Antiviral Chemistry & Chemotherapy 15:67–81

Design and synthesis of novel dihydroxyindole-2-carboxylic acids as HIV-1 integrase inhibitors

Mario Sechi[1]*, Gianfranco Angotzi[1], Roberto Dallocchio[2], Alessandro Dessì[2], Fabrizio Carta[1], Luciano Sannia[1], Alberto Mariani[3], Stefano Fiori[3], Tino Sanchez[4], Leah Movsessian[4], Carmen Plasencia[4] and Nouri Neamati[4†]

[1]Dipartimento Farmaco Chimico Tossicologico, Università di Sassari, Sassari, Italy
[2]CNR-Istituto di Chimica Biomolecolare, Li Punti, Italy
[3]Dipartimento di Chimica & Local INSTM Research Unit, Sassari, Italy
[4]Department of Pharmaceutical Sciences, University of Southern California, School of Pharmacy, Los Angeles, Calif., USA

Corresponding authors: *Tel: +39 079 228 753; Fax: +39 079 228 720, E-mail: mario.sechi@uniss.it; †Tel: +1 323 442 2341; Fax: +1 323 442 1390; E-mail: neamati@usc.edu

This work is dedicated to the memory of Professor Paolo Sanna

In a search for new HIV-1 integrase (IN) inhibitors, we synthesized and evaluated the biological activity of 5,6-dihydroxyindole-2-carboxylic acid (DHICA) and a series of its derivatives. These compounds were designed as conformationally constrained analogues of the acrylate moiety of caffeic acid phenethyl ester (CAPE). DHICA, an intermediate in the biosynthesis of melanins, was prepared as a monomeric unit by a novel synthetic route. In order to perform coherent SAR studies, two series of DHICA amides were synthesized. First, to validate the utility of a previously identified three-point pharmacophore based on CAPE in inhibitor design, we prepared a series of benzyl- or phenylethylamine substituted derivatives lacking and containing hydroxyl groups. Second, dimers of DHICA containing various aminoalkylamine linkers were also prepared with a goal to increase potency. All compounds were tested against purified IN and the C65S mutant in enzyme-based assays. They were also tested for cytotoxicity in an ovarian carcinoma cell line and antiviral activity in HIV-1-infected CEM cells. Seven compounds inhibited catalytic activities of purified IN with IC_{50} values below 10 μM. Further computational docking studies were performed to determine the title compounds' mode of interaction with the IN active site. The residues K156, K159 and D64 were the most important for potency against purified IN.

Keywords: 5,6-dihydroxyindole-2-carboxylic acid, catechol derivatives, HIV-1 integrase inhibitors, docking studies

Introduction

The HIV-1 integrase (IN) is one of the virally encoded enzymes essential for retroviral replication (Brown, 1998) and has emerged as an attractive target for the development of new anti-AIDS drugs. IN catalyses the integration of retro-transcribed viral DNA into the host cell's genome through two coordinated reactions (Asante-Appiah *et al.*, 1997). In the first step, IN recognizes the viral DNA ends and cuts the conserved terminal dinucleotide (5′-…CA-GT-3′) in a reaction known as 3′-processing. This reaction occurs in the cytoplasm within a large viral nucleoprotein complex known as the preintegration complex. In the second step, IN catalyses the strand transfer reaction (integration process). The 3′ OH terminal of the recessed viral DNA (5′-…CA-OH–3′) attacks host DNA as a nucleophile. The integration process is completed by cleavage of the unpaired dinucleotides from the 5′-ends of the viral DNA and repair of the gaps between the viral and target DNA. Since no human counterpart of IN is known, there is considerable interest in developing effective and selective IN inhibitors (Pommier *et al.*, 2000; Neamati, 2001; De Clercq, 2000).

In recent years a systematic screening of natural and synthetic products as potential IN inhibitors has been performed using purified IN (Chen *et al.*, 2002). Several inhibitors have emerged (Neamati *et al.,* 2000; Neamati, 2002) and two compounds, S-1360 (Shionogi & Co. Ltd.; Yoshinaga T *et al.*, *In vitro* activity of a new HIV-1 integrase inhibitor in clinical development, presented at the *9th Conference on Retroviruses and Opportunistic Infections,* Seattle, USA, 2002) and L-870810 (Merck and Co. Inc.;

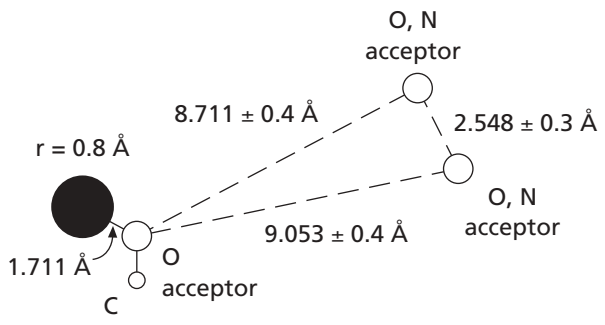
Pais *et al.*, 2002), have shown convincing therapeutic potential and are currently under clinical evaluations.

A common characteristic of many compounds active against IN is the presence of multiple aromatic rings with polyaryl hydroxylation, frequently of the catechol type (Fesen *et al.*, 1994; LaFemina *et al.*, 1995; Robinson *et al.*, 1996; Pommier *et al.*, 1997). Indeed, based on SAR and other studies (Farnet *et al.*, 1998; Burke *et al.*, 1995; Zhao *et al.*, 1997), the catechol moiety has been postulated to be the pharmacophore of polyhydroxylated inhibitors. Their likely site of action has been located in the catalytic core domain of IN, as suggested by their activity on the enzyme of deletion mutants (aminoacids 50–212) and dependence on divalent cations (Hazuda *et al.*, 1997).

Caffeic acid phenethyl ester (CAPE) was one of the first reported natural product inhibitor of IN (Fesen *et al.*, 1993, 1994). Several follow-up studies demonstrated the importance of the catechol moiety for activity (Burke *et al.*, 1995; Zhao *et al.*, 1997). It was also shown that a majority of reported IN inhibitors contain a three-point pharmacophore feature present in CAPE structure (Nicklaus *et al.*, 1997; Figure 1). In an attempt to identify new IN inhibitors of natural origin, we fixed CAPE's acrylate moiety (**I**) (Fesen *et al.*, 1993) in a constrained form by incorporating its vinyl bond into an indole ring, thereby obtaining the 5,6-dihydroxyindole-2-carboxylic acid (DHICA **II**) (Figure 2).

Interestingly, **II** has been recognized as the key monomer intermediate in the biosynthesis of melanins, the primary pigments of skin, hair and eyes in mammals (Nicolaus, 1968; Nicolaus *et al.*, 1964; Prota, 1992; D'Ischia *et al.*, 1996; Tsukamoto *et al.*, 1992). Moreover, this diffusible melanin precursor is a potent stimulator of lipopolysaccharide-induced production of nitric oxide.

Figure 1. Three-point pharmacophore used in the 3D database searching based on CAPE structure (Nicklaus *et al.*, 1997)



Furthermore, it has been hypothesized to play a role as a chemical messenger mediating interaction between active melanocytes and macrophages in epidermal inflammatory and immune responses (D'Aquisto *et al.*, 1995).

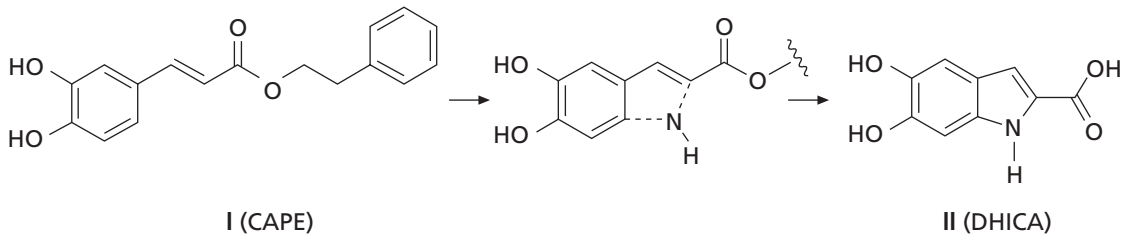
A novel synthetic route to **II** was performed followed by the synthesis of a series of derivatives such as: i) amides characterized by non-hydroxylated (**IIIa** and **IIIb**) or catechol-containing (**IIIc** and **IIId**) aromatic groups; ii) dimeric forms of **II** connected by linkers of various lengths (**III**e-**III**i) (Figure 3). In this study we present the inhibitory potency of the listed compounds against purified IN. Their cytotoxicity and antiviral activity were studied in cell-based assays. Recently, we showed that mercaptosalicylhydrazides designed to chelate Mg^{2+} on the active site of IN also bind to C65. Therefore, some of the most potent compounds presented in this study were also evaluated in enzyme assays against the C56S mutant. We also performed docking studies to investigate the interactions between these compounds and the amino acid residues on the IN active site.

Materials and methods

Chemistry

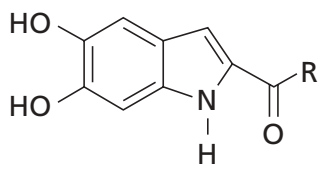
Anhydrous solvents and all reagents were purchased from Aldrich, Merck or Carlo Erba. Anhydrous diethyl ether was obtained by distillation from Na/benzophenone under nitrogen. All reactions involving air- or moisture-sensitive compounds were performed under nitrogen using oven-dried glassware and syringes to transfer solutions. Melting points (mp) were determined using an Electrothermal melting point or a Köfler apparatus and are uncorrected. Infrared (IR) spectra were recorded as thin films or nujol mulls on NaCl plates with a Perkin-Elmer 781 IR spectrophotometer and are expressed in ν (cm^{-1}). Nuclear magnetic resonance (^{1}H-NMR and ^{13}C-NMR) spectra were determined in $CDCl_3$, DMSO or $CDCl_3$/DMSO (in ratio 1:3) and were recorded on a Varian XL-200 (200 MHz). Chemical shifts (δ scale) are reported in parts per million (ppm) downfield from tetramethylsilane (TMS) used as an internal standard. Splitting patterns are designated as follows: s, singlet; d, doublet; t, triplet; q, quadruplet; m, multiplet; brs, broad singlet; dd, double doublet. The assignment of changeable protons (OH and NH) was confirmed by the addition of D_2O. Analytical thin-layer chromatography (TLC) was done on Merck silica gel F-254 plates. For flash chromatography, Merck Silica gel 60 was used with a particle size 0.040–0.063 mm (230–400 mesh ASTM). Elemental analyses were performed on a Perkin-Elmer 2400 spectrometer at Laboratorio di Microanalisi, Dipartimento di Chimica, Università di Sassari (Italy), and were within ±0.4% of the theoretical values.

