Abstract. Aminoglycoside antibiotics are known to be internalized via endocytosis and have been associated with subcellular organelle dysfunction; however, the route of intracellular trafficking and their distribution remain largely unknown. To address these questions, a Texas Red conjugate of gentamicin (TRG) was synthesized for dual-labeling experiments with the endoplasmic reticulum, Golgi, and lysosomal markers DiOC6-3, C6-NBD-ceramide, and fluorescent dextrans, respectively. Confocal images were overlaid to determine areas of colocalization. Initial characterization studies of the fluorescent gentamicin analogue revealed that both internalization and accumulation were inhibited by excess unlabeled gentamicin. Furthermore, the fluorescent gentamicin label was colocalized with unlabeled gentamicin, using immunologic techniques. LLC-PK1 cells were exposed to the fluorescent gentamicin in media containing 1 mg/ml labeled gentamicin for 8 h and then either fixed or chased with gentamicin-free media for an additional 16 or 40 h (24 to 48 h total). Studies with fluorescent dextrans revealed rapid intracellular colocalization within the endosomal and lysosomal systems. Neither endoplasmic reticulum nor mitochondrial colocalization could be detected. However, Golgi colocalization was revealed using both confocal and electron microscopic techniques at 8 h of TRG incubation, and continued to be present for an additional 40 h. Protein synthetic rates were quantified and revealed decreased synthesis at the 24-h chase mark. These results suggest that TRG can serve as a fluorescent tracer for aminoglycoside trafficking within cells. The fluorescent marker remained associated with vesicular structures at all times and colocalized with the Golgi apparatus. It is postulated that this early association of gentamicin with the Golgi complex may be an avenue for delivery of aminoglycosides to other intracellular compartments. (J Am Soc Nephrol 9: 167-174, 1998)

Aminoglycoside antibiotics remain highly efficacious in the treatment of gram-negative infections; however, nephrotoxicity continues to limit their use (1). Surface membrane binding of aminoglycoside antibiotics is well characterized (2-6), and cellular uptake occurs by fluid-phase and receptor-mediated endocytosis (3.6-8) across both apical and basolateral membranes of proximal tubule cells (9). Recently, a role for gentamicin binding and uptake by the apical surface receptor GP330 has been reported (10).

The postendocytic trafficking of aminoglycosides remains poorly understood. Through the use of immunocytochemistry, autoradiography, immunogold labeling, and transmission electron microscopy, alterations in intracellular morphology and the accumulation of gentamicin within endosomal and lysosomal structures have been well documented (3.7-9,11-13). The lysosome has remained the focus of studies involving the sequestration and accumulation of gentamicin in both cell culture and whole animal models. Association with and toxicity to other organelles have been examined after in vivo administration of radiolabeled gentamicin and cell fractionation techniques, using classical organelle enzyme markers to identify sites of association (14-17). These techniques have resulted in indirect evidence for the association of aminoglycoside antibiotics with specific intracellular organelles. However, the direct association of aminoglycosides with subcellular organelles has not been shown in vivo. In addition, the intracellular route by which endocytosed aminoglycosides traffic to other intracellular compartments has not been determined.

Therefore, the specific aim of this study was to delineate the intracellular trafficking of aminoglycosides. Specifically, we were interested in evaluating whether there is movement of aminoglycosides from the endosomal pool to the Golgi apparatus, as has been demonstrated for mannose-6-phosphate and the Shiga toxin (18-21). To accomplish this goal, a fluorescent analogue of the aminoglycoside gentamicin was synthesized and extensively characterized for use as a tracer during intracellular trafficking. This tracer was simultaneously used with organelle-specific fluorescent markers in LLC-PK1 proximal tubule cells. Association between the fluorescent gentamicin tracer and the labeled intracellular organelle markers was determined using confocal techniques and the overlaying of images to determine areas of colocalization. Furthermore, electron microscopic confirmation of intracellular localization was obtained.
Materials and Methods

Experimental Model

Porcine kidney proximal tubule cells (LLC-PK1, American Type Culture Collection, Rockville, MD) were grown on 0.1% porcine gelatin (300 bloom, Sigma, St. Louis, MO)-coated coverslips and cultured in a 1:1 mixture of Dulbecco’s modified Eagle’s medium and Ham’s nutrient mixture (F-12), supplemented with 10% fetal bovine serum and 1 mg/ml penicillin-streptomycin (Sigma). Cells were allowed to reach a state of confluence of approximately 40 to 80% and a height of 3 to 5 μm before being used in experimental protocols.

