Full Paper

Synthesis and Anti-HIV-1 Integrase Activitiy of Cyano Pyrimidinones

R. Ramajayam[1], Nilesh B. Mahera[1], Nouri Neamati[2], Mange Ram Yadav[1], and Rajani Giridhar[1]

[1] Pharmacy Department, Faculty of Technology and Engineering, Kalabhavan, The M.S. University of Baroda, Vadodara, India
[2] Department of Pharmacology and Pharmaceutical Sciences, School of Pharmacy, University of Southern California, Los Angeles, CA, USA

A series of 2-phenethyl/benzylthio-6-oxo-4-phenyl-1,6-dihydropyrimidine-5-carbonitrile were synthesized and tested against recombinant HIV-1 integrase in an enzyme assay. 2-(Phenethylthio)-4-(4-chlorophenyl)-6-oxo-1,6-dihydropyrimidine-5-carbonitrile **4m** and 2-(phenethylthio)-4-(3-chlorophenyl)-6-oxo-1,6-dihydropyrimidine-5-carbonitrile **4o** showed significant inhibition against integrase in the assay (strand transfer: IC_{50} values of 16 and 17 μM, respectively).

Keywords: Cyano pyrimidinone / HIV-1 integrase / Phenyl acrylonitrile

Received: March 30, 2009; accepted: April 29, 2009

DOI 10.1002/ardp.200900066

Introduction

The acquired immunodeficiency syndrome (AIDS), which is the final and most serious stage of human immunodeficiency virus (HIV) infection, renders the body susceptible to a variety of infections, cancers, and other diseases. To date FDA-approved anti-HIV therapies are primarily target at two viral enzymes, HIV reverse transcriptase [1] and HIV protease [2], to block the viral life cycle. Multi-drug cocktails consisting of a protease inhibitor (PI) or a non-nucleoside reverse transcriptase inhibitor (NNRTI) in combination with two nucleoside reverse transcriptase inhibitors (HAART, highly active antiretroviral therapy) are now the standard for treatment. HAART remarkably decreases the viral load and provides a significant improvement in the life expectancy of HIV / AIDS patients [3]. However, several factors [4], including the emergence of multi-drug-resistant HIV strains, drug toxicity, and costs are some of the reasons to develop novel drugs targeting other steps in the viral replication process [5]. In this context, HIV-1 integrase (IN), the enzyme which mediates a mandatory step in the viral replication process by catalyzing the integration of viral cDNA into the host genome, represents a validated target for the development of new drugs against HIV-1 infection [6 – 10]. The integration process is catalyzed by IN through two different reactions: 3′-processing and strand transfer [11 – 13]. Following reverse transcription, the viral cDNA is primed for integration in the cytoplasm by endonucleolytic cleavage of the 3′-viral DNA ends by removing the last two nucleotides (GT). This step is referred to as the 3′-processing reaction and generates two CA-3′-hydroxyl recessed ends, which are the reactive intermediates required for the next step, the strand transfer. After 3′-processing, IN remains bound to the viral cDNA as a multimeric complex that bridges both ends of the viral DNA within intracellular particles called pre-integration complexes. The pre-integration complex then translocates into the nucleus, where IN catalyzes joining of the processed 3′-OH DNA ends to the 5′-DNA phosphate of the host chromosome (strand-transfer reaction). Viral integration is completed by the removal of unpaired nucleotides and gap repair, carried out by cellular enzymes, leading to a stable provirus formation.

Correspondence: Rajani Giridhar, Pharmacy Department, Faculty of Technology and Engineering, Kalabhavan, The M.S. University of Baroda, Vadodara-390 001, India.
E-mail: rajanimsu@rediffmail.com
Fax: + 91 0265 2423898

Abbreviations: **HAART**, highly active antiretroviral therapy; **IN**, HIV-1 integrase; **NNRTI**, non-nucleoside reverse transcriptase inhibitor; **PI**, protease inhibitor

© 2009 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

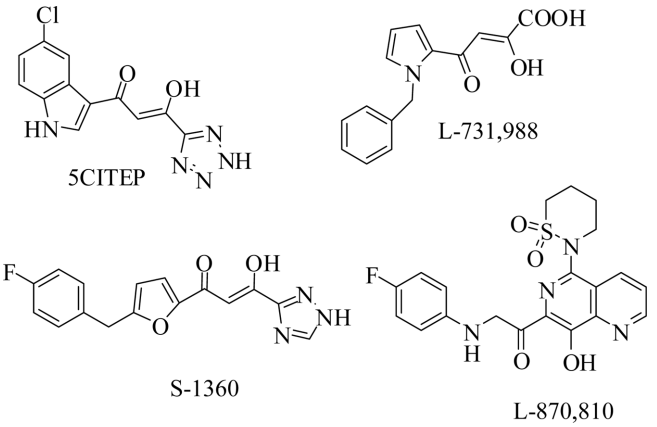


Figure 1. Structure of some representative HIV-1 integrase inhibitors.

