Drug Development and Industrial Pharmacy, 34:149–156, 2008

ISSN: 0363-9045 print / 1520-5762 online
DOI: 10.1080/03639040701484486

informa
healthcare

Molecular Encapsulation of Thalidomide with Sulfobutyl Ether-7 β-Cyclodextrin for Immediate Release Property: Enhanced In Vivo Antitumor and Antiangiogenesis Efficacy in Mice

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Thalidomide's reported ability to inhibit tumor angiogenesis has led to clinical trials determining its effectiveness in combating various types of cancer. Since thalidomide exhibits low oral bioavailability due to limitations in solubility, inclusion complexation using sulfobutyl ether-7 β-cyclodextrin was used to improve the delivery of thalidomide. Our main goals were to increase the solubility, bioavailability as well as chemical stability of thalidomide through complexation with anionic β-cyclodextrin, to characterize the complex in solid state using differential scanning calorimetry, X-ray powder diffractometry, and to explore thalidomide's antitumorigenic and antiangiogenesis potential when administered orally as free and in combination with cyclodextrin to experimental animals. The aqueous solubility and aqueous alkaline stability of thalidomide was markedly increased by the SBE7βCD complexation. Thalidomide administered orally in combination with SBE7βCD, led to a significant delay in tumor formation as a result of improved cellular drug absorption, distribution through solubilization in experimental animals. The improved pharmacological efficacy of the thalidomide-cyclodextrin complex compared to free thalidomide in mouse melanoma model suggest that such a delivery system may be useful for the improved therapeutics of thalidomide, in vivo.

Keywords thalidomide; sulfobutyl ether-7 β cyclodextrin; cancer chemotherapy; angiogenesis inhibition

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INTRODUCTION

If aqueous solubility of a drug is below 0.1–0.05 mg/mL then dissolution of the drug particles will be slow and the absorption will, in many cases, be dissolution rate-limited (Lipinski, Lombardo, & Dominy, 2001). Drug efflux transporters can also limit drug permeation through the mucosa, which then becomes the rate-limiting step in the drug absorption process from gastrointestinal tract. Various formulation techniques have been developed in an effort to overcome these and other obstacles in oral drug delivery. To design new drug delivery systems; suitable carrier materials are used to overcome the undesirable properties of the drug molecules (Hirayama & Uekama, 1999). Cyclodextrins (CDs) are a group of structurally related natural products able to form inclusion complexes with many drugs by incorporating commonly, a lipophilic moiety of the drug into its hydrophobic central cavity (Szejtli, 1998). Along with improvement of solubility and bioavailability, the cyclodextrin encapsulation protects the drug molecules against hydrolysis, oxidation and even enzymatic degradation (Loftsson & Brewster, 1996; Uekama, Hirayama, & Irie, 1998).

Thalidomide (THLD) was sold initially as a sleeping aid and to pregnant women as an antiemetic. It was later found to be teratogenic in fetal development. Although its role in teratogenesis is not fully defined, thalidomide's property to inhibit basal fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) induced angiogenesis in areas other than fetal tissue has been well documented. In addition, thalidomide reduces the amount of tumor necrosis factor-α (TNF-α) produced in the body and therefore may be used to reduce some of the unpleasant symptoms associated with cancer (Physicians Desk Reference, 2001). Unfortunately, when thalidomide's

antiangiogenic properties were applied to cancer research in rodent models, conflicting results were obtained (Pollard, 1996). Despite the lack of conclusive results in murine models, thalidomide entered human clinical trials where inconsistent and discouraging results were produced. This could be the result of very low aqueous solubility (2×10^{-4} mol/l) of thalidomide, which led to erratic and incomplete absorption in vivo. T_{max} ranges from 2.9–5.7 hr indicating that it is slowly absorbed from the GIT. There is lack of C_{max}-dose proportionality coupled with observed increase in T_{max} values. It suggests that the poor aqueous solubility of thalidomide may be hindering the rate of absorption.

