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ORIGINAL ARTICLE

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Hypericin—an inhibitor of proteasome function

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Abstract Hypericin is the presumed active moiety within Saint John's wort. Extracts of Saint John's wort are widely used as an effective treatment for depression. Available as "over-the-counter" drugs, they are frequently part of the self-medication of patients undergoing radiation therapy for malignant diseases. In addition to antidepressive properties, hypericin has been shown to be able to induce apoptosis and radiosensitize tumor cells, and to have antiinflammatory and phototoxic skin effects. However, the underlying mechanisms are not clear. In this study, we investigated possible inhibitory effects of hypericin on proteasome function and related pathways. Extracts from U373 human glioma cells were incubated with different concentrations of hypericin. Three proteasome activities were monitored using a fluorogenic peptide assay. Activity of the transcription factor NF-κB and protein levels of p65, p50, IκBα and caspase-3 were investigated by EMSA and Western blotting, respectively. Hypericin caused a dose-dependent and photoactivation-independent inhibition of proteasome function. Hypericin treatment (6.25–50 μM) inhibited NF-κB, caused accumulation of phosphorylated IκBα, decreased p50 protein levels and induced cleavage of p65 protein in U373 cells. These effects were observed in MCF-7 cells only at higher concentrations of hypericin (12.5–50 μM). Additionally, inhibition of NF-κB activity in U373 cells by hypericin was prevented by caspase inhibition. Although hypericin clearly inhibits proteasome function, its effect NF-κB DNA-binding activity was not exclusively proteasome-dependent. The underlying mechanism might also involve caspase activation, a consequence of proteasome inhibition.

Keywords St. John's wort · Hypericin · Proteasome · Apoptosis · NF-κB

Abbreviations AMC: 7-Amino-4-methylcoumarin · DMEM: Dulbecco's modified Eagle's medium · DMSO: Dimethyl sulfoxide · DTT: Dithiothreitol · EDTA: Ethylenediaminetetraacetic acid · HEPES: *N*-(2-Hydroxyethyl)piperazine *N*-2-ethanesulfonic acid · PMSF: Phenylmethane-sulfonyl fluoride · SDS: Sodium dodecyl sulfate · z-VAD-fmk: Benzyloxycarbonyl-Val-Ala-Asp-(OMe)-fluoromethylketone

Introduction

Extracts of the herb St. John's wort (*Hypericum perforatum*) have been used therapeutically in the treatment of mild to moderate depression [1] and are often part of the self-medication of patients. Because of their antidepressive action, these extracts are frequently used by patients suffering from chronic life-threatening disorders such as cancer, even without the knowledge of the physician [2], and thus may interfere with chemotherapy or radiation therapy. As a folk remedy, these extracts were used in the treatment of skin injuries and burns due to their antiinflammatory properties. Recent studies have indicated that these extracts are capable of inhibiting the synthesis of the cytokine interleukin-6 (IL-6) induced by substance P (SP) [3], which confirms the

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findings of earlier studies concerning the antiinflammatory properties of St. John's wort [4].

Herbal extracts of *H. perforatum* contain several active compounds including hypericin, hyperforin and flavonoids [5], which all contribute to different extents to the spectrum of effects of St. John's wort. However, as with all herbal extracts, the composition of different extracts may vary. Hypericin, the presumed active moiety within St. John's wort, exhibits potent pharmacological effects that include light-dependent antiretroviral activity against HIV infection [6], induction of apoptosis [7], inhibition of protein kinase C [8] and inhibition of the activation of the transcription factor nuclear factor kappa B (NF-κB) [9]. The phenanthroperylenequinone structure of hypericin allows it to be a natural tool for photodynamic therapy (PDT), a procedure that involves administration of a photosensitizing substance to the target cells, followed by selective irradiation that leads to the generation of reactive oxygen species (ROS) ultimately causing oxidative damage to the target cells [10]. In HeLa cells, PDT with hypericin causes caspase-independent activation of the c-Jun N-terminal kinase 1 (JNK1) and p38 mitogen-activated protein kinase (p38 MAPK), whose activation prevents apoptosis [11].

Although hypericin has been shown to localize in membranes of the endoplasmic reticulum and Golgi apparatus [12], it has also been shown to release mitochondrial cytochrome *c* into the cytosol leading to the activation of caspase-3 and apoptotic cell death [13]. This mechanism of apoptosis has been shown to be retarded, but not prevented, by overexpression of Bcl-2 protein [14]. Also, low-dose PDT with hypericin delays the onset of apoptosis by causing G_2/M phase arrest during which Bcl-2 becomes transiently phosphorylated probably through cyclin-dependent protein kinase 1 (CDK1) [15].

The NF-κB signaling pathway has been shown to be a key target for intervention in treating inflammatory conditions [16]. In most cell types, NF-κB is sequestered in the cytoplasm through its association with members of a family of inhibitory proteins known as Iκ-Bs. Potent activators of NF-κB, which include phorbol esters, proinflammatory cytokines, tumor necrosis factor, IL-1, bacterial endotoxin, viral double-stranded RNA and ionizing radiation, rapidly cause phosphorylation, polyubiquitination and proteolytic degradation of Iκ-B leading to the translocation of NF-κB into the nucleus where it regulates gene transcription. Several natural products and extracts have been identified that modulate the action of NF-κB [17]. Hypericin has been shown to inhibit NF-κB in HeLa and TC10 cells following stimulation with PMA and TNF-α, respectively, through a nonoxidative pathway [9].

