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Activity of melphalan in combination with the glutathione transferase inhibitor sulfasalazine

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Abstract Glutathione (GSH) transferases (GST), a family of detoxification enzyme proteins, are suggested to play an important role in tumor cell resistance to melphalan. The GST-activity inhibitor ethacrynic acid has been shown to increase the antitumor activity of melphaln in vitro as well as in vivo. In this study we determined the activity and toxicity of melphalan in combination with another GST-activity inhibitor, sulfasalazine, an agent used to treat ulcerative colitis. We entered 37 previously treated patients with advanced cancer of different histologies on sulfasalazine given at the individually calculated maximum tolerated dose (MTD) and melphalan given at doses beginning at 20 mg/m^2. The main toxicity arising from this combination was nausea and vomiting, whereas increased myelosuppression was not observed. A partial response was seen in 2/4 of the ovarian cancer patients only. Plasma sulfasalazine levels varied between 2.5 and 47.1 μg/ml. Although reductions in GSH/GST levels were observed in peripheral mononuclear cells of certain patients following sulfasalazine treatment, there was no correlation between the extent of reduction and the plasma sulfasalazine level. A larger patient population must be studied to determine the usefulness of this combination.

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Introduction

Bifunctional alkylating agents, including melphalan, are of considerable importance in cancer chemotherapy. However, the clinical effectiveness of these agents is often limited by the emergence of drug-resistant tumor cells [16]. Several different mechanisms have been proposed to account for tumor cell resistance to alkylating agents, including (a) reduced drug accumulation [23], (b) changes in subcellular distribution of the drug [6], and (c) reduced DNA cross-linking [24].

Recent studies have suggested that increased glutathione (GSH) transferase (GST)-mediated conjugation of bifunctional alkylating agents to GSH may also contribute to anticancer drug resistance [2, 17, 24, 32]. GSTs are a family of cytosolic proteins that catalyze the conjugation of electrophilic groups in xenobiotics with GSH [19]. Mammalian GST isoenzymes have been grouped into the three major classes α, μ, and π on the basis of their structural and functional characteristics [20]. Increased GST activity has been observed in tumor cells selected for resistance to various therapeutically important anticancer drugs, including bifunctional alkylating agents [5, 24, 28, 32]. In addition, resistance to alkylating agents has been shown to be associated with the overexpression of one or more individual GST isoenzymes. For example, elevated levels of α-class GST isoenzymes have been observed in cell lines resistant to nitrogen mustard [24, 32]. Similarly, overexpression of π-class GST protein has been observed in tumor cells resistant to doxorubicin [5] and to *cis*-diamminedichloroplatinum (II) (*cis*-DDP) [28]. The transfection of a rat α- or μ-class and human π-class GST genes conferred resistance to chlorambucil, melphalan, or *cis*-DDP in cultured cells [22]. However, transfection of a human

α-class GST gene into MCF-7 human breast cancer cells did not change their sensitivity to chlorambucil, melphalan, or cisplatin [18].

Further support for the involvement of GST in anticancer drug resistance derives from the findings that certain anticancer agents such as melphalan, hepsulfam, and chlorambucil are inactivated by a GST-catalyzed reaction [2, 8, 11] and that denitrosation of 1,3-bis-(2-chloroethyl)-1-nitrosourea occurs through mediation of the GSH/GST system [27]. The GST-activity inhibitor ethacrynic acid has been shown to increase the cytotoxicity in vitro of (a) chlorambucil in a rat mammary carcinoma and in HT-29 human colon carcinoma cells [29], (b) melphaln in a human melanoma cell line [15],, and (c) mitomycin C in a multidrug-resistant mouse leukemia cell line [34]. In addition, ethacrynic acid enhanced the activity of L-phenylalanine mustard in HT-29 human colon carcinomas implanted in *scid* mice [9]. In this study, we evaluated the toxicity of and response to melphalan in combination with sulfasalazine, another potent inhibitor of GST activity in vitro, in previously treated patients with advanced cancer of different histologies.

