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Short communication

Improved antiviral activity in vitro of ribavirin against measles virus after complexation with cyclodextrins

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Abstract

Despite vaccination, measles remains a burden in both developed and developing countries and complications may necessitate an efficient therapy. Measles virus (MEV) is susceptible to ribavirin (RBV), but the use of this drug is limited by its toxicity. Cyclodextrins (CDs) can form complexes with numerous molecules, improving their bioavailability and their biological properties. We have evaluated in vitro the antiviral effects of complexes of RBV with α-, β- or γ-CD against two clade A laboratory strains of MEV (Edmonston and CAM/RB) grown on Vero cells. Complexation of RBV with α-CD or β-CD lead to a five-fold or a two-fold decrease in the 50% inhibitory concentration, respectively, against both MEV strains. In contrast, γ-CD complexation showed no modification.

Keywords: Measles virus; Ribavirin; Cyclodextrins; Antiviral activity

With about one million dead children per year, measles virus (MEV) remains a major human pathogen and ranks 8th as the infectious cause of death worldwide, mostly in the developing countries (WHO/UNICEF, 2001). Because of insufficient vaccine coverage, MEV circulation persists even in most developed countries. No curative therapy is currently available, although ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide, RBV) has been reported to be effective against measles both after intravenous or oral administration (Gururangan et al., 1990) with or without hyperimmunoglobulin (Stogner et al., 1993).

RBV is a purine nucleoside analog with a broad antiviral activity spectrum (Sidwell et al., 1972). This antiviral agent is licensed as aerosol for the treatment of human respiratory syncytial virus and as an oral drug against hepatitis C in combination with α-interferon. Intravenous RBV is the only available treatment for haemorrhagic fever virus infections. However, the therapeutic use of RBV is limited by its toxicity, consisting mainly of anemia and teratogenicity.

CDs are cyclic oligosaccharides with a hydrophobic central cavity and a hydrophilic surface. CDs have been shown to be carriers for several different antiviral molecules such as antisense DNA (Abdou et al., 1997), ganciclovir (Nicolazzi et al., 2001, 2002) and cosalane (Udata et al., 2003). The most common native forms are α-, β- or γ-CD constituted by six, seven or eight α-1,4-linked glucopyranose units, respectively. Complexation with CD improves the solubility, the stability as well as the bioavailability and facilitates the absorption of the target molecule. Complexation of RBV by CDs has been suggested but its potential benefit not investigated (Loftsson, 1995). We have shown that an external molecular association exists between RBV and native CDs, although it is not a true host-guest inclusion complexation between the two molecules. α-, β- and γ-CD form complexes with a stability constant of 1493, 2606 and 1179 M^{-1}, respectively (Grancher et al., 2004). According to these results, we have evaluated the benefit brought by the complexation of native CDs (α-, β- or γ-CD) with RBV to its in vitro antiviral activity against MEV.

The RBV/CD complexes were prepared as described by Higuchi and Connors (1965). Native CDs (kindly provided by Wacker-Chemie GmbH, Lyon, France) and RBV (ICN Biomedical Inc., Aurora, OH) were dissolved in distilled

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water, sterilized by filtration and mixed at a molar ratio of 1:10. The solution was equilibrated overnight at 22 °C under continuous stirring. RBV/CD complexes were mixed v/v with double concentrated Eagle's minimum essential medium (EMEM).

Cytotoxicity of RBV and native CDs was determined by a modified MTT (3-[4,5-dimethylthiazol-2-yl]2,5-diphenyl tetrazolium bromide) assay (Carmichael et al., 1987). Therefore, 8×10^4 Vero cells were seeded in 96-well plates and grown to confluency. Increasing concentrations from 0 to 16 mM of RBV, CDs or RBV/CD complexes were added and plates were incubated for 7 days at 35 °C. Four wells were used for each point and experiments were repeated three times. Cell viability was measured by the addition of MTT 2.4 mM. After 4-h incubation at 35 °C, the medium was discarded and the absorbance of the formed formazan after dissolution in 50 μl of dimethyl sulfoxide was read at 540 nm on a microplate reader 3550-UV (Biorad) using 655 nm as reference. Results were expressed as the ratio of the absorbances of treated and untreated cultures. The 50% cytotoxic concentrations (CC_{50}) were determined graphically by interpolation (Carmichael et al., 1987).

Susceptibility to antiviral drugs was evaluated by the plaque reduction assay (PRA) using immunoperoxidase staining. Monolayers of Vero cells in Lumox^{TM} 96-well plates were grown at 8×10^4 cells per well. The cells were pretreated for 24 h with free or CD-complexed RBV from 0 to 256 μM, or native CDs from 0 to 2560 μM. Four wells were used for each point and experiments were made in triplicate. Each well was inoculated with 15 plaque-forming units of MEV suspension. After 2-h incubation at 37 °C, the inoculum was removed and 50 μl of free or CD-complexed RBV in culture medium with 2% CMC were added. After 3 (CAM/RB strain) or 5 days (Edmonston strain) at 35 °C, the medium was removed and the cells were fixed with 80% acetone for 20 min. After washing with phosphate-buffered saline (PBS), the cells were incubated for 30 min at 37 °C with 50 μl of an anti-measles mouse monoclonal antibody (3.75 μg ml^{-1}). Then the cells were washed with PBS and incubated for 30 min at 37 °C with 50 μl of peroxidase-conjugated polyclonal sheep anti-mouse IgG antibody (11.8 μg ml^{-1}). After washing with PBS, the cells were incubated for 10 min with 100 μl of diaminobenzidine peroxidase substrate. The reaction was stopped by washing the cells with PBS. The plaque-forming units were counted and then the 50% inhibitory concentration (IC_{50}) was determined graphically by interpolation (Isenberg, 1992). The selectivity indices (SI) for RBV and the RBV/CD complexes were calculated as: $SI = CC_{50}/IC_{50}$.

