Available online at www.sciencedirect.com
SCIENCE DIRECT
Bioorganic & Medicinal Chemistry Letters
Bioorganic & Medicinal Chemistry Letters 16 (2006) 2154–2157

Methylated β-cyclodextrin as P-gp modulators for deliverance of doxorubicin across an in vitro model of blood–brain barrier

Sébastien Tilloy,[b,*] Véronique Monnaert,[a] Laurence Fenart,[a] Hervé Bricout,[b] Roméo Cecchelli[a] and Eric Monflier[b]

[a]*Blood–Brain Barrier Laboratory, Université d'Artois, EA 2465, Faculté des Sciences Jean Perrin, rue Jean Souvraz, SP 18, 62307 Lens Cedex, France*
[b]*Laboratoire de Physico-Chimie des Interfaces et Applications, FRE CNRS 2485, Université d'Artois, rue Jean Souvraz, SP 18, 62307 Lens Cédex, France*

Received 25 November 2005; revised 13 January 2006; accepted 13 January 2006
Available online 7 February 2006

Abstract—Co-incubations of various β-cyclodextrins and doxorubicin have been evaluated on an in vitro model of blood–brain barrier in order to increase the delivery of this P-gp substrate to the brain. Among these cyclodextrins used, the Rame-β-cyclodextrin and Crysme-β-cyclodextrin increased the transport by a factor of 2 and 3.7, respectively. This increase was attributed to the cholesterol extraction property of these cyclodextrins from brain capillary endothelial cells leading to a modulation of the P-gp activity.

The blood–brain barrier (BBB) represents a major obstacle in the treatments of cerebral tumor since the majority of drugs do not reach the brain.[1] The BBB acts as an anatomical and transporter barrier notably due to the presence of tight junctions and the ATP-dependent efflux pump P-glycoprotein (P-gp), respectively. For example, the brain distribution of doxorubicin (DOX), an antitumoral agent widely used in the treatment of several cancers, is mainly restricted by P-gp on the BBB under normal physiological conditions.[2] To circumvent the brain limited access of DOX, different approaches were developed: drug delivery systems such as liposomes[3] or nanoparticles,[4] peptide-vector strategy using DOX linked to cationic peptides.[5] Coadministration of drug with a P-gp modulator, inhibiting the effect of the P-gp, has also been envisaged.[6] More invasive method such as temporary disruption of the BBB via osmotic pressure modification allowed to increase the DOX delivery.[7] Recently, we have described, on an in vitro model of BBB, a less drastic strategy using cyclodextrins (CD) since these compounds were able to modify the membrane composition of brain capillary endothelial cells (BCEC) by extracting lipid compounds.[8] As γ-CD was the less toxic CD and was the only native CD able to form an inclusion complex with DOX,[9] we have evaluated the ability of this association to improve the DOX transport to the brain.[10] The complexation by γ-CD did not succeed in increasing the brain DOX delivery. In fact, this inclusion complex crossed only slightly the BBB and no effect on P-gp activity was observed.

As α-CD and β-CD have more important interaction with BCEC[8] and are too small to include DOX, it was of great interest to evaluate their co-incubation in order to increase in DOX delivery to the brain. In this publication, the β-CD series was chosen to be incubated in the presence of DOX.

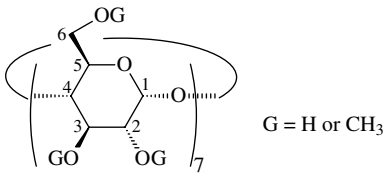
The β-CD and two methylated derivatives (Table 1) were used in order to increase the DOX delivery.

Before co-incubation, the threshold of toxicity of each CD, without DOX, has been determined. All experiments were realized on an in vitro model of BBB consisting of a co-culture of bovine BCEC with rat glial cells.[11] This model presents most of the in vivo BBB characteristics. Selected CD was placed on a monolayer of BCEC[12] and the integrity of this monolayer was checked by determination of the endothelial permeability of inulin across the BBB.

