Haemolytic activity of stonustoxin from stonefish (*Synanceja horrida*) venom: pore formation and the role of cationic amino acid residues

Desong CHEN*, R. Manjunatha KINI*, Raymond YUEN*† and Hoon Eng KHOO*†‡

*Bioscience Centre, School of Biological Sciences, Faculty of Science, National University of Singapore, 10 Kent Ridge Crescent, 119260 Singapore, and †Department of Biochemistry, Faculty of Medicine, National University of Singapore, 10 Kent Ridge Crescent, 119260 Singapore

Stonustoxin (SNTX) is a two-subunit protein toxin purified from the venom of the stonefish (*Synanceja horrida*), which induces potent haemolytic activity. We examined the pore-forming property of this non-enzymic protein by an osmotic protection assay. SNTX-induced haemolysis was completely prevented by osmotic protectants of adequate size [poly(ethylene) glycol 3000; molecular diameter approx. 3.2 nm]. Uncharged molecules of smaller size, such as raffinose and poly(ethylene) glycol 1000–2000, failed to protect against cell lysis. These findings indicate that SNTX induces the formation of hydrophilic pores in the cell membrane, which results in the lysis of erythrocytes. Since cationic residues contribute significantly to the cytolytic activity of several other pore-forming toxins, we examined the role of positively charged lysine and arginine residues in the haemolytic activity of SNTX. SNTX lost its haemolytic activity when the

positively charged side chains of lysine residues were neutralized or converted into negatively charged side chains upon carbamylation or succinylation respectively. The haemolytic activity of SNTX was also inhibited by the modification of positively charged arginine residues using 2,3-butanedione. The loss of haemolysis showed strong correlation with the number of Lys or Arg residues modified. CD analyses, however, showed that the conformation of SNTX was not significantly affected by these chemical modifications. Further, the haemolytic activity of SNTX was competitively inhibited by various negatively charged lipids, such as phosphatidylserine, cardiolipin and monosialogangliosides. These results indicate that SNTX induces potent haemolytic activity through the formation of pores in the cell membrane, and that cationic residues play a crucial role in its cytolytic mechanism.

INTRODUCTION

Cytolytic proteins and peptides play an important role in performing offensive and defensive actions in a number of organisms. They assist in the attack and digestion/degeneration of host (in the case of venoms and toxins), and furnish protection by killing any invader or preventing the invasion (in the case of antibacterial proteins and peptides). Both of these roles are achieved by lysing cells through enzymic and non-enzymic mechanisms. Many cytolytic toxins isolated from venoms and bacteria show various enzymic activities, such as phospholipase A₂, phospholipase C and sphingomyelinase [1,2]. They lyse cells directly or make cells more susceptible to damage by hydrolysing membrane lipids through enzymic action. On the other hand, a wide variety of non-enzymic proteins and peptides that possess cytolytic activity have been isolated from reptilian, amphibian, insect, microbial and mammalian origins [1–3]. Most of these cytolsins lyse the cells by formation of discrete transmembrane pores. Through these pores small osmoticants can move in or out, whereas the large molecules such as proteins cannot. Thus the cell interior is hyperosmotic, attracting a net influx of water, which results in a sustained cell swelling and subsequently cell lysis [4].

Pore-forming toxins bind to either lipids or proteins in the cell membrane. (a) A large number of cytolytic proteins and peptides, including pardaxin, cecropins, magainins and melittin, bind to negatively charged lipids [3,5–7]. Cationic amino acid residues appear to be essential for their interaction with the negatively charged lipids as well as for their cytolytic activity [5,8–11]. (b) A small group of bacterial cytolsins bind to cholesterol [1,12]. The

free thiol groups are critical for the biological activity of this group of cytolsins. (c) Cytolytic peptides from various species of sea anemone, however, appear to bind specifically to sphingomyelin [13]. Details of their interaction with sphingomyelin are not yet available. (d) Some toxins interact with erythrocyte-membrane glycoproteins, such as glycophorin or band 3 [14,15].

The venom of stonefish (*Synanceja horrida*), one of the most dangerous venomous fishes in the world, shows potent haemolytic activity against erythrocytes of various species [16]. Stonefish venom contains enzymes and non-enzymic proteins. Several enzymic activities detected in the venom of *S. horrida* include hyaluronidase, proteinase (thrombin-like), phosphodiesterase, alkaline phosphomonoesterase, arginine esterase, arginine amidinase, 5'-nucleotidase and acetylcholinesterase [17]. The hyaluronidase fraction of *S. horrida* has been purified and characterized [18,19]. We have purified a protein toxin with potent lethal and haemolytic activities from the venom of *S. horrida* [20] and designated it stonustoxin (SNTX). SNTX has a native *M_r* of 148000 and consists of two subunits named α and β of *M_r* 71000 and 79000 respectively. In addition to its haemolytic activity, the toxin possesses various other biological activities, such as oedema induction, vascular permeability, platelet aggregation and endothelium-dependent vasorelaxation and hypotension [20–23]. SNTX is devoid of enzymic activities, such as hyaluronidase, proteinase and phospholipase A₂ [20]. Therefore the potent haemolytic activity of SNTX is most probably mediated through a non-enzymic mechanism. Several related lethal proteins have been isolated from the venoms of other species of stonefish [24–26]. There is, however, very little information concerning the details of structure, function and mechanism of this group of

Abbreviations used: CL, cardiolipin; GM₁ and GM₂, monosialogangliosides; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PEG, poly(ethylene glycol); PS, phosphatidylserine; SNTX, stonustoxin.

