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# Probing inhibitory effects of destruxins from *Metarhizium anisopliae* using insect cell based impedance spectroscopy: inhibition vs chemical structure†

Keith B. Male,<sup>a</sup> Yew-Min Tzeng,<sup>\*b</sup> Johnny Montes,<sup>a</sup> Bing-Lan Liu,<sup>b</sup> Wan-Chun Liao,<sup>b</sup> Amine Kamen<sup>a</sup> and John H. T. Luong<sup>\*ac</sup>

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A noninvasive technique based on electric cell-substrate impedance sensing (ECIS) was demonstrated for on-line probing inhibitory effects of five destruxins on *Spodoptera frugiperda* Sf9 insect cells. Such chemically structurally similar cyclic hexadepsipeptides, were isolated and purified from the fungus *Metarhizium anisopliae*. Based on a response function, the inhibitory effect of the destruxins was established from determining the half-inhibition concentration (ECIS<sub>50</sub>), i.e., the level at which 50% inhibition of the cell response was obtained. Probing by cell based impedance spectroscopy indicated that only a slight change in their chemical structures provoked a significant effect on inhibition. Destruxin B was most inhibitory but replacement of a single methyl group with hydrogen (destruxin B2) or addition of a hydroxyl group (destruxin C) significantly reduced the inhibition. The removal of one methyl group and one hydrogen (destruxin A) lowered the inhibitory effect even more whereas the formation of an epoxy ring (destruxin E) in the structure nullified the inhibitory effect.

## Introduction

*Metarhizium anisopliae* has been widely used in the synthesis of cyclic hexadepsipeptides, known as destruxins which consist of an  $\alpha$ -hydroxyl acid and five amino acid residues. They are the only mycotoxins detected in the insect body at advance stages of infection in sufficient quantities to cause death.<sup>1–3</sup> To date, over 35 different structurally related destruxins have been isolated from cultures of *Aschersonia aleyrodis*,<sup>4</sup> *Alternaria brassica*,<sup>5</sup> *Oospora destructor*,<sup>6</sup> and *M. anisopliae*.<sup>7,8</sup> These forms differ in the R-group of the hydroxyl acid residue and appear to have overlapping but different biological effects. Destruxins A, B, and E are the most abounding components, which possess the same amino acid sequence but differ in the hydroxyl acid residue. The chemical structures of cyclic hexadepsipeptide destruxins A, B and E have the same backbone, with the general formula: –D-HA-L-Pro-L-Ile-L-MeVal-L-MeAla- $\beta$ -Ala–, where HA is a D- $\alpha$ -hydroxy acid residue. Five destruxin derivatives (Fig. 1) used in this study were isolated and identified as described in the literature.<sup>9–11</sup> Some important properties of these compounds have also been reviewed and can be found elsewhere.<sup>10,12</sup>

By virtue of their structure, destruxins exhibit a wide spectrum of biological and insecticidal activities.<sup>10,13</sup> The influence of destruxins on morphological and cytoskeletal changes in insect plasmatocytes *in vitro* has been reported. They adversely affect insect cellular immune responses such as encapsulation and phagocytosis.<sup>14,15</sup> Indeed, the destruxins also act as effective

insecticides against *Spodoptera litura* (Fab).<sup>16</sup> They mediate specific down-regulation of antimicrobial peptides by targeting the insect's innate immune signaling pathway.<sup>17</sup> Remarkable changes in the status of several antioxidant enzymes (catalase, peroxidase, ascorbate oxidase, and superoxide dismutase) and

Compound	Molecular Formula	R <sub>1</sub>	R <sub>2</sub>	Molecular Weight
Destruxin A (DA)	C <sub>29</sub> H <sub>47</sub> N <sub>5</sub> O <sub>6</sub>	Me		577
Destruxin B (DB)	C <sub>30</sub> H <sub>51</sub> N <sub>5</sub> O <sub>7</sub>	Me		593
Destruxin B2 (DB2)	C <sub>29</sub> H <sub>49</sub> N <sub>5</sub> O <sub>7</sub>	H		579
Destruxin C (DC)	C <sub>30</sub> H <sub>51</sub> N <sub>5</sub> O <sub>8</sub>	Me		609
Destruxin E (DE)	C <sub>29</sub> H <sub>47</sub> N <sub>5</sub> O <sub>8</sub>	Me		593

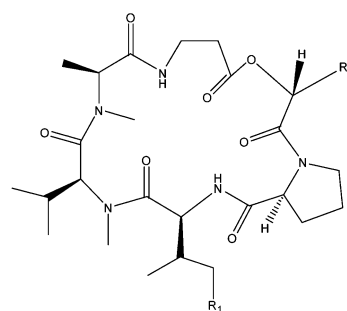


Fig. 1 Chemical structures of the five destruxins isolated and purified from *Metarhizium anisopliae*.

