Recognition ability and cytotoxicity of some oligosaccharidyl substituted β-cyclodextrins

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Summary - This paper reports a chemico-enzymatic synthesis of β-CD derivatives. The recognition properties of these derivatives were tested using flocculating yeast and isolated lectins. It was observed that the substitution of β-cyclodextrins with galactose end arms induces the better recognition by a cell-linked galactose-specific lectin. The physicochemical effects of the β-CD derivatives on membranes were estimated using red blood cells and the effects on the viability of yeast and human rectal tumor cells were appreciated by measuring the mitochondrial dehydrogenase activity. The substitutions of the β-CD ring by sugar antennae decrease the negative physicochemical effects of the β-CD, i.e., their hemolytic properties. However, these substitutions induce significant modifications of the biological properties of the molecules, particularly the cytotoxicity and the growth of eukaryotic cells.

Materials and methods

Chemicals

β-Cyclodextrin (β-CD) was a gift from Wacker GmbH (Lyon, France) and D-Gal and L-Fuc were purchased from Fluka (St Quentin Fallavier, France). The compounds Gal-Sp-β-CD and Fuc-Sp-β-CD were prepared as previously described [13]. The Glc-NAc-Sp-Ome was kindly donated by Dr D Charon (Chatenay-Malabry, France). The detailed synthesis of GlcNAcSp-β-CD, Gal-1-4-GlcNAc-Sp-β-CD and 7(Gal-Sp)-β-CD will be published elsewhere.

All the derivatives were soluble in distilled water except 7(Gal-Sp)-β-CD which was soluble at a concentration up to 200 mg/ml in a DMSO/H2O 1:10 v/v mixture. This mixture had no effect on the assays. All the derivatives were sterilized by filtration through a 0.22 µm membrane (Sartorius, Palaiseau, France) before use.

Micro-organisms and culture conditions

The following yeast strains were used: Kluyveromyces bulgaricus and Saccharomyces cerevisiae (uvarum) 806, two strains the flocculation of which was inhibited by galactose and mannose, respectively [5]. The yeast cells were grown aerobically (air flow 40 l/h) at 25°C during 24 h in 1.5 l liquid medium (4% glucose, 0.4% bactopeptone (Difco, Michigan, USA), 0.1% KH2PO4, 0.02% MgSO4·7H2O, 0.02% CaCl2) (Prolabo, Pads, France) contained in a 2 l capacity fermentor (Biolafitte, Poissy, France).

Eukaryotic cell strain and cell line maintenance conditions

Human rectal adenocarcinoma tumor cell line (HRT-18), obtained from Laporte (INRA, Jouy-en-Josas, France) was grown in RPMI 1640 medium without L-glutamine or phenol red (ICN Biomedicals Inc, Costa Mesa, CA, USA) supplemented with 20% fetal bovine serum (Life Technologies, Egany, France), 10 µg/ml ofloxacin (Roussel UCLAF, Romainville, France).
France) and incubated at 37°C for 3 or 4 days in a humidified atmosphere containing 5% CO₂ [8]. The cells were generally propagated upon trypsin treatment and the percentage of cell viability was determined by the trypan blue exclusion test. These HRT cells contain lectins which can be isolated by treatment with detergents in a 50 mM sodium phosphate buffer (pH 7.4).

**Red blood cells (RBC)**

Human O group erythrocytes (RBC) were washed three times in PBS buffer (137 mM NaCl, 2.7 mM KCl, 8 mM Na₂HPO₄, 1.5 mM KH₂PO₄, 0.5 mM MgCl₂, 1 mM CaCl₂, pH 7.4) and resuspended in the same buffer to a density of 1.6 x 10⁶ cells/ml and maintained at +4°C until use.

**Extraction of a galactose-specific lectin from Kluyveromyces bulgaricus**

The extraction of the galactose-specific lectin was performed according to Al Mahmood et al [1]. Cells harvested at 4°C by centrifugation at 3000 g for 10 min, were treated with galactose and extensively washed with 0.01 M phosphate buffer (pH 7). Cells then suspended at a concentration of 4% (w/v) in the same buffer containing 5 mM EDTA (Prolabo, Paris, France) and were generally treated twice in order to obtain non-flocculating cells.

