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# Effects of WF10 on Glycosaminoglycan Sulphation in Proinflammatory Monocytes and Macrophages

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## Abstract

The chlorite-based drug solution WF10 has been successfully applied to dampen strong inflammatory disease states and to improve wound healing processes. However, the molecular mechanisms of this drug are not well understood. This study is directed to investigate how WF10 and its components affect the expression of surface markers and sulphated proteoglycans and glycosaminoglycans in proinflammatory-stimulated monocytes and macrophages.

Human blood-derived macrophages were cultivated from monocytes in the presence of 50 U/ml granulocyte-macrophage colony-stimulating factor and activated by a mixture of 100 ng/ml lipopolysaccharide (LPS) and 10 ng/ml interferon  $\gamma$  (IFN $\gamma$ ). These cells were identified and characterised by their specific cell-surface receptors CD14, CD16, CD80, CD86, CD163, and CD206 using flow cytometry approaches. The sulphation level of proteoglycans and glycosaminoglycans was assessed by the Blyscan™ dye-binding assay. The expression of the surface marker CD44, a proteoglycan with sulphated glycosaminoglycan side chains, was followed by antibodies against CD44. The binding of fluorescence-labelled hyaluronan to CD44 was also investigated by flow cytometry. All analyses were performed after incubation of monocytes and macrophages with WF10 or with its main components chlorite and chlorate.

The drug substance WF10 inhibited the activation of LPS/IFN $\gamma$ -stimulated human monocyte-derived macrophages. Among them are the diminished expression of proinflammatory surface markers, the inhibition of the expression of the hyaluronan receptor CD44, and the binding of hyaluronan to CD44. Further, the overall amount of sulphated proteoglycans and glycosaminoglycans was down-regulated by WF10. These *in vitro* experiments indicate that WF10 is able to inhibit the proinflammatory activation of macrophages. The results suggested that chlorite is the active principle in WF10 as chlorite caused principally the same changes in targets as WF10. The WF10 component chlorate inhibited only the overall sulphation level of proteoglycans and glycosaminoglycans and the binding of hyaluronan to CD44.

To sum up, WF10 is a promising tool to inhibit proinflammatory states of immune cells. The inhibition of activation processes in monocytes and macrophages by WF10 coincides well with the results about clinical application of WF10.

**Keywords:** Inflammation, Macrophages, Hyaluronan, Sulphation, WF10

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## 1. Introduction

Macrophages are key players during inflammatory immune response. At inflammatory loci, they not only efficiently remove damaged tissue components, apoptotic cells, and cell debris but also secrete numerous cytokines and growth factors that either further promote the inflammatory process or terminate the attraction of immune cells as well as initiate proliferative activities. On murine macrophages, several patterns of macrophage activation such as M1 (classically activated) and M2 (alternatively activated) macrophages and their subsets are distinguished on the basis of gene expression profiles, the appearance of cell surface markers, and the release of cytokines and other mediators [1]. Despite clear differences in immunological relevant characteristics between man and mouse [2], most properties of this polarisation pattern can also be transferred to human macrophages.

Macrophages express on their surface several proteins bearing sulphated glycosaminoglycan side chains. These sulphated proteoglycans are responsible for the interaction with cytokines, growth factors, and other mediators of inflammation as well as for the interaction with components of the extracellular matrix [3,4]. One of the cell-surface glycoproteins is CD44, which is known to function as receptor for the nonsulphated glycosaminoglycan hyaluronan [5]. After the onset of inflammation, large amounts of hyaluronan are secreted by endothelial cells, fibroblasts, and other cells at inflammatory loci. Due to the high water binding capacity, hyaluronan secretion contributes to oedema formation, changes in vascular permeability, and leukocyte recruitment [6]. Hyaluronan is thereby fixed to the surface of macrophages and tissue cells by CD44. This interaction is among others regulated by carbohydrate sulphation of the receptor [7,8].

Many strong disease scenarios are accompanied by an uncontrolled activation of the immune system and long-lasting inflammatory states. The worst-case scenario is the development of a sepsis syndrome accompanied by multiple organ failure [9].

Among the therapeutic approaches against inflammatory diseases is the intravenous infusion of the chlorite-based immunomodulatory drug solution WF10. This drug solution and the more diluted form Oxoferin have been successfully applied to dampen strong inflammatory states and to improve wound healing processes in patients [10–13]. However, the fine mechanism of the action of WF10 remains unknown. WF10 is a tenfold diluted aqueous solution of the drug substance OXO-K933 (NUVO Research Inc., Mississauga, Canada) that is composed of chlorite (4.25%), chloride (1.9%), chlorate (1.5%), and sulphate (0.7%) with sodium as cationic component [11].

