

September 1995

Anti-human immunodeficiency virus (HIV) activities of halogenated gomisin J derivatives, new nonnucleoside inhibitors of HIV type 1 reverse transcriptase

Toshiaki Fujihashi
Thomas Jefferson University

Hiroto Hara
University of Pennsylvania

Toshiya Sakata
Thomas Jefferson University

Kazuya Mori
Institute for Molecular Genetics, Japan

Hiroataka Higuchi
University of Pennsylvania

[Let us know how access to this document benefits you](#)

[See next page for additional authors](#)

Follow this and additional works at: <http://jdc.jefferson.edu/bmpfp>

 Part of the [Medical Biochemistry Commons](#)

Recommended Citation

Fujihashi, Toshiaki; Hara, Hiroto; Sakata, Toshiya; Mori, Kazuya; Higuchi, Hiroataka; Tanaka, Akio; Kaji, Hideko; and Kaji, Akira, "Anti-human immunodeficiency virus (HIV) activities of halogenated gomisin J derivatives, new nonnucleoside inhibitors of HIV type 1 reverse transcriptase" (1995). *Department of Biochemistry and Molecular Biology Faculty Papers*. Paper 15.
<http://jdc.jefferson.edu/bmpfp/15>

Authors

Toshiaki Fujihashi, Hiroto Hara, Toshiya Sakata, Kazuya Mori, Hirotaka Higuchi, Akio Tanaka, Hideko Kaji,
and Akira Kaji

Anti-Human Immunodeficiency Virus (HIV) Activities of Halogenated Gomisin J Derivatives, New Nonnucleoside Inhibitors of HIV Type 1 Reverse Transcriptase

TOSHIKI FUJIHASHI,^{1†} HIROTO HARA,² TOSHIYA SAKATA,^{1†} KAZUYA MORI,^{3†}
HIROTAKA HIGUCHI,^{2†} AKIO TANAKA,² HIDEKO KAJI,¹ AND AKIRA KAJI^{2*}

Department of Pharmacology, Jefferson Medical College, Philadelphia, Pennsylvania 19107¹; Institute for Molecular Genetics, Tsumura and Co., 3586 Yoshiwara, Ami-machi, Inashiki-gun, Ibaraki, 300-11 Japan²; and Department of Microbiology, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104-6076²

Received 22 February 1995/Returned for modification 3 April 1995/Accepted 6 July 1995

Halogenated gomisin J (a derivative of lignan compound), represented by the bromine derivative 1506 {(6R, 7S, S-biar)-4,9-dibromo-3,10-dihydroxy-1,2,11,12-tetramethoxy-6,7-dimethyl-5,6,7,8-tetrahydrodibenzo[a,c]cyclooctene}, was found to be a potent inhibitor of the cytopathic effects of human immunodeficiency virus type 1 (HIV-1) on MT-4 human T cells (50% effective dose, 0.1 to 0.5 μM). Gomisin J derivatives were active in preventing p24 production from acutely HIV-1-infected H9 cells. The selective indices (toxic dose/effective dose) of these compounds were as high as >300 in some systems. 1506 was active against 3'-azido-3'-deoxythymidine-resistant HIV-1 and acted synergistically with AZT and 2',3'-ddC. 1506 inhibited HIV-1 reverse transcriptase (RT) in vitro but not HIV-1 protease. From the time-of-addition experiment, 1506 was found to inhibit the early phase of the HIV life cycle. A 1506-resistant HIV mutant was selected and shown to possess a mutation within the RT-coding region (at position 188 [Tyr to Leu]). The mutant RT expressed in *Escherichia coli* was resistant to 1506 in the in vitro RT assay. Some of the HIV strains resistant to other nonnucleoside HIV-1 RT inhibitors were also resistant to 1506. Comparison of various gomisin J derivatives with gomisin J showed that iodine, bromine, and chlorine in the fourth and ninth positions increased RT inhibitory activity as well as cytoprotective activity.

Lignans are phenylpropanoid dimers distributed very widely in the plant kingdom. There are various kinds of lignan depending on the mode of dimerization of phenylpropanoid. Gomisin is a dibenzocyclooctadiene-type lignan compound and a component of *Schizandra* fruit extracts (30–32, 70). There are various types of gomisins, A, B, C, D, E, F, G, J, and N; some possess central nervous system depressant, analgesic, antitussive, and/or Ca antagonist activities (42, 69). As for the antiviral activities of lignans, it has been reported that tetrahydronaphthalene-type lignans have selective inhibitory activities on herpes simplex virus and cytomegalovirus (41, 43) and that dibenzylbutanolide-type lignans have an inhibitory effect on human immunodeficiency virus (HIV) replication (63). However, the ratio of toxic dose to effective dose (selective index) of dibenzylbutanolide-type lignan was no more than 5, suggesting that this could not be used as a clinically effective anti-HIV agent.

In continuation of our search for anti-HIV agents (19, 20, 26, 52), we report in this paper new gomisin J derivatives which have potent anti-HIV activities with much higher selective indices than that of dibenzylbutanolide-type lignan. The highest selective index among gomisin J derivatives was over 300.

MATERIALS AND METHODS

Chemicals. Gomisin J was isolated as previously described (32). Halogenated derivatives (Fig. 1) of gomisin J were synthesized from gomisin J by using halogenating agents such as *N*-bromosuccinimide. Detailed descriptions of synthesis and characterization will be published elsewhere. Structures of pure, newly

synthesized compounds were determined by nuclear magnetic resonance and infrared analyses. These compounds were kindly provided by Tanaka, Tsumura Central Research Institute, Ibaraki, Japan. 3'-Azido-3'-deoxythymidine (AZT) and 2',3'-ddC were purchased from Sigma Chemical Co. (St. Louis, Mo.). Tetrahydroimidazo[4,5,1-*jk*] [1,4]-benzodiazepin-2(1*H*)-one (TIBO) derivative R82150 (53) and HIV protease (PR) inhibitor Ro 31-8959 (9) were kindly provided by M. Janssen (Janssen, Beerse, Belgium) and N. A. Roberts (Roche Products Limited, Herts, United Kingdom), respectively. All gomisin J derivatives were dissolved in dimethyl sulfoxide. The final concentration of dimethyl sulfoxide in culture medium was adjusted to 0.3%. AZT and ddC were dissolved in water.

Cells and viruses. Human T-cell line MT-4 cells (25), Sup-T1 cells (67), and H9 cells (56) were kindly provided by N. Yamamoto (Tokyo Medical and Dental University, Tokyo, Japan), J. A. Hoxie (University of Pennsylvania, Philadelphia, Pa.), and H. Mitsuya (National Cancer Institute, Bethesda, Md.), respectively. Chronically HIV type 1 (HIV-1)-infected H9 cells (H9/III_B) and MT-2 cells (25) were provided by the National Institutes of Health AIDS Research and Reference Reagent Program. Chronically HIV-1-infected Molt-4 cells (Molt-4/HTLV-III_B) were kindly provided by E. Henderson (Temple University, Philadelphia, Pa.). These cells were grown in 25 mM HEPES (*N*-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)-buffered RPMI 1640 (GIBCO) supplemented with 10% fetal calf serum, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 0.25 μg of amphotericin B per ml. At this concentration, amphotericin B is effective as an antimycotic in tissue culture but has no inhibitory activity for HIV replication (50, 64). HIV-1 was prepared from a culture supernatant of Molt-4/HTLV-III_B cells. The post-AZT isolates (A012D) of HIV-1 (38), simian immunodeficiency virus (SIV) MAC₂₅₁ (10), A17 variant strain of HIV_{III_B} resistant to pyridinone (51), and N119 nevirapine-resistant HIV-1 (61) were provided by the National Institutes of Health AIDS Research and Reference Reagent Program. A TIBO-resistant strain of HIV-1, L100I (46), was kindly supplied by J. W. Mellors (University of Pittsburgh Medical Center, Pittsburgh, Pa.).

