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Development of parenteral formulation for a novel angiogenesis inhibitor, CKD-732 through complexation with hydroxypropyl-β-cyclodextrin☆

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Abstract

The effect of hydroxypropyl-β-cyclodextrin (HP-β-CyD) on the aqueous solubility and chemical stability of *O*-(4-Dimethylaminoethoxycinnamoyl)fumagillol (CKD-732), a new angiogenesis inhibitor, was investigated with an aim of preparing a stable and effective parenteral formulation. The CKD-732/HP-β-CyD inclusion complex was obtained in solid state by freeze-drying and characterized in solution by proton nuclear magnetic resonance (^{1}H NMR). Then, the pharmacokinetic profile in rats and the in vivo tumor growth inhibitory activity in mice following the parenteral administration of aqueous CKD-732/HP-β-CyD complex were compared to those of CKD-732·hemioxalate solution having an equivalent concentration. The aqueous solubility of CKD-732 was markedly increased by the combination of pH adjustment and HP-β-CyD complexation through a soluble 1:1 inclusion complex formation, which was supported by NMR spectroscopy. The hydrolysis of CKD-732 following pseudo first-order kinetics was decelerated moderately but significantly in acidic and basic solutions in the presence of HP-β-CyD. The stability of lyophilized CKD-732/HP-β-CyD complex was also drastically improved after storage in various conditions. The intravenous pharmacokinetic profile and the subcutaneous in vivo tumor growth inhibitory activity of aqueous CKD-732/HP-β-CyD complex were not significantly different from those of CKD-732·hemioxalate solution with the favorable reduction of irritation. These results demonstrate that the CKD-732/HP-β-CyD complex is an attractive formulation for use in the parenteral delivery of CKD-732.

Keywords: *O*-(4-Dimethylaminoethoxycinnamoyl)fumagillol (CKD-732); Angiogenesis inhibitor; Hydroxypropyl-β-cyclodextrin (HP-β-CyD); Solubilization; Stabilization

1. Introduction

Angiogenesis is the process of endothelial cell division and migration into tissues to form new capillaries (Folkman, 1971). Endothelial cells are for the most part strictly regulated by a host of endogenesis promoters and inhibitors of endothelial cell growth in normal state whereas their growth rate is

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considerably faster in malignant state (Hobson and Denekamp, 1984; Fox et al., 1993). Several tumors secret angiogenic factors to elicit angiogenesis, and the inhibition of angiogenesis has been expected to provide a powerful and selective therapy for a wide variety of tumors (Wellstein et al., 1992).

Fumagillol derivatives have been reported to block the supply of nutritional and other needs of tumors by preventing tumor-induced neo-vascularization, and a considerable amount of interest has been focused on them as a promising avenue of cancer research due to little serious side effects in comparison with the cytotoxic anticancer agents (Ingber et al., 1990). *O*-(4-Dimethylaminoethoxycinnamoyl)fumagillol (CKD-732), a new type of anticancer agent with anti-angiogenic mechanism, has shown a potent anti-tumor and anti-metastatic activity at the preclinical stage (Lee et al., 2000). Despite these valuable features, however, CKD-732 exhibits a low aqueous solubility ($\sim$50 μg ml^{-1} at 25 °C) below the targeted concentration of 15–20 mg ml^{-1} and a chemically labile property at room temperature, which has been a serious obstacle to the formulation of CKD-732 into a parenteral dosage form. Therefore, the development of a new effective formulation should be considered for the early biological and clinical investigation.

Cyclodextrins (CyDs) are torus-shaped oligosaccharides consisting of 6, 7 or 8 (α-, β- and γ-CyD, respectively) glucopyranose units through α-1,4-linkages with hydrophobic central cavity and hydrophilic exterior surface. The entire or at least partial inclusion process of some drugs into CyDs has led to improvements in a variety of physicochemical and pharmaceutical properties such as aqueous solubility, chemical stability and bioavailability of drug molecules (Loftsson and Brewster, 1996). Although β-CyD is the most useful one of natural CyDs for pharmaceutical applications since its central cavity has good affinity for the hydrophobic structures of many compounds, it is not always ideal for drug formulations due to its relatively low aqueous solubility (i.e. 1.8% at 25 °C), renal toxicity and membrane destabilizing properties after parenteral administration. Recently, a number of chemically modified CyDs such as hydroxyalkylated, methylated or branched β-CyDs have been prepared to improve the inclusion capacity and physicochemical properties of natural CyDs, and widely studied in various pharmaceutical preparations. In particular, hydroxypropyl-β-cyclodextrin (HP-β-CyD) has been extensively investigated on account of its superior water-solubility and safety profile by the parenteral route as well as higher complexation potential relative to the parent β-CyD (Irie and Uekama, 1997; Szente and Szejtli, 1999). Recent commercialization of two aqueous itraconazole formulations for oral and intravenous administration containing as high as 40% (w/v) HP-β-CyD is noteworthy (Miyake et al., 1999).

The purpose of this study is to investigate the solubility and stability behavior of CKD-732 as a function of HP-β-CyD concentration, gain insight into the interaction between CKD-732 and HP-β-CyD by ^{1}H NMR spectroscopy and evaluate the pharmacokinetic profile in rats and the in vivo tumor growth inhibitory activity of the CKD-732/HP-β-CyD complex in mice as compared with those of a reference, CKD-732·hemioxalate for the development of a suitable parenteral formulation.

