

Anti-neoplastic efficacy of Haimiding on gastric carcinoma and its mechanisms

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Abstract

AIM: To study the anti-neoplastic effect of Haimiding and its mechanisms of action.

METHODS: Experiments using MTT and colony formation were carried out to study the *in vitro* anti-neoplastic action of Haimiding, its *in vivo* anti-neoplastic action was studied by observing its effect on the weight of tumors in FC mice and S₁₈₀, H₂₂ tumor bearing mice, as well as their life spans. The effect of Haimiding on cell apoptosis and different stages of cell cycles in human gastric carcinoma cells were studied by flow cytometry. Its effect on [Ca²⁺]_i of human gastric carcinoma cells and the source of Ca²⁺ during the change of [Ca²⁺]_i were observed by confocal laser scanning technique.

RESULTS: Haimiding showed a definite cytotoxicity to 8 human tumor cell lines, which was most prominent against BGC-823, E_{ca-109} and HCT-8 tumor cells. It also exhibited an obvious inhibition on colony formation of the above tumor cell lines, which was most prominent in E_{ca-109} tumor cells. It showed obvious inhibition on the growth of tumor in FC mice and S₁₈₀ bearing mice as well as prolonged the life span of H₂₂ bearing mice. It was able to induce apoptosis and elevate intracellular [Ca²⁺]_i concentration of tumor cells. The source of Ca²⁺ came from both extracellular Ca²⁺ influx and intracellular Ca²⁺ release.

CONCLUSION: Haimiding is composed of a TCM preparation and 5-fluorouracil. Its anti-neoplastic potency is highly enhanced by synergism as compared with either one of its components. Its mechanisms of anti-neoplastic action can be attributed to its action to initiate apoptosis of tumor cells by opening the membrane calcium channel and inducing intracellular Ca²⁺ release to elevate [Ca²⁺]_i of the tumor cells.

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INTRODUCTION

Haimiding (HMD) is an anti-neoplastic preparation made of a traditional Chinese medicine (TCM) preparation and 5-fluorouracil (5-Fu). Its TCM preparation consists of active ingredients from the extracts of *Sargassum fusiforme* (Harv.) Setch, *Ecklonia kurome* Okam, *Astragalus chrysopterus* Buge, and *Sophora flavescens* Aits. The present study was to observe the *in vivo* and *in vitro* anti-neoplastic activities of HMD, and the effect of HMD on apoptosis of human gastric carcinoma SGC-7901 and intracellular [Ca²⁺]_i, so to better understand the mechanism of its anti-neoplastic action.

MATERIALS AND METHODS

Materials

Drug samples Sterile HMD powder with or without 5-Fu for parenteral use (Lot No.010606) was prepared by the Pharmaceutical Laboratory, Institute of Materia Medica, Harbin Commercial University. 5-Fu (Lot No. 990304) was provided by Haipo Pharmaceutical Factory, Shanghai, China.

Cancer cell lines Human oral epithelial KB, human esophageal carcinoma cell E_{ca-109}, human proventriculus BGC-823, human pulmonary adenocarcinoma A-549, human colon HCT-8, human breast MCF-7, human ovary A₂₇₈₀ and human liver Bel-7402 were provided by the Department of Pharmacology, Institute of Materia Medica, Chinese Academy of Medical Sciences. SGC-7901 was from College of Public Health, Harbin Medical University.

Test animals Kunming strain mice were provided by Veterinary Department of Harbin Medical University, pure 615 strain mice were provided by Institute of Materia Medica, Chinese Academy of Medical Sciences.

Tumor bearing mice strain FC mice, S₁₈₀ A and H₂₂ mice of same sex weighing 20±2 g were supplied by Institute of Materia Medica, Chinese Academy of Medical Sciences.

Reagents Bovine serum and RPMI-1640 culture medium were products of Gibco. MTT was provided by Sigma. Fluo-3/AM fluorescence probe was from Molecular Probe Co., USA. Verapamil was a product of Heng Rui Pharmaceutical Factory, Jiangsu Province, China.

Methods

Anti-neoplastic study *in vitro*

MTT test^[1,2] About 1200 cells were added to each well of a 96 multi-well culture plate. After 24 h each dose of the drug was added to 3 different wells in the test group. Vehicle was added as control in another group. The plate was placed into a humidified incubator (CAN-111, Japan) containing 5% CO₂ and incubated at 37°C for 4 d. Ten μl MTT stock solution (5 mg/ml) was added to each well and shaken well, the incubation was continued for a further 4 h. One hundred μl

acidified 10% sodium dodecanoate sulfonate (SDS) (pH=4.7) was added to each well, which was then left overnight. The absorbance was determined by an EL-311 enzyme labeling apparatus (EL-311, Bio-Tek, USA) at a wave length of 570 nm and a reference wave length of 630 nm. The drug concentration inhibiting 50% of cell growth (IC_{50}) was measured.

