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Research Paper

Studies on cytotoxicity and antioxidant activities of 4-chloroacetylphenylacetic and 4-chlorophenoxyacetic acids

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Abstract

Inhibition of cell proliferation activity of acetic acid derivatives - 4-chloroacetylphenylacetic acid and 4-chlorophenoxyacetic acid using MTT, neutral red and LDH assays were assessed. 4-Cl-acetylphenylacetic acidexhibited a high cytotoxic activity against HeLa and HEp-2 cancer cells with IC_{50} value of 5.3 and 1.0 µg/ml respectively. Also these substances were studied for their antioxidant activities by changes in activity of GST, GR, SOD, MDA. It was found that these compounds do not exhibit antioxidant properties, as the increase of MDA and GR enzyme - markers of cellular damage.

Keywords: acetic acid, cancer cells, cytotoxicity, antioxidant activity

Introduction

Experimental and Clinical Oncology has a wide range of cancerolytics and anticancer drugs for the treatment of cancer[1]. Although many more drugs have been introduced into the market the response to therapy is still insufficient. Cytotoxic *medicines* are *toxic* to cancer *cells.* They kill cancer *cells*, but at the same time, they kill *healthy cells* (e.g. LD_{50} for cisplatin and carboplatin is 12.5 and 36 mg/kg, respectively)[2]. Therefore, there is an urgent need for new efficient anticancer drugs to be developed with high selectivity of action against cancer cells and low toxicity to normal cells.

In recent years, phenylacetic acid and its salts with promising activity against tumor cell lines have been synthesized and published[3]. Researchers have reported that complete colony reduction of the tumor cell lines HBL-100 and Ki No 1 was under the influence of 5.0 mg/ml of phenylacetic acid. By using 3-phenylacetylamino-2,6-piperidinedione at a concentration of 2.0 mg/ml authors observed the human tumor cytostas in breast carcinoma cell line MDA-MB-231[3]. Importantly, a good relationship was observed between the cytotoxic activity of the phenylacetic acid and their solubility. Phenylacetic acid (PA) is industrially used as a side chain precursor for the production of benzylpenicillin (penicillin G) by submerged fermentation of *Penicillium chrysogenum*[4].

A number of phenylacetic acid derivatives are found in nature. The culture filtrates from a submerged cultivation of *Penicillium chrysogenum* were found to contain 2-hydroxyphenylacetic acid. Later as a natural phenolic product 2-hydroxyphenylacetic acid was found in the genus *Astilbe* (Saxifragaceae), which derives from the shikimic acid pathway *via* phenylpyruvic acid[5]. Two distinct fractions of 4-hydroxyphenylacetic acid derivatives of inositol were isolated and characterised from an ethyl acetate fraction of *Taraxacum officinale* root[6]. 4-Hydroxyphenylacetic acid was identified from *Taraxacum officinale, Mellilotus officinalis* and yeast. 3,4-Dihydroxyphenylacetic acid is a metabolite of the neurotransmitter dopamine. It can also be found in the bark of *Eucalyptus globules*[7]. A novel mixed

anhydride based on dichloroacetic and aminoacetic acid which, for instance, enables to inhibit growth of carcinoma 755 (breast cancer) in monotherapy[8]. Also there are number of phenylacetic acid derivatives, which are offered for the treatment of neoplasia, formed from epithelial cells[9]. The aim of this study is to investigate the cytotoxic activity of 4-Cl- acetylphenylacetic acid and 4-Cl-phenoxyacetic acid, in particular the cytotoxicity in cancerous and normal non-transformed cells and antioxidant activity of these compounds.

Materials and Methods

Establishment of primary human fibroblast cultures

To establish primary cultures of human fibroblasts (PCF) the explants of human skin were used. The explants were placed in Petri dishes (Falcon, UK), dermis down in growth medium DMEM/F-12 (Invitrogen, UK) containing 10% fetal bovine serum FBS (SIGMA, USA) and 1% antibiotic-antimycotic solution (SIGMA, USA) and incubated at 37°C, 5% CO_2 and 100% humidity. Growth and migration of fibroblasts occurred during the week, and monolayer formed after 7-10 days, then subjected to passage of cells - subcultivation[3].