Materials and methods

Patient selection

Patients eligible for this study had a histological diagnosis of cancer and had failed standard chemotherapeutic options. This study was approved by the institutional review boards and all patients gave written informed consent. Eligibility criteria included the presence of measurable disease, a WBC of $> 4{,}000/mm^3$, a platelet count of $> 100{,}000/mm^3$, serum creatinine levels of > 2.0 mg/dl, bilirubin levels of < 2 times the normal value, and an Eastern Cooperative Oncology Group (ECOG) performance status of < 2. Patients were excluded if they had a history of allergy to sulfonamide or aspirin, bronchial asthma, of glucose 6-phosphate dehydrogenase deficiency or required radiation therapy.

Treatment plan

The maximum tolerated dose (MTD) of sulfasalazine was determined in each patient. The starting dose of sulfasalazine, 1 g p.o. q.i.d., was escalated by 0.5 g p.o. q.i.d. each day until the MTD, as indicated by nausea/vomiting, was reached. The dose of sulfasalazine was reduced by 0.5 g p.o. q.i.d for gastointestinal intolerance. This dose was then given for 4 days. Blood samples for various measurements were drawn prior to the start of sulfasalazine treatment and before melphalan administration. Melphalan ($20\ mg/m^2$) was given orally on day 3, and each cycle was repeated every 4 weeks. On subsequent cycles, the dose of sulfasalazine was kept the same, whereas the dose of melphalan was increased or decreased by $5\ mg/m^2$ to result in a nadir absolute neutrophil count of $1{,}000–1{,}500/mm^3$ (grade 2 toxicity).

Toxicity and response criteria

All side effects were graded according to ECOG toxicity criteria. Standard response criteria were as follows: complete response, the complete resolution of measurable disease; partial response, a decrease of more than 50% in tumor size; stable disease, a decrease of less than 50% in tumor size; and progressive disease, an increase of more than 25% in tumor size.

Estimation of plasma levels of sulfasalazine

Plasma sulfasalazine levels were determined according to the method of Sandberg and Hansson [25]. Briefly, 1 ml of plasma was acidified with 1 ml of 1 *N* HCl. The acidified plasma was mixed with 4 ml of isoamyl alcohol and vortexed vigorously for 30 s. The organic phase (3 ml) was separated by centrifugation at 3,000 rpm for 10 min and mixed with 4 ml of 0.5 *M* sodium hydroxide solution. After vigorous mixing and centrifugation, the aqueous phase was separated. The absorbance of the aqueous phase was measured at 455 nm using distilled water as a reagent blank. Plasma samples spiked with different amounts of sulfasalazine were also processed similarly to generate the standard curve.

Isolation of peripheral mononuclear cells

Blood samples were drawn into heparinized tubes. Mononuclear cells were isolated using Histopaque-1119 and Histopaque-1077 (Sigma Chemical Co., St. Louis, Mo.) according to the manufacturer's instructions. Plasma and mononuclear cells were stored at − 80°C until used.

GSH estimation and GST-activity determination

The GSH content in 14,000-*g* supernatant fractions of peripheral mononuclear blood cells was determined by the method of Ellman [12] as modified by Van Doorn et al. [31]. The protein content was determined by the method of Bradford [7]. The GST activity in 14,000-*g* supernatant fractions using 1-chloro-2,4-dinitrobenzene (CDNB) as an electrophilic substrate was determined according to the method of Habig et al. [14].

Effect of sulfasalazine on mononuclear-cell GST activity

Experiments were also undertaken to determine if sulfasalazine could inhibit GST activity in mononuclear cells in vitro in the presence of plasma. Blood was drawn from two normal human subjects for the isolation of peripheral mononuclear cells and plasma. The inhibitory effect of sulfasalazine on GST activity was assessed by an in situ GST-assay procedure. The in situ enzyme-activity determination was performed because sulfasalazine is a reversible inhibitor of GST activity [1] and, thus, may not show inhibition of this activity when measured in cell homogenates due to dilution of sulfasalazine during the process of cell lysis, homogenization, and GST assay. Briefly, the in situ GST-activity measurements were performed by incubating mononuclear cells, suspended in plasma, with 100 μ*M* CDNB, an electrophilic substrate of this enzyme. The reaction was terminated by the addition of an equal volume of ethanol containing 0.01 *N* HCl. The reaction mixture was centrifuged at 10,000 *g* and the supernatant fraction was subjected to high-performance liquid chromatography (HPLC) to isolate and quantitate the reaction product, *S*-dinitrophenyl GSH. The HPLC conditions were similar to those described previously [4]. To determine the effect of sulfasalazine on GST activity in situ, the peripheral mononuclear cells suspended in plasma were pretreated with 100 μ*M* sulfasalazine for 5 min prior to the addition of CDNB to the reaction mixture.