To address the issue of poor water-solubility and improving in vivo efficacy many water-soluble derivatives of thalidomide have been synthesized. Of these water-soluble analogues CC-3052 and CC-5013 (Lenalidomide) have been found to be successful as potent inhibitor of TNF-α production and as antiangiogenic in vivo, respectively (Dredge, Horsfall, Robinson et al., 2005; Marriot, Westby, Cookson et al., 1998). In another study it was found that synthetic N-methyl analogue of thalidomide exhibited higher water-solubility compared to other alkyl analogues and was the best skin permeant of the series (Goosen, Laing, Plessis et al., 2002). Improvement of solubility and stability of thalidomide using natural β-cyclodextrin and 2-hydroxy propyl β-cyclodextrin has also been reported previously (Koch & Steinacker, 1988; Krenn, Gamcsik, Vogelsang, et al., 1992). SBE7βCD, a highly soluble anionic derivative of β-cyclodextrin has attracted growing interest due to its greater intrinsic solubility, in vivo safety and improved complexing abilities. Investigations of oncogenicity of natural β cyclodextrin in laboratory animals have proved its safety (Toyada, Shoda, Uneyama, et al., 1997). Promotion of melanoma dormancy and impaired tumor vascularization in mice using quercetin-cyclodextrin binary system has been reported recently (Kale, Saraf, Juvekar, & Tayade, 2006). Therefore, the aim of present investigation was to evaluate complexing/solubilizing properties of SBE7βCD toward thalidomide to achieve improvement in solubility and chemical stability. Furthermore, in vivo tumor growth inhibition in mice and tumor microvessel density (tumor angiogenesis) evaluation using cyclodextrin inclusion complex of drug was studied in order to explore the ability of the carrier cyclodextrin to enhance the pharmacological efficacy through improved biopharmaceutics of thalidomide.

MATERIALS AND METHODS

Materials

THLD was purchased from Sigma Chemicals Co. (Mumbai, India). SBE7βCD with an average degree of substitution per anhydroglucose unit of 6.5 was generously donated by Cydex Inc. (Overland Park, Kansas). All other reagents and solvents used were of analytical grade.

Phase Solubility Study (Higuchi & Connors, 1965)

An excess amount of drug was added to 10 mL of phosphate buffer, pH 5 and aqueous buffered cyclodextrin solution (0.003–0.015 mol/L) in 15 mL glass test vials with screw caps and shaken at constant room temperature (27°C). After 24 hr, aliquots were withdrawn, filtered (0.22 μm pore size), appropriately diluted and analysed spectrophotometrically at 222 nm (Shimadzu-UV 160A Spectrophotometer). The apparent 1:1 stability constant (K_c) of the drug-CD complex was calculated from the slope and intercept of the phase-solubility diagrams according to the equation K_c = Slope/S_o (1–Slope), where S_o is the intrinsic solubility of the compound in the absence of complexing agent. Each experiment was carried out in triplicate (coefficient of variation [CV] < 3.0%).

Preparation of Drug-Cyclodextrin Solid Systems

Equimolar drug-cyclodextrin solid binary systems were prepared by following methods:

1. Physical mixture (PM) was prepared by gently admixing equimolar amounts of the respective components (80-mesh fractions) in a glass mortor for 2 min.
2. Kneaded (KN) complex was prepared in a 1:1 molar ratio by wetting physical mixture in a glass mortor with the minimum amount of 1:1 organic-aqueous mixture (ethanol-water) and kneading thoroughly with a glass pestle to obtain a paste which was then dried under vacuum at 60°C and stored in a dessicator until further evaluation.
3. Coevaporated (COE) inclusion complex was prepared by evaporation of equimolar drug-CD solution in ethanol-water (1:1 v/v) on a water bath at 60°C after continuous stirring for 2 hr. Each solid product was sieved through 80-mesh and same fraction was used for the following tests.

Differential Scanning Calorimetry (DSC)

The calorimetric measurements of the plain drug as well as 1:1 physical mixture and 1:1 drug-cyclodextrin complexes were made using Shimadzu-Thermal Analyzer DT 40 on 2–8 mg samples. Samples were heated in an open aluminium pans at a rate of 10°C min^{-1} in a 30–300°C temperature range under a nitrogen flow of 40 mL/min.

X-Ray Powder Diffractometry (XRD)

X-ray powder diffraction patterns of thalidomide raw material, the inclusion complexes and the physical mixture were recorded on a Jeol JDX 8030 powder X-ray diffractometer using Ni-filtered, CuKα radiation, a voltage of 40 kV, and a 25 mA current. The scanning rate employed was 1° min^{-1} over the 10–40° diffraction angle (2θ) range.