Cultivation of HeLa, CCRF-CEM, KML, HBL-100, and HEp-2 cells

To determine the cytotoxicity of obtained compounds the cultures of verified cell lines were used: laryngeal cells HEp-2 (collection number ATCC CCL23, ECACC 86-30501), breast cancer cells HBL-100 (collection number ATCC HTB 124), cervical carcinoma cells HeLa and T lymphoblastic leukemia CCRF-CEM (CCL-119, T-cell line, human ALL) were received from the Bank of Cell Cultures of the Institute of Cytology of Russia, the culture of normal skin cells - primary culture of fibroblasts (PCF)was obtained from the dermis of rats, both adult animals and embryos[10] and mouse melanoma cell primary culture (KML) was obtained in the Institute of Bioorganic Chemistry, Academy of Sciences of the Republic of Uzbekistan. Cells were grown in RPMI-1640 (SIGMA, USA) medium containing: 2 mM L-glutamine, 1% antibiotic-antimycotic solution, 10% FBS (SIGMA, USA) and incubated in CO_2 incubator (SHEL LAB, USA) at 37°C, 5% CO_2 and 100% humidity. For sub culturing cells were detached with Versen solution[2].

MTT-assay

For MTT assay the cells were seeded into 96-well plates (Costar, USA) in culture medium at the density of 2×10^3 per well and incubated for 24 hrs test and reference substances. After 24 hours, medium in each well was replaced with fresh medium containing 0.5 mg/ml MTT. After 3 hours incubation, the medium with MTT was discarded, and the wells were added 100 µl of DMSO per well (dimethyl sulfoxide), and after another 10 minutes absorbance was determined at 610nm. Proliferation index was calculated (PI) (in percentage %) using the following formula:

PI = (O-S/K-S) × 100, where O - optical density of the sample, K - optical density of control, S - absorbance of serum-free culture medium (RPMI-1640)[11]. Intact cells with solventin culture medium served as control. The effect was compared to antineoplastic drug «Ciplatin» (Fresenius Kabi, India) with cis-diammine dichloroplatinum as active ingredient and bioflavonoid dihydroquercetin (DHQ). Negative control was whole intact cells. The results are generated from three independent experiments each experiment was performed in triplicate.

Neutral red assay

For the assay, fibroblasts were seeded on 96 well plate at the density of 3×10^4 cells per well. Following 24-h incubation and attachment the growth medium was replaced with the medium containing a certain amount of Cisplatine (Pharmachemie, Netherlands) as a control and incubated in CO_2 incubator for 72 hours. Next, the plates were washed twice with HBSS (Hank's solution) and incubated in a freshly prepared solution of neutral red in DMEM medium (3.3 µg/ml) for 3 hours. After discarding the supernatant and washing off the neutral red dissolved in acidic ethanol with shaking for 45 minutes absorption was measured at 540 nm. The cell viability was determined as the ratio (in percentage) from the absorption of intact cells. All experiments were performed in triplicate at each concentration[2].

LDH test

We used LDH-Cytotoxicity Assay Kit (BioVision, USA)[10]. HBL-100 cells were seeded in 96-well plates at the density of 2×10^4 cells/well in 100 µl of complete growth medium/well and incubated overnight

in a humidified atmosphere at 37°C and 5% CO_2. Next, the medium was discarded and fresh medium containing the substances was added and incubated for 24 hours. Then 100µl of transferred cell medium was incubated with 50 µl NADH (25 mg/ml) and natrium sodium pyruvate in phosphate solution (1mg/ml) at 30°C for 6 min. The absorbance can be measured at 490 nm.

Determination of activity of glutathione-S-transferase (GST)

Principle of the method: activity of glutathione-S-transferase was determined from the rate of formation of glutathione-S-conjugates between glutathione (GSH) and 1-chloro-2,4-dinitrobenzene (CDNB). Increasing of the concentration of conjugates in the reaction was recorded spectrophotometrically at 340 nm (absorption maximum of glutathione-S- CDNB). As source of enzyme was homogenate of rat liver. In a cuvette with an optical path length of 1.0 cm containing 2.5 ml of 0.1 M potassium phosphate buffer pH 6.5 were added 0.2 ml of 0.015 M solution of reduced glutathione and 0.1 ml of the homogenate. The reaction was initiated by the introduction of the cuvette 0.2 ml 0.015M CDNB (prepared in 80% methanol). Parallel experimental test sample was prepared with adding of distilled H_2O instead of homogenate. Registration of absorbance was performed at t = 25°C and wavelength of 340 nm against water after stirring for three minutes. The enzyme activity was calculated using an extinction coefficient for the GS- CDNB at wavelength 340 nm of 9.6 $mM^{-1} \times cm^{-1}$ and expressed in mmoles for formed glutathione S-conjugates per minute per mg protein. Control was model system without tested substances.

