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Original Article

Comparison of PEG Interferon Loaded and non-Loaded Iron Oxide Nanoparticles on Hepatitis C Virus Replication in Cell Culture System

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Abstract

Background and Aims: Iron oxide nanoparticles are among the most effective tools which can replace current medical techniques for diagnosis and treatment of various diseases. Hepatitis C infection is one of the main health problems in the world, affecting around 3% of the world's population. This infection can develop into liver cirrhosis and liver cancer over the time in 80% of patients.

In this study, the effects of PEG interferon loaded iron oxide nanoparticles on hepatitis C virus infection compared with unloaded nanoparticles was studied in vitro.

Materials and Methods: First, Huh7.5 cells were cultured to replicate the hepatitis C virus. After loading the peg interferon alpha on iron oxide nanoparticles, their effects on the replication of hepatitis C virus was investigated by several methods.

Results: The results of this study showed that iron oxide nanoparticles and peg interferon loaded iron oxide nanoparticles were able to reduce the load of hepatitis C virus in infected cell culture, but differences were not statistically significant.

Conclusions: These data indicated that hepatitis C viral load was decreased in infected cells after induction of PEG interferon loaded iron oxide nanoparticles, but it needs more research to clarify in animal models or even to examine with other types of bare and drug-loaded nanoparticles in a similar way to our study.

Keywords: Hepatitis C Virus, Nanoparticles, Iron Oxide, Hepatitis C Virus Treatment, Peg Interferon.

Introduction

Hepatitis C virus is a member of the flaviviridae family that can cause a variety of acute and chronic infections (1, 2). Hepatitis C virus infection is one of the most challenging health problems in the world contributing to over 170 million infected people (3). In Iran, about 1% of people are infected with Hepatitis C virus (4). This virus targets the liver and causes severe diseases including chronic hepatitis, cirrhosis and liver cancer in humans. Hepatitis C infection leads to more than 80% chronic cases, which recovery from the disease does not lead to resilience (5). Chronic hepatitis C causes immune deficiency such as inadequate T cell function, inadequate antibody responses and metabolic disorders, such as hepatic steatosis, iron accumulation and insulin resistance associated with type 2 diabetes(6). Patients

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with liver cancer include 5.6% of the total cancer patients in the world. This cancer is the fastest growing cause of death in the United States of America between 1992 and 2010 (7). To date, no effective vaccine has been identified for this infection and its options for treatment are limited (1). Over the years, hepatitis C virus infection has been treated with interferon (IFN) and daily dose of ribavirin (8). Despite the advances in the treatment of chronic and acute hepatitis C virus infection using peg interferon and ribavirin, the infection continues to be a vast health problem worldwide, particularly patients who do not respond to current medications like, patients with relapsing diseases, those who co-infected with HIV or HBV, and patients with liver cirrhosis (9).

Nanotechnology refers to the knowledge of the use and control of matter in the dimensions of 1 to 100 nanometers (at least in one aspect of the nanoparticle). Nanoparticles have unique properties. One of these features can be their very small size, high surface-to-volume ratio, and high reactivity, which varies from nanoparticles to other materials of the same composition. These features have helped to overcome many of the limitations of a variety of diagnostic and therapeutic options (10).

Among the inorganic nanoparticles, iron oxide nanoparticles can be mentioned. Studies have shown that these nanoparticles have a fatal effect on cancer cells. While no toxic effects on healthy cells are induced (11).

The goal of this study was to determine the antiviral effect of iron oxide nanoparticles and peg interferon loaded iron oxide nanoparticles compared with peg interferon on hepatitis C virus replication in three pathways.

Methods

Huh 7.5 cells with the source of hepatic carcinoma are appropriate cells for hepatitis C virus. These cells were cultured in DMEM media (high glucose) with 10% FBS and 1% of penicillin and streptomycin. When confluency reached about 70-80%, virus confront to cells in three pathways.

First pathway. In the first pathway, 10,000 cells of Huh7.5 were cultured in a 96-well plate per well. Then, 50 µl of the virus was inoculated into each well and then incubated for one and a half hour at 37 ° C. In the next step, 200 µl of bare nanoparticles, peg interferon coated iron oxide nanoparticles, and peg interferon alone were added to the wells at the concentration of 1C50 (each with 4 repeats). No virus was added to the control wells.

