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SHORT COMMUNICATION

BCNU-sequestration by metallothioneins may contribute to resistance in a medulloblastoma cell line

Manny D. Bacolod · Randy Fehdrau · Stewart P. Johnson · Nancy S. Bullock · Darell D. Bigner · Michael Colvin · Henry S. Friedman

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Abstract
Purpose Resistance of neoplastic cells to the alkylating drug BCNU [1,3-bis(2-chloroethyl)-1-nitrosourea] has been correlated with expression of O^6-methylguanine-DNA methyltransferase, which repairs the O^6-chloroethylguanine produced by the drug. Other possible mechanisms of resistance include raised levels of glutathione or increased repair of the DNA interstrand cross-links formed by BCNU. Transcriptional profiling revealed the upregulation of several metallothionein (MT) genes in a BCNU-resistant medulloblastoma cell line [D341 MED (OBR)] relative to its parental line. Previous studies have shown that MTs, through their reactive thiol groups can quench nitrogen mustard-derived alkylating drugs. In this report, we evaluate whether MTs can also quench BCNU.
Methods To demonstrate the binding of BCNU to MT, we used an assay that measured the release of the MT-bound divalent cations (Zn^{2+}, Cd^{2+}) upon their displacement by the drug. We also measured the decomposition rates of BCNU at those reaction conditions.
Results The rate of release of the cations was higher in pH 7.4 than at pH 7.0, which is likely a result of more rapid decomposition of BCNU (thus faster release of MT-binding intermediate) at pH 7.4 than at pH 7.0.
Conclusion We demonstrate that resistance to BCNU may be a result of elevated levels of MTs which act by sequestering the drug's decomposition product(s).

Keywords Carmustine · BCNU · Metallothionein · Medulloblastoma · Brain tumor · Cancer drug resistance

M. D. Bacolod (✉) · S. P. Johnson · N. S. Bullock · H. S. Friedman
Department of Surgery, Duke University Medical Center, Durham, NC 27710, USA
e-mail: mdb2005@med.cornell.edu

Present Address:
M. D. Bacolod
Department of Microbiology and Immunology, Cornell University Weill Medical College, New York, NY 10021, USA

R. Fehdrau · M. Colvin
Department of Medicine, Duke University Medical Center, Durham, NC 27710, USA

D. D. Bigner
Department of Pathology, Duke University Medical Center, Durham, NC 27710, USA

Introduction

BCNU [1,3-bis(2-chloroethyl)-1-nitrosourea] or carmustine, a lipophilic chemotherapeutic drug which can efficiently cross the blood-brain barrier, is widely used in treating various types of brain tumors including medulloblastoma [1, 2]. However, the effectiveness of this alkylating drug can be moderated by various factors, foremost of which is increased O^6-methylguanine-DNA methyltransferase (MGMT) activity in target cells acquired during the course of treatment [3]. Repair by MGMT of the BCNU-derived chloroethyl lesion in the O^6 position of guanine halts the formation of cytotoxic DNA interstrand cross-links [4]. We have previously described a medulloblastoma subline, D341 MED (OBR), which has an acquired resistance to BCNU in combination with the MGMT pseudosubstrate, O^6-benzylguanine (O^6-BG) [5, 6]. The resistant cell line was not found to exhibit an elevated glutathione (GSH) level nor increased glutathione *S*-transferase (GST) activity, two factors that can also contribute to BCNU detoxification [7]. The D341 MED (OBR) cells did not

demonstrate enhanced repair of DNA interstrand cross-links relative to the parental D341 MED cells [5]. However, sequence analysis of the cDNAs indicated that the MGMT from the resistant cell line had acquired a mutation that potentially affected binding to O^6-BG [6]. Genome-wide expression analyses revealed altered expression of a number of genes including the upregulation of many members of the metallothionein (MT) gene family (MT2A, MT1X, MT1L, MT1A, MT1E, MT1F, MT1H, MT1B) in the BCNU-resistant cells [5]. These low molecular weight, thiol-rich proteins have been associated with resistance against platinum-based chemotherapy compounds [8], as well as against the alkylating agents, chlorambucil and melphalan [9, 10]. Fenselau and co-workers have demonstrated that specific thiol groups in MT can form covalent linkage with the chloroethyl group of each of the four nitrogen mustard-derived drugs (chlorambucil, melphalan, cyclophosphamide, and mechloroethamine) currently used in clinics [11–14]. Since BCNU and its reactive decomposition products have similar electrophilic centers, we examined if MTs do indeed sequester BCNU in solution. The observed sequestration suggests that upregulation of MTs is another potential mechanism of resistance to BCNU in this medulloblastoma cell line.

