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COMPARISON OF THE IN VITRO AND IN VIVO ANTI-HERPES ACTIVITIES OF 1-β-D-ARABINOFURANOSYLTHYMINE AND ITS 5′-MONOPHOSPHATE

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In vitro and in vivo anti-herpes activities of 1-β-D-arabinofuranosylthymine 5′-monophosphate (ara-TMP) were compared with those of 1-β-D-arabinofuranosylthymine (ara-T). On a molar basis ara-TMP was almost as active as ara-T against six strains of herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2) as monitored by a cytopathogenicity-inhibition and a plaque reduction assay in human embryonic lung fibroblast cells. When tested against experimental encephalitis in mice inoculated intracerebrally with HSV-1, intraperitoneal or intravenous treatment with 150 mg/kg/day of ara-TMP or 100 mg/kg/day of ara-T, for 5 days was effective in increasing in the mean survival time of mice. For a single dose of ara-TMP, intravenous administration was more effective than intraperitoneal or oral administration. However, oral administration of ara-T was the most effective of the treatment regimens used. Substantial plasma levels of ara-T were detected for a longer time after oral administration of ara-T than after intravenous administration of ara-TMP or ara-T, suggesting that the efficacy of oral administration of ara-T may be correlated with the maintenance of the substantial blood drug levels.

ara-T ara-TMP herpes simplex virus, types 1 and 2 mouse encephalitis

INTRODUCTION

A number of thymidine analogues exhibit anti-herpes activity in vitro [5,11]. Some of these are effective in the treatment of herpes simplex virus (HSV)-infected animals [3,6,10,12]. In a previous paper [8] we showed therapeutic efficacy of oral administration of 1-β-D-arabinofuranosylthymine (ara-T) for HSV type 1 (HSV-1)- and type 2 (HSV-2)-induced experimental encephalitis in mice. Ara-T was also effective in the treatment of equine abortion virus-infected hamsters [2]. There are some reports concerning the advantage of the 5′-monophosphate of anti-herpetic nucleosides [1,13,14] such as 1-β-D-arabinofuranosyladenine 5′-monophosphate (ara-AMP). Therefore, it was of interest to examine how the anti-HSV activity of ara-T 5′-monophosphate (ara-TMP) compared to that of ara-T. In this paper we evaluated the in vitro anti-HSV activity of ara-TMP and that of ara-T using a viral cytopathic effect (CPE) inhibition test and a plaque reduction

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assay. Their efficacy was also compared against experimental encephalitis in mice using different routes of administration. The benefits of oral administration of ara-T are discussed with respect to its concentration in the blood and urine.

MATERIALS AND METHODS

Cells and virus

Human embryonic lung fibroblast (HEL-F) cells were used in this study. HSV-1 strain VR-3 and HSV-2 strain MS were kindly supplied by Dr. S. Yamazaki, National Institute of Health of Japan, and HSV-1 strains MP and CHR-3 and HSV-2 strains UW-268 and HG-52 were provided by Dr. K. Hayashi, Institute of Medical Science, University of Tokyo. Methods for cultivation of the cells and propagation of the viruses have been described previously [9],

In vitro anti-herpesviral activity

In the viral CPE inhibition test, HEL-F cells were infected with 320 50% tissue culture infective doses of HSV. After 1 h incubation at 37°C, the inoculum was removed. Then the infected cells were incubated with maintenance medium containing an appropriate amount of the test compound in serial half-$\log_{10}$ dilutions for 2 or 3 days at 37°C until the score of HSV-induced CPE reached 4 (100% cell destruction) in the control infected cultures. The anti-HSV activity was expressed as the minimal virus-inhibitory concentration (MIC) of the compound at which viral CPE was reduced by 50%. The MICs are presented as the average values for two separate experiments. A plaque reduction assay was also employed to determine the anti-HSV activity. HEL-F cells grown in a multiwell plate (Linbro FB-12-TC) were infected with about 50 plaque-forming units of HSV-1 or HSV-2 per well. After 1 h absorption, the inoculum was removed and the cultures were overlaid with maintenance medium containing 0.5% Noble agar (Difco) and an appropriate amount of the test compound in serial half-$\log_{10}$ dilutions. Duplicate cultures were employed for each dilution of the compound. After 2 or 3 days of incubation, the number of plaques in each plate were counted microscopically without staining.