2. Materials and methods

2.1. Materials

CKD-732 and CKD-732·hemioxalate was synthesized at New Drug Research Labs in CKD Research Institute and used as received. HP-β-CyD (Kleptose-HPB®, degree of substitution = 4.41, M_w =$\sim$ 1390) was obtained from Roquette Korea (Seoul, South Korea). Other chemicals and solvents were of reagent or HPLC grade and used as supplied from commercial suppliers.

2.2. Methods

2.2.1. Solubility studies

An excess amount of CKD-732 was added to sealed glass vials (1.5 ml) containing distilled water or pH-adjusted buffer solutions with various concentrations of HP-β-CyD (from 0 to 0.2 M). The vials were shaken at 200 rpm for at least 72 h until the equilibrium was attained. After then, the solutions were centrifuged, filtered through a 0.2 μm membrane and analyzed for CKD-732 by high performance liquid chromatography (HPLC) under the following conditions: a Waters® HPLC system (Alliance

2690); a Waters® 2487 detector operated at 306 nm; a Kromasil® C_{18} column (4.6 mm × 250 mm, 5 μm); a column temperature of 30 °C; a mobile phase of acetonitrile/20 mM ammonium acetate buffer (pH 4.2) (45:55, v/v); a flow rate of 1.2 ml min^{-1}.

The 1:1 stability constant (K) were calculated from the slope of the linear phase solubility diagram according to Eq. (1) (Higuchi and Connors, 1965):

$$K = \frac{\text{Slope}}{S_o(1 - \text{Slope})} \quad (1)$$

where the apparent solubility values (S_o) of CKD-732 in the absence of HP-β-CyD were determined directly in aqueous media. All studies were carried out in triplicate.

2.2.2. Preparation of solid complex

CKD-732/HP-β-CyD complex for the solid-state stability, pharmacokinetic and in vivo tumor growth inhibitory activity studies was prepared using a freeze-drying method as follows. Based on the results of the solubility studies, the calculated amounts of CKD-732 and HP-β-CyD were shaken in phosphate buffer solution (pH 6.7) until the mixture was fully dissolved. The solution was filtered through a 0.2 μm membrane, filled into 10 ml glass vials and frozen at −40 °C for 24 h to ensure complete solidification. Primary freeze-drying was initiated at a temperature increment of 7 °C (chamber pressure of 50 mTorr) for 10 h and finally, secondary drying was carried out at 30 °C for 48 h at the same chamber vacuum.

2.2.3. ^1H NMR spectroscopic studies

NMR spectra were recorded on a Bruker DPX-400 ^1H NMR spectrometer operating at 400 MHz. The chemical shifts for free and complexed CKD-732 were reported in ppm (δ) relative to the residual solvent signal of HDO and H_2O (internal reference, $\delta = 4.780$ ppm) with an accuracy of ±0.001 ppm after CKD-732 and HP-β-CyD were dissolved in buffered D_2O (pD = 6.3, NaH_2PO_4–K_2HPO_4) at various ratios of CKD-732/HP-β-CyD to achieve a suitable solubility of CKD-732. The stoichiometry of complexation was assessed via the continuous variation method (Job's plot, Djedaïni et al., 1990). The operation conditions of two-dimensional rotating frame nuclear Overhauser effect spectroscopy (ROSEY) were as follows: acquisition time, 250 ms; pulse width, 11.9 μs; time domain, 2 K; Fourier number, 1 K; spectral width, 4085 Hz; mixing time, 350 ms; temperature, 300 K.

2.2.4. Chemical stability in solution and solid state

Solution kinetic studies were performed at 50 °C in two aqueous buffer solutions (0.05 M and μ = 0.2)—acetate buffer (pH 3.2) and borate buffer (pH 11.5), respectively. Test solutions were prepared by adding 0.5 ml of a stock solution of CKD-732 in acetonitrile (1 mg ml^{-1}) to 9.5 ml of each buffer solution containing various amounts of HP-β-CyD previously equilibrated at 50 °C to produce an initial concentration of 50 μg ml^{-1}. At predetermined time intervals, samples were taken and the remaining amount of CKD-732 was determined by HPLC. The observed first-order rate constants (k_o and k_{obs} in the absence and presence of HP-β-CyD) were obtained by linear regression analysis of the natural logarithm of remaining CKD-732 against time, and the mean values of the rate constants in duplicate measurement were determined.

To examine the long-term stability in solid state, sealed glass vials containing CKD-732 alone, a physical mixture of CKD-732 and HP-β-CyD, or the lyophilized CKD-732/HP-β-CyD complex were stored in light-protected chambers with controlled temperature and humidity. At various time points, samples were withdrawn, dissolved in acetonitrile, diluted to the proper concentration and analyzed by HPLC. All studies were performed in triplicate.

2.2.5. In vivo pharmacokinetic studies

For pharmacokinetic studies, 10 male Sprague–Dawley rats weighing 250–300 g were randomly divided into two groups of five rats. Under light ether anesthesia, the femoral arteries and veins of rats were cannulated with PE-50 polyethylene tubing. After complete recovery from anesthesia, CKD-732·hemioxalate or lyophilized CKD-732/HP-β-CyD complex dissolved in phosphate buffered saline (PBS) were administered intravenously to the femoral vein through a catheter at a dose of 20 mg kg^{-1} as CKD-732, respectively. Blood samples (0.2 ml) were collected from the femoral artery at predesignated time intervals after the dose, and the blood samples were centrifuged immediately at 3000 × g for 5 min. Plasma aliquot (50 μl) was transferred to an Eppendorf tube and kept frozen prior to the assay for CKD-732.