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the levels of lipid peroxidation and lipoxygenase enzyme are shown in the 9-day-old larvae of *S. litura* upon crude destruxin treatment.<sup>16</sup> Destruxin B and desmethyl-destruxin B are phytotoxic to the plants of *Brassica*.<sup>18</sup> Destruxins are also toxic to mammalian leukemia cells and spleen lymphocytes and have demonstrated anti-proliferative activity on mouse neoplasms *in vitro*.<sup>10,19</sup> Furthermore, destruxin A, destruxin B, roseotoxin B (RB), and roseocardin (RC) exhibit positive inotropic effects on heart muscles. This inotropic effect is accompanied by a negative chronotropic effect on the right atrium,<sup>20</sup> whereas destruxin B is a specific, dose dependent and reversible inhibitor of vacuolar-type ATPase, which maintains the acidity in the vacuolar organelles.<sup>10</sup>

Cell spreading, morphology, and micromotion, three important parameters in tissue culture, have been quantified using an electrical method referred to as electric cell-substrate impedance sensing (ECIS).<sup>21,22</sup> The resulting impedance, a coordination of many biochemical reactions, is very sensitive to pH, temperature, and chemical compounds added to the culture medium. This method serves as a general tool for probing cell spreading and motility as well as an alternative to animal testing for toxicology studies. To date, mammalian cells have been used extensively with ECIS to probe cell behavior including spreading, micromotion, and cytotoxicity.<sup>23–25</sup> Nevertheless, *Spodoptera frugiperda* Sf9 (fall armyworm) insect cells have been used with ECIS to probe the inhibitory effects of structurally similar compounds isolated and purified from the fruiting bodies of the fungus *Antrodia camphorata*.<sup>26</sup> This fall armyworm can be one of the more difficult insect pests to control in field corn. On the basis of its adherence and ease of maintenance, this *Spodoptera frugiperda* Sf9 cell line could be an ideal model to screen for new insecticides. Another potential application of this combination is the screening for new and more effective drugs for malaria with minimal side effects. This disease, transmitted by infected mosquitoes, still claims more than one million lives every year whereas children and pregnant women are particularly susceptible. There are an estimated 300 million acute cases of malaria each year globally, particularly prevalent in tropical and sub-tropical regions of the world, according to the World Health Organization (WHO).<sup>27</sup>

Destruxins A, B, and E have been shown to have antiviral properties in insect and human cell lines.<sup>17</sup> In particular, destruxin B displays a suppressive effect on hepatitis B surface antigen (HBsAg) in human hepatoma cells.<sup>28–30</sup> Destruxin-induced membrane depolarization due to the opening of Ca<sup>2+</sup> channels has been implicated as a cause of paralysis and death. These could be indirect results of the activation of Ca<sup>2+</sup> channels in insect muscles.<sup>31</sup> The influence of destruxins on morphological and cytoskeletal changes in insect plasmatocytes *in vitro*, which in turn adversely affects insect cellular immune responses such as encapsulation and phagocytosis were reported.<sup>14,15</sup> Therefore, ECIS can be exploited to probe the efficacy of these anti-hepatitis B drugs, a subject of future endeavor.

This paper demonstrates the applicability of impedance spectroscopy for continuous assessment of the behavior of *Spodoptera frugiperda* Sf9 insect cells exposed to five destruxins isolated and purified from *Metarhizium anisopliae*. A significant change in the measured impedance is anticipated due to attachment and spreading of the *Spodoptera frugiperda* Sf9 insect cells.

Alterations in cell behavior after exposure to these destruxins, whether cytotoxic or inhibitory at the substratum level, will lead to a resulting chemical effect which can be screened by measuring the impedance change.

