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Synthesis and biological evaluation of fatty acyl ester derivatives of 2′,3′-didehydro-2′,3′-dideoxythymidine

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ABSTRACT

A number of 5′-*O*-fatty acyl derivatives of 2′,3′-didehydro-2′,3′-dideoxythymidine (stavudine, d4T) were synthesized and evaluated for anti-HIV activities against cell-free and cell-associated virus, cellular cytotoxicity, and cellular uptake studies. The conjugates were found to be more potent than d4T. Among these conjugates, 5′-*O*-12-azidododecanoyl derivative of d4T (**2**), displaying EC_{50} = 3.1–22.4 μM, showed 4- to 9-fold higher activities than d4T against cell-free and cell-associated virus. Cellular uptake studies were conducted on CCRF-CEM cell line using 5(6)-carboxyfluorescein derivatives of d4T attached through β-alanine (**9**) or 12-aminododecanoic acid (**10**) as linkers. The fluorescein-substituted analog of d4T with long chain length (**10**) showed 12- to 15-fold higher cellular uptake profile than the corresponding analog with short chain length (**9**). These studies reveal that conjugation of fatty acids to d4T enhances the cellular uptake and anti-HIV activity of stavudine.

Stavudine (2′,3′-didehydro-2′,3′-dideoxythymidine, d4T) is a nucleoside reverse transcriptase (RT) inhibitor that is used as an anti-human immunodeficiency virus type 1 (HIV-1) drug. Stavudine was initially synthesized for evaluation against cancer and then identified to be active against HIV.[1–3] The compound was approved for clinical use in 1994. When compared with other thymidine analogs, such as zidovudine (AZT), d4T demonstrates comparable potency[4] and is well absorbed orally. Once inside the cells, d4T is converted to d4T 5′-triphosphate. Application of d4T as anti-HIV agent alone is limited due to the development of drug resistance. Different point mutations in RT, such as V75T and K65R, reduce d4T sensitivity against HIV-1.[5,6] d4T shows synergistic effect with other anti-HIV drugs and thus is generally used in anti-HIV-1 combination therapy.

The replication of HIV-1 can be inhibited by heteroatom-containing analogs of myristic acid without accompanying cellular toxicity.[7,8] It has been previously reported that several fatty acids, such as 2-methoxydodecanoic acid, 4-oxatetradecanoic acid, and 12-thioethyldodecanoic acid, reduce HIV-1 replication in acutely infected T-lymphocytes. For example, 12-thioethyldodecanoic acid was moderately active (EC_{50} = 9.4 μM) against HIV-infected T4 lymphocytes.[9] One potential mechanism for the anti-HIV activity of fatty acids is the inhibition of *N*-myristoyl transferase (NMT) enzyme, which is involved in catalyzing the myristoylation of several proteins in HIV life cycle (e.g., capsid protein p17, Pr160$^{gag-pol}$, Pr55^{gag}, p27^{nef}).[10] At N-terminal glycine, viral proteins (gag and nef) are covalently attached to myristic acid in the presence of NMT. Myristic acid attachment makes the proteins more hydrophobic, which improves protein–protein and protein–membrane interactions.[11] For example, after the N-myristoylation, p17 protein localizes itself towards the cell membrane, where new virus is produced.[12]

In continuation of our efforts to design novel compounds targeting different steps in HIV life cycle[13–19] and to improve the anti-HIV activity and cellular uptake of nucleoside analogs, herein we report the synthesis of fatty acyl derivatives of d4T, their anti-HIV activities, and their cellular uptake profiles. It is hypothesized that the attachment of nucleoside analogs to the long chain fatty acid analogs enhances their lipophilicity and thus their cellular uptake. Once the ester conjugate enters the cells; it gets hydrolyzed by esterases; and generates two active molecules, a nucleoside analog and a fatty acid, targeting RT and NMT enzymes, respectively.