The proteolytic degradation of Iκ-B is an ATP-dependent process that requires the 26S proteasome complex [18]. The proteasome is characterized by specific activities against short synthetic peptides; these include a "chymotrypsin-like" activity with preference for tyrosine or phenylalanine, a "trypsin-like" activity with preference for arginine or lysine, and a "post-glutamyl" hydrolyzing activity with a preference for glutamate or other acidic residues. The degradation of Iκ-B can be prevented by proteasome inhibitors [19]. The proteasome inhibitor, PS-341, has been shown in vitro to inhibit the chymotryptic activity, increase the intracellular levels of specific proteins including the CDK-1 inhibitor, p21, and cause a G_2/M-arrest, subsequently leading to apoptosis [20]. Apoptosis through proteasome inhibitors lactacystin and acetyl-leucinyl-leucinyl-norleucinal (AcLLNal) can be inhibited by the broad range caspase inhibitor, z-VAD-fmk, indicating the involvement of caspase-3 [21], which induces cleavage of Bcl-2 protein [22]. Some of the HIV-I protease inhibitors used clinically such as ritonavir and saquinavir have also been shown to induce apoptosis through the inhibition of proteasome function and blocking the activation of NF-κB [23, 24]. Inhibition of proteasome function has also shown to sensitize cells to radiation [25].

In the current study, we investigated the possible effects of hypericin on proteasome function. The functional coincidence of proapoptotic, radiosensitization and NF-κB inhibitory activities between hypericin and HIV-1 protease inhibitors led us to speculate about the possible inhibitory effect of hypericin on proteasome activity.

Materials and methods

Cell culture

U373 human glioma and MCF-7 human mammary carcinoma cells were obtained from the German Microorganism and Tissue Culture Collection (DSMZ, Braunschweig). Cells were grown in 75-cm^2 flasks (Greiner) at 37°C in a humidified atmosphere containing 5% CO_2in DMEM (Sigma) supplemented with 10% heat-inactivated fetal calf serum (Sigma) and 1% penicillin/streptomycin (Gibco BRL).

Drug treatment

Hypericin (Roth) was dissolved at 10 mg/ml in DMSO, and stored as small aliquots (10–30 µl) at −20°C. The pancaspase inhibitor z-VAD-fmk (Biomol) was dissolved in DMSO (25 m*M*) and stored as small aliquots (10 µl) at −20°C. Control cells received DMSO only. For gel-shift assays and Western blotting, cells were plated into six-well plates at a density of 3×10^5 cells/well and allowed to become adherent overnight. The following day cells were treated with hypericin or DMSO for 60 min.

Irradiation

Irradiation was carried out at room temperature using a ^{137}Cs laboratory irradiator (IBL 637, CIS Bio International) at a dose rate of 0.78 Gy/min. Corresponding controls were sham-irradiated.

Proteasome function assays

26S proteasome function was measured as described previously [20]. Briefly, confluent cell layers were washed with phosphate-buffered saline (PBS), then with buffer I (50 m*M* Tris, pH 7.4, 2 m*M* DTT, 5 m*M* $MgCl_2$, 2 m*M* ATP), and pelleted by centrifugation. Glass beads and homogenization buffer (50 m*M* Tris, pH 7.4, 1 m*M* DTT, 5 m*M* $MgCl_2$, 2 m*M* ATP and 250 m*M* sucrose) were added, which was followed by vortexing for 1 min. Beads and cell debris were removed by centrifugation at 1000 *g* for 5 min and 10,000 *g* for 20 min. Protein concentrations were determined by the BCA protocol (Pierce). Protein (100 µg) from each sample was diluted with buffer I to a final volume of 1000 µl and the fluorogenic proteasome substrates SucLLVY-7-amido-4-methylcoumarin (''chymotrypsin-like''; Sigma), Z-ARR-AMC (''trypsin-like''; Calbiochem) and Z-LLE-AMC (''peptidyl-glutamyl-like''; Calbiochem) were added in a final concentration of 80 µ*M* in 1% DMSO. To access 20S function, buffer I was replaced by buffer containing SDS (20 m*M* HEPES, pH 7.8, 0.5 m*M* EDTA, 0.03% SDS) [26]. Reaction mixtures were incubated at 37°C for 30 min. Free 7-amido-4-methylcoumarin (AMC) was assessed using a fluorescence plate reader (Gemini, Molecular Devices) at 380 nm (excitation) and 460 nm (emission). AMC (2 µ*M*) was incubated with hypericin in buffer I or 20S buffer without cell extracts, and measurements of proteasome function were corrected for direct effects of hypericin on AMC fluorescence.

Immunoblotting

Cells were lysed in RIPA buffer (50 m*M* Tris-HCl, pH 7.2, 150 m*M* NaCl, 1% Nonidet P-40, SDS, 10 m*M* PMSF, aprotinin and sodium vanadate). Protein concentrations were determined using the BCA protocol (Pierce) with bovine serum albumin (BSA, Sigma) as standard. Protein (50 µg) was electrophoresed in a SDS gel (0.1% SDS/12% polyacrylamide) and blotted to polyvinylidene fluoride membranes at 4°C. After blocking with Blotto buffer (Tris-buffered saline, 0.1% Tween 20, 5% skimmed milk) for 1 h at room temperature, the membranes were incubated with an antibody against human IκBα (rabbit polyclonal antibody, 1:2000; BD Biosciences), NF-κB p50 (rabbit polyclonal antibody, 1:1000; Santa Cruz Biotechnology) and NF-κB p65 (rabbit polyclonal antibody, 1:1000; Santa Cruz Biotechnology) and caspase-3 (polyclonal rabbit anti-human, 1:2000; BD Pharmingen) for 1 h at room temperature. A secondary HRP-conjugated antibody (goat anti-rabbit, 1:20,000; DAKO, and goat anti-mouse, 1:20,000; Serotec) and the ECLplus system (Amersham) were used for visualization. Uniformity of loading was confirmed by stripping and reprobing the membrane using an antibody against α-tubulin (mouse monoclonal antibody, 1:5000; Oncogene).

