Inhibition of the Ribonuclease H and DNA Polymerase Activities of HIV-1 Reverse Transcriptase by *N*-(4-*tert*-Butylbenzoyl)-2-hydroxy-1-naphthaldehyde Hydrazone[†]

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Received October 1, 1996; Revised Manuscript Received December 11, 1996[⊗]

ABSTRACT: HIV-1 reverse transcriptase (RT) is multifunctional, with RNA-dependent DNA polymerase (RDDP), DNA-dependent DNA polymerase (DDDP), and ribonuclease H (RNase H) activities. *N*-(4-*tert*-Butylbenzoyl)-2-hydroxy-1-naphthaldehyde hydrazone (BBNH) inhibited both the polymerase and the RNase H activities of HIV-1 RT *in vitro*. IC_{50} values for inhibition of RDDP were 0.8−3.4 μM, depending on the template/primer (T/P) used in the assay. The IC_{50} for DDDP inhibition was about 12 μM, while that for inhibition of RNase H was 3.5 μM. EC_{50} for inhibition of HIV-1 replication in cord blood mononuclear cells was 1.5 μM. BBNH inhibition of RNase H *in vitro* was time-dependent, whereas inhibition of RT polymerase activities was immediate. BBNH was a linear mixed-type inhibitor of RT RDDP activity with respect to both T/P and to dNTP, whereas BBNH inhibition of RT RNase H activity was linear competitive. Protection experiments using an azidonevirapine photolabel showed that BBNH binds to the non-nucleoside RT inhibitor (NNRTI) binding pocket. Importantly, the compound inhibited recombinant RT containing mutations associated with high-level resistance to other NNRTI. While BBNH did not inhibit the DNA polymerase activities of other retroviral reverse transcriptases and DNA polymerases, the compound inhibited *Escherichia coli* RNase HI and the RNase H activity of murine leukemia virus RT. BBNH also inhibited HIV-1 RT RNase H in the presence of high concentrations of other non-nucleoside inhibitors with higher affinities for the NNRTI binding pocket, and of RT in which the NNRTI binding pocket had been irreversibly blocked by the azidonevirapine photolabel. We conclude that BBNH may therefore bind to two sites on HIV-1 RT. One site is the polymerase non-nucleoside inhibitor binding site and the second may be located in the RNase H domain. BBNH is therefore a promising lead compound for the development of multisite inhibitors of HIV-1 RT.

Numerous drugs have been developed to hinder the replication of the human immunodeficiency virus (HIV-1).[1] Almost all anti-HIV agents thus far have been targeted to a single viral protein, primarily the DNA polymerase activity of reverse transcriptase (RT). Unfortunately, such drugs, although effective against HIV-1 reverse transcriptase *in vitro*, have proven clinically disappointing due to the appearance of drug-resistant viral strains (Larder & Kemp, 1989; Schinazi et al., 1993; Richman, 1993; Balzarini et al., 1994). Therefore, the discovery and development of new antivirals is crucial. One approach to minimize the development of drug resistance may be development of compounds that inhibit several viral targets, either different proteins and/or several different activities of the same enzyme.

Many HIV-1 proteins require metals for activity, for example, integrase (Engelman & Craigie, 1995), nucleocapsid protein (South et al., 1990), and reverse transcriptase (Hoffman et al., 1985; Cheng et al., 1987; Cirino et al., 1995). The latter is a multifunctional enzyme possessing RNA- and DNA-directed DNA polymerase activity (RDDP and DDDP, respectively) as well as intrinsic ribonuclease H (RNase H) activity (Le Grice, 1993), each of which is essential for HIV-1 replication. Both RT activities are metal-dependent, and several sites for the binding of metals have been identified on RT (Davies et al., 1991; Kohlstaedt et al., 1992; Rodgers et al., 1995). One useful approach for inhibiting metal-dependent enzymes is to identify agents capable either of extracting or of binding to the metal at the active site, thereby inactivating the protein. However, chelators must

[†] This work was supported in part by grants (to M.A.P.) from the National Health Research and Development Program, Health Canada (6605-4839-AIDS), the Medical Research Council of Canada (PG-11899/GR-13918), and CANFAR, the Canadian Foundation for AIDS Research. G.B. is recipient of a Fellowship from the Medical Research Council of Canada. M.A.P. is a Chercheur-boursier senior (Career Research Scholar) of the Fonds de la recherche en santé du Québec.

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[⊗] Abstract published in *Advance ACS Abstracts,* February 15, 1997.

