Inhibition by Mitoxantrone of In Vitro Migration of Immunocompetent Cells

A Possible Mechanism for Therapeutic Efficacy in the Treatment of Multiple Sclerosis

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Background: Damage of the blood-brain barrier and invasion of immunocompetent cells into the central nervous system represent key events in the immunopathogenesis of multiple sclerosis. Mitoxantrone hydrochloride reduces progression of disability and clinical exacerbations in patients with multiple sclerosis. Its precise mode of action is unclear.

Objective: To investigate the effects of mitoxantrone on the migratory capacity of immunocompetent cells ex vivo and in vitro.

Design: Case-control study.

Setting: Department of Neurology, Heinrich Heine University, Düsseldorf, Germany.

Participants: Peripheral blood mononuclear cells (PBMCs) were obtained from 11 patients with multiple sclerosis before and after intravenous mitoxantrone treatment; PBMCs from 5 healthy control donors were treated with mitoxantrone in vitro.

Main Outcome Measures: The migratory capacity was studied in an in vitro Boyden chamber assay; cells and their rates of migration were analyzed by light microscopy and flow cytometry. To determine the specificity of our findings, PBMCs were treated with perfosfamide in vitro.

Results: Mitoxantrone decreased the migratory capacity of CD14/HLA-DR positive monocytes and (to a lesser degree) of CD4/HLA-DR positive and CD8/HLA-DR positive T lymphocytes. These observations were confirmed when control PBMCs were treated with an equivalent dose of mitoxantrone in vitro. Similar effects were seen when PBMCs were preincubated with perfosfamide. The inhibitory effects of mitoxantrone on the migratory capacity of PBMCs were mediated by reduced matrix metalloproteinase 9 activity, as demonstrated by zymography, polymerase chain reaction, and inhibitory studies.

Conclusion: Mitoxantrone may inhibit the migration of inflammatory cells into and within the central nervous system.

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Mitoxantrone hydrochloride is an immunosuppressive drug approved for the treatment of worsening forms of multiple sclerosis (MS). Like other synthetic anthracyclines, mitoxantrone intercalates with DNA and via interaction with topoisomerase 2 causes DNA single- and double-strand fragmentation. As a result, apoptotic and necrotic cell death occurs in treated tissues. Studies demonstrated that mitoxantrone affects the innate and adaptive arms of the immune system: the proliferation of macrophages is reduced in vitro and in vivo, and mitoxantrone interferes with antigen function by inducing programmed cell death of antigen-presenting cells, including dendritic cells and macrophages. In addition, mitoxantrone reduces the proliferation of T cells and B cells and decreases the secretion of pro-inflammatory cytokines. However, the precise mechanism of action of mitoxantrone in the treatment of MS remains elusive to date.

Multiple sclerosis is an inflammatory demyelinating disorder of the central nervous system characterized by perivascular mononuclear infiltrates. Damage of the blood-brain barrier (BBB) and migration of immunocompetent cells across the BBB into and through the extracellular matrix of the central nervous system represent key events in the immunopathogenesis of MS. These critical processes are mediated, at least in part, by the secretion of enzymes of the so-called matrix metalloproteinases (MMPs).

Using an in vitro Boyden chamber assay, we studied the effects of mitoxantrone on the migratory capacity of immunocompetent cells.
trone on the migratory capacity of immunocompetent cells. Because this drug is given intravenously, it primarily affects cells within the systemic immune compartment. Therefore, the functional effects of mitoxantrone on the transmigration of proinflammatory cells across the BBB into the brain and spinal cord and through the extracellular matrix may be of therapeutic relevance. To our knowledge, this is the first report on the effects of mitoxantrone on migratory behavior of lymphoid and myeloid cells.

