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Inhibition of human immunodeficiency virus type I integrase by naphthamidines and 2-aminobenzimidazoles

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Abstract

Retroviral integrases catalyze two of the steps of insertion of proviral DNA into the host genomic DNA. Inhibitors that target the second step, strand transfer into the host DNA, have been demonstrated to have antiviral activity in cell culture. We describe two classes of HIV-1 integrase inhibitors that block strand transfer, one based on a naphthamidine core and one on a benzimidazole core. While the naphthamidine compounds showed some propensity to interact with the DNA substrate, both classes were shown to bind directly to integrase. The naphthamidine compounds showed activity in cell culture, and a direct effect on integrase was indicated by an increase in 2-LTR products in the presence of a naphthamidine compound. These two classes of compounds represent potential starting points for the development of new classes of integrase inhibitors.

Keywords: Human immunodeficiency virus type I; Naphthamidines; 2-Aminobenzimidazoles

1. Introduction

Human immunodeficiency virus encodes three essential enzymes, of which protease and reverse transcriptase have been effectively employed as drug targets. Integrase has proven a less tractable target; despite the identification of many integrase inhibitors, no drugs targeting integrase are currently available. Nevertheless, the complexity of current anti-HIV drug regimens, the side effects associated with some of those drugs, the propensity to develop resistance to treatment and the high cost of current treatments make additional drugs targeting integrase an attractive option.

Integrase catalyzes the insertion of a double-stranded DNA copy of the retroviral genome into a host chromosome, in three steps (reviewed in Craigie, 2001; Andrake and Skalka, 1996). A 3′-terminal dinucleotide is first removed from each strand of the retroviral DNA. The complex of trimmed viral DNA and integrase translocates to the nucleus, and viral DNA is ligated into the host chromosome (strand transfer step) at an essentially random location on the host DNA. Integration of both ends of the viral DNA occurs in a concerted reaction occurring at a 5 base interval on opposite strands. The junctions are then repaired to give fully double-stranded DNA with 5 base repeated sequences at the junctions. This final step is not fully understood, but is likely to be carried out by cellular repair enzymes rather than the viral integrase.

Integrase functions in an as-yet biochemically undefined complex of viral and cellular proteins referred to as the preintegration complex (Farnet and Haseltine, 1991; Brown et al., 1987). However, the strand transfer step can be recapitulated in biochemical assays using recombinant integrase purified from *Escherichia coli*. Inhibitors of integrase activity have been identified that inhibit either or both of the integrase catalytic steps. Assays using either isolated preintegration complexes or intact cells have demonstrated that inhibitors of the strand transfer step are more likely to show inhibitory activity in the context of the preintegration complex than inhibitors that only act at the initial step (Farnet et al., 1996). It has been proposed that this is due to a significant conformational change that occurs on assembly of the integrase–viral DNA complex (Espeseth et al., 2000). We

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have used the strand transfer reaction to identify inhibitors of integrase activity from a high throughput screen. Characterization of two classes of integrase inhibitors identified from the screen is described here. One class is based on a 2-aminobenzimidazole core, and the other on a naphthamidine core. We demonstrate binding of these inhibitors to integrase, and show that while activity of these compounds in cell culture is limited, the antiviral effect of at least one compound is mediated by integrase.

2. Methods

2.1. Integrase expression and purification

A synthetic integrase gene was constructed in order to change the codon usage profile to one favoring expression in *E. coli*. Synthesis was largely as described (Chen et al., 1994; Stemmer et al., 1995). Oligonucleotides of 50–54 nucleotides in length with 21 base overlaps were used, as shown in Fig. 1. The internal oligonucleotides were used at a concentration of 10 nM, while the concentration of the oligos at each 5′ end of the gene was 1 uM. PCR employed Pfu polymerase (0.05 U/ul, Stratagene) in buffer supplied with the polymerase, and 200 uM of each deoxynucleotide. Synthesis was for 30 cycles of 30 s at 94 °C, 30 s at 55 °C, and 3 min at 72 °C, followed by 72 °C for 10 min after the last cycle. PCR products were inserted into pCR2.1 (Invitrogen) for sequencing. Two errors introduced during PCR were eliminated by combining fragments from different clones that lacked the errors. The corrected gene was introduced into pET11a (Novagen) for expression and purification of integrase. Expression and purification were described previously (David et al., 2002).

2.2. Integrase assay

Oligonucleotide substrate preparation was as described (David et al., 2002). Briefly, the viral DNA, referred to as donor DNA, was a 40 nucleotide double-stranded oligonucleotide corresponding to the 3′ terminus of the HIV cDNA with a 2 base 5′ overhang at the terminal end. It was biotinylated at the opposite end, for binding to streptavidin plates. The chromosomal DNA equivalent (target DNA) was a double-stranded 60-mer, derivatized with fluorescein at each 3′ end.

Double-stranded oligonucleotides corresponding to the 3′ terminus of the HIV cDNA were bound via a biotin to streptavidin-coated plates (Noab). A 20 nM solution of donor DNA (100 ul/well) was added to each well in 25 mM Tris–Cl pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.02% NaN_3 and 1% bovine serum albumin (BSA, fraction V, Sigma). Plates were incubated for 1 h at room temperature, and washed twice with 200 ul/well of the same solution lacking BSA and DNA. Plates were stored at 4 °C in 150 ul of the wash buffer per well until used. Plates were stable on storage for at least several weeks.

