cleavage and inactivation of PARP (30,31). PARP is involved in repair of DNA damage induced by certain anti-cancer agents and/or radiation, and is important for the maintenance of cell viability (32,33). This was one of the first identified substrates of caspases, the main executioner of apoptosis. During apoptosis, caspase-3 cleaves PARP into two fragments, p89 and p24, thus suppressing PARP activity. As shown in Figs 5 and 6, PARP here was cleaved by K3O-ir and R3O-ir. Highest activity of caspase 3 was obtained at the lowest tested dose (400 μ g/ml).

These results suggest that apoptosis induced by the tested flavonoids in these human lymphoblastoid cells is associated with activation of caspase-3 and PARP cleavage.

It is thought that activation of caspases plays a central role in the execution stage of apoptosis. The extrinsic apoptotic pathway is triggered through the Fas death receptor, a member of the tumor necrosis factor (TNF) receptor superfamily. Death receptor-dependent apoptotic pathways are triggered at the cell surface and require activation of caspase-8. This caspase is an initiator protease that contributes to apoptotic cell commitment, and is regulated in both death receptor-dependent and -independent manners during apoptosis (34,35). It is known that caspase-3, which is the main executioner caspase, can be activated by caspase-8 (36).

As far as we obtained, at the same dose (400 μ g/ml of K3O-ir and R3O-ir), the highest caspase 3 and 8 inducing activities, as well as the more effective PARP cleavage effect, we can deduce that the two tested flavonoids activate the extrinsic pathway of apoptosis. Activation of caspase-8 leads to activation of caspase 3 and subsequently induces PARP cleavage and DNA fragmentation. However, we can not exclude participation of other pathways in the apoptotic effect exhibited by both of K3O-ir and R3O-ir.

It appears from our results that presence of a methoxyl group on C-7 of A ring (R3O-ir), instead of hydroxyl group (K3O-ir), influence conformation of the molecule and increases its inhibitory growth effect. These results are in accordance with those previously reported by Wang *et al.* (37) and Kawaii *et al.* (38). These authors suggested that position and number of hydroxyl groups on rings A and B strongly influence conformation of the compounds and modulate their growth inhibitory effect. Kawaii *et al.* (38) studied structure–activity relationships of four polymethoxylated flavonoids by comparing their antiproliferative effects. They concluded that the C2–C3 double bond

and 3-hydroxyl group of ring A are important factors for antiproliferative activity.

When comparing R3O-ir to K3O-ir inducing caspase-3 and caspase-8 activities, it appears that R3O-ir is a more potent inducer of caspases-3 and -8 than is K3O-ir. This result can be ascribed to presence of the 7-methoxyl group in ring A of R3O-ir instead of a hydroxyl group in ring A of K3O-ir. The OCH₃ group should enhance caspase activities, whereas Rusak *et al.* (39) have demonstrated that presence of a methoxyl group instead of a hydroxyl group as is in ring B, decreases antiproliferative and apoptotic activities of such molecules.

In summary, this study has shown that K3O-ir and R3O-ir induce apoptosis in human lymphoblastoid cells by activation of the extrinsic apoptotic pathway.

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