Aristoforin, a Novel Stable Derivative of Hyperforin, Is a Potent Anticancer Agent

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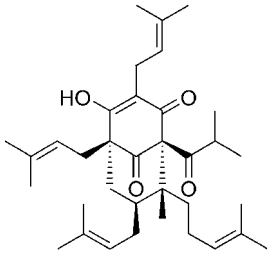
*Hyperforin, a natural product of St. John's wort (*Hypericum perforatum *L.), has a number of pharmacological activities, including antidepressive and antibacterial properties. Furthermore, hyperforin has pronounced antitumor properties against different tumor cell lines, both in vitro and in vivo. Despite being a promising novel anticancer agent, the poor solubility and stability of hyperforin in aqueous solution limits its potential clinical application. In this study, we present the synthesis of hyperforin derivatives with improved pharmacological activity. The synthesized compounds were tested for their solubility and stability properties. They were also investigated for their antitumor properties, both in vitro and in vivo. One of these hyperforin derivatives, Aristoforin, is more soluble in aqueous solution than hyperforin and is additionally highly stable. Importantly, it retains the antitumor properties of the parental compound without inducing toxicity in experimental animals. These data strongly suggest that Aristoforin has potential as an anticancer drug.*

Introduction

St. John's wort (*Hypericum perforatum* L.) is a member of the *hypericaceae* plant family. It is used for the treatment of depression and for the topical treatment of burns, skin injuries, and atopic dermatitis.[1–3] One of the active ingredients of St. John's wort is the phloroglucin derivative hyperforin **1**,[4,5] an



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acylphloroglucinol-type compound that consists of a phloroglucinol skeleton substituted with lipophilic isoprene chains. It is a natural antibiotic that inhibits the growth of several Gram-positive bacteria, including methicillin-resistant *Staphylococcus aureus*.[6,7] In previous studies, we have observed a dose-dependent antiproliferative effect of hyperforin in phytohemagglutinin-stimulated peripheral blood lymphocytes.[8] Most significantly, hyperforin has potent antitumor activity.[9,10] At the biochemical level, hyperforin is a ligand for the Pregnane X receptor (PXR) and its human homologue the steroid and xenobiotic receptor (SXR).[11,12] These orphan nuclear receptors regulate expression of cytochrome P450 3A4 and a number of other genes involved in the metabolism and elimination of xenobiotics from the body.[11,13,14]

Hyperforin inhibits the growth of several tumor cell lines in a dose-dependent manner with an IC_{50} value of 3–15 μM.[9] It exerts its antiproliferative effect by activating the intrinsic apoptosis pathway.[9,10] In an animal model, hyperforin was able to inhibit the growth of tumors in vivo without producing any overt toxicity, and its antitumor activity was comparable with that exerted by paclitaxel, a drug already in clinical use.[9]

As a pure compound, hyperforin is sensitive to light and oxygen, and it is also poorly soluble in aqueous solution.[15–18] These factors are major limitations to its therapeutic use. While hyperforin salts exhibit better stability and solubility, salt formation is likely to be reversed in the organism. In this study we have therefore developed chemical derivatives of hyperforin with improved stability and solubility properties. Significantly, some of these derivatives are more potent in suppressing tumor cell proliferation than the parental compound. One of the compounds, which we named Aristoforin, also has potent antitumor properties in vivo, pointing to a possible clinical use in cancer therapy.

Results and Discussion

To develop hyperforin derivatives with improved pharmacochemical properties, we designed chemical syntheses to

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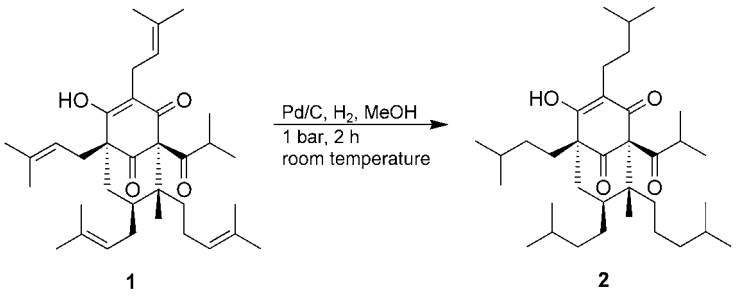
 DOI: 10.1002/cbic.200400195

modify the structure of hyperforin **1**. To provide sufficient hyperforin for these experiments, we used the method of Adam et al.[19] for its isolation. This method is rapid and cheap; this means that any future large-scale production for therapeutic purposes of the hyperforin derivatives described here should be economically viable.

Hyperforin was derivatized to produce 17,18,22,23,27,28,32,33-octahydrohyperforin (**2**), *O*-(carboxymethyl)-hyperforin (Aristoforin, **5**) and *O*-(carboxymethyl)-17,18,22,23,27,28,32,33-octahydrohyperforin (**6**). The hyperforin derivative **2** was obtained by catalytic hydrogenation of hyperforin with palladium on charcoal (Scheme 1). Aristoforin was produced in two steps. First, hyperforin was alkylated with ethyl bromoacetate to give the *C*-alkylated derivative **3** and the *O*-alkylated derivative **4**. The latter was then saponified with aqueous NaOH solution to afford **5** (Scheme 2). Finally derivative **6** was obtained from **5** by catalytic hydrogenation with palladium on charcoal (Scheme 3).

Afterwards we investigated whether compounds **2**, **5**, and **6** show enhanced solubility compared to hyperforin. We therefore tried to dissolve these compounds either in H_2O/2% DMSO or 100 mM aqueous $NaHCO_3$ solution/2% DMSO, at different concentrations. After ten minutes, the solubility of the compounds at different concentrations was assessed visually (Table 1). These data show that Aristoforin **5** and compound **6** have a distinct increased solubility compared to hyperforin, even in H_2O. In contrast, compound **2** shows poor solubility that is comparable to that of hyperforin. The solubility data show that an Aristoforin **5** concentration of at least 170 μM in H_2O and 420 μM in 100 mM aqueous $NaHCO_3$ solution can be reached. In sharp contrast, a hyperforin **1** concentration of only 20 μM in H_2O and 140 μM in 100 mM aqueous $NaHCO_3$ solution is possible.

