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A Novel Nonnucleoside Analogue That Inhibits Human Immunodeficiency Virus Type 1 Isolates Resistant to Current Nonnucleoside Reverse Transcriptase Inhibitors[▽]

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Nonnucleoside reverse transcriptase (RT) inhibitors (NNRTIs) are important components of current combination therapies for human immunodeficiency virus type 1 (HIV-1) infection. However, their low genetic barriers against resistance development, cross-resistance, and serious side effects can compromise the benefits of the two current drugs in this class (efavirenz and nevirapine). In this study, we report a novel and potent NNRTI, VRX-480773, that inhibits viruses from efavirenz-resistant molecular clones and most NNRTI-resistant clinical HIV-1 isolates tested. In vitro mutation selection experiments revealed that longer times were required for viruses to develop resistance to VRX-480773 than to efavirenz. RT mutations selected by VRX-480773 after 3 months of cell culture in the presence of 1 nM VRX-480773 carried the Y181C mutation, resulting in a less-than-twofold increase in resistance to the compound. A virus containing the double mutation V106I-Y181C emerged after 4 months, causing a sixfold increase in resistance. Viruses containing additional mutations of D123G, F227L, and T369I emerged when the cultures were incubated with increasing concentrations of VRX-480773. Most of the resistant viruses selected by VRX-480773 are susceptible to efavirenz. Oral administration of VRX-480773 to dogs resulted in plasma concentrations that were significantly higher than those required for the inhibition of wild-type and mutant viruses. These results warrant further clinical development of VRX-480773 for the treatment of HIV infection in both NNRTI-naive and -experienced patients.

Standard HIV therapies consist of combinations of nucleoside reverse transcriptase (RT) inhibitors (NRTIs) and nonnucleoside reverse transcriptase inhibitors (NNRTIs) or protease inhibitors. Although they are generally effective and have resulted in reduced AIDS-related morbidity and mortality, none of them is curative. Treatment failures often occur when viruses that are resistant to one or more components of the regimens arise. Compared to the large numbers of drugs in the NRTI and protease inhibitor classes, the NNRTI class has only two drugs (efavirenz and nevirapine) in extensive use; delavirdine is rarely used because of its low efficacy and its three-times-per-day dosing requirement. There are two important issues that impact the continued use of efavirenz or nevirapine and call for newer drugs in this class: a low genetic barrier against resistance development and cross-resistance among approved NNRTIs (2).

In this report, we characterize a novel and potent nonnucleoside RT inhibitor of human immunodeficiency virus type 1 (HIV-1), designated VRX-480773, that resulted from lead optimization of a substituted triazole discovered from high-throughput screenings (6). It inhibits HIV-1 derived from the molecular clones carrying the RT mutations commonly observed in plasma samples of patients who failed efavirenz treatment. More importantly, VRX-480773 exhibits activity superior to those of efavirenz and nevirapine against a majority of clinical NNRTI-resistant HIV-1 isolates. In addition, VRX-480773 seems to impose a higher genetic barrier for resistance development than does efavirenz. A majority of the viruses selected by VRX-480773 can be inhibited by efavirenz, indicating that there is a low level of cross-resistance between these two NNRTIs. Pharmacokinetic analysis in dogs showed that it is orally bioavailable and reaches plasma concentrations above the 50% effective concentration (EC_{50}) for both wild-type (wt) and mutant viruses. These data warrant further clinical development of VRX-480773 for its use in both naïve and NNRTI-experienced patients infected with HIV-1.

MATERIALS AND METHODS

Compounds. VRX-387902 [*N*-(2-bromo-4-methylphenyl)-2-(5-methyl-4-phenyl-4*H*-1,2,4-triazol-3-ylthio)acetamide] was purchased from SPECS (Rijswijk, The Netherlands). VRX-480773 {2-[5-bromo-4-(4-cyclopropylnaphthalen-1-yl)-4*H*-1,2,4-triazol-3-ylthio]-*N*-(2-chloro-4-sulfamoylphenyl)acetamide} was synthesized at Valeant Research and Development. Efavirenz was obtained from the NIH AIDS Research and Reference Reagent Program. All test compounds were prepared from dimethyl sulfoxide (DMSO) stocks.

Cell lines, plasmids, and virus isolates. HeLa-JC53 cells were obtained from David Kabat at the Oregon Health Sciences University (9). The HeLa-JC53-LTR-β-gal cell line was constructed by transduction of HeLa-JC53 cells with an HIV-1 vector carrying long terminal repeat (LTR)–β-galactosidase. The HeLa-JC53-LTR-Luci cell line was likewise constructed by transduction with an HIV-1 vector carrying LTR-luciferase.

The HIV-1 molecular clones pNL4-3 and pNL4-3.Luc.R−E− were obtained from the NIH AIDS Research and Reference Reagent Program (4). A panel of 20 HIV-1 isolates carrying NNRTI resistance mutations (Table 1) was con-

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TABLE 1. Activities of VRX-480773 against HIV-1 derived from molecular clones carrying RT mutations found in viruses from patients experiencing efavirenz treatment failure

RT mutation[a]	% of patients[b]	EC_{50} ± SD (nM)[c]		Mean change (fold) in EC_{50}[d]	
		VRX-480773	Efavirenz	VRX-480773	Efavirenz
wt		0.14 ± 0.032	0.17 ± 0.006		
K103N	88.5	0.09 ± 0.027	2.73 ± 1.774*	0.6	16.1
K103N-P225H	32.7	0.13 ± 0.000	34.88 ± 34.958*	0.9	205.2
K103N-V108I	28.8	0.14 ± 0.006	8.63 ± 0.750*	1.0	50.8
K103N-K101Q	16.3	0.15 ± 0.017	12.66 ± 4.328*	1.1	74.5
K103N-L100I	10.6	0.05 ± 0.023	258.37 ± 63.998*	0.4	1,519.8
G190S	10.6	0.23 ± 0.068	14.27 ± 9.963*	1.6	83.9
K103N-V108I-P225H	9.6	0.15 ± 0.017	77.06 ± 23.958*	1.1	458.3
Y188L	6.7	0.81 ± 0.026*	24.43 ± 8.130*	5.8	143.7
K101E	4.8	0.26 ± 0.025*	0.74 ± 0.275	1.9	4.4
K103N-F227L	4.8	0.08 ± 0.009	2.26 ± 1.178*	0.6	18.3
V106I-Y188L	4.8	2.27 ± 0.512*	135.57 ± 100.645*	16.2	797.5
G190A	3.8	0.19 ± 0.009	0.48 ± 0.238	1.4	2.8
K103N-Y188L	3.8	1.07 ± 0.138*	>2,500*	7.6	Max
K103N-G190A	3.8	0.32 ± 0.062*	25.14 ± 8.701*	2.3	147.9
K103N-K101Q-P225H	3.8	0.11 ± 0.007	78.39 ± 49.039*	0.8	461.1
K103N-V108I-A98G	3.8	0.24 ± 0.002	67.93 ± 18.038*	1.7	395.6
A98G	2.9	0.3 ± 0.055*	0.64 ± 0.123	2.1	3.8
K103N-Y181C	2.9	0.23 ± 0.025	4.24 ± 1.871*	1.6	24.9
V106I	1.9	0.09 ± 0.000	0.27 ± 0.056	0.6	1.6
Y181C	1.9	0.23 ± 0.029	0.33 ± 0.092	1.6	1.9

