



Statins inhibit aminoglycoside accumulation and cytotoxicity to renal proximal tubule cells

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ABSTRACT

Nephrotoxicity due to renal proximal tubule accumulation of aminoglycoside (AG) antibiotics, such as gentamicin, represents a major clinical problem. Receptor-mediated endocytosis via the multi-ligand receptor megalin is thought to be a key mechanism in the cellular uptake of AGs and nephrotoxicity. This process can be modulated by the intracellular concentration of isoprenoid pyrophosphates derived from the processing of mevalonate by 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase. Post-translation modifications by isoprenoid pyrophosphates are necessary for GTP-binding protein function. Given that statins inhibit HMG-CoA reductase and therefore affect the concentration of isoprenoid pyrophosphates, we have tested the hypothesis that statins will lead to a reduction in AG renal proximal tubule accumulation and cytotoxicity. Gentamicin accumulated within cultured proximal tubule derived opossum kidney (OK) cells and led to dose- and time-dependent cell death which was inhibited by non-toxic doses of simvastatin (IC_{50} 1.3 μ M), rosuvastatin (IC_{50} 16.3 μ M) and pravastatin (IC_{50} 38.8 μ M). The mechanism of inhibition was linked to the degree of cholesterol synthesis inhibition and GTP-binding protein unprenylation. Moreover, co-incubation with mevalonate or geranyl-geranyl pyrophosphate, products of HMG-CoA reductase, reversed the inhibitory effect of statins on cellular accumulation and cytotoxicity of gentamicin. In summary, our data suggest that the inhibition of the mevalonate pathway by statins may provide a potential therapeutic strategy to prevent AG-induced nephrotoxicity.

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1. Introduction

Aminoglycosides (AG), including gentamicin, are a widely prescribed and effective class of antibiotic which have low rates of resistance. They are used in the treatment of a number of severe bacterial infections including those affecting the lungs, heart, biliary and urinary tracts [1]. However, nephrotoxicity represents a serious clinical problem in 10–15% of patients and is the dose limiting factor for their therapeutic use [2,3]. To uncover mechanisms of AG-induced nephrotoxicity would thus be important to improve the therapeutic index and may also allow the development of novel intervention strategies. However, currently apart from once daily dosing [4], no other clinically effective strategies exist for the prevention of AG nephrotoxicity.

Following systemic administration, AGs remain relatively unchanged and have low tissue absorption and plasma protein binding. Around 80% of the systemic dose is excreted into the urine within 24 h by glomerular filtration. However, accumulation within the renal cortex, especially in the proximal tubule epithelial

cells, remains high compared to other organs (5–10% dose) [5]. This is thought to be important in the pathogenesis of nephrotoxicity, and thus attention has focused on the molecular and biochemical mechanisms underpinning AG accumulation. This may be key to developing potential strategies for the intervention of toxicity.

Binding of AGs to the multi-ligand receptor, megalin, and cellular uptake by receptor-mediated endocytosis has been demonstrated to be the principal pathway for accumulation [6]. The abundant negative charges on the extracellular receptor domain facilitate interactions between polybasic substances such as AGs [6]. Indeed, the rank order of AG toxicity (neomycin > amikacin > gentamicin = tobramycin > netilmicin) can be correlated with the ability to bind to the brush border membranes of the renal tubule [1].

Megalyn also constitutes the main endocytic pathway for the clearance of low molecular weight proteins from the glomerular filtrate, such as albumin, β 2-microglobulin, transthyretin, vitamin D-binding protein and retinol binding protein [7–10]. Because megalin is highly expressed in the renal cortex, it has been postulated to be a key factor in the organ specific uptake, accumulation and resulting toxicity of AGs [11]. In accordance with this, megalin knock-out mice are protected from the renal accumulation of AGs [12]. Furthermore, protein and peptide

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Table 1

Comparison of IC₅₀ values for the inhibitory effect of statins on cholesterol synthesis and gentamicin-induced inhibition of Rap1A prenylation, drug accumulation and cytotoxicity in OK cells. Values were obtained from dose-response curves generated for each statin incubated with and without gentamicin for 48 h. Data is given as mean IC₅₀ value \pm S.D. of three independent experiments.

Statin	IC ₅₀ (μ M)			
	Cholesterol synthesis	Rap1A prenylation	Gentamicin accumulation	Gentamicin cytotoxicity
Simvastatin	0.01 (0.003)	0.05 (0.01)	0.50 (0.20)	1.30 (0.40)
Rosuvastatin	0.08 (0.02)	1.70 (0.20)	3.40 (1.30)	16.30 (3.90)
Pravastatin	0.60 (0.10)	6.20 (1.20)	11.40 (4.70)	38.80 (6.20)

ligands which compete with AGs for binding to megalin are effective in reducing AG accumulation in cell lines expressing megalin and the appearance of urinary biomarkers of AG-induced tubular damage *in vivo* [13].

Several stable proximal tubule cell lines such as those derived from the opossum kidney (OK) can be utilised to study receptor-mediated endocytosis via megalin [14,15]. This process requires active GTP-binding proteins [16]. To function correctly, GTP-binding proteins require post-translational prenylation to anchor them and assist their translocation to cellular membranes. Isoprenoid pyrophosphates such as geranyl-geranyl pyrophosphate (GGPP) and farnesyl pyrophosphate (FPP) are mevalonate-derived intermediates formed during the biosynthesis of cholesterol which are used for the modification of GTP-binding proteins [17].

