Original Paper

Clozapine Induces Autophagic Cell Death in Non-Small Cell Lung Cancer Cells

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Key Words

Antipsychotic • Clozapine • Lung cancer • Cell cycle arrest • Autophagy

Abstract

Background/Aims: Previous studies have shown that patients with schizophrenia have a lower incidence of cancer than the general population, and several antipsychotics have been demonstrated to have cytotoxic effects on cancer cells. However, the mechanisms underlying these results remain unclear. The present study aimed to investigate the effect of clozapine, which is often used to treat patients with refractory schizophrenia, on the growth of non-small cell lung carcinoma cell lines and to examine whether autophagy contributes to its effects. **Methods:** A549 and H1299 cells were treated with clozapine, and cell cytotoxicity, cell cycle and autophagy were then assessed. The autophagy inhibitor bafilomycin A1 and siRNA-targeted *Atg7* were used to determine the role of autophagy in the effect of clozapine. **Results:** Clozapine inhibited A549 and H1299 proliferation and increased p21 and p27 expression levels, leading to cell cycle arrest. Clozapine also induced a high level of autophagy, but not apoptosis, in both cell lines, and the growth inhibitory effect of clozapine was blunted by treatment with the autophagy inhibitor bafilomycin A1 or with an siRNA targeting *atg7*. **Conclusions:** Clozapine inhibits cell proliferation by inducing autophagic cell death in two non-small cell lung carcinoma cell lines. These findings may provide insights into the relationship between clozapine use and the lower incidence of lung cancer among patients with schizophrenia.

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Introduction

The prevalence of smoking among patients with schizophrenia is approximately 3-fold higher than that in the general population [1]. Smoking is well known to increase the incidence of various types of cancer, including oral, stomach, liver, bladder, and lung cancer [2]; of these cancers, lung cancer has the highest mortality rate [3]. In addition, smoking and other cancer-related risk factors, such as alcohol abuse, obesity, and poor diet, are more common in schizophrenia patients than in the general population [4]. However, patients diagnosed with schizophrenia have a lower incidence of cancer than that observed in the general population [5-8]. A recent study based on the National Health Insurance Research Database in Taiwan showed that patients with schizophrenia exhibited an overall decrease in cancer risk compared with a control population [9]. Moreover, meta-analyses of patients with schizophrenia revealed a lower-than-expected cancer risk [10] and, after adjusting for smoking, a reduced incidence of lung cancer [6].

Although the mechanisms responsible for the reduced occurrence of lung cancer in patients with schizophrenia are unknown, several studies have demonstrated anticancer effects of antipsychotics in human and mouse cell lines [11-15]. For example, phenothiazines showed a strong cytotoxic effect on human leukemic cells, neuroblastoma and glioma cell lines, and a mouse melanoma cell line [11, 12, 15]. Additionally, a recent study that screened six different antipsychotics showed that only pimozide and olanzapine exhibited cytotoxic effects on non-small cell lung cancer (NSCLC) cells [14]. Although antipsychotic-related anticancer research is ongoing, the antiproliferative effect of antipsychotics on cancer remains unclear.

In recent years, autophagy has become a crucial topic in pharmacological studies [16, 17]. Autophagy is a dynamic process that is characterized by the formation of double-membrane autophagosomes that sequester cytoplasmic components and subsequently fuse with lysosomes to promote the degradation and recycling of autophagosome constituents [18]. Through the above mechanism, autophagy eliminates old or damaged organelles and long-lived proteins to maintain cellular homeostasis. In addition to protecting cell survival, autophagy can induce cell death. For example, antiestrogen-induced autophagy leads to cell death in the breast cancer cell line MCF-7 [19]. However, to date, there are no published articles regarding the role of autophagy in the anticancer effect of antipsychotics on NSCLC cells.

Clozapine, a second-generation antipsychotic, is an effective agent that has been widely used in the clinic to treat patients with refractory schizophrenia [20]. *In vitro* studies using liquid culture systems and immunofluorocytometry have revealed that clozapine specifically exhibits toxic effects against myeloid maturation [21]. However, only few studies have been conducted to study the drug's potential anticancer effects [22], and the possible mechanisms underlying the induction of autophagy have not been explored. In the present study, we hypothesized that clozapine could inhibit the proliferation of NSCLC cells. Therefore, the aims of the study were to investigate the effect of clozapine on the growth of NSCLC cells and to examine whether autophagy contributed to the antiproliferative effect.

