Antiviral Research 97 (2013) 108–111

Contents lists available at SciVerse ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

Short Communication

Catheterized guinea pigs infected with Ebola Zaire virus allows safer sequential sampling to determine the pharmacokinetic profile of a phosphatidylserine-targeting monoclonal antibody

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ARTICLE INFO

Article history:
Received 26 July 2012
Revised 5 November 2012
Accepted 6 November 2012
Available online 16 November 2012

Keywords:
Ebola
Catheterized
Model
Pharmacokinetic

ABSTRACT

Sequential sampling from animals challenged with highly pathogenic organisms, such as haemorrhagic fever viruses, is required for many pharmaceutical studies. Using the guinea pig model of Ebola virus infection, a catheterized system was used which had the benefits of allowing repeated sampling of the same cohort of animals, and also a reduction in the use of sharps at high biological containment. Levels of a PS-targeting antibody (Bavituximab) were measured in Ebola-infected animals and uninfected controls. Data showed that the pharmacokinetics were similar in both groups, therefore Ebola virus infection did not have an observable effect on the half-life of the antibody.

Bavituximab (PGN401) is a monoclonal human-mouse chimeric antibody, with the variable region from the mouse IgG3 monoclonal antibody 3G4 that targets phosphatidylserine (PS) joined to the human IgG1κ constant regions (Thorpe, 2010). Whilst PS resides predominantly in the inner leaflet of the plasma membrane in healthy cells, it externalizes under certain stress conditions, during cell activation and in cell death via apoptosis (Hengartner, 2000). During this process, PS becomes available for antibody binding. Initial work with PGN401 focused on its use to target endothelial cells in tumor vasculature via their PS surface expression (Huang et al., 2005). Bavituximab has completed Phase II clinical trials in patients with advanced breast cancer and non-small cell lung cancer (Thorpe, 2010), and other Phase II clinical trials are ongoing.

More recently, the effects of PGN401 have been studied in the context of viral infection. Virus-induced events also result in a loss of lipid asymmetry exposing PS on the plasma membrane (Pai et al., 2009). Binding of PGN401 to these cells results in their elimination through recognition by cells of the immune system. To date, many of the antiviral properties of PGN401 have been investigated with hepatitis C virus infections (Sakamoto and Watanabe, 2009), and this work has progressed to clinical trials (Tomillero and Moral, 2008, 2009). Studies have also been undertaken in other viral infections. In 2008, it was reported that PGN401 showed efficacy in guinea pigs infected with Pichinde virus, a model that closely resembles Lassa fever in humans (Jahrling et al., 1981), as well as having effects on cells infected with influenza A, vaccinia, vesicular stomatitis virus and mouse cytomegalovirus (Soares et al., 2008).

Virus induced externalization of PS raises the possibility that PGN401 has broad spectrum antiviral activity. The advantages of targeting PS include drug specificity for infected cells, and since PS is a feature of the host cell, effectiveness would potentially not be susceptible to viral escape mutations (Mir et al., 2009). One such application for a broad spectrum antiviral would be against biodefense pathogens, such as Ebola virus (EBOV). In addition to important public health problems stemming from severe disease outbreaks in rural parts of Africa, EBOV also poses a potential bioterrorism threat and in the past has been included in weapons development programmes (Borio et al., 2002). Case-fatality rates of the African EBOV species in man are as high as 90%, with no prophylaxis or treatment available. EBOV has been classified as a Category A biowarfare agent by the Centers for Disease Control (Bray, 2003) and the development of new strategies against EBOV infection are required urgently.

One of the stages of antiviral drug development is an evaluation of the pharmacokinetic profile of the compound *in vivo* in the presence of virus, which could be challenging if the virus is highly path-

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http://dx.doi.org/10.1016/j.antiviral.2012.11.003

ogenic. The animal model for such studies should replicate those used for subsequent efficacy studies. Whilst non-human primates are considered to be excellent models for EBOV studies, due to their susceptibility to human isolates and the associated disease and pathogen resembling human infection (reviewed by Bente et al., 2009), the high costs and limited facilities available for housing these often mean that smaller animal models are used for screening of compounds beforehand. For EBOV, one such suitable animal model is the guinea pig (Connolly et al., 1999). Whilst not susceptible to clinical isolates, passaging the virus in the host 3–7 times results in complete lethality (Chepurnov et al., 2003; Connolly et al., 1999; Ryabchikova et al., 1996; Subbotina et al., 2010; Volchkov et al., 2000). In this study, Ebola virus (EBOV) Zaire (strain ME718) was passaged five times via the spleen to create a guinea pig-adapted stock in a manner similar to what has been described previously (Connolly et al., 1999).