Electrophoretic mobility shift assays

For preparation of total cytosolic extracts, normal and treated cells were dislodged mechanically, washed with ice-cold PBS, and lysed in TOTEX-buffer (20 m*M* HEPES, pH 7.9, 0.35 m*M* NaCl, 20% glycerol, 1% Nonidet P-40, 0.5 m*M* EDTA, 0.1 m*M* ethyleneglycol-*bis*(β-aminoethyl)-*N*,*N*,*N*′,*N*′-tetraacetic acid, 0.5 m*M* DTT, PMSF and aprotinin) for 30 min on ice. Lysate was centrifuged at 12,000 *g* for 5 min at 4°C. Protein concentrations were determined using the BCA protocol (Pierce) with BSA as standard. Protein (15 µg) from the resulting supernatant was incubated for 25 min at room temperature with 2 µl BSA (10 µg/µl), 2 µl dIdC (1 µg/µl), 4 µl Ficoll buffer (20% Ficoll 400, 100 m*M* HEPES, 300 m*M* KCl, 10 m*M* DTT, 0.1 m*M* PMSF), 2 µl buffer D+ (20 m*M* HEPES, 20% glycerol, 100 m*M* KCl, 0.5 m*M* EDTA, 0.25% NP-40, 2 m*M* DTT and 0.1 m*M* PMSF) and 1 µl of the [γ^{32}P]-ATP-labeled oligonucleotide (Promega, NF-κB: AGTTGA GGG GAC TTT CCC AGG). For a negative control, unlabeled oligonucleotide was added in a 50-fold excess. Gel analysis was carried out in native 4% acrylamide/0.5% Tris-borate EDTA gels. Dried gels were placed on a phosphor screen for 24 h and analyzed on a phosphor imager (BAS-1500, Fujifilm).

Results

Hypericin is an inhibitor of the proteasome

Hypericin has a strong autofluorescence. To exclude a possible effect of hypericin on the AMC fluorescence signal, AMC was dissolved in buffer I at a final concentration of 2 µ*M*. Hypericin at concentrations from 0 to 50 µ*M* caused a dose-dependent decrease in the AMC fluorescence signal to 97.5 ± 0.2% at 3.125 µ*M*, 93.7 ± 3.3% at 6.25 µ*M*, 88.8 ± 3% at 12.5 µ*M*, 80.8 ± 0.4% at 25 µ*M* and 72.6 ± 1.1% at 50 µ*M*. In order to take this effect into account serial dilutions of hypericin in AMC-containing reaction buffer were included in each experiment, and measurements of proteasome activity were always corrected for this direct effect.

When protein extracts from U373 cells were incubated with different concentrations of hypericin, chymotryptic (Fig. 1a), tryptic and peptidyl-glutamyl 26S proteasome activity were decreased in a concentration-dependent manner (Table 1; IC_{50} values 8.2, 84 and

