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Susceptibility of *Autographa californica multiple nucleopolyhedrovirus* to inhibitors of DNA replication

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Abstract

The objectives of this study were to develop methods to evaluate the susceptibility of the type baculovirus AcMNPV to various antiviral compounds and to select potential inhibitors for investigating baculovirus DNA replication. In concert with the classical cytopathic effects (CPE) and cytotoxicity inhibition assays, two approaches, which could be amenable for high throughput application for evaluating several classes of known antiviral compounds were developed. (i) An indirect approach based on spectrofluorimetric analysis of EGFP expression in Sf21 cells infected with a recombinant AcMNPV (AcEGFP) and (ii) a direct DNA quantitative assay based on quantitative real time PCR (qPCR). Initial CPE results suggested that of 21 compounds tested, aphidicolin, abacavir, camptothecin, (*E*)-5-(2-bromovinyl)-2′-deoxyuridine (BVDU), L-mimosine, hydroxyurea and phosphonoacetic acid (PAA) were selective inhibitors of AcMNPV replication. Consistent with the CPE results, the EGFP fluorescence and the qPCR of viral DNA accumulation exhibited a dose dependent depression of EGFP expression and DNA accumulation, respectively, in infected cells exposed to them. The inhibitory effects of aphidicolin, abacavir, L-mimosine and hydroxyurea on AcMNPV DNA replication were reversible. Taken together, both spectrofluorimetric and qPCR assays are suitable and rapid quantitative approaches to investigate inhibitors of baculovirus DNA replication in infected cells.

Keywords: Baculovirus; DNA replication; Inhibitors; Screening assays

1. Introduction

The *Autographa californica multiple nucleopolyhedrovirus* (AcMNPV) is the type species of the family *Baculoviridae* characterized by a large circular duplex DNA genome of approximately 134 kb with a coding capacity of 154 proteins (Ayres et al., 1994). The baculoviruses are also distinguished by having a biphasic life cycle in which budded enveloped rod shaped virions are produced early in infection and later enveloped virions become occluded in large proteinic paracrystalline structures known as polyhedral inclusion bodies (PIBs), which are easily visualized by phase contrast microscopy (Blissard and Rohrmann, 1990). Viral DNA replication is a central component of baculovirus replication and its onset temporally separates early from late gene transcription. Like other biological entities, synergistic action among *cis*-acting elements and *trans*-acting factors is required to ensure fidelity of baculovirus DNA replication. Currently, knowledge of the molecular mechanisms involved in the initiation of baculovirus DNA replication and the conformation of the replication intermediates is still limited. Homologous repeat sequences (*hrs*), which are dispersed throughout the baculovirus genome, non-*hr* and early promoter regions have been implicated as potential *cis*-acting elements involved in the origin of baculovirus DNA replication (reviewed in Okano et al., 2006). In addition, previous studies using transient replication assays (Kool et al., 1994; Lu and Miller, 1995) and deletion analysis (Vanarsdall et al., 2005) have demonstrated that AcMNPV encodes six *trans*-acting factors viz., immediate early protein 1 (IE-1), helicase (P143), primase (LEF-1), primase accessory factor (LEF-2), DNA polymerase, and a single-stranded DNA binding protein (LEF-3) essential for DNA replication. In addition to their use in studying viral DNA replication, inhibitors of baculovirus replication could be applied to the protection against baculovirus infection in economically important insects such as the silkworms. For example, (*E*)-5-(2-bromovinyl)-2′-deoxyuridine (BVDU) reduced baculovirus infection in cell culture as well

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as baculovirus pathogenesis in economically useful insects or larvae reared for mass production of biopesticides (Kelly and Lescott, 1976; Kelly, 1984). Inhibitors of viral DNA synthesis are not only important as chemotherapeutic agents for combating viral diseases, but also as potential molecular probes for elucidating the components of the DNA replisome. Nucleotide and nucleoside analogues specific to DNA polymerases have been used to characterize the DNA polymerases involved in herpesvirus replication (Gonczol and Plotkin, 1985). In adenovirus replication, DNA polymerase α was implicated in the elongation of DNA based on preferential inhibition of DNA replication by aphidicolin (Ariga, 1983). Numerous studies have shown that camptothecin and antineoplastic compounds, which specifically target topoisomerase I and II, respectively could be used to delineate the functions of topoisomerases in DNA replication of adenovirus, simian virus 40 (SV 40) and herpesviruses (Elbert et al., 1990; Schaack et al., 1989; Tsui et al., 1989). Much information on the origins of viral DNA replication has been gathered by employing inhibitors that target the initiation of DNA synthesis. Dinter-Gottlieb and Kaufmann (1982) determined the priming activity of the SV40 replicon by synchronizing DNA replication at the origin through aphidicolin treatment and temperature shifts. Short replicons of human cytomegalovirus (HCMV) were also shown to accumulate in the presence of a nucleotide chain terminator, ganciclovir and were used to map the lytic origin of HCMV (Hamzeh et al., 1990). The antiviral activities of several compounds have generated some insight on the temporal cascade of baculovirus gene expression. For example, Rice and Miller (1986) demonstrated that in the presence of the protein synthesis inhibitor cycloheximide and the DNA replication inhibitor aphidicolin, early genes could be expressed, while the expression of late genes was abrogated. In a separate study, BVDU was shown to inhibit *Trichoplusia ni* MNPV DNA replication as well as synthesis of late virus-specific proteins (Wang et al., 1983). To date, there is only limited information on antibaculovirus compounds, particularly those that act at the initiation of DNA replication. The effectiveness of inhibitors of baculovirus replication could be assessed by virus production after treatment. However this approach is laborious and if virus replication is affected, the method does not discriminate between inhibition before or after viral DNA replication.

