Figure S1. Synthetic schemes to conjugate 3 different chemical moieties onto 2 amine sites on the aminoglycoside sisomicin.

A) Synthesis of N1 and N1,3" modified sisomicin derivatives. I=Amberlite IRA-400/MeOH; II=Zn(OAc)2 / MeOH; 1,3-dioxo-1,3,3a,4,7,7a-hexahydro-2H-4,7-methanoisooindol-2-yl (4-nitrobenzyl) carbonate; III=Zn(OAc)2 / MeOH; di-t-butyl dicarbonate / THF / Et3N; IV=PhSO2Cl / CHCl3 / NaHCO3 / H2O; V=MeSO2Cl / CHCl3 / di-isopropyl ethylamine; VI=PhCO2H / DMF / BOP; VII=EtOH / H2O / NaOH / Na2S2O4 / 70 degrees; VIII=CH2C12 / TFA.


C) Structures of moieties used to re-design sisomicin. (R in A-B) PNZ=para-nitrobenzyloxy carbonyl; Boc=t-butyloxy carbonyl; Nos=2-nitrophenylsulfonyl.
Figure S2. Structure and nuclear magnetic resonance (NMR) spectra of N1MS. A) The 1H NMR spectrum (H2SO4 salt / D2O) shows the expected 3 methyl singlets including the MeSO2 signal. The spectrum indicates a purity in excess of 95%. B) The 13C NMR shows the expected singlets for all 20 carbons.
Figure S3. Structure-ototoxicity relationships. Rat cochlear explants were treated with sisomicin and derivatives to determine hair cell toxicity in vitro. In this paradigm, sisomicin caused extensive hair cell loss in a basal-apical gradient. A) Modification of sisomicin with methylsulfonyl (MS) at either or both the N1 and N3" position effectively prevented hair cell loss. B) In contrast, addition of the benzoyl (BZ) structure only eliminated hair cell toxicity at the N3" position. C) Conjugating phenylsulfonyl (PS) to sisomicin also reduced ototoxicity, with modification at the N3" position being the most effective. D) When modification was made at the N1 position, the addition of MS resulted in the least ototoxicity, followed by PS and then BZ. E) When modification occurred at the N3" position, addition of either MS or BZ was more effective in preventing ototoxicity than PS. F) Similarly, when modification occurred at both N1 and N3", modification with MS or BZ more efficiently reduced ototoxicity than with PS. Data shown as average ± S.E. n=4-10.
Figure S4. A) Lethal dose of sisomicin and N1MS. P30 CBA/CaJ mice were injected with escalating doses of sisomicin or N1MS. Animal death within 24 hr of drug administration was quantified. Best fit curves show that the LD50 for N1MS is significantly higher than that for sisomicin. Most death occurred within 5 minutes of drug injection. n=5-10 animals. B) Mice were injected with N1MS or sisomicin (175 mg/kg IP), a subset of which also received furosemide (300 mg/kg). Blood was collected at the time points listed, plasma was extracted and mass spectroscopy analysis performed to identify the injected compounds and any of their metabolites. No metabolites were observed. n=5 for each time point.
Figure S5. Normal hearing and kidney function after treatment of urinary tract infection. A-B) After treatment with sisomicin or N1MS (625 µg for both) of *E. coli*-infected mice, animals with either treatment showed normal ABR and DPOAE thresholds. C) Serum collected from these animals showed creatinine levels indicative of normal kidney function. *n* = 5-10 for A-C.
Table S1: Outer hair cell survival after treatment with sisomicin and related derivatives in vitro*.

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<tr>
<th>Treatment</th>
<th>Apex</th>
<th>Middle</th>
<th>Base</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sisomicin (n=7)</td>
<td>38.6 ± 16.5%</td>
<td>10.2 ± 5.4%</td>
<td>4.5 ± 3.5%</td>
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<tr>
<td>N1BZ (n=10)</td>
<td>83.3 ± 11.3%*</td>
<td>28.0 ± 11.1%</td>
<td>26.5 ± 5.4%</td>
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<tr>
<td>N1MS (n=10)</td>
<td>100.0 ± 0.0%**</td>
<td>99.3 ± 0.5%**</td>
<td>98.2 ± 0.8%**</td>
</tr>
<tr>
<td>N1, 3”MS (n=10)</td>
<td>99.7 ± 0.2%**</td>
<td>99.8 ± 0.2%**</td>
<td>99.0 ± 0.7%**</td>
</tr>
<tr>
<td>N1, 3”BZ (n=5)</td>
<td>100.0 ± 0.0%**</td>
<td>99.0 ± 1.0%**</td>
<td>59.3 ± 10.5%**</td>
</tr>
<tr>
<td>N1, 3”PS (n=4)</td>
<td>86.7 ± 5.8%*</td>
<td>39.6 ± 16.1%</td>
<td>18.8 ± 11.3%</td>
</tr>
<tr>
<td>N1PS (n=6)</td>
<td>100.0 ± 0.0%**</td>
<td>75.8 ± 13.0%**</td>
<td>76.1 ± 10.4%**</td>
</tr>
<tr>
<td>N3”PS (n=4)</td>
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<td>30.8 ± 8.8%</td>
<td>2.1 ± 2.4%</td>
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<tr>
<td>N3”MS (n=4)</td>
<td>100.0 ± 0.0%**</td>
<td>99.6 ± 0.5%**</td>
<td>98.8 ± 1.4%**</td>
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<tr>
<td>N3”BZ (n=4)</td>
<td>98.8 ± 1.4%**</td>
<td>98.3 ± 1.4%**</td>
<td>95.8 ± 1.7%**</td>
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</tbody>
</table>

