Impact of the anti-inflammatory agent bindarit on the chemokinome: selective inhibition of the monocyte chemotactic proteins

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Accepted for publication July 30, 2008

ABSTRACT. Bindarit is an indazolic derivative that is devoid of any immunosuppressive effects and has no effect on arachidonic acid metabolism. However, it has been proved to have anti-inflammatory activity in a number of experimental diseases, including pancreatitis, arthritis, and lupus nephritis. This therapeutic effect has been associated with its ability to interfere selectively with monocyte recruitment, although the underlying molecular mechanisms are unknown. Here we comprehensively examine the effect of bindarit on the chemokine system, and report that in activated monocytes and endothelial cells, it selectively inhibits the production of the monocyte chemotactic protein subfamily of CC inflammatory chemokines (MCP-1/CCL2, MCP-3/CCL7, MCP-2/CCL8). The capacity of bindarit to inhibit the production of a defined set of related CC chemokines by monocytes and endothelial cells likely underlies the anti-inflammatory activity of this agent in disease. The exploitation of the chemokine system as drug target in inflammatory disease has relied mainly on the development of receptor antagonists and blocking antibodies. Here we report on the use of inhibition of synthesis as a potentially viable and selective approach to modify the chemokine system.

Keywords: inflammation, chemokines, lipopolysaccharide, gene regulation

Monocyte-macrophages play an important role in inflammatory diseases through their production of classical pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, IL-6, and IL-12. The archetypal inducers of the transcription of pro-inflammatory genes are microorganism products, many of which are recognized by pathogen recognition receptors of the Toll-like receptor (TLR) family. The most well studied TLR ligand is lipopolysaccharide (LPS), a component of the cell wall of Gram-negative bacteria, which initiates a complex signaling cascade culminating in nuclear translocation of the transcription factor NF-kB, transactivation of chemokine genes, and leukocyte recruitment [1, 2].

In humans, the chemokine system includes 42 ligands grouped in four subfamilies (C, CC, CXC and CX3C), on the basis of the number and position of N-terminal-conserved cysteine residues. Chemokines have chemotactic and cytokine-like activities and coordinate leukocyte recruitment through the interaction with 18 G protein-coupled receptors [3]. This system represents one of the most attractive drug targets in the huge battery of molecules that regulate inflammation and immunity. Mainstream attention has been devoted to the development of receptor antagonists and blocking antibodies, and clinical trials on inflammatory diseases involving chemokine-receptor antagonists are ongoing [4]. Here we report on the inhibition of s ynthesis as a potentially viable and selective approach to modifying the chemokine system.

Bindarit (2-methyl-2-1-(phenylmethyl)-1H-indazol-3yl] methoxy] propanoic acid) is an indazolic derivative devoid that is of any immunosuppressive effects and which is not active in arachidonic acid metabolism. It has been proved to be protective in a number of experimental diseases [5-7]. *In vivo* models of local inflammation have shown that the pharmacological activities of this compound are related to its ability to interfere with monocyte recruitment, which has been ascribed to a dose-dependent, inhibitory effect on MCP-1/CCL2 production [8]. Monocytes express a wide range of chemoattractant receptors, allowing them to be recruited under inflammatory conditions in response to

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doi: 10.1684/ecn.2008.0133

different types of chemoattractants, including chemokines, complement fragments, and chemotactic lipids [9]. To better define the molecular mechanisms responsible for the inhibitory effect of bindarit on monocyte recruitment, we investigated its effect on the LPS-dependent regulation of chemokines and chemoattractant receptors. Here we demonstrate that bindarit selectively inhibits the production of the monocyte chemotactic protein subfamily of CC inflammatory chemokines (MCP-1/CCL2, MCP-3/CCL7, MCP-2/CCL8), thus providing a potential mechanism underlying its anti-inflammatory activity.

METHODS AND MATERIALS

Reagents

LPS from *E. coli* (serotype 055:B5) was purchased from Sigma Chemicals.

Cell preparation and stimulation

Monocytes were isolated from buffy coats by Ficoll-Hypaque and Percoll gradients (GE Healthcare), followed by magnetic separation (Monocyte Isolation Kit II; Miltenyi Biotech), as previously described [10]. Monocytes were resuspended at $2x10^6$ cells/ml in RPMI 1640 medium supplemented with 1% FCS, plated in petriperm dishes (Sigma Chemicals), pretreated or not with bindarit for 30 min, and stimulated with 100 ng/mL LPS for the indicated time. Human umbilical vein endothelial cells (HUVEC) were obtained and cultured as described [11], maintained in M199 medium supplemented with 20% FCS, endothelial cell growth supplement (50 µg/mL; Collaborative Research) and heparin (100 µg/mL; Sigma Chemicals), and used between passage 2 and 6. Subconfluent HUVEC in 96-well plates were cultured overnight with 5% FCS, pretreated or not with bindarit for 30 min, and stimulated with 100 ng/mL LPS for 24 h.

