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Evaluation of the cytotoxicity of cyclodextrins and hydroxypropylated derivatives

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Summary

The haemolytic effect toward human erythrocytes and the cytotoxicity toward P388 cells of the three natural cyclodextrins and their hydroxypropylated derivatives have been compared. The cytotoxicity of the six cyclodextrins toward these two cell types follows a similar pattern, and the curve parameters are generally of the same order of magnitude for both cell types in spite of the biological differences. The in vitro cytotoxic effect decreases in the order βCD > αCD > γCD and HPβCD > HPγCD $\geq$ HPαCD. These results showed that phenomena involved in cyclodextrin cytotoxicity are not specific to the cell type, and bore out the hypothesis of destruction of membranes by the removal of basic membrane components. Moreover, they demonstrated the influence of parameters other than CD concentration, i.e., the presence of serum components, or the density of the cells, which can dramatically influence the cytotoxic effect of CDs. In fact, the cytotoxicity of a cyclodextrin is determined by the relative proportion of cellular and extracellular molecules likely to be included and cyclodextrins.

Introduction

Increasing interest in the parenteral use of cyclodextrins has led scientists to study the toxicity of these molecules. Frank et al. (1976) pointed out cyclodextrin nephrosis in the rat, characterized by the presence of crystals in tubular cells. Renal toxicity in rats treated with high levels of β-cyclodextrin has been confirmed by Perrin et al. (1978).

Cyclodextrins are of interest to many workers because of the numerous molecules which can be included. Their amorphous, hydroxypropylated derivatives have the same cavity size and greater water solubility. Consequently, many studies deal with these more soluble molecules, concerning the activity of complexes (Dietzel et al., 1990; Estes et al., 1990), pharmacokinetics (Frijlink, 1990; Monbaliu et al., 1990; Yamamoto et al., 1990) and safety (Pitha et al., 1988; Brewster et al. 1990a; Coussement et al., 1990; Pitha, 1990).

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Hydroxypropylated β-cyclodextrins showed much weaker toxicity in animals and few studies have been performed in humans (Brewster and Bodor, 1990; Seiler et al., 1990; Szathmary et al. 1990).

In vitro studies have dealt with the haemolytic effect of various cyclodextrins (Irie et al., 1982; Uekama et al. 1987; Jodal et al., 1988; Ohtani et al., 1989). They cannot be considered as a true approach to the in vivo toxicity, since in general no haematological trouble occurred after administration of cyclodextrins to various animals (Brewster et al., 1990; Seiler et al., 1990; Yamamoto et al., 1991). In only one publication was a regenerative anaemia described in a subchronic toxicity study using 2000 mg/kg of intravenous γ-cyclodextrin. This low cytotoxicity for such a high dose could be explained by the significant distribution volume and the consequent dilution of the cyclodextrin in the biological fluids (Antlsperger, 1992). Nevertheless, haemolysis studies were a simple and rapid means to classify the cyclodextrins according to their cytotoxicity and a way to examine the mechanism involved. All the authors agree with the mechanism proposed by Irie et al. in 1982: inclusion complexation of the membrane components induces their release and the lysis of the erythrocytes.

In this study, perturbations created by natural and hydroxypropylated cyclodextrins were investigated on two kinds of cell: human erythrocytes and P388 murine leukaemic cells. As mentioned above, few studies have been reported in the literature on cyclodextrin toxicity on erythrocytes, and only one investigation on fibroblasts (Pitha et al., 1988). Until now, no other cell type has been used to study cyclodextrin cytotoxicity. This work has been performed in order to verify, with a new cell type, the cytotoxic effect of the three natural cyclodextrins and their hydroxypropylated derivatives, and to determine the parameters involved in their cytotoxicity.

Materials and Methods

Chemicals

Commercial cyclodextrins (CD) were supplied by different companies, and were used as received: αCD (Chinoin, Budapest, Hungary), βCD (Roquette Frères, Lestrem, France), HPβCD MS 0.6 (Janssen, Olen, Belgium), and γCD, HPαCD and HPγCD MS 0.6 (Wacker, München, Germany). CD solutions were prepared by dissolution in phosphate-buffered saline 10× (Gibco, Cergy-Pontoise, France) first diluted 10-fold (PBS). All other materials and solvents were of analytical grade.

Haemolysis assays

Haemolysis assays were carried out on both human freshly drawn whole blood and on erythrocytes separated from blood by centrifugation. In the latter case, the cells were washed twice and suspended with PBS. Blood or erythrocytes were suitably diluted using PBS (1:5) before assays.