Statins are inhibitors of the enzyme 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase. They are relatively safe and are widely prescribed for the treatment of hyperlipidemia. They inhibit the rate limiting step in the sterol biochemical pathway, the conversion of HMG-CoA to mevalonate [18]. Therefore, by

decreasing the intracellular sterol pool, statins can also indirectly inhibit receptor-mediated endocytosis by blocking the post-translational modification of GTP-binding proteins [19]. Indeed, statins can inhibit receptor-mediated endocytosis of proteins *in vitro* [20] by a mechanism which correlates with the inhibition of cholesterol synthesis and an increase in the intracellular level of unprenylated GTP-binding proteins [21]. Since statins have a relatively safe clinical profile, they represent a possible strategy for the prevention of nephrotoxicity in man through the blockade of AG uptake and accumulation. Statins can also prevent other forms of renal injury through their inhibition of protein prenylation [22]. In this *in vitro* study, we have therefore tested the hypothesis that through the pharmacological inhibition of HMG-CoA reductase, statins can inhibit the receptor-mediated endocytosis of AGs into cultured renal proximal tubule cells and the resultant cytotoxicity.

2. Materials and methods

2.1. Materials

OK cells were purchased from the European Collection of Cell Cultures (ECACC). Cell culture reagents and test reagents (gentamicin, simvastatin, pravastatin, mevalonolactone, cholesterol and [2-¹⁴C] acetate were purchased from Sigma-Aldrich (Poole, UK). Rosuvastatin was purchased from Bosche Scientific (NJ, USA). For western blotting, the NuPAGE SDS-PAGE gels were obtained from Invitrogen life sciences (Paisley, UK), the ECL-Plus and hyperfilm from Amersham biosciences (Buckinghamshire, UK) and the antibodies (total and prenylated Rap1A and anti-rabbit actin) from Santa Cruz (CA, USA). All solvents were of high grade HPLC quality from Fischer scientific (Loughborough, UK). Lactate dehydrogenase assay kit was purchased from Roche diagnostics (West Sussex, UK).

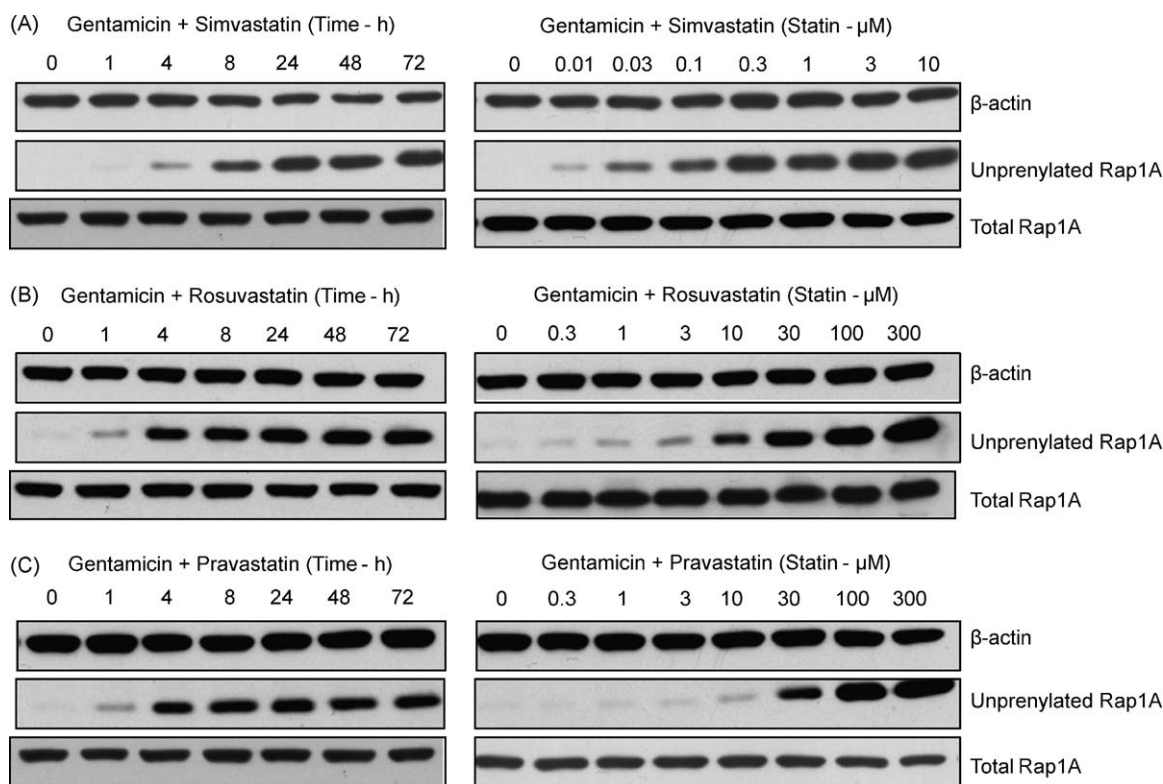


Fig. 1. Statins inhibit Rap1A prenylation in OK cells. Western blot analysis of the time and dose dependent effect of (A) simvastatin, (B) rosuvastatin and (C) pravastatin on the degree of unprenylated Rap1A in OK cells. OK cells were incubated with gentamicin (0.25 mg/ml) in the presence and absence of a statin for 24 h. 10 μ M simvastatin and 30 μ M of either rosuvastatin or pravastatin was used for the time course analysis. Equal amounts of protein were separated by SDS-PAGE from whole cell RIPA lysates. The presence unprenylated Rap1A was determined by western blot and compared to a β -actin loading control and the level of total Rap1A protein. Western blots are representative of three independent experiments.

2.2. Cell culture treatment and cytotoxicity assessment

OK cells were used between passages 40 and 55 and seeded at a density of 2×10^4 cells/cm² in cell culture plates containing transwell membrane inserts to promote polarity and megalin expression. Cells were cultured until confluent prior to dosing and maintained in DMEM-Ham's F-12 mix containing 10% FBS, 2 mmol/L L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin in a humidified atmosphere (5% CO₂, 37 °C). Dosing solutions were prepared in serum free media (mevalonate, gentamicin, GGPP) and as concentrated stock solutions in DMSO (pravastatin, rosuvastatin, simvastatin, GGTI-2147) or ethanol (cholesterol). Control cells were treated with 0.1% DMSO in serum free media. Cells were dosed in serum free media for up to 48 h and media containing 1% serum for later time points. Gentamicin-induced cytotoxicity was determined by quantification of lactate dehydrogenase (LDH) release according to the manufacturer's instructions.

