



HAL
open science

Statins inhibit aminoglycoside accumulation and cytotoxicity to renal proximal tubule cells

Daniel J. Antoine, Abhishek Srivastava, Munir Pirmohamed, B. Kevin Park

► To cite this version:

Daniel J. Antoine, Abhishek Srivastava, Munir Pirmohamed, B. Kevin Park. Statins inhibit aminoglycoside accumulation and cytotoxicity to renal proximal tubule cells. *Biochemical Pharmacology*, 2009, 79 (4), pp.647. <10.1016/j.bcp.2009.09.021>. <hal-00544818>

HAL Id: hal-00544818

<https://hal.science/hal-00544818v1>

Submitted on 9 Dec 2010

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



HAL Authorization

Accepted Manuscript

Title: Statins inhibit aminoglycoside accumulation and cytotoxicity to renal proximal tubule cells

Authors: Daniel J. Antoine, Abhishek Srivastava, Munir Pirmohamed, B. Kevin Park



PII: S0006-2952(09)00800-4
DOI: doi:10.1016/j.bcp.2009.09.021
Reference: BCP 10337

To appear in: *BCP*

Received date: 25-8-2009
Revised date: 16-9-2009
Accepted date: 17-9-2009

Please cite this article as: Antoine DJ, Srivastava A, Pirmohamed M, Park BK, Statins inhibit aminoglycoside accumulation and cytotoxicity to renal proximal tubule cells, *Biochemical Pharmacology* (2008), doi:10.1016/j.bcp.2009.09.021

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

TITLE PAGE

1
2
3
4
5 **Statins inhibit aminoglycoside accumulation and cytotoxicity to renal proximal tubule**
6
7 **cells**
8
9

10
11
12 Daniel J. Antoine, Abhishek Srivastava, Munir Pirmohamed & B. Kevin Park
13
14
15

AUTHOR AFFILIATION

16
17
18
19
20
21
22 MRC Centre for Drug Safety Science, Department of Pharmacology & Therapeutics,
23
24 University of Liverpool, Merseyside, UK
25
26
27

CORRESPONDING AUTHOR

28
29
30
31
32
33
34 Dr D.J Antoine
35

36 MRC Centre for Drug Safety Science
37

38 The Department of Pharmacology & Therapeutics
39

40 University of Liverpool, UK
41

42
43 L69 3GE
44

45
46 Tel: +44 151 795 0382.
47

48
49 E-mail: d.antoine@liv.ac.uk
50
51

ABBREVIATIONS

1 AG, aminoglycoside; HMG-CoA 3-hydroxy-3-methylglutaryl-CoA; IC₅₀, half-maximal
2 inhibitory concentration; LC-MS/MS, liquid chromatography tandem mass-spectrometry;
3
4 LC-MS/MRM, liquid chromatography-mass spectrometry / multiple reaction monitoring;
5
6
7 LDH, lactate dehydrogenase; OAT, organic anion transporter; OK, opossum kidney
8
9

10
11
12 **KEY WORDS**
13

14
15
16
17 Gentamicin; HMG-CoA reductase; Mevalonate; Megalin; Nephrotoxicity
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

ABSTRACT

1
2
3
4
5 Nephrotoxicity due to renal proximal tubule accumulation of aminoglycoside (AG)
6
7 antibiotics, such as gentamicin, represents a major clinical problem. Receptor-mediated
8
9 endocytosis via the multi-ligand receptor megalin is thought to be a key mechanism in the
10
11 cellular uptake of AGs and nephrotoxicity. This process can be modulated by the intracellular
12
13 concentration of isoprenoid pyrophosphates derived from the processing of mevalonate by 3-
14
15 hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase. Post-translation modifications by
16
17 isoprenoid pyrophosphates are necessary for GTP-binding protein function. Given that statins
18
19 inhibit HMG-CoA reductase and therefore affect the concentration of isoprenoid
20
21 pyrophosphates, we have tested the hypothesis that statins will lead to a reduction in AG
22
23 renal proximal tubule accumulation and cytotoxicity. Gentamicin accumulated within
24
25 cultured proximal tubule derived opossum kidney (OK) cells and led to dose- and time-
26
27 dependent cell death which was inhibited by non-toxic doses of simvastatin (IC_{50} 1.3 μ M),
28
29 rosuvastatin (IC_{50} 16.3 μ M) and pravastatin (IC_{50} 38.8 μ M). The mechanism of inhibition was
30
31 linked to the degree of cholesterol synthesis inhibition and GTP-binding protein
32
33 unprenylation. Moreover, co-incubation with mevalonate or geranyl-geranyl pyrophosphate,
34
35 products of HMG-CoA reductase, reversed the inhibitory effect of statins on cellular
36
37 accumulation and cytotoxicity of gentamicin. In summary, our data suggest that the inhibition
38
39 of the mevalonate pathway by statins may provide a potential therapeutic strategy to prevent
40
41 AG-induced nephrotoxicity.
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

1. INTRODUCTION

1
2
3
4
5 Aminoglycosides (AG), including gentamicin, are a widely prescribed and effective class of
6
7 antibiotic which have low rates of resistance. They are used in the treatment of a number of
8
9 severe bacterial infections including those affecting the lungs, heart, biliary and urinary tracts
10
11 [1]. However, nephrotoxicity represents a serious clinical problem in 10-15% of patients and
12
13 is the dose limiting factor for their therapeutic use [2, 3]. To uncover mechanisms of AG-
14
15 induced nephrotoxicity would thus be important to improve the therapeutic index and may
16
17 also allow the development of novel intervention strategies. However, currently apart from
18
19 once daily dosing [4], no other clinically effective strategies exist for the prevention of AG
20
21 nephrotoxicity.
22
23
24
25
26
27
28

29 Following systemic administration, AGs remain relatively unchanged and have low tissue
30
31 absorption and plasma protein binding. Around 80% of the systemic dose is excreted into the
32
33 urine within 24h by glomerular filtration. However, accumulation within the renal cortex,
34
35 especially in the proximal tubule epithelial cells, remains high compared to other organs (5-
36
37 10% dose) [5]. This is thought to be important in the pathogenesis of nephrotoxicity, and thus
38
39 attention has focused on the molecular and biochemical mechanisms underpinning AG
40
41 accumulation. This may be key to developing potential strategies for the intervention of
42
43 toxicity.
44
45
46
47
48
49
50

51 Binding of AGs to the multi-ligand receptor, megalin, and cellular uptake by receptor-
52
53 mediated endocytosis has been demonstrated to be the principal pathway for accumulation
54
55 [6]. The abundant negative charges on the extracellular receptor domain facilitate interactions
56
57 between polybasic substances such as AGs [6]. Indeed, the rank order of AG toxicity
58
59
60
61
62
63
64
65

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

(neomycin > amikacin > gentamicin = tobramycin > netilmicin) can be correlated with the ability to bind to the brush border membranes of the renal tubule [1].

Megalin 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 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

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

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- C^{14}] 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 solvent were of high grade HPLC quality from Fischer scientific (Loughborough, UK). Lactate dehydrogenase assay kit was purchased from Roche diagnostics (West Sussex, UK).

