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Ability of Prenylflavanones Present in Hops to Induce Apoptosis in a Human Burkitt Lymphoma Cell Line

Abstract

The identification of effective cancer preventive compounds from hops has become an important issue in public health-related research. We compared the antiproliferative and apoptosis-inducing effects of side chain variants of prenylflavanones, e.g., 8-prenylnaringenin (**7**) and 8-geranylnaringenin (**10**), which have been identified in hops (*Humulus lupulus*), and their synthetic variations 8-furanmethylnaringenin (**8**) and 8-cinnamylnaringenin (**9**). These were accessible by a Mitsunobu reaction and Claisen rearrangement. Flavanones **9** and **10** showed cytotoxic and apoptotic activities. Apoptosis was induced in a mitochondrial dependent manner. 8-Cinnamylnaringenin (**9**) displayed noticeably improved apoptotic effects when compared to 8-prenylnaringenin. The potential of 8-prenylnaringenin (**7**) is shown in an *ex vivo* experiment on a multi-drug resistant leukaemia blast.

Key words
Humulus lupulus · Cannabaceae · phyto-oestrogen · Burkitt lymphoma · apoptosis · mitochondrial permeability transition

Abbreviations

$Eu(Fod)_3$:	heptafluoro-7,7-dimethyloctanedionatoeuropium(III)
LDH:	lactate dehydrogenase
PBS:	phosphate-buffered saline
RPMI:	Roswell Park Memorial Institute

Supporting information available online at
http://www.thieme-connect.de/ejournals/toc/plantamedica

Introduction

8-Prenylnaringenin (**7**), a prenylated flavanone, is a molecule present in the female flower of hops (*Humulus lupulus*) and in some other plants [e.g., it has also been found in the heart wood of a tree (*Anaxagorea luzonensis*) indigenous to Thailand] [2], [3]. This compound is known to be a very potent phyto-oestrogen [4], [5] due to its affinity to the oestradiol receptor. Thus, it has been suggested as a substance useful for hormone replacement therapy. One major disadvantage of the application of 8-PN (**7**) for the improvement of osteoporosis is the danger of an unwanted induction of cell proliferation. In the case of mammary carcinomas, the growth of neoplastic cells in the initial stage is strongly dependent on activation by oestradiol receptors. Thus, in sensitive hormone-dependent neoplastic cell lines, 8-PN (**7**) may accelerate proliferation analogous to oestradiol. Information about this issue would be important in connection with a prospective hormone replacement therapy.

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We would like to report surprising antiproliferative and apoptosis-inducing effects of 8-prenylnaringenin. Some analogous compounds were then synthesised to find out which part of the structure is most important with respect to its apoptotic properties. Among the alterations of the side chain at the 8-position, 8-furan-2-ylmethyl-5,7-dihydroxy-2-(4-hydroxyphenyl)-chroman-4-one ("furanmethylnaringenin") (**8**) and 5,7-dihydroxy-2-(4-hydroxyphenyl)-8-(3-phenylallyl)-chroman-4-one ("cinnamylnaringenin") (**9**) were prepared for the first time using and adapting a published method [1], by which 8-(3,7-dimethylocta-2,6-dienyl)-5,7-dihydroxy-2-(4-hydroxyphenyl)-chroman-4-one, ("8-geranylnaringenin") (**10**) can be prepared also with advantage (Fig. **1**). 8-Geranylnaringenin (**10**), which is derived from hops only as a trace constituent [5], is accessible now in large amounts. It is already known as a very weak phyto-oestrogen.

