SHORT REPORT

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In vivo protection of spiral ganglion neurons by bryostatin 1: preliminary results

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Background: We aim to demonstrate the effect of bryostatin 1, a macrocyclic lactone that activates protein kinase C, on spiral ganglion neurons (SGNs) of adult guinea pigs deafened by aminoglycoside.
Methodology: Twenty-one guinea pigs were deafened by the aminoglycoside gentamicin and then treated by continuous infusion of experimental molecule for 1 month. The experimental molecule was bryostatin 1, artificial perilymph (negative control), or neurotrophins and an apoptosis inhibitor (positive control). Neuronal density in the spiral ganglia was quantified.
Results: Bryostatin 1 protected SGNs after a gentamicin challenge.
Conclusions: Bryostatin 1 has a neuroprotective effect when administered continuously at low doses in adult guinea pigs.

Keywords: *spiral ganglion*; *ototoxicity*; *gentamicin*; *aminoglycoside*; *protein kinase C*; *guinea pig*; *bryostatin 1*; *cochlea*

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Spiral ganglion neurons (SGNs) are essential to hear, or at least to effectively use a hearing aid. Growth factors are crucial for the survival, development, polarity, and homeostasis of SGNs (1–4). The pharmacological use of trophic factors is a promising approach to prevent SGN injury, but their clinical use is limited for several reasons: trophic factors are polypeptides with a short half-life, are subject to rapid proteolytic degradation, and may induce undesirable pleiotropic effects due to the ubiquitous expression of their receptors. The discovery of non-peptide molecules that have intrinsic trophic activities opens the way to alternative treatments.

Activation of protein kinase C beta type 1 (PKC-β1) creates a neurotrophic pathway for deafferented SGNs *in vitro* (5). Activation of protein kinase C (PKC) affects synaptic maturation, inhibits neuronal apoptosis, increases the neurotrophin level, and decreases β-amyloid aggregates, which are partly responsible for Alzheimer disease (6). Bryostatin 1 is a promising PKC activator (6). Bryostatin 1 induced SGN survival and neurite regrowth *in vitro* (5). The goal of our study was to validate the trophic effects of bryostatin 1 on SGNs *in vivo*. Guinea pigs deafened by aminoglycoside are a model of neuronal degeneration secondary to hair cell destruction in the organ of Corti. We studied the effects of low-dose bryostatin 1 administered locally for 1 month. The dose and prolonged administration were chosen to maintain a prolonged activation of PKC, in accordance with previous *in vitro* (5, 7) and *in vivo* (8) studies. We compared the effects of bryostatin 1 with those of artificial perilymph (bryostatin vehicle, negative control) and the association of neurotrophins and an apoptosis inhibitor (positive control).

The primary endpoint was the density of SGNs after 1 month of treatment by bryostatin 1. We also examined the effects on neurites between the spiral ganglion and the organ of Corti.

Material and methods

Animals and procedures

Twenty-one adult male albino Dunkin Hartley guinea pigs were supplied by Harlan (reference HsdPoc: DH, Horst, The Netherlands). All experiments were subject to the approval of the Animal Ethics Committee of the University of Liège. Animals were randomly distributed into 3 groups. For each animal, the left ear was operated