Keywords: Cyclodextrin; Doxorubicin; P-glycoprotein; Blood–brain barrier; Cholesterol.
* Corresponding author. Tel.: +33 3 21 79 17 54; fax: +33 3 21 79 17 55; e-mail: sebastien.tilloy@univ-artois.fr

0960-894X/$ - see front matter
doi:10.1016/j.bmcl.2006.01.049

Table 1. Structure of the various β-CDs



Abbreviation	Carbon bearing the OCH_3 group	Number of CH_3 groups by CD
β-CD	(−)	0
Rame-β-CD	2, 3, and 6	12.6
Crysme-β-CD	2, 3, and 6	5

[^{3}H]Inulin was used as an indicator of the functional integrity of the tight junctions. An endothelial permeability coefficient[13] for inulin ($Pe_{(Inulin)}$) higher than 0.4×10^{-3} cm min^{-1} was indicative of a leaky BBB. In our in vitro BBB model, the $Pe_{(Inulin)}$ across the monolayer was inferior to 0.2×10^{-3} cm min^{-1} (mean value of $0.15 \pm 0.03 \times 10^{-3}$ cm min^{-1}) in the control conditions. Figure 1 shows the evolution of $Pe_{(Inulin)}$ as a function of CD concentration and allows us to determine the CD concentration leading to a BBB breakdown. The CDs induced a toxicity in the order β-CD = Rame-β-CD > Crysme-β-CD with a threshold of toxicity equal to 2.5, 2.5, and 5 mM, respectively.

To evaluate the ability of these CDs to modify the brain delivery of DOX, β-CD and the two methylated derivatives have been used in this study at a concentration preserving the BBB integrity (1, 1, and 2.5 mM for β-CD, Rame-β-CD, and Crysme-β-CD, respectively). The DOX concentration was also fixed at 1 μM to avoid any toxic effect toward the BBB integrity.[10] DOX and selected CD were placed on a monolayer of BCEC and the transfer of DOX across this monolayer was observed after 2 h of incubation.[14] DOX transport to the brain at 1 μM without CD corresponds to 100% and is used as a reference (in this case, $Pe_{(DOX)} = 0.4 \times 10^{-3}$ cm min^{-1}). The integrity of the BCEC monolayer was checked by adding inulin. Figure 2 represents the evolution of DOX transport for the various β-CDs. Addition of each CD improved DOX transport through the BCEC monolayer. The increase was equal to 1.7, 2, and 3.7 in the presence of β-CD (not significant as demonstrated by one-way ANOVA), Rame-β-CD, and Crysme-β-CD, respectively. It is noteworthy that $Pe_{(Inulin)}$ was not significantly increased, indicating that this amelioration of DOX transport was not related to the loss of barrier integrity (data not shown).

The improvement in DOX delivery could be explained by two mechanisms. First, an interaction between the CDs and the BCEC components leading to a fragilization of the BBB, without loss of integrity, could be invoked. Second, an effect on the P-gp activity could be also envisaged. To gain an insight into these two questions, similar studies have been performed in the presence of urea (crossing the BBB only by passive diffusion) and in the presence of another P-gp substrate (vincristine, an antitumoral agent). These two compounds have been co-incubated with the Crysme-β-CD. Urea or vincristine and Crysme-β-CD were placed on a monolayer of BCEC and the transfer of each compound across this monolayer was observed after 2 h of incubation. Urea or vincristine transport to the brain at 0.78 μM or 50 nM without CD corresponds to 100% and is used as a reference (in this case $Pe_{(Urea)} = 2.2 \times 10^{-3}$ cm min^{-1} and $Pe_{(Vincristine)} = 0.2 \times 10^{-3}$ cm min^{-1}). Figure 3 represents the effect of Crysme-β-CD on urea and vincristine transport across the BBB.[15] The integrity of the BCEC monolayer was checked by adding inulin or sucrose.[16] In the presence of Crysme-β-CD, the urea transport is similar, whereas the vincristine transport is increased by a factor of two. In each case, it is important to notice that $Pe_{(Inulin)}$ or $Pe_{(Sucrose)}$ was not significantly modified, indicating that the BBB integrity was preserved (data not shown).

