Agmatine alleviates cisplatin-induced ototoxicity by activating PI3K/AKT signaling pathway

<https://doi.org/10.1523/ENEURO.0434-21.2022>

Cite as: eNeuro 2022; 10.1523/ENEURO.0434-21.2022

Received: 13 October 2021

Revised: 18 February 2022

Accepted: 23 February 2022

This Early Release article has been peer-reviewed and accepted, but has not been through the composition and copyediting processes. The final version may differ slightly in style or formatting and will contain links to any extended data.

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14 Running title: Agmatine alleviates cisplatin-induced ototoxicity

16 Funding: The study was supported by the Key Project of Medical Science Research in
17 Hebei Province in 2018 (20180338).

Abstract

Cisplatin-induced ototoxicity can be partially attributed to excessive reactive oxygen species (ROS) production, and agmatine is well-known for the activation of the phosphatidylinositol 3-kinase (PI3K)/ protein kinase B (AKT) pathway to inhibit ROS production. Whether agmatine could be utilized to alleviate cisplatin-induced ototoxicity is investigated. Cisplatin exposed House Ear Institute-Organ of Corti 1 (HEI-OC1) cells and cochlear explants showed increased ROS production detected by 2'-7'-dichlorofluorescein diacetate (DCFH-DA) staining and decreased cell viability detected by Cell Counting Kit-8 (CCK-8) or Myosin 7a staining, which could be reversed by the agmatine pre-treatment. Cisplatin intraperitoneally injected C57BL/6 mice demonstrated damaged auditory function as indicated by distortion products otoacoustic emissions (DPOAE) and auditory brainstem response (ABR) assays, and trans-tympanically administrated agmatine in the left ears could partly prevent the auditory function loss. Mechanistically, down-regulated B-cell lymphoma 2 (Bcl-2) expression, up-regulated Bcl2 Associated x (Bax) expression, and diminished p-PI3K and p-AKT expression were detected in cisplatin-exposed HEI-OC1 cells and cochlear explants, which could be prevented by the pre-treatment with agmatine. Our investigation demonstrates that agmatine pre-treatment could alleviate cisplatin-induced ototoxicity with the activation of PI3K/AKT signaling pathway.

Keywords: Agmatine; cisplatin-induced ototoxicity; PI3K/AKT

46 **Significance Statement**

47 The study demonstrated that agmatine pre-treatment could activate PI3K/AKT signaling
48 pathway to alleviate cisplatin-induced ototoxicity.

49

50

51 **Introduction**

52 Cisplatin was approved in 1978 by the US Food and Drug Administration (FDA) to treat
53 ovarian cancer and metastatic testicular patients(Farrell 2015). Although serious side
54 effects such as bone marrow depression, nephrotoxicity, and ototoxicity can occur,
55 cisplatin remains the most widely utilized and available chemotherapeutic drug to treat
56 solid malignant tumors (Glynne-Jones and Hoskin 2007, Chang and Chinosornvatana
57 2010, Johnstone, Suntharalingam et al. 2016, Rottenberg, Disler et al. 2021). The
58 incidence of cisplatin-induced ototoxicity can range from twenty percent to seventy
59 percent, which may manifest with progressive, irreversible, and bilateral hearing loss
60 (Tang, Wang et al. 2021). Young children are more inclined to cisplatin-induced
61 ototoxicity with delayed speech development and psychosocial and cognitive
62 development (Knight, Kraemer et al. 2005, Rybak, Mukherjea et al. 2019).

63 It is generally believed that cisplatin-induced ototoxicity may be attributed to the
64 excessive reactive oxygen species (ROS) production by the cochlea (Yu, Gu et al. 2020),
65 and endoplasmic reticulum stress is a target for treatment of hearing loss (Wang and Xu
66 2020). Multiple promising strategies have been performed to alleviate, treat, and prevent
67 cisplatin-induced ototoxicity, and none of these strategies has been confirmed or
68 recommended by the FDA (Gentilin, Simoni et al. 2019, Mukherjea, Dhukhwa et al.
69 2020).

70 Among the multiple signaling pathways contributing to the survival and differentiation of
71 hair cells, phosphoinositide-3-kinase (PI3K)/protein kinase B (AKT) pathway is well
72 investigated (Liu, Wei et al. 2021), which might play an essential role in inner ear hair
73 cells survival to resistance against harmful stimuli(He, Zheng et al. 2021). It is worth

74 noting that, in neonatal cochlear spiral ganglion explants, PI3K/AKT signaling mediates
75 brain-derived neurotrophic factor-induced neurite formation (Mullen, Pak et al. 2012). In
76 the noise-induced cochlea injury, PI3K/AKT pathway activation induced by
77 deferoxamine may promote mesenchymal stem cell homing (Peyvandi, Abbaszadeh et al.
78 2018).

