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Brain distribution of ribavirin after intranasal administration

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ABSTRACT

Ribavirin has proved to be effective *in vitro* against several RNA viruses responsible for encephalitis in humans and animals. However, the *in vivo* efficacy towards the cerebral viral load seems to be limited by the blood–brain barrier. Since the nose-to-brain pathway has been indicated for delivering drugs to the brain, we investigated here the distribution of ribavirin in the central nervous system (CNS) after intranasal administration. We first tested *in vitro* ribavirin diffusion from an aqueous solution across a biological membrane, using Franz cells and rabbit nasal mucosa. About 35% of ribavirin permeated in 4 h across the mucosa, after reaching steady-state flux in less than 30 min. In the first *in vivo* experiment, ribavirin aqueous solution was administered intranasally to Sprague Dawley rats (10 mg/kg). Animals were sacrificed at 10, 20 or 30 min after administration to collect brain areas (cerebellum, olfactory bulb, cerebral cortex, basal ganglia and hippocampus) and biological fluids (cerebrospinal fluid and plasma). Ribavirin, quantified by LC–MS/MS spectrometry, was detected at each time point in all compartments with the highest concentration in olfactory bulb and decreasing in rostro-caudal direction. Two subsequent *in vivo* experiments compared the nasal route (ribavirin solution) with the intravenous one and the nasal administration of ribavirin solution with ribavirin powder (10 mg/kg). It was found that 20 min after administration, ribavirin concentration in olfactory bulb was similar after intravenous or nasal administration of the ribavirin solution, whereas the powder led to significantly higher levels. Ribavirin was also present in deeper compartments, such as basal ganglia and hippocampus.

Even if the mechanisms involved in ribavirin nose-to-brain transport are not clear, these results suggest a rapid extracellular diffusive flux from the nasal epithelium to the olfactory bulb and different CNS areas.

1. Introduction

A number of animal and human infections involve the central nervous system (CNS) and result in various neurological complications including encephalitis, meningitis, myelitis or a combination of these inflammatory processes leading to neurological deficits (Strazielle and Ghersi-Egea, 2005).

Highly pathogenic RNA viruses include members of the family *Flaviviridae*, *Togaviridae* and *Paramyxoviridae*. Two members of the *Morbillivirus* genus of the family *Paramyxoviridae*, namely canine distemper virus (CDV) and measles virus (MV), are well-known for their ability to cause demyelinating disease of the CNS in their natural hosts, dogs and humans, respectively (Appel and Summers, 1995). Although with inconclusive results, both viruses have been studied for their possible involvement in the pathogenesis of the human demyelinating disease multiple sclerosis (MS) (Sips et al., 2007). Canine distemper virus has the highest incidence of cerebral complications among viruses of the genus *Morbillivirus*. Up to 30% of dogs exhibit signs of neurologic involvement during or after CDV infection (Rudd et al., 2010) and encephalomyelitis is the most common cause of death for CDV-infected animals, meaning that CDV is more neurotropic than MV (Singethan et al., 2010). CDV is also the causative agent of Old Dog Encephalitis (ODE), a rare chronic encephalomyelitis of mature dogs, which has been compared to the Subacute Sclerosing Panencephalitis (SSPE) caused in humans by measles virus. Studies on the neuropathogenesis of CDV might give insight into the disease mechanisms and suggest approaches for the control of other recently discovered paramyxovirus infections (Griot et al., 2003).

Along with others we have already shown that CDV is inhibited *in vitro* by the nucleoside analogue 1-(β-D-ribofuranosil)-1,2,4-triazole-3-carboxamide (ribavirin, RBV) (Elia et al., 2008; Scagliarini

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et al., 2006). To treat neurological distemper as well as other viral encephalitis manifestations *in vivo*, ribavirin should reach the CNS at the required therapeutic concentration. However, the efficacy of the antiviral therapy towards the cerebral viral load is often limited by blood–brain interfaces that impede drug distribution into the cerebral compartment. For this reason, there is great interest in seeking alternative administration routes (Gilbert and Wyde, 1988) or innovative formulation strategies (Jeulin et al., 2008, 2009) to increase the bioavailability of ribavirin in the brain.

