Aminoglycosides Induce Acute Cell Signaling and Chronic Cell Death in Renal Cells that Express the Calcium-Sensing Receptor

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The aminoglycoside antibiotics (AGAs) are calcium-sensing receptor (CaR) agonists that are toxic to the renal proximal tubule. Proximal tubule–derived opossum kidney (OK) cells express CaR-like proteins and respond to AGAs with intracellular Ca\(^{2+}\) mobilization and extracellular regulated protein kinase (ERK) phosphorylation. To examine the possible cellular basis of AGA toxicity, acute and chronic responses to AGA treatment in OK cells and in CaR stably transfected HEK-293 cells (CaR-HEK) were studied. Changes in cell-fate signaling, proliferation, and cell death were detected by semiquantitative Western blotting, Hoechst staining, cell counting, and FACS analysis. Confocal microscopy was used to study the relative internalization of fluoroaphore-labeled gentamicin in CaR-transfected and -nontransfected cells. Here it is reported that the AGA neomycin and gentamicin elicit acute, phosphatidylinositol-3 kinase–dependent phosphorylation of Akt, glycogen synthase kinase 3\(\beta\), and p38 mitogen-activated protein kinase. After 24 h of gentamicin treatment, OK cell proliferation was observed, whereas after 4 d, the OK cells underwent cell death, an effect that was mimicked by the CaR agonists spermine and polyarginine. Furthermore, gentamicin elicited substantially more cell death in CaR-HEK cells than in nontransfected HEK-293 cells. The pan-specific caspase inhibitor Z-VAD significantly inhibited cell death in both OK and CaR-HEK cells. Finally, the intracellular uptake of Texas Red–labeled gentamicin was equivalent in both CaR-transfected and vector-transfected HEK-293 cells, suggesting that the CaR does not enhance drug uptake. Together, these observations demonstrate that the AGAs induce both acute and chronic cell fate changes in OK cells and CaR-HEK cells and that the proximal tubular CaR is likely to contribute to signaling underlying the renal toxicity of the AGAs.

Aminoglycoside antibiotics (AGAs) are a mainstay therapy in the treatment of life-threatening, Gram-negative infections (1). They are an effective and economical, broad-spectrum antibiotic; however, their clinical usefulness is limited by their nephrotoxicity and ototoxicity (1). Renal impairment is commonly observed during or after therapy and is proportional to dose and duration of administration (2).

After glomerular filtration, AGAs bind to the apical surface of proximal tubule (PT) cells, initiating a process that leads ultimately to cell death and tubular injury (1,2). It is proposed that AGA nephrotoxicity results from endocytic retrieval of the drugs by megalin (3), with subsequent sequestration in PT cell lysosomes, leading ultimately to lysosomal rupture and necrotic cell death. However, chronic treatment of rats with low concentrations of gentamicin can elicit renal cortical apoptotic, rather than necrotic cell death (4). It is now well established that the extracellular calcium-sensing receptor (CaR) (5) is expressed on the apical surface of the PT (6–9) and is responsive to micromolar concentrations of certain AGAs (5,9,10). Specifically, the AGAs stimulate the CaR with a rank order of potency that correlates positively with the number of their attached amino groups and indeed their nephrotoxicity. It is interesting that in the reduced pH environment of the PT lumen, the sensitivity of the CaR to AGA challenge is actually enhanced (10). As a G protein–coupled receptor, the CaR is capable of responding rapidly to AGA treatment, initiating signal transduction cascades that could theoretically alter both acute cell physiology and chronic cell fate. In this regard, we reported recently that in a well-characterized cell culture model of the PT, namely opossum kidney (OK) cells, exposure of the OK cells to elevated extracellular calcium levels, gentamicin, or neomycin caused intracellular Ca\(^{2+}\) mobilization and activation of extracellular regulated protein kinase (ERK) 1,2 (9). In light of these circumstantial observations, to elucidate a possible cellular mechanism of AGA-induced toxicity, we investigated the consequences of acute and chronic aminoglycoside treatment on cell signaling in OK cells. The results in OK cells were compared with similar experiments performed in CaR-transfected (and nontransfected) HEK-293 cells.