In vivo efficacy against encephalitis induced by HSV in mice

Four-week-old male random-bred albino ICR-JCL Swiss mice, weighing 18–20 g, divided in groups of 10 for each drug dose, were inoculated intracerebrally (i.c.) with 10 μl of phosphate-buffered saline (PBS) containing 10 or 32 50% lethal doses of HSV-1 strain VR-3. The infected mice were treated intraperitoneally (i.p.), orally (p.o.) or intravenously (i.v.) with the test compound. Treatment was started at 4 h post-infection in the multiple treatment schedule. A total of 20 mice infected and treated with PBS were used as control. Mice were observed daily for 21 days to identify death and other reactions. Data on the comparative mortalities of control and drug-treated groups were eval-

uated by the Fisher exact test. Student's t test was used to evaluate the difference in mean survival times between the two groups. Only animals that died on or before day 21 were included in calculation of mean survival times.

Determination of blood and urine drug concentrations

A single dose of 200 mg of ara-T or 300 mg of ara-TMP per kg was administered to groups of four 6–8-week-old Swiss mice. The mice were bled with a heparinized syringe at appropriate times after administration. The concentration of the test compound in plasma was determined by a Waters Associates high performance liquid chromatograph (HPLC) model 440 with an ultraviolet detector at 254 nm equipped with an anion-exchange resin, μBondapak C18. The solvent for elution contained 8% Na_2SO_4/0.4% KH_2SO_4 solution and methanol at a volume ratio of 9 : 1. To prevent in vitro conversion of ara-TMP to ara-T, the plasma from mice injected with ara-TMP was heated at 65°C for 5 min. To examine urinary clearance of ara-T or ara-TMP, the groups of four or five mice were housed in metabolism cages, and the urines were collected at 4, 12 and 24 h after administration. The concentration of ara-T and ara-TMP in urine was determined by HPLC.

In vitro and in vivo toxicity

The in vitro toxicity of the compounds was determined by their inhibitory action on growth of HEL-F cells. The cells were plated in 35 mm plastic dishes at 1.5×10^5 cells per dish. After 6 h, the cells were refed with fresh growth medium containing an appropriate amount of the test compound. After 4 days of incubation, cells from duplicate cultures were dispersed by trypsin, and the number of cells was counted. The 50% inhibitory dose (ID_{50}) was estimated graphically as described previously [9]. In vivo acute toxicity was tested using groups of 10 uninfected, 6-week-old female ICR-JCL Swiss mice as described previously [8].

Compounds

The disodium salt of ara-TMP was prepared from ara-T by phosphorylation with phosphoryl chloride [15]. Only this salt was employed as ara-TMP. Therefore the disodium salt of ara-TMP (M_r = 382) is expressed simply as ara-TMP. Thus, a 1.5-fold dose of ara-TMP is equivalent to one dose of ara-T (M_r = 258) on a molar basis. Ara-TMP was characterized by elemental analysis, and ultraviolet and nuclear magnetic resonance spectroscopies. According to HPLC analysis the purity of ara-TMP was about 95%. No ultraviolet-absorbing contaminants were detected. Ara-T is a commercial product of Yamasa Shoyu Co., Ltd. The drugs were usually dissolved in PBS. When the size of the dose of ara-T precluded solution in PBS, the compound was suspended in PBS containing 0.5% carboxymethyl cellulose.

RESULTS

In vitro anti-herpesviral and anti-cellular activities

In the viral CPE inhibition test ara-TMP appeared almost as active as ara-T on a molar basis against six strains of HSV-1 and HSV-2 tested, although the mean MIC of the former (1.31 μg/ml, 3.43 μM) was a little greater than that of the latter (0.66 μg/ml, 2.56 μM) (Table 1). The MIC of ara-TMP for four out of six strains was 1.0 μg/ml, which was equivalent to the MIC of ara-T (0.66 μg/ml). Ara-TMP was less active than ara-T against the other two strains, HSV-1 strain MP and HSV-2 strain UW-268, even on a molar basis.

