A Journal of the Gesellschaft Deutscher Chemiker

Angewandte International Edition GDCh Chemie

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Accepted Article

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To be cited as: *Angew. Chem. Int. Ed.* 10.1002/anie.201902347
Angew. Chem. 10.1002/ange.201902347

Link to VoR: http://dx.doi.org/10.1002/anie.201902347
http://dx.doi.org/10.1002/ange.201902347

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10.1002/anie.201902347

COMMUNICATION

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A Single Atom Change Facilitates the Membrane Transport of Green Fluorescent Proteins in Mammalian Cells

Surendar R. Jakka, Vijayakumar Govindaraj and Govindasamy Mugesh*

Abstract: *Direct delivery of proteins into mammalian cells is a challenging problem in biological and biomedical applications. The most common strategies for the delivery of proteins into the cells include the use of cell-penetrating peptides or supercharged proteins. Herein, we show for the first time that a single atom change, hydrogen to halogen, at one of the tyrosine residues can increase the cellular entry of 28 kDa green fluorescent protein (GFP) in mammalian cells. The protein uptake is facilitated by a receptor-mediated endocytosis and the cargo can be released effectively into cytosol by co-treatment with the endosomolytic peptide ppTG21.*

The transport of macromolecules across the cell plasma membrane is a major challenge in biomedical applications, including the development of drug candidates,[1] and therefore, the biological applications of many exogenous proteins are restricted to extracellular targets.[2] The inability of almost all macromolecules to spontaneously enter cells led to the development of strategies for the delivery into mammalian cells. These methods are generally based on cationic cell-penetrating peptides (CPPs),[1a,3] antibodies,[4] nanoparticles,[5] receptor ligands,[6] and virus-like particles.[7] Another approach involves the use of supercharged proteins (SCPs) for the delivery of functional macromolecules.[8] Unfortunately, in most of these processes, large amounts of purified proteins are required for a reasonable cellular delivery. Although the attachment of cell permeable peptides enhances the cellular delivery of proteins, such modifications alter the protein function inside the cells.

Recently, we reported that the introduction of halogen atoms facilitates the active transport of small molecules across the plasma membrane in mammalian cells.[9] The cellular uptake of some of the commonly used fluorescent probes containing naphthalimide, coumarin, BODIPY and pyrene moieties has been shown to be enhanced significantly upon introduction of iodine atoms. Although this study provided a general strategy for enhancing the cellular uptake of small organic molecules, it is not known whether such strategy can be used for the delivery of difficult cargo such as proteins. In this paper, we report that a single atom change, hydrogen to halogen (Cl, Br or I), at one of the tyrosyl residues facilitates the delivery of a green fluorescent protein (GFP) having a molecular weight of 28 kDa into mammalian cells and the highest cellular uptake is observed for GFP having an iodine atom.

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Supporting information and the ORCID identification number(s) for the author(s) of this article can be found under: https://doi.org/10.1002/anie.2019xxxx.

The halo variants of GFP (EmGFP, Section 1.3, SI) were synthesized biochemically by expanding the genetic code of *E. coli* with 3-halo-L-tyrosine. Briefly, the *Methanococcus jannaschii* tyrosyl-tRNA synthetase and tRNA pair evolved for 3-iodo-L-tyrosine[10] was used to incorporate 3-halo-L-tyrosine to EmGFP using an amber codon (UAG) as shown in Figure 1A. The surface tyrosyl residue at 39-position of EmGFP was selected for the single atom modification to facilitate a favourable interaction between the halogen atom and the cell plasma membrane. Also, the introduction of heavier halogen atoms on the surface may not alter the secondary structure of the proteins. For the expression of the wild-type GFP, a construct having no amber codon at the 39-position was used. The recoded *E. coli* strain C321ΔA.exp was used for the protein expression. The fluorescence microscopy study showed significant expression of all four proteins in *E. coli* cells (Figure 1B). After lysing the cells,

