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In vitro and in vivo Anticancer Effects of a Novel 9-Phenyldibenzo[a,c]phenazin-9-ium Cation and Its Ligands

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Key Words
Anticancer effects · Apoptosis · Doxorubicin · Drug resistance · Ehrlich ascites carcinoma

Abstract
Background: Multidrug resistance (MDR) is a major problem in cancer treatment. Cu complexes possess the ability to overcome MDR in cancer. Therefore, the search for new Cu complexes is of great clinical significance and we address the anticancer effects of a previously synthesized novel 9-phenyldibenzo[a,c]phenazin-9-ium cation [1^+] as [1] [$CuCl_2$] and as [1] [I]. ***Methods:*** The existence of the monovalent Cu(I) in [1] [$CuCl_2$] was proven by electron paramagnetic resonance (EPR) studies and in vivo anticancer effects were studied in animals. ***Results:*** The monovalent nature of the Cu ion in [1] [$CuCl_2$] was determined through EPR. The mean survival time of mice bearing doxorubicin-resistant Ehrlich ascites carcinoma cells is longer when [1] [I] is injected intraperitoneally whereas [1] [$CuCl_2$] does not significantly increase the median survival in tumor-bearing mice. Compounds do not follow the immunomodulatory route and only [1] [I] shows cytotoxic activity in both MDR and drug-sensitive leukemia cell lines. ***Conclusion:*** An organic iodide complex rather than a cupric complex possesses direct cytotoxic potential.

Introduction

Multidrug resistance (MDR) remains a severe barrier in successful cancer treatment [1]. It may occur intrinsically without prior exposure to chemotherapy in some cancers, or it may be acquired when cancer cells become insensitive to treatment upon relapse [2]. Numerous attempts have been made to overcome MDR, and a number of MDR inhibitors have been identified and some are undergoing clinical trials [2–4], but the problem of MDR remains elusive.

A growing body of evidence disclosed that Cu chelates can overcome MDR through complex mechanisms [5–7]. Under biological conditions, copper exists in both (Cu^+) and (Cu^{2+}), which allows it to serve as a cofactor in redox reactions, such as cytochrome c oxidase (involved in the mitochondrial electron transport chain) and superoxide dismutase (involved in the detoxification of reactive oxygen species) [8, 9]. In physiological conditions, copper changes from a bivalent to a monovalent state and causes redox alterations. So far, bivalent Cu complexes were employed in the field of cancer. The role of monovalent Cu complexes has not yet been reported.

Previously, we synthesized and reported a novel 9-phenyldibenzo[a,c]phenazin-9-ium cation [1^+] as [1] [$CuCl_2$] and as [1] [I], and the 3D structure was also evalu-

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ated by us [10]. The existence of the monovalent Cu(I) in [1] [$CuCl_2$] was also ascertained. Herein, we further revealed the monovalent nature of Cu(I) in [1] [$CuCl_2$] through electron paramagnetic resonance (EPR) and also studied the effect of [1] [$CuCl_2$] and [1] [I] in a drug-resistant cancer model.

Materials and Methods

Electron Paramagnetic Resonance

The X-band EPR spectra at 298 K were measured on a Magnettech GmbH MiniScope MS 400 spectrometer (equipped with the temperature controller TC H03), where the microwave frequency was measured with an FC400 frequency counter.

Cytotoxicity Assay (MTT Assay)

Cell viability was determined by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay, which was carried out as described previously [11]. Briefly, cells were seeded in 96-well plates at a density 4×10^4 cells per well. For single-agent studies, cells were seeded and allowed to settle for 24 h before treatment with increasing drug concentrations and incubated for further 72 h with 5% CO_2 at 37°C. After completion of incubation, cells were incubated with MTT dye (5 mg/ml; Sigma, France) for 4 h at 37°C. The monolayer was suspended in 0.1 ml of DMSO and absorbance at 540 nm was read using an ELISA reader (Tecan 200). The control value corresponding to untreated cells was taken as 100% and the viability of treated samples was expressed as a percentage of the control. The IC_{50} values were determined as the concentration that reduced cell viability by 50%. The data generated were from three separate experiments, each performed in duplicate.