**METHODS**

**MONONUCLEAR CELLS EX VIVO**

Peripheral venous blood samples were obtained with informed consent from patients with MS (female-male ratio, 6:3; mean age, 41 years) at the Department of Neurology, Heinrich Heine University, Düsseldorf, Germany, where this study was approved by the local ethics committee. There were 2 patients with relapsing remitting MS and 9 patients with secondary progressive MS with superimposed relapses who had not received immunosuppressive therapy or corticosteroid treatment in the 3 months before blood draw. The median disability, as measured by the Expanded Disability Status Scale, was 5.5 (range, 3.5-6.5), the mean±SD disease duration was 12±6 years, and the mean cumulative dose of mitoxantrone administered was 44 mg/m² (range, 19-130 mg/m²). Blood samples were aseptically collected by standard venipuncture into vacuum tubes containing sodium EDTA as an anticoagulant before and immediately after intravenous mitoxantrone treatment. Peripheral blood mononuclear cells (PBMCs) were separated by gradient centrifugation using Ficoll-Paque Plus medium (Amersham Biosciences, Uppsala, Sweden) and were cultured in RPMI 1640 medium (Invitrogen, Karlsruhe, Germany).

**IN VITRO TREATMENT WITH MITOXANTRONE**

Peripheral venous blood samples were collected from 5 healthy control donors (female-male ratio, 1:4; mean age, 31 years) who were taking no medications known to affect the immune system, and PBMCs were obtained by density gradient centrifugation and suspended in RPMI 1640 medium. A total of 2×10⁶ PBMCs/mL were treated with 10µM mitoxantrone (Wyeth, Münster, Germany) in vitro, a dose that has been evaluated previously, and were incubated for 1 hour at 37°C and 5% carbon dioxide without further stimulation.

**BOYDEN CHAMBER ASSAY**

The migratory capacity of PBMCs before and after treatment with mitoxantrone ex vivo and in vitro was studied using an in vitro Boyden chamber assay as described previously. Briefly, the Boyden chamber assay (Becton Dickinson, Heidelberg, Germany) consists of 2 compartments separated by a polyethylene terephthalate membrane filter (10.5 mm in diameter with 3-µm pores) precoated with fibronectin, a major component of the basement membrane that surrounds cerebral endothelium. A total of 6×10⁶ PBMCs/mL, suspended in 600 µL of RPMI 1640 medium plus 2.5% fetal calf serum (FCS) (Invitrogen), was added to the upper chamber. The lower chamber was filled with 500 µL of RPMI 1640 medium plus 10% FCS. The higher concentration of FCS in the lower chamber served as a directional gradient for the transmigration of PBMCs. Alternatively, recombinant human RANTES (regulated on activation, normal T-cell expressed and secreted) (CCL5) and monococyte chemoattractant protein 1 (CCL2) (R&D Systems, Wiesbaden, Germany) were applied to the lower chamber to produce a chemotactic gradient. Each chemokine was reconstituted in PBS with 0.1% human serum albumin to 100 µg/mL, and aliquots were diluted in RPMI 1640 medium to the final concentration of 100 ng/mL, as described previously. Chambers were incubated for 90 minutes in 5% carbon dioxide at 37°C. Cells in both compartments were collected for cell counting by light microscopy and for flow cytometric analysis of cellular subsets.

**CELL COUNTING AND VIABILITY DETERMINATION BY LIGHT MICROSCOPY**

The collected PBMCs from the lower and upper chambers were diluted 1:1 in trypan blue. The viability of the cells was evaluated, and they were counted by light microscopy using a Neubauer chamber (Scherf Präzision, Meiningen, Germany).

**FLOW CYTOMETRY**

For the analysis of PBMCs and their rates of migration, cells were incubated for 30 minutes on ice and were stained with fluorescein isothiocyanate, phycoerythrin, peridinin chlorophyll protein, and allophycocyanin-labeled monoclonal antibodies directed against human CD14⁺, CD4⁺, CD8⁺, and CD19⁺ surface antigens (Becton Dickinson). Thereafter, cells were analyzed on a flow cytometer (FACScalibur; Becton Dickinson), using CellQuest software (Becton Dickinson). Mononuclear cells were analyzed in a lymphocyte/monocyte gate, which was set according to forward and sideward scatter properties. The results are given as percentage of all gated cells.

