Cytotoxic Effects of Haplamine and its Major Metabolites on Human Cancer Cell Lines

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- *trans/cis*-3,4-dihydroxyhaplamine
- cytotoxicity
- cancer cell lines
- Hapophyllum perforatum
- Rutaceae

Abstract

Haplamine, extracted from *Haplophyllum perforatum*, is widely used in Central Asia for treating various diseases, including testicular cancer. The purpose of the present study was to investigate *in vitro* the cytotoxic properties of haplamine and its major metabolites (*trans/cis*-3,4-dihydroxyhaplamine) on human pancreatic cancer, colorectal cancer and hepatic cancer cell lines. The efficacy of haplamine was compared with those of the respective reference drugs for treating digestive cancers (e.g., 5-FU, gemcitabine). Finally, the implication of apoptosis in haplamine-induced cell death was investigated. The IC_{50} values of of haplamine were 52.5 ± 2.6, 24.3 ± 0.7; 41.5 ± 2.5, 72 ± 2, 32 ± 2.2 and 59.7 ± 2.1 μM in human pancreatic cancer (Capan1 and Capan2), colorectal cancer (LS174T, HT29, and SW620) and hepatic cancer (HepG2) cells, respectively. The IC_{50} values of *trans/cis*-3,4-dihydroxyhaplamine were both > 200 μM, thus suggesting that the previously reported cytotoxic efficacy of haplamine was supported by the parent drug only. Besides, our data showed that haplamine leads to cell death through the induction of early/late apoptosis in the target cells. Interestingly, we found that haplamine showed significant antiproliferative efficacy on resistant SW620 colorectal cells, whereas the reference drug 5-FU was ineffective (32 vs. 73 μM, $p < 0.01$ t- test), thus suggesting that haplamine could be of interest for treating digestive cancers resistant to standard fluoropyrimidines. Similarly, haplamine proved to be significantly more potent in pancreatic cells than gemcitabine, the reference cytotoxic drug for treating pancreatic carcinomas. Overall, these results confirm the anticancer properties of haplamine suggested by its traditional use, and indicate that it could be further considered in various other solid tumours frequently encountered in adults, including those resistant to standard chemotherapy.

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Introduction

Plants have been an important source of anticancer agents. The use of plants as medicinal agents predates recorded history, and worldwide efforts are still ongoing to identify new anticancer compounds from plants. Haplamine is extracted from *Haplophyllum perforatum* (Rutaceae), which is widespread in Central Asia. This genus has been used for a long time in folk medicine by local populations for treating different diseases such as warts, herpes, lichens, erysipelas, and testicular cancer [1]. Haplamine has sedative and analgesic properties and low *in vivo* toxicity (LD_{50} = 1020 mg/kg, *i.p.*, mice) [2]. Of note, haplamine can be obtained by synthesis [3], a property that could be interesting for further studies as part of a future possible pharmaceutical development.

A preliminary study screening 14 alkaloids isolated from *Haplophyllum* A. Juss. for their cytotoxic properties showed that haplamine seemed to be significantly more cytotoxic than other selected alkaloids, with IC_{50} values < 100 μM on two cancer cell lines, and notable activity on a wide range of other cancer models [4].

In a previous study, we have identified by gas chromatography-mass spectrometry after silylation *trans/cis*-3,4-dihydroxyhaplamine (M3 and M4) as being the two major metabolites of haplamine [5]. Regarding the importance of the metabolization of haplamine, it was hypothesized that 3,4-dihydroxyhaplamine isomers could partly explain the cytotoxicity [6]. In this respect, assessing the antiproliferative properties of the parent drug and its metabolites could be critical for a better understanding of the pharmacology of this drug.

The aim of this work was therefore to study the cytotoxic effect of the previously identified metabolites of haplamine (M3 and M4) on various human digestive cancer cell lines, and to compare their activities with reference, standard anticancer drugs such as 5-FU and gemcitabine.

Materials and Methods

Chemicals and reagents

Haplamine was extracted from *Haplophyllum perforatum* Kar. and Kir. at the Institute of Plant Substances (Tashkent, Uzbekistan) according to the procedure cited previously [7] and identified by comparison with an authentic sample [8]. *Trans/cis*-3,4-dihydroxyhaplamines were synthesized from haplamine as described previously [5]. The purity of haplamine and *trans/cis*-3,4-dihydroxyhaplamines (98, 97, 96%, respectively) was determined by HPLC. RPMI medium, fetal calf serum, streptomycin, penicillin, kanamycin, 3-(4,5-dimethyltriazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and other chemicals and materials were purchased from Sigma Aldrich. The Annexin V-FITC kit was obtained from Stressgen.

Cell lines

Human colorectal adenocarcinoma (SW620, LS174 T and HT29), human pancreatic carcinoma (Capan1 and Capan2), and human hepatic carcinoma (HepG2) cells were obtained from the American Type Culture Collection (ATCC). SW620, Capan1 and Capan2 cells were resistant to 5-FU and gemcitabine, respectively. Cells were cultured at 37 °C in a fully humidified 5% carbon dioxide atmosphere in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS), 110 IU penicillin/mL, 100 μg streptomycin/mL and 50 μg kanamycin/mL.