A number of 5′-*O*-(fatty acyl) ester derivatives of d4T were synthesized (Scheme 1) through conjugation with fatty acids. Myristic acid, 12-azidododecanoic acid, 12-thioethyldodecanoic acid, and 12-bromododecanoic acid were selected based on the anti-HIV activities of the fatty acids and the corresponding fatty acyl derivatives of FLT and AZT.[9,16,18] Compounds were synthesized from the reaction of d4T with the commercially available myristoyl chloride or freshly prepared fatty acyl chloride (i.e., 12-azidododecanoyl chloride, 12-thioethyldodecanoyl chloride, and 12-bromododecanoyl chloride) in the presence of (dimethlyamino)pyridine (DMAP)

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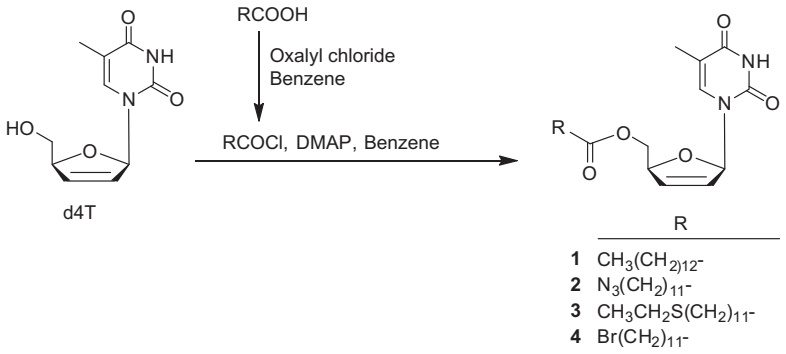


Scheme 1. Synthesis of 5′-*O*-(fatty acyl) ester derivatives of d4T (**1**–**4**).

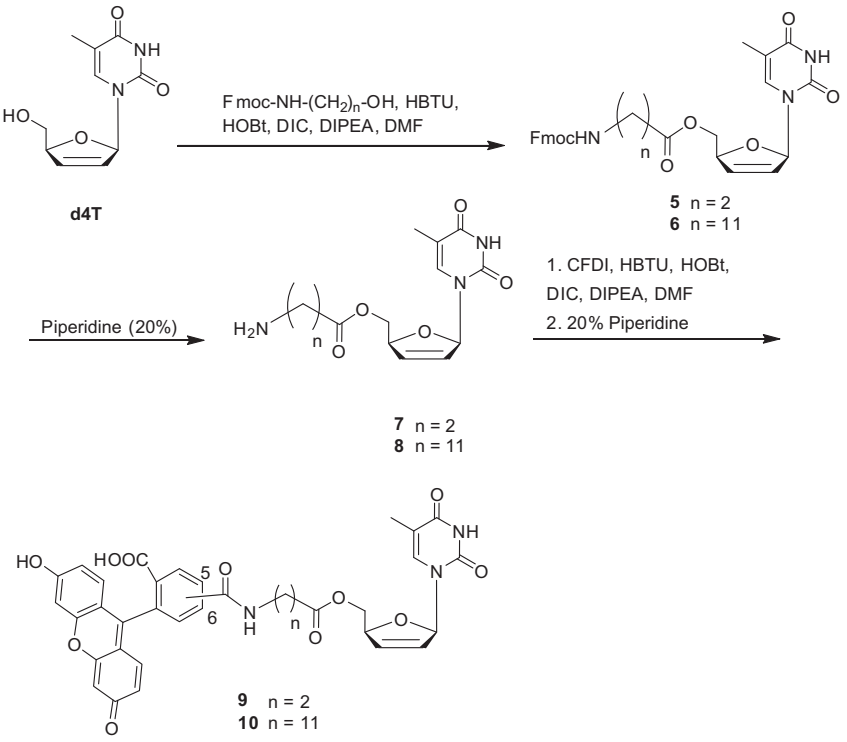
as a base. Fatty acyl chloride derivatives were synthesized from the reaction of fatty acids with oxalyl chloride in benzene in situ.

