BDNF Reduces the Retinal Toxicity of Verteporfin Photodynamic Therapy

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Purpose. Verteporfin photodynamic therapy (PDT) is the most effective treatment for age-related macular degeneration, using laser activation of a photosensitizing dye to achieve closure of choroidal neovascularization. Although PDT preferentially affects pathologic vessels, it can also cause collateral damage to the overlying retina. In the current study, it was found that the neuroprotective agent brain-derived neurotrophic factor (BDNF) reduces this retinal damage.

Methods. Normal adult rats received intravitreal BDNF in one eye and PBS or no injection in the other eye 2 days before PDT.

Results. Control eyes exhibited choroidal hypofluorescence, moderate to severe photoreceptor loss, and depression of local retinal function measured using multifocal ERG in the laser-treated area. BDNF-injected eyes had more surviving photoreceptors and improved multifocal ERG responses 1 week after PDT. BDNF did not diminish the effect of PDT on the choroidal circulation as assessed by fluorescein angiography, and there was no evidence of retinal toxicity due to BDNF treatment.

Conclusions. These results suggest that adjunctive neuroprotective therapy may reduce collateral damage to photoreceptors and improve visual outcome after PDT. (Invest Ophthalmol Vis Sci. 2004;45:4190–4196) DOI:10.1167/iovs.04-04076

Age-related macular degeneration (AMD) is the leading cause of blindness among the elderly in the United States and Europe.1–3 Severe vision loss in AMD usually results from choroidal neovascularization (CNV), the ingrowth of new vessels from the choroid through Bruch’s membrane and into the subretinal and sub-retinal pigment epithelial spaces. Photodynamic therapy (PDT) using the photosensitizing dye verteporfin is the current treatment of choice for subfoveal CNV.4 When activated by low energy laser with an absorption-specific wavelength, verteporfin generates oxygen radicals in the neovascular channels, resulting in their closure.5 Randomized trials have shown that PDT reduces the risk of vision loss in patients with CNV due to AMD5–9 or pathologic myopia.10,11 However, even with PDT, only 21% of patients retain vision better than 20/100 after 5 years.8 Preclinical and clinical studies demonstrate that PDT is not perfectly selective for CNV and can damage the overlying retina.5,7,12–16 In primate,5,12–14 rabbit,14 and rat15 models, PDT causes dose-dependent damage to normal retina, particularly to the retinal pigment epithelium (RPE) and photoreceptors.

Visual disturbances were reported in PDT clinical trials at higher rates by verteporfin-treated than by placebo-treated patients.5 In addition, acute severe vision loss was documented within 7 days of PDT in up to 4.9% of verteporfin-treated patients.5,17 Four studies have characterized the functional effects of PDT on the human retina using multifocal electroretinography (mERG).18–21 Three of these18–20 reported objective evidence of transient retinal dysfunction after PDT, which recovered over variable periods of up to 6 months.

Concerns about the toxicity of PDT are heightened by the fact that most patients require repeated treatment at 3-month intervals for recurrent CNV. In animal studies, collateral damage to normal retina is cumulative with successive treatments.13,14 Therefore, in addition to the effects of recurrent CNV, the retinal toxicity of PDT itself may contribute to loss of visual acuity over time.

Our goal is to improve PDT using adjuncts that reduce its toxicity without reducing its efficacy in closing CNV. As a first step toward this goal, we used rats without CNV to isolate the effects of PDT on normal retina. Previous studies to characterize PDT toxicity have used this approach,5,12,15,15 since experimental CNV and the laser treatment commonly used to induce it both damage the retina (see Discussion). Thus, laser induction of CNV would confound interpretation of both toxicity by PDT and protection by adjunctive therapies.