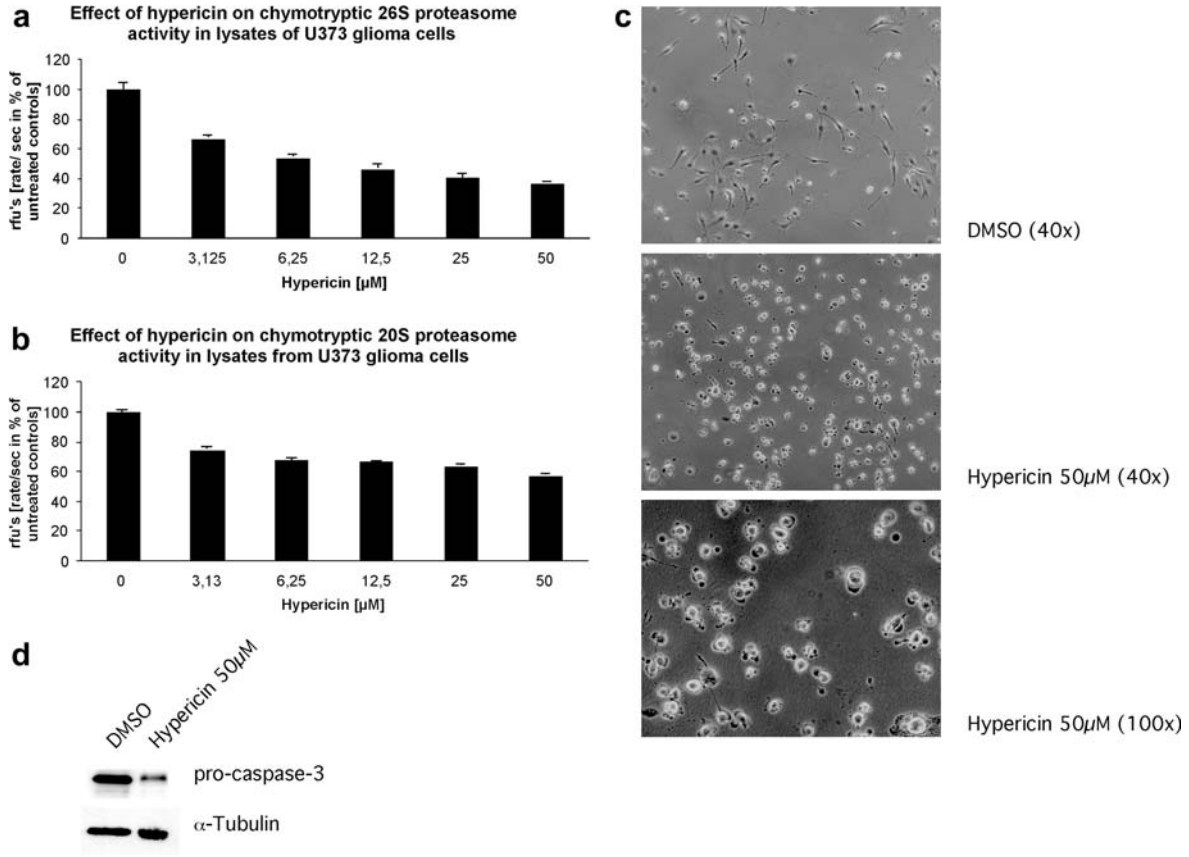

Fig. 1 Proteasome function assay using lysates from U373 cells. Hypericin caused a dose-dependent inhibition of chymotryptic 26S (**a**) and 20S (**b**) proteasome function. **c** Photoactivation of hypericin caused rapid induction of the apoptotic death program in U373 cells (*top* control cells, ×40; *centre* photoactivated hypericin, 50 μ*M*, ×40; *bottom* photoactivated hypericin, 50 μ*M*, ×100). Exposure of U373 cells to photoactivated hypericin led to membrane blebbing and chromatin condensation. **d** Morphological signs of apoptosis coincided with cleavage of procaspase-3 in Western blots using total cellular lysates from U373 cells

4.8 μ*M* for chymotryptic, tryptic and peptidyl-glutamyl activities, respectively) compared to untreated controls. Comparable results for chymotryptic 26S proteasome activity were obtained using lysates from MCF-7 (IC_{50} 3.8 μ*M*) and ECV304 (IC_{50} 2.1 μ*M*) cells.

Hypericin caused a concentration-dependent decrease in chymotryptic (Fig. 1b), tryptic and peptidyl-glutamyl 20S proteasome activity (in the absence of ATP and the presence of SDS) with IC_{50} values of 90.6, 2.2 and 0.9 μ*M*, respectively, compared to untreated controls (Table 1).

If hypericin was photoactivated it caused a rapid induction of apoptotic cell death (Fig. 1c), which coincided with cleavage of procaspase-3 (Fig. 1d). However, photoactivation of hypericin did not alter its effects on proteasome function (data not shown).

Hypericin inhibits NF-κB activation

When U373 cells were preincubated with different concentrations of hypericin (0–50 μ*M*) for 1 h, a dose-dependent inhibition of constitutive NF-κB

Table 1 Effect of hypericin on 26S and 20S proteasome function in extracts from U373 cells

Hypericin (μ*M*)	Proteasome activity (%)					
	26S			20S		
	Chymotryptic	Tryptic	Pedidyl-glutamyl	Chymotryptic	Tryptic	Pedidyl-glutamyl
0	100	100	100	100	100	100
3.125	66.9 ± 2.7	100.2 ± 3.7	56.4 ± 1.7	74.4 ± 2.8	43.1 ± 2	18.9 ± 0.7
6.25	54.1 ± 2.7	99.2 ± 2.8	48.8 ± 1.4	67.6 ± 1.6	38.8 ± 2.1	15.9 ± 0.4
12.5	46.5 ± 2.5	91.2 ± 2.8	41.0 ± 1	66.7 ± 1	33.8 ± 0.8	13.5 ± 0.4
25	40.6 ± 3	88.5 ± 2.5	35.3 ± 1.4	63.7 ± 1.5	33.3 ± 2.0	12.7 ± 0.3
50	36.7 ± 1.8	63.9 ± 1	32.8 ± 1.6	57.5 ± 1.5	31.8 ± 0.5	11.4 ± 0.3

