Inhibition of aflatoxin production by protein tyrosine phosphatase inhibitors, blasticidin A and dephostatin

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Abstract
Blasticidin A, a Streptomyces metabolite, inhibits aflatoxin production in aflatoxinogenic fungi. The mode of action of blasticidin A in the inhibition of aflatoxin production was investigated. Blasticidin A inhibited human PTP1B (a protein tyrosine phosphatase) with the IC₅₀ value of 27 µM, but not human PP1A (a protein serine/threonine phosphatase). Dephostatin, a known protein tyrosine phosphatase inhibitor, inhibited aflatoxin production without affecting fungal growth. Blasticidin A delayed expression of aflR, which encodes the key regulator of aflatoxin production, and aflatoxin biosynthetic enzyme genes, whereas dephostatin did not affect their mRNA levels. Aspergillus flavus has two genes encoding PTP1B homologs, AfPTP1B-1 and AfPTP1B-2. Blasticidin A inhibited recombinant AfPTP1B-1 more strongly than did dephostatin, and dephostatin inhibited recombinant AfPTP1B-2 much more strongly than did blasticidin A. These results suggest that aflatoxin production is regulated by some protein tyrosine phosphatases, and that protein tyrosine phosphatase inhibitors are potential aflatoxin production inhibitors.

1. Introduction
Aflatoxins form a group of mycotoxins with potent toxicity and carcinogenicity in mammals. Some strains of Aspergillus spp. produce aflatoxins that are found as contaminants in a wide variety of food and feed commodities. Aflatoxin accumulation in crops causes mycotoxicosis in humans and animals that ingest the crops¹,². Aflatoxin contamination of agricultural products is a serious problem in terms of not only food safety, but also economic loss³. However, resolution of this problem is difficult due to the lack of an effective method for the control of aflatoxin production by fungi.

We have been studying specific inhibitors of aflatoxin production as possible candidates for drugs to prevent aflatoxin contamination of foods without incurring the rapid spread of resistant strains⁴. These inhibitors are also useful biological probes to clarify the regulatory mechanism for aflatoxin production in aflatoxigenic fungi. To date, many compounds, including plant constituents, pesticides, and microbial metabolites have been shown to be aflatoxin production inhibitors⁵,⁶. Blasticidin A (Fig. 1) was identified as an antibiotic produced by Streptomyces griseochromogenes⁶,⁷ but its structure had not been determined. We rediscov- ered blasticidin A as an aflatoxin production inhibitor in Aspergillus parasiticus and determined its structure⁸,⁹ to be a congener of aflastatin A, an aflatoxin production inhibitor produced by Streptomyces sp.¹⁰. Proteome analysis in Saccharomyces cerevisiae revealed that blasticidin A reduced the abundance of ribosomal proteins, similar to the actions of known protein synthesis inhibitors¹¹. Blasticidin A inhibited protein synthesis in an in vivo galactose-induction protein synthesis system in S. cerevisiae, and protein synthesis inhibitors such as blasticidin S and G418 showed aflatoxin production inhibitory activity with selectivity. Thus, the aflatoxin production inhibitory activity of blasticidin A has been considered to potentially correlate with its protein synthesis inhibitory activity. The proteome analysis in S. cerevisiae also showed that blasticidin A
increased the phosphorylated/non-phosphorylated protein ratio in protein translation factors such as Sup45p and Tif5p, which was not observed for cycloheximide or blasticidin S \(^{11}\). These results suggest that blasticidin A affects protein phosphorylation, leading to the inhibition of protein synthesis and aflatoxin production. However, the target molecule of blasticidin A has not been identified.

In this study, we found the protein phosphatase inhibitory activity of blasticidin A and examined the effect of a known protein phosphatase inhibitor, dephostatin (Fig. 1), on aflatoxin production, to investigate the aflatoxin production inhibitory mechanism of protein phosphatase inhibitors.

