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The fungal neurotoxin penitrem A induces the production of reactive oxygen species in human neutrophils at submicromolar concentrations

H.F. Berntsen[a], I.L. Bogen[b], M.B. Wigestrand[c], F. Fonnum[c], S.I. Walaas[c], A. Moldes-Anaya[d,e,*,1]

[a] Department of Administration, Lab Animal Unit, National Institute of Occupational Health, P.O. Box 8149 Dep, 0033 Oslo, Norway
[b] Oslo University Hospital, Department of Forensic Sciences, Section of Drug Abuse Research, P.O. Box 4950 Nydalen, N-0424 Oslo, Norway
[c] Institute of Basic Medical Sciences, Department of Biochemistry, University of Oslo, P.O. Box 1112 Blindern, N-0317 Oslo, Norway
[d] Section of Chemistry and Toxicology, Norwegian Veterinary Institute, P.O. Box 750 Sentrum, N-0106 Oslo, Norway
[e] R & D Section, PET-center, University Hospital of North Norway (UNN), P.O. Box 100 Langnes, N-9038 Tromsø, Norway

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ABSTRACT

Penitrem A is a fungal neurotoxin that recurrently causes intoxication in animals, and occasionally also in humans. We have previously reported that penitrem A induced the production of reactive oxygen species (ROS) in rat cerebellar granule cells, opening for a new mechanism of action for the neurotoxin. The aim of this study was to examine the potential of penitrem A to induce ROS production in isolated human neutrophil granulocytes, and to study possible mechanisms involved.

Penitrem A significantly increased the production of ROS in human neutrophils at concentrations as low as 0.25 μM (40% increase over basal levels), as measured with the DCF fluorescence assay. The EC_{50} determined for the production of ROS by penitrem A was 3.8 μM. The maximal increase in ROS production was approximately 330% over basal levels at a concentration of 12.5 μM. ROS formation was significantly inhibited by the antioxidant vitamin E (50 μM), the intracellular Ca^{+2} chelator BAPTA-AM (5 μM), the mitogen activated protein kinase kinase (MEK) 1/2 and 5 inhibitor U0126 (1 and 10 μM), the p38 mitogen activated protein kinase (MAPK) inhibitor SB203580 (1 μM), the c-Jun amino-terminal kinase (JNK) inhibitor SP600125 (10 μM), and the calcineurin inhibitors FK-506 and cyclosporine A (1.5 and 0.5 μM, respectively).

These finding suggest that penitrem A is able to induce an increase in ROS production in neutrophils via the activation of several MAPK-signalling pathways. We suggest that this increase may partly explain the pathophysiology generated by penitrem A neuromycotoxicosis in both humans and animals.

1. Introduction

Penitrem A (Fig. 1) is a toxic secondary metabolite mainly produced by the fungal species *Penicillium crustosum* (Moldes-Anaya et al., 2011). Intoxication with the mycotoxin penitrem A in animals has been reported recurrently in the literature for decades (Arp and Richard, 1979; Boysen et al., 2002; Cysewski et al., 1975; Eriksen et al., 2009; Hocking et al., 1988; Lowes et al., 1992; Naudé et al., 2002; Richard et al., 1981; Schell, 2000; Tiwary et al., 2009; Walter, 2002; Young et al., 2003). A few cases of neurological disease in humans are also presumptively caused by penitrem A (Cole et al., 1983; Gordon et al., 1993; Lewis et al., 2005). The incidence of penitrem A-induced neuromycotoxicosis may be under-diagnosed, especially in humans, because of the lack of knowledge about penitrem A's deleterious effects and the difficult access to appropriate analytical tools for detection and determination of the neurotoxin.

The main route of penitrem A exposure is through consumption of contaminated food and/or feed in humans and animals (Eriksen et al., 2009). After ingestion, the toxin is rapidly absorbed into the bloodstream and distributed throughout the body (Moldes-Anaya et al., 2009). In particular, penitrem A crosses the blood-brain-barrier, where it exerts its toxicological effects (Moldes-Anaya et al., 2009). In animals, clinical signs of intoxication range from tremors, seizures and hyperthermia to ataxia and nystagmus, all signs of motor system dysfunction (Eriksen et al., 2013). In humans, hyperthermia, nausea and emesis, diplopia, severe tremors and bloody diarrhoea (Eriksen et al., 2013) have been observed. Existing evidence shows that the CNS is the primary target for penitrem A (Moldes-Anaya et al., 2011; Norris et al., 1980; Stern, 1971), however, the peripheral nervous system (PNS) may also be affected (Knaus et al., 1994; McLeay et al., 1999; Wilson et al.,

* Corresponding author at: R & D Section, PET-center, University Hospital of North Norway (UNN) P.O. Box 100 Langnes, N-9038, Tromsø, Norway.
E-mail address: angel.salvador.moldes.anaya@unn.no (A. Moldes-Anaya).
[1] URL: www.unn.no.