To test whether compounds **2**, **5**, and **6** have enhanced stability compared to the parental hyperforin, we dissolved the compounds either in DMSO or phosphate buffered saline solution (PBS)/20% DMSO and exposed them to light and a normal air atmosphere. Samples were taken at different time points and the amount of intact soluble compound remaining in solution was measured by HPLC (Table 2) or by NMR spectral analysis (compound **6**, data not shown). These data demonstrate that in contrast to hyperforin, the hyperforin derivatives **2**, **6** and Aristoforin are completely stable over a period of at least one week when dissolved in DMSO. Furthermore, compounds **5** and **6** remain completely stable in aqueous solution, in contrast to hyperforin which rapidly degrades, and to compound **2** which extensively precipitates after a week in solution.



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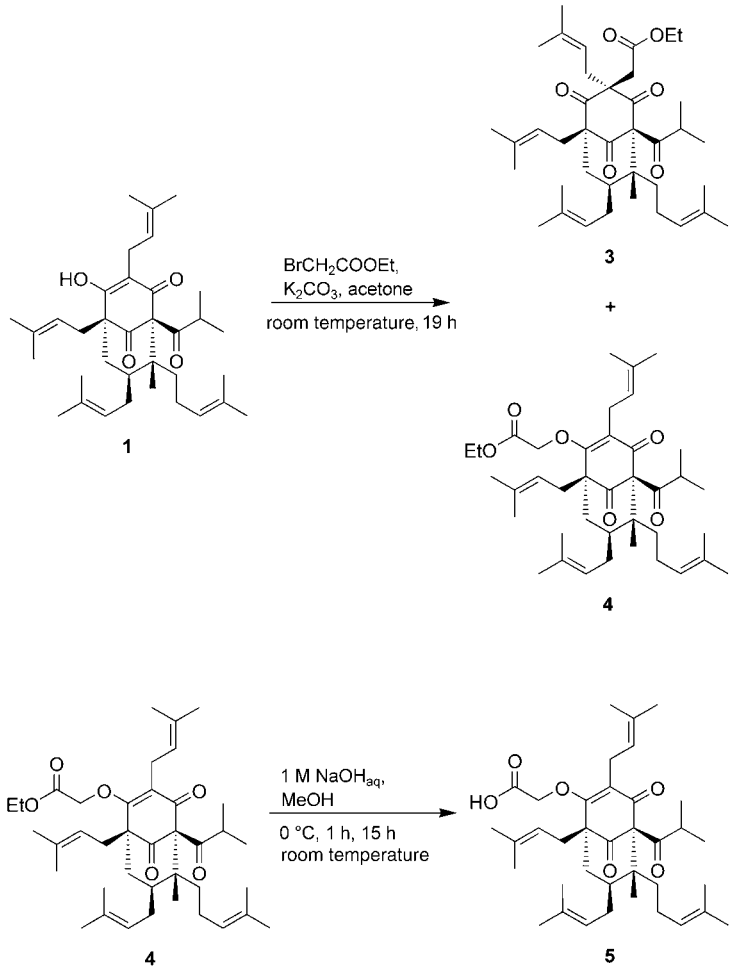
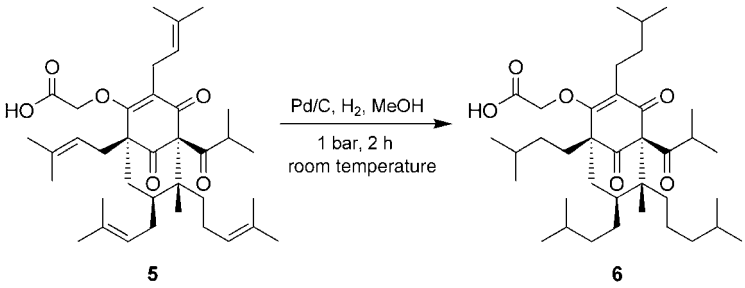
Scheme 1. *Synthesis of hyperforin derivative **2**.*



1 3 4

4 5

Scheme 2. *Synthesis of hyperforin derivative Aristoforin (**5**).*



5 6

Scheme 3. *Synthesis of hyperforin derivative **6**.*

Table 1. *Solubility of hyperforin and its derivatives.*[a]

Solvent	Compound	500 μg mL^{-1}	250 μg mL^{-1}	100 μg mL^{-1}	75 μg mL^{-1}	50 μg mL^{-1}	10 μg mL^{-1}
H_2O/2% DMSO	hyperforin	–	–	–	–	–	+
	2	–	–	–	–	–	+
	6	–	–	+	+	+	+
	Aristoforin	–	–	+	+	+	+
	hyperforin	–	–	–	+	+	+
100 mM aqueous $NaHCO_3$ solution/ 2% DMSO	**2**	–	–	–	–	–	+
	6	–	+	+	+	+	+
	Aristoforin	–	+	+	+	+	+

[a] + = compound is completely dissolved at the indicated concentration; – = compound is not fully dissolved at the indicated concentration. All measurements were performed at 20 °C.