**Flocculation and deflocculation test**

Yeast cells in stationary phase were harvested, washed with distilled water, then with 10 mM EDTA aqueous solution and twice with distilled water. Washed cells (about 20 mg dry weight) were placed in 10 ml Helm’s acetate buffer (pH 4.5). The flocculation degree (FD) was measured according to Hussein et al [5]. Yeast cells were suspended by vigorous shaking and the absorbance (A₅₅₀) at 620 nm was immediately measured; after a pause of 15 min, the absorbance of the suspension was again measured (A₅₅₀). The ratio R = (A₅₅₀)/(A₅₅₀) x 100 representing the percentage of cells still in suspension after 15 min was calculated and the FD was estimated (FD = 5, 4, 3, 2, 1 and 0) and associated with R values of 0, 0–10, 10–30, 30–65, 65–90, and 100.

**Inhibition of hemagglutination and yeast flocculating properties of the lectin**

The Kb CWL lectin was dissolved in Helm’s buffer at titers 16. All derivatives and carbohydrates to be tested for their inhibitory effect were dissolved in Helm’s buffer at concentrations up to 1 M. For inhibition of hemagglutination, a lectin solution (50 µl) was diluted (serial two-fold dilutions) and introduced in 96-well microtiter plates (Nunc, Kamstrup, Denmark) and an equal volume of yeast suspension (5 x 10⁵ cells/ml) were incubated at 25°C for 18 h under agitation. Absorbance at 25°C for 18 h under agitation the plates were centrifuged for 10 min at 2000 rpm and the supernatant discarded. Then, 50 µl of MTT (1 mg/ml) solution in the culture medium were added to the wells. The plate was agitated and incubated for 1 h at 25°C. After centrifugation, the supernatant was discarded and the insoluble blue formazan product was dissolved in 100 µl of DMSO. Toxic or stimulatory effect on the yeasts, corresponded to a decrease or increase of formazan production, respectively.

**Eukaryotic cellular viability assay**

The determination of the effect of β-CD and β-CD derivatives on the viability of eukaryotic cell line (HRT-18) in culture was performed by evaluation of the inhibition of cell proliferation measured by a mitochondrial deshydrogenase activity assay. Active mitochondrial succinate deshydrogenase of living HRT-cells causes cleavage and conversion of a soluble yellow tetrazolium solution (MTT) (3-[4.5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) (Sigma, Saint Louis, MO, USA) into an insoluble purple formazan. Dead cells do not cause this change. The cellular viability was evaluated with modified MTT following the technique of Carmichael et al [2]. Non-confluent HRT-18 cells, grown in 96-well flat bottom culture plates (Costar, Cambridge, USA) at 4 x 10⁴ cells per well, were treated with various concentrations of β-CD derivatives for 72 h at 37°C. At the end of the incubation period, cells were washed in RPMI-PR- (phenol red-free RPMI 1640) and 50 µl of 1 mg/ml solution of MTT in RPMI-PR-medium without fetal calf serum were added in each well. After 2 h incubation, medium was removed and insoluble blue formazan product was dissolved in 100 µl of DMSO (Sigma, Saint Louis, MO, USA).

The absorbance of the solubilized material was measured spectrophotometrically at 540 nm and 690 nm on a Titertek Multiscan MCC/340 MK II apparatus (Labsystems, Helsinki, Finland) and expressed as a function of concentration of converted dye. Results shown are average values from experiments done in triplicate. A cytotoxic effect corresponds to a decrease in purple formazan production.

**Yeasts viability and growth assay**

The determination of the effects of β-CD and β-CD derivatives on yeasts was performed by evaluation of the cell growth and their mitochondrial deshydrogenase activity following the technique of Carmichael et al [2] slightly modified. All the assays were performed in 96-well microtiter plates.

For growth evaluation, 200 µl of yeast suspension (5 x 10⁵ cells/ml) were incubated at 25°C for 18 h under stirring. Absorbance was measured at 620 nm. Toxic or stimulatory effect on the yeasts, corresponded to a decrease or increase of formazan production, respectively.

