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The fungal chimerolectin MOA inhibits protein and DNA synthesis in NIH/3T3 cells and may induce BAX-mediated apoptosis

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ABSTRACT

The *Marasmius oreades* mushroom agglutinin (MOA) is a blood group B-specific lectin carrying an active proteolytic domain. Its enzymatic activity has recently been shown to be critical for toxicity of MOA toward the fungivorous soil nematode *Caenorhabditis elegans*. Here we present evidence that MOA also induces cytotoxicity in a cellular model system (murine NIH/3T3 cells), by inhibiting protein synthesis, and that cytotoxicity correlates, at least in part, with proteolytic activity. A peptide-array screen identified the apoptosis mediator BAX as a potential proteolytic substrate and further suggests a variety of bacterial and fungal peptides as potential substrates. These findings are in line with the suggestion that MOA and related proteases may play a role for host defense.

1. Introduction

A lectin is defined as a protein or a glycoprotein that "possesses at least one noncatalytic domain that binds reversibly to a specific mono- or oligosaccharide" [1]. This definition stems from the original one dating back to the 1980s [2,3], which was subsequently extended to take into account the latest developments in the field. Often several carbohydrate-binding domains come together in one lectin, explaining why many lectins can agglutinate cells and are therefore alternatively referred to as *agglutinins*. Lectins are found in all kingdoms of life and take part in a variety of different physiological processes, ranging from protein trafficking to cell adhesion, innate immunity and host defense [4]. Mushrooms are known to contain a wide variety of lectins [5], playing a role in host defense against insects, nematodes, and a number of other external threats, and very often they show different kinds of bioactivities toward other organisms, including immunoenhancement, vasorelaxation, antiviral, mitogenic, antiproliferative and antitumor effects [5].

MOA is a 32.8 kDa homodimeric, histo-blood group B (Galα1,3(-Fucα1,2)Gal)-specific lectin extracted from the *Marasmius oreades* mushroom. The discovery of hemagglutination activity in extracts of *M. oreades* dates back to the 1950s [6,7], although MOA was isolated and characterized for the first time only 20 years later [8]. During the past few years, research on MOA has gained new impetus with the cloning of the MOA gene [9] and extensive characterization of its sugar binding affinity [12–14], followed by the X-ray structure determination of MOA in complexes with two sugar ligands [15,16]. Besides providing insights into the structural details of the sugar-binding sites, the crystal structures of MOA suggested a possible Ca^{2+}-dependent enzymatic activity associated with its dimerization domain, later discovered to be of a proteolytic nature [10,11].

The toxicity of MOA was first tested *in vivo* on mice, showing how parenterally administered MOA causes symptoms comparable to the human Hemolytic Uremic Syndrome [17], an often fatal disease characterized by hemolytic anemia, low platelet count and renal impairment [12]. At the same time, the lectin was tested on mouse-derived endothelial and dermal primary cell cultures, showing a cytotoxic effect of MOA leading to cell death and detachment [13]. A subsequent study published by Wohlschlager et al. demonstrated the toxic effect of MOA against the *Caenorhabditis elegans* nematode, reporting the proteolytic activity of MOA as the key mediator of toxicity [11].

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In the present work we established an *in vitro* NIH/3T3 cell-based model to shed further light on the connection between the enzymatic function of this fungal chimerolectin and its observed toxicity. The results presented show how the cytotoxic effect of MOA ultimately leads to the arrest of protein and DNA synthesis. Moreover, we explore the substrate specificity of MOA through a peptide array screen, providing three leads for a proteolytic target, which could act as additional mediators of the cytotoxicity.

