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Research paper

Hyperforin a constituent of St John's wort (*Hypericum perforatum* L.) extract induces apoptosis by triggering activation of caspases and with hypericin synergistically exerts cytotoxicity towards human malignant cell lines

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Abstract

Hyperforin (HP) is an abundant component of St John's wort with antibiotic and antidepressive activity. We report here the ability of HP and that of polyphenolic procyanidin B2 (PB-2) to inhibit the growth of leukemia K562 and U937 cells, brain glioblastoma cells LN229 and normal human astrocytes. HP inhibited the growth of cells in vitro with GI_{50} values between 14.9 and 19.9 μM. The growth inhibitory effect of PB-2 was more pronounced in leukemia cell lines K562 and U937, the GI_{50} concentrations being about 12.5 μM established after 48 h incubation differed significantly ($P < 0.05$) from those of LN229 and normal human astrocytes (103.1 and 96.7 μM), respectively. Further, HP and hypericin (HY) (a naphthodianthrone from St John's wort) acted synergistically in their inhibitory effect on leukemic (K562, U937) cell growth. Cell death occurred after 24 h treatment with HP and PB-2 by apoptosis. A dose-dependent loss of membrane phospholipid asymmetry associated with apoptosis was induced in all cell lines as evidenced by the externalization of phosphatidylserine (PS) and morphological changes in cell size and granulosity by scatter characteristics. In leukemia U937 cells, HP increased the activity of caspase-9 and caspase-3 and in K562 cells caspase-8 and caspase-3. In addition, the broad spectrum caspase inhibitor z-VAD-fmk inhibited both the appearance of PS exposure and the activation of caspases, illustrating the functional relevance of caspase activation during HP-induced apoptosis. Cytocidal effects of HP and its cooperation with HY on tumor growth inhibition in a synergistic manner make the St John's wort an interesting option in cancer warranting further in vitro and in vivo investigation.

Keywords: Hyperforin; Procyanidin B2; St John's wort; Apoptosis; Caspases; Malignant cells

1. Introduction

Apoptosis is a process that regulates cell number or eliminates damaged cells, which differs morphologically and biochemically from cellular necrosis. Apoptosis occurs in both normal and neoplastic tissues and is an active process. Defects in the apoptosis mechanisms can extend cell lifespan, thus contributing to neoplastic cell expansion independently of cell division. Deficiencies in apoptosis also contribute to carcinogenesis by creating a permissive environment for genetic instability and accumulation of gene mutations conferring resistance to cytotoxic anticancer drugs and radiation [1]. Apoptosis signal transduction and execution require the coordinated action of the cascade of caspases (asparate-specific cysteine proteases) [2]. Caspases are expressed as inactive proenzymes and become activated by proteolytic processing at internal aspartate residues when cells receive an apoptosis-inducing signal. At present 14 mammalian caspase family members have been described. Some, including caspases-2, -8, -9 and -10 are initiators of cell death (contain large prodomains). Once activated, these initiator caspases in turn activate the executioner caspases such as caspases-3, -6 and -7 (carry small prodomains). Thus, caspases can activate each other. It is of interest, therefore to identify the agents that are cytocidal and cytostatic rather than cytotoxic [3].

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The *Hypericum perforatum* L. (*H. perforatum*) extracts belong to mostly prescribed and clinically investigated plant extracts and have been shown to be an effective option in the treatment of mild to moderately severe depressive disorders [4,5]. *H. perforatum* contains a number of constituents with documented biological activity including a broad range of flavonoids, naphthodianthrones, phloroglucinols, proanthocyanidins, xanthones and essential oils [6]. There are numerous studies on the phototoxicity and apoptosis-inducing effect of hypericin (HY) [7,8] and inhibitory effects of flavonoids (quercetin) on the growth of malignant cells [9,10]. Several in vitro assays demonstrate potentially significant interactions between a series of proanthocyanidin dimers and biological systems, such as antiviral, antibacterial, antioxidant and radical-scavenging properties [11,12]. However those concerning phloroglucinol-hyperforin and procyanidins derived from *H. perforatum* herbs are limited. The acylphloroglucinol derivate hyperforin (HP) is the major non-nitrogenous secondary metabolite of *H. perforatum*. Apart from its characterized antibacterial properties [13], it was found that HP exerted inhibitory effects on human epidermal cells and on the proliferation of phytohemagglutinin-stimulated peripheral blood mononuclear cells [14]. In addition, it inhibited the growth of autologous MT-450 breast carcinoma in Wistar rats in vivo [15]. The tannin fraction from *H. perforatum* herbs contains oligomeric procyanidins, closely related to those of *Crataegus* species [16] and showed vasoactive properties in isolated porcine arteries [17]. In our previous paper [18] we reported on the cytostatic and apoptosis-inducing effect of *H. perforatum* extract without light exposure on various human malignant cell lines. In the present study, the effects of HP, its sodium salt and procyanidin B2 (PB-2) are investigated for their cell growth inhibitory and apoptosis-inducing activity on two leukemic cell lines (K562, U937), malignant glioblastoma cells LN229 and normal human brain astrocytes (NHA). As the biological effects of *H. perforatum* preparations are considered to be due to the mixture of the metabolites rather than to single constituents, we examined the effect of coexposure of HY and HP on the growth inhibition of K562 and U937 human hematological cell lines. Light-dependence was examined under exposure to 7.5 J/cm^2 white light using HY as a phototoxic control. This study provides evidence that HP-induced cytotoxicity toward human malignant cell lines (K562, U937, LN229) correlates with the induction of apoptosis and that caspases are involved in this process. Furthermore, the effect of combined treatment of cells with HY and HP in the dark results in a synergism of their antiproliferative effect.

