Monoclonal antibodies with subnanomolar affinity to tenofovir for monitoring adherence to antiretroviral therapies: from hapten synthesis to prototype development

Simone Cavalera\textsuperscript{a}, Consuelo Agulló\textsuperscript{b}, Josep V. Mercader\textsuperscript{c}, Fabio Di Nardo\textsuperscript{a}, Matteo Chiarello\textsuperscript{a}, Laura Anfossi\textsuperscript{*a}, Claudio Baggiani\textsuperscript{a}, Antonio D’Avolio\textsuperscript{d}, Antonio Abad-Somovilla\textsuperscript{b}, and Antonio Abad-Fuentes\textsuperscript{*c}

Characterization of TFV Derivatives

The progress of reactions was followed by thin-layer chromatography (TLC), using aluminum plates coated with silica gel (60F245 Merck). TLC plates were visualized by exposure to short wave ultraviolet light (254 nm) and irreversibly stained by treatment with an aqueous solution of ceric ammonium molybdate followed by heating. Purification of the synthesized compounds was achieved with a Merck silica gel 60 flash chromatography column (230-400 mesh), using the mobile phase solvent mixture indicated. \textsuperscript{1}H and \textsuperscript{13}C NMR spectra were recorded on a Bruker Avance DRX-300 MHz, in the solvent indicated, at 300 MHz and 75 MHz, respectively. \textsuperscript{31}P-NMR spectra were recorded under high-power proton decoupling conditions. The abbreviation used for NMR data are as follows: s = singlet, d = doublet, t = triplet, dd = double doublet, br = broad, m = multiplet, Pur = purine ring. High resolution mass spectra (HRMS) were obtained by electrospray ionization (ESI) mode in a TripleTOF™ 5600 LC/MS/MS System (ABSciex, Framingham, MA, USA) mass spectrometer equipped with an electrospray source (Waters, Manchester, United Kingdom). The obtained data are expressed as mass/charge ratio (m/z).

MALDI Mass Spectrometry Analysis of Bioconjugates

Sample preparation.

A 100 μL aliquot of protein conjugates purified by size-exclusion chromatography was dialyzed against Milli-Q water for 24 h. One microliter of every sample solution, containing about 1 μg/μL of protein conjugate, was spotted onto the MALDI plate, after the droplets were air-dried at room temperature, 1 μL of matrix [10 mg/mL sinapinic acid (Bruker) in 70% CH$_3$CN, 0.1% CF$_3$CO$_2$H] was added and allowed to air-dry at room temperature.

Mass spectrometry analysis.

The resulting mixtures were analyzed in a 5800 MALDI TOFTOF (ABSciex) in positive linear mode. Previously, the plate and the acquisition method were calibrated with 1 μL of the TOF-TOF calibration mixture (ABSciex), in 13 positions. Every sample was calibrated by ‘close external calibration’ method with a BSA, OVA or HRP spectrum acquired in a close position.
NMR Spectroscopic Characterization of TFVh and Intermediates

**Spectroscopic characterization data of methyl ester 1.**

$^1$H NMR (300 MHz, CD$_3$OD) δ 8.29 (1H, s, H-2 Pur), 8.20 (1H, s, H-8 Pur), 4.37 (1H, dd, $J = 14.4, 3.2$ Hz, H$_5$-1”), 4.22 (1H, dd, $J = 14.4, 6.8$ Hz, H$_5$-1”), 3.91 (1H, m, H-2”), 3.79-3.67 (2H, m, H$_2$-5), 3.71 (1H, dd, $J = 12.8, 9.6$ Hz, H$_5$-1”), 3.62 (3H, s, OCH$_3$), 3.45 (1H, dd, $J = 12.8, 10.2$ Hz, H$_5$-1”), 2.30 (2H, t, $J = 7.3$ Hz, H$_2$-2), 1.66-1.47 (4H, m, H$_2$-3, H$_2$-4), 1.17 (3H, d, $J = 6.2$ Hz, H$_3$-3”); $^{13}$C NMR (75 MHz, CD$_3$OD) δ 175.7 (C-1), 157.2 (C-6 Pur), 153.6 (C-2 Pur), 150.9 (C-4 Pur), 144.2 (C-8 Pur), 119.6 (C-5 Pur), 76.9 (d, $J = 13.1$ Hz, C-2”), 66.5 (d, $J = 160.3$ Hz, C-1”), 65.3 (d, $J = 5.9$ Hz, C-5), 51.9 (OCH$_3$), 49.1 (C-1”), 34.4 (C-2), 31.4 (d, $J = 6.6$ Hz, C-4), 22.4 (C-3), 16.8 (C-3”); $^{31}$P NMR (121 MHz, CD$_3$OD) δ 15.75; HRMS calculated for C$_{15}$H$_{29}$N$_5$O$_9$P [M+H]$^+$ 402.1537, found 402.1525.

