



Systemic aminoglycosides are trafficked via endolymph into cochlear hair cells

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Aminoglycoside antibiotics rapidly enter and kill cochlear hair cells via apical mechano-electrical transduction (MET) channels *in vitro*. *In vivo*, it remains unknown whether systemically-administered aminoglycosides cross the blood-labyrinth barrier into endolymph and enter hair cells. Here we show, for the first time, that systemic aminoglycosides are trafficked across the blood-endolymph barrier and preferentially enter hair cells across their apical membranes. This trafficking route is predominant compared to uptake via hair cell basolateral membranes during perilymph infusion.

Aminoglycoside antibiotics, like gentamicin, are widely-used and clinically essential for preventing or treating Gram-negative bacterial sepsis and tuberculosis¹. However, these drugs carry significant risk of nephrotoxicity and ototoxicity. In the inner ear, systemic aminoglycosides cross the blood-labyrinth barrier (BLB), and preferentially kill cochlear sensory hair cells, leading to lifelong hearing loss and deafness¹, the most prevalent sensory disability worldwide. The cochlear trafficking route(s) by which systemic aminoglycosides reach hair cells has remained an important, yet unanswered, question for over 60 years.

Cochlear hair cells are highly specialized neuro-epithelial cells immersed in two electrochemically distinct extracellular fluids. Their basolateral membranes are immersed in perilymph that fills the scala tympani and extracellular spaces of the organ of Corti (Fig. 1). Their apical membranes, including the mechanically-sensitive stereocilia, are bathed in endolymph. Tight junctions couple all cells lining the endolymphatic space, limiting paracellular ion movements between endolymph and perilymph (Fig. 1). Endolymph is rich in potassium and low in sodium and calcium (157, 1.3, 0.023 mM respectively); perilymph has typical extracellular levels of potassium, sodium and calcium (4.2, 148, 1.3 mM respectively)². The distinctive ionic composition of endolymph is regulated by the stria vascularis that actively transports potassium into endolymph; while the positive endolymphatic potential (+80 mV) drives sodium out through non-specific cation channels in sensory and non-sensory cells. The positive endolymphatic potential also provides a rapid potassium-rich depolarizing current into hair cells via mechanically-gated mechano-electrical transduction (MET) channels located at the tips of most hair cell stereocilia³. These MET channels are non-selective cation channels permeable to aminoglycosides *in vitro*^{4,5}, raising the question of whether systemic aminoglycosides are trafficked into endolymph prior to entering hair cells via MET channels *in vivo*. The most direct route for vascular aminoglycosides to enter endolymph is to cross the tight junction-coupled endothelial cells of strial capillaries into the intra-strial space and be taken up by marginal cells, prior to clearance into endolymph (Fig. 1). Previous *in vivo* studies^{6,7} revealed that systemic fluorescently-tagged gentamicin (GTTR) was preferentially taken up by the stria vascularis in the cochlea, providing suggestive, but inconclusive, evidence for trans-strial trafficking of systemic gentamicin into endolymph and hair cells.

Alternatively, vascular aminoglycosides could traffic through the tight junction-coupled endothelial cells of non-strial capillaries, in the spiral limbus, basilar membrane and spiral ligament, and load perilymph in the scala tympani⁸, with access to hair cell basolateral membranes (Fig. 1). However, there is little evidence that aminoglycosides cross hair cell basolateral membranes.

This study provides evidence that systemic gentamicin is trafficked across the blood-endolymph barrier and preferentially enter hair cells from endolymph *in vivo*. This has scientific and clinical implications for understanding the trafficking mechanisms involved, and developing novel, systemic approaches to preventing gentamicin-induced ototoxicity in humans.

