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Growth Inhibition and Differentiation of Murine Melanoma B16-BL6 Cells Caused by the Combination of Cisplatin and Caffeine

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We preliminarily investigated the combined effects of cisplatin and caffeine on murine melanoma B16-BL6 cells *in vitro*. When caffeine was added before or simultaneously with cisplatin, there was little growth inhibition. The addition of 2.0 m*M* caffeine after 1 h of exposure to cisplatin inhibited growth and induced cell differentiation. This treatment resulted in fewer cells, and the numbers of melanosomes and mitochondria and the amount of Golgi's complex and endoplasmic reticulum were increased. DNA histograms obtained by flow cytometry showed that cells treated with cisplatin alone accumulated in the G_2/M phase, with a partial G_2 block. The addition of 2.0 m*M* caffeine after 1 h of treatment with cisplatin reduced this block. Caffeine caused murine melanoma B16-BL6 cells treated with cisplatin to differentiate, and this inhibited growth.

Key words: Differentiation — Cytotoxicity — B16-BL6 melanoma cells — Caffeine — Cisplatin

Caffeine, which can inhibit DNA repair, increases the effect of DNA-damaging agents.[1-3] Caffeine was the most effective in potentiating cisplatin activity in cultured human osteosarcoma cells.[4] Cisplatin is often used for the chemotherapy of melanoma. Theophylline, a xanthine derivative can cause differentiation.[5,6] Here, we examined the effect of cisplatin and caffeine used in combination on murine melanoma B16-BL6 cells *in vitro*. Changes in DNA histograms obtained by flow cytometry were analyzed and morphological changes were studied by phase-contrast and electron microscopy. We preliminarily confirmed that, at a non-toxic concentration, caffeine inhibited cell growth and induced the differentiation of B16-BL6 melanoma cells in combination with cisplatin.

MATERIALS AND METHODS

Murine melanoma B16-BL6 cells were provided by Dr. T. Tsuruo (Cancer Chemotherapy Center, Japanese Foundation for Cancer Research, Tokyo). The cells (1×10^5/ml) were first cultured for 48 h in RPMI 1640 medium supplemented with 15% heat-inactivated fetal calf serum, 1% penicillin, and 1% streptomycin. The incubation was at 37°C in a humidified atmosphere of 7% CO_2. The cells were then treated with drugs in one of the following ways: 1) Treatment with caffeine (0.2 or 2.0 m*M*) for 48 h. 2) Treatment with cisplatin (0.2 or 2.0 μg/ml) for 1 h. 3) Treatment with caffeine (2.0 m*M*) for 48 h, and then with cisplatin (0.2 or 2.0 μg/ml) for 1 h. 4) Treatment with cisplatin (0.2 or 2.0 μg/ml) and caffeine (2.0 m*M*) simultaneously for 1 h. 5) Treatment with cisplatin (0.2 or 2.0 μg/ml) for 1 h, and then with caffeine (2.0 m*M*) for 96 h.

After treatment, the cells were rinsed twice with phosphate-buffered saline without Mg^{2+} and Ca^{2+}. A suspension of the treated tumor cells in 5 ml of RPMI 1640 medium was prepared and placed in a 10-ml centrifuge tube. Incubation of the cells was continued at 37°C in a CO_2 incubator. Viable cells were counted every 24 h by the trypan blue exclusion method until 96 h after the end of drug treatment. At these same times, changes in cell progression were analyzed with a flow cytometer (Showa Denko CS-20). For this purpose, cells were fixed in 70% alcohol for at least 30 min, and adjusted to the concentration of 5×10^5/ml of chromomycin A_3 solution (100 mg/500 ml of distilled water containing 1.5 g of $MgCl_2\cdot6H_2O$). The cells were passed through the flow cell at the rate of 500–1000 cells per second, and the fluorescence of the individual nuclei was measured with the use of 457.9 nm light from an argon ion laser. The data were then displayed on an X-Y plotter in the form of a histogram. The mitotic index (%) was obtained by calculation of the number of mitoses per 1000 cells treated with Papanicolaou's stain. The morphologic changes of the cells were examined by phase-contrast and electron microscopy. Tumor cells were fixed in 2% glutaraldehyde, postfixed in 2% osmium tetroxide, and processed in the usual way for the electron microscopic study. Fontana-Masson stain was also utilized.