Chlorite is known to interact preferentially with haem proteins affecting their catalytic cycles [14–16]. Furthermore, the chlorite component of WF10 converts oxyhemoglobin and ferryl hemoglobin into methemoglobin, which is also inactivated by chlorite/WF10 [17]. Chlorate is used as an inhibitor of the 3'-phosphoadenosine 5'-phosphosulphate (PAPS) synthase [18]. This enzyme is responsible for the production of PAPS, a common sulphur donor for all biological sulphation reactions that are catalysed by various sulphotransferases [19,20].

Here we addressed the question which effects WF10 and its components exhibit on human blood-derived monocytes and macrophages, which were stimulated by tumour necrosis factor  $\alpha$  (TNF $\alpha$ ) or a mixture of lipopolysaccharide (LPS) and interferon  $\gamma$  (IFN $\gamma$ ). In particular, we examined how WF10 influenced the expression of CD44 in these cells and the interaction of CD44 with hyaluronan. We demonstrated that WF10 inhibited the expression of surface markers in LPS/IFN $\gamma$ -activated macrophages, diminished the global expression of sulphated proteoglycans and glycosaminoglycans (GAGs), and interfered with the binding of hyaluronan to CD44.

## 2. Materials and methods

### 2.1. Chemicals

Blyscan<sup>TM</sup> sulphated glycosaminoglycan assay was obtained from Biocolor Ltd., Carrickfergus, United Kingdom. Monocyte isolation Kit II and MACS<sup>®</sup> separation columns were purchased from Miltenyi Biotec GmbH, Bergisch Gladbach, Germany. Biocoll separating solution was obtained from Biochrome AG, Berlin, Germany. RPMI 1640 medium and recombinant human granulocyte-macrophages colony-stimulating factor (GM-CSF) were purchased from Life Technologies GmbH, Darmstadt, Germany. Human recombinant interferon  $\gamma$  (IFN $\gamma$ ) and tumour necrosis factor-alpha (TNF $\alpha$ ) were supplied from Biomol, Hamburg, Germany. Lipopolysaccharide (LPS), papain from Papaya latex, foetal bovine serum (FBS), and penicillin/streptomycin were obtained from Sigma-Aldrich, Taufkirchen, Germany. Bovine serum albumin (BSA) was obtained from PAA Laboratories GmbH, Pasing, Germany. Hyaluronan conjugated with fluorescein isothiocyanate (FITC) was purchased from Calbiochem, Darmstadt, Germany. Antibodies conjugated with phycoerythrin (PE) and with PE-cyanine 5 (Cy5) for flow cytometry analysis were supplied from eBioscience, Frankfurt, Germany.

WF10 and special WF10 solutions free of either chlorate or chlorite were provided by NUVO Manufacturing GmbH, Wanzleben, Germany. In undiluted WF10, concentrations of chlorite and chlorate are 62.9 mM and 18 mM, respectively. In the chlorate-free WF10 solution (in the text mentioned as chlorite), chlorate was replaced by the equimolar amount of chloride. The same holds for the chlorite-free WF10 solution (named as chlorate).

### 2.2. Preparation and stimulation of monocytes and macrophages

Monocytes of human blood of healthy volunteers were isolated and purified by density gradient centrifugation followed by magnetic bead separation using the monocyte isolation

kit II and MACS<sup>®</sup> separation columns according to manufacturer's protocols. Monocytes ( $10^6$ ) were cultivated in tissue culture dishes in RPMI medium containing 10% FBS, 2 mM L-glutamine, and 1% penicillin/streptomycin at 37 °C and 5 vol.% CO<sub>2</sub>. The differentiation of monocytes towards macrophages was induced by addition of 100 ng/ml GM-CSF for 6 days followed by addition of a mixture of 100 ng/ml LPS and 10 ng/ml IFN $\gamma$  with or without WF10, chlorate, and chlorite for 24 h at 37 °C and 5 vol.% CO<sub>2</sub>.

Monocytes were stimulated with 2 ng/ml TNF $\alpha$  in the absence or presence of WF10, chlorate, and chlorite for 18 h at 37 °C and 5 vol.% CO<sub>2</sub>.