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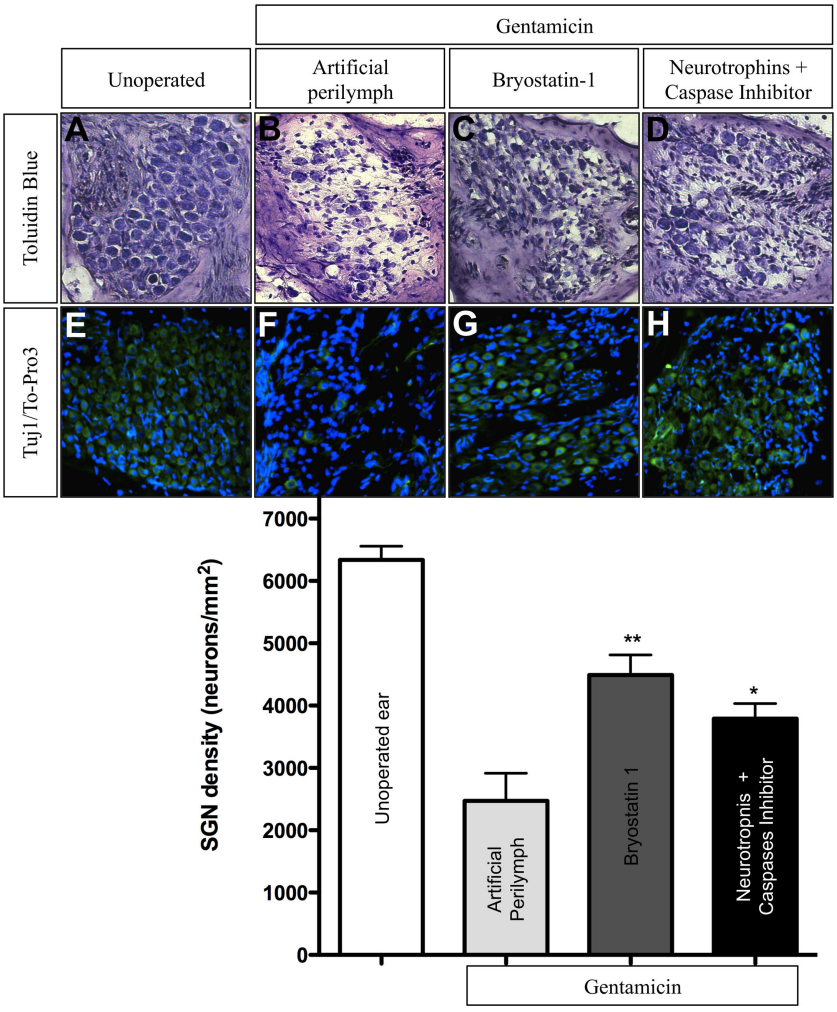

Fig. 1. Spiral ganglion neurons (SGNs) at the end of the experiment, stained by toluidine blue (A–D) and by immunofluorescence for βIII-tubulin (E–H). We observed normal spiral ganglia in non-operated ears (A and E) and dramatic neuronal loss after a gentamicin challenge in cochlea treated by artificial perilymph (B and F). Bryostatin 1 (C and G) and the positive control group's neurotrophins and apoptosis inhibitor (D and H) were able to relatively protect SGN after gentamicin challenge. Quantification of neuronal density is shown in this figure (mean ± standard error of mean). The white column shows the neuronal density in non-operated ears. We observed 61.01% neuronal loss after a gentamicin challenge in cochlea treated by artificial perilymph (light gray). The mean neuronal loss in spiral ganglia treated with gentamicin and bryostatin 1 was 29.13% (dark gray, $p < 01$ compared to artificial perilymph). The mean neuronal loss in spiral ganglia treated with gentamicin and neurotrophins plus a caspase inhibitor was 40.17% (black, $p < 05$ compared to artificial perilymph). There was no significant difference between bryostatin 1, on one hand, and neurotrophins with the caspase inhibitor z-VAD-fmk, on the other.

on, and the right ear was used as control (untreated ear, no operations). Anesthesia was performed using isoflurane (2%) and oxygen N25 (96%) at a rate of 1 L/min. Two animals died in the induction of anesthesia and were excluded from the study. Each left bulla was opened under sterile conditions through a retro-auricular approach. The cochlea was exposed ventrally and opened using a 26 gauge needle. We inserted a catheter connected to an electrical Hamilton syringe pump (Eicom microsyringe pump ESP-32, Eicom, Kyoto, Japan). The aminoglycoside gentamicin (gentamicin sulfate, Sigma-Aldrich, St. Louis, MO, USA) was administered at a concentration of 50 mg/ml for the cochlear lesion (25 µl of gentamicin in 5 min). Using the same cochlear opening, we inserted a catheter connected to a subcutaneous osmotic pump (Mouse Jugular Catheter adjustable length 7701 and 2004 Alzet Osmotic Minipump, 0.25 µl/h for 28 days, Alzet, Cupertino, CA, USA). According to the groups, the osmotic pumps were filled with the following products dissolved in artificial perilymph solution:

1) Experimental group: bryostatin 1 (1 µM; $n = 10$; Sigma-Aldrich)
2) Positive control group: the neurotrophins BDNF and NT-3 (Peprotech, Rocky Hill, NJ, USA) and

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the caspase inhibitor z-VAD-fmk (carbobenzoxy-valyl-alanyl-aspartyl-*O*-methyl-fluoromethylketone, Promega, Madison, WI, USA), in respective concentrations of 20 ng/ml, 20 ng/ml, and 20 µM ($n = 6$)

3) Negative control group: artificial perilymph (NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $NaHCO_3$, 11 mM glucose, pH 7.4; $n = 5$)

The osmotic pumps were designed to deliver a solution of 200 µl for 28 days continuously (i.e., 0.3 µl/h).

Tissue processing and neuron quantification

Temporal bones were dissected, fixed by 4% paraformaldehyde, and decalcified by 4% EDTA. The tissues were then cryoprotected by 20% sucrose and sliced in 14 µm cryostat sections. A series of cryostat sections were stained with toluidine blue, and another series was stained by immunofluorescence for βIII-tubulin. The sections were observed with our Olympus FV1000 confocal microscope (Olympus, Tokyo, Japan). Our primary endpoint was the SGN density after 1 month of treatment. We also examined the effects on neurites between the spiral ganglion and the organ of Corti.

We counted the immunopositive cells for βIII-tubulin in 4 non-adjacent sections through the middle of the cochlea. The number of neurons per square millimeter of spiral ganglia (mean ± standard deviation) was compared between groups using one-way ANOVA (GraphPad Prism program, San Diego, CA, USA).

Results

Ototoxicity

Each left cochlea received gentamicin before infusion by osmotic pump. The right cochlea received no treatment. After sacrifice, the correct positioning of the catheter in the left cochlea was verified. One guinea pig in the positive control group (i.e., those given BDNF, NT-3, and z-VAD-fmk) was excluded from the study because of incorrect insertion of the catheter. As seen in Fig. 2, we observed a massive hair cell loss in the operated ears of all groups, reflecting the gentamicin injury. The ears that did not undergo an operation had a normal appearance.

Spiral ganglion neurons

We observed a significant decrease of neuronal density in the operated cochlea that were treated by artificial perilymph. We observed a relative neuronal preservation in operated cochlea that were treated for 28 days by bryostatin 1 or by BDNF, NT-3, and z-VAD-fmk. The mean neuronal loss in spiral ganglia treated with gentamicin and artificial perilymph was 61.01%. The mean neuronal loss in spiral ganglia treated with gentamicin, neurotrophins, and a caspase inhibitor was 40.17%. The mean neuronal loss in spiral ganglia treated with gentamicin and bryostatin 1 was 29.13%. We observed a significant difference between the guinea pigs treated with a protective molecule and those treated with artificial perilymph ($p < 05$; Fig. 1).

