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Targeted delivery of a substrate for P-glycoprotein to renal cysts in vitro

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Abstract

Bodipy-verapamil has been tested as a fluorescent substrate for P-glycoprotein-mediated transepithelial secretion in MDCK-C5A epithelia. Net transepithelial secretion (J_{net}) of [^{3}H]vinblastine from basal-to-apical surfaces of monolayer epithelia is inhibited by taxotere, verapamil and Bodipy-verapamil primarily by a reduction in basal to apical vinblastine ($J_{b\text{-}a}$) transport. Bodipy-verapamil is itself subject to transepithelial net secretion by MDCK-C5A epithelia; at 5 μM a J_{net} of -310 ± 32 nmol cm^{-2} h^{-1} ($n = 3$) was observed. When MDCK-C5A cells are grown to form enclosed cysts in hydrated collagen gel, Bodipy-verapamil is accumulated within the cyst lumen showing that epithelial P-glycoprotein function may be used to target substrates to renal cysts.

Keywords: Renal cyst; P-glycoprotein; Glycoprotein; Vinblastine; Verapamil; MDCK; Drug delivery

1. Introduction

Cystic disease of the kidney is a major cause of renal failure. Autosomal dominant polycystic disease is associated with the formation in the renal parenchyma of enclosed fluid filled cysts [1,2]. The mechanism of fluid secretion and renal cyst expansion has been the subject of increasing study in order to indicate potential therapeutic opportunities [1,2]. Growth of renal epithelial cells in vitro within hydrated collagen gels is associated with the formation of cysts [1,2]. Salt and fluid secretion across the epithelial wall is associated with cyst enlargement; stimulation of epithelial fluid secretion by agents that raise intracellular cAMP such as forskolin, dibutyryl cAMP, arginine vasopressin, PGE_1, PGE_2 and cholera toxin is associated with increased cyst size and fluid secretion [1,3]. Inhibition of fluid secretion by ion transport inhibitors such as ouabain, amiloride and the stillbene, DIDS, have been documented [4]. Recently it has been shown, using in vitro cyst assays, that whilst inhibitors of DNA, RNA and protein synthesis do not prevent cyst formation, the microtubule inhibitors colchicine, vinblastine and taxol are effective [5]. In an in vivo recessive mouse model (cpk) of congenital polycystic disease, taxol was also able to prevent uraemic death of homozygous (cpk/cpk) animals with treated animals reaching normal size and with minimal loss of renal function [5].

P-glycoprotein is a 170–180 kDa membrane glycoprotein associated with the phenomenon of pleiotropic (multidrug) resistance (MDR). Substrates include a variety of cytotoxic agents such as colchicine, vinblastine and taxol [6]. Immunohistochemical techniques have demonstrated the presence of P-glycoprotein in the apical regions of several natural epithelia [7–10], including the kidney. Renal epithelial cell-lines capable of reforming intact epithelial layers when grown upon permeable matrices (e.g., MDCK, LLCPK1) mediate epithelial secretion (from basal to apical cell surfaces) of P-glycoprotein substrates such as vinblastine [11–14]. Retroviral transfection of the dog kidney epithelial cell-line (MDCK) with mdr1 cDNA results in polarised expression of P-glycoprotein to the apical plasma membrane domain [15] and an enhanced transepithelial secretion of vinblastine above intrinsic levels. The purpose of the present investigation was to determine if P-glycoprotein substrate transport could act to concentrate substrates for P-glycoprotein within cyst fluid so acting as a long-term reservoir.

We have chosen to examine transepithelial secretion of MDR substrates in a clonal MDCK line selected for its ability to form fluid-filled cysts when cultured within a hydrated collagen gel [16]. We show that this cell-line when reconstituted as a monolayer will maintain a highly

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polarised secretion of the MDR substrate vinblastine and that this is inhibited by verapamil, dideoxyforskolin and taxotere (a structural analogue of taxol, [17]). Moreover, a fluorescent verapamil derivative (Bodipy-verapamil) [18] inhibits vinblastine secretion, whilst being subject to transepithelial secretion itself. In MDCK cysts confocal laser-scanning microscopy of Bodipy-verapamil has been used to directly demonstrate accumulation within the cyst lumen after incubation, whilst there is retention within the cyst fluid after washing and incubation in substrate-free medium. The effective targeting and retention of P-glycoprotein substrates to renal cysts is likely to be an important factor in the efficiency of P-glycoprotein substrates such as vinblastine and taxol to inhibit cyst formation in vitro and in vivo [5].