Stability Assessment

A stock solution of thalidomide containing 1 mg/mL was prepared in acetonitrile. Sixty microliters of stock solution was added to 10 mL phosphate buffer (pH 5 or 8), buffer containing fixed concentration (0.015 mol/L) of SBE7βCD and equilibrated in a thermostatic shaking water bath at room temperature (27°C) and 37°C for 0.5 hr. After equilibration the solution was vortexed briefly, and aliquots (5 mL) of the solution were then withdrawn at predetermined time intervals (15 and 30 min), adjusted to pH 5 and mixed with an equal volume of acetonitrile:water (60:40). Twenty microliters of the resulting solution was then injected onto the HPLC for thalidomide analysis. Gradient elution HPLC analysis of thalidomide employed a Kromasil C_{18} reversed phase column (5 μm, 250 × 4.6 mm I.D.) and a Jasco LC system equipped with a Jasco 975 Intelligent UV detector. The analysis was carried out using mobile phase consisting of acetonitrile:water (60:40) at a flow rate of 1 mL/min.

IN VITRO DISSOLUTION STUDY

USP XXI/XXII Type 2 apparatus was employed to obtain the dissolution profiles of pure drug, 1:1 physical mixture, and cyclodextrin complexes of thalidomide. The dissolution medium consisted of 900 mL 0.2 N HCl thermostated at 37 ± 0.5°C and the paddle speed was 50 rpm. Solid products, each containing 50 mg of drug were subjected to dissolution. At fixed time intervals, samples withdrawn were filtered and concentration of drug was measured spectrophotometrically at 222 nm. Each test was carried out in triplicate. Dissolution efficiency (DE) was calculated from the area under the dissolution curve at time t (measured using the trapezoidal rule) and expressed as percentage of the area of the rectangle described by 100% dissolution in the time (Khan, 1975).

The effect of plain drug, its physical mixture, and inclusion complexes with cyclodextrin on dissolution efficiency was examined using one-way analysis of variance (ANOVA). Individual differences between various systems were then examined using Student's *t*-test.

Cytochrome P450 Microsomal Incubation

Rabbit liver microsomes were prepared and the spectral microsomal cytochrome P450 activity was assessed by the method reported earlier (Omura & Sato, 1964; Walawalkar, Serai, & Iyer, 2006). The spectral microsomal P450 content was estimated to be 1.88 nmole/mg protein. Drug was incubated with 3.0 μM (1.5 mL) of cytochrome P450, 6 mM of NADPH (1.0 mL) in 0.1M phosphate buffer pH 7.4. Incubation was initiated with NADPH and conducted at 37°C for a period of 30 min. After 30 min, the incubations were cooled in ice to 4°C and stored at –20°C until further evaluation.

Tumor Growth Inhibitory Activity in Mice Bearing B16F10 Melanoma

The Institutional Ethics Committee for 'Animal Care and Use' of Advanced Centre for Treatment, Research and Education, Navi Mumbai, India has approved the animal study protocol. The 'Oral Solution Formulation Protocol' for the use of SBE7βCD in experimental animals was also approved by the Cydex Inc.. Female BDF1 mice (18–20 g) received a subcutaneous injection of 10^6 B16F10 melanoma cells into the abdominal region. The cell injection day was considered day zero. The untreated control group was given orally 0.2 mL of phosphate buffer, while the test groups were administered orally with freshly thawed and thoroughly mixed rabbit liver microsomal cytochrome P450 preincubated plain drug or its cyclodextrin complex (200 and 100 mg/kg) every 48 hr beginning on day one and continuing for a total eight doses. Drug solutions were prepared freshly at each time prior to dosing. The day of tumor detection was recorded and tumor dimensions were measured every 48 hr with caliper. Tumor volume (T_{vol}) was calculated in accordance with the equation; $T_{vol} = L \times W^2 \times 0.5$, where L is the maximum length of the tumor and W is the minimum length. At the end of study the mice were sacrificed by cervical dislocation and the tumors were excised and fixed in 10% phosphate buffered formalin until further evaluation. The tumor development study lasted 16 days.

The tumor volume measurement results are expressed as mean ± standard error for three mice in each group. One-way analysis of variance (ANOVA) was used for multiple comparisons followed by Student's *t*-test to find out difference between individual treatments.