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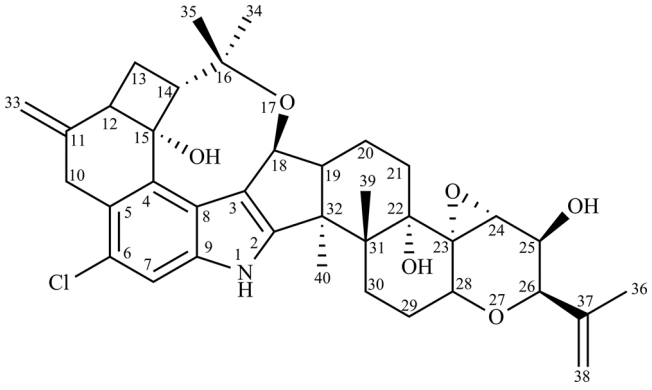

Fig. 1. Chemical structure of the neurotoxin penitrem A (Mw 634.2 Da).

1972). Penitrem A is able to impair GABAergic amino acid neurotransmission and selectively antagonise high-conductance Ca^{+2}-activated potassium (BK) channels. Dysfunctional neurotransmission and inhibition of neuronal BK channels are probably involved in the neurotoxic actions observed in both humans and animals (Moldes-Anaya et al., 2011; Eriksen et al., 2009). In a previous study, we have shown that penitrem A induces the production of reactive oxygen species (ROS) in primary cerebellar granule neurons from rats (Berntsen et al., 2013), opening for a new mechanism of action for the neurotoxin. Neuropathology has in general been limited to the cerebellum (Cavanagh et al., 1998), but massive pathological changes have also been reported in other organ systems of the thoracic and peritoneal cavities (Hayes et al., 1976). Hayes et al. (1976) also reported a significant increase in neutrophil levels in blood, while Cavanagh et al. (1998) reported a selective increase in brain cerebellar perfusion and neutrophil infiltration after penitrem A exposure.

Among circulating immune cells, neutrophils are one of the initial participants in the body's defence against external pathogens, and are involved in acute phases of inflammation. Neutrophils phagocyte, kill and digest invading pathogens by using proteinases, cytotoxic proteins, chelators and free radicals (Babior, 2000). Once in the blood, unstimulated neutrophils are mainly in free circulation. When needed, they exit the bloodstream and migrate actively towards the sites of inflammation, infiltrating the affected organs. Simultaneously, large oxygen consumption denoted as the respiratory burst takes place (Babior, 2000). During the respiratory burst NADPH oxidase catalyses the one-electron reaction of O_2 to $O_2^{\bullet -}$ (Babior, 2004). $O_2^{\bullet -}$ is highly reactive and unstable and will dismutate to H_2O_2, which may subsequently generate ROS such as OH^-, hydroxyl radical ($^{\bullet}OH$), singlet oxygen ($^1O_2$) and hypochlorous acid (HOCl). ROS are by-products of normal cellular metabolism, and cells possess several mechanisms to avoid damage induced by ROS and other free radicals. When generation of ROS exceed basal levels and the mechanisms of defence against ROS-induced injury are overwhelmed, oxidative stress occurs (Kehrer, 1993). Oxidative stress can cause cellular damage and subsequent cell death because ROS oxidise critical cellular components such as lipids, proteins and DNA (Kehrer, 1993). The activation of neutrophils may also stimulate ROS overproduction and cause inflammation and tissue injury.

The mycotoxin penitrem A recurrently causes intoxication in animals, and clinical signs of intoxication and even hospitalisation have also been reported in humans. In a previous study, we reported that penitrem A induced the production of ROS in rat cerebellar granule cells. The purpose of the present study was to examine the capability of penitrem A to induce the production of ROS in human neutrophil granulocytes, as well as to examine possible mechanisms involved in this induction. Human neutrophil granulocytes represent an easily available test system, relevant for its role in the body's defence mechanism against external pathogens.

2. Materials and methods

2.1. Chemicals and reagents

α-tocopherol (vitamin E), 5-amino-2,3-dihydro-1,4-phtalazindione (luminol; ≥ 97%), cyclosporine A (CsA; ≥ 98.5%), dimethyl sulfoxide (DMSO; ≥ 99.9%), N-ω-nitro-L-arginine methyl ester (L-NAME), methanol (MeOH; ≥ 99.9%) and penitrem A ≥ 95%, were supplied by Sigma-Aldrich (St. Louis, MO, USA). 2′,7′- dichlorodihydrofluorescein diacetat (DCFH-DA), Hanks' Balanced Salt Solution (HBSS; 10x) and HEPES buffer, came from GIBCO/Invitrogen (Oslo, Norway). 1,2-bis(o-aminophenoxy)-ethane-N,N,N'N'-tetraacetic acid tetraacetoxymethyl ester (BAPTA-AM), 4-[4-fluorophenyl]-2-[4-methylsulfinylphenyl]-5-[4-pyridyl]-1H-imidazole (SB203580) and anthra[1,9-cd]pyrazol-6(2H)-one (SP600125) were obtained from Calbiochem (San Diego, CA, USA). Tacrolimus (FK-506) was supplied by Cayman Chemicals (Ann Arbor, Michigan, USA) and 1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio]butadiene (U0126), CytoTox-ONE and TRITON-X from Promega (Madison, WI, USA). Lymphoprep™ was obtained from Medinor (Oslo, Norway). Stock solutions of BAPTA-AM, CsA, FK-506, SB203580, SP600125, U0126, vitamin E and luminol were all prepared by dissolution in DMSO. L-NAME was dissolved in phosphate buffered saline (PBS) with pH 7.4. DCFH-DA was dissolved in methanol. All other reagents used were analysis grade laboratory chemicals from standard commercial suppliers.