2. Materials and methods

2.1. Materials

[^{3}H]Vinblastine sulfate was purchased from Amersham International (Little Chalfont, Amersham, Bucks). Bodipy-verapamil was from Molecular Probes (Eugene, Oregon). Taxotere was a gift from Dr. J.L. Fabre, Rhône-Poulenc Rorer, Vitry-sur-Seine, France. All tissue culture media and reagents (Gibco BRL) and tissue culture plastics including tissue culture inserts (Nunc) were supplied by Life Technologies (Paisley, UK). All other chemicals were obtained from Sigma (Poole, Dorset) or BDH Chemicals (Poole, Dorset).

2.2. Cell culture

A cloned MDCK Strain C5A was a gift from Prof. J.J. Grantham (University of Kansas Medical Center, Kansas) [16]. MDCK-C5A has enhanced ability to form cysts in hydrated collagen gel, in the absence of cystogens. MDCK-C5A cells were cultured in minimum essential medium (Earle's salts) (MEME) supplemented with 10% (v/v) fetal calf serum, kanamycin antibiotic (1 μg/ml), non-essential amino acids and 2 mM glutamine. Stock cultures were subcultured every 7 days, by treatment with 0.05% trypsin and 0.2% EDTA in Ca^{2+}-and Mg^{2+}-free phosphate-buffered saline. Epithelial layers were prepared by high-density seeding (10^6 cells/cm^2) onto Nunc Anopore 25 mm culture inserts 4 cm^{-2} growth area. Epithelia were used 2–3 d after seeding, after transepithelial resistance (R_t), exceeded 750 Ω cm^2 measured using a WPI Evometer.

MDCK-C5A cysts were prepared by seeding ($0.3 \cdot 10^6$ cells/ml) into liquid collagen (prepared from rat tails)/MEME before gel formation [19]. All cultures were incubated at 37° C in a humidified atmosphere of 5% CO_2/95% air.

2.3. Transport experiments

Measurements of transepithelial solute flux were made essentially as described previously [12,13]. Briefly, the cell monolayers were extensively washed (four times in 500 ml of modified Krebs buffer of composition, all mmol/l: NaCl, 140; KCl, 5.4; $CaCl_2$, 2.8; $MgSO_4$, 1.2; NaH_2PO_4, 0.3; KH_2PO_4, 0.3; Hepes, 10; glucose, 5 (pH to 7.5 at 37° C with Tris base)), placed in 6-well plates, each well containing 2 ml of prewarmed modified Krebs. Aliquots of Krebs buffer were then placed in the upper filter cup (apical solution). Radiolabelled vinblastine was used at tracer concentrations (0.25 μCi/ml, 8–12 nM); Bodipy-verapamil was used alone or in conjunction with vinblastine at 1–10 μM. Matched pairs of epithelial layers were used to trace either apical-to-basal solute (vinblastine and or Bodipy-verapamil) flux ($J_{a\text{-}b}$) or basal-to-apical flux ($J_{b\text{-}a}$). Aliquots (200 μl) of fluid from each chamber were removed at predetermined times (1–3 h) for determination of ^{3}H activities (dpm) by liquid scintillation spectrometry, with appropriate corrections for quench and efficiency, using a Beckman LS 5000CE. The concentration of Bodipy-verapamil in sample aliquots was determined by fluorescence spectroscopy (Perkin-Elmer luminescence LS-5 spectrometer, excitation 480 nm, emission 510 nm). Solute permeation across the monolayers into the contralateral chamber is expressed as flux mol cm^{-2} h^{-1} or as a permeability ($P_{a\text{-}b} = J_{a\text{-}b}/C_a$), where C is the concentration of solute in the ipsilateral chamber. At the end of the incubation period cell monolayers were washed in 4 × 500 ml volumes of ice-cold Krebs buffer (pH 7.5) to remove any loosely-associated radiolabel, and removed from the insert. Cell monolayer-associated radiolabel was determined by solubilisation in scintillation fluid and scintillation counting. Cellular accumulation of vinblastine is expressed as mM or as a cell to solution (C/M) ratio.