A promising way to deliver drugs to the brain is the nasal route. Nasal drug delivery has many advantages from a clinical perspective for its non-invasiveness, accessibility, ease of administration and patient compliance. In addition, the olfactory region is the only site of the body where the CNS is somehow in contact with the external environment, due to the presence of the olfactory receptor neurons, whose axons end in the olfactory bulb.

Hence, a drug administered into the nasal cavity and deposited on the olfactory mucosa should have a good chance to reach the cerebrospinal fluid (CSF), upon diffusion across the mucosa itself. Afterwards, the drug could diffuse into the interstitial fluid and reach the olfactory and/or trigeminal nerve pathways, or the vascular, lymphatic or CSF pathways, eventually penetrating the brain parenchyma (Illum, 2004; Thorne and Frey, 2001). Moreover, the olfactory mucosa represents 77% and 50% of the total nasal mucosal surface, respectively, in dogs and rats (Illum et al., 1996), increasing the probability for the drug to be transported into the CNS. Recent reports confirm the positive outcome of nose-to-brain delivery not only for drug molecules with various molecular weights (Hanson et al., 2009; Yang et al., 2009), but also for living cells (Danielyan et al., 2009, 2011).

The aim of the present study was to assess *in vivo* availability and distribution of ribavirin in the brain after intranasal administration in rats, in comparison with intravenous injection. The effect of the physical form of ribavirin (solution or powder) on its transport across the nasal mucosa was also studied *in vitro* by diffusion experiments and *in vivo* as a preliminary step for the development of new formulations.

2. Materials and methods

2.1. Materials

Ribavirin analytical standard was purchased from Sigma Chemical Company (St. Louis, MO). The internal standard (IS) RBV $^{13}C_5$ was obtained from Campro Scientific GmbH (Berlin, Germany). Ammonium acetate, formic acid and acetonitrile were Fluka reagents of MS grade (Sigma–Aldrich Chemical Co., St. Louis, MO). Ultrapure water was produced in-house via a Human Power water purification system. The centrifugal filter devices (Amicon Ultra-0.5 3 kDa) were from Millipore Corporation (Billerica, MA).

Ribavirin raw material (batch #001-RIB-0908) was kindly donated by Euticals S.p.A. (Lodi, MI, Italy). This raw material was used as it was for the *in vivo* experiment with the powder as well as for preparing the solutions for the *in vitro* and *in vivo* experiments.

2.2. In vitro diffusion experiments

Rabbit nasal mucosa was used as model permeation tissue (Russo et al., 2006). Experiments were conducted according to Bortolotti et al. (2009). Briefly, rabbit heads were obtained from a local slaughterhouse (Pola, I-Finale Emilia) on the experiment day to dissect specimens of nasal mucosa. The specimens were then immediately inserted between the donor and receptor compartments of a Franz type vertical diffusion cell (0.58 cm^2 permeation area; VETROTECNICA S.r.l., I-Padova), with the mucosal side facing the donor. The receptor was filled with PBS pH 7.4, kept stirred, and the whole system maintained at 37 °C. Ribavirin was loaded in the donor compartment, either in the form of an aqueous solution (0.5 ml of a 10 mg/ml solution in deionized water, pH 5.5) or as a solid (about 5 mg of ribavirin powder raw material, in the presence of 0.1 ml of PBS pH 7.4 as solvent for powder dissolution *in situ*). All experiments lasted for 4 h. At fixed time-points, samples were withdrawn from the receptor and HPLC-analyzed. Experiments were replicated at least three times; results are expressed as the means ± SEM.

Ribavirin concentration in the samples was determined by reverse phase-HPLC. Isocratic elution was carried out with a 20 mM $(NH_4)_2HPO_4$ solution (pH 7.5) at room temperature. The detection wavelength was set at 225 nm. The column used was a Phenomenex Synergi Polar-RP 4 μm, 150 mm × 4.6 mm (Phenomenex, I-Castelmaggiore, BO). The flow rate was 0.8 ml/min. The injection volume was 20 μl. In these conditions, the retention time of ribavirin was around 3.7 min. The method was validated for linearity (r^2 = 0.999), limit of quantitation (LOQ) and limit of detection (LOD) (0.056 and 0.017 μg/ml, respectively).

