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The metallopeptide antibiotic bacitracin inhibits interleukin-12 $\alpha\beta$ and β_2 secretion

I. Alloza and K. Vandenbroeck

Abstract

The metalloantibiotic bacitracin is a known inhibitor of protein disulfide isomerase (PDI). The disulfide-linked interleukin-12 (IL-12) $\alpha\beta$-heterodimer and β_2-homodimer forms are crucial mediators of cell-mediated immune responses and inflammatory reactions. Bacitracin was found to potently block secretion of both the $\alpha\beta$- and β_2-dimer forms of IL-12, while it did not affect secretion of the β-monomer. This inhibition coincided with a reduction in the intracellular amount of PDI found in complex with the β-chain during intracellular transit. Bacitracin did not affect mRNA levels of the α- and β-chain. Similar to bacitracin, *N*-acetylcysteine blocked $\alpha\beta$- and β_2-secretion as well as PDI-β-chain complex formation. Thus, blocking PDI or shifting the endoplasmic reticulum towards a more reduced status disrupts the oxidative folding pathway or assembly of IL-12 dimer forms. The assembly stage of cytokines in the endoplasmic reticulum may represent a novel target for pharmacological intervention.

Introduction

Interleukin-12 (IL-12) is a heterodimeric cytokine composed of two disulfide-linked subunits, an α-chain or p35 subunit (35 kD) and a β-chain or p40 subunit (40 kD) (Brombacher et al 2003). IL-12 is involved in the regulation of cell-mediated immune responses and inflammatory reactions, and increased levels of this cytokine are typically observed in and associated with various chronic inflammatory disorders, including multiple sclerosis and rheumatoid arthritis. Production of disulfide-linked IL-12 β_2-homodimers was originally described as an endogenous antagonistic mechanism to counterbalance the pro-inflammatory effects of IL-12 (Gillesen et al 1995; Gately et al 1996). Increasing evidence is, however, emerging for the β_2-form to display pronounced agonistic effects. Especially, a role for the β_2-dimer as a regulator of macrophage chemotaxis has recently become convincingly clear (Brombacher et al 2003; Russell et al 2003). In addition, the β-chain is able to form covalently linked dimers with a distinct subunit, p19, to form the cytokine IL-23 (Brombacher et al 2003).

Given the prospects for therapeutic application, the search for pharmacological inhibitors of IL-12 production has received widespread attention and has resulted in the identification of numerous substances that block various stages in the cellular IL-12 production and effector machinery. Especially, the spectrum of drugs inhibiting nuclear factor kappa B (NF-κB)-mediated transcription of the β-chain, repressing IL-12 transcription by upregulating cAMP levels or interfering with post-receptor signal transduction pathways is becoming increasingly diversified (for review see Vandenbroeck et al 2004).

The unusual structural promiscuity of the β-chain of IL-12 suggests the existence of complex regulatory mechanisms operating at the level of folding, partner selection, maturation and assembly in the endoplasmic reticulum (ER) prior to secretion. We have recently demonstrated the presence of the ER chaperones GRP94 and calreticulin in folding complexes of the β-chain (Alloza et al 2004). Using cell-free refolding assays, we have also demonstrated that the ER folding catalyst protein disulfide isomerase (PDI) prevents unproductive aggregation reactions and facilitates oxidative assembly of β-monomers into β_2-dimers (Martens et al 2000). These data support the notion that

Applied Genomics Research Group, School of Pharmacy, Queen's University of Belfast, 97 Lisburn Road, Belfast BT9 7BL, UK

I. Alloza, K. Vandenbroeck

Correspondence:
K. Vandenbroeck, Applied Genomics Research Group, School of Pharmacy, Queen's University of Belfast, 97 Lisburn Road, Belfast BT9 7BL, UK. E-mail: k.vandenbroeck@qub.ac.uk

a subset of ER molecular chaperones and foldases is actively involved in orchestrating IL-12 dimer assembly. Importantly, the identification of PDI as a potential key factor in IL-12 assembly raises the question as to whether pharmacological inhibition of PDI could be employed as a valid strategy to block IL-12 secretion.

