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The Naphthyridinone GSK364735 Is a Novel, Potent Human Immunodeficiency Virus Type 1 Integrase Inhibitor and Antiretroviral▽

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The naphthyridinone GSK364735 potently inhibited recombinant human immunodeficiency virus type 1 (HIV-1) integrase in a strand transfer assay (mean 50% inhibitory concentration ± standard deviation, 8 ± 2 nM). As expected based on the structure of the drug, it bound competitively with another two-metal binding inhibitor (K_d [binding constant], 6 ± 4 nM). In a number of different cellular assays, GSK364735 inhibited HIV replication with potency at nanomolar concentrations (e.g., in peripheral blood mononuclear cells and MT-4 cells, 50% effective concentrations were 1.2 ± 0.4 and 5 ± 1 nM, respectively), with selectivity indexes of antiviral activity versus in-assay cytotoxicity of at least 2,200. When human serum was added, the antiviral potency decreased (e.g., a 35-fold decrease in the presence of 100% human serum was calculated by extrapolation from the results of the MT-4 cell assay). In cellular assays, GSK364735 blocked viral DNA integration, with a concomitant increase in two-long-terminal-repeat circles. As expected, this integrase inhibitor was equally active against wild-type viruses and mutant viruses resistant to approved drugs targeting either reverse transcriptase or protease. In contrast, some but not all viruses resistant to other integrase inhibitors were resistant to GSK364735. When virus was passaged in the presence of the inhibitor, we identified resistance mutations within the integrase active site that were the same as or similar to mutations arising in response to other two-metal binding inhibitors. Finally, either additive or synergistic effects were observed when GSK364735 was tested in combination with approved antiretrovirals (i.e., no antagonistic effects were seen). Thus, based on all the data, GSK364735 exerted potent antiviral activity through the inhibition of viral DNA integration by interacting at the two-metal binding site within the catalytic center of HIV integrase.

After an initial period of false starts, the advancements in the field of human immunodeficiency virus (HIV) integrase drug discovery since the late 1990s have been outstanding. Since the disclosure that molecules capable of binding two metals within the active site of integrase can potently inhibit the recombinant enzyme and virus replication in cells (16), a number of such integrase inhibitors (INIs) within different chemical templates have proceeded through preclinical and into clinical development (e.g., S-1360 [3], L-870,810 [12], MK-0518 [27, 28], and GS-9137 [10, 32]). Three have shown profound efficacy (e.g., 2-log reductions in viral load) as single agents in phase IIa studies, with MK-0518 and GS-9137 progressing into full development. The potential impact of this new class of antiretrovirals ranges from new components of initial combination therapy for drug-naïve patients through potent medicines for those in need of salvage therapy (7).

The limited safety data accumulated to date for MK-0518 and GS-9137 are encouraging, indicating little or no difference from the safety of a placebo. However, additional patient exposure and longer-term data will be required to establish definitive safety profiles. Furthermore, data presented at scientific meetings indicate that the treatment of a small but significant number of patients in phase II and III studies has failed due to the emergence of resistance to the INI (17, 29). Therefore, it is critical to identify new INI chemical scaffolds to (i) ensure that a subset are approved and that patients have options if compound-specific side effects materialize and (ii) identify scaffolds that have distinctively different resistance profiles.

Although no crystallographic system exists to directly study the interactions between the two-metal inhibitors and integrase, there is a reasonable understanding of both the chemical pharmacophore (2, 11, 23) and the biochemical mechanism of inhibition (13, 24). In the simplest description, the pharmacophore consists of two essential components: (i) the two-metal binding moiety comprising three heteroatoms with a central acidic hydroxyl and (ii) a hydrophobic aromatic ring on a flexible tether at a relatively well-defined distance and angle

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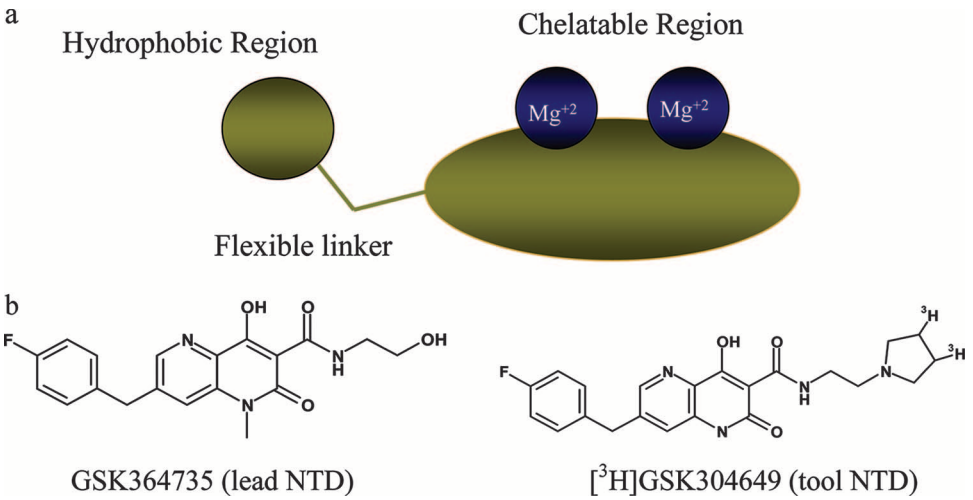

FIG. 1. Two-metal binding inhibitors of HIV integrase. (a) Schematic of a minimal pharmacophore; (b) structures of lead GSK364735 and tool GSK304649 NTDs.

from the metal binding center (Fig. 1a). Based on biochemical data, it is hypothesized that two-metal binding INIs interact with two magnesium ions within the integrase-viral DNA binary complex and selectively inhibit the second reaction catalyzed by integrase, strand transfer and the insertion of viral DNA into the host cell's genome.