The transport parameters, i.e., steady-state flux and permeability of ribavirin across the membrane, were calculated (Xiang et al., 2002) according to the steady-state solution of Fick Eq. (1):

$$J_{SS} = \frac{dM}{dt}\frac{1}{A} = P_e C \quad (1)$$

where P_e is the apparent permeability coefficient of diffusion (cm s^{-1}), C is the initial donor concentration and J_{SS} is the flux at steady-state (mg s^{-1} cm^{-2}); dM is the amount of drug (mg) transported across the membrane during the infinitesimal time dt and A is the diffusion area (cm^2). The permeability coefficient across the mucosa was calculated from the slope of the linear part of the line obtained by plotting mass transported per unit area against time.

2.3. Animal care

Male pathogen-free Sprague–Dawley rats (Harlan®, Udine, Italy) weighing 125–150 g were used in this study. Animals were housed in standard conditions at 22 °C with 12-h light/dark cycle and received a standard pellet diet (Mucedola 4RF21) with water available *ad libitum*. All experiments with animals described here were carried out according to the European Community Council Directive of November 24th, 1986 (86/609/EEC) and approved by intramural committee and the Ministero della Salute (prot. 34613-X/10) in compliance with the guidelines published in Guide for the Care and Use of Laboratory Animals (National Research Council, 1996).

2.4. In vivo ribavirin administration

All *in vivo* treatments with ribavirin were performed in anaesthetized rats. Surgical anesthesia was induced by intraperitoneal (i.p.) injection of ketamine (100 mg/kg). Depth of anesthesia was assessed by monitoring the loss of the hind limb withdrawal reflex after pinch stimulation of the foot and the loss of eyelids reflex. Core temperature was maintained at 37 °C using a heating pad.

2.4.1. Intranasal administration pilot study

A pilot study was carried out to identify the optimal conditions for intranasal administration (time-point for sacrifice) and CNS dissection for ribavirin quantification. Rats (n = 4 per group) received ribavirin in the form of an aqueous solution (100 mg/ml). The animal was placed in the right lateral recumbency and instillation took place unilaterally into the left nostril. For each animal a total volume of 10 μl of solution was administered, divided in two shots

of 5 μl each within a 3-min interval. Nasal instillation of the solution was performed using a polyethylene cannula (INTRAMEDIC, Clay Adams, PE 50) inserted for approximately 10–15 mm into the nasal cavity and connected to a 50 ml Hamilton micro-syringe (Hamilton Co., Reno, NV).

The animals were sacrificed at different time-points after administration (10, 20 and 30 min), to collect olfactory bulb (OB, halved in left and right olfactory bulb, namely OB Sx and OB Dx), cerebral cortex (CTX), cerebellum (CRB) and plasma. Samples were immediately frozen in liquid nitrogen and then stored at −80 °C until analysis.

2.4.2. Intranasal versus intravenous administration

For the second *in vivo* experiment two groups of animals were considered ($n = 4$ per group), one receiving intranasal administration of the 100 mg/ml ribavirin solution and the other an intravenous injection of a 35 mg/ml sterile ribavirin solution in distilled water. For the intranasal group, the administration procedure was the same as described for the pilot study. For the intravenous group, ribavirin was administered at a dosage of 10 mg/kg in a single shot (28.5 μl) into the femoral vein.

Both groups were sacrificed 20 min after administration to collect olfactory bulb, cerebral cortex (divided in anterior and posterior parts), basal ganglia, hippocampus, CSF and plasma for subsequent ribavirin quantification. After freezing in liquid nitrogen, samples were stored at −80 °C until analysis.

2.4.3. Intranasal administration of ribavirin solution versus ribavirin powder

In the third *in vivo* experiment, two groups of rats ($n = 11$ per group) received either ribavirin as an aqueous solution (100 mg/ml) or ribavirin raw material as a solid, intranasally. In both cases, a total dose of ribavirin of 1 mg was administered unilaterally to the left nostril with the animal placed in right lateral recumbency. The procedure with the solution was as described. Ribavirin raw material was administered using the DP-4 Dry Powder Insufflator™ (Penn-Century, Inc., Wyndmoor, PA), a drug delivery device designed to produce a puff of fine powder from the end of a small-diameter delivery tube. The tip of the delivery tube was inserted for approximately 10 mm into the treated nostril. In order to deliver the required ribavirin dose to the animal, 2–3 mg of powder were manually filled into the device's holding chamber immediately before administration. Based on the filled device's weight before and after use, the actual amount of ribavirin delivered (mg) was determined. For both groups, animals were sacrificed 20 min after administration to collect olfactory bulb, cortex (anterior and posterior), basal ganglia, hippocampus and plasma. After freezing in liquid nitrogen, samples were stored at −80 °C until analysis.