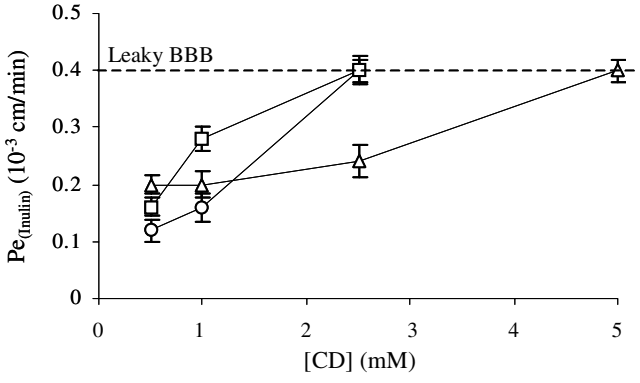


Figure 1. Effect of β-CD (□), Rame-β-CD (○), and Crysme-β-CD (△) concentrations on the endothelial permeability coefficient for inulin after 2 h of incubation. Transport studies were conducted at 37 °C, in buffered Ringer's solution at pH 7.4. Each point is the mean of three different filters and is representative of three series of independent experiments.

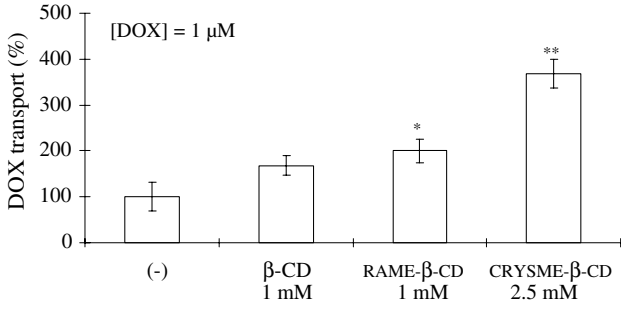


Figure 2. Effect of various β-CDs on DOX transport after 2 h of incubation. The transport of DOX to the brain was expressed according 100% to the reference (1 μM of DOX without CD). The 100% control value represents a Pe of 0.4×10^{-3} cm min^{-1} obtained after 2 h. Transport studies were conducted at 37 °C, in buffered Ringer's solution at pH 7.4. Each point is the mean of three different filters and is representative of three series of independent experiments. The mean scores were significant using one-way ANOVA ($p < 0.001$). $^*p < 0.05$, $^{**}p < 0.01$ versus control ($n = 9$ BCEC monolayers/treatment; Dunnet test).

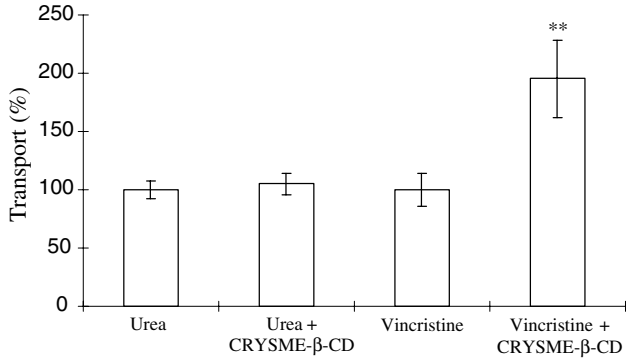


Figure 3. Effect of Crysme-β-CDs on urea and vincristine transport after 2 h of incubation. The transport of vincristine or urea to the brain was expressed according 100% to the reference (50 nM of vincristine and 0.78 μM of urea without CD). The 100% control value represents a Pe of 2.2×10^{-3} cm min^{-1} for urea and 0.2×10^{-3} cm min^{-1} for vincristine obtained after 2 h. Transport studies were conducted at 37 °C, in buffered Ringer's solution at pH 7.4. Each point is the mean of three different filters and is representative of three series of independent experiments. The mean scores were significant using one-way ANOVA ($p < 0.001$). $^{**}p < 0.01$ versus control ($n = 9$ BCEC monolayers/treatment; Dunnet test).

