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ORIGINAL ARTICLE

Effect of cyclodextrins on lonidamine release and in-vitro cytotoxicity

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Abstract The objective of this work was to study the effect of natural, hydroxypropyl and partially methylated cyclodextrins on the solubility of lonidamine and the cytotoxicity of the inclusion complex. The solubility study was conducted according to Higuchi et al. where HP-α-CD, HP-β-CD and HP-γ-CD showed type A_L solubility curves indicating the formation of inclusion complexes with molar ratio of 1:1 with lonidamine. However, A_P and A_N type phase solubility behaviors were observed with PM-CD and α-CD, β-CD and γ-CD, respectively. Solubility enhancement factors and stability constants ($K_{1:1}$ and $K_{1:2}$) were calculated from the phase diagrams as appropriate. The characterization of the above inclusion complexes was evaluated using differential scanning calorimetry, Fourier transform infrared, and dissolution. Lonidamine solubility was improved with all tested cyclodextrins and specifically with PM-β-CD (1148 mg/L) where the solubility increased by 380-fold compare to its solubility in water (3 mg/L) leading to a fast dissolution rate of the latter in less than 3 min. In addition, the in-vitro cytotoxicity studies showed a modest increase in the cytotoxicity of lonidamine complex with CD against the human glioblastoma cell line SNB-19. Cyclodextrins could be useful to improve the solubility of lonidamine and hence its bioavailability, which is a drug of interest in the cancer treatment.

Keywords Lonidamine · Cyclodextrins · Solubility · Cytotoxicity · Characterization

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Introduction

Lonidamine (LDN), an indazol-carboxylic acid derivative (Fig. 1), is an anticancer drug which exerts its effect by suppressing glycolysis in cancer cells through the inhibition of the mitochondrially-bound hexokinase and the electron transport chain.

In addition, Lonidamine inhibits the metabolism energy of neoplastic cells and increases the permeability of the cell membrane [1]. Lonidamine altered the cell plasma and mitochondrial membrane, resulting in inhibition of cellular respiration and depletion of cellular adenosine triphosphate (ATP); impeded DNA repair; and induced cellular acidification. It has been shown that the modification of the intracellular pH (pHi) markedly altered the subcellular distribution of hexokinase in glioma cells [2]. Treatment of xenografted human gliomas with lonidamine slowed the tumor growth rate in correlation with their mitochondria-bound hexokinase activity [3]. In-vitro studies have demonstrated that lonidamine could potentiate the oncolytic activity of cytotoxic drugs such as cisplatin, epirubicin, doxorubicin, mitomycin C and cyclophosphamide with the ability to reverse the acquired multidrug resistance of neoplastic cells [4]. Also, it induced apoptosis in-vitro in adriamycin and nitrosourea-resistant cells [5]. In vivo, the toxicity of lonidamine is highly noticeable after intravenous

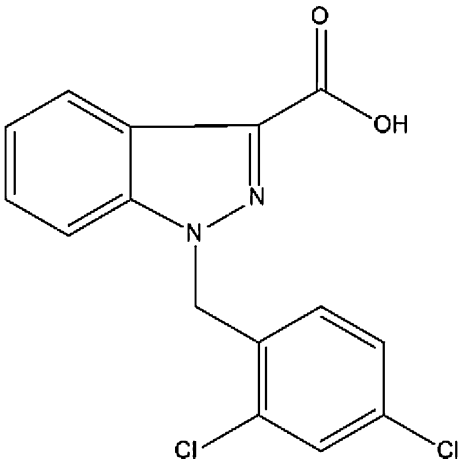

Fig. 1 Lonidamine chemical structure

injection than oral administration indicating a low oral bioavailability [6]. Apart from being a chemically labile drug, its poor aqueous solubility and dissolution rate negatively impacts its bioavailability [7].

The interaction of guest molecules with cyclodextrins might induce useful modifications of the chemical and physical properties of the guest molecule leading to an improvement of the stability, solubility in aqueous media and hence the bioavailability of such drugs [8]. Cyclodextrin (CD) inclusion complexation has been used by pharmaceutical formulators to improve the physico-chemical properties of many drug substances, and based on this fact, the solubilising potential and complexing tendencies of selected cyclodextrins with lonidamine in aqueous solution were evaluated using phase solubility methods [9]. This work reported the preparation of a lonidamine-cyclodextrin complex, using different methods. Selective physico-chemical properties of the inclusion complexes were determined using FTIR spectroscopy, differential scanning calorimetry (DSC), and dissolution along with the in-vitro cytotoxicity against the human glioblastoma cell line SNB-19 was evaluated.

