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Effects of Calcium Antagonists in Multidrug Resistant Primary Human Renal Cell Carcinomas

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ABSTRACT

Human renal cell carcinomas display a characteristically high degree of intrinsic chemoresistance to a multitude of chemotherapeutic agents. It was suggested previously, that P-170 glycoprotein contributes to this phenomenon in renal cell carcinoma indicated by elevated MDR-1 gene mRNA levels and by the expression of this specific resistance characteristic. The P-170-related efflux mechanism can be inactivated by certain calcium antagonists.

P-170 was traced immunohistochemically using monoclonal antibody C 219. Concomitantly, we studied the enhancement of vinblastine cytotoxicity with 4 major classes of calcium-blocking agents in a microculture tetrazolium assay. Seven different calcium antagonists were selected: verapamil (VPM, racemic form), its *R*-stereoisomer (*R*-VPM), diltiazem, flunarizine, nifedipine, and its derivatives nimodipine and nitrendipine.

Verapamil or *R*-verapamil causes a significant decrease of viable tumor cells as compared to vinblastine alone ($P < 0.001$). Similar effects were found with diltiazem, nifedipine, and its derivatives reaching approximately 70% of the VPM/*R*-VPM activity. Flunarizine showed only minor enhancement of cytotoxicity. P-170 expression was demonstrated in 18 of 32 tumors, and a relation to chemoresistance was evident. None of the chemoresponders, but 18 of 25 (72%) of the highly resistant tumors, revealed this resistance factor. It was concluded that certain calcium antagonists in combination with chemotherapy may well offer therapeutic options in renal cell carcinoma as they apparently inactivate the underlying mechanism conferring resistance. The new stereoisomer *R*-VPM, in particular, may be used in clinical trials since it combines strong enhancement of vinblastine drug responsiveness with a 10-fold lower cardiovascular activity as compared to racemic VPM, thus allowing higher concentrations to be applied.

INTRODUCTION

Intrinsic chemoresistance has major impact on the dismal prognosis of patients with disseminated RCC,[2] since reliable therapeutic alternatives are still lacking (1). P-170 glycoprotein was identified as a putative agent conferring drug resistance. Recently, a high expression of *MDR-1* gene mRNA (2) as well as of P-170 glycoprotein (3) was traced in human RCCs, which may partially explain the high degree of *de novo* resistance. CAs are substances which interfere with the calcium intake at the cytoplasmic membrane. They are mainly used in the treatment of cardiovascular disorders such as hypertension, arrhythmia, and angina pectoris. Lately, the interaction of CAs with chemotherapeutics to enhance cytotoxicity has gained particular interest. It was shown that CAs reverse acquired resistance of experimental animal tumors (4) to some anticancer drugs and increase the life span of tumor-bearing animals compared to those merely treated with *Vinca* alkaloids (5).

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[2] The abbreviations used are: RCC, renal cell carcinoma; CAs, calcium antagonists; VBL, vinblastine; NIF, nifedipine; DIL, diltiazem; NIT, nitrendipine; NIM, nimodipine; FLU, flunarizine; VPM, verapamil; MTT, microculture tetrazolium assay; MDR, multidrug resistance.

In solid human tumors like non-small cell lung cancer (6), colon cancer (7), or glioblastomas (8), CAs potentiate the effect of vincristine. The mechanisms are not yet fully understood, but there is substantial evidence that inhibition of the energy-dependent drug efflux related to P-170 glycoprotein in MDR cells plays an important role (9, 10).