2.3. Gentamicin intracellular quantification by LC-MS/MS

Remaining cells were washed twice with ice cold PBS, lysed with RIPA buffer and kept on ice for 15 min. A portion was removed for protein content determination by the methods of Bradford et al. [23] and samples were centrifuged at 4 °C for 5 min at 14,000 rpm. Intracellular gentamicin was detected by LC-MS/MS in multiple-reaction monitoring mode (MRM) with the main working parameters and product ion scanning based on methods described previously [24]. Analyses were performed using an Applied Biosystems/MDS Sciex API2000 mass spectrometer with TurbolonSpray source (Applied Biosystems/MDS Sciex, Foster City, CA). Analytes were resolved on a Gemini-NX 5 µm C18 110A 250 × 4.60 mm column (phenomenex). Eluate split-flow to the LC-MS interface was 200 µl/min. The following transitions were utilized to selectively detect gentamicin: C1 *m/z* 478 to *m/z* 322; C2/C2a *m/z* 464.1 to *m/z* 322; C1a *m/z* 450 to *m/z* 322. Data were acquired and processed with the Analyst Software (v.1.4; Sciex). Gentamicin levels were calculated by comparison with a standard curve and normalised to total cellular protein content of remaining cells. Coefficient of variation calculated from 5 standard curves ran on 5 separate days was <10% for each data point.

2.4. Cholesterol synthesis determination

Cells were incubated with test reagents for 24 h and 10 µCi [2-C¹⁴] acetate was added for 5 h. Cells were washed twice with ice cold PBS and lysed in 0.1 M NaOH. Labelled acetate incorporation in cholesterol was determined by scintillation counting following HPLC separation as described previously [25]. Percentage (%) inhibition of cholesterol synthesis was compared to control for test reagents.

2.5. Western blotting

OK cells were washed twice with ice cold PBS, lysed in RIPA buffer and incubated on ice for 15 min. Protein concentrations were determined by the Bradford assay [23] and 5 µg of the total whole cell lysate was separated by SDS-PAGE. Resolved proteins were transferred to nitrocellulose membranes and probed with antibodies raised against total or unphosphorylated Rap1A (both 1/1000; overnight) and β-actin (1/5000; 1 h). Secondary antibodies (1/10,000; 1 h) were visualised by exposure to hyperfilm after incubation with ECL-Plus. Unphosphorylated Rap1A levels were determined by densitometry and normalised to the level of β-actin.

2.6. Statistical analysis

Non-linear regression analysis was used to calculate inhibition constants (IC₅₀) from dose response curves recorded at 48 h post dose and statistical significance was assessed through unpaired *t*-test analysis, accepting *P* < 0.05 as being significant.

3. Results

3.1. Statins inhibit GTP-binding protein prenylation in OK cells

Statins can inhibit the function of GTP-binding proteins required for receptor mediated endocytosis of low molecular weight proteins in proximal tubule cells [17,19,21]. We therefore explored the relationship between the inhibition of the geranyl-geranylation of a model GTP-binding protein (Rap1A) and the inhibition of gentamicin-induced cytotoxicity. All 3 statins investigated, simvastatin, pravastatin and rosuvastatin, led to a time- and dose-dependent increase in the inhibition of Rap1A prenylation as determined by western blotting using an antibody which only recognises unphosphorylated Rap1A [26] (IC₅₀ values given in Table 1). Unphosphorylated Rap1A was absent at baseline, and in the absence of the statins, and furthermore was not induced by gentamicin treatment (Fig. 1). The total level of Rap1A remained unchanged with either gentamicin or statin treatment (Fig. 1).

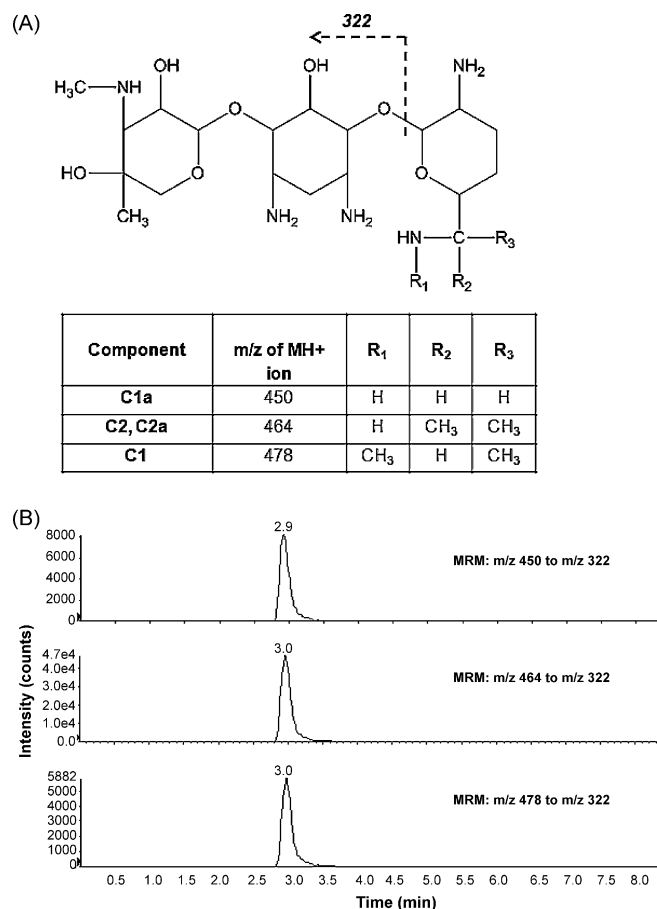


Fig. 2. Gentamicin intracellular quantification by LC-MS/MS. (A) Chemical structure and MS fragmentation of gentamicin components. (B) Individual ion chromatograms of the MRM detection of gentamicin components in OK cells incubated with gentamicin. MRM transitions given on each trace. Intracellular gentamicin was calculated based on the sum of total ion counts from a standard curve.

