In Search of a Novel Anti-HIV Drug: Multidisciplinary Coordination in the Discovery of 4-[[4-[[4-[(1*E*)-2-Cyanoethenyl]-2,6-dimethylphenyl]amino]-2-pyrimidinyl]amino]benzonitrile (R278474, Rilpivirine)

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Ideally, an anti-HIV drug should (1) be highly active against wild-type and mutant HIV without allowing breakthrough; (2) have high oral bioavailability and long elimination half-life, allowing once-daily oral treatment at low doses; (3) have minimal adverse effects; and (4) be easy to synthesize and formulate. R278474, a new diarylpyrimidine (DAPY) non-nucleoside reverse transcriptase inhibitor (NNRTI), appears to meet these criteria and to be suitable for high compliance oral treatment of HIV-1 infection. The discovery of R278474 was the result of a coordinated multidisciplinary effort involving medicinal chemists, virologists, crystallographers, molecular modelers, toxicologists, analytical chemists, pharmacists, and many others.

Introduction

The human immunodeficiency virus (HIV) is the cause of the acquired immunodeficiency syndrome (AIDS), which was first identified in the Western world in 1981. Since then, AIDS has developed into a worldwide pandemic of disastrous proportions,[1] with more than 42 million people infected, the vast majority of whom reside in resource-limited countries. The cumulative death toll from the epidemic is at least 23 million persons.[2]

Coming To Understand AIDS and Anti-HIV Therapies. As a retrovirus, HIV has a lipid bilayer membrane envelope and contains two copies of a single-stranded RNA genome that codes for structural proteins, surface glycoproteins, regulatory factors, and the enzymes reverse transcriptase (RT), protease, and integrase.[3]

In 1987, zidovudine (azidothymidine, AZT), a nucleoside RT inhibitor (NRTI), was approved in the USA as the first chemotherapeutic agent against HIV/AIDS.[4,5] However, resistance to anti-HIV compounds develops rapidly, sometimes within a few days of initiating treatment.[6,7] Errors made by the viral enzyme RT and cellular RNA polymerase II result in about one mutation per viral replication cycle (1 base change in 10 000 RNA nucleotides), which, together with the rapid replication of the virus, is responsible for rapid emergence of drug-resistant mutants.[8]

The "quasispecies" nature of HIV infection complicates development of drugs: successful therapy must anticipate the potential genetic flexibility of this moving target. This concept is part of highly active antiretroviral therapy (HAART),[9,10] in which potent combinations of inhibitors are used to maximally suppress viral replication, thereby reducing the number of viral variants generated and the opportunity to select for resistance mutations. The most common combinations used in HAART generally include two or three RT inhibitors and one or two protease inhibitors.

Twenty anti-HIV compounds have been approved in the USA for therapeutic use at the date of writing. Among these, 11 are RT inhibitors comprising seven nucleoside (NRTIs), one nucleotide (NtRTI), and three nonnucleoside RT inhibitors (NNRTIs); eight protease inhibitors; and a viral fusion inhibitor, enfuvirtide (Fuzeon).[11] Figure 1 shows the structures of the three approved NNRTIs: nevirapine (Viramune), delavirdine (Rescriptor), and efavirenz (Sustiva). Numerous other stages of the viral replication cycle are being investigated as possible targets of chemotherapy, including viral attachment to the host cell, integration of the

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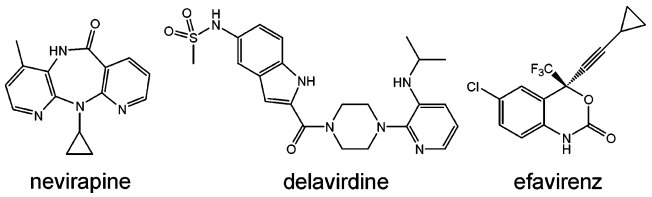

Figure 1. Nonnucleoside reverse transcriptase inhibitors (NNRTIs) that are approved for treating HIV/AIDS.

provirus into the host genome, packaging and assembly of viral particles, and budding from the host cell.[12]

Multidisciplinary Approach in a Long-Term Design Project

Sixteen Years of Research on NNRTIs. For the past 17 years, our multidisciplinary group of organic chemists, crystallographers, molecular modelers, virologists, and biologists has been striving to develop effective anti-HIV drugs. Our efforts have resulted in the discovery of the diarylpyrimidine (DAPY) family of NNRTIs.[13] By combining chemical synthesis with broad antiviral screening, bioavailability and safety assessments in animals, and analysis of three-dimensional structure−activity relationships, we identified the DAPY analogues TMC120 (R147681) and TMC125 (R165335) as promising drug candidates.[13] We report here the discovery of a new DAPY derivative, R278474.