d4T was attached to 5(6)-carboxyfluorescein (FAM) through β-alanine (**9**) and 12-aminododecanoyl (**10**) as linkers. Compounds **9** and **12** were used as representative fluorescein-conjugate of d4T and 5′-*O*-fatty acyl derivative of d4T, respectively, for cellular uptake studies. d4T was reacted with the corresponding Fmoc-amino acid in the presence of 1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), 1-hydroxybenzotriazole (HOBt), *N*,*N*′-diisopropylcarbodiimide (DIC), and *N*,*N*-diisopropylethylamine (DIPEA) followed by *N*-Fmoc deprotection to free amino group in the presence of piperidine. Finally, FAM was attached to free amino group in the presence of 5(6)-carboxyfluorescein diisobutyrate (CFDI), HBTU and DIPEA, followed by isobutyrate deprotection using 20% piperidine to afford 5(6)-carboxyfluorescein derivatives of d4T **9** and **10** (Scheme 2).

The anti-HIV activity of the compounds was evaluated according to the previously reported procedure.[13,19,20] Table 1 illustrates cytotoxicity and anti-HIV-1 activity against cell-free and cell-associated virus. 5′-*O*-(Fatty acyl) ester derivatives d4T **2–4** showed higher anti-HIV activity (EC_{50} = 2.3–14.9 μM) against cell-free virus than d4T (EC_{50} = 26.8–28.1 μM) in a single-round infection assay. The anti-HIV activity of 5′-substituted derivatives of d4T was dependent on the nature of the 5′-substituent. Among all fatty acyl ester derivatives of d4T, 12-azidododecanoyl derivative **2** was the most potent compound and showed 4- to 9-fold higher anti-HIV activity against cell-free and cell-associated virus than d4T (Table 1).

The differences in anti-HIV potency of conjugates **1–4** compared to each other and to d4T may be due to their increased rate of cellular uptake and intracellular hydrolysis, rendering higher amounts of parent nucleoside and fatty acids. Thus, cellular uptake profiles of 5′-*O*-fatty acyl derivatives were investigated in comparison with d4T using fluorescein-labeled compounds **9** and **10**. d4T attached to FAM through β-alanine (**9**) was used as a control d4T analog. d4T attached to FAM through 12-aminododecanoic acid (**10**) was used as an analog of 5′-*O*-fatty acid ester analogues of d4T.

Human T lymphoblastoid cells (CCRF-CEM, ATCC No. CCL-119) were grown to 70% confluency in culture medium. The cells were



Scheme 2. Synthesis of 5′-*O*-carboxyfluorescein derivatives of d4T (**9** and **10**) containing short and long linkers.

Table 1
Anti-HIV activity of fatty acyl ester derivatives of d4T

Compd	Cytotoxicity[a] EC_{50}[b] (μM)	Anti-viral activity		
		X4 virus[c] EC_{50} (μM)	R5 virus[d] EC_{50} (μM)	CA virus~[e] EC_{50} (μM)
d4T	>200	26.8	28.1	136.1
1	>200	78.3	12.4	>230.3
2	>200	6.7	3.1	22.4
3	>200	14.4	5.8	46.5
4	>200	14.9	2.3	>206.5
DMSO[f]	>1000	>1000	>1000	>1000

[a] Cytotoxicity assay (MTS).
[b] 50% effective concentration.
[c] Single-round infection assay (lymphocytotropic strain, IIIB).
[d] Single-round infection assay (monocytotropic strain, BaL).
[e] Cell-associated transmission assay (SupT1-IIIB).
[f] Solvent control.

incubated with fluorescein-substituted conjugates (**9** and **10**) at different time periods and in the presence or absence of trypsin (Figs. 1 and 2). DMSO and FAM were used as controls for the study. The cells were analyzed by flow cytometry (FACSCalibur: Becton Dickinson) using FITC channel and CellQuest software. The data presented are based on the mean fluorescence signal for 10,000 cells. All the assays were carried out in triplicate.

