

## Invited Paper: Animal Toxins of Asia and Australia

# BIOACTIVE PROTEINS FROM STONEFISH VENOM

Hoon Eng Khoo

Department of Biochemistry, Faculty of Medicine, National University of Singapore, Singapore

### SUMMARY

1. Of all the venomous fish known, the stonefish is one of the most commonly encountered by man. Studies on its venom started in the 1950s, but little work was performed after that until several groups revived interest in the venom in the 1980s after easier accessibility to the fish.

2. Stonefish venom is a mixture of proteins, containing several enzymes, including hyaluronidase of high specific activity. A purified stonefish hyaluronidase has been characterized.

3. Several of the effects of the crude venom have been isolated to a protein lethal factor that has cytolytic, neurotoxic and hypotensive activity. This protein is stonustoxin from *Synanceja horrida*, trachynilysin from *Synanceja trachynis* and verrucotoxin from *Synanceja verrucosa*.

4. The biochemical properties and activities of these protein lethal factors are reviewed.

**Key words:** hyaluronidase, stonefish venom, stonustoxin, trachynilysin, verrucotoxin.

### INTRODUCTION

'In Australia, the Aborigines perform an ancient dance ritual to educate their children. It relates a message which is as important today as it was hundreds of years ago. It starts out with a pantomime of a man wading in the tide pools looking for a fish. Suddenly, he steps on something which causes him to scream out in pain. It is a clay model of a fish with 13 wooden spines on its back. The dancer writhes on the ground, in apparent agony until the ritual ends sadly in a death song. The fish represented in the dance is the stonefish, a member of the scorpionfish family. It is said to be the deadliest fish in the world'.

This description was published by the National Aquarium in Baltimore's Department of Education in 1988 (URL: <http://www.aqua.org/animals/species/prvenom.html>). Indeed, as of today, there are many well-documented accounts of the severity of stonefish stings. In a prospective study<sup>1</sup> investigating the clinical effects and treatment of venomous fish stings in tropical northern Australia, of 22 fish stings, one was from a stonefish. However, a 1992 study<sup>2</sup> on antivenom use in Australia indicated that 26 cases of stonefish envenomation had required antivenom

treatment. Phoon and Alfred<sup>3</sup> have also reported 81 cases of stonefish envenomation in the Singapore region, but no fatalities, over a period of 4 years.

### GENERAL BIOLOGY AND DISTRIBUTION

Stonefish (genus *Synanceja*) are commonly found in shallow waters of the tropical Indo-pacific region and are considered the most dangerous and venomous of the scorpionfish family. As with other stonefish, *Synanceja horrida* has large pectoral fins that allow it to rapidly dredge sand or mud from beneath itself, allowing it to settle deeply with only its mouth and eyes fully exposed. *Synanceja horrida* have large upward-turning heads and scaled bodies (Fig. 1). As its name suggests, the colouring and shape of the stonefish camouflage it perfectly because it tends to be covered by algae. The stonefish lie half-buried among stones or in rock crevices, thus making them excellent ambush predators. Stonefish can live out of water for many hours and can grow to an average of approximately 30 cm in length. The names given to stonefish in general are self-explanatory and include *Ikan Hantu* (Devil Fish), the Warty Ghoul and *Nohu* (the Waiting One).

Within the genus *Synanceja*, there are five known species: *S. horrida*, *Synanceja trachynis*, *Synanceja verrucosa*, *Synanceja nana* and *Synanceja alula*.<sup>4</sup> Generally, stonefish are only dangerous if stepped on or caught. Following accidentally treading on a stonefish, large quantities of venom are injected into the limb through grooves in their dorsal fin spines. If left untreated, the envenomation can sometimes be deadly.

### SYNANCEJA HORRIDA

*Synanceja horrida* are distributed throughout the waters around Singapore, Malaysia, Indonesia, India and as far as Africa. One of the most striking features of this species is its spine of 13 grooved hypodermic-like projections, each capable of piercing through a shoe, but, in general, this system is used for defence only and never to get food.