Methodology

Cell lines and other materials

D341 MED was grown in Improved MEM Zinc Option (Richter's Modification) Medium (Invitrogen) supplemented with 10% FCS (Invitrogen). The generation, propagation, and maintenance of D341 MED (OBR) has been reported previously [5]. Rabbit liver MT2 was purchased from Sigma-Aldrich (St Louis, MO, USA). It was certified to have 1.6% Zn^{2+} and 6.5% Cd^{2+} or 3.8 mol and 1.6 mol Zn^{2+} and Cd^{2+} per mol protein, respectively. BCNU and 4-(2-pyridylazo) resorcinol (PAR) were also purchased from Sigma-Aldrich.

Quantification of metallothioneins in the cell lines

Cellular extracts were prepared by incubating the cells with lysis buffer (50 mM Tris–HCl, pH 7.8, 0.15 M NaCl, 1% NP-40, Roche complete mini EDTA-free protease-inhibitor cocktail) at 37°C for 10 min. The supernatant was isolated after centrifugation. Sixty micrograms of protein extracts from the cell lines, and 60 ng of pure rabbit liver MT2 were electrophoresed on NuPAGE 4–12% Bis–Tris gel (Invitrogen) and transferred onto nylon membrane (Pall). MT2 was then detected using 1:100 dilution of mouse anti-MT monoclonal antibody clone E9 (Dako) followed by 1:1000 dilution of horseradish peroxidase-conjugated anti-mouse IgG (GE Healthcare). Detection was performed using ECL Plus (GE Healthcare). The presence of MT2 mRNA was verified by Northern blot analysis. The *MT2* DNA probe was prepared from a plasmid clone containing the *MT2* gene (ATCC). Hybridization was performed using the NorthernMax Ultrasensitive Northern Blotting Kit (Ambion).

Metallothionein–BCNU binding assay

To test if BCNU binds to MT, we employed the assay originally developed by Laib et al. [15]. A 1-ml solution (either in 0.1 M Tris at pH 7.0, or 1× PBS at pH 7.4) containing 5 μM MT2, 100 μM PAR, and BCNU (at 0, 0.14, and 0.8 mM) was incubated at 37°C. Its absorbance at 485 nm was monitored every 10 min for a 150-min period, using the Beckman Coulter DU640 uv/vis spectrophotometer. In addition, solutions without MT2 were also run as controls.

BCNU decomposition assay

The decomposition of BCNU in the presence or absence of MT2 was monitored using a protocol originally intended to analyze the presence of BCNU in blood of patients undergoing chemotherapy [16]. Thirty micrograms per milliliter (140.2 μM) BCNU in 0.1 M Tris, pH 7.0, or 1× PBS, pH 7.4 was incubated at 37°C, in the absence or presence of 5 μM MT2. After every 30 min, 1 ml of the solution was transferred to a tube containing 1 ml ethyl acetate solution with 5,5′ diphenylhydantoin (internal standard). The collected samples were subsequently analyzed by reverse phase HPLC to quantitate the amount of intact BCNU in the solution.

Results

Our previous genome-wide transcriptional analysis had indicated elevated levels of several MTs in the D341 MED (OBR) cell line relative to the parental cell line (Fig. 1a) [5]. We confirmed this increase in expression of MT RNAs by Northern analysis (Fig. 1b), using a probe containing the complete MT2A cDNA sequence. The MT1 and MT2 isoforms, which were observed to be upregulated by our microarray analysis, have RNAs that are almost indistinguishable in size and nucleotide sequence. Thus, it is highly likely that the RNA band identified was actually a mixed population of MT1 and MT2 RNAs. We also measured the relative amounts of MT protein present in D341 MED (OBR) and D341 MED by immunoblot and found elevated MT protein in the BCNU-resistant cells (Fig. 1c). The increased MT protein expression that we observed likely represents enhanced expression of several MT isoforms as

