Systems Biology based studies on anti-inflammatory compounds

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Chapter 3

Categorization of anti-inflammatory compounds using transcriptomics, proteomics, and metabolomics in combination with multivariate data analysis.

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The discovery of new anti-inflammatory drugs is often based on an interaction with a specific target, although other pathways often play a primary or secondary role. Anti-inflammatory drugs can be categorized into classes, based on their mechanism of action. In this article we investigate the possibility to categorize novel anti-inflammatory compounds by three holistic methods. For this purpose, we make use of macrophage-like U937 cells which are stimulated with LPS in the absence or presence of an anti-inflammatory compound. Using microarrays, 2-D gel electrophoresis and a LC-MS method for lipids the effects on the transcriptome, proteome and metabolome of the exposed cells is investigated. The expression patterns are subsequently analyzed using in-house developed pattern recognition tools. Using the methods described above, we have examined the effects of 6 anti-inflammatory compounds. Our results demonstrate that different classes of anti-inflammatory compounds show distinct and characteristic mRNA, protein, and lipid expression patterns, which can be used to categorize known molecules and to discover and classify new leads. The potential of our approach is illustrated by the analysis of several beta$_2$-adrenergic receptor agonists (β_2-agonists). In addition to their primary pharmacological target, β_2-AR agonists posses certain anti-inflammatory properties. We were able to show that zilpaterol, a poorly characterized β_2-agonist, gives rise to an almost identical expression pattern as the β_2-agonists clenbuterol and salbutamol. Furthermore we have identified specific mRNA, protein and lipid markers for the anti-inflammatory compounds investigated in this study.

Introduction

Inflammation plays a pivotal role in the pathogenesis of many diseases. The development of novel inflammatory modulators to support treatment of such disorders is of major interest to the pharmaceutical industry. As a consequence, the number of anti-inflammatory drugs in the pharmaceutical pipeline is increasing rapidly. The discovery of these drugs is still often based on an interaction with a specific target or by measuring single or multiple cellular endpoints such as cytokine production, e.g. TNF-α. Current anti-inflammatory drugs can be categorized into a number of classes, based on their mechanism of action. These include among others NF-κB inhibitors, corticosteroids, anti-cytokine antibodies, anti-inflammatory cytokines,

cytokine-antagonists, enzyme inhibitors, kinase-inhibitors, proteasome inhibitors, and apoptosis inducers. However, inflammation is a complex process involving a variety of cell-types and hundreds of different inflammatory mediators. Although different signal transduction pathways may share similar endpoints, such as TNF-α inhibition, modulating these may give rise to completely different cellular reactions. TNF-α was found to be regulated by different MAP kinases (e.g. p38, ERK) [1] and transcription factors (e.g. NFκB, AP-1 and CREB) [2-5]. These MAP kinases and transcription factors are in turn activated by other kinases in different signalling pathways. Because of this, it is increasingly recognised that more holistic, systems biology methods should be introduced in the drug discovery process.

In this paper we integrate three biological levels, the transcriptome, proteome and the metabolome, to categorize inflammatory modulators on the basis of the biological responses that they elicit. Our methodology makes use of differentiated U937 macrophages as a model system, because macrophages are the major targets of anti-inflammatory agents. The U937 cell line is widely accepted as a model system for human macrophages as exemplified in our previously published observations on the expression of macrophage maturation markers in this cell line [6]. The U937 cells are stimulated with the endotoxin LPS, that induces a broad range of inflammatory pathways. By using oligonucleotide microarrays, 2-D gel electrophoresis and LC-MS, the effects of LPS-exposure on the transcriptome, proteome and metabolome are readily visualized. When this procedure is performed in the presence of an anti-inflammatory compound, this leads to characteristic changes in the U937 mRNA, protein and metabolite expression patterns. The patterns of the anti-inflammatory compound under investigation are compared to those of known inflammatory modulators using principal component discriminant analysis (PC-DA) [7]. This mathematical tool enables the rapid classification of anti-inflammatory compounds. In this study we have examined four different classes with anti-inflammatory properties, each of which has its own specific, partially overlapping inhibitory effect. We have studied dexamethasone, a corticosteroid [4, 5, 8, 9], a proteasome inhibitor (PSI) [10-12], the MAP kinase inhibitor SB203580 [13] and two β_2-adrenoreceptor agonists, clenbuterol [14, 15] and salbutamol [16]. In addition to their primary pharmacological target, β_2-agonists posses certain anti-inflammatory properties [14]. Our approach was evaluated with zilpaterol, a compound originally developed as β_2-agonist, but later specifically being introduced for yet another effect of this class, namely as a growth promoting (anabolic) agent for use in cattle [17, 18].

Materials and Methods

Chemicals

Lipopolysaccharide (LPS, *E.coli* 0111:B4), clenbuterol, salbutamol, formoterol, and dexamethasone were obtained from Sigma Aldrich (St. Louis, MO, USA). SB203580 and proteasome inhibitor (PSI) were purchased from Omnilabo international B.V. (Breda, The Netherlands) and zilpaterol from Intervet Inc. (Millsboro, US). The chemicals and equipment used for two-dimensional gel electrophoresis were obtained from Amersham biosciences (Uppsala, Sweden) unless stated otherwise.

Cell cultures and incubations

Human monocyte-like histiocytic lymphoma cells U937 [19] obtained from the ATCC (CRL-1593.2) were grown in RPMI-1640 medium, supplemented with 10% (v/v) fetal calf serum and 2 mM L-glutamine (Life technologies, Breda, The Netherlands) at 37 °C, 5% CO_2 in a humidified atmosphere. U937 monocytic cells were differentiated into macrophages using phorbol 12-myristate 13-acetate (PMA, 10 ng/ml, overnight, Omnilabo, Breda, The Netherlands) as described previously [20]. The PMA-differentiated macrophages were allowed to recover from PMA treatment for 48 h, during which the culture medium was replaced daily. At day three after PMA treatment, the macrophages were exposed for 6 h to LPS (E.Coli, 1µg/ml) in the presence or absence of an inflammatory inhibitor. The following inflammatory inhibitors were used: clenbuterol ($1x10^{-7}$ M), salbutamol ($1x10^{-6}$ M), zilpaterol ($1x10^{-6}$ M), dexamethasone ($1x10^{-7}$ M), SB203580 ($1x10^{-6}$ M), proteasome inhibitor (PSI, $1x10^{-5}$ M) and formoterol ($1x10^{-8}$ M). The concentrations of inhibitors used resulted in a 15 – 30 % inhibition of TNF-α release (see results section), as determined in a dose-response experiment in which the effect on TNF-α release by U937 macrophages was measured (data not shown). The incubations were performed in duplicate.

Transcriptomics

Incubations of U937 macrophages with LPS, with or without an inhibitor, where performed in triplicate and pooled afterwards. Total RNA was extracted from stimulated U937 macrophages using Trizol reagent (Life technologies, Rockville, USA) and RNeasy columns (Qiagen, Westburg, Leusden, The Netherlands) according to the manufacturer's protocol. DNase treatment (Qiagen) was performed on the RNeasy column. Each sample was analysed by oligonucleotide microarrays in triplicate, in which LPS was labelled with Cy-5 and LPS + inhibitor was labelled with Cy-3. In addition, a control experiment was performed

simultaneously by comparing LPS-Cy-5 with LPS-Cy-3 on the same array, to check the differences in labelling efficiency. Subsequently, the microarray experiments and data processing were performed as described before [6]. The resulting list of genes with their normalized log transformed spot volumes per array were analyzed by principal component analysis (PCA) and principal component discriminant analysis (PC-DA).

