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Short communication

Antiseptic properties of two calix[4]arenes derivatives on the human coronavirus 229E☆

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ABSTRACT

Facing the lack in specific antiviral treatment, it is necessary to develop new means of prevention. In the case of the *Coronaviridae* this family is now recognized as including potent human pathogens causing upper and lower respiratory tract infections as well as nosocomial ones. Within the purpose of developing new antiseptics molecules, the antiseptic virucidal activity of two calix[4]arene derivatives, the *tetra-para*-sulfonato-calix[4]arene (C[4]S) and the 1,3-bis(bithiazolyl)-*tetra-para*-sulfonato-calix[4]arene (C[4]S-BTZ) were evaluated toward the human coronavirus 229E (HCoV 229E). Comparing these results with some obtained previously with chlorhexidine and hexamidine, (i) these two calixarenes did not show any cytotoxicity contrary to chlorhexidine and hexamidine, (ii) C[4]S showed as did hexamidine, a very weak activity against HCoV 229E, and (iii) the C[4]S-BTZ showed a stronger activity than chlorhexidine, i.e. 2.7 and 1.4 $\log_{10}$ reduction in viral titer after 5 min of contact with 10^{-3} mol L^{-1} solutions of C[4]S-BTZ and chlorhexidine, respectively. Thus, the C[4]S-BTZ appeared as a promising virucidal (antiseptic) molecule.

The lack in specific antiviral treatments is still persisting, considering the large variety of viruses already circulating among human population and the potential emerging ones. The *Coronaviridae* family illustrates this problem. Indeed, no specific treatment is available to fight coronaviruses infections, while they are known to be responsible for upper and lower tract infections as well as nosocomial ones. Thus, efficient means of prevention, as an adapted antisepsis-disinfection (ATS-D), should be developed to prevent the environmental spread of such infections.

Human coronaviruses (HCoV) were historically known to be responsible for about 20% of common colds and other upper respiratory tract infections (Larson et al., 1980). Before the SARS (severe acute respiratory syndrome) epidemic in 2002–2003, only two HCoV were known, the 229E strain and the OC43 strain. This serious outbreak, due to a newly discovered HCoV, the SARS-CoV (Ksiazek et al., 2003; Peiris et al., 2003), reinforced the interest into the *Coronaviridae* family. Indeed, coronaviruses were since involved in more serious respiratory diseases, i.e. bronchitis, bronchiolitis or pneumonia, especially in young children and neonates (Gagneur et al., 2002; Gerna et al., 2006), elderly people (Falsey et al., 2002) and immunosuppressed patients (Gerna et al., 2007; Pene et al., 2003). Furthermore, they have been shown to survive for at least several hours under different environmental conditions (Ijaz et al., 1985; Lai et al., 2005; Rabenau et al., 2005a; Sizun et al., 2000). Finally, their adaptive properties and their ability of species barrier crossing, involve a significant possibility of new coronaviruses emergence (Laude et al., 1998; Li et al., 2005; Vijgen et al., 2005). Thus, these specificities (i.e. pathogenicity, potential environmental resistance and evolutionary ability) make the *Coronaviridae* family a pertinent model for studying ATS-D activity.

New antiviral molecules are urgently needed. Within this purpose, some macrocyclic compounds belonging to the calixarene family (de Fátima et al., 2009; Rodik et al., 2009), have already been shown to be interesting as anti-HIV and anti-HSV agents (Coveney and Costello, 2005; Harris, 1995, 2002; Hwang et al., 1994; Kral et al., 2005; Motornaya et al., 2006). In this field, our team described antiviral properties of various derivatives, such as 1,3-bis(bithiazolyl)-*tetra-para*-sulfonato-calix[4]arene (C[4]S-BTZ) and *tetra-para*-sulfonato-calix[4]arene (C[4]S) (Mourer et al., 2010) (Fig. 1).

Abbreviations: ATS-D, antiseptic-disinfectant or antisepsis-disinfection; C[4]S, tetra-para-sulfonato-calix[4]arene; C[4]S-BTZ, 1,3-bis(bithiazolyl)-tetra-para-sulfonato-calix[4]arene; CC_{50}, cytotoxic concentration 50%; CHX, chlorhexidine; HCoV, human coronavirus; HXM, hexamidine; IC_{50}, inhibitory concentration 50%; MTT, methyl thiazole tetrazolium; NR, neutral red; SARS, severe acute respiratory syndrome.

