Enhanced anticancer activity of 5-FU in combination with Bestatin: Evidence in human tumor-derived cell lines and an H22 tumor-bearing mouse

Jin Li¹, Xuejian Wang², Jinning Hou¹, Yongxue Huang³, Yingjie Zhang¹,*, Wenfang Xu¹,*

¹Department of Medicinal Chemistry, School of Pharmaceutical Sciences, Shandong University, Ji'nan, Shandong, China; ²Key Laboratory of Applied Pharmacology in Shandong Province, Department of Pharmacology, School of Pharmacy, Weifang Medical College, Weifang, Shandong, China; ³Bochuang International Medical Institute, Weifang, Shandong, China.

Summary
The clinical use of 5-fluorouracil (5-FU) is increasingly limited by low response rates, adverse reactions, and toxicity. A drug combination offers a new strategy for appropriate use of 5-FU. Bestatin, an aminopeptidase N (APN) inhibitor, has been used as an adjuvant chemotherapy drug because of its actions to suppress tumorigenesis and invasion. The current study evaluated the anticancer efficacy of 5-FU plus Bestatin at the cellular and animal level. The combination killed more colonic cancer, hepatic carcinoma, and ovarian cancer cells and fewer nonmalignant human embryonic kidney (HEK293) and Chinese hamster ovary (CHO) cells than 5-FU or Bestatin alone. Moreover, 41.58% of ES-2 and 20.86% of PLC/PRF/5 cell apoptosis was caused by the combination of the two, while 5-FU caused apoptosis of 20.86% of ES-2 cells and 8.85% of PLC/PRF/5 cells. The cell cycle was arrested in the S and G0/G1 phases when a combination of the two was used. In an experiment involving mice bearing tumors, a combination of the two had a rate of tumor inhibition of 61.98%, while 5-FU alone had a rate of tumor inhibition of just 49.17%. In addition, the combination of the two was safer than either drug alone and did not cause weight loss or death. In conclusion, combining 5-FU and Bestatin could enhance the anticancer activity of 5-FU and decrease its cytotoxicity. These results suggest that 5-FU plus Bestatin has greater efficacy as a tumor therapy.

Keywords: Drug combination, APN/CD13 inhibitor, Bestatin, 5-FU, anticancer or antitumor action

1. Introduction

Chemotherapy is an important form of cancer treatment that is widely used to treat malignant tumors in clinical practice. Cytotoxic drugs such as ADM, 5-fluorouracil (5-FU), cisplatin, and mitomycin are "star" drugs among chemotherapeutics. 5-FU is commonly used in clinical practice to treat oophoroma, breast cancer, neck and head cancer, and gastrointestinal malignancies (1). 5-FU inhibits cell proliferation and growth by pretending to be uracil, suppressing the activity of thymidylate synthase (TS) (2). Cancer cells are damaged when the synthesis of deoxyadenosine triphosphate (dTMP) is disturbed. However, 5-FU alone results in a low response rate, and adverse reactions to it and its toxicity have gradually forced it out of the limelight. 5-FU has been found to result in serious adverse reactions and toxicity to the healthy body by causing conditions such as myelo-suppression, nausea, emesis, and hand-foot syndrome (3,4). In addition, its clinical use is highly limited due to drug resistance. To overcome these shortcomings, a novel strategy in the form of a drug combination has been used to treat cancer using 5-FU. A combination of 5-FU with other cytotoxic drugs, such as oxaliplatin, or novel biological agents, such as monoclonal antibodies, are less toxic because of the reduced dose of each drug and can prolong survival.
(2,5-7). Murad et al. reported that a combination of paclitaxel and 5-FU provided an effective and safe regimen for the treatment of advanced gastric cancer (8). Identifying more appropriate agents for use in combination with 5-FU would improve the treatment of cancer.