The integrase–donor DNA complex was assembled in 20 mM HEPES pH 7.5, 10 mM $MgCl_2$, 20 mM NaCl, 2 uM $ZnCl_2$, 3 mg/ml bovine serum albumin, 0.3 mM DTT and 0.6 uM integrase, 100 ul/well, 30 min at 22 °C. Plates were washed twice, 5 min each in 100 ul of this solution lacking integrase to remove unbound integrase. The strand transfer reaction was then initiated by the addition of inhibitor and 2.5 nM target DNA in 20 mM HEPES pH 7.5, 10 mM $MgCl_2$, 20 mM NaCl, 10 mM CHAPS, 2 uM $ZnCl_2$, 5% polyethylene glycol (average molecular weight 8000 Da), 0.3 mM DTT and 10% DMSO. After incubation at 37 °C for 60 min, plates were washed three times for 10 min each with 200 ul 20 mM Tris–Cl pH 8.0, 1 mM EDTA, 0.15 M NaCl and 0.1% SDS, once at room temperature and twice at 60 °C.

Detection of integrase products was done using an antifluorescein antibody–alkaline phosphatase conjugate. Plates were blocked for 1 h to overnight in antibody binding buffer (100 mM Tris–Cl pH 7.5, 0.15 M NaCl, 3 mg/ml BSA and 0.3% Tween-20). A 1:10,000 dilution of antifluorescein-alkaline phosphatase conjugate (Roche) was added in antibody binding buffer and incubated for 60 min at room temperature. Plates were washed three times for 10 min each in antibody binding buffer lacking BSA, and

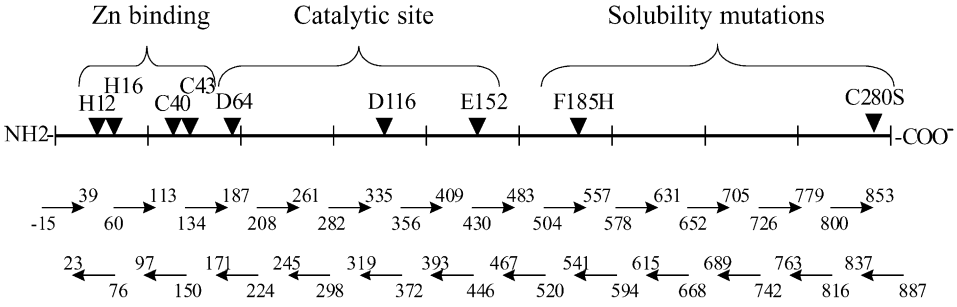


Fig. 1. Schematic for synthesis of integrase gene. Schematic of the integrase protein, with amino acids comprising the zinc binding site and catalytic site of integrase shown. Also, shown are the two mutations that were introduced to improve the solubility of the protein. Arrows indicate the position of oligonucleotides used for synthesis. The first coding nucleotide is position 1. The numbers at the beginning and end of the arrow represent the beginning and end of the oligonucleotides.

100 ul of 1 mM Attophos alkaline phosphatase substrate (Promega) in 2.4 M diethylamine, 57 uM $MgCl_2$, 0.005% NaN_3 was added. After 15 min of incubation at room temperature, the alkaline phosphatase reaction was stopped with 75 ul 0.5 M EDTA and fluorescence was quantitated (excitation at 450 nm, emission at 535 nm).

2.3. Gel retardation assay

A 30-mer double-stranded oligonucleotide of the sequence 5′-TTTTAGTCAGTGTGGAAAATCTCTAGCAGT was end labeled with ^{33}P to a specific activity of 7×10^7 dpm/nmol. A 10 nM solution of oligonucleotide in 20 mM HEPES pH 7.5, 10 mM $MgCl_2$, 20 mM NaCl, 2 uM $ZnCl_2$, 3 mg/ml bovine serum albumin, 0.3 mM DTT and 10% DMSO was incubated with serial dilutions of inhibitor. After 10 min of incubation at room temperature, mixtures were electrophoresed on 15% acrylamide, tris-borate-EDTA gels, which were dried and exposed to phosphorimager screen. Amount of product in each band was quantitated using a phosphorimager (Molecular Dynamics).

2.4. Binding of compounds to DNA

DNA from salmon testes was sheared and bound to AffiGel-15 resin (BioRad) according to the manufacturer's instructions. DNA was incubated with resin for 20 h at room temperature. The concentration of DNA on the resin was 4.0–4.5 mg/ml. Thirty microliters of this resin was incubated with 30 ul of 100 uM compound in 20 mM HEPES pH 7.5, 10 mM $MgCl_2$, 20 mM NaCl, 2 uM $ZnCl_2$, 3 mg/ml bovine serum albumin and 0.3 mM DTT for 30 min at room temperature. The unbound compound was recovered from the resin by washing with 3 volumes of the buffer used to apply the compound, and recovery was determined by comparison of a light absorbance peak with that from a standard curve derived from serial dilutions of the compound.

2.5. Methyl green displacement

Displacement of the dye methyl green from DNA by integrase inhibitors was determined as described (Burres et al., 1992).

