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# Hexahydrocolupulone and Its Antitumor Cell Proliferation Activity *In Vitro*

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**ABSTRACT.** The purpose of this study was to evaluate the ability of hexahydrocolupulone (HHC) to inhibit the growth of tumor cells *in vitro* and to investigate the potential mechanism(s) involved. HHC was demonstrated to have a wide spectrum of activity against a number of established human tumor cell lines, including some exhibiting drug resistance. Culturing human breast adenocarcinoma (MCF-7) cells in the presence of HHC for 18 hr resulted in a significant decrease in the incorporation of [$^3$H]uridine and [$^3$H]leucine into RNA and protein, respectively. MCF-7 cells cultured in the presence of 1.5 μM HHC for 48 hr demonstrated an increase in the amount of cells detected in $G_0/G_1$ and a decrease in the amount of cells detected in S phase. In contrast, treatment with 25 μM HHC decreased the amount of cells detected in $G_0/G_1$ and increased the amount of cells detected in S phase. HHC did not cause single-stranded or double-stranded DNA breaks, interfere with topoisomerase function, or generate free radicals. Mice injected intraperitoneally for 5 consecutive days with HHC to a final *in vivo* blood concentration of 200 μM survived and showed no obvious signs of toxicity. Mass spectroscopy analysis, crystal generation, and structure elucidation confirmed HHC purity. Consequently, all activity observed can be attributed to HHC, a metabolite, and/or a combination thereof. These data suggest that HHC inhibits tumor cell proliferation *in vitro* via a mechanism(s) that may involve effects on macromolecular synthesis, precursor metabolism/transport, and/or the cell cycle or cell cycle-dependent pathway(s). BIOCHEM PHARMACOL 55;4:505–514, 1998. © 1998 Elsevier Science Inc.

**KEY WORDS.** novel agents; hexahydrocolupulone; colupulone; cancer; hops

Uncontrolled cell growth is inherent in all neoplastic disease and, consequently, most current chemotherapy targets the cell cycle. This targeting strategy, however, confers toxicity not only to neoplastic cells but to normal host cells as well. Another significant problem in cancer chemotherapy is the development of malignancies that are drug resistant [1, 2]. These circumstances have led researchers to search for alternative anticancer agents with novel mechanisms of action that would limit host toxicity and bypass current drug-resistant mechanisms.

HHC§ is a semisynthetic derivative of colupulone [3]. Colupulone is a β-acid congener of lupulone that can be isolated from hops, the dried flower cone of the plant *Humulus lupulus* [4, 5]. The compounds of interest (also called total resins) represent approximately 15% of the total chemical composition of the hop extract [5]. These resins consist mainly of α- and β-acids. Humulone is the parent α-acid, and lupulone is the parent β-acid (see Fig. 1).

Our interest in these compounds (specifically the β-acid congener HHC) as antitumor cell proliferation agents, and consequently potential anticancer agents, revolves around observations made by other investigators. First, there is sufficient evidence to suggest that dietary constituents may have an inhibitory effect on the process of chemical carcinogenesis [6–9]. More specifically, when known carcinogens were administered concurrently, animals fed a crude diet were less likely to exhibit cancerous lesions than animals fed a purified diet [10–13]. One possible mechanism by which an inhibitory effect on chemical carcinogenesis could be obtained is by inducing the activity of carcinogen detoxification systems [14, 15]. These detoxification systems consist of phase I enzymes (the cytochrome P450 monooxygenase system) and phase II enzymes (those involved in conjugation reactions) [16, 17].

Second, the β-acid colupulone has been shown to induce the cytochrome P450 system [18, 19]. Investigators observed that the P450 system (specifically P4503A) was induced in animals fed the crude diet (i.e. the same diet

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§ *Abbreviations:* C1300, murine neuroblastoma; CEM, human acute lymphoblastic leukemia; CEM-V, human acute lymphoblastic leukemia overexpressing the multi-drug resistant protein; FACS, fluorescence-activated cell sorting; [$^3$H]Leu, tritiated leucine; [$^3$H]T, tritiated thymidine; [$^3$H]U, tritiated uridine; HHC, hexahydrocolupulone; HeLaS3, human cervical epitheloid carcinoma; KB, human oral epidermoid carcinoma; L1210, murine lymphoblastic leukemia; MCF-7, human breast adenocarcinoma; MCF-7-HHC, human breast adenocarcinoma resistant to hexahydrocolupulone; MCF-7-OAP, human breast adenocarcinoma resistant to oxazophosphorines; MDR, multi-drug resistance; MTD, maximal tolerated dose; PCA, perchloric acid; Raji, human Burkitt lymphoma; TPA, 12-O-tetradecanoylphorbol-13-acetate.

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shown to decrease the incidence of cancerous lesions) when compared with animals fed the purified diet. Further investigation led to the discovery that colupulone was the major constituent in the crude diet responsible for this enzyme induction [20, 21]. The inducible nature of the cytochrome P450 system and its varying substrate specificity make this enzyme system very important in the metabolism and subsequent activation and/or inactivation of many different xenobiotics [22]. Consequently, this enzyme system is an important mediator in the toxicity of xenobiotics and, therefore, is important in the process of carcinogenesis. The structural similarity of HHC to colupulone suggests that it may also induce cytochrome P450, which may consequently affect the metabolism of carcinogenic xenobiotics.

Finally, it has been observed in mice that the α-acid humulone inhibits the promotion of skin cancer by TPA [23], a tumor-promoting phorbol ester [24, 25]. The ability of humulone to inhibit TPA-promoted skin cancer *in vivo* and its structural similarity to colupulone suggest that colupulone and the congener HHC may also have similar effects.

Although the previously mentioned observations suggest that the compounds of interest are acting in a chemopreventive manner, we have also shown that colupulone inhibits the proliferation of a human tumor cell line *in vitro* [26]. In the present investigation, we demonstrated that HHC is more potent than colupulone in its ability to inhibit the growth of human tumor cells *in vitro*, and we have broadened the panel of cell lines investigated to include additional human tumor cells, murine tumor cells, and some human tumor cell lines exhibiting drug resistance. In addition, we have begun to investigate the mechanism(s) responsible for the inhibition of tumor cell proliferation observed using the β-acids colupulone and HHC.

