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A novel drug screening assay for papillomavirus specific antiviral activity

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Abstract

Discovery and development of human papillomavirus (HPV) specific antiviral agents have been hampered by the lack of an in vitro assay permissive to HPV replication. An experimental assay system for monitoring HPV-11 DNA replication has been optimized for use as a papillomavirus antiviral drug screening tool. Cloned HPV DNA was introduced into SCC-4 cells by electroporation and viral DNA replication monitored by Southern blot. Kinetic studies demonstrated an increased HPV genome copy number with time. Viral DNA replicated as episomal, unit length genome and remained episomal after multiple passages. These data suggested the basis for an in vitro replication assay for evaluating the antiviral activity of potential chemotherapeutic agents directly on HPV. This model was used to investigate antiviral activities of current anti-HPV therapies such as 5-fluorouracil (5-FU) and alpha-interferon (α-IFN) and potential therapies such as sodium butyrate, 5-bromo-2O-deoxyuridine (BrdU) and antisense oligonucleotides. HPV-11 replication is significantly inhibited by BrdU and sodium butyrate; however 5-FU and α-IFN did not give consistent dose response results. Finally, ISIS 2105, a 20-mer phosphorothioate antisense oligonucleotide, which targets HPV-11 E2 gene product, showed potent antiviral activity in this assay with an IC_{50} of ≈ 70 nM.

Keywords: Human papilloma virus type 11; DNA replication assay; Antiviral drug screening assay

1. Introduction

Currently, more than 70 HPV genotypes are recognized (Howley, 1996). Infection with certain HPV types is associated with epithelial neoplasms ranging from the benign common warts to invasive carcinoma of the cervix. However, it is infection of the anogenital region that is most

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significant. The importance of studying genital HPV infection is highlighted by its sexual mode of transmission, near epidemic proportions (Beutner et al., 1991), clear association of certain HPV types with the development of cervical dysplasia and progression to cervical cancer (Schiffman et al., 1993). Because of its sexual mode of transmission, genital HPV infection is particularly prevalent among the young sexually active population. In one study, 46% of women attending a university health center for routine gynecological examination were genital HPV DNA positive (Bauer et al., 1993). The magnitude of the problem is reinforced by the 1990 CDC estimate of 800 000 new cases of HPV infection diagnosed annually in the US (Krus and Stone, 1990).

Despite the prevalence of genital HPV infection and its association with malignant disease there is no effective antiviral therapy for HPV infection (Cowsert, 1994). The goal of current therapeutic strategies is wart removal, elimination of signs and symptoms, but not eradication of viral DNA. While many genital warts can be eliminated by such treatments as cryotherapy, electrodesiccation, CO_2 laser or surgical excision, recurrence rates are generally unacceptable. Recurrence is likely due to subclinical virus infection of adjacent tissue.

Chemotherapeutic approaches to control HPV infection have suffered from a lack of specificity. This may be due partly to the lack of traditional antiviral targets in papillomaviruses. For example, papillomaviruses do not encode a viral DNA polymerase and therefore rely solely upon cellular machinery for DNA replication. The dependence on cellular pathways for critical functions may explain, in part, the narrow host range and tissue tropism displayed by papillomaviruses. The tight association with cellular pathways, lack of traditional antiviral targets and absence of suitable in vitro assay systems have hampered efforts to identify chemotherapeutic agents with appropriate selectivity. These factors suggest that drugs with novel targets and mechanisms of action may be required to successfully treat papillomavirus infection.

Molecular studies have identified the products of the E1 and E2 open reading frames (ORFs) of papillomaviruses as essential for viral DNA replication (Ustav et al., 1991; Ustav and Stenlund, 1991; Yang et al., 1991; Chiang et al., 1992a,b). These studies were carried out by providing E1 and E2 in trans and observing replication of reporter plasmids containing an HPV origin of replication (ori) either in cell lines or in vitro. While these models provide important assay systems for characterization of the intermolecular interactions required for viral DNA replication, they do not address replication in the context of the entire viral genome. Furthermore, these systems are not able to identify compounds that do not act directly on E1 or E2 proteins or interfere with their interaction with the ori or other, as yet unidentified, targets.