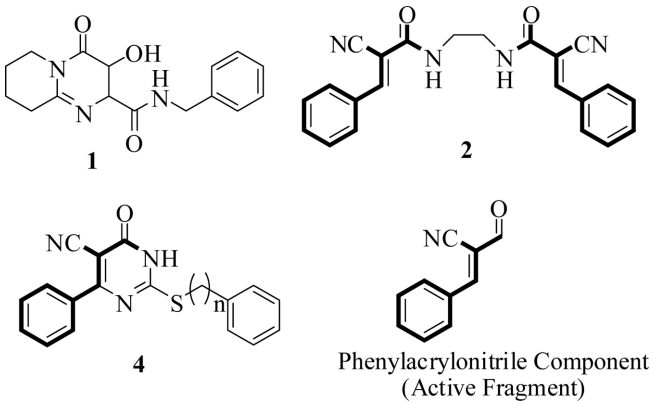


Figure 2. Drug design of HIV integrase inhibitors containing pyrimidinone and phenylacrylonitrile component.

In the past several years, a plethora of integrase inhibitors (β-diketo acid, hydrazides, catechols, DNA binders, and nucleotide based inhibitors) have been reported [14]. Several of them inhibit both the viral enzyme and viral replication in cell-based assays as well as in animal models. However, as of today, no inhibitor of HIV-1 IN has been approved. Figure 1 shows some potential HIV-1 IN inhibitors like 5CITEP, L-731,988, Shionogi / Glaxo-SmithKline's (S/GSK) S-136043, and Merck's L-870,810. Until now, only two compounds, the S-136043 [15, 16] and L-870,810 [17, 18] both belonging to β-diketo acid class of compounds, have entered clinical trials [19]. However, due to pharmacokinetic problems, S/GSK decided to end the development of S-1360 [20]. Although several synthetic and biological studies for diketo acid compounds have been reported, the mechanism by which they bind IN has not been well understood. So there is a great need to discover novel lead compounds with diverse structural scaffolds and promising pharmacokinetic properties, to overcome the difficulties observed with first-generation IN inhibitors.

Generally, most of the anti-HIV agents like NNRTIs were constructed by using pyrimidine as a common heterocyclic core but very few are reported as IN inhibitors. Recently, Summa *et al*. proposed the dihydroxypyrimidine-4-carboxylic acid, as a suitable replacement of the chelating motif for β-diketo acid, as HIV-1 IN inhibitors [21]. Whereas they found the dihydroxypyrimidine carboxylic acid derivatives were almost inactive in the HIV integrase-mediated strand transfer assay which was originally reported for HCV polymerase activity. Later, dihydroxypyrimidine-4-carboxamides **1** (Fig. 2) were discovered as potent reversible inhibitors of HIV integrase, by the same research group [22]. In continuation, very recently bicyclic pyrimidones were identified as potent inhibitors of HIV-1 integrase [23, 24].

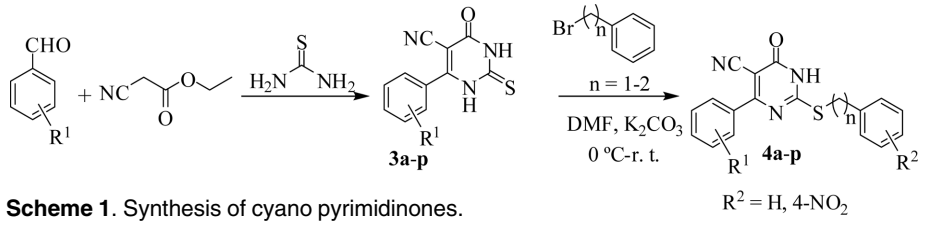
Herein, we report the preparation and preliminary biological evaluation of pyrimidine derivatives obtained by: (1) keeping the common structural motif, pyrimidine, as observed in literature; (2) replacement of the hydroxy by a cyano group, and (3) introduction of benzylthio or phenylpropylthio for *N*-benzyl carboxamide. Overall, 5-cyano-6-oxo-4-phenyl pyrimidine derivatives **4** can be viewed as simplified variants of tyrphositin **2**, HIV-1 integrase inhibitors (Fig. 2) [25, 26]. Tyrphositins belong to catechol derivatives of IN inhibitors. These compounds contain two aryl units, of which at least one contains a 1,2-dihydroxy substitution, separated by a linker. Most of the 1,2-dihdroxy-containing inhibitors display a toxic effect on the cell culture which may be related to the cross reactivity with other metal-requiring enzymes or covalent protein modification by the catechol unit [27–29]. Such alterations should cause negligible variation in both lipophilicity and overall topology of the rings, with a potential to coordinate a metal cofactor on the IN active site and be devoid of cytotoxicity.

Results and discussion

Synthesis

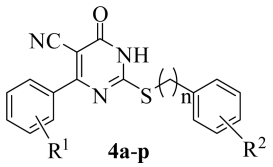
The synthesis of compounds **4a–p** was carried out via a two-step reaction which involved substituted benzaldehyde, ethyl cyanoacetate, and thiourea to afford the corresponding 6-aryl-5-cyano-2-thiouracil **3a–p** in accordance with the literature procedure [30, 31] followed by the regioselective alkylation of 2-thiouracil **3a–p** achieved by slow addition of the respective halides to a solution of **3a–p** in DMF using K_2CO_3 as a base at 0 to 5°C

© 2009 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim



Scheme 1. Synthesis of cyano pyrimidinones.

Table 1. Inhibition of HIV-1 integrase catalytic activities (μM) of the compounds **4a–p**.