### 2. Materials and Methods

#### 2.1. Chemicals

Blasticidin A (purified from the culture broth of \textit{S. greseochromogenes} IFO13413, purity >98% determined by HPLC), cycloheximide (Nacalai Tesque, Kyoto, Japan), dephostatin (Santa Cruz Biotechnology, Dallas, TX, USA) were dissolved in dimethyl sulfoxide (Kanto Chemical, Tokyo, Japan). Okadaic acid (FUJIFILM Wako Pure Chemical, Osaka, Japan) was dissolved in methanol (Kanto Chemical). The aflatoxin B\(_1\) standard was purchased from Sigma-Aldrich (St Louis, MO, USA).

#### 2.2. \textit{Aspergillus flavus} strain and culture conditions

The \textit{Aspergillus flavus} IMF47798 strain (obtained from the Agricultural research service of the USDA, USA), which produces mainly aflatoxin B\(_1\) was used throughout this study. A preserved glycerol stock of a spore suspension prepared from 1-week-old culture (stored at -80°C) was used as the inoculum. The spore suspension was inoculated into potato dextrose (BD, Sparks, MD, USA) liquid medium in 12-well microplates (2 mL/well) at 10\(^5\) spores/well. Blasticidin A or dephostatin was dissolved in dimethyl sulfoxide and added to the wells (final concentration of dimethyl sulfoxide, 0.1% v/v). The microplate was incubated at 28°C.

#### 2.3. Analysis of aflatoxin B\(_1\) production and mycelial weight

After incubation, the culture broth of \textit{A. flavus} from each well was centrifuged to obtain mycelia and culture supernatant. The mycelia were washed twice with 1.0 mL distilled water and collected by centrifugation in a 2.0-mL micro-tube. After freeze-drying of the mycelia, the mycelial weight was calculated by subtracting the weight of an empty 2.0-mL micro-tube from the total weight.

To analyze the aflatoxin B\(_1\) concentration in the culture supernatant, 0.5 mL supernatant was extracted with 0.5 mL chloroform (Kanto Chemical) twice, and the chloroform (total, 1.0 mL) solution was then distilled off by air-drying. The remaining residue was resolved in 0.1 mL of a 90% aqueous acetonitrile solution. The dissolved mixture was subjected to reverse-phase HPLC on a 250 × 4.6-mm inner diameter Capcell Pak C\(_{18}\) UG120 column (Osaka Soda, Osaka, Japan) by an isocratic elution of acetonitrile: methanol: water (10:30:60) over 20 min at a flow rate of 1.0 mL with detection at 365 nm (retention time of aflatoxin B\(_1\), 8.3 min).

#### 2.4. Analysis of protein synthesis inhibitory activity in a cell-free system

The inhibitory effects of test compounds on protein synthesis in a cell-free system were assayed using the Human Cell-Free Protein Expression System (TaKaRa Bio, Shiga, Japan). In a slight modification of the manufacturer’s protocol, 8.8 µL cell lysate, 6 µL mixture-1, and 1 µL mixture-2 were added to a 1.5-mL micro-tube. After incubation of the mixture for 10 min
at room temperature, 2 µL mixture-3, 1 µL control vector solution, and 1 µL T7 RNA Polymerase were added to the tube. Blasticidin A or cycloheximide dissolved in dimethyl sulfoxide was further added to the tube (final concentration of dimethyl sulfoxide, 1 % v/v). After incubation of the tube for four hours at 37°C, 15 µL of the solution in the tube was transferred to a new 1.5-mL micro-tube. One microliter of X-gal solution (25 mg/mL) and 18 µL distilled water were added to the new tube, followed by incubation for 30 min at 37°C. The color of the solution was observed visually.

2.5. Analysis of protein tyrosine phosphatase activity

The inhibitory effect of a test compound on a protein tyrosine phosphatase was assayed using the PTP1B Colorimetric Assay Kit (BPS Science, San Diego, CA, USA) according to the manufacturer’s protocol.

