Cytotoxicity of diplodiatoxin, dipmatol and diplonine, metabolites synthesized by *Stenocarpella maydis*

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Abstract

The cytotoxicity of three *Stenocarpella maydis* metabolites (diplodiatoxin, dipmatol and diplonine) was investigated on Neuro-2a, CHO-K1 and MDBK cell lines. Diplodiatoxin was the most cytotoxic followed by dipmatol. Conversely, diplonine was not cytotoxic. Diplodiatoxin and dipmatol affected mitochondrial succinate dehydrogenase (MTT assay) and the overall viability of cells as assessed in real-time (xCELLigence assay). The results obtained so far indicate that diplodiatoxin and dipmatol exert their toxicity possibly via the necrotic cell death pathway.

Keywords: *Stenocarpella maydis*, diplodiatoxin, diplonine, dipmatol, cytotoxicity, xCELLigence

Stenocarpella maydis (Berk.) Sutton, formerly known as *Diplodia maydis* (Berk.) Sacc., is encountered throughout the world as an economically important pathogen of maize. This fungus causes a seedling blight as well as stem and cob rot of maize (Marasas, 1977; Kellerman et al., 1985). Diplodiosis, a nervous disorder of cattle and sheep, results from the ingestion of mouldy cobs infected by *S. maydis*. Although this disease is most common in southern Africa (Kellerman et al., 1996), it has also been reported in Australia (Darvall, 1964), Argentina (Odriozola et al., 2005) and Brazil (Riet-Correa et al., 2013). Diplodiosis is characterized by reluctance of the animals to move, a wide-based stance, incoordination, tremors, paralysis and death (Kellerman et al., 1985). Gross pathological changes are rarely seen in ruminants suffering from diplodiosis.

Different toxic metabolites have been isolated from *S. maydis* or *S.maydis*-contaminated cultures by various research groups. These metabolites include diplodiatoxin (Steyn et al., 1972), dipmatol (Ackerman et al., 1995), chaetoglobosins K and L (Wicklow et al., 2011) and diplonine (Snyman et al., 2011).

The toxicity of diplodiatoxin has been evaluated in rats and poultry (Steyn et al., 1972; Rahman et al., 2002). Rahman et al. (2002) provided the first evidence, that diplodiatoxin could induce neurological clinical signs. Clinical signs reported include irritability, dullness, tremors and convulsions when purified diplodiatoxin was administered at a single oral dose of 5.7 mg/kg or 0.27 mg/kg/day for 21 days to male and female rats. In addition, a significant inhibition of brain acetylcholinesterase activity was observed in both groups, indicating a neurotoxic effect. The toxicity of dipmatol (Ackerman et al., 1995) and chaetoglobosin K (Cutler et al., 1980) has been evaluated in broiler chickens. Chaetoglobosin K was toxic to day-old chicks with an LD_{50} of between 25 and 62.5 mg/kg.

Guinea pigs dosed with 1580 mg diplonine per kg body weight per os developed incoordination, imbalance, paresis in the hindquarters, frequent falling and lateral recumbency which were followed by recovery (Snyman et al., 2011). It was suggested that the clinical signs were reminiscent of the neurological signs observed in cattle and sheep with diplodiosis.

However, none of these *S. maydis* metabolites have been administered to ruminants in an attempt to reproduce diplodiosis. Consequently it is not known whether these compounds play a role in the aetiology of diplodiosis. There is limited information available on the mechanism of action of these *S. maydis* metabolites. Understanding how the *S. maydis* metabolites exert their cellular effects will be valuable in elucidating the pathogenesis of diplodiosis.

The purpose of this study was to evaluate the cytotoxicity of diplodiatoxin, dipmatol and diplonine on the Neuro-2a (mouse neuroblastoma), CHO-K1 (Chinese hamster ovary) and MDBK (Mardin-Darby bovine kidney) cell lines. Cytotoxicity of the *S. maydis* metabolites was determined using the real-time cell analyzer (RTCA) xCELLigence and the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

All the materials were purchased from Sigma Aldrich (South Africa) unless otherwise stated. Diplodiatoxin ($C_{18}H_{28}O_4$; molecular weight = 308 g/mol) (Steyn et al., 1972), dipmatol ($C_{15}H_{27}O_5$; molecular weight = 287 g/mol) (Ackerman et al., 1995) and diplonine ($C_6H_{11}NO_3$; molecular weight = 145 g/mol) (Snyman et al., 2011) were utilized. The Neuro-2a, CHO-K1 and MDBK cells were obtained from the American Type Culture Collection (ATCC). The cells were grown in DMEM (Dulbecco's modified Eagle's medium) (Neuro-2a cells) or DMEM and Hams F-12 Nutrient Mixture (CHO-K1 and MDBK cells) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 µg/mL streptomycin and 2.5 µg/mL

amphotericin B (Fungizone) in a humidified atmosphere of 5% CO_2 at 37 °C. Cell lines seeded at a density of 20,000 cells/mL were exposed to the *S. maydis* toxic metabolites. After attaching to the wells (24h post culturing), cells were exposed to diplodiatoxin, dipmatol and diplonine at concentrations of 10, 100, 250, 350, 500 and 750 µM (750 µM concentrations were not included in xCELLigence assays for CHO-K1 cells). Control wells were prepared by adding 200 µL of the corresponding culture medium. The cytotoxic effect of the three *S. maydis* metabolites was evaluated by using the RTCA xCELLigence 16-well plate system (Roche Applied Sciences) and MTT assay (Mosmann, 1983). Three independent experiments were carried out with three replicate wells for each toxin concentration. A factorial analysis of variance (ANOVA) was used for analysis of the MTT assay data with exposure duration (24, 48 and 72 h), toxin (diplodiatoxin, dipmatol and diplonine), concentration (10, 100, 250, 350, 500 and 750 µM) and cell type (Neuro-2a, CHO-K1 and MDBK) as factors.

The dynamic cytotoxic responses of the Neuro-2a, CHO-K1 and MDBK cells after exposure to diplodiatoxin, dipmatol and diplonine are shown in Fig. 1. A concentration-dependent cytotoxic response was observed when the three cell lines were exposed to diplodiatoxin (Fig.1A-C) and dipmatol (Fig. 1D-F). A similar cytotoxic response was observed with the MTT assay when the three cell lines were exposed to diplodiatoxin and dipmatol (graphs not shown). The xCELLigence assay provided valuable and real-time information regarding the cell-toxin interactions. Real-time analysis indicated that at the highest concentrations (500 – 750 µM), diplodiatoxin and dipmatol induced a cytotoxic response that was irreversible on all three cell lines tested and most of the cells were unable to recover from the cytotoxic effects induced by the two toxins. However, partial concentration-dependent recovery of the three cell lines from the cytotoxic effects of diplodiatoxin and dipmatol was observed at the lower concentrations (10 – 350 µM). There

was no concentration-dependent cytotoxic response observed on the three cell lines after exposure to diplonine (Fig. 1G-I).

Depicted in Table 1 are the EC_{50} values obtained after exposure of Neuro-2a, CHO-K1 and MDBK cells to diplodiatoxin, dipmatol and diplonine. The EC_{50} could be calculated following exposure of Neuro-2a and CHO-K1 cells to diplodiatoxin for 48 and 72h, whereas EC_{50} values were obtained at all the time periods (24, 48 and 72h) when the MDBK cells were exposed to diplodiatoxin. An EC_{50} of ± 686 µM was determined when MDBK cells were exposed to dipmatol for 48 and 72h. No EC_{50} could be obtained after diplonine exposure to the three cell lines, indicating that diplonine was not cytotoxic at the concentration range used in this study. The lowest EC_{50} following exposure to the three *S. maydis* metabolites was recorded for diplodiatoxin (EC_{50} = 147±8.6 µM) when MDBK cells were exposed for 72h.

This is the first study where the diplodiatoxin, dipmatol and diplonine were compared using *in vitro* assays. There was a significant difference ($p < 0.05$) in the cytotoxicity induced by diplodiatoxin following exposure to the three cell lines at the different exposure periods and concentrations. Diplodiatoxin was the most cytotoxic *S. maydis* metabolite to the Neuro-2a, CHO-K1 and MDBK cells followed by dipmatol. Diplonine was not cytotoxic to the three cell lines. This lack of cytotoxicity was unexpected and indicates that diplonine is relatively safe at lower concentrations or might require metabolic activation to induce toxicity.

As diplodiosis is a neurotoxic disease of ruminants and mycotoxins synthesized by *S. maydis* induces spongiform degeneration in the white matter of the cerebrum and cerebellum of foetuses exposed during the second and third trimester of gestation (Prozesky et al., 1994), the neuroblastoma (Neuro-2a) and bovine-derived (MDBK) cell lines were selected to investigate the *in vitro* cytotoxicity of the three *S. maydis* metabolites.. The ovarian (CHO-

K1) cell line was added for comparison. Based on the EC_{50} results the MDBK cell line was the most sensitive for *in vitro* cytotoxicity testing of the *S. maydis* metabolites.

In conclusion, diplodiatoxin and dipmatol affected the activity of the mitochondrial succinate dehydrogenase enzyme and the overall viability and growth of the cells as demonstrated by the MTT and xCELLigence results, respectively. Mammalian succinate dehydrogenase plays a critical role in mitochondrial energy generation. Thus, the results obtained so far indicate that diplodiatoxin and dipmatol exert their toxicity possibly via the necrotic cell death pathway. Diplonine was not cytotoxic. Additional studies are planned to further investigate the cellular pathways and biochemical processes by which these metabolites exert their toxicity.

Table 1

EC_{50} values obtained from MTT assay after exposure of Neuro-2a, CHO-K1 and MDBK cell lines to diplodiatoxin, diplonine and dipmatol for 24, 48 and 72 h. (-) indicates no EC_{50} values (EC_{50} >750 µM) obtained with the toxin concentration range used.

	EC_{50} (µM)								
	Diplodiatoxin			Dipmatol			Diplonine		
Cell line	24h	48h	72h	24h	48h	72h	24h	48h	72h
Neuro-2a	-	614±10.6	466±7.3	-	-	-	-	-	-
CHO-K1	-	663±4.2	219±8.4	-	-	-	-	-	-
MDBK	660±7.9	230±11.9	147±8.6	-	686±17.5	686±15.4	-	-	-

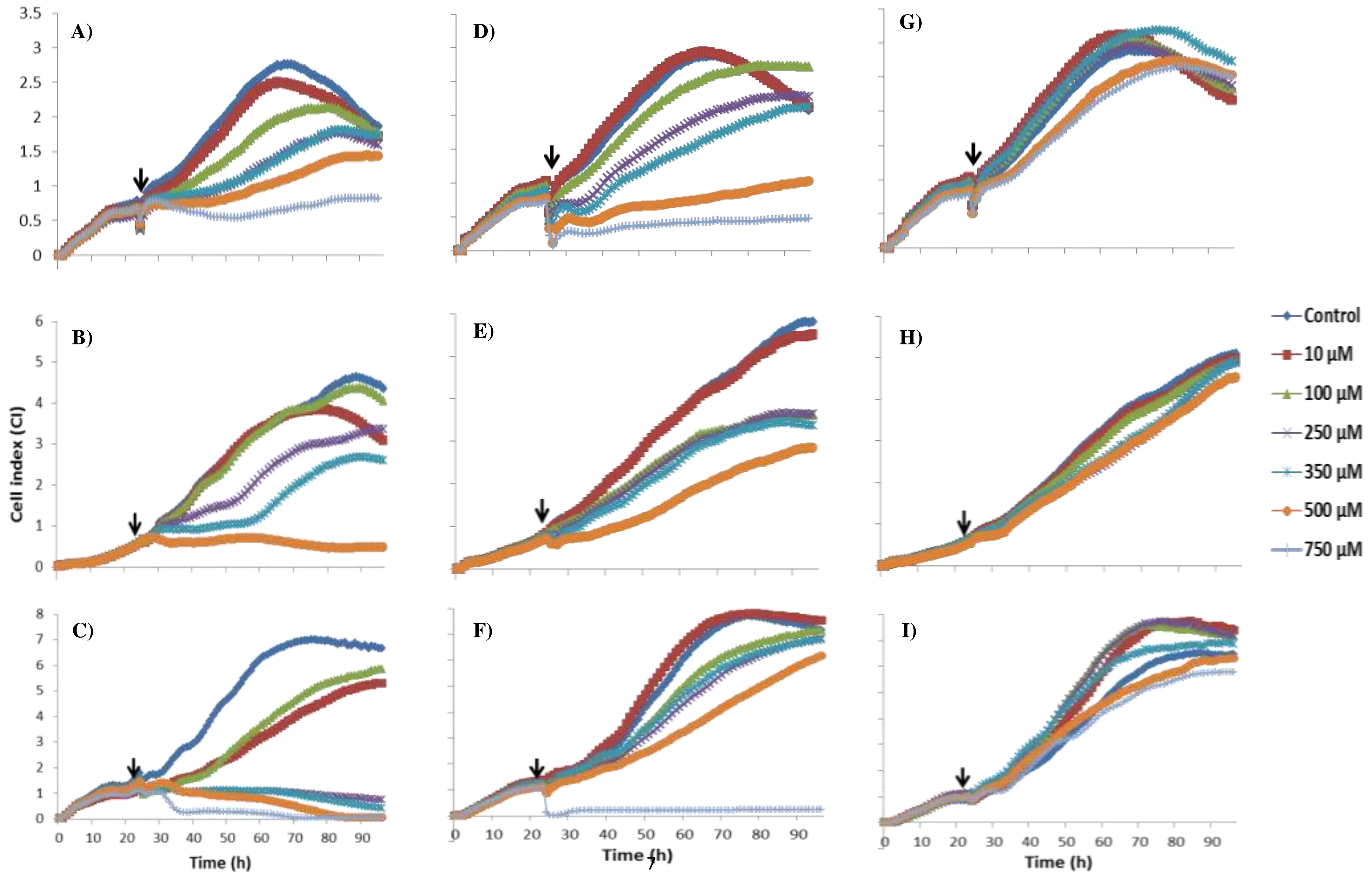

Fig. 1. The cytotoxic responses of Neuro-2a, CHO-K1 and MDBK cells were monitored in real-time following exposure for 72h to S. maydis metabolites using the xCELLigence system. Cells were cultured into the E-plate and allowed to attach to the plate overnight. 200 µL of the different concentrations of each S. maydis metabolite was added to the cultured cells after 24h (↓). Diplodiatoxin was added to Neuro-2 (A), CHO-K1 (B) and MDBK (C) cells. Dipmatol was added to Neuro-2 (D), CHO-K1 (E) and MDBK (F) cells. Diplonine was added to Neuro-2 (G), CHO-K1 (H) and MDBK (I) cells.

Conflict of interest statement

The authors declare that there are no conflicts of interest

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