Histone Deacetylase Inhibitor Entinostat Inhibits Tumor-Initiating Cells in Triple-Negative Breast Cancer Cells

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Abstract

Mortality following breast cancer diagnosis is mainly due to the development of distant metastasis. To escape from the primary site, tumor cells undergo the epithelial-to-mesenchymal transition (EMT), which helps them acquire a more motile and invasive phenotype. In our previous study, we showed that class I selective HDAC inhibitor entinostat reverses the EMT phenotype through reversal of epigenetic repression of E-cadherin. Recent evidence suggests that a subset of cells within a breast tumor may drive the metastatic outgrowth following escape from the primary site. These cells, termed tumor-initiating cells (TIC), represent a great threat to overall prognosis. They are critical in terms of drug resistance and tumor initiation at metastatic sites. Acquisition of EMT traits has also been shown to impart TIC phenotype to the cells, making EMT a “dual-threat” for prognosis. In the current study, we show that entinostat treatment can reduce the percentage of TIC cells from triple-negative breast cancer (TNBC) cells. Entinostat treatment was able to reduce the CD44high/CD24low cell population, ALDH-1 activity, as well as protein and mRNA expression of known TIC markers such as Bmi-1, Nanog, and Oct-4. Next, we inoculated MDA-MB-231 cells transfected with firefly luciferase (231/Luc) in mammary fat pad of NSG mice. The mice were then treated with entinostat (2.5 mg/kg/d), and tumor development and formation of metastasis were assessed by bioluminescence imaging. Treatment with entinostat significantly reduced tumor formation at the primary site as well as lung metastasis. As such, entinostat may help prevent development of distant metastasis. Mol Cancer Ther; 14(8); 1848–57. ©2015 AACR.

Introduction

Metastatic spread of disease is almost always responsible for mortality following a breast cancer diagnosis, as the primary tumor is rarely responsible for fatal clinical outcome. Epithelial-to-mesenchymal transition (EMT) and its reverse mesenchymal-to-epithelial transition (MET) are known to play a key role in embryogenesis (1) as well as wound healing (2). EMT is often activated in tumors during invasion and metastasis and helps cancer cells detach from solid tumors. EMT is characterized by loss of intracellular adhesion (loss of E-cadherin); loss of epithelial markers such as cytokeratins 8/18 and upregulation of mesenchymal markers such as vimentin and acquisition of fibroblast-like spindle morphology and increased motility (3, 4). Previously, we showed that treatment of triple-negative cells of basal origin that have a mesenchymal phenotype with HDAC inhibitor entinostat results in reversal of EMT phenotype and reduction in migratory capacity (5). Entinostat reversed the repression of E-cadherin promoter by reducing the binding of twist and snail to CDH-1 promoter. This was also accompanied by reduction in twist and snail protein expression. Other characteristics of EMT reversal, such as reduction in N-cadherin and increase in E-cadherin and cytokeratin 8/18 expression, were observed upon entinostat treatment, in vitro and in vivo.

Analysis of human tumor tissues has provided evidence of self-renewing, stem-like cells that have been labeled as cancer stem cells (CSC) or tumor-initiating cells (TIC). They are characterized by their ability to reseed a tumor in mice when inoculated in very few numbers (6). Al Hajj and colleagues showed that a certain fraction of the cells derived from pleural effusions (CD44high/CD24low) possessed the ability to form xenografts with just 100 cells whereas thousands of CD44high/CD24low cells failed (7). Thus, the CD44high/CD24low expression pattern is considered to be one of the characteristics of TICs. Studies have suggested that cells that activate EMT also acquire properties of TICs such as CD44high/CD24low expression (8–10). This connection between EMT and acquisition of TIC phenotype can prove to be worse for clinical outcome as a tumor will not only acquire invasive capability that allows the escape from primary site but also the ability to reseed at a new site. As such, treatments that can target both the EMT process and TICs will be critical.

In this study, we show that triple-negative breast cancer (TNBC) cells contain cells with tumor-initiating properties and treatment with entinostat (ENT) can reduce this population. The reduction in TIC population resulted in reduced mammosphere-forming ability and reduced tumor formation in vivo. Furthermore, entinostat treatment was able to reduce the dissemination of cancer.
cells and reseeding into the lungs, resulting in reduced metastatic lung colonization.