2.2. Isolation of human neutrophil granulocytes

Isolation of human neutrophil granulocytes for measurements of ROS was carried out by dextran sedimentation and density gradient centrifugation, as described by Boyum et al. (1991). EDTA blood from healthy, non-smoking, male volunteers (50 ml) was mixed with 6% dextran (5 ml) and left for 30 min at room temperature (RT) for the sedimentation of erythrocytes. The supernatant containing granulocytes was subjected to Lymphoprep™ density gradient centrifugation at 625*g* for 15 min at RT in 15 ml tubes. The resulting layer of mononuclear cells, formed at the interface between supernatant and separation fluid, was removed with the supernatant, leaving a pellet of granulocytes and erythrocytes at the bottom of the tubes. The pellets were re-suspended in 0.83% NH_4Cl, and left for 7 min at RT for lysis of remaining erythrocytes. The tubes were centrifuged for 7 min at 625*g*, the supernatant removed, and cells washed twice in 0.9% NaCl. The resulting pellets were re-suspended in HBSS buffer supplemented with 4.17 mM $NaHCO_3$, 20 mM HEPES and 5 mM glucose. The cell number was counted and the cells diluted in supplemented HBSS to a concentration of 2.5×10^6 cells/ml buffer.

2.3. Penitrem A exposure of human neutrophil granulocytes

A preliminary study was performed to examine whether penitrem A (10 μM) induced ROS production in human neutrophil granulocytes *in vitro*, measured by both the DCF assay and the luminol-chemiluminescence assays (from now referred to as the luminol assay). A positive control, 20 μM PCB 153, known to significantly induce ROS production measured with both assays, was included (Reistad and Mariussen, 2005; Berntsen et al., 2016).

Subsequently, in the main study cells were exposed to 1, 10, 250 and 750 nM, and 2, 10 and 12.5 μM penitrem A and ROS production assessed with the DCF assay. Concentrations of 20 and 40 μM penitrem A were also included in 2 and 1 experiments, respectively, but excluded from the rest of the experimental runs as they caused no further increase in ROS production than what was induced by 12.5 μM penitrem A. To rule out that any observed effects in the DCF assay was caused either by oxidation of the probe by penitrem A itself, or by penitrem A exhibiting autofluorescence, controls with penitrem A and the probe without cells, as well as penitrem A alone and penitrem A in

conjunction with cells without the probe, was included in a few experiments.

2.4. Detection of penitrem A-induced ROS production using the DCF assay

The assessment of ROS production with the DCF assay was carried out mainly as described by Myhre et al. (2000). The DCF-assay is based on the diffusion of the non-ionic probe 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) across the cell membrane, and its hydrolysis to the non-fluorescent DCFH inside the cell through the action of intracellular esterases. DCFH is oxidised to the fluorescent DCF amongst other when ROS such as $ONOO^-$, $^{\bullet}OH$ and H_2O_2 are present. Cells were preincubated (4 ml of cells/tube at a concentration of 2.5×10^6 cells/ml buffer) with DCFH-DA (2 μM) for 15 min at 37 °C under light protection, followed by centrifugation at 625*g*, and re-suspension of the cell pellet in 4 ml of buffer without DCFH-DA. Briefly, penitrem A was added to the 96-well plate in 150 μl buffer, followed by addition of 100 μl of cells (2.5×10^6 cells/ml). DCF fluorescence measurements were started immediately after addition of neutrophil granulocytes to the plate, and assessed every other minute for 2 h in a microplate spectrofluorometer (SpectraMax Gemini EM, Molecular Devices, Sunnyvale, CA, USA), with excitation/emission wavelengths of 485/530 nm. The area under the curve (AUC) was calculated, and values expressed as fluorescence relative to the DMSO control (set to 100%) in the case of the concentration-response studies, as well as the inhibitor studies in unstimulated cells. Due to the large inter-individual variation in fluorescence responses, for the study of the effects of inhibitors on the penitrem A-induced ROS production, results were expressed as % of 10 μM penitrem A (set to 100%).

