

## Assessing interactions of binary mixtures of *Penicillium* mycotoxins (PMs) by using a bovine macrophage cell line (BoMacs)

Se-Young Oh<sup>a,\*</sup>, Nina Cedergreen<sup>b</sup>, Alexandros Yiannikouris<sup>c</sup>, H.V.L.N. Swamy<sup>d</sup>, Niel A. Karrow<sup>a,\*</sup>

<sup>a</sup> Department of Animal Biosciences, Ontario Agriculture College (OAC), University of Guelph, Guelph, ON N1G 2W1, Canada

<sup>b</sup> Department of Life Sciences, University of Copenhagen, Frederiksberg, Denmark

<sup>c</sup> Alltech Inc., Nicholasville, KY, USA

<sup>d</sup> Trouw Nutrition Pvt. Ltd. India, Karnataka State 560065, India

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### ABSTRACT

*Penicillium* mycotoxins (PMs) are toxic contaminants commonly found as mixtures in animal feed. Therefore, it is important to investigate potential joint toxicity of PM mixtures. In the present study, we assessed the joint effect of binary combinations of the following PMs: citrinin (CIT), ochratoxin A (OTA), patulin (PAT), mycophenolic acid (MPA) and penicillic acid (PA) using independent action (IA) and concentration addition (CA) concepts. Previously published toxicity data (i.e. IC<sub>25</sub>; PM concentration that inhibited bovine macrophage (BoMacs) proliferation by 25%) were initially analyzed, and both concepts agreed that OTA + PA demonstrated synergism ( $p < 0.05$ ), while PAT + PA showed antagonism ( $p < 0.05$ ). When a follow-up dilution study was carried out using binary combinations of PMs at three different dilution levels (i.e. IC<sub>25</sub>, 0.5 \* IC<sub>25</sub>, 0.25 \* IC<sub>25</sub>), only the mixture of CIT + OTA at 0.5 \* IC<sub>25</sub> was determined to have synergism by both IA and CA concepts with Model Deviation Ratios (MDRs; the ratio of predicted versus observed effect concentrations) of 1.4 and 1.7, respectively. The joint effect of OTA + MPA, OTA + PA and CIT + PAT complied with the IA concept, while CIT + PA, PAT + MPA and PAT + PA were better predicted with the CA over the IA concept. The present study suggests to test both IA and CA concepts using multiple doses when assessing risk of mycotoxin mixtures if the mode of action is unknown. In addition, the study showed that the tested PMs could be predicted by IA or CA within an approximate two-fold certainty, raising the possibility for a joint risk assessment of mycotoxins in food and feed.

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### 1. Introduction

*Penicillium* mycotoxins (PMs) have been shown to cause a wide range of toxic effects in animals. Nephrotoxicity and hepatotoxicity are the most common clinical signs reported in animals fed PM-contaminated feed (Braunberg et al., 1992; Dickens and Jones, 1965; Sansing et al., 1976). Some studies have also reported immunomodulatory effect of PMs (Al-Anati and Petzinger, 2006; Ferrante et al., 2008; Herzog-Souares

and Freire, 2004; Oh et al., 2015). For example, our previous studies determined that PMs can differentially affect various macrophage biological activities, such as proliferation, viability, reactive oxygen production (ROS) production, phagocytosis as well as the gene expression of epigenetic enzymes and cytokines (Oh et al., 2012, 2013). The macrophage is one of the key regulators of immune system (Kelsall, 2008; Wynn et al., 2013), and therefore, changes in the function of macrophages could potentially predispose animals to secondary diseases (Wynn et al., 2013).

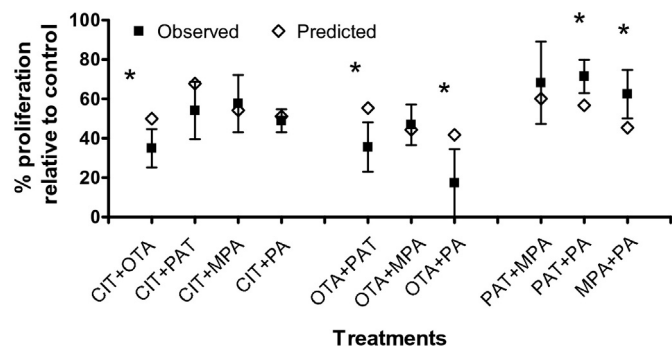
Until now, most toxicity studies involving mycotoxins have focused on toxicity of individual mycotoxins. However, contaminated animal feeds are in reality usually contaminated with various combinations of mycotoxins. Mansfield et al. (2008) for example, reported that >50% of maize silage collected from 30 different Pennsylvania dairies between 2001 and 2002 contained more than one mycotoxin. Therefore, the potential risk of exposure to mycotoxins may be underestimated when only assessing single mycotoxin presence and toxicity, and not their joint effect.