Animals

Swiss albino mice, originally obtained from the National Institute of Nutrition, Hyderabad, India, and reared in the animal facilities of the Institute, were used for all in vivo experiments with prior approval of the Institutional Animal Ethics Committee. The experimental protocols described herein were approved by the Committee (Registration No. 175/99/CPCSEA, dated 28/01/2000) in accordance with the ethical guidelines laid down by the Committee for the purpose of control and supervision of experiments on animals (CPCSEA) by the Ministry of Social Justice and Empowerment, Government of India. Adult male Swiss albino mice weighing 18–20 g were kept for a quarantine period of 1 week at a temperature of 25 ± 2°C, a relative humidity of 55 ± 2% and on a 12-hour light/12-hour dark photo cycle. Water and food pellets were provided ad libitum.

Cell Line, Tumor Implantation and Experimental Protocol

Ehrlich ascites carcinoma (EAC) cells were maintained as an ascitic tumor in male Swiss albino mice. A doxorubicin (Dox)-resistant subline was developed by sequential transfer of Dox-treated EAC cells to the subsequent generation of host mice with continuous Dox treatment [12–14]. Briefly, the treatment regime consisted of 2 mg/kg i.p. Dox per week. The daily treatment dose was 0.4 mg/kg for 5 days. Dox was started 24 h after intraperitoneal inoculation of 10^6 EAC cells into mice. Five groups (each group containing 6 mice) of animals were taken for animal survival studies. Each mouse was inoculated with 10^6 Dox-resistant EAC (EAC/Dox) cells intraperitoneally, and after 10 days either 15 or 20 mg/kg of body weight of either [1] [$CuCl_2$] or [1] [I] were injected intraperitoneally into EAC/Dox-bearing mice of appropriate groups. To check the infiltration of myeloid-derived suppressor cells (MDSCs) and IFN-γ positive CD4, CD8 T cells at the tumor site, EAC/Dox-bearing mice (9 days following peritoneal inoculation with 1×10^6 EAC/Dox cells) were kept either untreated or treated with a single dose of I complex (20 mg/kg of body weight) for 15 days.

Cell Culture

The human T-cell acute lymphoblastic CEM/ADR5000 leukemia cell lines [11, 12] were maintained in RPMI medium (GIBCO Invitrogen Corp., Carlsbad, Calif., USA) supplemented with 10% fetal bovine serum, additional glutamine (0.15%), HEPES (25 mM), 66.67 mg/l penicillin and 100 mg/l streptomycin. Cells were grown in plastic tissue culture flasks in a 5% CO_2 atmosphere at 37°C. Cells were passaged twice weekly. The Dox-resistant CEM/ADR5000 cell line was generated by treating CCRF-CEM cells with Dox doses up to a final concentration of 5,000 ng/ml [15]. These cell lines were kindly provided by Prof. Thomas Efferth, University of Mainz, Germany. The CEM/ADR5000 specifically overexpresses P-glycoprotein without concomitant overexpression of MRP1 or BCRP [16, 17]. Cells from exponentially growing cultures were used for all experiments. All experiments were repeated three times.

Results

Detection of the Cu(I) Ion by EPR Spectroscopy

The peaks in the EPR spectrum (fig. 1) in the compounds, viz $CuCl_2$, [1] [I] and [1] [$CuCl_2$], disclose the divalent nature of $CuCl_2$. As no EPR signal appears in case of [1] [I] or [1] [$CuCl_2$], we understand the presence of the Cu(I) ion in the reported drugs [1] [I]/[1] [$CuCl_2$].

In vivo Antiproliferative Effect of [1] [$CuCl_2$] and [1] [I] in Swiss Albino Mice Bearing EAC/Dox

Swiss albino mice were inoculated intraperitoneally with 1×10^6 cells of EAC/Dox and a considerable amount of tumor developed 10 days after inoculation. The animals were treated with either [1] [$CuCl_2$] or [1] [I] at a dose of 15 or 20 mg/kg body weight, and animals were monitored for any discomfort or pain. Table 1 shows that both [1] [I] doses significantly enhanced the survival of mice compared to the untreated control. However, 15 mg/kg [1] [$CuCl_2$] moderately increased the survivability of mice, while 20 mg/kg [1] [$CuCl_2$] failed to enhance the lifetime of mice.