### Stonefish envenomation and treatment

The venom apparatus of the stonefish consists of enlarged venom glands that adhere closely to the short, stout and thick 13 dorsal spines, two pelvic and three anal spines and are normally hidden within the thick warty skin of these fish. The dorsal spines (Fig. 1) that project from venom glands along the back of the fish are located such that venom is involuntarily expelled when the spine is pressed upon, with each venom sac containing approximately 10 mg venom.<sup>5</sup> Envenomation by stonefish is typified by a wide array of

Correspondence: Hoon Eng Khoo, Department of Biochemistry, Faculty of Medicine, National University of Singapore, 10 Medical Drive, Singapore 117597. Email: [bchkhe@nus.edu.sg](mailto:bchkhe@nus.edu.sg)

This is an invited paper, subjected to peer review.

Received 17 April 2002; accepted 19 April 2002.

responses, but one of the most striking symptoms is excruciating pain, with tremendous swelling and death of tissues.<sup>6</sup> Wounds produced by these fish are small, sometimes only 1.9 cm deep, but are sometimes fatal because the venom is extremely potent. Severity of the symptoms is related to the depth of penetration of the spines and the number of spines involved. Throughout history, stonefish has been associated with several fatalities. However, despite their ability to produce and discharge toxins and their sometimes formidable appearance, stonefish are said to be edible, with tender and tasty flesh.

### BIOLOGICAL ACTIVITIES OF STONEFISH VENOM

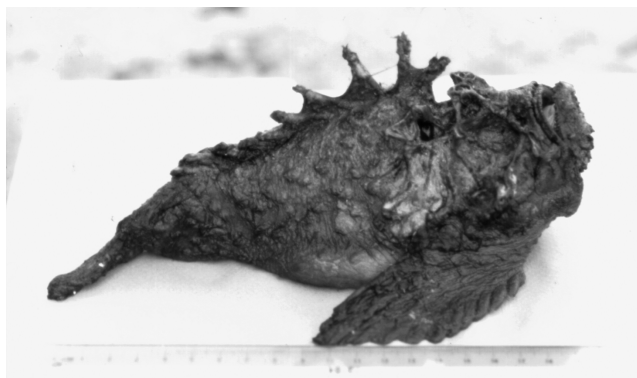
As with the venoms of many other organisms, stonefish venom is a complex mixture, containing 13% protein and 2% nitrogen.<sup>7</sup> The fresh venom is opalescent and has a pH of 6.8.<sup>8</sup> The LD<sub>50</sub> of the venom was estimated to be 0.36 µg/g in mice,<sup>5</sup> a value that is comparable to that of snake venoms. Venoms of other members of the scorpionfish family, such as *Scorpaena guttata*, have similar LD<sub>50</sub> values.<sup>9</sup> Extrapolated to a 60 kg human, this value suggests that 18 mg venom would cause death. This dose could be relayed from six intact spines.<sup>5</sup>

### ENZYMATIC PROPERTIES OF STONEFISH VENOM

Initial experiments investigating the enzymatic properties of *S. horrida* venom revealed only the presence of hyaluronidase activity.<sup>10</sup> Khoo *et al.*<sup>5</sup> have confirmed this and have shown that phospholipase A<sub>2</sub>, acetylcholine esterase, protease and Ca<sup>2+</sup>/Mg<sup>2+</sup>-activated triphosphatase activity are not present. However, other enzymes, such as alkaline phosphomonoesterase, 5' nucleotidase, arginine amidase, arginine esterase and proteinase, are present.<sup>5</sup> Garnier *et al.*<sup>11</sup> have also shown that the crude venom of *S. verrucosa* possesses numerous enzymatic activities. In addition to hyaluronidase activity, Garnier *et al.*<sup>11</sup> have reported the presence of esterase and aminopeptidase.

#### Hyaluronidase

The hyaluronidase component of stonefish venom has been purified and has a specific activity that is many-fold higher than the enzyme



**Fig. 1** A close-up view of the stonefish. Stonefish possess glaring, beady eyes and a wide, gaping mouth that makes them excellent ambush predators.