Proteomics

The stimulated U937 cell pellets were dissolved in lysis buffer containing 8 M urea, 2% (w/v) CHAPS, 0.02% (v/v) Pharmalytes, and 1% (w/v) dithiothreitol (Sigma-Aldrich chemie, Zwijndrecht, The Netherlands). The protein concentration was determined using Bradford reagent (Bio-Rad, Veenendaal, The Netherlands) according to the manufacturer's protocol. The protein extract (400 µg) was further diluted to 350 µl with rehydratation buffer containing 8 M urea, 0.5% (w/v) CHAPS, 2 mM tributyl phosphine (Fluka, Buchs SG, Switzerland), and 1% (v/v) IPG ampholytes pH 4-7. Per sample, two gels were processed and analyzed. In total 24 gels were run.

The first dimension was carried out on an IPGphor system using pH 4-7 IPG gel strips of 18 cm. The IEF was performed at 20 ºC under the following conditions: 12 h at 30 V; 30 min at 150 V; 1 h at 300 V; 1 h at 1500 V and 7.5 h at 8000V. After isoelectric focusing, the IPG strips were equilibrated for 30 min in a buffer containing 6 M urea, 30% (v/v) glycerol, 5 mM tributyl phosphine, and 2% (w/v) SDS in 0.05 M Tris-HCl buffer, pH 8.8. The second dimensional separations were carried out on custom made 12% SDS-polyacrylamide gels using a Hoefer DALT electrophoresis system. The gels were stained with RuBPS fluorescent staining using the protocol which is described in Rabilloud et al [21, 22]. The gels were scanned on the Typhoon laser scanner at 600 V with a green laser (532 nm) for excitation and 610 BP 30 emission filter.

Scanned TIFF images were analyzed using the Progenesis workstation software package (Nonlinear Dynamics, Newcastle upon Tyne, UK). Spots were automatically detected and visually checked for undetected or incorrectly detected spots. The protein spots detected in each experimental gel were matched to the corresponding spot in a digitized reference gel. Intensity levels were normalized between gels by dividing the spot intensity through the total intensity of all the spots in the gel. A list of spots with their normalized spot volumes per gel was analyzed by PCA and PC-DA. Protein spots were identified by in-gel digestion followed by MALDI mass mapping and/or MALDI MS/MS sequencing in combination with database search as described before [6].

Metabolomics

The treated U937 cell pellets containing 580 µg of protein were dissolved in 250 µl isopropanol and centrifuged at 1000 g for 10 min. The supernatant (75 µl) was injected in duplicate on a Inertsil ODS 3 (100 x 3 mm i.d. 5 µm, Varian Chrompack, Middelburg, The Netherlands) with S2 guard column (10 x 2 mm i.d., Varian Chrompack) and a flow of 0.7 ml/min. For elution the following solvents were used: 5 % acetonitrile in water (mobile phase A), 30 % isopropanol in acetonitrile (mobile phase B), and 50 % dichloromethane in isopropanol (mobile phase C). The gradient used was as follows: A:B (70:30) at 0 min until 2 min; A:B (5:95) at 15 min; A:B:C (5:35:60) at 35 min until 40 min; A:B (5:95) at 41 min and A:B (70:30) at 50 min. The separation was performed at 20 ºC. In total 24 injections were performed in randomized order. Detection was conducted on-line with a Thermo Finnigan TSQ 700 mass spectrometer (Thermo Finnigan, San Jose, USA) using electrospray interface in positive mode. The TSQ 700 operating conditions were as follows: heated capillary temperature, 225 ºC; spray voltage, 4 kV; sheath gas (nitrogen), 70 psi; auxiliary gas (nitrogen) 15 psi; scan range 300-1900, and scan speed 1.5 s/scan. Internal standards, lysophosphatidylcholine (C17:0), phosphatidylcholine (di-C12:0), and triglyceride (tri-C17:0) were used to normalize the data for peak intensity and retention time alignment. The data were processed using TNO IMPRESS version 1.9, an in-house developed software package to generate peak tables of all files. Alignment of retention times was performed using TNO – EQUEST 2.1. A list of aligned retention times, m/z values and peak areas were analyzed with PCA and PC-DA.

Data analysis

The datasets were analysed by PCA using the PLS tool box for Matlab (version 2.0; Eigenvector Research, Washington, USA). Only with the transcriptomics data we obtained clustering and separation of the different classes of anti-inflammatory inhibitors. Additionally, the datasets (transcriptomics, proteomics and metabolomics) were analysed with PC-DA using an in house developed function for Matlab (version 6.5.1, release 13, The Mathworks, Inc., 2003).

PCA describes data as a linear combination of so-called scores and loadings. These linear combinations are called principal components. The scores and loadings vectors give a concise and simplified description of the variance present in the dataset [23, 24].

PC-DA is based on the assumption that samples of the same group are more similar compared to samples of other groups [7]. The goal of PC-DA is to find and identify structures in the

original data that show large differences in the group means. This process involves a priori knowledge of which samples are similar. Therefore, PC-DA is said to be a supervised analysis technique. The data of the individual datasets were mean-centered [23, 24]. The combined dataset was scaled on the range of the variables followed by mean-centering.

Quantitative real time polymerase chain reaction (PCR)

Primers for human CXCR5 (forward primer; 5'-TCA GAC TGG TTG AGT TCA GGT AGC T-3', reverse primer; 5'ACC CAG GAT CCG GTG ACA T-3', TaqMan® probe; 5'CCC CTG GCT CTG ACC GAA ACA GC-3'), human CXCL6 (forward primer; 5'-AAT TTT GGA CAG TGG AAA CAA GAA A-3', reverse primer; 5'-AGA AAA CTG CTC CGC TGA AGA CT -3', TaqMan® probe; 5'-ACT GAG TAA CAA AAA AGA CCA TGC ATC ATA AAA TTG C-3'), and human ß-actin (forward primer; 5'CTG ACT GAC TAC CTC ATG AAG ATC CT 3', reverse primer; 5'CTT AAT GTC ACG CAC GAT TTC C 3', TaqMan® probe; TAC AGC TTC ACC ACC ACG GCC GA-3') were purchased from Applied Biosystems (Nieuwerkerk a/d IJssel, The Netherlands). The RT reaction was performed on 500 ng of total RNA with avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI, USA). Quantitative real time polymerase chain reactions were performed using TaqMan® probes. PCR reactions were performed in a total volume of 20 µl 1x TaqMan® Universal Mastermix in the iCycler iQ™ Real-Time PCR detection system (Bio Rad). The PCR program was as follows: 1 cycle 2 min at 50 °C, 1 cycle 10 min at 95 °C; 50 cycles 15 sec at 95 °C, 60 sec at 60 °C. The absolute number of copies of the gene of interest in the experimental cDNA samples was calculated from the linear regression of a standard curve. The expression of the measured genes in each sample was normalized for ß-actin expression.

Enzyme-Linked Immunosorbent Assay (ELISA) and enzyme activity measurements

Immunoassays for CXCL6 and oncostatin M were purchased from R&D systems (Oxon, UK). The VEGF ELISA was obtained from Prepotech (London, UK) and the cytoset antibody pair kit for TNF-α from Biosource (Etten-Leur, The Netherlands). The immunoassays were performed according to the manufactures instructions. All samples were analyzed in duplicate. The enzyme activity of lactate dehydrogenase (LDH) was quantitatively determined using the *in vitro* assay for LDH activity from Roche Diagnostics (Mannheim, Germany) for automated clinical chemistry analyzer Hitachi 911 (Hitachi, Japan).