Anti-HIV-1 assays of cultured cell lines. The procedure for measuring the cytoprotective activities of samples with MT-4 cells has previously been described (54) and was performed with slight modifications as described previously (20). The anti-HIV activity of the compound was expressed as the concentration required to reduce the number of dead cells to 50% of the control without an anti-HIV agent (ED₅₀). The cytotoxic effect on uninfected MT-4 cells was assessed by incubating uninfected cells with the compound under the same conditions as described above, except for the HIV addition, and expressed as the concentration required to kill 50% of cells (CD₅₀). Anti-HIV-1 activity with Sup-T1 cells was determined by counting the total number of multinuclear giant

* Corresponding author.

† Present address: New Drug Discovery Laboratories, Tsumura and Co., 3586 Yoshiwara, Ami-machi, Inashiki-gun, Ibaraki, 300-11 Japan.

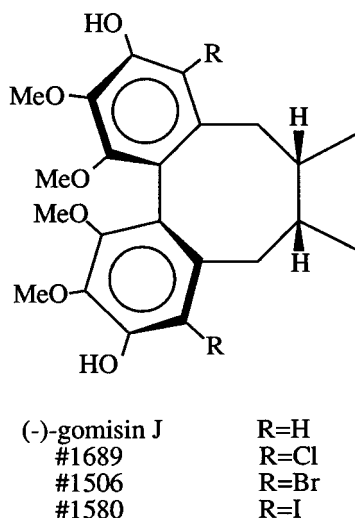


FIG. 1. Chemical structures of gomisin J and related compounds.

cells in each well 4 days after viral infection (1, 52). The cytotoxic effect against uninfected Sup-T1 cells was assessed by incubating uninfected cells with the compound for 7 days. The procedure for measuring viral p24 production by infected H9 cells was previously described (5, 20, 52). Cell number was determined by the MTT method (49).

Syncytium inhibition assay. The syncytium assay was performed on a 96-well microplate by mixing 5×10^4 Sup-T1 cells with 2.5×10^4 H9/III_B cells at 37°C in a CO₂ incubator for 18 h (44). The test compound was present in the medium throughout the test. The number of giant cells in each well was counted by microscopic examination.

Viral p24 expression in chronically HIV-1-infected cells. To study the inhibition of HIV-1 production by 1506 in chronically infected cells, 5×10^4 H9/III_B cells were incubated for 5 days in the presence of test compounds. The amount of viral p24 in supernatant fluid on the fifth day of culture was determined. The cytotoxicities of test compounds for H9/III_B cells were determined by the MTT method on day 5 after the addition of test compounds.

HIV-1 RT and PR assays. The inhibitory effects of 1506 on wild-type reverse transcriptase (RT) and mutant RT with an amino acid change at the 188th position (T-188→L [T188L]) were studied by using plasmids harboring either the wild-type or mutated RT gene kindly supplied by S. H. Hughes (National Cancer Institute). *Escherichia coli* DH5α was transformed with plasmids containing one of these genes, and crude RTs were prepared as previously described (4, 27). HIV-1 RT assays were performed as previously described (52, 71).

For HIV-1 PR assays (59), purified HIV-1 PR (2.5 μg/ml; Bachem) and 28 mM synthetic peptide (HIV substrate III; Bachem) [His-Lys-Ala-Arg-Val-Leu-(p-NO₂)Phe-Glu-Ala-Nle-Ser-amide] were incubated with various concentrations of test compound in 40 μl of reaction buffer (100 mM NaOAc [pH 5.5], 1.6 M NaCl, 1.2 mM dithiothreitol) for 3 h at 37°C. Reactions were terminated by the addition of an equal volume of 10% AcOH. The cleavage product was quantitated by high-pressure liquid chromatography with a YMC-AM312 column. The inhibitory effect of each test compound was expressed as the concentration required to reduce enzyme activity by 50% (IC₅₀).

Drug combination. The combined effects of 1506 and AZT or ddC were examined with MT-4 cells infected with HIV-1_{III_B} under the experimental conditions used for the determination of ED₅₀. To assess whether the drug combination resulted in synergistic, additive, or antagonistic effects, the isobologram technique was used (17, 60).

Time-of-addition experiment. To determine the target step of 1506 in the HIV life cycle, the time-of-addition experiment was performed according to the method previously described (14).

Isolation of 1506-resistant HIV-1 variants. The selection of HIV-1 variants resistant to 1506 was performed essentially as previously described for other resistant HIV mutants (21, 22). MT-4 cells (5×10^5 cells per ml) were infected with HIV_{III_B} in the presence of subeffective concentrations of drugs. The amount of 1506 was gradually increased during subsequent passages. For each passage, cultures were infected with the HIV obtained from the preceding passage. The resistant mutant thus obtained was named 1506R.

Identification of the nucleotide change causing resistance to 1506. MT-4 cells (10^7 cells) infected with wild-type HIV-1 or 1506R were cultured for 2 days. Cells were pelleted, and cellular DNA with integrated HIV-1 DNA was prepared by using the Qiagen genomic DNA preparation kit. A portion (0.5 μg) of cellular DNA was subjected to PCR with 21-mer primers (+ and - primers corresponding to positions 1869 through 1889 and 3974 through 3994 [58] of HIVhxb2 [55, 57, 65], respectively). PCR was performed with Vent polymerase (35 cycles)

(Perkin-Elmer Cetus). The 2.1-kb PCR product was electrophoretically eluted after separation by gel electrophoresis and was used for the second PCR (25 cycles). The amplified 2.1-kb PCR products were subjected to DNA sequencing without subcloning (Cycle DNA Sequencing; Bethesda Research Laboratories). Sequence analysis was repeated on DNA prepared from an independent PCR, and the results were identical.

Southern blot analysis of HIV-1 DNA. H9 cells were infected with HIV-1_{III_B} and cultured in the presence or absence of 1506 (7.4 μM [nontoxic dose]) for 24 h (34). DNA was prepared from 24-h-cultured cells by using the Qiagen genomic DNA preparation kit. Each undigested DNA sample (5 μg) was subjected to agarose gel electrophoresis, with subsequent Southern blotting analysis. The blotted filter was probed with ³²P-labeled HIV-1 genomic DNA (9.4-kb *Bst*EI-*Bst*EI fragment), prepared from λ DNA harboring HIVhxb2 (55, 57, 65) which was provided by the National Institutes of Health AIDS Research and Reference Reagent Program.

RESULTS

Antiretroviral activities of gomisin J derivatives in MT-4 cells. In the course of screening over 400 compounds of plant origin, we found that a lignan derivative, (-)-gomisin J, isolated from *Schizandra chinensis* BAILL had significant anti-HIV activity. Other lignan components [such as gomisins A, D, E, and N, deoxyshizandrin, and (+)-gomisin J] failed to exhibit anti-HIV action (data not shown). Among the various gomisin J derivatives tested for anti-HIV-1 activities in the MT-4 cell system, 4,9-dihalogenated gomisin J derivatives were found to be potent inhibitors (ED₅₀s of 0.39 to 1.23 μM) of HIV-1-induced cytopathicity (Table 1). The anti-HIV-1 activity of 1506 [(6*R*, 7*S*, 8*S*-biar)-4,9-dibromo-3,10-dihydroxy-1,2,11,12-tetramethoxy-6,7-dimethyl-5,6,7,8-tetrahydrodibenzo[*a,c*]cyclooctene] was 33-fold stronger than that of (-)-gomisin J. Other derivatives, 1580 (iodine in place of bromine) and 1689 (chlorine in place of bromine), were also effective.

The gomisin J derivatives were similar to other known non-nucleoside HIV RT inhibitors in that they were specific inhibitors of HIV-1 and did not inhibit HIV-2 or SIV replication (Table 2). In contrast, AZT was effective against all of these viruses, confirming previous reports (23, 35, 45, 47, 53).