2.6. Analysis of protein serine/threonine phosphatase activity

Human PPP1CA (PP1A) recombinant protein (Abcam, Cambridge, MA, USA) and disodium p-nitrophenyl phosphate hexahydrate (FUJIFILM Wako Pure Chemical) were used as the enzyme and substrate, respectively. After the addition of 40 µL 10 mM p-nitrophenyl phosphate in 1x colorimetric assay buffer 1 (100 mM Tris-HCl (pH7.5), 0.2 mM EDTA, 0.5 mM MnCl₂, 4 mM DTT, 0.4 mg/mL BSA) and 10 µL blasticidin A in dimethyl sulfoxide or okadaic acid in methanol to a well of a 96-well microplate, absorbance of the solution in the well at 415 nm was measured and 50 µL PP1A solution (5 ng/mL) was added to the well. After incubation of the well for 2 h at room temperature, absorbance of the solution at 415 nm was measured again. The increase in absorbance during incubation was used to assess the enzyme activity.

2.7. RT-qPCR

The lyophilized mycelia were ground under liquid nitrogen using a mortar and pestle. Total RNA was extracted with TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA) and purified with the PureLink RNA Mini Kit (Thermo Fisher Scientific), according to manufacturer’s protocol. The cDNA was prepared with the ReverTra Ace qPCR RT Master Mix (TOYOBO, Osaka, Japan) according to the manufacturer’s protocol. The cDNA derived from 0.05 µg total RNA was used as a qPCR template. qPCR was carried out using FastStart Universal SYBR Green Master (Roche; Roche, Basel, Switzerland) in a final volume of 25 µL for each reaction and an ABI7300 Real-Time PCR System (Thermo Fisher Scientific). Two-step PCR conditions were as follows: initial incubation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The amounts of qfLR, pksA, ver-1, omtA, ordA, AfPTP1B-1 and AfPTP1B-2 mRNA were normalized to the amount of β-tubulin mRNA (control gene) in each sample. PCR primers used for each gene were as follows: β-tubulin 5’-AGCTTCCACCCCCGTCTACG-3’ and 5’-TG AGCTGACGGAAGACG-3’; qfLR 5’-TCCGGCATCTTCTTC TCATCA-3’ and 5’-CCGAATTCGATCGACTTGA-3’; pksA 5’-TGCAAGGCGATGTAGTT-3’ and 5’-GAAG GCCGGAGAAGG-3’; ver-1 5’-TGAGCAATCGCAGA GTTATG-3’ and 5’-CCAGGCGATGACAGGACA-3’; omtA 5’-ACCAAGGAGTGGATTCTCTTAT-3’ and 5’-GC TTGCGGCCACCATCT-3’; ordA 5’-ATACCGGATTCGA CGGTCTTCT-3’ and 5’-CGTGGTCCTTTCCGAGAT-3’; AfPTP1B-1 5’-AAGGCAAGGAAACCAGATGTC-3’ and 5’-TC GCACTGCGCCATCTTAC-3’; AfPTP1B-2 5’-CATCGTGGCCCAAATCTGCT-3’ and 5’-GCC GGATTTCCCAAAAA-3’.

2.8. Classification of protein phosphatases

The amino acid sequences of human protein phosphatases were obtained from UniProt website (https://www.uniprot.org/). All the protein-coding DNA sequences of A. flavus were obtained from the website of Broad Institute (https://www.broadinstitute.org/fungal-genome-initiative/aspergillus-genome-projects). The searching and classification of protein phosphatases of A. flavus based on their amino acid sequences were conducted using Genetyx ver.10 (GENETYX, Tokyo, Japan) software equipped with the FASTA program.

