

Intracellular Expression of a Single Domain Antibody Reduces Cytotoxicity of 15-Acetyldeoxynivalenol in Yeast^{*S}

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15-Acetyldeoxynivalenol (15-AcDON) is a low molecular weight sesquiterpenoid trichothecene mycotoxin associated with *Fusarium* ear rot of maize and *Fusarium* head blight of small grain cereals. The accumulation of mycotoxins such as deoxynivalenol (DON) and 15-AcDON within harvested grain is subject to stringent regulation as both toxins pose dietary health risks to humans and animals. These toxins inhibit peptidyltransferase activity, which in turn limits eukaryotic protein synthesis. To assess the ability of intracellular antibodies (intrabodies) to modulate mycotoxin-specific cytotoxicity, a gene encoding a camelid single domain antibody fragment (V_HH) with specificity and affinity for 15-AcDON was expressed in the methylotropic yeast *Pichia pastoris*. Cytotoxicity and V_HH immunomodulation were assessed by continuous measurement of cellular growth. At equivalent doses, 15-AcDON was significantly more toxic to wild-type *P. pastoris* than was DON. In turn, DON was orders of magnitude more toxic than 3-acetyldeoxynivalenol. Intracellular expression of a mycotoxin-specific V_HH within *P. pastoris* conveyed significant ($p = 0.01$) resistance to 15-AcDON cytotoxicity at doses ranging from 20 to 100 $\mu\text{g}\cdot\text{ml}^{-1}$. We also documented a biochemical transformation of DON to 15-AcDON to account for the attenuation of DON cytotoxicity at 100 and 200 $\mu\text{g}\cdot\text{ml}^{-1}$. The proof of concept established within this eukaryotic system suggests that *in planta* V_HH expression may lead to enhanced tolerance to mycotoxins and thereby limit *Fusarium* infection of commercial agricultural crops.

Fusarium head blight of cereals and *Fusarium* ear rot of maize are caused by morphologically similar species (*Fusarium graminearum* and *Fusarium culmorum*, etc.) common throughout global agricultural regions. With few exceptions, *Fusarium* epidemics are characterized by cyclical and highly aggressive infection of commercial crops with economic impacts on food and feed industries that are immediate and far reaching. For example, losses associated with the most recent *Fusarium* outbreak in North America in the 1990s were estimated to range from 1.3 to 3.0 billion United States dollars (1).

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A toxin class commonly found within agricultural commodities infected by *Fusarium* are trichothecene mycotoxins. Trichothecenes represent a highly diverse group of over 180 sesquiterpenoid low molecular weight (typically 200–500 Da) mycotoxins characterized by a tricyclic ring structure containing a double bond at C-9,10 and an epoxide group at C-13 (2). Regardless of size and structural composition, trichothecenes are potent inhibitors of eukaryotic protein synthesis with specific activity on ribosomal protein L3 within the 60 S subunit resulting in inhibition of peptidyltransferase activity (3, 4). Although the capacity to inhibit protein synthesis is regarded as central to trichothecene cytotoxicity (5, 6), adverse effects on eukaryotic cells may actually be attributed to dysregulation of cellular signaling and alterations in downstream gene expression (7). As a result, trichothecenes such as deoxynivalenol (DON),² 15-acetyldeoxynivalenol (15-AcDON), and 3-acetyldeoxynivalenol (3-AcDON) (Fig. 1) are considered to be inherently hazardous feed- and food-borne contaminants (2, 8).

Numerous studies have demonstrated a correlation between *in planta* DON accumulation and *Fusarium* virulence in susceptible cultivars of wheat (9) and maize (10). Based on these findings, mechanisms that convey innate and acquired host plant resistance to DON and other trichothecene toxins have received considerable attention. To date, *in planta* trichothecene resistance has been achieved through mechanisms that alter targeted proteins within host cell ribosomes (11, 12), promote metabolic transformation to less toxic forms, e.g. DON-glucosyl conjugate (13) or to 3-AcDON (14), and/or reduce intracellular concentrations to effectively limit mycotoxin exposure to sensitive cellular targets. Collectively, such research can be applied to impart novel mechanisms of trichothecene resistance in higher order plants.

Yeast is well suited as a eukaryotic model organism to identify and validate mechanisms involved in host plant resistance to mycotoxins (12, 13, 15). Test systems based on yeast offer cost-effective convenience and flexibility as one can validate a wide range of novel detoxification mechanisms within a short period of time at a minimal cost using common/nonspecialized laboratory equipment. Assessment of mycotoxin resistance

² The abbreviations used are: DON, deoxynivalenol; 15-AcDON, 15-acetyldeoxynivalenol; 3-AcDON, 3-acetyldeoxynivalenol; ΔAUC , differential area under curve; HA, hemagglutinin; HPLC, high pressure liquid chromatography; Ab, antibody; PBS, phosphate-buffered saline.

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mechanisms is likewise straightforward as reproducible treatment-specific effects can be precisely determined based on simple measurements of cellular growth and function over time.

Single domain heavy chain antibody fragments (*i.e.* V_HH) from the camelidae heavy chain IgG subfamily are among the smallest functional recombinant antibody (Ab) fragments at 14–15 kDa. V_HH fragments exhibit the same *exquisite* specificity as larger immunoglobulins, with added biochemical advantages of high solubility, stability, and robust expression in various recombinant systems (16, 17). V_HH fragments have been generated against low molecular weight ligands (haptens) and toxins (18). Based on favorable physicochemical properties and efficacy against a wide range of antigens, single domain recombinant Ab fragments have been developed and tested as immunotherapeutic reagents with applications ranging from pharmacology (16, 19) to plant science (20).

This study demonstrates that intrabody expression of a V_HH fragment isolated from a hyper-immunized phagemid library with affinity for 15-AcDON (18) can impart real time immunomodulation of mycotoxin-specific cytotoxicity within a model eukaryotic system. *Pichia pastoris* was selected as the host organism based on an expected high level expression of functional V_HH intrabody fragments and anticipated sensitivity to 15-AcDON. This system was established as a “proof of concept” to demonstrate that intrabody expression of recombinant V_HH fragments could impart a novel means of mycotoxin-specific resistance.

EXPERIMENTAL PROCEDURES

V_HH Genes—NAT-267 V_HH DNA sequence (GenBank™ EU676170.1) (18) was used to design a *P. pastoris* codon optimized version of the gene to which was added 3' HA and His₆ epitope tags, as well as EcoRI- and XbaI-cloning sites at the respective 5' and 3' ends (GeneArt™, Toronto, Ontario, Canada). A nonspecific V_HH gene (B-24) isolated from a hyper-immunized phagemid llama library with confirmed nonspecificity for 15-AcDON, or any other trichothecene mycotoxin, was used as a V_HH intrabody control. Both V_HH fragments were of the same immunoglobulin family and had similar molecular weights and isoelectric points. Like NAT-267, B-24 V_HH DNA was PCR-amplified to include 3' HA and His₆ epitope tags and restriction cloning sites EcoRI and XbaI with the following gene-specific primers: VHH-B24For (5'-GGA-ATTCCATGCAGGTAAAGCTGGAGAG-5') and VHH-B24Rev (5'-GGGTCTAGACCCTATGCTCGGCCGGAACCGTAG-3'). A homology chart of nucleotide and peptide sequences of NAT-267 and B-24 V_HHs is provided to show respective framework and complementarity-determining regions, endonuclease sites, and epitope tags ([supplemental Fig. S1](#)). V_HH DNA inserts (~0.46 kb) were ligated into the pPICZB vector (Invitrogen). Constructs were sequenced by StemCore Labs (Ottawa, Ontario, Canada) using the pPICZB-specific primers 5' AOX1For (5'-GACTGGTTCCAATTGACAAGC-3') and 3' AOX1Rev (5'-GCAAATGGCATTCTGACA-TCC-3').

Transformation—Expression constructs (Fig. 2) were linearized with SacI and electroporated into *P. pastoris* strain KM71H (Invitrogen). Transformants were plated onto yeast extract,

peptone, dextrose, and sorbitol (YPDS) agar containing 100 μg ml⁻¹ Zeocin and incubated for 3 days at 30 °C until colonies formed. Ten colonies from each transformant were re-streaked on yeast extract, peptone, and dextrose (YPD) agar plates with 100 μg ml⁻¹ Zeocin to ensure pure clonal isolates.