Compound	R_1	R_2	n	3′ Strand Processing (IC_{50})	Strand Transfer (IC_{50})
4a	H	H	1	>100	>100
4b	H	H	2	>100	>100
4c	H	4-NO_2	1	>100	>100
4d	4-OCH_3	H	1	>100	>100
4e	4-OCH_3	H	2	>100	>100
4f	4-OCH_3	4-NO_2	1	52	75
4g	4-CH_3	H	1	>100	>100
4h	4-CH_3	H	2	>100	35
4i	4-CH_3	4-NO_2	1	67	35
4j	3-NO_2	H	1	>100	87
4k	3-NO_2	H	2	84	58
4l	3-NO_2	4-NO_2	1	>100	>100
4m	4-Cl	H	2	58	16
4n	4-Cl	4-NO_2	1	>100	>100
4o	3-Cl	H	1	51	17
4p	3-Cl	4-NO_2	1	>100	>100

to give the corresponding compounds **4a–p** (Scheme 1). All compounds were purified by crystallization and purity was ascertained by thin-layer chromatography. Structures of the compounds were established through IR, ^{1}H-NMR, and mass spectral analyses. The proton NMR of the compounds $-SCH_2$ shows singlet at around δ = 4.5–4.6 ppm. On the other hand, compounds possessing $-SCH_2CH_2$ show two sets of doublet of doublets at δ = 3.0–3.5 ppm, and their coupling constants for the ethylene protons are in the range of 15.2 to 15.5 Hz.

Biology

A series of substituted 2-benzylthio-6-oxo-4-phenyl-1,6-dihydro-pyrimidine-5-carbonitrile and 2-phenethylthio-6-oxo-4-phenyl-1,6-dihydropyrimidine-5-carbonitrile **4a–p** were synthesized with the aim to evaluate their integrase and cytotoxicity activity. The IC_{50} values of the compound **4a–p** are summarized in Table 1. In the integrase assay, compound **4m** showed significant strand transfer- and 3′-processing activity with IC_{50} values of 16 and 58 μM, respectively. Introduction of a chloro group at the meta position of the aryl ring as in compound **4o** showed a similar profile (strand transfer: IC_{50} = 16 μM and 3′-processing 51 μM, respectively). In parallel, all compounds were tested for their cytotoxicity against prostate cancer cell lines (data not shown). None of the compounds showed cytotoxicity (CC_{50} > 10 μM) at concentration values about 2.5 to 15-fold higher than their enzymatic inhibition-activity values. The cytotoxicity of the test compounds was tested by performing the MTT assay. All compounds are devoid of cytotoxicity towards the cell culture which is commonly observed in catechol derivatives.

Conclusion

In summary, we prepared a focused library of diversely substituted pyrimidin-4(3*H*)-ones containing the phenylacrylonitrile as a key pharmacophoric element. The biological evaluation of these compounds allowed the identification of new and potent anti-HIV agents. Further studies are in progress in our laboratories to exploit these preliminary results for the synthesis of a larger library incorporating substitution on/and bio-isosteric replacement of the phenyl group in the pyrimidinone system.

We thank the Indian Council of Medical Research (ICMR), New Delhi, India, for the award of a Senior Research Fellowship to RR, and the All India Council for Technical Education (AICTE), New Delhi, India, for financial support in form of a research promotion scheme [File No. 8023/RID/RPS.46/2004-05] to RG.

The authors have declared no conflict of interest.

Experimental

Chemistry

Melting points were determined on a Toshniwal melting point apparatus (Toshniwal, India) and are uncorrected. IR (in cm^{-1}) spectra in KBr pellets were performed on Shimadzu 8300 (Shi-

© 2009 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

madzu, Austria), and ^{1}H-NMR spectra were recorded on a Bruker spectrometer (400 MHz) (Bruker, Switzerland) using DMSO-d_6 as solvent and tetramethylsilane as an internal standard. Chemical shift data are reported in parts per million (δ in ppm) where s, br, d, dd, t, and m designate singlet, broad, doublet, doublet of doublet, triplet, and multiplet, respectively. Elemental analyses were recorded on PE 2400 CHNS analyzer (Perkin-Elmer, USA). Mass spectra were recorded on JEOL SX 102/DA-6000 mass spectrometer (Jeol, Japan). Thin-layer chromatography (TLC) was performed on precoated Silica gel Merck plates (Merck, Germany). Compounds were visualized by illuminating with UV light (254 nm) or exposure to iodine vapors. Solvents were purified using standard methods.

*General procedure for the synthesis of 2-phenethylthio/benzylthio-6-oxo-4-phenyl-1,6-dihydropyrimidine-5-carbonitrile **4a–p***

To a mixture of 6-aryl-5-cyano-2-thiouracil (1 mmol) and K_2CO_3 (1.5 mmol) in DMF (10 mL), alkyl iodide (1.2 mmol) was added dropwise with stirring while maintaining the temperature of the reaction mixture at 0 – 5°C. Stirring was continued for 3 h at this temperature and continued for additional 2 h at room temperature. Water was added to the mixture and filtered. The aqueous filtrate was neutralized with acetic acid and the precipitate was filtered and purified.