Materials and Methods

Cell culture and reagents

A549 and H1299 human NSCLC cells and IMR-90 normal human lung fibroblasts were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). The A549, H1299 and IMR-90 cells were maintained in F12K (Caisson Laboratories, North Logan, UT, USA), RPMI-1640 (Caisson Laboratories) and MEM (Caisson Laboratories), respectively, each supplemented with 10% fetal bovine serum (FBS; Thermo Scientific, Logan, UT, USA), 1x glutamine, and 1x antibiotic-antimycotic (Biowest, Nuaille, France). Additionally, clozapine and bafilomycin A1 were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA).

Cell viability assay

Cell viability was determined using PrestoBlue (Invitrogen, Carlsbad, CA, USA). After treatment as indicated in the figure legends, the cells were incubated with PrestoBlue for 1 h. The fluorescence values were then measured using a SYNERGY Mx microplate reader (BioTek, VT, USA) with an excitation wavelength of 560 nm (10 nm bandwidth) and an emission wavelength of 590 nm (10 nm bandwidth).

Apoptosis assay

Apoptosis was evaluated using the Dead Cell Apoptosis Kit (Invitrogen), which detects phosphatidylserine externalization (via annexin V staining) from the inner to the outer leaflet of the cell membrane [23]. After treatment as indicated, the cells were collected, washed twice with cold PBS, and resuspended in 1x annexin V binding buffer containing Alexa Fluor 488-labeled annexin V and propidium iodide (PI). The apoptotic cells were then assessed using a flow cytometer with 488 nm excitation and appropriate emission filters.

Cell proliferation and cell cycle analysis

We assessed the cell proliferation rate using carboxyfluorescein diacetate succinimidyl ester (CFSE) staining. Cells were first labeled with 5 μ M CFSE (Invitrogen) for 10 min at 37°C. Residual CFSE was then removed by washing twice with phosphate-buffered saline (PBS), followed by cell growth in culture medium with or without 50 μ M clozapine for 72 h. For cell cycle analysis, cells were fixed in 70% ethanol and stained with a PI/Triton X-100 DNA-staining solution (20 μ g/mL PI, 0.1% Triton X-100, and 0.2 mg/mL RNase A in PBS) at room temperature for 30 min. The cells labeled with CFSE or PI were then analyzed using a flow cytometer with 488 nm excitation and appropriate emission filters.

Western blotting

Cells were lysed with M-PER Mammalian Protein Extraction Reagent (Thermo Scientific) containing a 1x Halt protease and phosphatase inhibitor cocktail (Thermo Scientific). The cells were then centrifuged at 14,000 g for 15 min at 4°C to collect the supernatants. Next, equal amounts of protein were separated using SDS-PAGE before transfer onto polyvinylidene difluoride (PVDF) membranes. The membranes were blocked in a solution of 5% nonfat milk in PBS at room temperature for 1 h and then incubated with an anti- β -actin, anti-p21, anti-p27, anti-microtubule-associated protein light chain 3 (LC3 protein) or anti-ATG7 antibody (Cell Signaling Technology Inc., MA, USA) at a dilution of 1:1000 at 4°C overnight. After being washed three times with PBS containing 0.1% Tween 20 (PBST), the membranes were probed with the appropriate amount of secondary antibody for 1 h at room temperature. The membranes were then washed three times with 0.1% PBST and visualized using the Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare Biosciences, PA, USA).

Subcellular localization of LC3 fusion proteins

H1299 cells were transfected with the mRFP-EGFP-LC3 (Addgene, Cambridge, USA) fusion protein expression plasmid using TurboFect (Thermo Scientific) and selected with 500 μ g/mL G418 (Merck Millipore, Germany). Next, the cells were treated with DMSO or 50 μ M clozapine for 24 h and fixed in 4% paraformaldehyde at room temperature for 30 min. Confocal microscopy images were taken using a Zeiss LSM 780 plus ELYRA S.1 equipped with a Plan-Apochromat 100 $\times$ /1.4 oil objective (Carl Zeiss, Germany).

