Enhancement of Solubility and Bioavailability of β-Lapachone Using Cyclodextrin Inclusion Complexes

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Purpose. To explore the use of cyclodextrins (CD) to form inclusion complexes with β-lapachone (β-lap) to overcome solubility and bioavailability problems previously noted with this drug.

Methods. Inclusion complexes between β-lap and four cyclodextrins (α-, β-, γ-, and HPβ-CD) in aqueous solution were investigated by phase solubility studies, fluorescence, and ^{1}H-NMR spectroscopy. Biologic activity and bioavailability of β-lap inclusion complexes were investigated by *in vitro* cytotoxicity studies with MCF-7 cells and by *in vivo* lethality studies with C57Blk/6 mice (18–20 g).

Results. Phase solubility studies showed that β-lap solubility increased in a linear fashion as a function of α-, β-, or HPβ-CD concentrations but not γ-CD. Maximum solubility of β-lap was achieved at 16.0 mg/ml or 66.0 mM with HPβ-CD. Fluorescence and ^{1}H-NMR spectroscopy proved the formation of 1:1 inclusion complexes between β-CD and HPβ-CD with β-lap. Cytotoxicity assays with MCF-7 cells showed similar biologic activities of β-lap in β-CD or HPβ-CD inclusion complexes (TD_{50} = 2.1 μM). Animal studies in mice showed that the LD_{50} value of β-lap in an HPβ-CD inclusion complex is between 50 and 60 mg/kg.

Conclusions. Complexation of β-lap with HPβ-CD offers a major improvement in drug solubility and bioavailability.

KEY WORDS: β-lapachone; cyclodextrin; inclusion complex; solubility; bioavailability.

INTRODUCTION

β-Lapachone (β-lap) is a potent cytotoxic agent that demonstrates antitumor activity against a variety of human cancer cells. The drug was first isolated from the bark of the *Lapacho* tree (genus *Tabebuia*) in the rainforests of South America and has a long history as an herbal medicine. β-Lap is bioactivated by the enzyme, NQO1 [NAD(P)H:quinone oxidoreductase, E.C. 1.6.99.2], which is a ubiquitous flavoprotein found in most eukaryotic cells. This enzyme catalyzes a two-electron reduction of various quinones, utilizing either NADH or NADPH as electron donor. The human NQO1 gene encodes a 30-kd protein that is expressed in a tissue-dependent manner. More importantly, NQO1 is overexpressed (up to 20-fold) in a number of tumors, including breast, colon, and lung cancers, compared with adjacent normal tissue (1–4). Overexpression of NQO1 in cancerous cells makes it an ideal target for tumor-selective drug therapies with minimal toxicities to healthy cells. Despite the potency and selectivity of β-lap in killing NQO1-containing cancer cells *in vitro*, the low water solubility of β-lap (0.038 mg/ml or 0.16 mM) limits its systemic administration and clinical applications *in vivo*.

Cyclodextrins (CDs) are a well-known class of host molecules that can form inclusion complexes with a variety of drugs to improve drug solubility, stability, as well as bioavailability (5–11). Cyclodextrins consist of different number of glucopyranose units that are connected by α(1,4) glycosidic linkages. The shape of these molecules is similar to a truncated cone (Fig. 1). which has a hydrophilic outer surface and a hydrophobic inner cavity. α-Cyclodextrin (α-CD) comprises six glucopyranose units, β-cyclodextrin (β-CD) seven such units, and γ-cyclodextrin (γ-CD) eight such units. Different numbers of glucopyranose units lead to different cavity sizes. The inner diameter of the hydrophobic cavity is approximately 4.7–5.3, 6.0–6.5, and 7.5–8.3 Å for α-CD, β-CD, and γ-CD, respectively (7). Hydroxypropyl-β-cyclodextrin (HPβ-CD) is a modified β-CD obtained by treating a base-solubilized solution of β-CD with propylene oxide. This chemical modification significantly increases the solubility of HPβ-CD over β-CD (Table I). In addition, HPβ-CD is well tolerated and appears to be safe in clinical trials without observable renal toxicity as shown with β-CD (9).

The aim of this work was to explore the use of cyclodextrins to form inclusion complexes with β-lap to overcome the solubility and bioavailability problems of the drug. We hypothesize that binding of β-lap inside the hydrophobic cavity of cyclodextrins will drive the dynamic equilibrium of β-lap from solid state to the solution state (Fig. 1), thereby increasing the drug solubility. In this study, we used UV-Vis and fluorescence spectrometry to examine the effect of four types of cyclodextrins on the aqueous solubility of β-lap. The effect of β-CD and HPβ-CD on the resonance of β-lap protons and structure of the inclusion complexes were studied by ^{1}H-NMR spectroscopy. A maximal solubility of β-lap (16.0 mg/ml or 66.0 mM) was achieved with HPβ-CD, more than 400-fold increase over β-lap solubility in water. The effect of cyclodextrins on the biologic effectiveness of β-lap was investigated *in vitro* using antitumor activity assays against human MCF-7 breast cancer cells as well as *in vivo* using toxicity and weight loss measurements after intraperitoneal (i.p.) injections of β-lap inclusion complexes in C57Blk/6 mice.

