Antiviral Research 81 (2009) 261–266

Contents lists available at ScienceDirect

Antiviral Research

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

Effective ribavirin concentration in mice brain using cyclodextrin as a drug carrier: Evaluation in a measles encephalitis model

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ARTICLE INFO

Article history:
Received 22 September 2008
Received in revised form
26 November 2008
Accepted 10 December 2008

Keywords:
Ribavirin
Alpha-cyclodextrin
Blood–brain barrier
Measles virus
Encephalitis

ABSTRACT

Ribavirin (RBV) is a water-soluble synthetic nucleoside with broad spectrum antiviral properties, but it is ineffective against major viral encephalitis because of a failure to cross the blood–brain barrier (BBB). The antiviral activity of the complex ribavirin/alpha-cyclodextrin was previously demonstrated to be stronger than free ribavirin, in an in vivo model of measles virus (MV) encephalitis in mice. The role of cyclodextrin (CD) on ribavirin uptake into the brain needs to be defined. Ribavirin specific extraction from brain tissue was developed, based on a solid phase extraction. It was quantified by high performance liquid chromatography at different time points after intraperitoneal injection of single or multiple doses of free ribavirin or of the complex ribavirin/alpha-cyclodextrin. Whatever the tested dose (40 or 100 mg/kg), the amount of ribavirin in the brain was significantly higher ($p < 0.001$) when the drug was injected as a complex with alpha-cyclodextrin, in healthy or measles virus-infected mice.

1. Introduction

Ribavirin [1-(β-D-ribofuranosyl)-1H-1,2,4-triazole-3-carboxamide] is a water-soluble synthetic nucleoside with broad spectrum antiviral properties (Huffman et al., 1973). It has been shown to be ineffective against several encephalitis viruses in mice after intraperitoneal (i.p.), subcutaneous or intramuscular injection (Bussereau et al., 1988; Sidwell et al., 1973). In contrast, ribavirin (RBV) improved the survival of subacute sclerosing panencephalitis (SSPE) virus-infected hamsters but only when administered intracranially and did not improve their survival when administered intraperitoneally (Honda et al., 1994), suggesting a failure of RBV to access to the brain.

The central nervous system is protected by the blood–brain barrier (BBB), a particular tissue structure. The BBB controls the passage from the blood to the cerebral nervous system of neurological toxins as well as therapeutic molecules. Thus, a major challenge of treatment of brain disorders is to overcome this barrier.

Cyclodextrins (CDs) can form either inclusion complexes, where a lipophilic guest molecule, for example a drug molecule, is located within the lipophilic central cavity, or non-inclusion complexes, where the hydroxyl groups on the outer surface of CD molecules can form hydrogen bonds with other molecules (Loftsson and Duchêne, 2007), including RBV (Grancher et al., 2005). CDs were proposed as a solution to improve RBV bioavailability in the brain after i.p. administration. In pharmaceutical applications, CDs are generally used as solubilizers (Loftsson and Brewster, 1996). Since RBV is highly soluble in water, the interest of the use of cyclodextrin in a complex form with RBV is to improve bioavailability (Uekama and Otagiri, 1987). Moreover, studies on CDs behavior on biological membranes demonstrated enhancement of drug absorption across dermal, nasal, intestinal or blood–brain barrier by extracting cholesterol, phospholipids or proteins from membranes (Monnaert et al., 2004b). These properties of CD suggest that RBV/CD complexes have a potential to targeting RBV into the brain.

RBV-sensitive viruses responsible for encephalitis include measles, haemorragic fever, West Nile, tick born encephalitis and Japanese encephalitis. We chose measles virus (MV) since antiviral activity of RBV against MV had already been assessed in cotton rats, infected by either aerosol or i.p. route (Wyde et al., 2000) and in hamsters after intracranial injection (Honda et al., 1994).