**IN VITRO TREATMENT WITH PERFOSFAMIDE**

To compare the inhibitory effects of mitoxantrone with those of other immunosuppressive drugs used in escalating therapy of MS, control PBMCs were treated in vitro with perfosfamide (4-HPC), the active form of cyclophosphamide. A total of 2×10⁶ PBMCs/mL from 3 controls were cultured (at a concentration of 2×10⁶ cells/mL) in culture medium (RPMI 1640 medium supplemented with 10% FCS, 1% l-glutamine, and 1% penicillin-streptomycin). Cell cultures were kept untreated or were stimulated with the mitogenic lectin phytohemagglutinin (5 µg/mL; Sigma-Aldrich, Deisenhofen, Germany) for 24 hours at 37°C and 5% carbon dioxide, as reported previously. A total of 2×10⁶ PBMCs/mL (freshly obtained), as well as 24-hour phytohemagglutinin-stimulated and 24-hour phytohemagglutinin-unstimulated PBMCs, were treated with 2 µg/mL of 4-HPC (Niemeck, Bielefeld, Germany) in vitro and were incubated for 1 hour at 37°C and 5% carbon dioxide without further stimulation, as described previously. The migratory capacity of PBMCs before and after treatment with 4-HPC in vitro was studied using the in vitro Boyden chamber assay, as already described.

**ZYMOGRAPHY**

Because MMPs are known mediators of the transmigration of mononuclear cells, the enzymatic activity of MMP-2 and MMP-9 in supernatants from PBMCs of patients and controls was determined by zymography as described previously. Briefly, sodium dodecyl sulfate polyacrylamide gels (10% polyacrylamide) were copolymerized with 1 mg/mL of type A gelatin derived from porcine skin (Sigma-Aldrich). Diluted samples with sodium dodecyl sulfate sample buffer were electrophoresed at a constant voltage. After electrophoresis, the gel was washed in 2.5% Triton X-100 and was then incubated for 24 hours at
37°C in developing buffer (0.05M Tris–hydrochloric acid [pH 8.8], 5mM calcium chloride, and 0.02% sodium acid). After staining with Coomassie blue, proteolytic activity was identified as a clear band on a blue background.

INHIBITION OF MMPs IN VITRO

To compare the inhibitory effects of mitoxantrone with the effects of an MMP inhibitor, 2 × 10^6 PBMCs/mL from controls was treated with 0.5µM EDTA, which is a chelator and an effective MMP inhibitor,22,23 and was incubated for 1 hour at 37°C and 5% carbon dioxide. The migration of PBMCs across biological barriers after treatment with EDTA was assessed using the in vitro Boyden chamber assay as already described.

RNA EXTRACTION, COMPLEMENTARY DNA SYNTHESIS, AND QUANTITATIVE REAL-TIME POLYMERASE CHAIN REACTION

To delineate differences in regulation on the RNA level vs the protein level of MMP-9, RNA expression was investigated by quantitative real-time polymerase chain reaction (PCR). Total RNA was obtained from PBMCs of patients with MS before and after treatment with mitoxantrone using TriZOL RNA isolation reagent (Invitrogen). Reverse transcription was performed using TaqMan reverse transcription reagents (Applied Biosystems, Foster City, Calif) according to the manufacturer’s protocol. Conditions for the reverse transcription were 10 minutes at 23°C, 30 minutes at 48°C, and 5 minutes at 95°C using an automated thermocycler for the amplification of nucleic acids using PCR (GeneAmp PCR System 9700; Applied Biosystems). The PCR was performed as described previously24 using a complete, real-time PCR system that detects and quantitates nucleic acid sequences (ABI PRISM 7700 Sequence Detection System; Applied Biosystems). Each reaction was performed in a total volume of 30 µL, including 50% TaqMan Universal PCR Master Mix. Primers and probes for MMP-9 and the housekeeping gene 18S were purchased from Applied Biosystems. Conditions for the PCR were 50°C initially, 2 minutes at 95°C, followed after 10 minutes by 42 cycles of 15 seconds at 95°C and 60 seconds at 60°C. Data were analyzed on the ABI PRISM 7700 Sequence Detection System using the comparative C_t (threshold cycle) method. Samples were normalized to 18S ribosomal RNA to account for the variability in the initial concentration of the total RNA and the conversion efficiency of the reverse transcription reaction.