Results

To determine which intra-cochlear trafficking route aminoglycosides primarily follow to enter hair cells, we used both fluorescently-tagged gentamicin-Texas Red (GTTR) or immunodetection of gentamicin (Fig. 2c). Although

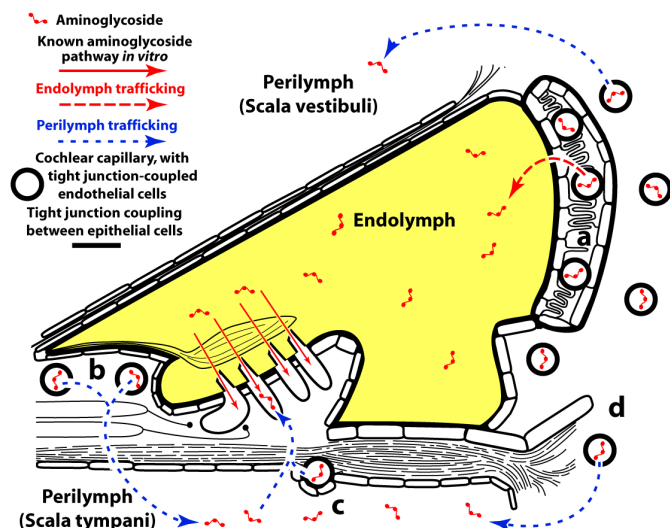


Figure 1 | Diagram illustrating potential intra-cochlear trafficking of systemic aminoglycosides to hair cells. Endolymph trafficking route is indicated by red dashed arrow, while perilymph trafficking route is indicated by blue dashed arrows. The endolymphatic space is encapsulated by tight junctions depicted by thick black lines. Cochlear capillaries are located in the stria vascularis (a), in the modiolus (b), on the scala tympani side of the basilar membrane (c) and within the spiral ligament of the lateral wall (d).

larger in size and mass than gentamicin, GTTR is cationic, permeates the MET channel and is rapidly localized at hair cell stereocilia following systemic injection, like gentamicin^{5,7}.

GTTR was given intravenously (1 mg/kg gentamicin base) while the scala tympani was perfused with artificial perilymph to reduce GTTR access to the basolateral membranes of hair cells (Fig. 2a). After 30 minutes, cochlear tissues were systemically perfusion-fixed and processed for confocal microscopy. Robust GTTR fluorescence was observed in single optical sections of the stria vascularis and organ of Corti (Fig. 2d and e respectively), similar to cochleae treated with systemic GTTR without perilymph wash (Supplementary Fig. 1). Serum levels of GTTR were ~ 7.0 $\mu\text{g/ml}$ ($n=5$) 15 minutes post-injection.

The reciprocal drug delivery paradigm was to infuse 1.4 $\mu\text{g/ml}$ GTTR in artificial perilymph through the scala tympani for 30 minutes, providing GTTR access only to the basolateral membranes of hair cells (Fig. 2b). This dose was derived from the perilymph-to-serum aminoglycoside ratio of 20% after a 6-hour i.v. infusion⁹, and likely higher than GTTR levels in perilymph 30 minutes after systemic injection⁷. After 30 minutes, there was negligible GTTR fluorescence in the stria vascularis and weak fluorescence in hair cells (Fig. 2f and g respectively). Two batches of GTTR were used, and cellular fluorescence intensities were found to be segregated by batch (Fig. 2l). A two-way ANOVA revealed fluorescence variation in the batches of GTTR used ($F[1,12] = 54.44$, $p < 0.0001$), and more importantly, fluorescence variation in the GTTR delivery route used ($F[1,12] = 245.35$, $p < 0.0001$). Post hoc unpaired t-tests (assuming unequal variances) confirmed that the intensity of systemic GTTR in hair cells after trafficking across the stria blood-endolymph barrier was significantly greater than that delivered by scala tympani infusion ($p < 0.001$, $n=8$; Fig. 2l), indicating that hair cells preferentially take up GTTR from endolymph.

The weak fluorescence in OHCs following scala tympani infusion with GTTR (Fig. 2g) was significantly greater than autofluorescence in OHCs without GTTR exposure (Fig. 2i, 2l; $p = 0.0009$, $n=8$, unpaired t-test). In contrast, stria tissues had negligible fluorescence following scala tympani infusion of GTTR that was not statistically different from the extremely weak autofluorescence observed in

untreated stria vascularis imaged at the same laser excitation intensities as in cochleae treated with systemic GTTR ($p = 0.22$, $n=6$, unpaired t-test; Fig. 2f and h), indicating negligible trafficking of GTTR from perilymph into endolymph via the stria vascularis. Cochleae treated systemically with hydrolyzed Texas Red also had negligible fluorescence in hair cells (Supplementary Fig. 1e and f). Thus, weak fluorescence in hair cells following scala tympani infusion of GTTR indicates that hair cells could take up GTTR across their basolateral membranes. However, this perilymph trafficking route is inefficient compared to the robust GTTR fluorescence observed in the stria vascularis and hair cells following systemic injection of GTTR implicating trans-stria trafficking into endolymph and hair cells.