from snake venoms.<sup>12</sup> Hyaluronidase is an important spreading factor present in many venoms that degrades hyaluronate, one of the major connective tissue constituents in animals. Thus, the high potency of the stonefish hyaluronidase is probably responsible for the extensive necrosis associated with envenomation. This enzyme is also different from mammalian hyaluronidase because it does not act on chondroitin sulphate or dermatan sulphate. It has been confirmed that the stonefish hyaluronidase is an endo- $\alpha$ -N-acetylglucosaminidase specific for hyaluronate.<sup>13</sup> The cDNA of *S. horrida* hyaluronidase has been elucidated and it encodes for a polypeptide of 477 amino acids. Results of BLASTX searches for homologues<sup>14</sup> seems to suggest that it is more closely related, at least in terms of its primary structure, to mammalian sperm-surface hyaluronidases (PH-20 family of hyaluronidases) compared with all other known forms of hyaluronidases, such as those found in mammalian lysosomes and serum. Stonefish hyaluronidase only has a 20% similarity with bee venom hyaluronidase (HE Khoo, unpubl. obs., 2000).

#### Haemolytic and oedema-inducing activity

Most scorpionfish venoms have potent *in vitro* haemolytic activity, stonefish venoms being no exception. Duhig and Jones<sup>15</sup> reported that *S. horrida* venom was strongly lytic for washed guinea-pig erythrocytes and weakly lytic for sheep and human erythrocytes. Two other reports<sup>5,16</sup> have demonstrated similar differential specificity of the venom, showing additional lysis against rabbit, dog, rat and pig erythrocytes, with inactivity for cow, monkey, mouse, goat, horse, burro and cat erythrocytes.

*Synanceja horrida* venom has also been shown to possess potent oedema-inducing activity, as evidenced by a 30% increase in the weight of a mouse leg upon injection.<sup>5</sup> This swelling persisted for more than 24 h after injection. The oedema-inducing effect was not inhibited by diphenhydramine, suggesting that histamine release did not mediate the increase in vascular permeability. Such venom-induced modifications of vascular permeability may account for the potent hypotension associated with envenomation.<sup>17</sup>

### CARDIOVASCULAR AND NEUROMUSCULAR EFFECTS

Some early studies using *S. trachynis* venom indicated a marked fall in blood pressure and bradycardia in mice.<sup>7</sup> More recently, Church and Hodgson<sup>18</sup> also showed that the cardiovascular effects of *S. trachynis* venom are mediated at muscarinic receptors and adrenoceptors. A common feature of experimental envenomation by stonefish venom is partial or complete paralysis of the limbs, as well as respiratory arrest attributed to skeletal muscle paralysis.<sup>10,19</sup> *In vitro* studies suggest that the skeletal muscle paralysis results from neuromuscular blockade.<sup>20</sup> Kreger *et al.*<sup>21</sup> showed that *S. trachynis* venom caused depolarization throughout the entire length of muscle fibres in murine and marked contracture of frog muscles. Their ultrastructural studies also showed marked pathological changes with damage to nerve terminals and muscle fibres.

### NEUROCHEMICAL EFFECTS

The venom of *S. horrida* has been reported to cause inhibition of the uptake of choline and aminobutyric acid into rat brain synaptosomes. It also causes a concentration-dependent release

of acetylcholine from rat brain synaptosomes.<sup>5</sup> This suggests that the venom of *S. horrida* may interfere with the synthesis and release of neurotransmitters from its storage sites. This has been confirmed with *S. trachynis* venom by Kreger *et al.*,<sup>21</sup> who showed that low concentrations of venom acted presynaptically by causing the release and depletion of neurotransmitter from the nerve terminal.

## PURIFIED LETHAL FACTOR FROM STONEFISH VENOM

### Purification of stonustoxin, the lethal factor from *S. horrida* venom

Stonustoxin (Stonefish National University of Singapore; SNTX) has been purified to homogeneity by a two-step procedure, using Sephacryl S-200 HR gel permeation and DEAE-Bio-Gel A chromatography.<sup>8</sup> High-performance size-exclusion liquid chromatography of SNTX showed a single protein peak with a molecular weight of 148 kDa. As determined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis, SNTX is comprised of two subunits,  $\alpha$  and  $\beta$ , with molecular weights of 71 and 79 kDa, respectively.