Inhibition of HIV-1-induced giant cell formation and p24 production by gomisin J derivatives. Gomisin J derivatives were effective in tests of their protective effects on giant cell formation induced by HIV-1 infection of an HIV-infected hu-

TABLE 1. Anti-HIV-1 activities of gomisin J and related compounds in culture cells^a

Compound	Assay system ^b	ED ₅₀ (μM)	CD ₅₀ (μM)	Selective index ^c
(-)-Gomisin J	Cell death	17.5	90.9	5.2
1689	Cell death	1.23 ± 0.20	41.9 ± 11.1	34.1
1506	Cell death	0.52 ± 0.06	21.1 ± 1.5	40.6
1580	Cell death	0.39 ± 0.05	21.0 ± 4.8	53.8
1689	Giant cell formation	1.52 ± 0.51	31.3 ± 3.2	20.6
1506	Giant cell formation	0.39 ± 0.09	14.9 ± 1.6	38.2
1580	Giant cell formation	0.16 ± 0.04	17.5 ± 4.2	109.4
1689	p24 production	0.68 ± 0.25	28.5 ± 3.5	41.9
1506	p24 production	0.13 ± 0.03	24.0 ± 3.6	184.6
1580	p24 production	0.06 ± 0.01	22.5 ± 5.3	375.0

^a Cells were infected with 50 HIV units per well (6×10^4 cells per well) for cell death, 500 HIV units per well (5×10^4 cells per well) for giant cell formation, and 80 HIV units per well (4×10^4 cells per well) for p24 production assays. Antiviral effective dose required to achieve 50% inhibition of HIV infection was measured. One HIV unit was defined as the amount of virus that killed 50% of infected MT-4 cells within 5 days of infection. Data are the means and standard errors of three or more determinations, except for the (-)-gomisin J data, which are the means of duplicate determinations.

^b MT-4, Sup-T1, and H9 cells were used in cell death, giant cell formation, and p24 production assays, respectively.

^c Ratio of CD₅₀ to ED₅₀.

TABLE 2. Lack of activities of gomisin J derivatives against HIV-2 and SIV in MT-4 target cells

Virus	Strain	ED (μM) ^a				
		1506	1689	1580	R82150	AZT
HIV-2	ROD	>18	>22	>16	>105	0.106
HIV-2	CBL-20	>18	>22	>16	>105	0.024
SIV	MAC ₂₅₁	18 (ED ₅₀)	22 (ED ₈)	16 (ED ₃₇)	>105	0.010

^a Data are ED₅₀s unless noted otherwise.

man T-cell line (Sup-T1) (Table 1). 1580 was the most potent compound for inhibiting giant cell formation among the three halogenated gomisin J derivatives, followed by 1506 and 1689. The iodine derivative (1580) of gomisin J gave the highest selective index (CD₅₀/ED₅₀). The gomisin J derivatives were shown to be effective in reducing p24 production in acutely infected H9 cells (Table 1). The IC₅₀s of these gomisin J derivatives for p24 production in this cell system were lower than those for the MT-4 and Sup-T1 cell systems. The iodine derivative (1580) was the most active, followed by the bromine (1506) and chlorine (1689) derivatives.

Lack of inhibitory effect on CD4-gp120 interaction and HIV production from chronically HIV-infected cells. When chronically infected H9 cells (H9/III_B) and Sup-T1 cells are mixed, the CD4 receptor on Sup-T1 cells and HIV-1 gp120 on the membrane of H9/III_B cells interact with each other, resulting in syncytium formation (68). No gomisin J derivative inhibited this system (data not shown). Tested under the same conditions, dextran sulfate (molecular weight, 8,000) inhibited syncytium formation by 50% at a concentration of 4.4 μM (2).

To investigate whether gomisin J derivatives inhibit the late stage of the HIV life cycle, we tested the effects of 1506 on p24 production by chronically infected H9 cells. Compound 1506 failed to reduce p24 production in this system at the dose which does not influence cell viability (data not shown). The positive control, PR inhibitor Ro 31-8959 (9), inhibited p24 production.

Time-of-addition experiment. Inhibitors were added at various times after HIV infection. Inhibitors, such as dextran sulfate, that act at the early step (2) must be added early to be effective, while those that act at the late step, such as HIV PR inhibitors (9), can be added later. As shown in Fig. 2, AZT, which acts at the RT step (48), was added to cells up to 6 h after infection without any loss of activity. The activity time courses of compounds 1506 and 1580 were comparable to that of AZT.

Effects of gomisin J derivatives on HIV-1 RT and HIV-1 PR activities. Although 1506 inhibited HIV-1 RT with poly(rA)·oligo(dT)₁₂₋₁₈, the IC₅₀ of 1506 for RT activity was 6- to 26-fold higher than the ED₅₀ for anti-HIV action (data not shown). Inhibitory activities were about 2-fold higher with poly(rC)·oligo(dG)₁₂₋₁₈, but the ED₅₀s of these anti-HIV agents were still lower than the IC₅₀s for HIV RT. The IC₅₀s of (-)-gomisin J, 1689, 1506, and 1580 were 45.0, 1.2, 1.1, and 0.8 μM , respectively. [These data are the means of two determinations, with poly(rC)·oligo(dG)₁₂₋₁₈ (1:1 mixture by weight) as the primer-template.] Although the exact correlation between the cytoprotective and RT inhibitory activities of halogenated derivatives was not obtained, 1580 was the most potent agent in both of these assays. The data show that the halogenation of gomisin J increased anti-HIV activity through its increase in the inhibitory activity of RT. Compound 1506 did not inhibit HIV-1 PR, even at a concentration that was

over 300-fold the effective concentration in this cell culture system (data not shown).

Kinetic studies with HIV-1 RT. Although 1580 (the iodine derivative) proved to be the most effective, it was found to be chemically more labile than the bromine derivative (1506). For this reason, most of the subsequent detailed studies were carried out with 1506 as the representative of halogenated gomisin J derivatives. Kinetic studies were performed with purified recombinant HIV-1 RT and various substrate (dGTP) and template-primer [poly(C)·oligo(dG)] concentrations. As shown in Fig. 3, the inhibition of RT by 1506 appeared to be noncompetitive with regard to the substrate and mixed (noncompetitive and uncompetitive) with respect to the template-primer. This contrasts with repandusinic acid, a nonnucleoside RT inhibitor, which gives competitive inhibition with respect to the primer-template of HIV RT (52). From the data presented in Fig. 3 and the Dixon plot of these data (15), the K_m for dGTP and K_i for 1506 were calculated to be 1.5 and 2.2 μM (at the template-primer complex concentration of 5 $\mu\text{g/ml}$), respectively. On the other hand, the K_m for the template-primer complex was 1.5 $\mu\text{g/ml}$ (1 μg contains 0.5 μg of primer and 0.5 μg of template), whereas the K_i for 1506 varied slightly, depending on the template-primer concentration (from 2.2 μM at 5 μg of template-primer per ml to 3.2 μM at 1 μg of template-primer per ml), in a manner similar to the K_i of TIBO (12).

HIV-1 mutant resistant to 1506. After 20 passages of HIV-1_{IIIB} in MT-4 cells in the presence of 1506, a virus strain, 1506R, for which the ED₅₀ of 1506 was higher than 10 μM was obtained. We found no difference in nucleotide sequence between wild-type HIV-1 and 1506R in the integrase-coding region (data not shown). On the other hand, four nucleotide changes were detected in the RT-coding region. Two changes at nucleotides 2691 (T to C) and 2692 (A to T) resulted in a substitution at amino acid 188 from Tyr to Leu. The other changes at nucleotides 2718 (G to A) and 2745 (G to A), corresponding to amino acids 196 (Gly) and 205 (Leu), resulted in no amino acid substitutions. These results confirmed the notion that the target step of gomisin J is the RT step of HIV.

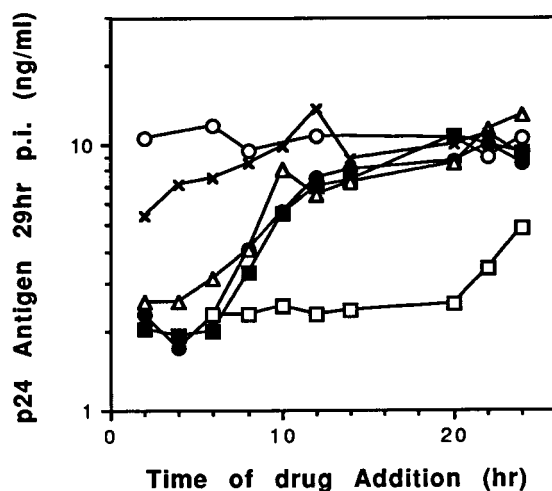


FIG. 2. Time-of-addition experiment. H9 cells were infected with HIV-1_{IIIB} at a multiplicity of infection of 0.1, and compounds were added at various times postinfection (p.i.) as described in Materials and Methods. The amounts of p24 accumulated at 29 h p.i. were determined with a Coulter enzyme assay kit. ○, no drug; ●, 1506 (7.4 μM); ■, 1580 (7 μM); △, AZT (1.9 μM); □, Ro 31-8959 (HIV PR inhibitor; 0.65 μM); ×, dextran sulfate (molecular weight, 8,000; 6.3 μM).