We previously developed a MV encephalitis model in mice, based on CAM/RB neurovirulent MV strain and measles-susceptible CBA/ca mice injected intracranially (Jeulin et al., 2006). Thus, previous studies on this MV encephalitis model in mice have demonstrated that i.p. injection of RBV/α-CD (1:3) complex (40 mg/kg of RBV) increased antiviral activity compared to free RBV injection at the same dose: the MV-viral load was reduced in mice brain and the morbidity (weight loss) and mortality rate decreased

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doi:10.1016/j.antiviral.2008.12.006

in the complex-treated group, from 80% to 40% for mortality rate of RBV- and RBV/α-CD treated mice, respectively (Jeulin et al., 2008). The aim of this work was to explain increased antiviral activity in the presence of CD: is there an increased quantity of RBV into the brain? To confirm influence of CD on drug concentration into the brain, a protocol of RBV extraction from the brain was developed; it was based on organ sample grinding, solid-phase extraction and extract freeze-drying. Quantification was achieved by high performance liquid chromatography (HPLC) with spectrophotometer detection, using calibration curve prepared ex vivo, at different time points after single or multiple i.p. injections of the RBV or the complex RBV/α-CD.

2. Materials and method

2.1. Chemicals and reagents

All chemicals were of HPLC or reagent grade. Acetonitrile and formic acid were obtained from Carlo Erba reagenti (Val de Reuil, France), and ammonium acetate and ammonia solution from Merck (Lyon, France). Ammonium phosphate and 3-methylcytidine methosulfate (as an internal standard) were purchased from Sigma–Aldrich (Lyon, France) and RBV from MP Biomedical (Illkirch, France). All chemicals where diluted with HPLC-grade water.

Phenyl boronic acid (PBA) cartridges (Bond Elute PBA [100 mg]) used for solid-phase extraction were obtained from Varian (Les Ulis, France).

The RBV/α-CD complex was prepared at a molar ratio of 1:3 in distilled water as previously described (Grancher et al., 2004). Concentrations of RBV/α-CD solutions are expressed as RBV concentrations. Both RBV and RBV/α-CD complex were dissolved in distilled water and sterilized by filtration through a 0.22 μm membrane.

2.2. High performance liquid chromatography

2.2.1. Apparatus and conditions

The HPLC system used in this study consisted of a vacuum degasser SCM1000 (Thermo-Finnigan, San Jose, CA) and a barrow-bore quarterly Spectra System P1000XR (Thermo-Finnigan) gradient pump with a loop of 20 μl. A C_{18} reversed phase column TSKgel ODS-120T (250 mm length × 4.6 mm i.d.) from Tosoh Bioscience (Lyon, France) was used and thermostated at 25 °C with a column temperature controller 560-CIL (Cluzeau Info Labo, Puteaux-la-Défense, France). The effluents were monitored with a double-beam spectrophotometric detector (Spectrasystem UV1000, Thermo-Finnigan) at 207 nm.

The mobile phase consisted on an aqueous ammonium phosphate buffer solution (0.087 M, pH 6.5). The flow rate of eluant was set at 1 ml/min.

2.2.2. Sample preparation for HPLC

Brain tissues were suspended in 1 ml of distilled water and grinded. A volume of 0.025 ml of a 100 mg/l solution of 3-methylcytidine methosulfate in distilled water was added to each sample as an internal standard. The homogenate was processed by centrifugation at 20 000 × *g*, for 30 min at +4 °C to remove debris. Sample supernatant was neutralized with 3 ml of aqueous ammonium acetate buffer (250 mM, pH 8.5).

The Bond Elute PBA cartridge was pre-treated with 1 ml of 100 mM formic acid followed by 5 ml of 250 mM ammonium acetate buffer (pH 8.5). The neutralized sample was loaded onto the treated-Bond Elute PBA cartridge and allowed to percolate.

RBV and the internal standard were collected by eluting the cartridge two times with 1 ml of 100 mM formic acid and then freeze-dried. Twenty-microliter of each sample, reconstituted in 1 ml of mobile phase, were injected onto the HPLC column.