Induction of V_HH Expression—Single colonies of V_HH and pPICZB control transformants were used to inoculate 5 ml of YPD media. Cultures were grown overnight (30 °C, 300 rpm). One ml of each culture was used to inoculate 100 ml of minimal glycerol medium with histidine (MGYH) and cultured in 1-liter baffled flasks (30 °C, 250–300 rpm) for 1 day. Cells were harvested by centrifugation (3000 × *g* for 5 min) at room temperature. To induce V_HH expression, *Pichia* cells were resuspended in 20 ml of minimal methanol medium with histidine (MMH), transferred to 125-ml baffled flasks, and incubated at 30 °C with shaking at 300 rpm. Methanol (100%) was added to a final concentration of 0.5% (% v/v) every 24 h. Western blot analysis of 10 V_HH (NAT-267 and B-24) transformants from 0, 24, 48, 72, 96, and 120 h post-induction was used to select clones and induction time points corresponding to the highest overall protein expression.

Preparation of Soluble V_HH Extracts and Western Blot Analysis—Cell pellets were thawed on ice and resuspended in 100 μl of lysis buffer (50 mM Tris, pH 7.5, 1 mM EDTA, 0.5% Triton X-100, 1 mM dithiothreitol, 1× Complete Protease Inhibitor Mixture (Roche Applied Science)). Equal volumes of acid-washed 0.5-mm glass beads were added to each resuspended pellet, followed by successive 30-s cycles of vortexing and incubation on ice for a total of 10 cycles. Cell lysate samples were clarified by centrifugation (14,000 rpm for 10 min) at 4 °C. One hundred microliters of 2× SDS loading buffer (Bio-Rad) was added to the supernatant followed by boiling (95 °C for 5 min) and electrophoresis on 12% SDS-polyacrylamide gels. Samples were transferred to polyvinylidene difluoride membrane (Bio-Rad) and blocked overnight in 1% blocking reagent (Roche Applied Science) in TBS at 4 °C. V_HH intrabody fragments were detected by probing for 1 h with rabbit anti-HA IgG primary antibody (Sigma) diluted 1:1000 in 0.5% blocking buffer followed by two washes with TBS + 0.1% Tween (TBST) and two washes with TBS + 0.5% blocking reagent. Secondary antibody (mouse anti-rabbit IgG horseradish peroxidase conjugate; Jackson ImmunoResearch, West Grove, PA), diluted 1:50,000 in 0.5% blocking buffer, was used to probe the membrane for 1 h, followed by four consecutive 15-min washes with TBST. V_HH proteins were visualized with ECL Plus Western blotting detection system (GE Healthcare). Transformants with the highest V_HH intrabody expression levels were used in subsequent *in vivo* cytotoxicity assays.

Cytotoxicity Assays—Cells were induced as described previously. Forty eight hours post-induction, *P. pastoris* cells were diluted to an A₆₀₀ of 0.25 in YPD and incubated for 4 h (30 °C, 300 rpm). After this “recovery period,” cells were diluted to an A₆₀₀ of 0.1 in YPD and immediately transferred (250 μl-well⁻¹) to an F96 microwell assay plate (Nalge Nunc Inc., Naperville, IL). Pre-calibrated concentrations of the four ribotoxin treatments (15-AcDON, DON, 3-AcDON, or cycloheximide) prepared in DMSO and a DMSO-only control were added to *P. pastoris* cells contained in the wells of assay plates. All treat-

ments, respective controls, and cell-free wells containing only YPD media (*i.e.* blank wells) were established in triplicate on each plate. Plates were sealed with Progene pressure-sensitive optical sealing film (Ultident, St.-Laurent, Quebec, Canada) prior to initiation of cytotoxicity assays. Cellular growth (30 °C, 300 rpm) was measured in real time based on measurement of A_{620} values at 25-min intervals, following 5 min of shaking (600 rpm) in a Polarstar Optima Microplate Reader (BMG Labtech, GmbH, Offenberg, Germany). Assay results were considered valid if similar and reproducible effects were observed in three separate experiments, conducted in triplicate under the same test parameters.

Data Analysis—Results were presented as *P. pastoris* growth curves over a 24-h time period. Mean ($n = 3$) A_{620} values for V_{HH} and pPICZB (empty vector) transformants used in ribotoxin and control treatments, minus A_{620} values of YPD media (blank) wells, were plotted against time (*i.e.* 25-min intervals). Standard error values and paired *t* tests ($p = 0.05$ and 0.01) were used to assess statistically significant differences at the end of each 25-min time interval.

The time to doubling of initial *P. pastoris* A_{620} cellular growth values was calculated from cytotoxicity assay data to assess relative toxicity of each ribotoxin treatment. A comparative ranking of various ribotoxin treatments on *P. pastoris* growth was calculated based on time required to double A_{620} values of $t = 0.0$ h pPICZB empty vector transformants.

Differential area under curve (ΔAUC) values were calculated to quantify relative differences in *P. pastoris* growth over the full time course of each cytotoxicity assay. Relative differences in cellular growth were established by subtracting mean A_{620} values of pPICZB (empty vector) wells from corresponding values for V_{HH} transformants. ΔAUC values for each toxophore treatment were calculated based on addition of differences in cellular growth between V_{HH} and control transformants at each 25-min time interval across the full time course of each assay.

Quantitative Western Blot Analysis—20-ml cultures were centrifuged ($1500 \times g$ for 5 min at 4 °C) 48 h after methanol induction. Culture pellets were lysed and prepared for SDS-PAGE as described above. V_{HH} lysate samples (500 μl) were diluted 1:5; 1:25; and 1:50 in nonreducing SDS sample buffer (Bio-Rad). pPICZB vector only (control) samples were not diluted. A reference V_{HH} recombinant Ab fragment of a precisely defined concentration, and of similar size to NAT-267 and B-24 V_{HH} with C-terminal HA and His₆ epitope tags, was used to establish a standard dilution series at final protein concentrations of 300.0, 150, 75, 37.5, and 18.75 ng·lane⁻¹. To decrease the bias of Western blot band intensities, V_{HH} standard dilution series was prepared in *P. pastoris* cell lysate from pPICZB empty vector sample.

Samples were loaded onto 12.5% SDS-polyacrylamide gels and electrophoresed followed by transfer to a polyvinylidene difluoride membrane (Millipore, Billerica, MA) followed by a 1-h blocking with 3% nonfat skimmed milk in PBST. Mouse anti-His₆ IgG primary antibody (GE Healthcare) diluted 1:2500 in 3% milk was added to membranes for 1 h followed by 1 h of blocking and three consecutive washes with PBST. Alkaline phosphatase-labeled goat anti-mouse IgG conjugate (Cedarlane Laboratories, Burlington, Ontario, Canada), diluted 1:3000

in blocking buffer, was used to probe the membrane for 1 h followed by three consecutive washes with PBST. V_{HH} proteins were visualized using an alkaline phosphatase conjugate substrate kit (Bio-Rad).

Quantitative Western blots were dried and photographed with an AlphaImager 3400 (Alpha Innotech Corp., San Leandro, CA). Analysis was performed using the AlphaEaseFc software package (version 7.0.1, Alpha Innotech Corp.). Densitometric values for a standard five data point V_{HH} dilution series (18.75–300 ng of V_{HH} ·lane⁻¹) were used to produce regression equations. Densitometric values measured for 1:5, 1:25, and 1:50 dilutions of NAT-267 or B-24 V_{HH} samples were expressed as V_{HH} mass per μl of *P. pastoris* cell pellet (ng· μl^{-1}) based on the following: V_{HH} quantity (nanogram) (from standard regression equation) divided by cell pellet lysis volume loaded into each well (microliter). Cellular densities for each cytotoxicity assay were determined by multiplication of A_{600} of 1.0 ml of culture by 5×10^7 cells per ml of culture (constant). V_{HH} concentration (fg·cell⁻¹) was calculated as V_{HH} mass per μl of cell pellets (ng· μl^{-1}) (above) divided by cell density (number of cells· μl^{-1} culture). V_{HH} intrabody concentration expressed as attomoles of V_{HH} per cell (amol·cell⁻¹) was calculated based on the following: V_{HH} mass per cell (fg·cell⁻¹) divided by molecular weight of NAT-267 or B-24 V_{HH} proteins (16,637 and 16,976 Da, respectively). Finally, intracellular V_{HH} molarity (μmol of V_{HH} ·liter⁻¹) was calculated as V_{HH} concentration (amol·cell⁻¹) divided by cell volume. *P. pastoris* cell volume was estimated to be 29 fl per cell (fl·cell⁻¹) based on the average of independent measurements of generic yeast cell volume as follows: 42, 37, 70, and 83 fl (21–24) multiplied by 0.5 to account for an estimate that the cytosol represents 50% of total yeast cell volume (25).

Mean and standard error values of intracellular V_{HH} expression values were calculated based on five independent quantitative Western blot assays. Additional details are summarized in [supplemental Table S1](#).