In the next step, the plate was kept in an incubator at 37 ° C for 48 hours. After this period of time, the supernatant of each well was collected for Real time PCR test. The purpose of the first study was to investigate their effect on the treatment of hepatitis C infection.

Second pathway. In the second pathway, 50 ml of virus with 200 ml of bare nanoparticles, peg interferon coated iron oxide nanoparticles, and peg interferon with concentration of 1C50 each were separately mixed. The incubation was carried out for 1.5 hr at 4°C. (Incubation was performed at this temperature to prevent damage to hepatitis C virus, which is a coated virus). Then mixtures containing virus, nanoparticles and compounds were separately grown each well containing Huh7.5 cells (50 ml of mixture was added to each well). For each compound, it was repeated four times, and for the control of the virus, four wells were considered. The plate was kept at 37°C for 48 hr, and then the supernatants of the wells and the control (in order to ensure the replication of the virus in the cells) were collected for Real time PCR test.

Third pathway. In the third pathway, 10,000 cells of Huh7.5 were first cultured in a 96-well plate per well. Then, 1C50 concentration of nanoparticles, peg interferon coated iron oxide nanoparticles and peg interferon alone were added to separate wells with amount of 200 ml. Four repeats were performed for each compound and 4 wells were considered for virus control.

After incubation for 24 hours at 37° C, 50 ml of the virus was added to all wells. Incubation was then performed at 37° C for 48 hours.

After this period, the supernatant fluids containing nanoparticles and compounds and supernatant fluids of control well were collected for Real time PCR test. The purpose of this pathway was to investigate the effect of nanoparticles on the entry of the virus into the cells.

MTT assay. To determine the cytotoxicity of peg interferon, peg interferon loaded iron oxide nanoparticles and iron oxide nanoparticles, MTT assay was used. Briefly, 10,000 cells of huh7.5 were cultured in a 96-well plate per well. The plate was incubated at 37ºC with 5% CO2 for 24 hours. Nanoparticles, Peg Interferon and peg interferon loaded iron oxide nanoparticles were added to the wells.

Six serial dilutions of each compound were made (1, 1:2, 1: 4, 1: 8, 1: 16 and 1:32). Each concentration of either treatment was tested in triplicate. Then, the plate was incubated for 24 hr. The media of each well was removed. Next, 100 ml of new media and 10 microliters of MTT reagent was added to each well. Then 100 microliters of DMSO was added to each well after 4 hour of incubation at 37ºC under 5% of CO2. Eventually, the optical density was read at 570 and 630 nm by an ELISA reader.

IC50 Determination. IC50 was calculated using Microsoft Office Excel and CalcuSyn software (Biosoft, version 2/1). First, 10,000 Huh7.5 cells were cultured in each well of a 96-well plate and put in an incubator at 37ºC under 5% of CO2 for 24 hours.

Fifty microliters of the virus and 200 microliters of 1 IC50 of each compound (peg interferon, peg interferon loaded iron oxide nanoparticles and nanoparticles alone) were mixed and kept at 4ºC. After discarding the media, the mixtures of the virus with mentioned compounds were added to the wells. Eventually, the supernatant of each well was collected after 48 hours and saved for RNA extraction.

RNA extraction and Real Time PCR. RNA extraction was performed using Genet Bio kit (South Korea). Then, Real Time PCR was done by Iranian Novin Gene kit (Iran) using Corbett Rotor Gene.

Results

Cell culture. Huh7.5 cells which are shown in figure 1 was taken by an invert microscope.

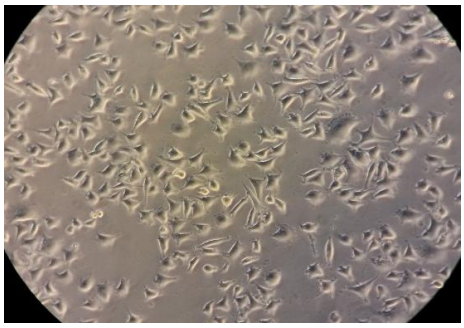


Fig. 1. Huh7.5 cells with 100 X magnification

MTT and IC50 determination results. Using MTT assay, the percentage of viable cells against different concentrations of peg interferon and peg interferon loaded iron oxide nanoparticles were determined.

