Enhanced antitumor effect of TM208 in combination with 5-fluorouracil in H_{22} transplanted mice

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Abstract: 4-Methylpiperazine-1-carbodithioc-acid-3-cyano-3,3-diphenylpropyl ester hydrochloride (TM208), a newly synthesized dithiocarbamate derivative, exhibits antitumor effect in vivo with low toxicity. However, the antitumor effect of TM208 in combination with drugs in clinical use for cytotoxic chemotherapy has not been identified. In our study, the antitumor effects and toxicities of TM208 in combination with cisplatin (DDP), cyclophosphamide (CTX) and 5-fluorouracil (5-Fu), respectively, were evaluated in vivo using a transplanted solid-type hepatocarcinoma H_{22} mice model. The results suggested that 5-Fu (5 mg/kg/2d) potentiated the antitumor effect of TM208 (100 mg/kg/d) with significantly higher tumor inhibition rates (P<0.01) and a slight elevation of toxicity; however, DDP and CTX in combination with TM208 did not exhibit similar enhanced antitumor effect. For further investigation, we found that the TM208 and 5-Fu combination therapy led to G_2/M cell cycle arrest of tumor cells in vivo by downregulating the protein expression of cyclin B1, cdc2, cdk7, and upregulating the expression of p21 and p53. The protein expression levels of cyclin D1 and cyclin E were also downregulated in tumor cells treated with TM208 and 5-Fu, while those of cdk4 and cdk2 remained unchanged. The change of mRNA expression level of cdc2 was consistent with that of its protein in each group, while the mRNA expression of cyclin B1 remained unchanged among each group. These results demonstrated the dosage regimen of TM208 for combination therapy and could serve as evidence for clinical use of TM208 as an antineoplastic drug.

Keywords: Combination therapy; Hepatocarcinoma H_{22}; Dithiocarbamate; 5-Fluorouracil; Cell cycle-related proteins

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1. Introduction

4-Methylpiperazine-1-carbodithioc-acid-3-cyano-3,3-diphenylpropyl ester hydrochloride (TM208), one of the new dithiocarbamate derivatives, showed significant cancer regressions in some preclinical models, including transplanted H_{22} and S_{180} in mice and xenograft BGC-823 and QGY-7703 tumors in immunodeficient nude mice[1,2]. While TM208 could inhibit tumor growth by oral administration, it did not cause significant body weight loss or immunosuppression, which indicated its low toxicity as an antitumor agent. However, doses of TM208 up to 200 mg/kg/d did not translate into higher tumor inhibition rate than 55% in the transplanted H_{22} tumor model. To obtain maximal anticancer effect in the treatment of hepatocellular carcinoma (HCC), combination chemotherapy of TM208 with other agents should be considered.

Combination chemotherapy plays an important role in clinical treatment of HCC, the fifth most common cancer in the world[3]. Results of multi-agent regimens, including the combination of 5-fluorouracil (5-Fu), doxorubicin, cisplatin (DDP) and α-IFN, have shown longer median survival times (7–10 months) in HCC patients than many single-agent studies[4]. Cyclophosphamide (CTX), a powerful cytotoxic agent, is also used in combination chemotherapy (together with vincristine, adriamycin and 5-Fu) in the treatment of children with malignant hepatoma[5]. However, HCC has a high incidence of expression of the multi-drug resistance gene (MDR1) and consequent high levels of *P*-glycoprotein (*P*-gp)[6], which is associated with a poor response to chemotherapy in this disease[7]. On the other hand, most antineoplastic drugs exhibit severe side effects, including leukocytopenia (adriamycin), gastrointestinal symptoms (5-Fu), nephrotoxicity (DDP), urinary symptoms (CTX),

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etc. To avoid the incidence of drug resistance and reduce side effects, combination chemotherapy, with each constituent being administered in a low dose, becomes a better choice than the single-agent therapy. Furthermore, a single-agent therapy can only eradicate a unique population of cells sensitive to that agent, and may be ineffective for cells that are resistant to it. For TM208, an effective antitumor agent with low toxicity, we are interested in whether the combination therapy of it with clinical antineoplastic drugs could achieve enhanced anticancer effect, while exerting no significant increase in side effects.

By examining the expressions of some MAPK signal pathway-related proteins and cell cycle-related proteins in tumor tissues from nude mice implanted with QGY-7703 cells, an earlier study revealed that the downregulation of PKCα, phospho-ERK1/2 and phospho-p38 might help to inhibit tumor growth in TM208 treated group. Downregulation of cdc2 and cyclin B1 implied that blocking the PKCα/ERK1/2 MAPK signal pathway might contribute to G_2/M cell cycle arrest, which further elucidated the mechanism of the tumor inhibition of TM208[2]. How the optimal drug combination exerts its action, in which the individual agents interact to potentiate the anticancer effect in an additive or synergistic manner, motivates us to investigate the underlying mechanism.

2. Materials and methods

2.1. Animals and tumor cells

Male ICR mice (body weight, 18–22 g) were obtained from the Department of Laboratory Animal Science of Peking University Health Science Center (Beijing, China) and kept with four mice in one cage ad libitum at (22±2) ºC on a 12 h light/dark cycle with food and water available freely. All animals were treated in accordance with the guidelines established by the Institutional Animal Care and Use Committee of Peking University. The murine H_{22} ascites tumor cells (supplied by the cell bank of the Pharmacology Group of State Key Laboratory of Natural and Biomimetic Drugs, Peking University) were diluted with 0.9% normal saline (NS) solution to 1×10^6 cells/mL and transplanted s.c. via trocar into the left armpits of ICR mice using an aseptic manipulation, 0.2 mL/mouse.

2.2. Drugs

TM208 was supplied by Professor Run-Tao Li (Peking University). DDP was purchased from Qilu Pharmaceutics Co., Ltd. (Shandong, China) as slight yellow sterile powder for injection. 5-Fu was purchased from Xudonghaipu Pharmaceutics Co., Ltd. (Shanghai, China) as a ready-to-use clinical formulation solution in 10-mL vials that contained 250 mg of drug (25 mg/mL). CTX was purchased from Hualian Pharmaceutical Co., Ltd. (Shanghai, China). TM208 was suspended by double-distilled water through sonication, while the other drugs were diluted in 0.9% NaCl.