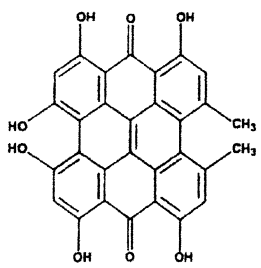
2. Materials and methods

2.1. Reagents

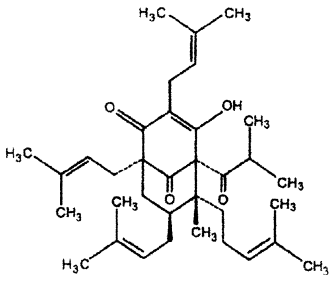
HY ($C_{30}H_{16}O_8$), quercetin ($C_{15}H_{10}O_72H_2O$) were from Alexis Corporation, Lausen, Switzerland. HP ($C_{35}H_{52}O_4$), HP–Na salt, PB-2 ($C_{30}H_{26}O_{12}$) [epicatechin-(4β- > 8)-epicatechin] (Fig. 1) were isolated and purchased from Natural Product Department of Dr W. Schwabe GmbH and Co. (Karlsruhe, Germany) as previously described [19,20]. The isolated HP fraction yielded about 18% adhyperforin identified as a homologue of HP [21] and characterized by spectroscopic methods [22]. Reagents were dissolved in DMSO and stored at −20°C in aliquots: of 5 mg/ml HY and 20 mg/ml PB-2. Phloroglucinols HP and HP–Na at concentrations of 20 mg/ml were stored at −80°C. The general caspase inhibitor benzyloxycarbonyl-Val-Ala-Asp fluoromethyl keton (z-VAD-fmk) was from Alexis Corporation. Cell proliferation reagent WST-1 was purchased from Roche Diagnostica (Rotkreuz, Switzerland).

2.2. Cell culture and media

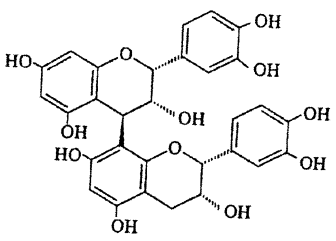
K562 human chronic myeloid leukemia and U937 human histiocytic lymphoma cell lines were obtained from the American Type Culture Collection (Rockville, MD, USA), normal human astrocytes NHA from Clonetics, BioWhittaker (Verviers, Belgium) and human malignant glioma cells LN229 characterized by Van Meir et al. [23] were provided by Dr N. de Tribollet (Lausanne, Switzerland). Cell culture media RPMI 1640, Dulbecco's modified Eagle's medium (DMEM), phosphate buffered salt solution (PBS), media supplements fetal calf serum (FCS), L-glutamine, penicillin–streptomycin (PenStrep) were purchased from Sigma (Buchs, Switzerland). Propagation



Hypericin (**1**)



Hyperforin (**2**)



Procyanidin B-2 (**3**)

Fig. 1. Chemical structures of HY (1), HP (2), PB-2 (3).

medium for K562 and U937 cells was RPMI 1640 supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine and 1% PenStrep. LN229 were cultured in DMEM containing 10% FCS 2 mM L-glutamine and 1% PenStrep. The NHA were grown in an Astrocyte Basal Medium supplemented with 5% FCS, 2 μg human recombinant epidermal growth factor (hEGF), 2 mg insulin, 2.5 μg progesterone, 5 mg transferrin, 5 mg gentamycin, and 5 μg amphotericin (AGM™ BulletKit) from BioWhittaker. Cells were cultured in a 5% CO_2, 95% air, fully humidified atmosphere at 37°C and were collected during the exponential growth phase. Cell viability was >95% before each experiment as estimated by trypan blue exclusion. The test medium for all experiments was 1640 RPMI or DMEM supplemented with 2% FCS.

2.3. Experimental design

Cytotoxicity and apoptosis-inducing activity of individually investigated compounds were determined by exposure of cells to their various concentrations for 24 and/or 48 h in the dark. Parallel, one test set was exposed to light after 1 h of treatment. Cell growth inhibition and apoptosis were estimated after a further 23 and/or 47 h incubation. In order to study the effect of caspase inhibitor, in some experiments cells were preincubated for 60 min with z-VAD-fmk (25, 50 μM) before the addition of HP. The final concentration of DMSO in the tests was less than 0.2% and did not affect the viability, cell growth or apoptosis.

2.4. Cell irradiation

Cells seeded at a concentration of 5×10^4/ml in microtiter plates were irradiated at a distance of 20 cm with 75 W tungsten lamp for 10 min as described [8]. The light dose was 7.5 J/cm^2. The temperature during irradiation (<30°C) did not influence the cell viability.

2.5. Cell growth assays

In order to determine the effect of individual compounds on cell growth, treated cells at a density of 5000 cells/well were incubated in microtiter plates for 44 h followed by 4 h with the tetrazolium salt WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate). The cleavage of WST-1 to formazan by metabolically active cells was quantified by scanning the plates at 450 and 650 nm reference wavelength in a microtiter plate reader. Test medium was used as background control [24]. Three independent sets of experiments performed in triplicates were evaluated. Controls with the solvent DMSO below 0.2% were tested in parallel. The growth inhibition rate was calculated as percentage of parallel untreated controls. As positive control, the flavonoid quercetin was used. Test compounds were compared using the GI_{50} value which was the concentration of individual compounds reducing cell growth by 50%.

2.6. Apoptotic death assays

2.6.1. Morphologic assessment of apoptosis

Tumor cell suspensions were washed with PBS and the pellet was spread on slides. After fixing the cells with methanol they were stained in Giemsa. Morphological analyses were performed on Giemsa stained slides and examined by oil-immersion light microscopy for occurrence of morphological changes consistent with apoptosis and by cell size scatter characteristics using flow cytometry.

2.6.2. Annexin V-binding analysis

Flow cytometric (FCM) analysis was performed to identify and quantify the apoptotic cells. One of the plasma membrane alterations in the early stages of apoptosis is the translocation of phosphatidylserine (PS) to the outer layer of cell membranes. Annexin V is a Ca^{2+}-dependent phospholipid-binding protein with high affinity for PS [25]. Apoptosis was detected and quantified in cells (5×10^4) by staining with the Annexin V–FITC (Roche Diagnostics, Rotkreuz, Switzerland) as previously described [26]. Plasma membrane permeability was estimated by trypan blue exclusion. All experiments were performed using FACScalibur flow cytometer (B–D, Mountain View, CA). A minimum of 10,000 events per sample was collected on log mode of green fluorescence and analyzed with CellQuest program. Histograms of treated cells were compared to untreated controls. Vehicle treated cells (0.2% DMSO) were always included and were not found to affect apoptosis. Each histogram is representative of two independent preparations.

