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Chemical library screen for novel inhibitors of Kaposi's sarcoma-associated herpesvirus processive DNA synthesis

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Abstract

Kaposi's sarcoma-associated herpesvirus (KSHV) is the causative agent of Kaposi's sarcoma and certain lymphoproliferative disorders. The role of KSHV lytic replication has been implicated in the tumor pathogenesis. A highly specific molecular complex formed by the KSHV DNA polymerase (POL8) and processivity factor (PF8) is indispensable for lytic viral DNA synthesis and may serve as an excellent molecular anti-KSHV target. The majority of conventional nucleoside-based anti-herpetic DNA synthesis inhibitors require intracellular phosphorylation/activation before they can exert inhibitory activity as competitive substrates for viral DNA polymerases. Novel and more potent inhibitors of KSHV DNA synthesis may be discovered through POL8/PF8-targeted high throughput screening (HTS) of small molecule chemical libraries. We developed a microplate-based KSHV POL8/PF8-mediated DNA synthesis inhibition assay suitable for HTS and screened the NCI Diversity Set that comprised 1992 synthetic compounds. Twenty-eight compounds exhibited greater than 50% inhibition. The inhibitory activity was confirmed for 25 of the 26 hit compounds available for further testing, with the 50% inhibitory concentrations ranging from 0.12 ± 0.07 μM (mean ± S.D.) to 10.83 ± 4.19 μM. Eighteen of the confirmed active compounds efficiently blocked KSHV processive DNA synthesis in vitro. One of the hit compounds, NSC 373989, a pyrimidoquinoline analog, was shown to dose-dependently reduce the levels of KSHV virion production and KSHV DNA in lytically induced KSHV-infected BCBL-1 cells, suggesting that the compound blocked lytic KSHV DNA synthesis. HTS for KSHV POL8/PF8 inhibitors is feasible and may lead to discovery of novel non-nucleoside KSHV DNA synthesis inhibitors.
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Keywords: Kaposi's sarcoma-associated herpesvirus; DNA polymerase; Processivity factor; Small molecule inhibitor screen

1. Introduction

Kaposi's sarcoma (KS) is a highly vascular malignant tumor of endothelial cell origin that typically manifests as multifocal cutaneous nodules, but can often involve internal organs in immunocompromised hosts (Lemlich et al., 1987; Friedman-Kien and Saltzman, 1990; Farge, 1993). Its infectious etiology had long been suspected (Giraldo et al., 1972) even before the epidemic form of KS was recognized in HIV-infected patients in the early 1980s (Hymes et al., 1981; MMWR, 1981). The γ-herpesvirus-like DNA sequences were first discovered in KS lesions obtained from patients with AIDS in 1994 (Chang et al., 1994). The viral DNA was subsequently detected in the majority of KS lesions of all types of KS: classic, endemic, post-transplant, and AIDS-associated epidemic KS (Ambroziak et al., 1995; Boshoff et al., 1995; Moore and Chang, 1995; Schalling et al., 1995; Chang et al., 1996), as well as in AIDS-related body cavity-based B cell lymphoma, termed primary effusion lymphoma (Cesarman et al., 1995), and a subset of multicentric

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Castleman's disease (Soulier et al., 1995). The agent termed KS-associated herpesvirus (KSHV, also called human herpesvirus 8 or HHV8) was shown to have sequence homologies with other γ-herpesviruses, including herpesvirus saimiri (γ2), equine herpesvirus 2 (γ2), and Epstein-Barr virus (EBV) (γ1), and was classified as a new γ2-herpesvirus (genus *Rhadinovirus*) (Moore et al., 1996).

Further evidence to suggest KSHV as the causative agent of KS and other lymphoproliferative malignancies came from a number of clinical studies that revealed a striking association between higher KSHV viral load in peripheral blood mononuclear cells and increased risk of KS in HIV-1-infected and uninfected individuals (Whitby et al., 1995; Farge et al., 1999; Pellet et al., 2002; Engels et al., 2003). Others also reported that increasing KSHV load was observed in patients with progressive KS or symptomatic lymphoproliferative disorders in HIV-infected individuals (Campbell et al., 2000; Lallemand et al., 2000; Oksenhendler et al., 2000; Quinlivan et al., 2002). These findings implied that KSHV infection was a prerequisite for KSHV tumorigenesis and/or tumor progression, and that anti-KSHV agents might provide therapeutic benefit. Soon after the discovery of KSHV, there were indeed reports that coincidentally found a decreased risk of KS in AIDS patients treated with anti-herpetic compounds for cytomegalovirus infection (Glesby et al., 1996; Mocroft et al., 1996; Martin et al., 1999). Anti-KSHV activities of licensed anti-herpetic compounds, including cidofovir (CDV) and ganciclovir (GCV), were also demonstrated in KSHV-infected cells lytically induced by phorbol ester (Kedes and Ganem, 1997; Medveczky et al., 1997; Neyts and De Clercq, 1997). These observations have prompted further exploration of therapeutic utilities of anti-herpetic compounds for KSHV-induced malignancies (Robles et al., 1999; Mazzi et al., 2001; Little et al., 2003; Casper et al., 2004). However, the majority of existing anti-herpetic agents are nucleoside-based compounds, which require intracellular conversion to phosphorylated active metabolites, often by virally encoded thymidine kinases or phosphotransferases before they can exert viral DNA synthesis inhibition (Furman et al., 1979; Derse et al., 1981; Keller et al., 1981; Ashton et al., 1982; Frank et al., 1984). Such a constraint may potentially give rise to varying antiviral effects due to differential metabolic profiles among treated individuals. It is possible that more potent non-nucleoside-based anti-KSHV agents may be discovered through cell-free molecular-targeted drug screening.

Lytic KSHV DNA synthesis is controlled by six virally encoded core DNA replication proteins: single stranded DNA-binding protein (open reading frame 6, ORF6), polymerase (POL8) (ORF9), primase-associated factor (ORF40/41), helicase (ORF44), primase (ORF56), and polymerase processivity factor (PF8) (ORF59) (Lin et al., 1998; Wu et al., 2001). KSHV POL8 and PF8, both successfully cloned in 1998 (Lin et al., 1998), are essential for viral DNA polymerization. KSHV PF8, the POL8-specific accessory protein, interacts with POL8 and tethers it onto extending DNA (Chen et al., 2004). PF8 also preferentially binds dsDNA over ssDNA, and its processive functionality is determined by both the POL8- and dsDNA-binding activities (Chan and Chandran, 2000). This highly specific POL8/PF8 complex may be effectively targeted by small molecule agents, which may inhibit POL8 enzymatic activity, cooperative molecular interaction of POL8 and PF8, or function of PF8 itself, without undergoing intracellular activation. For example, a small molecule inhibitor of herpes simplex virus type 1 (HSV-1) DNA polymerase (HSV-1 Pol) and its processivity factor UL42 interaction has recently been identified by a molecularly targeted screen using an HSV-1 Pol-derived peptide and UL42-based assay (Pilger et al., 2004). In the current study, we established a microplate-based POL8/PF8-mediated DNA synthesis inhibition assay suitable for high throughput screening (HTS) of small molecule chemical libraries. Of the 28 hit compounds selected from the primary screen of the NCI Diversity Set (http://dtp.nci.nih.gov/branches/dscb/diversity_explanation.html), one compound was shown to specifically block KSHV DNA synthesis in KSHV-infected cells. Our data provide proof-of-principle for a KSHV POL8/PF8 molecular targeting strategy and warrant future HTS campaigns of larger chemical libraries.