Viability of the cells following exposure to peg interferon is shown in chart 1. The highest concentration was 0.18 mg/ml.

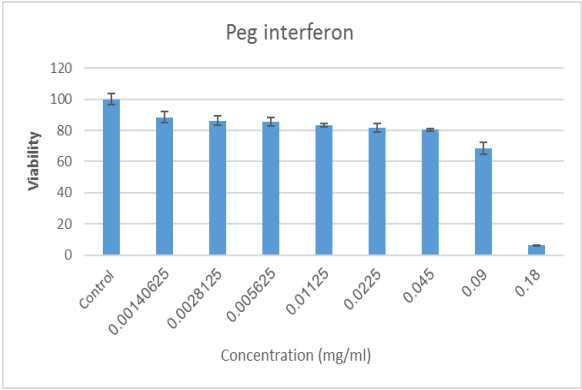


Chart. 1. Cytotoxicity effect of peg interferon on Huh7.5 cell line.

Chart 2 shows the viability of huh7.5 cells against different concentrations of peg interferon loaded iron oxide nanoparticles. The highest concentration was 0.25 mg/ml.

Chart.3. shows the viability of huh7.5 cells against different concentrations of iron oxide nanoparticles. The highest concentration was 0.25 mg/ml.

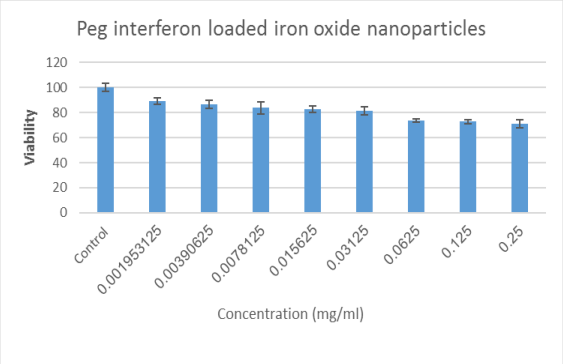


Chart.2. Cytotoxicity of peg interferon loaded iron oxide nanoparticles on huh7.5 cells.

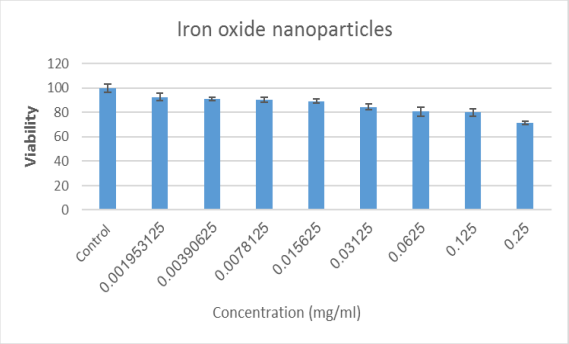


Chart.3. Cytotoxicity of iron oxide nanoparticles on huh7.5 cells.

The IC50 value of peg interferon and peg interferon loaded iron oxide nanoparticles, obtained from dose response curve, is shown in Figure 2 and figure 3, respectively.

Real time PCR results. RNA extraction was performed using Genet Bio kit (South Korea). Then, Real Time PCR was done by Iranian Novin Gene kit (Iran) using Corbett Rotor Gene for each pathway.

The statistical results of exposure of hepatitis C virus to compounds in the first pathway show that although the compounds have more reducing effect on the viral load than control, this effect was not statistically significant (P> 0.05). Other results also showed that there was no significant difference between the effects of different compounds on viral load (P = 0.867).

In the second pathway of the experiment, the results of real time PCR are shown in chart 4.

The statistical results related to the exposure of hepatitis C virus to the compounds, in the second method, show no statistically significant difference between each of the compounds with control (P> 0.05). Other results

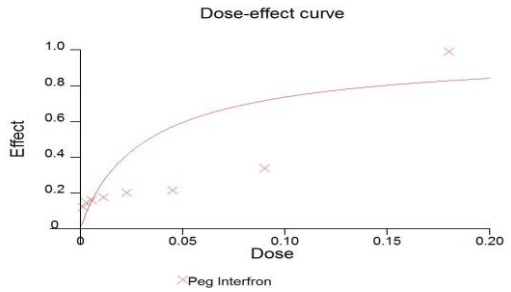


Fig. 2. The dose–effect curve of peg interferon.