## MATERIALS AND METHODS

### *Reagents*

HPLC-purified colupulone and HHC were gifts from Kalsec, Inc. The purity of each compound was confirmed by mass spectroscopy analysis performed by the mass spectroscopy facility at the University of Minnesota (data not shown). Radiolabeled [$^3$H]T, [$^3$H]U, and [$^3$H]Leu were purchased from ICN Pharmaceuticals. Radiolabeled [$^3$H]HHC was synthesized for our laboratory by Moravek Biochemicals, Inc. All other reagents were readily available commercially and were of the highest quality.

### *Animals/Cell Lines*

Female BDF1 mice, 11- to 14-weeks-old, were purchased from Harlan Sprague Dawley, Inc. Animals were maintained on standard pellet food and water available *ad lib.* The following cell lines were used: MCF-7, MCF-7-OAP, CEM, CEM-V, Raji, KB, HeLaS3, C1300, and L1210. All cell lines were acquired from the American Type Culture Collection (ATCC) with the exception of MCF-7, MCF-7-OAP, and L1210 cells, which were gifts from Dr. Norman Sladek (Department of Pharmacology, University of Minnesota). Established cell culture techniques [27] were used to maintain cells using the appropriate medium supplemented with 10% fetal bovine serum and kanamycin at a concentration of 0.1 mg/mL.

### *Crystal Generation and X-ray Crystallography Analysis*

HHC crystals determined to be appropriate for X-ray crystallography were obtained by dissolving 20–30 mg of HHC in 1 mL of 100% ethanol and leaving the solution at −20° until crystals formed (2–8 days). Crystals were dried under vacuum and submitted to the X-ray Crystallography Department at the University of Minnesota for crystal structure determination. All measurements were made using an Enraf-Nonius CAD-4 diffractometer at a temperature of −90 ± 1°, and the structure was solved by direct methods [28, 29].

### $IC_{50}$ *Determination*

TRYPAN BLUE EXCLUSION ASSAY. Monolayer cells were seeded into T-25 cm$^2$ flasks at a density of 2 × 10$^5$ cells/mL in a total volume of 5 mL. After a minimum of 14–18 hr, various concentrations of drug were added. When suspension cells were used, drug was added immediately after seeding. Cells were harvested and counted initially (zero time) and again after 24, 48, and/or 72 hr of continuous drug exposure. Briefly, a volume of 0.4% (w/v) trypan blue was mixed with an equal volume of cell suspension, and the cells in the resulting suspension were counted using a hemacytometer. The percentage of growth inhibition was calculated for each condition, and $IC_{50}$ values (i.e. the amount of drug required to inhibit 50% of cell growth) were determined by interpolation.

CLONOGENIC ASSAY. Briefly, MCF-7 cells were pulsed with various concentrations of drug for 6, 12, or 18 hr, harvested, and seeded into 60 × 15 mm culture dishes at a density of 300 or 900 cells/mL in a total volume of 5 mL. The cells were incubated for 5–8 days, after which time the cells were washed with PBS and fixed with ethanol for 30 min. Cells were then stained with Giemsa [1% (w/v) in 100% methanol] for 30 min. Colonies containing >50 cells were counted, a percent inhibition was calculated for each treatment using colony number instead of cell number, and $IC_{50}$ values were determined by interpolation. Plating efficiency was determined to be 9.5 ± 2.5% (mean ± SEM).

### *Development of MCF-7 HHC-Resistant Cells (MCF-7-HHC)*

Starting with 1.5 μM HHC, MCF-7 cells were exposed continuously to stepwise increments in HHC concentra-

tion over a period of 10 months. Three increments were made in total with a final increase to 7 μM.

### *FACS Analysis*

FACS analysis was used to determine the effect HHC had on the distribution of cells in the cell cycle. T-175 $cm^2$ flasks were seeded with approximately $2.5 \times 10^6$ MCF-7 cells. After 14–18 hr, 1.5 or 25 μM HHC was added to the flasks. After 48 and 72 hr of continuous exposure to HHC, the cells were harvested and prepared for FACS analysis. Untreated and solvent-treated (DMSO; 0.2%, v/v) cells under identical conditions were used as controls.

Briefly, $2 \times 10^6$ cells were suspended in 200 μL of citrate buffer (i.e. 250 mM sucrose, 40 mM trisodium citrate, and 5% DMSO, pH 7.6) and frozen/thawed once. The cells were then trypsinized for 10 min with 1.8 mL of stock solution (i.e. 3.4 mM trisodium citrate, 0.1% nonionic detergent NP-40, 1.5 mM sperminetetrahydrochloride, and 0.5 mM Tris base) containing 3 mg of trypsin. The reaction was terminated with 1.5 mL of stock solution containing 0.5 mg/mL of trypsin inhibitor and 0.1 mg/mL of RNase A. Propidium iodide was then added to the cells to a final concentration of 124.8 μg/mL. The cells were incubated for 15 min at 4°, centrifuged for 5 min at 600 × *g*, and resuspended in 0.5 to 1 mL of the supernatant. Then cell suspensions were submitted to the flow cytometry laboratory at the University of Minnesota for FACS analysis. The flow cytometer was calibrated to analyze the propidium iodide content of 10,000 cells. Propidium iodide intercalates into DNA and can be detected by fluorescence imaging techniques. The labeling pattern was used to determine the proportion of cells in the individual phases of the cell cycle [30, 31].

### *[$^3$H]Precursor Incorporation into Macromolecules*

The effect of HHC on DNA, RNA, and protein synthesis was evaluated by measuring the incorporation of [$^3$H]T, [$^3$H]U, and [$^3$H]Leu, respectively, into MCF-7 cells. Briefly, T-75 $cm^2$ flasks were seeded with $2 \times 10^6$ cells and incubated for 14–18 hr. After this time, HHC and the appropriate tritiated precursor were added to the flasks. Cells were harvested after 1.5 and 18 hr of incubation. Acid-soluble (unincorporated [$^3$H] precursor) and -insoluble (DNA, RNA, and protein) cellular fractions were separated by PCA extraction [32]. Samples were counted in a scintillation fluid using a Beckman scintillation counter (model LS2800).

