Enhancement of chemotherapy and radiotherapy of murine tumours by AQ4N, a bioreductively activated anti-tumour agent

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Summary AQ4 (1,4-Bis-[[2-(dimethylamino-N-oxide)ethyl]amino]5,8-dihydroxyanthracene-9, 10-dione) is a prodrug designed to be excluded from cell nuclei until bioreduced in hypoxic cells to AQ4, a DNA intercalator and topoisomerase II poison. Thus, AQ4N is a highly selective bioreductive drug that is activated in, and is preferentially toxic to, hypoxic cells in tumours. Five murine tumours (MAC16, MAC26, NT, SCCVII and RIF-1) have been used to investigate the anti-tumour effects of AQ4N. In only one tumour (MAC16) was AQ4N shown to be active as a single agent. However, when combined with methods to increase the hypoxic tumour fraction in RIF-1 (by physical clamping) and MAC26 tumours (using hydralazine) there was a substantial enhancement in anti-tumour effect. Notably, RIF-1 tumours treated with AQ4N (250 mg kg\(^{-1}\)) followed 15 min later by physically occluding the blood supply to the tumour for 90 min, resulted in a 13-fold increase in growth delay. When combined with radiation or chemotherapy, AQ4N substantially increased the effectiveness of these modalities in a range of in vivo model systems. AQ4N potentiates the action of radiation in both a drug and radiation dose-dependent manner. Further the enhancement observed is schedule-independent with AQ4N giving similar effects when given at any time within 16 h before or after the radiation treatment. In combination with chemotherapy it is shown that AQ4N potentiates the activity of cyclophosphamide, cisplatin and thiopeta. Both the chemotherapeutic drugs and AQ4N are given at doses which individually are close to their estimated maximum tolerated dose (data not included) which provides indirect evidence that in the combination chemotherapy experiments there is some tumour selectivity in the enhanced action of the drugs. © 2000 Cancer Research Campaign

Keywords: AQ4N; radiotherapy; bioreduction; cyclophosphamide; cisplatin; thiopeta

Hypoxia occurs in regions of solid tumours as a result of poorly formed micro-vasculature coupled with the metabolic demand of tumour cells close to the available blood supply; this compromises the oxygen availability to more distant tumour cells (Brown and Giaccia, 1994). Hypoxic regions are known to be resistant to radiotherapy and conventional chemotherapy and this has led to much interest in the discovery of cytotoxins that kill hypoxic tumour cells (reviewed in Stratford and Workman, 1998). These agents are designed to be bioreduced selectively in cells low in oxygen to produce potent cytotoxins. Synthetic compounds with chemotherapeutic potential as bioreductive agents are usually based on nitroaromatic or quinone structures, e.g. nitroimidazoles and mitosenes (indoloquinones) (Workman and Stratford, 1993; Denny et al, 1996; Patterson and Raleigh, 1998). These agents are cytotoxic, on reduction, due to their ability to covalently bind to DNA that will not occur in oxygenated cells. A benzotriazene di-N-oxide, tirapazamine, is currently the most promising bioreductive drug and is in phase II/III clinical trials. This agent can act as a chemosensitizer and radiation enhancer due primarily to toxicity of the free radical species formed transiently during its reduction to the non-toxic reduction product SR4317. The toxic effect is probably caused by direct local damage to DNA that results in DNA strand-breaks and chromosome breaks that are difficult to repair (Brown and Wang, 1998).

To date, therefore, bioreductive agents generate transient reactive species that are entirely dependent on hypoxia to generate the toxic species. In contrast, we report here preclinical data on a new class of bioreductive drug exemplified by AQ4N (1,4-bis-[[2-(dimethylamino-N-oxide)ethyl]amino]5,8-dihydroxyanthracene-9, 10-dione). This agent is a prodrug designed to be excluded from cell nuclei (Patterson, 1993; Smith et al, 1997) until metabolized in hypoxic cells to give AQ4, a stable, oxygen-insensitive metabolite (Smith et al, 1997) (Figure 1). AQ4 is a DNA intercalator and potent inhibitor of DNA type II topoisomerase (Patterson, 1993; Patterson et al, 1994; Smith et al, 1997). Previously we have shown that AQ4N is an effective enhancer of the anti-tumour effect of radiation in one murine tumour model, i.e. the T50/80 mammary carcinoma grown in BDF mice (McKeown et al, 1995, 1996). We now report on the efficacy of this drug in several murine models. These models were selected as they have previously been used successfully to show the efficacy of other bioreductive drugs when used in combination with radiation or chemotherapeutic agents (Stratford et al, 1989; Bremner et al, 1990; Bibby et al, 1989).
Figure 1  AQ4N and its reduction metabolites