GST activity was calculated by the following formula:

$$E = \frac{\text{(D beginning – D finishing)/ t (min) x volume of reaction mixture x sample dilution}}{\text{Molar extinction x sample volume x contain of protein in the sample x optically path length}}$$

Determination of glutathione reductase activity

Principle of the method: determining the activity of glutathione reductase is based on measuring the rate of oxidation of NADPH, which is recorded spectrophotometrically by a decrease in absorbance at 340 nm. To determine the activity of glutathione reductase used osmotic homogenate.

In a spectrophotometric cuvette with the distance between the working faces 10 mm successively introduced 2.7 ml of potassium phosphate buffer, 0.1 ml NADPH, 0.1 ml of the homogenate and 0.1 ml of GSSG (oxidized glutathione). The reaction is started by the addition of oxidized glutathione in the sample. The mixture was stirred. Changes in absorbance were recorded after 1 minute for 3 minutes against a sample containing all components except GSSG. Control was model system without tested substances.

Enzyme activity was expressed in µM×mg protein per minute. Calculations are made using the formula:

$$E = \frac{\text{(D beginning-D finishing)/ (t/60)} \times \text{volume of solution} \times \text{dilution of the sample}}{\text{molar extinction / contain of protein in the sample}}$$

Determination of superoxide dismutase

Method proposed by Ewinga and Janero[12] and based on the reaction of O_2 with NBT which appear as a result of dyed products. O_2 is formed in the nonenzymatic reduction reaction of phenazine methasulfate NADH, which acts as an electron donor. NBT has yellow color, and in the recovery process turns into a dark blue product. Control sample containes1875µl EDTA 10mM, 10µl NBT 93 mM, 10µl NADH 147 mM, and 1460 µl PBS buffer and 1µl PMS. And is measured at λ = 540nmfor 10 min every 30 sec.

Experimental sample containes1875 µl EDTA 10 mM, 10µl NBT 93 mM, 10µl NADH 147 mM, 1450µl PBS. Then 10µl of testing sample incubate for 5 minutes and then added 1µl PMS 4.95 mM and measure the absorbance at λ = 540 nm 10 min against PBS.

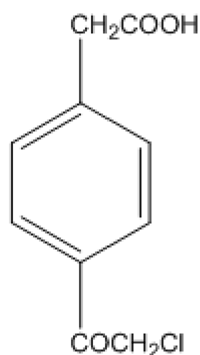
E = (100 – (D tested x100) / D control))/ 50 x contain of protein in the sample. The activity of SOD was expressed in y.e. /mg of protein.

Induction of lipid peroxidation
Induction of non enzymatic Fe^{2+} / ascorbate - dependent lipid peroxidation was carried out by addition of 10^{-5} M $FeSO_4$ and $2×10^{-4}$ M ascorbate into incubation medium containing 125 mM KCl, 10 mM Tris-HCl, pH = 75 and suspense of mitochondria in ratio of 0.5 mg protein per 1 ml of incubation medium. In the case of liposomes the lipid content was 2 mg per ml of medium. Incubation was carried out at 37°C in water bath with constant stirring. The reaction with mitochondria was quenched with adding of 150 µl of 70% trichloroacetic acid (3.5%), and liposomes – 15 µl of 10 mM EDTA (50 µM). The incubation mixture was centrifuged and the supernatant was examined for MDA content.