A

Probeset ID	Gene ID	Fold Change [D341 MED (OBR) vs D341 MED]	Detection p value
39120_at	MT1X	7.46	0.000266
39081_at	MT2A	6.06	0.000388
41446_f_at	MT1F	3.73	0.039365
31623_f_at	MT1A	3.48	0.001141
36130_f_at	MT1E	2.64	0.003585
31622_f_at	MT1F	2.46	0.019304
39594_f_at	MT1H	1.74	0.000959
609_f_at	MT1B	1.52	0.002617

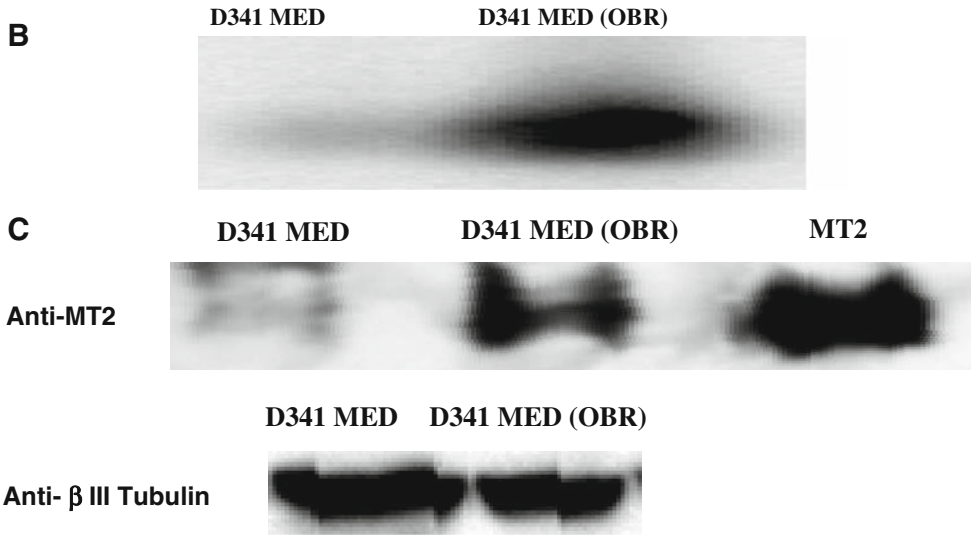

Fig. 1 Comparison of metallothionein (MT) expression levels in D341 MED (OBR) and its parental line, D341 MED using: (**a**) expression array (U95A ver 2) analysis [D341 MED (OBR) vs D341 MED] (some of these data were also reported in Bacolod et al., 2002), (**b**) northern blot, and (**c**) immunoblot

well since the E9 anti-MT antibody was originally raised for both MT1 and MT2 isoforms [17].

The binding of BCNU (or its decomposition product) to MT2 was then monitored by indirect measurement of Cd^{2+} and Zn^{2+} displaced by the drug. Upon their release from MT, these cations can bind to PAR causing the compound's spectral peak to shift from 410 to 485 nm [15]. The increase in absorbance at 485 nm over time (Fig. 2a) clearly indicated the dissociation of the divalent ions from MT2 upon the addition of BCNU. The rate of cation release was observed to be higher in pH 7.4 (PBS buffer) compared to pH 7.0 (0.1 M Tris).

We also measured the rate of decomposition of BCNU using these two buffer conditions. At pH 7.4, the drug's decomposition was faster ($t_{1/2} = 35.7$ min) than what was observed at pH 7.0 ($t_{1/2} = 79.7$ min). An earlier study [18] reported that the half-life of BCNU at pH 6.8 (0.1 M Tris) and pH 7.4 (0.1 M phosphate) were 112 and 52 min, respectively. We also tested the effect of MT on the decomposition of BCNU. As shown in Fig. 3, MT had no effect on the rate of BCNU decomposition. The combined results from the binding and decomposition experiments suggest that it is a BCNU decomposition product which binds to MT.