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RESULTS

When B16-BL6 melanoma cells were treated with 0.2 or 2.0 m*M* caffeine for 48 h, growth was not inhibited. When such cells were also treated with 0.2 μg/ml cisplatin, cytocidal effects were slight. Ninety-six hours after the end of treatment with 2.0 μg/ml cisplatin for 1 h, 90% of the cells were viable. However, treatment with both cisplatin and caffeine at these concentrations, which were non-toxic, had some cytotoxic effect. That is, when cells were first exposed to 0.2 μg/ml cisplatin for 1 h and then to 2.0 m*M* caffeine for 96 h, 73% of the cells were viable; when the concentration of cisplatin was 2.0 μg/ml instead, 52% of the cells survived (Fig. 1). When caffeine was added before or together with the cisplatin, the cytocidal effects and growth inhibition were slight; they were not significantly increased (data not shown).

There was no change in the DNA histograms when the cells were treated with caffeine alone. Treatment with 0.2 or 2.0 μg/ml cisplatin caused a partial G_2 block. The mitotic index was less than 0.1% at either concentration of cisplatin. However, when 2.0 m*M* caffeine was added after cisplatin treatment, the extent of the block decreased (Fig. 2).

The phase-contrast microscopic study showed that when cisplatin treatment at either concentration was followed by the use of caffeine, the cells swelled and became spherical; the ratio of nucleus to cytoplasm decreased (Fig. 3). Furthermore, in the Fontana-Masson-stained sections, the cells treated with caffeine after the addition of cisplatin showed many melanin granules compared to the cells treated with caffeine or cisplatin alone. Melanin granules were located in dendrite-like processes and in the cell periphery (Fig. 4). In the ultrastructural study, very few melanosomes were in stages II–III in the control group (treatment with saline) or the cells treated with cisplatin alone. When the cells were treated with 2.0 m*M* caffeine by method 1, 3, or 4, stage II or III melanosomes were found scattered in the dendrites, and the amount of rough endoplasmic reticulum and Golgi's complex was slightly increased. However, with the com-

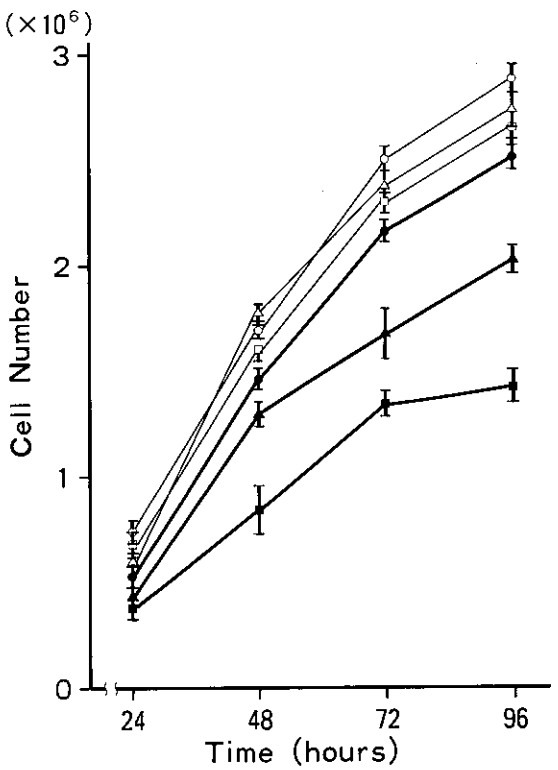

Fig. 1. Growth inhibition curve of murine melanoma B16-BL6 cells treated with cisplatin, caffeine, or both. Symbols: △, 0.2 m*M* caffeine; □, 2.0 m*M* caffeine; ○, 0.2 μg/ml cisplatin; ●, 2.0 μg/ml cisplatin; ▲, 1 h of exposure to 0.2 μg/ml cisplatin followed by treatment with 2.0 m*M* caffeine; ■, 1 h of exposure to 2.0 μg/ml cisplatin followed by treatment with 2.0 m*M* caffeine.