In the context of PMs, various interactions have been reported. Specifically, citrinin (CIT) and penicillic acid (PA) kidney and liver toxicity have been shown to increase in mice as well as embryotoxicity in chicks

**Abbreviation:** 95% CI, 95% confidence limit; BoMacs, bovine macrophage cell line; CA, concentration addition; CIT, citrinin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IA, independent action; IC<sub>25</sub>, concentration that inhibit 25% cell proliferation; IC<sub>50</sub>, concentration that inhibit 50% cell proliferation; MDR, Model Deviation Ratios (MDRs), the ratio of predicted versus observed effect concentrations of IC<sub>50</sub>s extrapolated from toxicity curves; MPA, mycophenolic acid; OTA, ochratoxin A;  $P_A$  or  $P_B$ , observed proportion proliferation of a individual PM; PA, penicillic acid; PAT, patulin;  $P_{PM}$ , predicted proportion proliferation of a PM mixture; PM, *Penicillium* mycotoxin;  $P_{OM}$ , observed percent proliferation of a PM mixture; RPMI 1640, Roswell Park Memorial Institute 1640 media.

\* Corresponding authors at: Centre for Genetic Improvement of Livestock (CGIL), Department of Animal Biosciences, Department of Toxicology, University of Guelph, Guelph, Ontario N1G 2W1, Canada.

E-mail addresses: [ohs@uoguelph.ca](mailto:ohs@uoguelph.ca) (S.-Y. Oh), [nkarrow@uoguelph.ca](mailto:nkarrow@uoguelph.ca) (N.A. Karrow).



**Fig. 1.** The comparison between  $P_{OM}$  (observed) and  $P_{PM}$  (predicted) of PM mixtures at their respective IC25s by using the IA approach from the previous proliferation data (Oh et al., 2012). Clear squares indicate the mean of 'predicted' proliferation from the PM mixtures. Black squares represent the mean of 'observed' proliferation, and the error-bars are 95% CI.

when these animals were co-exposed with ochratoxin A (OTA) (Sansing et al., 1976; Veselá et al., 1983). There is no other previously reported case of mycotoxin interaction on immune parameters. However, using bovine macrophages (BoMacs), we previously found potential synergistic interactions of binary PM mixtures at their respective IC25s, the concentration of PM that inhibited BoMac proliferation by 25% (Oh et al., 2012). BoMacs that were used in previous studies and this study originated from bovine macrophages isolated from the peritoneal region along the intestinal gut lining (Stabel and Stabel, 1995). Peritoneal macrophages may come into contact with mycotoxins even before detoxification occurs within the liver and kidney, which makes these cells a biologically relevant cell population to consider the effect of mycotoxins in addition to the liver and kidney. In this study, proliferation was used since BoMac proliferation was the only endpoint to be commonly affected by the following PMs, CIT, OTA, patulin (PAT), mycophenolic acid (MPA) and PA. The previous proliferation study, however, was lacking in both experimental and statistical design to more precisely quantify interactions between PMs. Specifically, conclusions were solely based on single concentration data and therefore, did not take into account binary interactions at different PM concentrations (Meadows et al., 2002; Oh et al., 2012).

There are several different ways of determining interaction of compounds in a mixture. For all methods, mixture toxicity data is compared to a predicted mixture effect that assumes no interactions between chemicals (Berenbaum, 1981; Cedergreen et al., 2008; Stork et al., 2007). Two basic toxicity concepts for estimating the combined effects of mixtures include independent action (IA) and concentration addition (CA) (Cedergreen et al., 2008; Kjaerstad et al., 2010; Kortenkamp and Altenburger, 1998). The IA concept assumes that compounds in a mixture have a completely independent mode of action that affects a common endpoint, while the CA concept assumes that compounds have a similar mode of action (Cedergreen et al., 2008); both concepts assume that the chemicals do not interact.

The application of IA or CA concepts determines the predicted additive effect from individual exposure data (Cedergreen et al., 2008). The comparison between the predicted additive effect and the observed joint effect provides a statistical means of distinguishing interactions of synergism and antagonism among PM mixtures. Previously, we assessed the observed effect of binary PM mixtures at their respective IC25s at their sub-lethal levels (Oh et al., 2012). The previous study described the interaction of the PM mixtures at a single concentration, but it did not investigate interactions of mixtures at other concentrations.

Therefore, in the present study, the mixture toxicity concepts were applied to re-analyze the proliferation data from the previous Oh et al. (2012) study, and were then used to re-examine potential interactions of binary mixtures of PMs at three different dilution levels ( $1C_{25}$ ,  $0.5 * IC_{25}$ , and  $0.25 * IC_{25}$ ) to test the interactions. Since the mode of action of these PMs on the proliferation of BoMacs is currently unknown, both IA and CA concepts were applied to predict joint effects of PM mixtures from the Oh et al. (2012) study, and were then compared to the results from the present dilution study.

## 2. Methods

### 2.1. Data analysis and plotting of the previous PM toxicity data

The previous proliferation data (Oh et al., 2012) were analyzed using IA and CA concepts (Figs. 1 and 2). The mixture data was plotted together with predicted effects based on concentration-response data of the individual chemicals.