2.6.3. Measurement of caspase activity

Caspase activities were assayed with synthetic substrates selective for different known caspases, DEVD–*p*-nitroanilide (pNa) (caspase-3), IETD–pNa (caspase-8) and LEDH–pNa (caspase-9) in the lysates from 1×10^6 cells according to the manufacturer's description. Colorimetric assay kits (Bio Vision) from Alexis Corporation were used. The assay is based on spectrophotometric detection of the chromophore pNa after cleavage from the labeled appropriate substrate. The pNa light emissions were quantified using a microtiter plate reader at 405 nm. Comparison of the absorbance of pNa from an apoptotic sample with an uninduced control allowed determination of the relative increase in caspase activity per milligram total protein. Following detergent solubilization, the cytosolic protein was determined with the Lowry assay [27] (Bio-Rad DC Protein Assay) with plasma bovine IgG as the standard. All samples were assayed in triplicate.

2.7. Statistical analysis

GI_{50} values were obtained by linear or exponential regression analysis of data using the Microsoft Excel software. Statistical evaluation of the results was performed with the Student's *t*-test. Probability values equal to or lesser than 0.05 were considered significant. Composite treatments were compared using one-way analysis of variances (ANOVA). The comparison of histograms was statistically evaluated using the Kolmogorov–Smirnov (K–S) two samples test for overlaid histograms.

3. Results and discussion

3.1. HP and PB-2 inhibited the growth of K562, U937, LN229 and NHA cells

In our previous studies [18], we found that *H. perforatum* extracts exerted growth inhibitory effects on malignant cell lines without light activation, which were at least HP-content related. The phloroglucinol content of herbal drugs depends on their pharmaceutical preparation. When the crude herbal drugs are improperly dried, HP content decreases, due to its chemical instability. HP must be protected from oxidation, because of the tendency to degrade. Stability of HP was extensively tested [28] and found that there were no differences between results obtained after 8 months at −20°C under nitrogen or at −30°C under normal atmosphere and those from a reference sample stored under liquid nitrogen (−196°C). We chose to store aliquots of 20 mg/ml HP at −80°C for these investigations. In spite of the instability of HP, it is an orally bioavailable component and is present in the plasma of blood samples from the subjects after administration of therapeutically used and clinically tested *hypericum* extracts [29–31]. Procyanidins are a diverse group of polyphenolics that are widely distributed in fruits and plants. Procyanidins from grape seeds caused growth inhibition and apoptotic death of human breast carcinoma and human prostate carcinoma cells [32,33]. In the present studies, HP and PB-2 treatment of cells resulted in significant inhibition of their growth in a dose-dependent manner (Fig. 2). Compared with untreated controls, 48 h of HP treatment at 10, 20, 50 and 100 μM doses, showed a strong effect accounting for 22.2–99.5% (K562), 31.7–99.8% (U937), 28.1–91.0% (LN229) and 28.2–74.1% (NHA) inhibition ($P < 0.05$). However, the effect of HP was not cell specific as expressed in the GI_{50} values between 14.9 and 19.9 μM in selected human cell lines. The tests performed in parallel with HP–Na showed effects similar to those of HP and there were no significant differences between HP and its HP–Na salt on cell growth inhibition in all tested cells (Table 1). Vehicle DMSO (0.2%) had no effect on cell growth compared to untreated cells. Mean values of cell growth inhibition for vehicle treated controls were 6.2 ± 3.4%.

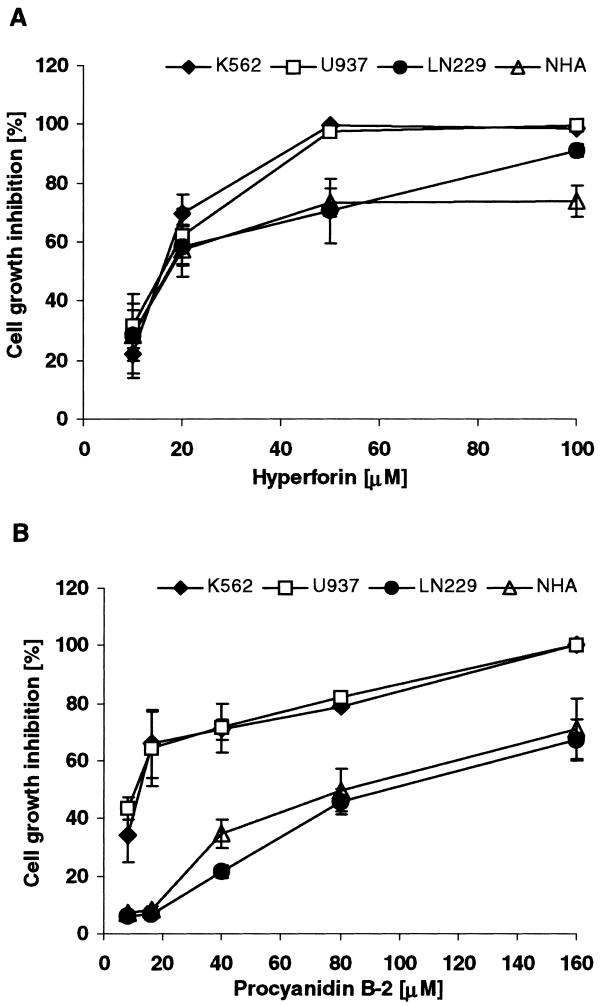


Fig. 2. Effect of exposure to HP and PB-2 on cell growth of K562, U937, LN229 and NHA cells. Cells (5×10^3/well) were grown for 48 h in the presence of various concentrations of HP (A) and PB-2 (B) and the cell growth inhibition was assessed by WST-1. Untreated cells (in medium only) and cells incubated in the presence of vehicle (0.2% DMSO) were used as controls. Individual dose–response curves for each cell line from triplicates repeated at least three times with similar results are expressed as mean growth inhibition percentage ± SD. The solvent 0.2% DMSO alone did not affect the growth inhibition of individual cells (mean = 6.2 ± 3.4%; $n = 4$).