Autophagic activity detection

We used the Cyto-ID Autophagy Detection Kit (Enzo Life Sciences, Villeurbanne, France) to specifically label autophagic vacuoles. Briefly, A549 and H1299 cells were seeded in twelve-well plates and treated as indicated. The cells were then collected, resuspended in Cyto-ID Green autophagy detection reagent, incubated at 37°C for 1 h, and analyzed using a flow cytometer with 488 nm excitation and appropriate emission filters.

RNA interference

Cells were seeded in a six-well plate at a final density of 5×10^4 and cultured for 48 h. Scramble (nonspecific) and Atg7 siRNA oligonucleotides were purchased from Thermo Scientific, and transfection was performed as indicated by the manufacturer. At 24 h after the transfection, the cells were trypsinized, reseeded, cultured for an additional 24 h, and then treated with clozapine.

Software and statistics

The CFSE staining, cell cycle analysis, autophagic activity detection, and apoptosis assay results were analyzed using a FACSaria flow cytometer and the accompanying software program (FCS Express 3.0). All of the statistical analyses were performed using SigmaPlot software (version 12.0; Systat Software, IL, USA).

Mean and standard deviation (SD) values are used to describe the continuous variables. A Student's *t* test was performed to compare the mean values of the results of various types of assays between cells with and without clozapine treatment. All of the statistical tests were two tailed, and the significance level was set at 0.05.

Results

Clozapine inhibits proliferation in NSCLC cells

To assess the effect of clozapine on lung cancer cell viability, we first treated the lung cancer cell lines A549 and H1299 with clozapine in a dose- and time-dependent manner. The results showed that 25 μ M clozapine inhibited the growth of A549 cells and that 50 μ M affected H1299 cell growth (Fig. 1A, upper panel). Because patients with schizophrenia usually require prolonged drug administration [24], we also determined the long-term effects of clozapine on the two NSCLC cell lines. Whereas 12.5 μ M clozapine did not affect cell viability over a 3-day period, treatment with 5 μ M clozapine for 7 days significantly reduced the viability of both A549 and H1299 cells (Fig. 1A, middle panel). To examine whether clozapine could induce cytotoxic effects on normal lung cells, we used lower doses of clozapine (because clozapine is used to treat schizophrenic patients at lower doses) to treat two NSCLC cell lines as well as the normal human lung fibroblasts IMR-90 for 7 days. No cytotoxic effects on the IMR-90 cells were observed in the treatment with up to 5 μ M clozapine for 7 days. However, the growth of A549 cells was inhibited when using 3–5 μ M clozapine, and 2–5 μ M inhibited the growth of H1299 cells (Fig. 1A, lower panel).

Previous research showed that clozapine could induce apoptosis in neutrophils [25, 26]. Thus, we next investigated whether apoptosis contributes to the growth inhibitory effect of clozapine on NSCLC cells. Surprisingly, the annexin V/PI double-staining assay revealed no apoptosis in the clozapine-treated group and the control group for both A549 and H1299 cells (Fig. 1B). Moreover, Z-VAD-FMK, a pan-caspase inhibitor, did not improve clozapine-inhibited cell viability (data not shown). These findings suggest that the cytotoxic effects of clozapine on lung cancer cells are not mediated by apoptosis.

Clozapine induces cell cycle arrest and regulates the protein levels of the related cyclin-dependent kinase inhibitors (CDKIs) p21 and p27

We next evaluated the effect of clozapine on cell division using a flow cytometry analysis of CFSE-labeled cells [27]. After 72 h, clozapine-treated cells exhibited significantly stronger fluorescence compared with the control, indicating that the cell proliferation rate was decreased among the clozapine-treated cells compared with the controls (Fig. 2A). Therefore, we also examined the cell cycle distribution. After A549 and H1299 cells were treated with 50 μ M clozapine, both cell lines showed an increased proportion of the number of cells retained in the G0/G1 phase (Fig. 2B). Interestingly, both A549 and H1299 cells nearly reached the maximal level of G0/G1 cell cycle arrest following 24 h of clozapine exposure. Consistent with the G0/G1 arrest, the western blotting results showed that p21 and p27, two CDKIs that regulate the G1 phase, were upregulated in both cell lines (Fig. 2C). Taken together, these data suggest that clozapine increases p21 and p27 expression and may therefore induce cell cycle arrest.