2.4.4. Sample collection and processing

Blood was collected from the abdominal aorta under surgical anesthesia and the plasma fraction was obtained by centrifugation. Afterwards, the animal was perfused with saline solution through the ascending aorta (40 ml, at 37 °C) to wash out the blood before removing the brain and dissecting it out. CSF was collected by puncture in the *cisterna magna*. Tissues were dissected out to expose the *dura mater* and the membrane was punctured. For sampling the various brain compartments, tweezers were kept clean in ethanol and changed for each region. Olfactory bulb, cortex (anterior and posterior), hippocampus, basal ganglia and cerebellum were isolated and collected separately into sterilized Eppendorf-type microtubes.

2.5. Ribavirin extraction and quantification by LC–MS/MS spectrometry

Biological samples were analyzed according to Zironi et al. (2011). Briefly, after the addition of $^{13}C_5$RBV (IS), ribavirin was extracted from 50 mg of rat brain tissue samples with ammonium acetate buffer. The analyses were carried out on an Alliance 2695 Separations Module coupled with triple quadrupole mass spectrometer Quattro Premiere XE, (Waters Corporation, Milford, MA). Chromatographic separation was performed on a Waters Atlantis T3 column (3 μm, 2.1 × 150 mm). All the analyses were conducted in positive electrospray ionization mode (ESI+) using selected reaction monitoring (SRM); the transitions monitored were: RBV m/z 245 → 113, $^{13}C_5$RBV m/z 250 → 113.

The limit of quantification (LOQ) was 5 ng/g. Linearity ($r^2 > 0.99$) was demonstrated over a concentration range of 5–1000 ng/g.

2.6. Statistical analysis of in vivo data

The overall data obtained from the *in vivo* experiments were analyzed using a two-way ANOVA. Univariate analyses of variables were also performed by unpaired Student *t*-test using GraphPad Prism version 4.00 (GraphPad Software, San Diego, CA). Statistical significance was accepted at $P < 0.05$.

3. Results and discussion

Ribavirin has a broad spectrum of antiviral properties and it is the only commercially available drug molecule with a well-known antiviral activity against several RNA viruses responsible of neurological disorders in animals and humans.

Ribavirin has pleiotropic modes of action (Benarroch et al., 2004; Crotty et al., 2001; Fang et al., 2000; Goswami et al., 1979; Leyssen et al., 2005; Maag et al., 2001; Patterson and Fernandez-Larsson, 1990) and this was also demonstrated for CDV (Dal Pozzo et al., 2010).

Despite its *in vitro* activity against morbilliviruses, systemic treatments fail *in vivo* likely due to limited drug uptake into the CNS as a consequence of its poor permeation across the blood brain barrier (BBB). Hosoya et al. (2004) proposed to pursue intratechal or intraventricular administration of ribavirin to by pass the BBB in patients with SSPE. Nonetheless, direct injection of ribavirin into the CNS is not the first-choice alternative for overcoming the barrier, because of its invasiveness. Gilbert et al. (1991) showed that ribavirin administered as an aerosol to the lungs reached the brain at higher concentrations than those obtained following intraperitoneal injection, indicating that ribavirin accessed the CNS by crossing the BBB. Indeed, increasing ribavirin plasma levels to increase brain levels may not be the best option, considering the safety concerns associated with systemic exposure to this drug (Riner et al., 2009). The challenge is to find a way to increase the uptake of ribavirin in the CNS to treat neurological disorders caused by ribavirin-sensitive viruses. Hence, we decided to explore the nasal route for ribavirin administration and the nose-to-brain delivery pathway (Dhuria et al., 2010). The drug accumulated at the infection site, without necessarily increasing plasma levels, allows a direct attack on the virus.

3.1. Ribavirin in vitro permeation across rabbit nasal mucosa

The capability of ribavirin to diffuse across the nasal mucosa after being deposited on it was first evaluated *in vitro*. Since aqueous solutions are the most common nasal dosage forms, an aqueous solution of ribavirin was used.

