Sultam Thiourea Inhibition of West Nile Virus

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We have identified sultam thioureas as novel inhibitors of West Nile virus (WNV) replication. One such compound inhibited WNV, with a 50% effective concentration of 0.7 μM, and reduced reporter expression from cells that harbored a WNV-based replicon. Our results demonstrate that sultam thioureas can block a postentry, preassembly step of WNV replication.

West Nile virus (WNV) and Japanese encephalitis virus (JEV) are members of the Flavivirus genus of the Flaviviridae family of viruses (9, 13). These viruses are considered emerging human pathogens (11, 12, 19, 29, 32, 37). They are closely related to the yellow fever and dengue flaviviruses, and together, these four pathogens are responsible for a significant percentage of virally induced human encephalitis cases worldwide (10–12, 19, 29, 32, 37). One line of defense against flaviviruses is the formulation of vaccines, usually directed against the viral surface envelope (E) proteins (12, 37). Another possible option is the intravenous administration of antiviral antibodies (25, 35). A complementary approach has been the development of small-molecule flavivirus inhibitors (7, 9, 13). These viruses are considered emerging human pathogens (10–12, 19, 29, 32, 36, 38, 39).

To assay for novel WNV inhibitors, we screened a diverse library of approximately 3,500 members for compounds that protected Vero cells from WNV-induced cytopathic effects (CPE). Cells were exposed continuously to a compound concentration of 10 μg/ml (10 to 50 μM) along with a 1% dimethyl sulfoxide (DMSO) carrier, infected with WNV (NY 1999) (19, 24) at a multiplicity of infection (MOI) of 0.2, and monitored for CPE at 3 to 5 days postinfection (p.i.). Of the candidate WNV inhibitors identified, the sultam thiourea TYT-1 (Fig. 1) appeared the most potent in replicate screens. TYT-1’s anti-WNV effects were confirmed in virus yield reduction assays (19, 29). Mock-treated and TYT-1-treated Vero cells were infected for 24 h, after which virus-containing medium samples were titrated by limiting dilution on fresh cells in the absence of new compound. An example of our results is shown in Fig. 2. As illustrated and expected, medium from mock-treated, mock-infected (“no virus”) cells yielded no deleterious effects on new cells. In contrast, dilutions of ≥105 from mock-treated infected (“no TYT-1”) cells generated virus sufficient to lyse new cell monolayers completely. However, treatment of cells with 2.3 or 23 μM TYT-1 reduced 24-h virus yields ≥100-fold (Fig. 2), substantiating the initial screen results.

Determination of the TYT-1 concentration needed to reduce WNV titers twofold (50% effective concentration [EC50]) followed the virus yield reduction regimen described above. As illustrated in Fig. 3 (black bars), the EC50 of TYT-1 against WNV was approximately 0.7 μM. Since our original screening protocol scored for protection of cells from virus-infused CPE, it appeared that TYT-1 was not toxic to cells, at least at 23 μM.

However, to test this directly, cells were treated with increasing concentrations of TYT-1 and assayed after 48 h for dehydrogenase levels in metabolically active cells, using MTS (3-[4,5-dimethylthiazol-2-yl]-5-[3-carboxymethoxyphenyl]-2-[4-sulfophenyl]-2H-tetrazolium] substrate (6). At the highest concentration tested (70 μM), TYT-1 did not reduce viability signals to the 50% level (Table 1). This result was confirmed microscopically by trypan blue (0.2%) exclusion (data not shown), indicating a 50% cytotoxic concentration (CC50) for TYT-1 against WNV in Vero cells is >100.