In addition to its direct role in viral DNA replication, E2 is also an essential viral transcription factor. Both transactivation and replication functions reside in the same amino-terminal domain of E2, however, they are separate and distinct functions (Winokur and McBride, 1992). Much of the initial characterization of E2 has been carried out in the bovine papillomavirus type-1 (BPV-1) system and later extended to the HPVs (Ham et al., 1991; McBride et al., 1991). E2 functions by binding to a 12 base pair DNA palindrome, $ACCN_6GGT$, through a DNA binding domain located near the carboxyl-terminus (Androphy et al., 1987). In BPV-1, three E2 polypeptides are transcribed from the E2 open reading frame. The full length E2 protein contains the amino-terminal transactivation domain and the carboxyl-terminal dimerization and DNA binding domains. This protein functions primarily as a transcriptional transactivator. Two truncated versions of E2 are produced by either alternative splicing or internal promotion. These proteins lack the amino-terminal transactivation domain but share the carboxyl-terminal DNA binding domain and therefore function as E2 transrepressors (Lambert et al., 1987). In the BPV-1 system, truncated E2 is not able to support replication (Ustav and Stenlund, 1991; Yang et al., 1991).

In HPV, both full-length and truncated versions of E2 are also produced and have been shown to

function in a manner similar to BPV-1 (Chiang et al., 1992a,b). However, it has also been shown that full length E2 can function as a transrepressor depending upon the proximity of its DNA binding site to the promoter (Romanczuk et al., 1990). E2 has been shown to repress the P_{97} promoter of HPV-16 and the P_{105} promoter of HPV-18 (Romanczuk et al., 1990). These promoters regulate the expression the oncoproteins of high risk HPVs, E6 and E7. It is hypothesized that disruption of E2 repression, by integration of the viral genome, is responsible for malignant progression of cervical lesions containing high risk HPV types.

Autonomous replication of intact HPV-6, HPV-11 and HPV-18 has already been reported (Del Vecchio et al., 1992; Mungal et al., 1992; Shadan et al., 1994). In this study, this system has been adapted and optimized to monitor HPV DNA replication in the presence of putative antiviral agents. Using this assay system, 5-FU did not have any specific effect on viral replication. α-IFN failed to give a consistent dose dependent response curve over a wide range of drug concentrations. Sodium butyrate and BrdU were found to be potent inhibitors of replication, while ISIS 2105 was shown to be a better inhibitor with an IC_{50} of ≈ 70 nM.

2. Materials and methods

2.1. Cell culture

SCC-4 cells, derived from a squamous cell carcinoma of the tongue, were obtained from the ATCC (Rockville, MD) and were grown and maintained at 37°C in the presence of 5% CO_2 in a basal medium consisting of 1:1 DMEM/F-12 supplemented with 10% heat-inactivated fetal bovine serum (FBS). Post-electroporation medium consisted of DMEM/F-12 (1:1), 10% FBS, 500 units/ml penicillin, 500 μg/ml streptomycin and 0.4 μg/ml hydrocortisone (Sigma). All replication and chemotherapeutic studies were conducted in post-electroporation medium.

2.2. DNA preparation

HPV-11 DNA (cloned in pUC19) (Cowsert et al., 1993), pUC19 (Life Technologies, Gaithersburg, MD) and pSV2neo (Clontech, Palo Alto, CA) DNAs were prepared using standard amplification and purification techniques. Prior to electroporation, HPV-11 DNA was digested with *Bam*HI, releasing the viral DNA from its cloning vector. pUC19 DNA was linearized by digestion with *Bam*HI. pSV2neo was linearized by digestion with *Pvu*I. Digested DNAs were purified by phenol/chloroform extraction, precipitated in ethanol and concentration determined by spectrophotometry. Stock DNA solutions, of 250 μg/ml, were prepared in water and stored frozen until use. All DNAs were visualized on ethidium bromide stained agarose gel to insure proper digestion.