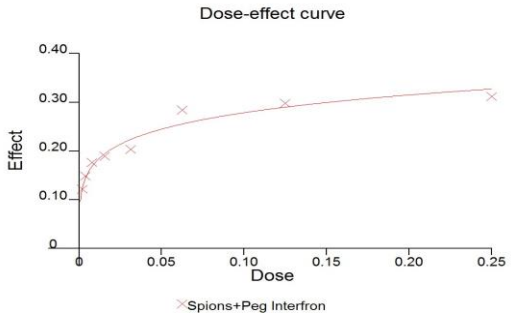


Fig. 3. Dose effect curve of peg interferon loaded iron oxide nanoparticles.

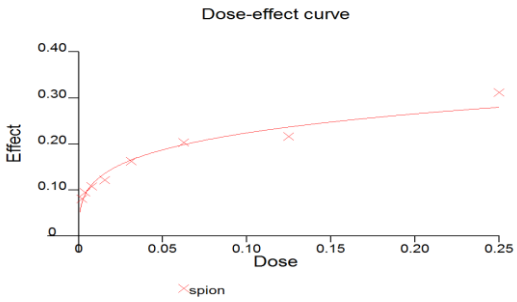


Fig.4. Dose effect curve of iron oxide nanoparticles

also show that there is no statistically significant difference between the effects of different compounds (P = 0.565).

In the third pathway of the experiment, real time PCR was performed to check the viral load, and chart.6 shows these results.

The statistical results related to the exposure of hepatitis C virus to the compounds in the third pathway show that although the compounds have a greater reduction effect on viral load than control, this effect is not statistically significant (P> 0.05). Other results also show that there is no statistically significant difference between the effects of different compounds (P = 0.895).

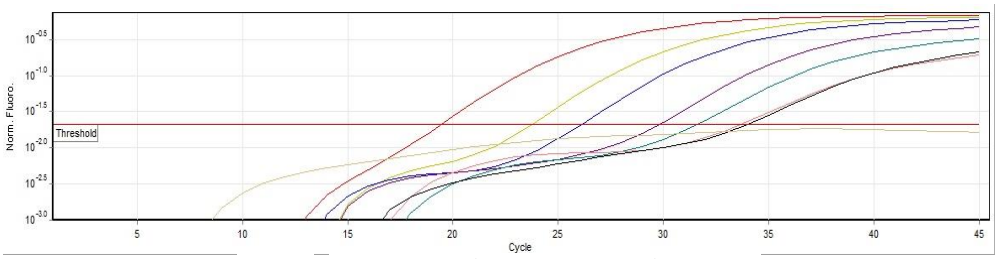


Chart.4. Sigmoid curve of virus replication in first pathway

Description: Peg interferon, Standard 1, Standard 3, Standard 2, Standard 4, Iron oxide nanoparticles Peg interferon loaded iron oxide nanoparticles, Virus control.

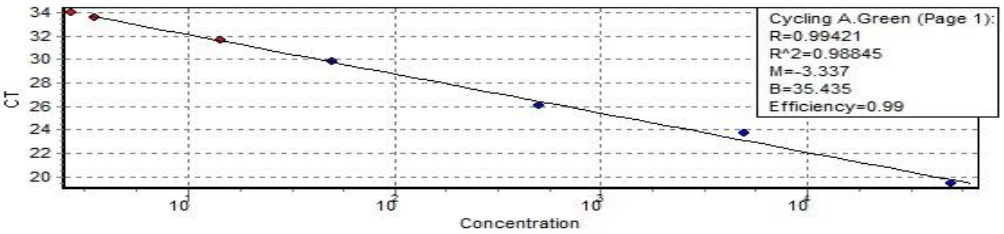


Chart.5. Linear standard Curve related to first pathway

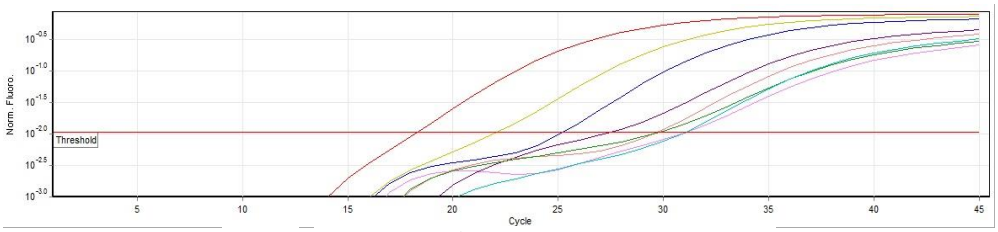


Chart.6. Sigmoid curve of virus replication in second pathway.

