

Original Article

Synergistic Action of fMLP-boanmycin Combination on the Growth of Mouse Colon Carcinoma and Its Action Mechanisms

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ABSTRACT

Objective: The inhibitory action of fMLP-boanmycin (BAM) combination on the growth of mouse colon carcinoma and its action mechanisms were observed in order to provide experimental proof for probing novel regimen of chemotactic modulation in combination with chemotherapy in the treatment of cancer.

Methods: Cytotoxicity of BAM-fMLP combination to tumor cells was determined by MTT assay *in vitro*. Antitumor activity of BAM-fMLP combination was assessed in mice subcutaneously transplanted colon carcinoma 26. The amount of superoxide anion ($O_2^{\cdot-}$) released from fMLP stimulated macrophages was determined by NBT assay. The amount of nitric oxide (NO) was indirectly determined by Griess method.

Results: BAM-fMLP combination had no synergistic effect on tumor cells (CDI>0.85), but BAM at the doses of 10 μ g/ml, 30 μ g/ml and 100 μ g/ml in combination with fMLP at the concentration 20 μ g/ml exhibited synergistic effect on tumor cells in the presence of macrophages (CDI<0.75). fMLP inhibited the growth of colon carcinoma 26 by 50.0% when it at dose of 1 mg/mouse was administered peritumorally. BAM (1 mg/kg, intraperitoneally, three times) alone and BAM - fMLP combination inhibited the growth of colon carcinoma 26 by 38.6% and 78.4%, respectively (CDI=0.71) on day 12. The amount of $O_2^{\cdot-}$ released from fMLP 4.6 $\times 10^{-7}$ mol/L (0.2 μ g/ml) stimulated macrophages which were treated by BAM *in vitro* increased significantly ($P<0.01$). fMLP 2.3 $\times 10^{-6}$ mol/L (1 μ g/ml) could not stimulate macrophages to release NO, but may stimulate macrophages treated with BAM 10 μ g/ml and 100 μ g/ml to release NO significantly ($P<0.01$).

Conclusion: The inhibitory action of fMLP-boanmycin combination on the growth of mouse colon carcinoma have synergism, which may associate with the increase of $O_2^{\cdot-}$ and NO released by macrophages. Chemotactic modulation in combination with chemotherapy may be a novel regimen in the treatment of cancer.

Key words: Chemotactic peptide; fMLP; Boanmycin; Colon carcinoma

Chemotactic peptide can attract and activate leukocytes including macrophages, which can interfere the process of tumor growth, invasion and metastasis^[1-3]. Bacterial chemotactic peptide fMLP (CHO-Met-Ile-Phe, fMLP) can induce the adhesion of leukocytes to endothelial cells, and promote them to migrate through endothelial cell space residing outside blood vessels^[4]. It was reported that fMLP induced immune inflammation of mucus membrane

when it was injected into mouse colon^[5]. Zhang L et al^[6] discovered that when fMLP was administered peritumorally in mice 48 h after tumor inoculation once every 2 days for 21 days, tumor growth delayed significantly. Ottonello L et al^[7-8] reported that monoclonal antibody (Lym-1) identified antigen HLA-DR on B type lymphoma cells could inhibit the proliferation of tumor cells by activating peripheral blood neutrophils only when fMLP was applied at the same time.

Boanmycin (BAM), a single A6 component of bleomycin, was isolated from *streptomyces verticillus*

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metabolites of China. It can inhibit the growth of subcutaneously transplanted human hepatoma Bel-7402^[9], human colon carcinoma HT-29 and cecum carcinoma Hce-8693^[10] in nude mice. It can also inhibit liver metastasis of subcutaneous, intracecal wall, intra-hepatic and intra-splenic transplanted colon carcinoma 26^[11]. It was reported that bleomycin at lower dose may restore the tumoricidal activity of macrophages from rats bearing KDH-8 hepatoma^[12].

This paper was set to investigate the synergistic action of fMLP-BAM combination on the growth of mouse colon cancer and its action mechanisms in order to provide experimental proof for probing novel regimen of chemotactic modulation in combination with chemotherapy in the treatment of cancer.

