

Effects of Imatinib Mesylate in Mouse Models of Multiple Sclerosis and In vitro Determinants

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ABSTRACT

Experimental autoimmune encephalomyelitis (EAE) is a mouse model for multiple sclerosis (MS). This autoimmune disease is mainly mediated by adaptive and innate immune responses that lead to an inflammatory demyelination and axonal damage. Imatinib mesylate is a selective protein tyrosine kinase inhibitor with immunomodulatory properties that abrogates multiple signal transduction pathways in immune cells. In the present research, our aim was to test the therapeutic efficacy of imatinib in experimental model of MS.

We performed EAE induction in 23 female C57 mice by myelin oligodendrocyte glycoprotein-₃₅₋₅₅ (MOG₃₅₋₅₅) in Complete Freund's Adjuvant (CFA) emulsion and used imatinib for treatment of EAE. The clinical evaluation and histopathology were assessed. Also for in vitro analysis, we used U-87 MG, C6 and WEHI-164 cell lines to evaluate the inhibitory effects of imatinib in cell proliferation, as well as pro-inflammatory cytokines (TNF- α , IL-1 β , IL-6) and matrix metalloproteinase (MMP) secretion.

Our findings demonstrated that this drug had beneficial effects on EAE by attenuation in the severity and a delay in the onset of disease. In vitro, imatinib inhibited cell proliferation, MMP-2 expression and activity and also attenuated the production of proinflammatory cytokines.

Imatinib with its potential therapeutic effects and immunomodulatory properties may be considered, after additional necessary tests and trials, for treatment of MS.

Keywords: Cytokine; Experimental autoimmune encephalomyelitis; Imatinib mesylate; Matrix metalloproteinase-2; Multiple Sclerosis

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INTRODUCTION

Experimental autoimmune encephalomyelitis

(EAE) is a mouse model for multiple sclerosis (MS), where disease is mediated by autoantigen-specific T cells. There is evidence linking CD4⁺ T cells secreting interleukin (IL)-17, termed T helper (Th) 17 cells, and Interferon (IFN)γ-secreting Th1 cells with the pathogenesis of EAE.¹⁻³ Th17 and Th1 cells are infiltrated in the CNS prior to the onset of clinical symptoms of EAE, in which they may mediate central nervous system (CNS) inflammation, in part, through microglia activation.¹ The activated microglia and the release of molecules which are detrimental to oligodendrocyte have been suggested as mechanisms by which innate immunity proceeds demyelination in MS. Therefore, microglia and reactive astrocytes play a central role in neuroinflammatory processes by releasing various kinds of noxious factors such as matrix metalloproteinase (MMP) and proinflammatory cytokines Tumor Necrosis Factor-α (TNF-α), IL-1β and IL-6.^{2,4,6}

It has been demonstrated that MMP-2 and MMP-9 have a crucial function in migration of inflammatory cells into the CNS and can be expressed by vascular endothelial cells, meninges or resident cells such as reactive astrocytes and accumulated inflammatory cells. Elevated expression of MMP-2 and MMP-9 may play a role in some stages of neuroinflammation and pathological changes in MS and EAE.⁷⁻⁹

Imatinib mesylate (Imatinib, Glivec/Gleevec, STI-571), an orally administered 2-phenylaminopyrimidine derivative which is a selective protein tyrosine kinase inhibitor was developed to inhibit BCR-ABL kinase activity in chronic myelogenous leukemia (CML). It can also block the activity of the tyrosine kinases such as FLT3, Lck and MAPK. Therefore, imatinib has antiproliferative activity and immunomodulatory effects on various cell types so that imatinib can act on normal immune cells and modulate the differentiation, proliferation, activation and function of T lymphocytes, macrophage and dendritic cells (DC).¹⁰⁻¹²

In autoimmune diseases such as Collagen-induced arthritis (CIA), MS and EAE, imatinib abrogates multiple signal transduction pathways that are implicated in pathogenesis, including TCR and p38 MAPK signaling, mast cell c-Kit (CD 117) signaling, macrophage c-Fms activation and cytokine production.^{13,14} In the present research, our aim was to test the therapeutic efficacy of imatinib in experimental model of MS.

