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Identification of novel metabolite and its cytotoxic effect on human lymphocyte cells in comparison to other mycotoxins

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ABSTRACT

A novel metabolite of *P. polonicum* was recently isolated from Cameroonian *Zea mays* by means of preparative silica gel thin layer chromatographic method. The potential cytotoxic effects of this novel compound was further tested, *in vitro*, against cultured human mononuclear lymphocyte cells over 18-hr period in comparison to ochratoxin A and T-2 toxin. At low concentrations (0.15, 0.31 and 0.63 µg/ml), the toxicity of the novel metabolite was similar to that of T-2 toxin, but was found to be significantly ($p<0.05$) less toxic than ochratoxin A. The increase of concentration levels of all investigated mycotoxins from 0.15 to 5.0 µg/ml significantly (R^2 =0.8; $p<0.001$) decreased the cell viability. These findings indicate that the newly identified compound has a toxic effect on lymphocyte cell viability and might be a potential health risk. Having in mind the circumstance, that the novel mycotoxin is frequently found in Cameroonian food commodities as well as in animal feeds from farms with nephropathy problems in Bulgaria, further studies are required on the impact of chronic dose administration of this mycotoxin on human and animal health.

Key words: Cytotoxicity, cell viability, T-2 toxin, ochratoxin A, nephropathy.

INTRODUCTION

Some groups of mycotoxins are toxic secondary metabolites produced by some fungi in agricultural products susceptible to mold infestation. Their production is unavoidable and depends on different environmental factors in the field and conditions of storage. Due to its unavoidable and unpredictable nature, mycotoxin contamination presents a unique challenge to food safety (Park, 2002). Only integrated approach to food safety, which includes systematic identification and assessment of hazards in foods and various control methods, could resolve the existing problems in this field. The moulds and mycotoxins have been associated with a variety of livestock diseases including ergotism, mycotoxic nephropathy in pigs and chicks, stachybotryotoxicosis, equine leukoencephalomalacia and many others

(Stoev, 2007). The lack of adequate evidences for the definitive relationship between mycotoxins and human disease does not necessarily imply that dietary exposure does not represent a potential risk (Stoev, 2007). In developing countries, many individuals are not only malnourished but are also chronically exposed to high levels of mycotoxins in their diet (Kyriakidis et al., 1981; Smedsgaard and Frisvad, 1996; Smedsgaard, 1997; Smedsgaard and Nielsen, 2004; Stoev, 2007).

It is also expected that mixtures of mycotoxins would have at least an additive, if not synergistic toxic effect. The presence of multiple toxins in various foods presents new concerns since toxicological information on the effects of simultaneous exposure is still very limited (Stoev, 2007). In a diverse human diet, exposure to multiple toxins at a low concentration on an intermittent rate over long periods of time can cause toxicological effect. The ultimate effect of this constant exposure is still unknown and has to be further investigated. The discovery of new mycotoxins and co-contamination of known mycotoxins is occurring also at a high rate (Stoev, 2007). Therefore, the present study looks into the toxic effect of some known mycotoxins in comparison to a novel metabolite of *Penicillium polonicum* isolated from Cameroonian human foods (Njobeh et al., 2009) as well as from feedstuffs known to provoke mycotoxic nephropathy in Bulgaria (Stoev, 2008a).

MATERIALS AND METHODS

Materials

All the reagents used were AR grade unless otherwise stated. The following materials and chemicals were also used: GeneAmp PCR System 9700, DYEamic ET Terminator Cycle Sequencing Kit, Dye terminator RR mix, GeneAmp PCR System 9700, Sephadex G-50 superfine columns, multiscreen HV plate MicroAmp Optical 96-well reaction plate and ABI PRISM 3700 Genetic analyzer, a Synapt HDMS Time-of-Flight mass spectrometer system equipped with Acquity-UPLC^{TM} Sample Manager, Sample Organizer and Photodiode Array (PDA) UV detector (Waters, Milford, USA), Microtitre 96-Well plates (Corning Cell Wells^{TM}, Corning, USA), DAS Microplate Reader (modello:A2; Rome, Italy), diphenyltetrazolium bromide (MTT) Assay Kit (Sigma, St Louis, USA), complete culture medium (RPMI-1640 supplemented with 10% FCS, 100 U/ml penicillin and 100 µg/ml streptomycin), haematocytometer (Neubauer), histopaque 1119 and 1077 (Sigma, St Louis, USA), phytohemagglutinin-p(PHA-p) (Sigma, Aldrich) and tetrazolium salt [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)].