In vitro ribavirin demonstrated high diffusivity across the rabbit nasal mucosa (Fig. 1). In 4 h, the amount permeated per unit area was 2.68 ± 0.24 mg cm^{-2} ($n = 9$), when the donor compartment contained 5 mg of drug as a 10 mg/ml ribavirin aqueous solution.

The linearity of the profile ($y = -0.019286 + 0.6919x$, $r^2 = 0.99697$), without a significant initial lag-time, indicates that drug diffusion took place in quasi steady-state conditions, i.e., at constant rate (drug flux = 0.691 ± 0.065 mg cm^{-2} h^{-1}, mean ± SEM, $n = 9$). The apparent permeability coefficient was calculated according to Eq. (1), being equal to $(1.92 \pm 0.18) \times 10^{-5}$ cm s^{-1}. High permeability across the mucosa is an advantage *in vivo*, considering that the mucociliary clearance rapidly removes the drug away from the nasal cavity, shortening the time available for absorption (Merkus et al., 1998).

Aqueous nasal systems may not always be preferred due to microbiological stability issues (addition of preservatives is required for multidose products) and the limited residence time in the nasal cavity after administration (Vidgren and Kublik, 1998). A nasal powder can provide longer residence time, thus improving drug absorption (Dyer et al., 2002). Thus, we also assessed ribavirin permeation *in vitro* by directly loading ribavirin powder inside the donor compartment of the diffusion cell.

Indeed, the amount permeated was almost three times as high (7.57 ± 0.48 mg cm^{-2}; $n = 3$) when 5 mg of ribavirin powder was loaded in the donor compartment (plus 100 μl of PBS as dissolution medium). Ribavirin permeated in 4 h was 34 ± 4% and 85 ± 2% of the loading, respectively, using the solution or the powder. The powder, being micronized ($d_{v,0.5} = 11.6$ μm), dissolved in the small volume of solvent forming *in situ* a saturated ribavirin solution, leading to maximum concentration gradient. Diffusion was very fast in the first hour (maximum drug flux) and then gradually slowed down. As permeation proceeded, the progressive reduction in the donor concentration reduced the flux to values more similar to those observed with the aqueous solution.

The good diffusivity of ribavirin across the nasal mucosa *in vitro* was not expected a priori. Hydrophilic small molecules might not be the best candidates for absorption across this mucosa, as permeability coefficient increases with increasing water/octanol partition coefficient (Costantino et al., 2007). Nonetheless, good aqueous solubility is also required for a drug to be administered intranasally and ribavirin is freely soluble in water. Likely, for this antiviral drug water solubility and small molecular weight counteract the apparently unfavorable hydrophobic/hydrophilic balance.

3.2. In vivo ribavirin distribution in the CNS

Various studies have been designed to investigate the bioavailability of antiviral drugs in the CNS (Strazielle and Ghersi-Egea, 2005). In the case of ribavirin, several authors studied *in vivo* its brain distribution and efficacy against different viral infections after systemic administration (Ferrara et al., 1981; Gilbert et al., 1991; Jeulin et al., 2008). Ribavirin was administered by intraperitoneal (i.p.) or intramuscular (i.m.) injection or by aerosol to the lungs, using different animal models. Indeed, in all these studies ribavirin levels were measured considering the brain *in toto*. To our knowledge, this is the first study aimed to determining ribavirin concentration in various brain compartments after intranasal administration. In our study ribavirin levels were measured shortly after the administration (within 30 min maximum). Our analytical method only allowed quantifying unmodified ribavirin, not its phosphorylated metabolites. Since intracellular ribavirin phosphorylation takes place soon after absorption, we wanted to detect as much ribavirin as possible before it was metabolized (Morse et al., 1993).

3.2.1. Intranasal administration of ribavirin solution (pilot experiment)

The first finding of our study was that intranasal administration allowed rapid ribavirin uptake into the CNS. In fact, when 10 μl of an aqueous solution (100 mg/ml) were administered intranasally, ribavirin was found in all analyzed compartments at each time-point (10, 20, 30 min), with the highest concentration in the olfactory bulb (between 700 and 1900 ng/g), decreasing in the rostro-caudal direction.