[1] Abbreviations: 9-AN, 9-azido-5,6-dihydro-11-ethyl-6-methyl-11*H*-pyrido[2,3-*b*][1,5]benzodiazepin-5-one; AMV, avian myeloblastosis virus; AZTMP, 3′-azido-3′-deoxythymidine monophosphate; BBNH, *N*-(4-*tert*-butylbenzoyl)-2-hydroxy-1-naphthaldehyde hydrazone; DDDP, DNA-dependent DNA polymerase; DMSO, dimethyl sulfoxide; HIV-1, human immunodeficiency virus type 1; Mo-MLV, Moloney murine leukemia virus; NNI, non-nucleoside inhibitor; NNRTI, non-nucleoside reverse transcriptase inhibitor; RDDP, RNA-dependent DNA polymerase; RT, reverse transcriptase; RNase H, ribonuclease H; TIBO, thiobenzimidazolone [(+)-*S*-4,5,6,7-tetrahydro-9-chloro-5-methyl-6-(3-methyl-2-butenyl)imidazo-[4,5,1-*jk*]1,4-benzodiazapine-2-thione]; T/P, template/primer; UC38, 2-chloro-5-[[(1-methylethoxy)thioxomethyl]-amino]benzoic acid 1-methylethyl ester; UC84, 2-chloro-5-[(5,6-dihydro-2-methyl-1,4-oxathiin-3-yl)carbonylamino]benzoic acid 1-methylethyl ester.

S0006-2960(96)02469-5 CCC: $14.00

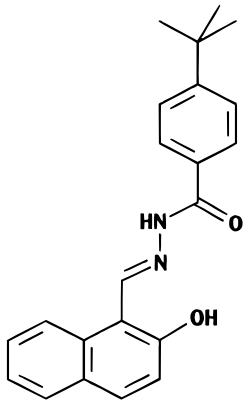

FIGURE 1: Structure of *N*-(4-*tert*-butylbenzoyl)-2-hydroxy-1-naphthaldehyde hydrazone (BBNH).

be designed to target the specific enzyme of interest, since many cellular enzymes are also metal-dependent. This approach has proven highly successful in the development of antihypertensives, namely, metal chelators targeted to angiotensin converting enzyme (Cushman et al., 1977).

In this report, we show that the metal chelator *N*-(4-*tert*-butylbenzoyl)-2-hydroxy-1-naphthaldehyde hydrazone (BBNH) (Figure 1) is a good inhibitor of all three enzymatic activities of HIV-1 RT *in vitro* and inhibits replication of HIV-1 in infected cells. The hydrazone also inhibits mutant RT that is resistant to other non-nucleoside inhibitors (NNI). Our data imply that BBNH may inhibit RT by binding to at least two sites on the enzyme, one of which is the non-nucleoside inhibitor (NNI) binding pocket involved in inhibition of RT DNA polymerase activity by compounds such as nevirapine, TIBO, etc. The other site may be at the RNase H catalytic site. Although inhibition of RT RNase H may be due to the metal chelation, this is apparently not a factor in the inhibition of RT DNA polymerase activity. BBNH therefore provides an interesting lead structure for the development of potential multitarget inhibitors of HIV-1.

MATERIALS AND METHODS

N-(4-*tert*-Butylbenzoyl)-2-hydroxynaphthaldehyde hydrazone (BBNH) was synthesized by standard methods (Edward et al., 1988). The nevirapine analog photolabel, 9-azido-5,6-dihydro-11-ethyl-6-methyl-11*H*-pyrido[2,3-*b*][1,5]-benzodiazepin-5-one (9-AN), was synthesized as described (Hargrave et al., 1991). [^{1}H]NMR and elemental analyses of BBNH and 9-AN were entirely consistent with the expected chemical structures. The carboxanilide non-nucleoside RT inhibitors UC38 and UC84 (Fletcher et al., 1995a,b) were a gift from Drs. W. Brouwer and A. W. Harrison, Uniroyal Chemical Ltd. Research Laboratories, Guelph, ON, Canada.

[^{3}H]dTTP and [^{3}H]dGTP were purchased from NEN-Dupont. The homopolymeric template/primers (T/P) poly(rC)-oligo(dG)$_{12-18}$, poly(rA)-oligo(dT)$_{12-18}$, and poly(dC)-oligo(dG)$_{12-18}$ were products of Pharmacia (Montreal, QC, Canada). Calf thymus activated DNA was from Sigma. Heteropolymeric RDDP T/P was prepared using an RNA transcript derived from pHIV-PBS and a synthetic 18-mer oligonucleotide primer (Arts et al., 1994). The RNase H substrate poly([^{3}H]rG)-poly(dC) was prepared as described (Starnes & Cheng, 1989).

The vectors for the expression of K103N, Y181I, Y181C, Y188L, and Y188H mutant RT were constructed by site-directed mutagenesis, using techniques similar to those previously described (Gu et al., 1994). Recombinant heterodimeric wild-type (wt) and mutant p51/p66 HIV-1 reverse transcriptases, and homodimeric HIV-2 RT, were purified as described (Fletcher et al., 1996). Avian myeloblastosis virus (AMV) and Moloney murine leukemia virus (Mo-MLV) reverse transcriptases, and bovine pancreatic RNase A, were from Pharmacia. *Taq* polymerase and Klenow polymerase were from Promega. *Escherichia coli* RNase HI was obtained from Sigma. HeLa cell nuclear extracts were prepared as described (Applegren et al., 1995) and were used as a source of eukaryotic cell DNA polymerases. All other reagents were of the highest quality available and were used without any further purification.