Isolation of the novel metabolite

A *Penicillium* culture was isolated from Cameroonian *Zea mays*, grown on potato dextrose agar and its nucleic acid profile determined using an automated sequencer according to Samson et al. (2004). The isolate was further cultured on 100 ml broth yeast extract sucrose agar in a 500 ml conical flask and incubated at 25 °C for one month. Extraction of the metabolite was done by adding 200 ml methanol/water (60/40, v/v) and placed on a mechanical shaker for 1 hr. The entire content was filtered through a Whatman n° 2V filter paper and the content poured into a 500 ml separation funnel. The content was defatted (x3) using 50 ml iso-octane, extracted (x3) with 50 ml of dichloromethane by passing the bottom layer through a bed of Na_2SO_4 anhydrous into a 500 ml round bottom flask and dried by rotary evaporation. The content was then reconstituted with 200 µl DCM into a 0.5 ml screw-cap vial, dried by passing through a stream of N_2 gas with the vial placed on the heating block set at 50 °C. Vial containing the extract was then stored at 4 °C until further analyzed.

The novel metabolite obtained from *P. polonicum* extracts was purified by thin layer chromatography. To this effect, dried extract was re-dissolved in 1 ml DCM and banded about 10 mm from the edge of a preparative silica gel TLC plate (20 x 20 mm) and developed as a one-dimensional thin layer chromatogram. This was done repeatedly with alternating mobile phase (CEI and methanol/DCM) concentrations. After each run, the silica containing unidentified metabolite (light-green fluorescence) was scraped from the plate into a flask, suspended in acetone, filtered through Whatmann n° 1 filter paper and dried by rotary evaporation. The residue was reconstituted with 2 ml

DCM, transferred to 0.5 ml screw-cap vial and dried with a stream of N_2 gas. The UV spectrum of the novel metabolite was therefore obtained on a photodiode array detector using acetonitrile/water (30:70, v/v) as mobile phase pumped at a flow-rate of 1.0 ml/min and at wavelength set between 190 and 300 nm.

Cytotoxicity assay

The method of isolation and purification of peripheral blood mononuclear cells (mainly lymphocytes) followed by 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed according to the modified method of Meky et al. (2001). Five ml venous blood was individually collected from three healthy donors using a 15 ml sterile syringe and transferred immediately into a 10 ml heparin tube. This was mixed with an equal volume of tissue culture medium. From each donor, the content was then overlaid on Histopaque 1077 and centrifuged at 800 x g for 30 min and the interface layer consisting of mononuclear cells was carefully removed with a sterile pipette. The cells were washed trice with culture medium and each time centrifuged at 800 x g for 10 min. The cells were mixed with trypan blue, counted using a Neubauer haemocytometer and % viability was determined as: Cell/ml = n/v x dilution factor (5) x 10^4, Where: n = number of cells counted; v = area (number of big squares counted) x depth (0.1). Dilution factor = 2 (equal volume of cell suspension and trypan blue).

% cell viability = (viable cell counted/total number of cells) x 100.

To obtain a known mass of the purified novel metabolite, the content was re-dissolved in methanol, put in a known mass of 0.5 ml screw-cap vial, dried by passing through a stream of N_2 gas, cold in a dessicator and weighed. The mycotoxin concentrations were prepared using 0.1% DMSO (mycotoxin stock preparatory solvent), which was also used as control. 200 µl of stock solution each mycotoxin standard was used.