STATISTICAL ANALYSIS

Differences in the migration of PBMCs across the fibronectin layer were analyzed by comparing the numbers of cells in the upper and lower chambers before and after treatment with mi-
toxantrone, using Wilcoxon signed rank test. Statistical significance was set at $P < .05$. Data are given as mean±SD.

## RESULTS

### MITOXANTRONE REDUCES THE MIGRATION OF PBMCs

The migratory capacity of mononuclear cells obtained from patients with MS was significantly inhibited after intravenous mitoxantrone treatment ($P = .002$). The percentage of cells that migrated across the fibronectin layer before treatment with mitoxantrone was 20%, whereas after treatment only 2% were detected in the lower chamber (Figure 1A). This inhibitory effect was most prominently seen in CD14$^+$ monocytes ($P = .003$) when analyzed by flow cytometry (Figure 1B). When comparing different subtypes of T lymphocytes, the transmigration of CD4$^+$ and CD8$^+$ T lymphocytes was significantly reduced. However, no statistically significant difference was observable between these 2 cell populations. We observed no effect of mitoxantrone on the migratory capacity of CD19$^+$ B lymphocytes, because too few B cells were recovered in the lower chamber after incubation before and after treatment with mitoxantrone to provide meaningful results.

There was no difference in the efficacy of mitoxantrone on PBMCs obtained from patients with relapsing remitting MS and secondary progressive MS, and there was no correlation with the duration of the disease. Neither the clinical disability nor the cumulative dosage of mitoxantrone affected the migratory capacity of cells (data not shown).

Similarly, the migration of PBMCs was significantly reduced after treatment with mitoxantrone in vitro. Approximately 27% of mononuclear cells migrated across the fibronectin layer before treatment with mitoxantrone, whereas after treatment only 4% were recovered in the lower chamber (Figure 1C). Flow cytometry detected that CD14$^+$ monocytes were most affected (Figure 1D-F). The migratory capacity of CD4$^+$ and CD8$^+$ T lymphocytes was reduced; however, these data were not statistically significant. The total number of recovered CD19$^+$ B lymphocytes before and after treatment with mitoxantrone was too low for further analysis. All mononuclear cells, especially those that were recovered from the lower chamber after transmigration, exhibited in vitro autofluorescence of mitoxantrone, as demonstrated by flow cytometry, indicating the uptake of mitoxantrone by PBMCs (Figure 1G).

### PERFOSFAMIDE INHIBITS THE MIGRATORY CAPACITY OF PBMCs

Approximately 6% of nonstimulated treatment-naïve PBMCs migrated across the fibronectin layer (Figure 2A).
In contrast, 25% of PBMCs activated with phytohemagglutinin for 24 hours migrated into the lower chamber, representing a 4.2-fold increase in migratory capacity (Figure 2A and C). In vitro treatment with 4-HPC revealed that only 1% of unstimulated and 4% of stimulated PBMCs were detectable in the lower chamber. Flow cytometry revealed that 4-HPC similarly affected different subtypes of T cells, including CD4\textsuperscript{T} lymphocytes, CD8\textsuperscript{T} lymphocytes, and CD14\textsuperscript{monocytes} (Figure 2B-D). The migration of freshly obtained PBMCs was similar to that of PBMCs after 24 hours of incubation in a nonstimulated environment (data not shown).