Table 2. *Stability of hyperforin and its derivatives. Samples were dissolved to 1 mg mL^{-1} in either DMSO or PBS/20% DMSO. Chromatographic purity of the samples was measured by HPLC at the indicated time points and is expressed as percent of the starting purity.*[a]

Solvent	Compound	Start	1 hour	1 day	1 week
DMSO	hyperforin	100.0	99.9	99.6	87.6
	2	100.0	100.0	100.0	99.8
	Aristoforin	100.0	100.1	100.0	100.1
PBS/20% DMSO	hyperforin	100.0	88.8	27.5	n.d.
	2	100.0	100.0	100.0	n.m.
	Aristoforin	100.0	99.9	98.8	100.7

[a] n.d. = not detectable; n.m. = not measurable due to extensive precipitation.

In previous work we have demonstrated the proapoptotic and antitumor effects of hyperforin in a wide range of different tumor cell types.[9] MT-450 mammary tumor cells were used in these studies and have the advantage that they can be used in syngeneic animal tumor model studies. We therefore used MT-450 cells as a baseline against which to characterize the antitumor properties of the new hyperforin derivatives in comparison to the parental compound. MT-450 cells were exposed to a range of concentrations of hyperforin, compound **2**, Aristoforin **5** and compound **6**. The effect of the compounds on cell growth was assessed by using ^{3}H-thymidine incorporation as a measure of proliferation (Figure 1 A–C), and the proapoptotic effects of the compounds were determined by using an ELISA assay that quantifies the concentration of oligonucleosomes that are released during the process of apoptosis (Figure 2). The hyperforin derivative **4** that is an intermediate in the synthesis of Aristoforin had no effect on proliferation and was not proapoptotic (data not shown). In sharp contrast, the hyperforin derivatives **2**, **5**, and **6** inhibit MT-450 cell proliferation and induce apoptosis to an equivalent extent as the parental hyperforin; this demonstrates that it is possible to chemically modify hyperforin and improve its solubility and stability without interfering with its antiproliferative and proapoptotic properties. Indeed, compounds **2** and **5** exert an enhanced antiproliferative effect on MT-450 cells in comparison to hyperforin.

To determine whether the novel hyperforin derivatives we describe here have antitumor properties in vivo, we tested their ability to inhibit the growth of MT-450 cell-derived tumors by using a syngeneic animal model (Figure 3). In preliminary experiments, we observed that compound **2** had no inhibitory effect on tumor growth (data not shown). We therefore focused our experiments on Aristoforin and compound **6**. MT-450 tumors were allowed to become established in vivo for 7 days, then tumor-bearing animals were treated with either hyperforin, Aristoforin, compound **6**, or with PBS/10% DMSO as a solvent control. The effect of the treatments was evaluated by measuring the tumor volume at regular intervals. Aristoforin had an equivalent inhibitory effect on tumor growth as the parental hyperforin, while the hyperforin derivative **6** had no inhibitory effect. After cessation of hyperforin and Aristoforin treatment, tumor growth accelerated in these animals to the same rate as tumors treated with compound **6** and the solvent control (data not shown). No overt signs of toxicity (loss of body weight, lethargy, anemia) were observed for any of the treatments.

In contrast to Aristoforin, hyperforin derivatives **2** and **6** had no antitumor activity in vivo, despite exerting strong proapoptotic and antiproliferative effects in cell-culture experiments. These results suggest poor bioavailability or stability for these compounds in vivo, possibly resulting from enhanced clearance and breakdown in the body. For derivative **2**, its poor solubility (Table 1) could also be a contributing factor. Furthermore, it is interesting to note that lack of the double bonds of the isoprene side chains in compounds **2** and **6** correlates with the distinct decrease of in vivo activity against MT-450 tumors, suggesting that these bonds might contribute to the in vivo effectiveness of the derivatives.

These data establish Aristoforin as a highly stable derivative of hyperforin that possesses enhanced solubility characteristics and retains the antitumor properties of the parental compound in vitro and in vivo. Furthermore, the method for isolation and derivatization of hyperforin into Aristoforin that we present here is rapid and cheap. Our findings therefore provide a strong impetus for further studies to determine the possible clinical utility of Aristoforin in cancer treatment, either in therapy or as a chemopreventive agent. Moreover, as the stimulation of drug metabolism by hyperforin could limit its combination with other anticancer drugs, these studies also give hope that it might be possible to remove its drug metabolism stimulatory activities through chemical modification without affecting its antitumor properties.