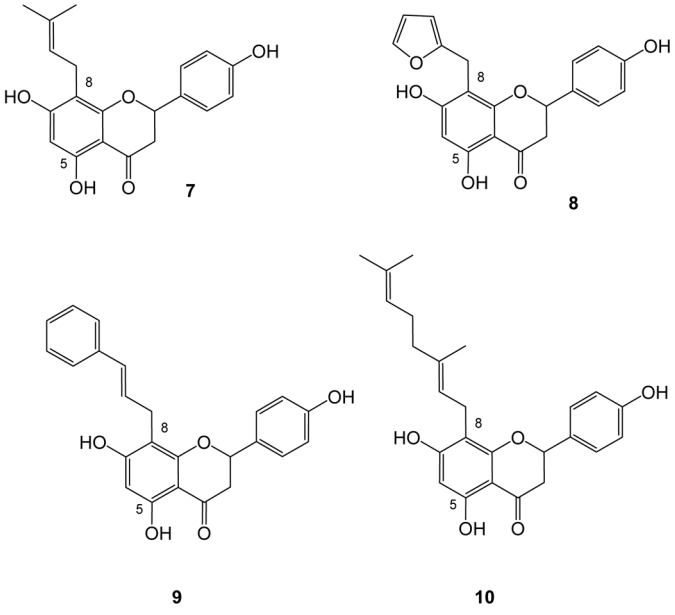

Fig. **1** Chemical structures of 8-prenylnaringenin (**7**), 8-furanmethylnaringenin (**8**), 8-cinnamylnaringenin (**9**) and 8-geranylnaringenin (**10**).

The antiproliferative effects of these flavanones were tested in BJAB cells, a human Burkitt lymphoma cell line with a density of oestradiol receptors of about 150 to 2000 per cell. We showed that these flavanones have noticeable antiproliferative and cytotoxic activities. Furthermore, we were able to demonstrate that the ability for inducing apoptosis was derived from a pathway dependent on mitochondria. After treatment with these agents, we observed a decrease in the mitochondrial membrane potential which was dose-dependent and which showed a release of cytochrome c [6] leading to the processing of the apoptosome, a complex with APAF-1 and pro-caspase 9.

The potential of 8-prenylnaringenin under "real" circumstances is shown in two cases of an *ex-vivo* experiment with multi-drug resistant clones of lymphoid blasts.

Materials and Methods

Preparation of 8-substituted naringenins

8-Prenylnaringenin (**7**) was synthesised according to Gester and Metz [1] from 4′,7-diacetylnaringenin (**2**), subsequently used in a Mitsunobu-reaction and then subjected to rearrangement using $Eu(Fod)_3$ (Fig. **2**) in a Claisen-type rearrangement to yield 8-prenylnaringenin diacetate. As an improvement, washing of the recrystallised Mitsunobu product **3** with 1% acetic acid was introduced in order to completely remove the hydrazine by-product. Spectroscopic data of **7** were identical to those of authentic samples from hops and to results that have been already published [1].

Similarly, the new compounds 8-furanmethylnaringenin (**8**), 8-cinnamylnaringenin (**9**) and 8-geranylnaringenin (**10**) were prepared. The compounds were obtained as a racemic mixture of *R* and *S* enantiomers from this reaction sequence and no attempt was made to resolve them to enantiopurity. Detailed experimental procedures and spectroscopic data can be found in the Supporting Information. Although all Claisen rearrangements tested gave acceptable to good yields, it was not possible to react either 5-allylnaringenin or 5-benzylnaringenin sufficiently.