2.3. In vivo quantification of ribavirin in the brain

2.3.1. Animals

CBA/ca mice of either sex (weight range 10–15 g at experimental onset) from Harlan France SARL (Gannat, France) were maintained in plastic cages under natural light cycle at 19 °C and 44–45% humidity. Food and water were provided ad libitum. All local legal and ethical requirements were observed. MV-infected and control animals were housed in separate rooms.

2.3.2. Intracerebral MV challenge

On day 0, CBA/ca mice (3–4 weeks-old) were anesthetized by intraperitoneal administration of 50 μl of a solution containing 6.25 mg/ml ketamine, 6.25 mg/ml xylazine and 0.2 mg/ml atropine (corresponding almost to 26 mg/kg of ketamine and xylazine and 0.8 mg/kg of atropine). The mice were injected intracerebrally with 1×10^3 PFU of the rodent brain-adapted CAM/RB strain of MV (kindly provided by Prof U. Liebert, Leipzig, Germany), as previously described by Jeulin et al. (2008). The same working stock of virus (titer of 7.40 $\log_{10}$ Eq. PFU/ml) was used for all in vivo experiments (Wyshak and Detre, 1972).

2.3.3. Intraperitoneal treatment

At least, three mice were used for each time point.

2.3.3.1. Single dose. Healthy CBA/ca mice were weighed and treated by i.p. injection of a single dose of free RBV (40, 100 or 200 mg/kg) or of the complex RBV/α-CD (corresponding to 40 or 100 mg/kg of RBV). Concentration of RBV in the brain was quantified from 30 min to 48 h after i.p. injection.

In MV infected mice, i.p. injection of a single dose of 40 mg/kg of free RBV or of the complex RBV/α-CD was performed on day 3 after intracerebral injection of MV, when viral load reached 2×10^7 equivalent copies/brain (quantification by polymerase chain reaction of the N gene of MV), according to Jeulin et al. (2008).

2.3.3.2. Multiple doses. Mice were weighed and treated once a day by i.p. injection of RBV (40 mg/kg) or RBV/α-CD (1:3) complex (corresponding to 40 mg/kg of RBV) from the day before challenge until the day 4. RBV was extracted from the brain 3 and 5 h after the last i.p. injection, depending on the T_{max} observed in single dose studies.

2.3.4. Ribavirin quantification

A stock solution of RBV was prepared at a concentration of 40 mM in HPLC-grade water and stored at 4 °C for 1 week. For ex vivo calibration curve preparation, reference samples containing 2, 4, 8, 12, 18, 24 and 30 nmol of RBV were prepared by diluting stock solution with drug-free grinded brain tissue. The calibration curve was determined by plotting the peak area ratio of RBV to the internal standard against the RBV added in the brain sample.

2.4. Statistical analyses

Two-tailed statistical analyses were performed using Statview software for windows (version 5; Abacus Concepts). Univariate analyses of variables were performed by unpaired *t*-test. $P < 0.05$ was considered to be significant in all analyses.

3. Results

3.1. Quantification of ribavirin in brain tissue

The proposed method to extract RBV from brain was an adaptation of the blood sample preparation procedure, previously described by Homma et al. (1999). The RBV extraction was preceded by brain grinding and a centrifugation step. The solid phase extraction was followed by a freeze-drying step before dissolution in mobile phase. RBV extraction was based on use of phenyl boronic acid bond elut cartridge which specifically retains oligosaccharides alditols via interactions of sterically unbindered vicinal hydroxyl group, present in RBV structure (Stoll and Hounsell, 1988). Indeed, the matrix bound phenyl boronic acid was activated in basic medium to give phenylboronate and permitted covalent bonding of planar vicinal diol to phenylboronate. Diols were released from the complex by diluted acid.

A typical chromatogram of RBV treated-mice brain sample is shown in Fig. 1. Sample pre-treatment and solid-phase extraction permitted RBV and internal standard peaks isolation on the chromatogram. The retention time of RBV and internal standard were 5.9 and 10.7 min, respectively. At those times, no interfering peaks were observed on the chromatogram for untreated mice brain (data not shown).