79 Agmatine is formed by L-arginine decarboxylation and hydrolyzation to putrescine,
80 which can bind to N-methyl-D-aspartate (NMDA) receptors and α 2-adrenergic receptors
81 to function as novel neurotransmitters and neuromodulators (Regunathan 2006, Piletz,
82 Aricioglu et al. 2013). Substantial preclinical and initial clinical evidence has indicated
83 the possibility to treat opioid addiction, mood disorders, neurotrauma, neurodegenerative
84 diseases, and cognitive disorders (Xu, Gao et al. 2018, Akasaka and Fujiwara 2020).

85 This investigation utilizes agmatine to treat cisplatin exposed HEI-OC1 cells, cochlear
86 explants, or cisplatin affected mice and finds that agmatine alleviates hearing loss with
87 reduced ROS production and cell loss and up-regulated PI3K/AKT signal pathway.
88 Therefore, as an adjuvant drug, agmatine has the potential value in reducing ototoxicity
89 caused by cisplatin chemotherapy.

90

91 **Methods & materials**

92 **Cell viability**

93 HEI-OC1 cells (5, 000/well) were seeded in 96-well plates in three replicates, and
94 relevant agmatine (10, 50, 100, 200 μ M) and or cisplatin (5, 10, 30, 50 μ M) were
95 incubated for indicated hours. CCK-8 (Dojindo Laboratories) was added to each well
96 with the final concentration of 10% for 4 hours. The optical density values were

97 measured at 450 nm with a Bio-Rad plate reader.

98 **Cisplatin exposed HEI-OC1 cells culture**

99 HEI-OC1 cell line was pre-treated with 100 μ M agmatine for 2 hours and then cotreated
100 with 30 μ M cisplatin for 24 hours in appropriate conditions (33°C, 5% CO₂, high-glucose
101 DMEM medium, 5% fetal bovine serum, Gibco).

102 **ROS detection**

103 2',7'-dichlorofluorescein diacetate (DCFH-DA) working solution (10 μ M, Beyotime,
104 S0033) was added into six-well plates and incubated the plates at 37°C for 30 minutes.
105 After the incubation, the fluorescence was observed with the LEXT OLS5100 laser
106 scanning confocal microscope.

107 **Cisplatin exposed cochlear explants**

108 Cochleae from C57BL/6 mice (three days postnatal) were dissected out and seeded intact
109 on Cell-Tak (BD Biosciences, Franklin Lakes, NJ, USA) coated glass coverslips, which
110 were further incubated with DMEM/F12 medium supplemented with 1 $\times$ N2/B27 as
111 recommended by the manufacturer (Invitrogen, Waltham, MA USA) at 37°C with 5%
112 CO₂. Agmatine (100 μ M) was used to pre-treat cochlear explants for 2 hours, and then
113 cisplatin (30 μ M) was added to induce the ototoxicity for 24 hours.

114 **Western blot**

115 HEI-OC1 or cochleae explant lysates were separated by 12% sodium dodecyl
116 sulfate-polyacrylamide gel and transferred to polyvinylidene fluoride membranes, which
117 was further blocked with 5% nonfat dry milk and incubated with primary antibodies
118 against GAPDH, Bax, Bcl2, p-PI3K, p-AKT, PI3K, and AKT (Santa Cruz).
119 Peroxidase-conjugated secondary antibody (Sigma-Aldrich, 1:1000 dilution, 2 hours, at

room temperature) was added, and an ECL system (Sigma-Aldrich) was utilized to obtain the signal. The intensity of protein bands was quantified with Image J software. GAPDH was utilized as the loading control to normalize the relative expression.

Cisplatin exposed mice

C57BL/6 male mice (four-week-old) purchased from Peking Vital River Laboratory Animal Ltd. (Beijing, China) were maintained. Agmatine (10 μ M, 5 μ L) was trans-tympanically injected into the left ears, while the same volume phosphate-buffered saline (PBS) was injected into the contralateral ears. Then cisplatin (30 mg/kg) was i.p. administered two hours later. Seven days post cisplatin administration, the auditory brainstem responses (ABR) and distortion product otoacoustic emission (DPOAE) measurements were done. All the procedure was approved by the Ethics Committee of the Second Hospital of Hebei Medical University.