Experiments on formation of colonies^[3,4] About 100 test cells were inoculated onto a 35 mm plastic petridish for 24 h and the assigned dose of test drugs was added to each dish. The culture liquid was discarded on the 9th day and the residue was washed 3 times in PBS. The cells were fixed in 10% formaldehyde solution and stained with 0.25% gentian violet. The number of colonies containing more than 50 cells in each dish was counted under a microscope. Vehicles were used instead of test drugs as blank control.

Anti-neoplastic studies *in vivo*

Effect of HMD on tumor weight of FC mice^[5] Tumor cells were transplanted to 615 FC mice. Nine days after transplantation, FC mice bearing the well growing tumor were placed in an ice basin inside an enclosed aseptic working table. Tumor tissues, were separated and then cut into small pieces and homogenized with a glass homogenizer. The homogenate was made into a suspension containing about 5.6×10^6 /ml of tumor cells with sterile normal saline in the conventional way^[6], and 0.2 ml of the suspension was transplanted to the right axilla of each 615 strain mouse. After 24 h, the mice were weighed and randomized into 3 HMD groups (treated respectively *ip* with high, medium and low doses of HMD, equivalent to 27.05 g/kg, 13.53 g/kg and 6.76 g/kg of crude drugs in 0.4 ml injectable solution, once daily), a 5-Fu group treated *ip* with 25 mg/kg 5-Fu in 0.4 ml solution once daily, and a compound TCM group treated *ip* with HMD (without 5-Fu) equivalent to 13.50 g/kg of crude drug in 0.4 ml solution daily. A blank control group was treated *ip* with 0.4 ml normal saline once daily. All drugs were withdrawn on the 11th day. The animals were sacrificed on the next day and their tumors were resected and weighed. Results of 3 repeated experiments were collected and the data were statistically analyzed. The rate of tumor inhibition was calculated according to the following equation:

$$\text{Rate of inhibition (\%)} = \frac{\text{Mean tumor wt of blank} - \text{mean wt of test group}}{\text{Mean tumor wt of blank}} \times 100$$

Effect of HMD on tumor weight of S_{180} bearing mice^[5] S_{180} tumor cells were transplanted into Kunming strain mice. After 7 days, ascites was drawn from the mice bearing well growing tumor under aseptic condition. The tumor cells were diluted with sterile normal saline in ice-bath to 4:1 to produce a suspension containing about 5.8×10^6 /ml of tumor cells. This suspension should have a semi-transparent creamy appearance. Any ascitic fluid with blood streaks should not be used. The mice were transplanted by *sc* injection of 0.2 ml of the suspension to the right axilla of each Kunming mouse according to the procedure described in "National Requirements for *in vivo* Screening of Anti-neoplastic Drugs"^[7]. After 24 h, the transplanted mice were weighed and randomized into groups to be treated with different doses of test drugs. Their tumors were resected, weighed and the rate of tumor inhibition was calculated as above (results of 3 repeated trials).

Effect of HMD on life span of H_{22} bearing mice^[8] Kunming mice were transplanted with H_{22} tumor cells. The mice bearing well growing transplanted tumor were selected after 7 d, and their ascites were drawn under aseptic condition and diluted in ice-cold sterile normal saline to a concentration of 4:1. The tumor cells were mixed well to generate a suspension containing about 6×10^6 tumor cells/ml, and 0.2 ml of the

suspension was transplanted *ip* to each Kunming mouse. After 24 h, the mice were weighed and randomized to different dosage groups as described above. The number of dead mice was recorded every day until no survival left. The rate of life prolongation was calculated according to the following equation:

$$\text{Rate of life prolongation (\%)} = \frac{\text{Survial days in treated group} - \text{survial days in blank}}{\text{Survial days in blank control}} \times 100$$

Effect of HMD on apoptosis of gastric carcinoma cells^[9]

Culturing of tumor cells: Human gastric carcinoma SGC-7901 cells were resuscitated conventionally and cultured in RPMI 1640 culture medium (Gibo) containing 0.1 mass fraction of bovine serum and incubated at 37°C with 95% relative humidity in an incubator containing a volume fraction of 0.05 CO_2 . The cells were generated every 2-3 days.

Experimental groups: Culture flasks containing human gastric carcinoma SGC-7901 cells were divided into 5 groups, 10 flasks each. HMD was added to each flask in the 3 dosage groups to a final concentration of 50 μ l/ml, 100 μ l/ml and 200 μ l/ml, respectively. 5-Fu was added to another group of 10 flasks to a final concentration of 100 μ l/ml. A similar amount of culture media was added to the remaining 10 flasks as blank control. They were incubated in an incubator containing 5% CO_2 for 24 h.

