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Molecular recognition and enhancement of aqueous solubility and bioactivity of CD437 by β-cyclodextrin

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ABSTRACT

CD437 (6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalene carboxylic acid) is a novel synthetic retinoic acid derivative that has been shown to selectively induce apoptosis in human lung cancer cells. This compound, however, is limited in its application due to its low solubility in aqueous solutions. One technique for increasing the solubility and bioavailability of a cytotoxic agent is the formation of inclusion complexes with cyclodextrins. Herein, we report the formation and characterization of a 2:1 complex between β-cyclodextrin (β-CD) and CD437. It is shown that CD437 is a tight binder of β-CD with an overall association constant of $2.6 \pm 0.6 \times 10^7\ M^{-2}$. In addition, we demonstrate (a) that β-CD-derived complexation enhances the aqueous solubility of CD437, and (b) that a significant increase in the toxicity of CD437 against a human lung adenocarcinoma cell line can be achieved by co-treatment with β-CD.

CD437 (6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalene carboxylic acid) is a synthetic retinoic acid (RA) derivative which is known to have antineoplastic properties across a variety of cancer cell lines, including lung,[1] prostate,[2] ovarian,[3,4] cervical,[5] and head and neck.[6] Typically, the molecular action of retinoids depends on the binding to and activation of a subset of the nuclear hormone receptor superfamily which contains both retinoic acid receptors (RARs) and retinoid X receptors (RXRs), each of which is encoded by three genes, α, β, and γ. CD437, like RA, can bind directly to a heterodimer formed between retinoic acid receptor γ (RARγ) and a retinoid X receptor (RXR). Growth arrest and cell differentiation then occur when the activated RARγ/RXR dimer binds to a RA response element on DNA.[7] In contrast to RA, however, CD437 also displays strong apoptotic properties. In addition to the RARγ-dependent pathway, CD437 is also able to exert its apoptotic effects through an RARγ-independent pathway.[8] Thus CD437 has been shown to induce apoptosis in both RA-resistant cell lines[8,9] and RARγ-negative cell lines.[8,10]

Despite the significant antineoplastic activity of CD437 it suffers from the drawback of being highly lipophilic and thus is poorly soluble in water. One manner in which the bioavailability of poorly soluble drugs can be increased is through encapsulation in a water-soluble host molecule.[11–15] One class of molecules commonly used for increasing the solubility of pharmacologically active compounds is cyclodextrins (CDs). CDs are cyclic oligosaccharides composed of repeating glucopyranoside units linked through α (1 → 4) glycosidic bonds. These macrocycles (see Fig. 1) are defined by a hydrophobic interior and hydrophilic exterior, and have shapes resembling that of truncated cones. The three naturally occurring CDs, α-, β-, and γ-CD, consist of 6, 7, and 8 glucopyranoside units, respectively, and have inner cavity diameters of 0.53, 0.65, and 0.83 nm across the larger rim.[16] CDs are well known to form inclusion complexes with a variety of organic guests, and host–guest complexes of both 1:1 and 2:1 stoichiometries have been reported in the literature.[17–23]

It has been previously shown that solubilization of the anticancer agent albendazole (ABZ) by hydroxypropyl-β-CD (Fig. 1) results in an enhancement in the cytotoxicity of ABZ against a SKOV-3 (human ovarian cystadenocarcinoma) cell line (IC_{50} = 1.94 μM, vs 3.88 μM for a formulation lacking CD).[21] In addition, it has been reported that pretreatment of human breast cancer cells with methyl-β-CD prior to treatment with carboplatin or 5-fluorouracil (5-FU) has a dramatic effect on the toxicity of these drugs. In fact, in a MCF-7 cell line, pretreatment of cells with 5 mM methyl-β-CD, followed by treatment with either 10 μM carboplatin or 10 μM 5-FU results in a decrease in cell survival to less than 50%. Neither of these drugs significantly alters cell survival at these concentrations without pretreatment with methyl-β-CD.[24] The enhancement in cytotoxicity of anticancer agents in the presence of CDs is most likely attributed to increased cellular uptake of drugs manifested by (a) enhanced solubility as a result of complex formation and/or (b) alteration of the cell membrane characteristics leading to increased drug permeability (e.g., CDs have been previously shown

