

TMC125, a novel next generation non-nucleoside reverse transcriptase inhibitor active against non-nucleoside reverse transcriptase inhibitor-resistant HIV-1

5 Running title: TMC125, a new non-nucleoside reverse transcriptase inhibitor

Koen Andries¹, Hilde Azijn², Theo Thielemans¹, Donald Ludovici³, Michael Kukla³, Jan Heeres⁴, Paul Janssen⁴, Bart de Corte³, Johan Vingerhoets², Rudi Pauwels² and Marie-Pierre de Béthune^{2*}

10

¹Johnson&Johnson Pharmaceutical Research & Development, Beerse, Belgium

²Tibotec, Mechelen, Belgium.

³Johnson&Johnson Pharmaceutical Research & Development, Spring House, USA.

⁴Johnson&Johnson Pharmaceutical Research & Development, Vosselaar, Belgium.

15

* Corresponding author

Telephone, +32 15 401 240

Fax, +32 15 286 347

Email: mdbethun@tibbe.jnj.com

20

Abstract

Non-nucleoside reverse transcriptase inhibitors (NNRTIs) are potent inhibitors of HIV-1, however, currently marketed NNRTIs rapidly select resistant virus and cross-resistance within the class is extensive. A parallel screening strategy was applied to test candidates from a series of diarylpyrimidines (DAPYs) against wild-type and resistant HIV carrying clinically relevant mutations. Serum protein binding and metabolic stability were addressed early in the selection process. The emerging clinical candidate, TMC125, was highly active against wild-type HIV-1 (EC_{50} 1.4–4.8 nM) and possessed some activity against HIV-2 (EC_{50} 3.8 μ M). TMC125 also inhibited a series of HIV-1 group M subtypes and circulating recombinant forms and a group O virus. Incubation of TMC125 with human liver microsomal fractions suggested good metabolic stability (15% decrease in drug concentration, 7% decrease in antiviral activity after 120 minutes). Although TMC125 is highly protein bound its antiviral effect was not reduced by the presence of 45 mg/ml human serum albumin, 1 mg/ml α_1 -acid glycoprotein or 50% human serum. In an initial screen for activity against a panel of 25 viruses carrying single and double reverse transcriptase amino acid substitutions associated with NNRTI resistance, the EC_{50} of TMC125 was < 5 nM for 19 viruses, including the double mutants K101E+K103N and K103N+Y181C. TMC125 also retained activity (EC_{50} < 100 nM) against 97% of 1081 recent clinically derived recombinant viruses resistant to at least one of the currently marketed NNRTIs. TMC125 is a potent next generation NNRTI, with the potential for use in individuals infected with NNRTI-resistant virus.

Introduction

Successful long-term treatment of HIV-1 by antiretrovirals is often hindered by incomplete viral suppression and the resulting emergence of drug resistance. There is now widespread resistance to all available classes of antiretrovirals and cross-resistance within classes is extensive, often severely limiting the treatment options available (15,19). Although currently marketed non-nucleoside reverse transcriptase inhibitors (NNRTIs) are highly selective and extremely potent, they rapidly select for resistant virus. Moreover, single mutations can lead to dramatic reductions in susceptibility, often to all available inhibitors within the class (2,9). This broad cross-resistance prevents the consecutive use of currently marketed NNRTIs in treatment regimens (1). Next generation agents with activity against NNRTI-resistant isolates would therefore offer new treatment options.

To increase the likelihood of identifying new compounds active against NNRTI-resistant strains, and having interesting drug-like properties, we developed the following strategies: The structure activity relationship (SAR), traditionally limited to activity against the wild type virus, was expanded to include concurrent evaluation of several NNRTI-resistant strains. To facilitate our understanding of the effect of new chemical substituents on the binding of the compounds to wild-type and resistant reverse transcriptase (RT), a panel of mutants was constructed harboring various NNRTI resistance associated mutations within exactly the same genetic background. A second step was to include assessment of metabolic stability early in the discovery process, thereby establishing a structure metabolization relationship (SMR). One of the early lead compounds was indeed prone to metabolization (21), leading to a short plasma half-life when tested *in vivo*. The combination of SAR and SMR allowed concurrent optimization of structures

for broad-spectrum anti-HIV activity and metabolic stability. Finally, functional protein binding assays and a large panel of NNRTI-resistant, clinically derived recombinant viruses were used to assess the activity of selected lead compounds.

- 5 Our strategy ensured a data-driven and rapid selection process that, along with structural insights, paved the road for the discovery of the imidoyl thiourea (ITU), diaryltriazines (DATA) and diarylpyrimidine (DAPY) series (20–22). Here we report the pharmacological characteristics of TMC125, (also known as R165335) (Fig 1), a DAPY compound identified using this screening strategy.

Methods

Cells and viruses

MT4 cells are human T-lymphoblastoid cells that are highly sensitive to HIV infection, producing a rapid and pronounced cytopathic effect. Peripheral blood mononuclear cells (PBMCs) for use in drug susceptibility assays were purified from HIV-negative donors and activated as previously described (11). Mature monocyte/macrophages (M/Ms) were separated out from freshly isolated PBMCs by adhesion as described by Perno *et al* (26). All cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum and antibiotics in a humidified incubator with a 5% CO₂ atmosphere at 37°C. Virus stocks of HIV-1 and HIV-2 strains were produced in MT4 cells, except for HIV-1 Ba-L which was cultured in M/Ms.

Site-directed mutants

Mutant RT coding sequences were generated from a pGEM vector containing the HIV-1 LAI (clone HXB2) protease (PR) and RT coding sequence, using the QuikChange[®] Site-Directed Mutagenesis Kit (Stratagene), and HPLC-purified primers (Genset Oligos). Plasmids were sequenced to confirm that they contained the desired mutations. Mutant viruses were created by recombination of the mutant PR-RT sequence with a PR-RT deleted HIV-1 HXB2 proviral clone (16).