MATERIALS AND METHODS

Materials

AQ4N and 1,4-bis-[[2-(dimethylamino)ethyl]amino]5,8-dihydroxy-anthracene-9,10-dione (AQ4) were synthesized as described previously (Patterson et al, 1994). Cisplatin, cyclophosphamide, thiotapec and hydralazine were obtained from commercial sources. Materials for cell culture were from Flow Labs (Irvine, UK) and all other chemicals were used as purchased from Sigma (Poole, Dorset, UK).

Murine tumour models

MAC 16, MAC26, RIF-1, NT and SCCV11 tumour-bearing animals

Pure strain NMRI male mice, aged 6–8 weeks were used for experiments with MAC16 and MAC26 tumours (Double and Ball, 1975; Bibby et al, 1987). Male C3H mice (8–12 weeks) were used for experiments using the RIF-1 and SCCVII tumours. NT tumours were grown in male CBA mice (Denekamp and Harris, 1975). RIF-1 and SCCVII tumours were maintained in mice by in vivo–in vitro passage as described previously by Twentyman et al (1980). Tumours were passaged using tumour brei from a donor mouse or by implantation of fragments. Tumours for experiments were implanted subcutaneously in the mid dorsal region of the back. All animal procedures were carried out under project licences issued by the Home Office, London, and UKCCCR guidelines (Workman et al, 1988) for the use of animals in experiments were adhered to throughout.

Tumour systems and treatments

Radiation treatments  To allow local irradiation of tumours, unanaesthetized mice were gently restrained in polyvinyl jigs with lead shielding and a cut-away section over the tumour. Irradiations were done with 250 kV X-rays as described by Stratford et al (1989). In most experiments, single high doses of radiation were used (20 Gy for the SCCVII tumour; 25 Gy for the RIF-1 tumour, 12 Gy for the NT tumour). These doses were chosen to kill most of the oxic cells in these tumours, thereby creating tumours which contained predominantly radio-resistant hypoxic cells. This mode of treatment should reveal the cytotoxicity of bioreductive drugs selectively toxic to hypoxic cells when injected into mice after local irradiation of the tumours.

Drug treatments  AQ4N was usually given at its maximum tolerated single dose (MTD) which was 200–250 mg kg⁻¹ (data not shown). However, in drug combination experiments, toxicity was observed with some chemotherapy agents, most notably cisplatin. The doses used were therefore at or close to the MTD of the combination as determined from pilot experiments (data not shown). All drugs were dissolved in phosphate-buffered saline (PBS) and administered to mice by the intraperitoneal (i.p.) route in a 0.1 ml injection volume.

Induction of hypoxia in tumours

Two methods were employed to temporarily reduce the oxygen status of the tumours. The first of these was physically to occlude the blood supply to the tumours by applying a hinged, metal D-shaped clamp around the base of the tumour to occlude the vascularity for 90 min (Denekemp et al, 1983). The second was to treat mice with 10 mg kg⁻¹ hydralazine. Both these methods can induce 100% radiobiological hypoxia in experimental tumours (Acker and Chaplin, 1987). In these experiments, mice were treated with AQ4N, then 15 min later, when it is presumed that tumour levels of drug will be high, the tumours were clamped or mice treated with hydralazine.

Measurement of tumour response to treatment

Tumour growth delay

For NT tumours, treatment was initiated when the tumour reached 6.5–7.5 mm GMD (geometric mean of three orthogonal diameters). Tumours were measured three times weekly and the time taken to reach double its treatment volume was used as a measure of anti-tumour efficacy. For RIF-1 tumours, treatment was initiated when the tumours reached 100–200 mm³ and their growth followed until they had reached 4 × the treatment volume. MAC16 and MAC26 tumours were treated when the two longest orthogonal diameters were at least 4 and 5 mm. Tumour response was measured twice weekly by two-dimensional caliper measurements. For RIF-1, MAC16 and MAC26, tumour growth delay was calculated by subtracting the mean time to grow to 4 × treatment volume for control tumours from that obtained after drug exposure.

