Cytotoxic Activities of Hypocretenolides from *Leontodon hispidus*

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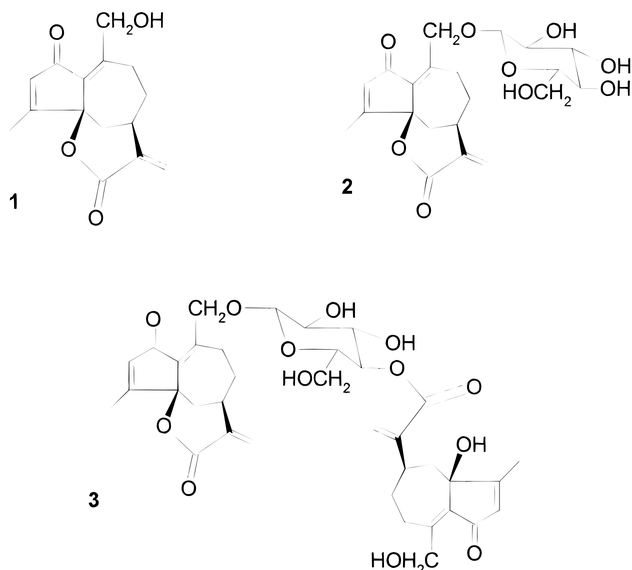
The hypocretenolides are a small group of sesquiterpene lactones with an unusual ring structure and are constituents of several species from the tribe Lactuceae of the family Asteraceae. In the present communication we report on the cytotoxic effects of three hypocretenolides (**1**−**3**) from *Leontodon hispidus* on: (a) eight solid-tumor cell lines (A431, HEP2, MCF7, OVCAR3, SK28, SK37, SW872, ZR75-1), tested by a ^{3}H-thymidine incorporation assay; (b) two different leukemia cell lines (GTB, HL60), measured by a MTT assay; and (c) CD34$^+$ bone-marrow cells, assessed by scoring the number of colonies derived from primitive and late erythroid progenitors (BFU-E and CFU-E) as well as from granulocytic/macrophagic progenitor cells (CFU-GM). The aglycon 14-hydroxyhypocretenolide (**1**) exhibited pronounced activities, although its β-D-glucoside (**2**) showed no activity, even at the highest concentration tested (2 μM). 14-Hydroxyhypocretenolide-β-D-glucoside-4′,14′′-hydroxyhypocretenoate (**3**), the ester of the glucoside esterified with the open-chain form of the aglycon, was the most potently cytotoxic substance and proved to be even more active than the positive-control substance helenalin.

Sesquiterpene lactones are a large class of natural compounds with many types of biological activities. They exhibit cytotoxic and/or antitumor activities; are toxic to vertebrates and invertebrates; have antiphlogistic, antimicrobial, and antiplasmodial effects; and cause allergic contact dermatitis.[1−8] The cytotoxicity of several sesquiterpene lactones has been evaluated thus far, but guaianolides of the hypocretenolide-type, like compounds **1**−**3**, have not yet been tested. Hypocretenolides occur in members of the tribe Lactuceae of the family Asteraceae and differ from other guaianolides by an unusual ring closure of the lactone moiety.



The biological activities mentioned require a Michael addition site in the molecule; thus, the presence of an α-methylene-γ-lactone moiety seems to be a prerequisite for all sesquiterpene lactones to have cytotoxic effects.[1] Further functional groups such as α,β-unsaturated carbonyl functions, conjugated esters, epoxides, or additional alkylating groups may enhance the cytotoxic activity.[1] Because hypocretenolides **1**−**3** isolated from *Leontodon hispidus* L. fulfill these essential structural requirements, we tested these compounds for cytotoxicity on various leukemic and solid-tumor cell lines.[9] In addition, the effects of the most potent cytotoxic agent, substance **3**, were tested in a clonogenic assay on immature human stem cells.

