Hypoestoxide, a Natural Nonmutagenic Diterpenoid with Antiangiogenic and Antitumor Activity: Possible Mechanisms of Action


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ABSTRACT

We have shown previously that hypoestoxide (HE), a natural diterpenoid [a bicyclo (9, 3, 1) pentadecane], is a potent nonsteroidal antiinflammatory drug. In this report, we demonstrate that HE also inhibits the growth of a variety of human and murine tumor cell lines in vitro at concentrations ranging from 0.3 to 10 μM and was inactive as a mutagen in the Ames test. HE exhibited highly potent (0.3–10 mg/kg dose ranges) activities against B16 melanoma growth in C57BL/6 mice and P388D1 leukemia in C57BL/6 × DBA/2 F1 mice, respectively. At a low maximal effective dose of 5 mg/kg, HE induced significant in vivo antitumor activities that were better than or comparable with most of the standard chemotherapeutic antiangiogenic agents tested: cortisone acetate, vincristine, bleomycin, Adriamycin, 5-fluorouracil, cyclophosphamide, and etoposide. All of the agents, except vincristine, had much higher maximal effective doses than HE. HE arrested the growth of human Burkitt lymphoma CA46 cells and HeLa (cervical epitheloid carcinoma) cells in the G2-M phase of the cell cycle, which was caused by interference, either direct or indirect, with actin assembly. Thus, the cell cycle arrest occurred at cytokinesis, as demonstrated by an increase in the number of binucleate cells. Moreover, HE inhibited vascular endothelial growth factor-induced cell proliferation in vitro, with an IC50 of 28.6 μM, and it significantly inhibited basic fibroblast growth factor-induced angiogenesis on the chick chorioallantoic membrane, with an IC50 of 10 μM. Furthermore, HE inhibited endothelial cell migration on vitronectin, collagen, and fibronectin. Besides its activity as a nonsteroidal anti-inflammatory drug, HE also has promise for the chemotherapy of cancer.

INTRODUCTION

There has been a continuing search for compounds useful in the prevention or treatment of cancer, especially for agents with reduced toxicity or activity against drug-resistant tumors (1, 2). Toward this goal, we report here the antitumor and biological activities of HE,3 a novel diterpene, isolated from the shrub HR (Acanthaceae; Refs. 3–5; Fig. 1). The leaf extracts of HR are used for medicinal purposes in many parts of Nigeria (6). We recently described anti-inflammatory effects of HE, and these effects derived in part from the ability of HE to inhibit nuclear factor-κB activation through direct inhibition of IkB kinase activity (7).

Nonsteroidal anti-inflammatory drugs have long been associated with tumor chemoprevention and inhibition of the growth of established tumors (8, 9). These properties may result from inhibition of nuclear factor-κB/rel A activity, causing inhibition of angiogenesis through suppression of the expression of VEGF (10). Consequently, we decided to evaluate HE for potential antitumor, antiangiogenic, and anti-VEGF activities. Although angiogenesis is critical in normal physiological processes (11), it also is required for tumor growth and other pathological conditions (12, 13). The complexity of the angiogenic process provides a number of potential targets for therapy. Many positive regulators, including VEGF, have been correlated with increased vascularity of tumors, poor prognosis for patient survival, and progressive tumor growth (14–18).

A number of anticancer agents have been shown to be antiangiogenic in an in vivo model of tumor angiogenesis (19, 20). However, the resistance of slow-growing tumors to antiangiogenic drugs (19), coupled with the side effects of standard anticancer drugs (21), make it desirable to discover well tolerated angiogenesis inhibitors with a wide effective dose range. In this study, we demonstrate that HE significantly inhibits tumor growth in vitro and in vivo, VEGF-induced cell proliferation in vitro, and angiogenesis in ovo.

Furthermore, treatment with HE induced ≥50% block in the G2-M phase of the CA46 Burkitt lymphoma line. The increase in G2-M cells caused by HE treatment appears to result from inhibition of cytokinesis through interference with actin assembly. After HE treatment, binucleate cells increase in number, as is commonly observed with agents that interfere with actin polymerization (22, 23). Several studies have shown that the organization of the actin cytoskeleton is essential for a variety of cellular processes, including cell cycle progression, cytokinesis, tumor growth, cell movement, and capillary cell growth during angiogenesis (24–26). Thus, our results suggest certain mechanisms of action of HE and, perhaps, of structurally similar compounds and indicate its potential usefulness as an antitumor and antiangiogenic agent.