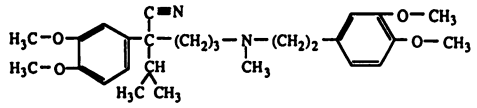
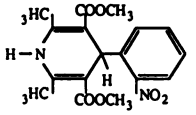
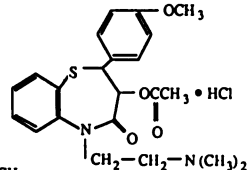
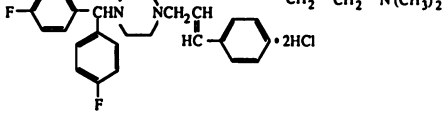
Recent publications have demonstrated that, in at least 5 cases of multiple myeloma and one case of non-Hodgkin's lymphoma, clinical drug resistance is associated with P-170 glycoprotein expression and that the addition of verapamil can partially reverse chemoresistance (11). VBL is accepted to be the most potent single cytostatic drug used in RCCs (1), even though its clinical use is very limited at present as not more than 20% of the patients with metastatic RCC show a response. We were, therefore, interested in evaluating the effects of VBL in combination with certain CAs in primary human RCCs. We selected representatives of 4 major classes of calcium-blocking agents (12) such as VPM (racemic form), NIF, DIL, and FLU (Table 1). In addition, we assessed *R*-VPM, the *R*(+)-stereoisomer of VPM, since it revealed a 10-fold lower cardiovascular activity than racemic VPM (13) as well as NIT and NIM, modern derivatives of NIF.

MATERIALS AND METHODS

Fresh tumor specimens from 32 primary human RCCs were placed in chilled Hanks' balanced salt solution (Seromed, Berlin, Federal Republic of Germany) and processed under sterile conditions within 1 h after nephrectomy. Because of the biological heterogenity of the tumors, large and representative segments of separate parts were selected and carefully freed from fat, connective tissue, and necrotic areas. Hematoxylin and eosin-stained smears were used as a matter of routine in order to avoid a major admixture of nontumorous material. Aliquots of the tumor probes served to determine the degree of chemoresistance and for examining the reversal of drug resistance by calcium antagonists or were shock-frozen in liquid nitrogen and preserved below −80°C for further immunohistochemical studies.

MTT. The basic feature of the MTT is the cell-generated conversion of a yellow tetrazolium salt into a purple formazan precipitate. This reaction directly indicates cell viability (14). Single cell suspensions were prepared by mechanical disaggregation. Tumor cells were filtrated through textile multilayer gauze (pore size, 50–100 μm; Schweizerische Seidengaze Fabrik, Zurich, Switzerland) and purified by Ficoll density centrifugation (density, 1.07; 400 × *g*, 10 min, 4°C; Seromed). Specimens from well-defined and small RCCs (maximum diameter, 5 cm) were used exclusively, and the primary cell suspensions contained approximately 80% viable cells (trypan dye exclusion test); more than 90% were classified cytologically (hematoxylin-eosin stain) as tumor cells. Cell suspensions were plated in monolayer flasks (Flow Laboratories GmbH, Meckenheim, Federal Republic of Germany) containing culture medium (RPMI 1640 without phenolred, supplemented with 20% synthetic NU serum; Flow) and maintained until an exponential growing phase was reached. Tumor cells were then distributed by multichannel pipettes into 96-well, flat-bottomed microculture plates (Flow) and exposed to antineoplastic agents. After the desired incubation period, MTT (Sigma Chemical Co.) was added and incubation continued for an additional 4 h. The formazan crystals were solubilized with dimethyl sulfoxide (Merck) and the plates read at 540 nm in a

Table 1 *Structures and properties of calcium antagonists*

Class[a]	Name	Derived from	Activity: Cardiac	Activity: Peripheral	Structure
I	VPM	Papaverine	++	(+)	
II	NIF	Dihydropyridine	+	+	
III	DIL	Benzothiazepine	+	+	
IV	FLU	Piperazine	–	+	

[a] From Ref. 12.

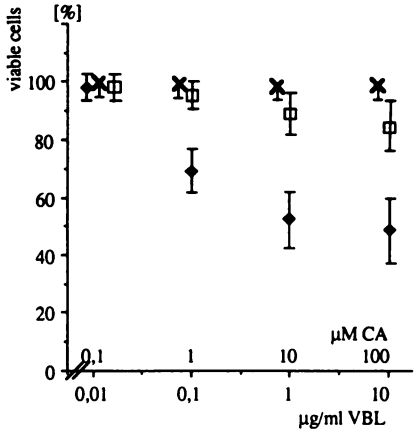


Fig. 1. Determination of the degree of chemoresistance in human renal cell carcinomas (n = 32) using an MTT. Values are expressed as a percentage of viable tumor cells as compared to untreated groups. VBL was applied in final concentrations of 0.01–10 µg/ml cell suspension for 16 h. MTT was then added for an additional 4 h and absorbence measured at 540 nm. The effect of CAs was studied in final concentrations of 0.1–100 µM under the same conditions. □, highly resistant (n = 25); ◆, less resistant (n = 7); X, only calcium antagonists (VPM, *R*-VPM, DIL, NIF, NIT, NIM, FLU).

microculture plate reader (Titertek Multiscan Plus MK II).