### *Determination of the MTD for HHC*

Female BDF1 mice (11–14 weeks old) were separated indiscriminately into groups of 5, tagged, and weighed to determine the amount of drug necessary to inject into each animal to reach the desired final HHC concentration(s) within the blood. Calculations were made based on the assumption that 100% of the injected HHC reaches the blood supply. A value of 8% body weight was used to approximate total blood volume. HHC was dissolved in DMSO (100%) and injected intraperitoneally. Animals were injected once every 3 days for 9 consecutive days (3 injections total), and their ability to survive for 90 days was observed. The maximal dose at which all animals survived (0.5 mg/kg) was injected via the same route, once per day for 5 consecutive days, into 5 new animals, and their ability to tolerate the dosing procedure was observed.

## RESULTS

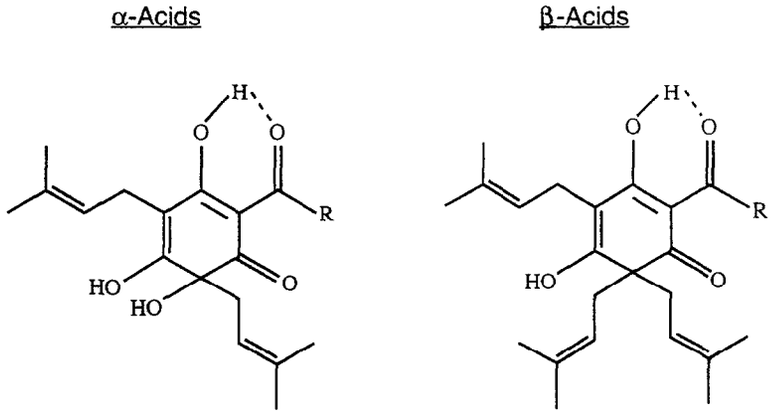
### *Antitumor Cell Proliferation Activity of Colupulone and HHC*

Colupulone and HHC differ only in the saturation of their side chains, HHC side chains being saturated completely (Fig. 1). These structural differences make HHC more hydrophobic than colupulone. To investigate the effect of increasing the hydrophobicity of colupulone on the antitumor cell proliferation activity previously observed [26], the abilities of colupulone and HHC to inhibit the growth of CEM and CEM-V cells *in vitro* were compared (Table 1).

CEM-V cells overexpress the MDR protein. This membrane protein has been demonstrated to extrude xenobiotics from cells [33–35]. Although both colupulone and HHC were effective at inhibiting the growth of CEM and CEM-V cells, HHC was approximately 2.3-fold more potent than colupulone ($P < 0.001$). Thus, our laboratory has focused the investigation specifically on HHC activity and the elucidation of its spectrum and potential mechanism(s) of action.

### *Crystal Structure of HHC*

The crystal structure of HHC was elucidated to confirm its purity, to determine any potential interactions occurring between and/or among HHC molecules, and to determine any unique structural characteristics that may exist within the HHC molecule. Figure 2A is a schematic representation of the crystal structure of a single HHC molecule. A unique characteristic is the "folding" of the side chains attached to carbon 6 into a hydrophobic moiety. This portion of the molecule, as well as intermolecular hydrogen bonding, allows for the association of the HHC molecules into a crystal lattice represented in Fig. 2B. The crystal structure and lattice organization represented in Fig. 2 are a static depiction of HHC molecular interactions and atomic positioning. Experimentally, HHC is in a very dynamic environment (solution state) and, therefore, may not adopt such an ordered state. The hydrophobic moiety, however, may exist in an aqueous environment and could contribute to any HHC molecular interactions occurring with other HHC molecules and/or cellular constituents.




| | R | | R |
|---|---|---|---|
| HUMULONE | $CH_2CH(CH_3)_2$ | LUPULONE | $CH_2CH(CH_3)_2$ |
| COHUMULONE | $CH(CH_3)_2$ | COLUPULONE | $CH(CH_3)_2$ |
| ADHUMULONE | $CH(CH_3)CH_2CH_3$ | ADLUPULONE | $CH(CH_3)CH_2CH_3$ |
| PREHUMULONE | $CH_2CH_2CH(CH_3)_2$ | PRELUPULONE | $CH_2CH_2CH(CH_3)_2$ |
| POSTHUMULONE | $CH_2CH_3$ | POSTLUPULONE | $CH_2CH_3$ |
| HH-COHUMULONE | $CH(CH_3)_2$ (with saturated side chains) | HH-COLUPULONE | $CH(CH_3)_2$ (with saturated side chains) |

**FIG. 1. Structures of α- and β-acids. Modified from Ref. 26. HH = hexahydro.**

### $IC_{50}$ *for HHC in Selected Tumor Cell Lines*

The two different methods used to measure the $IC_{50}$ of HHC were the trypan blue exclusion assay (measuring the effect of continuous drug exposure) and the clonogenic assay (measuring the effect of pulse drug exposure). The ability of cells to exclude trypan blue is an indicator of cell membrane integrity and was performed whenever cell counts were necessary. Cells that did not exclude trypan blue were determined to be cells that had their membrane compromised. Although membrane destabilization may lead to cell death, it is also possible for these cells to recover and continue to function normally. For this reason, cells that did not exclude trypan blue (i.e., never more than 20% total) were also counted as live. Counting the blue cells as live may lead to an artificially increased value for any calculated $IC_{50}$ and suggests that the actual $IC_{50}$ values are no more and possibly less than those values reported. Although continuous drug exposure was used to investigate the spectrum of action of HHC, a time-dependent or pulse exposure to HHC may more accurately reflect the clinical administration of many currently used anticancer chemotherapeutic protocols [36]. The clonogenic assay also allows us to follow individual cells after a pulse of drug exposure. This cannot be measured using the trypan blue exclusion assay.