The cells having 98, 98.5 and 99% viability for each donor were separately put in a flask containing 100 ml culture medium, mixed gently and then incubated in a 5% CO_2-buffered and humidified incubator at 37 °C for 24 hrs. The 180 µl of cell suspension from each donor, stimulated with 10 µg/ml phyto-haemagglutinin-p (PHA-p), was put in each well (in triplicate) and the same cells were further exposed to 20 µl of individual mycotoxins at various concentrations (0, 0.15, 0.31, 0.63, 1.25, 2.5, 5.0 µg/ml). The content was then incubated for 18 hrs and cytotoxicity determined by MTT assay. In this case, 30 µl MTT solution previously prepared via dissolving MTT salt in 0.14 M phosphate bovine saline (PBS) (pH 7.4) at 5 mg/ml and filtered using a 0.22 µM pore size was added to each well and thoroughly mixed. This was then incubated for 3 hrs after which, to each well, 50 µl of 20% (w/v) sodium dodecyl sulphate/1 M hydrochloric acid solution was added and allowed to solubilise the formazan crystals formed for 6 hrs. The absorbance optical density (OD) was then read using a microplate reader at wavelengths of 540 and 620 nm. Cell viability as influenced by mycotoxin exposure was determined as % cell stimulation.

% cell stimulation = (ODM/ODN) x 100; Where ODM is the OD value of mycotoxin-treated PHA stimulated cells and ODN is the OD value of control (no mycotoxin) PHA-stimulated cells.

Statistical analysis

A one-way analysis of variance (ANOVA) was done using a pairwise multiple comparison procedures (Holm-Sidak method) on cytotoxicity data. A linear regression analysis was done on SigmaStat 3.5 for Windows (Systat Inc., 2006a). The data were then graphically represented using SigmaPlot for Windows Version 10.0 (Systat Inc., 2006b). Mean values among treatment groups were deemed to be different if the level of probability was ≤ 0.05.

RESULTS

Novel metabolite

Microbiological results confirmed that the *Penicillium* culture used in the present study was *P. polonicum* and the novel metabolite isolated from it was found to be light green under TLC. Further results obtained showed this metabolite produced a UV spectrum which shows the molecule can be detected at maximum wavelength of 204 nm (Figure 1). The spectrum also confirmed the purity of the molecule.

Cytotoxicity assay

It was found that the viability of lymphocyte cells, after 18 hrs incubation, was strongly influenced by both the type and concentration level of toxin when compared to the control. A significant mycotoxin-induced-cell mortality with dose increment was observed. Accordingly, the cell viability decreased ($P<0.001$) significantly with increasing concentration levels of all the mycotoxins tested in the study (Table 1 and Figure 2). For instance, increasing the concentration of T-2 toxin from 0.15 to 5.0 µg/ml significantly ($p<0.001$, $R^2=0.79$) decreased cell viability from 64.8 to 23.6% ($y = 26.9 + 0.9x$) (y is the cell viability and x is toxin concentration). In addition, increasing the level of novel metabolite from 0.15 to 5.0 µg/ml resulted in a significant ($p<0.001$, $R^2=0.79$) decrease in cell viability from 63.3 to 25.8% ($y = 34.0 + 0.7x$) and a similar trend of 55 to 12.4% ($p<0.01$, $R^2=0.80$) was observed for OTA ($y=18.9+0.8x$). In other words, T-2 toxin, novel metabolite and OTA exposures resulted in a dramatic decrease in cell viability by about 80, 74 and 87%, respectively. The lowest cell viability value (12.4%) was recorded when cells were exposed to 5.0 µg/ml of OTA, while cells were most viable (64.8%) when exposed to 0.15 µg/ml of T-2 toxin.