2.2 Cell culture treatment and cytotoxicity assessment

OK cells were used between passages 40 – 55 and seeded at a density of 2×10^4 cells/cm² in cell culture plates containing trans-well 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, 2mmol/L L-glutamine, 100U/ml penicillin and 100µg/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 48h and media containing 1% serum for later time points.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

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 15min. A portion was removed for protein content determination by the methods of Bradford et al [23] and samples were centrifuged at 4°C for 5min at 14,000rpm. 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 TurboIonSpray source (Applied Biosystems/MDS Sciex, Foster City, CA). Analytes were resolved on a Gemini-NX 5µm C18 110A 250x4.60mm column (phenomenex). Eluate split-flow to the LC-MS interface was 200µl/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 24h and 10µCi [2-C¹⁴] acetate was added for 5h. Cells were washed twice with ice cold PBS and lysed in 0.1M NaOH. Labelled acetate

1 incorporation in cholesterol was determined by scintillation counting following HPLC
2 separation as described previously [25]. Percentage (%) inhibition of cholesterol synthesis
3 was compared to control for test reagents.
4
5
6
7
8

9 **2.5 Western blotting**

10
11
12
13
14 OK cells were washed twice with ice cold PBS, lysed in RIPA buffer and incubated on ice for
15 15min. Protein concentrations were determined by the Bradford assay [23] and 5 μ g of the
16 total whole cell lysate was separated by SDS-PAGE. Resolved proteins were transferred to
17 nitrocellulose membranes and probed with antibodies raised against total or unprenylated
18 Rap1A (both 1/1000; overnight) and β -actin (1/5000; 1h). Secondary antibodies (1/10,000;
19 1h) were visualised by exposure to hyperfilm after incubation with ECL-Plus. Unprenylated
20 Rap1A levels were determined by densitometry and normalised to the level of β -actin.
21
22
23
24
25
26
27
28
29
30
31
32
33

34 **2.6 Statistical analysis**

35
36
37
38
39 Non-linear regression analysis was used to calculate inhibition constants (IC_{50}) from dose
40 response curves recorded at 48hr post dose and statistical significance was assessed through
41 unpaired t-test analysis, accepting $P < 0.05$ as being significant.
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

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 unprenylated Rap1A [26] (IC₅₀ values given in table 1). Unprenylated 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).

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 its self on cholesterol synthesis (Fig.3A). Gentamicin accumulated within OK cells in a time-dependent manner which first reached significance at 2h post dose. Maximum accumulation was reached 24h 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 24h (0.25mg/ml). There

1 was also a direct relationship between gentamicin accumulation and cytotoxicity ($R^2=0.85$)
2 observed in OK cells (Fig.3D). Co-administration of simvastatin blocked the intracellular
3 accumulation of gentamicin and its cytotoxicity. Simvastatin alone did not cause a
4 significant increase in the release of LDH ($11.5\pm 4.1\%$) total amount in culture media)
5 compared to vehicle control levels ($7.5\pm 2.3\%$). This indicates that the inhibitory effect on
6 gentamicin accumulation and cytotoxicity cannot be explained by statin toxicity to the OK
7 cells. This is consistent with literature reports [21]. The effects of pravastatin and rosuvastatin
8 were similar to those of simvastatin. Simvastatin, pravastatin and rosuvastatin were
9 pharmacologically active in OK cells and induced a dose- and time-dependent inhibition of
10 cholesterol synthesis from acetate (Fig.3A and 4A). All statins tested also induced a dose
11 dependent inhibitory effect on the cellular unprenylation of Rap1A, accumulation of
12 gentamicin and resulting cytotoxicity in OK cells 48hr after dosing (Fig.4B-D). The IC_{50}
13 values are given in table 1 for each statin.
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30

31 **3.3 The relationship between statin-induced HMG-CoA reductase inhibition, Rap1A** 32 **prenylation and gentamicin accumulation and cytotoxicity** 33

34 For simvastatin, pravastatin and rosuvastatin, figure 5A shows the correlation between the
35 inhibition of cholesterol synthesis (surrogate marker for HMG-CoA reductase activity) and
36 gentamicin cellular accumulation. Figure 5B also shows the relationship between the
37 inhibition of cholesterol synthesis by statins and the resulting cytotoxicity induced by
38 gentamicin. An inhibition in cholesterol synthesis of more than 60% was required to have a
39 significant inhibitory effect on gentamicin accumulation and more than 70% to have a
40 significant inhibitory effect on cytotoxicity in OK cells. This relationship was also mirrored
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

1 through the correlation between gentamicin accumulation (Fig.5C) and cytotoxicity (Fig.5D)
2 and the degree to which the GTP-binding protein remained unmodified.
3
4
5
6

7 **3.4 Addition of isoprenoid products of HMG-CoA reductase reverses the inhibitory** 8 **effect of statins on gentamicin accumulation and cytotoxicity** 9

10 To further define the mechanism of statin inhibition of gentamicin uptake and cytotoxicity
11 and link it to the pharmacological action of the statin, mevalonate, GGPP and cholesterol
12 (downstream products of HMG-CoA reductase) were co-incubated with gentamicin and
13 simvastatin. Only mevalonate and GGPP reversed the inhibitory effect of simvastatin on
14 gentamicin accumulation (Fig.6A), cytotoxicity (Fig.6B) and Rap1A unprenylation (Fig.6C).
15 The level of gentamicin accumulation, cytotoxicity and Rap1A unprenylation was
16 comparable to that seen in cellular incubations where simvastatin was omitted. The effect of
17 mevalonate and GGPP when co-incubated with pravastatin and rosuvastatin was the same as
18 that seen with simvastatin.
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

4. DISCUSSION

1
2
3
4
5 AG-induced nephrotoxicity represents a clinically important adverse drug reaction affecting
6
7 10-15% of patients. The selective uptake and accumulation of AGs into renal proximal
8
9 tubular cells plays a key role in the development of toxicity [2, 3]. Schmitz *et al* demonstrated
10
11 that megalin-dependent receptor mediated endocytosis is the pathway exclusively responsible
12
13 for AG renal uptake and accumulation by proximal tubule cells *in vivo* [12]. Moreover,
14
15 extracellular peptide competitors of AG/megalín binding also prevent AG uptake *in vivo* [13].
16
17 However, endogenous peptides and proteins are relatively unstable within *in vivo* systems
18
19 and may elicit a potentially damaging immune response. Thus, extracellular targeting of
20
21 megalín with protein and peptide inhibitors cannot be considered as a therapeutic strategy at
22
23 present and requires further study. Therefore, inhibition of AG uptake with widely used drugs
24
25 that had a well understood clinical safety profile would represent a potentially important
26
27 mode of therapeutic intervention for the nephrotoxicity, particularly if it was accompanied by
28
29 an understanding of the mechanisms responsible for AG uptake, accumulation and
30
31 cytotoxicity.
32
33
34
35
36
37
38
39
40

41 Receptor-mediated endocytosis requires functional GTP-binding proteins which in turn are
42
43 dependent upon C-terminal post-translational modification derived from products of the
44
45 mevalonate pathway for correct function and membrane association [16, 17]. The highly
46
47 potent statin, rosuvastatin, has been shown to inhibit proximal tubular uptake of low
48
49 molecular weight proteins without altering renal function by a mechanism linked to the
50
51 inhibition of receptor-mediated endocytosis [20, 21, 27, 28]. We hypothesised that through
52
53 the pharmacological inhibition of HMG-CoA reductase, and hence mevalonate production,
54
55 AG accumulation and cytotoxicity could be inhibited by licensed statins.
56
57
58
59
60
61
62
63
64
65

1
2 We utilised the *in vitro* OK cell line derived from the proximal tubule of the American
3
4 opossum, to investigate the mechanism. OK cells retain megalin-mediated protein binding
5
6 and uptake, dependent on GTP-protein function, comparable to *in vivo* situations [14, 15, 29,
7
8 30] and have previously been used to investigate AG cytotoxicity [31] and the biochemistry
9
10 of statin renal transport [32]. We also cultured OK cells on trans-well membrane inserts to
11
12 promote polarity and megalin expression [33]. Using this model, our data shows that
13
14 gentamicin (a model nephrotoxic AG) was cytotoxic *in vitro* in a dose-and time-dependent
15
16 manner and this was directly proportional to drug accumulation. By 24hr 100% cell death
17
18 was observed with a gentamicin concentration of 1mg/ml which is comparable to literature
19
20 values seen with gentamicin dosed renal tubular cells [34]. Furthermore, cytotoxicity was
21
22 inhibited by statins, and this was directly due to the pharmacological action of statins and the
23
24 degree of GTP-binding protein unprenylation, rather than being a function of the inhibition of
25
26 AG receptor binding.
27
28
29
30
31
32
33
34
35