### 2.3. Determining of cell-surface marker expression by flow cytometry

Cells were harvested by washing with PBS at room temperature followed by incubation with ice-cold PBS supplemented with 2.5 mM EDTA for 20 min. Cells rounded up and could be scraped off using a cell scraper. After centrifugation for 5 min (400 $\times$ g), cells were incubated with PBS supplemented with 1% BSA and fluorescence-labelled antibodies (antiCD1a-PE, antiCD14-PE, antiCD16-PE, antiCD80-PE, antiCD86-PE, antiCD163-PE, antiCD206-PE, and antiTLR-4-PE), and the corresponding isotype controls for 30 min in the dark. Thereafter, cells were washed by centrifugation (400 $\times$ g, 5 min) and fluorescence intensity of the cells was detected by flow cytometry using a FACSCalibur (Becton Dickinson, Franklin Lakes, USA) with a laser excitation wavelength of 488 nm (PE detection in channel FL-2). Ten thousand events were analysed for each measurement using Flowing Software 2.4.1 by Perttu Terho.

### 2.4. CD44-hyaluronan-binding assay

The expression of the surface marker CD44 and the binding of its ligand hyaluronan were determined using a flow cytometry assay. Harvested cells were either dissolved in PBS supplemented with 1% BSA and incubated with antibodies against CD44 (anti-CD44-PE-Cy5, eBioscience) for 30 min in the dark or dissolved in PBS supplemented with 2% FBS, 2 mM EDTA, and 10  $\mu$ g FITC-labelled hyaluronan. Cells were washed with PBS (400 $\times$ g, 5 min), and the fluorescence intensity of the cells was detected using a FACSCalibur (Becton Dickinson, Franklin Lakes, USA) with a laser excitation of 488 nm (FITC detection in channel FL-1, PE-Cy5 in channel FL-3). Ten thousand events were analysed for each measurement using Flowing Software 2.4.1 by Perttu Terho.

### 2.5. Blyscan dye staining of sulphated proteoglycans and glycosaminoglycans

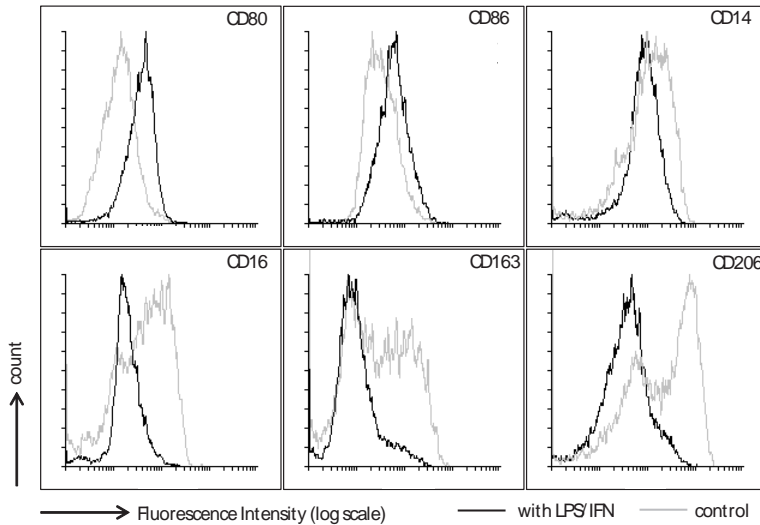
The analysis of the total amount of sulphated proteoglycans and glycosaminoglycans (GAGs) was performed using the Blyscan<sup>™</sup> dye-binding assay (Biocolor Ltd., Carrickfergus, United Kingdom) according to the manufacturer's protocol. Sulphated GAGs of the cells were extracted using a papain extraction reagent in 0.2 M sodium phosphate buffer (Na<sub>2</sub>HPO<sub>4</sub>-NaH<sub>2</sub>PO<sub>4</sub>), pH 6.4, containing 0.1 mg/ml papain at 65 °C for 3 h. After centrifugation at 10000 $\times$ g for 10 min, supernatant was added to the Blyscan dye reagent and incubated in a mechanical shaker for 30 min. After centrifugation (12000 $\times$ g, 10 min), addition of a dissociation reagent, and further centrifugation (12000 $\times$ g, 10 min) the absorbance at 656 nm was

measured. The content of sulphated GAG can be calculated using a standard curve with known concentrations.