Experiments

Materials

α-Cyclodextrin (α-CD), β-cyclodextrin (β-CD), γ-cyclodextrin (γ-CD), derivate cyclodextrins hydroxypropyl alpha, beta, gamma cyclodextrins and permethyl beta cyclodextrin (HPα-CD, HPβ-CD, HPγ-CD, PMβ-CD) were supplied by Wacker, Munnchen, Germany. Lonidamine was provided by A.C.R.F., Ancona, Italy.

All other ingredients and solvents were of analytical grade.

Methods

Phase solubility studies

Phase solubility studies were carried out according to the method described by Higuchi and Connors [10]. Briefly, suspensions were shaken in a steam room at 37 °C for 7 days and supernatants were filtered using 0.45 μm filter membrane and injected into HPLC system by which the concentration of lonidamine (LND) was determined.

Quantification of lonidamine by HPLC

The HPLC system was equipped with a Jasco 950 pump, Merck multichannel photo detector L3000 and a Cromasil® Column C18, 5 μm. The HPLC method consisted of mobile phase of 50% Acetonitrile/buffer (0.1 M Acetate Buffer at pH 3.50 adjusted with sodium hydroxide), a wavelength set at 300 nm, and injection volume of 20 μL.

Physical mixtures preparation and inclusion complex preparation

Fine powdered for physical mixtures of LND and β-CD or HP-β-CD or PM-β-CD with molar ratio of 1:1 were prepared by blending in a mortar for 5 min. Inclusion complex powders were prepared by mixing a mixture of LND and β-CD or HP-β-CD or PM-β-CD, with molar ratio of 1:1 or 2:1, dissolved and stirred for 24 h in a tris-(hydroxymethyl)-amino methane–glycine buffer (TAM-Gly). The solutions were evaporated under vacuum using a rotary evaporator, frozen and then lyophilized for 30 h under the following parameters: vacuum <200 mTorr, condenser <–40 °C, shelf at +30 °C. The lyophilized samples were stored in desiccators until further analysis.

Fourier Transform Infrared Spectroscopy

Infrared (IR) spectroscopy was carried out using a Perkin Elmer Spectrum™ One Fourier-Transform Infrared (FTIR) Spectrophotometer. Sample was used as it is. Scans were run from 4000 to 650 cm^{-1} a resolution spectral of 8 $cm^{-1,}$ 0.2 cm–1 scanning speed and 16 scans.

Differential scanning calorimetry

The inclusion complexes of lonidamine and cyclodextrin were subjected to DSC using a differential scanning calorimetry (Perkin-Elmer 6 DSC, USA). Samples (5 mg) were hermetically sealed in a flat-bottomed aluminum pan and heated from 30 to 260 °C at flow rate of 8 °C/min and Nitrogen flow set at 30 ml/min against an empty aluminum pan as a reference.

Dissolution studies

Drug release was evaluated by dissolution studies using apparatus #2, Varian Instrument. Based on the sink conditions for lonidamine, a 50 mg (powder, $n = 6$) of LND or inclusion complex of LND-PMβ-CD (LND/PM-β-Cd 1:1 physical mixture, LND/PM-β-CD 1:1 rotator evaporator, LND/PM-β-CD 1:1 freeze dry and LND/PM-β-CD 2:1 rotator evaporator) was placed into a vessel containing one liter of purified water set at temperature of 37 ± 0.5 °C and stirring speed of 100 rpm. A define sample volume was sampled at 3, 5, 15 and 30 min and filtered using 0.45 μm nylon membrane prior injection into HPLC by which LND content was determined.

In-vitro cytotoxicity assay

The cytotoxicity of free LND and its complex with PM-β-CD, prepared under vacuum, were evaluated against the human glioblastoma cell line SNB-19. On day 1, the cells were incubated in DMEM media, which was supplemented with 10% fetal calf serum, 2 mM of L-Gln and 1% Pen/strep in a 96-well-plate (2, 5 × 10^3/well). On day 2, the cells were washed and incubated at 37 °C in 5% CO_2 with different concentration (e.g. 1, 10, 100, 1000 μM) of free LND, PM-β-CD and inclusion complex of LND-PM-β-CD. Stock solutions (10000 μM) for all compounds were prepared with a TRIS 0, 5 M/Glycine 1, 5 M buffer, and then diluting with the culture media to obtain the desired concentration. On day 3, a fresh DMEM media was added into the well-plate of the cells. On day 4, J4, a mix of Methanol/Methylene blue was used to fix viable cells and after rinsing then adding HCl, the percent of survival cells was determined spectrophotometrically.

