Synthesis, biological activity, pharmacokinetic properties and molecular modelling studies of novel 1*H*,3*H*-oxazolo[3,4-*a*]benzimidazoles: non-nucleoside HIV-1 reverse transcriptase inhibitors

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New 1*H*,3*H*-oxazolo[3,4-*a*]benzimidazoles (OBZs) were synthesized as HIV-1 non-nucleoside reverse transcriptase inhibitors (NNRTI) to extend the structure–activity relationships observed for an early series of related 1*H*,3*H*-thiazolo[3,4-a]benzimidazole derivatives (TBZs). The new compounds showed inhibitory activity against the replication of various HIV-1 strains, including NNRTI-resistant strains. Testing of a representative OBZ derivative in an HPLC assay on biological fluids, indicated that the sulphur substitution appreciably improved the metabolic stability of the TBZ compound. In addition, molecular modelling studies demonstrated that OBZs, TBZs and other NNRTIs have similar structural properties, that is a butter-fly-like conformation, which is a key structural requirement for reverse transcriptase inhibition.

Keywords: 1*H*,3*H*-oxazolo[3,4-*a*]benzimidazoles, anti-HIV activity, NNRTIs
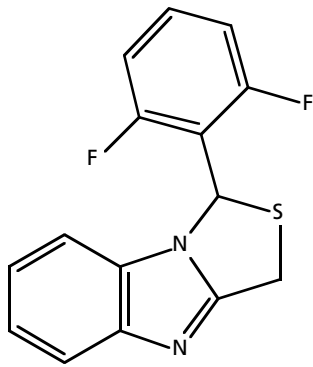
Introduction

The development of chemotherapeutic agents for the treatment of HIV-1 infection has focused on three enzymes essential for the viral replication cycle: reverse transcriptase (RT), protease and integrase, which are considered to be important targets for the development of anti-AIDS drugs. In recent years, effective inhibitors have been developed against the RT and protease enzymes, and 15 compounds have now been formally approved (De Clercq *et al.*, 1999). Various other compounds that interact with different targets, such as entry of the virus into the cell (binding/fusion), are being actively pursued as well. Viral RT has remained an interesting target in the treatment of AIDS, because it is not expressed in mammalian cells. RT inhibitors can be divided into nucleoside RT inhibitors (NRTIs) and non-nucleoside RT inhibitors (NNRTIs). NRTIs include 2′,3′-dideoxynucleosides analogues (ddNs), such as zidovudine, didanosine, zalcitabine, stavudine, lamiduvine and abacavir, acyclic nucleoside phosphonates (ANPs), such as adefovir and tenofovir.

NNRTIs differ mechanistically from NRTIs (chain terminators) and include nevirapine, delavirdine and efavirenz (De Clercq, 1996, 1998; Pedersen *et al.*, 1999; Hajos, *et al.*, 2000). Efavirenz, together with some thiocarboxanilides

and quinoxalines have been referred to as 'second generation' NNRTIs, because multiple mutations are required before virus drug resistance develops (De Clercq, 1996, 1998; Pedersen *et al.*, 1999; Hajos, *et al.*, 2000). The emerging therapeutic role of the NNRTIs has led to an increased interest in developing further drugs of this class, with improved potency and resistance profile. The NNRTIs interact with a specific pocket site of HIV-1 RT, which is closely associated with, but distinct from, the NRTI binding site. By contrast to NRTIs and ANPs, NNRTIs are highly specific HIV-1 inhibitors, and because their mode of action is entirely different they can be usefully combined with NRTIs to delay viral drug resistance, particularly if these combinations are used from the beginning at sufficiently high concentrations (De Clercq, 1996).

