Intracellular Expression of a Single Domain Antibody Reduces Cytotoxicity of 15-Acetyldeoxynivalenol in Yeast*

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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 (VHH) with specificity and affinity for 15-AcDON was expressed in the methylotrophic yeast Pichia pastoris. Cytotoxicity and VHH 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 VHH within P. pastoris conveyed significant (p = 0.01) resistance to 15-AcDON cytotoxicity at doses ranging from 20 to 100 μg/ml. We also documented a biochemical transformation of DON to 15-AcDON to account for the attenuation of DON cytotoxicity at 100 and 200 μg/ml. The proof of concept established within this eukaryotic system suggests that in planta VHH expression may lead to enhanced tolerance to mycotoxins and thereby limit Fusarium infection of commercial agricultural crops.

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. 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), 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

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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., V₁H) from the camelidae heavy chain IgG subfamily are among the smallest functional recombinant antibody (Ab) fragments at 14–15 kDa. V₁H 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). V₁H 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 V₁H 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 V₁H intrabody fragments and anticipated sensitivity to 15-AcDON. This system was established as a "proof of concept" to demonstrate that intrabody expression of recombinant V₁H fragments could impart a novel means of mycotoxin-specific resistance.

EXPERIMENTAL PROCEDURES

V₁H Genes—NAT-267 V₁H 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 Xbal-cloning sites at the respective 5’ and 3’ ends (GeneArt™, Toronto, Ontario, Canada). A nonspecific V₁H gene (B-24) isolated from a hyper-immunized phagemid llama with confirmed nonspecificity for 15-AcDON, or any other trichothecene mycotoxin, was used as a V₁H intrabody control. Both V₁H fragments were of the same immunoglobulin family and had similar molecular weights and isoelectric points. Like NAT-267, B-24 V₁H DNA was PCR-amplified to include 3’ HA and His₆ epitope tags and restriction cloning sites EcoRI and XbaI with epitope tags, as well as EcoRI- and XbaI-cloning sites at the smallest functional recombinant antibody (Ab) fragments (16, 17). V₁H 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).

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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 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 Zeocin to ensure pure clonal isolates.

Induction of V₁H Expression—Single colonies of V₁H 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 V₁H 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 V₁H (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 V₁H 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. V₁H 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. V₁H proteins were visualized with ECL Plus Western blotting detection system (GE Healthcare). Transformants with the highest V₁H 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 96-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 (Ulltident, 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 \( V_{14}H \) 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 (DAUC) 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 \( V_{14}H \) transformants. DAUC values for each toxophore treatment were calculated based on addition of differences in cellular growth between \( V_{14}H \) 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. \( V_{14}H \) lysate samples \((500 \mu l)\) 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 \( V_{14}H \) recombinant Ab fragment of a precisely defined concentration, and of similar size to NAT-267 and B-24 \( V_{14}H \) with C-terminal HA and His6 epitope tags, was used to establish a standard dilution series at final protein concentration \((1.3 \mu g/ml). Cells were observed after 5 min using a Carl Zeiss LSM 510 Meta confocal microscope equipped with diode 488-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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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 μg·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 mycoxin addition and were used to measure A 600 and culture pH. Samples were pelleted (3500 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 H₂O and passed through a 0.8-μm filter (Millipore). Filtrates were passed through Chromosep C₁₈ columns (C₁₈ Sep-Park cartridge, Waters) and pre-washed with 10 ml of 100% (w/v) HPLC grade methanol and 10 ml of double distilled H₂O. 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 N₂. Lysate samples and subsequent HPLC fractions of those samples were obtained on a Bruker AM 500 NMR 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 CDCl₃. Chemical shifts are referenced to residual CHCl₃ 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 μg·ml⁻¹) (Table 1).

Transformation and V₄H Intrabody Expression

P. pastoris KM71H cells were successfully transformed with linearized NAT-267 (treatment) or B-24 (control) V₄H 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 V₄H 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 V₄H 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 V₄H in P. pastoris cells within cytotoxicity assays of 15-AcDON (Fig. 4) was 4.01 ± 0.31 amol of V₄H·cell⁻¹ (Table 2). Accounting for an estimated mean P. pastoris cytosol volume of 29 fL·cell⁻¹ (as previously described), initial expression of NAT-267 V₄H was equivalent to an intracellular concentration of 138 ± 10.8 μmol of V₄H·liter⁻¹. Likewise, mean NAT-267 intrabody concentra-
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TABLE 1
Sensitivity of *P. pastoris* pPICZB empty vector transformants to ribotoxin treatments tested relative to toxin-free DMSO media

| Dose (μg ml⁻¹) | Time to doubling of initial A₆₂₀ cellular growth (min) |
|---------------|-------------------------------------------------------|
|               | 15-AcDON | DON | 3-AcDON | Cycloheximide | L.S.D.² |
| 0³           | 205 (2.2) | 221 (14.4) | 219 (21.7) | 221 (14.4) | 14.5 |
| 20           | 322 (4.4) | ND  | ND      | ND            | ND      |
| 30           | 384 (4.8) | 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         |

¹ L.S.D. means least significant difference (p = 0.05).
² Ribotoxin-free DMSO media were used.
³ ND means not determined.