Assay of RT RDDP and DDDP Activities. HIV-1 RT polymerase activity was determined by a fixed time assay. Briefly, reaction mixtures (100 μL total volume) contained 50 mM Tris-HCl (pH 7.8, 37 °C), 60 mM KCl, 10 mM $MgCl_2$, 10 ng of p51/p66 RT, 0.1−0.5 units of template/primer, and 0.5−25 μM of [^{3}H]dNTP substrate. Aliquots of a BBNH stock solution in dimethyl sulfoxide (DMSO) were added such that the final DMSO concentration never exceeded 2%. Control experiments showed that neither RT RDDP nor DDDP activity was affected by this concentration of DMSO (data not shown). Reaction assays were incubated at 37 °C for 10−20 min and then quenched with 500 μL of cold 20 mM sodium pyrophosphate in 10% trichloroacetic acid. After 30 min on ice, the samples were filtered on Whatman 934-AH glass fiber filters and washed with 10% TCA and ethanol, and the radioactivity determined by liquid scintillation analysis.

Assay of RNase H Activity. RT RNase H was assayed essentially as described (Starnes & Cheng, 1989). Briefly, reaction mixtures (50 μL total volume) contained 50 mM Tris-HCl (pH 8.0, 37 °C), 60 mM KCl, 8 mM $MgCl_2$, and variable amounts of poly([^{3}H]rG)-poly(dC). Generally, 10 ng of p51/p66 heterodimeric RT were used. Aliquots of a BBNH stock solution in DMSO were added such that the final DMSO concentration never exceeded 2%. Control experiments showed that RT RNase H activity was not affected by this concentration of DMSO. Reactions were carried out for 20 min at 37 °C and then quenched by placing the tubes on ice followed by the addition of 100 μL of cold 7% perchloric acid. After 30 min on ice, the reaction mixtures were centrifuged at 12000*g* for 15 min. 100 μL of the supernatants were carefully removed, and the radioactivity determined by liquid scintillation analysis.

Optimal inhibition of RT RNase H by BBNH required preincubation of the enzyme with the inhibitor. In these experiments, RT was preincubated with the inhibitor in 25 μL of 50 mM Tris-HCl (pH 8.0, 37 °C) containing 60 mM KCl. Aliquots of a BBNH stock solution in DMSO were added such that the final DMSO concentration never exceeded 2%. After preincubation for up to 30 min at 37 °C, 25 μL of a solution containing the RNase H assay components was added, so that the final concentrations of reactants were as indicated above. Studies of BBNH inhibition of *E. coli* RNase HI were carried out in a similar manner.

Bovine pancreatic RNase A activity was measured by following the degradation of total human placental tRNA, using agarose gel electrophoresis, essentially as described (Silberklang et al., 1979).

Table 1: Inhibition of Multiple Activities of HIV-1 RT and Other Enzymes by *N*-(4-*tert*-Butylbenzoyl)-2-hydroxy-1-naphthaldehyde Hydrazone

enzyme (template/primer or substrate)	IC_{50} (μM)[a] RDDP	DDDP	RNase H[b]
HIV-1 RT			
poly(rA)-oligo(dT)$_{12-18}$	1.9 ± 0.1	–[c]	–
poly(rC)-oligo(dG)$_{12-18}$	0.8 ± 0.5	–	–
heteropolymeric RNA	3.4 ± 0.5	–	–
poly(dC)-oligo(dG)$_{12-18}$	–	12.5 ± 3.5	–
calf thymus activated DNA	–	14.1 ± 0.8	–
poly([^{3}H]rG)-poly(dC)	–	–	3.5 ± 0.8
HIV-2 RT	>50	>50	>50
AMV RT			
poly(rC)-oligo(dG)$_{12-18}$	>50[d]	–	–
poly([^{3}H]rG)-poly(dC)	–	–	>50
Mo-MLV RT			
poly(rC)-oligo(dG)$_{12-18}$	>50	–	–
poly([^{3}H]rG)-poly(dC)	–	–	0.8 ± 0.2
Taq DNA polymerase			
poly(dC)-oligo(dG)$_{12-18}$	–	>50	–
Klenow DNA polymerase			
poly(dC)-oligo(dG)$_{12-18}$	–	>50	–
HeLa cell nuclear extract DNA polymerases			
calf thymus activated DNA	–	>50	–
E. coli RNase HI			
poly([^{3}H]rG)-poly(dC)	–	–	2.7 ± 0.9
bovine pancreatic RNase A[e]			
human placental tRNA	–	–	>50

[a] Data are the means ± SD of three to four separate determinations. [b] Concentration of BBNH in the preincubation mix. The concentration of inhibitor in the final reaction assay was one-half that indicated. See Materials and Methods for details. [c] Not applicable. [d] No inhibition was noted at 50 μM BBNH. [e] Measured by degradation of human placental tRNA as described (Silberklang et al., 1979).

Inhibition Kinetic Analysis. Kinetic parameters were determined using concentrations of dNTP ranging from 0.25 to 10 K_m, with a fixed concentration of template/primer (2 K_m), or a similar concentration range for T/P with a fixed concentration of dNTP (2 K_m). The kinetic parameters and inhibition constants were calculated according to standard techniques (Segel, 1975) using the programs EZFIT (Perella Scientific, Glenolden, PA) and ENZFITTER (Biosoft, Cambridge, U.K.).