## Materials and methods

### Production of destruxins

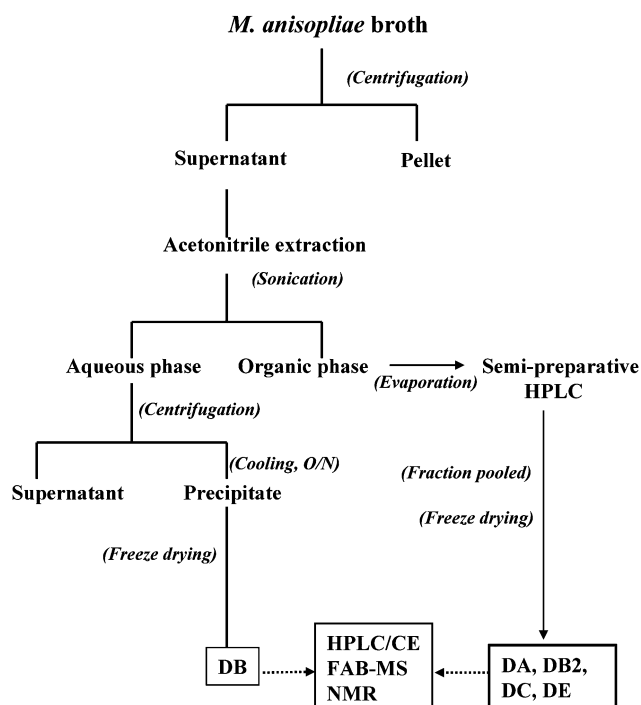
The seed culture (the spore density  $\sim 1 \times 10^7$  mL<sup>-1</sup>) from  $-80^\circ\text{C}$  was thawed at room temperature and inoculated into a 200 mL culture medium of 3% (w/v) Czapek-Dox broth with 0.75% Bacto-peptone (Difco) in a 500 mL Erlenmeyer flask with a baffle. The flask was cultivated in an incubator (LM-575R, Yih-Der Co., Taipei, Taiwan) at 200 rpm, 28 °C for 4 days. For the stirred-tank cultivation, the inoculum (10% of the working volume) was transferred from the flask of the 4-day-old seed culture to the reactor, which contained 3 L of the desired medium. Cultivations were conducted in a 5 L stirred-tank reactor (BTF-600T, Bio-Top Inc, Taichung, Taiwan) at 28 °C with the aeration rate regulated at 0.3 vvm (volume air volume liquid<sup>-1</sup> min<sup>-1</sup>). The culture medium pH 4 was maintained by automatic addition of 1 N NaOH or 1 N H<sub>2</sub>SO<sub>4</sub>. Unless otherwise indicated, the agitation rate was maintained at 150 rpm.<sup>7,8</sup>

### Isolation of destruxins

The destruxins were isolated and purified according to the method of Chen *et al.*<sup>32</sup> with minor modification. The culture medium was harvested after incubation for 14 days and centrifuged at 8000 rpm for 10 min. The supernatant was extracted three times with ethyl acetate (sample : EA = 5 : 2, v/v) under sonication, and the organic phase was evaporated with a rotary vacuum evaporator (model N-1, Eyela, Tokyo, Japan) at 40 °C. The concentrate was diluted with 4 times volume of water and incubated at 4 °C overnight until the precipitation of crystal was observed (destruxin B). The crystal was washed with reverse osmosis water and freeze-dried. The supernatant was further purified using a semi-preparative HPLC. After filtration through a 0.22  $\mu\text{m}$  chromatodisc unit, the sample (0.5 mL) was injected into a column (Merck LiChrosorb RP-18, 10  $\times$  250 mm, 7  $\mu\text{m}$ ). The eluent from the column was monitored at 215 nm with an L-7100 pump and L-7400 UV detector (Hitachi, Tokyo, Japan). The running gradient was: 0 min (0% acetonitrile, ACN), 30 min (40% ACN), 40 min (50% ACN) and 60 min (50% ACN). The eluting solvent was set at 2.5 mL min<sup>-1</sup>. The purification procedure is depicted in Fig. 2. Fractionated samples were characterized by FAB-MS and <sup>1</sup>H NMR spectroscopies.