2. Materials and methods

2.1. Compounds

CDV was kindly provided by Dr. M. Hitchcock (Gilead Sciences, Inc. Foster City, CA, USA). CDV-diphosphate (CDV-DP) was synthesized by TriLink BioTechnologies, Inc. (San Diego, CA, USA). The NCI Training and Diversity Sets were obtained from the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, NCI (http://dtp.nci.nih.gov/). The NCI Training Set contains 230 well-characterized anti-cancer compounds covering major mechanistic modes of actions, frequently used to validate the reproducibility of HTS assays (Shoemaker et al., 2002). The NCI Diversity Set comprises 1992 synthetic compounds selected from nearly 140,000 compounds available in sufficient quantity from the NCI DTP Repository. These Diversity Set compounds were chosen based on specific pharmacophores, as defined by the Chem-X program (Accelrys, San Diego, CA, USA), to represent diverse structure subsets of the NCI library (http://dtp.nci.nih.gov/branches/dscb/diversity_explanation.html).

2.2. Production of KSHV POL8 and PF8 proteins

In vitro-translated KSHV POL8 (ivt POL8) and PF8 (ivtPF8) were synthesized from pTM1-Pol8 and pTM1-PF8, respectively (Lin et al., 1998), using TNT® T7 Quick Coupled Transcription/Translation System (Promega, Madison, WI, USA). Recombinant KSHV POL8 (rPOL8) was expressed as previously described (Dorjsuren et al., 2003) and purified using a fast protein liquid chromatography system (AKTA™FPLC™, GE Healthcare, formerly Amersham Biosciences, Piscataway, NJ, USA). Recombinant KSHV PF8 (rPF8) was similarly produced by using a baculovirus vector system. Briefly, the full-length KSHV *pf8*-encoding gene insert was prepared from the PF8-expression plasmid, pTM1-PF8 (Lin et al., 1998), and cloned into pFastBacHTb (Bac-to-Bac®

Baculovirus Expression System, Invitrogen, Carlsbad, CA, USA) to generate pFastBacHTb-PF8 donor plasmid. The *pf8* fragment from the donor plasmid was transposed in vivo into bacmid DNA to produce the recombinant PF8-bacmid DNA (Bac-to-Bac® Baculovirus Expression System, Invitrogen). Sf9 cells were transfected with the PF8-bacmid DNA, and infectious baculovirus expressing PF8 was harvested after 72 h. KSHV rPF8 was purified from total cell extracts of Sf9 cells infected with the recombinant PF8-expressing baculovirus by using AKTA^{TM}FPLC^{TM}system (GE Healthcare).

2.3. Microplate-based POL8/PF8-mediated DNA synthesis assay

A rapid microplate-based DNA synthesis assay was carried out using KSHV POL8 and PF8 as previously described (Lin and Ricciardi, 2000) with modifications. Briefly, a biotinylated DNA primer-template (P-T) was prepared by incubating equimolar quantities of a 20-mer oligonucleotide primer (5′-GCCAATGAATGACCGCTGAC-3′) and a 5′-biotinylated 100-mer oligonucleotide template (5′-GCACTTATTGCATTCGC-TAGTCCACCTTGGATCTCAGGCTATTCGTAGCGAGCTA-CGCGTACGTTAGCTTCGGTCATCCCGTCAGCGGTCAT-TCATTGGC-3′) (Bionexus, Inc., Oakland, CA, USA) at 90 °C for 5 min, followed by a gradual cooling to room temperature. The annealed P-T solution was diluted to 1 pmol/μL with PBS. Streptavidin-coated plates (StreptaWell plates) (Roche Applied Science, Indianapolis, IN, USA) were coated with 0.2 pmol/well of P-T at 4 °C overnight. The DNA synthesis was carried out in the P-T coated plates, using 0.5 μL each of ivtPOL8 and ivtPF8, or 10 ng each of rPOL8 and rPF8 in a reaction mixture containing 50 mM $(NH_4)_2SO_4$, 20 mM Tris–HCl (pH 7.5), 3 mM $MgCl_2$, 0.1 mM EDTA, 0.5 mM DTT, 2% glycerol, 40 μg/mL BSA, 0.625 μM dNTPs, 0.125 μM digoxigenin-11-2′-deoxyuridine-5′-triphosphate (DIG-dUTP) (Roche Applied Science) in the absence or presence of test compounds for 60 min at 37 °C. The amounts of DIG-dUTP incorporated into DNA, which reflected the levels of total DNA synthesis, were determined by DIG detection ELISA (ABTS) kit (Roche Applied Science) according to the manufacturer's instructions. DNA synthesis inhibitory activity was expressed as $\%[1 - [OD_{405\,nm}$ (test drug) $- OD_{405\,nm}$ (buffer only background)]/[$OD_{405\,nm}$ (no drug) $- OD_{405\,nm}$ (background)]].

2.4. Processive DNA synthesis assay

To confirm the inhibitory activity of selected hit compounds against processive DNA synthesis mediated by KSHV POL8 and PF8, in vitro processive DNA synthesis assay was carried out using primed M13 template as described (Lin et al., 1998; Dorjsuren et al., 2003). Briefly, 10 ng each of rPOL8 and rPF8 were added to a final 50 μL reaction mixture, containing 100 mM $(NH_4)_2SO_4$, 20 mM Tris–Cl (pH 7.5), 3 mM $MgCl_2$, 0.1 mM EDTA, 0.5 mM DTT, 4% glycerol, 40 μg/mL of BSA, 60 μM (each) dATP, dGTP, and dTTP, 20 μCi [α-^{32}P]dCTP (3000 Ci/mmol) (GE Healthcare), and 50 fmol of primed M13 ssDNA template, and incubated in the absence or presence of test compounds at 37 °C for 60 min, followed by addition of a 50 μL stop solution consisting of 1% SDS, 10 mM EDTA, 10 mM Tris–Cl (pH 8), and 200 μg/mL of proteinase K. After 60 min incubation, the radiolabeled DNA products were extracted by phenol–chloroform followed by ethanol precipitation in the presence of 1 M ammonium acetate. The precipitated DNA products were fractionated on a 1.3% alkaline agarose gel. The dried gel was examined by autoradiography.

