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A novel action of minocycline: Inhibition of human immunodeficiency virus type 1 infection in microglia

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Human immunodeficiency virus type 1 (HIV-1) infection of the brain produces a characteristic disease called acquired immunodeficiency syndrome (AIDS) dementia in which productive infection and inflammatory activation of microglia and macrophages play a central role. In this report, the authors demonstrate that minocycline (MC), a second-generation tetracycline with proven safety and penetration to the central nervous system, potently inhibited viral production from microglia. Inhibition of viral release was sustained through the entire course of infection and even when the drug exposure was limited to the first day of infection. Minocycline was effective even at low viral doses, and against R5- and X4R5-HIV, as well as in single-cycle reporter virus assays. Electrophoretic mobility shift analysis showed that minocycline inhibited nuclear factor (NF)-κB activation in microglia. HIV-1 long terminal repeat (LTR)-promoter activity in U38 cells was also inhibited. These results, combined with recently demonstrated *in vivo* anti-inflammatory effects of MC on microglia, suggest a potential utility for MC as an effective adjunct therapy for AIDS dementia. *Journal of NeuroVirology* (2004) **10,** 284–292.

Keywords: brain; dementia; HIV; inflammation; microglia; NF-κB; tetracycline

Introduction

Human immunodeficiency virus type 1 (HIV-1) infects the central nervous system (CNS) in a significant number of patients and produces a characteristic clinical and pathological entity termed the acquired immunodeficiency syndrome (AIDS) dementia/HIV-1 encephalitis (Navia *et al*, 1986; Lipton and Gendelman, 1995; Kolson *et al*, 1998). Macrophages (monocyte-derived) and microglia (resident brain macrophages) play a central role in AIDS dementia as they are the primary targets of productive infection in the brain (Dickson *et al*, 1993; Gonzalez-Scarano and Baltuch, 1999; Cosenza *et al*, 2002). In addition, infected and uninfected microglia show diffuse immune activation, which may underlie neural damage in AIDS dementia (Glass *et al*, 1995; Merrill *et al*, 1992; Tyor *et al*, 1992; Andersson *et al*, 1992; Lipton, 1998). Therefore, treatment aimed at disabling microglial activation may possibly alter the progression of AIDS dementia. Ideal therapeutic agents for AIDS dementia would attenuate both viral replication and immune activation of glia. Evidence suggests that even with highly active antiretroviral therapy (HAART) therapy, HIV-1 is not eradicated and that infected macrophages, such as those within the CNS, may serve as a viral reservoir (Sharkey *et al*, 2000). Thus, additional antiviral agents that can penetrate the CNS would be of significant value.

Tetracyclines (TCs) are a class of broad-spectrum antibiotics. The TC derivatives minocycline (MC) and doxycycline (DC) have been demonstrated to exert anti-inflammatory effect independent of their

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antimicrobial activity (Golub *et al*, 1983; Gabler and Creamer, 1991; Tamargo *et al*, 1991; Amin *et al*, 1996). Recently it has been demonstrated that they have neuroprotective roles in animal models of various neurological diseases. After experimental ischemia, tetracyclines decrease infarct size and neuronal death by reducing ischemia-induced microglial activation and subsequent expression of proinflammatory mediators such as inducible nitric oxide synthase and caspase-1 (Yrjanheikki *et al*, 1999). In a transgenic mouse model of Huntington's disease, MC also inhibits caspase-1 and caspase-3 expression and delays disease progression (Chen *et al*, 2000), and its potential therapeutic value in experimental Parkinson's disease has also been presented (Du *et al*, 2001). More recently, MC treatment has also been shown to inhibit or delay experimental autoimmune encephalomyelitis, an animal model of multiple sclerosis (Popovic *et al*, 2002; Brundula *et al*, 2002). MC promotes the survival of transplanted oligodendroglial progenitor cells by suppressing microglial activation (Zhang *et al*, 2003), and furthermore, reverses impaired adult neurogenesis during inflammation by suppressing microglial activation (Ekdahl *et al*, 2003). As microglia and their cellular products play an important role in HIV-1 infection in the CNS and the pathogenesis of AIDS dementia, we evaluated the effect of MC on HIV-1 infection of human fetal microglia. We report here that MC suppresses viral production from microglia and astrocytes through diverse mechanisms. The results presented here argue for a potential use for MC in the therapy of HIV infection.