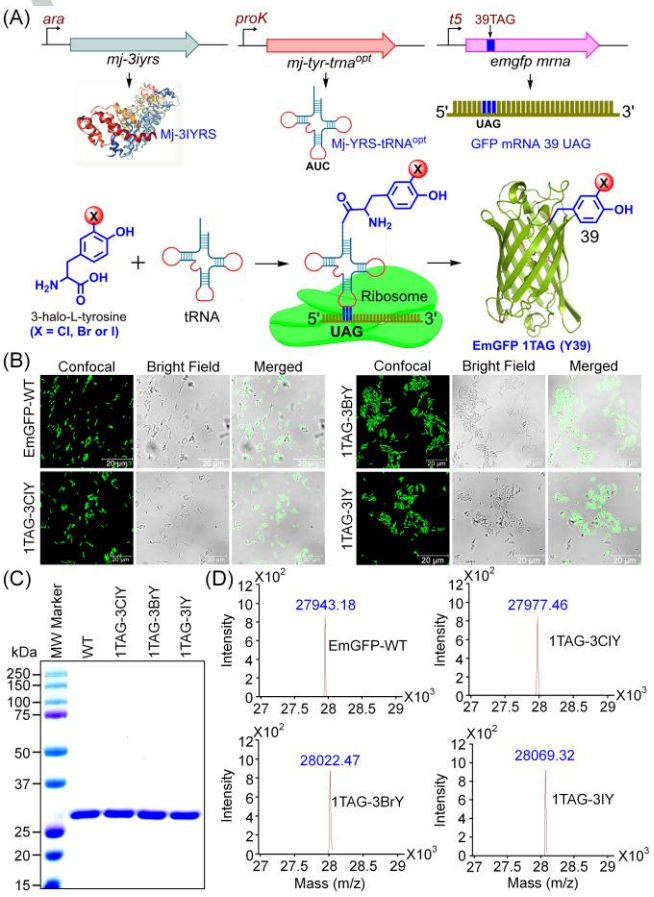

Figure 1. (A) The strategy for site-specific incorporation of 3-halo-L-tyrosine at 39-position of EmGFP utilizing amber stop codon suppression by an engineered orthogonal aaRS/tRNA pair. The proteins are represented as 1TAG-3ClY, 1TAG-3BrY or 1TAG-3IY, respectively. (B) Confocal images confirming the expression of 1TAG-3ClY or -3BrY or -3IY EmGFP in *E.coli*. (C-D) Confirmation of purified proteins by SDS-PAGE and mass spectrometry.

the histidine-tagged proteins were purified by using an affinity chromatography. The purity of the proteins was confirmed by the SDS-PAGE (Figure 1C) and the successful introduction of the halogen atoms into the proteins was confirmed by mass spectrometry (Figure 1D).

The absorption and fluorescence spectra of all four proteins indicated that there is no change in the spectral properties (Figure 2A). No quenching of the fluorescence was observed upon introduction of heavier halogen atoms. This is probably because the 3-halo-L-tyrosine residue is located on the surface of the modified proteins away from the GFP fluorophore. The circular dichroism (CD) studies indicated that the heavier halogen atoms do not alter the secondary structures of the proteins (Figure 2B). As the substitution of halogen atoms into GFP may increase the toxicity, the cell viability was determined by using HepG2 (human liver carcinoma) cells. The toxicity of all three halogenated proteins was found to be almost identical to that of the wild-type protein (Figure 2C), indicating that the substitution of a hydrogen atom with a heavier halogen does not lead to toxicity in mammalian cells. The interesting fluorescent properties of the EmGFPs prompted us to investigate the cellular uptake in mammalian cells. We studied the uptake of the wild-type (WT) as well as the modified proteins by using laser scanning microscopy and fluorescence microplate reader techniques. The HepG2 cells treated with 1 μM concentration of EmGFP-WT for 90 min showed a very low fluorescence (Figure 2D-F and S11-12), indicating that the WT protein is not taken up readily by the cells. The cellular uptake was marginally increased for 1TAG-3ClY, suggesting that the replacement of a hydrogen atom with a chlorine atom at 39-position increases the cellular uptake. A further increase in the uptake was observed for the bromo analogue (1TAG-3BrY) and the amount of protein entered the cells was found to be almost 2 times higher than that of the WT. Remarkably, a much higher uptake (almost 6-fold increase with respect to WT) was observed for 1TAG-3IY, indicating that the iodine atom facilitates the transport of the protein as observed earlier for the iodinated small molecules.