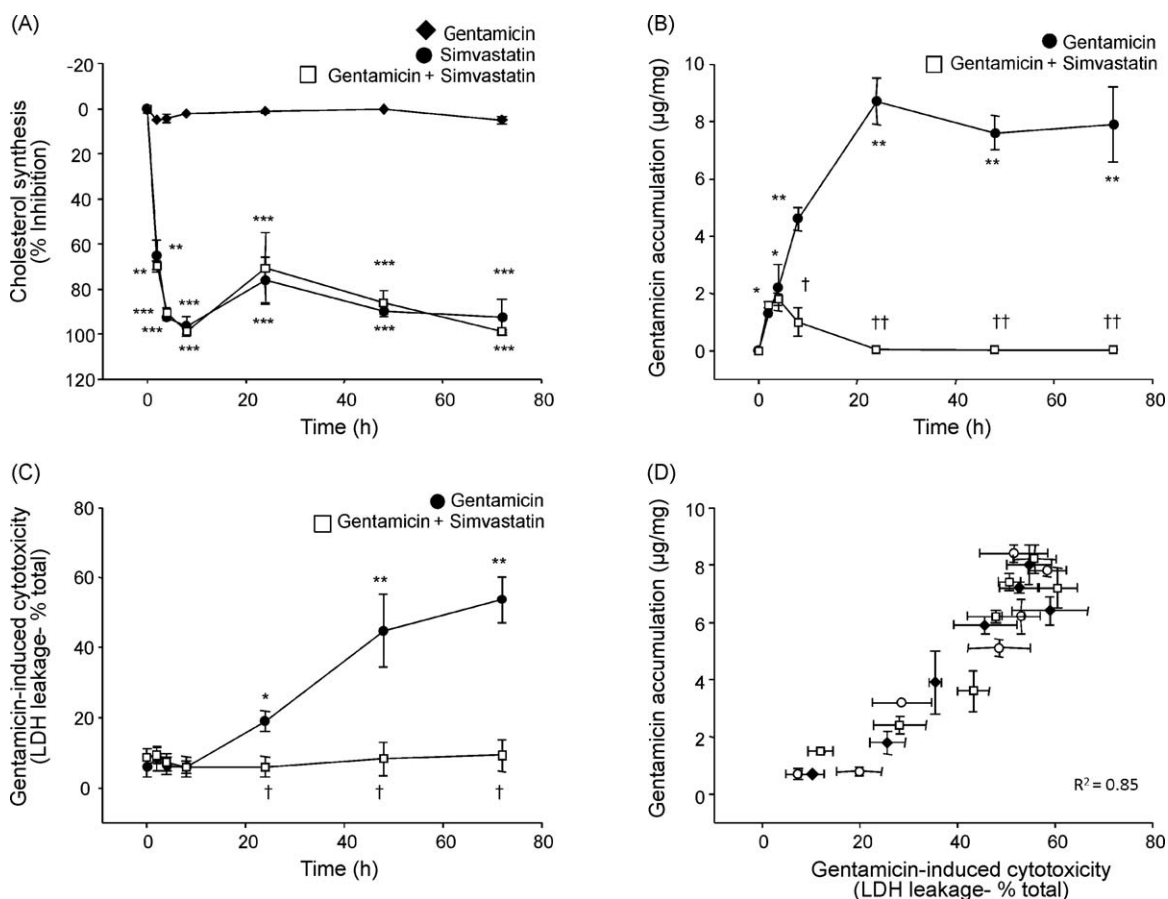


Fig. 3. Gentamicin is accumulated and causes toxicity to OK cells which can be inhibited by simvastatin. (A) The effect of gentamicin (0.25 mg/ml), simvastatin (10 μM) and simvastatin (10 μM) + gentamicin (0.25 mg/ml) on cholesterol synthesis (inhibition of HMG-CoA reductase) in OK cells over time. The effect on cholesterol synthesis was determined by the addition of [2- C^{14}] acetate for the final 5 h and the inhibitory effect of statins on the incorporation into cholesterol was determined as % inhibition 24 h after the statin dose. (B) The effect of simvastatin (10 μM) on the time dependent intracellular accumulation of gentamicin (0.25 mg/ml) in OK cells. (C) The effect of simvastatin (10 μM) on the time (0–72 h, 0.25 mg/ml) dependent cytotoxicity induced by gentamicin in OK cells. Gentamicin accumulation was determined by LC–MS/MS (μg gentamicin/mg cellular protein) and cytotoxicity was determined by LDH leakage into cell culture media (U/l). (D) The linear relationship between gentamicin accumulation and cytotoxicity in OK cells. Correlation coefficient is given where required (□; OK cells incubated with 30 μM pravastatin and 0.25 mg/ml gentamicin over 0–72 h) (●; OK cells incubated with 10 μM simvastatin and 0.25 mg/ml gentamicin over 0–72 h). Data is given as mean \pm S.D. of three independent experiments. Statistical significance was assigned relative to time matched vehicle dosed controls * $p < 0.05$, ** $p < 0.01$ or simvastatin + gentamicin compared to gentamicin alone † $p < 0.05$, †† $p < 0.01$.

