

Original Article

Inhibition of LN-308 glioma cell proliferation and migration by retinoic acid amide through activation of Akt pathway

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Abstract: The present study was performed to investigate the effect of retinoic acid amide (RAA) on the expression of integrin $\alpha 3\beta 1$, rate of cell proliferation and migration in p53-deficient glioma cell line, LN-308. The results revealed promotion of integrin $\alpha 3$ expression, reduction in proliferation and migration in RAA treated cells compared to the control LN-308 glioma cells. Promotion of RAA induced integrin $\alpha 3\beta 1$ expression led to the enhancement in cyclin-dependent kinase nuclear localization and activation of Akt pathway. In addition, RAA treatment inhibited the expression of nuclear factor- κ B, Bcl-2 and epidermal growth factor receptor (EGFR). These factors are responsible for promoting the rate of cell proliferation and survival in the carcinoma cells. Thus RAA treatment inhibits rate of LN-308 glioma cell proliferation and migration through increase in integrin $\alpha 3\beta 1$ expression and activation of Akt pathway. Therefore, RAA can be of therapeutic importance for the treatment of glioma.

Keywords: Retinoic acid amide, glioma, proliferation, migration, localization

Introduction

Retinoic acid exhibits inhibitory effect on the growth and progression of several types of carcinomas [1]. It is reported that retinoic acid treatment suppresses the rate of proliferation, induces differentiation and apoptosis in colon [2], prostate [3], lung [4] and leukemia [5] cancer cell lines. In neuroblastoma and astrocytoma cells exposure to retinoic acid alters morphological features as well as inhibits proliferation and gene expression [6, 7]. Furthermore, retinoic acid treatment exhibits inhibitory effect on the recurrent malignant cerebral gliomas [8-10].

Integrins are present on the surface of glioma cells and regulate various cellular processes including transfer of information between cells, proliferation, survival and migration [11-13]. The activation of phosphatidylinositol 3-kinase (PI3K)/Akt and extracellular signal-regulated kinase (ERK) control the process of cell signaling is also regulated by the integrins [11, 13].

One of the most commonly observed surface receptor, integrins, $\alpha 3$ plays a dual role in the regulation of carcinoma progression [13, 14]. In cases of pulmonary carcinoma decrease in integrin $\alpha 3$ expression promotes the cancer progression and reduces rate of prognosis [15, 16]. The tyrosine kinase receptor known as epidermal growth factor receptor (EGFR) is also found on the surface of many types of carcinoma cells [17-19]. It plays a vital role in cell signaling process through phosphorylation of Akt and ERK [17].

Various studies have demonstrated that cellular processes like proliferation, apoptosis and inflammation are mediated by the nuclear factor- κ B (NF- κ B) [20, 21]. NF- κ B plays a dual role in the mode of action in the carcinoma cell proliferation and migration based on the nature of tissue and p53, tumor suppressor protein expression [20]. The present study was performed to investigate the effect of retinoic acid amide (RAA, **Figure 1**) on the expression of integrin $\alpha 3\beta 1$, cell proliferation and extent of migration in p53-deficient glioma cells, LN-308.

Retinoic acid amide inhibits glioma cell proliferation and migration

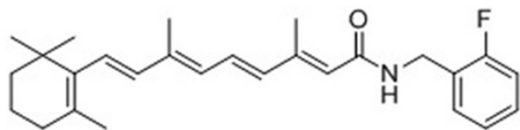


Figure 1. Structure of retinoic acid amide (RAA).

Materials and methods

Cell lines and culture

The p53-deficient glioma cell line, LN-308 was obtained from American Type Culture Collection (Manassas, VA, USA). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (HyClone Laboratories, Logan, UT, USA).

Reagents

Dulbecco's modified Eagle's medium and dimethyl sulphoxide were purchased from Sigma (St. Louis, MO, USA). MEK 1/2 inhibitor (PD-98059) and PI3K inhibitor (LY294002) were obtained from R&D Systems (Minneapolis, MN, USA).