Effect of Treatment on Tumor Angiogenesis

In order to evaluate microvessel density (angiogenesis) within the tumor mass, histological sections of 5 μm thick were obtained. Sections were stained with hematoxylin-eosin. The slides were observed independently under low-power microscopy and the angiogenesis response (tumor microvessel density) was scored as 4 (marked), 3 (moderate), 2 (mild), 1 (minimal), and 0 (complete absence).

Effect of different treatments on degree of angiogenesis was examined using Kruskal-Wallis test followed by Tukey's HSD and Dunnett's post hoc test for determining differences between individual treatments. A value of $P < 0.05$ was considered significant.

RESULTS AND DISCUSSION

Solubility Study of Drug-CD Binary System

Most of the pharmaceutically active molecules form 1:1 inclusion complexes with β-cyclodextrins, although complexes of higher order equilibrium with respect to drug and cyclodextrin almost always occur simultaneously. Phase solubility

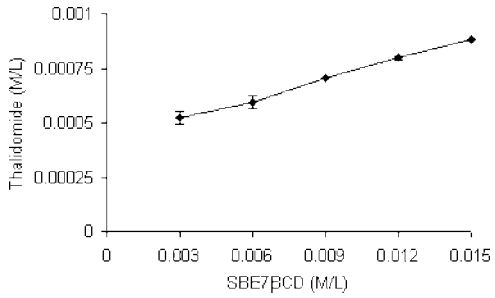

FIGURE 1. Phase solubility diagram of Thalidomide-SBE7βCD system. Each point represents $M \pm SD$ of three separate experiments.

diagram obtained with SBE7βCD, as shown in Figure 1 depicted a linear relationship between the amount of THLD solubilized and the concentration of SBE7βCD in solution. The intrinsic solubility of THLD was found to be 100 µg/mL in phosphate buffer, pH 5 at room temperature. In accordance with the previous results (Krenn et al., 1992), there observed a 2.2 fold increase in the solubility of THLD in presence of HPβCD (≈3% w/v). Since A_L type phase solubility diagram was obtained for THLD-CD system, it was assumed that the solubility increase of THLD is due to formation of 1:1 molar inclusion complex with the stability constant of 86 M^{-1}.

Solid-State Characterization of Drug-CD Binary Systems

The inclusion complexes formed by method of kneading and coevaporation was characterized qualitatively in the solid-state using DSC and XRD (Mura, Faucci, Parrini, Furlanetto, & Pizauti, 1999). DSC thermograms of plain THLD and of various solid binary systems with SBE7βCD are shown in Figure 2. DSC thermogram of plain THLD exhibited a sharp endothermic peak corresponding to its melting point (T_{onset} = 273.7°C, T_{peak} = 277.8°C). The characteristic thermal profile of the drug appeared to lower temperature but was still well

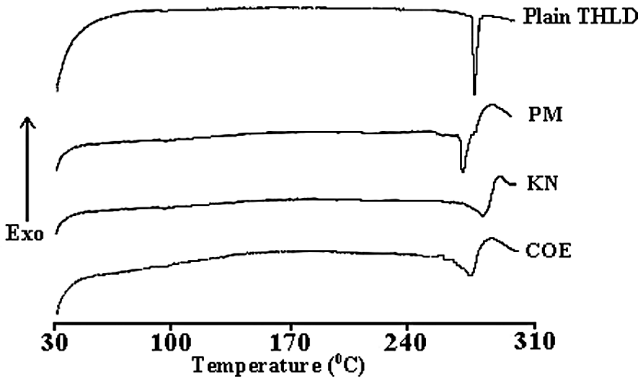

FIGURE 2. DSC thermograms of plain Thalidomide (plain THLD) and Equimolar drug-CD physical mixtures (PM), kneaded (KN) and coevaporated (COE) products with SBE7βCD.