Results and discussions

Lonidamine solubility was improved with all tested cyclodextrins and the highest observed value was obtained

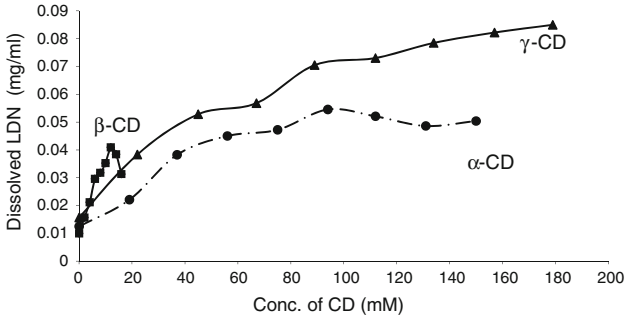

Fig. 2 Phase solubility diagram of *LDN/α-CD, β-CD* and *γ-CD* system in water at 37 °C

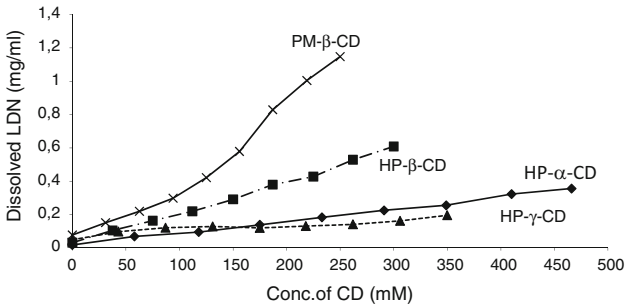

Fig. 3 Phase solubility diagram of *LDN/HPα-CD, HPβ-CD, HPγ-CD* and *PMβ-CD* system in water at 37 °C

with PM-β-CD (1148 mg/L) which increased by 380 fold compare to its solubility in water (3 mg/L) (Figs. 2, 3) and the data are tabulated in Table 1. Fig. 4.

Native Cyclodextrins were less effective to increase the solubility of LND, due to their limited solubility. Nevertheless, β-CD had the best apparent binding constant and was chosen with its derivatives HP-β-CD and PM-β-CD to characterize the inclusion complex of LND and CD. Figure 5 illustrates a thermogram of freeze-dried and rotavapored complexes of LND-PM-β-CD at two molar ratios of 1:1 and 2:1 where the peak of lonidamine disappeared and the appearance of new peak at different melting point temperature of pure lonidamine indicating the presence of

Table 1 LND/CDs complexes: apparent solubility and apparent binding constant

	LND	α-CD	β-CD	γ-CD	HP-α-CD	HP-β-CD	HP-γ-CD	PM-β-CD
Max concentration CD (mM)	0	150	16	179	466	300	350	250
LND solubility max. (mg/L)	3	59,4	44,3	84,9	356	608,4	195,3	1148
LND solubility max. (mM)	0,93 × 10^{-2}	18,5 × 10^{-2}	13,8 × 10^{-2}	26,4 × 10^{-2}	110,8 × 10^{-2}	189,4 × 10^{-2}	60,8 × 10^{-2}	357,5 × 10^{-2}
Solubility factor (Sf)		19,80	14,77	28,30	118,67	202,80	65,10	382,67
Apparent binding constant Kc $(M)^{-1}$		4 × 10^{-3}	2.3 × 10^{-4}	5.15 × 10^{-3}	6,3 × 10^{-3}	1.72 × 10^{-4}	5.44 × 10^{-3}	2.89 × 10^{-4}

