International Journal of Antimicrobial Agents 33 (2009) 168–173

Contents lists available at ScienceDirect

International Journal of Antimicrobial Agents

journal homepage: http://www.elsevier.com/locate/ijantimicag

Pentoxifylline inhibits replication of Japanese encephalitis virus: a comparative study with ribavirin

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ARTICLE INFO

Article history:
Received 23 May 2008
Accepted 17 July 2008

Keywords:
Antiviral agents
Japanese encephalitis virus
Pentoxifylline
Ribavirin

ABSTRACT

Several investigations have shown that pentoxifylline possesses broad-spectrum antiviral activity against a range of RNA and DNA viruses. However, its ability to inhibit Japanese encephalitis virus (JEV) replication has not yet been studied. The present study was designed to investigate the antiviral activity of pentoxifylline against JEV in vitro and in vivo. The activity of pentoxifylline against JEV was evaluated in vitro using cytopathic effect inhibition and plaque reduction assays. Pentoxifylline was able to inhibit JEV replication in a dose-dependent manner at a 50% inhibitory concentration (IC_{50}) of 50.3 μg/mL (0.00018 μM) and a therapeutic index (TI) of 10. Experiments to study the mechanism of antiviral action of pentoxifylline using in vitro translation of viral mRNA suggested that the drug did not interfere either with early or late protein synthesis but most likely exerted its action on virus assembly and/or release. Furthermore, the in vivo study showed that pentoxifylline at a concentration of 100 mg/kg and 200 mg/kg body weight was able to protect completely mice challenged with 50 × 50% lethal dose (LD_{50}) of JEV.

1. Introduction

Flaviviruses are important human pathogens causing a variety of diseases ranging from mild febrile illness to severe encephalitis and haemorrhagic fever. Among them, Japanese encephalitis virus (JEV) is a neuropathogenic virus commonly infecting children and is associated with acute encephalitis [1]. The disease burden of Japanese encephalitis (JE) is an increasing public health problem with an average of 50 000 cases per year in Asia [2,3]. Despite this, the prospects for therapy of flavivirus infections are not encouraging, which has led to the unavailability of a specific and efficient antiviral agent against JEV [4]. This has rekindled the search for a drug that can inhibit JEV replication.

Pentoxifylline is a methylxanthine derivative and has been used for treating human vascular diseases [5]. Despite being a cardiovascular drug, pentoxifylline also demonstrated high antiviral activity against herpes simplex virus, vaccinia virus, rotavirus and tick-borne encephalitis virus, suggesting that this drug also has broad-spectrum virus inhibitory properties [6]. Furthermore, pentoxifylline also showed inhibition of human immunodeficiency virus (HIV) expression in acutely and chronically infected cells in vitro and in human peripheral blood mononuclear cells [7,8]. However, the ability of pentoxifylline to inhibit JEV replication has not yet been studied. Therefore, the present study was designed to evaluate and specifically to understand the role of pentoxifylline as a potential therapeutic agent against JEV infection.

2. Materials and methods

2.1. Compounds

The compounds studied were an injectable form of pentoxifylline (20 mg/mL) (Trental™; Aventis, Paris, France) and ribavirin (200 mg) (Neaman, New York, NY).

2.2. Viruses and cells

A standard strain of JEV (P20778) was obtained from the National Institute of Virology, Pune, India. The *Aedes albopictus* (C6/36) mosquito cell line and porcine stable kidney (PS) cells were obtained from the National Centre for Cell Sciences, Pune, India, and maintained in minimum essential medium (MEM) with 10% foetal calf serum (FCS).

2.3. Animals

Random-bred Swiss albino mice (4 weeks old) were obtained from the Central Animal Research Facility, National Institute of Mental Health and Neurosciences (NIMHANS), Bangalore, India.

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doi:10.1016/j.ijantimicag.2008.07.013

2.4. Cytotoxicity of pentoxifylline and ribavirin

The cytotoxicity of pentoxifylline and ribavirin was evaluated using a trypan blue exclusion assay [9,10]. Briefly, PS cells grown to semiconfluence were exposed to five different concentrations of the compounds for 4 days and viable cells were counted using 2.5% trypan blue dye and a haemocytometer [10]. The concentration of compound that reduced cell growth by 50% was estimated as the 50% cytotoxic concentration (CC_{50}). The effect of the compounds on cellular proliferation was also studied as described previously [10]. Ribavirin, a known inhibitor of flavivirus replication, was used in this study as a standard for comparing the results in all the experiments.

2.5. Screening for inhibition of virus-induced cytopathic effect (CPE)

The antiviral activity of pentoxifylline and ribavirin was initially determined using a CPE inhibition assay as described previously [10]. Briefly, a PS cell monolayer was infected with 1 multiplicity of infection (MoI) of virus and incubated for 2 h at 37 °C. At the end of the incubation period, the monolayer was rinsed with sterile phosphate-buffered saline (PBS) and then doubling dilutions of ribavirin and pentoxifylline (beginning with the CC_{50}) were added and incubated for 3 days. The experiment was terminated when the virus control showed maximum CPE. The presence or absence of CPE was recorded microscopically every day and the plates were stained using crystal violet and compared with the virus control and drug control. All the experiments were run in triplicate to ensure reproducibility.