2.4. Microscopy

Renal cysts in hydrated collagen gel were visualised directly by confocal laser scanning microscopy (CLSM, Bio-Rad MRC-600, argon-ion laser, Hemel Hempstead, U.K., attached to a Nikon Diaphot microscope). The objective used was a Nikon oil immersion ×60, numerical aperture 1.4 giving an optical section of approx. 1 μm depth. Cell height was determined by confocal imaging of intact cell layers and this value was used in the determination of intracellular water for calculation of C/M ratios.

2.5. Statistics

Data are expressed as means ± S.E. of n replicates. Tests of significance of differences between mean values were made using a two-tailed Student's t-test (for paired or unpaired mean data) or Mann-Whitney U-tests (two-tailed tests, unpaired data), where appropriate.

3. Results

MDCK-C5A epithelia have high electrical resistance (2451 ± 194 (S.E.) $n = 24$ Ω cm^2, note that values of resistance for filter cups, 300 Ω cm^2 are subtracted from all data), similar to Strain 1-MDCK and typical of 'tight' epithelia [12]. This ability to form an epithelial barrier is also evident in the ability of MDCK-C5A epithelia to maintain a marked asymmetry in the bidirectional transepithelial fluxes of vinblastine. $J_{b\text{-}a}$ (0.325 ± 0.09 nmol cm^{-2} h^{-1} ($n = 6$)) exceeds $J_{a\text{-}b}$ (0.006 ± 0.001 nmol cm^2 h^{-1} ($n = 6$) $P < 0.001$) (Fig. 1), indicating a significant net secretion of vinblastine from basal to apical surfaces. In control conditions and in the presence of vinblastine the spontaneous transepithelial electrical p.d. is low (< 1 mV basal solution electro-positive) for MDCK-C5A epithelia. The observed asymmetry for vinblastine flux ($J_{b\text{-}a}/J_{a\text{-}b}$ = 58.0) cannot be accounted for by passive electrical forces and must represent active transport by the epithelium.

Both verapamil (100 μM) and taxotere (10 μM) present in both bathing solutions effectively abolish the net secretion of vinblastine by reducing the permeability of vinblastine in the secretory direction $P_{b\text{-}a}$ towards $P_{a\text{-}b}$ (Fig. 1) and increasing $P_{a\text{-}b}$ (Fig. 1). Bodipy-verapamil is a fluorescent analog of verapamil which retains the ability to competitively inhibit vinblastine secretion, being of similar molar potency to taxotere (Fig. 1). In addition to being a competitive inhibitor of P-glycoprotein function, Bodipy-verapamil is itself subject to epithelial secretion by MDCK-C5A epithelia. At 1 μM Bodipy-verapamil $J_{b\text{-}a}$ (37.7 ± 4.5 nmol cm^{-2} h^{-1} ($n = 9$)) exceeds $J_{a\text{-}b}$ (2.9 ± 0.3 nmol cm^{-2} h^{-1} ($n = 9$) $P < 0.001$), a 13-fold asymmetry. Both the unidirectional absorptive permeability for Bodipy-verapamil $P_{a\text{-}b}$ ((0.29 ± 0.04) · 10^{-2} cm h^{-1} n = 3), and that for vinblastine ((0.047 ± 0.005) · 10^{-2} cm h^{-1}, $n = 6$), are low compared to that for the secretory permeability for Bodipy-verapamil $P_{b\text{-}a}$ ((3.7 ± 0.4) · 10^{-2} cm h^{-1} $n = 3$), and vinblastine ((3.95 ± 0.25) · 10^{-2} cm h^{-1}, $n = 6$). Apparent saturation of net secretion of Bodipy-verapamil concentration is observed; at 5 μM J_{net} of −310 ± 32 nmol cm^{-2} h^{-1} $n = 3$ is similar to that at 10 μM (−345 ± 38 nmol cm^{-2} h^{-1} $n = 3$). Net Bodipy-verapamil secretion at 1 μM (−34.5 ± 3.4 nmol cm^{-2} h^{-1}) is subject to competitive inhibition by 20 μM dideoxyforskolin (to −23.4 ± 1.9 nmol cm^{-2} h^{-1}, $P <$ 0.05), and 20 μM taxotere (to −5.7 ± 0.2 nmol cm^{-2} h^{-1}, $P < 0.01$) added to both apical and basal bathing solutions.