2.6. Binding of compounds to integrase

Donor DNA was bound to streptavidin–agarose (Sigma) as described for binding to plates, but with 50 nmol of DNA per ml of resin. Final DNA concentration of bound DNA was 24 nmol/ml of resin. Integrase was added to 20 ul of resin in a 4:1 molar ratio of integrase to DNA (1.92 nmol per sample) and incubated for 30 min. Unbound integrase was removed by washing and 30 volumes of a 30 uM compound solution were passed through the resin. After three washes with 5 volumes each of 20 mM HEPES pH 7.5, 10 mM $MgCl_2$, 20 mM NaCl, 2 uM $ZnCl_2$, 0.3 mM DTT, the resin was extracted with either methylene chloride or ethyl acetate to recover bound compound. An identical sample was prepared using resin to which DNA, but not integrase, was bound, in order to measure nonspecific binding of compound to the resin or DNA. Binding of compound to integrase was not diminished by the addition of BSA at 0.1 mg/ml.

Mass spectrometry analysis was done on a Waters (Milford, MA) LCT mass spectrometer with an electrospray ionization source. Dried samples were reconstituted in 40 ul 50%MeOH in deionized water containing 2 uM [D-Ser^2]-leucine enkephalin-Thr (Sigma, MW = 686.8) as an internal standard. Flow injection analysis was done by injection of a 10 ul sample using a Shimadzu (Columbia, MD) LC-10ADvp pump and SIL-10Advp autosampler with 50%MeOH as mobile phase solvent. The analysis cycle was 1.65 min at a flow rate of 150 ml/min. Mass spectra were typically acquired from 150 to 900 Da for 1.5 min at 1 s/spectrum in profile mode. The single ion chromatogram for the compound of interest was integrated using the MassLynx software provided by Waters and peak areas of the compound were used in the quantitaton. Compounds were surveyed to determine the optimal ionization polarity for the mass spectrometry analysis. Each set contained three control samples: (1) compound titration at 1 and 5 uM dilution from 1 mM DMSO stock, to provide information about visibility of compounds in the mass spectrometry, (2) extraction control where compound stock solutions were extracted without going through the binding experiment, to determine the solvent extraction recovery, (3) extract from resin lacking integrase to determine nonspecific binding to the resin.

2.7. Cell culture assay

Potency and mechanism of action of compounds in a cell-based assay was determined using HIV strain NL4-3 psuedotyped with VSV-g protein. Briefly, 293T cells were cotransfected with 10 μg of the pNL4-3-Luc plasmid and 3 μg of VSV-g protein expression vector by a calcium phosphate method. The pNL4-3-Luc plasmid contains an HIV pNL4-3 provirus carrying a frameshift in the envelope gene, and with a firefly luciferase gene replacing the nef gene. At 48 h following transfection, supernatants containing recombinant viruses were harvested and filtered (0.2 μm pore-size filter). Fresh 293T cells were infected with recombinant reporter virus stock containing 1 ng of p24, in the absence and presence of the compound. The cells were lysed 2 days after infection, and the luciferase activity was determined, and used for IC_{50} calculation. In order to assess the mechanism of action of compound in infected cells, a real time PCR method was used for measuring the total HIV DNA and 2-LTR forms of HIV DNA from the 293 cells 16 h after infection, when the 2-LTR products peaked. The TaqMan Universal PCR Master Mix (Applied Biosystems) was used as recommended. Assays were carried out using the ABI Prism 7900 HT Sequence Detection System, for

50 cycles. PCR primers for 2-LTR product detection were, S-2LTR-586: 5′-TAACTAGAGATCCCTCAGAC; A-2LTR-712: 5′-GTGTAGTTCTGCCAATCA; P-2LTR-671: 5′-FAM-ATCCTTGATCTGTGGATCTACCA-TAMRA. PCR primers for total proviral DNA detection were: GagF: 5′-GACCATCAATGAGGAAGCTGC; GagR: 5′-TCTGGCCTGGTGCAATAGG; GagP: 5′-FAM-ATGGGATAGATTGCATCCAGTGCATGC-TAMRA.

3. Results

3.1. Expression and purification of integrase

Integrase is a 288 amino acid protein with a codon usage that is skewed against high-level expression in *E. coli*. For 15 of the 20 amino acids, the codons that account for over 50% of the codon usage for that amino acid by highly expressed *E. coli* genes (Sharp et al., 1988) represent a subset distinct from the codons that account for over 50% of the codon usage for the integrase gene. Holler et al. (1993) demonstrated that a significantly higher level of integrase expression in *E. coli* could be achieved with a more optimal codon usage, and we constructed a synthetic gene encoding the integrase gene from the HXB2 clone of HIV with this observation in mind. The synthetic scheme is outlined in Fig. 1. We also included two mutations shown previously to increase the solubility of integrase without affecting its activity (F185H and C280S, Fig. 1; Bujacz et al., 1996; Chen et al., 2000). This gene was expressed in a pET vector without an affinity tag.

An example of the purification of integrase is shown in Fig. 2. The integrase protein was insoluble in extracts of *E. coli* at low to moderate NaCl concentrations, but it was not in inclusion bodies. It could be extracted from 30,000 × *g* pellets by resolubilization in buffer containing 1 M NaCl, resulting in a good initial purification step. This material, after removal of nucleic acids by precipitation with polyethyleneimine, was enriched in a single additional step using cation exchange chromatography to approximately 90% purity.

An assay for integrase activity was developed that uses an immobilized viral DNA fragment (donor DNA) complexed with integrase. This complex is incubated with a DNA fragment into which the donor DNA is inserted (target DNA). The assay only performs the strand transfer integration step, because a preformed complex of integrase and donor DNA is used and the donor DNA lacks the two 3′-terminal nucleotides that are removed by integrase in vivo. This reaction carries out one half of the complete integrase reaction, in that only one integration event occurs rather than the concerted integration of both ends of the DNA.

