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Research paper

Synergistic activity of tenofovir and nevirapine combinations released from polycaprolactone matrices for potential enhanced prevention of HIV infection through the vaginal route

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ABSTRACT

Polycaprolactone (PCL) matrices were simultaneously loaded with the antiviral agents, tenofovir (TFV) and nevirapine (NVP), in combination to provide synergistic activity in the prevention of HIV transmission through the vaginal route. TFV and NVP were incorporated in PCL matrices at theoretical loadings of 10%TFV–10% NVP, 5%TFV–5%NVP and 5%TFV–10%NVP, measured with respect to the PCL content of the matrices. Actual TFV loadings ranged from 2.1% to 4.2% equating to loading efficiencies of about 41–42%. The actual loadings of NVP were around half those of TFV (1.2–1.9%), resulting in loading efficiencies ranging from 17.2% to 23.5%. Approximately 80% of the initial content of TFV was released from the PCL matrices into simulated vaginal fluid (SVF) over a period of 30 days, which was almost double the cumulative release of NVP (40–45%). The release kinetics of both antivirals over 30 days were found to be described most satisfactorily by the Higuchi model. *In vitro* assay of release media containing combinations of TFV and NVP released from PCL matrices confirmed a potential synergistic/additive effect of the released antivirals on HIV-1 infection of HeLa cells. These findings indicate that PCL matrices loaded with combinations of TFV and NVP provide an effective strategy for the sustained vaginal delivery of antivirals with synergistic/additive activity.

1. Introduction

Despite increasing access to HIV/AIDS services, women in the developing world remain disproportionately at risk of HIV infection [1]. Sub-Saharan Africa, for example, remains the most severely affected region, with nearly 1 in every 20 adults (4.9%) living with HIV and accounting for 69% of global incidence [2]. In the absence of a preventative vaccine, vaginal microbicides are receiving increasing attention as a prophylactic method for protecting women from HIV infection. The CAPRISA 004 clinical trial reported the modest effectiveness of a 1% tenofovir (TFV) vaginal gel formulation, resulting in a 39% reduction in HIV transmission compared with participants receiving a placebo gel [3]. However, the larger scale VOICE trial (MTN-003) found that TFV gel performed no better than a placebo [4]. The low compliance of volunteers was considered to be one reason for the ineffectiveness of the 1% TFV gel observed in the VOICE study [5].

Intra-vaginal ring (IVR) devices are widely acknowledged to present major advantages, compared with semi-solid formulations, for achieving sustained release of anti-viral microbicides in the vagina [6–9], thereby increasing efficacy and adherence of users to the regime [10]. IVRs are conventionally manufactured from silicone elastomer materials (polydimethylsiloxane) or thermoplastic poly(ethylene vinyl acetate) (pEVA). Woolfson et al. [11] incorporated the hydrophobic antiviral TMC120 (dapivirine) in silicone elastomer IVRs and achieved sustained release over 30 days. Silicone elastomer IVRs loaded with 200 mg or 25 mg of dapivirine were reported to be safe and well tolerated when evaluated over a

Abbreviations: CI, combination index; IVR, intravaginal ring; NVP, nevirapine; NNRTI, non-nucleoside reverse transcriptase inhibitor; NRTI, nucleoside reverse transcriptase inhibitor; PCL, polycaprolactone; pEVA, poly(ethylene vinyl acetate); SEM, scanning electron microscope; SVF, simulated vaginal fluid; TFV, tenofovir.

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7-day period in phase 1 safety trials (IPM001 and IPM008, respectively) involving HIV$^-$ women [12]. Dapivirine-loaded IVRs are currently being evaluated in phase III clinical trials (MTN020 or ASPIRE) [13]. Other investigators [14] loaded UC781 antiviral in pEVA IVRs and demonstrated greater drug release (over 12 mg) when compared with silicone elastomer rings (about 1.5 mg) after 14 days *in vitro* in simulated vaginal fluid (SVF). However, the use of silicone elastomer or pEVA is generally restricted to drug compounds possessing high thermal stability due to the heating requirements of ring manufacture. Curing of silicone elastomer is carried out at 80 °C for example [15] while hot melt extrusion of pEVA is usually carried out at around 110 °C [16]. More recently, thermoplastic polyurethane materials have been investigated for IVR production, resulting in controlled delivery of dapivirine over 30 days [8]. However, these materials also require high processing temperature in the region of 170 °C.

We have previously shown the potential of microporous PCL matrices for achieving vaginal delivery of hydrophilic antibacterial (ciprofloxacin) and antifungal (miconazole) agents in the treatment of gonorrhoea and candida infections [17]. We have recently incorporated the nucleoside reverse transcriptase inhibitor (NRTI) TFV in PCL matrices intended for the prevention of HIV infection through the vaginal route [18]. Delivery of TFV was sustained in SVF for 30 days and the released drug retained over 70% of TFV control activity against infectious-HIV1 *in vitro*.

The standard treatment for HIV/AIDS currently involves combinations of oral anti-HIV drugs since drugs with different mechanisms of action can target the virus at different stages of the infection/replication cycle. As such, drug combinations are widely recognised to offer distinct advantages over single microbicide approaches in enhancing antiviral activity through synergistic effects at potentially lower concentrations. As well as increasing potency, the incorporation of multiple anti-HIV drugs in a microbicide formulation [19] could decrease the formation of resistant viral strains [20,21]. In 2012, the FDA approved the use of fixed dose oral combinations of tenofovir disoproxil fumarate (300 mg) and emtricitabine (200 mg) (Truvada) as the first pre-exposure prophylaxis for the prevention of sexually transmitted HIV amongst high risk individuals. (http://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm312210.htm).

Dual segment polyurethane IVRs have been produced to exploit the advantages of vaginal delivery of combination microbicides by bonding separate rods containing dapivirine and TFV respectively [22]. *In vitro* release of both antivirals was sustained over 30 days. A silicone elastomer matrix-type vaginal ring formulation containing 25 mg dapivirine and 100 mg maraviroc was developed by Fetherston et al. [23]. Both microbicides were continuously released *in vitro* into a 1:1 mixture of isopropanol:water over 29 days. IVRs have also been loaded with TFV and acyclovir [24]. Saxena et al. [9] combined the reverse transcriptase inhibitor, TMC120 with a newly characterised anti-HIV agent, 3′-azido-3′-deoxythymidine in IVRs composed of soluble *Acacia* gum or a non-biodegradable hydrogel comprising 2-hydroxyethyl methacrylate and sodium methacrylate. In a recent study [42], pod-type IVRs incorporating multiple drugs (the anti-retroviral agents, tenofovir, nevirapine, and saquinavir along with the contraceptives, etonogestrel and estradiol) provided sustained delivery of each compound when evaluated in sheep.