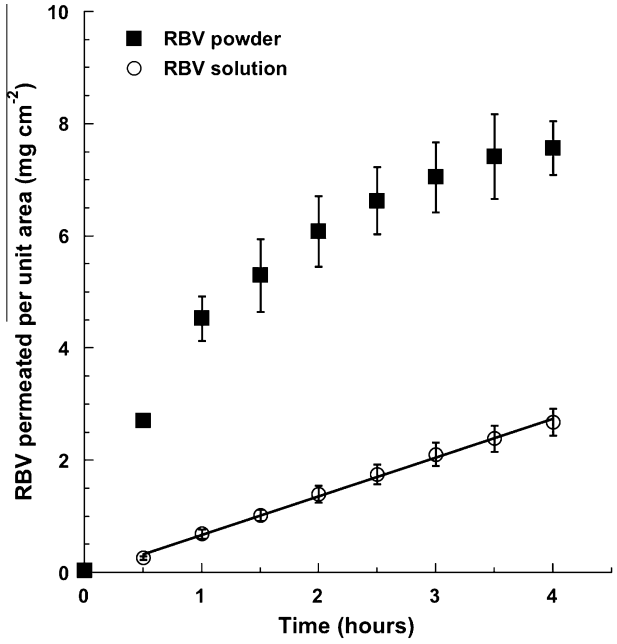

Fig. 1. *In vitro* ribavirin permeation profiles across rabbit nasal mucosa from an aqueous solution (empty circle, $n = 9$) in comparison with ribavirin powder (filled square, $n = 3$). Data are expressed as the mean ± SEM. Experimental points and theoretical straight line according to Eq. (1).

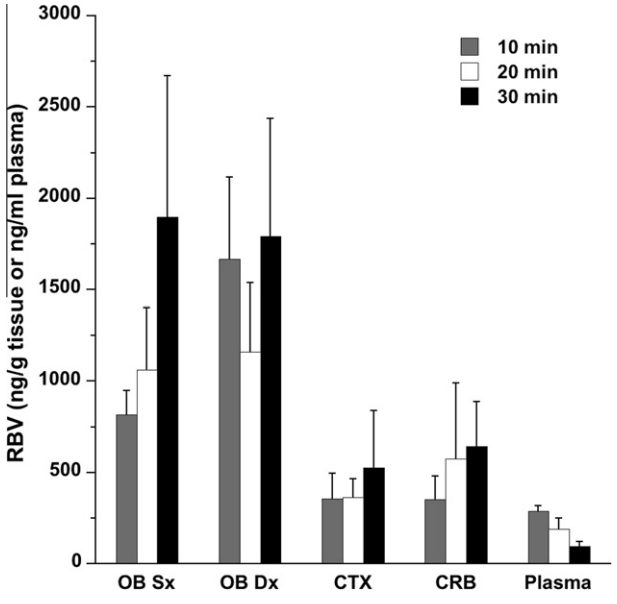

Fig. 2. Ribavirin brain concentrations after *in vivo* nasal administration of an aqueous solution as a function of the time between animal treatment and sacrifice: 10 min (gray bars), 20 min (white bars) or 30 min (black bars). OB Sx and OB Dx: left and right olfactory bulb; CTX: cerebral cortex; CRB: cerebellum. Data are expressed as the mean ± SEM ($n = 4$).

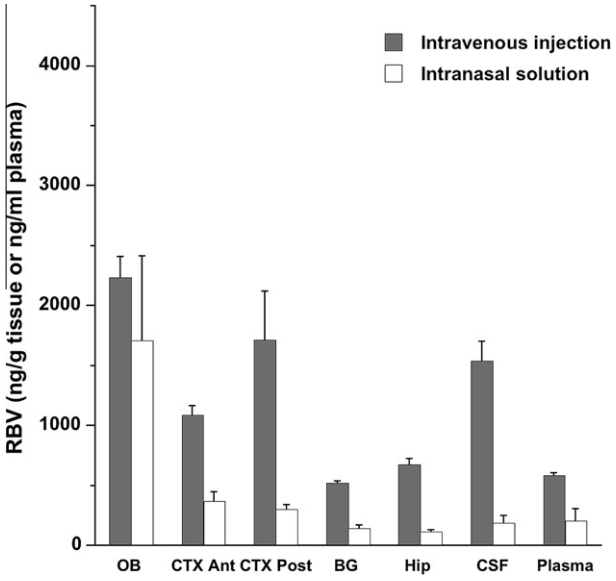

Fig. 3. Ribavirin brain concentrations after *in vivo* nasal administration of an aqueous solution (white bars) versus intravenous injection (gray bars). OB: olfactory bulb; CTX Ant and CTX Post: anterior and posterior cerebral cortex; BG: basal ganglia; Hip: hippocampus; CSF: cerebrospinal fluid. Data are expressed as the mean ± SEM ($n = 4$).