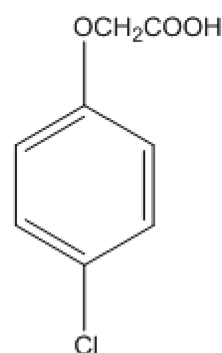
Determination of the amount of malondialdehyde (MDA) in the reaction with TBA (thiobarbituric acid)
Method is based on a color reaction between MDA and TBA that occurs at high temperature and acidic pH with formation of a complex containing one molecule of MDA and two molecules of TBA. 1 ml of 0.7% TBA (TBA 70 mg dissolved in 5 ml of distilled water under heating and after cooling, was added 5 ml of glacial acetic acid (CH_3CHOOH))was added to 2 ml of the supernatant. Samples were heated for 15 min in a boiling water bath. After cooling, the sample volume was to 3 ml and measured on colorimeter KFK-2MP at 540 nm. The quantity of MDA was determined using a molar extinction coefficient value equal to $1.56×10^5 M^{-1}$ $cm^{-1.}$The concentration of MDA was expressed in µM MDA/mg of protein or lipid. Control was model system without tested substances.

Statistical data analysis
Data were expressed as mean ± standard deviation (SD). Statistical analysis was performed by one-way analysis of variance (ANOVA).

Results and Discussion



4-Cl- acetylphenylacetic acid



4-Cl- phenoxyacetic acid

Cytotoxic activity
Table 1 shows the results of cytotoxic activity of 4-Cl- acetylphenylacetic acid and 4-Cl- phenoxyacetic acid on five cell lines

Table 1: Cytotoxic activity of derivatives of phenoxy- and phenylacetic acid on cancer and normal cells (MTT and neutral red assays)

Cells	IC_{50} µg/ml		
	4-Cl-acetylphenylacetic acid	4-Cl-phenoxyacetic acid	Control
PCF	20.0±1.2	90.0±3.4	5.0±0.5
HEp-2	1.0±0.1	10.0±3.2	4.5±1.0
HeLa	5.3±0.9	> 100	3.0±1.4
CCRF-CEM	55.0±11.5	> 100	6.0±2.2
KML	> 100	> 100	7.5±1.9

Note: significant difference from control P <0.05

From the table 1 it is shown that phenyl- and phenoxyacetic acid derivatives in comparison with anticancer drug "Cisplatin" showed a selective activity inhibited the growth of cancer cells of larynx

and didnot inhibit the growth of hormone-dependent cancer cells of the cervix, were low toxic to normal fibroblast cells, which is very important for substances having antitumor activity.
In our studies phenylacetic acid derivative - 4-Cl- acetylphenylacetic acid showed cytotoxic activity at a very low concentrations (1 mg/ml), inhibited the growth of cancer cells of larynx more than 50%, with low cytotoxicity to normal cells - fibroblasts. It is known that several derivatives of phenylacetic acid inhibit the cyclooxygenase-1 and 2. Therefore, they are particularly suitable for the treatment of cyclooxygenase-2 dependent disorders, including inflammation, pain of various etiologies, cancers (such as cancer of the digestive tract, such as colorectal cancer and melanoma) and others, with the exception of unwanted ulceration of the gastrointestinal tract (GIT), which manifests itself when using conventional cyclooxygenase inhibitors[13]. In connection with these data, our studies suggest that phenyl and phenoxyacetic acid derivatives inhibit laryngeal cancer related to gastrointestinal tract cancer and have a low cytotoxicity to normal cells.

The biological activity of phenoxyacetic acid derivatives investigated worldwide. Comparatively recently it has been shown that adenosine, which accumulates in large amounts near the tumor tissue, by binding to adenosine receptors of A_2d located on the cell membrane of lymphocyte adenosine transmits a signal into cells by activating intracellular accumulation of cyclic adenosine monophosphate (cAMP), which in turn reduces ability of lymphocytes to attack the tumor tissue and kill the cancer cells[14]. Based on these observations, there is provided an innovative approach to enhance the efficiency of vaccines using anticancer adjuvant A_2d an inhibitor of adenosine receptor, and as active compounds in these adjuvants used substituted phenoxyacetic acid, their esters and amides[15]. As seen from Table 1, T-lymphoblastic leukemia growth inhibition of 50% by 4-Cl-acetylphenylacetic acid at the concentration of 43.5-66.5 µg/ml was observed. The inhibition of growth of mouse melanoma cells in the studied concentrations was not observed.

