GABA_A Receptor Modulation by Phenyl Ring Compounds Is Associated with a Water Solubility Cut-Off Value

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Key Words

Benzene · GABA · Phenol · Propofol · Selectivity

Abstract

Background: The modulation of N-methyl-D-aspartate receptors is associated with a molar water solubility cut-off effect of approximately 1.1 mmol/l and hence are unaffected by significantly less soluble compounds. However, compounds with this molar water solubility are still able to modulate γ -aminobutyric acid type A (GABA_A) receptors. We hypothesized that GABA_A receptor modulation by phenolic compounds would exhibit cut-off at a molar water solubility value less than 1.1 mmol/l. **Methods:** GABA_A receptors consisting of human α_1 and rat β_2 and γ_{25} subunits were expressed in *Xenopus laevis* oocytes, and drug responses were measured using standard 2-electrode voltage clamp techniques. Twenty substituted phenols and benzenes of similar size and molecular volume were studied at saturated aqueous concentrations. Reversible and statistically significant change in GABA_A receptor current that was 10% or greater in magnitude from the baseline response defined a positive drug effect. **Results:** All phenyl ring compounds with a molar water solubility value equal to or greater than 0.46 mmol/l positively modulated GABA_A receptor currents. No compounds with a molar water solubility value equal to or less

than 0.10 mmol/l had any effect on GABA_A receptor currents. Saturated solutions of phenols with 2,6-dimethyl and 2,6-diisopropyl substituents also caused channel opening in the absence of GABA. **Conclusions:** The molar water solubility cut-off for GABA_A receptor modulation by phenyl ring compounds lies between 0.10 and 0.46 mmol/l. Data suggest that hydrocarbons, perhaps including inhaled anesthetics, might modulate GABA_A receptors by displacing water from one or more low-affinity amphipathic binding sites to induce conformational changes that increase ion conductance.

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Introduction

In contrast to volatile inhaled agents, such as halothane and isoflurane, which interact with multiple diverse receptors to produce immobility, injectable anesthetics tend to act primarily through a single or small number of receptors [1]. In addition, the anesthetic EC₅₀ is typically many times lower for injectable agents, indicating that interactions between the drug and critical receptor targets occur with much greater affinity for injectable agents than for inhaled ones. Hence, relative receptor specificity of injectable agents may simply be a function of their high efficacy at a single receptor target relevant to anesthesia

rather than a function of the chemical structure of the drug itself that prevents promiscuous low-affinity receptor interactions.

Propofol, an alkylphenol injectable anesthetic, is a potent and high-affinity positive allosteric modulator of the γ -aminobutyric acid type A (GABA_A) receptor. Receptor mutation studies suggest that high-affinity propofol binding of GABA_A receptors occurs at sites that are distinctly different from those that bind low-affinity volatile anesthetics, such as isoflurane [2]. Low-affinity binding sites are postulated to be amphipathic and water filled pockets within the receptor [3, 4]. At least in the case of N-methyl-D-aspartate (NMDA) receptors, this low-affinity protein binding is subject to a cut-off effect associated with molar water solubility, but not molecular structure or size [5].

Although only some alkylphenols bind GABA_A receptors with high affinity, many other phenyl ring compounds are still capable of augmenting GABA-induced currents at high concentrations, presumably at low-affinity binding sites [6–8]. If low-affinity GABA_A binding pockets are functionally analogous to NMDA low-affinity binding pockets, then GABA_A receptors should exhibit a molar water solubility cut-off effect just as NMDA receptors do. If the drug molar water solubility is less than the GABA_A receptor cut-off value, then even saturated drug concentrations should not be able to affect chloride conductance through the channel. Since GABA_A and NMDA receptors are phylogenetically unrelated proteins, they likely also have amphipathic pockets that differ in their affinity for water. Therefore, the bulk aqueous drug concentration necessary to compete with water in a low-affinity GABA_A-binding site would be different than in an analogous NMDA-binding site. Consequently, the GABA_A receptor molar water solubility cut-off value is predicted to be different than the cut-off value previously reported for NMDA receptors [5].