Results

The effect of the inhibitors on LPS activated U937 cells was tested by measuring the TNF-α release and LDH activity in the cell culture media (Fig. 1.). After 6 h of LPS stimulation both early inflammatory effects (e.g. TNF-α release) and late inflammatory effects (e.g. IL-10 release) were detectable (data not shown). The concentrations of the various inhibitors were chosen as such to achieve similar levels of TNF-α inhibition. At these concentrations, none of the inhibitors induced LDH release significantly above the level obtained with LPS alone. The one exception to this is PSI, which did lead to a 2 to 3-fold increase in LDH release. The PSI concentration used in this experiment was chosen in order to minimise the apoptotic effect while still generating a clear anti-inflammatory effect, although it was not possible to separate the two effects (data not shown).

Transcriptomics

The same experimental conditions were used to test the effects of the inhibitors on the mRNA expression levels in U937 macrophages stimulated by LPS. The microarrays used contained 21,529 oligonucleotides which correspond to 21,316 genes. After data pre-processing and normalisation a dataset of 9,436 genes remained and was used in the PC-DA. Figure 2 shows the results of the PC-DA of the microarray dataset.

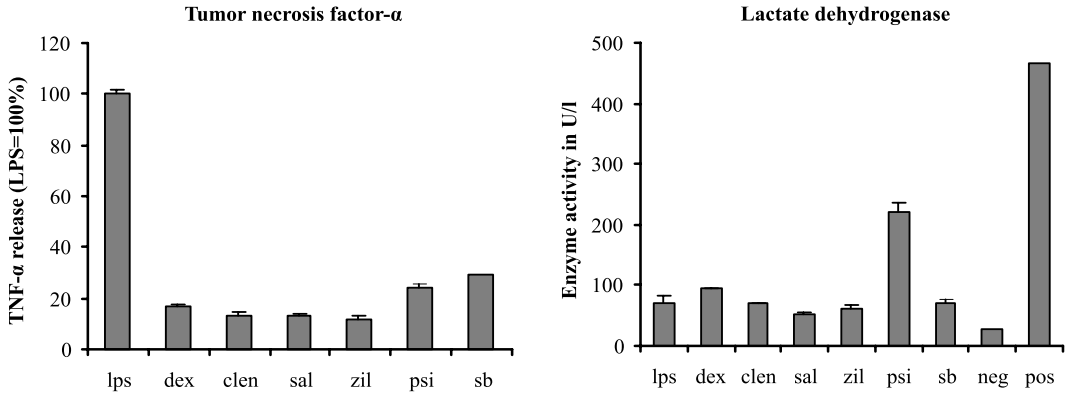

Figure 1 Release of TNF-α (left panel) and LDH enzyme activity (right panel) from U937 macrophages incubated for 6 h with LPS with or without an anti-inflammatory inhibitor, dexamethasone (dex, $1x10^{-7}$ M), clenbuterol (clen, $1x10^{-7}$ M), salbutamol (sal, $1x10^{-6}$ M), zilpaterol (zil, $1x10^{-6}$ M), proteasome inhibitor (psi, $1x10^{-5}$ M) and SB203580 (sb, $1x10^{-6}$ M). The release of TNF-α is given with respect to the TNF-α release of LPS stimulated cells. The enzyme activity of LDH in the culture media reflects the extent of cell death. As control incubations either culture media not incubated with cells (negative control, neg) or culture media from U937 cells lysed by repeated freeze-thawing (positive control, pos) were used. The results are presented as means ± SD of duplicate measurements.

The four classes of inhibitors were separated from each other. The two known β_2-agonists clenbuterol and salbutamol gave rise to overlapping clusters, which illustrates that the separation of the datasets reflects the biological responses elicited by the various inhibitors. Zilpaterol showed a similar pattern as clenbuterol and salbutamol. This was illustrated by the clustering of the array data from zilpaterol near to the array data from clenbuterol and salbutamol (Fig. 2). The data points of the PSI array data were positioned at a relatively great distance from the other data points, which may reflect the fact that the U937 transcriptome in response to PSI is very different from the transcriptome obtained after incubation with the other inflammatory inhibitors.

To further explore the specificity of the response to β_2-agonists compared to the other inhibitors, the microarray data was examined using PC-DA by defining two new groups (Fig. 3). Group one contained the microarray data from the two β_2-agonists, clenbuterol, and salbutamol and the poorly characterized β_2-agonist, zilpaterol. The second group consisted of the microarray data of dexamethasone, SB203580, and PSI. The results show that the data was separated in only one dimension in a score plot of two groups (Fig. 3A). From the loading plot, the individual genes responsible for the separation of the complete datasets could be derived (Fig. 3B).

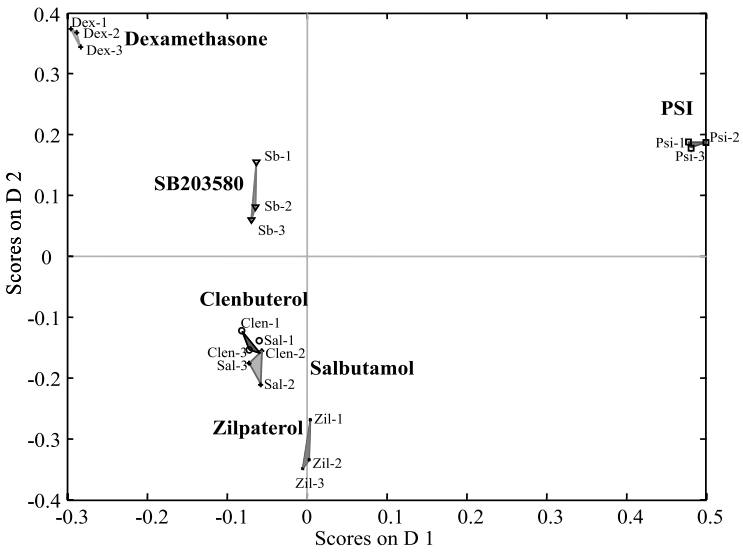

Figure 2 Graphical presentation of the microarray data analyzed by PC-DA. The arrays of zilpaterol (zil ×), clenbuterol (clen ○), salbutamol (sal*) SB203580 (sb Δ), dexamethasone (dex +), and proteasome inhibitor (PSI □) are represented as dots, asterisks or crosses and array ID code. For every inhibitor three symbols are shown, each representing a separate micro-array. The anti-inflammatory inhibitors show different mRNA expression patterns. The arrays form the three β_2-agonists are clustered in the lower left corner in the score plot. This indicates that the mRNA expression patterns are comparable.

Negative loadings indicated the genes that are important in the clustering of the array data of the β_2-agonists, and are putative markers for this class of inhibitors. Figure 4 represents the differential expression of the first nine genes that showed the highest negative loadings in the loading plot. The three β_2-agonists were found to regulate seven out of nine genes (CXCR5, CXCL6, VEGF, oncostatin M, Mitogen induced nuclear orphan receptor, and small breast epithelial mucin) in a similar manner. The effects of the other inflammatory inhibitors on the

expression of these genes, was either opposite to the effect of the β_2-agonists or showed no regulation at all. These seven genes could therefore be specific markers for the β_2-agonists compared to PSI, dexamethasone and SB203580.