RT-PCR

The cells at a density of 2.5×10^5 were cultured in 100 mm dishes with 10 ml medium, treated with RAA for 72 h and then collected. From the cell pellets RNA was extracted using an RNeasy-Plus Mini kit (Qiagen, TX, USA). Superscript III First-strand Synthesis SuperMix for qRT-PCR (Invitrogen, Carlsbad, CA, USA) was employed to perform the RT-PCR. For the determination of concentration of cDNA NanoDrop1000 (Thermo Fisher Scientific, DE, USA) was used and adjusted to 40 ng/ml with diethylpyrocarbonate (DPEC) water. The Chromo4 (Bio-Rad, Cambridge, MA, USA) was employed for performing real-time PCR using FAM-labeled TaqMan probes and TaqMan Universal Master Mix (Applied Biosystems). For the purpose of PCR initially incubation was carried out for 2 min at 50°C, followed by denaturation for 10 min at 95°C and 50 cycles for 15 sec at 95°C and for 1 min at 60°C.

Cell growth assay

LN-308 glioma cells treated with RAA or DMSO as control were distributed at a density of $2.5 \times$

10^5 cells per well onto the 6-well plates. The cells were serum-starved for 24 h for synchronization into G1/G0 phase of cell cycle. After synchronization the cells were incubated either in MEK-inhibitor supplemented FBS-DMEM or PI3K-inhibitor supplemented FBS-DMEM for 48 h. Following incubation the cells were rinsed in ice-cold PBS and detached using trypsin. Cell counting was performed using trypan blue exclusion method in triplicates independently.

Migration assay

LN-308 glioma cells at a density of 2×10^6 per well were dispersed onto 48-well plates and allowed to attain confluence. RAA at various concentrations was added to each well followed by incubation for 48 h. After incubation, pipette tip was pierced gently into the cell monolayer to make a small single wound. The cells were incubated with inhibitor for MEK or PI3K or with DMSO as control for 48 h. Following methyl alcohol fixing, the cells were stained using Giemsa stain. The cells were then examined for the analysis of migration into the wounded area.

Western blot analysis

LN-308 glioma cells (2×10^6) after incubation with various concentrations of RAA were treated with 200 μ l lysis buffer (40 mmol/l Tris-HCl, 1 mmol/l EDTA, 150 mmol/l KCl, 100 mmol/l NaVO₃, 1% Triton X-100 and 1 mmol/l PMSF, pH 7.5). NucBuster™ Protein Extraction kit (Novagen®; Merck KGaA, Darmstadt, Germany) was used for harvesting nuclear lysates according to the manufacturer's instructions. Proteins samples (60 μ g) after separation using 10% SDS-PAGE were transferred onto polyvinylidene fluoride membranes (Millipore Corp., Billerica, MA, USA). The non-specific binding sites were blocked by incubation of the membranes with non-fat milk [5% in Tris-buffered saline with Tween®-20 (TBST) buffer] for 1 h at 37°C. Incubation of the membranes with primary antibodies was performed overnight at 4°C. The membranes were washed in PBS and then incubated with horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit immunoglobulin G (Zhuangzhi Bio, Xi'an, China) for 1 h. Enhanced Chemiluminescence kit (ECL Plus; GE Healthcare Europe GmbH, Freiburg, Germany) was used for visualization of the bands. ImageJ software (version 1.42q; National In-

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Figure 2. Effect of RAA and siRNA-targeting integrin $\alpha 3$ on the expression of integrin $\alpha 3$ in LN-308 glioma cells determined using RT-PCR and western blot analysis. Statistical significance is indicated as $P < 0.05$.