This study describes the development and *in vitro* performance of PCL matrices simultaneously loaded with the hydrophilic NRTI, tenofovir, and the hydrophobic, non-nucleoside reverse transcriptase inhibitor (NNRTI), nevirapine (NVP). The drug combination released from the matrices into SVF was evaluated for synergistic activity using an *in vitro* assay based on infectious HIV-1.

2. Materials and methods

2.1. Materials

PCL (Mw 115,000 Da, Capa 650) was obtained from Solvay Interox, Warrington, UK. TFV (Mw 287 g/mol) was supplied by Taizhou Crene Chempharm Co., China. NVP (Mw 267 g/mol) was supplied by Huahai Pharmaceutical Co., China. Sodium chloride, potassium hydroxide, calcium chloride, bovine serum albumin, glucose, glycerol, urea, lactic acid, and acetic acid were purchased from Sigma–Aldrich, Australia. All solvents (acetone, methanol, ethanol, and dichloromethane) were of analytical grade and obtained from Sigma–Aldrich, Australia. SVF (pH4.2) was prepared following the method of Owen and Katz [25] and consisted of 3.51 g NaCl; 1.40 g KOH; 0.222 g $Ca(OH)_2$; 0.018 g bovine serum albumin; 2.00 g lactic acid; 1.00 g acetic acid; 0.16 g glycerol; 0.40 g urea and 5.00 g glucose in 1 L of distilled water.

2.2. Preparation of drug-loaded PCL matrices

PCL solution (15% w/v) was prepared by dissolving the polymer in acetone at 50 °C for 30 min. TFV powder and NVP powder were dispersed in the PCL solution to produce loadings equivalent to 10% (w/w) TFV and 10% (w/w) NVP measured with respect to the PCL content of the matrices (M1). Other combinations consisted of 5% TFV, 5% NVP (M2) and 5% TFV, 10% NVP (M3). The resulting suspensions were poured into a polypropylene syringe body (3 mL) and rapidly cooled in ethanol at −80 °C for 24 h to allow crystallization of PCL. Following the hardening process, the matrices were removed from the moulds and immersed in methanol for 24 h to extract the acetone. Residual solvents (acetone and methanol) in the matrices were allowed to evaporate under ambient conditions for 24 h prior to testing.

2.3. Determination of the drug content of PCL matrices

Sections (approximately 100 mg) were cut from each end of the matrix sample, weighed and dissolved in dichloromethane (2 mL). Precipitation of PCL was induced by adding 5 mL of SVF and 5 mL of methanol, followed by vortex mixing (Vibrax, IKA) at 1000 rpm overnight to allow dichloromethane to evaporate and the drug content to partition into the aqueous phase. The residue was washed twice with a mixture of SVF and methanol 1:1 (10 mL) to extract residual drug and the washings were combined. TFV and NVP concentrations in the aqueous phase were assayed using an Agilent 1200 HPLC system (Agilent Technologies, Australia) equipped with a binary pump and photodiode array detector. Separation was achieved using an Eclipse XDB-C18 (150 × 4.6 mm ID, 5 μm) column (Agilent Technologies, Australia). The solvent system comprised 0.1% trifluoroacetic acid in water (eluent A) and acetonitrile (eluent B). Gradient elution was performed as follows: initial condition 95% eluent A and 5% eluent B for 3 min, flow rate 1 mL/min, followed by 70% eluent A and 30% eluent B for 3.5 min, flow rate 1.5 mL/min. The column was then returned to the initial condition and equilibrated for 5.5 min. The sample injection volume was 10 μL. The quantitation of TFV and NVP was performed, by reference to standard curves, at 260 and 280 nm, respectively. The measured drug loading (w/w) of the matrices was compared with the theoretical loading and expressed as loading efficiency (%). The experiment was conducted using triplicate samples.

2.4. In vitro TFV and NVP release behaviour

A release study was implemented with cylindrical segments of drug-loaded PCL matrices (approximately 15 mm in length, 0.15 g

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in weight) taken from the middle of the moulding. Triplicate samples of PCL matrix were tested for each drug loading. Both ends of the samples were sealed by dipping in a 5% (w/v) solution of PCL in acetone followed by air drying at room temperature. Individual samples were placed in 10 mL SVF at 37 °C and the release media were collected totally and replaced with fresh SVF every day for a period of 30 days. The drug concentration in the release media was analysed using HPLC under the conditions described in Section 2.3. Drug release behaviour was expressed as cumulative release (%) versus time. Separate release samples were stored at 0 °C prior to anti-HIV testing.

2.5. Morphology of PCL matrices

The morphology of the surface and interior of drug-free and drug-loaded PCL matrices before and after drug release were examined using a JEOL JSM-6610LV scanning electron microscope (SEM). Specimens were mounted on SEM stubs using carbon tabs and sputter coated with platinum using an Eiko-Sputter coater automatic mounting press prior to examination in the SEM at a voltage of 15 keV.

2.6. Hardness testing

A CT3 Texture Analyzer (Brookfield Engineering Laboratories Inc., Middleboro, MA, USA) was used for hardness testing of drug-free and drug-loaded matrices. As-moulded cylinders were mounted horizontally and compressed locally at a speed of 0.1 mm/s over a distance of 2.0 mm using a flat cylindrical probe (TA39, 2 mm in diameter). The hardness (or indentation resistance) of each sample was calculated from the applied force measured at a depth of travel of 2 mm. A pEVA IVR (Nuvaring®, Schering-Plough Pty limited, NSW, Australia) was subjected to the same test procedure for comparison. The outer diameter of Nuvaring was 54 mm and the cross-sectional diameter was 4 mm.