Synthesis and Characterization of the Texas Red Gentamicin

Gentamicin (Fluka Biochemica, Ronkonkoma, NY) was dissolved in 100 mM K2CO3 buffer, pH 8.5, to a final concentration of 10 mg/ml. One milligram of Texas Red (Molecular Probes, Eugene, OR) was dissolved in 50 μl of high quality anhydrous N,N-dimethylformamide (Sigma) and slowly added to a 30-molar excess of gentamicin at 4°C (10). This 30:1 conjugation ratio was used to maximize the discernible fluorescent signal. As a control, an equimolar solution of hydrolyzed Texas Red was prepared by dissolving 1 mg of Texas Red in 50 μl of high-quality anhydrous N,N-dimethylformamide, followed by slow addition to 100 mM K2CO3 buffer, pH 8.5, without gentamicin. The calculated molecular weight of Texas Red gentamicin (TRG) is 1125.

To evaluate whether unconjugated Texas Red (TR) was taken up and underwent intracellular processing, LLC-PK1 cells were incubated in media containing either 1.0 mg/ml TRG or a molar equivalent of the hydrolyzed fluorochrome TR for 8 h. The cells were then washed briefly, fixed, and observed using confocal microscopy.

To determine whether TRG would follow a trafficking pathway similar to unlabeled gentamicin, LLC-PK1 cells were incubated in media containing 1.0 mg/ml tracer for 8 h, washed briefly, fixed, and permeabilized. The cells were then processed for indirect immunolocalization of gentamicin with a polyclonal goat-anti-gentamicin antibody (Jackson ImmunoResearch, West Grove, PA) and a polyclonal FITC-conjugated donkey-anti-goat secondary antibody (Jackson ImmunoResearch). Fluorescent signals from both the TRG and the immunolocalized gentamicin were compared using confocal microscopy.

Finally, to determine whether binding and internalization of TRG were specific, LLC-PK1 cells were incubated with TRG and increasing amounts of excess unlabeled gentamicin to determine whether uptake could be inhibited. Cells were incubated in media containing 50 μg/ml TRG and a 0-fold, 10-fold, or 40-fold excess of unlabeled gentamicin for 4 h. The cells were washed briefly, fixed, and observed using confocal microscopy.

Cell Fixation and Immunolabeling

LLC-PK1 cells were washed twice briefly in phosphate-buffered saline (PBS) with 0.1 mM CaCl2 and 1.0 mM MgCl2 (PBS-Mg/Ca), pH 7.4, at room temperature twice, followed by fixation. The cells were fixed in freshly thawed 4% paraformaldehyde in PBS, pH 7.4, for 1 h at room temperature or overnight at 4°C.

To prepare cells for immunofluorescence, they were permeabilized in PBS, pH 7.4, containing 0.1% Triton X-100 for 10 min. The cells were then incubated in a buffered blocking solution containing 2% defatted bovine serum albumin (BSA) and 0.5% dry nonfat milk in PBS-Mg/Ca for 30 to 60 min at 37°C. Antibodies, either primary or secondary, were diluted in the blocking solution and applied for 60 min at 37°C. Washes of 30 min were performed after each of the antibody incubations. The cells were then inverted over a drop of mounting media on a coverslip, sealed, and observed using confocal microscopy.

 Trafficking of TRG

To maximize our ability to follow gentamicin as it trafficked through the cell, as opposed to observing steady-state distributions at different time periods, we performed TRG pulse-chase experiments. LLC-PK1 cells were incubated in media containing 1.0 mg/ml TRG followed by a chase in TRG-free, but gentamicin-containing, media, to generate the following time points. (1) TRG for 8 h; (2) T24-LLC-PK1, TRG for 8 h, followed by chase media for 16 h; and (3) T48-TRG for 8 h, followed by chase media for 40 h.