3.2. Statins inhibit gentamicin accumulation and cytotoxicity in OK cells

Gentamicin was utilised as a clinically relevant model nephrotoxic aminoglycoside. Gentamicin intra-cellular accumulation was determined by LC–MS/MS (MRM) (Fig. 2). Gentamicin had no inhibitory effect on statin pharmacology (i.e., cholesterol synthesis inhibition) or had no inhibitory effect itself on cholesterol synthesis (Fig. 3A). Gentamicin accumulated within OK cells in a time-dependent manner which first reached significance at 2 h post dose. Maximum accumulation was reached 24 h post dose (Fig. 3B). The cytotoxic effect of gentamicin, analysed through LDH leakage, also showed a time dependent pattern (Fig. 3C). Cytotoxicity was first significant at time points later than 24 h (0.25 mg/ml). There was also a direct relationship between gentamicin accumulation and cytotoxicity ($R^2 = 0.85$) observed in OK cells (Fig. 3D). Co-administration of simvastatin blocked the intracellular accumulation of gentamicin and its cytotoxicity. Simvastatin alone did not cause a significant increase in the release of LDH ($11.5 \pm 4.1\%$ total amount in culture media) compared to vehicle control levels ($7.5 \pm 2.3\%$). This indicates that the inhibitory effect on gentamicin accumulation and cytotoxicity cannot be explained by statin toxicity to the OK cells. This is consistent with literature reports [21]. The effects of pravastatin and rosuvastatin were similar to those of simvastatin. Simvastatin, pravastatin and

rosuvastatin were pharmacologically active in OK cells and induced a dose- and time-dependent inhibition of cholesterol synthesis from acetate (Figs. 3A and 4A). All statins tested also induced a dose dependent inhibitory effect on the cellular unpreylation of Rap1A, accumulation of gentamicin and resulting cytotoxicity in OK cells 48 h after dosing (Fig. 4B–D). The IC_{50} values are given in Table 1 for each statin.

3.3. The relationship between statin-induced HMG-CoA reductase inhibition, Rap1A prenylation and gentamicin accumulation and cytotoxicity

For simvastatin, pravastatin and rosuvastatin, Fig. 5A shows the correlation between the inhibition of cholesterol synthesis (surrogate marker for HMG-CoA reductase activity) and gentamicin cellular accumulation. Fig. 5B also shows the relationship between the inhibition of cholesterol synthesis by statins and the resulting cytotoxicity induced by gentamicin. An inhibition in cholesterol synthesis of more than 60% was required to have a significant inhibitory effect on gentamicin accumulation and more than 70% to have a significant inhibitory effect on cytotoxicity in OK cells. This relationship was also mirrored through the correlation between gentamicin accumulation (Fig. 5C) and cytotoxicity (Fig. 5D) and the degree to which the GTP-binding protein remained unmodified.

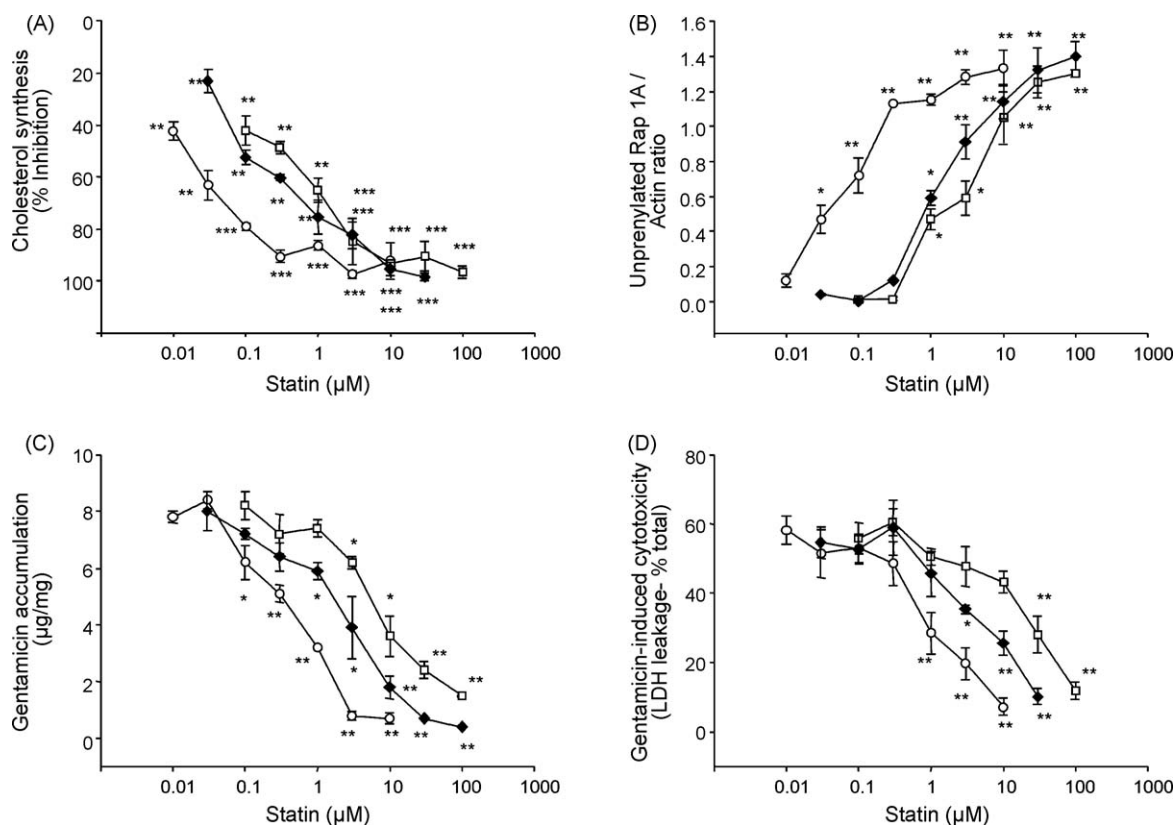


Fig. 4. Dose dependent inhibitory effects of statins in OK cells. The dose dependent inhibitory effect of statins in OK cells on (A) cholesterol synthesis, (B) Rap1A unprenylation, (C) gentamicin cellular accumulation and (D) gentamicin-induced cytotoxicity. OK cells were incubated with gentamicin (0.25 mg/ml) with simvastatin (○; 0–10 μ M), pravastatin (□; 0–100 μ M) or rosuvastatin (◆; 0–100 μ M) for 48 h. The effect on cholesterol synthesis was determined as in Fig. 3. Rap1A unprenylation was determined as described in Fig. 1. Gentamicin cellular accumulation was determined by LC–MS/MS (μ g gentamicin/mg cellular protein) and cytotoxicity was determined by LDH leakage into the cell culture media (U/l). Data is given as mean \pm S.D. of three independent experiments. Statistical significance was assigned relative to time matched gentamicin +0 μ M statin dosed controls * p < 0.05, ** p < 0.01 and *** p < 0.005.