### Analytical procedures

For destruxin analysis, a Micra (Northbrook, IL) NPS RP-C18 analytical column (33  $\times$  4.6 mm) was used. A gradient combination of the ACN/water system was set as: 0 min (0% ACN), 20 min (27% ACN), 25 min (90% ACN), and 30 min (90% ACN). The eluting solvent (1 mL min<sup>-1</sup>) was maintained throughout the studies. The injection volume was 5  $\mu\text{L}$  and the absorbance was recorded at 215 nm for 30 min. The details for analysis of destruxins by HPLC and capillary electrophoresis were described previously.<sup>33,34</sup> All mass spectrometric analyses were performed



**Fig. 2** Schematic for the preparation of the destruxins.

on a JMS-HX 110 (Joel, Tokyo, Japan) double-focusing magnetic sector mass spectrometer with modified Nier-Johnson geometry. Spectra were recorded by the Joel DA5000 data system. Destruxins samples for NMR studies were unbuffered by vacuum-drying and reconstituted in the  $\text{CDCl}_3$ .  $^1\text{H}$  NMR spectra were recorded at 300 MHz on a Bruker (Billerica, MA) DPX 300 spectrometer as described elsewhere.<sup>32</sup>

### Cell line and culture conditions

Erlenmeyer flasks (125 mL) with a 20 mL serum-free SF-900 II medium (Gibco BRL, Canadian Life Technologies, Burlington, ON, Canada) were used to maintain the *Spodoptera frugiperda* Sf9 cells. Cells cultured weekly at  $0.4 \times 10^6$  cells mL<sup>-1</sup> (27 °C, pH 6.2 and 110 rpm agitation) were monitored during growth for viability by the Trypan Blue exclusion assay using a CEDEX Innovatis cell counter (Bielefeld, Germany). Sf9 cells, inoculated at an initial cell density of  $0.4 \times 10^6$  cells mL<sup>-1</sup>, were grown to the mid-exponential phase ( $2.5\text{--}3 \times 10^6$  cells mL<sup>-1</sup>) and centrifuged at 1500 rpm for 4 min. A fresh medium was used to resuspend the cells to  $3 \times 10^6$  cells mL<sup>-1</sup>.

### Electrode coating and cell inoculation

As described by Luong *et al.*,<sup>35</sup> concanavalin A (Con A, 0.40 mL, 0.5 mg mL<sup>-1</sup>, prepared fresh daily in 50 mM PBS, pH 7.4 with the aid of sonication for 1 h) was added to coat the detecting gold electrodes (250  $\mu$ m diameter) in each of the 8 wells of a sensing chip (8W1E, Applied Biophysics, Troy, NY). Con A bound quickly ( $\sim$ 90% of the change occurs in the first 10 min) to the electrode surface as confirmed by an increase in the impedance and a decrease in the capacitance of each well. The attachment of Con A to the gold electrode was very stable as reflected by

constant impedance readings over 24 h. After protein adsorption ( $\sim 30\text{--}60$  min), the wells were washed 3 times with 0.85% NaCl and then 0.4 mL of culture medium was placed in each well and the resistance of the wells was monitored for 1 h at  $27^\circ\text{C}$  in a humidified chamber until it had stabilized. The wells were emptied and 0.4 mL of cell suspension ( $\sim 3 \times 10^6$  cells  $\text{mL}^{-1}$ ) was added into each well. Except for destruxin E which was prepared at 100 mM, the remaining destruxins (6–12 mg) were dissolved in ethanol (0.5–1.0 mL) with the aid of sonication to concentrations of 20 mM. Destruxin samples at various concentrations in ethanol (30  $\mu\text{L}$ ) were then added to cell suspensions (1.5 mL at  $3 \times 10^6$  cells  $\text{mL}^{-1}$ ) before adding 0.4 mL (from the same destruxin sample–cell suspension mixture) to 2 or 3 wells to test for possible inhibitory effects. For each destruxin, 6 concentrations including a control with 30  $\mu\text{L}$  ethanol were tested at the same time and each destruxin was analyzed 3–5 different times.

## Impedance measurement with ECIS

Detailed information on ECIS impedance measurement has been reported elsewhere.<sup>23–26,35</sup> For each experiment up to 16 sample wells (2 chips of 8 wells, each containing a singly addressable detecting electrode) can be monitored simultaneously by the system. The 8 detecting electrodes share a common counter gold electrode and the two electrodes (detecting gold electrode and counter gold electrode) of the well are connected to a lock-in amplifier of the ECIS system and the cell behavior should not be affected by the applied potential if it is less than 1 V AC.<sup>36,37</sup> The system acquires resistance, impedance and capacitance data such that the impedance of each well was measured every 2 min at 4 kHz. However, in this study we have focused on the resistance changes since they were largest. The  $ECIS_{50}$  value derived from the time response function,  $f(C, t)$ , was calculated as described by Xiao *et al.*<sup>23</sup> Data points at 30 min intervals were selected from the raw resistance data to simplify plots and calculations. The cells were observed during experimentation by temporarily removing the sensing chip (pause function from the software) from the ECIS system incubator and placing it on a Wilevert AFL 30 inverted microscope (Hund, Germany) equipped with a digital video camera (KP-D50U, Hitachi, Tokyo, Japan).