Discussion

Metallothioneins are present in a variety of central nervous system tissues since these cysteine-rich, low molecular weight proteins are involved in various neuroprotective, regenerative, and cognitive functions (reviewed in [19]). Among brain tumors (such as gliomas and meningeal tumors), the high expression levels of these proteins have been confirmed immunohistochemically [20]. The regulation of expression of these proteins (the MT1 and MT2 isoforms in particular) often involves the promoter elements, metal response element and antioxidant response element (ARE), which respond to stimuli by heavy metals and oxidative stress respectively [21]. The regulatory mechanism which explains how chemotherapeutic alkylating agents induce expression of these MTs is not yet well understood, but some evidence suggests that it may involve ARE [22]. Nonetheless, it is safe to assume that lengthy exposure to BCNU eventually led to the elevated expression levels of the MT1 and MT2 isoforms in our BCNU-resistant medulloblastoma subline [D341 MED (OBR)] [5]. Interestingly, the D341 MED (OBR) cell line exhibited the upregulation of only the MT1 and MT2 isoforms, while the known

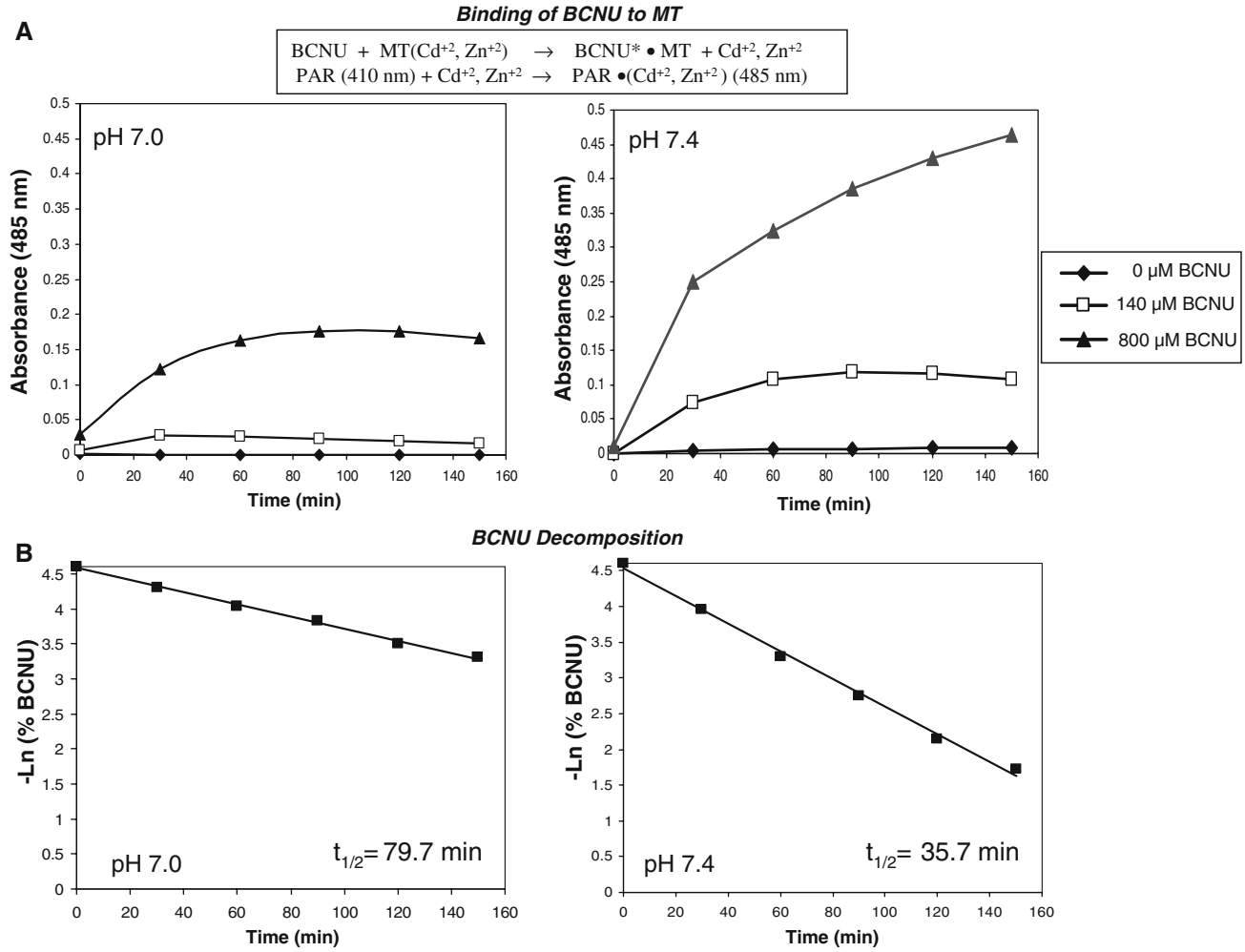