Effects of [1] [I] on Tumor-Infiltrating Lymphocytes and MDSC

Protective antitumor immunity largely depends on the activity of effector T-cell responses and MDSC, which are potent immune suppressors; to evade host immunosur-

Table 1. Effect of [1] [I] and [1] [$CuCl_2$] on drug-resistant tumor-bearing female Swiss mice

EAC/Dox groups	Drug concentration, mg/kg	Animals, n	Mean survival time, days	T/C value, %
Untreated control	–	12	22±1.3	100
[1] [I] treated	15	12	25±1.2$^{n.s.}$	113
[1] [I] treated	20	12	28±1.4**	127
[1] [$CuCl_2$] treated	15	12	24±1.1$^{n.s.}$	109
[1] [$CuCl_2$] treated	20	12	18±0.9*	81

T/C = Test/control; n.s. = nonsignificant; * $p < 0.05$; ** $p < 0.01$.

veillance, T-cell function is suppressed at the tumor site. Therefore, to study the effect of [1] [I] as an immunomodulator, we preliminary targeted these populations, and the 20 mg/kg body weight dose was chosen as it resulted in significantly increased survival of EAC/Dox-bearing mice. The data summarized in figure 2a show that injection of 20 mg/kg i.p. [1] [I] to EAC/Dox-bearing mice, followed by euthanasia 7 days later, does not increased the percentage IFN-γ producing CD4+ T cells in tumor ascites. However, the IFN-γ-secreting CD8 population was slightly (although not significantly) increased in tumors from the [1] [I]-treated group (fig. 2b). Furthermore, the population expressing GR1/CD11b, a marker of MDSC, remained unchanged in the [1] [I]-treated group (data not shown).

In vitro Cytotoxic Effect of [1] [$CuCl_2$] and [1] [I]

Next, to ascertain whether these complexes possess cytotoxic potential, the MTT assay was performed on human T-lymphoblastic leukemia cells, viz CEM/ADR5000 and CCRF-CEM. When cells were cultured for 72 h in vitro, [1] [$CuCl_2$] did not show an antiproliferative effect on both cell lines (fig. 3a, b). However, under the same condition, [1] [I] induced a cytotoxic effect in a dose-dependent manner on both CEM/ADR5000 and CCRF/CEM with IC_{50} values of 1.03×10^{-5} and 1.02×10^{-5}, respectively (fig. 1c, d). Figure 1a, b clearly suggests that [1] [$CuCl_2$] is not toxic for both CEM/ADR and CCRF/CEM cells, whereas both of these cell lines were almost equally sensitive to [1] [I] (fig. 3c, d).

