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Enhanced Replication of R5 HIV-1 Isolates in vitro by a Small-Molecule Reagent Targeting HIV-1 Protease

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Entry of human immunodeficiency virus (HIV) into host cells requires the initial attachment of virion envelope glycoprotein gp120 to cell-surface receptor CD4, and then interaction with chemokine receptor CXCR4 (expressed on T-lymphocytes) or CCR5 (expressed on monocytes/macrophages).[1] The chemokine-receptor subtype exploited for cell entry determines the tropism of HIV-1 strain involved;[2] those utilising CXCR4 are termed T-tropic X4 viruses, and those exploiting CCR5 are M-tropic R5 viruses. Dual-tropic R5X4 viruses are also known. R5 viruses are important in viral transmission,[3] being responsible for 95% of new infections, and are the predominant species replicating during asymptomatic stages of the disease. Thereafter, evolutionary emergence of X4 viruses characterises significant disease progression and the advent of symptomatic stages.[4] Hence use of clinically relevant R5 viruses in vaccine and drug development is important, motivating interest in addressing the practicalities of achieving R5 virus titres suitable for in vitro biological studies. In HIV replication assays, monocyte-derived macrophages (MDMs) are likely to be more physiologically representative than continuous cell lines.[5] CD8 T-cell-depleted, cytokine-stimulated peripheral blood mononuclear cells (PBMCs) are routinely used for R5 replication studies, containing high levels of activated memory cells (CD45RO+), which are permissive to R5 viruses.[6] However, R5 replication in stimulated PBMCs is slow, and the virus titres obtained are usually poor.[7] Hence scope exists for further development of in vitro R5 virus replication models.

Described here are the unexpected results of HIV replication studies using this model of bioevaluation in our investigation of structures targeting HIV-1 protease (HIV-1 PR). The original objective was to present electrophilic phosphonate functions in putative substrates of HIV-1 PR,[8,9] developed in pursuit of a viable mechanism-based approach[10] to inhibition. In this context, the γ-hydroxyphosphonate arrangement was of interest as potentially susceptible to acid-catalysed intramolecular (5-*exo*-P-tet) transesterification in the environment of the protease active site. The *O*-alkyl propylphostonate[11] function so formed would be highly activated to nucleophilic displacement at phosphorus, and reactive, therefore, towards critical enzymatic residues and structural features. "Molegro" virtual docking experiments[12] indicated docking of **1** within the protease active site, identifying H-bonding interactions involving P=O (O→H interatomic distance 2.6 Å) and hydroxy functions, respectively, with Asp 25 residues of both monomers of the enzyme (Figure 1). These interactions could themselves inhibit protease activity, however, proximity of the phosphonate function of **1** to enzymatic Ile 50/50′ N atoms (P→N interatomic distances 6.6 Å and 4.7 Å, respectively) also suggested scope for hydrolytic sequestration of a Ile 50/50′-bound structural water molecule critical to enzyme–substrate binding interactions.[9,13]

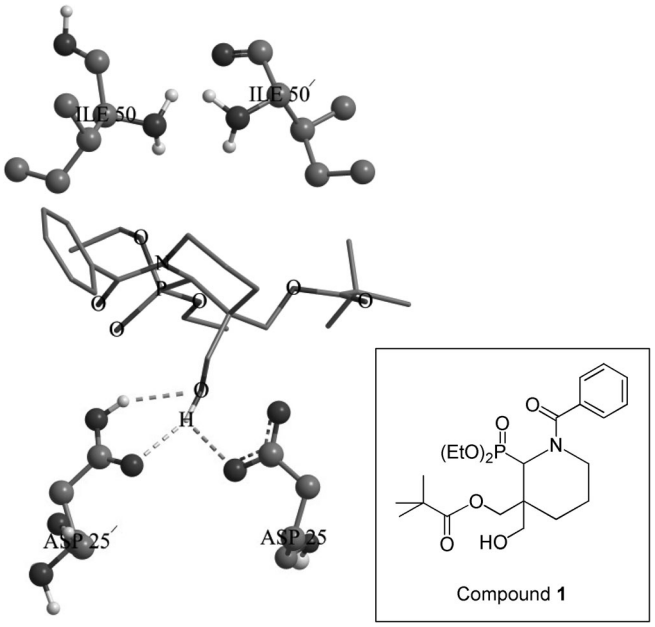

Figure 1. Lowest docked energy conformation of compound **1** in the active site binding pocket of HIV-1 PR. Residues of the second monomer of HIV-1 PR are given prime notation. Inset: structure of compound **1**.

The azacycloalkyl scaffold of **1** was developed from malonate **2**[14] via phase-transfer-catalysed alkylation. This employed 1,3-dibromopropane in a heterogeneous toluene/potassium carbonate system with 18-crown-6 and Aliquot 336 catalysts acting synergistically (Scheme 1).[15] Essential for successful

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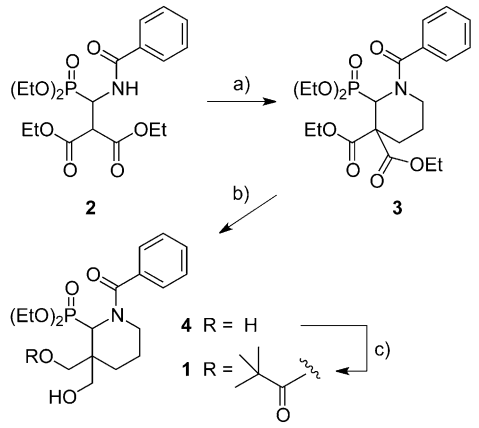