### 3. Results

#### 3.1. Effects of WF10 on the expression of surface markers in LPS/IFN $\gamma$ -stimulated macrophages

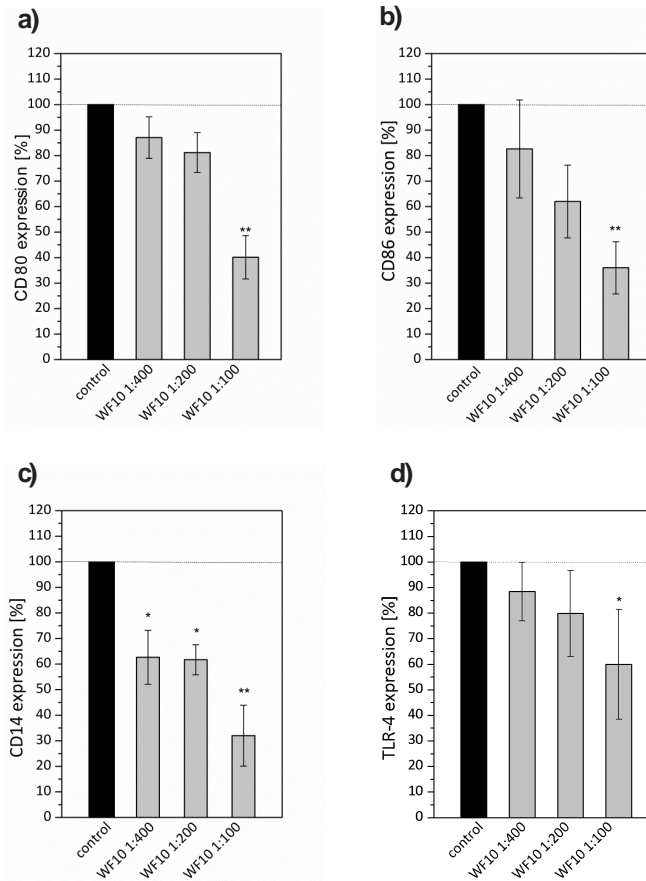
Macrophages were differentiated from peripheral blood-derived monocytes in the presence of the granulocyte-macrophage colony-stimulating factor (GM-CSF). These cells were stimulated by a mixture of LPS/IFN $\gamma$  for 24 h to obtain macrophages with proinflammatory properties corresponding to M1-type macrophages. The activation pattern of these macrophages was assessed by the expression of selected surface markers (Figure 1).



**Figure 1. Profile of selected cell-surface markers of LPS/IFN $\gamma$ -stimulated macrophages.** Whereas the markers for M1 activation (CD80 and CD86) were up-regulated, the markers for M2 activation (CD14, CD16, CD163, and CD206) were down-regulated after stimulation with LPS/IFN $\gamma$ . Representative examples of the fluorescence intensity of LPS/IFN $\gamma$ -stimulated macrophages (black) are given in comparison to the control samples (silver) without LPS/IFN $\gamma$ . Data were obtained from at least four different macrophage preparations.

As expected, there was an increased expression of the surface proteins CD80 and CD86 (Figure 1) upon exposure of macrophages to LPS/IFN $\gamma$ . We analysed also the flow cytometry pattern of surface markers that are known to be up-regulated by alternative activation but down-regulated in the sole presence of LPS/IFN $\gamma$ . The LPS/IFN $\gamma$ -induced down-regulation of CD14, CD16, CD163, and CD206 is clearly demonstrated in Figure 1.

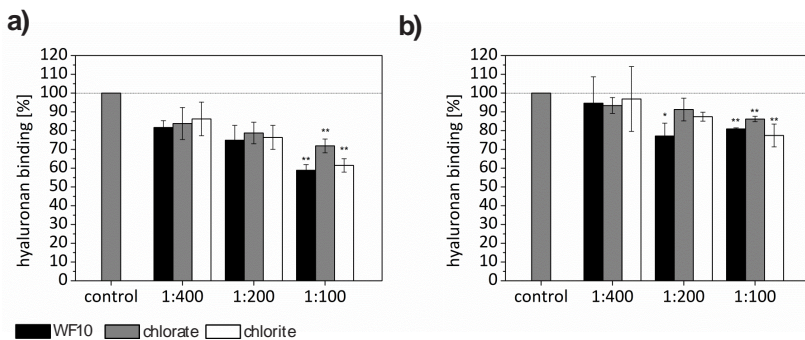
Next, the expression of several receptors was measured upon LPS/IFN $\gamma$ -mediated stimulation of macrophages in the presence of WF10 (Figure 2). There was a concentration-dependent decrease of CD80 expression by WF10. At a 1:100 dilution of WF10 corresponding to a final chlorite concentration of 629  $\mu$ M, only 40%  $\pm$  8% of the original amount of receptors could be detected. A similar pattern of receptor decrease was also observed for CD86, CD14, and the toll-like receptor 4 (TLR4). This indicates that WF10 exhibits a general effect on the proinflammatory activation mechanism of macrophages. These effects are caused by the WF10 component chlorite, as shown in experiments replacing WF10 by chlorite. The exposure of LPS/IFN $\gamma$ -stimulated macrophages to chlorate does not cause any changes in the expression of surface markers (data not shown).