MATERIALS AND METHODS

Animal Study

Animal Selection and Grouping

Twenty three female C57BL/6 mice (7- to 9-weeks-old), weighing 18–20 g, were purchased from the Experimental Animal Center of Pasteur institute of Iran. Mice were housed according to institutional guidelines with access to food (pelleted diet) and water. Mice were randomly divided into I- untreated (3 mice) II- Control (10 mice, myelin oligodendrocyte glycoprotein-35-55 (MOG₃₅₋₅₅)-induced EAE and sterile water treated) III- Treatment (10 mice, MOG₃₅₋₅₅-induced EAE and imatinib treated).

EAE Induction and Treatment Protocol

We performed EAE induction as most commonly used animal model for studying the pathogenesis and treatment of MS¹⁵ by Hooke Kit (Hooke Laboratories, Inc., USA). The kit consisted of two components each was delivered in separate pre-filled syringes consisting of antigen (MOG₃₅₋₅₅) in Complete Freund's Adjuvant (CFA) emulsion, and pertussis toxin (PTX) in PBS. The mice were injected subcutaneously on upper back and lower back with 0.1 ml of emulsion, respectively. Within 2 hours of injection of the emulsion, the first dose of PTX (0.1 ml per mouse) was injected intraperitoneally. Also 22-26 hours after injection of the emulsion, the intraperitoneal injection of second dose of PTX into the mice (0.1 ml to each mouse) was done. Imatinib was purchased from Mumbai, India and suspended in sterile water. Pharmacological dose of imatinib was calculated regarding its effective dose in human and animal studies.^{16,17} The mice (group II and III) were administered orally with vehicle (sterile water) and imatinib, respectively at the specified dose (60 mg/kg imatinib) on six consecutive days per week for 2 weeks, from day 7 post immunization through animal-feeding needles.

Clinical score of EAE was defined as follows: 0 = no sign; 1=Limp tail; 2=Limp tail and weakness of hind legs; 3=Limp tail and complete paralysis of hind legs (most common) or limp tail with paralysis of one front and one hind leg.; 4=Limp tail, complete hind legs and partial front legs paralysis; 5=Mouse is spontaneously rolling in the cage or mouse is found dead due to paralysis.

Histopathology

The mice were sacrificed on day 35 post-immunization. Brains, cerebellums and lumbar spinal cords from control and treatment and naive (healthy) mice were removed and fixed in formalin, embedded in paraffin, sectioned and then stained with hematoxylin-eosin (H&E) for meningeal and parenchymal inflammatory foci counting and Luxol fast blue (LFB) to distinguish demyelination. All stained slides were analyzed in a blinded manner by an expert pathologist and one neuropathologist.

Cytotoxicity Study

Cell Culture

Regarding the MMP-2, MMP-9 and pro-inflammatory cytokines which are significantly represented in glial cells, we decided to use glioblastoma and astrocytoma cell lines. U-87 MG glioblastoma, C6 Astrocytoma and Fibrosarcoma WEHI-164 cell lines were purchased from the Pasteur Institute of Iran. U-87 MG cell line was cultured in Eagle's minimal essential medium (EMEM) supplemented with 10% fetal bovine serum (FBS) (Sigma, Aldrich), 2mM glutamine, 1% non-essential amino acids (NEAA) and 1mM NaP. C6 and WEHI-164 cell lines were cultured in RPMI media supplemented with 10% FBS, L-glutamine and 100 U/ml penicillin/streptomycin (P/S). For in vitro studies, imatinib was dissolved in acetate buffer (pH: 5.5) at a 1000 μ M stock solution and stored at 4°C until the time of use.

In vitro Proliferation Assay

Cell viability was determined using a colorimetric microculture assay with the microculture tetrazolium test (MTT) end-point assessment. In this assay, the amount of MTT reduced to formazan is proportional to the number of viable cells.¹⁸ Three different cell lines, U87MG, C6 and WEHI-164 were seeded at 5×10^3 cells per well on a 96-well plate. Imatinib was diluted in standard buffer as described earlier and added at various concentrations (0, 10, 20, 30, 40, 50, 60, 70, 80 and 90 μ M for U87 MG and WEHI-164, as well as 0, 5, 10, 25, 50 and 100 μ M for C6 cells) 4 h after cell attachment. Cells were incubated for 48 h at 37°C and then the medium was removed. The number of viable cells was determined by the MTT colorimetric assay: with adding 200 μ l of MTT (0.5 mg/ml in tissue culture medium without phenol red) and incubating the plates

for a further 2–4 h, removing medium and adding 200 μ l of dimethyl sulfoxide (DMSO) to each well to solubilize the formazan dye and then reading the plates on an ELISA reader.