MATERIALS AND METHODS

Reagents

fMLP was purchased from Sigma Company. It was dissolved with tiny amount of DMSO and then diluted with PBS 0.05 mol/L (pH 8.5) so that DMSO content was less than 5%. BAM, was dissolved with 0.9% NaCl before experiment.

Animals

The animals used in the experiment were Balb/C mice (Female, 18-22 g, Grade II, certificate No. SCXK (Army) 2002-001, from Experimental Animal Institute, Chinese Military Medical Academy, Beijing, China)

Tumor cells

Mouse colon carcinoma 26 cell suspension of 0.2 ml (10^7 cells) stored in liquid nitrogen was placed in room temperature for some time, then centrifuged at $400\times g$ for 5 min. The pellete was diluted with RPMI-1640 medium supplemented with 10% inactivated fetal calf serum, and then the dilution was transferred into culture flask and cultivated in 5% CO₂ incubator at 37°C for 3 days. Cells in the phase of exponential growth were collected and resuspended at 1×10^5 cells/ml for next use.

Macrophages

Peritoneal macrophages were collected from peritoneal lavage of Balb/c mice injected intra-peritoneally with 2 ml RPMI-1640 medium. The macrophage suspension was centrifuged at $400\times g$ for 5 min, and then the macrophage pellet diluted with

about 10 ml RPMI-1640 medium was transferred into culture dishes to cultivate for 2 h in 5% CO₂ incubator at 37°C. After the supernatant was dislodged, the cells were collected by scraping with scraper and cell suspension at $5\sim 6\times 10^5$ cells/ml in RPMI-1640 medium was prepared for next use.

Evaluation of Cytotoxicity of BAM in Combination with fMLP to Tumor Cells by MTT Assay

Mouse colon carcinoma 26 cell suspension (10^4 cells/100 μ l) was seeded into each well of 96-well plate. Each well was further supplied with an equal volume of RPMI-1640 culture media. Vehicle control group, BAM group, fMLP group and BAM+ fMLP group were designed in the experiment. The plates were incubated in 5% CO₂ incubator at 37°C for 4 h after drugs of various concentrations were added into each well of drug groups. Stock MTT solution (2 g/L) of 50 μ l was added into each well and the plates were continued to incubate for 4 h in the same incubator. Supernatant from each well was drawn out carefully and 150 μ l DMSO was added. The plate was shaken strongly for 15 min. Absorbance was measured at 560 nm. To observe the cytotoxicity of the drugs to tumor cells in the presence of macrophages, 100 μ l tumor cell suspension (1×10^4 cells) and 100 μ l macrophage cell suspension (5×10^4 cells) were mixed, and then seeded into 96 well plate and cultivated for 4 h in 5% CO₂ incubator at 37°C. The inhibitory rate was calculated as follows:

$$\text{Inhibitory rate} = [(A_0 - A_1) / A_0] \times 100\%$$

A_0 represents total absorbance of tumor cell control,

A_1 represents the absorbance of drug treatment group.

Animal Experiment

Mouse colon carcinoma 26 cell suspension (10^6 cells) were injected subcutaneously into each mouse's right lateral chest wall near the axilla. The cells were passed every 13 days and the second passage was used for the experiment. At the time of animal experiment, mouse subcutaneous tumor was removed, weighed, cut up with eye scissors and transferred into homogenizer. After homogenization, the cell suspension was sedimented for 5 min and upper cell suspension was collected for next use.

The above cell suspension (0.2 ml) was injected subcutaneously into each mouse's right lateral chest wall near the axilla. Twenty four animals were averagely divided into vehicle control group, BAM

group, BAM + fMLP group and fMLP group. BAM was administered intraperitoneally and fMLP peritumorally once every 3 days for three times beginning at day 1. Tumor diameters were measured with a caliper. Tumor size was calculated by the formula " $a \times b^2/2$ ", where "a" represents long diameter and "b" short diameter. Inhibitory rate was calculated as follows: inhibitory rate (%) = $[(C-T)/C] \times 100\%$, where "C" represents tumor size of control group and "T" tumor size of treatment group.