Determination of cell apoptosis by flow cytometry^[7]: Nine volumes of 70% ethanol were added to 1 volume (about 10^6 cells) of cell suspension and fixed at -20°C for 12 h. The cells were kept in 70% ethanol at -20°C for 2-3 weeks, centrifuged and washed in PBS solution. The resulting cells were again suspended in 1 ml of PI staining solution, and stained at room temperature for 30 min. The red fluorescence FCM was detected with a laser (488 nm) or blue light (BG 12 light filter).

Effect of HMD on $[Ca^{2+}]_i$ of gastric cancer cells **Culturing of cells** Human gastric carcinoma SGC-7901 Cells, after resuscitated conventionally, were cultured in RPMI-1640 medium containing 0.1 mass fraction of bovine serum and incubated at 37°C with 95% relative humidity in an incubator containing 0.05 volume fraction of CO_2 . Every 2-3 days were taken for a generation.

Experimental grouping and treatment with test drugs Trypan blue was used to show the number of cells, which was adjusted to about 2×10^5 /ml with culture medium, and stored in different culture bottles.

The experiments consisted of 6 groups (A, B, C_1 , C_2 , C_3 and D) and 4 different periods of time (12, 24, 36 and 48 h). Groups A and B were negative and positive controls respectively. Groups C_1 , C_2 and C_3 were low, medium and high HMD dose groups, and group D was medium dose of HMD plus verapamil.

When the cells attached to the wall after culturing, the culture medium of each group was adjusted to different concentrations. Group B was adjusted to a final concentration of 50 μ g/mL of 5-Fu, groups C_1 , C_2 and C_3 to a final concentration of 200 μ g/ml, 100 μ g/ml and 50 μ g/ml of HMD, respectively and group D to a final concentration of 100 μ g/mL of HMD plus 20 μ g/mL of verapamil. Group A, as a negative control was cultured in RPMI-1640 medium containing 10% bovine serum. These media were cultured for 12, 24, 36 and 48 h respectively, and the resulting cells were collected.

Determination of free Ca^{2+} concentration in cells^[39] SGC-7901 human gastric carcinoma cells, digested after addition of test drugs at different times, were collected and washed twice with 0.1 mass fraction RPMI-1640 and then stained with 0.25 mass fraction of trypan blue. The viable cell count was over 95%. The cell suspension was preheated at 37°C for 5 min and then washed 2-3 times in PBS and calcium free Dakins solution, stained with 100 μ l Fluo-3/AM solution at 37°C for 45 min. A cell suspension was prepared with an appropriate amount of

calcium free Dakins solution. The change of intracellular Ca^{2+} concentration was examined under a confocal laser scanning microscope (Insight Plus-type IQ, meridian, USA), using the intensity of fluorescence light as the criteria of Ca^{2+} concentration, and was observed with a 40 \times objective lens at an exciting wavelength of 488 nm.

Statistical analysis

t test was used for statistical analysis and results were expressed as mean \pm SD.

RESULTS

Anti-neoplastic activity of HMD in vitro

Cytocidal activities of HMD against 8 human cancer cell lines are shown in Table 1. It demonstrated that the actions were more prominent in BGC-823, E_{ca-109} and HCT-8 cell lines in a dose dependent manner. Results from regression calculation showed that IC₅₀ of HMD against BGC-823, E_{ca-109} and HCT-8 was 25.28 mg/ml, 12.78 mg/ml and 28.29 mg/ml (equivalent to the crude drug) respectively.

Effect of HMD on formation of BGC-823, E_{ca-109} and HCT-8 cell colonies in cell culture

HMD had an obvious inhibitory effect on colony formation of BGC-823, E_{ca-109} and HCT-8 tumor cell lines, being most prominent in E_{ca-109} in a positive dose-effect manner. The data are shown in Table 2.

Anti-neoplastic action of HMD in vivo

Effect of HMD on tumor weight of FC mice HMD at doses of 27.05 g/kg \cdot d, 13.53 g/kg \cdot d and 6.76 g/kg \cdot d demonstrated

an obvious inhibitory effect on the growth of tumor in FC mice in a positive dose- effect manner. The inhibitory effect of 13.53 g/kg HMD (containing 25 mg 5-Fu) was more potent than 25 mg/kg of 5-Fu alone with statistical significance ($P<0.05$). Results are shown in Table 3.