Figure 2. Design of DHICA from CAPE

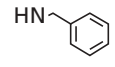
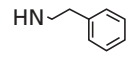
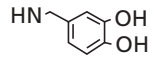
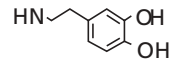


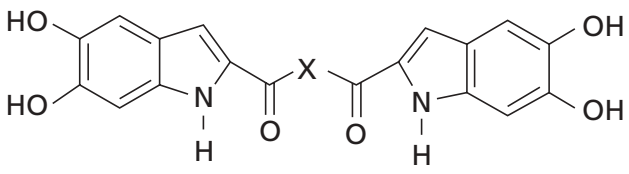
I (CAPE) **II** (DHICA)

Figure 3. DHICA derivatives



III a–d

	R
a	
b	
c	
d	



III e–i

	X
e	
f	
g	
h	
i	

Preparation of azido-acetic acid methyl ester ***(2)***. A solution of methyl bromoacetate (84.98 mmol) and sodium azide (9.0 mmol) in DMF (21 mL) was stirred at room temperature for 2.5 h. After the formation of a white solid, an equivalent quantity of water was added and the resulting solution was extracted three times with diethyl ether. The organic layer was washed six times with water and dried with sodium sulphate. The solvent was removed under reduced pressure to obtain a yellow oil. Yield=94%; IR (film) 2120 (N_3), 1750 (C=O) cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ 3.90 (s, 3H, OC*H*$_3$), 3.81 (s, 2H, C*H*$_2$).

General procedure for the preparation of azidocinnamates ***3a*** *and* ***3b.*** To a solution of the appropriate aldehyde (**1a** and **1b**) (1 mmol) and **2** (3.4 mmol) in methanol (9 mL) a cold solution of sodium methoxyde (3 mmol) was added. The yellow slurry was stirred at −15°C for 4 h and then ice-cold water (36 mL) was added. The resulting precipitate was filtered off and washed with water.

(E/Z)-2-Azido-3-benzo[1,3]dioxol-5-yl-acrylic acid methyl-ester ***(3a)***. Yellow crystals. Yield=91%; mp=61–62°C (from EtOH-H_2O); IR (nujol) 2210 (N_3), 1710 (C=O) cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ 7.57 (d, 1H, Ar-*H*), 7.17 (dd, 1H, Ar-*H*), 6.83 (s, 1H, C*H*=C), 6.81 (d, 1H, Ar-*H*) 6.00 (s, 2H, OC*H*$_2$O), 3.89 (s, 3H, OC*H*$_3$).

(E/Z)-2-Azido-3-(3,4-dimethoxyphenyl)-acrylic acid methyl-ester ***(3b)***. Yellow crystals. Yield=58%; mp=91–92°C (from EtOH-H_2O); IR (nujol) 2120 (N_3), 1700 (C=O) cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ 7.51 (d, 1H, Ar-*H*), 7.35 (dd, 1H, Ar-*H*), 6.88 (s, 1H, C*H*=C), 6.87 (d, 1H, Ar-*H*), 3.92 (s, 6H, OC*H*$_3$ ×2), 3.90 (s, 3H, OC*H*$_3$).

General procedures for the preparation of esters ***4a*** *and* ***4b***. A solution of the azidocinnamates **3a** and **3b** (4.04 mmol) in xylene (25 mL) was heated under reflux for 15 min. After cooling, the resulting pale yellow precipitate was filtered and washed with petroleum ether.

5H-[1,3]Dioxolo[4,5-f]indole-6-carboxylic acid methyl-ester ***(4a)***. Yellow crystals. Yield=79%; mp=194–195°C (from EtOH-H_2O); IR (nujol) 3319 (NH), 1690 (C=O) cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ 9.10 (brs, 1H, N*H*), 7.09 (s, 1H, Ar-*H*), 6.96 (s, 1H, Ar-*H*), 6.83 (s, 1H, Ar-*H*), 5.96 (s, 2H, OC*H*$_2$O), 3.91 (s, 3H, OC*H*$_3$). Anal. Calcd for $C_{11}H_9NO_4$: C, 60.27; H, 4.14; N, 6.39. Found: C, 60.31; H, 4.25; N, 6.19.

5,6-Dimethoxy-1H-indole-2-carboxylic acid methyl-ester ***(4b)***. Yellow crystals. Yield=67%; mp=167–168°C (from EtOH-H_2O); IR (nujol) 3320 (NH), 1680 (C=O) cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ 9.12 (brs, 1H, N*H*), 7.12 (d, 1H, Ar-*H*), 7.04 (s, 1H, Ar-*H*), 6.84 (s, 1H, Ar-*H*), 3.92 (s, 9H, OC*H*$_3$×3). Anal. Calcd for $C_{12}H_{13}NO_4$: C, 61.27; H, 5.57; N, 5.95. Found: C, 61.34; H, 5.70; N, 6.11.

General procedures for the preparation of acids ***5a*** *and* ***5b***. A suspension of the esters **4a** and **4b** (2.28 mmol) in 17 mL of 12% KOH solution was heated under reflux for 1 h. The clear solution was poured in ice-cold water and then acidified with HCl 6N. The resulting white precipitate was filtered and washed with water.

5H-[1,3]Dioxolo[4,5-f]indole-6-carboxylic acid ***(5a)***. White crystals. Yield=98%; mp=248–250°C (dec.) (from EtOH-H_2O); IR (nujol) 3320 (N*H*), 1700 (C=O) cm^{-1}; ^{1}H-NMR ($CDCl_3$:DMSO) δ 11.33 (brs, 1H, N*H*), 6.94 (s, 2H, Ar-*H*), 6.88 (s, 1H, Ar-*H*), 5.93 (s, 2H, OC*H*$_2$O), 4.08–2.75 (brs, 1H, COO*H*). Anal. Calcd for $C_{10}H_7NO_4$: C, 58.54; H, 3.44; N, 6.83. Found: C, 58.60; H, 3.21; N, 6.97.

5,6-Dimethoxy-1H-indole-2-carboxylic acid ***(5b)***. White crystals. Yield=98%; mp=210–212°C (dec.) (from EtOH-H_2O); IR (nujol) 3240 (NH), 1700 (C=O) cm^{-1}; ^{1}H-NMR ($CDCl_3$:DMSO) δ 9.90 (brs, 1H, N*H*), 7.11 (d, 1H, Ar-*H*), 7.04 (s, 1H, Ar-*H*), 6.91 (s, 1H, Ar-*H*), 4.65–3.82 (brs, 1H, COO*H*), 3.93, (s, 3H, OC*H*$_3$) 3.91, (s, 3H, OC*H*$_3$). Anal. Calcd for $C_{11}H_{11}NO_4$: C, 59.73; H, 5.01; N, 6.33. Found: C, 59.55; H, 4.97; N, 6.45.

Preparation of 5,6-dihydroxy-1H-indole-2-carboxylic acid ***(II)***. To a suspension of the acid **5a** or **5b** (1 mmol) in dichloromethane (20 mL) at −40°C and under nitrogen atmosphere, 1M BBr_3 solution in dichloromethane (4 mmol) was added. The mixture was stirred, at the same temperature, for 4 h. After the reaction was quenched with water, the resulting precipitate was filtered, washed with water and triturated with ether. The crude product was purified by silica gel flash column chromatography (eluents petroleum ether–ethyl acetate 3:7) and then recrystallized two times with isopropylic alcohol. Yield: 21% (from **5a**), 36% (from **5b**); mp 235°C dec; IR (nujol) 3480 (COOH), 3430 (OH), 3300 (NH), 1700 (C=O) cm^{-1}; ^{1}H-NMR (DMSO) δ 10.10 (brs, 1H, N*H*), 9.10 (brs, 1H, O*H*), 8.60 (brs, 1H, O*H*), 6.86 (s, 1H, Ar-*H*), 6.82 (s, 1H, Ar-*H*), 6.77 (s, 1H, Ar-*H*). ^{13}C-NMR (DMSO) δ 162.8, 146.2, 142.1, 132.7, 125.9, 119.9, 107.2, 104.9, 97.1. Anal. Calcd. for $C_9H_7NO_4$: C, 55.96; H, 3.65; N, 7.25. Found: C, 55.77; H, 3.69; N, 7.13.

General procedure for the preparation of the amides ***8–16***. A suspension of **5a** (3.65 mmol) and PCl_5 (5.48 mmol) in anhydrous diethyl ether (25 mL) was stirred at room temperature for 2 h. The solvent was removed under reduced pressure; the residue was washed twice with diethyl

ether and then washed three times with chloroform. The acyl chloride obtained was dissolved in anhydrous diethyl ether (25 mL) and a solution of the appropriate amine (5.48 mmol) in 5 mL of dioxane was added dropwise. The mixture was stirred at room temperature for 2 h and the resulting precipitate was filtered and washed with water.

5H-[1,3]Dioxolo[4,5-f]indole-6-carboxylic acid benzyl-amide ***(8)***. White powder. Yield=47%; mp=202–203°C; IR (nujol) 3240 (NH), 1620 (C=O) cm^{-1}; ^{1}H-NMR ($CDCl_3$:DMSO) δ 10.81 (brs, 1H, N*H*), 8.07 (brs, 1H, N*H*), 7.39–7.22 (m, 5H, Ar-*H*), 7.00 (s, 1H, Ar-*H*), 6.93 (s, 1H, Ar-*H*), 6.86 (s, 1H, Ar-*H*), 5.92 (s, 2H, OCH_2O); 4.62 (d, 2H, NHCH_2). Anal. Calcd for $C_{17}H_{14}N_2O_3$: C, 69.38; H, 4.79; N, 9.52. Found: C, 69.16; H, 4.61; N, 9.66.

5H-[1,3]Dioxolo[4,5-f]indole-6-carboxylic acid phenetyl-amide ***(9)***. White powder. Yield=68%; mp=203–204°C; IR (nujol) 3290 (NH), 1610 (C=O) cm^{-1}; ^{1}H-NMR ($CDCl_3$:DMSO) δ 10.47 (brs, 1H, NH), 8.90–8.30 (brs, 1H, *NH*), 7.45–7.20 (m, 5H, Ar-*H*), 6.94 (s, 1H, Ar-*H*), 6.91 (s, 1H, Ar-*H*), 6.83 (d, 1H, Ar-*H*); 5.93 (s, 2H, OCH_2O), 3.66 (q, 2H, NHCH_2), 2.94 (t, 2H, CH_2). Anal. Calcd for $C_{18}H_{16}N_2O_3$.0.15H_2O: C, 69.49; H, 5.29; N, 9.01. Found: C, 69.71; H, 4.98; N, 9.31.

