Relative quantification of human β-defensins by an SRM-based proteomics approach

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Abstract:	RATIONALE: The SRM-based targeted proteomics method is a relevant approach for the analysis of multiple analytes in biological samples. Defensins are phylogenetically conserved small antimicrobial peptides contributing to innate host defense and exhibiting low immunogenicity, resistance to proteolysis and a broad range of antimicrobial activities. The goal of the present study was to develop and optimize SRM-based targeted proteomics methods for the detection of human β-defensins 1 – 4 in various biological fluids. METHODS: An SRM-based targeted proteomics method was developed and validated for the detection of human β-defensins 1 – 4. The supernatants of resting and IL-1β stimulated Caco2, HT-29 and SW-1116 colonic epithelial cells (CEC), cell lysates of CECs and tear samples of human healthy individuals were analyzed and the feasibility of the developed method was validated by ELISA and dot-blot analysis complemented by RT-qPCR. RESULTS: Our results demonstrate that the developed SRM method offers an alternative approach for the cost-effective and rapid analysis of human β-defensins in samples with biological relevance. CONCLUSION: A semi-quantitative targeted mass spectrometry method was developed and validated for the relative quantification of β-defensins 1 – 4 in cell culture supernatants and body fluid analyses.

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ABSTRACT

RATIONALE: The SRM-based targeted proteomics method is a relevant approach for the analysis of multiple analytes in biological samples. Defensins are phylogenetically conserved small antimicrobial peptides contributing to innate host defense and exhibiting low immunogenicity, resistance to proteolysis and a broad range of antimicrobial activities. The goal of the present study was to develop and optimize SRM-based targeted proteomics methods for the detection of human β-defensins 1 – 4 in various biological fluids.

METHODS: An SRM-based targeted proteomics method was developed and validated for the detection of human β-defensins 1 – 4. The supernatants of resting and IL-1β stimulated Caco2, HT-29 and SW-1116 colonic epithelial cells (CEC), cell lysates of CECs and tear samples of human healthy individuals were analyzed and the feasibility of the developed method was validated by ELISA and dot-blot analysis complemented by RT-qPCR.

RESULTS: Our results demonstrate that the developed SRM method offers an alternative approach for the cost-effective and rapid analysis of human β-defensins in samples with biological relevance.

CONCLUSION: A semi-quantitative targeted mass spectrometry method was developed and validated for the relative quantification of β-defensins 1 – 4 in cell culture supernatants and body fluid analyses.

KEYWORDS

β-defensin, tear, relative quantification, targeted proteomics, SRM

ABBREVIATIONS

AUC, area under the curve; AMP, antimicrobial peptide; CD, Crohn's disease; CEC, colonic epithelial cells; hBD, human β-defensin; IBD, irritable bowel disease; MRM, Multiple Reaction Monitoring; PTP, proteotypic peptide; SELDI, Surface Enhanced Laser Desorption Ionization; SIL, stable isotope labelled peptide; SRM, Selected Reaction Monitoring; UC, ulcerative colitis

INTRODUCTION

Defensins represent a phylogenetically ancient and structurally conserved group of antimicrobial peptides (AMP) consisting of 16 – 50 amino acids. They exhibit multiple functional activities and act in a coordinated manner to provide a first line of defense against various pathogens [1]. The α and β families of human defensins differ in their precursor structures, peptide length and the site of secretion. In humans, four β-defensin types are expressed referred to as β-defensin 1 - 4 (hBD 1 - 4) [1-4]. Based on the UniProt database, β-defensin 5 and 6 also exist but no protein data have been published so far. The β-sheets of these molecules create a compact tertiary structure referred to as the β-defensin fold [5]. Due to this unique organization human defensins are resistant to proteolysis and can exert their effects even at harsh environmental conditions [6]. In epithelial cells β-defensins have been considered as potential AMPs providing a protective barrier against Gram-negative bacteria and Candida species [7]. Another important function of β-defensins is their chemotactic activity resulting in the recruitment of various immune cells towards the site of infection or inflammation [8]. Elevated levels of human β-defensin-2 (hBD2) was observed in patients with irritable bowel disease (IBD) and in ulcerative colitis (UC) indicating an activated immune system in these diseases. Increased levels of hBD2 was also detected in stool samples of the inflamed digestive tract [9], whereas increased hBD3 levels were described in the colonic mucosa of patients with UC [10]. hBD4 functions as a synergistic partner of lysozyme-C and together with hBD3 exerts additive effects [11] suggesting its protective role against different types of pathogens. Human β-defensin molecules are present in various body fluids such as saliva [12], sweat [13], tears [14], while hBD1 was also detected in the urogenital tract [15].