2.3. Drug doses and treatment

Drug treatments were initiated the day after s.c. tumor implantation. DDP (0.25 mg/kg/d), 5-Fu (5 mg/kg/2d) and CTX (10 mg/kg/2d) were delivered by intraperitoneal route, while TM208 was delivered intragastically at various doses (50, 100 mg/kg/d). The combination therapy for each drug includes 6 groups: the NS negative control group, TM208 groups (50, 100 mg/kg/d), clinical drug group, 2 combination therapy groups (TM208 (50, 100 mg/kg/d) combined with a drug in clinical use, respectively), with 8 or more mice in each group. Body weight was recorded once per 2 or 3 d.

2.4. Toxicity evaluation and dosage determination

The dosage of each clinical drug for combination therapy was determined by evaluating drug-induced toxicities (body weight, the number of white blood cells (WBC) in blood, the spleen and thymus index, drug-related lethality). After 9 or 10 d of single-agent administration of several dosage levels and when the average tumor weight of the negative control group exceeded 1.0 g and tumor-induced deaths were observed or can be predicted, mice were sacrificed and tumors together with immune organs (including spleens and thymuses) were harvested. Meanwhile, peripheral blood of each animal was collected and analyzed using Medonic-CA620 Vet Automated Cell

Analyzer (Boule Co., Sweden). Organ index was calculated according to the following formula: organ weight (mg)/ body weight when sacrificed (g). After identifying the dose-toxicity relationship of each agent, the dosage for combination therapy was determined as the highest dose level that shows low tumor inhibition rate with no significant toxicity in some aspects.

2.5. Antitumor effect and toxicity of combination therapy

Combination administration began on the day after tumor implantation with the previously determined dosage. Each group had 8 or more mice per experiment, and each experiment was repeated at least twice. After tumors and organs were harvested, as described previously, tumor inhibition rate was used to evaluate the antitumor effect, while organ index and the number of WBC in blood was used in the toxicity evaluation. Tumor inhibition rate was calculated according to the following formula: (mean tumor weight of negative group – mean tumor weight of treated group)/mean tumor weight of negative group × 100%. Organ index was calculated according to the formula stated above.

2.6. Histological and immunohistochemical staining of tumor tissue

Tumors were harvested, fixed in 10% formalin, embedded in paraffin, and then sliced into 4 μm-thick sections. Hematoxylin and eosin (H&E) staining was performed for conventional light microscopic observation. Immunostaining was also carried out by deparaffinizing and rehydrating the 4 μm-thick sections. An antigen-retrieval procedure was performed by boiling tissue sections in 10 mM citrate buffer (pH 6.0) for 20 min, followed by cooling the sections at room temperature for 20 min. Endogenous peroxidase activity was blocked with 3% H_2O_2. All sections were washed with a phosphate-buffered saline (PBS) solution and incubated with an anti-mouse Ki-67 monoclonal antibody (1:200, Lab Vision, Fremont, California, USA) at 4 ºC overnight. After being washed with PBS for 4 times (5 min/time), the sections were incubated with a second antibody (Lab Vision; Zhongshan Goldenbridge, Beijing, China) at 37 ºC for 3 h, and then washed with PBS, visualized with 3,3′-diaminobenzidine tetrahydrochloride (DAB) as a chromogen substrate. Finally, tissue sections were slightly counterstained with hematoxylin, differentiated by chlorhydric acid alcohol, dehydrated and mounted.

All sections of H&E and immunohistochemical staining were examined blindly by an observer with an Olympus BX 52 light microscope and images were recorded with Olympus DP50 digital camera system. On each slide of immunohistochemical staining, the Ki-67-positive nuclei were counted through a minimum of 300 cells per field in six different fields. The Ki-67 labeling index is the number (%) of positive cells.

2.7. Flow cytometric assay

The effect of combination therapy on cell cycle distribution was determined by flow cytometric analysis of DNA content of nuclei of cells following staining with propidium iodine (PI). After tumors were harvested, four H_{22} tumor samples per group were cut into small pieces and rubbed through a cell strainer to obtain single cell suspensions. The cells were washed with PBS and fixed with 70% ethanol overnight at –20 ºC. The cells were then centrifuged, washed again with PBS, and treated with 0.1 mg/mL RNase A at 37 ºC for 30 min. After DNA was stained (PI, 1 μg/mL PBS) for 15 min at room temperature, cell cycle was analyzed by flow cytometry. Cytometric data were collected for 10 000 cellular events per sample and analyzed using CELLQuest Pro software (BD Biosciences). Percentages of stained nuclei (2 N and 4 N) in different cell cycle phases were calculated using Modfit LT software (v 2.0, Verity Software House, Topsham, Maine, USA).

2.8. Western blot analysis

Frozen tumor samples from mice implanted with H_{22} were cut into small pieces and rubbed through a cell strainer (40 μm mesh) to obtain single cell suspensions. Cells were collected in cold PBS by centrifugation. After resuspension in an appropriate volume of lysis buffer (1% Nonidet P-40, 10 mM

Tris-HCl, 150 mM NaCl, 2 mM EDTA, 2 mg/mL leupeptin, 2 mg/mL aprotinin, 10 mg/mL phenylmethylsulfonyl fluoride, pH = 7.4), cells were incubated in ice for 30 min and vortexed every 10 min. Tumor lysates were centrifuged at 14 000 rpm and at 4 ºC for 30 min. Concentration of proteins in the supernatant was determined by Pierce protein assay (Pierce Biotechnology, Rockford, Illinois, USA). Equal amounts of supernatant protein (65 μg) were denatured by heating at 65 ºC for 10 min and loaded for SDS-PAGE on 10% gels. After proteins were electroblotted onto nitrocellulose membranes (Hybond C; Amersham, Little Chalfont, UK), membranes were blocked with 3% albumin fraction Ⅴ (BSA) in Tris-buffered saline (TBS), containing 0.05% Tween-20. Membranes were incubated with primary antibodies, diluted with 1.5% BSA in TBST at 4 °C overnight, specific to cyclin B1 (Cell Signaling Tech, Massachusetts, USA), cdc2, cyclin D1, cyclin E, cdk2, cdk4, p21, cdk7 (Santa Cruz Biotech, California, USA), p53 (Lab Vision Corporation, Fremont, California, USA), and β-actin (Sigma, Missouri, USA). After being washed four times with TBST for 10 min each, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotech, California, USA) for 1 h at room temperature, diluted 1:15 000, and ECL (Amersham, Biosciences, Little Chalfont, Buckinghamshire, UK) was used for immunodetection.