Bestatin, a commercially available APN/CD13 inhibitor, has been used as an adjuvant chemotherapy drug because of its anticancer activity to suppress tumor invasion and induce cell apoptosis. Bestatin also works as an immunoenhancer in oncotherapy. It prolonged the survival of patients with acute adult non lymphocytic leukemia (9). Aminopeptidase N (EC 3.4.11.2, APN), also known as CD13, is a zinc-dependent transmembrane metallo-peptidase belonging to the M1 family; APN degrades preferentially proteins with an N-terminal neutral amino acid, such as Phe, Leu, and Gly (10-12). Widely expressed in various tissues (13,14), APN/CD13 plays important roles in various biological processes, such as antigen presentation, signal transduction, and angiogenesis (15). APN/CD13 is overexpressed in many tumor cells, including melanoma, thyroid cancer, prostate cancer, pancreas cancer, and myeloid leukemia (16,17), and APN/CD13 has been implicated in angiogenesis and cancer progression (18). It assists the degradation of type IV collagen, possibly contributing to tumor invasion and metastasis (19,20). APN/CD13 has been identified as promoting the generation of tumor vasculature and other types of new blood vessels (21-24). Therefore, APN/CD13 has been regarded as a significant target for anticancer agent development (3).

5-FU is a well-known cytotoxic drug used in chemotherapy, but its use is limited due to its low response rates, adverse reactions, and considerable toxicity. The APN inhibitor Bestatin could enhance immunity and possesses antitumor activity to some extent. Recently, Haraguchi et al. found that 5-FU plus Bestatin strongly inhibited liver tumor growth in a NOD/SCID mouse model (25). Haraguchi et al. noted that APN/CD13 was a therapeutic target in human liver cancer stem cells (CSC). The treatment of liver cancer may be improved by combining Bestatin and 5-FU to disrupt APN/CD13+ cells (CSC) by respectively up-regulating the ROS level and inhibiting the proliferation of general cancer cells. In additional, treatment with 5-FU or doxorubicin could up-regulate APN/CD13 expression on the surface of PLC/PRF/5 cells. Therefore, combining the APN/CD13 inhibitor with a cytotoxic anticancer drug may prolong patient survival and greatly reduce the suffering of patients. A combination of 5-FU and Bestatin could serve as an effective therapy to treat cancer with a low level cytotoxicity. The current study evaluated the pharmaceutical activity of 5-FU plus Bestatin in colon cancer, hepatic carcinoma, and ovarian cancer cell lines and nonmalignant human embryonic kidney and Chinese hamster ovary cell lines. The anticancer activity of the two drugs was also assessed in animals. Results should indicate whether this drug combination has potential as a chemotherapy regimen.

2. Materials and Methods

2.1. Materials

5-FU was purchased from Hisun Pharmaceutical (Zhejiang, China). Bestatin and 5-FU were dissolved in DMSO and diluted with saline solution or cell culture medium before use. MTT [(3-[4, 5-dimethyl-2-thiazolyl]-2, 5-diphenyl-2H-tetrazolium bromide)] and L-leucine-p-nitro-anilide were from Solarbio (Beijing, China). PE anti-human CD13 was from BD (New York, the USA). Hoechst 33342 and an Annexin-V/PI apoptosis detection kit were purchased from Beyotime (Jiangsu, China). Microsomal aminopeptidase from porcine kidney microsomes was from Sigma-Aldrich (Shanghai, China). Male Kunming mice were supplied by the Experimental Animal Center of Shandong University. The ratio of combination of the two drugs means the molar concentration ratio.

2.2. Cell lines and culture

Cancer cells (HCT-116, HepG2, ES-2, and PLC/PRF/5 cells) were maintained in RPMI-1640 medium. In addition, human embryonic kidney (HEK293) cells were cultured in DMEM medium, and Chinese hamster ovary (CHO) cells were cultured in DMEM:F-12 (1:1) medium. All media were supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 100 IU/mL penicillin, and 100 IU/mL streptomycin in a 5% CO2 humidified atmosphere at 37°C. Cells were harvested with 0.25% EDTA-Trypsin.