MATERIALS AND METHODS

Materials

α-CD, β-CD, γ-CD, and HPβ-CD were obtained from Cyclodextrin Technologies Development, Inc. (CTD) (High Springs, FL) with >98% purity. β-Lap was synthesized following a previously reported procedure (12). Phosphate-buffered saline (PBS, pH 7.4) was purchased from Fisher Sci-

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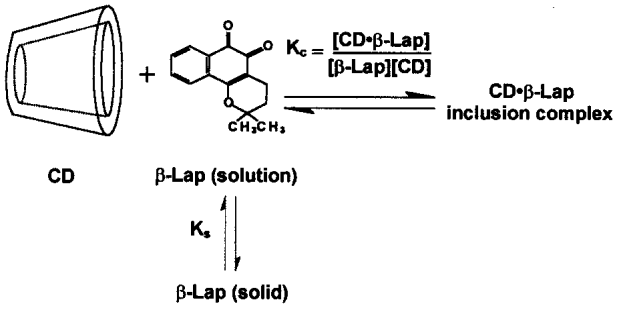

Fig. 1. Schematic diagram of the solubility equilibria of β-lap in aqueous solutions containing cyclodextrin (CD). K_s and K_c are the equilibrium constants for β-lap solubility and formation of inclusion complex, respectively. [CD·β-lap], [β-Lap], and [CD] are the concentrations of CD·β-lap complex, free β-lap, and free CD, respectively.

entific (Pittsburgh, PA). RPMI 1640 medium, fetal bovine serum, L-glutamine, penicillin, and streptomycin were purchased from Hyclone (Logan, UT) and Life Technologies, Inc. (Rockville, MD). MCF-7 breast cancer cells were routinely passed at 1:5 to 1:20 dilutions every 5 days using mycoplasma-free 0.05% trypsin as described (13).

Phase Solubility Studies of CD·β-Lap Inclusion Complexes

Solubility studies were performed by adding an excess amount of β-lap to a series of PBS buffers containing different concentrations of each CD molecule ranging from zero to its solubility limit (see Table I for the solubility limit of each CD molecule). The suspensions were stirred at 25°C until dissolution equilibrium was reached. Then aliquots were withdrawn, filtered (Nylon syringe filter, 0.2 μm pore size, from Fisher Scientific, Pittsburgh, PA), and analyzed for β-lap concentrations by UV-Vis spectrophotometry [λ_{max} = 257.2 nm, ε = 109.6 ml/(mg·cm)]. A phase solubility diagram for each CD was obtained by plotting the β-lap solubility at dissolution equilibrium as a function of the CD concentration. The association constant (K_c) for the complex formation was calculated based on Eq. (1) assuming a 1:1 ratio of complex formation (14).

$$K_c = \frac{\text{Slope}}{\text{Y} - \text{intercept} \times (1 - \text{Slope})} \quad (1)$$

^{1}H-NMR Study of CD·β-Lap Inclusion Complexes

All ^{1}H-NMR spectra were obtained on a Varian 600-MHz NMR spectrometer. The probe temperature was set at 25°C. ^{1}H-NMR spectrum of β-lap was assigned by homonuclear correlation spectoscopy (COSY) and heteronuclear multiple quantum coherence spectroscopy (HMBC). One-dimensional gradient-enhanced ROESY (GROESY) experiments were carried out by using the following pulse sequence: relaxation delay, 1 s; 90° pulse width, 8.2 μs; spin lock time, 400 ms; and acquisition time, 3.495 s. The concentrations of β-lap and HPβ-CD for the GROESY experiments were 10.6 and 58.8 mM, respectively, in D_2O.

The complex for the NMR shift titration study was prepared by adding 78 μl of β-lap stock solution (1.64 mM in MeOH). The solution was dried, and then variable amounts of β-CD and HPβ-CD solution in D_2O were added. The resulting β-lap (0.123 mM) and β-CD (0.1–14.7 mM) or HPβ-CD (0.5–430 mM) solutions were vigorously stirred at 25°C overnight to ensure the reaching of equilibrium. The association constants can be determined based on Eq. (2) (15).

$$\Delta\delta_{\text{Hc or Hd}} = \frac{K_C \Delta\delta_0 (\Delta\delta_0[\text{CD}] - \Delta\delta[\beta\text{-Lap}])}{\Delta\delta_0 + K_C (\Delta\delta_0[\text{CD}] - \Delta\delta[\beta\text{-Lap}])} \quad (2)$$

For methyl protons (Hc) on β-lap, $\Delta\delta_{Hc}$ denotes the difference of chemical shift between the two splitting methyl groups at a particular concentration of CD. For aromatic Hd protons, $\Delta\delta_{Hd}$ ($\Delta\delta_{Hd} = 7.787 - \delta_i$) is calculated as the difference between the chemical shift of pure β-lap (7.787 ppm) and that of CD·β-lap inclusion complex at a particular concentration of CD (δ_i). For both Hc and Hd protons, $\Delta\delta_0$ denotes the difference between pure β-lap and pure CD·β-lap inclusion complexes, [CD] stands for the concentration of cyclodextrin, and [β-lap] denotes the concentration of β-lap used in this experiment (0.123 mM).