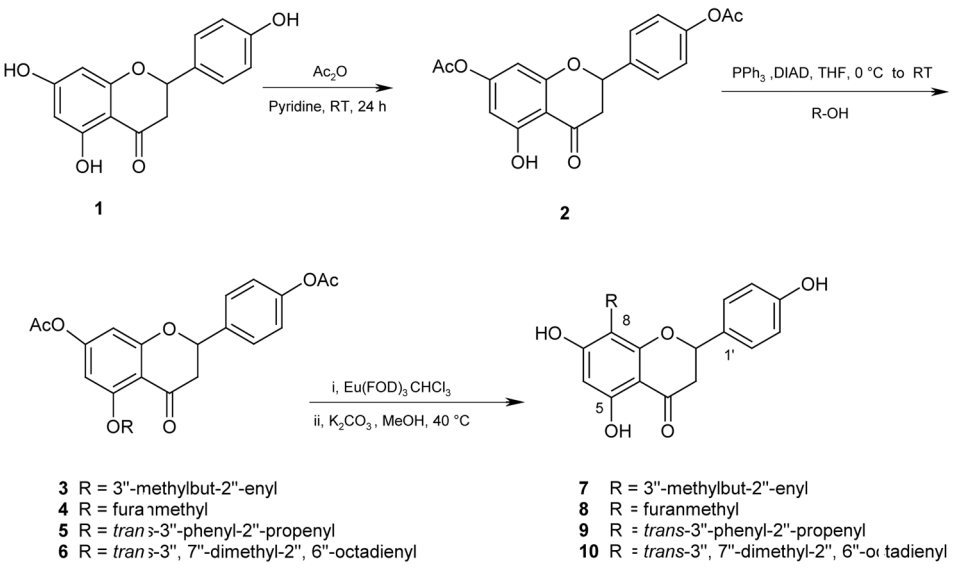

Fig. **2** Synthesis of alkylflavanones.

Cell culture

BJAB cells were grown in RPMI 1640 medium (Rosewell Park Memorial Institute) supplemented with 10% foetal calf serum, 0.56 g/L of L-glutamine, 100,000 U/L of penicillin and 0.1 g/L of streptomycin. Media and culture reagents were from Life Technologies GmbH (Karlsruhe, Germany). BJAB cells were subcultured every 3 – 4 days by dilution of the cells to a concentration of 1×10^5 cells/mL.

Cell counts, measurement of cytotoxicity

Cell counts were obtained by Casy (Schärfe System GmbH; Reutlingen, Germany) and using a Neubauer chamber after staining with trypan blue. Both quantification methods gave nearly equal results. Cytotoxicity was assessed by release of lactate dehydrogenase (LDH) as described previously [8]. After incubation with different concentrations of naringenins, LDH activity released by BJAB cells was measured in the cell culture supernatants using the cytotoxicity detection kit from Boehringer-Mannheim (Mannheim, Germany). The supernatants were centrifuged at 300 × *g* for 5 min. 20 μL of cell-free supernatants were diluted with 80 μL of phosphate-buffered saline (PBS) and 100 μL of the reaction mixture containing 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT), sodium lactate, NAD^+, and diaphorase were added. Then, the time-dependent formation of the reaction product was quantified photometrically at 492 nm. The maximum amount of LDH activity released by the cells was determined by cell lysis using 0.1% Triton X-100 in the culture medium and set as 100% cell death.

Measurement of DNA fragmentation

DNA fragmentation was measured essentially as described [9]. After treatment with different concentrations of naringenins for 72 h, cells were collected by centrifugation at 300 × *g* for 5 min and washed with PBS at 4 °C. Cells were fixed in PBS/2% (v/v) formaldehyde on ice for 30 min, pelleted, incubated with ethanol/PBS (2 : 1, v/v) for 15 min, pelleted and resuspended in PBS containing 40 μg/mL RNase A. RNA was digested for 30 min at 37 °C. Cells were pelleted again and finally resuspended in PBS containing 50 μg/mL of propidium iodide. Nuclear DNA fragmentation was quantified by flow cytometric determination of hypodiploid DNA. Data were collected and analysed using a FACScan (Becton Dickinson; Heidelberg, Germany) equipped with the CELLQuest software. Data are given in % hypoploidy (subG1) which reflects the number of apoptotic cells.

