1 **Bovine serum albumin as an immunogenic carrier facilitating the development of** 2 **hapten-specific monoclonal antibodies**

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4 **Abstract:**

5 There is a common misconception that the generation of hapten-specific monoclonal antibodies (mAbs)
6 requires the use of a heterologous conjugate to ensure carrier-specific antibodies not being detected. In
7 this study, salbutamol (SAL) was used as a model hapten to exhibit the benefits of bovine serum
8 albumin (BSA) as a carrier for developing hapten-specific mAbs. SAL-BSA conjugate would serve as
9 both an immune antigen and a screening antigen during the preparation of SAL-specific mAbs. Six
10 hybridomas were identified to secrete mAbs specific for free SAL with minor or negligible
11 cross-reactivity with other β -agonists. Meanwhile, none of hybridomas secreting anti-BSA antibodies
12 were screened out even though the fetal bovine serum (FBS) added to the medium decreased from 10%
13 to 1% (v/v). Based on one of the six mAbs, 3F12, a direct competitive enzyme-linked immunosorbent
14 assay (dcELISA) was developed for measuring SAL. Under the optimized assay, the quantitative
15 working range was from 312.5 to 20,000 pg/mL ($R^2 = 0.9959$), with a limit of detection (LOD) of 142.9
16 pg/mL. The results showed that BSA is an efficient and suitable protein carrier for facilitating the
17 development of hapten-specific mAbs.

18 **Keywords**

19 Bovine serum albumin; carrier protein; hapten; monoclonal antibody

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20 **1. Introduction**

21 To develop specific and sensitive immunoassays for small-molecule compounds (haptens) detection,
 22 the crucial step is to generate high quality antibodies against the target molecule (Chappey et al. 1992).
 23 Haptens, however, are usually nonimmunogenic unless they are conjugated with an immunogenic
 24 carrier. In the majority of hapten-carrier systems, the B cells will produce antibodies that are specific
 25 for both the hapten and carrier (Parker 2013). Accordingly, different carrier proteins required to be used
 26 in immunization and screening/purification for screening hapten-specific rather than carrier-specific
 27 antibodies (Tong et al. 2015; Zhang et al. 2007). Despite of numerous reviews outlining the theoretical
 28 advantages of monoclonal antibodies (mAbs) over polyclonal antibodies (pAbs), most competitive
 29 enzyme immunoassays for haptens were based on pAbs (Goodrow and Hammock 1998; Lei et al.
 30 2010; Darwish et al. 2012) since production of mAbs requires time-consuming and labor-intensive
 31 multistep screening procedures with heterologous conjugate (Chappey et al. 1992; Zhang et al. 2009).
 32 Therefore, eliminating the preparation of heterologous conjugates and simplifying the screening
 33 procedure would promote the development of hapten-specific antibodies.

34 Keyhole limpet hemocyanin (KLH) (Pak et al. 1995; Ling et al. 2014), bovine serum albumin (BSA)
 35 (Kim et al. 2004; López-Moreno et al. 2014) and ovalbumin (OVA) (Green et al. 2012; Parra et al.
 36 2014) are the three most common carrier proteins employed for hapten conjugation. In many cases,
 37 KLH (or BSA) conjugate is preferred as immunogen for immunization, while BSA (or OVA) conjugate is
 38 used as coating antigen to monitor the raised antibodies (Jin et al. 2014; Saeed et al. 2017; Gao et al.
 39 2007; Esteve-Turrillas et al. 2015). Hence, BSA or OVA, is often used as control carrier to verify that
 40 the antibodies are specific for the target hapten rather than the carrier itself (e.g. KLH or BSA). In order
 41 to avoid undesired cross-reactivity with the linker regions in antibody-screening procedures and final

42 applications, moreover, different coupling strategies must be employed for preparing immunogens and
43 coating antigens (Esteve-Turrillas et al. 2010; Vasylieva et al. 2015).
44 In our opinion, BSA is the most suitable carrier protein for preparing mAbs against haptens. Thus, the
45 present study was an attempt to use salbutamol (SAL), a β -adrenergic receptor agonist illegally used
46 for feed additive with a molecular weight of 239.31, as a model hapten to show the advantages of BSA
47 as a carrier protein for preparation of hapten-specific mAbs.

