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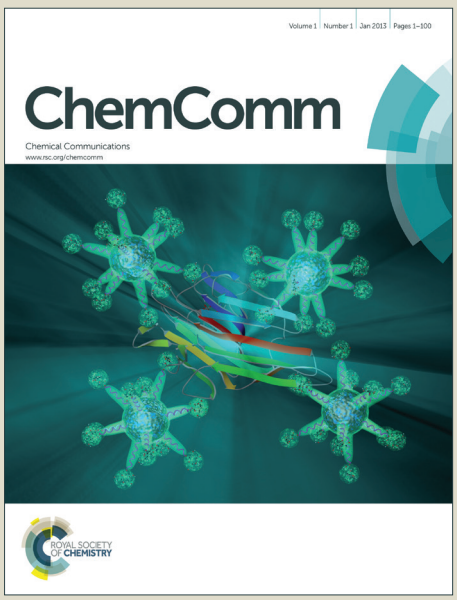

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ARTICLE TYPE

Large negatively charged organic host molecules as inhibitors of endonuclease enzymes

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Three large negatively charged organic host molecles; β-cyclodextrin sulphate, *para*-sulphonato-calix[6]arene and *para*-sulphonato-calix[8]arene have been shown to be effective inhibitors of endonuclease in the low micromolar range, additionally *para*-sulphonato-calix[8]arene is a partial inhibitor of rhDNase I.

The endonucleases are a class of enzymes whose biological role is to digest DNA.[1] As such, they play a role in human cell repair [2] but also are key elements in viral infection.[3] The endonucleases also act as protective elements in bacterial defense strategy against bacteriophages.[4] They represent a valid target in drug design for anti-cancer, anti-viral and antibiotic treatments, however new compounds compatible with pharmaceutical criteria (high solubility, non-toxic) are needed.[5]

A number of studies have pointed to endonucleases as potential targetsfor influenza treatment.[6] The few anti-influenza medications currently available are often associated with severe side-effects. Commercial treatments target the viral membrane protein M2 (amantadine and rimantadine);[7] or neuramidases, oseltamivir (Tamiflu) and zanamivir (Relenza).[8]

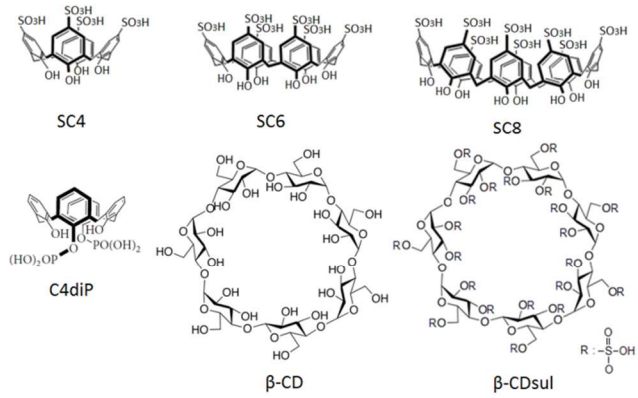
PA endonuclease is a domain belonging to the RNA-dependent RNA polymerase (RdRp) and it initiates the translation from viral mRNA to viral proteins. Its contribution is essential to viral production inside the infected cell.[9] Pharmaceutically active soluble endonuclease inhibitors would thus appear to be excellent target as antiviral medications.[10]

Secondly, human endonucleases present an interest as oncotherapeutic targets. AP endonuclease is a human enzyme involved in DNA lesion repairing system. This endonuclease is overexpressed in cancers such as glioblastoma leading to resistant to radio- and chemo-therapy. While development of AP endonuclease inhibitors is underway none are presently available due to their incompatibility with desirable clinical criteria (high solubility, non-toxic, low efflux transport, enzyme-resistant).[11]

Supramolecular organic macrocycles,[12] present considerable interest in biopharmaceutical science, the cyclodextrins are well known as transporters for bioactive compounds[13] but are somewhat less well known for direct biological activity against proteins.[14] The calix[n]arenes are well documented both as transporter molecules and also for their direct biological activity,[15] particularly with regard to protein complexation.[16]

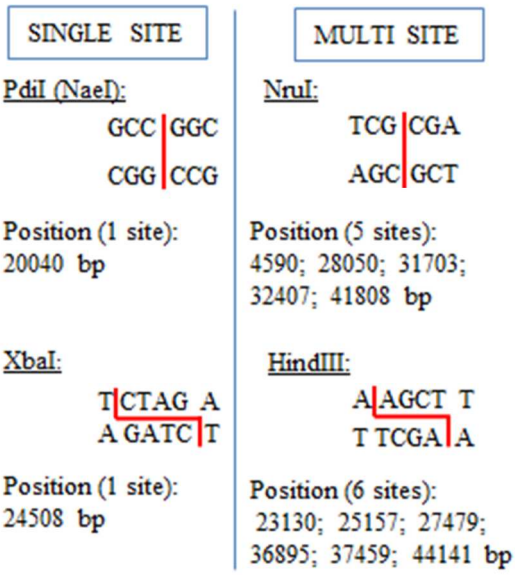
In the current paper we describe the inhibitory activity of a series of organic host moleceuls, Scheme 1, with regard to four site specific endonucleases, Scheme 2, and the non-specicific human rhDNase I enzyme.