Effect of HMD on tumor weight of S₁₈₀ bearing mice HMD at doses of 27.05 g/kg \cdot d, 13.53 g/kg \cdot d and 6.76 g/kg \cdot d significantly inhibited the growth of S₁₈₀ sarcoma in tumor-bearing mice in a positive dose-effect manner ($P<0.01$, $P<0.05$). Results are shown in Table 4.

Effect of HMD on life span of H₂₂ bearing mice HMD at doses of 27.05 g/kg \cdot d, 13.53 g/kg \cdot d, and 6.76 g/kg \cdot d obviously prolonged the life span of H₂₂ bearing mice in a positive dose-effect manner. HMD at 13.53 g/kg (containing 25mg 5-Fu) showed a more potent effect than that of 5-Fu alone ($P<0.05$), and HMD without 5-Fu ($P<0.05$). Results are shown in Table 5.

Effect of HMD on tumor cell apoptosis and their cell cycles

Studies on the effects of HMD on cell apoptosis and cell cycles of tumor cell lines showed that HMD was able to induce cell apoptosis to a certain degree (Tables 6 and 7), which was statistically significant ($P<0.01$) as compared with the blank control. 5-Fu, however, was devoid of such an effect. HMD caused a decrease of cells at stage S, resulting in cells differentiation at G₀-G₁ and G₂-M stages. But most cells remained at stage G₂-M with an increase of HMD doses.

Effect of HMD on intracellular $[\text{Ca}^{2+}]_i$ of tumor cells Changes of intracellular $[\text{Ca}^{2+}]_i$ in human gastric carcinoma cells after treated with HMD for 12 h. It showed that HMD significantly increased the intracellular free calcium ion concentration ($P<0.001$) in a dose dependent manner (Tables 8-11). Calcium ion concentration of the verapamil group

Table 1 Cytotoxicity of HMD to human tumor cell lines (MTT assay)

Drug	IC ₅₀ (mg/ml)							
	KB	BGC-823	A549	MCF-7	A ₂₇₈₀	Bel-7420	HCT-8	E _{ca-109}
HMD	83.89	25.28	292.41	34.14	16.84	36.57	28.29	12.78
5-Fu	0.12	0.06	0.19	0.10	0.08	0.09	0.08	0.04
HMD without 5-Fu	428.58	274.90	685.70	365.00	124.65	295.60	187.58	100.42

Table 2 Effect of HMD on formation of colonies in culture of BGC-823, E_{ca-109} and HCT-8 cell lines (n=8)

Drug	Percentage of colony formation (mean \pm SD)			
	Dose (mg/ml)	BGC-823	HCT-8	E _{ca-109}
HMD	10.00	72.18 \pm 7.29 ^c	83.18 \pm 9.01	61.58 \pm 4.12 ^a
HMD	20.00	50.21 \pm 5.18 ^d	59.45 \pm 6.78 ^c	22.43 \pm 3.65 ^d
HMD	40.00	28.43 \pm 3.42 ^d	30.15 \pm 4.20 ^d	0
5-Fu	0.04	61.18 \pm 4.27 ^c	68.70 \pm 5.15 ^a	69.27 \pm 5.48 ^a
HMD without 5-Fu	19.63	90.51 \pm 7.42	82.15 \pm 7.92	78.29 \pm 6.25 ^a
Blank control		98.27 \pm 6.58	95.68 \pm 9.12	97.14 \pm 8.26 ^c

^a $P<0.05$, ^b $P<0.01$ vs blank control; ^c $P<0.05$, ^d $P<0.01$ vs HMD without 5-Fu.

Table 3 Effect of HMD on tumor weight of FC mice

Drug	Dose (g/kg)	Route of administration	No. of animals	Average weight of tumor (g)	Rate of inhibition (%)
HMD	27.05	<i>ip</i>	30	0.42 \pm 0.21 ^{bd}	76.92
HMD	13.53	<i>ip</i>	30	0.58 \pm 0.30 ^{bc}	68.13
HMD	47.63	<i>ip</i>	30	0.88 \pm 0.34 ^a	51.65
HMD					
Without 5-Fu	13.50	<i>ip</i>	30	1.21 \pm 0.45	33.52
5-Fu	0.03	<i>ip</i>	30	0.89 \pm 0.40	51.10
Normal saline		<i>ip</i>	30	1.82 \pm 0.43	

^a $P<0.05$, ^b $P<0.01$ vs HMD without 5-Fu; ^c $P<0.05$, ^d $P<0.01$ vs 5-Fu.