ABR test

ABR assessment was performed as previously reported (McLean, Clamp et al. 2021). Briefly, anesthetized mice (25 mg/kg xylazine sodium and 100 mg/kg ketamine, i.p.) were kept warm during the ABR recordings process (highest intensity of acoustic stimuli, 90 dB SPL; decrements, 5 dB SPL) at 38°C on the thermostatic heating pad. The hearing threshold at five frequencies (4, 8, 16, 24, and 32 kHz) was detected with TDT System III apparatus (Tucker Davies Technologies).

DPOAE test

DPOAE was performed as previously reported with a TDT-RZ6 system (Tucker-Davis Technologies) (Li, Zhou et al. 2021). Two sine wave tones with different frequencies but equal intensities ($F_2=1.2F_1$, F_2 ranging from 4 to 40 kHz) were utilized with 1 s duration

143 to elicit DPOAE. Twenty adjacent frequency bins around the distortion product
144 frequency were averaged as the surrounding noise floor. DPOAE threshold was
145 determined when the signal was over 5 dB sound pressure level and over two standard
146 deviations above the surrounding noise floor.

147 **Immunofluorescence**

148 Intact cochleae were separated from the temporal bone, which were embedded in Optimal
149 Cutting Temperature O.C.T medium (Richard-Allan Scientific, Kalamazoo, MI), snap
150 frozen in liquid nitrogen, and stored at -80°C until use. Five-micrometer sections were
151 cut by a cryostat (Microm HM525, Walldorf, Germany). After being fixed with 4%
152 paraformaldehyde for 10 min, the sections were blocked with 10% normal goat serum
153 and permeabilized with 0.3% Triton X-100 for 2 h at room temperature. The Fast
154 ImmunoCytoChemistry Staining Kit (Protein Biotechnologies), anti-Myosin 7a antibody
155 (Proteus Bioscience, 25–6790), and DAPI were utilized for hair cell detection.

156 **Statistical Analysis**

157 The difference between groups was assessed using one or two-way ANOVA analysis
158 before corresponding post hoc tests. The significance level was set as $p\text{-value} < 0.05$. All
159 statistical analyses were performed with GraphPad Prism (GraphPad Software).

160

161 **Results**

162 **Agmatine alleviates cisplatin-induced ototoxicity in HEI-OC1 cells**

163 To optimize the dose of cisplatin, different concentrations of cisplatin (0, 5, 10, 30, or 50
164 μM) were utilized to treat HEI-OC1 cells for 24 hours, and the cell viability was analyzed
165 by CCK-8 assay. Cisplatin (at a dose greater than 30 μM) can markedly reduce cell

166 viability (Figure 1a). While as to agmatine, only 200 μ M agmatine could diminish the
167 viability of HEI-OC1 cells (Figure 1b), which indicated that the dose under 200 μ M was
168 safe. To determine the protective effect of agmatine on cisplatin-induced ototoxicity,
169 HEI-OC1 cells were pre-treated with different concentrations of agmatine for 2 hours and
170 then cotreated with 30 μ M cisplatin for 24 hours. A significant dose-dependent protective
171 effect was observed, and 100 μ M agmatine showed the maximal protective effect (Figure
172 1c). These results testified that agmatine could protect HEI-OC1 cells viability upon
173 cisplatin exposure, and 100 μ M agmatine and 30 μ M cisplatin were chosen in the
174 following experiment.

175 **Agmatine alleviates cisplatin-induced ROS in HEI-OC1 cells**

176 We examined ROS production with a mitochondria-specific ROS indicator, DCFH-DA,
177 to determine whether agmatine could alleviate cellular oxidative stress. Cisplatin-induced
178 up-regulated ROS production, while the pre-treatment with agmatine could diminish
179 ROS induction (Figure 2a and 2b). It was worth noting that agmatine alone could not
180 induce the production of ROS. All of these indicated the protection effect caused by
181 agmatine.