In addition, entinostat treatment was also able to reduce the expression of miRNA-181a. MicroRNAs (miRNA/miRs) are small (20–30 nucleotides) noncoding RNAs that posttranscriptionally control gene expression through canonical base pairing between miR seed sequence (nucleotides 2–8 at the 5’ end) and the complementary sequences in the 3’-untranslated region (UTR) of the target mRNA. The net effect of these events can be either translational repression or degradation of targeted miRNAs. Aberrant expression of specific miRNAs can be used as potential prognostic or predictive marker. Deregression of miRs is common in human cancer and correlates with initiation and progression of cancer. Such miRs, including miR-181a, are often termed as oncomiRs, as higher levels have been observed in breast cancer cell lines compared with normal breast epithelial cells and breast tumor samples compared with normal tissue. Specifically, the expression of miR-181a correlated more with highly proliferative tumors (high grade and high Ki67; refs. 11, 12). In colorectal cancer, miR-181a has been associated with poor prognosis (13). In addition, miR-181 family members have been implicated with cancer, miR-181a has been associated with poor prognosis (13). In colorectal tumors (high grade and high Ki67; refs. 11, 12). In colorectal cancer, miR-181a has been associated with poor prognosis (13).

Materials and Methods

Materials

Estrogen receptor (ER)α-negative MDA-MB-231, BT549, and Hs578T cells were obtained from ATCC and cultured in Dulbecco Modified Eagle Medium (DMEM) containing 5% FBS and penicillin/streptomycin solution (P/S; 10,000 IU each). DMEM, FBS, P/S, 0.25% trypsin–1 mmol/L EDTA solution, and Dulbecco PBS (DPBS) were obtained from Life Technologies. Matrigel was obtained from Sigma Chemical Company. Antibodies against β-catenin, Oct-4, Nanog, Bmi-1, and GAPDH were purchased from Cell Signaling Technology. Mammocult medium (Stem Cell Technologies) was used for mammosphere formation assays. Dr. Peter Ordentlich (Syndax Pharmaceuticals) provided entinostat (ENT/SNDX-275/MS-275). Entinostat for in vitro use was prepared in DMSO as a 10–2 mol/L stock and further diluted in 95% ethanol. Treatment with entinostat subjected cells to less than 0.5% of vehicle.

Cell line authentication

All cell lines used in this study were authenticated in August 2013, using CellCheck service [microsatellite marker (short tandem repeat, STR) analysis] provided by Idexx Radii. The cell lines were found to be identical to the genetic profile reported by ATCC. Frozen aliquots of cell lines were then used within 6 months of culturing.

Western blotting

Protein extracts from TNBC cells were made as described earlier, and 25 µg of protein was analyzed by SDS-PAGE as described previously (16, 17). Bands were quantitated by densitometry using ImageJ. Densitometric values are corrected for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) loading control.

Flow cytometry

TNBC cells were treated with entinostat (1 µmol/L) for 72 hours. The cells were harvested and resuspended in DPBS containing 2% BSA. One million cells were pelleted and resuspended with a cocktail of APC-anti human CD44 (BD Pharmingen), FITC-anti human CD24 (BD Pharmingen), and near-infrared viability dye (Invitrogen). Cells were incubated on ice for 40 minutes. Following incubation, cells were washed with DPBS containing 2% BSA and resuspended in DPBS containing 2% BSA. After resuspension, data were acquired on FACS Canto with appropriate compensation controls. At least 10,000 viable cells were acquired and data plotted. Data were analyzed using FlowJo software.

RNA extraction, reverse transcription, and PCR

RNA was extracted and purified using the RNeasy Mini Kit (Qiagen) and miRNA was isolated using miRNeasy Kit (Qiagen) per manufacturer’s protocol. MicroRNA from patient samples was isolated using miRNeasy FFPE Kit (Qiagen) according to manufacturer’s instructions. Total RNA concentration and purity were determined from 260 and 280 nm absorbance. RNA was diluted with DNase/RNase-free water to 0.08 µg/µL and 0.64 µg of RNA was reverse transcribed as per manufacturer’s protocol (Bio-Rad). Analysis of Nanog and Bmi-1 mRNA expression was carried out by real time qRT-PCR as described earlier (16, 18) using a Bio-Rad CFX Connect real-time system. Values were normalized to corresponding 18S rRNA values and expressed as the fold increase relative to control. Total RNA was diluted 1:10 before amplification of 18S ribosomal RNA (rRNA).