2.5. BCBL-1 culture for evaluation of anti-KSHV activity of hit compounds

The anti-KSHV activity of selected hit compounds was evaluated in KSHV-infected BCBL-1 cells by examining their effects on KSHV DNA synthesis and newly released KSHV virions, following lytic induction (Renne et al., 1996). BCBL-1, a latently KSHV-infected B cell line established from a primary effusion lymphoma, was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH; contributed by Drs. Michael McGrath and Don Ganem (Renne et al., 1996). Briefly, exponentially growing BCBL-1 cells were washed three times with phosphate-buffered saline (PBS) and resuspended in serum free AIM-V w/BSA medium (Invitrogen) at 2×10^5 cells/mL in the absence (unstimulated control) or presence of 20 ng/mL phorbol 12-myristate 13-acetate (PMA, also called 12-*O*-tetradecanoylphorbol 13-acetate, TPA) (Sigma-Aldrich, St. Louis, MO, USA). After 24 h, unstimulated and PMA-stimulated BCBL-1 were harvested, washed once with PBS and cultured in serum free AIM-V w/BSA medium at 2×10^5 cells/mL without PMA in the absence or presence of the test compounds at varying concentrations. After 3 days, the cells were counted by the trypan blue dye exclusion method and centrifuged at 1500 rpm for 5 min. The supernatants were centrifuged at 3000 rpm for 10 min before subjected to virion-derived KSHV DNA extraction and quantitation (see below). The cytotoxicity of the compounds was determined simultaneously in unstimulated and PMA-stimulated BCBL-1 cells in 96-well microplates, using the XTT assay (Weislow et al., 1989).

2.6. Measurements of cell- and virion-associated KSHV DNA by PCR

Low molecular weight (LMW) DNA was extracted from the pelleted cells according to Hirt's method (Hirt, 1967) and 0.1 μg of LMW DNA was used for KSHV late lytic gene ORF65 PCR by a primer pair, 5′-ACGGTTGTCCAATCGTTGCCTA-3′ and 5′-TCCAACTTTAAGGTGAGAGAC-3′, generating a 529 bp fragment. The ORF65 PCR reaction mixture, containing 20 mM Tris–HCl (pH 8.4), 50 mM KCl, 2.5 mM $MgCl_2$, 200 μM each dNTP, 0.25 U of Platinum® *Taq* DNA polymerase (Invitrogen), 200 nM of each primer and template DNA, was subjected to 25 cycles of PCR amplification at 94 °C for 60 s, 60 °C for 60 s and 72 °C for 60 s, followed by a final extension at 72 °C for 5 min. In addition, the mitochondrial DNA primer pair (5′-TGGAGCCGGAGCACCCTATGTC-3′ and 5′-ATGGGCGGGGGTTGTATTGATG-3′) was used as an internal control for each LMW DNA PCR sample (Yang et al., 2005).

The amplified products were visualized by electrophoresis on a 1.8% agarose gel.

KSHV virions were pelleted from 300 μL of BCBL-1 culture supernatants by a microcentrifugation at 37,000 × *g* for 2 h at 4 °C (Sei et al., 2000). The pelleted virions were resuspended in 150 μL PBS and treated with 20 units of DNase I (Promega) at 37 °C for 30 min to remove cellular DNA from the samples, followed by the incubation with stop solution (20 mM EGTA) at 70 °C for 5 min. Virion-associated KSHV DNA (vDNA) was then extracted by QIAamp DNA extraction kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's instructions. One microliter of vDNA eluted in 100 μL of elution buffer was subjected to real-time quantitative PCR using a LightCycler® instrument (Roche Applied Science). The 20-μL reaction mixture consisted of the LightCycler FastStart DNA Master SYBR Green I reagents mix (Roche Applied Science), 2.5 mM $MgCl_2$ and 500 nM each of KSHV ORF26 primer pair (5′-AGCCGAAAGGATTCCACCATT-3′ and 5′-TCCGTGTTGTCTACGTCCAGA-3′). Ten-fold serial dilutions of the plasmid, pKS330Bam (obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: contributed by Drs. Yuan Chang and Patrick Moore), which contains a 330 bp KSHV fragment encoding a portion of the ORF26 gene (Chang et al., 1994), were included in each assay as external standards to represent 10–10^7 KSHV DNA copies/tube. The number of KSHV vDNA in each supernatant sample was calculated by the LightCycler software version 3.5 (Roche Applied Science), adjusted by the cell count (copies/10^6 cells) to reflect the level of virion release from an equal number of viable cells, and depicted as percent of no drug control.

3. Results

3.1. Optimization of the microplate-based DNA synthesis assay for HTS mode

The feasibility of a microplate-based KSHV POL8/PF8 assay had previously been demonstrated in principle by using ivtPOL8 and ivtPF8 (Lin and Ricciardi, 2000). One of the critical determinants for successful molecular targeted screens is the use of lower test compound concentrations, as screening at higher concentrations can be associated with selection of "promiscuous inhibitors" of targeted molecules (McGovern et al., 2002). To adapt the assay for HTS mode, we first attempted to optimize the POL8/PF8-mediated DNA synthesis assay condition, wherein the DNA synthesis inhibitory activity could be demonstrated at low micromolar concentrations of test compounds. Using CDV-DP as a reference inhibitory agent, we modified the ionic strength of the buffer solution as well as the concentrations of dNTPs, DIG-dUTP, and ivtPOL8/ivtPF8 proteins in the reaction mixture. CDV-DP was selected as a reference inhibitor, because its precursor, CDV, had been shown to potently inhibit KSHV DNA synthesis and virion production in cell-based assays (Kedes and Ganem, 1997; Medveczky et al., 1997; Neyts and De Clercq, 1997). The optimal concentration of $(NH_4)_2SO_4$ in the assay buffer was titrated to 50 mM for the most efficient DNA synthesis by ivtPOL8/ivtPF8 as well as by rPOL8/rPF8 (see below). The concentrations of dNTPs were adjusted to the low micromolar range, as has been done in other DNA polymerization assays (Baba et al., 1991; Campiani et al., 1996). In the modified assay, CDV-DP consistently achieved ≥50% inhibition at 20 μM.

Once the optimal assay condition was determined, the assay suitability for HTS was examined by pilot screening of the NCI Training Set, a collection of 230 well-characterized compounds (Shoemaker et al., 2002). The reproducibility of the optimized assay was demonstrated by replicate testing of the Training Set compounds, yielding correlation coefficients (r^2) of 0.92 and 0.90 when tested at 200 and 20 μM concentrations, respectively. At 200 μM test concentration, we found that 25 of the 37 DNA–interacting compounds (including those referred to as topoisomerase II poisons (Pommier et al., 2001)) and 34 of the remaining 193 compounds exhibited greater than 50% inhibition of DNA synthesis (Fig. 1). While the majority of the compounds with DNA-binding capacity were expected to show varying degrees of DNA synthesis inhibition regardless of their inhibitory activity against KSHV DNA polymerase, many of the hit compounds from non-DNA-interacting compounds in the Training Set may have represented "non-specific hits" due to the high test concentration employed. Indeed, when the test concentration was lowered to 20 μM, only 5 of the 193 non-DNA-binding compounds exhibited greater than 50% inhibition, while 17 of the 37 DNA-binding compounds remained as hits (Fig. 1). These data suggested that the prevalence of non-specific inhibition of DNA synthesis in the current assay could be significantly reduced at the lower test concentration. Thus, we elected to initially screen the chemical libraries at 20 μM with 50% inhibition as a hit threshold. Inhibitors of DNA polymerase α included in the Training Set (NSC 303812 and NSC 639829) did not show any inhibitory activity at 20 μM.