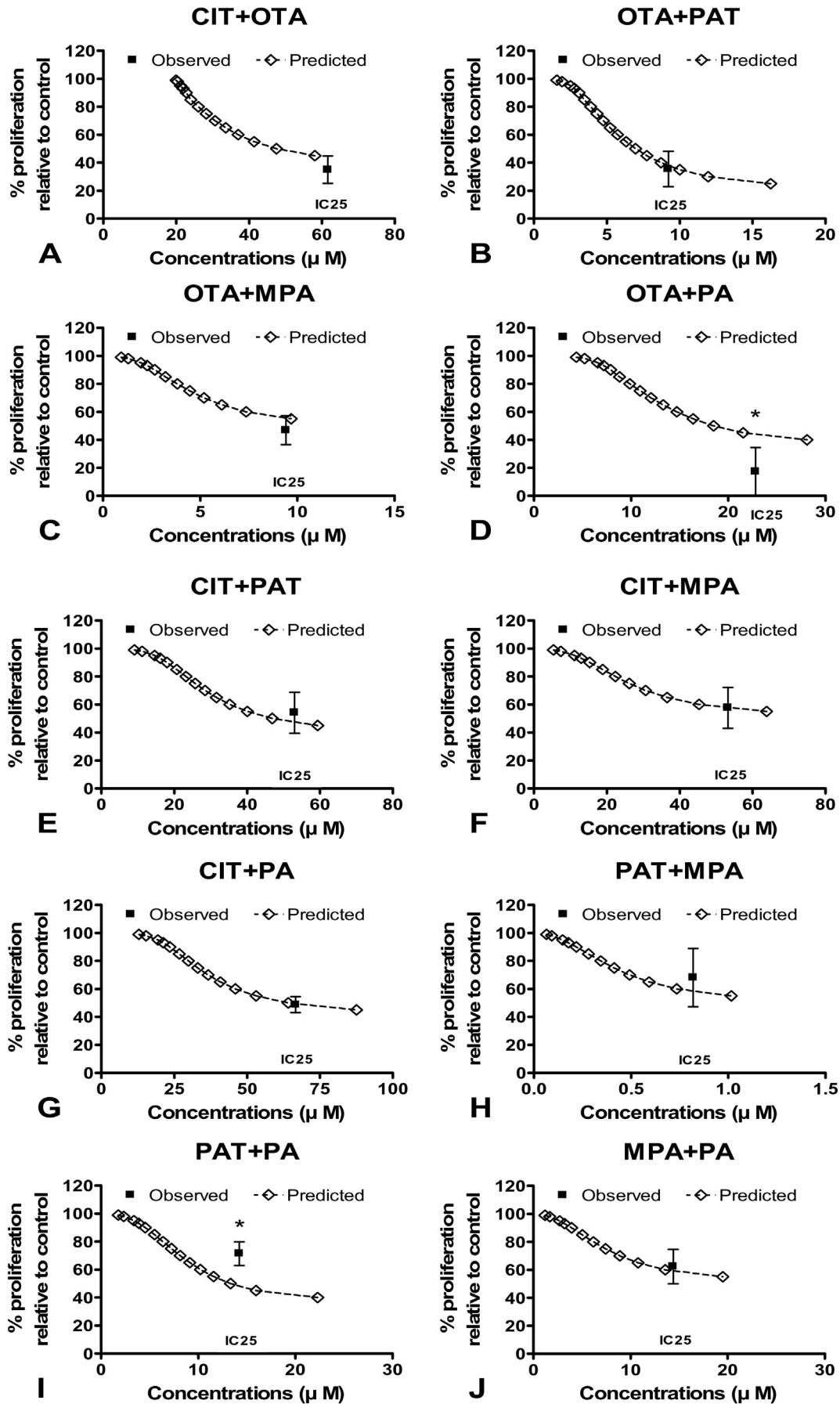
Based on the assumptions of the IA concept, the effect of the binary PM mixture was predicted from the observed effect of the individual PM exposure data using the following equation (Jørgensen, 2013):  $P_{PM} = P_A * P_B$ , where  $P_{PM}$  is the predicted proliferation of the administered mixture relative to the control treatment, and  $P_A$  and  $P_B$  are the relative proliferation of the individual PMs. The observed and predicted relative proliferation from the mixture ( $P_{OM}$ ) and  $P_{PM}$  were plotted in Fig. 1. If the predicted cell proliferation fell within the 95% confidence limits (CI) of the observed cell proliferation, then the assumption of additivity was accepted, and PMs within the mixture were said to have an additive effect. In contrast, when the mixture prediction was outside of the 95% CI of  $P_{OM}$ , then significant interaction between two PMs occurred ( $p < 0.05$ ) as either synergistic ( $P_{PM}$  being located above 95% CI of  $P_{OM}$ ), or antagonistic ( $P_{PM}$  being located below 95% CI of  $P_{OM}$ ).