Results and Discussion

We investigated the cytotoxic effects of three hypocretenolides isolated from *L. hispidus* on a panel of human leukemic and solid-tumor cell lines that were available to our laboratory. The leukemic cell lines used represent a myelogenic (HL60) and a monocytic (GTB) clone. Some solid-tumor cell lines originated from melanoma (SK28, SK37) and epithelial cancer (A431, HEP2), which are known to be resistant to alkylating agents. The liposarcoma line (SW872), which is known to be sensitive to such alkylating agents as cyclophosphamide, was also used. In addition, human breast cancer (MCF7, ZR75-1) and ovarian adenocarcinoma cell (OVCAR3) lines were tested, which are known to be sensitive to anthracyclines.[10] Clonogenic assays of CD34$^+$ progenitors from normal human donors were performed to examine the antiproliferative side effects of the various substances tested.

The sesquiterpenoids 14-hydroxyhypocretenolide (**1**) and 14-hydroxyhypocretenolide-β-D-glucopyranoside-4′,14′′-hydroxyhypocretenoate (**3**) from *L. hispidus*, as well as the positive control helenalin, exhibited pronounced dose-dependent effects on the GTB and HL60 cell lines using an MTT [3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide] assay (Table 1, Figure 1). Compounds **1**−**3** and helenalin were tested in concentrations ranging from 0.01 to 2.00 μM and with an incubation time of 5 h. Compound **3** was by far the most active substance with IC_{50} values of 0.10 μM (HL60) and 0.07 μM (GTB), respectively. Helenalin and compound **1** had significantly higher IC_{50} values, up to four times higher than the corresponding values of compound **3**. The glycoside of **1**,

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Table 1. IC_{50} Values (μM) of Compounds **1**−**3** in Comparison with Helenalin in Leukemic Cell Lines as Determined by an MTT Assay (incubation time 5 h)[a]

cell line	1	2	3	helenalin
GTB	0.26 ± 0.04	*b*	0.07 ± 0.01[c]	0.27 ± 0.03
HL60	0.26 ± 0.04	*b*	0.10 ± 0.01[c]	0.32 ± 0.05

[a] Determined graphically from dose−activity curves, curves not shown. [b] Showed no activity at the concentration range investigated. [c] Significantly more active than reference substance helenalin ($p < 0.05$).

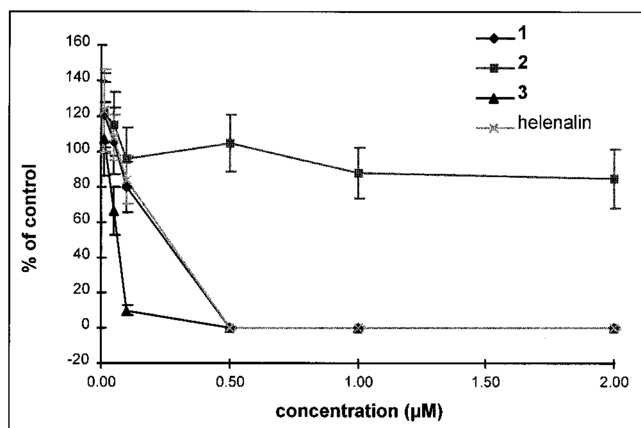

Figure 1. Inhibitory effects of hypocretenolides **1**−**3** and helenalin on GTB cells measured by the MTT assay.

14-hydroxyhypocretenolide-β-D-glucoside (**2**), showed no measurable cytotoxic effects up to a concentration of 2.00 μM.