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Materials and Methods
Unless otherwise stated, items were obtained from Sigma-Aldrich (Poole, Dorset, UK) or from sources described previously (9). Antisera were obtained from the following sources: phospho-Akt (Ser 473), phospho-Akt (Thr 308), pan-Akt 1/2/3, phospho-glycogen synthase kinase (GSK-3β, Ser 9) and phospho-p38 mitogen-activated protein kinase (MAPK) were from Cell Signaling (Beverly, MA).

Tissue Cell Culture
OK cells (used within 12 to 23 passages of purchase from the American Type Culture Collection, Rockville, MD) were cultured as described previously (9). HEK-293 cells, stably transfected with human parathyroid CaR (CaR-HEK) (11), were a gift from Dr. E.F. Nemeth (NPS Pharmaceuticals, Inc., Salt Lake City, UT), and empty vector-transfected HEK-293 cells were a gift from K. Croskery and Dr. R. Prince (University of Manchester, Manchester, UK) and were cultured as described previously (10).

Akt Activation Assay
Cells were grown to 80 to 90% confluence in 60-mm culture dishes, and Akt (protein kinase B) phosphorylation was assayed as described previously for ERK (9). Cells were incubated at 37°C before lysis on ice in RIPA buffer supplemented with protease and phosphatase inhibitors (9), and proteins were resolved by SDS-PAGE before immunoblotting using phospho-specific antibodies (9,12).

Cell Counting and Mitosis Estimation
OK cells were grown for 24 h in 60-mm culture dishes (for cell counting) or on 22-mm sterile coverslips (for DAPI staining) and then for an additional 24 h in serum-free medium ± gentamicin. OK cells were counted using a Neauber’s counting slide. For DAPI staining, cells were incubated in buffer that contained DAPI (1 µg/ml) and then mounted in ice-cold mounting medium. Viable cells (DAPI excluded) and nonviable cells (DAPI solution) were counted using a Neauber’s counting slide. For DAPI staining, cells were grown for 24 h in 60-mm culture dishes (for cell counting) or on 22-mm sterile coverslips (for DAPI staining) and then for an additional 24 h in serum-free medium ± gentamicin. OK cells were counted using a Neauber’s counting slide. For DAPI staining, cells were incubated in buffer that contained DAPI (1 µg/ml) and then mounted in ice-cold mounting medium.

Cell Death Counting
Cells (60 to 70% confluence, 60-mm dishes) were incubated in medium that contained 1% serum supplemented with various treatments for up to 4 d. Adherent cells were mixed 1:1 (vol/vol) with 0.4% Trypan Blue solution. Viable cells (Trypan Blue excluded) and nonviable cells (Trypan-Blue stained, including cells floating in the medium) were counted on a light microscope using a Neauber’s counting slide.

Flow Cytometric DNA Analysis
Cells that were grown in culture flasks were treated as above and then resuspended in serum-free medium that contained propidium iodide (10 µg/ml). Flow cytometric analysis was performed using a FACS Vantage flow cytometer equipped with an Enterprise laser (Innova Technology, Santa Clara, CA; laser excitation at 488 nm, 250 mW). Red fluorescence (DNA-bound propidium) was detected at 630 ± 22 nm and acquired using logarithmic amplifiers. Forward and side light scatter was also recorded to indicate cell size and granularity, respectively. A total of 10,000 cells were analyzed per sample, and Cell-Fit software (Becton-Dickinson, Santa Clara, CA) was used to evaluate the data.

Texas Red Gentamicin Internalization
HEK-293 cells were transfected with pcDNA3.1 or CaR–enhanced green fluorescent protein (EGFP) vectors using Eugene 6 reagent (Roche, Basel, Switzerland) and after 24 h plated onto glass coverslips. After an additional 24 h, cells (at 60 to 70% confluence) were incubated at 37°C in prewarmed HEPES-buffered physiologic saline that contained Texas Red–conjugated gentamicin (2 mg/ml; Molecular Probes, Eugene, OR) for 30 min (13). Cells were fixed and mounted in ice-cold solutions as described previously (14). Relative levels of Texas Red–conjugated gentamicin (TRG) uptake were studied by confocal microscopy using an Ultraview confocal optical scanner with a Kr/Ar laser (Perkin Elmer Life Sciences, Cambridge, UK) mounted on an Olympus IX70 inverted microscope. Images were acquired with an UltrapiX CCD digital camera and processed using Perkin Elmer UltraView software package. Laser intensity, shutter speed, and image capture speed were constant throughout the acquisition process of each experiment.

