Potent and selective inhibition of Tat-dependent HIV-1 replication in chronically infected cells by a novel naphthalene derivative JTK-101

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In search for effective human immunodeficiency virus type 1 (HIV-1) transcription inhibitors, we have evaluated more than 100,000 compounds for their inhibitory effects on HIV-1 long terminal repeat (LTR)-driven reporter gene expression, and identified a novel naphthalene derivative, JTK-101. This compound could suppress tumour necrosis factor (TNF)-α-induced HIV-1 production in latently infected OM-10.1 cells at nanomolar concentrations. JTK-101 could also potently inhibit constitutive HIV-1 production in MOTL-4/IIIB. However, the antiviral activity of JTK-101 was found to be much weaker in acutely infected cells and the chronically infected cells U937/IIIB cells than in OM-10.1 and MOLT-4/IIIB cells. JTK-101 selectively suppressed TNF-α-induced HIV-1 mRNA synthesis in OM-10.1 cells in a dose-dependent fashion. JTK-101 modestly inhibited TNF-α-induced HIV-1 LTR-driven reporter gene expression, but potently inhibited Tat-induced gene expression. Immunoblot analysis revealed that low-level expression of the Tat cofactors CDK9 and cyclin T1 might contribute to the diminished antiviral activity in U937/IIIB cells. Furthermore, JTK-101 could not inhibit HIV-1 replication in chronically infected monocytes/macrophages, in which CDK9 and cyclin T1 were undetectable. These results suggest that JTK-101 exerts its anti-HIV-1 activity through the inhibition of known or unknown Tat cofactors, presumably CDK9/cyclin T1.

Keywords: CDK9/cyclin T1, HIV-1, naphthalene derivative, NF-κB, Tat

Introduction

Significant progress in the treatment of human immunodeficiency virus type 1 (HIV-1) infection has been achieved by the advent of highly active antiretroviral therapy (HAART), which targets different steps in the viral replication cycle with multiple inhibitors (Yeni et al., 2004). At present, one entry inhibitor, eight nucleoside or nucleotide reverse transcriptase inhibitors (NRTIs), three non-NRTIs (NNRTIs) and eight protease inhibitors (PIs) are available for the treatment of HIV-1 infection. HAART with these inhibitors has significantly decreased plasma viraemia to undetectable levels and has considerably improved the survival of infected individuals (Pomerantz & Horn, 2003). However, considering the drug resistance and side effects of long-term HAART, discovery of novel HIV-1 agents with different mechanisms of action is still highly desirable. In addition, the reservoir cells containing latent HIV-1 are capable of producing infectious particles after cellular activation, which leads to a rebound of the viral load after interruption of HAART (Pierson et al., 2000). Therefore, HAART cannot be terminated unless such reservoir cells have been eradicated or viral recovery from the cells can be completely suppressed. In this regard, inhibitors that selectively prevent HIV-1 gene expression can potentially inhibit the recovery of latent virus from resting memory T cells as well as infected monocytes/macrophages (M/Ms), which are also considered to be a long-surviving chronically infected cell population in HIV-1-infected patients.

Molecular analyses of HIV-1 replication have revealed a concerted complexity that regulates the viral life cycle. Among the various steps of the HIV-1 life cycle, transcription from the integrated proviral DNA is considered to be a crucial step for viral replication, as amplification of the viral genetic information is attainable only through transcription (Cullen, 1991; Jones & Peterlin, 1994; Okamoto, 1995). The viral-encoded transactivator protein Tat stimulates transcriptional elongation through its interaction with the transactivation response (TAR) RNA structure. Tat also interacts with cellular cofactors, such as positive transcription elongation factor b (P-TEFb), a complex composed of cyclin T1 and cyclin-dependent
kinase 9 (CDK9; Peng et al., 1998; Price, 2000; Wei et al., 1998). CDK9 hyperphosphorylates the carboxy-terminal domain of RNA polymerase II, and induces efficient promoter clearance and transcriptional elongation. In addition to the viral protein Tat, several cellular factors are known to regulate HIV-1 gene expression (Peterlin & Trono, 2003). Among these factors, nuclear factor κB (NF-κB) is the most potent activator of HIV-1 gene expression (Nobel & Baltimore, 1987). In general, NF-κB exists in an inactive form in the cytoplasm, where it is bound to the inhibitory molecule IκBα. Stimulation of the cells with several cytokines, such as tumour necrosis factor-α (TNF-α), leads to the immediate degradation of IκBα and activates NF-κB, resulting in immediate translocation of NF-κB from the cytoplasm to the nucleus (Roulston et al., 1995). HIV-1 gene expression is initiated and enhanced by the activation of NF-κB and subsequent binding to the specific DNA motifs in the enhancer region of the HIV-1 long terminal repeat (LTR). However, complex and unknown machinery may also be involved in the regulation of HIV-1 gene expression.