MATERIALS AND METHODS

Materials. HE was prepared as described previously (3–5, 7). Recombinant human IFN-α/β was obtained from BioSource International (Camarillo, CA). Cell lines were obtained as reported previously (27), except that PtK2 cells, Burkitt lymphoma CA46 cells, and murine cell lines were obtained from the American Type Tissue Collection (Manassas, VA). Pathogen-free C57BL/6 mice, 6–8 weeks of age, were purchased from Charles River Laboratories (Wilmington, MA). Purified bovine brain tubulin was prepared as described previously (28). Purified rabbit muscle actin was obtained from Cytoskeleton. Lab-Tek II chambered slides were from Nalge Nunc International, and a fluorescent phallolidin derivative (phallolidin coupled to Alexa Fluor 488) and Antifade Mounting Solution were from Molecular Probes. DAPI, propidium iodide, RNase A, cytochalasin B, and a monoclonal antibody to β-tubulin conjugated to the chromophore Cy3 were from Sigma Chemical Co. HBSS, HEPES-buffered M199 medium, and other culture media were from Irvine Scientific (Irvine, CA). Ten-day-old chicken eggs were from McIntyre Poultry (Ramona, CA). VEGF and bFGF were from Genzyme, Inc. (Cambridge, MA). HUVECs were maintained in M199 medium containing sodium bicarbonate, HEPES, heparin, endothelial cell growth supplement

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4 Present address: SurroMed, Inc., 2375 Garcia Avenue, Mountainview, CA, 94030.

5 The abbreviations used are: HE, hypoestoxide; HR, Hypoestes rosea dried leaf powder; VEGF, vascular endothelial growth factor; DAPI, 4,6-diamidino-2-phenylindole; bFGF, basic fibroblast growth factor; HUVEC, human umbilical vein endothelial cell; FBS, fetal bovine serum; CA, cortisone acetate; VCR, vincristine; BLEO, bleomycin; ADR, Adriamycin; 5-FU, 5-fluoracil; CY, cyclophosphamide; ETP, etoposide; CAM, chorioallantoic membrane; MED, maximal effective dose; MST, mean survival time; % ILS, percentage of increased life span.
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Fig. 1. A bicyclic structure of HE which features two epoxide moieties, an α/β unsaturated ketone, and an acetyl ester function. B. HEA, the deacetylated form of HE.

Methods. The Ames test (29) and VEGF-induced cell proliferation assays (30) were performed commercially at MDS Panlabs Taiwan, Ltd. The in ovo chick CAM assay was performed as described by Kim et al. (31). CAMs were harvested on the fourth day of stimulation by fixation with a drop of 3% paraformaldehyde in PBS before excision of the stimulated area. Blood vessel branch points in the 5-mm disk were counted at 10 under fiber optic illumination in a blinded fashion. At least 10 embryos were used per treatment group. Each experiment was performed a minimum of three times.

Cell migration assays were performed as described previously (31). Cells were allowed to migrate from the upper to the lower chamber for 20 min at 37°C. Each experiment was performed in triplicate, with triplicate samples/condition.

Cell adhesion assays were performed as described previously (31). Cells (5 × 10^4) in 25 μl/ml of an anti-α,β function blocking antibody (JBS5), 25 μg/ml of an anti-α,β function blocking antibody (LM609), 25 μg/ml of an anti-β function blocking antibody (P4,10), or 0–100 μM HE in adhesion buffer were allowed to adhere to dishes for 20 min at 37°C. Each experiment was performed in triplicate, with triplicate samples/condition.

Cell lines were grown in appropriate medium containing 10% FBS and 1% t-glutamine. Cell lines that grow as monolayers were passaged, trypsinized, and harvested for experiments before reaching confluence. Cells that grow as suspension cultures were also passaged, harvested, and washed in culture medium before they were used for experiments. HUVECs and other normal epithelial cells were prepared by a method described previously (31) and grown in medium 199 containing 2.2 mg/ml of NaHCO₃ supplemented with 20% heat-inactivated FBS, endothelial cell growth supplement (150 μg/ml), penicillin (100 μg/ml), and streptomycin (100 μg/ml). The cells were grown in 6-well plates at 37°C under 5% CO₂, and the medium was replaced on the first day after seeding and every 2 days thereafter. Confluent primary cultured cell monolayers were used 4–5 days after the cells were seeded.