Irreversible Inactivation of RT by the Photolabel 9-Azido-5,6-dihydro-11-ethyl-6-methyl-11H-pyrido[2,3-b][1,5]-benzodiazepin-5-enone (9-AN). These studies were conducted essentially as we have previously described (Fletcher et al., 1995a). Briefly, RT (0.4 μM solution of 117 kDa heterodimer) was incubated with 9-AN (2 μM), in the absence or the presence of varying concentrations of BBNH, in 40 μL of 50 mM Tris-HCl (pH 8.0, 25 °C) and illuminated at 254 nm (20 μW/cm^2; Mineralight UVGL-25, UVP Inc., San Gabriel, CA). Inactivation of RT was followed by removing 2 μL aliquots of the mixture at different times and diluting the sample 250-fold into 50 mM Tris, pH 7.8 (37 °C), in order to dissociate any reversibly-bound photoaffinity probe. A 30 μL aliquot of this diluted sample was then assayed for RNA-dependent DNA polymerase activity as described above. The final dilution of photolabel and/or BBNH in these RT assays was 825-fold. This dilution factor resulted in final concentrations of approximately 2 nM for 9-AN and about 10 nM of BBNH, sufficiently low to prevent these compounds from interfering as reversible inhibitors of RT in the RDDP assay. No inhibition of RT RDDP, DDDP, or RNase H activities was found upon illumination of the enzyme at 254 nm in the absence of the photoaffinity probe, under the conditions used in our experiments. Similarly, no inactivation of RT was noted upon illumination of RT in the presence of BBNH alone, indicating that BBNH did not covalently cross-link to RT under the conditions of the photoinactivation experiments.

Photoinactivation of the RT-T/P binary complex was carried out as described above after preincubation of RT with 15 μg/mL poly(rC)-oligo(dG)$_{12-18}$ for 5 min prior to addition of 9-AN and/or BBNH, to ensure formation of the RT-T/P binary complex.

Cell Culture and Virus Replication. The following reagents were obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID: the CD4$^+$ MT-4 cell line (contributed by Dr. D. D. Richman) and the HTLV-III$_B$ laboratory strain of HIV-1 (contributed by Dr. R. C. Gallo). Cord blood mononuclear cells (CBMC) were isolated by Ficoll-Hypaque centrifugation from cord blood obtained from the Department of Obstetrics, SMBD-Jewish General Hospital. CBMC were activated by treatment with phytohemagglutinin before use (Fletcher et al., 1995b). Cells were infected with HIV-1 (multiplicity of infection 0.2−1.0) for 1 h, residual virus was removed by washing and centrifugation and the infected cells were then incubated in medium containing the appropriate concentrations of inhibitor. Every 2 days thereafter, one-half of the cell culture medium was replaced with fresh medium containing the same concentration of drug used initially. HIV-1 production was determined by measurement of viral p24 or RT activity in the culture supernatants, or by microscopic assessment of HIV-1 cytopathicity (MT-4 cells) (Yao et al., 1992).

RESULTS

Inhibition of RT RNA-Dependent and DNA-Dependent Polymerase Activities by BBNH. BBNH was found to be a good inhibitor of RT RDDP activity (Table 1). Similar extents of inhibition were noted with or without preincubation of RT with BBNH prior to assay. The degree of inhibition was influenced by the T/P used in the assay, with IC_{50} values ranging from 0.8 to 3.4 μM (Table 1). BBNH showed linear mixed-type inhibition of RT RDDP activity both with respect to T/P and to dNTP substrate (data not shown). BBNH was a less effective inhibitor of RT DDDP activity, with IC_{50}

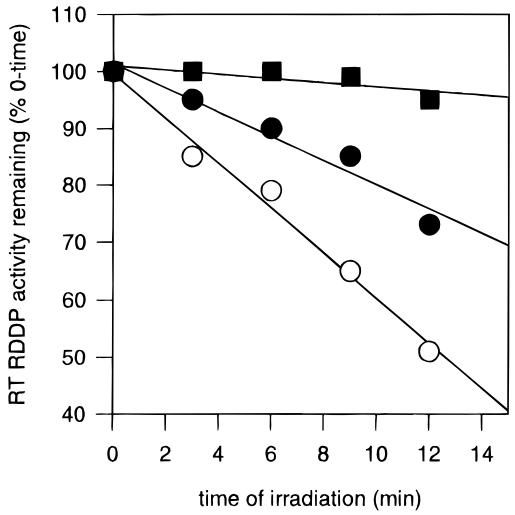

FIGURE 2: Irreversible photoinactivation of RT by 9-azidonevirapine in the absence (○) and the presence of 1 BBNH (●) and 10 μM BBNH (■).

values of 12−14 μM depending on the T/P used (Table 1). The compound was unable to inhibit other DNA polymerases, and was ineffective against the RDDP activity of HIV-2, AMV, and Mo-MLV reverse transcriptases (Table 1).