## Results and discussion

### Response of Sf9 insect cells

The gold electrode was fully covered by Con A; a lectin purified from *Concanavalis ensiformis*. Con A has been shown to provoke the best adhesion and spreading behavior for this insect cell line,<sup>26,35</sup> whereas fibronectin, collagen and laminin, are known to promote cell adhesion and spreading for mammalian cells. Con A is also adsorbed more strongly to a gold surface in comparison to fibronectin, a well-known cellular “glue” for various mammalian cells, as reflected by a significantly smaller  $K_d$  value, 0.89 nM (the binding constant,  $1/K_d = 1.12 \text{ nM}^{-1}$ ) for Con A in comparison to 63 nM for fibronectin ( $1/K_d = 0.015 \text{ nM}^{-1}$ ).<sup>35</sup> The resistance of the culture medium without cells was  $\sim 3.5 \text{ k}\Omega$  while with cells there was a slight increase to  $\sim 4.0 \text{ k}\Omega$ . Without the destruxins, as observed by the video-enhanced microscope, the cells descended to the bottom of the well within 20 min and as the cells spread, they changed to flattened forms with much

larger dimensions compared to their initial round shapes. Cell–substratum (cell–Con A) interactions including spreading, morphology and cell motility require a complex series of events to occur in a regulated and integrated fashion. The effective area available for current flow was altered as the cells spread causing a significant increase in the resistance of the well by  $\sim 6.6 \text{ k}\Omega$  as shown in Fig. 3 (curve a). The number of normal Sf9 cells to completely cover an 8W1E detecting electrode coated with Con A was 150–200 such that a resistance change of  $35\text{--}50 \text{ }\Omega \text{ cell}^{-1}$  was predicted for each attached cell. Note that without Con A bound to the electrode surface the resistance increase in the presence of insect cells was very small ( $< 2 \text{ k}\Omega$ ). Therefore, if the attachment of Con A to the gold surface was not strong the resistance increase would have been much closer to  $2 \text{ k}\Omega$ .

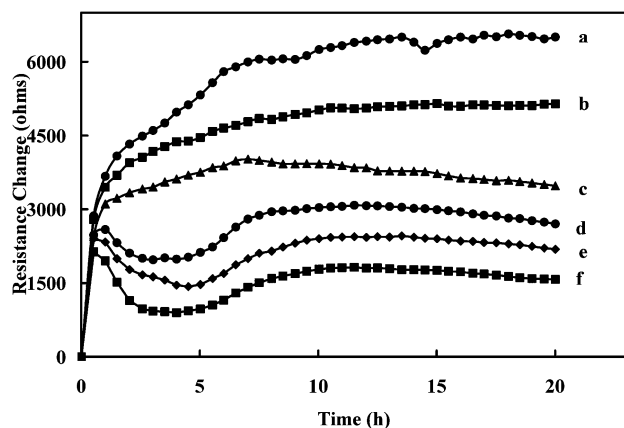
Addition of a low concentration ( $4 \text{ }\mu\text{M}$ ) of destruxin B (Fig. 3, curve b) to the cell suspension exhibited a minimal effect on the resistance signal compared to the control well (Fig. 3, curve a). However, as the concentration was increased ( $8\text{--}100 \text{ }\mu\text{M}$ ) the resistance change was significantly decreased (Fig. 3, curves c–f) as the time increased. The resistance change for all destruxin B concentrations was similar for the first 30 min, as during this time the cells are settling to the electrode surface followed by initial spreading. Without destruxins, the insect cells were intact and well spread on the Con A coated electrode surface even after washing the wells with saline 3 times as observed by inverted fluorescent microscopy (Fig. 4a). After 24 h into the experiment, there were  $\sim 197$  cells on the electrode surface. However, insect cells exposed to  $100 \text{ }\mu\text{M}$  destruxin B for 24 h were more spherical (Fig. 4b), compared to the control cells. Extensive washing of these ECIS wells with saline removed many of the effector cells indicating that they were no longer firmly attached to the Con A precoated electrode surface. In addition, destruxin B could bind directly to either bare gold surfaces or Con A coated gold surfaces (in the absence of insect cells) as illustrated by an impedance increase in both cases. Hydrophobic interaction is mainly anticipated between destruxin B and the gold surface whereas the interaction between destruxin B and Con A could be governed by both hydrogen bonding and hydrophobic interactions. The impedance increase was stable over time indicating a strong attachment of destruxin B to either surface. The

presence of destruxin B (up to  $200 \text{ }\mu\text{M}$ ) bound to the Con A coated electrode surface did not interfere with the resistance response to insect cells as the resistance response obtained with or without destruxin was virtually comparable. The wells were extensively washed to remove excess destruxin before adding the cells.