V_{HH} Immunolocalization—Cultures of *P. pastoris* cells expressing either NAT-267 V_{HH} or pPICZB (empty vector) were induced with methanol (as described). Cells (20 A_{600} units) were pelleted and washed three times with PBS and resuspended in 500 μl of PBS. Fifty- μl aliquots of *P. pastoris* cells were incubated in 500 μl of fixative solution (1:1 acetone:methanol) for 20 min at –20 °C. Cells were pelleted (1000 $\times g$ for 1 min), washed three times with PBS, and resuspended in 500 μl of PBS supplemented with Lyticase (5 units· μl^{-1}). After incubation (30 °C, 15 min.), cells were pelleted (as described) and incubated in 500 μl of permeabilization solution (3% bovine serum albumin, 0.5% Triton X-100 in PBS) for 30 min at room temperature. After washing with incubation solution (1% bovine serum albumin, 0.5% Tween 20), cells were probed with a 1:100 dilution of monoclonal anti-HA-fluorescein isothiocyanate antibody (Sigma) for 1–2 h at room temperature. Cells were mounted in PBS supplemented with 4',6-diamidino-2-phenylindole dihydrochloride stain (Sigma) at a final concentration of 1.3 $\mu\text{g}/\text{ml}$. Cells were observed after 5 min using a Carl Zeiss LSM 510 Meta confocal microscope equipped with diode 405-nm and argon/2 line 488-nm lasers for excitation. Emission images were taken at 420–480 and 505–565 nm for

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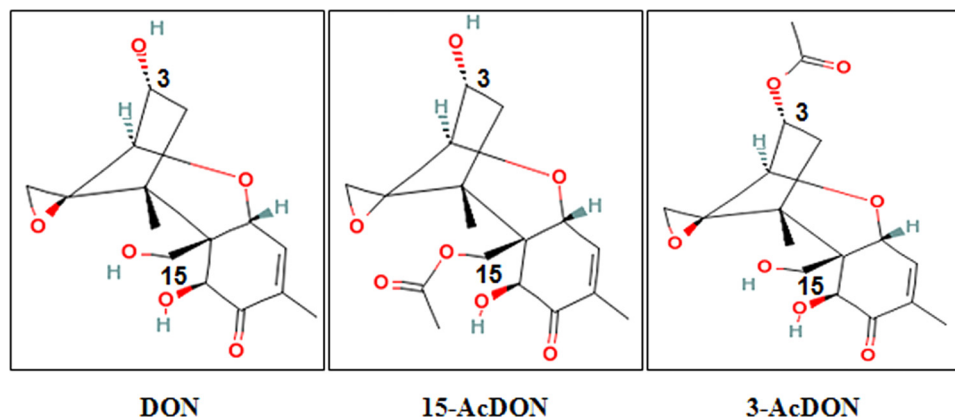


FIGURE 1. Structure, composition, and molecular weight of trichothecene mycotoxins used within this study (2).

4',6-diamidino-2-phenylindole dihydrochloride and fluorescein isothiocyanate, respectively.

Mycotoxin Biotransformation Assays—After 48 h of induction (see above), cultures were diluted to an A_{600} of 0.25 in YPD and incubated for 4 h (30 °C, 300 rpm). Cultures were diluted to A_{600} 0.1 in YPD, and 2.5-ml aliquots of each culture were transferred to 50-ml tubes. Highly purified DON was added to respective samples to a final concentration of 100 $\mu\text{g}\cdot\text{ml}^{-1}$ followed by incubation (30 °C, 300 rpm). Culture samples (0.5 ml) were taken at intervals of 30 min and 16 and 24 h after mycotoxin addition and were used to measure A_{600} and culture pH. Samples were pelleted (3500 $\times g$ for 5 min). Supernatant was removed and transferred to 2.0-ml tubes. Samples were frozen in liquid nitrogen and stored at -80 °C. Lysate samples were prepared from cell pellets (as described above). All samples were split to enable concurrent anti-HA Western blot analysis (as described above) and mycotoxin biotransformation assays.

Supernatant, cell lysate, and pellet of selected samples were washed with double distilled H_2O and passed through a 0.8- μm filter (Millipore). Filtrates were passed through Chromosep C_{18} columns (C_{18} Sep-Park cartridge, Waters) and pre-washed with 10 ml of 100% (w/v) HPLC grade methanol and 10 ml of double distilled H_2O . After washing with double distilled water (10 ml), mycotoxin fractions were eluted with 10 ml of 100% (v/v) HPLC grade methanol and dried under a stream of N_2 . Selected culture samples (as described in supplemental Table S2) were diluted in 0.5 ml of HPLC grade methanol (100%) and 50.0- μl subsamples were used for HPLC analysis.

Trichothecenes were separated on a Shimadzu HPLC system equipped with a 150 \times 4.6-mm C_{18} column (5 μm packing size) and a Hichrome UV flow cell. Standards and samples were eluted at a flow rate of 1.0 $\text{ml}\cdot\text{min}^{-1}$ with a 20-min linear gradient of 95% solvent A (degassed double distilled H_2O), 5% solvent B (acetonitrile) to 40% solvent A, 60% solvent B. The column was washed from 20 to 25 min by ramping the gradient to 100% solvent B, holding at 100% solvent B for 5 min, and then returning to the starting conditions over 2 min. Assessment of DON biotransformation was based on assessment of retention times of sample peaks relative to trichothecene standard peaks.

Gas chromatography/mass spectrometry analysis, based on a standard Canadian Food Inspection Agency approved method

(26), was used to further characterize and validate HPLC samples for structural composition of trichothecene analytes. Biotransformation samples from HPLC analysis were derivatized with 1.5 $\text{mg}\cdot\text{ml}^{-1}$ dimethylaminopyridine in toluene:acetonitrile (95:5) solution and trifluoroacetic anhydride. This solution was heated at 60 °C for 30 min and neutralized twice with 5% KH_2PO_4 . Derivatized samples were analyzed on a Saturn 2000 GC/ion-trap mass spectrometer equipped for chemical ionization using acetonitrile.

^1H NMR spectra of pooled *P. pastoris* lysate samples and subsequent HPLC fractions of those samples were obtained on a Bruker AM 500 NMR spectrometer in CDCl_3 . Chemical shifts are referenced to residual CHCl_3 at 7.24 ppm for ^1H spectra and reported (δ) relative to tetramethylsilane.

RESULTS

Sensitivity to Ribotoxin Treatments

The structure, composition, and molecular weight of the mycotoxins used in these experiments are shown in Fig. 1. The sensitivity of wild-type *P. pastoris* to trichothecene and cycloheximide (control) ribotoxin treatments, was governed by the dose and chemical structure tested. Treatments based on 15-AcDON resulted in the most immediate and largest overall reduction of cellular growth (Table 1). *P. pastoris* was comparatively less sensitive to equivalent doses of DON and cycloheximide. It was not possible to establish a dose-specific sensitivity to 3-AcDON relative to toxin-free DMSO media as cellular growth was not inhibited at the highest concentration (200 $\mu\text{g}\cdot\text{ml}^{-1}$) (Table 1).

Transformation and $V_{\text{H}^{\text{H}}}$ Intrabody Expression

P. pastoris KM71H cells were successfully transformed with linearized NAT-267 (treatment) or B-24 (control) $V_{\text{H}^{\text{H}}}$ DNA ligated into expression vector pPICZB (Fig. 2). Lysate fractions of methanol-induced transformants were assessed by anti-HA epitope Western blot analysis, and robust intracellular $V_{\text{H}^{\text{H}}}$ expression was confirmed by the presence of a 17-kDa band at 48 and 72 h post-methanol induction (data not shown).

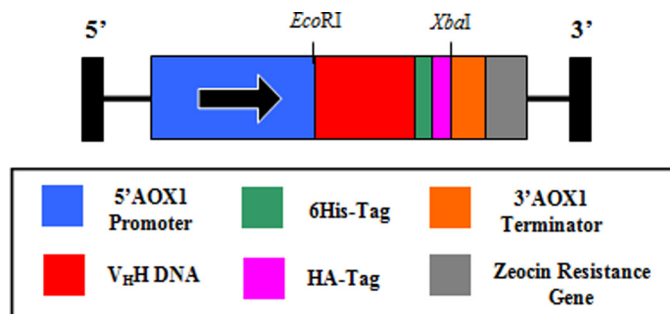
Quantitative Western Blot Assay

Mean levels of soluble intracellular $V_{\text{H}^{\text{H}}}$ expression ($t = 0$ h) within transformants tested within cytotoxicity assays were determined by quantitative Western blot analysis (Fig. 3). The initial concentration of NAT-267 $V_{\text{H}^{\text{H}}}$ in *P. pastoris* cells within cytotoxicity assays of 15-AcDON (Fig. 4) was 4.01 ± 0.31 amol of $V_{\text{H}^{\text{H}}}\cdot\text{cell}^{-1}$ (Table 2). Accounting for an estimated mean *P. pastoris* cytosol volume of 29 $\text{fl}\cdot\text{cell}^{-1}$ (as previously described), initial expression of NAT-267 $V_{\text{H}^{\text{H}}}$ was equivalent to an intracellular concentration of 138 ± 10.8 μmol of $V_{\text{H}^{\text{H}}}\cdot\text{liter}^{-1}$. Likewise, mean NAT-267 intrabody concentra-

TABLE 1

Sensitivity of *P. pastoris* pPICZB empty vector transformants to ribotoxin treatments tested relative to toxin-free DMSO mediaLeast significant differences are based on a significant analysis of variance ($p = 0.05$). Standard deviation (σ) for each mean value is shown in parentheses.