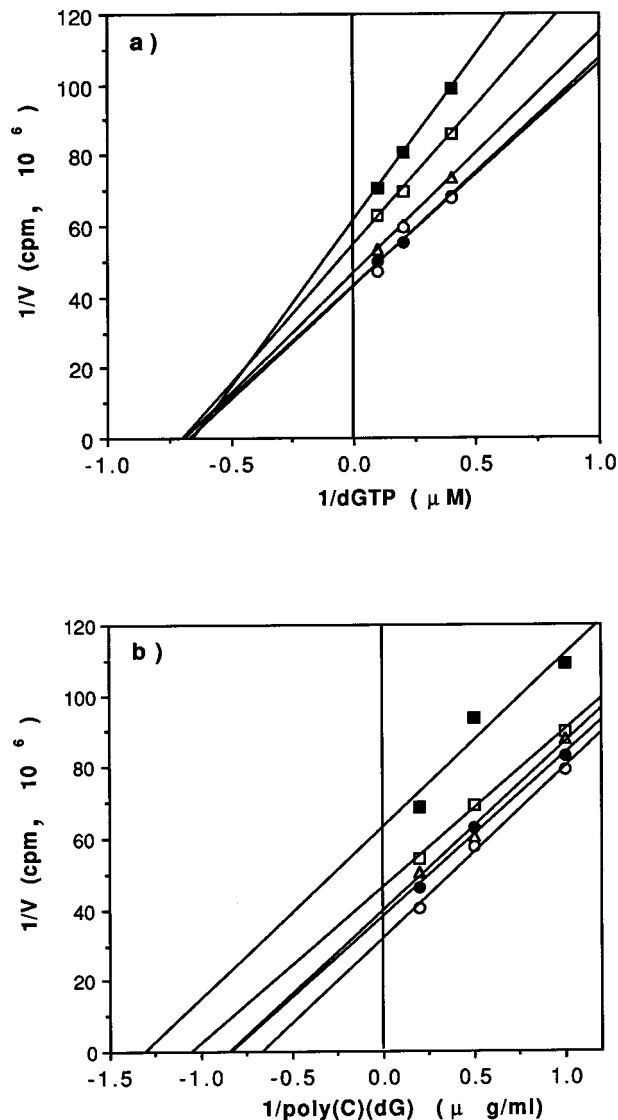


FIG. 3. Double-reciprocal plot analysis of purified recombinant HIV-1 RT inhibition by 1506. Reactions were performed in 50 μ l containing various concentrations of dGTP (a) or poly(C) \cdot oligo (dG) (b) and 1506 at 0 (\circ), 0.1 (\bullet), 0.2 (Δ), 0.5 (\square), or 1 (\blacksquare) μ M. Five micrograms of poly(C) \cdot oligo(dG) per ml (a) and 10 μ M dGTP (b) were also present.

Activities of gomisin J derivatives against various drug-resistant strains of HIV-1. In the experiments whose results are shown in Table 3, the effects of gomisin J derivatives on various HIV-1 strains with RTs which are resistant to various anti-HIV agents were studied. The HIV-1 mutants tested were L100I (resistant to TIBO) (46), N119 (resistant to nevirapine) (61), A17 (resistant to pyridinone) (51), and A012D (resistant to AZT) (38). All three compounds, 1506, 1689, and 1580, behaved identically toward the various HIV-1 mutants, suggesting that variations in halogen at the fourth and ninth positions of gomisin J do not change the basic mechanism of this action. Moreover, gomisin J derivatives did not exert any appreciable effect on mutants resistant to nevirapine, thus suggesting that the mode of action of our compounds is similar to that of nevirapine. In addition, gomisin J derivatives were effective against mutants resistant to AZT or TIBO. This is consistent with the fact that 1506R was sensitive to AZT. On the other hand, 1506R was resistant to TIBO. Mutants 1506R, N119 (nevirapine resistance), and A17 (pyridinone resistance) possess increased resistance to all other nonnucleoside inhibitors, although the degree of resistance varies depending on the mutant.

In vitro evidence that the RT of 1506R is resistant to 1506.

To confirm that the mutational change at the 188th position in the RT-coding region identified in 1506R confers on the mutated RT resistance to 1506, we compared the inhibitory effects of 1506 on crude preparations of wild-type and mutated RTs. As shown in Fig. 4, 20 μ M 1506 inhibited wild-type RT by about 50%, whereas the mutated RT was not inhibited at all, even at a concentration of 100 μ M. In confirmation of the resistance of 1506R to TIBO (R82150) (Table 3), the mutant RT was not inhibited by 40 μ M TIBO, while wild-type RT was inhibited by 50% by 0.03 μ M TIBO. As noted in this figure, a somewhat higher concentration of 1506 was required to inhibit the crude RT prepared from *E. coli*. This was perhaps due to interference by bacterial components. Nevertheless, the assay was valid because the mutated and wild-type enzymes were equally sensitive to foscarnet, a completely different RT inhibitor.

1506 acts synergistically with AZT. The results presented above (Table 3) showed that the mode of action of 1506 was different from those of nucleoside analogs such as AZT, suggesting that combinations of 1506 and nucleoside inhibitors may result in synergistic effects. In Fig. 5, the protection of MT-4 cells by 1506 alone was compared with that by a combination of 1506 and nucleoside inhibitors. Compound 1506 acted synergistically with nucleoside inhibitors.

TABLE 3. Activities of gomisin J derivatives against various drug-resistant HIV-1 strains

Strain	RT mutation(s)	Target cell	ED (μ M) ^a					
			1506	1689	1580	R82150	Nevirapine	AZT
IIIB	None	MT-4	0.63 \pm 0.20	1.42 \pm 0.49	0.26 \pm 0.05	0.18 \pm 0.05	0.17 \pm 0.03	0.05 \pm 0.03
L100I ^b	L100I	MT-4	1.25 \pm 0.05	1.42 \pm 0.17	0.66 \pm 0.20	47.2 \pm 10.2	0.71 \pm 0.35	0.01 \pm 0.005
N119 ^c	Y181C	MT-2	>20	>20	>20	69.3 \pm 33.0	>100	0.12 \pm 0.06
A17 ^d	K103N, Y181C	MT-4	20 (ED ₅₀)	20 (ED ₂₀)	8.71 \pm 1.80	>100	100 (ED ₂₅)	0.02 \pm 0.004
1506R ^e	Y188L	MT-4	>20	>20	>20	>100	>100	0.07 \pm 0.03
A012D ^f	D67N, K70R, T215F, K219Q	MT-2	2.19	4.38	0.81	ND	ND	>100

^a Data are ED₅₀s unless noted otherwise and are the means and standard errors of three determinations. ND, not determined.

^b Selected in the presence of TIBO (R82150) (46).

^c Selected in the presence of BI-RG-587 (nevirapine) (61).

^d Selected in the presence of pyridinone RT inhibitor (51).

^e Selected in the presence of 1506.

^f Isolated after AZT treatment (resistant to AZT) (38).

