Gene Therapy (2001) 8, 41–48

www.nature.com/gt

RESEARCH ARTICLE

Identification of polyamides that enhance adenovirus-mediated gene expression in the urothelium

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Adenovirus-mediated gene therapy of bladder diseases has been limited by the inability to transduce the urothelium successfully using adenoviral vectors. We have sought to identify agents that would increase adenovirus-mediated transgene expression in the bladder. We have utilized a rat model to screen compounds for their ability to enhance viral transgene expression in the rat bladder. Rats received intravesical administration of replication-deficient adenovirus (rAd) formulated in various agents, and transgene expression was evaluated after 48 h by determining the amount of lacZ expression in the luminal epithelium of the bladder. We report the identification of two different polyamides, each capable of dramatically increasing viral transgene expression in the bladder without causing detectable alteration of the umbrella cell layer of the urothelium. We have utilized a carcinogen-induced rat bladder tumor model to demonstrate that these polyamides are also capable of enhancing viral transgene expression in tumor tissue. The identification of these polyamides potentiates the use of adenovirus-mediated gene therapy for the treatment of superficial bladder cancer or other bladder diseases. Gene Therapy (2001) **8,** 41–48.

Keywords: *adenovirus; gene therapy; polyamides; bladder neoplasms; p53*

Introduction

One goal of gene therapy is the introduction of therapeutic genes into defective target cells. Currently viral vectors represent the optimal forum for the transfer of genetic material to a target cell. Recombinant, replication-deficient adenoviral vectors (rAd) are particularly attractive for this purpose due to their ease of manipulation and possible growth to high titers. For successful gene therapy treatment, efficient transduction of a high percentage of cells is desired; however, while many tissues exhibit a native tropism for adenovirus,[1] other tissues exhibit recalcitrance to infection. In particular, the bladder has been show to be resistant to infection.[2] Although the bladder urothelium can be transfected by rAd, initial efforts to infect the bladders of rats and mice using recombinant adenoviruses resulted in very low levels of transgene expression.[3] Although many mechanisms may prevent viral transgene expression, one possible explanation is that virus entry is prohibited due to the presence of an anti-adherence layer comprised of secreted glycosaminoglycans (GAG) reported to be present on the luminal epithelial surface of the bladder.[4] The glycosaminoglycan layer is a hydrophilic, polyanionic barrier that functions to prevent adherence of bacteria and viruses to the luminal epithelium, while providing an osmotic barrier to hypertonic urine and preventing reabsorption of water.[5] We hypothesized that abrogation of this barrier by organic reagents might enhance viral transgene expression. Indeed, previous work has shown that pretreatment of the bladder lumen with ethanol, a mucin-degrading organic reagent, significantly enhanced viral transgene expression.[6] Similarly, pretreatment of the luminal epithelium of the bladder with acetone has been shown to enhance adherence of bacillus Calmette–Guerin (BCG).[7]

Because modification of this barrier present at the luminal surface of the bladder can enhance viral transgene expression, we have sought to identify less aggressive agents that also enhance viral transduction of the urothelium. A variety of detergents were initially screened for their ability to enhance viral transgene expression to the urothelium. We report the identification of two different polyamide compounds, each capable of dramatically enhancing viral transgene expression in the urothelium when intravesically coadministered with rAd.

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Received 23 May 2000; accepted 14 September 2000

Results

rAd-mediated transgene expression in the rat urothelium

Initial attempts to infect the bladder using a recombinant adenovirus containing the *lacZ* reporter gene resulted in only low levels of transgene expression.[3] In an effort to abrogate existing barriers to virus entry, we tested various detergents at or above their critical micelle concentration (CMC) for their ability to enhance viral transgene expression, since detergents have been shown to enhance adenovirus gene transfer to the airway epithelium,[8]

increase intestinal uptake,[9] and may eradicate existing barriers of virus entry due to their solubilization properties. Rats received an intravesical administration of rAd-β-gal (0.5 ml at 7×10^{10} PN/ml; 2.1×10^{10} IU/ml) formulated in various detergents (Calbiochem, La Jolla, CA, USA), and transgene expression was assessed at 48 h by determining *lacZ* expression in the entire luminal surface of the bladder. For comparison, *lacZ* expression was determined in control bladders of rats that received intravesical administration of rAd-β-gal formulated in vPBS alone. Transgene expression was very low in the group of animals that received rAd in vPBS (<5% of the bladder luminal epithelium was transduced). In contrast, several cationic and some nonionic detergents demonstrated elevated transgene expression (Table 1). Interestingly, the nonionic detergent Tween-80 did not enhance viral transduction of the bladder as reported for intestinal uptake of small molecules,[10] even at 10× the CMC of this detergent. Many of the detergents that enhanced transduction were found to cause adverse events such as cystitis or bladder stones and were not evaluated further. One exception was the nonionic detergent Big CHAP. Using rAd-β-gal formulated in 7 mM Big CHAP, transgene expression was dramatically increased compared with rAd formulated in a vPBS buffer (Figure 1a and b, respectively). To evaluate the extent of viral transgene expression into the bladder wall, these bladders were paraffin embedded and 5-μm sections were stained using hematoxylin and eosin (H&E). *LacZ* expression was observed throughout the luminal epithelium of the bladder, primarily in the umbrella cell layer of the urothelium (Figure 1c).