Table 4 Effect of HMD on tumor weight of S₁₈₀ bearing mice

Drug	Dose (g/kg)	Route	No. of animals	Average weight of tumor (g)	Rate of inhibition (%)
HMD	27.05	<i>ip</i>	30	0.28±0.10 ^{bc}	73.33
HMD	13.53	<i>ip</i>	30	0.40±0.15 ^a	61.91
HMD	6.76	<i>ip</i>	30	0.49±0.31 ^a	53.33
HMD					
Without 5-Fu	13.50	<i>ip</i>	28	0.79±0.33	24.76
5-Fu	0.03	<i>ip</i>	28	0.54±0.21	48.57
Normal saline		<i>ip</i>	29	1.05±0.32 ^d	

^a*P*<0.05, ^b*P*<0.01 vs HMD without 5-Fu; ^c*P*<0.05, ^d*P*<0.01 vs 5-Fu.**Table 5** HMD at doses of 27.05 g/kg·d, 13.53 g/kg·d and 6.76 g/kg·d prolonged life span of H₂₂- bearing mice

Drug	Dose (g/kg)	Route	No. of animals	Life-span(d)	Rate of life prolongation (%)
HMD	27.05	<i>iv</i>	27	21.1±1.6 ^{ac}	74.4
HMD	13.53	<i>iv</i>	28	18.6±2.9 ^{ac}	53.7
HMD	6.76	<i>iv</i>	30	17.1±3.2	41.3
HMD					
Without 5-Fu	13.50	<i>iv</i>	30	15.8±2.5	30.6
5-Fu	0.03	<i>iv</i>	29	14.6±2.7	20.7
Normal saline		<i>iv</i>	30	12.1±3.1	

^a*P*<0.05, ^b*P*<0.01 vs HMD without 5-Fu; ^c*P*<0.05, ^d*P*<0.01 vs 5-Fu.**Table 6** Effect of HMD on cell cycle of human gastric carcinoma

Group	Dose(μg/ml)	No. of cases	G ₀ /G ₁ (%)	G ₂ /M (%)	S (%)
Blank	Media only	10	22.20±5.06	4.09±1.27	73.56±12.10
5-Fu	50	10	44.81±10.25	0.03±0.01	55.13±7.28 ^b
HMD	50	10	22.63±3.34	11.01±3.56	63.38±7.31 ^a
HMD	100	10	26.70±7.99	19.33±4.67	52.83±10.03 ^b
HMD	200	10	12.65±2.43	31.36±7.06	54.27±8.33 ^b

^a*P*<0.05 vs blank control; ^b*P*<0.01 vs blank control.

(group D) was between that of the negative control and medium dose HMD group, both of which were statistically significant (*P*<0.01).

Table 7 Effect of HMD on cell apoptosis of human gastric carcinoma

Group	Dose (μg/ml)	No. of cases	APO (%)
Blank control	Media only	10	0.15±0.04
5-Fu	50	10	0.17±0.06
HMD	50	10	1.16±0.23 ^b
HMD	100	10	1.14±0.20 ^b
HMD	200	10	1.72±0.41 ^b

^b*P*<0.01 vs blank control.**Table 8** Changes of intracellular [Ca²⁺]_i in human gastric carcinoma cells after treated with HMD for 12 h

Group	Concentration of drug (μg/ml)	No. of cells	[Ca ²⁺] _i (FI)
A	Same vol. of medium	20	320.26±147.99
B	50	22	386.00±163.85 ^b
C ₁	200	25	1 367.56±133.98 ^d
C ₂	100	28	916.78±151.46 ^d
C ₃	50	26	724.26±151.33 ^d
D	100+20	24	776.36±144.10 ^f

^b*P*<0.01 vs negative control; ^d*P*<0.001 vs negative control; ^f*P*<0.01 vs medium dose group and negative control.**Table 9** Changes of intracellular [Ca²⁺]_i in human gastric carcinoma cells after treated with HMD for 24 h

Group	Concentration (μg/ml)	No. of cells	[Ca ²⁺] _i (FI)
A	Same vol. of medium	22	323.50±141.35
B	50	25	382.67±135.02 ^b
C ₁	200	23	824.63±155.65 ^d
C ₂	100	23	672.65±165.16 ^d
C ₃	50	27	532.46±146.76 ^d
D	100+20	26	579.16±147.20 ^f

^b*P*<0.01, ^d*P*<0.001 vs negative control; ^f*P*<0.01 vs medium dose group and negative control, respectively.**Table 10** Changes of intracellular [Ca²⁺]_i in human gastric carcinoma cells after treated with HMD for 36 h

Group	Concentration of drug (μg/ml)	No. of cells	[Ca ²⁺] _i (FI)
A	Same vol of medium	30	339.97±150.19
B	50	23	399.83±159.52 ^b
C ₁	200	25	833.84±133.10 ^d
C ₂	100	26	684.31±148.45 ^d
C ₃	50	23	519.00±170.92 ^d
D	100+20	32	583.67±144.80 ^f

^b*P*<0.01, ^d*P*<0.001 vs negative control; ^f*P*<0.01 vs medium dose group and negative control group separately.