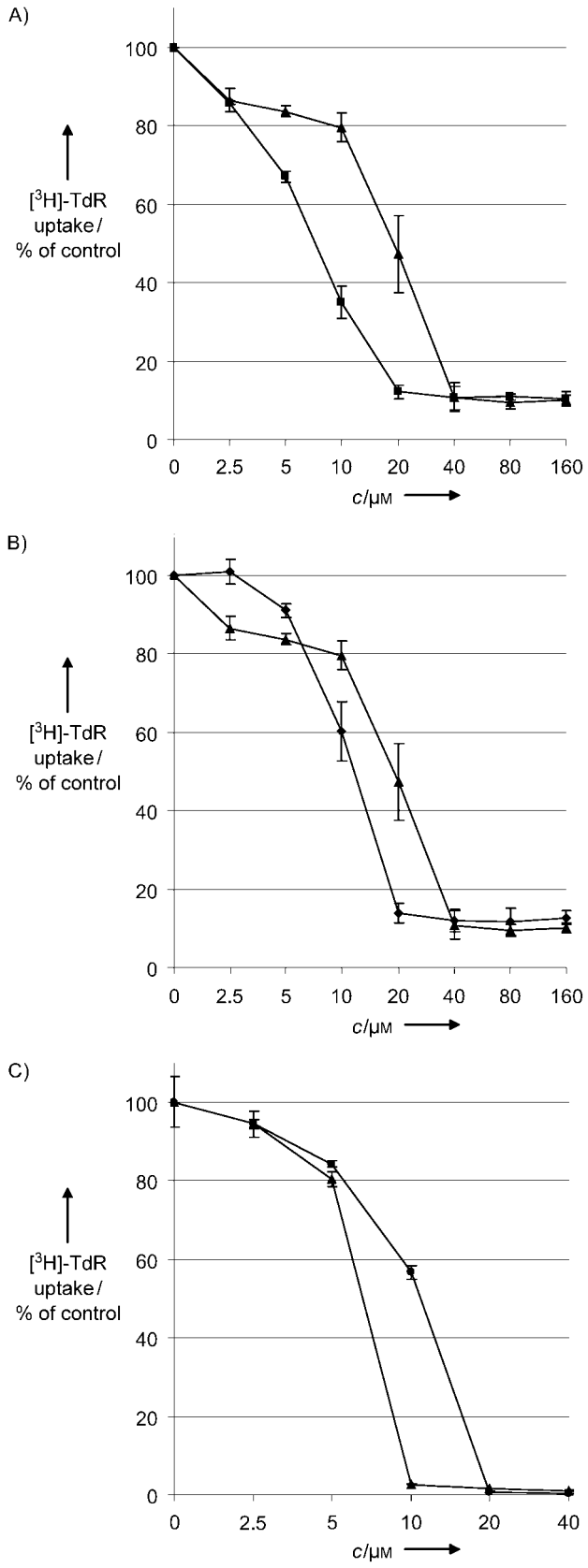


Figure 1. *Inhibition of MT-450 tumor cell proliferation by A) hyperforin (▲) and compound **2** (■), B) hyperforin (▲) and Aristoforin (◆), C) hyperforin (▲) and compound **6** (●); ^{3}H-TdR uptake = incorporation of ^{3}H-thymidine into MT-450 tumor cells as percent of proliferation relative to the solvent-only control; data points are presented as mean and standard error of quadruplicate samples.*

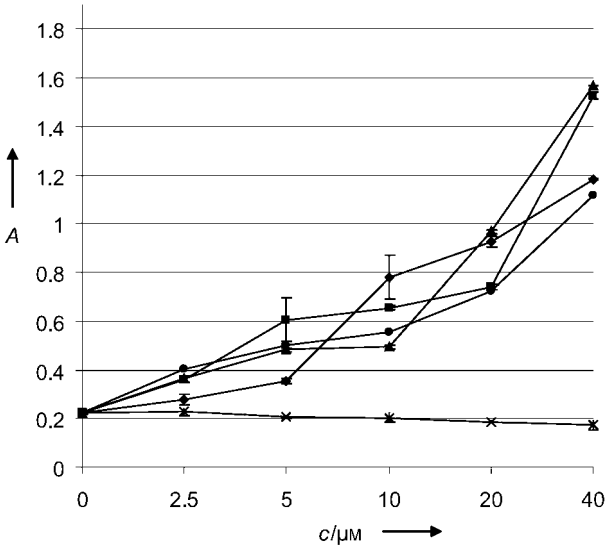


Figure 2. *Induction of MT-450 tumor cell apoptosis by hyperforin (▲), compounds **2** (■), **5** (◆), and **6** (●), and the equivalent amount of solvent (DMSO) as control (×); A = $A_{405nm} - A_{490nm}$; data points are presented as mean and standard error of quadruplicate samples.*

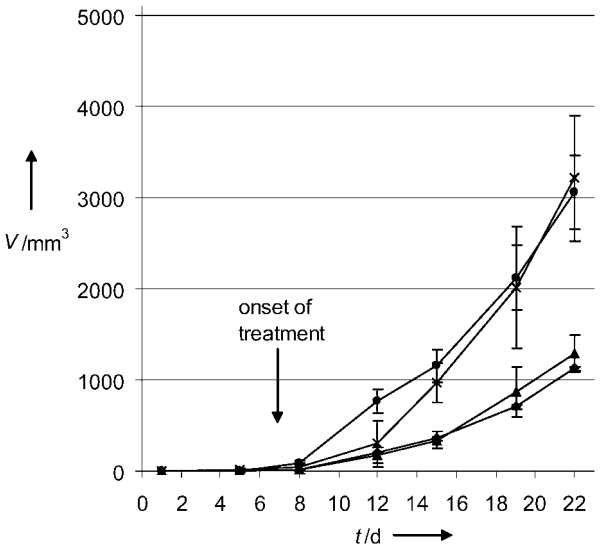


Figure 3. *Inhibition of MT-450 tumor cell growth* in vivo *by hyperforin (▲), Aristoforin **5** (◆), compound **6** (●) and the solvent (PBS/10% DMSO) (×); V = tumor volume, t = time after tumor cell injection; data points are presented as mean and standard error of tumor volumes in eight rats.*

Experimental Section

General methods: Hyperforin was isolated directly from St. John's wort according to Adam et al.[19] Hyperforin and all synthesized compounds were stored at −78 °C under argon atmosphere and light exclusion.

^{1}H and ^{13}C NMR spectra were recorded on Bruker DRX 500 or Bruker DRX 600 NMR spectrometers. The stereochemistries of the synthesized compounds were determined by NOE and 2D NMR spectroscopy. The solvents stated were used as internal standards. High-resolution (HR) mass spectra were obtained with a Finnigan

MAT MS 70 mass spectrometer. Commercially available reagents were used without further purification. In general, reactions were carried out in dry solvents under argon atmosphere and light exclusion, unless otherwise noted. All reactions were monitored by thin-layer chromatography (TLC) carried out on Merck silica gel 60 F_{254} aluminium sheets and viewed with UV light. Flash chromatography was performed on Merck silica gel 60.

MT-450 rat mammary carcinoma cells[20] were cultured in a cell medium containing Dulbecco's modified Eagle medium (DMEM), which was supplemented with 10% fetal calf serum (FCS) and 1% Penicillin/Streptomycin (all from Gibco, Eggenstein, Germany). Cells were incubated in a humidified atmosphere (95% air humidity, 5% CO_2) at 37°C.