Since tumor lesions may have altered glycosaminoglycan expression, as well as up-regulation of integrin expression,[11] we desired to determine if the Big CHAP formulation is needed for enhancement of viral transgene expression in tumor tissue in addition to normal urothelium. Bladder tumors were induced in Fisher rats by addition of the carcinogen BBN (N-N-Butyl-N-butane-4d-nitrosamine; TCI America, Portland, OR, USA) to their drinking water.[12] When these tumor-bearing animals received intravesical administration of rAd-β-gal formulated in 7 mM Big CHAP, significant enhancement of viral transgene expression was observed (Figure 2a). In contrast, no transduction of either normal or neoplastic tissue was seen when rAd was delivered in a vPBS formulation (Figure 2b). Histological examination of sections from these bladders that received rAd-β-gal in the Big CHAP formulation revealed that the *lacZ* expression occurs in both tumor tissue and in normal urothelium (Figure 2c), with differential transduction observed within the same tumor. These variations in transduction within the same tumor may reflect intrinsic differences in the transducability of the various cell types in the tumor. Indeed, cells grown from tumors excised from male Fisher rats with advanced BBN-induced tumors showed variation in rAd-tropism when tested *in vitro* (data not shown). The enhancement of viral transgene expression by Big CHAP in tumor tissue suggests that either the tumor tissue still retains a similar GAG layer as normal urothelium, or that the enhancement mechanism is independent of either the glycosaminoglycan composition or integrin expression.

Further characterization of the enhancement activity of Big CHAP revealed variability among different commercially available preparations, suggesting heterogeneity in the detergent's composition. In fact, some preparations of Big CHAP failed to enhance viral transgene expression altogether. When a bioactive lot of Big CHAP was compared with an inactive lot by thin layer chromatography, at least three additional compounds were present in the

Table 1 Enhancement of viral transgene expression by detergent formulations

Charge of detergent	*Type of detergent*	*Levels of viral transgene expression*	*Adverse events*	*Virus stability*
—	vPBS	0	none	+
Cationic	Benzalkonium chloride	0/+	none	–
	Cetylpyridinium	+	none	–
Anionic	Taurocholate	+	none	ND
	Deoxycholate	+	Cystitis	ND
	Taurodeoxycholate	+++	Cystitis	+
Zwitterionic	Zwittergent 3-14	+++	Stone formation	ND
	CHAPS	+++	Stone formation	+
Nonionic	Big CHAP	+++	none	+
	Deoxy Big CHAP	+++	Cystitis	ND
	C12E8	++	none	ND
	Octyl-β-D-glucopyranoside	++	none	ND
	Pluronic F68	+	none	+
	Tween-20	+	none	+
	Tween-80	+	none	+

Various detergents were assayed for their ability to enhance viral transgene expression relative to virus formulated in vPBS alone. Recombinant adenovirus (0.5 ml at 7×10^{10} PN/ml) was intravesically administered in a detergent solution prepared in vPBS at their CMC. (All detergents were obtained from Calbiochem, La Jolla, CA, USA.) After 48 h, the bladders were harvested and transgene expression estimated based upon the percentage of the luminal urothelium exhibiting β-galactosidase expression. Levels of transgene expression (column 3) in whole bladders were scored as follows: 0–25% transduction: (0); 25–50% transduced: (+); 50–75% transduced: (++); >75% transduction: (+++). Image analysis performed using Image-Pro software. Adverse events that arose as a result of detergent treatment are also indicated (column 4). To determine virus stability (column 5), rAd was diluted into detergent for 60 min in the indicated detergent, and stability measured by evaluating elution/retention from an HPLC Resource Q column:[19] –, not stable; +, stable; ND, not determined.