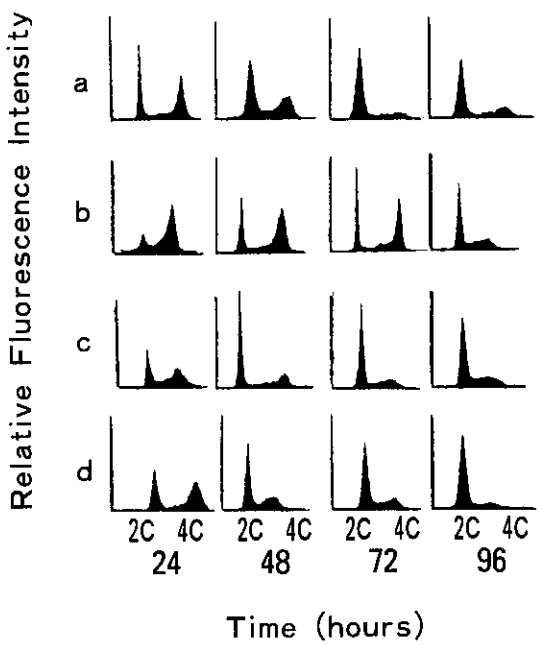

Fig. 2. Movement of B16-BL6 cells treated with cisplatin, caffeine, or both through the cell cycle. (a) 0.2 μg/ml cisplatin; (b) 2.0 μg/ml cisplatin; (c) 1 h of exposure to 0.2 μg/ml cisplatin followed by 2.0 m*M* caffeine; (d) 1 h of exposure to 2.0 μg/ml cisplatin followed by 2.0 m*M* caffeine. X-axis, relative amount of DNA per cell; Y-axis, number of cells. Hours are hours after the end of drug treatment. Caffeine reduced the partial G_2 block caused when cisplatin alone was used.