Dose ($\mu\text{g}\cdot\text{ml}^{-1}$)	Time to doubling of initial A_{620} cellular growth (min)				L.S.D. ^a
	15-AcDON	DON	3-AcDON	Cycloheximide	
0 ^b	205 (2.2)	221 (14.4)	219 (21.7)	221 (14.4)	14.5
20	322 (4.4)	ND ^c	ND	ND	ND
30	384 (4.4)	ND	ND	ND	ND
40	439 (6.6)	ND	ND	ND	ND
50	494 (8.8)	308 (7.2)	190 (124)	283 (7.2)	71.7
100	642 (138)	417 (47.3)	194 (119)	310 (20.1)	99.3
200	996 (312)	590 (58.1)	217 (13.0)	341 (7.2)	166.6
L.S.D.	117.2	40.2	91.3	14.0	

^a L.S.D. means least significant difference ($p = 0.05$).^b Ribotoxin-free DMSO media were used.^c ND means not determined.FIGURE 2. Diagram of pPICZB expression vector (3745 bp) with $V_{\text{H}}\text{H}$ gene (NAT-267 = 387 bp, B-24 = 390 bp). Legend in the box shows schematic representation for each component of transgenic DNA for expression in *P. pastoris*.

tion within cells used in cytotoxicity assays of DON, 3-AcDON, and cycloheximide (Fig. 5) was $3.18 \pm 0.42 \text{ amol}\cdot\text{cell}^{-1}$ or $110 \pm 14.4 \mu\text{mol}\cdot\text{liter}^{-1}$ (Table 2). The average initial concentration of B-24 control $V_{\text{H}}\text{H}$ intrabody in cytotoxicity assays of 15-AcDON and cycloheximide (Fig. 6) was $1.93 \pm 0.14 \text{ amol}\cdot\text{cell}^{-1}$ or $66.6 \pm 4.3 \mu\text{mol}\cdot\text{liter}^{-1}$ (Table 2).

Cytotoxicity Assays

Intrabody $V_{\text{H}}\text{H}$ expression did not influence the cellular growth of transformed *P. pastoris* cultures in mycotoxin-free DMSO media, as A_{620} values and overall growth rate of NAT-267 and B-24 $V_{\text{H}}\text{H}$ transformants were similar to pPICZB empty vector control cells (Figs. 4–6).

15-AcDON—Significant differences ($p = 0.01$) in cellular growth were observed between NAT-267 $V_{\text{H}}\text{H}$ and pPICZB control transformants grown in media spiked with 20–100 $\mu\text{g}\cdot\text{ml}^{-1}$ 15-AcDON (Fig. 4). The impact of NAT-267 $V_{\text{H}}\text{H}$ intrabody expression on cellular growth was dependent on the concentration of 15-AcDON used. At the lowest dose (20 $\mu\text{g}\cdot\text{ml}^{-1}$), growth of NAT-267 $V_{\text{H}}\text{H}$ -expressing cells was similar to cell lines grown in DMSO (control) media.

Time to significantly improved ($p = 0.01$) growth of NAT-267 $V_{\text{H}}\text{H}$ intrabody transformants was dose-dependent. Significant ($p = 0.01$) differences in A_{620} values were observed after 7.5 h of growth for cells cultured in medium supplemented with 15-AcDON at both 20 and 50 $\mu\text{g}\cdot\text{ml}^{-1}$ (Fig. 4). Assays spiked with 30, 40, and 100 $\mu\text{g}\cdot\text{ml}^{-1}$ 15-AcDON had significant differences in A_{620} values after 4.6, 2.9, and 11.3 h of growth, respectively. No differences in A_{620} values were observed between NAT-267 $V_{\text{H}}\text{H}$ and control transformants at 200 $\mu\text{g}\cdot\text{ml}^{-1}$ 15-AcDON (Fig. 4).

ΔAUC analysis was used to quantify relative differences in cellular growth between NAT-267 and B-24 (control) $V_{\text{H}}\text{H}$ transformants. The largest ΔAUC value (5.25) corresponded to NAT-267 $V_{\text{H}}\text{H}$ -expressing *P. pastoris* grown with 15-AcDON at 30 $\mu\text{g}\cdot\text{ml}^{-1}$. Cytotoxicity assays based on 15-AcDON at 20, 40, and 50 $\mu\text{g}\cdot\text{ml}^{-1}$ had similar ΔAUC values (4.48, 4.50, and 4.45, respectively) (Fig. 4). Accordingly, assays at 100 $\mu\text{g}\cdot\text{ml}^{-1}$ produced a substantially lower ΔAUC value (1.20), whereas NAT-267 $V_{\text{H}}\text{H}$ intrabody expression conferred no immunomodulation when spiked with 200 $\mu\text{g}\cdot\text{ml}^{-1}$ 15-AcDON (Fig. 4).

DON, 3-AcDON, and Cycloheximide—NAT-267 $V_{\text{H}}\text{H}$ intrabody expression resulted in a dose-dependent response to DON. Although no beneficial impact on cellular growth was observed in cultures supplemented with 50 $\mu\text{g}\cdot\text{ml}^{-1}$ DON, NAT-267 $V_{\text{H}}\text{H}$ expression resulted in significantly improved cellular growth at 100 and 200 $\mu\text{g}\cdot\text{ml}^{-1}$ DON relative to pPICZB control transformants (Fig. 5A). Comparative differences in growth were greater for yeast spiked with 100 relative to 200 $\mu\text{g}\cdot\text{ml}^{-1}$ DON (ΔAUC values = 2.11 and 1.68, respectively). In addition, time to significant differences in A_{620} values was several hours later for the higher dose (Fig. 5A).

Differences in cellular growth between NAT-267 $V_{\text{H}}\text{H}$ and pPICZB control *P. pastoris* cells could not be established for cytotoxicity assays spiked with 3-AcDON because this trichothecene had little or no adverse effect on *P. pastoris* growth (Table 1). Assays based on cycloheximide (control ribotoxin) exhibited a dose-dependent effect on cellular growth; however, no differences were observed between *P. pastoris* NAT-267 $V_{\text{H}}\text{H}$ and pPICZB transformants spiked with doses of 50, 100, and 200 $\mu\text{g}\cdot\text{ml}^{-1}$ (Fig. 5B).

B-24 $V_{\text{H}}\text{H}$ Control—Expression of B-24 control $V_{\text{H}}\text{H}$ intrabody had no effect on cellular growth rate relative to pPICZB control transformants in DMSO or in media supplemented with either 15-AcDON or cycloheximide (Fig. 6). These results suggest that trichothecene-specific immunomodulation imparted by NAT-267 $V_{\text{H}}\text{H}$ intrabody expression was trichothecene-specific, as *Pichia* cells expressing a nonspecific $V_{\text{H}}\text{H}$ intrabody were equally as sensitive as pPICZB control transformants.

$V_{\text{H}}\text{H}$ Immunolocalization

NAT-267 $V_{\text{H}}\text{H}$ intrabody expression and distribution within *P. pastoris* transformants were further validated with fluores-

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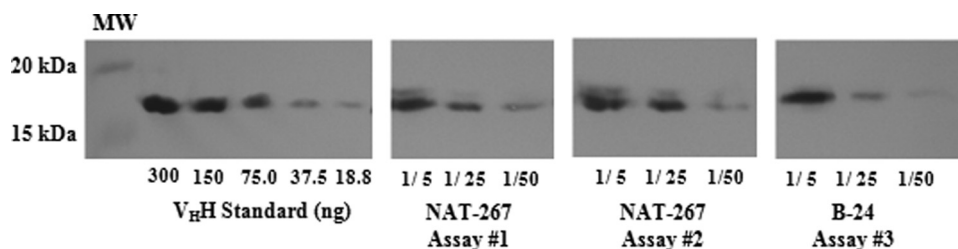


FIGURE 3. Quantitative Western blot analysis of $V_{\text{H}}\text{H}$ intrabody expression within cytotoxicity assays. 1st blot is $V_{\text{H}}\text{H}$ standard dilution series from 300.0 to 18.75 $\text{ng}\cdot\text{well}^{-1}$. 2nd blot is NAT-267 $V_{\text{H}}\text{H}$ expression for cytotoxicity assay 1 (Fig. 4). 3rd blot is NAT-267 $V_{\text{H}}\text{H}$ expression for cytotoxicity assay 2 (Fig. 5). 4th blot is B-24 (control) $V_{\text{H}}\text{H}$ expression for cytotoxicity assay 3 (Fig. 6). Bio-Rad molecular weight marker (MW) is indicated on the left. Protein dilutions are shown along the bottom of 2nd to 4th blots.