3.2. Screening of the NCI Diversity Set by the microplate assay and confirmation of the activity of hit compounds

To further explore feasibility of HTS campaigns using the modified ivtPOL8/ivtPF8-DNA synthesis assay, we screened the NCI Diversity Set, which comprises 1992 synthetic compounds representing diverse structure subsets of the 140,000 compounds available from the NCI DTP chemical library (Shoemaker et al., 2002). After the primary screen at 20 μM, 28 compounds were identified as hits exhibiting ≥50% inhibition (1.4% hit rate) (Table 1). Screening window coefficients, *Z*′-factors (Zhang et al., 1999), ranged from 0.683 to 0.951 (median 0.845). These data suggested that the modified assay was well suited for HTS applications. However, HTS campaigns for novel inhibitors of KSHV POL8/PF8 require large quantities of homogeneous and pure POL8 and PF8 proteins. Therefore, we constructed the recombinant baculovirus vectors, and successfully expressed and purified functionally active recombinant KSHV POL8 and PF8 from the viral vector-infected Sf9 insect cells (Dorjsuren et al., 2003) (Fig. 2). Functional integrities of the purified rPOL8 and rPF8 were verified by their in vitro processive DNA

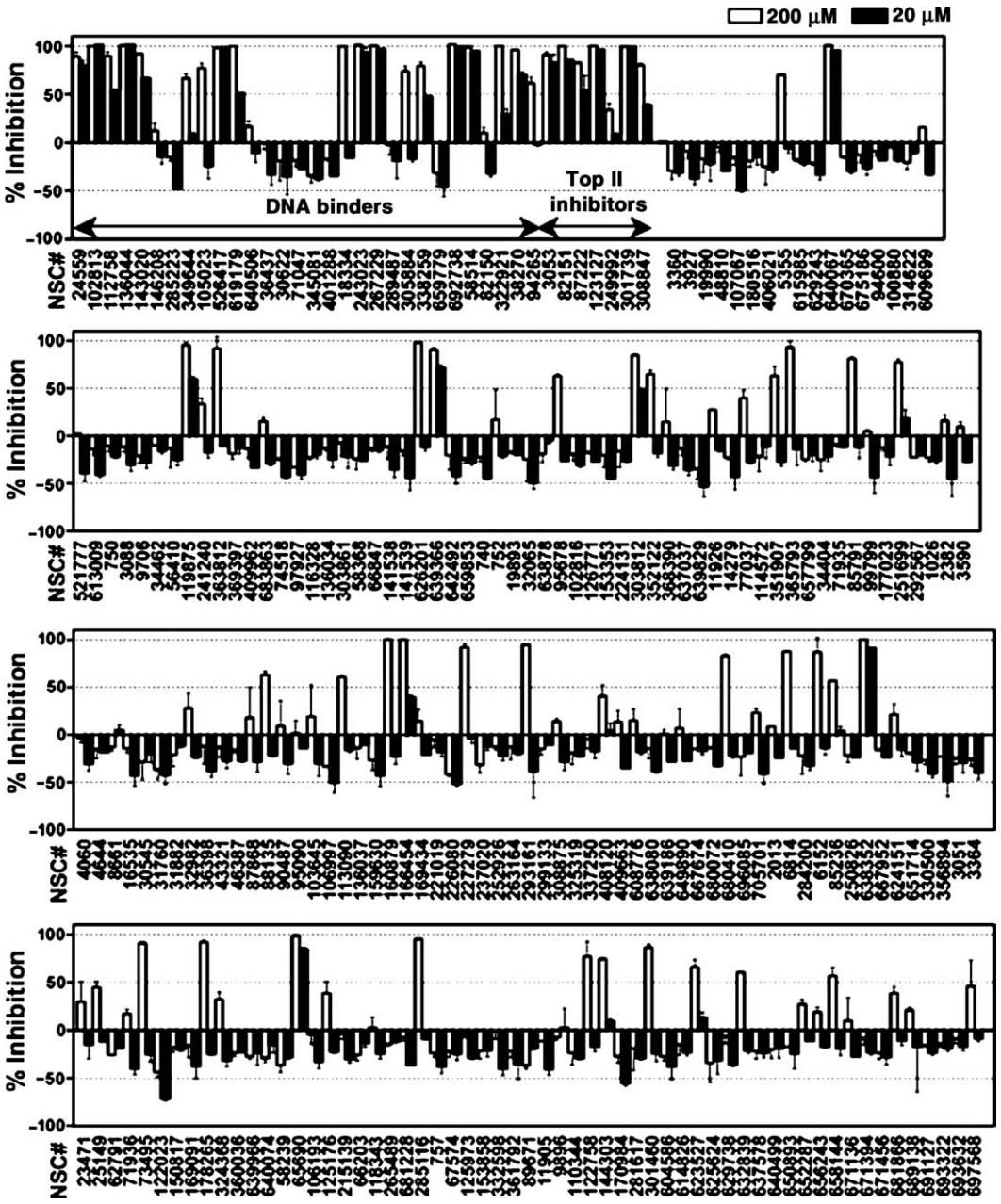


Fig. 1. The inhibitory activities of the NCI Training Set compounds, identified by NSC number (http://dtp.nci.nih.gov/), were compared between DNA-interacting (including topoisomerase II inhibitors or Top II inhibitors) and non-DNA-interacting compounds, defined based on known mechanisms of action or target molecules (Shoemaker et al., 2002), tested at 200 and 20 μM. The data shown are mean ± S.D. of two separate assays. The prevalence of DNA synthesis inhibition by non-DNA binders was significantly reduced at 20 μM.

synthesis activity, which was effectively blocked by CDV-DP in a dose-dependent manner (Dorjsuren et al., 2003). The Training Set compounds were screened at 20 μM in the microplate DNA synthesis assay using rPOL8/rPF8 in place of ivtPOL8/ivtPF8. Of the 225 compounds examined by both ivtPOL8/ivtPF8-based and rPOL8/rPF8-based DNA synthesis assays, all 21 positive hits (≥50%inhibition) selected from the ivtPOL8/ivtPF8 assay also exhibited greater than 50% inhibition in rPOL8/rPF8-based assay, while all 198 inactive compounds identified by rPOL8/rPF8-based assay were also deemed negative in ivtPOL8/ivtPF8-based assay, resulting in the assay concordance of 97.3%. Z'-factors for the rPOL8/rPF8-assay were comparable to those of ivtPOL8/ivtPF8-based assay (0.750 ± 0.126 versus 0.799 ± 0.055: mean ± S.D. of Z'-factors from three separate rPOL8/rPF8-based versus ivtPOL8/ivtPF8-based assays, respectively).

All but two (NSC 176328 and NSC 357756) of the 28 hit compounds identified from the NCI Diversity Set were available in sufficient quantities for further testing. The activity was confirmed for 25 of the 26 compounds in the rPOL8/rPF8-based microplate DNA synthesis inhibition assay, with the 50% inhibitory concentrations (IC50s) ranging from 0.12 ± 0.07 μM to 10.83 ± 4.19 μM (mean ± S.D. of three separate experiments, Table 1).