Examination of HE for potential cytotoxicity against various normal human cells was determined by a colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay after growth for 72 h. Cytotoxicity or growth-inhibitory effect of HE against three human cell lines were determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, flow cytometric, and sulfonfrodamine B protein assays. The sulfonfrodamine B assay was performed by the National Cancer Institute Drug screen on 23 of these lines.

For in vivo drug toxicity evaluation, 40 C57BL/6 female mice, 8 weeks of age, were divided into eight groups of 5 mice/group. Each group (except vehicle control) was treated i.p. with various doses of HE in 0.5 ml (0.25 ml × 2) of 2% Tween 80/PBS (0, 1.5, 15, 75, 100, 250, 500, and 750 mg/kg, respectively). All mice were observed over an 8-day period for acute toxicity and maintained for 3 months for further observation for deaths or any untoward effects. A similar experiment was conducted using p.o. HE administration.

The B16F, mouse melanoma line was used for the study of experimental metastasis (32). The cells were grown in DMEM containing 10% FBS and 1% t-glutamine. The cells were passaged several times and harvested by 15 min of trypsinization (0.25% trypsin-EDTA) before reaching confluence. An aliquot of 1 × 10^6 melanoma cells was injected into the lateral tail vein of each of 40 female C57BL/6 mice, 7 weeks of age. Before treatment, mice were randomized into eight groups of 5 mice/group. The negative vehicle control group was gavaged with 0.2 ml of 2% Tween 80/PBS. Groups 2–7 were treated p.o. with various doses of HE (0.5–100 mg/kg) in 2% Tween 80/PBS. Finally, a positive drug control group received an oral dose of 100 units of recombinant human IFN-α/β, suspended in 3 mg/ml BSA in 2% Tween 80/PBS. Drugs and vehicle in 0.2 ml volumes were administered once daily (beginning 1 h after tumor inoculation) for 10 days. Seventeen days after tumor inoculation, the mice were killed by cervical dislocation, and their lungs were removed and fixed in 10% formaldehyde. The number of surface melanoma colonies were counted.

At the conclusion of the foregoing experiment, a comparative study was conducted to compare HE with seven standard antiangiogenic drugs at equipotential doses (HE, 5 mg/kg; CY, 50 mg/kg; BEOLE, 50 mg/kg; ADR, 7.5 mg/kg; 5-FU, 50 mg/kg; CY, 100 mg/kg; and ETP, 20 mg/kg). A MED of 5 mg/kg was chosen for HE on the basis of results shown in Table 1. The MEDs of the other drugs were based on published reports (19).

Female C57BL/6 mice, 6–8 weeks of age, were used to study inhibition of tumor growth in vivo. The mice were maintained according to institutional regulations in facilities approved by the American Association for Accreditation of Laboratory Animal Care in accordance with current regulations and standards of the United States Department of Agriculture.

A suspension of 1 × 10^5 viable B16F, mouse melanoma cells as described above was administered s.c. into the left flanks of 45 mice. When the tumors had developed to about 15–20 mm³ in volume, the mice were randomized into nine groups that received the following treatments: group 1, i.p. injection of 0.2 ml of 2% Tween 80/PBS; group 2–8, various doses of HE (0.3125–100 mg/kg) suspended in 0.2 ml of 2% Tween 80/PBS; and group 9, 1000 units of recombinant human IFN-α/β suspended in 0.2 ml of 3 mg/ml BSA in 2% Tween 80/PBS. Treatment was once daily for 10 days. The tumors were measured with microcalipers once a week for 4 weeks, and the tumor volume was calculated (33). The day of death was recorded for each mouse, and the MST was calculated from the period between tumor inoculation and the day of death. In addition, the % ILS was calculated using MST for each drug-treated mouse as follows:

\[
\% \text{ ILS} = \frac{\text{MST of drug-treated mouse} - \text{MST of vehicle control}}{\text{MST of vehicle control}} \times 100
\]

At the conclusion of the foregoing experiment, a separate experiment was conducted to compare HE with seven standard antiangiogenic drugs at their