‡ To whom correspondence should be addressed.

proteins. In this regard, we recently isolated and characterized the cDNA clones encoding the α - and β -subunits of SNTX from a venom-gland cDNA library [27]. The deduced amino acid sequences of both subunits contain 15 cysteine residues (seven in the α - and eight in the β -subunit) with five free thiol groups, as determined by titration with 5,5'-dithiobis-(2-nitrobenzoic acid) [27]. Under both reducing and non-reducing conditions, SDS/PAGE analysis showed that SNTX can be separated into two subunits, indicating that the 10 remaining cysteines are involved in intrachain linkages. Thus the two subunits are associated via some non-covalent interactions rather than by disulphide bridges. The deduced amino acid sequences of both subunits also show no structural similarity to any known protein, indicating that SNTX may be a novel protein toxin.

As a first step towards understanding the cytolytic mechanism and structure–function relationships of SNTX, we have examined the pore-forming property of SNTX and the role of cationic residues in the cytolytic activity of the toxin by chemical modification of Lys and Arg residues. The results show that SNTX-induced haemolytic activity is due to its ability to form pores in the cell membrane and that cationic residues indeed play an important part in the cytolytic activity of SNTX.

EXPERIMENTAL

Materials

Stonefish venom was obtained from *S. horrida*, supplied by local fishermen. The crude venom was extracted from the venom sacs located at the dorsal spines, freeze-dried and stored at -70°C . Cardiolipin (CL), dansyl chloride, potassium cyanate, phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), cholesterol, raffinose, sphingomyelin, monosialogangliosides (GM_1 and GM_2) and succinic anhydride were purchased from Sigma (St. Louis, MO, U.S.A.). 2,3-Butanedione was obtained from Aldrich (Milwaukee, WI, U.S.A.). Poly(ethylene glycol) (PEG) 1000, 1500, 2000, 3000 and 4000 were from Fluka (Buchs, Switzerland; the numbers represent their average M_r). Normal rat blood for haemolytic assay was freshly obtained from healthy rats. All other reagents used were of analytical grade.

Purification of SNTX

SNTX was purified from the crude venom by a two-step procedure on Sephacryl S-200 high-resolution gel-permeation and DEAE Bio-Gel A anion-exchange chromatography [20]. Briefly, the freeze-dried venom was dissolved in 0.05 M sodium phosphate buffer, pH 7.4 and centrifuged at 3000 g for 5 min to remove insoluble material. The solution was then applied to a Sephacryl S-200 HR gel-filtration column (1.5 cm $\times$ 84 cm). Elution was at a flow rate of 12 ml/h with the same buffer. The fractions with haemolytic activity were pooled and further fractionated on a DEAE Bio-Gel A anion-exchange column (2.0 cm $\times$ 20 cm). Unbound proteins were eluted with the same buffer. Then a linear (0–0.15 M) NaCl gradient was initiated. SNTX was eluted at 0.08 M. The homogeneity of SNTX was confirmed by SDS/PAGE, which showed the presence of only two bands corresponding to the α - and β -subunits.

Modification of free amino groups

Free amino groups of SNTX were modified by the following two methods.

(a) Succinylation. Native SNTX was succinylated according to the method described by Klotz [28]. Succinic anhydride (100 mol/

mol of amino groups; 110 ϵ -amino groups and one free α -amino group of the β -subunit; the N-terminus of the α -subunit is blocked [20]) was added in small aliquots to rapidly stirred PBS containing SNTX. The pH was maintained between 8.0 and 8.5 by the addition of 1 M NaOH manually. Excess reagent was removed by dialysis for 24 h at 4°C against PBS for the haemolytic assay or 50 mM acetate buffer for CD analyses.

(b) Carbamylation. Native SNTX was carbamylated as described by Stark [29] at a potassium cyanate/lysine molar ratio of 100:1 in PBS adjusted to pH 8.0. After incubation for 24 h and 48 h at room temperature ($\approx 25^{\circ}\text{C}$), excess reagent was removed by dialysis as described above.