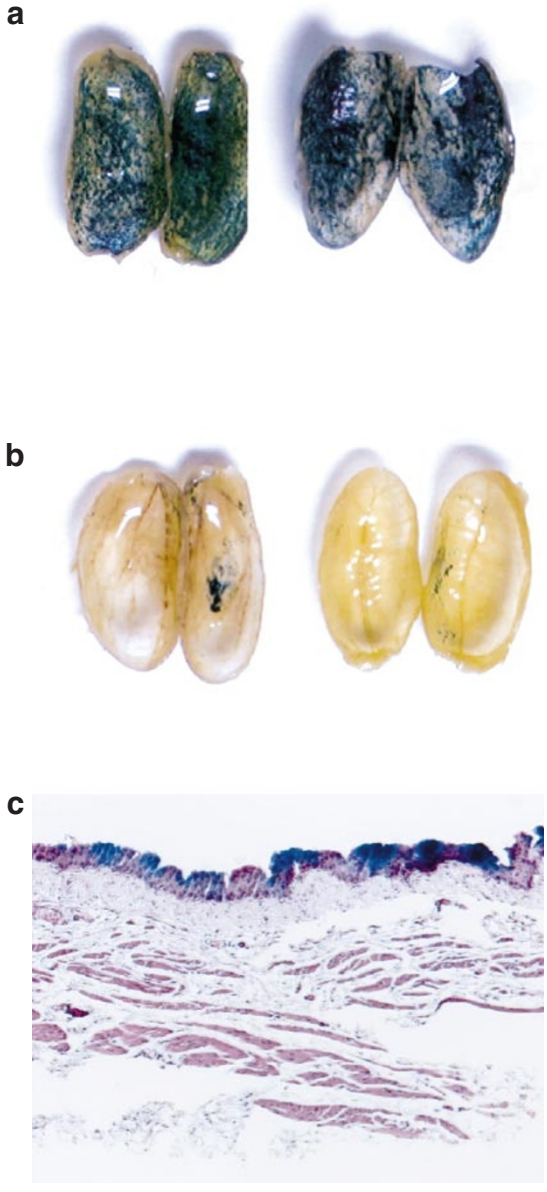


Figure 1 *Enhancement of viral transgene expression in the bladders of normal rats. Normal Sprague–Dawley rats were intravesically administered rAd-β-gal (7×10^{10} PN/ml; 0.5 ml) formulated in either 7 mM Big CHAP (a) or vPBS (b). Animals were killed 48 h after treatment, their bladders harvested, fixed and transgene expression determined by whole organ X-gal staining. Depicted are two representative whole rat bladders (10 animals tested per group) from each group. Bladders from animals that received rAd-β-gal formulated in Big CHAP had significantly higher viral transgene expression in the luminal epithelium compared had control animals that received virus formulated in vPBS. To determine the extent of lacZ expression, bladders from rAd-β-gal/Big CHAP animals were paraffin embedded and 5-μm sections stained with hematoxylin and eosin (H&E) (c). Transgene expression was primarily confined to the umbrella cell layer of the urothelium, with some minor expression observed in the lamina propria of the bladder.*

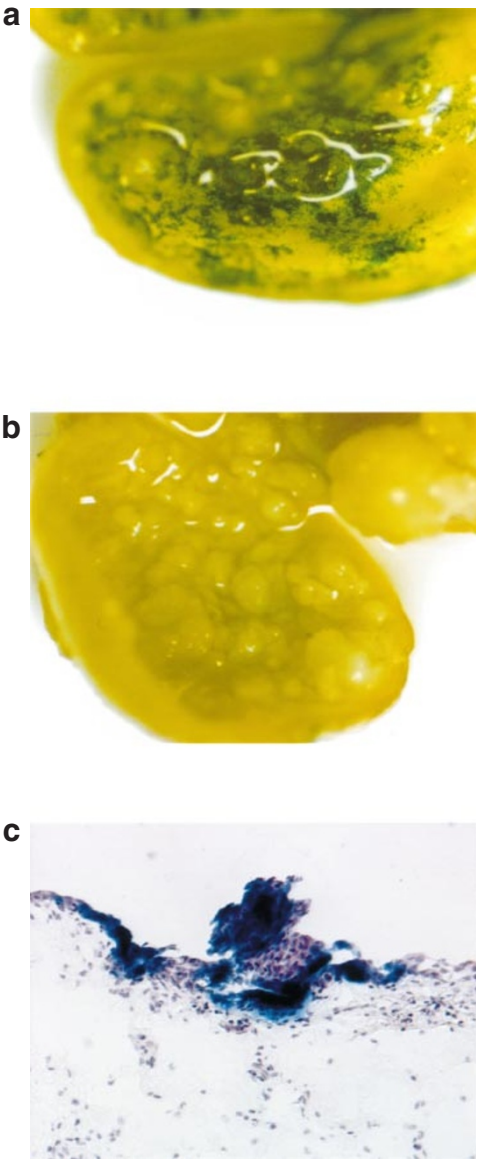


Figure 2 *Enhancement of viral transgene expression in the bladders of tumor-bearing rats. Bladder tumors were induced in male Fisher rats by the introduction of the carcinogen BBN to the drinking water (0.05%). After development of tumors these animals were intravesically administered rAd-β-gal formulated in 7 mM Big CHAP. After 48 h, animals were killed and transgene expression evaluated by whole organ X-gal staining. (a) Bladders that received rAd-β-gal formulated in 7 mM Big CHAP had significant enhancement of viral transgene expression compared to (b) control bladders that received rAd-β-gal formulated in vPBS. (c) Histological examination of a tumor-bearing bladder that received rAd-β-gal formulated in Big CHAP demonstrates lacZ expression in both tumor tissue as well as in normal urothelium.*