<table>
<thead>
<tr>
<th>HE drug dose (mg/kg)</th>
<th>Mean number of colonized melanocytes (±SE)</th>
<th>% inhibition</th>
</tr>
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<tbody>
<tr>
<td>Vehicle control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(2% Tween 80/PBS)</td>
<td>96.8 ± 10.6</td>
<td>0</td>
</tr>
<tr>
<td>0.5 mg/kg</td>
<td>65.0 ± 2.9</td>
<td>33</td>
</tr>
<tr>
<td>1.0 mg/kg</td>
<td>51.6 ± 5.2</td>
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<tr>
<td>2.5 mg/kg</td>
<td>37.3 ± 12.0</td>
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<td>5.0 mg/kg</td>
<td>32.8 ± 3.7</td>
<td>66</td>
</tr>
<tr>
<td>10.0 mg/kg</td>
<td>57.0 ± 5.7</td>
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</tr>
<tr>
<td>100.0 mg/kg</td>
<td>67.5 ± 10.5</td>
<td>31</td>
</tr>
<tr>
<td>Positive control</td>
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</tr>
<tr>
<td>100 units rhIFN-α/β</td>
<td>43.5 ± 6.4</td>
<td>55</td>
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</tbody>
</table>

Table 1 Oral administration of HE inhibits lung colonization of B16F1 melanoma cells in C57BL/6 mice

Drugs were administered once daily for 10 days. Treatment began 1 h after i.v. inoculation of tumor cells and was stopped on day 10. Lungs were removed and fixed in formaldehyde on day 17, and the number of surface melanoma colonies for each mouse within a group of 5 mice/group was counted.
respective MEDs as shown on Table 4. Tubulin (34) and actin (23) assembly assays were performed as previously described.

For flow cytometry studies, CA46 (Burkitt lymphoma) and HeLa (cervical epitheloid carcinoma) cells were treated for 24 h with 10 μM HE. The cells were harvested by centrifugation, resuspended in PBS, and fixed in 70% ethanol for 30 min at 4°C. The cells were recollected by centrifugation and suspended in 1 ml of PBS containing 100 μg each of propidium iodide and RNase A. DNA content of the cells was analyzed with a Becton Dickinson FACScan flow cytometer, and the proportion of cells in G2-M was quantitated using Modfit LT V2.0 software.

Immunofluorescence studies were performed with PtK2 cells as described previously (23). Cells were examined with a Nikon Model Eclipse E800 microscope equipped with epifluorescence and the appropriate filters. Images were captured with a Spot Digital Camera, model 2.3.0, using version 3.0.2 software. All images shown here were obtained with the ×100 oil objective (N.A. 1.30).

RESULTS

Nonmutagenic Activity of HE as Determined by Ames Test. HE was evaluated in the Ames test for its mutagenic activity to induce reverse mutation at five specific histidine loci in Salmonella typhimurium. Positive mutagenicity was indicated by reversion significantly higher than spontaneous reversions by 3-fold of the vehicle controls. On the basis of this criterion, HE was negative in mutagenicity at concentrations as high as 3000 μM for the strains examined in either the presence or absence of S9, an exogenous metabolic activating fraction from Aroclor 1254-induced rat liver (data not presented).

Effect of HE on Tubulin and Actin Assembly. Consistent with the absence of an apparent effect on mitosis, HE had no effect on the assembly of purified tubulin (data not presented; highest concentration examined, 40 μM) in glutamate (34). We also could see no effect on the assembly of purified actin, measured both by the fluorescence assay (copolymerization of actin and pyrenyl actin; Ref. 35) and by a sedimentation assay (23).

Nevertheless, when PtK2 cells treated with HE were examined by fluorescence microscopy, we observed a dramatic disruption of their

Lack of Toxicity of HE against Various Cultured Normal and Malignant Human Cells. Cytotoxic effects of HE were measured against various normal human cells (cervical ectoepithelial cells, mammary epithelial cells, HUVECs, and peripheral blood mononuclear cells) and 31 human tumor cell lines (data not presented). Although HE exhibited some toxicity at high concentrations (50–100 μM) against ectoepithelial cells, mammary epithelial cells, and HUVECs, the half-maximal growth inhibitory (GI50) values for HE against the human tumor lines were in the 0.3–10 μM range, values substantially lower than the toxic concentrations for the normal cells examined. As before, HE was not toxic to peripheral blood mononuclear cells (7).

Of the 33 tumor lines examined, the most sensitive was the CCRF-CEM (T-cell leukemia) line, and the least sensitive were the NCI-H460 (non-small cell lung cancer), HCC-2998 (colon cancer), HeLa S3 (cervical carcinoma), OVCAR-3 (ovarian carcinoma), and three melanoma (M14, SK-Mel-28, and UACC-62) lines. The mean GI50 for the 31 lines was 6.0 μM ± 3.4 SE.