The aim of this study was to measure GABA_A receptor responses to 20 different substituted phenyl compounds having either hydrogen, hydroxyl or halogen (F, Cl, or Br) at the X position and 2 hydrogen or identical alkyl groups (methyl, ethyl, or isopropyl) at the R position (fig. 1). Although these substituted phenyl compounds differ modestly from each other with respect to molecular shape and volume, the group spans nearly a 100,000-fold range of calculated water solubility values. We hypothesize that GABA_A receptors exhibit a cut-off effect at high phenyl drug concentrations necessary for low-affinity interactions, and that this cut-off is associated with the drug molar water solubility.

Fig. 1. General structure of substituted benzenes and phenols.

Methods

Xenopus laevis frogs were surgically ovariectomized using a protocol approved by the Institutional Animal Care and Use Committee at the University of California, Davis. Ovaries were incubated in 0.2% type I collagenase (Worthington Biochemical, Lakewood, N.J., USA) to defolliculate oocytes, which were washed and stored in fresh and filtered modified Barth's solution composed of 88 mmol/l NaCl, 1 mmol/l KCl, 2.4 mmol/l NaHCO₃, 20 mmol/l HEPES, 0.82 mmol/l MgSO₄, 0.33 mmol/l Ca(NO₃)₂, 0.41 mmol/l CaCl₂, 5 mmol/l sodium pyruvate, gentamycin, penicillin, streptomycin, and corrected to pH 7.4. All salts and antibiotics were A.C.S. grade (Fisher Scientific, Pittsburgh, Pa., USA).

GABA_A receptors were expressed using clones for the human GABA_A α_1 and the rat GABA_A β_2 and γ_{2s} subunits in pCIS-II vectors. Clones were a gift from Dr. R.A. Harris (University of Texas, Austin) and were sequenced and compared to references in the National Center for Biotechnology Information database to confirm the identity of each gene. Approximately 0.25–1 ng total plasmid mixture containing α_1 , β_2 , and γ_2 genes in a respective ratio of 1:1:10 was injected intranuclearly through the oocyte animal pole and studied 2–4 days later. These plasmid ratios ensured incorporation of the γ subunit into expressed receptors, as confirmed via receptor potentiation to 10 μ mol/l chlordiazepoxide or insensitivity to 10 μ mol/l zinc chloride during co-application with GABA [9, 10].

Oocytes were studied in a 250 μ l linear-flow perfusion chamber with solutions administered by a syringe pump at 1.5 ml/min with gastight glass syringes polytetrafluoroethylene tubing. Whole cell currents were studied with a –80 mV holding potential using standard 2-electrode voltage clamping techniques. Methods for measuring whole-cell GABA_A receptor currents have been described [5, 11–13]. Briefly, pairs of identical concentrations of the phenyl compound were prepared in glass syringes containing frog ringer's (FR) solution (115 mmol/l NaCl, 2.5 mmol/l KCl, 1.8 mmol/l CaCl₂, and 10 mmol/l HEPES in 18.2 M Ω H₂O, filtered and adjusted to pH 7.4) or FR + agonist (FR-GABA; FR plus 20–40 μ mol/l, GABA, equal to an EC_{10–20}). After FR perfusion for 5 min, oocytes were exposed to 30 s FR-GABA followed by another 5 min FR washout; this was repeated until stable GABA_A receptor-elicited peaks were obtained. Next, the oocyte chamber was perfused with FR containing the study phenol or benzene for 2 min followed by 30 s perfusion with an equal concentration of the same phenol or benzene dissolved in FR-GABA solution. FR was next

perfused for 5 min to allow drug washout, and oocytes were finally perfused with FR-GABA for 30 s to confirm return of currents to within 10% of the initial baseline response. Phenyl compounds (table 1) had a purity of 98% or greater and were obtained from Sigma Aldrich (St. Louis, Mo., USA) with the following exceptions: 2-halo-m-xylenes (TCI America, Portland, Oreg., USA), 2-halo-1,3-diethylbenzenes and 2-halo-1,3-diisopropylbenzenes (Richman Chemical, Lower Gwynedd, Pa., USA), and all alkylphenols (Alfa Aesar, Ward Hill, Mass., USA). All compounds were studied at saturated aqueous concentrations of up to 10 mmol/l. The alkylphenols were also studied at the maximum concentrations that did not elicit GABA_A receptor currents in the absence of GABA.