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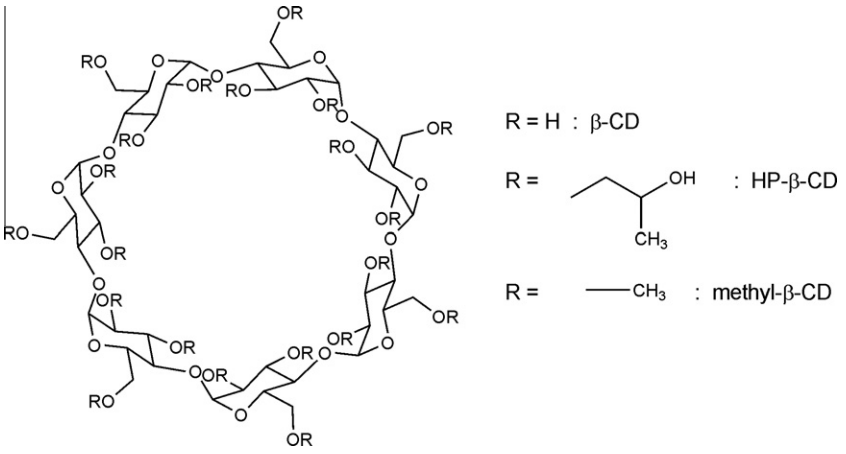

Figure 1. Chemical structure of β-CDs.

to interact with cholesterols and phospholipids resulting in cholesterol depletion of the cell membrane[25,26]). The abovementioned literature precedence gave us impetus to study the solubility and bioavailability of CD437 in the presence of β-CDs. For our studies we chose to use the parent non-derivatized β-CD.

The structure of CD437 (Fig. 2) contains a central phenol ring flanked by adamantane and naphthalene carboxylic acid units. Since both adamantyl[27–31] and naphthyl[32–36] groups are known to serve as guest molecules for β-CD, it was reasoned that β-CD could bind to CD437 in a 2:1 stoichiometry. A convenient way to test this hypothesis is through proton NMR titrations since the aromatic naphthyl protons and aliphatic adamantyl protons appear in distinct regions of the NMR spectrum. Upon increasing the concentration of β-CD in aqueous solutions (40% DMSO in phosphate buffer) of CD437, the adamantyl proton signals are all observed to experience strong downfield complexation-induced chemical shifts (CISs) when binding into the hydrophobic cavity of β-CD, with the Hγ protons experiencing the greatest shifts (Fig. 3).[37] Further, upon addition of β-CD the previously overlapping signals of the Hδ protons are observed to exhibit different CISs. Specifically, these signals appear as an unresolved broad singlet in the absence of β-CD, while in the presence of β-CD geminal coupling (^2J = 18.7 Hz) can be observed between the axial ($H\delta_A$) and equatorial ($H\delta_E$) protons, indicating distinct chemical environments for the two protons. These results are consistent with what is observed

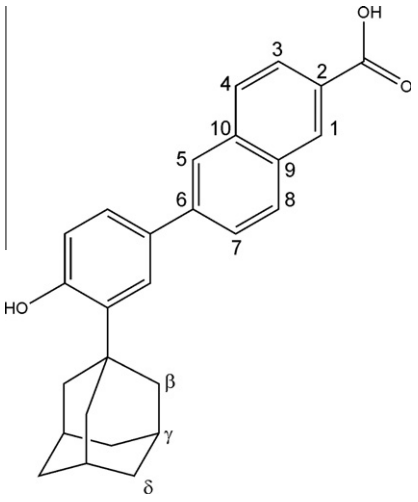

Figure 2. Chemical structure of CD437.