Fluorescence Study of CD·β-Lap Inclusion Complexes

Fluorescence study was performed on a LS45 Luminescence Spectrometer (Perkin Elmer Instruments) with 100 nm/min scan speed and 10 nm for both excitation and emission slit widths. Initially, emission spectra of β-lap (0.015 mg/ml) in PBS buffer were obtained at different excitation wavelengths to determine the optimal values of λ_{ex} and λ_{em} for spectrophotometry measurements. The effect of CD concentrations on the fluorescence spectra of β-lap was studied. In these studies, each sample was prepared by adding the same volume (4 ml) of a stock solution of β-lap (0.005 mg/ml) but different quantities of CD inside a 5 ml volumetric flask filled with PBS buffer. The resulting solutions were vigorously stirred at 25°C overnight to ensure the reaching of equilibrium. Emission spectra of β-lap at different CD concentrations were obtained at λ_{ex} = 330 nm. The fluorescence intensity at λ_{em} = 436 nm was measured and used to determine the value of K_c of CD·β-lap inclusion complex.

In Vitro Cytotoxicity Assays

The cytotoxicity of β-CD·β-lap and HPβ-CD·β-lap inclusion complexes to MCF-7 breast cancer cells was determined

Table I. Physical Properties of α-CD, β-CD, HPβ-CD, and γ-CD (7)

Properties	Cyclodextrins			
	α-CD	β-CD	HPβ-CD	γ-CD
No. of glucose units	6	7	7	8
Molecular formula	$(C_6H_{10}O_5)_6$	$(C_6H_{10}O_5)_7$	$(C_6H_{10}O_5)_7(C_3H_6O)_{4-5}$	$(C_6H_{10}O_5)_8$
Molecular weight	972	1135	1390	1297
Cavity diameter (Å)	4.7–5.3	6.0–6.5	6.0–6.5	7.5–8.3
Solubility (mg/ml)	145	18.5	500	232

following a previously published procedure (16). The MCF-7 cells were grown in RPMI 1640 medium supplemented with 5% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin. In cytotoxicity studies, cells were first seeded into 96-well plates at 1×10^4 cells/well in 1 ml medium and allowed to attach overnight. Medium was removed 24 h later, and new medium (1 ml) containing different concentrations of CD alone or β-lap in CD inclusion complex was added to each well. After 4 h, the medium was removed and replaced with drug-free growth medium. Cells were allowed to grow for an additional 6 days. On day 7, cells were washed with PBS after medium removal, and 250 μl double-distilled Milli Q H_2O was added to each well. After one freeze-thaw cycle, TNE buffer (2 M NaCl, 1 mM EDTA, 10 mM Tris-HCl, pH 7.4) with 10 μg/ml Hoechst 33258 (Sigma) fluorescent dye was added to each well. Changes in cell number, measured as DNA content, were then determined by an adaptation of the method of Labarca and Paigen (17) and analyzed with a Perkin Elmer HTS 7000 Bio Assay Reader with excitation wavelength of 360 nm and emission wavelength of 460 nm. Data were expressed as relative growth (T/C) by dividing DNA content of treated cells (T) by that of untreated cells (C) at identical times. The reproducibility of each data point is represented by the means ± SEM of at least six replicate wells. β-Lap in dimethylsulfoxide (DMSO) was used as a positive control to compare the drug cytotoxicity to MCF-7 cells.

Animal Toxicity Studies

C57Blk/6 female mice (3–4 weeks old, 18–20 g) (Jackson Labs, Maine) were used to study the morbidity and mortality of mice treated with HPβ-CD·β-lap inclusion complex. Four mice per group were used for each dose, which varied from 20 to 100 mg/kg. Two groups of four mice were used for 60 mg/kg because this dose proved to be near the LD50 (lethal dose that kills 50% of the mice population) of the β-lap in HPβ-CD inclusion complex. Mice were injected (i.p.) every Monday, Wednesday, and Friday for a total of 10 injections. Control animals (four mice/group) were injected with 5000 mg/kg of HPβ-CD alone to evaluate its toxicity. This HPβ-CD dose is approximately 10 times the HPβ-CD amount introduced at the highest dose of β-lap (100 mg/kg) via the HPβ-CD·β-lap inclusion complex. The higher dose of HPβ-CD was used to ensure the lack of toxicity of this compound. Weight and lethality were measured on a daily basis following initial drug administration. All animals were maintained in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care according to the "Principles of Laboratory Animal Care" of the National Institutes of Health.

RESULTS AND DISCUSSION

Solubility Study

The effect of cyclodextrins on the aqueous solubility of β-lap was evaluated using the phase solubility method (14). Fig. 2 shows the phase diagrams of β-lap with four different types of CDs in PBS buffer. The solubility of β-lap increased linearly as a function of α-, β-, or HPβ-CD concentrations.