Recombinant clinical isolates

Recombinant viruses derived from clinical samples (r-HIV) were constructed as previously described by co-transfection of MT4 cells with sample derived viral PR and RT coding sequences and an HIV-1 HXB2 derived proviral clone deleted in the PR and RT coding region (16).

Drug sensitivity assays

The antiviral activity of compounds against laboratory adapted strains, site-directed mutants and clinical sample derived recombinant viruses was tested using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay as previously described (16,25). Briefly, various concentrations of the test compounds were added to wells of a flat-bottom microtiter plate. Subsequently, virus and MT4 cells were added to a final concentration of 200 50% cell culture infectious dose (CCID₅₀)/well and 30,000 cells/well, respectively. In order to determine the toxicity of the test compound, mock-infected cell cultures containing an identical compound concentration range, were incubated in parallel with the virus infected cell cultures. After 5 days of incubation (37°C, 5% CO₂), the viability of the cells was determined using MTT. The results of drug susceptibility assays were expressed as an EC₅₀ defined as the concentration of drug at which there was 50% infection compared with the drug-free control. In some cases a fold change in susceptibility was calculated by dividing the EC₅₀ for the tested virus by the EC₅₀ for the wild-type virus (HIV-1 LAI) tested in parallel. Toxicity results are expressed as CC₅₀, defined as the concentration of drug at which the cell viability was reduced by 50% compared to the drug-free control.

PBMC-based drug susceptibility assays were carried out as previously described (11). Briefly, PHA-stimulated PBMCs from healthy seronegative donors were incubated with serial dilutions of the test compounds and infected with virus at a multiplicity of infection (MOI) of 0.001 CCID₅₀ per cell. Infected cells were washed and incubated in media containing the same

concentration of drug. The production of virus was quantified after 7 days using a p24 ELISA (NEN Life Science products).

5 To test the drug sensitivity of HIV-1 Ba-L, adherent M/Ms cultured in 48-well plates were exposed to various concentrations of drug for 1 hour, before incubation with 300 TCID₅₀/ml of virus for 2 hours. Cells were then washed and cultured for 15 days in the same concentration of drug. The production of virus was quantified using a p24 ELISA as above (26).

Genotyping and subtype determination

10 Genotypic analysis was performed by automated population-based full-sequence analysis (ABI PRISM[®] BigDye[™] Terminator cycle sequencing). Sequencing results are reported as amino acid changes compared to the wild-type (HXB2) reference sequence (18). Subtypes were determined by heteroduplex mobility assay (HMA) (10) or sequencing.

15 *Mechanism of action studies*

For time of addition assays, a high MOI of HIV-1 LAI was used to infect MT4-LTR-EGFP cells which were then incubated on ice for 30 minutes. Before transferring the cells 37°C, two washing steps at 4°C were included to remove non-adsorbed virus and to synchronize the infection. From 1 hour post-infection onwards, the compounds to be tested were added to parallel cultures at
20 different time points. After 24 hours, cultures were examined for fluorescence, and supernatants were tested for p24 concentration.

Inhibition of RT was assessed by using a poly r(A) RT-scintillation proximity assay (SPA) (Amersham Biosciences). This assay consists of a biotinylated DNA/RNA primer template, which is annealed to streptavidin-SPA beads. The radiolabelled compound [³H]TTP is incorporated, and extension of the primer is proportional to the amount of RT present. This assay mimics the RNA-dependent DNA-polymerase reaction during reverse transcription. The IC₅₀ is the concentration of compound that inhibits RT activity by 50%.

Metabolic stability assay

Aliquots of human liver microsomal fractions, prepared as previously described (5), were incubated with TMC125 at a final concentration of 30 μM and a co-factor solution (1 mg/ml glucose-6-phosphate, 1 mg/ml MgCl₂.6H₂O and 0.5 units/ml glucose-6-phosphate dehydrogenase in 0.5 M phosphate buffer pH 7.4). After 5 minutes at 37°C the reactions were initiated by adding a 1.25 mg/ml solution of nicotinamide adenine dinucleotide phosphate in homogenization buffer (1.15% KCl in 0.01 M phosphate buffer pH 7.4). After 15, 30 and 120 minutes of incubation at 37°C with constant shaking, reactions were terminated by addition of an equal volume of DMSO. The degree of metabolisation of TMC125 was determined by measurement of the residual parent compound in the reaction mixture using a liquid chromatography – mass spectrometry (LC-MS) method. Residual anti-HIV activity was also measured using a colorimetric anti-HIV assay as described previously (25).

Antiviral activity in the presence of human serum proteins

MT4 cells were infected with HIV-1 LAI at an MOI of 0.001–0.01 CCID₅₀ per cell. Following 1 hour of incubation, cells were washed and plated into a 96-well plate containing serial dilutions

of TMC125 in the presence of 10% fetal calf serum (FCS), 10% FCS + 1 mg/ml α_1 -acid glycoprotein (AAG), 10% FCS + 45 mg/ml human serum albumin (HSA) or 50% human serum. After 5 to 6 days, the EC₅₀ was determined by cell viability assay using resazurin (12). Briefly this involved adding 1/10 volume of resazurin solution (0.1 mg/ml resazurin, 1 mM K₄Fe(CN)₆, 1 mM K₃Fe(CN)₆ in 0.1 mM potassium phosphate buffer pH7.4) and measuring the fluorescence of the formed resofurin after 5 or 25 hours of incubation at 37°C. In some cases data were confirmed by quantifying the level of HIV replication by p24 ELISA (NEN Life Science Products).