5H-[1,3]Dioxolo[4,5-f]indole-6-carboxylic acid-(benzo-[1,3]dioxol-5-yl-methyl)-amide ***(10)***. White powder. Yield=66%; mp=210–211°C (dec.); IR (nujol) 3280 (NH), 1600 (C=O) cm^{-1}; ^{1}H-NMR ($CDCl_3$:DMSO) δ 10.71 (brs, 1H, N*H*), 7.95 (brs, 1H, N*H*), 6.92–6.75 (m, 6H, Ar-*H*), 5.92 (s, 4*H*,OCH_2O ×2), 3.66 (d, 2H, NHCH_2). Anal. Calcd for $C_{18}H_{14}N_2O_5$.0.25H_2O: C, 63.06; H, 4.26; N, 8.17. Found: C, 63.33; H, 4.35; N, 8.44.

5H-[1,3]Dioxolo[4,5-f]indole-6-carboxylic acid-[2-(3,4-dimethoxy-phenyl)-ethyl]-amide ***(11)***. White powder. Yield=64%; mp=201–202°C; IR (nujol) 3290 (NH), 1630 (C=O) cm^{-1}; ^{1}H-NMR ($CDCl_3$:DMSO) δ 10.80 (brs, 1H, N*H*), 7.56 (brs, 1*H*, N*H*), 6.94-6.91 (m, 3*H*, Ar-*H*), 6.80–6.75 (m, 3*H*, Ar-*H*), 5.92 (s, 2*H*, OCH_2O), 3.84 (s, 3*H*, OCH_3), 3.82 (s, 3*H*, OCH_3), 3.63 (q, 2*H*, N*H*CH_2), 2.88 (t, 2H, CH_2). Anal. Calcd for $C_{20}H_{20}N_2O_5$.0.40H_2O: C, 63.96; H, 5.58; N, 7.46. Found: C, 63.88; H, 5.37; N, 7.65.

5H-[1,3]Dioxolo[4,5-f]indole-6-carboxylic acid-{3-[(5H-[1,3]dioxolo[4,5-f]indole-6-carbonyl)-amino]-propyl}-amide ***(12)***. Beige powder. Yield=42%; mp=268–269°C (dec.); IR (nujol) 3280 (NH), 1630 (C=O) cm^{-1}; ^{1}H-NMR ($CDCl_3$:DMSO) δ 11.25 (brs, 2H, N*H*×2), 8.30 (brs, 2H, N*H*×2), 6.97 (s, 2H, Ar-*H*), 6.93 (s, 2H, Ar-*H*); 6.91 (s, 2H, Ar-*H*×2), 5.93 (s, 4H, OCH_2O×2), 4.72–3.72 (m, 4H, NHCH_2×2), 1.92–1.83 (m, 2H, CH_2). Anal. Calcd for $C_{23}H_{20}N_4O_6$: C, 61.60; H, 4.50; N, 12.49. Found: C, 61.83; H, 4.31; N, 12.52.

5H-[1,3]Dioxolo[4,5-f]indole-6-carboxylic acid-{3-[(5H-[1,3]dioxolo[4,5-f]indole-6-carbonyl)-amino]-butyl}-amide ***(13)***. Beige powder. Yield=49%; mp=297–299°C (dec.); IR (nujol) 3220 (NH), 1630 (C=O) cm^{-1}; ^{1}H-NMR ($CDCl_3$:DMSO) δ 11.20 (brs, 2H, N*H*×2), 8.16 (brs, 2H, N*H*×2), 6.97 (s, 2H, Ar-*H*), 6.92 (s, 2H, Ar-*H*), 6.90 (s, 2H, Ar-*H*), 5.91 (s, 4H, OCH_2O×2), 3.44–3.29 (m, 4H, NHCH_2×2), 1.80–1.52 (m, 4H, CH_2×2). Anal. Calcd for $C_{24}H_{22}N_4O_6$: C, 62.33; H, 4.79; N, 12.12. Found: C, 62.31; H, 4.75; N, 12.34.

5H-[1,3]Dioxolo[4,5-f]indole-6-carboxylic acid-{3-[(5H-[1,3]dioxolo[4,5-f]indole-6-carbonyl)-amino]-esyl}-amide ***(14)***. Beige powder. Yield=39%; mp=252–254°C (dec.); IR (nujol) 3320 (NH), 1620 (C=O) cm^{-1}; ^{1}H-NMR ($CDCl_3$:DMSO) δ 10.90 (brs, 2H, N*H* ×2), 7.80 (s, 2H, N*H*×2), 6.94 (s, 2H, Ar-*H*), 6.91 (s, 2H, Ar-*H*), 6.89 (s, 2H, Ar-*H*), 5.92 (s, 4H, OCH_2O×2), 3.42-3.30 (m, 4H, NHCH_2 ×2), 1.75–1.55 (m, 4H, CH_2 ×2), 1.51–1.25 (m, 4H, CH_2 ×2). Anal. Calcd for $C_{26}H_{26}N_4O_6$.0.25H_2O: C, 63.09; H, 5.40; N, 11.32. Found: C, 63.33; H, 5.21; N, 11.40.

{4-[(5H-[1,3]Dioxolo[4,5-f]indole-6-carbonyl)-piperazin-1-yl}-{5H-[1,3]dioxolo[4,5-f]indol-6-yl}-methanone ***(15)***. White powder. Yield=37%; mp=309–310°C; IR (nujol) 3290 (NH), 1580 (C=O) cm^{-1}; ^{1}H-NMR ($CDCl_3$:DMSO) δ 11.39 (brs, 2H, N*H* ×2), 6.97 (s, 2H, Ar-*H*), 6.90 (s, 2H, Ar-*H*), 6.73 (s, 2H, Ar-*H*), 5.95 (s, 4H, OCH_2O ×2), 3.92 (brs, 8H, NCH_2 ×4). Anal. Calcd for $C_{24}H_{20}N_4O_6$: C, 62.60; H, 4.38; N, 12.17. Found: C, 62.67; H, 4.52; N, 11.91.

5H-[1,3]Dioxolo[4,5-f]indole-6-carboxylic acid-[3-(4-{2-[(5H-[1,3]dioxolo[4,5-f]indole-6-carbonyl)-amino]-propyl}-piperazin-1-yl)-propyl]-amide ***(16)***.
White powder. Yield=39%; mp=234–235=°C (dec.); IR (nujol) 3200 (NH), 1630 (C=O) cm^{-1}; ^{1}H-NMR ($CDCl_3$:DMSO) δ 11.25 (brs, 1H, N*H*×2), 8.28 (brs, 2H, N*H*×2), 6.97 (s, 2H, Ar-*H*), 6.94 (s, 2H, Ar-*H*), 6.88 (s, 2H, Ar-*H*), 5.94 (s, 4H, OCH_2O×2), 4.05–3.22 (m, 12H, CH_2×2), 2.64–2.09 (m, 4H, CH_2×2), 1.81–1.59 (m, 4H, CH_2×2). Anal. Calcd for $C_{30}H_{34}N_6O_6$: C, 62.71; H, 5.96; N, 14.63. Found: C, 62.55; H, 6.07; N, 14.84.

General procedure for the preparation of catechols ***IIIa–IIIh***. To a solution of the amides **8–15** (1 mmol) in dichloromethane (70 mL for **8**, 100 mL for **9–11**, **15** and 200 mL for **12–14**) at −40°C and under nitrogen

atmosphere, 1M BBr_3 solution in dichloromethane (4 mmol for 8–11 and 8 mmol for 12–15) was added. The mixture was stirred under nitrogen atmosphere at the indicated temperature (−40°C for the amides **8–11** and 0°C for **12–14**) for 4 h. The reaction was quenched with methanol, washed three times with methanol and the solvents were removed under reduced pressure. The crude product was purified following method A) or B), depending on the amides. Method A) consisted of silica gel flash column chromatography (eluent petroleum ether–ethyl acetate 4:6 for **IIIa** and **IIIb** or ethyl acetate for **IIIc** and **IIId** or chloroform–methanol 7:3 for **IIIg**) and trituration of the solid with diethyl ether-petroleum ether to provide a powder that was filtered off and collected. Method B) consisted of washing with water and trituration of the solid with diethyl ether for **IIIe**, **IIIf** and **IIIh**.

*5,6-Dihydroxy-1H-indole-2-carboxylic acid-benzyl-amide **(IIIa)**.* White powder. Yield=42%; mp=219–221°C; IR (nujol) 3380 (OH), 3280 (NH), 1630 (C=O) cm^{-1}; ^{1}H-NMR ($CDCl_3$:DMSO) δ 10.05 (brs, 1H, N*H*), 8.02 (brs, 1H, O*H*), 7.86–7.69 (m, 2H, OH and N*H*), 7.42–7.19 (m, 5H, Ar-*H*), 7.00 (s, 1H, Ar-*H*), 6.90 (s, 2H, Ar-*H*), 4.60 (d, 2H, NHCH_2). Anal. Calcd for $C_6H_{14}N_2O_3$: C, 68.07; H, 5.00; N, 9.92. Found: C, 68.21; H, 5.09; N, 9.77.

*5,6-Dihydroxy-1H-indole-2-carboxylic acid-phenetyl-amide **(IIIb)**.* White powder. Yield=14%; mp=174–176°C; IR (nujol) δ 3240 (OH), 3300 (NH), 1620 (C=O) cm^{-1}; ^{1}H-NMR ($CDCl_3$:DMSO) δ 9.45 (brs, 1H, NH), 7.79 (brs, 1H, O*H*), 7.47 (brs, 1H, O*H*), 7.42–7.15 (m, 6H, Ar-*H* and N*H*), 7.02 (s,1H, Ar-*H*), 6.91 (s, 1H, Ar-*H*), 6.66 (s, 1*H*, Ar-*H*), 3.68 (q, 2H, NHCH_2), 2.93 (t, 2H, CH_2). Anal. Calcd for $C_{17}H_{16}N_2O_3$: C, 68.91; H, 5.44; N, 9.45. Found: C, 69.05; H, 5.57; N, 9.30.