3.4. Addition of isoprenoid products of HMG-CoA reductase reverses the inhibitory effect of statins on gentamicin accumulation and cytotoxicity

To further define the mechanism of statin inhibition of gentamicin uptake and cytotoxicity and link it to the pharmacological action of the statin, mevalonate, GGPP and cholesterol (downstream products of HMG-CoA reductase) were co-incubated with gentamicin and simvastatin. Only mevalonate and GGPP reversed the inhibitory effect of simvastatin on gentamicin accumulation (Fig. 6A), cytotoxicity (Fig. 6B) and Rap1A unprenylation (Fig. 6C). The level of gentamicin accumulation, cytotoxicity and Rap1A unprenylation was comparable to that seen in cellular incubations where simvastatin was omitted. The effect of mevalonate and GGPP when co-incubated with pravastatin and rosuvastatin was the same as that seen with simvastatin.

4. Discussion

AG-induced nephrotoxicity represents a clinically important adverse drug reaction affecting 10–15% of patients. The selective uptake and accumulation of AGs into renal proximal tubular cells plays a key role in the development of toxicity [2,3]. Schmitz et al. demonstrated that megalin-dependent receptor mediated endocytosis is the pathway exclusively responsible for AG renal uptake and accumulation by proximal tubule cells *in vivo* [12]. Moreover, extracellular peptide competitors of AG/megalín binding also prevent AG uptake *in vivo* [13]. However, endogenous peptides and proteins are relatively unstable within *in vivo* systems and may elicit a potentially damaging immune response. Thus, extracellular

targeting of megalín with protein and peptide inhibitors cannot be considered as a therapeutic strategy at present and requires further study. Therefore, inhibition of AG uptake with widely used drugs that had a well understood clinical safety profile would represent a potentially important mode of therapeutic intervention for the nephrotoxicity, particularly if it was accompanied by an understanding of the mechanisms responsible for AG uptake, accumulation and cytotoxicity.

Receptor-mediated endocytosis requires functional GTP-binding proteins which in turn are dependent upon C-terminal post-translational modification derived from products of the mevalonate pathway for correct function and membrane association [16,17]. The highly potent statin, rosuvastatin, has been shown to inhibit proximal tubular uptake of low molecular weight proteins without altering renal function by a mechanism linked to the inhibition of receptor-mediated endocytosis [20,21,27,28]. We hypothesised that through the pharmacological inhibition of HMG-CoA reductase, and hence mevalonate production, AG accumulation and cytotoxicity could be inhibited by licensed statins.

We utilised the *in vitro* OK cell line derived from the proximal tubule of the American opossum, to investigate the mechanism. OK cells retain megalín-mediated protein binding and uptake, dependent on GTP-protein function, comparable to *in vivo* situations [14,15,29,30] and have previously been used to investigate AG cytotoxicity [31] and the biochemistry of statin renal transport [32]. We also cultured OK cells on trans-well membrane inserts to promote polarity and megalín expression [33]. Using this model, our data shows that gentamicin (a model nephrotoxic AG) was cytotoxic *in vitro* in a dose- and