Combination with other antiretrovirals

10 Combinations of compounds were tested against HIV-1 LAI in MT4 cells at three different molar ratios – 3x, 1x and 1/3x the estimated EC₅₀ ratio of the compounds. Drugs tested in combination with TMC125 included the nucleoside RT inhibitors (NRTIs) abacavir, didanosine, lamivudine, stavudine, zalcitabine and zidovudine; the nucleotide RT inhibitor (NtRTI) tenofovir; the PR inhibitors (PIs) amprenavir, indinavir, lopinavir, nelfinavir, ritonavir and saquinavir; as well as
15 the currently marketed NNRTIs delavirdine, efavirenz and nevirapine. A combination index (CI) at 50% protection was calculated using a formula derived from the classical isobologram model for combinations (7). Each combination was tested nine times. A drug combination was scored as synergistic when the mean CI was less than 0.8 and as antagonistic when the mean CI was greater than 1.2. Control combinations were the sham combination zidovudine/zidovudine, and the
20 combination zidovudine/emivirine, which has previously been reported as synergistic (6).

Compounds

Marketed anti-HIV compounds were extracted and HPLC purified from the commercial formulation. Emivirine was synthesized and purified according to published methods (28).

Results

Lead optimization process

Compounds were simultaneously optimized for activity against wild type HIV-1 and selected
5 NNRTI-resistant mutants and for increased metabolic stability (Table 1). Increased potency
against one NNRTI-resistant mutant generally correlated with increased potency against the other
mutants, but did not correlate with potency versus the wild-type virus. Whilst the activity of
TMC125 against wild-type virus was similar to the activity of the other compounds, it was more
active than the other tested compounds against all the NNRTI-resistant mutants. This increased
10 activity was particularly notable for the double mutant L100I + K103N. TMC125 was selected
for further study on the basis of its increased antiviral activity and of the improved metabolic
stability compared with the other compounds tested (Table 1).

Anti-HIV activity of TMC125

15 The activity of TMC125 against three different strains of HIV-1 (LAI, SF2 and the
monocytotropic HIV-1 Ba-L) and one strain of HIV-2 (ROD) was compared with the activity of
the three currently marketed NNRTIs: delavirdine, efavirenz and nevirapine (Table 2). The EC₅₀
values for TMC125 against HIV-1 LAI (in both MT4 cells and PBMC), HIV-1 SF and HIV-1
Ba-L were in the nanomolar range (1.4–4.8 nM) and were similar to those obtained for efavirenz
20 (1.0–3.4 nM). Delavirdine and nevirapine were at least ten-fold less potent than TMC125 or
efavirenz against HIV-1 in all the virus–cell combinations tested. In contrast to the other three
NNRTIs tested, TMC125 also showed activity in the micromolar range against HIV-2 (EC₅₀ 3.5

μM). In this assay, TMC125 did not show cytotoxicity ($\text{CC}_{50} > 100 \mu\text{M}$) as opposed to efavirenz ($\text{CC}_{50} = 42 \mu\text{M}$).

Activity of TMC125 against NNRTI-resistant viruses

5 An initial screen for activity against NNRTI-resistant HIV-1 was carried out on a panel of viruses constructed by site-directed mutagenesis to carry a selection of single ($n = 20$) and double ($n = 5$) RT amino acid substitutions known to be associated with resistance to marketed and investigational NNRTIs. When compared to currently prescribed NNRTIs, TMC125 showed excellent activity against this panel of mutant viruses with EC_{50} values below 5 nM (Fig 2) and
10 fold change in EC_{50} values below four for 18 of the viruses, including four carrying double amino acid substitutions. TMC125 EC_{50} values above 10 nM and fold resistance values greater than ten were observed for only three of the viruses tested (L100I + K103N, Y181I and F227C). These 3 variants are present at very low frequency in the clinical population. An analysis of a database of over 74,000 clinical isolates showed that the prevalence of Y181I is 0.51%, that of F227C is
15 nearly 0 (17 isolates) and the combination of L100I and K103N is present in only 3% of these isolates.

To further evaluate the antiviral activity of TMC125 on the virus populations currently present in patients, drug sensitivity assays were performed on a broad, random selection of recent clinical
20 samples. A total of 2,157 samples were tested, of which 1,081 (50.1%) were resistant to at least one NNRTI (\geq ten-fold change in EC_{50} as compared to wild type). The distribution of the EC_{50} concentrations within the resistant samples is illustrated in Figure 3, which compares TMC125 and the three approved NNRTIs. TMC125 inhibited 98% of all samples, and 97% of the NNRTI-

resistant strains, with an EC_{50} below 100 nM. For efavirenz, a compound equally potent against wild-type HIV-1, these percentages were 77% and 54%, respectively. Moreover TMC125 inhibited 77% of NNRTI-resistant strains with an EC_{50} below 10 nM, whereas this was the case for only 23% for efavirenz.

5

Activity of TMC125 against a range of HIV-1 subtypes

TMC125 was tested in parallel with nevirapine, delavirdine and efavirenz for activity against a panel of 32 clinically derived recombinant viruses (M.-P. de Béthune, K. Hertogs, L. Heyndrickx, J. Vingerhoets, K. Fransen, H. Azijn, L. Michiels, W. Janssens, A. Scholliers, B. Larder, S. Bloor, R. Pauwels and G. van der Groen. 3rd Int. Workshop on HIV Drug Resistance, Treatment Strategies and Eradication, Abstr. 18, 1999). These viruses represented HIV-1 group M subtypes A, B, C, D, F and H as well as circulating recombinant forms (CRF) CRF01_AE, CRF02_AG, CRF05_DF and HIV-1 group O. The strains were from different geographical origins. The results are summarized in Table 3. All the group M viruses tested were sensitive to TMC125 with EC_{50} values below 5 nM and fold change in EC_{50} values below 4. Seven of the group M viruses carried mutations in the RT coding region at positions associated with NNRTI resistance (positions 98, 101, 106 and 179). Except for one virus carrying a V179I substitution, which showed slightly reduced sensitivity to delavirdine (5-fold change), all these viruses remained sensitive to all the inhibitors tested. The one group O virus tested naturally harbored amino acids at positions 98 (G), 179 (E) and 181 (C), which in group M HIV-1 strains are associated with NNRTI resistance. This virus displayed significantly reduced sensitivity to nevirapine (89-fold change), delavirdine (144-fold change) and efavirenz (42-fold change) but only moderately reduced sensitivity to TMC125 (10-fold change).