Test Conditions. The calcium antagonists [*R*-VPM, VPM (Knoll, Ludwigshafen, FRG), NIF, NIT, NIM (Bayer, Leverkusen, FRG), DIL (Goedecke, Berlin, FRG), and FLU (Janssen, Neuss, FRG)] were dissolved in ethanol:sterile water (1:1) at 10^{-2} M, light protected, and serially diluted in culture medium immediately before use. VBL (E. Lilly, Giessen, FRG) was prepared in accordance with the manufacturer's instructions at 1 mg/ml as stock solution and serially diluted in culture medium. Prior to the addition of VBL, 10^4 tumor cells/well were preincubated for 1 h with the calcium antagonist to achieve a good membrane saturation. The exposure time to VBL was 16 h with a maximum concentration of 1 µg/ml VBL in the presence of the chemosensitizing agents. Subsequently, the MTT staining assay was performed. The cell viability of the untreated group was concomitantly analyzed. Less than 10% of the tumor cells died naturally during this culture period. Since human RCCs behave like floating cells in microculture plates, neither the CAs nor VBL were removed prior to the application of the MTT dye to prevent differences in the residual volume of the test vials or the loss of cells. Synthetic NU serum (Flow) as a supplement to the culture medium was required for this procedure, because this compound did not cause false reading of the absorbance owing to serum precipitates in organic solvents. When validating the test conditions, we applied VBL for 72 h at a maximum concentration of 0.1 µg/ml and the results were confirmed, but the number of viable cells in the untreated control group decreased to approximately 75%. Therefore, the shorter incubation period was preferred as the standard assay protocol.

Evaluation. Peripheral wells served as blanks (MTT + VBL + calcium antagonists + culture medium without cells). The mean of 4 identical wells determined each point. Values were expressed as a percentage of the untreated trial groups in order to determine the effect of the chemotherapeutic VBL alone or of groups containing the maximum concentration of VBL in order to measure the reversal of resistance by chemosensitizers:

$$\%\ \text{viable cells} = \frac{\text{absorption of test} - \text{absorption of blank}}{\text{absorption of control groups} - \text{absorption of blank}} \times 100$$

Student's t test was used for statistical analysis, and the Bonferroni-Holm correction for curves was applied (15).

Demonstration of P-170 Glycoprotein. Cryostat cuts (6 µm) of the RCCs were air-dried and fixed in acetone. The primary monoclonal antibody C 219 (Centocor, Brussels, Belgium) (16) was applied (5–20 µg/ml; 2 h). After the tumor tissues were washed, they were incubated with biotinylated sheep anti-mouse immunoglobin second antibody (dilution 1:50, 30 min; Amersham, Braunschweig, FRG) and the streptavidine-biotinylated horseradish peroxidase complex binding (Amersham) (dilution 1:100; 30 min) was carried out. Peroxidase activity was visualized by 3-amino-9-ethylcarbazole/H_2O_2 (5–10 min). Counterstaining was performed with hematoxylin. Negative trials were run without a primary antibody. MDR/Chinese hamster ovary cells served as positive control groups. The tissues were scored as positive or negative in accordance with the color reaction. A minimum of 10 different cryostat sections from various parts of the tumor were analyzed. Although the staining reaction appeared focal rather than diffuse and the color intensity varied, a positive signal at the cytoplasmic membrane was, when present, clearly visible in positive samples. The decision, however, to declare a tumor definitely negative was based on the absence of positive staining in any cell of any part of the tumor examined.

All reagents were of pro analysii grade and were, if not otherwise stated, purchased from Boehringer Mannheim (Mannheim, FRG).

RESULTS

Of 32 RCCs, 25 proved to be highly refractory to VBL in the MTT (Fig. 1, Table 2), *e.g.*, the number of viable tumor cells in

Table 2 *Effect of 1 μm of various CAs combined with 1 μg/ml VBL on human RCCs*

Exposure of CAs was 1 h; VBL was then added for an additional 16 h, followed by MTT addition for another period of 4 h, and absorbance was measured at 540 nm immediately after dimethy sulfoxide solubilization of formazan crystals.

