J Incl Phenom Macrocycl Chem (2007) 57:303–308
DOI 10.1007/s10847-006-9177-z

ORIGINAL ARTICLE

Contribution of cyclodextrins in the development of different pharmaceutical formulations of a new matrix metalloproteinase inhibitor

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Received: 15 May 2006 / Accepted: 20 October 2006 / Published online: 14 February 2007

Abstract Ro 28-2653 is a new synthetic inhibitor of matrix metalloproteinases. The ability of these enzymes to degrade various components of the extracellular matrix seems to play a major role in tumors progression and is potentially effective against bronchial remodeling in asthma and BPCO. Ro 28-2653 is very poorly soluble in water. This low solubility estimated at about 0.56 μg/ml in water at 25 °C gives rise to difficulties in pharmaceutical formulation of oral, injectable or nebulizable solutions. The purpose of our study is to prepare and to characterize inclusion complexes between Ro 28-2653 and cyclodextrins and to investigate the biopharmaceutical repercussion of the inclusion of the active substance.The complex formation was investigated by phase solubility studies. ^{1}H-NMR spectroscopy and molecular modeling studies were carried out to elucidate the structure of the inclusion complex between Ro 28-2653 and cyclodextrin. Oral, intravenous and nebulizable solutions of Ro 28-2653 were developed with cyclodextrin. The in vivo studies were performed on healthy sheep for the pharmacokinetic evaluation of the oral and intravenous formulations while the nebulization of the complex solution was studied by using an asthma model in mouse.

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Keywords Bioavailability · Cyclodextrins · Pharmacokinetics · Pulmonary administration · Solubility · Ro 28-2653

Introduction

Ro 28-2653 (5-biphenyl-4-yl-5-[4-(4-nitro-phenyl)-piperazin-1-yl]-pyrimidine-2,4,6-trione) (RO) is a new synthetic inhibitor of matrix metalloproteinases (MMPs) with a high selectivity towards MMP2, MMP9 and membrane type 1-MMP. The ability of these enzymes to degrade various components of the extracellular matrix seems to play a major role in tumors progression and is potentially effective against bronchial remodeling in asthma and BPCO [1, 2]. Unfortunately, RO is a drug that exhibits poor water-solubility. This low solubility (about 0.56 μg/ml in water at 25 °C) makes the pharmaceutical formulation of oral or injectable solutions difficult and reduces flexibility in terms of administration [3]. Furthermore, the lack of aqueous solubility may lead to poor and erratic absorption from the gastrointestinal tract and, consequently, low and variable bioavailability may occur. In order to overcome these drawbacks, increasing the aqueous solubility of RO is an important goal. The purpose of our study is to prepare and to characterize inclusion complexes between RO and cyclodextrins (CD) and to investigate the biopharmaceutical repercussion of the inclusion of the active substance.

Materials

RO was synthetized by Syntheval (Caen, France). Hydroxypropyl-β-CD (HP-β-CD, Kleptose HPB)

(Eur. Ph.) was provided by Roquette (Lestrem, France). Randomly methylated-β-CD (RAMEB) was given by Wacker Chemie GmbH (Munich, Germany). 2,6-dimethyl-β-CD (DIMEB) was synthesized by CEA (Saclay, France). Apyrogenic phosphate buffered saline (PBS) and water for injection were purchased from Bio-Wittaker (Verviers, Belgium). CDs and RO were tested following the Bacterial Endotoxin Test described in USP XXVI using Limulus Amebocyte Lysate (LAL).

Methods

Complex formation analysis [3]

The complex formation was investigated by phase solubility studies. Solubility studies were performed as described by Higuchi and Connors [4].