Modification of arginyl residues

Arginyl residues of SNTX were modified with 2,3-butanedione in 0.05 M borate buffer, pH 8.1, according to the method of Riordan [30]. Three different molar ratios (25, 50 or 100) of butanedione to arginyl groups were used for modification. The reaction mixtures were incubated for 60 min at room temperature. Periodically, 2.5 μl aliquots were removed from the reaction mixtures and tested directly for residual haemolytic activity as described below. At these concentrations butanedione did not affect the haemolytic or lethality assay. The modified toxin (after 60 min reaction at a molar ratio of 50:1), dialysed against the same buffer for 24 h at 4°C , was used for the subsequent CD measurements.

Determination of extent of modification

To determine the extent of modification of Lys and Arg residues, amino acid analyses of native and modified SNTX were carried out using an Applied Biosystems Model 420A Derivatizer/Amino Acid Analyser. The content of free Arg or Lys residues lost upon modification was estimated. The extent of modification of Lys residues upon succinylation was determined by dansylation [31], since succinyl-lysine is hydrolysed during amino acid analysis. Briefly, 2 nmol of native or modified SNTX in 0.5 ml of 0.1 M sodium bicarbonate with 8 M urea was treated with 0.5 ml of dansyl chloride (20 mg/ml in acetone) for 5 h at 37°C . Excess reagent was removed by dialysis. The fluorescence emission was measured at 520 nm after excitation at 365 nm. The number of free amino groups was calculated from the fluorescence emission of dansylated product of the modified SNTX, using the fluorescence emission of dansylated native SNTX as 100% for the free amino groups.

CD studies

To examine secondary structural changes induced by chemical modifications of Lys and Arg residues, CD studies on native and modified SNTX were carried out with a Jasco J-715 spectropolarimeter. The cuvette chamber of the spectropolarimeter was continuously purged with pure nitrogen before and during the experiments. Measurements for Lys- and Arg-modified SNTX were made in 50 mM acetate buffer, pH 7.4, and in 50 mM borate buffer, pH 8.1, respectively. The spectra were obtained by using a 1 mm path-length cell at room temperature. The results were expressed as the mean residue ellipticity (θ). The α -helix, β -sheet, β -turn and random-coil contents were estimated by the method of Chang et al. [32].

Assay of haemolytic activity

Haemolytic activity of the native and modified SNTX was assayed as described earlier [20]. Rat blood was collected into a

Figure 1 Osmotic protection studies of SNTX-induced haemolysis

(A) Kinetics of SNTX-induced haemolysis. Rat erythrocytes (0.05%), suspended in PBS or PBS supplemented with 30 mM of the indicated sugars and continuously stirred, were exposed to SNTX (1 nM). Haemolysis was followed at room temperature by measuring the decrease of turbidity at 700 nm. (B) Relationship between SNTX-induced haemolysis in the presence of osmotic protectants and the molecular diameters of these protectants. The reciprocal of half-times of haemolysis ($t_{1/2}$) was taken as a measure for the rate of permeability of the sugar through the pores. Hydrated diameters of osmoticants were taken from Kuga [36]. Data are means of triplicate analyses.

solution of 3.8% sodium citrate (9:1, v/v). After centrifugation, the erythrocytes were washed 3 times with PBS, pH 7.4. Samples (200 μ l) containing various concentrations of native or modified SNTX were incubated with 2 ml of resuspended erythrocytes (0.5%, v/v) for 20 min at 37 °C. Tubes were then centrifuged at room temperature and 3000 g for 5 min. The concentration of haemoglobin released in the supernatant was measured at 540 nm. A 100% haemolysis value was obtained by the addition of saponin at a final concentration of 0.1 mg/ml.

Osmotic protection assay

An osmotic protection assay was carried out to estimate the size of pores generated by SNTX. Briefly, washed rat erythrocytes obtained as above were resuspended at a concentration of 0.05% (v/v) in PBS in the absence and presence of various sugars (30 mM), as specified in Figure 1, and continuously stirred. The time course of erythrocyte lysis induced by SNTX (1 nM) was followed spectrophotometrically at 700 nm in a 1 cm cuvette at room temperature. The initial A_{700} was approx. 0.5.

Competitive inhibition studies

PC, PE, PS, sphingomyelin, cholesterol and CL were dissolved separately in a chloroform/methanol (2:1) mixture and evaporated to dryness in clean glass tubes. Lipid suspensions were prepared by adding PBS and sonicating for 2 min. GM₁ and GM₂ were dissolved in PBS. Diluted suspensions were used for

the studies on competitive inhibition of haemolysis caused by SNTX. Individual lipids (2–3000 nM) were mixed with SNTX for 2 min at room temperature and the haemolytic activity of the mixture was measured by the method described above.

Protein estimation

Protein concentrations were estimated by amino acid analysis or by UV absorption at 280 nm. Native and modified SNTX with a concentration of 1 mg/ml showed an absorbance of 0.40 at 280 nm in a 1 cm path-length cell.

Statistical analyses

Results are expressed as means $\pm$ S.D. and compared by unpaired, two-tailed Student's t test. A probability level of less than 0.05 was taken as significant. The correlation coefficient (r) between the haemolysis induced by SNTX and the number of modified Lys or Arg residues was calculated by the method of Pearson [32a].