No.	Age (yr)	Sex	pTNM[a]	Grade[a]	VBL[b] (1 μg/ml)	VPM[c] (1 μm)	R-VPM[c] (1 μm)	NIF[c] (1 μm)	DIL[c] (1 μm)	FLU[c] (1 μm)	P-170[d]
1	57	M	pT2	2	92	41	41	49	48	81	+
2	48	F	pT1	1	96	42	40	51	48	83	+
3	68	M	pT3a	2	87	44	45	49	49	77	+
4	76	M	pT3a	1	91	41	42	50	49	82	+
5	55	F	pT3aM1	2	77	37	39	47	48	73	−
6	61	M	pT2	2	90	42	40	47	47	80	+
7	58	F	pT2	1	96	45	45	50	49	84	+
8	66	M	pT3b	1	92	46	44	49	48	79	−
9	69	M	pT2	1	98	40	39	45	45	86	+
10	73	F	pT2	2	87	45	48	55	52	73	−
11	61	M	pT3bN2	1	94	41	42	48	48	82	+
12	66	F	pT2	2	92	42	40	47	46	80	+
13	59	M	pT3a	3	72	37	38	44	42	65	−
14	77	M	pT2	2	89	40	43	48	46	76	+
15	68	M	pT2	1	95	41	45	52	51	82	+
16	62	F	pT3aN2	2	75	42	38	44	45	67	−
17	52	M	pT2	1	90	37	39	45	45	81	+
18	78	M	pT2	2	94	36	44	53	51	80	+
19	72	M	pT2	2	96	47	45	52	51	84	+
20	67	M	pT2	3	70	41	39	44	44	62	−
21	65	M	pT3a	1	97	42	42	50	49	87	+
22	59	M	pT2	2	89	47	43	49	48	79	+
23	73	F	pT3b	3	74	37	39	44	44	69	−
24	69	F	pT2	2	90	44	40	50	48	80	+
25	67	M	pT1	2	95	42	42	50	50	82	+

[a] Tumor classification (17).
[b] Values are expressed as % of viable cells as compared to untreated controls.
[c] Combined with 1 μg/ml VBL, values are expressed as % of viable cells as compared to controls containing 1 μg/ml VBL. The mean of 4 replicate wells determined each point.
[d] Monoclonal antibody C 219 (16).

Table 3 *Effect of 1 μm of various CAs combined with 1 μg/ml VBL on less VBL resistant human RCCs*

Exposure of CAs was 1 h; VBL was then added for an additional 16 h, followed by MTT addition for another period of 4 h, and absorbance was measured at 540 nm immediately after dimethyl sulfoxide solubilization of formazan crystals.

No.	Age (yr)	Sex	pTNM[a]	Grade[a]	VBL[b] (1 μg/ml)	VPM[c] (1 μm)	R-VPM[c] (1 μm)	NIF[c] (1 μm)	DIL[c] (1 μm)	FLU[c] (1 μm)	P-170[d]
S1	44	F	pT2	2	58	40	38	43	44	54	−
S2	67	F	pT2	2	56	34	35	44	41	56	−
S3	72	M	pT3b	3	49	39	41	45	44	48	−
S4	61	F	pT2	1	62	42	41	49	49	52	−
S5	76	M	pT3a	3	53	34	34	41	44	49	−
S6	58	M	pT3b	2	59	38	39	46	50	55	−
S7	66	M	pT2	3	55	39	38	47	52	55	−

[a] Tumor classification (17).
[b] Values are expressed as % of viable cells as compared to untreated controls.
[c] Combined with 1 μg/ml VBL; values are expressed as % of viable cells as compared to controls containing 1 μg/ml VBL. The mean of 4 replicate wells determined each point.
[d] Monoclonal antibody C 219 (16).

the presence of this chemotherapeutic agent did not distinctly differ from that of the untreated control group, whereas in 7 cases (Fig. 1, Table 3), a clear reduction was measured. This division into highly resistant and less resistant tumors is significant from a concentration of 0.1 μg/ml VBL ($P < 0.001$). All CAs examined in concentrations of ≤100 μM did not affect the ability of RCCs to produce MTT formazans (Fig. 1).