Mechanism of action assays

A time of addition study was carried out in order to define the stage in the HIV life cycle at which TMC125 acts. The binding inhibitor DS5000, the NRTI zidovudine, the PI saquinavir and the NNRTI efavirenz were used as controls. DS5000 had no inhibitory effect when added 1 hour post-infection, whereas zidovudine lost activity from 4 hours post infection on. Saquinavir, which acts at the end of the viral life cycle, retained activity even when added 20 hours post-infection (data not shown). The time of addition versus activity profile for TMC125 was very similar to that of efavirenz, and did not overlap with zidovudine, with a significant loss of activity when drug addition was delayed for 7 hours post-infection. In a single experiment measuring the effect of the compounds on RT enzymatic activity, TMC125 gave an IC_{50} of 38 nM, in comparison to an IC_{50} for efavirenz of 16 nM.

Metabolic stability assays

The metabolic stability of compounds was tested by incubation with human microsomal fractions. The residual compound remaining was determined by LC-MS and residual anti-HIV activity was assessed in an *in vitro* assay. The results of the LC-MS analysis correlated well with those of the antiviral assay (Table1). While compound 6 (the lead compound) was rapidly degraded, minimal degradation was seen for TMC125 after 30 minutes of incubation with no reduction in concentration and only a 2% reduction in antiviral activity (data not shown). After 120 minutes there was a 15% decrease in TMC125 concentration and a 7% decrease in antiviral activity.

Antiviral activity of TMC125 in the presence of human serum proteins

In uninfected individuals the average HSA concentration is 40 mg/ml and the AAG concentration ranges from 0.4 to 0.8 mg/ml. Studies have shown that AAG levels in HIV-infected individuals may be elevated to 0.8–1.8 mg/ml (24). For this reason, a functional assay was performed to
5 determine the antiviral activity of TMC125 against HIV-1 LAI in the presence of 45 mg/ml HSA or 1 mg/ml AAG as well as 50% human serum. The ratio between the EC_{50} in the presence of the protein and the EC_{50} in the absence of the protein was calculated (Table 4). The antiviral effect of TMC125 was not significantly affected by presence of 50% human serum (two-fold increase in EC_{50}) or by the presence of HSA (three-fold increase in EC_{50}). AAG had no measurable affect on
10 the activity of TMC125. Nevirapine gave similar results whereas the activity of efavirenz was significantly reduced in the presence of HSA (20-fold increase in EC_{50}), as described previously (17).

Combination assays

15 Both the positive control zidovudine/emivirine and the combination of TMC125/zidovudine gave a mean CI of less than 0.8 (0.63 and 0.72, respectively), suggesting synergy between TMC125 and zidovudine. All other combinations tested gave mean CIs that were between 0.8 and 1.2 (data not shown) indicating an additive effect with no evidence of synergy or antagonism.

Discussion

We have applied a novel parallel screening strategy to simultaneously optimize a series of compounds for activity against wild type HIV-1, selected NNRTI mutants, and increased metabolic stability. Interestingly, increased potency against the wild type virus was not predictive
5 for increased potency against NNRTI mutants. This highlights the importance of including NNRTI mutants in first line screening protocols to facilitate the identification and selection of broad-spectrum NNRTIs.

TMC125 was found to be a highly potent inhibitor of HIV-1, with activity in the nanomolar
10 range comparable to that of the commonly prescribed NNRTI, efavirenz. Unlike efavirenz and the majority of marketed and investigational NNRTIs, TMC125 also showed some activity against HIV-2, which, while not clinically relevant, is a reflection of its broader spectrum of activity. Cytotoxicity of TMC125 was low in a variety of human cell lines (data not shown) resulting in a selectivity index of $> 70,000$ (HIV-1 LAI in MT4 cells). Mechanism of action
15 studies confirmed that the activity of TMC125 is principally due to the inhibition of HIV RT. TMC125 acted at the same stage of replication as efavirenz and was an active inhibitor of recombinant HIV-1 RT with an IC_{50} comparable to that of efavirenz. No inhibitory activity of TMC125 was seen in assays monitoring the entry of HIV into the host cell or the enzymatic activity of HIV-1 PR (data not shown).

20 Since orally administered drugs pass through the liver before reaching the systemic circulation, molecules stable in the presence of liver enzymes are more likely to exhibit higher systemic exposure on oral administration than molecules that are rapidly metabolized. The most important

metabolic pathway is the cytochrome P450 system (CYP), found in high concentrations on the endoplasmic reticulum of the hepatocytes. The stability of compounds in the presence of human liver microsomes should be considered early in any drug selection procedure. The limited decrease in concentration (15%) and in antiviral activity (7%) measured after 120 minutes of incubation of TMC125 with human liver microsomal fractions suggests a slow hepatic metabolism and therefore good metabolic stability.

Binding of some antiretrovirals to human serum proteins is known to inhibit their entry into cells (4) and can reduce their activity *in vivo*. TMC125, as other NNRTIs, is highly bound to human serum proteins (> 99%, unpublished observations). However, in the functional assay described in this paper, the activity of TMC125 was not significantly affected by the presence of either human serum proteins at physiological concentrations (1 mg/ml AAG or 45 mg/ml HSA) or human serum (50%). Of the other NNRTIs tested, nevirapine showed a similar pattern in the presence of protein, while the activity of efavirenz was strongly affected.

Although the HIV-1 group M subtype B is predominant in Europe and the United States, recent studies have indicated that the prevalence of non-B subtypes and circulating recombinant forms is increasing to significant levels, particularly in immigrants and heterosexually infected individuals (3,27). For this reason it is essential to determine a drug candidate's activity against other subtypes and groups. TMC125 showed comparable activity to a range of clinically derived recombinant viruses representing HIV-1 group M subtypes A through H, including several circulating recombinant forms. TMC125 also showed significantly improved activity against Group O viruses when compared with the other NNRTIs tested.