The cell viability was significantly ($P<0.05$) affected by the type of mycotoxin as the lowest % cell viability (mean 32.6±6.9) was seen in the presence of OTA and the highest % cell viability (mean 45.1±5.6) was seen in the presence of the novel compound. Comparing the concentrations of the separate mycotoxins tested resulted in a significant difference on cell viability at 5.0, 1.25 and 0.15 µg/ml ($p<0.05$) and 2.5 µg/ml ($p<0.001$). On the other hand, there was no significant difference between the viability of cells at exposure levels of 0.63 and 0.31 µg/ml concentrations.

DISCUSSION

Penicillium crustosum (Rundberget et al., 2004) and *P. polonicum* are common fungal species predominantly associated with food spoilage worldwide. Their metabolic profiles (production of roquefortine C, the penitrems) have been also investigated (Kyriakidis et al., 1981). The novel metabolite presented in this study, in addition to some other mycotoxins as ochratoxin A and fumonisin B_1 (Stoev et al., 1998; 2002), was also found in Bulgarian feed samples taken from farms with mycotoxic porcine nephropathy (unpublished results), which suggests its possible involvement in this nephropathy.

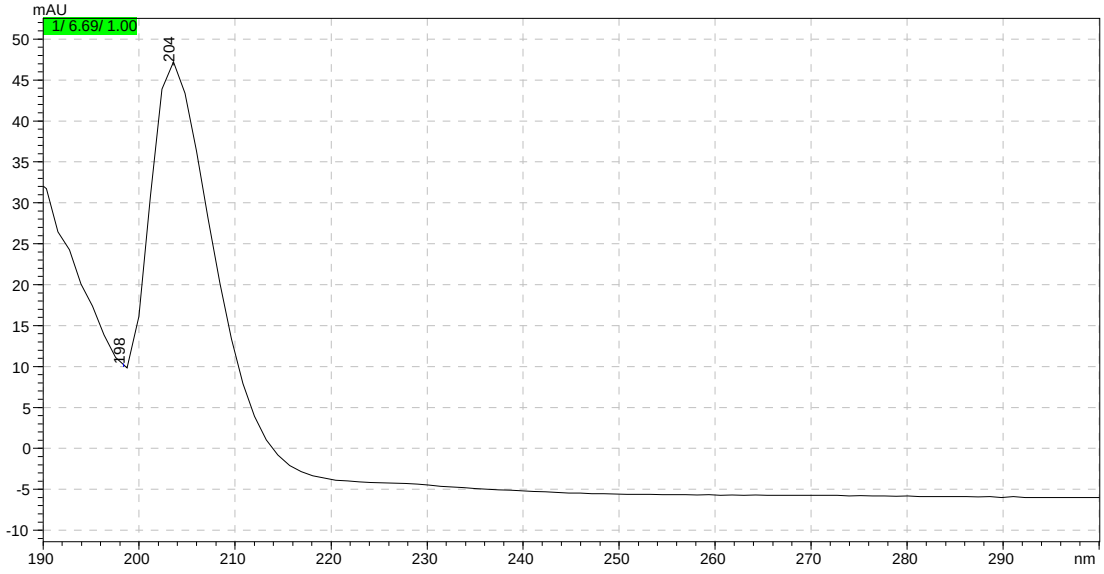

Figure 1: UV spectrum of Novel metabolite.

Table 1: Effects of mycotoxin level on cell viability of human lymphocytes.

Mycotoxin Level (µg/ml)	%Viability of cells treated with mycotoxins		
	T-2 Toxin	Novel Metabolite	Ochratoxin A
5.0	23.60 ± 2.83[a]	25.84 ± 1.95[a]	12.36 ± 1.95[a]
2.5	26.22 ± 1.63[a]	35.96 ± 2.25[b]	17.98 ± 0.65[ab]
1.25	31.09 ± 2.09[ab]	40.82 ± 1.98[bc]	27.72 ± 3.20[bc]
0.63	44.94 ± 8.48[abc]	47.57 ± 1.98[c]	33.33 ± 0.99[c]
0.31	54.31 ± 0.75[cd]	56.93 ± 0.38[d]	49.06 ± 4.00[d]
0.15	64.79 ± 1.98[d]	63.30 ± 0.99[e]	55.06 ± 2.34[d]
Probability Level	***	***	**

[a, b, c, d & e] Mean values in the same column not sharing the same superscript are significantly different ** p<0.01, *** p<0.001. Values within columns are means (left) and standard error of the mean (right). Linear equation of y=mx + c.