**Hemolytic activity**

Human erythrocytes from freshly drawn blood were supplied by CTS, Nancy, France. Hemolytic effect determination of β-cyclo-dextrin derivatives was performed by mixing serial dilutions of cyclo-dextrin derivatives in PBS and human O group erythrocyte (RBC) suspension. The final red blood cells concentration was of 8 x 10^⁹ cells/ml and incubation was carried out under slight agitation at 37°C for 60 min [7].

After incubation, suspensions were centrifuged (1000 g for 10 min), the optical density of the supernatant was measured for hemoglobin at 540 nm, and the percentage of hemolysis was evaluated versus a 100% hemolyzed erythrocyte suspension (erythrocyte suspension/distilled water 1:20, v/v).
Chemico-enzymatic synthesis of β-cyclodextrin (β-CD) derivatives

All the modifications concern substitution at the more reactive primary hydroxyl face (OH-6). The Gal-Sp-β-CD, Fuc-Sp-β-CD and GlcNAc-Sp-β-CD derivatives were obtained via peptide coupling of the respective saccharide spacer-carboxylic acid with the mono-6-amino-6-deoxy-β-CD derivative, obtained by reduction of the mono-azido compound [17]. The coupling was carried out under soft conditions using DCC/HOBt as the activating system. Similar conditions were used for 7(Gal-Sp)-β-CD where full substitution of per-6-amino-6-deoxy-β-CD was achieved.

The stereospecific transfer of a β-D-Gal group to the O4 of GlcNAc-Sp-β-CD from UDP, generated in situ from UDP-Glc and UDP-glucose-4-epimerase, was achieved by use of galactosyl transferase [3] to give in high yield the desired lactosylamine derivative. The synthesis will be described in detail elsewhere [9].

Use of the epimerase allows to avoid the extremely expensive UDP-Gal, which being consumed in the next step does not affect the overall yield of the synthesis.

The different derivatives used and tested are presented in table I.

Flocculation and deflocculation activity

Flocculation degree of fully flocculating yeasts was evaluated in Helm’s acetate buffer according to the procedure described by Hussain et al [5]. Under these conditions, the flocculation degrees of K bulgaricus and S cerevisiae were estimated to be 4 and 3, respectively.

In table II are reported the deflocculating effects of β-CD derivatives on fully flocculating yeasts. Results show no deflocculating effect on S cerevisiae, the flocculation of which involves a mannose-specific lectin [15]. On K bulgaricus, which flocculation involves a galactose-specific lectin, the most efficient derivatives were Gal-β-1,4-GlcNAc-Sp-β-CD and Fuc-Sp-β-CD. The weaker activity of Gal-Sp-β-CD and 7(Gal-Sp)-β-CD may result from their lower water-solubility than has the former. All the other β-CD products and GlcNAc-Sp-OMe, bearing no galactose residue, presented lower or even no activity.

Hemagglutination and flocculation inhibition effects

It has been shown that the Kb CWL lectin induced O group red blood cell’s (RBC) agglutination as well as K bulgaricus flocculation. Galactose derivatives inhibit these agglutination and flocculation effects [1]. When β-CD derivatives were used as inhibitors of the Kb CWL lectin agglutinating activity on RBC and on previously deflocculated K bulgaricus yeast cells, a parallel inhibiting effect was observed in both tests. Indeed, as reported in table III, Gal-β-1,4-GlcNAc-Sp-β-CD and Gal-Sp-β-CD were the most efficient either on red blood cells or on yeasts flocculation. Unsubstituted β-CD, GlcNAc-Sp-β-CD and the GlcNAc-Sp-OMe had no effect on K bulgaricus flocculation. These compounds presented however an inhibitory activity on hemagglutination which was slightly less efficient than the galactose β-CD derivatives.

Hemolytic test

The results of the hemolytic effects of the β-CD derivatives versus the natural β-CD are presented in figure 1. Substitution of the ring strongly diminished the hemolytic properties of β-cyclodextrins. When the ring is substituted with a galactose or fully substituted with 7 Gal arms, its hemolytic activity was about three times lower than the natural β-CD. With the other derivatives the hemolytic activity was much weaker.