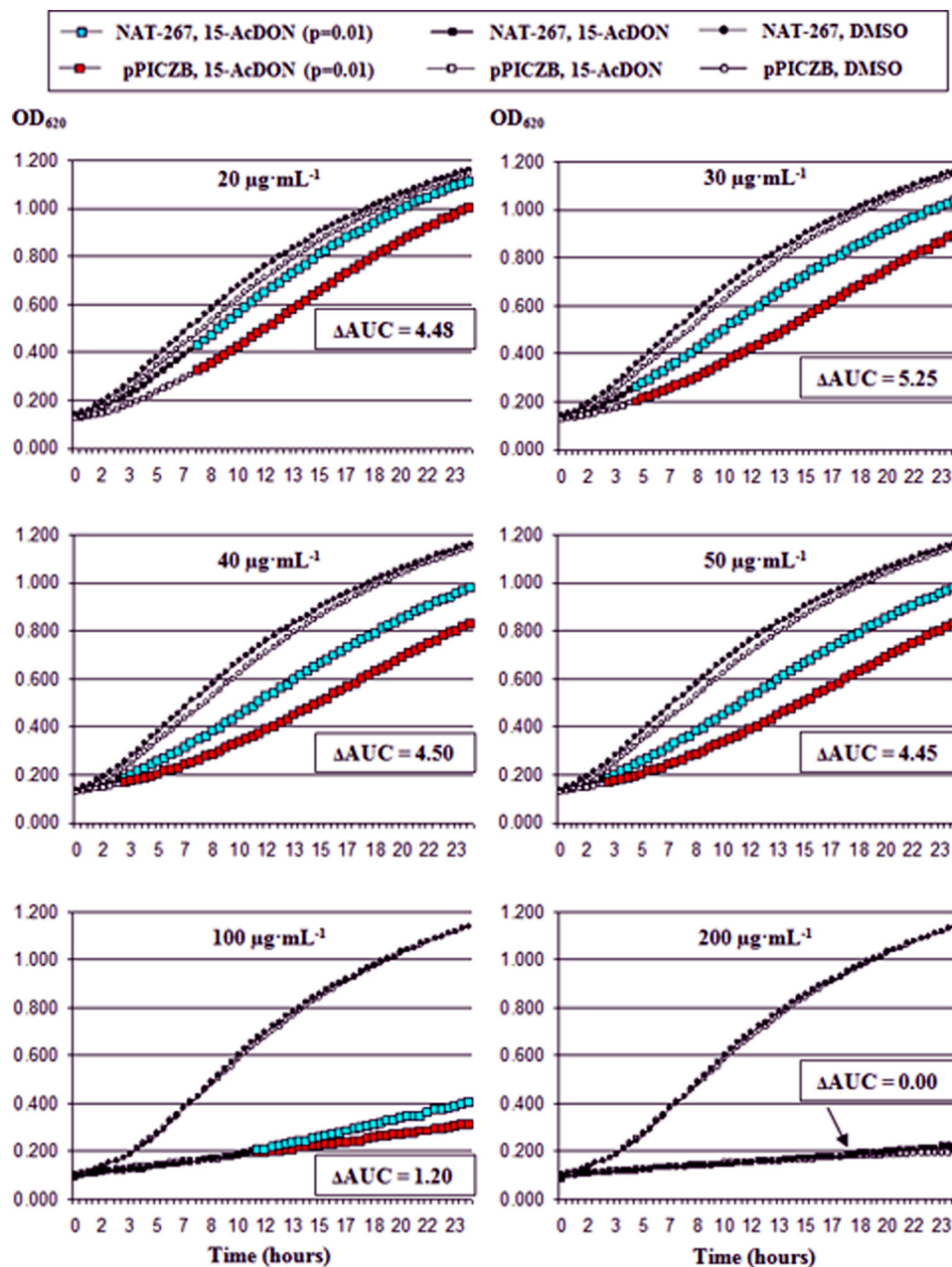


FIGURE 4. Cytotoxicity assays showing the effect of various concentrations (20–200 $\mu\text{g}\cdot\text{mL}^{-1}$) of 15-AcDON on the growth of *P. pastoris* pPICZB and NAT-267 $V_{\text{H}}\text{H}$ transformants as measured by absorbance at 620 nm (OD_{620}). ΔAUC values for NAT-267 $V_{\text{H}}\text{H}$ minus pPICZB empty vector are shown on each graph. At all concentrations of 15-AcDON (except 200 $\mu\text{g}\cdot\text{mL}^{-1}$) where blue versus red symbols are used, there was a significant difference ($p = 0.01$) at each time between NAT-267 and pPICZB transformants.

cent confocal microscopy (Fig. 7). Detection of the HA tag on NAT-267 $V_{\text{H}}\text{H}$ intrabody fragments within transformants grown in YPD media to $A_{600} = 20$ further confirmed robust intrabody expression within our test system.

Mycotoxin Biotransformation Assays

Given that NAT-267 $V_{\text{H}}\text{H}$ was previously confirmed to have binding affinity limited to 15-AcDON (18), we were surprised to observe a dose-dependent amelioration to DON cytotoxicity within NAT-267 $V_{\text{H}}\text{H}$ transformants (Fig. 5A). To validate this observation and reconfirm *in vivo* antigen specificity of NAT-267 $V_{\text{H}}\text{H}$ intrabody expression, we assessed the potential biotransformation of DON within our *P. pastoris* test system by spiking with DON. HPLC analysis of 16- and 24-h cultures grown in DON-supplemented YPD media consistently yielded another peak in addition to the DON peak at ~ 5.85 min (supplemental Table S2). This peak appeared at ~ 9.5 min and matched the retention time of 15-AcDON (supplemental Table S2). HPLC of cell lysate and supernatant samples of pPICZB and NAT-267 $V_{\text{H}}\text{H}$ transformants provided clear evidence of a metabolic conversion of DON to 15-AcDON. No trichothecenes were found within cell pellet fractions (supplemental Table S2). HPLC analysis revealed no evidence of DON-glucosyl metabolites or any structurally similar compounds within the samples tested. Gas chromatography/mass spectrometry analysis confirmed the presence of 15-AcDON within pooled cell lysate samples (data not shown). The GC retention time and mass spectrum of the peak confirmed the presence of 15-AcDON and absence of 3-AcDON within the samples tested (data not shown). Finally, the ^1H NMR spectra of the total filtrate extract was dominated by DON and other impurities from the medium. However, the presence of 15-AcDON was confirmed

TABLE 2

Mean $V_{\text{H}}\text{H}$ intrabody concentration ($t = 0$ h) within *P. pastoris* transformants used in cytotoxicity assays (Figs. 4–6)

Values were derived from data generated from quantitative Western blot assays (Fig. 3) as summarized in supplemental Table S1. $V_{\text{H}}\text{H}$ concentration values are expressed in femtomoles and attomoles of $V_{\text{H}}\text{H}$ per cell, as well as micromoles-liter⁻¹ $V_{\text{H}}\text{H}$ equivalent within *P. pastoris* cytosol. Standard errors for all means ($n = 9$) are shown in parentheses.

$V_{\text{H}}\text{H}$ gene	Cytotoxicity assay	$V_{\text{H}}\text{H}$ intrabody concentration		
		fgcell^{-1}	amolcell^{-1}	$\mu\text{mol}\cdot\text{liter}^{-1}$
NAT-267	15-AcDON (Fig. 4)	66.8 (± 5.2)	4.01 (± 0.31)	138 (± 10.8)
NAT-267	DON, 3-AcDON, and cycloheximide (Fig. 5)	53.0 (± 7.0)	3.18 (± 0.42)	110 (± 14.4)
B-24	15-AcDON and cycloheximide (Fig. 6)	32.8 (± 2.3)	1.93 (± 0.14)	66.6 (± 4.3)