*2-(Benzylthio)-6-oxo-4-phenyl-1,6-dihydropyrimidine-5-carbonitrile **4a***

Yield: 54%; m.p.: 202 – 204°C; IR (KBr): 3000, 2219, 1660, 1531, 1475, 1378, 1261, 1115, 1002, 912, 848, 765 cm^{-1}; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 4.51 (s, 2H, CH_2), 7.25 – 7.33 (m, 3H, ArH), 7.37 – 7.39 (d, *J* = 8.04 Hz, 2H ArH), 7.51 – 7.60 (m, 3H, ArH), 7.96 – 7.98 (d, *J* = 8.48 Hz, 2H, ArH), 12.56 (br, 1H, NH); MS (CI) *m/z*: 320 [M + H$^+$]. Anal. calcd. for $C_{18}H_{13}N_3OS$: C, 67.69; H, 4.10; N, 13.16. Found: C, 67.74; H, 3.98; N, 13.24.

*2-(Phenethylthio)-6-oxo-4-phenyl-1,6-dihydropyrimidine-5-carbonitrile **4b***

Yield: 62%; m.p.: 236 – 237°C; IR (KBr): 3008, 2223, 1650, 1523, 1473, 1375, 1257, 1120, 999, 852, 763 cm^{-1}; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 3.07 – 3.10 (dd, *J* = 15.52 Hz, 2H, CH_2), 3.52 – 3.56 (dd, *J* = 15.56 Hz, 2H, CH_2), 7.20 – 7.31 (m, 5H, ArH), 7.52 – 7.63 (m, 3H, ArH), 8.07 – 8.10 (m, 2H, ArH), 12.98 (br, 1H, NH,); MS (CI) *m/z*: 334 [M + H$^+$]. Anal. calcd. for $C_{19}H_{15}N_3OS$: C, 68.45; H, 4.53; N, 12.60. Found: C, 68.48; H, 4.48; N, 12.72.

*2-(4-Nitrobenzylthio)-6-oxo-4-phenyl-1,6-dihydropyrimidine-5-carbonitrile **4c***

Yield: 63%; m.p.: 247 – 248°C; IR (KBr): 2980, 2219, 1654, 1515, 1413, 1346, 1257, 1107, 1001, 929, 852, 763 cm^{-1}; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 4.60 (s, 2H, CH_2), 7.51 – 7.63 (m, 5H, ArH), 7.90 – 7.92 (d, *J* = 8.64 Hz, 2H, ArH), 8.12 – 8.14 (d, *J* = 8.76 Hz, 2H, ArH), 12.85 (br, 1H, NH); MS (CI) *m/z*: 365 [M + H$^+$]. Anal. calcd. for $C_{18}H_{12}N_4O_3S$: C, 59.33; H, 3.32; N, 15.38. Found: C, 59.30; H, 3.44; N, 15.16.

*2-(Benzylthio)-4-(4-methoxyphenyl)-6-oxo-1,6-dihydropyrimidine-5-carbonitrile **4d***

Yield: 52%; m.p.: 248 – 250°C; IR (KBr): 3018, 2214, 1651, 1598, 1506, 1473, 1369, 1263, 1180, 1024, 929, 840, 781 cm^{-1}; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 3.89 (s, 3H, OCH_3), 4.52 (s, 2H, CH_2), 7.03 – 7.06 (d, *J* = 8.92 Hz, 2H, ArH), 7.26 – 7.33 (m, 5H, ArH), 8.02 – 8.04 (d, *J* = 8.88 Hz, 2H, ArH), 12.88 (br, 1H, NH); MS (CI) *m/z*: 350 [M + H$^+$]. Anal. calcd. for $C_{19}H_{15}N_3O_2S$: C, 65.31; H, 4.33; N, 12.03. Found: C, 65.61; H, 4.27; N, 11.98.

*2-(Phenethylthio)-4-(4-methoxyphenyl)-6-oxo-1,6-dihydropyrimidine-5-carbonitrile **4e***

Yield: 64%; m.p.: 210 – 212°C; IR (KBr): 3000, 2223, 1658, 1602, 1533, 1456, 1373, 1251, 1174, 1022, 997, 840, 788 cm^{-1}; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 3.07 – 3.11 (dd, J = 15.52 Hz, 2H, CH_2), 3.52–3.56 (dd, *J* = 15.56 Hz, 2H, CH_2), 3.91, (s, 3H, OCH_3), 7.01 – 7.04 (dd, *J* = 9.0 Hz, 2H, ArH), 7.22 – 7.26 (m, 3H, ArH), 7.30 – 7.33 (m, 2H, ArH), 8.15 – 8.17 (dd, *J* = 8.8 Hz, 2H, ArH), 12.7 (br, 1H, NH); MS (CI) *m/z*: 364 [M + H$^+$]. Anal. calcd. for $C_{20}H_{17}N_3O_2S$: C, 66.10; H, 4.71; N, 11.56. Found: C, 66.04; H, 4.82; N, 11.56.