ΔAUC analysis was used to quantify relative differences in cellular growth between NAT-267 and B-24 (control) V₄₄H transformants. The largest ΔAUC value (5.25) corresponded to NAT-267 V₄₄H-expressing *P. pastoris* grown with 15-AcDON at 30 μg ml⁻¹. Cytotoxicity assays based on 15-AcDON at 20, 40, and 50 μg ml⁻¹ had similar ΔAUC values (4.48, 4.50, and 4.45, respectively) (Fig. 4). Accordingly, assays at 100 μg ml⁻¹ produced a substantially lower ΔAUC value (1.20), whereas NAT-267 V₄₄H intrabody expression conferred no immunomodulation when spiked with 200 μg ml⁻¹ 15-AcDON (Fig. 4).

**DON, 3-AcDON, and Cycloheximide—**NAT-267 V₄₄H intrabody expression resulted in a dose-dependent response to DON. Although no beneficial impact on cellular growth was observed in cultures supplemented with 50 μg ml⁻¹ DON, NAT-267 V₄₄H expression resulted in significantly improved cellular growth at 100 and 200 μg ml⁻¹ DON relative to pPICZB control transformants (Fig. 5A). Comparative differences in growth were greater for yeast spiked with 100 relative to 200 μg ml⁻¹ DON (ΔAUC values = 2.11 and 1.68, respectively). In addition, time to significant differences in A₆₂₀ values was several hours later for the higher dose (Fig. 5A).

Differences in cellular growth between NAT-267 V₄₄H 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 V₄₄H and pPICZB transformants spiked with doses of 50, 100, and 200 μg ml⁻¹ (Fig. 5B).

**B-24 V₄₄H Control—**Expression of B-24 control V₄₄H 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 V₄₄H intrabody expression was trichothecene-specific, as *Pichia* cells expressing a nonspecific V₄₄H intrabody were equally as sensitive as pPICZB control transformants.

**V₄₄H Immunolocalization**

NAT-267 V₄₄H intrabody expression and distribution within *P. pastoris* transformants were further validated with fluores-
Detection of the HA tag on NAT-267 VHH 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$_{14}H$ 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$_{14}H$ transformants (Fig. 5A). To validate this observation and reconfirm in vivo antigen specificity of NAT-267 V$_{14}H$ 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$_{14}H$ 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 within the samples tested (data not shown). Finally, the $^1$H 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...
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 VHH 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 methylotrophic 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 cytotoxicity.

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**TABLE 2**

Mean VHH 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. VHH concentration values are expressed in femtograms and attomoles of VHH per cell, as well as micromoles/liter VHH equivalent within *P. pastoris* cytosol. Standard errors for all means (*n* = 9) are shown in parentheses.

| VHH gene | Cytotoxicity assay | VHH intrabody concentration |
|----------|--------------------|-----------------------------|
|          |                    | fg cell⁻¹ | amol cell⁻¹ | μmol 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) |

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**FIGURE 5.** Cytotoxicity assays showing the effect of various concentrations (50–200 μg ml⁻¹) of DON and cycloheximide on the growth of *P. pastoris* pPICZB and NAT-267 VHH transformants as measured by absorbance at 620 nm (OD₆₂₀). ΔAUC values for NAT-267 VHH 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.
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 \textit{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 \textit{V}_{14}H intrabody expression, \textit{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 synthesis, it was an obvious necessity to induce \textit{V}_{14}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 \textit{V}_{14}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 \textit{V}_{14}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 \textit{V}_{14}H intrabody fragments were well expressed within \textit{P. pastoris}. Average protein expression levels (Table 2) were found to be much higher than previously reported in \textit{P. pastoris} (38–40). Furthermore, images generated by confocal immunomicroscopy confirmed that NAT-267 \textit{V}_{14}H fragments were well distributed within the cytosol of \textit{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 \textit{V}_{14}H intrabody fragments would effectively reduce (i.e. immunomodulate) bioavailable 15-AcDON concentrations within the cell and thereby limit cellular toxicity, thereby resulting in enhanced growth of \textit{P. pastoris}. \textit{V}_{14}H-mediated mycotoxin binding was assessed by time-to-significant immunomodulation and \( \Delta \text{AUC} \) values between \textit{V}_{14}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 	extit{Fusarium} infection of agricultural crops. For example, intracellular levels of DON have been reported at concentrations ranging from 1.3 to 88.7 \( \mu \text{g g}^{-1} \) within \textit{F. graminearum}-inoculated cereal tissue (41, 42).