When RT was incubated with BBNH (at twice the appropriate IC_{50} value) for varying periods of time and then diluted 100-fold into reaction assays, complete recovery of both RDDP and DDDP activities was noted (data not shown). Inhibition of RT polymerase by BBNH is therefore reversible.

Ability of BBNH to Photoprotect RT from Irreversible Inactivation by 9-AN. The kinetics of inhibition of RT RDDP by BBNH were similar to those of other nonnucleoside RT inhibitors (NNRTI) (Balzarini et al., 1992; Fletcher et al., 1995a), suggesting that BBNH might inhibit RT polymerase by interacting with the NNRTI binding site. We therefore examined the ability of BBNH to protect RT from irreversible inactivation by 9-AN, a photoactivatable analog of the well-characterized NNRTI, nevirapine. We have previously used this technique to identify the binding pocket for carboxanilide type NNRTI (Fletcher et al., 1995a). Under the conditions of the photolabeling experiments, 9-AN inactivated RT at a rate of 0.035 min^{-1}. The concomitant presence of BBNH reduced the rate of photoinactivation in a concentration dependent manner, with inactivation rates of 0.018 and 0.011 min^{-1} in the presence of 1 μM and 10 μM BBNH, respectively (Figure 2).

BBNH was unable to protect either the RT-T/P binary complex or the RT-T/P-dNTP ternary complex from photoinactivation by 9-AN (data not shown), suggesting that the hydrazone was unable to bind to either of these enzyme mechanistic forms. BBNH therefore may bind only to the NNRTI pocket in the free enzyme form of RT.

Inhibition of RT RNase H Activity. In contrast to our observations of BBNH inhibition of RT DNA polymerase activity, little inhibition of RNase H activity (<20%) was noted when BBNH was added directly to complete RT RNase H assay mixtures. However, significant inhibition was noted when RT was first preincubated with BBNH and then assayed for RNase H activity. This inhibition of RNase H activity was time dependent, with maximum inhibition requiring approximately 15 min preincubation with BBNH.

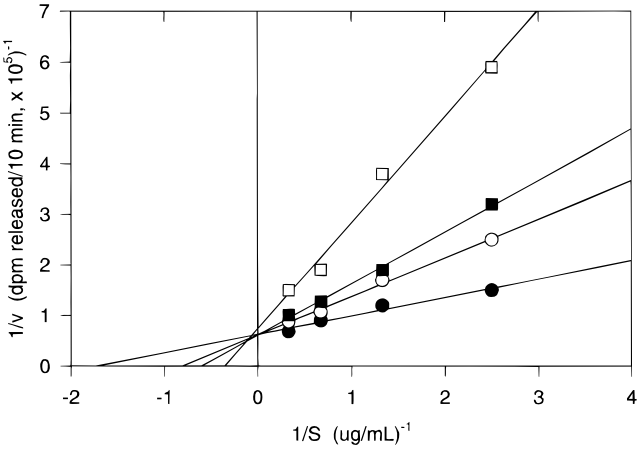

FIGURE 3: Kinetics of BBNH inhibition of RT RNase H activity. RT was preincubated in the absence (●) or the presence of 2 μM (○), 4 μM (■), or 8 μM (□) BBNH for 15 mins at 20 °C prior to initiation of reactions by the addition of the RNase H substrate poly([^{3}H]rG)-poly(dC). Reactions were allowed to proceed for 20 min at 37 °C and were then quenched and analyzed as described in Materials and Methods. The data points are the averages of duplicate determinations.

Under these conditions, IC_{50} values of about 3.5 μM were noted (Table 1). Inhibition of RT RNase H activity by BBNH was linear competitive with respect to the RNA−DNA duplex substrate (Figure 3).

When RT was preincubated with 20 μM BBNH for 20 min and then diluted at least 200-fold into RNase H reaction assays, complete recovery of nuclease activity was noted (data not shown). Inhibition of RT RNase H by BBNH is therefore reversible, as found for inhibition of RT polymerase activity.

BBNH also inhibited both *E. coli* RNase H and the RNase H activity of Mo-MLV RT, with similar or better potency than that noted for inhibition of HIV-1 RT RNase H (Table 1). As noted for HIV-1 RT RNase H, maximal inhibition of *E. coli* and Mo-MLV RT RNase H by BBNH also required preincubation of the enzyme and inhibitor. However, BBNH was unable to inhibit the RNase H activities of either AMV RT or HIV-2 RT under our assay conditions (Table 1).

Inhibition of NNRTI-Resistant Mutant RT by BBNH. The K103N, Y181C, Y181I, Y188H, and Y188L amino acid substitutions in HIV-1 RT result in more than 100-fold decreased sensitivity to NNRTI such as nevirapine and the carboxanilide UC84.[2] Interestingly, BBNH was a good inhibitor of the RDDP activity of all these mutants (Table 2). In addition, BBNH was an effective inhibitor of the RNase H activity of each of the mutants, with the exception of the Y181C RT (Table 2).