X-ray crystallography was used throughout the program to determine structures of HIV-1 RT complexed with key analogues from each class. Molecular modeling based on the structures was systematically used to evaluate the structural determinants of inhibitor potency and antiviral resistance and to guide further synthesis. Our procedure for optimizing lead compounds used parallel, rather than sequential, screening of derivatives against wild-type and NNRTI-resistant viral strains that contained clinically relevant mutations. We also explored bioavailability and safety assessments for selected derivatives in parallel.

Figure 2 provides a historical synopsis of the developments that led to the discovery of R278474. TIBO (*t*etrahydro*i*midazo*b*enzodiazepin*o*ne) analogues, the first NNRTIs, were discovered in 1987 by screening a subset of the Janssen compound library of pharmacologically "inactive" compounds in a cell-based anti-HIV test at the Rega Institute.[14] Subsequent screening of the Janssen compounds led to the discovery of the α-APA (α-*a*nilino*p*henyl*a*cetamide) class of NNRTIs.[15] Further chemical modification[16] led to the class of potent ITU (*i*mino*t*hio*u*rea) NNRTIs. In an attempt to synthesize the corresponding imino-*N*-cyanoguanidine derivatives of ITU analogues, an unexpected ring closure occurred, producing R106168, the first compound of the DATA (*di*a*r*yl*tri*a*z*ine) class of NNRTIs.[17]

In 1996, molecular modeling studies suggested replacing the central aminotriazine ring of DATA with a pyrimidine ring. This led to the class of DAPY (*di*a*r*yl*py*rimidine) NNRTIs, of which TMC120 (R147681) is the prototype. In phase II studies on treatment-naïve HIV-infected patients, TMC120 and TMC125 proved to be highly active in reducing viral loads.[18,19] TMC125 was also found to significantly reduce viral load after 7 days of treatment in anti-retroviral-experienced patients whose HIV viruses carried RT mutations selected by previous exposure to NNRTIs.[20]

Further collaboration among medicinal chemists, crystallographers, and molecular modelers led in 2001 to the discovery of the cyanovinyl DAPY compounds, of