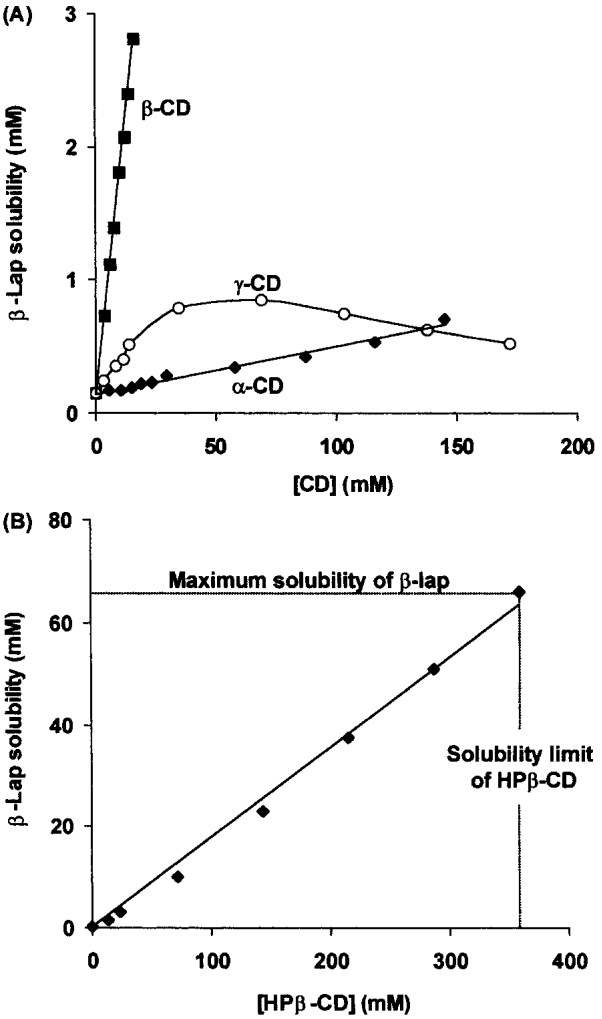

Fig. 2. Phase solubility diagrams of β-lap as a function of cyclodextrin concentrations at 25°C. A, α-CD, β-CD, and γ-CD. B, HPβ-CD.

These phase diagrams are classified as type A_L by Higuchi (14), which denotes a linear increase in solubility. In contrast, γ-CD showed a typical B_S-type solubility curve (14), which denotes an initial rise in the solubility of the solute followed by a plateau and a decreasing region because of the limited solubility of the complexes.

Increases in β-lap solubility in aqueous CD solutions are consistent with the formation of inclusion complexes between β-lap and CD molecules. In general, the main driving force for the complex formation is the hydrophobic interactions between a poorly soluble guest compound, such as β-lap, and the apolar cavity of the CD molecule. The hydrophobicity and geometry of the guest molecule as well as the cavity size of the CD molecule are important parameters for the complex formation. In the current study, the enhancement of β-lap solubility is highly dependent on the type of CD molecule. For example, the phase diagram for β-CD shows a much higher slope (0.16) than that of α-CD (0.0035) and the linear region ([γ-CD] < 20 mM) of γ-CD (0.024, Fig. 2A), demonstrating that β-CD is more effective in solubilizing β-lap. Based on the phase solubility diagrams, the association constants for the different inclusion complexes are determined using Eq. (1). The values of K_c are 20.0 ± 0.7, (1.23 ±

0.01) × 10^3, (0.94 ± 0.08) × 10^3, and 160 ± 5 M^{-1} for α–CD, β–CD, HPβ-CD, and γ-CD, respectively.

The different association constants for different cyclodextrin molecules indicate the importance of cavity size to encapsulate the β-lap molecule. α-CD has the lowest affinity to associate with β-lap, presumably because β-lap cannot fit into the relatively small hydrophobic cavity of α-CD (diameter ~5 Å, Table I). This is in agreement with other studies (10) in which guest molecules carried a phenyl moiety. On the other hand, although the wider cavity size of γ-CD (diameter ~8 Å) allows room for encapsulation (K_c increased by a factor of 8 for γ-CD over α-CD), it has lower affinity to associate with β-lap than that of β-CD and HPβ-CD, which have smaller cavity size. Therefore, β-CD and HPβ-CD appear to be significantly better host molecules for β-lap encapsulation. The much higher association constants of HPβ-CD and β-CD show the importance of appropriate cavity size in facilitating the interactions between β-lap and HPβ-CD or β-CD, as further supported by molecular recognition studies of host–guest chemistry (18).

Even though β-CD is a better host molecule for β-lap than α-CD and γ-CD, its application to maximize the solubility of β-lap is limited by the solubility of β-CD vehicle itself (16.3 mM). Consequently, the maximal solubility of β-lap in β-CD solution is limited to 2.8 mM or 0.68 mg/ml. This concentration is still relatively low for systemic administrations of this drug. To overcome this problem, we used HPβ-CD molecule as a β-lap carrier. HPβ-CD is formed by covalent modification of the external hydroxyl groups on β-CD by hydroxylpropyl groups. The modification significantly increased the solubility limit of HPβ-CD (360 mM, a factor of 22 over β-CD). The maximal solubility of β-lap in HPβ-CD solution reached 66.0 mM or 16.0 mg/ml, a 24-fold increase over that in β-CD vehicle and a 413-fold increase over β-lap aqueous solubility (0.16 mM). HPβ-CD provides the most effective candidate to solubilize β-lap.