Combination therapy is the current standard of care for antiretroviral therapy. Since mixtures of antiretroviral agents may be synergistic, additive or antagonistic it is important to test compounds of interest in combination with all the currently prescribed antiretrovirals. Zidovudine has been previously shown to act in synergy with other NNRTIs, for example, delavirdine (6) and emivirine (5). TMC125 in combination with zidovudine was synergistic whilst all other combinations tested (NRTI, NNRTI and PI) were additive with no evidence of antagonism.

One of the greatest challenges facing clinicians treating HIV today is overcoming the increasing levels of resistance and cross-resistance that develop at each successive therapeutic failure. The extensive cross-resistance that exists between currently approved NNRTIs has severely limited the sequential use of these potent agents. While not fully predictive of the *in vivo* situation, *in vitro* selection of resistant HIV is a valuable tool for better understanding of emerging resistance and drug susceptibility. *In vitro* selection for TMC125 resistance at both a high and low MOI is in progress and the results of these selection experiments will be published separately. In the meantime we have assessed the potential activity of TMC125 against a very large pool of NNRTI-resistant viruses, generated by site-directed mutagenesis or clinically derived. The data presented here show that TMC125 remains active against a broad panel of NNRTI-resistant recombinant viruses chosen to represent a range of mutations selected by currently marketed and investigational NNRTIs. For example, L234I, F227L and V106A + F227L are associated with resistance to capravirine (formerly S-1153 and AG-1549) whilst V106A, E138K and F227L are associated with resistance to the new GlaxoSmithKline benzophenone compounds (GW4751, GW4511 and GW3011) (J. Chan, R. Ferris, G. Roberts, S. Short, K. Weaver, R. Hazen, K.

Creech, M. St Clair, R. Dornsife, G. Freeman, J. Tidwell, K. Romines, L. Schaller, J. Cowan, L. Boone. Abstr. 10th Conf. Retrovir. Opport. Infect., abstr. 6, 2003). Importantly, TMC125 inhibited not only viruses with single amino acid substitutions in the RT enzyme but also several common double mutants such as K101E + K103N and K103N + Y181C (8,9,23). Activity was slightly reduced against virus carrying L100I + K103N (EC_{50} 19.4 nM), but to a much lesser extent than the activity of the other NNRTIs tested, leaving the potential for *in vivo* efficacy. High throughput cell-based screening assays allowed for the testing of TMC125 against a panel of more than 1000 NNRTI-resistant recombinant viruses derived from recent clinical samples. TMC125 showed a high level of activity against this panel of resistant viruses, with EC_{50} values of less than 100 nM for 97% of the samples. Attempts to crystallize TMC125 bound to wild-type RT have failed so far due to poor resolution (K. Das, A.D. Clark, P.L. Boyer, D.W. Ludovici, M.-P. de Béthune., K. Andries, P. Lewi, E. Arnold, S.H. Hughes, B.L. De Corte, R.W. Kavash, M.J. Kukla, R. Pauwels, M. de Jonge, F. Daeyaert, L. Koymans, M. Vinkers, J. Heeres, P.A. Janssen. Abstr. 10th Conf. Retrovir. Opport. Infect., abstr. 613, 2003). A crystal structure has been obtained with the K103N mutant in which the mode of binding of TMC125 appears to be different from other DAPY and DATA compounds. The lack of success with crystallizing TMC125 with the wild-type enzyme is possibly due to the drug having several alternative docking modes in the wild-type enzyme. Interestingly, this binding mode flexibility may explain the drug's potent activity against resistant viruses.

We have shown in a 7 day Phase IIa proof of concept study (TMC125-C208) that treatment with TMC125 of antiretroviral-naive HIV-infected subjects resulted in a very rapid decline in viral load (mean 1.99 \log_{10} decrease in HIV-1 RNA) when compared to placebo treatment. Two of 12

TMC125 treated subjects reached a viral load of less than 50 HIV-1 RNA copies/ml and 8 of 12 reached a viral load of less than 400 copies/ml (14). The excellent *in vitro* activity of TMC125 against a wide range of NNRTI-resistant viruses suggests that this compound could be used to treat NNRTI-experienced patients. This is the first time that it has been considered possible to effectively use an NNRTI sequentially following NNRTI failure. This potential has been confirmed in a separate clinical study (TMC125-C207) in which 16 subjects who were failing on an NNRTI-containing regimen and whose virus displayed between 10- and 500-fold resistance to efavirenz had their NNRTI substituted for TMC125. Treatment with TMC125 was associated with a rapid and substantial drop in viral load (median 0.89 log₁₀ decrease in HIV-1 RNA over 7 days)(13).

We conclude that TMC125 is a potent next generation NNRTI that holds promise for the treatment of NNRTI-resistant virus. Our results validate the parallel screening approach that was designed and applied to the identification of TMC125 as a drug candidate. TMC125 has the potential to significantly improve treatment options for HIV-infected individuals.

Acknowledgements

We thank Hilde Bohets for performing the metabolic stability experiments, Dirk Jochmans for the combination experiments, Chih Y Ho and Robert W Kavash for the synthesis of the compound and Carlo F Perno for the assessment of the activity of TMC125 against HIV-1/BaL in

5 primary monocytes/macrophages.

