Hypoestoxide inhibits tumor growth in the mouse CT26 colon tumor model

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Abstract

AIM: To evaluate the effect of the natural diterpenoid, hypoestoxide (HE) on the growth of established colon cancer in mice.

METHODS: The CT26.WT mouse colon carcinoma cell line was grown and expanded in vitro. Following the expansion, BALB/c mice were inoculated s.c. with viable tumor cells. After the tumors had established and developed to about 80-90 mm³, the mice were started on chemotherapy by oral administration of HE, 5-fluorouracil (5-FU) or combination.

RESULTS: The antiangiogenic HE has previously been shown to inhibit the growth of melanoma in the B16F- tumor model in C57BL/6 mice. Our results demonstrate that mean volume of tumors in mice treated with oral HE as a single agent or in combination with 5-FU, were significantly smaller (> 60%) than those in vehicle control mice (471.2 mm³ vs 1542.8 mm³, P < 0.01). The significant reductions in tumor burden resulted in pronounced mean survival times (MST) and increased life spans (ILS) in the treated mice.

CONCLUSION: These results indicate that HE is an effective chemotherapeutic agent for colorectal cancer in mice and that HE may be used alone or in combination with 5-FU.

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Key words: Hypoestoxide; 5-Fluorouracil; Colon, Cancer; Mice

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**Table 1** Oral administration of HE inhibits the growth of s.c. implanted CT26 colon tumor in BALB/c mice

| Drug          | Dose (mg/kg per day) | (d 18) (mean ± SE) | MST (d) (mean ± SE) | ILS (%) |
|---------------|----------------------|--------------------|---------------------|---------|
| Vehicle control (PBS/DMSO) | 0                  | 1542.8 ± 330       | 34.0 ± 6.2          | 0       |
| HE            | 1 × 10               | 738.7 ± 158        | 43.0 ± 1.2          | 25      |
| HE            | 5 × 10               | 582.3 ± 116        | 77.0 ± 4.2          | 126     |
| HE            | 50 × 1               | 288.6 ± 188        | 52.7 ± 3.0          | 54      |
| 5-FU          | 25 × 1               | 911.1 ± 144        | 48.0 ± 3.6          | 40      |
| 5-FU          | 100 × 1              | 486.7 ± 171        | 59.7 ± 4.8          | 75      |
| 5-FU + HE     | (5-FU) 25 × 1 + (HE) 1 × 10 | 57.7 ± 4.0         | 69                  |

PBS/DMSO: phosphate buffered saline/dimethyl sulfoxide; MST: mean survival times; ILS: increased life spans. MST: 75.0 ± 3.8 (HE-treated mice at 5 mg/kg × 10) vs Vehicle control (30.0 ± 4.1). ILS: 150% (HE-treated mice) vs 0% (Vehicle control mice), P < 0.01.

Aldrich (St. Louis, MO). Phosphate buffered saline (PBS), Dulbecco’s minimal essential medium (DMEM), and other culture media components were purchased from Irvine Scientific (Irvine, CA).

**Cell line**

CT26.WT mouse colon carcinoma cell line was purchased from ATCC, Manassas, VA. The cell line was grown as monolayer in DMEM culture medium containing 10% FBS and 1% L-glutamine. Full grown monolayer cultures were trypsinized for 15 min (0.25% trypsin-EDTA), harvested and passaged several times for expansion.

**Experimental procedure**

Following the growth and expansion of the cell line in *vitro*, trypsinized cells were harvested, washed, counted by trypan blue dye exclusion method and cell density was adjusted to 10 × 10⁶/mL in PBS. A suspension of 2 × 10⁶ viable tumor cells in 0.2 mL PBS was inoculated s.c. into the left flanks of 35 mice. After tumors developed to about (80-90) ± 10 mm³ volume, the mice were randomized into seven groups as depicted on Table 1. They were treated with varying doses of HE alone, 5-FU alone or combination of lower doses of HE (1 mg/kg) and 5-FU (25 mg/kg) *via* oral administration with a gavage needle attached to a 1.0 cc syringe.

**Statistical analysis**

A student *t* test was used to determine significance of difference between tumor burdens in vehicle control mice and mice treated with HE as a single agent or in combination with 5-FU. Survival data are presented in days as mean ± SE.