The plasma concentrations of CKD-732 were determined by HPLC, and the non-compartmental pharmacokinetics parameters such as area under the drug concentration-time curve (AUC), biological half-life ($t_{1/2}$), mean resident time (MRT) and total clearance (CL) were calculated using WINNOLIN® (V.1.1). The systemic bioavailability (F) was determined by dividing the AUC value of CKD-732 from the aqueous CKD-732/HP-β-CyD complex into that from the CKD-732·hemioxalate solution. Levels of statistical significance ($P < 0.05$) were assessed using the one-way ANOVA to compare the data sets. All results were expressed as mean ± standard deviation (S.D.).

2.2.6. Tumor growth inhibitory activity in mice bearing Lewis lung carcinoma

Pre-prepared tumor mass (Lewis lung carcinoma) of 8 mm^3 was subcutaneously inoculated into the right axillary region of BDF1 mice (20–30 g, 4 weeks old). When the tumor size was 100–200 mm^3, the mice were divided randomly into three groups of six mice: untreated control group, reference group and test group. The untreated control group was given injections of 0.2 ml of PBS subcutaneously, while the reference group and the test group were administered with CKD-732·hemioxalate or CKD-732/HP-β-CyD complex dissolved in PBS at a dose of 30 or 120 mg kg^{-1} as CKD-732 every other day for five injections, respectively. The tumor volume was taken using the following Eq. (2) as measured with calipers at predetermined time points and was expressed as a ratio to the volume just before treatment (tumor volume ratio):

$$\text{Tumor volume } (mm^3) = a \times b^2 \times 0.5 \quad (2)$$

where a denotes the longest diameter of tumor mass and b is the shortest. The T/C values (%) were calculated as tumor volume $\text{ratio}_{(\text{treated})}$/tumor volume $\text{ratio}_{(\text{untreated control})}$.

3. Results and discussion

3.1. Solubility studies

Solubilization approaches for the parenteral administration of poorly water-soluble drugs have been conventionally attempted by pH-control, cosolvency, surfactant micellization, CyD complexation, emulsification or their combination (Kim et al., 1997; Li et al., 1999). Although CKD-732 is quite lipophilic with a measured partition coefficient ($\log P$) value of 3.58, solubilization attempts using cosolvents or surfactants could not provide stable formulations. Furthermore, the infusion of non-aqueous formulations of CKD-732 as with many other anticancer agents may cause a precipitation where the equilibrium drug solubility is lower than the dilution concentration line in cosolvency systems, and add pain to the intrinsic vascular irritancy effect of the compound upon administration. The presence of ionizable nitrogen of CKD-732 made a solubilization via pH adjustment possible, and the solubility increased from 6×10^{-5} M for the neutral species to 1.8×10^{-2} M for the cationic species as the pH value was reduced below 6.0. This change represents an approximate 300-fold increase in solubility due to ionization and is consistent with the pK_a value of 8.48 measured by the potentiometric approach. However, a solubility over 15 mg ml^{-1} requires the pH value below 5.0, in which condition the compound was found to undergo rapid acid-catalyzed hydrolysis from the preliminary study. Since solubilization by pH adjustment alone was also ineffective for a stable formulation, complexation with highly water-soluble and parenterally well-tolerated HP-β-CyD was chosen as a feasible method to circumvent the above limitations.

The phase solubility behavior of CKD-732 in HP-β-CyD solution was investigated at 4, 15 and 25 °C and presented in Fig. 1. These diagrams show that the aqueous solubility of CKD-732 increased in a linear manner as a function of HP-β-CyD concentration, suggesting the formation of 1:1 complex classified as A_L-type (Higuchi and Connors, 1965). The interaction parameters obtained were as follows: $S_o = 9.12(\pm 0.27) \times 10^{-5}$ M, $K = 3113 \pm 127\ M^{-1}$ at 4 °C, $S_o = 9.76(\pm 0.26) \times 10^{-5}$ M, $K = 2961 \pm 281\ M^{-1}$ at 15 °C and $S_o = 10.12(\pm 0.29) \times 10^{-5}$ M, $K = 2678 \pm 252\ M^{-1}$ at 25 °C, respectively. The intrinsic solubility of the compound increased with the rise of temperature while the stability constants decreased, as is usual with the CyD complexation of many other organic guest molecules. The slightly lower stability constant at higher temperature presumably stems from the weakening of driving force involved in complex formation such as van der Waals interactions or hydrophobic association. Unfortunately, the

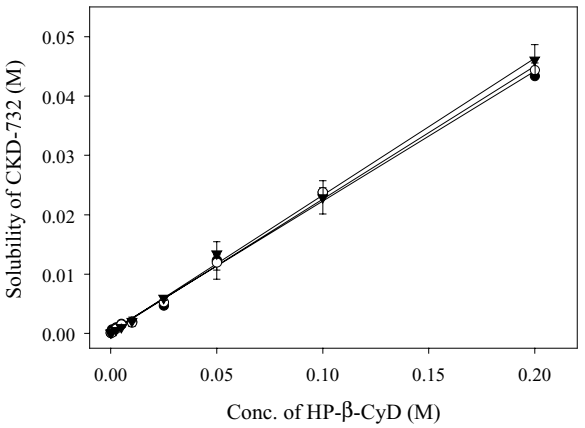

Fig. 1. Phase solubility diagrams of CKD-732 in the presence of HP-β-CyD in distilled water at 4 °C (●), 15 °C (○) and 25 °C (▼).