Scheme 1. Structures of the organic host molecules evaluated as endonucleases inhibitors



The negatively charged organic host molecules, were chosen for the possible binding affinity for the DNA binding site and cleavage site, using the crystallographic information on the influenza virus PA endonuclease as the lead structure. The endonuclease enzymes were chosen for their known cleavage properties on the lambda phage DNA. Two, NruI, (CG site cleavage) and HindIII (AA) give rise to multisite cleavage of the DNA chain. The other two PdiI (CG) and Xbal (TT) cause cleavage at only a single site on the DNA chain.[17] Our aim was to determine the factors which influence the inhibitory effects of the organic host molecules for possible use as therapeutic agents for influenza treatment.

In the inhibition experiments, the half maximal inhibitory concentration (IC_{50}) was measured. The digestion activity of endonuclease was evaluated using agarose gel electrophoresis at varying inhibitor concentrations. After quantifying the intensity of the digested bands on the gel the concentration of inhibitor needed for 50% (IC_{50}) inhibition of the endonuclease activity, was determined. See SI



Scheme 2. Sequence of cleavage sit for different restriction enzymes and their positions on Lambda DNA phage

The IC_{50} concentrations for the six organic host molecules tested are given below in Table 1, of these three, β-CD, C4diP and SC4 show no inhibitory activity. The other three β-CDsul, SC6 and SC8 all show IC_{50} values in the low micromolar range with regard to NruI and slightly lower values for HindIII. All three molecules are characterised by a combination of high negative charge and a size capable of spanning both the DNA binding site and the cleavage site in an endonuclease.[10] As both these sites are characterised, in influenza PA Endonuclease by the presence of basic amino-acids (DNA binding site, K34 and R124) and (Catalytic site, R84 and K184), blockage of the sites, by large anionic macrocycles, is not unexpected and is a requirement for enzyme inhibition.[15]

Table 1. Half maximal inhibitory concentration (IC_{50}) of different organic host molecules determined for restriction enzymes NruI and HindIII. N.I. corresponds to an absence of endonuclease inhibition.

Molecules	IC_{50} (μM)	
	NruI	HindIII
β-CD	N.I.*	N.I.*
β-CDsul	3	6
C4diP	N.I.*	N.I.*
SC4	N.I.*	N.I.*
SC6	3	1.1
SC8	1.8	0.6

N.I.*: No Inhibition

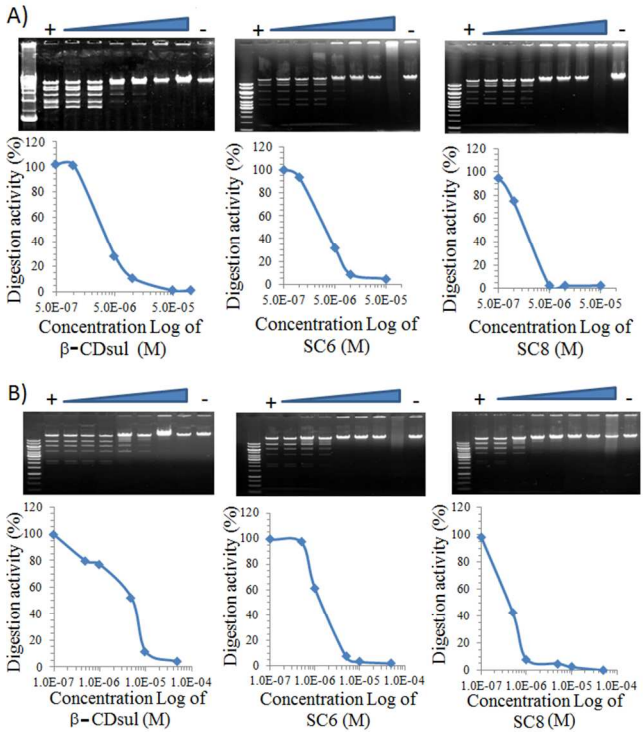


Figure 1. IC_{50} values for three different supramolecular organic macrocycles acting on the restriction enzymes A) NruI and B) HindIII. Gel electrophoresis was used to determine the activity of the enzyme in the presence of increasing concentration of inhibitor. After quantification of the band intensities the digestion activity is then plotted as a factor of inhibitor concentration.