Mammosphere formation assay

TNBC cells were treated with entinostat (1 µmol/L) for 72 hours, collected by trypsinization and counted. Viable cells (2,000) were seeded in Mammocult medium [Mammocult media + supplements (STEMCELL Technologies)], 4 µg/mL heparin, and 0.48 µg/mL hydrocortisone (Sigma-Aldrich) in ultra-low attachment plates (Corning) in specified treatment conditions and allowed to propagate for 3 weeks at 37°C in 5% CO₂. Two independent operators counted mammospheres manually and the average was taken. Spheres with a colony count of at least 50 cells were considered mammospheres.

ALDEFLUOR assay

ALDEFLUOR assay was performed using the ALDEFLUOR Kit (Stem Cell Technologies). Hs578T cells were treated with entinostat (1 µmol/L) for 24 hours, and 1 × 10⁶ cells were resuspended in 1-mL ALDEFLUOR buffer containing 1 µL/mL propidium iodide. ALDEFLUOR reagent (5 µL; Bodipy-aminoacetaldehyde, BAAA) was added to the cell suspension. Cell suspension (500 µL)
was transferred to a new tube and 5 μL DEAB (diethylaminobenzaldehyde, inhibitor of aldehyde dehydrogenase enzyme) was added to the tube. Cells were incubated for 40 minutes at 37°C, centrifuged and resuspended in ALDH buffer. Cells were acquired using BD LSRII and FACS CANTO. Data were analyzed by FlowJo software.

**In vivo tumor formation with bioluminescence imaging**

All animal studies were performed according to the guidelines and approval of the Animal Care Committee of the University of Maryland (Baltimore, MD). Female ovariectomized NSG (Nonobese diabetic-Severe combined immunodeficient lacking interleukin 2 receptor Gamma or NOD.Cg-Prkdc<sup>scidIl2rg<sup>tm1Wjl</sup></sup>/Sjcl or NOD-SCID gamma) mice, 4–6 weeks of age, were obtained from the University of Maryland School of Medicine Veterinary Resources. The mice were housed in a pathogen-free environment under controlled conditions of light and humidity; received food and water ad libitum. The fat pads of the mice were allowed to form and mature in presence of 50 μg/d E<sub>2</sub> (in 0.3% HPC-0.9%NaCl) subcutaneously for about 8 weeks. Mice were then injected with 500,000 (2 sites per mouse) luciferase transfected MDA-MB-231 (231/Luc) cells (in Matrigel) in the mammary fat pad. Treatment with entinostat began the following day. Mice were injected with 2.5 mg/kg/d entinostat given po in 30% HPβ-cyclodextrin (5 times/wk). To measure tumor formation, mice were imaged using Xenogen IVIS imaging system. Mice were injected with 150 mg/kg of ν-luciferin intraperitoneally and anesthetized. Mice were placed in imaging chamber and bioluminescence was measured for 3 minutes and superimposed on photographic image of the mouse. The intensity of the bioluminescence is reported in relative luciferase units (RLU).

**Patient-derived xenografts**

Br-001 was derived from an African-American woman aged 74 years at University of Maryland Translational Core Laboratory (TCL). The tumor is triple-negative; ER-negative, PR-negative; HER-2-negative. TCL used excess tissue that would have been discarded thus the tissue was taken under the IRB status "not for human research." The tumor was excised from the patient and within 3 hours was implanted into the mammary fat pad (MFP) of 3 female NSG mice (UMB Vet Res) with estrogen supplementation. After approximately 4 weeks, the tumor was large enough to excise from the mouse, transplant, and freeze back tumor pieces (2 mm<sup>2</sup>) viably. Typically, the Br-001 tumor pieces are thawed from frozen stock (F<sub>0</sub>) and implanted into female NSG mice subcutaneously or in the MFP. The tumors are then grown and passaged into mice to be used for the efficacy study. When the tumors were passaged into final experimental mice, treatment with entinostat was started. Mice received entinostat for 8 weeks after which the mice were euthanized.