### Biological and pharmacological properties of SNTX

The estimated LD<sub>50</sub> (i.v.) of SNTX was found to be 0.017  $\mu\text{g/g}$ , which represented a 22-fold increase in toxicity over the crude venom.<sup>8</sup> Many of the symptoms associated with envenomation by *S. horrida* have been accredited to SNTX, thus suggesting that SNTX is, indeed, the lethal component of the venom. Stonustoxin possesses various biological activities that include potent species-specific haemolysis, vascular permeability, platelet aggregation, oedema induction, endothelium-dependent vasorelaxation and lethality.<sup>5,17,22</sup> This toxin causes species-specific potent haemolytic activity on washed erythrocytes and diluted blood of rat, guinea-pig and rabbit, but not on human or rat red blood cells.<sup>8</sup> There was, however, no phospholipase A<sub>2</sub>, proteinase activity or hyaluronidase in the lethal factor. In the whole blood of rabbit or rat, SNTX induced a concentration-dependent and irreversible platelet aggregation.<sup>23</sup> However, the primary lethal action of SNTX is attributed to the potent hypotensive activity of this lethal factor, because studies using the rat aortic strips have shown that SNTX mediated its vasorelaxant activity via the L-arginine-nitric oxide synthase pathway.<sup>17</sup> Stonustoxin is also neurotoxic, producing a rapid and concentration-dependent inhibition of neuromuscular function in the mouse hemidiaphragm and chick biventer cervicis muscle.<sup>22</sup> Stonustoxin causes the formation of pores in erythrocyte membranes of approximately 3.2 nm diameter.<sup>24</sup>

Eight monoclonal antibodies (mAbs) against SNTX have been developed with four of the mAbs having similar epitope specificity, whereas the rest were directed at different epitopes on the SNTX molecule.<sup>25</sup> Although six of these monospecific antibodies were able to protect mice from a challenge of a lethal dose of SNTX, not all the protective mAbs were able to neutralize the haemolytic effect of SNTX *in vitro*, which suggests that the domain(s) responsible for both biological activity may be different.<sup>25</sup>

The vasorelaxant effect of SNTX has been further characterized. It was found that a component of SNTX-mediated vasorelaxation is via binding of either SNTX or substance P to substance P receptors that are located on the endothelial cells. Occupation of these substance P receptors caused subsequent production of nitric oxide

and activation of K<sup>+</sup> channels, thus leading to vasorelaxation of rat aortic rings.<sup>26</sup>

### Biochemical characterization of SNTX

As a first step towards elucidating the structure-function relationship of SNTX, detailed genomic characterization of genes encoding SNTX had been performed by Ghadessy *et al.*<sup>27</sup> cDNA clones from a venom gland cDNA library encoding the  $\alpha$ - and  $\beta$ -subunits of SNTX were isolated and sequenced, with the deduced amino acid sequence for the  $\alpha$ - and  $\beta$ -subunits of SNTX containing 699 and 702 amino acids, respectively.<sup>28</sup> Although both the subunits are 50% homologous to each other, a homology search in GenBank indicated that SNTX is a novel lethal factor that shares no significant homology with other proteins. However, a recent report has indicated that SNTX shares a domain, B30.2, with several other proteins.<sup>29</sup>

The B30.2 domain is a conserved region of approximately 170 amino acids associated with several different protein domains. This globular domain is found in the C-terminal parts of three different types of proteins, each with distinct N-terminal regions. The first type comprises a subset of RING (Really Interesting New Gene) finger proteins with BBox and coiled-coil domains, which includes the ret finger protein (RFP),<sup>30-32</sup> the 52 kDa Sjoren's syndrome nuclear antigen A (SSA/Ro),<sup>33</sup> acid finger protein (AFP),<sup>34,35</sup> RING finger B30 protein (RFB30),<sup>36</sup> nuclear factor 7 of *Xenopus laevis* (xnf7),<sup>37</sup> nuclear factor pwa-33 of *Pleurodeles waltl*,<sup>38</sup> pyrin/marenostrin<sup>39</sup> and the oestrogen-responsive protein (EBBP).<sup>40</sup> The second category of N-terminal region is present in the extra-cytoplasmic region of butyrophilin (BT),<sup>41</sup> which is a glycoprotein associated with the milk fat globule membrane; the third type of N-terminal region associated with B30.2-like domains is found in the 71 and 79 kDa of SNTX  $\alpha$ - and  $\beta$ -subunits, respectively.

