Original Article
mTOR inhibitor AZD8055 inhibits proliferation and induces apoptosis in laryngeal carcinoma

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Abstract: The mammalian target of rapamycin (mTOR) kinase forms two multiprotein complexes, mTORC1 and mTORC2, which regulate cell growth, survival, and autophagy. Allosteric inhibitors of mTORC1, such as rapamycin, have been extensively used to study tumor cell growth, proliferation, and autophagy but have shown only limited clinical utility. Here, we describe AZD8055, a novel ATP-competitive inhibitor of mTOR kinase activity, against all class I phosphatidylinositol3-kinase (PI3K) and other members of the PI3K-like kinase family. The study was to determine the effect of AZD8055 on proliferation and apoptosis on Hep-2, a human laryngeal cancer cell line and to investigate the underlying mechanism(s) of action. Hep-2 cells were treated with AZD8055 for 24, 48 or 72 h. MTT was used to determine cell proliferation. Rhodamine 123 and TUNEL staining were used to determine mitochondrial membrane potential and cell apoptosis analyzed by fluorescence-activated cell sorting (FACS). Protein expressions were examined by western blotting. Treatment with AZD8055 inhibited proliferation and induced apoptosis in Hep-2 cells in a dose- and time-dependent manner. During the prolonged treatment with AZD8055, AZD8055 inhibits the mammalian target of rapamycin mTOR. Further experiments showed which signaling cascade p-4EBP1 and substrate EIF4E as well as downstream proteins were down regulated. Furthermore, our study showed that the expression profiles of various BH3-only proteins including Bid, Bad, and Bim, apoptosis regulatory protein cleaved caspase3 was up regulated in a time-dependent manner in Hep-2 cells treated with AZD8055. Thus, in vitro, AZD8055 potently inhibits proliferation and induces apoptosis in head and neck squamous cell carcinoma.

Keywords: mTOR, AZD8055, Hep-2, apoptosis, laryngeal carcinoma

Introduction

Laryngeal carcinoma (LC) is one of the most prevalent malignant tumors in the head and neck area. Approximately a global population of 190,000 people are diagnosed this disease per year [1], more importantly, the number is increasing year by year [2]. Therefore, LC is still the major cause of cancer-related death, which owns significant threat on human’s health on a worldwide scale. Since 1873, after Billroth performed the very first total laryngectomy, surgical treatment of LC has been developing for more than 100 years. However, until now, advanced LC treatment is still a huge challenge. With surgery and post-surgical adjuvant radiotherapy or chemotherapy, only less than 60% of the patients achieved 5-year survival [3-5]. In addition, surgery might lead to complete or partial loss of swallowing and vocal function, so many patients have to maintain a tracheal cannula on a long-term basis due to laryngeal stenosis after surgery; such problems have impaired their quality of life remarkably [6]. Therefore, we are seeking for a promising treatment strategy for the treatment of middle and advanced stage of LC. A promising treatment strategy should ensure treatment efficacy, reduce treatment-related toxicity reaction and improve quality of life. More importantly, these aspects have been climbed into the top priority consideration. Thus, developing an effective drug is of utmost importance.

Abnormal signaling pathways play crucial roles in the pathogenesis and progression of cancer...
The PI3K/AKT/mTOR signaling axis is widely recognized as a critical mediator of cancer-cell survival and resistance to therapeutic agents. Mammalian target of rapamycin (mTOR) is a serine/threonine protein kinase. Mitogenic signals are transmitted to mTOR via PI3K and AKT [8, 9], which forms two distinct multiprotein complexes, mTORC1 and mTORC2. mTORC1, the sensitive target of rapamycin, phosphorylates downstream targets of S6K1 (p70S6K1) and 4E-BP1 which control the cap-dependent protein translation, while mTORC2 is insensitive to rapamycin, and its main substrates are AKT and related kinases [9, 10]. Furthermore, mTOR functions as a sensor of mitogen, energy and nutrient levels, and is a central controller of cell growth and a negative regulator of autophagy [11].