Based on this knowledge, we have focused on identifying and developing potent novel scaffolds of INIs. We report here the antiviral properties of a lead compound of one such scaffold, the naphthyridinone (NTD) GSK364735 (also known as S-364735) (Fig. 1b) jointly discovered by GlaxoSmithKline and Shionogi. The other properties pertaining to drug developability, such as pharmacokinetics and results from initial clinical trial studies, will be reported elsewhere.

(Some of these data were presented in poster H-1048 at the 47th Interscience Conference on Antimicrobial Agents and Chemotherapy, Chicago, IL, 17 to 20 September 2007.)

MATERIALS AND METHODS

Compounds. The syntheses of GSK364735 and GSK304649 were first described in the patent literature (22), and the medicinal chemistry effort that led to these and other NTDs will be described in a separate publication. [^{3}H]GSK304649 was synthesized at GE Healthcare UK Limited, Buckinghamshire, United Kingdom. All other antiretrovirals were synthesized according to procedures previously described in the literature.

In vitro HIV integrase biochemical assays. In vitro recombinant HIV integrase activity was measured as previously described (4) in a strand transfer scintillation proximity assay (SPA). Purified integrase was first combined in a complex with a biotinylated viral DNA oligonucleotide duplex, the complex was preincubated with the inhibitor, and strand transfer was initiated with a radiolabeled target DNA substrate. A separate SPA was utilized for mechanism studies in which the binary biotinylated viral DNA oligonucleotide-integrase complex was formed under conditions identical to those in the strand transfer assay, after which the test compound was added to the assay mixture and then [^{3}H]GSK304649 (Fig. 1b) was incorporated to give a final ligand concentration of 20 nM. The final volume of each assay mixture was 20 μl, and the mixtures contained the following components: 20 mM sodium MOPS (morpholinepropanesulfonic acid; pH 7.2), 23 mM NaCl, and 10 mM $MgCl_2$. When compounds were tested for inhibition, 1 μl of the compound (in 100% dimethyl sulfoxide) was added to 14 μl of the binary complex, the mixture was preincubated at 37°C for 60 min, 5 μl of either 28 nM ^{3}H-labeled target DNA substrate or 100 nM [^{3}H]GSK304649 was added, and the reaction proceeded for 45 min at 37°C. To the strand transfer reaction mixture, a 60-μl volume containing the following ingredients was added to quench the activity: 50 mM sodium EDTA (pH 8), 25 mM sodium MOPS (pH 7.2), 0.1 mg of salmon testis DNA/ml, and 500 mM NaCl. The ligand binding assay mixture reached equilibrium within 45 min, and the wells were read without any further addition. The signal was read in a Wallac MicroBeta scintillation plate reader using a counting protocol that corrected for optical cross talk between wells.

Cells and viruses. MT-4 cells, a human T-cell line transformed with human T-cell leukemia virus type 1 (HTLV-1), were maintained as described previously (8). 293T cells were maintained in Dulbecco's modified Eagle's medium-F12 medium containing 10% fetal bovine serum (FBS). Normal-donor peripheral blood mononuclear cells (PBMCs) were isolated from random buffy coats (35 to 40 ml of elutriated whole blood in anticoagulant from HIV-negative donors) received from the Carolina Division of the American Red Cross. PBMCs were isolated by density gradient centrifugation over lymphocyte separation medium (catalog no. 25-072-CL; Mediatech) and stimulated by the addition of 5-μg/ml phytohemagglutinin (PHA [catalog no. L9017; Sigma]) for 24 to 48 h. Molt-4 cells persistently infected with HIV type 1 (HIV-1) strain IIIB and MT-2 cells (14) were obtained from S. Harada (Kumamoto University, Kumamoto, Japan). HeLa-CD4 cells containing an HIV-1 long terminal repeat (LTR)-driven β-galactosidase reporter gene were described previously (20). HIV-1 strain IIIB was derived from cell-free supernatants of cultures of the chronically infected cell line H93B (derived from H9 cells infected with HTLV-IIIB). HIV-1 strain Ba-L was purchased from Advanced Biotechnologies (Columbia, MD) and expanded in PHA-activated PBMCs. HIV-1 NL432 (1) was obtained from A. Adachi (Tokushima University, Tokushima, Japan).

MT-4 cell HIV replication assay. Anti-HIV activity and compound-induced cytotoxicity in the MT-4 cell line transformed with HTLV-1 were measured in parallel as described previously (15), except that the detection method was changed from dye staining to a luminescence-based procedure. Cells were infected with HIV-1 strain IIIB and incubated for 1 h at 37°C in 5% CO_2. Aliquots were added to each well of the plate containing prediluted compound. Plates were incubated at 37°C with 5% CO_2 for 5 days. HIV-induced cytopathic effects (CPEs) were assessed by a CellTiter-Glo luminescence cell viability assay (catalog no. G7573; Promega, Madison, WI). Sample luminescence was measured by using a microplate luminescence reader (Topcount NXT HTS microplate scintillation and luminescence counter; Packard Instrument Co., Downers Grove, IL). To determine the effect of added human serum on GSK364735 antiviral potency, 0, 4, 8, 12, 16, or 20% human serum (catalog no. H4522; Sigma, St. Louis, MO) was added to the standard assay mixture. (We note that the serum titration study could not be performed with PBMCs due to human serum toxicity against those cells in culture.)