Inhibition of HIV Replication by BBNH. BBNH inhibited replication of HIV-1 in activated cord blood mononuclear cells with an EC_{50} of 1.5 μM (Figure 4). This value is consistent with the IC_{50} values for inhibition of recombinant HIV-1 RT RDDP and RNase H activities *in vitro* (Table 1). BBNH was also effective against HIV-1 replication in acutely-infected MT4 cells, with an EC_{50} of about 5 μM (data not shown). At concentrations above 10 μM, BBNH exhibited cytostatic activity, inhibiting cell proliferation. Nonetheless, these "arrested" cells remained viable, and resumed proliferation upon removal of BBNH from the

[2] G. Borkow, D. Motakis, and M. A. Parniak, unpublished data.

Table 2: Inhibition of NNI-Resistant Mutant RT by BBNH

RT	IC_{50} (μM)[a]	
	RDDP	RNase H[b]
wild type	0.8 ± 0.5	3.5 ± 0.8
K103N	2.4 ± 1.0	10.0 ± 2.1
Y181C	1.6 ± 0.3	>50
Y181I	3.6 ± 1.1	3.3 ± 0.6
Y188H	2.2 ± 0.5	3.7 ± 1.6
Y188L	3.3 ± 1.3	3.8 ± 0.3

[a] Values are the means ± SD of three separate experiments. RDDP activity was measured using poly(rC)-oligo$(dG)_{12-18}$ as T/P and [^{3}H]dGTP as substrate, and RNase H activity was measured using poly([^{3}H]rG)-poly(dC) as substrate, as described in Materials and Methods. [b] The concentrations are those of BBNH in the preincubation mix. The concentration of inhibitor in the final reaction assay was one-half that indicated. See Materials and Methods for details.

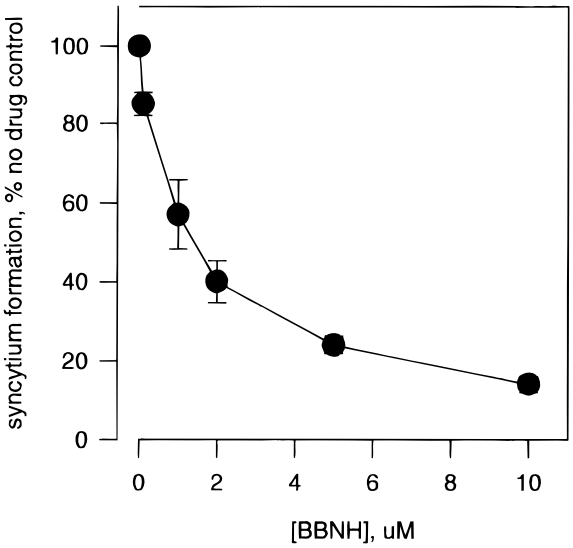

FIGURE 4: BBNH inhibition of HIV-1 replication in cord blood mononuclear cells. The data are the means ± SD of triplicate determinations from two separate experiments.

culture medium. Cytotoxicity resulting in cell death was noted only at concentrations above 25 μM.

Does BBNH Bind to Two Different Sites on RT? The differences in BBNH inhibition kinetics for RT polymerase compared to RNase H implied that inhibition of RT polymerase and RT RNase H activities might be due to BBNH interacting with different sites on the enzyme. However, the need for preincubation to attain substantial inhibition of RNase H could also be due to a slow conformational change resulting from BBNH binding to the NNI binding pocket in the polymerase domain, that results in alteration of the RNase H domain. Such effects are not without precedence, since TIBO has been found to "activate" RT RNase H activity after binding to the polymerase domain (Gopalakrishnan & Benkovic, 1994), and the cleavage specificity of HIV-1 RT RNase H is affected by the interaction of nevirapine with the NNRTI site (Palaniappan et al., 1995). In contrast to BBNH inhibition of RNase H, we found that the stimulation of RT RNase H by TIBO did not require preincubation of RT with the NNRTI.

We reasoned that if BBNH inhibition of RNase H resulted from binding only to the NNRTI site in the polymerase domain, then this inhibition should be attenuated in the presence of other compounds that compete for binding to this site. We therefore examined the ability of BBNH to inhibit RT RNase H activity in the absence and in the presence of other NNRTI, namely, TIBO (De Vreese et al., 1992) and the carboxanilides UC38 and UC84 (Fletcher et al., 1995a). Each of these inhibits RT RDDP activity with IC_{50} values much lower than that of BBNH. For example, the IC_{50} for UC38 inhibition of RT RDDP is about 0.05 μM and that for TIBO and UC84 is about 0.2 μM (De Vreese et al., 1992; Fletcher et al., 1995a,b). As shown in Table 3, neither UC38 nor UC84 affects RT RNase H activity, whereas TIBO activates RT RNase H. BBNH was able to inhibit RNase H activity equally well both in the absence and in the presence of all these NNI, suggesting that BBNH inhibits RT RNase H by interacting with a site other than the NNI binding pocket in the RT polymerase domain. This was further tested using RT in which the NNI binding pocket had been irreversibly blocked by the photolabel 9-AN. This photoinactivated enzyme had less than 20% residual DNA polymerase activity, but retained more than 80% RNase H activity. BBNH inhibited this residual RNase H activity with a potency similar to that noted with wt RT (Table 3).

