

## Relation of 8-Ketotrichothecene and Zearalenone Analog Structure to Inhibition of Mitogen-Induced Human Lymphocyte Blastogenesis†

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**The 50% effective doses of fusarenon X, nivalenol, deoxynivalenol, and 15-acetyldeoxynivalenol required to reduce [<sup>3</sup>H]thymidine uptake in mitogen-stimulated human lymphocytes by 50% were 18, 72, 140, and 240 ng/ml, respectively. These results indicated that lymphotoxicity of 8-ketotrichothecenes decreased according to the C-4 substituent order acetyl > hydroxyl > hydrogen, whereas acetylation of position C-15 of deoxynivalenol caused a slight decrease in in vitro toxicity. The 50% effective doses for zearalenone,  $\alpha$ -zearalenol,  $\beta$ -zearalenol,  $\alpha$ -zearalanol, and  $\beta$ -zearalanol were 3,500, 6,300, 36,000, 3,750, and 33,000 ng/ml, respectively, suggesting that a keto group or  $\alpha$ -hydroxyl at the position C-6' contributed to the lymphotoxicity of the parent molecule. The inhibitory effects of zearalenone analogs observed in the blastogenesis assay did not correlate with the estrogenic potencies of these compounds. All 8-ketotrichothecenes and zearalenone analogs tested were capable of inhibiting B- and T-cell subsets stimulated by a mitogen panel of leucoagglutinin, concanavalin A, and pokeweed mitogen.**

Mycotoxins of the 8-ketotrichothecene and zearalenone (ZEN) groups are commonly found in cereal grains produced in the United States, Canada, and Japan (13, 14, 21, 27, 30-33). Two predominant chemotypes exist among the fusaria which elaborate 8-ketotrichothecenes: (i) nivalenol (NIV) and fusarenon (FX) producers and (ii) deoxynivalenol (DON) and 3- or 15-acetyldeoxynivalenol (3- or 15-ADON) producers (12). While both chemotypes are encountered in Japan, the latter group appears most commonly in North America (12, 18, 20). Depending on environmental and nutritional conditions, both chemotypes can produce the estrogen ZEN.  $\alpha$ -Zearalenol ( $\alpha$ -ZEL) and  $\beta$ -zearalenol ( $\beta$ -ZEL) are ZEN metabolites and can occur simultaneously with ZEN in *Fusarium* cultures and in infected corn (4, 29).

Trichothecenes and estrogens have been shown to have deleterious effects on immune function (3, 8, 10, 11, 15-17, 23, 24). Because the 8-ketotrichothecenes and ZEN analogs enter the food chain, their immunotoxicity should be evaluated. Inhibition of lymphocyte blastogenesis is a valid in vitro model for determining the effect of environmental contaminants on immunocompetent cells from humans, and both DON and ZEN inhibit proliferation of phytohemagglutinin-stimulated peripheral human lymphocytes (1, 5). The purpose of this paper is to investigate the relationship of 8-ketotrichothecene and ZEN analog structure to inhibitory activity in the human lymphocyte blastogenesis assay.

DON was produced in culture and purified as described by Witt et al. (34). 15-ADON was produced in culture (34) and purified on a 10% (wt/vol) water-deactivated silica gel column, using toluene-ethyl acetate (1:3) as the elution solvent. Purity of DON and 15-ADON was verified by melting point, high-pressure liquid chromatography, and UV spectra (20, 34). FX and NIV standards were purchased from Romer Laboratories (Washington, Mo.). Diethylstilbestrol and

estradiol were purchased from the Sigma Chemical Co. (St. Louis, Mo.). ZEN,  $\alpha$ -ZEL,  $\beta$ -ZEL,  $\alpha$ -zearalanol ( $\alpha$ -ZAL), and  $\beta$ -zearalanol ( $\beta$ -ZAL) were kindly donated by M. Bachman (International Minerals and Chemical Corp., Terre Haute, Ind.). The ability of the various compounds to inhibit [<sup>3</sup>H]thymidine ([<sup>3</sup>H]TdR) incorporation in mitogen-stimulated lymphocytes was tested as previously described (9). To evaluate the effect of the toxin on various lymphocyte subsets, a panel of mitogens was used. These included leucoagglutinin (LA) (5.0  $\mu$ g/ml), which stimulates T cells; concanavalin A (10.0  $\mu$ g/ml), which stimulates a different T-cell subset than does LA; and pokeweed mitogen (10.0  $\mu$ g/ml), which stimulates primarily B cells with some T cells (25). Inhibition data were expressed as percentage of DNA synthesis in control lymphocytes that were stimulated by a mitogen in the absence of toxin.