[a] RT mutations were built upon wt pNL4-3.Luc.R−E−.
[b] Appearance frequency among plasma HIV-1 samples from patients for whom combination therapies including efavirenz failed (1).
[c] From two tests. *, EC_{50} was significantly higher than that against wild-type virus ($P < 0.05$).
[d] Changes (*n*-fold) in EC_{50} relative to that against wt virus.

structed as described previously based on the molecular clone pNL4-3.Luc.R−E− (15).

A panel of 94 clinical HIV-1 isolates carrying the most prevalent NNRTI-resistant mutations (Table 2) and 5 non-B wt clinical HIV-1 isolates (belonging to clades A, AC, C, D, and F) were generated at Monogram Biosciences Inc. (formerly ViroLogic, Inc., South San Francisco, CA) (12, 14). HIV-$2_{(NIH-Z)}$ was obtained from ABI Advanced Biotechnologies Inc. (Columbia, MD).

In vitro anti-HIV-1 activity assays. The antiviral activity of VRX-480773 against NNRTI-resistant HIV-1 strains derived from both molecular clones and clinical isolates was evaluated. HeLa-JC53 cells were used for vesicular stomatitis virus G (VSV-G)-pseudotyped pNL4-3.Luc.R−E−-derived virus infections, while HeLa-JC53-LTR-Luci cells (expressing luciferase under the control of the HIV-1 LTR) were used for infections by HIV-2 (7, 9, 10, 13). Serial dilutions (1:4) of test compounds were performed in DMSO and then in culture medium (phenol red-free Dulbecco's modified Eagle's medium, 2% fetal bovine serum, and 1% penicillin-streptomycin) to yield a final DMSO concentration of 0.2% in the cell culture. Cells (15,000 cells in 85 μl of culture medium) were mixed with 5 μl of virus (multiplicity of infection of 0.03) and 10 μl of the serially diluted compounds in 96-well plates. All cultures were maintained at 37°C in 5% CO_2 for 48 h. Following incubation, an equal volume (100 μl) of Bright-Glo luciferase reagent (Promega, Madison, WI) was added to each well, and chemiluminescence was read on an LJL Analyst (LJL BioSystems, Sunnyvale, CA). EC_{50} values (defined as the concentration of an inhibitor required to inhibit luciferase activity by 50%) were determined by nonlinear regression using XLfit4 or GraphPad Prism 4. One-way analysis of variance to compare mutant EC_{50} values to wild-type virus EC_{50} values was performed with GraphPad Prism4 after logarithmic transformation of the EC_{50} values.

The antiviral activity of VRX-480773 against a panel of 94 clinical HIV-1 isolates carrying NNRTI-resistant mutations and 5 wt non-B clinical HIV-1 isolates (belonging to clades A, AC, C, D, and F) was evaluated by Monogram Biosciences Inc. (South San Francisco, CA) (8).

In vitro cellular toxicity assay. The cellular toxicity of VRX-480773 against a panel of tissue culture cell lines (HeLa, MT-2, SupT1, Panc 10.05, Hep3B, and ACHN) was determined by measuring cellular ATP levels in the presence of the compound. Cells (10,000 to 15,000 cells/well) were cultured with VRX-480773 in culture medium (phenol red-free Dulbecco's modified Eagle's medium or RPMI-1640 medium, 10% fetal bovine serum, and 1% penicillin-streptomycin) for 48 h at 37°C and 5% CO_2, after which time an equal volume of CellTiter-Glo reagent (Promega, Madison, WI) was added to each well, and chemiluminescence was determined using an LJL Analyst system. The CC_{50} was defined as the concentration of inhibitor required to decrease cellular ATP levels by 50%.

In vitro RT assay. HIV-1 RT was expressed in *Escherichia coli* and purified according to a procedure described previously by Boretto et al. (3). Expression plasmid p66RTB was a gift of B. Canard. Inhibition of HIV-1 RT was performed as described previously (15). Briefly, in vitro RT reactions were carried out for 1 h at 25°C in the presence of 16 μg/ml poly(rA)/oligo$(dT)_{18}$, 2 μM TTP (labeled with 0.5 μCi of α-^{33}P), 1 nM RT, and 0 to 100 μM inhibitor in a buffer containing 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 1 mM dithiothreitol, and 60 μg/ml bovine serum albumin. Equal volumes of 20% trichloroacetic acid–1% sodium pyrophosphate were added, and radioactivity in the precipitated product was analyzed. The 50% inhibitory concentration was defined as the concentration of inhibitor required to inhibit RT activity by 50%.

Selection and determination of VRX-480773 resistance mutations. SupT1 cells (2×10^6 cells in 1 ml of RPMI 1640 containing 10% fetal bovine serum) were exposed to wt NL4.3 virus (multiplicity of infection of 0.05) for 3 h. The virus culture was subsequently maintained in 1 ml of growth medium containing 1 nM VRX-480773 or 2 nM efavirenz. Every 3 to 4 days, 100 μl of infected culture was transferred into 900 μl of medium containing fresh drug and 9×10^5 SupT1 cells. Virus replication was monitored microscopically by observing the formation of syncytia. At each virus breakthrough (massive syncytium formation), the concentration of inhibitor was doubled. Culture media and cell pellets from each breakthrough point were collected. Cellular DNA was purified with a Wizard genomic DNA isolation kit (Promega, Madison, WI). The protease and RT coding regions of proviruses were amplified using high-fidelity *Pfu* Turbo DNA polymerase (Stratagene, La Jolla, CA) and cloned into the TOPO TA cloning vector (Invitrogen, Carlsbad, CA). The entire protease and RT coding sequences of breakthrough viruses were determined by sequencing 10 to 15 individually recovered PCR clones at each point.