In the plaque reduction assay ara-TMP exhibited an activity against four strains of HSV that was almost equal to ara-T (Fig. 1). The 50% plaque reduction doses of ara-TMP for HSV-1 strains VR-3 and CHR-3 and HSV-2 strains MS and HG-52 were 0.14, 0.17, 0.40 and 0.58 μg/ml, respectively. The doses of ara-T for the four strains were 0.11, 0.15, 0.29 and 0.50 μg/ml, respectively. These figures indicate that ara-TMP is as active as ara-T against the four strains on a molar basis. On the other hand, 50% plaque reduction doses of ara-TMP for HSV-1 strain MP and HSV-2 strain UW-268 were about 3-fold greater than those of ara-T. The mean 50% plaque reduction doses of ara-TMP for HSV-1 and HSV-2 were 0.22 μg/ml (0.58 μM) and 0.60 μg/ml (1.57 μM), respectively. The doses of ara-T were 0.15 μg/ml (0.58 μM) and 0.28 μg/ml (1.09 μM), respectively. Strain MP, which was the most susceptible to both ara-T and ara-TMP in the CPE inhibition test, was more resistant to ara-TMP than the other strains of HSV-1 in the plaque reduction assay. This may relate to the particular nature of the CPE produced by strain MP, because this strain induced syncytia rather than focal CPE. With respect to the activities of the compounds, however, the results of the plaque reduction assay were parallel with those of the CPE inhibition test.

Neither ara-T nor ara-TMP showed any cytotoxicity for confluent HEL-F monolayers at a concentration as high as 500 μg per ml, when assayed under the same conditions as the antiviral assays. The in vitro toxicity of the drugs was also examined with exponentially growing HEL-F cells. The ID_{50} values of ara-T and ara-TMP were 75 and 50 μg/ml, respectively (average of three separate experiments). Ara-TMP was about twice more inhibitory than ara-T on a molar basis.

TABLE I

Anti-herpes activity of ara-TMP and ara-T as determined by the CPE inhibition test

Compound	MIC[a] (μg/ml)					
	HSV-1			HSV-2		
	VR-3	CHR-3	MP	MS	UW-268	HG-52
Ara-TMP	1.0	1.0	0.66	1.0	3.2	1.0
Ara-T	0.66	0.66	0.32	0.66	1.0	0.66

[a] MIC = 50% inhibitory dose.

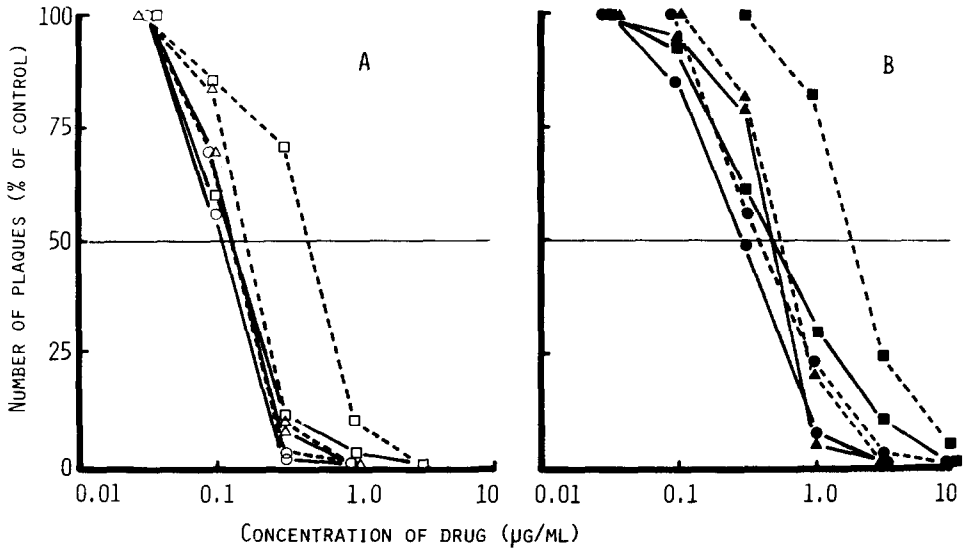

Fig. 1. Effect of ara-T (——) and ara-TMP (- - - -) on the plaque formation of HSV-1 strains VR-3 (○), CHR-3 (△) and MP (□) (A) and HSV-2 strains MS (●), HG-52 (▲) and UW-268 (■) (B). The values are averages for two observations.