2.5.1. Confirmation of antiviral activity by the plaque reduction assay

The antiviral activity of pentoxifylline noted in the screening experiments was confirmed by the plaque reduction assay as described previously [10]. Briefly, PS cells grown to a confluent monolayer were infected with 1 MoI of JEV and adsorbed for 2 h at 37 °C. At the end of adsorption the monolayer was rinsed and 100 μL of MEM containing varying concentrations of pentoxifylline (500, 250, 125, 62.5 and 31.25 μg/mL) or ribavirin (50, 25, 12.5, 6.25 and 3.12 μg/mL) was added. The monolayer was then overlaid with maintenance medium containing 0.2% molten agarose (Sigma–Aldrich, St. Louis, MO). Appropriate controls were included in each run of the assay. Incubation was carried out at 37 °C for 3 days. At the end of the incubation period, monolayers were fixed and stained using 1% crystal violet and plaques were counted using a hand lens. All experiments were run in triplicate. Percentage inhibition of plaques was determined using the following formula:

$$\%\,\text{inhibition} = \frac{\text{no. of plaques in virus control} - \text{no. of plaques in drug-treated}}{\text{no. of plaques in virus control}} \times 100$$

The antiviral activity was expressed as 50% inhibitory concentration (IC_{50}), which is the concentration of compound required to inhibit viral plaques by 50% compared with the virus control. The therapeutic potential and the specificity of action of the compounds were calculated as the therapeutic index (TI), which is the ratio of CC_{50} to IC_{50}.

2.6. Determining the mechanism of action of pentoxifylline in relation to JEV replication

To understand the possible mechanism of action of pentoxifylline in relation to the replicative cycle of JEV, various in vitro experiments detailed below were carried out.

2.6.1. Determining the kinetics of JEV replication in PS cells

A 24-well plate containing sterile coverslips in each well was seeded with 4×10^4 cells/well and incubated to attain confluence. The monolayer was then infected with JEV (MoI = 1) for 1 h at 37 °C. Following incubation, the monolayer was rinsed and replenished with medium containing 1% FCS. This time point was considered as 0 h post infection. Subsequently at 2, 4, 6, 8, 10, 12, 14, 16 and 24 h post infection, the medium was harvested to determine the amount of extracellular virus released into the supernatant. At each time point, the coverslip containing cells was also removed, fixed in chilled acetone and stained by immunofluorescence assay (IFA) using a monoclonal antibody against the envelope protein of JEV to detect the cell-bound antigen [11].

2.6.2. Understanding the kinetics of antiviral activity of pentoxifylline

A 24-well plate was seeded with PS cells and incubated at 37 °C overnight. JEV was added to this monolayer and incubated for 2 h at 37 °C. At the end of virus adsorption, the monolayer was rinsed using sterile PBS and replenished with MEM containing 1% FCS. This time point was considered as 0 h post infection. Starting from the 0 h time point, 0.0017 μM pentoxifylline was added at 0, 2, 4, 6, 8, 10, 12, 14, 16 and 24 h post infection and incubated at 37 °C. The supernatant fluid was harvested from the respective wells at 48 h post infection. The fluid was divided into two parts. One part was used to determine the virus yield (50% tissue culture infective dose ($TCID_{50}$)/mL) and the second part was used to detect the presence of soluble JEV antigen by antigen capture enzyme-linked immunosorbent assay (ELISA) as described elsewhere [12]. To detect cell-bound antigen, the coverslip cultures were fixed in chilled acetone for 30 min at 4 °C and stained using monoclonal antibody to JEV (clone F2C2) and anti-mouse IgG–fluorescein isothiocyanate (FITC) conjugate by indirect IFA.

2.6.3. Confirmation of the mechanism of action of pentoxifylline

To understand the mechanism of action of pentoxifylline, an in vitro translation experiment was carried out using commercially available Transcend™ Non-Radioactive Translation Detection System and Rabbit Reticulocyte Lysate (Promega, Madison, WI). A PS cell monolayer was adsorbed with JEV (MoI = 1) for 1 h. Following adsorption, monolayer was rinsed and 0.0017 μM pentoxifylline was added to one set of JEV-infected cells and incubated for 4 h. Pentoxifylline at the same concentration was added to a second set of monolayer cultures at 10 h. The plates were further incubated for 48 h at 37 °C. Appropriate virus and cell controls were included. At the end of incubation, the cells were treated with 750 μL of TRIzol reagent (Gibco, Rockville, MD) and viral RNA was extracted as per the manufacturer's instruction. The extracted RNA was subjected to real-time reverse transcriptase polymerase chain reaction (RT-PCR) using SYBR green I chemistry as described previously [13] with minor modifications [10] to ensure the presence of JEV RNA. The viral RNA obtained from JEV-infected cells, which encodes a 50-kDa protein, was subjected to in vitro translation using a commercial kit (Promega) as described previously [10]. After completion of the translation reaction, 1 μL of the product was subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The gel was electroblotted onto a polyvinylidene fluoride (PVDF) membrane. The membrane was reacted with specific monoclonal antibody to JEV and developed using diaminobenzidine and H_2O_2.