The optimal reaction conditions for the strand transfer assay are given in Section 2. Integrase had a broad optimum of 5–15 mM for $MgCl_2$. NaCl stimulated activity up to 20 mM but was strongly inhibitory at higher concentrations. $ZnCl_2$ and DTT were required at 1–2 uM and

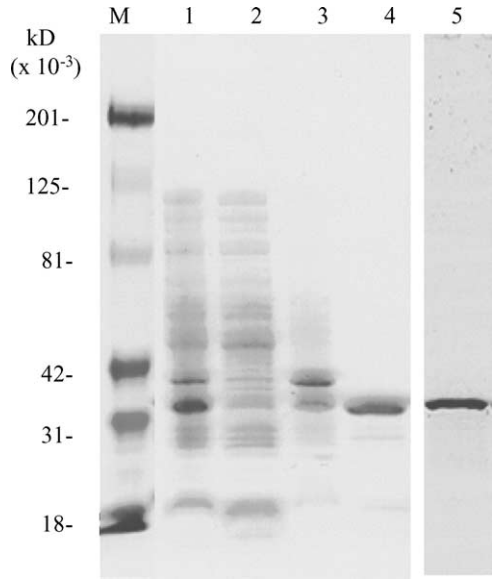


Fig. 2. Purification of integrase protein from *E. coli* extracts. Samples were electrophoresed on a 4–15% acrylamide SDS gel (Ready gel, BioRad). M: molecular weight markers; 1: whole cell lysate; 2: 30,000 × *g* supernatant; 3: 100,000 × *g* pellet; 4: 100,000 × *g* supernatant; 5: eluate from S column.

0.3–1 mM, respectively. PEG enhanced integrase activity at 6–8%, as did moderate concentrations of glycerol (4–5%). Removal of unbound integrase from the donor–integrase complex was essential to prevent nonspecific binding of integrase to the target DNA, blocking integration. Assembly of a donor–integrase complex was very inefficient at 4 °C, optimal at 22 °C and 70% of the optimal amount at 37 °C. The lower limit of detection of strand transfer products was 0.1 fmol.

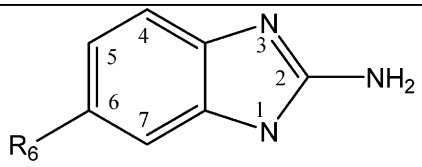
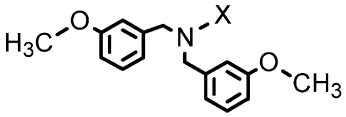
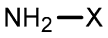
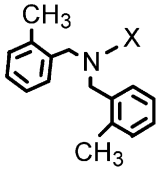
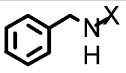
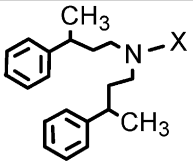
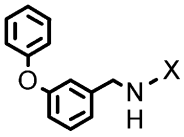
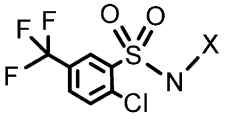
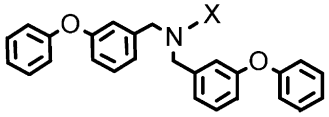
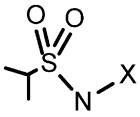
Approximately 250,000 compounds were screened using a high throughput assay described elsewhere (David et al., 2002). Several classes of inhibitors were identified from this screen; two of them, the 2-aminobenzimidazoles and the naphthamidines, are described here.

3.2. 2-Aminobenzimidazole inhibitors

The aminobenzimidazole compounds are shown in Table 1. The 2-aminobenzimidazole core showed no activity (IC_{50} > 300 uM). However, the addition of an amino group in position 6 gave weak activity (IC_{50} = 170 uM). Substitution on the amine produced compounds which exhibited improved integrase potency. A benzyl group increased potency two-fold. A single 3-phenoxybenzyl substitution had no marked effect on potency compared to the benzyl group, however, addition of a second 3-phenoxybenzyl group to the nitrogen exhibited a 19-fold improvement, from IC_{50} = 93 to 5 uM. Replacement of the terminal phenoxy groups with methoxy groups improved potency further to IC_{50} = 1.8 uM.

A second series of compounds were discovered in the screen, containing a sulfonamide in place of the methy-

Table 1
Effect of substitutions of 2-amino aminobenzimidazole at the C6 position on inhibition of integrase activity