PHIV cellular assay. A vesicular stomatitis virus G-pseudotyped HIV (PHIV) vector expressing luciferase was generated by cotransfecting 293T cells with pGJ3-Luci (21) and pVSV-G (Clontech) by the calcium phosphate method. Approximately 5 h following transfection, the medium was exchanged. Sodium butyrate was added at 10 mM, and the cultures were incubated for approximately 40 h, after which the cell supernatants were harvested, filtered through a 0.45- or

1.0-μm-pore-size filter, and stored at −80°C. Compounds were dissolved in dimethyl sulfoxide, diluted in medium, and plated at 100 μl per well in 96-well black, clear-bottom tissue culture plates (Costar). 293T cells were harvested by trypsinization, counted, and mixed with PHIV viral vector. The amount of PHIV was adjusted to give approximately 100,000 counts per min in the assay. One hundred microliters of the cell-virus mixture was then plated on top of the compounds to give 2×10^4 cells per well. The plates were incubated at 37°C and 5% CO_2 for 2 days. The medium was aspirated from the plates, and 100 μl of prepared Steady Glo reagent (Promega) mixed 1:1 with medium was added per well. The plates were then read in a Topcount instrument (PerkinElmer) for 1 s per well. To assess the effect of serum proteins on GSK364735 antiviral potency, the standard assay was performed with the addition of either 40-mg/ml human serum albumin (HSA [catalog no. A1653; Sigma, St. Louis, MO]) or 2-mg/ml human α1-acid glycoprotein (AAG [catalog no. G9885; Sigma, St. Louis, MO]).

PBMC HIV replication assay. HIV-1 Ba-L replication in PBMCs was quantitated by measuring reverse transcriptase (RT) activity present in the supernatant as previously described (15). PHA-stimulated PBMCs were grown in RPMI 1640 medium containing 15% FBS, 10% interleukin-2, and 50 μg of gentamicin/ml and distributed into 96-well tissue culture plates. Test compounds, serially diluted in medium, were added, and the plates were incubated in a humidified incubator at 37°C and 5% CO_2 for 1 h. A separate aliquot of a diluted compound was added to HIV-1 Ba-L, which was then added to the PBMC-compound mixture. Infection proceeded in a humidified incubator at 37°C and 5% CO_2 for 7 days. Fifty microliters of cell-free culture supernatant was transferred into a new 96-well plate. Ten microliters of RT extraction buffer (500 mM KCl, 50 mM dithiothreitol, 0.5% NP-40) was added and mixed, followed by 40 μl of RT assay buffer {1.25 mM EGTA, 125 mM Tris-HCl, 12.5 mM $MgCl_2$, 68 Ci of methyl-[^{3}H]deoxythymidine-5′-triphosphate/mmol, 0.62 optical density U of poly(rA) · poly(dT)$_{12–18}$/ml}. After mixing, the RT reaction proceeded in a humidified incubator at 37°C and 5% CO_2 for 2 h. Radioactivity was captured on Unifilter DE-81 96-well plates (Whatman; catalog no. 7700-4313) by using a Univac vacuum manifold (Whatman; catalog no. 7705-102). Wells were washed a total of three times: first with 5% Na_2HPO_4, then with distilled water, and finally with 95% ethanol. The plates were read in a Topcount luminometer (PerkinElmer) at 10 s per well.

Mechanistic cellular studies. 293T cells were transfected with the NL432 plasmid to generate infectious virus. Supernatant was filtered through 0.45-μm-pore-size filters and treated with DNase I (Roche) at 20 U/ml for 60 min at room temperature to prevent plasmid DNA carryover. To demonstrate a low level of residual plasmid DNA after DNase I treatment, the total viral DNA PCR signal in NL432-infected MT-4 cells was reduced to 0.1% of the control with the addition of 1.5 μM efavirenz. MT-4 cells were infected with HIV-1 NL432 virus for 1 h in mixtures with dilutions of compounds and collected after 6 or 18 h of incubation. Samples collected after 6 h of incubation were prepared for total-DNA PCR to detect late RT products. Samples collected after 18 h of incubation were prepared for nested *Alu* PCR to detect integrated provirus and for two-LTR PCR to detect two-LTR circles. All cells were incubated with 1 μM nelfinavir to limit HIV replication to a single cycle. Total cell DNA was extracted from infected cells with a DNeasy blood and tissue kit (Qiagen). The copy numbers of late RT products, two-LTR circles, and integrated provirus were determined using specific quantitative PCR methods previously reported by Butler et al. (6) and Brussel and Sonigo (5). Reactions were analyzed using the ABI Prism 7900HT-3 sequence detection system (Applied Biosystems).

Cross-resistance profiling of GSK364735. GSK364735 was evaluated against molecular clones with mutations in the integrase, RT, and protease coding regions. The reporter assay based on HeLa-CD4 cells was used with INI- and RT inhibitor (RTI)-resistant mutants. HIV infection of MT-4 cells, monitored by RT activity, was used with protease inhibitor (PI)-resistant mutants. The HIV-1 wild-type infectious molecular clone pNL432 was used for site-directed mutagenesis to generate HIV clones containing T66I, T66K, E92Q, F121Y, T124A, P145S, Q146R, Q148K, Q148R, S153Y, M154I, and N155S mutations in the integrase coding region. The molecular clones with K103N, Y188L, M184V, D67-K70R-T215Y, and R4 (V75I-F77L-F116Y-Q151M) mutations were used as RTI-resistant viruses, and PI-resistant mutants carrying D30N-A71V, M46I-I84V, D30N-N37S-M46L-G57R-L63P-I85V, and L24I-M46I-L63P-A71V-G73S-V82T mutations were used. 293T cells were subsequently transfected with the plasmids to generate infectious virus. Supernatants were harvested 2 to 3 days after transfection and were stored as cell-free culture supernatants at −80°C. In the HeLa-CD4 reporter assay, the compounds were incubated with HeLa-CD4 cells for 1 h prior to infection with the particular HIV-1 clone. After 3 days of incubation, the cells were lysed with cell lysis buffer and the supernatant from each well was used for the measurement of luminescence activity with a β-galactosidase reporter assay kit (TOYOBO). The luminescence activity (in relative light units) of each well was measured using a MicroBeta TRILUX instrument (Amersham Pharmacia Biotech). In MT-4 cell assays with PI-resistant mutants, cells were infected in bulk with mutant virus, incubated for 2 h, and washed and the cell suspensions (2×10^4 cells/well) were dispensed into assay plates containing diluted compounds. The plates were incubated at 37°C and 5% CO_2 for 4 to 5 days, and RT was quantitated by incubating supernatant with 10-μCi/ml methyl-[^{3}H]deoxythymidine-5′-triphosphate as a substrate in a mixture of 50 mM Tris-HCl (pH 8.3), 150 mM KCl, 10 mM $MgCl_2$, 0.1% Nonidet P-40, 10 mM dithiothreitol, 5 μg of poly(rA)/ml, and 5 μg of oligo(dT)$_{12-18}$/ml for 3 h. Radioactivity was bound to a DEAE filtermat by using a cell harvester. After sequential washing with 0.25 M phosphate buffer and H_2O, filter plates were subjected to counting using a MicroBeta TRILUX instrument (Amersham Pharmacia Biotech).