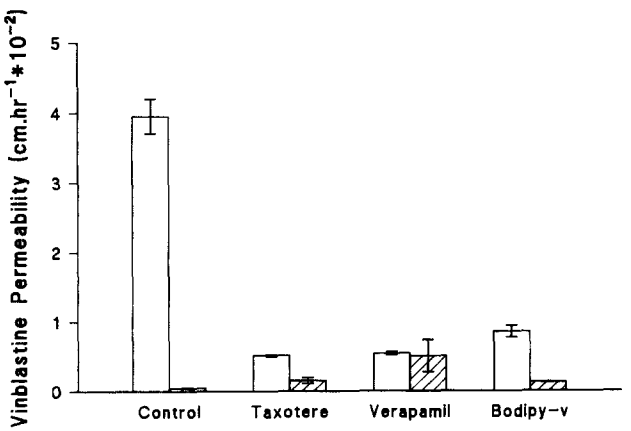

Fig. 1. Transepithelial vinblastine sulphate permeabilities measured in MDCK-C5A epithelial layers. [^{3}H]Vinblastine flux was measured in the basolateral-to-apical (b-a) (open bars), and in the apical-to-basolateral (a-b) (hatched bars) directions in control conditions and in the presence of 10 μM taxotere, 100 μM verapamil and 10 μM Bodipy-verapamil added to both apical and basal bathing solutions. Data are mean values ± S.E. from three to six separate epithelial layers.

The cellular accumulation of [^{3}H]vinblastine from either the apical or basal bathing solutions of MDCK-C5A epithelia, also shows asymmetry, accumulation across the basal cell aspects (C/M 7.3 ± 0.8, $n = 3$) exceeding that across the apical cell aspects (C/M 0.4 ± 0.1). This is in agreement to that found previously [11]. Inhibition of P-glycoprotein function is associated with increased cellular accumulation of vinblastine; 10 μM taxotere (added to both bathing solutions) increases C/M across the basal surface from 22.8 ± 2.7 (control $n = 3$) to 63.9 ± 1.8 $P <$ 0.01 (paired data). Similarly 10 μM Bodipy-verapamil (added to both bathing solutions) increases C/M from 7.3 ± 0.8 to 24.7 ± 1.6 ($P < 0.01$ $n = 3$, paired data).

Taken together, these data are consistent with expression of P-glycoprotein in MDCK-C5A epithelia and with Bodipy-verapamil being an effective substrate for P-glycoprotein.

Fig. 2a shows a series of confocal images through an MDCK-C5A cyst grown in hydrated collagen gel after incubation with 5 μM Bodipy-verapamil for 16 h. It is apparent that there is marked intracellular accumulation of the fluorophore and this is mainly vesicular in nature. It is also apparent that in order to assess whether Bodipy-verapamil is accumulated within the cyst lumen it is necessary to eliminate fluorescence from out of focus structures by an appropriate optical section. Fig. 2b shows an optical section which has been placed to transect the cyst lumen; by averaging pixel intensity over the whole image on a 0–256 scale (including cell fluorescence) and then delimiting areas of cyst lumen and extracellular fluid over which average pixel intensity is computed, it is apparent that an increased average fluorescence intensity is observed within the lumen compared (35.7 units/pixel) with that in the external medium (20.6 units/pixel). Relating the known external concentration to pixel intensity, we estimate accumulation of Bodipy-verapamil within the cyst lumen in Fig. 2b to be 5.3-fold. Absolute calibration of luminal Bodipy-verapamil was not possible since the dark current set on control cysts (18.4 units/pixel) was the major fraction of intensity levels recorded with external Bodipy-verapamil, sometimes exceeding external average intensity. After prolonged incubation times (16 h) at 5 μM Bodipy-verapamil of 14 cysts in two separate preparations

(51–157 μm outer diameter) 13 out of 14 showed significant accumulations with respect to incubation medium. Extensive washing and incubation of cysts in Bodipy-verapamil free media and incubation for up to 3 h following overnight incubation failed to reduce either cell associated Bodipy-verapamil or lumen Bodipy-verapamil (not shown), confirming that the effective barrier to equilibration afforded by the epithelium observed in monolayer culture is maintained within the cyst. Assuming net transport rates for Bodipy-verapamil observed at 5 μM for intact layers, it can be calculated that equilibration of the cyst lumen should be achieved for an 80 μm cyst within 5 min. Fig. 3 shows that when cysts are incubated at 0.5 μM Bodipy-verapamil for 15 min marked accumulation of Bodipy-verapamil is evident in cysts of smaller diameter; the larger cyst shown fails to demonstrate Bodipy-verapamil accumulation at this incubation time.

