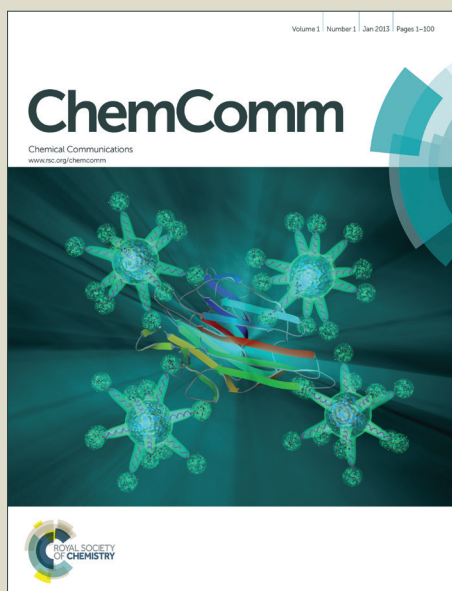


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COMMUNICATION

***para*-Sulphonato-calix[n]arenes as selective activators for the passage of molecules across the Caco-2 model intestinal membrane**

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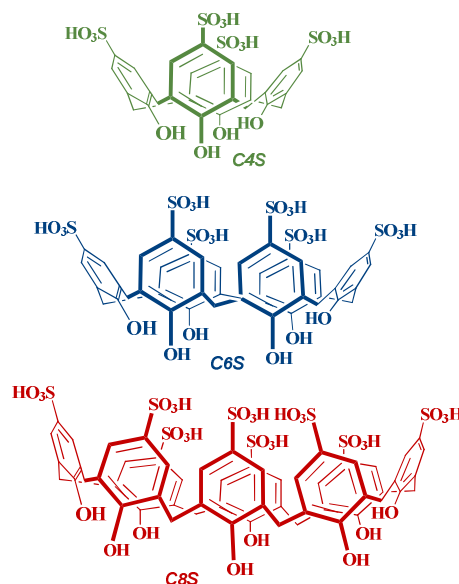
The passage of Lucifer Yellow across the Caco-2 intestinal model membrane has been studied for the *para*-sulphonato-calix[n]arenes, the results show that *para*-sulphonato-calix[4]arene and *para*-sulphonato-calix[8]arene activate membrane passage when used simultaneously with a transport probe, Lucifer Yellow, whereas *para*-sulphonato-calix[6]arene has no effect.

The formation of a confluent monolayer of Caco-2 cells was developed by Fogh in 1975.¹ This cell line consists of heterogenous human epithelial colorectal adenocarcinoma cells, which when cultured under certain conditions become differentiated and polarized. Under these conditions the cells resemble in their morphology and functionality the enterocytes forming the lining of the small intestine.² In the confluent monolayers the Caco-2 cells form tight junctions, and give rise to microvilli, they also express enzymes and transport systems typical of such intestinal cells. When they are cultured on a cell culture insert filter to yield the confluent monolayer, they form a polarised epithelial cell monolayer that provides a physical and biochemical barrier similar to that of the gut.³ This monolayer is widely used as *an in vitro* model of the human small intestine mucosa thus allowing prediction for the absorption of orally administered Active Pharmaceutical Ingredients, (APIs). The adoption of the Caco-2 model undoubtedly contributed to the reduction in API failures due to pharmacokinetic problems from 40% in the early 1990s to about 10% in 2009.

A second major problem in the development of APIs is their often low solubility in physiological systems. Much research has been devoted to the use of supramolecular systems to complex, solubilise and transport APIs.⁴ The two major molecular systems are the cyclodextrins,⁵ and the water soluble calix[n]arenes.⁶ Cyclodextrins have been widely studied for their transport properties,⁵ however their inherent hematotoxicity,⁷ and their complex chemistry have acted to reduce interest in these molecules. A number of studies have been undertaken on the transport capacities of the cyclodextrin-API

complexes across the Caco-2 model membrane and indeed transport has been shown.⁸ With regard to the calix[n]arenes their use as transport agents was initially effectively blocked by a lack of aqueous solubility. With the synthesis of the *para*-sulphonato-calix[n]arenes by Shinkai in 1984,⁹ their use in biological systems became possible. Subsequently a large body of work has accumulated on the biochemistry and API complexation in solution¹⁰ and in the solid state.¹¹ In contrast to the cyclodextrins the *para*-sulphonato-calix[n]arenes appear to present no haemolytic activity¹² and have shown no *in vivo* toxicity.¹³

In the current publication we present the first study of the transport properties of the *para*-sulphonato-calix[n]arenes with regard to the Caco-2 model of the intestinal membrane. It is of interest that we observe clear differences between the transport activity as a function of macrocycle size.



