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Elsamicin A binding to DNA. A comparative thermodynamic characterization

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Abstract The antitumor drug elsamicin A contains a coumarin-related chartarin chromophore that intercalates into DNA. It differs from other related molecules in its disaccharide moiety, which bears an amino sugar. Its binding to DNA was analyzed using isothermal titration calorimetry and UV thermal denaturation, and characterized thermodynamically. For the association of elsamicin A with DNA we found $\Delta G^{\circ} = -8.6$ kcal mol^{-1}, $\Delta H = -10.4$ kcal mol^{-1}, $\Delta S = -6.1$ cal mol^{-1} K^{-1}, and $K_{obs} = 2.8(\pm 0.2) \times 10^6$ M^{-1} at 20 °C in 18 mM Na$^+$. The contributions to the free energy of binding that lead to the DNA–elsamicin complex are compared with the binding to DNA of chartreusin, another chartarin-containing drug. The results are discussed in terms of the contributions of the disaccharide moieties into the strength of binding.

Keywords: DNA–drug interaction; Chartreusin; Elsamicin A; Binding; Calorimetry; Drug design

1. Introduction

The interaction of small ligands like antitumour drugs, with DNA sequences or structures is the focus of considerable research. Understanding DNA–drug interactions requires not only knowledge of the structures, but also a detailed thermodynamic characterization of free energy contributions to the interaction between DNA and ligands [1–5]. The energetic characterization of drug binding to DNA provides new insights into DNA–drug interactions [1,3,4] and this, together with structural studies, may be used in rational drug design [6].

We aimed to shed some light on a group of antitumor drugs containing the coumarin-related chromophore chartarin but different sugar moieties. These drugs can be considered a paradigm of intercalating molecules, in which disaccharide moieties could change sequence-binding preferences and alter the strength of binding to DNA. Elsamicin A and chartreusin (Fig. 1) are well-characterized members of this group [7]. Binding to DNA is believed to be central to the mechanism by which these drugs exert their antitumoral effects [7] and the drug-binding sites have been mapped in DNA regions containing CpG steps [7–9]. Both the rate and the extent of the B-to-Z transition were reduced by elsamicin A, in conditions that would otherwise favor the left-handed conformation [10]. Moreover, elsamicin A alters the interaction of the Sp1 factor with DNA, and it inhibits the transcription from the P1 promoter of *c-myc* in vitro [11].

Elsamicin A has broad-spectrum of activity against various tumors, in which it is more potent than chartreusin in terms of minimum effective dose [7,12], and it is undergoing phase II clinical trials [13]. The structure of elsamicin A and related drugs stimulated interest in both natural and semi-synthetic analogs, which may have much better pharmacological profiles [7,14]. One such analog, IST-622, is under clinical trials in Japan [15].

Despite the pharmacological interest of elsamicin A, there is no experimental data on elsamicin complexes with DNA in the crystalline state. This shortcoming has been partially met by a thorough theoretical study [16], which provided some details about the DNA–drug interactions. Among charteusin analogs, A132 (3′,4′-*O*-exobenzilidene chartreusin) [14] has been crystallized, and the A132–DNA complex was modeled to study the role of the digitalose sugar moiety in DNA recognition [17].

We characterize thermodynamically the binding of elsamicin A to DNA. An analysis of the binding to DNA of the related drug chartreusin has been reported elsewhere [18]. The data we have obtained from the analyses of drug binding to DNA are used to dissect some components of the free energy of binding, and to compare the contribution of the various disaccharide moieties to the strength of binding, in the pursuit of rational drug design of new chartarin-containing drugs.

2. Materials and methods

2.1. Materials

Elsamicin A was a gift of Bristol-Myers Squibb. Salmon testes DNA (Sigma) was sonicated, phenol extracted twice, and dialyzed against 10 mM cacodylate buffer (pH 7.0) containing 8 mM NaCl and 0.1 mM EDTA ([Na$^+$] = 18 mM). DNA concentration, in base pairs, was determined spectrophotometrically at 260 nm using a molar extinction coefficient of 12 824 M^{-1} cm^{-1}.

2.2. Differential scanning calorimetry (DSC)

Differential scanning calorimetry (DSC) measurements of the enthalpy of salmon testes DNA melting were performed using a Microcal MC-2 Scanning Calorimeter (Microcal Inc.) as described in detail elsewhere [18].