2.3. Assay of enzyme activity

The enzyme activity of the drugs were determined using L-leu-p-nitro-anilide as a substrate and microsomal aminopeptidase from porcine kidney microsomes in 50 mM PBS buffer, pH 7.2, at 37°C. The mixture of the APN/CD13 enzyme and drug compounds was incubated at 37°C for 5 min. The substrate was then added and the resulting mixture was incubated for another 30 min at 37°C. The hydrolysis of the substrate was measured by following the change in absorbance monitored at 405 nm with a Micro-plate Reader (ThermoFisher, Shanghai, China). The experiment was repeated more than three times.

APN/CD13 activity on the surface of ES-2 cells was estimated by measuring the hydrolysis of L-leucine-p-nitro-anilide. A cell suspension (2.86 × 10⁶/mL) in 1× PBS buffer was added to a 96-well plate (70 μL) containing 1–256 μM of 5-FU or 3.125–400 μM of the combined drugs. Subsequently, L-leucine-p-nitro-anilide
was added to a final concentration of 1.6 mM and the mixture was incubated for 1 h in a thermostatic shaker at 37°C, 100 rpm. APN/CD13 activity was then estimated by measuring the absorbance at 405 nm as described before. The experiment was repeated more than three times.

2.4. MTT assay

The cell viability of four cancer cell lines exposed to drugs was evaluated using an MTT assay and 96-well plates. HEK293 and CHO cells were chosen to evaluate the toxicity of compounds with the MTT assay. In brief, cells were seeded on 96-well plates at 4000/well. After incubation for 24 h, cells were treated with Bestatin, 5-FU, or a combination of the two (the ratio of the concentration of 5-FU to Bestatin was 1:1, 2.5:1, 5:1, and 10:1). After incubation for another 48h, 0.5% MTT was added to each well. Four hours later, 200 μL DMSO was added and mixed for 10 min. The absorbance values at 490 nm were then recorded using the Micro-plate Reader (ThermoFisher). The experiment was repeated more than three times.

2.5. Clonogenic assay

A clonogenic assay was performed to evaluate the effect of the compounds on cancer cell growth over a relatively long duration of exposure. PLC/PRF/5 and ES-2 cells, seeded on six-well plates (250 cells per well), were exposed to 5-FU, Bestatin, and a combination of the two (5-FU:Bestatin, 5:1) for two consecutive weeks at 37°C. Cells were fixed with fresh 4% paraformaldehyde at room temperature for 15 min and stained with Giemsa. Cell images were captured using an inverted microscope (magnification ×4, Olympus, Beijing, China). The colonies (more than 50 cells) in each experimental group were counted and were then compared to the control. The experiment was repeated more than three times.

2.6. Assay of Hoechst 33342 staining

PLC/PRF/5 and ES-2 cells seeded on 6-well plates (1 × 10⁵ per well) were exposed to 5-FU (10 μM for PLC/PRF/5, 4 μM for ES-2) and 5-FU plus Bestatin (5:1, 10 μM for PLC/PRF/5, 4 μM for ES-2) for 24 h. Cells were then stained with Hoechst 33342 at 37°C for 15 min in the dark. The apoptotic cells were stained blue and were observed using the Olympus inverted microscope (magnification ×20).

2.7. Flow cytometry

Flow cytometry was used to evaluate the expression of APN/CD13 on cells. Cells were harvested and washed with cold 1× PBS buffer. PE anti-human CD13 was added to 1 × 10⁶ cells in 100 μL 1× PBS. After incubation for 15 min in 4°C, cells were washed and analyzed using flow cytometry.