Preparation of product solutions

Compounds were dissolved in DMSO to give the desired stock solutions. Each solution was diluted at different concentrations with culture medium. The maximum concentration of DMSO (0.02%) used in the experiments did not affect cell viability (data not shown). The chemical structure of these compounds is shown in **Fig. 1**.

Antiproliferative assays

Cells were plated in 96-well flat bottom plates at 5,000 to 10,000 cells/well in standard medium. The difference in cell numbers plated adjusts for differences in growth rates of the various cell lines. After allowing the cells to attach overnight, they were exposed to increasing concentrations of haplamine (0.01 – 100 μM) and *trans/cis*-3,4-dihydroxyhaplamines (0.01 – 500 μM). Drug-free medium supplemented in DMSO (0.02%) was added to the control wells. Plates were incubated at 37 °C, 5% CO_2 for 72 h. The cell growth was evaluated using the classic colorimetric 3-(4,5-dimethyltriazol-2-yl)-2,5-diphenyltetrazolium bromide assay (MTT) [9]. After 2 h exposure to MTT, optical densities were analyzed by reading absorbance at 550 nm with a multiwell spectrophotometer. The respective IC_{50} values were defined as the haplamine, M3 and M4 concentrations inhibiting 50% of cell growth. The absorbance level in control wells was considered as 100% viability. Each experiment was performed on two separate plates (duplicate). Results were finally expressed as mean ± standard deviation of three independent experiments. 5-FU was used as a reference anticancer drug for human colon and liver cancer cells, and gemcitabine as a standard for treating human pancreatic cancer cells. Determination of the respective IC_{50} values of 5-FU and gemcitabine was performed as described above.

Apoptosis studies

Capan2 cells in the exponential phase were exposed to 25 μM haplamine for 72 h. Cells were then harvested, necrosis, early

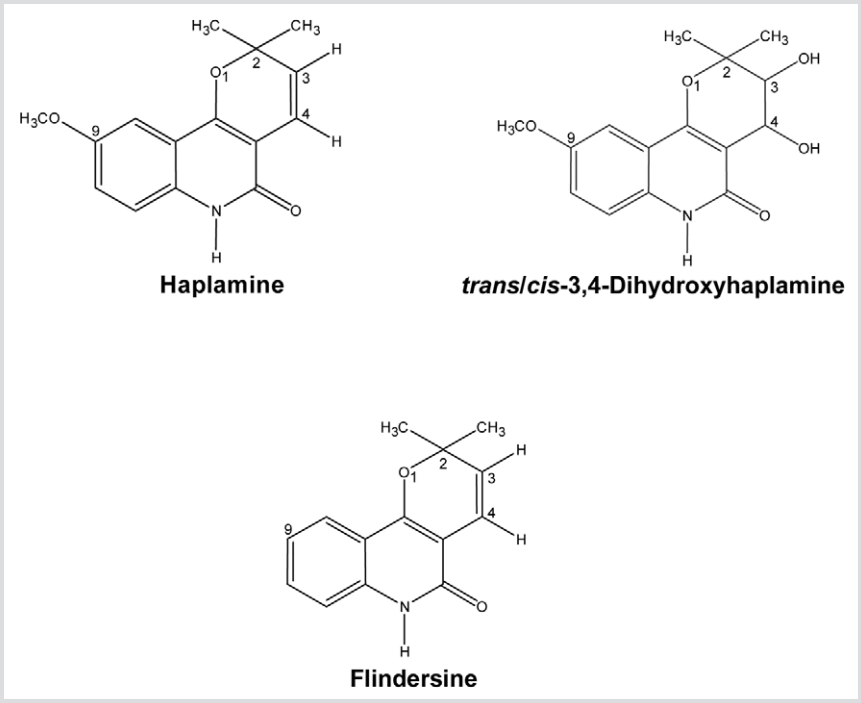

Fig. 1 Chemical structures of haplamine, metabolites M3/M4 and flindersine.

apoptotic changes and late apoptosis were detected by simultaneous staining with annexin V and propidium iodide using Annexin V FITC staining kit. Cells were treated following the manufacturer's guideline. FACS analysis was carried out in an FACScan flow cytometer (Becton Dickinson) using Cell Quest Software.

Statistical analysis

Differences in cytotoxic efficacy between treatments were evaluated by performing t tests using Sigma Stat software (Systat) after checking appropriate statistical prerequisites (e.g., normality test and equal variance test). A p value of 0.01 was regarded as statistically significant.

Results and Discussion

Results of the antiproliferative assays are shown in **Table 1**. Little efficacy was observed for the *trans/cis*-3,4-dihydroxyhaplamine metabolites, regardless of the cell lines used, thus suggesting that the anticancer properties of haplamine reported in traditional use were not supported by its metabolites but solely by the parent drug. The differences in IC_{50} values observed between haplamine and its metabolites demonstrate that the cytotoxicity of alkaloid drugs depends on their degree of substitution and their stereochemistry. This observation is in agreement with a previous study showing such differences between haplamine and flindersine (**Fig. 1**), an alkaloid similar to haplamine but with no methoxy function [4]. These reported differences warrant the need for further research on the different function and position of substituents on the basic structure to generate more potent compounds in the future.