Several compounds have been reported to suppress HIV-1 gene expression and replication through the inhibition of Tat or NF-κB. In our previous studies, the fluoroquinoline derivative K-37 proved to be a potent and selective HIV-1 transcription inhibitor in both acutely and chronically infected cells at nanomolar concentrations (Baba et al., 1998). K-37 was an inhibitor of not only Tat but also other RNA-dependent transactivators. Although its target molecule remains to be elucidated, the aminoquinolone WM5, which is structurally related to K-37, was found to interact with the bulge region of the TAR (Parolin et al., 2003; Richter et al., 2004).

In our recent extensive search programme for novel HIV-1 transcription inhibitors, more than 100,000 compounds have been examined for their inhibitory effects on HIV-1 LTR-driven reporter gene expression in cell cultures. Among the test compounds, several compounds showed selective inhibition of HIV-1 replication in chronically infected cells; the novel naphthalene derivative JTK-101 (Figure 1) was selected as the representative of the active compounds because it exhibited the highest selectivity. JTK-101 is a more potent and selective transcription inhibitor of HIV-1 than K-37 in latently and chronically infected cells. Studies of its mechanism of action suggest that JTK-101 is an inhibitor of Tat cofactors, presumably CDK9/cyclin T1.

**Materials and methods**

**Compounds**

JTK-101 (Figure 1) was synthesized by Japan Tobacco Co. (Takatsuki, Japan) and the fluoroquinoline derivative K-37 (Figure 1) was provided by Daiichi Pharmaceutical Co. (Tokyo, Japan). Lamivudine (3TC), zidovudine (AZT), and the histone deacetylase inhibitor trichostatin A (TSA) were purchased from Sigma (St. Louis, MO, USA). All compounds were dissolved in DMSO at 10 mM or higher concentrations to exclude any antiviral or cytotoxic effect of DMSO and stored at -20°C until use.

**Cells and virus**

Peripheral blood mononuclear cells (PBMCs), CEM, MOLT-4, OM-10.1 cells, MOLT-4/IIIb, and U937/IIIb cells were used in antiviral assays. OM-10.1 cells are a clone of HL-60 cells latently infected with HIV-1. MOLT-4/IIIb and U937/IIIb cells are MOLT-4 and U937 cells chronically infected with HIV-1 (IIIb strain), respectively. PBMCs were obtained from healthy donors and stimulated with phytohaemagglutinin (PHA; Sigma). W-3 and KM-3 cells were clones of CEM cells that stably integrate an HIV-1 LTR-driven secreted alkaline phosphatase (SEAP) gene. The integrated HIV-1 LTR contains two intact NF-κB-binding sites in W-3 cells, whereas both of the sites are mutated in KM-3 cells. M/Ms were isolated from healthy donors and cultivated according to the procedure described previously (Perno et al., 1988). Two strains of HIV-1 (IIIb and Ba-L) were used in antiviral assays. IIIb and Ba-L are CXCR4- and CCR5-using strains,
respectively. One CCR5-using HIV-1 isolate (CTV), and one CCR5- and CXCR4-using HIV-1 isolate (HE) were also used in antiviral assays.