Cell apoptosis and cell cycles were determined using flow cytometry. PLC/PRF/5 and ES-2 cells seeded on 6-well plates (1 × 10⁵ per well) were treated with 5-FU (2 μM) and Bestatin (2 μM) and 5-FU plus Bestatin (5:1, 2 μM) for 48 h. Cells were harvested and washed with cold binding buffer. Phosphatidylserine on the surface of apoptotic cells was quantitatively detected using an Annexin-V/FITC and PI apoptosis detection kit in accordance with the manufacturer’s instruction. Cell cycles were detected using PI staining. Cell apoptosis and cell cycles were analyzed using flow cytometry.

2.8. Animal experiment

All experiments concerning living laboratory animals were performed the approval of the local ethics committee. Mice were inoculated subcutaneously with injections of 1 × 10⁴/mL H22 cells. When the tumor was approximately 0.2 cm in size, mice were divided randomly into five groups. A: Control group; B: Combination group 1 treated with 5-FU (15 mg/kg/day) plus Bestatin (7.5 mg/kg/day); C: Combination group 2 treated with 5-FU (7.5 mg/kg/day) plus Bestatin (3.65 mg/kg/day); D: Bestatin group (50 mg/kg/day); and E: 5-FU group (15 mg/kg/day). 5-FU and Bestatin were dissolved in DMSO and diluted with 1× PBS to the appropriate concentration. Each mouse was administered 100 μL of the drug solution in the abdominal cavity every time. All groups were treated with drugs for 2 weeks. Inhibition of tumor growth was calculated at the end of the treatment to determine antitumor action.

3. Results

3.1. Expression of APN/CD13 and inhibition of APN/CD13 enzyme activity

The extent to which compounds inhibited APN/CD13 enzyme activity was estimated by quantifying the APN/CD13 enzymatic cleavage of the substrate L-leucine-p-nitro-anilide. As shown in Table 1, APN/CD13 enzymatic activity

<table>
<thead>
<tr>
<th>Compounds</th>
<th>IC₅₀ (μM, mean ± S.D.)</th>
<th>IC₅₀ of Bestatin</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-FU</td>
<td>&gt; 256</td>
<td></td>
</tr>
<tr>
<td>Bestatin</td>
<td>3.50 ± 0.39</td>
<td>3.50</td>
</tr>
<tr>
<td>5FU:Bestatin (1:1)</td>
<td>9.35 ± 2.98</td>
<td>4.68</td>
</tr>
<tr>
<td>5FU:Bestatin (2.5:1)</td>
<td>19.3 ± 3.94</td>
<td>5.51</td>
</tr>
<tr>
<td>5FU:Bestatin (5:1)</td>
<td>21.9 ± 6.88</td>
<td>3.65</td>
</tr>
<tr>
<td>5FU:Bestatin (10:1)</td>
<td>37.5 ± 6.99</td>
<td>3.41</td>
</tr>
</tbody>
</table>

Data are expressed as mean values from three independent experiments. aIC₅₀ Values for Bestatin were calculated from the corresponding IC₅₀ for Bestatin and 5-FU.
was significantly inhibited in all groups treated with Bestatin, as indicated by IC\textsubscript{50} values at the micromole level. 5-FU did not suppress APN/CD13 activity because of its large IC\textsubscript{50} with respect to APN/CD13. The four groups treated with a combination of Bestatin and 5-FU had an IC\textsubscript{50} like that for Bestatin alone, indicating that 5-FU did not affect interaction between Bestatin and APN/CD13.

Moreover, the influence of 5-FU on the binding of Bestatin to APN/CD13 was investigated at the cellular level. First, flow cytometry was used to detect the level of APN/CD13 expression in a panel of cells, including PLC/PRF/5, HepG2, HCT-116, and ES-2 cells (Figure 1). Over-expressing APN/CD13, ES-2 cells were selected to evaluate the APN/CD13 activity on the cell surface in groups treated with drugs. The results are shown in Table 2 and lead to a similar conclusion as before, i.e. that 5-FU did not inhibit APN/CD13 activity and that it did not influence the binding of Bestatin to APN/CD13.