*5,6-Dihydroxy-1H-indole-2-carboxylic acid-(3,4-dihydroxy-benzyl-)-amide **(IIIc)**.* Grey powder. Yield=15%; mp=265–267°C (dec.); IR (nujol) 3400 (OH), 3320 (NH), 1630 (C=O) cm^{-1}. ^{1}H-NMR ($CDCl_3$:DMSO) δ 10.76 (brs, 1H, N*H*), 8.74 (brs, 2H, O*H*×2), 8.31 (brs, 2H, O*H*×2), 7.60 (brs, 1H, N*H*), 6.98–6.71 (m, 4H, Ar-*H*), 6.70–6.52 (m, 2H, Ar-*H*), 4.32–4.05 (m, 2H, NHCH_2). Anal. Calcd for $C_{16}H_{14}N_2O_5$: C, 61.14; H, 4.49; N, 8.91. Found: C, 61.22; H, 4.44; N, 8.99.

*5,6-Dihydroxy-1H-indole-2-carboxylic acid-(3,4-dihydroxy-phenyl-ethyl)-amide **(IIId)**.* White powder. Yield=47%; mp=221–223°C (dec.); IR (nujol) 3280 (NH), 3320 (OH), 1620 (C=O) cm^{-1}; ^{1}H-NMR ($CDCl_3$:DMSO) δ 10.59 (brs, 1H, N), 8.48 (brs, 4H, O*H*×4), 7.92 (brs, 1H, N*H*), 6.91–6.62 (m, 5H, Ar-*H*), 6.50 (d, 1H, Ar-*H*), 3.60–3.42 (m, 2H, NHCH_2), 2.71 (t, 2H, CH_2). Anal. Calcd for $C_{17}H_{16}N_2O_5$: C, 62.19; H, 4.91; N, 8.53. Found: C, 61.97; H, 5.04; N, 8.66.

*5,6-Dihydroxy-1H-indole-2-carboxylic acid-{3-[(5,6-dihydroxy-1H-indole-2-carbonyl)-amino]-propyl}-amide **(IIIe)**.* Grey powder. Yield=29%; mp=208–210°C (dec.); IR (nujol) 3380 (OH), 3280 (NH), 1630 (C=O) cm^{-1}; ^{1}H-NMR ($CDCl_3$:DMSO) δ 10.85 (brs, 2H, N*H*×2), 8.28 (brs, *4H*, O*H*×4), 7.92 (brs, 2H, N*H*×2), 6.89 (s, 4H, Ar-*H*), 6.86 (s, 2H, Ar-*H*), 3.62–3.34 (m, 4H, CH_2×2), 2.05–2.76 (m, 2H, CH_2). Anal. Calcd for $C_{21}H_{20}N_4O_6.0.15H_2O$: C, 59.05; H, 4.79; N, 13.12. Found: C, 59.26; H, 4.87; N, 13.11.

*5,6-Dihydroxy-1H-indole-2-carboxylic acid-{3-[(5,6-dihydroxy-1H-indole-2-carbonyl)-amino]-butyl}-amide **(IIIf)**.* Grey powder. Yield=59%; mp=304–306°C (dec.); IR (nujol) 3400 (OH), 3200 (NH), 1630 (C=O) cm^{-1}; ^{1}H-NMR ($CDCl_3$:DMSO) δ 10.71 (brs, 2H, N*H*×2), 8.80–8.00 (brs, 4H, O*H*×4), 8.04 (brs, 2H, N*H*×2), 6.90 (s, 2H, Ar-*H*), 6.86 (s, 4H, Ar-*H*), 3.48–3.23 (m, 4H, NHCH_2×2), 1.80–1.55 (m, 4H, CH_2×2). Anal. Calcd for $C_{22}H_{22}N_4O_6$: C, 60.27; H, 5.06; N, 12.78. Found: C, 60.38; H, 4.89; N, 12.82.

*5,6-Dihydroxy-1H-indole-2-carboxylic acid-{3-[(5,6-dihydroxy-1H-indole-2-carbonyl)-amino]-esyl}-amide **(IIIg)**.* Red powder. Yield=26%; mp=201–204°C (dec.); IR (nujol) 3300 (OH), 3200 (NH), 1610 (C=O) cm^{-1}; ^{1}H-NMR ($CDCl_3$:DMSO) δ 10.75 (brs, 2H, N*H*×2), 8.10–7.98 (brs, 2H, O*H*×2), 7.90–7.65 (brs, 4H, O*H*×2 and N*H*×2), 6.86 (s, 2H, Ar-*H*), 6.83 (s, 4H, Ar-*H*), 3.43–3.22 (m, 4H, NHCH_2×2), 1.75–1.55 (m, 4H, CH_2×2), 1.50–1.25 (m, 4H, CH_2×2). Anal. Calcd for $C_{24}H_{26}N_4O_6.0.30H_2O$: C, 61.09; H, 5.68; N, 11.87. Found: C, 61.28; H, 5.88; N, 12.08.

*{4-[(5,6-Dihydroxy-1H-indole-2-carbonyl)-piperazin-1-yl}-{5,6-dihydroxy-1H-indol-2-yl}-methanone **(IIIh)**.* Grey powder. Yield=55%; mp=242–244°C (dec.); IR (nujol) 3380 (OH), 3280 (NH), 1640 (C=O) cm^{-1}; ^{1}H-NMR (DMSO) δ 11.25 (brs, 2H, N*H* ×2), 9.23 (s, 2H, O*H*), 8.76 (s, 2H, O*H*), 7.12 (s, 2H, Ar-*H*), 6.91 (s, 2H, Ar-*H*), 4.13 (m, 8H, NCH_2×4). Anal. Calcd for $C_{22}H_{20}N_4O_6$: C, 65.55; H, 4.62; N, 12.84. Found: C, 65.71; H, 4.45; N, 12.95.

*Preparation of 5,6-dimethoxy-1H-indole-2-carboxylic acid-pentafluorophenyl-ester **(6)**.* A solution of the acid **5b** (9.94 mmol), pentafluorophenol (10.93 mmol) and dicycloexylcarbod**ii**mide (9.94 mmol) in 80 mL of dioxane was stirred at room temperature for 4 h. After the suspen-

sion of dicycloexylurea was filtered off, the filtrate was evaporated under reduced pressure. The yellow residue obtained was purified by silica gel flash column chromatography (eluent, petroleum ether-ethyl acetate 8:2). Yield=88%; mp=207–209°C; IR (nujol) 3340 (NH), 1700 (C=O) cm^{-1}; ^{1}H-NMR ($CDCL_3$) δ9.22 (brs, 1H, N*H*), 7.43 (s, 1H, Ar-*H*), 7.07 (s, 1H, Ar-*H*), 6.85 (s, 1H, Ar-*H*), 3.94 (s, 6H, OCH_3 ×2). Anal. Calcd for $C_{17}H_{10}F_5NO_4$: C, 52.73; H, 2.60; N, 3.62. Found: C, 52.81; H, 2.75; N, 3.68.

Preparation of 5,6-dihydroxy-1H-indole-2-carboxylic acid-pentafluorophenyl ester ***(7)****.* 1M BBr_3 solution in dichloromethane (3.7 mmol was added to a solution of **6** (1.23 mmol) in 20 mL dichloromethane at −40°C under nitrogen atmosphere. The solution was stirred at the same temperature for 4 h. After addition of water the resulting precipitate was filtered and thoroughly washed with water. Yield=32%; mp=220–221°C (dec.); IR (nujol) 3420 (OH), 3340 (NH), 1700 (C=O) cm^{-1}; ^{1}H-NMR ($CDCl_3$:DMSO) δ 10.68 (brs, 1H, N*H*), 8.23 (brs, 1H, O*H*), 8.01 (brs, 1H, O*H*), 7.31 (d, 1H, Ar-*H*), 7.07 (s, 1H, Ar-*H*), 6.96 (s, 1H, Ar-*H*). Anal. Calcd for $C_{15}H_6F_5NO_4$: C, 50.16; H, 1.68; N, 3.90. Found: C, 49.95; H, 1.73; N, 3.99.

Preparation of 5,6-Dihydroxy-1H-indole-2-carboxylic acid-[3-(4-{2-[(5,6-dihydroxy-1H-indole-2-carbonyl)-amino]-propyl}-piperazin-1-yl)-propyl]-amide ***(IIIi)****.* A solution of **7** (0.83 mmol) and 1,4-bis-(3-aminopropyl)-piperazine (0.27 mmol) in 5 mL of anhydrous DMF was stirred, at 65°C under nitrogen atmosphere, for 16 h. After addition of water the resulting precipitate was filtered, washed with petroleum ether and triturated with diethyl ether to obtain a grey powder. Yield=21%; mp=278–281°C (dec.); IR (nujol) 3300 (OH), 1620 (C=O) cm^{-1}; ^{1}H-NMR ($CDCl_3$:DMSO) δ 11.02 (brs, 2H, N*H*), 6.92–6.81 (s, 4H, Ar-*H*), 6.60 (s, 2H, Ar-*H*), 4.10–3.32 (m, 12H, CH_2×6), 2.72–2.34 (m, 4H, NHCH_2×2), 1.62–1.88 (m, 4H, CH_2×2). Anal. Calcd for $C_{28}H_{34}N_6O_6$: C, 61.08; H, 6.22; N, 15.26. Found: C, 61.27; H, 6.26; N, 15.03.

Biology

Materials, chemicals and enzymes. All compounds were dissolved in DMSO and the stock solutions were stored at –20°C. The γ [^{32}P]-ATP was purchased from either Amersham Biosciences or ICN. The expression systems for the wild-type IN and soluble mutant $IN^{F185KC280S}$ were generous gifts of Dr Robert Craigie, Laboratory of Molecular Biology, NIDDK, NIH, Bethesda, Md., USA. The expression system for the cysteine mutant enzymes was a generous gift of Dr Anna Marie Skalka, Fox Chase Cancer Center, Philadelphia, Pa., USA and the proteins were prepared as previously described (Yi *et al.*, 1999; Asante-Appiah *et al.*, 1998).

Preparation of oligonucleotide substrates. The oligonucleotides 21top, 5′-GTGTGGAAAATCTCTAGCAGT-3′ and 21bot, 5′-ACTGCTAGAGATTTTCCACAC-3′ were purchased from Norris Cancer Center Core Facility (University of Southern California) and purified by UV shadowing on polyacrylamide gel. To analyse the extent of 3′-processing and strand transfer using 5′-end labelled substrates, 21top was 5′-end labelled using T4 polynucleotide kinase (Epicentre, Madison, Wis., USA) and γ [^{32}P]-ATP (Amersham Biosciences or ICN). The kinase was heat-inactivated and 21bot was added in 1.5-molar excess. The mixture was heated at 95°C, allowed slowly cool to room temperature, and run through a spin 25 mini-column (USA Scientific) to separate annealed double-stranded oligonucleotide from unincorporated material.