^{1}H-NMR spectroscopy and molecular modeling studies were carried out to elucidate the structure of the inclusion complex between RO and DIMEB. The ^{1}H-NMR spectrum of RO in D_2O could not be performed due to its very low aqueous solubility, therefore ^{1}H-NMR signal assignments for RO were performed in DMSO. Solutions containing RO and DIMEB were prepared as follows: an excess amount of RO was added to a 10 mM Dimeb D_2O solution. After shaking for 7 days at 37 °C, the suspension was filtered (Millex-HV, Millipore). All ^{1}H-NMR experiments were performed on a Bruker DRX500 spectrometer operating at 500 MHz for protons. The temperature was set at 25 °C. Calibration was achieved using the residual resonance of the solvent as secondary reference (4.80 ppm for HDO) corresponding to external TMS at 0 ppm. For T-ROESY experiments, a 300-ms mixing time was used. All processings were carried out on a Silicon Graphics INDY data station using the WINNMR program from Bruker. For molecular modeling, calculations were performed at the approximate quantum chemistry AM1 level using the Gaussian 98 suite of programs [3]. The thermochemical results at 298.15°K and 1 atm were computed from the numerically derived frequencies using statistical mechanics formulas. This procedure allows the determination of consistent energetic data, as each relative energy is calculated by reference to the geometry of the complex. Interatomic distances were measured by Mercury 1.2.1 Software (Cambridge, UK).

Biopharmaceutical studies

Intravenous and oral formulations

The RO-β-CD intravenous solution was obtained by dissolving RO (10 mg/ml) in a solution containing HP-β-CD (200 mM), L-lysine (20 mM) and water for injection [5]. The osmolality (about 325 mOsmol/kg) and the pH (about 8.2) values of this solution are compatible with an intravenous injection. The solution was sterilized by being passed through a sterile 0.20 μm cellulose acetate filter under aseptic conditions. The RO suspension was composed of RO (15 mg/ml), with polysorbate 80 (0.1 mg/ml) as a wetting agent, together with simaldrate (VEEGUM HV®, 1% m/v) and methylcellulose (METHOCEL A400®, 0.4% m/v) as viscosifying agents.

Animal experimental protocol and drug administration for bioavailability studies

Six healthy sheep ranging from 45 to 82 kg of body weight were used as experimental animals. During the test, the animals were fed and watered ad libitum. The experimental study included a randomized two-way cross-over design for oral administration followed by intravenous administration. A wash-out period of 3 weeks was allowed between each administration. For the oral dosage forms, each animal received a RO dose equal to 15 mg/kg of body weight from both formulations. Sheep were weighed on the day of drug administration in order to adapt the dosage form volume. Blood samples were taken from jugular vein before oral administration and at intervals of 0.25, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 10, 12, 24, 28, 32, 48, 72, 96, 120, 144, 168 h afterwards. For the intravenous dosage form, all six sheep received 5 mg of RO/kg of body weight. The solution was administered through the left jugular vein. Blood samples were taken from the right jugular vein before intravenous administration and at intervals of 5, 10, 15, 20, 30, 45 min, 1, 1.5, 2, 3, 4, 5, 6, 8, 10, 12, 24, 28, 32, 48, 72, 96, 120, 144, 168 h afterwards. All blood samples were centrifuged and the serums were stored at –80 °C until assayed. A fully automated method was developed for the LC determination of this compound in serum. The operating conditions are described in a previous paper [6].

Pulmonary administration of the RO:CD complex

Nebulizable solutions of RO at a concentration of 30 and 300 μg/ml were developed with HP-(-CD [3]. They were evaluated regarding peribronchial inflammation and peribronchial cells infiltration in an asthma model in mice in comparison with fluticasone dipropionate (Flixotide, GSK). The pulmonary histology and tissue processing was similar to the method previously described by Cataldo [7, 8]. The extent of peribronchial inflammation was estimated by a score calculated by

quantification of peribronchial inflammatory cells. A value of 0 was adjudged when no inflammation was detectable, a value of 1 when there was occasionally inflammatory cells, a value of 2 when most bronchi were surrounded by a thin layer (1–5 cells) of inflammatory cells and a value of 3 when most bronchi were surrounded by a thick layer (>5 cells) of inflammatory cells. Since 5–7 randomly selected tissue sections per mouse were scored, inflammation scores are expressed as a mean value and can be compared between groups. After Congo Red staining, the eosinophilic infiltration in the airway walls was quantified by manual count and reported to the perimeter of epithelial basement membrane defining an eosinophilic inflammatory score.