Results

Minocycline inhibits HIV-1 production in microglia
We determined the effect of MC at concentrations of 3 to 30 μg/ml, chosen based on previous reports on experimental animal systems, in HIV-exposed microglial cultures. The inhibitory effect of MC on HIV-1 p24 production was observed in more than 20 separate experiments. A representative experiment is shown in Figure 1. MC inhibited p24 production in a dose-dependent manner, with IC_{50} at approximately 20 μg/ml. The degree of inhibition at 30 μg/ml of MC (screening dose) ranged from 71% to 96% (not shown). In contrast, TC failed to show an effect at 30 μg/ml. Formation of multinucleated giant cells (MGCs) and expression of intracellular HIV-1 p24 antigen were also inhibited by MC but not TC (Figure 2 and Table 1). In MC-treated cultures, microglial cells were prevented from forming syncytia but remained healthy in morphology (Figure 2). The highest concentration of the vehicle alone (0.3 mM HCl) did not affect HIV-1 replication in microglia (not shown).

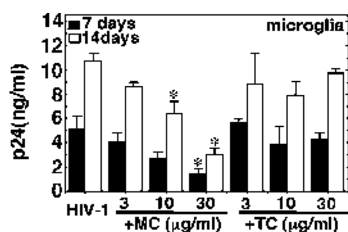

Figure 1 MC inhibits HIV-1 infection in microglia. Human fetal microglia were exposed to 2000 $TCID_{50}$HIV-1_{ADA} with and without MC or TC at indicated concentrations. HIV-1 p24 levels in the culture supernatants were determined by ELISA. MC but not TC inhibited p24 release at concentrations tested. Mean ± SD from triplicate wells. MC effect was repeated in more than 20, and TC effect in 4, separate experiments using cells from different brains. $^{*}P < 0.05$ versus no drug.

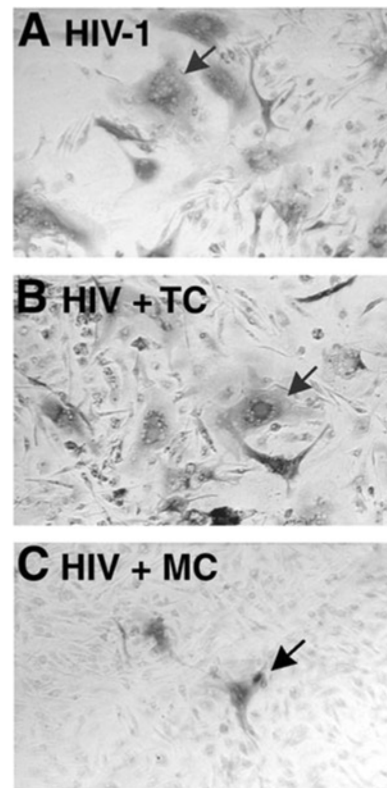

Figure 2 MC inhibits multinucleated giant cell (MGC) formation and intracellular p24 expression. Microglial cultures were exposed to HIV-1_{ADA} in the absence (**A**) or presence of TC (**B**) or MC (**C**) at 30 μg/ml, and then examined for MGC formation and p24 expression by immunocytochemistry at day. Formation of MGC (*arrows*) and p24 immunoreactivity are greatly reduced by MC. Magnification: ×400. Note that due to lack of syncytia formation, microglial cells in MC-treated cultures remain small.

Effect of minocycline on microglial cell survival and metabolism
We determined whether MC affected microglial cell survival, proliferation, or metabolism. We show that MC (up to 30 μg/ml) was not toxic to microglia, as judged by morphology, lactate dehy-drogenase (LDH), and 3-4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide (MTT) assay (Table 2). Microglia do not proliferate under the standard culture conditions (used for this study), but

Table 1 Inhibition of microglial MGC formation by MC

Cell treatment	*Number of MGCs*[a]
HIV-1 alone	492.2 ± 89.8
HIV-1 + 3 μg/ml MC	410.7 ± 61.5
HIV-1 + 10 μg/ml MC	231.3 ± 8.3*
HIV-1 + 30 μg/ml MC	46.25 ± 7.3*

[a]The number of multinucleated giant cells was used as an index of HIV-1–induced microglial syncytia formation and was calculated by counting the total number of nuclei within the multinucleated giant cells (MCGs) per well (see Figure 2, for example). Microglial cultures in triplicate were infected with HIV-1_{ADA} for 14 days, and then immunostained for HIV-1 p24 as described. The results are mean ± SD from triplicate and represent one out of three experiments with similar results. $^*P < .05$ by Student's *t* test.

do proliferate following granulocyte-macrophage colony-stimulating factor (GM-CSF) treatment (Scholzen and Gerdes, 2000; Lee *et al*, 1994); MC suppressed microglial proliferation induced by GM-CSF, as determined by Ki-67 labeling (Q Si and SC Lee, unpublished data). MC did not change the total protein content in either microglia or astrocyte cultures (not shown). Together, these data exclude toxic or antimetabolic effect as the basis for MC-mediated antiviral activity described in this report.