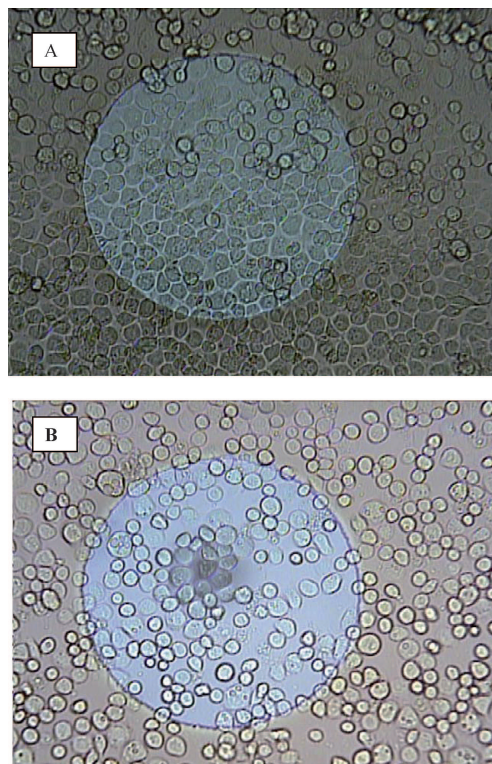
### Half-inhibition concentration (ECIS<sub>50</sub>) for destruxin B

For the effector cells, the resistance change ( $\Delta R_s$ ) of the well is dependent on the number ( $N_0$ ) of initial cells attached on the detecting electrode, the toxicant concentration ( $C$ ) and the exposure time ( $t$ ) as reported by Xiao *et al.*<sup>23</sup> The resistance change normalized by  $N_0$  is defined as the cell response to the toxicant measured by ECIS,  $f(C, t) = \Delta R_s/N_0$ . As a control with no toxicant,  $C$  is equal to zero and  $f(0, t)$  increases as the cells spread on the electrode and reaches a plateau. In the presence of toxicant,  $f(C, t)$  after an initial increase the value decreases and even approaches zero, indicating total cell death at high toxicant concentrations. The inhibitor concentration required to achieve 50% inhibition of the response is defined as the half-inhibition concentration (ECIS<sub>50</sub>) or  $f(\text{ECIS}_{50}, t)/f(0, t) = 50\%$ .

The data obtained in Fig. 3 was used to calculate the ECIS<sub>50</sub> for destruxin B. The time response function  $f(C, t)$  was used to construct a series of inhibition curves at any given time  $t_0$  ( $> 2.5 \text{ h}$ ) for the series of destruxin B concentrations used in Fig. 3. As confirmed by inverted fluorescent microscopy, the insect cells settled to the electrode surface coated with Con A very rapidly (20 min). Very few cells ( $< 10\%$ ) were observed in the medium of



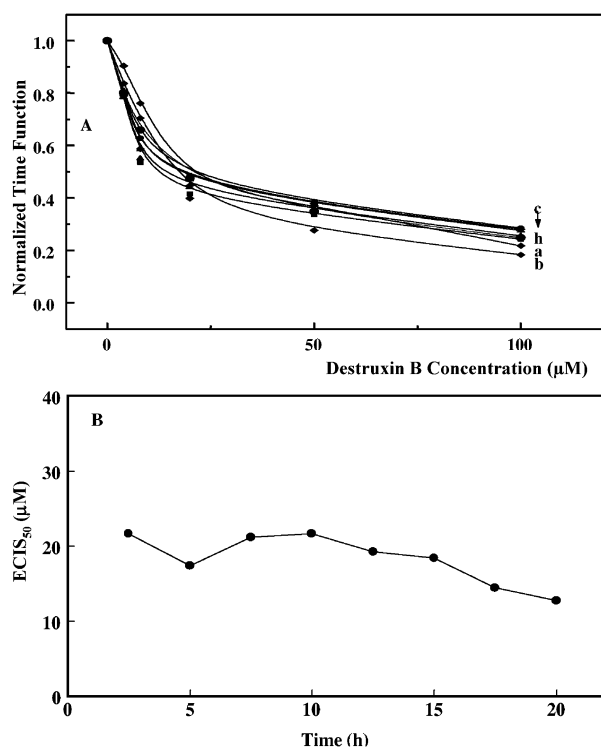
**Fig. 3** (A) Resistance response change ( $\Delta\Omega$ ) of Sf9 insect cells to various concentrations ( $\mu\text{M}$ ) of destruxin B: (a) 0, (b) 4, (c) 8, (d) 20, (e) 50 and (f) 100.