36 Studies in OK cells have shown that statins do not inhibit the cell surface binding of low
37
38 molecular weight proteins or fluid phase endocytosis of proteins [21]. Trafficking of megalin-
39
40 ligand containing vesicles within cells is preceded by the acidification of fused early
41
42 endosomes with endocytic clathrin-coated vesicles. These early events are directly disrupted
43
44 by chlorpromazine and cytochalasin D through actions on clathrin and the cytoskeleton [29].
45
46 However, statin inhibition of gentamicin uptake was not rapid in this investigation. Instead,
47
48 as suggested by Sidaway *et al.*, [21] statins caused a gradual depletion or inactivation of the
49
50 co-factors required for early uptake. A high threshold level in the degree of the inhibition of
51
52 cholesterol synthesis and unmodification of Rap1A was required to significantly inhibit
53
54 gentamicin accumulation and toxicity. In addition, the time course for the appearance of the
55
56
57
58
59
60
61
62
63
64
65

1 surrogate unprenylated GTP-binding protein (Rap1A) mirrored the time course of inhibition
2 of gentamicin toxicity, further supporting our hypothesis that statin treatment results in a
3 gradual depletion of the intracellular isoprenoid pool and progressive inactivation of GTP-
4 binding proteins involved in megalin-mediated AG uptake.
5
6
7
8
9

10
11 The key finding in this investigation which supports the hypothesis tested was that the
12 protective effect of statins was reversed by mevalonate. Mevalonate is the downstream
13 product of HMG-CoA reductase and a precursor for the necessary isoprenoid products
14 required for GTP-binding protein geranyl-geranylation. Moreover, the reversal of the statin
15 protective effect was also seen with the addition of GGPP, but not with cholesterol (from
16 which GTP-binding protein modifications are not derived). Mevalonate and GGPP
17 modulation further provides strong evidence to link the protective effect of statins on
18 gentamicin cytotoxicity to the inhibition of HMG-CoA reductase and the mevalonate
19 biochemical pathway.
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35

36 Although the OK cell model represents a suitable *in vitro* model to investigate receptor-
37 mediated endocytosis and statin pharmacology, it is possible that the findings seen here, with
38 respect to the effect of statins on the inhibition of gentamicin cytotoxicity, may differ *in vivo*
39 because of the different pharmacokinetic properties of the statins. It is important to have a
40 comprehensive understanding of the factors that determine the disposition of a drug *in vivo*
41 when investigating the effect of pharmacodynamically compounds *in vitro* [21]. For instance,
42 it is possible that lipophilic statins such as simvastatin may not be as effective as hydrophilic
43 statins. Within this *in vitro* study, the relative potency of each statin in the inhibition of
44 cholesterol synthesis, Rap1A modification and the inhibition of gentamicin cytotoxicity could
45 be ranked in terms of the relative lipophilicity of each statin (simvastatin > rosuvastatin >
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

pravastatin) [35]. Simvastatin undergoes greater than 80% hepatic extraction compared to 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

1 on accumulation of 5-10% of the dose. Calculations from our *in vitro* model show that
2 approximately 6.5% of the dose accumulated prior to the induction of cell death which, taken
3
4 together with megalin expression and the ability to inhibit cholesterol synthesis, supports the
5
6 use of this *in vitro* model as a test of the hypothesis.
7
8
9

10
11 The results presented in this investigation provide evidence that through the inhibition of
12
13 HMG-CoA reductase, statins can inhibit the receptor-mediated endocytosis of AGs into renal
14
15 proximal tubule cells and the resulting toxicity. This was linked to the depletion of
16
17 intracellular isoprenoid pyrophosphates derived from mevalonate required for GTP-binding
18
19 protein prenylation and function. This now needs to be taken further, first of all in an animal
20
21 model of AG-nephrotoxicity, and secondly in proof of concept studies in patients who receive
22
23 repeated courses of AG, and therefore are most susceptible to renal damage, such as cystic
24
25 fibrosis. The main advantage of using this approach is the large amount of knowledge we
26
27 have gained about the efficacy and safety of statins, and is also consistent with their
28
29 increasing use and investigation in other non-lipid diseases [40], including in cystic fibrosis
30
31 to reduce bronchiolar inflammation
32
33 (<http://clinicaltrials.gov/ct2/show/NCT00255242?term=statin+and+cystic+fibrosis&rank=1>).
34
35
36
37
38
39
40
41
42
43
44
45

46 5. ACKNOWLEDGMENTS

47
48
49
50

51 This work was supported by the Medical Research Council (grant number G0700654)
52
53 through the Centre for Drug Safety Science (CDSS).
54
55
56
57
58
59
60
61
62
63
64
65

6. REFERENCES

- 1
2
3
4
5 [1] Tan KH, Mulheran M, Knox AJ, Smyth AR. Aminoglycoside prescribing and
6 surveillance in cystic fibrosis. *Am J Respir Crit Care Med* 2003;167:819-23.
7
8
9 [2] Begg EJ, Barclay ML. Aminoglycosides--50 years on. *British Journal of Clinical*
10 *Pharmacology* 1995;39:597-603.
11
12 [3] Slaughter RL, Cappelletty DM. Economic impact of aminoglycoside toxicity and its
13 prevention through therapeutic drug monitoring. *Pharmacoeconomics* 1998;14:385-
14 94.
15
16 [4] Smyth A, Walters S. Prophylactic antibiotics for cystic fibrosis.[update of Cochrane
17 *Database Syst Rev.* 2001;(3):CD001912; PMID: 11687002]. *Cochrane Database Syst*
18 *Rev* 2003:CD001912.
19
20 [5] Mathews A, Bailie GR. Clinical pharmacokinetics, toxicity and cost effectiveness
21 analysis of aminoglycosides and aminoglycoside dosing services. *J Clin Pharm Ther*
22 1987;12:273-91.
23
24 [6] Moestrup SK, Cui S, Vorum H, Bregengard C, Bjorn SE, Norris K, et al. Evidence
25 that epithelial glycoprotein 330/megalin mediates uptake of polybasic drugs.[see
26 comment]. *Journal of Clinical Investigation* 1995;96:1404-13.
27
28 [7] Christensen EI, Moskaug JO, Vorum H, Jacobsen C, Gundersen TE, Nykjaer A, et al.
29 Evidence for an essential role of megalin in transepithelial transport of retinol. *J Am*
30 *Soc Nephrol* 1999;10:685-95.
31
32 [8] Nykjaer A, Dragun D, Walther D, Vorum H, Jacobsen C, Herz J, et al. An endocytic
33 pathway essential for renal uptake and activation of the steroid 25-(OH) vitamin D3.
34 *Cell* 1999;96:507-15.
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

- 1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65
- [9] Orlando RA, Rader K, Authier F, Yamazaki H, Posner BI, Bergeron JJ, et al. Megalin is an endocytic receptor for insulin. *J Am Soc Nephrol* 1998;9:1759-66.
- [10] Sousa MM, Norden AG, Jacobsen C, Willnow TE, Christensen EI, Thakker RV, et al. Evidence for the role of megalin in renal uptake of transthyretin. *Journal of Biological Chemistry* 2000;275:38176-81.
- [11] Nagai J, Tanaka H, Nakanishi N, Murakami T, Takano M. Role of megalin in renal handling of aminoglycosides. *Am J Physiol Renal Physiol* 2001;281:F337-44.
- [12] Schmitz C, Hilpert J, Jacobsen C, Boensch C, Christensen EI, Luft FC, et al. Megalin deficiency offers protection from renal aminoglycoside accumulation. *Journal of Biological Chemistry* 2002;277:618-22.
- [13] Watanabe A, Nagai J, Adachi Y, Katsube T, Kitahara Y, Murakami T, et al. Targeted prevention of renal accumulation and toxicity of gentamicin by aminoglycoside binding receptor antagonists. *J Controlled Release* 2004;95:423-33.
- [14] Brunskill NJ, Cockcroft N, Nahorski S, Walls J. Albumin endocytosis is regulated by heterotrimeric GTP-binding protein G alpha i-3 in opossum kidney cells. *American Journal of Physiology* 1996;271:F356-64.
- [15] Malstrom K, Stange G, Murer H. Identification of proximal tubular transport functions in the established kidney cell line, OK. *Biochim Biophys Acta* 1987;902:269-77.
- [16] Clague MJ. Molecular aspects of the endocytic pathway. *Biochemical Journal* 1998;336:271-82.
- [17] Magee T, Marshall C. New insights into the interaction of Ras with the plasma membrane. *Cell* 1999;98:9-12.
- [18] Goldstein JL, Brown MS. Regulation of the mevalonate pathway. *Nature* 1990;343:425-30.