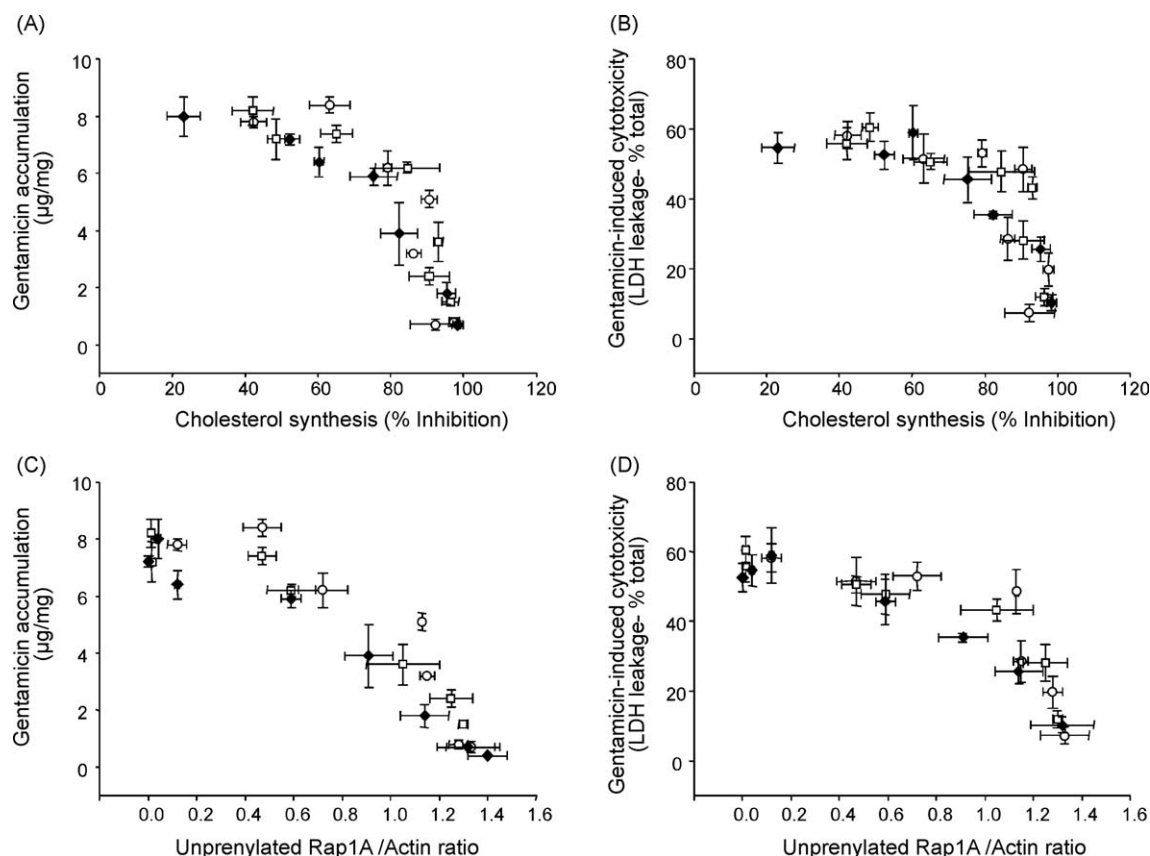


Fig. 5. The relationship between statin pharmacology and the protective effect on gentamicin cytotoxicity. The relationship between the statin-induced inhibition of (A) gentamicin accumulation (μg/mg) and (B) gentamicin-induced cytotoxicity (LDH leakage; U/l) in OK cells with the degree of the inhibition of HMG-CoA reductase (% inhibition of cholesterol synthesis). The relationship between the statin-induced inhibition of (C) gentamicin accumulation (μg/mg) and (D) gentamicin-induced cytotoxicity (LDH leakage; U/l) in OK cells with the degree of Rap1A unprenylation. OK cells were incubated with gentamicin (0.25 mg/ml) and either simvastatin (O; 0–10 μM), pravastatin (□; 0–100 μM) or rosuvastatin (◆; 0–100 μM) for 48 h. Cholesterol synthesis inhibition, gentamicin accumulation and resulting cytotoxicity were determined as described for Fig. 3. Rap1A unprenylation was determined by western blot and normalised to β-actin as described in Fig. 1. Data is given as mean ± S.D. of three independent experiments (horizontal error bars cholesterol synthesis or unprenylation of Rap1A, vertical error bars gentamicin accumulation or cytotoxicity were required).

time-dependent manner and this was directly proportional to drug accumulation. By 24 h 100% cell death was observed with a gentamicin concentration of 1 mg/ml which is comparable to literature values seen with gentamicin dosed renal tubular cells [34]. Furthermore, cytotoxicity was inhibited by statins, and this was directly due to the pharmacological action of statins and the degree of GTP-binding protein unprenylation, rather than being a function of the inhibition of AG receptor binding.

Studies in OK cells have shown that statins do not inhibit the cell surface binding of low molecular weight proteins or fluid phase endocytosis of proteins [21]. Trafficking of megalin-ligand containing vesicles within cells is preceded by the acidification of fused early endosomes with endocytic clathrin-coated vesicles. These early events are directly disrupted by chlorpromazine and cytochalasin D through actions on clathrin and the cytoskeleton [29]. However, statin inhibition of gentamicin uptake was not rapid in this investigation. Instead, as suggested by Sidaway et al., [21] statins caused a gradual depletion or inactivation of the co-factors required for early uptake. A high threshold level in the degree of the inhibition of cholesterol synthesis and unmodification of Rap1A was required to significantly inhibit gentamicin accumulation and toxicity. In addition, the time course for the appearance of the surrogate unprenylated GTP-binding protein (Rap1A) mirrored the time course of inhibition of gentamicin toxicity, further supporting our hypothesis that statin treatment results in a gradual depletion of the intracellular isoprenoid pool and progressive inactivation of GTP-binding proteins involved in megalin-mediated AG uptake.

The key finding in this investigation which supports the hypothesis tested was that the protective effect of statins was reversed by mevalonate. Mevalonate is the downstream product of HMG-CoA reductase and a precursor for the necessary isoprenoid products required for GTP-binding protein geranylgeranylation. Moreover, the reversal of the statin protective effect was also seen with the addition of GGPP, but not with cholesterol (from which GTP-binding protein modifications are not derived). Mevalonate and GGPP modulation further provides strong evidence to link the protective effect of statins on gentamicin cytotoxicity to the inhibition of HMG-CoA reductase and the mevalonate biochemical pathway.

Although the OK cell model represents a suitable *in vitro* model to investigate receptor-mediated endocytosis and statin pharmacology, it is possible that the findings seen here, with respect to the effect of statins on the inhibition of gentamicin cytotoxicity, may differ *in vivo* because of the different pharmacokinetic properties of the statins. It is important to have a comprehensive understanding of the factors that determine the disposition of a drug *in vivo* when investigating the effect of pharmacodynamically compounds *in vitro* [21]. For instance, it is possible that lipophilic statins such as simvastatin may not be as effective as hydrophilic statins. Within this *in vitro* study, the relative potency of each statin in the inhibition of cholesterol synthesis, Rap1A modification and the inhibition of gentamicin cytotoxicity could be ranked in terms of the relative lipophilicity of each statin (simvastatin > rosuvastatin > pravastatin) [35]. Simvastatin undergoes greater than 80% hepatic extraction compared to