Table 1 Patients characteristics and response to the melphalan/sulfasalazine combination

Patients entered	37
Male/Female	17/20
Prior therapy:	
Chemotherapy	26
Radiotherapy	13
Immunotherapy	8
Histology:	
Lung	15
Colorectal	10
Ovarian	4
Breast	4
Other	4
Number of sulfasalazine/melphalan cycles:	
1	19
2	5
3	7
4 or more	6
Response:	
Partial response	2
Stable disease	2
Disease progression	33

Results

A total of 37 patients were entered onto the study (Table 1). The vast majority of the patients had received prior chemotherapy, radiation therapy, or immunotherapy. Six patients with non-small-cell lung cancer and one patient with colon cancer had not received prior chemotherapy. Two patients did not receive any melphalan, and two others were taken off the study for radiation therapy. These patients were considered as failures of therapy. Overall, 33 patients had progressive disease, and most of them had received 1–2 cycles of melphalan. A total of 13 patients received 3 or more cycles of sulfasalazine-melphalan; among these were 2 partial responders, 2 patients with stable disease, and 9 patients with progressive disease.

Of the four ovarian-cancer patients entered onto the study, two were partial responders who had failed prior platinum-based chemotherapy, one had progressive disease, and one did not receive any melphalan. One of the responders had a marked decrease in the size of a pelvic mass that was associated with a decrease in CA-125 antigen from 261 to 29. After five cycles of sulfasalazine and melphalan, an exploratory laparotomy revealed no gross disease; however, there was residual microscopic carcinoma on biopsy specimens. Following two more cycles of treatment, the CA-125 value recorded for this patient increased to 83. The other partial responder had complete regression of multiple pulmonary nodules and partial regression of a large hilar mass. After completing five cycles of sulfasalazine (12–16 g/day), this patient refused to take sulfasalazine any further secondary to nausea. At the highest dose of melphalan (40 mg/m^2), this patient did not develop any neutropenia, whereas the previous responder (at a melphalan dose of 30 mg/m^2) developed grade 3 neutropenia.

Table 2 Toxic effects of combined treatment with melphalan and sulfasalazine

	Toxicity grade				
	0	1	2	3	4
Nausea[a]	1	4	15	9	–
Vomiting[b]	5	2	17	6	–
Neutropenia[c]	27	1	1	3	–
Thrombocytopenia[d]	28	1	2	–	–
Anemia[e]	23	4	3	2	–

[a] Data not available on 8 patients
[b] Data not available on 7 patients
[c] Data not available on 3 patients
[d] Data not available on 4 patients
[e] Data not available on 3 patients

The major side effects encountered are shown in Table 2. The MTD of sulfasalazine ranged between 4 and 20 g/day. In all, 24 and 23 patients developed grade 2 and 3 nausea/vomiting, respectively, which was uniformly associated with the administration of sulfasalazine and did not appear to be secondary to melphalan. Five patients went off the study due to nausea associated with sulfasalazine. Only four patients developed significant neutropenia, which suggests that the MTD of melphalan was not achieved and argues against the occurrence of enhanced myelosuppression from the combination of sulfasalazine and melphalan.

Plasma sulfasalazine levels were analyzed in 21 patients and the results are summarized in Fig. 1. The MTD of sulfasalazine in these patients ranged between 1.0 and 5.0 g p.o. q.i.d. Sulfasalazine could not be detected in the plasma samples obtained from three subjects (patients 6, 17, and 20). Plasma sulfasalazine levels varied between 2.5 and 47.1 μg/ml (mean, 15.06 ± 13.86 μg/ml). There was no correlation between the plasma sulfasalazine level and either the MTD or the duration of sulfasalazine treatment.

