Pyridine N-oxide derivatives: unusual anti-HIV compounds with multiple mechanisms of antiviral action

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Pyridine N-oxide derivatives represent a new class of anti-HIV compounds, for which some members exclusively act through inhibition of HIV-1 reverse transcriptase and thus characteristically behave as non-nucleoside reverse transcriptase inhibitors. Other members act, additionally or alternatively, at a post-integralional event in the replication cycle of HIV, that is, at the level of HIV gene expression. Repeated administration of one of the prototype compounds (JPL-32) to DBA/2 and hu-PBMC-SCID mice demonstrated, in the absence of any acute toxicity, protective activity against HIV-induced destruction of CD4 human T lymphocytes.

Keywords: NF-κB, reverse transcriptase, NNRTI;transcription inhibitors, HIV chemotherapy

Introduction

Up to 19 different drugs have been approved for the treatment of HIV-infected individuals, including seven nucleoside reverse transcriptase inhibitor (NRTIs), one nucleotide RT inhibitor (NtRTI), three non-nucleoside RT inhibitors (NNRTIs), seven protease inhibitors (PIs) and one fusion inhibitor. Nevertheless, long-term side effects and virus–drug resistance emergence, together with compliance problems in accurately adhering to the often complicated time schedules of drug uptake, have made it imperative to develop additional drugs, preferably acting at a novel target in the replication cycle of the virus.

The pyridine N-oxide derivatives represent a peculiar class of antiviral compound that qualify as promising novel drugs for exploration as potential anti-HIV agents. They have an entirely new mechanism of antiviral action and the capacity to retain antiviral activity against virus strains that have gained resistance to clinically used drugs, such as NRTIs, NtRTIs, NNRTIs (depending on the particular structure of the pyridine N-oxide derivative) and PIs. Indeed, it is clear that, whereas several members of this class of compounds functionally interact with HIV-1 RT as NNRTIs, other distinct members inhibit HIV replication. They do this by interacting, additionally or alternatively, with a target in the HIV replication cycle that is situated, following proviral DNA integration in the host cell chromosome(s).

An extensive study on the anti-HIV activity of a wide variety of pyridine N-oxide derivatives has been performed and revealed that several functional subclasses of compound derivatives within the pyridine N-oxides seem to exist. Indeed, whereas a number of pyridine N-oxides are specifically and exclusively inhibitory towards HIV-1 strains targeting HIV-1 RT, but not HIV-2 RT, other closely related compounds are inhibitory to both HIV-1 and HIV-2 strains as well as the simian immunodeficiency virus (SIV) strain SIVMAC251. Other pyridine N-oxides are also active against human cytomegalovirus (but not other DNA viruses, such as herpes simplex virus or vaccinia virus). Intriguingly, there is no clear structure–antiviral activity relationship between the functionally distinct subgroups within the pyridine N-oxide derivatives (Figure 1).

First target: HIV-1 RT

The most active pyridine N-oxide congener, JPL-133 (Figure 2), has an EC50 of 0.05 μg/mL against HIV-1(IIIb), with a selectivity (ratio CC50/EC50) of ~760 in CEM cell cultures (Table 1). It behaves as a characteristic NNRTI: it is not inhibitory to HIV-2 (i.e. ROD, EHO) strains and it selects for characteristic NNRTI amino acid changes in HIV-1 RT. Typically, amino acid changes that occur in the presence of the NNRTI-like pyridine N-oxide derivatives include Lys-103 → Asn, Val-108 → Ile, Glu-138 → Lys, Tyr-181 → Cys and Tyr-188 → His. In the presence of these RT mutations, the inhibitory activity of the pyridine N-oxides against mutated virus strains is partially or completely lost, but pronounced antiviral activity is retained against mutant virus strains that contain other NNRTI-specific RT mutations such as Leu-100 → Ile and Val-179 → Asp. Interestingly, several novel amino acid mutations were found in the RT of pyridine N-oxide-exposed virus strains (i.e. Pro-313 → Ser, Arg-83 → Lys, Asp-237 → Asn), but their role in drug resistance (or increased viral fitness of the mutant virus strains) is currently unclear.
Second target: a post-integration event in the HIV replication cycle