**TABLE 1. Inhibition of tumor cell growth by colupulone and HHC***

| | $IC_{50}$ (μM) | |
|---|---|---|
| Agent | CEM cells | CEM-V cells |
| Colupulone | 4.25 ± 0.34 | 6.41 ± 0.27 |
| HHC | 1.85 ± 0.04 | 2.77 ± 0.10 |

* Acute lymphoblastic leukemia cells (CEM) and a subline of CEM made resistant to vinblastine (CEM-V) were cultured in the presence of HHC for 48 hr. Cells were then harvested and counted, and $IC_{50}$ values were determined as described in "Materials and Methods." The vehicle (DMSO; 0.2%, v/v) was determined to have no effect on cell growth. Values are means ± SEM obtained from 3 experiments, each done in duplicate.

**HHC Spectrum Of Action.** Before any detailed investigation of the potential mechanism(s) responsible for the antitumor cell proliferation activity of HHC is performed, it is important to evaluate the ability of HHC to inhibit the growth of many different tumor cell lines (including those exhibiting drug resistance) *in vitro*. This information allows us to speculate on the clinical relevance of this compound in the potential treatment of many different cancers, including solid tumors from different origins and leukemias. Evaluating the effect of HHC in drug-resistant tumor cells might also suggest a potential mechanism(s) and cellular pathways and/or components that may or may not be involved with HHC activity.

Selected tumor cell lines were evaluated for their sensitivity to HHC using the trypan blue exclusion assay to determine the $IC_{50}$ in cells continuously exposed to HHC for a period of time (Table 2). A number of different human tumor cell lines (including breast carcinoma, Burkitt lymphoma, oral epidermoid carcinoma, cervical epithelioid carcinoma and acute lymphoblastic leukemia) and murine cell lines (including lymphocytic leukemia and neuroblastoma) were evaluated. The $IC_{50}$ of HHC against these cells ranged from approximately 0.85 to 2.19 μM and suggests that HHC has a reasonably wide spectrum of action. More interestingly, HHC was effective against tumor cells that are resistant to agents used clinically. These observations *in vitro* suggest that HHC may have clinical relevance.

**Pulse Exposure To HHC.** The effect that different exposure times with HHC had on the proliferation of

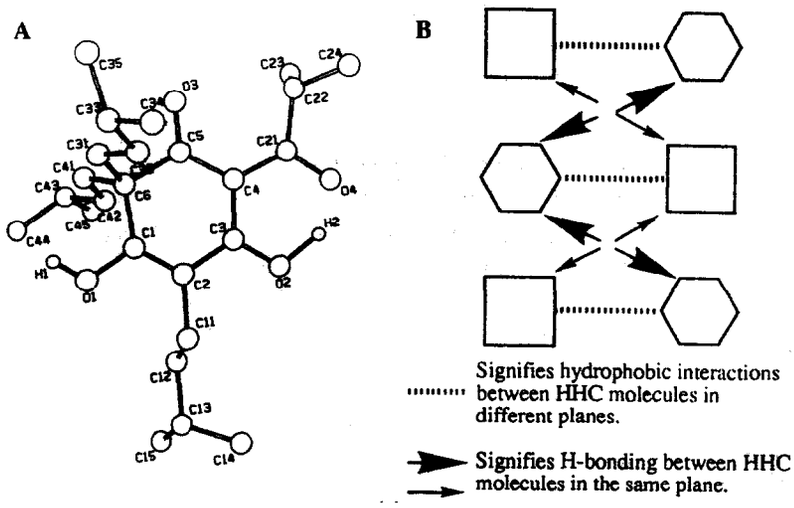



**FIG. 2. Crystal structure of HHC and its crystal lattice organization. HHC crystals appropriate for X-ray diffraction were prepared by dissolving 20–30 mg of HHC in 100% ethanol and allowing the solution to sit at −20° for 2–8 days. X-ray crystallography experiments were performed at the University of Minnesota X-ray crystallography facility. All measurements were made in an Enraf-Nonius CAD-4 diffractometer. (A) Crystal structure of a single HHC molecule. (B) Organization of the crystal lattice of HHC. Hexagons represent HHC molecules in one plane and squares represent HHC molecules in another parallel plane.**

MCF-7 cells was evaluated using the clonogenic assay. A longer pulse with HHC led to a decrease in the $IC_{50}$ (Fig. 3). A linear relationship between the log of the response ($IC_{50}$) and the log of the pulse time was observed specifically within the experimental pulse range. This supports the hypothesis that HHC is effective at inhibiting the growth of human tumor cells *in vitro* and also suggests that a time-dependent mechanism(s) is involved. The drug must be present >18 hr to achieve an $IC_{50}$ value that approaches the $IC_{50}$ value observed with continuous exposure. This time dependence observed on the maximal antiproliferative effect suggests that the activity may be due to a metabolite(s) of HHC and/or that HHC may be affecting the cell cycle or a cell cycle-dependent pathway(s).

### *Development of HHC-Resistant MCF-7 Cells*

Identifying the mechanism(s) of resistance and cross-resistance that MCF-7-HHC cells have for other pharmacological agents of known mechanism(s) may contribute to the elucidation of the mechanism(s) by which HHC inhibits tumor cell proliferation *in vitro*. The ability of these cells to develop resistance to HHC may also be an indicator of the ability of cancer cells, in general, to acquire resistance to this compound and is clinically very important. At present, we possess MCF-7-HHC cells that grow in the presence of 7 μM HHC. The $IC_{50}$ of HHC using these cells, as determined by continuous exposure to HHC for 72 hr, was 4.46 ± 0.41 μM (Table 2). These cells grow more slowly, appear larger in size, and are approximately 3.6-fold more resistant to HHC than the parent cell line. Interestingly, the MCF-7-HHC cells do not grow to confluence but instead begin to detach and die when they are approximately 60–70% confluent.