In the ^{3}H-thymidine incorporation assay, as in the MTT assay for the leukemic cells, helenalin and compounds **1** and **3** showed marked cytotoxic activities for all cell lines. In contrast, substance **2** was inactive at each concentration (0.25, 0.50, 1.00, and 2.00 μM) tested. The dose-dependent activities of helenalin and compounds **1** and **3** were in the μM range with varying IC_{50} values for the different cell lines. Different incubation times (24, 48, and 72 h) hardly influenced the cytotoxic effect, as shown in Table 2. In Figure 2 it is clearly shown that HEP2 cells were inhibited in a dose-dependent manner by helenalin and compounds **1** and **3** to a similar extent, and this held true also for the other cell lines tested (data not shown). However, on comparison of the IC_{50} values of the substances tested, compound **1** was significantly less active than helenalin in all cell lines except ZR75-1. In contrast, compound **3** was significantly more active than helenalin in this cell line and also in the MCF7 and OVCAR3 cell lines. In the remaining cell lines, the activity of compound **3** did not differ significantly from the activity of helenalin.

To determine whether substance **3** could inhibit CSF (colony stimulating factor)- and rhEPO (recombinant human erythropoietin)-stimulated proliferation of immature hematopoetic progenitor cells, CD34$^+$ cells were cultured with agar-stimulated human leukocyte-conditioned medium, rhEPO, and varying concentrations of substance **3**. As shown in Figure 3, growth factor-induced colony formation derived from granulomacrophagic (CFU-GM) and erythroid [BFU-E (burst-forming unit erythroid) and CFU-E (colony-forming unit erythroid)] progenitors were inhibited by substance **3** in a dose-dependent manner. There was no significant difference in the sensitivity of the various progenitor cells to substance **3**. Thus, CFU-GM, BFU-E, and CFU-E colony formation was inhibited up to 60% at a concentration of 0.5 μM by substance **3** (Figure 3). The complete inhibition of CFU-E and BFU-E was obtained in all experiments at 1 μM of substance **3**, whereas for the IC_{100} of CFU-E, a concentration of 2 μM was required.

Table 2. IC_{50} Values (μM) of Compounds **1**−**3** in Comparison with Helenalin in Solid-Tumor Cell Lines Determined by a ^{3}H-Thymidine Incorporation Assay at Various Incubation Times[a]

cell line	incubation time (h)	1	2	3	helenalin
A431	24	1.5 ± 0.6[b]	*c*	0.7 ± 0.4	0.9 ± 0.3
A431	48	1.3 ± 0.4[b]	*c*	0.7 ± 0.3	0.9 ± 0.1
A431	72	0.9 ± 0.2	*c*	0.5 ± 0.1[d]	0.8 ± 0.1
HEP2	24	1.5 ± 0.3[b]	*c*	0.8 ± 0.1	0.9 ± 0.1
HEP2	48	1.3 ± 0.4[b]	*c*	0.7 ± 0.3	0.9 ± 0.3
HEP2	72	1.1 ± 0.1[b]	*c*	0.6 ± 0.2	0.8 ± 0.1
MCF7	24	0.8 ± 0.2	*c*	0.5 ± 0.2[d]	0.8 ± 0.2
MCF7	48	0.7 ± 0.1[b]	*c*	0.3 ± 0.1[d]	0.5 ± 0.2
MCF7	72	1.0 ± 0.2[b]	*c*	0.4 ± 0.1[d]	0.7 ± 0.1
OVCAR3	24	1.9 ± 0.4[b]	*c*	0.9 ± 0.2[d]	1.4 ± 0.3
OVCAR3	48	2.1 ± 0.3[b]	*c*	1.0 ± 0.2[d]	1.6 ± 0.2
OVCAR3	72	1.8 ± 0.2	*c*	1.0 ± 0.1[d]	1.7 ± 0.1
SK28	24	2.8 ± 0.7[b]	*c*	1.3 ± 0.4	1.3 ± 0.3
SK28	48	2.6 ± 1.1[b]	*c*	0.9 ± 0.5	0.9 ± 0.3
SK28	72	1.2 ± 0.6[b]	*c*	0.4 ± 0.1	0.5 ± 0.2
SK37	24	3.2 ± 0.4[b]	*c*	1.3 ± 0.4	1.3 ± 0.3
SK37	48	2.4 ± 0.6[b]	*c*	0.9 ± 0.1[d]	1.2 ± 0.2
SK37	72	1.8 ± 0.9[b]	*c*	0.7 ± 0.2	0.7 ± 0.2
SW872	24	3.1 ± 0.4[b]	*c*	1.5 ± 0.2	1.4 ± 0.3
SW872	48	2.2 ± 1.1[b]	*c*	1.4 ± 0.3	1.2 ± 0.3
SW872	72	2.8 ± 0.2[b]	*c*	1.4 ± 0.3	1.2 ± 0.2
ZR75-1	24	0.6 ± 0.1	*c*	0.3 ± 0.1[d]	0.7 ± 0.2
ZR75-1	48	0.9 ± 0.2	*c*	0.3 ± 0.1[d]	0.8 ± 0.2
ZR75-1	72	1.0 ± 0.1	*c*	0.5 ± 0.1[d]	1.1 ± 0.1