Integrase assays. To determine the extent of 3′-processing and strand transfer, wild-type IN was preincubated at a final concentration of 200 nM with the inhibitor in reaction buffer (50 mM NaCl, 1 mM HEPES, pH 7.5, 50 μM EDTA, 50 μM dithiothreitol, 10% glycerol (w/v), 7.5 mM $MnCl_2$, 0.1 mg/ml bovine serum albumin, 10 mM 2-mercaptoethanol, 10% dimethyl sulphoxide and 25 mM MOPS, pH 7.2) at 30°C for 30 min. Then, 20 nM of the 5′-end ^{32}P-labelled linear oligonucleotide substrate was added, and incubation was continued for an additional 1 h. Reactions were quenched by the addition of an equal volume (16 μl) of loading dye (98% deionized formamide, 10 mM EDTA, 0.025% xylene cyanol and 0.025% bromophenol blue). An aliquot (5 μl) was electrophoresed on a denaturing 20% polyacrylamide gel (0.09 M tris-borate pH 8.3, 2 mM EDTA, 20% acrylamide, 8M urea).

Gels were dried, exposed in a PhosphorImager cassette, analysed using a Typhoon 8610 Variable Mode Imager (Amersham Biosciences) and quantitated using ImageQuant 5.2. Percent inhibition (%I) was calculated using the equation *% I=100 X [1–D–C)/(N –C)],* where C, N and D are the fractions of 21-mer substrate converted to 19-mer (3′-processing product) or strand transfer products for DNA alone, DNA plus IN and IN plus drug, respectively. The IC_{50} values were determined by plotting the logarithm of drug concentration versus percent inhibition to obtain concentration that produced 50% inhibition.

Cell culture and drug preparation

Human tumour-derived ovarian (HEY) cells were cultured in RPMI 1640 media supplemented with 10% fetal bovine serum (FBS) and 2 mM L-glutamine at 37°C in a humidified atmosphere of 5% CO_2. Drugs were prepared in DMSO at a concentration of 10 mM stock solution and stored at –20°C. Further dilutions were freshly made in PBS.

Table 1. Inhibition of HIV-1 integrase catalytic activities, cytotoxicity and antiviral activities of title compounds **I, II**, **IIIa–i** and intermediates **8–16**

Cpds	3′-Processing IC_{50} (μM)	Strand transfer IC_{50} (μM)		CC_{50}(μM) CEM cells	EC_{50}(μM) CEM cells	CC_{50}(μM) HEY cells
	Soluble mutant	Soluble mutant	C65S			
I	5	5	10	–	–	–
II	6.5 ±1	6.0 ±1	–	ND	ND	>20
IIIa	25 ±5	29 ±11	–	29	>29	>20
IIIb	3.5 ±0.2	2.6 ±0.4	–	24	>24	>20
IIIc	11 ±2	3 ±1	–	33	>33	>20
IIId	16 ±2	12 ±1	–	10	>10	>20
IIIe	4.2 ±1.2	4 ±2	6	3.6	> 3.6	>20
IIIf	3 ±1	2.3 ±1.3	4	26	>26	>20
IIIg	46 ±6	18 ±1	–	26	>26	>20
IIIh	3.6 ±2.2	4.4 ±0.7	0.4	ND	ND	>20
IIIi	5 ±1	2.6 ±1.1	–	88	>88	>20
8	>100	>100	–	>200	>200	>20
9	>100	>100	–	>200	>200	>20
10	>100	>100	–	>200	>200	>20
11	>100	>100	–	>200	>200	>20
12	>100	>100	–	>200	>200	ND
13	>100	>100	–	>200	>200	3 ±1
14	>100	>100	–	>200	>200	0.1 ±0.01
15	>100	>100	–	>200	>200	20 ±8
16	ND	ND	–	ND	ND	ND

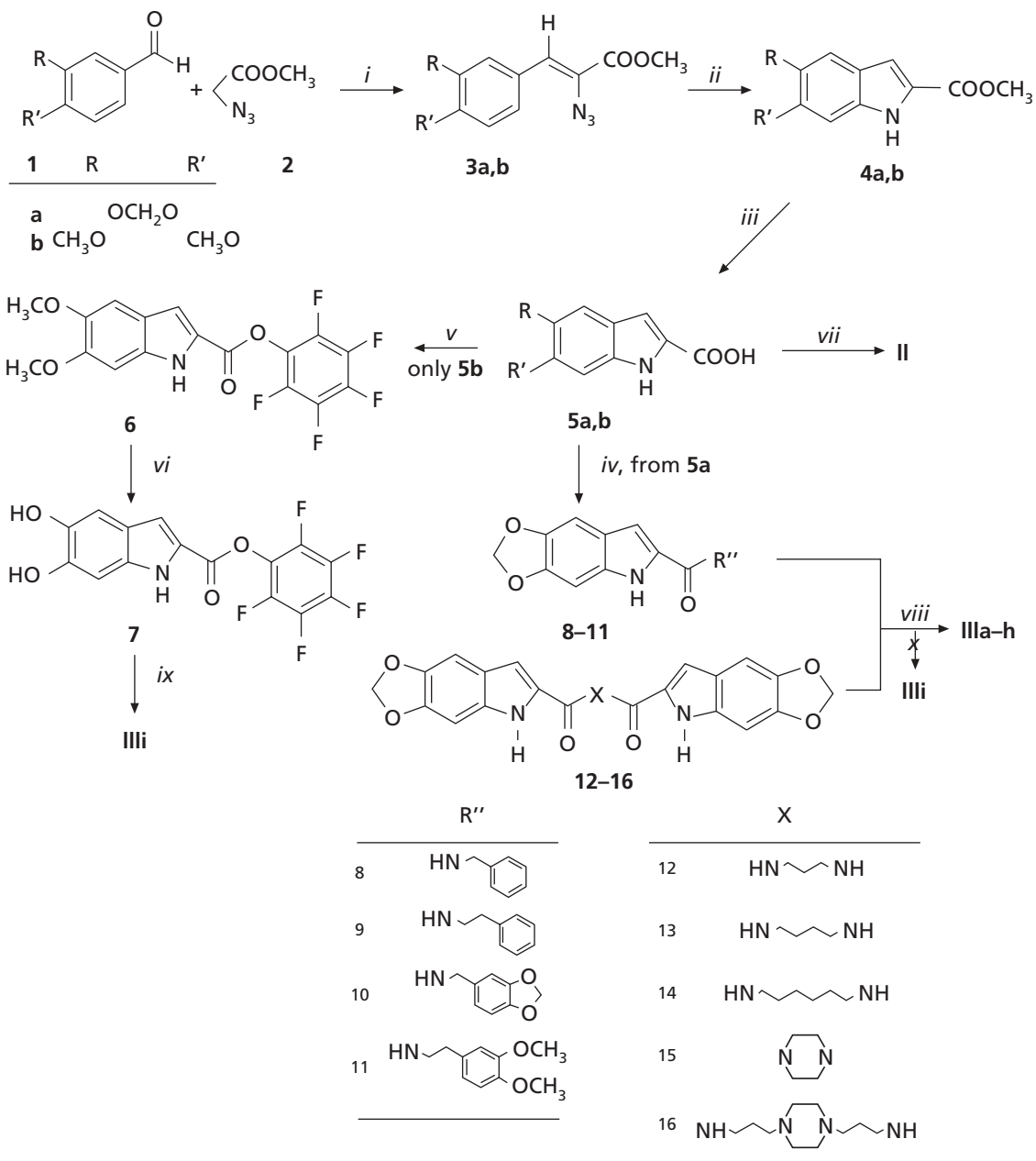
CC_{50} cytotoxic concentration 50%; EC_{50}, effective concentration 50%; MTT, cytotoxicity assay using ovarian carcinoma cells; ND, not determined.

Table 2. Results of 50 independent runs for compounds **II** and **IIIa–IIIi**

Ligand	*N_{tot}	†f_{occ}	‡ΔG_{bind}	H-bonds
II	27	5/5	–5.81	D64, C65, T66, K156, K159
IIIa	14	14/8	–5.26	D64, H67, N155, K159
IIIb	6	21/11	–5.42	D64, C65, K159
IIIc	12	22/10	–5.06	D64, T66, H67, Q148, N155, K159
IIId	6	28/9	–5.39	D64, T66, T115, F139, K156
IIIe	4	35/9	–6.74	C65, T66, P142, N144, Q148, N155
IIIf	6	40/5	–6.40	T66, P142, N144, K159
IIIg	2	48/2	–5.60	T66, N144, S147, N155
IIIh	13	19/9	–6.30	N155, K156, K159
IIIi	2	46/4	–6.09	D64, T66, H67, Q146, K159

*Total number of clusters. †Number of distinct conformational clusters found out of 50 runs/number of multi-member conformational clusters. ‡Estimated free binding energy (kcal/mol).

Figure 4. Experimental conditions



i) CH_3ONa, CH_3OH, –15°C for 4 h; ii) xylene, reflux for 15 min; iii) 12% KOH reflux for 1 h; iv) PCl5, Et_2O, appropriate amine, rt; v) pentafluorophenol, dioxane, r.t. for 4 h; vi) BBr_3, CH_2Cl_2, H_2O, –40°C for 4 h; vii) and viii) BBr_3, CH_2Cl_2, CH_3OH, –40°C for 4 h; ix) DMF, 1,4-bis(aminopropyl)-piperazine, 65°C for 16 h.

Cell viability assay

The cytotoxicity of all drugs was assessed by MTT assay. Briefly, ovarian cancer cells were seeded at a density of 4000 cells/well in 96-well microtitre plates and allowed to attach. Cells were subsequently treated with a continuous exposure to the drugs for 72 h. An MTT solution (at a final concentration of 0.5 mg/ml) was added to each well and cells were incubated for 4 h at 37°C. After removal of the media, DMSO was added and the absorbance was read at 570 nm.