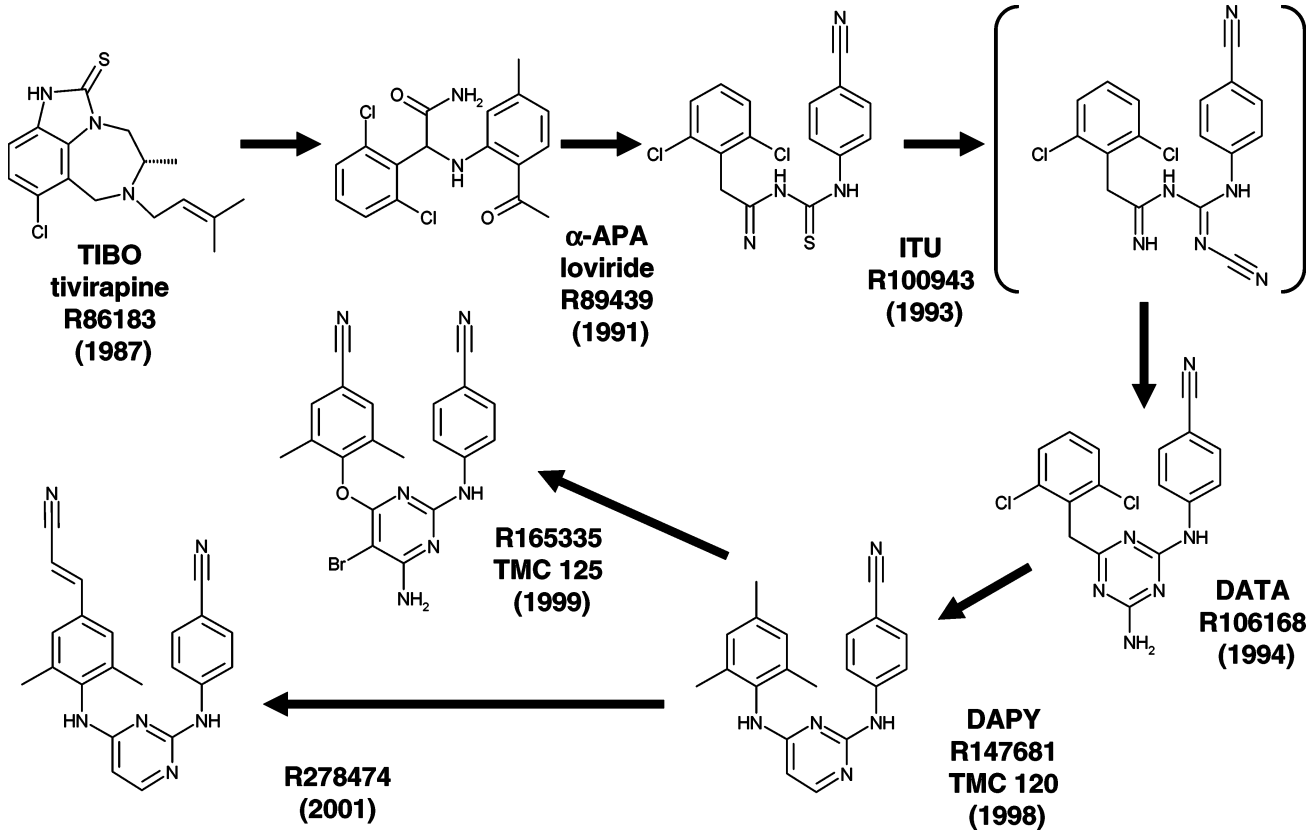

Figure 2. Chemical evolution from TIBO to R278474, starting in 1987.

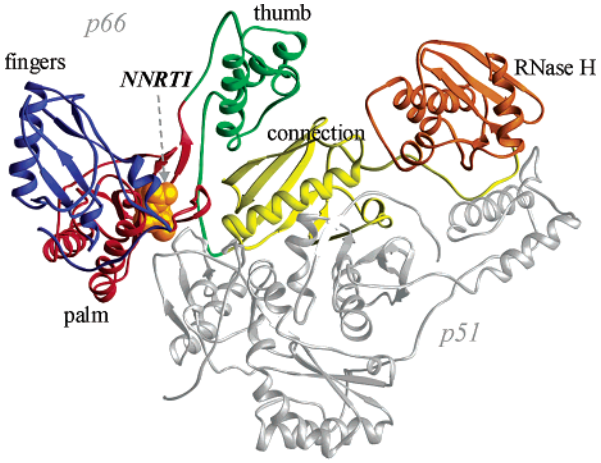

Figure 3. Ribbon representation of the three-dimensional structure of HIV-1 RT p66/p51 heterodimer showing the subdomains and the location of the NNRTI-binding pocket.

which R278474 is the prototype. The latter is the *E*-isomer of the *p*-cyanovinyl analogue of TMC120.

Crystal Structure Analysis. HIV-1 RT is a heterodimer containing two subunits, p66 and p51 (Figure 3). The overall shape of the HIV-1 RT p66/p51 heterodimer resembles a human right-hand, with the p66 fingers, palm, and thumb domains forming a cleft that binds a template-primer; the polymerase active site is located at the base of the cleft. RT is able to catalyze DNA polymerization using either RNA or DNA templates.

In 1992, Steitz and collaborators determined an X-ray crystallographic structure of HIV-1 RT complexed with nevirapine (Viramune) at 3.5-Å resolution, the first approved NNRTI.[21] In 1993, Arnold and collaborators published the structure of HIV-1 RT complexed with a DNA template-primer.[22] Since that time, additional HIV-1 RT crystal structures have been described, among which are structures of the unliganded enzyme,[23,24] various complexes with NNRTIs,[25−31] and drug-resistant mutants.[28,30,32−36]

Analysis and comparison of the crystallographic structures obtained from HIV-1 RT and its complexes with chemically diverse NNRTIs opened the way to structure-based design of NNRTIs. The allosteric binding site of NNRTIs is a lipophilic cavity situated near the catalytic site where template-directed nucleotide polymerization takes place. This NNRTI-binding pocket (NNIBP) contains amino acids whose side chains interact with bound NNRTIs: key pocket side chains include Tyr181, Tyr188, Trp229, Lys103, Leu100, Leu234, and Tyr318 (Figure 4). The hydrophobic binding pocket does not exist in structures of unliganded HIV-1 RT that do not have a bound NNRTI.

The crystallographic structures also suggested criteria for improving NNRTI potency, such as optimization of hydrogen bonding with the main-chain backbone of Lys101 and optimization of hydrophobic interactions with the aromatic side chains of Tyr181, Tyr188, and Trp229. An important pharmacophore of NNRTIs is the presence of a hydrogen-bond donor (usually NH) interacting with the Lys101 main-chain carbonyl oxygen as acceptor. Many NNRTIs, including TIBO and α-APA derivatives, adopt typical butterfly-like conformations at the binding site, with "wing I" (left wing) interacting