Statistical Analyses
Unless otherwise stated, data are presented as means ± SE, and statistical significance was determined by one-way ANOVA (Tukey post hoc test).

Results
Aminoglycoside-Induced Akt Signaling in OK Cells
We showed previously that in proximal tubular OK cells, stimulation with AGA elicits phosphatidylinositol-3 kinase (PI3K)-dependent ERK1,2 activation (9). A well-established downstream effector of PI3K activity is the antiapoptotic protein kinase Akt (PKB); thus, we studied the effect of AGA treatment on Akt phosphorylation in OK cells. As shown in Figure 1A, acute exposure of the cells to neomycin (300 µM; 5 min) resulted in marked elevation of Akt phosphorylation on residues Thr-308 (116 ± 27% increase; P < 0.01; n = 7) and Ser-473 (519 ± 227% increase; P < 0.001; n = 7), believed to be activation signals for Akt (15). To confirm the PI3K dependence of neomycin-induced Akt phosphorylation, we pretreated the cells with wortmannin (30 nM; Figure 1Aii) or LY294002 (30 µM; Figure 1Bii). These two structurally unrelated inhibitors of PI3K fully ablated the neomycin response. Immunoblotting with a nonphosphospecific Akt antibody revealed similar levels of Akt protein in each sample. Treatment of the cells with gentamicin elicited an identical stimulation (Figure 1Aii). Neomycin-induced Akt phosphorylation was unaffected by pretreatment with the protein kinase C inhibitor GF109203X (500 nM; Figure 1Bii) or by the MEK1 inhibitor PD98059 (10 µM; Figure 1Biii). However, the phosphatidylinositol-4,5-bisphosphate (PIP2)–phospholipase C (PLC) inhibitor U73122 (5 µM) did fully inhibit neomycin-induced Akt phosphorylation (Figure 1Biii).

A downstream effector of Akt activation is GSK-3β, which is inhibited upon Akt-mediated phosphorylation of Ser-9. Using a phospho-specific antibody to this residue, we determined that neomycin stimulates phosphorylation of GSK-3β by a mechanism that was again fully inhibited by LY294002 (Figure 1C).

Next, we examined the effect of AGA exposure on the phos-
phorylation of p38 MAPK on its activation residues. Both neomycin and gentamicin elicited p38 MAPK activation (neomycin, 658 ± 89% increase; P<0.01; n=3), and the neomycin response was inhibited by co-treatment with 30 nM wortmannin, suggesting that the response is also PI3K mediated (Figure 1D).

Because the AGAs are known agonists for CaR and because we demonstrated previously the molecular and functional expression of a CaR-like molecule in OK cells, we examined whether elevation of extracellular Ca\(^{2+}\) (Ca\(^{2+}\)o) levels could mimic the response to the AGAs. As is shown in Figure 1E, stimulation of OK cells with high Ca\(^{2+}\)o levels elicits phosphorylation of ERK1,2 as well as Akt (Ser-473, 540% increase; P<0.05; n=4) and GSK-3β (Ser-9).

**Aminoglycosides Induce OK Cell Proliferation**

Given the acute activation of the pro-proliferative ERK and antiapoptotic Akt pathways, we next examined whether chronic gentamicin treatment results in increased cell number. As shown in Figure 2A, gentamicin (500 µM) induced modest but significant increases in OK cell numbers after 24 h (7.6 ± 1.8%; P<0.01; n=7) when co-treated with 1% serum. Similar increases in cell number were observed when treatment occurred under serum-free conditions (data not shown).