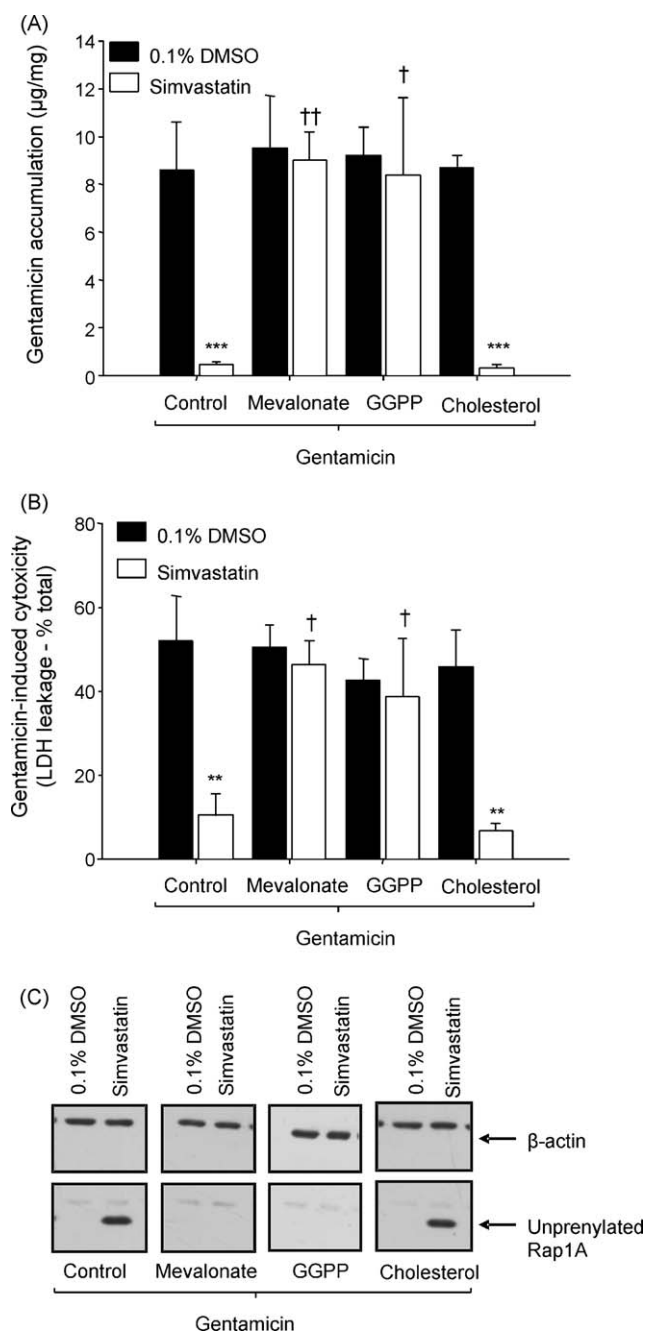


Fig. 6. Modulation of statin protective effects by isoprenoid pyrophosphates. The effect of mevalonate, geranyl-geranyl pyrophosphate (GGPP) or cholesterol addition on the inhibitory effect of simvastatin on (A) gentamicin cellular accumulation, (B) gentamicin-induced cytotoxicity and (C) Rap1A prenylation in OK cells. Cells were incubated with gentamicin (0.25 mg/ml) and either a solvent vehicle control (0.1% DMSO), mevalonate (10 µM), GGPP (10 µM) or cholesterol (100 µM) for 48 h with and without simvastatin (10 µM). Gentamicin cellular accumulation and resulting cytotoxicity was determined as described in Fig. 2. Rap1A unprenylation was determined as described in Fig. 1. Data is given as mean \pm S.D. of three independent experiments. Statistical significance was assigned $^{**}p < 0.01$ and $^{***}p < 0.005$ for comparisons between simvastatin and control or $^{\dagger}p < 0.05$ and $^{\dagger\dagger}p < 0.01$ for comparisons between simvastatin/mevalonate with simvastatin/vehicle or simvastatin/cholesterol or simvastatin/GGPP with simvastatin/vehicle or simvastatin/GGPP.

more hydrophilic statins such as pravastatin which undergo around 45% hepatic extraction and 80% renal clearance [36]. Active tubular secretion can account for 40% of the renal clearance of pravastatin [37,38]. Rosuvastatin undergoes 28% renal clearance, of which 90% can be accounted for by active uptake by

proximal tubule cells [39]. For hydrophilic statins, such as pravastatin and rosuvastatin, OATs (organic anion transporters) localised on the basolateral membrane of proximal tubule cells have been shown to play a central role in their renal uptake. However, the expression patterns of the different sub-types of the transporters are also important. For instance, rosuvastatin is a good substrate for OAT3 and not for OAT1 [21], whereas OK cells mainly express OAT1 [32]. It is possible that if the OK cells stably expressed the full complement of transporters observed *in vivo*, the inhibitory effect of the hydrophilic statins, with respect to inhibition of toxicity, would be more pronounced and the rank order of potency, with respect to inhibition of HMG-CoA reductase, would better reflect that seen clinically.

In man, animal models and the *in vitro* system investigated here, gentamicin nephrotoxicity is concentration-dependent being related to the degree of drug cellular uptake [1,12]. In man and in animal models, gentamicin may take much longer to induce renal damage than that observed in our cell model. Additionally, the optimal concentration required *in vivo* for efficacy is around 20 times lower than the concentration used *in vitro*. Thus, one possible refinement of our *in vitro* model might be to use lower AG concentration over longer time periods [31]. However, it is important to note that (a) although the concentrations we have used are higher than those attained for efficacy, many patients achieve much higher concentrations, which is when they run into problems with nephrotoxicity; and (b) gentamicin toxicity *in vivo* does not solely depend on the plasma concentration, but more so on accumulation of 5–10% of the dose. Calculations from our *in vitro* model show that approximately 6.5% of the dose accumulated prior to the induction of cell death which, taken together with megalin expression and the ability to inhibit cholesterol synthesis, supports the use of this *in vitro* model as a test of the hypothesis.

The results presented in this investigation provide evidence that through the inhibition of HMG-CoA reductase, statins can inhibit the receptor-mediated endocytosis of AGs into renal proximal tubule cells and the resulting toxicity. This was linked to the depletion of intracellular isoprenoid pyrophosphates derived from mevalonate required for GTP-binding protein prenylation and function. This now needs to be taken further, first of all in an animal model of AG-nephrotoxicity, and secondly in proof of concept studies in patients who receive repeated courses of AG, and therefore are most susceptible to renal damage, such as cystic fibrosis. The main advantage of using this approach is the large amount of knowledge we have gained about the efficacy and safety of statins, and is also consistent with their increasing use and investigation in other non-lipid diseases [40], including in cystic fibrosis to reduce bronchiolar inflammation (<http://clinicaltrials.gov/ct2/show/NCT00255242?term=statin+and+cystic+fibrosis&rank=1>).

Conflict of interest

The authors wish to report no competing financial interests.

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