Descriptions: Standard 1, Standard 2, Standard 3 , Virus control, Peg interferon loaded iron oxide nanoparticles, Standard 4, Iron oxide nanoparticles

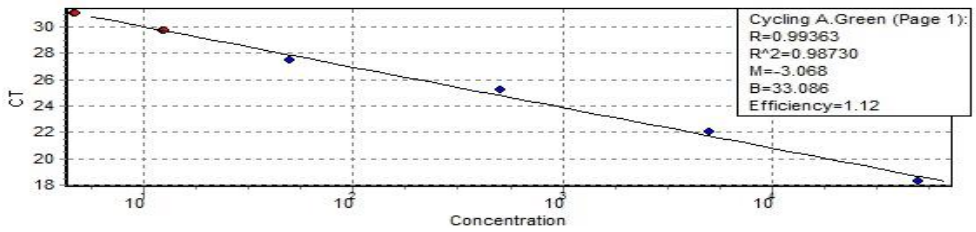


Chart.7. Linear standard Curve related to second pathway.

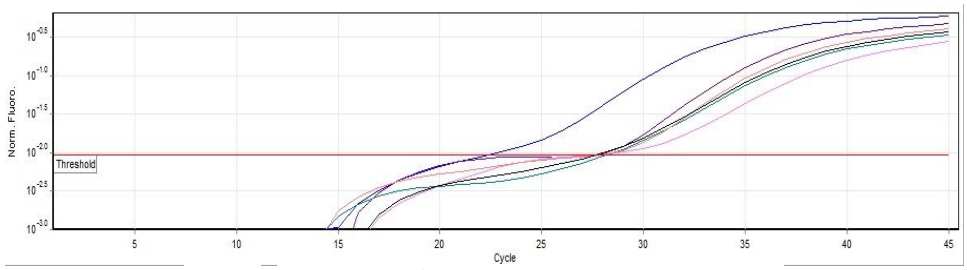


Chart.8. Sigmoid curve of virus replication related to third pathway.

Descriptions: Peg interferon loaded iron oxide nanoparticles, Peg interferon, Standard 3, Standard 4, Iron oxide nanoparticles, Virus control

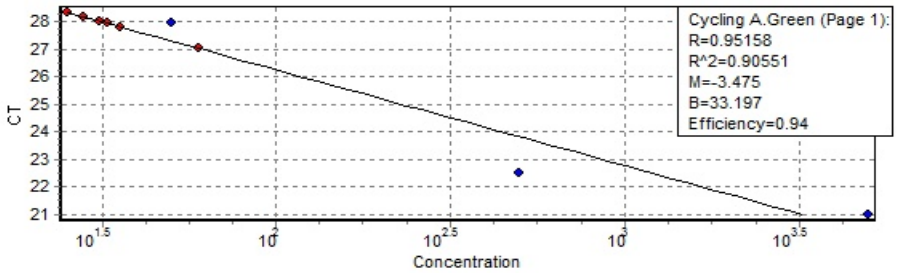


Chart.8. standard curve related to third pathway.

Discussion

Hepatitis C virus infection is a major health problem in the world. 80 to 85 % of the people with hepatitis C virus infection will get chronic hepatitis, of which about 25 % will progress to cirrhosis of the liver and about 20 % to hepatocellular carcinoma (12). Liver cancer is the 5th most common cancer in men, the seventh most common cancer in women, and is the second leading cancer death in the world (6). As a result of a study done in mashhad, it was found that about 1% of the population of this city were infected with hepatitis C virus and among them, 67% were man and 33% were women (13). In Iran, the genotypes 1a (with 61.2% frequency) and 3a (with 25.2% frequency) are dominant (3). In Mashhad, genotypes 1a and 3a have a frequency of 14.7% and 33.0% (14). Despite the fact that around 170 million people are carriers of hepatitis C virus infection in the world, developing better and more effective treatment options for this infection is very important (15).

