Supplementary Material (ESI) for Chemical Communications


# Tetra-End-Linked oligonucleotides forming DNA G-Quadruplexes: a new class of aptamers showing anti-HIV activity

Giorgia Oliviero,[a] Jussara Amato,[a] Nicola Borbone,[*a] Stefano D'Errico,[a] Aldo Galeone,[*a] Luciano Mayol,[a] Shozeb Heider,[b] Olujide Olubiyi,[b] Bart Hoorelbeke,[c] Jan Balzarini[c] and Gennaro Piccialli[a]

[a] Dipartimento di Chimica delle Sostanze Naturali, Università degli Studi di Napoli "Federico II", Via D. Montesano 49, I-80131 Napoli, Italy. E-mail: galeone@unina.it; borbone@unina.it.

[b] CRUK Biomolecular Structure Group, The School of Pharmacy, University of London, 29-39 Brunswick Square, London, UK.

[c] Rega Institute for Medical Research, K.U.Leuven, Minderbroedersstraat 10, B-3000 Leuven, Belgium

**Contents** **Page Number**

## General instrumentations

Automated solid-phase oligonucleotide synthesis was performed on a 8909 DNA-Synthesizer (Applied Biosystems). Mass spectra of the oligonucleotides were performed on a Bruker Reflectron MALDI TOF. CD measurements were performed on a JASCO J-715 spectropolarimeter equipped with a Peltier Thermostat JASCO ETC-505T.

## Buffer solution (PBS 1x)

8 g NaCl, 0.2 g KCl, 1.44 g $Na_2HPO_4$, 0.24 g $KH_2PO_4$ were dissolved in 800 mL of distilled $H_2O$. The pH was adjusted to 7.4 with diluted HCl. Then $H_2O$ was added up to 1 liter. The resulting solution was sterilized by autoclave.

## Preparation of ODNs

The synthesis of unmodified ODNs was performed using the standard solid phase phosphoramidite chemistry on controlled pore glass support using an Expedite 8909 DNA synthesizer (15 μmol) (Applied Biosystems). The syntheses of TEL-ODNs **2L**, **2S** and **3L**, **3S** wer performed according to the previously described procedure (G. Oliviero, N. Borbone, A. Galeone, M. Varra, G. Piccialli and L. Mayol, *Tetrahedron Lett.*, 2004, **45**, 4869-4872). In the case of oligomers **TH**, **1L** and **1S**, 5'TBDPS-thymidine-3'-phosphoramidite has been used in the last coupling step of the chain assembly. The 5'-O-*tert*-butyldiphenylsilyl-thymidine-3'-O-(2-cyanoethyl)-N,N'-diisopropylphosphoramidite was, in turn, obtained by following the procedure reported in literature (H. Hotoda, M. Koizumi, R. Koga, M. Kaneko, K. Momota, T. Ohmine, H. Furukawa, T. Agatsuma, T. Nishigaki, J. Sone, S. Tsutsumi, T. Kosaka, K. Abe, S. Kimura and K. Shimada, *J. Med. Chem.*, 1998, **41**, 3655-3663). After automated synthesis, the oligonucleotides were detached and deprotected from the support by using concentrated aqueous ammonia at 55 °C for 17 h. The combined filtrates and washings were dried, redissolved in water, analyzed and purified by HPLC on an anion exchange column (Macherey-Nagel, 1000-8/46, 4.4x50 mm, 5 μm) using a linear gradient from 0 to 100% B in 30 min, flow rate = 1 mL/min and detection at 260 nm,

buffer A: 20 mM $NaH_2PO_4$ aq.solution pH 7.0, containing 20% (v/v) $CH_3CN$.

buffer B: 20 mM $NaH_2PO_4$ aq.solution pH 7.0, containing 1M NaCl and 20% (v/v) $CH_3CN$.