Fig. 2. Untreated PtK2 cells (A and B) and cells exposed to 8 μM HE (C) or 1 μM cytochalasin B (D) for 16 h. These concentrations represent the IC50 of the two drugs. A, C, and D, phalloidin and DAPI fluorescence. B, tubulin antibody and DAPI fluorescence. Note that A and B show the same cells. Arrows, binucleate cells.
microfilament network as well as an increase in binucleate cells, indicating a failure of cytokinesis, with less effect on the microtubule network (Figs. 2 and 3). The cells were examined with both an antibody to β-actin derivatized with fluorescein and a fluorescent phalloidin derivative. With the antibody, the drug-treated cells took on an overall luminescent appearance, whereas staining was more discreet with the phalloidin derivative. Only the latter results are presented here. We performed comparative studies with a number of agents known to affect actin polymerization, and of those examined, the effects of HE most closely resembled those of the cytochalasins. Cytochalasin B was chosen for a more detailed comparative study.

Growth of the PtK2 cells, as measured by cell protein, was inhibited 50% by 1.0 μM cytochalasin B and 8.0 μM HE. Time course evaluations were therefore performed at these IC50s (Fig. 2) and at 10-fold higher concentrations (Fig. 3).

Fig. 2, A and B, display a group of control PtK2, with their microfilament and microtubule networks revealed by staining with a fluorescent phalloidin derivative and a fluorescently derivatized antibody against β-tubulin, respectively (nuclei are stained with the fluorescent DNA binding agent DAPI). Fig. 2, C and D, show cells treated for 16 h with μM HE or 1 μM cytochalasin B, respectively, and stained with the phalloidin derivative and DAPI. Note the persistence of significant amounts of stress fibers in most of the cells treated with either drug and the single binucleate cell (arrows) in each field. In addition to the stained microfilaments, with both drugs there was both punctate staining and somewhat larger roundish clumps, presumably representing very short filaments, perhaps bundled, of f-actin. This rearrangement was moderately greater with cytochalasin B than with HE. The microtubule networks in these cells were not greatly affected (data not shown, but see below).

Cells treated with both drugs for 4 h were also examined. They differed only slightly in appearance from the control cells shown in Fig. 2, A and B.

Cells treated at 10 times the IC50s are shown in Fig. 3. In A–C are shown cells exposed to 80 μM HE, and in D–F, cells exposed to 10 μM cytochalasin B. Fig. 3, A and D, display the results of 4-h exposure to the drugs (phalloidin stain), whereas B and E (phalloidin stain) and C and F (tubulin antibody stain) show cells exposed to drug for 16 h. With both agents, as noted above, there was little effect on the microtubule networks. After 4 h of treatment with HE, most of the stress fibers had disappeared, and there was an abundance of the roundish clumps of f-actin. With 4 h of cytochalasin B, there were even fewer stress fibers, and the clumps of f-actin were somewhat more punctate than those observed after HE treatment. After 16 h of treatment with 80 μM HE, there were still a few persisting stress fibers, but the roundish clumps of f-actin appeared both reduced in number and more punctate.

**Induction of Cell Cycle Arrest by Hypoestoxide.** Initial studies on HeLa cells had indicated that HE might interfere with cell division in G2-M at an IC50 of 10 μM (data not shown). This effect was more marked in human Burkitt lymphoma CA46 cells. When examined by
HE Inhibits VEGF-induced HUVEC Proliferation. We next evaluated whether HE exerted inhibitory effects on VEGF functions, because VEGF is expressed by most cancers and blocking its activity can inhibit tumor growth in vivo but not in vitro (36). We found that HE significantly inhibited VEGF-induced HUVEC proliferation, with an IC_{50} of 29 μM, whereas tyrphostin AG1478, the standard antagonist, had an IC_{50} of 37 μM (Fig. 4).

HE Inhibits bFGF-induced Angiogenesis in Ovo Chick CAM. Because expression of bFGF by tumor cells directly correlates with angiogenesis and tumor growth (36), we evaluated HE for potential antiangiogenic activity. HE reproducibly inhibited bFGF-induced angiogenesis on the chick CAM with an IC_{50} of 10 μM (Fig. 5).

Cell Migration, but not Cell Adhesion, Is Inhibited by HE. The angiogenic process involves the growth and migration of endothelial cells; therefore, we next determined whether HE was able to inhibit endothelial cell migration or attachment (adhesion). We found that HE blocked endothelial cell migration but had no effect on attachment.