Attenuation of 15-AcDON activity clearly demonstrates that NAT-267 \textit{V}_{14}H intrabody expression conveys significant (\( p < 0.01 \)) resistance to mycotoxin-specific cytotoxicity (Fig. 4) by regulating the availability of free and \textit{V}_{14}H-bound 15-AcDON. Consequently, results were dose-dependent as time to immunomodulation was optimal for 30 and 40 \( \mu \text{g ml}^{-1} \) 15-AcDON, when compared with the lower (20 \( \mu \text{g ml}^{-1} \)) and higher concentrations.
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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 μg·ml⁻¹ (or 148 μM) (Fig. 4), this model predicts (138 × 148 μM) / (138 + 1.24 μM) = 147 μM 15-AcDON (or >99.9%) toxin was bound, and ~1.3 μM (or <0.1%) of 15-AcDON was free. This simple model indicates that 15-AcDON cytotoxicity at 50 μg·ml⁻¹ should be completely eliminated by expression of NAT-267 V₄₁H; however, although the effects of 15-AcDON were significantly ameliorated by V₄₁H expression at this concentration, they were not completely eliminated (Fig. 4).

A more complete assessment of NAT-267 V₄₁H 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 V₄₁H has a relatively low affinity (Kᵦ = 1.24 μM) 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 V₄₁H. In other words, the efficiency of NAT-267 V₄₁H in terms of limiting the cytotoxicity of 15-AcDON is limited by the short “residence time” of hapten binding to the V₄₁H (47).

Further evidence for this hypothesis resides in the fact that even when the V₄₁H intrabody is expressed in excess (138 μmol·liter⁻¹; Table 2) compared with 15-AcDON, i.e. at 89 μM (30 μg ml⁻¹), NAT-267 cannot fully immunomodulate the cytotoxic effects of 15-AcDON when compared with controls (Fig. 4). Thus, at higher doses of ≥30 μg·ml⁻¹ 15-AcDON, we postulate that the V₄₁H simply cannot compete with a stronger

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**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 A₆₀₀ = 20 (OD₆₀₀). A, control, pPICZB empty vector; B, *P. pastoris* transformants expressing NAT-267 V₄₁H intrabody shown by fluorescent green cells.

Centrations (i.e. 50 and 100 μg·ml⁻¹) of the toxophore. At 15-AcDON concentrations of 100 μg·ml⁻¹, immunomodulation was substantially reduced and delayed, although at 200 μg·ml⁻¹, there was no benefit associated with V₄₁H expression (Fig. 4).

NAT-267 V₄₁H 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 V₄₁H on the cytotoxicity of 3-AcDON because all doses tested were not toxic to *P. pastoris* cells (Table 1). Further confirmation of NAT-267 V₄₁H specificity was shown with cytotoxicity assays using *P. pastoris* transformants expressing a control V₄₁H 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 μg·ml⁻¹ (Fig. 5) was an unexpected result because we previously reported that NAT-267 V₁₁H 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 V₄₁H 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.

\[
\text{[bound toxin]} = \frac{[V_{41}] \times [\text{total toxin}]}{([V_{41}] + K_D)} \quad \text{(Eq. 1)}
\]

We assumed a linear, dose-independent binding of V₄₁H to 15-AcDON within an aqueous environment, where 1 g = 1 ml. The dissociation constant (Kᵦ) of NAT-267 V₄₁H was taken as 1.24 μM (18) with 1:1 stoichiometry of Abantigen binding. NAT-267 ([V₄₁H]) concentration within the *P. pastoris* cytosol was estimated to be 138 μmol·liter⁻¹ (supplemental Table 2). We assumed optimal disulfide bond formation and post-translational V₄₁H folding within the endoplasmic reticulum and cytosol (45) with no NAT-267 V₄₁H intrabody leakage from the *P. pastoris* cell membrane.
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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$_{H}$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 tri-chothecene-specific cytotoxicity is not a simple process is in agreement with previous work that demonstrated only partial 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$_{H}$H within the cytosol before tri-chothecene 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$_{H}$H with an improved dissociation constant ($K_d$) to ensure a longer association between the V$_{H}$H target ligand for improved *in vivo* efficacy (47). It would also be of great interest to develop and test novel V$_{H}$H fragments with affinity for various other trichothecenes (e.g. neosolaniol, diacetoxyscirpenol, 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 expression of optimized mycotoxin-specific V$_{H}$H fragments, possibly with catalytic activity, to bind and deactivate/degrade mycotoxins during critical initial periods of plant pathogenesis.

**FIGURE 8.** A, representative 500-mHz $^1$H 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.

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