Although TYT-1 showed antiviral effects against WNV, at 23 μM it did not inhibit adenovirus 5, the Prospect Hill (2) hantavirus, or a human immunodeficiency virus type 1 (HIV-1) expression vector (data not shown). However, in virus yield reduction tests with JEV (SA14-2-8) (30), TYT-1 again inhibited virus replication, albeit with an EC50 of 7 μM, which is 10-fold higher than its EC50 against WNV (Table 1). Because very few analogues of TYT-1 have been described (28), our ability to probe structure-activity relationships is currently limited. However, we have examined the cytotoxicity and antiflavivirus effects of three available TYT-1 analogues, TYT-2, TYT-3, and TYT-4 (Fig. 1). As shown in Fig. 3 and Table 1, none of these showed impressive antiviral effects against WNV, with EC50 values of ≥20 μM. Moreover, TYT-2 and TYT-4 appeared to be cytotoxic at 50 to 100 μM (Table 1). However, TYT-3 was not cytotoxic at the highest concentration tested and gave some level of protection against JEV (Table 1).

To ascertain how TYT-1 might inhibit WNV, we initially explored the ability of TYT-1 to block WNV entry into Vero cells.
probed viral protein levels in treated and untreated acutely infected cells. Vero cells that were mock treated or treated with TYT-1 were infected with WNV and processed for either immunofluorescence (4, 18) or immunoblot (18, 23) detection of the viral E protein. Importantly, regardless of the detection method employed, we found that TYT-1 treatment dramatically reduced E protein levels in infected cells (data not shown). We also addressed whether WNV RNA levels are reduced by TYT-1 treatment through quantitation of RNA levels by real-time PCR (8, 19). With mock-treated, mock-infected, negative control Vero cells, no WNV RNA signals were observed (data not shown). With mock-treated, infected, positive control cells, real-time PCR signals were halfway through their exponential increase phase by cycle number 15 (Fig. 4), corresponding to 3,255 ± 325.8 WNV RNA copies per cell, as quantitated relative to an in vitro-transcribed NS3 RNA standard. Treatment of infected cells with TYT-1 clearly shifted the amplification signals to higher cycle numbers (Fig. 4), corresponding to 27.2 ± 1.6 WNV RNA copies per cell. Thus, TYT-1-mediated inhibition of WNV E expression was accompanied by a 100-fold reduction in WNV RNA levels.

The observed reductions of WNV protein and RNA levels imply that TYT-1 exerts its antiviral activity prior to the assembly stage of virus replication. However, these experiments did not discriminate whether inhibition occurs at viral entry or postentry steps. One way to distinguish between these possibilities is to screen for antiviral activity when an inhibitor is added after the onset of infection. When such time course experiments were undertaken, using a virus yield reduction readout, we found that TYT-1 application as late as 2 h p.i. gave similar levels of virus inhibition to those obtained when cells were pretreated with the drug (data not shown). Addi-

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### Table 1. Characteristics of sultam thiourea compounds

<table>
<thead>
<tr>
<th>Compound</th>
<th>EC₅₀ (μM)</th>
<th>CC₅₀ (μM)</th>
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<tbody>
<tr>
<td></td>
<td>WNV</td>
<td>JEV</td>
</tr>
<tr>
<td>TYT-1</td>
<td>0.7</td>
<td>7</td>
</tr>
<tr>
<td>TYT-2</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>TYT-3</td>
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<td>8</td>
</tr>
<tr>
<td>TYT-4</td>
<td>22</td>
<td>65</td>
</tr>
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*EC₅₀ and CC₅₀ values were derived from virus yield reduction results and MTS cytotoxicity assays performed in quadruplicate. Note that 50% cytotoxicity was defined as a 50% drop in background-subtracted MTS signals and that for TYT-1 and TYT-3, 50% cytotoxicity was not obtained with the highest drug concentrations employed.*
FIG. 4. WNV RNA levels in treated and untreated cells. Vero cells were mock treated with DMSO ("mock"; final concentration, 0.5% DMSO) or treated with 11 μM TYT-1 in DMSO ("TYT-1") and then mock infected (not shown) or infected with WNV at an MOI of 5. At 16 h p.i., RNAs were isolated, and equivalent input RNA amounts were reverse transcribed and subjected to real-time PCR quantitation of WNV RNA levels following previously described protocols (8, 19). The results, plotted as relative fluorescence signals versus PCR cycle numbers, indicate the following average (n = 4) WNV RNA copy numbers per cell, as quantitated relative to an in vitro-transcribed NS3 RNA standard: for uninfected cells, 0; for untreated cells, 3,255 ± 325.8; and for TYT-1-treated cells, 27.2 ± 1.6. Reverse transcription-PCR cycle parameters were 30 min at 48°C for the reverse transcription step, 10 min at 95°C for a denaturation step, and 40 cycles of 13 s at 95°C and 1 min at 60°C.