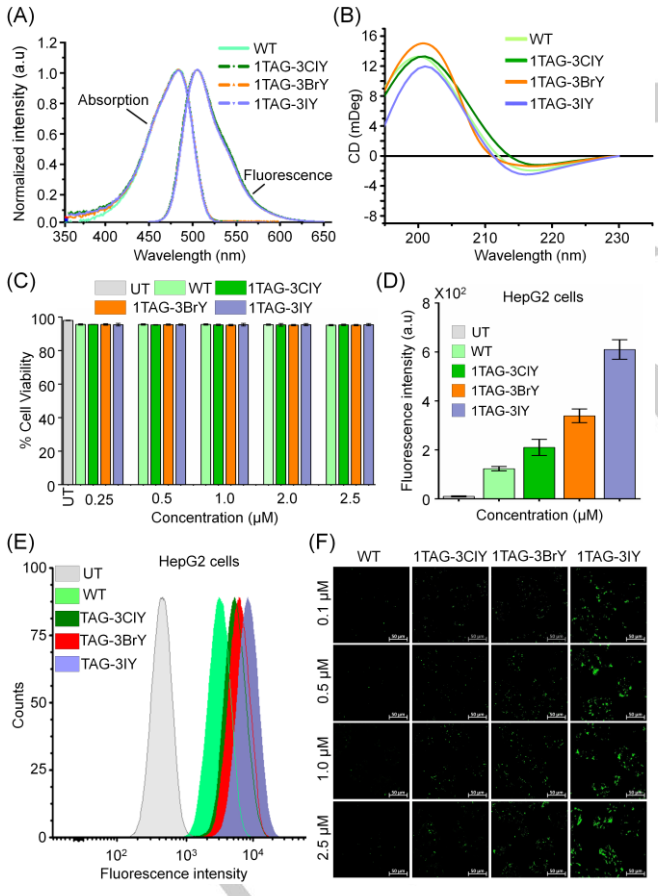

Figure 2. (A-B) Normalized fluorescence intensity and CD spectra of the WT and modified proteins. (C) Cell viability of HepG2 cells incubated with halo-EmGFP (1TAG) for 90 min. (D) The fluorescence measured by a plate reader, (E) Mean fluorescence as determined by flow cytometry and (F) confocal images after 90 min treatment of HepG2 cells with WT and modified EmGFP.

It is known that hydrophobic small molecules such as benzene and gaseous molecules such as O_2 and CO_2 can cross the cell membrane by simple diffusion. However, such simple diffusion through the plasma membrane is extremely difficult for macromolecules. When the cellular uptake studies were carried out at 4 ºC, essentially no fluorescence was observed for all four proteins (Figure 3B), indicating that the WT or the modified proteins do not enter the cells by diffusion. In our earlier studies, we showed that the iodine-containing small molecules such as **1**-**5** (Figure 3A) enter the cells through the monocarboxylate transporter 8 (MCT8),[9] which is a very specific transporter for the iodine-containing thyroid hormone, thyroxine (T4).[11] The 2-iodoanisole moiety serves as an efficient receptor recognition unit for compound **1**-**5** as the iodine atoms can form halogen bonding with the receptor, facilitating their cellular entry.[9,12] As the GFPs, particularly the iodo-GFP having the ability to form halogen bonding with the receptor,[12] may enter the cells through MCT8, we studied the cellular uptake in the presence of silychristin (SY), which is a potent and selective inhibitor of MCT8-mediated uptake.[13] Interestingly, no inhibition of cellular uptake was observed in the presence of SY (Figure 3A,B), indicating that MCT8 is a specific small-molecule transporter and it does not mediate the uptake of proteins such as GFP even when they contain iodine atoms. Further, the cellular uptake was not inhibited by 3-iodo-L-tyrosine (Figure S13).