**TABLE 2. Inhibition of tumor cell growth by HHC***

| Cell line | $IC_{50}$ (μM) |
|---|---|
| MCF-7 (human breast adenocarcinoma) | 1.23 ± 0.10†‡ |
| Raji (human Burkitt lymphoma) | 2.19 ± 0.20§‖ |
| KB (human oral epidermoid carcinoma) | 0.85 ± 0.04†‡ |
| HeLaS3 (human cervical epithelioid carcinoma) | 1.21 ± 0.02†‖ |
| MCF-7-OAP (oxazaphosphorine resistant) | 1.26 ± 0.28†‡ |
| MCF-7-HHC (HHC resistant) | 4.46 ± 0.41†¶ |
| C1300 (mouse neuroblastoma) | 1.78 ± 0.28†‡ |
| L1210 (mouse lymphocytic leukemia) | 1.77 ± 0.27†‖ |

* Cells were incubated in the presence of HHC for 24, 48, or 72 hr, and harvested at the end of each incubation; $IC_{50}$ values were determined as described in "Materials and Methods." The vehicle (DMSO; 0.2%, v/v) was determined to have a −2.1 ± 2.6% inhibitory effect on cell growth. Values are means ± SEM for N = 3 or 4 experiments, each done in duplicate.

† N = 3.

‡ Experiments were done with continuous exposure to HHC for 48 hr.

§ N = 4.

‖ Experiments were done with continuous exposure to HHC for 24 hr.

¶ Experiments were done with continuous exposure to HHC for 72 hr.

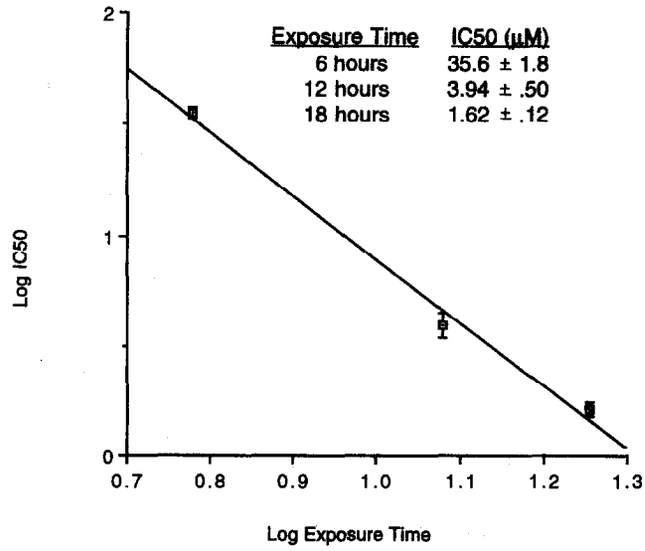



**FIG. 3. The $IC_{50}$ of HHC in MCF-7 cells after 6, 12, and 18 hr of exposure. MCF-7 cells were exposed to HHC, the drug was removed, and the cells were plated at a density of 300 or 900 cells/mL (5 mL total) into 60 × 15 mm culture plates. After 6–8 days, the cells were stained and colonies of >50 cells were counted for $IC_{50}$ determination. The $IC_{50}$ values are means ± SEM obtained from 3 experiments, each done in duplicate. The vehicle control (DMSO; 0.2%, v/v) was evaluated to have a 5.6 ± 5.3% inhibitory effect on cell growth.**

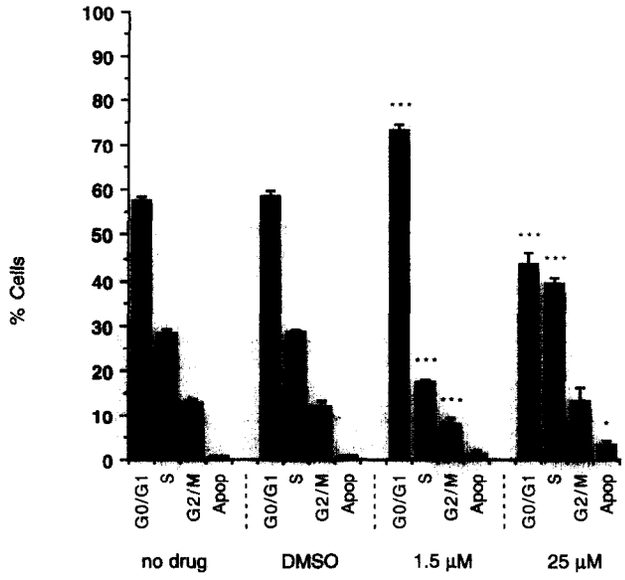



**FIG. 4. Effect of HHC on the distribution of MCF-7 cells throughout the cell cycle. Cells were exposed continuously to HHC for a period of 48 hr, after which time they were harvested and processed as described in "Materials and Methods." Approximately 10,000 cells were scored by FACS analysis to determine the percentage of cells in defined stages of the cell cycle ($G_0/G_1$, S, and $G_2/M$) or undergoing apoptosis (Apop). Values are means ± SEM from 3 individual experiments, each done in duplicate. P values were determined using Student's *t*-test. For both treatments, the distribution profiles of $G_0/G_1$ and S phase were significantly different from vehicle controls ($P < 0.001$; ***). The profiles for $G_2/M$ from the 1.5 μM treatment ($P < 0.001$; ***) and for apoptosis from the 25 μM treatment ($P < 0.01$; *) were also significantly different from the vehicle controls.**

### *HHC Effects on the Cell Cycle*

The pulse exposure data in Fig. 3 suggest that HHC may elicit its antitumor cell proliferation activity by affecting the cell cycle or a cell cycle-dependent pathway(s). To determine if HHC had any gross effect on the distribution of cells throughout the cell cycle, FACS analysis was performed. The distribution of cells throughout the cell cycle after 48 hr of continuous exposure to HHC is shown in Fig. 4. It is clear that DMSO (0.2%, v/v) has no significant effect on the distribution profile; however, both 1.5 and 25 μM HHC exposures showed different profiles from the controls and each other ($P < 0.001$). With 1.5 μM HHC there was a 25.7% increase in the number of cells detected in $G_0/G_1$, a 34.7% decrease in the number of cells detected in $G_2/M$, and a 39.2% decrease in the number of cells detected in S phase. With 25 μM HHC there was a 25.2% decrease in the number of cells detected in $G_0/G_1$ and a 38.1% increase in the number of cells detected in S phase. Although there was a significant increase in the amount of cells detected undergoing apoptosis in the presence of 25 μM HHC ($P < 0.01$), the percentage of the total (3%) was not enough to suggest that it is a major mechanism involved in the antitumor cell proliferation activity observed at low HHC concentrations (0.85 to 2.19 μM) where there appears to be no significant effect on apoptosis. The profile for 72 hr of continuous drug exposure was similar (data not shown).