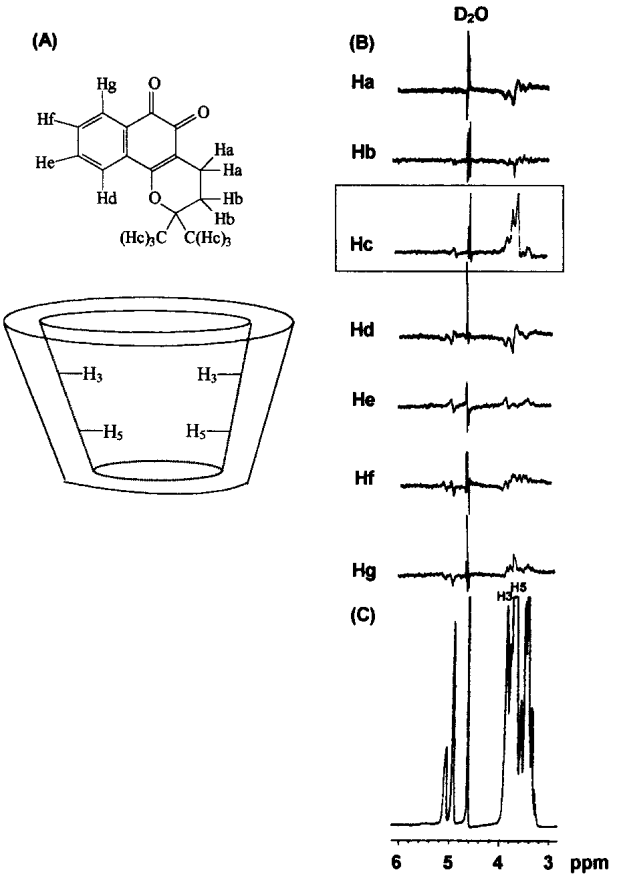

Fig. 3. A, Chemical structure of β-lap and general geometry of HPβ-CD. B, GROESY spectra of HPβ-CD·β-lap inclusion complex in D_2O at 25°C. C, The ^{1}H-NMR spectrum of HPβ-CD·β-lap inclusion complex. The concentrations of HPβ-CD and β-lap are 58.8 and 10.6 mM, respectively.

NMR Study of CD·β-Lap Inclusion Complexes

NMR spectroscopy is a powerful tool to study the inclusion phenomena. It has been shown that GROESY spectroscopy can be used to accurately detect the nuclear Overhauser effect (NOE) (19,20). In this study, we carried out the GROESY experiment to gain insight regarding the molecular structure of HPβ-CD•β-lap inclusion complex. Fig. 3 shows the GROESY spectra of the HPβ-CD•β-lap inclusion complex obtained by exciting every proton of β-lap (Ha to Hg). The significant NOE enhancement of the H5 and H3 protons located inside the HPβ-CD cavity was observed with the selective excitation of the Hc protons from β-lap. In contrast, no obvious NOE enhancement was observed with the selective excitation of the rest of β-lap protons, suggesting that the methyl moiety of β-lap is bound inside the cavity. This result also suggests that HPβ-CD forms a 1:1 inclusion complex with β-lap.

It is well known that the insertion of a guest molecule into the hydrophobic cavity of cyclodextrin can effect chemical shifts of the guest protons. In this experiment, we studied the effect of β-CD and HPβ-CD on the resonance of β-lap protons. Fig. 4A A shows the effect of increasing β-CD concentration on the ^{1}H-NMR spectra of phenyl protons of β-lap. Interestingly, Hd was the only proton that showed upfield shifts as a result of increasing β-CD concentrations. Above [β-CD] = 11.4 mM, no further changes of the upfield shift were observed (data not shown). Upfield shifts of Hd as a result of increasing of HPβ-CD concentrations were also found. Figure 4B shows the effect of β-CD on the ^{1}H-NMR spectra of methyl (Hc) and methylene (Ha, Hb) protons of β-lap. A splitting of these three groups of proton peaks was observed to result from the formation of an inclusion complex. This effect was most pronounced with the methyl protons (Hc), whereas the Ha protons had the least effect, suggesting the formation of diastereomeric complexes between β-lap and CD. A splitting of Ha, Hb, and Hc was also found with HPβ-CD, but the signal was interfered with by the methylene protons from hydroxypropyl groups on HPβ-CD.

The upfield shift of Hd (but not of other phenyl protons) and the splittings of Ha, Hb, and Hc indicate that these changes are the result of inclusion complex formation but not of the nonspecific interaction between cyclodextrin and β-lap. Chemical shift changes of Hd (Fig. 5A) A as a function of β-CD and HPβ-CD concentrations and the splitting of Hc (Fig. 5B) as a function of β-CD gave good fits with a 1:1 complex model as shown in Eq. (2) (15). The association constants determined from these data are 774 ± 52 M^{-1} (Hd shift) and 734 ± 20 M^{-1} (Hc splitting) for the β-CD·β-lap inclusion complex, and 662 ± 27 M^{-1} (Hd shift) for the HPβ-CD·β-lap inclusion complex.