**RESULTS**

Mice receiving varying doses of HE, 5-FU or combination experienced significant tumor growth inhibition as compared to controls. Mean % tumor growth inhibition obtained for HE was 65%; 5-FU, 55%; and HE + 5-FU, 82% relative to vehicle control. The additive effect of HE + 5-FU combination resulted in 69% ILS as compared to 25% ILS for HE and 40% ILS for 5-FU when tested alone at each of their respective lowest doses (Table 1). Treatment was started when tumors had a mean volume of 80-90 mm³. Five female BALB/c mice were allocated to each group. Tumor volume at the start of treatment (d 0) and on d 18 after tumor implantation is shown. MST and ILS (%) were calculated for each group. MST was calculated from the period between tumor implantation and the day of death. ILS (%) was calculated using MST for each drug-treated mouse as follows:

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

This experiment was conducted twice with similar results.

**DISCUSSION**

HE is a novel and unique nonsteroidal antiinflammatory drug (NSAID) because it does not inhibit cyclooxygenase (COX) activity. The mechanism of action that defines NSAIDs as a class is their ability to inhibit COX activity. Several studies have established that numerous NSAIDs such as sulindac, aspirin, celecoxib, piroxicam and ibuprofen inhibit or prevent colorectal neoplasia in rodents and humans because of their ability to inhibit COX activity. HE has been shown in this report to be effective at reducing tumor burden in mice with colorectal cancer and it is thus similar to sulindac sulfide, a metabolite of sulindac sulfoxide, an NSAID which has also been shown to be effective against murine and human colorectal cancer without inhibitory effect on COX activity. The various mechanisms by which HE inhibits tumor growth include its ability to arrest cell cycle at G2-M phase by interference with actin assembly, inhibit angiogenesis, vascular endothelial growth factor (VEGF)-induced cell proliferation and endothelial cell migration. All of these mechanisms have been shown to contribute to the treatment of colon cancer. Conversely, 5-FU is known to trigger apoptosis by depleting thymidine, partially through inhibition of thymidine synthase and partially through direct incorporation into RNA and DNA. Because HE lacks alkylating properties, toxicity, and uses several other aforementioned mechanisms, it is therefore consistent that the combination of low doses of HE with low doses of 5-FU enhances the anti-tumor responses of 5-FU.

Interestingly, consumption of the dried leaf powder of *Hypoestes rosea* (the parent plant of HE), as a dietary supplement, resulted in the elimination of existing intestinal polyps in human subjects (Nchekwube, unpublished results). Collectively, these results indicate that HE may be a promising chemotherapeutic agent either alone or in combination with 5-FU against colorectal cancer.

**COMMENTS**

**Background**

Plants and their products have been used for medicinal purposes for thousands of years. Drugs from natural bio-resources have often been discovered on the basis of ethno-botanical information provided by herbalists living in regions of the world rich in bio-resources. Hypoestoxide is an investigational new drug isolated from the plant *Hypoestes rosea* (Acanthaceae) which is indigenous to the rain forest

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regions of Nigeria. The natives have long used the $H$ rosea leaf extracts in folk medicine to treat various ailments. In this article, old world and new world medicine are brought together.

**Research frontiers**

The findings in this article relate well to the present state of the field in regard to the use of natural products as pharmaceutical agents. Artemisinin is an example of a natural product isolated from the Chinese plant, Artemisia annua. Currently, Artemisinin is used for the treatment of malaria. Interestingly, Hypoestoxide has also been found to possess anti-malarial activity. Curcumin, a polyphenolic antioxidant from a dietary spice is in clinical development for anti-cancer and anti-inflammatory activities. Vincristine is another example of a natural product (isolated from the rose periwinkle flower) which is used as a standard anti-cancer agent.

**Innovations and breakthroughs**

The findings are significant and novel because Hypoestoxide is an investigational new drug for which new indications are being sought. This is the first report on both the chemotherapeutic effect of Hypoestoxide on colon cancer and its additive effect with a standard chemotherapeutic agent, 5-Fluorouracil.

**Applications**

Future applications of the findings in this article will be in the areas of single and combination drug therapies for colorectal cancer. The findings also lend support to the use of NSAIDs such as Hypoestoxide, as anti-cancer agents. NSAIDs have long been associated with tumor chemoprevention and inhibition of the growth of established tumors.

**Terminology**

NSAIDs are anti-inflammatory drugs that are not steroids. Anti-angiogenesis is the process by which a drug inhibits the growth of an established tumor by shutting off the blood supply to the tumor. CT26 is an N-nitroso-N-methylurethane-(NMU)-induced, undifferentiated colon carcinoma cell line. It was cloned to generate the cell line CT26.WT (ATCC CRL-2638).

**Peer review**

The paper is well written. Using the simple and reliable methods, the authors studied the effect of hypoestoxide (HE) on mouse colon adenocarcinoma, and give us a definite conclusion: HE is an effective chemotherapeutic agent for colorectal cancer in mice and that HE may be used alone or in combination with 5-FU. The conclusion will help us to deeply understand HE’s value in the treatment of colorectal cancer.

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