Percent increase in GABA_A receptor current was calculated as follows:

$$\% = 100 \times \frac{I_p - I_o}{I_o}$$

where I_p and I_o are whole cell currents with and without the phenyl compound, respectively. Data are described as mean $\pm$ SD, and 95% CIs were calculated to determine whether current responses were significantly different from zero.

Results

A sample voltage clamp tracing is shown in figure 2, and a summary of GABA_A receptor responses to phenyl compounds is shown in table 1. At saturated concentrations, all of the dialkylphenols produced 500–1,000% receptor potentiation; however, these concentrations also produced direct receptor activation. At dimethyl-, diethyl-, and diisopropyl-phenol concentrations equal or less than 250, 2,000, and 0.75 μ mol/l, respectively, dialkylphenols potentiated GABA_A receptor currents without direct channel activation.

All phenolic compounds potentiated GABA_A receptor currents, and the magnitude of potentiation increased with the size of the 2,6-alkyl chain at the doses studied. In contrast, less than 40% of the benzene compounds had any effect on GABA_A receptors. All unalkylated halobenzenes potentiated GABA_A currents, as did the 1,3-dimethyl benzenes except for the 2-chloro and 2-bromo analogues. An addition of 1,3-diethyl or 1,3-diisopropyl groups to benzene – whether surrounding a hydrogen or a halogen at the second phenyl ring carbon – eliminated all efficacy at the GABA_A receptor.

When compounds are ranked in the order of increasing molar water solubility (table 2), there appears an abrupt change between substituted phenyls that lack receptor efficacy, ending with 2-bromo-1,3-dimethylbenzene (CAS# 576-22-7), and those that potentiate GABA_A receptors, starting with 2-fluoro-1,3-dimethylbenzene (CAS# 443-

88-9). Unlike molecular volume or other physicochemical properties, no outlier responses exist within the data set studied. Low-affinity GABA_A receptor modulation is associated with a cut-off effect when water solubility of the drug is between 0.10 and 0.46 mmol/l, corresponding to the molar water solubility values of 2-bromo-1,3-dimethylbenzene and 2-fluoro-1,3-dimethylbenzene, respectively.

No single phenyl ring substitution was consistently associated with either a gain or a loss of pharmacologic action. Diethyl and diisopropyl groups eliminated receptor modulation when present on benzene, but increased drug efficacy when present on phenol. All halobenzenes potentiated GABA_A receptors in their unalkylated forms, but often did not when dialkylated. For compounds with the dimethyl substituent, fluorinated and hydroxylated rings exhibited potency, whereas chlorinated and brominated counterparts did not. The relative electronegativity of these substituents in descending order is F > O > Cl > H > Br. However, because diethyl and diisopropyl substituents exhibited potency only when a hydroxyl group was present, but not F or Cl, electronegativity of the second carbon on the phenyl ring cannot explain a cut-off effect. Similarly, the relatively electron affinity can be ranked in the descending order: Cl > F > Br > O > H. Only 2-fluoro-1,3-dimethylbenzene and 2,6-dimethylphenol, but not their chlorinated or brominated analogues, exhibited efficacy; thus, electron attraction cannot explain a cut-off.

There was no consistent correlation between substituent or molecular size and GABA_A receptor modulation. The radius of a halogen increases as its atomic number increases; the radius of the hydroxyl group is intermediate between measurements for chloride and bromide atoms. Although m-xylene compounds with a fluorine or hydroxyl substituent increased GABA_A currents, 2-chloro-m-xylene did not increase GABA_A currents even though the chlorine atom size lies between that of fluorine and the hydroxyl group. There was also no pattern to receptor modulation when considering the size of substituted phenyl compounds in their entirety. Although many of the larger molecules did not affect GABA_A currents, propofol (CAS# 2078-54-8) and 2,6-diethylphenol (CAS# 1006-59-3) are noted exceptions with very potent GABA_A receptor effects (table 3).