2.5. Detection of penitrem A-induced ROS production using the luminol assay

Detection of ROS formation with the luminol assay was performed as described by Voie et al. (1998) with minor modifications. Chemiluminescence in the presence of luminol, which penetrates the cell membrane, is in neutrophil granulocytes dependent on the myeloperoxidase-H_2O_2 $-Cl^-$ system and detects the presence of HOCl (Dahlgren and Karlsson, 1999; Myhre et al., 2003), but has also been claimed to potentially be oxidised in the presence of $ONOO^-$, $^{\bullet}OH$ and $O_2^{\bullet -}$ (Myhre et al., 2003). In short, penitrem A was added to the 96 well-plate in 50 μl buffer, followed by 100 μl luminol (0.25 mM) and 100 μl cells (2.5×10^6 cells/ml). Luminescence readings were started immediately after the addition of cells, and performed every other minute for 1 h in a multidetection microplate reader (Synergy 4, BioTek Instruments, Inc., Winooski, VT, USA). The AUC was calculated and values expressed as relative luminescence (% of DMSO control).

2.6. Mechanistic studies of penitrem A-induced ROS production

The DCF assay was used, as described in Section 2.4, to investigate the potential mechanisms involved in penitrem A-induced ROS production. Briefly, human neutrophil granulocytes were incubated with 10 μM penitrem A alone and in conjunction with specific inhibitors of intracellular signalling pathways and other factors acting upstream of the NADPH-oxidase to determine if they were able to inhibit penitrem A-induced ROS production. The following compounds and concentrations were used; two antioxidants (50 μM vitamin E, and 300 μM L-NAME), one intracellular calcium chelator (5 μM BAPTA-AM), three inhibitors of different mitogen activated protein kinase (MAPK) signalling cascades (1 and 10 μM U0126, 1 μM SB203580, 10 μM SP600125) and two calcineurin inhibitors (0.5 μM CsA and 1.5 μM FK-506). The inhibitors were also tested in absence of penitrem A to elucidate effects on basal ROS production. The concentrations of the inhibitors were based on concentrations previously reported in the literature (Myhre et al., 2009; Reistad and Mariussen, 2005; Reistad et al., 2005), and in previous experiments performed in our laboratory (Berntsen et al., 2016). Each experiment included a control containing cells and buffer, as well as a DMSO control. The content of DMSO used in the experiments never exceeded 0.4%.

2.7. LDH assay

Assessment of cell integrity after exposure of human neutrophil granulocytes to 10 μM penitrem A, was performed with the LDH homogenous membrane integrity assay CytoTox-ONE (Promega, Madison, WI, USA) as described by the manufacturer kit-protocol. 2.5×10^6 cells/ml buffer were exposed to the chemicals in triplicates for 2 h (n = 3).

2.8. Statistical analysis

Statistical analyses were carried out using GraphPad Prism 5 and PAWS Statistics 18 software. Prior to statistical testing, homogeneity of variances between group means was tested with Bartlett's test. For multiple pairwise comparisons between groups, in the case of homoscedasticity, an ANOVA test was performed, followed by a Dunnett's post hoc test for comparison of each group mean with control. Where heteroscedasticity was detected, a Welch's ANOVA followed by a Games-Howell post hoc test was used. A *t*-test was used for statistical comparison of the LDH response in exposed vs unexposed cells. In all the experiments conducted, a *p*-value lower than 0.05 was regarded as statistically significant.

3. Results

3.1. Studies of penitrem A-induced ROS production in human neutrophil granulocytes

Preliminary studies revealed that penitrem A (10 μM) significantly increased the production of ROS (314% of DMSO control) measured by the DCF assay, while no increase in ROS production was measured using the luminol assay (Fig. 2). The polychlorinated biphenyl PCB 153 was included as a positive control. Based on these results, further studies of penitrem A-induced ROS production, such as concentration-response studies and mechanistic studies, were performed using the DCF assay. The absence of oxidation of the DCFH-DA probe by penitrem A itself and absence of penitrem A autofluorescence confirmed that the observed increases in ROS levels were caused by penitrem A-induction of cellular ROS production in human neutrophil granulocytes.

Human neutrophil granulocytes were exposed to increasing concentrations of penitrem A, and a concentration-dependent increase in ROS production was observed. Penitrem A concentrations ≥250 nM significantly increased ROS production compared to the DMSO control ($p < 0.05$) (Fig. 3) and a maximum increase was measured after exposure to 12.5 μM. Higher concentrations of the toxin (20 and 40 μM) were only tested in two and one experiments, respectively, but did not further increase the ROS levels. For the concentration-response curve, a best-fit approach was applied using a four parameter logistic fit where the Hill slope was set to unit and no constrains applied. EC_{50} was estimated to be 3.8 μM.

3.2. Mechanistic studies of penitrem A-induced ROS production

For mechanistic studies of penitrem A-induced ROS production, neutrophil granulocytes were co-incubated with penitrem A (10 μM) and various substances known to potentially inhibit ROS production. Effects on basal ROS production in cells treated with inhibitors only were also examined (Fig. 4).