More recently it was reported that HP exerts antiproliferative activity toward various human and rat cells in the concentration range comparable to that presented here [15]. The growth inhibitory effect of PB-2 was dose-dependent and close to that of HP in the two leukemia cells K562 and U937 as shown in Fig. 2 and by comparison of GI_{50} values of both compounds (Table 1). However, the GI_{50} values for NHA and LN229 human brain cells differed significantly ($P < 0.05$) from those of K562 and U937 cells as summarized in Table 1. The growth of K562 and U937 cells was suppressed completely after 48 h treatment with 160 μM PB-2, while the same concentration of PB-2 caused about 70% cell growth inhibition of the brain cell lines. The cytotoxicity of different types of flavonoids showed great

Table 1
Cell growth inhibitory concentration 50% of tested compounds in individual cell lines ($n = 3$)

Compounds	Cell lines			
	K562	U937	LN229	NHA
HY (a)	37.5 ± 6.3	21.0 ± 2.8	ND	ND
(b)	1.1 ± 0.1	1.2 ± 0.2	1.5 ± 0.2	3.8 ± 0.3*
HP (a)	14.9 ± 1.5	15.8 ± 1.3	19.2 ± 5.9	19.9 ± 2.8
(b)	13.4 ± 1.8	14.3 ± 1.7	18.5 ± 3.4	20.1 ± 4.3
HP–Na (a)	15.7 ± 2.9	18.3 ± 3.7	27.8 ± 4.2	29.4 ± 6.6
(b)	16.2 ± 3.4	19.9 ± 3.2	26.9 ± 5.1	27.6 ± 4.2
PB-2 (a)	12.2 ± 2.1	12.4 ± 3.5	103.1 ± 6.6**	96.7 ± 5.4**
(b)	12.2 ± 2.6	12.1 ± 3.3	96.5 ± 9.3**	88.9 ± 8.7**
Quercetin (a)	28.4 ± 2.0	36.3 ± 7.2	45.3 ± 5.1	44.8 ± 3.6
(b)	29.5 ± 2.0	36.9 ± 7.0	43.2 ± 5.8	47.5 ± 5.2

Values are reported as GI_{50} (μM) ± SD. (a) Experiments performed in the dark; (b) experiments performed under 7.5 J/cm^2 white light exposure. *$P < 0.05$ vs. K562, U937 and LN229 cells; **$P < 0.05$ vs. K562 and U937 cells.

variation in their GI_{50} concentration within the same cell line, and in relation to distinct cell types [9,34]. The growth of leukemia cells is strongly inhibited by quercetin [9,10,35] and therefore we used this flavonoid as a positive control in our experiments (Table 1). Further we tested the toxicity of both compounds (HP and PB-2) under white light (7.5 J/cm^2) exposure and compared it to that of the known phototoxic agent HY. HY showed strong light-dependent increase in growth inhibition, but there was no potentiation of the inhibitory effect on HP and PB-2. There are differences of about threefold ($P < 0.05$) in the GI_{50} values of light-activated HY between malignant cells (K562, U937 and LN229) and NHA cells (Table 1). The 5 μM light-exposed HY inhibited the cell growth almost completely. The photo-dependent cytotoxic activity of HY in a concentration range (1–5 μM) has been reported in a variety of human cancer cell lines [7,8,36]. Without light exposure, concentrations of HY up to 10 μM caused 10 and 23% inhibition of growth of K562 and U937, respectively. Above 10 μM concentration, HY exerted a toxicity toward leukemia cells K562 and U937, which was mirrored in their GI_{50} values, namely 37.5 and 21 μM, respectively. Additionally, the cells became more necrotic. In spite of this, it could be shown that HY had an antitumoral effect in rats in the dark, which was apparently cytostatic in nature compared to the cytocidal effects of the light-activated compound [37].

3.2. Combined treatment of HY and HP resulted in a synergistic cell (K562, U937) growth inhibition

Data resulting from cell growth experiments support the importance of HP as a potentially active compound not only with antimicrobial and antidepressive properties as previously reported [14,38,39] but also with antineoplastic activity. Since *H. perforatum* extracts consist of a number of pharmacologically active constituents, which could interact with each another, we further investigated the combined effect of HY and HP, the two main active ingredients of *H. perforatum* extracts, on the growth of leukemia cells K562 and U937 in the treatment conditions in the dark and on exposure to white (7.5 J/cm^2) light. The two leukemia cell lines were treated with various concentrations of either HY and HP individually or in combinations (Table 2). Either HY (2 μM) or HP (10 μM) alone had no (HY) – or only a marginal effect (HP). Experiments to assess the biological significance of the combined effects observed with HY and HP showed that they cause the inhibition of human leukemia cells K562 and U937 and that this cooperation was synergistic at the concentrations of 2 μM (1 μg/ml) HY and 10 μM (5 μg/ml) HP. This cooperative effect of HY and HP without exposure of cells to white light was found to be significant by one-way ANOVA ($P = 0.00077$; $F = 5.36$; K562) and ($P = 0.0067$ $F = 3.86$; U937). No synergism was observed on light (7.5 J/cm^2) exposure ($P = 0.122$; $F = 1.89$; K562 and $P = 0.41$; $F = 1.00$; U937), although the activities of the two compounds were approximately additive. For this effect the phototoxicity of HY was therefore decisive. The present data indicate that the individual constituents HY and HP cooperate with each other in the cytotoxicity in leukemia cell lines K562 and U937 and support the idea that cytotoxicity of total *hypericum* extracts [18] cannot be explained solely by the toxicity of single compounds. Therefore we hypothesize that a network of several distinct compounds acting in an additive or synergistic manner may be responsible for the growth inhibitory effect of St John's wort. Therapeutically, combination of different compounds is often advantageous by limiting unspecific toxicities frequently observed with a very high single treatment regimens in vivo. So far, only the additive actions of PB-2 on the effect of HY and pseudohypericin in the antidepressant activity of *H. perforatum* extracts in vivo have been reported [40].