In this manuscript, we show that the metalloantibiotic and PDI inhibitor bacitracin potently blocks secretion of both the β_2- and the $\alpha\beta$-dimer forms of IL-12 at concentrations at which β-monomer secretion remains unaffected. This inhibition coincides with a reduction in the intracellular amount of PDI found in complex with IL-12 in co-capture assays. Real-time reverse transcription-polymerase chain reaction (RT-PCR) analysis of mRNA levels revealed that the lack of IL-12 secretion in the presence of bacitracin is not due to inhibition of gene transcription. Thus, our data substantiate a mode of action of bacitracin involving interference with PDI-mediated IL-12 assembly in the ER. Though bacitracin is primarily known for its potent bactericidal activity against Gram-positive organisms (Ming & Epperson 2002), our work opens perspectives to employ bacitracin or its derivatives with increased selectivity for PDI as drugs targeting the ER-dependent assembly stage in the cellular IL-12 $\alpha\beta$- or β_2-production machinery.

Materials and Methods

Cell culture conditions and bacitracin treatment

The HEK EcR-293 (Invitrogen, Paisley, UK)-derived 3H10 (Alloza et al 2004) cell line was cultivated in Dulbecco's modified Eagle's medium (Invitrogen, Paisley, UK) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 400 μg mL^{-1} zeocin and 600 μg mL^{-1} G418. Cells were maintained in a CO_2 incubator at 37°C (5% CO_2). Cells were treated with various concentrations of bacitracin (Sigma-Aldrich, UK) or *N*-acetylcysteine (Sigma-Aldrich, UK). Bacitracin was dissolved in phosphate-buffered saline (PBS) to make up a stock concentration of 0.3 M and a final concentration range of 0.1–10 mM was used for the treatment of cells. Cells were plated in 12-well plates and bacitracin or *N*-acetylcysteine was added to the culture medium 2 h before induction with Ponasterone A (Invitrogen, Paisley, UK).

Transient transfection of 3H10 cells with pINDSP1-p35 to produce IL-12 $\alpha\beta$-secreting cells

The development of a stable recombinant HEK293 cell line (named 3H10) that produces high levels of hexahistidine-tagged IL-12 monomeric and homodimeric β-chains from a ponasterone A-inducible pIND vector has been described in detail (Alloza et al 2004). To produce IL-12 $\alpha\beta$-secreting cells, 3H10 cells (3H10) were transiently transfected with an α-chain expression vector. 3H10 cells were plated the day before transfection at a confluency of 10^5 cells per well. Vector DNA (1 μg) containing the α-chain coding sequence (pINDSP1-p35; Alloza et al 2004) was used to transfect these cells using 3 μL of FuGENE-6 reagent (Roche Diagnostics, Basel, Switzerland). Cells were allowed to recover for 24 h before induction with ponasterone A.

Isolation of hexahistidine-tagged β_2- and $\alpha\beta$-secreted protein and folding complexes

Cells were treated with bacitracin for 24 h and lysed. Purification was performed using Ni^{2+}-nitrilotriacetic acid (NTA) affinity chromatography (Qiagen, West Sussex, UK) as described previously (Alloza et al 2004). Cultured medium was collected and mixed with an equal amount of 2× binding buffer (100 mM NaH_2PO_4, 600 mM NaCl, 40 mM imidazole, 2% protease inhibitors and 20 mM *N*-ethylmaleimide (NEM), pH 8) together with 60 μL of pre-washed Ni^{2+}-NTA agarose. Cells were lysed in 1× binding buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, 0.5% Triton-X-100, 1× of protease inhibitors and 10 mM NEM, pH 8) on ice for 30 min. After 15 min of centrifugation at 15 000 rev min^{-1} the supernatants were mixed with 10 μL of pre-washed Ni^{2+}-NTA agarose. Both cultured medium and lysate supernatants were incubated for 1 h at 4°C on a rotating wheel. Agarose beads were washed three times with 1× binding buffer (pH 6.3) before elution in 10 μL of 50 mM ethylenediamine tetraacetic acid (EDTA).

Western blot and immunodetection

Cultured medium fractions and Ni^{2+}-NTA agarose eluates were separated on non-reducing 4–20% Tris-glycine gels using the Novex System (Novex, Nuernberg, Germany). Following electroblot, membranes were decorated with primary antibodies (mouse α-p35 antibody G161-566 or mouse α-p40 antibody C8.6; BD Biosciences, Erembodegem-Aalst, Belgium). For detection of PDI, the rabbit anti-PDI antibody SPA-890 was used (Stressgen Biotechnologies, San Diego, CA). The secondary antibodies used were goat anti-mouse or anti-rabbit horseradish peroxidase-conjugated antibody obtained from Jackson&ImmunoResearch (West Grove, PA). Protein bands were detected using ECL or ECL-plus chemiluminiscence detection reagents (Amersham Biosciences, Bucks, UK).