First, cells were incubated with 10 μM of the compounds for 1, 2, 4, and 8 h (Fig. 1). Compound **10** exhibited 10- to 16-fold higher cellular uptake than that of **9** and FAM alone. The results clearly indicate that presence of long chain enhances the cellular uptake of d4T, by increasing lipophilicity. The continuous incubation of cells with compounds for up to 8 h did not show significant difference in the cellular uptake, suggesting that most of the fatty acyl ester derivative is absorbed into cells within the first hour and that cellular uptake was not time dependent.

To confirm that the enhanced uptake of 5(6)-carboxyfluorescein derivative of d4T, **10**, is not due to the absorption of the compound to the cell membrane surface, cells were incubated with 10 μM DMSO, FAM, **9**, or **10** for 1 h, and then half of the cells were treated with trypsin for 5 min to wash off the adsorbed molecules (if any) from the cell membrane. These studies showed that cellular uptake of **10** was still much higher than those of control compounds, FAM and **9** (Fig. 2). Cellular uptake for the trypsin-treated cells with **10** was approximately seven times higher than that of **9**.

CCRF-CEM cells were incubated with 10 μM DMSO, FAM, **9**, and **10** for 1 h, and were imaged using fluorescent light microscopy (ZEISS Axioplan 2). Cells showed no significant fluorescence when incubated with DMSO, FAM, and **9** (Fig. 3). On the other hand, cells incubated with **10** showed clearly evident fluorescence. The results further confirm the higher cellular uptake of **10**, a fatty acyl derivative of d4T, in comparison to **9** and FAM alone. In general, these data indicate that the fatty acyl derivatives of d4T have better cellular uptake than the parent nucleoside.

These fatty acyl derivatives of anti-HIV nucleosides represent potential candidates for active ingredients in topical microbicidal formulations being developed for the prevention of HIV sexual transmission.[21] Since fatty acids intercalate into the sperm membrane altering its functionality[22], and vaginal microbicides are likely to come in contact with sperm, we investigated the effects of our conjugates on sperm motility and viability.[23] In addition, a compound that displays anti-HIV and sperm-immobilizing properties would be an ideal candidate for a dual-protection, microbicidal and contraceptive, technology.[24] The spermicidal activity of d4T and their fatty acyl ester derivatives **1–3** was compared (Fig. 4) with that of nonoxynol-9 (N-9), a commercially available spermicidal product. In a dose–response study, unlike N-9, compounds **1–3** did not show any significant sperm immobilizing or spermicidal activity, even at their maximum concentrations (1 mg/mL).

In general, the conjugation of selected fatty acids with d4T to generate bifunctional 5′-*O*-substituted fatty acyl derivatives of d4T resulted in better anti-HIV profile than the parent nucleoside, possibly through improved cellular uptake of the conjugates. It is also possible that the fatty acyl moiety might have had a direct inhibitory effect, either on the myristoylation of critical HIV proteins or the entry of the virus.[9,25] Among all the ester derivatives, 5′-*O*-12-azidododecanoyl derivative of d4T (**2**) was found to have better anti-HIV activity profile than d4T and other fatty acyl derivatives. The presence of long chain fatty acid at 5′-position enhanced the lipophilicity of d4T and the cellular uptake. These results showed increased inhibition of HIV-1 infection by fatty acyl ester derivatives of d4T and suggest that the increased potency may be due to their higher rate of uptake and intracellular