3.3. Death-inducing effect of HP and PB-2 on leukemia cells K562, U937, glioma cells LN229 and brain NHA, is mediated by triggering of apoptosis

It is increasingly evident, that the potentially effective therapeutic agents against tumors should be cytocidal and cytostatic rather than cytotoxic. We studied whether HP and PB-2 induced apoptosis in human malignant cells K562, U937, LN229 and NHA. Tendency to apoptotic cell death was estimated from three types of data (cell morphology following Giemsa staining, cell size according to FACS forward (FSC) and side scatter (SSC) and PS exposure (detected by Annexin V-binding)) all of which point to a dose-dependent apoptosis-inducing effect of HP on the cells investigated. Tumor cells treated with HP displayed the characteristics of apoptosis induced by other agents [41].

Table 2
Modulation of the combined effect of HY and HP after 48 h treatment of cells investigated by WST-1 assay

Reagents (μM)	Cell growth inhibition (%)			
	K562		U937	
	Without light	7.5 J/cm^2 light	Without light	7.5 J/cm^2 light
HY				
0.5	2.9	7.9	0.7	3.5
1.0	2.7	23.0	1.2	4.1
2.0	**6.0**	**40.2**	**2.1**	**50.4**
5.0	8.3	94.5	1.9	98.3
HP				
5.0	5.4	8.6	−2.6	−1.1
10.0	**22.3**	**30.9**	**0.6**	**4.8**
20.0	58.7	62.4	46.2	50.3
HY + HP				
0.5 + 5.0	7.4	10.1	2.1	−1.2
0.5 + 10.0	27.8	27.9	2.9	2.9
2.0 + 5.0	5.6	42.7	5.2	43.0
2.0 + 10.0	**43.6***	**72.7#**	**20.2****	**46.2##**

Mean values from triplicates of five independent experiments are expressed. One-way ANOVA was used for statistical evaluation of the combined treatment. *$P < 0.00077$, $F = 5.36$; **$P < 0.0067$, $F = 3.86$; #$P < 0.122$, $F = 1.89$; ##$P < 0.41$, $F = 1.00$. Figures in bold indicate the concentrations and related values in which the synergisms between both substances occurred.

Morphological changes in the early phase of apoptosis were well reflected by reduced ability to scatter light in the forward direction and an increase or no change in the 90° light scatter. A representative experiment of HP-induced apoptotic death in K562 cells after 24 h is shown in Fig. 3A, B. This effect was not potentiated by light exposure (7.5 J/cm^2). Surprisingly, potentiation of the apoptosis-inducing effect of PB-2 on K562 cells occurred on exposure to light (Fig. 3C, D). However, in other cell lines (U937, LN229 and NHA), the light-exposed PB-2 did not potentiate cell apoptosis. Redistribution of membrane PS from the inner leaflet of the plasma membrane to the outer surface is common in many apoptotic cells and was detected by Annexin V staining [24]; the plasma membrane integrity was investigated by trypan blue stain. In K562 and U937 cells, the apoptosis-inducing effect of HP was dose-dependent and light independent, being 43 and 55%, respectively at 10 μM and reaching a maximum of 68%, 85% at 50 μM. However, at the 100 μM dose of HP, there was a decrease in apoptotic cells (49.8 and 66.5%) that was possibly associated with an increased proportion of necrotic cells (Fig. 4). Compared with the untreated controls showing 5–8% death cells after 24 h, a maximum of 53.6% was reached in LN229 and 45.2% in NHA at 20 μM HP. Further increase of HP concentration resulted in a decline of apoptotic cells to 28.7% (LN229) and 23.6% (NHA).

A concentration of 200 μM HP showed no toxicity and only about 18% of tumor cells revealed a damaged cell membrane and took up trypan blue dye. PB-2 induced a dose-dependent generation of apoptotic cells. Leukemic K562 and U937 cells responded to 80 μM PB-2 treatment with apoptotic death in 84.5 and 88.7% of cells, respectively, while this concentration only caused apoptosis in 26.7% of LN229 and 22.7% NHA cells. HY requires illumination for apoptosis induction [7,8]. The range in which apoptosis took place was 0.5–5.0 μM HY; above 5 μM light-activated HY, the dying cells were morphologically necrotic.

3.4. HP-induced cell apoptosis is a caspase-dependent mechanism

Recent evidence suggests that apoptotic mechanisms mediate drug-induced cell death and that the activation of caspases plays a key role. Depending on the stimulus that initiates a death program, different caspase cascades, the core of the apoptotic program, are activated [42]. The possible role of caspases as mediators of HP-induced apoptosis was investigated by reversing their effects on cells with the broad caspase inhibitor z-VAD-fmk. z-VAD-fmk attenuated HP-induced apoptosis, confirming the requirement of caspase activation for cell death to occur. The translocation of PS and cell shrinkage were also partly blocked by this pretreatment (50 μM z-VAD-fmk). Evidence is now accumulating that there is also a caspase-independent cell death [43] and recent studies demonstrated that z-VAD-fmk could not always abrogate the process involving exposure of PS [44,45]. Therefore we cannot rule out the possibility that a caspase-independent pathway is involved in HP-induced cell death. Prevention of HP-induced apoptosis by z-VAD-fmk in K562 cells is shown in Fig. 5. Caspases are expressed as inactive proforms and require processing to active subunits either by autoprocessing or via activation by other caspases. To date, several different caspases have been