MC inhibits HIV production in microglia: kinetics, effect of input viral concentrations and viral strains
Because microglia can sustain viral infection for weeks *in vitro*, we examined the kinetics of the MC effect by weekly examination of the p24 levels. In addition, we determined the minimum MC exposure time required for inhibition of viral production. As shown in Figure 3A, the inhibition of p24 production by MC observed during the first week was sustained for 3 weeks (shown as cumulative p24 value in each well). Interestingly, wash-out experiments showed that exposure to MC for only 1 day was as effective as exposure for 7 or 21 days. To determine the effect of MC on productively infected cells, we also tested MC at variable times after HIV-1 exposure (Figure 3B). Interestingly, MC was not effective if added after day 1 (see Discussion). We next determined whether MC could inhibit infection induced by low viral dose. Serial dilution of the input virus resulted in the reduction of the output p24 levels, but MC inhibited p24 production in all viral input doses (Figure 3C). We also examined additional strains of HIV-1, including another R5-tropic virus (BaL) and a dual-tropic (R5X4) virus (89.6). BaL produced high and 89.6 produced much lower p24 in microglia as expected, but MC potently inhibited p24 production in all (Figure 3D).

Table 2 LDH efflux and MTT assay following MC treatment

MC (μg/ml)	*0–7 days (LDH OD_{490})*	*0–7 days (MTT OD_{570})*	*7–14 days (LDH OD_{490})*
0 (control)	0.21 ± 0.006	1.5 ± 0.07	0.18 ± 0.002
3	0.23 ± 0.017	1.6 ± 0.08	0.19 ± 0.004
10	0.20 ± 0.015	1.5 ± 0.11	0.18 ± 0.003
30	0.21 ± 0.013	1.6 ± 0.12	0.19 ± 0.01

Note. Microglia in complete medium containing 5% FCS were treated with indicated doses of MC for indicated time periods and the LDH and MTT assays were performed using the culture supernatants and cell lysates, respectively. Mean ± SD from triplicate samples. There were no statistically significant changes in either in MC-treated cultures ($P > .05$ versus control).

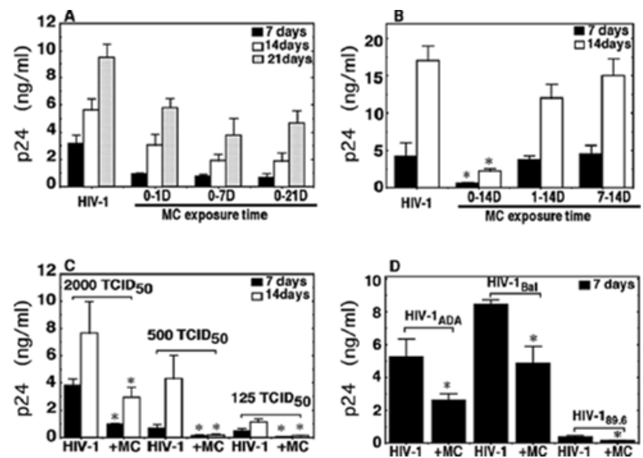

Figure 3 (**A**) Time–dependent effect of MC on HIV-1 infection in microglia. Microglia infected with HIV-1_{ADA} were exposed to MC at 30 μg/ml for varying time intervals as indicated in the figure. Weekly determination of p24 concentrations showed that exposure to MC for the first 24 h was sufficient for its HIV-inhibitory effect. P24 levels (shown as cumulative values in each well) are significantly different from control (HIV-1) at all time points ($P < .05$). (**B**) Wash-out experiment: Cultures were treated as in (**A**) except that MC exposure time varied from 0–14 to 1–14 to 7–14 days. (**C**) Effect of viral concentration: Microglial cultures were treated with different amounts of input virus (ADA). Data show that MC is effective in inhibiting p24 release at all concentrations. (**D**) MC inhibits microglial infection by various HIV isolates. In addition to ADA, MC also inhibits BaL and 89.6. Data are Mean ± SD in all. $^*P < .05$ versus no MC. Results (**A–D**) are representative of three independent experiments with similar results.

MC inhibits glial infection by replication-defective reporter HIV-1
To further delineate the specific steps of viral life cycle affected by MC, we tested MC in single-cycle HIV infection using replication-defective reporter viruses (Deng *et al*, 1996; He *et al*, 1997; Dragic *et al*, 1996; Albright *et al*, 1999). HIV-1 *env* (JR-FL) and non-HIV-1 *env* (VSV-G or AML-V) enter the cells via the CD4/CCR5 and endocytic pathway, respectively. As shown in Figure 4, MC inhibited luciferase expression in microglia infected with HIV-$1_{JR\text{-}FL}$ pseudotype (range 80% to 100%; data not shown), and to a lesser degree when infected by VSV-G or AML-V *env* viruses (~40% to 50%; Figure 4B, C). These results suggest that MC inhibits HIV-1 production by suppressing the intracellular step(s) of the viral cycle, and possibly the entry step as well (see Discussion). Because astrocytes can be efficiently infected with VSV-G *env*-HIV-1 (Canki *et al*, 2001), we also