2.9. Reverse transcription-PCR analysis

Reverse transcription (RT)-PCR was used to quantify mRNA expression of tumor cells. Total RNA was prepared from frozen tumor samples using Trizol reagent (Invitrogen Life Technologies, Carlsbad, California, USA) according to the general protocol. To prepare cDNA sample, 1 μg total RNA was reverse-transcribed with a ReverTra Ace reverse transcriptase using a high-fidelity RT-PCR kit (TOYOBO Co., Ltd., Osaka, Japan) in a 20 μL reaction buffer. To amplify mRNA, 18 or 20-mer primers were designed. β-Actin was used as an internal control. The primer sequences were as follows:

Cyclin B1 (234 bp) forward: 5′-TTTGGTTGATAATCCCTC-3′; and reverse: 5′-GTCACTTCACGACCCTGT-3′; cdc2 (240 bp) forward: 5′- GAGGTAGTGACGCTGTGGTA-3′; and reverse: 5′-GGGAAAGGTGTTCTTGTAGT-3′; β-actin (400 bp) forward: 5′-GTGATGGTGGGAATGGGT-3′; and reverse: 5′-GGATGGCGTGAGGGAGAG-3′. PCR reaction was carried out for one cycle of 94 ºC for 2 min, followed by 30 cycles of 94 ºC for 10 s, T_m-5 ºC for 30 s, and 72 ºC for 30 s, and one cycle of 72 ºC for 5 min. The products were analyzed by gel electrophoresis on 2% agarose gels, stained with ethidium bromide (EB) and visualized on the UV transilluminator.

2.10. Statistical analysis

The differences between the mean values were analyzed for significance using the unpaired two-tailed Student's *t*-test for independent samples; $P<0.05$ was considered statistically significant.

3. Results

3.1. Determination of the doses of DDP, CTX and 5-Fu

The dosage-effect relationships for DDP, CTX and 5-Fu were established as shown in Table 1. Significant reduction of spleen index was observed at the 0.5 mg/kg/d DDP dose level. The dosage of DDP for combination therapy was determined to be 0.25 mg/kg/d, with no toxicity-related death or significant reduction of the immune organ index, the number of WBC in blood, as well as the increase in body weight.

After evaluating the effect and toxicity of CTX, dosages of 30 mg/kg/2d and 20 mg/kg/2d were found to be too high for combination chemotherapy because of the reduction of the immune organ index and the number of WBC in blood. Hence the dosage of CTX for combination chemotherapy was determined to be 10 mg/kg/2d.

With 5-Fu, severe immunosuppression was observed at 30 mg/kg/d and 15 mg/kg/d. After dose level was reduced and the schedule was adjusted to 1 time/2d, a significant reduction of spleen index, thymus index and WBC was observed at the 15 mg/kg/2d dose level. As for 10 mg/kg/2d, the reduction of

Table 1. Effects of DDP, CTX and 5-Fu on ICR mice with implanted solid-type H_{22}

	Dosage (mg/kg)	Increase in body weight (g)	Spleen index (mg/g)	Thymus index (mg/g)	WBC ($\times 10^9$/L)
DDP[1]	0	9.1±2.5	8.0±1.9	2.3±0.8	9.3±6.9
	0.5	7.4±1.3	5.9±1.1[a]	2.1±0.8	10.1±4.3
	0.25	10.8±3.0	7.3±1.0	2.2±0.4	8.1±2.3
CTX[2]	0	12.1±2.2	8.8±1.6	2.3±0.5	7.7±3.1
	30	10.3±0.4	5.6±1.1[b]	1.0±0.4[b]	2.1±0.9[b]
	20	12.2±2.6	7.2±1.0[b]	1.4±0.3[b]	5.2±1.6
	10	12.9±1.4	8.0±2.3	1.8±0.4	7.8±2.6
5-Fu[2]	0	6.0±2.6	7.3±1.9	2.1±1.0	6.9±1.7
	15	6.3±1.5	5.2±1.2[c]	1.2±0.3[c]	3.3±1.6[c]
	10	4.1±2.8	5.7±1.4	1.5±0.5	5.3±1.8
	5	6.3±1.2	6.4±1.0	1.7±0.4	7.1±2.1

Separate experiments for single use of DDP, CTX and 5-Fu were delivered with the respective negative control groups (treated with NS in the same schedule as the drug-treated group). Animals were observed for 9 or 10 d. 8 mice were treated at each tested dose;
[1]DDP was treated once a day, [2]CTX and 5-Fu were treated once per 2 d;
[a]$P<0.05$ versus DDP (0 mg/kg/d); [b]$P<0.05$ versus CTX (0 mg/kg/2d); [c]$P<0.05$ versus 5-Fu (0 mg/kg/2d).

these parameters was still more severe than the 5 mg/kg/2d group. The dosage of 5-Fu for combination therapy was therefore determined to be 5 mg/kg/2d.

3.2. Antitumor effect of TM208 in combination with DDP, CTX and 5-Fu respectively

According to a preliminary study, the dosage of TM208 for combination therapy was determined to be 100 mg/kg/d and 50 mg/kg/d. After determination of the dosages of DDP, CTX and 5-Fu as single agents, studies were initiated to combine TM208 (100, 50 mg/kg/d, respectively) with these three agents. The agents in combination regimens were administered simultaneously.

As shown in Figure 1A, when combining TM208 with DDP simultaneously every day, the tumor inhibition rates were 57.6% and 48.6% for the combination therapy groups of DDP with TM208 at 100 and 50 mg/kg/d, respectively. Compared with the tumor inhibition rates of single-dose groups, especially with the DDP group (tumor inhibition rate 52.0%), the inhibition rates of the combination therapy groups were not increased appreciably.

A similar result was observed for the combination therapy using CTX and TM208, as Figure 1B describes. Co-treatment of CTX with TM208 did not translate into a statistically higher antitumor efficacy. The tumor inhibition rates of the two combination groups were 44.6% and 43.9%, only a little higher than the CTX group (33.4%).

When combining TM208 with 5-Fu, as shown in Figure 1C, significant differences were observed between the combination group and respective single dose group in tumor inhibition rates. Tumor inhibition rate was raised up to 64.9% in the combination group (5-Fu together with TM208 (100 mg/kg/d)), while it could only reach 21.4% or less in single-agent treatment (TM208 100 mg/kg/d, 50 mg/kg/d and 5-Fu 5 mg/kg/2d).

3.3. Toxicity of TM208 in combination with DDP, 5-Fu, and CTX

Body weight was recorded once every 3 d when mice were treated with TM208 in combination with DDP. The NS group had a higher increase in body weight than the other agent-treated groups. On days 1, 4, 7, there was no significant difference between each agent-treated group, while on day 10, combination therapy group with a high dose of TM208 exhibited significant difference compared to its corresponding single-agent groups ($P<0.05$). This result indicated that combination therapy has some side effects in body weight change, compared to the single-agent regimen.

