J Incl Phenom Macrocycl Chem (2009) 65:431–435
DOI 10.1007/s10847-009-9594-x

Water soluble 5 FC complexes, preliminary pharmacological studies

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Received: 14 November 2008 / Accepted: 12 May 2009 / Published online: 30 May 2009

Abstract Flucytosine-β-cyclodextrin and hydroxypropyl β-cyclodextrin water soluble inclusion complex were prepared by freeze drying method in distilled water. The formation of inclusion complex between β-cyclodextrins and flucytosine has been studied and fully described in our previous work [1]. In this paper we are describing the results obtained concerning the antifungal activity of this new compounds. As expected the new inclusion complexes presents a semnificative increase of the antifungal activity, illustrated by the reduction of the minimal inhibitory concentrations for 50 and 90% of the tested strains decreased. Also, the acute toxicity of the flucytosine β cyclodextrin and hydroxypropyl β cyclodextrin complex is smaller comparing with the pure drug, analyzed alone. These results recommend the described conjugates as future promising therapeutic agents.

Keywords Flucytosine · Antifungal activity · MIC 50 and MIC 90

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Introduction

In the last decades, because the range of clinically important fungi has broadened, and the number of immunosuppressed patients increased the prevalence of resistance to antifungal agents is increasing [2]. In this context the interest in developing new antifungal agents or reducing the dosage, with the resistance reducing has continuously increased.

Cyclodextrins (CDs) and their derivatives are well known host molecules, able to form inclusion complexes rather nonspecifically with a wide variety of guest molecules. Complexation process with native or modified CDs increases guest solubility and stability against the effects of light, heat, and oxidation. The most common application of CDs in the pharmaceutical industry is to enhance the solubility, the dissolution rate, and the bioavailability of poorly water-soluble drugs [3]. That is why a large variety of drugs encapsulated through noncovalent interactions into unmodified or modified cavity are described in the literature [4–6].

Flucytosine (5-FC), a fluorinated analogue of cytosine, the oldest synthetic antifungal agents, received a special attention in the last years, because of increasingly usage in combination with a number of antifungal agents for lethal invasive mycosis treatment and alone as a possible new therapeutic for colorectal carcinoma [7]. 5-FC usage is limited by its major side effects including hepatotoxicity, causing severe liver necrosis [8] and bone-marrow depression inducing life-threatening leucicytopenia, thrombocytopenia, and pancytopenia [9].

Complexation of 5-FC with cyclodextrin offers the possibility to improve the aqueous solubility of 5-FC, without modifying the drug original structure, increasing 5-FC bioavailability, and reducing its toxicity [1, 5, 6].

We synthesized cyclodextrin-5-FC inclusion complexes, in order to increase the bioavailability of 5-FC, with the consequent reduction of the dosage, the treatment period and the gravity of all possible side effects [1]. These new complexes were tested from antifungal activity efficiency point of view, in order to evaluate their potential applications as therapeutic agents.

Materials and methods

Materials

Flucytosine (5 FC) (Fluka), β-cyclodextrin (β-CD) and hydroxypropyl β-cyclodextrin (HP β-CD) (Aldrich) were used as received. Double distilled water was used throughout the study. RPMI 1640 medium (Sigma) buffered to a pH of 7.0 with 0.165 M morpholinepropanesulfonic acid (MOPS) buffer (Sigma) and stored at −80 °C until use. Antifungals was solubilized in dimethyl sulfoxide (DMSO) (Sigma). RPMI 1640 Medium was developed at Roswell Park Memorial Institute in 1966 by Moore and his co-workers and contains the carbon and nitrogen source, all the mineral salts in order to support a wide variety of cells that are anchorage dependent. Originally intended to be used with a serum supplement, RPMI 1640 has been shown to support several cell lines in the absence of serum. It has also been widely used in fusion protocols and in the growth of hybrid cells. 0.5 McFarland standard was prepared by mixing 0.05 mL of 1.175% barium chloride dihydrate ($BaCl_2 \cdot 2H_2O$), with 9.95 mL of 1% sulfuric acid (H_2SO_4) and its turbidity can be compared visually to a suspension of bacteria in sterile saline or nutrient broth.