Resistance passage studies. MT-2 cells were cocultivated with Molt-4 cells persistently infected with HIV-1 IIIB, and the combined cell samples were subsequently resuspended into each well of a 24-well tissue culture plate in 2 ml of medium containing the appropriate dilution of the test compound. If no CPE was observed, infected cells were passaged every 3 to 4 days. If CPEs were observed, the supernatants were used to infect new MT-2 cells and the concentration of the dose of compounds was increased fivefold. When the replication of viruses was assessed visually, the infected cells were collected and used for sequence analysis of HIV proviral DNA.

Combination antiviral activity assay with MT-4 cells. For combination testing, aliquots of GSK364735 were serially diluted vertically in a 96-well master assay plate in a mixture of RPMI 1640 medium, 10% [vol/vol] FBS, and 10 μg of gentamicin/ml. Approved HIV inhibitors were diluted horizontally across separate master assay plates. Checkerboard-style dilutions were arranged by combining aliquots from both the horizontally and vertically diluted master plates into daughter plates, so that every concentration of GSK364735 was tested in the presence and absence of every concentration of the approved HIV inhibitor. Anti-HIV activity tests were performed with a minimum of three replicate assays of each combination. Cell infection, incubation, and assay readout were carried out by the same methods used in the standard MT-4 cell assay.

Data analyses. The 50% inhibitory concentrations (IC_{50}s) and 50% effective concentrations (EC_{50}) were concentrations that gave 50% inhibition in biochemical assays and 50% antiviral efficacy in cellular assays, respectively. In both instances, the data were fit to the following equation: $y = V_{max} \cdot \{1-[x^n/(K^n + x^n)]\}$, where y is the percent inhibition, x is the concentration of the inhibitor, V_{max} is the maximum activity, K is the concentration that gives 50% effect, and n is the Hill coefficient, or the slope of the curve. For analyses of data from competition experiments in the ligand binding SPA, full titrations of the inhibitor were done at 10, 20, 40, and 80 nM [^{3}H]GSK304649 ligand and the data were analyzed by two methods. (i) Data were globally fit to the following equation for competitive inhibition: $y = x/K_d \cdot (1 + L/K_L) + x$, where y is the percent inhibition, x is the concentration of the inhibitor, K_d is the binding constant for the inhibitor, L is the concentration of the radiolabeled ligand, and K_L is the binding constant for the radiolabeled ligand. (ii) IC_{50}s corresponding to each concentration of the radiolabeled ligand were determined, plotted versus the concentration of [^{3}H]GSK304649, and fit to the following linear function: $y = mx + b$, where b is equal to the K_d for the inhibitor and m is the slope of the line. For drug combination studies, EC_{50}s were calculated by curve fitting the data to the Hill equation (18) by using a nonlinear least-squares curve-fitting program based on the Marquardt-Levenberg algorithm (26). The interaction of each pair of compound combinations was analyzed by the methods described by Selleseth et al. (30), which provide an estimation of the strength of any interaction and of its statistical significance. Interactions with an average deviation from additivity ranging from 0.1 to −0.1 were considered to be additive. Values in the range of −0.1 to −0.2 indicate weak synergy, and values between −0.2 and −0.5 indicate strong synergy in the interaction. Conversely, values between 0.1 and 0.2 indicate that weak antagonism exists between the compounds.

RESULTS

In vitro biochemical inhibition of HIV integrase. GSK364735 inhibited recombinant HIV integrase in an in vitro strand transfer assay, with a mean IC_{50} ± standard deviation of 7.8 ± 0.8 nM. This potency was similar to the potencies of clinical INIs tested in the same assay (Table 1).

To test whether GSK364735 bound to the same site as previously described INIs, an INI ligand binding assay was developed using another NTD inhibitor, GSK304649 (Fig. 1b). To

TABLE 1. Inhibition of recombinant HIV integrase and HIV replication by GSK364735 and clinically relevant INIs

INI	IC_{50} (nM) for:		Antiviral EC_{50} (nM) for:		
	Strand transfer	^{3}H-labeled-INI binding	PBMCs	MT-4 cells	PHIV
GSK364735	7.8 ± 0.8	11 ± 3	2 ± 1	5 ± 2	1.7 ± 0.1
L-870,810	9.9 ± 0.4	13 ± 5	3 ± 1	11 ± 3	2.2 ± 0.3
MK-0518	3.3 ± 0.6	16 ± 4	2 ± 1	1.8 ± 0.2	2 ± 1
GS-9137	6 ± 1	11 ± 3	2 ± 1	2 ± 3	1.0 ± 0.1

Values are the geometric means ± standard deviations from at least three determinations.

characterize this assay, the [^{3}H]GSK304649 concentration was varied and the SPA signal displayed saturation kinetics, with a K_d of 26 ± 5 nM (Fig. 2a). We note that this potency was the same as the potency (IC_{50} of 23 nM) in the strand transfer assay (data not shown). When unlabeled GSK304649 was titrated in association with different concentrations of [^{3}H]GSK304649 and the data were fit to a competitive model, a K_d of 27 ± 6 nM was determined. Secondly, when the concentration of L-870,810, a known two-metal binding INI (12), was varied in association with various fixed concentrations of [^{3}H]GSK304649 (e.g., 20 nM radiolabeled ligand) (Fig. 2b), the data were fit to the competitive model with a K_d of 3.3 ± 0.5 nM for L-870,810 (Fig. 2c). When the concentration of GSK364735 was varied in association with various fixed concentrations of the labeled ligand, competitive inhibition was likewise observed, with a K_i of 6 ± 4 nM (data not shown). Finally, when various INIs were tested with 20 nM [^{3}H]GSK304649, IC_{50}s consistent with the potencies in the strand transfer assay were observed (Table 1).