After HPLC purification, the oligonucleotide samples were desalted on a biogel column (BIORAD) eluted with $H_2O/CH_3CH_2OH$ (9:1 v/v). The isolated oligomers were characterized by MALDI TOF mass:

**H** calculated: [M] = 7489.6; found $[M+H]^+ = 7490$

**TH** calculated: [M] = 8442.4; found $[M+H]^+ = 8443$

**1L** calculated: [M] = 9765.3; found $[M+H]^+ = 9766$

**1S** calculated: [M] = 9170.5; found $[M+H]^+ = 9171$

**2L** calculated: [M] = 8811.7; found $[M+H]^+ = 8913$

**2S** calculated: [M] = 8216.9; found $[M+H]^+ = 8218$

**3L** calculated: [M] = 8811.7; found $[M+H]^+ = 8913$

**3S** calculated: [M] = 8216.9; found $[M+H]^+ = 8218$

## Preparation of quadruple helices (annealing).

The ODN samples were dissolved in PBS buffer and the corresponding quadruplexes were formed by heating at 90°C for 5 min and slowly cooling at room temperature for 12 h. The concentration of the dissolved oligonucleotides **2L**, **3L**, **2S** and **3S** were determined by UV measurements at 260 nm and at 90°C using the nearest-neighbour calculated molar extinction coefficient of 5'-TGGGAG-3' multiplied by 4 (the number of strands in each TEL-ODN complex). For oligomers **TH**, **1L** and **1S**, a contribute of 8658 $cm^{-1}M^{-1}$ was added for each group 5'-TBDPS (J. D'Onofrio, L. Petraccone, E. Erra, L. Martino, G. Di Fabio, L. De Napoli, C. Giancola and D. Montesarchio, *Bioconjugate Chem.*, 2007, **18**, 1194-1204).

**H**, ε calculated = 250000 $cm^{-1}M^{-1}$

**TH**, ε calculated = 284632 $cm^{-1}M^{-1}$

**1L**, ε calculated = 284632 $cm^{-1}M^{-1}$

**1S**, ε calculated = 284632 $cm^{-1}M^{-1}$

**2L**, ε calculated = 250000 $cm^{-1}M^{-1}$

**2S**, ε calculated = 250000 $cm^{-1}M^{-1}$

**3L**, ε calculated = 250000 $cm^{-1}M^{-1}$

**3S**, ε calculated = 250000 $cm^{-1}M^{-1}$

## CD spectroscopy measurements

Oligonucleotides were suspended in PBS 1x. Samples (2 x $10^{-5}$ M quadruplex concentration) were annealed as described above and stored at 4°C overnight before measurements. CD spectra were recorded using a wavelenght range of 350-200 nm, using 5 scans at 100 nm/min, 1 s response time, 1 nm bandwidth. Cuvettes of 1 mm were used for the measurements. A buffer baseline was collected in the same cuvette and subtracted from each spectrum. Thermal denaturation-renaturation experiments were carried out in a temperature range of 5-90 °C, by monitoring CD values at 264 nm at a heating/cooling rate of 0.5 °C/min. The $T_m$ is the mid-point of the melting curve at which the complex is 50% dissociated. This was estimated from the maximum in the first derivative of the melting profile.