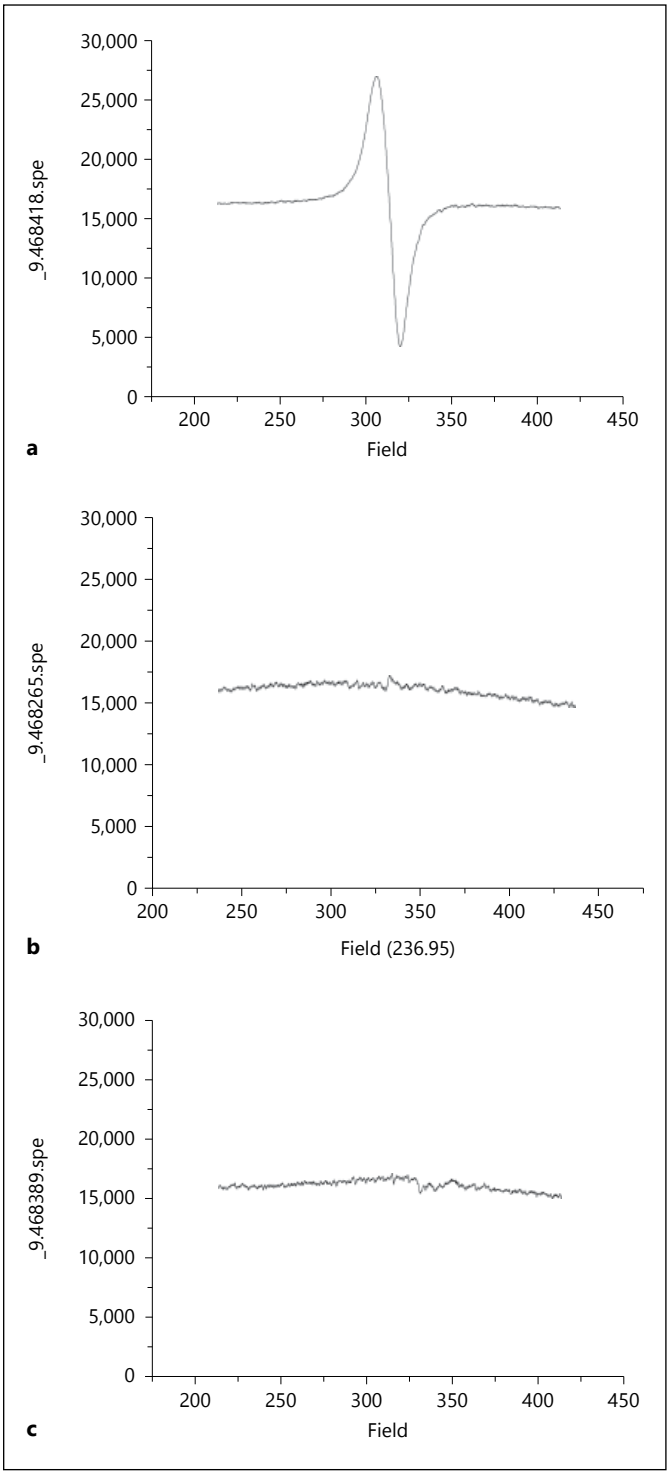

Fig. 1. EPR spectra of $Cu(II)Cl_2$ (**a**), [1] [I] (**b**) and [1] [$CuCl_2$] (**c**). EPR spectral evidence is significant to prove the presence of copper (I) ion in $1^+CuCl_2^-$. Presence of the Cu(II) ion shows the characteristic EPR signal (**a**), whereas absence of the paramagnetic signal (**b**) ensures the diamagnetic nature of the corresponding ligand. **c** Absence of a remarkable paramagnetic EPR peak from which we can conclude the presence of the Cu(I) ion in the reported drug.

Discussion

Despite numerous attempts, the MDR problem remains unanswered [18, 19]. A number of mechanisms have been associated with MDR and emerging evidence suggests the