Construction of VRX-480773-resistant viruses. The major patterns of HIV-1 RT mutations selected by VRX-480773 from the wt virus were engineered into the parental pNL4-3.Luc.R−E− molecular clone by exchanging a 1485-bp ApaI/AgeI RT fragment (nucleotides 2006 to 3490 in NL4.3) with recovered mutant PCR fragments. The resulting plasmids were sequenced throughout the insertion region. Virus stocks with the RT mutations were generated by transfection as previously described (15). The resulting viruses were tested for their susceptibilities to VRX-480773 and efavirenz.

Determination of replication capacities of VRX-480773-selected viruses. The replication capacities (RCs) of viruses carrying VRX-480773-selected RT muta-

TABLE 2. Activity of VRX-480773 against a panel of 94 clinical NNRTI-resistant HIV-1 isolates

Isolate	NNRTI mutation(s)	% of NNRTI-resistant isolates[a]	Change (fold) in EC_{50}[b]		
			VRX-480773	Efavirenz	Nevirapine
1	103N	21.571	0.7	14.0	51.0
2	103N, 181C	6.145	10.0	>100	>100
3	181C	4.941	10.0	6.7	>100
4	100I, 103N	4.498	0.9	>100	42.0
5	106I	3.611	0.9	0.7	1.1
6	98G	2.597	2.4	2.9	5.9
7	101Q	2.534	2.2	2.5	2.8
8	103N, 225H	2.407	1.6	>100	>100
9	103N, 225H	2.407	1.0	89.0	93.0
10	188L	2.312	11.0	86.0	>100
11	103N, 108I	2.249	1.3	>100	>100
12	179D	1.932	3.3	5.7	4.2
13	190A	1.457	13.0	18.0	>100
14	181C, 190A	1.457	>100	14.0	>100
15	103N, 190A	1.425	76.0	>100	>100
16	101E	1.362	11.0	16.0	42.0
17	108I	1.362	1.5	1.7	2.3
18	103N, 181C, 190A	1.172	>100	>100	>100
19	101E, 190A	1.109	83.0	>100	>100
20	103N, 238T	1.077	0.6	31.0	>100
21	98G, 103N	1.014	4.4	>100	>100
22	101Q, 103N	0.887	5.2	>100	>100
23	101P, 103N	0.855	2.2	>100	>100
24	179E	0.824	2.4	4.1	1.9
25	103N, 108I, 181C	0.697	6.2	72.0	>100
26	103N, 188L	0.665	3.9	>10.0	>100
27	106A	0.634	6.0	5.7	>100
28	108I, 181C	0.602	14.0	5.3	>100
29	101E, 181C, 190A	0.602	>100	>100	>100
30	179A	0.570	0.6	0.5	0.4
31	106A, F227L	0.570	>100	>100	>100
32	98G, 181C	0.570	11.0	17.0	>100
33	101Q, 181C, 190A	0.570	>100	43.0	>100
34	190S	0.507	3.9	97.0	>100
35	101E, 190S	0.475	16.0	>100	>100
36	190E	0.443	>100	>100	>100
37	106I, 188L	0.443	62.0	>100	>100
38	103N, 106A	0.412	2.6	26.0	>100
39	179G	0.348	0.3	0.2	0.1
40	227L	0.348	0.47	0.80	2.10
41	103R, 179D, 188L	0.348	>100	>100	>100
42	101E, 181C	0.317	>100	>100	>100
43	103N, 106M	0.317	12.0	>100	>100
44	103N, 106I	0.317	1.8	28.0	122.0
45	103S, 190A	0.285	>100	>100	>100
46	181I	0.285	5.4	1.4	>100
47	179T	0.285	1.3	0.8	0.5
48	101E, 181C, 190S	0.285	>100	>100	>100
49	101Q, 103R	0.285	0.6	1.2	1.3
50	103R, 179D	0.285	0.3	0.9	1.3
51	103N, 108I, 225H	0.253	2.6	>100	>100
52	98G, 103N, 108I	0.253	2.2	>100	>100
53	101P, 190A	0.253	92.0	>100	>100
54	101R, 103N	0.253	1.2	37.0	>100
55	101Q, 190S	0.253	2.4	>100	>100
56	98G, 190A	0.253	16.0	64.0	>100
57	106A, 190A	0.253	>100	>100	>100
58	101Q, 181C, 190S	0.253	>100	>100	>100
59	101E, 103N	0.222	0.9	52.0	>100
60	101Q, 181C	0.222	8.9	4.4	>100
61	236L	0.222	0.9	0.2	0.5
62	101H, 190A	0.222	32.0	77.0	>100
63	98G, 188L	0.222	>100	>100	>100
64	98G, 103N, 181C	0.190	4.2	>100	>100
65	101E, 108I, 181C, 190A	0.190	>100	>100	>100
66	103N, 108I, 238T	0.190	2.5	>100	>100

Continued on following page

TABLE 2—*Continued*

Isolate	NNRTI mutation(s)	% of NNRTI-resistant isolates[a]	Change (fold) in EC_{50}[b]		
			VRX-480773	Efavirenz	Nevirapine
67	98G, 100I, 103N	0.190	1.4	>100	>100
68	100I, 103N, 108I	0.190	1.9	>100	>100
69	103N, 188H	0.190	3.5	>100	>100
70	188H	0.190	1.6	8.7	>100
71	98G, 101E, 181C, 190A	0.190	65.0	71.0	>100
72	103Q	0.190	1.4	1.7	1.0
73	101H	0.190	2.5	4.1	12.0
74	103N, 108I, 181C, 190A	0.190	>100	>100	>100
75	179D, 188L	0.158	>100	>100	>100
76	103N, 108I, 190A	0.158	20.0	>100	>100
77	108I, 181C, 190A	0.158	>100	400	>100
78	101Q, 103N, 225H	0.158	3.9	>100	>100
79	98G, 103N, 181C, 190A	0.158	82.0	>100	>100
80	103N, 190S	0.158	18.0	>100	>100
81	106A, 181C	0.158	16.0	8.2	>100
82	98G, 108I, 181C	0.158	15.0	47.0	>100
83	238N	0.158	0.7	0.8	1.9
84	238T	0.127	2.2	3.0	100
85	103N, 238N	0.127	2.3	>100	>100
86	103S	0.127	2.7	8.6	39.0
87	230L	0.127	11.0	16.0	46.0
88	101R, 103N, 181C, 190A	0.127	>100	>100	>100
89	101E, 103N, 190A	0.127	50.0	>100	>100
90	101N, 103N	0.127	0.6	26.0	32.0
91	181V	0.127	20.0	4.5	>100
92	106L, 188L	0.127	>100	>100	>100
93	179D, 181C	0.127	>100	>100	>100
94	100F	0.127	1.8	1.6	2.4
Total		88.312			