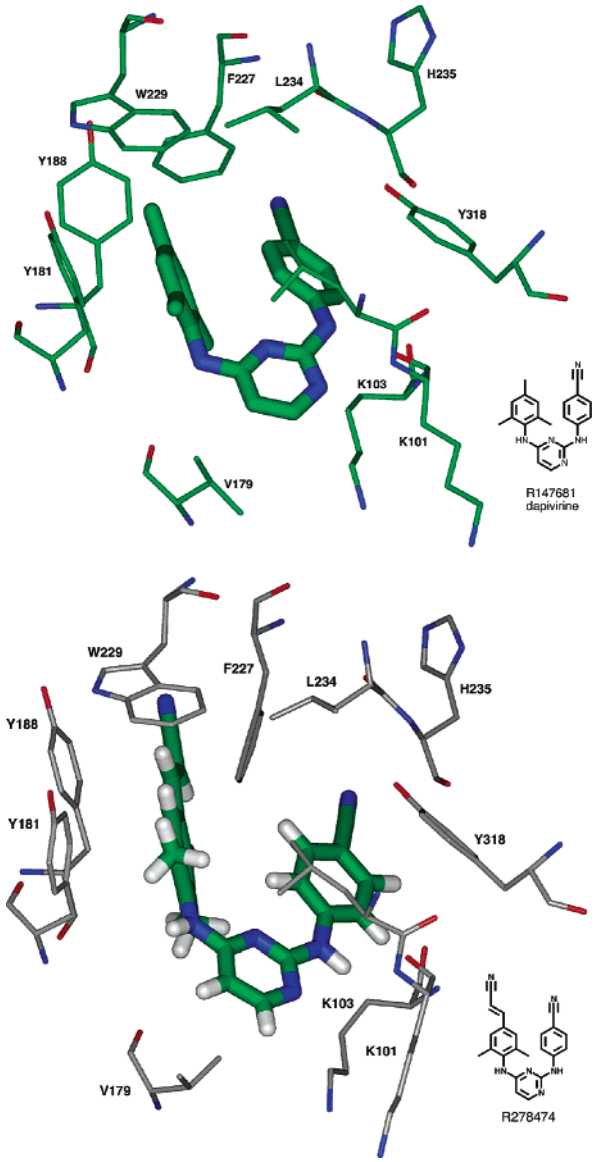

Figure 4. (a) TMC120 in the NNRTI-binding pocket (X-ray crystallographic structure). (b) R278474 in the NNRTI-binding pocket (modeled structure).

with Tyr181 and Tyr188 and oriented in the direction of Trp229. Molecular modeling studies suggested that extension of the wing I pharmacophore in the direction of the conserved Trp229 residue could greatly enhance the activity of the ligand against the wild-type virus and its resilience to mutation. Also since substitutions of Tyr181 and Tyr188 led to resistance to many NNRTIs, the plan favored reduction of binding dependence on interactions with Tyr181 and Tyr188.

R278474 is a cyanovinyl analogue of the prototype DAPY TMC120. Figure 4 shows the structure of R278474 within the nonnucleoside binding site of RT, as modeled from the crystallographic structure of RT complexed with the prototype DAPY compound TMC120.[37] The improved activity of R278474 on wild-type and mutant HIV-1 strains may involve a specific interaction of the cyano group in wing I of the molecule with the indole ring of Trp229 (Max Perutz, personal communication to P.A.J.J., 1999). The presence of an additional torsional degree of freedom in R278474 (the flexible dihedral angle between the anilino ring and cyanovinyl

moiety) relative to earlier DAPY analogs is also likely to contribute to the excellent resistance profile of R278474.[37]

Surprisingly, it has been difficult to obtain well-ordered crystals of HIV-1 RT complexes with a number of the highly potent NNRTIs of the DAPY class that are effective against NNRTI-resistant RTs. This has especially been the case for TMC125, R278474, and analogues of R278474. A possible explanation for this phenomenon is that very potent and resilient DAPY compounds can bind in multiple modes within the highly flexible NNRTI binding site of RT. This phenomenon can be described in terms of the internal conformational flexibility of the inhibitor ("wiggling") and of the plasticity of the interactions between the inhibitor and the protein in the binding site ("jiggling").[37] The "wiggling" and "jiggling" degrees of freedom enable the DAPY compounds to escape the effects of resistance mutations. In this sense, a single compound binding in different modes can have an effect comparable to that attained when different compounds, each binding in its distinctive mode, are used in combination. A multivariate statistical analysis of available virological, structural, and modeling results supports the hypothesis that the highly potent and resilient DAPY compounds bind in different modes.[38]

Molecular Modeling. Molecular modeling, which led to the design of the DAPY NNRTIs, involved docking a ligand into the NNRTI binding site of RT and minimizing the energy of the ligand−protein complex. In this procedure, between 10 and 20 distinct ligand conformers, each within 5 kcal/mol of the minimal energy conformation, are docked into a binding site held rigid for the initial calculations. These conformations are identified by means of a genetic algorithm that efficiently searches conformational space for suitable candidates. Conformations that bump into the walls of the site are eliminated. The energies of the remaining conformations are minimized and the amino acid side chains are allowed flexibility. At the same time, the main-chain backbones of the amino acids that form the wall of the pocket are tethered. Optimization is performed by means of simulated annealing, followed by a local minimization algorithm in the neighborhood of the minimum.