17,18,22,23,27,28,32,33-Octahydrohyperforin (2): Hyperforin (75 mg, 140 μmol) was dissolved in methanol (4.0 mL). Palladium on activated charcoal (15 mg) was then added and the suspension was stirred for 2 h at room temperature under an atmosphere of hydrogen (1 bar). After the reaction was complete, the suspension was diluted with methanol (30 mL) and the charcoal was removed by filtration over celite. The solvent was removed under reduced pressure and the residue was purified by column chromatography (*n*-hexane/diethyl ether 5:1) to yield **2** (61 mg, 112 μmol, 80%) as a slightly yellow oil. $R_f = 0.54$ (*n*-hexane/diethyl ether 3:1); ^{1}H NMR (500 MHz, [D$_4$]methanol, 25°C): $\delta = 0.90$ (s, 3H; CH_3, H-14), 1.05 (d, ^3J(H,H) = 6.5 Hz, 3H; CH_3, H-13), 1.10 (d, ^3J(H,H) = 6.5 Hz, 3H; CH_3, H-12), 0.76–1.18 (m, 32H; 8CH_3, 4CH_2, H-19, H-20, H-24, H-25, H-29, H-30, H-34, H-35, H-17, H-22, H-27, H-32), 1.18–1.60 (m, 10H; 4CH, 3CH_2, H-18, H-23, H-28, H-33, H-15, H-16, H-21), 1.62–1.78 (m, 4H; CH_2, 2CH, H-31, H-4, H-5), 1.91–1.97 (m, 1H; CH, H-5′), 2.13–2.21 (m, 1H; CH, H-11), 2.34–2.46 ppm (m, 2H; CH_2, H-26); ^{13}C NMR (125.8 MHz, [D$_4$]methanol, 25°C): $\delta = 14.4$ (CH_3, C-14), 19.9 (CH_3, C-12), 20.1 (CH_2, C-16), 20.7 (CH_3, C-13), 21.4, 21.5, 21.8 (8CH_3, C-19, C-20, C-24, C-25, C-29, C-30, C-34, C-35), 23.1 (CH_2, C-26), 26.3 (CH_2, C-21), 27.7 (CH, C-18), 27.8 (CH, C-33), 27.9 (CH, C-28), 28.6 (CH, C-23), 29.1 (CH_2, C-31), 33.4 (CH_2, C-32), 36.9 (CH_2, C-27), 37.3 (CH_2, C-15), 37.4 (2CH_2, C-17, C-22), 39.7 (CH_2, C-5), 40.6 (CH, C-11), 41.8 (CH, C-4), 48.8 (C_q, C-3), 59.3 (C_q, C-6), 81.3 (C_q, C-2), 121.4 (C_q, C-8), 169.1 (C_q, C-7), 171.4 (C_q, C-9), 207.9 (C=O, C-1), 210.1 ppm (C=O, C-10); HR-MS (EI, 70 eV): *m/z*: calcd for $C_{35}H_{60}O_4$: 544.4492 $[M]^+$; found: 544.4495.

***C*-[(Ethoxycarbonyl)methyl]-hyperforin (3) and *O*-[(Ethoxycarbonyl)methyl]-hyperforin (4):** Hyperforin (305 mg, 568 μmol) was dissolved in acetone (5.0 mL). Potassium carbonate (314 mg, 2272 μmol) and ethyl bromoacetate (63 μL, 568 μmol) were then added and the reaction mixture was stirred for 19 h at room temperature. The suspension was diluted with water (50 mL) and the aqueous phase was extracted with diethyl ether (4×50 mL). The combined organic layers were dried over Na_2SO_4. Afterwards, the solvent was removed in vacuo and the residue was purified by flash chromatography (*n*-hexane/diethyl ether 25:1) to give **3** (100 mg, 161 μmol, 28%) and **4** (171 mg, 275 μmol, 48%) both as slightly yellow oils.

Compound 3: $R_f = 0.76$ (*n*-hexane/diethyl ether 3:1); ^{1}H NMR (500 MHz, [D]chloroform, 25°C): $\delta = 1.01$ (d, ^3J(H,H) = 6.5 Hz, 3H; CH_3, H-13), 1.03 (s, 3H; CH_3, H-14), 1.08–1.14 (m, 1H; CH, H-15), 1.19 (d, ^3J(H,H) = 6.5 Hz, 3H; CH_3, H-12), 1.23 (t, ^3J(H,H) = 7.1 Hz, 3H; OCH_2CH_3), 1.54 (s, 3H; CH_3, H-25), 1.62 (s, 3H; CH_3, H-20), 1.68–1.70 (m, 18H; 6CH_3, H-19, H-24, H-29, H-30, H-34, H-35), 1.71–1.78 (m, 3H; 3CH, H-4, H-5, H-21), 1.91–1.95 (m, 1H; CH, H-16), 2.00–2.05 (m, 1H; CH, H-15′), 2.10–2.15 (m, 2H; 2CH, H-5′, H-21′), 2.21–2.30 (m, 2H; 2CH, H-11, H-16′), 2.62 (d, ^3J(H,H) = 7.5 Hz, 2H; CH_2, H-31), 2.71 (d, ^2J(H,H) = 15.5 Hz, 1H; C*H*HCOOEt), 2.70–2.73 (m, 1H; CH, H-26), 2.77 (d, ^2J(H,H) = 15.5 Hz, 1H; CH*H*COOEt), 2.81–2.83 (m, 1H; CH, H-26′), 4.04–4.08 (m, 1H; OC*H*HCH$_3$), 4.08–4.12 (m, 1H; OCH*H*CH$_3$), 4.88 (br, 1H; CH, H-22), 5.04–5.07 (m, 1H; CH, H-17), 5.13–5.16 (m, 1H; CH, H-27), 5.31–5.34 ppm (m, 1H; CH, H-32); ^{13}C NMR (125.8 MHz, [D]chloroform, 25°C): $\delta = 13.8$ (CH_3, C-14), 14.2 (OCH_2CH_3), 17.9 (CH_3, C-20), 18.1 (2CH_3, C-25, C-35), 18.2 (CH_3, C-30), 20.8 (CH_3, C-13), 22.0 (CH_3, C-12), 25.5 (CH_2, C-16), 25.8 (CH_3, C-19), 26.0 (CH_3, C-24), 26.1 (CH_3, C-34), 26.3 (CH_3, C-29), 28.4 (CH_2, C-21), 30.7 (CH_2, C-31), 32.1 (CH_2, C-26), 37.3 (CH_2, C-15), 41.4 (CH, C-11), 43.6 (CH_2, C-5), 44.1 (*C*H$_2$COOEt), 45.4 (CH, C-4), 55.7 (C_q, C-3), 61.9 (OCH_2CH_3), 64.0 (C_q, C-8), 66.5 (C_q, C-6), 87.9 (C_q, C-2), 118.4 (CH, C-27), 119.5 (CH, C-32), 121.9 (CH, C-22), 124.5 (CH, C-17), 131.7 (C_q, C-18), 133.9 (C_q, C-23), 135.0 (C_q, C-33), 136.7 (C_q, C-28), 169.3 (CH_2*C*OOEt), 205.8 (C=O, C-9), 207.0 (C=O, C-1), 208.5 (C=O, C-10), 208.7 ppm (C=O, C-7); HR-MS (EI, 70 eV): *m/z*: calcd for $C_{39}H_{58}O_6$: 622.4233 $[M]^+$; found: 622.4222.