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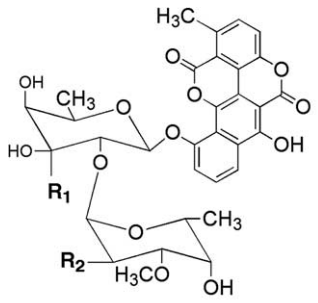

Fig. 1. Structures of the chartarin-containing drugs elsamicin A and chartreusin.

2.3. Determination of binding enthalpy for elsamicin A using isothermal titration calorimetry (ITC)

Experiments were carried at 20 °C using a Microcal MCS-ITC calorimeter (Microcal Inc., Northampton, MA). The Origin software (Microcal) was used for data acquisition and analysis. In a typical experiment, 1.34 ml of a 0.75 mM (bp) DNA in 10 mM cacodylate buffer (pH 7.0) containing 8 mM NaCl and 0.1 mM EDTA ($[Na^+]=18$ mM) was titrated using a 50 μM elsamicin A solution in the same buffer (10–15 injections of 10 μl each), using a 250 μl syringe rotating at 400 rpm. The injection time was 25 s, and the delay between injections was 2.5 min. The peaks produced during titration were converted to heat output per injection by integration and correction for the cell volume and sample concentration. Binding enthalpies for elsamicin were determined from the heats of reaction obtained after each injection to obtain multiple estimates of ΔH° without any fitting bias. Using a high DNA concentration ensured that all the titrated drug was effectively bound after each addition [19,20]. The enthalpy due to drug dilution was determined by 15 serial injections of elsamicin A solution into the reaction cell loaded with buffer alone, and the values averaged. It was subtracted from the ΔH value determined for titration into DNA to render a corrected value for the binding-induced enthalpy change ΔH_b [19].

2.4. Determination of binding constants by DNA ultraviolet-melting analysis

Profiles of absorbance at 260 nm versus temperature were measured between 25 and 98 °C, at a heating rate of 0.5 °C min^{-1}, in the presence of different concentrations of NaCl, in 10 mM cacodylate buffer (pH 7.0) and saturating concentrations of elsamicin A (20 μM DNA (bp); 10 μM elsamicin A), using a Shimadzu UV-2101PC spectrophotometer equipped with a Shimadzu SPR-8 temperature controller.

DNA melting temperatures (T_m) were used to calculate the binding constant to salmon DNA at the DNA melting temperature (K_{T_m}), using the equation derived by Crothers [21]:

$$1/T_m^\circ - 1/T_m = (R/n\Delta H_{wc}) \ln(1 + K_{T_m} a) \quad (1)$$

where T_m° is the UV-melting temperature of salmon testes DNA alone, T_m is the melting temperature in the presence of saturating amounts of the drug, ΔH_{wc} is the enthalpy of DNA melting obtained by DSC, R is the gas constant, K_{T_m} is the drug binding constant at T_m, a is the free drug activity, which is estimated by one half of the total drug concentration, and n is the size of the drug binding site.

The calculated apparent binding constant at T_m can be extrapolated to a reference temperature using the standard relationship:

$$\delta[\ln(K_{obs})]/\delta(1/T) = -(\Delta H_b/R) \quad (2)$$

where K_{obs} is the DNA binding constant of the drug at the reference temperature T (Kelvin) and ΔH_b the binding enthalpy, which was directly determined by ITC.

2.5. Electrostatic contributions in elsamicin A binding to DNA

The effects of different NaCl concentrations upon the binding constant of elsamicin A was calculated from the spectrophotometric UV-melting profiles, as described above, and used to determine the ionic strength dependence of the equilibrium binding constants, at 20 °C, according to the polyelectrolyte theory of Record et al. [22]. The observed linear dependence is described by the relationship:

$$SK = \delta \log K/\delta \log[Na^+] = -Z\psi \quad (3)$$

where ψ is the fraction of sodium counterion associated per DNA phosphate ($\psi = 0.88$ for B-DNA), and Z is the apparent charge of the drug. The quantity SK is equivalent to the number of counterions released upon binding of the ligand with net charge Z.