Previous studies have identified several neurotrophic factors that protect retinal photoreceptors from the damaging effects of constant light or slow photoreceptor loss in inherited retinal degenerations. They include brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF), basic fibroblast growth factor (bFGF), lens epithelium-derived growth factor (LEDGF), pigment epithelial cell-derived growth factor (PEDF), and glial cell line-derived growth factor (GDNF).22–29 In choosing a neurotrophic factor as a potential adjunct to PDT, we were aware that most of these agents demonstrate a degree of specificity, i.e., they are protective in some forms of retinal degeneration, but not in others.24,25,30–34 Moreover, protection from damage caused by laser alone, or by the unique combination of verteporfin and laser used in PDT, had never been demonstrated. We chose BDNF as a potential adjunct to PDT for these initial studies, because although it fails to rescue several forms of retinal degeneration,24,35 it protects the overlying retina. In the current study, it was found that the neuroprotective agent brain-derived neurotrophic factor (BDNF) reduces this retinal damage.

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photoreceptors from constant light when injected intravitreally.\textsuperscript{22,32} We hoped that comparable protection would occur after intravitreal injection of BDNF before PDT. Moreover, BDNF is nontoxic to photoreceptors even when overexpressed in the retina for several months.\textsuperscript{23} In this study, we have tested whether the neuroprotective activity of BDNF can reduce collateral damage to the overlying retina caused by PDT. We have correlated histologic damage from PDT with a localized reduction in retinal function using mfERG, and have demonstrated that BDNF injection before PDT increases photoreceptor survival and improves retinal function.

**Methods**

**Animals and Injections**

All studies were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the guidelines of the University of California San Francisco (UCSF) Committee on Animal Research. Brown-Norway rats 4 to 6 months of age were maintained in a 12-hour light–dark cycle at in-cage illuminance of <150 lux. For all experiments, rats were anesthetized with intramuscular xylazine (13 mg/kg) and ketamine (87 mg/kg). Corneas were anesthetized with 0.5% proparacaine, and pupils were dilated with 2.5% phenylephrine and 1.0% atropine.

For some animals, 2 mL BDNF (2 mg/mL in PBS; gift of Regeneron Pharmaceuticals, Tarrytown, NY) was injected into the superior aspect of the intravitreal cavity of one eye, using a transscleral approach and a 10-μL syringe (Hamilton, Reno, NV) with a 0.5-in., 52-gauge beveled needle. The other eye was injected with 2 μL PBS or was uninjected. Half of all animals received BDNF in the right eye, half in the left. Eyes were checked after injection for vitreous hemorrhage, cataract, and other complications. All subsequent experiments were performed and evaluated by experimenters who were masked to the BDNF-injected eye.

**Photodynamic Therapy**

Our protocol was adapted from that described previously.\textsuperscript{15} For animals that received intraocular injections, PDT was performed 2 days later. Body surface area was calculated as described.\textsuperscript{33} For most experiments, 6.0 mg/m\textsuperscript{2} verteporfin (QLT PhotoTherapeutics, Seattle, WA) was diluted to a final volume of 0.33 mL in water with 5% dextrose and injected as a bolus into the saphenous or tail vein. Verteporfin was protected from light at all times except during dilution and injection. A laser (Opal Photovactivator; Lumenis, Santa Clara, CA) with a slit lamp adaptor delivered a 3.0-μm spot of 680-nm light at 600 mW/cm\textsuperscript{2} for 17 seconds, resulting in a total fluence of 10 J/cm\textsuperscript{2}. The spot was delivered to the superior retina, with its inferior border adjacent to the optic disc and its center on the vertical meridian. Because the rat eye does not have a macula and photoreceptors are distributed uniformly throughout the retina, the placement of the laser spot was chosen to facilitate reproducible treatment and its identification angiographically and histologically. The right eye was treated beginning 3 minutes after injection of verteporfin and the left eye at 4 minutes.

Where noted in the text, some experiments used 3.0 mg/m\textsuperscript{2} verteporfin or no verteporfin injection; laser delivery times of 42 seconds (fluence 25 J/cm\textsuperscript{2}) or 83 seconds (50 J/cm\textsuperscript{2}); or laser delivered 15 to 20 minutes after verteporfin injection.