The differential expression of the two marker genes that were found to be regulated in a similar way by zilpaterol, clenbuterol, and salbutamol according to the microarray data analyzed by PC-DA, was confirmed by real time PCR experiments (Fig. 5). For this experiment, cells were incubated with the same inflammatory inhibitors as used in the microarray experiment. In addition, a fourth well-characterized β_2-agonist, formoterol [25], was tested to further confirm the specificity of the markers found. The results clearly showed that CXCL6 and CXCR5 are specifically induced in cells exposed to LPS and a β_2-agonist (clenbuterol, salbutamol, zilpaterol, and formoterol), compared to cells treated with LPS alone. Cells incubated with LPS and dexamethasone, PSI or SB203580 showed a down-regulation compared to cells treated with LPS alone. This confirms the observations made in the microarray experiments.

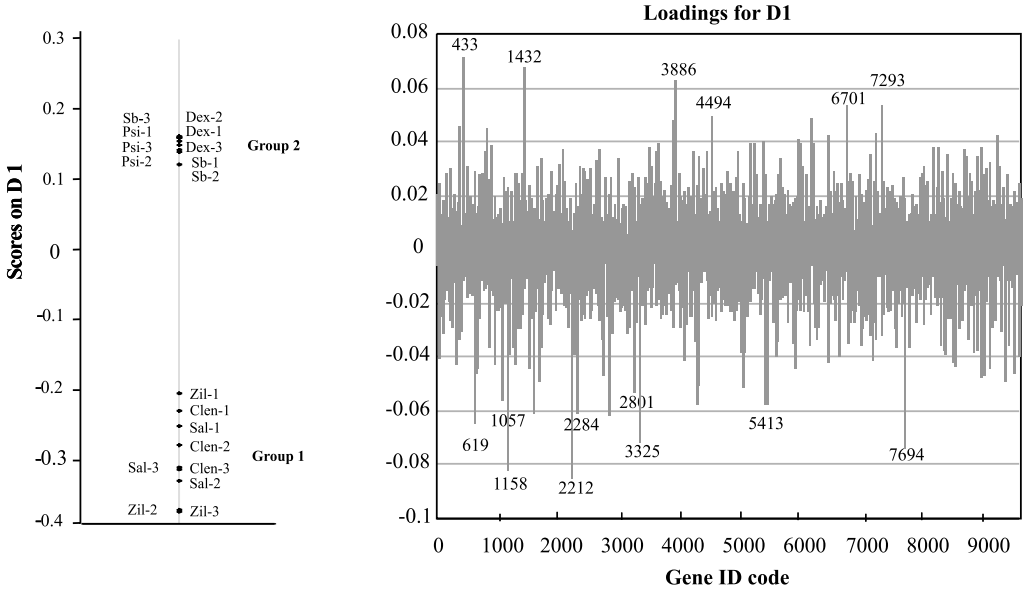

Figure 3 PC-DA of the microarray data whereby the arrays from the three β_2-agonists are combined in group 1. Group 2 contains the array data from dexamethasone, SB203580 and PSI. Left panel: The score plot gives an indication about clustering and trends present in the mRNA profiles. The individual arrays are represented as asterisks. The result shows that the arrays from group 1 cluster between -0.2 and -0.4 on the D1 axis, and the arrays from group 2 cluster between 0.1 and 0.2, indicating that the two groups can be separated on the basis of their mRNA expression profiles. Right panel: The loadings for D1 from the score plot (left panel). The loading plot indicates which genes are responsible for the difference between the two defined groups. Genes with high positive and high negative loadings represent the genes that are differentially regulated between the two groups. The negative loadings represent genes that are involved in group 1. Genes coded 1158 and 2212 have high loadings and are therefore important determinants for the separation of the two groups by PC-DA.

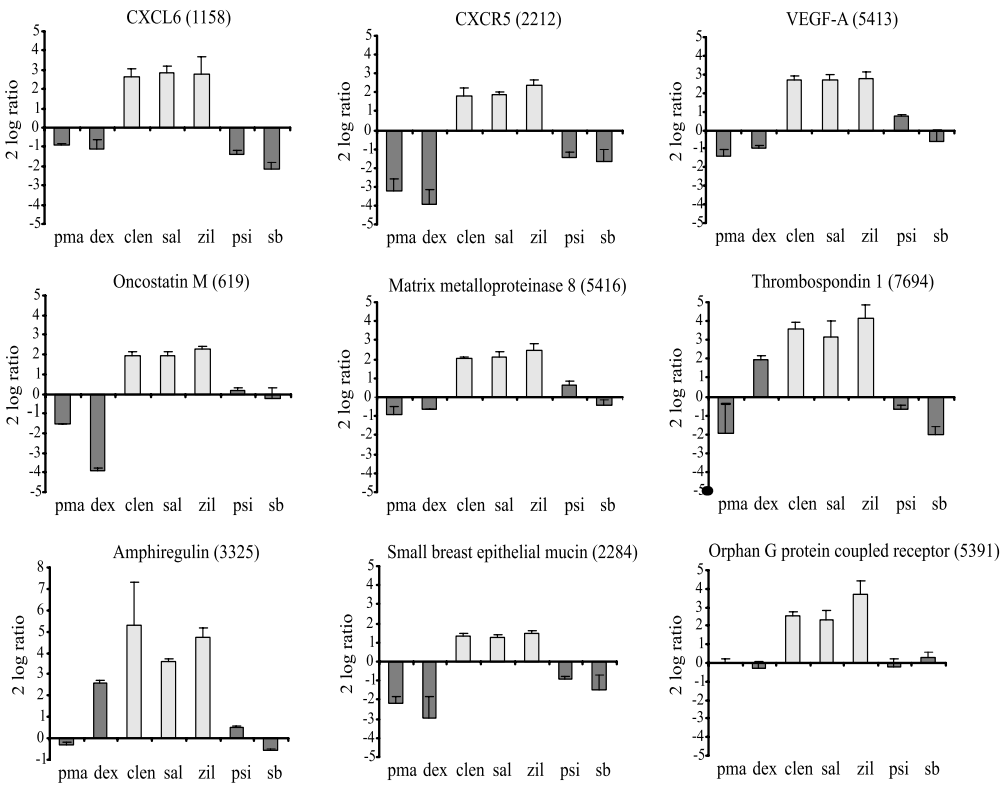

Figure 4 Differential mRNA expression of the 9 genes that showed the highest negative loadings according to PC-DA. The induction factor is presented as a 2 log ratio value. The 9 genes were found to be regulated in a similar manner by zilpaterol (zil), salbutamol (sal) and clenbuterol (clen) according to PC-DA analysis (light grey bars). The expression levels are compared to proteasome inhibitor (PSI), dexamethasone (dex), and SB203580 (sb) (dark grey bars). The numbers between brackets indicate the gene ID code used in PC-DA.

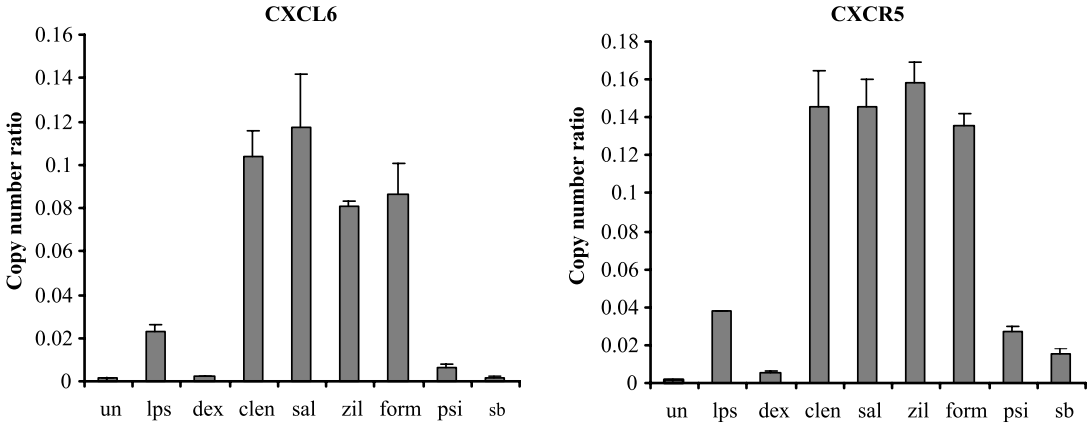

Figure 5 mRNA expression levels of CXCL6 and CXCR5 determined in untreated U937 macrophages (un) and after a 6 hours incubation with LPS with or without an anti-inflammatory inhibitor, dexamethasone (dex), clenbuterol (clen), salbutamol (sal), zilpaterol (zil), formoterol (form), proteasome inhibitor (PSI), and SB203580 (sb). The expression levels of CXCL6 and CXCR5 mRNA were determined by real time real time PCR. The copy number given was corrected for the expression levels of the reference mRNA β-actin and is the mean + SD of two experiments.