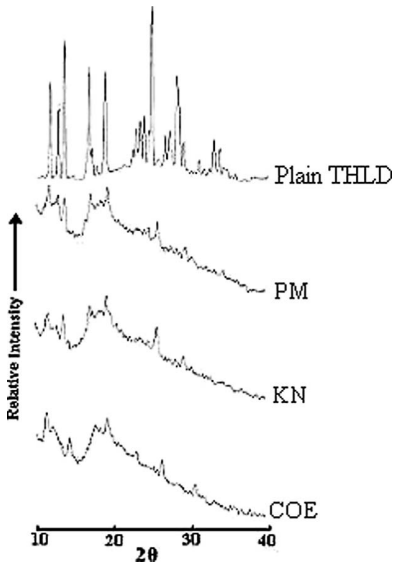

FIGURE 3. X-ray diffractograms of plain Thalidomide (plain THLD) and Equimolar drug-CD physical mixtures (PM), kneaded (KN) and coevaporated (COE) products with SBE7βCD.

recognizable in the physical mixture with SBE7βCD. The DSC thermogram of the inclusion complex, when compared to those of the plain THLD and its physical mixture with SBE7βCD, lacked the characteristic endothermic peak attributed to the melting of THLD. This phenomenon is generally considered as indicative of drug amorphization/complex formation due to the stronger interaction in the solid state between guest and cyclodextrin (Kim, Frank, & Henderson, 1985).

X-ray diffraction patterns of plain THLD and of the drug-cyclodextrin systems are shown in Figure 3. In the X-ray diffractogram of plain THLD sharp peaks were present and it suggests that the drug was present as a crystalline material. Diffraction peaks relevant to crystalline THLD were detectable in the physical mixture with SBE7βCD as few broad peaks of low intensity which emerged on the diffused background due to the amorphous carrier, indicating a partial loss of crystallinity of THLD. The X-ray diffraction patterns of inclusion complexes showed the formation of a new amorphous phase indicated by the absence of characteristic crystallinity peaks present in the plain THLD diffraction pattern. Thus, the results of solid-state characterization of various solid THLD-CD binary systems, confirms the formation of a new solid amorphous phase.

Chemical Stability Study

As demonstrated by the phase solubility study, thalidomide was chemically stable in aqueous media at pH 5. Thalidomide undergoes rapid spontaneous hydrolysis in aqueous solutions at a pH of 6 or above to form three primary products and eight minor products as shown in Figure 5 and the rate of hydrolytic

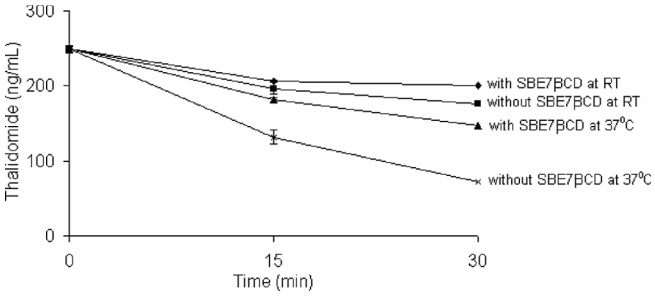

FIGURE 4. Observed hydrolytic degradation pattern of Thalidomide with and without SBE7βCD as a function of time in phosphate buffer (pH 8) at room temperature (RT) and 37°C.

degradation are highly dependent on temperature (Krenn et al., 1992; Simmons, Lush, & Figg, 1997). Likewise, the parent compound is degraded rapidly in an in vivo system by spontaneous hydrolysis (assuming a pH greater than 6). Less than 15% of thalidomide is present at 24 hr following an oral dose with terminal half-life ≈8 hr (Chen, Vogelsang, Petty, et al., 1989). As depicted in Figure 4, in addition to improving the aqueous solubility of thalidomide, complexation with SBE7βCD is highly effective in enhancing the stability of the drug. The THLD-SBE7βCD solution was more stable with ≈78% and ≈60% of the parent drug detected after 30 min at room temperature and at 37°C, respectively. In contrast, < 25% of the drug was intact in aqueous alkaline solution (pH 8) at the same time point at 37°C in the absence of SBE7βCD. These results are in well agreement with the data reported earlier where experiment was performed on saturated thalidomide-HPβCD solution in pH 7.4 phosphate buffer (0.1 M) and found that drug was stable, with ≈75% of the parent compound detected after 8 hr and less that 10% of the free drug was intact at the same time point (Krenn et al., 1992).

