Dioctatin A is a strong inhibitor of aflatoxin production by Aspergillus parasiticus

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INTRODUCTION

Aflatoxins are a group of mycotoxins with potent toxicity and carcinogenicity toward mammals. They are produced by some strains of Aspergillus spp., such as strains of Aspergillus parasiticus and Aspergillus flavus, and they can be found as contaminants in a wide variety of food and feed commodities. Aflatoxin contamination in agricultural products is a serious problem from the viewpoint of food safety, and also that of economical loss (Payne, 1998; Bennett & Klich, 2003). However, it is difficult to resolve the problem because of the lack of an effective method to control aflatoxin production.

We have been studying specific inhibitors of aflatoxin production by A. parasiticus, based on the idea that they may be useful in preventing foods and feeds from contamination by aflatoxin, without incurring a rapid spread of resistant strains. We also wished to determine the mechanism of aflatoxin production by the fungus at the molecular level. We have found that aflastatins A and B (AsA and AsB), and blasticidin A (BcA), are inhibitors of aflatoxin production, and we have determined their structures; they are produced by Streptomyces, and share a similar structure (Sakuda et al., 1996, 2000a; Ono et al., 1997, 1998; Ikeda et al., 2000). They strongly inhibit aflatoxin production by A. parasiticus by disturbing the primary metabolism of the fungus, which may regulate a pathway leading to expression of aflatoxin biosynthetic enzymes (Kondo et al., 2001). However, a detailed molecular mechanism of inhibition of aflatoxin production by these compounds has not yet been clarified.

BcA has antibiotic activity toward the yeast Saccharomyces cerevisiae (Sakuda et al., 2000b). During the course of a recent investigation into the mode of action of BcA in the yeast, we observed that the amount of a diketopiperazine produced in yeast cells was drastically reduced in the presence of BcA (unpublished data). Since the diketopiperazine, which is composed of methionine and proline, was thought to be synthesized through a dipeptide produced by the action of a dipeptidyl peptidase (DPP) on a certain protein or peptide, it was assumed that there might be a relationship between the action of BcA and the DPP. Thus, the inhibitory activity of BcA toward DPP, and that of several known DPP inhibitors toward aflatoxin...
production, was tested. The results showed that BcA exhibited no inhibition toward human DPP II or DPP IV, but we found that one of the DPP inhibitors, diocatin A (DotA; Fig. 1), strongly inhibited aflatoxin production of *A. parasiticus*, without affecting fungal growth. Although it has not become clear why production of the diketopiperazine is reduced by BcA, we appeared to have discovered a new biological activity of DotA.

DotA was isolated from the culture broth of *Streptomyces* sp. SA-2581, as an inhibitor of human dipeptidyl peptidase II (DPP II), in 1991 (Takeuchi et al., 1991). DPP II acts at acidic pH, and releases an N-terminal dipeptide from a protein with proline or alanine in the penultimate position. In mammals, DPP II is known to be an intracellular protein with proline or alanine in the penultimate position.

New aflatoxin production inhibitor

**METHODS**

**Strains, growth media and chemicals.** *Aspergillus parasiticus* strains NRRL 2999 (ATCC 26691) and ATCC 24690 were used as producers of aflatoxin and norsolorinic acid (NA), respectively. Aflatoxins B1 and G1 are the main aflatoxins produced by the NRRL 2999 strain. *Aspergillus nidulans* FGSC A4 was used as a sterigmatoctyin producer. The strains were maintained on potato dextrose agar (PDA; Difco), and subcultured monthly. A spore suspension was prepared from a 2-week-old culture, at a concentration of 2.5 × 10⁶ c.f.u. ml⁻¹, and used as inoculum for this study. All incubations were done at 28 °C. DotA was obtained from the culture broth of *Streptomyces* sp. SA-2581 (Takeuchi et al., 1991). Orn-Pip and Dab-Pip were synthesized according to the method of Senten et al. (2002).

**Analysis of growth and aflatoxin production.** DotA was dissolved in a solution of methanol/HCl (100:0.01, v/v) at appropriate concentrations, and the solution was added to potato dextrose broth (PD; Difco) at a final concentration of 0.1 % (v/v). As a control culture, methanol/HCl (100:0.01, v/v) solution was added to a medium at 0.1 % . A 3 ml volume of medium was put into each well of a six-well microplate. The spore suspension of *A. parasiticus* NRRL 2999 (10 μl) was inoculated into each well, and the plate was incubated statically for 4 days. The culture broth was separated into mycelium and culture filtrate by filtration. After washing with 5 ml distilled water, the mycelium was collected into a 1.5 ml microtube, and dried at 100 °C for 3 h. Mycelial weight was calculated by subtracting the weight of a 1.5 ml microtube without the mycelium from the total weight. The culture filtrate was subjected to HPLC analysis of aflatoxin [COSMOSIL 5-ph column, 4.6 mm × 150 mm (Nacalai Co.); isocratic elution of tetrahydrofuran-H₂O (20:80, v/v); flow rate, 1.0 ml min⁻¹; detection at 365 nm]. The retention times of aflatoxins B1 and G1 were 5.0 and 6.3 min, respectively. When the amount of aflatoxin in the mycelium had been analysed, the mycelial cake that had been separated from the culture broth by filtration was extracted with chloroform, and the chloroform extracts were subjected to HPLC analysis of aflatoxin under the conditions described above.

**Analysis of NA production.** DotA was added to PD, according to the method used for analysis of aflatoxin. A 1 ml volume of the medium was put into each well of a 24-well microplate. The spore suspension of *A. parasiticus* ATCC 24690 (10 μl) was inoculated into the plate, and it was incubated statically for 5 days. The mycelial cake was harvested by filtration, and extracted with chloroform (1 ml). The chloroform extract was evaporated to dryness. The residue was dissolved in methanol (100 μl), and subjected to HPLC analysis [Capcell-Pak C₁₈ column, 4.6 mm × 250 mm (Shiseido); gradient elution of 70–100 % methanol in water containing 0.1 % TFA for 15 min, followed by isocratic elution of 100 % methanol containing
0.1% TFA; flow rate, 0.8 ml min⁻¹; detection at 315 nm). The retention time of NA was 21 min.

**Analysis of sterigmatocystin production.** The wells of a 24-well microplate, each containing 1 ml PDA, were inoculated with the spore suspension of *A. nidulans* FGSC A4 (10 μl), and incubated for 6 days. The agar medium in each well, together with the mycelial cake grown on it, was extracted with methanol (10 ml). The methanol extract was filtered, and evaporated to dryness. Water and chloroform (1 ml each) were added to the residue, and the mixture was vortexed. The chloroform layer was collected, air-dried, and dissolved in chloroform (100 μl), and subjected to HPLC analysis using the same conditions as those described for NA. The retention time of sterigmatocystin was 16 min.

**RT-PCR analysis of the genes encoding proteins involved in aflatoxin biosynthesis and conidiation.** The wells of a 24-well plate, each containing 1 ml PDA, were inoculated with the spore suspension of *A. parasiticus* NRRL 2999 (10 μl), and incubated for 3 days. The mycelial cake was harvested by filtration. Total RNA was isolated from the mycelia and was used as a template. Real-time quantitative RT-PCR was carried out using the SYBR Green Master Mix (Applied Biosystems), in a final volume of 25 μl for each reaction, and an ABI PRISM 7300 thermal cycler (Applied Biosystems). Two-step PCR conditions were as follows: after an initial incubation at 95 °C for 10 min, 40 cycles of 95 °C for 15 s and 60 °C for 1 min were performed. The PCR primers for each gene were as follows: *pksA*, 5'-TCGATGCGATGTTAGTT-3' and 5'-GTAAGGCCCCGGAGAAGG-3'; *omtA*, 5'-GGCAGCATATCGCGAGTGT-3' and 5'-CGATGACACCATCCAAAT-3'; *ver-1*, 5'-CCATGCGGTGGTTGTG-3' and 5'-TGAGGAAACGCGACCGAATGAA-3'; aflR, 5'-GCGTCGTCAGGAGCAAACC-3' and 5'-CCCGAATTTCGAATCG-3'; brIA, 5'-CCCTCCTATGTTGCGCA-GTAAC and 5'-GGCCGGCCCTTGGCAGATAC-3'; *β-actin* (control gene), 5'-TGCTCTCGTFATCGCAATGTTG-3' and 5'-CATGTGCACCGCGAAA-3'.

**Determination of spore numbers.** The spore suspensions of *A. parasiticus* NRRL 2999 (50 μl) and *A. nidulans* (50 μl) were inoculated onto PDA, with and without 50 μM DotA. After 7 days cultivation, 3 ml distilled water containing 0.1% Triton X-100 was added to the plate. Conidia were harvested by scraping the surface of the agar with a bent glass rod. This procedure was repeated twice. The spor suspensions obtained were combined, and the number of conidia was counted using a haemocytometer.

**Analysis of the production of yellow pigment and kojic acid.** The spore suspension of *A. parasiticus* NRRL 2999 (50 μl) was inoculated onto PDA plates, with and without 50 μM DotA. After 7 days cultivation, the agar and the mycelial cake were extracted with methanol. The methanol extract was filtered, and evaporated to dryness. The residue was dissolved in 1 ml water, and fractionated by methanol. The methanol extract was filtered, and evaporated to dryness. Water and chloroform (1 ml each) were added to the residue, and the mixture was vortexed. The chloroform layer was collected, air-dried, and dissolved in chloroform (100 μl), and subjected to HPLC analysis using the same conditions as those described for NA. The retention time of sterigmatocystin was 16 min.

**RESULTS**

**Effects of DotA on aflatoxin production and fungal growth.** We tested the effects of DotA on aflatoxin production and growth of *A. parasiticus* NRRL 2999. The fungus was cultured in PD, with and without DotA, at 28 °C for 4 days; in the control culture without DotA, it is known that both the amount of aflatoxin and the mycelial weight reach a maximum under these culture conditions (Kondo et al., 2001). Fig. 2(a) shows the amount of aflatoxin produced in each culture filtrate of the fungus. DotA produced dose-dependent inhibition of aflatoxin production by the NRRL 2999, with an IC₅₀ value of 4.0 μM. However, the mycelial weight of the fungus was not affected by DotA, even at a concentration of 50 μM (Fig. 2b). A small amount of aflatoxin (less than 3% of the total amount in the culture broth) was obtained from the mycelium of the fungus in the control culture, but aflatoxin was not detected in the mycelium cultured in the presence of DotA at a concentration of 0.2 μM, indicating that DotA inhibited aflatoxin production without causing accumulation of aflatoxin in the mycelium. Unlike AsA and BcA, DotA showed no antibiotic activity toward *S. cerevisiae* at a concentration of 50 μM (data not shown) (Sakuda et al., 2000b).

**Effect of DotA on NA production**

NA is a very early intermediate in the aflatoxin biosynthetic pathway. *A. parasiticus* ATCC 24690, a mutant blocked in aflatoxin biosynthesis, can accumulate NA in the mycelium (Lee et al., 1971). When the ATCC 24690 strain was cultured in PD with DotA, NA production in the mycelium was inhibited more strongly than aflatoxin production by the NRRL 2999 strain, and showed an IC₅₀ value of 0.8 μM (Fig. 2c). This result shows that DotA inhibits an early step of aflatoxin biosynthesis, before the synthesis of NA.

**Effects of DotA on the transcription of genes encoding proteins involved in aflatoxin biosynthesis**

Next, we examined whether DotA affects the expression of genes encoding proteins involved in aflatoxin biosynthesis. The NRRL 2999 strain was cultured in PD, with and without DotA, for 3 days, when mRNA levels of aflatoxin biosynthetic genes were known to be maximal (Kondo et al., 2001). The mycelium was obtained by filtration, and total RNA was extracted. We analysed the mRNA levels of *pksA*, *pksB*, and *afrR* by quantitative PCR. The genes *pksA*, *ver-1* and *afrR* encode enzyme proteins involved in the
aflatoxin biosynthetic pathway (Yabe & Nakajima, 2004), and aflR is a regulatory gene whose product regulates transcription of some genes, including pksA, ver-1 and omtA (Woloshuk et al., 1994). When DotA was added to the culture, there was a strong dose-dependent repression of the transcription of pksA, ver-1 and omtA (Fig. 3a–c), and a significant repression of aflR (Fig. 3d).

**Effects of DotA on conidiation**

When NRRL 2999 was cultured in a liquid medium, DotA did not influence the morphology of the fungus or the mycelial weight. However, on solid medium, DotA strongly affected differentiation and morphology of the fungus. Seven days after cultivation, the control culture without DotA germinated green conidia (Fig. 4a), while inhibition of conidium formation (Fig. 4b) and induction of fluffy morphology (Fig. 4c) were observed in the culture with DotA (50 μM). The inhibition of conidiation was confirmed by counting conidia numbers, and these were strongly reduced by the addition of DotA (Fig. 4d). The mRNA level of brlA, which encodes one of three conidiation-specific transcription factors (Boylan et al., 1987), was significantly reduced by the addition of DotA: mRNA levels were (mean ± SD) 63 ± 24, 59 ± 10, 55 ± 21 and 45 ± 20% in 0.8, 3.2, 12.5 and 50 μM DotA, respectively, and they all showed a significant difference (P<0.01, n=7–9) from the control that did not contain DotA (100 % mRNA).

**Promotion of kojic acid production by DotA**

We noticed that production of two compounds was clearly increased by addition of DotA in the solid culture of the NRRL 2999 strain. To identify the compounds, the strain was cultured on PDA for 7 days, and the compounds were extracted with methanol from the agar, and quantified by a colorimetric method. One compound, a yellow pigment observed in Fig. 4(b), was not identified because of its instability, but the other was identified as kojic acid. The effect of DotA on the increase of kojic acid production was very marked: kojic acid levels (mean ± SD, n=3) were 0.06 ± 0.03, 0.5 ± 0.1, 2.2 ± 0.5, 4.1 ± 0.4 and 4.6 ± 0.4 mg ml⁻¹ in 0, 0.8 3.2 12.5 and 50 μM DotA, respectively.

**Effect of DotA on sterigmatocystin production and conidiation in A. nidulans**

Sterigmatocystin is known to be a biosynthetic intermediate of aflatoxin, and also a mycotoxin produced by many fungi, including A. nidulans. Since A. nidulans is a genetic model organism whose whole genome has been sequenced, and there is more information available on the regulatory mechanism of sterigmatocystin production in A. nidulans than information on aflatoxin production in A. parasiticus, the effect of DotA on the sterigmatocystin production was tested to investigate the mode of action of DotA. When A. nidulans FGSC A4 was cultured on PDA with DotA, production of sterigmatocystin was strongly inhibited, with an IC₅₀ value of 0.3 μM: sterigmatocystin levels (mean ± SD, n=3) were 17.2 ± 7.0, 8.9 ± 3.0, 5.7 ± 1.3, 1.1 ± 0.7, 0.6 ± 0.6 and 0.2 ± 0.5 mg ml⁻¹ in 0, 0.2, 0.8 3.2 12.5 and 50 μM DotA, respectively. DotA also inhibited conidiation of A. nidulans: 1.0 × 10⁹ ± 3.0 × 10⁸ conidia per plate were produced in 50 μM DotA, compared with

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**Fig. 2.** Effects of DotA on aflatoxin production, fungal growth and NA production. (a, b) A. parasiticus NRRL 2999 was cultured in PD, with and without DotA, at 28 °C for 4 days. (a) The amount of aflatoxin (total amount of aflatoxins B₁ and G₁) in the culture filtrate was analysed by HPLC. (b) The mycelial cake was harvested by filtration, dried at 100 °C for 3 h, and weighed. Data are means ± SD (n=6). (c) A. parasiticus ATCC 24690 was cultured under the same conditions used in the cultivation of the NRRL 2999 strain. NA was extracted from the mycelial cake with chloroform, and analysed by HPLC. Data are means ± SD (n=4).

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3.3 × 10^9 ± 2.9 × 10^8 conidia per plate in the control containing no DotA (mean ± SD, n=3). This effect on conidiation was weaker than that observed for A. parasiticus (Fig. 4d).

**DISCUSSION**

The discovery of the aflatoxin production inhibitory activity of DotA was a surprise finding of our study on the mode of action of BcA (unpublished data). Since DotA is a strong inhibitor of human DPP II, it is possible to speculate that the activity of DotA toward aflatoxin production could be attributed to inhibition of a function of a DPP-II-like protein present in A. parasiticus. Some synthetic compounds, such as Orn-Pip and Dab-Pip (Fig. 1), are known to be human DPP II inhibitors (Senten et al., 2002), and they can inhibit the enzyme to the same extent as DotA. We tested the effects of Orn-Pip and Dab-Pip on aflatoxin production of the NRRL 2999 strain, but they showed no inhibition, even at concentrations as high as 500 μM (data not shown). At present, it is not clear if the mode of action of DotA in A. parasiticus is related to a function of a DPP-II-like protein that has low homology to human DPP II, and which may be present in the fungus, as it is in other Aspergillus spp. mentioned in the Introduction. It has been reported that cyclo(L-leucyl-L-prolyl) (Fig. 1) can inhibit aflatoxin production weakly, with an IC50 value of 0.2 mg ml^-1 (Yan et al., 2004). Because this diketopiperazine contains a proline residue, and DPP II releases an N-terminal dipeptide from a protein with a proline residue in the penultimate position, it is possible that some DPP-II-like proteins of the fungus are concerned with the action of the diketopiperazine compound.

DotA inhibited aflatoxin production, and also conidiation of A. parasiticus. Similar inhibition by DotA was also observed in sterigmatocystin production and conidiation of A. nidulans. Some phenomena have been observed that suggest that there is a relationship between conidiation and mycotoxin production in Aspergillus spp. (Calvo et al., 2003).

**Fig. 3.** Effects of DotA on the transcription of genes encoding aflatoxin biosynthesis enzymes. Total RNA was prepared from the mycelium of A. parasiticus NRRL 2999 cultured in PD, with and without DotA, for 3 days. Transcription of pksA (a), ver-1 (b), omtA (c), and aflR (d), was analysed by real-time quantitative PCR. We used β-actin mRNA as an endogenous reference because its copy number is not influenced by DotA. The amount of each mRNA was normalized to the amount of β-actin mRNA in each sample. Data are means ± SD (n=7–9). The differences between the amounts of mRNA were assessed by one-way ANOVA, followed by the Dunnett test. *P<0.05, **P<0.01, versus the control.

**Fig. 4.** Effect of DotA on conidiation. A. parasiticus NRRL 2999 was cultured on PDA without DotA (a), and with 50 μM DotA (b, c), for 7 days. Part of (b) is magnified in (c). Bars, 1 cm (a, b); 0.2 cm (c). (d) Conidia numbers were counted with a haemocytometer. Data are means ± SD (n=3).
2002). For example, many of the fluffy mutant strains of A. nidulans that are unable to form conidia cannot produce sterigmatocystin either (Wieser et al., 1997). It is also known that conidiation-inhibiting compounds can sometimes decrease mycotoxin production. For example, ethylene can inhibit both conidiation and sterigmatocystin accumulation in A. nidulans (Roze et al., 2004a), and 1,4-diamino-2-butanone (DAB), a competitive inhibitor of ornithine decarboxylase (ODC), can repress both conidiation and aflatoxin biosynthesis of A. parasiticus, at a concentration of 50 mM. This effect of DAB is counteracted by addition of putrescine, which is a product of the action of ODC, and spermidine, which is a polyamine biosynthesized from putrescine (Guzman-de-Pena & Ruiz-Herrera, 1997). We tested the effect of spermidine on the activity of DotA, but the activity of DotA on both aflatoxin production and conidiation was not counteracted at all (data not shown), suggesting that the mode of action of DotA may be different from that of DAB.

Because DotA inhibited NA production, and reduced the mRNA levels of aflR and brlA, it may affect a regulatory system that controls both aflatoxin production and conidiation. Recently, an adenyl cyclase/cAMP/cAMP-dependent protein kinase cascade has been suggested to be important for both sterigmatocystin production and sporulation in A. nidulans (Shimizu et al., 2003; Roze et al., 2004b). In the cascade, G-protein-mediated signalling regulates the activity of adenyl cyclase, and mutation of a protein, e.g. FadA or FlbA, involved in the signalling pathway causes the fluffy phenotype. The effects of DotA observed in A. nidulans suggest that this G-protein-signalling pathway may be a possible target for DotA.

Aflatoxin production by A. parasiticus was inhibited by DotA, but production of kojic acid and a yellow pigment was markedly increased. This suggests that DotA may influence secondary metabolism of the fungus. If DotA can affect secondary metabolism of other fungi in a similar way to that for A. parasiticus, DotA may have the potential to reduce the production of unwanted compounds, and also to increase production of useful compounds.

DotA strongly inhibits aflatoxin production and conidiation, without affecting fungal growth. These features are very advantageous in prevention of aflatoxin contamination of foods and feeds, without a rapid spread of resistant strains, and also without wide diffusion of conidia. DotA inhibited aflatoxin production in a model infection system consisting of A. parasiticus on raw peanuts (Sakuda et al., 2006), suggesting that it may be effective in preventing aflatoxin contamination of peanuts during storage. We are currently testing the effect of DotA on the whole peanut plant by using a method that was previously used for confirming the effect of aflastatin A in prevention of aflatoxin contamination (Sakuda et al., 1999). DotA has a relatively simple structure, and shows no toxicity to mammals. Therefore, DotA may be a good lead compound for developing practically effective drugs. From the viewpoint of basic research, DotA is a very important probe to use in the investigation of the regulatory mechanism of fungal secondary metabolite production and differentiation. Work to clarify the mode of action of DotA with a photo-affinity probe, and to develop more effective DotA derivatives, is now in progress.

ACKNOWLEDGEMENTS

We are grateful to K. Yabe (National Food Research Institute, Tsukuba, Japan) and H. Horiuchi (University of Tokyo, Japan) for the kind gift of fungal strains.

REFERENCES


Edited by: J.-R. Xu