*2-(4-Nitrobenzylthio)-4-(4-methoxyphenyl)-6-oxo-1,6-dihydropyrimidine-5-carbonitrile **4f***

Yield: 70%; m.p.: 241 – 243°C; IR (KBr): 2920, 2214, 1656, 1598, 1467, 1379, 1259, 1178, 997, 844, 781 cm^{-1}; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 3.89 (s, 3H, OCH_3), 4.61 (s, 2H, CH_2), 7.01 – 7.03 (d, *J* = 8.92 Hz, 2H, ArH), 7.61 – 7.63 (d, *J* = 8.72 Hz, 2H, ArH), 7.98 – 8.00 (d, *J* = 8.88 Hz, 2H, ArH), 8.12 – 8.15 (d, *J* = 8.72 Hz, 2H, ArH,), 12.56 (br, 1H, NH); MS (CI) *m/z*: 395 [M + H$^+$]. Anal. calcd. for $C_{19}H_{14}N_4O_4S$: C, 57.86; H, 3.58; N, 14.21. Found: C, 58.01; H, 3.56; N, 14.12.

*2-(Benzylthio)-6-oxo-4-(4-methylphenyl)-1,6-dihydropyrimidine-5-carbonitrile **4g***

Yield: 51%; m.p.: 229 – 232°C; IR (KBr): 3000, 2221, 1652, 1539, 1456, 1377, 1244, 1186, 1001, 900, 829, 779 cm^{-1}; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 2.44 (s, 3H, CH_3), 4.51 (s, 2H, CH_2), 7.24 – 7.38 (m, 7H, ArH), 7.86 – 7.90 (m, 2H, ArH), 12.42 (br, 1H, NH); MS (CI) *m/z*: 334 [M + H$^+$]. Anal. calcd. for $C_{19}H_{15}N_3OS$: C, 68.45; H, 4.53; N, 12.60. Found: C, 68.48; H, 4.58; N, 12.74.

*2-(Phenethylthio)-6-oxo-4-(4-methylphenyl)-1,6-dihydropyrimidine-5-carbonitrile **4h***

Yield: 73%; m.p.: 213 – 214°C; IR (KBr): 3018, 2223, 1657, 1533, 1473, 1377, 1257, 1182, 1001, 918, 858, 781 cm^{-1}; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 2.46 (s, 3H, CH_3), 3.04 – 3.07 (dd, *J* = 15.48 Hz, 2H, CH_2), 3.46 – 3.49 (dd, *J* = 15.44 Hz, 2H, CH_2), 7.20 – 7.24 (m, 3H, ArH), 7.27 – 7.29 (dd, *J* = 7.12 Hz, 2H, ArH), 7.31 – 7.33 (dd, *J* = 7.84 Hz, 2H, ArH), 7.96 – 7.98 (dd, *J* = 8.12, 2H, ArH), 12.84 (br, 1H, NH); MS (CI) *m/z*: 348 [M + H$^+$]. Anal. calcd. for $C_{20}H_{17}N_3OS$: C, 69.14; H, 4.93; N, 12.09. Found: C, 69.10; H, 4.93; N, 12.00.

*2-(4-Nitrobenzylthio)-6-oxo-4-(4-methylphenyl)-1,6-dihydropyrimidine-5-carbonitrile **4i***

Yield: 55%; m.p.: 253 – 254°C; IR (KBr): 3000, 2219, 1660, 1539, 1479, 1348, 1267, 1120, 1004, 914, 833, 783 cm^{-1}; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 2.44 (s, 3H, CH_3), 4.62 (s, 2H, CH_2), 7.33 – 7.35 (d, *J* = 8.08 Hz, 2H, ArH), 7.64 – 7.66 (d, *J* = 8.76 Hz, 2H, ArH), 7.81 – 7.84 (d, *J* = 8.24 Hz, 2H, ArH), 8.12 – 8.15 (d, *J* = 8.76 Hz, 2H,

© 2009 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

ArH), 13.12 (br, 1H, NH); MS (CI) *m/z*: 379 [M + H$^+$]. Anal. calcd. for $C_{19}H_{14}N_4O_3S$: C, 60.31; H, 3.73; N, 14.81. Found: C, 60.32; H, 3.68; N, 14.94.

2-(Benzylthio)-4-(3-nitrophenyl)-6-oxo-1,6-dihydropyrimidine-5-carbonitrile ***4j***

Yield: 27%; m.p.: 256–257°C; IR (KBr): 3008, 2218, 1651, 1544, 1514, 1473, 1344, 1257, 1190, 1001, 906, 856, 777 cm^{-1}; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 4.50 (s, 2H, CH_2), 7.26–7.40 (m, 5H, ArH), 7.77 (t, *J* = 8.04 Hz, 1H, ArH), 8.33–8.35 (d, *J* = 7.88 Hz, 1H, ArH), 8.43–8.41 (dd, *J* = 8.16 Hz, 1H, ArH), 8.82 (m, 1H, ArH), 12.64 (br, 1H, NH); MS (CI) *m/z*: 365 [M + H$^+$]. Anal. calcd. for $C_{18}H_{12}N_4O_3S$: C, 59.33; H, 3.32; N, 15.38. Found: C, 59.08; H, 3.48; N, 15.28.