Measurement of the mitochondrial permeability transition

After incubation with different concentrations of the naringenins **7 – 10**, BJAB cells were collected by centrifugation at 300 × *g* and 4 °C for 5 min. Mitochondrial permeability transition was then determined by staining the cells with 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanin iodide (JC-1; Molecular Probes; Leiden, The Netherlands) as described [10]. 1×10^5 cells were resuspended in 500 μL of phenol red-free RPMI 1640 without supplements and JC-1 was added to give a final concentration of 2.5 μg/mL. The cells were incubated for 30 min at 37 °C with moderate shaking. Control cells were likewise incubated in the absence of JC-1 dye. The cells were harvested by centrifugation at 300 × *g* and 4 °C for 5 min, washed with ice-cold of PBS and resuspended in 200 μL of PBS at 4 °C. The mitochondrial permeability transition was then quantified using the flow cytometric determination of cells with decreased fluorescence, i. e., with mitochondria displaying a lower membrane potential. Data were collected and analysed using a FACScan (Becton Dickinson; Heidelberg, Germany) equipped with the CELLQuest software (Becton-Dickinson). Data are given in % cells showing a decrease in mitochondrial membrane potential which reflects the number of cells undergoing apoptosis via a mitochondrial dependent pathway.

Ex vivo studies

Samples containing at least 5×10^7 leukaemic cells of 2 children with relapsed acute myeloid leukaemia (ALL) were analysed in this study. Both children were male. The ages at diagnosis were 7 and 15 years. Leukaemic cells were obtained after bone marrow aspiration and Ficoll preparation. *Ex vivo* drug resistance analysis of lymphoblasts from children with relapsed acute lymphoblastic leukaemia (ALL) is a part of the ALL REZ BFM 2002 study for treatment of children with relapsed ALL. For this study we have obtained ethics approval on 14.12.2000.

Supporting information

Full analytical data of the compounds are available in the Supporting information.

Results and Discussion

The antiproliferative effects of 8-prenylnaringenin (7), 8-furfurylnaringenin (**8**), 8-cinnamylnaringenin (**9**) and 8-geranylnaringenin (**10**) in BJAB cells were tested. 8-Prenylnaringenin (**7**) inhibited proliferation at concentrations higher than 50 μM (data not shown). The inhibition at 50 μM was 17%, and at 100 μM it was 41%. 8-Furfurylnaringenin (**8**) showed no antiproliferative effect (data not shown). 50 μM of 8-geranylnaringenin (**10**) inhibited the proliferation in 34% of the BJAB cells. The effect increased to 56% inhibition at 100 μM (Fig. **3**). The strongest antiproliferative effect was induced by 8-cinnamylnaringenin (**9**) at a concentration of 50 μM which reduced proliferation to about 41% (Fig. **4**).

In contrast to 8-prenylnaringenin (Fig. **5**), 8-cinnamylnaringenin significantly induced apoptosis in BJAB cells at a concentration of 50 μM (Fig. **6**). The apoptotic effect of 8-cinnamylnaringenin exceeded those of all other naringenins tested in this study. The induction of apoptosis depended on the concentration of 8-cinnamylnaringenin (11% apoptotic cells at 50 μM and 38% at 100 μM).

We were able to show that, in our experiments, apoptosis induction took place via the mitochondrial pathway (Fig. **7**). All flavanones were able to decrease the mitochondrial membrane potential in BJAB cells after treatment for 48 h. Despite a low capacity of 8-prenylnaringenin to induce apoptosis (Fig. **5**), it also induced a decrease of the mitochondrial membrane potential. However, 8-geranylnaringenin caused a change in the membrane potential at much lower concentrations. At 100 μM we noticed a saturation effect in the decrease of mitochondrial membrane potential. But the greatest effect was demonstrated with 8-cinnamylnaringenin. Even at a concentration of 50 μM, we observed a transition in 77% of the BJAB cells. A kind of saturation effect could explain why this percentage does not increase at 100 μM.