In previous publications, we reported a series of 1-aryl substituted 1*H*,3*H*-thiazolo[3,4-*a*]benzimidazole derivatives (TBZs) exhibiting anti-HIV-1 activity (Chimirri *et al.*, 1991a,b, 1994, 1996, 1997a,b, 1998, 1999). Their mechanism of action was elucidated and found to be allosteric inhibition of viral RT with subsequent inhibition of viral replication (Schultz *et al.*, 1992; Chimirri *et al.*, 1997a,b, 1998; 1999). The lead compound in this thia-

Figure 1. Structure of 1H,3H-thiazolo[3,4-a]benzimidazole derivatives derivative

Table 1. Anti-HIV activity, cytotoxicity and inhibitory effects on HIV-1 RT enzymatic activity of compounds 6–8

	EC_{50} (µM)			
Compound	HIV-1 (IIIB)	HIV-2 (ROD)	CC_{50} µM	IC_{50} µM
6	>30	>17	32.2±20.3	775
7	7.9±3.9	>62	32.6±5.5	90
8	6.1±2.7	>71	34.9±28.7	57
1	0.6±0.5	>49	49±5.5	15

EC_{50}, concentration required to reduce HIV-induced cytopathic effect by 50% in MT-4 cells; CC_{50}, concentration required to inhibit cell proliferation by 50%; IC_{50}, poly(C)/oligo(dG) was used as the template/primer and [^{3}H]dGTP as the radiolabelled substrate.

zolobenzimidazole series of RT inhibitors was 1-(2,6-difluorophenyl)-1*H*,3*H*-thiazolo[3,4-*a*]benzimidazole [(**1**), Figure 1)]. This compound proved to be a highly potent inhibitor of HIV-1-induced cytopathic effect and inhibited the replication of various strains of HIV-1 in a variety of human cell lines including a zidovudine-resistant strain (G910-6). Both the racemate and single enantiomers showed inhibition of HIV-1 replication (Chimirri *et al.*, 1997a). In addition, combination of TBZ with either zidovudine or didanosine synergistically inhibited HIV replication and HIV-1-induced cytopathicity. It also inhibited the replication of the Rauscher murine leukemia retrovirus and for these reasons TBZ derivatives have been defined as a new class of NNRTIs with an enhanced range of anti-retroviral activity (Buckheit *et al.*, 1993).

The potential therapeutic utility of TBZ derivatives was hampered both by the metabolic oxidation of the thiazole ring leading to the formation of less potent sulphoxide and sulphone metabolites and the loss of activity against HIV-1 strains with mutation in their RT genes. This behaviour has continued to fuel research efforts to improve the metabolic stability and spectrum of TBZ activity as well as cross-resistance profile. Therefore, the synthesis of analogous 1H,3H-oxazolo[3,4-*a*]benzimidazoles (OBZs), in which the sulphur atom was replaced by an isosteric oxygen, was carried out and is herein reported. The metabolic stability of a representative OBZ derivative was evaluated by an HPLC assay on biological fluids (such as, serum). OBZs were screened for their HIV-1 and HIV-2 inhibitory activity. In addition, the inhibitory effects on HIV-1 RT, as well as the antiviral activity against NNRTI-resistant mutant HIV-1 strains, were examined. A further aim of this study was to demonstrate a possible common mode of interaction of OBZs and other NNRTIs with RT. To reach our research objective, molecular modelling studies were performed and the conformational features of the new derivatives were determined and compared with those of

TBZs and other structurally diverse NNRTIs.

Materials and Methods

Chemistry

Melting points were determined on a Kofler hot stage apparatus and were not corrected. Elemental analyses were carried out on a C Erba Model 1106 Elemental Analyzer (C, H, N) and the results were within ±0.4% of the theoretical values. Merck silica gel 60 F_{254} plates were used for thin layer chromatography (TLC) and column chromatography was performed on Merck silica gel 60 (70–230 mesh). ^{1}H- and ^{13}C-NMR spectra were measured with a Varian Gemini 300 spectrometer (Palo Alto, Calif., USA) in $CDCl_3$ with TMS as internal standard. Chemical shifts are expressed in δ (p.p.m.) and coupling constants (J) in Hertz. Mass spectra were recorded with a GC-17A/MS-QP5050A Shimadzu. 2-(Hydroxymethyl)-benzimidazole (**2**) and aromatic aldehyde dimethylacetals (**3–5**) were synthesized according standard procedures.