For the CA concept, whole dose-response mixture predictions were constructed from the cell proliferation data of individual PM (Oh et al., 2012). The effect concentrations (ECs) for each proliferation response (e.g. the concentrations that inhibited BoMacs proliferation by 99%... 1%) were calculated from the curves giving the best fit, including biphasic curves using SigmaPlot 13 (Systat, CA, USA). To calculate a mixture CA curve from several curves with different slopes, the predicted curve is calculated from several EC concentrations. The mixture prediction for each effect concentration were then calculated by  $1 / (\frac{pA}{EC_{CA}^A} + \frac{pB}{EC_{CA}^B})$ , where A and B represent different PMs with p being the proportion of the individual PM in the mixture ( $\Sigma(pA + pB) = 1$ ) (Ohlson et al., 2010). The predicted data points were connected with a curve and plotted together with  $P_{OM}$  including 95% CI (Figs. 1 and 2). Significant deviations between observed and predicted data were evaluated as above, with predictions falling outside the 95% CI being considered as either synergistic ( $P_{PM}$  being located above 95% CI of  $P_{OM}$ ) or antagonistic ( $P_{PM}$  being located below 95% CI of  $P_{OM}$ ).

### 2.2. Assessing the interactions of PMs at a range of concentrations

**2.2.1. Cell preparation.** The BoMacs, provided by Stabel and Stabel (1995), were cultured in Roswell Park Memorial Institute (RPMI) in 1640 medium, supplemented with 2.0 mM L-glutamine, 10% heat inactivated fetal bovine serum (FBS), 100 unit/ml of penicillin, 100  $\mu$ g/ml of streptomycin, 0.25  $\mu$ g/ml of amphotericin B, and 25 mM HEPES buffer. All cell culture products were purchased from Invitrogen, Canada. Cells were incubated at 37 °C with 5% CO<sub>2</sub> in a 75 cm<sup>2</sup> flask, and were grown to above 80% confluence prior to use for the study.

**Fig. 2.** (A–J). The comparison between theoretical curve of  $P_{PM}$  (predicted) and  $P_{OM}$  (observed) of PM mixtures at their respective IC25s by using CA approach from the previous proliferation data (Oh et al., 2012). Clear diamond with dotted lines indicate the mean of observed mean of 'predicted' proliferation from PM mixtures. Black squares represent the observed proliferation from IC25 mixtures of PMs, and the error-bars are 95% CI.



**2.2.2. BoMacs exposure to mycotoxins.** BoMacs ( $1.0 \times 10^4$ ) were seeded into 96-well flat-bottom plates, centrifuged at 100g for 2 min and incubated at 37 °C with 5% CO<sub>2</sub> for 2 h until the cells completely adhered to the bottom of the plates. The BoMacs were then exposed to individual PMs and binary combinations of PMs at three dilutions: IC25, 0.5 \* IC25 and 0.25 \* IC25. The IC25-values for the single compounds were: CIT (52.72 μM), OTA (8.91 μM), patulin (PAT; 0.32 μM), mycophenolic acid (MPA; 0.50 μM) and PA (13.90 μM) as determined by [Oh et al. \(2012\)](#). These concentrations of PMs were previously determined to only inhibit cell proliferation, but no effect on the cell death when exposed to the BoMacs individually or in combination as a binary mixture for 48 h.

The IC25s of individual mycotoxins were exposed to BoMacs to calculate  $P_{PM}$ , and the mixtures of IC25s were exposed to BoMacs to obtain  $P_{OM}$ . All PMs were ordered from Sigma, Aldrich, U.S.A., and dissolved in ethanol prior to use. After 47 h of PM exposure, a cell proliferation standard was prepared by seeding another batch of BoMacs ( $1.0 \times 10^5$ ,  $7.5 \times 10^4$ ,  $5.0 \times 10^4$ ,  $2.5 \times 10^4$ ,  $1.0 \times 10^4$  and  $5.0 \times 10^3$  cells per well) in each plate, centrifuging at 100g for 2 min, then incubating for 1 h at 37 °C with 5% CO<sub>2</sub>.

The proliferation assay was carried out as described previously ([Oh et al., 2012](#)). The media was removed from all wells by blotting, and the plates were frozen in a -80 °C freezer for at least 24 h to lyse cells. The number of cells was estimated using a commercially available cell proliferation kit (Invitrogen, ON, Canada) that uses CyQUANT® GR dye (excitation 494/emission 517 nm) to label nucleic acids. The fluorescence intensity was measured with a 1420 Victor<sub>2</sub> Multi-label Counter (PerkinElmer, U.S.A.).

**2.2.3. Data and statistical analyses.** All raw data were normalized to cell number based on the standard curve of each plate, and presented as percent cell proliferation compared to the control. For the IA concept,  $P_{PM}$  was predicted from the individual PM proliferation data at their IC25, 0.50 \* IC25 and 0.25 \* IC25-values, which were done at the same time as  $P_{OM}$ . For the CA concept, whole dose-response curves for the binary mixtures were constructed from the individual PM proliferation curves generated by [Oh et al. \(2012\)](#) as described above. The difference between  $P_{OM}$  and  $P_{PM}$ , indicated as “observed and predicted”, respectively in [Figs. 3 and 4](#), for each respective dilution were evaluated using the 95% CI of  $P_{OM}$  in comparison to the mean of  $P_{PM}$ . If the mean of predicted proliferation overlaps with 95% CI of observed proliferation, then it indicates there is no significant interaction between two PMs. If the means of predicted proliferation falls above or below the 95% CI of observed proliferation, then it indicates a significant synergism or antagonism, respectively ( $p < 0.05$ ). (\*) denoted significant differences at  $p$ -value  $< 0.05$  ([Figs. 3 and 4](#)). Model Deviation Ratios (MDRs; [Belden et al., 2007](#)) for each binary mixture from the present data were calculated from the ratio of predicted versus observed effect concentrations of IC50s extrapolated from toxicity curves.