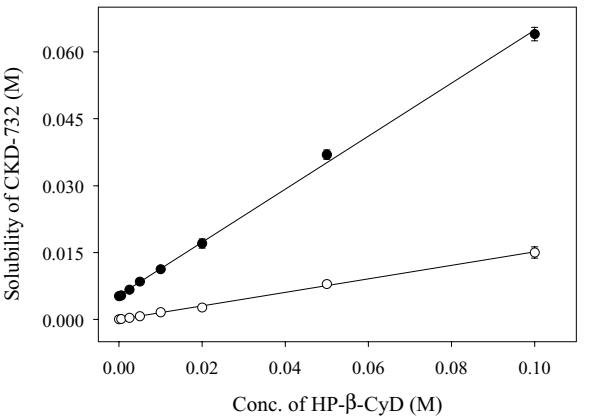

Fig. 2. Phase solubility diagrams of CKD-732 in the presence of HP-β-CyD in 0.05 M phosphate buffer solution (pH 6.7, ●) and 0.05 M borate buffer solution (pH 9.7, ○) at 25 °C.

thermodynamic parameters could not be determined accurately because of the relatively large standard deviations of the stability constants.

Jain et al. (2001) reported that the use of pH along with cosolvency, micellization or complexation could efficiently enhance the solubility of poorly water-soluble drugs with ionizable moieties. The synergic effect of pH adjustment and complexation has been described to be as follows:

$$S_{tot} = [D_u] + \frac{K_u \times [D_u]}{1 + K_u \times [D_u]} \times [L_{tot}] + [D_u] \times 10^{pK_a - pH} + \frac{K_i \times [D_u] \times 10^{pK_a - pH}}{1 + K_i \times [D_u] \times 10^{pK_a - pH}} \times [L_{tot}] \quad (3)$$

where S_{tot} is the total drug solubility, $[D_u]$ is the solubility of free unionized species, $[L_{tot}]$ is the CyD concentration, K_u is the stability constant for unionized species, and K_i is the stability constant for ionized species, respectively. The solubility of CKD-732 in the absence of HP-β-CyD is approximately 5×10^{-3} M at pH 6.7 and 6×10^{-5} M at pH 9.7, in which conditions the compound exists predominantly in the ionized and unionized form, respectively. Although the solubility increases linearly as a function of HP-β-CyD concentrations at both pH values in Fig. 2, the solubilization slopes are quite different. The solubilization slope at pH 6.7 is approximately four-fold greater than that at pH 9.7. Nonionic CyDs including HP-β-CyD have shown a general trend that hydrophobic or lipophilic guests are preferably included in the hydrophobic cavity, but the unfavorable interaction of protonated CKD-732 with the hydrophobic cavity was observed. The stability constants, K_u and K_i were calculated to be $2959 \pm 136\ M^{-1}$ and $294 \pm 19\ M^{-1}$, respectively. The lower stability constant for the cationic species than for the unionized species is attributed to the increase in the hydrophilicity of ionized substrate or the reduction of driving force for inclusion into the apolar HP-β-CyD cavity. This observation is in good agreement with the fact that the ionization of a drug molecule increases its apparent S_o, so ionized species makes more contribution to the improvement of overall solubility though it forms a weaker complex with CyDs in comparison with unionized species (Johnson et al., 1994).

3.2. ^1H NMR spectroscopic studies

NMR spectroscopy has become one of the most useful techniques for deriving information on the inclusion of drugs into CyDs cavity since the chemical and electronic environments of protons are affected during complexation, which is reflected by changes in the chemical shifts ($\Delta\delta = \delta_{complex} - \delta_{free}$) (Djedaïni et al., 1990; Bongiorno et al., 2002).

In spectra for CKD-732 in the presence of HP-β-CyD, appreciable chemical shift changes were observed with respect to the spectra for the free

Table 1
Chemical shifts (ppm) of the protons of CKD-732 and HP-β-CyD in the free (δ_o) and the complexed (δ_c) states at the equimolar ratio

Proton	δ_o (free)	δ_c (complex)	$\Delta\delta$ ($\delta_c - \delta_o$)
CKD-732			
H_a	1.224	1.273	0.049
H_{b2}	1.748	1.751	0.003
H_f	2.109	2.064	−0.045
H_{g2}	3.107	3.112	0.005
H_p	4.420	4.402	−0.018
H_e	5.249	5.202	−0.047
H_s	5.715	5.749	0.034
H_m	6.481	6.509	0.028
H_n	7.050	7.077	0.027
H_o	7.630	7.604	−0.026
HP-β-CyD			
H_1	5.049	5.043	−0.006
H_2	3.595	3.593	−0.002
H_3	3.962	3.903	−0.059
H_4	3.542	3.544	0.002
H_5	3.761	3.697	−0.064
H_6	3.849	3.831	−0.018