Antiviral activity in cellular HIV replication assays. When GSK364735 was tested in a number of cellular assays using laboratory strains of HIV, it inhibited viral replication with potency at nanomolar concentrations, commensurate with its potency in biochemical HIV integrase assays (Table 1). This antiviral activity was separate from cellular toxicity, as GSK364735 cell culture IC_{50}s ranged from 5 to 190 μM in these and similar cell assays (data not shown). The smallest selectivity index of antiviral activity versus in-assay cytotoxicity was 2,200 in the MT-4 cell assay (an EC_{50} of 5 nM versus a cell culture IC_{50} of 11 μM).

When either human serum or human serum proteins were added to the cellular antiviral assay mixtures, the potency of GSK364735 decreased. When the potency of the inhibitor was measured in the presence of 0 to 20% human serum in the MT-4 cell assay, the IC_{50}s were linearly proportional to the amount of human serum added (Fig. 3). The extrapolated IC_{50}s corresponding to 100% serum as determined from four separate experiments were averaged to calculate a 35-fold shift in potency. To explore the predominant protein(s) to which GSK364735 bound, either HSA (40 mg/ml) or AAG (2 mg/ml) was added to the PHIV assay mixture. HSA induced a 17-fold shift in potency, compared to only a 2-fold shift caused by AAG, indicating that HSA was the predominant serum protein that bound the inhibitor. The 35-fold shift with 100% human serum was used to calculate a therapeutic target for future clinical trials by multiplying this effect by the PBMC antiviral potency, which was considered to be the most relevant cellular assay result. (In equilibrium dialysis experiments with human plasma, the free fraction of GSK364735 was 1%, predicting a 100-fold shift. The 35-fold shift determined in the antiviral assays was considered more relevant because it accounted for the existence of equilibrium among multiple binding partners of a drug [e.g., plasma proteins and HIV integrase].) Thus, the

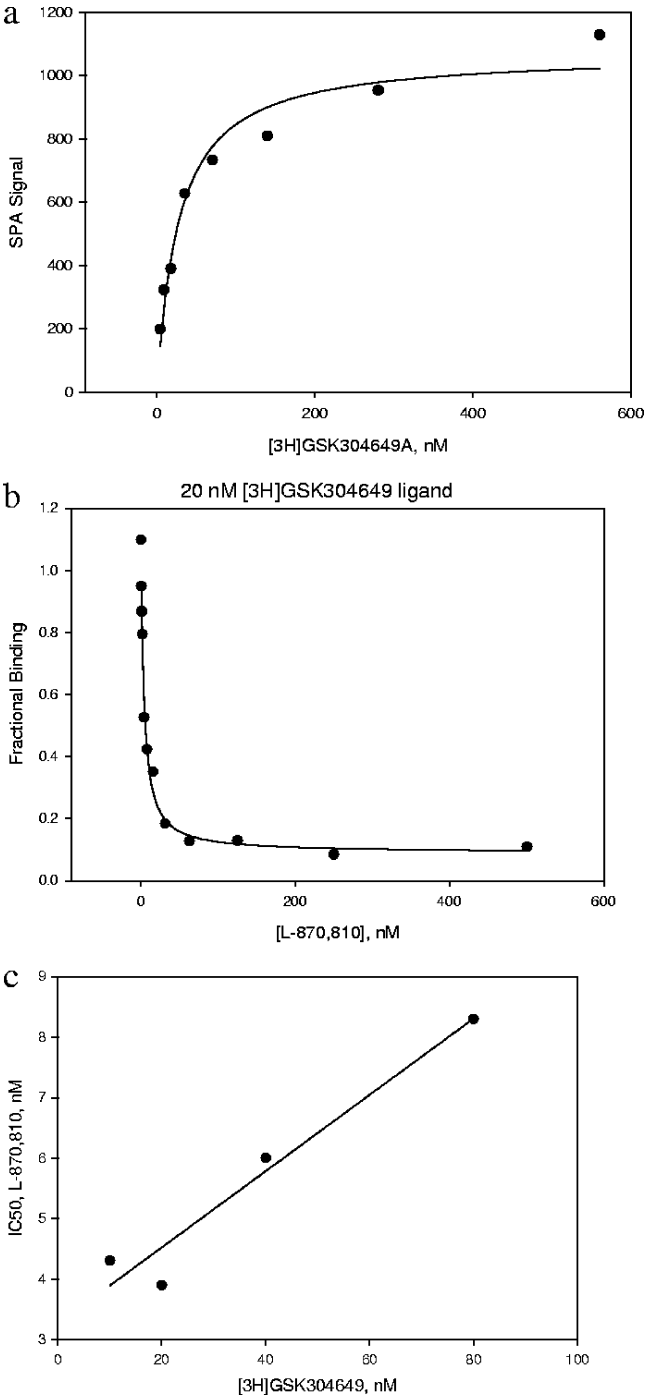

FIG. 2. Results from NTD ligand binding SPA. (a) Dependency of SPA signal on the concentration of [^{3}H]GSK304649; (b) inhibition of 20 nM [^{3}H]GSK304649 binding by the two-metal binding INI L-870,810; (c) linear dependence of the potency of inhibition on the concentration of L-870,810, demonstrating competitive inhibition.