**Antiviral assays**

The activities of the compounds against chronic HIV-1 infection were based on the inhibition of HIV-1 p24 antigen production. OM-10.1 cells (1×10⁵ cells/ml) were incubated in the absence or presence of the compounds for 2 h and stimulated with 1 ng/ml TNF-α (Boehringer-Mannheim, Mannheim, Germany), whereas MOLT-4/IIIb and U937/IIIb cells (1×10⁵ cells/ml) were cultured in the absence or presence of the test compounds without any stimulation. After 3 days of incubation at 37°C, the culture supernatants were collected and their p24 antigen levels were determined with a sandwich enzyme-linked immunosorbent assay kit (Cellular Products, Buffalo, NY, USA). The cytotoxicity of the test compounds for M/Ms was also determined by the MTT method (Pauwels *et al.*, 1988).

The assay procedure for measuring the anti-HIV-1 activity of the compounds in chronically infected M/Ms was also based on the quantitative detection of p24 antigen in the culture supernatants. The isolated M/Ms (5×10⁴ cells/ml) were cultured in RPMI 1640 medium supplemented with 10% heat-activated fetal calf serum, 10% heat-inactivated human AB serum, penicillin G (100 U/ml), and streptomycin (100 μg/ml). At day 7, differentiated M/Ms were infected with HIV-1, and their p24 antigen levels were determined by chemiluminescence. The test compounds for M/Ms was also determined by the MTT method.

The compounds’ activities against acute HIV-1 infection were based on the inhibition of virus-induced cytopathicity in CEM cells and p24 antigen production in PBMCs, as described previously (Baba *et al.*, 1998). CEM cells (1×10⁵ cells/ml) were infected with the virus at a multiplicity of infection of 0.01 and cultured in the presence of various concentrations of the compounds. After 4 days of incubation at 37°C, the CEM cells were subcultured at a ratio of 1:5 with fresh culture medium containing appropriate concentrations of the test compounds and further cultured. For the assays in PBMCs, the cells (1×10⁵ cells/ml) were infected with HIV-1 at a multiplicity of infection of 0.1. After virus adsorption for 2 h, the cells were extensively washed to remove unadsorbed virus particles and cultured in the presence of various concentrations of the test compounds. After 6 days incubation at 37°C, the culture supernatants were collected and examined for their p24 antigen levels. The cytotoxicity of the test compounds were also determined by the MTT method.

**Quantitative RT-PCR analysis**

OM-10.1 cells (2.5×10⁵ cells/ml) were incubated in the absence or presence of the JTK-101 for 2 h, stimulated with 1 ng/ml TNF-α, and further incubated for 24 h. Total RNA was extracted from the cells with an RNA extraction kit (Promega, Madison, WI, USA). The extracted RNA was subjected to quantitative RT-PCR analysis to determine HIV-1 mRNA, using GeneAmp 5700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). For quantitative RT-PCR, Taqman One-Step RT-PCR Master Mix Reagents Kit (Applied Biosystems) was used according to the manufacturer’s instructions. Using the sequence of the HIV-1 molecular clone HXB2, a primer and a probe were designed downstream of the transcription initiation site for HIV-1 mRNA. The primer and the probes were 581F (5′-TGTTAATGTTTCAATCCAGAC-3′, nucleotide position 582–605), 683R (5′-AGCTCTCTGTGTTCCCTTCT-3′, nucleotide position 662–682) and 620T (5′-TGGAATTCCTCTAGCTGGCAGAC-3′, nucleotide position 619–647). Non-specific inhibition of host cellular RNA synthesis by JTK-101 was determined with Taqman GAPDH Control Reagents kit (Applied Biosystems).

**Reporter gene assays**

W-3 and KM-3 cells were either treated with 10 ng/ml TNF-α or transfected with 1 μg of plasmid expressing HIV-1 Tat, which contains the second exon under the control of the simian virus 40 promoter (modification of pSV2tat72), by Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). The cells were cultured in the presence of various concentrations of the test compounds. After 2 days of incubation at 37°C, the SEAP activities in the culture supernatants were determined by chemiluminescence. The SEAP activities were measured using the GreatEscape SEAP detection kit (CLONTECH, Palo Alto, CA, USA), according to the manufacturer’s instructions. The chemiluminescence intensity was measured with a LB96P luminometer (Berthold, Wildbad, Germany). At the same time, the number of viable cells was determined by the MTT method.