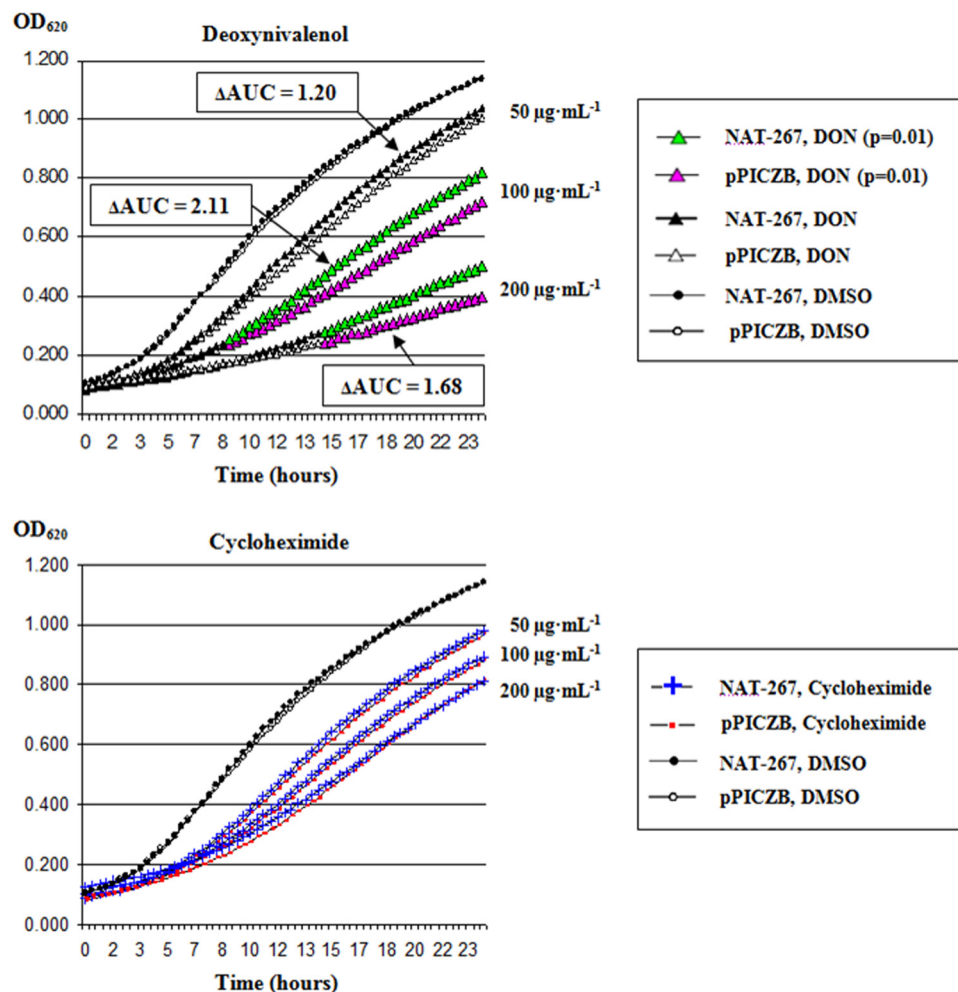


FIGURE 5. Cytotoxicity assays showing the effect of various concentrations ($50\text{--}200\ \mu\text{g}\cdot\text{mL}^{-1}$) of DON and cycloheximide on the growth of *P. pastoris* pPICZB and NAT-267 $V_{\text{H}}\text{H}$ transformants as measured by absorbance at 620 nm (OD_{620}). ΔAUC values for NAT-267 $V_{\text{H}}\text{H}$ minus pPICZB empty vector are shown for each concentration of DON. ΔAUC values for cycloheximide treatments were nil. At all concentrations of DON where green versus red symbols are used, there was a significant difference ($p = 0.01$) at each time between the NAT-267 and pPICZB transformants; this was not the case for all cycloheximide treatments.

in the NMR spectrum of the HPLC fraction isolated at ~ 9.5 min from the pooled lysate samples (Fig. 8).

DISCUSSION

Various strategies have been used to develop plants with enhanced resistance to trichothecene cytotoxicity and associated *Fusarium* pathogenesis. These approaches tend to be based on mechanisms that alter host cellular targets (11, 12), reduce mycotoxin cytotoxicity (13, 14), or enhance innate host resistance through crop breeding techniques (27). Our goal was to evaluate the ability of a mycotoxin-specific $V_{\text{H}}\text{H}$ intrabody to

immunomodulate, or attenuate, the cytotoxic effects of 15-AcDON using a yeast model.

Aside from application as a bioassay-indicator organism (28, 29), several species of yeast have been used to evaluate the *in situ* function of various trichothecene-specific genes (13, 30) or to assess the efficacy of mycotoxin-specific transgenes prior to *in planta* application (12, 13, 15). The methylotropic yeast *P. pastoris* (strain KM71H) was selected as our eukaryotic model based on expected sensitivity to target ribotoxins and known capacity to express high levels of functional heterologous proteins (31). We demonstrated that it was possible to transform, assess, and validate *P. pastoris* cells within a matter of weeks, thus creating an ideal platform for screening mycotoxin-specific constructs before *in planta* evaluation.

Trichothecene cytotoxicity is determined by the C-12,13 epoxide group common to this class of mycotoxins (3, 4, 6). However, the number and position of hydroxyl and acetyl ester groups on the trichothecene structure can also influence the mechanism of protein synthesis inhibition and relative toxicities within eukaryotic cells (32–34). In this regard, we found that the position of an acetyl ester group on either carbon 3 or 15 of

DON (Fig. 1) had a profound effect on their cytotoxicity to *P. pastoris* (Table 1). Our findings align with results established in other yeast species where 15-AcDON was significantly more toxic than DON (12, 13), which was more toxic than 3-AcDON (35). These results also support the findings of structure-activity studies established *in planta* that demonstrated 15-AcDON is more toxic than DON (13, 34) and 3-AcDON (2, 14). Our observation of no measurable cytotoxicity of 3-AcDON is in agreement with previous studies that demonstrated that addition of an acetyl group at C-3 (Fig. 1) serves to eliminate cyto-

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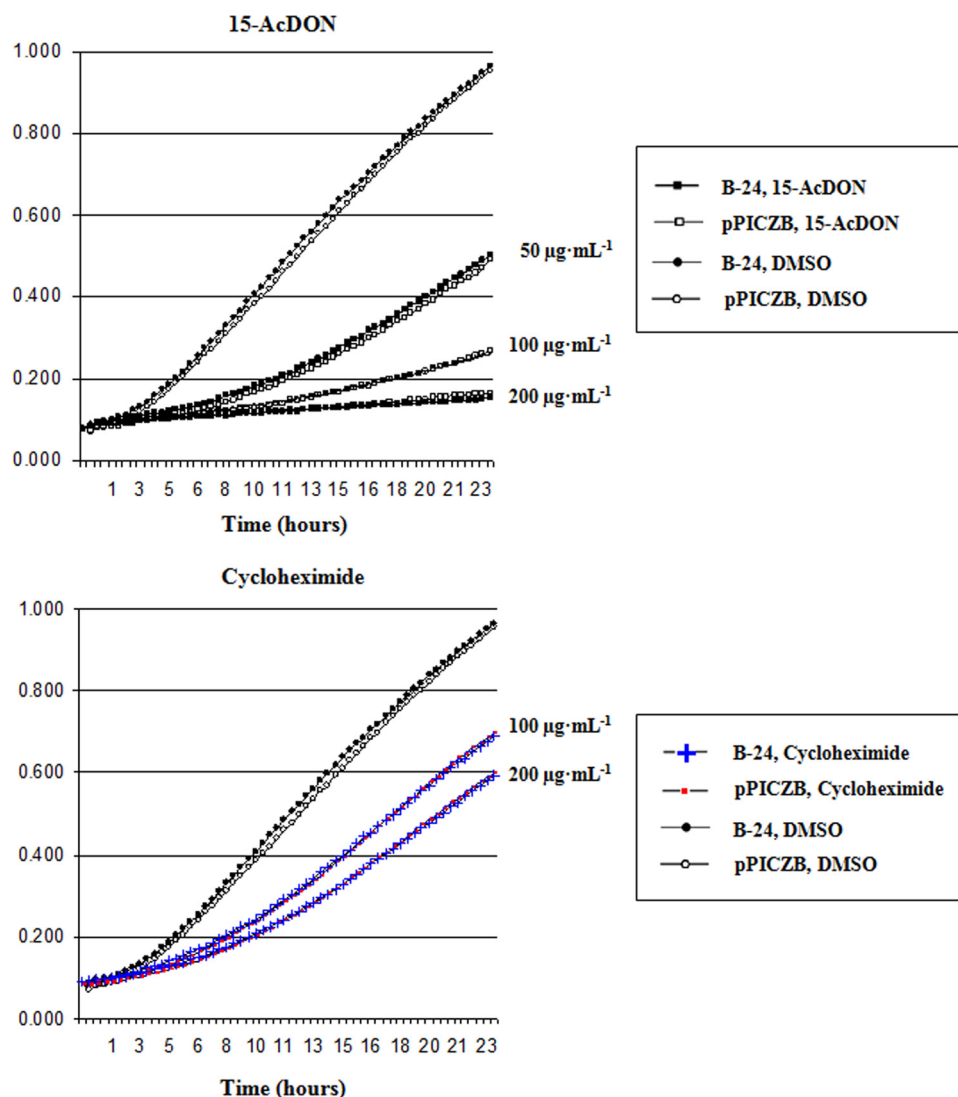


FIGURE 6. Cytotoxicity assays showing the effect of various concentrations (50–200 $\mu\text{g}\cdot\text{mL}^{-1}$) of 15-AcDON and cycloheximide on the growth of *P. pastoris* pPICZB and nonspecific B-24 $V_{\text{H}}\text{H}$ transformants as measured by absorbance at 620 nm (OD_{620}). ΔAUC values for B-24 $V_{\text{H}}\text{H}$ minus pPICZB empty vector for both 15-AcDON and cycloheximide are nil. At all concentrations of DON and cycloheximide, there was no significant difference at each time between the B-24 and pPICZB transformants.

toxicity as part of a metabolic defense mechanism during trichothecene synthesis (36, 37).