For each 8-ketotrichothecene or ZEN analog tested, effective doses that caused 50% reduction (ED<sub>50</sub>) of [<sup>3</sup>H]TdR incorporation were very similar for all three mitogens (Table 1), indicating that these compounds were capable of inhibiting all of the B- and T-cell subsets stimulated by the mitogen panel. Average dose-response effects of the various 8-ketotrichothecenes on the blastogenesis assay are summarized in Fig. 1. In order of decreasing toxicity, ED<sub>50</sub>s (in nanograms per milliliter) were: FX, 18; NIV, 72; DON, 140; and 15-ADON, 240.

Trichothecenes inhibit DNA and protein synthesis by interaction with polyribosomes (19). 8-Ketotrichothecene concentrations required for inhibition of human lymphocytes blastogenesis were approximately 10-fold less than those required to inhibit cell-free protein synthesis (28). As found previously for T-2 toxin, a type A trichothecene (9), lymphotoxicity of the 8-ketotrichothecenes (type B trichothecenes) was dependent on the degree of acylation in substituent groups. Replacement of the position C-4 acetyl of FX with the hydroxyl in NIV resulted in an approximately fourfold decrease in toxicity, whereas replacement of the position C-4 hydroxyl of NIV with the hydrogen in DON

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TABLE 1. Inhibition of incorporation of [<sup>3</sup>H]TdR in mitogen-stimulated human lymphocytes by 8-ketotrichothecenes and ZEN analogs

Toxin	ED <sub>50</sub> (ng/ml)		
	LA (5 μg/ml)	Concanavalin A (10 μg/ml)	Pokeweed mitogen (10 μg/ml)
8-Ketotrichothecenes			
FX	16	18	21
NIV	94	78	48
DON	140	160	140
15-ADON	300	220	150
ZEN analogs			
ZEN	3,200	3,800	4,200
α-ZEL	>10,000 <sup>a</sup>	3,400	2,700
α-ZAL	5,300	2,300	3,000
β-ZEL	33,000	36,000	40,000
β-ZAL	50,000	18,000	26,000

<sup>a</sup> Higher concentrations of α-ZEL were not tested because of solubility limitations.

resulted in a further twofold decrease in toxicity. In an analogous manner, hydrolysis of T-2 toxin at the position C-4 acetyl to HT-2 toxin and hydrolysis of 3'OH T-2 toxin at the position C-4 acetyl to 3'OH HT-2 toxin decreases lymphotoxicity 2.3-fold and 12.5-fold, respectively (9). Substitution of the position hydroxyl C-15 of DON with the acetyl in 15-ADON resulted in a 1.7-fold decrease in in vitro toxicity. In contrast, replacement of the position C-15 hydroxyl of T-2 triol with the acetyl in HT-2 toxin resulted in a 43-fold increase in toxicity. This suggests that dependence of lymphotoxic activity on the C-15 substituent is very different for the type A and B trichothecenes. Although not determined in this study, it has been noted previously that acetylation of DON at position C-3 to 3-ADON results in an approximately fivefold decrease in lymphotoxicity (1), indicating that 3-ADON is less immunotoxic than 15-ADON. This is significant because 15-ADON rather than 3-ADON is

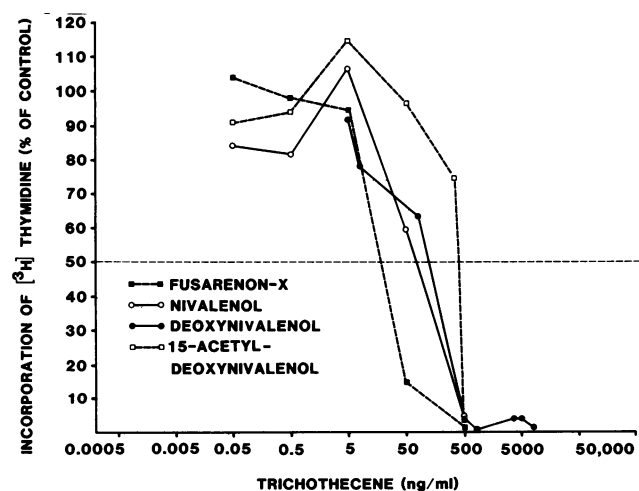


FIG. 1. Inhibition of [<sup>3</sup>H]TdR incorporation in mitogen-stimulated human lymphocytes by 8-ketotrichothecenes. Each point represents the mean inhibition in lymphocytes stimulated by LA, concanavalin A, and pokeweed mitogen performed in triplicate, or a total of nine determinations.