References

1. **Antinori, A., M. Zaccarelli, A. Cingolani, F. Forbici, M. G. Rizzo, M. P. Trotta, S. Di Giambenedetto, P. Narciso, A. Ammassari, E. Girardi, A. De Luca, and C. F. Perno.** 2002. Cross-resistance among nonnucleoside reverse transcriptase inhibitors limits recycling efavirenz after nevirapine failure. *AIDS Res. Hum. Retroviruses* **18**:835–838.
2. **Bachelor, L., S. Jeffrey, G. Hanna, R. D'Aquila, L. Wallace, K. Logue, B. Cordova, K. Hertogs, B. Larder, R. Buckery, D. Baker, K. Gallagher, H. Scarnati, R. Tritch, and C. Rizzo.** 2001. Genotypic correlates of phenotypic resistance to efavirenz in virus isolates from patients failing nonnucleoside reverse transcriptase inhibitor therapy. *J. Virol.* **75**:4999–5008.
3. **Balotta, C., G. Facchi, M. Violin, S. Van Dooren, A. Cozzi-Lepri, F. Forbici, A. Bertoli, C. Riva, D. Senese, P. Caramello, G. Carnevale, G. Rizzardini, L. Cremonini, L. Monno, G. Rezza, C. F. Perno, G. Ippolito, A. d'Arminio-Monforte, A. M. Vandamme, and M. Moroni.** 2001. Increasing prevalence of non-clade B HIV-1 strains in heterosexual men and women, as monitored by analysis of reverse transcriptase and protease sequences. *J. Acquir. Immune Defic. Syndr.* **27**:499–505.
4. **Bilello, J. A., P. A. Bilello, K. Stellrecht, J. Leonard, D. W. Norbeck, D. J. Kempf, T. Robins, and G. L. Drusano.** 1996. Human serum alpha 1 acid glycoprotein reduces uptake, intracellular concentration, and antiviral activity of A-80987, an inhibitor of the human immunodeficiency virus type 1 protease. *Antimicrob. Agents Chemother.* **40**:1491–1497.
5. **Bohets, H., K. Lavrijsen, J. Hendrickx, J. van Houdt, V. van Genechten, P. Verboven, W. Meuldermans, and J. Heykants.** 2000. Identification of the cytochrome P450 enzymes

- involved in the metabolism of cisapride: in vitro studies of potential co-medication interactions. *Br. J. Pharmacol.* **129**:1655–1667.
6. **Brennan, T. M., D. L. Taylor, C. G. Bridges, J. P. Leyda, and A. S. Tyms.** 1995. The inhibition of human immunodeficiency virus type 1 in vitro by a non-nucleoside reverse transcriptase inhibitor MKC-442, alone and in combination with other anti-HIV compounds. *Antiviral Res.* **26**:173–187.
 7. **Chou, T. C. and P. Talalay.** 1984. Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. *Adv. Enzyme Regul.* **22**:27–55.
 8. **Deeks, S. G.** 2001. International perspectives on antiretroviral resistance. Nonnucleoside reverse transcriptase inhibitor resistance. *J. Acquir. Immune Defic. Syndr.* **26 (Suppl 1)**:S25–33.
 9. **Delaugerre, C., R. Rohban, A. Simon, M. Mouroux, C. Tricot, R. Agher, J. M. Huraux, C. Katlama, and V. Calvez.** 2001. Resistance profile and cross-resistance of HIV-1 among patients failing a non-nucleoside reverse transcriptase inhibitor-containing regimen. *J. Med. Virol.* **65**:445–448.
 10. **Delwart, E. L., E. G. Shpaer, J. Louwagie, F. E. McCutchan, M. Grez, H. Rubsamen-Waigmann, and J. I. Mullins.** 1993. Genetic relationships determined by a DNA heteroduplex mobility assay: analysis of HIV-1 env genes. *Science* **262**:1257–1261.
 11. **Division of AIDS, National Institute of Allergy and Infectious Diseases.** DAIDS Virology Manual for HIV Laboratories. Publication NIH-97-3828. Publication NIH-97-3828. 1997. U.S. Department of Health and Human Services, Washington. D.C.
 12. **Fields, R. D. and M. V. Lancaster.** 1993. Dual-attribute continuous monitoring of cell proliferation/cytotoxicity. *Am. Biotechnol.Lab.* **11**:48–50.

13. **Gazzard, B., A. Pozniak, W. Rozenbaum, P. Yeni, S. Staszewski, K. Arasteh, K. De Dier, M. Peeters, B. Woodfall, J. Stebbing, G. A. E. van't Klooster.** 2003. An open-label assessment of TMC 125—a new, next generation NNRTI, for 7 days in HIV-1 infected individuals with NNRTI resistance. *AIDS* **17**: F49–F54.
14. **Gruzdev, B., A. Rakhmanova, E. Doubovskaya, A. Yakovlev, M. Peeters, A. Rinehart, K. De Dier, P. Baede-Van Dijk, W. Parys, and G. van 't Klooster.** 2003. A randomized, double-blind, placebo-controlled trial of TMC125 as 7-day monotherapy in antiretroviral naïve, HIV-1 infected subjects. *AIDS* **17**:2487–2494.
15. **Harrigan, P. R. and B. A. Larder.** 2002. Extent of cross-resistance between agents used to treat human immunodeficiency virus type 1 infection in clinically derived isolates. *Antimicrob. Agents Chemother.* **46**:909–912.
16. **Hertogs, K., M. P. de Bethune, V. Miller, T. Ivens, P. Schel, A. Van Cauwenberge, C. Van Den Eynde, V. Van Gerwen, H. Azijn, M. Van Houtte, F. Peeters, S. Staszewski, M. Conant, S. Bloor, S. Kemp, B. Larder, and R. Pauwels.** 1998. A rapid method for simultaneous detection of phenotypic resistance to inhibitors of protease and reverse transcriptase in recombinant human immunodeficiency virus type 1 isolates from patients treated with antiretroviral drugs. *Antimicrob. Agents Chemother.* **42**:269–276.
17. **Corbett J.W., S.S. Ko, J.D. Rodgers, S. Jeffrey, L.T. Bachelier, R.M. Klabe, S. Diamond, C-M. Lai, S.R. Rabel, J.A. Saye, S.P. Adams, G.L. Trainor, P.S. Anderson, and S.K. Erickson-Viitanen.** 1999. Expanded-Spectrum Nonnucleoside Reverse Transcriptase Inhibitors Inhibit Clinically Relevant Mutant Variants of Human Immunodeficiency Virus Type 1. *Antimicrob. Agents Chemother.* **43**:2893-2897.