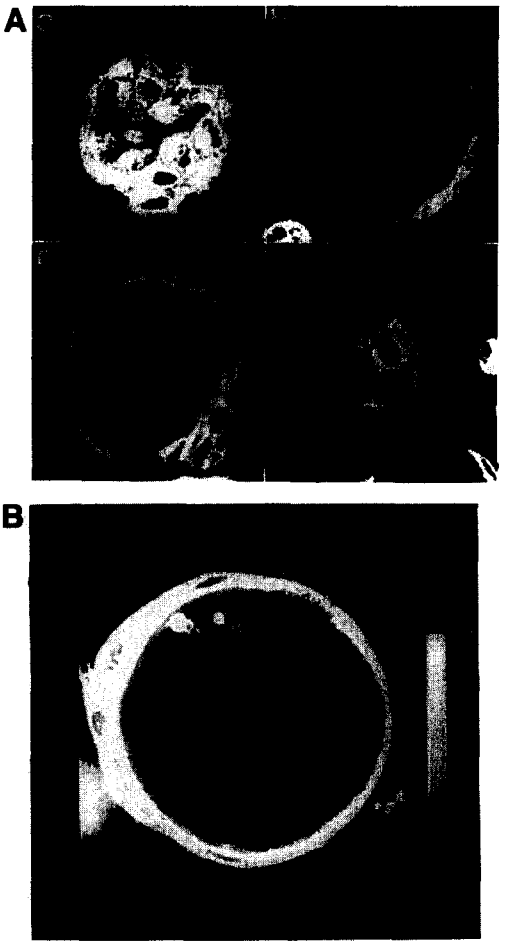

Fig. 2. (A) Series of four CLSM optical sections at 15 μM steps of a single MDCK-C5A cyst (a–d) grown in hydrated collagen gel and incubated with 5 μM Bodipy-verapamil for 16 h in serum free Hams F12/DMEM 1:1, Hepes-buffered medium minus Phenol red. Note the clear section through the lumen obtained in the second and third images. Fluorescence intensity is linear (1–256) Scale bar = 25 μm. (B) Analysis of luminal Bodipy-verapamil concentration. Renal cysts were incubated for 16 h with 5 μM Bodipy-verapamil. Average fluorescence intensity over the whole image is represented on a 1–256 scale. 0–30 is set as black; note that this encompasses the bulk of the collagen gel equilibrated with 5 μM Bodipy-verapamil. 30–256 is coded by the grey-white non-linear wedge indicated by the intensity scale at the right of the panel. Scale bar = 25 μM.

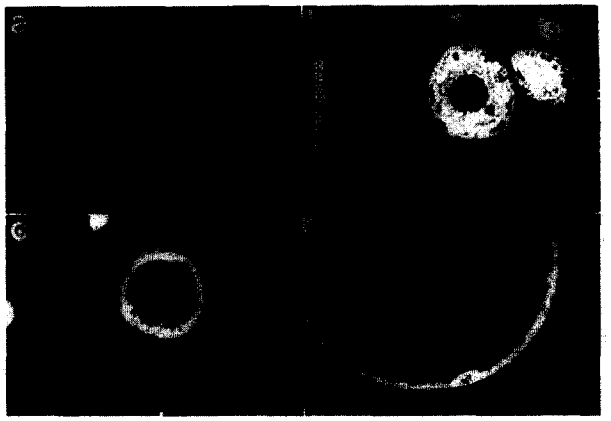

Fig. 3. Analysis of luminal Bodipy-verapamil concentration with short incubation. Renal cysts were incubated for 15 min with 0.5 μM Bodipy-verapamil. Average fluorescence intensity over the whole image is represented on a 1–256 scale. 0–20 is set as black; note that this encompasses the bulk of the collagen gel equilibrated with 0.5 μM Bodipy-verapamil. 20–256 is coded by the grey-white non-linear wedge indicated by the intensity scale. Scale bar = 50 μM in panel (a). (a) Control cyst incubated without Bodipy-verapamil, (b–d) cysts of differing luminal diameter.

4. Discussion

Epithelial expression of P-glycoprotein in the apical brush-border of normal epithelial tissues such as intestine and kidney has physiological relevance. In intestinal tissue it has been suggested that ATP-dependent extrusion of P-glycoprotein substrates results in maintenance of an effective barrier despite the relative lipophilicity of many of these substrates [6,20]. In kidney P-glycoprotein will serve to maintain epithelial secretion of substrates such as vinblastine for subsequent elimination in urine. Indeed, high renal clearance of P-glycoprotein substrates has been convincingly documented [21].