Imaging data were collected and analyzed from only sensitive cochleae with near-normal compound action potentials (CAPs). Sealing the perfusion catheter occasionally induced mild CAP threshold shifts in the 12 to 24 kHz region at the base of the cochlea (e.g. Fig. 2j). However, cochlear sensitivity was well-maintained in the 2–9 kHz frequency range corresponding to the third cochlear coil (from the apex) in either experimental paradigm (Fig. 2j and k) derived from the murine frequency-place map¹⁰. This indicates that relatively normal cochlear physiological function was maintained during perfusion (see Discussion).

Since GTTR has a larger molecular mass than gentamicin, we repeated the same experimental paradigms using gentamicin and processed the tissues for immunofluorescence. The results were qualitatively similar to that with GTTR, with characteristic gentamicin immunolabeling in hair cells following systemic gentamicin delivery and perilymph wash (Fig. 3a and b). Following infusion of gentamicin through the scala tympani, the immunofluorescence signal was weak and largely non-specifically distributed throughout the organ of Corti (i.e., not localized to specific cell types; Fig. 3c and d). Significantly, CAP thresholds, and thus cochlear sensitivity, were maintained in the cochlear segment of interest (Fig. 3e and f).

Discussion

Previous studies reported only very low levels of aminoglycosides in endolymph compared to perilymph *in vivo*^{8,9}. The +80-mV endolymphatic potential will electrophoretically drive the cationic aminoglycosides present in endolymph through hair cell MET channels, or other aminoglycoside-permeant cation channels into supporting cells^{4,5,7}. This will selectively increase the risk of hair cell toxicity^{5,11}, and simultaneously reduce aminoglycoside levels in endolymph compared to perilymph.

The sensitive hearing monitored by CAP recording during these experiments indicates that the stria BLB maintained its integrity. Loss of BLB integrity, or paracellular extravasation of drug-laden serum into the intra-stria space, would short-circuit the electrophysiological environment in the cochlea with consequent loss of both endolymphatic potential and cochlear sensitivity^{12,13}. This was not observed in these experiments. Furthermore, non-selective, aminoglycoside-permeant cation channels cannot be present in the basolateral membrane of marginal cells^{14,15}, suggesting that uptake of aminoglycosides from the intra-stria space into marginal cells requires active or electrogenic transport across their basolateral membranes. Identifying this molecular mechanism will initiate new, systemic strategies to pharmacologically block aminoglycoside trafficking across the blood-endolymph barrier and into hair cells, and subsequent cochleotoxicity.

In this study, we used fluorescently-tagged and native gentamicin. Gentamicin induces both cochleotoxicity and vestibulotoxicity, and intratympanic administration of gentamicin is used to manage Meniere's disease and vertigo¹⁶. Our data show that, in relatively normal physiological conditions, hair cells take up little gentamicin from perilymph compared to trans-stria trafficking of systemic gentamicin from stria capillaries into endolymph and

