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Structure-activity relationships of nucleoside analogues for inhibition of tick-borne encephalitis virus

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ABSTRACT

Tick-borne encephalitis (TBE) represents one of the most serious arboviral neuro-infections in Europe and northern Asia. As no specific antiviral therapy is available at present, there is an urgent need for efficient drugs to treat patients with TBE virus (TBEV) infection. Using two standardised *in vitro* assay systems, we evaluated a series of 29 nucleoside derivatives for their ability to inhibit TBEV replication in cell lines of neuronal as well as extraneural origin. The series of tested compounds included 2′-*C*- or 2′-*O*-methyl substituted nucleosides, 2′-*C*-fluoro-2′-*C*-methyl substituted nucleosides, 3′-*O*-methyl substituted nucleosides, 3′-deoxynucleosides, derivatives with 4′-*C*-azido substitution, heterobase modified nucleosides and neplanocins. Our data demonstrate a relatively stringent structure-activity relationship for modifications at the 2′, 3′, and 4′ nucleoside positions. Whereas nucleoside derivatives with the methylation at the C2′ position or azido modification at the C4′position exerted a strong TBEV inhibition activity (EC_{50} from 0.3 to 11.1 μM) and low cytotoxicity *in vitro*, substitutions of the O2′ and O3′ positions led to a complete loss of anti-TBEV activity (EC_{50} > 50 μM). Moreover, some structural modifications of the heterobase moiety resulted in a high increase of cytotoxicity *in vitro*. High antiviral activity and low cytotoxicity of C2′ methylated or C4′ azido substituted pharmacophores suggest that such compounds might represent promising candidates for further development of potential therapeutic agents in treating TBEV infection.

1. Introduction

Tick-borne encephalitis virus (TBEV), a causative agent of tick-borne encephalitis (TBE), is a member of the *Flaviviridae* family, which includes many medically important viruses, such as hepatitis C virus (HCV), West Nile virus, Zika virus, dengue virus, Japanese encephalitis virus, yellow fever virus, and several haemorrhagic fever-associated flaviviruses (Baier, 2011). TBE represents one of the most serious arboviral neuro-infections in Europe and northern Asia with thousands of TBEV-infected people and many reported deaths annually (Dumpis et al., 1999; Heinz and Mandl, 1993). The characteristic clinical symptoms of acute TBE range from a mild meningitis to severe meningoencephalitis/myelitis with the risk of temporary or permanent neurologic sequelae after TBE infection (Ruzek et al., 2010). Although safe and efficient vaccines against TBEV are available, the number of TBE patients in the endemic regions of Europe continuously increases (Heinz et al., 2013; Zavadska et al., 2013). As no specific antiviral therapy is available at present, there is an urgent need for efficient drugs to treat patients with TBEV infection (Puig-Basagoiti et al., 2006).

Inhibitors of viral polymerases are the largest class of approved antiviral drugs, of which the largest number is represented by nucleoside analogue inhibitors (De Clercq, 2011). Mode of action of nucleoside inhibitors is based on the premature termination of viral RNA synthesis (De Clercq and Neyts, 2009). Following intracellular phosphorylation, the 5′-triphosphate metabolites are competitively incorporated into the viral RNA nascent chains, which

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prevents further extension of the incorporated analogue by addition of the next nucleoside triphosphate resulting in formation of incomplete (non-functional) viral RNA chains. In general, the antiviral activity of nucleoside inhibitors is predominantly determined by steric interference (hydrogen bonding capability) between the nucleoside triphosphate and the viral polymerase active site. Moreover, the effect of the absence, conformational constraints, or steric/electronical hindrance of the nucleoside 3′-hydroxyl function on formation of a phosphodiester linkage with the incoming nucleoside triphosphate could also play an important role in the efficient termination of viral RNA synthesis (De Clercq, 2004; De Clercq and Neyts, 2009). Cellular uptake and intracellular metabolism (such as deamination, phosphorolysis or phosphorylation) can also considerably influence the antiviral activity of a nucleoside analogue (Eldrup et al., 2004; Tomassini et al., 2005; Ma et al., 2007).

Previously, we identified three 2′-*C*-methylated nucleoside analogues (i.e., 2′-*C*-methyladenosine, 2′-*C*-methylcytidine and 7-deaza-2′-*C*-methyladenosine) as effective inhibitors of TBEV replication *in vitro* (Eyer et al., 2015). In connection with these results, we report here a structure-activity relationship study based on the antiviral/cytotoxicity profile of 29 nucleoside derivatives, each differing in chemical substituents on the ribose ring and in the type and chemical modifications of the heterobase. We focused our attention on the evaluation of 2′, 3′, and 4′-modified nucleosides, for which antiviral activity was previously reported for other viruses, especially HCV (Eldrup et al., 2004; Klumpp et al., 2008; Sofia et al., 2012), Zika (Eyer et al., 2016), dengue (Lee et al., 2015), yellow fever (Julander et al., 2010), and haemorrhagic fever-associated flaviviruses (Flint et al., 2014). The tested compounds were characterized using standardised *in vitro* assay systems in terms of inhibition of TBEV replication, inhibition of virus-induced cytopathic effect (CPE) formation, suppression of viral antigen expression in TBEV-infected cell cultures, and evaluation of viability on compound-treated host cells. Based on these screens, we identified the 2′-*C*-methyl or 4′-*C*-azido substituents as important for a selective TBEV inhibition and a low cytotoxicity *in vitro*.