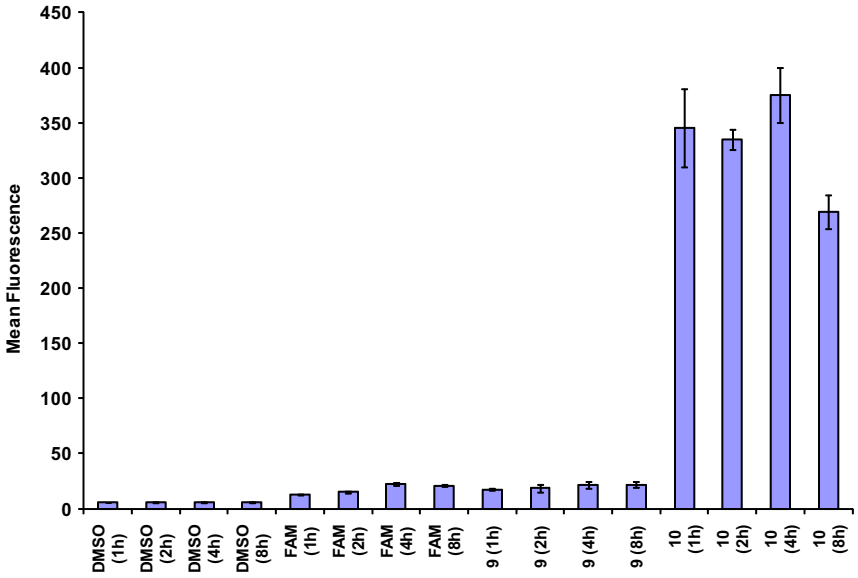


Figure 1. Cellular uptake studies for 5(6)-carboxyfluorescein derivatives of d4T (**9** and **10**) along with FAM and DMSO as controls at different time intervals.

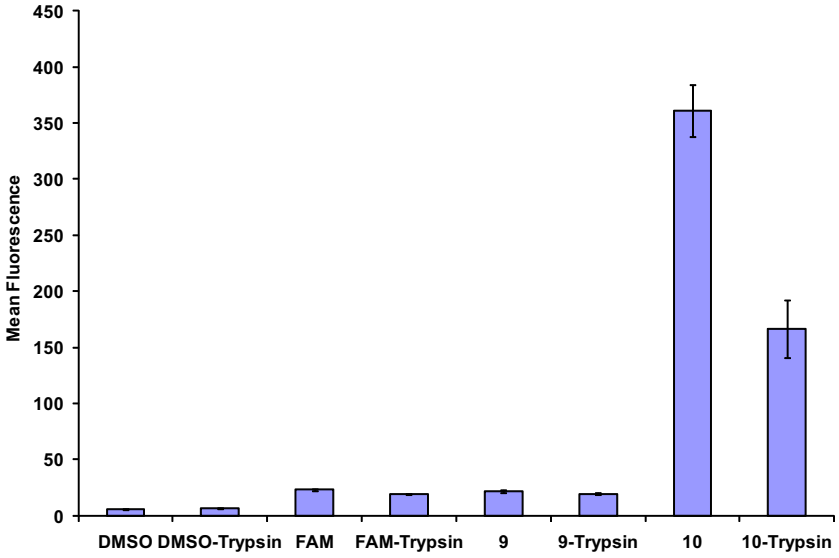


Figure 2. Cellular uptake studies for **9** and **10** with DMSO as controls with and without treatment with trypsin.

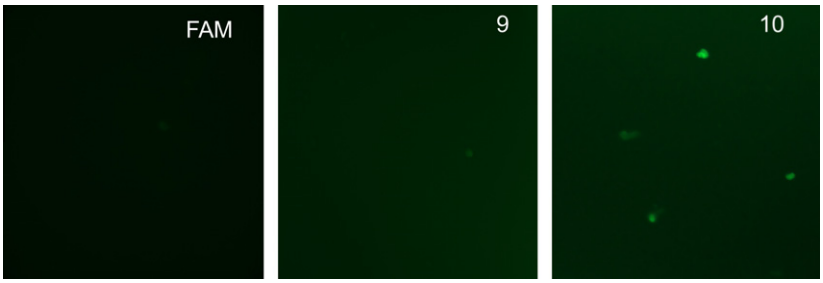


Figure 3. Real time fluorescence microscopy of **9** and **10** in live CCRF-CEM cell line. Control = DMSO, FAM = 5(6)-carboxyfluorescein.