The large mucosal surface of the intestinal epithelium acts as an important physical and chemical barrier and confers a first line of defense against pathogenic bacteria, viruses and other microorganisms [16, 17]. In the small intestine hBD1 was described as a constitutively expressed AMP, the expression of which is not regulated by proinflammatory cytokines [46]. However, the level of hBD4 is upregulated as a result of bacterial infection but not by classical proinflammatory signals such as IL-1β [11]. On the contrary, hBD2 and hBD3 were described as inducible AMPs, which respond to stimulation by elevated proinflammatory cytokine secretion [16-18]. Inflammatory bowel diseases such as UC and CD are associated with increased levels of inducible β-defensins [19] leading to epithelial cell proliferation, immune cell migration and enhanced production of proinflammatory cytokines [20]. In addition to their antimicrobial and immune modulatory effects, some defensins have been identified as cancer-associated molecules with anti-tumor effects [21].

Basal tear fluid contains proteins, lipids, metabolites, salts and organic molecules [22]. Studies aimed to characterize the tear proteome have been performed and more than 500 tear proteins have been identified [23, 24]. Literature data suggest that changes in tear proteome composition can be of diagnostic value in some diseases such as diabetic retinopathy [25, 26], keratoconus [27], blepharitis [28], dry-eye [29] and Sjögren's syndrome [30]. Changes in tear hBD levels have also been reported after ocular surgery [31] and in patients with pterygium [32].

Targeted proteomics preferentially relies on the SRM/MRM scan mode of triple quadrupole-containing mass spectrometers. In the course of sample analysis the first quadrupole transmits ions with a specified m/z, which is fragmented in the second quadrupole functioning as a collision cell, while the third quadrupole transmits a single specified fragment resulting in signal, when the precursor ion and its selected fragment is present at the same time [23, 34]. SRM-based

methods ensure high specificity on one hand and quantitative data on the other hand, since the AUC of the specific signal corresponds to the amount of the compound entering the mass spectrometer. When functioning in a semi-quantitative setup, the targeted approach allows the relative quantification of specified analytes [35], but absolute quantification may also be possible [36] The usual requirement for absolute quantification include at least three protein-specific sequences (proteotyptic peptides, PTPs) with five transitions for each PTP [37], while in case of relative quantification one peptide with two transitions could be satisfactory.

Identification of β-defensins can be performed by gene expression profiling [38], ELISA, Western-blot [39-41] or SELDI [42, 43]. To our best knowledge the targeted proteomic approach has not been used so far for the detection of human β-defensins. Although the classical antibody-based methods are widely used for the quantitative or semi-quantitative analyses of proteins and peptides, the dynamic range in the quantitative settings can be a potential limiting factor and the determination of more than one analyte as well as lack of suitable antigens may restrict the utilization of this method. The SRM-based methods also offer multiplexing, but are more cost-effective than the classical antibody-based techniques. Another advantage of the targeted methods is their high flexibility, the possibility to design and validate SRM transitions together with the simultaneous analysis of multiple proteins [44, 45]. However, the limitation of the SRM based approach could be the restricted availability of PTPs and the possible co-elution of interfering compounds [37, 46].

In order to establish a cost-effective and flexible multiplex method for the analysis of the most common β-defensins, we have developed an SRM-based method that could be applied for the determination of hBD 1 – 4 levels in different biological samples.