Our results also quantify relative differences in trichothecene cytotoxicity over time, not at a specific assay end point. Our *P. pastoris* model system enables real time evaluation of cellular effects during the initial phases of ribotoxin exposure. We propose that this biological system is very sensitive (*i.e.* compared with whole plant systems) and shows clear effects on cellular growth within the first hours of exposure thereby indicating the potency of trichothecene-mediated cytotoxicity.

A sequential two-step process was required before commencement of each cytotoxicity assay. First, transformants were induced with methanol for 48 h. After induction of $V_{\text{H}}\text{H}$ intrabody expression, *P. pastoris* cells were transferred to YPD media for a 4-h recovery period to re-stimulate cellular growth. Mycotoxin or cycloheximide (control) treatments were then added (at $t = 0$ h) to initiate each cytotoxicity assay. Given the well established negative impact on eukaryotic protein synthe-

sis, it was an obvious necessity to induce $V_{\text{H}}\text{H}$ expression prior to addition of ribotoxin treatments. The 4-h recovery in YPD media was required because induction conditions did not support an optimal cellular division and growth over time. We also noted that, by virtue of its design, our test system ensured a fixed ratio of $V_{\text{H}}\text{H}$ antibody to ribotoxin antigen throughout the course of each cytotoxicity assay. We recognized the potential disadvantage of culturing induced cells in YPD as leading to a continuous dilution of $V_{\text{H}}\text{H}$ concentration within the cytosol of rapidly dividing cells. However, we sought to maintain a fixed ratio of Ab:antigen within each microtiter well to ensure unbiased measurements of overall cellular growth.

Soluble NAT-267 and B-24 control $V_{\text{H}}\text{H}$ intrabody fragments were well expressed within *P. pastoris*. Average protein expression levels (Table 2) were found to be much higher than previously reported in *P. pastoris* (38–40). Furthermore, images generated by confocal immunomicroscopy confirmed that NAT-267 $V_{\text{H}}\text{H}$ fragments were well distributed within the cytosol of *P. pastoris* transformants even after growth to a very high A_{600} value (Fig. 7).

Our principal hypothesis was that the expression of mycotoxin-specific $V_{\text{H}}\text{H}$ intrabody fragments would effectively reduce (*i.e.* immu-

nomodulate) bioavailable 15-AcDON concentrations within the cell and thereby limit cellular toxicity, thereby resulting in enhanced growth of *P. pastoris*. $V_{\text{H}}\text{H}$ -mediated mycotoxin binding was assessed by time-to-significant immunomodulation and ΔAUC values between $V_{\text{H}}\text{H}$ transformants and pPICZB (control) cell lines for each ribotoxin treatment tested (Figs. 4–6). The initial doses of 15-AcDON tested are generally equivalent to those found during *Fusarium* infection of agricultural crops. For example, intracellular levels of DON have been reported at concentrations ranging from 13.3 to 88.7 $\mu\text{g}\cdot\text{g}^{-1}$ within *F. graminearum*-inoculated cereal tissue (41, 42).

Attenuation of 15-AcDON activity clearly demonstrates that NAT-267 $V_{\text{H}}\text{H}$ intrabody expression conveys significant ($p = 0.01$) resistance to mycotoxin-specific cytotoxicity (Fig. 4) by regulating the availability of free and $V_{\text{H}}\text{H}$ -bound 15-AcDON. Consequently, results were dose-dependent as time to immunomodulation was optimal for 30 and 40 $\mu\text{g}\cdot\text{mL}^{-1}$ 15-AcDON, when compared with the lower (20 $\mu\text{g}\cdot\text{mL}^{-1}$) and higher con-

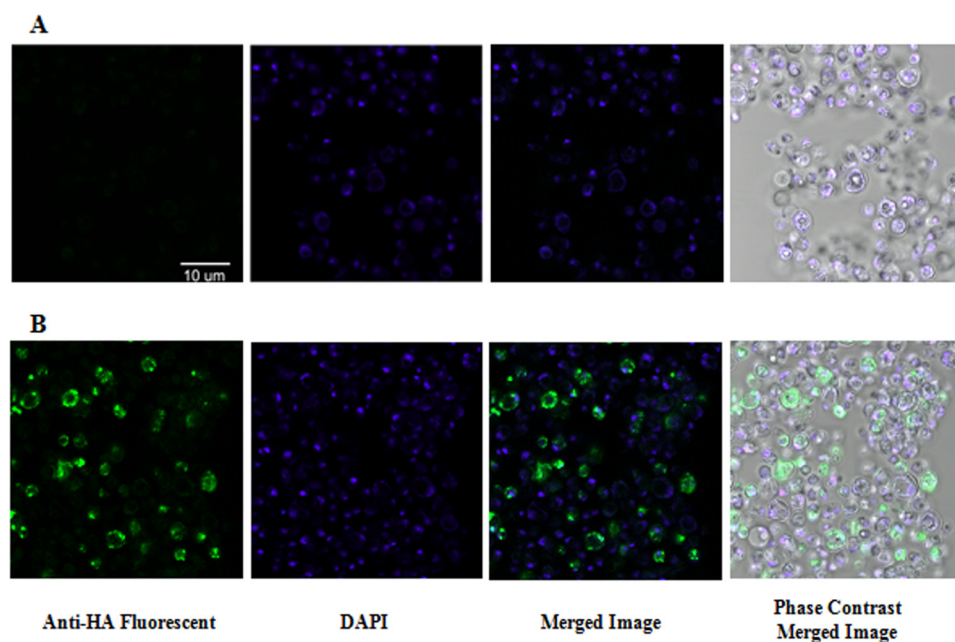


FIGURE 7. Representative confocal microscopy photographs of *P. pastoris* (KM71H) cells isolated, washed, and immunoprobed with anti-HA epitope monoclonal antibody-fluorescein isothiocyanate conjugate and 4',6-diamidino-2-phenylindole (DAPI) stain after growing in YPD media to $A_{600} = 20$ (OD_{600}). A, control, pPICZB empty vector. B, *P. pastoris* transformants expressing NAT-267 $V_{H}H$ intrabody shown by fluorescent green cells.

centrations (i.e. 50 and 100 $\mu\text{g}\cdot\text{ml}^{-1}$) of the toxophore. At 15-AcDON concentrations of 100 $\mu\text{g}\cdot\text{ml}^{-1}$, immunomodulation was substantially reduced and delayed, although at 200 $\mu\text{g}\cdot\text{ml}^{-1}$, there was no benefit associated with $V_{H}H$ expression (Fig. 4).

NAT-267 $V_{H}H$ intrabody immunomodulation was trichothecene-specific as no attenuation of 15-AcDON toxicity was observed in assays supplemented with control ribotoxin (cycloheximide) (Fig. 5). It was not possible to assess the effect of NAT-267 $V_{H}H$ on the cytotoxicity of 3-AcDON because all doses tested were not toxic to *P. pastoris* cells (Table 1). Further confirmation of NAT-267 $V_{H}H$ specificity was shown with cytotoxicity assays using *P. pastoris* transformants expressing a control $V_{H}H$ intrabody, i.e. B-24. Furthermore, there was no immunomodulation of either 15-AcDON- or cycloheximide-specific cytotoxicity when pPICZB control cells were used (Fig. 6).

The attenuation of DON cytotoxicity at 100 and 200 $\mu\text{g}\cdot\text{ml}^{-1}$ (Fig. 5) was an unexpected result because we previously reported that NAT-267 $V_{H}H$ has no affinity for DON (18). *In vivo* immunomodulation of DON was explained by confirmation of biotransformation of DON to 15-AcDON in *P. pastoris* cell lysate and culture supernatant samples of NAT-267 and pPICZB transformants (supplemental Table S2 and Fig. 8). Data mining of the genome of NRRL Y-11430 *P. pastoris* (the parent strain of KM71H) by Integrated Genomics Inc. (Chicago) revealed no similarities or related homologs to a previously characterized fungal gene responsible for acetylation of DON to 15-AcDON (43). We therefore attributed this biochemical conversion to a previously uncharacterized acetyltransferase gene within the *P. pastoris* genome.