IC50, uM		IC50, uM	
> 300		1.8	
170		24	
88		9.2	
93		1.8	
4.9		2.6	

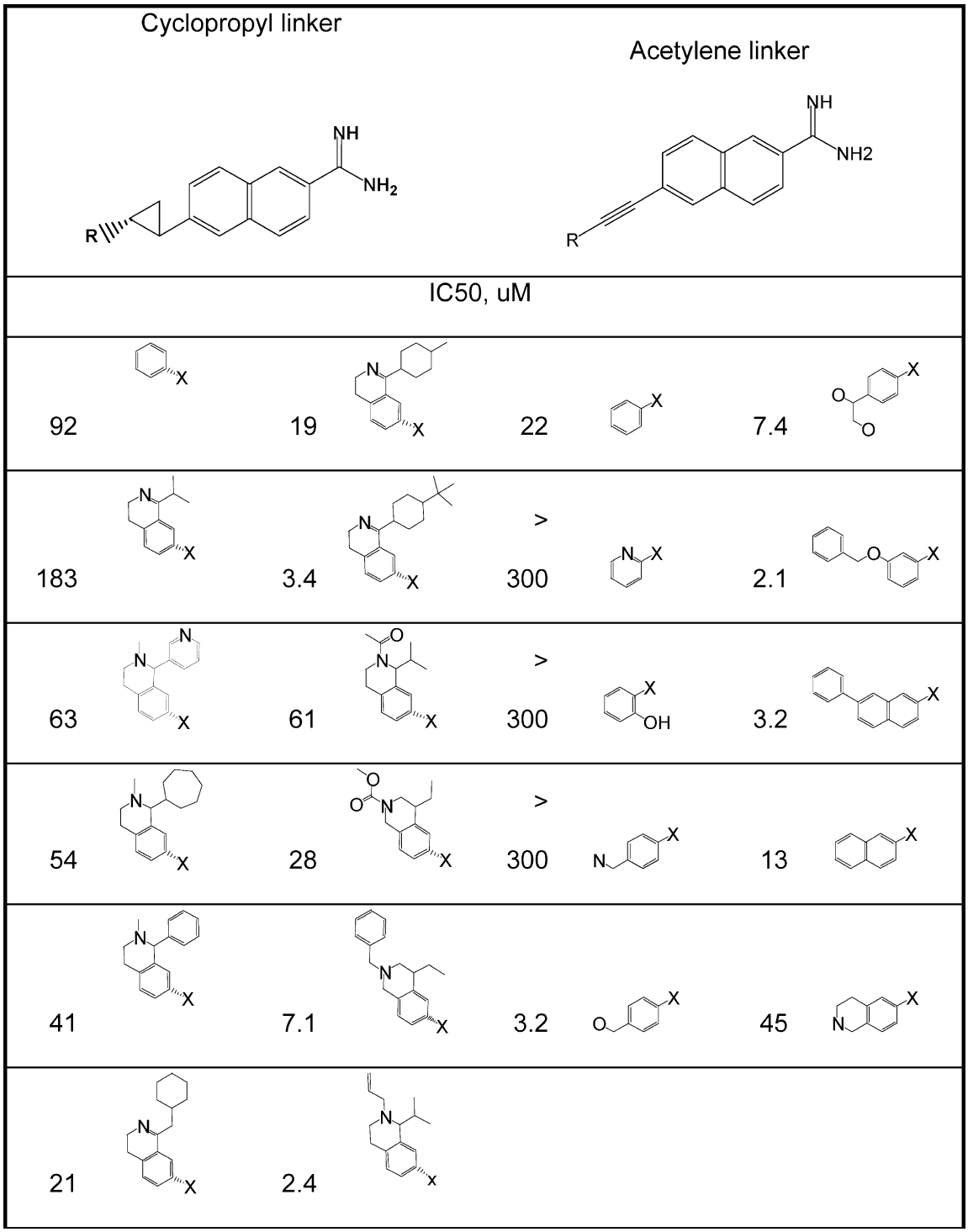
lene linker (Table 1). This series generated two potent compounds, containing a 1-chloro,4-trifluoromethyl phenyl group (IC_{50} = 1.8 uM), or an isopropyl group (IC_{50} = 2.6 uM). Other substitutions tested were 50- to at least 160-fold less potent. *Para* substitutions of the phenyl ring were inactive, as was the addition of a second *meta*-trifluoromethyl group.

3.3. Naphthamidines

While naphthamidine itself was inactive against integrase, a number of 6-substituted compounds exhibited potent integrase inhibition (Table 2). These compounds had three components, the naphthamidine, an R6 group and a "linker" connecting the two. Several types of linkers were screened and evaluated for integrase potency, including amide, urea, ether, cyclopropyl, acetylene and ketone. Compounds containing the cyclopropyl and acetylene linkers proved to be the best inhibitors. These two linkers differ in the vector created for the attached substituent (see Table 2). The acetylene-linked substituent is approximately 180°, relative to the naphthamidine C6 position, while the direction of the cyclopropyl-linked substituents are approximately 116–120°.

Substituents attached to the cyclopropyl linker are shown in Table 2. A phenyl group alone gave weak activity (IC_{50} = 92 uM). A 3-dihydro-isoquinoline substituent was two-fold weaker than the phenyl alone. However, larger hydrophobic groups attached at the 3 position of the dihydroisoquinoline moiety increased potency, with a *t*-butyl cyclohexyl group showing the greatest potency increase at this position (IC_{50} = 3.4 uM). Substituents on the nitrogen

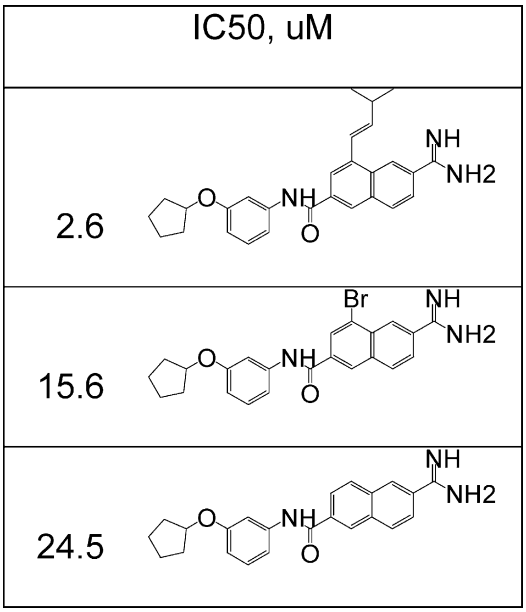
Table 2
Effect of substitutions of naphthamidines with two types of linkers at the C6 position on inhibition of integrase activity



of the tetrahydroisoquinoline ring gave a further increase in potency, with an N-allyl group showing a 76-fold potency increase when combined with the isopropyl group, over the isopropyl group itself.