First 50 μl of diluted sample was added in 2 ml of CD solution in increasing concentrations depending on the CD solubility. In a second assay, 30–200 ml of diluted blood sample was added in 2 ml of βCD solution, in order to vary the cell number for the same βCD concentration. Three concentrations of 3, 4 and 5 mmol/l were then used. The mixtures were stirred and incubated for 30 min at 37°C, then centrifuged at $1000 \times g$ for 10 min. The haemoglobin concentration in the supernatant was assayed spectrophotometrically at 543 nm. The percentage haemolysis was expressed as the ratio of the absorbance of the samples to the absorbance of a control (complete haemolysis of erythrocytes in water).

P388 cytotoxicity assays

P388 murine leukaemic cells were grown in RPMI 1640 medium with glutamine supplemented with 10% foetal calf serum (Gibco, Cergy-Pontoise, France), 0.0015% of mercaptoethanol (Sigma, La Verpillière, France) and 100 mg/ml of streptomycin and 100 IU/ml of penicillin (Eurobio, Les Ulis, France).

P388 cells were distributed in cell culture tubes at the rate of about 80000 cells per tube. The samples underwent a 24 h incubation period (37°C, 5% CO_2, 95% relative humidity). A quantity of 1 ml of CD solution in increasing concentrations was then added in each tube. The mix-

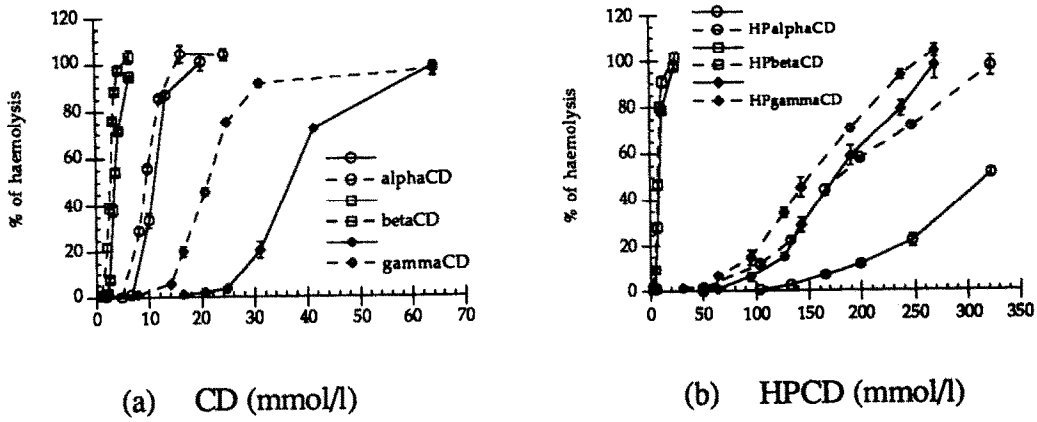


Fig. 1. Cytotoxicity curves against erythrocytes and whole blood ($n \geq 3$).

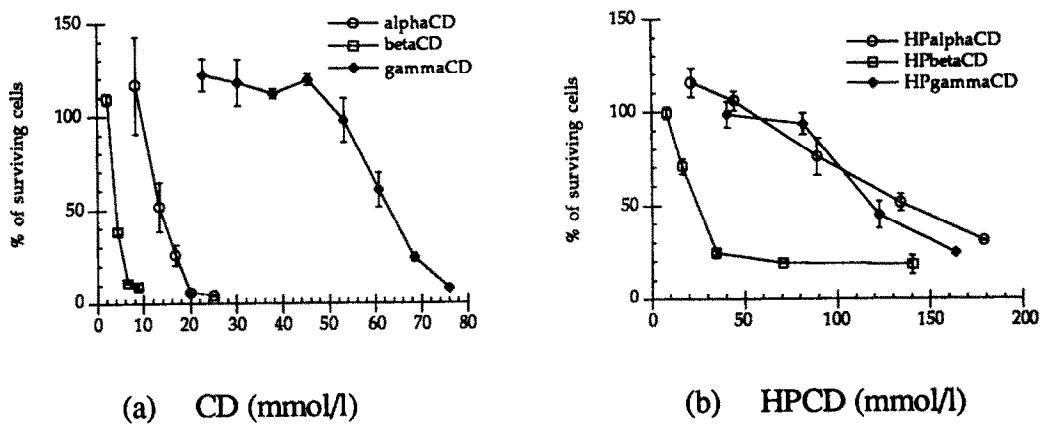


Fig. 2. Cytotoxicity curves against P388 cells ($n = 3$).