**Immunoblot analysis**

Immunoblot analysis was performed as described previously (Wang *et al.*, 2002). Briefly, cells extracts were prepared by incubating cells in lysis buffer (50 mM Tris [pH 8.0], 120 mM NaCl and 0.5% NP-40) containing protease inhibitor cocktail and phenylmethylsulphonyl fluoride (Sigma, St. Louis, MO, USA). Protein concentration...
Figure 2. Inhibitory effects of JTK-101 and K-37 on HIV-1 replication in TNF-α-stimulated OM-10.1 cells

(A) Inhibitory effects of JTK-101. (B) Inhibitory effects of K-37. OM-10.1 cells were incubated in the absence or presence of the test compounds for 2 h, stimulated with TNF-α (1 ng/ml), and further incubated. After 3 days of incubation, the p24 antigen levels of culture supernatants (lines) were measured by ELISA. At the same time, the number of viable cell was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method (columns). The experiments were repeated three times and representative results are shown.
Inhibitory effects of JTK-101 and other selected compounds on HIV-1 replication in chronically infected cells*

| Compound | Cells | EC50, mM | CC50, mM | SI
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>JTK101</td>
<td>OM-10.1</td>
<td>0.0014 ±0.0005</td>
<td>3.8 ±0.2</td>
<td>2,714</td>
</tr>
<tr>
<td></td>
<td>MOLT-4/IIIB</td>
<td>0.0057 ±0.0025</td>
<td>13 ±0.4</td>
<td>228</td>
</tr>
<tr>
<td>K-37</td>
<td>OM-10.1</td>
<td>0.033 ±0.012</td>
<td>2.1 ±0.3</td>
<td>63</td>
</tr>
<tr>
<td></td>
<td>MOLT-4/IIIB</td>
<td>0.074 ±0.033</td>
<td>&gt;5.0</td>
<td>&gt;68</td>
</tr>
<tr>
<td>3TC</td>
<td>OM-10.1</td>
<td>&gt;20</td>
<td>&gt;20</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>MOLT-4/IIIB</td>
<td>&gt;20</td>
<td>&gt;20</td>
<td>–</td>
</tr>
</tbody>
</table>

*All data represent means ±SD for three separate experiments. †Concentration required for 50% inhibition of p24 antigen production in culture supernatants. ‡Concentration required for 50% inhibition of cell proliferation and viability. §Selectivity index (ratio of CC50 to EC50).

Antiviral activity in acutely infected cells

In the next experiment, JTK-101 was examined for its inhibition of HIV-1 (IIIg strain) replication in acutely infected CEM cells and PBMCs. Although JTK-101 could suppress p24 antigen production in culture supernatants at non-toxic concentrations, the compound was found to be less inhibitory to HIV-1 replication in acutely infected cells than in chronically infected cells (Tables 1 and 2). The EC50 and CC50 of JTK-101 were 0.0014 and 3.8 μM, respectively. K-37 (Figure 1), another potent HIV-1 transcription inhibitor, could also suppress the production of p24 antigen in a dose-dependent fashion (Figure 2B). The EC50 and CC50 of K-37 was 0.033 and 2.1 μM, respectively (Table 1). Thus, their selectivity indexes, based on the ratio of their CC50 to EC50, were 2,714 and 63 for JTK-101 and K-37, respectively, indicating that JTK-101 is a much more potent and selective inhibitor of HIV-1 replication in chronically infected cells than K-37.

The inhibitory effects of JTK-101 on HIV-1 replication were also evaluated in MOLT-4/IIIg and U937/IIIg cells, both of which continuously produce a large amount of virus without any stimuli (data not shown). As shown in Figure 3A, JTK-101 efficiently suppressed HIV-1 production in MOLT-4/IIIg cells at very low concentrations, yet higher concentrations are required to completely block viral production in MOLT-4/IIIg cells than in OM-10.1 cells. Again, K-37 was less active than JTK-101. The EC50 of K-37 in MOLT-4/IIIg cells was 0.0057 and 0.074 μM, respectively (Table 1). Interestingly, little, if any, suppression of HIV-1 production by JTK-101 was observed in U937/IIIg cells even at high concentrations (Figure 3B). In contrast, K-37 had similar inhibitory effect on HIV-1 production in MOLT-4/IIIg and U937/IIIg cells (Figure 3C and 3D). The NRTI 3TC was totally inactive in these chronically infected cells, such as OM-10.1 and MOLT-4/IIIg (Table 1).