Compound 4: $R_f = 0.67$ (*n*-hexane/diethyl ether 3:1); ^{1}H NMR (500 MHz, [D]chloroform, 25°C): $\delta = 0.99$ (s, 3H; CH_3, H-14), 1.00 (d, ^3J(H,H) = 6.5 Hz, 3H; CH_3, H-13), 1.10 (d, ^3J(H,H) = 6.5 Hz, 3H; CH_3, H-12), 1.31 (t, ^3J(H,H) = 7.7 Hz, 3H; OCH_2CH_3), 1.36–1.39 (m, 1H; CH, H-15), 1.41–1.45 (m, 1H; CH, H-5), 1.55 (s, 3H; CH_3, H-25), 1.52–1.56 (m, 1H; CH, H-4), 1.58 (s, 3H; CH_3, H-20), 1.64 (s, 3H; CH_3, H-35), 1.66 (s, 9H; 3CH_3, H-19, H-29, H-30), 1.67 (s, 6H; 2CH_3, H-24, H-34), 1.71–1.76 (m, 1H; CH, H-21), 1.87–1.91 (m, 2H; 2CH, H-15′, H-16), 1.92–1.98 (m, 1H; CH, H-11), 2.01–2.04 (m, 1H; CH, H-5′), 2.06–2.11 (m, 2H; 2CH, H-16′, H-21′), 2.53–2.60 (m, 2H; CH_2, H-31), 3.06–3.09 (m, 1H; CH, H-26), 3.14–3.17 (m, 1H; CH, H-26′), 4.23–4.30 (m, 2H; OCH_2CH$_3$), 4.48 (d, ^2J(H,H) = 16.1 Hz, 1H; C*H*HCOOEt), 4.69 (d, ^2J(H,H) = 16.1 Hz, 1H; CH*H*COOEt), 4.93–4.95 (m, 1H; CH, H-22), 5.00–5.03 ppm (m, 3H; 3CH, H-17, H-27, H-32); ^{13}C NMR (125.8 MHz, [D]chloroform, 25°C): $\delta = 13.6$ (CH_3, C-14), 14.3 (OCH_2CH_3), 17.8 (CH_3, C-20), 18.1 (2CH_3, C-25, C-30), 18.2 (CH_3, C-35), 20.5 (CH_3, C-12), 21.4 (CH_3, C-13), 23.8 (CH_2, C-26), 25.1 (CH_2, C-16), 25.7 (CH_3, C-34), 25.8 (CH_3, C-29), 25.9 (CH_3, C-19), 26.1 (CH_3, C-24), 27.2 (CH_2, C-21), 30.0 (CH_2, C-31), 36.7 (CH_2, C-15), 38.6 (CH_2, C-5), 42.9 (CH, C-11), 43.6 (CH, C-4), 49.6 (C_q, C-3), 58.8 (C_q, C-6), 61.7 (OCH_2CH_3), 71.1 (*C*H$_2$COOEt), 84.4 (C_q, C-2), 119.6 (CH, C-32), 121.3 (CH, C-27), 122.6 (CH, C-22), 124.9 (CH, C-17), 128.1 (C_q, C-8), 131.3 (C_q, C-18), 133.5 (C_q, C-23), 133.9 (C_q, C-28), 134.4 (C_q, C-33), 167.7 (CH_2COOEt), 173.0 (C_q, C-7), 194.1 (C=O, C-9), 207.1 (C=O, C-1), 209.0 ppm (C=O, C-10); HR-MS (EI, 70 eV): *m/z*: calcd for $C_{39}H_{58}O_6$: 622.4233 $[M]^+$; found: 622.4251.

