Minocycline inhibits West Nile virus replication and apoptosis in human neuronal cells

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Objectives: West Nile virus (WNV) infection causes severe meningitis and encephalitis in a subset of patients. WNV-induced apoptosis has been suggested to contribute to WNV pathogenesis. Tetracyclines exert antiviral effects against HIV and inhibit apoptosis in different models of neuronal disease. Here, the effects of the tetracyclines minocycline, demeclocycline and chlortetracycline were observed on WNV replication and WNV-induced apoptosis in different human CNS-derived cell types (primary human brain neurons, primary human retinal pigment epithelial cells and T98G human glioma cell line).

Methods: WNV replication was studied by cytopathic effects and virus yield reduction assay. Cell viability was examined by MTT assay. Apoptosis was investigated by immunostaining for activated caspase 3 and cleaved poly(ADP-ribose) polymerase. Expression and phosphorylation of cellular proteins were examined by western blot.

Results: Minocycline exerted the strongest anti-WNV activity. Non-toxic minocycline concentrations that can be achieved in human tissues significantly reduced WNV titres in all cell types tested. Minocycline inhibited WNV-induced apoptosis and suppressed virus-induced activation of c-Jun N-terminal kinase (JNK) and its target c-jun. The JNK inhibitor L-JNKi exerted similar effects to minocycline.

Conclusions: These data suggest that minocycline-induced inhibition of JNK activation contributes to minocycline-induced inhibition of WNV replication and WNV-induced apoptosis. Minocycline is a clinically available, inexpensive and generally very well-tolerated drug. It could be readily evaluated for the treatment of humans with serious WNV infection.

Keywords: antiviral therapy, brain, central nervous system, antibiotic, encephalitis

Introduction

West Nile virus (WNV) is a neurotropic RNA virus of the Flaviviridae family that is mainly transmitted by Culex mosquitoes and causes West Nile encephalitis in humans, animals and birds.1,2 WNV infections typically result in subclinical or non-specific, mild febrile illnesses. However, WNV infection may also result in severe meningitis and encephalitis. Three thousand one hundred and thirty-five cases of human WNV disease were reported to CDC from 41 states of the USA and from the District of Columbia from 6 January to 10 October in 2006.3

There is no vaccine against WNV infection licensed for humans, and therapy of WNV encephalitis is limited to supportive care as no effective antiviral option has been established.

Different clinically approved antiviral agents, including ribavirin and α-interferon, showed some degree of inhibition of WNV replication in vitro; however, none of them was proved to be beneficial in vivo.4

WNV-induced cell death includes caspase-dependent apoptosis in neuronal cells in vitro and in vivo, suggesting caspase/apoptosis inhibition as a possible novel therapeutic strategy to restrict WNV-induced CNS injury.5,6 Tetracyclines have been used for decades for the treatment of various Gram-positive and Gram-negative infections.7 Minocycline was shown to possess anti-apoptotic (neuroprotective) as well as antiviral and anti-inflammatory properties against HIV-induced encephalitis.8 In the present study, we observed effects of the tetracyclines minocycline, demeclocycline and chlortetracycline on replication of two...
different WNV strains (NY385-99 and Ug37) in different human CNS-derived cell cultures including primary cultures of neurons.

Materials and methods

Cell culture

Human brain neurons (HBNs) and neuronal medium were obtained from Sciencell, CA, USA and grown in accordance with the manufacturer’s protocol. Cells were seeded at a density of $4 \times 10^5$ cells per well in a 96-multiwell plate with a complete medium change every 24 h. After ~3 days, when cells had formed a complex neuritic network, cultures were used for the experiments.

Human retinal pigment epithelial (HRPE1) cells were isolated and cultured as described previously.9 T98G cells, derived from a patient with stage IV glioma, were obtained from the American Type Culture Collection (Rockville, MD, USA). Cells were grown at 37°C in Iscove’s modified Dulbecco’s medium supplemented with 10% heat-inactivated fetal calf serum, containing 100 IU/mL penicillin and 100 µg/mL streptomycin.