MATERIALS AND METHODS

Cell culture

In this study, we have used the human HT-29, SW-1116 and Caco2 colonic epithelial cell lines (CECs) derived from various types of human colorectal adenocarcinomas. The tested cell lines are adherent and resemble the features of the human colon. The HT-29 and SW-1116 cells were grown in RPMI medium supplemented with 10% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine at 5% CO_2 and 37 ^{o}C. Caco2 cells were grown in 20% FCS containing RPMI medium supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine and 1% non-essential amino acids at 5% CO_2 and 37 ^{o}C. $5x10^5$/ml resting CECs were stimulated with 10 ng/ml IL-1β for 1 hour followed by the removal of the supernatant that was replaced by fresh medium and the cells were incubated for 5 hours before harvesting. The cell culture supernatants were collected without disturbing the cellular monolayer and trypsin-EDTA was added to collect the cells. The detached cells were washed twice with PBS in order to avoid contamination by the cell culture supernatant, and the lysis buffer (50 mM Tris-HCl pH 8.3, 1 mM EDTA, 17 mM β-mercaptoethanol, 0.5% TritonX) was added to collect the cell lysates.

ELISA

Determination of hBD2 protein levels in 100 µl cell lysate or in cell culture supernatants was performed by sandwich ELISA in three biological replicates using the EK-072-37 kit (Phoenix Pharmaceuticals Inc.) according to the suggested protocol. Determination of hBD2 levels in tear samples was performed in a volume corresponding to 5 µg total tear protein.

Development of the SRM-based targeted proteomics method

Amino acid sequences of hBD 1- 4 were retrieved from the UniProt database (www.uniprot.org, Accession numbers: P60022, O15263, P81534, Q8WTQ1) and were subjected to analysis by the PeptideCutter software (http://web.expasy.org/peptide_cutter). Tryptic fragments with ≥95% cleavage probability were selected for BLASTp analysis (http://blast.ncbi.nlm.nih.gov) and the NCBI non-redundant protein sequence database was searched to determine the protein-specific, unique tryptic peptide sequences. Design of the SRM transitions was performed by the Skyline software (www.brendanx-uw1.gs.washington.edu). Stable isotope-labeled synthetic (SIL) crude peptides were obtained from JPT Peptide Technologies GmbH, Germany while purified hBD2 SIL was obtained from PepscanPresto, The Netherlands. The quality of the synthetic peptides was assessed by MALDI-TOF spectra recorded in our laboratory (data not shown) and the SRM spectra of y ions were recorded.

Sample preparation for mass spectrometry

Protein concentration of the tested samples was determined with the Bradford method [47]. The proteins were denatured with 6 M urea and were reduced with 10 mM dithiothreitol. The reduced samples were alkylated with 20 mM iodo-acetamide and diluted with 25 mM ammonium bicarbonate to decrease the urea concentration to 1 M. Trypsin digestion was performed at 37 ^{o}C overnight by adding MS grade modified trypsin (ABSciex) in 1:25 enzyme to protein ratio. The digested peptides were lyophilized and dissolved in 1% formic acid. The samples were desalted with C18 ZipTip (Millipore), lyophilized and re-dissolved in 1% formic acid. Cell lysates and supernatants from each cell line were processed together on the same day, while in case of the tears, samples belonging to each set were digested and analyzed in parallel on the same day.

Mass spectrometry analysis

SRM-based analysis of biological fluids was carried out on a 4000 QTRAP (ABSciex) mass spectrometer using a NanoSpray II MicroIon Source and was controlled by the Analyst 1.4.2 software (ABSciex). The spray voltage was 2800 V, the ion source gas was 50 psi, the curtain gas was 10 psi and the source temperature was 110 ^{o}C. The cycle time was 1.15 sec, which provides near 30 data points/peak. Chromatographic separation was performed with an EasynLC II system (Bruker). Desalting was performed on a Zorbax 300SB-C18 column (5 x 0.3 mm, 5 µm pore size, Agilent) followed by separation on a Zorbax 300SB-C18 analytical column (150 mm x 75 µm 3.5 µm pore size, Agilent). Solvent A was 0.1% formic acid in LC water, solvent B was acetonitrile containing 0.1% formic acid. The flow rate was 300 nl/min, and a 30 min water/acetonitrile gradient combined with a continuous increase of solvent B from 0% to 100% was applied. Sample blocking was carried out by a randomly selected control and a randomly selected IL1β-treated sample that were paired and digested together and analyzed one after the other using the same conditions. In case of the cell culture supernatants a randomly selected control-IL1β-treated sample pair followed by two other randomly selected control-IL1β-treated pairs were analyzed (three replicates). The same procedure was applied for cell lysates and tear samples.