In vivo–in vitro clonogenic assay

C3H mice bearing SCCVII or RIF-1 tumours were treated upon reaching a volume of 100–200 mm³. After 24 h tumours were excised, minced and digested with an enzyme cocktail (6 mg DNase, 2 mg collagenase and 2 mg pronase per 10 ml PBS) to produce a single cell suspension. The cells were counted, diluted as necessary, plated and incubated at 37°C in 95% air/5% carbon dioxide for 12–14 days. Colonies were fixed, stained with 0.4% crystal violet in methanol and scored by eye. Surviving fraction (SF) was calculated as the number of colonies counted divided by the number of cells plated for a given treatment, divided by the same fraction determined for untreated control tumours.

RESULTS

Activity of AQ4N when combined with methods to induce hypoxia

To investigate the effect of tumour hypoxia on AQ4N activity a physical clamp and a vascular ‘steal’ agent, hydralazine were used. Table 1 shows that AQ4N, clamping or hydralazine alone does not significantly delay tumour growth in RIF-1 or MAC26 tumours. However, making the tumours completely hypoxic for 90 min, using a physical clamp, after AQ4N administration does significantly delay tumour growth by 13-fold. Hydralazine, which
acts as a partial pharmacological clamp also, increases the anti-
tumour effect of AQ4N in MAC26 tumours.

Activity of AQ4N in combination chemotherapy

Table 2 shows that when AQ4N (50 mg kg\(^{-1}\)) or thiopeta
(10 mg kg\(^{-1}\)) alone were tested for anti-tumour efficacy against the
MAC16 tumour only a small effect (approx. 4 days delay) on
tumour growth was seen; combination of AQ4N and thiopeta at
these concentrations significantly delayed tumour growth by over
15 days (\(P < 0.01\)). Table 2 also shows that against RIF-1 tumours,
a combination of AQ4N (200 mg kg\(^{-1}\)) and cyclophosphamide
(50 mg kg\(^{-1}\)) significantly delays tumour growth whereas, as single
agents, neither drug has a measurable effect. Similar results are
seen with SCCVII where clonogenic cell survival was below the
limit of detection following a combination of AQ4N (250 mg kg\(^{-1}\))
and cyclophosphamide (100 mg kg\(^{-1}\)). Scheduling of drug adminis-
tration is important since tumour regression (in RIF-1) and clono-
genic cell kill (in SCCVII) is greater when cyclophosphamide is
administered 1 h prior to AQ4N compared to 1 h following AQ4N
administration. See Materials and Methods for experimental details.
Mann–Whitney U-test: * \(P < 0.05\), ** \(P < 0.01\).

Table 1  Activity of AQ4N in combination with hypoxia inducing protocols

| Treatment         | Tumour model | Growth delay\(^a\) (days) | n  |
|-------------------|--------------|--------------------------|----|
| Hypoxia           | RIF-1        | 0.6±0.5                  | 5  |
| AQ4N              | RIF-1        | 1.1±1.0                  | 14 |
| QLD alone         | MAC26        | 14.1±1.7                 | 8  |
| AQ4N + QLD        | MAC26        | 5.5\(^*\)               | 5  |
| Hydralazine       | MAC26        | 0                        | 5  |

\(^a\) Time to 4 \(\times\) initial tumour volume (treated group minus untreated group). Growth delay = time to reach 4 \(\times\) tumour volume at treatment (treated group minus untreated group) for RIF-1 and MAC26; NT tumours were followed to 4 \(\times\) treatment volume. All drugs were dosed i.p. See Materials and Methods for experimental details. CPM = cyclophosphamide. All results (\(n \geq 5\) per treatment group). Mann–Whitney U-test: * \(P < 0.05\), ** \(P < 0.01\), *** \(P < 0.001\).