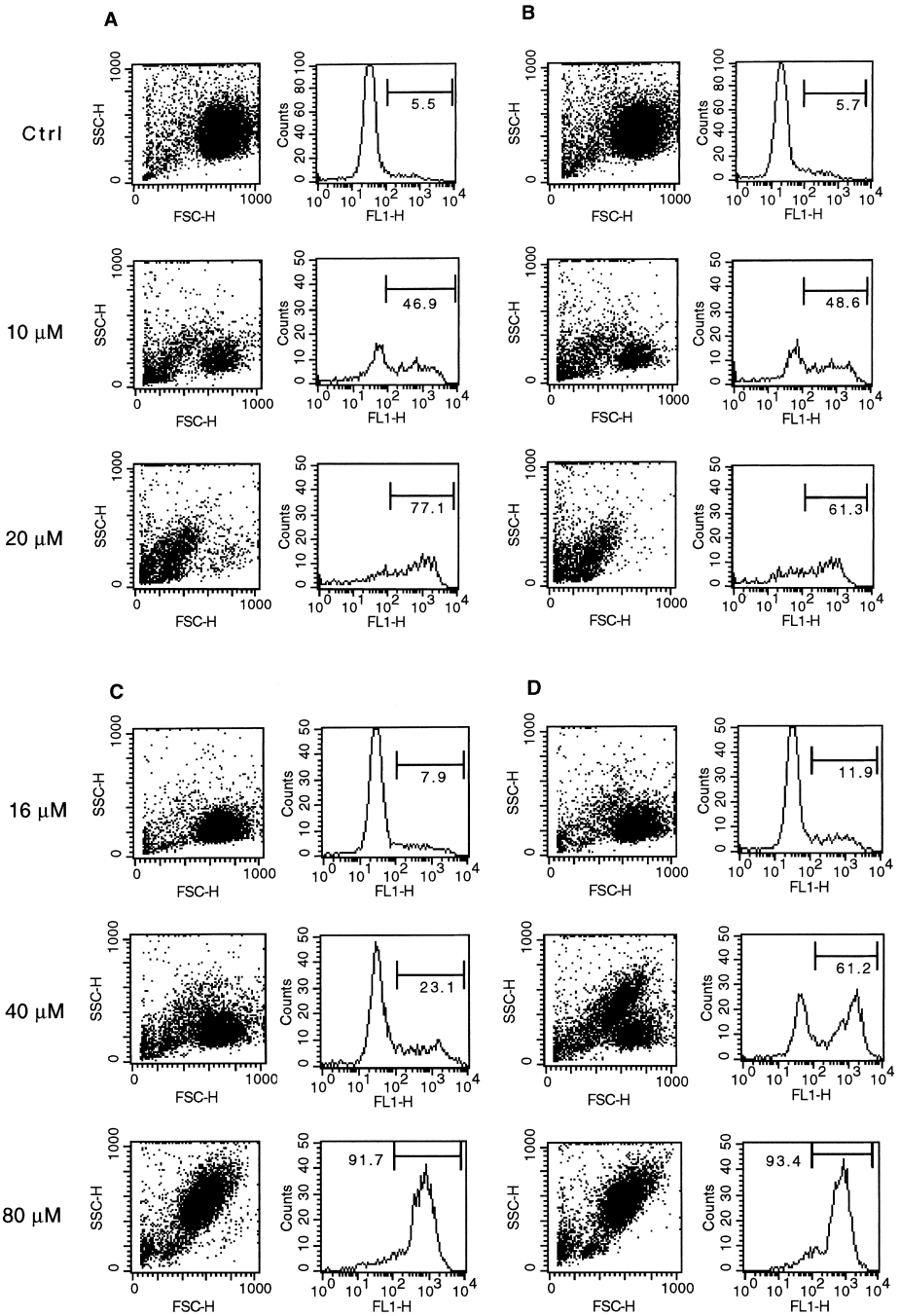


Fig. 3. HP and PB-2-mediated morphological changes accompanied with increased PS exposure. K562 cells were incubated for 24 h with indicated concentrations of HP (A,B) and PB-2 (C,D) in the absence of light (A,C) or upon 7.5 J/cm^2 irradiation (B,D) and the induction of Annexin V–FITC binding (right) and cell shrinkage (left) were evaluated. Biparametric histograms represent the scattering properties of the cells, cell size (measured as forward scatter (FSC) vs. cell granulosity (measured as side scatter (SSC))). PS externalization was assessed by staining with Annexin V–FITC (FL1-fluorescence) as described in Section 2 and is indicated in each histogram. Experiment is one of three repetitions.

identified in humans. Some, including caspases-2, -8, -9, or -10 which are initiators of cell death and contain large prodomains can act either directly or indirectly by activating the executioner caspases such as caspases-3, -6 and -7 which carry small prodomains [2]. To examine involvement of caspases in HP-induced apoptosis caspase activities were assessed in the same in vitro assay using the specific chromogenic substrates. Treatment of K562 and U937 cells with HP (20 μM) was accompanied by different increases in the enzymatic activities detected with the substrates specific for caspases-3, -8 and -9. Increases in enzymatic activity of twofold or more compared to uninduced controls were