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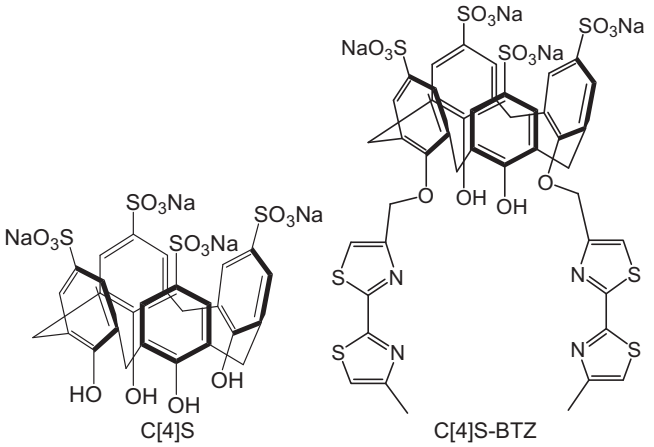

Fig. 1. Structures of the *tetra-para*-sulfonato-calix[4]arene (C[4]S) and the 1,3-bis(bithiazolyl)-*tetra-para*-sulfonato-calix[4]arene (C[4]S-BTZ).

Table 1
Results of controls (non retention and neutralization) for antiseptic virucidal assays validation.

Non retention of virus after filtration on Sephadex™ G-25 columns		
Contact times	C[4]S[a]	C[4]S-BTZ[a]
5 min	ND[b]	0.3 ± 0.2
15 min	ND[b]	0.3 ± 0.2
30 min	0.3 ± 0.2	0.3 ± 0.2
60 min	0.3 ± 0.1	0.2 ± 0.1
Neutralization controls		
Concentrations (mol L^{-1})	C[4]S[a]	C[4]S-BTZ[a]
10^{-3}	0.3 ± 0.2	0.2 ± 0.2
10^{-4}	ND[b]	0.4 ± 0.1

Non retention of viruses (HCoV 229E) by Sephadex™ G-25 columns has been checked out for each tested contact times and neutralization efficiency for each tested concentrations. To be accepted, a control should present a $\log_{10}$ difference lower than 0.5 $\log_{10}$.

[a] Expressed as $\log_{10}$ reduction.
[b] Not determined.

The potential ATS-D activities of C[4]S and C[4]S-BTZ were then estimated on the HCoV 229E (ATCC VR 740), cultivated on L-132 cells (ATCC CCL-5).

To evaluate these properties, a protocol described elsewhere (Geller et al., 2009) has been implemented. It responded to the general imperatives of the only European standard existing (NF EN 14476 + A1) to evaluate ATS-D antiviral activity in human medicine (AFNOR, 2007). According to this standard, a product should induce a 4 $\log_{10}$ reduction in viral titers to quality as an ATS-D antiviral activity. For comparison, American standards recommend a reduction of 3 $\log_{10}$ as efficiency criterion (ASTM, 1996, 1997).

The general principle of our protocol is: (i) to incubate viruses with the test product, at room temperature, for a defined contact time, (ii) to neutralize product activity and (iii) to estimate the loss in viral titers. The neutralization process allows: (i) to stop the potential antiviral activity of the product, (ii) to remove its eventual cytotoxicity and (iii) to prevent interference, due to the test itself, in viral infectivity. It was achieved thanks to a gel filtration method, using Sephadex™ G-25 columns, developed and validated previously (Geller et al., 2009). These assays required appropriate controls especially to check the non retention of viruses by Sephadex™ columns, the absence of interference with viral infectivity, the efficiency of neutralization and the absence of cytotoxicity (Supp. data 1). As recommended by the European Standard NF EN 14476 + A1, controls are validated if the difference between viral titers, with and without treatment, is less than 0.5 $\log_{10}$ (AFNOR, 2007).

Molecular masses of C[4]S and C[4]S-BTZ were 1069.80 g mol^{-1} and 1365.22 g mol^{-1} respectively. Thus, they were susceptible to be retained by the Sephadex™ G-25 columns. To assess the non cytotoxicity of the filtrates, cytotoxic assays and spectrophotometric measurements were conducted. Two concentrations for both molecules were tested, i.e. 10^{-4} and 10^{-3} mol L^{-1}.

MTT (methylthiazole tetrazolium) and NR (neutral red) assays were first performed to evaluate cytotoxicity of C[4]S and C[4]S-BTZ on L-132 cells. For both molecules, IC_{50} (inhibitory concentration 50%) and CC_{50} (cytotoxic concentration 50%) were higher than 10^{-4} mol L^{-1}, even after 168 h, the time required for obtaining the HCoV 229E cytopathogenic effect. The same assays were then performed with the filtrates obtained after filtration of both molecules on Sephadex™ G-25 columns and cytotoxicity was also higher than 10^{-4} mol L^{-1} even after 168 h of incubation.