Colocalization of TRG with the Endosomal/Lysosomal Marker FITC-Dextran

After each of the three time points, cells were incubated in culture medium containing 10 mg/ml F-10, 10,000 fixable amine-dextran conjugated to FITC for 30 min, followed by a chase in dextran-free medium for 60 min. The cells were then washed briefly, fixed, and imaged.

Colocalization of TRG with the Golgi Complex Marker C6-NBD-Ceramide

After each of the three time points, cells were washed briefly and fixed. They were then incubated in 5 μM C-6-NBD-ceramide/defatted-BSA complex (prepared as described by Pagano et al. (22)) for 30 min at room temperature, followed by several washes in PBS-Mg/Ca containing 2.0 mg/ml defatted-BSA for 30 to 90 min.

Image Acquisition and Processing

Images were acquired on a Bio-Rad MRC-1024 confocal microscope equipped with a krypton/argon laser on a Nikon Diaphot 200 platform using a 100× planApo 1.4 numerical aperture oil immersion objective. To avoid possible spectral overlap from the green channel (FITC or NBD fluorophores) into the red channel (TR fluorophore), images were taken sequentially by exciting the fluorophores using only single lines (488 nm or 568 nm, respectively) from the Kr/Ar laser. The Golgi marker (NBD-ceramide) images were “thresholded” to remove background lipid staining, and all images were contrast-enhanced using a 3 × 3 low-pass filter. Images from corresponding focal planes were then overlaid to determine areas of colocalization. All image processing was done using Metamorph version 2.5 image processing software (Universal Imaging, West Chester, PA).

Cytchemical Localization of TRG

To visualize gentamicin at the electron microscope level, LLC-PK1 cells were grown on filters and incubated for 8 h in Dulbecco’s modified Eagle’s medium/Ham’s F-12 media with 10% FBS with 1% penicillin/streptomycin containing 0.5 mg/ml TRG. The cells were then washed with PBS and fixed in freshly thawed 4% paraformaldehyde in PBS. The TRG was then photopolymerized in the presence of diaminobenzidine (DAB) (DAB substrate kit no. SK-4100, Vector Laboratories, Burlingame, CA) under light specific for the excitation wavelength of Texas Red using a protocol modified from Sandell and Masland (23). The cells were then fixed in 1% glutaraldehyde (Ted Pella, Redding, CA) in PBS for 1 h and washed in PBS. Postfixation in 0.14% OsO4, 0.04% tannic acid, and 0.5% K3Fe(CN)6 in PBS...
followed. The cells were then dehydrated in increasing levels of ethanol (25, 50, 75, and 95%) before tissue infiltration in LR White resin (London Resin Co., Berkshire, United Kingdom). The cells were embedded in LR White and polymerized at 65°C. Ultrathin sections were cut using an RMC-MT6000XL ultramicrotome and were not counterstained with uranium or lead. Visualization was done under a Phillips CM 12 electron microscope using 100-kV accelerating voltage.

**Determination of Protein Synthesis Rates after Gentamicin Exposure**

To determine whether the findings observed via confocal microscopy had significant biological relevance, rates of protein synthesis in LLC-PK1 cells were determined at each of the three time points, using [35S]-methionine incorporation, essentially as described by Bonifacino (24). In brief, LLC-PK1 cells grown in 10-cm culture dishes were rinsed, incubated in methionine-free medium for 10 min, followed by incubation with 35S-labeled protein hydrolysate (American Radiolabeled Chemicals, St. Louis, MO) for 30 min. The cells were then washed, resuspended in PBS with 0.1 mg/ml BSA as a carrier protein, and total cellular protein was precipitated in 10% trichloroacetic acid (TCA) and collected onto glass microfiber filters (Fischer Scientific, Itasca, IL). The filters were rinsed with additional TCA and with ethanol, and label incorporation was quantified via liquid scintillation counting. Background was measured using cells in which exposure to 35S-labeled protein hydrolysate was immediately fol-