2. Material and methods

2.1. Cell cultures, virus strains and antiviral compounds

Porcine kidney stable (PS) cells, a cell line widely used for TBEV isolation, multiplication, and for conducting plaque assays (Kozuch and Mayer, 1975), were cultured at 37 °C in Leibovitz (L-15) medium supplemented with 3% precolostral calf serum and a 1% mixture of penicillin and glutamine (Sigma-Aldrich, Prague, Czech Republic). Human neuroblastoma UKF-NB-4 cells, a valuable model for neuropathogenesis studies of TBEV (Ruzek et al., 2009), were cultured at 37 °C in 5% CO_2 in Iscove's modified Dulbecco's medium (IMDM) with 10% foetal bovine serum and a 1% mixture of antibiotics (Sigma-Aldrich, Prague, Czech Republic). Hypr and Neudoerfl TBEV strains, typical representatives of the West European TBEV subtype, were used for evaluation of the antiviral activity of the test compounds. Nucleoside analogues were purchased as follows: 2′-*C*-methyl, 2′-*O*-methyl, and 3′-*O*-methyl substituted nucleosides, 3′-deoxynucleosides, sofosbuvir, and 6-azauridine from Carbosynth (Compton, UK), 4′-azidocytidine, balapiravir and RO-9187 from Medchemexpress (Stockholm, Sweden), neplanocin A from Cayman Chemical Company (Ann Arbor, Michigan), 3-deazaneplanocin A from Selleckchem (Munich, Germany), mericitabine from ChemScene (Monmouth Junction, NJ), PSI-6206 from ApexBio (Boston, MA), tubercidin, toyocamycin, sangivamycin, ribavirin and 2′-deoxynucleosides from Sigma-Aldrich (Prague, Czech Republic). Nucleotide triphosphate standards for HPLC analysis were purchased from TriLink Biotechnologies (San Diego, CA). The test compounds were solubilised in 100% DMSO to yield 10 mM stock solutions.

2.2. In vitro antiviral assays

A viral titre inhibition assay was performed to measure the antiviral efficacy of nucleoside analogues in cell culture. PS or UKF-NB-4 cells were seeded in 96-well plates (approximately 2×10^4 cells per well) and incubated for 24 h to form a confluent monolayer. Following incubation, the medium was aspirated from the wells and replaced with 200 μl of fresh medium containing 50 μM of the test compound (three wells per compound), which was inoculated with the Hypr or Neudoerfl TBEV strain at a multiplicity of infection (MOI) of 0.1. As a negative control, DMSO was added to virus- and mock-infected cells at a final concentration of 0.5% (v/v). Culture medium was collected 3 days postinfection (p.i.) to yield a 40–50% CPE in virus control wells. The CPE was monitored visually using the Olympus BX-5 microscope equipped with an Olympus DP-70 CCD camera. Viral titres were determined by plaque assays and expressed as PFU ml^{-1} (De Madrid and Porterfield, 1969). For dose-response studies, PS cell monolayers were cultured with 200 μl of medium containing the test compounds over the concentration range of 0–50 μM and TBEV (Hypr strain) at an MOI of 0.1. The medium was collected from the wells at 2-day intervals (post-infection days 1, 3 and 5), the viral titres were determined by plaque assays and used to construct TBEV dose-response curves. The dose-response curves on post-infection days 3 were used to estimate the 50% effective concentration (EC_{50}).

2.3. Immunofluorescence staining

To measure the compound-induced inhibition of viral surface antigen expression, a cell-based flavivirus immunostaining assay was performed as previously described (Eyer et al., 2015). Briefly, PS cells were seeded on slides, infected with the TBEV Hypr strain at an MOI of 0.1, treated with the test compound (50 μM) and cultured for 4 days at 37 °C. After a cold acetone-methanol (1:1) fixation and blocking with 10% foetal bovine serum, the cells were incubated with a mouse monoclonal antibody recognising the flavivirus group surface antigen (1:250, Sigma-Aldrich, Prague, Czech Republic) and subsequently labelled with an anti-mouse goat secondary antibody conjugated with FITC (1:500) by incubation for 1 h at 37 °C. The cells were counterstained with DAPI (1 μg ml^{-1}) to visualise the cell nuclei. Finally, the cells were mounted in 2.5% DABCO (Sigma-Aldrich, Czech Republic) and the fluorescence signal was recorded with an Olympus IX71 epifluorescence microscope.

2.4. Cytotoxicity assays

PS cells were grown in 96-well plates overnight to form a confluent monolayer and subsequently treated with test compounds over the concentration range of 0–50 μM. At day 3 p.i., the medium was collected and the potential cytotoxicity of test compounds was determined in terms of cell viability using the Cell Counting Kit-8 (Dojindo Molecular Technologies, Munich, Germany) according to the manufacturer's instructions. The concentration of compound that reduced cell viability by 50% was considered the 50% cytotoxic concentration (CC_{50}).

2.5. Quantification of 2′-C-methylated nucleosides in compound-treated cells

The UKF-NB4 cells were plated at a density of 2×10^5 cells/ml in 6-well plates for 48 h and subsequently exposed to tested