Table 1
Structures and activities of the hit compounds identified from KSHV POL8/PF8-DNA synthesis inhibition screen of the NCI Diversity Set

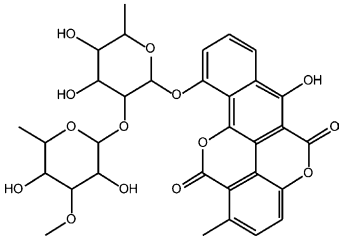
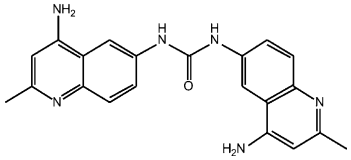
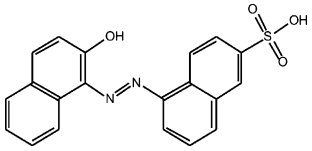
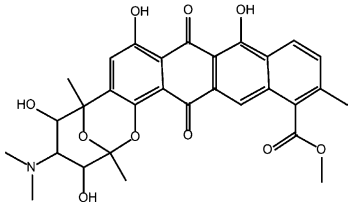
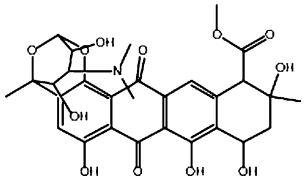
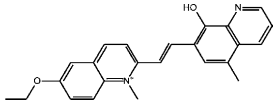
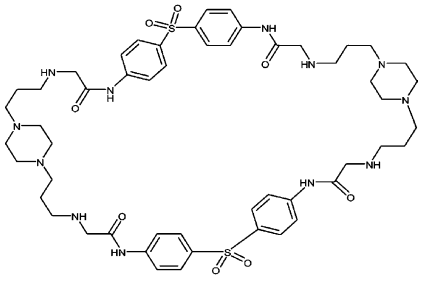
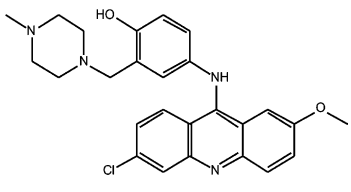
NSC#	Structure	Primary screen tested @ 20 μM (percent inhibition)	rPOL8/rPF8 DNA synthesis assay (IC50 (μM): mean ± S.D.)
5159		68.06	2.74 ± 0.40
12155		80.81	1.59 ± 0.17
45576		84.25	5.64 ± 1.31
82892		68.61	4.14 ± 1.19
86005		51.26	4.95 ± 1.19
86372		57.88	3.86 ± 1.01
95609		72.47	3.53 ± 0.17
130813		79.54	1.90 ± 0.43

Table 1 (*Continued*)

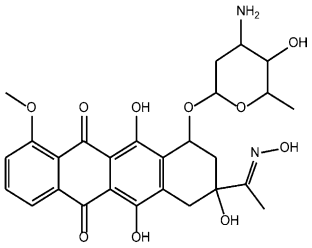
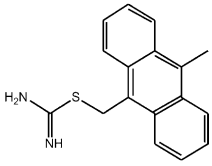
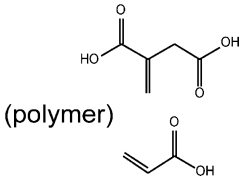
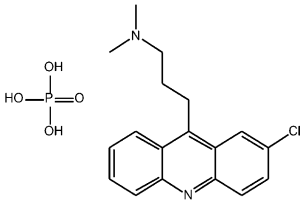
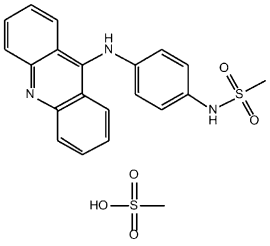
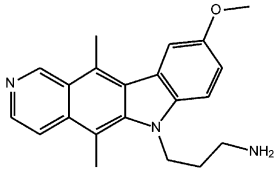
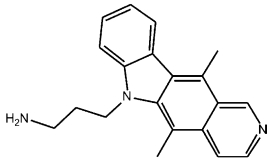
NSC#	Structure	Primary screen tested @ 20 μM (percent inhibition)	rPOL8/rPF8 DNA synthesis assay (IC50 (μM): mean ± S.D.)
143491		76.63	1.39 ± 0.37
146109		52.09	5.14 ± 2.32
146443[a]	 (polymer)	96.04	0.04 ± 0.01[a]
147744		51.07	>20
156305		59.19	2.81 ± 0.63
176327		100.00	0.12 ± 0.07
176328[b]		100.00	N/D

Table 1 (*Continued*)

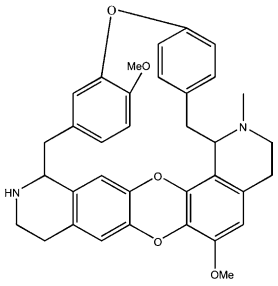
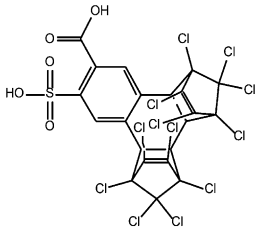
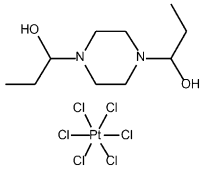
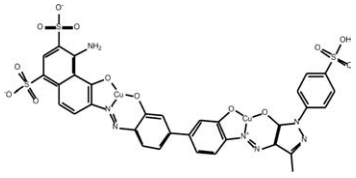
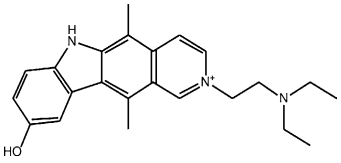
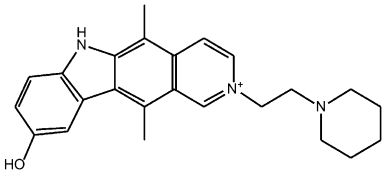
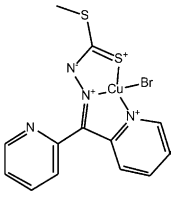
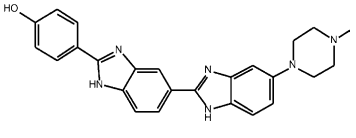
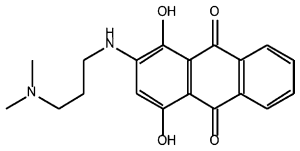
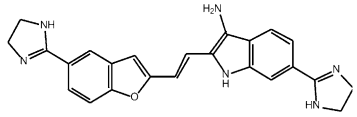
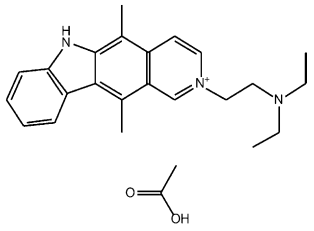
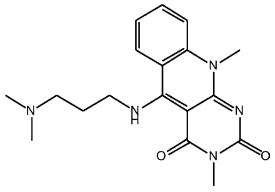
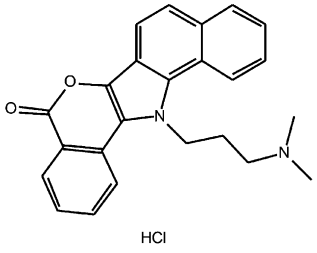
NSC#	Structure	Primary screen tested @ 20 μM (percent inhibition)	rPOL8/rPF8 DNA synthesis assay (IC50 (μM): mean ± S.D.)
181486		73.23	10.83 ± 4.19
270718		89.65	2.38 ± 0.31
295558		68.28	2.63 ± 0.54
306711		55.66	1.46 ± 0.32
311152		78.80	0.50 ± 0.12
311153		73.21	1.68 ± 0.30
321206		96.54	1.28 ± 0.16
322921		54.99	3.61 ± 0.09