18. **Larder, B. A., A. Kohli, P. Kellam, S. D. Kemp, M. Kronick, and R. D. Henfrey.** 1993. Quantitative detection of HIV-1 drug resistance mutations by automated DNA sequencing. *Nature* **365**:671–673.
19. **Leigh Brown, A. J., S. D. Frost, W. C. Mathews, K. Dawson, N. S. Hellmann, E. S. Daar, D. D. Richman, and S. J. Little.** 2003. Transmission fitness of drug-resistant human immunodeficiency virus and the prevalence of resistance in the antiretroviral-treated population. *J. Infect. Dis.* **187**:683–686.
20. **Ludovici, D. W., B. L. De Corte, M. J. Kukla, H. Ye, C. Y. Ho, M. A. Lichtenstein, R. W. Kavash, K. Andries, M. P. de Bethune, H. Azijn, R. Pauwels, P. J. Lewi, J. Heeres, L. M. Koymans, M. R. de Jonge, K. J. Van Aken, F. F. Daeyaert, K. Das, E. Arnold, and P. A. Janssen.** 2001. Evolution of anti-HIV drug candidates. Part 3: Diarylpyrimidine (DAPY) analogues. *Bioorg. Med. Chem. Lett.* **11**:2235–2239.
21. **Ludovici, D. W., R. W. Kavash, M. J. Kukla, C. Y. Ho, H. Ye, B. L. De Corte, K. Andries, M. P. de Bethune, H. Azijn, R. Pauwels, H. E. Moereels, J. Heeres, L. M. Koymans, M. R. de Jonge, K. J. Van Aken, F. F. Daeyaert, P. J. Lewi, K. Das, E. Arnold, and P. A. Janssen.** 2001. Evolution of anti-HIV drug candidates. Part 2: Diaryltriazine (DATA) analogues. *Bioorg. Med. Chem. Lett.* **11**:2229–2234.
22. **Ludovici, D. W., M. J. Kukla, P. G. Grous, S. Krishnan, K. Andries, M. P. de Bethune, H. Azijn, R. Pauwels, E. De Clercq, E. Arnold, and P. A. Janssen.** 2001. Evolution of anti-HIV drug candidates. Part 1: From alpha-anilinophenylacetamide (alpha-APA) to imidoyl thiourea (ITU). *Bioorg. Med. Chem. Lett.* **11**:2225–2228.
23. **Moyle, G.** 2001. The emerging roles of non-nucleoside reverse transcriptase inhibitors in antiretroviral therapy. *Drugs* **61**:19–26.

24. **Oie, S., M. A. Jacobson, and D. I. Abrams.** 1993. Alpha 1-acid glycoprotein levels in AIDS patients before and after short-term treatment with zidovudine (ZDV). *J. Acquir. Immune Defic. Syndr. Hum. Retrovirol.* **6**:531–533.
25. **Pauwels, R., J. Balzarini, M. Baba, R. Snoeck, D. Schols, P. Herdewijn, J. Desmyter, and E. De Clercq.** 1988. Rapid and automated tetrazolium-based colorimetric assay for the detection of anti-HIV compounds. *J. Virol. Methods* **20**:309–321.
26. **Perno, C. F., and R. Yarchoan.** 1993. Culture of HIV in monocytes and macrophages. pp. 12.4.1–12.4.11. *In* Coligan JE, Kruisbeek AM, Margulies DH, Shevach EM, and Strober W (eds.), *Current protocols in immunology*. Vol 3. John Wiley & Sons, New York.
27. **Perrin, L., L. Kaiser, and S. Yerly.** 2003. Travel and the spread of HIV-1 genetic variants. *Lancet Infect. Dis.* **3**:22–27.
28. **Tanaka, H., H. Takashima, M. Ubasawa, K. Sekiya, N. Inouye, M. Baba, S. Shigeta, R. T. Walker, E. De Clercq, and T. Miyasaka.** 1995. Synthesis and antiviral activity of 6-benzyl analogs of 1-[(2-Hydroxyethoxy)methyl]-5-(phenylthio)thymine (HEPT) as potent and selective anti-HIV-1 agents. *J. Med. Chem.* **38**:2860–2865.

Tables and Figures

FIG 1. Chemical structure of TMC125. Chemical formula: $C_{20}H_{15}BrN_6O$ or 4-[[6-amino-5-bromo-2-[(4-cyanophenyl)amino]-4-pyrimidinyl]oxy]-3,5-dimethylbenzonitrile.

FIG 2. Antiviral activity of TMC125 and current NNRTIs tested against a panel of 25 HIV-1 strains harboring one or two NNRTI resistance associated mutations. HIV-1 strains were constructed by site-directed mutagenesis as described in Materials and Methods. NVP, nevirapine; DLV, delavridine, EFV, efavirenz.

FIG 3. Antiviral activity of different NNRTIs tested against a panel of 1,081 resistant recombinant clinical isolates. Black shading, $EC_{50} > 100\text{nM}$; grey shading, $EC_{50} < 100 \text{ nM} > 10 \text{ nM}$; white, $EC_{50} < 10 \text{ nM}$. NVP, nevirapine; DLV, delavridine, EFV, efavirenz.