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Inhibitory effect on HIV-1 transcription

As JTK-101 was selected through screening in an HIV-1 LTR-driven reporter gene expression system and showed potent anti-HIV-1 activity in chronically infected cells, the compound was assumed to be an HIV-1 transcription inhibitor. Therefore, quantitative RT-PCR analysis was conducted to determine whether JTK-101 could prevent HIV-1 mRNA synthesis in TNF-α-stimulated OM-10.1 cells. As shown in Figure 4, JTK-101 selectively suppressed TNF-α-induced HIV-1 mRNA synthesis in a dose-dependent fashion. Even at a concentration of 1 mM, the compound could prevent HIV-1 mRNA synthesis by 60% in the cells. By contrast, it did not affect glyceraldehydes-3-phosphate dehydrogenase (GAPDH) mRNA synthesis at concentrations up to 100 nM, indicating that JTK-101 selectively inhibits HIV-1 growth at the transcriptional level.

Inhibitory effects on TNF-α- and Tat-induced transactivation

To elucidate whether JTK-101 primarily inhibits Tat or the cellular transcriptional factor NF-κB, transfection experiments with a Tat expression plasmid into W-3 and KM-3 cells were conducted. Transfection with the Tat expression plasmid induced an increase of SEAP production in both W-3 and KM-3 cells. In contrast, treatment with TNF-α
(10 ng/ml) induced an increase of SEAP production in W-3 cells, but not KM-3 cells, because two NF-κB binding sites of the HIV-1 LTR were mutated in KM-3 cells (Baba et al., 1999). In both W-3 and KM-3 cells, JTK-101 could reduce the Tat-induced SEAP production in a dose-dependent fashion (Figure 5A). Interestingly, JTK-101 reduced the Tat-induced SEAP production more efficiently in KM-3 cells than in W-3 cells – its IC50 values in W-3 and KM-3 cells were 110 and 4.5 nM, respectively. K-37 inhibited Tat-induced SEAP production less than JTK-101. However, there was no substantial difference between K-37’s activity in W-3 and KM-3 cells. The IC50 of K-37 in W-3 and KM-3 cells were 318 and 236 nM, respectively (Figure 5B). Furthermore, JTK-101 could reduce the TNF-α-induced SEAP production in W-3 cells with an IC50 of 229 nM, whereas K-37 had no effect on the

Table 2. Inhibitory effects of JTK-101 and other selected compounds on HIV-1 replication in acutely infected cells

<table>
<thead>
<tr>
<th>Compound</th>
<th>Cells</th>
<th>EC50, µM</th>
<th>CC50, µM</th>
<th>SI</th>
</tr>
</thead>
<tbody>
<tr>
<td>JTK101</td>
<td>CEM</td>
<td>0.031 ±0.007</td>
<td>1.0 ±0.5</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>PBMC</td>
<td>0.39 ±0.25</td>
<td>1.2 ±0.9</td>
<td>3.1</td>
</tr>
<tr>
<td>K-37</td>
<td>CEM</td>
<td>0.11 ±0.07</td>
<td>1.8 ±0.6</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>PBMC</td>
<td>0.095 ±0.074</td>
<td>3.2 ±0.5</td>
<td>34</td>
</tr>
<tr>
<td>AZT</td>
<td>CEM</td>
<td>0.0026 ±0.0005</td>
<td>&gt;1</td>
<td>&gt;385</td>
</tr>
</tbody>
</table>

*All data represent means ±SD for three separate experiments. †Concentration required for 50% inhibition of p24 antigen production in culture supernatants. ‡Concentration required for 50% inhibition of cell proliferation and viability. § Selectivity index (ratio of CC50 to EC50).
JTK-101, nM mRNA level, %

The cells were incubated with the compound for 2 h, stimulated (+) with tumour necrosis factor (TNF-α (1 ng/ml)), and further incubated. After 24 h incubation, total RNA was extracted from the cells, and quantitative RT-PCR for HIV-1 mRNA was performed. The cytotoxic effects of the test compounds on host cellular mRNA synthesis were determined by quantitative RT-PCR for glyceraldehydes-3-phosphate dehydrogenase (GAPDH) mRNA. Representative results for two independent experiments are shown.