**Figure 2. Regulation of selected cell-surface markers of macrophages by WF10.** LPS/IFN $\gamma$ -stimulated macrophages were incubated with or without (control) different concentrations of WF10 for 24 h. WF10 reduced the expression of CD80 (a), CD86 (b), CD14 (c), and TLR-4 (d) in a concentration-dependent manner. Fluorescence intensities (means  $\pm$  standard deviation) are given as a function of the content of WF10. Fluorescence of samples without WF10 (control) was set to 100%. Data from three independent experiments are given. \* $p \leq 0.05$ , \*\* $p \leq 0.01$ .

### 3.2. Binding of hyaluronan to CD44 in the presence of WF10

The binding of fluorescence-labelled hyaluronan to CD44 is well described [7,8]. WF10 exposure diminished the binding of hyaluronan to LPS/IFN $\gamma$ -stimulated macrophages and TNF $\alpha$ -stimulated monocytes in a concentration-dependent manner (Figure 3). At the highest applied WF10 concentration (1:100 dilution), the binding of hyaluronan was decreased by 41.2%  $\pm$  3% in macrophages and 22.6%  $\pm$  6% in monocytes. Chlorite (applied as chlorate-free WF10) caused a similar inhibition of hyaluronan binding as WF10. Chlorate (applied as chlorite-free WF10) diminished also the binding of this nonsulphated GAG. Thus, both chlorite and chlorate are involved in inhibition of the interaction between hyaluronan and CD44.

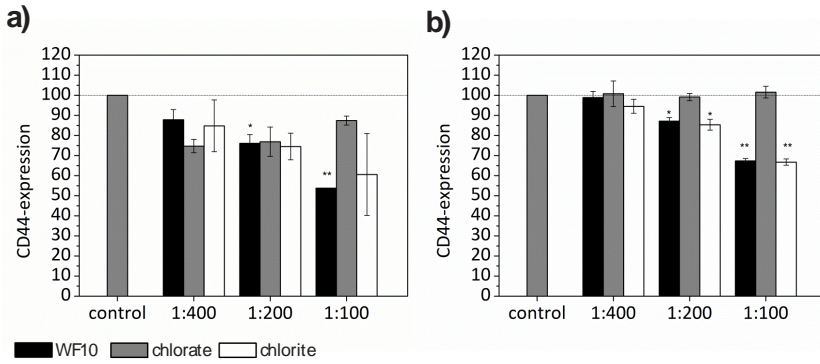


**Figure 3. Binding of fluorescence-labelled hyaluronan to the surface of macrophages (a) and monocytes (b).** LPS/IFN $\gamma$ -stimulated macrophages and TNF $\alpha$ -stimulated monocytes were incubated for 24 or 18 h, respectively, with or without (control) different concentrations of WF10/chlorate/chlorite. In these experiments, the applied concentrations of chlorite or chlorate were the same as in WF10. WF10, chlorate, and chlorite reduced the binding of hyaluronan to monocytes and macrophages. Fluorescence intensities (means  $\pm$  standard deviation) are given as a function of the content of WF10. Fluorescence of samples without WF10 (control) was set to 100%. Data from three independent experiments are given. \* $p \leq 0.05$ , \*\* $p \leq 0.01$ .

In addition, the expression of CD44 was assessed with anti-CD44 antibodies. The expression of CD44 was also significantly decreased by WF10 in both macrophages and monocytes (Figure 4). In both cell types, the WF10 effect coincides well with the application of chlorite. In monocytes, chlorate did not cause any changes in fluorescence values of CD44. In macrophages, the expression of CD44 was slightly decreased at the highest applied concentration of chlorate. However, this diminution was not significant.