Table 2  Activity of AQ4N in combination chemotherapy against murine tumours in vivo

| Treatment and Time schedule in minutes | Drug dose (mg kg\(^{-1}\)) | Tumour | Growth delay\(^a\) (days) | Surviving fractions\(^a\) |
|----------------------------------------|---------------------------|--------|--------------------------|--------------------------|
| AQ4N                                  | 50                        | MAC16  | 4.6                      | –                        |
| Thiopeta                              | 10                        | MAC16  | 3.7                      | –                        |
| AQ4N + thiopeta                        | 50/10                     | MAC1615.2\(^**\) | –                      | –                        |
| CPM                                   | 200                       | RIF-1  | 0.5                      | –                        |
| AQ4N + CPM                            | 50                        | RIF-1  | 0.1                      | –                        |
| Radiation (25 Gy) + AQ4N               | RIF-1                    | 31.1±2.0 | 19                      |                          |
| Radiation (25 Gy)                      | RIF-1                    | 59.7±4.8 | 5                        |                          |
| Radiation (25 Gy) + AQ4N               | RIF-1                    | 0.05±0.6 | 8                        |                          |
| Radiation (12 Gy) + AQ4N               | NT                       | 3.76±2.1 | 8                        |                          |
| Radiation (12 Gy) + AQ4N               | NT                       | 13.76±3.8\(^**\) | 8                        |                          |

\(^a\) Time to 4 \(\times\) initial tumour volume (treated group minus untreated group). Growth delay = time to reach 4 \(\times\) tumour volume at treatment (treated group minus untreated group). \(^b\) Surviving fraction of clonogenic cells following treatment.

All drugs were dosed i.p. See Materials and Methods for experimental details. CPM = cyclophosphamide. All results (\(n \geq 5\) per treatment group). Mann–Whitney U-test: * \(P < 0.05\), ** \(P < 0.01\), *** \(P < 0.001\).
Materials and Methods. Results are the means excised and the number of clonogenic cells determined as described in 30 min after exposure to radiation. Mice were sacrificed 24 h later, the tumour were treated with a range of X-radiation doses with (open circles) or of AQ4N after various doses of X-radiation. C3H mice bearing the SCCVII tumour were treated with a range of AQ4N doses 30 min after exposure to 20 Gy X-radiation. They were also administered with a single i.p. dose of AQ4N (250 mg kg\(^{-1}\)) at known times intervals up to 24 h before (o) or after radiation (c). Mice were sacrificed 24 h after each dosing interval, the tumour excised and the number of clonogenic cells determined as described in Materials and Methods. Results are given for individual tumours.

Previously we have shown (McKeown et al, 1995, 1996) that in the T50/80 tumour model in vivo we have observed potent anti-tumour activity of AQ4N when used in 10 Gy. This will be because, at doses of 10 Gy and below, the response of the tumour will be dominated by the aerobic population of tumour cells, i.e. killing of the radiation-resistant hypoxic cells will only be revealed with single doses of radiation > 10 Gy. Since the level of tumour cell killing following 15 Gy plus AQ4N is only seen when radiation alone is given at 30 Gy, we can calculate a dose modification of 2.0 at this level of cell kill. The effect of administering AQ4N at various times before or after irradiation of SCCVII tumour-bearing mice was subsequently investigated (see Figure 4). This shows that i.p. injection of AQ4N (250 mg kg\(^{-1}\)) up to 16 h before irradiation or up to 16 h following irradiation (20 Gy) produced a substantial increase in cell kill when these intervals were increased to 24 h the potentiating effect is lost.

DISCUSSION

The rationale underlying the development of AQ4N was to design a drug with potential for targeting DNA and DNA associated enzymes, but which was not active until metabolized in hypoxic tumour cells. The DNA affinity of several clinically useful agents, including the anthracyclines and mitoxantrone, is associated with a planar, electron-deficient chromophore bearing cationic amino side arm(s). It was proposed that an N-oxide of a tertiary aliphatic amine is electrically neutral and thus will exhibit a markedly decreased DNA binding affinity compared to the reduced cationic tertiary amine (Patterson, 1993). In fact previous studies have shown that AQ4 has excellent DNA binding affinity (Patterson et al, 1993) and is a DNA type II topoisomerase inhibitor (Smith et al, 1997a). In contrast AQ4N had an equilibrium binding to DNA which was too low to measure and AQ4N exhibited a 50-fold decrease in topoisomerase II inhibitory activity as compared to AQ4. AQ4N, in combination with hypoxia was a very poor cytotoxin against rodent and human cell lines whilst its reduction product, AQ4 is a potent cytotoxic agent (Smith et al, 1997a). This was confirmed in several other cell lines (Wilson et al, 1996).