Body weight was recorded once every 2 d when mice were treated with TM208 in combination with CTX and 5-Fu, respectively. With CTX, no significant difference was observed between the NS negative control group and each agent-treated group. This result implied that the combination therapy of TM208 and CTX did not exhibit more severe side effects in body weight change than the single-agent treatments.

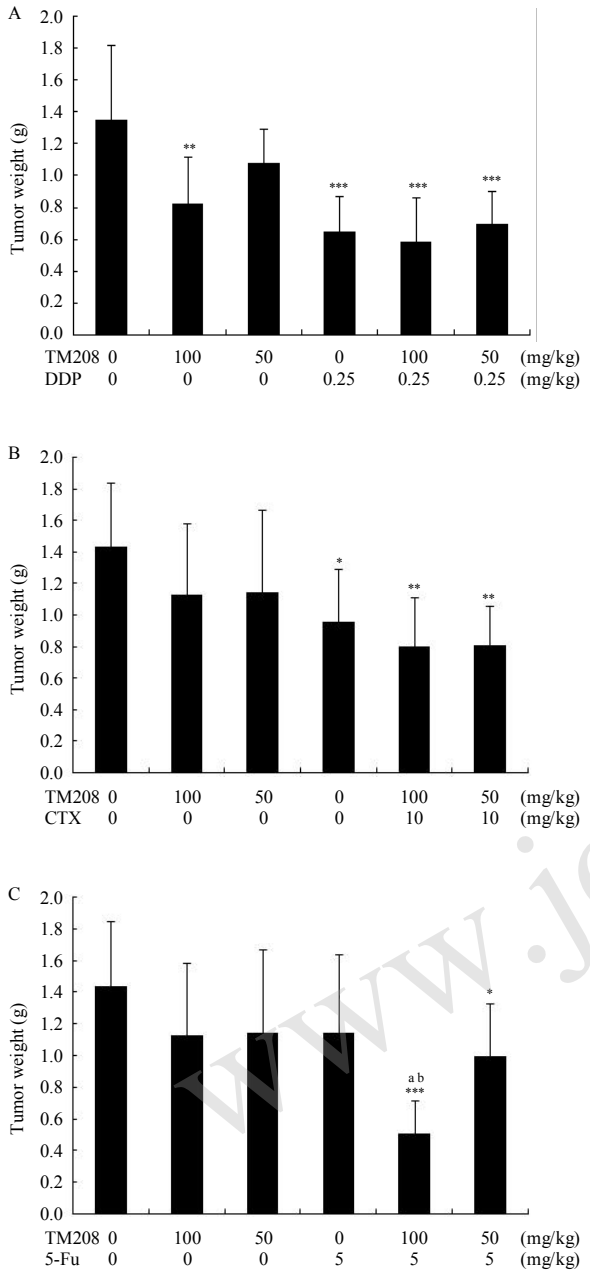

Figure 1. Antitumor effect of TM208 on the growth of H_{22} transplanted tumor in combination with DDP (A), CTX (B) and 5-Fu (C), respectively. (A) Two agents were administrated simultaneously every single day. Mice (n = 16) were treated in the negative control group for 9 d, with 10 mice in every other drug-treated group. Significant differences of tumor weight were observed between the NS negative control group and the three groups treated with DDP. $^{**}P$<0.01; $^{***}P$<0.001, versus NS group. (B, C) Antitumor effect of TM208 on the growth of solid-type H_{22} tumor in combination with CTX and 5-Fu, respectively. Mice (n = 12) were treated in the negative control group for 8 d, with 8 mice in every other drug-treated group. On days 1, 3, 5, 7, two agents were administrated simultaneously; on days 2, 4, 6, 8, H_{22} mice were treated only with TM208. Significant differences of tumor weight were observed between the NS negative control group and the three groups treated with CTX. $^{*}P$<0.05; $^{**}P$<0.01, versus NS group. Significant differences of tumor weight were also observed between the NS negative control group and the combination therapy group with TM208 and 5-Fu. $^{*}P$<0.05; $^{***}P$<0.001, versus NS group. ^{a}P<0.01 versus TM208 (100 mg/kg/d) group; ^{b}P<0.01, versus 5-Fu (5 mg/kg/2d) group.

As for 5-Fu, which showed the possibility to potentiate the antitumor effect of TM208, a significant difference between the body weight of combination therapy groups and that of the NS control group was observed since day 5 (P<0.01). But no significant differences between the combination therapy groups and single-agent groups, respectively, were observed, except on d 5, when the combination therapy group showed a lower body weight compared to the 5-Fu group (P<0.05). Through the curve of body weight (Fig. 2), we can clearly observe the difference between the body weight of combination therapy groups and that of the other groups on day 5. However this difference became smaller on day 9. This result indicated that combination therapy may exert slight side effects on body weight in the early phase of administration.

To evaluate the effect of combination therapy on the immune system, we calculated the spleen and thymus index. When DDP was co-treated with TM208 (100 mg/kg/d), a significant reduction in both the spleen index and thymus index was observed compared to the NS negative control group, which indicated side effects on the immune organs of combination therapy. No significant difference was observed when TM208 was co-treated with CTX, compared with the NS negative control group. As Table 2

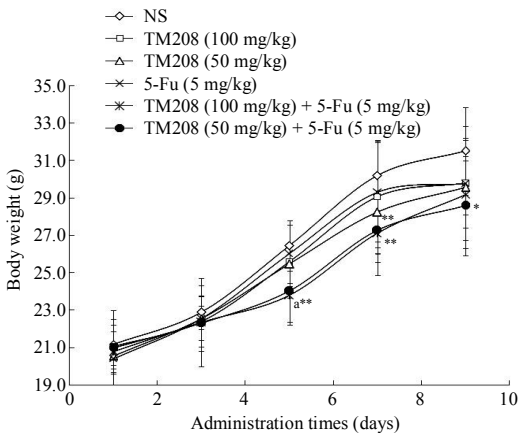

Figure 2. The change of body weight when TM208 was treated in combination with 5-Fu in solid-type H_{22} mice model. Twelve mice were treated in the negative control group for 8 d, with 8 mice in every other drug-treated group. Significant differences were observed between combination therapy groups and control group since day 5. $^{*}P$<0.05; $^{**}P$<0.01, versus NS group. Significant differences were observed between combination therapy groups and 5-Fu group on day 5. ^{a}P<0.05, versus 5-Fu group.