Virus preparation

WNV strain NY385-99 was kindly provided by Dr Jan ter Meulen (Institut für Virologie, Phillips-Universität, Marburg, Germany).10 WNV strain Ug37 was kindly provided by Dr Christian Drosten (Department of Virology, Bernhard Nocht Institute for Tropical Medicine, Hamburg, Germany). Viruses were propagated on Vero cells and stocks were stored at −80°C. Virus titres were determined as 50% tissue culture infectious dose (TCID$_{50}$/mL) on Vero cell monolayers in 96-well microtitre plates. For viral infection, cells were incubated with virus at a multiplicity of infection (MOI) of 0.1 for 60 min followed by washing four times with PBS.

Drugs

Minocycline, demeclocycline and chlortetracycline were from Sigma-Aldrich (Taufkirchen, Germany). The c-Jun N-terminal kinase (JNK) inhibitor L-JNKi was obtained from Calbiochem (Heidelberg, Germany). It was used at non-toxic concentrations.

Cytopathic effect (CPE) reduction assay

Confluent T98G monolayers grown in 96-well microtitre plates were pre-treated with the respective tetracycline derivative for 24 h prior to infection with WNV at an MOI of 0.1. Following a 1 h virus incubation period, the medium was removed and infected cells were incubated in medium containing different concentrations of drugs at the respective concentration. The virus-induced CPE was recorded at 48 h post-infection using an inverted light microscope.

Virus yield reduction assay

Cells were pre-treated with different concentrations of minocycline for 24 h prior to infection with WNV at an MOI of 0.1. Following a 1 h virus incubation period, the medium was removed and infected cultures were incubated with medium containing respective concentrations of minocycline. At 48 h post-infection cultures were deep-frozen at −80°C. After thawing of the cultures, infectious virus titres were determined as TCID$_{50}$/mL on Vero cell monolayers in 96-well microtitre plates.

Western blots

Western blots were performed using the enhanced chemiluminescence method, as described previously.11 The following primary antibodies were used: anti-JNK, anti-phospho-JNK, anti-c-jun and anti-phospho-c-jun (all obtained from Cell Signalling, Frankfurt, Germany).

Viability assay

The cellular viability was assessed on confluent cell layers with the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay method, as described previously.11 The viability was expressed as percentage of non-treated control.

Immunostaining

Virus envelope-antigen, activated caspase 3 and the 85 kDa fragment of cleaved poly(ADP-ribose) polymerase (PARP) were detected by immunostaining. For this purpose, cells were fixed with 60:40 methanol/acetone for 15 min. The following primary antibodies were used: WNV-E1 (Chemicon, Hofheim, Germany), caspase 3 (active) (R&D Systems, Wiesbaden, Germany) and PARP p85-fragment (Promega, Mannheim, Germany). Biotin-conjugated secondary monoclonal antibodies were used and visualization was performed with streptavidin–peroxidase complex with 3-amino-9-ethylcarbazole as a substrate.

Statistics

Results are expressed as mean values ± SD of at least three experiments. Comparisons between two groups were performed using Student’s t-test; three or more groups were compared by analysis of variance followed by the Student–Newman–Keuls test. P values less than 0.05 were considered to be significant.

Results

Effect of different tetracycline derivatives on WNV-induced CPE in T98G cells

T98G cells are highly susceptible to WNV-induced CPE, as characterized by cell rounding and detachment (Figure 1). In contrast, WNV infection of HBN (data not shown) or HRPE11 does not result in CPE formation. This makes T98G cells useful for rapid screening of antiviral substances. Therefore, the influence of minocycline, demeclocycline or chlortetracycline on WNV-induced CPE was investigated at different concentrations ranging from 0 to 40 µg/mL in T98G human glioma cells. Minocycline and demeclocycline exerted anti-WNV activity with minocycline showing the highest selectivity index (SI) for both WNV strains (NY385-99 and Ug37; Table 1). All further experiments used minocycline.