The solutions were prepared in DMSO, due to the reduced solubility of 5 FC that can affect the final results.

Methods

Preparation of the solid complex

The inclusion complexes (C) were prepared by freeze drying method. An aqueous solution containing 5 FC and β-CD, or HP β-CD in a 1:1 molar ratio was frozen by immersion in liquid nitrogen and freeze-dried in a Martin Christ, ALPHA 1-2LD Freeze-Dryer. The aqueous solution was obtained by dissolving 7.74×10^{-4} mol 5 FC and 7.74×10^{-4} mol cyclodextrin in 25 mL distilled water and stirring it at room temperature for 48 h.

Pharmacological studies

Antifungal activity studies Antifungal activity studies were performed on 32 yeast strains belonging to *Candida* genus, isolated from fungemia episodes from patients hospitalized in Iasi, using the testing method M27-A2 recommended by CLSI (NCCLS) to evaluate the antifungal susceptibility of the yeasts [10].

The yeast strain subcultures were obtained by incubating the initial strains at 35 °C for 48 h. Yeast strain inocula were obtained using stock cultures, by the suspension of five colonies of approximately 1 mm in diameter in saline solution. Pure 5 FC, β-CD-5 FC and HP β-CD-5 FC inclusion complexes solutions (3.2 (pure 5 FC) and (3.2 (complexed 5 FC) μmol/mL, containing 3.2 μmol active substance) in DMSO were diluted to the final concentrations (32; 16; 8; 4; 2; 1; 0.5; 0.125; and 0.0625 μg/mL) in DMSO and each solution was inoculated on the yeast strain inoculum. Suspension density adjustment was performed spectrophotometrically, so that the absorbance of each suspension to correspond to that produced by McFarland standard 0.5 at 530 nm. The final density of the suspensions varied from 1×10^6 to 2.5×10^6. These suspensions were diluted 1:100 with sterile saline solution and 1:20 in RPMI 1640-MOPS, so that the final cell density to vary from 0.5×10^3 to 2.5×10^3. The witness suspension was obtain by adding 900 μL yeast inoculum to 100 μL RPMI 1640-MOPS DMSO 1:10 dilution. The growth was evaluated by comparing the turbidity from each vial containing the antifungal agent to the turbidity of a 1:4 dilution in RPMI of the witness. Minimal inhibitory concentrations (MIC) were calculated as the minimal antifungal agent concentration that caused 50 and 90% (MIC90 and MIC50) growth inhibition. As already described in the literature the CDs have no antifungal effect [3].

The best results were obtained on *C. Krusei* and C. *norvegensis*, isolated from fungemia episodes obtained from Cantacuzino Institute, Bucharest.

Acute toxicity studies Acute toxicity studies were performed on laboratory mice, on nulliparous and nonpregnant healthy young females with age between 8 and 12 weeks old, and weight around 20 ± 0.2 g. The animals were housed individually, respecting the same microclimate (temperature around 22 ± 3 °C, the relative humidity 55% and an alternation 12 h artificial light, 12 h darkness) and feeding conditions. The animals were randomly selected, marked to permit individual identification, and kept in their cages for at least 5 days prior to dosing, in order to allow the acclimatization to the laboratory conditions. The administration of the pure and complexed 5 FC was made in a constant volume of 2 mg/100 g body weight in aqueous solutions [11]. The dose volume was administered by gavage using a stomach tube. The experiments were performed on Wistar SD1 NRM1 White/C57Bi6 mice, offered by the Cantacuzino Institute, Bucharest, on groups composed of 20 animals. All the results were reported to a control group.