compound due to some conformational change occurred via the complexation (Table 1). No new peak was present in the spectra of CKD-732/HP-β-CyD complex, indicating that the complexation is a dynamic process with the compound undergoing rapid exchange between the free and included state relative to the NMR timescale (Loukas et al., 1997). The most markedly affected H_a, H_e, H_f and H_s protons (assigned in Fig. 4) in the equimolar solution of CKD-732 and HP-β-CyD showed a $\Delta\delta$ value of 0.049, −0.047, −0.045 and 0.034 ppm, respectively, while 0.004 ppm for H_b proton unlikely to be included in the HP-β-CyD cavity. As expected, a diagnostic CKD-732-induced chemical shift change was also observed for the HP-β-CyD proton signals between the free and complexed state, and the main difference was reported about the H_3, H_5 and H_6 protons located within or near the HP-β-CyD cavity with $\Delta\delta$ of −0.059, −0.064, and −0.018 ppm, respectively, while the signals of H_1, H_2 and H_4 protons on the outer surface of HP-β-CyD changed only slightly. The change of protons other than those listed in Table 1 could not be accurately measured because of the overlapping and broadening of signals. According to Ganza-Gonzalez et al. (1994), the upfield shifts of the protons in the drug following complexation are due to the association with the oxygen atoms of the CyD, rich in π electrons, while the downfield shifts are probably attributed to a variation of local polarity when these protons are inside in the cavity or a deshielding effect due to van der Waals forces between the drug and carbohydrate chains.

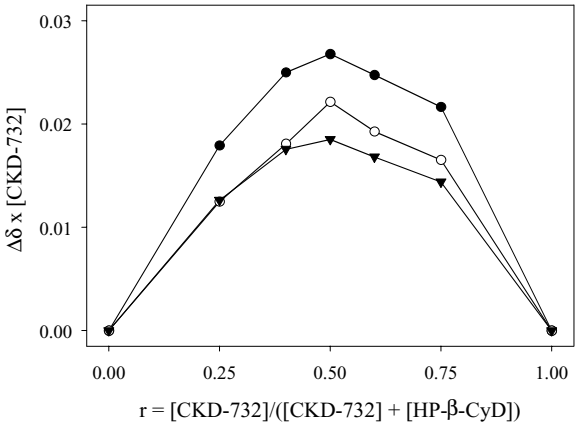

Fig. 3. Continuous variation plots for the most markedly affected protons (H_a: ●, H_f: ○ and H_s: ▼) of CKD-732 in the presence of different concentrations of HP-β-CyD.

The continuous variation plot of $\Delta\delta \cdot [\text{CKD-732}]_t$ against r for the markedly affected protons (H_a, H_f, and H_s) of CKD-732 in Fig. 3, where $\Delta\delta$ is the difference in chemical shifts, $[\text{CKD-732}]_t$ is the total concentration of CKD-732 and r is the mole fraction of CKD-732, indicates the presence of complex with 1:1 stoichiometry since the maximum is at $r = 0.5$. This stoichiometry is in agreement with the previous result from phase-solubility studies.

In order to gain additional insight into the interaction between CKD-732 and HP-β-CyD, ROSEY spectroscopic study was performed. Fig. 4 shows a partial contour plot of ROSEY spectrum for CKD-732/HP-β-CyD system in buffered D_2O. The H_a, H_e, H_f and H_s protons of CKD-732 gave correlation peaks with the glucose skeleton protons of HP-β-CyD, namely, H_3, H_5 and H_6 protons, denoting effective interactions. These cross-peaks were not observed for CKD-732 alone. In the light of these findings, it may thus be hypothesized that the cyclohexane ring moiety of CKD-732 is preferably involved in the complexation with HP-β-CyD.