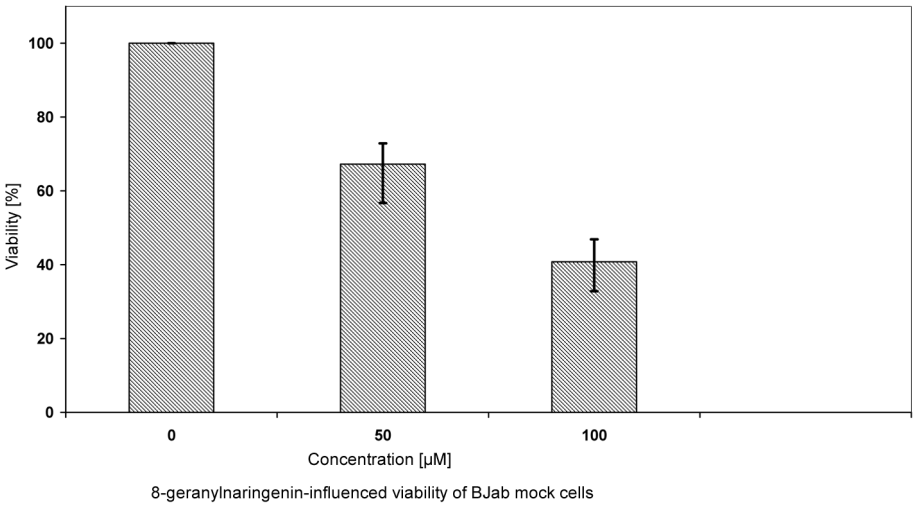

Fig. **3** Viability of BJAB cells after 4 h of treatment with 8-geranylnaringenin (**10**). The viability decreased in a dose dependent manner. The bars represent the mean of three determinations. The experiments were repeated twice and yielded similar results.

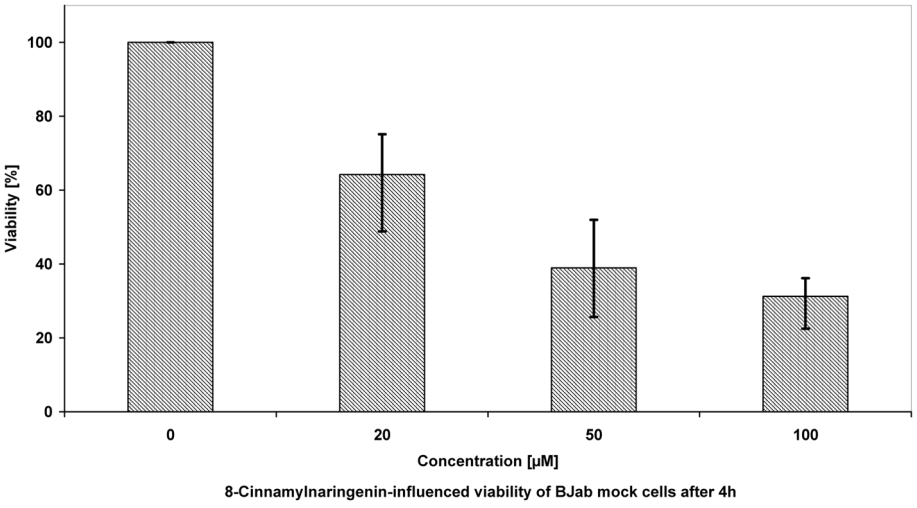

Fig. **4** Viability of BJAB after 4 h under influence of 8-cinnamylnaringenin (**9**). It decreased in a dose dependent manner with IC_{50} = 37.0 μM. The bars represent the mean of three determinations. The experiments were repeated twice and yielded similar results.