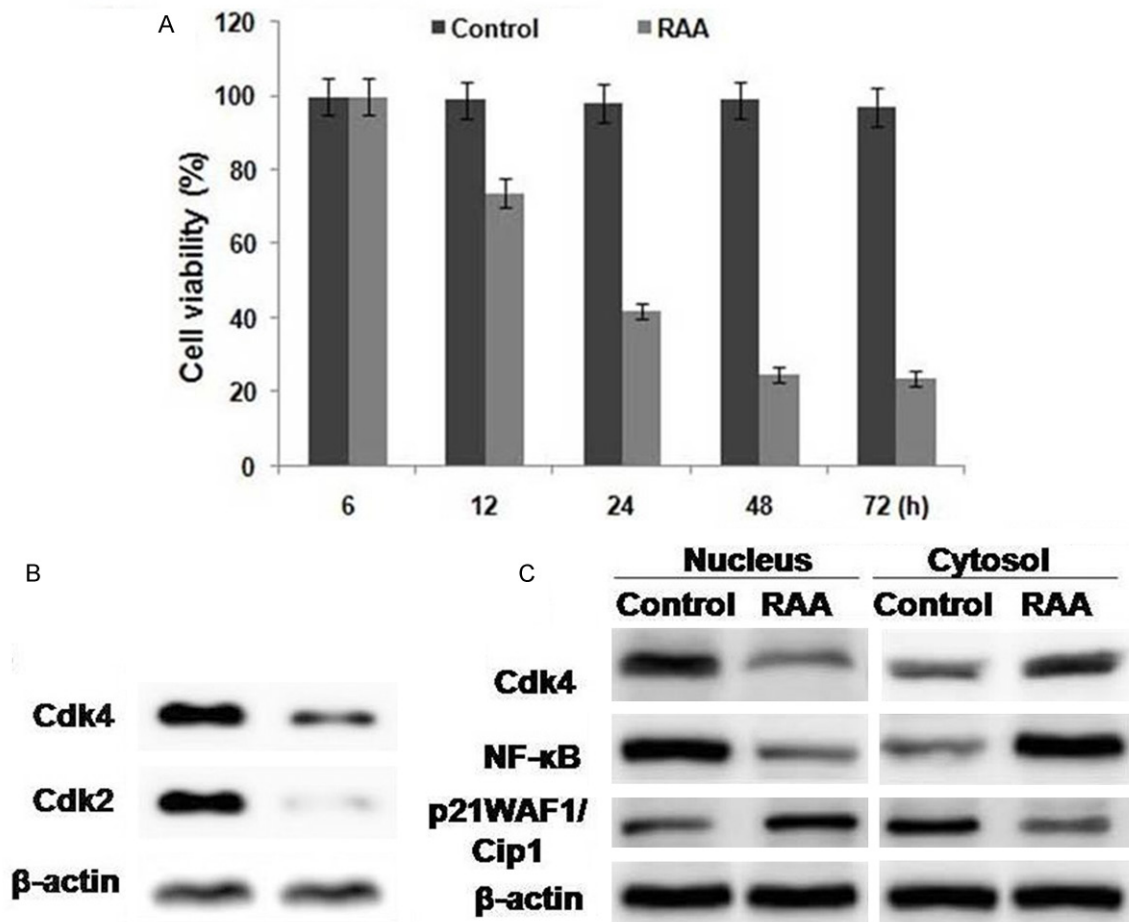


Figure 3. RAA treatment inhibited cell proliferation in LN-308 glioma cells by decreasing nuclear localization of Cdks and NF- κ B. The values expressed are the mean \pm SD for three independent experiments. Statistical significance is indicated ($P < 0.05$).

stitute of Health, Bethesda, MD, USA) was used for the densitometric analysis of the bands.

Analysis of matrix metalloproteinase activity

We used zymography for determination of the activity of matrix metalloproteinases. Briefly, the cell samples diluted with sample buffer were separated by electrophoresis on 10% polyacrylamide gel supplemented with gelatin. Re-

moval of SDS and re-naturalization of MMPs was performed by incubation of the gels in 2.5% Triton X-100 for 2 h. The gels were incubated again in developing buffer supplemented with 50 mM Tris-HCl (pH 7.5), 10 mM CaCl_2 , and 150 mM NaCl at 37°C for 24 h. Coomassie brilliant blue R-250 in 30% methanol-10% acetic acid was used for the staining of gels. The gel was de-stained using a binary system of methanol (30%) and acetic acid (10%).