48 **2. Materials and methods**

49 **2.1 Reagents**

50 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), bovine serum albumin (BSA), ovalbumin
51 (OVA) horseradish peroxidase (HRP), N-hydroxysuccinimide (NHS), Freund's complete and
52 incomplete adjuvants, polyethylene glycol (PEG, Mw 1450), HT and HAT Media Supplement were
53 supplied by Sigma-Aldrich (Madrid, Spain). Dulbecco's modified Eagle's medium (DMEM) and fetal
54 bovine serum (FBS) were from HyClone Laboratories (Logan, UT, USA). Salbutamol sulfate (SAL),
55 ractopamine hydrochloride, clenbuterol hydrochloride and phenylethanolamine A were from Green
56 Stone Switzerland Co., Ltd. All other chemicals and organic solvents were of analytical grade or better.

57 **2.2 Conjugates of SAL-BSA and SAL-OVA preparation**

58 The introduction of carboxylic group into the structure of SAL, required for protein conjugation, was
59 achieved as described by Beaulieu N, et al. (Beaulieu et al. 1985) with a slight modification. In brief, 5
60 mg of succinic anhydride was added dropwise to the solution of 10 mg SAL in 5 mL at room
61 temperature (RT), and the reaction mixture became orange in color. After centrifugation, the
62 precipitates were thoroughly washed three times with ethanol to remove the unreacted SAL and
63 succinic anhydride, and dried under a nitrogen atmosphere to yield about 15 mg of yellow amorphous

64 solid.

65 Conjugates of SAL-BSA and SAL-OVA were prepared according to the mixed anhydride method
66 (Erlanger et al. 1957) In this procedure, 5 mg of the dry derivative was dissolved in 2 mL of distilled
67 water followed by the dropwise addition of 1.4 mg of EDC and 1 mg of NHS with constant stirring.
68 The reaction mixture stood at RT for 24 h. Subsequently, BSA or OVA (10 mg) dissolved in 5 mL of
69 0.13 M sodium bicarbonate solution was dropwise added to the above solution with continuous stirring,
70 and then further stirred at RT for 24 h. The conjugates were then purified by dialysis against PBS for
71 one day with two changes of fresh buffer. Insoluble precipitates were removed by centrifugation (3,000
72 rpm, 10 min). The dialyzed solutions were frozen at -20 °C until use. Ultraviolet (UV) and sodium
73 dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) were used to identify the conjugates.

74 **2.3 Immunization and antibody generation**

75 Seven-week-old BALB/c female mice (Comparative medicine center, Yangzhou University, Yangzhou,
76 China) were vaccinated intraperitoneally with 0.2 mL of an emulsion containing Freund's complete
77 adjuvant and SAL-BSA conjugate (1:1; at a dose of 5 µg SAL-BSA conjugate per mouse). One booster
78 vaccination was given in two weeks later except for the conjugate emulsified in Freund's incomplete
79 adjuvant.

80 Serum was collected ten days later and tested for antibody titres by indirect enzyme-linked
81 immunosorbent assay (iELISA) and indirect competitive ELISA (icELISA) to determine specific
82 IgM or IgG-class antibody for SAL-OVA and free SAL, respectively.

83 **2.4 Cell fusion and screening**

84 Three days before cell fusion, the mouse was challenged ip with SAL-BSA conjugate alone (0.1
85 mg/mL in PBS, 0.2 mL/mouse). Splenocytes from the immunized mouse were fused with SP2/0

86 myeloma cells according to standard protocols. Fused cells were resuspended gently in 100 mL of
87 DMEM containing 15% FBS and 1× HAT media supplement, and evenly distributed into 96-well cell
88 plates and incubated at 37°C in 5% CO₂ (day 0). At day 5, half of the medium was replaced with fresh
89 DMEM containing 10% FBS and 1× HT media supplement. At day 7, fresh DMEM containing 10%
90 FBS and 1× HT media supplement completely substituted for the medium.