[a] Prevalance of mutation patterns was determined from sequence data in the Stanford HIV Resistance database (14).
[b] Changes (*n*-fold) in EC_{50} relative to that against wt virus.

tions relative to that of wt virus were studied in the absence of inhibitor in a single-round infection assay. Transfection-generated, VSV-G-pseudotyped viruses (normalized to 50 ng/ml p24) were used to infect 1.5×10^4 HeLa-JC53 cells. Infected cells were cultured at 37°C and 5% CO_2 for 48 h. Following incubation, an equal volume of Bright-Glo luciferase reagent was added to each well, and chemiluminescence was read using an LJL Analyst system. The RC of the mutant viruses was defined as the percent luciferase activity relative to that of the wt virus control.

Determination of EC_{50} and EC_{90} values of VRX-480773 in 100% human serum. The EC_{50} and EC_{90} values of VRX-480773 against wt and mutant Y188L viruses in 100% human serum were derived by fitting the increases in EC_{50} and EC_{90} values associated with increasing serum concentrations to a one-site binding model (hyperbola) and extrapolation to 100% serum using GraphPad Prism 4. HeLa-JC53 cells (1.5×10^4 cells in 35 μl of phenol red-free Dulbecco's modified Eagle's medium containing 2% fetal bovine serum and 1% penicillin-streptomycin) were mixed with 5 μl of VSV-G-pseudotyped wt or Y188L NL4-3.Luci.R−E− virus (multiplicity of infection of 0.03) and 10 μl of serially diluted VRX-480773. The cell-virus compound mixture was then mixed with heat-inactivated human serum (Bioreclamation Inc., Meadow, NY) to give final concentrations of human serum of 0, 5, 10, 20, 30, 40, and 50% in the culture medium in a final volume of 100 μl. Cultures were incubated at 37°C in 5% CO_2 for 48 h. Following incubation, an equal volume of Bright-Glo luciferase reagent (Promega, Madison, WI) was added to each well, and chemiluminescence was read using an LJL Analyst system (LJL BioSystems, Sunnyvale, CA). The EC_{50} and EC_{90} values at different human serum concentrations were determined by nonlinear regression using GraphPad Prism 4.

Pharmacokinetics of VRX-480773 in dogs. VRX-480773 was given to dogs intravenously at 10 mg/kg or orally at 20 mg/kg. Plasma samples were collected at predose and at 0.25, 1, 2, 3, 4, 6, 8, 12, and 24 h following administration. Bioanalytical methods have been validated for quantification of VRX-480773 in plasma containing tripotassium EDTA (K_3EDTA) as an anticoagulant. [D_6]VRX-480773 was used as internal reference standard. Plasma proteins were precipitated with acetonitrile. The extract was evaporated to dryness, reconstituted, and analyzed by high-performance liquid chromatography with tandem mass spectrometry. An API 4000 triple-quadruple mass spectrometer, operated in positive electrospray ion mode, was used to monitor the precursor→product ion transitions of *m/z* 592.0→386.0 and 598.0→392.0 for VRX-480773 and [D_6]VRX-480773, respectively. The methods were linear over the concentration range of 1.00 to 1,000 ng/ml with a validated lower limit of quantitation of 1.00 ng/ml. Intra- and interbatch assay accuracy (percentage of nominal) and precision (percent coefficient of variation) were within ±15% for all levels and <±20% for the lower limit of quantitation, respectively.

RESULTS

Discovery of VRX-480773 as a novel and potent HIV-1 RT inhibitor. The small molecule VRX-387902 (Fig. 1) was originally discovered from a cell-based high-throughput screen for inhibitors of HIV-1 replication. Although VRX-387902 was a moderate inhibitor of wt HIV-1, with an in vitro EC_{50} value of 0.1 μM, it had reduced activity against an NNRTI-resistant virus containing the K103N-Y181C mutation (EC_{50} = 1.3 μM) (6). Lead optimization to increase potency against NNRTI-resistant mutants led to the synthesis of VRX-480773. VRX-480773 exhibited greatly increased in vitro activity, with wild-type EC_{50} values of 0.14 nM and K103N-Y181C mutant EC_{50} values of 0.23 nM (Table 1), and low cytotoxicity (CC_{50} of 3 to >100 μM). VRX-480773 was specific for HIV-1 inhibition, as it did not inhibit hepatitis B virus or hepatitis C virus replication, and, similar to other NNRTIs, it did not inhibit an HIV-$2_{(NIH-Z)}$ isolate (data not shown). However, VRX-480773 inhibited five non-B HIV-1 isolates (belonging to clades A, AC, C, D, and F) with an activity similar to that against wt clade B

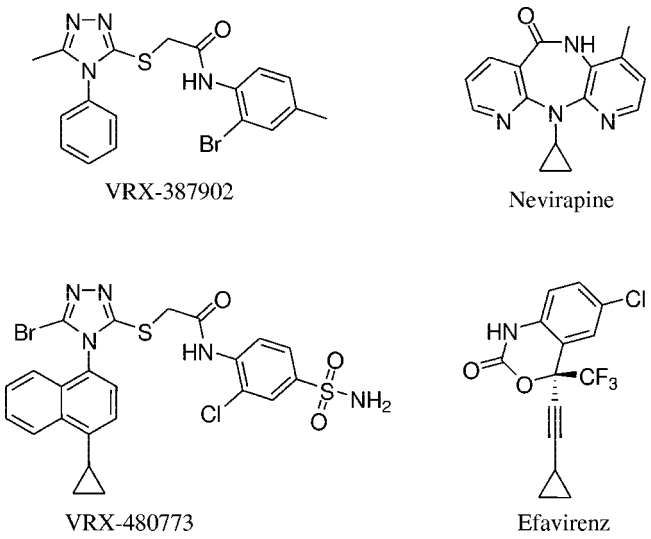

FIG. 1. Chemical structures of VRX-387902, VRX-480773, nevirapine, and efavirenz.

isolate NL4-3 (data not shown). In addition, enzymatic experiments demonstrated that VRX-480773 inhibits HIV-1 RT activity with wild-type 50% inhibitory concentration value of 4.0 nM, while it did not inhibit human DNA polymerases α and β at up to 45 μM (data not shown). Taken together, these results indicate that VRX-480773 is a potent and specific HIV-1 RT inhibitor.