### *HHC Effects on Macromolecules and Macromolecular Synthesis*

Due to the novel nature of the use of hop-derived α- and β-acids as antitumor cell proliferation agents and consequently the limited data base, a broad approach was taken to investigate potential mechanisms responsible for this activity of HHC *in vitro*. Initial experiments were performed to investigate some of the mechanisms of action known to be involved in the activity of currently used anticancer agents [37, 38]. Through these investigations it was determined that HHC does not induce double-stranded DNA breaks or protein-linked DNA breaks (data not shown). This suggests that HHC is not a gross DNA-damaging agent (similar to cyclophosphamide and mitomycin) or a compound that interferes with topoisomerase (similar to etoposide and camptothecin). HHC was also determined not to generate free radicals via $H_2O_2$ production (data not shown), which suggests that its antitumor cell proliferation activity does not involve free radical generation (as is often observed with quinones).

To further elucidate potential mechanisms responsible for the antitumor cell proliferation activity observed *in vitro*, the effect of HHC on the incorporation of [$^3$H]precursors into their respective macromolecules was investigated. The results can be interpreted as the effect HHC has on macromolecular synthesis (DNA, RNA, and/or protein) as well as specific effects on the transport and/or metabolism of the precursors ([$^3$H]T, [$^3$H]U and/or [$^3$H]Leu). Table 3 summarizes the effects HHC had on the amount of [$^3$H]precursor detected in the acid-soluble fraction (i.e. the unincorporated fraction) and the acid-insoluble fraction (i.e. DNA, RNA, and protein) of MCF-7 cells after 1.5 and 18 hr of HHC exposure.

A 1.5-hr exposure to 1.5 μM HHC led to a significant decrease in the insoluble fractions of all precursors, a decrease in the soluble fraction of uridine, and an increase in the soluble fraction of leucine. There was no significant effect on the soluble fraction of thymidine. A 1.5-hr exposure to 25 μM HHC led to a significant decrease in the insoluble fractions of all precursors, a decrease in the soluble fraction of uridine, and an increase in the soluble fraction of thymidine. There was no significant effect observed on the soluble fraction of leucine. An 18-hr exposure to 1.5 μM HHC led to a significant decrease in the insoluble fractions of uridine and leucine and in the soluble fraction of uridine. There was no significant effect on the soluble fractions of thymidine and leucine or the insoluble fraction of thymidine. An 18-hr exposure to 25 μM HHC led to a significant decrease in the soluble and insoluble fractions of all precursors. The vehicle (DMSO; 0.2%, v/v) had little/no effect on the acid-soluble or -insoluble fractions of all precursors (data not shown).

**TABLE 3. Effect of HHC on the unincorporated and incorporated fractions of radiolabeled macromolecular precursors***

| HHC (μM) | Thymidine (% of control) | | Uridine (% of control) | | Leucine (% of control) | |
|---|---|---|---|---|---|---|
| | Soluble† | Insoluble‡ | Soluble | Insoluble | Soluble | Insoluble |
| 1.5§ | 107.2 ± 6.4 | 68.8 ± 9.1[a] | 67.0 ± 6.9[a] | 66.6 ± 8.0[a] | 161.6 ± 6.9[a] | 80.1 ± 6.5[b] |
| 25§ | 127.2 ± 9.4[b] | 58.1 ± 5.7[a] | 84.8 ± 3.6[b] | 52.1 ± 6.3[a] | 93.4 ± 6.0 | 31.5 ± 2.5[a] |
| 1.5‖ | 94.8 ± 22.8 | 88.6 ± 20.2 | 61.4 ± 5.6[a] | 59.3 ± 6.8[a] | 101.9 ± 3.6 | 68.8 ± 1.3[a] |
| 25‖ | 52.6 ± 9.4[a] | 25.7 ± 5.9[a] | 72.8 ± 3.8[a] | 25.5 ± 2.6[a] | 51.8 ± 3.5[a] | 16.5 ± 1.7[a] |

*MCF-7 cells were treated with HHC for 1.5 and 18 hr after which time PCA extraction was used to separate the unincorporated precursor from the incorporated. Student's *t*-test was used to evaluate the significant difference between the drug-treated effect and the control effect ($P < 0.001$ = a; $P < 0.01$ = b). Values are means ± SEM obtained from 3 experiments, each done in duplicate.

† Acid-soluble fraction (i.e. unincorporated precursor).

‡ Acid-insoluble precursor (i.e. incorporated precursor; DNA, RNA, and protein).

§ 1.5-hr HHC exposure.

‖ 18-hr HHC exposure.

### *Determining the MTD of HHC*

Experiments were conducted to determine the MTD of HHC that could be delivered intraperitoneally to female BDF1 mice (Table 4). All animals survived 100, 150, and 200 μM HHC. However, 40% of the animals given 400 μM HHC died and none of the animals in the 800 μM group survived. These results suggest that BDF1 mice could tolerate an intraperitoneal injection of 200 μM HHC. To further confirm these results, 5 female BDF1 mice were injected 5 times (once each day for 5 consecutive days) with HHC to an estimated final *in vivo* concentration of 200 μM and their ability to tolerate the dosing schedule was evaluated. One hundred and twenty days post injection all animals were alive and appeared to be healthy.