**Fig. 4** Microscopic photos of the electrode surface ( $250 \text{ }\mu\text{m}$  diameter) after 24 h: (A) electrode at  $0 \text{ }\mu\text{M}$  destruxin B, (B) electrode at  $100 \text{ }\mu\text{M}$  destruxin B.

the well, while 180 cells were counted on the electrode surface after the removal of this medium. The cells spread on the electrode surface over the next 2 h to form a confluent layer resulting in an increase in the resistance. Similarly, cells could also settle on the gold electrode surface in the absence of the Con A coating, however spreading was not observed. In the presence of destruxin B at 4, 8, 20 and 50  $\mu\text{M}$ , the number of insect cells observed on the electrode surface after 20 min was similar to the control without destruxin B. Therefore, the presence of destruxin B did not interfere with the initial settling of the insect cells, although the spreading was not as evident as time progressed especially at high concentrations of destruxin B. As a result,  $N_0$  for each well was assumed to be equivalent, therefore no adjustment for  $\Delta R_s$  was required due to different  $N_0$  values. The time response function,  $f(C, t)$ , was then normalized by simply taking the  $\Delta R_s$ , *i.e.*,  $R_t - R_0$  at different destruxin B concentrations and dividing these values by the  $\Delta R_s$  value at  $f(0, t)$ . As expected, as the concentration of destruxin B increased for all exposure times considered the normalized time response function decreased (Fig. 5A).

The  $\text{ECIS}_{50}$  for destruxin B was determined for each exposure time by extrapolating the value on the concentration axis when

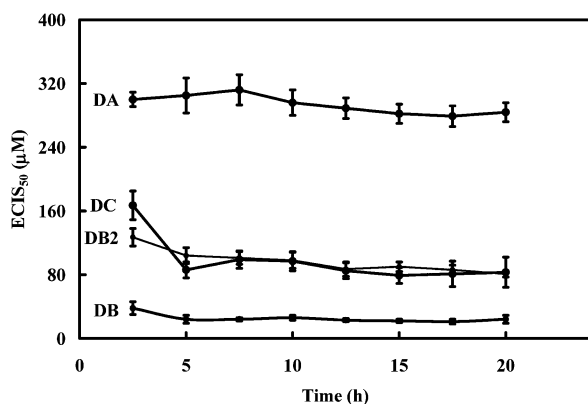


**Fig. 5** (A) Destruxin B inhibition curves were obtained for each destruxin B concentration (Fig. 3, curves a–f) at various exposure times (h): (a) 2.5, (b) 5.0, (c) 7.5, (d) 10, (e) 12.5, (f) 15, (g) 17.5 and (h) 20. The normalized time response function (*y*-axis),  $f(C, t)$ , was determined by taking the  $\Delta R_s$  (Fig. 2, curves b–f), *i.e.*,  $R_t - R_0$  at different destruxin B concentrations and dividing the values by the  $\Delta R_s$  ( $\sim 6.6 \text{ k}\Omega$ , Fig. 3, curve a) at  $f(0, t)$ . (B) Relationship between the half-inhibition concentration ( $\text{ECIS}_{50}$ ) and exposure time during cell culture for destruxin B. The  $\text{ECIS}_{50}$  value obtained for destruxin B was determined for each exposure time (curves a–h) by extrapolating the value for the *x*-axis from the *y*-axis (0.5).