Figure 4. Inhibitory effects of JTK-101 on HIV-1 mRNA synthesis in OM-10.1 cells

The presence of reservoir cells that contain latent viruses results in the production of infectious particles upon cellular activation, which leads to a rebound of the viral load after interruption of HAART (Pierson et al., 2000). The persistence of these virus reservoirs, despite prolonged HAART treatments, represents a major obstacle to the eradication of HIV-1 in infected patients (Finzi et al., 1997; Wong et al., 1997). Therefore, therapeutic targets for HIV-1 replication at the level of transcriptional activation hold great potential for further attempts at clearing viral latency. In this study, we have identified JTK-101, a novel naphthalene derivative, as a potent and selective transcription inhibitor of HIV-1 in latently and chronically infected cells. JTK-101 did not prevent proviral DNA synthesis (data not shown), suggesting that viral entry, uncoating, reverse transcription and integration are not the target of this compound. Furthermore, the inhibition of HIV-1 transcription by JTK-101 is potent and selective. Quantitative RT-PCR analysis revealed that JTK-101 almost completely inhibited HIV-1 mRNA synthesis without altering the level of GAPDH mRNA in TNF-α-treated OM-10.1 cells at a concentration of 100 nM (Figure 4).

K-37, an anti-HIV-1 fluoroquinoline derivative, also displayed selective inhibition of HIV-1 replication in acutely and chronically infected cells (Tables 1 and 2). However, its antiviral activity was weaker than that of JTK-101. K-37 was capable of inhibiting RNA-dependent transactivation mediated by Tat, but did not inhibit DNA-dependent transactivation mediated by NF-κB (Baba et al., 1998; Okamoto et al., 2000). Furthermore, K-37 did not inhibit but stimulated the NF-κB-mediated transactivation at the highest concentration tested (1,000 nM), yet the mechanism was still unknown (Figure 5). Unlike K-37, JTK-101 could inhibit not only Tat but also NF-κB-mediated transactivation of the HIV-1 LTR (Figure 5). Although JTK-101 was highly inhibitory to Tat-mediated transactivation, a much higher concentration

Inhibition of HIV-1 transcription by JTK-101

CDK9/cyclin T1 level and JTK-101 activity

As the expression of CDK9 and cyclin T1 affect the transactivation by Tat, the protein levels of CDK9 and cyclin T1 were evaluated in several chronically infected cells. As shown in Figure 6, CDK9 and cyclin T1 were highly expressed in the T-lymphoblastoid cell lines CEM and MOLT-4. However, only low levels of the molecules were detected in the promonocytic cell line U937. Chronic infection of MOLT-4 and U937 cells with HIV-1 did not significantly alter the expression of CDK9 and cyclin T1. Furthermore, like MOLT-4/IIIb cells, OM-10.1 cells displayed high level expression of CDK9 and cyclin T1. These results suggest that the poor activity of JTK-101 against HIV-1 production in U937/IIIb is partly attributed to the low level expression of CDK9 and cyclin T1.

It was reported that the downregulation of cyclin T1 expression at a late stage of M/Ms differentiation contributed to low or absent Tat transactivation function at this stage (Liou et al., 2002). Therefore, the level of CDK9 and cyclin T1 expression and the anti-HIV-1 activity of JTK-101 were examined in differentiated and chronically infected M/Ms. As expected, the expression of cyclin T1 was an undetectable level in both uninfected and chronically infected M/Ms after 17 days of cultivation (Figure 7A). CD9K was also undetectable, although activated PBMCs obtained from the same donor displayed a high level of cyclin T1 and CDK9 expression. Furthermore, JTK-101 did not inhibit HIV-1 production in chronically infected M/Ms (Figure 7B), whereas K-37 did inhibit HIV-1 production in a dose-dependent fashion (data not shown). These results suggest that the interaction of JTK-101 with either cyclin T1 or CDK9, or both, is needed to exert its anti-HIV-1 activity.