Table 2. Effect of TM208 on immune organ and WBC in peripheral blood of H_{22} transplanted mice in combination with 5-Fu

	Dose regimen (mg/kg)	Spleen index (mg/g)	Thymus index (mg/g)	WBC ($\times 10^9$/L)
NS	–	7.7±1.5	2.2±0.6	6.3±2.3
TM208	100	6.6±1.2	2.0±0.6	5.3±2.8
TM208	50	7.1±0.6	2.0±0.8	8.0±3.3
5-Fu	5	6.8±1.2	2.3±0.7	4.8±2.2
TM208+5-Fu	100+5	7.6±1.9	1.7±0.5	7.3±2.2
TM208+5-Fu	50+5	6.3±1.6	1.8±0.7	4.1±1.3

Twelve mice were treated in the negative control group for 8 d, with 8 mice in every other agent-treated group. Peripheral blood of each animal was collected on day 9 for analyzing. No significant difference was observed between any two groups.

describes, the combination therapy of TM208 and 5-Fu, which showed significantly enhanced antitumor effect compared to single-agent therapy, did not exhibit significant side effect on immune organs.

Peripheral blood of each animal was collected before it was sacrificed. By analyzing the number of WBC in the blood, we evaluated the effect of combination therapy on the hematological system. There was no significant difference in the number of WBC between single-agent groups (TM208, DDP, CTX and 5-Fu) and the NS negative control groups, respectively. Meanwhile, no significant differences were observed between groups of combination therapy and the corresponding single-agent groups, or between the groups of combination therapy and the NS negative control groups. Table 2 shows the result of WBC analysis of combination therapy of TM208 and 5-Fu.

3.4. Pathological examination of tumor tissue

To investigate the morphology of H_{22} cells treated with combination therapy of TM208 (100 mg/kg/d) and 5-Fu (5 mg/kg/2d), a pathological examination of the tumor tissue was performed, and the results are shown in Figure 3. The NS group was characterized by well-developed proliferation of H_{22} cells, with a compact arrangement; the nuclei were of various sizes, and staining color was variable; the cell membranes were clear, and there was no apparent necrosis in the tumor tissue. Following treatment with TM208, 5-Fu or both, there was evidence of dying tumor cells undergoing vacuolar degeneration of the cytoplasm; cellular arrangements were seen as discrete and incompact; nucleus pycnosis of some cells was observed. Especially in combination group, coagulative necrosis of tumor cells around capillary vessel was observed; the area of necrosis tumor cells was larger than those of single-agent groups. These results demonstrated that treatment with TM208, 5-Fu and combination therapy led to histopathological changes in the H_{22} tumor tissue that were associated with the death of the tumor cells.

3.5. Immunohistochemical staining of Ki-67

Ki-67 protein is a nuclear protein, which is associated with somatic cell proliferation and is used as an immunohistological marker for assessment of cancer cell proliferation. Reduction in Ki-67 expression correlates with responsiveness to chemotherapy[8,9]. As shown in Figure 4, the Ki-67 labeling index was significantly higher in sections of untreated tumors (84.7±2.5), than in sections of the TM208 (100 mg/kg/d) treated tumors (69.9±11.4) with $P<0.05$, the 5-Fu (5 mg/kg/2d) treated tumors (60.4±5.9) with $P<0.001$ and the combination group (34.4±11.9) with $P<0.001$. The index was significantly lower in combination therapy group than single-agent groups with $P<0.001$. The results indicated that combination therapy of TM208 and 5-Fu effectively inhibited cellular proliferation in the tumor, and it was more effective than single-agent treatment.

3.6. Cell cycle analysis

To determine whether the enhanced antitumor effect of the combination therapy of TM208 and 5-Fu was mediated through an alteration of the cell cycle, the cell cycle distribution of cells from H_{22} transplanted tumor tissue was analyzed. Cell cycle analysis showed that after treated with single agents, the percentages of H_{22} cells in the G_2/M phase increased significantly, while those of cells in the G_1 phase decreased substantially (Table 3). The percentage of tumor cells in G_2/M phase treated with

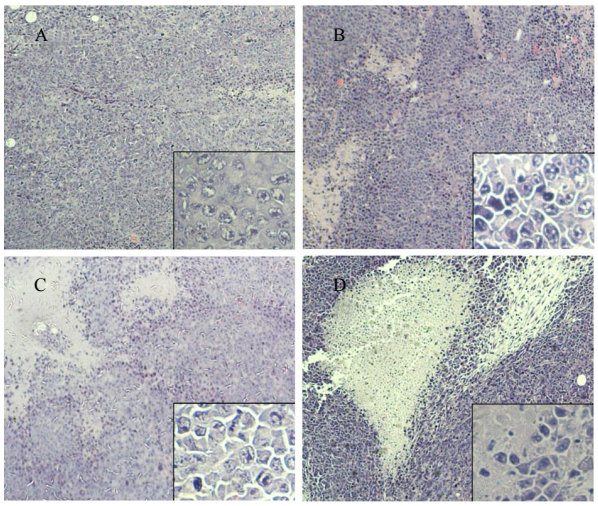

Figure 3. Photomicrographs of H_{22} tumor samples with H&E staining. (A) Control group. (B) TM208 (100 mg/kg/d). (C) 5-Fu (5 mg/kg/2d). (D) Combination group. H&E staining, original magnifications, ×40; insets, ×200.