### 3. Results

The proliferation data from the previous [Oh et al. \(2012\)](#) study were re-analyzed based on single concentration of IC25s and plotted using the IA and CA concepts to determine the interactions between binary PM mixtures. From the IA, the mixtures of OTA with CIT, PAT, or PA showed significant synergism, where the observed inhibition effect on BoMacs proliferation was 1.4, 1.6 and 2.4-fold higher than the predicted effect, respectively. In contrast, the mixtures of PA with PAT or MPA showed significant antagonism, showing 0.8 and 0.7-fold lower observed effect compared to the predicted effect on proliferation, respectively ( $p < 0.05$ ; [Fig. 1](#)). From the CA, OTA + PA was the only mixture that showed a synergistic interaction with a 2.5-fold higher observed effect than predicted effect of BoMac proliferation ( $p < 0.05$ ; [Fig. 2D](#)). PAT + PA showed an antagonistic interaction with 1.5-fold lower observed effect than the predicted effect ( $p < 0.05$ ; [Fig. 2I](#)).

In the follow-up dilution study, mixtures of three different dilutions were tested for each binary mixture of PMs ([Fig. 3](#)). Both IA and CA concepts confirmed that CIT + OTA exhibited significant synergistic inhibition of BoMacs proliferation at the 0.5 \* IC25 mixture ( $p < 0.05$ ), but not for IC25 or 0.25 \* IC25 ([Fig. 3A and 4A](#)). The MDRs at their IC50s were predicted to be 1.4 and 1.7 using IA and CA, respectively.

The IA concept confirmed the antagonism between MPA and PA for IC25s and 0.25 \* IC25s levels with MDR of 0.7 ( $p < 0.05$ ; [Fig. 3J](#)). The IA concept also showed antagonism between CIT + MPA and CIT + PA at their IC25s and 0.5 \* IC25s ( $p < 0.05$ ; [Fig. 3F and G](#)) as well as PAT + MPA across all tested dilutions ( $p < 0.05$ ; [Fig. 3H](#)). The MDRs at their IC50s were 0.5 for both CIT + MPA and PAT + MPA, and 0.9 for CIT + PA combinations. The antagonism between PAT and PA was also confirmed for IC25 ( $p < 0.05$ ), but synergism occurred at 0.5 \* IC25 ( $p < 0.05$ ; [Fig. 3I](#)). For PAT and PA mixture, the MDR could not be calculated since the observed toxicity curve did not reach an IC50 in the present dilution study.

Synergism between OTA and PA was not observed using IA; however, CA demonstrated the synergism between these two PMs at IC25 and 0.25 \* IC25 with a MDR of 1.1 at their IC50s ( $p < 0.05$ ; [Fig. 4D](#)). In contrast to the IA results from [Oh et al. \(2012\)](#) study, the CA concept also revealed synergism between CIT + MPA at IC25 and 0.25 \* IC25 ( $p < 0.05$ ; [Fig. 4F](#)), as well as OTA + MPA and MPA + PA across all tested dilutions, with MDRs of 2.0, 2.0 and 3.3, respectively ( $p < 0.05$ ; [Fig. 4C and J](#)). CIT + PAT showed antagonism at 0.5 \* IC25 with a MDR of 0.8 when analyzed using CA concept ( $p < 0.05$ ; [Fig. 4E](#)).