Anti-HIV assays in cultured cell lines

The anti-HIV drug testing performed at the NCI was based on a protocol described by Weislow *et al.* (1989). In brief, all compounds were dissolved in DMSO and diluted in 1:100 in cell culture medium. Exponentially growing T4 lymphocytes (CEM cell line) were added at 5000 cells per well. Frozen virus stock solutions were thawed immediately before use, suspended in complete medium to yield the desired multiplicity of infection (~0.1) and added to the microtitre wells, resulting in a 1:200 final dilution of the compound. Uninfected cells with the compound served as a toxicity control, and infected and uninfected cells without the compound served as basic controls. Cultures were incubated at 37°C in a 5% CO_2 atmosphere for 6 days. The tetrazolium salt, XTT [2,3-bis (2-methoxy-4-nitro-5-sulphenyl)-2H-tetrazolium-5-carboxamide], was added to all wells and cultures were incubated to allow formazan colour development by viable cells. Individual wells were analysed spectrophotometrically to quantitate formazan production, and in addition were viewed microscopically for detection of viable cells and confirmation of protective activity.

Molecular modelling

Title compounds **II** and **IIIa–IIIi** were constructed with standard bond lengths and angles from the fragment database with MacroModel 6.0 (1997) using a Silicon Graphics O2 workstation running IRIX 6.3. Sybyl 6.2 (2001) was used as graphics platform. Compounds were modelled in neutral form by standard molecular mechanics procedures, with the single exception of DHICA (**II**). Because the carboxylic group of compound **II** has a pKa of ~5 under physiological conditions, we used the deprotonated form for our modelling studies. Docking calculations were performed on HP Exemplar Parallel Server V2200 running HP UX 11.0. The atomic charges were assigned using the Gasteiger-Marsili procedure (Gasteiger *et al.*, 1980). Minimization of structures was performed with the MacroModel/BachMin 6.0 program using the AMBER force fields. Extensive conformational search was carried out using the Monte Carlo/Energy minimization (Chang *et al.*, 1989) for all the compounds considered in the study (Ei-Emin<5 Kcal/mol, energy difference between the generated conformation and the current minimum).

The subunit A of (PDB 1QS4) of the IN-5CITEP complex was selected for all docking studies. The missing residues at positions 141–144 in this subunit were incorporated from monomer B of the IN structure PDB 1BIS after superimposition of the backbones of residues 135–140 and 145–150, as reported previously (Sotriffer *et al.*, 2000). Docking was performed with version 3.05 of the program AutoDock (Morris *et al.*, 1998), using the new empirical free energy function and the Lamarckian protocol as described (Morris *et al.*, 1996).

Results

Chemistry

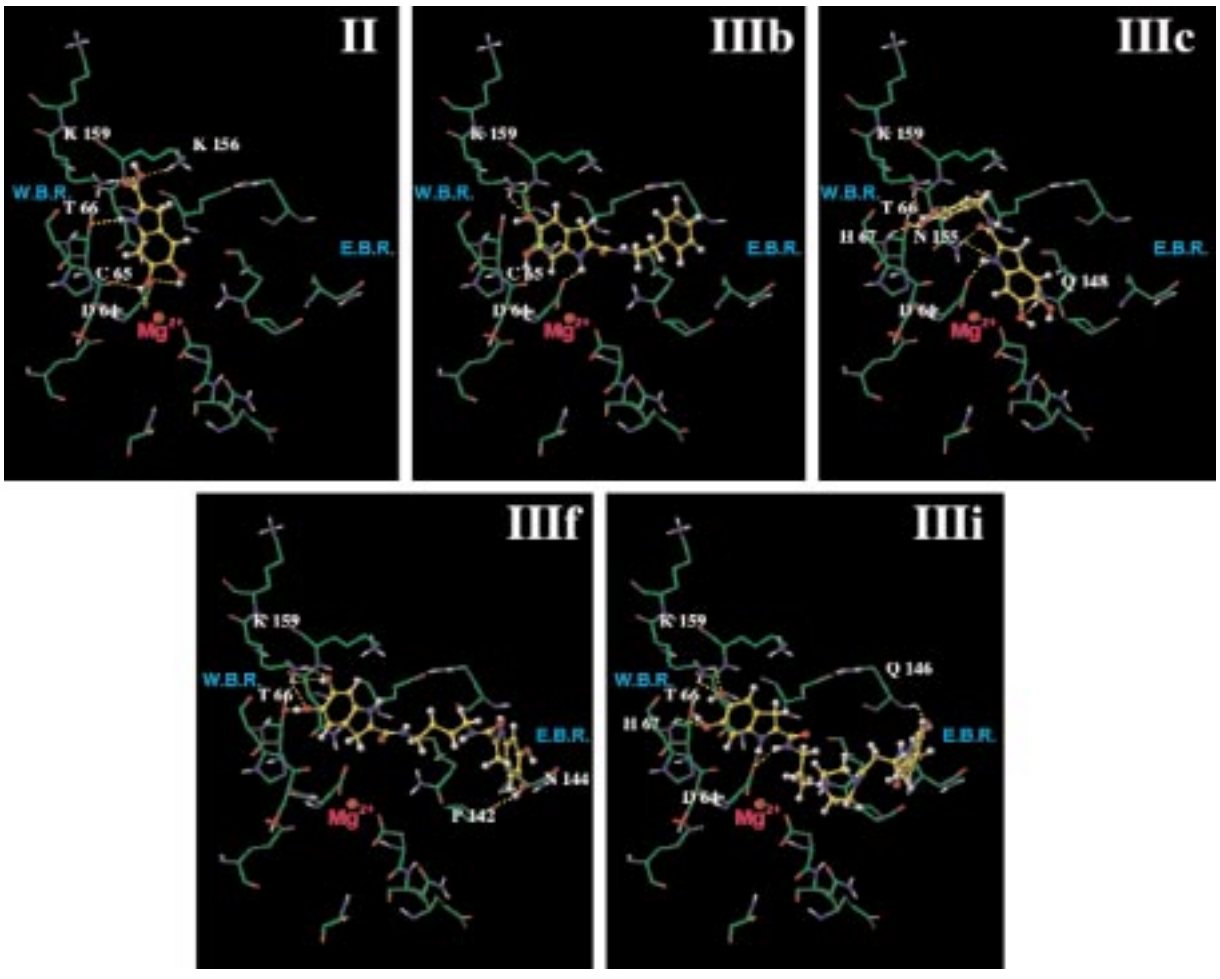
The synthetic approach to compounds **5a** and **5b** and **8–16** used as starting material for the preparation of **II**, **IIIa–IIId** and **IIIe–IIIi** is depicted in Figure 4. Starting from the aldehydes **1a** and **1b** and methyl azidoacetate **2**, azidocinnamates **3a** and **3b** were prepared in high yield according to the Hemetsberger reaction (Hemetsberger *et al.*, 1970; Knittel, 1985; Sechi *et al.*, Design and synthesis of novel indole β-diketo acid derivatives as HIV-1 integrase inhibitors, under review). Intermediates **3a** and **3b** were converted into the esters **4a** and **4b** in refluxing xylene from which, on alkaline hydrolysis, the expected acids **5a** and **5b** were obtained in 98% yields. Deprotection of the catechol moiety with BBr_3 in dichloromethane at −40°C gave the expected DHICA II (Figure 4).

The intermediate **5a** was treated with PCl_5 and the acyl chloride was then coupled with the appropriate amines to give the amides **8–16**. Deprotection of the catechol moiety was carried out as described above to obtain the desired compounds **IIIa-IIIh**. Since compound **IIIi** could not be obtained by this route, we used the intermediate **5b** that was converted into the pentaflurophenyl ester **6**. The latter was first deprotected into **7** and then reacted with 1,4-bis(aminopropyl)-piperazine in DMF at 65°C to give **IIIi** in 21% yield.

Inhibition of HIV-1 IN catalytic activities

Table 1 summarizes the *in-vitro* results of the title compounds **II**, **IIIa–IIIi** and intermediates **8–16**. Inhibition of IN catalytic activities, 3′-processing and strand transfer reactions were carried out using oligonucleotide-based assays as described (Neamati *et al.*, 2002). All target compounds (**II** and **IIIa–IIIi**) showed anti-IN activity in enzyme assays at low micromolar concentrations. In general, all the tested compounds showed equal potency against both 3′-processing and strand-transfer activities of purified IN. This fact prompted us to postulate that most of these compounds are tightly bound to the pre-assembled IN-DNA complex.

Figure 5. Graphical representation of hypothetical binding modes for monomers **II, IIIb, IIIc** and dimers **IIIf, IIIi**



WBR, west binding region; EBR, east binding region. Mg^{2+} ion is shown in magenta.

Table 3. Analysis of elements

		Theoretical			Found		
No.	Formula	C%	H%	N%	C%	H%	N%
4a	$C_{11}H_9NO_4$	60.27	4.14	6.39	60.31	4.25	6.19
4b	$C_{12}H_{13}NO_4$	61.27	5.57	5.95	61.34	5.70	6.11
5a	$C_{10}H_7NO_4$	58.54	3.44	6.83	58.60	3.21	6.97
5b	$C_{11}H_{11}NO_4$	59.73	5.01	6.33	59.55	4.97	6.45
6	$C_{17}H_{10}F_5NO_4$	52.73	2.60	3.62	52.81	2.75	3.68
7	$C_{15}H_6F_5NO_4$	50.16	1.68	3.90	49.95	1.73	3.99
8	$C_{17}H_{14}N_2O_3$	69.38	4.79	9.52	69.16	4.61	9.66
9	$C_{18}H_{16}N_2O_3 0.15H_2O$	69.49	5.29	9.01	69.71	4.98	9.31
10	$C_{18}H_{14}N_2O_5.0.25H_2O$	63.06	4.26	8.17	63.33	4.35	8.44
11	$C_{20}H_{20}N_2O_5.0.40H_2O$	63.96	5.58	7.46	63.88	5.37	7.65
12	$C_{23}H_{20}N_4O_6$	61.60	4.50	12.49	61.83	4.31	12.52
13	$C_{24}H_{22}N_4O_6$	62.33	4.79	12.12	62.31	4.75	12.34
14	$C_{26}H_{26}N_4O_6.0.25H_2O$	63.09	5.40	11.32	63.33	5.21	11.40
15	$C_{24}H_{20}N_4O_6$	62.60	4.38	12.17	62.67	4.52	11.91
16	$C_{30}H_{34}N_6O_6$	62.71	5.96	14.63	62.55	6.07	14.84
II	$C_9H_7NO_4$	55.96	3.65	7.25	55.77	3.69	7.13
IIIa	$C_{16}H_{14}N_2O_3$	68.07	5.00	9.92	68.21	5.09	9.77
IIIb	$C_{17}H_{16}N_2O_3$	68.91	5.44	9.45	69.05	5.57	9.30
IIIc	$C_{16}H_{14}N_2O_5$	61.14	4.49	8.91	61.22	4.44	8.99
IIId	$C_{17}H_{16}N_2O_5$	62.19	4.91	8.53	61.97	5.04	8.66
IIIe	$C_{21}H_{20}N_4O_6.0.15H_2O$	59.05	4.79	13.12	59.26	4.87	13.11
IIIf	$C_{22}H_{22}N_4O_6$	60.27	5.06	12.78	60.38	4.89	12.82
IIIg	$C_{24}H_{26}N_4O_6.0.30H_2O$	61.09	5.68	11.87	61.28	5.88	12.08
IIIh	$C_{22}H_{20}N_4O_6$	65.55	4.62	12.84	65.71	4,45	12.95
IIIi	$C_{28}H_{34}N_6O_6$	61.08	6.22	15.26	61.27	6.26	15.03

Discussion

II, **IIIb**, **IIIc**, **IIIe**, **IIIf**, **IIIh** and **IIIi** with IC_{50} values ranging from 2–11 μM showed similar potency to that of reference compound **I** (IC_{50}=5 μM) (Zhang *et al.*, 2001) against purified IN.