- 1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65
- [19] Cordle A, Koenigsnecht-Talboo J, Wilkinson B, Limpert A, Landreth G. Mechanisms of statin-mediated inhibition of small G-protein function. *Journal of Biological Chemistry* 2005;280:34202-9.
- [20] Verhulst A, D'Haese PC, De Broe ME. Inhibitors of HMG-CoA reductase reduce receptor-mediated endocytosis in human kidney proximal tubular cells. *J Am Soc Nephrol* 2004;15:2249-57.
- [21] Sidaway JE, Davidson RG, McTaggart F, Orton TC, Scott RC, Smith GJ, et al. Inhibitors of 3-hydroxy-3-methylglutaryl-CoA reductase reduce receptor-mediated endocytosis in opossum kidney cells. *J Am Soc Nephrol* 2004;15:2258-65.
- [22] Khwaja A, O'Connell J, Hendry BM. Prenylation inhibitors in renal disease. *Lancet* 2000;355:741-4.
- [23] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* 1976;72:248-54.
- [24] Heller DN, Peggins JO, Nochetto CB, Smith ML, Chiesa OA, Moulton K. LC/MS/MS measurement of gentamicin in bovine plasma, urine, milk, and biopsy samples taken from kidneys of standing animals. *J Chromatogr B Analyt Technol Biomed Life Sci* 2005;821:22-30.
- [25] McTaggart F, Brown GR, Davidson RG, Freeman S, Holdgate GA, Mallion KB, et al. Inhibition of squalene synthase of rat liver by novel 3' substituted quinuclidines. *Biochemical Pharmacology* 1996;51:1477-87.
- [26] Staal A, Frith JC, French MH, Swartz J, Gungor T, Harrity TW, et al. The ability of statins to inhibit bone resorption is directly related to their inhibitory effect on HMG-CoA reductase activity. *J Bone Miner Res* 2003;18:88-96.

- 1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65
- [27] Brewer HB, Jr. Benefit-risk assessment of Rosuvastatin 10 to 40 milligrams. *Am J Cardiol* 2003;92:23K-9K.
- [28] Olsson AG, McTaggart F, Raza A. Rosuvastatin: a highly effective new HMG-CoA reductase inhibitor. *Cardiovasc Drug Rev* 2002;20:303-28.
- [29] Gekle M, Mildenerger S, Freudinger R, Schwerdt G, Silbernagl S. Albumin endocytosis in OK cells: dependence on actin and microtubules and regulation by protein kinases. *American Journal of Physiology* 1997;272:F668-77.
- [30] Schwegler JS, Heppelmann B, Mildenerger S, Silbernagl S. Receptor-mediated endocytosis of albumin in cultured opossum kidney cells: a model for proximal tubular protein reabsorption. *Pflugers Arch* 1991;418:383-92.
- [31] Gibbons CE, Maldonado-Perez D, Shah AN, Riccardi D, Ward DT. Calcium-sensing receptor antagonism or lithium treatment ameliorates aminoglycoside-induced cell death in renal epithelial cells. *Biochim Biophys Acta* 2008;1782:188-95.
- [32] Sauvant C, Holzinger H, Gekle M. Modulation of the basolateral and apical step of transepithelial organic anion secretion in proximal tubular opossum kidney cells. Acute effects of epidermal growth factor and mitogen-activated protein kinase. *Journal of Biological Chemistry* 2001;276:14695 - 703.
- [33] Nielsen R, Birn H, Moestrup SK, Nielsen M, Verroust P, Christensen EI. Characterization of a kidney proximal tubule cell line, LLC-PK1, expressing endocytotic active megalin. *J Am Soc Nephrol* 1998;9:1767-76.
- [34] Sandoval RM, Reilly JP, Running W, Campos SB, Santos JR, Phillips CL, et al. A non-nephrotoxic gentamicin congener that retains antimicrobial efficacy. *J Am Soc Nephrol* 2006;17:2697-705.

- 1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
- [35] McTaggart F, Bucket L, Davidson R, Holdgate G, McCormick A, Schneck D, et al. Preclinical and clinical pharmacology of Rosuvastatin, a new 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor. *Am J Cardiol* 2001;87:28B-32B.
- [36] Neuvonen PJ, Backman JT, Niemi M. Pharmacokinetic comparison of the potential over-the-counter statins simvastatin, lovastatin, fluvastatin and pravastatin. *Clin Pharmacokinet* 2008;47:463-74.
- [37] White CM. A review of the pharmacologic and pharmacokinetic aspects of rosuvastatin. *J Clin Pharmacol* 2002;42:963-70.
- [38] Hatanaka T. Clinical pharmacokinetics of pravastatin: mechanisms of pharmacokinetic events. *Clin Pharmacokinet* 2000;39:397-412.
- [39] Martin PD, Warwick MJ, Dane AL, Brindley C, Short T. Absolute oral bioavailability of rosuvastatin in healthy white adult male volunteers. *Clinical Therapeutics* 2003;25:2553-63.
- [40] Steffens S, Mach F. Drug insight: Immunomodulatory effects of statins--potential benefits for renal patients? *Nature Clinical Practice Nephrology* 2006;2:378-87.

42 7. CONFLICT OF INTEREST

43
44
45
46
47 The authors wish to report no competing financial interests

48 49 50 51 8. FIGURE LEGENDS

52
53
54
55
56
57 Fig.1.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

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 (0.25mg/ml) in the presence and absence of a statin for 24hr. 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 3 independent experiments.

Fig.2.

Gentamicin intracellular quantification by LC-MS/MRM. (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 (0.25mg/ml), simvastatin (10 μ M) and simvastatin (10 μ M) + gentamicin (0.25mg/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 5hr and the inhibitory effect of statins on the incorporation into cholesterol was determined as % inhibition 24hr after the statin dose. (B) The effect of simvastatin (10 μ M) on the time dependent intracellular accumulation of gentamicin (0.25mg/ml) in OK cells. (C) The effect of simvastatin (10 μ M) on the time (0-

1 72hr, 0.25mg/ml) dependent cytotoxicity induced by gentamicin in OK cells. Gentamicin
2 accumulation was determined by LC-MS/MRM (μg gentamicin/mg cellular protein) and
3
4 cytotoxicity was determined by LDH leakage into cell culture media (U/l). (D) The linear
5
6 relationship between gentamicin accumulation and cytotoxicity in OK cells. Correlation
7
8 coefficient is given where required (\square ; OK cells incubated with $30\mu\text{M}$ pravastatin and
9
10 0.25mg/ml gentamicin over 0-72hr) (\bullet ; OK cells incubated with $10\mu\text{M}$ simvastatin and
11
12 0.25mg/ml gentamicin over 0-72hr). Data is given as mean \pm S.D of 3 independent
13
14 experiments. Statistical significance was assigned relative to time matched vehicle dosed
15
16 controls $*p < 0.05$, $**p < 0.01$ or simvastatin + gentamicin compared to gentamicin alone $\dagger p$
17
18 < 0.05 , $\dagger\dagger p < 0.01$.

23
24
25
26
27 Fig.4.