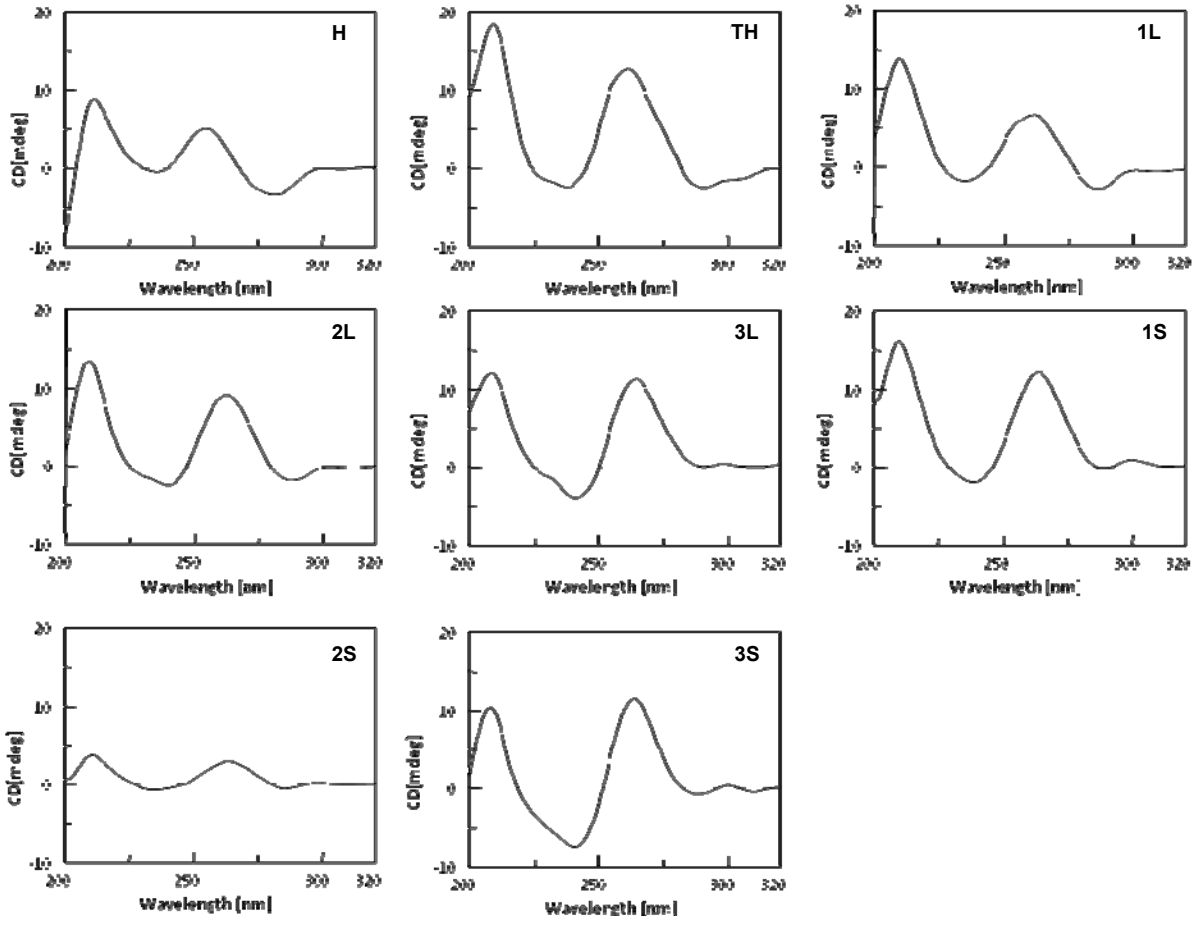


**Fig. S1:** CD profiles of **H**, **TH**, **1L**, **2L**, **3L**, **1S**, **2S** and **3S** in PBS 1x at 25°C.

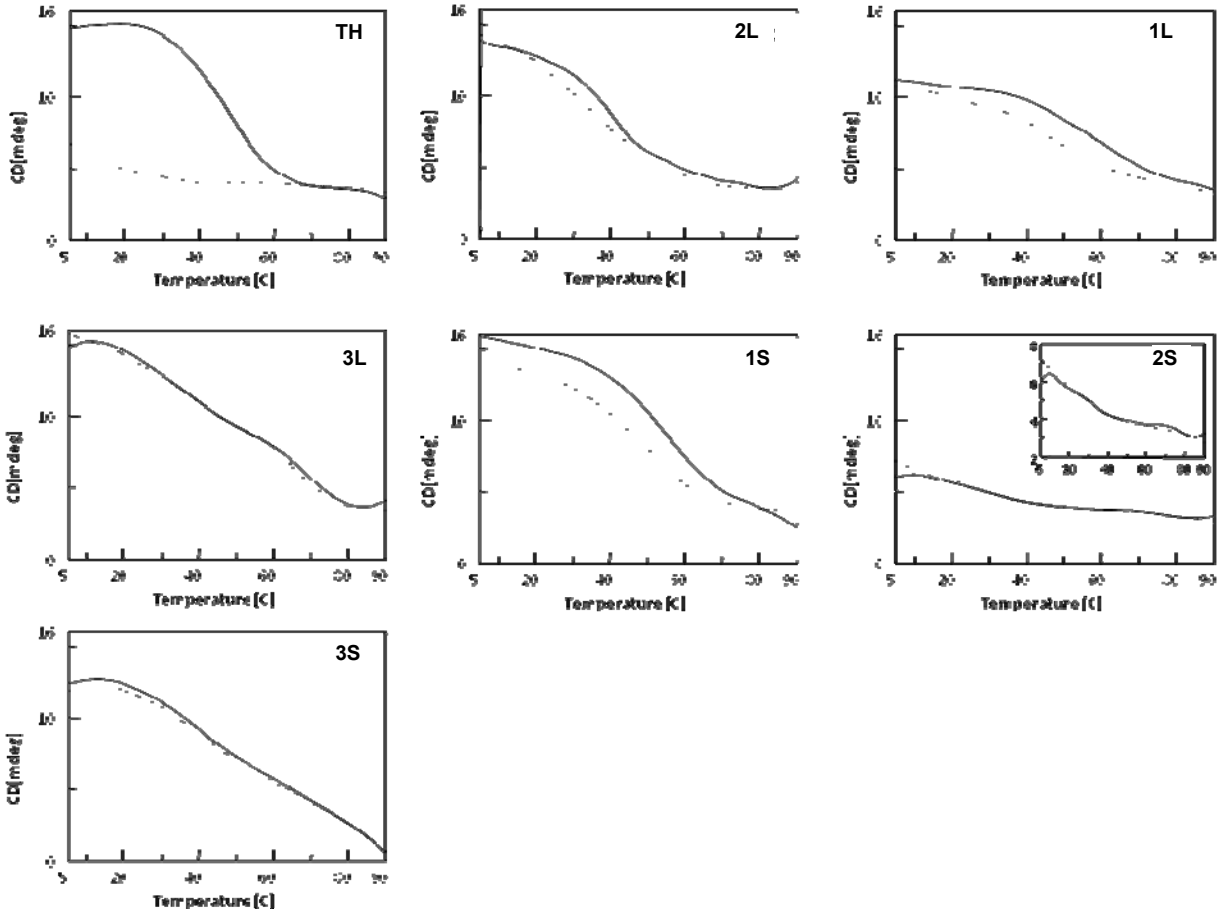


**Fig. S2**: CD-melting and annealing curves recorded at 264 nm for of **TH**, **1L**, **2L**, **3L**, **1S**, **2S** and **3S** in PBS 1x (——— from 5 to 90°C; --------- from 90 to 5°C)

## Docking protocol

The molecular models of TEL-ODNs were generated using the Insight Software (Accelrys, Inc.). Modified linkers were made using the Builder tool provided in the Insight suite. The main aim of the study was to identify the mode of interactions of the aptamers (**1L**, **1S**, **2L** and **2S**) with the hypervariable V3 loop. The hypervariable V3 loop of HIV gp120 protein was extracted from a complex with IGG1 Fab fragment (Pdb id 3F58).

V3 loop in gp120 was docked with TEL-ODN using the program ZDOCK, that employs fourier transformation to search multiple binding possibilities for receptor and ligand. The scoring function is based on shape complementarity, desolvation energy and electrostatics. The output from the program is a list of transformation (rotation and translation) values for the docks, from which models can be generated. A total of 100 transformations were calculated, ranked based on internal scoring functions and presented through a global energy term. The top solution from each dock was chosen and further minimised to relieve any steric clashes that might have arisen in docking. No atoms were restrained during the minimisation process. The minimised models were then chosen for visualization and further studies. Visualization of the models and figures were generated using the Pymol software.

In **2L**-**2S**/V3 loop complexes (Fig. S3 (C, D)), R190 from V3 loop interacts by sliding in the grooves created by the phosphodiester backbone atoms of **2S** and **2L** aptamers. Although this is analogous to the interactions observed in **1L** (Fig. 3) and **1S** (Fig. S3 (A, B)), the orientation of the V3 loop interacting with **2S** and **2L** is different. The main contributors of electrostatic interactions are the backbone atoms of G189, A191, T194, T195 and the side chain of E197 that interact with phosphodiester backbone atoms of the **2S**-**2L** aptamers.