Discussion

Many 2,6-dialkylphenols exhibit high-affinity interactions with GABA_A receptors, possibly in part via hydrogen bonding between the hydroxyl group and amino ac-

Table 1. Names, structures, and physical characteristics of phenyl ring compounds applied to voltage clamped oocytes expressing GABA_A receptors

IUPAC name	CAS#	R	X	MW	Volume, cm ³ /mol	Solubility, mol/l	Concentration	Receptor potentiation, %	SD	Oocytes, n	Receptor effect
Benzene	71-43-2	-H	-H	78.11	89.4	1.2E-02	Saturated	30.5	6.0	5	+
Benzene, fluoro-	462-06-6	-H	-F	96.1	93.6	3.6E-03	Saturated	34.7	10.6	6	+
Benzene, chloro-	108-90-7	-H	-Cl	112.56	101.3	7.6E-04	Saturated	148.4	10.9	5	+
Benzene, bromo-	108-86-1	-H	-Br	157.01	105.6	8.7E-04	Saturated	85.7	9.5	5	+
Benzene, 1,3-dimethyl-	108-38-3	Methyl	-H	106.17	121.9	9.3E-04	Saturated	266.4	52.4	6	+
Benzene, 2-fluoro-1,3-dimethyl-	443-88-9	Methyl	-F	124.16	126.1	4.6E-04	Saturated	57.5	11.7	6	+
Benzene, 2-chloro-1,3-dimethyl-	6781-98-2	Methyl	-Cl	140.61	133.9	9.3E-05	Saturated	7.9	4.5	5	0
Benzene, 2-bromo-1,3-dimethyl-	576-22-7	Methyl	-Br	185.06	138.1	1.0E-04	Saturated	-1.6	10.6	5	0
Benzene, 1,3-diethyl-	141-93-5	Ethyl	-H	134.22	155.0	5.5E-05	Saturated	0.4	0.7	6	0
Benzene, 1,3-diethyl-2-fluoro-	84604-67-1	Ethyl	-F	152.21	159.2	6.6E-05	Saturated	2.3	2.0	5	0
Benzene, 2-chloro-1,3-diethyl-	288402-30-2	Ethyl	-Cl	168.66	167.0	1.7E-05	Saturated	2.4	2.4	5	0
Benzene, 2-bromo-1,3-diethyl-	65232-57-7	Ethyl	-Br	213.11	171.2	1.6E-05	Saturated	-0.4	1.4	6	0
Benzene, 1,3-bis(1-methylethyl)-	99-62-7	Isopropyl	-H	162.27	189.5	1.3E-05	Saturated	-1.1	4.5	6	0
Benzene, 2-fluoro-1,3-bis(1-methylethyl)-	87591-05-7	Isopropyl	-F	180.26	193.7	3.4E-05	Saturated	-1.3	5.0	5	0
Benzene, 2-chloro-1,3-bis(1-methylethyl)-	54845-36-2	Isopropyl	-Cl	196.72	201.5	1.1E-05	Saturated	1.1	2.9	6	0
Benzene, 2-bromo-1,3-bis(1-methylethyl)-	57190-17-7	Isopropyl	-Br	241.17	205.7	1.8E-05	Saturated	3.3	9.6	9	0
Phenol	108-95-2	-H	-OH	94.11	87.8	1.0E+00	10 mmol/l	187.7	35.2	5	+
Phenol, 2,6-dimethyl-	576-26-1	Methyl	-OH	122.16	120.4	5.7E-02	0.25 mmol/l*	397.3	59.9	5	+
Phenol, 2,6-diethyl-	1006-59-3	Ethyl	-OH	150.22	153.4	4.7E-03	2 mol/l*	1435.4	134.5	5	+
Phenol, 2,6-bis(1-methylethyl)-	2078-54-8	Isopropyl	-OH	178.27	188.0	1.6E-03	0.75 µmol/l*	179.1	59.2	6	+

Refer to figure 1 for locations of -R and -X substituents. Propofol is 2,6-bis-(1-methylethyl)-phenol, CAS# 2078-54-8. Percent increases in normalized GABA_A receptor currents are summarized as mean and SDs and as binary responses (potentiation '+', or no effect '0'). Substituted phenols causing channel opening in the absence of GABA are identified by an asterisk (*); these compounds were studied at concentrations below the threshold for direct receptor activation. Values for molecular volume and molar water solubility (25°C, pH 7) were calculated by SciFinder (Chemical Abstracts Service, Columbus, Ohio, USA).

Fig. 2. Sample 2-electrode voltage clamp tracing of oocyte-expressed GABA_A receptor currents before, during, and after exposure to a 10 mmol/l phenol solution.