**Statistical analysis**

The Student t test (for 2 samples) and one-way ANOVA with Tukey post hoc test (for >2 samples) was used. P < 0.05 was considered statistically significant. The graph represents mean ± SEM.

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**Figure 1.** Flow cytometric analysis of MDA-MB-231 and BT-549 cells treated with entinostat (ENT) for the expression of CD44 and CD24. MDA-MB-231 (top) and BT-549 (bottom) cells were treated with entinostat (1 μmol/L) for 72 hours, and expression of CD44 and CD24 was analyzed by flow cytometry. CD44 expression is plotted on x-axis and CD24 is plotted on y-axis.
Results

Reduction in CD44<sup>high</sup>/CD24<sup>low</sup> population

Expression of cell surface markers such as CD44 with low expression of CD24 is considered an EMT trait. TNBC cell lines such as Hs578T have high level of CD44<sup>high</sup>/CD24<sup>low</sup> cell population. Previously, we reported that when Hs578T cells were treated with entinostat, a significant reduction in CD44<sup>high</sup>/CD24<sup>low</sup> fraction was observed (5). Similar results were also observed in other TNBC cell lines such as MDA-MB-231, BT-549 (Fig. 1), and MDA-MB-436 (data not shown). This change was due to increase in CD24 expression as well as reduction in CD44. Reduction in CD44 was also observed using immunofluorescence (Supplementary Fig. S1).

Entinostat reduces markers of TIC

CD44<sup>high</sup>/CD24<sup>low</sup> is also considered a trait of TICs. To examine whether entinostat regulated other characteristics of TICs, we evaluated the effect of entinostat on markers of TICs, such as the expression of Oct-4, Bmi-1, and β-catenin proteins using Western blotting (Fig. 2A). Treatment of Hs578T and MDA-MB-231 cells with entinostat (1 μmol/L) resulted in reduction in expression of these proteins compared with vehicle-treated control. Furthermore, we measured the mRNA expression of Bmi-1 and Nanog using real-time qRT-PCR (Fig. 2B). Treatment with entinostat (1 μmol/L) resulted in statistically significant reduction in Bmi-1 (P = 0.018) and Nanog (P = 0.0027) in Hs578T cells compared with vehicle-treated control.

Next, we measured the ALDH-1 activity using ALDEFLUOR assay in Hs578T cells (Fig. 2C). High ALDH activity has been shown in stem-like cells of various lineages (19), and its expression in breast cancer correlates with poor clinical outcome (20, 21). Entinostat treatment reduced the ALDH-1 activity by 79% (from 4% in control to 0.84% with entinostat treatment). We also measured the ability of TIC fraction of the cells to efflux Hoechst dye. TICs are known to have high expression of ABCG2 transporters such as BCRP, which enable them to escape therapeutic assault (22, 23). Entinostat treatment was also able to significantly (P = 0.009) reduce this efflux compared with vehicle-treated control, suggesting inhibition of BCRP (Supplementary Fig. S2).

Entinostat reduces mammosphere formation

A characteristic of mammary stem cells to escape anoikis and grow into mammospheres in anchorage-independent environments has been successfully used to enrich functional stem cells from both cancerous breast tissue and breast cancer cell lines. Formation of mammospheres when plated in nonadherent conditions is a marker that distinguishes CSCs (TICs). Hs578T cells were plated (2,000 cells/well) in nonadherent conditions and treated with entinostat (1 μmol/L) while in nonadherent conditions. Mammosphere formation was assessed after 3 weeks. Mammospheres were counted (Fig. 3A) and pictured (Fig. 3B). Treatment with entinostat resulted in significant reduction in mammosphere formation (P < 0.0001). Entinostat treatment also caused reduced mammosphere formation in MDA-MB-231 cells (Supplementary Fig. S3). Alternatively, Hs578T cells were pretreated with entinostat (1 μmol/L) for 72 hours and then plated (2,000 cells/well) in nonadherent conditions. These pretreated cells also exhibited significantly (P = 0.0186) reduced mammosphere-forming ability (Supplementary Fig. S4). Entinostat treatment for 72 hours did not reduce the cell viability significantly (P = 0.1044) and as such, the effect on mammospheres is independent of the cell killing properties of entinostat (Supplementary Fig. S5). Next, we evaluated the effect of entinostat on TGFB-induced mammosphere formation in immortalized normal epithelial cells (non-cancerous) such as MCF-10A and 184B5. As reported elsewhere,
treatment of normal mammary epithelial cells with TGF\(\beta\) resulted in morphological changes (Supplementary Fig. S6; refs. 8, 24, 25). Furthermore, the cells exhibited increase of TIC population as measured by mammosphere-forming ability upon treatment with TGF\(\beta\). This increase in TIC population was significantly inhibited by entinostat as well as LY2109761, an inhibitor of TGF\(\beta\) signaling (Fig. 3C and D).