Moreover, $Z\psi$ can be used to evaluate the polyelectrolyte contribution to the free energy of binding to salmon testes DNA following the equation [22]:

$$\Delta G_{pe} = -Z\psi T \ln[Na^+] \quad (4)$$

and the ΔG_{pe} obtained (Table 2) allows us to calculate ΔG_t: the 'non-polyelectrolyte' (non-electrostatic) contribution to binding [23] according to:

$$\Delta G_{obs} = \Delta G_t + \Delta G_{pe} \quad (5)$$

3. Results and discussion

The enthalpy of salmon testes DNA melting was determined by DSC (Fig. 2A). Eq. (1) was used to obtain the elsamicin A binding constant at the DNA melting temperature, considering the enthalpy of DNA melting together with the results of DNA ultraviolet melting studies in the presence of saturating concentrations of the drug and different salt concentrations (Figs. 2B and C and Table 1). A value of $n=3$ for elsamicin A was used in Eq. (1), based on the size of the drug binding-site determined by molecular dynamics [16]. Experimentally, a Job plot analysis has shown a binding mode corresponding to 3 mol of DNA per mol of the structurally related chartreusin [18]. Correction of the observed binding constant to lower temperatures requires knowledge of the binding enthalpy, which we determined by ITC (Fig. 3).

Fig. 3A shows representative primary data from the ITC titration of elsamicin A into salmon testes DNA at 20 °C. A distribution of binding enthalpy estimates was obtained from three independent titrations and the different values averaged (Fig. 3B). The ΔH_b measured by ITC ($\Delta H_b = -10.4(\pm 0.3)$ kcal mol^{-1}, at 20 °C) was used in Eq. (2) to calculate the binding constant for the elsamicin–DNA interaction ($K_{obs} = 2.8(\pm 0.2) \times 10^6$ M^{-1}, at 20 °C). This direct determination of ΔH_b has the advantage that it does not require to assume that ΔH_b was constant with the temperature [18,20,24].

The experimental K_{obs} and the binding enthalpy for elsamicin binding to DNA, permitted to obtain complete thermodynamic profiles. Table 2 shows the free energy of binding, which was obtained from the standard equation $\Delta G^\circ = -RT \ln K_{obs}$ and the entropy calculated using the standard thermodynamic relationship: $\Delta S^\circ = -(\Delta G^\circ + \Delta H^\circ)/T$. The favorable free energy of binding ($\Delta G_{obs} = -8.6(\pm 0.1)$ kcal mol^{-1} at 20 °C) was derived from a large negative enthalpic contribution ($\Delta H_b = -10.4(\pm 0.3)$ kcal mol^{-1}) but was opposed by the entropic contribution ($\Delta S = -6.1(\pm 0.8)$ cal mol^{-1} K^{-1}). For the easy of comparison, Table 2 also shows thermodynamic parameters for chartreusin binding to DNA, which we had determined previously [18]. The entropy term is *favorable* for the interaction of chartreusin and *unfavorable* for elsamicin A (Table 2). This may be due to increased bonding, including H-bonds formed by the amino sugar of elsamicin [16], which produced a loss in translational and rotational