2.7. HIV infection assay

The synergy of antiviral activity produced by the mixture of TFV and NVP released from PCL matrices into SVF was evaluated using the combination index (CI) derived by Chou and Talalay [26]. The analysis is based on the median effect principle of the mass action law which is characterised by the median effect equation

$$\frac{f_a}{f_u} = \left(\frac{D}{D_m}\right)^m \tag{1}$$

where D is the dose (drug concentration), f_a and f_u are the fractions of the viral infection event affected (i.e. antiviral activity) and unaffected, respectively, by the dose concentration of the drug (D), D_m is the dose (concentration) required to produce the median effect (analogous to the IC_{50} value) and m is a Hill-type coefficient signifying the sigmoidicity of the dose–effect curve. Since by definition, $f_a + f_u = 1$, Eq. (1) can be written in the following form:

$$f_a = \frac{1}{1 + \left(\frac{D_m}{D}\right)^m} \tag{2}$$

The dose–effect curve was constructed based on Eq. (2). In this study, f_a was considered as the anti-HIV activity of drugs measured by luciferase-production during infectious HIV-1 replication as previously described [18]. Briefly, the infectivity assay of each drug was performed using HeLa cells (ATCC number CCL-2) cultured in RPMI 1640 (Invitrogen Corporation) supplemented with 10% foetal bovine serum (Invitrogen Corporation), 100 mg/mL streptomycin and 100 U/mL penicillin at 37 °C and 5% CO_2. HeLa cells (10^5 cells/mL) were first seeded in 24-well plates to achieve greater than 50% confluence after 24 h. Aliquots (100 μL) of SVF release medium containing TFV and NVP released from PCL matrices at days 7, 15 and 23 or mixtures of the unformulated drugs (standard) were filtered through a 0.22 μm membrane and twofold dilutions were added to each well. Cells exposed to the release media were incubated at 37 °C for 24 h to allow TVF to enter the cells and metabolize to an active form. The following day, HIV (10 ng of CAp24 equivalent per well) and hexadimethrine bromide (0.8 μg/mL, for enhancing HIV infection) were added. At 1 day post-infection, the culture was harvested, the cells were lysed in 100 μL Glo Lysis Buffer (Promega Corporation) and the amount of luciferase activity (expressed in relative light units (RLU)) in 20 μL of each lysate was determined using the Steady-Glo Luciferase Assay System (Promega Corporation). Luminescence was detected and measured using a luminescence-compatible spectrophotometer (Synergy H4 Hybrid Reader).

The level of reduction in luciferase production correlating with the anti-viral activity of TFV or NVP (f_a) was determined using Eq. (3)

$$\text{Level of reduction of RLU} = \left(1 - \frac{\text{RLU}_{\text{test}} - \text{RLU}_{\text{cell}}}{\text{RLU}_{\text{virus}} - \text{RLU}_{\text{cell}}}\right) \tag{3}$$

RLU_{test} – RLU of wells containing infected HeLa cells and TFV or NVP in SVF; RLU_{virus} – RLU of control wells containing infected HeLa cells and drug-free SVF; RLU_{cell} – RLU of control wells containing uninfected HeLa cells.

The median effect equation (Eq. (1)) can be transformed linearly by taking the logarithms of both sides:

$$\log(f_a/f_u) = m \log D - m \log D_m \tag{4}$$

The median effect plot based on Eq. (4) gives the slope, m, and when $f_a = f_u$, $f_a/f_u = 1$ and hence $y = \log(f_a/f_u) = 0$ which gives $\log(D_m)$ and consequently the D_m value (IC_{50} value).

The synergistic action of the two drugs in combination was investigated by measuring the combination index (CI) corresponding to the IC_{50} values (D_m value) (or other inhibition concentrations at selected inhibition levels) of each drug in isolation and at least one fixed ratio mixture of TFV and NVP [27].

$$CI_x = \frac{D_1}{Dx_1} + \frac{D_2}{Dx_2}$$

where CI_x is the combination index calculated at a specific inhibition level (x) which is typically 50, 75 or 95%, giving CI_{50}, CI_{75} or CI_{95} values; D_1 and D_2 are doses (concentrations) of drug 1 and drug 2 in the mixture; Dx_1 and Dx_2 are the corresponding doses (concentrations) of drug 1 and drug 2 in isolation that affect the system at corresponding inhibition level (x).

CI values reflect the nature of the interaction between the two drugs: <1, synergistic activity; 1, additive; >1, antagonism and were calculated in this study for antiviral effect (or inhibition levels) corresponding to 50%, 75% and 95%.

The first fixed ratio mixture used a 1:1 IC_{50}:IC_{50} ratio for the two drugs. The second ratio used a 1:2 TFV:NVP ratio (IC_{50}:$2IC_{50}$). The third ratio used a 2:1 TVF:NVP ratio ($2IC_{50}$:IC_{50}). Release samples obtained from drug-loaded PCL matrices were selected with drug mixtures equivalent to the mixtures of drug standards. Released samples collected at day 21 from 4.2% TFV and 1.9% NVP matrices exhibited the first fixed ratio (IC_{50}:IC_{50}). Release samples collected at day 15 from 4.2% TFV and 1.9% NVP matrices exhibited the second fixed ratio of TFV and NVP (IC_{50}:$2IC_{50}$) while release samples collected at day 7 from 4.2% TFV and 1.9% NVP matrices correlated with the third fixed ratio of TFV and NVP ($2IC_{50}$:IC_{50}). The combination index (CI) values were estimated using the CompuSyn software (ComboSyn, Inc. Paramus, NJ 2007 [www.combosyn.com]).

A toxicity study was conducted using an MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay (CellTiter 96® AQueous One Solution Cell

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Proliferation Assay, Promega) to confirm that the decrease in luminescence in the luciferase assay was associated with antiviral activity of TFV and/or NVP rather than their cytotoxicity towards HeLa cells. Samples of TFV and NVP either in isolation or as combinations of drug standards or release samples were incubated with HeLa cells. The number of viable cells that presents after incubation under the same conditions used for the anti-viral activity assay (Section 2.7) was determined by UV quantification of formazan production. One-way ANOVA was performed to establish whether statistical differences existed between the mean absorbance values of blank samples (cells only) and the test samples (cells incubated with TFV and/or NVP).

3. Results and discussion

3.1. Drug loading of matrices

The dried PCL matrices were in the form of microporous cylinders approximately 40–45 mm in length and 6–6.5 mm in diameter and were free from cracks and voids in the surface and interior. TFV and NVP were incorporated in the PCL matrices with theoretical loadings of 5% or 10% measured with reference to the PCL weight (Table 1). PCL matrices (M1) produced using 10% TFV resulted in an actual loading of 4.2% w/w while the actual loading of TFV in matrices M2 and M3 was 2.1%, reflecting the lower starting weight of drug in the formulation (5%). Loading efficiencies of 41–42% were obtained regardless of the initial TFV content. Actual loadings of NVP of 1.2% were measured when the matrices were formulated using 5% NVP and this value increased to 1.7% and 1.9% when the starting weight of NVP was raised to 10% of the PCL component (Table 1).