In normal physiology, mTOR activity is tightly regulated: Phosphorylation of S6K by mTOR induces the degradation of IRS1, decreasing insulin-driven AKT activity and, therefore, mTOR activity [12, 13]. The activity of mTOR is also regulated by the energy sensor AMP-activated protein kinase, which stabilizes the TSC1/TSC2 complex and decreases mTOR activity [14]. In cancers, increased signaling through mTOR can be due to enhanced upstream signaling through activating mutations in receptor tyrosine kinases or PI3K, or through loss-of-function mutations in PTEN or LKB1, all of which are associated with an increase in phosphorylated AKT [15]. mTORC1 controls essential functions in cells, such as protein translation, cell growth, and autophagy. Rapamycin is an allosteric inhibitor of mTORC1 that does not directly affect mTORC2, except in a small subset of cell lines where, after prolonged exposure, it decreases mTORC2 function by decreasing mTOR protein levels [16]. Perhaps surprisingly, considering its major effect on cell growth and autophagy in yeast, rapamycin has limited effect on overall protein synthesis, induces only partial growth inhibition, and is a poor inducer of autophagy in cancer cell lines [17, 18].

Recent reports using ATP-competitive inhibitors of mTOR kinase suggest that allosteric inhibition of mTORC1 by rapamycin does not recapitulate inhibition of mTOR kinase activity [19-21]. In particular, a decrease in phosphorylation of 4E-BP1 at position 37 and 46 is observed with mTOR kinase inhibitors but not with rapamycin [20]. This leads to a greater inhibition of cap-dependent translation compared with rapamycin. Additionally, inhibition of mTORC1 by rapamycin and analogues results in the release of the negative feedback loop between S6K and IRS1, leading to hyperactivation of AKT [22]. In contrast, mTOR kinase inhibitors inhibit AKT phosphorylation. Rapamycin and analogues have only shown clinical activity as a single agent in a limited number of tumor types [23]. Cloughesy and colleagues [24] showed that in PTEN-deficient patients with glioblastoma, hyperactivation of AKT after rapamycin treatment was associated with shorter time to progression, suggesting that the absence of AKT inhibition through mTORC2 targeting limited antitumor activity.

Historically, several molecules inhibiting both PI3K and mTOR, such as LY294002 or PI-103, have been used as “probe compounds” to investigate the biology of the PI3K pathway. Novel agents with dual PI3K and mTOR pharmacology, such as NVP-BEZ235 and XL-765, are now in clinical development in oncology [25]. Given that feedback and cross talk of signaling networks significantly influence the efficacy of cancer therapy. Recently, new selective ATP-competitive mTOR kinase inhibitors (mTOR-Kis) have been developed that are able to completely suppress both mTORC1/C2 complex-mediated signaling, thereby suppressing the feedback activation of AKT [19, 26]. In addition, in vitro data have been reported for the ATP-competitive small-molecule inhibitors of mTOR kinase activity PP242 or Torin1. Herein, we described the preclinical pharmacology of AZD8055, a first-in-class orally available, potent, and specific inhibitor of mTOR kinase activity in vitro. Understanding the molecular mechanisms of AZD8055 on Hep-2 cells may facilitate the development of strategies.

Materials and methods

Chemicals

(5-{2,4-Bis[(3S)-3-methylmorpholin-4-yl]pyrido [2,3-d]pyrimidin-7-yl}-2-methoxyphenyl)methanol (AZD8055) was purchased from Chemietek (Indianapolis, IN, USA). For in vitro studies, AZD8055 was prepared as 200 mg/L stock solution in DMSO and stored under nitrogen. All antibodies were obtained from Proteintech group, USA.
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**Cell culture**

The Hep-2 human laryngocarcinoma cell line obtained from the American Type Culture Collection (ATCC). Hep-2 cells were incubated in RPMI 1640 medium (Gibco, Grand island, New York, USA) supplemented with 5% fetal bovine serum (FBS), 100 IU/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified atmosphere containing 5% CO₂ in the air.

**Cell proliferation assay**

The effect of AZD8055 on proliferation of Hep-2 cells was measured by MTT assay. 5×10³ cells per well were grown in 96-well plates overnight. Cells were incubated with 0.8, 2.6, 8, 26, 80 and 260 μg/L AZD8055 for 24, 48 or 72 h, then 10 μl of MTT (1 mg/ml) reagent was added to each well, and the cells were further incubated at 37°C for 4 h.