the normalized time response function was 0.5. Fig. 5B shows the relationship between the half-inhibition concentration and exposure time, indicating that the  $\text{ECIS}_{50}$  for destruxin B was  $\sim 20 \mu\text{M}$  at all exposure times greater than 2.5 h. In this study, due to the rapid settling of the cells on the surface at high cell concentration, the  $\text{ECIS}_{50}$  value was a reflection of the interference or inhibition capacity of destruxin B with respect to the cell spreading on the substratum. After 20 min into the experiment, if the medium containing  $100 \mu\text{M}$  of destruxin B was removed and replaced by a control medium, the resistance increased rapidly to that of the control. Such behavior indicated that the inhibitory effect could be reversed after the initial binding of the cells to the surface by removing the destruxin B. However, after a contact time of 4 h, the resistance effect could not be reversed, implying that after a longer period of time the cells could not easily spread even after the removal of destruxin B. Cell viability testing by the Trypan Blue exclusion assay confirmed that destruxin B was not toxic to the cells at the concentrations used during the experiment. The cell viability in the absence of destruxin was 85% (1533 viable cells of 1804 cells counted) after 4 h, while in the presence of destruxin B at  $20 \mu\text{M}$  (1546 viable cells of 1798 cells counted), and  $100 \mu\text{M}$  (1584 viable cells of 1800 cells counted) the cell viability was still 86% and 88%, respectively. Even at a concentration ( $400 \mu\text{M}$ ) of destruxin B much higher than that used for the ECIS experiments, the cells were still viable (87% viable cells of 1850 cells counted). Hence, the effect of destruxin on the ECIS response was not likely cytotoxic, but rather an inhibitory effect at the level of cell adherence and spreading on the substratum layer. In addition, an experiment was performed by adding destruxin ( $100 \mu\text{M}$ ) to a well which had reached confluency (resistance maximum) after a few hours. The resistance decreased immediately and the effector cells were more spherical in morphology. This finding revealed that destruxin could still have an inhibitory effect at the substratum level even after the cells had been spread on the Con A coated electrode surface.

### Inhibitory effect vs chemical structural change

A series of experiments were conducted using the four destruxins whose chemical structures are very similar to destruxin B. Destruxin B2 only differs from destruxin B in the replacement of the methyl group at position  $R_1$  by hydrogen. Impedance analysis indicated that this destruxin was less inhibitory as the  $\text{ECIS}_{50}$  value increased from  $23 \mu\text{M}$  to  $92 \mu\text{M}$  (Fig. 6). Destruxin C differs from destruxin B in that one of the methyl groups at position  $R_2$  has been hydroxylated. Similar to destruxin B2, the  $\text{ECIS}_{50}$  value for destruxin C increased compared to destruxin B from  $20 \mu\text{M}$  to  $87 \mu\text{M}$  (Fig. 6). These two findings indicate that increased levels of methylation (3 for destruxin B vs 2 for destruxin B2 or C) seem to correlate to increased inhibition. Destruxin A differs from destruxin B in that the  $R_2$  group has lost one methyl group and that there is also a  $\text{C}=\text{C}$  group. The removal of these two methyl groups drastically reduced the inhibitory effect as the  $\text{ECIS}_{50}$  was determined to be  $293 \mu\text{M}$  as shown in Fig. 6. It should be noted that sometimes at lower exposure times the  $\text{ECIS}_{50}$  value was higher, indicating there was a lag time before the destruxin generates the inhibitory effect. The last destruxin tested, destruxin E, has an epoxy ring as part



**Fig. 6** Relationship between the half-inhibition concentration and time, during cell culture for destruxins: DA, DB, DB2, and DC. Data expressed as SEM,  $n = 3$ –5.

of R<sub>2</sub> and only one methyl group and this compound was observed to have no significant inhibitory effect up to 2 mM.

## Conclusions

In brief, an on-line and continuous technique based on electric cell–substrate impedance sensing (ECIS) has been developed for measuring the concentration and time response function of *Sf9* insect cells exposed to a series of destruxins from *Metarhizium anisopliae*. The half inhibition concentration, (ECIS<sub>50</sub>), the required concentration to achieve 50% inhibition, can be estimated from the response function to ascertain inhibition during the course of the assay. The technique is extremely sensitive to detecting inhibition in response to slight changes in the chemical structures of such destruxins. An obvious application of ECIS with *Sf9* insect cells is the screening of anti-malarial drugs and their plausible side effects. On the basis of their anti-viral activity for hepatitis B, ECIS with pertinent cells is extremely useful to assess the efficacy of destruxin analogs which are modified chemically or enzymatically to improve their effectiveness and/or suppress any drug resistance from the virus.

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