Real-time RT-PCR analysis

Cells were stimulated as described and total RNA was extracted from 5 x 10^6 cells using TRIzol (Invitrogen Life Technologies), retrotranscribed, and prepared for a custom-designed 384-well TaqMan Low Density Arrays (LDA) to investigate transcripts of chemokines and chemoattractant receptors in duplicate. Samples were analyzed using the 7900HT system with a TaqMan LDA Upgrade (Applied Biosystems) and SDS2.1 software, according to the manufacturer's instructions. In short, 5 µL of single-stranded cDNA (to a final concentration of 100 ng starting RNA) was combined with 45 µL water and 50 µL TaqMan Universal PCR Master Mix, followed by loading of 100 µL of sample per port. Thermal cycling conditions were as follows: 50°C for 2 min, 95°C for 10 min, 95°C for 15 s, and 60°C for 1 min. The expression level of each gene was normalized to 18S and calibrated against the control sample to obtain the relative expression level. Each gene was assessed in duplicate in every experiment and only the genes with reproducible amplification curves were analyzed. Experiments were conducted in triplicate.

Cytokine measurement by ELISA assay

Supernatants were collected, and chemokines were measured using commercially available sandwich ELISA kits (R&D Systems) according to the manufacturer's instructions.

RESULTS AND DISCUSSION

The anti-inflammatory activity of bindarit has been previously ascribed to its ability to impair monocyte recruitment in inflamed tissues [8]. To better define the molecular mechanisms involved, we investigated the effect of bindarit on the regulation of chemokines and chemoattractant receptors by inflammatory mediators on monocytes. Using LPS as a prototypic inflammatory stimulus, the effect of bindarit on chemokines transcripts was investigated in Q-PCR experiments using a custom-made LDA investigating all known human chemokines. As expected, LPS was able to induce (> 2-fold as compared to control) a significant number of chemokines in monocytes (not shown). Bindarit did not show any significant inhibitory effect (< 0.5-fold as compared to LPS) on the LPS-dependent induction of most inflammatory chemokines, with the remarkable exception of the monocyte chemotactic protein subfamily of inflammatory chemokines MCP-1/CCL2, MCP-2/CCL8, and MPC-3/CCL7 *(figure 1)*. Preliminary results using a comprehensive, whole genome transcriptional profiling approach [12] show a similar selective effect of bindarit on the chemokinome of activated human monocytes (data not shown).

The selective inhibition of monocyte chemotactic proteins was then investigated at the protein level. Monocytes were stimulated with 100 ng/mL LPS in the presence of increasing doses of bindarit for 18 h and chemokines released into the supernatant were measured. Consistent with data at transcript level, bindarit exerted a dose-dependent inhibi-

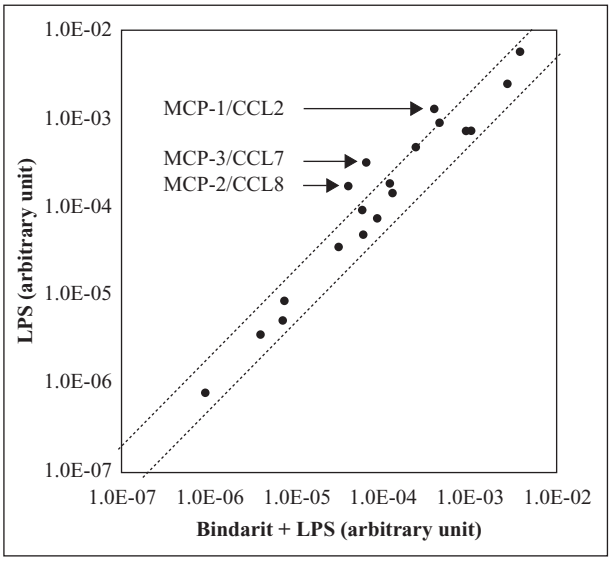

Figure 1

Effects of bindarit on LPS-dependent chemokine induction in monocytes.