RESULTS AND DISCUSSION

Pore-forming mechanism in the haemolytic activity of SNTX

To test whether SNTX induces its haemolytic activity by pore formation in the cell membrane and to examine the role of colloid-osmotic shock in SNTX-induced haemolysis, we studied the effect of various osmotic protectants on SNTX-induced haemolysis. This approach is based on the concept that colloid-osmotic lysis can be suppressed by an osmotic protectant of appropriate size which, being too large to penetrate the induced membrane pores, is capable of balancing the osmotic drag of intracellular impermeant solutes such as haemoglobin and organic phosphates [4]. This simple method for assessing pore diameter has proved its validity in many studies, as shown in the case of *Pseudomonas aeruginosa* cytotoxin [33], *Staphylococcus aureus* α -toxin [34] and equinatoxin II [35]. We studied the kinetics of haemolysis of erythrocytes exposed to SNTX in the presence of sugars of increasing size at a concentration of 30 mM as used by Menestrina et al. [4]. As shown in Figure 1(A), $t_{1/2}$ (the time necessary to reach 50% of haemolysis) increased with increasing size of osmoticants. The smallest osmoticant, raffinose, did not prevent the haemolysis, but PEG 1000, 1500 and 2000 reduced the rate of haemolysis induced by SNTX. In the presence of PEG 3000 the haemolytic rate was very slow ($t_{1/2}$ 100 min), whereas $t_{1/2}$ for the native SNTX or in the presence of raffinose or PEG 1000, 1500 and 2000 was within 2.6–6 min. Thus the addition of osmotic protectants prolongs the lag time before the onset of haemolysis and reduces its rate. In the colloid-osmotic hypothesis, this delay is due to the time necessary for the osmoticant to diffuse inside the cell through toxin-induced lesions [35]. As expected, when cells incubated with SNTX and PEG 3000 were washed and resuspended in PBS, rapid haemolysis was observed. These results indicate that PEG does not primarily inhibit the binding of SNTX to erythrocytes, but acts as an osmotic protectant by counterbalancing the intracellular osmotic pressure of haemoglobin.

The relative permeability of the sugar through the pore was estimated by the reciprocal of $t_{1/2}$ [4], and the radii of the osmoticants were obtained from Kuga [36]. Using a Renkin plot [37,38], the relative permeability of the sugar molecule versus its size (Figure 1B), yielded an estimate of the functional diameter of the pores generated by SNTX to be 2.5–3.2 nm. The pore size generated by SNTX is similar to that (3 nm) caused by *Escherichia coli* haemolysin (M_r 107000) [39], but is bigger than those generated by jellyfish CARTOX (M_r 107000; 0.84–1.08 nm) [40]

Table 1 Summary of chemical modifications of Lys and Arg residues based on amino acid analyses

Overall charge column: the overall charge on the SNTX molecule is the difference between the positively charged and negatively charged residues. Based on the deduced amino acid sequence derived from cDNA cloning [27], native SNTX contains 190 positively charged residues (49 Arg, 110 Lys and 31 His), 168 negatively charged residues (74 Asp and 94 Glu), one free N-terminal amino group and two free C-terminal carboxylate groups. Arg residues were modified by butanedione at a molar ratio of 50:1 (butanedione/Arg).

Modifications		Charge on Lys/Arg side chain modified	No. of Lys/Arg modified	Overall charge
Native	None	Positive	0	+21
Lys	Succinylation	Negative	80*	-139
	Carbamylation (24 h)	Neutral	46	-25
	Carbamylation (48 h)	Neutral	79	-58
Arg	Butanedione (30 min)	Negative	9	+3
	Butanedione (60 min)	Negative	37	-53

* Determined by dansylation method (succinyl-lysine is hydrolysed to lysine and succinic acid during amino acid analysis).

and other smaller cytolytins such as *Staph. aureus* α -toxin (M_r 34000; 1–1.5 nm) [34], *P. aeruginosa* cytotoxin (M_r 25000; approx. 2.4 nm) [33] or equinatoxin II (M_r 20000; approx. 2.2 nm) [35]. SNTX is one of the largest toxins that form pores. This is the first demonstration of pore-formation by SNTX and its role in the haemolytic activity of the toxin. A similar mechanism may account for the lytic activity of several related protein toxins isolated from the venoms of other species of stonefish [24–26].

Melittin, a potent pore-forming peptide, induces endothelium-dependent vasorelaxation [41]. It creates pores or channels in the endothelial cell membranes. The subsequent influx of calcium into endothelium cells then triggers the release of nitric oxide, resulting in endothelium-dependent vasorelaxation and hypotension [41]. Similarly, we have shown that SNTX induces endothelium-dependent vasorelaxation of isolated rat aorta, which appears to be mediated by endothelium-dependent relaxant factor, probably nitric oxide or nitric oxide-yielding substances [23]. Such a vasorelaxant effect of SNTX is thought to be responsible for its marked hypotensive action *in vivo*. Thus the potent hypotensive effect of SNTX could be correlated with its pore-forming property. Further studies are necessary to clarify whether SNTX functions in a similar manner to melittin to induce endothelium-dependent vasorelaxation.