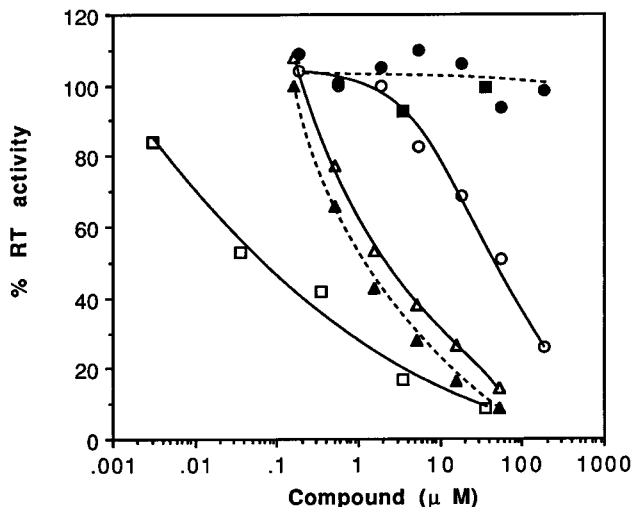


FIG. 4. Inhibitory effects of 1506 and other RT inhibitors on wild-type (open symbols) and mutant HIV-1 (T188L; closed symbols) RTs. Assays were performed with poly(rC) · oligo(dG) as the template-primer in the presence of 1506 (circles), R82150 (TIBO) (rectangles), and foscarnet (triangles). RT activities in the presence of various concentrations of inhibitor are expressed as percentages of controls measured without inhibitors.

Detection of HIV-1 linear DNA after HIV-1 infection. To further establish that 1506 inhibits HIV-1 RT, the presence of linear HIV-1 DNA in infected cells was examined by Southern blotting (data not shown). Viral linear DNA was detected in control HIV-1-infected cells and cells treated with a PR inhibitor (Ro 31-8959). In contrast, AZT and 1506 reduced the amount of HIV-1 DNA to an undetectable level. The viability of drug-treated cells under these conditions was comparable to that of control cells on day 1 (at the time of DNA preparation) and day 4 (at the time when the cytoprotective effect was confirmed). These results indicate that 1506 interferes with the RT step in HIV-1-infected cells (just as AZT does).

DISCUSSION

In our continued attempts to develop an effective anti-HIV agent (19, 20, 26, 52), we found that 4,9-halogenated gomisin J derivatives have potent anti-HIV activities with high selective indices (some of them are over 300). We conclude that these derivatives exert their anti-HIV activities through their inhibitory effects on HIV-1 RT. The mode of action of these gomisin J derivatives is completely different from that of other anti-HIV ligand derivatives, which have been reported to inhibit topoisomerase II (63).

Though nonnucleoside HIV RT inhibitors have common properties and cross-resistance exists (51, 61), the exact binding site for each inhibitor must vary because the degree of cross-resistance varies, depending on the inhibitor (3, 16, 24, 28, 35). Our data also support this notion. 1506 was completely inactive against HIV strain N119 (nevirapine resistant), while it had a modest effect on strain A17 (pyridinone resistant). On the other hand, the opposite is true with R82150 (TIBO). In confirmation of previous reports (51, 61, 62), TIBO was completely inactive against A17, while it had weak activity against N119. Furthermore, 1506 was active against TIBO-resistant mutant L100I (46). On the basis of these observations and other experimental data described below, we postulate that 1506 binds to HIV-1 RT in a fashion similar to that of nevirapine. X-ray crystallography analysis has shown that nevirapine

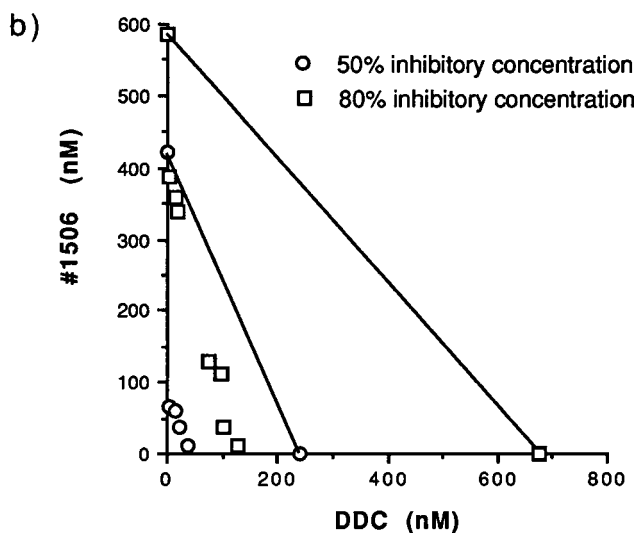
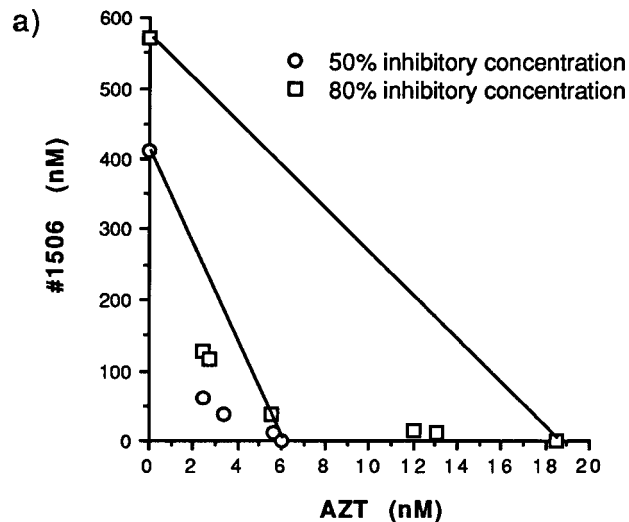


FIG. 5. Isobologram plots of the inhibition of HIV cytopathic effects on MT-4 cells by combinations of 1506 and nucleoside RT inhibitors. The combined effects of 1506 and AZT (a) or ddC (b) on MT-4 cells infected with HIV-1_{IIIB} were evaluated. The line between values for individual drugs indicates the values at which additive effects occur. Values below and to the left of this line indicate synergy.

ine binds to a conserved hydrophobic pocket (amino acids 180 through 190) that is near but not at the substrate binding site (11, 36, 39). A photoaffinity binding experiment showed that residues Tyr-181 and Tyr-188 are the nevirapine binding sites (7). This region has been suggested to be involved in the "precise positioning of the template-primer relative to the active site" (33). As described below, our experimental data and other considerations are consistent with our hypothesis. (i) An HIV-1 mutant resistant to 1506 had an amino acid change at the 188th position (Tyr to Leu), and this gave complete resistance to nevirapine and other nonnucleoside inhibitors (4, 62,

72). (ii) 1506 and nevirapine have a common structural feature of two aromatic rings bridged by an eight- or seven-membered ring. Like many other nonnucleoside RT inhibitors, nevirapine has a ketone-containing heterocyclic ring structure (23, 37, 40, 47); 1506 does not. However, the halogen or hydroxy groups of 1506 may yield electronegativity in a way similar to that of the heterocycle. (iii) The electronegative group of nonnucleoside inhibitors has been postulated to bind to the Lys at positions 101 through 104 (40, 66). The halogen group of 1506 may also bind to this region. (iv) Like nevirapine, 1506 inhibits the TIBO-resistant HIV-1 mutant L100I. This suggests that bound 1506 or nevirapine does not extend to cover the Leu at position 100 and that the binding of TIBO is different from that of 1506 or nevirapine.

The most striking effect of the gomisin J modifications was the large increase in anti-HIV activity caused by halogenation of positions 4 and 9. This increase in activity was not accompanied by a parallel increase in toxicity. An increase in anti-HIV activity caused by halogenation has also been reported for TIBO derivatives (53). The increase in anti-HIV activity for TIBO may be due to facilitated cellular uptake of halogenated derivatives, because *in vitro* RT inhibitory activities remain unchanged by halogenation (53). On the other hand, the halogenation of gomisin J as well as pyridinone derivatives (24) results in increased *in vitro* inhibitory activity for HIV RT. The introduction of a methyl or ethyl group into positions 4 and 9 increased anti-HIV activity, but cell toxicity also increased. The introduction of an *n*-propyl or *n*-butoxy group to positions 4 and 9 reduced anti-HIV activity dramatically. Furthermore, the introduction of a methyl or ethyl group into the hydroxy groups at positions 3 and 10 resulted in a loss of activity, indicating the importance of these hydroxy groups (data not shown).

In general, nonnucleoside RT inhibitors are absorbable through the gastrointestinal tract to maintain a high concentration in serum (13, 47, 53). In accordance with this general rule, in a preliminary experiment, a single oral administration of 30 mg of 1506 per kg to individual rats resulted in a 1.8 μ M concentration in serum without apparent toxicity to these animals. Together with the fact that AZT-resistant HIV-1 mutants are susceptible to gomisin J derivatives and that 1506 works synergistically with AZT, these observations suggest that further studies of gomisin J derivatives are warranted, especially in light of the recent evidence that HIV actively multiplies continuously until the infected individual dies (18, 29).