### Chemical modification studies of SNTX

Chemical modification of proteins in solution can provide important structural and functional information. In many ways, chemical modification is complementary to site-directed mutagenesis and protein engineering as a methodology for the study of protein variants. Although the latter method is favourable to modification of specific residues by chemicals, chemical modification is still useful when a biologically active recombinant protein of interest is not available for site-directed mutagenesis studies. Stonustoxin has been characterized by chemical modification and several amino acids, such as cationic lysine and arginine residues,<sup>24,42</sup> free thiol<sup>43</sup> and tryptophan,<sup>44</sup> have been found to play important roles in its function. Stonustoxin lost its lethal activity upon succinylation of the positively charged side chains of lysines to negative charges and, similarly, modification of arginine and lysine residues of SNTX by 2,3-butadione and 5,5'-dithiobis (2-nitrobenzoic acid; DTBN), respectively, also led to the loss of the toxin's lethality.<sup>42</sup> In contrast, the haemolytic activity of SNTX was affected by modification of tryptophan and lysine residues.<sup>24,44</sup> It has also been shown recently that modification of histidine residues in SNTX by diethylpyrocarbonate (DEPC) also affects its cytolytic and haemolytic activity (HE Khoo, unpubl. obs., 2001).

### Crystallization of SNTX

Crystals of SNTX have been obtained and preliminary data indicate that there is one native stonustoxin molecule per asymmetric



unit. The crystals belong to the tetragonal space group *P422*, with unit cell constants  $a = b = 109.0 \text{ \AA}$ ,  $c = 245.7 \text{ \AA}$ .<sup>45</sup>

### LETHAL FACTOR FROM OTHER SYNANCEJA SPECIES

The only other reported purified toxins from other species are trachynilysin (TLY) from *S. trachynis* venom<sup>16</sup> and verrucotoxin (VTX) from *S. verrucosa* venom.<sup>11</sup> Trachynilysin has a pI of 5.7 and a molecular weight of 158 kDa. It contains the venom's haemolytic, as well as the lethal and vascular permeability increasing, activity.<sup>16</sup> Further work on this toxin has shown that TLY also forms pores in planar lipid bilayers.<sup>46</sup> Trachynilysin also mediates soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein receptor (SNARE)-dependent release of catecholamines from chromaffin cells via external and stored  $\text{Ca}^{2+}$ .<sup>47</sup> Trachynilysin increased spontaneous quantal acetylcholine release from neuromuscular junctions.<sup>48</sup> Moreover, TLY also enhanced the release of acetylcholine from atrial cholinergic nerve terminals and indirectly activated muscarinic receptors.<sup>49</sup> An earlier report had shown that TLY selectively stimulated the release of small clear synaptic vesicles and impaired the recycling of small clear synaptic vesicles, but did not affect the release of large dense-core vesicles.<sup>50</sup>

Unlike SNTX and TLY, VTX was found to be a glycoprotein with a higher molecular weight of 322 kDa, comprising four subunits, two  $\alpha$  (83 000) and two  $\beta$  (78 000). Just like the crude venom, VTX was lethal in mice, lysed washed rabbit erythrocytes and induced a fall in blood pressure.<sup>11</sup> Verrucotoxin was found to inhibit calcium channels and may activate ATP-sensitive  $\text{K}^+$  channels in frog atrial heart muscle.<sup>51</sup> The cDNA of the  $\beta$ -subunit of VTX was found to encode an identical N-terminal sequence and had 96% homology with the beta subunit of SNTX.<sup>52</sup>

### NON-PROTEIN COMPONENTS OF STONEFISH VENOM

The only study on the presence of non-proteinaceous compounds in *S. verrucosa* was performed by Garnier *et al.*,<sup>53</sup> who showed that the venom contained noradrenaline and other biogenic amines, such as dopamine and tryptophan, but no 5-hydroxytryptamine.

### CONCLUSIONS

Most of the work on stonefish venom has been conducted on the three species that are available in the Indo-pacific (*S. horrida*), Australia (*S. trachynis*) and the Mediterranean (*S. verrucosa*). The lethal factors seem to be similar. Thus, even though VTX appeared to have four subunits (two  $\alpha$  and two  $\beta$ ), its  $\beta$ -subunit shared 96% homology with the  $\beta$ -subunit of SNTX.<sup>52</sup> Trachynilysin (158 kDa) is similar to SNTX (148 kDa) in molecular weight.<sup>16</sup> While some preliminary data on a protein crystal of SNTX have been reported,<sup>45</sup> more work needs to be performed to tease out the structure-function characteristics of this molecule because the mAb work seems to indicate that it has separate domains for its different activities.