Spectrophotometric analyses, coupled with regression analyses, allowed to determine the specific parameters of each molecule (Supp. data 2). Retention rates by Sephadex™ G-25 columns were then estimated after evaluation of residual concentration in the filtrates. Retention rates of C[4]S solutions by Sephadex™ G-25 columns were 98.9% and 88.5% for solutions at 10^{-3} mol L^{-1} and 10^{-4} mol L^{-1}, respectively. The lower retention rate of 10^{-4} mol L^{-1} solution was due to calculation limitations. Retention rate of the solution at 10^{-3} mol L^{-1} was considered as significant. In the case of C[4]S-BTZ, retention rates were 94.8% and 99.4% for solutions of 10^{-3} and 10^{-4} mol L^{-1}, respectively (Supp. data 2).

ATS-D antiviral assays were then conducted. Each experiment was performed in triplicate. To validate these tests, controls, mentioned above, were done the same time, and results are shown for both molecules in Table 1.

The C[4]S was tested at a concentration of 10^{-3} mol L^{-1} and contact times of 30 min and 60 min. Because of the very weak activity against HCoV 229E, i.e. 0.5 and 0.6 $\log_{10}$ reduction for contact times of 30 min and 60 min, respectively, no further experiments were performed with C[4]S (Fig. 2).

Results obtained with the C[4]S-BTZ were markedly better. Indeed, at a concentration of 10^{-4} mol L^{-1}, it induced $\log_{10}$ reductions of 0.3, 0.6, 0.8 and 1.0 after contact times of 5, 15, 30 and 60 min, respectively. At 10^{-3} mol L^{-1}, it induced reductions of 2.7, 2.7, 2.4 and 2.8 $\log_{10}$ in viral titers for the same contact times (Fig. 2).

Previous experiments have been conducted with two largely used antiseptics in human medicine, hexamidine (HXM) and chlorhexidine (CHX) (Geller et al., 2009). First of all, both of these molecules showed cytotoxicity towards L-132 cells. Indeed, CHX showed IC_{50} and CC_{50} values of 4.3×10^{-6} and 6.0×10^{-6} mol L^{-1}, respectively, after 24 h. In the same way, IC_{50} and CC_{50} of HXM were 3.8×10^{-5} and 5.5×10^{-5} mol L^{-1} after 24 h of contact time. Thus, the first interest of these two calix[4]arenes was the absence of cytotoxicity (>10^{-4} mol L^{-1} after 168 h), contrary to HXM and CHX.

The HXM, in the manner of the C[4]S, showed a very weak activity on the HCoV 229E, i.e. 0.6 and 0.9 $\log_{10}$ reduction after contact times of 30 min and 60 min, respectively. The CHX showed a better activity, since it induced 0.8, 0.5, 1.4 and 2.1 $\log_{10}$ reduction at 10^{-4} mol L^{-1} for contact times of 5, 15, 30 and 60 min, respectively, and 1.4, 2.1, 2.4 and 3 $\log_{10}$ reduction at 10^{-3} mol L^{-1} and for the same contact times (Fig. 2).

When comparing C[4]S-BTZ and CHX activities, the first important point is that, even if they showed a certain anti-HCoV 229E activity, they did not reach the threshold fixed by European and American standards, except for CHX at 10^{-3} mol L^{-1} and 60 min of contact time. However, this contact time could not be considered as really representative time for ATS-D in field conditions.

Thus, a really attractive characteristic of the C[4]S-BTZ was its fast action at 10^{-3} mol L^{-1}, as soon as 5 min, compared to CHX,