Binding energy is computed by means of a scoring function obtained from a molecular mechanics force field developed at the Center for Molecular Design from the MMF94 force field.[39] This force field is specifically parametrized for ligand−protein interaction. It accounts for Coulomb (electrostatic) and van der Waals (dispersive) potentials and hydrogen bonding. The scoring function is a linear combination of contributions from selected amino acid residues. A genetic algorithm guides the selection from among several hundred possible interactions (side chain, backbone, electrostatic, hydrophobic, and hydrogen bonding), so that interactions producing the best possible prediction of observed virological activity can be selected from the computed binding energies. The reliability of the prediction depends on the extent, diversity, and quality of the virological data.

Virological Screening. To date, the HIV−NNRTI project of Janssen has generated about 4000 diverse chemical compounds, each of which has been tested for anti-HIV activity at Tibotec.

Table 1. Activity of R278474 and Reference Compounds[a]

	nevirapine	efavirenz	TMC120	R278474
wild-type	81	1.4	1.2	0.4
L100I	597	35	11	0.4
K103N	2 879	28	2	0.3
Y181C	10 000	2	7	1.3
Y188L	10 000	78	37	2.0
G190S	1 000	275	2	0.1
K103N+Y181C	10 000	37	54	1.0

[a] Activity (EC_{50}, nM) of R278474 and reference compounds against wild-type HIV-1, and selected single and double HIV-1 site-directed mutants, in a cell-based assay.

Compounds were tested in a cell-based assay, using a human T-cell line, MT-4. These MT-4 cells were infected with wild-type or mutant HIV-1 and exposed to different concentrations of the antiviral compound in culture medium supplemented with 10% fetal calf serum. Compounds have to penetrate the cell membrane in order to interfere with replication steps inside the cell. Cytotoxicity was determined in parallel with the antiviral activity. After 5 days of incubation at 37 °C, the viability of the HIV-infected and of the mock-infected cells was assessed by the MTT method, an automated tetrazolium-based colorimetric assay.[40,41] This method permitted simultaneous determination of the 50% inhibitory concentration (EC_{50}) for inhibiting viral cytopathicity and the 50% cytotoxic concentration (CC_{50}). The ratio CC_{50}/EC_{50}, also called the selectivity index, is an indication of the specificity of the antiviral effect. EC_{50} values vary over time. From the standard deviation of the log EC_{50} the limits of the 95% confidence of EC_{50} are derived to be approximately ($EC_{50}/4 - EC_{50} \times 4$).

Requirements for a Novel Antiviral Drug

The desirable features that a novel anti-HIV drug should display are the following:

(1) high antiviral activity against wild-type and mutant viruses,

(2) high oral bioavailability, allowing once-daily administration,

(3) minimal adverse effects, and

(4) ease of synthesis and formulation.

Maximal suppression of viral replication is especially important with a virus such as HIV, which has high rates of replication and genetic variation. The problem can be likened to a game of chess, where winning strategies require consideration of all possibilities for as many moves ahead as feasible. A very good drug may require five or more mutations to convert a wild-type virus to a resistant virus, but if previous drug treatment experience has already selected mutants corresponding to the first few steps in the path to resistance, the virus now needs only to make three or four changes to achieve replicative fitness.

Requirement 1: High Antiviral Activity.

Wild-Type and Site-Directed Mutants. R278474 is more active against wild-type HIV-1 and against all single and double mutants tested than are nevirapine, efavirenz, TMC120, or TMC125[37] (Table 1 and Figure 5). R278474 is on average about 10−20 times more active than efavirenz.

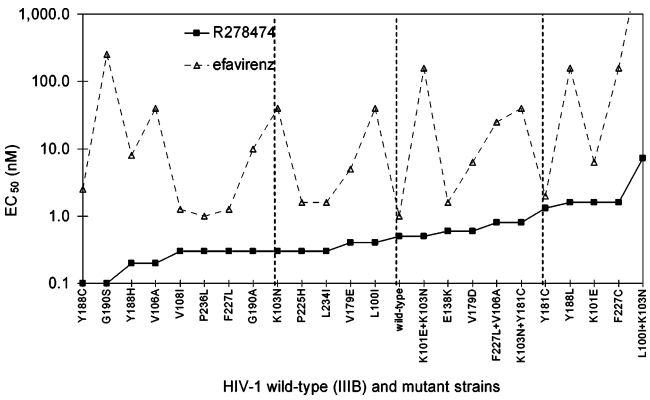

Figure 5. Virological spectra of R278474 and efavirenz, showing antiviral activity (EC_{50}) plotted on a log scale [$pEC_{50} = -\log(EC_{50})$] for wild-type and selected site-directed single and double mutants of HIV-1. The positions of wild-type (IIIB) and single mutants K103N and Y181C are identified on the spectrum by interrupted vertical lines.