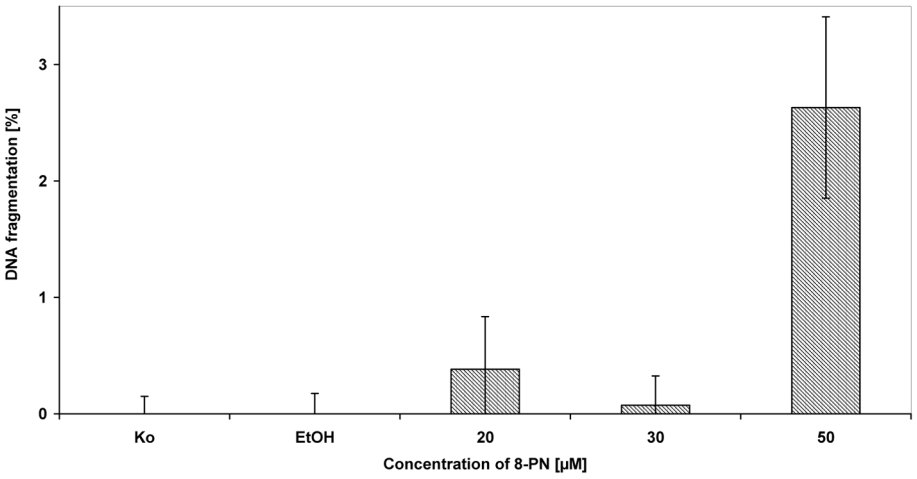

Fig. **5** Apoptosis induction (number of apoptotic cells in %) after treatment with 8-prenylnaringenin (**7**) in different concentrations over 72 h in human Burkitt lymphoma cells (BJAB). Apoptosis induction was detected by DNA fragmentation via FACS measurement after staining the cells with propidium iodide. Bars represent the mean of three determinations from separate cultures. The experiments were repeated twice and yielded similar results.

To demonstrate the apoptosis inducing potency of 8-prenylnaringenin, primary leukaemic cells from children with acute myeloid leukaemia (AML) were examined. The primary leukaemia cells of a 7-year-old boy (Fig. **8**) were multi-drug-resistant. Idarubicin was able to induce significant apoptosis *ex vivo* (15 %). 8-Prenylnaringenin (100 μM, lane 1) caused remarkable apoptosis. Thirty-four percent of the patient's myeloblasts underwent apoptosis after *ex vivo* treatment for 72 h.

Previous studies showed no antiproliferative and apoptotic activities of naturally occurring naringenins in human cancer cell lines [7]. The potency of the naringenins **7 – 10** as inhibitors of

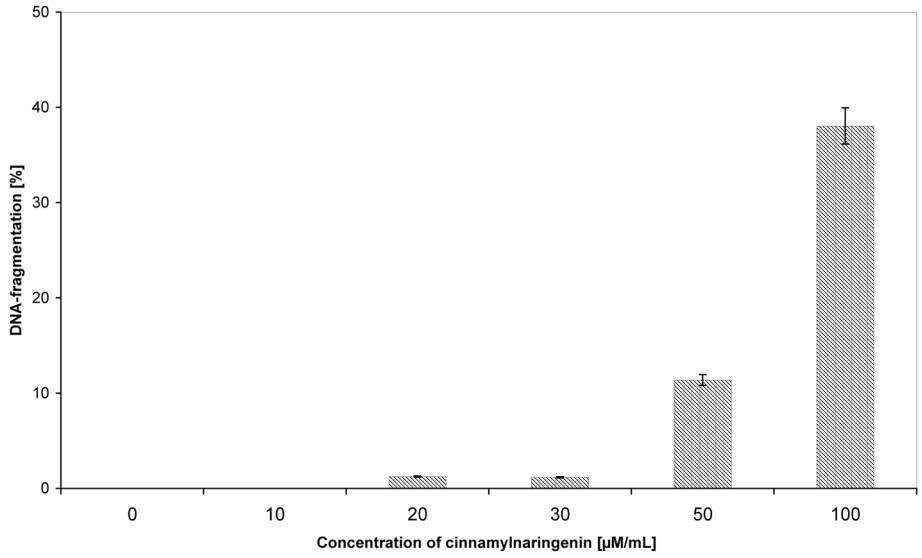

Fig. **6** Apoptosis induction in BJAB cells after treatment for 72 h depends on the concentration of 8-cinnamylnaringenin (**9**). It showed the strongest apoptosis inducing effects of all tested naringenins. The amount of hypodiploid cells increased from 11 % at 50 μM to 38 % at 100 μM. Apoptosis induction was detected by DNA fragmentation via FACS measurements after staining the cells with propidium iodide. Bars represent the mean of three determinations from separate cultures. The experiments were repeated twice and yielded similar results.