After 4 h, media were removed and 100 μL DMSO was added to each well to dissolve purple crystals of formazan. Spectrophotometric absorbance at 490 nm was read on a 96-well plate reader. Cell cytotoxicity was thus expressed as the relative viability against control cells treated only with the solvent. Cell survival rate was detected by 3 repeated measurements.

**Mitochondrial membrane potential (Δψm) assay**

To observe the changes in Δψm, fluorescence microscopy was performed with mitochondrial probe rhodamine 123. Briefly, Hep-2 cells treated with AZD8055 (0, 8, 26, and 80 μg/L) for 48 h were harvested, washed in PBS and then cell samples were incubated with 10 μg/L rhodamine 123 (Sigma-Aldrich, St. Louis, MO, USA) at 37°C in the dark for at least 15 min and then photographs were taken under fluorescence microscopy (Olympus, Tokyo, Japan) using a fluorescein filter and a 200× objective.

**Immunohistochemistry**

A total of 81 LC patients (57 males and 24 females) that received laryngeal cancer resection at the Second Affiliated Hospital of Jilin University were enrolled in this study. Their ages ranged from 42 years to 70 years with an average of 56. All patients were confirmed pathologically with LC. They had not received chemotherapy or radiotherapy and had complete clinical, pathological, and follow-up data. 21 para-LC non-carcinous tissue specimens were collected randomly (1 cm within the border of the tumor and pathologically excluded from cancer cell infiltration). Clinical classification and staging were carried out in line with the tumor, neck, and metastasis (TNM) classification scheme by the Italian-international Union for the Control of Cancer. According to differentiation, 43 cases were well differentiated, 23 well-moderately differentiated, and 15 were undifferentiated. This study was conducted in accordance with the declaration of Helsinki. This study was conducted with approval from the Ethics Committee of the Second Affiliated Hospital of Jilin University. Written informed consent was obtained from all participants. Those for immunohistochemistry were fixed, dehydrated, and embedded routinely, then for determination of mTOR, Elf-4E and p-4EBP1 expression. Paraffin-embedded tissue sections of tumors and normal tissues were used for immunohistochemical studies. Immunostaining was carried out using Vectastain Elite ABC avidin/biotin staining kit (Vector Laboratories Inc., Burlingame, CA, USA). The criteria for this assay results are as follows: the percentage of cells positively stained in each section were categorized as follows: intensity of staining, none (0), mild (2), strong (3) and the percentage of positive staining, <5% (0), 5-25% (1), 25-50% (2), >50% (3) of cells. The combination of the intensity and the percentage of staining resulted in the following score: 0-1, negative (-) and 2-6, positive (+) (original magnification, 400×).

**Western blot analysis**

Hep-2 cells (1×10⁵/well) were seeded in 6-well plates. Cells were treated as described above. The media was aspirated, and cells were washed with cold PBS. The cells were scraped and washed twice by centrifugation at 500×g for 5 min at 4°C. The pellet was resuspended in lysis buffer supplemented with proteases and phosphatase inhibitors and incubated for 1 h at 4°C. The lysate was collected by centrifugation at 14,000×g for 40 min at 4°C, and the supernatant (total cell lysate) was stored at -80°C. For Western blot analysis, 30 μg proteins was resolved over 12% polyacrylamide gels and transferred to a nitrocellulose membrane. The blot was blocked in blocking buffer (5% non-fat
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dry milk/1‰ Tween 20 in TBS) for 1 h at room temperature, incubated with appropriate polyclonal primary antibodies in blocking buffer overnight at 4°C. The blot was then incubated with appropriate horseradish peroxidase conjugated secondary antibody and detected by enhanced chemiluminescence and autoradiography using X-ray film. β-actin was detected on the same membrane and used as a loading control.

**Immunofluorescence staining**

Nuclear DNA in treated cells contained in 24-well plates was visualized by staining with the DNA-specific dye Hoechst 33258 (Invitrogen, Carlsbad, CA, USA) at a final concentration of 5 μg/ml. Cells were observed immediately with filters for blue fluorescence.