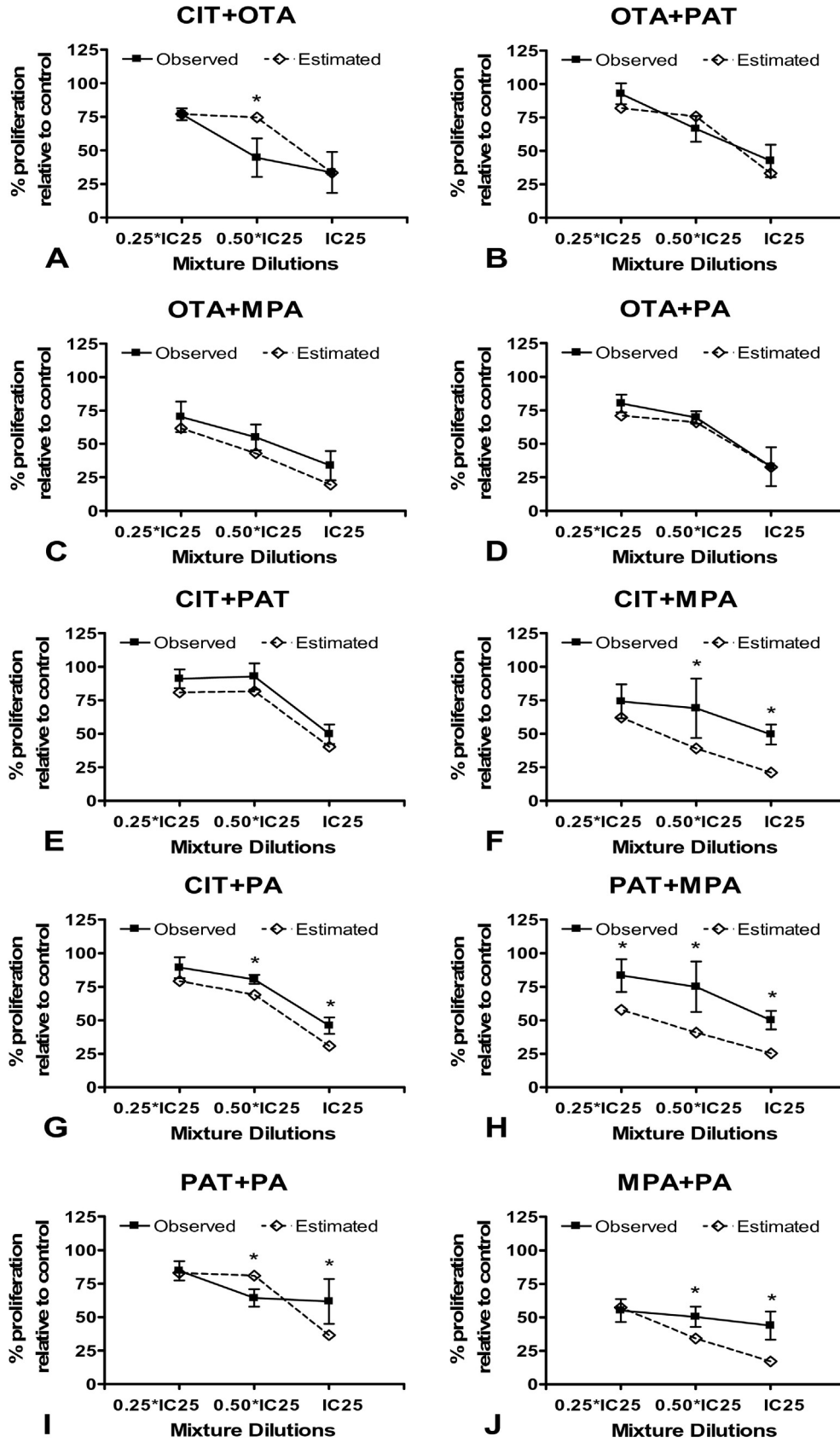
### 4. Discussion

When the BoMacs proliferation data from the study of [Oh et al. \(2012\)](#) were reanalyzed using the IA and CA concepts, both approaches showed that the OTA + PA mixture exhibited synergism with up to 2.5-fold difference between observed and predicted values, whereas PAT + PA had an antagonistic effect on proliferation. Synergism between OTA and PA has also been reported in other studies. [Sansing et al. \(1976\)](#) described synergistic toxicity of this mixture combination on murine liver and kidney tissues, and [Shepherd et al. \(1981\)](#) observed significant synergistic nephrotoxicity along with increased mortality rate in an in vivo feeding study with mice consuming both OTA and PA compared to OTA or PA alone ([Shepherd et al., 1981](#)). Considering that some common mold species in grain, such as *Penicillium viridicatum* and *Aspergillus ochraceus*, can produce both OTA and PA ([Bacon et al., 1973](#); [Sansing et al., 1976](#)), co-occurrence of the toxins is very likely to occur in animal silage ([Oh et al., 2015](#)). Assessing the risk of joint exposure to these two mycotoxins, therefore needs to take into account their potential synergism, if an approximate two-fold difference is considered in the overall risk assessment.