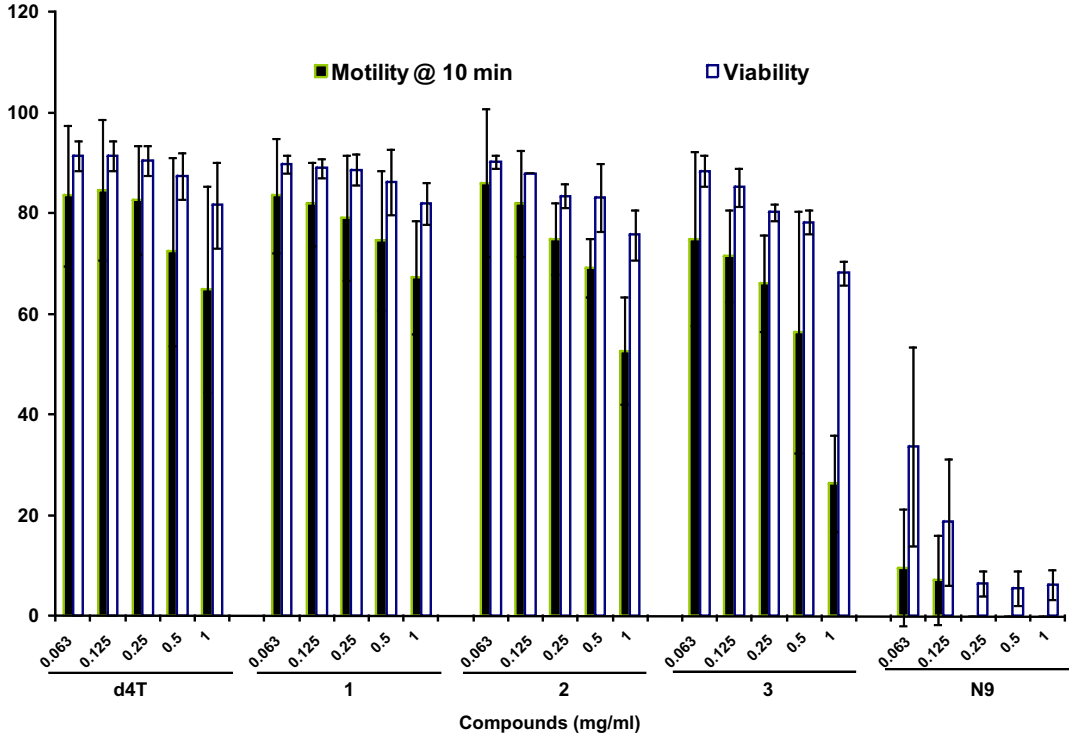


Figure 4. In vitro assays for spermicidal activity of d4T, **1**, **2**, **3** and N-9 (control).

hydrolysis yielding two anti-viral agents, parent nucleoside and fatty acid analog, with different targets. These data provide insights in the design of prodrug esters of other nucleosides to improve their biological activities.

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Supplementary data

Supplementary data (experimental procedures and characterization of compounds, and anti-HIV and cytotoxicity assays) associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2011.02.070.