Densitometric scanning

Densitometric scanning of protein bands on western blots was done with a Syngene GeneSnap apparatus and bands were subsequently analysed with GeneTools software.

Cytotoxicity test

10^5 cells per well were treated with a concentration series of bacitracin or *N*-acetylcysteine and induced with Ponasterone A. After 24 h of induction, 10 μg mL^{-1} 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich, UK) was added to the cells. After 2 h of incubation, the formed formazan crystals were dissolved in 200 μL of dimethyl sulfoxide (DMSO). The obtained solution was measured in a spectrophotometer at 550 nm.

Primers and probes

Real-time quantitative PCR (QuantiProbes) primers and probes for the IL-12 α- and β-chains were purchased from Qiagen (West Sussex, UK). A FAM-labelled α-chain probe (241047) and a FAM-labelled β-chain probe (241049) were used. Glyceraldehyde-3-phosphate dehydrogenase (GADPH) primers and probe were obtained as pre-developed TaqMan assay reagents from Applied Biosystems (4333764) (Warrington, UK) and were also FAM labelled.

Quantification of α-chain and β-chain mRNA levels by real-time RT-PCR

Transfected or untransfected H310 cells (10^6 cells/well) were treated with a concentration series of bacitracin or *N*-acetylcysteine. After induction for 24 h, total RNA was extracted using the Absolutely RNA RT-PCR Miniprep kit (Stratagene, Amsterdam, The Netherlands). mRNA levels of α- and β-chain were quantified using the RT-PCR Quantitect Gene Expression Assay kit (Qiagen, West Sussex, UK) on a DNA Engine Opticon 2 (MJ Research, Essex, UK). Real-time RT-PCR was performed following the supplier's protocol (Qiagen, West Sussex, UK): 30 min at 50°C, 15 min at 95°C, 45 cycles of 15 s at 94°C, 30 s at 56°C and 30 s at 76°C. Assay conditions were as follows: 12.5 μL of 2× QuantiTect Probe RT-PCR Master Mix, 2.5 μL of 10× Quantiprobe and primer Mix Solution, 0.25 μL of QuantiTect RT Mix and 10–200 ng of RNA. All reactions were replicated in separate tubes for detection of α-chain, β-chain and GADPH. Control reactions without template were performed to detect non-specific amplification due to contamination.

Statistical analysis

Statistical analysis of the effects of increasing concentrations of bacitracin (0.001, 0.01, 0.1, 1, 3 mM) or *N*-acetylcysteine (0.001, 0.01, 0.1, 1, 10 mM) on the level of α- or β-chain-specific mRNA was performed using Kruskal–Wallis Test (Jones 2002). Statistical analysis of the effect of the individual concentrations on the above parameters was performed using a non-parametric post-hoc test (Dunn's test). $P < 0.05$ denoted significance in all cases.

Results

Bacitracin and *N*-acetylcysteine inhibit secretion of IL-12 $\alpha\beta$- and β_2-dimers

Oxidative formation of disulfide bridges in secretory proteins is driven by thiol–disulfide exchange with other disulfides, such as the coupled cystine/cysteine and oxidised glutathione/reduced glutathione (GSSG/GSH). This reaction is generally catalysed by PDI or related oxidoreductases (Freedman et al 1994). The precursor of GSH synthesis, *N*-acetylcysteine, is a membrane-permeable amino acid that contains a free sulfydryl group and acts as a thiol antioxidant. We used 3H10 cells to assess the effects of *N*-acetylcysteine and bacitracin on secretion of IL-12 dimer forms. 3H10 cells produce the β-chain as a mixture of monomers and homodimers (Alloza et al 2004), while 3H10 cells transiently transfected with an α-chain expression plasmid produce the $\alpha\beta$-heterodimer form. Figure 1A shows that bacitracin completely inhibited IL-12 $\alpha\beta$-secretion at a concentration of 3 mM. Bacitracin also potently decreased β_2-secretion when used at a concentration of 3–5 mM (Figure 1B). Remarkably, secretion of the β-monomer under these conditions was virtually unaffected, indicating that the inhibitory effect of bacitracin was not due to inhibition of transcription of the β-chain. Similar observations were made for cells treated with *N*-acetylcysteine (complete inhibition of $\alpha\beta$-and β_2-secretion at ≥ 10 mM; Figure 1C, D).