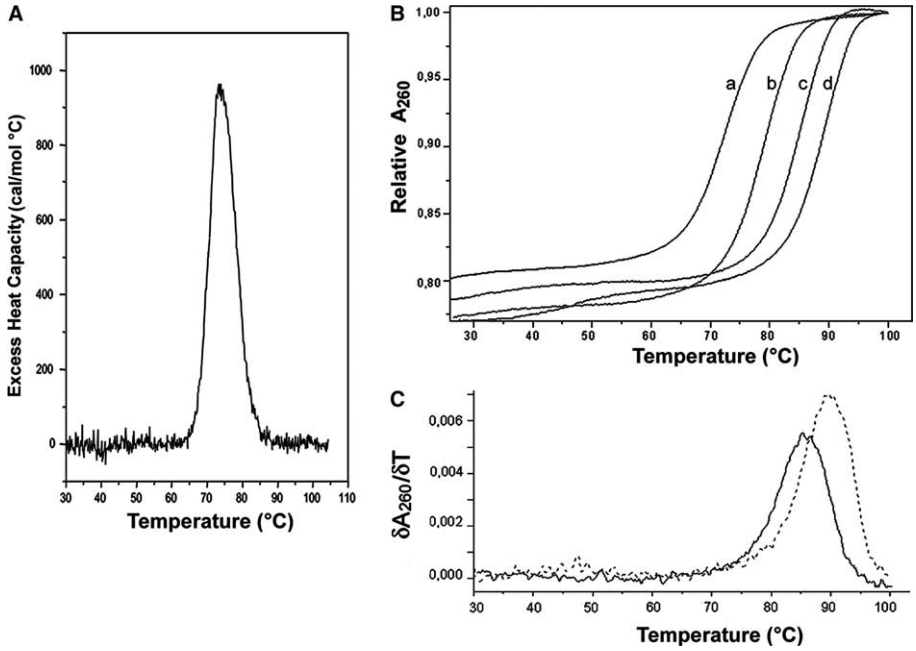

Fig. 2. (A) DSC curve of salmon testes DNA (excess of heat capacity plotted as a function of temperature). T_m: 74.4(±0.3) °C, ΔH_{wc}: 8.8(±0.2) kcal mol^{-1}. The estimated error is the standard deviation of the mean (three experiments). (B) Examples of UV-melting profiles at 260 nm of salmon testes DNA and its complexes with elsamicin A. Experiments in the presence of the drug were performed in conditions that ensured complete saturation of DNA (20 μM DNA (bp) plus 10 μM elsamicin A). DNA melting curves correspond to the following salt concentrations: (a) 18 mM Na^+ (b) 30 mM Na^+ (c) 18 mM Na^+, in the presence of saturating concentrations of elsamicin, (d) 30 mM Na^+, in the presence of saturating concentrations of elsamicin. A compilation of the DNA melting results is shown in Table 1. (C) Differential melting curves for the elsamicin–DNA complexes at 18 nM Na^+ (straight line) and 30 Na^+ (dashed line) in the presence of sufficient ligand to ensure the saturation of the DNA lattice. A unique melting transition is observed.

Table 1
UV-melting temperatures of DNA and DNA saturated with elsamicin A under different salt conditions[a]

[Na^+] mM	T_m° (°C)	T_m (°C)	K_{T_m} (M^{-1})	K_{obs} (M^{-1})
8	69.0	85.4	$9.9(\pm0.5)\times10^5$	$5.2(\pm0.3)\times10^6$
18	74.4	86.6	$5.4(\pm0.3)\times10^5$	$2.8(\pm0.2)\times10^6$
30	80.0	90.1	$3.7(\pm0.2)\times10^5$	$1.9(\pm0.1)\times10^6$
60	85.8	92.4	$1.9(\pm0.1)\times10^5$	$1.0(\pm0.1)\times10^6$
120	92.6	97.0	$1.1(\pm0.1)\times10^5$	$5.7(\pm0.2)\times10^5$

[a] Salt dependence of the binding constants at the melting temperature (K_{T_m}), and at 20 °C (K_{obs}) for elsamicin A binding to DNA. T_m° is the UV-melting temperature of salmon testes DNA alone, T_m is the melting temperature in the presence of saturating amounts of elsamicin (Fig. 2). K_{T_m} the drug binding constant at T_m and K_{obs} drug binding constant determined at 20 °C (mean values ± S.D.), were calculated using Eqs. (1) and (2) as described in the main text. For application of Eq. (2) the elsamicin binding enthalpy, ΔH_b at 20 °C was used (Fig. 3 and Table 2).

degrees of freedom of both DNA and elsamicin A, because of a tight binding to DNA, as discussed below.

Fig. 4 shows the dependence of the DNA-binding constant of elsamicin A and chartreusin on salt concentration. The salt-dependent changes in the binding constant were used, according to the polyelectrolyte theory [22], to calculate the 'actual' charge of the ligands and the salt dependence of the binding constant using Eqs. (3) and (4). For elsamicin, the slope (SK) of the double logarithmic Record's plot in Fig. 4 was −0.82 (Table 2), which is equivalent to the number of counterions released upon drug binding, and corresponds to a calculated apparent change ($Z = +0.93$) consistent with the positive charge carried by elsamicin at neutral pH (Fig. 1). The binding constant of elsamicin A decreased with increasing salt concentrations and the binding was thermodynamically linked to the release of counterions bound to DNA. However, the binding constant for the uncharged chartreusin varied only slightly with salt concentrations (Fig. 4), and it was accompanied by a rather low counterion release [18], which is consistent with the absence of net charge in this molecule (Fig. 1).