Gelatinase Zymography

The cells (4×10^4) were seeded in 24-well plates and 4h after cell attachment, complete growth medium in the absence (control) or presence of the imatinib (0-50 μ M) was added with total volume 1 mL per well and incubated for 48h at 37°C in 5% CO₂, and saturated humidity. Cell culture supernatants were then collected and stored at -20°C for zymoanalysis.

To evaluate the MMP-2 enzymatic activity, a sensitive procedure that was modified according to previous publication was used.¹⁹ Briefly, protein-content adjusted aliquots of conditioned media, in duplicate, were subjected to SDS-PAGE containing 0.5 mg/mL gelatin (Merck, Germany). Electrophoresis was performed for 3h at a constant voltage of 100 volts in tris-borate buffer media. After electrophoresis, gels were gently washed two times with 2.5 % Triton X-100 solution to remove SDS (2 \times 30 min) and incubated overnight at 37°C in 0.1 M Tris HCL gelatinase activation buffer (pH 7.4) containing 10 mM CaCl₂. After incubation, the gels were stained with 0.1% Coomassie Brilliant Blue G-250 (0.1% in 25% methanol and 10% acetic acid in water) for 60 min at room temperature and then destained for 2h in 10% methanol and 10% acetic acid in water. MMP-2 proteolysis areas appeared as clear bands against a blue background in the gels. Using AlphaEaseFC densitometry software (Alpha Innotech, Miami, USA), quantitative evaluation of both surface and intensity of lysing bands (mean area density) were compared relative to non-treated control wells and expressed as relative expression of gelatinolytic activity.

Enzyme-linked Immunosorbent Assays for Proinflammatory Cytokines and MMPs Activation of C6 Rat Astrocyte with LPS and Treatment with Imatinib

The C6 rat astrocyte cell lines were seeding at a density of 1×10^4 cells per well in 96-well plates with 1 μ g/well of LPS (sigma, USA), in the presence (5, 10 and 25 μ M) or absence of imatinib. Cells were incubated for 48 h, and then cultured. Supernatants were collected, centrifuged, protein-content adjusted and stored in aliquots at -20° C until use.

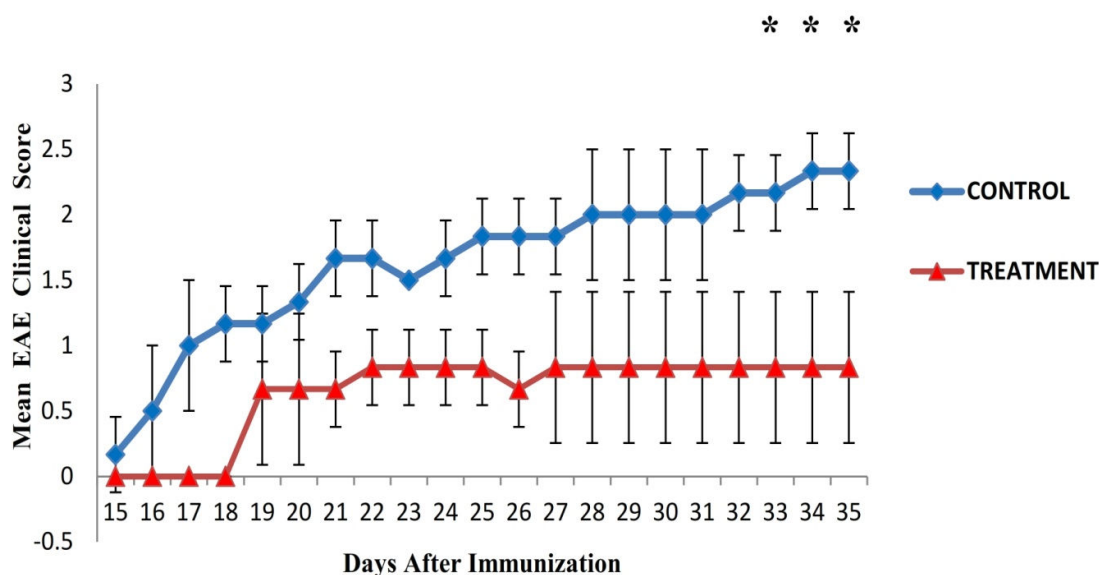


Figure 1. Effect of imatinib on clinical score of EAE.