Effects of chemical modification on the structure of SNTX

Studies have shown that a large number of cytolytic proteins or peptides create pores through a 'barrel-stave' mechanism [42]. The toxin monomers bind to the membrane and a hydrophobic surface in the toxin inserts into the membrane, resulting in the formation of pores. In this model, a hydrophobic surface such as an amphiphilic α -helix or β -sheet is very critical [42,43]. Recently, a 'carpet-like' mechanism has been suggested to regulate the pore-forming property of some cytolytic peptides [7,44]. In this model, peptide monomers bind to the cell membrane by electrostatic force between the cationic residues and the anionic cell surface. The peptides may rotate and the subsequent reorientation of hydrophobic residues towards the hydrophobic core of the membrane results in the formation of transmembrane pores. Despite the differences in both pore-forming models,

Figure 2 CD spectra of native and modified SNTX.

CD spectra were recorded using a 1 mm-path-length cell as described in the Experimental section. Measurements for Lys- and Arg-modified SNTX were made in 50 mM acetate buffer, pH 7.4, and in 50 mM borate buffer, pH 8.1, respectively. The concentrations of SNTX and its derivatives were 3–4 μ M. Native SNTX in acetate buffer (—); succinylated SNTX (---); carbamylated SNTX (···); native SNTX in borate buffer (----); butanedione-modified SNTX (-.-.-).

cationic charges and a hydrophobic surface appear to be important in the pore-formation. Interestingly, Kini and Evans [3] have shown the presence of a hydrophobic surface flanked by a cationic site in a large number of cytolytic proteins and peptides derived from various organisms. The cationic site consists of several positively charged residues distributed at either the C- or N-terminal side of the hydrophobic surface. This cationic site has been shown to determine the lytic activity by enhancing the electrostatic interaction of cytolytins with negatively charged vesicles or cell surfaces [3,5].

To examine the role of cationic amino acid residues in the pore-forming or haemolytic activity of SNTX, we studied the effect of chemical modification of the positive charge on the side chains of Lys and Arg residues on the haemolytic activity of SNTX. Lys residues were succinylated or carbamylated with succinic anhydride or potassium cyanate to obtain negatively charged succinyl-lysine residues or neutral homocitrulline residues respectively [28,29]. Arg residues were modified with butanedione to give the negatively charged borate-dihydroxy-imidazoline complex [30]. The extent of Lys and Arg modification based on the amino acid analysis and dansylation is summarized in Table 1. Upon carbamylation with potassium cyanate for 24 and 48 h, 46 (42%) and 79 (72%) Lys residues were neutralized respectively, whereas 80 (73%) Lys residues were succinylated. Thus approx. 25% of Lys residues were not accessible with these chemical modifications. Similarly, nine (18%) and 37 (76%) Arg residues were modified upon interaction with butanedione for 30 and 60 min respectively. The overall net charges on the SNTX molecule were significantly changed from +21 into -139 charges by succinylation or -58 charges by carbamylation (48 h) or -53 charges by modification with butanedione (60 min) (Table 1).

To determine the effect of chemical modifications on the conformation of the molecule, the secondary structures of the native and modified SNTX samples were evaluated by CD spectral analyses. The CD spectrum of native SNTX showed negative ellipticity extrema near 220 nm and 208 nm (Figure 2). The content of the secondary protein structures calculated from the CD spectra is shown in Table 2. Native SNTX contains almost equal amounts of α -helix, β -sheet and random coil. This is similar to the secondary-structure predictions that SNTX contains approx. 34% α -helix, 25% β -sheet and 20% random

coil, based on the deduced amino acid sequence [27]. No significant differences were observed between the CD spectra of native and Lys- or Arg-modified SNTX (Figure 2; Table 2). Only small increases in the content of α -helix and β -sheet were observed after modification of Lys residues by succinylation. Therefore although modification of Lys or Arg residues changed the overall charges, it did not significantly affect the secondary structure of the SNTX molecule. The effects of these modifications on the tertiary or quaternary structure of SNTX, although undetermined, may not be significant.