Scheme 1 : colour coded molecular structures of the three *para*-sulphonato-calix[n]arenes used in the current work.

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The initial study involved determination of the IC₅₀ values of the three *para*-sulphonato-calix[n]arenes regard to the Caco-2 cells used in the transport studies. The IC₅₀ values were determined using a standard MTT test.¹⁴

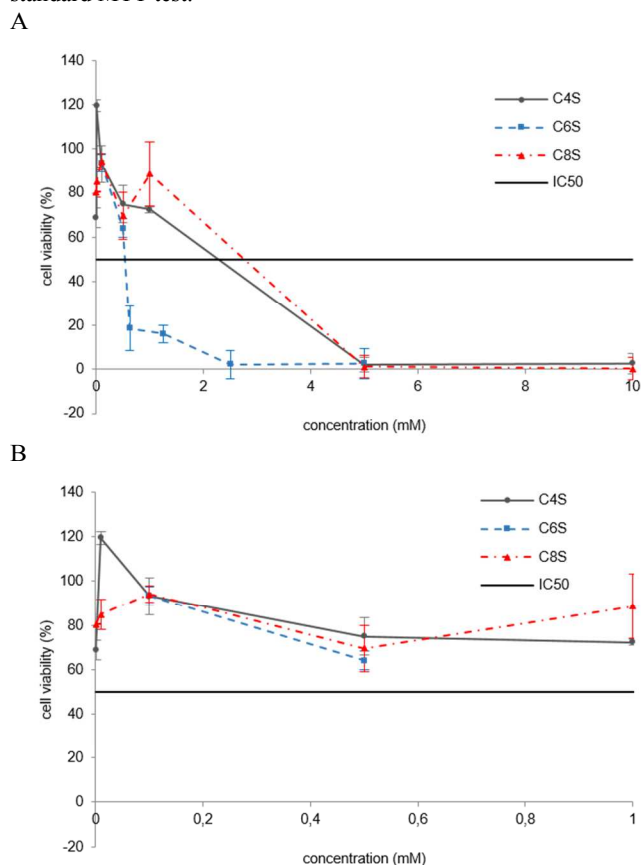


Figure 1: MTT test on the Caco-2 cells in the presence of three *para*-sulphonato-calix[n]arenes, **A** over the concentration range 1mM–10mM, **B** over the concentration range above the IC₅₀ values.

The MTT assay was performed on Caco-2 cells seeded in 96 well plate until cell monolayer become confluent. After the medium was removed and the cells were washed with phosphate buffer (PBS). They were then treated with increasing concentrations, up to 10 mM of the three *para*-sulphonato-calix[n]arenes dissolved in PBS. A control group was treated in the same manner without the application of any calixarenes. After incubation at 37°C during 30 minutes the cells were washed and treated with MTT dye ((3-(4,5-dimethylthiazol-2-yl))-2,5-diphenyltetrazolium bromide, 5mg/ml) during 3 hours. MTT solution was removed and isopropanol : hydrochloric acid (25:1) was added to dissolve the formed formazan crystals. The absorbance was measured at 570 nm against a 690 nm reference. The cell viability was expressed as the percentage of untreated control.¹⁵

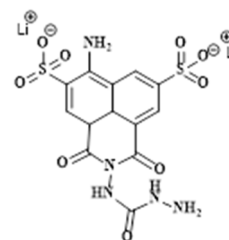
The behaviour of the three compounds is quite different. For *para*-sulphonato-calix[4]arene a cell viability of 100% is maintained up to 1mM and then decreases to 0% at 5mM. The observed IC₅₀ is at 2mM. With regard to *para*-sulphonato-calix[6]arene the behaviour is very different, here an IC₅₀ value of about 0.5mM is observed with 0% viability at 2mM. It should be noted that the purity of the compound was verified and more importantly this synthetic batch was used in our recent paper on ROS activity where no anomalous cell toxicity was observed.¹⁶ For *para*-sulphonato-calix[8]arene,

100% cell viability is maintained until 1mM, decreasing to 0% at 5mM. The observed IC₅₀ value here was 2.6 mM.

For the transport experiments the concentrations of the *para*-sulphonato-calix[n]arenes were set below the IC₅₀ value to ensure effects were not due to cell mortality.