Table 11 Changes of intracellular $[Ca^{2+}]_i$ in human gastric carcinoma cells after treated with HMD for 48 h

Group	Concentration of drug given ($\mu\text{g/ml}$)	No. of cells	$[Ca^{2+}]_i$ (FI)
A	Same vol. of medium	22	328.45 ± 135.15
B	50	36	380.42 ± 151.46^b
C ₁	200	33	1119.55 ± 193.94^d
C ₂	100	28	787.00 ± 178.19^d
C ₃	50	21	547.29 ± 142.25^d
D	100+20	26	693.38 ± 168.15^f

^b $P < 0.01$, ^d $P < 0.001$ vs negative control; ^f $P < 0.01$ vs separately medium dose group and negative control group.

Table 12 Changes of $[Ca^{2+}]_i$ in human gastric carcinoma cells after treated with HMD for different times

Different durations of treatment (h)	No. of cells	$[Ca^{2+}]_i$ (FI)
12	28	916.78 ± 151.46
24	23	672.65 ± 165.16
36	26	684.31 ± 148.41
48	28	787.00 ± 178.19

The experimental results indicated that when HMD was used for treatment of tumor cells, an increase of intracellular free calcium ion concentration was observed regardless of the duration of treatment. But comparatively, at the early stage of treatment (12 h), the range of increase was most evident (Table 12).

DISCUSSION

Gastric carcinoma, as one of the most common human malignant tumors, ranks the first leading cause of gastrointestinal cancer-related mortality worldwide. In China, it now ranks the second. It has been shown that tumor apoptosis played an important role in its growth, invasion, metastasis and recurrence^[10-29].

HMD is a compound TCM preparation for injection in combination with modern synthetic drugs. It has an obvious anti-neoplastic activity shown by extensive pharmacological studies. In the present study *in vitro* experiments with MTT and cell colony formation were carried out to assess its inhibition on 8 human cancer cell lines. It showed that HMD could inhibit the growth of cancer cell lines. The anti-neoplastic activity of HMD was most prominent against human gastric carcinoma BGC-823, human esophagus carcinoma E_{ca}-109, and human colon carcinoma HCT-8 cell lines in a dose dependent manner. These results were consistent with the clinical effects of HMD on dysphagia and regurgitation, suggesting that HMD was extremely sensitive to carcinomas of the digestive tract. *In vivo* anti-neoplastic activity of HMD was studied by transplanting various cancer cell lines (proventricular FC, S₁₈₀ sarcoma and H₂₂ liver cancer) into experimental animals to observe its inhibition on tumor growth. It showed that HMD could inhibit the growth of FC and S₁₈₀ tumors in a positive dose-effect manner. It also significantly prolonged the life span of H₂₂ bearing mice, though its potency was not comparable to those obtained in FC and S₁₈₀-bearing mice. This indicates that HMD is more potent for solid tumors than ascitic carcinoma, which also conforms with the clinical observations.

HMD injection is composed of 2 parts, a compound TCM formula and a synthetic drug 5-fluorouracil (5-Fu). In the above *in vivo* and *in vitro* anti-neoplastic studies, controls with TCM formula or 5-Fu alone were included to compare their individual and combined efficacy. Results showed that the anti-

neoplastic action of HMD injection exceeded that of TCM formula or 5-Fu alone (at the same dosage level). Thus, it seemed that TCM formula and 5-Fu had an excellent synergism when used in combination.

To obtain a deeper understanding of the mechanism of the anti-neoplastic action of HMD on human gastric carcinoma, we carried out further studies on the induction of cell apoptosis of human gastric carcinoma SGC-7901 by HMD.

Cell apoptosis, as an autonomic process of organisms, is intrinsically different from necrosis in pathological conditions. Cell apoptosis is a "suicidal" process on its own accord under physiological condition, though it may also involve some pathological events. But, undoubtedly it plays an important role in the recovery and maintenance of normal physiological function. Carcinogenesis is not merely due to abnormal cell proliferation and differentiation, but the mechanisms of cell apoptosis, which leads to neoplastic occurrence, development, evolution, metastasis and eventually death of tumor-bearing hosts should also be duly considered. The mechanism of cell apoptosis in the etiology of carcinogenesis might be induced by many factors, such as inactivation and mutagenesis of apoptotic gene or inhibition of the process of apoptotic gene expression, apoptotic mechanism caused by chemical carcinogens or virus, apoptosis insufficiency of precancerous cell, apoptosis insufficiency of cancer cells due to immunological response of the organism, inhibition of tumor cell apoptosis by adhesion factor and growth factor of the host, attempts of tumor cells to avoid apoptosis by lowering their dependence on survival signal while enhancing their survival competence thereby aggravating the disease with metastasis^[30].