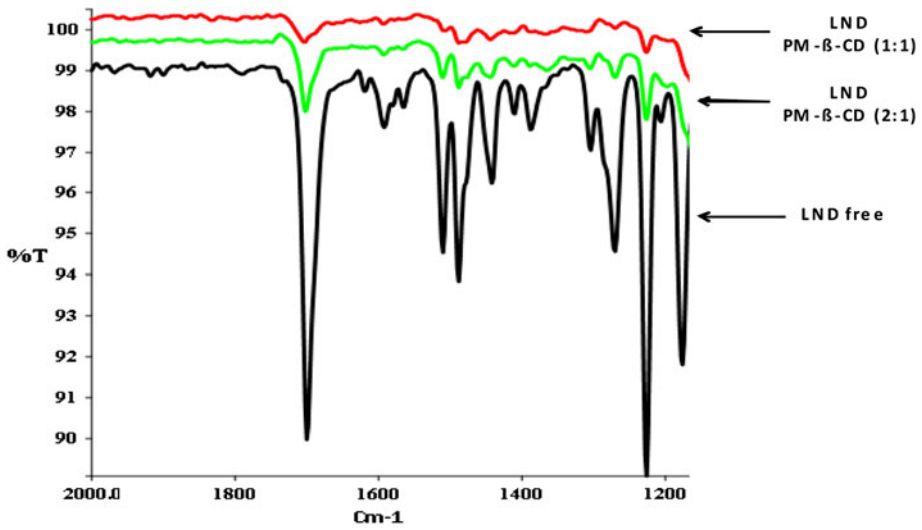

Fig. 4 FTIR with *PM-β-CD* freeze-dried complexes

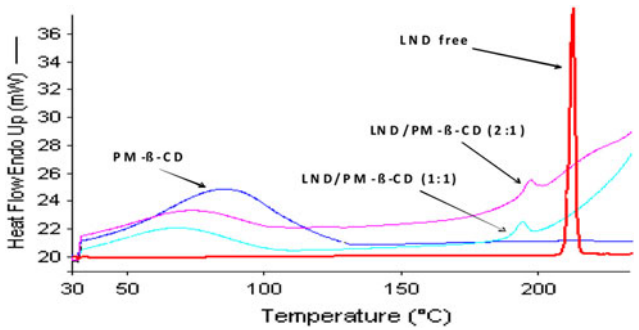

Fig. 5 DSC with freeze-dried *PM-β-CD* complexes

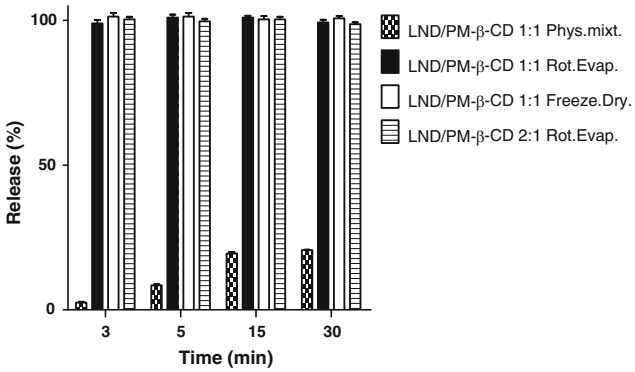

Fig. 6 Dissolution of *LND/PM-β-CD* complexes

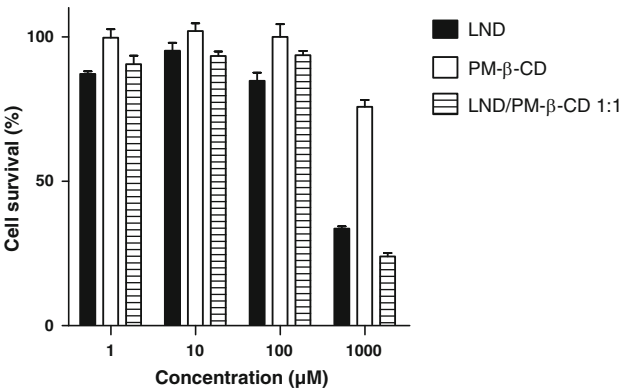

Fig. 7 In-vitro cytotoxicity assay

an amorphous form. This data suggest the formation of either an inclusion complex between LND and PM-*β*-CD or lost of crystallinity of lonidamine.

FTIR spectra showed the disappearance of peaks between 1500 and 1600 cm^{-1} with freeze-dried (Fig. 2) and rotavapored complexes with PM-*β*-CD versus free LND, probably due the entrapment of the aromatic group in the cyclodextrin cavity. DSC spectra with physical mixtures with PM-*β*-CD showed no difference with free LND, indicating no complex formation (I did not understand this statement). The same results of DSC and FTIR, not shown in this paper, were obtained with *β*-CD and HP-*β*-CD than with PM-*β*-CD.

Dissolution study showed a flash release of lonidamine in less than 3 min (Q ~ 100%), with freeze-dried and rotavapored LND/PM-*β*-CD complexes, which were not observed with physical mixtures (Fig. 6). The same results were obtained with HP-*β*-CD. It is clearly obvious that the formation of the inclusion complex between LND-PM-*β*-CD and LND-HP-*β*-CD seemed to increase the release rate of lonidamine and such system could be useful for the oral administration.

The in-vitro cytotoxicity of LND against the human glioblastoma cell line SNB-19 was slightly increased with 1:1 freeze-dried LND/PM-*β*-CD complex versus free LND (Fig. 7), indicating such inclusion complex could also be useful to improve the LND efficiency in cancer treatment.

Conclusion

LND and all cyclodextrins used could form an inclusion complex which was supported by FTIR and DSC. Solubility was increased with all cyclodextrins, especially PM-β-CD, allowing a very fast dissolution in less than 3 min. Furthermore, in-vitro cytotoxicity against the human glioblastoma cell line SNB-19 was improved with PM-β-CD. Cyclodextrins could be useful to improve the solubility of lonidamine and hence its bioavailability, which is a drug of interest in the cancer treatment and more recently like a male contraceptive.

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