Two types of additions to the acetylene linker showed good inhibitor potency, shown in Table 2. A phenyl group in this position was about four-fold more active than it was with the cyclopropyl linker. Polar substituents such as pyridyl, *o*-phenol or *p*-aminomethyl phenyl attached to the acetylene linker resulted in a significant loss of activity. However, a hydroxymethyl phenyl substituent *para* to the linker increased potency to $IC_{50} = 3.2$ uM. The other active group of compounds had more extended aromatic ring structures. Naphthalene or tetrahydroisoquinoline groups

Table 3
Effect of additions to the C8 position of the naphthamidine core



resulted in moderate potency. Further extension with a phenyl group, either from the naphthalene or a phenyl ether, increased potency an additional four- to six-fold.

In addition to the effect of substituents at the 6 position of the naphthamidine, groups at the 8 position also increased potency, as illustrated with an amide linker in Table 3. A 3-methyl-1-butenyl group increased potency by approximately 10-fold compared to the unsubstituted compound.

3.4. Inhibition of topoisomerase I, binding of compounds to DNA

As a test of specificity, both classes of compounds were tested against human topoisomerase I (Tables 4 and 5 for structures). Three compounds of each class were tested. The aminobenzimidazoles showed no reactivity with topoisomerase I. However, the naphthamidines inhibit topoisomerase I activity with a range of 6–10-fold weaker activity than for integrase inhibition. This inhibitory activity does not distinguish between a direct interaction with the topoisomerase and indirect inhibition of topoisomerase activity by binding to the DNA substrate. To assess this possibility, several methods of measuring an interaction between DNA and two of the naphthamidines used in the experiment were applied (Table 4). First, a direct binding assay with an immobilized DNA molecule was used, with detection of unbound compound by light absorbance. Neither compound was detected as binding by this method (IC_{50} > 100 uM). A second method used displacement of the dye methyl green from the DNA as an indirect method of detecting binding (Burres et al., 1992). Neither compound displaced methyl green from DNA (IC_{50} > 300 uM). Finally, the compounds were tested in a gel retardation assay. A double-stranded 30-mer oligonucleotide was incubated with increasing amounts of compound and electrophoresed in a nondenaturing acrylamide gel. The results are shown in Fig. 3 and Table 4. Both of the naphthamidine compounds resulted in a shift of the DNA to the well of the gel, with half-maximal responses at 9–21 uM, approximately equivalent to their IC_{50} values for inhibition of integrase. In contrast, Merck L-708,906 (Hazuda et al., 2000), a diketo acid integrase inhibitor did not retard the DNA fragment (Table 4 and Fig. 3). A-54188 is a compound that was shown by the direct binding assay with immobilized DNA to bind DNA, and it also results in disappearance of the input bands. The two bands found at the lowest concentration of compound are also present in the control lacking compound (data not shown). The origin of the second band was not determined. This assay raises the possibility that at least some of the integrase inhibition noted by naphthamidine compounds could be due to an effect on the substrate DNAs. However, as demonstrated below, these compounds also bind to integrase directly.

3.5. Binding to integrase

In order to get a more direct indication of whether these compounds interacted with integrase, we asked whether binding of these compounds to integrase could be detected. We first tried to detect binding by assessing the capacity of these compounds to compete with L-708,906 in the integrase activity assay. We were unable detect competition between the classes of compounds using this assay (not shown), so we developed a direct means of measuring binding to integrase. Donor DNA was immobilized on agarose resin, and a complex of integrase bound to donor DNA was formed. Compound was incubated with this complex, and unbound compound was removed by extensive washing. The bound compound was stripped from the resin by extraction in organic solvent, and detected by mass spectrometry. The ratio of compound recovered from resin containing integrase to that lacking integrase was determined after correction for recovery of an internal standard, and is shown in Table 4 for the compounds. The two naphthamidine compounds were enriched 4.5- and 7.6-fold on the resin containing bound integrase. Use of this technique with the aminobenzimidazole compounds was limited somewhat by their propensity to bind to the resin. A compound with a large R6 group gave a ratio of less than 2, but that ratio is misleading due to the high background in the absence of integrase. A-201735 (Table 5), an aminobenzimidazole compound with a small R6 group gave a ratio of 5, similar to the naphthamidines. Thus, while the inhibition of integrase by at least some of the naphthamidines may have been partially mediated by interaction with DNA, both the benzimidazoles and the naphthamidines are capable of binding directly to integrase. Binding of the compounds to integrase was unaffected by the presence of 0.1 mg/ml BSA, indicating that a specific binding effect was being measured.