HE Inhibited endothelial cell migration on fibronectin (Fig. 6A; IC_{50}, 30 μM), collagen (IC_{50}, 100 μM; data not shown), and vitronectin (IC_{50}, 100 μM; data not shown). In contrast, HE did not inhibit endothelial cell adhesion on fibronectin (Fig. 6B), the substrate for endothelial cell integrin α5β1, which is a key receptor regulating angiogenesis in vivo. Nor did HE block cell attachment to collagen, the substrate for integrin α2β1, nor to vitronectin, the substrate for integrin αvβ3 (data not presented).

Lack of Toxicity of HE in Vivo. C57BL/6 mice given HE by i.p. or oral administration showed no toxicity at tested doses (1.5–750 mg/kg). The mice were observed over an 8-day period for normal behavior, vocalization, slow breathing, hypoactivity, coolness to touch, and neural integrity. The mice were kept for an additional 3

Flow cytometry for DNA content, these cells accumulated at G_{2}-M when treated with 10 μM HE. Analysis of the data obtained yielded (data not shown) 16% G_{2}-M cells for the control population versus 49% G_{2}-M cells for the HE-treated population. Morphological evaluation of similarly treated Burkitt cells showed no increase in the mitotic index of 3%.

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Figure 4. HE inhibits VEGF-induced HUVEC proliferation. VEGF-stimulated HUVECs were treated with either HE or tyrphostin AG1478 for 48 h at the indicated concentrations. Cell proliferation was determined by fluorescent quantitation. This experiment was reproduced three times.

Figure 5. HE inhibits bFGF-induced angiogenesis in embryonated chicken eggs. A. photos illustrate the formation of blood vessel branch points in either control (saline), bFGF, or bFGF + 250 μM HE-treated CAMs of 10-day old embryonated chicken eggs stimulated with CA pretreated filter discs. Note the significant inhibition of the formation of blood vessel branch points in the egg exposed to 250 μM HE. B, dose-dependent inhibitory effect of HE on the number of blood vessel branch points formed (percentage of control ± SE).

The IC_{50} of HE for VEGF-induced HUVEC proliferation was determined to be 29 μM.
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Fig. 6. HE inhibits cell migration but not cell adhesion. A. HUVECs in either medium containing the functional blocking antibody (anti-α5β1), medium alone, or medium containing 0–100 μM HE were allowed to migrate from the upper to the lower chamber of Transwell inserts coated with fibronectin. The number of cells that had migrated to the bottom of the insert were counted. SEs are identical. B, HUVECs in either medium containing functional blocking antibody (anti-αβ1), medium alone, or medium containing 1–100 μM HE were allowed to adhere to fibronectin-coated plates for 20 min. The data are presented as a percentage of adhesion exhibited by the positive control (adhesion medium alone; bars, SE).

The results on the s.c. injections of tumor cells, followed by p.o. HE (data not shown), were similar to the results obtained by i.p. treatment. Thus, although oral administration of HE resulted in a marked reduction (66%) of lung metastatic colonies (Table 1), i.p. administration also produced significant inhibition (70%) of tumor volume, which resulted in 145% ILS (Table 2). Identical results were also obtained on ascitic tumors injected in C57BL/6 × DBA/2 F1 mice by i.p. injections of a different murine cell line, P388D1, which is a lymphoid neoplasm (data not shown). In this experiment, CY, a standard immuno-suppressant in common use as an antitumor agent, served as a positive drug control. In separate experiments, HE was compared with standard antiangiogenic drugs in use. Oral administration of HE at a MED of 5 mg/kg resulted in significant reduction of lung metastatic colonies (63%), which is comparable with the results obtained with six other agents with a mean percentage of inhibition of 61.7 ± 3.8 (Table 3). Similarly, comparable results were also obtained in the s.c. solid tumor model in which a MED of 5 mg/kg HE induced 80% inhibition of tumor volume as compared with a mean MED of 40 mg/kg for seven drugs, which induced a mean of 77% (±8.3) inhibition of tumor volume and was reflected in the % ILS (Table 4).

Although histological examinations of the s.c. solid tumors were not performed, we did prepare single-cell suspensions from excised tumors and found no difference in the number of infiltrating mononuclear cells in treated versus untreated tumors. Furthermore, the untreated s.c. solid tumors contained more apoptotic cells than the treated tumors. Therefore, it is unlikely that the antitumor properties observed in vivo are attributable to a reduction in inflammatory cells within and around the tumor.