bioactive lot. MALDI-MS, ^{1}H-NMR and ^{13}C-NMR structural analysis indicated that the three additional components were most likely byproducts from Big CHAP manufacturing processes. Each impurity was isolated and assayed for its ability to enhance viral transgene expression in the bladder by coadministration with rAd-β-gal (Table 2). Impurity No. 1, the methyl ester of cholic acid, was found to be inactive. Although impurity No. 2 had limited ability to enhance viral transgene expression, by far the most potent compound for enhancing gene expression was impurity No. 3. Structurally, impurity No. 3 resembled Big CHAP with a substitution of a second cholyl group for one of the gluconic acid moieties of Big CHAP. However, this impurity was found to be extremely insoluble, probably due to an increase in hydrophobicity resulting form the addition of the second

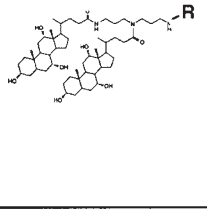
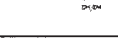
Table 2 Assessment of Big CHAP components for their ability to enhance viral transgene expression.

Chemical composition	*MW*	*Enhancement of viral transgene expression*	*Solubility in vPBS*
Unpurified Big CHAP	—	++	++
Impurity No. 1	422.6	0	0
Impurity No. 2	1268.2	+	+
Impurity No. 3	1090.2	++	0/+
Purified Big CHAP	878.1	0	++

Compounds were isolated by flash chromatography on silica gel 60 from a bioactive lot of Big CHAP, and assayed for their ability to enhance viral transgene expression. The Table summarizes the different compounds obtained by fractionation of Big CHAP by TLC. Shown are their molecular weights as determined by MALDI-MS (column 2), the approximate ability of the impurity to enhance viral transgene expression compared with control-treated bladders (column 3), and the relative solubility of these compounds in aqueous solution (column 4). Enhancement of viral transduction of the urothelium and solubility were quantified as follows: 0, low viral expression/insoluble; +, moderate viral expression/low solubility; ++, high viral expression/complete solubility.

cholyl group. In an effort to prepare a more soluble analog of impurity No. 3, we hypothesized that the introduction of additional carbohydrate might increase hydrogen bonding and increase the solubility of the analog in aqueous solution. Therefore, two synthetic analogs were prepared by substitution of either lactobionic or melibionic acid for the single gluconic acid moiety of impurity No. 3: Synthetic Lactobionic analog No. 3 (Syn3-Lac) and Synthetic-Melibionic analog No. 3 (Syn3-Mel) (Table 3). When tested for solubility, Syn3-Lac and Syn3-Mel (Syn3 analogs) had greater solubility than impurity No. 3, but both still required a detergent formulation for dissolution (eg Tween-80). Syn3-Mel had the greatest solubility, and could also be dissoluted into a non-detergent formulation.

Table 3 Structure of Syn3 analogs Syn3-Lac, Syn3-Mel, and Syn3-Suc

Syn3 Analog	Donor Group	R-Group
Syn3-Lac		
Syn3-Mel		
Syn3-Suc		

Various analogs were prepared by conjugation of either lactobionic acid, melibionic acid, or succinic anhydride (column 3) to the conserved dicholyltriamine of Syn3 (column 2).

Enhancement of viral transgene expression in the urothelium by Syn3-Lac and Syn3-Mel

Syn3-Lac and Syn3-Mel were tested for their ability to enhance transduction of the urothelium by administration of rAd-β-gal formulated in a solution of the Syn3 analog (1 mg/ml in 0.1% Tween-80/vPBS; 0.8 mM). For comparison, control rats received an intravesical administration of rAd-β-gal formulated in the vehicle control (0.1% Tween-80/vPBS). After 48 h, bladders were harvested and evaluated for *lacZ* expression by whole organ X-gal staining. Dramatic enhancement of viral transgene expression was obtained for bladders treated with rAd-β-gal in Syn3-Lac (Figure 3a) and Syn3-Mel (Figure 3b) compared with virus administered in vehicle control (Figure 3c). No *lacZ* expression was observed in bladders that were treated with the Syn3-Lac alone (Figure 3d) or in untreated bladders (Figure 3e). To determine the extent of transgene expression, 5 μm sections of bladders treated with rAd-β-gal formulated in 0.8 mM Syn3-Lac were hematoxylin and eosin stained. β-Galactosidase activity was detected throughout the umbrella cell layer of the urothelium as well as in the underlying epithelial layer, and at low levels in the lamina propria of the bladder (Figure 4a). No *lacZ* expression was observed in sections of bladders that received rAd-β-gal formulated in vPBS (Figure 4b) or the Syn3 formulation alone (Figure 4c). No alteration or removal of the umbrella cell layer was observed for the bladders treated with rAd formulated in a Syn3 analog 48 h after treatment, the time of transgene expression evaluation.