91 The antibody-producing hybridoma were screened by a two-step procedure. On the 10th day of fusion,
92 hybridoma culture supernatants were screened by iELISA. The antibody secreting hybridoma cells
93 were expanded into 48-well plates for being further confirmed to be SAL-specific by indirect
94 competitive ELISA (icELISA). Hybridomas with desired characteristics were subcloned and expanded
95 for antibody production by being injected intraperitoneally into the pristine primed BALB/c mice to
96 obtain ascetic fluids.

97 **2.5 First step: iELISA screening**

98 The 384-well microplates were coated with 10 µL/well of SAL-BSA or SAL-OVA at concentration of
99 10 µg/mL in carbonate-buffered saline (CBS, 0.05 M, pH 9.6) at 37°C for 2 h. After washing three
100 times with phosphate-buffered saline (0.01 M, pH 7.4) containing 0.05% Tween-20 (PBST), the culture
101 supernatants (10 µL/well) were respectively added into the SAL-BSA conjugate coated plates and
102 SAL-OVA conjugate coated plates, and incubated at 37°C for 1 h. The fresh complete medium was
103 used as negative controls. After another washing, the plates were incubated at 37°C for another 1 h with
104 HRP conjugated goat anti-mouse IgG and IgM antibodies diluted 1/10,000 in PBST (10 µL/well). The
105 final washing procedure was followed by a color development, which was initiated by adding 10
106 µL/well of 3,3',5,5'-Tetramethylbenzidine liquid substrate to measure the HRP tracer activity. After
107 incubating at 37°C for 30 min, the enzymatic reaction was terminated by adding 10 µL/well of 1 M

108 H₂SO₄, and the absorbance was measured at 450 nm with a Multiskan Spectrum (Thermo Electron
109 Corporation, San Jose, CA, USA).

110 **2.6 Second step: icELISA screening**

111 Each well of 96-well microplates was coated with 100 µl of SAL–BSA conjugate (1.0 µg/mL) diluted
112 with SCB at 37°C for 2 h. After washing three times with PBST, 50 µL of SAL (1.0 µg/mL diluted in
113 PBS) and 50 µL of the supernatants of positive hybridomas were respectively added to each well. The
114 subsequent steps were performed as above. Relative absorbance was calculated using the formula $100 \times B/B_0$, where B and B₀ were the absorbance of the well with and without SAL. The relative
115 absorbances are around 100% for antibodies not reacting with free SAL.
116

117 **2.7 Effect of FCS contents in culture medium on anti-BSA antibody detection**

118 During the experiment, 96 SAL-BSA-negative hybridoma, each hybridoma divided into three equal
119 parts, were transferred from 96-well cell plates to 48-well cell plates for further BSA-specific antibody
120 screening. On the 3rd day, the medium was replaced with the medium containing 1%, 2% or 5% of
121 FBS. After that the hybridoma cells would be allowed to grow until all died, and the culture
122 supernatants were collected for BSA-specific antibody detection by iELISA as above with BSA as
123 coating antigen.

124 **2.8 Characterization of SAL-specific mAbs**

125 The ascetic fluids were obtained and purified by the caprylic acid/ammonium sulfate method of
126 McKinney and Parkinson (McKinney and Parkinson 1987). The protein content was determined by
127 BCA kit (Pierce, USA). The mAbs were isotyped using a mouse mAb isotyping test kit (Sigma-Aldrich,
128 USA). The icELISA described above was used to evaluate the affinity (K_a) and sensitivity of each
129 mAb. The median inhibitory concentration (IC₅₀) values were calculated by Graph pad Prism 5 to

130 determine the sensitivity. Specificity was defined as the ability of structurally related analogs, including
131 ractopamine, clenbuterol and phenylethanolamine A, to bind the specific mAb and the CR was
132 calculated as: $(IC_{50} \text{ of SAL}) / (IC_{50} \text{ of analogs}) \times 100$.

133 **2.9 A direct competitive ELISA for SAL**

134 After checkerboard optimization of the SAL-BSA coating amount and HRP-conjugated antibody
135 concentration, a direct competitive ELISA (dcELISA) was developed for SAL using SAL-BSA
136 conjugate at 10 µg/mL and HRP-conjugated mAb 3F12 at 1:2,500. Results were reported as the mean
137 of two separate light absorbance values subtracted from the negative control value.