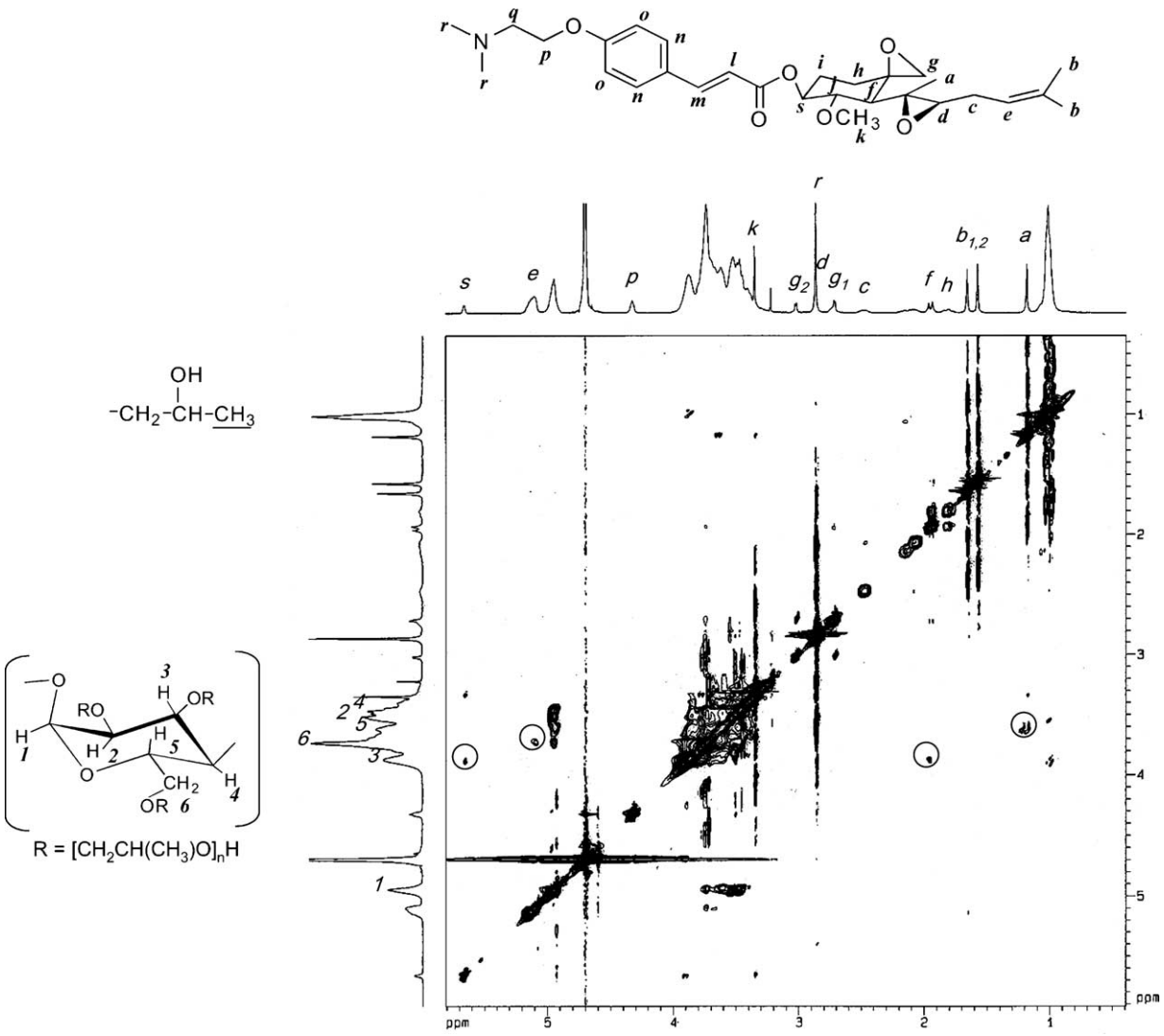

Fig. 4. Partial contour plot of ROSEY spectrum of CKD-732/HP-β-CyD mixture at the molar ratio of 2:3.

3.3. Effect of HP-β-CyD on the stability

The effects of CyDs on the chemical stability of drug molecules have been extensively documented in the literature. The hydrolytic or photolytic decomposition of partly or completely molecular-encapsulated compounds can be decelerated by virtue of the shielding of labile moieties from potential corrosive factors in aqueous media or from ultraviolet light (Loftsson, 1995; Loftsson and Brewster, 1996).

Fig. 5 shows the effects of HP-CyD concentration on the first-order hydrolysis rate constants of CKD-732 in acidic and basic conditions at 50 °C. The hydrolysis rate of CKD-732 was moderately decelerated in the presence of HP-β-CyD, and the rate constants decreased with the rise of HP-β-CyD concentration in both conditions. That is, the observed first-order rate constants (k_o) of CKD-732 in absence of HP-β-CyD were determined to be 0.2042 and 0.0918 min^{-1} at pH 3.2 and pH 11.5,

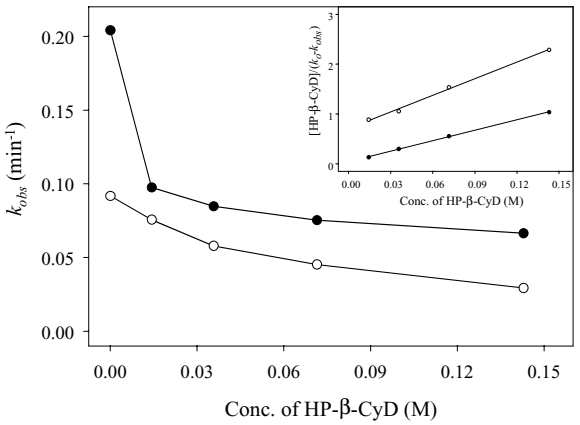

Fig. 5. Observed rate constants for the hydrolysis of CKD-732 as a function of HP-β-CyD concentration in 0.05 M acetate buffer (pH 3.2, ●) and 0.05 M borate buffer (pH 11.5, ○). The linearized form of Eq. (4) is shown in the inset.

respectively. However, k_{obs} values of CKD-732 at 0.14 M HP-β-CyD were reduced by a factor of approximately 3 to be 0.0665 and 0.0294 min^{-1} at pH 3.2 and pH 11.5, respectively. It is evident that k_{obs} is not a linear function of HP-β-CyD concentration, but rather asymptotically approaches a minimum value with increasing HP-β-CyD concentrations. This saturation behavior is consistent with some earlier findings that the degradation rate of drugs forming 1:1 inclusion complexes with CyDs depends on the CyD concentration (Gorecka et al., 1995; Roy and Guillory, 1996). Therefore, the HP-β-CyD concentration dependency of k_{obs} was treated by the following Eq. (4) (Uekama et al., 2001):

$$\frac{[\mathrm{CyD}]}{k_o - k_{obs}} = \frac{[\mathrm{CyD}]}{k_o - k_c} + \frac{1}{K \times (k_o - k_c)} \quad (4)$$

where k_o and k_c are rate constants of free and complexed CKD-732, respectively, k_{obs} is apparent rate constant in the presence of CyDs, and [CyD] is total concentration of CyDs. The plots of the left-hand side versus HP-β-CyD concentration according to Eq. (4) gave straight lines ($r^2 > 0.99$), from which $k_c = 0.0612$ and $0.0019\,\mathrm{min}^{-1}$ at pH 3.2 and pH 11.5, respectively, were obtained for each complex (the inset of Fig. 5). However, the estimation of stability constant from the kinetic method here is not suitable because the degradation becomes more rapid at higher temperature and the degradation product may interact with HP-β-CyD, thus leading to a lower stability constant below than expected.