28
29 Dose dependent inhibitory effects of statins in OK cells. The dose dependent inhibitory effect
30
31 of statins in OK cells on (A) cholesterol synthesis, (B) Rap1A unprenylation, (C) gentamicin
32
33 cellular accumulation and (D) gentamicin-induced cytotoxicity. OK cells were incubated with
34
35 gentamicin (0.25mg/ml) with simvastatin (O; $0-10\mu\text{M}$), pravastatin (\square ; $0-100\mu\text{M}$) or
36
37 rosuvastatin (\blacklozenge ; $0-100\mu\text{M}$) for 48hr. The effect on cholesterol synthesis was determined as in
38
39 fig.3. Rap1A unprenylation was determined as described in fig.1. Gentamicin cellular
40
41 accumulation was determined by LC-MS/MRM (μg gentamicin/mg cellular protein) and
42
43 cytotoxicity was determined by LDH leakage into the cell culture media (U/l). Data is given
44
45 as mean \pm S.D of 3 independent experiments. Statistical significance was assigned relative to
46
47 time matched gentamicin + $0\mu\text{M}$ statin dosed controls $*p < 0.05$, $**p < 0.01$ and $***p <$
48
49 0.005 .

50
51
52
53
54
55
56
57
58 Fig.5.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

The relationship between statin pharmacology and the protective effect on gentamicin cytotoxicity. The relationship between the statin-induced inhibition of (A) gentamicin accumulation ($\mu\text{g}/\text{mg}$) 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 ($\mu\text{g}/\text{mg}$) and (D) gentamicin-induced cytotoxicity (LDH leakage; U/l) in OK cells with the degree of Rap1A unprenylation. OK cells were incubated with gentamicin (0.25mg/ml) and either simvastatin (O; 0-10 μM), pravastatin (\square ; 0-100 μM) or rosuvastatin (\blacklozenge ; 0-100 μM) for 48hr. 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 \pm S.D of 3 independent experiments (horizontal error bars cholesterol synthesis or unprenylation of Rap1A, vertical error bars gentamicin accumulation or cytotoxicity were required).

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 (0.25mg/ml) and either a solvent vehicle control (0.1% DMSO), mevalonate (10 μM), GGPP (10 μM) or cholesterol (100 μM) for 48hr with and without simvastatin (10 μM). Gentamicin cellular accumulation and resulting cytotoxicity was determined as described in figure 2. Rap1A unprenylation was determined as described in figure 1. Data is given as mean \pm S.D of 3 independent experiments. Statistical significance was assigned $**p < 0.01$ and $***p <$

1 0.005 for comparisons between simvastatin and control or † $p < 0.05$ and †† $p < 0.01$ for
2 comparisons between simvastatin/mevalonate with simvastatin/vehicle or
3 simvastatin/cholesterol or simvastatin/GGPP with simvastatin/vehicle or simvastatin/GGPP.
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

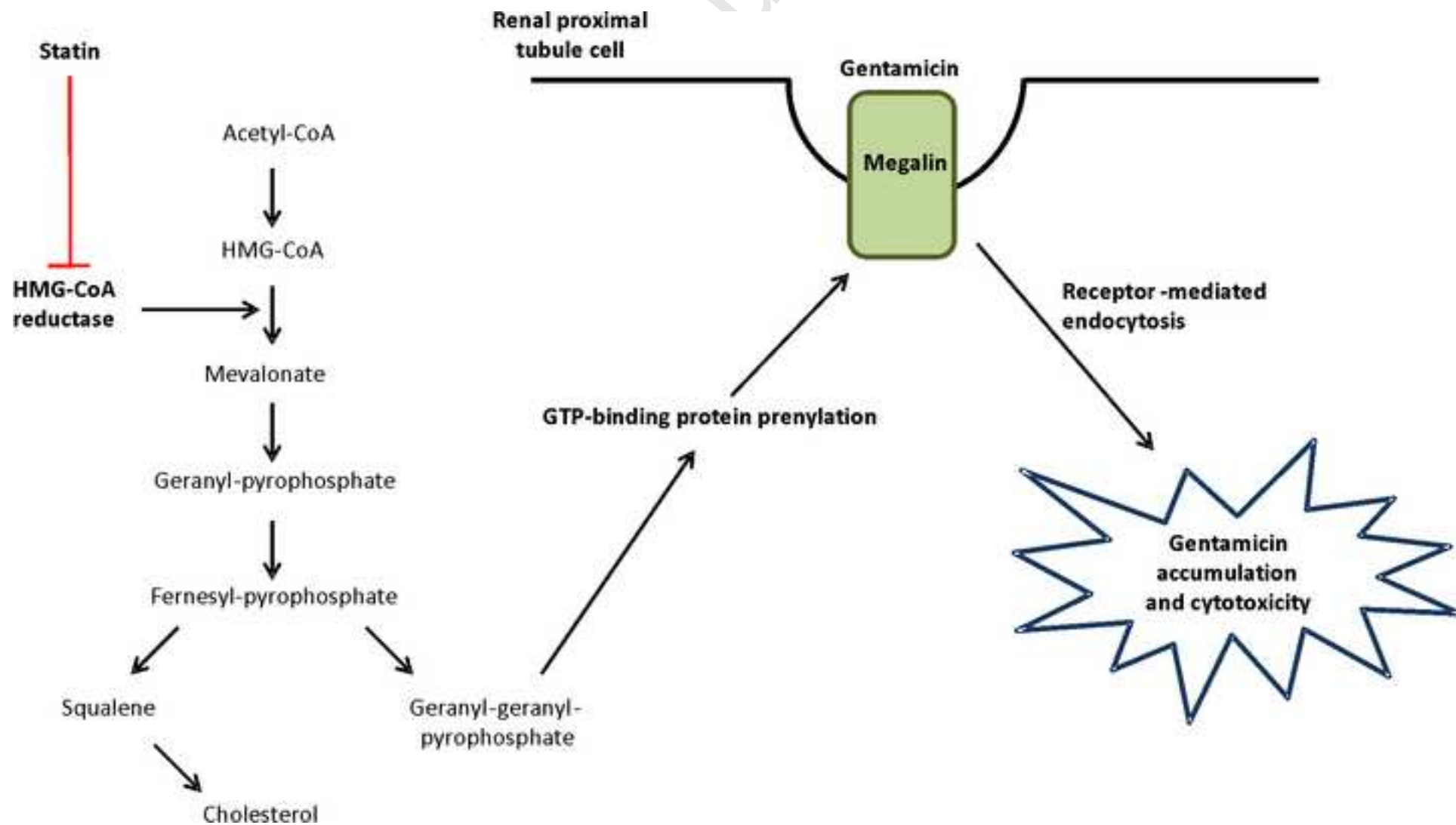
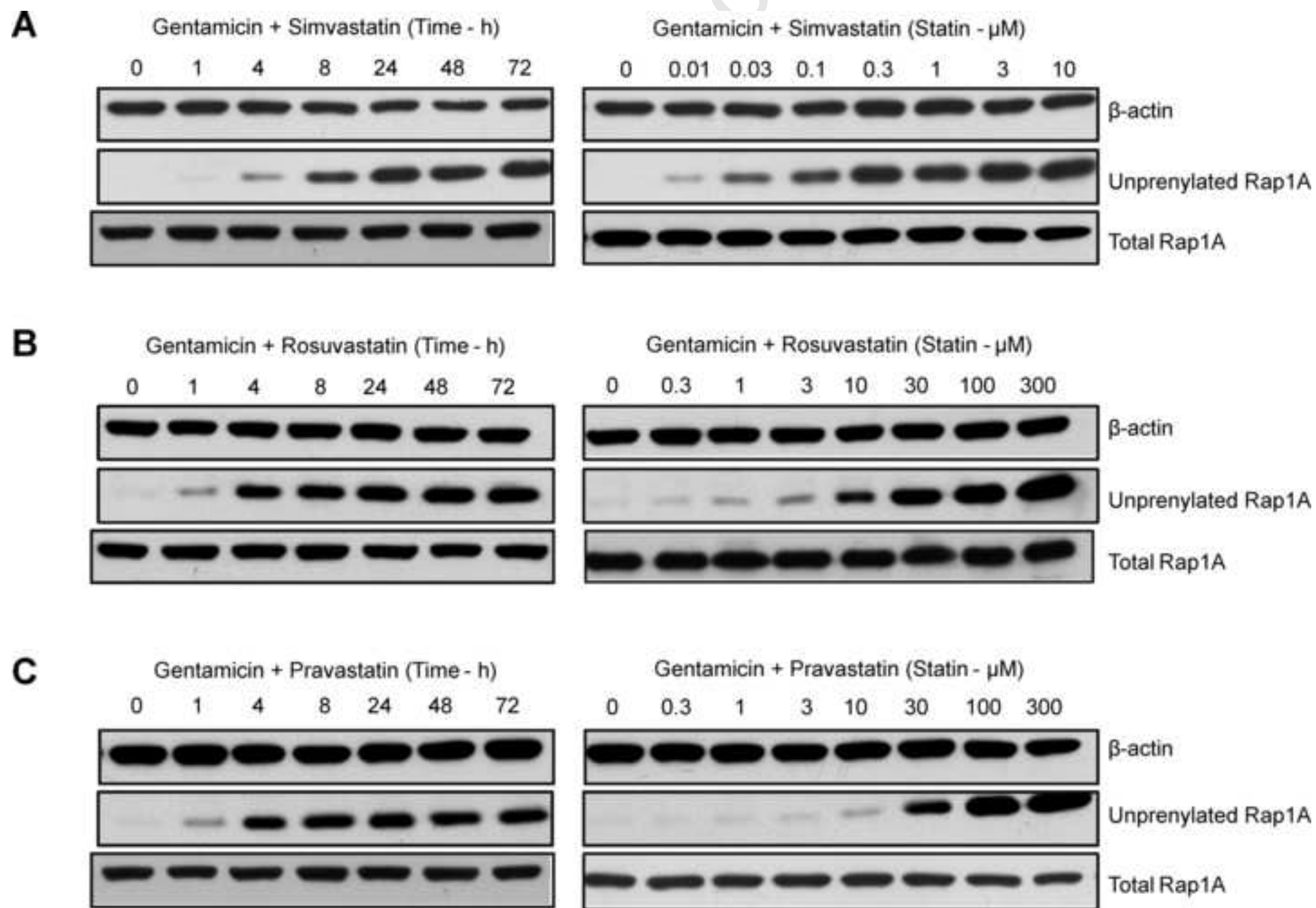