## DISCUSSION

In the present report, HHC, a semisynthetic derivative of the hop-isolated β-acid colupulone, was shown to inhibit the proliferation of tumor cells *in vitro*. When the abilities of colupulone and HHC to inhibit the growth of CEM and CEM-V cells are compared, a number of conclusions can be reached. First, the compounds are efficacious at inhibiting the growth of both tumor cell lines *in vitro*. Second, the CEM-V cell line is approximately 1.5-fold more resistant to colupulone and HHC than the CEM cell line. However, this resistance may not be enough to suggest that these compounds are good substrates for the MDR protein, as demonstrated by the approximately 11-fold resistance of CEM-V cells to a known substrate (doxorubicin) (data not shown). To further investigate the involvement of the MDR protein in HHC resistance, we intend to look for increased expression of this protein in the MCF-7-HHC cell line developed by our laboratory. Finally, HHC appears to be more potent (2.3-fold) than colupulone. This suggests that derivatization of colupulone, and possibly HHC, may lead to other novel compounds with an enhanced ability to inhibit tumor cell proliferation. The enhancement seen in the activity due to saturation of the side chains of colupulone during the synthesis of HHC may be due to a better ability of HHC to interact with its target(s). This enhanced activity could also be due to the more hydrophobic quality of HHC, allowing it to enter the cell more easily than colupulone and consequently reach its target(s) more efficiently.

Further investigation of the activity of HHC against a number of other well established human tumor cell lines (i.e. breast carcinoma, Burkitt lymphoma, oral epidermoid carcinoma, and cervical epitheloid carcinoma) suggests that this compound has a reasonably wide spectrum of activity, including activity against solid tumors and leukemias as well as cell lines that are resistant to currently used chemotherapeutic agents (Tables 1 and 2). The MCF-7-OAP cell line was shown previously to be resistant to cyclophosphamide through a mechanism involving increased aldehyde dehydrogenase activity [39]. The fact that the $IC_{50}$ of HHC in the MCF-7-OAP cell line was similar to that of the parent MCF-7 cell line suggests that the mechanism(s) responsible for the antitumor cell proliferation activity of HHC appears not to be affected by an increase in aldehyde dehydrogenase activity. As previously discussed, the lack of apparent resistance to HHC treat-

**TABLE 4. Effect of intraperitoneal injections of HHC on mouse survival**

| Group | Average weight (mg) | [HHC] Delivered* (mg/kg) | Survival after 90 days |
|---|---|---|---|
| Not injected | 18.7 ± 1.1 | 0.00 | 5/5 |
| DMSO | 19.6 ± 0.3 | 0.00 | 5/5 |
| 50 μM | 19.6 ± 0.7 | 0.81 | 3/5† |
| 100 μM | 19.6 ± 1.0 | 1.63 | 5/5 |
| 150 μM | 19.3 ± 0.7 | 3.25 | 5/5 |
| 200 μM | 19.4 ± 0.8 | 6.50 | 5/5 |
| 400 μM | 19.9 ± 1.0 | 13.00 | 3/5‡ |
| 800 μM | 19.4 ± 1.2 | 26.00 | 0/5§ |

* Female BDF1 mice, approximately 11-weeks-old, were injected intraperitoneally with HHC dissolved in 100% DMSO. Final HHC concentrations were calculated using 8% of mouse body weight as blood volume and assuming that 100% of the injected HHC reached the blood. Approximately 100 μL was injected into each animal once every 3 days for 9 consecutive days (3 injections total), and their ability to survive was observed.

† One animal died after the first injection and another animal died after the second injection.

‡ Both animals died after the first injection.

§ All animals died within 45 min after the first injection.

ment observed in CEM-V cells suggests that HHC may not be a substrate for the MDR protein.

When investigating the effects of HHC using a pulse exposure protocol, it was observed that with increasing exposure time to the drug, there was a decrease in the $IC_{50}$ of HHC in MCF-7 cells (Fig. 3). This clearly demonstrates that there is a drug-induced inhibition of tumor cell growth observed with HHC exposures and that a time-dependent mechanism(s) is involved. A pulse of 18 hr led to an $IC_{50}$ approximately 1.3-fold that observed with continuous exposure to HHC and a 6-hr pulse led to an $IC_{50}$ approximately 28.9-fold that observed with continuous exposure. This suggests that the antitumor cell proliferation activity of HHC may be due to time-dependent metabolism (i.e. a metabolite) of HHC. It is also possible that HHC may be affecting the cell cycle and/or a cell cycle-dependent pathway(s).

Indeed, the effects of HHC on the distribution of cells throughout the cell cycle after a given time of exposure clearly suggest that the antitumor cell proliferation activity of HHC may involve the cell cycle and/or a cell cycle-dependent pathway(s) (Fig. 4). These cells were not synchronized; therefore, the effect on the distribution profile is an overall summary of the effect HHC has on the distribution of cells throughout the cell cycle at a particular time. Further investigations using synchronized cells are necessary to determine what stage(s) is being affected specifically. The reasons for the differences observed in the distribution of cells at low versus high HHC concentrations are not clear at this time.

The $IC_{50}$ values for HHC in all the tumor cell lines evaluated were approximately 0.85 to 2.19 μM. It is possible that the effects observed using 25 μM HHC may not be specific and consequently may not be as important to the mechanism(s) responsible for the activity seen using 1.5 μM HHC. For this reason, a possible mechanism by which 1.5 μM HHC inhibits tumor cell proliferation is by increasing movement of cells to arrest, therefore increasing the number of cells detected in $G_0/G_1$ and decreasing the number of cells detected in S phase. Interestingly, our investigation of the $IC_{50}$ of HHC at concentrations ranging from 1.5 to 25 μM suggests that the drug effect is mostly cytostatic and not cytotoxic. This is supported by the observation that cell counts after HHC treatment do not decrease significantly below the initial seeding density during the time of investigation (data not shown). This also suggests a potential cell cycle-dependent mechanism for HHC and lends support for the previously discussed explanation for the cell cycle distribution profile observed with 1.5 μM HHC treatment. We are currently investigating these possibilities.