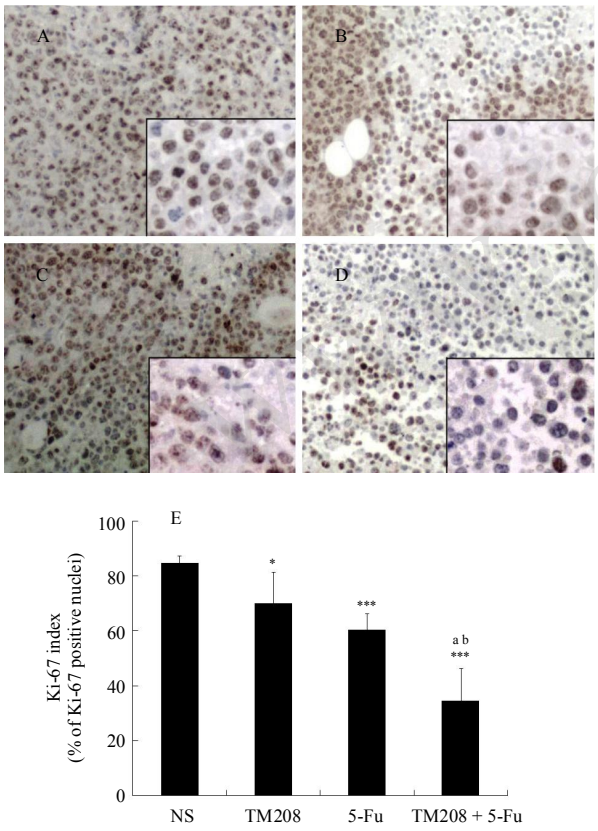

Figure 4. Effect of TM208 on Ki-67 activity in H_{22} transplanted tumor in combination with 5-Fu. (A–D) The presence of Ki-67 in H_{22} transplanted tumors of NS group, TM208 (100 mg/kg/d) group, 5-Fu (5 mg/kg/2d) group and the combination group, respectively. Ki-67-positive nuclei were stained brown, while the Ki-67-negative nuclei were stained light blue. Original magnifications, ×100; insets, ×200. (E) Summarized graphical representation as the positive cell-labeling index of Ki-67 expression in each group. Data are given as mean±S.D. from 24 fields (2 mice/group, 2 sections/mouse, 6 fields/section). $^{*}P<0.05$; $^{***}P<0.001$, versus NS group. $^{a}P<0.05$, versus TM208 (100 mg/kg/d) group. $^{b}P<0.05$, versus 5-Fu (5 mg/kg/2d) group.

Table 3. Cell cycle analysis of H_{22} transplanted tumor treated with the combination therapy of TM208 and 5-Fu

	Dosage (mg/kg)	G_1 (%)	S (%)	G_2/M (%)
NS	–	38.5±2.6	55.0±4.3	5.8±3.1
TM208	100	33.0±3.8	51.5±5.2	15.5±4.8*
5-Fu	5	29.7±4.1*	53.3±4.9	17.1±0.9***
TM208+5-Fu	100+5	18.8±2.4***	56.9±3.2	24.3±2.8$^{***, a, b}$

Four mice were treated in each group. TM208 (100 mg/kg/d) was treated with 5-Fu (5 mg/kg/2d) spontaneously. Cell cycle distribution of H_{22} transplanted tumors was analyzed. $^{*}P<0.05$; $^{***}P<0.001$, versus NS group. $^{a}P<0.05$, versus TM208 group. $^{b}P<0.01$, versus 5-Fu group.

two agents further significantly increased compared with that of either drug alone. This observation suggested that the combination of TM208 and 5-Fu had an enhanced effect on the G_2/M cell cycle arrest.

3.7. Regulation of the expression of cell cycle-related proteins

To investigate the mechanism of tumor inhibition in combination chemotherapy, we examined the expression levels of some specific regulatory proteins including cyclin D1, cdk4, cyclin E, cdk2, cyclin B1, cdc2 (p34), p21, p53, and cdk7, which may be involved in the cell cycle arrest in H_{22} transplanted tumor treated with TM208 and 5-Fu. As Figure 5A and 5C shown, single-agent treatment resulted in a significant reduction in the expression of cyclin E and cyclin D1 compared to the control group, while combination therapy led to a further reduction of these two proteins. No significant changes in cdk4 and cdk2 were observed. As Figure 5B and 5D shown, compared to the negative control group, the expression of cyclin B1, cdc2 (p34), and cdk7 decreased in TM208 group and 5-Fu group, while that of p53 in 5-Fu group increased. No significant difference was observed between the expression of p21 of single-agent groups and the negative control group. Further decrease in the expression of cyclin B1, cdc2 and cdk7 was observed by comparing the gray-levels of these proteins of the combination group and the single-agent groups. A significant increase in p21 was observed in combination group, compared to the control group. The gray-level of p53 in the combination group was significantly higher than that of NS negative control group, but no significant difference was observed between the combination group and the single-agent groups, especially the 5-Fu group.

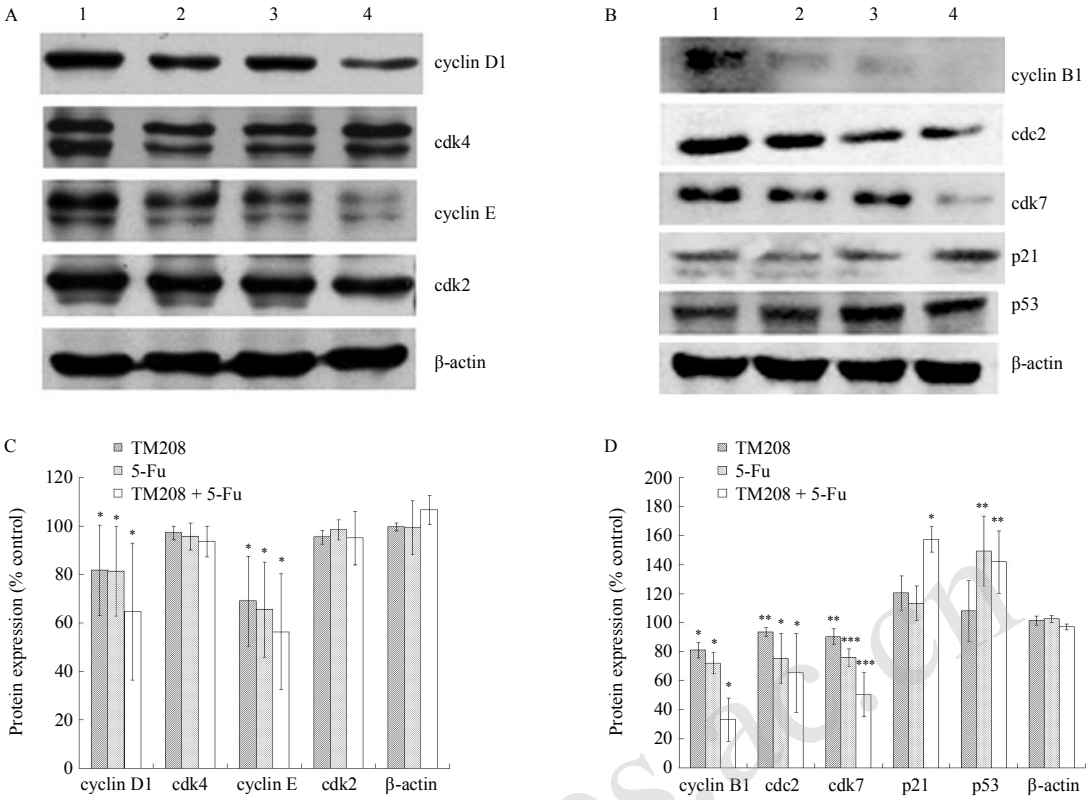

Figure 5. Effects of combination chemotherapy of TM208 and 5-Fu on the expression of cyclin D1, cdk4, cyclin E, cdk2, cyclin B1, cdc2, p21, p53, and cdk7 in tumor tissues of H_{22} transplanted mice. Lanes 1–4: NS, TM208 (100 mg/kg/d), 5-Fu (5 mg/kg/2d), combination group. Cellular lysate protein (65 μg) from solid-type H_{22} tumors in vivo was loaded on a 10% sodium dodecyl sulfate-polyacrylamide gel, electrophoresed and subsequently transferred onto nitrocellulose membranes. Immunoblots were probed with antibodies specific for main cell cycle related proteins. β-Actin level was examined as a loading control. The results shown were representative blot of four animals per group.