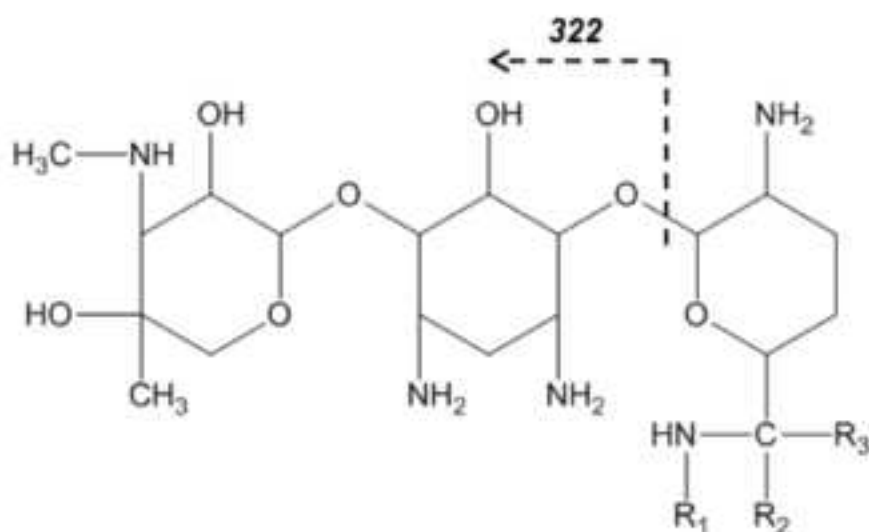
Table 1.

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 48hr. Data is given as mean IC₅₀ value ±S.D of 3 independent experiments.

Statin	IC ₅₀ (μM)			
	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)



A



Component	m/z of MH ⁺ ion	R ₁	R ₂	R ₃
C1a	450	H	H	H
C2, C2a	464	H	CH ₃	CH ₃
C1	478	CH ₃	H	CH ₃