Table 1 (*Continued*)

NSC#	Structure	Primary screen tested @ 20 μM (percent inhibition)	rPOL8/rPF8 DNA synthesis assay (IC50 (μM): mean ± S.D.)
339585		74.39	9.05 ± 2.97
357756[b]		89.48	N/D
359449		71.61	2.03 ± 0.13
373989		52.55	9.75 ± 1.98
638432		49.89	3.27 ± 0.40

rPOL8: recombinant KSHV DNA polymerase; rPF8: recombinant KSHV polymerase processivity factor, IC50: inhibitory concentration to achieve 50% reduction in DNA synthesis.

[a] This polymer compound was tested at 4 μg/mL. The IC50 value shown is in μg/mL.

[b] Inhibitory activity not confirmed due to insufficient quantity, N/D: not done.

3.3. Inhibitory activity of hit compounds in the M13 nucleotide extension assay

In order to corroborate that primary hits identified by the current microplate assay comprised the agents inhibitory to functional activities of POL8 and/or PF8, the hit compounds from the NCI Diversity Set were evaluated for inhibitory activity against POL8/PF8-mediated in vitro processive DNA synthesis, using the M13 nucleotide extension assay (Lin et al., 1998; Lin and Ricciardi, 2000), wherein POL8/PF8 can synthesize long-chain DNA of up to 7249 nucleotides (nts) in length. As shown in Fig. 3, 18 of the 26 compounds blocked rPOL8/rPF8-mediated processive DNA synthesis at 20 μM (except for NSC 146443, a polymer compound as shown in Table 1, used at 4 μg/mL). Most notably, all of ellipticine/ellipticinium derivatives (NSC 176327, NSC 311152, NSC 311153, and NSC 359449), anthracycline derivatives (NSC 82892, NSC 86005, and NSC 143491) and quinoline-containing derivatives (NSC 12155, NSC 86372, and NSC 373989) examined exhibited highly potent inhibition (Fig. 3). The remaining active compounds consisted of an acridine-containing derivative (NSC 130813), a benzimidazole DNA binder NSC 322921 (Hoe-33258) and compounds with various other structures (NSC 95609, NSC 146443, NSC 270718, NSC 306711, NSC 321206, and NSC 638432, Fig. 3).

3.4. Anti-KSHV activity of one of the hit compounds in lytically induced BCBL-1 cells

Lastly, we examined whether the active compounds selected from the current molecular targeted screen exerted inhibitory

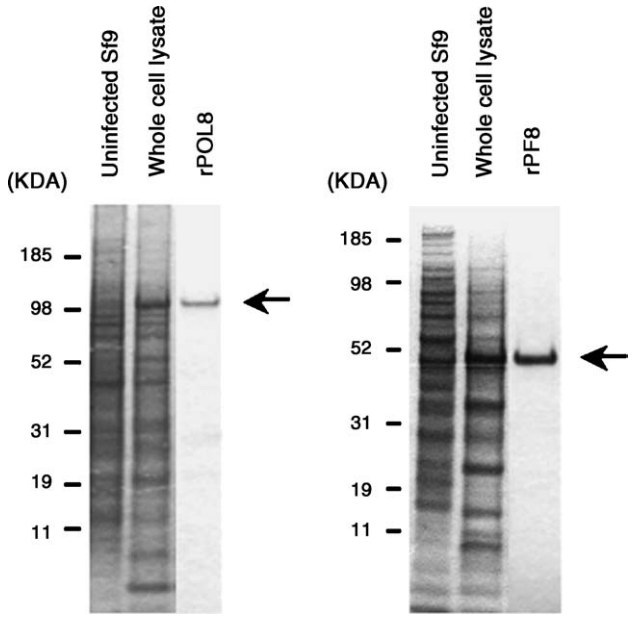


Fig. 2. Expression and purification of recombinant KSHV POL8 (rPOL8) and PF8 (rPF8) using a baculovirus vector system (Dorjsuren et al., 2003). Sf9 cells were infected with the recombinant POL8- or PF8-expressing baculovirus and harvested at 72 h post infection. KSHV rPOL8 and rPF8 were purified from total cell extracts of infected Sf9 cells by using AKTA^{TM}FPLC^{TM}system (GE Healthcare). Shown are the whole cell lysates of uninfected and infected Sf9 cells as well as the purified protein analyzed by SDS–PAGE with Coomassie blue staining. Molecular weight markers are indicated on the left in kilodaltons (kDa).

activity on lytic KSHV DNA replication in KSHV-infected cells. In the BCBL-1 cell-based assay employed in our study, the number of newly released KSHV virion-associated DNA copies determined by quantitative PCR was consistently 10–50-fold increased in PMA-induced cells over uninduced control with a corresponding increase in the amount of KSHV DNA in the Hirt supernatant DNA (Hirt DNA) (Fig. 4A and B, see PMA (−) and PMA+). The 18 hit compounds, which efficiently blocked in vitro processive DNA synthesis mediated by rPOL8/rPF8 (Fig. 3), were added to the BCBL-1 culture at various concentrations after the lytic cycle was fully induced by PMA for 24 h in order to determine their effects specifically on lytic KSHV DNA replication. Of the 18 compounds tested, 1 compound, NSC 373989, dose-dependently inhibited KSHV vDNA production in PMA-stimulated BCBL-1 cells (Fig. 4A), with IC50 ranging from 1.4 to 2.7 μM in three independent experiments (mean ± S.D., 1.9 ± 0.8 μM). The reduction in KSHV vDNA level was accompanied by a corresponding decrease in KSHV DNA in the Hirt DNA without affecting control mitochondrial DNA levels (Fig. 4B), suggesting that the compound specifically blocked lytic KSHV DNA replication. However, NSC 373989 exerted cytotoxicity at relatively low concentrations (50% cytotoxic concentration, CC50, mean ± S.D. from three separate experiments: 8.0 ± 1.7 μM). The remaining compounds did not significantly inhibit KSHV DNA synthesis at non-cytotoxic concentrations (data not shown).