The aim of this study was to identify antiviral compounds, which act at the level of viral DNA replication for use in DNA replication studies. For that, two new rapid and sensitive approaches based on a spectrofluorimetric analysis of EGFP expressed by a recombinant AcEGFP virus and quantitative PCR (qPCR) were developed to select useful targets of AcMNPV DNA replication and assess their potential as reagents for investigating the molecular mechanisms of baculovirus DNA replication.

2. Materials and methods

2.1. Cells and viruses

Spodoptera frugiperda clonal isolate 21 (Sf21) cell line was used in all experiments. The cells were cultured in spinner flasks containing Grace's insect medium supplemented with 10% fetal calf serum. The spinner cultures were maintained at 28 °C under constant stirring on a low profile multi-stir 4-position magnetic stirrer (Bellco Biotechnology, Bellco glass, Inc. Vineland, NJ, USA). Parental AcMNPV virus and a recombinant AcMNPV (AcEGFP) harbouring an enhanced green fluorescent protein (EGFP) gene driven by a polyhedrin promoter were used to infect Sf21 cells. The recombinant AcEGFP virus, kindly provided by Jeff Hodgson (Hodgson et al., 2007), was originally generated by inserting an EGFP cassette under the control of a polyhedrin (ph) promoter at the overlapping and divergent promoter loci of the AcMNPV *cathepsin* and *chitinase* genes.

2.2. Antiviral compounds

A total of 21 compounds were evaluated for their effect on AcMNPV replication. The test compounds aphidicolin (APC), (*E*)-5-(2-bromovinyl)-2′-deoxyuridine (BVDU), camptothecin (CPT), actinomycin-D, hydroxyurea (HU), phosphonoacetic acid (PAA), L-mimosine (L-MS), ciclopirox olamine (CPX), cytosine 1-β-D-arabinofuranoside (Ara-C), 2-aminothiazole, novobiocin, amsacrine and etoposide were purchased from Sigma-Aldrich (Canada). BAY 1257, was provided by Gerald Kleymann (Bayer, Germany) and nucleotide reverse transcriptase inhibitors abacavir (ABC), didanosine (ddI), ementricitabine, (FTC), lamivudine (3TC), stavudine (d4T), tenofovir (TEN) and zidovudine (AZT) were obtained from the NIH AIDS Research and Reference Reagent Program. Stock solutions were prepared by dissolving each compound at a known concentration in their recommended solvents as depicted in Table 1. Solubilized stock solutions were aliquoted into small amounts and stored at −20 °C for future use. All the working dilutions for each inhibitor were prepared in Grace's insect medium to the desired concentrations.

2.3. Cytopathic effect (CPE) inhibition assay

The ability of different compounds to inhibit AcMNPV replication was initially determined by following inhibition of AcMNPV-induced CPE in Sf21 cells. Briefly, 0.5 ml of Sf21 cells from spinner cultures were seeded into 24-well plates at a concentration of 1×10^6 cells/ml and allowed to attach for 1 h at 28 °C. Attached cells were infected with AcMNPV and AcEGFP virus at a multiplicity of infection (MOI) of 10, rocked gently for 10 min and incubated at 28 °C. After 1 h, the medium containing unadsorbed virus was aspirated, and the wells were replenished with fresh medium containing varying concentrations of inhibitors (500 μl per well). Control cells received a similar treatment but were not infected while mock-infected cells were not exposed to the antiviral compounds. To assess the inhibitory effects at different dilutions of each inhibitor, virus-induced CPE and production of polyhedra were initially examined by scoring CPE in wells of both untreated infected cells and treated infected cells using an inverted light microscope (Zeiss, Telaval 31). At 48 h post-infection, a time when AcMNPV infected cells exhibited highest CPE, cells were resuspended at a concentration of about 5×10^5 cells/well. For each