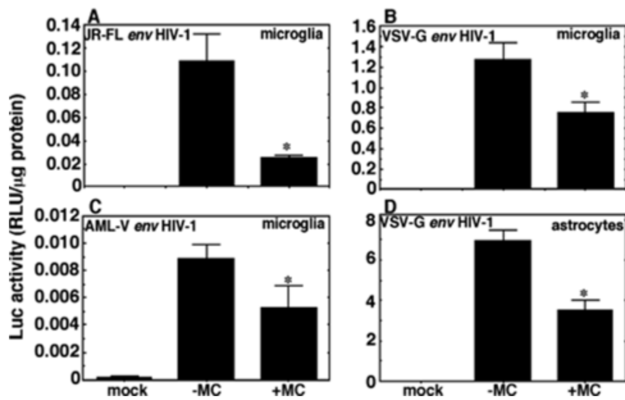

Figure 4 Effect of MC on single-cycle HIV-1 infection in microglia and astrocytes. Microglia were infected with reporter viruses (NL4.3-luc-R^-E^- pseudotyped with HIV-1_{JR-FL}, VSV-G, or AML-V *env*) in the presence or absence of MC (**A–C**). Astrocytes were infected by VSV-G *env*–pseudotyped virus (**D**). Luciferase activity in the cell lysates was determined as a measure of viral infection and expressed as RLU per μg of total protein. MC inhibited luciferase expression in all ($^*P < .05$). Mean ± SD.

examined the MC effect on astrocytes. The results show that HIV replication in astrocytes is also inhibited (~50%) by MC (Figure 4D). These results are in keeping with those obtained in microglia and demonstrate that MC blocks HIV-1 replication by inhibiting viral life stage(s) after the entry. Suppression of HIV-1 production in both cell types is encouraging and suggests that MC could inhibit HIV-1 expression in the CNS regardless of the cell type infected.

MC's effects on long terminal repeat (LTR) activity

MC effect was monitored in U38 cells, a cell line stably transfected with the HIV LTR-CAT reporter construct (Felber and Pavlakis, 1988). The transcriptional activation of the LTR, determined by the reporter gene expression (chloramphenicol acetyl transferase, CAT) was dose-dependently reduced by MC (Figure 5). There was no significant change in total protein content of the U38 cells following treatment with MC at 3 or 10 μg/ml (99 ± 7, 119 ± 8, 91 ± 13, at 0, 3, and 10 μg/ml of MC, respectively).

MC inhibits nuclear factor (NF)-κB activation induced by HIV-1

U38 cell data demonstrated that MC directly inhibits the HIV-1 LTR transcriptional activation. HIV-1 LTR contains two copies of κB sites within the enhancer

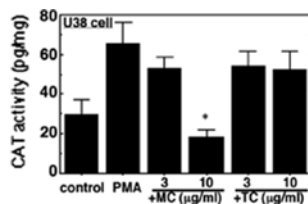

Figure 5 MC effect on LTR transactivation: Monolayers of U38 cells stably transfected with a LTR-CAT reporter construct were treated with PMA (10 nM) in the presence of MC or TC. CAT activity was determined 48 h later. $^*P < .05$ versus PMA alone.

region and NF-κB has been shown to play an important role in HIV replication (Rabson and Lin, 2000). We therefore determined NF-κB nuclear binding activity in microglial cells by gel shift analysis. Microglial cells were exposed to HIV-1 with and without MC, and then nuclei were harvested at 0 h, 1 h, 3 h, 1 day, 3 days, and 7 days after viral exposure. As shown in Figure 6, NF-κB:DNA complex formation was induced following HIV-1 infection beginning at 1 h and reaching maximum at 1 day. Supershift analysis with the subunit specific antibodies demonstrated that both the p65/p50 heterodimers (*top band*) as well as the p50 homodimers (*bottom band*) were induced following HIV-1 infection of microglia. Treatment of microglia with MC reduced the amounts