Procedure for 1-aryl-1H,3H-oxazolo[3,4-a]benzimidazoles 6–8

To a stirred solution of 2-(hydroxymethyl)benzimidazole (0.74 g, 5 mmol) in dry toluene (150 ml) the appropriate aromatic aldehyde dimethylacetal (5 mmol) and a catalytic amount of p-toluenesulphonic acid were added. The reaction mixture was refluxed for 48 h. After removal of the solvent under reduced pressure, the oily residue was purified by silica gel column chromatography eluting with cyclohexane/ethyl acetate 4:6. All compounds were recrystallized from ethanol.

1-Phenyl-1*H*,3*H*-oxazolo[3,4-*a*]benzimidazole (**6**)
Yield 74%, melting point 103–105 °C; ^{1}H-NMR: 5.19 (d, 1H, −13.2, H_A-3), 5.32 (dd, 1H, J=1.6 and −13.2, H_B-3), 6.90–7.80 (m, 9H, ArH and H-1). ^{13}C-NMR: 64.72 (C-

Figure 2. Synthesis of compounds 6–8

	R1	R2
3,6	H	H
4, 7	Cl	F
5,8	F	F
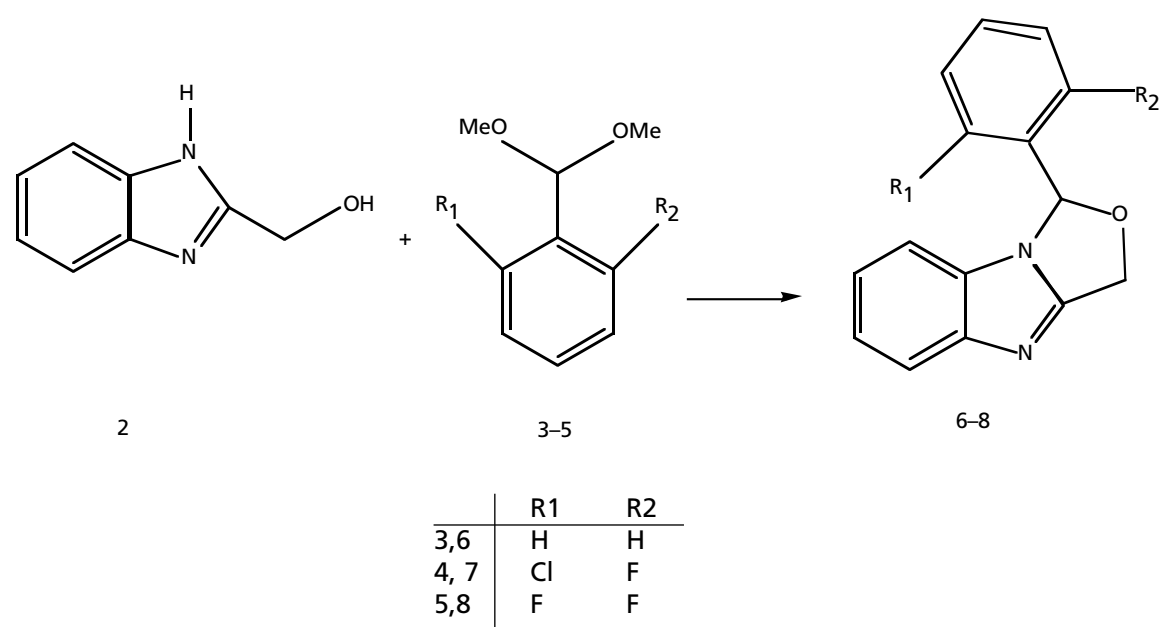
Reagents and conditions: dry toluene, p-TsOH, Δ, 48 h

3), 89.56 (C-1), 109.90 (C-8), 120.19 (C-5), 122.49 and 122.55 (C-6,7), 127.02 (C-2′,6′), 129.04 (C-3′,5′), 130.31 (C-8a), 130.52 (C-4′), 135.50 (C-1′), 149.72 (C-4a), 156.69 (C-3a), MS m/z (%): 236 (M$^+$, 48), 131 (11), 130 (100), 105 (20), 103 (43), 90 (23), 77 (22), 51 (11). Anal. Calcd for $C_{15}H_{12}N_2O$: C, 76.25; H, 5.12; N, 11.86; found: C, 76.46; H, 5.23; N, 11.58.