Female C57BL/6 mice (n=10) in treatment group were administered orally with imatinib at a dose of 60 mg/kg from day 7 after immunization on six consecutive days per week for 2 weeks through animal-feeding needles. Disease severity was assessed by a visual cumulative scoring system. Cumulative scores from day 15 until day 35 are given as mean±SEM. * $P < 0.05$ at each data point by Mann–Whitney U test comparing treatment versus control.

Quantification of TNF- α , IL-1 β and IL-6

The levels of pro-inflammatory cytokines were determined in the supernatants of C6 cell cultures by a sandwich enzyme-linked immunosorbent assay (ELISA) technique. To evaluate TNF- α , we used ID Labs Kit (ID Labs inc. London, Canada), also the amount of IL-1 β and IL-6 were determined using the kits from Boster (Boster Biological Technology, Ltd. USA). All assays were performed according to the manufacturers' instructions. Absorbance was read at 450 nm in a 96-well microplate ELISA reader.

Quantification of MMP-2 and MMP-9

MMP-2 and MMP-9 concentrations in culture supernatants were determined after 48h incubation of U87MG cells with 0, 10, 20, 30, 40 and 50 μ M imatinib by ELISA kit (RayBiotech, Inc. Norcross, USA) using the manufacturer's instructions. All standards and samples were carried out in duplicate. Optical density (OD) was determined using a microplate reader at a wavelength of 450 nm and concentrations of MMP-2 and MMP-9 were reported as ng/ml.

Statistical Analysis

Data were expressed as mean±SD, except for histological scores, which were calculated as mean±SEM. Statistical analysis was performed with Mann-Whitney U-test for nonparametric data and Student's t test for parametric data. A p -value < 0.05 was considered statistically significant.

RESULTS

Clinical Findings

Imatinib Could Reduce the Severity and Onset of EAE

The mice in treatment group that were administered orally with 60 mg/kg imatinib, displayed significant reductions in the severity of EAE compared to control group (Figure 1). Also in the treatment group, imatinib caused a significant delay at onset of EAE ($P=0.046$) but had no effect on EAE incidence.

Histological Findings

Histopathologic analysis was performed on brains, cerebellums and spinal cords in EAE mice receiving

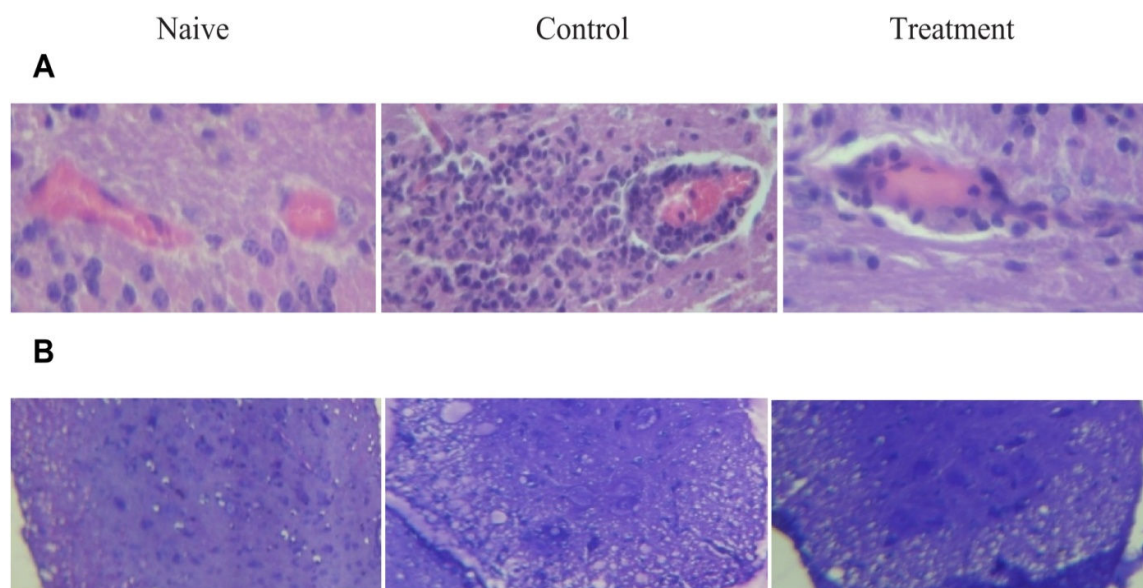


Figure 2. Histological examination of CNS during the course of EAE in different groups. (A) H&E staining of cerebellums showed that imatinib therapy could suppress the formation of inflammatory foci and vasculitis as well as limit the infiltration rate of inflammatory cells into the CNS. (B) LFB-PAS staining of spinal cords showed lower demyelination sites in imatinib treated mice compared to control group.