Activity of VRX-480773 against efavirenz-resistant HIV-1 derived from molecular clones. The anti-HIV-1 activity of VRX-480773 against the wt and a panel of 20 HIV-1 isolates (Table 1) that carry the HIV-1 RT gene mutations most frequently observed in patients who have failed efavirenz-containing regimens was evaluated (1). As shown in Table 1, VRX-480773 and efavirenz showed similar activities against wt virus. However, VRX-480773 was active against most (18 of 20) mutant isolates with EC_{50} values of less than 1.0 nM, while efavirenz was active against only five isolates at similar EC_{50} levels. More importantly, VRX-480773 was active against viruses that are most prevalent and highly resistant to efavirenz, such as those that carry RT mutations K103N, G190S, K103N-L100I, K103N-K101Q, K103N-V108I, and K103N-P225H. This result suggests that VRX-480773 may be used in both

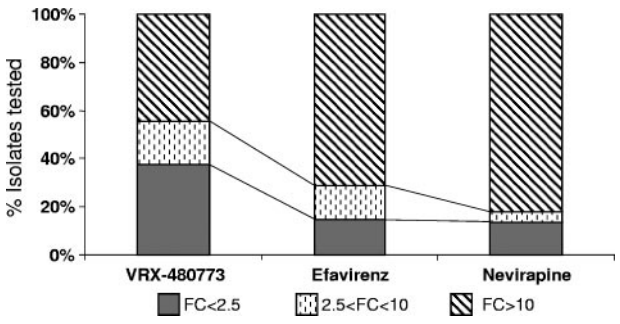

FIG. 2. Profiles of activity of VRX-480773, efavirenz, and nevirapine against 94 clinical NNRTI-resistant HIV-1 isolates. FC, change (*n*-fold) in EC_{50} relative to EC_{50} against wt virus.

TABLE 3. Cross-resistance of 94 clinical HIV-1 isolates between efavirenz and VRX-480773

Efavirenz fold change in EC_{50} value	No. (%) of isolates with VRX-480773 fold change in EC_{50} value of[a]:	
	<10	>10
<10	24 (22.4)	3 (0.9)
>10	28 (45.9)	39 (19.1)

[a] Numbers in parentheses correspond to the percentage of total isolates in the Stanford database.

NNRTI-naïve patients and patients who are experiencing efavirenz treatment failure.

Activity of VRX-480773 against clinical HIV-1 isolates carrying NNRTI resistance mutations. The Stanford HIV Resistance database contains 3,157 HIV-1 sequences associated with NNRTI resistance (12, 14). These sequences can be clustered into 218 different patterns of amino acid changes in the RT gene, with the K103N mutation being the most prevalent one (681 sequences or 21.6% of the total). The activity of VRX-480773 against a panel of 94 clinical isolates containing the most prevalent NNRTI resistance mutation patterns was tested (Table 2). These 94 mutant viruses represent 88.3% of the total NNRTI resistance isolates (prevalence) in the Stanford HIV Resistance database. As summarized in Fig. 2, there are significantly more isolates in this panel that are susceptible (<2.5-fold change) to VRX-480773 than to the current NNRTIs efavirenz or nevirapine (37% versus 15% or 14%, respectively). This represents 49% of the total NNRTI-resistant isolates present in the Stanford database that can be effectively inhibited (<2.5-fold change) by VRX-480773, compared to only 10.6% and 8.6% for efavirenz and nevirapine, respectively. Conversely, significantly fewer isolates showed high resistance (>10-fold change) to VRX-480773 than to efavirenz or nevirapine (45% versus 71% or 82%, respectively, representing 20%, 65%, and 72.5% of the total NNRTI-resistant

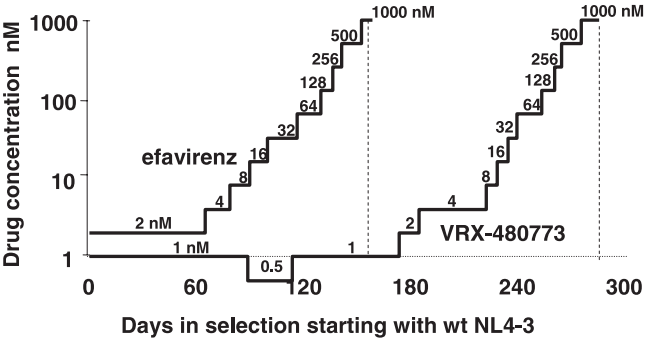

FIG. 3. Time courses of resistance mutation selection for VRX-480773 and efavirenz. Wild-type NL4.3 was used to infect SupT1 cells (2×10^6 cells in 1 ml of RPMI 1640 medium containing 10% fetal bovine serum) in the initial presence of 1 nM VRX-480773 or 2 nM efavirenz. Subsequently, every 3 to 4 days, 100 μl of infected culture was transferred into 900 μl of medium containing fresh drug and 9×10^5 SupT1 cells. Virus replication was monitored microscopically by observing the formation of syncytia. At each virus breakthrough (massive syncytium formation), the concentration of the inhibitor was doubled.