2.9. Preparation of recombinant AfPTP1B proteins

cDNA of A. flavus was prepared as described above. PCR was performed with the cDNA serving as a template using the following primers: AfPTP1B-1 5’-TATAATAGAAAGCTGCTCATCCCACCAT CAAGATCG-3’ and 5’-TTGATAGCTGATGCTGACATTCC TTGAGGAGACGTCATGCGGC-3’; AfPTP1B-2 5’- TATAATA TTAGCTTAAATTTTGCGAGGTGGAAATATTGCC-3’ and 5’- TTATCGGAGACTTCTGGCG-3’; AfPTP1B-2 5’- TATAATA TTAGCTTAAATTTTGCGAGGTGGAAATATTGCC-3’ and 5’- TTATCGGAGACTTCTGGCG-3’.

To insert PCR products into the pT7-FLAG-2 Expression Vector (Sigma-Aldrich), the vector was digested with HindIII and KpnI and purified by Monarch PCR&DNA Clean up Kit (BioLabs, Ipswich, MA, USA). PCR products were ligated into the vector with an In-Fusion HD Cloning Kit (TaKaRa Bio), according to the manufacturer’s protocol. After ligation, Escherichia coli Competent Quick DH5α (TOYOBO) was transformed with the pT7-FLAG-2-based expression plasmid and selected with LB agar medium containing ampicillin (50 µg/mL). After confirming the accuracy of sequence inserts, Escherichia coli BL21 (DE3) pLysS cells (BioDynamics Laboratory Inc., Tokyo, Japan) or Escherichia coli BL21 (DE3) cells (BioDynamics laboratory) were transformed with the AfPTP1B-1 or AfPTP1B-2 expression
plasmid, respectively. Transformed cells were cultured in 1 L LB containing ampicillin (50 µg/mL) and chloramphenicol (30 µg/mL) or only ampicillin (50 µg/mL) and grown to a final OD_{600} of 0.4 at 37 °C. Isopropyl β-D-1-thiogalactopyranoside (0.1 mM) was added to the culture, which was incubated for an additional 4 h at 26.5 °C.

Bacterial cells were collected by centrifugation and resuspended in phosphate buffered saline (10 mM potassium phosphate buffer (pH 7.4), 150 mM NaCl). After disruption of the cells by sonication, recombinant proteins in the soluble fraction were purified with an ANTI-FLAG M2 Affinity Gel (Sigma-Aldrich), according to the manufacturer’s protocol. Recombinant AFPTP1B-1 and AFPTP1B-2 were obtained in TBS (50 mM Tris-HCl (pH 7.5), 138 mM NaCl, 2.7 mM KCl) containing 200 µg/mL FLAG peptide (DYKDDDDK peptide; FUJIFILM Wako Pure Chemical). The protein concentration was determined with the BCA Protein Assay Reagent (Thermo Fisher Scientific), with BSA used as a standard.

Solutions of AFPTP1B-1 (0.2 ng/mL) and AFPTP1B-2 (15 ng/mL) in 1×colorimetric assay buffer 2 (20 mM Tris-HCl (pH 7.5), 5 mM MgCl₂, 1 mM EGTA, 0.02 % β-mercaptoethanol, 0.1 mg/mL BSA) were used as enzyme solutions, and the enzyme assay was carried out as described in 2.5.

2.10. Western blotting

Proteins were separated on 12% polyacrylamide gel and transblotted onto a PVDF (polyvinylidene difluoride) membrane (ATTO, Tokyo, Japan). The membrane was blocked with 5% skim milk in TBS-T (50 mM Tris-HCl (pH 7.5), 138 mM NaCl, 2.7 mM KCl, 0.05% Tween-20) for 1 h at room temperature. After washing with TBS-T, the membrane was incubated with TBS-T containing mouse monoclonal anti-FLAG M2 antibody (F1804; Sigma-Aldrich) at a dilution of 1:1000 for 2 h at room temperature. After washing with TBS-T, the membrane was incubated with TBS-T containing horse radish peroxide-conjugated goat polyclonal antimouse IgG (H+L) secondary antibody (7024; Cell Signaling Technology, Danvers, MA, USA) at a dilution of 1:1000 for 2 h at room temperature. After washing with TBS-T, the membrane was treated with ECL Prime Western Blotting Detection Reagent (GE Healthcare, Little Chalfont, UK), according to the manufacturer’s protocol. Chemiluminescent signal bands were visualized with an ImageQuant LAS 4000 system (GE Healthcare).