Initial studies utilized the *lacZ* reporter gene. However, we desired to determine whether the Syn3 analogs might enhance expression of other nuclear localized therapeutic proteins. Since one approach for the treatment of superficial bladder cancer could be the delivery of the p53

Figure 3 *Enhancement of viral transgene expression by the synthetic polyamides Syn3-Lac and Syn3-Mel. Rats that received intravesical rAd-β-gal formulated in either Syn3-Lac or Syn3-Mel had dramatic enhancement of lacZ expression throughout the luminal surface of the urothelium. Shown are representative X-gal stained whole rat bladders (12 animals tested per group) from animals that received a single bolus of rAd-β-gal (7×10^{10} PN/ml; 0.5 ml) formulated in a 0.8 mM solution of either (a) Syn3-Lac or (b) Syn3-Mel or (c) vehicle control. No lacZ expression was observed in rats that were intravesically administered a 1 mg/ml solution of Syn3 alone (d), or in an untreated rat bladder (e).*

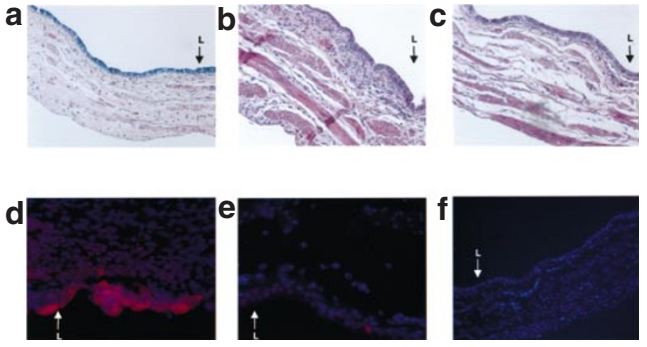


Figure 4 *β-Galactosidase and p53 transgene expression in situ. Enhancement of viral transgene expression by Syn3-Lac was determined in rat bladders following intravesical administration of either rAd-β-gal (a–c) or rAd-p53 (d–f). Formalin-glurtaraldehyde fixed bladders were paraffin embedded, and 5-μm sections prepared for either H&E staining, or p53 detection by immunohistochemistry. (a) Transduction of the entire luminal surface of the urothelium occurs when adenovirus is delivered in a Syn3-Lac formulation. (b) No lacZ expression was observed following administration of adenovirus in a vehicle (vPBS/Tween-80) formulation. (c) No lacZ expression is observed following intravesical administration of the Syn3-Lac formulation only. (d) Immunohistochemistry of bladder tissue revealed that high levels of p53 expression (red) resulted from intravesical administration of rAd-p53 in a Syn3-Lac formulation, primarily in the luminal epithelium. (e) No detectable expression of p53 was seen in bladders that received rAd-p53 in the vehicle formulation, or in bladders that received an empty vector adenovirus (rAd-EV) formulated in Syn3-Lac (f). In all panels (L) denotes the luminal epithelium. Nuclei are shown counterstained using DAPI (blue).*

tumor suppressor gene, we wanted to determine if Syn3-Lac could enhance the expression of a recombinant adenovirus containing human p53 (rAd-p53).[13] Rats received an intravesical administration of rAd-p53 (0.5 ml at 7×10^{10} PN/ml; 3.7×10^9 IU/ml) formulated in either Syn3-Lac (0.8 mM) or vehicle control and their bladders harvested at 48 h to determine p53 transgene expression. Rat bladders that were administered rAd-p53 formulated in Syn3-Lac (Figure 4d) had significantly higher viral transgene expression compared with rats that received rAd-p53 formulated in vehicle control (Figure 4e), analogous to results obtained with the *lacZ* reporter gene. Control rat bladders that were administered an adenovirus with no transgene (rAd-empty vector) formulated in Syn3-Lac (0.8 mM) had no detectable p53 transgene expression (Figure 4f).

In order to determine if the Syn3 analogs increase viral expression by transcriptional activation or by better distribution of rAd within the bladder, we took advantage of the observation that the Syn3-mediated enhancement of gene expression did not require coadministration of rAd with the Syn3 analog. Pretreatment of the urothelium with Syn3-Lac followed by intravesical administration of rAd-β-gal (45 min per treatment) gave equally high levels of viral transgene expression as coadministration (Figure 5a). However, when bladders were first preadministered rAd-β-gal and subsequently administered Syn3-Lac analog (45 min per treatment) only minimal viral transgene expression was observed (Figure 5b), suggesting that the polyamides do not function by transcriptional activation of rAd. Therefore, the Syn3 analogs likely enhance viral gene expression by facilitating greater viral attachment to the urothelium. Indeed, electron microscopy reveals that the urothelium treated with rAd formulated in Syn3-Lac has many virus particles