Prior to and following sulfasalazine treatment, 16 samples were analyzed for GSH levels in peripheral mononuclear cells (Fig. 2). A large interindividual variation was observed in the GSH content of peripheral mononuclear cells obtained from patients prior to sulfasalazine administration. GSH levels in the untreated mononuclear cells varied between 4.6 and 109.8 nmol/mg protein, whereas the level of this thiol detected in mononuclear cells after sulfasalazine treatment ranged from 1.2 to 141.7 nmol/mg protein (Fig. 2). Whereas GSH depletion was observed in the mononuclear cells of 12 patients, its level increased in 3 patients and remained unaffected in 1 patient following sulfasalazine treatment. The extent of GSH depletion varied between 25.0% and 87.1%. There was no correlation between the plasma sulfasalazine level and the

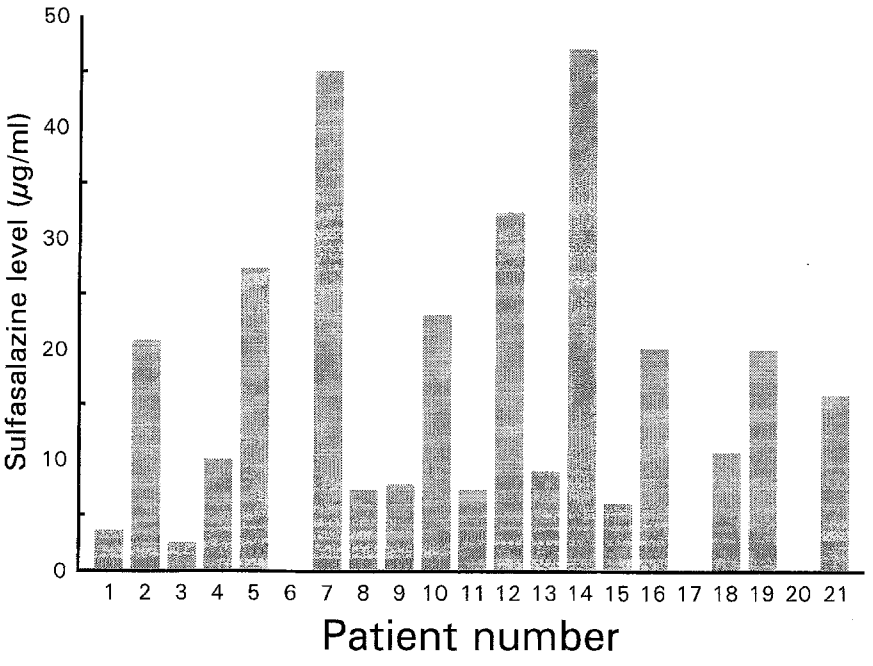

Fig. 1 Plasma sulfasalazine levels measured in 21 patients

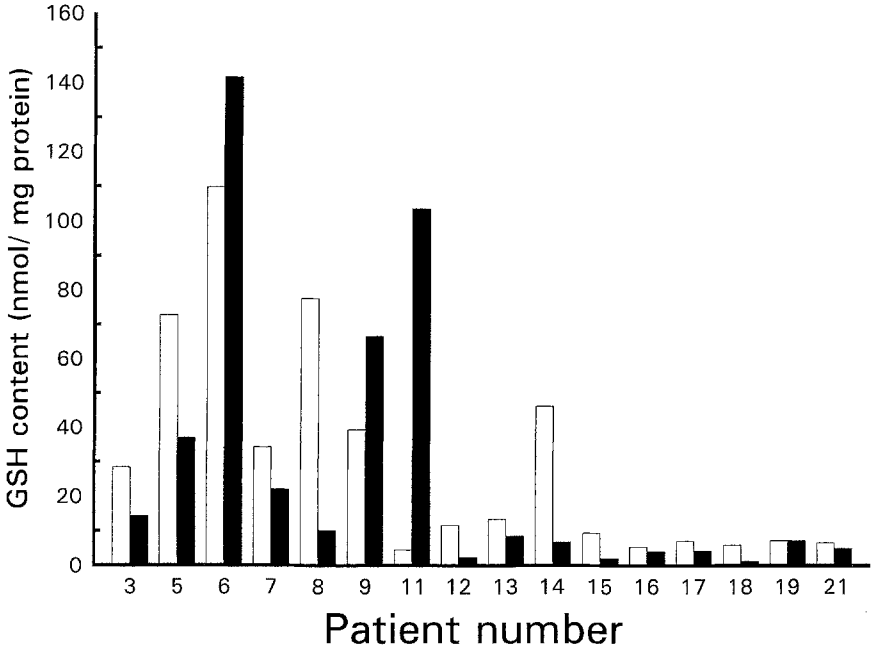

Fig. 2 GSH levels detected in the peripheral mononuclear cells of 16 patients prior to (□) and follwing (■) sulfasalazine administration