1-(2-Chloro,6-fluorophenyl)-1H,3H-oxazolo[3,4-a]benzimidazole (7)

Yield 25%, melting point 142−144 °C; ^{1}H-NMR: 5.25 (d, 1H, J=−12.9, H$_A$-3), 5.36 (dd, 1H, J=1.5 and −12.9, H$_B$-3), 6.99-7.78 (m, 8H, ArH and H-1). ^{13}C-NMR: 65.36 (C-3), 83.49 (C-1), 109.37 (C-8), 115.50 (C-5′), 122.42 (C-1′), 120.29 (C-5), 122.62 (C-6,7), 126.34 (C-3′), 129.83 (C-8a), 132.17 (C-4′), 149.70 (C-4a), 156.64 (C-3a), 160.11 (C-2′,6′). MS m/z (%): 290 (M$^+$+2, 14), 288 (M$^+$, 38), 157 (10), 131 (13), 130 (100), 129 (10), 103 (36), 90 (17). Anal. Calcd for $C_{15}H_{10}N_2ClFO$: C, 62.40; H, 3.49; N, 9.70; found: C, 62.56; H, 3.63; N, 9.58.

1-(2,6-Difluorophenyl)-1H,3H-oxazolo[3,4-a]benzimidazole (8)

Yield 44%, melting point 132-134 °C; ^{1}H-NMR: 5.22 (d, 1H, J=−12.8, H$_A$-3), 5.35 (dd, 1H, J=2.1 and −12.8, H$_B$-3), 6.91−7.78 (m, 8H, ArH and H-1). ^{13}C-NMR: 65.04 (C-3), 80.67 (C-1), 109.19 (C-8), 112.22 (C-3′,5′), 112.54 (C-1′), 120.34 (C-5), 122.61 (C-6,7), 129.78 (C-8a),

132.32 (C-4′), 149.84 (C-4a), 156.49 (C-3a), 161.41 (C-2′,6′). MS m/z(%): 272 (m$^+$, 49), 141 (9), 131 (12), 130 (100), 103 (36). Anal. Calcd for $C_{15}H_{10}N_2F_2O$: C, 66.18; H, 3.70; N, 10.29; found: C, 66.46; H, 3.63; N,10.38.

HPLC analysis

Sample preparation. Derivatives **8** and **1** were administered intraperitoneally to Sprague−Dawley rats (10 mg/kg body mass). Blood samples were taken from the animals by an indwelling catheter at selected times after dosing. Blood cells were removed by centrifugation and the separated plasma was stored at −20°C until analysis. A plasma sample (1 ml) was applied to an Oasis HLB solid phase extraction column (Waters, Milan, Italy). Before use, the column was primed with 1 ml of methanol, followed by 1 ml of water. The sample was loaded and drawn through the cartridge at atmospheric pressure. The cartridge was washed with 1 ml of 5% methanol in water; analytes retained in the column were eluted with 1 ml of methanol. The methanol was evaporated under reduced pressure and the sample was reconstituted in 500 μl of acetonitrile. A portion of 40 μl of the resulting solution was injected into the HPLC system.

HPLC system. The HPLC system consisted of the following components: LC-10AD pumps, CTO-10AS column oven, SCL-10A system controller, SIL-10AD autoinjector and SPD-10A UV-detector, all from Shimadzu, Kyoto, Japan. The column was a Hypersil BDS/C18 reversed

Table 2. Inhibitory effect of compounds **6–8** against HIV-1 mutant strains in CEM cells