MITOXANTRONE INHIBITS THE PROTEOLYTIC ACTIVITY OF GELATINASES

Because MMPs are implicated as important mediators in the transmigration of immunocompetent cells, the proteolytic activity of the gelatinases MMP-2 and MMP-9 were assessed by gelatin zymography. In the supernatants from PBMCs, a decrease (compared with cells before therapy) in the proteolytic activity at 92 kDa (indicative of MMP-2) and at 72 kDa (indicative of MMP-9) was observable in samples that were treated with mitoxantrone in vitro and in samples that were obtained from patients after intravenous mitoxantrone treatment (Figure 3A).

MITOXANTRONE INHIBITS MMP-9 RNA EXPRESSION

Our finding of a striking reduction of the proteolytic activity of MMP-9 raised the question of whether this effect is mirrored on the RNA level as well. The PCR revealed a reduction of the RNA expression level of MMP-9 after the application of mitoxantrone to mononuclear cells ex vivo and in vitro (Figure 3B).

MMP INHIBITION REDUCES THE MIGRATION OF PBMCs

To compare the decrease in migratory capacity of PBMCs treated with mitoxantrone with the effects of an MMP inhibitor, mononuclear cells were treated with EDTA in vitro. Compared with mitoxantrone, EDTA similarly decreased the migration of PBMCs in the Boyden chamber assay; 2% of the mononuclear cells were recovered after the addition of EDTA, emphasizing the involvement of MMPs (at least in part) in the process of transmigration across the fibronectin layer (Figure 3C).

COMMENT

The migration of mononuclear cells across the BBB into the central nervous system is considered a key event in the immunopathogenesis of MS.\textsuperscript{12,22} In clinical trials, mitoxantrone reduced disability and clinical exacerbations in patients with secondary progressive MS and with worsening relapsing remitting MS.\textsuperscript{1,26,27} In the present study, we investigated whether mitoxantrone exhibits an effect on the migratory capacity of mononuclear cells across a fibronectin layer in a Boyden chamber assay as a BBB model. Unlike other noncollagenous components of the basement membrane, fibronectin is present in the fibrous tissue that surrounds blood vessels. It is a major structural component of the leptomeninges and the epithelial layer of the choroid plexus.\textsuperscript{28} In active MS plaques, fibronectin expression was increased up to 57% compared with inactive MS plaques, nonlesion areas of MS brains, and normal control white matter.\textsuperscript{29} Other investigations revealed that fibronectin acts as a stimulus for the migration of mononuclear cells and can be successfully used for in vitro migration assay.\textsuperscript{17}
During the 90-minute migration assay, approximately 20% of freshly obtained ex vivo PBMCs migrated across the fibronectin layer overlaying 3-µm pores before treatment with mitoxantrone. This magnitude seems somewhat high compared with previous reports for unstimulated cells. However, methodological differences render a direct comparison of these studies difficult. In our study, various chemokines tested in the assay did not improve the migratory rate. The combination of chemokines plus serum did not produce superior results (data not shown). The migration rates in our study are similar to the results published by Stuße et al in a study in which specific chemokines were used as chemotaxtractants. The pattern of chemokines expressed within MS lesions, which may attract mononuclear cells from the periphery into the central nervous system, seems to be complex and is not fully understood. Therefore, we decided to build a chemotactic gradient using FCS, which may reflect a more physiological scenario than targeting specific cell subtypes by selecting particular chemokines.