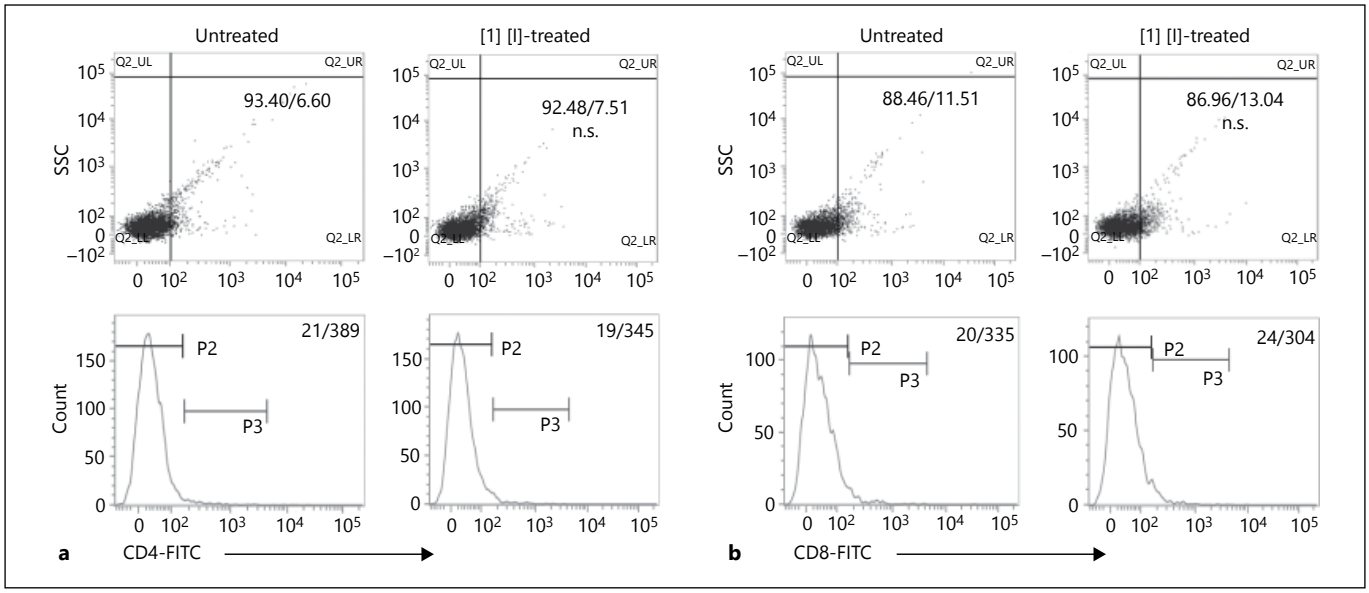

Fig. 2. Effect of [1] [I] on tumor-infiltrating T cells of drug-resistant tumor-bearing mice. Ascitic fluid from [1] [I]-treated or untreated EAC/Dox-bearing mice was collected after 7 days and total cell populations were counted. Magnetic-sorted CD4+ (**a**) or CD8+ (**b**) cell populations were permeabilized with permeabilizing solution and stained for intracellular anti-IFN-γ PE. Lower panels depict the representative histograms. Immunofluorescence analysis was performed using FACSCalibur (BD Biosciences) with CellQuest software. Representative data of three independent experiments are shown. ** $p < 0.01$; *** $p < 0.001$; n.s. = nonsignificant.