The effect of HP-β-CyD on the chemical reactivity of CKD-732 in solid state, when a lyophilized complex was prepared, was summarized in Table 2. Significant inhibition of degradation was observed for CKD-732 alone stored at 4 °C and the lyophilized complex of CKD-732/HP-β-CyD in conditions studied, whereas the physical mixture of CKD-732 and HP-β-CyD exhibited no inhibiting effect similar to the compound alone at 25 °C and 60%RH. Most solid-state chemical reactions are generally recognized to require sufficient molecular mobility to occur over a practical timescale. Therefore, these results appears to be related to the reduction of the chemical reaction rate in low temperature condition and the reduction of molecular mobility through specific molecular complexation, indicating that HP-β-CyD is closely associated with CKD-732 in the lyophilized complex, but not involved in any reaction with CKD-732 in the physical mixture. That is, HP-β-CyD with a high collapse temperature and intrinsic amorphous property as a freeze-drying additive may be able to contribute to the

Table 2
Solid-state stability of CKD-732 alone, the physical mixture of CKD-732 and HP-β-CyD, and the CKD-732/HP-β-CyD complex

Storage conditions[a]	Duration	Relative latency (%)		
		CKD-732 alone	Physical mixture	Complex
	Initial	100	100	100
4 °C	12 months	98.05 ± 1.57	–	–
25 °C/60%RH	2 months	63.37 ± 1.10	64.54 ± 1.81	–
	12 months	–	–	96.55 ± 1.11
40 °C	3 months	Melted	–	98.65 ± 1.42
50 °C	3 months	Melted	–	96.29 ± 0.56
40 °C/75%RH	3 months	Melted	–	98.97 ± 1.79

[a] Container: sealed 15 ml glass vials.

stabilization of CKD-732 in the lyophilized complex (Pikal, 1990).

Based on these observations, it can be concluded that the labile moiety of CKD-732 may be included in the hydrophobic environment of HP-β-CyD cavity and protected from the attack of aqueous buffered media or moisture in the air and thus, a lyophilized stable formulation may be possible.

3.4. Pharmacokinetic studies

The use of CyDs in the parenteral delivery of a drug raises the problem of a potential alteration of drug pharmacokinetics by an incomplete or a delayed release of drug from a complex. Drug release from a CyD complex has been assessed by a comparative pharmacokinetic study of the drug with a control formulation since there are few well-designed studies assessing drug release mechanism from CyD complexes but hypothetical simulations (Stella et al., 1999).

Fig. 6 shows mean plasma concentration-time profiles (with standard deviation, ±S.D.) for the aqueous CKD-732/HP-β-CD complex and CKD-732·hemioxalate solution, respectively, after the intravenous dose of 20 mg kg^{-1} as CKD-732. There were no significant differences in the overall plasma levels of CKD-732 from both two preparations. The non-compartmental pharmacokinetic parameters obtained with two preparations are listed in Table 3. No statistical differences were also observed in AUC value and other parameters between them. The systemic bioavailability (F) of CKD-732/HP-β-CyD complex was essentially comparable with that of the reference solution. These results are consistent with the earlier study on the miconazole–CyD complex showing that the CyD derivatives had no effects on the i.v. pharmacokinetics of miconazole by comparison with its commercial micellar formulation (Piel et al., 1999).

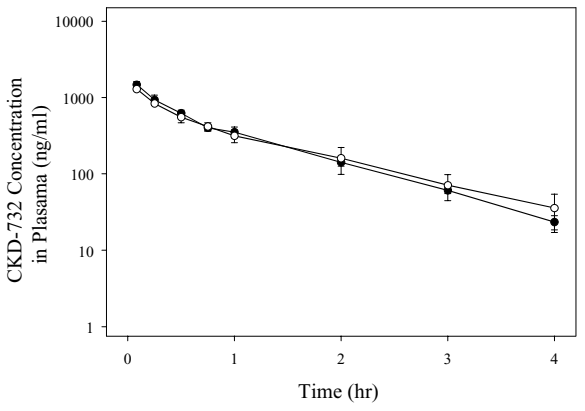

Fig. 6. Mean plasma concentration–time profile of CKD-732 after intravenous administration of CKD-732·hemioxalate (●) and CKD-732/HP-β-CyD complex (○) dissolved in PBS to rats at a dose of 20 mg kg^{-1} as CKD-732 (mean ± S.D., $n = 5$).

Table 3
Non-compartmental pharmacokinetic parameters of CKD-732 after intravenous administration of CKD-732·hemioxalate and CKD-732/HP-β-CyD complex dissolved in PBS to rats at a dose of 20 mg kg^{-1} as CKD-732 (mean ± S.D., $n = 5$)

Parameters	Preparations	
	CKD-732·hemioxalate	CKD-732/HP-β-CyD
$t_{1/2}$ (h)	0.80 ± 0.06	0.90 ± 0.13
$AUC_{0\rightarrow\infty}$ (ng h ml^{-1})	1125.15 ± 59.55	1114.11 ± 105.70
MRT (h)	0.97 ± 0.07	1.12 ± 0.22
CL (ml min^{-1} kg^{-1})	17.81 ± 0.91	18.28 ± 2.09
F (%)[a]	100	99.02 ± 9.39

[a] Systemic availability.