**Table S1** Interaction energies of top docks

| Aptamer | Interactions Energy (kcal/mol) |
|---|---|
| **1L** | -29.14 |
| **1S** | -26.69 |
| **2L** | -21.94 |
| **2S** | -18.21 |

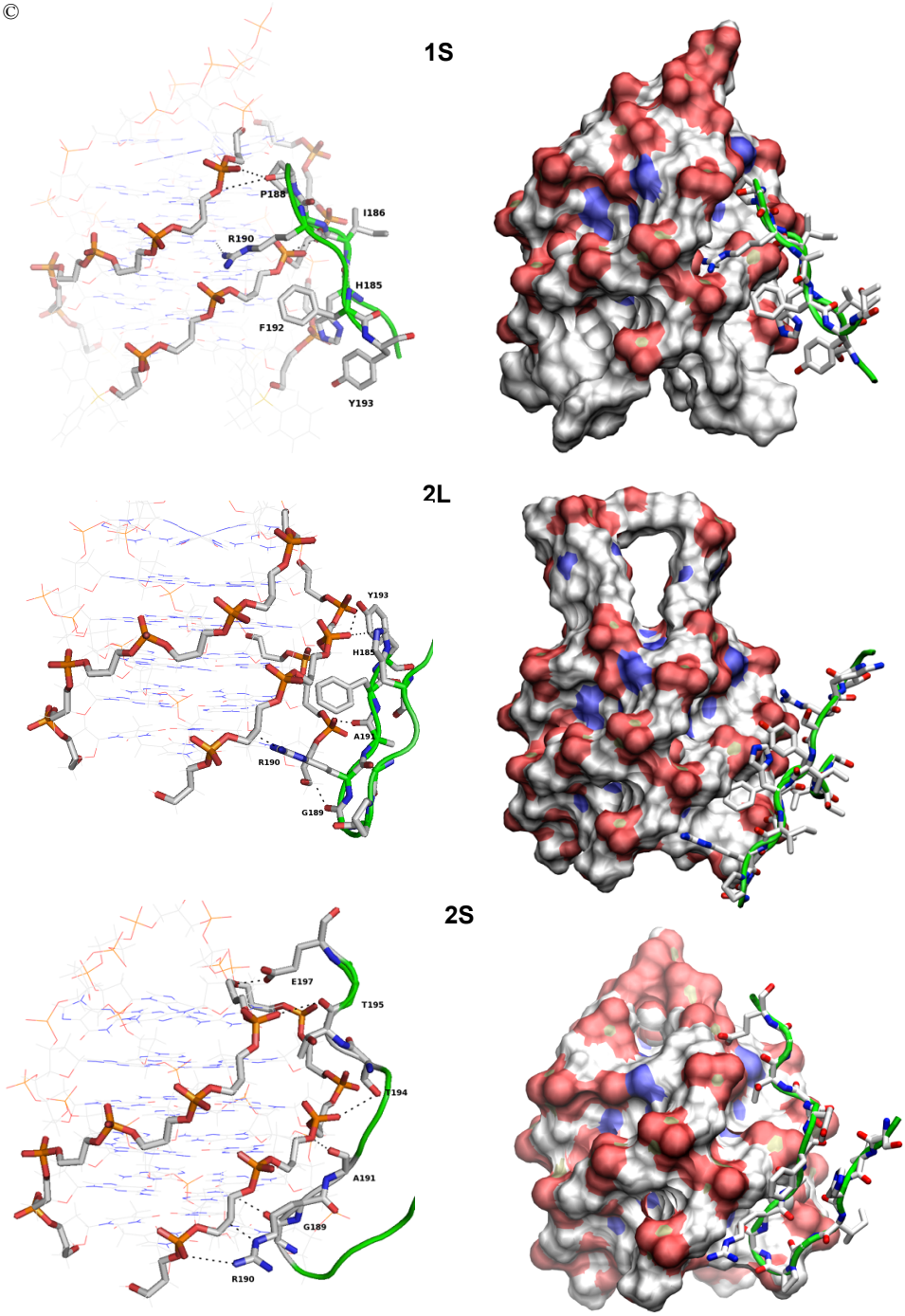


**Fig. S3** Molecular models of interactions between aptamers **1S**, **2L**, **2S** and V3 loop (green). Two different modes of interactions are observed. (Left) The backbone of the aptamer is drawn in liquorice. Hydrogen bonding interactions between gp120 V3 loop and the aptamer are drawn as dashed lines. (Right) The aptamer is represented as charged molecular surface and highlights how residues from the V3 loop slides in the grooves generated by the backbones in the aptamer to maximise the interactions.