The emergence of strains resistant to anti-HIV agents does not necessarily mean that these agents are useless, because some resistant mutations are bound to retard the multiplication rate of the virus (6). Thus, proper combinations of various anti-HIV RT and/or anti-HIV PR agents may cause attenuated forms of the virus and therefore prolong the survival of individuals. This approach is especially important because of the recent emergence of HIV strains resistant to various PR inhibitors (8).

ACKNOWLEDGMENTS

We are grateful to Rosemarie Buenaflor and Gina Lee for their assistance in preparation of the manuscript.

REFERENCES

- Agrawal, S., J. Goodchild, M. P. Civeira, A. H. Thornton, P. S. Sarin, and P. C. Zamecnik. 1988. Oligodeoxynucleoside phosphoramidates and phosphorothioates as inhibitors of human immunodeficiency virus. *Proc. Natl. Acad. Sci. USA* **85**:7079-7083.
- Baba, M., R. Pauwel, J. Balzarini, J. Arnout, and J. Desmyte. 1988. Mechanism of inhibitory effect of dextran sulfate and heparin on replication of human immunodeficiency virus *in vitro*. *Proc. Natl. Acad. Sci. USA* **85**:3132-3136.
- Balzarini, J., A. Karlsson, M. J. Perez-Perez, L. Vrang, J. Walbers, H. Zhang, B. Oberg, A. M. Vandamme, M. J. Camarasa, and E. De Clercq. 1993. HIV-1-specific reverse transcriptase inhibitors show differential activity against HIV-1 mutant strains containing different amino acid substitutions in the reverse transcriptase. *Virology* **192**:246-253.
- Boyer, P. L., M. H. Currens, J. B. McMahon, M. R. Boyd, and S. H. Hughes. 1993. Analysis of nonnucleoside drug-resistant variants of human immunodeficiency virus type 1 reverse transcriptase. *J. Virol.* **67**:2412-2420.
- Coates, J. A. V., N. Cammack, H. J. Jenkinson, I. M. Mutton, B. A. Pearson, R. Storer, J. M. Cameron, and C. R. Penn. 1992. The separated enantiomers of 2'-deoxy-3'-thiacytidine (BCH 189) both inhibit human immunodeficiency virus replication *in vitro*. *Antimicrob. Agents Chemother.* **36**:202-205.
- Coffin, J. M. 1995. HIV population dynamics *in vivo*: implications for genetic variation, pathogenesis, and therapy. *Science* **267**:483-489.
- Cohen, K. A., J. Hopkins, R. H. Ingraham, C. Pargellis, J. C. Wu, D. E. H. Palladino, P. Kinkade, T. C. Warren, S. Rogers, J. Adams, P. R. Farina, and P. M. Grob. 1991. Characterization of the binding site for nevirapine (BI-RG-587), a nonnucleoside inhibitor of human immunodeficiency virus type-1 reverse transcriptase. *J. Biol. Chem.* **266**:14670-14674.
- Condra, J. H., W. A. Schleif, O. M. Blahy, L. J. Gabryelski, D. J. Graham, J. C. Quintero, A. Rhodes, H. L. Robbins, E. Roth, M. Shivaprakash, D. Titus, T. Yang, H. Tepler, K. E. Squires, P. J. Deutsch, and E. A. Emini. 1995. *In vivo* emergence of HIV-1 variants resistant to multiple protease inhibitors. *Nature (London)* **374**:569-571.
- Craig, J. C., C. Grief, J. S. Mills, D. Hockley, I. B. Duncan, and N. A. Roberts. 1991. Effects of a specific inhibitor of HIV proteinase (Ro 31-8959) on virus maturation in a chronically infected promonocytic cell line (U1). *Antivir. Chem. Chemother.* **2**:181-186.
- Daniel, M. D., N. L. Levin, N. W. King, M. Kannagi, P. K. Sehgal, and R. D. Hunt. 1985. Isolation of T-cell tropic HTLV-III-like retrovirus from macaques. *Science* **228**:1201-1204.
- Darby, G. 1992. Novel approaches in HIV chemotherapy. Reverse transcriptase of HIV as a target for anti-viral drugs. *Biochem. Soc. Trans.* **20**:505.
- Debyser, Z., R. Pauwels, K. Andries, J. Desmyter, M. Kukla, P. A. Janssen, and E. De Clercq. 1991. An antiviral target on reverse transcriptase of human immunodeficiency virus type 1 revealed by tetrahydroimidazo-[4,5,1-*j*][1,4]benzodiazepin-2(1*H*)-one and -thione derivatives. *Proc. Natl. Acad. Sci. USA* **88**:1451-1455.
- De Clercq, E. 1993. HIV-1 specific RT inhibitors: highly selective inhibitors of human immunodeficiency virus type 1 that are specifically targeted at the viral reverse transcriptase. *Med. Res. Rev.* **13**:229-258.
- De Clercq, E., N. Yamamoto, R. Pauwels, M. Baba, D. Schols, H. Nakashima, J. Balzarini, Z. Debyser, B. A. Murrer, D. Schwartz, D. Thornton, G. Bridger, S. Fricker, G. Henson, M. Abrams, and D. Picker. 1992. Potent and selective inhibition of human immunodeficiency virus (HIV)-1 and HIV-2 replication by a class of bicyclams interacting with a viral uncoating event. *Proc. Natl. Acad. Sci. USA* **89**:5286-5290.
- Dixon, M., and E. C. Webb. 1979. Enzyme inhibition and activation, p. 332-381. *In* M. Dixon and E. C. Webb (ed.), *Enzymes*. Academic Press, Inc., Orlando, Fla.
- Dueweke, T. J., T. Pushkarskaya, S. M. Poppe, S. M. Swaney, J. Q. Zhao, I. S. Y. Chen, M. Stevenson, and W. G. Tarpley. 1993. A mutation in reverse transcriptase of bis(heteroaryl)piperazine-resistant human immunodeficiency virus type 1 that confers increased sensitivity to other nonnucleoside inhibitors. *Proc. Natl. Acad. Sci. USA* **90**:4713-4717.
- Eliou, G. B., S. Singer, and G. H. Hitchings. 1954. Antagonists of nucleic acid derivatives. VIII. Synergism in combinations of biochemically related antimetabolites. *J. Biol. Chem.* **208**:477-488.
- Embretson, J., M. Zupancic, J. L. Ribas, A. Burke, P. Racz, K. Tenner-Racz, and A. T. Haase. 1993. Massive covert infection of helper T lymphocytes and macrophages by HIV during the incubation period of AIDS. *Nature (London)* **362**:359-362.
- Fujihashi, T., T. Sakata, A. Kaji, and H. Kaji. 1994. Short, terminally phosphorylated oligoriboguanilyc acids effectively inhibit cytopathicity caused by human immunodeficiency virus. *Biochem. Biophys. Res. Commun.* **203**:1244-1250.
- Fujihashi, T., T. Sakata, A. Kaji, and H. Kaji. 1995. Antiviral action of oligodeoxyguanylic acids against human immunodeficiency virus-1. *AIDS Res. Hum. Retroviruses* **11**:461-471.
- Gao, Q., Z. Gu, M. A. Parniak, X. Li, and M. A. Wainberg. 1992. *In vitro* selection of variants of human immunodeficiency virus type 1 resistant to 3'-azido-2'-deoxythymidine and 2',3'-dideoxyninosine. *J. Virol.* **66**:12-19.
- Gao, Q., M. A. Parniak, Z. Gu, and M. A. Wainberg. 1993. Generation of nucleoside-resistant variants for HIV-1 by *in vitro* selection in the presence of AZT or DDI but not by combinations. *Lancet* **6**(Suppl.):192-195.
- Goldman, M. E., J. H. Nunberg, J. A. O'Brien, J. C. Quintero, W. A. Schleif, K. F. Freund, S. L. Gaul, W. S. Saari, J. S. Wai, J. M. Goffman, P. S. Anderson, D. J. Hope, E. A. Emini, and A. M. Stern. 1991. Pyridinone derivatives: specific human immunodeficiency virus type 1 reverse transcriptase inhibitors with antiviral activity. *Proc. Natl. Acad. Sci. USA* **88**:6863-6867.
- Goldman, M. E., J. A. O'Brien, T. L. Ruffing, W. A. Schleif, V. V. Sardana,