138 **2.10 Statistical Analysis**

139 Mean and standard deviation (SD) were calculated using Microsoft Excel 2007 software.
140 All values in the text, tables, and figures were presented as mean ± standard deviation.

141 **3. Results**

142 **3.1 Conjugates of SAL-BSA and SAL-OVA verification**

143 UV spectra and SDS-PAGE were employed to obtain evidences of successful conjugation of SAL to
144 proteins. Full-scan experiments over the range of 230–390 nm were performed to analyze the UV
145 absorption characteristics of SAL, BSA, OVA and the conjugates. UV spectra in Fig. 1A and 1C
146 showed a red-shift at 280 nm for SAL-BSA compared with 278 nm for BSA, and a red-shift at 279 nm
147 for SAL-OVA compared with 277 nm for OVA. Analysis of SAL conjugates by SDS-PAGE (Fig. 1B
148 and 1D) revealed an increase in the molecular weight of the conjugates when compared with BSA and
149 OVA, respectively. These results clearly indicate the successful conjugation of SAL to BSA and OVA,
150 respectively.

151 **3.2 Determination of SAL-specific antibody titres**

152 Anti-SAL IgM and IgG antibody responses were determined ten days after the third immunization and
153 typical antibody dilution curves were shown in Fig. 2. The antiserum had an IgM antibody binding
154 activity to the SAL-OVA conjugate of 2.049 at 1:1,000 and bound at a dilution of up to 1:32,000.
155 However, after competition with free SAL, the activity was reduced to 1.330 at 1:1,000, and the serum
156 reacted at a dilution of up to 1:32,000 (Fig. 2A). Similar results were obtained with IgG. The antiserum
157 without SAL had an antibody binding activity of 2.642 at a dilution of 1:1,000 and reacted at dilutions
158 of up to 1:32,000. After competition, the binding activity was reduced to 1.479 at a 1:1,000 dilution and
159 bound at a dilution of up to 1:32,000 (Fig. 2B). These results show a successful immunogenic response
160 induced by SAL-BSA conjugate.

161 3.3 SAL-specific hybridoma screening

162 Following fusion of splenocytes with SP2/0 cells and outgrowth of primary hybridoma, the hybridoma
163 culture supernatants from 351 wells were evaluated by iELISA on the 10th day after fusion. Fifteen
164 hybridomas culture supernatants were found to contain antibody-specific to SAL-BSA conjugate,
165 which was consistent with that of SAL-OVA conjugate as coating antigen. After expansion into 48-well
166 plates, nine primary hybridomas were still positive for anti-SAL-BSA antibodies confirmed by iELISA.
167 The positive hybridomas were subsequently tested by icELISA using SAL as competitive inhibitor (1
168 µg/mL). Eventually, five hybridomas secreting mAbs specific for free SAL were cloned and stabilized
169 (Table 1). The mAbs derived from the rest four hybridomas targeted the linker region of SAL-BSA due
170 to their binding activity being only partially or negligibly inhibited by free SAL.

171 3.4 BSA-specific hybridoma screening

172 A total of 96 hybridoma clones were expanded into 48-well plates with the medium decreased in FBS
173 contents from 5% to 1% (v/v). No hybridoma was found to produce BSA-specific mAbs.

174 3.5 Characterization of SAL-specific mAbs

175 The characteristics of five mAbs in terms of titre, affinity, class, and subclass were summarized in
176 Table 1. Except for mAb 6A6 belonging to IgG2a, all other mAbs were IgG1 with λ light chain, and the
177 affinity constants of the mAbs ranged from 10^8 to 10^{11} per mole. The IC₅₀ values of the mAbs for SAL
178 ranged from 1.02 to 5.09 ng/mL with mAb 3F12 being the lowest. Therefore, the specificity of mAb
179 3F12 was further evaluated using the other β -adrenergic receptor agonists, and showed that the CRs
180 were 0.51%, 31.4% and 0.92% for ractopamine hydrochloride, clenbuterol hydrochloride and
181 phenylethanolamine A, respectively. The mAb 3F12 was conjugated to horseradish peroxidase (HRP)
182 through the periodate method (Tijssen and Kurstak 1984).