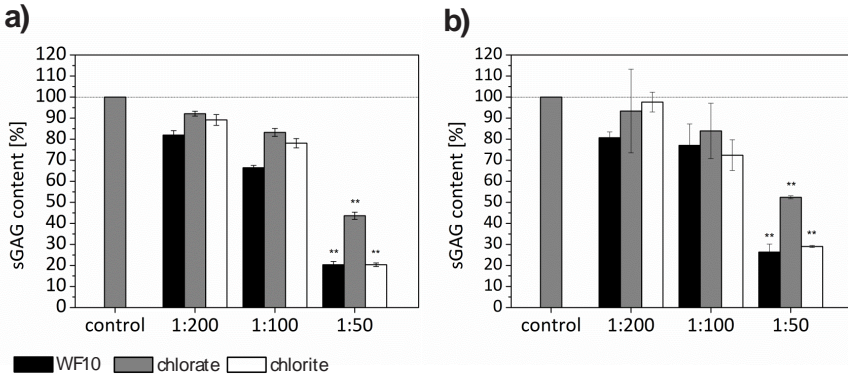
### 3.3. Effects of WF10 on the sulphation of glycosaminoglycans

Due to the strong effects of WF10 on the activation pattern and binding of hyaluronan, we next investigated which effects exhibit WF10 on the total formation of sulphated proteoglycans and GAGs in monocytes and macrophages. After activation of macrophages with LPS/IFN $\gamma$  or monocytes with TNF $\alpha$ , the total amount of sulphated GAGs was analysed in cell extracts by the Blyscan assay. This reagent interacts with sulphated proteoglycans and GAGs yielding a blue-coloured complex.



**Figure 4.** CD44 expression of macrophages (a) and monocytes (b) on cell surface. LPS/IFN $\gamma$ -stimulated macrophages and TNF $\alpha$ -stimulated monocytes were incubated for 24 h or 18 h, respectively, with or without (control) different concentrations of WF10/chlorate/chlorite. WF10 and chlorite reduced the CD44 expression in a concentration-dependent manner. Fluorescence intensities (means  $\pm$  standard deviation) are given as a function of the content of WF10. Fluorescence of samples without WF10 (control) was set to 100%. Data from three independent experiments are given. \* $p \leq 0.05$ , \*\* $p \leq 0.01$ .

WF10 considerably diminished the amount of sulphated proteoglycans and GAGs in macrophages and also in monocytes (Figure 5). Again, chlorite caused the same effects as WF10. A strong inhibition of the total sulphation degree was also observed for chlorate both in monocytes and macrophages. However, chlorate was less efficient than chlorite.



**Figure 5.** Effects of WF10 on the sulphation of glycosaminoglycans in macrophages (a) and monocytes (b). LPS/IFN $\gamma$ -stimulated macrophages and TNF $\alpha$ -stimulated monocytes were incubated for 24 h or 18 h, respectively, with or without (control) different concentrations of WF10/chlorate/chlorite. WF10, chlorate, and chlorite reduced the sGAG content in a concentration-dependent manner. Absorbance values for sulphated GAGs are given as a function of the content of WF10, chlorite or chlorate. Absorbance of the control samples (without WF10 or components) was set to 100%. Data from at least three independent experiments are shown. \* $p \leq 0.05$ , \*\* $p \leq 0.01$ .



## 4. Discussion

The drug substance WF10 inhibits the activation of LPS/IFN $\gamma$ -stimulated human monocyte-derived macrophages. Among them are the diminished expression of proinflammatory surface markers, the inhibition of the expression of the hyaluronan receptor CD44, and the binding of hyaluronan to CD44. Further, the overall amount of sulphated proteoglycans and GAGs was down-regulated by WF10. These *in vitro* experiments indicate that WF10 is able to inhibit the proinflammatory activation of the M1-type in macrophages.

These data are in line with other WF10 effects observed on monocytes, macrophages, and the interaction of these cells with fibroblasts and T-cells. In macrophages, WF10 is known to decrease the expression of TNF $\alpha$  [11], to diminish the antigen presentation that diminishes T-cell proliferation [21,22], and to enhance the interaction with fibroblasts [11]. WF10 inactivates AIDS viruses [10,23], enhances the cytotoxicity of natural killer cells [24], and prolongs cardiac graft survival in a rodent xenotransplantation model [25]. Further, the application of WF10 attenuates the disease state in radiation-associated tissue damage [12,26,27], in diabetic foot ulcers [13], and in wound healing [28,29].