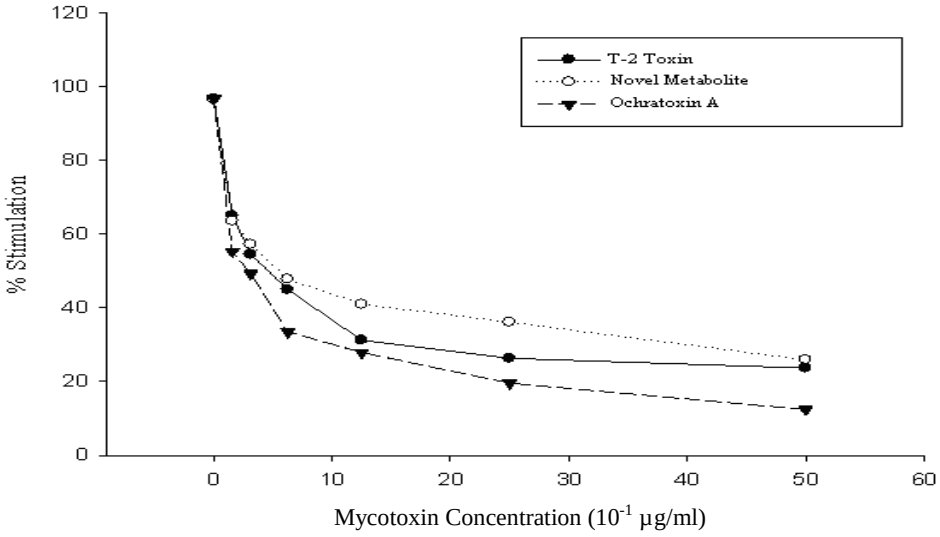

Figure 2: Influence of mycotoxin level on cell viability of human lymphocytes.

The importance of mycotoxins as food-borne contaminants implicated in human health disorders cannot be overemphasized. Human mycotoxin exposure by direct route is of great concern owing to the various health related problems associated with different mycotoxins, which can exacerbate with the increase of their food levels. One of the biological effects of mycotoxin intake by both animal and man is the generation of mycotoxin-induced apoptosis. Several studies have been performed to evaluate the effects of mycotoxin on cell viability and proliferation of mammalian lymphocytes or cell lines. Such data for T-2 toxin (Visconti et al., 1991; Meky et al., 2001), OTA (Lioi et al., 2004), FB_1 (Dombrink-Kurtzman et al., 1993) and AFB_1 (Meky et al., 2001) are well documented.

The tested metabolites in this study were able to provoke significant lymphocyte mortality (by decreasing cell viability). A direct inverse relationship between the level of mycotoxin exposure and cell viability of human lymphocytes was seen for the tested mycotoxins. These results are consistent with those obtained by Lioi et al. (2004) in studying the effects of OTA and ZEA on bovine lymphocytes. Exposure of cells to mycotoxin may cause cell injury and eventual cell death depending on the toxicological properties of the toxin in question, with additional variation among different animal

species (Smith and Moss, 1985; Piva and Fabio, 1999) when mycotoxins exhibit their toxicity.