**Entinostat reduces expression of miRNA-181a**

Although the role of miRNAs in regulating EMT or metastasis is not yet clearly understood, expression of miR-181a has been shown to be high in TNBC cell lines and high-grade tumors (11, 12, 26). Furthermore, miR-181a has also been associated with chemoresistance and TGF\(\beta\)-induced EMT transition. We also observed that miR-181a was overexpressed in TNBC cell lines compared with normal breast epithelial cells and ER-positive breast cancer cell line MCF-7Ca (Fig. 4A). Furthermore, TNBC patient samples expressed significantly more miR-181a (Fig. 4B) compared with ER-positive/Her-2–negative patient tissues \((P = 0.0195)\) and ER-negative/Her-2–positive specimens \((P = 0.0301)\). Next, we examined the effect of entinostat of expression of miR-181a and observed that entinostat treatment can significantly \((P = 0.0317)\) downregulate miR-181a expression in Hs578T cells (Fig. 4C). Similar results were also observed in other TNBC cell line such as MDA-MB-157 and BT-549 (data not shown).

**Entinostat reduces tumor formation and development of metastasis**

MDA-MB-231 cells have been shown to produce spontaneous lung metastasis upon inoculation in the mammary fat pad.
To evaluate the effect of entinostat on tumor formation and metastasis, we inoculated 231/Luc cells into mammary fat pads of NSG mice (500,000/site). The mice were then grouped to receive vehicle (n = 9) or entinostat (n = 9) at 2.5 mg/kg/d po. Three weeks later, mice were imaged using Xenogen bioluminescence imaging (Fig. 5A, Supplementary Fig. S7). The mice treated with entinostat had significantly lower bioluminescence intensity (RLU) than control mice (P = 0.0068) at the primary tumor site (fat pad). We also measured cell infiltration into the lungs and saw that entinostat treatment significantly reduced lung colonization of cells (P = 0.0461).

We repeated the imaging at 8 weeks after inoculation. Entinostat treatment was still effective at controlling the growth of tumors at primary site (P = 0.0326; data not shown). Next, the primary tumors were surgically excised and chest area of the mice was imaged again. In the control group, the 231/Luc cells had colonized in the lungs, whereas entinostat treatment significantly reduced lung colonization (P = 0.0303; Fig. 5B, Supplementary Fig. S8). These results suggest that entinostat treatment can reduce the tumor-initiating ability of 231/Luc cells as well as the ability of the 231/Luc cells to colonize and metastasize (Supplementary Fig. S9). Next, the excised tumors were digested to produce a single-cell suspension and 10,000 cells were plated in nonadherent...
conditions to assess the mammosphere-forming ability of the cells. The cells from the entinostat-treated tumors formed less mammospheres than the vehicle-treated control group (Fig. 5C).

Entinostat reduces formation of patient-derived tumor xenografts

The experiment was also repeated using patient-derived xenografts (PDX). Br-001 tumors collected from a TNBC patient's discarded tumor tissue were inoculated subcutaneously (Fig. 6A) or in the MFP (Fig. 6B) in ovariectomized NSG mice. The mice received entinostat from the day of inoculation for 8 weeks, when the mice were euthanized. Treatment with entinostat significantly reduced formation of Br-001 tumors. Furthermore, we examined the entinostat-treated tumors for expression of TIC markers using Western blotting (Fig. 6C) and qRT-PCR (Fig. 6D). We observed that expression of TIC markers such as Bmi-1, Nanog, Oct-4, and β-catenin was significantly downregulated in entinostat-treated tumors. We also observed that entinostat treatment of Br-001 tumors resulted in significantly reduced expression of miRNA-181a (data not shown).