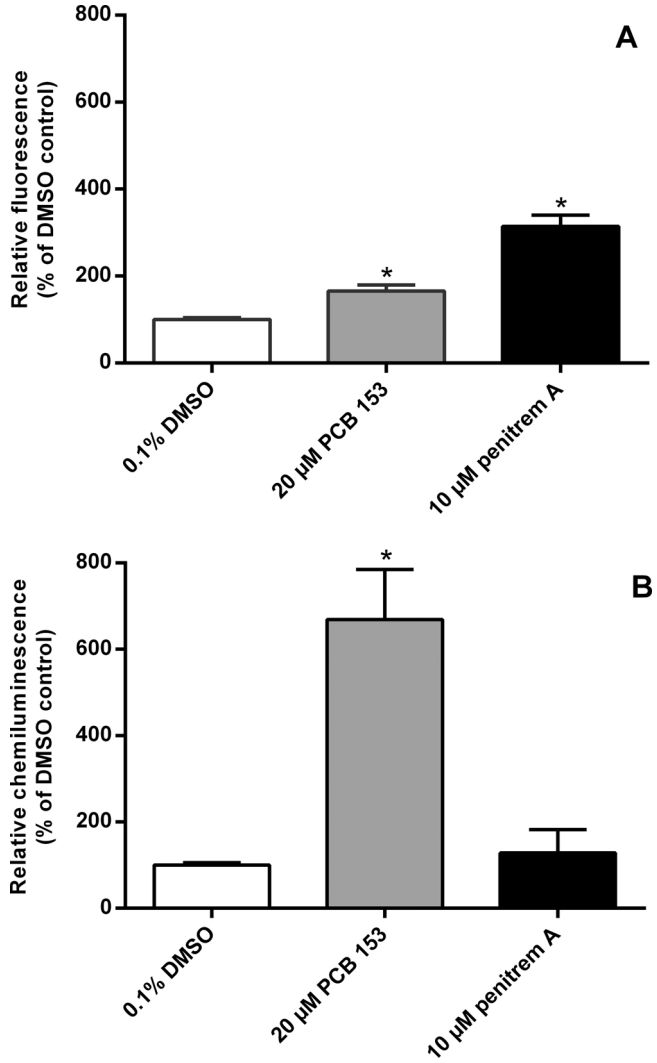

Fig. 2. ROS production in human neutrophil granulocytes after exposure to 10 µM penitrem A measured by (A) the DCF assay and (B) the luminol assay. Data are shown as relative fluorescence or chemiluminescence (% of DMSO control set to 100%). 20 µM PCB 153 was included as a positive control. Mean and SEM for n = 9-11 individual experiments are shown. Values significantly different from the DMSO control are indicated with * representing $p < 0.05$, assessed with a Welch test and a Games-Howell post hoc test.

3.2.1. Effects of antioxidants and calcium chelators on penitrem A-induced ROS production

The effects of two antioxidants on penitrem A-induced ROS production were examined. Vitamin E (50 µM) reduced ROS production induced by penitrem A (10 µM) with 64% ($p < 0.001$), whereas the nitric oxide synthase (NOS) inhibitor L-NAME (300 µM) had no significant effect. The intracellular calcium chelator BAPTA-AM (5 µM) reduced penitrem A-induced ROS production with 39% ($p < 0.001$).

3.2.2. Effects of mitogen activated protein kinase (MAPK) inhibitors on penitrem A-induced ROS production

To study the involvement of the extracellular signal regulated kinase (ERK) 1/2 and/or ERK 5 signalling pathways in penitrem A-induced ROS production, two concentrations of the inhibitor U0126 (1 and 10 µM) were applied, as both have previously been reported in the literature. 22 and 59% reductions in penitrem A-induced ROS production were observed, respectively ($p < 0.05$ and $p < 0.001$). The p38 MAPK inhibitor SB203580 (1 µM) and c-Jun amino-terminal kinase (JNK) inhibitor SP600125 (10 µM) reduced penitrem A-induced ROS production with 41 and 61%, respectively ($p < 0.001$).

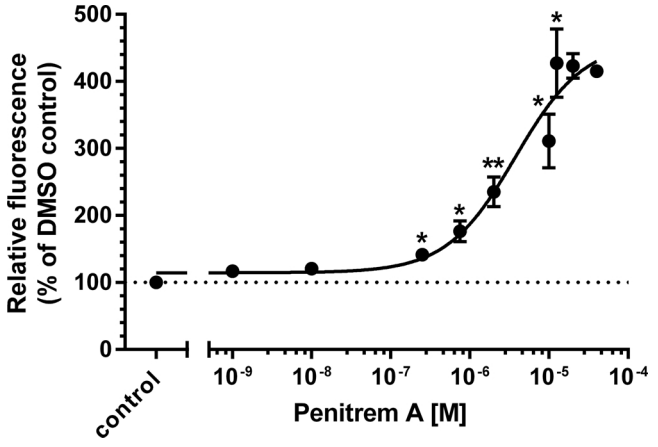

Fig. 3. ROS production in human neutrophil granulocytes, after exposure to 0.001–40 µM penitrem A. Values were calculated as AUC relative to the DMSO control after 2 h of readings. Mean values and SEM are displayed (n = 5–9 independent experiments for 0.001–12.5 µM). Values significantly different from DMSO control are indicated with * or **, representing $p < 0.05$ and $p < 0.01$, respectively, assessed with a Welch test and a Games-Howell post hoc test. The relative fluorescence for 20 and 40 µM penitrem A were included for completeness of the concentrations-response curve, but were not included in the statistical analysis due to the low number of independent experiments (n = 1-2).