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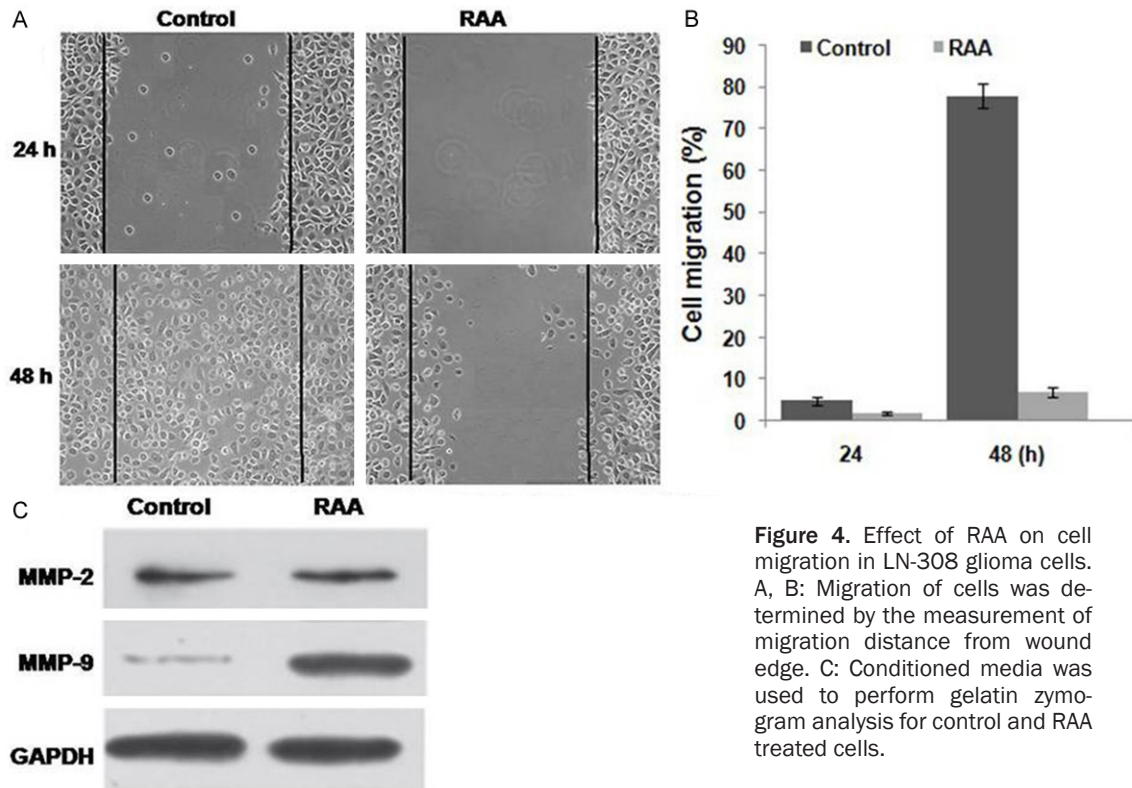


Figure 4. Effect of RAA on cell migration in LN-308 glioma cells. A, B: Migration of cells was determined by the measurement of migration distance from wound edge. C: Conditioned media was used to perform gelatin zymogram analysis for control and RAA treated cells.

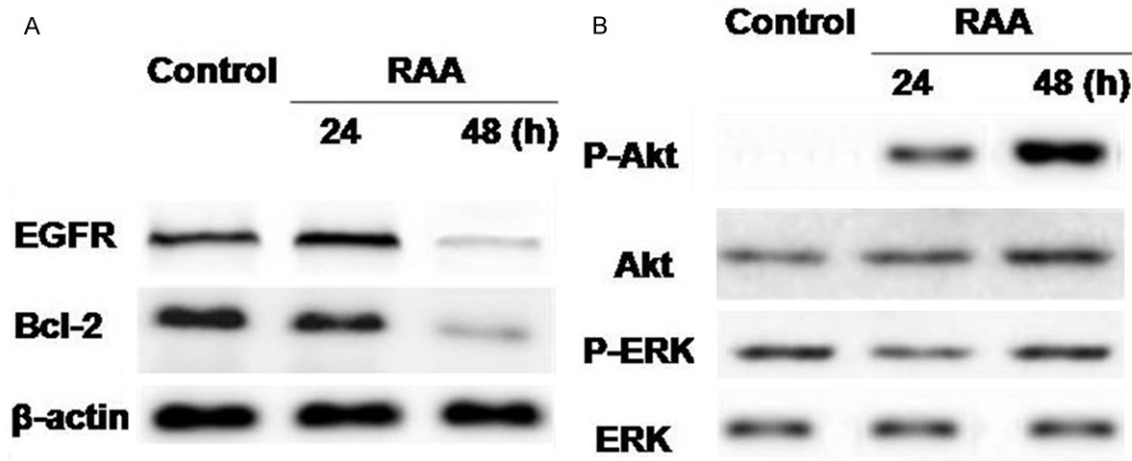


Figure 5. Effect of RAA on EGFR expression and signaling pathways in LN-308 glioma cells using western blot analysis.

