The dual inhibitor of lipoxygenase and cyclooxygenase ML3000 decreases the expression of CXCR3 ligands

Ospelt, C; Kurowska-Stolarska, M; Neidhart, M; Michel, B A; Gay, R E; Laufer, S; Gay, S

Postprint available at:
http://www.zora.uzh.ch

Posted at the Zurich Open Repository and Archive, University of Zurich.
http://www.zora.uzh.ch

Originally published at:
The dual inhibitor of lipoxygenase and cyclooxygenase ML3000 decreases the expression of CXCR3 ligands

Abstract

OBJECTIVE: To find previously unknown properties of ML3000, a competitive inhibitor of the cyclooxygenase and the lipoxygenase (LO) pathway. METHODS: Gene expression of ML3000 treated and untreated rheumatoid arthritis synovial fibroblasts were measured with Affymetrix gene arrays. Downregulation of chemokine (C-X-C motif) ligands CXCL9, CXCL10 and CXCL11 was verified with Real-time polymerase chain reaction, CXCL10 protein levels were determined with ELISA. Rheumatoid arthritis synovial fibroblasts were treated with the cyclooxygenase inhibitor naproxen, the 5-LO inhibitor BWA4C and the 5-lipoxygenase-activating protein (FLAP) inhibitor MK886, and consecutive changes in CXCL10 protein levels measured. 5-LO expression was determined by polymerase chain reaction and Western blot. RESULTS: In synovial fibroblasts and monocyte-derived macrophages ML3000 inhibited the tumour necrosis factor induced expression of CXCL9, CXCL10 and CXCL11, which are all ligands of the chemokine receptor CXCR3. No effect was observed in monocytes. Whereas inhibition of the cyclooxygenase pathway or the FLAP protein showed no effect, blockade of 5-LO significantly downregulated CXCL10 protein levels. 5-LO mRNA was detected in monocytes and in monocyte-derived macrophages. All tested cell types expressed 5-LO protein. CONCLUSIONS: ML3000 effectively downregulates CXCR3 ligands. This study confirms that a thorough analysis of the impact of a drug on its target cells cannot only reveal unexpected properties of a substance, but also helps to understand the underlying molecular mechanisms. Accordingly, our data provide the basis for further clinical studies testing the application of ML3000 in diseases such as rheumatoid arthritis or multiple sclerosis.
The dual inhibitor of lipoxygenase and cyclooxygenase ML3000 decreases the expression of CXCR3 ligands

C Ospelt, M Kurowska-Stolarska, M Neidhart, B A Michel, R E Gay, S Laufer and S Gay

Ann Rheum Dis 2008;67;524-529; originally published online 31 Jul 2007; doi:10.1136/ard.2007.071589

Updated information and services can be found at:
http://ard.bmj.com/cgi/content/full/67/4/524

These include:

References
This article cites 31 articles, 8 of which can be accessed free at:
http://ard.bmj.com/cgi/content/full/67/4/524#BIBL

Rapid responses
You can respond to this article at:
http://ard.bmj.com/cgi/eletter-submit/67/4/524

Email alerting service
Receive free email alerts when new articles cite this article - sign up in the box at the top right corner of the article

Notes

To order reprints of this article go to:
http://journals.bmj.com/cgi/reprintform

To subscribe to Annals of the Rheumatic Diseases go to:
http://journals.bmj.com/subscriptions/
The dual inhibitor of lipoxygenase and cyclooxygenase ML3000 decreases the expression of CXCR3 ligands

C Ospelt,1 M Kurowska-Stolarska,1 M Neidhart,1 B A Michel, R E Gay,1 S Laufer,2 S Gay1

ABSTRACT

Objective: To find previously unknown properties of ML3000, a competitive inhibitor of the cyclooxygenase and the lipoxygenase (LO) pathway.