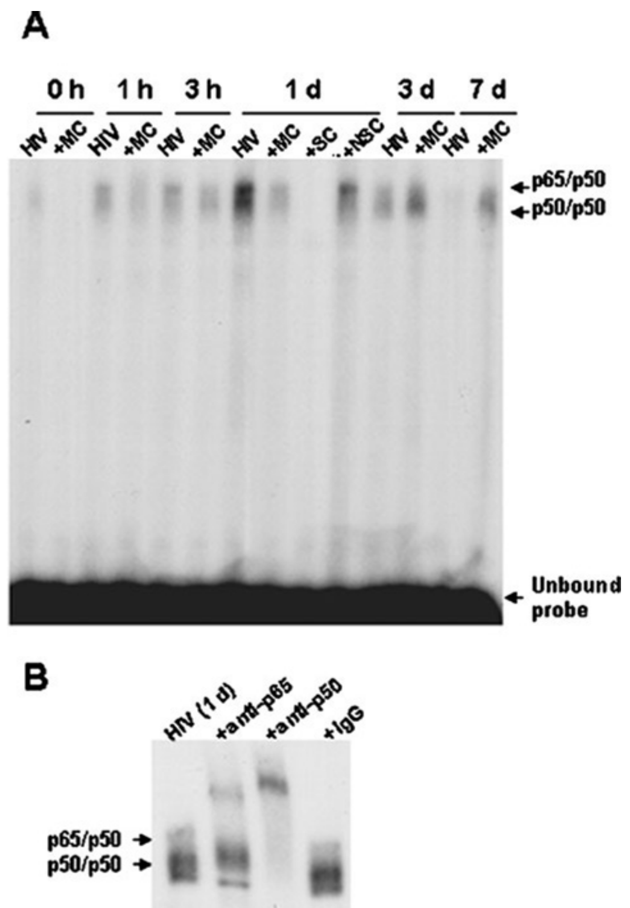

Figure 6 MC suppresses HIV-1-induced NF-κB activation in microglia. (**A**) EMSA was performed with microglial cell nuclear extracts using an oligonucleotide consensus sequence specific for NF-κB, as described in Materials and Methods. MC at 30 μg/ml was added simultaneously with HIV-1 and nuclear extracts were harvested at time points indicated. NF-κB activation occurred in microglia following HIV-1 exposure in a time-dependent manner (0 h = control) and MC inhibited NF-κB activation. Excess specific (SC) and nonspecific (NS) unlabeled oligonucleotides were used to compete out binding. Two NF-κB bands are present in HIV-1–infected microglia. (**B**) Supershift analysis with the NF-κB subunit–specific antibodies (p65, p50, or control IgG) demonstrates that the top band consists of the p65/p50 heterodimers and the bottom band consists of the p50 homodimers. Identical results were obtained in two separate experiments.

of both complexes at 1 day. At 3 and 7 days after HIV-1 exposure, NF-κB induction was reduced compared to 1 day and MC appeared to slightly increase the NF-κB complexes, particularly the p50 homodimers (see 3 days, for example). These results show that one of the mechanisms of MC-induced suppression of microglial activation and viral production might be through inhibition of NF-κB activation; however, MC may not have universal inhibitory effects on all NF-κB subunits (see Discussion).

Discussion

In this report, we document a novel anti–HIV-1 effect of MC in microglia. MC is a semisynthetic, long-acting, second-generation TC that exerts anti-inflammatory effects that are completely separate from its antimicrobial activity. The drug is clinically well tolerated and is known to penetrate the blood-brain barrier efficiently. MC's reported anti-inflammatory effects include down-regulation of nitric oxide, cyclooxygenase (COX)-2, matrix metalloproteinases, and superoxide formation, making it a potential candidate drug for treatment of inflammatory, degenerative, vascular, traumatic, and neoplastic diseases (Gabler and Creamer, 1991; Tamargo *et al*, 1991; Amin *et al*, 1996; Yrjanheikki *et al*, 1999; Teng *et al*, 2004; Chen *et al*, 2000). To our knowledge, we are the first to report an antiviral effect of MC.

In our experiments, MC at 3 to 30 μg/ml range showed an anti-HIV effect in microglia. Although MC inhibited proliferation of rat microglia (Tikka *et al*, 2001), human fetal microglia do not proliferate under our tissue culture conditions (Lee *et al*, 1992, 1994; Si *et al*, 2002), therefore the anti-HIV effects cannot be attributed to reported inhibition of proliferation. We observe, however, when we induce microglial proliferation with GM-CSF (Lee *et al*, 1994), MC treatment suppresses microglial proliferation (Si and Lee, unpublished data), in keeping with the reported antiproliferative effect of MC on rodent microglial cells. In the current study, we measured microglial cell number and viability employing several methods and found no evidence that MC caused cell toxicity. The concentrations of TC and TC derivatives that are reported to show immunomodulatory activity *in vitro* range from low (3 to 5 μg/ml) to high (500 μg/ml), with most studies reporting effects at approximately the 10 to 50-μg/ml range. Therefore, microglial cell response reported here is consistent with the previously documented effects of MC (Tikka *et al*, 2001; Brundula *et al*, 2002).