2.3. Transient replication assays

SCC-4 cells were trypsinized, counted and diluted to 1.2×10^7 cells/ml in DMEM/F12 supplemented to 10% FBS. Cells (250 μl) and DNA (20 μl of 250 μg/ml) were gently mixed prior to electroporation. The mixture was transferred to a 2 mm electroporation cuvette and exposed to a 140 V/1600 μF pulse (BTX, San Diego, CA). After electroporation, cells were diluted in post-electroporation medium to a concentration of 1.6×10^6 cells/ml and 3 ml of this dilution were plated per well in 6-well plates. After 24 h, these cells were treated with drug for 4 h. After the treatment period, drug containing medium was aspirated and replaced with post-electroporation medium.

At either 72 or 96 h post-electroporation, low molecular weight DNA was prepared and analyzed for replicated HPV genomes (Hirt, 1967; Ustav et al., 1991). Cell monolayers were washed once with PBS, after which 0.5 ml of lysis buffer (10 mM Tris pH 8.0, 1 mM EDTA, 0.5% SDS) was added to each well. Cell lysates were collected in 1.5 ml microcentrifuge tubes, adjusted to 1.5 M NaCl, mixed gently and refrigerated for at least 4 h. Samples were centrifuged at $10\,000 \times g$ for 15 min, supernatants collected and extracted twice with phenol/chloroform and once with chloro-

form, then precipitated in ethanol with 0.2 M NaCl. DNA samples were resuspended and digested overnight using 60 U *Dpn*I and 40 U *Hin*dIII. *Dpn*I is a methylation dependent enzyme that digests only methylated and therefore nonreplicated DNA (Peden et al., 1980). *Hin*dIII has a single recognition site in HPV-11 DNA. Samples were precipitated in ethanol, resuspended in 30 μl of water, electrophoresed through a 0.7% agarose gel, transferred to Amersham Hybond-N + in 1 M ammonium acetate and crosslinked using the AutoLink setting on a Stratagene Stratalinker. Blots were rinsed in $2 \times$ SSC and pre-hybridized for 10 min at 68°C in QuikHyb (Stratagene, San Diego, CA). High specific activity (1×10^9 cpm/μg) random primed probe was prepared using gel purified unit-length HPV-11, linearized pUC19, or a commercially available mix of *Hin*dIII digested lambda and *Hae*III digested ϕX174 DNAs. Target probe and molecular weight marker probe were added to the hybridization reactions at 1×10^6 and 1×10^5 cpm/ml, respectively. Hybridization was continued for an additional 18–20 h. After hybridization, membranes were washed in $0.1 \times$ SSC, 0.1% SDS at 60°C for 30 min. The amount of radioactivity associated to every band was quantitated on the PhosphorImager (Molecular Dynamics, Sunnyvale, CA) and expressed in PhosphoImager units. No cross-hybridization between HPV-11, molecular weight probe or pUC19 was detected.

2.4. Antisense oligonucleotides and antiviral compounds

ISIS 2105 is a 20-mer phosphorothioate antisense oligonucleotide designed to hybridize to the initiation of translation region of HPV-6 and HPV-11 E2 (5O-TTGCTTCCAT CTTCCTCGTC-3O) (Cowsert et al., 1993). ISIS 13564 (5O-TTACTCCCGTCGTCTTCTTC-3O) is a mismatched control for 2105 and ISIS 3925 (5O-AGCAAAAGCAGGGGAAAATA-3O) is a 20-residue phosphorothioate oligonucleotide designed to hybridize to influenza A virus. They have no homology to HPV and are used as a negative control.

Twenty-four hours after electroporation, cells were treated with one of the oligonucleotides in serum free medium containing Lipofectin (Gibco/BRL, Gaithersburg, MD) at 10 μg/ml for 4 h. After treatment, drug-containing medium was aspirated and replaced with post-electroporation medium.