Fig. 2 (**a**) The binding of BCNU to MT2 at pH 7.0 and pH 7.4. Addition of BCNU to a solution containing PAR and MT2 leads to an increase in absorbance at 485 nm, due to the conjugation of PAR to the divalent cations Cd^{2+} and Zn^{2+}. (**b**) The rate of BCNU decomposition at pH 7.0 and pH 7.4 was determined as described in Sect. "Methods"

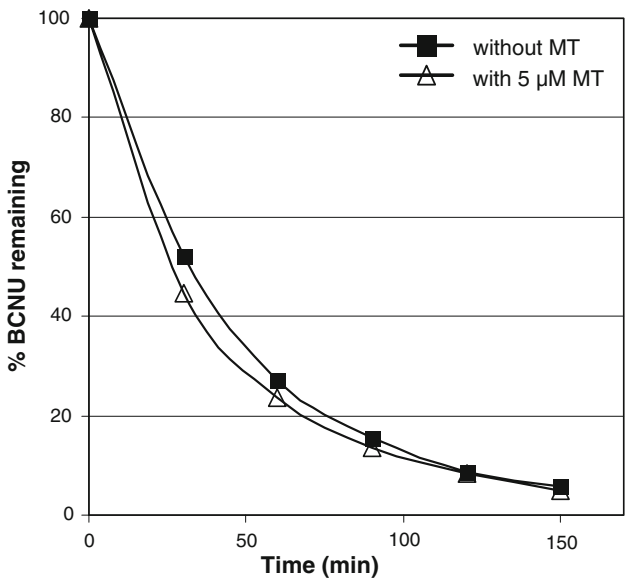

Fig. 3 The lack of effect of MT2 on BCNU decomposition at pH 7.4

brain-specific isoform (MT3) and the other minor isoform (MT4) did not register any change in expression level relative to the parental line.

Whether these MTs are expressed transiently (as a response to stimuli), or due to changes in the target cell genome (e.g., gene amplification, changes in promoter methylation) [21], the elevated levels of these proteins should be a concern since it is well documented that MTs can decrease the activities of various alkylating agents. Indeed, several studies have correlated elevated levels of MTs in biopsies to poor prognosis in different types of cancer [23–26]. The chemotherapy regimens used in these studies included platinum-based drugs [23, 24].

The experimental results that we present here link elevated MT expression and BCNU resistance in a cancer cell line. We have also demonstrated that the rate of binding of MT to BCNU mirrored the rate of BCNU decomposition. This suggests that it is the product(s) of BCNU decomposition that binds to MT. In aqueous solution and physiological pH,