Effect of 300 µM bindarit on the regulation of chemokine transcripts following monocyte stimulation with 100 ng/mL LPS for 4 h. Results are expressed on a log scale as $2^{-\Delta ct}$ and represent mean values of independent experiments performed in triplicate. Dotted lines represent the 2 and 0.5 boundary values.

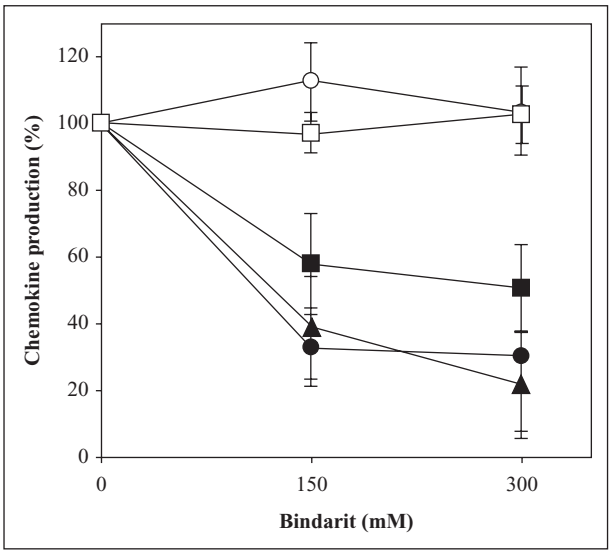

Figure 2
Effects of bindarit on MCP production in response to LPS.
Chemokines secreted by monocytes stimulated for 18h with 100 ng/mL LPS, in the presence of increasing concentrations of bindarit, were measured by ELISA. Results are mean ± SD of independent experiments performed in triplicate. ■, MCP-1/CCL2; ▲, MCP-2/CCL8; ●, MCP-3/CCL7; ○, MIP-1α/CCL3; □, IL-8/CXCL8.

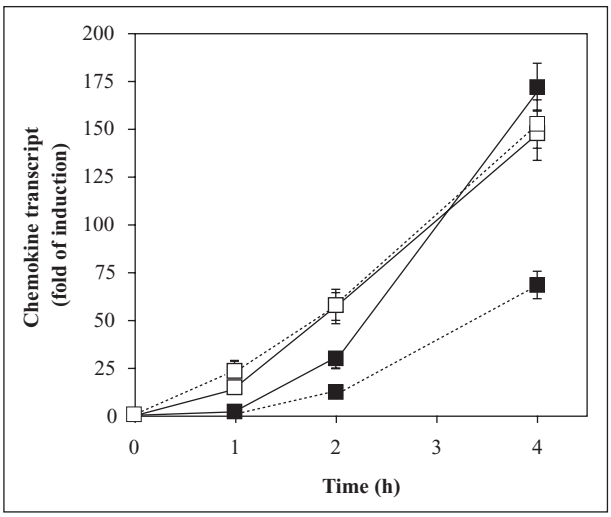

Figure 3
Time-course of the bindarit effect on LPS-dependent MCP-1/CCL2 transcript induction.
Chemokine transcript induction in monocytes stimulated with 100 ng/mL LPS for increasing amounts of time were evaluated in Q-PCR experiments and plotted as fold of induction over control levels. ■, MCP-1/CCL2; □, IL-8/CXCL8; full line, medium; dotted line, 300 µM bindarit. Results are shown as fold of induction over control (calculated using the ΔΔct method), and represent the mean ± SD of independent experiments performed in triplicate.

tory effect on MCP-1/CCL2, MCP-2/CCL8, and MPC-3/CCL7, while production of MIP-1α/CCL3 and IL-8/CXCL8 was unaffected *(figure 2)*. As observed in monocytes, bindarit also inhibited MCP-1/CCL2 production in LPS-activated HUVEC cells (13.5 ± 5.9 *versus* 8.9 ± 3.6 ng/mL for LPS and bindarit plus LPS, respectively; $p < 0.05$). MCP-2/CCL8 and MCP-3/CCL7 were not induced in HUVEC cells.

To gain insight into the mechanisms accounting for the effect of bindarit on LPS-dependent MCP-1/CCL2 induction, a time-course experiment was conducted. In monocytes exposed to LPS, the MCP-1/CCL2 transcript was readily detectable at the 2 h time-point and then progressively accumulated. Bindarit treatment resulted in a consistent inhibition of about 50% at each time-point tested, starting from the earliest time-point evaluated *(figure 3)*. Again, bindarit was completely inactive in LPS-dependent IL-8/CXCL8 induction. Although not formal proof, the short time-point and the consistent level of inhibition over time argue against a potential indirect effect of bindarit on LPS-dependent MCP-1/CCL2 induction.