Statistical analysis

Student's t-test was used for the statistical analysis of the data obtained. All the experiments were performed in triplicates independently. The statistically significant differences were considered at $P < 0.05$.

Results

Effect of RAA on the expression of integrin $\alpha 3\beta 1$ in p53-deficient LN-308 glioma cells

Treatment of LN-308 glioma cells with RAA caused a significant increase in the expression

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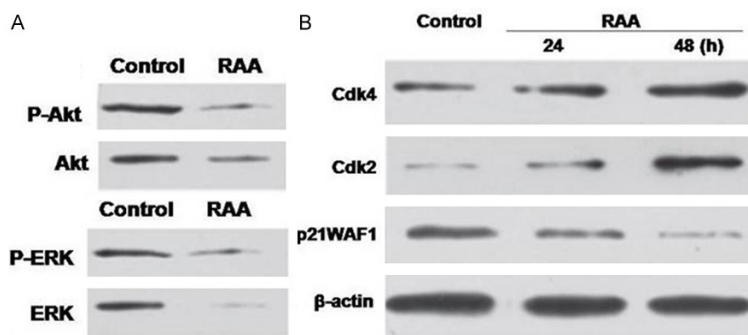


Figure 6. Effects of RAA on the expression of Cdk4 and LN-308 cell proliferation. (A) The cells were pretreated with LY294002 or PD98059 for 24 h (A) or 48 h (B) followed by cell proliferation and western blot analysis.

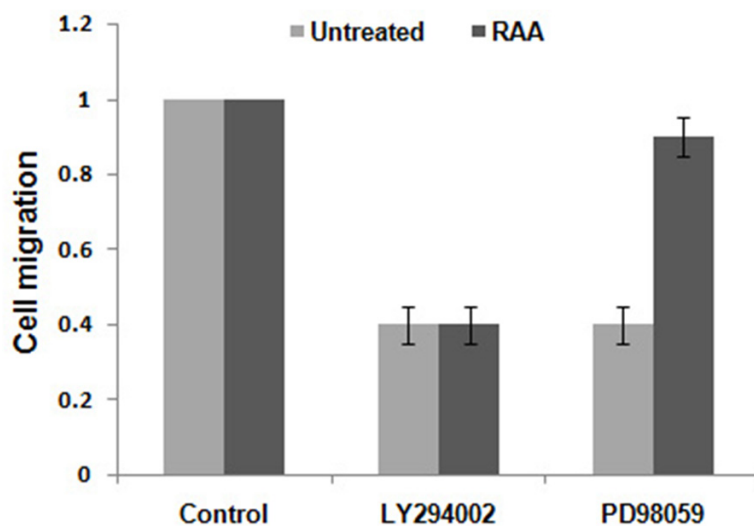


Figure 7. Inhibition of cell migration in RAA treated cells involves Akt- and ERK-independent pathways.

of integrin $\alpha 3$ compared to the control cells. Comparison of the RAA treated, control and siRNA-targeting integrin $\alpha 3$ transfected cells revealed that the level of integrin $\alpha 3$ was significantly higher in RAA treated compared to the control cells. Moreover, in control cells the level of integrin $\alpha 3$ was higher than in the cells treated with siRNA-targeting integrin $\alpha 3$ (Figure 2).

RAA reduced proliferation of LN-308 cells

Treatment of LN-308 cells with 20 μM concentration of RAA for 48 h led to a significant reduction in the rate of cell proliferation compared to the control cells (Figure 3A). Analysis of the expression of cell cycle proteins revealed that RAA treatment significantly reduced the expres-

sion level of Cdk4 and nuclear localization of Cdk4 in LN-308 cells compared to the control cells. However, the expression of p21WAF1/Cip1, Cdk inhibitor was increased in the nuclear compartments of the RAA treated cells (Figure 3B, 3C). Furthermore, RAA treatment in LN-308 cells significantly inhibited the expression levels of NF- κB in the cytosolic compartments (Figure 3C).