## Biological evaluation assays

*Activity assay of test compounds against HIV-1 and HIV-2 in cell cultures*. A total number of 4 x $10^5$ CEM cells per millilitre were infected with HIV-1($III_B$) or HIV-2 (ROD) at ~ 100 $CCID_{50}$ (50% cell culture infective dose) per mL of cell suspension. Then, an amount of 100 µL of the infected cell suspension was transferred to 96-well microtiter plates and mixed with 100 µL of the appropriate dilutions of the test compounds. Giant cell formation was recorded microscopically in the HIV-infected cell cultures after 4 days. The 50% effective concentration ($EC_{50}$) of the test compounds was defined as the compound concentration required to inhibit virus-induced cytopathicity. The 50% cytostatic concentration ($CC_{50}$) was defined as the compound concentration required to inhibit CEM cell proliferation by 50%.

*HIV-1 RT RNA-dependent DNA polymerase activity assay.* RNA-dependent DNA polymerase activity was assayed as follows: a final volume of 25 µL contained reaction buffer (50 mM Tris-HCl, pH 7.5, 1 mM DTT, 0.2 mg/mL BSA, 4% glycerol), 10 mM MgC2L, 0.5 µg of poly(rA)/oligo(dT) or poly(rC)/oligo(dG), 10 µM [$^3$H]dTTP or [$^3$H]dGTP and 2−4 nM RT. Mixtures were incubated at 37 °C for 30 min. Then 20 µL aliquots were spotted on glass fiber filters GF/C which were immediately immersed in 5% ice-cold TCA. Filters were washed twice in 5% ice-cold TCA and once in ethanol for 5 min and dried, and acid-precipitable radioactivity was quantitated by scintillation counting.

## Surface plasmon resonance (SPR) analysis

Recombinant gp120 protein from HIV-1($III_B$) (ImmunoDiagnostics Inc., Woburn, MA), produced by CHO cell cultures was covalently immobilized on a CM5 sensor chip in 10 mM sodium acetate, pH 4.0, using standard amine coupling chemistry. The chip density for gp120 was 820 RU (~ 6.8 fmol of gp120). A reference flow cell was used as a control for non-specific binding and refractive index changes. The experiment was performed at 25°C on a Biacore T100 instrument (GE Healthcare, Uppsala, Sweden). Compound **1L** was serially diluted in HBS-P (10 mM HEPES, 150 mM NaCl and 0.05% surfactant P20; pH 7.4), covering a wide concentration range by using serial two-fold analyte dilutions (covering a concentration range from 269 to 4300 nM). Samples were injected for 2 minutes at a flow rate of 30 µL/min and the dissociation was followed for 5 minutes. One duplicate sample and several buffer blanks were used as a positive control and as double referencing, respectively. The CM5 sensor chip surface was regenerated by 1 injection of 50 mM NaOH. The studied interaction resulted in specific binding signals. The shape of the association and dissociation phases revealed that the curves are not 1:1 Langmuir. The experimental data were fit using the 1:1 binding model (Biacore T100 Evaluation software 2.0.2) to determine the binding kinetics. These kinetic values are apparent values since the injected concentrations of the evaluated compounds did result in biphasic binding signals. However, the obtained kinetic data were considered to be acceptable and reliable since the $\psi^2$ value of the fitting of the data for the interaction of the compound with gp120 proved to be 0.444. The $\psi^2$ value is a statistical measure of how closely the model fits the experimental data and must be as low as possible and at least lower than 10 for a good fit. In comparison, a reference compound Pradimicin S, that specifically binds to HIV-1 gp120 and shows antiviral activity at an EC50 of ~ 9 µM, has a $K_D$ of 0.40 µM and a $k_a$ and $k_d$ of 1.27 x $10^4$ $M^{-1}s^{-1}$ and 5.1 x $10^{-3}s^{-1}$, respectively.[1] Although the $K_D$'s are very similar for HIV-1 gp120, **1L** is 100-fold more inhibitory to HIV-1 than Pradimicin S. However, it should be noticed that Pradimicin S directly interacts with the glycans of gp120, whereas **1L** most likely interacts with the protein part of the gp120, resulting in a different eventual antiviral activity.

[1] J. Balzarini, K.O. François, K. Van Laethem, B. Hoorelbeke, M. Renders, J. Auwerx, S. Liekens, T. Oki, Y. Igarashi and D. Schols. *Antimicrob. Agents Chemother.*, 2010, **54**, 1425-1435