To confirm these observations, we treated OK cells with various concentrations of gentamicin for either 12 (Figure 2Bi) or 24 h (Figure 2Bii) in the absence of serum and then processed them for the XTT assay. After 12 h, gentamicin elicited a small but significant dose-dependent increase in proliferation. By 24 h, the gentamicin-induced rise in XTT product was still evident but no longer dose dependent with the concentrations tested. As a positive control, we also treated OK cells with 2% serum-containing medium and found, as expected, that the serum elicited a greater increase in cell number (Figure 2A) and XTT product (Figure 2B). Co-treatment with the PI3K inhibitor wortmannin (100 nM) significantly inhibited (46%) this gentamicin-induced rise in OK cell proliferation (Figure 2Ci). In addition, the gentamicin effect was substantially inhibited after treatment with PIP2-PLC inhibitor U73122 (2 µM) but not with its inactive analogue U73343 (Figure 2Cii). In agreement with these findings, a greater number of mitotic nuclei (detected using the nuclear dye Hoechst-33342) were observed on glass coverslips that were treated for 24 h with gentamicin (Figure 2D, indicated by arrow) than in control samples (quantification not shown). Similarly, in a separate but identical experiment in which the nuclei were stained with DAPI, there was a significant increase in the proportion of mitotic cells, thus confirming proliferation (control, 3.0 ± 0.3%; gentamicin, 4.8 ± 0.2%; n=5 [each counted in quadruplicate]; P<0.01). Importantly, gentamicin did not significantly increase or decrease ethidium bromide cellular uptake, *i.e.*, cell death, within those first 24 h of treatment.
Aminoglycoside-Induced Cell Death in OK Cells

To examine the effect of AGA on cell death in OK cells, we incubated the cells for 4 d in 1% serum-containing medium supplemented with various AGA treatments. Dead cells, both floating and adherent, were collected and stained either with trypan blue for counting with a Neubauer’s counting slide (Figure 3A) or with propidium iodide for counting by FACS analysis (Figure 3B). Thus, by these two independent methods, we demonstrated gentamicin-induced cell death in OK cells after 4 d of treatment (Figure 3). To test whether other CaR agonists elicit OK cell death, we incubated cells for 4 d with spermine (1 mM) or polyarginine (100 nM; 5 to 15 kD), which caused almost identical levels of cell death as for gentamicin.

Aminoglycoside-Induced Cell Death in CaR-Transfected HEK Cells

Nontransfected HEK-293 (nt-HEK) cells, empty vector stably-transfected HEK-293 cells (vect-HEK, a control for possible transfection artifacts), and HEK-293 stably transfected with human CaR (CaR-HEK) were treated with AGA for 4 d in medium that contained 1% FBS, and the proportion of dead cells was assessed by trypan blue exclusion as before. In all experiments, gentamicin-induced cell death was significantly greater in the CaR-transfected cells than in either the nt-HEK or the vect-HEK cells. In the experiment shown in Figure 4Ai, 200 μM gentamicin treatment for 4 d had no effect on cell death in nt-HEK cells, whereas in CaR-HEK cells, gentamicin treatment increased cell death by 76%. Raising the gentamicin concentration to 500 μM caused a small (nonsignificant in this experiment) rise in cell death in the nt-HEK cells but a 165% increase.
in the CaR-HEK cells. In another experiment, 500 μM gentamicin treatment for 3 d increased cell death by 31% in vect-HEK but by 151% in CaR-HEK cells. By day 4, the increase in gentamicin-induced cell death was 127% in vect-HEK cells but again was substantially higher (706%) in CaR-HEK cells. Thus, although gentamicin elicits baseline increases in cell death in all of the HEK cell lines studied, as would be expected given the use of the aminoglycosides in vector-selection studies, the rise in cell death was always significantly greater in the CaR-HEK cells.

The time dependence described above is further illustrated in a different CaR-HEK cell experiment (Figure 4Aii) in which gentamicin-induced cell death was detectable initially after 3 d of treatment but more marked after 4 d of treatment. In support of these data, the dose-dependent increase in CaR-HEK cell death was reproduced by flow cytometry performed after 4 d of gentamicin exposure (Figure 4B).

Assessment of Cell Death by Light Scatter and Caspase Inhibition

To determine the likely mechanism of death that gentamicin induced in OK and CaR-HEK cells, we further analyzed the flow cytometry data to show the size (forward scatter) and...
granularity (side scatter) of the treated cells. Figure 5 shows that in both OK cells (A) and CaR-HEK cells (B), cells that were highly stained with propidium iodide as a result of gentamicin treatment are ultimately smaller and exhibit greater granularity than cells that were treated under control conditions. Such a finding is more usually associated with apoptosis rather than necrosis. Furthermore, the increases in OK and CaR-HEK cell death elicited by chronic gentamicin exposure were both significantly inhibited by co-treatment with the pan-specific caspase inhibitor Z-VAD (Figure 5).