Table 2. Phenyl compounds sorted in order of ascending molar water solubility

CAS#	Solubility, mol/l	GABA
54845-36-2	1.1E-05	0
99-62-7	1.3E-05	0
65232-57-7	1.6E-05	0
288402-30-2	1.7E-05	0
57190-17-7	1.8E-05	0
87591-05-7	3.4E-05	0
141-93-5	5.5E-05	0
84604-67-1	6.6E-05	0
6781-98-2	9.3E-05	0
576-22-7	1.0E-04	0
443-88-9	4.6E-04	+
108-90-7	7.6E-04	+
108-86-1	8.7E-04	+
108-38-3	9.3E-04	+
2078-54-8	1.6E-03	+
462-06-6	3.6E-03	+
1006-59-3	4.7E-03	+
71-43-2	1.2E-02	+
576-26-1	5.7E-02	+
108-95-2	1.0E+00	+

Rows shaded grey contain compounds that potentiate GABA_A receptor currents. A discrete cut-off in the modulation pattern for phenyl compounds is consistent with water solubility as the critical determinant of the GABA_A receptor cut-off effect.

ids within a binding cavity on the β subunit [14–17]. However, positive allosteric modulation of GABA_A receptor function is also possible through lower-affinity interactions by high concentrations of chemically related substituted phenyl compounds. Low-affinity GABA_A receptor modulation is associated with a molar water solubility cut-off. Phenyl compounds with a solubility ≥ 0.46

Table 3. Phenyl compounds sorted in the order of descending molar volume

CAS#	Volume, cm ³ /mol	GABA
57190-17-7	205.7	0
54845-36-2	201.5	0
87591-05-7	193.7	0
99-62-7	189.5	0
2078-54-8	188.0	+
65232-57-7	171.2	0
288402-30-2	167.0	0
84604-67-1	159.2	0
141-93-5	155.0	0
1006-59-3	153.4	+
576-22-7	138.1	0
6781-98-2	133.9	0
443-88-9	126.1	+
108-38-3	121.9	+
576-26-1	120.4	+
108-86-1	105.6	+
108-90-7	101.3	+
462-06-6	93.6	+
71-43-2	89.4	+
108-95-2	87.8	+

Rows shaded grey contain compounds that potentiate GABA_A receptor currents. Discontinuity in the modulation pattern for phenyl compounds indicates that GABA_A receptor cut-off effects are not entirely explained by molecular size.

mmol/l potentiate GABA_A currents, whereas similar compounds with a solubility ≤ 0.1 mmol/l do not.

These data support the presence of at least one water-filled amphipathic binding pocket within the GABA_A receptor at which a conformational change that increases ion conductance can be induced when the water molecules are displaced by a phenyl compound. When the drug molar

water solubility falls below the cut-off value, it is impossible to achieve drug concentrations sufficient to effectively compete with water at this binding site, even when the solution is saturated with drug. Complete loss of drug potency, or absolute receptor selectivity, results from drug failure to displace water from the amphipathic modulatory site on the receptor (or the site that has the highest water dissociation constant if multiple such amphipathic pockets are present).

NMDA receptors also exhibit a cut-off effect that is associated with the hydrocarbon molar water solubility [5]. Similar amphipathic, water-filled binding pockets have been postulated to explain why poorly soluble drugs fail to inhibit NMDA receptors. However, low-affinity modulation of NMDA receptors is lost when aqueous solubility is less than 1.1 mmol/l, a value that is 2.4–11 times higher than the GABA_A receptor cut-off value. Since the low-affinity receptor cut-off value is a function of the ease (or difficulty) with which water in the amphipathic pocket can be displaced, the modulatory site with the highest water dissociation constant on the GABA_A receptor is therefore predicted to be between 2.4 and 11 times greater than the analogous site on the NMDA receptor.

A cut-off effect predicted only by molar water solubility implies that the dissociation constant for water in the binding pocket is more critical than the shape of the binding pocket itself. However, this molar water solubility hypothesis predicts GABA_A receptor modulation by phenyl compounds as a binary event; it does not predict the magnitude or even the direction of modulation. Once bound, the types, number and strength of intermolecular forces (hydrophobic, dipole, and hydrogen bonding) should still determine what type and to what extent a conformational change in protein structure will be induced. For example, many of the dialkylated phenyl ring compounds were positive allosteric modulators of GABA_A receptors, but only those with a 1-hydroxyl group were capable of direct receptor activation. This is consistent with structure-activity relationships showing alkyl groups must be present at the ortho position relative to the hydroxyl group to allow a phenolic compound to activate chloride currents in the absence of GABA [18].