Acyclovir, α-INF, BrdU, ribavirin, sodium butyrate and 5-FU were added directly to the growth medium 24 h after electroporation.

3. Results

3.1. Kinetics of HPV-11 DNA replication

SCC-4 cells have previously been shown to support transient replication of HPV DNA (Del Vecchio et al., 1992; Shadan et al., 1994). This system has been modified and adapted to provide a quantitative assay for HPV-11 viral DNA replication.

Replicating *Dpn*I resistant, unit length, HPV-11 DNA is first detected at 24 h and accumulates with time after electroporation (Fig. 1A). *Dpn*I sensitive viral DNA is gradually lost over the course of the experiment (Fig. 1A). Quantitation of the *Dpn*I resistant viral DNA band confirmed the accumulation of HPV-11 DNA (Fig. 1B). The accumulation of *Dpn*I resistant HPV-11 DNA is the result of replication and not simply demethylation of input DNA. This was supported by the observations that neither viral DNA, that has not been cleaved from its vector (Fig. 2), nor pUC19 DNA (data not shown) resulted in accumulation of *Dpn*I resistant DNAs. These results show that HPV-11 DNA is capable of autonomously replicating in SCC-4 cells and that replication is dependent upon an intact viral genome.

3.2. HPV-11 DNA remains episomal in transient and long term cultures

To characterize the state of the viral genome in both transient and long term cultures, Hirt DNAs were prepared and analyzed by a panel of restric-

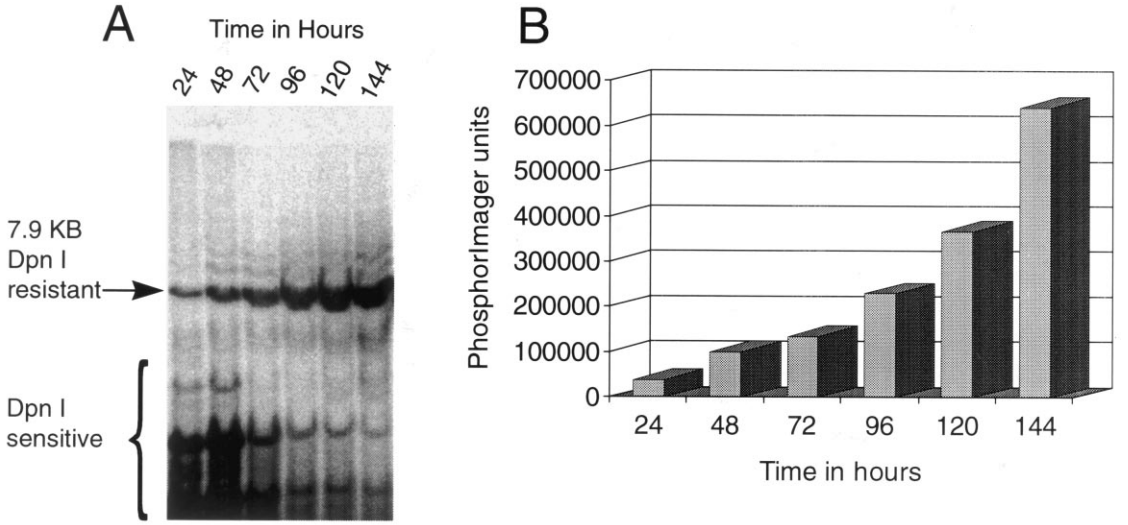

Fig. 1. Kinetics of HPV-11 DNA replication. HPV-11 DNA was cleaved from its cloning vector and electroporated into SCC-4 cells. At various time points Hirt DNA was prepared and digested with *Dpn*I and *Hin*dIII. DNA was analyzed by Southern blot (A) and the 7.9 kb *Dpn*I resistant band quantitated by PhosphorImager (B).

tion endonucleases (Fig. 3). Digestion of Hirt DNA with *Xho*I, which has no recognition sequence in HPV-11, resulted in a single band with a mobility consistent with supercoiled DNA. Digestion with *Hin*dIII, which has a single recognition site, resulted in only one band with a molecular weight of ≈ 7.9 kilobases, consistent with full-length HPV-11 DNA. Analysis with three enzymes with multiple recognition sites gave results consistent with full-length episomal viral DNA with no detectable rearrangements or deletions.