The combined application of calcium antagonists and VBL, however, led to a varying significant decrease of viable tumor cells (Fig. 2), depending on the substance applied. The strongest reversal of chemoresistance was obtained with VPM or *R*-VPM ($P < 0.001$), whereas FLU displayed only minor effects.

NIF, NIT, NIM, and DIL exhibited similar behavior and reached about 70% of the VPM/*R*-VPM activity. High conformity between NIF and its derivatives NIT and NIM, as well as between VPM and *R*-VPM, was ascertained (Fig. 2, Tables 2–4). Furthermore, Fig. 2 demonstrates that a minimum concentration of 1 μM VPM/*R*-VPM, NIF, or DIL was required to halve tumor cell viability in the presence of 1 μg/ml VBL, as depicted in Table 2. The data indicate that all primary RCCs were influenced by VBL + VPM/*R*-VPM and also by DIL and NIF (Tables 2 and 3).

The degree of VBL potentiation by CAs showed a positive correlation to the extent of intrinsic chemoresistance to this drug, *i.e.*, the highest enhancement was obtained in the most drug-refractive RCCs and vice versa. The accumulated results regarding *R*-VPM, in Tables 2 and 3, respectively, have been depicted in Fig. 3.

P-170 glycoprotein was traced in 18 of 32 RCCs using monoclonal antibody C 219. Seventy-two percent of the highly resistant tumors expressed this specific resistance characteristic, but none of the less resistant cases did so (Tables 2 and 3). Eight of 10 well-differentiated (G_1) (17) and 10 of 16 quite well-differentiated (G_2), but none of 6 undifferentiated (G_3), RCCs were detected to be P-170 positive (Tables 2 and 3).

DISCUSSION

So far, clinical trials for enhancing the cytotoxicity of various chemotherapeutic agents by the combination with VPM have not been very rewarding. The limiting factor proved to be the inherent cardiovascular activity of racemic VPM with subsequent hypotension and/or prolonged atrioventricular conduc-

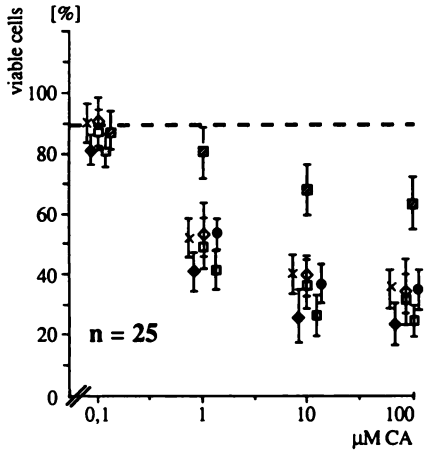


Fig. 2. Reversal of chemoresistance by calcium antagonists in human RCCs (n = 25, group of highly resistant tumors from Fig. 1). Values are expressed as a percentage of viable tumor cells exposed to different concentrations of calcium antagonists and to a constant concentration of 1 μg/ml VBL as compared to groups which only contain the same amount of chemotherapeutic agent. *Dashed line*, effect of 1 μg/ml VBL alone on this group of RCCs (data derived from Fig. 1); —, vinblastine (1 μg/ml); ■, VBL (1 μg/ml) + NIF; ■, VBL (1 μg/ml) + DIL, ◆, VBL (1 μg/ml) + VPM, ■, VBL (1 μg/ml) + FLU; X, VBL (1 μg/ml) + NIT; ●, VBL (1 μg/ml) + NIM; □, VBL (1 μg/ml) + *R*-VPM; *bar*, SD.

Table 4 *Effect of 1 μm of dihydropyridine derivatives combined with 1 μg/ml VBL on human RCCs*

Exposure of CAs was 1 h; VBL was then added for an additional 16 h, followed by MTT addition for another period of 4 h, and absorbance was measured at 540 nm immediately after dimethyl sulfoxide solubilization of formazan crystals.