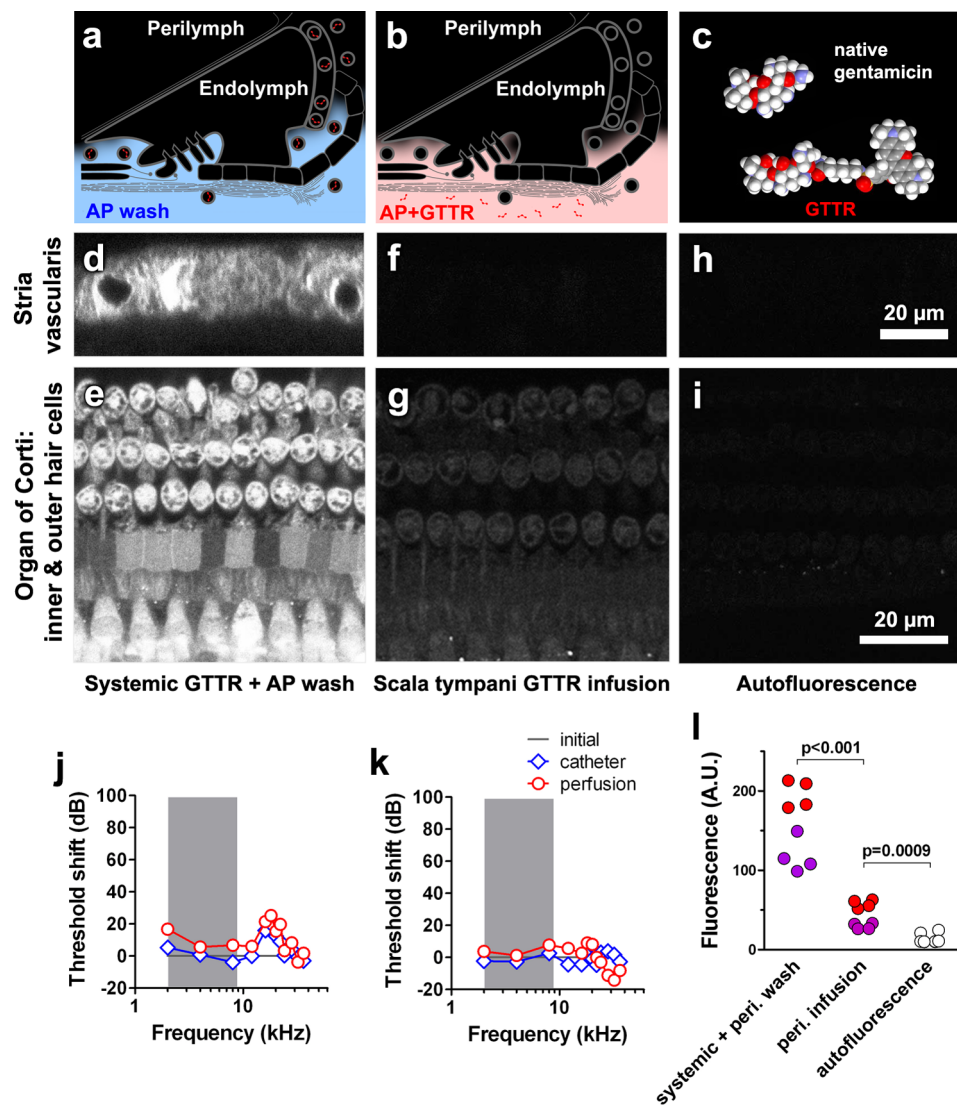


Figure 2 | Greater hair cell uptake of GTTR via endolymph trafficking. (a) Diagram illustrating systemic GTTR delivery with scala tympani perilymph wash. AP, artificial perilymph. (b) Diagram illustrating perilymph infusion with GTTR. (c) Space-fill models of gentamicin and GTTR. Robust GTTR fluorescence in the stria vascularis (d) and in OHCs, IHCs and pillar cells of the organ of Corti (e) after systemic GTTR delivery with perilymph wash. Negligible GTTR fluorescence in the stria vascularis (f) and weak fluorescence in the organ of Corti (g) after scala tympani infusion of GTTR. Negligible autofluorescence ($\lambda > 615$ nm) in the stria vascularis (h) and organ of Corti (i). Scale bars = 20 μ m. All images were acquired and processed using identical settings. (j) CAPs were largely maintained in the 2–9 kHz region (gray region) corresponding to the third coil from the cochlear apex¹⁰. (k) Cochlear sensitivity was preserved during scala tympani infusion of GTTR. (l) The intensity of GTTR fluorescence in OHCs was quantified from single confocal planes. More robust fluorescence was present in cochleae exposed to systemic GTTR plus perilymph wash compared to scala tympani infusion of GTTR ($p < 0.001$; $n = 8$ per condition, post hoc unpaired t-test). Red (or magenta) circles indicate the same batch of GTTR used among experiments. After scala tympani infusion of GTTR, fluorescence in OHCs was weak, but detectable, and statistically greater than in untreated OHCs ($p = 0.0009$; $n = 8$ per condition, unpaired t-test). A.U., arbitrary units.

hair cells. However, perilymph is a reservoir for gentamicin deposition⁸, and pathophysiological conditions, such as Meniere's Disease, could potentially enhance cochlear hair cell uptake of gentamicin from perilymph¹⁷. Similarly, trafficking of systemic aminoglycosides to hair cells can also be modulated by acoustic trauma, bacterial sepsis or exposure to loop diuretics^{18–21}. Although loop diuretics block the sodium-potassium-chloride co-transporter prevalent on the basolateral membrane or marginal cells, the integrity of the blood-labyrinth and perilymph-endolymph barriers are maintained²². This further implicates a transcellular trafficking pathway for gentamicin across the strial blood-endolymph barriers.