Clozapine induces autophagic cell death in NSCLC cells

During clozapine exposure, we noticed that both the A549 and the H1299 cell lines displayed an enlarged, flattened morphology. Visualization by light microscopy also revealed a distinct accumulation of vacuolar structures (Fig. 3A). Thus, we hypothesized that clozapine could induce autophagy and tested this hypothesis. We first treated A549 and H1299

Fig. 1. The inhibitory effects of clozapine on the proliferation of NSCLC cells. (A) A549 and H1299 cells were treated with control or different concentrations of clozapine at the indicated times (upper panel) and for 7 days (middle panel). Lower doses of clozapine were used to treat the two NSCLC cell lines and the normal human lung fibroblasts IMR-90 for 7 days (lower panel). Cell viability was determined at the indicated times using a PrestoBlue assay. The data are represented as the mean $\pm$ SD of three independent experiments. * $P < 0.05$, ** $P < 0.01$ compared with the control. (B) Cells were treated with 50 μ M clozapine for the indicated times and stained with Alexa Fluor 488-labeled annexin V and PI, as described in the Materials and Methods section. Cells that underwent serum starvation for 72 h were used as the positive control. Q1: damaged during harvesting, Q2: late-stage apoptosis and necrosis, Q3: live, Q4: apoptotic.

cells with 50 μ M clozapine and monitored LC3 protein levels. LC3 is an autophagosome-associated protein that is used as an autophagy marker; it exists in two forms, LC3I and

Fig. 2. Clozapine induces cell cycle arrest and regulates the protein levels of the CDKIs p21 and p27. (A) A549 and H1299 cells were labeled with CFSE and cultured in the presence or absence of 50 μ M clozapine for 72 h. Fluorescence intensity was determined using flow cytometry analysis (upper) and plotted (lower). The data are expressed as the mean $\pm$ SD of three independent experiments. * $P < 0.05$, ** $P < 0.01$ compared with the control. (B) Cells were treated with 50 μ M clozapine for the indicated times before they were fixed, stained with PI, and subjected to an analysis of DNA content using flow cytometry, as described in the Materials and Methods section. Each data point represents the mean $\pm$ SD of three independent experiments. * $P < 0.05$, ** $P < 0.01$ compared with the control. (C) Following treatment with 50 μ M clozapine for the indicated times, whole-cell lysates were subjected to immunoblotting with anti-p21, anti-p27, and anti- β -actin antibodies.

LC3II. LC3I is localized in the cytoplasm when autophagy is not occurring, whereas when autophagy is activated, LC3I is processed and conjugated with phosphatidylethanolamine to form LC3II, which then localizes to the autophagosomal membrane. Our results showed that the LC3II protein levels increased in a time-dependent manner in the clozapine-treated A549 and H1299 cell lines (Fig. 3B). Furthermore, as shown in Fig. 3C, significant increases in autophagic activity were detected in the clozapine-treated A549 and H1299 cells after 24 h of clozapine treatment compared with the control cells. Because the increase in LC3-II levels may have been due to either increased autophagosome formation or a blockade of autophagosome maturation, we transfected H1299 cells with mRFP-EGFP-LC3 and observed the localization of the fluorescent protein to monitor whether there was an increase in autophagic influx or autophagolysosome fusion impairment. Because low pH quenches the fluorescent signal from GFP, but not mRFP, LC3 in autophagosomes should appear yellow (mRFP and GFP), whereas LC3 in autophagolysosomes (mRFP only) should appear red [28]. Our results showed that 24 h after the clozapine treatment, H1299 cells contained an increased number of both yellow and red LC3 punctae (Fig. 3D). Clearly, clozapine increased LC3II levels by increasing autophagic influx.