Biological effects of mycotoxins vary depending on the type of mycotoxin (Al-azzawi et al., 1978) with some of them being more toxic than others as observed in the works of Lioi et al. (2004) and de Lorenzi et al. (2005). A similar trend was noted in this study, where, variation among toxins was observed when the cytotoxic potential of each of these compounds was compared. Ochratoxin A was found to be the most toxic of all of the tested mycotoxins at concentrations greater than 1.25 µg/ml with reduced cell viability of about 70%. Cell mortality was influenced by the quantity of mycotoxin used. Ochratoxin A (IARC, 1993) and T-2 toxin are among the mycotoxins classified as possible human carcinogens. Cell mortality recorded as a result of the mycotoxin exposure therefore may also lead to carcinogenesis in some cases (Dragon et al., 2001). It is therefore necessary, the novel metabolite be tested in further experiments in order to ascertain all possible toxicological effects for it may be carcinogenic, although this may depend on a number of other circumstances such as the ability to provoke direct mitogenesis, the regenerative response resulting from oncotic or apoptotic necrosis (Dragon et al., 2001; Timbrell, 2002), as well as the type of animal species.

The resultant loss of lymphocyte cells provoked by all of the mycotoxins tested in this study may cause immunosupression as indicated by Sharma (1993), since cell mortality is the initial step of immunomodulation in animal species (Forsell et al., 1985) thus favouring infections. It is known, that T lymphocytes play a pivotal role in the immune system by being responsible for immune response (Stormer and Lea, 1995), acting as a natural defence mechanism against host invasion of diseases. While significant variations were noted at some concentrations, the tested mycotoxins were found to have similar effects with regards to causing cell mortality. This could possibly be attributed to the rate of uptake of mycotoxins by cells. On the other hand, the rate of uptake according to Yiannikouris and Jouany (2002) depends on the gastrointestinal absorption and distribution of toxins, which occurs according to the polarity of the compound and active transport.

It is important to mention, that our results were in agreement with those earlier reported by Miljkovic et al. (2003). It has been found that administration of *P. polonicum* extract (not containing OTA or other known mycotoxins) to rats can provoke profound and persistent histopathological damages such as apoptic and karyomegalic or mitotic changes in the nuclei of tubular epithelium in kidneys of rats, including DNA-adducts formation (Miljkovic et al., 2003). The same *P. polonicum* strain, which is a common food/feed spoilage mould in warm temperate latitudes, has been found as a frequent contaminant in Bulgarian feeds suspected of causing spontaneous porcine nephropathy (Mantle and McHugh, 1993; Stoev et al., 1998). The same changes (apoptosis and karyomegaly in tubular epithelium), provoked by *P. polonicum* extract, possibly are induced by the same novel metabolite, which need to be further proven. This would be of great interest, because the same metabolite could be partly responsible for the nephrotoxic damages described in Bulgarian animal nephropathy (Stoev et al., 1998; 2002), which at present remains with unclear etiology. Moreover, the apoptosis, induced by *P. polonicum* nephrotoxin (unidentified metabolite at present), could be responsible for the cryptic and clinically-silent onset of renal atrophy in the idiopathic Balkan endemic nephropathy in humans (Stoev, 1998b; Mantle et al., 1998). On the other hand, it has been reported that a strong dose of OTA induces apoptosis and DNA-adducts *in vitro* (Obrecht-Pflumio and Dirheimer, 2000) and in rodent *in vivo* (Atroshi et al., 2000). Therefore, it is of great importance to investigate the combined effect and interaction between various mycotoxins in further *in vivo* and *in vitro* studies, because this is the real situation occurring in field conditions.

Conclusion

In view of the study, mycotoxins are generally cytotoxic to human lymphocytes. Strategies to minimize food contamination by *P. polonicum* (and *P. crustosum*) responsible for the production of this novel mycotoxin must be explored in order to prevent the

hazards it may cause to humans. Particular attention has to be paid to the high incidences of these *Penicillium* spp. responsible for the high contamination levels of this metabolite in human foods from Cameroon and also from Bulgarian animal feeds, where the same mycotoxin would be involved in well known animal and human nephropathy. Therefore, it is essential to investigate the other potential biological effects of the novel metabolite on mammalian cells. Some further investigations, including nuclear magnetic resonance, must be also undertaken in order to clarify and prove the chemical structure of this compound.

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