[a] Determined graphically from dose−activity curves; curves not shown with the exception of HEP2 (Figure 2). [b] Significantly less active than reference substance helenalin ($p < 0.05$). [c] Showed no activity in the investigated concentration range. [d] Significantly more active than reference substance helenalin ($p < 0.05$).

To our knowledge this is the first report comparing the cytotoxic activities of various hypocretenolides (**1**−**3**) to the well-known activity of helenalin against human leukemic and solid-tumor cell lines. Our results reveal that in the ^{3}H-thymidine and MTT assays, only the more nonpolar 14-hydroxyhypocretenolides (**1** and **3**) from *L. hispidus* exhibited pronounced cytotoxic activities, while compound **2**, the most polar of the investigated substances was inactive. The IC_{50} values in the leukemic cell lines were between < 0.1 and 0.4 μM, whereas IC_{50} values for the solid tumor cell lines were between 0.3 and 3.2 μM. Thus, the leukemic cell lines (GTB, HL60) were, on the whole, significantly more sensitive ($p < 0.05$) to the active sesquiterpene lactones than the solid-tumor cell lines (A431, HEP2, MCF7, OVCAR3, SK28, SK37, SW872, and ZR75-1). When comparing the activity of the hypocretenolides with that of helenalin, only substance **3** was significantly more active against the leukemic cell lines and some of the solid-tumor cell lines (MCF7, OVCAR3, ZR75-1). Substance **1**, however, displayed less cytotoxicity than helenalin in all human solid tumor cell lines and exhibited an antileukemic activity similar to that of helenalin (Table 2).

Previous structure−activity-relationship investigations of sesquiterpene lactones have revealed that irreversible Michael-type additions on biological nucleophiles are one of the main types of pharmacological actions of the cytotoxic representatives.[1,11] In this respect, the presented results are in agreement with earlier published work. However, it seems that, besides the reactive centers, the polarity of the compounds also plays a major role in cytotoxicity.[11,12] Thus, the less polar substances **1** and **3** showed strong biological activities, whereas the more polar compound **2** was absolutely inactive under the test conditions used in this investigation.