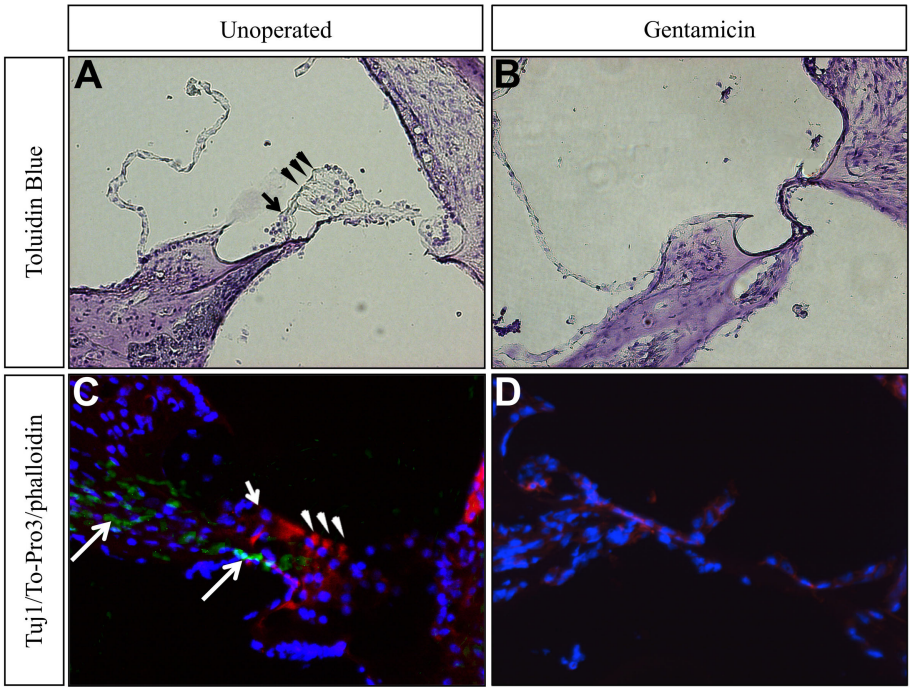

Fig. 2. In ears that were not operated on (A: toluidine blue; C: βIII-tubulin immunostaining and phalloidin), we observed normal organs of Corti with inner hair cells (arrow), outer hair cells (arrowheads), and neurites (long arrows). After a gentamicin challenge (left ear), we observed complete hair cell and neurite loss (B: toluidine blue; D: βIII-tubulin immunostaining and phalloidin) in all groups.

Neurite outgrowth

Nerve endings in the organs of Corti were marked using an antibody directed against βIII-tubulin. In non-operated ears, neurites are clearly observed at the base of the hair cells. In operated ears after the gentamicin challenge, we did not detect neuronal extensions, regardless of the experimental group (artificial perilymph, neurotrophins with a caspase inhibitor, or bryostatin 1; Fig. 2).

Discussion

The key finding of this study is the neuroprotective effect of prolonged administration of low-dose bryostatin 1. After a gentamicin challenge, we observed hair cells and SGN loss, which is consistent with previous data from the literature (9–14). Our surgical technique to induce ototoxicity and to continuously infuse the inner ear has been validated in previous studies (15–22).

Bryostatin 1 has never been administered in the inner ear *in vivo*, and it has never been studied in adult cochlea. The protective effect of bryostatin 1 was first demonstrated on deafferented postnatal SGNs of rats (5). We studied for the first time the effect of bryostatin 1 administered locally for 1 month in the cochlea of adult guinea pigs after injection of gentamicin. We compared the effects of bryostatin 1 with those of the artificial perilymph (negative control) and of molecules known for their protective effect (neurotrophins and a caspase inhibitor) (4, 23–28). By implementing the osmotic pump directly after the gentamicin challenge, we ensured that the SGNs were infused by bryostatin 1 before the first detectable neuronal lesion. Hair cells lesions occurred earlier, and this study did not show any protective effect of bryostatin 1 on the organ of Corti. Bryostatin 1 did not show any protective effect on hair cells *in vitro*. In our deafness model, we demonstrated a massive loss of hair cells in the organ of Corti in all groups.

Bryostatin 1 showed a significant protective effect on the number of SGNs, similar to the effect of neurotrophins with an apoptosis inhibitor. The molecular mechanisms are currently hypothetical, but they would involve PKC in the protection of adult spiral ganglia (5). The activation of PKC could lead to the activation of two major pathways of neuronal survival: the phosphatidylinositol 3-kinase (PI3K) pathway and mitogen extracellular regulated kinase with extracellular regulated kinase (MEK–ERK) (5). We were unable to demonstrate any effect on neuritogenesis.

Conclusion

Bryostatin 1 has a protective effect on SGNs when administered continuously at low doses in adult guinea pigs. This effect is probably due to activation of PKC, especially PKC-β1. The terms and the administration dose in humans have yet to be determined.

Financial disclosure information

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Conflict of interest and funding

The authors have not received any funding or benefits from industry to conduct this study.

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