Table 3: Inhibition of RT RDDP and RNase H by BBNH in the Presence of Other Non-Nucleoside Inhibitors

inhibitor	activity (% control)[a]	
	RDDP[b]	RNase H[c]
none	100	100
BBNH (10 μM)	<10	15
UC38 (10 μM)	<10	100
UC38 (10 μM) + BBNH (10 μM)	<10	15
UC84 (10 μM)	<20	100
UC34 (10 μM) + BBNH (10 μM)	<10	15
TIBO (10 μM)	nd[d]	140
TIBO (10 μM) + BBNH (10 μM)	nd	32
photoinactivated RT[e]	<20	82
photoinactivated RT + BBNH (10 μM)	<20	18

[a] Data are the averages of two separate determinations. [b] RT RDDP activity was determined using poly(rC)-oligo$(dG)_{12-18}$ and dGTP as described in Materials and Methods. [c] RNase H activity was assayed after 10 min preincubation with the indicated concentration of BBNH in the absence or the presence of other inhibitors as indicated prior to addition of reaction assay components. [d] Not determined. [e] RT was irradiated at 254 nm in the presence of 6 μM 9-AN for 20 min. RT was separated from unreacted 9-AN prior to use.

DISCUSSION

Each of the activities of the multifunctional reverse transcriptase of HIV-1 is crucial for virus replication. Although numerous inhibitors of HIV-1 RT polymerase activity have been developed, very few inhibitors of RT RNase H activity have been described. Covalent modification of RT with the sulfhydryl reactive agent *N*-ethylmaleimide inhibits RT RNase H without affecting RT polymerase activity (Hizi et al., 1992). The natural product illimaquinone inhibits RT RNase H, although rather poorly (Loya et al., 1990). AZTMP inhibits RT RNase H, although very high concentrations are required. This inhibitory activity of AZTMP is strongly dependent on metal cation identity (Tan et al., 1991; Zhan et al., 1994). Interestingly, the metal chelator *o*-phenanthroline inhibits RT RNase H (Hizi et al., 1991), although only at exceptionally high concentrations in excess of 5 mM. Certain NNI of RT polymerase activity can also affect RT RNase H activity. For example, TIBO inhibits RT polymerase activity while concomitantly activating RNase H activity (Gopalakrishnan & Benkovic, 1994). Naphthylenesulfonic acid derivatives inhibit RT RNase H, but only at concentrations significantly higher than those at which BBNH is effective (Mohan et al., 1994).

The *N*-acyl hydrazone BBNH inhibited both the DNA polymerase and the RNase H activities of HIV-1 RT. The inhibition kinetics for HIV-1 RT DNA polymerase activity were similar to those of NNRTI such as nevirapine (Merluzzi et al., 1990), TIBO (De Vreese et al., 1992), and the carboxanilides (Fletcher et al., 1995a). BBNH also protected RT from irreversible inactivation by the photolabel 9-AN that inhibits RT by covalent interaction with amino acid residue Y181 (Cohen et al., 1991), an important component of the NNRTI binding pocket (Smerdon et al., 1994; Ding et al., 1995; Ren et al., 1995). NNRTI such as nevirapine and the carboxanilides prevent inactivation of RT by 9-AN (Wu et al., 1991; Fletcher et al., 1995a). The photoprotection of RT by the hydrazone BBNH thus demonstrates that this inhibitor can bind at or near the NNRTI binding pocket in the polymerase domain of RT. Consistent with this is our observation that BBNH was unable to inhibit the DNA polymerase activity of other retroviral reverse transcriptases or DNA polymerases that do not possess a binding pocket for NNRTI compounds effective against HIV-1. Interestingly, BBNH showed good inhibition of K103N, Y181C/I, and Y188H/L mutant RT. The K103, Y181, and Y188 residues are important components of the NNRTI binding pocket (Smerdon et al., 1994; Ding et al., 1995; Ren et al., 1995), and the mutants show high-level resistance to nevirapine and to the carboxanilide UC84. Thus, BBNH must bind to RT by interacting with residues other than those which are important contacts for nevirapine and UC84. Interestingly, although BBNH was a good inhibitor of the DNA polymerase activity of the Y181C mutant RT, it was unable to inhibit the RNase H activity of this mutant. We are unable to readily account for the "resistance" of the RNase H of this particular mutant to inhibition by BBNH, especially when the RNase H activity of another mutant, Y181I, was sensitive to BBNH inhibition. However, The RNase H domain of HIV-1 RT is quite sensitive to conformational changes in the polymerase domain (Volkmann et al., 1993; Mizrahi et al., 1994), and perhaps the changes induced by the Y181C substitution result in long-range alterations in the RNase H domain, thereby preventing BBNH binding to this site.