2-(Phenethylthio)-4-(3-nitrophenyl)-6-oxo-1,6-dihydropyrimidine-5-carbonitrile ***4k***

Yield: 46%; m.p.: 267–268°C; IR (KBr): 3018, 2221, 1645, 1537, 1475, 1352, 1255, 1153, 1012, 914, 833, 786 cm^{-1}; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 3.04–3.08 (dd, *J* = 15.28 Hz, 2H, CH_2), 3.48–3.52 (dd, *J* = 15.32 Hz, 2H, CH_2), 7.18–7.27 (m, 5H, ArH), 7.82 (t, 1H, ArH), 8.39–8.46 (m, 2H, ArH), 8.85–8.86 (m, 1H, ArH), 12.78 (br, 1H, NH); MS (CI) *m/z*: 401 [M + Na$^+$]. Anal. calcd. for $C_{19}H_{14}N_4O_3S$: C, 60.31; H, 3.73; N, 14.81. Found: C, 60.42; H, 3.70; N, 14.94.

2-(4-Nitrobenzylthio)-4-(3-nitrophenyl)-6-oxo-1,6-dihydropyrimidine-5-carbonitrile ***4l***

Yield: 51%; m.p.: 236–237°C; IR (KBr): 3078, 2221, 1666, 1521, 1473, 1346, 1249, 1112, 1010, 916, 891, 785 cm^{-1}; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 4.59 (s, 2H, CH_2), 7.63–7.65 (d, *J* = 8.72 Hz, 2H, ArH), 7.79 (t, *J* = 8.04 Hz, 1H, ArH), 8.17–8.20 (d, *J* = 8.72 Hz, 2H, ArH), 8.31–8.33 (d, *J* = 8.0 Hz, 1H, ArH), 8.41–8.44 (m, 1H, ArH), 8.69–8.70 (m, 1H, ArH), 12.9 (br, 1H, NH); MS (CI) *m/z*: 410 [M + H$^+$]. Anal. calcd. for $C_{18}H_{11}N_5O_5S$: C, 52.81; H, 2.71; N, 17.11. Found: C, 52.68; H, 2.85; N, 17.04.

2-(Phenethylthio)-4-(4-chlorophenyl)-6-oxo-1,6-dihydropyrimidine-5-carbonitrile ***4m***

Yield: 61%; m.p.: 199–200°C; IR (KBr): 3025, 2218, 1678, 1533, 1471, 1375, 1257, 1093, 1001, 914, 840, 783 cm^{-1}; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 3.02–3.06 (dd, *J* = 15.28 Hz, 2H, CH_2), 3.44–3.48 (dd, *J* = 15.32 Hz, 2H, CH_2), 7.19–7.23 (m, 3H, ArH), 7.26–7.30 (m, 2H, ArH), 7.48–7.50 (dd, *J*= 8.64 Hz, 2H, ArH), 7.98–8.00 (dd, *J* = 8.64 Hz, 2H, ArH), 12.92 (br, 1H, NH); MS (CI) *m/z*: 368 [M + H$^+$]. Anal. calcd. for $C_{19}H_{14}ClN_3OS$: C, 62.04; H, 3.84; N, 11.42. Found: C, 62.08; H, 3.98; N, 11.40.

2-(4-Nitrobenzylthio)-4-(4-chlorophenyl)-6-oxo-1,6-dihydropyrimidine-5-carbonitrile ***4n***

Yield: 66%; m.p.: 254–257°C; IR (KBr): 3000, 2218, 1651, 1517, 1467, 1346, 1244, 1009, 1004, 997, 856, 779 cm^{-1}; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 4.60 (s, 2H, CH_2), 7.51–7.53 (d, *J* = 8.64 Hz, 2H, ArH), 7.60–7.62 (d, *J* = 8.76 Hz, 2H, ArH), 7.89–7.91 (d, *J* = 8.64 Hz, 2H, ArH), 8.13–8.15 (d, *J* = 8.72 Hz, 2H, ArH), 12.88 (br, 1H, NH); MS (CI) *m/z*: 399 [M + H$^+$]. Anal. calcd. for $C_{18}H_{11}ClN_4O_3S$: C, 54.21; H, 2.78; N, 14.05. Found: C, 54.18; H, 2.85; N, 14.01.

2-(Phenethylthio)-4-(3-chlorophenyl)-6-oxo-1,6-dihydropyrimidine-5-carbonitrile ***4o***

Yield: 69%; m.p.: 241–242°C; IR (KBr): 2997, 2221, 1643, 1523, 1473, 1375, 1255, 1120, 1008, 887, 779 cm^{-1}; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 3.02–3.06 (dd, *J* = 15.52 Hz, 2H, CH_2), 3.44–3.48 (dd, *J* = 15.52 Hz, 2H, CH_2), 7.18–7.30 (m, 5H, ArH), 7.52–7.62 (m, 2H, ArH), 7.93–8.00 (m, 2H, ArH), 13.1 (br, 1H, NH); MS (CI) *m/z*: 368 [M + H$^+$]. Anal. calcd. for $C_{19}H_{14}ClN_3OS$: C, 62.04; H, 3.84; N, 11.42. Found: C, 61.98; H, 3.78; N, 11.58.