Haplamine showed weak efficacy in the hepatocarcinoma model HepG2, and contradictory effects on colorectal cells as compared with the reference 5-FU, ranging from 2.3-fold more potent (SW620, $p < 0.001$, t test) to 101-fold less cytotoxic (HT29) than the fluoropyrimidine drug. Data on SW620 are of particular interest since this is a canonical model for studying resistance to fluoropyrimidine drugs by thymidylate synthase (TS) overexpression, a frequent mechanism of drug-resistance observed in clinical oncology [10]. The fact that haplamine shows marked, significant antiproliferative efficacy on these resistant cells whereas the standard 5-FU shows little effect suggests that the cytotoxicity of haplamine is probably not supported by an anti-TS mechanism. Resistance to 5-FU in relation with TS overexpression is a major concern in clinical oncology, this preliminary observation warrants further studies to elucidate whether haplamine could be of interest in digestive tumours resistant to standard fluoropyrimidine drugs [11]. Beside resistant colorectal cancer cells, our data showed that haplamine displayed notable efficacy on resistant pancreatic cells as well, with IC_{50} values up to 4-fold lower than the ones measured with gemcitabine ($p < 0.01$, t test), the reference drug for treating pancreatic cancer [12]. Regarding the frequent, spontaneous chemoresistance of most pancreatic adenocarcinomas, this observation is of particular interest and warrants further studies to be performed in other pancreatic models to confirm the better efficacy of haplamine as compared with canonical gemcitabine.

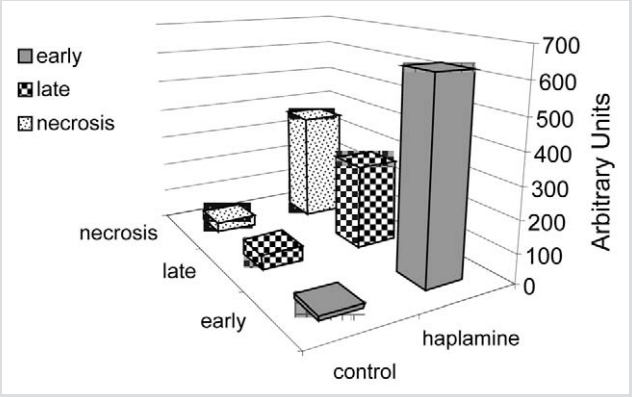

Fig. 2 Apoptosis induced by 25 μM haplamine (72 h) in Capan2 cells. Necrosis and early/late apoptosis were discriminated by simultaneous propidium iodide + annexin V double staining with subsequent flow cytometry analysis.

Further apoptosis studies have been performed on resistant Capan2 cells, to evaluate the part played by apoptosis induction in the cytotoxic effect observed. Apoptosis was measured at 72 h, a time-window defined previously when our group evaluated the effects of various anticancer drugs on pancreatic or colorectal cells [13]. Results are summarized in **Fig. 2** and showed that cells exposed to haplamine displayed a 628% increase in early apoptotis, a 275% increase in late apoptosis and a 360% increase in necrosis. Our data indicate therefore that the antiproliferative properties of haplamine are supported by a higher induction of both early and late apoptosis, and not solely arrest of cell proliferation or necrosis. This finding can explain retrospectively why haplamine showed little efficacy on colorectal HT29 cells, a model with mutated p53 and subsequent disruption in apoptosis signalling pathways. This suggests therefore that haplamine triggers apoptosis in a p53-dependent fashion. Overall, although only preliminary, these *in vitro* data are promising since hapla-

Table 1 *In vitro* cytotoxicity (IC_{50} μM, mean ± S.D.) of haplamine and *trans/cis*-3,4-dihydroxyhaplamine (M3 and M4) on human cancer cell lines

Cell lines	IC_{50} (μM), mean ± S.D.				
	Haplamine	M3	M4	5-FU	Gemcitabine
HT29	72.8 ± 2.0	460 ± 15	450 ± 20	0.72 ± 0.02	
LS174T	41.5 ± 2.5	342.5 ± 22.5	238 ± 8	54.3 ± 5.0	
SW620	32 ± 2	> 500	> 500	73.3 ± 2.9	
Capan1	52.5 ± 2.6	> 500	> 500	> 100	
Capan2	24.3 ± 0.7	240.7 ± 33.5	204 ± 34	> 100	93 ± 3
HepG2	59.7 ± 2.1			3.8 ± 0.5	

S.D.: Standard deviation.

HT29, LS174T, SW620: human colon cancer cells; Capan1, Capan2: human pancreatic cancer cells; HepG2: human liver cancer cells; M3, M4: *trans/cis*-3,4-dihydroxyhaplamines.

mine proved here to be significantly more effective than the respective reference drugs in human, 5-FU-refractory, colorectal cancers, as well as in pancreatic cancer cell lines.

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