A linear calibration curve was obtained ex vivo from 2 to 30 nmol/brain. The equation for the line, calculated by regression analysis, was $y = 0.0111x - 0.0262$ ($r^2 = 0.9927$) where y is the mean peak area ratio of RBV/internal standard and x the quantity of RBV per brain. The RBV concentration of each sample was estimated from this standard curve.

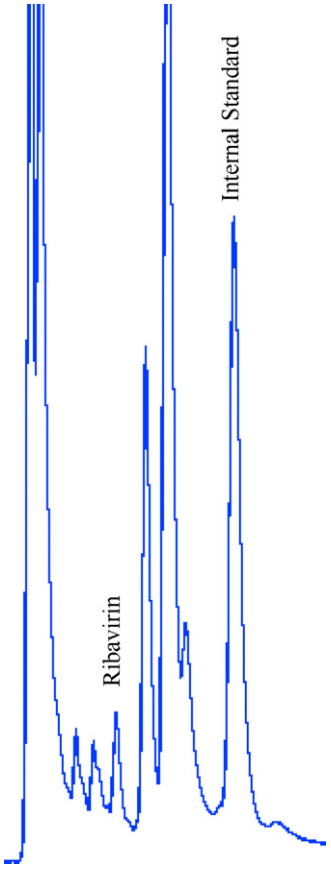

Fig. 1. Chromatogram of a brain extract from intraperitoneal ribavirin-treated mice (40 mg/kg), at 12 h after injection. Internal standard (3-methylcytidine methosulfate) was added before solid-phase extraction.

3.2. Ribavirin pharmacokinetics following ribavirin or ribavirin/α-cyclodextrin intraperitoneal administration

3.2.1. Single dose in healthy mice

In the first trial, RBV pharmacokinetic parameters in brain were determined in healthy mice, using the method described above. Intraperitoneal injection of healthy mice with a single dose of free RBV at 40, 100 or 200 mg/kg resulted in a maximal concentration (C_{max}) of 5.23 ± 0.56, 9.39 ± 0.64 or 25.31 ± 0.86 nmol/brain, respectively, at a maximal time (T_{max}) of 3–6 h after injection. RBV is present in the brain for up to 24 h (Fig. 2).

After i.p injection of a single dose of RBV/α-CD complex at 40 or 100 mg/kg, the quantity of RBV in the brain was maximal at 2–3 h, respectively, after inoculation and then decreased up to 24 h. The C_{max} reaches 11.49 ± 1.28 and 14.39 ± 0.35 nmol/brain, respectively.

Regardless of the dose used, the quantity of RBV in the brain was 1.5–2 times above, thus significantly higher ($p < 0.0001$ and $p = 0.002$, respectively) when the drug was administered as a complex of RBV/α-CD (Fig. 2). After RBV/α-CD-treatment (100 mg/kg), the maximal quantity was obtained earlier (2 h) than after free RBV-treatment (6 h).

3.2.2. Single dose in measles virus-infected mice

When RBV was administered as a single dose of free ribavirin at 40 mg/kg, on day 4 after intracranial injection of MV, RBV in