Cells were fixed with 4% paraformaldehyde in PBS (15 min, room temperature), blocked for 30 min with 10% normal goat serum in PBS containing 0.1% saponin, and incubated with primary antibodies diluted in blocking buffer (1:500) for 1 h at room temperature. After washing with PBS, cells were incubated with Alexa 488 Fluor-labeled secondary antibodies (diluted 1:5,000 in blocking buffer) for 1 h and washed with PBS. Thereafter, the samples were counterstained with PPD mounting medium containing DAPI and inspected with a confocal microscope (Olympus, Tokyo, Japan).

Treated cells grown on 8-well chamber slides were washed twice with PBS, and then fixed with 4% paraformaldehyde in PBS for 60 min at room temperature. After permeabilization with 0.1% Triton-X-100 (Sigma-Aldrich) for 2 min on ice, cells were stained with TUNEL reagent (Promega Corp., Madison, WI, USA) for 60 min at room temperature in the dark for in situ apoptosis detection. Coverslips were mounted in Prolong Gold anti-fade reagent with DAPI (Molecular Probes) and inspected with a confocal microscope (Olympus, Tokyo, Japan).

**Results**

**Expression of mTOR, Elf-4E, p-4EBP1 and its relationship with clinical parameters in LC**

Immunohistochemistry revealed that 50 out of the 81 (62%) tumor tissues were mTOR, Elf-4E, p-4EBP1-positive, while 8 out of the 81 (10%) clear surgical margin tissues were positively stained (P<0.01, Figure 1). The mTOR, Elf-4E and p-4EBP1 protein expression levels were significantly lower in the normal squamous epithelium tissues than those in the LC tissues (Figure 1). The positive expression rates of mTOR, Elf-4E, p-4EBP1 in patients with poorly differentiated LC was noticeably higher than that in patients with well differentiated LC (P<0.01), whereas no significant differences were observed with regard to gender, age, and growth sites (P>0.05).

**The mTOR inhibitor AZD8055 inhibits mTOR and the downstream proteins of EIF4E and p-4EBP1 in Hep-2 cells**

To investigate the molecular mechanism of AZD8055 on Hep-2 cells, we examined the effect of AZD8055 on the activity of mTOR, an important component of the PI3K/Akt/mTOR signaling pathway that plays a crucial role in the pathogenesis of Laryngeal squamous cell carcinoma [27]. Our data showed that the mTOR and the downstream proteins of EIF4E and p-4EBP1 were significantly reduced by AZD8055 in a concentration-dependent manner in Hep-2 cells (Figure 2), which are the best characterized targets of the mTOR complex cascade. These findings suggest that AZD8055 may inhibit Hep-2 cell proliferation by repressing the mTOR pathway.

To determine the effects of AZD8055 treatment on the expression of apoptosis-associated genes, Western blot assays were performed. We next examined the expression of some apoptosis-related genes including ERK, Bim, Bid, Bad, p-Bad, Caspase3, and Bcl-2. We observed that the expressions of Bim, Bid, p-Bad, and Caspase3 proteins were markedly elevated when mTOR was down regulated in Hep-2 cells, but the levels of Bcl-2 and ERK proteins were dramatically down regulated (Figure 3).

**AZD8055 inhibits proliferation of Hep-2 cells**

Using MTT assay, the cytotoxicity of AZD8055 in Hep-2 cells was shown in Figure 4. After exposure to AZD8055 (8, 26 and 80 μg/L) for 24 h, 48 h and 72 h, inhibition of cell viability by AZD8055 happened in a dose-dependent manner. Reduction in cell viability with AZD8055 treatment at concentrations 8 to 80 μg/L
increased from 11.5% to 61.9%, with significant difference beyond the concentration of 8 μg/L as compared to control group (P<0.05 or P<0.01, Figure 4A).

**AZD8055 causes apoptosis in Hep-2 cells**

After exposure to AZD8055 at the concentration of 80 μg/L, Hep-2 cells were floating and round in shape with shrinkage of the cell membrane under an ordinary inverted microscope (Figure 4I).