To understand whether the cellular uptake is an energy-dependent process, we depleted the adenosine triphosphate (ATP) level in the cells by using sodium azide and deoxyglucose in glucose-free medium.[14] The uptake is blocked under these conditions (Figure 3B, S14), suggesting that the proteins are taken up by the cells *via* an energy-dependent pathway, involving either the clathrin-mediated or caveolar endocytosis. As several proteins are transported *via* the clathrin-mediated endocytosis (CME),[15,16] we studied the uptake in the presence chlorpromazine (CPZ), a cationic amphipathic compound that inhibits the CME by depleting clathrin from the plasma membrane.[16a] As observed for SY, no inhibition of cellular uptake was observed in the presence of CPZ (Figure 3A,B, S14), suggesting that a clathrin-independent endocytosis (CIE) is probably responsible for the cellular uptake. Further support for CIE came from the cellular uptake experiments with methyl-β-cyclodextrin (MβCD), which is known to disrupt lipid rafts by removing cholesterol from the plasma membrane.[16] While the uptake of WT protein is not affected, the MβCD treatment

significantly inhibited the uptake of the halogenated GFPs (Figure 3B, S14), indicating that the proteins enter the cells *via* the caveolar endocytosis. The caveolae-mediated internalization was further confirmed by carrying out the uptake experiments in the presence of genistein (GST), a tyrosine-kinase inhibitor known not only to cause local disruption of the actin network at the site of endocytosis, but also to inhibit the recruitment of dynamin II.[16a] In the presence of GST, an almost complete inhibition of the cellular uptake was observed (Figure 3B, S14) confirming that all four proteins are taken up by the cells through the ATP-dependent, caveolae-mediated endocytosis.

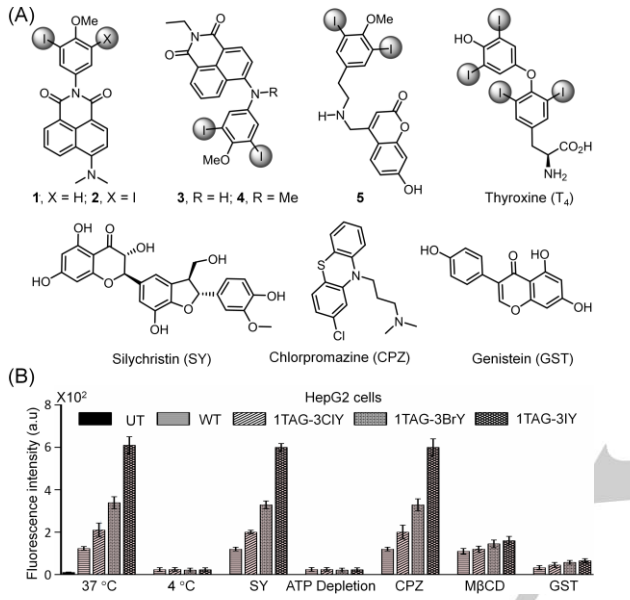

Figure 3. (A) Chemical structures of compounds **1**-**5**, thyroxine and various inhibitors used. (B) The fluorescence measured by a plate reader after 90 min treatment of HepG2 cells with 1 μM of 1TAG-3ClY, 1TAG-3BrY and 1TAG-3IY EmGFP at 37 °C, 4 °C, under ATP depletion or after pre-treatment with MCT8 inhibitor or endocytosis inhibitors. After treatment with the inhibitors, the cells were washed thoroughly with PBS buffer before incubating with EmGFP.

To understand whether the introduction of an additional 3-iodo-L-tyrosine residue at the other end of the EmGFP can increase the cellular uptake by pushing the tail-end through the receptor by forming a halogen bonding, we generated 2TAG-3IY having the iodinated tyrosine residues at 39- and 214-positions by using two amber codons (Figure 4A). The protein was expressed in the recoded *E. coli* strain C321ΔA.exp and the resulting His-Tag protein was purified by an affinity chromatography. The mass spectrometry study confirmed the incorporation of two iodotyrosyl residues (Figure 4D). The protein expression levels and spectral features are quite like that of 1TAG-3IY (Figure 4B-E). Although the secondary structure is not affected by the substitution of two iodotyrosyl residues (Figure 4F), only a marginal increase in the cellular uptake was observed in HepG2 cells (Figure 4G-I, S15), indicating that the initial recognition of the 3-iodo-L-tyrosine (3IY) moiety by the receptor is sufficient for the cellular uptake. The transport under ATP depleted conditions or in the presence of various inhibitors indicated that 2TAG-3IY also enters the cells through caveolae-mediated endocytosis pathway (Figure 5A,B, S16).