The results obtained can be compared to known inhibition activities, for example for small molecule inhibitors of Apurinic/apyrimidinic (AP) endonuclease 1 (Ape1) four were reported to have IC_{50} values of less than 10μM and one, Ape1 repair inhibitor 03 [2,4,9-trimethylbenzo[*b*][1,8]-naphthyridin-5-amine; AR03), inhibited cleavage of AP sites in SF767 glioblastoma cells, in whole cell extracts and inhibited purified human Ape1 in vitro.[18] With regard to influenza PA Endonuclease inhibition, values are in the range high nanomolar to sub 10 μM for effective inhibitors.[10] The observed values in this work are in the same range and IC_{50} of SC8 with regard to HindIII is comparable to the best published value.

We have previously shown that supramolecular hybrid silver nanoparticles have anti-bacterial activity,[19] thus it was of interest to investigate if such systems possess enzyme inhibitory activity. However, of the current systems only hybrid nanoparticles capped by β-CDsul proved stable under the conditions of the enzyme inhibition experiments.

The results are given in Figure 2 below. In order to ascertain that free β-CDsul was not responsible for the inhibitory effect the suspension was dialysed; the observed values decrease from an IC_{50} of 3 μM for the free ligand to 3.8 μM for the β-CDsul capped silver nanoparticles. This decrease is similar to the small decrease in the plasmon resonance intensity observed, given in SI

Figure S1.

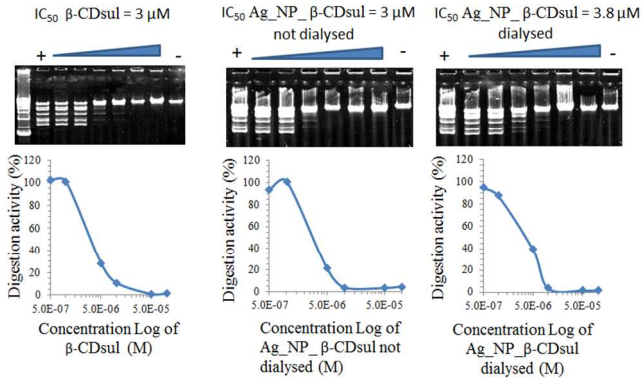


Figure 2. IC_{50} of β-cyclodextrin sulphate and β-cyclodextrin sulphate capped on silver nanoparticles (dialysed in DI water or not) have been determined on the restriction enzyme NruI.

In contrast to the endonucleases, the super family of the DNases are a family of enzymes that non-specifically cleave phosphodiester bonds. It is, also, to be noted that these enzymes do not conserve the aminoacid geometry around the active site. Thus rhDNase I has an active site with histidine, asparagine and aspartic acid and glutamic acid residues. In contrast, in bDNase I there are additional basic (Arg) residues. Both are characterised by a need for divalent cations in the active sites.[20]

The inhibition experiments, using rhDNase I, were initially carried out at the same concentration as the Endonuclease experiments. However, the evidence for enzyme inhibition was un-convincing. Reducing the rhDNase I concentration to 100µM and observing the kinetics of digestion led to the results shown in Figure 3, below. Here SC8 inhibits the action of rhDNase I during 60 minutes. The partial inhibition at low enzyme concentrations is not unexpected as rhDNase I only contains histidine residues, which bind weakly to SC8, in the active site.

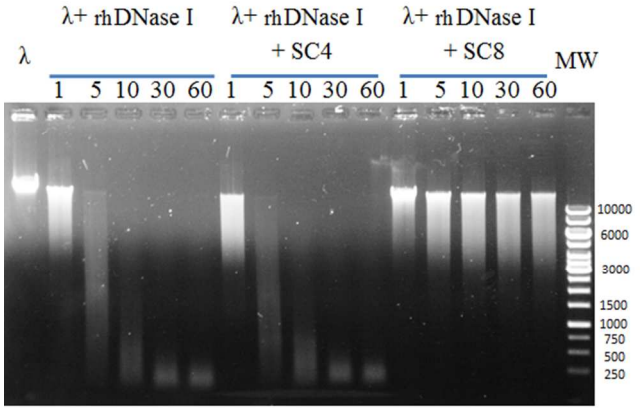


Figure 3. Agarose gel electrophoresis showing the kinetics of Lambda DNA (annoted λ) digestion by rhDNase I in the presence of SC4 and SC8 at 100 µM. Time of incubation was 1, 5 , 10 , 30 and 60 minutes. MW correspond to the Molecular Weight ladder (bp is shown on the right).

In conclusion we have demonstrated that large organic host molecules with a size above four units in the macrocycle and possessing strong negative charge are effective inhibitors of endonuclease enzymes. Work is currently underway to extend the work to influenza endonucleases and to study the cellular efficacy of the molecules.

Notes and references

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