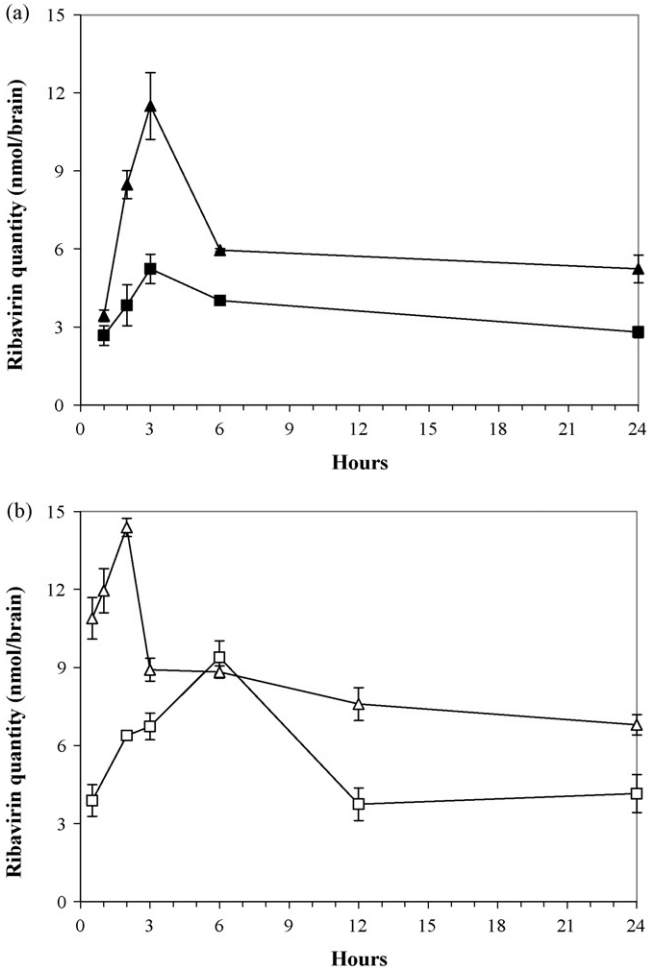

Fig. 2. Pharmacokinetics of ribavirin in brain tissue of healthy mice after (a) a single intraperitoneal injection of (■) ribavirin (40 mg/kg) or (▲) ribavirin/alpha-cyclodextrin complex (40 mg/kg of ribavirin), or (b) after a single intraperiotoneal injection of (□) ribavirin (100 mg/kg) or (△) ribavirin/alpha-cyclodextrin complex (100 mg/kg of ribavirin). Values represent the means found for three mice ± standard deviation.

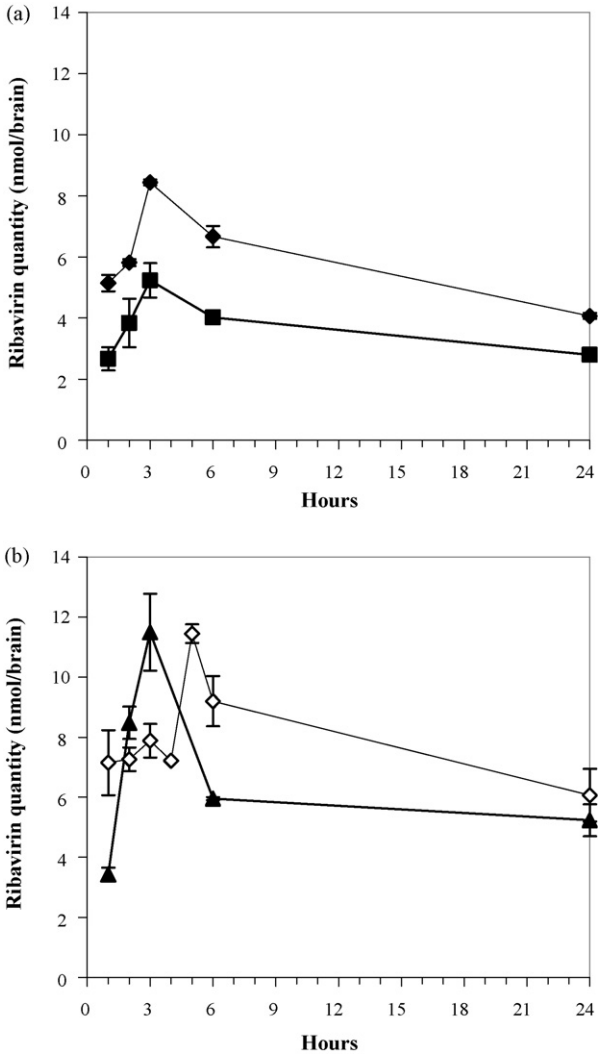

Fig. 3. Pharmacokinetics of ribavirin in brain tissue after (a) a single intraperitoneal injection of ribavirin (40 mg/kg) in healthy (■) or measles virus CAM/RB strain intracranially-infected mice (♦), or (b) after a single intraperitoneal injection ribavirin/alpha-cyclodextrin complex (40 mg/kg of ribavirin) in healthy (▲) or measles virus CAM/RB strain intracranially-infected mice (◊). Values represent the means found for three mice ± standard deviation.

the brain reached its highest concentration at 3 h after drug injection (Fig. 3a). The mean brain concentration for RBV from 30 min to 24 h after drug injection was significantly higher in mice developing measles encephalitis than in healthy mice ($p < 0.0001$).