To predict the impact of NAT-267 $V_{H}H$ intrabody binding to reduce effective 15-AcDON toxin concentrations within the

cytosol, we adopted a previously described (44) mass-balance model based on Equation 1.

$$[\text{bound toxin}] = \frac{[V_{H}H] \times [\text{total toxin}]}{([V_{H}H] + K_D)} \quad (\text{Eq. 1})$$

We assumed a linear, dose-independent binding of $V_{H}H$ to 15-AcDON within an aqueous environment, where 1 g = 1 ml. The dissociation constant (K_D) of NAT-267 $V_{H}H$ was taken as 1.24 μM (18) with 1:1 stoichiometry of Ab:antigen binding. NAT-267 ($[V_{H}H]$) concentration within the *P. pastoris* cytosol was estimated to be 138 $\mu\text{mol}\cdot\text{liter}^{-1}$ (supplemental Table 2). We assumed optimal disulfide bond formation and post-translational $V_{H}H$ folding within the endoplasmic reticulum and cytosol (45) with no NAT-267 $V_{H}H$ intrabody leakage from the *P. pastoris* cell membrane.

The concentration of 15-AcDON within the cytosol was taken as equivalent to culture media, and we also assumed that there were no trichothecene targets outside of the cells.

Based on total 15-AcDON concentration of 50 $\mu\text{g}\cdot\text{ml}^{-1}$ (or 148 μM) (Fig. 4), this model predicts $(138 \times 148 \mu\text{M}) \div (138 + 1.24 \mu\text{M}) = 147 \mu\text{M}$ 15-AcDON (or >99.9%) toxin was bound, and $\sim 1.3 \mu\text{M}$ (or <0.1%) of 15-AcDON was free. This simple model indicates that 15-AcDON cytotoxicity at 50 $\mu\text{g}\cdot\text{ml}^{-1}$ should be completely eliminated by expression of NAT-267 $V_{H}H$; however, although the effects of 15-AcDON were significantly ameliorated by $V_{H}H$ expression at this concentration, they were not completely eliminated (Fig. 4).

A more complete assessment of NAT-267 $V_{H}H$ immunomodulation should also account for *in situ* competition effects and binding affinity between 15-AcDON and its major cellular target, i.e. ribosome binding. Binding affinity is also an important consideration because NAT-267 $V_{H}H$ has a relatively low affinity ($K_D = 1.24 \mu\text{M}$) for 15-AcDON that is mediated by weak, noncovalent interactions (e.g. van der Waals forces, hydrogen bonding, etc.) (46). Due to the rapid dissociation rate constant for NAT-267, we hypothesize that 15-AcDON is subject to continuous turnover in terms of mycotoxin binding to the $V_{H}H$. In other words, the efficiency of NAT-267 $V_{H}H$ in terms of limiting the cytotoxicity of 15-AcDON is limited by the short "residence time" of hapten binding to the $V_{H}H$ (47). Further evidence for this hypothesis resides in the fact that even when the $V_{H}H$ intrabody is expressed in excess (138 $\mu\text{mol}\cdot\text{liter}^{-1}$; Table 2) compared with 15-AcDON, i.e. at 89 μM (30 $\mu\text{g}\cdot\text{ml}^{-1}$), NAT-267 cannot fully immunomodulate the cytotoxic effects of 15-AcDON when compared with controls (Fig. 4). Thus, at higher doses of $\geq 30 \mu\text{g}\cdot\text{ml}^{-1}$ 15-AcDON, we postulate that the $V_{H}H$ simply cannot compete with a stronger

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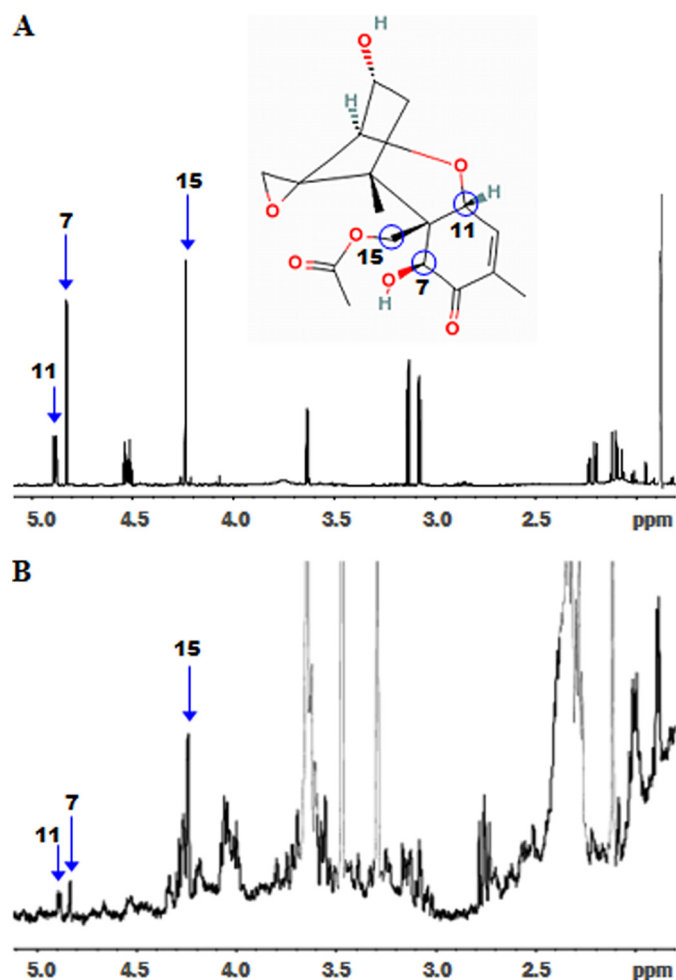


FIGURE 8. A, representative 500-mHz ^1H NMR spectrum of 15-AcDON standard. B, HPLC fraction of *P. pastoris* cell lysate sample (24 h). Unique chemical shifts of 15-AcDON are labeled due to H-11 (4.87 ppm), H-7 (4.81 ppm), and H-15 (4.21 ppm) protons, which confirm the presence of 15-AcDON within the cell lysate.

and quite possibly longer residence time of 15-AcDON on the 60 S ribosomal protein subunit L3 (Rpl3) of *P. pastoris*.

We also assert that, at very high doses, 15-AcDON may cause other potentially irreversible effects such as membrane disruption, inhibited RNA and DNA synthesis, and various other apoptotic effects (reviewed in Refs. 4–6), which may also severely limit cellular growth. Thus, to accurately determine the efficacy of NAT-267 $V_{\text{H}}\text{H}$ intrabody in *P. pastoris* cells, one must account for the dynamic nature of mycotoxin-mediated cytotoxicity and *in vivo* binding kinetics of 15-AcDON for other cellular binding targets. Our assertion that attenuation of trichothecene-specific cytotoxicity is not a simple process is in agreement with previous work that demonstrated only partial remediation of DON cytotoxicity based on *in planta* expression of an altered Rpl3 target protein (11, 12) or the expression of *Tri101* (14, 36).

Most phytopathogenic *Fusarium* species are believed to produce 15-AcDON or 3-AcDON as acetylated precursors to DON (15). Production of each acetylated compound has been described as geography-dependent; 3-AcDON chemotypes dominate in Asia and Europe, although *Fusarium* species, which produce 15-AcDON, are more prevalent in North Amer-

ica (48, 49). Therefore, the accumulation of DON within plant tissue is believed to be a metabolic process conferred by fungal and plant carboxylesterases that continuously deacetylate either 15-AcDON or 3-AcDON within plant tissue (15). Confirmation of NAT-267 efficacy observed in this work is very significant because 15-AcDON was the most cytotoxic compound tested. If 15-AcDON is as toxic to plants as it is to yeast, then expressing NAT-267 $V_{\text{H}}\text{H}$ within the cytosol before trichothecene accumulation may help limit *in vivo* pathogenesis and metabolism to DON during *Fusarium* infection of plants such as corn and wheat.

Future experiments will focus on the development of an anti-15-AcDON $V_{\text{H}}\text{H}$ with an improved dissociation constant (K_D) to ensure a longer association between the $V_{\text{H}}\text{H}$ and target ligand for improved *in vivo* efficacy (47). It would also be of great interest to develop and test novel $V_{\text{H}}\text{H}$ fragments with affinity for various other trichothecenes (e.g. neosolaniol, diacetoxyscripenol, T-2 toxin, etc.) and various other mycotoxin classes (e.g. fumonisins, aflatoxins, etc.) using this test system. A logical subsequent application would be constitutive *in planta* expression of optimized mycotoxin-specific $V_{\text{H}}\text{H}$ fragments, possibly with catalytic activity, to bind and deactivate/degrade mycotoxins during critical initial periods of plant pathogenesis.

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