It has been reported that only the earliest pharmacokinetic time points will be perturbed by CyD complexation, and then only for strongly bound ($K > 10^5$ M^{-1}) drugs (Stella and Rajewsky, 1997). The stability constant of CKD-732/HP-β-CyD (294 M^{-1} at pH 6.7 and 25 °C) indicates a relatively weak binding that will lead to a rapid dissociation due to the dilution effect, the complete release of CKD-732 from the complex and a rapid equilibrium between the released free drug molecules and the complexed drug molecules within the CyD cavity under physiological conditions after the intravenous administration. However, one might intuitively anticipate that a precipitation would occur upon the dissociation of a poorly water-soluble drug (e.g. CKD-732) from a CyD complex, but changes in the free/complexed ratio of a sparingly water-soluble drug have been shown to depend on the phase solubility behavior of the system. In the case of CKD-732/HP-β-CyD complex, an 1:1 complex formation with a linear solubility profile was observed as discussed previously, so no precipitation would be anticipated regardless of the extension of dilution by a large volume of parenteral fluid or body

fluid even if the concentration of drug exceeds its water solubility limit.

Another potential advantage was that the animals apparently did not feel pain on the injection of CKD-732/HP-β-CyD complex in contrast to the reference, CKD-732·hemioxalate solution, which implies the alleviation of the intrinsic irritancy effect of the compound against blood vessels via a complexation. This can be probably attributed to the amorphous nature of HP-β-CyD, which makes its parenteral use possible by providing high compatibility with biological components such as skeletal muscles or mucous membrane. Detailed toxicological studies have shown that HP-β-CyD is well-tolerated as a parenteral carrier even at extremely high doses in contrast to limited administration routes of parent β-CyD (Brewster et al., 1990).

3.5. In vivo tumor growth inhibitory activity

Specific angiogenesis inhibitors should have an inhibitory activity against the growth of tumors in vivo on the concept that solid tumor growth beyond a certain size requires newly formed blood vessels for the transport of nutrients and oxygen, which is called to be angiogenesis-dependent.

Fig. 7 shows a comparison of tumor growth inhibitory activities expressed as tumor volume ratio after the subcutaneous administration of aqueous CKD-732/HP-β-CD complex and CKD-732·hemioxalate solution into mice bearing Lewis lung carcinoma. Subcutaneous injection of both two preparations on days 0, 2, 4, 6 and 8 markedly inhibited the growth of carcinoma whilst the tumors in the untreated control group grew extensively and their volume increased approximately 41-fold during 2 weeks. The tumors in the reference group—mice receiving CKD-732·hemioxalate dissolved in PBS at a dose of 30 mg kg^{-1}—were persistently suppressed, and the T/C value at the final day was 56.1% to that of the untreated control. The tumors in the test group administered with the CKD-732/HP-β-CD complex at an equivalent dose were also persistently suppressed, and the T/C value was 55.1%. When CKD-732 was given at a dose of 120 mg kg^{-1} through both two preparations, each tumor volume of the reference group and the test group was considerably reduced and the T/C values were also comparable each other

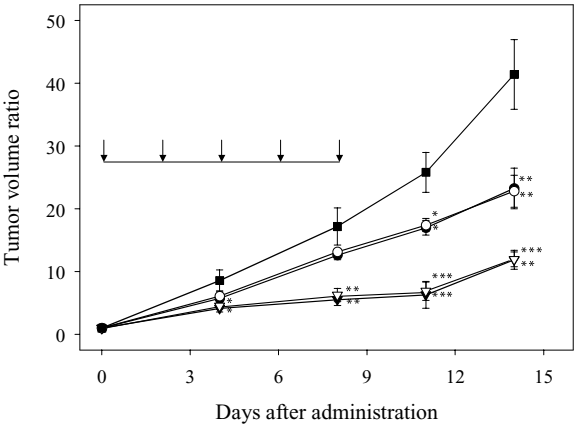

Fig. 7. Antitumor inhibitory activities of CKD-732·hemioxalate solution and aqueous CKD-732/HP-β-CyD complex after repeated injection in mice bearing Lewis lung carcinoma (■: untreated control; ●: CKD-732·hemioxalate at 30 mg kg^{-1}; ○: CKD-732/HP-β-CyD complex at 30 mg kg^{-1}; ▼: CKD-732·hemioxalate at 120 mg kg^{-1}; ∇: CKD-732/HP-β-CyD complex at 120 mg kg^{-1}). Arrows indicate injections. Each point represents the mean ± S.E. ($n = 6$). Significant different from the corresponding point for the untreated control by Student's t-test ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

(28.7 and 28.9%, respectively). Thus, there found to be no significant mitigation of the pharmacological potency of CKD-732 delivered by the complexation with HP-β-CyD. This observation, together with results obtained from pharmacokinetic studies, indicates the immediate in vivo complex dissociation and instantaneous availability of CKD-732 from the HP-β-CyD complex.

4. Conclusion

The present study could rationalize the development of a parenteral delivery form for CKD-732. It was possible to enhance the solubility of CKD-732 through the combination strategy of pH adjustment and HP-β-CyD complexation, and the lyophilized CKD-732/HP-β-CyD complex showed fairly good long-term stability. After reconstitution and parenteral administration of the lyophilized CKD-732/HP-β-CyD complex, CKD-732 was rapidly released from the complex with no loss of pharmacological activity but with better tolerance. Thus, it can be concluded that the described HP-β-CyD formulation of CKD-732

may provide a physiologically acceptable dosage form as a parenteral therapeutic system for the treatment of tumors.

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