The only enzyme that has been isolated from stonefish venom is hyaluronidase from *S. horrida*. This has been characterized and it should be interesting to compare its structure-function properties

with other fish hyaluronidases, as well as similar enzymes from other venoms.

### REFERENCES

1. Isbister GK. Venomous fish stings in tropical northern Australia. *Am. J. Emerg. Med.* 2001; **19**: 561–5.
2. Sutherland SK. Antivenom use in Australia. Premedication, adverse reactions and the use of venom detection kits. *Med. J. Aust.* 1992; **157**: 734–9.
3. Phoon WO, Alfred ER. A study of stonefish (*Synanceja*) stings in Singapore with a review of the venomous fishes of Malaysia. *Sing. Med. J.* 1965; **6**: 158–63.
4. Burnett JW. Aquatic adversaries: Stonefish. *Cutis* 1998; **62**: 269–70.
5. Khoo HE, Yuen R, Poh CH, Tan CH. Biological activities of *Synanceja horrida* (Stonefish) venom. *Nat. Toxins* 1992; **1**: 54–60.
6. Saunders PR, Rothman S, Medrano VA, Chin HP. Cardiovascular actions of venom of the stonefish *Synanceja horrida*. *Am. J. Physiol.* 1962; **203**: 429–32.
7. Wiener S. Observations of the venom of the stonefish (*Synanceja trachynis*). *Med. J. Aust.* 1959; **1**: 620–7.
8. Poh CH, Yuen R, Khoo HE, Chung M, Gwee M, Gopalakrishnakone P. Purification and partial characterization of stonustoxin (lethal factor) from *Synanceja-horrida* venom. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* 1991; **99**: 793–8.
9. Carlson RW, Schaeffer Jr RC, La Grange RG, Roberts CM, Russell FE. Some pharmacological properties of the venom of the scorpionfish *Scorpaena guttata*. I. *Toxicon* 1971; **9**: 379–91.
10. Austin L, Gillis RG, Youatt G. Stonefish venom: Some biochemical and chemical observations. *Aust. J. Exp. Biol. Med. Sci.* 1965; **43**: 79–90.
11. Garnier P, Goudeyepierre F, Breton P, Dewulf C, Petek F, Perriere C. Enzymatic-properties of the stonefish (*Synanceia verrucosa* Bloch and Schneider, 1801) venom and purification of a lethal, hypotensive and cytolytic factor. *Toxicon* 1995; **33**: 143–55.
12. Poh CH, Yuen R, Chung MCM, Khoo HE. Purification and partial characterization of hyaluronidase from stonefish (*Synanceja horrida*) venom. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* 1992; **101**: 159–63.
13. Sugahara K, Yamada S, Sugiura M *et al.* Identification of the reaction-products of the purified hyaluronidase from stonefish (*Synanceja horrida*) venom. *Biochem. J.* 1992; **283**: 99–104.
14. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. *J. Mol. Biol.* 1990; **215**: 403–10.
15. Duhig JV, Jones G. Haemotoxin of the stonefish *Synceja horrida*. *Aust. J. Exp. Biol.* 1928; **5**: 173–9.
16. Kreger AS. Detection of a cytolytic toxin in the venom of the stonefish (*Synanceia trachynis*). *Toxicon* 1991; **29**: 733–3.
17. Low KS, Gwee MC, Yuen R, Gopalakrishnakone P, Khoo HE. Stonustoxin: A highly potent endothelium-dependent vasorelaxant in the rat. *Toxicon* 1993; **31**: 1471–8.
18. Church JE, Hodgson WC. Dose-dependent cardiovascular and neuromuscular effects of stonefish (*Synanceja trachynis*) venom. *Toxicon* 2000; **38**: 391–407.
19. Russell FE. Marine toxins and venomous and poisonous marine animals. *Adv. Mar. Biol.* 1965; **3**: 255–85.
20. Low KS, Gwee MC, Yuen R. Neuromuscular effects of the venom of the stonefish *Synanceja horrida*. *Eur. J. Pharmacol.* 1990; **183**: 574 (Abstract).
21. Kreger AS, Molgo J, Comella JX, Hansson B, Thesleff S. Effects of stonefish (*Synanceia trachynis*) venom on murine and frog neuromuscular-junctions. *Toxicon* 1993; **31**: 307–17.
22. Low KS, Gwee MC, Yuen R, Gopalakrishnakone P, Khoo HE. Stonustoxin: Effects on neuromuscular function *in vitro* and *in vivo*. *Toxicon* 1994; **32**: 573–81.
23. Khoo HE, Hon WM, Lee SH, Yuen R. Effects of stonustoxin (lethal factor from *Synanceja horrida* venom) on platelet aggregation. *Toxicon* 1995; **33**: 1033–41.