2. Materials and methods

2.1. Expression and purification

The DNA constructs described in this work are the same used in a previous publication [10]. The expression and purification of MOA and its functional variants have been performed with small variations of the protocol described therein. Briefly, the IPTG-inducible pT7 vectors (pT7-LO) containing the cDNA for wild-type MOA or the Cys215Ala variants, were transformed in *Escherichia coli* strain BL21 (DE3) cells. Bacteria were induced using 1 mM IPTG, grown at 18 °C for 24 h and subsequently collected by centrifugation (6000 rcf, 15 min). The pellet was resuspended in a lysis buffer composed of 50 mM Tris pH 8.0, 2 mM EDTA, 5 mM DTT, cOmplete protease inhibitor cocktail EDTA-free (Roche Diagnostics Ltd.), 4 mg/ml hen egg white lysozyme and Benzonase nuclease (Sigma–Aldrich). After incubation on a shaker for 2 h at 20 °C, the insoluble fraction was removed by centrifugation (18,000 rcf, 40 min).

The recombinant proteins were purified using a three-step chromatographic protocol. As a capture step, the clarified cell lysate was passed through a D-Gal-Sepharose affinity resin (Thermo Scientific), followed by extensive washing with 20 mM Tris pH 7.5 buffer and elution of the protein using a 1.0 M D-Gal single step gradient. The eluate was dialyzed overnight against 20 mM acetate pH 4.2 (Sigma–Aldrich), 2 mM EDTA, 2 mM DTT. Further purification was achieved through cation exchange on a Mono Q HR 5/50 GL column (Tricorn, GE Healthcare Life Sciences), followed by size exclusion chromatography (SEC) using a Superdex 200 10/300 GL gel-filtration column (Tricorn, GE Healthcare Life Sciences). The fractions containing the purified protein were pooled and concentrated to a final concentration of ≈10 mg/ml using concentrator tubes with a 10000 MWCO PES membrane (Vivaspin, Sartorius AG).

2.2. Cytotoxic activity tests

The cytotoxicity of MOA was determined as the 50% reduction of protein biosynthesis in toxin-treated cells. $3{\cdot}10^4$ cells/well were seeded the day before the experiment in fibronectin-coated or untreated 24-well plates (Falcon) and cultured in medium supplied with 10% fetal calf serum, 2 mM L-glutamine, penicillin (50 units/ml), and streptomycin (50 mg/ml) at 37 °C and 5% CO_2. Cells were washed twice in leucine-free HEPES medium and MOA was added at increasing concentrations or at different time intervals. After 3 h of incubation at 37 °C, the HEPES medium was removed and replaced with leucine-free HEPES medium complemented with 2 μCi/ml [^{3}H]leucine (GE Healthcare), after which the incubation was continued for 20 more minutes. Proteins were precipitated with 5% TCA, washed once in 5% TCA, and then dissolved in 0.1 M KOH. Incorporation of radioactively labeled leucine was quantified using a beta counter. Decrease in the level of DNA synthesis was evaluated using the same procedure described above, adding an amount of 2 μCi/ml of [^{3}H]thymidine instead of [^{3}H]leucine to the HEPES medium. The cytotoxic effect of MOA and the Cys215Ala variant was evaluated as the percentage of residual protein biosynthesis in toxin-treated cells in presence or absence of the proteolytic activity inhibitor E-64 (Sigma). $4{\cdot}10^4$ cells/well were seeded the day before the experiment in fibronectin-coated 24-well plates (Falcon) and cultured in medium supplied with 10% fetal calf serum, 2 mM L-glutamine, penicillin (50 units/ml), and streptomycin (50 mg/ml) at 37 °C and 5% CO_2. Cells were washed twice in leucine-free HEPES, incubated 1 h with leucine-free HEPES complemented with 10 mM E-64, and treated with either MOA wild-type or the Cys215Ala variant at increasing concentrations. After 4 h of incubation at 37 °C, the HEPES medium was removed and replaced with leucine-free HEPES medium complemented with 2 μCi/ml [^{3}H]leucine (GE Healthcare), after which the incubation was continued for 20 more minutes. Cell fixation with TCA and the [^{3}H]leucine incorporation was evaluated as described before.