---

**Figure 1.** Characterization of Texas Red gentamicin (TRG). Porcine kidney proximal tubule (LLC-PK1) cells were exposed to either 1 mg/ml TRG for 8 h (A) or hydrolyzed Texas Red sulfonyl chloride for 8 h (B). (C and D) LLC-PK1 cells were exposed to 1 mg/ml TRG for 8 h and then processed for indirect immunofluorescent localization of gentamicin with an FITC-conjugated 2° antibody. TRG channel (C) and FITC channel (D) for the same field.
lowed by washing, resuspension, and TCA precipitation. Total cellular protein was measured in parallel plates using a bicinchoninic acid assay (Pierce, Rockford, IL) with BSA standards, and was used to normalize label incorporation rates. Within each experiment, these rates were then normalized to the average value observed for cells not treated with gentamicin (i.e., control cells).

**Statistical Analyses**

Protein synthesis rates were compared using ANOVA. Results are expressed as mean and a 95% confidence interval, and the level for statistical significance was chosen as $P < 0.05$.

**Results**

**Characterization of Tracer TRG**

Several initial studies were directed toward determining whether the fluorescent TRG probe behaved similarly to native gentamicin. First, to evaluate whether unconjugated TR was internalized and resulted in intracellular fluorescence, LLC-PK1 cells were treated with either 1.0 mg/ml TRG or an equimolar amount of hydrolyzed TR. The cells incubated in the presence of hydrolyzed TR (Figure 1B) exhibited staining similar to autofluorescence and failed to produce any localized or concentrated staining patterns. Cells incubated in media containing TRG displayed a punctate, cytosolic staining pattern that was readily discernible over the minimal background fluorescence observed (Figure 1A). These data are consistent with endocytic uptake of TRG, and lack of binding or uptake of unconjugated TR.

To evaluate whether the fluorescent probe behaved similar to native gentamicin, TRG and anti-gentamicin antibody-labeled native gentamicin distributions were compared. TRG (Figure 1C) and indirect immunofluorescence of native gentamicin (Figure 1D) staining patterns were strikingly similar, and areas exhibiting identical staining patterns are readily discernible. These data indicate that the fluorescent probe colocalized with native gentamicin in endocytic compartments.

Finally, to confirm that the specificity of gentamicin binding and uptake remained unchanged by the conjugation procedure, cells were incubated in media containing TRG and increasing amounts of excess unlabeled gentamicin to evaluate for competitive inhibition. Increasing amounts of unlabeled gentamicin (no excess, 10-fold excess, and 40-fold excess in Figure 2, A, B, and C, respectively) resulted in a dose-dependent diminution of uptake and subsequent accumulation of TRG into the punctate cytosolic compartments seen previously. Taken together, these data indicate that the synthetic TRG behaves similarly to native gentamicin and can be used as a fluorescent tracer of gentamicin.

**Uptake and Intracellular Trafficking of TRG: Colocalization with Intracellular Organelles**

In the next phase of this study, colocalization of TRG with fluorescent intracellular organelle markers was used to follow gentamicin trafficking in LLC-PK1 cells. In all experiments and at every time point, TRG was found to colocalize with well-defined organelles (Figure 3). Characteristic features included the retention of TRG within vesicle-like structures and decreased TRG fluorescence signal as time progressed from T8 to T48 h.

Initial studies were undertaken to localize TRG within the endosomal/lysosomal system, using FITC dextrans as a specific marker for the endocytic compartment. Previous studies indicated that uptake occurred via endocytosis and, as expected, a high degree of colocalization of TRG and FITC dextrans was observed (Figure 3, A through C). Colocalization was most marked in small endosomal/lysosomal structures at all time points. At later time points, there was also accumulation of both TRG and FITC dextrans in larger vesicular structures consistent with lysosomal accumulation. However, although fluorescent vesicular dextran staining remained punctate or distributed throughout the cytosol, TRG staining eventually also localized to the perinuclear region, suggesting Golgi localization.