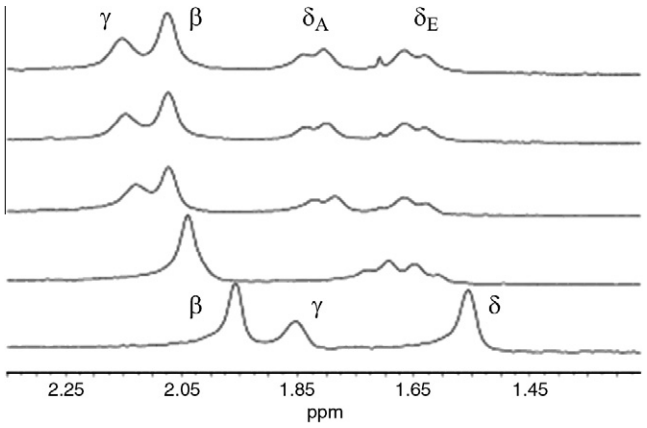

Figure 3. Proton NMR spectrum of the adamantyl region of CD437 at varying concentrations of β-CD, CD concentration from top to bottom: 0 mM, 3.6 mM, 9.4 mM, 15.8 mM, 24.0 mM.

Table 1
Maximum observed complexation-induced chemical shifts (ppm) of AdCA and CD437 upon inclusion complex formation with β-CD

	Hβ	Hγ	$H\delta_A$	$H\delta_E$
AdCA	+0.10	+0.14	+0.10	+0.01
CD437	+0.12	+0.30	+0.26	+0.08

for the binding of adamantanecarboxylic acid (AdCA) to β-CD.[28] Interestingly, however, the magnitude of the CISs (see Table 1) for the signals arising from the adamantyl protons of CD437 are greater than for the corresponding protons in AdCA, possibly indicating deeper inclusion of the adamantyl group into the CD cavity for CD437.

The naphthyl protons of CD437 also all exhibit noticeable CISs, which lead us to suggest that the naphthyl group is included into a second molecule of β-CD. However, in general the naphthyl group protons exhibit much smaller CISs than the adamantyl protons, with CISs between −0.08 and +0.07 ppm. It is interesting that the proton attached to the C5 carbon of the naphthyl group appears to be the only one which exhibits an upfield CIS (−0.08 ppm). Since this behavior is different than that observed for the other protons, it is reasoned that this proton is not included in the cavity of cyclodextrin. Indeed, computational studies (described below) suggest that this proton rests on the larger secondary rim of one of the CDs as shown in Figure 4.

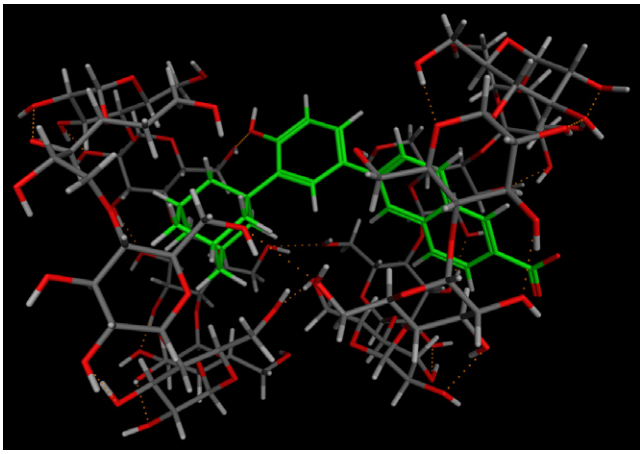

Figure 4. Lowest energy conformation of β-CD/CD437 2:1 complex as determined by molecular modeling simulations. Carbons on CD437 are colored in green and carbons on β-CD are colored in gray for clarity.