***O*-(Carboxymethyl)-hyperforin (Aristoforin; 5):** A solution of **4** (171 mg, 275 μmol) in methanol (5.0 mL) was cooled with an ice/water bath. Then an aqueous NaOH solution (1 M, 1375 μL) was slowly added. The reaction mixture was stirred for 1 h at 0°C and afterwards 15 h at room temperature. The solution was diluted with water (20 mL) and the methanol was removed in vacuo. The remaining aqueous solution was acidified to pH 1 by addition of hydrochloric acid (0.2 M); this resulted in the precipitation of a white solid. The aqueous suspension was extracted four times with diethyl ether and the combined organic layers were dried over Na_2SO_4. The solvent was removed under reduced pressure. Purification of the residue by flash chromatography (diethyl ether/*n*-hexane/acetic acid 6:4:0.03) yielded Aristoforin (**5**, 127 mg, 214 μmol, 78%) as a slightly yellow oil. $R_f = 0.44$ (diethyl ether/*n*-hexane/acetic acid 6:4:0.03); ^{1}H NMR (600 MHz, [D]chloroform, 25°C): $\delta = 1.00$ (s, 3H; CH_3, H-14), 1.01 (d, ^3J(H,H) = 6.5 Hz, 3H; CH_3, H-13), 1.11 (d, ^3J(H,H) = 6.5 Hz, 3H; CH_3, H-12), 1.36–1.40 (m, 1H; CH, H-15), 1.43–1.47 (m, 1H; CH, H-5), 1.55 (s, 3H; CH_3, H-25), 1.54–1.56 (m, 1H; CH, H-4), 1.59 (s, 3H; CH_3, H-20), 1.65 (s, 3H; CH_3, H-

35), 1.66 (s, 9H; $3CH_3$, H-19, H-29, H-30), 1.67 (s, 6H; $2CH_3$, H-24, H-34), 1.71–1.77 (m, 1H; CH, H-21), 1.87–1.91 (m, 2H; 2CH, H-15′, H-16), 1.94–1.97 (m, 1H; CH, H-11), 1.97–2.00 (m, 1H; CH, H-5′), 2.07–2.12 (m, 2H; 2CH, H-16′, H-21′), 2.53–2.60 (m, 2H; CH_2, H-31), 3.09–3.12 (m, 1H; CH, H-26), 3.15–3.19 (m, 1H; CH, H-26′), 4.57 (d, ^2J(H,H) = 16.2 Hz, 1H; C*H*HCOOH), 4.75 (d, ^2J(H,H) = 16.2 Hz, 1H; CH*H*COOH), 4.93–4.95 (m, 1H; CH, H-22), 5.00–5.04 ppm (m, 3H; 3CH, H-17, H-27, H-32); ^{13}C NMR (150.9 MHz, [D]chloroform, 25 °C): δ = 13.6 (CH_3, C-14), 17.8 (CH_3, C-20), 18.0 (CH_3, C-30), 18.1 (CH_3, C-25), 18.2 (CH_3, C-35), 20.5 (CH_3, C-12), 21.4 (CH_3, C-13), 23.8 (CH_2, C-26), 25.1 (CH_2, C-16), 25.7 (CH_3, C-34), 25.8 (CH_3, C-29), 25.9 (CH_3, C-19), 26.0 (CH_3, C-24), 27.1 (CH_2, C-21), 30.0 (CH_2, C-31), 36.7 (CH_2, C-15), 38.6 (CH_2, C-5), 43.0 (CH, C-11), 43.7 (CH, C-4), 49.7 (C_q, C-3), 58.7 (C_q, C-6), 70.2 (CH_2COOH), 84.4 (C_q, C-2), 119.5 (CH, C-32), 121.0 (CH, C-27), 122.5 (CH, C-22), 124.7 (CH, C-17), 128.5 (C_q, C-8), 131.4 (C_q, C-18), 133.7 (C_q, C-23), 134.2 (C_q, C-28), 134.7 (C_q, C-33), 172.3 (C_q, C-7), 172.5 (CH_2*C*OOH), 194.0 (C=O, C-9), 206.8 (C=O, C-1), 208.9 ppm (C=O, C-10); HR-MS (EI, 70 eV): *m/z*: calcd for $C_{37}H_{54}O_6$: 594.3920 $[M]^+$; found: 594.3930.

***O*-(Carboxymethyl)-17,18,22,23,27,28,32,33-octahydrohyperforin (6):** Aristoforin **5** (54 mg, 91 μmol) was dissolved in methanol (3.0 mL). Palladium on activated charcoal (10 mg) was then added and the suspension was stirred for 2 h at room temperature under an atmosphere of hydrogen (1 bar). After the reaction was complete, the suspension was diluted with methanol (20 mL) and the charcoal was removed by filtration over celite. The solvent was removed under reduced pressure and the residue was purified by column chromatography (*n*-hexane/diethyl ether/acetic acid 5:5:0.03) to give **6** (43 mg, 71 μmol, 78%) as a slightly yellow oil. R_f = 0.41 (*n*-hexane/diethyl ether/acetic acid 5:5:0.03); ^{1}H NMR (600 MHz, [D]chloroform, 25 °C): δ = 0.93 (s, 3H; CH_3, H-14), 1.02 (d, ^3J(H,H) = 6.6 Hz, 3H; CH_3, H-13), 1.12 (d, ^3J(H,H) = 6.6 Hz, 3H; CH_3, H-12), 0.78–0.98 (m, 24H; 8CH_3, H-19, H-20, H-24, H-25, H-29, H-30, H-34, H-35), 0.98–1.30 (m, 8H; 4CH_2, H-17, H-22, H-27, H-32), 1.30–1.67 (m, 10H; 4CH, 3CH_2, H-18, H-23, H-28, H-33, H-15, H-16, H-21), 1.67–2.08 (m, 5H; 3CH, CH_2, H-4, H-5, H-5′, H-31), 2.13 (br, 1H; CH, H-11), 2.27–2.65 (m, 2H; CH_2, H-26), 4.59 (d, ^2J(H,H) = 16.2 Hz, 1H; C*H*HCOOH), 4.85 (d, ^2J(H,H) = 16.2 Hz, 1H; CH*H*COOH), 7.80 ppm (br, 1H; COO*H*); ^{13}C NMR (150.9 MHz, [D]chloroform, 25 °C): δ = 14.2 (CH_3, C-14), 20.7 (CH_3, C-12), 21.6 (CH_3, C-13), 22.4, 22.5, 22.6, 22.8, 22.9 (8CH_3, C-19, C-20, C-24, C-25, C-29, C-30, C-34, C-35), 22.7 (CH_2, C-16), 24.0 (CH_2, C-26), 26.3 (CH_2, C-21), 28.0 (CH, C-18), 28.3 (CH, C-33), 28.4 (CH, C-28), 28.9 (CH, C-23), 29.7 (CH_2, C-31), 33.6 (CH_2, C-32), 36.9 (CH_2, C-27), 37.7 (CH_2, C-15), 38.1 (CH_2, C-22), 39.9 (CH_2, C-17), 40.0 (CH_2, C-5), 42.6 (CH, C-11), 43.2 (CH, C-4), 50.6 (C_q, C-3), 58.7 (C_q, C-6), 70.2 (CH_2COOH), 84.8 (C_q, C-2), 129.4 (C_q, C-8), 172.4 (C_q, C-7), 172.8 (CH_2COOH), 194.2 (C=O, C-9), 207.3 (C=O, C-1), 209.0 ppm (C=O, C-10); HR-MS (EI, 70 eV): *m/z*: calcd for $C_{37}H_{62}O_6$: 602.4546 $[M]^+$; found: 602.4553.