2.3. Peptide array screening

The search for a peptide substrate was performed through the peptide array scanning service provided by the company JPT (Jerini Peptide Technologies GmbH, Berlin, Germany). The detailed procedures for the production of peptide microarrays [14] and samples of their applications [15] have been described in the literature. In brief, the screen was performed with PepStar microarrays, displaying approximately 3500 phosphotyrosine-carrying peptide substrates, immobilized on a glass slide support using the SPOT synthesis method. The microarrays were incubated at RT up to 12 h with either wild type MOA or the inactive Cys215Ala variant at a final concentration of 0.1 mg/ml in presence of 50 mM HEPES, 10 mM $CaCl_2$ and 5 mM DTT. The readout was performed by incubation with a fluorophore-conjugate anti-phosphotyrosine antibody and subsequent detection of the fluorescent signal.

3. Results

3.1. MOA induces the arrest of protein synthesis in NIH/3T3 cells

The cytotoxicity of MOA was primarily assessed by quantifying the level of protein biosynthesis of cultured cell lines exposed to varying amounts of MOA. The results, summarized in Table 1, show that the NIH/3T3 cell line (mouse embryo fibroblasts) is the most sensitive among the cell lines tested, with the amount of MOA required to inhibit 50% of the protein biosynthesis (EC_{50}) being only 0.5 μg/ml. After a lag time, the incubation of NIH/3T3 cells with MOA leads to cell detachment (data not shown), as previously observed on different mouse cell lines by Warner et al. [13]. A parallel evaluation of both the level of protein and DNA synthesis by incubating NIH/3T3 cells with MOA at a concentration of 3 μg/ml shows a drop in the level of protein synthesis, followed by a concomitant decrease in DNA synthesis (Fig. 1).

3.2. The cytotoxicity of MOA only partially depends on its proteolytic activity

To probe if the cytotoxicity of MOA (*i.e.* its ability to arrest protein synthesis) depends on its proteolytic activity, we compared wild-type MOA with the proteolytically inactive MOA variant

Table 1
Cytotoxic effect of MOA on different cell lines.

Cell line	Origin	EC_{50} [μg/ml]
NIH/3T3	Mouse embryo, fibroblasts	0.5
SK-BR-3	Human mammary tumor, epithelial	20.3
HEp-2	Human laryngeal cancer, epithelial	69.6
SW480	Human colorectal cancer, epithelial	10.7
HeLa	Human cervical cancer	42.8

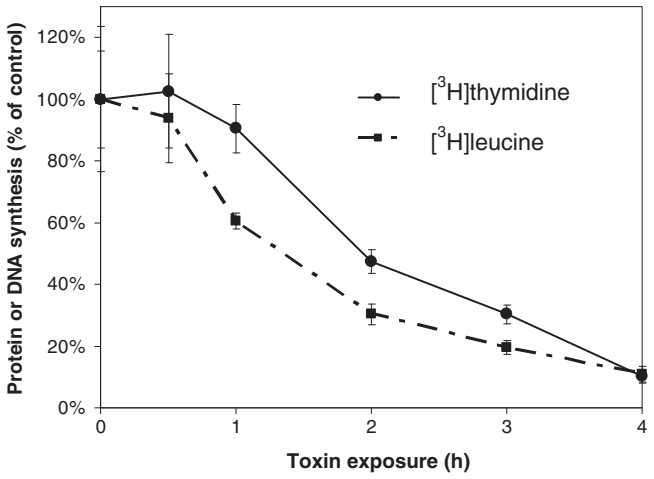

Fig. 1. Cytotoxic effect of MOA on NIH/3T3 cells, represented as percentage of residual protein synthesis and DNA synthesis (compared to a negative control) in cells incubated with 3 μg/ml of MOA. The curves for [^{3}H]Leu and [^{3}H]thymidine show how the decrease in the level of DNA synthesis lags behind the level of protein synthesis; standard deviations are shown for five independent experimental points.

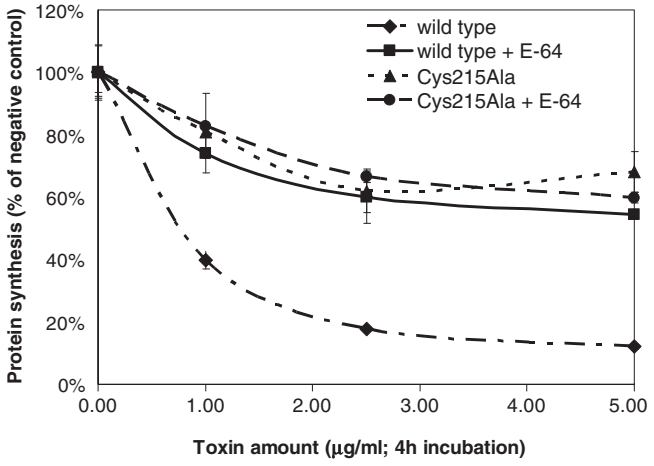

Fig. 2. Comparison of the cytotoxic effect of different MOA variants on NIH/3T3 cells plated on fibronectin and incubated with an increasing amount of lectin. Compared to the wild type lectin, the catalytic inactive Cys215Ala MOA variant shows a significantly weaker cytotoxic effect. In presence of the E-64 inhibitor both the Cys215Ala variant of MOA and the inactivated wild type protein exert the same cytotoxic effect, which is comparable to the cytotoxicity of the Cys215Ala MOA variant. All experiments are normalized to a negative control.

C215A. Indeed, the catalytically incompetent MOA variant elicited a considerably lower decrease of the level of protein synthesis (40% compared to 80% inhibition; Fig. 2), consistent with the hypothesis that the proteolytic activity of MOA is the main determinant of its cytotoxic effect. However, the fact that also the enzymatically inactive MOA variant induces a small effect on protein synthesis, suggests that other properties of the lectin also contribute to its toxicity.

In a second attempt to confirm the role of proteolytic activity in mediating cytotoxicity, the toxic effect of MOA and the Cys215Ala variant was tested in the presence of E-64, an irreversible Cys-protease inhibitor and a known inhibitor of the proteolytic activity of MOA. In the presence of E-64, the level of protein synthesis in NIH/3T3 cells incubated either with the wild type or the Cys215Ala variant of MOA decreased to the same extent (Fig. 2).

3.3. Search for a potential substrate

In an effort to search for potential cellular mediators of the cytotoxic effect, the substrate preference of MOA was probed by a peptide microarray carrying 1989 annotated 8-meric cleavage sites and 1536 randomly generated 15-meric peptides. The screen, performed by the company JPT GmbH (Berlin, Germany), reported a list of positive hits, three of which (reported in Table 2) show a signal significantly above the background noise level.

The three cleaved sequences, corresponding to peptides derived from Malayan tapir kappa casein, human BAX (BCL-2-associated X protein) and a random 15-mer, bear little to no sequence similarity to each other, with the exception of a conserved phenylalanine-isoleucine motif between the first two cleaved substrates. Although a sequence-based alignment of the first two peptides with the putative sequence autoproteolytically cleaved by MOA [9] points towards the phenylalanine-isoleucine bond as the possible cleavage site (Fig. S1), the iceLogo diagram reported by Wohlschlager et al. identifies a preference of MOA for either a proline, a valine, an alanine or a histidine residue in the P2 position of the proteolytic substrate, which does not match the proposed analysis, making it clear that there is currently no unambiguous way to identify the correct bond cleaved by MOA in the three substrates.

Due to the murine origin of the NIH/3T3 cell line, the three peptides were used for a pattern search by BLAST [16] against the known *Mus musculus* proteome. The first hundred records resulting from each search were parsed, pruned from redundant entries and sorted by decreasing *E*-value: the results, reported in Tables S1–S3, provides a list of potential targets cleaved by MOA.

4. Discussion

While the class of plant and fungal lectins includes a growing number of known members showing a toxic activity. MOA, together with the structurally unrelated subtilase cytotoxin, constitutes one of the few examples of toxic lectins carrying a proteolytically active domain [17].

Our NIH/3T3 *in vitro* model confirms and extends the previously published data, showing the same MOA-induced cell detachment observed by Warner et al. [13] and the correlation between the proteolytic activity of MOA and its cytotoxic effect outlined by Wohlschlager et al. [11]. However, the availability of an *in vitro* cell-based system allowed us to gain further insight into the role played by different functional components of MOA in mediating its cytotoxic activity at the cellular level. While cytotoxicity relies on the presence of the enzymatically active dimerization domain, the use of the proteolytically inactive Cys215Ala variant or the inhibited MOA-E-64 complex shows a residual toxic effect, suggesting the presence of two concurrent mechanisms leading to cell death, only one of which requiring the proteolytic activity of MOA. While Wohlschlager et al. associated the residual cytotoxicity to the presence of the ricin B chain-like sugar binding domain, other options remain. Examples like the *Agrocybe aegerita* lectin (AAL),

Table 2
Proteolytic substrates resulting from a peptide array screening.

Attenuation (neg. ctrl)	Peptide	Origin
82.17	RRPSFIAI	kappa-casein (*Tapirus indicus*)
19.31	LLLQGFIQ	BAX (*Homo sapiens*)
9.76	EQVASVKTAHATIML	Random peptide

triggering apoptosis in the human colon cancer cell line HT29 and the breast cancer cell line MCF-7 by engaging cellular partners through a hydrophobic patch on the protein surface [18], suggest that a specific portion of MOA could act in a similar fashion, possibly after a calcium-induced conformational change.

The search for a proteolytic substrate cleavable by MOA through a peptide microarray reported three candidates bearing little to no sequence similarity to each other, except for an F-I motif. The three peptides were used for a BLAST search on the mouse proteome, returning a list of potential cellular targets for the proteolytic action of MOA. While many entries in the list constitute potentially interesting targets, BAX stands out since it contains the full sequence of the proteolytic peptide and is a well-known mediator of intrinsic apoptosis [19].

While it is not a fundamental step in the apoptotic process, BAX is cleaved at the residue Asp33 by calpain (another Ca-depended papain-like Cys protease) during stress-induced apoptosis to yield p18 BAX, a process which has been shown to enhance drug induced apoptosis by 25% [20]. The sequence identified by the peptide array screen as a MOA-cleavable substrate corresponds to the stretch Leu26-Gln32 of the full length BAX protein: intriguingly, the proximity of the physiological calpain-mediated cleavage site to the target sequence for MOA suggests a potential connection with the observed cytotoxic activity.

In spite of a very low degree of sequence conservation, structural homology and novel structural data of complexes with the E-64 and the zVAD-fmk inhibitors (manuscripts in preparation) suggest that MOA belongs to the clan CA of the Cys proteases family, characterized by the conserved papain-like fold of the hydrolytic core [21–23]. Clan CA, and the family C1 in particular, include a large number of proteases playing fundamental roles in both physiology and disease, with many of them involved in parasite–host interactions, often targeting key mediators of cell signaling during host invasion [24].

Plant and mushroom lectins have an acknowledged role as defense proteins [1], exerting their role against predators through different mechanisms, often through an enzymatically active domain [25]. This picture agrees well with the behavior observed for MOA, suggesting its role as a fungal defense protein, and it is further reinforced by extending the search for potential targets to the rest of the known proteome. A proteome-wide BLAST search using the proteolytic substrates resulting from the peptide array scan (Tables S4–S6) shows a prevalence of partial matches from proteins belonging to bacterial and fungal genomes. While the presence of bacterial targets fits well with the role of a defense protein against predators, the inclusion of fungal proteins suggests that MOA may also be of advantage for the mushroom when competing against other fungi.

In conclusion, the experimental work presented here confirms and extends the hypothesis on the role of MOA and the previous insights into the dependence of its toxicity on the enzymatic activity, while at the same time showing the viability of NIH/3T3 cells as an *in vitro* model system and providing a series of leads as potential cellular protein targets for further cytotoxicity studies.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.04.043.

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