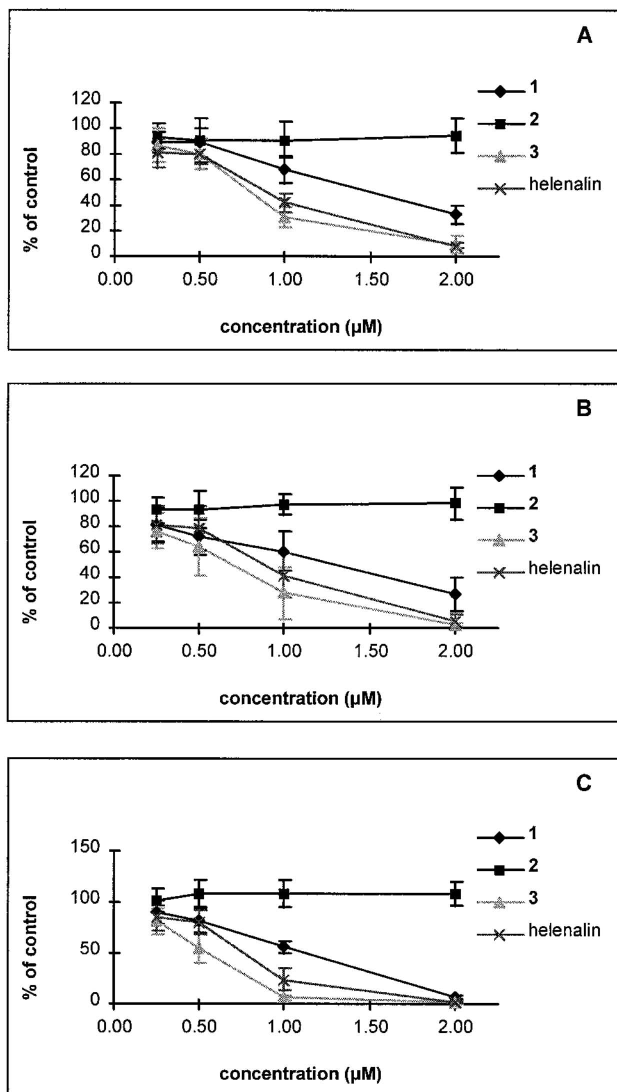

Figure 2. Inhibitory effects of hypocretenolides **1**–**3** and helenalin on ^{3}H-thymidine incorporation of HEP2 cells after incubation for 24h (**A**), 48h (**B**), and 72h (**C**).

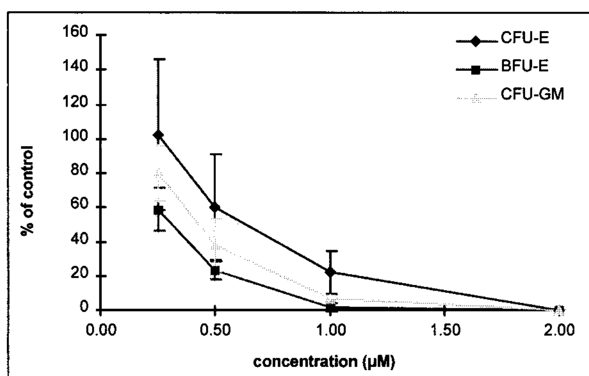

Figure 3. Influence of compound **3** on different bone marrow-derived cell types.

The more pronounced activity of substance **3** when compared to substance **1** is probably related to the presence of a higher number of active sites in the molecule. This hypothesis is also in accordance with the observation that compound **3** shows stronger antileukemic and antitumor activities than helenalin, with helenalin and compound **1** having only one exocyclic double bond and one cyclopentenone moiety, and substance **3** having four possible active centers (Figure 1). The difference in activity between helenalin and compound **1** remains unexplained; the different carbon skeletons and the positions of OH groups may play a role.

In addition, we report here for the first time that substance **3** exhibited dose-dependent effects on erythroid and myeloid progenitor cells. However, there was no significant difference in the sensitivity of immature (CFU-GM, BFU-E) and mature progenitor cells (CFU-E) (Figure 3). Nor could significant differences between the sensitivities of human stem cells and of leukemic and solid-tumor cell lines to substance **3** be demonstrated. This lack of selectivity toward the tumor cell lines investigated, together with the cytotoxicity of **3**, indicates the need for further investigations into in vivo models such as L-1210 leukemia and B16 melanoma mice, before its possible employment as an anticancer agent.

Experimental Section

Materials. Helenalin, isolated from *Psilostrophe cooperi* (A. Gray) Greene, was obtained as a kind gift from Dr. E. Rodriguez (Cornell University, Ithaca, NY). MTT was purchased from Sigma (Vienna, Austria).