Solubility assays: Solubility assays were performed at 20 °C. Hyperforin and its derivatives were dissolved in DMSO and further diluted to different concentrations ranging from 10 μg mL^{-1} to 500 μg mL^{-1} in either H_2O or 100 mM aqueous $NaHCO_3$ solution (pH 8.2). The final concentration of DMSO was 2% for each sample. Every sample was mixed thoroughly and, after 10 min, the solubility of the corresponding compound was assessed visually. A clear solution means that the compound is completely dissolved, a cloudy or opalescent solution means that the compound is not fully dissolved at the corresponding concentration.

Stability assays: Hyperforin and the hyperforin derivatives were dissolved to a concentration of 1 mg mL^{-1} in either DMSO or PBS containing 20% DMSO. The samples were exposed to light at room temperature in a normal air atmosphere for the indicated times. At each time point the chromatographic purity of the samples was measured by HPLC using the validated method SAM 0022–3. For **6**, the stability of the compound in DMSO was assessed by using ^{1}H and ^{13}C NMR spectroscopy methods.

Proliferation assays: Hyperforin and the hyperforin derivatives were dissolved in DMSO and further diluted with cell medium to obtain final concentrations as indicated. The maximal final DMSO concentration was 1%. MT-450 cells were seeded into each well of a 96-well cell culture plate at 10^4 cells per well and incubated at 37 °C for 24 h with the indicated concentrations of hyperforin and hyperforin derivatives. Control samples were incubated with the equivalent amount of solvent alone. Each sample was repeated in quadruplicate. Afterwards ^{3}H-thymidine (Amersham Pharmacia Biotech, Freiburg, Germany) was added to the cells at 1 μCi per well and incubation was resumed at 37 °C for a further 18 h. To analyze the amount of incorporated radioactivity, the cells were harvested onto a glass fiber filter (Wallac Oy, Turku, Finland) with aid of the Harvester 96 cell harvester (Tomtec, Hamden, USA). The filter-immobilized radioactivity was quantified by using scintillation liquid and a MicroBeta TriLux Liquid Scintillation and Luminescence counter (Wallac).

Apoptosis assays: Hyperforin and the hyperforin derivatives were dissolved in DMSO and further diluted with cell medium to obtain final concentrations as indicated. The maximum final DMSO concentration was 1%. MT-450 cells were seeded into each well of a 96-well cell culture plate at 10^4 cells per well and incubated at 37 °C for 24 h with the indicated concentrations of hyperforin and hyperforin derivatives. Control samples were incubated with the equivalent amount of solvent alone. Each sample was repeated in quadruplicate. The proapoptotic effect of the substances was detected by using the Cell Death Detection ELISA^{PLUS} kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. The kit constitutes a photometric enzyme-immunoassay for the qualitative and quantitative in vitro determination of cytoplasmic histone-associated DNA fragments (mono- and oligonucleosomes) after apoptosis. The absorption values measured ($A = A_{405nm} - A_{490nm}$) give a quantitative indication of the induced amount of apoptosis. The higher the absorption value *A*, the higher the induction of apoptosis at the corresponding concentrations of the compounds.

In vivo tumor experiments: MT-450 cells were resuspended in PBS and injected subcutaneously into groups of Wistar Furth rats (8 animals per group, 5×10^5 cells per animal). Drug treatment was initiated 7 days after tumor cell injection. The different groups of animals received 100 μL of hyperforin (**1**), Aristoforin (**5**), or compound **6**, each dissolved in PBS/10% DMSO (1 mg mL^{-1}), equating to a dose of 0.4 mg kg^{-1} of each compound. Furthermore, one group of animals received 100 μL PBS containing 10% DMSO as solvent control. Daily injections of the drugs or control were administered subcutaneously at the site of the tumor cell injection for fifteen days. Tumors were measured with a micrometer calliper every third to fourth day throughout the study. These studies were approved by the local Ethical Review Board.

Acknowledgements

We thank the animal house staff at the Forschungszentrum Karlsruhe, Institut für Toxikologie und Genetik, for their expert help. Furthermore we thank Dr. Lothar Hennig at the Institut für

Organische Chemie, Universität Leipzig, for recording and interpreting the NMR spectra.

Keywords: antitumor agents · drug design · hyperforin · natural products · St. John's wort

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Received: June 10, 2004