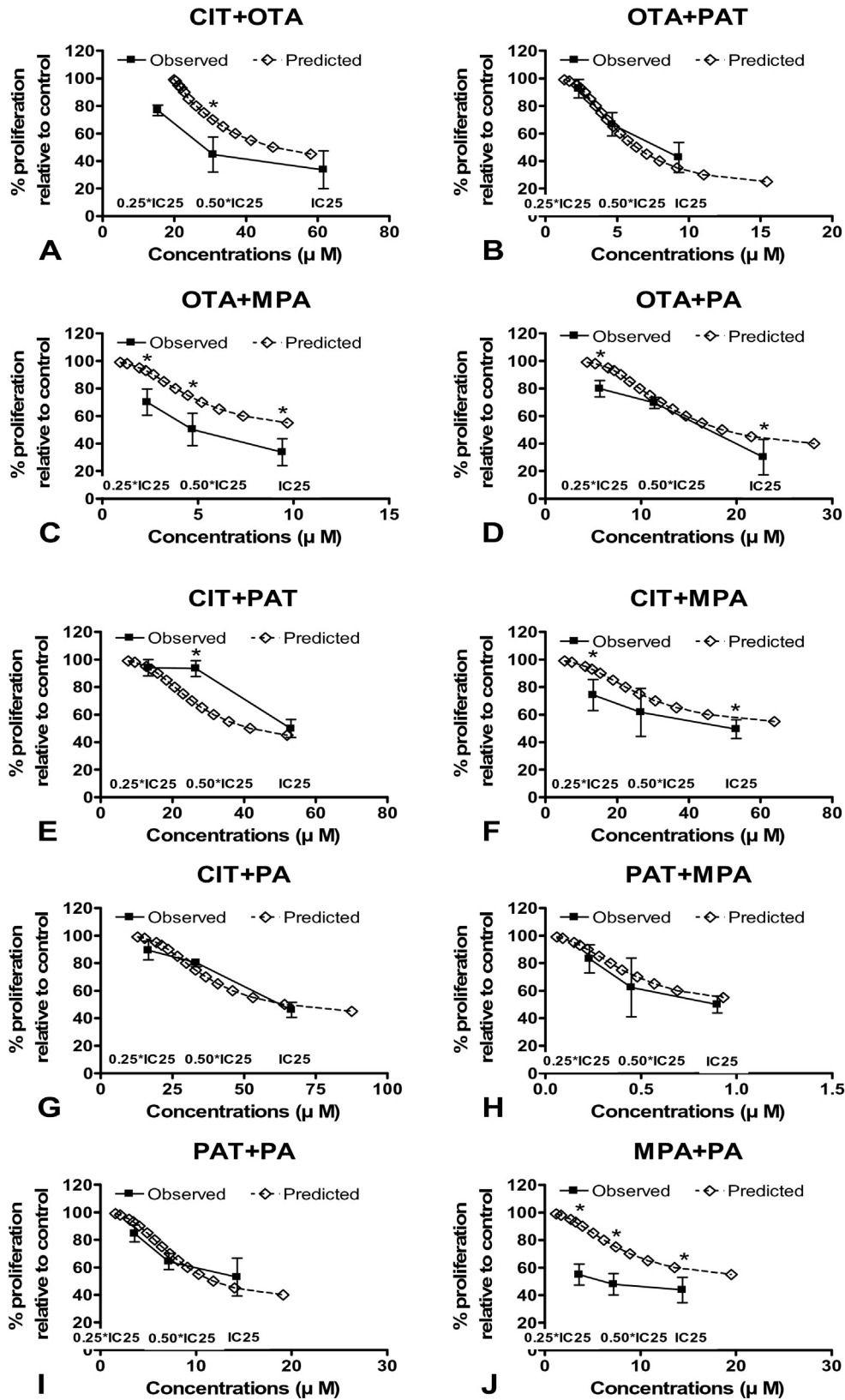
The PAT + PA mixture from [Oh et al. \(2012\)](#) study was consistently identified as antagonistic based on both IA and CA concepts with 0.8- and 0.7-fold differences for IA and CA concepts, respectively ([Table 1](#)). We are unaware of PAT + PA interactions being previously reported.

When we previously investigated these PM mixtures, synergism was defined solely based on significant difference between the observed effects of a mixture and its corresponding individual PMs using a Tukey's test ([Oh et al., 2012](#)). This simple statistical test only determined a significant joint effect of PM mixtures, and there was no clear way to define synergism, additivity and antagonism of the mixtures. In contrast, both IA and CA concepts allowed us to calculate the predicted effect of a mixture, allowing us to easily distinguish between synergism, additivity and antagonism of a mixture. In addition to this, the current application of both concepts instead of using either one of the approaches individually appeared to reduce the risk of false positives for PM interactions.

When IA and CA toxicity concepts were used to analyze the data from the present titration study, the results differed in comparison to the re-analyzed results from the [Oh et al. \(2012\)](#) study ([Table 1](#)). Also,



**Fig. 3.** (A–J). The comparison between  $P_{OM}$  (observed) and  $P_{PM}$  (predicted) of PM mixtures at three different dilutions of IC25s by using IA approach. The mixture of IC25s, the first dilution of IC25 mixtures and the second dilution of IC25s are denoted as 'IC25', '0.5 \* IC25' and '0.25 \* IC25' in Fig. 3, respectively. Clear squares with dotted lines indicate the mean of 'predicted' proliferation from PM mixtures. Black squares with continuous lines represent the mean of observed proliferation with 95% CI.



**Fig. 4.** (A–J). The comparison between theoretical curve of  $P_{PM}$  (predicted) and  $P_{OM}$  (observed) of PM mixtures at three different dilutions of IC25s by using CA approach. The mixture of IC25s, the first dilution of IC25s mixtures and the second dilution of IC25s are denoted as 'IC25', '0.5 \* IC25' and '0.25 \* IC25' in Fig. 4. Clear squares with dotted lines indicate the mean of 'predicted' proliferation from PM mixtures. Black squares with continuous lines represent the mean of observed proliferation with 95% CI.

it was more common to see antagonism with IA than CA, whereas CA reached conclusion more commonly toward synergism. Previous reviews of the reproducibility of mixture studies have shown that small

deviations from the reference model (<two-fold) are difficult to reproduce and therefore might not be of great biological importance (Cedergreen et al., 2007, 2008). It has therefore been recommended

**Table 1**

The summary of the mycotoxin mixtures analyzed with either IA or CA approaches. Figs. 1 and 2 are reanalyzed data from the previous proliferation study (Oh et al., 2012). Figs. 3 and 4 are analyzed data from the present study. IC25s, 0.50 \* IC25s, and 0.25 \* IC25s represent binary combinations of PMs at three different dilution levels. IC25s indicate the binary mycotoxin mixture at their IC25s; 0.50 \* IC25s indicate the binary mycotoxin mixture at their two-fold dilution of IC25s; and 0.25 \* IC25s indicate the binary mycotoxin mixture at their four-fold dilution of IC25s. 'S' denotes the synergism, and 'A' denotes the antagonism.