**Electroretinography**

Full-field ERGs were recorded 5 days after PDT, as described previously.\textsuperscript{34} Using a visual diagnostic system (UTAS-E 3000; LKC Technologies, Gaithersburg, MD), the responses to two flashes of +0.4 log cd-sec/m\textsuperscript{2} with an interstimulus interval of 120 seconds were averaged. Multifocal ERGs were also recorded (VERIS 5.0.7; EDI, San Mateo, CA), after full-field ERGs after 10 minutes of adaptation to 10 cd/m\textsuperscript{2} room light. This resulted in a retinal illumination of 200 scotopic trolands, based on a dilated pupil size of 19.6 mm\textsuperscript{2}.\textsuperscript{35} Light adaptation increased the consistency and uniformity of recordings from control animals, probably by reducing responses to scattered light (Sutter E, personal communication, 2003). A 61-hexagon stimulus array subtending a visual angle of 45° was projected onto the fundus and its location was monitored using an infrared charge-coupled device (CCD) camera. The optic nerve head fell within the same hexagon in all recordings. Stimulus intensity was 200 cd/m\textsuperscript{2} for “on” hexagons and 10 cd/m\textsuperscript{2} for the background. Twelve dark frames were inserted between m-frames. The m-sequence length was set to $2^{12} - 1$, resulting in a total recording time of 11 minutes, 50 seconds. The minimum interstimulus interval for a given hexagon was 172.9 (13 × 13.3) ms. Recording was performed using a gold loop electrode attached to a 15-D contact lens (Hansen Laboratories, Iowa City, IA). The raw signal was sampled 16 times within each frame interval and amplified 50,000-fold. Frequency cutoffs were set at 1 and 300 Hz. The first order kernel was filtered 1 to 150 Hz, and one iteration of artifact removal was performed over the first 150 ms. One iteration of 17% spatial averaging was used on the resulting trace array, and the scalar product was used for comparisons.

To quantify mfERG data, we generated a reference trace array by averaging results from six untreated eyes. We calculated the depression of each experimental trace as the difference of the scalar product from the corresponding trace in the reference array. We used choriocapillars to align fundus photographs showing the PDT-treated area with infrared images taken during recordings. The PDT-treated areas overlapped 16 to 18 hexagons in a stimulus array schematic included in the infrared images. Therefore, we averaged the depressions of the 16 most depressed traces in each eye, which clustered tightly in the expected area (see Figs. 5B–D).

**Fundus Imaging**

Retinal photography and fluorescein angiography were performed 6 days after PDT (TRC-50EX digital camera and IMAGEnet 2000 software; Topcon). For angiography, 1 mL of 10% fluorescein was injected intraperitoneally. Hypofluorescent areas were measured with NIH Image software (US Department of Health and Human Services, National Institute of Mental Health; available at rsb.info.nih.gov/nih-image).

**Histology**

To aid in localization of the PDT-treated area in histologic sections, a diode green laser (Novus Verdi; Lumenis) was used 7 days after PDT to place a small burn outside the superior border of the PDT-treated area. Vascular landmarks noted at the time of PDT were used to place the burns accurately. After recovery from anesthesia, rats were euthanized by carbon dioxide inhalation and perfused intracardially with 2% paraformaldehyde and 2.5% glutaraldehyde. Perfusion was performed under diode laser burns accurately. After overnight fixation, eyes were removed and the cornea and lens were dissected away. Eyecups were bisected near the vertical meridian (the greatest diameter of the PDT-treated area) using the optic disc and diode laser burns as landmarks. The hemispheres were postfixed in osmium tetroxide, embedded in epoxy resin, sectioned at 1 μm thickness, and stained as described.\textsuperscript{37}

We quantified photoreceptor survival in histologic sections in two ways. First, because the number of surviving photoreceptors is proportional to outer nuclear layer (ONL) thickness,\textsuperscript{38} we measured the area of the ONL between the optic disc and the diode laser burn and divided by its length to obtain the mean ONL thickness. The area was measured using Bioquant software with a digitizing tablet and camera lucida. The density of photoreceptor nuclei per unit area was indistinguishable between BDNF-injected and control eyes. Second, to assess the maximum damage, the rows of photoreceptor nuclei were counted at the five points in each eye at which the ONL was thinnest. The counts were averaged and compared to those of the contralateral eye.
For all quantitative data, interocular comparisons between BDNF-injected and control eyes used Student’s paired, two-tailed t-test. Comparisons between PBS-injected and uninjected eyes used the unpaired test.