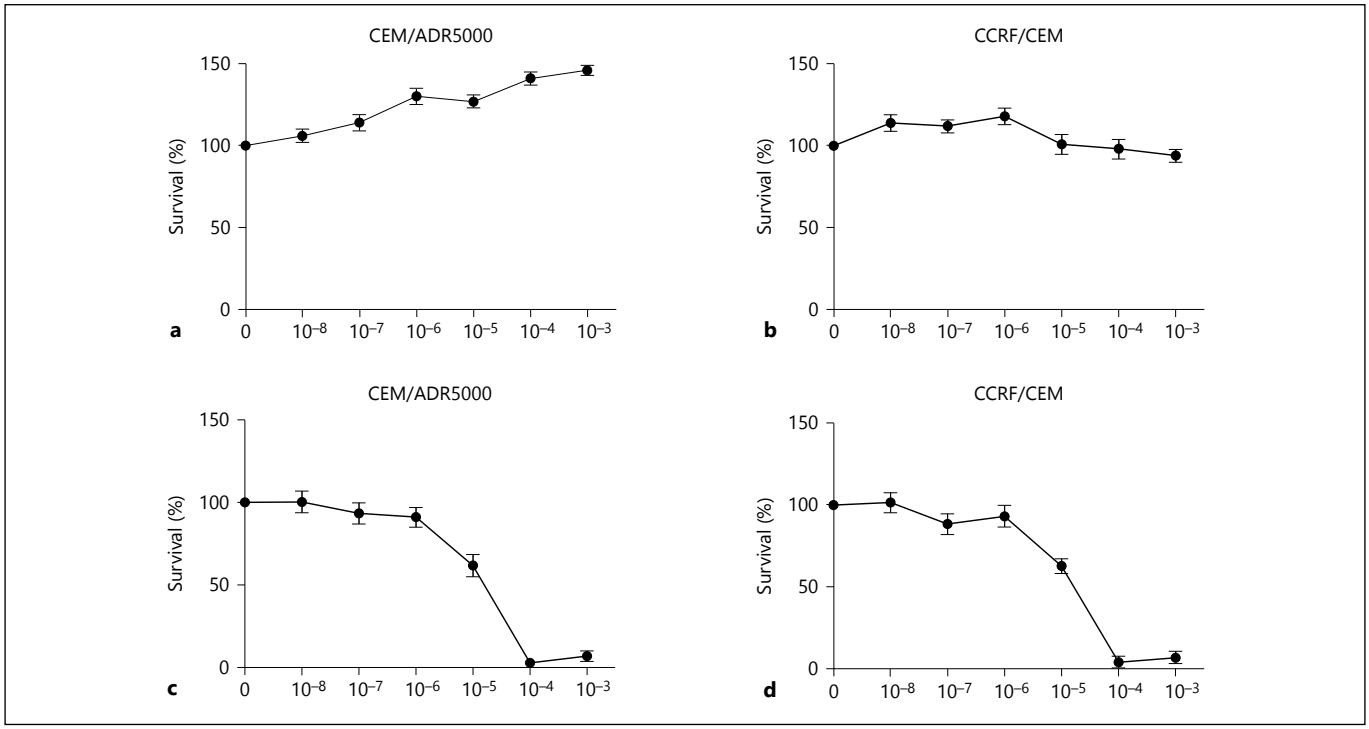

Fig. 3. Comparison of the cytotoxic effect of [1] [$CuCl_2$] and [1] [I] on different cell types. Dose-response curves for [1] [$CuCl_2$] (**a**, **b**) and [1] [I] (**c**, **d**) using CEM/ADR5000 and CCRF-CEM cells, as assessed by MTT assay. Cells were seeded into 96-well plates (4×10^4 cells/well) and incubated overnight at 37°C in 5% CO_2. The next day, cells were treated with increasing concentrations of either [1] [$CuCl_2$] or [1] [I] for 72 h. Results are expressed as percent viability of solvent-treated control cells. Value represents the mean ± SD of three independent experiments performed in quadruplicate.

involvement of the tumor microenvironment in MDR cancer [19]. Besides classical mechanisms, the tumor microenvironment creates an additional impact that imparts further protection for tumor cells from chemotherapeutic drugs [20, 21]. Therefore, new drugs or agents are being introduced to modulate or overcome the problem of MDR.

We earlier synthesized and evaluated the structure of a novel 9-phenyldibenzo[a,c]phenazin-9-ium cation [1^+] as [1] [$CuCl_2$] and as [1] [I]. We also investigated the DNA-binding potential of [1] [$CuCl_2$]. Herein, we report the evaluation of the anticancer as well as immunomodulatory activity of a novel 9-phenyldibenzo[a,c]phenazin-9-ium cation [1^+] as [1] [$CuCl_2$] and as [1] [I] both in vivo and in vitro. The potential of [1] [$CuCl_2$] and [1] [I] to increase the survivability of EAC/Dox-bearing mice was investigated. The study revealed that the intraperitoneal treatment with [1] [I] significantly increases the life span of tumor-bearing mice, which is 1.27 times that of the untreated counterpart (table 1). However, at the lower dose, it also helps to increase the survivability of mice nonsignificantly, whereas [1] [$CuCl_2$] fails to increase the survivability of tumor-bearing mice.

Emerging evidence disclosed that the immune cells of the tumor microenvironment often determine the severity and outcome of the disease [6]. Hence, to explore whether [1] [I] acts as an immunomodulator, we investigated its effect on IFN-γ-secreting tumor-infiltrating CD4+, CD8+ T cells and MDSCs. The compound [1] [I] does not augment the number of CD4+ T cells stained for IFN-γ (fig. 2a). However, the number of IFN-γ-specific CD8+ T cells is slightly (although nonsignificantly) increased following treatment with [1] [I] at 20 mg/kg body weight (as this dose was found to be effective in vivo; fig. 2b). No significant changes were found in the MDSC number when treated with [1] [I] compared with the untreated control group.

Next, we evaluated the cytotoxic potential of both [1] [$CuCl_2$] and [1] [I] in vitro using drug-resistant and drug-sensitive human T-lymphoblastic leukemia cells using the MTT assay. The cytotoxic ability of [1] [I] surpasses that of [1] [$CuCl_2$]. The resistant cell line as well as its parental counterpart is almost equally sensitive to [1] [I] in a dose-dependent manner, whereas [1] [$CuCl_2$] does not impart its antiproliferative effect to any of these cell lines. However, further study is needed to evaluate why [1] [I] is more efficacious than [1] [$CuCl_2$].

In conclusion, the present results suggest that complexes of the novel 9-phenyldibenzo[a,c]phenazin-9-ium $[1]^+$ cation as [1] [I] exert antitumor activities both in vivo in drug-resistant tumor-bearing mice and in vitro in T-lymphoblastic leukemia cell lines.

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Disclosure Statement

The authors find that there is no conflict of interest regarding the publication of this research article.

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