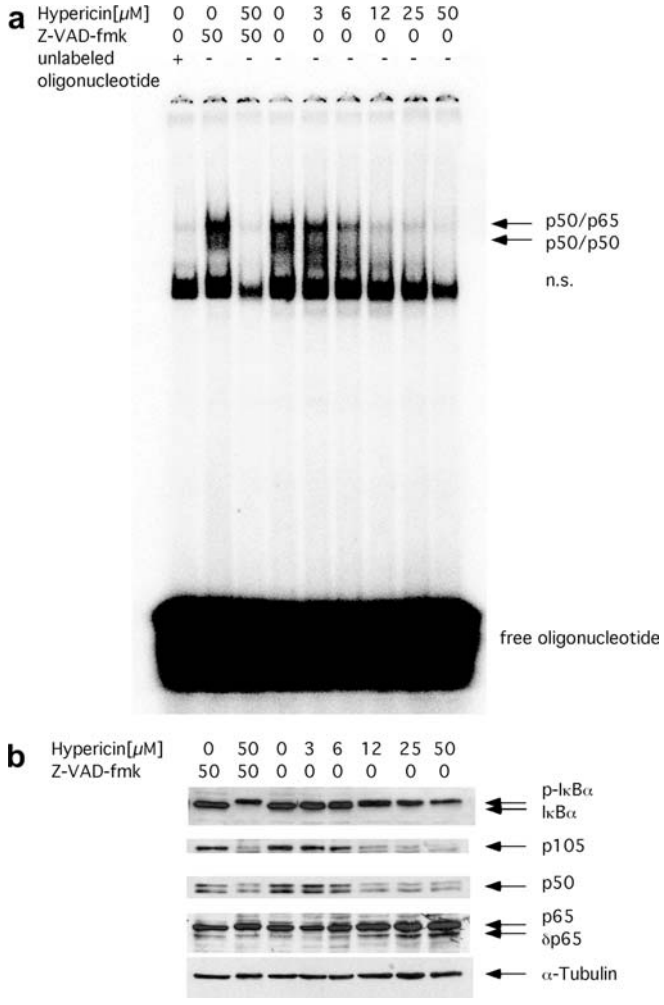

Fig. 2 **a** Electrophoretic mobility shift assay (EMSA) using total cellular extracts from U373 glioma cells. Non-photoactivated hypericin caused a dose-dependent inhibition of NF-κB DNA-binding activity, which was almost complete at concentrations ≥12 μ*M*. Preincubation with the pan-caspase inhibitor z-VAD-fmk (50 μ*M*) was not able to prevent this effect of hypericin at 50 μ*M*. **b** Western blots using total cellular extracts from EMSAs. Hypericin caused a dose-dependent decrease in IκBα, p105 and p50, and led to the accumulation of phosphorylated IκBα. Additionally, a cleavage product of p65 (δp65) was present. Cleavage of p65 was prevented by preincubation with z-VAD-fmk (50 μ*M*)

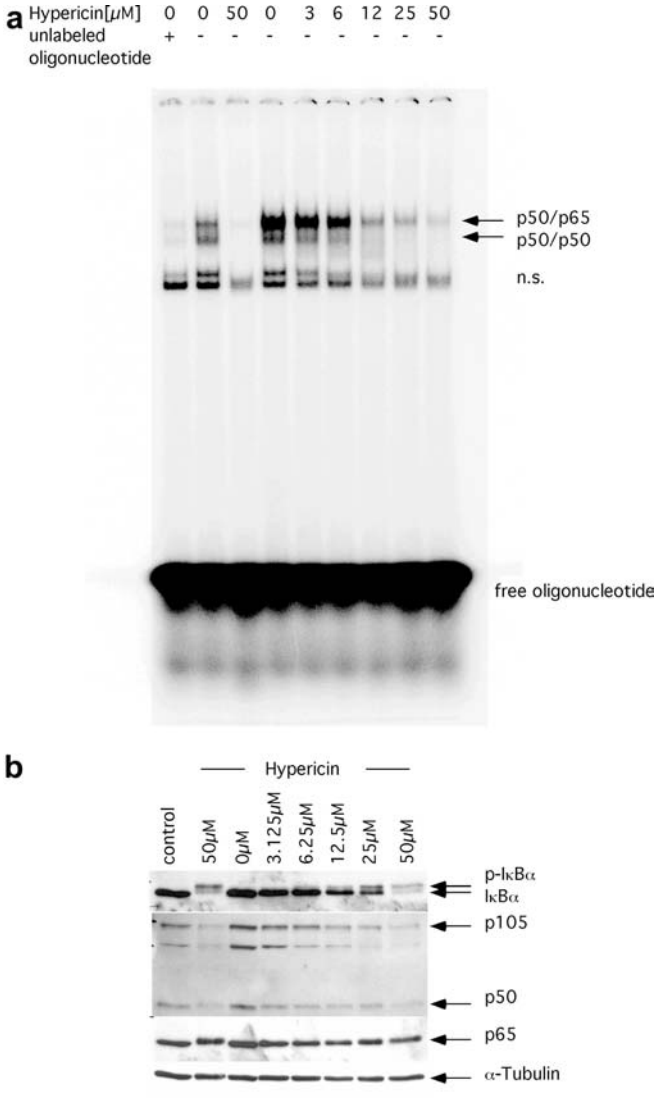

Fig. 3 **a** EMSA using total cellular extracts from MCF-7 breast cancer cells, functionally deficient in caspase-3. DNA-binding activity of NF-κB was inhibited in a dose-dependent manner but was nearly complete only at high concentrations (>25 μ*M*) of hypericin. **b** Western blots using total cellular extracts from EMSAs. Hypericin caused a dose-dependent decrease in IκBα, p105 and p50, and led to the accumulation of phosphorylated IκBα. However, a cleavage product of p65 was not detectable even at a hypericin concentration of 50 μ*M*. Preincubation with z-VAD-fmk (50 μ*M*) did not have any effect

DNA-binding activity was observed with almost complete inhibition at concentrations as low as 12.5 μ*M* (Fig. 2a). Inhibition of NF-κB was accompanied by an overall loss of IκBα and accumulation of phosphorylated forms of IκBα, which supports our observation of inhibitory effects of hypericin on proteasome function. Additionally, dose-dependent decreases in p105 and p50 protein levels were also observed, indicating an additional proteasome-independent mechanism of hypericin-induced inhibition of NF-κB. p65 protein levels remained unchanged during the course of treatment but hypericin treatment led to the occurrence of a p65 cleavage product (Fig. 2b). As hypericin activated caspase-3 in U373 cells, we repeated this experiment using MCF-7 breast cancer cells, which are known to be functionally deficient in caspase-3 [27]. Again, hypericin treatment caused a dose-dependent accumulation of phosphorylated forms of IκBα, decreases in p105 and p50 (Fig. 3b) protein levels, and inhibition of constitutive NF-κB DNA-binding activity (Fig. 3a). However, this effect was less pronounced and occurred only at high concentrations of hypericin. Additionally, hypericin did not lead to cleavage of p65 (Fig. 3b).