On the other hand, in RBV/α-CD-treated mice, MV encephalitis infection does not impact on RBV concentration in the brain ($C_{max} = 11.5 \pm 0.31$ nmol/brain). RBV maximal concentration occurred 5 h after drug administration instead of 3 h in healthy mice (Fig. 3b).

3.2.3. *Multiple doses in measles virus-infected mice*

RBV level in brain tissue was determined after mice were treated with either RBV or RBV/α-CD complex (40 mg/kg/day) of RBV) for 6 days. The treatment began 1 day before MV intracranial injection. Since RBV concentration is maximal at 3 and 5 h after administration of free RBV or the complex, respectively, both periods were selected for determination of drug concentration in the brain after multiple doses in infected mice. Three hours after the last i.p. injection, mean RBV concentration was determined to be 12.74 ± 0.29 nmol/brain in free RBV-treated mice and

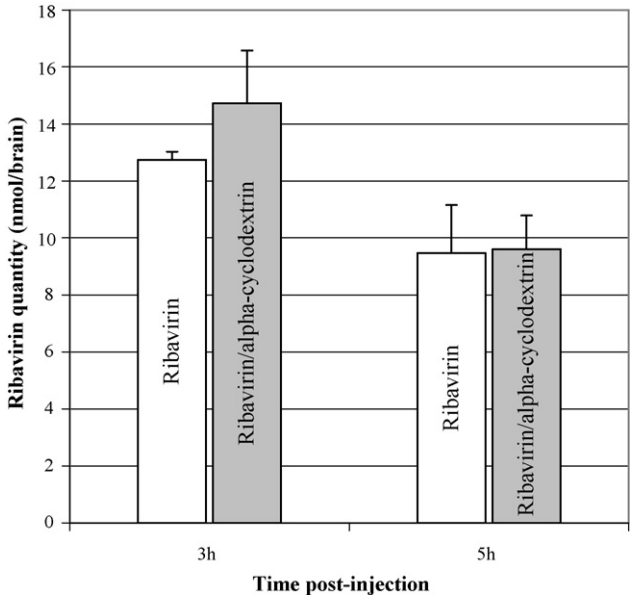

Fig. 4. Ribavirin concentrations in brain tissue of infected mice, treated daily (from day 1 to day 4) by intraperitoneal injection of free ribavirin (40 mg/kg) or the complex ribavirin/alpha-cyclodextrin (40 mg/kg of ribavirin). Mice were intracranially injected with the measles virus CAM/RB strain on day 0. Ribavirin was extracted 3 or 5 h after the last injection, on day 4. Values represent the means found for three mice ± standard deviation.

14.73 ± 1.85 nmol/brain in complex-treated mice. At $t = 5$ h, mean RBV content in the brain was 9.47 ± 1.68 and 9.61 ± 1.18 nmol/brain for RBV and complex-treated mice, respectively (Fig. 4). The differences were not significant.

4. Discussion

In the present study, we have adapted an HPLC method for RBV quantification to ex vivo mice brain samples. This method was first validated on RBV stock solution dilutions.

HPLC determination of drugs in solid tissues is problematic and requires multiple steps extraction procedure. Especially brain tissue is rich of both insoluble materials such as lipids and soluble tissue compounds such as proteins. Samples thus need to be homogenized and clarified from tissue debris and compounds before RBV separation.