2.11. Data Analysis

Normalized values were used for statistical analysis. Data are presented as means ± standard deviations. Differences between groups were assessed by Dunnett’s test. Values of $P < 0.05$ were considered to be significant.

3. Results

3.1. Effect of blasticidin A on protein synthesis in a cell-free system

The effect of blasticidin A on protein synthesis was examined with a commercially available in vitro transcription/translation system derived from a human cell line. Expression of β-galactosidase in the in vitro system was strongly inhibited by cycloheximide at the concentration of 100 µM, but not by blasticidin A, even at the concentration of 500 µM (Supplementary Fig. 1). This result suggested that the target molecule of blasticidin A was not involved in transcription/translation reactions in the in vitro system.

3.2. Protein phosphatase inhibitory activity of blasticidin A

As blasticidin A may affect protein phosphorylation, its effect on protein phosphatases was examined. Protein phosphatase activities of human PTP1B, a commercially available typical protein tyrosine phosphatase, and human PP1A, a commercially available typical protein serine/threonine phosphatase, were measured using p-nitrophenyl phosphate as a substrate (Fig. 2). Blasticidin A inhibited enzymatic activity of human PTP1B with the IC₅₀ value of 27 µM much more strongly than did dephostatin, which reduced enzymatic activity of human PTP1B to 55% at the concentration of 1 mM. On the other hand, blasticidin A did not inhibit the phosphatase activity of PP1A significantly, even at the concentration of 300 µM. These results showed that blasticidin A is a protein tyrosine phosphatase inhibitor.

3.3. Effects of blasticidin A and dephostatin on aflatoxin production and fungal growth

Next, the effects of dephostatin on aflatoxin production of A. flavus and fungal growth were compared with those of blasticidin A. When strain IMF 47798 was cultured in a liquid medium, fungal mycelial weight peaked after 48 h cultivation, and aflatoxin production was measured after 48 h cultivation (Fig. 3). It almost completely inhibited aflatoxin production at the concentration of 100 µM, but it did not affect the fungal mycelial weight at the same concentration. Blasticidin A was added at the beginning of culture similarly to the case of dephostatin, but the aflatoxin amount and fungal mycelial weight were measured after 96 h cultivation because the delay of
expression of aflR and aflatoxin biosynthetic genes were observed by addition of blasticidin A as described below. Blasticidin A inhibited aflatoxin production with the IC50 value of 0.17 µM, and the fungal mycelial weight was significantly reduced when aflatoxin production was completely inhibited by blasticidin A (Fig. 3).

3.4. Effects of blasticidin A and dephostatin on the mRNA levels of genes responsible for aflatoxin production.

The effects of blasticidin A and dephostatin on the mRNA levels of aflR and four genes encoding aflatoxin biosynthetic enzymes (pksA, ver-1, omtA, and ordA) were examined by real-time PCR. AflR regulates the transcription of pksA, ver-1, omtA, and ordA13. Strain
IMF 47798 was cultured for 24, 48, or 72 h with or without blasticidin A (300 nM) or dephostatin (100 μM), at which aflatoxin production was almost completely inhibited (Fig. 3). In the control culture with no drug, the mRNA levels of aflR increased gradually from 24 h to 72 h and those of pksA, ver-1, omtA, and ordA peaked on the second day (Fig. 4). In the culture with blasticidin A, the mRNA levels of aflR on the first and second days were lower than those in the control culture, but the mRNA level of aflR on the third day did not differ from that of the control. Blasticidin A strongly reduced the mRNA levels of pksA, ver-1, omtA, and ordA on the second day, but levels on the third day were much higher than those in the control culture.