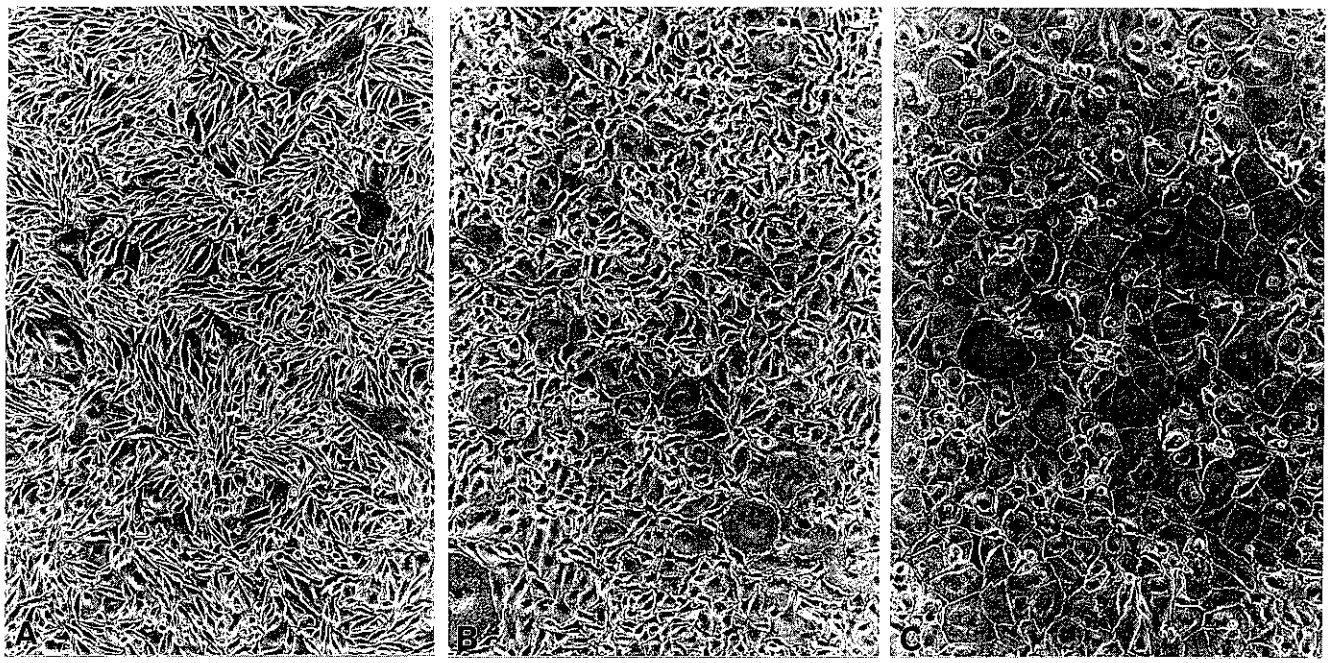

Fig. 3. Phase-contrast photomicrographs of B16-BL6 cells treated with (A) 2.0 μg/ml cisplatin for 1 h, (B) 2.0 mM caffeine for 96 h, or (C) 2.0 μg/ml cisplatin for 1 h and then 2.0 mM caffeine for 96 h. Magnification, $\times$100.

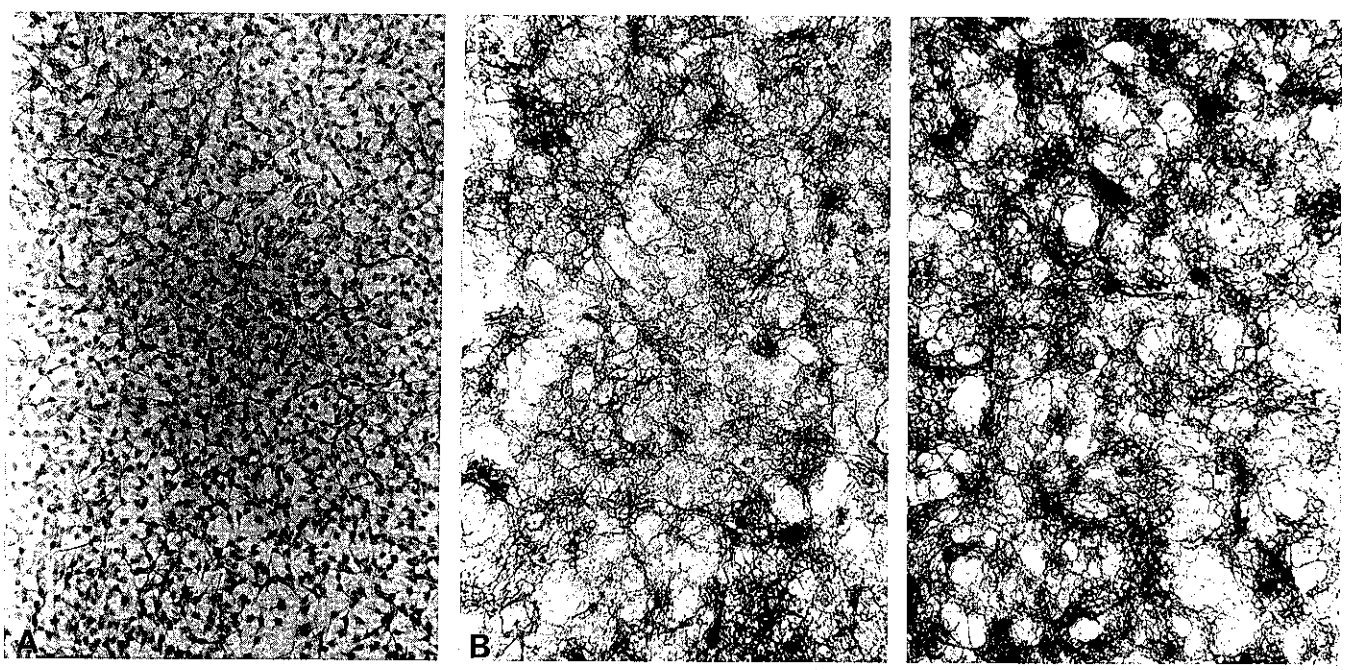

Fig. 4. Murine B16-BL6 melanoma cells stained according to the Fontana-Masson method. (A) When the cells were treated with 2.0 μg/ml cisplatin alone, melanin granules were scarcely observed. (B) When the cells were treated with 2.0 mM caffeine alone, there was a moderate increase of melanin granules. (C) The cells treated with 2.0 mM caffeine and 2.0 μg/ml cisplatin showed a marked increase of melanin granules compared to the cells treated with caffeine or cisplatin alone. Magnification, $\times$100.