The CaCo2 cells and tears were analyzed in two experimental settings. In the first set of experiments purified non labeled synthetic peptides were utilized for hBD1, 3 and 4 and a purified SIL peptide was utilized for hBD2. In the second set of the experiments crude SIL peptides for hBD1-4 were spiked into the CaCo2 cell lysate and the supernatant samples.

Data analysis

The SRM data were analyzed by using the Skyline software. The endogenous/SIL peptide ratios were calculated by the software. The Skyline data are publicly available through the Panorama web site:

(https://panoramaweb.org/labkey/project/University%20of%20Debrecen/Defensin/begin.view?).

For the calculation of the linear dynamic range the log AUC values were plotted against the log concentration values and logistic regression was applied for curve calculation. The linear dynamic range was defined based on these curves.

Statistical analysis was performed with the SigmaPlot 12.0 software (Systat Software Inc.) using Student's t-test. Correlation coefficients were determined by the linear regression method [48], the level of significance was set to $p \leq 0.05$.

RESULTS AND DISCUSSION

In order to better understand the role of β-defensins in innate defense mechanisms we sought to develop an SRM-based targeted proteomics approach for the determination of the relative levels of hBD 1 – 4. The amino acid sequence of these proteins was retrieved from the UniProt database, subjected to the PeptideCutter software, and the tryptic fragments with ≥ 95% cleavage probability were selected for BLASTp analysis. The results demonstrated the presence of one peptide specific for each hBD including hBD 1, 2 and 3, respectively. Thus hBD1 could be identified by the IQGTCYR sequence, hBD2 by GIGDPVTCLK and hBD3 by the GIINTLQK peptide underscoring the conserved structure of these small molecules (Table 1).

As an exception, four specific peptide sequences could be identified for hBD4 (LVLLLAISLLLYQDLPVR, ICGYGTAR, CPNTYACCLR, WDESLLNR). In order to select

from the four available peptides a prediction using the CONSeQuence software [49] was applied. Based on our previous experiments and literature data [50], from the peptides ICGYGTAR and WDESLLNR offered by the software the ICGYGTAR sequence was selected for the SRM design.

Optimization of SRM parameters and determination of the linear dynamic range for hBD peptides

SRM transitions for the selected β-defensin peptides were designed by using Skyline and were tested with SIL peptides. Signals for all transitions of y ions were recorded and examined manually. All transitions showing co-elution in case of each individual peptide were selected and used for further analyses (Table 1). To determine the linear dynamic range increasing amounts of stable isotope labeled (SIL) synthetic hBD peptides in the dilution range of 1000 - 5 fold were spiked either into the cell culture supernatant or to the tear fluid. Using the developed SRM method 1000 fold dilution (approximately 75 fmol) of the tested hBD peptides could be detected, but the linear dynamic range of the peptides was different in the studied matrices (Table 2). In case of the SIL hBD1 peptide, the linear range in the cell culture supernatant matrix was at 250-5 fold dilution (approximately 300 fmol-15 pmol), while in tears it was in the range of 500-25 fold dilution (approximately 150 fmol-3 pmol). The linear range of the SIL hBD2 peptide in the cell culture supernatant and tears was between 250-10 fold dilution (approximately 300 fmol-7.5 pmol) and 500-10 fold dilution (approximately 150 fmol-7.5 pmol), respectively. The SRM analyses of the SIL hBD3 peptide showed that the linear range in the cell culture supernatant matrix was at the 250-5 fold dilution range (approximately 300 fmol-15 pmol) and in tears between 250-25 fold dilution (approximately 300 fmol-3 pmol). The linear range of the selected SIL hBD4 peptide was observed between 250-5 fold dilution (approximately 300 fmol-15 pmol)

both in the cell culture supernatants and the tear matrix. Based on these results, the designed SRM method exhibited a broad dynamic range and it allowed the analysis within a wide range of changes often observed in biological systems.