These results demonstrate that upon electroporation HPV-11 DNA establishes itself as an autonomously replicating plasmid, achieves a steady-state level of plasmid and can persist over several cell passages without selection.

In addition, 6 months after HPV-11 and pSVneo electroporation in SCC-4 cells, analysis of G418-selected clones indicated that the viral DNA remained episomal. Some of the cell lines maintained HPV-11 at high copy number (30–40 copies per cell), while other clones maintained HPV-11 at extremely low copy number (1–2 copies per cell) (data not shown).

3.3. Inhibition of HPV-11 viral DNA replication by antiviral agents

5-FU and α-INF have been used as treatments for papillomavirus infection with mixed results (Cowsert, 1994). These compounds were evaluated for antiviral activity using the transient replication assay. 5-FU failed to inhibit HPV replication in a dose-dependent manner (Fig. 4A) with the only inhibitory dose simultaneously inducing toxicity, as measured by tripan blue exclusion. The increase of viral DNA replication at 100 mM was characteristic of 5-FU in all assays. α-INF induced a general reduction in HPV replication, however results from different assays were not consistent: we measured different percent inhibition of DNA replication (35–70% control at the lower concentration) and obtained different inhibition curve profiles (Fig. 4C). Acyclovir and ribavirin failed to inhibit viral DNA replication at subtoxic doses (Fig. 4B and 4D).

Both sodium butyrate and BrdU were found to have significant antiviral activity against HPV-11 (Fig. 5A and 5B). Sodium butyrate was a potent inhibitor of HPV-11 replication at subtoxic doses with an approximate IC_{50} of 2 mM (Fig. 5A).

BrdU had potent antiviral activity in this assay with an approximate IC_{50} of 10 μM. These results are representative of at least three similar studies.

3.4. Inhibition of HPV-11 DNA replication by antisense oligonucleotides

ISIS 2105, a 20 residue phosphorothioate oligonucleotide, is complementary to the initiation of translation codon for the E2 open reading frame (Cowsert et al., 1993). The sequence of this region is conserved between HPV-6 and HPV-11. In transfection assays ISIS 2105 inhibits HPV-11 E2 transactivation in a sequence dependent and dose dependent-manner (Cowsert et al., 1993). In replication assays ISIS 2105 inhibited replication of HPV-11 viral DNA in a dose-dependent and sequence-dependent manner (Fig. 6). The apparent IC_{50} of ISIS 2105 in this assay is 70 nM.

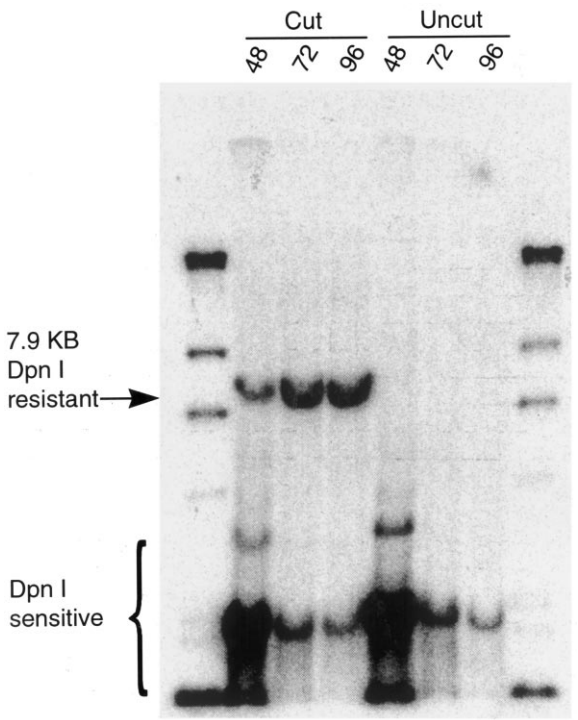

Fig. 2. Replication of HPV-11 DNA requires an intact genome. SCC-4 cells were electroporated with HPV-11 DNA, either released from vector sequences (cut) or containing vector sequences (uncut). Hirt DNA was prepared at 96 h post-electroporation and analyzed for HPV DNA.