Isolation of Hypocretenolides (1–3). 14-Hydroxyhypocretenolide (**1**); 14-hydroxyhypocretenolide-β-D-glucopyranoside (**2**), and 14-hydroxyhypocretenolide-β-D-glucopyranoside-4′,14″-hydroxyhypocretenoate (**3**) were isolated from the CH_2Cl_2 extract of air-dried entire plants of *L. hispidus* by repeated Si gel column chromatography and medium-pressure column chromatography as described previously.[9] The plant material was collected in the vicinity of Innsbruck (Tyrol, Austria); a voucher specimen is deposited at the Institute of Pharmacognosy, University of Innsbruck.

Preparation of Stock Solutions. From each test compound, stock solutions in DMSO with a concentration of 1.00 M were made. Before the actual tests these solutions were prediluted 1:25 with the respective culture media and subsequently given to the test cultures. This resulted in a final DMSO concentration of 0.1%. In a series of preliminary experiments we determined that this concentration exhibits no cytotoxicity on the cell lines tested.

Cell Lines. GTB and HL60, A431, HEP2, MCF7, OVCAR3, SK28, SK37, SW872, and ZR75-1, and bone marrow CD34$^+$ cells were studied. The cell lines were grown in 10% fetal calf serum and RPMI 1640 (PAA Laboratories GmbH, Linz, Austria) supplemented with 2 mmol/L glutamine, 100 U/mL penicillin G, and 100 μg/mL streptomycin (Gibco BRL, Life Technologies, Vienna, Austria).

GTB was established from malignant cells from the pleural effusion of a 37-year-old Caucasian male with diffuse histeocytic lymphoma. It is one of only a few human cell lines still expressing many of the monocytic-like characteristics exhibited by cells of histeocytic origin. HL60 is a promyelocytic cell line derived from peripheral blood leukocytes obtained from a 36-year-old female Caucasian with acute promyelocytic leukemia.

A431 is a human epidermis carcinoma cell line obtained from an 85-year-old female patient. HEP2 is a squamous human carcinoma cell line obtained from the larynx of a 56-year-old male Caucasian. MCF7 is a human mammary gland adenocarcinoma cell line obtained from the pleural effusion of a 69-year-old female Caucasian. OVCAR3 is an ovarian adenocarcinoma cell line from a 60-year-old female Caucasian. SK28 is a human melanoma cell line obtained from a 51-year-old male. SK37 is a human melanoma cell line kindly provided by Memorial Sloan–Kettering Institute (New York). SW872 is a liposarcoma cell line obtained from a 36-year-old male Caucasian. ZR75-1 is an mammary gland ductal carcinoma cell line obtained from a 63-year-old female Caucasian.

After obtaining informed consent, CD34$^+$ cells were isolated from normal bone marrow from donors undergoing hematological assessment. For isolation, direct antibody binding to

immunomagnetic beads was used as previously described.[13] In short, mononuclear cells (MNC) were incubated with Dynabeads (Dynal CD34 Progenitor Cell Selection System, #113.02, Dynal AS, Oslo, Norway) coated with the $CD34^+$ antibody (BIC35) for 45 min with constant rotation. The rosetted $CD34^+$ cells were washed by placing the tube on a magnet, and the nonrosetting $CD34^+$ cells were removed by aspiration. This washing procedure was repeated at least seven times. The $CD34^+$ cells were released from the beads by incubation time for 45 to 60 with Detachabead (Dynal AS, Oslo, Norway) at a dilution of 1:10, and the beads were washed several times to collect residual cells. This positive population was labeled with an HPCA-2 $CD34^+$ antibody (Becton Dickinson, Vienna, Austria) and analyzed for purity by fluorescence-activated cell sorter analysis.