Determination of hBD1, hBD3 and hBD4 levels in human colonic epithelial cells

Considering that β-defensins are produced in the cytosol but exert their biological activities outside the cell, where their secreted forms can exhibit various biological activities, we measured the levels of hBD1, hBD3 and hBD4 both in cell lysates and cell culture supernatants of three different CEC lines. SIL peptides were spiked either into the epithelial cell lysates or the supernatants right before the analysis (Supplementary Figure 1). Upon IL-1β stimulation the level of hBD1 did not change significantly in SW-1116 and HT-29 cells, however, significant decrease was detected only in the Caco2 cell lysates (Figure 1a). Considering the secreted hBD1 levels the decrease was significant in stimulated HT-29 cells but not in SW-1116 CECs (Figure 1d) [51, 52].

Changes of endogenous:SIL peptide ratio showed significantly higher hBD3 levels in all stimulated cells as compared to the cell lysates of their unstimulated counterparts (Figure 1c), and these changes could also be detected at the level of secreted hBD3 (Figure 1e). As a result of stimulation the level of intracellular hBD4 did not change significantly and a similar tendency has been observed as shown for hBD1 (Figure 1c). The secreted form of hBD4 changed significantly only in the HT-29 cell culture supernatants, while in SW-1116 and Caco2 cells hBD4 levels followed the pattern of hBD1 (Figure 1f). García et. al. has shown that hBD4 collaborates with hBD3 [11], but this functional interplay likely does not require elevated hBD4 expression. It seems that hBD1 and hBD4 exhibited similar kinetics when challenged by

inflammatory stimuli such as IL-1β. As a conclusion, our data demonstrate the feasibility of the SRM-based method in the comparative analysis of hBD levels and provide further evidence for the inducible feature of hBD3 in an inflammatory environment [17, 51].

Validation of SRM data measured for hBD2 with ELISA and RT-qPCR

To further validate the results obtained for hBD2 by the SRM approach, the mRNA and protein levels of IL-1β stimulated and control CaCo2 cells was determined by the SRM, RT-qPCR and ELISA methods (Figure 2 and Supplementary Figure 2). The CaCo2 cells responded to the IL-1β stimulation with increased hBD2 levels both in the cytosol and in the cell culture supernatant as indicated by the SRM-based method (Figure 2a and 2b). The RT-qPCR data indicated higher hBD2 mRNA levels in IL-1β stimulated cells than in controls *(Figure 2c)*. In line with previous results the concentration of intracellular and secreted hBD2, determined by ELISA also showed significantly higher hBD2 levels when the cells were stimulated by IL-1β (*Figure 2d and 2e).* Similar results were obtained with dot-blot analysis and ELISA showing robust upregulation of secreted hBD2 in the supernatants of IL-1β activated CaCo2 cells (Supplementary figure 2) demonstrating the activation-induced secretion of hBD2 under inflammatory conditions [53-56]. It is important to note that despite the similar tendency of the changes determined with ELISA and SRM, the ELISA method resulted in higher changes as compared to those determined by using SRM. This may be due to the amplification steps making that technique more sensitive than the SRM approach. Comparison of the magnitude of changes obtained with the SRM, RT-qPCR and ELISA revealed the ELISA method as the most sensitive method, while the RT-qPCR and SRM-based assays provided changes with similar magnitudes. In a next step we also compared the efficacy of the ELISA and the newly developed SRM method for the analysis of hBD2 levels derived from the same samples (Figure 2f)). The correlation coefficient of the data

obtained by the SRM and ELISA techniques (0.937) indicated that these methods provide comparable results. Based on these results the developed SRM approach can be evaluated as an alternative quantification method that is cheaper than the antibody-based techniques. It could be especially useful in those cases when the determination of more than one hBD is needed and the sample volume does not permit the administration of consecutive ELISA measurements.