To measure transport across the confluent monolayer, Caco-2 cells (2×10^5) were seeded in 12-well Transwell inserts at a density of 2×10^5 cells cm^{-2} and grown for 30–35 days. Trans-endothelial electrical resistance (TEER) was measured when the medium was changed every third day. The inserts were used for experiments when the TEER values reached $1000 \Omega \times \text{cm}^2$. The culture medium was removed and the apical and basolateral compartments of the Caco-2 cell monolayers were washed with Hank's Balanced Salt Solution (HBSS).

In the experiments involving pre-treatment with calixarenes, the cells were exposed to 500 μl of calixarene solution in HBSS and applied in the apical chamber 1.5 ml of HBSS solution was placed the basal chamber. The pre-treatment was carried out during 30 minutes at 37°C. After treatment cells were washed with HBSS solution and then Lucifer Yellow dye was applied at 40 $\mu\text{g}/\text{ml}$ concentration diluted with HBSS into the apical chamber and HBSS into the basal chamber.



Scheme 2 Molecular structure of Lucifer Yellow

In the case of testing without pre-treatment, the calixarene concentrations used were set at, *para*-sulphonato-calix[4]arene = 2.5 mM, *para*-sulphonato-calix[6]arene = 0.5 mM and *para*-sulphonato-calix[8]arene = 1.25 mM, and Lucifer Yellow (40 $\mu\text{g}/\text{ml}$) in HBSS was applied into the apical chamber, and only HBSS into the basal chamber. In each cases samples were taken from the basal chamber in the 5, 10, 20, 30, 40, 50, 60, 90 and 120 minutes and were replaced by fresh HBSS. Samples were collected in 96 well black plate and fluorescence was measured at an excitation wavelength of 450 nm by FLUOstar OPTIMA Microplate Reader. The values of TEER was measured in the beginning of the experiment and after 120 minutes.¹⁷

Concerning TEER measurements; when pre-treatment with *para*-sulphonato-calix[n]arenes was used, the TEER value remained above $1000 \Omega \times \text{cm}^2$, with no significant difference from the starting value, showing that the calix[n]arenes do not degrade the Caco-2 monolayer structure. For the combined transport experiments using the *para*-sulphonato-calix[n]arenes and Lucifer Yellow dye together, the TEER value seriously decreased to, under $200 \Omega \times \text{cm}^2$ for C4S and C8S and around $600 \Omega \times \text{cm}^2$ for C6S compared to the starting value above $1000 \Omega \times \text{cm}^2$.

The results of the transport experiments are shown in Figure 2 below, colour coded to correspond to those used in Scheme 1.

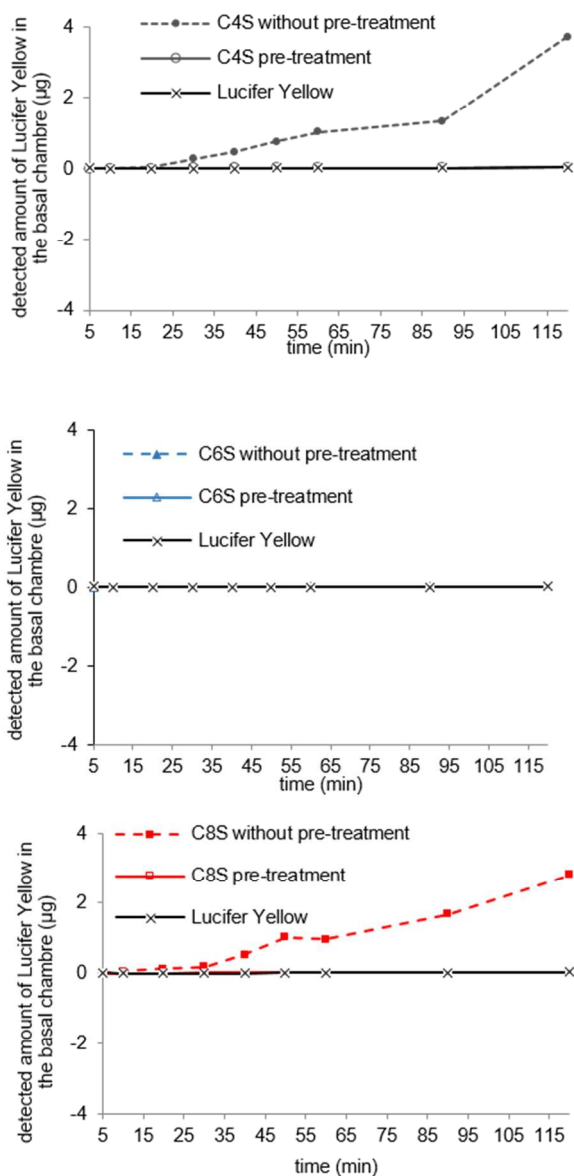


Figure 2 Transport of Lucifer yellow across the confluent Caco-2 monolayer using *para*-sulphonato-calix[n]arenes as transporter molecules, the Lucifer Yellow control is carried out in the absence of *para*-sulphonato-calix[n]arenes but with Lucifer Yellow concentrations the same as used in the combined transport experiments.