An important aspect of pharmacokinetic analysis is the requirement for sequential sample collection over the evaluation period. For small animal models such as rodents, size and difficulties in blood collection represent significant obstacles. These obstacles are further compounded, when working with highly pathogenic viruses such as EBOV, a Biosafety Level 4 (BSL-4) pathogen, since efforts to reduce the use of sharps and risks to operators also become important requirements of working at maximum biosafety containment; referred to as containment level 4 (CL4) in the UK. To overcome these difficulties, guinea pigs with a catheter pre-inserted into the jugular vein (Charles River Laboratories, France) were utilized. The surgery involved is not dangerous to the animals, as the approach of the jugular vein is relatively simple. The catheter is exteriorized in the back through a small incision, and as such animals are housed singly for 24–48 h after surgery. Due to a rapid recovery process, studies typically started 6 days post-surgery. During experimental procedures, catheters were covered with a nylon band to prevent dislodging.

Catheterization enabled PGN401 to be administered intravenously, and for subsequent blood collection from the same animals. While enabling work to be carried out at CL4, this methodology also avoided the alternative approach of serial sacrifice, which would have resulted in higher numbers of animals being used; in contrast to the principles of the UK's National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3R's) (Holmes et al., 2010). In addition to the ethical and scientific merits of using the same groups of animals for pharmacokinetic analysis, the use of catheterized guinea pigs also eliminates the need to anesthetize animals before any procedures are carried out. In pharmacokinetic studies, samples are required at short time intervals, thus making multiple anesthetizations unacceptable in relation to animal welfare and the study. Catheterization bypasses these problems and EBOV-infected guinea pigs can be safely handled due to the easy access of the catheter port, simultaneously avoiding added animal stress of anesthetization.

The aim of this study was to determine the half-life of PGN401 in EBOV-infected and uninfected guinea pigs. Guinea pigs were infected subcutaneously with a host adapted strain of EBOV Zaire (ME718), at a dose of 10^3 $TCID_{50}$. This dose had previously been determined to cause 100% lethality in preliminary studies (data not shown). Two days post-infection, 3 mg/kg PGN401 (supplied by Peregrine Pharmaceuticals, Inc.) was delivered via the intravenous route. Blood samples were taken from cohorts of three guinea pigs at regular time intervals, and sera samples prepared by allowing the blood to clot in sera separation tubes and centrifuging at 1340*g* for 10 min. Samples were stored at −80 °C until analysis. A timecourse of samples was also prepared from uninfected animals that received 4 mg/kg PGN401 at identical timepoints post-administration.

For use of the catheter port, a small plug is removed from the end of the tube to allow access (Fig. 1A). Following this, a syringe adaptor is inserted to allow a syringe to be connected (Fig. 1B). This allows solutions to be injected intravenously, or blood samples collected (Fig. 1C). When the port is not in use, the tube is filled with blocking buffer (sodium chloride containing heparin) to prevent clot formation. If catheters are not used for a prolonged period of time, lines are flushed with blocking buffer at least every 48 h. Before administration or sample collection, the blocking solution is withdrawn until blood fills the length of the tube. After use, the tube is washed with sterile sodium chloride solution to ensure all substance is delivered or no blood is left that may form clots, before the blocking buffer is replaced followed by the small plug.

To determine the circulating concentration of PGN401 in the sera samples, high binding microtitre plates were coated with 1 μg/mL anti-human antibody (Jackson ImmunoResearch, USA) diluted in phosphate buffered saline (PBS) (Severn Biotech Ltd., UK) for 1 h at room temperature. Unbound antibody was washed off with three washes of 300 μL/well wash buffer consisting of PBS containing 0.05% Tween-20 (Sigma, UK). Wells were blocked by the addition of PBS containing 5% dialyzed fetal bovine sera (FBS) (Invitrogen, UK) for 1 h at room temperature. Blocking buffer was washed off, and samples and standards that had been pre-diluted in non-binding plates were transferred to the appropriate wells of the ELISA plate. Samples were tested at dilutions of 1:500, 1:2500 and 1:12500, with triplicate wells per dilution tested. A standard curve of PGN401 was produced by using a starting concentration of 1280 ng/ml and a twofold dilution series made to 1.25 ng/ml. This standard curve was run on each plate to control for any interplate variation. Plates were incubated for 1 h at room temperature. Wells were washed and horseradish peroxidase-conjugated goat anti-human IgG detector antibody (Jackson ImmunoResearch, US) added at a 1:2500 dilution and left for one hour at room temperature. Unbound antibody was removed by washing, and 3,3′,5,5′ tetramethyl benzidine (TMB) substrate (KPL, UK) added and left for approximately 10–15 min to allow the colorimetric reaction to occur. The reaction was stopped by the addition

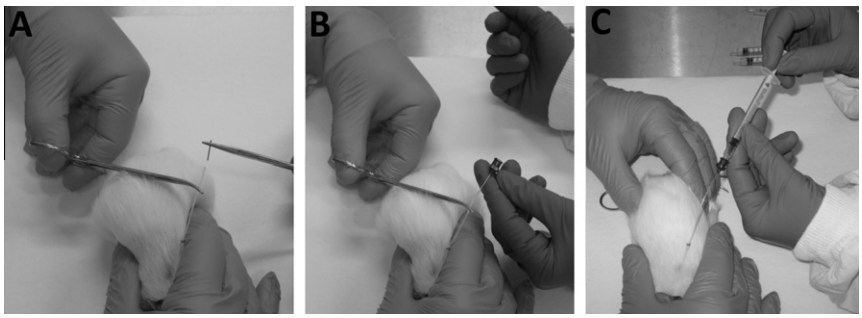

Fig. 1. Use of the catheter port in guinea pigs. (A) Removal of the plug to allow access to the tube. (B) Insertion of a syringe adaptor. (C) Connection of syringe to allow intravenous access for administration or sampling.