### 3.2. In vitro assay of inhibition of proliferation and cytotoxicity

To explore the effects of compounds on human cancer cell lines and non-malignant cells in vitro, the IC\textsubscript{50} was assessed with an MTT assay using a panel of human cancer cell lines, namely HCT-116 (colonic cancer), HepG2 (hepatic carcinoma), ES-2 (ovarian cancer), and PLC/PRF/5 (hepatic carcinoma). The IC\textsubscript{50} was also assessed with an MTT assay using a panel of non-malignant cells, namely CHO and HEK293 cells. As shown in Table 3, combining Bestatin with 5-FU inhibited the growth of cancer cells at a lower concentration than Bestatin or 5-FU alone. With high IC\textsubscript{50} values, Bestatin displayed little cytotoxicity to a panel of cancer cells and non-malignant cells. 5-FU strongly inhibited non-malignant CHO and HEK293 cells, but 5-FU plus Bestatin had relatively lower cytotoxicity (Table 4). When the ratio of the concentration of 5-FU to Bestatin was 5:1, the combination had a relatively higher efficacy and lower toxicity. At a ratio of 10:1, the combined drugs did not have a marked increase in

### Table 2. APN/CD13 enzymatic activity on the cell surface of cells treated with 5-FU, Bestatin, and a combination of the two

<table>
<thead>
<tr>
<th>Compounds</th>
<th>IC\textsubscript{50} (μM, mean ± S.D.\textsuperscript{a})</th>
<th>IC\textsubscript{50} of Bestatin\textsuperscript{b}</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-FU</td>
<td>&gt; 256</td>
<td></td>
</tr>
<tr>
<td>Bestatin</td>
<td>57.2 ± 5.8</td>
<td>57.2</td>
</tr>
<tr>
<td>5-FU:Bestatin (1:1)</td>
<td>98.9 ± 16.5</td>
<td>49.4</td>
</tr>
<tr>
<td>5-FU:Bestatin (2.5:1)</td>
<td>112 ± 19.8</td>
<td>32.0</td>
</tr>
<tr>
<td>5-FU:Bestatin (5:1)</td>
<td>164 ± 49.8</td>
<td>27.3</td>
</tr>
<tr>
<td>5-FU:Bestatin (10:1)</td>
<td>1051 ± 41.1</td>
<td>95.5</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Data are expressed as the mean values from three independent experiments. \textsuperscript{b} IC\textsubscript{50} values for Bestatin were calculated from the corresponding IC\textsubscript{50} for Bestatin and 5-FU.

### Table 3. The anti-proliferative action (IC\textsubscript{50}) of Bestatin plus 5-FU in 4 strains of cancer cells and 2 strains of non-malignant cells

<table>
<thead>
<tr>
<th>Cells</th>
<th>Compds/IC\textsubscript{50} (μM, mean ± S.D.)\textsuperscript{a}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1:1\textsuperscript{b}</td>
</tr>
<tr>
<td>HCT-116</td>
<td>20.9 ± 4.81</td>
</tr>
<tr>
<td>HepG2</td>
<td>17.3 ± 1.65</td>
</tr>
<tr>
<td>ES-2</td>
<td>24.5 ± 0.85</td>
</tr>
<tr>
<td>PLC/PRF/5</td>
<td>61.4 ± 7.73</td>
</tr>
<tr>
<td>HEK293</td>
<td>ND\textsuperscript{c}</td>
</tr>
<tr>
<td>CHO</td>
<td>ND\textsuperscript{c}</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Data from an MTT assay and the mean from 3 repetitions and the standard deviation. \textsuperscript{b} Ratio refers to the molar ratio of 5-FU and Besatin. \textsuperscript{c} Not detected.