The relationship between cell apoptosis and carcinogenesis provides us a new pharmacological mechanism and target for the study of anti-neoplastic drugs. As an anti-neoplastic drug, if it is very potent to induce tumor cell apoptosis and to activate the programmed cell death process, then it can avoid a great number of untoward side effects due to the release of waste material resulted from cell necrosis, and alleviate the damage of normal cells caused by chemotherapy and prolong the life of patients.

Experimental studies of the effect of HMD on apoptosis of human gastric cancer cell line showed that there was a definite trend to promote cell apoptosis, but with the increase of dosage no further increase of cell apoptosis could be observed. This might be due to the fact that the increased dose caused a sufficient amount of tissue necrosis, which overshadowed cell apoptosis. The positive control 5-Fu was found unable to induce cell apoptosis which coincided with the finding that 5-Fu could enhance the tolerability of cells toward apoptosis as reported in the literature^[30]. Therefore it seems to be possible that the effective ingredient responsible for the induction of apoptosis is the polysaccharides present in HMD formula. Sea weed polysaccharides is known to possess cytotoxicity that can inhibit and kill cancer cells directly, and when excited by low doses of a cytotoxic agent it can induce cell apoptosis, while polysaccharides from *astragalus chrysopterur* can enhance the cytotoxicity of NK cells of mice. Such NK cell induced death of the target cell is known as apoptosis.

It has been found that the majority of active cells were proliferating at the S stage of DNA synthesis, while the differentiated cells were stagnant at the G₁/G₀ stage^[31], when DNA synthesis was not in progress. Anti-neoplastic agents can markedly change the kinetic features of cell cycle such as that occurred in tumor cells from S₁₈₀ mice by the action of HMD. Moreover, different doses of HMD had different effects on cell cycle kinetics. At small (50 $\mu\text{g/ml}$) and medium (100 $\mu\text{g/ml}$) doses, HMD could block cell growth at G₁/G₀ stage and S stage. Larger doses of HMD could cause the cells to remain at G₂ and M stage resulting in an obvious increase of G₂ and M cells,

indicating that different doses of HMD showed different anti-neoplastic mechanisms. Regarding its induction of cell apoptosis, HMD also showed several different mechanisms.

HMD exerts its anti-neoplastic action by inducing cell apoptosis of the tumor cells. Ca^{2+} has been found to play an important role in the transmission system of this pathway^[37]. To clarify the relationship between the mechanisms of the anti-neoplastic activity of HMD and the intracellular Ca^{2+} concentration, we tried to observe the effect of HMD on the Ca^{2+} concentration in cancer cells by confocal laser scanning microscopic technique.

Calcium presents in the body in two different forms: the combined form and the ionic form (Ca^{2+}). Only the ionic form shows physiological activity. Such calcium ions are divided into intracellular and extracellular types. It was shown that Ca^{2+} concentration in extracellular fluid was about 10^{-3} mol/L, while the intracellular concentration was only 10^{-7} mol/L. They were always maintained at this definite level with intracellular concentration being only 1/10 000 of that of the extracellular one. It has become increasingly evident nowadays that this seemingly negligible change of intracellular Ca^{2+} concentration interferes with many physiological and metabolic functions, especially transmission of cellular signals. At present, Ca^{2+} is regarded as a rather important second messenger and at the same time, participates in or coordinates with the metabolism of other second messengers and the regulation of cell functions.

In the transmission of cell signals, either intrinsic or extrinsic signals pass through the cell membrane to get into cytoplasm and enter into cell nuclei to induce changes of gene expression. Three pathways for a signal to cross over cell membrane have been found^[38]: through the mediation of tyrosine protein kinase, G-protein or the ionic channel^[32]. The signal transmission in cytoplasm was mainly accomplished by the messenger adenylate cyclase, lipositol and calcium calmodulin^[33]. Five corresponding transmission pathways which can induce apoptosis may be derived from the above 3 messengers. They are the Ca^{2+} signal system in cells, the cAMP/PKA signal system, the DG/PKC signal system, the tyrosine protein kinase system, and the acylsphingosine pathway. The main signal molecules in these messengers include cAMP, IP_3 , PKC and Ca^{2+} , in which Ca^{2+} could play a pivotal role in the transmission^[7,38]. Therefore, in our study on the mechanism of transmission of apoptosis signal to tumor cells, Ca^{2+} was first selected.