Results and discussion

Complex formation analysis

Figure 1 shows diagrams obtained with HP-β-CD and RAMEB. Diagrams are classified as A_P diagrams indicating that these derivatives of β-CD form a 1:2 complex with RO. The stability constant $K_{1:1}$ and $K_{1:2}$ are shown in Table 1. The phase solubility diagram of RO obtained at 25 °C in the presence of HP-β-CD in purified water, in a 50 mM L-lysine solution and in a 500 mM L-lysine solution show that the aqueous solubility of RO increased as a function of CD concentration. The solubility diagram obtained in the absence of L-lysine confirms the previously mentioned results: the solubility of RO in a 200 mM HP-β-CD solution was approximately 5 mg/ml which corresponds to a 10,000 times increase in RO aqueous solubility. In the presence of L-lysine, RO solubility in HP-β-CD

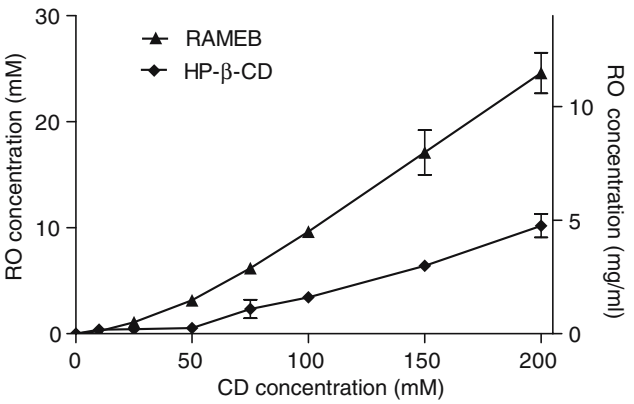

Fig. 1 Phase solubility diagrams of RO with RAMEB (▲) and HP-β-CD (◆)

Table 1 Stoichiometry and stability constant of RO–CD complexes in water

CD	Stoichiometry	$K_{1:1}$ (M^{-1})	$K_{1:2}$ (M^{-1})
HP-β-CD	1:1, 1:2	12575	15
RAMEB	1:1, 1:2	27595	23

solutions was even higher. Solubility in a 200 mM HP-β-CD solution was increased by about two and seven times in the presence of 50 and 500 mM of L-lysine respectively. Table 2 shows solubility data of RO in the different media and pH values of the solutions obtained at equilibrium. Results show a synergistic effect between L-lysine and HP-β-CD. Solubility in the presence of both 500 mM L-lysine and 200 mM HP-β-CD (38 mg/ml) was higher than had been expected by adding separately the effect of HP-β-CD and L-lysine (5 and 0.09 mg/ml). This synergistic effect between L-lysine and HP-β-CD allowed a significant increase in RO aqueous solubility (70,000 times with 500 mM of L-lysine and 200 mM of HP-β-CD).

As methylated β-CD seems to give the highest increase in solubility, further investigations of the chemical structure of the complex were undertaken with DIMEB. The spectrum of pure DIMEB was compared with the spectrum of DIMEB in the presence of RO. The signals corresponding to H-3 and H-5 protons were shifted upfield. Since these protons are inside the cyclodextrin cavity, their shift suggests that RO or part of RO is included inside the DIMEB. T-ROESY spectra were performed to provide more information on the structure of the complex (Fig. 2). Correlation spots indicated interactions between b, c, e, f, g and h RO protons and H-3 and H-5. Other interactions between RO protons and DIMEB $-CH_3$ 6^* and 2^* (corresponding to primary and secondary hydroxyl faces respectively) were observed. On the one hand, H-b only interfered with $-CH_3$ 2^*, whereas H-c only showed correlation spots with $-CH_3$ 6^*. On the other hand, all biphenyl protons interfered with $-CH_3$

Table 2 Solubility of Ro 28-2653 (mg/ml) in purified water and in L-lysine (50 and 500 mM) without or with HP-β-CD (200 mM) and pH values of the corresponding solutions