RBV was thus quantified in the whole brain. This raises the question of sample contamination by circulating blood which might interfere with RBV quantification into the brain tissue. The rate of RBV contained in biological fluids into the brain and the influence upon RBV quantification was estimated to have minor impact, due to the small amount of liquid volume within the brain (0.0252 ml) (Gilbert and Wyde, 1988). In fact, when comparing RBV and RBV/α-CD-treated mice, RBV amount in the brain due to biological fluids contamination may remain the same in both cases.

In healthy mice, after a single dose of free drug, the maximal concentration of RBV in the brain was observed at 3 h after i.p. injection of free ribavirin, whereas it was observed later (at 8 h) after intramuscular injection in rats (Ferrara et al., 1981), or earlier (at 30 min) after RBV i.p. injection (25 mg/kg) in mice (Gilbert et al., 1991). Differences observed were probably due to the site of injection and to the dose used. Determined concentrations of RBV in the brain were in nanomolar range, which is in good correlation with previous findings (Gilbert et al., 1991). Moreover, the RBV quantity in the brain (5, 9 to 25 nmol/brain) was correlated to the injected dose (40, 100 or 200 mg/kg), proving that, up to the maximal tested dose, RBV system of transport is not saturated.

After i.p. injection of the complex RBV/α-CD in healthy mice, the RBV content in the brain is up to 2 times higher than the RBV content in the brain after free drug injection at the same dose and in the same conditions. Previously described improved in vivo antiviral activity (Jeulin et al., 2008) is related to increased RBV quantity in neuronal tissue and suggests that the CD act as a ribavirin permeation enhancer through the BBB. Various effects of CDs on drug delivery through biological membranes have been already proven on intestinal, nasal, buccal and lung mucosa, skin, cell cultures (caco-2 cells) or other artificial membranes (Loftsson et al., 2007). Moreover permeation enhancement through the BBB has been demonstrated in vitro by Monnaert et al. (2004a). Our results confirm in vivo permeation enhancement through the BBB previously demonstrated in vitro.

The consequences of an infection on the BBB needed to be evaluated. In MV-challenge mice (day 4 post-inoculation, viral load of 8.10 $\log_{10}$ Eq. copies/ml) the mean RBV content in the brain (8.4 nmol/brain) after a single dose of free drug (40 mg/kg) is significantly higher ($p < 0.0001$) than in healthy mice (C_{max} = 5.2 nmol/brain). It is known that in central nervous system diseases states, changes of tight junctions occur in the brain and that neuroinflammatory conditions (including encephalitis) are characterized by BBB disruption and tight junction opening via cytokines and chemokines secreted by macrophages and microglia (Avison et al., 2004; Persidsky et al., 2006). The changes in BBB structure and function under pathologic conditions can explain the improved RBV content in the brain, after free drug injection, due to the diffusion of the unbound drug through released tight junction. Moreover, it could not be ignored that lesions in the brains of intracerebrally injected mice might affect RBV pharmacokinetics but it would not distort comparison between treatments within infected mice.

Nevertheless, in the complex-treated group of mice (40 mg/kg of RBV) the maximal RBV content in the brain of measles virus challenged mice (day 4 post-challenge) was delayed but remained equal (around 11 nmol/brain) to the one in healthy mice. But RBV content was still higher from 6 to 24 h post-injection. So, measles encephalitis influenced in a different way RBV passage through the BBB after free RBV or complex treatment. Because of their chemical structure (large number of hydrogen bond donors and acceptors, high molecular weight and low octanol/water partition coefficient), CDs do not penetrate biological membranes (Loftsson et al., 2007). Even if RBV quantity in the brain increased after free drug injection during measles infection, the benefit of the complex still occurs in mice with measles virus encephalitis. Thus, despite BBB disruption during diseases states, cyclodextrins may act in another way to increase RBV bioavailability into the brain.

Thus, in our in vivo model, RBV content in the brain increased when it was intraperitoneally administered as a complex with α-CD, probably following an increased transport through the blood–brain barrier. In one hand, despite CDs properties to extract membrane compounds and to disrupt BBB cohesion, Monnaert et al. have demonstrated that γ-CD and derivatives were not able to increase the delivery of doxorubicin across an in vitro model of BBB, probably due to the low complex penetration through the model of BBB (Monnaert et al., 2004a). On the other hand, co-incubation of various β-CDs and doxorubicin in the same in vitro model of BBB improved doxorubicin delivery into the brain (Tilloy et al., 2006).