4. Discussion

The rapid advances in functional genomics, proteomics, combinatorial chemistry and bioinformatics have dramatically streamlined the strategic approach to discovery and development of novel therapeutics for various human diseases in recent years. As soon as exploitable molecular targets are identified and validated for therapeutic intervention, small molecule inhibitors of the new targets can be discovered from vast collections of chemical compounds (chemical libraries) through HTS at an unprecedented rate. Although cell-free biochemical HTS assays do not provide information on potential toxicity (unwanted effects), toxicity evaluations may be integrated in the secondary screening once candidate compounds are identified. Such a rational, molecularly targeted approach can potentially lead to discovery of novel therapeutic agents that are highly specific and effective against the targeted diseases. The first critical step toward successful identification of new efficacious agents is to establish assay methodologies, which will selectively identify compounds with desired activities. Once specific functional assays are developed, the assays should be applied to screening of diverse groups of chemical compounds in order to accelerate the discovery process for potentially useful drug-like compounds. However, these prototype assays are not necessarily optimized for screening

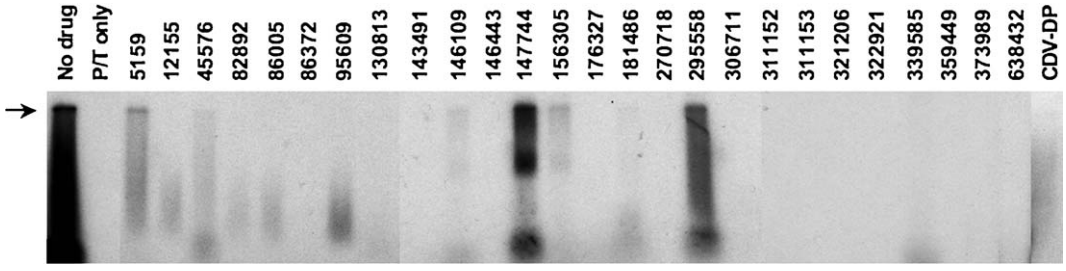


Fig. 3. Inhibitory activity of the primary hit compounds from the NCI Diversity Set on in vitro processive DNA synthesis by KSHV rPOL8 and rPF8. Processive DNA synthesis assay was carried out using primed M13 template (Lin et al., 1998; Dorjsuren et al., 2003) and 10 ng each of KSHV rPOL8 and rPF8 in the absence or presence of 20 μM test compounds. The radiolabeled, synthesized DNA products were extracted and fractionated on a 1.3% alkaline agarose gel. Shown is an autoradiogram of the dried gel. Note, compared to no drug control, which generated the full-length DNA strands, processive DNA synthesis was completely blocked by 18 compounds added at 20 μM (except for NSC 146443, a polymer compound, used at 4 μg/mL). Twenty micromolars CDV-DP, the active form of CDV, was included as a reference inhibitory agent. Numbers above each lane are NSC numbers identifying the compounds from the NCI DTP repository. The arrow denotes a full-length (7249 nts) DNA product synthesized from the M13 template. The smear below represents size-range of processively synthesized long-chain DNA products.

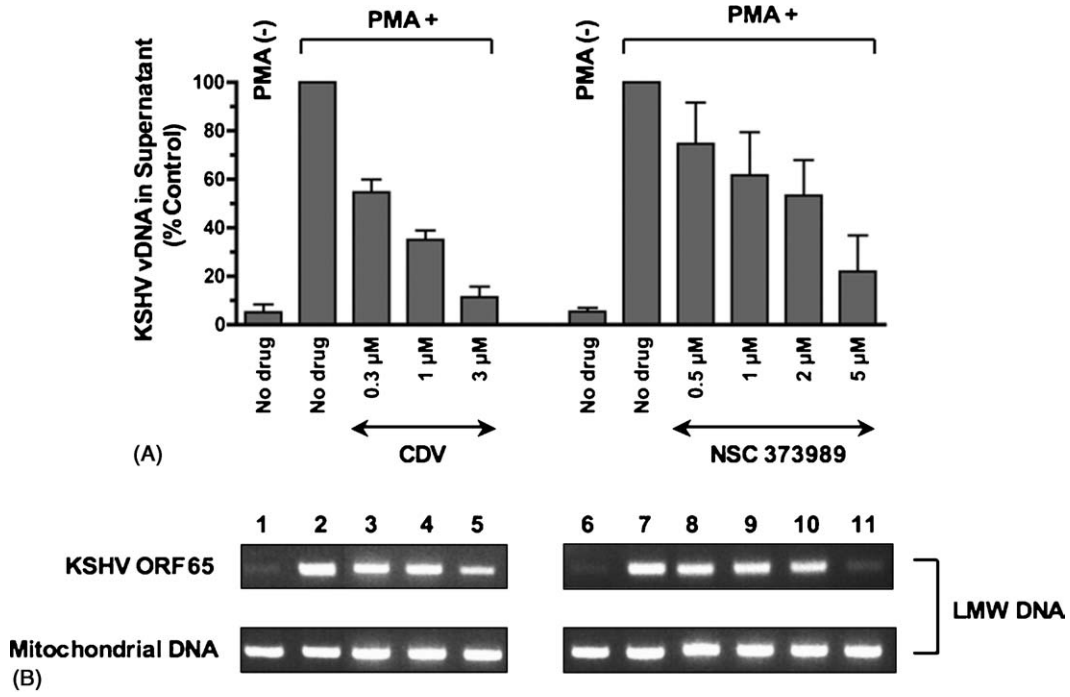


Fig. 4. Anti-KSHV activity of one hit compound selected from the library screen. (A) The antiviral effects of the active compounds were evaluated in PMA-stimulated BCBL-1 cells, which typically produced over 10- to 50-fold higher levels of KSHV virions than unstimulated BCBL-1 (PMA (−)). Of the 18 compounds tested, one compound, NSC 373989, dose-dependently decreased KSHV virion production from lytically induced BCBL-1 cells. KSHV virion-associated DNA (vDNA) was assayed by real time quantitative PCR. The levels of KSHV vDNA were adjusted by the cell count (copies/10^6 cells) and depicted as percent of no drug control. The data shown are mean ± S.D. from three separate experiments. The dose-response effect of cidofovir (CDV) is included as a reference. (B) The amounts of KSHV DNA were examined by late gene ORF65 PCR, using low molecular weight (LMW) DNA. Dose-dependent decreases in KSHV DNA were demonstrated in PMA-stimulated BCBL-1 cells treated with CDV (left) or NSC 373989 (right). The mitochondrial DNA fragment is shown as an internal control for each sample. The data shown are representative of three independent experiments. Lanes 1 and 6, no PMA/no drug control; lanes 2 and 7, PMA-stimulated/no drug control; lanes 3–5, CDV 0.3, 1, and 3 μM, respectively; lanes 8–11, NSC 373989 0.5, 1, 2, and 5 μM, respectively.

of large chemical libraries constructed through combinatorial chemistry. The adaptation of original assays to high-throughput applications, therefore, has become a fundamental requirement for drug screening campaigns.

In the current study, we attempted to develop an HTS assay for identification of inhibitors of KSHV POL8/PF8-mediated processive DNA synthesis. We initially employed in vitro translated proteins in the assay, while we vigorously attempted to express and purify functionally active recombinant KSHV POL8 and PF8 in large scale (Dorjsuren et al., 2003). We have shown that the microplate-based DNA synthesis assay using two cooperatively functioning proteins, KSHV POL8 and PF8, produced preliminarily by in vitro translation (Lin et al., 1998) and subsequently by using a baculovirus vector system (Dorjsuren et al., 2003), could be optimized for an HTS mode with high assay reproducibility ($r^2 \geq 0.9$) and excellent levels of screening window co-efficient, Z'-factor (Zhang et al., 1999). The hit threshold was set at 50% inhibition based on the preliminary screening profile of the NCI Training Set, which indicated that at low micromolar test concentrations non-specific inhibitors would be less likely to be selected with this cut-off. Using the hit threshold of ≥50% inhibition, we identified 28 active compounds from the NCI Diversity Set, resulting in a primary hit rate of 1.4%.