Table I. Effect of Cisplatin and Caffeine on the Numbers of Melanosomes in B16-BL6 Cells

Treatment	Mean melanosomes/nucleus[a]
Untreated (control)	5.9±5.0
Cisplatin, 2.0 μg/ml	7.1±4.5
Caffeine, 2.0 m*M*	138.0±42.1[b]
Caffeine, 2.0 m*M* → Cisplatin, 2.0 μg/ml	141.2±32.8[b]
Cisplatin, 2.0 μg/ml + Caffeine, 2.0 m*M* (simultaneously)	29.5±8.6[b]
Cisplatin, 2.0 μg/ml → Caffeine, 2.0 m*M*	208.0±59.2[b]

a) Mean number of melanosomes per nucleus was calculated from melanosomes seen in 20 cells in electron micrographs.
b) $P<0.05$ vs. control and with cisplatin (Student's *t* test).

bined use of cisplatin and caffeine by method 5, many mature melanosomes in stages III or IV were seen in the long dendrites and in the periphery of the B16-BL6 cells (Table I). The amount of rough endoplasmic reticulum and Golgi's complex, and the number of mitochondria were clearly increased (Fig. 5).

DISCUSSION

In this study, 2.0 m*M* caffeine, a non-toxic concentration, increased the cytotoxicity of cisplatin to B16-BL6 cells. Continuous exposure to caffeine after cisplatin treatment enhanced the cytocidal effect and the inhibition of B16-BL6 cell growth compared to treatment with cisplatin alone. This result may relate to a DNA-repair-inhibiting effect of caffeine. Although caffeine inhibits post-replication repair in mammalian cells,[7] the precise