Concepts Used	Data source (Oh et al., 2012)		Analyzed by using the recent data						Complying model
	IA (Fig. 1)	CA (Fig. 2)	IA (Fig. 3)			CA (Fig. 4)			
Concentration	IC25s	IC25s	0.25 * IC25s	0.5 * IC25s	IC25s	0.25 * IC25s	0.5 * IC25s	IC25s	
CIT + OTA	S			S			S		Interaction
OTA + PAT	S								IA, CA
OTA + MPA						S	S	S	IA
OTA + PA	S	S				S		S	IA
CIT + PAT							A		IA
CIT + MPA				A	A	S		S	N/A <sup>a</sup>
CIT + PA				A	A				CA
PAT + MPA			A	A	A				CA
PAT + PA	A	A		S	A				CA
MPA + PA	A			A	A	S	S	S	N/A <sup>a</sup>

<sup>a</sup> More data is required to make any conclusion.

that a MDR limit be set at >2 or <0.5 for synergistic and antagonistic interactions, respectively, to exclude false positivity (Belden et al., 2007; Cedergreen, 2014).

Regardless of their potential interactions indicated by either toxicity concept with MDR in the present titration study, 7 out of 10 PM mixtures were considered to have no significant interaction since they were in compliance with either of the two concepts tested in this study as shown in Table 1 (Cedergreen et al., 2008). The observed curves for the following mixtures (3 out of 10), including OTA combined with either MPA and PA as well as CIT + PAT, complied with the estimated curves by IA. In contrast, the joint toxicity of the PM mixtures (3 out of 10), including CIT + PA, PAT + MPA and PAT + PA appeared to be better represented by CA, since their observed curves complied well with the estimated curves. The observed curve of OTA + PAT complied with the predicted curve by both IA and CA concepts, even though it appears to be more suited with CA concept.

Although the joint toxicity of most PM mixtures were well characterized with either IA or CA concepts, there were three combinations: CIT + OTA, CIT + MPA and MPA + PA, where their joint toxicity could not be described with either concept. The MDR values for CIT + OTA were calculated to be 1.4 and 1.7 for IA and CA concepts at their IC50s, respectively, which did not reach a recommended MDR limit defining interaction. However, it can't be ignored that the synergism for CIT + OTA was observed with both concepts. Therefore, it seems reasonable to conclude that the mixture of CIT + OTA is likely to have a true synergistic interaction, at least with respect to BoMacs proliferation.

It is worth noting that the concentration of OTA that was implicated in synergism in this study (4.5 μM) was determined to be much higher than the biological circulating concentrations of OTA (estimated to be 0.025 μM) from in vivo studies (Blank et al., 2003; Höhler et al., 1999). This suggests that the chance of finding synergism between CIT and OTA may be low in animal studies. However, OTA can potentially bioaccumulate in certain target tissues, such as kidney, intestine, and cutaneous fat (Galtier et al., 1981; Prelusky, 1986). Furthermore, it is also possible that synergism between CIT and OTA could occur at much lower concentrations in other tissues, such as liver, kidney, and embryos, which could be more sensitive to PMs than BoMacs (Sansing et al., 1976; Veselá et al., 1983).

Unlike the CIT + OTA mixture, the two other PM mixtures, CIT + MPA and MPA + PA, showed inconsistent results (Table 1). Hence, more data are needed to draw conclusions for these mixtures.

## 5. Conclusion

In the present study, IA and CA concepts were applied to determine the potential interactions among binary mixtures of PMs. Since the

interaction differed depending on the titration levels tested, it is important to test at least three dilutions or concentrations of mycotoxins to estimate the joint toxicity of mixtures mycotoxins if the mode of action is unknown. In the dilution study, we determined that the toxicity of most binary PM mixtures (7 out of 10) followed the additive behavior described by either IA or CA concepts, whereas only one PM mixture (CIT + OTA) showed synergistic interaction by both concepts. This study indicated that different PM mixtures follow different joint toxicity concepts, IA versus CA; therefore, there is a need for testing both IA and CA concepts for the risk assessment of PM mixtures when the mode of action is unknown. The present study may also be used for providing an informative initial screening to determine interactions of not only the ten PM mixtures tested in this study but also other mycotoxin mixtures prior to animal study to validate the joint toxicity behavior of PM mixtures in other target tissues and cells.

## Conflict of interest

The authors declare that there are no conflicts of interest.

## Transparency document

The Transparency document associated with this article can be found, in online version.

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