Figure 5 *Temporal administration of the Syn3 analog suggests that enhancement of viral transgene expression is not mediated by rAd transcriptional activation. In order to understand the mechanism whereby Syn3-Lac enhances viral transgene expression, bladders were treated with the polyamide either before or after exposure to rAd-β-gal. Bladders depicted are representative of two separate experiments. (a) Although highest levels of gene expression occurs with coadministration of rAd and the Syn3 analog, rats that received intravesical administration Syn3-Lac before administration of rAd-β-gal had significantly elevated levels of lacZ expression compared with controls. (b) Rat bladders that received intravesical administration of rAd-β-gal before Syn3-Lac gave minimal transduction, suggesting that transcriptional activation of the viral transgene is not the primary mechanism of enhanced viral transduction of the urothelium.*

attached to the epithelium and taken up by cellular vesicles. (Figure 6a) compared with urothelium treated with rAd formulated in vehicle control (Figure 6b).

In an effort to identify a more soluble analog of Syn3-Lac/Mel, we replaced the disaccharide group of Syn3-Lac/Mel with succinate (Suc) to obtain the sodium salt: Syn3-Suc (see Table 3). However, when Syn3-Suc was intravesically administered to rat bladders, it did not enhance viral transgene expression, even at concentrations as high as 2 mM (data not shown). Therefore, not only do the dicholyl moieties of the Syn3 analogs appear to be necessary for enhancing activity, but the saccharide group of the Syn3 analogs may be an additional structural requirement for bioactivity.

Discussion

Adenovirus-mediated gene therapy for the treatment of bladder disease has been impeded by the inability to transduce the urothelium successfully. It has been hypothesized that viral binding and internalization is prevented by the presence of an anti-adherence barrier comprised of secreted glycosaminoglycans (GAG).[6] The GAG layer is believed to function as an impenetrability barrier, preventing dehydration from hypertonic urine, as well as protecting the transitional epithelium from the toxic effects of hyperosmotic urine and bacterial adhesion.[4] Water molecules bind tightly to sulfated polysaccharides, forming a barrier between urine and the bladder luminal

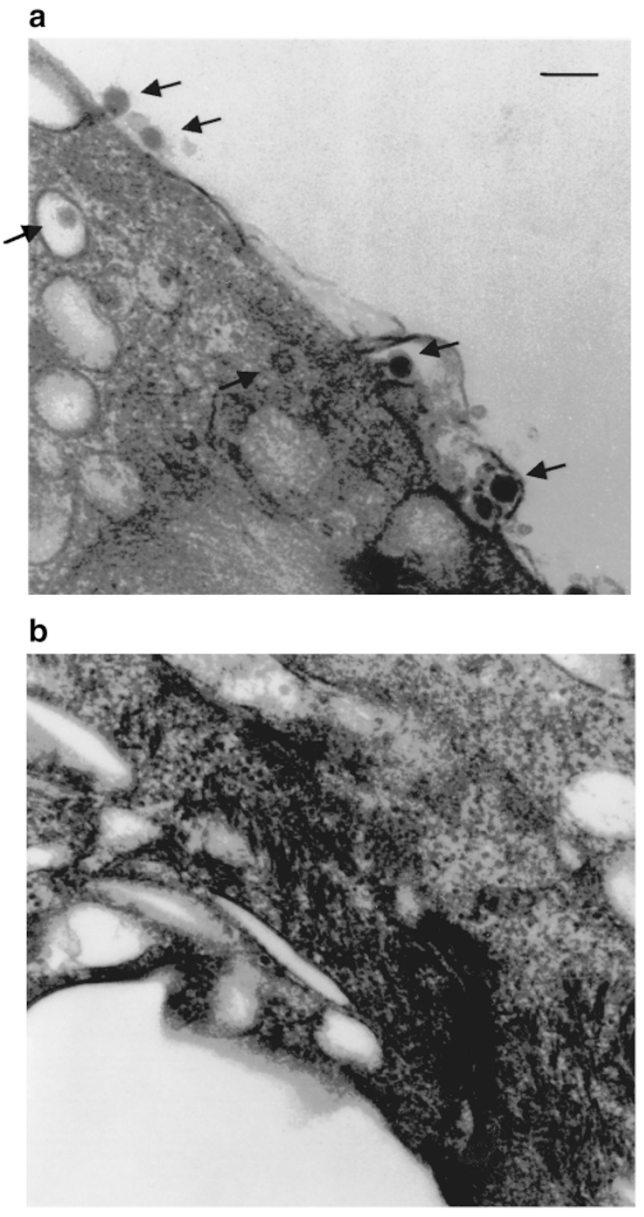


Figure 6 *Electron microscopy demonstrates that the Syn3 analogs facilitate increased viral attachment to the urothelium. Rats received an intravesical administration of rAd-β-gal formulated in either Syn3-Lac or vehicle control for 45 min and their bladders were harvested immediately after removal of the dosing material. Shown are negatively stained electron micrographs depicting the umbrella cell layer present on the luminal epithelium. Typically shaped viral particles are recognizable by the hexagonal shape of the virus particle (arrows). (a) Viral particles attached to the epithelium and taken up into vesicles of the umbrella cell layer were only observed in the Syn3-Lac treated animals. (b) No virus is observed in the urothelium of animals that received rAd formulated in the vehicle control. Scale bar, 200 nm.*