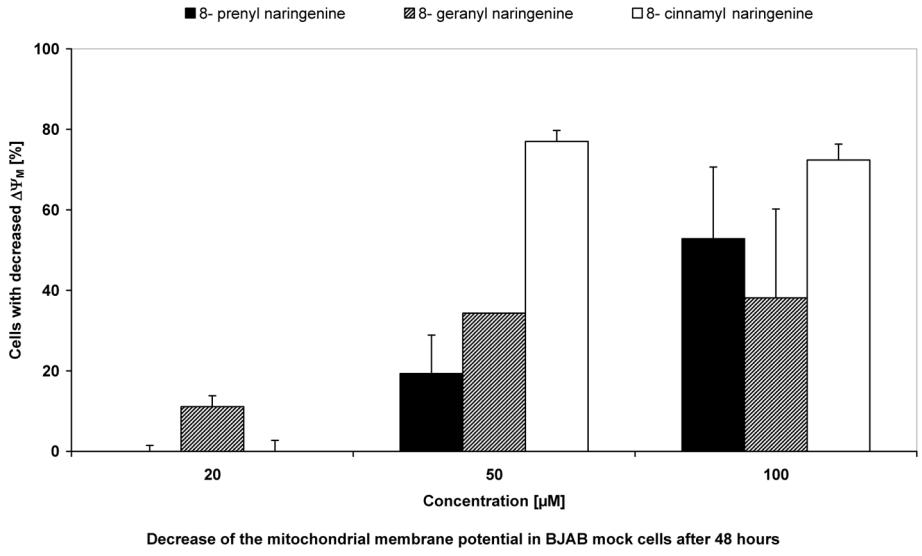

Fig. **7** Decrease of mitochondrial membrane potential in BJAB cells induced after treatment with **7**, **9** and **10** in different concentrations for 48 h. The mitochondrial permeability transition was determined by FACS measurements after staining the cells with JC-1. The bars represent the mean of three determinations from separate cultures. The experiments were repeated twice and yielded similar results.

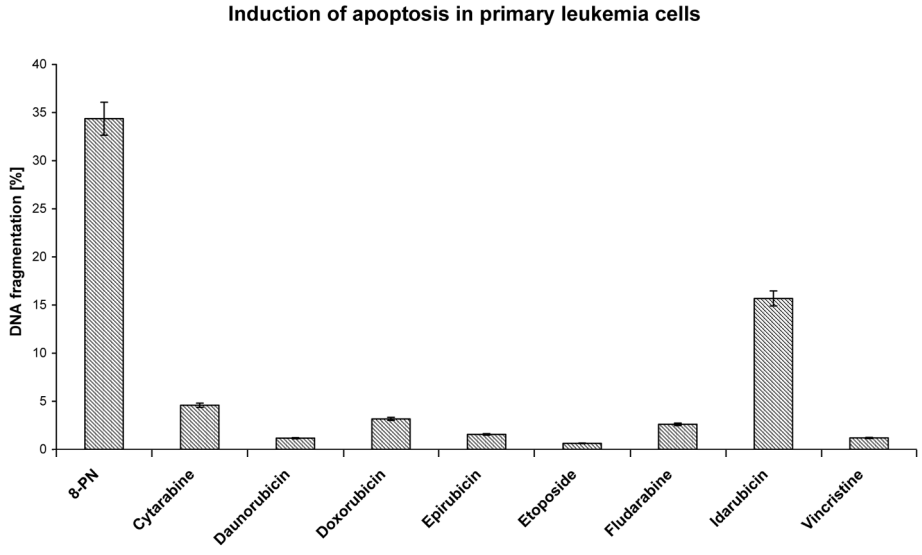

Fig. **8** Induction of apoptosis after treatment with 8-prenylnaringenin (**7**) and eight cytostatic drugs under same conditions in primary myeloblasts from a 7-year-old boy with acute myeloblastic leukaemia (AML). In a concentration of 100 μM, 8-prenylnaringenin showed the strongest apoptosis inducing effect. Apoptosis induction was detected by DNA fragmentation via FACS measurements after staining with propidium iodide. The bars represent the mean of three determinations from separate cultures.