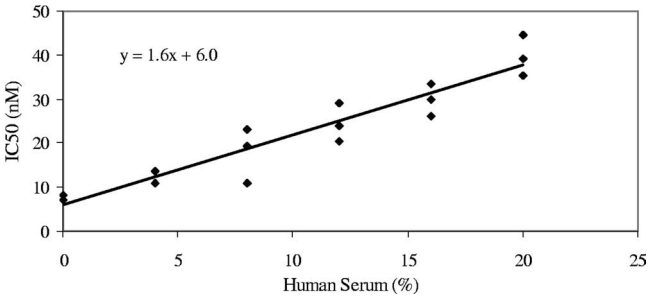

FIG. 3. Effects of human serum on antiviral EC_{50}s of GSK364735. The concentrations of GSK364735 were varied in association with different fixed amounts of human serum in the standard MT-4 cell antiviral assay. Within each experiment, titrations at each amount of human serum were done in triplicate. The indicated equation gives the linear fit of the data and generated a 27-fold shift when extrapolated to 100% human serum.

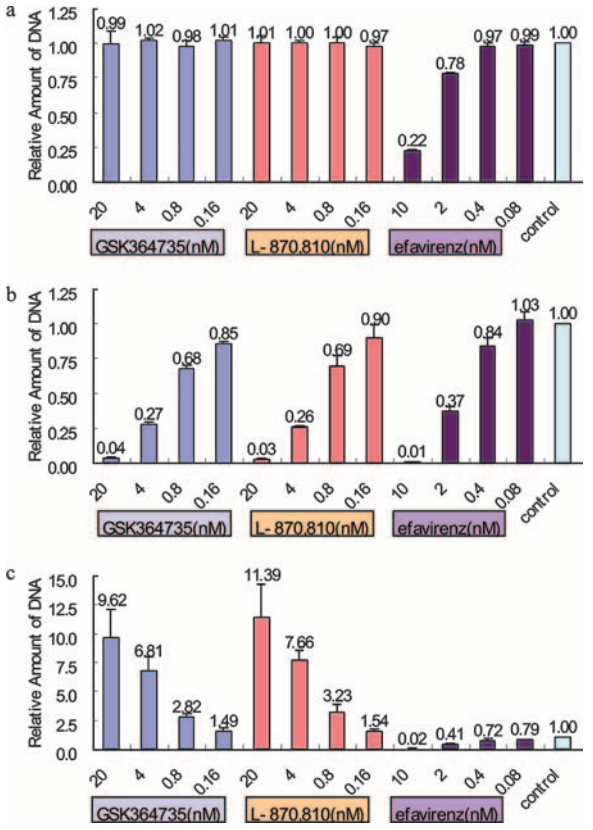

FIG. 4. Effects of inhibitors on various forms of viral DNA in MT-4 cells. The INIs GSK364735 and L-870,810 and the NNRTI efavirenz were each tested separately with MT-4 cells infected with HIV-1 NL432 to determine their effects on total viral DNA (a), integrated viral DNA (b), and two-LTR circular viral DNA (c). The *y* axis of each graph represents amounts of DNA relative to the control. Each bar represents the mean value of results from three independent experiments. Error bars represent standard deviations.

EC_{50} of GSK364735 as adjusted for protein was estimated to be 42 nM.

Cellular mechanistic studies. To demonstrate that GSK364735 was inhibiting HIV replication in cellular assays through an integrase mechanism, effects on total viral DNA production, viral DNA integration, and HIV two-LTR circle production in MT-4 cells infected with HIV NL432 were measured. As shown in Fig. 4, GSK364735 indeed showed all the effects of an INI: (i) no effect on total viral DNA (Fig. 4a), (ii) a concentration-dependent decrease in the amount of integrated viral DNA (Fig. 4b), and (iii) a concentration-dependent increase in levels of viral two-LTR circles (Fig. 4c). Furthermore, the concentration dependency of the effects, when observed, was within the range of error for the potency observed in the inhibition of viral replication in MT-4 cells. Finally, these effects were similar to the effects observed with L-870,810 and contrasted with the effects of the nonnucleoside RTI (NNRTI) efavirenz.

Cross-resistance profiling of GSK364735. It would be expected that an antiretroviral with a novel mechanism would be insensitive to the effects of mutations in viruses resistant to established classes of inhibitors. GSK364735 was tested and retained full potency against HIV strains resistant to marketed drugs. GSK364735 showed efficacy against five different RTI-resistant viruses, with activity equivalent to that against wild-type virus (IC_{50}s of 2.2 to 3.7 nM). Likewise, GSK364735 showed efficacy against four different PI-resistant viruses, with activity equivalent to that against wild-type virus (IC_{50}s of 2.0 to 3.2 nM).

The activity of GSK364735 against HIV-1 with known INI resistance mutations was also profiled. GSK364735 demonstrated activity against seven INI-resistant mutant viruses relatively equivalent to its activity against wild-type virus: T66I (1.2-fold increase in activity), E92Q (3.7-fold increase in activity), T124A (1.0-fold the level of activity against the wild type), P145S (1.4-fold increase in activity), Q146R (1.7-fold increase in activity), S153Y (1.4-fold increase in activity), and M154 (0.8-fold the level of activity against the wild type). It also exhibited greatly reduced activity against five other mutant viruses: T66K (17-fold reduction), F121Y (25-fold reduction), Q148K (210-fold reduction), Q148R (73-fold reduction), and N155S (23-fold reduction).

Resistance passage studies. HIV was passaged in medium containing increasing concentrations of GSK364735, GS-9137, or lamivudine (3TC; as a control for rapid resistance). Rapid resistance with pronounced phenotypic impact was confirmed with the M184I mutation arising by day 13 of passage in 3TC-containing medium (Table 2). Mutations within the integrase-encoding region were generated by both GSK364735 and GS-9137, consistent with both molecules' targeting the inhibition of integrase as the antiviral mechanism. A number of the mutations observed in the virus incubated with GS-9137 (e.g., Q148K/R, T66I/A, and E92Q) have been observed previously in a phase IIb study with this INI (29), suggesting the clinical validity of this in vitro cellular system.