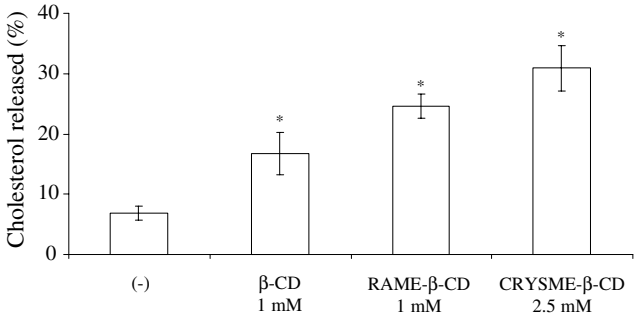


Figure 4. Cholesterol released from BCEC after 2 h of incubation in the presence of various β-CDs. The quantity of [^{3}H]cholesterol in the BCEC for the control was equal to 55400 ± 2280 d.p.m/well. Results are expressed as a percentage of cholesterol released from BCEC compared to the control. Each percentage is the mean of three different filters and is representative of three series of independent experiments. The mean scores were significant using one-way ANOVA ($p < 0.001$). $^{*}p < 0.05$, versus control ($n = 9$ BCEC monolayers/treatment; Dunnet test).

The methylated β-CDs are able to increase the transport of a P-gp substrate and have no effect on the urea transport. These results indicate that this phenomenon is P-gp specific and it is not due to a fragilization of the BBB. A modification of P-gp activity probably takes place.

P-gp was observed to be located in the caveolae of BCEC on our in vitro model of BBB.[17] Caveolae are flask-shaped plasma membrane invaginations involved in many cellular events such as signal transduction, lipid and protein sorting, endocytosis, and potocytosis. Caveolar microdomains are enriched in glycosphingolipids, cholesterol, sphingomyelin, and a number of signal transduction molecules.[18] On our in vitro model of BBB, it was demonstrated that cholesterol is important for the transport activity of P-gp because in the presence of cholesterol-depletion agents, P-gp activity was reduced.[17]

So, in our study, the modification of P-gp activity is probably due to a cholesterol extraction from the plasma membrane in the presence of these CDs. Indeed, we have already demonstrated that β-CD released cholesterol from BCEC[8] and in this way cholesterol efflux studies were envisaged in the presence of methylated β-CDs. Figure 4 represents the percentage of cholesterol extracted from endothelial cells by the β-CD and its methylated derivatives at the concentration used above.[19]

Release of cholesterol from BCEC was clearly dependent on the nature and the concentration of the CDs used. Indeed, the cholesterol efflux was equal to 16%, 21%, and 31% for β-CD (at 1 mM), Rame-β-CD (at 1 mM), and Crysme-β-CD (at 2.5 mM), respectively. The most important efflux induced by methylated-β-cyclodextrin can be explained by the values of association constants with cholesterol (β-CD/cholesterol = 20,000 M^{-1} compared to Rame-β-CD/cholesterol = 57,000 M^{-1})[20] and the higher concentration used in the case of Crysme-β-CD. In addition, other studies of cholesterol efflux mediated by β-cyclodextrin or Rame-β-cyclodextrin have shown that Rame-β-cyclodextrin was a greater cholesterol extractor compared to β-cyclodextrin.[21] In addition, it is very important to notice that the DOX transport increase is related to this efflux. The more significant the cholesterol efflux is, the higher the DOX delivery is. These results demonstrate that the methylated β-CDs used are implicated in the deliverance of P-gp substrates by modifying the P-gp activity.