Among the non-phenolics, the magnitude of GABA_A receptor potentiation is parabolic in shape, reaching a peak at 1,3-dimethylbenzene with its molar water solubility of 0.93 mmol/l (table 1). The ability of a drug to passively penetrate a lipid membrane is described by a parabolic function of its hydrophobic bonding capacity, and this same activity relationship may underlie benzene efficacy at GABA_A receptors [19]. However, increased numbers and strength of hydrophobic interactions be-

tween drug and the binding pocket – which differ the most from the types of interactions between water molecules and the binding pocket – might instead allow greater protein conformational change and produce greater drug efficacy. At the same time, within a given chemical group, increasing hydrophobicity usually accompanies decreasing molar water solubility. As progressively hydrophobic substituents push a series of compounds closer to the receptor molar water solubility cut-off value, increased efficacy of the bound drug may be partially offset by increasing difficulty for the drug to displace water and bind the receptor, thereby accounting for the downslope of the parabolic efficacy-solubility relationship. In theory, some very hydrophobic and non-efficacious phenyl compounds might strongly potentiate GABA_A receptors if only they could achieve aqueous concentrations sufficient to displace the water molecules that block drug binding to these modulatory sites.

Propofol (2,6-diisopropylphenol, CAS# 2078-54-8) exerts its anesthetic effects primarily through the potentiation of GABA_A chloride currents [20]. Capable of modulating of GABA_A receptor function at even low aqueous phase concentrations (table 1), propofol exhibits high-affinity binding in addition to other possible low-affinity interactions. In vitro, injectable anesthetics such as propofol and its structurally similar congeners can modulate other unrelated receptors, including NMDA receptors, given sufficient drug molar water solubility and high aqueous drug concentrations [5]. However, high affinity drug-receptor binding at targets relevant to anesthetic action increases drug potency in vivo; consequently, the probability of low-affinity receptor interactions with low drug concentrations is reduced. The anesthetic EC₅₀ for propofol in humans is 45 µmol/l [21]; the IC₅₀ for NMDA receptors is 160 µmol/l [22]. Hence, propofol only modestly inhibits NMDA receptors at an anesthetic EC₅₀ because the drug is delivered at low concentrations under clinical conditions, resulting in relative receptor specificity. Presumably, other low-affinity receptor interactions with other receptors are possible as well, some of which may result in undesirable effects during anesthesia [6].

In contrast to propofol, benzene and fluorobenzene have much higher anesthetic EC₅₀ values of 1.3 and 1.1 mmol/l, respectively, in rats [23]. These agents inhibit NMDA receptors by 60–74% at one minimum alveolar concentration of drug [24], and the immobilizing potency of these and other substituted benzenes is postulated to depend on NMDA receptor inhibition [25]. However, at these near-saturated concentrations, low-affinity GABA_A receptor interactions also occur. Receptor selectivity with

high aqueous drug concentrations might feasibly be attained by modifying the drug to decrease its molar water solubility below the cut-off value for the receptor.

Other conventional volatile inhaled anesthetics, such as halothane and isoflurane, are administered at high aqueous phase concentrations and exhibit a lack of receptor specificity [1]. At such concentrations, volatile agents may only interact with ion channels and receptors through low-affinity binding sites at amphipathic pockets containing water with a sufficiently large K_d . The very presence of such motifs may, in fact, be what renders a volatile anesthetic-sensitive receptor sensitive to volatile anesthetics. In contrast, conventional injectable anesthetics, such as propofol, typically exhibit much greater receptor

specificity and affinity; consequently, they need not have high water solubility and high aqueous concentrations to bind the target receptor. This study shows that when delivered at high concentrations, phenyl compounds similar to propofol exhibit molar water solubility cut-off effects reminiscent of other low-affinity hydrocarbon-receptor interactions, and that these cut-off effects even extend to interactions with GABA_A receptors.

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