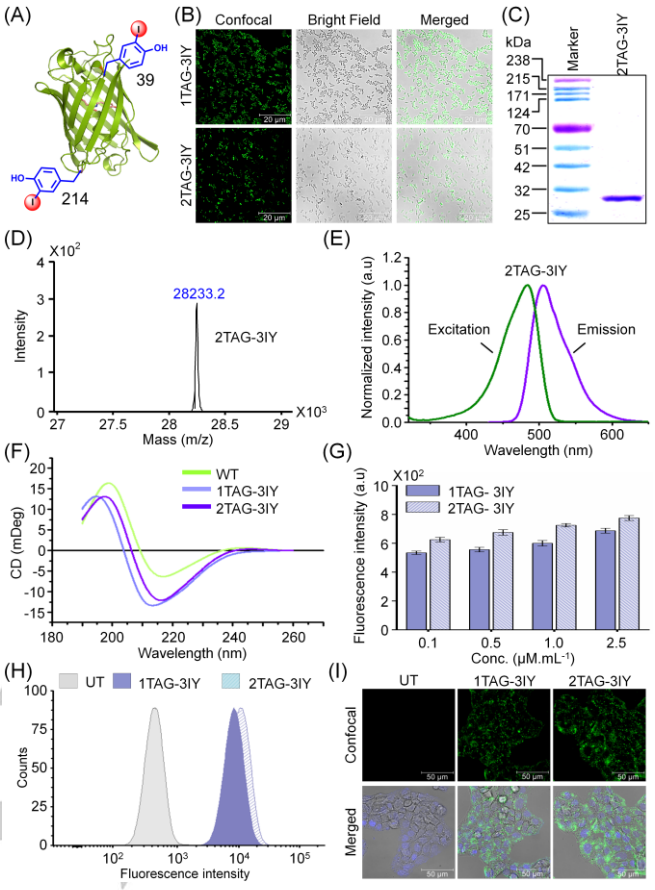

Figure 4. (A) Representation of EmGFP having 3-IY at positions 39 and 214. (B) Confocal images showing the relative expression of 2TAG-3IY and 1TAG-3IY in *E. coli* cells. (C-D) Confirmation of purified 2TAG-3IY by SDS-PAGE and mass spectrometry. (H-I) Mean fluorescence intensity as determined by flow cytometry and the corresponding confocal images after treating HepG2 cells with the proteins for 90 min. DAPI was used for staining the nucleus.

It is known that biomolecules, particularly proteins, that enter the cells by endocytosis pathways get entrapped in endosomes, transported to lysosomes and are subsequently degraded by specific enzymes. The time-dependent cellular uptake experiments indicate that both 1TAG-3IY and 2TAG-3IY rapidly enter the cells through the receptor-mediated endocytosis, but a significant decrease in the fluorescence intensity was observed after 24 h (Figure 5D-E, S17), probably due to degradation of the proteins in lysosomes (Figure 5F). To release the entrapped proteins from the endosomes before their degradation, we co-treated the cells with the histidine-rich 20-mer peptide, ppTG21 (pI 7.7, charge +1.3 @ pH 7.4), which has been reported as a promising endosomolytic agent for plasmid-based gene delivery.[17] Interestingly, a remarkable enhancement in the fluorescence intensity was observed for both 1TAG-3IY and 2TAG-3IY in the presence of ppTG21 after 90 min, and there was no decrease in the fluorescence was observed even after 24 h (Figure 5E, S17). We obtained similar results when the cells were treated with the peptide after 60 min treatment with 1TAG-3IY (Figure S18), indicating the intracellular effect of ppTG21 in the cellular uptake of 1TAG-3IY. These observations confirm that the iodinated proteins are taken up by the cells

preferentially through a receptor-mediated endocytosis and the proteins can be released effectively into cytosol by using endosomolytic agents such as ppTG21.