Although the effects of enzymatic or metabolic degradation were not considered, this experiment approximated hydrolytic degradation as would occur in vivo. From the HPLC data it is evident that the presence of SBE7βCD substantially decelerated the hydrolysis of the THLD under alkaline condition. Two major primary hydrolytic products of thalidomide are the result

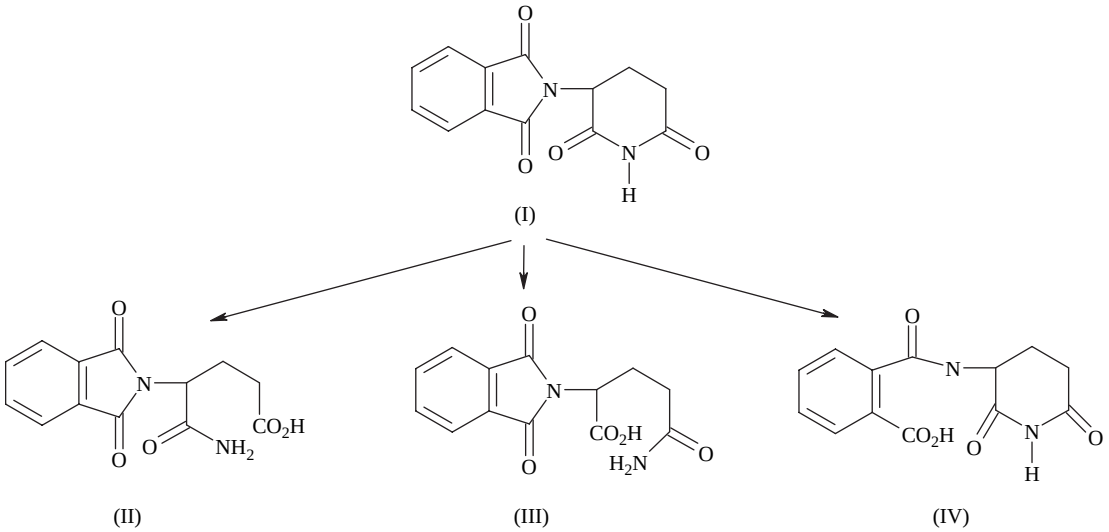

FIGURE 5. Thalidomide and its primary products after hydrolysis in aqueous alkaline solution. Key: (I) Thalidomide; (II) 4-Phthalimidoglutaramic Acid; (III) 2- Phthalimidoglutaramic Acid; (IV) α-(*O*-Carboxybenzamido) Glutarimide.

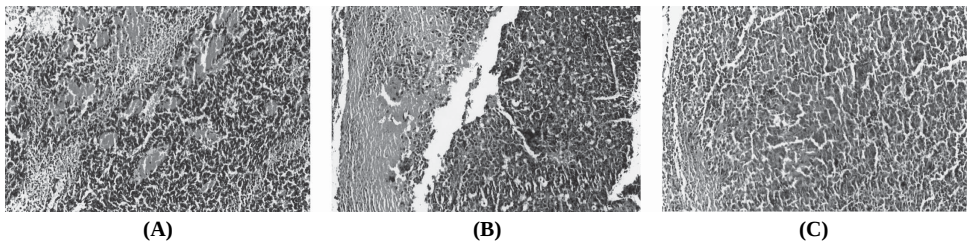

FIGURE 6. Microvessel density in tumor sections after Hematoxylin-Eosin staining. at day 16, in control tumors (A); Dense microvasculature (light grey patches) appeared through out the tumor section whereas; tumor microvessel density was significantly reduced in tumors treated with plain THLD (B); and THLD-SBE7βCD complex (C).

of hydrolytic attack on dioxo-piperidinyl ring of thalidomide In accordance with the initial observation of the structure of thalidomide suggesting that dioxo-piperidinyl moiety would be expected to be included within the cyclodextrin cavity due to its hydrophobicity. Therefore, decreased hydrolytic cleavage of thalidomide in presence of SBE7βCD can be considered the effect of inclusion of dioxo-piperidinyl moiety of thalidomide into the apolar cavity of SBE7βCD protecting it from hydrolysis.