It is interesting that among several TC compounds, MC has been most extensively tested and has consistently shown antimicroglial and immune modulatory activities. We find that in contrast to MC, the same concentration of TC has no anti-HIV effect. TC compounds are reported to have different potency in part because of the differences in lipid solubility (Barza *et al*, 1975). These results collectively suggest that MC may be the most active TC compound. Clinically, oral administration of MC 100 mg twice a day results in serum concentration of 2 to 5 μg/ml within 1 to 4 h in humans (www.wyeth.com/products). Because of the lipophilic property, tissue concentration of MC may exceed serum concentration. Animal studies have demonstrated that high concentrations of MC are well tolerated and produce amelioration of several experimental CNS diseases (Yrjanheikki *et al*, 1999; Chen *et al*, 2000; Brundula *et al*, 2002). Furthermore, clinical trials of MC have shown promising results for rheumatoid arthritis (Langevitz *et al*, 2000). Therefore, it is conceivable that effective antiviral concentrations of MC may be achieved *in vivo* without causing significant side effects.

Our observation may have implications for the pathogenesis of HIV infection/AIDS. Infected macrophages and microglia could serve as the viral reservoirs in individuals with HAART (Sonza *et al*, 2001; Lambotte *et al*, 2000; Dickson *et al*, 1994; Budka, 1990). There is also mounting evidence that supports that HIV-1–infected macrophages control T-cell activation, infection, and survival (Stevenson and Gendelman, 1994; Sharkey *et al*, 2000; Swingler *et al*, 1999; Mahlknecht and Herbein, 2001; Mahlknecht *et al*, 2000), indicating that macrophages play a central role in the pathogenesis of HIV infection. We show that MC is effective in reducing viral production from chronically infected U1 cells, a model of latent infection (not shown), as well as in microglia, suggesting that MC would be effective against both acute infection as well as reactivation of latent infection. We show that MC inhibited viral production even at low input viral concentrations, suggesting its efficacy in individuals with low viral load, such as in post-HAART individuals. We show that MC was effective against a broad range of HIV-1, including R5, R5X4, and X4 viral pseudotypes (pNL4.3) bearing R5 HIV-, VSV-G-, or AML-V-*env*. These results suggest that MC could inhibit HIV production in multiple settings, against a variety of HIV strains, and against cells infected via HIV receptor/coreceptor-dependent or -independent manner.

We show that MC inhibited HIV expression in astrocytes infected with VSV-G–pseudotyped virus. Although astrocytes are generally resistant to HIV-1 infection compared to microglia, a low level "restrictive" infection has been shown to occur *in vivo* and *in vitro* that results in transcription and expression of the early regulatory viral proteins (Tornatore *et al*, 1994; Takahashi *et al*, 1996). Furthermore, as shown by Canki *et al* (2001) and by this study, human astrocytes sustain high levels of HIV-1 infection *in vitro* if viruses are introduced via VSV-G *env*. These results support that astrocytes do not have the intracellular block to HIV replication, and the major block to wild-type HIV-1 infection is probably at the entry/fusion stage. Regardless, our astrocyte data support that MC can block intracellular step(s) of viral cycle. The ability of MC to suppress HIV-1 infection

in a broad range of cell types offers a therapeutic advantage.

Several aspects of our results are intriguing and need further explanation. In spreading infection of microglial cells, the kinetics of drug exposure relative to HIV inoculation show that MC exerts its inhibition early during the course of infection. Pretreatment of microglia with MC confers no further advantage (not shown), whereas MC added 1 day after HIV inoculation showed little or no effect. Because these data point to inhibition during early HIV-1 life cycle, we tested whether MC inhibits HIV-1 binding: (1) We first examined whether MC can alter gp120 binding to CCR5 in an artificial assay utilizing a CCR5-expressing cell line, as described (Olson *et al*, 1999). The results showed that MC had no effect (data not shown). Although direct binding assay on microglial cell CCR5 would be more relevant in this context, we could not test this in microglia because of their low to undetectable levels of CCR5 (Zhao and Lee, unpublished data) (2) As an alternative approach, we tested MC in a microglial HIV-1 binding assay: microglia were incubated with HIV-1 with and without MC at 4°C for 2 h, then cell-based p24 enzyme-linked immunosorbent assay (ELISA) was performed after extensive washing of the cells. By this method, MC decreased the amount of cell bound p24 by ∼50% (55% ± 10% [mean ± SD], $P < .05$). (3) CCR5 mRNA expression was examined by ribonuclease protection assay with and without MC treatment. MC did not inhibit CCR5 mRNA expression; rather, it slightly increased CCR5 mRNA level by 24 h (data not shown). Together, these results suggest that MC might alter HIV-1 binding to microglia, without suppressing the coreceptor expression. The results of the viral pseudotype experiments also support this notion because the inhibition was always greater when cells were infected with HIV-1 *env*–bearing, as opposed to non-HIV *env*–bearing, virus.