**Gentamicin Internalization in HEK-293 Cells**

Because AGA toxicity is believed to involve cellular uptake of the drug, we then studied the uptake of TRG into HEK-293 cells that were transiently transfected with EGFP-tagged CaR. Figure 6A shows the intracellular accumulation of TRG in cells that exhibit green fluorescence, i.e., CaR expression, on or at the cell surface. Finally, when empty vector-transfected HEK-293 cells and HEK-293 cells that were transfected with EGFP-tagged CaR were incubated for 30 min in a solution that contained TRG, there was no apparent difference in the amount of gentamicin uptake in the presence or absence of CaR expression (Figure 6B).

**Discussion**

This study demonstrates that, in the PT-derived OK cell line, the aminoglycoside antibiotics induce contrasting biphasic responses: Short-term, they evoke an increase in cell number accompanied by proliferative signaling followed, long-term, by time- and dose-dependent cell death. Furthermore, overexpression of CaR in a kidney-derived cell line, HEK-293, substantially increases these cells' susceptibility to AGA-induced toxicity. Given the presence of the CaR on the apical membrane of the PT (8) and given its responsiveness to these antibiotics, which exhibit a similar rank order of potency for CaR activation as for their nephrotoxicity (5,10), we propose, therefore, that the CaR may contribute to AGA nephrotoxicity.

In OK cells, acute AGA exposure elicited PI3K-dependent Akt phosphorylation, resulting in GSK3β phosphorylation.

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**Figure 5.** Gentamicin elicits apoptotic cell death in OK and CaR-HEK cells. OK cells (A) and CaR-HEK cells (B) were treated in the absence or presence of gentamicin (500 μM) as described in the legends to Figures 3 and 4, respectively. The cell size and granularity profiles of the cells from these experiments are shown here as two-dimensional plots. In both OK and CaR-HEK cells, gentamicin increased the proportion of smaller, more granular cells. These subpopulations, marked by polygons, consisted almost exclusively of propidium iodide–positive cells, suggesting that the cells undergo apoptotic cell death (from a minimum of three independent experiments). Alternatively, cells were exposed to gentamicin (500 μM) for 3 (OK cells; A) or 4 d (CaR-HEK cells; B) in the presence or absence of the pan-specific caspase inhibitor Z-VAD (20 μM; Z-V-A-D-(OMe)-FMK; R&D Systems; after a 10-min inhibitor pretreatment), and cell death was counted as before; n = 5. *P < 0.05 gent versus zvad/gent by unpaired t test.
Phosphorylation of GSK3β on Ser-9 inhibits its enzyme activity and subsequent apoptosis (15). Consistent with the promitogenic activation of ERK and antiapoptotic activation of Akt, the initial cell-fate effect of gentamicin treatment on OK cells is an increase in cell number and XTT product. Indeed, this gentamicin-elicited increase in XTT product was significantly inhibited by co-treatment with the PI3K inhibitor wortmannin and nearly abolished by the PLCβ inhibitor U73122, a treatment that also inhibited the phosphorylation of Akt and ERK (9). ERK1,2 activation is known to enhance the cellular repair capacity of renal epithelial cells (16); therefore, the observed kinase signaling could represent the physiologic response of the cells to a perceived injury by AGA. It is possible that the 1% serum "background" reduces the magnitude of the gentamicin-induced proliferation; nevertheless, the OK cell proliferative response to the drug is real, reproducible, highly significant, and observed under different experimental conditions (1% serum, 12 and 24 h) and by different methods of quantification (cell counting, XTT assay, and Hoechst dye/DAPI nuclear staining).

The activation of p38 MAPK is associated with the progression of renal lesion formation in various experimental models of kidney failure, including agonist-induced apoptosis in rat proximal tubular cells (17,18). Here we show that acute neomycin or gentamicin treatment stimulated the phosphorylation/activation of p38 MAPK, suggesting the initiation of a stress response in OK cells to the AGA after only 5 min. These signals, together with the previously documented AGA-induced Ca^{2+} mobilization in OK cells (9), represent the earliest responses of proximal tubular cells to AGA challenge so far reported. Furthermore, gentamicin-induced OK cell proliferation is itself an entirely novel observation and unexpected if indeed the AGA exert their nephrotoxicity by disrupting protein translation. An important issue to address next will be how or indeed whether the chronic cell death is related to the signaling and proliferation events seen in the first 24 h.