In our studies, the compound-4-Cl-phenoxyacetic acid showed the highest inhibiting activity of cancer cells and in particular the larynx cancer cells, compared with the compound of 4-Cl-acetylphenylacetic acid. The derivative of 4-Cl-phenoxyacetic acid stably suppress cancer cells in the investigated concentration and in concentration of 10 and 1 µg/ml had very low cytotoxicity to normal cell cultures, in particular cell cultures of fibroblasts. Activity of the compounds at the low and very low concentrations is important for medicine in chemotherapy of patients, and for the preparation of pharmaceutical drugs.

The presence of cytosolic enzyme lactate dehydrogenase (LDH in the incubation supernatant was found. LDH is a marker of cell membrane integrity disorders (Table 2).

Table 2: Inhibition of breast cancer cells (LDH-assay)

Substances	LDH activity on cells HBL-100 mM/L* sec		
	100 µg/ml	10 µg/ml	1 µg/ml
4-Cl- acetylphenylacetic acid	0.197±0.05	0.199±0.06	0.205±0.05
4-Cl- phenoxyacetic acid	0.194±0.09	0.209±0.07	0.232±0.07
Cisplatin	0.236±0.07	0.221±0.06	0.207±0.06

As shown in Table 2 the acetic acid derivatives at low concentrations of 1 -10 µg/ml, and especially 4-Cl-phenoxyacetic acid at the concentration of 1 µg/ml resulting in leakage of LDH on the breast cancer cells.

Antioxidant activity

We have investigated anantioxidant activity of these compounds. In particular changes of activity of as malondialdehyde (MDA), glutathione-S-transferase (GST), glutathione reductase (GR) and superoxide dismutase (SOD) were observed.

Malondialdehyde is one of indicators of lipid peroxidation, it is a cleavage product of fatty acids. The aldehyde forms a Schiff base with amino groups of proteins, resulting in formation of insoluble complexes of lipid-proteins. MDA is also able to react with DNA to form DNA-adducts. On the rate of formation of malondialdehyde it is possible to define the damage degree of cell structures (Table 3).

As a comparison preparation we used a flavanoid - quercetin. At dose of 100 µg/ml this compound reduces the content of MDA by 55% compared to the control (pure lipid peroxidation). Reduction of the concentration of the substance entails a small decrease in activity - 46%. The mechanism of action of quercetin is based on utilization of free radicals and binding transition metals responsible for activation of free radical processes. High concentration of 4-Cl- acetylphenylacetic acid (100 µg/ml) increased the content of malondialdehyde to 2.5 times comparedtothe control, but at the lower concentrations of active compound, its prooxidant activity was replaced by a low antioxidant activity. Derivative of 4-Cl-phenoxyacetic acid in the represented doses did not affect the growth of malondialdehyde.

Table 3: The content of malondialdehyde (MDA), µmol/mg in mouse liver homogenate

Substances	MDA content, µM/mg	
	100 µg/ml	10 µg/ml
4-Cl- acetylphenylacetic acid	91±1.5	26.4±0.1
4-Cl- phenoxyaceticacid	38.4±0.6	32.8±0.8
Quercetin	22.6±0.4	22.6±0.6
Ionol	22.4±0.8	23.2±0.9
Control	50.2±2.0	

Thus, 4-Cl- acetylphenylacetic acid and 4-Cl- phenoxyacetic acid exhibit weak antioxidant properties, probably by activating theenzyme glutathione peroxidase, responsible for utilization of peroxide in cells or mechanism of their protective action based on direct utilization of free radicals.

Glutathione reductase - an enzyme catalyzing the reduction reaction of oxidized glutathione and most active found in red blood cells - cells that are constantly exposed to oxidative stress. It also present in liver, heart, kidney.

The activity of glutathione reductase depends on the concentration of reduced glutathione and NADPH levels in the cell. The content of glutathione reductase is determined by the activity of pentose phosphate pathway, and the activity of certain enzymes, providing an alternative production of NADPH.

As a model system for this experiment we used a rat liver homogenate. As control served a model system under the influence of inducers without the addition of test substances. As a standard of comparison was natural antioxidant - quercetin (Table 4).