Discussion

The presence of reservoir cells that contain latent viruses results in the production of infectious particles upon cellular activation, which leads to a rebound of the viral load after interruption of HAART (Pierson et al., 2000). The persistence of these virus reservoirs, despite prolonged HAART treatments, represents a major obstacle to the eradication of HIV-1 in infected patients (Finzi et al., 1997; Wong et al., 1997). Therefore, therapeutic targets for HIV-1 replication at the level of transcriptional activation hold great potential for further attempts at clearing viral latency. In this study, we have identified JTK-101, a novel naphthalene derivative, as a potent and selective transcription inhibitor of HIV-1 in latently and chronically infected cells. JTK-101 did not prevent proviral DNA synthesis (data not shown), suggesting that viral entry, uncoating, reverse transcription and integration are not the target of this compound. Furthermore, the inhibition of HIV-1 transcription by JTK-101 is potent and selective. Quantitative RT-PCR analysis revealed that JTK-101 almost completely inhibited HIV-1 mRNA synthesis without altering the level of GAPDH mRNA in TNF-α-treated OM-10.1 cells at a concentration of 100 nM (Figure 4).

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was required to inhibit NF-κB-mediated transactivation. Thus, it is assumed that JTK-101 exerts its potent anti-HIV-1 activity primarily through the inhibition of Tat function rather than NF-κB. Furthermore, JTK-101 was still active against HIV-1 replication in OM-10.1 cells, even when added to culture medium 24 h after stimulation with TNF-α (data not shown). The compound showed a similar inhibitory effect on TSA-induced HIV-1 production in OM-10.1 cells (data not shown), further suggesting that NF-κB was not a major target. Although the cellular transcription factor NF-κB plays an important role in triggering HIV-1 gene expression, the activation of NF-κB leads to rapid production of Tat, which may be necessary to maintain continuous HIV-1 gene expression in latently infected cells. Therefore, as well as NF-κB inhibitors, a Tat inhibitor could be effective in restricting the recovery of latent virus from resting T cells in vivo. Stevens et al. (2007) recently reported that N-aminoimidazole derivatives interfered with viral replication at a post-integrational level by inhibiting HIV-1 mRNA transcription. However,

Figure 5. Inhibitory effects of JTK-101 and K-37 on HIV-1 Tat-induced or TNF-α-induced transactivation in W-3 and KM-3 cells

For HIV-1 Tat-induced transactivation (A and B), W-3 and KM-3 cells were transfected with the Tat expression plasmid (1 μg). For tumour necrosis factor (TNF-α)-induced transactivation (C and D), W-3 and KM-3 cells were treated with or without TNF-α (10 ng/ml). The cells were cultured in the presence of various concentrations of the compounds. After 2 days of incubation, the culture supernatants were collected and examined for their secreted alkaline phosphatase (SEAP) levels. At the same time, the number of viable cells was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) methods. Transfection with the Tat expression plasmid induced 8.8- and 6.3-fold increase of SEAP production in W-3 and KM-3 cells, respectively. While TNF-α stimulation induced 3.5- and 0.9-fold increases of SEAP production in W-3 and KM-3 cells, respectively. Effects of JTK-101 (A and C) and K-37 (B and D) on TNF-α- or HIV-1 Tat-induced transactivation were expressed as percent inhibition of SEAP activity. All experiments were carried out in duplicate and expressed as means (ranges). Representative results for two independent experiments are shown.
Inhibition of HIV-1 transcription by JTK-101

Figure 6. Western blot analysis for CDK9 and cyclin T1 expression in various cell lines

Whole cell-lysates were fractionated by 10% sodium dodecyl sulphate-polyacrylamide gels, and western blot analysis was performed with anti-CDK9, anti-cyclin T1 or anti-actin polyclonal antibodies. The analysed samples were CEM cells (lane 1), MOLT-4 cells (lane 2), MOLT-4/IIIb cells (lane 3), U937 cells (lane 4), U937/IIIb cells (lane 5) and OM-10.1 cells (lane 6).