	Without CD		With HP-β-CD (200 mM)	
	Drug solubility (mg/ml)	pH value	Drug solubility (mg/ml)	pH value
Purified water	About 0.56×10^{-3}	–	5.53	–
L-lysine 50 mM	0.05	9.8	17.08	8.8
L-lysine 500 mM	0.09	10.0	38.14	9.8

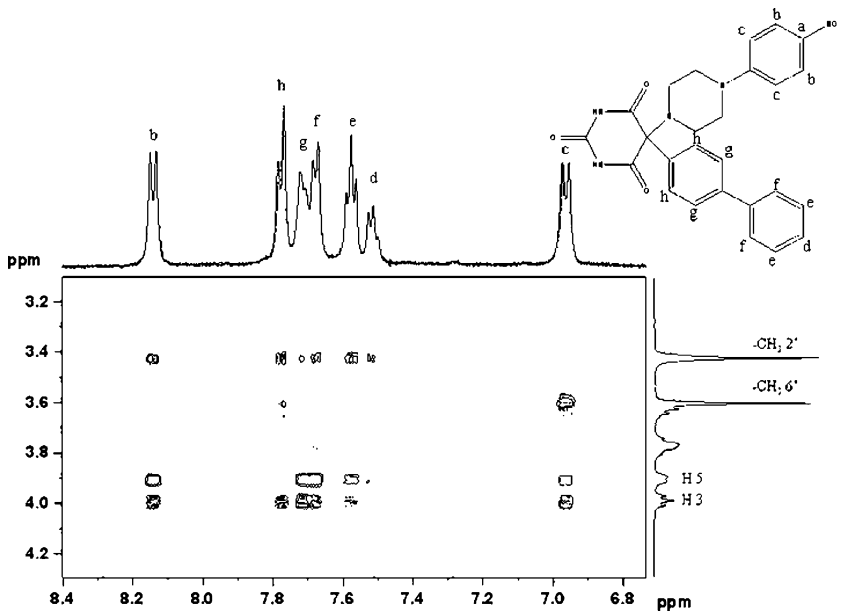

Fig. 2 2D-NMR spectroscopy: RO/DIMEB

2* and 6*. All these observations suggest that both barbituric substituents of RO are complexed by DIMEB. The nitrophenyl group is included by DIMEB with primary hydroxyls oriented towards the piperazin cycle, whereas the biphenyl group is included by a second DIMEB molecule, but with two possible inclusion directions, primary or secondary hydroxyls oriented towards the barbituric ring (Fig. 3).

Molecular modeling was performed to calculate some of the most energetically favorable conformations and confirm the ^{1}H-NMR observations. The 1:2 complex formation exhibits great entropy variation. The closeness of partners in this complex requires that RO and DIMEB undergo some geometry deformations to form the complex, thereby explaining the high level of deformation energies. The interatomic measurements again show H-bonds between the barbituric ring and DIMEB, but surprisingly between both DIMEBs too [3].

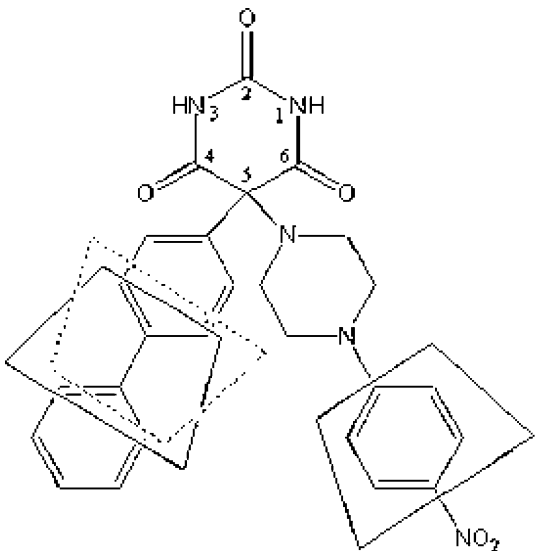

Fig. 3 Schematic representation of the complex

Biopharmaceutical studies

Pharmacokinetics of RO after intravenous administration

The mean RO serum concentration versus time curve obtained after a single administration of the intravenous solution (5 mg/kg) to sheep shows that RO pharmacokinetics follow a two-compartment model. The different pharmacokinetic parameters calculated after this intravenous administration are listed in Table 3. The distribution phase was short (about 30 min), showing that RO is rapidly distributed trough the organism. The overall volume of distribution was small (about 8 l), indicating that RO distribution could