- V. W. Byrnes, J. H. Condra, J. M. Hoffman, and E. A. Emini. 1993. A nonnucleoside reverse transcriptase inhibitor active on human immunodeficiency virus type 1 isolates resistant to related inhibitors. *Antimicrob. Agents Chemother.* **37**:947-949.
25. Harada, S., Y. Koyanagi, and N. Yamamoto. 1985. Infection of HTLV-III/LAV in HTLV-1-carrying cells MT-2 and MT-4 and application in a plaque assay. *Science* **229**:563-565.
26. Higuchi, H., K. Mori, A. Kato, T. Ohkuma, T. Endo, H. Kaji, and A. Kaji. 1991. Antiretroviral activities of anthraquinones and their inhibitory effects on reverse transcriptase. *Antivir. Res.* **15**:205-216.
27. Hizi, A., C. McGill, and S. H. Hughes. 1988. Expression of soluble, enzymatically active human immunodeficiency virus reverse transcriptase in *Escherichia coli* and analysis of mutants. *Proc. Natl. Acad. Sci. USA* **85**:1218-1222.
28. Hizi, A., R. Tal, M. Shaharabany, M. J. Currens, M. R. Boyd, S. H. Hughes, and J. B. McMahon. 1993. Specific inhibition of the reverse transcriptase of human immunodeficiency virus type 1 and the chimeric enzymes of human immunodeficiency virus type 1 and type 2 by nonnucleoside inhibitors. *Antimicrob. Agents Chemother.* **37**:1037-1042.
29. Ho, D. D., A. U. Neumann, A. S. Perelson, W. Chen, J. M. Leonard, and M. Markowitz. 1995. Rapid turnover of plasma virions and CD4 lymphocytes in HIV-1 infection. *Nature (London)* **373**:123-126.
30. Ikeya, Y., H. Taguchi, I. Yosioka, Y. Iitaka, and H. Kobayashi. 1979. The constituents of *Schizandra chinensis* BAILL. II. The structure of a new lignan, gomisin D. *Chem. Pharm. Bull. (Tokyo)* **37**:1395-1401.
31. Ikeya, Y., H. Taguchi, I. Yosioka, and H. Kobayashi. 1979. The constituents of *Schizandra chinensis* BAILL. I. Isolation and structure determination of five new lignans, gomisin A, B, C, F and G, and the absolute structure of schizandrin. *Chem. Pharm. Bull. (Tokyo)* **27**:1383-1394.
32. Ikeya, Y., H. Taguchi, I. Yosioka, and H. Kobayashi. 1979. The constituents of *Schizandra chinensis* BAILL. IV. The structures of two new lignans, pre-gomisin and gomisin J. *Chem. Pharm. Bull. (Tokyo)* **27**:1583-1588.
33. Jacob-Molina, A., J. Ding, R. G. Nanni, A. D. Clark, Jr., X. Ju, C. Tantillo, R. L. Williams, G. Kamer, A. L. Ferris, P. Clark, A. Hizi, S. H. Hughes, and E. Arnold. 1993. Crystal structure of human immunodeficiency virus type 1 reverse transcriptase complexed with double-stranded DNA at 3.0 Å resolution shows bent DNA. *Proc. Natl. Acad. Sci. USA* **90**:6320-6324.
34. Jacobsen, H., L. Ahlborn-Laake, R. Gugel, and J. Mous. 1992. Progression of early steps of human immunodeficiency virus type 1 replication in the presence of an inhibitor of viral protease. *J. Virol.* **66**:5087-5091.
35. Kashman, Y., K. R. Gustafson, R. W. Fuller, J. H. Cardellina, J. B. McMahon, M. J. Currens, R. W. Buckheit, S. H. Hughes, G. M. Cragg, and M. R. Boyd. 1992. The calanolides, a novel HIV-inhibitory class of coumarin derivatives from the tropical rainforest tree, *Calophyllum lanigerum*. *J. Med. Chem.* **35**:2735-2743.
36. Kohlstaedt, L. A., J. Wang, J. M. Friedman, P. A. Rice, and T. A. Steitz. 1992. Crystal structure at 3.5 Å resolution of HIV-1 reverse transcriptase complexed with an inhibitor. *Science* **256**:1783-1790.
37. Kukla, M. J., H. J. Breslin, R. Pauwels, C. L. Fedde, M. Miranda, M. K. Scott, R. G. Sherril, A. Raeymaekers, J. Van Gelder, K. Andries, M. A. C. Janssen, E. De Clercq, and P. A. J. Janssen. 1991. Synthesis and anti-HIV-1 activity of 4,5,6,7-tetrahydro-5-methylimidazo[4,5,1-jk][1,4]benzodiazepin-2(1H)-one (TIBO) derivatives. *J. Med. Chem.* **34**:746-751.
38. Larder, B. A., and S. D. Kemp. 1989. Multiple mutations in HIV-1 reverse transcriptase confer high-level resistance to zidovudine (AZT). *Science* **246**:1155-1158.
39. Larder, B. A., J. M. Purifoy, K. L. Powell, and G. Darby. 1987. Site-specific mutagenesis of AIDS virus reverse transcriptase. *Nature (London)* **327**:716-717.
40. Maass, G., U. Immendoerfer, B. Koenig, U. Leser, B. Mueller, R. Goody, and E. Pfaff. 1993. Viral resistance to the thiazolo-iso-indolinones, a new class of nonnucleoside inhibitors of human immunodeficiency virus type 1 reverse transcriptase. *Antimicrob. Agents Chemother.* **37**:2612-2617.
41. MacRae, W. D., J. B. Hudson, and H. N. Towers. 1989. The antiviral action of lignans. *Planta Med.* **55**:531-535.
42. Maeda, S., K. Sudo, M. Aburada, Y. Ikeya, H. Taguchi, I. Yoshioka, and M. Harada. 1981. Pharmacological studies on schizandra fruit. I. General pharmacological effects of gomisin A and schizandrin. *Yakugaku Zasshi* **101**:1030-1041.
43. Markkanen, T., M. L. Mäkinen, E. Maunukela, and P. Himanen. 1981. Podophyllotoxin lignans under experimental antiviral research. *Drugs Exp. Clin. Res.* **7**:711-718.
44. Matsushita, S., M. Robert-Guroff, J. Rusche, A. Koito, T. Hattori, H. Hoshino, K. Javaherian, K. Takatsuki, and S. Putney. 1988. Characterization of a human immunodeficiency virus neutralizing monoclonal antibody and mapping of the neutralizing epitope. *J. Virol.* **62**:2107-2114.
45. McMahon, J. B., R. J. Gulakowski, O. S. Weislow, R. J. Schultz, V. L. Narayanan, D. J. Clanton, R. Pedemonte, F. W. Wassmundt, R. W. Buckheit, Jr., W. D. Decker, E. L. White, J. P. Bader, and M. R. Boyd. 1993. Diaryl-sulfones, a new chemical class of nonnucleoside antiviral inhibitors of human immunodeficiency virus type 1 reverse transcriptase. *Antimicrob. Agents Chemother.* **37**:754-760.
46. Mellors, J. W., G.-J. Im, E. Tramontano, S. R. Winkler, D. J. Medina, G. E. Dutschman, H. Z. Bazmi, G. Piras, C. J. Gonzalez, and Y.-C. Cheng. 1992. A single conservative amino acid substitution in the reverse transcriptase of human immunodeficiency virus-1 confers resistance to (+)-(5S)-4,5,6,7-tetrahydro-5-methyl-6-(3-methyl-2-butenyl)imidazo[4,5,1-jk][1,4]benzodiazepin-2(1H)-thione (TIBO R82150). *Mol. Pharmacol.* **43**:11-16.
47. Merluzzi, V. J., K. D. Hargrave, M. Labadia, K. Grozinger, M. Skoog, J. C. Wu, C.-K. Shih, K. Eckner, S. Hattox, J. Adams, A. S. Rosenthal, R. Faanes, R. J. Eckner, R. A. Koup, and J. L. Sullivan. 1990. Inhibition of HIV-1 replication by a nonnucleoside reverse transcriptase inhibitor. *Science* **250**:1411-1413.
48. Mitsuya, H., K. J. Weinhold, P. A. Furman, M. H. St. Clair, S. Nusinoff-Lehrman, R. C. Gallo, D. Bolognesi, D. W. Barry, and S. Broder. 1985. 3'-Azido-3'-deoxythymidine (BW A509U): an antiviral agent that inhibits the infectivity and cytopathic effect of human T-lymphotropic virus type III/lymphadenopathy-associated virus *in vitro*. *Proc. Natl. Acad. Sci. USA* **82**:7096-7100.
49. Mosmann, T. 1983. Rapid colorimetric assay for the detection of anti-HIV compounds. *J. Immunol. Methods* **65**:55-63.
50. Nielson, C., C. M. Nielson, J. L. Petersen, P. C. Gotzsche, C. Pedersen, M. Arendrup, and B. F. Vestergaard. 1991. Isolation of HIV from cultures of purified CD4+ lymphocytes. *J. Virol. Methods* **35**:15-25.
51. Nunberg, J. H., W. A. Schleif, E. J. Boots, J. A. O'Brien, J. C. Quintero, J. M. Hoffman, E. A. Emini, and M. E. Goldman. 1991. Viral resistance to human immunodeficiency virus type 1-specific pyridinone reverse transcriptase inhibitors. *J. Virol.* **65**:4887-4892.
52. Ogata, T., H. Higuchi, S. Mochida, H. Matsumoto, A. Kato, T. Endo, A. Kaji, and H. Kaji. 1992. HIV-1 reverse transcriptase inhibitor from *Phyllanthus niruri*. *AIDS Res. Hum. Retroviruses* **8**:1937-1944.
53. Pauwels, R., K. Andries, J. Desmyter, D. Schols, M. J. Kukla, H. J. Breslin, A. Raeymaekers, J. Van Gelder, R. Woestenborghs, J. Heykants, K. Schellekens, M. A. C. Janssen, E. De Clercq, and P. A. J. Janssen. 1990. Potent and selective inhibition of HIV-1 replication *in vitro* by a novel series of TIBO derivatives. *Nature (London)* **343**:470-474.
54. 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.
55. Popovic, M., P. S. Sarin, M. Robert-Guroff, V. S. Kalyanaraman, D. Mann, J. Minowada, and R. C. Gallo. 1983. Isolation and transmission of human retrovirus (human T-cell leukemia virus). *Science* **219**:856-859.
56. Popovic, M., M. G. Saryngadharan, E. Read, and R. C. Gallo. 1984. Detection, isolation and continuous production of cytopathic retroviruses (HTLV-III) from patients with AIDS and pre-AIDS. *Science* **224**:497-500.
57. Ratner, L., A. Fisher, L. L. Jagodzinski, H. Mitsuya, R.-S. Liou, R. C. Gallo, and F. Wong-Staal. 1987. Complete nucleotide sequences of functional clones of the AIDS virus. *AIDS Res. Hum. Retroviruses* **3**:57-69.
58. Ratner, L., W. Haseltine, R. Patarca, K. J. Livak, B. Starcich, S. F. Josephs, E. R. Doran, J. A. Rafalski, E. A. Whitehorn, K. Baumeister, L. Ivanoff, S. R. Petteway, Jr., M. L. Pearson, J. A. Lautenberger, T. S. Papas, J. Ghayee, N. T. Chang, R. C. Gallo, and F. Wong-Staal. 1985. Complete nucleotide sequence of the AIDS virus, HTLV-III. *Nature (London)* **313**:277-284.
59. Richards, A. D., R. Roberts, B. M. Dunn, M. C. Graves, and J. Kay. 1989. Effective blocking of HIV-1 proteinase activity by characteristic inhibitors of aspartic proteinases. *FEBS Lett.* **247**:113-117.
60. Richman, D., A. S. Rosenthal, M. Skoog, R. J. Eckner, T.-C. Chou, J. P. Sabo, and V. J. Merluzzi. 1991. BI-RG-587 is active against zidovudine-resistant human immunodeficiency virus type 1 and synergistic with zidovudine. *Antimicrob. Agents Chemother.* **35**:305-308.
61. Richman, D., C. K. Shih, I. Lowy, J. Rose, P. Prodanovich, S. Goff, and J. Griffin. 1991. Human immunodeficiency virus type 1 mutants resistant to nonnucleoside inhibitors of reverse transcriptase arise in tissue culture. *Proc. Natl. Acad. Sci. USA* **88**:11241-11245.
62. Sardana, V. V., E. A. Emini, L. Gotlib, D. J. Graham, D. W. Lineberger, W. J. Long, A. J. Schlabach, J. A. Wolfgang, and J. H. Condra. 1992. Functional analysis of HIV-1 reverse transcriptase amino acids involved in resistance to multiple nonnucleoside inhibitors. *J. Biol. Chem.* **267**:17526-17530.
63. Schröder, H. C., H. Merz, R. Steffen, W. E. G. Müller, P. S. Sarin, S. Trumm, J. Schulz, and E. Eich. 1990. Differential *in vitro* anti-HIV activity of natural lignans. *Z. Naturforsch. Sect. C* **45**:1215-1221.
64. Selvam, M. P., R. A. Blay, S. Geyer, S. M. Buck, L. Pollock, R. E. Mayner, and J. S. Epstein. 1993. Inhibition of HIV-1 replication in H9 cells by nystatin-A compared with other antiviral agents. *AIDS Res. Hum. Retroviruses* **9**:475-479.
65. Shaw, G. M., B. H. Hahn, S. K. Arya, J. E. Groopman, R. C. Gallo, and F. Wong-Staal. 1984. Molecular characterization of human T-cell leukemia (lymphotropic) virus type III in the acquired immune deficiency syndrome. *Science* **226**:1165-1171.
66. Smerdon, S. J., J. Jäger, J. Wang, L. A. Kohlstaedt, A. J. Chirino, J. M. Friedman, P. A. Rice, and T. A. Steitz. 1994. Structure of the binding site for nonnucleoside inhibitors of the reverse transcriptase of human immunode-