To further confirm the involvement of caspases, we preincubated U373 cells with the pan-caspase inhibitor z-VAD-fmk. At a hypericin concentration of 10 μ*M*,

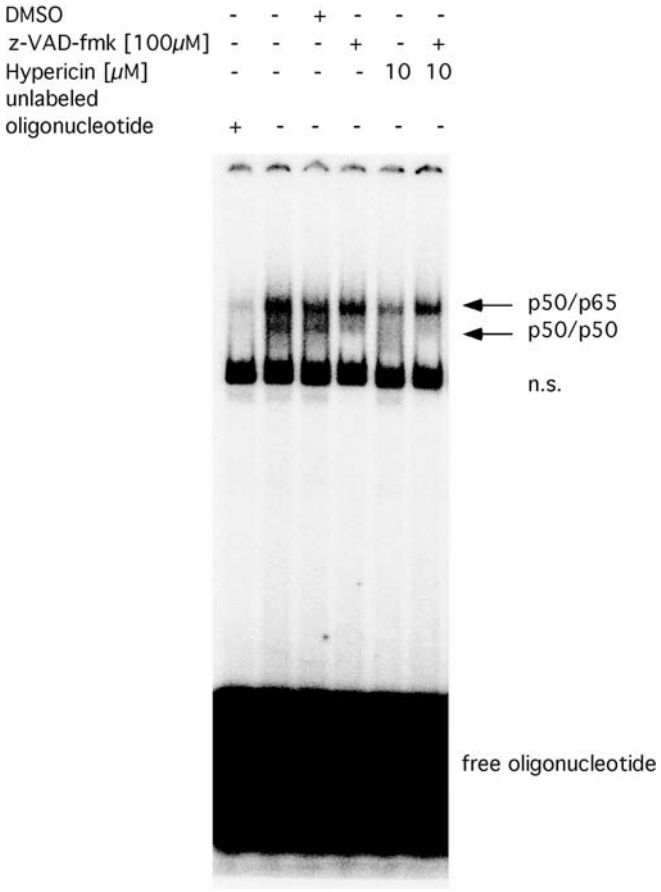

Fig. 4 EMSA using total cellular extracts from U373 glioma cells. Non-photoactivated hypericin at a concentration of 10 μ*M* caused a partial inhibition of NF-κB DNA-binding activity. Preincubation with the pan-caspase inhibitor z-VAD-fmk (100 μ*M*) was able to reverse this effect

hypericin-induced inhibition of NF-κB activation was partially prevented by z-VAD-fmk (Fig. 4).

Discussion

The antitumor activity of hypericin, the active moiety within Saint John's wort, has recently been attributed to activation of the caspase cascade, although the mechanisms by which hypericin activates the apoptotic death program are incompletely understood [28]. Caspase-activation alone is not sufficient to explain the remaining broad spectrum of antiinflammatory [29], antibacterial [4] and antidepressive [1] effects of this drug, and do not explain how hypericin acts to lower serum levels of HIV-I protease inhibitors [30] by induction of P-glycoprotein (P-gp), the gene product of the multidrug-resistance gene 1 (mdr-1) [31]. Additionally, St. John's wort has been shown to interfere with chemotherapy in cancer patients [32, 33]. This is of special interest as the majority of cancer patients use vitamins or herbal extracts as a complementary medication for cancer treatment [34]. Herbal extracts of St. John's wort contain a variety of substances including flavonoids such as quercitin, rutin and astilbin, phoroglucinols such as hyperforin and adhyperforin, and naphthodiathrones such as hypericin and pesudohypericin, which might all contribute to the broad spectrum of effects of these herbal extracts [5]. In this study, our investigation was focused on an alternative molecular mechanisms of action of hypericin.

A major transcription factor responsible for proinflammatory gene regulation and inhibition of apoptosis is NF-κB and there is strong evidence that target genes of NF-κB play a major role in depression [35]. Treatment of cells with hypericin is known to decrease DNA-binding activity of NF-κB by a non-antioxidative pathway, but at present this pathway is not fully understood [9]. Since NF-κB is under the tight control of the ubiquitin/26S proteasome system, we investigated the possible interaction of hypericin with this system. Our results demonstrate a direct effect of hypericin on chymotryptic, tryptic and peptidyl-glutamyl cleavage activity of the 20S and 26S proteasome, suggesting an interaction of this drug with the 20S core unit of this protease. However, because IC_{50} values for chymotryptic 26S activity were much lower than for 20S activity, hypericin might also interact with one or more subunits of the 19S regulatory unit of the proteasome. This was further supported by the observation that tryptic 26S activity was almost unaffected while 20S tryptic activity was inhibited with an IC_{50} of 2.2 μ*M*.

As expected, DNA-binding activity of NF-κB was strongly inhibited by hypericin treatment in U373 cells. These effects easily explain the antiinflammatory, immune-suppressive and proapoptotic effects of hypericin since inhibition of the proteasome alters MHC class I presentation [36] and inhibits DNA-binding activity of NF-κB, which terminates the proinflammatory gene program and induces apoptosis [37, 38]. We have previously demonstrated radiosensitizing effects of proteasome inhibitors [24, 25]. Radiosensitization of glioma cells by hypericin treatment has been reported by Zhang and coworkers [39]. Although the underlying mechanism of radiosensitization by proteasome inhibition still remains elusive, proteasome inhibition may also serve as an explanation for the radiosensitizing effects of hypericin.

In U373 cells hypericin treatment decreased p105 and p50 protein levels and induced the occurrence of a p65 cleavage product. Our analysis of the published p105/IκBα protein sequence (Swissprot, P19838) revealed a potential caspase-6 cleavage site at position 693-VEHD-N-697 that cuts off parts of the ankyrin-like repeat 5 (aa 684–714), the ankyrin-like repeat 6 (719–751) and the ankyrin-like repeat 7 (aa 772–804). This part of the protein has been shown to control cytoplasmatic retention of p105 [40] and contains the PEST sequence with its putative serine phosphorylation sites that lead to polyubiquitination and subsequent posttranslational processing to p50 by the proteasome [41]. Cleavage of p65 by caspase-3 has been reported previously [42].