extent of GSH depletion. However, it is noteworthy that both of the patients with ovarian cancer (patients 12 and 13) who responded partially to this combination had GSH depletion (80.6% and 37.2%, respectively) in their mononuclear cells.

Figure 3 shows the GST activity detected in peripheral mononuclear cells of 15 patients before and after sulfasalazine treatment. The GST activity measured in untreated mononuclear cells ranged between 0.7 and 125.4 nmol min^{-1}mg $protein^{-1}$, whereas that detected in mononuclear cells following sulfasalazine treatment varied between 5.7 and 147.5nmol min^{-1} mg $protein^{-1}$. Whereas sulfasalazine treatment caused a marked reduction in mononuclear GST activity in five patients, the enzyme activity increased in nine patients.

The in vitro experiments were also performed to determine if sulfasalazine could inhibit the GST activity of peripheral mononuclear cells in the presence of plasma, as sulfa drugs are known to bind extensively to plasma proteins. The mean GST activity measured by an in situ GST-assay procedure in the peripheral mononuclear cells of two normal human subjects was 120 nmol min^{-1} mg $protein^{-1}$. The GST activity

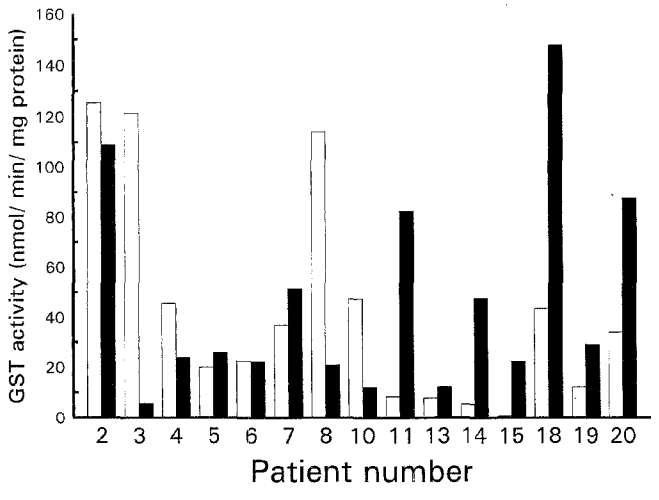

Fig. 3 GST activity detected in the peripheral mononuclear cells of 15 patients prior to (□) and following (■) sulfasalazine treatment. The enzyme activity was determined using CDNB as the electrophilic substrate

measured in cell homogenate was found to be comparable with that observed by the in situ enzyme-assay method. Pretreatment of mononuclear cells with 100 μ*M* sulfasalazine for 5 min caused about 70% inhibition of GST activity.

Discussion

Recent studies suggest that cellular resistance to anticancer drugs may be manifested by multiple mechanisms and that resistant tumor may have elevated levels of one or more proteins that normally provide cellular protection against toxicants [13, 16, 17]. One such mechanism involves enhanced inactivation of anticancer drugs or their active metabolites through elevated levels of GSH and/or GST in resistant tumors [2, 3, 17, 32, 33]. Wang and Tew [32] were the first investigators to describe an elevation in GST activity in a nitrogen mustard-resistant rat mammary-carcinoma cell line. Subsequently, many investigators have reported elevated levels of GSH and/or GSTs in cancer cells selected for resistance to many structurally and functionally different anticancer agents, including melphalan [5, 24, 28, 33]. These studies suggest that the effective treatment of resistant tumors may require reversal of the GSH/GST-mediated drug-resistance mechanism. However, it has to be emphasized that the pharmacological strategies directed toward reversal of these mechanisms should be relatively nontoxic.