Table 1 The frequency of MIC of 5 FC for the 32 tested strains

	Concentration of 5 FC (μg/mL)							
	0.5	1	2	4	8	16	32	64
Absolute frequency (*n*)	19	5	2	0	3	1	1	1
Cumulative frequency (%)	59.37	75.00	81.25	81.25	90.62	93.75	96.87	100

The acute toxicity (LD50) was established using the Dixon and Mood method [12, 13].

Results and discussions

As already described in a previous paper, the inclusion complex obtained by freeze drying between 5 FC and β-CD or HP β-CD were characterized and it was proved their formation. The authors observed an improving of the dissolution rate of the pure drug after complexation, making the new obtained compound a proper candidate for therapy [1]. All the results can be summarized in Tables 1 and 2 and Fig. 1.

In vitro antifungal activity studies

The data presented in Table 1 indicate that 5 FC is efficient in antifungal treatments, the growth inhibition is 100% for different Candida strains tested in our experiment The determined sensibility rate was 93.73% for all analyzed stains, the best results are obtained for two strains

Table 2 In vitro susceptibility of the analyzed 32 strains at pure and complexed 5 FC (the synthesis of the data)

Antifungal agent	Concentration (μmol/mL)	Geometric average (μmol/mL)	CMI50 (μmol/mL)	CMI90 (μmol/mL)
5 FC	0.0005–0.032	0.0001163	0.0005	0.0008
5 FC β CD	0.00025–0.008	0.0000262	0.00025	0.0002
5 FC HP β CD	0.0002–0.0076	0.0000245	0.000083	0.0001

[a] 5 FC is efficient in the treatment of the Candida species in concentrations higher than 32 μg/mL (0.032 μmol/mL)

[b] The minimal inhibitory concentration of the antifungal agent required for growth inhibition is reduced in the presence of the cyclodextrin

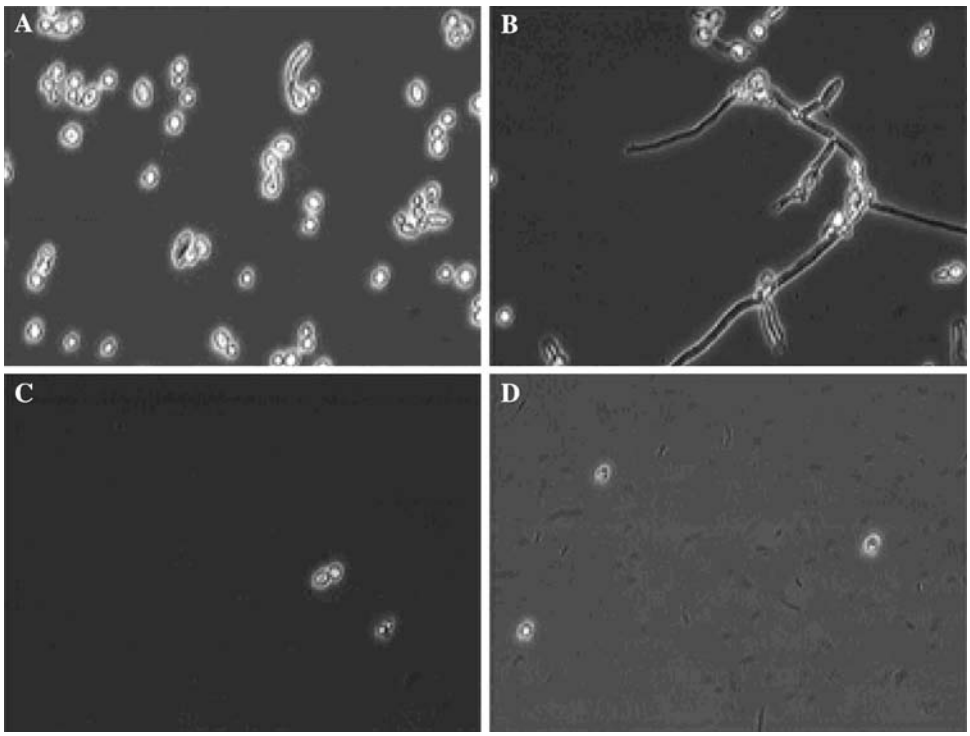

Fig. 1 Candida albicans cultures—witness at 35 °C (**a**), in the presence of 5 FC (0.0032 mmol/mL) (**b**), in the presence of β-CD-5 FC (0.008 mmol/mL) (**c**), and HP β-CD-5 FC (0.0076 mmol/mL) (**d**)