In summary, gentamicin is trafficked across the strial BLB into endolymph preferentially enter hair cells across their apical

membranes, compared to perilymph trafficking, answering a long-standing question in ototoxicity.

Methods

Animal preparation. Albino guinea pigs (270–500 g, Dunkin-Hartley from Charles River Laboratories International, Inc.) of either sex with Preyer's reflex were anesthetized (ketamine 30 mg/kg and xylazine 5 mg/kg, i.m.). Supplemental doses were given on recovery of toe-pinch withdrawal reflex. Body temperature was maintained at 37°C with a servo-regulated heating blanket. Experimental protocols were approved by the Animal Care and Use Committee at Oregon Health & Science University (IS00000351).

The animal was supine to expose the ventral aspect for surgical procedures. The head was held firmly in place, and a tracheotomy performed to insert a ventilation tube for unobstructed breathing. A ventral-postauricular approach exposed the left auditory bulla, with the medial portion of the external auditory canal preserved to allow placement of a sound delivery tube. The temporal bulla was opened to access the cochlea.

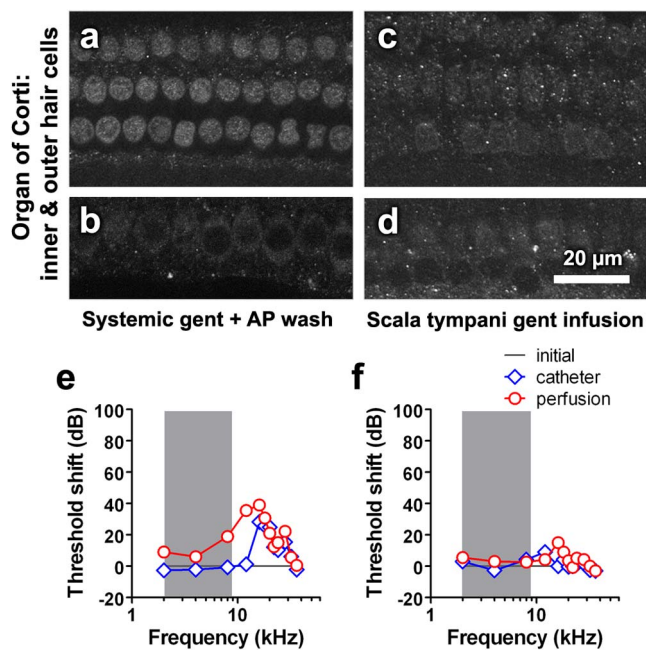


Figure 3 | Gentamicin immunofluorescence also supports a primary endolymph trafficking route for aminoglycosides. (a) Characteristic gentamicin immunofluorescence in hair cells in the organ of Corti after systemic gentamicin plus perilymph wash. Inner hair cell bodies are not in the same focal plane (b). (c) Gentamicin immunofluorescence had little cell specificity in the organ of Corti after perilymph infusion. Inner hair cell bodies are not in the same focal plane (d). Scale bar = 20 μm. All confocal images acquired and processed using identical settings. (e) Cochlear sensitivity was largely maintained in a cochlea receiving systemic gentamicin plus perilymph wash. Gray area indicates the frequency region corresponding to the third coil of the guinea pig cochlea. (f) Cochlear sensitivity was also preserved in a cochlea receiving scala tympani infusion of gentamicin.

Measurement of CAPs. A ball electrode made of Teflon-coated silver wire was placed in the round window niche and fixed on the bulla with carboxylate cement. Tone bursts (10 ms duration, 1-ms rise/fall) generated using a 16-bit D/A converter (Tucker Davis Technologies) were delivered to the ear canal to evoke CAPs. The round window signal was amplified 1,000x by an AC amplifier (CWE Inc, model BMA-200) and a custom-designed AC amplifier. After A/D conversion and averaging, the evoked electrical responses were displayed in real time. The minimum sound level-evoked CAP was documented as threshold at each frequency from 0.5 to 32 kHz. The CAP thresholds were obtained prior to procedures on the cochlea, after fixing the perfusion inlet catheter and creating the efflux orifice, and after scala tympani perfusion.