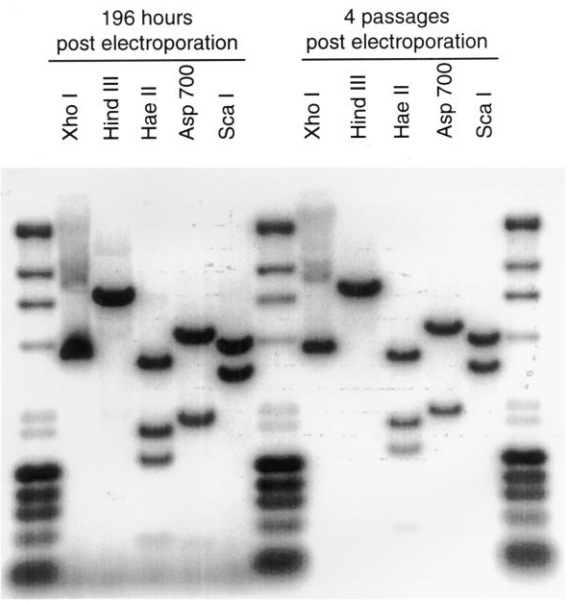

Fig. 3. Analysis of HPV DNA in transient and stable transfection experiments. Hirt DNA from either transient or stable transfection experiments were digested with a panel of restriction endonucleases and analyzed by Southern blot using HPV specific probes.

ISIS 3925, a 20 residue phosphorothioate oligonucleotide designed to be complementary to influenza A virus, has no significant homology to HPV-6 and -11. As expected, ISIS 3925 failed to inhibit HPV-11 DNA replication in this assay (Fig. 6).

In addition, ISIS 13564, a mismatched control oligo for ISIS 2105, did not have any effect on HPV-11 replication up to the concentration of 100 nM (not shown).

4. Discussion

In benign papillomavirus-induced disease, the viral genome is maintained as a low-copy episome in the nucleus of basal cells of infected epithelia. As infected cells differentiate, viral DNA copy number increases, late genes are expressed, viral DNA is encapsidated and infectious virions are released into the environment. It is thought that infection is maintained by the continued presence

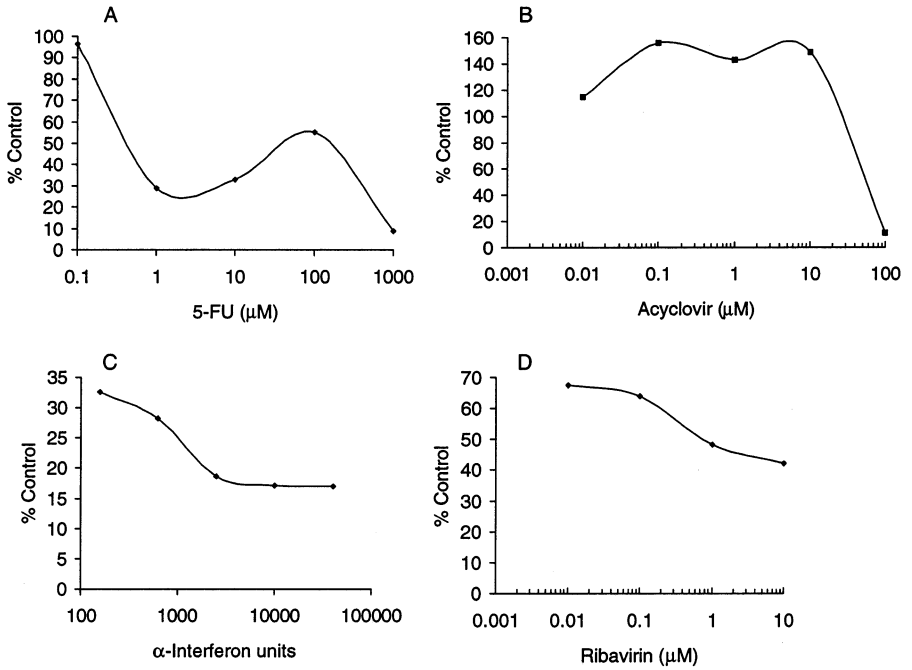

Fig. 4. Effects of select antiviral agents on HPV DNA replication. SCC-4 cells were electroporated with HPV-11 DNA. Twenty-four hours after electroporation, cells were treated with individual antiviral agents for 4 h. The drug was aspirated and replaced with standard growth medium. Cells were harvested 72 h post-electroporation and analyzed for replicated HPV DNA. (A) 5-fluoro uracil; (B) Acyclovir; (C) α-interferon; (D) Ribavirin. Every data point was obtained from a single sample and is plotted as percent untreated control. All the experiments were repeated at least three times.

of low-copy viral DNA in the basal cells rather than reinfection. Thus inhibition of viral DNA replication may provide the opportunity for viral clearance from the epithelium.

One of the major hindrances to the identification and development of papillomavirus-specific antiviral compounds has been the lack of suitable in vitro models for testing compounds for antiviral activity. The results reported here show that HPV-11 can autonomously replicate in SCC-4 cells, that replication can be measured and quantitated and that this assay can be used to screen compounds for the ability to inhibit papillomavirus DNA replication.

An important feature of any antiviral screen is the ability to predict in vivo activity from in vitro results. Currently there are no proven antiviral therapeutics available for the treatment of papillomavirus infection. Therefore, it is not yet possible to determine the true predictability of this assay. This may be approached experimentally by taking active compounds identified in this assay and evaluating them in the HPV-11 human xenograft severe combined immunodeficiency (SCID) mouse model (Bonnez et al., 1993). However, the true predictability of this assay will only be determined when compounds are tested in clinical trials.

The utility of this assay is demonstrated by comparing the antiviral activity of ISIS 2105 to the activity of different drugs. ISIS 2105 was initially identified as an antisense oligonucleotide capable of sequence-specific, dose-dependent inhibition of HPV-11 E2 expression as measured by E2 transactivation assays (Cowsert et al., 1993). Because E2 is obligatory for viral replication it was hypothesized that ISIS 2105 would exhibit antiviral activity. However, because of the sequence-dependence of this antisense oligonucleotide and the sequence divergence of papillomaviruses, it was not possible to evaluate ISIS 2105 in papillomavirus animal models such the cotton tailed rabbit model or the bovine papil-

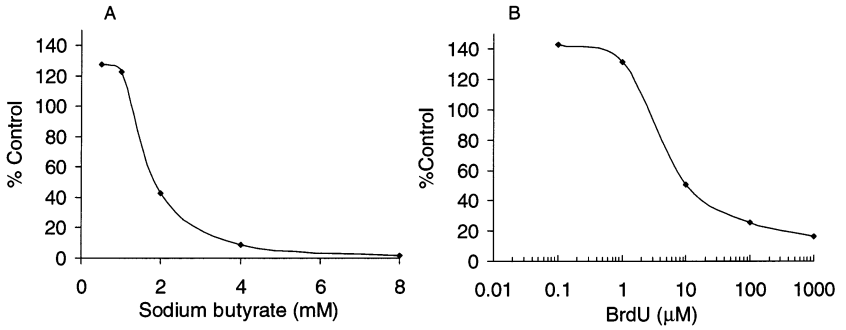

Fig. 5. Effect of sodium butyrate and 5′-bromo-2′-deoxyuridine on HPV-11 replication. SCC-4 cells were electroporated with HPV-11 DNA. Twenty-four hours after electroporation, cells were treated with sodium butyrate or BrdU for 4 h. The drug was aspirated and replaced with standard growth medium. Cells were harvested 72 h post-electroporation and analyzed for replicated HPV-11 DNA. Every data point was obtained from a single sample and is plotted as percent untreated control. All the experiments were repeated at least three times.

lomavirus model. Activity in this in vitro model demonstrates the antiviral activity of ISIS 2105.

In previous studies, antisense oligonucleotides were identified that inhibited bovine papillomavirus type-1 (BPV-1) E2 transactivation

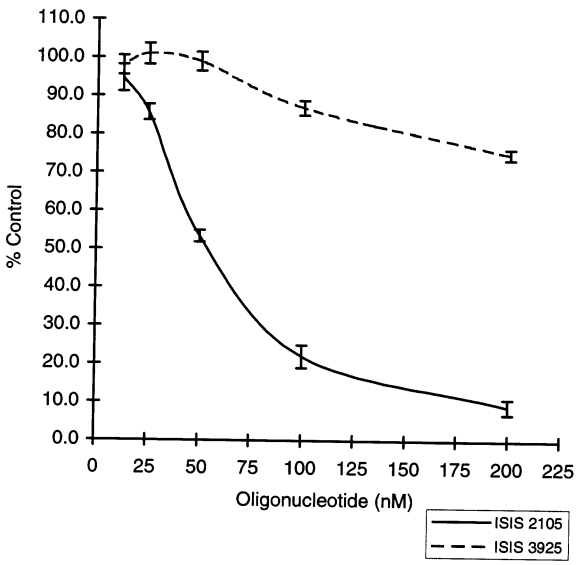

Fig. 6. Effect of ISIS 2105 and ISIS 3925 on HPV DNA replication. SCC-4 cells were electroporated with HPV-11 DNA. Twenty-four hours after electroporation, cells were treated with increasing doses of either ISIS 2105 (——) or ISIS 3925 (- - -). Cells were harvested 72 h post-electroporation and analyzed for HPV DNA. Data are plotted as percent untreated control. Error bars rapresent the avarage between three different experiments.

(Cowsert et al., 1993). These compounds were also shown to inhibit infection as measured by the BPV-1 focus forming assay. The BPV-1 E2 data and the antiviral activity of ISIS 2105 in this assay provide a strong rationale for targeting E2 of low risk HPV types as an antiviral strategy. However, because E2 of high risk HPV types such as HPV-16 and HPV-18 has been shown to function as a repressor of E6 and E7 expression, there is no rationale for targeting E2 of these viruses in antiviral strategies (Romanczuk et al., 1990).

α-INF and 5-FU have been used in the clinic for treatment of papillomavirus-associated disease. Both have demonstrated only marginal efficacy. Based on the shallow dose responses, we conclude that both failed to show convincing direct effects in the replication assay. These data suggest that activity observed in vivo may not be due to antiviral activity but rather due to indirect mechanisms such as antiproliferative activity, local toxicity, or immunostimulation. Other antiviral compounds, such as ribavirin and acyclovir, also failed to show convincing activity in this assay. Since these compounds are either activated by or inhibit viral encoded enzymes, not encoded by papillomaviruses, these results are not unexpected.

In previous studies it has been shown that preparations of mouse L-cell interferon induced morphological reversion and loss of BPV-1 DNA from BPV-1 transformed mouse C127 cells (Turek

et al., 1982). We were not able to reproduce these results using the HPV replication assay. The discrepancy may lie in the interferon preparations, cell lines used or viruses. The data generated using the HPV replication assay are, however, consistent with clinical observations.

The cell culture assay system described here represents the first in vitro assay system available to screen compounds for HPV antiviral activity. When tested in this assay system, ISIS 2105, an antisense inhibitor of expression of E2, an obligatory viral replication gene, was found to be a potent inhibitor of viral DNA replication. Other compounds, such as 5-FU and α-INF, that have shown marginal activity in the clinic, show no selective antiviral activity in this assay.

The influence of 2105 on the transcription of E1, E2, E6 and E7, in stable and transient transfected SCC-4 cells, is under investigation.

Acknowledgements

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