The resultant comparatively low loading efficiencies, ranging from 17.2% to 23.5% were around 50% of the values found for TFV. The above findings of higher loading efficiency of TFV compared with NVP are explained by the practical insolubility of TFV in methanol that limits partition of the drug into methanol during the solvent extraction stage of matrix production. The higher solubility of NVP in methanol (9.7 μg/mL) facilitates partition of NVP during solvent extraction and subsequently gives rise to loss of drug from the matrix.

Table 1
Loading of tenofovir (TFV) and nevirapine (NVP) in PCL matrices.

	Theoretical loading (% PCL)		Actual loading (% PCL)		Loading efficiency (%)	
	TFV	NVP	TFV	NVP	TFV	NVP
M1	10	10	4.2 (0.2)	1.9 (4.1)	42.1	19.4
M2	5	5	2.1 (5.8)	1.2 (3.9)	41.0	23.5
M3	5	10	2.1 (5.2)	1.7 (1.9)	42.3	17.2

Values in parentheses indicate standard deviations.

3.2. Matrix morphology

SEM examination revealed the irregular porous morphology of matrices formed by crystallization and hardening of the PCL phase. The surfaces of the drug-free matrices (Fig. 1A) exhibit a nodular morphology with pore dimensions of 3–5 μm and flattened areas probably formed by contact of the material with the mould surface. The surfaces of drug-loaded matrices (Fig. 1B) exhibit larger pore dimensions of 5–20 μm and drug particles distributed on the surfaces (arrow in Fig. 1B). Changes in morphology of the TFV and NVP particles (Fig. 1C and D) may occur during matrix fabrication, which makes it difficult to distinguish the individual drugs. However drug particles were not clearly evident in the interior of drug-loaded matrices (Fig. 1E).

3.3. Hardness testing

Hardness values for drug-free PCL matrices, drug-loaded matrices and pEVA (Nuvaring®) are shown in Fig. 2. The hardness value of 1990 mN/mm^2 recorded for drug-free PCL matrices (Fig. 2) indicates a relatively soft material. The incorporation of TFV and NVP particles in the PCL phase provided a reinforcing effect, resulting in increased hardness of about 50% for matrices M2 and M3 (M2 with 3.3% total drug loading, 2986 mN/mm^2 and M3 with 3.8% total drug loading, 3225 mN/mm^2) when compared with the drug-free PCL matrices. M1 matrices, featuring 6% total drug loading, exhibited an increase of 115% of hardness value of the drug-free matrices. However, the hardness values of the drug-loaded PCL matrices were less than half the value of a pEVA IVR (Nuvaring) indicating a potential advantage of PCL matrices for

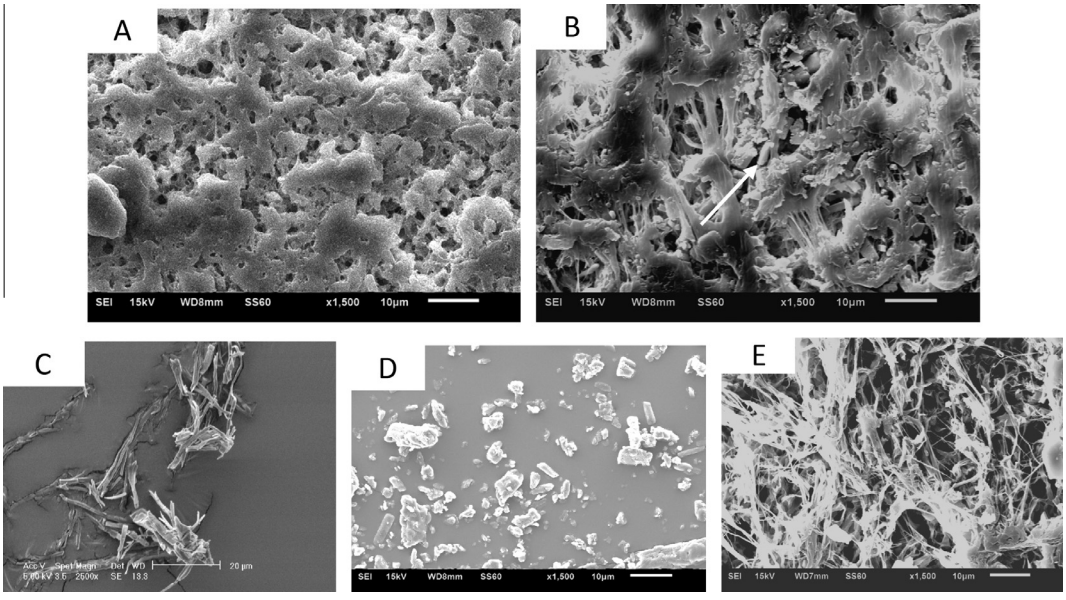

Fig. 1. Scanning electron micrographs of PCL matrices before release testing: (A) external surface of drug-free PCL matrix, (B) external surface of 4.2% TVF and 1.9% NVP-loaded matrix (M1), (C) tenofovir powder, (D) nevirapine powder and (E) interior of 4.2% TFV and 1.9% NVP loaded matrix (M1).

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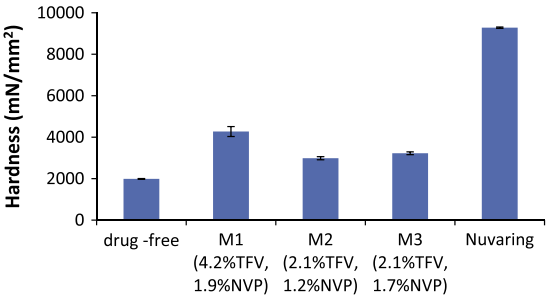

Fig. 2. Hardness of drug-free PCL matrices, drug-loaded matrices and polyethylene vinyl acetate (Nuvaring); error bars indicate sample variance (standard deviation of mean measurement of three different test samples). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

improving the user comfort of IVRs compared with conventional devices. While the TFV loadings of matrices M2 and M3 were similar (around 2%), the higher NVP loading of matrices M3 (1.7%) compared with matrices M2 (1.2%) had no significant influence on the matrix hardness (M2, 2986 mN/mm^2, M3, 3225 mN/mm^2). The increased hardness of around 20% for matrices M1 in comparison with matrices M2 and M3 (Fig. 2) may be explained by the higher overall drug loading and its reinforcing effect on the PCL phase.