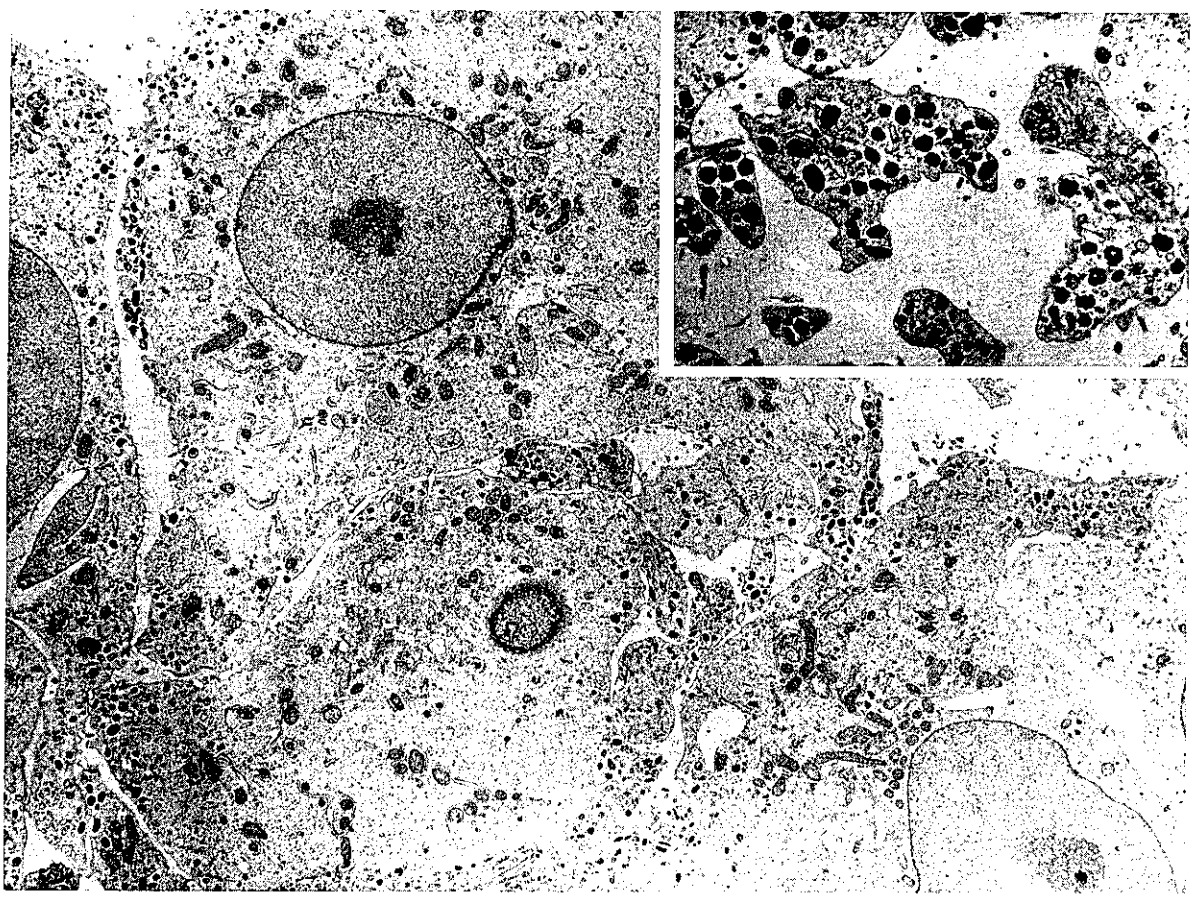

Fig. 5. Electron micrographs 96 h after the treatment of B16-BL6 cells treated with 2.0 μg/ml cisplatin for 1 h and 2.0 m*M* caffeine for 96 h. The cytoplasm increased, and the dendrites lengthened. Many mature stage III–IV melanosomes appeared, mostly in the altered dendrites. The amount of endoplasmic reticulum and Golgi's complex and the number of mitochondria increased. Magnification, ×5400. Inset: Higher magnification (×9000) showing the mature melanosomes in elongated dendrites.

mechanism is still unclear. Another xanthine analogue, pentoxifylline, also enhances the cytocidal effect of DNA-damaging agents.[8] It was reported that cultured cells repaired their DNA damaged by cisplatin, based on measurements of the extent of platinum binding to template DNA in relation to the size of low-molecular-weight nascent DNA.[9] The results of analysis of the cell cycle by flow cytometry suggested that caffeine promoted cell cycle progression, because it reduced the accumulation of cells in the G_2 phase caused by treatment with cisplatin alone. Lau *et al.* concluded that caffeine inhibited DNA repair after treatment with nitrogen mustard based on cell cycle analysis, morphological changes of the nucleus (nuclear fragmentation), and protein synthesis.[2] Although nuclear fragmentation was not prominent in the sheeted cells, when caffeine enhanced the cytotoxicity of cisplatin it might allow mitosis to occur before DNA repair is complete, leading to cell death and growth inhibition after mitosis. Namely, caffeine may accelerate the G_2 phase in some way, although tumor cells ordinarily prolong the G_2 phase to repair damaged DNA.[7]

Marked morphological changes were caused by treatment with 2.0 m*M* caffeine after a 1-h treatment with cisplatin (0.2 or 2.0 μg/ml). Treatment with 2.0 m*M* caffeine alone induced moderate differentiation of the B16-BL6 cells, but continuous exposure to caffeine after cisplatin treatment strongly induced differentiation. The ratio of nucleus to cytoplasm decreased, and the number of dendrites increased. The production of more mature melanosomes was increased by the use of both cisplatin and caffeine. Namely, organelles increased in extent or number. Therefore, not only was there an increase in the cytocidal effect but also differentiation of B16-BL6 cells was affected markedly when caffeine was used after cisplatin treatment. The differentiating effect of caffeine was time-dependent, because simultaneous addition of cisplatin and caffeine for 1 h slightly increased melanin synthesis compared to the treatment with caffeine for 96 h after the addition of cisplatin.

Many agents that cause differentiation have been identified,[10–12] but their mechanisms of action are unknown. Kreider *et al.* reported that theophylline causes the differentiation of B16 melanoma cells; this compound inhibits cyclic nucleotide phosphodiesterase, causing an increase of cyclic-AMP, which suppresses mitosis and melanin production.[6] Harris suggested that differentiation is induced by the suppression of the activity of poly-ADP-ribose polymerase.[13] Further investigation is required to confirm the mechanism of the differentiation of B16-BL6 cells. For the present, it is not clear why inhibition of DNA repair also leads to the differentiation of tumor cells.

This current study is very preliminary, and further work should be carried out. However, this finding that differentiation led to growth inhibition of B16-BL6 cells suggests that caffeine could be a useful differentiating agent for cancer treatment.

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