### Table 4. The rate of tumor inhibition for 5-FU, Bestatin, and a combination of the two in a Kunming mouse model with subcutaneous H22 tumor cells

| Compounds | Dose | Survived/total mice | Tumor weight (mean ± S.D.) | Inhibition rate\textsuperscript{c} (%)
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>PBS</td>
<td>7/7</td>
<td>2.42 ± 0.94 g</td>
<td>0</td>
</tr>
<tr>
<td>5-FU</td>
<td>15 mg/kg/day</td>
<td>5/7</td>
<td>1.23 ± 0.54 g</td>
<td>49.2</td>
</tr>
<tr>
<td>Bestatin</td>
<td>50 mg/kg/day</td>
<td>7/7</td>
<td>1.68 ± 0.89 g</td>
<td>30.6</td>
</tr>
<tr>
<td>5-FU:Bestatin (5:1)</td>
<td>7.5 mg/kg/day+3.65 mg/kg/day</td>
<td>7/7</td>
<td>1.20 ± 0.66 g</td>
<td>50.4</td>
</tr>
<tr>
<td>5-FU:Bestatin (5:1)</td>
<td>15.0 mg/kg/day+7.5 mg/kg/day</td>
<td>7/7</td>
<td>0.92 ± 0.47 g</td>
<td>62.0</td>
</tr>
</tbody>
</table>

\textsuperscript{c} The tumors were weighed after mice were sacrificed, and the inhibitory effect in each group was defined as a percentage of the control tumor weight.
anticancer but they did have greater cytotoxicity to non-malignant cells. Therefore, the appropriate molar ratio of 5-FU to Bestatin was determined to be 5:1.

A colony assay revealed similar results, as shown in Figure 2A. PLC/PRF/5 and ES-2 cells were chosen for the colony-forming assay. The colony-forming ability of cancer cells was effectively inhibited by a combination of the two drugs. Bestatin plus 5-FU inhibited cancer cells by 54.1% in PLC/PRF/5 cells and 92.8% in ES-2 cells, while 5-FU alone inhibited cancer cells by 39.2% in PLC/PRF/5 cells and 95.2% in ES-2 cells. In contrast, Bestatin alone had no effect on colony formation in the two cell lines, which may have been due to its low concentration.

3.3. Induction of apoptosis and prevention of cell cycle progression

Hoechst 3342 staining was performed to determine whether 5-FU plus Bestatin induced cell apoptosis. Significant morphological changes in PLC/PRF/5 and ES-2 cells were observed after treatment with combined drugs for 24 h. Cells with a bright blue nucleus after Hoechst staining are known to have cellular shrinkage and nuclear fragmentation, which are typical features of apoptotic cells. Figure 3 indicates that the combination of Bestatin plus 5-FU resulted in more apoptotic cells than 5-FU or Bestatin alone. To further measure the percentage of apoptotic and necrotic cancer cells, Annexin-V/PI staining was analyzed using flow cytometry. Figure 4 shows the specific percentage of PLC/PRF/5 and ES-2 cells. The combination of the two drugs significantly induced cell apoptosis at a rate of 20.86% in PLC/PRF/5 cells and 41.58% in ES-2 cells, while 5-FU or Bestatin alone resulted in relatively low rates of apoptosis in both PLC/PRF/5 cells (< 10%) and ES-2 cells (< 30%). These results indicate that the drug combination inhibited cell growth by greatly inducing apoptosis.