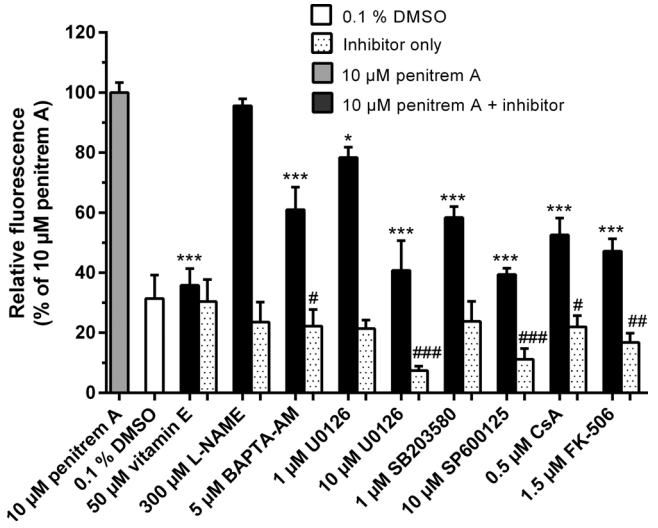

Fig. 4. ROS production in human neutrophil granulocytes measured by the DCF assay after exposure to 10 µM penitrem A, alone or in conjunction with different potential inhibitors of ROS production (gray or black columns, respectively). Effect of the different potential inhibitors on basal ROS production, and basal ROS production in cells exposed to DMSO only are also shown (dotted and white columns, respectively). Mean values and SEM are displayed (n = 5 independent experiments, performed in triplicate). The antioxidants L-NAME (300 µM) and vitamin E (50 µM), the intracellular calcium chelator BAPTA-AM (5 µM), the MEK 1/2 and MEK 5 inhibitor U0126 (1 and 10 µM), the p38 MAPK inhibitor SB203580 (1 µM), the JNK inhibitor SP600125 (10 µM), and the calcineurin inhibitors cyclosporine A (0.5 µM) and FK-506 (1.5 µM) were tested.
Values were calculated as area under the curve (AUC) and expressed for graphical presentation relative to 10 µM penitrem A (set to 100 %) after 2 h of readings. For statistical testing inhibitors inducing significant effects on ROS production in cells treated with penitrem A as compared to penitrem A only, are indicated with * and ***, representing $p < 0.05$ and $p < 0.001$, respectively. Inhibitors exerting a significant effect on basal ROS production in cells treated with DMSO (set to 100 %), as compared to cells treated with 0.1 % DMSO alone are indicated with #, ## or ###, representing $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively. Statistical significance was assessed with ANOVA and Dunnett's post hoc test in both cases.

3.2.3. Effects of calcineurin inhibitors on penitrem A-induced ROS production

Co-incubation of cells with penitrem A and the immunosuppressants and calcineurin inhibitors CsA (0.5 µM) and FK-506 (1.5 µM), reduced

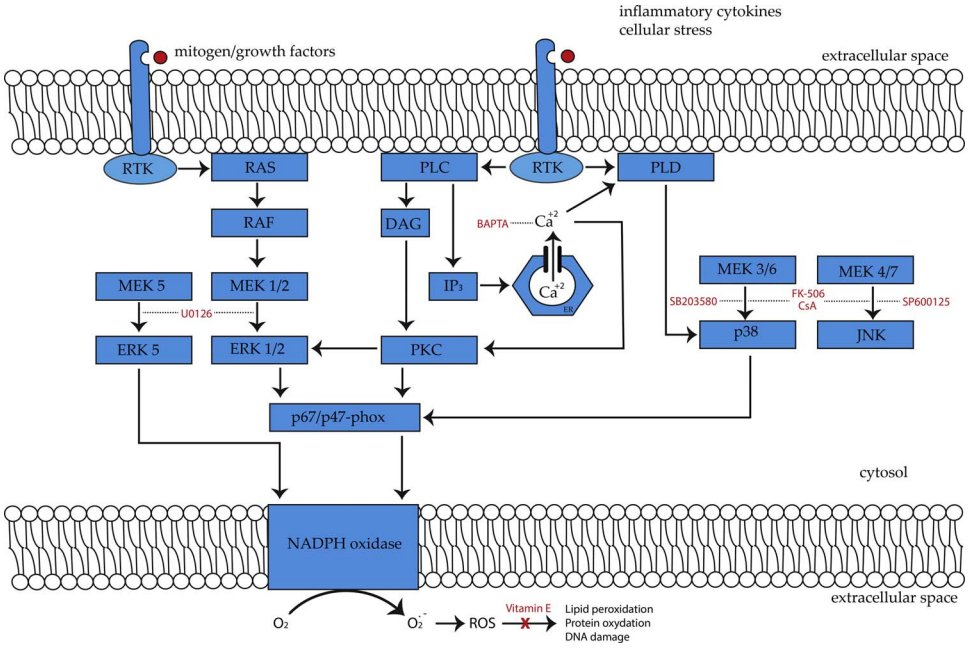

Fig. 5. Possible intracellular signalling pathways involved in penitrem A-induced production of reactive oxygen species (ROS) in human neutrophil granulocytes. The illustration shows the MAP kinase pathways ERK 1/2, ERK 5, p38 and JNK. The ERK 1/2 pathway is activated through tyrosine kinases (TRK) by extracellular signal substances. The p38 and JNK pathways are mostly activated in situations of cellular stress. The phospholipases C and D (PLC/PLD) may activate protein kinase C (PKC) via intermediate signal substances. IP_3 produced by phospholipases causes the release of Ca^{+2} from the endoplasmic reticulum (ER). PLD may be involved in activation of the p38 pathway. Site of action of the inhibitors used in the present study are shown (adapted from Berntsen et al., 2016).

ROS production with 47 and 53% as compared to penitrem A (10 μM) alone ($p < 0.001$).

3.2.4. *Effects of inhibitors on unstimulated cells*

The effect of the different inhibitors on basal ROS production in unstimulated cells was also studied (Fig. 4). BAPTA-AM (5 μM), the highest concentration of U0126 (10 μM) as well as SP600125 (10 μM), CsA (0.5 μM) and FK-506 (1.5 μM) significantly affected ROS production in unstimulated cells (treated with 0.1% DMSO), whereas no effect was seen for U0126 (1 μM), vitamin E (50 μM), L-NAME (300 μM) or SB203580 (1 μM).

3.3. *Cell integrity after penitrem A exposure*

The LDH assay showed no significant damage to the cell membrane of the human neutrophil granulocytes during 2 h of incubation with 10 μM penitrem A (data not shown).

4. Discussion

To date, there is no report on whether the mycotoxin penitrem A is able to affect the oxidative function of neutrophil granulocytes. The present study aimed to advance in this area by studying the effects of penitrem A on the production of ROS by neutrophils. We have found that penitrem A is able to induce a concentration-dependent boost of ROS production even at submicromolar levels without causing significant damage to the cells. Further we have also unravelled some of the mechanism that may be involved in the increased production of ROS in neutrophil granulocytes when exposed to penitrem A.

Penitrem A exposure caused a significant increase in ROS production when assessed with the DCF assay, whereas no significant increase was observed when measured with the luminol assay (Fig. 2A and B). The two probes applied in the present study are both reported to be unspecific, potentially being reduced by various reactive species (Myhre et al., 2003; Wardman, 2007; Kalyanaraman et al., 2012) and are not suitable to conclude on the origin of the oxidising agent formed (Kalyanaraman et al., 2017). Therefore, the exact reactive species generated in penitrem A-exposed granulocytes would have to be confirmed in additional studies. Despite it limitations, however Wojtala et al. (2014) indicated that the DCF assay is still useful as a screening to provide an overview over general ROS production.

In the mechanistic studies inhibitors of intracellular signalling pathways and other factors acting upstream of the NADPH-oxidase were applied (Fig. 4). The fat-soluble antioxidant vitamin E, which is believed to act in a fast and non-enzymatic manner primarily by the scavenging of lipid peroxyl radicals (Wang and Quinn, 2000), but also by involvement in reactions with $ONOO^-$ (Hogg et al., 1994) and $O_2^{\bullet -}$ (Ha and Csallany, 1992), significantly reduced penitrem A-induced ROS production. No protective effect was observed for the NOS inhibitor L-NAME (Moncada and Higgs, 1991). The effect of decreased ROS production with the use of the intracellular calcium-chelator BAPTA-AM (Ndountse and Chan, 2009), may indicate that an increase in intracellular levels of free calcium is occurring after penitrem A exposure.

We also applied inhibitors of MAPK signalling pathways to examine their involvement in penitrem A-induced ROS production. Effects of co-incubation with the mitogen activated protein kinase kinase (MEK) 1/2 and MEK 5 inhibitor U0126 (Favata et al., 1998; Kamakura et al., 1999), confirmed the involvement of the ERK 1/2 and/or ERK 5 signalling pathways in penitrem A-induced ROS production, whereas a reduction in ROS after application of SB203580, a selective inhibitor of the p38 MAPK (Cuenda et al., 1995), and SP600125 a selective inhibitor of JNK (Bennett et al., 2001), suggests the involvement also of these pathways. The ERK 1/2 signalling pathway is in general thought to be activated by extracellular ligands such as growth factors and mitogens, whereas the p38 and JNK pathways are activated by cellular stress and exposure to inflammatory cytokines. However, there is often cross-talk between the different signalling pathways, which may be activated simultaneously. The immunosuppressants and calcineurin inhibitors CsA and FK-506 also significantly reduced ROS production. In addition, these drugs have also been described to inhibit the p38 and JNK pathways (Schreiber and Crabtree, 1992; Matsuda et al., 2000). An illustration of possible signalling pathways involved in penitrem A-induced ROS production is presented in Fig. 5 (adapted from Berntsen et al., 2016). The site of action of the different inhibitors used is also shown.