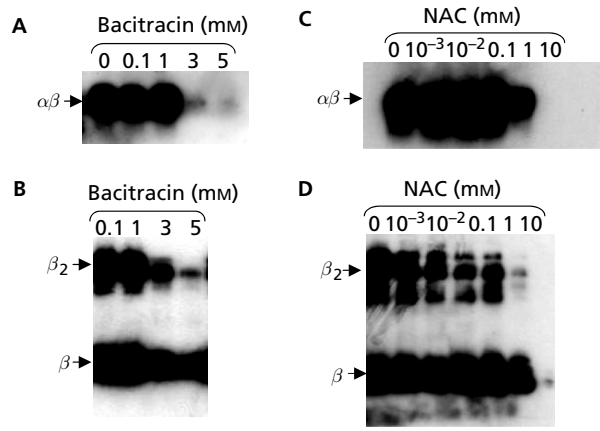

Figure 1 Effect of bacitracin (A, B) and *N*-acetylcysteine (NAC) (C, D) on secretion of the $\alpha\beta$-form (A, C) and β-monomer and β_2-dimer (B, D) of IL-12. Untransfected (B, D) or transiently transfected (A, C) 3H10 cells were treated for 24 h with the concentrations of bacitracin or NAC indicated. Hexahistidine-tagged IL-12 forms were immunoprecipitated from the culture medium by Ni^{2+}-NTA agarose chromatography and identified by non-reducing SDS-PAGE and immunoblot with the antibody G161-566 (A, C) or C8.6 (B, D).

Bacitracin and *N*-acetylcysteine decrease complex formation between PDI and the IL-12 β-chain

3H10 cells were treated with 3 mM bacitracin or 5 mM *N*-acetylcysteine for 24 h, lysed, and the β-chain folding complexes were extracted from the cell lysates and purified using Ni^{2+}-NTA agarose. The purified complex was submitted to reducing sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and co-captured PDI was detected in immunoblot. Figure 2 shows that bacitracin or *N*-acetylcysteine reduced the amount of PDI co-captured with the β-chain compared with untreated induced control cells ($P < 0.05$, bacitracin- or *N*-acetylcysteine-treated vs non-treated cells in three independently replicated experiments). To rule out the possibility that the inhibitory effects of bacitracin and

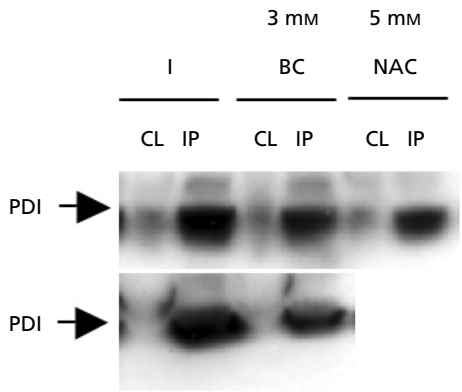

Figure 2 Co-immunoprecipitation of protein disulfide isomerase (PDI) with the β-chain of IL-12 from 3H10 cells treated with 3 mM bacitracin (BC) or 5 mM *N*-acetylcysteine (NAC). β-chain–PDI complexes were isolated by affinity capture on Ni^{2+}-NTA agarose through the hexahistidine-tagged β-chain, and resolved by reducing SDS-PAGE and immunoblot. Results from two independent experiments are given. Fractions of cell lysates (CL) and immunoprecipitates (IP; eluates from the Ni^{2+}-NTA agarose) were analysed.

N-acetylcysteine on IL-12 secretion and complex formation with PDI were due a decrease in cell metabolism or viability, we measured cytotoxicity by means of the MTT assay. Neither bacitracin nor *N*-acetylcysteine were cytoxic at IL-12 inhibitory concentrations (Figure 3).