2-(4-Nitrobenzylthio)-4-(3-chlorophenyl)-6-oxo-1,6-dihydropyrimidine-5-carbonitrile ***4p***

Yield: 33%; m.p.: 245–246°C; IR (KBr): 2980, 2227, 1658, 1520, 1471, 1343, 1217, 1109, 1008, 887, 785 cm^{-1}; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 4.57 (s, 2H, CH_2), 7.47–7.51 (m, 1H, ArH), 7.54–7.57 (m, 1H, ArH), 7.60–7.62 (d, *J* = 8.68 Hz, 2H, ArH), 7.74 (t, 1H, ArH), 7.83–7.85 (m, 1H, ArH), 8.16–8.18 (d, *J* = 8.72 Hz, 2H, ArH), 12.96 (br, 1H, NH); MS (CI) *m/z*: 399 [M + H$^+$]. Anal. calcd. for $C_{18}H_{11}ClN_4O_3S$: C, 54.21; H, 2.78; N, 14.05. Found: C, 54.36; H, 2.64; N, 14.24.

Biology

All compounds were dissolved in DMSO and the stock solutions were stored at 20°C. The γ[32P]-ATP was purchased from either Amersham Biosciences or ICN (Germany). The expression system for wild-type IN was a generous gift of Dr. Robert Craigie, Laboratory of Molecular Biology, NIDDK, NIH, Bethesda, MD, USA.

Preparation of oligonucleotide substrates

The oligonucleotides 21top, 5′-GTGTGGAAAATCTCTAGCAGT-3′ and 21bot, 5′-ACTGCTAGAGATTTTCCACAC-3′ were purchased from Norris Cancer Center Core Facility (University of Southern California) and purified by UV shadowing on polyacrylamide gel. To analyze the extent of 3′-processing and strand transfer using 5′-end labeled substrates, 21top was 5′-end labeled using T4 polynucleotide kinase (Epicentre, Madison, WI, USA) and γ[32P]-ATP (Amersham Biosciences or ICN). The kinase was heat-inactivated and 21bot was added in 1.5-molar excess. The mixture was heated at 95°C, allowed to cool slowly to room temperature, and run through a spin 25 mini-column (USA Scientific) to separate annealed double-stranded oligonucleotide from unincorporated material.

Integrase assay [32]

To determine the extent of 3′-processing and strand transfer, wild-type IN was preincubated at a final concentration of 200 nM with the inhibitor in reaction buffer (50 mM NaCl, 1 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.5, 50 μM EDTA, 50 μM dithiothreitol, 10% glycerol (w/v), 7.5 mM $MnCl_2$, 0.1 mg/mL bovine serum albumin, 10 mM 2-mercaptoethanol, 10% DMSO, and 25 mM 3-(*N*-morpholino)-propanesulfonic acid (MOPS), pH 7.2) at 30°C for 30 min. Then, 20 nM of the 5′-end ^{32}P-labeled linear oligonucleotide substrate was added, and incubation was continued for an additional 1 h. Reactions were quenched by the addition of an equal volume (16 μL) of loading dye (98% deionized formamide, 10 mM EDTA, 0.025% xylene cyanol, and 0.025% bromophenol blue). An aliquot (5 μL) was electrophoresed on a denaturing 20% polyacrylamide gel (0.09 M Tris-borate pH 8.3, 2 mM EDTA, 20% acrylamide, 8 M urea). Gels were dried, exposed in a PhosphorImager

© 2009 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

cassette, analyzed using a Typhoon 8610 Variable Mode Imager (Amersham Biosciences), and quantitated using Image Quant 5.2. Percent inhibition (% I) was calculated using the following equation:

$$\% I = 100 \times [1 - (D - C)/(N - C)] \quad (1)$$

where C, N, and D are the fractions of 21-mer substrate converted to 19-mer (3′-processing product) or strand transfer products for DNA alone, DNA plus IN, and IN plus drug, respectively. The IC_{50} values were determined by plotting the logarithm of drug concentration versus percent inhibition to obtain concentration that produced 50% inhibition.

Cell culture

Human prostate cancer cells were obtained from the American Type Cell Culture (ATCC). Cells were maintained as monolayer cultures in RPMI 1640 media supplemented with 10% fetal bovine serum (Gemini-Bioproducts, Woodland, CA, USA) and 2 mM L-Glutamine at 37°C in a humidified atmosphere of 5% CO_2. To remove the adherent cells from the flask for passaging and counting, cells were washed with phosphate buffered saline (PBS) without calcium or magnesium, incubated with a small volume of 0.25% trypsin-EDTA solution (Sigma) for 5 – 10 min, and washed with culture medium and centrifuged. All experiments were performed using cells in exponential cell growth.

Stock solution

A 10 mM stock solution of all compounds were prepared in dimethylsulfoxide (DMSO) and stored at 20°C. Further dilutions were freshly made in PBS.

Cytotoxicity assay

Cytotoxicity was assessed by MTT assay as previously described [33]. Briefly, cells were seeded in 96-well microtiter plates and allowed to attach. Cells were subsequently treated with a continuous exposure to the corresponding drug for 72 h. An MTT solution (at a final concentration of 0.5 mg/mL) was added to each well and cells were incubated for 4 h at 37°C. After removal of the media, DMSO was added and the absorbance was read at 570 nm. All assays were performed in triplicate. The 50% inhibitory concentration (IC_{50}) was then determined for each drug from a plot of log (drug concentration) versus percentage of cell kill.

References

[1] M. Hogberg, I. Morrison, *Expert Opin. Ther. Pat.* **2000**, *10*, 1189 – 1199; H. Jonckheere, J. Anne, E. De Clercq, *Med. Res. Rev.* **2000**, *20*, 129 – 154.