To test whether the identified β_2-agonists marker genes are also specifically regulated at the protein level, a limited number of identified marker genes were selected to determine their regulation at the protein level. These included three secreted proteins, Oncostatin M, VEGF, and CXCL6, which expression in U937 cells was analysed by using specific immunoassays. U937 macrophages where stimulated in duplicate according to a time series (6, 8, 16, and 24 h) without LPS or with LPS in the presence or absence of an inflammatory inhibitor. The results show that Oncostatin M, VEGF, and CXCL-6 were all up-regulated in cells treated with the combination of LPS and a β_2-agonist compared to cells treated with LPS alone (Fig. 6). The highest level of expression of all three proteins was detected after 16 to 24 h incubation.

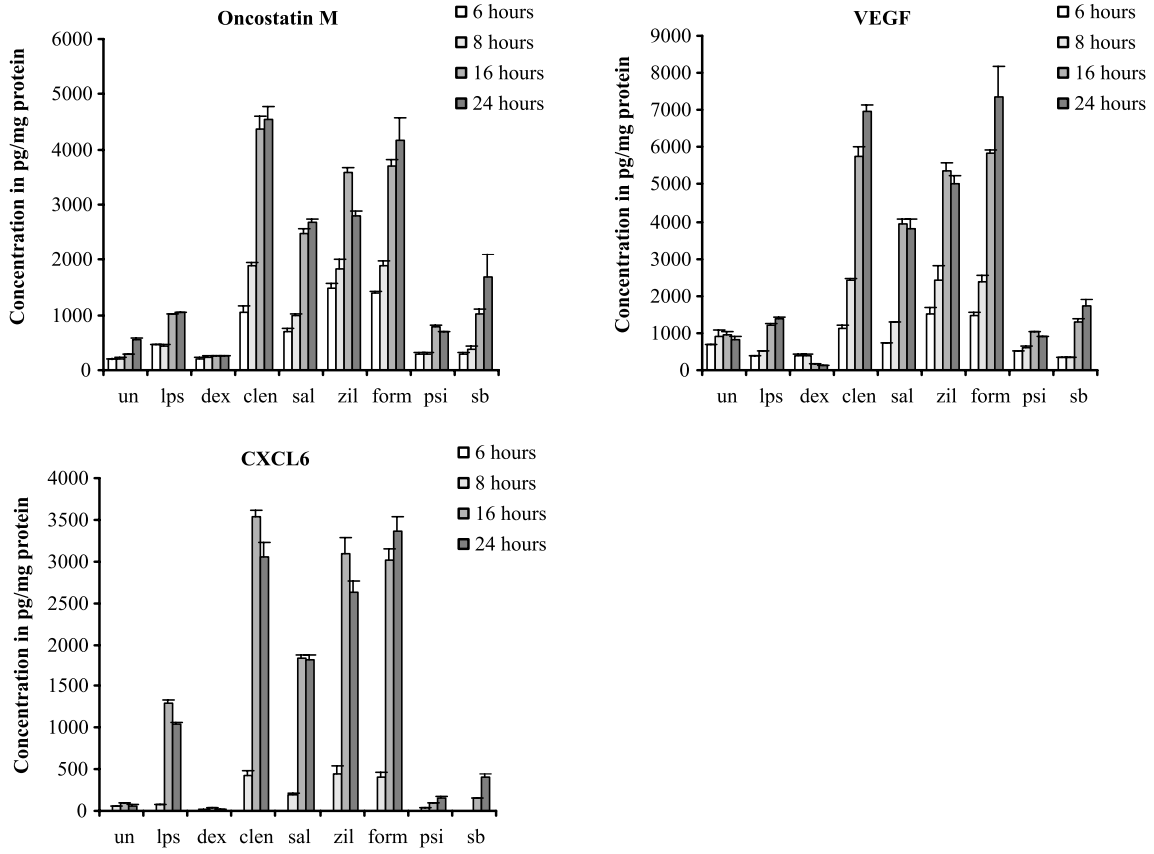

Figure 6 Protein expression levels of oncostatin M, VEGF, and CXCL6 determined using specific immunoassays. The levels of these proteins released by the macrophages in the culture medium were determined at the time points indicated. The following incubations were performed: untreated macrophages (un), macrophages treated with LPS alone (LPS) and LPS with an anti-inflammatory inhibitor, dexamethasone (dex), clenbuterol (clen), salbutamol (sal), zilpaterol (zil), formoterol (form), proteasome inhibitor (PSI), and SB203580 (sb). The expression levels given are the average + SD of duplicate incubations.

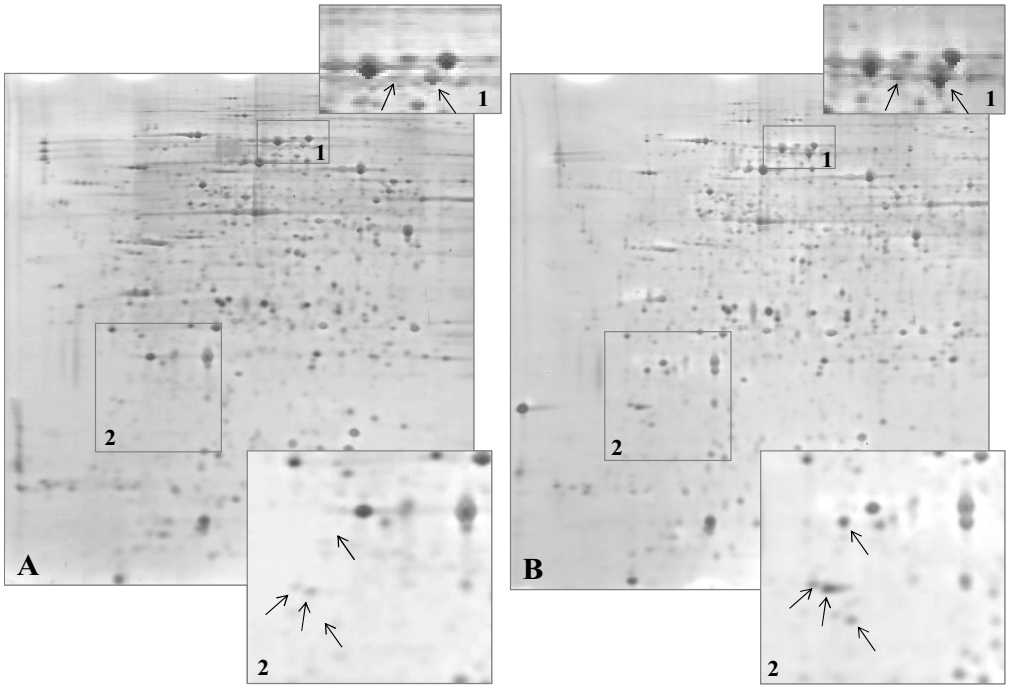

Figure 7 Representative 2-D gel image for U937 macrophages stimulated with LPS (Panel A) and stimulated with LPS and PSI (Panel B). Protein extracts of 400 µg were separated on a 12 % polyacrylamide gel, pH 4-7 and stained with RuBps stain. The arrows indicate the marker protein spots for PSI, according to PC-DA. The protein spots in panel 1 were identified by mass spectrometry as Heat shock protein 70 and in panel 2 as fragments of vimentin.