The observed free energy was partitioned into the non-electrostatic (ΔG_t) and polyelectrolyte (electrostatic) (ΔG_{pe}) contributions using Eq. (5). A ΔG_{pe} of 1.4 kcal mol^{-1} and a ΔG_t of −7.2 kcal mol^{-1} for elsamicin A indicated that the non-electrostatic component of binding was fundamental in the elsamicin–DNA complex (it represented about 85% of the free energy of binding). ΔG_t was used to compare the 'non-electrostatic' contribution to the free energy of binding for both drugs, which was almost the same (Table 2). For elsamicin, the polyelectrolyte component of binding increased by about fivefolds owing to the presence of the charged amino sugar. At the same time, the charged amino would also anchor the disaccharide more snugly into the DNA minor groove [16]. This

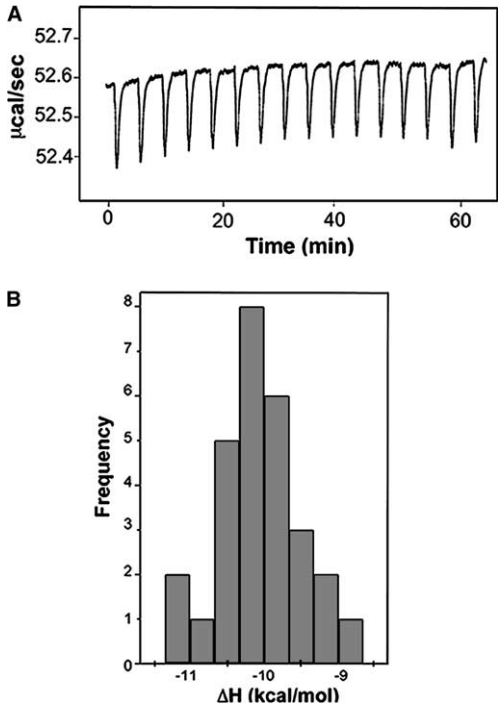

Fig. 3. (A) Representative primary data from an isothermal titration calorimetry experiment, at 20 °C, used to obtain multiple estimates of ΔH° in a titration of elsamicin A on a large excess of DNA, without any assumptions regarding the binding model [20]. The enthalpy associated with elsamicin dilution was averaged from 15 drug injections into buffer, and this value ($\Delta H_{dil} = 1.46(\pm 0.11)$ kcal mol^{-1}) subtracted from the raw calorimetric data. (B) Distribution of DNA-binding enthalpy values obtained after integration of peaks and normalization for the total of elsamicin A added per injection. Data accumulated from three independent isothermal titration calorimetry analyses at 20 °C, with 10–15 drug injections in each experiment.

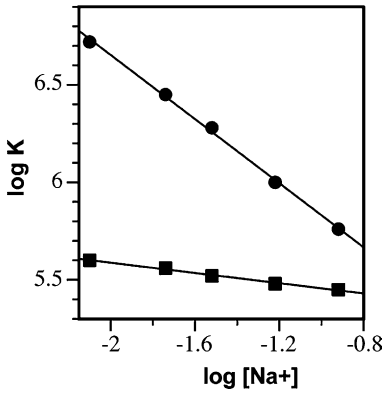

Fig. 4. Salt dependence of elsamicin A and chartreusin binding constants at 20 °C. Data are presented according to the Record's theory [22]. Elsamicin A (circles) and chartreusin (squares). For the easy of comparison, data for chartreusin were adapted from [18]. The linear least-squares fit of the data yields slopes (SK) of −0.82 for elsamicin A and −0.13 for chartreusin, respectively. From these values, it is estimated that elsamicin A is positively charged, while chartreusin is uncharged at neutral pH (see the main text for details).