Analysis of β-defensin levels in tears

Most of the body fluids contain a wide array of antimicrobial peptides as a part of the innate immune defense mechanisms. In this context the major function of β-defensins is to rapidly kill and/or inactivate microorganisms [57]. AMPs also differ in the spatial area of secretion and this type of distribution depends on the actual environmental challenges, which could have an impact on the efficacy of combating against a large variety of microorganisms. In order to assess whether the newly developed SRM method could also be utilized for the analysis of biological fluids, such as human tears we collected tear samples from healthy volunteers and analyzed them. The quantification of hBD1, hBD2, hBD3 and hBD4 peptides was performed by the SRM method *(Figure 3a*) and the SRM analyses of hBD2 were confirmed by ELISA *(Figure 3b and Supplementary Figure 3).* The level of hBD2 was the lowest and the level of hBD3 was the highest, while the levels of hBD1 and hBD4 were almost the same highlighting the non-inducible feature of hBD1 and hBD4 [11,46]. The β-defensins defensin levels in tear samples were found to be lower than those detected in the supernatants of CECs indicating differences in the tissue specific expression of β-defensins [58]. The data indicate that the developed SRM method is suitable for the relative quantification of β-defensins also in body fluids typically of low sample volume such as tears, although the antibody based method seems to be more sensitive than the developed mass spectrometry-based method.

Analysis of accuracy

For the determination of accuracy of the developed SRM method the coefficients of variance (CV) were calculated. Based on literature data no consensus CV value is available for validating the accuracy of datasets. The most widely used recommendations involve CV <20% [59, 60] or CV <25% [61, 62]. This type of analysis showed that the CV between the biological replicates of all SRM experiments was below 20%. Considering that CVs for SRM analyses were acceptable, we concluded that the developed and validated SRM method provides an accurate approach for the relative quantification of hBDs in different biological samples.

CONCLUSION

In this study we present a semi–quantitative targeted proteomics method appropriate for the relative quantification of hBD 1-4 levels in biological samples as an alternative approach of the classical antibody-based detection and quantification systems. The ultimate advantage of the newly developed SRM method compared to ELISA or Western blot relies on its multiplex feature that allows a cost-effective simultaneous analysis of multiple analytes derived from the same sample, having relevance for cell biology and medical applications, where the amount of the sample to be studied is limited. The inducible feature of hBD2 and hBD3 confirmed at the mRNA and protein levels in case of hBD2 together with the utility of the developed SRM-based targeted proteomic method for semi-quantitative analysis of hBD 1-4 in cell lysates, culture supernatants and tear samples is highlighted.

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Supplementary Information Available

The authors declare no competing financial interest.

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Table 1: SRM transition parameters of representative proteotypic peptides for hBD1, hBD2, hBD3 and hBD4. Amino acids in *italics* represent carbamidomethylated cysteines. DP: de-clustering potential, CE: collision energy.

Protein	Proteotypic Peptide	Q1 (m/z)	Q3 (m/z)	Ion	Dwell Time (msec)	DP	CE
hBD1	IQGT***C***YR	420.705	727.319	y6	250	61.8	19.7
		420.705	599.261	y5	250	61.8	19.7
		420.705	542.239	y4	250	61.8	19.7
		420.705	441.191	y3	250	61.8	19.7
		420.705	338.182	y2	250	61.8	19.7
hBD2	GIGDPVT***C***LK	522.773	945.507	y9	250	69.2	25.5
		522.773	832.423	y8	250	69.2	25.5
		522.773	775.401	y7	250	69.2	25.5
		522.773	660.374	y6	250	69.2	25.5
		522.773	563.322	y5	250	69.2	25.5
		522.773	464.253	y4	250	69.2	25.5
		522.773	363.206	y3	250	69.2	25.5
		522.773	260.196	y2	250	69.2	25.5
hBD3	GIINTLQK	464.776	829.514	y7	250	65.0	22.2
		464.776	716.430	y6	250	65.0	22.2
		464.776	603.346	y5	250	65.0	22.2
		464.776	489.303	y4	250	65.0	22.2
		464.776	388.255	y3	250	65.0	22.2
		464.776	275.171	y2	250	65.0	22.2
hBD4	I***C***GYGTAR	449.216	784.341	y7	250	63.9	21.3
		449.216	624.310	y6	250	63.9	21.3
		449.216	567.288	y5	250	63.9	21.3
		449.216	404.225	y4	250	63.9	21.3
		449.216	347.203	y3	250	63.9	21.3
		449.216	246.156	y2	250	63.9	21.3

Table 2: Linear dynamic range of the hBD SIL peptides in cell culture supernatant and tear matrix. The values represent the dilution factors.

