# Glucosaminylmuramyl Dipeptide Potentiates the Effects of Tumor Necrosis Factor-α and Cisplatin on Transformed Cells *in Vitro*

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We studied the effect of combined treatment with cisplatin, glucosaminylmuramyl dipeptide, and TNF-α on viability of MCF-7, U-937, B16, and L-929 tumor cells, Ehrlich ascites carcinoma cells, and normal cells (human peripheral blood lymphocytes, peritoneal macrophages, and mouse bone marrow cells). Glucosaminylmuramyl dipeptide was nontoxic for normal and tumor cells, but promoted death of tumor cells after administration in combination with cisplatin and/or TNF-α. At the same time, glucosaminylmuramyl dipeptide did not modulate the cytotoxic effect of individual or combined treatment with cisplatin and TNF-α on normal cells. Administration of glucosaminylmuramyl dipeptide to cultured MCF-7 cells 20 h before the study increased the potentiating effect of muramyl peptide.

**Key Words:** *cisplatin; glucosaminylmuramyl dipeptide; tumor necrosis factor; tumor cells; cytotoxicity*

New data on human genome, immune mechanisms for antitumor protection, and programmed cell death (apoptosis) substantiate complex approach to anticancer therapy. Nevertheless, chemotherapeutics are still the main antitumor drugs. Antimetabolites, alkylating agents, and antibiotics act via different mechanisms, but in all cases they cause death of actively proliferating cells, not only tumor cells, but also normal cells (primarily in the bone marrow). These specific features limit the use of cytostatics. The search for new agents that selectively affect tumor cells is an urgent problem. An alternative approach is the use of cytostatics in combination with drugs potentiating their effect on tumor cells and decreasing the influence on normal cells. Drugs with immunoregulatory activity (*e.g.*, muramyl peptides and cytokines) hold much promise in this respect. The use of combinations of drugs characterized by synergistic effects allows reducing the doses of constituents, which decreases their toxicity against normal cells. The necessity to adjust the dose of constituents makes it difficult to synthesize combination drugs. Hence, the initial stage of development of combination drugs involves *in vitro* studies on tumor cells of different etiology. There are published data on *in vitro* and *in vivo* antitumor effect of combinations including cytostatics (*e.g.*, cisplatin and doxorubicin), cytokines, and muramyl peptides [3-5].

Here we studied the effect of glucosaminylmuramyl dipeptide (GMDP) on cytotoxic activity of TNF-α in combination with cisplatin (CP) of without CP on some lines of tumor cells and on normal cells.

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## MATERIALS AND METHODS

The following cell lines were used: U-937 (human histiocytic lymphoma), MCF-7 (human breast adenocarcinoma), L-929 (mouse fibrosarcoma), mouse melanoma B16 cells, and mouse Ehrlich cells (EAT). MCF-7 and B16 cells were cultured in DMEM (Paneko) containing 10% fetal bovine serum (FBS, Gibco) and 10 μg/ml gentamicin (Paneko). L-929, U-937, and EAT cells were cultured in RPMI-1640 medium (Paneko) containing 10% FBS and 10 μg/ml gentamicin. The cells were cultured in a 5% $CO_2$ atmosphere at 37°C using Costar flasks.

CP was manufactured by Sigma. GMDP was synthesized by E. A. Makarov at the Laboratory of Peptide Chemistry (M. M. Shemyakin and Yu. A. Ovchinnikov Institute of Bioorganic Chemistry). Recombinant TNF-α was kindly provided by L. N. Shingarova.

For evaluation of cytostatic activity of preparations, the tumor cells ($10^5$ cells/ml) were placed in 96-well plates (Costar) with complete culture medium. The test preparations were administered alone or in combination after 24-h culturing of cells.

Venous blood from healthy donors was centrifuged in a Ficoll-Paque density gradient (Amersham Biosciences) to remove erythrocytes. Interphase leukocytes were suspended in RPMI-1640 medium with 10% FBS ($2\times10^5$ cells/ml) and placed in 96-well plates. The test preparations were added to the culture after 24-h incubation.