*All compounds were tested at 200 µM. Outer hair cells were quantified per cochlear length and normalized to cultured, undamaged controls, shown as mean±S.E.

*p<0.05 in comparison to the same region of sisomicin-treated organs.

**p<0.01 in comparison to the same region of sisomicin-treated organs.
Table S2: Dose response (outer hair cell survival) for sisomicin and N1MS*.

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<tr>
<th>Drug</th>
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<th>Base</th>
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<tr>
<td></td>
<td>20 (n=8)</td>
<td>99.0 ± 0.9%</td>
<td>98.8 ± 0.9%</td>
<td>96.7 ± 1.7%</td>
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<tr>
<td></td>
<td>50 (n=9)</td>
<td>97.6 ± 1.3%</td>
<td>87.1 ± 4.5%</td>
<td>50.6 ± 5.1%</td>
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<tr>
<td>Sisomicin</td>
<td>200 (n=7)</td>
<td>77.5 ± 2.4%</td>
<td>41.7 ± 4.6%</td>
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<td>500 (n=9)</td>
<td>54.4 ± 4.1%</td>
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<td>2000 (n=9)</td>
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<td>5000 (n=4)</td>
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<td>50 (n=4)</td>
<td>100 ± 0.0%</td>
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<tr>
<td>N1MS</td>
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<td>99.6 ± 0.6%</td>
<td>98.9 ± 1.4%</td>
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<td>500 (n=5)</td>
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<td>2000 (n=5)</td>
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<td>5000 (n=5)</td>
<td>60.8 ± 2.5%</td>
<td>45.4 ± 5.5%</td>
<td>27.1 ± 3.3%</td>
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</table>

*Hair cells were quantified per cochlear length and normalized to cultured, undamaged controls, shown as mean±S.E.
# Table S3: Normalized cochlear outer hair cell survival after sisomicin and N1MS in vivo*. 

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose*</th>
<th>Apex</th>
<th>Middle</th>
<th>Base</th>
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<tr>
<td>Sisomicin (1 week later)</td>
<td>175 mg/kg (n=9)</td>
<td>83.7 ± 6.3%</td>
<td>16.9 ± 10.1%</td>
<td>11.1 ± 10.5%</td>
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<td>N1MS (1 week later)</td>
<td>175 mg/kg (n=14)</td>
<td>100.0 ± 0.0%</td>
<td>99.2 ± 0.0%</td>
<td>87.9 ± 4.0%</td>
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<tr>
<td>Sisomicin (5-6 weeks later)</td>
<td>175 mg/kg (n=10)</td>
<td>86.4 ± 8.7%</td>
<td>24.1 ± 12.8%</td>
<td>20.0 ± 13.3%</td>
</tr>
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<td>N1MS (5-6 weeks later)</td>
<td>175 mg/kg (n=9)</td>
<td>100.0 ± 0.0%</td>
<td>100.0 ± 0.0%</td>
<td>97.9 ± 1.1%</td>
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<td>N1MS (1 week later)</td>
<td>400 mg/kg (n=10)</td>
<td>96.9 ± 2.3%</td>
<td>49.5 ± 13.5%</td>
<td>15.2 ± 10.4%</td>
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</table>

*Hair cells were quantified per cochlear length and normalized to saline-treated animals, shown as mean±S.E. n represents mice (CBA/CaJ) examined.

*Furosemide (300 mg/kg) was administered 30 min after sisomicin.
Table S4: Histologic analyses of organs from mice treated with sisomicin or N1MS#.

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<th>Organs/Animal #</th>
<th>Control 1</th>
<th>Control 2</th>
<th>Sisomicin group 1</th>
<th>Sisomicin group 2</th>
<th>N1MS group 1</th>
<th>N1MS group 2</th>
<th>N1MS group 3</th>
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#P30 CBA/CaJ mice were examined 3 days after injection with saline (control), sisomicin and furosemide (sisomicin group), or N1MS and furosemide (N1MS group).

- nl = within normal microscopic limits
- np = not present
- ne = not examined
- A1 = benign intraventricular choroid meningioma or benign melanocytoma, likely incidental to this study.
- A2 = multifocal acute tubular epithelial degeneration and necrosis with evidence of renal tubular regeneration (mitosis). This is consistent with acute aminoglycoside toxicity.
- A3 = Multifocal epicardial mineralization (cardiac calcinosis) of the right ventricular epicardium. This lesion is likely incidental to this study.