Proteomics

The proteomes of macrophages incubated with LPS or with LPS combined with one of the 6 anti-inflammatory inhibitors were compared using 2-D gel electrophoresis. In total 999 different spots were detected in the various gels. Figure 7 shows two representative 2-D gel images of U937 macrophages stimulated with LPS and LPS in combination with PSI. PC-DA was performed to find differences in the protein patterns.

The PC-DA of the proteomics data (Fig. 8) revealed distinct positioning of clenbuterol from salbutamol and zilpaterol. Similar to what was seen with the transcriptomics data, the gels from PSI were placed at a greater mutual distance in the score plot compared to the gels from the other inhibitors. The effect of PSI on the protein dataset was stronger than seen with the microarray dataset. In the score plot of discriminant 1 against discriminant 2 (data not shown), there was no separation between dexamethasone, SB203580 and the three β_2-agonists, meaning that PSI has a great impact on the model. Plotting discriminant 1 against discriminant 3 (Fig. 8) showed a better separation between the 6 inhibitors. Further analysis by defining new groups in the PC-DA, revealed only specific markers for PSI, e.g. vimentin and heat shock protein 70. These markers were confirmed by univariate analysis of the gel

spots in the 2-D gels (Fig. 7) and identified by in-gel digestion followed by MALDI mass mapping and/or MALDI MS/MS sequencing in combination with database search. From the results of these experiments we concluded that with the exception of PSI, no specific marker proteins could be detected.

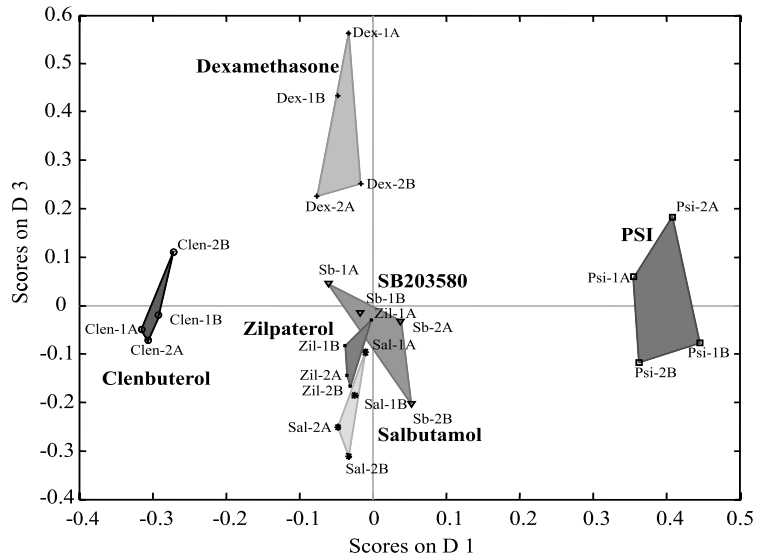

Figure 8 Score plot of the 24 2-D gels analyzed with PC-DA. The graphic represents the gels of zilpaterol (zil ×), clenbuterol (clen ○), salbutamol (sal*) SB203580 (sb Δ), dexamethasone (dex +), and proteasome inhibitor (PSI □) as dots, asterisks or crosses. For every inhibitor four symbols can be found, each representing the results of a single gel. In all cases, the four gels of the anti-inflammatory inhibitors are clustered, showing that the gel-to-gel variation is acceptable. The PC-DA revealed distinct positioning of the two $ß_2$-agonists, clen and sal.

Metabolomics

The lipid profiles of U937 cells after incubation with LPS in the absence or presence of an inflammatory inhibitor were analyzed by LC-MS. Figure 9 shows a representative chromatogram of the lipid extract of macrophages incubated with LPS and zilpaterol.

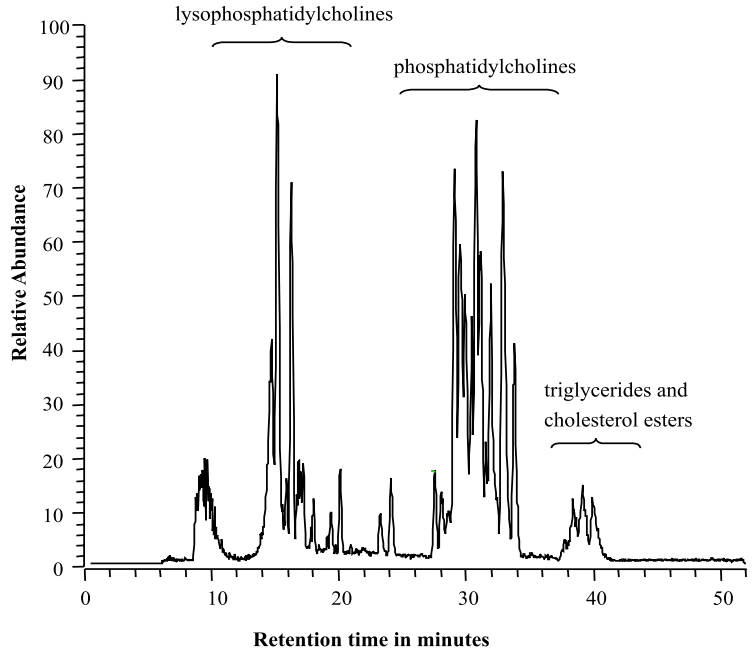

Figure 9 Base peak chromatogram (m/z 400-1900) of the lipid extract obtained from macrophages incubated with LPS and zilpaterol. The elution order of the lipid compounds was as follows: lysophosphatidylcholines, phosphatidylcholines, triglycerides and cholesterol esters.

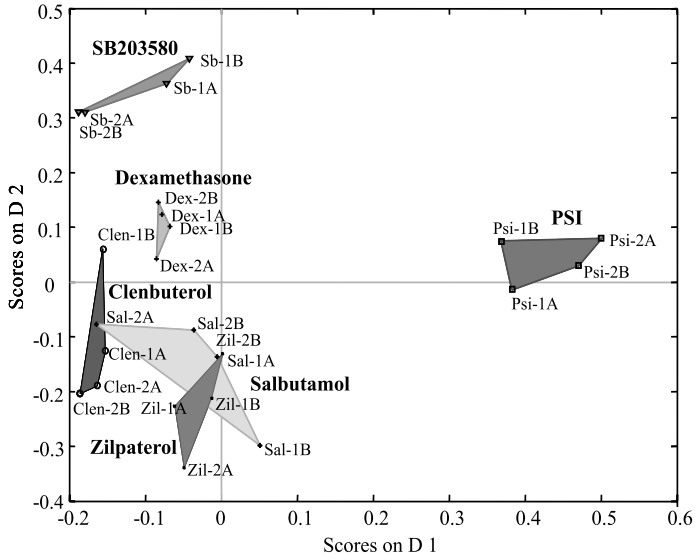

Figure 10 Score plot of the lipid patterns of zilpaterol (zil ×), clenbuterol (clen ○), salbutamol (sal*) SB203580 (sb Δ), dexamethasone (dex +), and proteasome inhibitor (PSI □). The lipid expression patterns of zil, sal and clen are clustered together in the lower left corner; this indicates a similarity in lipid expression pattern of the three β_2-agonists.