Role of cationic residues in the haemolytic activity of SNTX

The effects of modification of Lys residues on the lytic activity of SNTX on rat erythrocytes are shown in Figure 3(A). SNTX lost its cytolytic activity when positively charged side chains of Lys residues were neutralized or changed to negatively charged side chains. The inhibition of SNTX-induced haemolytic activity and the number of Lys residues modified were correlated significantly ($r = -0.996$, $P = 0.004$) (Figure 3B). SNTX at 2.5 nM only induced 47.5 % haemolysis even when 46 (42 %) Lys residues were neutralized (Figure 3B), whereas the native toxin at 1 nM caused 90 % haemolysis (Figure 3A). SNTX completely lost its haemolytic activity upon carbamylation of Lys for 48 h, although only 79 (72 %) Lys residues were modified (Figure 3B). Similarly, the haemolytic activity of SNTX was abrogated by the modification of Arg residues with butanedione (Figure 4). The loss of haemolytic activity of SNTX was found to be dependent on the molar ratio of butanedione and Arg residues (Figure 4A). SNTX lost 50 % of its native haemolytic activity upon modification with a 25-, 50- or 100-fold molar excess of butanedione after 60, 30 and 15 min respectively. No haemolytic activity was observed after interaction of Arg residues with butanedione for 60 min, although 12 (24 %) Arg residues remained unmodified. The loss of cytolytic activity was found to be correlated significantly with the number of Arg residues modified ($r = -0.958$, $P = 0.01$) (Figure 4B). Thus chemical modification of positively charged amino acid residues resulted in the loss of the cytolytic activity of SNTX.

These results are consistent with the studies of other pore-forming peptides such as cardiotoxin, cecropins, magainins, melittin, pardaxin and amoebapore [3,5,6,8–11]. The lytic or pore-forming activity of these cytotoxins is reduced or abolished by chemical modification of cationic residues or by substituting negative or neutral charges for positive charges. Interestingly, the cytolytic activity is unchanged when the positive charges of Lys residues in melittin or cardiotoxin were retained upon guanidination [5,11]. Furthermore, the cytolytic activity could be enhanced by attachment of a positively charged chain at the N- or C-terminus of peptides such as magainin, cecropin and synthetic amoebapore analogue [9,45,46], or by addition of amines at the C-terminus of pardaxin [7]. Therefore all these above observations strongly support a significant role for positive charges in the cytolytic activity of proteins or peptides including SNTX.

In our earlier paper, we have predicted that both the α - and β -subunits of SNTX contain one amphiphilic α -helix with a length of at least 20 residues flanked by a region rich in basic residues at the N- and C- terminal side of the α -helix respectively [27]. These structural motifs may be crucial for the haemolytic activity of SNTX. Because of the large number of cationic residues (110 Lys and 49 Arg residues), we have not been able to identify the specific cationic residues involved in the haemolytic activity of SNTX. It is difficult to obtain mono-derivatives by controlling the chemical modification or identifying the residues modified.

Table 2 Effect of chemical modification of Lys and Arg residues on the secondary structure of SNTX

Secondary structural contents columns: The structural content was determined by CD according to the method of Chang et al. [32]. For Lys: CD measurements for native and Lys-modified SNTX were made in 50 mM acetate buffer, pH 7.4. For Control SNTX (48 h): SNTX was incubated at room temperature for 48 h in the absence of potassium cyanate. For Arg: CD measurements were made in 50 mM borate buffer, pH 8.1. Molar ratio of butanedione/Arg residues was 50:1. For Control SNTX (60 min): SNTX was incubated at room temperature for 60 min in the absence of butanedione.

		Secondary structural contents (%)			
	Modifications	α -Helix	β -Sheet	β -Turn	Random coil
Native	None	25.4	34.2	13.3	27.0
Lys	Succinylation	29.0	40.2	11.6	29.2
	Carbamylation (48 h)	29.9	25.1	19.3	25.7
	Control SNTX (48 h)	27.4	25.7	17.6	28.8
Arg	Butanedione (60 min)	30.6	30.6	15.0	23.8
	Control SNTX (60 min)	29.2	29.2	16.5	25.0

Figure 3 Effect of chemical modification of Lys residues in SNTX

(A) Direct haemolytic activity of native and modified SNTX on rat erythrocytes. Carbamylation was carried out at room temperature for 24 h and 48 h. Native SNTX (24 h) (■); native SNTX (48 h) (□); carbamylated SNTX (24 h) (●); carbamylated SNTX (48 h) (○); succinylated SNTX (△). (B) Correlation between the haemolytic activity of SNTX (2.5 nM) and the number of Lys residues modified by carbamylation. Data are means $\pm$ S.D. of triplicate analyses.

Further studies using site-directed mutagenesis and/or synthetic peptides should contribute to our understanding of the structure–function relationships of SNTX and related toxins.

Figure 4 Effect of chemical modification of Arg residues in SNTX

(A) Direct haemolytic activity of native and butanedione-modified SNTX on rat erythrocytes. The molar ratios of butanedione/Arg residues were 25:1 ($\square$), 50:1 ($\bullet$) and 100:1 ($\circ$) respectively. At the defined time, 5 μ l aliquots were tested for haemolytic activity. (B) Correlation between the loss of haemolysis by SNTX (0.5 nM) and the number of modified Arg residues. The molar ratio of butanedione/Arg is 50:1. Data are means $\pm$ S.D. of triplicate analyses.