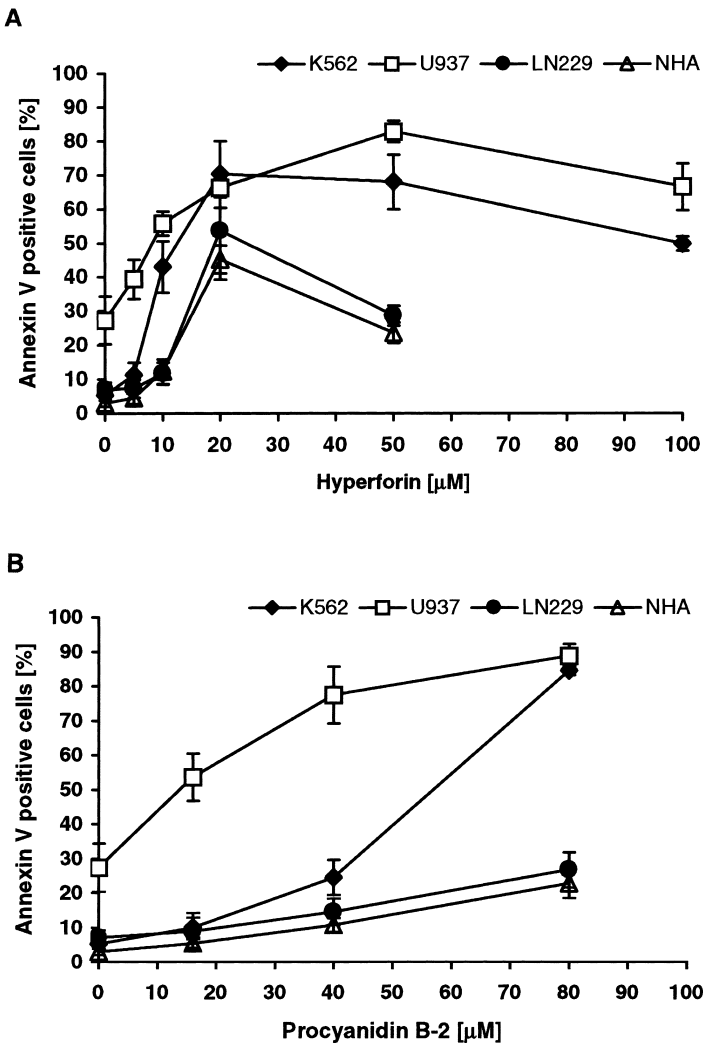


Fig. 4. Dose-dependent course of induction of apoptosis in K562 cells. After 24 h incubation of cells with the indicated concentrations of HP (A) and PB-2 (B) the PS expressing cells were expressed by Annexin V-binding. Each data point was repeated independently at least three times and represents mean ± SD. The solvent control DMSO did not increase the spontaneous apoptotic rate in any of the malignant cell lines tested.

considered relevant. Activation of specific caspases during HP-induced U937 cell apoptosis was demonstrated by a strong increase of the initiator caspase-9 activity (14.7-fold) after 24 h. When normalized to milligram total protein of lysed cells, the level of the effector caspase-3 increased 2.4-fold although the modulation of caspase-8 level was lower (2.1-fold) (Fig. 6). Our observations suggest, that activated caspase-9 initially activates caspase-3. Since activated caspase-3 may subsequently cleave caspase-8 and in addition feeds back to process caspase-9 [46]. The low modulation of caspase-8 activation in the presence of a strong caspase-9 activation during HP-induced apoptosis could be due to activation of the intrinsic mitochondria-related cell death pathway. It was found, that HP induced apoptosis in rat MT-450 cells triggering the mitochondria-mediated pathway (loss of mitochondrial membrane potential, cytochrome *c* release) and increasing the activity of caspase-3 and -9 [15]. In the K562 cells the level of caspases-3 and -8 activation was comparable to that of U937. An approximately 2.7-fold increase in the level of caspase 8 and 3.9-fold increase in the activation of effector caspase 3 compared to untreated cells were detected. In contrast, the lysates of leukemic K562 cells treated for 24 h with 20 μM HP featured levels of caspase-9-like activity similar to those detected in cell lysates from control cells. It may be possible, that in leukemia K562 cells HP induced a signaling pathway involving activation of caspase-8 as an initiator caspase and subsequent caspase-8 dependent triggering of a caspase cascade, which recruits effector caspase-3 [47]. Pretreatment of leukemia cells (K562, U937) for 1 h with z-VAD-fmk, inhibited both the appearance of PS exposure (Fig. 5) and the activation of caspases (Fig. 6), illustrating the functional relevance of caspase activation during HP-induced apoptosis.

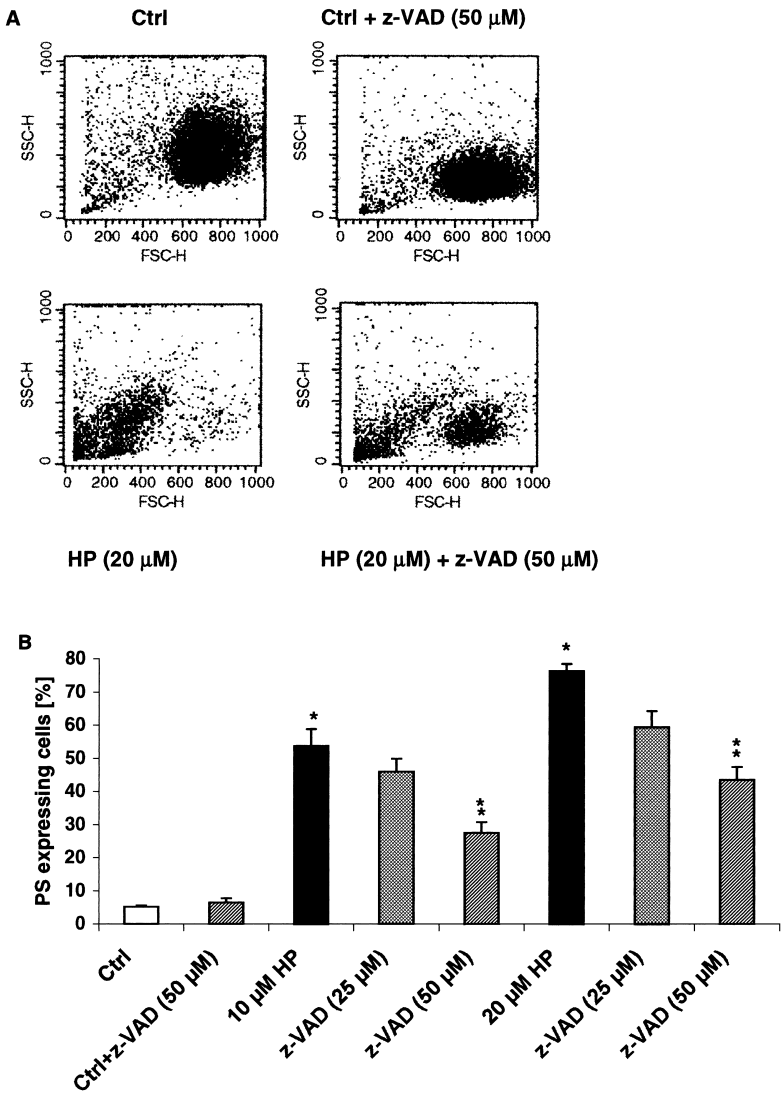


Fig. 5. Effect of z-VAD-fmk on HP-induced apoptosis. The reversion of apoptosis in K562 cells induced by HP (10, 20 μM, 24 h) was investigated after pretreatment of cells with inhibitor z-VAD-fmk (25, 50 μM, 1 h). A representative experiment of cell scatter characteristics (FSC vs. SSC) from three independent observations (A) and the percentages of cells expressing PS (B) as the mean of three independent experiments + SD are shown. (*$P < 0.01$ vs. untreated control, Ctrl; **$P < 0.01$ vs. cell treated with 20 μM HP, *t*-test).