Effect of β-CD derivatives on eukaryotic HRT-18 cells

The effect of β-CD derivatives on the cellular viability of non-confluent HRT-18 cells was evaluated after 72 h treatment by MTT assay as described in Materials and methods.

It must be noted that natural β-CD did not affect the cellular viability at any concentration tested (0.1 to 4 mM) over a 72 h period compared to untreated cells.

On the contrary, β-CD derivatives exhibited some inhibiting effects on the mitochondrial deshydrogenase activity of the HRT-cells.

From figure 2a, a significant cytotoxic effect can be observed, 50% decrease of cell viability in the presence of only 1 mM of GlcNAc-Sp-β-CD or Fuc-Sp-β-CD and 40% decrease with 4 mM. However, the mitochondrial enzymatic activity of the cells was poorly affected by the presence of GlcNAc, no more than 12% decrease was observed at the highest concentration tested (4 mM).

The results obtained with the β-CD derivatives bearing a galactose arm are shown in figure 2b. They all have a cyto-

Table I. Structure of the β-CD derivatives.

| β-CD        | R1 = R2 = OH |
|-------------|-------------|
| Gal-Sp-β-CD | R1 = D-Gal-NHCO-(CH2)32-CONH- |
|             | R2 = OH     |
| Fuc-Sp-β-CD | R1 = L-Fuc-NHCO-(CH2)32-CONH- |
|             | R2 = OH     |
| GlcNAc-Sp-β-CD | Glc NAco-(CH2)32-CONH- |
|             | R2 = OH     |
| Gal-β-1,4-GlcNAc-Sp-β-CD | Gal-β-1,4-GlcNAco-(CH2)32-CONH- |
|             | R2 = OH     |
| 7(Gal-Sp)-β-CD | R1 = R2 = D-Gal-NHCO-(CH2)32-CONH- |

Cyclodextrins are regular cyclic oligosaccharidyl molecules exhibiting a tronconic ring which are for simplicity represented by a truncated cone with the wider side formed by the secondary 2- and 3-hydroxyl groups and the narrower side by the primary 6-hydroxyl [11]. Sp = Spacer = NHCO-(CH2)7-CONH- or O-(CH2)7-CONH-.
Table II. Deflocculating effect of β-CD and sugar derivatives on fully flocculating \( K \) bulgaricus and \( S \) cerevisiae cells.

| Derivatives | β-CD | GlcNAc-Sp-β-CD | Fuc-Sp-β-CD | Gal-β-1,4-GlcNAc-Sp-β-CD | Gal-Sp-β-CD | 7(Gal-Sp)-β-CD | Gal | Fuc |
|-------------|------|----------------|-------------|-------------------------|------------|----------------|-----|-----|
| Deflocculation of \( K \) bulgaricus | – | – | 475 | 165 | 65 | 185 | 290 | 350 | 350 |
| \( S \) cerevisiae var uvarum | – | – | >1000 | >1000 | – | – | – | – | – |

Results are expressed in mM. They are the minimum concentration which deflocculates fully flocculating yeasts. The degree of flocculation was measured following the procedure described by Hussain et al [5]: \( K \) bulgaricus had a FD of 4. \( S \) cerevisiae var uvarum had a FD of 3. -: no effect detected at 15 mM for the β-CD, and 1 M for the other derivatives. SD ± 10%.

Table III. Inhibition of the \( K \) bulgaricus lectin induced hemagglutination and flocculation.

| Derivatives | β-CD | GlcNAc-Sp-OMe | GlcNAc-Sp-β-CD | Fuc-Sp-β-CD | Gal-β-1,4-GlcNAc-Sp-β-CD | Gal-Sp-β-CD | 7(Gal-Sp)-β-CD | Gal | Fuc |
|-------------|------|---------------|----------------|-------------|-------------------------|------------|----------------|-----|-----|
| O group red blood cells | 2.2 | 6.7 | 2.5 | 0.8 | 0.2 | 0.8 | 1.1 | 1.5 | 1.5 |
| \( K \) bulgaricus cells | – | – | – | 3.8 | 1.8 | 2.5 | 3.9 | 3.5 | 3 |

Results are expressed in mM. They are the minimum concentration of β-CD, β-CD and sugar derivatives or sugars which inhibit hemagglutination or yeast flocculation by Kb CWL lectin. -: no effect detected at 15 mM. SD ± 10%.