TABLE 4. VRX-480773-selected RT mutations starting from wt HIV-$1_{(NL\text{-}4.3)}$

Concn (nM)	Clone	Known NNRTI resistance mutation(s)	Novel mutation(s)	Other mutation(s)
2	181			E240G, V559I
	183			E240G, T131A
	185			G18D, A371T
	186	Y181C		V241I, A437T
	187			P176L
	188			K220E, V245A, A376V, Q509R, S515L
	189	V106I	D123G	D471N
	231			V118I, Q207R, V254I
	233			G15R, T470A
	235			G555R
	237			V10I, E300G, A327T
	238	Y181C		A129V, K259R, V518I
	240			F116S
	243			E240G, A446V
3	280	Y181C		
	283	Y181C		L303P, E430K, I556T
	285	Y181C		C162Y
	287			I37N
	288	Y181C		R172K, E204G, E328G
	290	Y181C		A445V
	292			V8I, K82R, V189I, Q407R
	293			D121N, H221R
	296	Y181C		E29K, V559I
	297		D123G	E194K, P225S, V276A, A304T, K395R
	298	Y181C		
	300			V8I, Q197R, D250G
	301	Y181C		V108A
32	205	Y181C		T409A
	213	V179A		E204G
	214			E204G
	251	Y181C		
	252	Y181C		V276I, E291G, V317A
	253			F214L
	254	Y181C		
	255			V261M, E291G, P313S, E430G
	256	Y181C		K43R, E404G, P433L, A446V
	259	V106I, Y181C	D123G	
	260	Y181C	D123G	K43R
	261	V106I, Y181C		Q242R, D471N
	262	V106I, Y181C		
64	151	V106I, Y181C, F227L	T369I	D471N
	153	V106I, Y181C, F227L	T369I	K103R, CI62R, K424R, S519N
	155	V106I, Y181C	D123G	
	156	V106I, Y181C, F227L	D123G	
	157	V106I, Y181C, F227L	D123G, T369I	
	158	V106I, Y181C, F227L	T369I	T296A, I326M
	159	V106I, Y181C, F227L	T369I	E204G, A267T
	160	V106I, Y181C	D123G, T369I	E204G
	161	V106I, Y181C, F227L	T369I	A408T, Q524R
	162	V106I, Y181C, F227L	D123G, T369I	
	163	V106I, Y181C, F227L	D123G, T369I	M184V
128	61	V106I, Y181C, F227L	D123G, T369I	
	62	V106I, Y181C, F227L	D123G, T369I	F61S
	63	V106I, Y181C, F227L	D123G, T369I	W71R
	65	V106I, Y181C, F227L	T369I	T351A
	66	V106I, Y181C, F227L		
	67	V106I, Y181C		P4T, G99E, K353R, M357I, P412S
	68	V106I, Y181C, F227L	D123G, T369I	P4T, H198Q
	69	V106I, Y181C, F227L	D123G, T369I	V189A
	70	V106I, Y181C, F227L	T369I	S513N
	71	V106I, Y181C, F227L	T369I	G40R
500	91	V106I, Y181C, F227L	D123G, T369I	
	92	V106I, Y181C, F227L	D123G, T369I	H221Y

Continued on facing page

TABLE 4—*Continued*

Concn (nM)	Clone	Known NNRTI resistance mutation(s)	Novel mutation(s)	Other mutation(s)
	93	V106I, Y181C, F227L	D123G, T369I	Y188H, V467I
	95	V106I, Y181C, F227L	D123G, T369I	V118A, V467I
	96	V106I, Y181C, F227L	D123G, T369I	I257V, D488G, L551S
	98	V106I, Y181C	D123G	P236L
	99	V106I, Y181C, F227L	T369I	
	100	V106I, Y181C, F227L	T369I	
	101	V106I, Y181C, F227L	T369I	
	102	V106I, Y181C, F227L	D123G, T369I	P236L, I329T
	103	V106I, Y181C, F227L	T369I	
1,000	3	V106I, V179D, Y181C, F227L	T369I	
	5	V106I, Y181C, F227L	D123G, T369I	V314I, G384R
	8	V106I, Y181C, F227L	T369I	D460N
	11	V106I, V179D, Y181C, F227L	D123G, T369I	D17N
	13	V106I, Y181C, F227L	T369I	I495T
	14	V106I, Y181C, F227L	T369I	
	15	V106I, V179D, Y181C, F227L	D123G, T369I	
	18	V106I, Y181C, F227L	D123G, T369I	
	23	V106I, Y181C, F227L	T369I	
	28	V106I, V179D, Y181C, F227L	D123G, T369I	
	31	V106I, Y181C, F227L	D123G, T369I	K431R

isolates in the Stanford database, respectively) (Fig. 2). Table 3 further breaks down the 94 isolates into those with less-than- or greater-than-10-fold changes in resistance against VRX-480773 and efavirenz. Among the 67 isolates with which efavirenz has a greater-than-10-fold reduction in potency, 28 isolates are susceptible to VRX-480773 (with less than a 10-fold change in potency). Although there were 39 mutants with a greater-than-10-fold change in resistance against both VRX-480773 and efavirenz, viruses with these mutation patterns represent less than 20% of the total NNRTI-resistant isolates in the Stanford database (Table 3). The susceptibility or resistance of viruses containing the remaining 125 mutation patterns (11.7% of the isolates) is not known and requires further testing.

Mutation selection and durability of VRX-480773 suppression. Wild-type virus strain NL4-3 was cultured in the presence of increasing concentrations of VRX-480773 or efavirenz to determine the times required for viral breakthrough and resistance pathways. This experiment also allowed us to compare the genetic barriers imposed by VRX-480773 and efavirenz against resistance development in cell culture conditions. As shown in Fig. 3, VRX-480773 exhibited a more durable pressure on the virus than efavirenz. It took 180 days for mutant viruses to emerge and break through the initial selection of VRX-480773 at 1 nM. In contrast, mutant virus emerged in 60 days under the selection of 2 nM efavirenz (Fig. 3). Note that the virus replicated so slowly in the presence of VRX-480773 that we had to drop the initial concentration of VRX-480773 from 1 nM to 0.5 nM after 90 days of selection. This 0.5 nM concentration was kept for 30 days to allow the recovery of virus replication (Fig. 3).