imatinib or vehicle. All sections were scored by two investigators blinded to the study by light microscopic examination. Representative images of LFB and H&E-stained tissue sections from naive, control and treatment groups (Figure 2) demonstrated that treated mice with imatinib had significantly fewer inflammatory foci, vasculitis, mononuclear cells in perivascular cuffs and demyelination than control mice.

Effects of Imatinib on Cell Proliferation

To examine the cytotoxicity and anti-proliferative effects of imatinib in glioblastoma, fibrosarcoma and astrocytoma cell line, we used MTT assay (Figure 3). The half maximal inhibitory concentration (IC_{50}) values for imatinib-inhibited U-87 MG, WEHI-164 and C6 proliferation were 50 μ M (Figure 3A), 30-40 μ M (Figure 3 B) and 25 μ M (Figure 3 C), respectively.

Gelatinase Activity

We determined the activity of MMP-2 by gelatin zymography after 48h incubation with raising imatinib concentration (0-50 μ M) in U-87 MG, C6 and WEHI-164 culture supernatants in consecutive wells after protein-content adjustment. The results of our

experiments in figure 4 showed that increasing amount of imatinib induced a remarkable decrease in the activities of MMP-2 on U-87 MG and C6 cells whereas its effect on MMP-2 activity in WEHI-164 cells was low.

Effect of Imatinib on Proinflammatory Cytokines

The effect of imatinib on IL-1 β , IL-6 and TNF- α cytokine secretion by C6 cells which were stimulated by 1 μ g/well LPS was assessed. The analysis was performed on culture supernatants using an ELISA cytokine assay. Imatinib at 10 and 25 μ M dramatically reduced TNF- α and IL-1 β production, whereas, there was no differences in IL-6 levels in untreated and treated cells (Figure 5).

Effect of Imatinib on MMP-2 and MMP-9 Secretion

The effect of rising concentration of imatinib on MMP-2 expression in U87 MG cell line is illustrated in figure 6. As shown, imatinib in tested doses reduced MMP-2 secretion. However, with respect to the fact that MMP-9 secretion levels in cell supernatants was very low; we could not show a statistically significant difference in untreated and treated cells.

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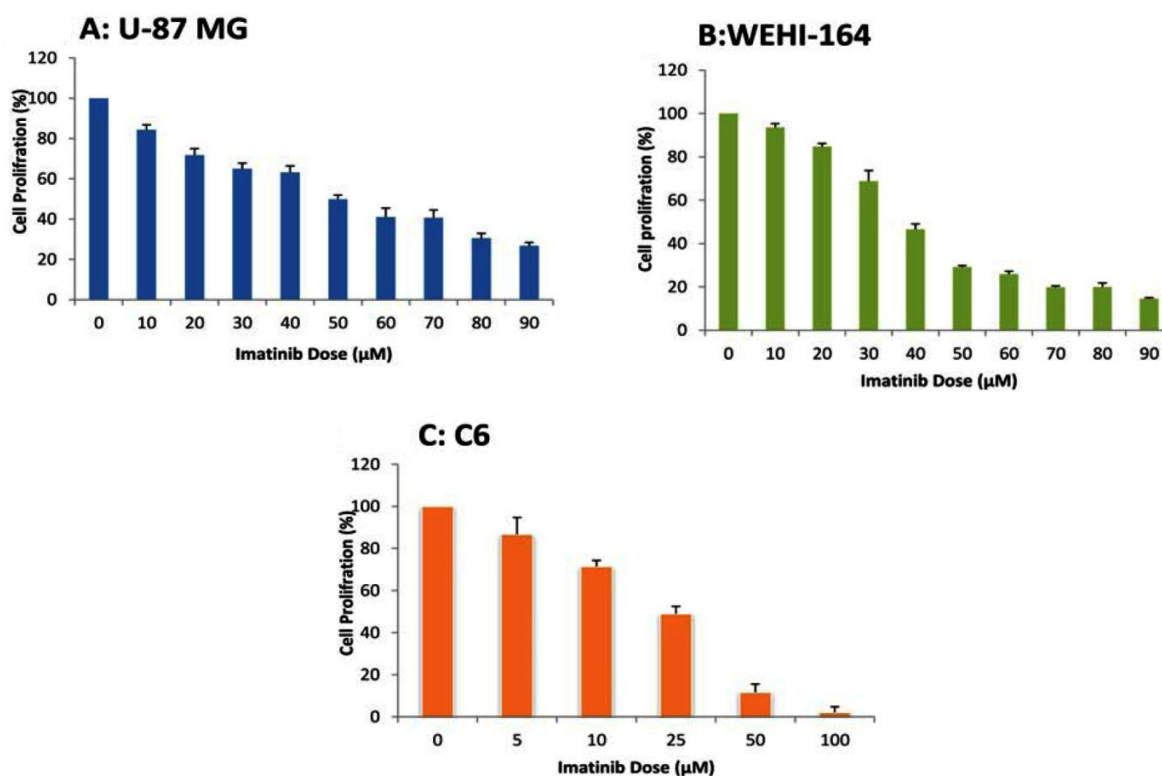