is likely to produce two qualitatively different effects: first, an additional entropic penalty (Table 2), given the loss of molecular flexibility in both the drug and the DNA, and second, some favorable van der Waals and hydrophobic contacts, as well as the presence of additional H-bonding with DNA bases and phosphates. A main difference in the hydrophobic contribution to binding can occur because of the removal of water and counterions from the disaccharide moieties. Several molecular interactions between either elsamicin A or chartreusin and DNA have been suggested from molecular dynamics studies [16,17], which, in the absence of co-crystallographic structures, is the most reliable representation of DNA–drug interactions. These interactions should contribute to both negative enthalpy and entropy [25–27]. A hydroxyl group of the digitalose sugar of chartreusin (R_2 in Fig. 1) has been suggested to form a H-bond with the guanine 2-amino group (N-2) and the carbonyl group (O-2) of cytosine in the base pair on one side of the intercalation site [17], while for elsamicin, the amino group (R_2 in Fig. 1) of the sugar was bonded directly to one of the phosphates, and it also formed a water bridge with the adjacent cytosine and guanine [16].

Differences in the fluorescence spectral characteristics of the complexes formed by elsamicin A and chartreusin with different deoxyribonucleic acids have indicated a significant participation of the guanine 2-amino and the amino sugar of elsamicin in DNA–drug interaction [28]. For these structurally related drugs ΔG_t only differed in 0.1 kcal mol^{-1}, which suggests that differences in molecular interactions with DNA like hydrogen-bonds that involve the amino group of the sugar of elsamicin or the hydroxyl group of chartreusin at the same sugar position (Fig. 1) may contribute equally to the non-electrostatic strength of binding. In a comprehensive study about the energetic cost of structural alterations in anthracyclines [2] the calculations of ΔG_t for doxorubicin and hydroxyrubicin, in which the amino group of the sugar is replaced by a hydroxyl, showed values that differed by 0.7 kcal mol^{-1}. Taking into account that the error for this type of determination was estimated to be 0.5 kcal mol^{-1} [2], we tentatively consider that the difference we observed in the non-electrostatic part of the binding ($\Delta G_t = 0.1$ kcal mol^{-1}) was mainly due to the distinct H-bonds provided by the amino group in the R_2-position of elsamicin A (Fig. 1). Other non-bonded drug–DNA interactions may be at the expense of existing molecular interactions between water and the

Table 2
Comparison of thermodynamic parameters for chartreusin and elsamicin A binding to salmon testes DNA[a]

	K_{obs} (M^{-1})	ΔG_{obs} (kcal mol^{-1})	SK	ΔG_{pe} (kcal mol^{-1})	ΔG_t (kcal mol^{-1})	ΔH (kcal mol^{-1})	$T\Delta S$ (kcal mol^{-1})
Elsamicin A	$2.8(\pm 0.2)\times 10^6$	−8.6(±0.1)	−0.82	−1.4	−7.2	−10.4(±0.3)	−1.8(±0.3)
Chartreusin	$3.6(\pm 0.3)\times 10^5$	−7.4(±0.1)	−0.13	−0.3	−7.1	−7.1(±0.2)	0.4(±0.1)

[a] K_{obs} (M^{-1}) is the binding constant for the interaction of the ligand with salmon testes DNA in 18 mM Na^+ at 20 °C. ΔG_{obs} is the binding free energy calculated from $\Delta G_{obs} = -RT \ln K$. SK is the slope of the plot of log K_{obs} versus log[Na^+] shown in Fig. 4. ΔG_{pe} and ΔG_t are the polyelectrolyte and non-electrostatic contributions to the binding free energy ($\Delta G_{pe} = SKRT\ln[Na^+]$) evaluated at 18 mM Na^+. Enthalpy values were determined by ITC at 20 °C (Fig. 3 and [18]). The estimated errors are the standard deviations of the mean.

deoxyribonucleic acid without net gain of H-bonding [16], and thus basically isoenergetic.

The charged amino group appears to favor both the long-range and short-range elsamicin–DNA interactions, which can participate in both direct and indirect H-bonding to DNA [16]. In this context, differences in the interactions between elsamicin or chartreusin and DNA can be used to design new chartarin-containing molecules with improved DNA-binding affinity. The role of disaccharide moieties may have implications for rational drug design, not only because of the disaccharide binding to DNA studied here, and its effects on drug solubility [7], but also because the sugar moieties of some useful antitumor drugs are considered essential in several aspects of their biological activity [29].

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