Several pyridine N-oxide derivatives, although structurally very closely related to their NNRTI-like congeners, have acquired an activity spectrum broadened to HIV-2 (i.e. ROD, EHO) and SIVMAC251 strains (Figure 3). Thus, JPL-88 and JPL-153, which inhibit HIV-1 and HIV-2 at EC\textsubscript{50} values that rank between 1.5 and 12 \textmu g/mL, depending on the nature of the virus strain (Table 1), also inhibit SIV\textsubscript{MAC251} in CEM 174X cell cultures at an EC\textsubscript{50} of 2.1 and 2.5 \textmu g/mL, respectively (CC\textsubscript{50}: 66 and 68 \textmu g/mL, respectively). Time-of-addition studies, in which the pyridine N-oxides (i.e. JPL-32, JPL-153) were added at several time points after virus infection, revealed that addition of these compounds can be delayed for a much longer period after virus infection of the cell cultures than the NNRTI-like pyridine N-oxides, such as JPL-133. These data point to a post-integrational step in the replication cycle of HIV as an additional or alternative target for these compounds. The time-of-addition experiments were performed in HeLa-CD4-LTR-β-galactosidase cell cultures (MAGI) and the effect of the pyridine N-oxides could be demonstrated for both HIV-1(III\textsubscript{b}) and HIV-2(ROD). Moreover, it could also be shown that these compounds were active not only in acutely HIV-1-infected but also in chronically HIV-infected CEM cell cultures, where infectious progeny HIV-1 yield, as well as HIV-1 p24 production and viral RNA load in the cell culture supernatants, were suppressed by the pyridine N-oxide derivatives. Such an inhibitory effect is clearly independent from inhibition of reverse transcriptase. Indeed, in chronically infected cells, RT activity no longer contributes to the efficient production of virus particles, since the new virus...
progeny is generated from cell cultures that contain HIV in the integrated (proviral DNA) form. The catalytic activity of RT that is needed to convert viral RNA to proviral DNA prior to integration is no longer required in such chronically HIV-infected cells.

It must be pointed out that those pyridine N-oxide derivatives that are active against both HIV-1 and HIV-2 are, in general, more cytotoxic than the NNRTI-like pyridine N-oxide derivatives, and, likewise, their selectivity index (ratio CC50/EC50) is limited (Table 1). Whereas with some compounds (i.e. JPL-10) HIV-1 RT could be inhibited to a moderate extent, others (i.e. JPL-88, JPL-153) showed absolutely no inhibitory activity against HIV-1 RT at the highest concentrations tested. It is not clear whether these compounds are intrinsically inactive against HIV-1 RT or whether no activity against HIV-1 RT can be detected, due to limited solubility of the compounds and/or the nature of the (artificial) template/primers used in the assays.

Interestingly, the antiviral activities of JPL-10, JPL-88, JPL-153 and other pyridine N-oxide congeners were virtually the same when tested against wild-type HIV-1, mutant HIV-1 strains (containing NNRTI-characteristic mutations such as Leu-100 → Ile, Lys-103 → Asn, Glu-138 → Lys and Tyr-181 → Cys) and HIV-2. These observations are in agreement with a mechanism of antiviral action that is different from the inhibitory spectrum of NNRTIs.