As for the TEER measurements, a very clear difference is observed between C4S and C8S on one hand and C6S on the other hand. For C6S there is no passage of Lucifer Yellow across the Caco-2 membrane neither with pre-treatment nor for the combined transport experiment. For C4S, no passage is induced by pre-treatment. however, for the combined transport experiment, after an induction period of 20 minutes transport commences giving 1 µg detected after 90 minutes. Above this time passage increases sharply to about 3.5 µg after 120 minutes. The behaviour of C8S is generally similar, with no Lucifer Yellow passage induced by pre-treatment. However for combined transport there is a small but real passage in the 5 minutes to 30 minutes zone and an effectively linear increase until 120 minutes where passage of about 2.8 µg is observed.

Conclusions

Determination of the induction of molecular passage of the model compound Lucifer Yellow across the Caco-2 model of the human small intestine mucosa, by certain *para*-sulphonato-calix[n]arenes represents an important step in their future use as transporter systems for APIs. The difference between C4S and C8S on one hand and C6S on the other is of interest and suggests that there may be a different mode of action with Caco-2 confluent monolayers which is in agreement with a difference in the effect on Trans-Endothelial Electrical Resistance for C4S and C8S compared to the effect of C6S. Of particular impact is the requirement for the combined use of the *para*-sulphonato-calix[n]arenes and Lucifer Yellow to induce transport. Work is currently being undertaken to understand these differences in action.

Notes and references

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1 J. Fogh, and G. Trempe. *Human Cancer Cells in Vitro*, ed. J.Fogh, Plenum, 1975, 115-141

2 I. J. Hidalgo, T.J. Raub, and R.T. Borchardt, *Gastroenterology*, 1989, **96**, 736-749.

3 P. Artursson, *J.Pharm.Sci.*, 1990, **79**, 476-82

4 J.W. Steed and J.L. Atwood, *Supramolecular Chemistry*, 2nd Edition, 2009, J.Wiley, Chichester, U.K.

5 Z.-Y. Jin, *Cyclodextrin Chemistry: Preparation and Application*, 2013, World Scientific Publishing Co. Singapore,

6 F. Perret, and A. W. Coleman, *Chem. Commun.*, 2011, **47**, 7303 - 7319, F. Perret, A. N. Lazar and A. W. Coleman, *Chem. Commun.*, 2006, 2425 - 2438

7 T. Kiss, F. Fenyvesi, I. Bacskay, J. Varadi, E. Fenyvesi, R. Ivanvi, L. Szente, A. Tsaki and M. Vecsernyes, *Eur. J. Pharm. Sci.*, 2010, **40**, 376-380

8 A.E. Daruhazi, T. Kiss, T.M. Vecsernyes, L. Szente, E. Szoke, and E. Lemberkovics, *J. Pharm. Biomed. Anal.*, 2013, **84**, 112-116, M. Okawara, Y. Tokudome, H. Todo, K. Sugibayashi and F.Hashimoto, *Biol. Pharm. Bull.*, 2014, **37**, 54-59

9 S. Shinkai, S. Mori, T. Tsubaki, T. Sone and O. Manabe, *Tetrahedron Lett.*, 1984, **25**, 5315-5318.

10 D.-S. Guo and Y. Liu, *Acc. Chem. Res.*, 2014, **47**, 1925-34

11 O. Danyluk and K. Suwinska, *Chem. Commun.*, 2009, 5799-5813

12 E. Da Silva, P. Shahgaldian and A.W. Coleman, Anthony W., *Int. J. Pharm.*, 2004, **273**, 57-62.

13 A. W. Coleman, F. Perret, S. Cecillon, A. Moussa, A. Martin, M. Dupin and H. Perron, *New J. Chem.*, 2007, **31**, 711 - 717

14 T.F.Slater, B. Sawyer and U. Straeuli, *Biochim. Biophys. Acta*, 1963, **77**, 383-393.

15 T. Mosmann *J. Immunol. Meth.*, 1983, **65**, 55-63.

16 E.K. Stephens, Y. Tauran, A.W. Coleman and M. Fitzgerald, *Chem. Commun.*, 2015, **51**, 851-854

17 R. Konsoula and F.A. Barile, F.A., *Toxicol. in Vitro*, 2005, **19**, 675-684.