To determine the mutation pathway to VRX-480773 resistance from wild-type virus, the sequences of 10 to 15 proviral DNA clones at each breakthrough concentration point were examined. Table 4 lists the individually cloned sequences. Although it needs to be further confirmed, the virus seemed to evolve in the presence of increasing concentrations of VRX-480773 through the following pathway: wt to Y181C to V106I-

TABLE 5. Replication capacity and cross-resistance of VRX-480773-selected viruses

VRX-480773-selected mutation[a]	% wt RC[b]	Mean change (fold) in EC_{50} ± SD[c]	
		VRX-480773	Efavirenz
wt	100		
V106I	54.56 ± 5.02	1.0 ± 0.11	0.7 ± 0.07
D123G	89.5 ± 19.09	1.0 ± 0.02	1.0 ± 0.03
Y181C	87.8 ± 10.96	1.9 ± 0.37	1.3 ± 0.03
F227L	114.5 ± 2.12	0.7 ± 0.09	0.5 ± 0.07
T369I	30.5 ± 3.46	1.3 ± 0.08	2.1 ± 0.07
V106I-Y181C	43.8 ± 10.25	5.4 ± 0.72*	3.3 ± 1.05*
V106I-Y181C-F227L	0.635 ± 0.09	18.7 ± 3.95*	2.5 ± 0.90
V106I-Y181C-F227L-T369I	0.2 ± 0.00	111.9 ± 22.28*	8.0 ± 1.72*
V106I-D123G-Y181C-F227L	64.0 ± 14.14	9.1 ± 0.08*	1.5 ± 0.18
V106I-D123G-Y181C-F227L-T369I	11.3 ± 2.40	31.2 ± 13.96*	2.9 ± 0.09

[a] Molecular clones were built in the reporter vector pNL4-3.Luc.R−E−, and EC_{50} values were determined by using Hela-JC-53 cells.
[b] Virus replication capacity was determined in the absence of inhibitor.
[c] Mean change (*n*-fold) in EC_{50} values ± standard deviations were from two tests. *, EC_{50} was statistically significantly higher than that against wild-type virus ($P < 0.05$).

TABLE 6. EC_{50} values of VRX-480773 and efavirenz under different concentrations of human serum

Strain	Treatment	Mean EC_{50} (nM) ± SD under human serum concn in culture medium[a] (%) of:							
		0	5	10	20	30	40	50	100[b]
WT NL4-3	VRX-480773	0.6 ± 0.21	2.4 ± 0.44	3.9 ± 0.92	7.1 ± 3.82	8.6 ± 3.10	9.5 ± 2.58	13.0 ± 1.98	23 ± 3.0
	Efavirenz	0.8 ± 0.04	2.1 ± 0.76	3.5 ± 1.21	5.6 ± 1.38	8.5 ± 3.82	10.3 ± 3.80	12.8 ± 3.58	24.2 ± 0.07
Y188L NL4-3	VRX-480773	3.7 ± 0.15	13.7 ± 2.60	20.7 ± 1.45	43.3 ± 1.95	45.9 ± 7.88	54.0 ± 2.28	79.9 ± 1.51	89.1 ± 0.07
	Efavirenz	85.9 ± 1.56	298.5 ± 6.77	416.6 ± 41.55	814.9 ± 160.71	1,016.1 ± 107.10	1,382.1 ± 240.47	1,608.4 ± 22.34	2,524 ± 0.07

[a] Culture medium contained basal 2% fetal bovine serum.
[b] EC_{50} values in 100% human serum were derived by fitting the increases in EC_{50} values associated with increasing serum concentrations to a one-site binding model (hyperbola) and extrapolation to 100% serum using GraphPad Prism 4.

Y181C to V106I-Y181C-F227L to V106I-D123G-Y181C-F227L or V106I-Y181C-F227L-T369I to V106I-D123G-Y181C-F227L-T369I and, finally, to V106I-D123G-V179D-Y181C-F227L-T369I (Table 4). Notably, the D123G and T369I mutations are two mutations that have not previously been reported to be associated with NNRTI resistance.

Resistance conferred by VRX-480773-selected mutations. In order to determine the levels of resistance to VRX-480773 and to efavirenz conferred by VRX-480773-selected mutations, these mutations, individually or in combination, were engineered back into the wt NL4-3 molecular clone by site-directed mutagenesis. The resulting viruses were tested for their resistance to VRX-480773 and efavirenz and for their replication capacity in the absence of an inhibitor (Table 5). The single mutations V106I, D123G, Y181C, F227L, and T369I did not confer much resistance (<2-fold change) to VRX-480773. Multiple mutations were required before significant shifts in susceptibility were observed. Importantly, all mutant viruses remained susceptible to efavirenz (≤8-fold change). These data suggest a reduced level of cross-resistance between VRX-480773 and efavirenz.

Prediction of EC_{50} and EC_{90} values of VRX-480773 in 100% human serum. The EC_{50} and EC_{90} values of VRX-480773 against wt and mutant Y188L NL4-3 viruses determined with 0 to 50% human serum in cell culture were used to project its EC_{50} and EC_{90} values in 100% human serum (Table 6 and data not shown). Using this method, the EC_{50} and EC_{90} of VRX-480773 in 100% human serum are predicted to be 23 nM and 68.5 nM, respectively, for wt virus. For Y188L mutant virus, the predicted EC_{50} and EC_{90} values in 100% human serum were 89 nM and 840.2 nM, respectively. Notably, the EC_{50} values of efavirenz were similarly affected by increasing amounts of human serum in culture medium (Table 6), indicating that the potency of these two inhibitors is similarly affected by binding to serum proteins. The EC_{50} values predicted for efavirenz in 100% human serum are 24 nM and 2,524 nM for wt and Y188L mutant viruses, respectively.

Pharmacokinetics of VRX-480773 in dogs. Pharmacokinetic studies demonstrated that VRX-480773 was rapidly absorbed in dogs. Following a single oral administration of 20 mg/kg, an average time to maximum concentration of drug in serum of 5.3 h and a maximum concentration of drug in serum of 1,980 ng/ml were observed (Table 7). The bioavailability was estimated to be 47% for VRX-480773. The plasma concentration-versus-time profile of VRX-480773 in dogs is presented in Fig. 4. At 12 h postadministration, a plasma concentration of VRX-480773 of 742 ng/ml (1,251 nM) was observed in dogs (Table 7). Based on the predicted EC_{50} values of VRX-480773 of 23 nM for wt virus and 89 nM for Y188L virus, the inhibitory quotient (IQ) (IQ = concentration of drug in plasma at 12 h/predicted EC_{50} in 100% human serum) for VRX-480773 given 20 mg/kg twice daily was calculated to be 54 for wt virus and 14 for Y188L virus (Table 7).