183 3.6 Determination of SAL by dcELISA

184 After checkerboard optimization of the coating antigen (SAL-BSA) and detection antibody
185 concentration (HRP-3F12), a representative standard curve for the dcELISA was depicted in Fig. 3. A
186 strong negative linear relationship between the relative absorbance and log concentration of SAL was
187 obtained in the range of 312.5–20,000 pg/mL ($R^2 = 0.9959$). The limit of detection (LOD) was 142.9
188 pg/mL of SAL (LOD was calculated as mean – $3 \times$ SD, where mean was based on the responses of
189 blank samples and SD was the standard deviation of analytical responses.). The limit of quantitation
190 (LOQ) was 193.9 pg/mL SAL (mean – $10 \times$ SD, $n = 3$).
191 Intra- and inter-assay precisions were qualified with three standard solutions of SAL at 625, 2,500 and
192 10,000 pg/mL, which was calculated by the relative standard deviation (RSD) resulting from well to
193 well ($n = 6$) within one plate, and inter-assay precision obtaining from different plates ($n = 3$). The
194 analytical performance of the assay was demonstrated in Table 2. Precision analysis showed low RSD
195 ($\leq 9.7\%$) and good accuracy (95.6–108.8%).

196 4. Discussion

197 A hapten is a small molecule that can elicit an immune response only when covalently attached to a
198 large carrier such as a protein. As an ideal carrier of hapten, single polypeptide protein BSA has
199 numerous functional groups (amines, hydroxyls, carboxyls, or sulfhydryl groups) for effective
200 conjugation.^(12, 13) In addition, BSA has been proved to be heterologous enough to evoke an adequate
201 immune response in mice (Hirayama et al. 1990).

202 During immunization with hapten-BSA conjugates, the immunized mice will produce antibodies
203 against both the hapten and BSA. In order to avoid the false-positive results in screening tests, there is
204 a misconception that heterologous conjugates are necessary for screening of hapten-specific
205 antibody-secreting hybridoma. Accordingly, the hepten-OVA conjugates, for instance, should be used
206 to detect the hapten-specific antibodies, and vice versa (Jin et al. 2014; Saeed et al. 2017; Gao et al.
207 2007; Esteve-Turrillas et al. 2015; Esteve-Turrillas et al. 2010). However, in the present study, no
208 hybridoma secreting anti-BSA mAb was screened out using the SAL-BSA conjugate for
209 antibody screening, even though the FBS content in the supernatant was reduced from 10% to 1.0%.

210 Typically, the mAb content in the supernatant of hybridoma is less than 10 µg/mL, which is equivalent
211 to 70 nmol mAb per liter (Kuhne et al. 2014; Tomita et al. 2011). Meanwhile, the average content of
212 albumin in FBS is about 23g/L, which is equivalent to 350 µmol BSA per liter of the hybridoma
213 culture supernatant (10% FBS) (Yang and Xiong 2012). In other words, the molar number of BSA in
214 the supernatant is 5,000 times that of mAb. Considering that each antibody molecule has two identical
215 antigen binding sites (IgM has 10 binding sites), and each BSA molecule has only one identical epitope.
216 Therefore, BSA in the supernatant can fully saturate the binding sites of anti-BSA antibody.
217 Considering the results of another experiment obtained (data not shown), there were many hybridomas

218 secreting anti-OVA mAbs established by using SAL-OVA conjugate as immunogen. The results
219 showed that using immunogen hapten-BSA conjugates as coating antigens can specifically screen out
220 the hapten-specific hybridomas.

221 Due to the small size of hapten and the immunogenicity of cross linker, the bridging group connecting
222 BSA and hapten may be involved in hybridoma screening. To avoid this situation, heterologous
223 conjugates are always used as screening antigens in conventional hybridoma procedure
224 (Esteve-Turrillas et al. 2010). However, the preparation of heterologous conjugates is usually laborious,
225 time-consuming and expensive. More importantly, the coupling process may modify the antigenicity of
226 haptens via influencing their original steric and electronic characteristics (Chappey et al. 1992),
227 and thus reduce the screening efficiency of hapten-specific hybridoma. To avert such problems, an
228 icELISA (as seen in this study) could be used to further confirm free hapten-specific hybridomas by
229 using the hapten-BSA conjugate as a coating antigen and the hapten as a competitive inhibitor.