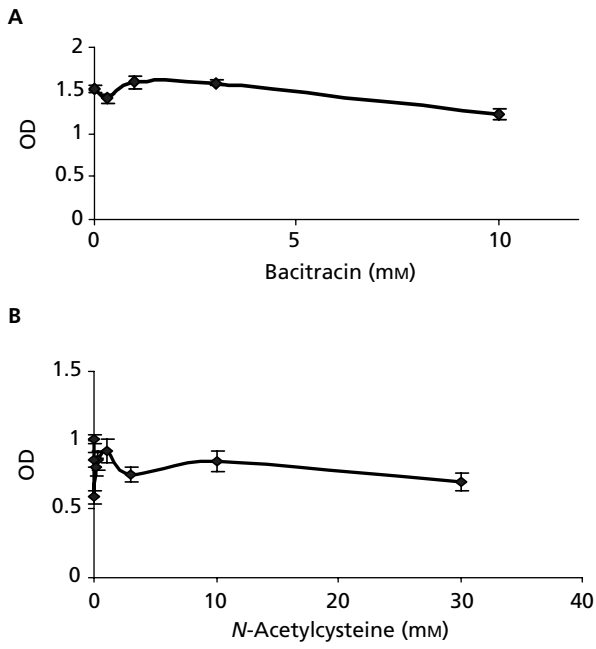

Figure 3 Cytotoxicity test of bacitracin (A) and *N*-acetylcysteine (B) on 3H10 cells. Cells were treated for 24 h with bacitracin or *N*-acetylcysteine at the concentrations indicated, and cytotoxicity was determined by means of the MTT assay. Each value is the mean of three experiments (± s.d.).

Bacitracin does not alter α- and β-chain mRNA levels

To investigate whether bacitracin treatment interferes with transcription of the α- and β-chain genes, total RNA was extracted from cells treated for 24 h with a concentration series of bacitracin. Real-time RT-PCR was performed to determine mRNA levels of the α-chain, β-chain and housekeeping enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH). As shown in Figure 4A, bacitracin did not significantly affect mRNA levels of either subunit ($P > 0.05$). In contrast, while *N*-acetylcysteine did not alter mRNA levels of the β-chain ($P > 0.05$), it significantly reduced α-chain mRNA levels when used at a concentration of 0.1 mM or higher (Figure 4B; Kruskal–Wallis Statistic $KW = 17.205$; $P = 0.0086$). Levels of α-chain mRNA in cells treated with 0.1, 1 or 10 mM *N*-acetylcysteine were significantly lower than those in induced non-treated cells (Dunn's Test P values < 0.05). When *N*-acetylcysteine was used at lower concentrations (0.001 or 0.01 mM), α-chain mRNA levels were not significantly different from those in induced non-treated cells ($P > 0.05$).

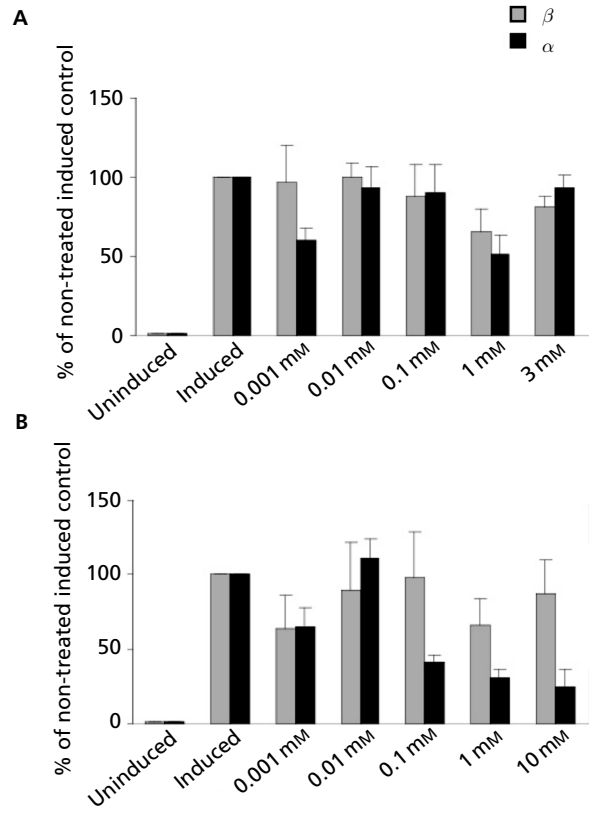

Figure 4 Quantification of α- (p35) and β-chain (p40) gene transcription levels by real-time RT-PCR. 3H10 cells were treated for 24 h with bacitracin (A) or *N*-acetylcysteine (B), using the concentrations indicated. The mRNA levels of each gene were determined relative to GADPH house-keeper gene transcription. Values represent percentages of pg p35 or p40 per μg of total RNA relative to induced, non-treated cells. Each value is the mean of four experiments (± s.d.).