In comparison to these studies on the complex doxorubicin/γ-CD, our positive results could be explained by the greater interaction existing between α-CD with brain capillary endothelial cells compared to γ-CD and a greater ability to extract membrane compounds such as phospholipids, cholesterol and proteins (Monnaert et al., 2004b). Secondly, our model of RBV targeting to the brain was probably closer to the co-incubation of doxorubicin and β-CD of Tilloy et al. (2006), since RBV/CDs complexes have been proven to be rather external molecular associations than true host guest inclusions (Grancher et al., 2005), with apparent better availability of the guest molecule. Likewise, when the treatment solutions are administered parenterally, drugs are rapidly and quantitatively released from CD solutions (Uekama and Otagiri, 1987), so the benefits of the complexation of RBV with α-CD may include nucleoside analogue protection within the organism and blood–brain barrier permeation enhancement. Given that, in aqueous solution, the equilibrium between guest and host molecule and the degree of the dissociation are dependant on the magnitude of the stability constant of the complex, the lack of antiviral activity improvement of the complex RBV/β-CD (Jeulin et al., 2006) may be due to the RBV/β-CD complex higher stability constant compared to RBV/α-CD complex (2606 M^{-1} vs. 1493 M^{-1} for RBV/β- and α-CD, respectively) (Grancher et al., 2005).

After multiple doses, the RBV content in the brain at 3 h after the last injection is higher than 3 h after a single dose, in agreement with the long half-life of the drug. But, in those conditions, previously demonstrated benefit of the complex on antiviral activity is not enlightened by improved RBV quantity in the brain. Indeed, 3 h after the last treatment injection, RBV quantified in the brain is not significantly higher in the complex-treated mice group. This could not be explained by a delayed response, since it has been proved 5 h after the last injection. Accumulation of RBV in mouse brain, even in the free RBV-treated group, could explain that the difference between pharmacokinetic profiles diminished after several days of treatment. RBV content in the brain has also been demonstrated to be higher at day 5 compared to day 1 after daily small particle aerosol administration (Gilbert et al., 1991). Moreover, effects of cyclodextrin multiple injections or long-term toxicity on the BBB have not yet been studied. The hypothesis of an efflux of RBV due to cyclodextrin-mediated changes of the BBB should be explored in further in vitro studies.

Our results confirm that RBV reaches effective concentration into mouse brain only when it is administered in a complex formulation form and explain the advantage of the complex RBV/α-CD over RBV in protecting mice from intracerebral infection with MV (Jeulin et al., 2008). These results confirm that cyclodextrins are of a great interest for the treatment of human infectious diseases of central nervous system, in particular, viral encephalitis which often is due to highly pathogenic RNA virus sensitive to RBV, including West Nile, tick borne encephalitis, Japanese encephalitis or Rift valley fever virus (Canonico et al., 1984; Crance et al., 2003; Day et al., 2005). In the same way, cyclodextrin could improve SSPE treatment, since the success of treatment has been demonstrated to rest on RBV concentration in cerebrospinal fluid (Hosoya et al., 2004).

To our knowledge, no other in vivo study has described benefit of alpha-cyclodextrin for drug delivery to the brain. Even if the mechanism of action of cyclodextrin needs to be elucidated, this animal model revealed the virological and pharmacological potential of α-CD as a drug carrier for central nervous system disorders.

Acknowledgements

We are grateful to Professor Alain Nicolas (UMR UHP-CNRS 7561) for helpful discussions in development of sample preparation for HPLC. We would like to thank Mr. Stephane Fontanay for technical assistance and Mrs. Adeline Parenty for editing the English of this manuscript.

This work was supported by the Ministère de l'Enseignement Supérieur et de la Recherche and the Centre National de la Recherche Scientifique (CNRS).

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