Clinically, gentamicin-induced nephrotoxicity is most commonly reported after approximately 6 d of treatment (1,2). Here we observed significant toxicity after only 3 (HEK cells) or 4 d (OK cells) of gentamicin exposure. Regarding the potential involvement of the CaR in AGA-induced cell death, two alternative, membrane-impermeant CaR agonists, spermine and polyarginine (19,20), closely mimicked gentamicin-induced OK cell death. In addition, significantly more cell death was observed in HEK-293 cells expressing the CaR than in nontransfected or vector-transfected HEK-293 cells. This indicates that transfection per se does not affect the sensitivity of the cells to the drug and that the presence of the CaR increases the susceptibility of these cells to AGA toxicity. In CaR-HEK cells, gentamicin elicited cell death with a time-course, dose-response range and light scatter profile similar to its effects in OK cells. Our current explanation for these observed effects is that the proliferative stimulus, possibly sustained by ERK, Akt, and p38 signals, represents the initial cellular response to AGA damage. Long-term, the prolonged activation of the CaR by AGA may lead to sustained elevation of intracellular calcium levels, with subsequent mitochondrial and/or endoplasmic reticulum calcium overload (21), resulting in apoptotic cell death (22,23).
will be important to determine the relative contributions of the various signaling pathways to AGA proximal tubular toxicity. It is generally believed that gentamicin induces necrosis; however, the cell size and granularity profiles that we observed are more indicative of apoptosis. In addition, the attenuation of AGA cell death by caspase inhibition further supports this. Such findings are in apparent agreement with the data of El Moudden et al. (4), who demonstrated apoptotic cell death in gentamicin-treated rats. Because evidence exists for both AGA-induced apoptosis and necrosis, it is possible that the necrosis occurs secondary to the apoptosis or that the apoptosis occurs via lysosomal destabilization, as has been demonstrated for quinolone antibiotics (24). The relative contributions of apoptosis and necrosis to acute renal injury are a current area of active investigation (reviewed in reference 25). The concentrations of gentamicin used here are approximately 15 to 30 times higher than the clinical target blood concentration. However, it should be noted that the cell death reported here is observed after only 3 to 4 d, whereas clinical nephrotoxicity is usually observed later than this. It is perfectly possible that lower concentrations of gentamicin will elicit cell death in culture given longer treatment periods. As the AGAs are polycationic and cannot freely cross the membrane, it has been assumed that the mechanism of cellular toxicity must involve endocytic uptake of the drug. This occurs via drug binding to a specific, cell-surface receptor complex, involving megalin and cubilin (3,26), followed by internalization of the receptor-substrate complex and its accumulation into lysosomes (2,27). A reduced accumulation of AGA in the renal cortex of mice in which the megalin gene has been selectively disrupted (28) underlines the importance of megalin in the drug uptake process. Thus, in theory, the CaR could contribute to AGA toxicity by increasing uptake of the drug into the cell. However, the uptake of fluorophore-labeled gentamicin was equivalent in vector-transfected and CaR (EGFP)-transfected HEK-293 cells, the presence of the CaR alone does not seem to stimulate endocytic uptake of the drug. It is also interesting in this regard that the empty vector-transfected HEK-293 cells exhibited acute TRG uptake but with substantially less toxicity. Hence, it is likely that, in CaR-HEK cells, AGA toxicity is substantially mediated by receptor-elicited signaling events rather than by drug uptake.

In summary, the current data, together with our previous study (9), indicate that the AGAs exert a biphasic response in OK cells. First, pro-proliferative (ERK) and antiapoptotic (Akt phosphorylation) signaling and an increase in cell number are seen that could represent a response to a perceived damage. Then, as the drug exposure continues, cell death that more closely resembles apoptosis than necrosis occurs. Given that the observed gentamicin-induced effects are (1) attenuated by PI3K and PLCβ signaling inhibitors, (2) mimicked by other membrane-impermeant CaR agonists, (3) reproduced selectively in CaR-expressing HEK-293 cells, and (4) not due to enhanced drug uptake, these observations strongly suggest that AGAs act on OK cells most likely via a membrane receptor, e.g., a GPCR. Thus, we believe that an overstimulation of the CaR present on the apical surface of PT cells could contribute significantly to AGA toxicity in renal cells.

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