3.8. Regulation of the mRNA expression of cyclin B1 and cdc2

The results described above suggested that the downregulation of cyclin B1 and cdc2 by the TM208 and 5-Fu combination therapy may be the main cause of the G_2/M phase arrest. To further understand the mechanism of the combination therapy-induced protein expression changes, we analyzed the mRNA expression of cyclin B1 and cdc2 in H_{22} transplanted tumor tissues using RT-PCR. The expression of cdc2 mRNA decreased in single-agent group compared to the control group, and further reduction was observed in combination group; however, the difference in cyclin B1 mRNA expression was not detected between every agent-treated group and the control group (Fig. 6).

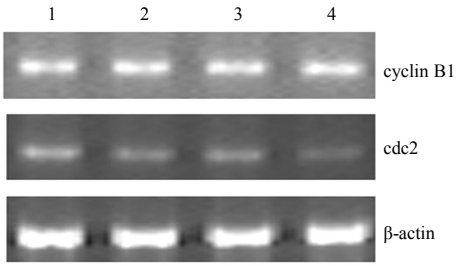

Figure 6. mRNA expression of cyclin B1 and cdc2 in tumor tissues of H_{22} transplanted mice when treated with TM208 and 5-Fu. Lanes 1–4: NS, TM208 (100 mg/kg/d), 5-Fu (5 mg/kg/2d), combination group. After treatment for 9 d, H_{22} tumors were harvested and the PCR products were analyzed by gel electrophoresis on 2% agarose gels for analysis of mRNA expression of cyclin B1 and cdc2. β-Actin was examined as a loading control.

4. Discussion

Combination therapies, which have been proven to be effective in treating a wide variety of aggressive human cancers, have become a general approach in cancer treatment[10–12]. The goal of developing combination therapy regimens is to obtain optimal drug combinations in which the individual agents interact to achieve augmentation of antitumor effects, with side effects or toxicities as low as possible.

As a potential chemotherapeutic agent that can be administrated orally, TM208 exhibits tumor growth inhibition with low toxicity in several preclinical mice models. Based on the principles of combination therapy, which include prevention of overlapping side effects and promotion of therapeutic action on cells of different sensitivities via distinct mechanisms, we are interested in the performance of TM208 on the H_{22} mice model in combination with drugs in wide clinical use, e.g. DDP, CTX and 5-Fu. This research is expected to provide evidence for further clinical use of TM208.

The toxicity of each single agent (DDP, CTX and 5-Fu) was evaluated. The results indicated that these cytotoxic agents at the high dose level exerted severe side effects on the immune organs, and also affected body weight slightly. To limit the toxicity at the lowest level, low dosages of these cytotoxic agents were chosen for combination therapy.

Our results reveal that the combination therapy of TM208 and 5-Fu has achieved significant augmentation of the antitumor effect on H_{22} transplanted ICR mice. When mice were treated with TM208 (100 mg/kg/d) in combination with 5-Fu (5 mg/kg/2d) simultaneously, tumor inhibition rate exceeded 60%, while single-agent therapy inhibited the tumor growth with inhibition rates less than 25%. Significant differences between the combination group and the single-agent groups were observed (P<0.01). However, similar effects were not achieved when mice were treated with TM208 in combination with DDP and CTX, respectively, on the H_{22} mice model. Only a slight elevation of the tumor inhibition rate was observed in the combination therapy of TM208 and CTX. Meanwhile the tumor inhibition of the combination group of TM208 and DDP remained at the same level as that of the DDP group. The pathological examination of the tumor tissues in the combination therapy of TM208 (100 mg/kg/d) and 5-Fu (5 mg/kg/2d) revealed that the H_{22} cells in the NS treated group had clear cell membranes with a compact arrangement, while different extents of coagulative necrosis were observed in TM208, 5-Fu, and the combination groups, with vacuolar degeneration of the cytoplasm and indistinct cell membranes. In addition, the Ki-67 nuclear protein staining further demonstrated a significant decrease in the number of positive cells in animals receiving the combined treatment compared to single agents, which indicated that 5-Fu could effectively increase inhibition of cellular proliferation in the tumors, induced by TM208.

The toxicity of combination therapy was also evaluated by analyzing body weight, the immune organ index and the number of WBC in blood. The combination therapy did not provide DDP with a higher tumor inhibition rate but with more severe side effects on the immune organs and body weight than the corresponding two single-agent groups. As for CTX, the combination therapy did not lead to an accumulation of toxicity, with no significant difference between combination group and single-agent group. In contrast, the antitumor effect was augmented in the combination therapy of TM208 and 5-Fu, and side effects on body weight change were observed only in the early phase of administration (P<0.01), with no other significant detectable toxicity. Our results indicate that 5-Fu can potentiate the antitumor effect of TM208, with a slight elevation of side effects, while DDP and CTX cannot.

Given the significantly enhanced tumor growth inhibition rate when mice were treated with TM208 (100 mg/kg/d) in combination with 5-Fu (5 mg/kg/2d), we are interested in the mechanism of how 5-Fu potentiates the antitumor activity of TM208. According to an earlier study[2], which showed TM208 significantly inhibited tumor growth in an xenograft tumor model with QGY-7703 cells by inducing G_2/M cell cycle arrest (at least partly), we examined the cell cycle distribution of H_{22} transplanted tumor cells and the expression of some key proteins to investigate whether this enhancement of combination chemotherapy of TM208 and 5-Fu was related to cell cycle arrest.