Methods: Gene expression of ML3000 treated and untreated rheumatoid arthritis synovial fibroblasts were measured with Affymetrix gene arrays. Downregulation of chemokine (C-X-C motif) ligands CXCL9, CXCL10 and CXCL11 was verified with Real-time polymerase chain reaction, CXCL10 protein levels were determined with ELISA. Rheumatoid arthritis synovial fibroblasts were treated with the cyclooxygenase inhibitor naproxen, the 5-LO inhibitor BWA4C and the 5-lipoxygenase-activating protein (FLAP) inhibitor MK886, and consecutive changes in CXCL10 protein levels measured. 5-LO expression was determined by polymerase chain reaction and Western blot.

Results: In synovial fibroblasts and monocyte-derived macrophages ML3000 inhibited the tumour necrosis factor induced expression of CXCL9, CXCL10 and CXCL11, which are all ligands of the chemokine receptor CXCR3. No effect was observed in monocytes. Whereas inhibition of the cyclooxygenase pathway or the FLAP protein showed no effect, blockade of 5-LO significantly downregulated CXCL10 protein levels. 5-LO mRNA was detected in monocytes and in monocyte-derived macrophages. All tested cell types expressed 5-LO protein.

Conclusions: ML3000 effectively downregulates CXCR3 ligands. This study confirms that a thorough analysis of the impact of a drug on its target cells cannot only reveal unexpected properties of a substance, but also helps to understand the underlying molecular mechanisms. Accordingly, our data provide the basis for further clinical studies testing the application of ML3000 in diseases such as rheumatoid arthritis or multiple sclerosis.
Ficol-Paque PLUS (Amersham Biosciences, Uppsala, Sweden) gradient centrifugation. Peripheral blood monocytes were then positively separated with CD14 microbeads (Miltenyi Biotec, Auburn, CA, USA) according to the manufacturer’s protocol. Monocyte-derived macrophages (MDMs) were generated by treatment of the isolated peripheral blood monocytes with 15 ng/ml macrophage colony-stimulating factor (R&D Systems, Minneapolis, MN, USA) every 48 h for 7 days. MDMs and monocytes were cultivated in RPMI 1640 (Gibco In Vitrogen) supplemented with 10% FCS, 50 IU/ml penicillin–streptomycin, 2 mM L-glutamine, 10 mM HEPES and 0.2% fungicide.

All cell cultures were maintained in a 5% CO₂ humidified incubator at 37°C.

**Stimulation**

ML3000 (Merckle GmbH, Blaubeuren, Germany) was reconstituted with DMSO to a stock concentration of 45 mg/ml, naproxen to a concentration of 150 mg/ml, BWA4C to 1.4 mg/ml and MK886 to 2.5 mg/ml (all Sigma, Basel, Switzerland). The drugs were further diluted with 2% human serum albumin (1:50), 0.5% human serum albumin (1:10), and with the corresponding medium without antibiotics, fungicides and with 0.5% FCS to the final working concentration. For control, DMSO alone was diluted correspondingly. Viability of RA SFs (RASF) after treatment with the above-mentioned drugs was assessed by trypan blue exclusion and by luminometric measurement of adenosine triphosphate with the ViaLight MDA Plus kit according to the manufacturer’s instructions (Cambrex, Taufkirchen, Germany).

In the chosen working concentrations all the drugs showed comparable effectiveness; ie, 3 μg/ml ML3000, 0.05 μg/ml MK886 and 0.03 μg/ml BWA4C similarly reduced the production of leucotriene B₄ in MDMs by 60–65%, and 3 μg/ml ML3000 and 30 μg/ml naproxen similarly reduced prostaglandin E₂ production in SFs by 85–90%. Leucotriene and prostaglandin 5-LO, but has no effect on the activity of 12-LO or 15-LO.