To date, the main treatment options for Hepatitis C virus therapy have been the combination of peg interferon alfa and ribavirin. This treatment approach is not optimal for some reason, including their harmful side effects and high cost (16, 17). The use of ribavirin alone has no effect on patients with hepatitis C infection. The most common side effects of peg interferon include fatigue, muscle aches, and physiological problems such as depression, tiredness, anxiety and sleep disorders. Interferon also causes pancytopenia due to its bone marrow suppressing activity. The most common side effects of ribavirin are hemolysis and anemia. Since ribavirin is teratogenic in women and men, it is recommended to use the contraceptives during treatment and at least 6 months after treatment. In addition, milking should also be stopped (9). After a while, new treatments called DAA[†] were introduced, drugs such as Boceprevir and Telaprevir which were against protease of hepatitis C virus, and are more commonly used to treat genotype 1. Using DAAs can increase SVR[‡]. Of course, there are still concerns about the creation of resistant strains of the virus. Because resistance to both Boceprevir and Telaprevir drugs has been seen in many studies (18).

With the development of nanotechnology over the last decade, golden opportunities have been created to discover the antimicrobial effects of metallic nanoparticles. The small size and high surface-to-volume ratio of these nanoparticles, cause them to solve many of the limitations of a variety diagnostic and therapeutic options (19). A study showed that Iron ion alone can interfere with the virus replication by preventing the function of the RNA poly-merase of virus (20). Also, according to a study, metallic nanoparticles, especially iron oxide nanoparticles, They can help to better targeting different types of medications (21).

Superparamagnetic iron oxide nanoparticles increase the half-life of the drug in the body or increase drug entry into infectious cells (22).

These nanoparticles have several advantages over gadolinium-based contrast agents, such as lower toxicity and lower diagnostic limits (23). According to a study, Iron oxide nanoparticles did not have any toxicity on the HLA cells at low concentrations and had not effected on DNA (24).

[†] Direct acting antivirals
[‡] Sustained virologic response

It was found that Fe3O4, TiO2 and Al2O3 nanoparticles at concentrations below 200 µg / ml have no toxic effects on the cells. iron nanoparticles were also found to have the lowest toxicity for mammalian cells (25).

These nanoparticles that are injected into the body, for the purpose of drug delivery, diagnosis, etc. and can be used as iron stores for body (21).

It should also be noted that iron oxide nanoparticles have antimicrobial effects against a variety of bacteria such as bacteria in food, Staphylococcus aureus and Escherichia coli and can be used to prevent the proliferation of microbial agents (26-29).

Considering these findings and multiple side effects or high cost of drugs for hepatitis c infection treatment, we decided to produce a combination drug with better targeting capabilities and a greater reduction in viral load by using peg interferon iron oxide nanoparticles that do not have high toxicity to the cells.

In addition to the results of three different pathways that combined iron oxide nanoparticles, peg interferon loaded iron oxide nanoparticles and interferon peg alone with hepatitis C virus, the effect of each compound with itself was also investigated. In the third pathway, peg Interferon reduced viral load more than first pathway. From these results, it can be concluded that peg interferon is likely to have a better performance than treatment in prevention. Iron oxide nanoparticles also reduced viral load more than first pathway, which suggests that iron oxide nanoparticles may also have better preventive effects compared to therapeutic effects. The same comparison was made with peg interferon loaded iron oxide nanoparticles. Peg interferon loaded iron oxide nanoparticles in the third pathway resulted in more reduction in loading of viruses compared to the first pathway.

Conclusion

Despite of the fact that hepatitis C viral load was decreased in the infected cells after induction of PEG interferon loaded iron oxide nanoparticles, no statistically significant results were found between bare iron oxide nanoparticles, peg interferon loaded iron oxide nanoparticles and peg interferon alone.

According to these results, more research in vivo is needed to clarify if there is a true reduction of viral load using peg interferon loaded iron oxide nanoparticles or not. It is suggested that other types of bare and drug-loaded nanoparticles may be examined in a similar method to our study.

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