Peritoneal macrophages were isolated from BALB/c mice [1] and placed in 96-well plates ($3\times10^5$ cells/ml). The test preparations were added after 24-h culturing. Bone marrow cells were isolated from BALB/c mice [1], placed in 96-well plates ($5\times10^6$ cells/ml), and incubated for 24 h before addition of preparations. Peripheral blood lymphocytes, macrophages, and bone marrow cells were incubated with test preparations in a 5% $CO_2$ atmosphere at 37°C for 48 h.

The experiments were performed at constant concentrations of TNF-α (500 U/ml) and GMDP (1 μg/ml). The concentration of CP varied from 1 to 10 μM. Cytotoxicity of preparations to cell lines was evaluated at various time intervals, which depended on cell sensitivity to CP and TNF-α. GMDP (0.01-1.00 μg/ml) and TNF-α (10 and 500 U/ml for L-929 and MCF-7 cells, respectively) were added to cell cultures at various time intervals to evaluate the possibility of increasing the potentiating effect of GMDP. Cell viability was estimated in tests with

**TABLE 1.** Effect of Individual and Combined Treatment with GMDP, CP, and TNF-α on Tumor Cell Viability, %

| Incubation conditions | Cell line, time of incubation | | | | |
|---|---|---|---|---|---|
| | L-929, 48 h | B16, 48 h | EAT, 72 h | MCF-7, 120 h | U-937, 48 h |
| GMDP | 98±1 | 98±1 | 98±2 | 98±1 | 99±1 |
| TNF-α | 65±2 | 87±2 | 93±1 | 55±2 | 72±2 |
| TNF-α and GMDP | 35±2* | 84±3 | 89±2* | 40±3* | 69±2 |
| CP, 1 μM | 90±1 | 81±2 | 92±2 | 79±1 | 96±1 |
| +GMDP | 86±1* | 78±2* | 86±2* | 78±1 | 89±2* |
| +TNF-α | 60±2 | 75±2 | 89±1 | 49±2 | 76±2 |
| +GMDP and TNF-α | 30±3* | 73±3* | 79±2* | 37±3* | 70±2* |
| CP, 3 μM | 86±1 | 77±3 | 78±2 | 72±1 | 81±1 |
| +GMDP | 78±2* | 71±3* | 69±2* | 72±2 | 73±2* |
| +TNF-α | 50±3 | 72±2 | 73±2 | 39±3 | 57±2 |
| +GMDP and TNF-α | 25±3* | 65±3* | 59±3* | 31±3* | 51±2* |
| CP, 6 μM | 80±2 | 63±3 | 52±3 | 68±2 | 65±2 |
| +GMDP | 70±2* | 62±3 | 46±3* | 68±3 | 58±2* |
| +TNF-α | 30±3 | 63±3 | 42±3 | 34±2.5 | 30±2 |
| +GMDP and TNF-α | 20±3* | 53±3* | 33±3* | 28±3* | 22±3* |
| CP, 10 μM | 69±2 | 42±3 | 30±3 | 66±2 | 43±3 |
| +GMDP | 55±2* | 33±3* | 22±3* | 66±2 | 40±2 |
| +TNF-α | 25±3 | 28±3 | 21±3 | 30±3 | 23±3 |
| +GMDP and TNF-α | 10±4* | 26±3 | 18±3 | 26±2 | 20±3 |

**Note.** Results of the MTT test. TNF-α, 500 U/ml; GMDP, 1 μg/ml (100 μg/ml for B16). *$p<0.05$ compared to the sample without GMDP.

trypan blue and 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT). The MTT test was used to measure dehydrogenase activity, which decreased during cell death. Viability of untreated cells was taken as 100%.

The results were analyzed by Student's *t* test.

## RESULTS

The results of MTT and trypan blue tests coincided. Although MTT test based on measurement of mitochondrial reductase activity often showed greater viability of cells because of partial preservation of enzyme activity in dead cells, in contrast to the trypan blue test based on evaluation of cell membrane integrity.