**Real-time polymerase chain reaction**

Total RNA was isolated from the RNeasy MiniPrep kit, including treatment with RNase-free DNase (Qiagen) and reverse transcribed using random hexamers and multiscribe reverse transcriptase (both Applied Biosystems, Rotkreuz, Switzerland). Non reverse transcribed samples were used as negative controls. Quantification of mRNA was performed by single-reporter Real-time polymerase chain reaction (PCR) using the ABI Prism 7700 Sequence Detection system (Applied Biosystems). Eukaryotic 18S rRNA levels, measured with a pre-developed primer/probe system (Applied Biosystems) were used as endogenous control for relative quantification. The differences of the comparative threshold cycle (Ct) values of sample and 18S cDNA were calculated (ddCt). Relative expression levels were calculated following the formula $dCt = dCt (sample \text{untreated}) - dCt (sample \text{stimulated})$. Relative expression was calculated using the expression $2^{-\Delta\Delta Ct}$. Only samples with a difference of at least four cycles between cDNA and non-reverse, transcribed samples were considered for calculations.

Primers were designed with Primer 3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi, accessed 1 June 2007). Dissociation curve analysis for each SYBR green primer pair and reaction was performed to verify specific amplification.

CXCL9: forward primer $5'$-GGC ATC ATC TTG CTT GGT CT-3', reverse primer $5'$-TCA CTA CTG GGG TTC CTG GC-3'; CXCL10: forward primer $5'$-AAC CAG AGG GGA GCA AAA TC-3', reverse primer $5'$-CTG TGT GGT CCA TCC TTT G-3'; CXCL11: forward primer $5'$-TGG CAA CAG TGC ACA TAT TTC A-3', reverse primer $5'$-CAA ATT AAG ACC GGT GCT GCT A-3'.

**Enzyme-linked immunoabsorbent assay**

CXCL10 protein was detected in the supernatant using DuoSet ELISA Development Systems (R&D Systems) according to the manufacturer’s instructions. Absorption was measured at 450 nm and data were analysed using Revelation v4.22 software (Dynex Technologies, Denkendorf, Germany).

**Conventional polymerase chain reaction**

Total RNA was isolated as described above for real-time PCR. Conventional PCR was performed on a GenAmp PCR System 9700 (Applied Biosystems) with the following primer pairs and protocols: 5-LO: forward primer $5'$-TAC ATG GAG TTC CCC TGC TAC-3', reverse primer $5'$-CTT TAC TAC GTT GGT TCT-3'; β-microglobulin forward primer $5'$-AGG ATT CAG GTT TAC TCA CTT GCT C-3', reverse primer $5'$-TGA TGC TGC TTA CAT GTT TCG-3'; 94°C 5 min, 25 cycles with 94°C 50 s, 57°C (for 5-LO)/55°C (for β-microglobulin) 30 s, 72°C 30 s and a final elongation of 5 min with 72°C. Reaction products were separated on a 1% agarose gel and signals were visualised using ethidium bromide. As a negative control, PCR was carried out in the absence of cDNA for each set of primers.
versus untreated RASF, both stimulated with TNF-α. To screen for genes that are regulated by treatment with ML3000, we used an Affymetrix high-density oligonucleotide microarray. 

**RESULTS**

Expression levels were measured by real-time polymerase chain reaction. Values are shown as mean percentage expression (SEM) as compared with control.

Western blot

Cells were lysed in 2× Laemmli buffer, denatured for 3 min at 95°C and separated on 10% SDS–PAGE. After transfer on to Protran nitrocellulose transfer membranes (Schleicher & Schüll, Dassel, Germany) and blocking with 5% non-fat dry milk, membranes were incubated with mouse anti-human 5-LO antibodies (BD Transduction Laboratories, San Diego, CA, USA) overnight at 4°C (dilution 1:250). Signals were visualised by incubation with peroxidase-conjugated goat anti-mouse antibodies (Jackson ImmunoResearch, West Grove, PA, USA) and enhanced chemiluminescence system (Amersham Biosciences, Otelfingen, Switzerland). For normalisation, membranes were stripped and probed with mouse anti-human α-tubulin antibodies (Sigma).

Statistical analysis

Values are presented as mean (SEM); n = number of stimulations. Wilcoxon matched-pairs signed-ranks test was used for statistical evaluation of the data by SPSS software; p<0.05 was considered significant.