**Spectroscopic characterization data of dimethyl diester 2.**

$^1$H NMR (300 MHz, CDC$_3$) δ 8.29 (1H, s, H-2 Pur), 7.92 (1H, s, H-8 Pur), 6.19 (2H, br s, NH$_2$), 4.32 (1H, dd, $J = 14.4, 3.0$ Hz, H$_5$-1”), 4.09 (1H, dd, $J = 14.4, 7.8$ Hz, H$_5$-1”), 4.03-3.87 (5H, m, 2H$_2$-5, H-2”), 3.82 (1H, dd, $J = 13.6, 9.1$ Hz, H$_5$-1”), 3.62 and 3.61 (3H each, each s, 2xOCH$_3$), 3.55 (1H, dd, $J = 13.6, 9.7$ Hz, H$_5$-1”), 2.31 and 2.28 (2H each, each t, $J = 6.9$ Hz, 2xH$_2$-2), 1.70-1.55 (8H, m, 2xH$_2$-3, 2xH$_2$-4), 1.20 (3H, d, $J = 6.2$ Hz, H$_3$-3”); $^{13}$C NMR (75 MHz, CDC$_3$) δ 173.7 (C-1), 155.7 (C-6 Pur), 152.9 (C-2 Pur), 150.1 (C-4 Pur), 141.8 (C-8 Pur), 119.2 (C-5 Pur), 76.4 (d, $J = 12.3$ Hz, C-2”), 65.9 and 65.8 (two d, $J = 5.3$ Hz, 2xC-5), 62.6 (d, $J = 168.4$ Hz, C-1”), 51.6 (2xOCH$_3$), 48.2 (C-1”), 33.4 and 33.3 (2xC-2), 29.9 and 29.8 (two d, $J = 5.9$ Hz, 2xC-4), 21.0 and 20.9 (2xC-3), 16.5 (C-3”); $^{31}$P NMR (121 MHz, CDC$_3$) δ 21.02; HRMS calcld for C$_{31}$H$_{58}$N$_6$O$_{13}$P [M+H]$^+$ 516.2218, found 516.2209.

**Spectroscopic characterization data of TFVh.**

$^1$H NMR (300 MHz, CD$_3$OD) δ 8.43 (1H, s, H-2 Pur), 8.36 (1H, s, H-8 Pur), 4.48 (1H, dd, $J = 14.4, 3.1$ Hz, H$_5$-1”), 4.30 (1H, dd, $J = 14.4, 7.0$ Hz, H$_5$-1”), 4.00 (1H, dt, $J = 6.6, 3.0$ Hz, H-2”), 3.87 (2H, dt, 5.6, 5.6 Hz H$_2$-5), 3.82 (1H, $J = 13.1, 9.3$ Hz, H$_5$-1”), 3.62 (1H, dd, $J = 13.1, 9.8$ Hz, H$_5$-1”), 2.30 (2H, t, $J = 7.1$ Hz, H$_2$-2), 1.68-1.56 (4H, m, H$_2$-3, H$_2$-4), 1.21 (3H, d, $J = 6.2$ Hz, H$_3$-3”); $^{13}$C NMR (75 MHz, CD$_3$OD) δ 177.2 (C-1), 152.0 (C-6 Pur), 150.5 (C-4 Pur), 146.5 (C-2 Pur), 145.7 (C-8 Pur), 119.2 (C-5 Pur), 76.9 (d, $J = 12.6$ Hz, C-2”), 66.0 (d, $J = 5.9$ Hz, C-5), 64.6 (d, $J = 162.1$ Hz, C-1”), 49.4 (C-1”), 34.4 (C-2), 31.2 (d, $J = 6.2$ Hz, C-4), 22.4 (C-3), 16.9 (C-3”); $^{31}$P NMR (121 MHz, CD$_3$OD) δ 17.51; HRMS calcld for C$_{18}$H$_{23}$N$_5$O$_8$P [M+H]$^+$ 388.1380, found 388.1371.
$^1$H NMR spectrum of ester 1 (300 MHz, CD$_3$OD)
$^{13}$C NMR spectrum of ester 1 (75 MHz, CD$_3$OD)
$^1$H NMR spectrum of diester 2 (300 MHz, CDCl$_3$)
$^{13}$C NMR spectrum of diester 2 (75 MHz, CDCl$_3$)
\(^1\)H NMR spectrum of TFVh (300 MHz, CD\(_2\)OD)
$^{13}\text{C}$ NMR spectrum of TFVh (75 MHz, CD$_3$OD)
Edited HSQC NMR spectrum of TFVh (75 MHz, CD$_3$OD)

Lateral Flow Strip Production
A 300-mm long nitrocellulose membrane was dispensed with immunoreagents using a XZ1010 Dispense Platform (BioDot, Irvine, CA, USA) dispenser at 0.5 µL/cm. The test line comprised BSA–TFVh conjugate (1 mg/mL), whereas the control line was drawn by dispensing GAM solution (1 mg/mL). Both reagents were diluted in PBS for dispensing. The membrane was dried at rt before assembling. The LFIA strips were assembled by sequentially pasting the dry nitrocellulose membrane, the sample pad, and the adsorbent pad on a backing support. Then, the membrane was cut into 4-mm width strips using a CM5000 Guillotine Cutter (BioDot, Irvine, CA, USA) and stored sealed in dry tubes at 4 °C. Signal from lateral flow assays was read using an EPSON Perfection V39 ultra-compact color image scanner from Seiko Epson Corp. (Suwa, Japan).

Effect of ionic strength and pH on the binding and sensitivity of MAbs #13, #321, and #322
Calibration curves in ic competitive ELISA were prepared by diluting TFV and the mAb in buffers with different composition. In details, the reference buffer comprises 20 mM phosphate buffer, pH 7.4, with 130 mM NaCl added, the low and high ionic strength buffers were obtained by adding 0 and 230 mM of NaCl rather than 130, while acid and basic conditions were obtained by adjusting the pH to 6.5 and 8.5, respectively.
All other experimental details were kept as described.
Results of the experiments are shown in Figure S1.

**Figure S1**: Effect of salt amount (low I and high I represent 0 and 230 mM of NaCl added to the phosphate buffer) and pH (acid and basic correspond to 6.5 and 8.5, respectively) on the binding to the antigen and on the assay sensitivity, as measured by $A_{\text{max}}$ variation (a) and $IC_{50}$ value (b). The reference buffer (ref) was composed as follows: 20 mM phosphate, pH 7.4, with 130 mM of NaCl added.