surface.[5] We have sought to identify agents that could render this barrier susceptible to adenovirus infection, and report the identification of two polyamides that dramatically increase adenovirus-mediated transgene expression to the bladder epithelium *in vivo*.

Various explanations for this increase in transgene expression can be proffered. First, to discern the mechanism of the Syn3 analogs, structural comparison of the analogs to the parent compound, Big CHAP, is useful. Highly purified preparations of Big CHAP have no enhancement of viral transgene expression; in contrast, substitution of one of the gluconic acid moieties of Big CHAP with a second cholyl group yields potent enhancing agents (Syn3-Lac and Syn3-Mel). If an interaction exists between the Syn3-Lac/Mel analog and adenovirus, then the addition of a second cholyl group may promote viral adhesion to the urothelium, in effect causing a greater local concentration of virus that consequently results in greater viral uptake and transgene expression.

Another possible explanation could be that the Syn3 analogs enhance transduction of the urothelium by removal of a nonspecific anti-adherent barrier on the urothelium. Big CHAP and Syn3-Suc possibly fail to enhance viral transgene expression *in vivo* because they are too hydrophilic to partition into the water-excluding GAG. In contrast, Syn3-Lac and Syn3-Mel could be sufficiently hydrophobic that they can solubilize the GAG layer and consequently render this polyanionic barrier susceptible to viral infection. Indeed, bacterial adherence has been shown to be increased by removal of GAG either by ethanol treatment,[14] or by mild acid treatment.[15,16] We have observed that rAd-β-gal coadministered with ethanol results in enhanced viral transgene expression in the urothelium.[6] Since ethanol is known to alter transiently mucins of the gastro-intestinal mucosa,[9] it could likely alter the mucins of the urinary tract system, consistent with abrogation of the bladder GAG barrier.

The GAG may not be solely responsible for the inability to transduce the urothelium. Adenovirus cellular entry involves two separate and uncoupled events. Virus binds first to the host cell through the high affinity interaction between the knob-shaped globular carboxy-terminal domain of the fiber protein that protrudes from the viral capsid and the coxsackie-adenovirus receptor (CAR).[17] Following binding of the virus to CAR, internalization of the virus is mediated via the RGD sequences in the penton base with αvβ3 integrins.[18] The Syn3 analogs may enhance the avidity of rAd for the binding of these proteins on the target cells. Further experiments are ongoing to determine the involvement of these mechanisms.

For clinical cancer gene therapy, reintroduction of the p53 gene using rAd has already been shown to be a viable approach.[19] Superficial bladder cancer is an attractive target for p53 gene therapy due to the easy accessibility of the bladder and the frequent p53 mutations found in tumor tissue.[20] Our results demonstrate that coadministration of rAd with these polyamides potentiates the delivery of therapeutic genes to bladder tumor tissue as well as in normal urothelium. These compounds may facilitate rAd-mediated-p53 gene therapy treatment of superficial bladder cancer and may be useful for gene therapy of other bladder diseases. The tumor suppressor gene p53 is frequently altered in bladder cancer, and is often used as an indication of the progression of the disease.[21] Syn3 analogs have been shown to increase transgene expression in tumor-bearing bladder tissue and thus may facilitate p53 gene therapy of superficial bladder cancer.

It is possible that these compounds may facilitate enhancement of adenoviral gene transfer to other epithelium that contain glycosaminoglycans (eg synovium), or to other epithelial tissues that have previously shown recalcitrance to adenovirus infection, such as airway epi-

thelium.[22] Future work determining the mechanism of enhancement for these polyamides could reveal other indications where the Syn3 analogs may potentiate adenovirus-mediated gene therapy.

Materials and methods

Preparation of virus stocks

Recombinant adenovirus (rAd) utilized for these experiments was derived from human type 5 adenovirus that was rendered replication deficient by deletion of its E1A and E1B regions. rAd-β-gal and rAd-p53 were prepared by insertion of the *lacZ* reporter gene or human p53 gene into the deleted E1 region respectively, both driven by a CMV promoter. rAd-empty vector (rAd-EV) contained the same viral backbone as rAd-β-gal, with only a CMV promoter inserted into the deleted E1 region (no transgene). Recombinant adenoviruses were purified by column chromatography, and purified virus concentrations were determined in particles per milliliter (PN/ml) and infectious particles (IU)/ml upon elution from a Resource Q anion exchange HPLC column.[23] The purified rAd concentration was formulated in Dulbecco's PBS buffer containing 3% sucrose and 2 mM $MgCl_2$. (vPBS). The concentration of all rAd-β-gal used was approximately 7×10^{11} PN/ml/2.1×10^{10} IU/ml, and the concentration of rAd-p53 was 7×10^{11} PN/ml/3.7×10^{9} IU/ml.