Thus, these pyridine N-oxide derivatives inhibit HIV replication by a novel mechanism of action, different from that of the NNRTI-like pyridine N-oxides. On the one hand, some of the compounds may act as typical NNRTIs, but on the other, compounds may also act at a step in the replication cycle that follows proviral integration, i.e. at the HIV gene expression level. The interaction of these compounds with this post-integrational step in the HIV replication cycle has been confirmed by the observed inhibition of the expression of a reporter gene (green fluorescent protein) from the HIV LTR promoter in a transactivation assay. Investigations are now being performed to delineate whether the pyridine N-oxides that inhibit both HIV-1 and HIV-2 are targeting a viral and/or a cellular protein in the HIV transactivation process so as to exert their anti-HIV activity. It was found that the pyridine N-oxides can inhibit virus production stimulated by TNF-α in latently HIV-infected cell lines. Electrophoretic mobility shift assays revealed that the drugs inhibit the TNF-α-induced DNA binding of nuclear NF-κB, but not the binding of NF-κB to DNA directly. As a working hypothesis, we are now trying to demonstrate that NF-κB activation is inhibited by pyridine N-oxides at the level of nuclear translocation.

**In vivo toxicity and antiviral activity of pyridine N-oxides**

For drugs that act at a post-integrational event, for which a cellular target is most likely involved, and whose selectivity indices in cell culture are moderate, toxic side effects *in vivo* may be expected. Therefore, JPL-32, a prototype pyridine N-oxide whose CC50 against CEM cell proliferation was not higher than 1.6 µg/mL, was administered intraperitoneally to adult DBA/2 mice (10–22 g) for 10 subsequent days at 100 and 50 mg/kg/day (four male and four female mice/treatment condition). However, no signs of visible toxicity or side effects were observed. Drug-treated mice gained weight in a comparable manner to control mice that received placebo. When a similar drug treatment was given to DBA/2 mice, in which 5 x 10⁶ murine P388 leukaemia cells had been inoculated intraperitoneally, no delay in time of animal death due to P388 leukaemic growth was noted in the drug-treated mice. However, in a preliminary experiment, SCID mice were reconstituted with human PBMC (hu-PBMC SCID mice), infected intraperitoneally with HIV-1(IIIb) and treated with JPL-32 at 15 mg/kg/day for 10 days. The drug was released continuously at 12.5 µg/h by

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**Figure 3.** Four prototype pyridine N-oxide derivatives endowed with activity against both HIV-1 and HIV-2 strains.

**Figure 4.** Inhibitory effect of JPL-32 on human CD4⁺ T lymphocyte destruction in PBMC-reconstituted SCID mice infected with HIV-1(IIIb). Symbols: filled squares, mice inoculated with PBMC only (cell control); filled triangles, mice inoculated with PBMC and challenged with HIV-1 (virus control); filled circles, mice inoculated with PBMC, challenged with HIV-1 and treated with JPL-32 at 15 mg/kg/day for 10 days.
subcutaneously implanted 200 µL Alzet pumps. In this experiment, HIV-1-induced destruction of CD4 T lymphocytes was markedly prevented in four out of five HIV-1-infected mice treated with the drug (Figure 4). Again, no signs of drug-related side effects were observed in the uninfected control group of mice that received the drug under similar experimental conditions. These data revealed that JPL-32 showed in vivo anti-HIV efficacy at drug doses that were not harmful to, and easily tolerable by, the animals.

Perspectives on the future potential of pyridine N-oxide derivatives

Those pyridine N-oxide derivatives that show inhibitory potential against HIV-1, HIV-2 and SIV strains, including mutant HIV-1 strains bearing characteristic NNRTI resistance mutations in their RT, exert their antiviral activity by a novel mechanism of action, presumably by interfering with HIV gene expression through interaction with the NF-κB activation pathway. The potential of these drugs to inhibit HIV replication, in both acutely and chronically HIV-infected as well as TNF-α-activated latently HIV-infected cell cultures, add to the therapeutic potential of these compounds. The activity data obtained in HIV-1-infected hu-PBMC SCID mice at non-toxic doses of the pyridine N-oxide derivatives support this view. The interaction of the drugs with the NF-κB activation pathway may further broaden the potential usefulness of this type of compound. Here, a possible role in the treatment of inflammatory disorders, such as rheumatoid arthritis, could be envisaged. Further investigations are currently being carried out in this direction.

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