Table 2. Resilience of R278474 and Efavirenz[a]

	5000 nM	1000 nM	200 nM	40 nM	10 nM
efavirenz	6	6	6	3	3
R278474	NT	>30	>30	>30	10

[a] Time to breakthrough (days) for wild-type HIV-1 as a function of concentration of R278474 and efavirenz. NT: not tested.

No sign of virus breakthrough could be observed with R278474 at 1 μM within 30 days (Table 2). This outcome differs from that for efavirenz, which shows virus breakthrough after 6 days. At 40 or 200 nM concentrations, R278474 did not select for resistant virus within 30 days using wild-type virus. Starting from the single mutant Y181C (EC_{50} = 1.3 nM) or K103N (EC_{50} = 0.3 nM), virus breakthrough did not occur at 40 and 200 nM but did occur at 10 nM. If a double resistant K103N+Y181C mutant (EC_{50} = 1.0 nM) was used instead of wild-type virus, resistance did emerge at all tested concentrations (data not shown).

The *Z*-isomer of R278474 has relatively high potency against wild-type HIV-1 (EC_{50} = 0.6 nM), but it is on average about 5 times less active against single and double mutants in comparison to the *E*-isomer.

Mutants Isolated in Vivo. About 1200 recombinant clinical isolates were collected from HIV-infected patients and these isolates were tested for sensitivity to nevirapine, efavirenz, TMC120, and R278474. The distributions of the activities (pEC_{50}) are plotted in Figure 6. R278474 inhibits 81% of these clinical isolates at a 50% inhibitory concentration (EC_{50}) less than 1 nM (pEC_{50} > 9); it inhibits 94% at EC_{50} less than 10 nM. For comparison, the percentages of clinical isolates inhibited at EC_{50} at or below 10 nM are 18% for nevirapine, 69% for efavirenz, and 79% for TMC120.

Requirement 2: High Oral Bioavailability and Long Elimination Half-Life. Absorption and Distribution in Animals. Pharmacokinetic studies have been performed in several species including Sprague–Dawley rat, beagle dog, white New Zealand rabbit, and cynomolgus monkey.[42]

Pharmacokinetic parameters such as elimination half-life ($t_{1/2}$) and exposure (AUC_{inf}) were calculated after iv administration of R278474 formulated in poly(ethylene glycol) (PEG) 400. Elimination half-life ranged from 4.4 h in rat to 31 h in dog, and exposure (AUC_{inf}) amounted

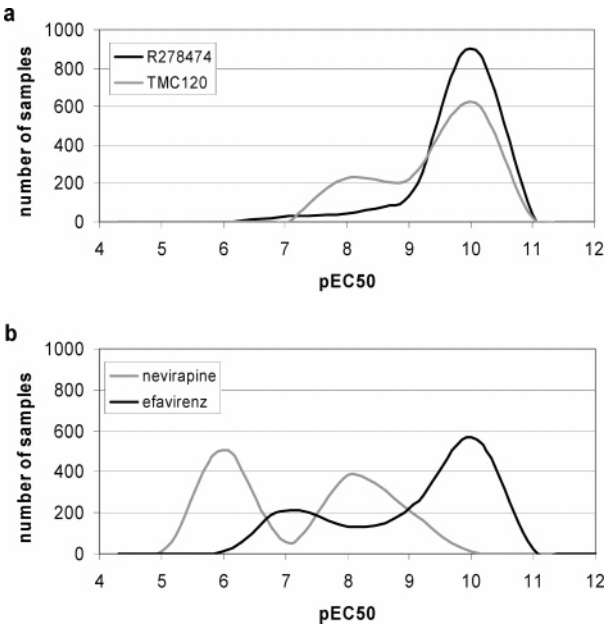

Figure 6. Distributions of antiviral activity (pEC_{50}) against 1200 clinical isolates (a) for R278474 and TMC120 and (b) for efavirenz and nevirapine.

to 3.1 μg h/mL (iv dose of 4 mg/kg) in rat, 8.7 μg h/mL (iv dose of 1.25 mg/kg) in dog, 1.4 μg h/mL (iv dose of 1.25 mg/ kg) in monkey, and 44 μg h/mL (iv dose of 1.25 mg/kg) in rabbit. Tissue plasma ratios ranged between 0.47 and 3.4. Tissue levels declined in parallel with plasma concentrations, indicative of no undue retention or accumulation of R278474 in tissue. The brain/plasma AUC ratio in rats was found to be 0.5.