Although HHC does affect the incorporation of [$^3$H]precursors into their respective macromolecules, it may not be enough to suggest that these effects are responsible for the antitumor cell proliferation activity observed, though they may be involved. Just as for the effects observed on the cell cycle, the effects observed for 1.5 μM HHC treatment on

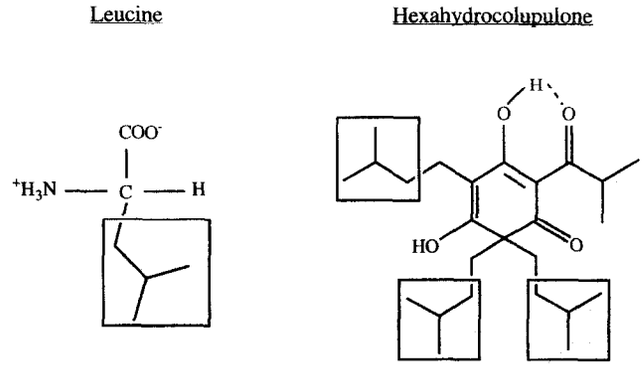



**FIG. 5. Possible explanation for the more pronounced effect of HHC on [$^3$H]Leu incorporation into MCF-7 cells. The boxed areas of the HHC molecule are identical to the boxed area on the leucine molecule.**

[$^3$H]precursor incorporation may be more relevant to the antitumor cell proliferation activity than the observations for 25 μM HHC treatment (Table 3). The pulse exposure data suggest that there is a time-dependent effect on tumor cell growth. HHC needs to be present for at least 18 hr to achieve an $IC_{50}$ value which approaches that for continuous exposure. Extrapolation of the curve (Fig. 3) to determine the theoretical value for a 1.5-hr $IC_{50}$ for HHC in MCF-7 cells gives an $IC_{50}$ of 1740 μM. It is technically impossible at this time (i.e. due to solubility issues) to determine if this is true. If the theoretical $IC_{50}$ value is true, the effects of much lower concentrations of HHC given for only 1.5 hr may not be that important to the mechanism(s) responsible for the antitumor cell proliferation activity observed and, therefore, the incorporation data obtained for 18 hr of HHC exposure are more important. Consequently, an 18-hr exposure of MCF-7 cells to HHC (1.5 μM) appears not to affect the acid-soluble or -insoluble fractions of thymidine. This suggests that HHC does not affect thymidine transport and/or metabolism or DNA synthesis. In contrast to thymidine, both the acid-soluble and -insoluble fractions of uridine appear to be decreased to the same degree with HHC (1.5 μM) treatment. This suggests that HHC may be affecting the transport and/or metabolism of uridine as well as RNA synthesis. It is also possible that the decrease in the acid-soluble fraction becomes the limiting factor for the incorporation of uridine into RNA and consequently the insoluble fraction is decreased. The effect of HHC (1.5 μM) on leucine appears to be specific to a decrease in the acid-insoluble fraction, while there is no effect on the soluble fraction. This suggests that HHC may affect protein synthesis and/or leucine incorporation more specifically. We propose that HHC may affect leucine more specifically because the hydrophobic side chains on the HHC molecule are very similar to the structure of leucine (Fig. 5). Side chains of the intact HHC molecule or side chains cleaved via metabolism may ultimately compete for the [$^3$H]Leu utilized in the incorporation studies. We are presently investigating these possibilities.

We are currently taking advantage of two recently

acquired "tools" that can be used to further investigate the mechanism(s) responsible for the antitumor cell proliferation activity of HHC. They are the MCF-7-HHC resistant cell line developed by our laboratory and our possession of [$^3$H]HHC. It is our intention to evaluate the effects of xenobiotics with known mechanisms of action on the survival of MCF-7-HHC resistant cells. This information may suggest potential pathways by which HHC elicits its antitumor cell proliferation activity. Elucidating the mechanism(s) of resistance may also suggest a potential mechanism(s) for the activity of HHC. [$^3$H]HHC can be used for a number of investigations including subcellular distribution of HHC, metabolism of HHC, and binding of HHC to cellular constituents.

Clearly, HHC is a novel compound that has been demonstrated to inhibit the proliferation of many different human tumor cell lines *in vitro*, including those exhibiting resistance to currently used chemotherapeutic agents. Closer evaluation of potential mechanisms suggests that HHC has an effect on the cell cycle and/or a cell cycle-dependent pathway(s). HHC has also been demonstrated to have an effect on the incorporation of radiolabeled precursors into their respective macromolecules, which can be interpreted as effects on the transport and/or metabolism of the precursors as well as effects on macromolecular synthesis. Investigations by us have shown that HHC is not a gross DNA-damaging agent, does not interfere with topoisomerase, and does not generate free radicals. MTD determinations of HHC *in vivo* have demonstrated that a concentration of >100-fold the $IC_{50}$ value determined *in vitro* can be injected intraperitoneally into mice and be well tolerated. Mass spectroscopy analysis and X-ray crystallography have confirmed the purity of HHC and, consequently, all observed effects can be attributed to HHC and/or a metabolite(s) acting either alone or in combination. The existence of the hydrophobic moiety located on ring carbon 6 of the HHC molecule dramatically changes the charge distribution when compared with colupulone. Consequently, the enhanced antitumor cell proliferation activity seen with HHC suggests that this portion of the molecule may be important in the increased activity observed when compared with colupulone. Although the toxicity of this compound is yet to be determined in humans, the fact that HHC is a congener of natural products that have been ingested for millennia suggests that toxicity of at least moderate amounts of this compound may be minimal. Finally, our recent investigations of the spectrum of activity of HHC as an antimicrobial agent (Stephan *et al.*, unpublished results) suggest that HHC is a unique entity. Specifically, HHC possesses a selective spectrum of activity against infectious agents (gram positive bacteria and mycobacteria), which may relate to its unique structure and potential antimicrobial mechanism(s) of action. The aforementioned and the as of yet unidentified and potentially unique mechanism(s) of action responsible for the antitumor cell proliferation activity of HHC suggest that this compound may be clinically useful, especially with respect to drug-resistant tumors and potentially against microbial infections. Collectively, the antitumor activity of HHC observed in this study in conjunction with the recently obtained data demonstrating the antimicrobial activity of HHC indicates that further studies are mandated.

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