The cell cycles of PLC/PRF/5 cells treated with 5-FU or Bestatin alone or in combination were also examined. Flow cytometry (Figure 5) revealed that 5-FU exposure induced cells to remain in the S phase while Bestatin increased the number of cells in the G0/G1 phase. The results of the combination of the two drugs indicated that the cell cycle was arrested in the G0/G1 and S phase.
To evaluate the antitumor action of 5-FU plus Bestatin in vivo, a Kunming mouse model was established with subcutaneous H22 tumor cells using 5-FU and Bestatin as the positive control. Figure 6A and Table 4 show that a combination of the two significantly inhibited tumor growth in contrast to the control drugs. In comparison...
to 5-FU (15 mg/kg/day) alone, the combination of 5-FU and Bestatin used half the dose of 5-FU (7.5 mg/kg/day), but the two had similar rates of inhibition (about 50%). Bestatin inhibited the tumor by only ~30% at a large dose (50 mg/kg/day). In contrast to 5-FU alone, a combination of 5-FU and a low dose of Bestatin (7.5 mg/kg/day) increased the rate of inhibition from 49.2% to 62.0%. In addition, two mice in the 5-FU group died while no mice in the other groups died, indicating the substantial toxicity of 5-FU and its reduced toxicity when used with Bestatin. The mean weight of mice in each group is shown in Figure 6B. 5-FU resulted in weight loss after three days of drug delivery while the Bestatin group and the combination groups tolerated the treatment well.

4. Discussion

Although 5-FU is commonly used as an anticancer drug, its clinical use is increasingly limited due to drug resistance, adverse reactions, and toxicity. A drug combination provides a novel strategy to enhance its efficacy and reduce its toxicity when treating cancer. APN, also known as CD13, is a zinc-dependent M1-class metalloprotease that is over-expressed in cancer cells; APN plays important roles in various biological processes, such as antigen presentation, signal transduction, and angiogenesis (22, 26). APN/CD13 is considered to be an important target for developing anticancer agents. Moreover, APN/CD13 has been found to be a biomarker of liver cancer stem cells (27, 28). Bestatin is widely used as an APN/CD13 inhibitor in adjuvant chemotherapy and it prolongs patient survival.

The current study investigated the anticancer activity of 5-FU plus Bestatin in vitro and in vivo. An assay of enzyme activity at the protein and cell level revealed that 5-FU did not disturb APN/CD13 activity and that it did not influence the binding of Bestatin to APN/CD13. This signals the possibility that 5-FU and Bestatin could work along both lines. An assay of inhibition of proliferation and cytotoxicity in vitro revealed that a combination of 5-FU and Bestatin had greater anti-proliferative action on human cancer cells with a lower level of cytotoxicity in normal cells than 5-FU or Bestatin alone. Moreover, Hoechst 3342 staining of PLC/PRF/5 and ES-2 cells revealed that the combined drugs had greater activity in triggering apoptosis. Flow cytometry revealed that the drug combination resulted in 10% more apoptotic cells than 5-FU or Bestatin alone. In an analysis of cell cycles, exposure to 5-FU and Bestatin induced cells to remain in the S and G0/G1 phases. All of these results in vitro indicate that the combined drugs had greater efficacy than either drug alone. In an experiment involving H22 tumor-bearing mice, 5-FU in combination with a small quantity of Bestatin significantly inhibited tumor growth. 5-FU killed two mice and resulted in weight loss while the combination of the two resulted in no deaths and no loss of weight. 5-FU plus Bestatin shines because of its lower level of toxicity and superior therapeutic action. A study in vivo revealed that combining 5-FU and Bestatin ensured the survival rate for mice and also significantly inhibited tumor growth.

In conclusion, this study has described the broad-spectrum anticancer activity of 5-FU in combination with the APN/CD13 inhibitor Bestatin. A combination of 5-FU and Bestatin has superior anticancer action and a lower cytotoxicity in normal cells than 5-FU or Bestatin alone in vitro and in vivo. The preliminary mechanisms for cell apoptosis and cell cycles induced by this combination have been discussed as well. The current results provide important information for clinical cancer therapy. Combining Bestatin with 5-FU therapy may improve the treatment of cancer.

Acknowledgements

This work was supported by the National High-tech R&D Program of China (Program 863) (Grant No.2014AA020523), Major National Scientific and Technological Projects of the Ministry of Science and Technology of China (Grant no. 2011ZX09401-015), the National Natural Science Foundation of China (Grant nos. 21302111, 81373282 and 21172134), and the China Postdoctoral Science Foundation (Grant no. 2014T70654).

References