**MECHANISMS OF CXCR3 LIGAND REGULATION**

To find out whether the blockade of the COX or the 5-LO pathway is responsible for the described effects of ML3000, we treated TNF-α-stimulated RASF with the COX inhibitor naproxen, the 5-LO inhibitor BWA4C and with the inhibitor of the 5-LO activating protein (FLAP) MK886. The COX inhibitor naproxen, had no effect on CXCL10 production by TNF-α-stimulated RASF. Treatment with the FLAP inhibitor only slightly reduced the production of CXCL10 protein in some of the tested patient cells; therefore, no statistical significance was reached. In contrast, downregulation of CXCL10 protein levels after treatment with BWA4C was consistent and reached statistical significance (fig 4). The combination of either 5-LO inhibitor, BWA4C or MK886 with naproxen, had the same inhibitory effect on the production of CXCL10 as treatment with the 5-LO inhibitor alone (n = 2; data not shown). Thereby, we concluded that inhibition of CXCR3 ligand production by ML3000 must be an effect, that is independent of its ability to block the COX pathway, but is linked to its blockade of 5-LO.

Furthermore, mRNA and protein levels of 5-LO were measured. We could detect a strong band for 5-LO mRNA in monocytes, only a weak band in MDMs and no band was visible in SF (fig 5a). All measured cell types had detectable levels of 5-LO protein, albeit they were noticeably higher in monocytes compared with MDMs or SF (fig 5b). Thus, we assume that different regulation of 5-LO transcription and de novo synthesis in the analysed cell types could not be explained by differences in the expression of 5-LO mRNA or protein.
Influence their sensitivity to treatment with ML3000 in respect to the measured outcomes.

**DISCUSSION**

In the present study, we analysed the impact of ML3000 on the transcriptome of SFs and found a previously unknown, anti-inflammatory action. Thereby, we used microarray technology as starting point for further experiments, characterising the newly found properties in-depth. This simplified microarray approach is not motivated by the generation of a complete gene expression profile with clusters of genes that correlate with a specific condition like in large-scale microarray experiments, but serves as a basic screening technique for novel, unexpected properties of a drug.

In our screening, we found evidence that ML3000 has an impact on the transcriptional regulation of the chemokines CXCL9, CXCL10 and CXCL11, all three ligands of CXCR3. This effect was measurable on the mRNA as well as on the protein level and was not restricted to SF or stimulation with TNF-α. Chemokines in general play a fundamental role in the migration of leucocytes to the site of inflammation. In the subgroup of CXC chemokines, the CXCR3 ligands take an exceptional position. While most CXC chemokines are clustered at chromosome 4q12–13 and act on neutrophils, CXCL9, CXCL10 and CXCL11 are found at 4q21.21 and mainly attract T lymphocytes. High levels of CXCL9, 10 and 11 could be detected in the synovial fluid of patients with RA. They are mainly produced by fibroblasts and macrophages in the synovium and are thought to be involved in the recruitment of T helper 1 cells into the joint (for review). CXCR3 ligands were also found to play a crucial part in the accumulation of activated lymphocytes in the brain of patients with multiple sclerosis. Moreover, it is suggested that T cell attraction in autoimmune liver disease, myasthenia gravis and acute renal allograft rejection is mediated by CXCR3 and its ligands.

Thus, this group of chemokines has been suggested as drug targets in a variety of diseases. In fact, the application of a decoy chemokine receptor DNA for the binding site of CXCR3 was shown to suppress the relapse of experimental autoimmune encephalomyelitis in rats. Therefore, our data provide the basis for further experiments testing the application and effectiveness of ML3000 in diseases, such as multiple sclerosis and acute allograft rejection, and thus a new field of indications for ML3000 could be opened up.