Unlike JTK-101, these compounds enhanced NF-κB binding at the HIV-1 promoter. Furthermore, they suppressed viral transcription via potent inhibitory effects on the recruitment of Tat to the HIV-1 promoter and on the transcriptional processivity of RNA polymerase II during the viral transcription process (Stevens et al., 2007).

In spite of robust inhibition of HIV-1 replication in the chronically infected cells OM-10.1 and MOLT-4/IIIb, JTK-101 did not produce a significant inhibitory effect in U937/IIIb cells and primary M/Ms. It showed less inhibitory to HIV-1 replication in acutely infected cells, especially in acutely infected PBMCs, than in chronically infected cells. This difference in anti-HIV-1 activity between acute and chronic stages of infection could be attributed to a distinct role of Tat in the infection stage or to the possibility that the compound interacts with known or unknown cellular factors involved in Tat-mediated transactivation.

Several lines of evidence have suggested that Tat function is largely dependent upon the interaction with the cellular transcription factor TAK/P-TEFb, a complex containing cyclin T1 and CDK9 (Herrmann & Rice, 1995; Mancebo et al., 1997). In fact, several CDK inhibitors were found to potently suppress Tat functions and inhibited HIV-1 replication in cell cultures (Heredia et al., 2005; Wang et al., 2001). Our preliminary experiments demonstrated that JTK-101 could inhibit CDK9 with an IC50 of 0.3 μM, whereas it did not show any inhibition of CDK7 and casein kinase II (data not shown). Although direct interaction between JTK-101 and CDK9 has not been demonstrated, its activity was totally dependent on the expression of CDK9 and cyclin T1 in infected cells. The expression of TAK/P-TEFb, especially cyclin T1, was regulated during differentiation from monocytes to macrophages. PMA, vitamin D3 and other agents cause the human myelomonocytic cell line HL-60 and the promonocytic cell line U937 to differentiate into terminal cells exhibiting macrophage characteristics, accompanied by a dramatic increase in their cyclin T1 levels (Herrmann et al.,

Antiviral Chemistry & Chemotherapy 18.4 209
enhancer, especially NF-κB HIV-1 LTR was strictly dependent on the HIV-1 study showed that Tat-mediated transactivation of the removal from the HIV-1 LTR (Figure 5). A previous effect of JTK-101 on Tat-mediated transactivation was weak or absent when cyclin T1 expression was very low. In such cells as U937 and primary M/Ms, JTK-101 may not be able to intervene between Tat and cyclin T1/CDK9 and exert its anti-HIV-1 activity. Another interesting finding is that the inhibitory effect of JTK-101 on Tat-mediated transactivation was still affected by the function of NF-κB. JTK-101 was more efficient in suppressing Tat-mediated transactivation when the functional NF-κB binding sites were removed from the HIV-1 LTR (Figure 5). A previous study showed that Tat-mediated transactivation of the HIV-1 LTR was strictly dependent on the HIV-1 enhancer, especially NF-κB, in human blood CD4+ T-lymphocytes (Alcamí et al., 1995). NF-κB-independent Tat transactivation could occur in transformed lymphoblastoid T-cell lines, but not in normal T lymphocytes (Alcamí et al., 1995). Thus, the absolute dependence of Tat function on NFκB responsive elements in T lymphocytes might bring about the much reduced anti-HIV-1 activity of JTK-101 observed in acutely infected PBMCs as compared with acutely infected CEM cells. Furthermore, Tat upregulates cytokine gene expression via a TAR-independent pathway and induces the production of proinflammatory cytokines, such as TNF-α and interleukin 1β, which activate NFκB signal transduction pathways (Biswas et al., 1995).

In conclusion, the novel naphthalene derivative JTK-101 is a potent and selective inhibitor of HIV-1 replication in cell cultures. Although its precise target molecule remains to be elucidated, the compound suppresses HIV-1 gene expression through the inhibition of known or unknown Tat cofactors, presumably CDK9/cyclin T1.

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References


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