FIG. 5. WNV replicon inhibition. BHK cells expressing a WNV luciferase replicon (WNV) (33) or 293 cells transfected with an HIV-based luciferase expression vector (HIV) (34) were mock treated with DMSO (0.1% [final concentration]) or treated with 23 μM TYT-1 in DMSO. At 48 h posttreatment, cells were processed for detection of total protein levels (white bars) or luciferase activities (black bars). Protein levels and luciferase levels are expressed as percentages of the values obtained for mock-treated samples; background luciferase levels with parental BHK and untransfected 293 cells were <0.1% of the 100% values shown. Values obtained for WNV replicon samples were averaged from four separate experiments and are shown with standard deviations.

ventionally, we tested TYT-1 effects on baby hamster kidney (BHK) 26.5 cells (33), which stably harbor a WNV replicon expressing a luciferase reporter gene. To do so, BHK or BHK 26.5 cells were mock treated for 48 h with DMSO (0.1% final concentration) or with 23 μM TYT-1 (final concentration) in DMSO and processed for determination of luciferase activities (34) and total protein levels (Bio-Rad). Significantly, TYT-1 treatment of these WNV replicon-expressing cells reduced luciferase reporter levels >20-fold but did not alter cellular total protein levels (Fig. 5, left panel). In contrast, TYT-1 did not reduce luciferase levels in control cells expressing the protein from an HIV-1-based (34) vector (Fig. 5, right panel).

The above results demonstrate that TYT-1 blocks a postentry, preassembly step of WNV replication. However, the precise mechanism by which TYT-1 exerts its antiviral effects is not known. Since the compound reduced virus levels in African green monkey Vero cells and viral replicon levels in BHK 26.5 cells, its effects are not specific to one cell type or species. Another observation which suggests that our sulfam thioureas interfere with a virus-specific target is that TYT-1 and TYT-3 showed opposite differential effects on WNV versus JEV (Table 1); it is difficult to reconcile how these results might occur if the two compounds were to act on a common cellular factor. Thus, the accumulated data (Table 1; Fig. 2 to 5) suggest that TYT-1 targets a sensitive step somewhere in the middle of the virus replication cycle. Conceivably, inhibition could occur via a block to viral translation, polyprotein processing, or RNA replication, but further investigation will be needed to dissect the mechanism in greater detail and to determine whether the potency of TYT-1 will be sufficient for therapeutic purposes in vivo.

We could find no reports concerning the potential biological activities of sulfam thioureas closely related to TYT1-4. However, numerous sulfonamides have been employed as inhibitors of a diverse set of proteases (1). Moreover, several sulfamts have been considered excellent antiarthritic drug candidates by virtue of their activities against matrix metalloproteinases (1, 14, 21, 22, 31). While these reports might point to the WNV protease as the TYT-1 target, sulfams have been reported to block other enzyme activities. For instance, sulfam derivatives have been shown to inhibit histone deacetylase (3), HIV reverse transcriptase (5), and HIV integrase (16) activities. Thus, available data on sulfam activities do not help to implicate a particular TYT-1 target. Indeed, given the limited number of available TYT-1 analogues (Fig. 1), it is important to emphasize that even the requirement of a sulfam ring for TYT-1 or TYT-3 antiflavivirus activity is uncertain. The results suggest that replacement of the TYT-1 thiourea nitrogen phenyl groups with isopropyl (TYT-3) or methylphenyl (TYT-4) substituents has a significant impact on antiviral activity (Table 1), but considerably more study will be needed to determine how and how well these inhibitors act as antivirals.

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