Previously using the T50/80 murine tumour model in vivo we have observed potent anti-tumour activity of AQ4N when used in
combination with radiation (McKeown et al, 1995, 1996). For all bioreductive drugs to be useful clinically, tumour expression in vivo of appropriate reductases will be an important determinant of efficacy. Although not addressed in this study AQ4N is known to be reductively metabolized by haem containing enzymes including cytochrome P450s (CYPs). Specifically, AQ4N is a substrate for the CYP3A subfamily under hypoxic conditions (Raleigh et al, 1998) and CYP3A has been measured in RIF-1 and SCCVII tumours (Murray et al, 1998). CYP3A isoforms have been detected in a broad spectrum of human cancers including colon (Massaad et al, 1993; McKay et al, 1993) breast (Murray et al, 1993) and lung (Kivistö et al, 1995). Unlike tirapazamine it has not proved possible to metabolize AQ4N in cultured tumour cells, this is almost certainly due to the fact that cytochrome P450s are known to be down-regulated ex vivo (Patterson et al, 1998). We have previously shown that the potential to metabolize AQ4N disappears within 24 h of excision in the T50/80 tumour (Hejmadi et al, 1996). The present studies were undertaken to further examine the potential of AQ4N to enhance radiation and chemotherapy in a number of in vivo murine tumours and hence support its use as a hypoxia activated bioreductive agent in the clinic.

Enhancement of AQ4N metabolism in vivo by hypoxia

Evidence that AQ4N is acting as a hypoxia-activated cytotoxin comes from the combination of this agent with clamping or hydralazine (Table 1). Clearly, the production of 100% hypoxia with a tumour clamp enhances the anti-tumour effect of AQ4N administered as a single agent and supports the contention that AQ4N can be bioreductively activated in vivo. Enhancement of the anti-tumour activity of bioreductive drugs with the vasoactive agent, hydralazine, has previously been shown with RSU 1069 (Acker and Chaplin, 1987; Stratford et al, 1997; Brown et al, 1987) and mitomycin-C (Adams et al, 1989; Quinn et al, 1992). It is known that the treatment of mice bearing the MAC26 colon tumour with hydralazine produces 80% shut down in functional tumour vasculature and greater than 60% reduction in tumour blood perfusion (Quinn et al, 1992). The present results show that hydralazine significantly enhances the anti-tumour efficacy of AQ4N again supporting the hypothesis that it is bioreductively activated in vivo despite poor activation in vitro. Our interpretation of the present data supports other published studies. Notably, AQ4N was active against the mouse mammary tumour MDAH-Mca-4 when combined with the tumour blood flow inhibitor 5, 6-dimethylxanthenone-4-acetic acid (DMXAA) (Wilson et al, 1996). In addition, the anti-tumour effect of AQ4N in vivo was potentiated in T50/80 tumours by combination with hypobaric hypoxia with a dose enhancement ratio of 5.1 (McKeown et al, 1995).

Enhancement of chemotherapy by AQ4N

From the results presented in Table 2 it can clearly be seen that AQ4N significantly enhances the effect of three standard chemotherapy agents (cisplatin, cyclophosphamide and thiotepa). This supports previous results that showed that AQ4N enhances the anti-tumour effect of cyclophosphamide and cisplatin in the T50/80 tumour (McKeown et al, 1998). The results with the RIF-1 and SCCVII model systems are consistent with the chemotherapeutic enhancement effects of other bioreductive agents, including tirapazamine (Kim and Brown, 1994; Slom et al, 1997) and E09 (Bibby et al, 1993) in these tumour types. MAC16 is an unusual model since it is poorly responsive to most conventional anti-cancer drugs (Bibby et al, 1988). This may be in part because MAC16 is a relatively slow growing tumour becoming necrotic as it grows suggesting the presence of a significant level of hypoxia. The significantly enhanced effect of thiopeta in combination with AQ4N suggests that this approach might have particular value in control of colon tumours.