Crystallographic and modeling studies show that the RNase H domain of HIV-1 RT is structurally similar to *E. coli* RNase H (Katayanagi et al., 1990; Yang et al., 1990; Kohlstaedt et al., 1992) and to Mo-MLV RT RNase H (Nakamura et al., 1991). *E. coli* RNase H possesses distinct sites for the binding of essential divalent metal cations (Katayanagi et al., 1993). We considered that the metal chelating properties of BBNH might enable interaction with the RNase H metal ions. The binding of chelators to metal-dependent enzymes often demonstrates a time-dependence of inhibition (Coombs et al., 1962). Indeed, we found that BBNH inhibition of each of HIV-1 RT RNase H, Mo-MLV RT RNase H, and *E. coli* RNase H was time-dependent, with maximal inhibition attained after approximately 10 min preincubation of enzyme and inhibitor. In contrast, BBNH inhibition of RT RDDP and DDDP needed no preincubation. Thus, BBNH may inhibit RT RNase H by binding to metal ions in the RNase H active site (Kohlstaedt et al., 1992; Rodgers et al., 1995), whereas inhibition of RT polymerase activities results from interaction with the NNRTI pocket in a non-metal-dependent manner. However, BBNH was unable to inhibit the RNase H activity of AMV or HIV-2 RT, and had no effect on other metal-dependent DNA polymerases. Thus, interactions other than BBNH-metal binding must be important for the observed inhibition of the multiple activities of HIV-1 RT.

It is possible that the time-dependent BBNH inhibition of RT RNase H results from a slow conformational change in the RNase H domain of RT due to binding of BBNH to the NNRTI pocket. The RNase H domain of HIV-1 RT is highly flexible and sensitive to conformational changes in the polymerase domain (Volkmann et al., 1993; Mizrahi et al., 1994). For example, binding of TIBO to the NNRTI pocket leads to stimulation of RT RNase H activity (Gopalakrishnan & Benkovic, 1994), and the interaction of nevirapine with the NNRTI site in the polymerase domain of HIV-1 RT alters the cleavage specificity of the RNase H (Palaniappan et al., 1995). However, we believe that the inhibition of RT RNase H activity by BBNH likely results from interaction of the hydrazone at a site distinct from the NNRTI binding pocket, for several reasons. First, BBNH is a competitive inhibitor of RT RNase H, implying that the inhibitor interacts with the RNase H active site. This site is well removed from the NNRTI binding pocket (Kohlstaedt et al., 1992). Second, BBNH inhibits RT RNase H activity in the presence of equimolar amounts of NNRTI that have affinities for the NNI-binding pocket at least 5−10-fold greater than that of BBNH. Under these conditions, interaction of BBNH with the NNRTI pocket should be significantly attenuated. Third, BBNH was a good inhibitor of Mo-MLV RT RNase H activity. Mo-MLV RT has no NNRTI binding pocket and thus NNRTI with good activity against HIV-1 RT are unable to inhibit the DNA polymerase activity of this enzyme. Indeed, BBNH was unable to inhibit Mo-MLV RT DNA polymerase activity (Table 1). Thus, BBNH inhibition of Mo-MLV RT RNase H may be due to interaction with the RNAse H domain of the enzyme. Finally, the most compelling evidence for our conjecture is the fact that BBNH was able to inhibit the RNase H activity of RT in which the polymerase NNRTI site was irreversibly blocked by covalent modification with the photoaffinity label 9-AN. In this case, BBNH inhibition of RT RNase H must be due to interaction with a site other than the RT polymerase NNRTI site. Preliminary molecular modeling studies[3] imply a good fit between BBNH and the RT RNase H catalytic site, in contact with one of the divalent metal cations in this site. More rigorous analyses are in progress. Nonetheless, definitive proof that BBNH interacts with two sites on HIV-1 RT must await direct binding analysis.

Our initial rationale for studying BBNH was to investigate the metal-binding of this compound as an inhibitory mechanism against the several metal-dependent enzymatic activities of HIV-1 RT. Although BBNH is indeed a good inhibitor of all activities of HIV-1 RT, we feel that metal-binding probably plays only a minor role. Inhibition likely results from other types of BBNH-RT interactions, since the inhibitory activity of BBNH was highly specific for HIV-1 RT, with no measurable effect on other metal-dependent DNA polymerases and retroviral reverse transcriptases.

In conclusion, we believe that *N*-(4-*tert*-butylbenzoyl)-2-hydroxy-1-naphthaldehyde hydrazone (BBNH) is the first example of an inhibitor with reasonable activity against each of the enzymatic activities of the multifunctional HIV-1 reverse transcriptase. This multiple target inhibition may

[3] R. S. Fletcher, G. Borkow, M. A. Parniak, and G. I. Dmitrienko, unpublished data.

result from interaction of BBNH with at least two different sites on the enzyme, including the RNase H catalytic site. The inhibitor is also effective against several mutant RT that have high-level resistance to other NNRTI. BBNH therefore represents a novel lead structure for the development not only of inhibitors of RT RNase H, but also of effective multitarget inhibitors of HIV-1.

ACKNOWLEDGMENT

We are grateful to Prof. Amnon Hizi, Tel Aviv University, for supplying us with the plasmid for the expression of HIV-2 reverse transcriptase. We thank Dr. Prem Ponka for providing us with initial samples of BBNH. We also thank Ms. M. T. Ruiz and Dr. M. Zannis- Hadjopoulos of the McGill University Cancer Centre for providing the HeLa cell nuclear extracts.

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BI9624696