Immunocytochemical evidence suggests that P-glycoprotein expression is present in proximal renal tubules [8–10]. In situ hybridisations suggest that renal medullary tissue possess P-glycoprotein mRNA levels in excess of renal cortex [22]. Thus, it is likely that expression is present in more than one renal segment. MDCK epithelial cells display antigenic properties consistent with a distal tubular or collecting duct location [12]. Since renal cysts may arise from several tubular sites [1,3] the expression of P-glycoprotein in human renal cystic epithelia will need to be explored.

The present data show that epithelial secretion of P-glycoprotein substrates is present in epithelial monolayers of MDCK-C5A monolayers, consistent with P-glycoprotein expression to the apical membranes of these cells.

MDCK-C5A epithelia form effective epithelial barriers with high electrical resistance by 2 days post seeding at high density. They are thus similar to Strain 1 MDCK epithelia [11]. A model for vinblastine secretion by epithelial layers is passive accumulation of this basic molecule across the basolateral membrane driven by the electrochemical gradient, binding (sequestration) of substrate within the cystosol followed by ATP-dependent extrusion of free cytosolic drug across the apical membrane [11]. The observation in MDCK-C5A epithelia of cellular accumulation of vinblastine across the basal membrane together with the action of inhibitors is consistent with this model.

In renal cystic epithelia basic cell polarity is maintained [1–3]. The epithelial lining of the cyst displays tight-junctions, an apical brush-border at the cyst lumen and a basolateral membrane opposed to the renal parenchyma (in vivo) or to the collagen substrate in vitro [1–5]. However, there is evidence that certain membrane proteins such as the Na/K-ATPase may be mislocated in cystic renal epithelia [23]. The present data show that the P-glycoprotein substrate, Bodipy-verapamil, is accumulated avidly within the cell cystosol, the fluorescence being vesicular in nature. Bodipy-verapamil is also accumulated within the lumen of renal cysts. This pattern of substrate deposition (passive flux at the basolateral membrane driven by the electrochemical gradient and resulting in cellular accumulation, with extrusion against the prevailing electrochemical gradient at the apical membrane), is consistent with the maintenance of the apical polarised expression of P-glycoprotein. Confirmation of this will require the use of dog-specific antibodies to P-glycoprotein, or antibodies that cross-react with dog P-glycoprotein. It should be noted that endogenous levels of P-glycoprotein expression in MDCK cultures required to give the measured level of maximal substrate flux may be small compared to intestinal cell-lines expressing P-glycoprotein [11,20].

The ability of P-glycoprotein to target substrate delivery to the enclosed cyst lumen will provide a reservoir of cytotoxic/cytostatic agents. In this study Bodipy-verapamil is used as a fluorescent marker to trace the deposition of a cytotoxic substrate such as vinblastine or taxotere. In MDCK cells cytotoxic effects of vinblastine are recorded that are enhanced by vinblastine [11] in actively growing cultures. The present study has not attempted to investigate cytotoxic effects of P-glycoprotein substrates upon cyst expansion and integrity. The ability of taxotere to reverse cystic disease in an animal model suggests that this may also be the case in vivo [5]. The retention of Bodipy-verapamil within the cyst after washing suggests that under the conditions used here, no frank cytotoxic effect is observed with this agent. Clearance of cellular substrate after external levels are reduced to zero will be directed to the cyst lumen due to the adverse electrochemical gradient at the basolateral membrane and the apical localisation of P-glycoprotein. An enclosed cyst may be contrasted to an intact tubule where urine flow will remove transported substrate to be voided in urine. We have investigated retention of Bodipy-verapamil over incubation periods up to 3 h; subsequent effects of cytotoxic/cytostatic agents on epithelial integrity will need to be explored with longer term incubation.

The size of the renal cyst is likely to be crucial in delivery of P-glycoprotein substrates to the cyst to obtain optimal therapeutic action. In progressive renal cystic disease, cysts are initially small, ultimately being separated from the filtering nephron of origin, and progressively increase in size, compressing and destroying the renal parenchyma [1–3]. From a consideration of cyst volume/surface area relationship small cysts will show the highest accumulation of substrate after the shortest exposure to external P-glycoprotein substrates. Thus, it is likely that targeted delivery will be optimal in early disease.

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