The monomer **II** with IC_{50}=6.5 ±1 μM and 6 ±1 μM against 3′-processing and strand transfer activities, respectively, proved to be one of the most potent compounds. The high potency of DHICA (**II**) could be explained by its unique arrangement in the IN active site, as detailed below. The interaction of catechol with K159, as well as D64 with indolic N-H and coordination with a Mg^{2+} cation in the IN active site, could be responsible for high inhibitory potency of compounds **IIIb** and **IIIc**. Substitution of the carboxyl group of **II** with amide functionality led to compound **IIIa**, which was about 5-fold less active. SAR studies suggested that the anti-IN activity of **IIIa** could be slightly increased, either by substitution of the aromatic hydrogen with a cathecol system (compare **IIIa** and **IIIc**), or by elongation of the alkylamide chain by one methylene unit (compare **IIIa** and **IIIb**). A decrease in anti-IN potency was observed when a cathecol system was added to **IIIb** to obtain **IIId**. With the exception of **IIIg**, dimerization of **II** to obtain compounds **IIIe**–**IIIi** did not lead to a significant variation in activity. In the case of **IIIe**–**IIIg**, a rough correlation between length of linker and activity could be observed. In fact, the best activity was exhibited by compound **IIIf** with IC_{50s}=3 ±1 μM and 2.3 ±1.3 μM for 3′-processing and strand transfer, respectively). Analogue **IIIg** was significantly less potent. Also, **IIIh** and its corresponding flexible analogue **IIIi** displayed potent inhibitory activities. On the other hand, the protected catechol intermediates 8–16 did not show significant activity at the highest tested concentration (200 μM). This clearly indicates that the free catechol functionality is necessary for a strong interaction with the catalytic residues.

The above compounds potentially chelate divalent metal ions. In addition it has been proposed that compounds, such as mercaptosalicylhydrazides that chelate Mg^{2+} ions in the active site of IN, bind to the C65 residue (Neamati *et al.*, 2002). Therefore we wanted to test the hypothesis

that C65 binding is important for activity. The top-ranking compounds **IIIe**, **IIIf** and **IIIh** were active against the C65S (Yi *et al.*, 1999) mutant, which implies that this residue does not bind to these compounds. Interestingly, while **I** was half as active against the C65S mutant as the wild type IN (Zhang *et al.*, 2001), **IIIh** showed 10 times greater potency against the C65S mutant than the wild type IN. In this case, the mutation of Cys to Ser could be favourable for the inhibitory activity of **IIIh**. **IIIe** and **IIIf** inhibited both enzymes with similar potency. It is also noteworthy that **IIIe**, **IIIf** and **IIIh** were 1.6-, 2.5- and 25-fold more active than I against C65S, respectively.

Molecular modelling

Previously, several computational docking studies using the IN-5CITEP co-crystal structure (Goldgur *et al.*, 1999) were reported (Sotriffer *et al.*, 2000; Polanski *et al.*, 2002; Ouali *et al.*, 2000). We applied similar docking procedure to investigate the interactions of **II** and **IIIa–IIIi**, with the amino acid residues of the IN catalytic site. The results of clusterized docking runs with the most favourable free binding energy for each compound are given in Table 2. Graphical representations of top-ranking binding modes obtained for **II**, **IIIb**, **IIIc**, **IIIf** and **IIIi** showing the important residues involved in binding are depicted in Figure 5 and Table 3. According to docking results, the amino acid residues involved in the binding of title compounds located near the catalytic site were as follows: D64, C65, T66, H67, T115, F139, P142, N144, Q146, S147, Q148, N155, K156 and K159. Several of these were considered to be very important for the activity of IN (Neamati *et al.*, 2000) and to some have been previously shown to play a role in substrate binding (Sotriffer *et al.*, 2000). In general, different ligands showed different binding modes with some overlapping features, which were predicted as potential H-bonds and van der Waals interactions.

In order to better analyse the docking results, the structures of title compounds were divided into two main groups constituted by monomers **II**, **IIIa–IIId** and dimers **IIIe–IIIi**. Compound **II** was found to bind to both K156 and K159, two sites important for DNA and mononucleotide binding (Jenkins *et al.*, 1997; Esposito *et al.*, 1998; Drake *et al.*, 1998). In addition, **II** also interacts with T66 (through the indole NH), D64 and C65, both residues located near the Mg^{2+} (through the indole catechol group). On the other hand, the active monomeric derivatives (**IIIa–IIId**) showed different orientation patterns and binding modes within the IN active site. In fact, they form H-bonds with either K156 or K159 through the indole catechol (**IIIa**, **IIIb** and **IIId**) or the phenyl (**IIIc**) moieties. Moreover, they were found to share similar binding with D64 through their indole NH (**IIIb–IIId**) or carboxamide group (**IIIa**), and with either C65 (**IIIb**) or T66 (**IIIc** and **IIId**) through the catechol groups of the indole (**IIIb** and **IIId**) or phenyl (**IIIc**) moieties.

The plane of the indole of **IIIa** is rotated by approximately 180° with respect to that of **IIIb** and **IIId**, and D64 interacts with the NH of the amide function rather than with the indolic NH. This could be the reason for the significantly lower anti-IN activity of **IIIa** with respect to **IIIb** and **IIId**. However, the opposite disposition in the **IIIc** site did not influence the activity. This observation can be consistent with the additional interaction by the indolic catechol with Q148. This confirms that the HIV-1 IN active site is quite large and relatively shallow and therefore able to accommodate large variations in ligand size and shape with different binding modes. The lower activity of the dimer **IIIg** may be explained considering that this compound does not interact with K156, K159 and D64, a recurrent motif important for activity.

Dimeric compounds **IIIe–IIIi** interact with additional binding regions and significantly differ on their positions on the IN active site. While half of the molecule occupies the same space as monomers (the west binding region, as discussed by Buolamwini *et al.*, 2002), the other half uses the area in front of the two catalytic aspartates but without contacting them (east binding region). With the exception of **IIIh**, all dimers establish H-bonds with T66 through one of the cathecol oxygens (interaction on the west region) while the other oxygen interacts with either C65 (**IIIe**) or K159 (**IIIf** and **IIIi**). The indole catechol of **IIIh** instead H-bonds with both K156 and K159. Moreover, indolic NH displayed H-bonding with either N155 (**IIIe** and **IIIh**) or D64 (**IIIi**) while the carboxamide group interacts with Q148 (**IIIe**) or D64 (**IIIi**). On the east binding region the other indole catechol portion establishes H-bonds with both P142 and N144 (**IIIe** and **IIIf**) and with either N144 (**IIIg**) or Q146 (**IIIi**). Our docking results indicated that the estimated free binding energy values and corresponding inhibition constant (Ki) values were, in general, higher than the IC_{50} values obtained experimentally.

Cytotoxicity and antiviral activity

Previously, it was shown that hydroxylated aromatics show significant cytotoxicity and some can cross-link proteins (Stanwell *et al.*, 1996). It was recently shown that catechol-containing compounds are potent inhibitors of lipoxygenase and inhibit cell growth in low micromolar concentration (Simpson *et al.*, 2003). In our cancer screen, none of the target compounds showed significant activity against ovarian carcinoma cells. Interestingly, some of the protected compounds (**13**, **14** and **15**) showed significant cytotoxicity. Surprisingly **14** displayed high cytotoxicity against HEY cells (CC_{50}=0.1 ±0.01 μM) and studies are underway to

determine its mechanism of cytotoxicity.

When we expanded our study to include cytoprotection activity of the title compounds **II** and **IIIa–i** as well as **8–16** against HIV-1-infected CEM cells we observed that the target compounds **II** and **IIIa–IIIi** were toxic and did not yield therapeutic efficacy. These observations are consistent with previous studies (Burke *et al.*, 1995; Neamati, 2002). We also observed that none of the protected compounds **8–16** showed antiviral activity at the highest tested concentration (200 μM).

Acknowledgements

We thank Dr Christoph Sotriffer for help with the initial modelling studies, Dr Maria Orecchioni for assistance with NMR spectroscopy, Ms Paola Manconi for mass spectrometric analysis, Mr Domenico Serra for HPLC analyses and Mr Hossein Rezajan and Mr Franco Fiori for their partial financial support. This work was supported by funds from the Ministero dell'Istruzione, dell'Università e della Ricerca (MIUR), Rome, Italy. We thank Dr Robert Shoemaker and Dr Shizuko Sei for antiviral testing. We also thank Paula Roberts, Quan-En Yang, Omar Ragab and Carney Chen for their excellent technical assistance. The work in NN's laboratory was supported by funds from the GlaxoSmithKline Drug Discovery Award.

References

Asante-Appiah E & Skalka AM (1997) Molecular mechanism in retrovirus DNA integration. *Antiviral Research* **36:**139–156.

Asante-Appiah E, Merkel G & Skalka AM (1998) Purification of untagged retroviral integrases by immobilized metal ion affinity chromatography. *Protein Expression & Purification* **12:**105–110.

Brown PO (1998) In *Retroviruses*. Edited by JM Coffin, SH Hughes and HE Varmus. Cold Spring Harbor: Cold Spring Harbor Press, pp. 161–203.

Buolamwini JK & Assefa H (2002) CoMFA and CoMSIA 3D Docking studies on conformationally-restrained cinnamoyl HIV-1 integrase inhibitors: exploration of a binding mode at the active site. *Journal of Medicinal Chemistry* **45:**841–852.