3.4. In vitro release behaviour

The cumulative release profiles of TFV and NVP from PCL matrices in SVF at 37 °C are presented in Fig. 3 and provide an indication of delivery efficiency. Similar release profiles of TFV for all matrices indicated that doubling the initial loading of TFV in matrices (M1, 4.2%, M2, M3, 2.1%) did not influence the percentage cumulative drug release. Over the first 24 h, around 8–9% of the initial TFV content was released from the matrices (Fig 3A) and approximately 80% of the content was released gradually over 30 days, demonstrating high delivery efficiency. This behaviour indicates that the microporous structure of the PCL phase possesses a high degree of continuity that facilitates drug transport from the matrix. NVP release from the three types of PCL matrix (Fig 3B) followed a similar pattern; about 10% of the initial NVP loading was extracted in SVF in 24 h but release was limited to 40–45% in 30 days, equating to approximately half the delivery efficiency of TFV.

The amount of drug released daily from dual-loaded PCL matrices is shown in Fig. 4. Overall, the amount of TFV and NVP released daily from PCL matrices containing similar drug loadings were similar. The reason for the large fluctuation in TFV release from the highly loaded matrices (M, 4.2%) is unclear but may indicate differences in pore inter-connectivity within the matrix due to increasing numbers of drug particles. The extent of TFV release increased with initial TFV loading in the matrices (Fig. 4A). For example, M2 and M3 loaded with 2.1% TFV released similar amounts of drug each day which was about half the amount released daily from 4.2% TFV-loaded matrices (M1). In contrast, differences in initial loading of NVP in the matrices did not notably affect the amount of NVP released daily (Fig. 4B).

This behaviour suggests the operation of a rate-defining step controlled by the lower solubility of NVP in SVF compared with TFV.

Various release models were fitted to the drug release data and these provide information on the possible underlying release mechanisms. The process of drug release from matrix-type devices in which solid drug particles are distributed in a polymer phase is frequently controlled by Fickian diffusion and is described effectively by the Higuchi model. A dependency of drug release on the

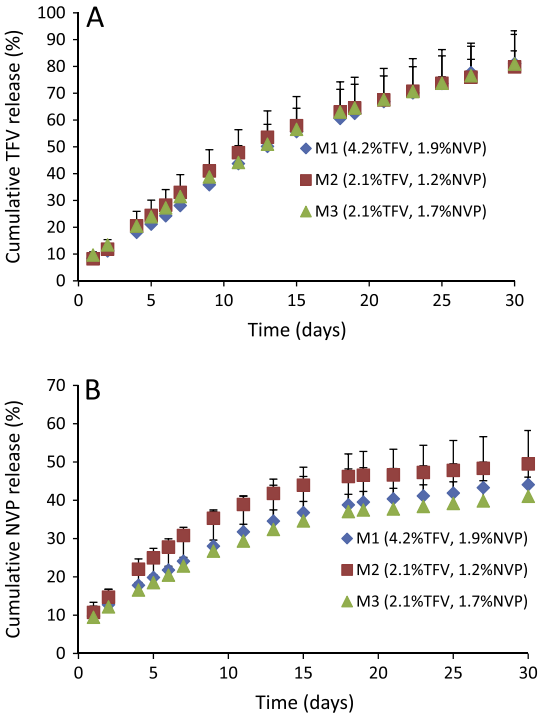

Fig. 3. Cumulative release (%) of tenofovir (TFV) (A) and nevirapine (NVP) (B) from dual-loaded PCL matrices in simulated vaginal fluid at 37 °C; error bars indicate sample variance (standard deviation of mean measurement of three different test samples). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

square root of time is found and a decreasing rate of drug release over time observed as the diffusion path length increases [28]. The Korsmeyer–Peppas model [29] representing a dependency of

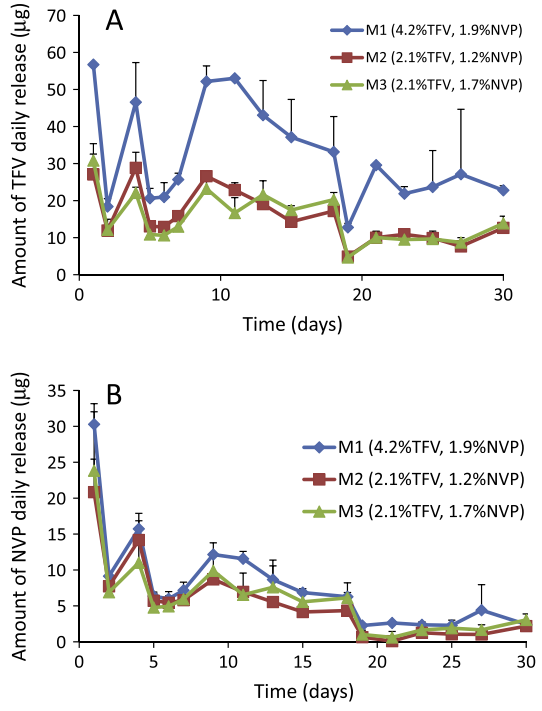

Fig. 4. Release amounts (µg) of tenofovir (TFV) (A) and nevirapine (NVP) (B) from dual-loaded PCL matrices in simulated vaginal fluid at 37 °C; error bars indicate sample variance (standard deviation of mean measurement of three different test samples). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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Table 2
Modelling tenofovir (TFV) and nevirapine (NVP) release from PCL matrices in simulated vaginal fluid at 37 °C.

Drug	Matrix type	Theoretical loading (%w/w)	Actual loading (%w/w)	Zero order kinetics		Higuchi model		Korsmeyer–Peppas		
				k_0	R^2	K_d	R^2	k	n	R^2
TFV	M1	10	4.2	0.176 (0.012)	0.974	1.195 (0.073)	0.990	0.089 (0.033)	0.724 (0.013)	0.990
	M2	5	2.1	0.083 (0.002)	0.951	0.571 (0.017)	0.993	0.100 (0.071)	0.704 (0.052)	0.991
	M3	5	2.1	0.079 (0.003)	0.970	0.539 (0.025)	0.993	0.101 (0.066)	0.661 (0.049)	0.994
NVP	M1	10	1.9	0.036 (0.001)	0.906	0.253 (0.008)	0.978	0.218 (0.017)	0.471 (0.008)	0.990
	M2	5	1.2	0.024 (0.001)	0.838	0.172 (0.059)	0.944	0.237 (0.060)	0.461 (0.042)	0.972
	M3	10	1.7	0.027 (0.010)	0.898	0.189 (0.030)	0.972	0.225 (0.048)	0.461 (0.032)	0.987

Zero order: $M_t = k_0 * t$, k_0 – zero order release constant (mg/day), M_t – amount of drug released at time (t).
Higuchi: $M_t = K_d * t^{0.5}$; K_d – release rate constant (mg/day$^{0.5}$).
Korsmeyer–Peppas: $M_t/M_\infty = k * t^n$, M_t – the amount of drug release at time t, M_∞ – the total drug released over a long time period, k – kinetic constant, n – release exponent.
Values in parentheses indicate standard deviations.