Figure 3. Cytotoxicity and antiproliferative effects of imatinib on cell lines in culture.

The effect of imatinib on cell proliferation using 5×10^3 U-87 MG, WEHI-164 and C6 cells in 96-well plates was assessed by MTT test. The cells were incubated with imatinib for 48h at various concentrations. Imatinib inhibited cell proliferation in three cell lines in a dose-dependent manner. Data are expressed as mean \pm SD.

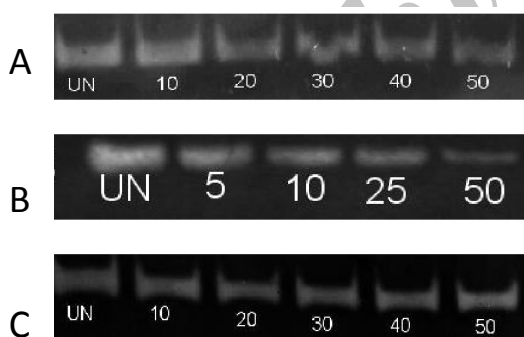


Figure 4. Effect of imatinib on MMP-2 activity.

This figure demonstrates that the lowest inhibitory concentration of imatinib for MMP-2 activity is 20 μ M on U-87 MG cells (A) and 5 μ M on C6 cells (B). In contrast, WEHI-164 cell line showed a relative tolerability against raising concentration of imatinib on the inhibition of MMP-2 activity (C).

Un-treated=0 μ M, treated=10-50 μ M

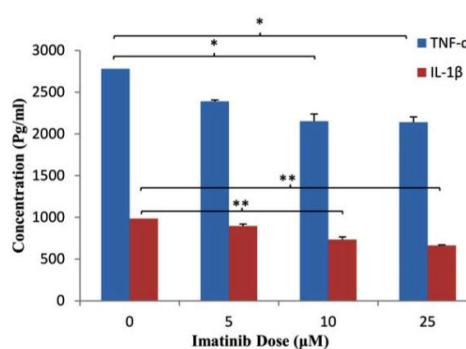


Figure 5. Inhibitory effects of imatinib in proinflammatory cytokine secretion in C6 cells.

C6 astrocyte cells were stimulated by LPS in the presence or absence of imatinib for 48h. Imatinib at 10 and 25 μ M dramatically reduced TNF- α and IL-1 β production whereas, there was no differences in IL-6 levels in untreated and treated cells. Error bars, mean \pm SD statistics were calculated using t-test and $P < 0.05$ were considered significant (*= $P < 0.05$, **= $P < 0.01$).

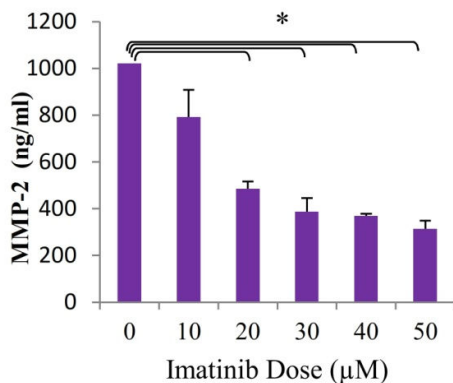


Figure 6. MMP-2 expression in U87 MG after 48h incubation in the presence or absence of imatinib using ELISA method. As shown in this figure, 20 μM imatinib could increasingly inhibit MMP-2 expression. Data are expressed as mean ±SD (*= $P < 0.05$ was considered statistically significant).