Burke TR Jr, Fesen MR, Mazumder A, Wang J, Carothers A, Grumberger D, Driscoll J, Kohn K & Pommier Y (1995) Hydroxylated aromatic inhibitors of HIV-1 Integrase. *Journal of Medicinal Chemistry* **38:**4171–4178.

Chang G, Guida WC & Still WC (1989) An internal coordinate Monte Carlo method for searching conformational space. *Journal of the American Chemical Society* **111:**4379–4386.

Chen I-J, Neamati N & MacKerrel A (2002) Structure-based inhibitor design targeting HIV-1 integrase. *Current Drug Targets* **2:**217–234.

D'Aquisto F, Carruccio M, D'Ischia M & Misuraca G (1995) 5,6-Dihydroxyindole-2-carboxylic acid, a diffusible melanin precursor, is a potent stimulator of lipopolysaccharide-induced production of nitric oxide by J774 macrophages. *Life Science* **57:**401–406.

D'Ischia M, Napoletano A & Prota G (1996) [Oxidative polymerization of 5,6-dihydroxyindoles. Tracking the biosynthetic pathway to melanin pigments]. *Gazzetta Chimica Italiana* **126:**783–789.

De Clercq E (2000) Current lead natural products for the chemotherapy of human immunodeficiency virus (HIV) infection. *Medicinal Research Reviews* **20:**323–349.

Drake RR, Neamati N, Hong H, Pilon AA, Sunthankar P, Hume SD, Milne GWA & Pommier Y (1998) Identification of a nucleotide binding site in HIV-1 integrase. *Proceedings of the National Academy of Science, USA* **95:**4170–4175.

Esposito D & Craigie R (1998) Sequence specificity of viral end DNA binding by HIV-1 integrase reveals critical regions for protein-DNA interaction. *EMBO Journal.* **17:**5832–5843.

Farnet CM, Wang B, Hansen M, Lipford JR, Zalkow L, Robinson WE Jr, Siegel J & Bushman F (1998) Human immunodeficiency virus type 1 cDNA integration: new aromatic hydroxylated inhibitors and studies of the inhibition mechanism. *Antimicrobical Agents & Chemotherapy* **42:**2245–2253.

Fesen MR, Kohn KW, Leteurtre F & Pommier Y (1993) Inhibitors of human immunodeficiency virus integrase. *Proceedings of the National Academy of Science, USA* **90:**2399–2403.

Fesen MR, Pommier Y, Leteurtre F, Hiroguchi S, Young SD & Kohn KW (1994) Inhibition of HIV-1 integrase by flavones, caffeic acid phenethyl ester (CAPE) and related compounds. *Biochemical Pharmacology* **48:**595–608.

Gasteiger J & Marsili M (1980) Iterative partial equilization of orbital electronegativity - a rapid access to atomic charges. *Tetrahedron* **36:**3219–3228.

Goldgur Y, Craigie R, Cohen GH, Fujiwara T, Yoshinaga T, Fujishita T, Sugimoto H, Endo T, Murai H & Davies DR (1999) Structure of the HIV-1 Integrase catalytic domain complexed with an inhibitor: a platform for antiviral drug design. *Proceedings of the National Academy of Science, USA* **96:**13040–13043.

Hazuda DJ, Felock PJ, Hastimgs JC, Pramanick B & Wolfe AL (1997) Differential divalent cation requirements uncouple the assembly and catalityc reactions of human immunodeficiency virus type 1 integrase. *Journal of Virology* **71:**7005–7011.

Hemetsberger H, Knittel D & Weidmann H (1970) [Enazides Part 3: Thermolysis of α-azidocinnamates; synthesis of indole derivatives]. *Monatschefte für Chemie* **101:**161–165. German.

Jenkins TM, Esposito D, Engelman A & Craigie R (1997) Critical contacts between HIV-1 integrase and viral DNA identified by structure-based analysis and photo-crosslinking. *EMBO Journal* **16:**6849–6859.

Knittel D (1985) Improved synthesis of α-azidocinnamates and 2*H*-azirines. *Synthesis* 186–188.

LaFemina RL, Graham PL, LeGrow K, Hastings JC, Wolfe A, Young SD, Emini EA & Hazuda DJ (1995) Inhibition of human immunodeficiency virus integrase by bis-catechols. *Antimicrobical Agents & Chemotherapy* **39:**320–324.

Macromodel, version 6.0; Columbia University, New York, 1997.

Morris GM, Goodsell DS, Halliday RS, Huey R, Hart WE, Belew RK & Olson AJ (1998) Automated docking using a Lamarckian genetic algorithm as an empirical binding free energy function. *Journal of Computational Chemistry* **19:**163–166.

Morris GM, Goodsell DS, Huey R & Olson A (1996) Distributed automated docking of flexible ligands to proteins: Parallel application of AutoDock 2.4*. *Journal of Computational-Aided Molecular Design* **10:**293–304.

Neamati N (2001) Structure-based HIV-1 integrase inhibitor design: a future perspective. *Expert Opinion on Investigational Drugs*. **10:**281–296.

Neamati N (2002) Patented small molecule inhibitors of HIV-1 integrase: a ten-year saga. *Expert Opinion Therapeutic Patents* **12:** 709–724.

Neamati N, Marchand C & Pommier Y (2000) HIV-1 integrase inhibitors: past, present, and future. In *Advances in Pharmacology*, vol. 49. Academic Press; pp. 147–165.

Neamati N, Marchand C, Winslow H & Pommier Y (2001) Human

immunodeficiency virus type 1 integrase-targeted inhibitor design. *Antiretroviral Therapy* **5:**87–104.

Neamati N, Lin Z, Karki RG, Orr A, Cowansage K, Strumberg D, Pais CGP, Voigt JH, Nicklaus MC, Winslow HE, Zhao H, Turpin JA, Yi J, Skalka AM, Burke TR Jr & Pommier Y (2002) Metal-dependent inhibition of HIV-1 integrase. *Journal of Medicinal Chemistry* **45:**5661–5670.

Nicklaus MC, Neamati N, Hong H, Mazumder A, Sunder S, Chen J, Milne GWA & Pommier Y (1997) HIV-1 Integrase pharmacophore: discovery of inhibitors through three-dimensional database searching. *Journal of Medicinal Chemistry* **40:**920–929.

Nicolaus RA (1968). In *Melanins*. Paris: Hermann, pp. 155–215.

Nicolaus RA, Piattelli M & Fattorusso E (1964) The structure of melanins and melanogenesis. IV. On some natural melanins. *Tetrahedron* **20:**1163–1172.

Ouali M, Laboulais C, Leh H, Gill D, Xhuvani E, Zouhiri F, Desmaele D, d'Angelo J, Auclair C & Mouscadet JF (2000) Tautomers of styrylquinoline derivatives containing a methoxy substituent: computation of their population in aqueous solution and their interaction with RSV integrase catalytic core. *Acta Biochimica Polonica* **47:**11–22.

Pais GCG & Burke TR Jr (2002) Novel aryl diketo-containing inhibitors of HIV-1 integrase. *Drugs of the Future* **27:**1101–1111.

Polanski J, Zohuri F, Jeanson L, Desmaele D, d'Angelo J, Mouscadet JF, Gieleciak R, Gasteiger J & Le Bret M (2002) Use of the Kohonen neural network for rapid screening of *ex vivo* anti-HIV activity of styrylquinolines. *Journal of Medicinal Chemistry* **45:**4647–4654.

Pommier Y, Marchand C & Neamati N (2000) Retroviral integrase inhibitors year 2000: update and perspectives. *Antiviral Research* **47:**139–148.

Pommier Y, Pilon A, Bajaj K, Mazumder A & Neamati N (1997) HIV-1 integrase as a target for antiviral drugs. *Antiviral Chemistry & Chemotherapy* **8:**483–503.

Prota G (1992). In *Melanins & Melanogenesis*. San Diego: Academic Press, pp. 93–148.

Robinson WE Jr, Reinicke MG, Abdel-Malek S, Jia Q & Chow SA (1996) Inhibitors of HIV-1 replication that inhibit HIV integrase. *Proceedings of the National Academy of Science, USA* **93:**6326–6331.

Simpson J, Forrester R, Tisdale MJ, Billington DC & Rathbone DL (2003) Effect of catechol derivatives on cell growth and lipoxygenase activity. *Bioorganic & Medicinal Chemistry Letters* **13:**2435–2439.

Stanwell C, Ye B, Yuspa SH & Burke TR Jr (1996) Cell protein cross-linking by erbstatin and related compounds. *Biochemical Pharmacology* **52:**475–480.

Sotriffer CA, Ni H & McCammon AJ (2000) Active site binding modes of HIV-1 Integrase inhibitors. *Journal of Medicinal Chemistry* **43:**4109–4117.

Sotriffer CA, Ni H & McCammon AJ (2000) HIV-1 Integrase inhibitor interactions at the active site: prediction of binding modes unaffected by crystal packing. *Journal of the American Chemical Society* **122:**6136–6137.

SYBYL, version 6.2; Tripos Inc., St Louis, Mo., USA, 2001.

Tsukamoto K, Palumbo A, D'Ischia M, Hearing VJ & Prota G (1992) 5,6-Dihydroxyindole-2-carboxylic acid is incorporated in mammalian melanin. *Biochemical Journal* **286:**491-495.

Weislow OW, Kiser R, Fine D, Bader J, Shoemaker RH & Boyd MR (1989) New soluble-formazan assay for HIV-1 cytopathic effects: application to high-flux screening of synthetic and natural products for AIDS antiviral activity. *Journal of National Cancer Institute* **81:**577–586.

Yi J, Asante-Appiah E & Skalka AM (1999) Divalent cations stimulate preferential recognition of a viral DNA end by HIV-1 integrase. *Biochemistry* **38:**8458–8468.

Zhao H, Neamati N, Mazumder A, Sunder S, Pommier Y & Burke TR Jr (1997) Hydrazide-containing inhibitors of HIV-1 Integrase. *Journal of Medicinal Chemistry* **40:**1186–1194.

Zhang X, Neamati N, Lee YK, Orr A, Brown RD, Whitaker N, Pommier Y & Burke TR Jr (2001) Arylisothiocyanate-containing esters of caffeic acid designed as affinity ligands for HIV-1 integrase. *Bioorganic & Medicinal Chemistry* **9:**1649–1657.

Received 15 January 2004; accepted 18 March 2004