Compound	EC$_{50}$ (μM)						CC$_{50}$ (μM)
	HIV-1$_{IIIB}$	100 Leu'Ile	103 Lys'Asn	106 Val'Ala	138 Glu'Lys	181 Tyr'Cys	
6	8.5 ±0.0	11.0 ±5.9	>3.4	≥17	>3.4	≥17	13 ±4.1
7	3.8 ±0.5	6.6 ±2.4	≥14	≥14	6.9 ±2.4	≥14	31 ±8.2
8	6.2 ±2.6	7.7 ±1.5	>15	≥15	≥15	≥15	38 ±3.7
1	1.4 ±0.0	3.1 ±1.5	16 ±2.4	7.6 ±4.6	1.7 ±0.5	14 ±6.9	–

EC$_{50}$, concentration required to reduce HIV-induced cytopathic effect by 50%; CC$_{50}$, concentration required to inhibit cell proliferation by 50%.

phase column, particle size 5 μm, 250 mm×4.6 mm ID. The mobile phase was 100 mM ammonium formate:acetonitrile (55:45), with a flow rate of 1 ml/min; detection was by UV absorption at 254 nm.

Molecular modelling

Molecular modelling was performed with use of the software package SYBYL 6.5 (TRIPOS Assoc., St Louis, Mo., USA) running on a Silicon Graphics Octane 10 000 workstation. Conformational energies were calculated through the molecular mechanics TRIPOS force field (Clark *et al.*, 1989). Full geometry optimizations were realized with the semiempirical molecular orbital method AM1. The geometries of nevirapine, α-APA, and TIBO were extracted from structures of the HIV-1/complexes retrieved from the Brookhaven Protein Data Bank. All molecules were superimposed by least-square fitting of some selected atoms.

Virology

In vitro anti-HIV assay. The antiviral experiments using MT-4 cell cultures and HIV-1 (III$_B$) and HIV-2 (ROD) or CEM cell cultures and NNRTI-resistant mutant HIV-1 strains were performed as previously described (Pauwels *et al.*, 1988; Witvrouw *et al.*, 1991).

RT assay. The RT assay using recombinant HIV-1 RT was

essentially performed as described (Balzarini *et al.*, 1993).

Results

Chemistry

The synthesis of 1-aryl-1*H*,3*H*-oxazolo[3,4-*a*]benzimidazoles **6–8** reported here, was accomplished by reacting 2-(hydroxymethyl)benzimidazole **2** with the appropriate aromatic aldehyde acetals **3–5** (Figure 2). Preparation of **2** was achieved by condensation–cyclization of glycolic acid with o-phenylenediamine in acidic medium. Acetals **3–5** were obtained by commercially available aromatic aldehydes and dimethyl sulphate in refluxing MeOH.

The structures of the synthesized compounds **6–8** were assigned on the basis of spectroscopic data (MS, ^{1}H- and ^{13}C-NMR) and supported by satisfactory elemental analysis. Similar to observations of the ^{1}H NMR spectra of isosteric 1-aryl-1*H*,3*H*-thiazolo[3,4-*a*]benzimidazoles (Chimirri *et al.*, 1991a), the spectral features of OBZs suggest a *cis* 1,3-diequatorial relationship between the H-1 and one of the methylene protons at C-3 and a pseudoaxial disposition of the phenyl ring at C-1. These conformational characteristics are indicative of the butterfly-like orientation of the π-systems of OBZ molecules.

In vitro anti-HIV activity

The antiviral activities of compounds **6–8** were evaluated in MT-4 cells acutely infected with HIV-1 (III$_B$) or HIV-2 (ROD) and compared with the results of compound **1** (Table 1). Derivatives **7** and **8** were found to inhibit HIV-1 (III$_B$) replication (EC$_{50}$ 7.9 and 6.1 μM, respectively) whereas none of the compounds inhibited the replication of HIV-2 (ROD) in MT-4 cells at subtoxic concentrations.

In Table 1 we also report the results of the evaluation of the inhibitory effects on HIV-1 RT enzymatic activity. Compounds **6–8** exhibited 50% inhibition of the RT enzymatic activity (IC$_{50}$) at concentration approximately 10-fold higher than their inhibitory activity in cell culture (IC$_{50}$ 57–775 μM).