To directly investigate this possibility, colocalization studies using C-6-NBD-ceramide were performed. This fluorescent marker had been characterized previously as a marker for the trans-Golgi complex and, using this probe, colocalization of

![Figure 2. Competitive inhibition of TRG uptake by gentamicin. LLC-PK1 cells were incubated with increasing concentrations of excess unlabeled gentamicin for 4 h. Cells were exposed to 50 μg/ml TRG without excess gentamicin (A), with a 10-fold excess of unlabeled gentamicin (B; 500 μg/ml), or with a 40-fold excess of unlabeled gentamicin (C; 2 mg/ml). Magnification, ×815.](image-url)
Figure 3. Gentamicin colocalization with endosomal/lysosomal and Golgi compartments. Colocalization of TRG with the endosomal/lysosomal fluid-phase fluorescent marker FITC-dextran was determined at 8 h (A), T24 (B), and T48 (C). Colocalization is evidenced by yellow structures. In separate studies, colocalization of TRG within the trans-Golgi-specific marker NBD-ceramide (green signal) at 8 h (D), T24 (E), and T48 (F) was undertaken. For all studies, TRG (1 mg/ml) was incubated with cells for 8 h, and then the media was exchanged and cells were imaged at 8, 24, and 48 h.
TRG with the Golgi complex was observed at all time points (Figure 3, D through F). The degree of colocalization within the Golgi complex was not as extensive as that exhibited within endosomal/lysosomal structures. Furthermore, the Golgi-colocalization signal appeared to increase over the T8 to T24 time points, but then decreased at the T48 time point (Figure 3, D through F).

An electron microscopic study was then undertaken to confirm Golgi localization of TRG. For this study, an electron-dense DAB reaction product was produced at specific cellular sites by exciting Texas Red molecules in the presence of DAB (23). Figure 4 shows these reaction products at different sites within the cell. In Figure 4, A through C, cells were exposed to DAB without previous exposure to TRG, thus serving as negative controls. Figure 4B shows a lysosome (L)-containing reaction product, indicating the presence of TRG within. Note that the reaction product was seen as discrete accumulations (arrow) and as linear staining of the membrane (arrowhead). Figure 4D shows two stacks of Golgi stained with DAB reaction product in TRG-exposed cells; again, both discrete accumulations and linear membrane staining were observed.

Additional studies examined colocalization of TRG with the endoplasmic reticulum (ER) or mitochondrial compartments. Rabbit-anti-K-DEL (StressGen Biotechnologies, Victoria, British Columbia, Canada) and mitotracker Green FM (Molecular Probes) were used as labels for the ER and mitochondrial compartments, respectively. Excellent labeling of these compartments was achieved in both cases, but no colocalization with TRG was observed (data not shown).

Finally, to determine whether gentamicin treatment under this protocol was associated with functional consequences, protein synthetic rates were quantified using [35S]-methionine incorporation (Figure 5). [35S]-Methionine incorporation was significantly decreased below control values at T24 ($P < 0.05$),
cells. LLC-PK1 cells exposed to unlabeled gentamicin for 8 h, followed by measurement of \([^{35}S]\)-methionine incorporation either immediately or after return to nongentamicin-containing medium for 24 and 48 h. Rates were normalized to total protein and to control cells (not exposed to gentamicin). A trend toward decreased incorporation was seen at 8 h, but was statistically significant (*P < 0.05) only at 24 h. Error bars denote 95 percentile confidence ranges.

but by T48, it had recovered to near initial rates. This is in agreement with previous studies showing gentamicin inhibits protein synthesis (14,25,26).

Discussion

Aminoglycoside nephrotoxicity results directly from uptake and accumulation of the nonmetabolizable antibiotic within renal proximal tubular cells (3,7,9,11), primarily within the S1 and S2 segments of the nephron (3). Aminoglycosides are minimally protein bound and, after glomerular filtration, bind to acidic surface membrane phospholipids, principally phosphatidylinositol (3,5). Binding to the apical receptor GP330 has also been reported (10). Autoradiographic, immunofluorescence, and immunogold studies indicate that aminoglycosides are then internalized across the apical membrane of renal proximal tubule cells by endocytosis and sequestered within endosomal/lysosomal compartments (3,7,9,11).