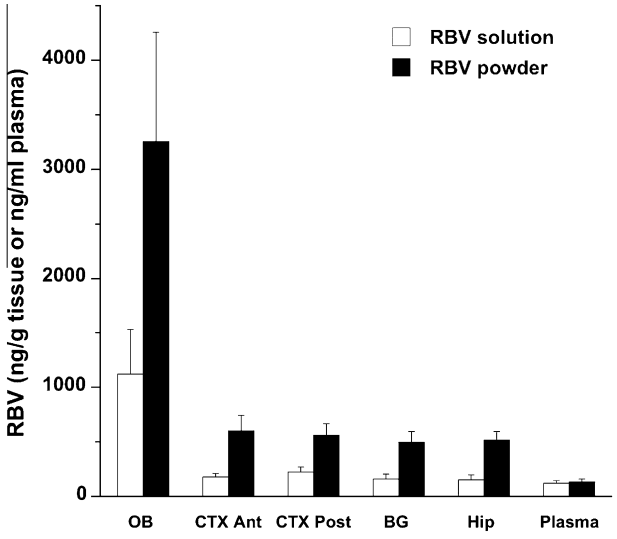

Fig. 4. Ribavirin brain concentrations after *in vivo* nasal administration of an aqueous solution (white bars) versus ribavirin powder (black bars). The measured values were normalized by the dose administered. OB: olfactory bulb; CTX Ant and CTX Post: anterior and posterior cerebral cortex; BG: basal ganglia; Hip: hippocampus. Data are expressed as the mean ± SEM ($n = 11$).

The actual amounts measured in the left side of the olfactory bulb (OB Sx) after 10, 20 and 30 min were the highest among the analyzed compartments (olfactory bulb, cerebral cortex and cerebellum), being 817 ± 133, 1061 ± 342 and 1898 ± 776 ng/g tissue, respectively. This was somehow expected, considering the bulb anatomical localization immediately behind the nasal mucosa. The unilateral administration into the left nostril did not influence ribavirin localization, as no significant difference existed between the right and left olfactory bulb. Fig. 2 also shows that ribavirin concentration increased in cerebral compartments with time. However, within each compartment, concentrations were not significantly different ($P > 0.05$) among the three time points, with the exception of plasma levels between 10 and 30 min. For this reason, in the subsequent *in vivo* experiments the animals were sacrificed 20 min after the treatment.

The presence of ribavirin in the cerebellum was noteworthy, considering that this compartment is far from the administration site, and suggested possible rapid CSF-mediated ribavirin diffusion. The overall results confirm *in vivo* permeation through the nasal mucosa as previously demonstrated *in vitro*. The presence of ribavirin in plasma is not surprising since this route can also lead to systemic drug absorption, but the measured plasma concentrations were well below those found in the CNS compartments (Fig. 2). Low ribavirin plasma levels are favorable with respect to the treatment safety as the risk of systemic side-effects should be reduced.

3.2.2. Intranasal and intravenous ribavirin administrations

The intranasal (i.n.) and intravenous (i.v.) routes were compared administering the same drug dose.

Fig. 3 shows that ribavirin was detected in all brain compartments after i.v. injection, at concentration levels that were significantly higher ($P < 0.0001$, two-way ANOVA) than those measured after i.n. instillation. The only exception was represented by the olfactory bulb in which the concentrations ranged from 0.1 to 3.5 μg/g after i.n. administration and from 1.8 to 2.6 μg/g after i.v. treatment, without statistically significant differences between the two groups (Student *t*-test). Hence, the second finding from our study was that ribavirin reached the CNS after i.v. injection. As different authors have been stating that ribavirin did not cross the blood brain barrier (Livonesi et al., 2006; Rockx et al., 2010), our data show the opposite. Indeed, transcellular diffusion of drugs across the barrier is limited to small, lipophilic compounds (Liu et al., 2011). For hydrophilic molecules, the blood–brain interface tight junctions hinder paracellular diffusion (Strazielle and Ghersi-Egea, 2005). As ribavirin is highly hydrophilic, an active transport mechanism could justify its presence in the CNS following i.v. injection. Recently, the involvement of nucleotide transporters Ent1 in the uptake of ribavirin into the mouse brain has been shown (Endres et al., 2009). Hence, the apparently contradicting data could be explained by considering that other authors did not directly measure ribavirin levels in the brain, but based their statement on the *in vivo* failure of the treatment with ribavirin of different viral infections. It may be argued that such a lack of efficacy *in vivo* was not due to ribavirin not crossing the BBB at all, but to ribavirin crossing the BBB in amounts insufficient to reach/maintain the therapeutic concentration required to defeat the viral load.