Interaction of negatively charged lipids with SNTX

The positively charged residues in cytolytic proteins or peptides possibly determine the electrostatic interaction of proteins with anionic phospholipid vesicles or cell surfaces [3,5,47]. The net positive charge of various antimicrobial peptides is responsible for their initial electrostatic contact with target organisms [47]. Therefore negatively charged lipids inhibit the cytolytic effect of this group of cytotoxins by masking the binding sites [43,46]. In the present study, neutral or zwitterionic lipids, such as PC, PE, sphingomyelin and cholesterol, did not affect the haemolytic activity of SNTX. However, the lytic activity of SNTX was inhibited by anionic lipids such as CL, PS, GM₁ and GM₂ SNTX (results not shown). The order of inhibitory potencies was CL > PS > GM₂ > GM₁. The IC₅₀ values (the concentration of lipid that inhibited 50% of the haemolytic activity) for CL, PS, GM₂ and GM₁ were 12, 200, 488 and 1740 nM respectively in the presence of 0.5 nM SNTX. GM₂ was more potent than GM₁ in inhibiting the haemolytic activity of SNTX despite their structural similarity. The negatively charged carboxylate group in GM₁ is oriented towards an additional terminal galactose residue and is thus shielded by a bulky hydrophilic group [48]. This may explain the differences in the potencies of inhibition by GM₁ and GM₂. The differences in the potencies between GM₂, PS and CL can be related to the number of negative charges on the lipids. GM₂ and PS have only one net negative charge, whereas CL has

two negatively charged groups at pH 7.4. This explains the higher potency of CL compared with that of GM₂ and PS. The difference in inhibitory potencies also reflects the pK_a values of the acid groups (carboxylate, 4–5 and phosphate, 2) on the lipids. At pH 5, the haemolytic activity of SNTX could not be inhibited by GM₂, but was inhibited approx. 40% and 55% by PS and CL respectively. GM₂, with only one negatively charged carboxylate group, would have no negative charge at pH 5 and hence failed to inhibit the lytic activity of SNTX. On the other hand, PS and CL retained their negatively charged group(s) (phosphate) at pH 5 and hence could inhibit the lytic activity of SNTX. Thus the electrostatic interaction of anionic lipids and SNTX is influenced by the number and pK_a of acid groups in the lipids, as indicated by the inhibitory potencies of different lipids.

In summary, we have demonstrated the pore-forming property of SNTX, which is responsible for its potent haemolytic activity, and cationic residues play an important role in this cytolytic mechanism by regulating the electrostatic interaction between SNTX and the negatively charged cell surface.

We thank Mr. W. F. Lau from the Biotechnology Processing Centre for performing the amino acid analysis, and Ms. R. Lim of Biosains (Singapore) and Mr. T. Takakuwa of JASCO International (Japan) for performing the CD analysis. We also thank Dr. J. F. Ghadessy for his critical reading of the manuscript and Ms. B. K. Gui and Ms. K. L. Lim for their technical assistance. This work was supported by National University of Singapore research grant RP910461, awarded to R.Y. and H.E.K. D.C. is the recipient of a postdoctoral fellowship from the National Science and Technology Board of Singapore.