We found that the ability of ML3000 to inhibit CXCR3 ligand production does not stem from its ability to block the COX pathway, but seems to be linked to the inhibition of 5-LO. In accordance with our experiments, it was previously described in macrophages and cancer cells that prostaglandin E2 can suppress CXCL10 production and consequently, inhibition of the COX pathway rather enhances production of CXCL10. Stimulation of SFs with prostaglandin E2 and/or leucotriene B4 had no effect on the expression of either CXCL9, 10 or 11, which remained under the detection limit (data not shown). Additionally there was no difference between the tested 5-LO inhibitors in their potential to inhibit leucotriene B4 synthesis in

**Figure 2**

(A) Downregulation of CXCL9 mRNA after treatment with ML3000 in stimulated rheumatoid arthritis synovial fibroblasts (RASF; n = 8). (B) Time course of the effect of ML3000 on the expression of CXCL9, 10 and 11 (n = 3). Downregulation of CXCL9, CXCL10 and CXCL11 mRNA after treatment with ML3000 in stimulated (C) OA SF (n = 5), (D) monocytes (n = 5) and monocyte-derived macrophages (MDMs; n = 6). Expression levels were measured by real-time polymerase chain reaction. TNF, tumour necrosis factor; IFN, interferon.
monocytes. Therefore the downregulation of CXCR3 ligands is probably independent of the role of 5-LO in leucotriene biosynthesis. We assume that inhibition of the production of this specific group of chemokines could be a direct effect of inhibiting other functions of the 5-LO protein. Functions of 5-LO beyond the leucotriene pathway have been suggested before and are supported by studies showing 5-LO to localise at sites of active gene transcription in the nucleus even in cells that are not producing leucotrienes.27 By means of a yeast two-hybrid system 5-LO was found to interact with the transforming growth factor type b receptor-I-associated protein 1 (TRAP-1). It was suggested that via TRAP-1, 5-LO is associated with the activated transforming growth factor-β receptor and modulates its signalling.28 However, up to now the association between the expression of CXCL10 and transforming growth factor-β receptor has not been described yet, and further studies have to be conducted to elucidate these cellular functions of 5-LO.

Regulation, localisation and translocation of 5-LO have been found to be highly variable between different cell types.29 In particular it has been shown that while monocytes express high amounts of 5-LO mRNA, this production is lost during the maturation to MDMs30. In resting cells 5-LO is stored, and only upon activation translocated and bound to membranes.31 These findings can explain why we could only detect mRNA for 5-LO in monocytes, but not in MDMs or SFs. In contrast, stored 5-LO protein could be found in all tested cell types. Inhibition of 5-LO activity by blocking its active centre could possibly be overcome in monocytes via increased de novo production.

In summary, we show that the COX/5-LO inhibitor ML3000 inhibits the production of the CXCR3 ligands CXCL10, CXCL9 and CXCL11. Furthermore, we found evidence for new cellular roles of 5-LO. Our study proves that by analysing the molecular impact of a drug on its target cells, off-target effects and their underlying molecular mechanisms can be revealed. In our case, off-target effects of the analysed drug turned out to be beneficial, as CXCR3 ligands have repeatedly been suggested as possible drug targets for a variety of inflammatory diseases. Accordingly, animal and clinical studies testing the application of ML3000 in diseases such as RA and multiple sclerosis or acute allograft rejection should be taken into consideration. On the other hand, off-target effects are often the cause of adverse side-effects, which makes it even more important to include such molecular studies early on in the development of a drug.

Figure 3 Regulation of CXCL10 protein levels as measured by ELISA in supernatants of stimulated rheumatoid arthritis synovial fibroblasts (RASFs; n = 7), osteoarthritis synovial fibroblasts (OASF; n = 5), monocytes (n = 4) and monocyte-derived macrophages (MDMs; n = 6) after treatment with 3 μg/ml ML3000. (A) Values are shown as mean percentage expression (SEM) as compared with control. *p<0.05. (B) Levels of CXCL10 in each individual measurement are shown before and after treatment.
REFERENCES

None.

Acknowledgements: We thank Maria Comazzi and Ferenc Pataky for their excellent technical assistance. We thank the Functional Genomic Center Zurich for their technical support.

Funding: The study was financially supported by a research grant from Merckle, Germany. The company did not exert any influence on study design, analysis and interpretation of the data or in the writing of the report, nor in the decision to submit the paper for publication.

Competing interests: None.

REFERENCES