What remains unknown is whether and how aminoglycosides traffic to other intracellular organelles within the cell after endocytosis. Although it has been well documented that direct in vitro exposure of cellular organelles to aminoglycosides results in organelle dysfunction, the degree, route, and rate of drug delivery to specific organelles remains unknown. Techniques such as cell fractionation and the use of radiolabeled gentamicin have advanced the understanding of gentamicin binding and uptake by proximal tubule cells (14–17,26), but employ the use of cell lysates and tissue homogenates. This imposes potential problems of rupture of gentamicin-sequestering organelles, particularly in studies using rapid-freezing liquid N2. This can then result in artifactual binding of gentamicin (and polycationic compounds in general) to subcellular fractions during processing. This may also have been a factor in the increased distribution of gentamicin previously reported in necrotic cells (11). Thus, fluorescence microscopy was chosen to study the uptake and trafficking of gentamicin, because this method could provide compelling evidence for direct association with various intracellular organelles without cell fractionation or processing. A fluorescent tracer for gentamicin was synthesized, extensively characterized, and then used in conjunction with specific organelar probes. Of particular note is avoidance of the disruption of the Golgi complex often encountered with permeabilization steps required during typical indirect immunofluorescent localization (22).

In the present studies, TRG was preferentially located to the greatest extent in the endosomal/lysosomal system. This is consistent with previous data and supports the use of TRG as a fluorescent tracer of gentamicin. A major finding in this study was the colocalization of TRG with the Golgi complex at time points as early as 8 h of TRG exposure. The confocal findings were confirmed at the electron microscope level, using a well accepted method of subcellular localization of a fluorescent probe by selective excitation. This method leads to direct visualization of the intracellular location of the fluorescent marker (22,23). Therefore, it minimizes potential differences that can occur when different visualization techniques are used for fluorescent and electron microscope studies. The presence of TRG in the Golgi at such early time points and the high viability of the cells suggest that these associations are indeed specific and not due to subsequent associations that might arise from the rupture and release of TRG from sequestering organelles. A possible route of entry for gentamicin into the Golgi complex may lie in the recycling events that occur between the late endosome and the trans-Golgi network (18,19). Recent evidence indicates that, after binding to the surface membrane, the protein toxin Shiga traffics to the Golgi via late endosomes after binding to the surface membrane (20,21).

Data regarding handling of this protein toxin may also explain how gentamicin results in protein synthesis inhibition.

Once in the Golgi, the Shiga toxin moved in a retrograde manner into the ER (20,21). It is possible that gentamicin follows a similar pathway; however, we were unable to show ER colocalization in our present studies. This may relate to the relatively poor specific activity of our fluorescent probe (1:30), the large intracellular pool of the ER compartment, or the small amount of compound necessary to mediate toxicity. However, we did document transient inhibition of protein synthesis consistent with ER association. These cell culture biochemical data support previous data from other groups (14,25,26), demonstrating that aminoglycosides lower the rate of protein synthesis. More importantly, this protocol showed that an 8-h exposure of gentamicin was sufficient to mediate these changes, and it lends support to the associations visualized in the Golgi complex.

We postulate that aminoglycoside trafficking from the Golgi complex to other intracellular organelles could serve as the route of transport allowing for direct organelle delivery and subsequent toxicity. Utilization of this pathway would keep
aminoglycosides within the vesicular compartment and is consistent with the lack of directly observable free cytosolic aminoglycoside (3,9).

In summary, a well characterized fluorescent gentamicin marker colocalized with both endosomal/lysosomal vesicles and with the Golgi complex, concurrent with inhibition of protein synthesis. This represents the first documentation of aminoglycoside trafficking to the Golgi complex.

Acknowledgments
These studies were supported by grants from the National Institute of Diabetes and Digestive and Kidney Diseases (DK-41126), the Veterans Affairs Research Service, and the American Heart Association. Dr. Molitoris is an Established Investigator for the American Heart Association.

References
20. Sandvig K, Ryd M, Oystern G, Schweda E, Holm PK, van Deurs B: Retrograde transport from the Golgi complex to the ER of both Shiga toxin and the nontoxic Shiga β-fragment is regulated by butyric acid and cAMP. *J Cell Biol* 126: 53–64, 1994