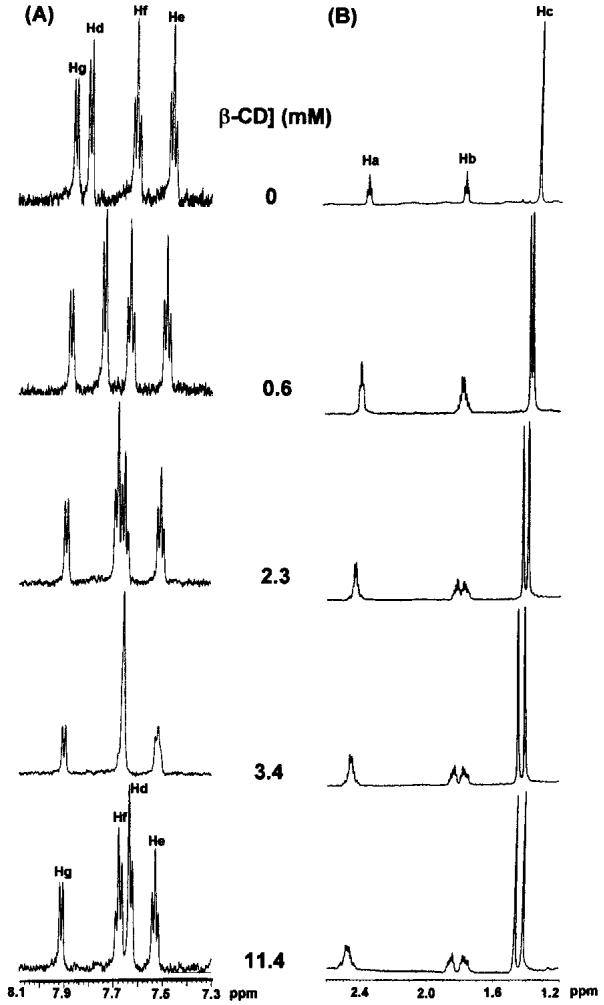

Fig. 4. ^{1}H-NMR (600 MHz) spectra of β-lap ([β-lap] = 0.123 mM) as a function of β-CD concentrations in D_2O. A, Phenyl protons (Hd, He, Hf, and Hg). B, Methyl and methylene protons (Ha, Hb, and Hc).

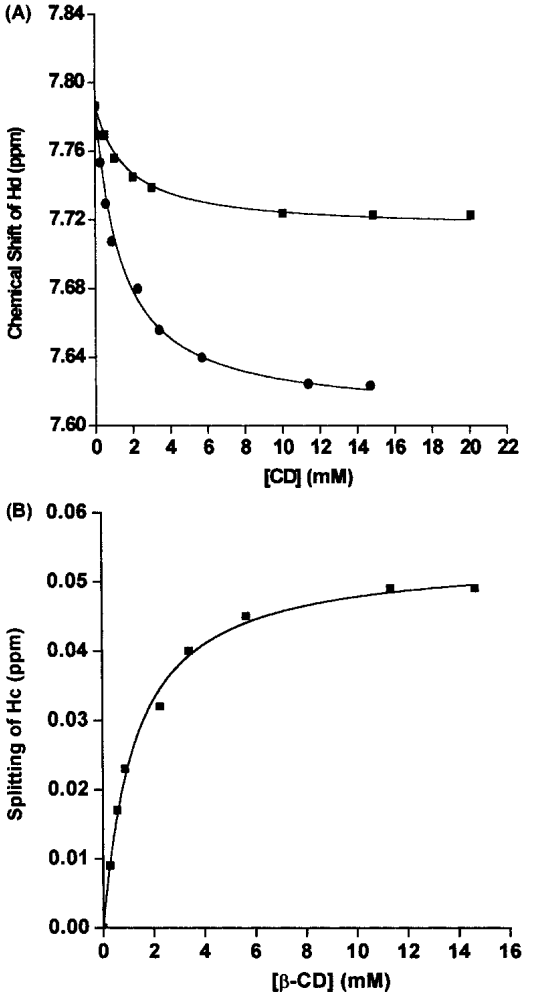

Fig. 5. Nonlinear curve fitting of Eq. (2) to experimental data using (A) chemical shift of Hd in β-lap ([β-lap] = 0.123 mM) as a function of HPβ-CD (■) and β-CD (●) concentrations in D_2O. B, Splitting of Hc as a function of β-CD concentrations in D_2O.

Fluorescence Studies of β-Lap Inclusion Complex

In the course of this study, we discovered that β-lap was a fluorescent molecule, and we used fluorescence spectroscopy to further study the association of HPβ-CD•β-lap and β-CD•β-lap inclusion complexes. Fig. 6A A shows a series of emission spectra of β-lap alone in PBS buffer at different excitation wavelengths ranging from 257 to 360 nm. These data showed that an excitation wavelength at 330 nm gave the highest emission intensity. For all the excitation wavelengths, the maximum emission wavelength was located at 436 nm. These experiments established the optimal spectroscopy conditions for β-lap complexation studies (λ_{ex} = 330 nm, λ_{em} = 436 nm).

Figure 6B shows the dependence of β-lap emission spectra as a function of HPβ-CD concentrations in PBS buffer. All the experiments were carried out at the same excitation wavelength (λ_{ex} = 330 nm) and same β-lap concentration (18 μM). Results showed that the β-lap emission intensity decreased when the HPβ-CD concentration increased (Fig. 6B). In addition, there is a slight blue shift (~6 nm) of the maximum emission wavelength in solution containing HPβ-CD. The change in fluorescence intensity and maximum emission wavelength of guest β-lap compound by addition of cyclodextrins is another indication of the formation of inclusion complexes between these two compounds. On encapsulation inside the hydrophobic cavity of CD molecules, the β-lap compound encounters a different chemical environment compared to aqueous solution. Geometric restrictions caused by space limitations in the CD cavity and reduced polarity because of the hydrophobic cavity of CD are found to alter the energetics and dynamics of the photophysical and photochemical processes of the guest molecule (21). The blue shift is consistent with the fact that β-lap experiences a less-polar environment in the hydrophobic cavity of HPβ-CD.