24. Chen DS, Kini RM, Yuen R, Khoo HE. Haemolytic activity of stonustoxin from stonefish (*Synanceja horrida*) venom: Pore formation and the role of cationic amino acid residues. *Biochem. J.* 1997; **325**: 685–91.
25. Yuen R, Cai B, Khoo HE. Production and characterization of monoclonal antibodies against stonustoxin from *Synanceja horrida*. *Toxicon* 1995; **33**: 1557–64.
26. Sung JML, Low KS, Khoo HE. Characterization of the mechanism underlying stonustoxin-mediated relaxant response in the rat aorta *in vivo*. *Biochem. Pharmacol.* 2002; **63**: 1113–18.
27. Ghadessy FJ, Jeyaseelan K, Chung MCM, Khoo HE, Yuen R. A genomic region encoding stonefish (*Synanceja horrida*) stonustoxin beta-subunit contains an intron. *Toxicon* 1994; **32**: 1684–8.
28. Ghadessy FJ, Chen DS, Kini RM *et al*. Stonustoxin is a novel lethal factor from stonefish (*Synanceja horrida*) venom: cDNA cloning and characterization. *J. Biol. Chem.* 1996; **271**: 25 575–81.
29. Seto MH, Lin HL, Zajchowski DA, Whitlow M. Protein fold analysis of the B30.2-like domain. *Proteins* 1999; **35**: 235–49.
30. Takahashi M, Inaguma Y, Hiai H, Hirose F. Developmentally regulated expression of a human 'finger'-containing gene encoded by the 5' half of the ret transforming gene. *Mol. Cell Biol.* 1988; **8**: 1853–6.
31. Iwata Y, Nakayama A, Murakami H *et al*. Characterization of the promoter region of the human RFP gene. *Biochem. Biophys. Res. Commun.* 1999; **261**: 381–4.
32. Cao T, Borden KL, Freemont PS, Etkin LD. Involvement of the rfp tripartite motif in protein–protein interactions and subcellular distribution. *J. Cell Sci.* 1997; **110**: 1563–71.
33. Itoh K, Itoh Y, Frank MB. Protein heterogeneity in the human Ro/SSA ribonucleoproteins. The 52- and 60-kD Ro/SSA autoantigens are encoded by separate genes. *J. Clin. Invest.* 1991; **87**: 177–86.
34. Chu TW, Capossela A, Coleman R, Goei VL, Nallur G, Gruen JR. Cloning of a new 'finger' protein gene (ZNF173) within the class I region of the human MHC. *Genomics* 1995; **29**: 229–39.
35. Chan EK, Di Donato F, Hamel JC, Tseng CE, Buyon JP. 52-kD SS-A/Ro. Genomic structure and identification of an alternatively spliced transcript encoding a novel leucine zipper-minus autoantigen expressed in fetal and adult heart. *J. Exp. Med.* 1995; **182**: 983–92.
36. Henry J, Ribouchon M, Depetris D *et al*. Cloning, structural analysis, and mapping of the B30 and B7 multigenic families to the major histocompatibility complex (MHC) and other chromosomal regions. *Immunogenetics* 1997; **46**: 383–95.
37. Reddy BA, Kloc M, Etkin L. The cloning and characterization of a maternally expressed novel zinc finger nuclear phosphoprotein (xnf7) in *Xenopus laevis*. *Dev. Biol.* 1991; **148**: 107–16.
38. Bellini M, Lacroix JC, Gall JG. A putative zinc-binding protein on lampbrush chromosome loops. *EMBO J.* 1993; **12**: 107–14.
39. The International FMF Consortium. Ancient missense mutations in a new member of the RoRet gene family are likely to cause familial Mediterranean fever. *Cell* 1997; **90**: 797–807.