Mitochondria play an essential role in the propagation of apoptosis and mitochondrial dysfunction usually triggers specific cellular signaling to induce apoptosis. Increasing evidence suggests that the disruption of mitochondrial integrity is a critical step occurring in cells undergoing apoptosis and a decreasing mitochondrial membrane potential is associated with mitochondrial dysfunction. Therefore, loss of mitochondrial membrane potential is an important event during the mitochondrial-mediated apoptosis. So we investigated whether AZD8055 could induce loss of mitochondrial membrane potential in Hep-2 cells by measuring mitochondrial membrane polarity using mitochondrial probe rhodamine 123. After treatment with various concentrations of AZD8055 for 48 h, as showed in Figure 4B, the fluorescence intensity was significantly decreased in the Hep-2 cells treated with AZD8055 compared with the control groups, respectively, suggesting mitochondrial membrane depolarization. This observation suggests that the mitochondria are involved in AZD8055-induced apoptosis.

Immunofluorescence with TUNEL staining was simultaneously performed to detect apoptotic morphology alteration of individual Hep-2 cells in the cell population. Apoptotic cells demonstrating nuclear condensation and DNA fragmentation can be detected by TUNEL staining and fluorescence microscopy. Obvious differences were observed in the nuclei of AZD8055-treated and untreated Hep-2 cells after staining with TUNEL. Fragmented DNA in nuclei was revealed as a green fluorescence signal, treatment with 80 μg/L of AZD8055 for 48 h showed predominantly increased DNA fragmentation compared with the control group (Figure 4B).
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Figure 2. AZD8055 inhibits rapamycin-resistant functions of mTOR, and the downstream proteins of EIF4E and p-4EBP1. (A) Inhibition of mTOR, EIF4E and p-4EBP1 in Hep-2 cells, exposed for 48 h to increasing concentrations of AZD8055, determined by immunoblotting of whole cell lysates with the indicated antibodies. (B) Quantitative data from A by densitometry using Quantity One Software (Bio-Rad). *indicates significant difference as compared to
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Discussion

mTOR is a protein kinase involved in the PI3K/Akt signaling pathway with a central role in the control of cell growth, proliferation, metabolism, survival and angiogenesis [28, 29]. It has been well established that the PI3K/Akt/mTOR signaling pathway plays a crucial role in cancer development. Multiple studies have reported that this signaling pathway is aberrantly activated in multiple types of cancer [30, 31].

As a result, the frequent dysregulation of this signaling pathway in cancer make it an important target in the treatment of multiple types of malignant tumors [32, 33].

Inhibitors of the mTOR pathway have been used individually and in combination with a variety of cancer therapeutic agents, including chemotherapy, IGF-IR inhibitors, and trastuzumab [34-36].

In recent years numerous agents targeting the Ras/Raf/MEK or PI3K/AKT/mTOR pathways have entered clinical development and dual targeting of these pathways is now been invested clinically. It had been generally accepted and shown, that direct inhibition of targets subject to mutational activation is effective and this expectation has to some extent translated to cases where the mutationally activated pathway is targeted at a non-mutated node (e.g. MEK inhibitors and Rapalogues). However, in some settings these initial observations have been shown to be more complicated and the dependency of cancer cells for survival on both of these pathways is becoming better understood. In this study we report that targeting of the mTOR pathways, Furthermore, pharmacodynamic analysis of tumor cells demonstrated MAPK and mTOR pathway suppression, enhanced apoptotic signaling and the modulation of MEK functional output pathways following exposure to this agent.

ERK has been the best characterized MAPK and the Raf-MEK-ERK pathway represents one of the best characterized MAPK signaling pathways. Then the activated ERKs translocate to the nucleus and transactivate transcription factors, changing gene expression to promote growth, differentiation or mitosis. Interfering with components of the ERK signaling pathway

Figure 3. AZD8055 induces apoptosis-related genes expression. Cellular proteins were extracted from Hep-2 cells treated with AZD8055 at 0 to 80 μg/L for 48 h. A: Expression of ERK, bim, bid, p-bad, bad, bcl-2 and cleaved-caspase3 were determined by western blot. B: Quantitative data from a by densitometry using Quantity One Software (Bio-Rad). *indicates significant difference as compared to the control group (*P<0.05, **P<0.01).
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with dominant negative mutants or antisense constructs for raf-1 or ERK1 shows significant inhibition of cell proliferation. On the contrary, stimulating ERK1 activity results in enhanced cell proliferation [37].