Some of the inhibitors used in the present study did also have effect on basal ROS production (Fig. 4). As previously discussed in Berntsen et al. (2016) one cannot rule out that some activation of granulocytes may occur during the isolation process, resulting in activation of some of the same signalling pathways as after penitrem A exposure. However, the decreases in ROS production for all the inhibitors that had an effect in unstimulated cells was considerably smaller than the decreases in the

penitrem A stimulated cells (visible when results are expressed on the same axis), and should not be the result only of inhibition of basal ROS production.

In a previous study in cerebellar granule cells, we have shown that penitrem A is able to significantly increase ROS production over basal levels at a concentration of 25 μM, when assessed with the DCF assay as applied in the present study (Berntsen et al., 2013). In the same study, an LC_{50} value of 10.5 μM was observed, and significant decreases in viability were seen at concentrations ≥2 μM. In the present study, ROS production was significantly increased already at a concentration of 250 nM, while no effect on neutrophil viability was observed at a concentration of 10 μM (Fig. 3). The absence of LDH leakage indicates that the penitrem A-induced ROS produced is the result of activation and production of ROS in an intact, undamaged cell.

The information on penitrem A concentrations in blood or other tissues is scarce, especially regarding human material. The diagnosis of penitrem A-induced neuromycotoxicosis is mainly limited to the analysis of penitrem A in source materials (mouldy food and feed) or gastrointestinal contents. In animals, as far as we know, only two reports give semiquantitative levels of penitrem A in tissues (Eriksen et al., 2009; Moldes-Anaya et al., 2012). In Moldes-Anaya et al. (2012), brain and kidneys from mice dosed with 8 mg/kg penitrem A presented penitrem A levels of around 4 ng/g tissue (corresponding to 6.3 nM), while the liver showed levels of approximately 15 ng/g (24 nM) one hour after oral administration. In the washed gastrointestinal tract (from the same study), approximately 90 ng/g (142 nM) of penitrem A were found 1 h post administration. Another study reported serum levels of 4.2 ng/ml (corresponding to 6.6 nM) penitrem A in a dog presenting with penitrem A-induced neuromycotoxicosis (Tiwary et al., 2009). However, in this case, no information was given about the time elapsed from intoxication to sample withdrawal. Thus, it is possible that most of the neurotoxin was already metabolised and eliminated from the body prior to sampling. No other tissues were analysed for penitrem A in this study. Regarding humans there is no information on penitrem A levels in blood or other tissues available (Eriksen et al., 2013).

ROS are important pathophysiological mediators of ischemia-induced toxicity (Luer et al., 1996). While cerebellar granule neurons generate ROS mainly in the mitochondrial respiratory chain, as well as through the activation of neuron specific enzymes, neutrophils infiltrate regions with transiently increased blood flow and release ROS after activation of the NADPH oxidase system (Matsuo et al., 1995; Coyoy et al., 2008). A remarkable biphasic increase in regional cerebellar perfusion has been reported coinciding with tremor and ataxic episodes in rats treated with penitrem A (Cavanagh et al., 1998). Cavanagh et al. (1998) also reported leucocyte infiltrations in the cerebellum. In another study in dogs administered with penitrem A, intense infiltration of neutrophils was reported in portal areas of the liver as well as a marked increase in blood neutrophil numbers (Hayes et al., 1976). In that study, a dose-related liver damage showing vacuolated hepatocytes indicated fatty degeneration of the liver. Lipid peroxidation caused by oxidative stress has been related to liver steatosis (Muriel, 2009). Hayes et al. (1976) also observed severe congestion and focal areas of haemorrhages in the liver, spleen, gastrointestinal tract, urinary bladder and pancreas. Additionally, in a more recent study, *post mortem* histopathological examination of a dog poisoned with *Penicillium* toxins (mainly penitrem A, but also traces of penitrem B, D, E) again revealed multifocal haemorrhages in cerebellum and focal haemorrhages in the stomach and liver (Eriksen et al., 2009). It is therefore likely that an acute inflammatory state after penitrem A poisoning leads to an elevation of regional perfusion which in turn increases neutrophil recruitment. The subsequent release of cytotoxic metabolites, including ROS, by neutrophils and other cells may explain the pathological changes observed in both cerebellum and other organ systems.

5. Conclusion

Our study has shown that penitrem A significantly increased the production of ROS by neutrophil granulocytes at low concentrations (0.25 μM), without causing damage to the cells. A concentration-response curve yielded an EC_{50} value of 3.8 μM. The maximal increase in ROS production was approximately 330% at a concentration of 12.5 μM. The mechanism of ROS formation seems to involve an activation of several MAPK signalling pathways as well as an increase in intracellular calcium.

We suggest that the increased neutrophil ROS production may, at least in part, explain the pathological changes observed in both brain and other tissues of animals intoxicated with penitrem A.

Conflict of interest statement

The authors declare that they have no conflict of interest.

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