The treatment with TM208 resulted in the accumulation of cells at the G_2/M phase suggesting a G_2 to M block in the cell cycle, which is consistent with the preliminary report. The cytotoxic effect of 5-Fu has been principally ascribed to the misincorporation of fluoronucleotides into DNA and RNA during their synthesis, and the inhibition of thymidylate synthase (TS) by FdUMP (one of the active metabolites of

5-Fu), which leads to nucleotide pool imbalance[13]. The results of cell cycle analysis revealed that the treatment with 5-Fu led to G_2/M cell cycle arrest of H_{22} transplanted tumor in vivo. Meanwhile, the number of cells arrested at the G_2/M phase by combined TM208 and 5-Fu treatment was markedly higher (24.3% accumulation, i.e. about 50% more) than that caused by either agent alone.

The cell cycle is mediated by the activation and inactivation of a large family of proteins. G_1 progression and G_1/S transition are regulated by cdk2 and cdk4, which assemble with cyclin E and cyclin D1. We found that single-agent treatment caused a slight decrease in cyclin D1 and cyclin E expression, while combination therapy further downregulated the expression of cyclin D1 and cyclin E. No significant change in cdk2 and cdk4 expression was observed between control group and agent-treated groups. Cyclin B1 is an activator of cyclin-dependent kinase1 (cdc2; p34), and both proteins work in a complex that plays an important role in the progression from G_2 to the M phase of the cell cycle[14,15]. Downregulation of cyclin B1 and cdc2 in TM208 (100 mg/kg/day) group as our result revealed, in agreement with an earlier study, may be the main cause of the G_2/M phase arrest. When mice were treated with TM208 in combination with 5-Fu, a further downregulation of cyclin B1 and cdc2 was observed, compared with the single-agent groups, which might finally lead to the inhibition of tumor growth.

Progression through G_2, culminating in mitosis, requires the formation of cdc2 and cyclin B1 complexes. The threonine residue 161 of mammalian cdc2 is implicated in cyclin binding, and phosphorylation at this position may be a prerequisite of the combination between cdc2 and cyclin B1[16,17]. This activation is dependent upon cdk7 kinase. Our experiments further revealed that the expression of cdk7 was inhibited by TM208 and 5-Fu, treated alone. Combination therapy enhanced the inhibition, which hamperd cdk7 from phosphorylating Thr^{161} of cdc2 and contributed to the G_2/M arrest.

While cell cycle progression is strictly regulated by a class of cyclins and cyclin-dependent kinases (CDKs), constraints upon CDK activity and cell cycle progression are provided by CDK inhibitors (CKIs), which can bind and inactivate the CDK-cyclin complexes[18]. $p21^{WAF1/Cip1}$ is a general inhibitor of CDKs. It can accumulate in the nuclei during the onset of G_2/M and cause a transient block in late G_2 phase[19]. Meanwhile, p53 serves as a major barrier for carcinogenesis by inducing cell cycle arrest and apoptosis in response to various endogenous and exogenous stimulations. p53-Mediated cell cycle arrest is partly achieved through the transcriptional activation of p21[20]. In our study, we further analyzed the role of p21 and p53 in the TM208 and 5-Fu combination therapy. The results showed combination therapy upregulated the expression of p21. The upregulation of p53 was also observed in the three agent-treated groups, compared to the negative control group. This result suggested that TM208 and 5-Fu combination therapy induced G_2/M arrest in a p53, p21-mediated manner.

Since protein downregulation of cyclin B1 and cdc2 has been identified as the main cause of G_2/M cell cycle arrest, it is of great interest to investigate whether transcriptional inhibition was involved in the TM208 and 5-Fu combination therapy. RT-PCR was employed to analyze the mRNA expression of cyclin B1 and cdc2. No significant change of cyclin B1 mRNA expression was observed between the control group and agent-treated groups, which indicated that the combination therapy of TM208 and 5-Fu may affect the cyclin B1 protein synthesis or reduce the protein stability followed by degradation through the ubiquitin/proteasome pathway. The change of cdc2 mRNA expression was consistent with that of protein expression, which indicated that the regulation of cdc2 is mainly mediated at the transcriptional level.

In summary, we showed that 5-Fu can potentiate the antitumor effect of TM208 on the H_{22} solid-type mice model, with a slight elevation of side effects, based on the mechanism related to G_2/M cell cycle arrest. The further mechanism by which TM208 and 5-Fu combination therapy shows enhanced antitumor effect still needs to be clarified. The dose-sequence relationship for TM208 and 5-Fu combination therapy on H_{22} also needs exploration. How TM208 inhibits tumor growth on some other preclinical cancer model in combination with more clinically used drugs (e.g. adriamycin and mitomycin) or hormone therapy should also be investigated to achieve maximal

antitumor efficacy and selectivity, and to provide a rationale for the clinical development of TM208.

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TM208与5-氟尿嘧啶联合应用对小鼠肝癌H_{22}移植瘤的抗肿瘤作用及其机制研究

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摘要: 4-甲基哌嗪-1-二硫代甲酸-(3-氰基-3,3-二苯基)丙酯盐酸盐 (TM208)是一种新合成的氨基二硫代甲酸酯类化合物, 具有良好的体内抗肿瘤作用, 且毒性较低。探讨TM208与临床已知抗癌药联用能否提高疗效并降低毒性, 可为TM208的临床试验提供依据。本实验通过小鼠肝癌H_{22}移植瘤模型考察了TM208与顺铂 (DDP)、环磷酰胺 (CTX)、5-氟尿嘧啶(5-Fu)分别联合用药的体内抗肿瘤作用及毒性。体内实验结果证实, 5-Fu (5 mg/kg/2d) 与TM208 (100 mg/kg/d) 联用后可显著增强对H_{22}肿瘤的抑制作用 (P<0.01), 且几乎不增加毒性; 而DDP和CTX则不能。进一步研究表明, TM208与5-Fu联用可通过下调cyclin B1, cdc2, cdk7和上调p21, p53的表达引起H_{22}实体瘤细胞发生G_2/M周期阻滞。同时此联合用药方案也可下调cyclin D1, cyclin E的表达, 对cdk4, cdk2的表达则没有影响。Cdc2 mRNA的表达与其蛋白表达趋势一致, 而cyclin B1 mRNA表达在各组间没有差异。总之, TM208与5-Fu联用可提高抗肿瘤疗效, 毒性不变, 其抗肿瘤作用与细胞周期阻滞及其相关蛋白的表达变化有关。

关键词: 联合用药; 小鼠肝癌H_{22}; 氨基二硫代甲酸酯类化合物; 5-氟尿嘧啶; 细胞周期相关蛋白