182 **Agmatine alleviates cisplatin-induced cochleae cell apoptosis in vitro with up-regulated**
183 **PI3K/AKT pathway**

184 In order to decipher the relevant pathway for apoptosis, Bcl-2 family proteins expression
185 was detected with Western blot in cisplatin exposed cells. Elevated Bax (pro-apoptotic,
186 Figure 3a and 3b) and decreased Bcl-2 (anti-apoptotic, Figure 3a and 3c) were observed,
187 which could be reversed by the pre-treatment of agmatine. At the same time, the
188 upstream PI3K/AKT signaling pathway molecules were detected. After cisplatin

189 treatment, down-regulated p-PI3K (Figure 3a and 3d) and p-AKT expression (Figure 3a
190 and 3e) were detected. As expected, agmatine administration could up-regulate the
191 PI3K/AKT pathway as indicated by the up-regulation of p-PI3K and p-AKT. These data
192 demonstrated that agmatine had the ability to inhibit cisplatin-induced apoptosis with
193 up-regulated PI3K/AKT signaling pathway in HEI-OC1 cells.

194 *Agmatine alleviates cisplatin-induced cochleae explants apoptosis*

195 Myosin 7a staining showed that cisplatin treatment could lead to the conspicuous loss of
196 mature hair cells in the apex (data not shown), basal turns (data not shown), and the
197 middle turn of cochlea. The most significant damage effect was observed on the middle
198 turn of the cochlea, which could be alleviated by the pre-treatment of agmatine (Figure 4a
199 and 4b). Accumulation of ROS may lead to the apoptosis of hair cells. No
200 DCFH-DA-positive cells were detected in the untreated or the agmatine-treated cochlear
201 explants (Figure 4c). While significantly increased DCFH-DA-positive cells were
202 detected after cisplatin exposure, this increase was reversed by agmatine pre-treatment
203 (Figure 4c and 4d).

204 *Agmatine stimulates PI3K/AKT signaling to inhibit the apoptosis in cochleae explant* 205 *induced by cisplatin exposure*

206 Cochleae explant was further utilized to confirm the apoptosis induced by cisplatin
207 exposure. Increased Bax expression (pro-apoptotic, Figure 5a and 5b) and decreased
208 Bcl-2 expression (anti-apoptotic, Figure 5a and 5c) were observed in cisplatin exposed
209 cochleae explant, which could be reversed by the agmatine pre-treatment. At the same
210 time, down-regulated p-PI3K (Figure 5a and 5d) and p-AKT expression (Figure 5a and
211 5e) were detected after cisplatin treatment. As expected, agmatine administration could

up-regulate the PI3K/AKT pathway. All of these data confirmed that agmatine could stimulate PI3K/AKT signaling pathway to inhibit cisplatin-induced intrinsic apoptosis pathway in cochleae explant and HEI-OC1 cells.

Agmatine prevents auditory function loss in cisplatin exposed mice

ABR and DPOAE measurements were utilized to indicate the auditory function. Hearing thresholds were significantly elevated at all frequencies tested 14 days after cisplatin exposure, whereas pre-treatment with agmatine could diminish the thresholds (Figure 6a and 6b). All of these indicated that agmatine could partially prevent auditory function loss in cisplatin exposed mice.

Discussion

In order to minimize cisplatin-induced ototoxicity, it is vital to find an appropriate strategy to prevent auditory function loss or restore auditory function (Tang, Wang et al. 2021). Cisplatin exposed HEI-OC1 cells, cochleae explant, and mice are utilized in this investigation, and we confirm that agmatine alleviates cisplatin-induced ototoxicity with up-regulated PI3K/AKT signaling pathway. Agmatine supplement may help to reduce cisplatin-induced ototoxicity in clinic.

Cisplatin can transport into cochlea cells and retain for months to years to undergo hydrolysis to form highly reactive aqua cisplatin complexes, which can induce hair cells apoptosis, inflammation, and permanent hearing loss (Rybak, Mukherjea et al. 2019). Our results demonstrate the beneficial effect of agmatine pre-treatment on cisplatin-induced cochleae cells apoptosis with the inhibition of the downstream mitochondrial apoptotic pathway, thereby protecting cochleae cells from cisplatin-induced ototoxicity in the acute

235 phase. A long-term effect of agmatine administration should be performed in future
236 investigations.

237 Cisplatin-induced ototoxicity usually appears in the early stages after exposure to
238 cisplatin, primarily affecting the high frequencies and leading to progressive, permanent,
239 and cumulative hearing loss. As indicated in previous reports, DPOAE (Breglio, Rusheen
240 et al. 2017) and ABR (Rybak, Husain et al. 2000) are dysregulated in cisplatin-affected
241 mice. Our investigation testifies that agmatine could improve the degenerative auditory
242 responses ranging from 4 kHz and 32 kHz.