DISCUSSION

In this study, we observed that imatinib could reduce EAE severity and delayed the onset of EAE in C57BL/6 mice.

After mice immunization and impairment in blood brain barrier (BBB), imatinib can traverse from BBB and target cells in CNS. The infiltrating inflammatory cells including T cells and macrophages contribute in stimulating the glial cells to cause acute plaques and neuroinflammation.^{1,2,20} In this study, we found that oral application of 60 mg/kg imatinib daily could decrease the number of inflammatory cells and foci in the CNS of mice with EAE.

Previous data showed that T cells which secreted IL-17 or IL-17 and IFN-γ infiltrated to CNS prior to the onset of clinical symptoms of EAE, where they may mediate CNS inflammation, in part, through microglial activation and local production of TNF-α, IL-1β and IL-6 in the CNS.¹

Other findings suggest that imatinib dose-dependently attenuates inflammatory cytokine production in mononuclear cells. Current findings showed that imatinib could prevent EAE, essentially via distinct mechanisms, in that imatinib inhibited both inflammatory and T-cell-derived cytokine production which correlated to decreased levels of activated NF-κB and an impairment in phosphorylation of ZAP-70 and p38 MAPK pathway.^{21,22}

Moreover, other receptor tyrosine kinases FLT-3, c-

Kit and c-fms are also expressed in resident and nonresident cells in CNS. Inhibition of signaling in these cells by imatinib leads to decreased production of pro-inflammatory cytokines, Hence the receptor tyrosine kinases may be a therapeutic target to mitigate cytokine mediated disorders during CNS inflammation in EAE and MS, and/or inhibit recruitment of inflammatory cells to the CNS by decrease in TNF-α, IL-1β and IL-6 production.²³⁻²⁶

In this experiment, we demonstrated that imatinib could abrogate the production of pro-inflammatory cytokines in LPS treated C6 astrocyte cell line. Our in vitro data indicated that imatinib could inhibit the production of TNF-α and IL-1β that led to inhibition of macrophage migration ability and activity, as well as an immunomodulatory effect on glial cells.

Although the correlation between the presence of macrophages and reactive glial cells with axon injury in the acute plaques is well established,^{27,28} however, little is known about the involvement of MMP-2 activity in MS.²⁹ Anthony et al.³⁰ and Maeda et al.³¹ showed that MMP-2 was upregulated not only in plaques but also in normal appearing white matter adjacent to acute plaques. Further reports by Newman et al. showed that microinjection of MMP -2 into rat subcortical white matter resulted in axonal injury.³²

Thus, MMP-2 seems to play a key role in BBB disruption which facilitates immune cell transmigration into the CNS. Also the over expression of MMP-2 is associated with fragmentation of myelin basic protein (MBP), degrading the myelin sheath, and damaging axons.^{9,33-35} Whereas down regulation of MMP-2 could inhibit BBB disruption and migration ability of inflammatory cells to CNS. To evaluate the inhibitory effect of imatinib on cell proliferation, MMP-2 activity and expression, we used an in vitro experiment by glioblastoma, astrocytoma and fibrosarcoma cell lines based on recent study by Schultz et al.³⁶ that showed MMP-2 expression was suppressed in the presence of imatinib in HNSCC cell lines.

Our data using ELISA and zymoanalysis method showed that the inhibitory effect of imatinib in MMP2 activity was increased in two glial cell lines with rising concentrations of this drug.

Our results along with other researchers demonstrated that imatinib could robustly attenuate EAE clinical symptoms (severity) in mice and modulate proliferation and activation of microglia and astrocytes involve in neuroinflammation by selectively

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inhibiting a spectrum of signal transduction pathways central to the pathogenesis of EAE.³⁷

On the other hand, imatinib can be administered orally to the patients and comparatively limited toxic side effects have been reported for this immunomodulatory drug than usual drug use for MS treatment.³⁸

Therefore, we used animal model to show a reduced side-effect profile while maintaining the anti-inflammatory and immunomodulatory properties of imatinib in MS.

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