The present study described AZD8055 as a potent mTOR inhibitor involved in multiple cellular responses, including inhibiting proliferation and inducing apoptosis in human cervical cancer HeLa cells. Our study suggested that AZD8055-mediated human laryngeal cancer cell proliferation inhibition may occur through deregulating mTOR activity and up-regulating the expression of Bim. A previous report showed that administration of histone deacetylase inhibitors (HDACis) cause apoptosis through activation of the proapoptotic protein Bim [38, 39].

In that study, the administration of mTOR kinase inhibitors (mTORKi) was seen to enhance HDACi-triggered upregulation of Bim thus resulting in apoptosis in hepatocellular carcinoma (HCC) cells. These results indicated that the deregulation of the mTOR signaling pathway may be involved in the AZD8055-induced antiproliferative effects and apoptosis in human laryngeal cancer cells. In conclusion, the present study provides a novel antitumor mechanism of AZD8055 in human laryngeal cancer cells.

Our results showed that the levels of mTOR protein was down regulated in Hep-2 cells after AZD8055 treatment, indicating that AZD8055 can inhibit the expression of mTOR in Hep-2 cancer cells. Our immunohistochemical examination showed that mTOR, Elf-4E and p-4EBP1-

Figure 4. A: AZD8055 inhibits cell proliferation in Hep-2 cells. An MTT assay was applied to examine the relative proliferation rate of Hep-2 cells after treatment with AZD8055 for 24, 48, 72 and 96 h. During prolonged treatment with AZD8055, the relative cell proliferation rate gradually decreased. The difference between each group is statistically significant. *P<0.05, **P<0.01, compared with the control. B-E: Analysis of mitochondrial membrane potential by Rhodamine 123 staining in Hep-2 cells under the fluorescence microscope. F-I: Morphological images of Hep-2 cells in the presence of increasing concentrations of AZD8055 for 48 h. J-M: TUNEL staining. The apoptotic cells were marked by green fluorescence.
positive cells were increased with the poor differentiation. The TUNEL assay showed that apoptosis in the AZD8055 treatment group was significantly increased. These findings reveal that the mTOR inhibitor AZD8055 can exert a potent anti-tumor effect in vitro by suppressing proliferation and promoting apoptosis of cancer cells. Furthermore, our results showed that the expression of pro-apoptotic factors including Bax and Caspase3 was markedly up regulated in the AZD8055 treatment group. However, anti-apoptotic factor Bcl-2 was reduced significantly. Studies have demonstrated that mTOR can up regulate the expression of several anti-apoptotic proteins, including Bcl-2 and Bcl-xL, which are key components of mitochondrial apoptotic pathways. Therefore, the mTOR inhibitor AZD8055 induced apoptosis of cancer cells may be caused in part by the activation of mitochondrial apoptosis pathways.

Recently, it has been shown that phosphorylation of Bad at Thr-201 by JNK1 promotes glycolysis through activation of phosphofructokinase-1 [40]. There is growing information in the literature that the BH3-only protein plays an essential role in cytokine deprivation induced apoptosis in mast cells [41]. BH3-only members may initiate apoptosis by directly binding to the essential cell-death mediators Bax and Bak. Alternatively, they can act by engaging their pro-survival Bcl-2-like relatives [42].

In this study, we detected the member of BH3-only proteins Bid, Bim, Bad, and p-Bad. The result showed that the expression of cleaved-caspase3, Bid, Bim, and p-Bad were increased in AZD8055 treatment Hep-2 cells. We demonstrated that BH3-only proteins play critical roles in AZD8055-induced apoptosis. This study indicated that inhibit of mTOR can induce expression of the BH3-only proteins Bid, Bim, Bad, p-Bad, BH3-only proteins further engage directly and activate Bax, this led to changes in the mitochondrial membrane potential, cytochrome C release into the cytosol, and enhanced caspase-3 activities, thereby inducing apoptosis.

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Disclosure of conflict of interest

None.

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