TABLE 1

Characteristic values of the cytotoxicity curves of cyclodextrins

	TD_0 (mmol/l)			TD_{50} (mmol/l)			TD_{100} (mmol/l)		
	Red blood cells	Whole blood	P388 cells	Red blood cells	Whole blood	P388 cells	Red blood cells	Whole blood	P388 cells
αCD	5	7	11	9.5	11	15	16	20	20
βCD	1	2.2	2.5	2.8	3.6	4	4.5	6.5	6.6
γCD	15	25	55	21	37	65	32	64	76
HPαCD	50	100	50	180	320	140	320	> 320	≧ 180
HPβCD	2.5	5	9	7.5	9	25	25	25	35
HPγCD	30	65	40	150	175	120	255	270	≧ 165

TABLE 2

P388 cell multiplication (48 h) according to cell density

Cell density (cells/ml)	150000	100000	50000	25000
Multiplication rate	2.3	2.9	3.7	4.5

tures were incubated for 2 h. After centrifugation ($380 \times g$, 4°C, 5 min), the cells were washed with 2 ml of PBS before suspending in a 3 ml culture medium, and were then incubated again for 48 h. Finally, the samples were suitably diluted in isoton II, and the cells were counted using a Coulter Counter® ZM (Coultronics, Margency, France). The percentage of surviving cells was calculated in comparison with a control without CD. Other culture tubes received $0.25–1.5 \times 10^6$ cells. After the 24 h incubation period, 1 ml of βCD solution of 3, 4 and 5 mmol/l was added.

Results

Influence of CD concentration

Fig. 1 shows cytotoxicity curves for CD (a), and HPCD (b). The percentages of haemolytic activity are plotted against the CD concentrations.

Fig. 2 shows cytotoxicity curves for CD (a), and HPCD (b). The percentages of surviving P388 cells are plotted against the CD concentrations.

Three characteristic values have been obtained from every cytotoxicity curve. They are defined as follows: TD_0 = CD concentration at the beginning of the cytotoxic effect, TD_{50} = CD concentration for 50% cytotoxic effect, and TD_{100} = CD concentration for maximal cytotoxic effect. The mean characteristic values of the six CDs tested are summarized in Table 1.

Influence of cell density

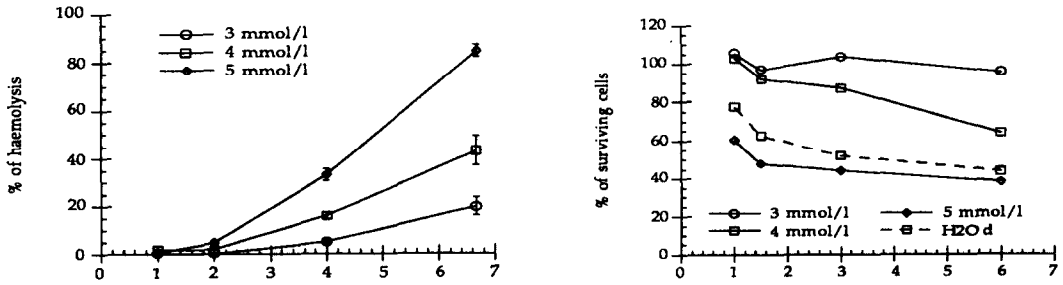
Fig. 3 shows cytotoxicity curves of βCD for different cell densities of erythrocytes (a), and for P388 cells (b). Dilution factors are within the range between 1 and 6.66 for both cell types.

Table 2 groups the multiplication rate of the P388 cell in 48 h for the different initial cell densities.

Discussion

The cytotoxicity of the six cyclodextrins toward P388 cells and erythrocytes follows similar patterns (Figs 1 and 2). The values of the TD_0, TD_{50} and TD_{100} parameters are generally of the same order of magnitude for both cell types in spite of the biological differences (Table 1). Irrespective of the parameter considered, the in vitro cytotoxic effect of cyclodextrins decreases in the order: $\beta CD > \alpha CD > \gamma CD$ for the natural cyclodextrins, and $HP\beta CD > HP\gamma CD \geq HP\alpha CD$ for the hydroxypropylated cyclodextrins (Leroy-Lechat et al., 1992).

The inclusion complexation of membrane components, particularly cholesterol (Djedaïni, 1991;



(a) Whole blood (b) P388 cells

Fig. 3. Cytotoxicity curves for βCD as a function of dilution $1/x$ ($n = 3$).