The inhibitory effect of hypericin on proteasome function in extracts from U373 cells was less pronounced than in extracts from MCF-7 cells. However, hypericin inhibited NF-κB at lower concentrations in U373 cells compared to MCF-7 cells, which also indicates involvement of caspase-dependent degradation of p65 and p50 subunits in addition to proteasome inhibition as a mechanism to terminate NF-κB signal transduction.

Hypericin treatment may thus inhibit NF-κB by two different mechanisms. First, it stabilizes phosphorylated IκBα by proteasome inhibition. Second, this downregulates NF-κB with the consequence that loss of its antiapoptotic function causes sensitization of cells to proapoptotic stimuli, e.g., TNF-related apoptosis inducing ligand, via downregulation of NF-κB-dependent cellular FLICE-inhibitory protein and X-linked inhibitor of apoptosis expression. This in turn activates caspase-dependent apoptosis, which leads to degradation of p65 by caspase-3 and cleavage of the p50 precursor protein p105 by caspase-6 resulting in a decrease in p50 protein levels. The possible involvement of caspase-3 is supported by the observation that in MCF-7 cells, deficient in caspase-3, much higher concentrations of hypericin were necessary to inhibit NF-κB and no cleavage product of p65 was detectable. Additionally, the pancaspase inhibitor z-VAD-fmk was—at least in part—able to prevent the effect of hypericin treatment on NF-κB DNA-binding activity.

Taken together our observation of inhibition of proteasome function gives a new insight into how hypericin acts to terminate inflammatory conditions, interferes with HIV protease inhibitor treatment and activates the apoptotic death program in cancer cells.

References

1. Josey ES, Tackett RL (1999) St John's wort: a new alternative for depression? Int J Clin Pharmacol Ther 37(3):111–119
2. Martin-Facklam M, Rieger K, Riedel KD, Burhenne J, Walter-Sack I, Haefeli WE (2004) Undeclared exposure to St John's wort in hospitalized patients. Br J Clin Pharmacol 58(4):437–441
3. Fiebich BL, Hoellig A, Lieb K (2001) Inhibition of substance P-induced cytokine synthesis by St John's wort extracts. Pharmacopsychiatry 34 [Suppl 1]:S26–S28
4. Fiebich BL, Heinrich M, Langosch JM, Kammerer N, Lieb K (1999) Antibacterial activity of hyperforin from St John's wort. Lancet 354(9180):777
5. Zou Y, Lu Y, Wei D (2004) Antioxidant activity of a flavonoid-rich extract of Hypericum perforatum L. in vitro. J Agric Food Chem 52(16):5032–5039
6. Miskovsky P (2002) Hypericin—a new antiviral and antitumor photosensitizer: mechanism of action and interaction with biological macromolecules. Curr Drug Targets 3(1):55–84
7. Couldwell WT, Gopalakrishna R, Hinton DR, He S, Weiss MH, Law RE, Apuzzo ML (1994) Hypericin: a potential antiglioma therapy. Neurosurgery 35(4):705–709 (discussion pp. 709–710)
8. Takahashi I, Nakanishi S, Kobayashi E, Nakano H, Suzuki K, Tamaoki T (1989) Hypericin and pseudohypericin specifically inhibit protein kinase C: possible relation to their antiretroviral activity. Biochem Biophys Res Commun 165(3):1207–1212
9. Bork PM, Bacher S, Schmitz ML, Kaspers U, Heinrich M (1999) Hypericin as a non-antioxidant inhibitor of NF-kappa B. Planta Med 65(4):297–300
10. Dougherty TJ, Gomer CJ, Henderson BW, Jori G, Kessel D, Korbelik M, Moan J, Peng Q (1998) Photodynamic therapy. J Natl Cancer Inst 90(12):889–905
11. Assefa Z, Vantieghem A, Declercq W, Vandenabeele P, Vandenheede JR, Merlevede W, de Witte P, Agostinis P (1999) The activation of the c-Jun N-terminal kinase and p38 mitogen-activated protein kinase signaling pathways protects HeLa cells from apoptosis following photodynamic therapy with hypericin. J Biol Chem 274(13):8788–8796
12. Uzdensky AB, Ma LW, Iani V, Hjortland GO, Steen HB, Moan J (2001) Intracellular localization of hypericin in human glioblastoma and carcinoma cell lines. Lasers Med Sci 16(4):276–283
13. Vantieghem A, Assefa Z, Vandenabeele P, Declercq W, Courtois S, Vandenheede JR, Merlevede W, de Witte P, Agostinis P (1998) Hypericin-induced photosensitization of HeLa cells leads to apoptosis or necrosis. Involvement of cytochrome c and procaspase-3 activation in the mechanism of apoptosis. FEBS Lett 440(1–2):19–24
14. Chaloupka R, Petit PX, Israel N, Sureau F (1999) Overexpression of Bcl-2 does not protect cells from hypericin photoinduced mitochondrial membrane depolarization, but delays subsequent events in the apoptotic pathway. FEBS Lett 462(3):295–301
15. Vantieghem A, Xu Y, Assefa Z, Piette J, Vandenheede JR, Merlevede W, De Witte PA, Agostinis P (2002) Phosphorylation of Bcl-2 in G2/M phase-arrested cells following photodynamic therapy with hypericin involves a CDK1-mediated signal and delays the onset of apoptosis. J Biol Chem 277(40):37718–37731
16. Karin M, Ben-Neriah Y (2000) Phosphorylation meets ubiquitination: the control of NF-[kappa]B activity. Annu Rev Immunol 18:621–663
17. Bremner P, Heinrich M (2002) Natural products as targeted modulators of the nuclear factor-kappaB pathway. J Pharm Pharmacol 54(4):453–472
18. Coux O, Tanaka K, Goldberg AL (1996) Structure and functions of the 20S and 26S proteasomes. Annu Rev Biochem 65:801–847
19. Palombella VJ, Rando OJ, Goldberg AL, Maniatis T (1994) The ubiquitin–proteasome pathway is required for processing the NF-kappa B1 precursor protein and the activation of NF-kappa B. Cell 78(5):773–785
20. Adams J, Palombella VJ, Sausville EA, Johnson J, Destree A, Lazarus DD, Maas J, Pien CS, Prakash S, Elliott PJ (1999) Proteasome inhibitors: a novel class of potent and effective antitumor agents. Cancer Res 59(11):2615–2622
21. Kitagawa H, Tani E, Ikemoto H, Ozaki I, Nakano A, Omura S (1999) Proteasome inhibitors induce mitochondria-independent apoptosis in human glioma cells. FEBS Lett 443(2):181–186
22. Zhang XM, Lin H, Chen C, Chen BD (1999) Inhibition of ubiquitin–proteasome pathway activates a caspase-3-like protease and induces Bcl-2 cleavage in human M-07e leukaemic cells. Biochem J 340(Pt 1):127–133
23. Schmidtke G, Holzhutter HG, Bogyo M, Kairies N, Groll M, de Giuli R, Emch S, Groettrup M (1999) How an inhibitor of the HIV-I protease modulates proteasome activity. J Biol Chem 274(50):35734–35740
24. Pajonk F, Himmelsbach J, Riess K, Sommer A, McBride WH (2002) The human immunodeficiency virus (HIV)-1 protease inhibitor saquinavir inhibits proteasome function and causes apoptosis and radiosensitization in non-HIV-associated human cancer cells. Cancer Res 62(18):5230–5235
25. Pajonk F, Pajonk K, McBride W (2000) Apoptosis and radiosensitization of Hodgkin's cells by proteasome inhibition. Int J Radiat Oncol Biol Phys 47(4):1025–1032
26. Glas R, Bogyo M, McMaster JS, Gaczynska M, Ploegh HL (1998) A proteolytic system that compensates for loss of proteasome function. Nature 392(6676):618–622