Scheme 1. *Reagents and conditions*: a) $Br(CH_2)_3Br$ (1.5 equiv), K_2CO_3 (3 equiv), 18C6/Aliquot 336 (cat), PhMe, 95–100 °C, 24 h, 26%; b) $LiBH_4$–MeOH (4 equiv), Et_2O, N_2, 0 °C→RT, 1 h, then Δ, 2 h, 25%; c) PivCl (1 equiv), *N*-methylmorpholine, CH_2Cl_2, N_2, 0 °C, 5 min, then RT, 72 h, 29%.

one-step cyclisation is the gradual introduction of base to liquid phase, eliciting initial deprotonation and alkylation at malonate carbon C_β. Kinetic preference for 6-*exo*-tet cyclisation over both O-alkylation (8-*exo*-tet annulation) and intermolecular processes then directs site-selectivity to favour intramolecular, amide N-alkylation, generating the azacyclohexyl structure. Slow, concomitant phosphonate dealkylation limited reaction times, the optimum conditions being 24 hours at 95–100 °C (26% yield).

Chemoselective reduction of a non-enolisable malonate function in the presence of amide required a lithium borohydride/methanol system[16] (use caution for this protocol) in order to generate diol **4** (15–25% yield). Steric factors rendered the malonate function of **3** more refractory to reduction than anticipated, even with lithium borohydride in large excess and, along with the slow development of an unidentified polar by-product, this limited reaction times. Derivatisation of one hydroxy function to pivaloate then generated **1** in 29% yield over 3 days.

HIV-1 PR binding studies conducted on **1** indicated moderate inhibitory activity towards the protease ($IC_{50} = 1.15$ μM). Meanwhile, the effect of compound **1** on HIV-1 replication was examined in vitro at a compound concentration of 50 μM using a panel of clinically relevant primary isolates of HIV-1 in primary cell (CD8-depleted PBMC) cultures.[7,17,18] Unexpectedly, virus replication was significantly enhanced in R5 isolates by factors ranging from 6.7- to 12.6-fold (Table 1), as determined by measurement of virus core protein p24 (determined using a p24 ELISA; supplied by Innogenetics NV, Gent, Belgium). Little or no enhancement was seen with the single X4 virus ($HIV\text{-}1_{PE106}$)[19] tested (data not shown).

The selective action of **1** towards R5 viruses over their X4 counterparts suggested interaction with the CCR5 receptor as a potential cause. Compound **1** also contains a 1-arylcarbamoyl-piperidyl motif common to some therapeutically relevant CCR5 ligands,[22] although other critical pharmacophores are absent.[23] Hence, further investigation of this putative mode of action was undertaken (assays performed by Euroscreen S.A., Gosselies, Belgium). Compound **1** was tested for functional activity towards human CCR5 receptor using an aequorin assay[24] for GPCR-mediated Ca^{2+} signalling, and it was also tested in a [^{125}I]-macrophage inflammatory protein (MIP)-1β radioligand[25] binding competition assay.

Table 1. Compound **1** enhances replication of R5 HIV isolates in peripheral blood mononuclear cells (PBMCs).

Virus[a]	Fold change[b]	Virus[a]	Fold change[b]
PE124	11.5 ± 0.39	041	9.7 ± 0.43
010	10.4 ± 0.19	048	12.6 ± 0.60
031	6.7 ± 0.16	094	9.2 ± 0.86
037	9.1 ± 0.28	114	6.9 ± 0.10

[a] For details, see Ref. [19] (PE124) or Ref. [20] (all other viruses); [b] Fold change in virus yield; for details, see Ref. [21]. Data represent the mean ± SEM of three or more independent experiments.

While no change in activity was observed in the aequorin assay at a compound concentration of 50 μM, compound **1** gave rise to a modest (21%) enhancement in radioligand binding at this concentration. These results are consistent with the concept that **1** binds CCR5 at an alternative site to that of chemokines, causing a conformational change in the receptor by an allosteric mechanism. Noncompetitive, allosteric modes of interaction have been proposed for a number of low-molecular weight CCR5 antagonists.[26] However, in light of the specificity of action of **1** towards R5-type viruses over their X4 counterparts, enhanced rates of HIV replication *without* promotion of intracellular Ca^{2+} release implies facilitation of HIV cell entry without modulation of CCR5 expression or transcriptional activity. In the physiological situation, HIV gp120 competes with chemokines for binding at receptor CCR5 (and CXCR4).[27] Hence, potentiation of CCR5 binding of chemokine MIP-1β indicates a capacity for reagent **1** to facilitate HIV gp120 binding in macrophages cultivated in vitro in the absence of chemokines. To our knowledge, this constitutes the first instance of a small molecule potentiating HIV replication in this way.

These unexpected results are significant with respect to facilitating in vitro biological studies with the R5 virus group. Monocytic cell line Mono Mac 1 is now regarded as a viable model for R5 HIV-1 infection,[5,28] and human acute monocytic leukaemia cell line THP-1 offers qualified utility as such.[5] However, these systems are seen as complementary to physiologically representative infection models relying on MDMs. As current methods produce poor R5 replication profiles in PBMCs,[6] a reagent enhancing replication in this system would facilitate the use of clinically relevant isolates in biological studies.

Supporting Information

Chemical and biological experimental protocols and details of the computational methods used are given in the Supporting Information.

Keywords: antiviral agents · CCR5 · chemokines · HIV-1 · R5 isolates · viral replication · vaccine and drug development

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