Comparing intravenous and intranasal administration of ribavirin solution, the levels measured were significantly different in all compartments, being lower after intranasal administration. It must be borne in mind that ribavirin was administered at the same dose by the two routes (10 mg/kg). While drug bioavailability is complete with i.v. injection, this is not the case with all other administration routes. Hence, the dose of a drug given by another than the intranasal route has to be adjusted to take into account the different bioavailability.

Again, ribavirin was detected in deeper regions such as basal ganglia and hippocampus (BG: 144 ± 29 and 521 ± 19 ng/g respectively after i.n. and i.v. administration; Hip: 114 ± 17 and 675 ± 47 ng/g, respectively, after i.n. and i.v. administration).

3.2.3. Intranasal administration of ribavirin solution versus ribavirin powder

Administration of ribavirin powder was compared with the aqueous solution (Fig. 4). Ribavirin powder enhanced the uptake into the brain with respect to the solution and this even if the actual dose administered with the powder was lower than 1 mg (0.3–0.7 mg), due to the difficulties with the insufflator. For this

reason, the concentrations measured were normalized by the actual ribavirin dose calculated based on the mg of powder leaving the device (average delivered dose 0.58 ± 0.05 mg, mean ± SEM, $n = 11$).

In all brain compartments the powder led to ribavirin concentrations much higher than with the solution. In the olfactory bulb the amounts were 1123 ± 409 and 3255 ± 1003 ng/g, respectively. Similarly, ribavirin levels were significantly higher ($P < 0.0001$, two-way ANOVA) in the cerebral cortex, hippocampus and basal ganglia, whereas they were not in the case of plasma (124 ± 26 and 140 ± 21 ng/ml, respectively).

The *in vivo* behavior of the solid powder thus reflected what already was observed *in vitro*: the stronger and longer contact between powder and mucosa and the higher concentration gradient across the mucosa increased drug absorption and brain bioavailability. In comparison with intravenous ribavirin, the concentrations reached in the various compartments with the powder were still lower, except for the olfactory bulb, where the concentration was 1.5-time as high (3255 ± 1003 and 2236 ± 171 ng/g, respectively, for intranasal powder and intravenous injection). Hence, comparing the two administration routes, the olfactory bulb was the compartment where ribavirin concentrations were either higher (nasal powder versus i.v.) or at least not significantly different (nasal solution versus i.v.). Finally, regardless the administration route, ribavirin was present also in hippocampus and basal ganglia, two deeper brain compartments. If ribavirin also reached these areas in 20 min, this could be due not only to the circulating cerebrospinal fluid, but also to intra-parenchyma passive diffusion, thus creating a gradient-like effect from the olfactory bulb. Further *in vivo* studies would be required to investigate this hypothesis.

4. Conclusions

This work was the first part of a research project aiming to develop an innovative antiviral treatment to be applied intranasally to treat neurologic viral disorders. In the veterinary field, this could be particularly important for the therapy of neurologic distemper as no treatment is available at present and the intraventricular ribavirin treatment is not straightforward in clinical practice. Our overall data suggest that the nasal route could be exploited to increase the availability of ribavirin inside the brain. We also believe that nasal administration could be easier to perform in animals (e.g., no sterile setting required) and safer with respect to the physiology of the administration site (Han et al., 1990). The physical form in which ribavirin was given (aqueous solution or powder) had a significant effect on the measured brain concentrations. This observation represents the beginning of the formulative study that will develop a solid dosage form from which ribavirin uptake into the CNS may be further enhanced.

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