Ethacrynic acid, a diuretic drug, is a potent inhibitor of GST activity. The cytotoxic activity of several alkylating agents, including melphalan, have been shown to be enhanced by ethacrynic acid both in vitro [15, 29, 34, 35] and in vivo [9]. Ethacrynic acid interferes with GST-mediated conjugation of drugs with GSH by two related mechanisms: (1) ethacrynic acid acts as a direct inhibitor of GST activity, and (2) ethacrynic acid serves as a substrate for GST-catalyzed conjugation reactions. As a result, ethacrynic acid treatment reduces cellular GSH levels, which may result in further impairment of GST-dependent detoxification reactions. A phase I study of ethacrynic acid and thiotepa was also recently conducted [21] to determine the utility of this agent as a modifier of GSH/GST-dependent anticancer drug resistance.

It has been documented that sulfasalazine, a relatively nontoxic agent used for the treatment of ulcerative colitis, is a potent inhibitor of GST activity in vitro [1]. Sulfasalazine effectively inhibits all the three major classes of GST isoenzymes: α, μ, and π [1]. The concentrations of sulfasalazine required (28 μ*M* or less) to inhibit 50% of the enzyme activity in vitro of all the three major classes of GSTs [1] are in the range that can readily be achieved by conventional oral doses of sulfasalazine. An average daily dose 3–6 g results in steady-state serum concentrations of approximately 45 μ*M* sulfasalazine [10]. Therefore, sulfasalazine appeared to be a good candidate for modulation of the GSH/GST-mediated drug-resistance mechanism. In this study, we evaluated the toxicity of and response to the sulfasalazine-melphalan combination and determined the effect of sulfasalazine administration on GSH and GST levels in acceptable surrogate normal tissue, peripheral mononuclear blood cells.

A large interpatient variability was observed not only in the basal level of GST activity but also in the extent of GST-activity inhibition in mononuclear cells of sulfasalazine-treated patients. Whereas a marked reduction in mononuclear GST activity was observed in five patients following sulfasalazine treatment, the remaining samples reflected either higher or unchanged levels of GST activity after such treatment. As suggested by O'Dwyer et al. [21], variation in the degree of GST-activity inhibition may result from two factors: (1) differences in the pharmacokinetics of the GST-activity inhibitor or (2) phenotypic variation in the expression of individual GST isoenzymes. Since plasma sulfasalazine levels did not correlate with the extent of GST-activity inhibition, the first possibility seems rather unlikely.

Phenotypic differences in the expression of GST isoenzymes has previously been documented in certain human tissues [19]. Similar diversity in the GST-isoenzyme expression in peripheral mononuclear cells has also been reported [26]. Whereas about 40% of the population expresses only the μ-type GST subunit, the majority of subjects express both the α and μ-classes of GST isoenzymes. Phenotypic differences in the expression of GST isoenzymes in tumors of various histologies have been documented as well [30]. Since the 50% inhibitory concentration of sulfasalazine for α- μ- and π-class human GST isoenzymes varies considerably (20, 0.2, and 13 μ*M*, respectively) [1], the overall degree

of GST-activity inhibition caused by this drug in any tissue, including the tumor, is likely to be dependent upon the GST-isoenzyme profile. It is also noteworthy that the GST isoenzymes of different classes have overlapping substrate specificity and that the substrate used to measure GST activity in this study is not specific for any class of isoenzyme [20]. Therefore, measuring total GST activity may not reveal subtle differences in a particular class of isoenzyme.

Similar to GST activity, a large interindividual variation was also observed in the basal level of GSH in mononuclear cells. However, the frequency of GSH depletion by sulfasalazine treatment appeared to be higher than that of GST-activity inhibition. Although the mechanism by which sulfasalazine causes GSH depletion remains to be determined, two possibil ities exist for this effect. Similar to ethacrynic acid, sulfasalazine and/or its metabolites (sulfapyridine and 5-aminosalicylate) may act as a substrate for GST, thereby reducing the GSH level. Alternatively, sulfasalazine or its metabolites may deplete GSH by inhibiting the enzymes of GSH bio-synthesis. In any case, concomitant GSH depletion and GST-activity inhibition by sulfasalazine may be advantageous in the reversal of GSH/GST-mediated drug-resistance mechanisms. In conclusion, further studies are needed to identify more potent/less toxic GST-activity inhibitors/GSH depletors for use in reversing GSH/GST-mediated drug resistance.

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