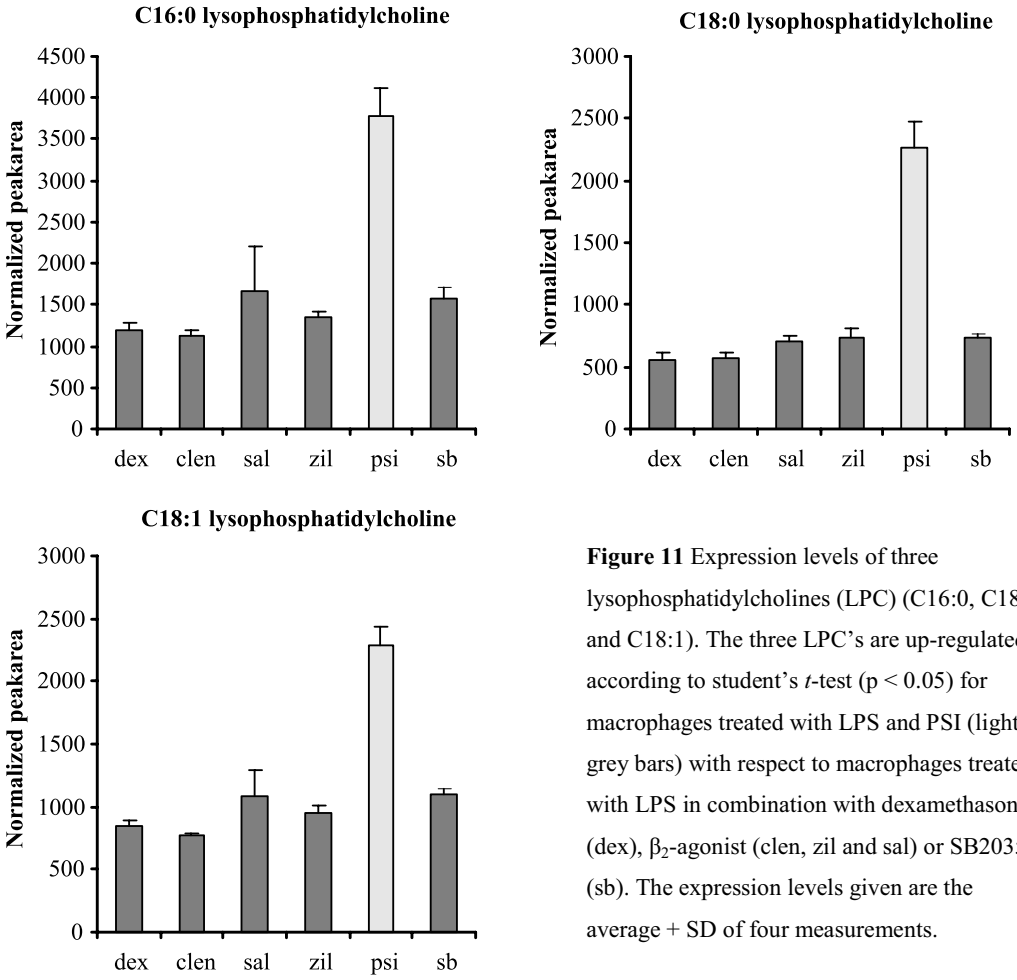

Figure 11 Expression levels of three lysophosphatidylcholines (LPC) (C16:0, C18:0 and C18:1). The three LPC's are up-regulated according to student's *t*-test ($p < 0.05$) for macrophages treated with LPS and PSI (light grey bars) with respect to macrophages treated with LPS in combination with dexamethasone (dex), β_2-agonist (clen, zil and sal) or SB203580 (sb). The expression levels given are the average + SD of four measurements.

Comparison of the lipid expression patterns of the 6 anti-inflammatory compounds using PC-DA (Fig. 10) resulted in a separation of the inflammatory inhibitors SB203580, PSI, dexamethasone and β_2-agonists. The lipid expression patterns of zilpaterol, clenbuterol and salbutamol were similar, which is demonstrated by their clustering in the lower left corner of the coordinate system. Similar to the transcriptomics and the proteomics data, PSI showed a completely different expression pattern. However, definition of two new groups in the PC-DA in order to detect markers for the different inhibitors did not reveal individual marker lipids. For the β_2-agonists we found a group of lipids that were regulated in a similar way. This group was identified as triglycerides. The induction of the triglycerides was not statistically different from the other anti-inflammatory compounds according to the univariate Student's *t*-test (assuming normal distributions and equal variance). Nevertheless, by using multivariate analysis we were able to separate the β_2-agonists from the other inhibitors. Only PSI gave rise to an induction of several individual lipids compared to the other inhibitors (Fig. 11). By applying LC-MS/MS, the lipids shown in Figure 11 were identified as various forms of lysophosphatidylcholine (LPC).

Figure 12 shows the PC-DA of the combination of the transcriptomics, proteomics en metabolomics dataset. As expected, the score plot again revealed a clustering of the three β_2-agonists in the upper left corner, indicating that there is a clear resemblance in mRNA, protein, and lipid patterns for the three compounds. The combination of the three datasets showed the best separation of the different anti-inflammatory inhibitors after PC-DA with respect to the separate techniques. However, evaluation of the data for individual marker molecules did not reveal important marker molecules in addition to those found on the basis of transcriptomics, proteomics or metabolomics datasets alone.

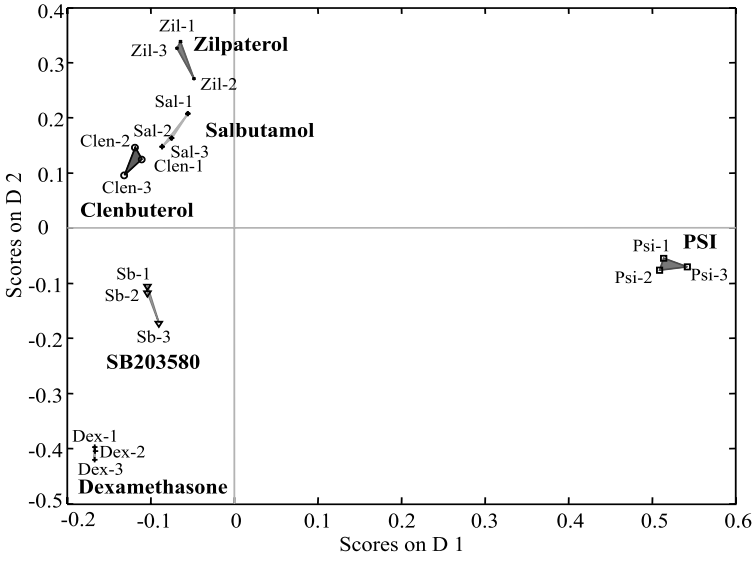

Figure 12 PC-DA score plot of the combination of the mRNA, protein and lipid datasets. Data points are represented as dots, asterisks and crosses; zilpaterol (zil ×), clenbuterol (clen ○), salbutamol (sal*) SB203580 (sb Δ), dexamethasone (dex +), and proteasome inhibitor (PSI □). The combination of expression patterns shows a clustering of clen, sal en zil in the upper left corner; this indicates a similarity of the three β_2-agonists.