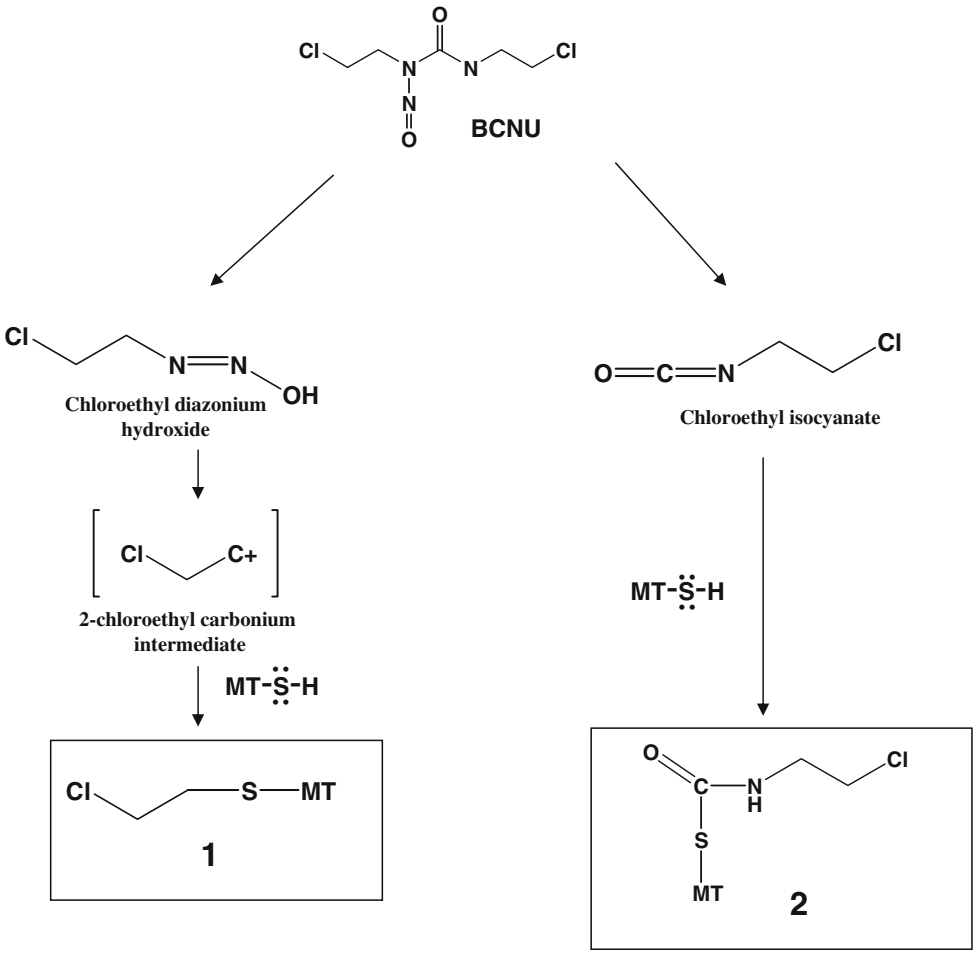

Fig. 4 Possible reaction of MT with the BCNU decomposition products: thiol capture of the carbonium intermediate to form the chloroethyl adduct (*1*), or CEIC carbamoylation of a thiol group in MT (*2*)

BCNU quickly decomposes to chloroethyl isocyanate (CEIC) and chloroethyl carbonium intermediate [27]. The carbonium intermediate is generally considered the primary genotoxic (cancer-killing) by-product since it is the precursor to the DNA interstrand cross-link formation [28]. However, studies have shown that CEIC may also contribute to overall BCNU genotoxicity by inhibiting DNA repair [29, 30], presumably through CEIC's carbamoylation of the repair enzymes [31, 32]. In theory, both of the BCNU decomposition products have electrophilic carbons capable of reacting to sulfhydryl groups in MTs (Fig. 4). Other studies have demonstrated the interaction of BCNU with glutathione (GSH), whose elevated level has also been linked to BCNU-resistance in brain tumors [33–35]. Similar to MT, GSH has a reactive thiol group. When Stahl et al. reacted BCNU with GSH, the CEIC/GSH *S*-linked conjugate, *S*-[(2-chloroethyl)carbamoyl] glutathione, was identified as the major product, while the alkylated derivative formed only in low yield [36]. In a similar experiment conducted by Carbone et al., a decapeptide with cysteine in the middle also reacted with BCNU [37]. They, too, have identified the *S*-carbamoylation derivative at cysteine as the primary adduct. Although it is more likely for CEIC, rather than the chloroethyl carbonium intermediate to covalently modify MTs, further experiments will be necessary to detect the actual adducts.

The results from this study, as well as those by other groups, point to the potential importance of MTs in resistance to DNA alkylators such as BCNU. The clinical manifestation of these results is that the level of MTs may be a factor to consider in medulloblastoma (or other types of cancer) chemotherapy that includes BCNU in the regimen. For example, an earlier study demonstrated that inhibition of MT synthesis by propargylglycine increased the activity of an anti-cancer cocktail which included the alkylating drug melphalan [38].

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