RESULTS

Retinal Toxicity of Verteporfin PDT

Our first objective was to establish a reproducible model of retinal damage due to PDT. Zacks et al.15 demonstrated damage to normal rat retina at laser doses from 10 to 100 J/cm² and verteporfin doses of 3 or 6 mg/m². We found that laser treatment at 25 or 50 J/cm² without verteporfin injection caused loss of photoreceptor nuclei in the treated area (not shown).

Treatment at 10 J/cm² without verteporfin, however, caused no apparent damage (Fig. 1A). Angiography, full-field ERG, and mFERG also revealed no abnormalities in these animals (not shown). Since our goal was to study verteporfin-dependent toxicity, we conducted all further experiments at 10 J/cm².

Fundus photography 6 days after PDT with fluence 10 J/cm² and 6 mg/m² verteporfin showed hypopigmentation in the treated area (Fig. 2A). Fluorescein angiography revealed choroidal hypofluorescence (Fig. 2B). A ring of mild hyperfluorescence around the hypofluorescent zone (Fig. 2B) suggested damage to RPE and choroid without complete choroidal closure.

Histologic analysis showed loss of 60% to 70% of photoreceptors 1 week after PDT (Fig. 1B). The ONL was reduced from the normal 10 rows of photoreceptor nuclei in the outer nuclear layer (ONL). Photoreceptor inner segments (IS) and outer segments (OS) and the retinal pigment epithelium (RPE) appear normal. (B) Verteporfin PDT 2 days after PBS injection causes photoreceptor loss, thinning the ONL to three to four irregular rows. The ONL is scalloped forming partial rosettes, and some photoreceptor nuclei lie apposed to the highly attenuated RPE (white arrowhead). Some IS are present, and pigmented cells are found adjacent to the RPE (black arrowhead). (C) BDNF injection 2 days before PDT rescued many photoreceptors resulting in an ONL of approximately 6 rows of nuclei. IS and OS (asterisk) appear somewhat longer in BDNF-treated eyes than in controls, although some photoreceptor nuclei are still apposed to the RPE (arrow). Scale bar, 20 μm.

We also assessed the effects of the relative timing between verteporfin injection and laser treatment. Application of laser light to the two eyes of a single animal at 3 and 4 minutes after injection, respectively, resulted in damage of the degree shown in Figure 1B that was highly consistent between the two eyes.

Anatomic Rescue by BDNF

We next asked whether adjunctive treatment with BDNF could reduce damage due to verteporfin PDT. For these experiments, 2 days before PDT each animal received intravitreal BDNF in one eye, and either PBS (n = 9) or no injection (n = 8), chosen...
microscopy demonstrated disorganized outer segments in damage to staining, possibly due to the PDT. However, electron microscopy was nearly restored in BDNF-injected eyes, the combined inner and outer segment lengths of photoreceptors appeared to be somewhat greater in BDNF-injected eyes (Figs. 1B, C), but the specific lengths were difficult to quantify because the outer segments were refractory to staining, possibly due to the PDT. However, electron microscopy demonstrated disorganized outer segments in damaged areas of both BDNF-injected and control eyes (Fig. 4 and data not shown).

We used fluorescein angiography to assess the effect of BDNF on choroidal vessels (Fig. 2D). Choroidal hypofluorescence after PDT in BDNF-injected eyes was similar to control eyes. Measurement of the hypofluorescent area in images taken at the same magnification revealed no significant effect of BDNF (mean ± SEM in arbitrary units normalized to controls: control eyes (n = 4), 1.00 ± 0.11; BDNF-injected eyes (n = 4), 1.11 ± 0.20; P = 0.61). Although we cannot exclude more subtle changes in the choroidal circulation, our analysis at this level suggests that BDNF does not alter the effects of PDT on choroidal vessels.

**Functional Rescue by BDNF**

The foregoing experiments show that BDNF increases the number of photoreceptors surviving 1 week after verteporfin PDT. To determine whether BDNF also preserves retinal function, we used full-field ERG and mfERG.

Full-field ERG, which measures the global response of the retina to light, revealed no significant difference between BDNF-injected and control eyes (not shown). Functional rescue within the focal PDT lesion would not be expected to result in a measurable difference in full-field responses. These results suggest that BDNF itself does not affect retinal function, consistent with previous observations.