Table 4
Specificity, binding characteristics, and activity in cell culture of aminobenzimidazoles and naphthamidines[a]

	Integrase[b]	Topo I[c]	DNA bind[d]	Me. gr.[e]	Gel shift[f]	IN bind., ratio[g]	EC_{50} (uM)[h]	TD_{50} (uM)[h]	2-LTR ratio[i]
2-aminobenzimidazoles									
A-206115	1.5	>300					11	21	
A-206116	4.9	>300				1.8	4.4	4.0	
A-206123	9.2	>300					7.2	11	
A-201735	6.0					4.9	>100	>100	
Naphthamidines									
A-224812	12	75	>300	>300	9.1	4.5	>100	>100	
A-249411	2.1	20					1.7	1.9	
A-184441	16	200	>300	>300	21	7.6	16	48	2–5
Diketoacids[j]									
L-708,906	0.4	>100			>300		0.4	>100	
L-731,988	0.6	>100	>300	>300	>300		1.7	>100	10
Control compounds									
A-88151	2.6	85	>300	>300	69		12	69	0.8
Doxorubicin			27						
A-82232				40					

[a] Structures of these compounds are shown in Table 5.
[b] IC_{50} determined from biochemical integrase assay (uM).
[c] IC_{50} against topoisomerase I (uM).
[d] DNA (in nmol) needed for 50% binding of 3 nmol of compound.
[e] IC_{50} for displacement of methyl green from DNA (uM).
[f] IC_{50} for change in mobility of DNA on gel electrophoresis (uM).
[g] Binding to integrase. Value represents the ratio of resin containing integrase to resin lacking integrase, for the peak areas corresponding to the mass of the compound. The peak areas are corrected for recovery by normalizing to an internal standard.
[h] Antiviral activity and toxicity determined in a single cycle replication assay in HEK 293 cells, as described in Section 2.
[i] Ratio of 2-LTR products to total viral cDNA in infected HEK 293 cells.
[j] Compounds described in (Hazuda et al., 2000).

3.6. Activity in a cell culture assay

As a further test of the interaction between integrase and these compounds, they were used in an HIV infectivity assay (Table 4). One compound out of each class had both undetectable antiviral activity and undetectable toxicity. One of the aminobenzimidazole compounds was highly toxic. The other naphthamidine compound, A-184441, had a small

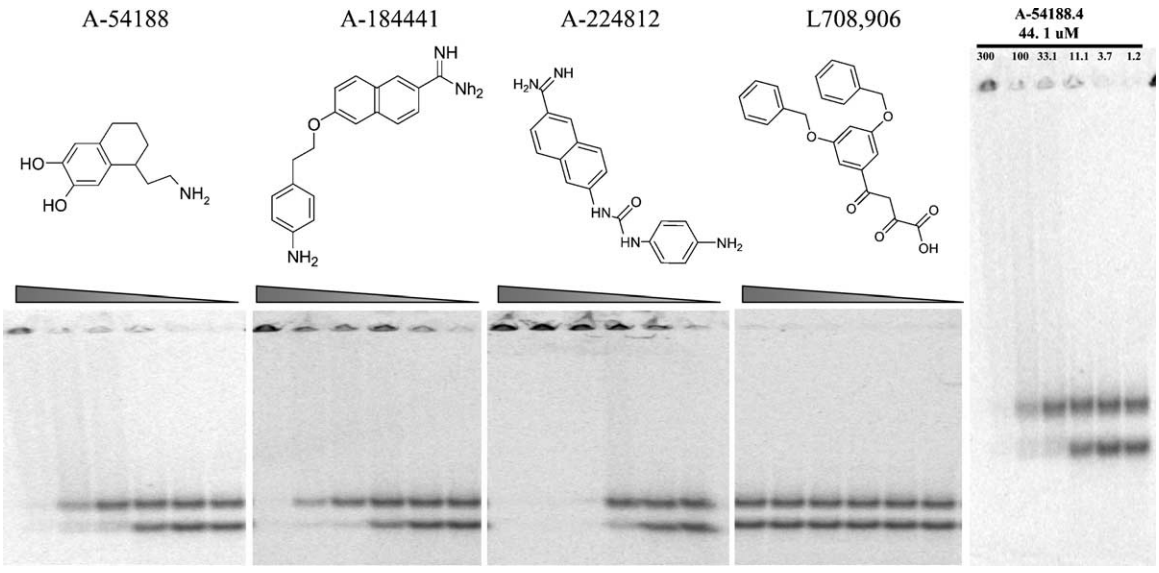
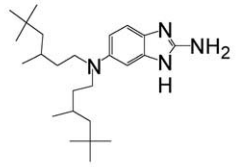
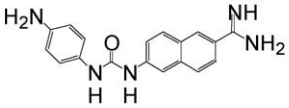
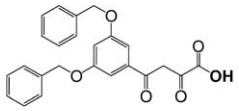
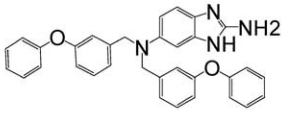
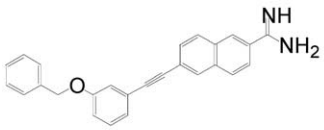
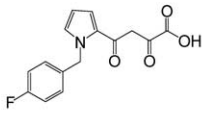
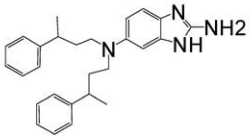
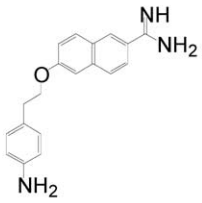
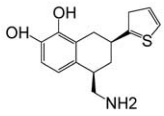
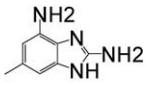
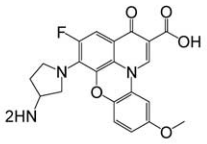


Fig. 3. Effect of naphthamidine compounds on electrophoretic mobility of a DNA fragment. A 33P-labelled 30-mer double-stranded oligonucleotide was incubated with increasing amounts of the indicated compound, from 0 to 300 uM, followed by resolution of the products on a 15% nondenaturing acrylamide gel. Half-maximal concentrations for loss of DNA from the doublet are given in Table 4.