We next examined whether MC can suppress HIV-1 LTR transactivation and found that MC inhibited LTR activation in stably transfected U38 cells. These experiments support that the major inhibition conferred by MC is at the viral transcription. We therefore determined the level of a key transcription factor, NF-κB, in HIV-1–infected microglia and show that MC indeed inhibited NF-κB activation. Kinetic analysis demonstrated that NF-κB activation following HIV infection of microglia peaked at 24 h, then diminished afterwards. Because viral production from microglia occurs several days after HIV infection, these results suggest a significant delay between NF-κB activation and viral production. These data are also interesting in light of the fact that MC's antiviral effect takes place within the first day of viral exposure (see Figure 3, for example). Analysis of the NF-κB subunit composition demonstrated that both the p65/p50 heterodimers as well as the p50 homodimers are induced as a result of HIV-1 infection. It has been well-established that the p65/p50 heterodimers positively regulate the viral and macrophage gene expression; however, the role of p50 homodimer is less well known and the data suggest that it might inhibit gene transcription (see Lewin *et al* [1997] and Baer *et al*, [1998] for example). Thus, it is intriguing that MC appears to increase the NF-κB complex formation with time (p50 homodimers, in particular; see Figure 6), suggesting that it may actually induce the inhibitory type of NF-κB.

In summary, our results show a novel effect of MC on HIV production from microglia and astrocytes. The results suggest NF-κB as a cellular target of MC. Because NF-κB is a transcriptional factor involved in the production of cytokines, chemokines, and other substances implicated in AIDS dementia (Glass *et al*, 1995; Lipton and Gendelman, 1995), our data suggest that MC could suppress both arms (viral production and inflammation) of the microglia and macrophage activation that critically contribute to neurodegeneration in AIDS dementia.

Materials and methods

Human fetal microglial and astrocyte culture
Human fetal CNS cell cultures were prepared from second trimester abortuses as described previously (Lee *et al*, 1992, 1993a). Primary CNS mixed cultures were prepared by enzymatic and mechanical dissociation of the cerebral tissue followed by filtration through nylon meshes of 230 and 130 pore sizes. Single cell suspensions were plated at 10^6 to 10^7 cells per ml in Dulbecco's modified Eagle medium (DMEM) (Cellgro, Herndon, VA; supplemented with 450 mg/L glucose, L-glutamine, and 25 mM HEPES) supplemented 5% fetal calf serum (Gemini Bio-products, Woodland, CA), penicillin (100 U/ml), streptomycin (100 μg/ml), and fungizone (0.25 μg/ml) for 2 weeks, then microglial cells were collected by aspiration of the culture medium. Monolayers of microglia were prepared in 100-mm tissue culture dishes at 10^6 cells per 10 ml medium, in 96-well tissue culture plates at 4×10^4 cells per 0.1 ml medium, or in 24-well plates at 10^5 cells per 1 ml medium. Two to four hours later, cultures were washed twice to remove nonadherent cells (neurons and astrocytes). Microglial cultures were highly pure consisting of >98% CD68$^+$ cells, as described (Lee *et al*, 1992).

Human fetal astrocyte cultures were prepared by repeated passage of mixed CNS cultures at approximately 2-week intervals until a population of >99% glial fibrillary acidic protein$^+$ was obtained (Lee *et al*, 1992, 1993b). Astrocytes were plated in 24-well plates at 2×10^5 cells per 1 ml medium and used for infection with pseudotype viruses.

HIV-1 infection
HIV-1 isolates (ADA, BaL, and 89.6) were obtained from the NIH AIDS Research and Reference Reagent Program (ARRRP) and propagated following the instructions with minor modifications. Briefly, normal

PBMCs were exposed to HIV-1_{ADA} in the presence of IL-2 and phytohemagglutinin (PHA), in RPMI containing 10% fetal calf serum (FCS). HIV-1 was washed out after 16 h, and then cells were cultured in the presence of IL-2. On days 7 and 14, culture supernatants were collected and kept frozen at −80°C until use. HIV-1 p24 levels were determined by ELISA (NEN Life Science Products Inc., Boston, MA). Control culture supernatants (mock-infection) were prepared in PBMCs stimulated with PHA and IL-2 without HIV-1. Tissue culture infective dose 50 ($TCID_{50}$) was determined by serial dilution of the viral stock in fresh PBMCs. Microglial cultures were infected with HIV-1_{ADA} at 2000 $TCID_{50}$ (unless otherwise indicated) per well in 96-well plates for 16 h at 37°C, then washed and incubated with fresh medium. Fungizone was omitted from the microglial medium once HIV-1 was added.

Drug treatment and determination of toxicity
Stock solutions of MC and TC (all from Sigma Chemicals) were made in 0.1 N HCl and further dilutions were made in DMEM/5% FCS. Drugs at 30 μg/ml were administered at the time of viral exposure (0 h) unless indicated otherwise. Drugs were added back following medium change, except in wash out experiments. Cultures were monitored for potential drug toxicity for up to 2 weeks by LDH assay as described previously (Downen *et al*, 1999). Live cell number was also determined by MTT assay (Promega, Madison, WI) following the manufacturer's protocol.