Fig. 4  Effects of blasticidin A and dephostatin on expression of genes involved in aflatoxin biosynthesis of A. flavus.
(a): aflR  (b): pksA  (c): ver-1  (d): omtA  (e): ordA. Transcription of each gene was analyzed by real-time quantitative PCR. White, gray, and black bars indicate control culture, culture with 300 µM blasticidin A, and culture with 100 µM dephostatin, respectively. Each mRNA level was normalized to the amount of β-tubulin mRNA in each sample. Data are presented as means and standard deviations from three biological replicates. Asterisks indicate significant differences (* P < 0.05, ** P < 0.01 vs. control group, Dunnett test).
Although the mRNA level of afIR in the culture with dephostatin was lower than that in the control culture on the first day, the mRNA levels of afIR, pksA, ver-1, omtA, and ordA on the second and third days were not affected by dephostatin.

3.5. Classification of protein phosphatases of A. flavus.

To identify a protein tyrosine phosphatase concerning aflatoxin production in A. flavus, protein phosphatases of A. flavus were searched from published genome data of A. flavus and were classified by comparing their amino acid sequences with those of human protein phosphatases. Classification was conducted by the following two methods using the FASTA homology search tool. 1) Each human protein phosphatase was used as a query sequence and searched. The proteins showing 76% or more homology were inferred as protein phosphatases homologous to each human protein phosphatase. 2) For A. flavus proteins already annotated as protein phosphatases in the genome database, using these proteins as queries, human protein phosphatases showing 60% or more homology were searched. The A. flavus proteins used as a query were considered to belong to the same group. Results are shown in Table S1. The total 28 putative protein phosphatases of A. flavus were classified to 9 protein serine/threonine phosphatases, 13 protein tyrosine phosphatases, and 6 Asp-based protein phosphatases. Two proteins that belong to the protein tyrosine phosphatase group and that show high similarity to both PTP1B and CD45 were named AFPTPB-1 and AFPTPB-2. AFPTPB-1 and AFPTPB-2 had 66% and 68% amino acid sequence similarity with human PTP1B, respectively.

3.6. Effects of blasticidin A and dephostatin on phosphatase activities of AFPTPB-1 and AFPTPB-2.

The time course of the mRNA levels of AFPTPB-1 and AFPTPB-2, which increased from 24 h to 48 h cultivation, was similar to that of aflatoxin production (Fig. 5). Recombinant proteins of AFPTPB-1 and AFPTPB-2 were prepared to examine the inhibitory activities of blasticidin A and dephostatin toward them. FLAG-tagged recombinant AFPTPB-1 and AFPTPB-2, named rAFPTPB-1 and rAFPTPB-2, were bacterially expressed and purified with an anti-FLAG M2 antibody-immobilized agarose gel (Supplementary Fig. 2). Purified rAFPTPB-1 and rAFPTPB-2 showed phosphatase activity when p-nitrophenyl phosphate was used as a substrate. Blasticidin A and dephostatin inhibited the phosphatase activity of rAFPTPB-1 with the IC50 values of 170 µM and 346 µM, respectively (Fig. 6). On the other hand, dephostatin inhibited rAFPTPB-2 with the IC50 value of 587 µM, but blasticidin A only reduced its activity by about 20% at the concentration of 1 mM.

4. Discussion

In 1997, we found that blasticidin A strongly inhibited the aflatoxin production of A. parasiticus and had a structure very similar to that of aflastatin A [4]. Blasticidin A inhibited aflatoxin production without significantly affecting fungal growth at low concentrations in A. parasiticus. However, the anti-fungal activity of blasticidin A toward A. parasiticus at high concentrations suggested that this activity was correlated with the aflatoxin production inhibitory activity of blasticidin A [9]. As blasticidin A strongly inhibited S. cerevisiae growth, its mode of action in inhibiting yeast growth was examined [10]. Proteome analysis and in vivo protein synthesis assays showed that blasticidin A may affect protein synthesis in S. cerevisiae. Furthermore, as some known protein synthesis inhibitors inhibited aflatoxin production of A. flavus with selectivity, we concluded that blasticidin A inhibited aflatoxin production as a protein synthesis inhibitor, although its target molecule was unknown. In this study, we further investigated the mode of action of blasticidin A.