Superoxide Anion Free Radical ($O_2^{\cdot -}$) Determination^[13]

The macrophages were pretreated by BAM at concentrations of 1 $\mu\text{g/ml}$, 10 $\mu\text{g/ml}$ and 100 $\mu\text{g/ml}$ for 30 min, followed by incubation for 30 min after addition of NBT (1 mg/ml) 500 $\mu\text{l/well}$ and fMLP 4.6×10^{-7} mol/L (0.2 $\mu\text{g/ml}$). The macrophages were stained with Giemsa for 10 min, washed with distilled water and air-dried. NBT positive macrophages contained over 3 blue-black particles were counted under microscope. Positive percentage was calculated.

Qualification of Nitric Oxide (NO)^[14]

The macrophage cell suspension at 1×10^6 cells/0.2 ml and BAM at various concentrations were added into each well of 96-well plate. The plate was incubated in 5% CO_2 incubator at 37°C for 30 min. After fMLP at final concentration of 2.3×10^{-6} mol/L (1 $\mu\text{g/ml}$) was added into the wells, the plate was continuously incubated for another 24 h. One hundred μl supernatants were mixed with an equal volume of Griess's reagent (1% sulfanilamine/0.1% naohthylethylenediamine dihydrochloride, Sigma

Chemical Co.) and incubated at room temperature for 15 min. The absorbance at 560 nm was measured in an ELISA reader. The NO concentration was determined using sodium nitrite as a standard and fresh medium as a blank.

Data Analysis

The synergism of drug combination was evaluated as follows^[15]: CDI (combination drug index) = $AB/A \times B$, where AB was the ratio of the data of combination groups to that of control group and A or B was the ratio of the data of drug group to that of control group. $\text{CDI} < 0.85$ represents synergism and $\text{CDI} < 0.75$ indicates significant synergism in the experiment.

Data were expressed as mean \pm standard deviation ($\bar{x} \pm s$). The significance of the results was determined by "F" test using SAS 6.12 & 8.0 software.

RESULTS

Cytotoxicity of fMLP-BAM Combination to Tumor Cells

fMLP at the concentration of 20 $\mu\text{g/ml}$ showed no cytotoxicity to mouse colon carcinoma 26 cells. CDI of cytotoxicity of fMLP at the concentration of 20 $\mu\text{g/ml}$ in combination with BAM at the concentrations of 10 $\mu\text{g/ml}$ and 100 $\mu\text{g/ml}$ to mouse colon carcinoma 26 cells was more than 0.85 (Table 1). These experiments indicated that fMLP-BAM combination had no synergism in cytotoxicity to mouse colon carcinoma 26 cells.

Table 1. Cytotoxicity of BAM in combination with fMLP to colon carcinoma 26 cells

BAM ($\mu\text{g/ml}$)	fMLP ($\mu\text{g/ml}$)	A_{560} ($\bar{x} \pm s$)	Inhibition (%)	CDI
0	0	0.892 \pm 0.101		
-	20	0.986 \pm 0.054	-10.5	
10	-	0.714 \pm 0.067	20.0	
100	-	0.647 \pm 0.043	27.5	
10	20	0.782 \pm 0.064	12.3	1.11
100	20	0.685 \pm 0.082	23.2	1.07

Note: The cells exposed to drugs for 4 h, determined by MTT assay. n=3

Cytotoxicity of fMLP-BAM Combination to Tumor Cells in the Presence of Macrophages

CDI of cytotoxicity of fMLP at the concentration of 20 $\mu\text{g/ml}$ in combination with BAM at the concentrations of 10 $\mu\text{g/ml}$, 30 $\mu\text{g/ml}$ and 100 $\mu\text{g/ml}$,

in the presence of macrophages, was all lower than 0.75 (Table 2). These experiments indicated that fMLP-BAM combination had synergism in cytotoxicity to mouse colon carcinoma 26 cells in the presence of macrophages.