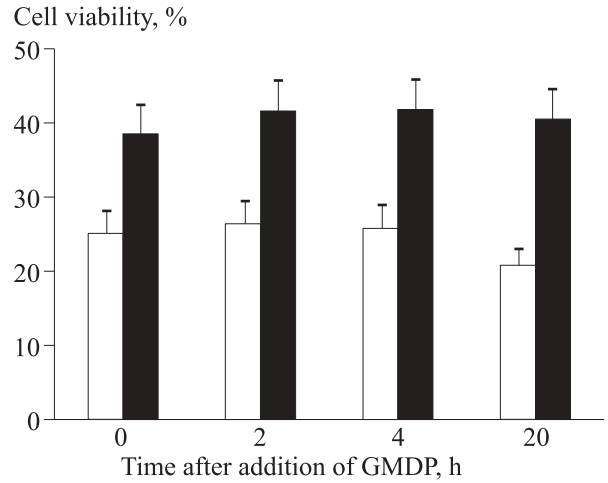


**Fig. 1.** Cytotoxicity of TNF-α (500 U/ml) for MCF-7 cells in dependence on the time of preincubation with GMDP. Light bars, TNF-α; dark bars, TNF-α and GMDP. Control, 100%.

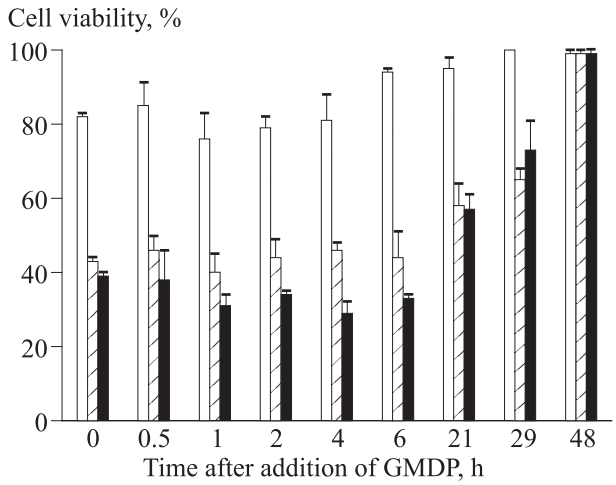


**Fig. 2.** Cytotoxicity of TNF-α (10 U/ml) for L-929 cells in dependence on the time of preincubation with GMDP. Light bars, 0.01 μg/ml GMDP; shaded bars, 0.1 μg/ml GMDP; dark bars, 1 μg/ml GMDP. Viability of TNF-α-treated cells is taken as 100%.

GMDP potentiated the cytotoxic effects of TNF-α and CP on L-929, EAT, and U-937 cells, but had no intrinsic effect on viability of these cells (Table 1). GMDP did not decrease viability of MCF-7 cells treated with CP. However, GMDP potentiated the cytotoxic effect of TNF-α on these cells. Besides this, GMDP potentiated the cytotoxic effect of TNF-α alone or in combination with CP on all lines of cells. The potentiating effect of GMDP depended on cell line. Administration of CP in low concentration after addition of GMDP as the third component decreased cell viability, which was similar to that observed during treatment with this cytostatic in high concentration in the absence of GMDP. Administration of CP (3-6 μM) in combination with TNF-α and GMDP produced the most potent cytotoxic effect on cell lines. GMDP in a concentration of 1 μg/ml insignificantly potentiated the cytotoxic effect of combined treatment with these preparations on B16 cells (similarly to experiments with L-929, EAT, U-937, and MCF-7 cells). Increasing the concentration of GMDP to 100 μg/ml potentiated the effect of combined treatment.

Preincubation of MCF-7 cells with GMDP slightly increased the potentiating effect of muramyl peptide on TNF-α in a concentration of 500 U/ml (compared to simultaneous addition of preparations, Fig. 1). Simultaneous addition of TNF-α and GMDP was followed by a decrease in cell viability from 55 (for TNF-α) to 40% (for TNF-α and GMDP). However, administration of muramyl peptide 20 h before addition of TNF-α decreased cell viability from 55 to 30%. Shortened interval between addition of GMDP and TNF-α (2-4 h) did not have any advantage over simultaneous addition of these preparations.