Cellular combination studies. It is critical, prior to initiating combination studies in clinical trials, to demonstrate that a particular combination has no or low potential for being antagonistic, at least at the level of antiviral activity. Therefore, GSK364735 was tested in cellular combination studies with the 18 HIV antiretrovirals approved at the time of the study, as well as with adefovir and ribavirin, which are likely to be coadministered to HIV patients coinfected with hepatitis B

TABLE 2. Mutations generated by passage of virus in inhibitor-containing medium

Inhibitor	Mutation(s)[a] detected on culture day:			
	13	18	42	56
GSK364735	No mutation	T124A	T124A **Q148R** F121Y-T124A	T124A Q146R **Q148R** F121Y-T124A **E10D-N17S-Q148R**
GS-9137	**V151I**	T66I T124A **P145S** **Q148K** T661-T124A	T66A T66I T124A **P145S** **Q148K** **Q148R** **T661-T124A** **T66K-T124A** **Q148R-T124A**	T66I **E92Q** T124A **P145S** Q148K **Q148R** T66I-T124A T66K-T124A E92V-T124A P145S-T124A Q146S-T124A **Q148R-T124A** T66I-V72A-A128T **T66I-E92Q-T124A** T66I-T124A-Q146L
3TC	M184I	M184I M184V	Not determined	Not determined

[a] Mutations listed in boldface (integrase mutations) and underlined mutations (RT mutations) corresponded to a >10-fold decrease in potency.

TABLE 3. Summary of results from combination studies of GSK364735 and approved antiretrovirals[a]

Compound	Deviation from additivity			Interaction with GSK364735
	Average	SE	*P* (*t* test)[b]	
GSK364735	−0.013	0.035	0.357	Additive
NRTIs				
Zidovudine	−0.388	0.016	3.4E-08	Synergistic
Stavudine	−0.064	0.034	0.051	Additive
Dideoxycytosine	0.063	0.060	0.159	Additive
Dideoxyinosine	−0.162	0.062	0.0002	Synergistic
Abacavir	−0.111	0.039	0.010	Synergistic
3TC	−0.315	0.027	4.20E-06	Synergistic
Emtricitabine	−0.009	0.062	0.442	Additive
Tenofovir	−0.092	0.030	0.008	Synergistic
NNRTIs				
Efavirenz	−0.006	0.051	0.452	Additive
Nevirapine	−0.108	0.032	0.004	Synergistic
Delavirdine	−0.144	0.027	0.00036	Synergistic
PIs				
Indinavir	0.092	0.072	0.117	Additive
Lopinavir	−0.029	0.047	0.277	Additive
Nelfinavir	−0.239	0.051	0.0027	Synergistic
Ritonavir	0.050	0.049	0.169	Additive
Amprenavir	0.049	0.031	0.079	Additive
Saquinavir	0.025	0.054	0.327	Additive
Atazanavir	−0.058	0.072	0.223	Additive
Fusion inhibitor				
Enfuvirtide (Fuzeon)	−0.197	0.047	0.003	Synergistic

[a] Average values and standard errors are from at least three determinations.
[b] *P* is the probability that the deviation from additivity is equal to zero.

virus or hepatitis C virus. Firstly, there was no antagonism observed with any combination (Table 3), and there was no enhanced cytotoxicity observed with the concentrations tested for antiviral activity (data not shown). As a control for antagonism, ribavirin suppressed the antiviral activity of stavudine (d4T) by increasing the IC_{50} by fourfold at the highest noncytotoxic concentration of ribavirin tested. Further, ribavirin did not suppress the antiviral activity of GSK364735. (Note that ribavirin itself has no anti-HIV activity and that, therefore, the data were not analyzed in the same manner as those obtained when two antiviral compounds were combined). Nine of the 19 anti-HIV drug combinations displayed statistically significant synergism, with the remaining 10 showing additivity, and the combination of this INI with adefovir was synergistic (data not shown). Example isobolograms for these data are shown in Fig. 5. The clinical relevance of possible synergistic combinations remains to be determined.

DISCUSSION

GSK364735 displayed potent anti-HIV activity at single-digit nanomolar concentrations independent of the virus type and independent of the cell lines infected. Based on the following data, this antiviral inhibition was due to binding to the active site of HIV integrase and the inhibition of its catalysis of the strand transfer step of viral DNA insertion into host DNA. (i) GSK364735 inhibited both HIV integrase and HIV replication with similar potencies. (ii) GSK364735 bound competitively to the two-metal binding site within the integrase-viral DNA complex (see below), and its chemical structure contained the classic metal binding moiety. (iii) The inhibitor had no effect on total viral DNA synthesis but blocked the integration of viral DNA into host DNA. In addition, this INI increased the appearance of viral two-LTR circles, a predicted by-product of INIs. (iv) The compound had potency against mutant viruses resistant to nucleoside RTIs (NRTIs), NNRTIs, and PIs similar to its potency against the wild type, consistent with its acting on a novel antiretroviral target. (v) GSK364735 had decreased potency against several mutant viruses resistant to known INIs. (vi) Finally, the passage of virus in GSK364735-containing medium selected for resistant virus with mutations within the active site and, specifically, the two-metal binding site of integrase.

As alluded to above, GSK364735 was studied in a competitive binding assay. This novel assay was characterized using previously described INIs, with L-870,810 binding competitively with the ligand employed. Furthermore, IC_{50}s of several known two-metal binders were consistent with their potencies in a strand transfer enzymatic assay and with their antiviral activities. Because both GSK364735 and L-870,810 bound competitively with the ligand, the data support the notion that these INIs bind at the same site.