Protein	Peptide	Linear dynamic range in cell culture supernatant	Linear dynamic range in tears
hBD1	IQGTCYR	250x-5x	500x-25x
hBD2	GIGDPVTCLK	250x-10x	500x-10x
hBD3	GIINTLQK	250x-5x	250x-25x
hBD4	ICGYGTAR	250x-5x	500x-25x

FIGURE 1

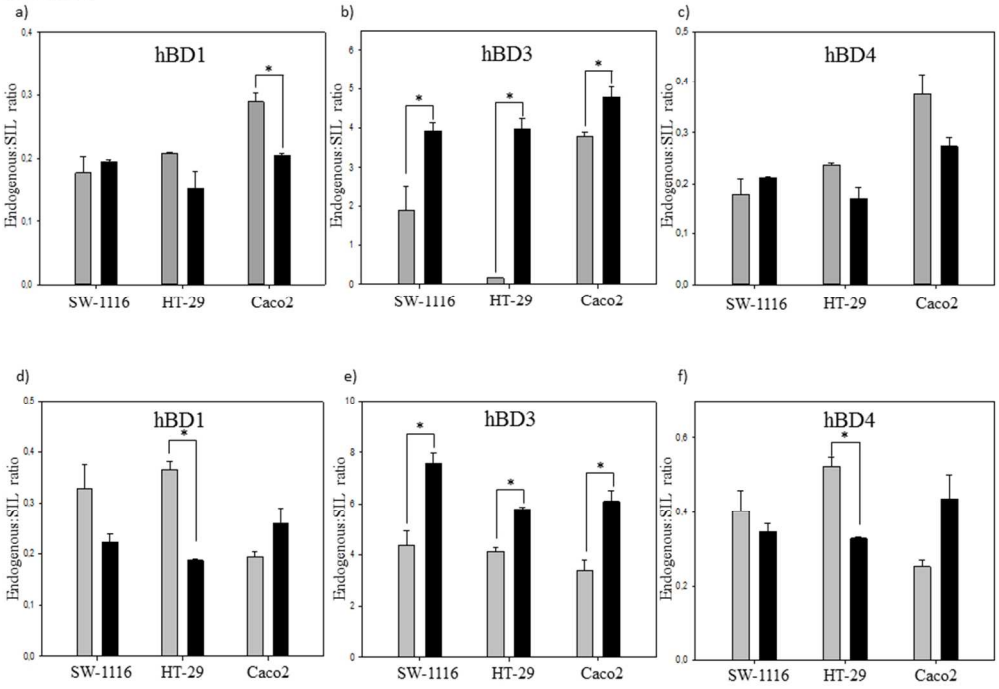

Figure 1: Determination of hBD1, hBD3 and hBD4 levels in colonic epithelial cells.
a) Determination of β-defensin 1 IQGTCYR levels in cell lysates. b) Determination of β-defensin 3 GIINTLQK levels in cell lysates. c) Determination of β-defensin 4 ICGYGTAR levels in cell lysates. d) Determination of secreted β-defensin 1 IQGTCYR levels in cell culture supernatants. e) Determination of secreted β-defensin 3 GIINTLQK levels in cell culture supernatants. f) Determination of secreted β-defensin 4 ICGYGTAR levels in cell culture supernatants. The bars represent the mean values of three independent experiments with the standard error of the means, the grey bars show the values for the control group and the black bars correspond to the IL-1β treated group; * indicates $p < 0.05$.