Fig. 3. Clozapine induces autophagy in NSCLC cells. (A) A549 and H1299 cells were treated with 50 μ M clozapine for 72 h before their cellular morphology was photographed under a phase-contrast microscope. The scale bar represents 20 μ m. (B) Cells were treated with 50 μ M clozapine for the indicated periods. Whole-cell lysates were then subjected to immunoblotting with anti-LC3B and anti- β actin antibodies. (C) A549 and H1299 cells were treated with 50 μ M clozapine for 24 h, and autophagic activity was assessed with the Cyto-ID Autophagy Detection Kit. The data are expressed as the mean $\pm$ SD of three independent experiments. ** $P < 0.01$ compared with the control group. (D) H1299 cells expressing ptfLC3 (mRFP-GFP-LC3) were treated with or without 50 μ M clozapine for 24 h and visualized using confocal microscopy. The scale bar represents 10 μ m.

Because autophagy can promote cell survival or induce cell death, we next investigated the role of clozapine-induced autophagy among lung cancer cells. To do so, we used bafilomycin A1, a vacuolar type H(+)-ATPase (V-ATPase) inhibitor that impairs the fusion of autophagosomes with lysosomes [29, 30], to inhibit autophagy. We then analyzed the cytotoxic effects of clozapine on A549 and H1299 cells. To compare the two cell types, we adjusted the clozapine concentration used to treat the H1299 cells to reach the IC_{50} dose so that it was consistent with the dose administered to the A549 cells. As shown in Figure 4A, pretreatment with bafilomycin A1 effectively decreased clozapine-induced autophagy, and the cytotoxic effects of clozapine were mitigated by bafilomycin A1 (Fig. 4C). To validate these results, we also measured the effects of the introduction of an siRNA targeting *atg7*, a key regulator during the elongation step of autophagy. Consistent with the results using bafilomycin A1, *atg7* knockdown also decreased the autophagic activity (Fig. 4B) and the cytotoxicity (Fig. 4D) induced by clozapine, although the level of reduction was modest. Taken together, these data indicate that autophagic cell death may partially mediate clozapine-induced cytotoxicity in lung cancer cells.

Discussion

As a class, antipsychotics exhibit an antiproliferative effect on cancer cells [11-15, 31]. However, the effect of clozapine on cancer cells is poorly understood. The results of the

Fig. 4. Clozapine induces autophagic cell death in NSCLC cells. (A) Autophagic activity after bafilomycin A1 treatment. A549 and H1299 cells were pretreated with vehicle or 5 nM bafilomycin A1 for 1 h and subsequently treated for 48 h with the IC_{50} dose of clozapine (50 μ M and 60 μ M for A549 and H1299 cells, respectively). (B) Autophagic activity after siRNA transfection. A549 and H1299 cells were transfected with scrambled or *atg7*-specific siRNA. At 48 h after transfection, the cells were treated with the IC_{50} dose of clozapine for 48 h. (C) & (D) Cell numbers in the bafilomycin A1 (C) and siRNA experiments (D), assessed using PrestoBlue. The data are expressed as the mean $\pm$ SD of three independent experiments. * $P < 0.05$; ** $P < 0.01$.

present study support our hypothesis that clozapine is able to inhibit lung cancer cell growth by reducing the rate of cell proliferation; by inducing cell cycle arrest in the G0/G1 phase; and, most importantly, by triggering autophagic cell death.

Studies of the effects of antipsychotics on lung cancer demonstrated that two antipsychotics, pimozide and olanzapine, were cytotoxic to NSCLC cells [14]. However, there are no related reports regarding the effects of pimozide and olanzapine on the cell cycle, apoptosis, and autophagy in NSCLC cells [14]. Recently, an autophagy assay system in SH-SY5Y neuroblastoma cells was built to screen 1120 chemicals and found that first-

generation antipsychotics (pimozide, triflupromazine, chlorpromazine, and fluphenazine) and second-generation antipsychotics (such as sertindole) could induce autophagy. It was also discovered that sertindole can cause autophagic cell death in SH-SY5Y neuroblastoma cells [32], which is consistent with our findings in NSCLC cells.