Table 5
Structures of compounds cited in Table 4

A-206115 	A-224812 	L-708906 (Merck) 
A-206116 	A-249411 	L-731,988 (Merck) 
A-206123 	A-184441 	A-88151 
A-201735 	A-82232 	

therapeutic window of three-fold. It is not surprising that these compounds lack a good antiviral profile in cell culture, since they are unlikely to enter cells efficiently due to their positive charge. Use of these classes of compounds would likely require delivery as a prodrug.

Since A-184441 had a small therapeutic window in cultured cells, it provided an opportunity to ask whether inhibition was mediated directly by integrase. In addition to integration into the host chromosome, the retroviral cDNA can undergo several other fates, including degradation or ligation into dead-end products by cellular ligases. One of the dead-end ligation products results in ligation of viral DNA ends together, giving a product with two long terminal repeat regions ligated to each other. This product can be detected by a PCR amplification that spans the junction of the two repeats (2-LTR product). Inhibition of integrase shifts the equilibrium of products toward the dead-end products, and can be measured as an increase in 2-LTR products. When A-184441 was tested in a 2-LTR assay, it resulted in a two- to five-fold increase in the ratio of 2-LTR products to total HIV cDNA. As a comparison, L-731,988, one of the diketo acids identified previously by Hazuda et al. (2000) gave a 10-fold increase in 2-LTR products in this assay. In contrast to these compounds, A-88151 (Tables 4 and 5) showed no increase in 2-LTR products. While the increase in 2-LTR products was modest with A-184441, this likely reflects the weak activity of this compound in cell culture. A-184441 is 40-fold less potent in cell culture than is L-731,988. This result indicates that the cellular target of A-184441 is integrase.

4. Discussion

Derivatives of 2-aminobenzimidazole and naphthamdine yielded inhibitors of integrase strand transfer activity with half-maximal inhibitory concentrations as low as 1.5 uM. Several compounds with diverse structures had IC_{50} values

less than 5 uM, indicating that there is substantial potential for optimizing this series of compounds to improve potency. While results of the gel retardation assay indicated that the naphthamidines might interact with the substrate DNAs, both classes of compounds were shown to bind to integrase itself (Table 4). It is likely that the assay for detecting binding to integrase would result in some false negatives. The samples were extensively washed before the extraction to recover bound compound, so compounds with weak or moderate binding affinity would be likely to be removed in the washes. A positive result indicates binding to integrase, but the minimal binding affinity needed to be detected in this assay has not been established.

Evidence of binding to integrase does not necessarily imply a mechanism for inhibition of integrase activity, since binding could be unrelated to inhibition. Previous studies have indicated that several compounds that were inhibitors of integrase in biochemical assays inhibited other targets in vivo (De Clercq, 2000; Pluymers et al., 2000). The aminobenzimidazole compounds were inactive in cell culture. However, the naphthamidine A-184441 showed an antiviral effect in cell culture, with an increase in 2-LTR products. The accumulation of 2-LTR products in cell culture is evidence for a direct effect on integrase. The cell culture-derived EC_{50} and biochemically determined IC_{50} values for A-184441 were essentially equivalent. Unlike the biochemical assay, the local concentration of DNA in the nucleus is high, making it unlikely that the inhibitory effect would be mediated by binding to substrate DNAs without a large decrease in potency. Extending these observations to other members of these two classes of compounds will require additional refinements to minimize poor cell uptake and toxicity properties.

Results with several aminobenzimidazole compounds indicate that a tertiary amine at the C6 position, with similar or identical substituents on the nitrogen may give optimal potency. These structures bear some similarity to the diketo acid L-708,906 (e.g., compare L708,906 and A-206116 in Table 5). Both of these inhibitors target the strand transfer step, as opposed to assembly of the integrase–DNA complex or processing of the viral 3′ end. These similarities raise the question of whether they share binding sites as well.

The active site of integrase contains a strictly conserved motif composed of two aspartates with a glutamate separated from the second aspartate by 35 amino acids (DD35E motif). Crystallographic studies have shown that this motif coordinates a divalent metal ion in the active site (Wlodawer, 1999; Goldgur et al., 1998; Maignan et al., 1998). The serine protease urokinase-type plasminogen activator (uPA) binds 5-hydroxyl-2-aminobenzimidazole (Nienaber et al., 2000). Binding required an interaction by the 2-amino group with an aspartate, and by the N1 imidazole nitrogen with a backbone carbonyl. By analogy with this interaction, the aminobenzimidazole moiety could interact with the DD35E motif of integrase. The diketo acid L-708906, shown in Table 5, also has the potential to interact with the DD35E–metal ion complex. The mode of binding of the naphthamidine and aminobenzimidazole integrase inhibitors has not been established, nor have we determined whether they are capable of displacing the metal ion from the active site. However, the inhibitory activity of these compounds against recombinant integrase and in cell culture, their demonstrated binding to integrase, and the potential mechanistic similarities to the diketoacid inhibitors indicate that they may provide useful cores for further development of integrase inhibitors.

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