Minocycline inhibits WNV replication in different human brain-derived cell types

The effect of minocycline on WNV replication was investigated in different human brain-derived cells types including HBN, HRPE and T98G cells using the WNV strain NY385-99 (MOI of 0.1). To compare effects of minocycline on WNV replication,
a virus yield reduction assay was performed to compare infectious virus titres of infected cultures treated with 0, 2.5, 5, 10, 20 and 40 μg/mL minocycline. Maximum virus titres were detected in all three cell types 48 h post-infection (4.4 ± 0.105, 2.2 ± 0.0105 and 4.9 ± 0.0105 TCID50/mL for HBN, HRPE and T98G, respectively). Minocycline inhibited CPE formation in T98G cells (Figure 1) and significantly reduced infectious WNV titres in all three cell types tested in a dose-dependent manner (Figure 2). IC50 values obtained were 5.56 ± 0.45, 5.01 ± 1.23 and 4.23 ± 0.99 μg/mL for HBN, HRPE and T98G, respectively. Immunostaining of WNV strain NY385-99-infected (MOI of 0.1) T98G cells for WNV-E1 indicated that minocycline reduces virus spread in WNV-infected cell cultures (Figure 1). Infection of non-treated cells resulted in 88 ± 9% WNV-infected cells after 48 h. Minocycline (40 μg/mL) treatment resulted in 8 ± 4% infected cells.

Treatment of T98G cells with 40 μg/mL minocycline resulted in a low but significant decrease in cell viability. Lower concentrations did not affect T98G cell viability. In contrast, minocycline did not influence cellular viability of HBN or HRPE cells at concentrations up to 40 μg/mL (data not shown).

**Minocycline inhibits WNV-induced apoptosis**

WNV infection was shown to induce apoptosis as indicated by the detection of activated caspase 3 and PARP cleavage. Caspase 3 activation and PARP cleavage were detected in WNV strain NY385-99-infected T98G cells after 72 h of incubation by immunostaining. Minocycline decreased the number of cells stained for activated caspase 3 (Figure 3a) or for the 85 kDa fragment of cleaved PARP (Figure 3b) in a dose-dependent manner.

**Minocycline inhibits WNV-induced JNK phosphorylation**

JNK signalling was shown to have a pro-apoptotic role in neurons. Moreover, minocycline inhibited JNK signalling in microglial cells. Therefore, we investigated the influence of minocycline on JNK signalling in our system. T98G cells were treated with minocycline (10–40 μg/mL). Cellular levels of JNK, its phosphorylated form (phospho-JNK) and those of the JNK downstream c-jun and its phosphorylated form (phospho-c-jun) were determined by western blot. Infection with WNV strain NY385-99 did not influence expression of JNK, but induced expression and phosphorylation/activation of c-jun. Minocycline did not influence the expression of either JNK or c-jun in mock-infected cells (data not shown), but suppressed virus-induced JNK phosphorylation as well as phosphorylation/activation and expression of c-jun (Figure 4a). The relative numbers of WNV-infected T98G cells positively immunostained for phosphorylated c-jun are shown in Figure 4(b).

**Table 1. Inhibitory activity of tetracycline derivatives on CPEs in T98G glioma cells infected with WNV at an MOI of 0.1**

<table>
<thead>
<tr>
<th>Tetracycline derivative</th>
<th>CC50 a (μg/mL)</th>
<th>EC50 b (μg/mL)</th>
<th>SI c</th>
<th>EC50 b (μg/mL)</th>
<th>SI c</th>
</tr>
</thead>
<tbody>
<tr>
<td>Minocycline</td>
<td>62.9 ± 3.59d</td>
<td>8.18 ± 0.99</td>
<td>7.69</td>
<td>11.23 ± 1.12</td>
<td>5.6</td>
</tr>
<tr>
<td>Democycline</td>
<td>27.3 ± 1.62</td>
<td>7.34 ± 1.2</td>
<td>3.72</td>
<td>9.67 ± 1.53</td>
<td>2.82</td>
</tr>
<tr>
<td>Chlortetracycline</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>—</td>
<td>&gt;50</td>
<td>—</td>
</tr>
</tbody>
</table>

a Determined as the concentration that reduced the cell viability to 50% of control (cells without addition of drug).

b Determined as the concentration of drug required to inhibit virus-induced CPE to 50% of control value (untreated infected cells).

c Selectivity index (CC50/EC50).