While the NMR studies indicate a potential 2:1 stoichiometry, in order to confirm such a binding phenomenon fluorescence spectroscopy was employed. Due to its naphthalene carboxylic acid terminus, CD437 is fluorescent with an emission maximum observed near 440 nm in aqueous solutions (λ_{ex} = 309 nm). Moreover, the fluorescence intensity of CD437 is strongly dependent on solvent polarity, with a marked increase in fluorescence intensity in solvents with low dielectric constants (see ESI Fig. 1). These preliminary studies suggest that complexation of CD437 by β-CD should be accompanied by a significant increase in fluorescence as the naphthyl group moves into the hydrophobic cavity of β-CD. A Job's plot obtained based on fluorescence emission of CD437 with increasing mole fraction of β-CD displays a maximum near 0.66, which is indicative of the formation of a 2:1 complex (Fig. 5). Furthermore, fluorescence titrations in which the concentration of β-CD was varied in aqueous solutions of CD437 resulted in a binding isotherm that could be readily fit (R^2 = 0.998) via non-linear regression to an equation describing a 2:1 binding mode[38] (see Supplementary data) to give association constants of $Ka_1 = 1.8 \pm 0.4 \times 10^5\ M^{-1}$ and $Ka_2 = 1.5 \pm 0.1 \times 10^2\ M^{-1}$ (Fig. 6). This indicates that the first binding event (most likely to the adamantyl group) is three orders of magnitude stronger than the second binding event.

In order to further validate the 2:1 stoichiometry of binding and to gain more insight into the molecular recognition event, the self-assembly was modeled using a ligand–receptor docking simulation with the molecular operating environment (MOE) software as described in the Supplementary data. The modeling studies show that when ranked by estimated binding energies using the London dG function, a 2:1 binding conformation with the adamantyl group included in one β-cyclodextrin ring and the naphthalene moiety included in the second β-cyclodextrin ring is one of the most ener-

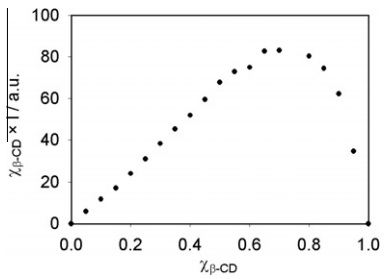

Figure 5. Job's plot showing a 2:1 stoichiometry for the β-CD/CD437 inclusion complex.

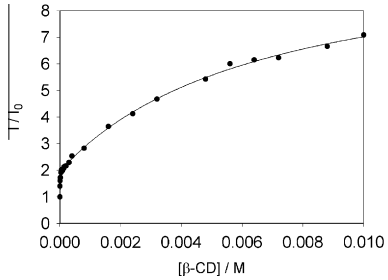

Figure 6. Fluorescence enhancement of CD437 (440 nm) with increasing concentration of β-CD along with the best-fit curve from a least-squares regression analysis of a 2:1 binding model.

getically favorable. Not only is this 2:1 binding conformation found to be the most stable, but with minor deviations in torsional angles and positions relative to the cyclodextrin rings, it is found to be the predominant binding stoichiometry of the 29 next most energetically favorable binding conformations as well.

In addition to examining the mode of the binding interaction between β-CD and CD437, we wished to probe how complexation could improve the aqueous solubility of CD437. Hence, a solubility experiment was carried out via the method described by Connors[38] by addition of an excess of CD437 (0.2 mg) to solutions containing varying concentrations of β-CD in phosphate buffer. After agitation for 48 h, undissolved CD437 was removed by filtration and the UV absorbance of the solutions at 312 nm was recorded. This absorbance maximum was chosen since the absorbance at this wavelength does not vary significantly on complexation with β-CD at concentrations tested (ESI Fig. 2), so any change in absorbance intensity could be attributed to increased concentration of soluble CD437. The absorbance of CD437 at 312 nm versus β-CD concentration is shown in Figure 7. It can be seen that the solubility of CD437 increases more than three-fold on going from 0 to 100 μM β-CD.

Given that the abovementioned studies clearly show that β-CD can bind to CD437 and enhance its water solubility, we were interested to determine whether complexation of CD437 enhanced the cytotoxicity of CD437. Thus the growth inhibitory activity of a solution of CD437 in the presence of 2 equiv of β-CD was compared with that of either CD437 or β-CD alone in A549 cells, a human lung adenocarcinoma cell line whose growth arrest response to CD437 has been well characterized.[39] A549 cells were exposed to CD437, β-CD, or a 2:1 mixture of β-CD/CD437 for 48 h. The number of live cells in each treated group was determined using an XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide) assay and the percentage of growth was determined