References and notes

1. Baba, M.; Pauwels, R.; Herdewijn, P.; De Clercq, E.; Desmyter, J.; Vandeputte, M. *Biochem. Biophys. Res. Commun.* **1987**, *142*, 128.
2. Balzarini, J.; Kang, G. J.; Dalal, M.; Herdewijn, P.; De Clercq, E.; Broder, S.; Johns, D. G. *Mol. Pharmcol.* **1987**, *32*, 162.
3. Herdewijn, P.; Balzarini, J.; De Clercq, E.; Pauwels, R.; Baba, M.; Broder, S.; Vanderhaeghe, H. *J. Med. Chem.* **1987**, *30*, 1270.
4. Mansuri, M. M.; Hitchcock, M. J. M.; Buroker, R. A.; Bregman, C. L.; Ghazzouli, I.; Desiderio, J. V.; Starrett, J. E.; Sterzycki, R. Z.; Martin, J. C. *Antimicrob. Agents Chemother.* **1990**, *34*, 637.
5. García-Lerma, J. G.; MacInnes, H.; Bennett, D.; Reid, P.; Nidtha, S.; Weinstock, H.; Kaplan, J. E.; Heneine, W. *J. Virol.* **2003**, *77*, 5685.
6. Hurst, M.; Noble, S. *Drugs* **1999**, *58*, 919.
7. Bryant, M. L.; McWherter, C. A.; Kishore, N. S.; Gokel, G. W.; Gordon, J. I. *Perspect. Drug Discov. Des.* **1993**, *1*, 193.
8. Takamune, N.; Hamada, H.; Misumi, S.; Shoji, S. *FEBS Lett.* **2002**, *527*, 138.
9. Parang, K.; Wiebe, L. I.; Knaus, E. E.; Huang, J.-S.; Tyrrell, D. L.; Csizmadia, F. *Antiviral Res.* **1997**, *34*, 75.
10. Langner, C. A.; Travis, J. K.; Caldwell, S. J.; Tianbao, J. E.; Li, Q.; Bryant, M. L.; Devadas, B.; Gokel, G. W.; Kobayashi, G. S.; Gordon, J. I. *J. Biol. Chem.* **1992**, *267*, 17159.
11. Farazi, T. A.; Waksman, G.; Gordon, J. I. *J. Biol. Chem.* **2001**, *276*, 39501.
12. Wu, Z.; Alexandratos, J.; Ericksen, B.; Lubkowshi, J.; Gallo, R. C.; Lu, W. *PNAS* **2004**, *101*, 11587.
13. Agarwal, H. K.; Kumar, A.; Doncel, G. F.; Parang, K. *Bioorg. Med. Chem. Lett.* **2010**, *20*, 6993.
14. Agarwal, H. K.; Doncel, G.; Parang, K. *Tetrahedron Lett.* **2008**, *49*, 4905.
15. Parang, K.; Wiebe, L. I.; Knaus, E. E.; Huang, J. S.; Tyrrell, D. L. *J. Pharm. Pharm. Sci.* **1998**, *1*, 107.
16. Parang, K.; Knaus, E. E.; Wiebe, L. I. *Nucleosides Nucleotides* **1998**, *17*, 987.
17. Parang, K.; Wiebe, L. I.; Knaus, E. E. *J. Pharm. Pharmacol.* **1998**, *50*, 989.
18. Parang, K.; Knaus, E. E.; Wiebe, L. I. *Antiviral Chem. Chemother.* **1998**, *9*, 311.
19. Ahmadibeni, Y.; Tiwari, R.; Swepson, C.; Pandhare, J.; Dash, C.; Doncel, G. F.; Parang, K. *Tetrahedron Lett.* **2011**, *52*, 802.
20. Krebs, F. C.; Miller, S. R.; Ferguson, M. L.; Labib, M.; Rando, R. F.; Wigdahl, B. *Biomed. Pharmacother.* **2005**, *59*, 438.
21. Hladik, F.; Doncel, G. F. *Antiviral Res.* **2010**, *88*(Suppl 1), S3.
22. Brown-Woodman, P. D. C.; Post, E. J.; Chow, P. Y. W.; White, I. G. *Int. J. Fertility* **1985**, *30*, 38.
23. Wood, B. L.; Doncel, G. F.; Reddy, P. R.; Sokal, D. C. *Contraception* **2003**, *67*, 241.
24. Friend, D. R.; Doncel, G. F. *Antiviral Res.* **2010**, *88*(Suppl 1), S47.
25. Paskaleva, E. E.; Xue, J.; Lee, D. Y.; Shekhtman, A.; Canki, M. *PLoS One* **2010**, *5*, e12168.