Table 2. Cytotoxicity of BAM in combination with fMLP on colon carcinoma26 cells in the presence of macrophages

BAM ($\mu\text{g/ml}$)	fMLP ($\mu\text{g/ml}$)	A_{560} ($\bar{x} \pm s$)	Inhibition (%)	CDI
-	-	1.084 \pm 0.073	3.8	
-	20	0.806 \pm 0.067	28.5	
10	-	0.950 \pm 0.029	15.7	
30	-	0.875 \pm 0.085	22.4	
100	-	0.800 \pm 0.037	29.0	
10	20	0.399 \pm 0.140	64.6	0.52
30	20	0.313 \pm 0.080	72.2	0.44
100	20	0.160 \pm 0.030	85.8	0.25

Note: The cells exposed to drugs for 4 h, determined by MTT assay. n=3

Tumor Growth Inhibition of fMLP and BAM Applied 24 h after Tumor Inoculation

Mouse colon carcinoma 26 cells were injected s.c into each mouse at right lateral chest wall near the axilla 24 h before first administration. fMLP (peritumorally) and BAM (intraperitoneally) were all

administered for three times. On day 12, fMLP 1 mg/kg inhibited tumor growth by 50.0%. BAM 1mg/kg alone and BAM-fMLP combination suppressed the tumor growth by 38.6% and 78.4% ($P<0.05$, CDI=0.71), respectively. This result showed fMLP-BAM combination had synergism (Table 3).

Table 3. The effect of BAM in combination with fMLP on the growth of coloncarcinoma in mice

BAM ($\mu\text{g/ml}$)	fMLP (mg/mouse)	Weight (g)	Tumor (cm^3) ($\bar{x} \pm s$)	Inhibition (%)
0	0	+3.2	0.88 \pm 0.31	
1	0	+1.1	0.54 \pm 0.22	38.6
0	1	+3.5	0.44 \pm 0.24 [#]	50.0
1	1	+2.2	0.19 \pm 0.10 [*]	78.4

* $P<0.05$, as compared with BAM group. [#] $P<0.05$, as compared with control group. n=6

$\text{O}_2 \cdot^-$ Production of Macrophages

The amount of $\text{O}_2 \cdot^-$ released from fMLP stimulated macrophages was determined by NBT assay. The result showed that the amount of $\text{O}_2 \cdot^-$ released from fMLP (4.6×10^{-7} mol/L, 0.2 $\mu\text{g/ml}$) stimulated macrophages which were treated by BAM in vitro increased significantly. Positive rate(%) ascended from 24.4 \pm 6.6 in fMLP group to 62.0 \pm 9.4, 57.2 \pm 7.4 and 48.8 \pm 10 in those treated with BAM 1 $\mu\text{g/ml}$, 10 $\mu\text{g/ml}$ and 100 $\mu\text{g/ml}$, respectively ($P<0.01$) (Table 4).

NO Production of Macrophages

Nitric oxide (NO) becomes nitrite ($\text{NO}_2 \cdot^-$) soon in vitro. The amount of $\text{NO}_2 \cdot^-$ determined by Griess method can represent that of NO indirectly. When murine peritoneal macrophages were incubated with drugs for 24h, fMLP 2.3×10^{-6} mol/L(1 $\mu\text{g/ml}$) could not stimulate macrophages to release NO, but did

stimulate macrophages treated with BAM 10 $\mu\text{g/ml}$ and 100 $\mu\text{g/ml}$ to release NO significantly. The amount of NO was increased from 18.8 \pm 2.1 $\mu\text{mol/L}$ per 10^6 cells in fMLP group to 30.8 \pm 3.9 $\mu\text{mol/L}$ per 10^6 cells and 46.6 \pm 3.4 $\mu\text{mol/L}$ per 10^6 cells in groups treated with BAM 10 $\mu\text{g/ml}$ and 100 $\mu\text{g/ml}$, respectively ($P<0.01$) (Figure 1).

Table 4. The effect of fMLP on the release of $\text{O}_2 \cdot^-$ from macrophages treated by BAM, in vitro

BAM ($\mu\text{g/ml}$)	Positive rate (%) ($\bar{x} \pm s$)
0	23.7 \pm 4.5
1	62.7 \pm 8.1 ^{**}
10	54.4 \pm 8.4 ^{**}
100	49.5 \pm 7.7 ^{**}

** $P<0.01$, compared with control. fMLP: 4.6×10^{-7} mol/L. n=3.

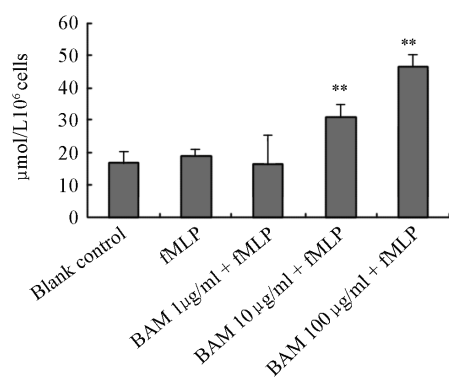


Figure 1. The effect of fMLP on NO release of macrophages treated by BAM. ** $P < 0.01$, as compared with fMLP group. $n = 3$, $\bar{x} \pm s$. BAM: $\mu\text{g/ml}$. fMLP: 2.3×10^{-6} mol/L ($1 \mu\text{g/ml}$).