Table 3 RO pharmacokinetic parameters (mean ± SD) obtained after intravenous administration (5 mg/kg) to sheep (n = 6)

	IV solution
$AUC_{0-168\ h}$ (μg h/ml)	858.11 ± 211.58
$AUC_{0-\infty}$ (μg h/ml)	858.87 ± 212.08
Cl_t (ml/h)	358.76 ± 67.47
Vd_t (l)	8.18 ± 2.16
$T_{1/2\beta}$ (h)	15.76 ± 2.34

Table 4 Ro 28-2653 pharmacokinetic parameters (mean ± SD, except for *F*) obtained after oral administration (15 mg/kg) to sheep

	Oral solution (n = 5)	Suspension (n = 6)	p-Value (n = 5)
$AUC_{0-168\ h}$ (μg h/ml)	1848.66 ± 854.97	208.94 ± 103.82	0.0049
$AUC_{0-\infty}$ (μg h/ml)	2070.13 ± 943.79	214.65 ± 103.04	0.0035
$C_{max\ experimental}$ (μg/ml)	51.84 ± 23.73	4.84 ± 1.95	0.0009
$C_{max\ calculated}$ (μg/ml)	56.85 ± 24.67	5.34 ± 2.24	0.0010
$T_{max\ experimental}$ (h)	3.59 ± 1.52	12.34 ± 5.99	0.0094
$T_{max\ calculated}$ (h)	3.98 ± 0.57	10.42 ± 3.01	0.0046
F_{absol}	0.80	0.08	–

be limited to extracellular fluids and that RO diffusion into tissues may not be very significant. On the other hand, RO biologic half-life was shown to be long (about 15.5 h), with drug elimination consequently being very slow.

Pharmacokinetics of RO after oral administration of a suspension and a solution

The pharmacokinetic parameters of RO obtained after oral administration of a single dose (15 mg/kg) of solution and suspension are summarized in Table 4.

The serum concentrations of RO after administration of the solution were clearly higher than those obtained with an equal dose administered as a suspension. The absorption phase observed with the solution (about 4 h) was shorter than that achieved after administration of the suspension (about 10 h). It can also be seen that the pharmacokinetic parameters of the solution and the suspension were significantly different ($p < 0.05$). The mean RO serum peak concentrations were approximately 54 and 5 μg/ml after administration of the solution and the suspension respectively. C_{max} of the solution was about 10 times higher than that of the suspension. A three times earlier T_{max} was obtained with the solution (about 3.8 h) than with the suspension (about 11 h). The AUC values followed the same trend as the C_{max} values: the AUCs after administration of the solution were about 10 times higher than those after administration of the suspension. Consequently, after comparison with the IV solution, absolute bioavailability was much higher with the solution (80%) than with the suspension (8%).

Pulmonary administration of the RO:CD complex

Concerning the inhalation route of administration, we have previously demonstrated that RAMEB, Crysmeb and HP-β-CD can undergo aerosolization and that the resulting droplet size range are compatible with pulmonary deposition. Moreover, we have demonstrated in vivo, that short-term exposure to these inhaled CDs solutions are non-toxic after assessing bronchoalveolar lavage, lung and kidney histology, bronchial responsiveness to methacholine and blood urea [8].