Dissolution Study of Drug-CD Solid Binary Systems

The dissolution parameters of thalidomide from various binary systems with amorphous βCD derivative are tabulated in Table 1 as mean ± *SD* of three independent measurements. As can be noted, complexation allowed a significant improvement of dissolution rate of drug. The improvement of dissolution rate obtained with physical mixtures can be attributed to both, improved drug wettability due to presence of hydrophilic cyclodextrin that can reduce the interfacial tension between poorly soluble drug and dissolution medium, and formation of readily soluble complexes in dissolution medium (Corrigan & Stanley, 1982). As for the other methods of complexation, kneading found to be better in achieving the higher drug dissolution where ≈50% of drug was dissolved in first 5 min (DP_5).

The dissolution efficiency of kneaded product was 1.8 times higher than that of the corresponding physical mixture and about 2 times higher than that of plain drug as evident from Table 1. A statistically significant difference was found for DP_5 (% drug dissolved at 5 min) and DE_{60} (dissolution efficiency over a period of 60 min) between plain drug, its physical mixture, and cyclodextrin complexes ($P < 0.05$) whereas the individual difference between plain drug and its physical mixture with SBE7βCD was statistically insignificant ($P > 0.05$). The better performance of the cyclodextrin complexes may be attributed to drug amorphization due to its deeper interaction with SBE7βCD as confirmed by DSC and X-ray diffraction analysis. The effectiveness of βCD derivative can also be explained on the basis of its greater hydrosolubility, higher wetting, greater solubilizing, and complexing ability towards THLD in accordance with the results of phase solubility study.

TABLE 1
In Vitro Dissolution Parameters of Thalidomide Alone and Its Equimolar Physical Mixture (PM), Kneaded (KN), and Coevaporated (COE) Complex with SBE7βCD

Sample		DP_5	DE_{60}
Plain THLD	–	6.15 ± 2.86	42.48 ± 5.23
THLD-SBE7βCD	PM	14.9 ± 1.75	46.8 ± 2.40
	KN	73.8 ± 10.95	88.91 ± 4.14
	COE	59.1 ± 2.56	76.9 ± 2.19

Data are represented as $M \pm SD$ ($n = 3$).

Tumor Growth Inhibition with THLD and its CD Binary System

Thalidomide has been shown to be antiangiogenic in a rabbit cornea micropocket model but failed to demonstrate this activity in other models (Gutman, Szold, Ravid, et al., 1996). These results suggest that the antiangiogenic effects of thalidomide may only be observed following metabolic activation of the compound and this activation process may be species specific. In an in vitro model of angiogenesis involving use of thalidomide preincubated with either human, rabbit and rat liver microsomes, thalidomide demonstrated inhibition of microvessel formation in the presence of human and rabbit microsomes, but not with rat microsomes. Thus, metabolite of thalidomide is responsible for its antiangiogenesis effects and this metabolite can be formed in both humans and rabbits, but not in rodents (Bauer, Dixon, & Figg, 1998). Therefore, in our present investigation we have used thalidomide preincubated with rabbit liver micrsomes for its metabolic activation before ingesting to the rodents.

From the results it is evident that when B16F10 melanoma cells injected subcutaneously into mice, grew to an average size of 450 mm^3 as tabulated in Table 2. From the tumor development pattern it is evident that the large increases in tumor volume between time points was observed among the four treatment groups. At the end of study, both drug treatments

TABLE 2
Results of the Tumor Development Study Wherein BDF1 Mice Received THLD, Its Equivalent of SBE7βCD Complex or Neither

	Untreated Control	Plain THLD (mg/kg)		THLD-SBE7βCD (mg/kg Equivalent of Plain THLD)	
		200	100	200	100
Mean tumor detection day	3.7 ± 0.34	4.7 ± 0.34	4.0 ± 0.58	6.7 ± 0.33	4.66 ± 0.34
Mean tumor volume (mm^3) at death	459.32 ±16.83	31.31 ± 3.0	45.44 ± 4.1	13.88 ± 1.3	38.17 ± 2.01

Data are represented as $M \pm SE$ ($n = 3$).

resulted in significant inhibition of tumor growth as compared to control ($P < 0.05$). When compared to the untreated control and plain drug treatment, the administration of THLD-SBE7βCD complex produced a significant delay in tumor formation in experimental animals. In all the animals receiving THLD-SBE7βCD complex (eqv. to 200 mg/kg of THLD), tumors were detectable only on day 7 in contrast to animals in control as well as plain drug treatment groups. The significantly delayed tumor growth ($P < 0.05$) of THLD-SBE7βCD (eqv. to 200 mg/kg free THLD) treated group versus untreated control and free drug treatment at the same dose level caused the tumor growth in this group to be offset from that of the untreated control and plain drug treatment by about 4 days.