Standard chemotherapy drugs typically target the well-perfused and hence oxygenated tumour regions. The enhancement by AQ4N on the anti-tumour effects of these drugs may be explained by the existence in the hypoxic regions of AQ4 which would exert its cytotoxicity on cells as they attempt to re-enter the cell cycle, in response to the damage accrued by the well oxygenated cells. Recently, AQ4N over a range of concentrations has been shown to enhance the anti-tumour effects of cyclophosphamide in T50/80 implants. In particular, the anti-tumour effect of AQ4N (100 mg kg⁻¹) combined with cyclophosphamide (100 mg kg⁻¹) was shown to be equivalent to that produced by a single 200 mg kg⁻¹ dose of cyclophosphamide alone, i.e. a 50% reduction in cyclophosphamide dose (McKeown et al, 1998).

Enhancement of radiation therapy by AQ4N

Combination of AQ4N (200 mg kg⁻¹) with ionizing radiation (25 Gy) slowed RIF-1 tumour growth by over 40% compared to radiation alone (Table 1). This encouraging result was explored more thoroughly using SCCVII implants, which showed that AQ4N, over a range of concentrations, greatly enhanced the positive effects of radiation (20 Gy). When AQ4N and radiation are administered at approximately the same time, a positive interaction can be explained on the basis that the two modalities are cytotoxic to different (i.e. oxic or hypoxic) cell populations. However, with AQ4N the observed enhancement is maintained even when drug is administered up to 16 h before radiation in the SCCVII tumour model (Figure 4); the stability and DNA affinity of AQ4 is likely to explain this phenomenon (Smith et al, 1997a; 1997b). Once produced, AQ4 will persist in the hypoxic cell fraction and kill any cell which attempts to re-enter the cell cycle following destruction of oxic cells by irradiation. When AQ4N was administered up to 16 h after irradiation, an enhancement was also observed. This suggests that formation of AQ4 in hypoxic cells that have not yet re-oxygenated, can still prevent them from repopulating the tumour. In T50/80 tumours a similar, although even longer, positive interaction time was observed with a 50% dose sparing effect reported for up to 4 days before and 6 h after irradiation (Hejmadi et al, 1996; McKeown et al, 1996). An enhanced anti-tumour activity post radiation was also observed in MDAH-Mca-4 tumours (Wilson et al, 1996). Overall this and previous studies are consistent with AQ4N acting as a bioreductive radiation enhancer. Differences in the time scales of the radiation enhancement by AQ4N in SCCVII compared to T50/80 tumours may be related to tumour clearance and/or host animal clearance of compound. The ability of cells to survive in the hypoxic compartment of tumours may also be a factor. Moore (1988) has reported that T50/80 tumours have a particularly high hypoxic fraction (60%) which suggests that T50/80 cells are capable of remaining viable, when hypoxic, for some considerable time; a
factor which might also account for the very long times of interaction in this model previously observed (McKeown et al., 1995). In contrast, SCCVII tumours are significantly better oxygenated and have a faster doubling time than T50/80. This may explain the shorter time of maximal interaction, i.e. 16 h versus 96 h in the T50/80 tumor. The stable DNA-affinic agent AQ4N, which is produced in the hypoxic cells, will compromise the cells in which it is generated for as long as they are viable. Once these cells die and become necrotic the cells, and the associated AQ4, are no longer capable of influencing tumour responses. This is likely to occur in fast growing tumours (e.g. SCCVII) more quickly than slow growing tumours especially those with a high proportion of hypoxic cells (e.g. T50/80).

In conclusion AQ4N acts like a bioreductive drug when combined with hypoxia, chemotherapy or radiation treatment in vivo. The efficacy of the drug has now been shown against MAC16, MAC 26, NT, RIF-1 and SCCVII, T50/80 (McKeown et al., 1995, 1996) and MDAH-Mca-4 (Wilson et al., 1996). They all support the use of AQ4N as a bioreductive agent that has little or no intrinsic activity per se but is a very efficient enhancer of radiation as well as standard chemotherapy agents such as cyclophosphamide, cisplatin and thiopeta. An additional benefit of this bioreductively activated cytotoxin is the stable nature of the reduction product, AQ4, which may be capable of eliciting a bystander cell killing effect on proximate non-hypoxic tumour regions. In support of this confocal microscopy shows that viable cells preloaded with AQ4, the authentic reduction product of AQ4N liberate drug within 2 h despite the high DNA affinity of the latter (Smith et al., 1997b).

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