27. Kurokawa H, Nishio K, Fukumoto H, Tomonari A, Suzuki T, Saijo N (1999) Alteration of caspase-3 (CPP32/Yama/apopain) in wild-type MCF-7, breast cancer cells. Oncol Rep 6(1):33–37
28. Schempp CM, Simon-Haarhaus B, Termeer CC, Simon JC (2001) Hypericin photo-induced apoptosis involves the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and activation of caspase-8. FEBS Lett 493(1):26–30
29. Kang BY, Chung SW, Kim TS (2001) Inhibition of interleukin-12 production in lipopolysaccharide-activated mouse macrophages by hypericin, an active component of Hypericum perforatum. Planta Med 67(4):364–366
30. Piscitelli SC, Burstein AH, Chaitt D, Alfaro RM, Falloon J (2000) Indinavir concentrations and St John's wort. Lancet 355(9203):547–548
31. Hennessy M, Kelleher D, Spiers JP, Barry M, Kavanagh P, Back D, Mulcahy F, Feely J (2002) St Johns wort increases expression of P-glycoprotein: implications for drug interactions. Br J Clin Pharmacol 53(1):75–82
32. Mathijssen RH, Verweij J, de Bruijn P, Loos WJ, Sparreboom A (2002) Effects of St. John's wort on irinotecan metabolism. J Natl Cancer Inst 94(16):1247–1249
33. Mannel M (2004) Drug interactions with St John's wort: mechanisms and clinical implications. Drug Saf 27(11):773–797
34. Richardson MA, Sanders T, Palmer JL, Greisinger A, Singletary SE (2000) Complementary/alternative medicine use in a comprehensive cancer center and the implications for oncology. J Clin Oncol 18(13):2505–2514
35. Leonard BE (2001) The immune system, depression and the action of antidepressants. Prog Neuropsychopharmacol Biol Psychiatry 25(4):767–780
36. Niedermann G, Butz S, Ihlenfeldt HG, Grimm R, Lucchiari M, Hoschutzky H, Jung G, Maier B, Eichmann K (1995) Contribution of proteasome-mediated proteolysis to the hierarchy of epitopes presented by major histocompatibility complex class I molecules. Immunity 2(3):289–299
37. Pajonk F, Pajonk K, McBride WH (1999) Inhibition of NF-kappaB, clonogenicity, and radiosensitivity of human cancer cells. J Natl Cancer Inst 91(22):1956–1960
38. Yang Y, Yu X (2003) Regulation of apoptosis: the ubiquitous way. FASEB J 17(8):790–799
39. Zhang W, Anker L, Law RE, Hinton DR, Gopalakrishna R, Pu Q, Gundimeda U, Weiss MH, Couldwell WT (1996) Enhancement of radiosensitivity in human malignant glioma cells by hypericin in vitro. Clin Cancer Res 2(5):843–846
40. Blank V, Kourilsky P, Israel A (1991) Cytoplasmic retention, DNA binding and processing of the NF-kappa B p50 precursor are controlled by a small region in its C-terminus. EMBO J 10(13):4159–4167
41. MacKichan ML, Logeat F, Israel A (1996) Phosphorylation of p105 PEST sequence via a redox-insensitive pathway up-regulates processing of p50 NF-kappaB. J Biol Chem 271(11):6084–6091
42. Kang KH, Lee KH, Kim MY, Choi KH (2001) Caspase-3-mediated cleavage of the NF-kappa B subunit p65 at the NH2 terminus potentiates naphthoquinone analog-induced apoptosis. J Biol Chem 276(27):24638–24644