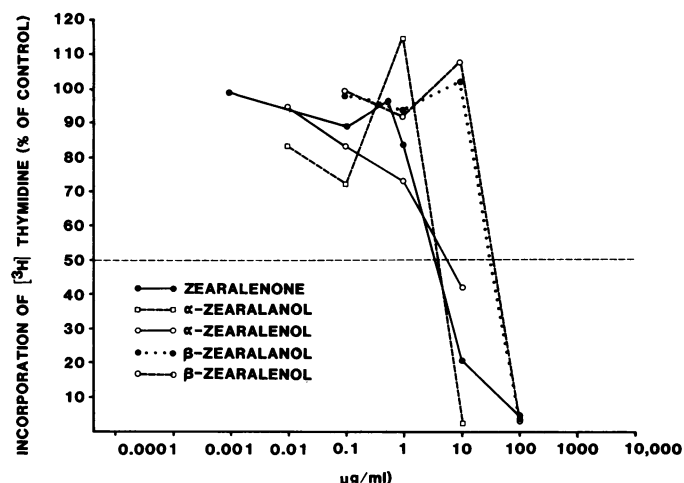


FIG. 2. Inhibition of [<sup>3</sup>H]TdR incorporation in mitogen-stimulated human lymphocytes by ZEN analogs. Data points calculated as described in the legend to Fig. 1.

typically coproduced with DON by *Fusarium* isolates from North America (6a, 20).

Fifty percent lethal doses (intraperitoneal) in mice for FX, NIV, DON, 3-ADON (28), and 15-ADON (unpublished data) are 3.4, 4.1, 70, 49, and 113 mg/kg, respectively. Both these data and ED<sub>50</sub>s for inhibition of human lymphocyte blastogenesis indicate that FX and NIV are more toxic than DON, 15-ADON, and 3-ADON. However, the relative in vivo and in vitro toxicities within these two trichothecene groups are not comparable. Assuming negligible contribution due to species variation, these differences are likely to be attributable to the fact that 50% lethal doses reflect the combined effect of absorption, metabolism, and toxin-target cell interaction, whereas the blastogenesis assay reflects only the toxin-target cell interaction. Thus, the in vitro data described herein might be most applicable to predicting direct trichothecene-lymphocyte interactions such as might occur between dietary DON and the gut-associated lymphoid tissue.

Dose-response effects of ZEN analogs are summarized in Fig. 2. ED<sub>50</sub>s (in nanograms per milliliter) were: ZEN, 3,500; α-ZEL, 6,300; β-ZEL, 36,000; α-ZAL, 3,750; and β-ZAL, 33,000. These compounds were much less toxic in the blastogenesis assay than were the 8-ketotrichothecenes, and the levels at which ZEN analogs had an effect are unlikely to be encountered physiologically. The presence of a single bond (ZEN) or double bond (ZAL) at positions C-1' and C-2' did not affect the toxicity of these analogs. However, keto (ZEN) or α-hydroxyl substituents (α-ZEL and α-ZAL) at position C-6' of the parent molecule resulted in 10-fold-higher toxicity than when a β-hydroxyl (β-ZEL and β-ZAL) was the C-6' substituent. Thus, metabolism of ZEN to α-ZEL would not decrease lymphotoxicity, while reduction to β-ZEL would result in detoxification of ZEN. Since DON and ZEN often occur simultaneously in nature (12, 18), the potential for synergism between these two toxins in the blastogenesis assay was tested by determining the dose-response effects of DON in the presence of an ED<sub>80</sub> concentration (1 μg/ml) of ZEN. The observation that inhibitory effects of DON were not enhanced by the presence of ZEN is consistent with the results of Atkinson and Miller (1).

Hypothetical reasons for inhibition of lymphoblastogene-

sis by estrogens include interaction with B- and T-cell binding sites (17), induction of adherent-cell suppressor activity (3), interference with glucocorticoid binding (22), and interaction with DNA polymerase (2, 7). ZEN analogs bind to cytoplasmic estrogen receptors according to the following rank order:  $\alpha$ -ZAL >  $\alpha$ -ZEL >  $\beta$ -ZAL > ZEN >  $\beta$ -ZEL. They have approximately 20 to 100 times less uterotrophic activity than does diethylstilbestrol or estradiol (26, 29). However, in the human lymphocyte blastogenesis assay, we found that diethylstilbestrol and estradiol had ED<sub>50</sub>s of 1,950 and 3,500 ng/ml, respectively, indicating that these estrogens were equivalent to or only slightly more toxic than ZEN or its  $\alpha$ -hydroxyl analogs. The inhibition of blastogenesis by ZEN analogs did not correlate to the estrogenic potencies of these compounds but rather exhibited a distinct structure-activity relationship. Future perspectives should therefore include understanding both the in vitro mechanism of action of ZEN and its analogs on lymphocyte function and the relation of this activity to in vivo immune function.

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