No.[a]	NIF[b]	Nitrendipine[b]	Nimodipine[b]
8	49	50	52
9	45	44	43
10	55	55	54
11	48	46	44
12	47	45	51
13	44	44	43
14	48	47	49
15	52	54	52
16	44	44	45
17	45	48	46
18	53	50	51
19	52	54	53
20	44	47	45
21	50	49	51
22	49	49	48
23	44	43	46
24	50	49	51
25	50	48	49
S6	46	47	44
S7	47	44	46

[a] Numbers refer to Tables 2 and 3.

[b] 1 μm combined with 1 μg/ml VBL; values are expressed as % of viable cells as compared to controls containing 1 μg/ml VBL. The mean of 4 replicate wells determined each point.

tion time. No objective tumor remissions after i.v. injection (18), or only partial responses after oral administration of VPM (19, 20), were observed. One possible explanation for the differing clinical results as a consequence of the route of administration may be that higher doses can be tolerated after oral uptake of racemic VPM (21). This fact has been attributed to the inherent properties of the components of racemic VPM. With i.v. application, both stereoisomers (*R*-VPM + *L*-VPM) exert their effects simultaneously, whereas with oral administration, *R*-VPM, as well as the metabolite norverapamil, becomes efficaceous owing to diverse resorption and to a different so-called "first pass" effect in the liver (22). However, *R*-VPM reveals 10-fold lower cardiovascular activity than racemic VPM (13).

The experimental data on human RCCs show that *in vitro* concentrations of at least 1 μM VPM are required to enhance

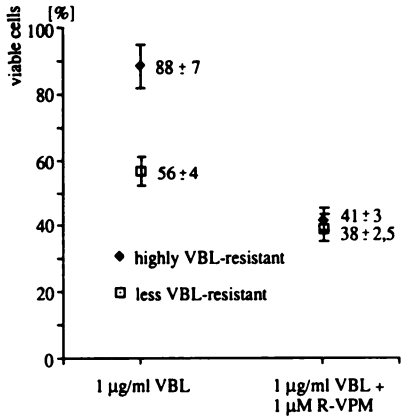


Fig. 3. Effect of 1 μg/ml VBL in combination with 1 μM *R*-VPM on human RCCs with regard to the degree of chemoresistance. Highly resistant (n = 25), detailed in Table 2; less resistant (n = 7), detailed in Table 3. Values are expressed as a percentage of viable tumor cells exposed to untreated control groups to determine the effect of vinblastine and of control groups containing 1 μg/ml VBL to measure the effect of *R*-VPM. *Bar*, SD.

the effect of VBL significantly (Fig. 2). These concentrations are difficult to realize *in vivo* (21). Therefore, the clinical application of chemosensitizing agents such as CAs requires reduced cardiovascular activity of the CAs while they maintain their influence on chemoresistance. Among the evaluated CAs, only the newly available isomer *R*-VPM (Fig. 2, Tables 2 and 3) fulfills these criteria with adequate potentiation of VBL cytotoxicity (Table 2, Figs. 2 and 3) and with 10-fold lower cardiovascular activity (13). But even in high concentrations of up to 100 μM, none of the CAs evaluated overcame VBL resistance totally in any of the 32 human RCCs, and a substantial percentage of tumor cells survived (Fig. 2, Tables 2 and 3).

Apparently, undifferentiated RCCs in this series (13, 20, 23, S3, S5, S7) appear to respond with a stronger decrease of viable tumor cells under the influence of VBL than G_1 or G_2 tumors. The explanation for this could be the lack of P-170 expression in these cases (Tables 2 and 3). In fact, P-170 expression seems to prevail in G_1 and G_2 tumors (Table 2). Indirect support may be deduced from previous reports referring to 42 renal cell carcinomas displaying a correlation between tumor grading and the expression of MDR-1 RNA. Elevated levels were traced in well-differentiated RCCs in contrast to poorly differentiated carcinomas (23). Furthermore, these authors report that the reversal of chemoresistance effected by quinidine in human RCCs showed a relationship to elevated MDR-1 RNA levels. Our data using different CAs suggest that the circumvention of resistance was more pronounced in those cases which exhibited the resistance characteristic of P-170 (Tables 2 and 3). However, the fact that the enhancement of VBL cytotoxicity by CAs could even be detected in tumors where P-170 could not be traced may lead to the interpretation that the applied immunohistochemical method may be limited to P-170 overexpression, and low P-170 levels may be missed as demonstrated in human cell lines (24).

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