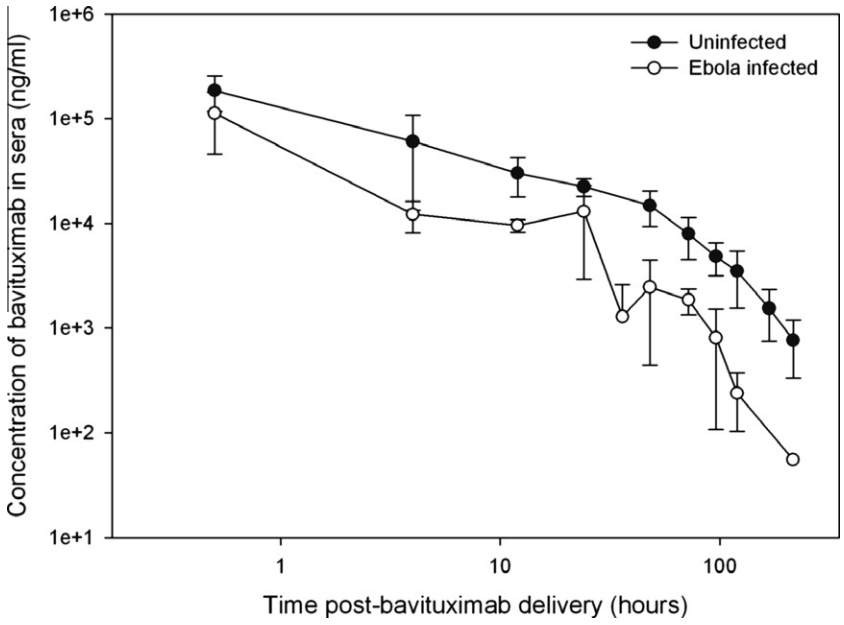

Fig. 2. Concentrations of PGN401 detected in uninfected and Ebola-infected guinea pigs at different times post-administration. Data points show mean results with error bars denoting standard deviation.

of 2 M H_2SO_4 (Fisher Scientific, UK). Absorbancies were read at a wavelength of 450 nm within 30 min. Results were analyzed using SoftMax Pro analysis software (Molecular Devices, UK).

The data showed that the concentration of PGN401 in the sera peaked soon after administration with the highest levels seen on the first timepoint; 30 min post-delivery, for both EBOV-infected and uninfected groups (Fig. 2). Concentrations declined with similar kinetics for both groups.

The half-life of PGN401 was determined to be 30.1 h in uninfected animals, and 28.4 h in the EBOV-infected group, showing similar decays of the monoclonal antibody in serum of guinea pigs irrespective of EBOV infection status. This will be important for determining optimum dosing strategies and concentrations of the compound in order to obtain the levels required for effective antiviral activity. When tested previously in guinea pigs infected with Pichinde virus, PGN401 was delivered intraperitoneally (Soares et al., 2008). However, this route will be difficult to apply in humans, so intravenous delivery is preferable and was the principal reason for studying PGN401 pharmacokinetics following this route of administration.

To our knowledge, this is the first report of catheterized guinea pigs being used for sequential sampling after infection with a highly pathogenic organism requiring maximum containment (CL4) facilities. Whilst in the larger non-human primate animals species the use of catheterization has been well documented (Graham et al., 2010; Kitagawa et al., 1992; Scalese et al., 1990; Wojnicki et al., 1994), for the smaller rodent models it has not been. Our approach was to initially use the uninfected GP model to generate a control data set. This also provided valuable experience of working with these animals before carrying out procedures at CL4. This experience allowed the catheters to be improved in consultation with the supplier, including a larger length of catheter for easier manipulation and a stronger tube composition due to the requirement for clamping during procedures. The updated design was used subsequently for the CL4 EBOV studies.

Ultimately, we have shown catheterized guinea pigs provide many advantages for work in high containment (CL4) facilities. Risk to laboratory workers are minimized by removing requirements for sharp needles. Ethical considerations are improved by reducing the number of animals required in studies and by eliminating the times animals are anesthetized. Finally, the scientific benefits of using this approach includes the potential that therapeutic compounds can be administered to and then assessed sequentially in the same groups of animals being studied, rather than being assessed in different animals as in the alternative approach of serial sacrifice. Importantly the catheter methodology described here is therefore able to reduce group variation which can become an issue when using outbred strains. All of these traits strengthen and expand the use of the guinea pig model of EBOV infection for use in the testing of new antivirals.

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