Our observations suggest that HP, an abundant component of extracts of St John's wort (*H. perforatum*) with antiproliferative and apoptosis-inducing activities might be a useful plant-derived agent against malignancy. Further we showed that, HY and HP acted synergistically in their antiproliferative activity. HP-induced cell death was confirmed as apoptotic following activation of a cascade of caspases which occurred in the dark. In contrast, only light-activated HY caused apoptosis in various tumor cells due to activation of caspases-8 and -3 [48,49]. The tumoricidal properties of HY commonly used in photodynamic therapy were evaluated in various tumors in vivo. However, this therapy caused extensive tumor necrosis that was followed by local, intratumoral, and systemic inflammatory reactions [50–52]. As recently published [15], HP inhibited the growth of autologous MT-450 breast carcinoma in immunocompetent Wistar rats to a similar extent as paclitaxel, but without any sign of toxicity. Both HY and HP belong to the main groups of components of *H. perforatum* extracts. In this study, the half-maximal concentrations needed for antiproliferative effects of HY in the dark on leukemia cells (21–37.5 μM) as well as for HP on leukemia cells (14.9–19.9 μM) or brain cells (96.7–103.1 μM) were higher than those needed for their neurochemical effects in in vitro binding assays. The latter were more than ten times lower being between 0.08–2 μM for HP and 0.3–3 μM for HY [39,53–55]. In our previous study, however, we described the cytostatic and apoptosis-inducing ability of *H. perforatum* extract on tumor cells which had very low toxicity [18]. Cancer patients often use *H. perforatum*

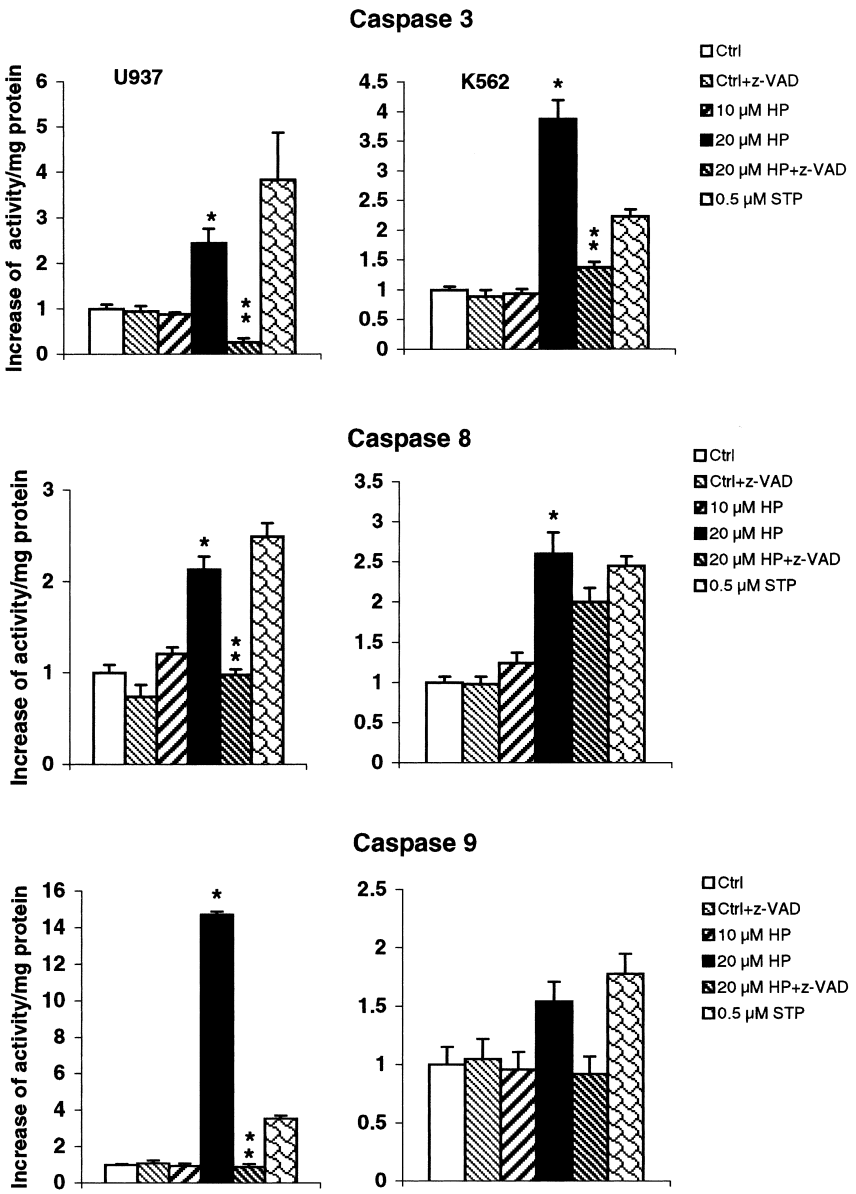


Fig. 6. Involvement of caspases in HP-induced apoptosis of U937 and K562 cells. Cells were treated with HP (10, 20 μM) without or with pretreatment by z-VAD-fmk (50 μM for 1 h) for 24 h. Cell lysates were tested for protease activity by the addition of caspase-specific peptides. The cleavage of the peptide by the caspase released a chromophore, which was quantitated at 405 nm. Data are presented as increase of caspase activity per milligram protein normalized to untreated controls and represent mean + SD of three independent experiments. Staurosporine (STP, 0.5 μM) was used as positive control (*$P < 0.01$ vs. untreated control; **$P < 0.01$ vs. 20 μM HP, *t*-test).

extract for therapy of depression. As it is well tolerated even at high doses and has no mutagenic potential [56,57], it could therefore be an interesting option in the management of malignancies.

4. Conclusion

In conclusion, our present data demonstrate: (i) the anticarcinogenic property of HP, which triggers cell death by apoptosis involving a caspase-dependent pathway; (ii) evidence, that compounds HY and HP cooperate in a synergistic manner in their antiproliferative activity without light work on; (iii) the cytostatic and apoptotic activities of the polyphenolic component PB-2 of *H. perforatum* extract toward malignant cells.

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