230 A blocking step is necessary to prevent any nonspecific binding of an antibody to the surface of
231 microplates. If the blocking step can be omitted, the operation time and labor intensity of
232 antigen-specific hybridoma screening can be further reduced. BSA is also a common blocking agent in
233 ELISA methods (Lee et al. 2009). Therefore, when BSA is used as a carrier for immunogens, other
234 blocking agents must be used, including casein, skimmed milk powder, and non-protein blocking
235 solutions (such as Ficoll or polyvinyl alcohol) (Kim et al. 2004; López-Moreno et al. 2014; Huber et al.
236 2009). In this study, it was found that omitting the blocking step would not increase the nonspecific
237 adsorption. The reason was presumably that the amount of BSA in the culture supernatant of
238 hybridoma was far more than that of mAbs, which would give BSA more opportunities to occupy the
239 residual active sites on the microwell surface.

240 **5. Conclusion**

241 The main disadvantages of the anti-hapten mAb preparation are the need to prepare heterologous
242 conjugates and block microplates, which increase much workload and cost of the production, and even
243 reduce the screening efficiency. As a carrier of haptens, this study showed the unique advantages of
244 BSA in the development of haptic-specific mAbs, which greatly simplified the preparation process,
245 reduced the preparation cost, and improved the screening efficiency of anti-hapten mAbs. In addition,
246 the dcELISA is sensitive enough to be used for the determination of SAL in animal feed.

247 **Author Disclosure Statement**

248 The authors have no financial interests to disclose.

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349 Table 1. Characteristics of anti-SAL mAbs

MAbs	Idiotyp	Titres of ascetic fluid	Affinity constant (per mole)	IC ₅₀ (ng/mL)
1A2	IgG1	1:1,280,000	2.5×10^{10}	1.83
1B7	IgG1	1:640,000	4.2×10^8	5.09
3F12	IgG1	1:2,560,000	1.5×10^{11}	1.02
6A6	IgG2a	1:640,000	8.1×10^{10}	1.57
8D2	IgG1	1:1,280,000	5.6×10^9	4.21

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369 Table 2. Intra-assay and inter-assay precisions of SAL detection by the dcELISA

Assay	Standards (pg/mL)	Mean $\pm$ SD	RSD (%)	Accuracy (%)
Intra-assay	625	665.0 $\pm$ 35.1	5.3	106.4
	2,500	2,489.7 $\pm$ 110.9	4.5	99.6
	10,000	10,879.2 $\pm$ 243.5	2.2	108.8
Inter-assay	625	645.0 $\pm$ 62.5	9.7	103.2
	2,500	2,390.2 $\pm$ 163.1	6.8	95.6
	10,000	10,199.5 $\pm$ 535.8	5.3	102.0

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Fig. 1 UV spectra and SDS-PAGE analysis of the conjugates of SAL-BSA and SAL-OVA

Comparative UV absorption spectra with the conjugate and controls. Two microliters of the samples prepared in PBS at about 0.2 mg/ml were injected into NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific Inc. USA) (a) and (c). The SDS-PAGE analysis of the conjugates stained with Coomassie brilliant blue. The SDS-PAGE of the conjugates and carrier proteins were performed using Mini-PROTEAN Tetra System (Bio-Rad Laboratories, Inc. USA) (b) and (d). The SDS-PAGE procedure was performed as previously described by Sambrook 15% separated gel and 5% concentrated gel was used (Sambrook and Russell 2006).

Fig. 2 Dilution curve of a mouse antiserum raised against the SAL-BSA conjugate

The specific titres of IgM (a) and IgG (b) to the SAL-BSA conjugate and SAL alone were assayed with serial dilutions of antiserum by iELISA and icELISA, respectively. The iELISA and icELISA were performed as described in “Materials and Methods” except the serial dilutions of antiserum instead of the supernatants of hybridomas.

Fig. 3 The standard curve for SAL in PBS was determined by the dcELISA

Vertical bars represent standard deviations for three independent assays.

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