The animal experiments using two different dose levels were performed to investigate if the anti-tumorigenic efficacy of THLD-SBE7βCD system was significant at low equivalent dose of drug. After oral administration, THLD-SBE7βCD kneaded product at the low equivalent dose (100 mg/kg of plain THLD) reduced tumor growth and the effect obtained with the lower equivalent dose of drug in presence of cyclodextrin was not significantly different to that observed with higher dose (200 mg/kg) of plain THLD ($P > 0.05$). Thus a reduction of drug oral dose is feasible if cyclodextrin carriers are used in the formulation. This observation indicates the faster in vivo complex dissociation and instantaneous availability of drug at the absorption site that causes faster and complete drug absorption from the inclusion complex. Consequently, cyclodextrin potentially serve as a drug reservoir, replenishing the free drug concentration at a membrane surface by rapid dissociation as equilibrium is disturbed following drug absorption. The driving concentration for drug absorption can thus be maintained, as the formulation/drug is retained at the membrane surface in its solubilized state in presence of cyclodextrin. Thus in accordance with the reported data, it could be assumed that the effect of cyclodextrin on the higher anti-tumorigenic efficacy of thalidomide could be due atleast in part to an increase in cellular drug incorporation (Grosse, Francoise, & Pinguet, 1997).

Anti-Angiogenic Efficacy of THLD and its SBE7βCD Complex

Representative photomicrographs of hematoxylin-eosin stained tumors as presented in Figure 6 showed that a dense vascularization was observed in the control tumors. Tumors treated with plain THLD and THLD-SBE7βCD complex had significantly fewer microvessels compared with the control ($\alpha = 0.05$; Kruskal-Wallis test & Dunnett's test) as shown in Table 3. Among the various molecular players involved in different mechanisms of vascular growth, members of the VEGF family have a predominant role. By inhibiting VEGF and other angiogenic growth factors including bFGF, tumor growth and metastasis can be blocked (Tayade, Saraf, & Kale, 2003). Since thalidomide has ability to inhibit VEGF and bFGF induced angiogenesis; the anti-tumor effect by thalidomide could be ascribed to the inhibition of pro-angiogenic vascular growth factors.

TABLE 3
Effect of THLD with and without SBE7βCD on Tumor Angiogenesis

Groups	Degree of Angiogenesis
Untreated control	4, 3, 4
Plain THLD (200 mg/kg)	3, 1, 2
THLD-SBE7βCD complex (Eqv. to 200 mg/kg plain THLD)	1, 1, 2

However, the microvessel density difference between THLD and SBE7βCD combination treatment was not significant at the end of study ($\alpha = 0.05$; Tukey's HSD test). It is well known that without blood vessels tumours cannot grow beyond a critical size or metastasize to another organ. Thus, if angiogenesis is inhibited at the initial stages of tumor development by the administration of molecules that specifically suppress the growth of vascular endothelial cells, tumours in animals can be limited to a dormant size at which they are essentially harmless (Tayade et al., 2003). On the similar lines, in the present investigation the significant difference in the tumor microvessel density of two different drug treatment groups must have been evident at the initial stages of treatment instead at the end of study. This could be, then attributed to the high drug accumulation inside the tumor because of high drug availability at the absorption site and improved cell permeation of the drug as a consequence of solubilization by cyclodextrin.

CONCLUSION

SBE7βCD showed better solubilizing and stabilizing effect toward THLD. After peroral administration of the THLD-SBE7βCD binary system, the THLD was found to be rapidly released from the complex with improved potential in maintaining/enhancing therapeutic effects of THLD. Reduction of the doses of common antitumoral agents so as to decrease unwanted side effects without compromising efficacy, in combination with new less toxic molecules such as SBE7βCD, could offer new perspectives in cancer treatment. Thus, SBE7βCD is useful in improving solubility, stability, and therefore the bioavailability of THLD in pharmaceutical formulation.

ACKNOWLEDGMENTS

The author thanks members of Anti-cancer Drug Screening Facility, ACTREC for help in carring out in vivo experiments. Financial support from University Grants Commission (UGC), Government of India, New Delhi is gratefully acknowledged.

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