The CC_{50} of RBV alone was 6.8 ± 0.1 mM (Table 1). The CC_{50} of α- and β-CD was in the same range although γ-CD was found of a lower cytotoxicity. Complexes of RBV and α-, β- or γ-CD were found to be respectively 10, 15 and 8 times more cytotoxic than the RBV alone.

Native CDs were without antiviral activity against the Edmonston or CAM/RB strain. The IC_{50} of the free RBV was quasi identical for the Edmonston and/or CAM/RB strain (Table 1). For both strains, complexation of RBV with γ-CD did not improve the antiviral activity of RBV, but a two-fold decrease in IC_{50} was observed with β-CD-complexed RBV. Complexation with α-CD decreased by five-fold the IC_{50} of RBV. However, this improvement of activity of RBV was associated with a decrease of its CC_{50}, more pronounced with β-CD than with α-CD, thus decreasing the SI.

To the best of our knowledge, this in vitro study represents the first evaluation of antiviral activity of RBV complexed to native CDs. Besides CDs, other carriers of RBV have been studied in vivo. The lipophilic dihydropyridine (DHP)-hydrophilic pyridinium salt was used as chemical delivery system for RBV to target the central nervous system in a murine model with Japanese encephalitis virus (Prokai et al., 2000). Treatment with RBV-DHP resulted in 40–50% survival, whereas RBV or the vehicle alone were ineffective. Contradictory results have been found with liposome-encapsulated RBV. With an encapsulation efficiency of 20%, liposomal RBV proved more efficacious than free RBV in treatment of mice infected with Rift Valley fever, influenza or herpes simplex viruses (Kende et al., 1985; Gangemi et al., 1987). In contrast, in a study of kittens infected with feline infectious peritonitis virus, a

Table 1
Evaluation of cytotoxicity on Vero cells and antiviral activity against two MEV strains of RBV, RBV/CD complexes, and native CDs

Molecules	CC_{50} (mM)	Edmonston			CAM/RB		
		IC_{50} (μM)	Improvement[a]	SI[b]	IC_{50} (μM)	Improvement[a]	SI[b]
α-CD	8.0 ± 0.8	>2560	–	–	>2560	–	–
β-CD	6.8 ± 0.2	>2560	–	–	>2560	–	–
γ-CD	>8.0	>2560	–	–	>2560	–	–
Free RBV	6.8 ± 0.1	48.2 ± 3.3		141	49.4 ± 4.9		138
RBV/α-CD	0.61 ± 0.02[c]	10.6 ± 3.6[c]	4.6	58	10.4 ± 2.6[c]	4.8	59
RBV/β-CD	0.45 ± 0.02[c]	19.7 ± 2.4[c]	2.4	23	24.9 ± 2.6[c]	2.0	18
RBV/γ-CD	>0.8[c]	45.4 ± 2.0[c]	1.1	>18	49.6 ± 2.9[c]	1.0	>16

All experiments were performed in triplicate.
[a] Ratio of IC_{50} of the uncomplexed RBV to IC_{50} of CD-complexed RBV.
[b] Ratio of CC_{50} on IC_{50}.
[c] Expressed as RBV concentration.

higher encapsulation efficiency was correlated with a lower activity as compared to the free RBV at equivalent concentrations. In this case, the loss of efficacy has been related to low stability of the complex in the bloodstream and counterproductive enhanced uptake by the reticuloendothelial system (Weiss et al., 1993).

RBV has been evaluated against different MEV strains and the IC_{50} values ranging from 41 to 410 μM, did not necessarily correlate with the viral genotypes (Barnard et al., 2002). The IC_{50} of RBV (41.0 μM) with the Edmonston strain in these studies was very similar to the ones observed with both the CAM/RB and Edmonston strain.

Complexation with α-CD and β-CD improved the RBV efficacy against both MEV strains. As native CDs were devoid of antiviral activity and cytotoxicity on both MEV strains at the tested concentrations, the improvement of the IC_{50} of RBV is likely brought by its complexation. The 1:10 molar ratio used in this study was adopted according to the observations made in previous studies (Al-Omar et al., 1999; Nicolazzi et al., 2001, 2002). This ratio was also chosen as it ensured an almost complete association of the two compounds. The greater benefit obtained with α-CD was surprising as the carrier of choice for in vitro studies was β-CD for ganciclovir (Nicolazzi et al., 2001, 2002) and for doxorubicin as well (Al-Omar et al., 1999).

The complexation of RBV to CD increased its toxicity. This may be due to a facilitation of the penetration of RBV into the cell. This possibility has to be further investigated using red blood cells which are known to accumulate RBV (Glue, 1999). The toxicity of the complexes RBV/CD will be evaluated by determining the concentrations which induce lysis in comparison to RBV alone. Such a study has to be performed if an animal model were to be developed.

Thus, complexation of RBV to α and β native CDs proved to be beneficial as it improved the IC_{50} values of RBV. The CDs could be an alternative to other delivery systems of RBV. However, the benefit of this complexation has to be explored in vivo and it seems necessary to compare RBV with its α- and β-CD complexes, using an animal model. For this purpose, an encephalitis model is currently being developed in newborn mice, challenged intracranially with the neuroadapted CAM/RB strain of MEV.

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