Our results showed that clozapine had a greater potency to inhibit cell growth in A549 cells than in H1299 cells. The reasons for the difference may be that H1299 is more malignant than A549 and that different tumor-related genotypes exist between A549 and H1299. Wiklund's study also suggested that H1299 cells which lacked p53 protein were more resistant to antipsychotics as compared to A549 cells that harbored the functional tumor suppressor protein p53 [14]. In our study, both yellow and red punctae were detected, which is different from the study of Park et al. in which only yellow punctae were observed [33]. Autophagy is a functional influx process. The presence of red punctae in our study indicates the increased formation of the autophagolysosome and, therefore, increased autophagic influx. Only yellow punctae were observed in the study of Park et al., without the presence of red punctae, suggesting blockage of the formation of autophagolysosomes and therefore blockage of autophagic influx. The major difference between the methods of Park et al. and ours is that they used rat primary neurons, while we used human non-small cell lung cancer cells. In addition to quiescent and terminal differentiation, neurons are different from other cell types with respect to autophagy. For example, the brain does not upregulate autophagy upon starvation [34]. Additionally, rapamycin and lithium chloride treatment, used to induce mTOR-dependent and -independent autophagy, respectively, do not increase LC3 levels in cultured primary neurons [35]. Clozapine is recognized as an effective antipsychotic in treating refractory schizophrenia, and has a complex pharmacology linked to 5-HT_{2A/2C}, dopaminergic receptors, histamine receptors or muscarinic acetylcholine receptor. So far there is no evidence of clozapine-induced autophagic cell death in NSCLC cells being mediated by these various receptors. However, in prostate cancer cells, one possible molecular mechanism for clozapine-induced autophagic cell death could be inhibition of the PI3K/Akt/mTOR pathway (our unpublished data).

Historically, autophagy has been regarded as promoting survival by recycling basic building blocks, such as amino acids and lipids, to allow cell vitality under nutrient-restricted conditions. Currently, the involvement of autophagy in the processes of cell death and cell survival is also known to be important in therapeutic responses [36, 37]. Additionally, accumulating evidence has shown that a number of anticancer agents induce autophagy, such as tamoxifen; rapamycin; temozolomide; histone deacetylase inhibitors; and DNA-damaging agents, including camptothecin, etoposide, and ionizing radiation [38]. Thus, autophagic cell death can be exploited as another attractive therapeutic strategy. Regarding our results, first, clozapine exclusively induces autophagic cell death, without the involvement of apoptosis. Second, clozapine causes an increase in autophagic influx, not only an increase in levels of the autophagic marker LC3II, in the dying cells. Third, both the pharmacological inhibitor bafilomycin A1 and the knockdown of Atg7 using a specific siRNA can significantly reduce cell death. Taken together, the results suggest that clozapine-induced autophagy in NSCLC cells meets the new definition of autophagic cell death [39].

Although we have provided compelling evidence documenting that clozapine can induce autophagic cell death and inhibit cell proliferation, several topics warrant further investigation. First, further studies are required to suppress autophagy more effectively and investigate whether other mechanisms are involved in clozapine-induced cancer cell death. Techniques like transmission electron microscopy or molecular markers of RIPK1 and RIPK3, for example, can be applied to examine whether necrosis or necroptosis is involved in clozapine-induced cell death in NSCLC cells. Second, these results make us question whether other antipsychotics exert similar tumor suppressive effects. Additionally, it is critical to investigate whether clozapine also induces autophagic cell death in other cancer cell types. Third, because autophagy can regulate the cell cycle [40, 41] and because we have demonstrated that clozapine arrests the cell cycle and induces autophagic cell death, we will next elucidate whether clozapine-induced autophagy also mediates cell cycle arrest in

NSCLC cells. Finally, in this study, we assessed the short-term effect of clozapine (over 2 to 3 days), but not the long-term effect. Although the dose of clozapine that we used was rational and has been implemented in many studies [22, 42, 43], we also demonstrated that even at a lower concentration, clozapine continued to impair NSCLC cell growth during treatment for a longer period. Not only is this finding consistent with the clinical observation that patients with schizophrenia require prolonged antipsychotic administration [24], but the dose that we used for long-term treatment was also closer to the actual dose used in the clinic [44, 45]. Therefore, it will be important to further elucidate the role of autophagy in long-term treatment with clozapine. Most importantly, it remains unknown whether the observed results can be applied clinically. Thus, further investigations, such as animal studies and epidemiologic studies, should be performed to better understand the link between clozapine and cancer inhibition, and particularly lung cancer inhibition.

The results of this study provide novel cellular evidence supporting the anticancer effects of antipsychotics and may partially explain the clinical finding that patients with schizophrenia have a lower than expected risk of lung cancer.

Disclosure Statement

The authors have none to declare.

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