RAA treatment inhibits cell migration

RAA treatment caused a marked reduction in the migration potential of LN-308 cells into the wounded area after 48 h (Figure 4A, 4B). Examination of the expression of MMP-2 and MMP-9 revealed a significant increase in the MMP-9 activity without any effect on MMP-2 on exposure to RAA (Figure 4C).

Effect of RAA on ERK and Akt activation in LN-308 glioma cells

The results showed that RAA treatment inhibited the expression of EGFR and anti-apoptotic Bcl-2 protein in LN-308 cells significantly compared to the control cells (Figure 5A). Analysis of the alterations in PI3K/Akt and ERK activation revealed that RAA treatment induced phosphorylation of Akt significantly without any effect on ERK phosphorylation at 48 h (Figure 5B).

LN-308 cells when pretreated with LY294002, an inhibitor of PI3K-Akt pathway or PD98059, an inhibitor of ERK pathway showed inhibition of ERK and Akt activation in both control and RAA treated cells (Figure 6A). Treatment of RAA treated glioma cells with LY294002 enhanced the expression level of Cdk4 and Cdk2 and reduced the expression of Cdk inhibitor p27^{Kip1} (Figure 6B).

LN-308 glioma cells on pretreatment with LY294002 or PD98059 significantly showed decrease in the migration of control cells (Figure 7). However, in RAA treated cells LY294002 but not PD98059 treatment decreased cell migration (Figure 7).

Discussion

The present study demonstrates that RAA treatment enhances the expression of integrin $\alpha 3\beta 1$, inhibits the rate of proliferation and migration in p53-deficient LN-308 glioma cells. Furthermore, RAA treatment suppressed the expression level of EGFR, Cdks, NF- κ B, Bcl-2 and induced the activation of Akt. Integrin $\alpha 3\beta 1$ plays a dual role in the progression of tumor metastasis and invasion in various types of cancers either by promoting or inhibiting it [14, 22, 23]. In brain tumor metastasis the expression of integrin $\alpha 3\beta 1$ is enhanced [23] whereas in small cell lung cancer and NSCLC the expression of integrin $\alpha 3\beta 1$ is inhibited [15, 16]. It is reported that suppression of integrin $\alpha 3$ expression leads to enhancement in rate of carcinoma cell proliferation and survival along with the reduction in the survival rate of cancer patients [16, 24]. Results from the present study revealed that treatment of LN-308 cells with RAA caused a significant enhancement in the expression of Integrin $\alpha 3\beta 1$.

Progression of cell cycle is mediated by activation of cyclin-dependent kinases (Cdks) and localization of Cdks from cytoplasm to nucleus [25]. Analysis of the expression of cell cycle proteins in LN-308 cells revealed a significant reduction in the expression levels and nuclear localization of Cdks by RAA treatment. The expression of Cdk inhibitor, p21WAF1/Cip1 was increased in the nuclear compartments of RAA treated cells. NF- κ B transcription factor is known for regulation of expression of a variety of genes in response to inflammation, cell cycle progression, survival, and anti-apoptosis [20, 21, 26]. In the present study RAA treatment significantly inhibited the expression levels of NF- κ B in the cytosolic compartments in LN-308 cells. Therefore, it appears that RAA induces inhibition of cell cycle progression and proliferation through suppression in the expression and nuclear localization of Cdks and NF- κ B.

Activation of the matrix metalloproteinases (MMPs) leads to increase in the extent of cell

migration [27]. Results from the present study revealed that RAA significantly reduced cell migration and increased MMP-9 activity without any effect on MMP-2.

Enhancement in the expression of anti-apoptotic regulators like Bcl-2 is mediated by NF- κ B [21]. Our results revealed that RAA treatment enhanced the phosphorylation of Akt without any effect on phosphorylation of ERK.

Conclusion

Therefore, RAA plays an important role in the inhibition of glioma cell migration through increase in the expression of integrin $\alpha 3\beta 1$ and activation of Akt pathway.

Acknowledgements

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Disclosure of conflict of interest

None.

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