As with all classes of anti-HIV agents, INIs will be used in combination regimens. Because of that circumstance, it is important to show little or, ideally, no potential for antagonism with any approved drug. In addition, synergism between drugs would be desired, although clinical synergism is difficult to prove, let alone predict. Thus, GSK364735 was tested in cellular assays in dual combinations with all approved HIV therapeutics, as well as with adefovir and ribavirin, drugs likely to be coadministered to HIV patients coinfected with hepatitis B

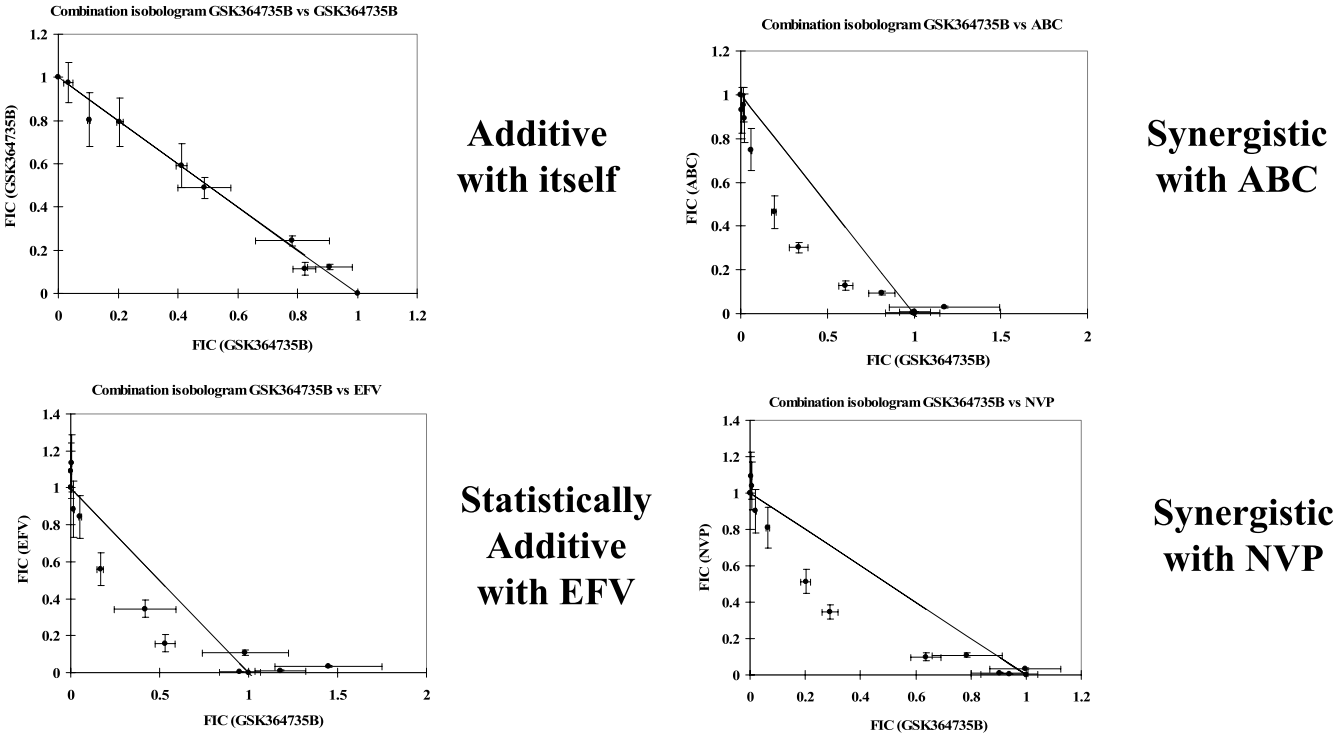

FIG. 5. Examples of results from combination studies of GSK364735 with approved anti-HIV drugs. Shown are isobolograms for GSK364735 in combination with itself (as a control for additivity), abacavir (ABC), efavirenz (EFV), and nevirapine (NVP). The solid line is for visualization of strict additivity. FIC, fractional inhibitory concentration.

virus or hepatitis C virus. To characterize our in vitro cellular system, we used ribavirin and d4T in a combination study as positive controls for antagonism (19). Ribavirin has no anti-HIV activity itself, and when added to d4T, it led to a decrease in the potency of d4T, thus demonstrating antagonism. No antagonism, or trend toward antagonism, was observed when GSK364735 was tested with any marketed HIV drug, adefovir, or ribavirin, the positive antagonism control compound. On the other hand, with several of the combinations, either statistically significant synergism or a trend toward synergism was seen. Similar data for other INIs have been presented previously (25) and raise the possibility of highly potent combinations in the clinic.

It is well accepted that HIV can overcome drug therapy through the accumulation of resistance mutations. Preliminary clinical data indicate that resistance to INIs will indeed arise. In phase II studies with drug-experienced patients, failure rates due to resistance to MK-0518 (17) and GS-9137 (29) have ranged around 25 and 40%, respectively. In addition, a relatively high level of cross-resistance between these two compounds has been reported in these studies. In fact, a recent report described a lack of prolonged efficacy of MK-0518 when this drug was used to treat two patients that had experienced GS-9137 treatment failure due to resistance (9). Although GSK364735 showed potency against several integrase-resistant mutants similar to that against the wild type, a high level of cross-resistance conferred by the key mutations observed in the MK-0518 and GS-9137 phase II studies (e.g., mutations at Q148 and N155) was detected, and furthermore, when virus was passaged in medium containing this INI, mutations similar to those described in the recent clinical studies arose. It is interesting that these three structurally diverse two-metal binders have such similar resistance profiles, suggesting that this pocket may be relatively restrictive to distinctly different profiles. In contrast, a recent report (31) has illustrated that novel scaffolds may have the potential to retain sufficient activity to treat clinically resistant virus.

Finally, it is disclosed that the clinical development of GSK364735 has recently been terminated due to hepatotoxicity observed in a long-term safety study with cynomolgus monkeys. Ironically, this toxicity was discovered at the same time that phase IIa data showing a 2.2-log reduction in viral load at the highest tested dose of GSK364735 were being generated (S. Piscitelli, S. Min, et al., unpublished data). It is noted that no significant side effects of GSK364735 were observed in any of the clinical trials but that the animal toxicity precludes development due to a lack of adequate safety coverage for longer-term phase IIb and III studies.

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