Compounds **6–8** were also evaluated for their inhibitory effects on a variety of mutant HIV-1 strains containing the following NNRTI-specific amino acid substitution in their

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Figure 3. Superimposition of **1** (white) and **8** (magenta) resulting from QXP fitting

Figure 4. Superimposition of **8** (magenta) and NNRTIs

TIBO, green; Nevirapine, cyan; α-APA, yellow).

RT: 100 Leu'Ile, 103 Lys'Asn, 106 Val'Ala, 138 Glu'Lys and 181 Tyr'Cys (Table 2). These mutant HIV-1 strains were obtained under selective passage in cell culture in the presence of other NNRTIs (TIBO R82150 for 100 Leu'Ile, TIBO R82913 for 103 Lys'Asn, nevirapine for 106 Val'Ala, TSAO-m³T for 138 Glu'Lys, pyridinone for 181 Tyr'Cys and a HEPT derivative for 188 Tyr'His; Balzarini *et al.*, 1993). Compounds **6–8** retained virtually full activity against 100 Leu'Ile RT mutant strain, but lost inhibitory activity against the other mutant viruses.

HPLC analysis
To evaluate whether the substitution of the sulphur atom by an isosteric oxygen engendered an improvement in metabolic stability, we performed HPLC analysis of plasma samples of rats intraperitoneally treated with the 1-(2,6-difluorophenyl)-derivative **8** and with the prototype analogue **1**.

In the chromatograms of plasma samples, taken 4 and 6 h after intraperitoneal administration of derivative **8**, only a peak at 9.2 min retention time was observed. This corresponded to the unchanged compound. No other significant peak was detected. On the contrary, HPLC analysis, carried out in the same experimental conditions on plasma samples of rats treated with **1**, revealed that 4 h after intraperitoneal administration **1** was entirely biotransformed. The peaks present in the chromatograms corresponded to two metabolites identified as the axial and equatorial sulphoxide isomers of **1**, while a third minor metabolite was identified as the sulphone, as already reported (El Dareer *et al.*, 1993).

Molecular modelling studies
In a previous publication (Barreca *et al.*, 1999) we carried out molecular modelling studies on TBZ derivatives and other NNRTIs by comparative molecular field analysis (CoMFA) and flexible docking experiments. An informative and statistically significant CoMFA model was developed for a large series of TIBO derivatives endowed with interesting inhibitory potency towards the HIV RT. The main molecular determinants responsible for high inhibitory activity were identified and proved valid also for TBZ derivatives. The butterfly-like conformation and a suitable spatial location of lipophilic and electron-rich groups are the key structural requirements for a potent inhibitory effect of the NNRTIs through a tight binding in a region close to the RT active site.

On this basis, the 3-D structure of OBZ derivatives was compared with that of **1** and other NNRTIs (Ding *et al.*, 1995; Barreca *et al.*, 1999) aiming at the maximum overlap of the most plausible pharmacophoric components. The molecular model of the OBZ derivative **8** was constructed using the Sybyl 6.5 molecular modelling software and fitted with that of the TBZ derivative. As shown in Figure 3, considerable similarity exists between the energy-minimized butterfly-like shape of compounds **8** and **1**. The alignment with other NNRTIs such as TIBO, nevirapine and α-APA (Figure 4), points out overlapping of the conformation of these ligands.

Discussion

In this paper, the anti-HIV activity and the metabolic pathway of the OBZs were investigated and compared with isoster TBZs. Furthermore, modelling studies were carried out in order to demonstrate a common binding mode of OBZs, TBZs and other NNRTIs.

Our data clearly suggest that OBZs are anti-HIV-1 agents and their mechanism of action, similar to TBZs, proved to be RT enzyme inhibition. We achieved our aim of obtaining compounds with enhanced metabolic stability. This behaviour suggests that the substitution of the sulphur by the oxygen atom positively influences the pharmacokinetic properties of this kind of compounds. In addition, OBZs appear to retain inhibitory activity against some HIV-1 strains that engender resistance to other NNRTIs. Finally, the results of molecular modelling studies allowed us to confirm that also for OBZs, the butterfly-like conformation and suitable spatial location of lipophilic and electron-rich groups are the key structural requirements for RT inhibition.

Acknowledgements

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