Table 2 Intraperitoneal administration of HE inhibits the growth of s.c. implanted B16F1 melanoma in C57BL/6 mice

<table>
<thead>
<tr>
<th>Drug dose (mg/kg)</th>
<th>Vehicle control</th>
<th>HE 0.3125</th>
<th>HE 0.625</th>
<th>HE 1.25</th>
<th>HE 2.50</th>
<th>HE 5.0</th>
<th>HE 10.0</th>
<th>HE 100.0</th>
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<tr>
<td>Dose (mg/kg)</td>
<td>2% Tween 80/PBS</td>
<td>2% Tween 80/PBS</td>
<td>20</td>
<td>18</td>
<td>100</td>
<td>20</td>
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<tr>
<td>Tumor volume (mm³)</td>
<td>2252 ± 128</td>
<td>2264 ± 134</td>
<td>2246 ± 177</td>
<td>2104 ± 60</td>
<td>996 ± 54</td>
<td>693 ± 47</td>
<td>1369 ± 95</td>
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<tr>
<td>% inhibition</td>
<td>174 ± 1.0</td>
<td>23.5 ± 1.2</td>
<td>24.6 ± 1.4</td>
<td>26.3 ± 2.1</td>
<td>32.2 ± 2.7</td>
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<td>32.1 ± 2.2</td>
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Table 3 Effect of oral administration of HE in comparison with standard anticancer agents on lung colonization of B16F1 melanoma cells in C57BL/6 mice

<table>
<thead>
<tr>
<th>Drug</th>
<th>Dose (mg/kg)</th>
<th>Mean number of colonized melanocytes (=SE)</th>
<th>% inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle control</td>
<td>2% Tween 80/PBS</td>
<td>47.3 ± 4.0</td>
<td>0</td>
</tr>
<tr>
<td>HE</td>
<td>5</td>
<td>17.7 ± 4.5</td>
<td>63</td>
</tr>
<tr>
<td>CA</td>
<td>50</td>
<td>21.3 ± 5.2</td>
<td>55</td>
</tr>
<tr>
<td>BLEO</td>
<td>50</td>
<td>16.5 ± 3.7</td>
<td>65</td>
</tr>
<tr>
<td>ADR</td>
<td>7.5</td>
<td>16.4 ± 4.6</td>
<td>65</td>
</tr>
<tr>
<td>5-FU</td>
<td>50</td>
<td>17.5 ± 4.3</td>
<td>63</td>
</tr>
<tr>
<td>CY</td>
<td>100</td>
<td>17.9 ± 4.2</td>
<td>62</td>
</tr>
<tr>
<td>ETP</td>
<td>20</td>
<td>19.1 ± 3.5</td>
<td>60</td>
</tr>
</tbody>
</table>
HYPOESTOXIDE: A NATURAL ANTITUMOR DITERPENOID

Drugs were administered i. p. once daily for 10 days (except for HR, which was administered p.o.), starting when tumors had a mean tumor volume of 18–20 mm³. Five mice were allocated to each group. Tumor volume (mean ± SE) at the start of treatment (day 0) and on day 17 after the start of treatment is shown (tumor volume for the vehicle control group on day 13 is shown in the table because their poor condition and shorter life span). Drug treatment for the experimental groups was stopped on day 10, except for VCR, which was stopped on day 6 because toxicity. MST and % ILS were calculated for each group.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Dose (mg/kg)</th>
<th>Tumor volume (mm³)</th>
<th>MST ± SE (days)</th>
<th>% ILS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle control</td>
<td>2% Tween 80/PBS 0 20</td>
<td>2828 ± 135 (day 13)</td>
<td>4013 ± 50</td>
<td>0</td>
</tr>
<tr>
<td>HE</td>
<td>5.0</td>
<td>18</td>
<td>545 ± 45</td>
<td>38.7 ± 1.2</td>
</tr>
<tr>
<td>CA</td>
<td>50</td>
<td>20</td>
<td>497 ± 34</td>
<td>39.0 ± 4.2</td>
</tr>
<tr>
<td>VCR</td>
<td>1.2</td>
<td>20</td>
<td>724 ± 93</td>
<td>34.0 ± 1.4</td>
</tr>
<tr>
<td>BLEO</td>
<td>50</td>
<td>20</td>
<td>536 ± 54</td>
<td>43.0 ± 2.8</td>
</tr>
<tr>
<td>ADR</td>
<td>7.5</td>
<td>20</td>
<td>621 ± 99</td>
<td>27.0 ± 1.3</td>
</tr>
<tr>
<td>5-FU</td>
<td>50</td>
<td>20</td>
<td>1142 ± 59</td>
<td>25.5 ± 0.7</td>
</tr>
<tr>
<td>CY</td>
<td>100</td>
<td>18</td>
<td>451 ± 62</td>
<td>49.0 ± 7.5</td>
</tr>
<tr>
<td>ETP</td>
<td>20</td>
<td>18</td>
<td>514 ± 57</td>
<td>30.0 ± 1.0</td>
</tr>
<tr>
<td>Control for HE</td>
<td>100</td>
<td>18</td>
<td>1137 ± 31</td>
<td>38.0 ± 3.0</td>
</tr>
</tbody>
</table>