Measurements of viral production
Culture supernatants collected at a weekly interval were examined for HIV-1 p24 levels using an ELISA kit (NEN Life Sciences). Cells were also fixed with methanol and immunostained for HIV p24 using a monoclonal antibody (DAKO, Carpinteria, CA) as described (Lee *et al*, 1993a; Zhao *et al*, 2001).

Infection with replication-defective, env-pseudotyped viruses
Human embryonic kidney 293T cells were cotransfected with pNL4.3 Luc R-E- and *env* plasmids (HIV-$1_{JR\text{-}FL}$, vesicular stomatitis virus, VSV-G, or amphotropic murine leukemia virus, AML-V) provided by Dr. N. Landau (Salk Institute, San Diego, CA) for 3 days (Deng *et al*, 1996; Di Marzio *et al*, 2000). Culture supernatants containing viruses were collected and used to infect microglial or astrocyte cultures at approximately 10 ng/ml of p24. Cell lysates were prepared in 7-day cultures using a kit from Promega. Luciferase activity was measured by a luminometer (Lumat LB luminometer; Berthold, Bad Wildbad, Germany), and expressed as relative light unit (RLU) per microgram of total protein.

Propagation of U38 cells and treatment with minocycline
U38 cells are a subclone of U937 cells that are stably transfected with the HIV-1 LTR CAT gene as a reporter (Felber and Pavlakis, 1988). U38 cells are also obtained from ARRRP and propagated in RPMI containing 10% FCS at 2×10^6 cells per well in quadruplicate in 12-well plates in the presence of 10 nM of phorbol 12-myristate 13-acetate (PMA; Sigma). Minocycline at 3 or 10 μg/ml was added simultaneously with PMA. After 48 h, CAT activity was measured using a kit from Roche (Indianapolis, IN) following the manufacturer's instructions. Results are expressed as per milligram total protein.

Electrophoretic mobility shift assay (EMSA)
Microglial cell nuclear extracts were prepared and EMSA performed as described (Kim *et al*, 2002; Liu *et al*, 2000). Briefly, nuclear extracts were prepared using a modified Dignam method. Buffers were supplemented with 1 mM phenyl methyl sulfonyl fluoride (PMSF), 1 mM dithiothreitol (DTT), and a protease inhibitor cocktail (Boehringer-Mannheim, Indianapolis, IN). Cells (approximately 1×10^6) were scraped off in 1mM PMSF/PBS and pelleted. Pellets were resuspended in low salt buffer (10 mM Hepes, pH 7.9/1.5 mM $MgCl_2$/10 mM KCl), and allowed to sit on ice before addition of 10% Nonidet P-40 (Igepal CA-630 [Sigma]). Samples were again pelleted and resuspended in high salt buffer (20 mM Hepes, pH 7.9/25% glycerol/420 mM NaCl/1.5 mM $MgCl_2$); samples were rocked gently before a final centrifugation. The supernatant was saved, and the protein was quantified using the Bradford assay. Oligonucleotides containing concensus binding sequences for NF-κB (5′-AGT TGA GGG GAC TTT CCT AGG C-3′) were radiolabeled with γ-^{32}P ATP using polynucleotide T4 kinase according to the manufacturer's instructions (Gel Shift Assay Core System kit; Promega). Labeled probe was purified on a G25 spin column (Boehringer-Mannheim). Three micrograms of nuclear extracts were incubated in binding buffer (4% glycerol, 1 mM MgCl2, 0.5 mM EDTA, 50 mM NaCl, 10 mM This-HCl, 50 μg/ml poly [dI-dC]) with 1.75 pmol of specific (NF-κB) and nonspecific (AP-1: 5′-CGCTTGATGAGTCAGCCGGAA-3′) competitor oligonucleotides for 15 min at room temperature prior to addition of labeled probe. Supershift analysis was performed using antibodies from Santa Cruz (p65 and p50) as described previously (Hua *et al*, 2002; Kim *et al*, 2002). Samples were loaded unto gels containing 5.5% polyacrylamide, 5% glycerol, and 1 × TGE (Tris-glycine-EDTA buffer), then electrophoresed at 200 V for approximately 1.5 h. Gels were then dried and exposed to film at −80°C.

Data analysis
Each figure displays data from triplicate wells (mean ± SD) from a single representative experiment. Experiments were repeated 2 to 7 times using different brain cases with similar results. For statistical analysis, one-way analysis of variance (ANOVA) followed by Scheffer's multiple comparison procedure was used (SigmaStat). Differences between treatments were considered statistically significant when $P < .05$.

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