Next, we determined the association constant for the formation of the inclusion complex based on the fluorescence data. Emission intensity at 436 nm was used for these studies. Scatchard analysis by Eq. (3) (22) was used to determine the association constant (K_c) of the inclusion complex.

$$R/[CD]_f = n K_c - R K_c \quad (3)$$

where $[CD]_f$ is the unbound (free) molar concentration of CD, n is the number of binding sites, i.e., the stoichiometry

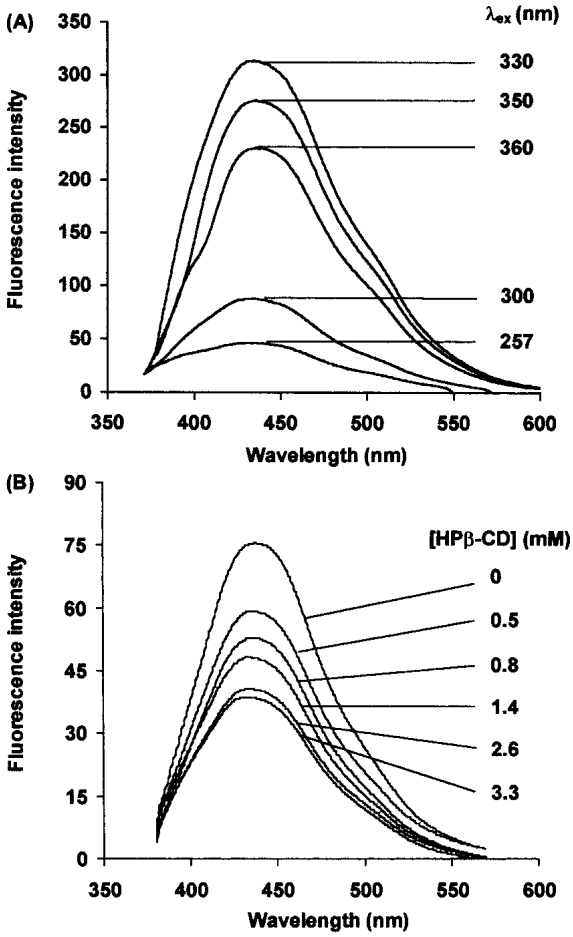

Fig. 6. A, Emission spectra of β-lap (61 μM) at different excitation wavelengths ranging from 257 to 360 nm. B, Emission spectra of β-lap (18 μM) in different HPβ-CD concentrations at 25°C (λ_{ex} = 330 nm).

of the complex, and R is the molar fraction of β-lap bound to CD.

The values of K_c are $(1.10 \pm 0.06) \times 10^3$ M^{-1} (R^2 = 0.97) and $(1.06 \pm 0.06) \times 10^3$ M^{-1} (R^2 = 0.98) for β-CD·β-lap and HPβ-CD·β-lap complexes, respectively. The numbers of binding sites (n) of β-CD·β-lap and HPβ-CD·β-lap inclusion complexes were found to be 1.04 ± 0.02 and 1.01 ± 0.02, respectively, which confirm the formation of 1:1 inclusion complexes. The values of K_c from fluorescence measurement are consistent with those from phase solubility studies but are higher than the data from NMR measurement. This difference is most likely a result of the different solvents used (e.g., PBS buffer was used in fluorescence and phase solubility studies, in comparison to D_2O in NMR studies).

In Vitro Cytotoxicity Studies in MCF-7 Cells

In order to evaluate the biologic activity of β-lap when it forms inclusion complexes with cyclodextrin, initial cytotoxicity DNA assays using MCF-7 human breast cancer cells were performed. Previous studies (13,16,23) have demonstrated that NQO1-expressing MCF-7 cells treated under these conditions not only showed growth inhibition, but the results can be equated to loss of survival according to colony-forming ability assays. Log-phase MCF-7 cells were exposed to different concentrations of β-lap in HPβ-CD inclusion complexes, β-lap in β-CD inclusion complexes, or with HPβ-CD and β-CD alone for 4 h. Drugs were then removed, and DNA content as a measure of cell survival was determined. β-Lap in DMSO was used as a positive control for comparison. Fig. 7 shows the viability of MCF-7 cells exposed to HPβ-CD·β-lap, β-CD·β-lap inclusion complexes, or with HPβ-CD and β-CD alone. The primary x-axis is the β-lap concentration used in this experiment, and the secondary x-axis is the concentration of HPβ-CD and β-CD required to solubilize β-lap. Cell viability of MCF-7 cells was statistically identical for cells treated with vehicles (HPβ-CD, β-CD) alone or with PBS for 4 h. These data showed that pure HPβ-CD (0 to 18.8 μM) and β-CD (0 to 20.8 μM) alone showed no cytotoxicity or growth inhibition. β-Lap in HPβ-CD and β-lap in β-CD inclusion complexes showed similar cytotoxic responses for the entire range of β-lap–equivalent doses (Fig. 7). Quantitatively, the drug potency was measured as TD_{50}, the toxic dose that kills 50% of the cell population. The TD_{50} values of β-lap in HPβ-CD and β-CD inclusion complexes were found to be the same at 2.1 μM for a 4-h transient drug exposure. These values were slightly higher than that from β-lap in DMSO, whose TD_{50} value is 1.7 μM.

In Vivo Analyses of β-Lap Toxicity

To evaluate the bioavailability of β-lap in CD inclusion complexes, C57Blk/6 mice were injected with increasing concentrations of β-lap in HPβ-CD inclusion complex 3 days per week for 3 weeks, and changes in weight and survival were recorded. Results showed no morbidity (decreases in weight loss) or lethality of mice for the control group injected i.p. with vehicles alone, or for mice injected i.p with 20 to 50 mg/kg of β-lap in HPβ-CD inclusion complex. In contrast, mice injected i.p. with 70 to 100 mg/kg showed both morbidity and 100% lethality (Fig. 8). Finally, mice treated with 60 mg/kg β-lap in HPβ-CD inclusion complex i.p. showed significant morbidity (loss of >15% body weight in most animals) and