After oral administration of R278474 in PEG 400, half-life ranged between 2.8 h in rat and 39 h in dog. Oral bioavailability (F_{abs}) was calculated by comparing exposures (AUC_{inf}) obtained after iv administration, which represents 100% bioavailability, with exposures after oral administration. Oral bioavailability (F_{abs}) was calculated to be 32% and 31% in rat and dog, respectively. Addition of 100 mg/mL citric acid to PEG 400 had no effect on the absorption in rat, but increased the absorption fraction in dog from 31% to 80%.

Aggregate Formation and Lymphatic Absorption. Despite intensive modeling and chemical syntheses to improve the potency of the DAPY compounds against wild-type virus, it has not been possible to find compounds with EC_{50} values below 0.1 nM in cell-based screening tests. It thus appears that there is a minimum threshold on the number of molecules that are required to inhibit virus reproduction in an infected cell.[42,43] It is known that lipophilic compounds (such as TMC120 and R278474) can form aggregates.[44] Formation of aggregates is common, and it may be the cause of nonspecific binding to proteins that has been observed in high-throughput screening (HTS) tests.[45]

In a simplified model we assumed that inhibition of RT results from endocytosis of a single aggregate of inhibitor molecules by the infected cell. Knowing the EC_{90} of the DAPY compounds (about 5 times the EC_{50}) and the concentration of infected cells in the test medium (150 000 cells/mL), it is possible to estimate the number of inhibitor molecules per aggregate as approximately 2 000 000. Taking into account the average

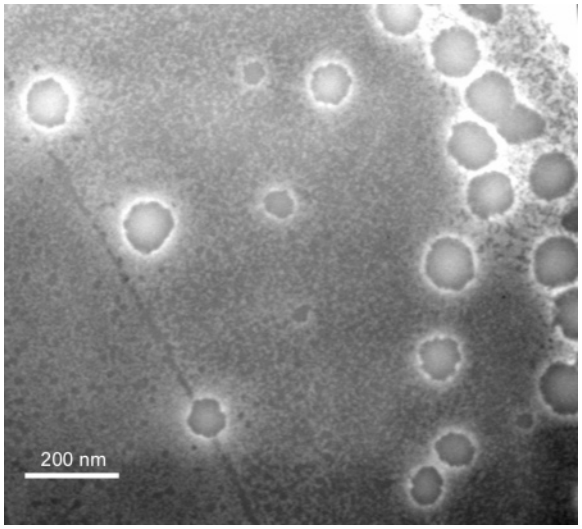

Figure 7. Electron micrograph showing aggregates prepared from a solution of R278474 with 0.1% tyloxapol and stained with uranyl acetate (Wang, Y.-H., RWJMS/UMDNJ, personal communication).

volume of a DAPY molecule (0.38 nm^3) yielded an estimate of 57 nm for the theoretical radius of an aggregate.

Dynamic light-scattering measurements (by photon correlation spectroscopy) at the CABM showed that many DAPY compounds form aggregates. From the shape and intensity of the scattering pattern and using the Stokes−Einstein formula, it is possible to calculate the hydrodynamic radius of the aggregates in the test sample.[46] Given conditions that simulated the environments of both gastric (stomach) and gut (small intestine) lumen environments, experimental aggregate radii of DAPY compounds ranged between 30 and 116 nm.

Aggregates of R278474 can also be observed directly by means of electron microscopy as prepared from a 0.5 mM solution in 0.1% tyloxapol and stained with uranyl acetate (Figure 7). The average radius observed in the micrograph of these aggregates agrees with the dynamic light scattering data (58 nm).

Drug aggregates may be absorbed via the transcellular pathway through epithelial cells (enterocytes) that line the intestinal wall or through specialized microvilli (M-) cells that cover lymphoid follicles (Peyer's patches).[47,48] Highly fat-soluble substances (such a vitamins A and D) are known to be well-absorbed[49] and transported via the lymphatic system.[50] The gastrointestinal system is extensively permeated by a lymphatic network that drains via the cisterna chyli into the thoracic duct, which in turn unites with the blood circulatory system at the junction of the left internal jugular and subclavian veins.[51] These absorption mechanisms may explain the good oral bioavailability of some highly lipophilic and poorly water-soluble compounds such as R278474.

In Vitro Metabolism. R278474 was metabolized slowly in hepatocytes of humans and of the various species used in toxicological studies. Glutathione-dependent conjugative metabolism was the primary pathway observed in hepatocytes of rodents (rat, mouse) and humans. Metabolism studies in dog, rabbit, and monkey showed mainly oxidative metabolism followed by sulfate conjugation or O-glucuronidation. N-Glucuronidation was the primary metabolism pathway in rabbit. All metabolites observed in humans were also found in dog, rat, and mouse.[47,52]

There was no major difference in the metabolic stability of R278474, expressed as percent metabolized after 120 min of incubation in S9 subcellular liver fractions of rat, dog, and humans, which amounted to 48, 25, and 38%, respectively.