Table 3
Diffusion coefficient (D) of tenofovir (TFV) and nevirapine (NVP) in simulated vaginal fluid at 37 °C in PCL matrices.

	Theoretical loading (%w/w)		Actual loading (%w/w)		D ($\times 10^{-9}$ cm^2/s)	
	TFV	NVP	TFV	NVP	TFV	NVP
M1	10	10	4.2	1.9	12.5 (0.36)	8.9 (0.23)
M2	5	5	2.1	1.2	12.1 (0.61)	8.5 (0.86)
M3	5	10	2.1	1.7	11.6 (0.71)	8.8 (0.57)

Values in parentheses indicate standard deviations.

logarithm of cumulative per cent drug released on the logarithm of time was applied in a further attempt to correlate the release data with underlying controlling factors and to identify release kinetics arising from multiple phenomena. The data analysis conducted on the release profiles of TFV and NVP from PCL matrices is shown in Table 2.

The release kinetics of TFV and NVP from PCL matrices over 30 days were found to be described most satisfactorily by the Higuchi model with high correlation coefficients ($R^2 > 0.99$ and 0.94, respectively). The value of release rate constant (K_d) for TFV in M1 samples (1.2 mg/day$^{0.5}$) was double those obtained in M2 and M3 matrices while the K_d values of NVP were similar amongst M1, M2 and M3 matrices (around 0.2 mg/day$^{0.5}$). The higher loading of TFV in M1 matrices (4.2%) in association with its high aqueous solubility is expected to result in a high concentration gradient for drug diffusion. The exponent value of TFV and NVP in the Korsmeyer–Peppas model was close to 0.45 (0.7 and 0.46) providing further support for a diffusion controlled release mechanism. The diffusion coefficient (D) of tenofovir and NVP in PCL matrices was subsequently estimated (Table 3) using the Korsmeyer–Peppas relationship:

$$\frac{M_t}{M_\infty} = \frac{2\sqrt{Dt}}{\delta}$$

where M_t/M_∞ is the fractional amount of drug released at time (t) and δ is the radius of the cylindrical PCL matrix [30].

The diffusion co-efficient of TFV was similar for the three types of PCL matrix with an average of 12.1×10^{-9} cm^2/s. This figure is around 10 times less than the value of D of 0.3–1.5 $\times 10^{-7}$ cm^2/s obtained by Cost et al. [31] for TFV release from a 1% drug-loaded intra-vaginal gel prepared from hydroxymethylcellulose and carbomer 940P. The comparison highlights the potential for sustained drug release from intravaginal matrices compared with the rapid release characteristic of semi-solid formulations, which necessitate frequent re-application.

The average value of TFV diffusion coefficient measured for dual-loaded PCL matrices in the present study is lower by a factor of 6–36 than the reported diffusion coefficient of TFV in PCL matrices singly-loaded with 4–12% TFV (0.8×10^{-7} cm^2/s in phase 1 and 4.5×10^{-7} cm^2/s in phase 2) [32]. A value of 6.7×10^{-9} cm^2/s was obtained for the diffusion coefficient of NVP in PCL matrices, singly-loaded with 1.3% NVP (unpublished data), which is similar to the mean value obtained in the present study for dual-loaded matrices (8.7×10^{-9} cm^2/s). This finding suggests that inclusion of TFV in the matrix with NVP has no significant effect on diffusion of NVP. However, the presence of NVP appears to dramatically reduce the diffusion behaviour of TFV and may be explained by interference of co-dissolved NVP molecules with the dissolution and transport processes of TFV in the release medium within the matrix pore structure.

3.5. Anti-HIV activity of tenofovir and nevirapine released from PCL matrices

The combination of two or more therapeutic agents is acknowledged to be advantageous compared with a single entity for lowering drug dosage and consequently toxicity and for avoiding the development of drug resistance. Several approaches have been used to assess the effectiveness of drug combinations. The isobologram method [33] has several drawbacks such as requiring large number of measurements for analysis or assuming the drugs have a similar mode of action to obtain a valid measure of drug effects [27]. The fractional product method [34], shows other limitations when applied to the analysis multiple drug effects. The method is valid only when the two drugs are mutually non-exclusive and the dose–response curves of both drugs follow a hyperbolic relationship. In contrast, the method of proposed by Chou and Talalay [26] is based on the 'median-effect principle' of the mass action law and offers advantages in that examination of the relationship between concentration (or dose) and effect is carried out using a simple graphical method that does not require large numbers of measurements. There are several conditions to be satisfied in the Chou and Talalay method. Dose–effect relationships for each drug and their mixtures (at known ratios of the two drugs) are required since the parameters of median doses (D_m or IC_{50}) of each drug and of a mixture are necessary for calculation of CI values. The dose–effect relationships would be accepted if they follow the basic mass-action principle relatively well (e.g. median-effect plots having correlation coefficients for the regression lines >0.9). Twofold serial dilutions are usually performed for *in vitro* experiments for each drug alone and their mixtures to create 5–6 concentrations, with the Dm values (IC_{50} values) located approximately in the middle of the concentration ranges [35].

Empirical dose–response curves for TFV and NVP in isolation and in the mixture at ratios IC_{50}:IC_{50} (1:1), $IC_{50/TFV}$:2×$IC_{50/NVP}$ (1:2) and 2×$IC_{50/TFV}$:$IC_{50/NVP}$ (2:1) against infectious HIV-1 (plotted using CompuSyn software) are presented in Fig. 5.