DISCUSSION

Tumor treatment by chemotactic modulation is to attract immune cells of peripheral blood into tumor tissue and to activate the cells to inhibit tumor growth. Chemotactic peptide fMLP has the function of attracting and activating peripheral leukocyte including macrophages. It may induce immune inflammation reaction of intestinal mucous membrane when it is injected into colon^[5]. fMLP may stimulate human colon carcinoma HT29 - MTX cell line to release IL-8 which may attract peripheral leukocyte further^[16]. So, mouse colon carcinoma 26 transplanted model was selected in this experiment to observe the effect of fMLP or fMLP-BAM combination on tumor growth. Our results *in vivo* showed that fMLP inhibited tumor growth by 50%, similar to the results reported by Zhang L et al^[6], and fMLP-BAM combination had synergism in inhibiting the growth of tumor. This indicated that chemotactic modulation in combination with chemotherapy may be a new regimen in tumor treatment.

Our experiment *in vitro* showed that the cytotoxicity of BAM alone to mouse colon carcinoma cells increased with concentration and the cytotoxicity of fMLP-BAM combination did not show further increase, but in the presence of macrophages, cytotoxicity of fMLP-BAM combination had synergism, which indicated that the antitumor action of fMLP-BAM combination need the participation of macrophages.

The mechanism of the synergism of BAM-fMLP combination was studied. The experiment was set on the basis that fMLP may stimulate macrophages to release free radicals. The results showed that fMLP at

4.6×10^{-7} mol/L *in vitro* stimulated BAM-pretreated macrophages to release $\text{O}_2 \cdot^-$ significantly and fMLP at 2.3×10^{-6} mol/L could not stimulate macrophages to release nitric oxide (NO), but could stimulate macrophages pretreated with BAM of $10 \mu\text{g/ml}$ and $100 \mu\text{g/ml}$ to release NO significantly *in vitro*, similar to the result reported by Yuan L et al^[12] that lipopolysaccharide, one of bacterial immunomodulators, stimulated macrophages from rats bearing KDH-8 hepatoma treated by bleomycin *in vivo* to release NO. Above results indicated that one of the mechanisms of antitumor action of fMLP-BAM combination may be related to the production of $\text{O}_2 \cdot^-$ and NO.

It is well known^[17] that NO is formed enzymatically from the terminal guanidine -nitrogen of L-arginine by the Ca^{2+} -independent, inducible NO synthase (iNOS). There are regulatory sites in iNOS gene promoter region for the transcription factor, nuclear factor- κB (NF- κB). NF- κB and I κB , an inhibitory protein, coexist in the cytoplasm in their inactive complex form. Activation of signaling cascade in response to external stimuli such as fMLP induces phosphorylation of I κB and its dissociation from the NF- κB complex; proteolytic degradation of I κB by I κB protease; and translocation of the activated NF- κB and its binding to the relevant gene promoter. It is reported that the fMLP-induced signal transduction for macrophage NO production is involved in NF- κB ^[17] and BLM induced the transcriptional activation of NF- κB signaling in bronchial epithelial cells^[18]. These results suggested that fMLP-BAM combination may synergistically activate NF- κB signaling pathway to cause NO release from fMLP-stimulated BAM-pretreated macrophages.

In conclusion, Besides cytotoxic action of BAM on tumor cells, chemotactic peptide fMLP may stimulate leukocyte including macrophages pretreated with BAM to produce active oxygen ($\text{O}_2 \cdot^-$ and NO) to suppress the growth of tumor further. Chemotactic modulation in combination with chemotherapy may be a novel regimen in tumor treatment.

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