243 Multiple intracellular signaling pathways, including PI3K/AKT pathway, can
244 phosphorylate Bad (serine-136) to inhibit apoptosis (Wang and Youle 2009, Lu and
245 Imlay 2021, Muri and Kopf 2021). Our present study demonstrates that agmatine could
246 increase phosphorylation of PI3K and AKT diminished by cisplatin exposure. The
247 activation of PI3K/AKT may contribute to the anti-ototoxicity effect of agmatine on
248 cisplatin exposure.

249 The antioxidant effect of agmatine may act as a scavenger against ROS in human
250 neuronal-like SH-SY5Y cells to maintain mitochondrial membrane potential (Condello,
251 Currò et al. 2011). In RAW 264.7 cells, agmatine has antioxidant activity against
252 lipopolysaccharides-induced ROS accumulation *via* the activation of PI3K/Akt
253 pathway (Chai, Luo et al. 2016). It is worth noting that agmatine has anti-inflammatory
254 effects, effectively inhibiting the transcription factor NF- κ B (Li, Zhu et al. 2020). As to
255 the safe dose identified in our study (200 μ M agmatine), a preprint paper indicates that
256 the safe dose of agmatine can reach to 10 mM (Park, Lee et al. 2020). Such discrepancy
257 may need further detailed analysis.

258 In summation, we demonstrate that agmatine significantly affects the protection against
259 cisplatin-induced ototoxicity by inhibiting ROS production and mitochondrial apoptosis.
260 Our findings further indicate that agmatine can function as a therapeutic or preventive
261 agent in cisplatin-induced ototoxicity.

262

263 **Conclusions**

264 Agmatine can be utilized to alleviate cisplatin-induced ototoxicity with up-regulated
265 PI3K/AKT signaling.

266

267 **Declaration of conflict of interest**

268 None.

269

270 **References**

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- 356

Figure legends

Figure 1. The viability of HEI-OC1 cells with the treatment of designed concentration of cisplatin (a), agmatine (b) and different concentration of agmatine and 30 μ M cisplatin for 24 hours. Data were represented as mean $\pm$ SD. $n = 3$. $*P < 0.05$, $**P < 0.01$ compared with control group; $\#P < 0.05$, $\##P < 0.01$ compared with 30 μ M cisplatin only group.

Figure 2. The intracellular level of reactive oxygen species (ROS) in HEI-OC1 cells was detected with 2',7'-Dichlorodihydrofluorescein diacetate (DCFH-DA) staining. a, represent fluorescent images from different groups. b, fluorescence intensity was measured with Image J software. Data were represented as mean $\pm$ SD. $n = 3$. $**P < 0.01$, $***P < 0.001$ compared with control group; $\##P < 0.01$ compared with cisplatin group. Scale bar = 50 μ m.

Figure 3. Effect of agmatine on the Bcl-2 family proteins expressions and PI3K/AKT signaling activation in HEI-OC1 cells. Representative Western blot images (a) and quantitative analysis of Bax (b), Bcl-2 (c), p-PI3K (d), and p-AKT (e). Data were represented as mean $\pm$ SD. $n = 3$. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ compared with control group; $\##P < 0.01$ compared with cisplatin group.

Figure 4. Effects of agmatine on cisplatin-induced hair cell loss (a) and ROS level (c) in the middle turns of the cochleae explant. Quantification of myosin 7a-positive (b) and DCFH-DA-positive (d) hair cells. Scale bar = 20 μ m. Data were represented as mean $\pm$

SD. $n = 6$ for each group. $*P < 0.05$, $**P < 0.01$ compared with control group; $\#P < 0.05$,
 $\#\#P < 0.01$ compared with cisplatin group.

382

383 Figure 5. Effect of agmatine on the Bcl-2 family proteins expression and PI3K/AKT
384 signaling pathway activation in the middle turns of the cochleae explant. Representative
385 Western blot images (a) and quantitative analysis of Bax (b), Bcl-2 (c), p-PI3K (d), and
386 p-AKT (e). Data were represented as mean $\pm$ SD. $n = 3$. $**P < 0.01$, $***P < 0.001$
387 compared with control group; $\#\#P < 0.01$, $###P < 0.001$ compared with cisplatin group.

388

389 Figure 6. Agmatine prevents auditory function loss in cisplatin exposed mice. ABR (a)
390 and DPOAE (b) thresholds were analyzed. Data were represented as mean $\pm$ SD. $n = 6$
391 for each group. $*P < 0.05$, $**P < 0.01$ compared with control group; $\#P < 0.05$ compared
392 with cisplatin group.

393

a

b