The treatment of mononuclear cells with mitoxantrone reduced their migration rates to 2% when cells were studied ex vivo and to 4% when studied in vitro. This significant reduction in migratory capacity was seen for CD14+ monocytes, CD4+ T lymphocytes, and CD8+ T lymphocytes. All cells studied incorporated mitoxantrone, which was demonstrated by detecting the autofluorescence of the drug by flow cytometry. The inhibitory effect of mitoxantrone was most prominent in CD14+ monocytes. This is probably based on the overall better migrational performance of CD14+ monocytes compared with other cell populations studied in our assay. It is unlikely that this reflects a selective targeting of cell types with different susceptibilities and fragilities by mitoxantrone. A significant reduction in migration across the fibronectin layer was seen for T lymphocytes ex vivo. This effect was not observable for in vitro-treated T cells, perhaps because of differences in the preactivation of these cells before being added to the Boyden chamber assay. Because no differences were observed between the CD4+ and CD8+ T lymphocytes, it seems that mitoxantrone similarly affects both subtypes of T lymphocytes.

To compare the observed inhibitory effect of mitoxantrone with that of other immunosuppressive drugs in the treatment of MS, we applied 4-HPC (the activated form of cyclophosphamide) in vitro. Cyclophosphamide has been widely studied for the treatment of MS, and effective stabilization among selected patients receiving this therapy has been suggested. Our data demonstrate that cyclophosphamide significantly reduced the migratory capacity of PBMCs in vitro.

This effect can be detected in other disease entities besides MS. We studied PBMCs ex vivo from 2 patients with chronic inflammatory demyelinating polyradiculoneuropathy who had been treated with mitoxantrone and 4-HPC. In both patients, the migratory rates were similarly reduced and were comparable to the results obtained among patients with MS (data not shown). Therefore, immunosuppressive drugs act (at least in part) on mononuclear cell migration independent of the disease entity.

Because of methodological limitations, we were unable to study the effects of mitoxantrone on the migratory capacity of CD19+ B lymphocytes. We believe that our serum gradient did not contain enough chemokines attracting B cells to recruit sufficient numbers of B lymphocytes into the lower compartment of the chamber. Chan et al reported preferentially induced cell death in CD19+ B lymphocytes that is mediated by mitoxantrone. This may represent an additional factor that could have negatively affected the migration of treated B cells in our study; however, we were unable to acquire data to address this issue. Further studies are warranted to clarify this question.

Previous investigations demonstrated that cell migration across the subendothelial basement membrane was mediated by the secretion of MMP-2 and MMP-9, the production of which is regulated by independent genes. We analyzed the expression of these 2 gelatinases after treatment with mitoxantrone and found a mild reduction of the proteolytic activity of MMP-2 and a more pronounced reduction of MMP-9 activity, indicating reduced protein expression of this protease. Similarly, MMP-9 RNA was reduced in the treated cells. Blocking MMP activity with EDTA resulted in an equivalent effect on cell migration, underlining that mitoxantrone (at least in part) exhibits its effects on the migratory capacity of PBMCs via the transcriptional inhibition of MMPs. The effects of mitoxantrone on other key molecules, such as adhesion molecules or chemokines, in this process of transmigration has been studied; however, mitoxantrone affected neither the gene transcription of various α- and β-chemokine receptors (as demonstrated by ribonuclease protection assays) nor the surface expression of very late antigen 4 or of intercellular adhesion molecule 1 (as shown by flow cytometry).

**CONCLUSIONS**

Mitoxantrone inhibits the migration of immunocompetent mononuclear cells across a fibronectin layer but does not specifically affect certain cell subtypes. This effect is not specific for cells obtained from patients with MS, as it was similarly observed in PBMCs from patients with chronic inflammatory demyelinating polyradiculoneuropathy and in vitro controls. The absolute and cumulative dosages of mitoxantrone had no effect on the degree of inhibition of cell migration, suggesting that the effects of mitoxantrone are not reduced by tachyphylaxis and that a dose of 5 mg/m² is sufficient to inhibit cell migration. However, the number of patients included in the present study is too low to draw final conclusions. Greater numbers of patients should be investigated to obtain deeper insights into this issue.

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