TABLE 1. Antiviral activity and metabolic stability of ITU, DATA and DAPY analogs

Compound ^a	EC ₅₀ (nM)								
	6 (II)	14f (II)	21a (II)	13a (III)	20a (II)	13c (III)	20c (III)	15b (III)	TMC125
wild type HIV-1	0.006	0.002	0.001	0.001	0.0003	0.0004	0.003	0.001	0.001
L100I	0.398	0.107	0.037	0.018	0.013	0.034	0.017	0.007	0.003
K103N	0.040	0.013	0.002	0.004	0.003	0.002	0.006	0.001	0.001
Y181C	0.200	0.038	0.011	0.008	0.008	0.007	0.032	0.022	0.007
Y188L	0.316	0.026	0.124	0.048	0.040	0.008	0.034	0.006	0.005
L100I + K103N	>10.0	5.0	1.0	>10.0	1.26	1.09	0.32	0.049	0.019
K103N + Y181C	1.4	0.353	0.037	0.044	0.050	0.037	0.114	0.025	0.004
Percent decrease in parent compound after 2 hour incubation with human or rat microsomes									
Human ^b	97%	89%	74%	36%	69%	44%	nt	-7%	7%
Human ^c	99%	91%	76%	38%	48%	36%	nt	nt	15%
Rat ^b	78%	44%	56%	23%	18%	25%	nt	36%	34%
Rat ^c	93%	59%	60%	42%	20%	50%	nt	nt	35%

^a compound numbers refer to the numbers in the publications by Ludovici et al (20–21). The roman numerals in parentheses indicate the specific publication in which the compound was introduced (II, reference 21; III, reference 20).

^b as assayed by bioassay for residual anti-HIV activity.

^c as assessed by liquid chromatography – mass spectrometry.

ITU, imidoyl thiourea; DATA, diaryltriazines; DAPY, diarylpyrimidine; nt, not tested

TABLE 2. Anti-HIV activity of TMC125 in comparison with other NNRTIs

Compound	Median EC ₅₀ , nM (range) ^a					
	MT4 CC ₅₀ (μM)	MT4		PBMC		M/M
		LAI (HIV-1)	ROD (HIV-2)	LAI (X4)	SF2 (X4 – R5)	Ba-L (R5)
Nevirapine	> 100	76.3 (30.8–202.0)	> 100,000	22.1	129.8	45.0 (18.0–65.0)
Delavridine	73	16.1 (8.3–531.8)	> 100,000	nd	nd	nd
Efavirenz	42	1.0 (0.2–3.2)	> 41,000	1.2	3.4	2.0 (0.9–3.0)
TMC125	> 100	1.4 (0.3–1.6)	3,479	1.4	4.8	2.0 (0.8–4.0)

^a Median and range of several determinations for MT4 LAI and M/M Ba-L experiments. For MT4 ROD and PBMC experiments the result of a single determination is shown.

NNRTI, non-nucleoside reverse transcriptase inhibitor; PBMC, peripheral blood mononuclear cell; M/M, monocyte/macrophage; nd, not determined.

TABLE 3. Activity of NNRTIs against recombinant viruses of different subtypes

pol subtype ^a	n ^b	NVP		DLV		EFV		TMC125	
		EC ₅₀ ^c	FC ^d	EC ₅₀	FC	EC ₅₀	FC	EC ₅₀	FC
CRF02_AG	4	13 (5.6-19)	0.75 (0.4-1.3)	9.3 (5.6-16)	1.1 (0.6-1.9)	0.7 (<0.3-1.1)	0.9 (<0.4-1.2)	1.3 (0.9-1.9)	1.1 (0.7-1.5)
CRF01_AE	5	19 (12-98)	1.3 (0.8-7)	29 (20-170)	3.3 (2.3-20)	1.2 (0.6-1.9)	1.3 (0.7-2.1)	1.8 (1-4.3)	1.4 (0.8-3.3)
B	8	29 (11-70)	2.1 (0.8-5)	28 (5.7-52)	3.2 (0.7-6)	1.2 (<0.3-2.6)	1.1 (0.3-2.9)	1.5 (0.9-3)	1.2 (0.7-2.3)
C	4	13 (7.7-53)	0.9 (0.6-3.8)	34 (22-46)	3.9 (2.6-5.3)	0.6 (<0.3-1.4)	0.6 (0.4-1.5)	1.5 (1.0-2.4)	1.2 (0.8-1.9)
D	3	12 (7.0-15)	0.9 (0.5-1.0)	9.7 (7.7-20)	1.1 (0.9-2.3)	0.4 (<0.3-0.4)	0.4 (0.3-0.5)	1.7 (1.2-1.7)	1.3 (1.0-1.3)
CRF05_DF	3	33 (18-43)	1.7 (0.9-3.1)	6.1 (5.6-12)	0.6 (0.6-1.3)	0.4 (<0.3-0.4)	0.8 (0.3-0.9)	0.9 (0.8-1.0)	0.7 (0.7-0.8)
F	1	30	2.1	27	3.1	0.9	1.0	1.3	1.0
H	3	15 (5.0-19)	1.1 (0.4-1.4)	18 (2.8-22)	2.0 (0.3-2.6)	0.6 (<0.3-0.7)	0.5 (0.3-0.8)	1.1 (0.9-1.8)	0.9 (0.7-1.4)
O	1	>1,300	89	>1,300	140	38	42	13	9.9

^a Polymerase subtype as determined by full sequencing of the protease and reverse transcriptase (first 400 amino acids) genes. For some of the strains, mutations at amino acid positions implicated in NNRTI resistance were found; subtype B, two strains (A98S/A + K101R and V179I); subtype CRF05_DF, two strains (V106I and V179I); subtype H, three strains (V179I and twice K101Q + V179I); subtype O, one strain (A98G + Y179E + Y181C).

^b n = number of strains tested in each subtype

^c EC₅₀ = median (range) EC₅₀ expressed in nM.

^d FC, fold change in EC₅₀, calculated as the EC₅₀ for the tested virus divided by the EC₅₀ for the wild-type virus (HIV-1 HXB2) tested in parallel, according to the Antivirogram™ method.

TABLE 4. Influence of human serum proteins on the anti-HIV potency of selected NNRTIs

Compound	Median ^a EC ₅₀ with human serum protein / EC ₅₀ in 10% FCS		
	AAG 1 mg/ml	HSA 45 mg/ml	HS 50%
Nevirapine	1	3	1
Efavirenz	4	20	6
TMC125	1	3	2

^a Median of at least four determinations.

NNRTI, non-nucleoside reverse transcriptase inhibitor; FCS, fetal calf serum; AAG, α_1 -acid glycoprotein; HSA, human serum albumin; HS, human serum.