BJAB cell-proliferation was dependent on the chemical structure of the alkyl side chain in position 8. We observed a meaningful apoptosis inducing effect for 8-geranylnaringenin (**10**) and especially for 8-cinnamylnaringenin (**9**) than for compound (**7**) (Fig. **5**). In contrast to this result, the furanmethyl residue conferred no antiproliferative activity. Therefore, one could draw the cautious conclusion for further research that a larger alkyl side chain at position 8 increases the antiproliferative properties. The differences in the cytotoxicity of 8-geranylnaringenin and 8-cinnamylnaringenin demonstrate possibly the effect of a folding in the side chain.

The observations with compounds **9** and **10** on the transition point of the mitochondrial membrane potential suggests that BJAB cells underwent apoptosis initiated by intrinsic signals. The extent of the transition increases with the size of the alkyl side chain in the 8-position. 8-Cinnamylnaringenin caused a stronger decrease of the mitochondrial membrane potential than both 8-geranylnaringenin and 8-prenylnaringenin. This finding can be explained by the stronger apoptotic effects of compound **9**.

Antiproliferative and apoptotic effects of prenylated chalcones derived from hops extracts have already been reported [7], [12]. Xanthohumol inhibits the proliferation of MCF-7 cells with an IC_{50} of 13.3 μM and the cell growth of MDA-MB-435 human mammary adenocarcinoma cells with an IC_{50} of 3.7 μM. Miranda et al. [7] reported that the conversion of a chalcone to a flavanone (**7** to demethylxanthohumol) resulted in reduced antiproliferative activity. In our experiments 8-prenylnaringenin showed nearly similar antiproliferative activity as demethylxanthohumol [17]. Our example suggested that the antiproliferative effects depend on the chemical structure of the alkyl side chain in position 8 rather than on the chalcone or flavanone structure. Whether a substance is apoptosis inducing or inhibiting, will be decided in the context of other regulatory receptor mechanisms involved in the cells, which may be counteracting each other. Many flavanones and flavones like genistein and daidzein are known as phyto-oestrogens [2], [4], [13], [14]. Up to now, 8-PN resembles oestradiol more closely than any other flavone (EC_{50} = 4.4 nmol/L compared to oestradiol (0.82 nmol/L), although 8-PN is considered to be a partially ER-α selective oestradiol receptor agonist. 8-Geranylnaringenin is a very low potency oestrogen agonist. With 8-PN, but not with 8-geranylnaringenin, one can expect some proliferation inducing activity in cell lines sensitive to oestrogen receptor modulation. Instead, both are particularly apoptosis inducing that is, antiproliferative.

However, in our case there is a significant instance which, at the moment, hampers the clarification of this point. A racemic 8-PN mixture of *R* and *S* enantiomers is about three times more active on ERα [18] compared to ERβ. At present, the ratio of ERα/ERβ in BJAB cells is unknown. The chalcones xanthohumol and demethylxanthohumol show apoptosis inducing activity without cytotoxic effects [17]. 8-Geranylnaringenin (**10**) and 8-cinnamylnaringenin (**9**) show – besides the apoptosis induction – considerable cytotoxic activity.

The Flavopiridol© example, a semi-synthetic flavone with its core structure originally derived from the Indian plant *Dysoxylum binectariferium*, shows how promising the structure of substituted flavanones can be. It induces growth arrest and apoptosis in several chronic B-cell leukaemia lines [15]. It selectively inhibits cycline-dependent kinase 1 (CDK 1) a serine/threonine protein kinase, the driving force behind cell proliferation [16], with an IC_{50} of 110 and 130 nM. The quest for a potent apoptotic agent might lead to inspecting chalcones from hops like xanthohumol that possesses a larger alkyl side chain in position 8.

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