Monocytes express a wide range of chemoattractant receptors allowing them to be recruited under inflammatory conditions in response to different types of chemoattractants, including chemokines, complement fragments, and chemotactic lipids [9]. To investigate the possibility that bindarit may modify the pro-inflammatory activity of LPS by interfering with chemoattractant receptors expression, we investigated the effect of bindarit on the LPS-dependent regulation of chemoattractant receptor transcripts. As shown in *figure 4*, Q-PCR experiments, using a custom-made LDA, investigating all known human chemoattractant receptors expressed on monocytes, showed that bindarit did not significantly influence the effect of LPS.

CC chemokines are crucial molecules in monocyte recruitment, and are encoded by a gene cluster located on

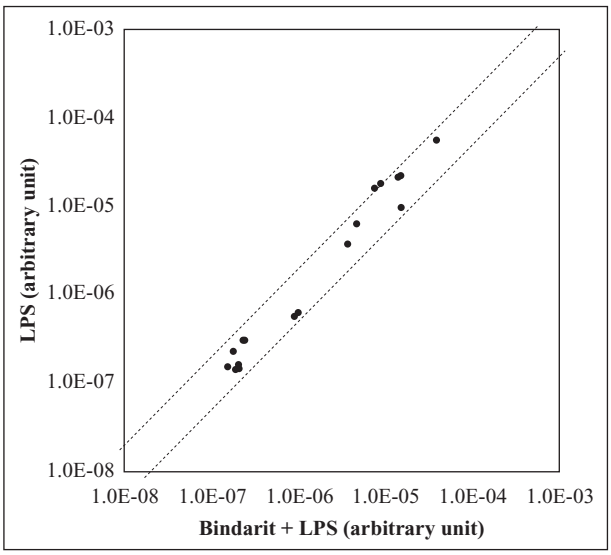

Figure 4
Effect of bindarit on chemoattractant receptor transcripts.
Effect of 300 µM bindarit on the regulation of chemoattractant receptor transcripts following monocyte stimulation with 100 ng/mL LPS for 4 h. Results are expressed on a log scale as $2^{-\Delta ct}$ and represent mean values of independent experiments performed in triplicate. Dotted lines represent the 2 and 0.5 boundary values.

17q11.2 where two distinct groups of genes can be distinguished. The macrophage inflammatory protein (MIP) group includes potent monocyte chemoattractants agonists at CCR1 and CCR5, such as MIP-1α/CCL3, MIP-1β/CCL4, MIP-3/CCL23, and RANTES/CCL5 among others. These chemoattractants are regulated in the experimental conditions tested, and are resistant to the inhibitory activity of bindarit. Conversely, the inhibitory activity of bindarit selectively targeted all the CC chemokines regulated in the experimental conditions tested encoded by the

MCP group, which includes MCP-1/CCL2, MCP-2/CCL8, and MCP-3/CCL7. These chemoattractants share a significant degree of homology as regards gene organization, primary sequence (58 to 71% identity), and tertiary structure, and most interestingly are the sole activators of CCR2 [13]. Thus, the anti-inflammatory activity of this agent in disease models, including pancreatitis, arthritis, and lupus nephritis [5-7, 14-16] is likely mediated by the selective inhibition of the production by monocytes and endothelial cells of a distinct group of inflammatory CC chemokines, which play a prominent role in monocyte recruitment through the activation of CCR2. Although the molecular mechanisms involved have still to be defined, it is interesting to note that experimental results indicate that bindarit activity acts on MCP production by interfering with their induction at the transcriptional level, and MCP also share homologies in their transcriptional regulation [13].

The exploitation of the chemokine system as drug target for inflammatory diseases has mainly relied on the development of receptor antagonists and blocking antibodies [17]. Bindarit has been shown to be active in several animal models of inflammation, and preliminary clinical studies have shown a significant reduction in albumin and MCP-1/CCL2 urinary excretion in orally-treated lupus nephritis patients (A. Guglielmotti, personal communication), suggesting that the use of small molecules that are able to inhibit cytokine/chemokine production should be considered as a potentially viable and selective approach to modifying the chemokine system.

Acknowledgments. *This study was supported by the EC research grant INNOCHEM (project 518167) and was conducted in the context and with the support of the Fondazione Humanitas per la Ricerca.. AG, GM and GB are Angelini employees. AM and ML have received support form Angelini to study the pharmacological activity of bindarit.*

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