Suspension Culture. Suspensions of 200-μL aliquots containing 0.5×10^6 cells/mL medium (RPMI) were dispensed into 96-well flat-bottomed microtiter plates along with the test compounds at concentrations of 0.01, 0.05, 0.10, 0.50, 1.00, and 2.00 μM, respectively. Cells were incubated at 37 °C in a completely humidified atmosphere with 5% CO_2. In the experiment three replicate wells were used at each point, and experiments were performed in triplicate on different days ($n = 9$). Plates were incubated for 7 days. After incubation the MTT assay was performed.

MTT Assay. The MTT assay is a widely employed viability test and uses the potential of living cells to transform soluble MTT into an insoluble purple formazan precipitate, which can be measured photometrically.[14] In short, viable cells convert a soluble tetrazolium salt [3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide] (MTT) into an insoluble formazan precipitate. The resulting purple formazan crystals are dissolved in DMSO, and the optical density of the solution is measured on a multiwell spectrophotometer at 550 and 630 nm (ELISA plate reader). Decreased cell viability due to cytotoxic substances can be detected and quantified by the reduced optical densities of the respective solutions.

^{3}H-Thymidine Incorporation Assay. To determine the cytotoxic effects of the test compounds on adherent tumor cells, a ^{3}H-thymidine assay was performed. In this assay the cytostatic effect of the test substances is based on a decrease of ^{3}H-thymidine incorporation into the cells. In brief, a 100-μL aliquot of the cell suspension (1×10^6 cells/mL) was placed into each well of a microtiter plate and layered by 100 μL of medium containing various concentrations of substances.

A concentration range between 0.25 and 4.00 μM was tested. Controls contained 100 μL of pure medium instead of the test substances. Plates were then incubated at 37 °C, in an atmosphere of 5% CO_2 and high humidity for 24, 48, and 72 h, respectively. All cultures were then pulsed for 24 h with 0.5 mCi of ^{3}H-thymidine per well. The samples were collected on glass filter paper using a multiple automated harvester. The filters were dried at 55 °C and then transferred to scintillation vials containing 4 mL of scintillation fluid. Radioactivity was measured in a liquid scintillation counter.[15] As for the MTT assay, three replicate wells were used at each point, and experiments were performed in triplicate on different days ($n = 9$).

Clonal Progenitor Assays. Erythropoietic (CFU-E, BFU-E) and granulopoietic (CFU-GM) progenitors were assayed as previously described.[16] Briefly, $CD34^+$ cells were plated at a final concentration of 2×10^3 cells/dish (1.1 mL of culture medium per dish) in Isocove's medium with 0.8% methylcellulose containing 30% fetal calf serum, 10% bovine serum albumin, 1% 2-mercaptoethanol (1×10^{-4} M), and 200 mM L-glutamine, using 3 U/mL recombinant human erythropoietin (rhEPO; Cilag, Vienna, Austria) and 10% of agar-stimulated human leukocyte-conditioned medium as stimulants. Compound **3** was added in the concentration range of 0.25 to 2.00 μM (final concentrations). Each assay was set up in duplicate and repeated once ($n = 4$).

Colonies derived from late-stage erythroid progenitor cells (1 to 2 clusters) (CFU-E) and small erythroid bursts thought to be derived from mature subclasses of BFU-E (3 to 8 clusters) were scored under an inverted microscope after 12 days of incubation. Colonies derived from primitive subclasses of BFU-E ($\geq$9 clusters) as well as colonies derived from granulocytic/macrophagic progenitor cells (CFU-GM) containing more than 50 cells were scored in the same assay dishes after 18 days of incubation.

Statistical Analysis. Significant differences between experiment groups were assessed by applying the Student's two-tailed *t*-test. IC_{50} values were obtained graphically from the dose-activity curves, which have been drawn separately for each experiment (nine for each compound of the MTT and the ^{3}H-thymidine incorporation assay and four for the clonal progenitor assay).

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