TABLE 7. Pharmacokinetic parameters of VRX-480773 in dogs following single intravenous or oral administrations

Parameter	Dosing	
	Intravenous	Oral
Dose (mg/kg)	10	20
No. of animals[a]	3	3
T_{max} (h)[b]	0.28 (0.083–0.50)	5.3 (4.0–8.0)
C_{max} (ng/ml)[c]	4,420 ± 465	1,980 ± 246
$T_{1/2}$ (h)[d]	2.89 ± 0.490	3.36 ± 0.558
AUC_{last} (ng · h/ml)[e]	20,900 ± 224	19,600 ± 4,480
$C_{12\ h}$ (ng/ml)[f]	283 ± 73	742 ± 343
IQ_{wt}[g]	20.7	54.4
IQ_{Y188L}[h]	5.4	14.1
Bioavailability (%)[i]		47.1

[a] $n = 3$ for each time point with multiple groups due to limit of sample volume.
[b] T_{max}, time at which dosed drug reached maximum concentration in plasma.
[c] C_{max}, maximum concentration of dosed drug in plasma.
[d] $T_{1/2}$, elimination half-time of dosed drug in plasma.
[e] AUC_{last}, total area under concentration-time curve.
[f] $C_{12\ h}$, drug concentration in plasma at 12 h postadministration.
[g] $IQ_{wt} = C_{12\ h}$ (nM)/23 nM (EC_{50} against wt virus predicted in 100% human serum).
[h] $IQ_{Y188L} = C_{12\ h}$ (nM)/89 nM (EC_{50} against Y188L virus predicted in 100% human serum).
[i] Bioavailability (percent) = (oral $AUC_{0-\infty}$/intravenous $AUC_{0-\infty}$) × 100.

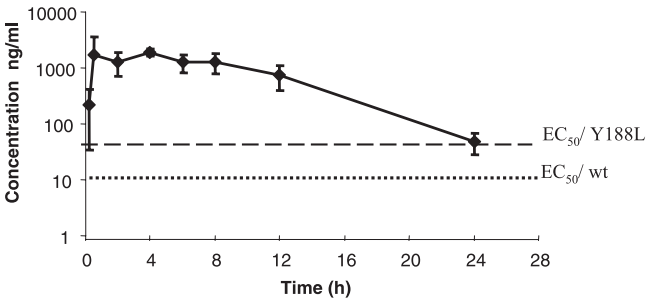

FIG. 4. Plasma concentration-versus-time profile of VRX-480773 following oral administration in dogs. EC_{50}/wt and EC_{50}/Y188L are the predicted EC_{50} values of VRX-480773 in 100% human serum for wt and Y188L HIV-1, respectively.

DISCUSSION

In this report, we have described VRX-480773 as being a novel nonnucleoside RT inhibitor of HIV-1. VRX-480773 was as potent as efavirenz against wt virus but was more potent than efavirenz against HIV-1 carrying the RT mutations most commonly observed in plasma samples from patients experiencing efavirenz treatment failure. More importantly, VRX-480773 was superior to efavirenz and nevirapine against a majority of NNRTI-resistant HIV-1 clinical isolates. In addition, the longer time for mutant viruses to break through suppression by VRX-480773 suggests that it has a higher genetic barrier for resistance development than efavirenz. Most of the resistant viruses selected by VRX-480773 can be inhibited by efavirenz, indicating a low level of cross-resistance between these two NNRTIs. Finally, levels of VRX-480773 that were higher than its EC_{50} values for inhibiting wt and mutant Y188L viruses were observed in dog plasma following oral administration. These data support the further clinical development of VRX-480773 for its use in both naïve and NNRTI-experienced patients infected by HIV-1.

The more durable suppression of virus replication exerted by VRX-480773 than that by efavirenz seems to stem from the more flexible nature of VRX-480773. In contrast to the rigid structures of efavirenz and nevirapine, VRX-480773 has multiple linkers connecting the "ring systems," which allows them to rotate within the hydrophobic pocket of the HIV-1 RT to accommodate amino acid changes, similar to another NNRTI, TMC-125, which has been described as being able to bind the RT enzyme in more than one conformationally distinct mode (5). This improved molecular flexibility seems to enable the inhibitor to adapt to conformational changes due to mutations. Thus, single amino acid changes selected by VRX-480773, such as Y181C, V106I, or F227L, do not cause significant resistance to VRX-480773. The V106I-Y181C double mutation caused merely a 5.4-fold increase in resistance to VRX-480773 (Table 5).

During the in vitro selection for viruses that were resistant to VRX-480773, Y181C seemed to be the first single mutation selected; the other four mutations (V106I, D123G, F227L, and T369I) appeared in association with either this mutation or other unreported mutations in the RT (Table 4). Analysis of the replication and resistance patterns of the reconstituted viruses carrying these mutations suggests that V106I, F227L, and T369I appear to contribute directly to virus resistance, since adding V106I toY181C increased the resistance from 1.9- to 5.4-fold and decreased the RC from 88% to 44% (Table 5). Similarly, adding F227L to V106I-Y181C resulted in an increase in resistance from 5.4- to 18.7-fold, while dramatically decreasing the RC from 44% to 0.6% of that of wt virus. Adding T369I to V106I-Y181C-F227L further increased the resistance from 18.7- to 112-fold and further dropped the virus RC from 0.6% to 0.2%. Although T369I is clearly associated with increased resistance to VRX-480773, its mechanism is unclear, since this residue is located far from the NNRTI-binding pocket (11).

In contrast to the other mutations, the D123G mutation seems to play a compensatory role that increases the virus RC. Adding the D123G mutation to V106I-Y181C-F227L dramatically increased the virus RC from 0.6% to 64%, while the virus resistance decreased from 18.7- to 9-fold (Table 5). Similarly, adding the D123G mutation to V106I-Y181C-F227L-T369I increased the virus RC from 0.2% to 11.3%, while decreasing the virus resistance from 112- to 31-fold. Although the Y181C mutation alone occurs frequently in the Stanford Drug Resistance database (156 sequences, or 4.9% of the total NNRTI-resistant sequences), the prevalence of the Y181C mutation in combination with the other mutations is rather low. There are only two sequences containing the V106I-Y181C combination and none containing V106I-Y181C-F227L (12, 14).

Although, like efavirenz, VRX-480773 is highly protein bound, the VRX-480773 level in dogs seems to be high enough at 12 h to effectively inhibit both wt and mutant Y188L viruses (with predicted IQ values of 54 and 14, respectively) (Table 7). VRX-480773 is currently under phase I clinical studies to determine its pharmacokinetics and safety profile in humans.

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