B

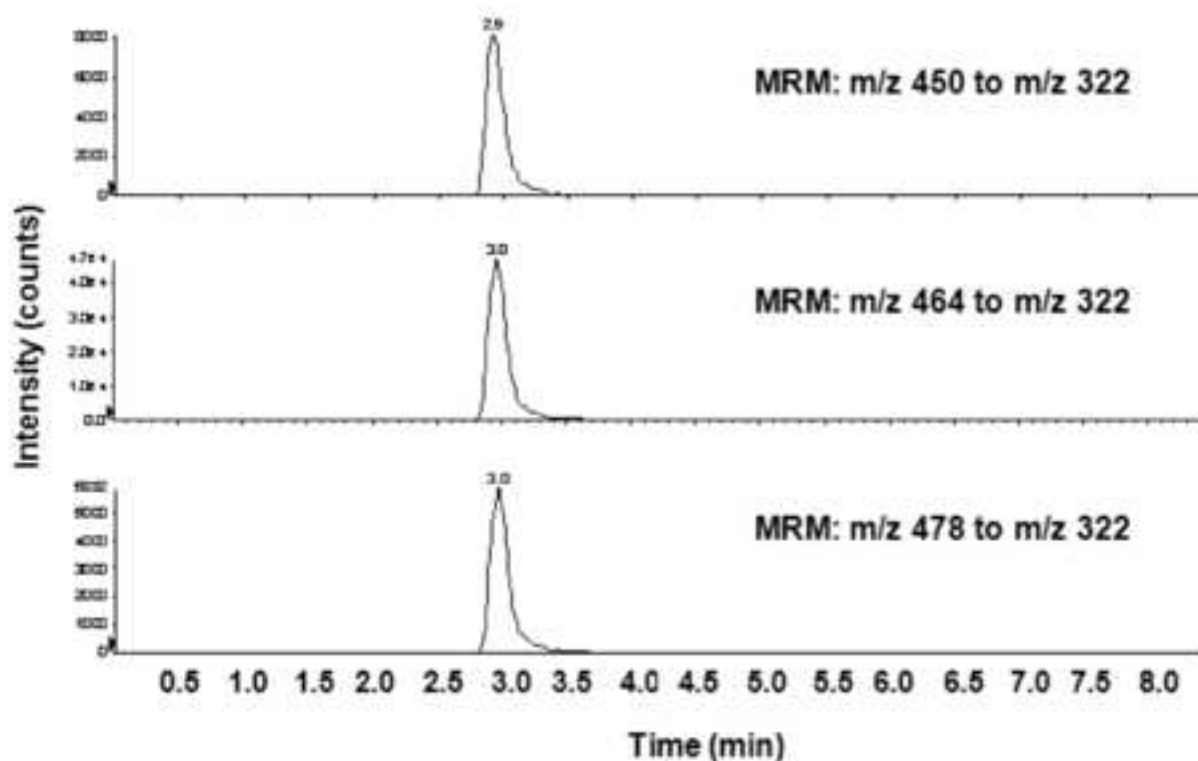


Figure 3

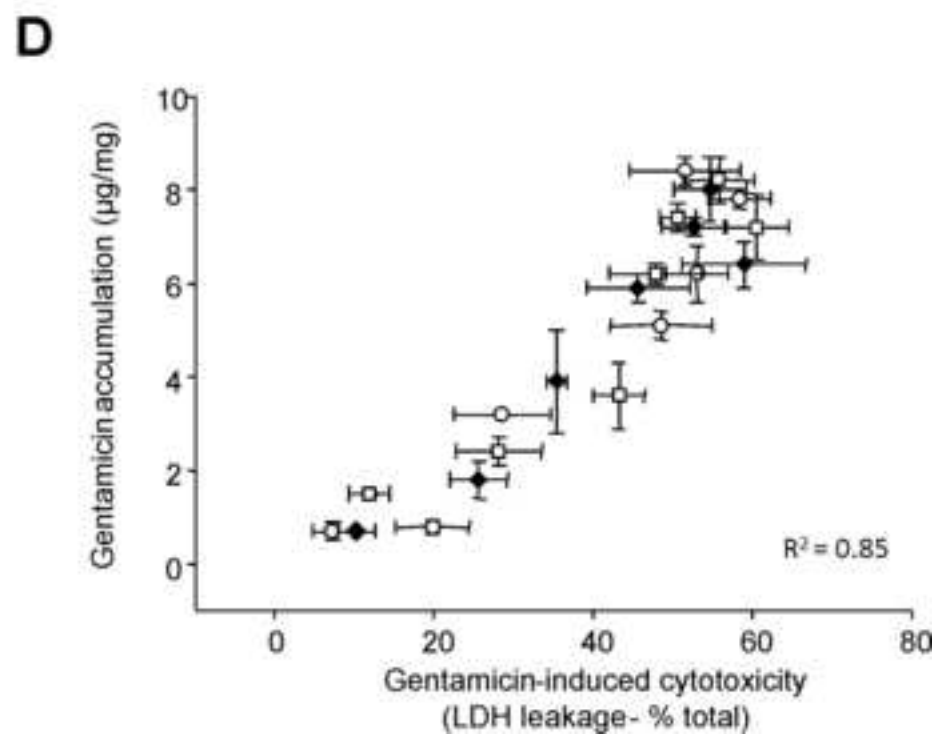
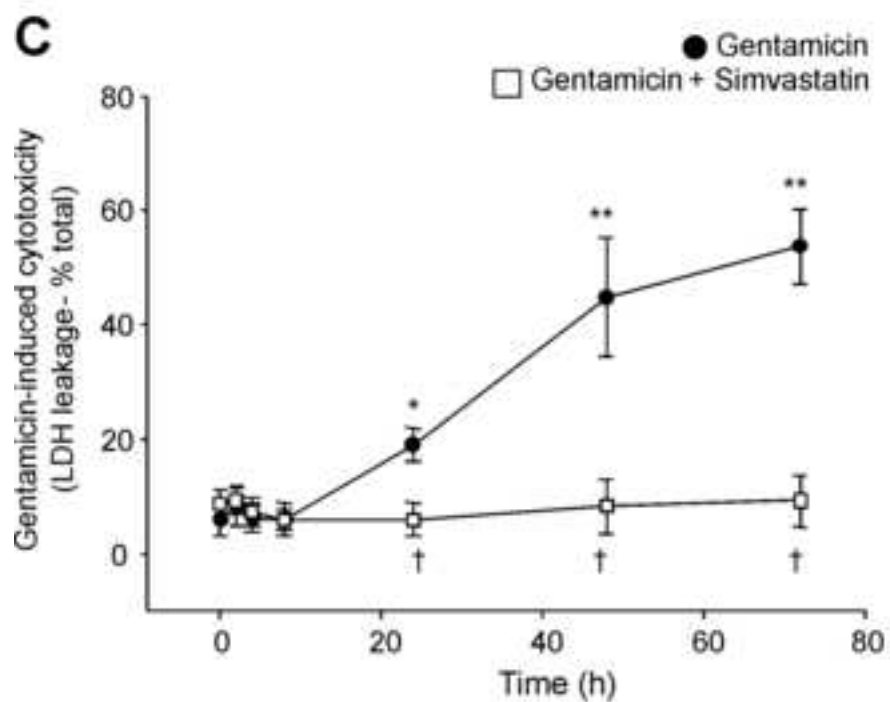
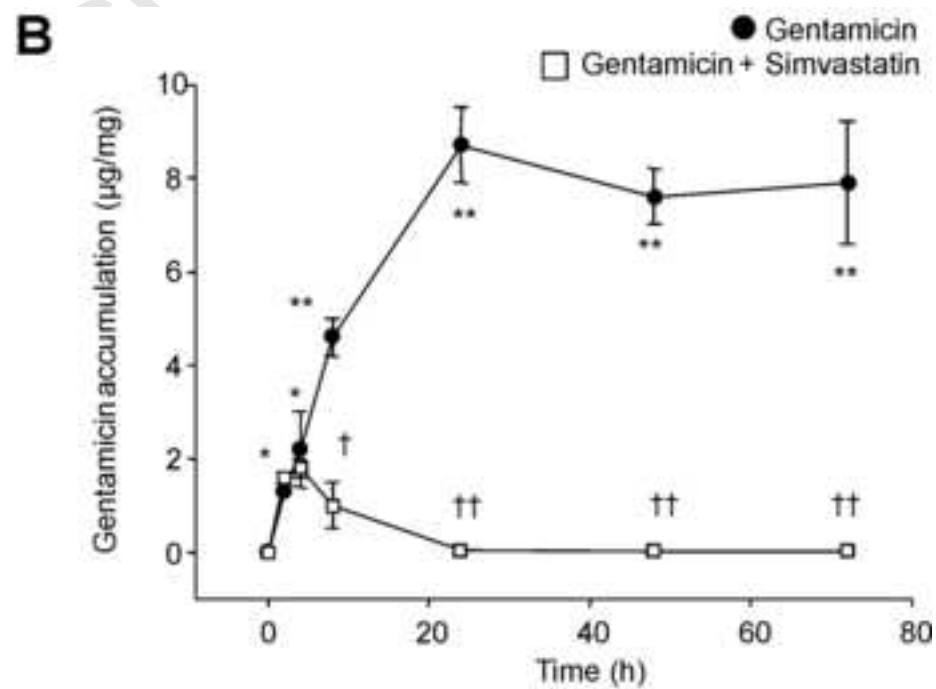
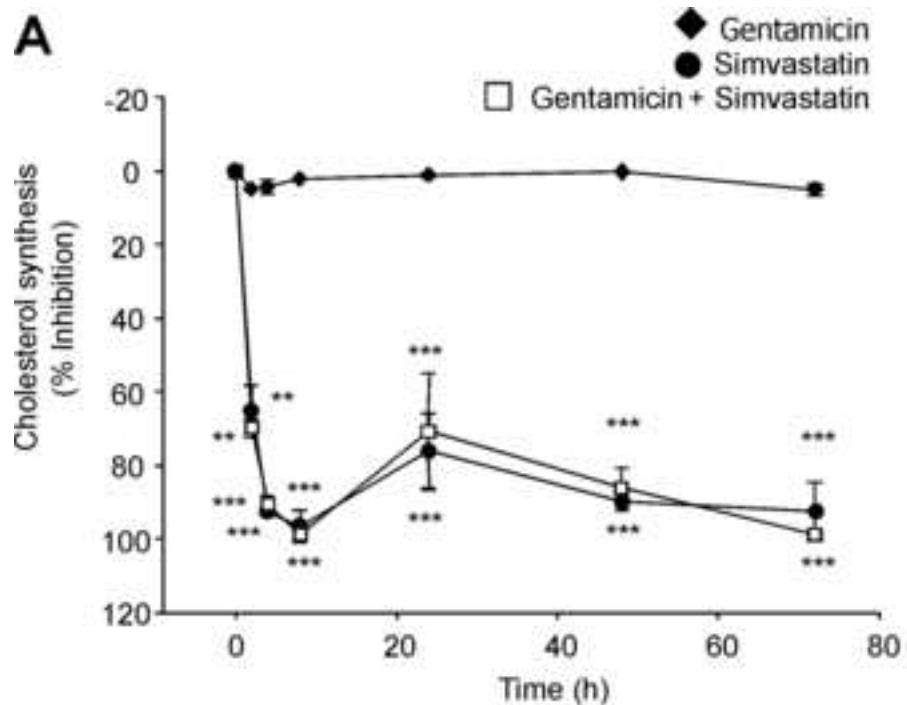
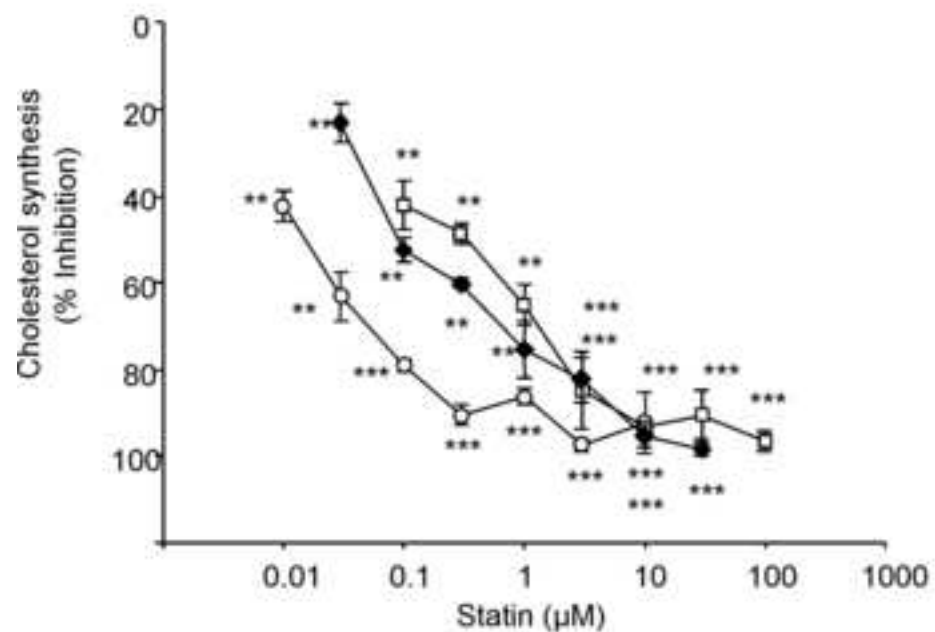
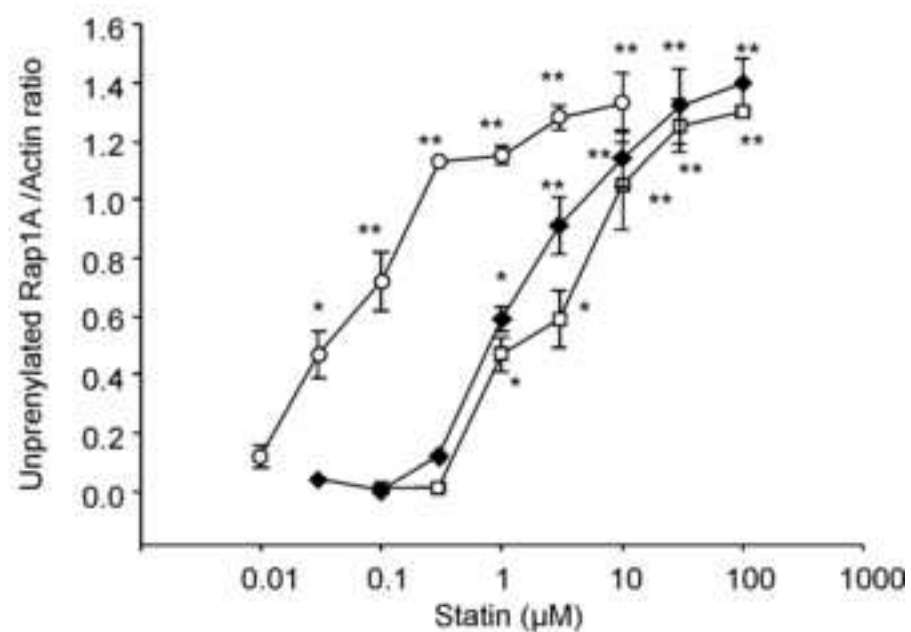