(*C. krusei* and C. *norvegensis*) known to behave as resistant fungi. As an example we present the microscopic images of the culture of *Candida albicans* on the culture media in the absence and presence of the 5 FC in concentration of 0.032 μmol/mL.

The percentage variability of the inactivated strains depends in 89.57% proportion on 5 FC concentration, the multiple correlation coefficient having the value $R^2 = 0.8957$. The regression of the inactivated strains depending on the antifungal substance concentration has the following equation: $y = 0.4068\text{Ln}(x) + 0.1749$.

From Table 2 one can see that the inclusion complexes β-CD-5 FC and HP β-CD-5 FC present an higher antifungal activity comparing with the free drug, due to the increase of the hydrosolubility of the complex comparing with the pure 5 FC. It can be observed that MIC 50 is reduced 2 times and 6 times for the inclusion complexes of 5 FC with β-CD and HP- β-CD. Also, MIC90 is reduced four and respectively, eight times for the complexed 5 FC with β-CD and HP β-CD, comparing with the free drug alone, indicating also the possible reduction of the treatment dosage and of the gravity of all side effects in case of using these compounds for mycosis treatment. These results are also sustained by the microscopic images of the culture of *Candida albicans* on the culture media in the absence and presence of 5 FC, β-CD-5 FC and HP β-CD-5 FC in concentration of 0.032, 0.008 and 0.0076 μmol 5 FC/mL. The growth inhibition is clear in the four images, sustained by the reduction of the fungal cells density on the culture media (Fig. 1).

The performed tests proved that there are significant differences between the antifungal effect of the native substance and of the complexed substance. The improvement of in vitro antifungal effect of complexed 5 FC, characterized by the decrease of the active concentrations necessary to inactivate the yeasts and also by the decrease of the minimal inhibitory concentrations for 50 and 90% of the strains, can be observed.

Acute toxicity studies

The acute toxicity studies data show, that the acute toxicity of the β-CD-5 FC and HP β-CD-5 FC inclusion complex is smaller, comparing with the free 5 FC, analyzed separately. The oral LD50 for the β-CD-5 FC lyophilized complex is 1.4, and respectively 1.55 μmol 5 FC/kg body weight, higher than the value of LD50 obtained for free 5 FC 1.25 μmol/kg body weight.

The inclusion complex showed an improved bioavailability, and a reduction of the toxicity comparing with the pure 5 FC, aspect sustained by the absence of any hepatic, hematological and/or renal anomalies at 1.4 and 1.55 μmol 5 FC complexed/kg body weight.

Conclusions

Inclusion complex of flucytosine and β-CD, or HP β-CD was prepared by freeze-drying method in a molar ratio 1:1. The inclusion efficacy was confirmed by all the data already described in the literature [1, 5, 6]. Complexation by inclusion increases flucytosine solubility and dissolution in water for the two analyzed cyclodextrins, due to the low crystallinity of the complex. Due to this aspect, cyclodextrin based supramolecular systems represent an interesting formulation platform for delivery of drugs with poor physicochemical and biopharmaceutical properties.

The inclusion complex shows an acute toxicity smaller than the pure drug, oral 1.4 and 1.5 μmol 5 FC complexed/kg body weight, due to higher solubility and bioavailability of the complexed drug. Also, the in vitro antifungal activity of the complexed drug is higher (MIC50 is half and MIC90 is four time smaller), than of the pure biological active compound.

The increase of the bioavailability of the drug in β-CD-5 FC inclusion complexes, combined with the decrease of the active dose and of the toxic effects proves the efficacy of the therapeutic usage of these inclusion complexes.

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