Similar observations have been reported on Caco-2 cell monolayers. The enhancing effect of dimethyl-β-cyclodextrin (DM-β-CD) on the oral bioavailability of tacrolimus (an immunosuppressor, P-gp substrate) on Caco-2 cell monolayer is due, at least in part, to its inhibitory effect on the P-gp-mediated efflux.[22] The inhibitory effect of DM-β-CD on P-gp could be attributed to the release of this transporter from apical membranes into the medium as secondary effects through cholesterol depletion in caveolae.[23] Another study has shown that the treatment of Madin Dardy Canine Kidney (MDCK) cells with Rame-β-CD did not affect viability but altered the structural appearance of the MDCK cells and abolished efflux of rhodamine 123 (a P-gp substrate).[24] The presence of Rame-β-CD reduced significantly the amount of cholesterol in the raft fractions. These results show that removal of cholesterol modulates the membrane lipid composition, changes the localization of P-gp, and results in loss of P-gp function.

In conclusion, methylated β-CDs are able to reduce the P-gp activity by extracting the cholesterol from BCEC. This behavior allows to increase the delivery of P-gp substrates. The effect obtained for doxorubicin deliverance is in the same order of magnitude as those depicted

in vivo in the absence of P-gp.[25] Now, further works are necessary to target specifically the BBB.

References and notes

1. Pardrige, W. *Mol. Interv.* **2003**, *100*, 90.
2. Ohnishi, T.; Tamai, I.; Sakanaka, K.; Sakata, A.; Yamashima, T.; Yamashita, J.; Tsuji, A. *Biochem. Pharmacol.* **1995**, *17*, 1541.
3. Huwyler, J.; Wu, D.; Pardridge, W. M. *Proc. Natl. Acad. Sci.* **1996**, *93*, 14164.
4. Kreuter, J.; Shamenkov, D.; Petrov, V.; Ramge, P.; Cychutek, K.; Koch-Brandt, C.; Alyautdin, R. *J. Drug Target.* **2002**, *10*, 317.
5. Rousselle, C.; Smirnova, M.; Clair, P.; Lefauconnier, J. M.; Chavanieu, A.; Calas, B.; Scherrmann, J. M.; Temsamani, J. *J. Pharmacol. Exp. Ther.* **2001**, *296*, 124.
6. Fenart, L.; Buee-Scherrer, V.; Descamps, L.; Duhem, C.; Poullain, M. G.; Cecchelli, R.; Dehouck, M. P. *Pharm. Res.* **1998**, *15*, 993.
7. Neuwelt, E. A.; Pagel, M.; Barnett, P.; Glassberg, M.; Frenkel, E. P. *Cancer Res.* **1981**, *41*, 4466.
8. Monnaert, V.; Tilloy, S.; Bricout, H.; Fenart, L.; Cecchelli, R.; Monflier, E. *J. Pharmacol. Exp. Ther.* **2004**, *310*, 745.
9. Bekers, O.; Beijnen, J. H.; Otagiri, M.; Bult, A.; Underberg, W. J. M. *J. Pharm. Biomed. Anal.* **1990**, *8*, 671.
10. Monnaert, V.; Betbeder, D.; Fenart, L.; Bricout, H.; Lenfant, A. M.; Landry, C.; Cecchelli, R.; Monflier, E.; Tilloy, S. *J. Pharmacol. Exp. Ther.* **2004**, *311*, 1115.
11. Cecchelli, R.; Dehouck, B.; Descamps, L.; Fenart, L.; Buee-Scherrer, V.; Duhem, C.; Lundquist, S.; Rentfel, M.; Torpier, G.; Dehouck, M. P. *Adv. Drug Del. Rev.* **1999**, *36*, 165.
12. For further information on the in vitro model of BBB, see: Dehouck, M. P.; Jolliet-Riant, P.; Brée, F.; Fruchart, J. C.; Cecchelli, R.; Tillement, J. P. *J. Neurochem.* **1992**, *58*, 1790.
13. The amount of product crossing the BBB was expressed in endothelial permeability (Pe, cm/min). The cleared volume was calculated by dividing the amount of compound in the receiver compartment by the product concentration in the donor compartment at each time point. The average cumulative volume cleared was plotted versus time and the slope was estimated by linear regression analysis to give the mean and the standard deviation of the estimate. The slope of the clearance curve with inserts alone and inserts with BCEC monolayer is equal to PSf and PSt, respectively, where PS = the permeability surface area product. The units of PS and S are microliters/minute and square centimeters, respectively. The PS value for endothelial monolayer (PSe) was obtained as follows:

$$1/\mathrm{PSe} = 1/\mathrm{PSt} - 1/\mathrm{PSf}.$$

To generate the endothelial permeability coefficient, Pe (cm/min), the PSe value were divided by the surface area of the insert. Amounts of [^{3}H]inulin in each compartment was determined with a liquid scintillation counter (Tri-Carb 2100TR).
14. All transport studies were conducted at 37 °C, in buffered Ringer's solution at pH 7.4. Ringer Hepes (RH) solution containing DOX, inulin, and CD, at the required concentrations, was placed at time zero in the apical compartment. The plates were then placed on a rocking platform. At selected times, 30, 60, and 120 min after the addition of the solution, the insert was moved to other wells of the plate to minimize back diffusion of compound to the upper compartment. Three inserts with BCEC monolayer and three without cells were assayed for each solution.Amounts of [^{3}H]inulin and [^{14}C]doxorubicin in each compartment was determined with a liquid scintillation counter (Tri-Carb 2100TR).
15. Amounts of [^{14}C]urea and [^{3}H]vincristine in each compartment were determined with a liquid scintillation counter (Tri-Carb 2100TR).
16. [^{3}H]Inulin or [^{14}C]sucrose was used as an indicator of the functional integrity of the tight junctions for [^{14}C]urea or [^{3}H]vincristine, respectively.
17. Jodoin, J.; Demeule, M.; Fenart, L.; Cecchelli, R.; Farmer, S.; Linton, K. J.; Higgins, C. F.; Beliveau, R. *J. Neurochem.* **2003**, *87*, 1010.
18. Shaul, P. W.; Anderson, R. G. W. *Am. J. Physiol.* **1998**, *275*, L843.
19. At the 10th day of co-culture, [^{3}H]cholesterol (0.66 μCi/mL) was added in the cell-conditioned medium in the upper compartment. BCEC were incubated in this radioactive medium for 48 h, and under these conditions cells were radiolabeled with cholesterol. BCEC were washed with PBS-CMF and incubated in DMEM supplemented with 5% (v/v) heat-inactivated calf serum and horse serum for 4 h. The cell monolayers were washed with PBS-CMF and incubated in transport buffer containing CDs or not (control) for 2 h. The control was incubated only with the transport buffer for 2 h. The concentration of [^{3}H]cholesterol in BCEC in the presence and in the absence (control) of CDs after 2 h of experiment was determined in a liquid scintillation counter (Tri-Carb 2100TR). Results are expressed as a percentage of the total cholesterol released from BCEC in the presence of CD compared to the control.
20. (a) Frijlink, H. W.; Eissens, A. C.; Helfting, N. R.; Poelstra, K.; Lerk, C. F.; Meijer, D. K. F. *Pharm. Res.* **1991**, *8*, 9; (b) Nishijo, J.; Moriyama, S.; Shiota, S. *Chem. Pharm. Bull.* **2003**, *51*, 1253.
21. Kilsdonk, E. P. C.; Yancey, P. G.; Stoudt, G. W.; Bangerter, F. W.; Johnson, W. J.; Phillips, M. C.; Rothblat, G. H. *J. Biol. Chem.* **1995**, *270*, 17250.
22. Arima, H.; Yunomae, K.; Hirayama, F.; Uekama, K. *J. Pharmacol. Exp. Ther.* **2001**, *297*, 547.
23. Yunomae, K.; Arima, H.; Hirayama, F.; Uekama, K. *FEBS Lett.* **2003**, *536*, 225.
24. Kamau, S. W.; Kramer, S. D.; Gunther, M.; Wunderli-Allenspach, H. *In vitro Cell. Dev. Biol. Anim.* **2005**, *41*, 207.
25. Cisternino, S.; Rousselle, C.; Dagenais, C.; Schermann, J. M. *Pharm. Res.* **2001**, *18*, 183.