On the basis of metabolism data in hepatocytes (in vitro), a slow metabolic clearance of the compound is to be expected. In vivo studies in rat and dog show a slow metabolic clearance for dog and a faster metabolic clearance for rat, which is in good agreement with the in vitro finding. Metabolism in human hepatocytes appeared to be slow, and extrapolating in vitro data to humans suggests a low metabolic clearance in humans. Drugs with a low metabolic clearance are expected to result in good exposures and longer elimination half-life, which can potentially result in a once daily dosing. Since oxidative metabolic clearance is of minor importance, the potential for drug−drug interactions is estimated to be low. Only minor, oxidative, cytochrome P450 (CYP) dependent metabolites were observed. Therefore, the metabolism of R278474 by cytochrome P450 is likely to be of minor importance in vivo. The IC_{50} values for inhibition of CYP450 isoforms 3A4, 2C9, and 2D6 expressed in *Escherichia coli* amounted to 4.6, 5.0, and >10 μM, respectively. There were no major differences in the ability of TMC120 and R278474 to inhibit these CYP450 isoforms.

Plasma Protein Binding. Plasma protein binding is high within the DAPY-class of compounds.[52] More than 99% of R278474 binds to human plasma proteins in a concentration-independent manner. The addition of α-acid glycoprotein (2 mg/mL) had no effect on the EC_{50} for TMC120 and R278474. Human serum albumin (45 mg/mL) increased the EC_{50} values of these compounds 20 and 30 times, respectively. The addition of human serum (50%) increased the EC_{50} values of the same compounds 6- and 9-fold, respectively, suggesting that the effect of human serum may be related to the presence of human serum albumin.

The clinical relevance of reversible plasma protein binding has not been established for NNRTIs.

Requirement 3: Minimal Adverse Effects. Cytotoxicity was determined in the cell-based assay used for virological screening. The selectivity index of R278474 was 20 000 for wild-type HIV-1.

R278474 was not mutagenic in the Ames reverse mutation test up to bacteriotoxic concentrations. The compound tested negative for mutagenicity in the mouse lymphoma[53] test and did not increase the number of micronucleated[54] polychromatic erythrocytes in the in vivo chromosomal aberration test in mice.

No relevant effect of R278474 was observed in in vitro radioligand binding[55] on various bioamine receptors. The compound has low binding affinity for sodium and calcium channels from rat and for the human HERG channel.

Twelve-hour monitoring of awake telemetered dogs after single oral doses of 20, 80, and 160 mg/kg demonstrated that R278474 had no effect on cardiovascular, pulmonary, electrophysiological, and behavioral parameters.[56]

No drug-induced neurological aberrations were observed in rats up to 400 mg/kg. A single oral dose of 800 mg/kg of R278474 did not produce mortality or abnormal clinical observations[57,58] in rat (apart from salivation at the day of the dosing). Consecutive[59] oral administration for 1 month of doses ranging from 10 to 160 mg/kg formulated in PEG400 did not produce abnormal effects in rat, apart from liver weight increase and species-specific thyroid hypertrophy, both at the higher dose levels.

A one month of orally administered R278474, formulated with PEG400, in male and female dogs produced a no-toxic-effect dose (NOEL) of 5 mg/kg.

Requirement 4: Ease of Synthesis and Formulation. R278474 is the *E*-isomer of 4-[[4-[[4-(2-cyanoethenyl)-2,6-dimethylphenyl]amino]-2-pyrimidinyl]amino]-benzonitrile, which can be synthesized in six high-yield reaction steps.[60] The end product contains minimal amounts (less than 0.5%) of the *Z*-isomer.

R278474 is a slightly yellow crystalline powder with molecular mass of 366.4 Da and a melting point of 242 °C. It is practically insoluble in water (20 ng/mL at pH 7.0), moderately soluble in poly(ethylene glycol) (PEG 400, 40 mg/mL), and readily soluble in dimethyl sulfoxide (>50 mg/mL). The compound is ionizable in aqueous solution ($pK_a = 5.6$) and is very lipophilic (log $P = 4.8$ at pH 8.0). For comparison, the pK_a value for TMC120 is 5.8 and the corresponding log P value amounts to 5.3.

Under daylight and in weak acid solution a conversion of 8% of the *E*-isomer of R278474 into the *Z*-isomer has been observed.

Preliminary experiments indicate that the compound can be formulated in solid form without substantial loss of bioavailability.

Conclusion

R278474 is an NNRTI of the DAPY class of anti-HIV compounds which has a superior activity profile against wild-type and mutant HIV-1 strains when compared to all currently approved NNRTIs. R278474 is efficiently absorbed and has a satisfactory safety profile as determined in various animal species. R278474 can be easily synthesized and formulated. With R278474 we have closely approached our stated criteria for a novel anti-HIV drug.

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