[2] F. Lebon, M. Ledecq, *Curr. Med. Chem.* **2000**, *7*, 455 – 477; J. P. Vacca, J. H. Condra, *Drug Discov. Today* **1997**, *2*, 261 – 272.

[3] F. J. Palella, K. M. Delaney, A. C. Moorman, M. O. Loveless, *et al.*, *N. Engl. J. Med.* **1998**, *338*, 853 – 860.

[4] J. Cohen, *Science* **2002**, *296*, 2320 – 2324.

[5] E. De Clercq, *Nat. Rev. Drug Discov.* **2002**, *1*, 13 – 25.

[6] N. Neamati, C. Marchand, Y. Pommier, *Adv. Pharmacol.* **2000**, *49*, 147 – 165.

[7] J. d'Angelo, J. F. Mouscadet, D. Desmaele, F. Zouhiri, H. Leh, *Pathol. Biol.* **2001**, *49*, 237 – 246.

[8] N. Neamati, *Expert Opin. Inv. Drug* **2001**, *10*, 281 – 296.

[9] N. J. Anthony, *Curr. Top. Med. Chem.* **2004**, *4*, 979 – 990.

[10] Y. Pommier, A. A. Johnson, C. Marchand, *Nat. Rev. Drug Discov.* **2005**, *4*, 236 – 248.

[11] A. Engelman, K. Mizuuchi, R. Craigie, *Cell* **1991**, *67*, 1211 – 1221.

[12] P. O. Brown, *Integration*, Cold Spring Harbor Press, Cold Spring Harbor, NY, USA, **1999**.

[13] E. Asante-Appiah, A. M. Skalka, *Adv. Virus Res.* **1999**, *52*, 351 – 369.

[14] S. Dubey, Y. D. Satyanaraya, H. Lavania, *Eur. J. Med. Chem.* **2007**, *42*, 1159 – 1168.

[15] T. S. Yoshinaga, T. Fujishita, T. Fujiwara, Presented at the 9th Conference on "Retroviruses and Opportunistic Infections", Seattle, WA, **2002**.

[16] A. Billich, *Curr. Opin. Invest. Drugs* **2003**, *4*, 206 – 209.

[17] S. Young, Presented at the "XIV International AIDS Conference", West Point, PA, **2002**.

[18] D. J. Hazuda, S. D. Young, J. P. Guare, N. J. Anthony, *et al.*, *Science* **2004**, *305*, 528 – 532.

[19] A. A. Johnson, C. Marchand, Y. Pommier, *Curr. Top Med. Chem.* **2004**, *4*, 671 – 686.

[20] M. Witvrouw, B. van Maele, J. Vercammen, A. Hantson, *et al.*, *Curr. Drug. Metab.* **2004**, *5*, 291 – 304.

[21] V. Summa, A. Petrocchi, V. G. Matassa, M. Taliani, *et al.*, *J. Med. Chem.* **2004**, *47*, 5336 – 5339; U. Koch, B. Attenni, S. Malancona, S. Colarusso, *et al.*, *J. Med. Chem.* **2006**, *49*, 1693 – 1707.

[22] P. Pace, M. E. Di Francesco, C. Gardelli, S. Harper, *et al.*, *J. Med. Chem.* **2007**, *50*, 2225 – 2239.

[23] E. Muraglia, O. D. Kinzel, C. Gardelli, B. Crescenzi, *et al.*, *J. Med. Chem.* **2008**, *51*, 861 – 874.

[24] M. R. Rico Ferreira, G. Cecere, P. Pace, V. Summa, *Tetrahedron Lett.* **2009**, *50*, 148 – 151.

[25] A. Mazumder, A. Gazit, A. Levitzki, M. Nicklaus, *et al.*, *Biochemistry* **1995**, *34*, 15111 – 15122.

[26] H. Hongbin Yuan, A. L. Parrill, *Bioorg. Med. Chem.* **2002**, *10*, 4169 – 4183.

[27] T. R. Burke Jr., M. R. Fesen, A. Mazumder, J. Wang, *et al.*, *J. Med. Chem.* **1995**, *38*, 4171 – 4178.

[28] C. M. Farnet, R. Lipford, B. Wang, F. D. Bushman, *Proc. Natl. Acad. Sci. U. S. A.* **1996**, *93*, 9742 – 9747.

[29] C. M. Farnet, B. Wang, M. Hansen, J. R. Lipford, *et al.*, *Antimicrob. Agents Chemother.* **1998**, *42*, 2245 – 2253.

[30] V. J. Ram, D. A. Vanden Berghe, A. J. Vlietinck, *J. Heterocyclic Chem.* **1984**, *21*, 1307 – 1312.

[31] N. Agarwal, P. Srivastava, S. K. Raghuwanshi, D. N. Upadhyay, *et al.*, *Bioorg. Med. Chem.* **2002**, *10*, 869 – 874.

[32] C. Marchand, N. Neamati, Y. Pommier, *Methods Enzymol.* **2001**, *340*, 624 – 633.

[33] J. Carmichael, W. G. DeGraff, A. F. Gazdar, J. D. Minna, J. B. Mitchell, *Cancer Res.* **1987**, *47*, 936 – 942.

© 2009 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim