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# Therapeutic evaluation of compounds in the SCID-RA papillomavirus model

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**Abstract**

A previous study by Kreider (Kreider et al., 1979) indicated that rabbit skin, which had been transplanted to immunodeficient nude mice, could be successfully infected with cottontail rabbit papillomavirus (CRPV). We have extended this observation in developing a rodent model for evaluation of compounds for activity against the papillomaviruses. In this model (called the SCID-Ra model), rabbit ear skin is transplanted to the dorsum of SCID mice and allowed to heal for 3 weeks. Infection with CRPV by scarification leads to the growth of warty lesions within 2–3 weeks in > 95% of the animals. Topical and/or systemic therapy can be initiated at various times post infection (PI). Weekly lesion scores are recorded and compounds are evaluated for their ability to suppress wart growth when compared to untreated control mice. Ribavirin, which has had a suppressive effect both in the clinic for the treatment of respiratory papillomatosis and on the growth of warts in the rabbit back model, was evaluated and showed significant anti-proliferative activity with oral dosing. Both antiviral and antiproliferative compounds including podophyllin and 5-fluorouracil, which have been used clinically for the treatment of human papillomavirus (HPV) infections, were evaluated in this model. The anti-mitotic compound, Navelbine™ (vinorelbine tartrate), which is used for the treatment of non-small cell lung carcinoma was evaluated in this system and showed significant inhibition of wart growth with somewhat less topical cytotoxicity when compared to podophyllotoxin. 

*Keywords:* Papillomavirus; Cottontail rabbit papillomavirus; SCID; Animal models; Antivirals

## 1. Introduction

The most common and familiar result of HPV infection is the development of plantar and palmar warts on the hands and feet. Cutaneous warts

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caused by HPV types 1–4 are virtually ubiquitous in the population although the incidence is somewhat higher in children and young adults. Benign cutaneous warts can persist or grow slowly for many months or years, and then abruptly disappear without apparent cause or deliberate therapy. In addition to cutaneous disease, a distinct group of HPVs infects mucosal epithelium causing benign oral or genital warts (Gissmann and zur Hausen, 1980; Gissmann et al., 1983; De Villiers, 1994) and a subgroup of these viruses has been strongly associated with genital cancer (zur Hausen and Schneider, 1987; Koutsky et al., 1988; zur Hausen, 1994; Howley, 1996).

Although HPV infection is widespread in the population and associated with a wide spectrum of human disease ranging from benign warts to invasive, metastatic and lethal cancer, there is currently no viral-specific drug therapy available. Standard treatments rely primarily on the excision or destruction of infected tissue through controlled physical or chemical ablation. The search for a specific antiviral therapy for PV infections has been severely hampered by the lack of a productive tissue culture system which can be used as a primary screen for potential antiviral inhibitors (Phelps and Alexander, 1995). The life cycle of the PVs is closely associated with the process of keratinocyte differentiation in infected epithelium. Efforts to reproduce vertical epidermal differentiation in raft culture systems has met with some success (Laimins, 1996); however, such systems remain technically difficult and thus far, only limited viral particle production has been seen.

The lack of a productive cell-based system has placed added importance on the use of animal models for the study of the PVs and for the evaluation of potential antiviral therapies. The classical animal models for the study of the PVs have been the cottontail rabbit papillomavirus (CRPV), the bovine (BPV) system and canine oral PV (COPV). CRPV infection and its neoplastic potential was first described more than 50 years ago (Shope, 1933) and since then, has been the favored model for the study of the genetic and immunological factors associated with infection and progression to carcinoma (Wettstein, 1987). CRPV induces lesions on the skin of wild cottontail rabbits and is enzootic in certain geographically restricted locations in the US. Laboratory infection of domestic rabbits with CRPV will induce benign cutaneous lesions similar to those found in wild rabbits. CRPV infection of domestic rabbits has been used to evaluate the efficacy of several anti-PV therapies including ribavirin (Ostrow et al., 1992), retinoids (McMichael, 1965; Ito, 1981) and PMEG (Kreider et al., 1990a).

A number of conventional animal models of human viral diseases have been developed based on direct in vivo infection of rodent tissues with arenaviruses, togaviruses, reoviruses, poxviruses, rhabdoviruses, orthomyxoviruses, and herpesviruses (Field, 1988). Unfortunately in this regard, the HPVs are very host specific and thus far, have been shown to productively infect only human epithelial cells. Due to their impaired immunological functions, immunodeficient mice are generally able to accept most grafted, xenogeneic tissues without subsequent immune-mediated rejection. This property has been particularly useful in the establishment of animal models for the study of infectious diseases where an appropriate model cannot be readily derived through direct inoculation of mouse tissues (Hendrickson, 1993). Infectious HPV11 virions have been produced in this way through implantation of HPV11 infected human foreskin tissue under the renal capsule of nude or SCID mice (Kreider et al., 1985; Bonnez et al., 1993). This model has been valuable both to study the molecular events associated with infection (Stoler et al., 1990) and as a renewable source of virus for HPV11 (Kreider et al., 1987), HPV1 (Kreider et al., 1990b), HPV16 (Bonnez et al., 1998) and HPV40 and HPVLVX82/MM7 (Christensen et al., 1997). Recently, Brandsma et al. (1995) have successfully produced warts on human foreskin grafted onto SCID mice through inoculation with HPV16 DNA. It is reasonable to expect that with continued technical advances, HPV infection of xenografted human tissue will become a standard tool both for the study of the virus life cycle and for the evaluation of potential antiviral drugs.

The replication functions of the PVs are highly conserved among the animal and human viruses;

indeed, many of the components are qualitatively interchangeable (Chiang et al., 1992; Del Vecchio et al., 1992). Therefore, it is likely that inhibitors of PV replication will show a broad spectrum of activity when measured against many of the animal and human viruses. As an intermediate step in the establishment of a small animal model for the evaluation of potential inhibitors of HPV replication, we further explored a previously published CRPV model. A number of years ago, Kreider demonstrated that CRPV could induce cutaneous papillomas on rabbit skin orthotopically grafted onto nude mice (Kreider et al., 1979). As a practical extension of this observation, we have developed a model using SCID mice, which allows for the efficient evaluation of compounds for inhibitory activity against the PVs. The model was validated through the analysis of ribavirin, which has shown activity in the clinic in the treatment of juvenile respiratory papillomatosis (McGlennen et al., 1993). Further, since several antitumor compounds have seen clinical uses for the treatment of warts, a number of antiproliferative compounds including Navelbine™ (vinorelbine tartrate), were evaluated in this system. The Navelbine results are compared to efficacy studies done in the rabbit back model with this compound. Finally, a selection of antiviral compounds with well-defined activities against HIV or Herpesviruses was evaluated. We anticipate that the techniques developed during the establishment of this model will be directly transferable to the infection of xenografted human epithelial tissues with HPVs.

## 2. Materials and methods

### *2.1. Animals*

The research complied with national legislation and with company policy on the care and use of animals and with related codes of practice. Female C.B-17 SCID mice were obtained at 3 weeks of age from Taconic Labs. Nude mice were purchased form Charles River. After transplantation mice were housed individually in microisolator cages with sterile bedding, food and water to minimize infection or disruption of transplanted tissue or chewing of developing warts. New Zealand White (NZW) rabbits (2–3 kg) were purchased from Hazelton Labs.

### *2.2. Preparation of donor tissue*

In experiments designed to assess treatment efficacy, donor skin was removed from the dorsal surface of the ears of euthanized NZW rabbits. Harvested skin was placed in cold phosphate buffered saline (PBS) until processed (usually within 2 h). After removing excessive underlying connective tissue, a skin punch was used to create circular grafts (7 mm diameter). The grafts were sterilized by adding cold 70% ethanol to cover the grafts and vortexing for 10 s. Cold, sterile PBS was quickly added, the tube vortexed and decanted. The grafts were washed twice more with PBS, and transferred to sterile Eppendorf tubes, which were kept on ice until transplant. Residual PBS kept the grafts moist until transplantation.

### *2.3. Transplantation of rabbit skin grafts*

All surgical procedures were done under aseptic conditions using a hood. Mice were anesthetized by subcutaneous injection of ketamine (100 mg/kg) and xylazine (10 mg/kg) and the dorsum shaved with surgical clippers then sterilized with alternating ethanol and iodine swabs. Sterile glycerol ointment was placed in each eye to prevent drying. Using the skin punch, two circular graft beds were created on contralateral sides of the dorsum. Sterile graft tissue was placed in each receptive graft bed and positioned with a cotton-tipped swab. The mice were placed in individual cages and maintained on a slide warmer until recovery from anesthesia.

### *2.4. Preparation of CRPV inoculum and infection of grafted rabbit tissue*

The grafted tissue was allowed to heal for approximately 3 weeks prior to infection with CRPV. A 10% (w/v) homogenate of a wart collected from a wild cottontail rabbit trapped in Minnesota was prepared by standard methods

(Watts et al., 1983) and stored at −70°C for use as a stock virus. Virus was diluted 1:10 in sterile PBS for infecting grafted tissue. For infection, mice were anesthetized with the eyes protected as described above and the grafts wiped with an ethanol swab. Each graft was thoroughly scarified (~100 strokes) with a 27 gauge hypodermic needle through a 4 μl droplet of inoculum. After scarification, an additional 4 μl of the inoculum was applied to each graft, and evenly distributed over the surface of the graft with the pipeter tip. The mice were returned to their cages and placed on a slide warmer until recovery from the anesthetic.

### 2.5. Preparation of drugs and treatment of mice

Acyclovir (ACV), zidovudine (AZT), Wellferon™, Navelbine™, Condylox™ (5% podofilox), *cis*-5-flouro-1-[2-(hydroxymethyl)-,3-oxathiolan-5-yl]cytosine (FTC), 9-((2*R*,5*S*)-tetrahydro-5-(hydroxymethyl)-2-furyl)guanine 2′,3′-dideoxyguanosine (ddG), 882C87 (1-β-D-arabinofuranosyl-5-(1-propynyl)uracil), 1954U89 (1,3-Diamino-7-(1-ethylpropyl)-8-methyl-7H-pyrrolo (3,2-F)quinazoline and ganciclovir (GCV) were obtained from Glaxo Wellcome compound stores. Ribavirin was purchased from Pharmatec. 5-Iodo-2′-deoxyuridine (IDU), 2′-deoxy-5-trifluoromethyluridine (TFT; trifluorothymidine), phosphonoacetic acid (PAA), phosphonoformic acid (PFA; foscarnet), 5-fluorouridine (5-FU), forskolin, camptothecin, colchicine, vinblastine, vincristine and tubercidin were purchased from Sigma. Compounds for topical delivery were either dissolved in 100% DMSO and stirred into a modified aqueous cream (final DMSO concentration 10%) or added directly to the cream into which DMSO was added to a final concentration of 10% to aid in drug penetration. Topical drugs were applied with sterile swabs either once or twice daily (Monday–Friday). Systemic treatment was administered either by oral delivery of drug given ad libitum in sterile drinking water or via mini-osmotic pumps (Alzet model # 2002) implanted subcutaneously on the dorsum. The solvent used for drug delivery in the mini-pumps was either water or 80% DMSO. Treatment of mice usually began 1–5 days post-infection (PI) and continued for 6 weeks.

### 2.6. Scoring of lesions and determination of efficacy

Weekly lesion scores were recorded according to the criteria described in Table 2 (Syverton et al., 1950). Lesion development is nearly linear after the appearance of the lesions at about week 2 or 3 PI and progresses through each of the scores noted in Table 2.

Typically, the scores were recorded for approximately 6–8 weeks, since after this time the lesions became very large and scratching or gnawing of the warts affected scoring. Therefore, long term studies of regression or involution could not be evaluated in this system. Any grafts or lesions that became difficult to score due to scratching or irritation were eliminated from the scoring. The area under the curve (AUC) of mean lesion scores versus weeks PI for each treatment group was computed and the percent reduction in the AUC for each treated group compared to the untreated control group was then calculated. In practice we observed that a compound needed to reduce the AUC by greater than 20% before any clinical improvement in lesion severity was seen. Treatment toxicity was noted as either mild (slight erythema of treated sites), moderate (erythema with some scabbing or ulceration of treated sites) or severe (extreme burning of treated area; systemic effects or death)

### 2.7. Rabbit back model

The rabbit back model has been previously described (Kreider et al., 1990a). Briefly, NZW rabbits were anesthetized and the fur removed from the dorsum with surgical clippers. Two dilutions (1:10 and 1:100) of CRPV stock virus prepared as described above, were used to scratch infect two contralateral sites on the dorsum. The sites on the left dorsum (high and low inoculum) were treated with anti-PV therapies while the sites on the right dorsum served as untreated controls. Drugs were prepared as described above for the

Table 1
Efficiency of transplant and infection procedures in the SCID-Ra model

| Number of mice | Total number of transplants | Graft success (%) | Successful infection (%) |
|---|---|---|---|
| 218 | 436 | 414 (95) | 86/94[a] (91) |

[a] Untreated controls.

mouse model. Weekly measurements of the lesions were made in 3 axes ($L \times W \times H$) and the geometric mean diameter of each lesion calculated. Percent reductions in AUCs of the geometric mean diameters of the treated sites relative to untreated sites or placebo treated animals were calculated.

## 3. Results

An ideal animal model for the study of human disease should be efficient, reproducible, and pathologically representative of the human disease on a reasonable time scale. To appropriately model human epithelial warts, the infection in animals should be superficial to allow for continuous observation, localized treatment of the infection, and for facile collection of tissue samples as required. Furthermore, to assess whether the model is predictive of compound efficacy in humans, clinically active drugs should be evaluated.

### *3.1. Preliminary characterization of the mouse model*

Female SCID mice were used throughout these studies. For preparation of the donor tissues and the recipient graft beds, a cutting tool was designed and routinely utilized with the result that graft tissue and the graft beds were reproducibly of equal size. With experience, we found that suturing or gluing of the size-matched transplanted tissue was not necessary greatly facilitating the transplant procedure. The transplanted tissues completely healed in about 3 weeks at which time they could be infected with CRPV.

Various procedures were investigated for infection of the grafted tissues including use of a tattoo gun or vaccination gun; however, none proved to be more reproducible or technically easier in our hands than traditional scratch-infection. Since the model was being developed as a moderate throughput system for assessing the efficacy of potential therapies, and in order that treatments might be initiated prior to any evidence of infection, the efficiency of the transplant and infection procedures was critical. Shown in Table 1 is a compilation of seven typical experiments illustrating the high rates of successful transplantation and subsequent infection of the transplanted tissues. In these experiments, 414 of 436 grafts (95%) were successfully transplanted while successful infection was achieved in 86 of the 94 grafts (91%) in untreated control mice in these same experiments. The efficiency of the model permits the use of relatively small treatment groups for primary evaluation of compound activity. Furthermore, since $> 90\%$ of the animals that are inoculated will develop lesions, candidate therapies may be initiated prior to the appearance of macroscopic lesions.

### *3.2. CRPV infection of transplanted rabbit skin induces benign warts*

The progression of benign lesion development in transplanted NZW rabbit tissue infected with CRPV is shown in Fig. 1. Evidence of infection is visually apparent at 2–3 weeks PI with initial thickening of the infected graft. Small, discrete papillomas are visible at about 3 weeks PI and become larger over the next few weeks. The individual papillomas become confluent at about 4–5 weeks PI and eventually involve the entire graft. Over the next 2 weeks the warts continue to increase in size and become highly keratinized. To allow for semi-quantitative evaluation of potential therapies a lesion scoring system (grades 1–6) was developed and is described in Table 2. Typically,

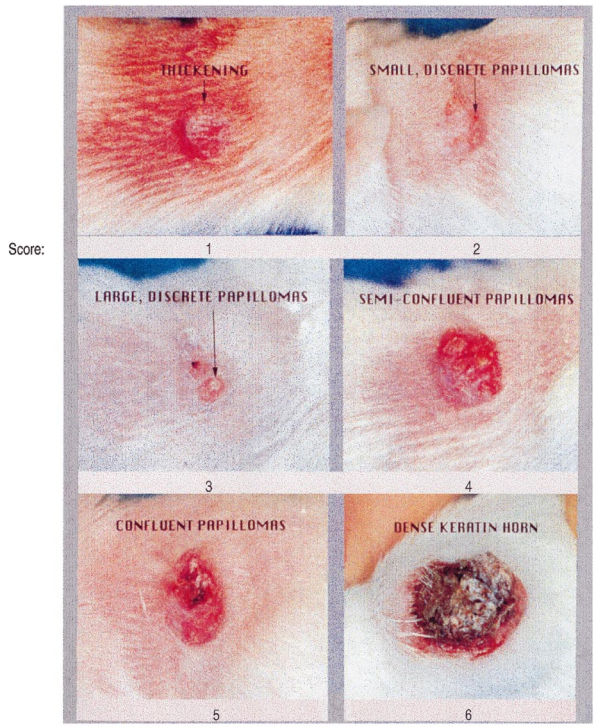


Fig. 1

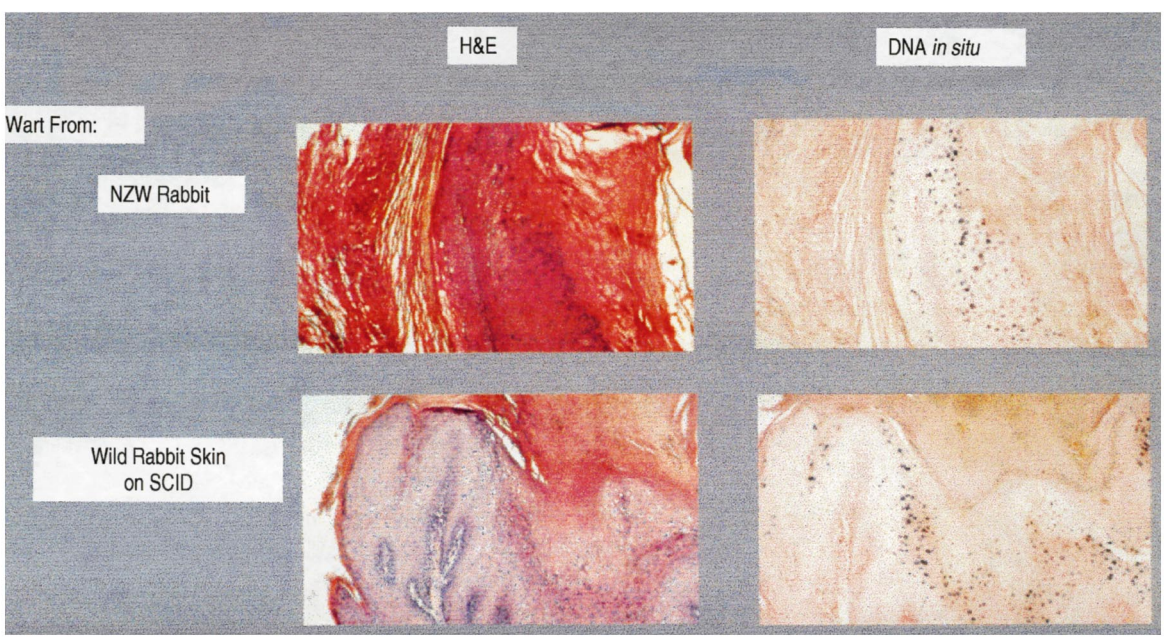


Fig. 2

Fig. 1. Lesion development and scoring system in the SCID-Ra model.
Fig. 2. Histological comparison of warts from a NZW rabbit and wild rabbit xenograft on SCID mouse. Tissues were collected and fixed in 10% formalin for 24 h prior to processing. For DNA in situ hybridization, the Digene Tissue Hybridization kit (cat. # 4206 0100) was used with a biotinylated CRPV probe (BioNick® Labeling System; Life Technologies # 18247-015).

animal lesions were visually scored on a weekly basis, and the average lesion scores were calculated using five animals per treatment group, each carrying two infected transplants. Therefore, each data point represents lesion scores from 10 individual skin grafts.

### 3.3. Transplanted wild rabbit skin can be productively infected

It has been known for many years that CRPV infection of wild rabbits produces infectious virions while induction of benign warts in domestic NZW rabbits does not generally result in the production of infectious virus particles. In order to determine if the mouse model recapitulated this characteristic host specificity, wild rabbit ear skin was transplanted to mice and infected with CRPV. Shown in Fig. 2 are the H and E and DNA in situ hybridizations of a benign wart produced on wild rabbit skin transplanted to SCID mice and, for comparison, a wart from a NZW rabbit. The H and E staining of the SCID wart reveals features typical of PV infection including a thickened, hyperplastic epidermal layer with excessive keratin production. Capsid antigen staining confirmed that viral capsid antigens were abundantly expressed in the benign wart of the wild rabbit xenograft (data not shown). To determine whether infectious particles were produced, a lesion was harvested 56 days after infection, homogenized, and used to infect naive NZW rabbit skin, which had been transplanted to mice. Warts were evident several weeks PI and progressed to stage 6 lesions indicating the presence of high titer infectious virus in the inoculum. As expected, infection with extracts from benign warts on NZW transplanted skin failed to induce subsequent lesions. These results indicate that this system may also be used to passage virus and in the production of laboratory stocks of CRPV as in the renal capsule model.

Table 2
Scoring system used for determination of treatment efficacy in the SCID-Ra model

| Score | Clinical description of infected sites |
|---|---|
| 0 | No infection visible |
| 1 | Thickening of infected skin |
| 2 | Small, discrete papillomas |
| 3 | Large, discrete papillomas |
| 4 | Semi-confluent papillomas, some keratinization |
| 5 | Confluent papillomas; more keratinization |
| 6 | Dense keratinized surface on wart |

### 3.4. Effect of antiviral compounds on developing lesions

Since there is no known, specific antiviral inhibitor of HPV for use as a positive control in the model, the efficacy of ribavirin was evaluated. Ribavirin, a broad-spectrum antiviral, has been used successfully in the clinic for the treatment of juvenile laryngeal papillomatosis (McGlennen et al., 1993). Fig. 3 illustrates the effect of therapy with ribavirin on developing CRPV-induced lesions. Oral therapy with 1 mg/ml ribavirin reduced the AUC by 71% when compared to untreated control animals. As the amount of ribavirin in the drinking water was reduced to 0.5 and 0.1 mg/ml there was a significant reduction in efficacy (38 and 3% reduction in AUC, respectively). A combination of 5% topical therapy and 1 mg/ml oral treatment was slightly more effective than oral therapy alone (85% reduction in AUC), however, there may have been some toxicity associated with this treatment as 1 of the six mice in this group died during the treatment period. Furthermore, it should be noted that lesion scores rebounded once treatment was stopped on day 35 PI, although the score of the combination group appeared to be rising more slowly than the scores of the other treatment groups. In the combination group 5/10 grafts showed no evidence of infection at the end of the study while the group receiving 1 mg/ml in the drinking water had 1/12 grafts that appeared to be uninfected. As a measure of the reproducibility of the model, another experiment with the same combination of topical and oral ribavirin therapy resulted in 87% suppression of lesion scores and was also associated with toxicity (data not shown).

To gain additional, pilot experience with this model system, we examined the effects of oral, topical, and systemic treatments with various an-

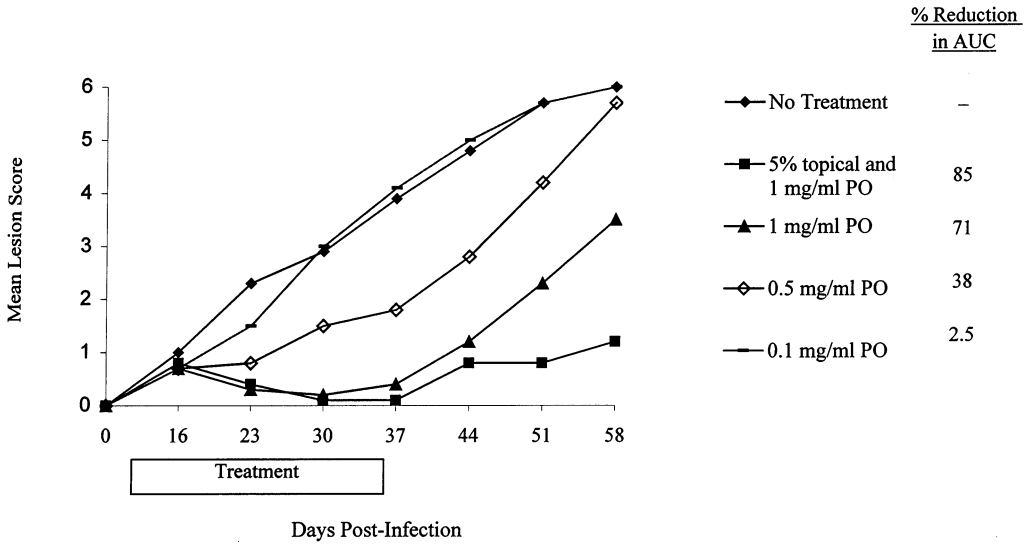


Fig. 3. Effect of ribavirin treatment on developing lesions in the SCID-Ra model. Treatments were initiated 24 h post-infection and continued through day 35. Topical drug was applied once daily (Monday–Friday). Oral drug was given in the drinking water (24 h/day).

tiviral compounds as summarized in Table 3. Little inhibition of wart growth was noted with IDU, TFT, AZT, ddG, GCV, FTC or 882C at the dosing regimens tested (0–35% reduction in AUC). A combined oral and topical regimen of PAA produced good suppression of wart growth (73% reduction of AUC); however, at this therapeutic dose, significant toxicity was associated with treatment. Oral PAA alone was only slightly suppressive (36% reduction in AUC); PFA (foscarnet) given both orally and topically also showed little efficacy (22% reduction). ACV when given topically and orally, or the prodrug Valtrex™ (data not shown) produced a modest reduction in lesion scores (33 and 26%). With experience it was observed that an unequivocal therapeutic benefit was associated with a reduction in AUC $> 25\%$ in this model system.

### *3.5. Effect of antitumor compounds on developing lesions*

A range of antitumor compounds was also evaluated since several antiproliferative drugs have been used clinically for the treatment of HPV-associated benign disease. As illustrated in Table 4, treatment with a varied selection of antitumor compounds resulted in variable degrees of suppression of lesion growth. Unfortunately, however, many of these therapies were also quite toxic at the efficacious doses.

Podophyllotoxin, the active ingredient in crude extracts of podophyllin, is approved for topical treatment of external genital warts (Condylox™, Oclassen Pharmaceuticals). The antiproliferative activity of podophyllotoxin is thought to be primarily mediated through anti-mitotic effects resulting from disruption of cellular microtubules. In this model system, podophyllotoxin demonstrated little efficacy, and treatment resulted in significant cutaneous erosion. Another common class of antimitotic compounds, the vinca alkaloids including both vincristine and vinblastine, significantly suppressed lesion growth (62 and 73% reduction of AUC), but were associated with redness, swelling, and topical erosion.

Navelbine, a related vinca alkaloid, showed comparable efficacy (up to 58% reduction in AUC), and somewhat reduced cutaneous toxicity when compared to podophyllotoxin, vincristine or

Table 3
Antiviral compounds tested in the SCID-Ra model

| Compound | Treatment | | | | Supression (%) | Toxicity[a] |
|---|---|---|---|---|---|---|
| | Topical (%) | Oral (mg/ml) | Pump (mg/kg/d) | IP (U/day) | | |
| ACV | 5 | — | 46 | — | 33 | − |
| AZT | 5 | 1 | — | — | 35 | +++[b] |
| IDU | 5 | 1 | — | — | 13 | − |
| TFT | 1 | — | — | — | 21 | − |
| PAA | - | 1 | — | — | 36[c] | − |
| | 5 | — | 80 | — | 73[c] | ++ |
| PFA | 5 | 1 | — | — | 22 | − |
| FTC | 5 | 1 | — | — | 9 | − |
| GCV | 1 | — | — | — | 0 | − |
| ddG | 2.5 | — | 80 | — | | |
| 882C87 | 5 | 1 | — | — | 15 | − |
| Wellferon | — | — | — | $1 \times 10^4$ | 44 | − |
| Ribavirin | 4 | — | — | — | 22 | − |
| | — | 0.1 | — | — | 3[d] | − |
| | — | 0.5 | — | — | 38[d] | − |
| | — | 1 | — | — | 71[d] | − |
| | 5 | 1 | — | — | 85[d] | + |

[a] Toxicity recorded as negative (−), mild (+), moderate (++) or severe (+++).
[b] One mg/ml in drinking water is equal to an approximate dose of 200 mg per kg $day^{-1}$ (assuming consumption of 5 ml of water/day and mouse weight of 25 g). Current AZT therapies in human give dose of approximately 5 mg $kg^{-1}$ per day or less.
[c] Evaluated in separate experiments.
[d] Evaluated in the same experiment.

vinblastine. The dose dependent efficacy of Navelbine in this model is illustrated in Fig. 4. Once daily topical therapy with 0.3% Navelbine reduced AUCs by 58%. Similar treatment with 0.1% Navelbine only resulted in a 23% reduction in AUC compared to untreated grafts. Twice weekly (Monday, Friday) application of 1% Navelbine produced a 42% reduction in AUC. Some dermal erosion was evident with both daily and twice weekly therapies; indeed, daily application of 1% Navelbine was too erosive to be evaluated in this system. As further validation of the utility of the mouse model, Navelbine was tested in the conventional immunocompetent rabbit back model of PV infection against 1:10 and 1:100 dilutions of CRPV inoculum. As shown in Table 5, once daily topical therapy with 0.3% Navelbine reduced the AUC by 66% when compared to lesions on the untreated flank. A similar treatment regimen with 0.9% Navelbine resulted in a 90% inhibition of AUC. Twice weekly application of 1.5% Navelbine resulted in a 73% reduction in AUC. Although there again was evidence of dermal toxicity, these results confirmed the results obtained from the SCID-Ra system and further support the utility of the mouse model.

### *3.6. A significant reduction in compound required for evaluation*

Compound evaluation in the SCID-Ra system typically required $< 1$ g of pure compound to complete a 6–8 week primary treatment study. Obviously, depending upon the potency and the route of administration more or less compound might be needed in individual circumstances. As suggested by the comparison to the rabbit back model in Table 6, this may represent a 10–100-fold reduction in the amount of compound required for a preliminary in vivo evaluation. In the absence of a well validated in vitro or cell culture model of HPV infection, significant reductions in

Table 4
Antitumor compounds tested in the SCID-Ra model

| Compound | Treatment (%) | Frequency applied (%) | Suppression (%) | Toxicity[a] |
|---|---|---|---|---|
| 5-FU | 0.8 | 5 | 9 | − |
| Forskolin | 0.3 | 5 | 6[b] | − |
| | 0.6 | 10 | 27[b] | − |
| Camptothecin | 0.3 | 5 | 31 | + |
| Colchicine | 0.3 | 5 | 22 | ++ |
| Vinblastine | 1.5 | 1 | 73[c] | ++ |
| | 0.3 | 3 | 13[c] | + |
| Vincristine | 0.3 | 2 | 62 | ++ |
| Condylox | 0.025 | 5 | 0 | − |
| | 0.05 | 5 | 19 | + |
| | 0.5 | 3[d] | nc[e] | +++ |
| Navelbine | 0.1 | 5 | 23[f] | +/− |
| | 0.3 | 5 | 58[f] | + |
| | 1 | 2 | 42[f] | + |
| Tubercidin | 1 | 5 | nc | ++ |
| 1954U89 | 0.5 | 3 | 26 | ++ |

All treatments were topical and were formulated in an aqueous cream with 10% DMSO added.
Suppression indicates the percent reduction in AUC compared to untreated controls.
[a] Toxicity recorded as negative (−), mild (+), moderate (++) or severe (+++) at the doses tested.
[b] Evaluated in separate experiments.
[c] Evaluated in separate experiments.
[d] Applied once/day for 3 consecutive days
[e] Not calculated, evaluated in separate experiment from others shown for Condylox.
[f] Evaluated in same experiment.

compound requirements for in vivo evaluation could be invaluable prior to broader preclinical assessment or synthetic scale-up.

## 4. Discussion

In this work, we describe the development of a small animal model that can be used both in the assessment of inhibitory effects of compounds on PV infections and for the in vivo study of certain biological aspects of virus infection. The lack of a routine, productive cell culture system for the PVs places added importance on the use of animal model systems for the study of these viruses, and thus, any model with improved capacity and efficiency represents an important advance for the field.

Although the PVs infect most vertebrate species, there is no well-characterized mouse or rat PV that can be readily adapted for evaluation of potential therapies in a small animal system. Two PVs have been isolated from rodents (O'Banion et al., 1988; Tan et al., 1994), however, infection of laboratory strains using these isolates has thus far been unsuccessful. Several other well-defined models in larger animals have been studied in some depth during the last 50 years including the CRPV, BPV, and COPV papillomaviruses (Lancaster and Olson, 1982; Stanley et al., 1997). More recently, PVs have been isolated from several nonhuman primates including rhesus monkeys (RhPV), long-tailed macaques, and pygmy chimpanzees (PCPV) (Kloster et al., 1988; Ostrow et al., 1990; Van Ranst et al., 1992; Ostrow et al., 1995; Chan et al., 1997). Each of these can be considered classical animal model systems that rely on infection of the natural host which supports both virus replication and archetypal disease. With regard to human papillomaviruses, the characteristic stringent species specificity and inherent ethical considerations demand that, in the near term at least, such surrogate animal systems will continue to be quite useful for late stage

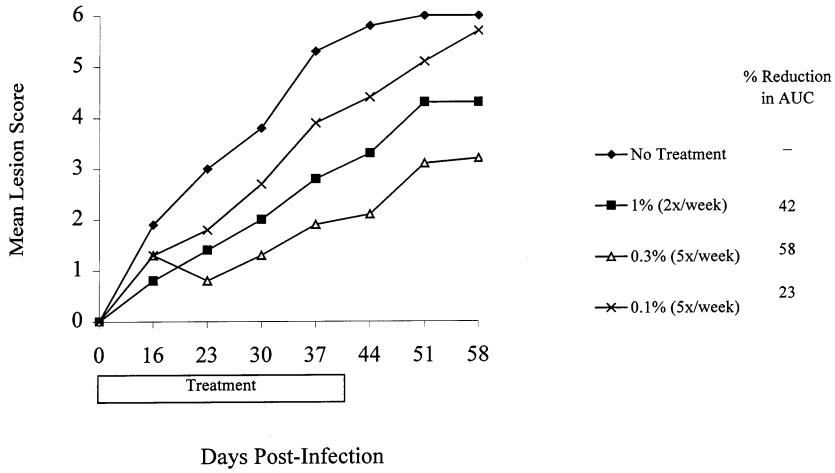


Fig. 4. Effect of Navelbine treatment on developing lesions in the SCID-Ra model. Treatments were initiated 24 h PI and continued through day 40. All treatments were topical and applied as indicated in the legend.

clinical development of antiviral drugs and vaccines.

Two strains of immunodeficient mice, nude and SCID, have been used to support infection of human epithelial tissue with human PVs (Kreider et al., 1987; Bonnez et al., 1993; Brandsma et al., 1995). In the earliest example, a pooled clinical stock of HPV11 virus was used to infect human foreskin tissue prior to surgical implantation within the renal capsule of anesthetized nude mice (Howett et al., 1997). The renal capsule provides an enclosed, aseptic, and well-vascularized site for subsequent development of HPV-infected condylomatous cysts. Viral gene expression is detectable by 4 weeks PI, late gene expression seen at about 8 weeks, and abundant virus particle production evident after about 3 months (Stoler et al., 1990). HPV particles can be purified from the epithelial cysts and serially passaged by infection (Howett et al., 1990). Antiviral efficacy of candidate drugs can be assessed in this system provided sufficient compound is available for long-term, systemic exposure (Kreider et al., 1990a). Furthermore, to assess activity in the renal capsule, candidate compounds must possess favorable pharmacokinetic/pharmacodynamic and toxicity profiles to permit exposure of the renal capsule to therapeutically relevant concentrations of drug. As a practical matter, these conditions are typically only met at late stages of preclinical drug development. Finally, lesion progression and/or chemotherapeutically-induced resolution can not be readily observed except at necropsy.

Although we and others have orthotopically transplanted human tissue including neonatal foreskin to immunodeficient mice, infection of these tissues with HPV does not consistently or efficiently produce clinically overt disease. Recent studies suggest that utilizing recombinant viral DNA as an inoculum to initiate the virus life may circumvent the variability in infectivity of clinical samples and provide an exploitable system for genetic studies of virus replication, expression, and neoplastic progression (Brandsma et al., 1995; Frattini et al., 1997).

Since PV infection in wild rabbits is a very robust infection producing uniform stocks of high titer infectious virus, we chose to establish a preclinical model using CRPV as the infecting agent. This is an appropriate system for assessment of antiviral inhibitors since the disease in rabbits is pathologically representative of human disease in that virus infection results in benign epithelial disease which can regress, persist or progress to malignancy. Furthermore, the viral replication and transcription functions in the human and animal PVs are structurally and functionally well conserved (Hirochika et al., 1987; Phelps and

Table 5
Effect of therapy with Navelbine in the treatment of CRPV induced lesions on NZW rabbits infected with a $10^{-2}$ dilution of virus

| Dose | Suppression (%) | Toxicity | |
|---|---|---|---|
| | | Systemic[a] | Dermal |
| 0.3% Navelbine (5×/wks 3–5) | 66[c] | − | +[b] |
| 0.9% Navelbine (5×/wks 3–5) | 90[c] | − | +[b] |
| 1.5% Navelbine (2×/wks 3–5) | 73[d] | − | − |
| 3% Navelbine (2×/wks 3–5) | 52[e] | − | − |

All treatments were initiated on day 14 PI and applied topically for 3 weeks either once daily on Monday through Friday or once daily on Monday and Friday.
Therapies were formulated (w/w) in an aqueous cream with no DMSO added.
Suppression indicates the percent reduction in AUC compared to untreated sites on the same animal.
There were five rabbits/treatment group with one treated and one untreated site at the $10^{-2}$ dilution on each rabbit.
[a] Difference in weight gain in treated rabbits compared to placebo treated rabbits.
[b] Sites treated daily were red, irritated and scabbed after 1 week of treatment.
[c] Evaluated in same experiment.
[d,e] Evaluated in separate experiments.

Howley, 1987; Chiang et al., 1992; Del Vecchio et al., 1992; Liang et al., 1996) suggesting that inhibitors of these functions are likely to be active in both animal and human systems. In contrast, therapies that target the infected host cell such as retinoids and podofilox, do not demonstrate comparable activities in animals and humans.

Without an in vitro system for selecting among the best of a series of potential antiviral inhibitors for animal model testing, it is important to maximize a compound's ability to inhibit lesion development in animals. One way to accomplish this is to initiate treatment soon after infection of the grafted tissue. Since warts are not evident for several weeks after infection with CRPV, a high proportion of uninfected grafts can be statistically problematic. Therefore, a model used for routine screening of compounds for efficacy against the PVs requires high rates of transplant and infection success. If evaluation of potential inhibitors is to be done in animals where both compound supply and cost (both time and money) are a concern, the advantages of a small animal model are obvious. The typical amounts of compounds required in this study for topical, oral and systemic treatment in the SCID-Ra model and the rabbit back model are compared in Table 6. The SCID-Ra model requires about 10 × less compound for topical therapy than the rabbit back model. Even greater reductions in the amount of required compound are seen with both oral (drinking water) and systemic (osmotic pump) therapies when comparing the SCID model and the rabbit back model (30 and 100 ×, respectively). This is a vital practical consideration as the vast majority of

Table 6
Comparison of compound requirements for evaluation in the SCID-Ra model and the rabbit back model of papillomavirus infection

| Treatment | Compound quantity (g) required for testing in | | Approximate fold reduction |
|---|---|---|---|
| | SCID-Ra Model | Rabbit Back Model | |
| Topical[a] | 0.5 | 5 | 10 |
| Oral (drinking water)[b] | 1 | 30 | 30 |
| Systemic (osmotic pumps)[c] | 0.5 | 50 | 100 |

[a] Assuming a dose of 2.5% (w/w) and 20 g of cream for the SCID model. Since lesion area is approximately 39 $mm^2$ in the SCID-Ra model and 500 $mm^2$ in the rabbit back model the amount of compound required for the treatment of rabbits would be about 10×.
[b] Assuming daily consumption of 4 ml/mouse and 120 ml/rabbit with 1 mg/ml of drug in the drinking water for a 6 week treatment period and 6 animals per treatment group.
[c] Assuming treatment for 6 weeks at a dose of 80 mg kg $day^{-1}$ and weights of 0.025 kg (mouse) and 2.5 kg (rabbit) with six animals/group.

compounds available in the chemical libraries of pharmaceutical companies are available in < 1 g quantities.

Since the antiviral ribavirin had been shown to be clinically effective in the treatment of laryngeal papillomatosis (McGlennen et al., 1993) and has shown activity in the rabbit back model of PV infection (Ostrow et al., 1992), we used this compound to clinically validate our model as well as an indicator of reproducibility. Our results show that oral therapy with 1 mg/ml of ribavirin in the drinking water resulted in significantly reduced lesion scores. The therapeutic response was dose-dependent as a reduction in efficacy was seen when the amount of ribavirin was decreased. We also found that a rebound in lesion scores was seen when therapy was discontinued in the treated groups, however, in the group receiving combination therapy with 1 mg/ml orally and 5% topically half of the grafts appeared uninfected at the end of the study.

In the past, several antitumor compounds have been used for the treatment of warts (Gross, 1995). To determine if these compounds would be inhibitory in the SCID-Ra model, various classical anti-proliferative compounds were evaluated including anti-mitotic, anti-metabolites, and topoisomerase inhibitors. As expected with this class of compounds, some suppression of the growth of benign warts was observed; however, dermal erosion was also typically seen indicative of non-specific cytotoxic activities. One anti-mitotic compound of interest was Navelbine currently used for the treatment of non-small cell lung carcinoma. Navelbine treatment resulted in a good reduction in lesion scores although treatment was associated, under these assay conditions, with mild to moderate dermal erosion or irritation. To corroborate and extend these results, Navelbine was also tested in the rabbit back model where it also showed comparable suppression of lesion development and dermal toxicity.

Since the major use of this model is expected to be in the evaluation of inhibitors of PV replication, we also evaluated a collection of nucleoside antiviral compounds. Bearing in mind that the papillomaviruses do not encode a viral nucleotide kinase or a viral polymerase, the anabolic activation and selective incorporation seen with nucleosides such as ACV (Furman et al., 1984) in the inhibition of the herpesviruses would not be anticipated with papillomaviruses. As expected, purine and pyrimidine nucleoside analogs were essentially inactive for inhibition of lesion growth.

In summary, we have established a reproducible, relatively easy mouse model for the evaluation of anti-PV therapies. The model is efficient with both a high rate of transplant and infection successes, and an infection that is evident in weeks rather than months. High titer viral stocks are available from warts harvested from both trapped wild rabbits and from warts produced on wild rabbit skin transplanted to mice. Although the model can not be used for the evaluation of compounds that require an intact immune system, a variety of treatment schemes can be evaluated and, since the infection is external, the course of infection is easily monitored on a daily basis. Depending on the route of treatment the model reduces the amount of compound required for testing by 10–100-fold over the conventional rabbit system, and candidate compounds can be evaluated with less than 500 mg. This model is likely to become an invaluable tool in the preclinical evaluation of candidate antiviral compounds for inhibition of HPV replication.

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## References

Bonnez, W., Darin, C., Borkhuis, C., De Mesy Jensen, K., Reichman, R.C., Rose, R.C., 1998. Isolation and propagation of human papillomavirus type 16 in human xenografts implanted in the severe combined immunodeficiency mouse. J. Virol. 72, 5256–5261.

Bonnez, W., Rose, R.C., Da Rin, C., Borkhuis, C., Jensen,

K.L.M., Reichman, R.C., 1993. Propagation of human papillomavirus type 11 in human xenografts using the severe combined immunodeficiency (SCID) mouse and comparison to the nude mouse model. Virology 197, 455–458.

Brandsma, J.L., Brownstein, D.G., Xiao, W., Longley, B.J., 1995. Papilloma formation in human foreskin xenografts after inoculation of human papillomavirus type 16 DNA. J. Virol. 69, 2716–2721.

Chan, S.-Y., Bernard, H.-U., Ratterree, M., Birkebak, T.A., Faras, A.J., Ostrow, R.S., 1997. Genomic diversity and evolution of papillomaviruses in rhesus monkeys. J. Virol. 71, 4938–4943.

Chiang, C.-M., Ustav, M., Stenlund, A., Ho, T.F., Broker, T.R., Chow, L.T., 1992. Viral E1 and E2 proteins support replication of homologous and heterologous papillomaviral origins. Proc. Nat. Acad. Sci. USA 89, 5799–5803.

Christensen, N.D., Koltun, W.A., Cladel, N.M., Budgeon, L.R., Reed, C.A., Kreider, J.W., Welsh, P.A., Patrick, S.D., Yang, H., 1997. Coinfection of human foreskin fragments with multiple human papillomavirus types (HPV-11, -40, and –LVX82/MM7) produces regionally separate HPV infections within the same athymic mouse xenograft. J. Virol. 71, 7337–7344.

Del Vecchio, A.M., Romanczuk, H., Howley, P.M., Baker, C.C., 1992. Transient replication of human papillomavirus DNAs. J. Virol. 66, 5949–5958.

De Villiers, E.M., 1994. Human pathogenic papillomavirus types: an update. Curr. Top. Microbiol. Immunol. 186, 1–12.

Field, H.J., 1988. Animal models in the evaluation of antiviral chemotherapy. In: Field, H.J. (Ed.), Antiviral Agents: The Development and Assessment of Antiviral Chemotherapy, vols. 1–2. CRC Press, Boca Raton, pp. 67–84.

Frattini, M.G., Lim, H.B., Doorbar, J., Laimins, L.A., 1997. Induction of human papillomavirus type 18 late gene expression and genomic amplification in organotypic cultures from transfected DNA templates. J. Virol. 71 (9), 7068–7072.

Furman, P.A., St. Clair, M.H., Spector, T., 1984. Acyclovir triphosphate is a suicide inactivator of the herpes simplex virus DNA polymerase. J. Biol. Chem. 259, 9575–9579.

Gissmann, L., Wolnik, L., Ikenberg, H., Koldovsky, U., Schnurch, H.G., zur Hausen, H., 1983. Human papillomavirus type 6 and 11 DNA sequences in genital and laryngeal papillomas and in some cervical cancers. Proc. Nat. Acad. Sci. 80, 560–563.

Gissmann, L., zur Hausen, H., 1980. Partial characterization of viral DNA from human genital warts (*condylomata acuminata*). Int. J. Cancer 25, 605–609.

Gross, G., 1995. Treatment of human papillomavirus infection. In: Mindel, A. (Ed.), Genital Warts: Human Papillomavirus Infection. Edward Arnold, London, pp. 198–236.

Hendrickson, E.A., 1993. The SCID mouse: relevance as an animal model system for studying human disease. Am. J. Pathol. 143, 1511–1522.

Hirochika, H., Broker, T.R., Chow, L.T., 1987. Enhancers and *trans*-acting E2 transcriptional factors of papillomaviruses. J. Virol. 61, 2599–2606.

Howett, M.K., Christensen, N.D., Kreider, J.W., 1997. Tissue xenografts as a model system for study of the pathogenesis of papillomaviruses. Clin. Dermatol. 15, 229–236.

Howley, P.M., 1996. Papillomavirinae: the viruses and their replication. In: Fields, B.N. (Ed.), Fields Virology. Lippincort-Raven, Philadelphia, pp. 2045–2076.

Howett, M.K., Kreider, J.W., Cockley, K.D., 1990. Human xenografts. A model system for human papillomavirus infection. Intervirology 31, 109–115.

Ito, Y., 1981. Effect of an aromatic retinoic acid analog (Ro 10-9359) on growth of virus-induced papilloma (Shope) and related neoplasia of rabbits. Eur. J. Cancer 17, 35–42.

Kloster, B.E., Manias, D.A., Ostrow, R.S., Shaver, M.K., McPherson, S.W., Rangen, S.R.S., Uno, H., Faras, A.J., 1988. Molecular cloning and characterization of the DNA of two papillomaviruses from monkeys. Virology 166, 30–40.

Koutsky, L.A., Galloway, D.A., Holmes, K.K., 1988. Epidemiology of genital human papillomavirus infection. Epidemiol. Rev. 10, 122–162.

Kreider, J.W., Balogh, K., Olson, R.O., Martin, J.C., 1990a. Treatment of latent rabbit and human papillomavirus infections with 9-(2-phosphonylmethoxy) ethyl guanine (PMEG). Antivir. Res. 14, 51–58.

Kreider, J.W., Bartlett, G.L., Sharkey, F.E., 1979. Primary neoplastic transformation in vivo of xenogeneic skin grafts on nude mice. Cancer Res. 39, 273–276.

Kreider, J.W., Howett, M.K., Leure-Dupree, A.E., Zaino, R.J., Weber, J.A., 1987. Laboratory production in vivo of infectious human papillomavirus type 11. J. Virol. 61, 590–593.

Kreider, J.W., Howett, M.K., Wolfe, S.A., Bartlett, G.L., Zaino, R.J., Sedlacek, T.V., Mortelk, R., 1985. Morphological transformation in vivo of human uterine cervix with papillomavirus from condylomata acuminata. Nature 317, 639–641.

Kreider, J.W., Patrick, S.D., Cladel, N.M., Welsh, P.A., 1990b. Experimental infection with human papillomavirus type 1 of human hand and foot skin. Virology 177, 415–417.

Laimins, L.A., 1996. Human papillomaviruses target differentiating epithelia for virion production and malignant conversion. Semin. Virol. 7, 305–313.

Lancaster, W.D., Olson, C., 1982. Animal papillomaviruses. Microbiol. Rev. 46, 191–207.

Liang, H., Petros, A.M., Meadows, R.P., Yoon, H.S., Egan, D.A., Walter, K., Hozman, T.F., Robins, T., Fesik, S.W., 1996. Solution structure of the DNA-binding domain of a human papillomavirus E2 protein: Evidence for flexible DNA-binding regions. Biochemistry 35, 2095–2103.

McGlennen, R.C., Adams, G.L., Lewis, C.M., Faras, A.J., Ostrow, R.S., 1993. Pilot trial of ribavirin for the treatment of laryngeal papillomatosis. Head Neck 15, 504–513.

McMichael, H., 1965. Inhibition of growth of Shope rabbit papilloma by hypervitaminosis A. Cancer Res. 25 (7), 947–955.

O'Banion, M.K., Reichmann, M.E., Sundberg, J.P., 1988.

Cloning and characterization of a papillomavirus associated with papillomas and carcinomas in the European harvest mouse (*Micromys minutus*). J. Virol. 62, 226–233.

Ostrow, R.S., Coughlin, S.M., McGlennen, R.C., Johnson, A.N., Ratterree, M.S., Scheffler, J., Yaegashi, N., Galloway, D.A., Faras, A.J., 1995. Serological and molecular evidence of rhesus papillomavirus type 1 infections in tissues from geographically distinct institutions. J. Gen. Virol. 76, 293–299.

Ostrow, R.S., Forslund, K.M., McGlennen, R.C., Shaw, D.P., Schlievert, P.M., Ussery, M.A., Huggins, J.W., Faras, A.J., 1992. Ribavirin mitigates wart growth in rabbits at early stages of infection with cottontail rabbit papillomavirus. Antivir. Res. 17, 99–113.

Ostrow, R.S., McGlennen, R.C., Shaver, M.K., Kloster, B.E., Houser, D., Faras, A.J., 1990. A rhesus monkey model for sexual transmission of a papillomavirus isolated from a squamous cell carcinoma. Proc. Nat. Acad. Sci. USA 87, 8170–8174.

Phelps, W.C., Alexander, K.A., 1995. Antiviral therapy for human papillomaviruses: rationale and prospects. Annals Intern. Med. 123, 368–382.

Phelps, W.C., Howley, P.M., 1987. Transcriptional *trans*-activation by the human papillomavirus type 16 E2 gene product. J. Virol. 61, 1630–1638.

Shope, R.E., 1933. Infectious papillomatosis of rabbits with a note on the histopathology. J. Exp. Med. 58, 607–624.

Stanley, M.A., Masterson, P.J., Nicholls, P.K., 1997. In vitro and animal models for antiviral therapy in papillomavirus infections. Antivir. Chem. Chemother. 8 (5), 381–400.

Syverton, J.T., Dascomb, H.E., Koomen, J., Wells, E.B., Berry, G.P., 1950. The virus-induced papilloma-to-carcinoma sequence I. The growth pattern in natural and experimental infections. Cancer Res. 10, 379–384.

Stoler, M.H., Whitbeck, A., Wolinsky, S.M., Broker, T.R., Chow, L.T., Howett, M.K., Kreider, J.W., 1990. Infectious cycle of human papillomavirus type 11 in human foreskin xenografts in nude mice. J. Virol. 64, 3310–3318.

Tan, C.-H., Tachezy, R., Ranst, M.V., Chan, S.-Y., Bernard, H.-U., Burk, R.D., 1994. The mastomys natalensis papillomavirus: nucleotide sequence, genome organization, and phylogenetic relationship of a rodent papillomavirus involved in tumorigenesis of cutaneous epithelia. Virology 198, 534–541.

Van Ranst, M., Fuse, A., Fiten, P., Beuken, E., Pfister, H., Burk, R.D., Opdenakker, G., 1992. Human papillomavirus type 13 and pygmy chimpanzee papillomavirus type 1: comparison of the genome organizations. Virology 190, 587–596.

Watts, S.L., Ostrow, R.S., Phelps, W.C., Prince, J.T., Faras, A.J., 1983. Free cottontail rabbit papillomavirus DNA persists in warts and carcinomas of infected rabbits and in cells in culture transformed with virus or viral DNA. Virology 125, 127–138.

Wettstein, F.O., 1987. Papillomaviruses and carcinogenic progression. I: CRPV (Shope) papillomavirus. In: Salzman, N.P., Howley, P.M. (Eds.), The Papillomaviruses, vol. 2. Plenum, New York, pp. 167–186.

zur Hausen, H., 1994. Molecular pathogenesis of cancer of the cervix and its causation by specific human papillomavirus types. In: zur Hausen, H. (Ed.), Human Pathogenic Papillomaviruses. Springer Verlag, Heidelberg, pp. 131–156.

zur Hausen, H., Schneider, A., 1987. The role of papillomaviruses in human anogenital cancers. In: Salzman, N., Howley, P.M. (Eds.), The Papovaviridae, vol. 2. Plenum Press, New York, pp. 245–263.