REFERENCES

- Harvey, A. L. (1990) in *Handbook of Toxicology* (Shier, W. T. and Mebs, D., eds.), pp. 1–66, Marcel Dekker, New York
- Bernheimer, A. W. and Rudy, B. (1986) *Biochim. Biophys. Acta* **864**, 123–141
- Kini, R. M. and Evans, H. J. (1989) *Int. J. Pept. Protein Res.* **34**, 277–286
- Menestrina, G., Moser, C., Pellet, S. and Welch, R. (1994) *Toxicology* **87**, 249–267
- Kini, R. M. and Evans, H. J. (1989) *Biochemistry* **28**, 9209–9215
- Andra, J. and Leippe, M. (1994) *FEBS Lett.* **354**, 97–102
- Oren, Z. and Shai, Y. (1996) *Eur. J. Biochem.* **237**, 303–310
- Shai, Y., Hadari, Y. R. and Finkels, A. (1991) *J. Biol. Chem.* **266**, 22346–22354
- Maloy, W. L. and Kari, U. P. (1995) *Biopolymers* **37**, 105–122
- Blondelle, S. E. and Houghten, R. A. (1991) *Biochemistry* **30**, 4671–4678
- Habermann, E. and Kowallek, H. (1970) *Hoppe-Seyler's Z. Physiol. Chem.* **351**, 884–890
- Geoffroy, C. and Aouf, J. E. (1983) *J. Biol. Chem.* **258**, 9968–9972
- Turk, J. (1991) *J. Toxicol. Toxin Rev.* **10**, 223–263
- Garland, W. J. and Buckley, J. T. (1988) *Infect. Immun.* **56**, 1249–1253
- Maharaj, I. and Frakell, H. (1980) *Can. J. Microbiol.* **26**, 524–531
- Duhig, J. V. and Jones, G. (1928) *Aust. J. Exp. Biol. Med. Sci.* **5**, 173–179
- Khoo, H. E., Yuen, R. and Tan, C. H. (1992) *Natural Toxins* **1**, 54–60
- Poh, C. H., Yuen, R., Chung, M. and Khoo, H. E. (1991) *Comp. Biochem. Physiol., B: Comp. Biochem.* **101**, 159–163
- Sugahara, K., Yamada, S., Sugiura, M., Takeda, K., Yuen, R., Khoo, H. E. and Poh, C. H. (1992) *Biochem. J.* **283**, 99–104
- Poh, C. H., Yuen, R., Khoo, H. E., Chung, M., Gwee, M. and Gopalakrishnakone, P. (1991) *Comp. Biochem. Physiol., B: Comp. Biochem.* **99**, 793–798
- Gwee, M. C. E., Gopalakrishnakone, P., Yuen, R., Khoo, H. E. and Low, K. S. Y. (1994) *Pharmacol. Ther.* **64**, 509–528
- Khoo, H. E., Hon, W. M., Lee, S. H. and Yuen, R. (1995) *Toxicon* **33**, 1033–1041
- Low, K. S. Y., Gwee, M. C. E., Yuen, R., Gopalakrishnakone, P. and Khoo, H. E. (1993) *Toxicon* **31**, 1471–1478
- Kreger, A. S. (1991) *Toxicon* **29**, 733–743
- Shiomi, K., Hosaka, M. and Kituchi, T. (1993) *Nippon Suisan Gakkaishi* **59**, 1099–1103
- Garnier, P., Goudey-Perriere, F., Breton, P., Dewulf, C., Petek, F. and Perriere, C. (1995) *Toxicon* **33**, 143–155
- Ghadessy, F. J., Chen, D., Kini, R. M., Chung, M. C. M., Jeyaseelan, K., Khoo, H. E. and Yuen, R. (1996) *J. Biol. Chem.* **271**, 25575–25581

-
- 28 Klotz, I. M. (1967) *Methods Enzymol.* **11**, 576–580
- 29 Stark, G. R. (1972) *Methods Enzymol.* **25**, 579–584
- 30 Riordan, J. F. (1973) *Biochemistry* **12**, 3915–3923
- 31 Gray, W. R. (1967) *Methods Enzymol.* **11**, 139–155
- 32 Chang, C. T., Wu, C. C. and Yang, J. T. (1978) *Anal. Biochem.* **91**, 13–31
- 32a Snedecor, G. W. and Cochran, W. G. (1980) in *Statistical Methods*, 8th edn, Iowa State University Press, Ames
- 33 Weiner, R. N., Schneider, E., Haest, C. W. M., Deuticke, B., Benz, R. and Frimmer, M. (1985) *Biochim. Biophys. Acta* **820**, 173–182
- 34 Palmer, M., Weller, U., Meßner, M. and Bhakdi, S. (1993) *J. Biol. Chem.* **268**, 11963–11967
- 35 Belmonte, G., Pederzoli, C., Macek, P. and Menestrina, G. (1993) *J. Membr. Biol.* **131**, 11–22
- 36 Kuga, S. (1981) *J. Chromatogr.* **206**, 449–461
- 37 Renkin, E. M. (1954) *J. Gen. Physiol.* **38**, 225–243
- 38 Ginsburg, H. and Stein, W. D. (1987) *J. Membr. Biol.* **96**, 1–10
- 39 Bhakdi, S., Mackman, N., Nicaud, J.-M. and Holland, I. B. (1986) *Infect. Immun.* **52**, 63–69
- 40 Rottini, G., Gusmani, L., Parovel, E., Avian, M. and Patriarca, P. (1995) *Toxicon* **33**, 315–326
- 41 Rapoport, R. M., Ashraf, M. and Murad, F. (1989) *Circ. Res.* **64**, 463–473
- 42 Ojcius, D. M. and Young, J. D. E. (1991) *Trends Biochem. Sci.* **16**, 225–229
- 43 Leippe, M., Ebel, S., Shoenberger, O. L., Horstmann, R. D. and Muller-Eberhard, H. J. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 7659–7663
- 44 Shai, Y. (1995) *Trends Biochem. Sci.* **20**, 460–464
- 45 Bessalle, R., Haas, H., Gorla, A., Ahalit, I. and Fridkin, M. (1992) *Antimicrob. Agents Chemother.* **36**, 313–317
- 46 Leippe, M., Andra, J. and Muller-Eberhard, H. J. (1994) *Proc. Natl. Acad. Sci. U.S.A.* **91**, 2602–2606
- 47 Gabay, J. E. (1994) *Science* **264**, 373–374
- 48 Gabizon, A. and Papahadjopoulos, D. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 6949–6953