Discussion

Mortality following a breast cancer diagnosis is not due to the primary solid tumor but due to the development of metastasis. Metastasis is a complex multistep process that requires tumor cells to possess the ability to invade local stromal microenvironment and intravasate into the circulation, survive the journey through the circulation, extravasate and invade a foreign tissue, and undergo metastatic outgrowth at a new distant site such as lungs, bone, liver, and brain. EMT is a cellular program activated by cancer cells that enables them to accomplish most of the steps of the metastatic cascade (10, 28). The last step of the cascade of colonization requires the cells to possess a tumor-initiating trait. Growing number of reports have shown that cancers contain a small subset of cells within the tumor with the ability to regenerate the tumor; these cells are termed GSCs or TICs. These cells have the ability to produce unlimited number of progenitor cells, which may lack this self-renewal capacity, but can form the bulk of the tumor. Furthermore, activation of EMT phenotype leads to activation of TIC traits, which may result in more cells from the tumor dedifferentiating into TICs (8). This connection between EMT and epithelial TICs indicates that the EMT could be doubly dangerous: by imparting mesenchymal traits to carcinoma cells and generating cellular traits associated with high-grade malignancy, including motility, invasiveness, and a resistance to apoptosis; the cells are primed for metastatic dissemination (10, 28). In addition, by affording the trait of self-renewal to carcinoma cells, EMT generates cancer cells that are capable of generating large colonies of cancer cells that form macroscopic and clinical metastases. This makes the TICs the ultimate cause of metastatic formation was assessed 8 weeks later with bioluminescence imaging. Entinostat treatment significantly reduced formation of lung metastasis (P = 0.0303). C, ex vivo mammosphere formation from tumors of mice treated with entinostat: 231Luc cells were inoculated in fat pads of NSG mice and allowed to form tumors with or without treatment with entinostat (2.5 mg/kg/d). Tumor formation was assessed 8 weeks later with bioluminescence imaging.
outgrowth and a critical target for future therapeutic interventions. In our previous study, we showed that HDAC inhibitor entinostat has the ability to reverse EMT by reversing the epigenetic repression of E-cadherin promoter. In this current study, we show that reversal of EMT following entinostat was accompanied by inhibition of TICs.

Entinostat treatment reduced the CD44\textsuperscript{high}/CD24\textsuperscript{low} cell population in several TNBC cell lines. Furthermore, entinostat treatment reduced the formation of mammospheres \textit{in vitro}, reduced tumor formation \textit{in vivo}, and inhibited metastatic outgrowth in the lungs. The results obtained in human cell lines were also corroborated in human PDX. This suggests that HDAC inhibitor entinostat may help in reducing the formation of new metastasis.

HDAC-dependent histone acetylation may be required to repress epithelial genes such as E-cadherin during EMT (5, 29). Furthermore, HDACs can affect the activity of non-histone proteins such as HIF-1α, which have been implicated in drug resistance, EMT, and development of TICs (30, 31). Thus, HDAC inhibitors have a potential to affect EMT and TICs. HDAC inhibitor TSA was able to reduce CSC population in head and neck cancer cells, although it did cause increase in EMT traits (32). Moreover, HDAC inhibitors valproic acid and SAHA (vorinostat)
have been shown to induce TIC phenotype in breast cancer cells (33). However, all 3 of the above-mentioned HDACis block classes I and II of HDACs, whereas entinostat is a class I specific HDACi. As such, it is important to study the role of specific HDACs that may be regulating EMT and TICs to devise better therapeutic options. Furthermore, miRNAs can also play a role in regulating TICs and/or EMT, which can be exploited as either therapeutic agents or prognostic tools.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: P. Shah, G. Sabnis

Development of methodology: P. Shah

Acquisition of data (provided animals, acquired and managed patients, facilities, etc.): A. Schech, A. Kazi, S. Yu, P. Shah, G. Sabnis

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A. Schech, A. Kazi, P. Shah, G. Sabnis

References

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