The experimental data suggest that chlorite is the active principle in WF10 and in the more diluted form Oxoferin as chlorite caused principally the same changes in targets as WF10 [30]. In our experiments, a chlorate-free preparation of WF10 showed the same results as WF10 confirming the fact that chlorite is the active component. Chlorate was insensitive in all experiments concerning the expression of surface proteins in macrophages. Only the binding of hyaluronan to CD44 and the formation of sulphated proteoglycans and GAGs responded to chlorate. In experimental cell biology, chlorate is usually used as an inhibitor of sulphation reactions [18,31] because it inhibits the formation of the common sulphur transfer reagent PAPS. As chlorate and sulphate share a similar geometry concerning the arrangement of oxygen–chlorine and oxygen–sulphur bonds, respectively, chlorate reversibly blocks the sulphate-binding side in PAPS synthase [32]. The stronger impact of chlorite over chlorate on the total amount of sulphated proteoglycans and GAGs can be explained by the fact that chlorite inhibits important signalling pathways in proinflammatory cells that apparently affect also sulphation reactions. A 50% inhibition of PAPS production by PAPS synthase occurs by 0.11 mM chlorate in the presence of physiological relevant concentrations of ATP and sulphate [32].

In leukocytes, the binding of hyaluronan to CD44 is tightly regulated by additional glycosylation, sulphation and sialylation [8]. Upon stimulation of blood-derived monocytes with TNF $\alpha$ , the overall degree of CD44 sulphation increased [7,33]. This increased sulphation of CD44 in TNF $\alpha$ - or IFN $\gamma$ -stimulated monocytes correlates well with the enhanced binding of hyaluronan that is inhibited by chlorate [7].

Our observation about the inhibition of hyaluronan binding and overall sulphation in stimulated monocytes and macrophages by WF10 can only partially be explained by the chlorate effect, as a chlorate-free preparation of WF10 exhibited the same inhibitory efficiency as the complete WF10 solution. Thus, the chlorite component of WF10 should preferential-

ly act on upstream targets of signalling pathways in stimulated leukocytes. In the result of this activity, not only the induction of PAPS synthase will be dampened but also other signalling cascades that induce the expression of proinflammatory receptors as well as the secretion of cytokines and other inflammatory mediators.

At the moment, we can only speculate about the molecular targets for chlorite in signalling cascades of macrophages. As a redox-active component, chlorite is well known to interact with the porphyrin iron in haem proteins [14–16, 34–37]. In monocytes and macrophages, a number of different cytochromes and other haem proteins are involved in signalling pathways. In human macrophages, IFN $\gamma$  is a strong inducer of indoleamine-2,3-dioxygenase, a haem protein that catalyses the oxidation of the amino acid tryptophan to kynurenine [38]. Contrary to this, IFN $\gamma$  up-regulates the expression of the haem protein inducible nitric oxide synthase in murine macrophages [39]. There are also several redox-sensitive elements in signalling cascades that respond to changes in oxidative homeostasis [40]. Different effects of WF10 on monocytes and THP-cells have been reported such as inhibition of translocation of the transcription factor NFAT to the nucleus or the activation of AP1 and NF $\kappa$ B signalling [22].

## 5. Conclusions

Taken together, WF10 is a promising tool to inhibit proinflammatory states of immune cells. By general inhibition of macrophage activation in the presence of inflammatory mediators, WF10 affects not only the activation state of these key immune cells but also the interaction of macrophages with T-cells, fibroblasts, and other cells at inflammatory sites [11,21,22,24]. Many diseases are accompanied by long-lasting chronic inflammations with disturbed processes of immune regulation. In a number of cases, the application of WF10 improved the disease state of these patients [10–13, 25–28]. Thus, our data about the inhibition of activation processes in monocytes and macrophages by WF10 coincide well with the results about clinical application of WF10.

The molecular mechanisms of the action of WF10 components are only poorly understood at present. Despite clear effects of chlorate on the overall sulphation in monocytes and macrophage, the chlorite component is the main active principle of WF10. Chlorite caused principally the same effects as WF10. Many efforts are necessary to deepen our knowledge about the cellular and tissue targets for chlorite, to understand how this WF10 component affects signalling pathways in inflamed tissues, and to give an answer on the question how WF10 contributes to resolution of chronic inflammatory states.

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