- iciency virus type 1. Proc. Natl. Acad. Sci. USA **91**:3911–3915.
67. **Smith, S. D., M. Shatsky, P. S. Cohen, R. Warnke, M. P. Link, and B. E. Glader.** 1984. Monoclonal antibody and enzymatic profiles of human malignant T-lymphoid cells and derived cell line. Cancer Res. **44**:5657–5660.
68. **Sodroski, J., W. C. Goh, C. Rosen, K. Campbell, and W. A. Haseltine.** 1986. Role of the HTLV-III/LAV envelope in syncytium formation and cytopathicity. Nature (London) **322**:470–474.
69. **Suekawa, M., T. Shiga, H. Sone, Y. Ikeya, H. Taguchi, M. Aburada, and E. Hosoya.** 1987. Effects of gomisins J and analogous lignan compounds in schizandra fruits on isolated smooth muscles. Yakugaku Zasshi **107**:720–726.
70. **Taguchi, H., and Y. Ikeya.** 1975. The constituents of *Schizandra chinensis* BAILL. I. The structures of gomisins A, B and C. Chem. Pharm. Bull. (Tokyo) **23**:3296–3298.
71. **Tan, G. T., A. D. Kinghorn, S. H. Hughes, and J. M. Pezzuto.** 1991. Psychotrine and its O-methyl ether are selective inhibitors of human immunodeficiency virus-1 reverse transcriptase. J. Biol. Chem. **266**:23529–23536.
72. **Vandamme, A.-M., Z. Debyser, R. Pauwels, K. De Vreese, P. Goubau, M. Youle, B. Gazzard, P. A. Stoffels, G. F. Cauwenbergh, J. Anne, K. Andries, P. A. J. Janssen, J. Desmyter, and E. De Clercq.** 1994. Characterization of HIV-1 strains isolated from patients treated with TIBO R82913. AIDS Res. Hum. Retroviruses **10**:39–46.