Values are mean values ± SD from three independent experiments.
JNK inhibitor L-JNKi inhibits WNV replication and WNV-induced apoptosis

To investigate whether JNK inhibition is suitable to inhibit WNV replication and WNV-induced apoptosis, the JNK inhibitor L-JNKi was used. L-JNKi (2 μg/mL) was shown to inhibit JNK phosphorylation, c-jun expression and c-jun phosphorylation in WNV strain NY385-99-infected T98G cells (Figure 4a). L-JNKi inhibited WNV-induced CPE formation (data not shown) and reduced infectious virus titres (Figure 5a) in T98G cells. Moreover, L-JNKi inhibited WNV-induced apoptosis in T98G cells as indicated by a decreased number of cells stained positively for cleaved PARP (Figure 5b).

Discussion

In the present study, minocycline is demonstrated to inhibit WNV replication in both transformed and primary human CNS-derived cells, including human neurons. Minocycline was earlier shown to exert neuroprotective, antiviral and anti-inflammatory properties in different models of virus-induced encephalitis. In a murine model of reovirus encephalitis, minocycline delayed disease onset and progression. Virus-induced
CNS injury, apoptosis, virus titre and antigen expression were significantly decreased in the brains of minocycline-treated mice. In an in vivo simian immunodeficiency virus model of HIV CNS disease, minocycline reduced the severity of encephalitis, suppressed viral load in the brain and decreased the expression of CNS inflammatory markers.

Minocycline inhibited WNV replication at concentrations between 5 and 40 \( \mu \text{g/mL} \). These concentrations are achievable in human tissue. Levels of minocycline in the sera of patients treated with a standard dosage of 100 mg/day reached up to 10 \( \mu \text{g/mL} \). In two double-blind, randomized, placebo-controlled feasibility trials of minocycline in patients with amyotrophic lateral sclerosis, the mean tolerated dose was 387 mg/day (5.5 mg/kg per day for a 79 kg individual). Furthermore, it should be noted that minocycline has a high degree of lipid solubility that results in concentrations in tissue that exceed those in serum.

WNV-induced caspase-dependent apoptosis was suggested to contribute directly to the pathogenesis of WNV encephalitis. Minocycline also inhibited WNV-induced apoptosis, as indicated by inhibition of caspase 3 activation and PARP cleavage. Previous studies demonstrated that WNV induces apoptosis in infected neuronal cells, but not in non-infected cells. Moreover, repression of WNV-induced apoptosis was shown previously to protect WNV-infected cells without affecting virus replication or spread.

Sindbis virus is an RNA virus belonging to the alphaviruses, which is related to the Flaviviruses and also causes epidemic, mosquito-borne encephalitis in humans. Previous studies revealed that minocycline does not interfere with replication of Sindbis virus, indicating that the antiviral activity of minocycline may vary even between related RNA viruses. Despite lack of antiviral effects, minocycline protected mice from fatal Sindbis virus encephalitis by neuroprotective effects. Thus, both mechanisms—inhibition of WNV replication and neuroprotective effects (that do not depend on antiviral activity)—are likely to contribute to inhibition of WNV-induced apoptosis by minocycline. Therefore, individuals suffering from severe WNV disease of the CNS may benefit from minocycline by antiviral effects and by neuroprotective effects that are independent of antiviral activity.

Minocycline inhibited JNK signalling in microglial cells. Moreover, there is strong evidence that JNK signalling has a pro-apoptotic role in neurons. Our data show that minocycline suppresses JNK signalling, probably by activity upstream of JNK in T98G cells. The JNK inhibitor L-JNKi exerted effects similar to minocycline. It inhibited WNV-induced CPE formation and decreased WNV infectious titres. In addition, L-JNKi inhibited WNV-induced apoptosis. Therefore, minocycline-induced anti-WNV effects are likely to be due to JNK inhibition. These findings are in concordance with previous results, in which the JNK inhibitor SP600125 was found to reduce infectious WNV titres in the mosquito-derived cell line C6/36.21

In conclusion, minocycline inhibits WNV replication and WNV-induced apoptosis, probably by inhibition of JNK signalling. It represents a clinically approved drug that shows high penetration across the blood–brain barrier. Because it is a safe, frequently used, inexpensive drug, minocycline could be readily considered for testing in patients suffering from WNV encephalitis.

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**Transparency declarations**

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