40. Liu HL, Golder-Novoselsky E, Seto MH, Webster L, McClary J, Zajchowski DA. The novel estrogen-responsive B-box protein (EBBP) gene is tamoxifen-regulated in cells expressing an estrogen receptor DNA-binding domain mutant. *Mol. Endocrinol.* 1998; **12**: 1733–48.
41. Jack LJ, Mather IH. Cloning and analysis of cDNA encoding bovine butyrophilin, an apical glycoprotein expressed in mammary tissue and secreted in association with the milk-fat globule membrane during lactation. *J. Biol. Chem.* 1990; **265**: 14 481–6.
42. Khoo HE, Chen DS, Yuen R. The role of cationic amino acid residues in the lethal activity of stonustoxin from stonefish (*Synanceja horrida*) venom. *Biochem. Mol. Biol. Int.* 1998; **44**: 643–6.
43. Khoo HE, Chen DS, Yuen R. Role of free thiol groups in the biological activities of stonustoxin, a lethal factor from stonefish (*Synanceja horrida*) venom. *Toxicon* 1998; **36**: 469–76.
44. Yew WS, Khoo HE. The role of tryptophan residues in the hemolytic activity of stonustoxin, a lethal factor from stonefish (*Synanceja horrida*) venom. *Biochimie* 2000; **82**: 251–7.
45. Yew WS, Kolatkar PR, Kuhn P, Khoo HE. Crystallization and preliminary crystallographic study of stonustoxin, a protein lethal factor isolated from the stonefish (*Synanceja horrida*) venom. *J. Struct. Biol.* 1999; **128**: 216–18.
46. Mattei C, Pompa C, Thieffry M, Kreger AS, Molgo J. Trachynilysin, a toxin isolated from stonefish *Synanceia trachynis* venom, forms pores in planar lipid bilayers. *Cybiurn* 2000; **24**: 141–8.
47. Meunier FA, Mattei C, Chameau P *et al*. Trachynilysin mediates SNARE-dependent release of catecholamines from chromaffin cells via external and stored Ca<sup>2+</sup>. *J. Cell Sci.* 2000; **113**: 1119–25.
48. Ouanounou G, Mattei C, Meunier FA, Kreger AS, Molgo J. Trachynilysin, a protein neurotoxin isolated from stonefish (*Synanceia trachynis*) venom, increases spontaneous quantal acetylcholine release from *Torpedo marmorata* neuromuscular junctions. *Cybiurn* 2000; **24**: 149–56.
49. Sauviat MP, Meunier FA, Kreger A, Molgo J. Effects of trachynilysin, a protein isolated from stonefish (*Synanceia trachynis*) venom, on frog atrial heart muscle. *Toxicon* 2000; **38**: 945–59.
50. Colasante C, Meunier FA, Kreger AS, Molgo J. Selective depletion of clear synaptic vesicles and enhanced quantal transmitter release at frog motor nerve endings produced by trachynilysin, a protein toxin isolated from stonefish (*Synanceia trachynis*) venom. *Eur. J. Neurosci.* 1996; **8**: 2149–56.
51. Garnier P, Sauviat MP, GoudeyPerriere F, Perriere C. Cardiotoxicity of verrucotoxin, a protein isolated from the venom of *Synanceia verrucosa*. *Toxicon* 1997; **35**: 47–55.
52. Garnier P, Ducancel F, Ogawa T *et al*. Complete amino-acid sequence of the beta-subunit of VTX from venom of the stonefish (*Synanceia verrucosa*) as identified from cDNA cloning experiments. *Biochim. Biophys. Acta Prot. Struct. Mol. Enzymol.* 1997; **1337**: 1–5.
53. Garnier P, Grosclaude JM, GoudeyPerriere F *et al*. Presence of norepinephrine and other biogenic amines in stonefish venom. *J. Chrom. B Biomed. Appl.* 1996; **685**: 364–9.