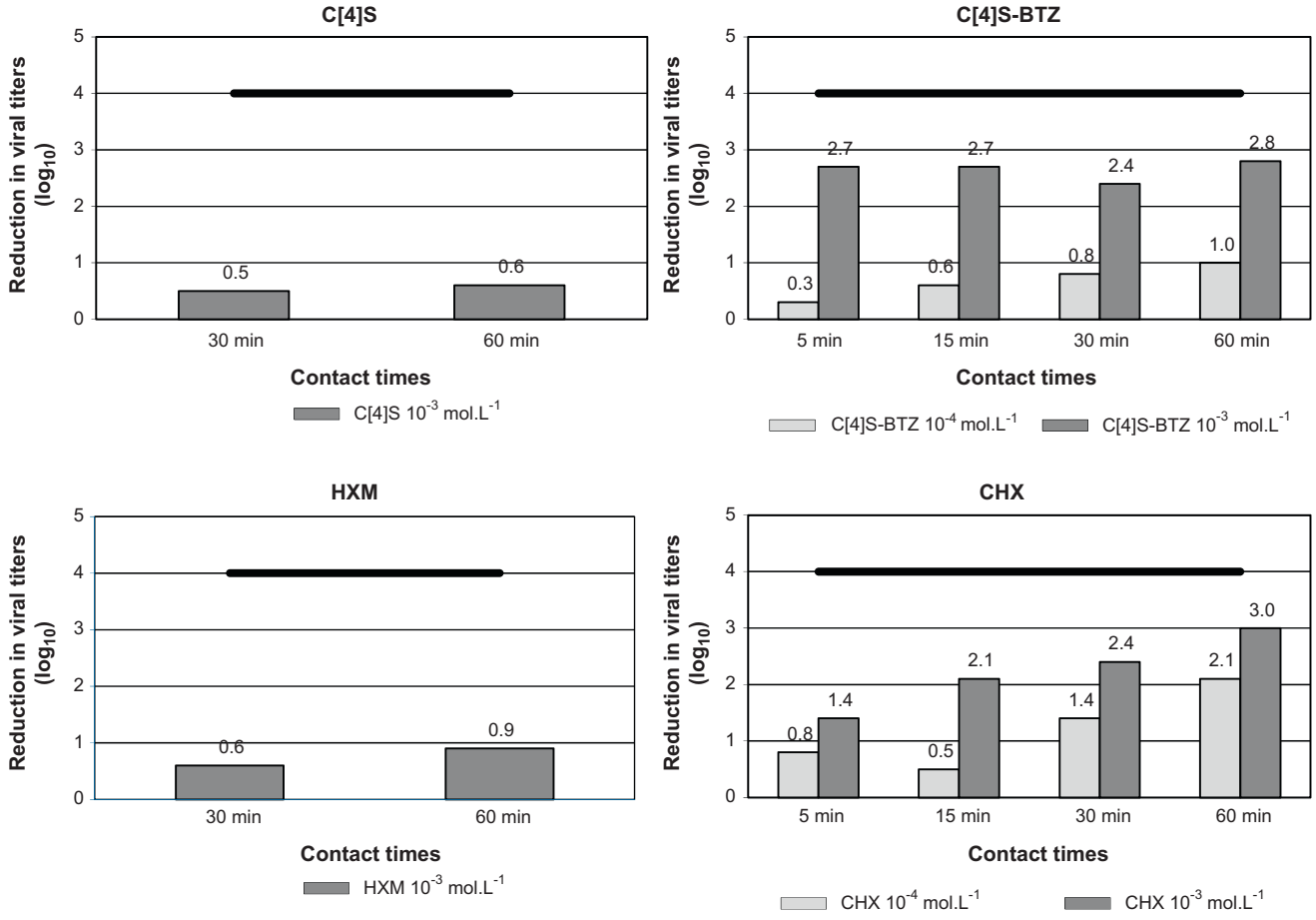

Fig. 2. Comparison of antiseptic activities of C[4]S, C[4]S-BTZ, HXM and CHX toward the HCoV 229E. ▬ : Threshold of ATS-D antiviral activity according to the European standard NF EN 14476+A1.

which appeared concentration- and time-dependent. Furthermore, this activity persisted until 60 min of contact time.

Several items should be yet taken into consideration when analyzing these results. First, the different molecules were tested alone, i.e. without any additive as alcohol and without any interfering substances. In this way, their own anti-coronavirus activity could be estimated, but this was not really representative of field conditions, since viruses are normally found embedded in organic materials, preventing them from the action of ATS-D. These results are consistent with previous studies, which showed that CHX did not have ATS-D anticoronavirus activity unless it was associated with cetrimide and 70% (v/v) ethanol (Sattar et al., 1989).

It would be of interest to associate the fast and persistent action of C[4]S-BTZ with that of alcoholic solutions. Indeed, even if ethanol showed a good ATS-D activity, in particular against coronaviruses (Rabenau et al., 2005b; Sattar et al., 1989), its volatile nature involves a transient action, which could potentially be improved by C[4]S-BTZ activities.

Furthermore, the absence of cytotoxicity made the C[4]S-BTZ even more promising, considering toxicity risks involved with the currently used ATS-D (skin reactions, allergy or occupational diseases).

Conflict of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.antiviral.2010.09.009.

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