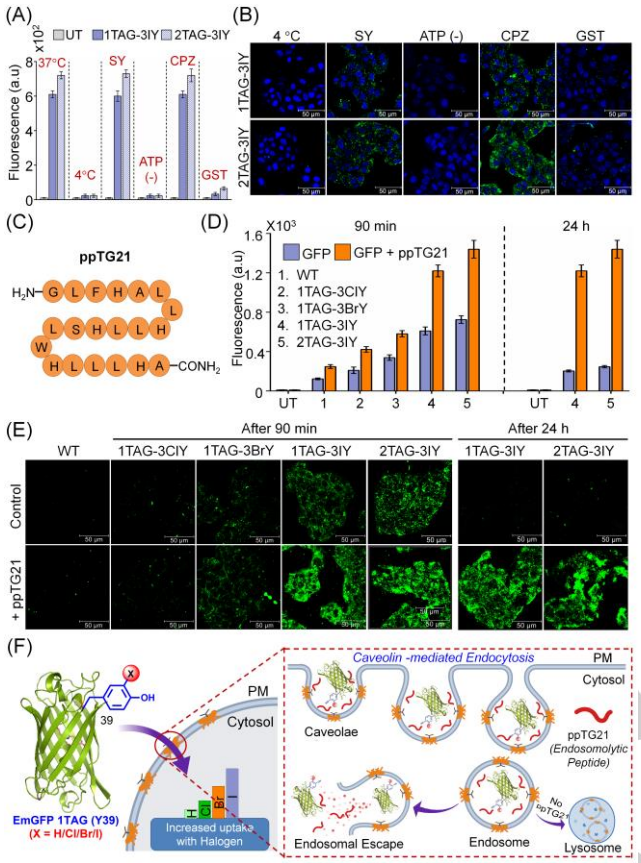

Figure 5. (A-B) The fluorescence measured by a plate reader and the corresponding confocal images after 90 min of treatment of HepG2 cells with 1 μM proteins under various conditions. (C) Amino acid sequence of ppTG21. (D-E) The fluorescence measured by a plate reader and the corresponding confocal images in HepG2 cells after 90 min or 24 h of co-treatment with 1 μM EmGFP and 30 μM ppTG21. (F) A schematic representation of caveolae-mediated cellular uptake and proposed endosomal escape route for EmGFP.

In summary, we showed for the first time that proteins can be transported across the cell plasma membrane by a single atom substitution. The introduction of iodine atom to green fluorescent proteins remarkably enhances the cellular uptake and halogen bonding may play key roles in the caveolae-mediated endocytosis. The proteins can be released effectively into cytosol by co-treatment with the histidine-rich 20-mer peptide ppTG21, which can mediate the rupture of endosomal membrane by altering the proton gradient in endosomes. This study provides a novel finding that proteins can be transported across the cell membrane by the introduction of iodine atoms on the protein surface.

Acknowledgements

This study was supported by the Science and Engineering Research Board (SERB), New Delhi. S. R. J. and V. G. acknowledge IISc, Bangalore, and SERB, respectively, for a research fellowship. We thank Mary Nirmala Sarkar, M. Kishorkumar Reddy, Sritama Bose and Aswin Govindan for their help with some of the experiments. G. M. thanks the SERB for the J. C. Bose fellowship (SB/S2/JCB-067/2015).

Keywords: cellular uptake • endocytosis • green fluorescent protein • halogen • membrane transport • tRNA synthetase

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10.1002/anie.201902347

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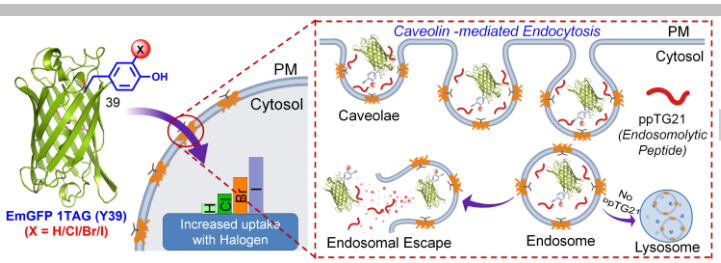
Introduction of an iodine atom to one of the tyrosine residues facilitates the cellular entry of green fluorescent proteins, providing a strategy for the delivery of macromolecules into mammalian cells.



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