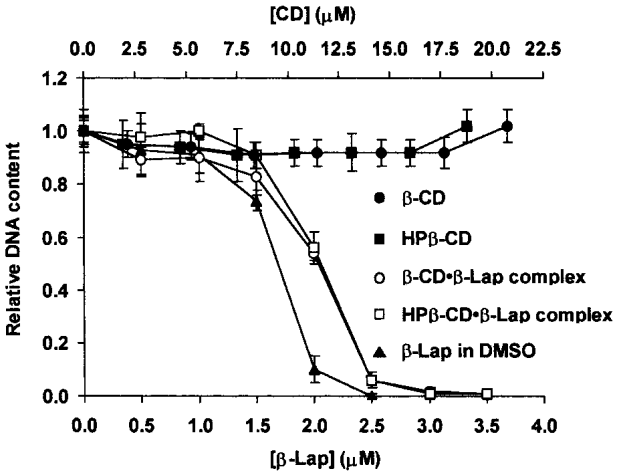

Fig. 7. Viability of log-phase MCF-7 cells exposed to β-lap in DMSO, HPβ-CD or β-CD inclusion complexes, as well as β-CD and HPβ-CD vehicles alone. For β-lap in DMSO and β-lap inclusion complexes, the bottom horizontal axis denotes the β-lap concentrations. The top horizontal axis denotes the cyclodextrin concentrations in β-lap inclusion complexes as well as for vehicles (β-CD and HPβ-CD) alone. Experiments were performed at least two times in triplicate to provide the standard deviation.

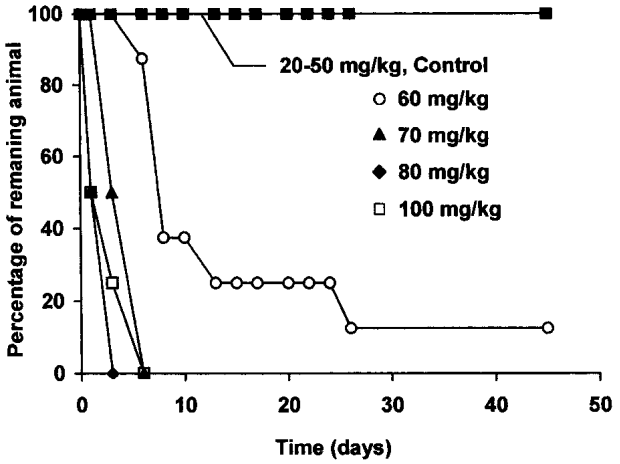

Fig. 8. Effect of varied doses of β-lap in HPβ-CD inclusion complex and HPβ-CD (control group injected with 5000 mg of HPβ-CD/kg) on the survival of C57Blk/6 mice. Animals were injected i.p. on days 1, 3, 6, 8, 10, 13, 15, 17, 20, and 22.

lethality (seven of eight animals died within 45 days of the treatment regimen). Consequently, the LD_{50} (lethal dose that kills 50% of mice population) value of β-lap in HPβ-CD inclusion complex was estimated to be 50–60 mg/kg in 18- to 20-g C57Blk/6 mice. This was determined by considering that 50 mg/kg kills 0% of mice, and 60 mg/kg kills 85% of mice in the course of this experiment. Interestingly, mice responded to doses above 50 mg/kg β-lap in HPβ-CD inclusion complex, but not to HPβ-CD vehicle alone, with unusual but temporary drug reactions. Within 15 min post-i.p.-injection, mice were observed to have a shivering reflex and difficulty in breathing. These drug responses lasted approximately 2 h, with mice exposed to 40–50 mg/kg recovering completely with essentially no weight loss noted over time. In contrast, most mice exposed to >60 mg/kg exhibited similar drug responses that resulted in lethality. Preliminary autopsies with mice that ultimately died did not result in the detection of major damage to vital organs, and more detailed analyses of cause of death are ongoing. Our studies indicate a nearly threefold greater bioavailability of β-lap *in vivo* compared to previous animal studies using Cremophor as a vehicle for β-lap administration, where an LD_{50} of >150 mg/kg was reported (24).

CONCLUSION

Phase solubility studies of β-lap in complexation with α-CD, β-CD, HPβ-CD, or γ-CD were carried out to overcome the problems of β-lap solubility and bioavailability. HPβ-CD demonstrated the maximum enhancement of β-lap solubility to 16.0 mg/ml or 66.0 mM, more than a 400-fold increase over β-lap solubility in water (0.038 mg/ml or 0.16 mM). The association constants of β-lap with cyclodextrins were determined by the phase solubility method, ^{1}H-NMR, and fluorescence spectroscopy (λ_{ex} = 330 nm, λ_{em} = 436 nm). β-CD and HPβ-CD showed higher binding affinity (K_c = 0.9–1.2 × 10^3 M^{-1}) to β-lap than α-CD (20 M^{-1}) and γ-CD (160 M^{-1}). Cytotoxicity assays indicated little differences in biologic activity between β-lap in HPβ-CD or β-CD inclusion complexes, with nearly identical cell responses (cell death in induced apoptosis) and TD_{50} values (2.1 μM). Finally, studies of morbidity and mortality in C57Blk/6 mice suggested a LD_{50} of 50–60 mg/kg, with no morbidity or mortality following 20–50 mg/kg β-lap in HPβ-CD inclusion complex. Complexation of β-lap with HPβ-CD offers a major advancement in improvement of bioavailability of this very active anticancer agent, and the antitumor activity of these complexes against human breast and prostate cancer xenografts are under investigation.

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