Because the damage and rescue were focal, we used mfERG to assess localized retinal function. With the mfERG, multiple small areas of retina are stimulated simultaneously, and local responses are extracted from the massed electrical potential. These appear to have the same cellular origins as the full-field ERG.

Figure 5A shows averaged responses from six untreated rat eyes. A depression at the location of the optic nerve head confirms both the local nature of the responses and consistent placement of the stimulus array on the superior retina. Figure 5B shows the effect of PDT, representing a typical result for a PBS-injected eye. The broad depression corresponds to the treatment spot seen by fundus photography. BDNF injection in the contralateral eye of the same animal partially rescued retinal function within the PDT-treated area (Fig. 5C). Superimposing the trace arrays used to generate the color plots for both eyes demonstrates improved function in the BDNF-injected eye (Fig. 5D).

To quantify the mfERG data, we subtracted each response from the average response at the same position from the set of 6 normal eyes shown in Figure 5A to derive the depression in nanovolts. Figure 6A shows sums of the 16 most depressed responses in each eye, corresponding to the PDT-treated areas, for the animal shown in Figures 5B–D. The BDNF-injected eye exhibits less depression than the control eye. This animal demonstrated the median degree of rescue among seven rats for which mfERGs were recorded. Interocular comparisons for

**Table 1. Comparison between PBS-Injected and Uninjected Control Eyes**

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>PBS Injected</th>
<th>Uninjected</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>ONL thickness (µm)</td>
<td>27.4 ± 1.4</td>
<td>26.2 ± 1.8</td>
<td>0.59</td>
</tr>
<tr>
<td>ONL minima (rows of nuclei)</td>
<td>5.6 ± 0.3</td>
<td>3.7 ± 0.5</td>
<td>0.75</td>
</tr>
<tr>
<td>Choroidal hypofluorescence</td>
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<tr>
<td>(arbitrary area units)</td>
<td>1.13 ± 0.18</td>
<td>0.87 ± 0.03</td>
<td>0.19</td>
</tr>
<tr>
<td>mfERG depression (nV)</td>
<td>94.9 ± 5.1</td>
<td>104.2 ± 12.0</td>
<td>0.50</td>
</tr>
<tr>
<td>mfERG a-wave amplitude (µV)</td>
<td>129.6 ± 12.8</td>
<td>122.1 ± 7.8</td>
<td>0.58</td>
</tr>
<tr>
<td>mfERG b-wave amplitude (µV)</td>
<td>428.4 ± 61.8</td>
<td>435.8 ± 22.6</td>
<td>0.90</td>
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</tbody>
</table>

Data shown are the means ± SEM. The number in each group is in parentheses.
the entire group of seven animals show that mfERG responses were significantly less depressed in BDNF-injected eyes (Figs. 6B, 6C).

These results demonstrate that adjunctive treatment with BDNF before PDT not only improves photoreceptor survival, but also preserves retinal function within the PDT-treated area.

**DISCUSSION**

Our goal was to test whether a neuroprotective adjunct could reduce the collateral retinal damage caused by verteporfin PDT. We found that BDNF injection before PDT protected photoreceptors in the treated area and preserved local retinal function in normal rats.

The damage that we observed was comparable to that in a previous study of PDT in rats. Using the same verteporfin and laser doses to treat normal retina that we did, the authors noted the death of over 50% of photoreceptors, with severe disruption of inner and outer segments and RPE damage. The changes they observed were quite similar to those in our study, given that we examined eyes histologically 7 days after PDT, while they examined eyes after 24 hours. We found that damage 7 days after PDT was most consistent among eyes with laser application at 3 and 4 minutes after verteporfin injection rather than at 15 to 20 minutes, but the consistency with results published previously suggests that the precise timing does not markedly affect the degree of damage in rats.