Comparison of the therapeutic effects of ara-TMP and ara-T on encephalitis induced by HSV-1 in mice

In the multiple i.p. treatments, ara-TMP (75–300 mg/kg) as well as ara-T (50–200 mg/kg) effectively increased the mean survival time of mice infected with HSV-1 (Table 1, Expt. 1). Treatment with 200 mg of ara-T per kg twice a day for 5 days reduced the final mortality in the group of mice infected with 10 LD_{50} of HSV-1, while the treatment with 300 mg of ara-TMP did not. Using i.v. treatment once a day for 5 days, dosages of 50, 100 and 200 mg of ara-T or 150 and 300 mg of ara-TMP per kg increased the mean survival time; a dosage of 75 mg of ara-TMP did not cause a statistically significant difference (Table 2, Expt. 2). There was no difference in therapeutic efficacy between i.p. and i.v. treatment of ara-TMP.

A large single dose of ara-TMP (2400 mg/kg) was effective in increasing the mean survival time irrespective of the route of delivery of the drug (Table 3, Expt. 1). However, only i.v. treatment reduced the final mortality in the group of mice infected with 32 LD_{50} of HSV-1. Although i.v. treatment with ara-TMP seems to be more effective than either i.p. or p.o. treatment, p.o treatment with ara-T was superior to i.v. treatment with ara-TMP (Table 3, Expt. 2).

TABLE 2

Comparison of therapeutic effects of i.p. and i.v. treatment with are-T and ara-TMP on HSV-1-induced encephalitis in mice[a]

Expt.	Treatment/dose (mg/kg)		Administration route	Survivors/total	Mean survival time (days)
1	PBS		i.p.	1/20	4.9 ± 0.23[b]
	Ara-T	50	i.p.	0/10	7.1 ± 0.74[c]
		100	i.p.	3/10	7.4 ± 0.72[d]
		200	i.p.	7/10[d]	13.0 ± 2.08[d]
	Ara-TMP	75	i.p.	1/10	6.8 ± 1.23[e]
		150	i.p.	2/10	6.9 ± 0.55[d]
		300	i.p.	1/10	8.7 ± 0.76[d]
2	PBS		i.v.	0/20	4.5 ± 0.22
	Ara-T	50	i.v.	0/10	5.9 ± 0.48[c]
		100	i.v.	1/10	6.4 ± 0.53[d]
		200	i.v.	1/10	6.2 ± 0.52[c]
	Ara-TMP	75	i.v.	0/10	5.2 ± 0.52
		150	i.v.	0/10	5.4 ± 0.44[e]
		300	i.v.	0/10	6.0 ± 0.44[c]

[a] Groups of mice were inoculated (i.c.) with 10 LD_{50} (Expt. 1) or 32 LD_{50} (Expt. 2) of HSV-1 VR-3 and treated with drug twice in Expt. 1 or once in Expt. 2 daily for 5 days.
[b] Standard error.
[c] $P < 0.01$.
[d] $P < 0.001$.
[e] $P < 0.05$.

Blood drug level and urinary excretions of ara-T after administration of the compounds in mice

When 200 mg of ara-T per kg were administered i.v. to mice, the blood concentration of ara-T 15 min after administration was higher than 200 μg per ml of plasma; then it rapidly decreased (Fig. 2A). Six hours after administration ara-T was not longer detected in the plasma. The blood drug concentration after i.p. administration of ara-T was similar to that after i.v. administration (data not shown). On the other hand, upon p.o. dosage of ara-T, active drug concentration (1 μg/ml) persisted in the blood for about 8 h, although the maximal concentration was relatively low, compared with i.v. administration. The blood drug concentrations following i.v. administration of 300 mg of ara-TMP per kg were almost identical to those following i.v. administration of 200 mg of ara-T per kg (Fig. 2B). Ara-TMP must be dephosphorylated immediately after administration since only ara-T, and not ara-TMP, was found in the plasma of mice, even as early as 15 min after administration.