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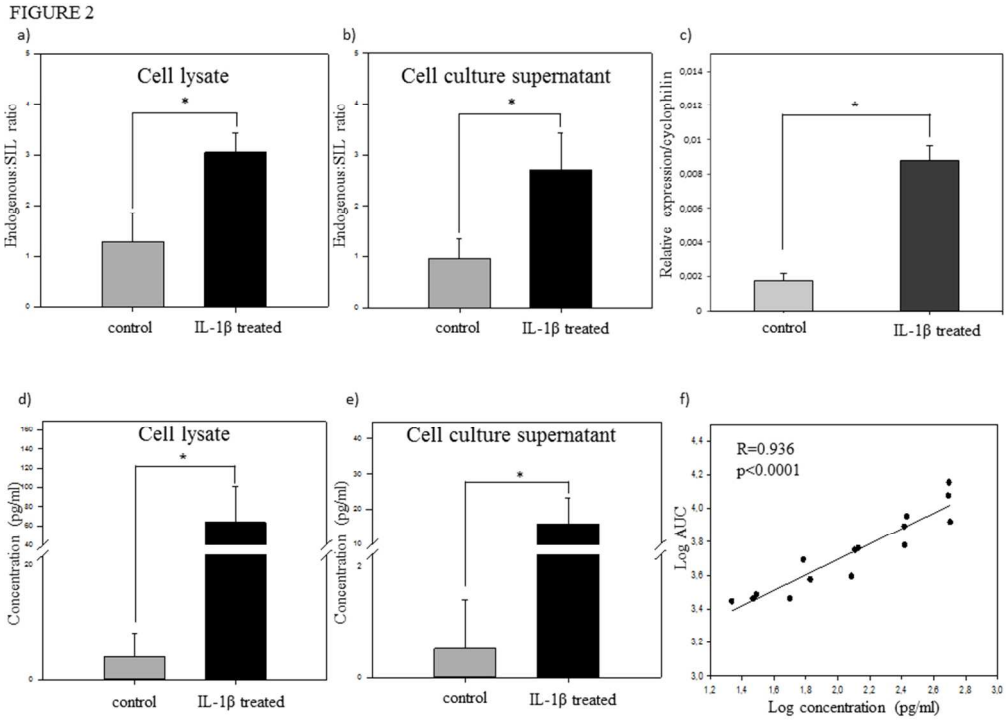

Figure 2: Analysis of hBD2 levels in Caco2 cell culture.
a) Determination of β--defensin 2 GIGDPVTCLK levels in Caco2 cell lysates. b) Determination of secreted β-defensin 2 GIGDPVTCLK levels in Caco2 cell culture supernatants. c) qPCR data for β-defensin 2 mRNA levels normalized to cyclophylin. d) Determination of β-defensin 2 protein concentrations (pg/ml) in cell lysate using ELISA. e) Determination of secreted β-defensin 2 protein concentrations (pg/ml) in cell culture supernatant with ELISA. The bars represent the mean values of three independent experiments with the standard error of the means, the grey bars show the values for the control group and the black bars correspond to the IL-1β treated group; * indicates $p < 0.05$. f) Correlation analysis of the ELISA and SRM methods. The x axis contains the log concentration (pg/ml) of hBD2-containing samples determined using ELISA and the y axis contains the log area under the curve (AUC) values determined for the same samples using the SRM method. The correlation coefficient (R) and p value is indicated.

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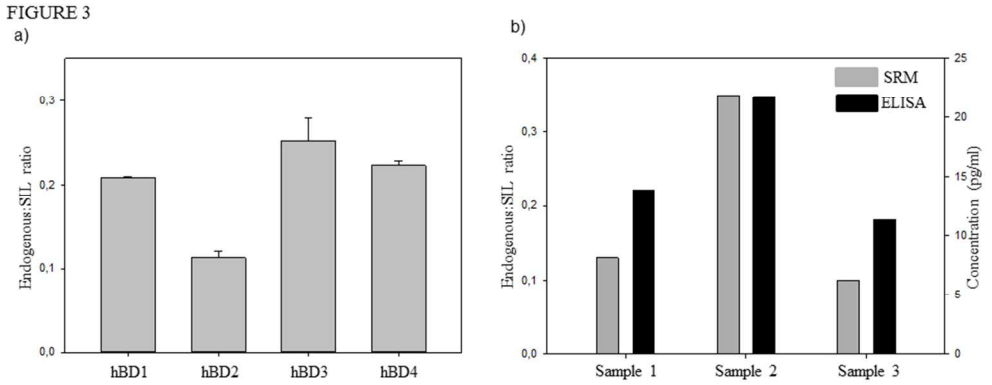

Figure 3: Analysis of β-defensin levels in tears.
a) Examination of β-defensin 1-4 levels in tears. The bars represent the mean values of three independent experiments with the standard error of means. b) Comparison of tear β-defensin 2 amounts determined by ELISA (black bars) and SRM (grey bars).

254x100mm (96 x 96 DPI)