Discussion

The metalloantibiotic bacitracin is a known inhibitor of PDI (Mizunaga et al 1990; Mandel et al 1993; Higuchi et al 2004). Here, we show that bacitracin potently inhibits both IL-12 $\alpha\beta$- and β_2-dimer but not β-monomer secretion by a mechanism involving reduced complex formation between PDI and the IL-12 β-chain during intracellular transit. This inhibitory effect did not correlate to a decreased level of transcription or stability of α- and β-chain mRNA, and was seen at concentrations = 3 mM. Others have reported PDI-inhibitory effects of bacitracin occurring at similar millimolar concentration ranges in different assay systems (Mandel et al 1993; Davis et al 2002; Higuchi et al 2004). Similar to bacitracin, the thiol antioxidant *N*-acetylcysteine , when used at a concentration of 10 mM, completely inhibited IL-12 heterodimer or homodimer secretion without affecting that of the β-monomer. This conforms to the study of Mazzeo et al (2002), who showed that 5–10 mM *N*-acetylcysteine inhibited IL-12 heterodimer secretion without affecting β-chain secretion. However, in their study, Mazzeo et al (2002) used a p40-specific ELISA to measure β-chain production, which does not allow for discrimination between β-monomers and -homodimers. They may therefore have missed the inhibitory effect of *N*-acetylcysteine on β_2-secretion.

According to the crystal structure of the IL-12 $\alpha\beta$-form, the unique intermolecular disulfide bond in the quaternary structure may not be essential for linking both subunits (Yoon et al 2000). Rather than this disulfide bond, interlocking of an arginine residue on the α-chain with a hydrophobic pocket on the β-chain was suggested to drive tight association of both subunits. The disulfide bond, then, is merely involved in stabilization of the heterodimer (Yoon et al 2000). This model is difficult to reconcile with the data presented in this study. Under this assumption, neither an *N*-acetylcysteine-induced enhancement of the reducing potential of the ER, nor inhibition of PDI with bacitracin should affect heterodimer assembly and secretion. On the other hand, in a previous study we found that at a molar ratio of PDI to β-chain of 5, PDI stimulated cell-free β_2-formation by a mechanism involving thiol-independent suppression of misfolding and aggregation reactions (Martens et al 2000). This is consistent with the notion of PDI being a multifunctional protein capable of both enzyme-like isomerase and chaperone-like anti-aggregation effects (Wang & Tsou 1993; Yao et al 1997). Primm & Gilbert (2001) showed that relatively low concentrations of bacitracin can inhibit the anti-aggregation chaperone effect of PDI in-vitro. In the context of these findings, a model in which bacitracin inhibits IL-12 secretion by blockage of the chaperone effect of PDI would reconcile our data with those of Yoon et al (2000). At any rate, further studies are needed to clarify the mechanistic pathway by which bacitracin inhibits IL-12 secretion at the folding stage.

Due to its toxicity bacitracin is no longer used internally, even though it is widely used in dermatological ointment for topical therapy in skin and eye infections. Application of bacitracin or other anti-PDI drugs as IL-12-blocking reagents, therefore, may be of special interest for treatment of those inflammatory skin conditions, in the pathogenesis of which IL-12 is known to play a crucial role, such as psoriasis (Hong et al 1999). The bacitracin preparation used in this study is a mixture of at least 9 isoforms of which bacitracin A is the major component. Further work is needed to identify the isoform that is most active with regard to inhibition of IL-12 secretion.

In conclusion, our data imply that either targetting PDI or shifting the ER towards a more reduced status disrupts the oxidative folding pathway or assembly of IL-12 dimer forms. Folding of cytokines in the ER is likely to be actively regulated by chaperones (Vandenbroeck et al 2002). Pharmacological targetting of the ER as the site of cytokine production may provide an attractive alternative or complement to more conventional and widespread drug strategies aiming at the transcriptional stage of cytokine production.

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