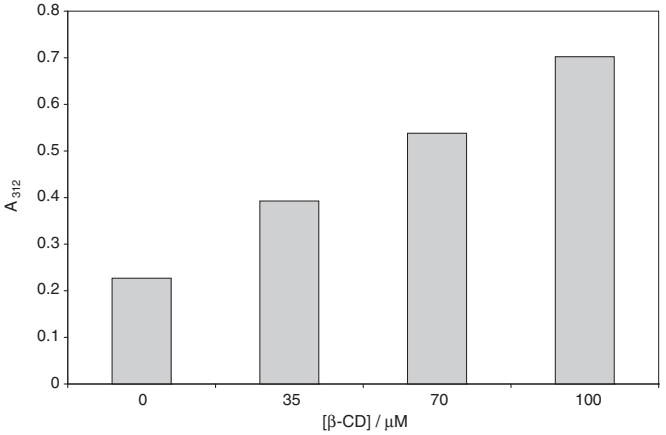

Figure 7. Enhancement of solubility of CD437 in solutions containing increasing concentration of β-CD as shown by the increase in absorbance at 312 nm.

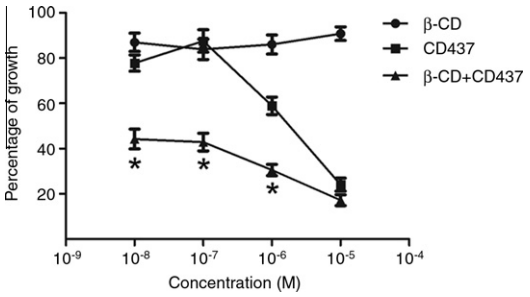

Figure 8. Growth inhibition studies: A549 cells were exposed for 48 h to the indicated doses of β-CD, or CD437, or 2:1 β-CD/CD437. Growth inhibition by the compounds was determined using XTT assays as described in the Supplementary data. Percentage of cell growth of the treated groups relative to that of the control group was presented in mean ± sem obtained from 12 data points collected from four independent experiments. * Denotes a *P* value <0.001 as determined using two-way Anova test.

by comparing the values between the treated groups and the control group exposed to the vehicle DMSO and by setting values of the control group to 100%. Similar to a previous report,[39] CD437 alone at 1 μM and 10 μM reduced growth to 60% and 20% of the control group, respectively (Fig. 8). Lower doses of CD437 at 100 nM and 10 nM caused only a slight reduction in cell growth (to around 80% of the control group). As expected, β-CD at all doses tested exhibited minimal effect on cell growth. Importantly, the addition of β-CD in conjunction with CD437 substantially enhanced growth arrest induced by CD437 especially at lower concentrations of CD437. For instance, a 2:1 mixture of β-CD and CD437 at 10 nM achieved a growth arrest percentage of around 40% that was much greater than that by 10 nM CD437 alone and comparable to that by 1 μM CD437 alone (Fig. 8). The greater potency of the 2:1 mixture in the range of 10 nM–1 μM than that of CD437 alone is statistically significant with a *P* value <0.001.

This work shows that CD437 is capable of forming an inclusion complex with β-CD, with an apparent host–guest stoichiometry of 2:1 based on both the method of continuous variation and on a non-linear curve fitting procedure. This complex formation is characterized by an overall association constant of $Ka_1Ka_2 = 2.6 \pm 0.6 \times 10^7\ M^{-2}$, and coincides with a significant increase in fluorescence intensity of the guest molecule as well as a marked increase in aqueous solubility. In addition, co-treatment of A549 cells with both CD437 and β-CD results in increased growth inhibition. It is possible that this phenomenon is due to increased cellular uptake of CD437 due to disruption of the cell membrane by β-CD; however, it should be noted that in our study much lower concentrations of β-CD were used than in previous reports (20 μM vs 5 mM[22]–200 mM[21]). Inclusion complex formation between CD437 and β-CD may also be significant in explaining the observed increase in toxicity, as in a 2:1 aqueous mixture of β-CD and CD437, greater than 55% of CD437 is expected to exist as either a 1:1 or 2:1 inclusion complex with β-CD. It is expected that this work will have implications on future pharmaceutical compositions of this novel apoptotic agent.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.11.073.

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