In the present study, Fluo-3/AM was used as the fluorescence indicator, and confocal laser scanning microscopy technique was used to determine the dynamic change of $[\text{Ca}^{2+}]_i$ in human gastric carcinoma SGC-7901 cell line after different durations of drug administration. Therefore, the study was performed at 4 different stages of 12, 24, 36 and 48 h. In each stage, the dose-dependent manner of the effect of HMD on intracellular $[\text{Ca}^{2+}]_i$ was observed. Experimental results showed that the Ca^{2+} concentration of all the HMD treated groups, regardless of the stage, was higher than that of the negative blank control ($P < 0.001$), and the extent of elevation by high, medium and low dosage of HMD was dose-dependent. As to groups dosed for different length of time, the group dosed for 12 h showed a most drastic elevation of Ca^{2+} concentration. All the other time groups showed a larger Ca^{2+} concentration change than the negative control though smaller than that of the 12 h group. This result conformed with literature reports that the elevation of Ca^{2+} was closely related to the early apoptosis.

Cohen and Duke^[34] found a continual increase of $[\text{Ca}^{2+}]_i$ when apoptosis of thymocytes was induced by glucocorticoid. Further studies found that the intrinsic endonuclease which caused DNA fragmentation was Ca^{2+} and Mg^{2+} dependent and the activity of this enzyme was obviously enhanced as the

concentration in cytoplasm was elevated. Similarly, when VP-16 or TNF was used to induce apoptosis of breast cancer cells, $[\text{Ca}^{2+}]_i$ was also elevated to activate the Ca^{2+} dependent endonuclease, causing DNA fragmentation and cell apoptosis^[35]. As shown in the present study HMD could induce apoptosis of cancer cells, and cause an increase of intracellular $[\text{Ca}^{2+}]_i$ similar to the changes of $[\text{Ca}^{2+}]_i$ when apoptosis of other cancer cell lines occurred^[38], which could explain why Ca^{2+} could activate Ca^{2+} dependent endonuclease leading to apoptosis.

While Ca^{2+} , with its pivotal role in those signal transmission systems may induce cell apoptosis through several different approaches: (1) to activate the Ca^{2+} dependent endonuclease causing apoptosis; (2) the elevation of Ca^{2+} activates PKC directly and at the same time helps DG to activate PKC, which is a bifunctional kinase that not only regulates cell differentiation but also induces cell apoptosis; (3) after the elevation of Ca^{2+} , it combines with CaM to form a complex Ca-CaM, which activates adenylate cyclase (AC) resulting in the activation of cAMP/PKA signal system, thereby activating PKA to induce tumor cell apoptosis; (4) Ca^{2+} could control the activity of glutamyl transferase resulting in cell apoptosis, though its exact mechanism is still unclear. In brief, Ca^{2+} plays a specific role in transmission of cell signals of apoptosis. HMD is capable of inducing tumor cell apoptosis by elevating intracellular $[\text{Ca}^{2+}]_i$.

Ca^{2+} comes from two sources when cellular $[\text{Ca}^{2+}]_i$ increases. One is the inflow of Ca^{2+} from extracellular sources, mainly by opening calcium channels. The other is the release of Ca^{2+} from intracellular calcium storage. Ca^{2+} in cells is mainly stored in endoplasmic reticulum/saroplasmic reticulum and is mainly regulated by IP_3 . To trace the source of Ca^{2+} when intracellular $[\text{Ca}^{2+}]_i$ in gastric carcinoma cells was elevated by HMD, we tried on an additional "verapamil group", with the use of a calcium channel blocker added to the medium dosage group of HMD. We deduced that one of the following results might occur. (1) If Ca^{2+} only came from extracellular calcium inflow, the $[\text{Ca}^{2+}]_i$ value should be similar to that of the negative control. (2) If Ca^{2+} came only from the release of intracellular storage, the $[\text{Ca}^{2+}]_i$ value should be similar to that of the medium dose of HMD group. (3) If the Ca^{2+} came from both extracellular Ca^{2+} inflow and intracellular Ca^{2+} release, the $[\text{Ca}^{2+}]_i$ value should be between those of negative control and medium dose HMD with statistical significance. Results of the experiment showed that the $[\text{Ca}^{2+}]_i$ value was higher than that of the negative control, but lower than that of the medium dose HMD group with statistical significance ($P < 0.001$). It indicated that HMD elevated intracellular $[\text{Ca}^{2+}]_i$ to induce tumor cell apoptosis and caused the opening of calcium channels, which facilitated Ca^{2+} inflow and interacted with receptors on the membrane to produce IP_3 for the release of Ca^{2+} from its storage.

From this study we conclude that HMD shows a remarkable cytotoxicity against tumor cells. Its mechanism of action is to increase intracellular $[\text{Ca}^{2+}]_i$ by opening the calcium channels of cell membrane, and initiate the release of Ca^{2+} from its storage to induce tumor cell apoptosis.

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