DISCUSSION

New agents are required both for the prevention and therapy of malignancies. To this end, we report on HE, a new nonmutagenic natural antitumor agent with greater cytotoxic effects on cancer cells than on nonmalignant cells. HE also has antiangiogenic activity, probably caused by its ability to inhibit bFGF production by tumor cells.

Because HE contains two epoxide moieties and an α,β unsaturated ketone, structural features often associated with DNA alkylation, we evaluated HE for possible mutagenic activity. It was negative in the Ames test, consistent with our previous finding that HE lacked alkylating properties (7). Moreover, HE is nontoxic to mice (maximally tolerated dose, >750 mg/kg).

Angiogenesis plays a pivotal role in progression of malignant tumors (reviewed in Ref. 35). This process requires proliferation, migration, and tube formation by endothelial cells, and it is stimulated by bFGF and VEGF (37). The activities of both factors were shown to be inhibited by HE.

We examined HE for antitumor activity in vivo against a murine melanoma, using the i.v. lung metastasis and s.c. solid tumor models. HE was p.o. bioactive, and significant inhibition of lung colonization by B16F₁ melanocytes was observed over a wide dose range, 0.5–100 mg/kg. The MED of 5 mg/kg induced 66% inhibition of colonization of melanocytes \((P < 0.01\) versus control), and this same dose was the most effective in the s.c. solid tumor model. HE induced a 70% inhibition of tumor volume and a 145% ILS \((P < 0.01\) versus control). HE was less effective at higher doses, with 2.5 and 100 mg/kg inducing equivalent responses. In both experimental models, the activity of HE was better than that of the positive drug control, IFN-α/β. We chose IFN-α/β because IFN-α/β has been used in human therapy as an antiangiogenic drug (38, 39).

Furthermore, the activity of HE in the lung metastatic melanoma model was comparable with six other standard antiangiogenic drugs tested, whereas HE was more active than four of the seven agents (VCR, ADR, 5-FU, and ETP) tested in the s.c. solid melanoma model. Our results also demonstrate the efficacy of HR in vivo and thus provide a scientific basis for the use of the leaf extracts of HR in folk medicine.

The exact mechanism(s) of action of HE is unknown, but it inhibits endothelial cell migration. This could occur through inhibition of the cellular motility machinery or of a signal transduction cascade required for effective cell migration. In support of this notion are the findings demonstrating that HE interferes, either directly or indirectly, with actin assembly and cytokinesis.

With HE, we have been unable to demonstrate an effect on the assembly of purified actin. This could reflect the relatively weak cytotoxicity \((in vitro)\) of the agent, with cells concentrating it to higher concentrations than we were able to evaluate in our biochemical assays. It is also possible that the actual target of HE could be an actin-associated protein (reviewed in Ref. 40). A final possibility is that HE acts upstream from actin assembly so that the dramatic changes in the actin cytoskeleton represent an indirect effect of the drug.

From our results, HE is more potent \(in vivo\) than \(in vitro\). The reasons for this difference in potency are most likely attributable to the inhibitory effects of HE on angiogenesis, which is required \(in vivo\) but not \(in vitro\). We have found that the initial metabolite of HE, the deacetylated HE (see Fig. 1 for structure), also induces cell cycle arrest and apoptosis in the HeLa, CAKI-1, and B16F₁, tumor cell lines. Moreover, HE has a large volume of distribution and high clearance from the circulation and, thus, an extensive tissue distribution and metabolism (7).

In a previous report, we described HE as a novel nonsteroidal anti-inflammatory drug (7). In this report, we present data describing its antitumor properties. These appear to result from several mechanisms, a property that should make HE an ideal candidate for use in combination with other drugs in the treatment of human neoplasms.

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REFERENCES

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