Figure 4

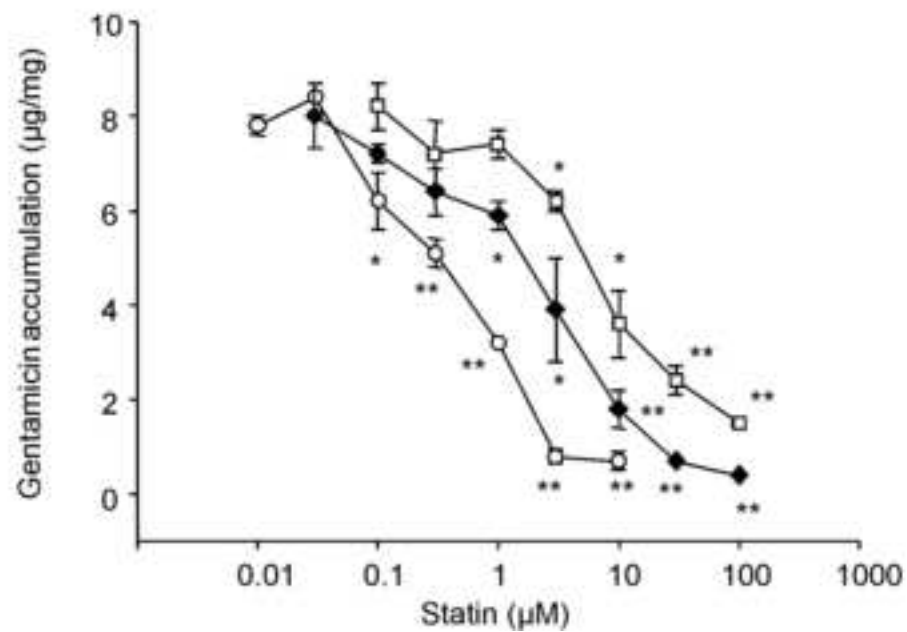
A



B



C



D

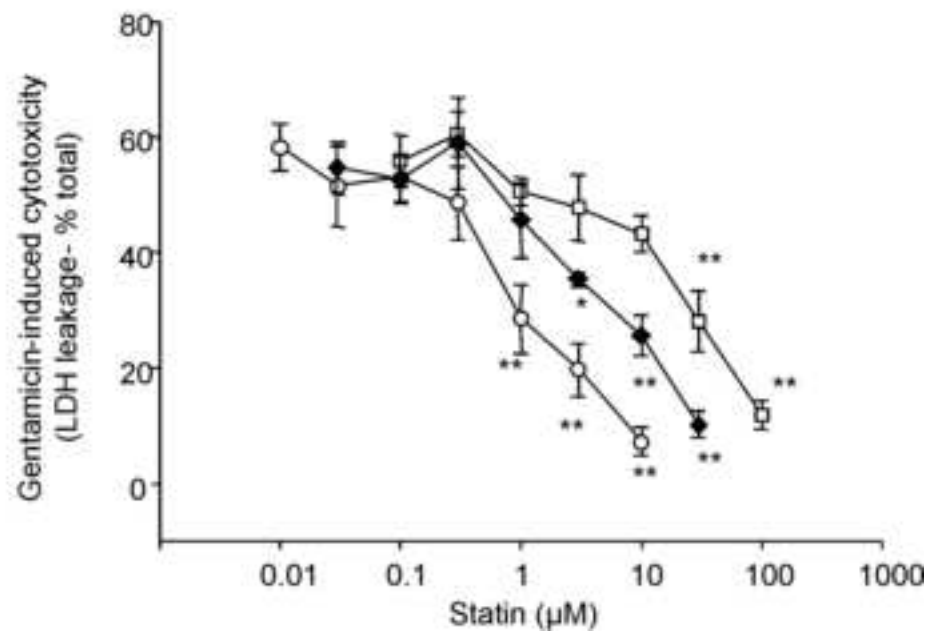


Figure 5