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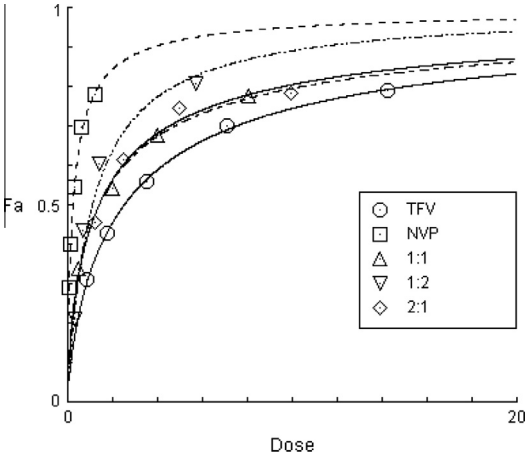

Fig. 5. Dose–response curves of tenofovir (TFV) and nevirapine (NVP) and their mixture at ratios IC_{50}:IC_{50} (1:1), $IC_{50/TFV}$:2×$IC_{50/NVP}$ (1:2) and 2×$IC_{50/TFV}$:$IC_{50/NVP}$ (2:1) prepared in SVF (*x*-axis represents concentrations of TFV or NVP (μM), *y*-axis represents the level of reduction in luciferase production correlating with the antiviral activity of TFV or NVP or their mixture in HeLa cells). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Dose–response curves were transformed linearly to give median-effect plots which allowed the determination of the following parameters of the system: the median dose value (D_m or IC50), the coefficient signifying the shape of the dose–effect relationship, m, and applicability of the method, *R* (correlation coefficient). The corresponding median-effect plots for TFV, NVP and their combinations are presented in Fig. 6.

Correlation coefficients (*R*) for the regression lines were greater than 0.998 (Table 4) which provided strong evidence for anti-HIV activity following a dose–effect relationship. The median dose effect values D_m (IC_{50} values) of TFV and NVP were 2.52 μM and 0.245 μM, respectively. Values of m less than 1 indicated that a flat sigmoidal dose–effect response is obtained for both TFV and NVP.

All samples including TFV or NVP in isolation or in combination as mixtures of standards or in release samples were determined to be non-toxic to HeLa cells. These findings confirm that the reduction in luciferase expression resulted from an inhibition of HIV infectivity due to exposure to TFV and/or NVP.

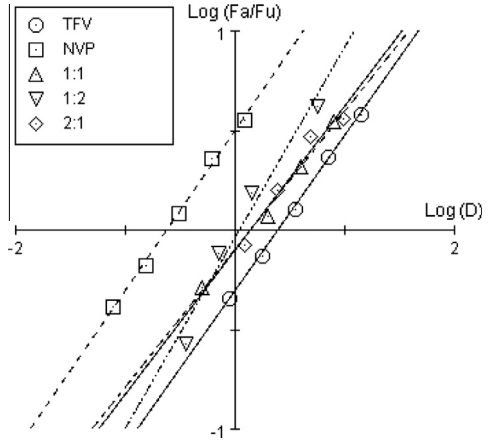

Fig. 6. Median-effect plot of tenofovir (TFV) and nevirapine (NVP) and their mixture at ratios IC_{50}:IC_{50} (1:1), $IC_{50/TFV}$:2×$IC_{50/NVP}$ (1:2) and 2×$IC_{50/TFV}$:$IC_{50/NVP}$ (2:1) prepared in SVF (*x*-axis represents logarithm of concentrations of TFV or NVP or their mixture (μM), *y*-axis presents logarithms of fraction of affected (i.e. antiviral activity in HeLa cells) and unaffected system). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

The synergistic action of the two drugs in combination was investigated by measurement of the combination index based on the IC_{50} values (D_m) of each drug in isolation and at least one fixed ratio mixture of TFV and NVP [27]. An equipotent ratio (IC_{50}:IC_{50}) of the two drugs is usually the first choice for the analysis. Since the IC_{50} of TFV is 10 times higher than the IC_{50} of NVP, the equipotent ratio of TFV and NVP is approximated to a mass ratio of 10:1. Ratios of TFV:NVP in the release samples obtained from PCL matrices ranged from 4.2 to 25.1. In order to investigate the anti-HIV activity of the drug combinations which had been released from the PCL matrices into SVF, the IC_{50} of release samples was compared with mixtures of drug standards having equivalent ratios. To simplify the preparation of standard drug combinations, three ratios were used, $IC_{50/TFV}$:$IC_{50/NVP}$ (mass ratio 9.7:1), 2×$IC_{50/TFV}$:$IC_{50/NVP}$ (mass ratio 21.2:1) and $IC_{50/TFV}$:2×$IC_{50/NVP}$ (mass ratio 5.2:1). Release samples collected at day 21 from 4.2% TFV and 1.9% NVP matrices (M1) exhibited the first fixed ratio of TFV:NVP (IC_{50}:IC_{50}, mass ratio 10.5:1). Release samples collected at day 7 from 4.2%TFV and 1.9% NVP matrices correlated with the second fixed ratio of TFV and NVP (2IC_{50}:IC_{50}, mass ratio of 21:1) while release samples collected at day 15 from 4.2% TFV and 1.9% NVP matrices (M1) exhibited the third fixed ratio of TFV and NVP (IC_{50}:2IC_{50}, a mass ratio 5.3:1). A similar approach was used to construct dose–response curves and corresponding median-effect plots for standard drug combinations and for mixtures of TFV and NVP in release samples.

Good linearity ($R > 0.9$) (Table 4) was obtained for median-effect plots constructed using mixtures of standard drugs or mixtures released from PCL matrices in SVF; confirming the applicability of the median effect plots to determine IC_{50} for all samples. IC_{50} values of release samples comprising mixtures of TFV and NVP ranged from 1.11 to 1.46 μM (Table 4) and were similar to the values obtained for the corresponding standard fixed ratio mixtures of TFV and NVP. These findings demonstrate that the anti-HIV activity of TFV and NVP is maintained following co-incorporation in PCL matrices and following release in SVF.

Combination index (CI) values were determined to investigate possible synergistic antiviral activity in TFV and NVP mixtures and are presented in Table 5.

Synergy was indicated (CI = 0.4–0.8) when TFV was combined with NVP at a 2×$IC_{50/TFV}$:$IC_{50/NVP}$ ratio for both the mixture of drugs in solution (standards) and following release from PCL matrices into SVF. An $IC_{50/TFV}$:$IC_{50/NVP}$ mixture resulted in an additive effect (CI = 0.96–1.12) at all calculated inhibitory concentrations (50%, 75% and 95%). Combination of TFV and NVP in the ratio of $IC_{50/TFV}$:2×$IC_{50/NVP}$ demonstrated an additive effect (CI = 1) at 50% inhibitory effect, moderate synergy (CI = 0.8) at 75% inhibitory effect and synergy (CI = 0.7) at 95% inhibitory effect. These findings of added and synergistic activity of TFV and NVP are in agreement with a study on the inhibitory potential of antiviral drug combinations using the Loewe and Bliss model [36]. Combinations of tenofovir disoproxil fumarate (a prodrug of TFV) and NVP were tested on HIV infection in primary CD4+ T-lymphoblasts. The authors reported the Bliss independence of the two drugs, indicating an additive effect of the mixture.