Ara-T administered i.p. or i.v. was much more rapidly excreted into the urine than

TABLE 3

Effect of single-dose treatment with ara-TMP on HSV-1-induced encephalitis in mice[a]

Expt.	Treatment/dose (mg/kg)		Administration route	Survivors/total	Mean survival time (days)
1	PBS		i.p.	0/20	5.0 ± 0.34[b]
	Ara-TMP	600	i.p.	1/10	4.7 ± 0.33
		2400	i.p.	1/10	6.9 ± 0.89[c]
		600	p.o.	0/10	5.9 ± 0.31
		2400	p.o.	0/10	6.3 ± 0.37[c]
		600	i.v.	0/10	6.0 ± 0.45
		2400	i.v.	3/10[c]	7.8 ± 1.11[d]
2	PBS		i.v.	0/20	6.1 ± 0.34
	Ara-TMP	600	i.v.	1/10	7.9 ± 0.42[d]
		2400	i.v.	3/10[c]	8.6 ± 1.02[d]
	Ara-T	400	i.v.	0/10	7.6 ± 0.43[c]
		1600	i.v.	3/10[c]	8.9 ± 0.86[d]
		400	p.o.	3/10[c]	6.6 ± 0.37
		1600	p.o.	6/10[e]	8.8 ± 1.59[c]

[a] Groups of mice were inoculated (i.c.) with 32 LD_{50} (Expt. 1) or 10 LD_{50} (Expt. 2) of HSV-1 VR-3 and treated with drug once at 12 h after virus inoculation.
[b] Standard error.
[c] $P < 0.05$.
[d] $P < 0.01$.
[e] $P < 0.001$.

that administered p.o. (Table 4). About 82% of ara-T administered (200 mg/kg) i.p. or i.v. was recovered from the urine within 4 h. About 85–90% of ara-T administered i.p. or i.v. was recovered from the urine within 24 h. It should be noted that still a rather large amount of ara-T was recovered in 12–24 h urine when administered p.o., indicating that ara-T was excreted into urine continuously for more than 12 h after administration. Interestingly, the amounts of ara-T recovered from the urine of mice given ara-TMP were almost equal to those given ara-T by the same injection route. Ara-TMP was not detected at all in the urine. The minimal detectable level of ara-TMP in urine by the HPLC analysis was as high as about 500 μg per mice. However, since almost all ara-TMP (upon i.p. or i.v. administration) and half of the ara-TMP (upon p.o. administration) was recovered as ara-T from the urine, ara-TMP must be readily converted to ara-T within the organism.

Toxicity of ara-TMP for mice

In vivo toxicity of ara-TMP was studied by evaluation of the single-dose acute toxicity

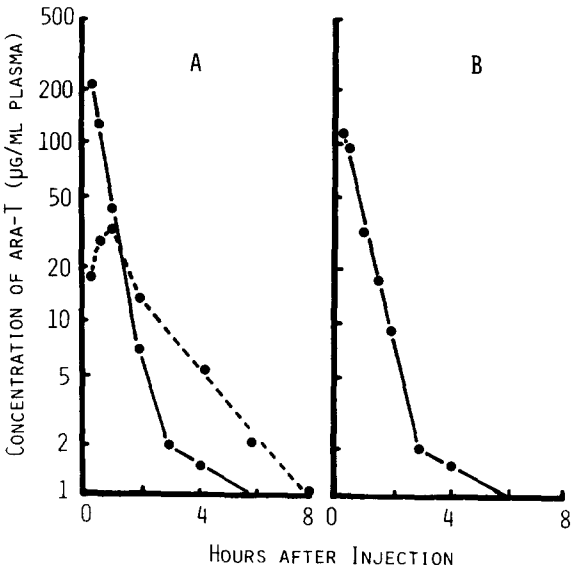

Fig. 2. Blood concentrations of ara-T after i.v. (——) and p.o. (- - -) administration of 200 mg of ara-T per kg (A) and i.v. administration of 300 mg of ara-TMP per kg (B). Groups of four mice were treated with the drug. The concentrations at each point represent the average values for four mice.

for mice. The 50% lethal doses (LD_{50}) of ara-TMP were 3.90, 3.90 and more than 10 g per kg following i.p., i.v. and p.o. administration, respectively. Although the LD_{50} for i.p. administration was lower than that of ara-T [8], ara-TMP can be considered relatively non-toxic for mice.