Table 5 Peribronchial eosinophil infiltration in mice after allergen exposure (n = 8)

	PBS	Flixotide	RO/HP300	RO/HP30
Epithelial cell	6.6 ± 2.8	11.9 ± 6.5	3.5 ± 2.5	2.5 ± 1.1
Eosinophils	70.3 ± 38.0	20.5 ± 15.6	8.5 ± 6.7	13.9 ± 1.1
Neutrophils	0.1 ± 0.3	0.1 ± 0.1	0.3 ± 0.2	0.5 ± 0.1
Lymphocytes	1.43 ± 0.7	0.5 ± 0.3	0.4 ± 0.3	0.1 ± 0.0
Macrophages	26.6 ± 10.0	48.68 ± 6.7	18.7 ± 9.3	20.0 ± 6.7

Nebulizable solutions of the complex HP-β-CD-RO were also evaluated in the same asthma model in mice. Table 5 and Fig. 4 demonstrate that the sensitized mice having received only PBS, present a rather high inflammatory score. The inhalation of the solutions containing RO produces a reduction in this score, comparable with that obtained with propionate of fluticasone. This confirms that the inhalation of the solutions containing RO significantly reduces the pulmonary ignition induced by an allergen, probably by inhibition of the MMP-9.

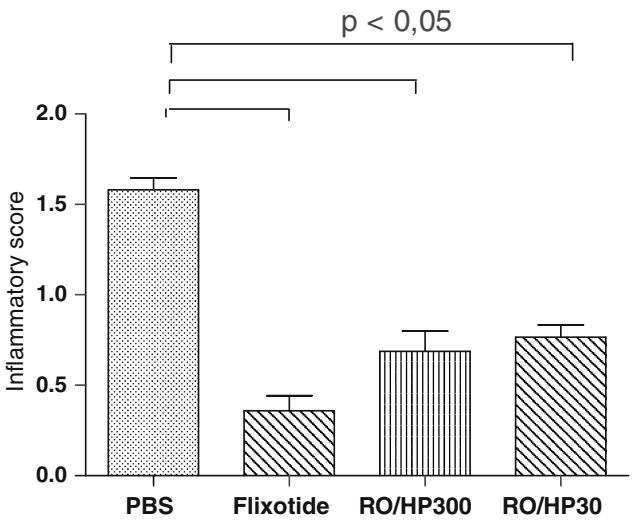

Fig. 4 Peribronchial inflammation score in mice after allergen exposure (n = 8)

Conclusions

This study shows that RO aqueous solubility can be improved by the use of CDs RAMEB and HP-β-CD form a mixture of 1:1 and 1:2 complexes with high 1:1 stability constant values. The ^{1}H-NMR study demonstrated that DIMEB interacts with RO through the nitrophenyl and biphenyl groups with preferential inclusion directions. The molecular modeling study demonstrated that inclusion modes observed with ^{1}H-NMR were energetically possible and that these complexes are notably stabilized by H-bonds formation between complex partners. In this study, it has also been shown that a synergistic effect between L-lysine and HP-β-CD allows a significant increase in RO aqueous solubility (70,000 times). The use of this multicomponent system permitted the preparation of oral and intravenous solutions of RO Consequently, intravenous administration of RO is possible with less toxic excipients than when using other usual solubilizing agents as, for example, surfactants. Absolute bioavailability of this oral solution of RO was significantly higher (about 10 times) than that obtained with a suspension of the same drug. In addition, this solution was characterized by a higher C_{max} and a lower T_{max}. The solubilization of the drug before oral administration allowed for rapid and more significant gastrointestinal absorption of RO.

Moreover, the in vivo administration of RO solutions highlighted some information about the pharmacokinetic behavior of this new drug. RO was shown to be rapidly distributed in the organism, its biologic half-life was long (about 15.5 h) and the total volume of distribution very small (about 8 l). These new intravenous and oral solutions open new opportunities to test in vivo efficacy of this new MMP inhibitor with regard both to its antitumoral activities and its anti-angiogenic properties. A first efficiency study has also been realized by using the pulmonary route of administration and has shown that the complex RO/CD has a similar local anti-inflammatory activity to that of fluticasone propionate in an in vivo asthma model in mice.

Acknowledgment This work is supported by the Ministry of the Walloon Region of Belgium (DGTRE).

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