Drug combinations have been used widely for treating aggressive diseases, such as cancer and AIDS [37] for the reasons mentioned above (enhanced efficacy or synergy, decreased dosage and toxicity and avoidance of drug resistance). Among all the anti-HIV drugs in development or in the clinic, combinations of nucleoside or nucleotide reverse transcriptase inhibitors (NRTIs) and non-nucleoside reverse transcriptase inhibitors (NNRTIs) have been the most extensively studied [38]. The findings of the present study clearly suggest an additive/synergistic effect of the NRTI, tenofovir and the NNRTI, nevirapine. Both NRTIs and NNRTIs target the reverse transcriptase enzyme, an essential viral enzyme which transcribes viral RNA into DNA. However, while TFV attacks the

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Table 4
Analysis of parameters based on median-dose plots of anti-HIV activity for mixtures of tenofovir (TFV) and nevirapine (NVP) as standards or when released from PCL matrices into SVF.

Sample	Mass ratio of TFV/NVP	IC_{50} (μM) (D_m)	m	R
TFV		2.522	0.78	0.999
NVP		0.245	0.80	0.998
Std1 ($IC_{50/TFV}$:$IC_{50/NVP}$)	9.7:1	1.41 (0.176)	0.69	0.9946
Std2 ($IC_{50/TFV}$:2×$IC_{50/NVP}$)	5.2:1	1.11 (0.079)	0.96	0.9679
Std3 (2×$IC_{50/TFV}$:$IC_{50/NVP}$)	21.2:1	1.42 (0.222)	0.72	0.9823
RS1 ($IC_{50/TFV}$:$IC_{50/NVP}$)	10.5:1	1.40 (0.195)	0.73	0.9089
RS2 ($IC_{50/TFV}$:2×$IC_{50/NVP}$)	5.3:1	1.11 (0.061)	1.06	0.9924
RS3 (2×$IC_{50/TFV}$:$IC_{50/NVP}$)	21:1	1.46 (0.338)	0.87	0.9522

IC_{50} calculated as median effect dose (D_m) from median-effect plot; Std1, 2, 3: fixed ratio mixture; RS1, 2, 3: release samples.
Values in parentheses indicate standard deviations.

Table 5
Combination indices (CI) for fixed ratio mixtures of tenofovir (TFV) and nevirapine (NVP) in solution and released from PCL matrices into SVF.

Samples	Mass ratio of TFV/NVP	CI at varying HIV-1 inhibition levels			Combination effect
		50%	75%	95%	
Std1 ($IC_{50/TFV}$:$IC_{50/NVP}$)	9.7:1	0.96 (0.067)	0.99 (0.092)	1.05 (0.102)	Additive
Std2 ($IC_{50/TFV}$:2×$IC_{50/NVP}$)	5.2:1	1.06 (0.029)	0.71 (0.081)	0.37 (0.088)	Additive/synergy
Std3 (2×$IC_{50/TFV}$:$IC_{50/NVP}$)	21.2:1	0.78 (0.051)	0.61 (0.074)	0.41 (0.011)	Synergy
RS1 ($IC_{50/TFV}$:$IC_{50/NVP}$)	10.5:1	0.99 (0.083)	1.12 (0.098)	1.09 (0.054)	Additive
RS2 ($IC_{50/TFV}$:2×$IC_{50/NVP}$)	5.3:1	1.04 (0.013)	0.77 (0.018)	0.47 (0.032)	Additive/synergy
RS3 (2×$IC_{50/TFV}$:$IC_{50/NVP}$)	21:1	0.73 (0.008)	0.75 (0.010)	0.78 (0.016)	Synergy

Values in parentheses indicate standard deviations.

enzyme's active site (acting as a chain terminator) [39], NVP binds allosterically at a distinct site away from the active site [40]. The mechanism of action of NVP is considered to be non-competitive with regard to both the template primer and the nucleotide triphosphate [40]. However, the present study indicates synergy in antiviral activity when HeLa cells were exposed to released TFV:NVP combinations in the ratio 2×$IC_{50/TFV}$:$IC_{50/NVP}$ (21:1 mass ratio) prior to infection with HIV. The antiviral effect was reduced to 'additive' at an $IC_{50/TFV}$:$IC_{50/NVP}$ ratio, suggesting a complex, competitive interplay between allosteric binding of NVP and direct binding of TVF at the reverse transcriptase enzyme active site and the resulting anti-HIV activity. These findings suggest that dual-loaded PCL IVRs need to be designed such that TFV and NVP are released in a 20:1 mass ratio for maximum antiviral effect. This may be achieved by incorporating TVF and NVP in PCL matrices at a theoretical w/w loading of 10% for each drug to provide an actual loading of 4% and 2% respectively and by exploiting the greater release efficiency of TFV. Over the 30 days release period, the ratio of TFV and NVP concentrations in the SVF release medium was maintained in the range from 2×$IC_{50/TFV}$:$IC_{50/NVP}$ ratio (2.1TFV:1NVP mass ratio) to $IC_{50/TFV}$:2×$IC_{50/NVP}$ (5.3TFV:1NVP mass ratio), indicating that a potential synergistic/additive antiviral effect may be sustained for up to a month *in vivo*.

This study did not attempt to model the potential effects of semen, either as a single event or repeated exposure, upon drug release or antiviral activity. Drug release could potentially be affected, for example by the high pH (7.2–8.0) of semen affecting drug solubility. Regarding the latter issue, a previous *in vitro* [41] reported unchanged antiviral activity of 1% TFV gel when cells were challenged with virus in the presence of semen; suggesting that semen has little effect upon the antiviral potency of TFV (effects upon NVP have not been reported).

4. Conclusion

The hydrophilic NRTI, tenofovir and the hydrophobic NNRTI, nevirapine can be incorporated simultaneously in PCL matrices for potential application as intravaginal devices in the prevention of heterosexual HIV transmission. Matrices loaded with around 4% TFV and 2% NVP gradually released up to 80% and 45% of each drug respectively in SVF over 30 days. Values of the combination index (CI) calculated using the antiviral activity of TFV and NVP mixtures in the release media were around or below 1 indicating an additive or synergistic interaction which offers advantages in controlling heterosexual HIV transmission.

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