Toxic effect at a concentration of 1 mM. In cells treated with 1 mM of Gal-β-1,4-GlcNAc-Sp-β-CD and 7(Gal-Sp)-β-CD a decrease of cell viability of 60% was obtained. However, Gal-Sp-β-CD at the same concentration led to a decrease of 30% of the deshydrogenase activity. The derivative 7(Gal-Sp)-β-CD showed the higher cytotoxic effect, the incubation of the cells with 4 mM of this derivative inhibited 95% of the enzymatic activity.

**Effect of β-CD derivatives on yeast viability and growth**

When \( K \) bulgaricus cells were grown in the presence of β-CD and Gal-Sp-β-CD, no toxic effect could be observed. We observed instead, as shown in figure 3a, an increase of deshydrogenase activity (MTT test) which was concomitant with an increase of cell growth. These effects were observed with the substituted β-CD ring bearing a Gal arm. Free β-CD ring as well as free arm had a much lower effect. When \( S \) cerevisiae yeast cells were grown under the same conditions (fig 3b) no significant effect was observed neither on growth nor on the MTT activity.

In order to control whether the stimulation was in relation with a recognition process of the Gal-Sp-β-CD by the galactose specific lectin of \( K \) bulgaricus, the effect of the latter derivative was tested on resting yeast cells. The tests were performed in the presence of mannose, galactose and methyl galactoside. As shown in figure 4a, Gal-Sp-β-CD enhanced the deshydrogenase activity of \( K \) bulgaricus. When the test was performed in the presence of mannose, its activity was not affected, but in the presence of methyl galactoside and galactose the activity was diminished by 50 to 60% respectively. This indicated a competition between Gal-Sp-β-CD and galactose derivatives on the recognition site of the yeast lectin. When the β-CD ring did not bear any galactose arm, no effect on the MTT activity was observed (fig 4b).
fl-CD chemico-enzymatic synthesis and cytotoxicity

Fig 2. Effect of fl-CD and galactose fl-CD derivatives on HRT-cell's viability at 37°C at a density of 410^4 cells/well, evaluated by dehydrogenase activity (MTT assay). a. Non-galactose substituted derivatives: , fl-CD; , Glc NAc; , Glc NAc-Sp-fCD; , Fuc-fCD. b. Galactose substituted derivatives: , fl-CD; , Gal-fCD; , Gal-1,4-Glc NAc Sp-fCD; , Gal-Sp-fCD; , 7(Gal-Sp)-fCD.

Fig 3. Effect of fl-CD and Gal-Sp-fCD on the growth and dehydrogenase activity (MTT assay) of yeast cells. a. Effect on K. bulgaricus. b. Effect on S. cerevisiae. Growth of the yeast in the presence of: Gal-Sp-fCD (■) fl-CD ( ) MTT assay on yeast in the presence of: Gal-Sp-fCD (■) fl-CD ( ). Results are expressed in percentage of variation versus control (assay without derivatives).

Discussion

One of the limitations for the application of natural cyclodextrins in medical situations is related to some of their physicochemical properties such as solubility, hemolytic activity, which are particularly important in the case of fl-cyclodextrin [6].

Several chemical modifications have been proposed for many different purposes such as decreasing the cytotoxic effects, improvement of the solubility, and recognition phenomenon.