TABLE 4

Urinary excretion of ara-T after administration of ara-T and ara-TMP in mice

Administration[a]	Average of the amounts of ara-T recovered from urines (μg/mouse)		
	0–4 h[b]	4–12 h	12–24 h
Ara-T, 200 mg/kg, i.p.	4960 (82.7)[c]	177 (3.0)	13 (0.2)
Ara-T, 200 mg/kg, i.v.	5911 (82.1)	529 (7.3)	<3[d]
Ara-T, 200 mg/kg, p.o.	2473 (41.2)	304 (5.1)	163 (2.7)
Ara-TMP, 300 mg/kg, i.p.	5282 (75.5)	1060 (15.0)	6[e] (0.1)
Ara-TMP, 300 mg/kg, i.v.	5969 (82.9)	291 (4.0)	<3
Ara-TMP, 300 mg/kg, p.o.	2385 (33.5)	983 (13.8)	<3

[a] Groups of four or five mice, weighing 30–36 g, were given ara-T or ara-TMP at the indicated doses.
[b] Urines were collected at the times indicated.
[c] In parentheses percent of the administered amount.
[d] Ara-T was not detected by HPLC analysis.
[e] Ara-T (24 μg) was recovered from only one out of four specimens.

DISCUSSION

In the present paper, we have compared anti-HSV activities of ara-TMP and ara-T. In vitro activity of ara-TMP against four of six strains of HSV-1 and HSV-2 tested was as high as that of ara-T on a molar basis, although ara-TMP was less active than ara-T against the two other strains, strain MP and UW-268. This result stemmed from two different assay systems, the viral CPE inhibition test and the plaque reduction test. Ara-TMP was readily converted to ara-T during incubation with the culture medium, both in the presence and in the absence of the cells (data not shown). Therefore, ara-TMP, even if it exhibited low permeability through cell membrane, could show antiviral activity due to its conversion to ara-T in the medium or on the surface of cell membrane. Sidwell et al. [13] reported that in vitro antiviral activity of ara-AMP appeared equivalent to that of the corresponding nucleoside, 9-β-D-arabionfuranosyladenine (ara-A). Our findings on ara-TMP and ara-T agree with their results on ara-AMP and ara-A.

In mice inoculated i.c. with HSV-1, a single i.v. injection of ara-TMP was more effective than other routes of administration. 9-β-D-Arabinofuranosylhypoxanthine 5′-monophosphate (ara-HxMP) is also suitable for i.v. therapy because of its high solubility in water [1]. Ara-TMP as well as ara-HxMP [1] and ara-AMP [5] may be advantageous because of this high solubility (more than 30% for ara-TMP; unpublished data). In addition, ara-TMP, like ara-T [8] showed extremely low toxicity for mice. Water-soluble derivatives of 1-β-D-arabinofuranosylpurine were shown to be significantly effective against experimental HSV-induced keratitis in rabbits [14]. Ara-TMP may be clinically useful since it is highly soluble and relatively non-toxic. Actually, Mizuno et al. (1979, General Meeting of the Japanese Clinical Ophthalmology, Tokyo) showed therapeutic efficacy of ara-TMP eye drops in the treatment of herpes keratitis in humans.

A single p.o treatment with ara-T was the most effective among single-dose treatments with ara-T or ara-TMP. The benefit of oral ara-T administration may be correlated to its persistence in the blood. Likewise, De Clercq et al. [4] have reported that active drug concentrations persisted longer in the blood of mice after p.o. administration of *E*-5-(2-bromovinyl)-2′-deoxyuridine than after i.p. or subcutaneous treatment. The longer persistence of ara-T in the blood of mice given ara-T p.o. may be the reason why substantial amounts of the drug were excreted into the urine within 12–24 h after oral administration (Table 4). On the other hand, neither are-TMP nor ara-T was detected in the urine of mice given ara-TMP either p.o., i.v. or i.p. Several micrograms of ara-T per ml of serum of rabbits were found as late as 24 h after p.o. dosage of 200 mg of ara-T per kg (Shiota and Machida, in preparation). The rate of absorption and clearance of the drug may be different from one animal species to another. Further pharmacokinetic studies in higher animals would be necessary, should ara-T or ara-TMP be pursued for their clinical use.

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