Chemical analysis

TLC analysis of Big CHAP detergent composition: Thin layer chromatography (TLC) analysis was utilized for assessing the purity of Big CHAP preparations. Material was separated on silica gel plates using a 1:6:5 water/chloroform/methanol mobile phase, and visualized by heating. Bands were isolated preparatively by HPLC and analyzed by mass spectrometry. Analogs were synthesized by a modification of the synthesis of Big CHAP.[24] In brief, the glycosidic R-group must be converted from lactobionic acid or melibionic acid to the lactone. This lactone is then coupled to one of the primary amines of diethylenetriamine (Sigma, St Louis, MO, USA) by using an excess of the triamine. After addition of the R-group, two chloyl groups are added to the derivatized triamine and the compound-purified using silica gel and reverse phase chromatography. To prepare the Syn3-Suc analog, succinic anhydride was substituted for the lactone.

Animal studies

Induction of bladder tumors in rats by addition of BBN into drinking water: Bladder tumors were induced in adult female Fisher rats by the addition of the carcinogen BBN (N-N-Butyl-N-butane-4d-nitrosamine (TCI America, St Louis, MO, USA)) to their drinking water (0.05%).[12] Bladder tumors developed in these animals after approximately 3 to 4 months.

Evaluation of transgene expression in normal and tumor bearing rats: Enhancement of adenovirus-mediated transgene expression was evaluated in female rats (approximately 250 g) as previously described.[6] Briefly, rats were anesthetized with isofluorane and a PE50 catheter inserted via the urethra into the bladder. Bladders were preflushed using 0.5 ml of Dulbecco's PBS and emptied before administration of the 0.5 ml bolus of either rAd-β-gal or rAd-p53 diluted in the appropriate detergent formulation. After 45 min, the dosing material was removed, and the bladder flushed again with 0.5 ml of PBS. Animals were allowed to recover, and killed 48 h later. The bladders were fixed by clamping the urethra and injecting whole organ fixative (2% neutral buffered formalin, 2% glutaraldehyde, 2 mM $MgCl_2$, 10 mM PBS, pH 7.4) through the bladder wall. Bladders were then removed and immersed in whole organ fixative for 1 h. Following fixation, the bladders were sectioned longitudinally, and washed for 24 h at 4°C in rinse buffer (2 mM $MgCl_2$, 0.1% sodium deoxycholate, 0.2% IGEPAL-20, 10 mM PBS, pH 7.4). To detect transgene expression, the bladders were submerged in chromogen (X-gal: 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, Gibco, Rockville, MD, USA) for 4 h. Following X-gal staining, bladders were photographed and paraffin embedded for histological examination.

To detect rAd-mediated p53 expression, bladders were fixed in whole organ fixative as described above, embedded in paraffin, and slides were prepared (5-μm sections). Before immunohistochemical staining, antigen retrieval was performed by microwaving rehydrated sections using citrate buffer, pH 6. For immunofluoresence, primary human specific polyclonal antibody CM1 (Novocastra (Vector Laboratories), Burlingame, CA, USA) was diluted 1:5000 in 0.1% BSA/PBS (rabbit IgG diluted 1:5000 was used for negative controls) and incubated on the section for 90 min, followed by a Texas-Red goat anti-rabbit secondary antibody diluted 1:200. DAPI nuclear stain was utilized (diluted 1:1000) in antifade (Molecular Probes, Eugene, OR, USA). Photographs were obtained using a Nikon Optiphot microscope (100 ×) and a Hamamatsu CCD video system (C5810) (Canji, San Diego, CA, USA).

Electron microscopy

For electron microscopy, rats received an intravesical dose of rAd-β-gal formulated in either 1 mg/ml Syn3-Lac or vehicle. Bladders were treated for 45 min and harvested immediately after the removal of the dosing material. Following tissue harvest, the bladders were fixed in glutaraldehyde, negatively stained with uranyl acetate and ultrathin sections prepared and imaged using a Hitachi H-600 scanning transmission electron microscope at 74 kV (The Burnham Institute, La Jolla, CA, USA).

Acknowledgements

We would like to thank Stephen Chang, PhD Scientific Director, Canji, Inc. for scientific advice, Ken Wills, PhD, Molecular Biology, Canji, Inc., and Beth Hutchins, PhD, Process Sciences, Canji, Inc. for providing the rAd constructs and Debbie Muilwijk for assistance with the development of the BBN rat tumor model.

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