Our results show that the chemical modification of fl-CD by substitution of primary hydroxyl on the ring with galactose end arms induces their recognition by carbohydrate recognition proteins. Indeed, a galactose-specific lectin, which provokes yeast aggregation and hemagglutination, is inhibited by the galactose substituted fl-CD. Furthermore, this recognition is specific as only a galactose-specific lectin is inhibited by these derivatives and not a mannose-specific one.

fl-CD at sufficient high concentrations causes hemolysis [4, 11]. We observed that the substituted fl-CD have weaker hemolytic activities than that of the unsubstituted fl-CD. Although the chemical structures of the substitutions are different from those studied by Yamamoto et al [19], our results are in agreement with the data of these authors.

Nevertheless, if the chemical modifications of the cyclodextrin ring decrease its hemolytic properties, the
substitutions induce changes of the biological activities in the cells.

As the surfaces of numerous normal and malignant eukaryotic cells are covered with galactose-specific lectins, galactose-substituted β-CD may be used as drug carriers in vitro and in vivo. Therefore it appeared necessary to test the effect of these carrier molecules on such cells.

Tumour cells can be resistant to a variety of chemotherapeutic drugs of different structures (multidrug resistance or MDR) by expressing a transmembrane P-glycoprotein (P-gp) on their cell surface. Colon and renal tumors are relatively refractory to chemotherapy and are known to express Pgp. Drug carrier systems are widely studied to circumvent this obstacle [13]. In order to investigate the ability of cyclodextrins as carriers to enhance the anti-cellular activity of antineoplastic drugs and to reverse multidrug resistance, we have evaluated the effect of cyclodextrin derivatives on the P-glycoprotein-positive MDR HRT-18 cell line (human rectal adenocarcinoma cell line).

Native β-CD, over a concentration range from 0.1 to 4 mM and for 72 h exposure time, has no effect on the deshydrogenase activity of the human cell line HRT-18 grown on cell culture containing 20% foetal calf serum. However, sugar substituted β-CD rings with saccharide antennae (GlcNAc, galactose, fucose) have a significant cytotoxic effect on these cells and so about 0.5 mM of each molecule is the maximum recommended concentration for 72 h exposure. Pitha [14] proposed to limit the use of 2-hydroxypropyl β-CD to about 0.5% for exposures of only 1 h on cells grown in serum-free media but up to 2% of this cyclodextrin may be used without limitation on exposure time in media containing 10% serum.

When β-CD derivatives such as Gal-Sp-β-CD are present in a culture of the yeast K. bulgaricus, which also bears a galactose-specific lectin on its surface, they increase the growth of the cells and deshydrogenase activity as shown by the increase of absorbance of the cell suspension and by the increase of formazan formation. The higher deshydrogenase activity does not result from a growth increase since the activity was strongly enhanced when the test was achieved with resting cells (in a phosphate buffer without nitrogen source). This phenomenon appears specific as: i) galactose derivatives diminished the effect; ii) no effect was observed with S. uvarum which bears a mannose specific lectin; and iii) free β-CD without galactose arm had no stimulatory effect; in the last case, a toxic effect may be observed.

The way by which the derivatives affect the intracellular mitochondrial succinate deshydrogenase activity remains to be demonstrated. The substitution of the ring with a galactose arm improves the recognition process by the peripheral cell receptors, also it can not be excluded that the derivatives, after their association with the cell surface lectin, may activate transmembrane molecules which induce cellular reactions. Further studies have to be achieved to confirm this hypothesis.

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Fig 4. Variation of deshydrogenase activity (MTT assay) of K. bulgaricus resting cells. a. Influence of Gal-Sp-β-CD in the absence or presence of mannose, methyl α-D-galactopyranoside and galactose, 200 mM. 1, buffer + Gal-Sp-β-CD; 2, mannose + Gal-Sp-β-CD; 3, methyl-β-D-galactopyranoside + Gal-Sp-β-CD; 4, galactose + Gal-Sp-β-CD. b. Influence of β-CD in the absence and in the presence of mannose, Methyl α-D-galactopyranoside and galactose, 200 mM. 1, β-CD + methyl-α-D-galactopyranoside; 2, β-CD + galactose; 3, β-CD + mannose; 4, β-CD + buffer. □ 0.55 mM; ▣ 1.1 mM; □ 2.2 mM. Results are expressed as percentage of variation versus control (assay without Gal-Sp-β-CD or β-CD).
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