GMDP in concentrations of 0.1 and 1.0 μg/ml potentiated the cytotoxic effect of TNF-α (10 U/ml) on L-929 cells. These results were obtained in experiments with the simultaneous addition of GMDP and TNF-α, as well as under conditions of TNF-α administration within 6 h after GMDP treatment (Fig. 2). The interval decreased to 4 h after administration of GMDP in a concentration of 0.01 μg/ml. Further increase in the interval between administration of TNF-α and GMDP to the culture was followed by a decrease in cytotoxic activity of these agents. After increasing the interval between treatment with TNF-α and GMDP to 24 h, cytotoxic activity of the combination practically did not differ from that of TNF-α alone.

Incubation of L-929 cells with TNF-α less than 2 h before administration of GMDP induced the total cytotoxic effect, which did not differ from that observed after simultaneous addition of these pre-

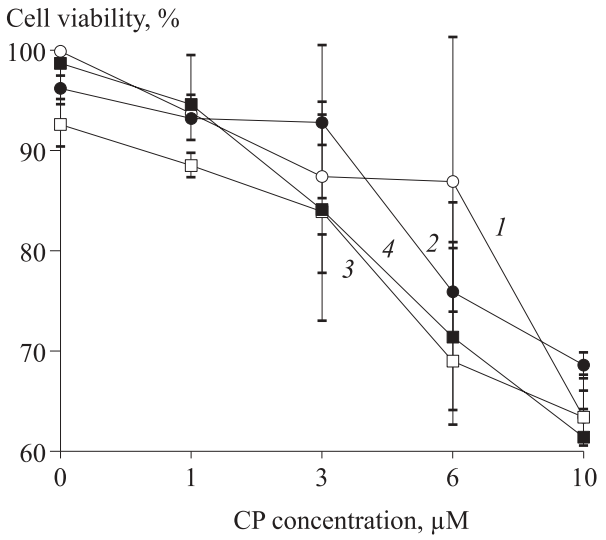


**Fig. 3.** Effect of 48-h incubation with GMDP (1 μg/ml), CP, and TNF-α (500 U/ml) on viability of normal mouse bone marrow cells in the MTT test. CP (*1*), CP+GMDP (*2*), CP+TNF-α (*3*), CP+TNF-α+ GMDP (*4*). Mean values of 3 experiments. Standard deviation does not exceed 10%. $p<0.05$.

parations (data not shown). The total cytotoxic effect of preparations decreased with increasing the interval between administration of TNF-α and GMDP. Cytotoxic activity of preparations in experiments with administration of GMDP 24 h after addition of TNF-α was similar to that of TNF-α alone. GMDP produced no potentiating effect under these conditions. Hence, total cytotoxic activity of GMDP and TNF-α for L-929 cells depends on the sequence and interval between administration of these preparations to the culture. Under these conditions the potentiating effect of GMDP on cytotoxic activity of TNF-α did not exceed that observed in experiments with the simultaneous addition of preparations.

Normal peripheral blood leukocytes and peritoneal macrophages were incubated with preparations. The tests with MTT and trypan blue showed that individual and combined administration of CP, TNF-α, and GMDP has little effect on cell viability (data not shown). The MTT test revealed that individual and combined administration of TNF-α and GMDP did not modulate viability of bone marrow cells (Fig. 3). CP in a concentration of 1 μM did not induce the cytotoxic effect on bone marrow cells. However, CP in concentrations of 3 and 6 μM slightly decreased cell viability (87 and 77%, respectively). CP in a concentration of 10 μM had a strong effect on cell viability, which decreased to 63%. Administration of CP in combination with TNF-α (or TNF-α and GMDP) did not modulate the cytotoxic effect on bone marrow cells.

Our results show that tumor cells of different etiology are more sensitive to combined treatment with GMDP, TNF-α, and CP than normal cells. It should be emphasized that GMDP decreases the effective concentration of cytotoxic agents. We conclude that administration of GMDP reduces the therapeutic doses of CP and TNF-α and potentiates the effect of these agents on tumor cells.

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