Several issues remain for further study. First, a clinically useful adjunct to PDT must protect the retina without reducing CNV closure. The present study was not designed to address this issue, since we applied PDT to normal retina to avoid confounding damage due to CNV. For example, the most widely studied experimental CNV models require the use of high-energy laser burns to induce CNV; yet this sort of laser results in the endogenous release of neurotrophic factors and protection from retinal degeneration. Furthermore, the use of normal animals as we have done is well-established for study of PDT in animal models. Clearly, however, further work is needed to determine whether BDNF modifies the effects of PDT on CNV. Two considerations suggest that this possibility is unlikely. The high-affinity BDNF receptor TrkB is not expressed in the choroid, so BDNF probably cannot exert direct protective effects in this tissue. Moreover, BDNF did not change the effect of PDT on choroidal vessels, as determined by fluorescein angiography. Nevertheless, the effect on CNV of BDNF with PDT must be determined experimentally in future studies.

Second, can neuroprotection be demonstrated at higher laser doses? The previous rat study found that the efficacy and toxicity of PDT both rise with increasing laser fluence. A more detailed study would be required to determine the effect of BDNF on CNV under different laser conditions.

**FIGURE 4.** Electron micrograph of a BDNF-injected eye, showing that photoreceptor outer segment membranes (OS) are present in the region of PDT laser treatment, albeit shorter and more disorganized than normal. IS, photoreceptor inner segments; PR, rod photoreceptor nucleus; RPE, retinal pigment epithelium. Scale bar, 5 μm.

**FIGURE 5.** BDNF improves retinal function after PDT. (A) Pseudocolor plot of mfERG showing averaged responses from six untreated rat eyes. A physiologic depression due to the optic nerve head appears inferiorly (white circle) and is similarly positioned in all other recordings. (B) A typical result for PDT preceded by PBS injection, showing a broad depression in retinal responses. Outside the treated area, in the periphery of this recording, retinal function is essentially normal. (C) BDNF injection before PDT increases responses in the contralateral eye of the same animal shown in (B). (D) Overlay of the trace arrays used to generate plots in (B) and (C). The array in black represents the PBS-injected eye; red, the BDNF-injected eye. Most responses in the central region, corresponding to the PDT-treated areas, were larger in the BDNF-injected eye. Note that these traces are shown on a slightly different scale than that used in (A–C), since pseudocolor plots use the scalar product of each trace rather than absolute amplitude.
neuroprotective adjunct could allow a higher fluence to be used without increasing collateral damage to the retina, perhaps improving the efficacy of treatment.

Third, what is the effect of BDNF over multiple treatments? Most patients require multiple courses of PDT for recurrent CNV. In animal models, damage to normal retina is cumulative with repeated treatment. Effective neuroprotection could be especially valuable as investigators consider earlier retreatment regimens.

Fourth, what is the long-term protective effect of BDNF? In photoreceptors protected from cell death by FGF-2, the surviving cells had regenerated some outer segments when examined 10 days after light exposure, and even longer outer segments were found 20 days after light exposure (LaVail M, unpublished observations, 1993). These and the present findings raise the possibility that photoreceptors protected from verteporfin PDT by BDNF may regenerate light-sensitive outer segments, further improving visual outcome over time.

Finally, we note that the protective effect of BDNF on retinal structure and function was incomplete. A higher dose of BDNF, other neurotrophic factors, or combinations of factors might further reduce collateral damage due to PDT.

To our knowledge, there is only one previous report of mFERG recordings in rats. Our study demonstrates that mFERG is useful for correlating the histologic and functional effects of PDT in animal models, and is sensitive and reproducible enough to detect a therapeutic effect. The use of a fundus camera to monitor stimulus placement on the retina improved our ability to identify local features such as the optic nerve depression and PDT lesion.

As noted previously, the cell types that produce rat mFERG responses cannot be identified with certainty. Our light-adapted protocol probably enhances cone-mediated responses, but two considerations suggest that rod-driven pathways also contribute. First, cones represent only approximately 1.5% of rat photoreceptors, and are unlikely to mediate robust mFERG responses alone. Second, the response latencies in our recordings were on the order of 50 to 60 ms (Fig. 6A), consistent with rod-driven responses.

Our results demonstrate the potential of neuroprotective adjuncts to improve PDT. By protecting the retina through multiple courses of treatment and perhaps by reducing barriers to earlier or more aggressive retreatment, neuroprotective agents may make PDT safer and more effective in preserving long-term visual function in patients with CNV.

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