Gentamicin-Texas Red. GTTR was produced in large batches as described earlier²⁰, and two batches were used at the same relative fluorescence intensity.

Scala tympani perfusion. Perfusion of the scala tympani with artificial perilymph was performed to remove GTTR or gentamicin from the scala tympani following systemic injection, or to infuse aminoglycosides through the scala tympani. An inlet hole (diameter: 70 μm) was made in the scala tympani of the basal turn, and the efflux orifice (diameter: 80 μm) at the apex turn of the cochlea. The inlet catheter (MRE-040, Braintree Scientific) with a fine tip (diameter: 60 μm) was inserted into the inlet hole. Tissue glue fastened the tube in position and sealed the inlet hole. GTTR (0.5 mg/ml) and gentamicin (100 mg/ml) were dissolved in 0.9% sodium chloride (Baxter Health Corp.), and further diluted with artificial perilymph (in mM: 125 NaCl, 3.5 KCl, 1.3 CaCl₂, 1.5 MgCl₂, 0.15 NaH₂PO₄, 10 HEPES, 5.0 glucose; pH 7.4; 300 ± 10 mOsm) as needed prior to use. Perfusates were infused into the scala tympani for 30 minutes at 2 μl/min using a syringe pump (Harvard Apparatus, PHD 2000), and efflux absorbed using cotton wicks.

Drug delivery. For systemic delivery, 1 mg/kg GTTR (gentamicin base) was injected into the jugular vein, or 200 mg/kg gentamicin intraperitoneally. Serum levels of GTTR or gentamicin, obtained using turbidimetric inhibition immunoassay²³ by Lab Services at OHSU (sensitivity: 0.5 μg/ml)⁷ were ~7.0 μg/ml (n=5) or ~57 μg/ml (n=3), respectively. Thus, 1.4 μg/ml GTTR or 11 μg/ml gentamicin was delivered by scala tympani infusion (20% of serum level by systemic injection⁹).

Confocal analysis. After 30 minutes of drug exposure and CAP measurement, guinea pigs were cardiac-perfused with PBS, then 4% formaldehyde in 0.1 M PBS. Cochleae were excised and fixed overnight, rinsed in PBS, and permeabilized with 0.5% Triton X-100 for unmasking GTTR fluorescence²⁴ or gentamicin immunolabeling, as follows. Fixed gentamicin-treated cochleae were then immunoblocked in 10% goat serum in PBS for 30 minutes and incubated with 5 μg/ml mouse monoclonal (Fitzgerald Industries, Concord, MA) gentamicin antisera for 2 hours. After washing with 1% goat serum in PBS, specimens were further incubated with 20 μg/ml Alexa-488-conjugated goat anti-mouse antisera (Molecular Probes, Eugene, OR), then washed, and post-fixed with 4% formaldehyde for at least 15 minutes, as before²⁴. The stria vascularis and organ of Corti from the third coil were excised and whole-mounted in VectaShield (Vector Labs, Burlingame, CA) for confocal microscopy (Bio-Rad MRC 1024 ES).

Imaging and data analysis. Fluorescent images were collected sequentially. Each set of experimental and control tissues were imaged at the same confocal settings. Two sites from the third coil of each cochlea were imaged, and identically processed using Adobe Photoshop. High resolution xz optical sections (0.2 μm in z dimension) of the whole-mounted stria vascularis were acquired by confocal image acquisition software. For statistical analysis, GTTR fluorescence intensities were obtained²⁰ for two-way ANOVA (delivery route X GTTR batch) and post hoc t-tests to identify any statistically significant effect of delivery routes. The statistical analysis was performed with Prism (GraphPad Software, Inc.).

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Author contributions

HL built experimental platform, collected and analyzed the experimental data, and prepared the manuscript. PSS conceived the study and edited the manuscript.

Additional information

Supplementary information accompanies this paper at <http://www.nature.com/scientificreports>

Competing financial interests: The authors declare no competing financial interests.

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