Discussion

The data of the microarray analysis of U937 cells incubated with the various anti-inflammatory compounds, which was analysed by PC-DA, showed that the different classes of inhibitors could be separated from each other. In spite of the marked structural differences, zilpaterol appears to evoke a response in U937 cells very similar to salbutamol and clenbuterol. This, combined with the fact that zilpaterol is reported to be a β_2-agonist [17, 18], suggest that this effect is $ß_2$-receptor associated. Further studies, for example including specific antagonists, should elucidate this. By defining two new groups in the PC-DA, group 1; clenbuterol, salbutamol and zilpaterol and group 2; dexamethasone, PSI, and SB203580, we were able to find specific markers for the β_2-agonists with respect to PSI, dexamethasone and SB203580, (e.g. CXCL6, CXCR5, VEGF, and oncostatin M). These markers were confirmed by real time PCR and ELISA experiments to be specific for β_2-agonists. Since PC-DA is a mathematical tool, finding a false positive marker can't be ruled out beforehand, especially when the number of data points is at the lower limit. For example amphiregulin and thrombospondin (Fig. 4) showed the same regulation for clenbuterol, salbutamol and zilpaterol, but was also up-regulated by dexamethasone, although to a lesser extent than achieved by the β_2-agonists (induction factor is 2-8 times lower). For this reason the markers found by PC-DA were verified by univariate analysis of the original dataset. Specific markers for the other inhibitors, dexamethasone, PSI and SB203580 were also found by using the same approach (data not shown). It can be envisioned that these markers could be useful in screenings methods, where these markers are used for the screening of newly synthesized anti inflammatory compounds.

Remarkably, the proteome of the cell lysates obtained from U937 macrophages incubated with LPS and an anti-inflammatory compound revealed no similarities between clenbuterol and salbutamol, although both are known to be β_2-agonists and also evoked a very similar transcriptome in U937 cells. We speculate that the differences in the proteomes are not related to β_2-receptor effects. In the score plot of discriminant 1 versus discriminant 2 the proteome data from PSI was the most dominant factor. PSI was separated from the other inhibitors that were all clustered together (data not shown). Even in the score plot of discriminant 3 versus 1 (Fig. 8) the effect of PSI is still quite dominant, although there is a better separation of the other inhibitors as well. Interestingly, the PSI-marker proteins we identified by mass spectrometry included heat shock protein 70 and different fragments of vimentin. Vimentin is known to be cleaved in different fragments during apoptosis [26, 27], while heat shock protein 70 is up-regulated in apoptotic cells to perform their protective function against cell death [28-30].

These two markers indicate that apoptosis is a major determinant for the separation by PC-DA of the proteome data. This is compatible with the notion that PSI is able to induce apoptosis in various cell types [10, 11]. Apart from PSI, the 2-D PAGE technique appeared to be not very suitable for categorization of the anti-inflammatory compounds based on their anti-inflammatory mechanism of action. When this technique is applied to whole cell lysates, only abundant proteins can be detected, e.g. cytoskeleton proteins like actin. Many of these ubiquitously expressed proteins are modified during apoptosis. Unfortunately, the proteins involved in other more specific pathways (e.g. the pathways involved in the inflammatory response) are more difficult to detect, because of their low abundance [31]. Possibly, pre-fractionation of the protein sample may overcome this. The markers found with the microarray experiment were mostly mRNAs encoding secreted or membrane-bound proteins. Membrane bound proteins are difficult to dissolve in the standard buffers used for 2-D gel electrophoresis [31, 32]. The secreted proteins are present at the pg/ml level in the culture media. Therefore, the culture medium has to be concentrated at least a thousand times before secreted proteins are detectable on a 2-D gel. It has already been shown that this concentrating step will cause problems in the first dimension of 2-D gel electrophoresis (e.g. high salt concentration) [33]. Moreover, pre-fractionation will make 2-D gel electrophoresis not suitable for relatively fast screening of inflammatory inhibitors. Whether alternative proteomics techniques (e.g. 2-D LC-MS), are more suitable than 2-D gel electrophoresis for this type of screening approach remains to be established.

The LC-MS method for lipids was found to be the fastest method for categorizing anti-inflammatory compounds. The PC-DA of the lipid expression patterns of the 6 anti-inflammatory compounds gave a similar result as the PC-DA of the mRNA expression patterns. The lipid expression patterns of zilpaterol, clenbuterol and salbutamol are similar according to multivariate analysis and are therefore clustered in the score plot. Surprisingly further analysis of the lipid data by PC-DA by defining two new groups (group1; clenbuterol, zilpaterol, and salbutamol, group 2; dexamethasone, PSI, and SB203580) revealed no specific markers for the β_2-agonists. There is no significant up- or down-regulation of individual lipids with respect to the other inhibitors according to univariate analysis. This means that the combination of several lipids together is responsible for the differences in the lipid expression patterns found by PC-DA. This underlines the power of multivariate analysis, namely the capacity to find hidden correlation and trends. By contrast, the PC-DA of PSI versus the other inhibitors did reveal a few individual marker lipids. These markers where identified as lysophosphatidylcholines (LPC). These lipids are known to be involved in apoptosis [34-36].

LPC is generated upon phospholipase A_2 (PLA_2) mediated hydrolysis of membranous phosphatidylcholine into LPC and arachidonic acid. PLA_2 is activated by means of caspase-3 cleavage during apoptosis. The secreted forms of LPC are involved in attraction of phagocytes to the apoptotic cells (secreted form) [35], in the recognition of apoptotic cells (membrane bound form) [37] and is even known to be able to induce apoptosis by itself [34, 36] . The PC-DA of the combination of the three datasets showed the best separation. The three β_2-agonists, clenbuterol, salbutamol and zilpaterol are clustered as one group, whereas the other inhibitors are clearly separated from the β_2-agonists and from each other. The transcriptomics data is the dominant factor in this model. This is not only explained by the large amount of data points (9585 of the 13208 variables belong to the microarray dataset) but is also influenced by the number of variables that are specifically regulated by the different anti-inflammatory compounds. In other words, the number of mRNA's that is regulated by the anti-inflammatory compounds is much greater than the number of detectable proteins or lipids. This is compatible with the notion that there are more pathways that have the same end point. For example TNF-α, all tested anti-inflammatory compounds inhibited the TNF-α expression at the protein level, but the metabolic pathways leading to this effect are different. Nevertheless, we expect that the number of proteins that is regulated is larger than we have found in our experiment, but may have been missed due to the limited fraction of proteins that was analysed. Although the microarray data dominated the PC-DA model, the incorporation of the lipid and protein data clearly improved the clustering of the datasets. From a practical point of view, PC-DA of microarray data alone already gives satisfactory results.

In conclusion, our results demonstrate that different classes of anti-inflammatory compounds show distinct and characteristic mRNA, protein and lipid expression patterns in our model system. Furthermore, our model system shows that zilpaterol, a compound originally developed as a β_2-agonist but later specifically introduced as a growth promoting (anabolic) agent, gives rise to an almost identical mRNA and lipid expression pattern as the well-characterized β_2-agonists clenbuterol and salbutamol. This shows that the approach presented here is suitable to classify new or poorly characterized anti-inflammatory compounds that act on macrophages by using mRNA and lipid expression data both in combination with PC-DA. However it should be mentioned that the success of classification depends on the compounds that are used as reference compounds in the experiment and the effect on other cells of the immune system has to be tested additionally, but with the results of the categorization method, it is possible to apply a more targeted research.

More rapid screening is possible when the specific markers from the microarray experiments are used in simplified experiments. For example CXCL6, VEGF, and oncostatin M could be used as markers for β_2-agonists.

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