To further validate the assay's suitability for future HTS campaigns, the inhibitory activity of the primary hit compounds from the Diversity Set was verified by secondary testing: the microplate-based DNA synthesis assay performed in the absence or presence of serially diluted test compounds and in vitro processive DNA synthesis inhibition assay. We confirmed that 25 of the 26 available compounds dose-dependently blocked DNA synthesis in the microplate assay, and that 18 of the 25 compounds efficiently blocked in vitro processive DNA synthesis mediated by rPOL8/rPF8. Furthermore, of the 18 active compounds examined, one agent, NSC 373989 (5-((3-(dimethylamino)propyl)amino)-3,10-dimethylpyrimido[4,5-b]quinoline-2,4(3*H*,10*H*)-dione), was shown to specifically block lytic KSHV DNA replication in PMA-stimulated KSHV-infected BCBL-1 cells. These data indicated that our primary microplate-based screening assay was optimally designed for identification of potential inhibitors of processive KSHV DNA synthesis. Of note, the primary hit compounds selected from the Diversity Set included known DNA binders (NSC 5159 and NSC 321206) (Jorgenson et al., 1978; Uramoto et al., 1983; Rao and Lown, 1991; Barcelo et al., 2002), and compounds with structures similar to various classes of topoisomerase II inhibitors, such as anthracycline derivatives (NSC 82892, NSC 86005, and

NSC 143491), anthracene derivatives (NSC 146109 and NSC 339585), acridine-containing derivatives (NSC 130813 and NSC 156305), and ellipticine/ellipticinium derivatives (NSC 176327, NSC 311152, NSC 311153, and NSC 359449). Other topoisomerase II inhibitors included in the NCI Training Set, actinomycins (NSC 3053 and NSC 87222), anthracyclines (NSC 82151 and NSC 123127), and anthracenedione (NSC 301739), also demonstrated potent inhibitory activity in the current study (Fig. 1). Of these topoisomerase II inhibitors, ellipticines are generally recognized as potent DNA intercalators, while anthracyclines, anthracenes, and acridines (e.g., amsacrine) are known to possess mixed features of DNA intercalators and groove binders (Capranico and Binaschi, 1998). Because the current assay employs a primed DNA template for DNA synthesis, it is not surprising to observe various degrees of DNA synthesis inhibition by compounds that are expected to bind to DNA. When analyzing primary hit compounds selected from large chemical libraries, such presumed "non-specific" hits can be readily recognized by structure search (Flower, 1998; Roberts et al., 2000) and excluded from further characterization and development, unless they are a unique class of anti-cancer agents such as topoisomerase II inhibitors as discussed below.

Topoisomerase II inhibitors are important anti-cancer therapeutics widely used in clinic for a variety of cancers, including Kaposi's sarcoma (Gill et al., 1991; Hande, 1998; Newell et al., 1998; Evans et al., 2002). It is interesting to note that all of ellipticines and anthracycline analogs examined in the current study exhibited potent inhibitory activity against rPOL8/rPF8-mediated processive DNA synthesis in vitro. As expected, these compounds were highly toxic to KSHV-infected, actively proliferating lymphoma cell line, BCBL-1. Our data suggest that certain topoisomerase II-targeting agents may serve as dual inhibitors of human DNA topoisomerase II as well as KSHV DNA synthesis, and potentially play a critical role in the treatment of KSHV-induced malignancies. Antiviral activity of various topoisomerase II inhibitors, including DNA intercalators and catalytic inhibitors, has previously been reported against HSV-1, HSV-2, cytomegalovirus (CMV) and EBV (Nishiyama et al., 1987; Benson and Huang, 1988; Huang et al., 1992; Kawanishi, 1993; Hammarsten et al., 1996; Akanitapichat et al., 2000). It was initially speculated that host topoisomerase II was required for efficient herpesvirus DNA replication in infected cells and that the inhibition of the host enzyme activity hindered viral replication (Ebert et al., 1990; Hammarsten et al., 1996). However, it is possible that some topoisomerase II inhibitors may directly block processive DNA synthesis of various herpesviruses, as has been demonstrated in our study. Whether the inhibition of viral DNA synthesis by these agents is primarily mediated by their tight binding to DNA or involves the formation of the enzyme-drug-DNA complex, as seen in the inhibition of topoisomerase II (Burden and Osheroff, 1998), has yet to be determined. Further efforts to characterize physiological properties of the POL8/PF8/DNA/drug conformational relationship may provide new insights into the mode of processive DNA synthesis inhibition by the topoisomerase II inhibitors and may help develop more efficacious anti-KSHV agents.

In the current study, we found that one of the primary hit compounds, NSC 373989 (5-((3-(dimethylamino)propyl)amino)-3,10-dimethylpyrimido[4,5-b]quinoline-2,4(3*H*,10*H*)-dione), potently inhibited in vitro processive DNA synthesis mediated by KSHV rPOL8/rPF8 and significantly reduced lytic KSHV DNA replication in cells. For KSHV vDNA quantitation, the number of KSHV vDNA copies was standardized by the cell count of each sample in order to compare the level of virion particles released from an equal number of viable cells. The amounts of KSHV DNA were also evaluated in the equivalent quantity of Hirt LMW DNA as controlled by the amounts of mitochondrial DNA. Therefore, the observed decreases in KSHV virion production and KSHV DNA in the Hirt DNA most likely represented the inhibition of lytic KSHV replication by the compound. Antiviral properties of various quinoline or hydroxyquinoline (quinolone) derivatives have previously been demonstrated against various human viral pathogens, including HSV-1, HSV-2, CMV, varicella-zoster virus, and HIV-1 (Nasr et al., 1988; Althaus et al., 1993; McCormick et al., 1996; Albin et al., 1997; Baba et al., 1997; Wentland et al., 1997; Hagihara et al., 1999; Brideau et al., 2002; Oien et al., 2002; Parolin et al., 2003). Although the mechanism of antiviral action by the majority of these quinoline compounds is still undefined, one of the 4-oxo-dihydroquinoline analogs has recently been shown to exert broad anti-herpetic activity against HSV-1, HSV-2, CMV, VZV, EBV, and KSHV by inhibiting viral DNA polymerases (Thomsen et al., 2003). NSC 373989, a pyrimido-quinoline, does not possess a similar structure to any of the previously reported antiviral quinolines. The compound may primarily target KSHV POL8, PF8 and/or their cooperative interaction. Although this compound itself is not suited for therapeutic development because of the narrow therapeutic index, further studies are warranted to elucidate the mechanism of anti-KSHV activity exhibited by NSC 373989, and to define the key pharmacophores by structure activity relationship studies. In summary, our current study demonstrates that KSHV POL8/PF8 molecular targeting HTS is feasible and may lead to identification of novel non-nucleoside inhibitors of KSHV DNA synthesis.

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