As our experiment showed that the target molecule of blasticidin A was not involved in the transcription/translation cell-free system, we focused on the effect of blasticidin A on protein phosphorylation. We determined that blasticidin A was a protein tyrosine phosphatase inhibitor.

As PTP1B functions as a negative regulator of the insulin signaling [15, 16], inhibitors specific for PTP1B have been studied as drug candidates for diabetes and obesity [17]. Protein tyrosine phosphatases may have important roles in the phosphate cascade leading to obesity.
the phosphorylation of protein translation factors\textsuperscript{18,19}. Therefore, blasticidin A might inhibit a protein tyrosine phosphatase involved in this cascade to cause protein synthesis inhibition.

We previously reported that the mRNA levels of aflR, omtA, pksA, and ver-1 were reduced by blasticidin A on the second day of cultivation in A. parasiticus\textsuperscript{20}. In the current study, these mRNA levels were increased on the third day in the same experiment conducted with A. flavus, suggesting that blasticidin A delayed the expression of aflR and aflatoxin biosynthetic genes. This delay may be correlated with the reduction of aflatoxin production.

Dephostatin is the first natural product inhibiting CD45, a receptor-like protein tyrosine phosphatase that serves as a positive regulator of the immune system\textsuperscript{21}. Dephostatin inhibited aflatoxin production of A. flavus without affecting fungal growth or the mRNA levels of aflR and aflatoxin biosynthetic genes. These findings suggest that the target protein tyrosine phosphatase of dephostatin for the inhibition of aflatoxin production have roles in the regulation of aflatoxin production. In Aspergillus nidulans, which produces sterigmatocystin, an intermediate in the aflatoxin biosynthetic pathway, the promotion of AflR phosphorylation reduced sterigmatocystin production\textsuperscript{22}. Work examining the effect of dephostatin on AflR phosphorylation is currently in progress.

AfPTP1B-1 and AfPTP1B-2 had homology to PTP1B and CD45. Blasticidin A inhibited rAfPTP1B-1 more strongly than did dephostatin, whereas rAfPTP1B-2 was inhibited by dephostatin much more strongly than by blasticidin A. To clarify whether AfPTP1B-1 and/or AfPTP1B-2 are targets of blasticidin A and/or dephostatin
for the inhibition of aflatoxin production in *A. flavus*, gene disruption experiments with protein tyrosine phosphatase genes, including *AfPTP1B-1* and *AfPTP1B-2*, may be beneficial. The identification of target protein tyrosine phosphatases is important for clarification of the regulatory mechanism for aflatoxin production and the development of effective aflatoxin production inhibitors.

**Supplementary Materials**

Supplementary materials may be found in the online version of this article:

**Supplementary Fig. 1** Effect of blasticidin A on protein synthesis in cell-free system.

**Supplementary Fig. 2** Expression and purification of recombinant *AfPTP1B-1* and *AfPTP1B-2*.

**Supplementary Table 1** Putative classification of protein phosphatases in *A. flavus* based on amino acid sequence homology with human proteins.

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Supplementary Fig. 1  Effect of blasticidin A on protein synthesis in cell-free system. The factors necessary for transcription and translation in the cell-free system were mixed with test compound and incubated. After incubation, X-gal solution were added to the mixture and its color was observed to investigate inhibition of protein synthesis. Clear solution indicated that translation was inhibited.
Supplementary Fig. 2  Expression and purification of recombinant AfPTP1B-1 and AfPTP1B-2.
(a, b) Expression conditions of FLAG-tagged recombinant AfPTP1B-1 (a) and AfPTP1B-2 (b), rAfPTP1B-1 and rAfPTP1B-2, were optimized. Recombinant proteins were detected by western blotting using anti-FLAG antibody.
(c, d) Expressed rAfPTP1B-1 (c) and rAfPTP1B-2 (d) were purified with anti-FLAG M2 affinity gel. These proteins were eluted from the affinity gel by competition with FLAG peptide. Red arrows indicate bands of recombinant proteins predicted from their theoretical molecular weights.
**Supplementary Table 1** Putative classification of protein phosphatases in *A. flavus* based on amino acid sequence homology with human proteins.

| Protein family | Human proteins | *A. flavus* protein number | Identity (%) | Similarity (%) |
|----------------|----------------|---------------------------|-------------|---------------|
| serine/threonine protein phosphatase | PP1A | 05986 | 86 | 96 |
| | PP1A | 01420 | 66 | 89 |
| | PP2A | 06146 | 85 | 96 |
| | PP4 | 03082 | 64 | 89 |
| | PP6 | 06146 | 59 | 84 |
| | PP4 | 06146 | 117 | 89 |
| | PP6 | 06146 | 89 | 84 |
| | PP5 | 05986 | 49 | 86 |
| | PP6C | 04277 | 64 | 90 |
| | PP2CA | 01488 | 34 | 76 |
| tyrosine protein phosphatase | CD45 | 02156 | 29 | 64 |
| | PTP1B | 02456 | 25 | 66 |
| | CD45 | 12456 | 31 | 66 |
| | PTP1B | 12456 | 33 | 68 |
| | PTPN10 | 08294 | 28 | 68 |
| | VHR | 08294 | 30 | 69 |
| | KIAA1298 | 08294 | 23 | 67 |
| | PTPN10 | 08294 | 40 | 78 |
| | VHR | 08294 | 32 | 70 |
| | KIAA1298 | 08294 | 36 | 72 |
| | PTPN10 | 08294 | 35 | 77 |
| | VHR | 08294 | 40 | 76 |
| | KIAA1298 | 08294 | 30 | 73 |
| | PTPN10 | 08294 | 33 | 66 |
| | PTPN10 | 08294 | 27 | 69 |
| | PTEN2 | 08294 | 27 | 70 |
| | VHR | 08294 | 24 | 61 |
| | PTP4A | 02229 | 24 | 63 |
| | CDC14 | 02229 | 34 | 73 |
| | VHR | 02229 | 19 | 67 |
| | PTP4A | 02229 | 33 | 67 |
| | CDC14 | 02229 | 36 | 71 |
| | PTP4A | 02229 | 12159 | 30 | 75 |
| | LMW-PTP | 05222 | 45 | 81 |
| | CDC25 | 02846 | 34 | 81 |
| Asp-based protein phosphatase | MGC5987 | 02554 | 35 | 71 |
| | MGC5987 | 05220 | 33 | 62 |
| | CIN | 01345 | 33 | 71 |
| | FCP1 | 03314 | 36 | 73 |
| | FCP1 | 03314 | 35 | 74 |
| | SCP1 | 03790 | 47 | 83 |
| | SCP1 | 02160 | 56 | 86 |

a) Numbers allocated in the genome data obtained from http://aspgd.org/.
b) Percentage of identity calculated by the FASTA program.
c) Percentage of similarity calculated by the FASTA program.

The amino acid sequences of human protein phosphatases were obtained from UniProt website (https://www.uniprot.org/). All the protein-coding DNA sequences of *A. flavus* were obtained from the website of Broad Institute (https://www.broadinstitute.org/fungal-genome-initiative/aspergillus-genome-projects). The searching and classification of protein phosphatases of *A. flavus* based on their amino acid sequences were conducted using Genetyx ver.10 software equipped with the FASTA program.