Table 4: Activity of glutathione reductase, µM/min in mouse liver homogenate

Substances	Activity of glutathione reductase, µM/min	
	100 µg/ml	10 µg/ml
n-Cl- acetylphenylacetic acid	0.132±0.01	0.013±0.00
n-Cl- phenoxyaceticacid	0.125±0.08	0.112±0.07
Quercetin	0.027±0.02	0.023±0.01
Ionol	0.014±0.00	0.043±0.06
Control	0,026±0.03	

As seen from Table 4, quercetin at the concentration of 100 µg/ml did not affect the enzyme activity - 3.8% compared to the control. By reducing the concentration of quercetintoto10 µg/ml the activity of glutathione reductase was slightly reduced – to 11.5% and was 0.023 µM/min. As it is known from the literature, the mechanism of antioxidant action of quercetin is conditioned to the formation of inactive complexes with iron ions, as well as its direct interaction with free radicals, in this connection we chose en it for comparison. However, as the results of our study, antioxidant activity of quercetin does not apply to the activation of glutathione reductase.

4-Cl- acetylphenylacetic acid at a dose of 100 µg/ml increases the activity of glutathione reductase 5 times compared to the controls and is 0.132 µM/min. Reducing the concentration to 10 µg/ml decreased the activity of enzyme sharply - increase of only 50% compared to the control.

Other derivative - 4-Cl-phenoxyacetic acid at a high concentration (100 μg/ml) increased the enzyme activity 4.8 times. By reducing the concentration of the substance a slight reduction of the enzyme activity of 50% compared to the high concentration was observed.
These results demonstrate an excessive increase of glutathione under the influence of these compounds. This could be caused by a sharp decrease of reduced glutathione, or sharp decrease of NADPH in the cell. It is possible that these compounds have a damaging effect on the enzymes providing NADPH production or reduce the activity of pentose phosphate pathway.

The effect of the compounds at the concentrations of 100 μg/ml and 10 μg/ml was on enzyme activity of glutathione-S-transferase (GST) and superoxide dismutase (SOD). GST is one of the antioxidant enzymes, which utilizes xenobiotics in cells. SOD is an enzyme of antioxidant defense, which plays an important role in protecting cells from the action of superoxide anion radical, stabilizing the cell membranes, preventing LPO (lipid peroxidation), and reducing the level of O^{2-}. Superoxide dismutase protects catalase and glutathione peroxidase against deactivating action. A rat liver homogenate under the influence of inducers without test substances was used as a negative control. As reference preparations we used ionol and quercetin in concentrations of 100 μg/ml and 10 μg/ml respectively.

In controls the activity of enzymes was observed. At aconcentration of 100 μg/ml quercetin showed SOD enzyme activity of 3.6% compared to control, however, the lowest concentration of quercetin at(10 μg/ml) did not contribute to decrease of superoxide dismutase. Our results for quercetin correlated with published data. At these concentrations 4-Cl-acetylphenylacetic acid and 4-Cl-phenoxyacetic acid did not affect the activity of SOD and GST enzymes.

Thus, 4-Cl-acetylphenylacetic acid and 4-Cl-phenoxyacetic acid inhibit cells proliferation at a very low concentrations of 1 μg/ml, and particularly the cells of laryngeal cancer (HEp-2) (the most active is 4-Cl-phenoxyacetic acid), while these compounds are low- or not cytotoxic to normal cells. Decreasing the concentrations of these derivatives increased their negative influence on the membrane, particularly at the concentration of 1 μg/ml, a sharp increase in LDH release was observed. The 4-Cl-acetylphenylacetic and 4-Cl-phenoxyacetic acids exhibit weak antioxidant properties, probably by activating of the enzymes of catalase and glutathione peroxidase, responsible for the utilization of peroxide in the cells or mechanism of their protective action based on direct l utilization of free radicals.

In addition, all compounds showed an excessive increase of glutathione. This can be caused by sharp reduction of reduced glutathione or sharp decrease of NADPH in cells. Possibly, these compounds have a damaging effect on the enzymes providing of NADPH production or reduce the activity of pentose phosphate pathway.

Conclusion

4-Cl-acetylphenylacetic acid and 4-Cl-phenoxyacetic acid have a selective antitumor activity; in particular they significantly inhibit proliferation of larynx cancer cells, and demonstrate a weak inhibiting activity of cervical cancer, T lymphoblastic leukemia cancer and normal cells which is very important in antitumor chemotherapy of gastrointestinal diseases. As for the antioxidant activity, these compounds did not exhibit antioxidant properties, as the increase MDA and GR enzyme - markers of cellular damage.

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Conflict of interests

The authors declare no conflict of interest.

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