Frijlink et al., 1991) and phospholipids (Szejtli et al., 1986; Okada et al., 1989), could be the cause of the destruction of the cell membrane. By the same mechanism, cyclodextrins create perturbation of the surface tension of lipid monolayers and destruction of liposomes (Irie et al., 1982; Miyajima et al., 1987; Ohtani et al., 1989). Liposomes made of as much cholesterol as phospholipids are degraded by CD as follows: βCD > αCD > γCD, the same pattern as for cytotoxicity of CD toward erythrocytes and P388 cells. βCD is the most cytotoxic of the natural cyclodextrins. This can be explained by its affinity for phospholipids and even more for cholesterol.

Every hydroxypropylated cyclodextrin showed reduced cytotoxicity in comparison with its natural parent cyclodextrin (Figs 1 and 2). This phenomenon is particularly marked for the hydroxypropylated derivatives of αCD and γCD. These derivatives are characterized by high water solubilities, however, this physicochemical difference cannot explain the cytotoxicity difference. In fact, methylated cyclodextrins also have high water solubilities, but induce more pronounced haemolysis than βCD. According to Jodal et al. (1988), their in vitro haemolytic effect decreases in the order: dimethyl-βCD > trimethyl-βCD > βCD. The surfactant property of methylated cyclodextrins could not play a prominent role since, conversely, trimethyl-βCD is more tensioactive than dimethyl-βCD. Considering that the cytotoxicity phenomenon arises from the inclusion ability of cyclodextrins toward membrane lipids, the more or less significant cytotoxicity of the derivatives could result from the differences in affinity of the cyclodextrins for the molecules included. In fact, dimethyl-βCD often has a higher affinity for steroids than βCD and the hydroxypropylated derivatives (Uekama and Irie, 1987; Yoshida et al., 1988, 1989). Moreover, Szente et al. (1992) showed that methylated cyclodextrins have a much higher affinity for fatty acids than hydroxypropylated cyclodextrins. These latter derivatives, with lipids, generally give inclusion compounds with a weak stability constant (Irie et al., 1992).

Haemolysis assays demonstrated lowered cytotoxicity of all the cyclodextrins toward whole blood rather than isolated erythrocytes (Fig. 1). Serum components appear to protect cells from the inclusion attack of the cyclodextrin. Similar results have been obtained by Pitha et al. (1988) on human fibroblasts in vitro when adding 10% of serum in the culture medium. Serum contains lipid carriers such as lipoproteins responsible for the transport of various molecules, including cholesterol. Thus, serum contains the same lipids as cell membrane and maybe numerous molecules able to be included in the cyclodextrin cavity. The lowered cytotoxicity could come from the competition between serum and membrane components for complexation with cyclodextrins. The ratio between cyclodextrin and membrane components would be reduced as a function of the serum components included. The amount of cyclodextrin available to complex membrane components would be reduced proportionally, and cytotoxicity also.

Fig. 3 shows that cell density plays a part in the cytotoxicity results. In the case of erythrocytes, the percentages of haemolysis due to β-cyclodextrin increased dramatically with blood dilution. In the case of P388 cells, percentages of surviving cells decreased with dilution of the cell suspension. Thus, the same β-cyclodextrin concentration caused greater cytotoxicity, the lower the cell density, irrespective of cell type. Nevertheless, percentage variations observed for P388 cells are much weaker than for erythrocytes. This might be explained by the fundamental differences between both cell types and between both experiments. Erythrocytes cannot multiply or ensure protein synthesis on account of the lack of a nucleus, and haemolysis was measured after 30 min. P388 cells multiply rapidly, and were counted after 48 h. Table 2 shows that the multiplication rate decreases with cell density. This phenomenon might enable the cells to compensate for more pronounced initial differences in cell numbers.

Thus, the cytotoxic effect depends both on cyclodextrin concentration and on cell density, and also on the concentration of all the molecules able to be included present in the cell medium. To summarise, cyclodextrin cytotoxicity depends on its inclusion ability for the membrane components and on its avaibility, i.e., on the ratio be-

tween cyclodextrin and all the molecules able to be included.

In this work, for the first time, a comparison between haemolytic effect and cytotoxicity of the three natural cyclodextrins and their hydroxypropylated derivatives has been undertaken. The use of a new cell type, which has never before been considered in cyclodextrin studies, murine leukaemic P388 cells, as well as erythrocytes, showed that phenomena involved in cyclodextrin cytotoxicity are not specific to the cell type, and bore out the hypothesis of destruction of membranes by the removal of basic membrane components. However, some experiments showed that cytotoxicity results do not have absolute significance unless certain parameters are defined. Over and above cyclodextrin concentration, it has been shown that the environment of the cells, such as the presence of serum lipids, and the density of the cells, can dramatically influence the cytotoxic effect of cyclodextrins. In fact, the cytotoxicity of cyclodextrins is determined by the relative proportion of cellular and extracellular molecules likely to be included and cyclodextrins.

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