Antitumour and Anti-Inflammatory Effects of Palladium(II) Complexes on Ehrlich Tumour

Marcela B. Quilles^a, Camila B. A. Carli^a, Sandra R. Ananias^b, Lucas S. Ferreira^a, Livia C. A. Ribeiro^a, Danielle C. G. Maia^a, Flávia A. Resende^a, Antônio C. Moro^b, Eliana A. Varanda^a, Marisa Campos Polesi Placeres^a, Antonio E. Mauro^b, and Iracilda Z. Carlos^{a,*}

^a UNESP – São Paulo State University, Pharmaceutical Sciences Faculty, C. Postal 502, 14801–902, Araraquara, SP, Brazil. Fax: +55 16 3322-0073. E-mail: carlosiz@fcar.com.br

^b UNESP – São Paulo State University, Chemical Institute of Araraquara, C. Postal 355, 14801–970, Araraquara, SP, Brazil

* Author for correspondence and reprint requests

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Palladium(II) complexes are an important class of cyclopalladated compounds that play a pivotal role in various pharmaceutical applications. Here, we investigated the antitumour, anti-inflammatory, and mutagenic effects of two complexes: [Pd(dmmba)(Cl)tu] (**1**) and [Pd(dmmba)(N₃)tu] (**2**) (dmmba = *N,N*-dimethylbenzylamine and tu = thiourea), on Ehrlich ascites tumour (EAT) cells and peritoneal exudate cells (PECs) from mice bearing solid Ehrlich tumour. The cytotoxic effects of the complexes on EAT cells and PECs were assessed using the 3-(4,5-dimethylthiazol-3-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. The effects of the complexes on the immune system were assessed based on the production of nitric oxide (NO) (Griess assay) and tumour necrosis factor- α (TNF- α), interleukin-12 (IL-12), and interleukin-10 (IL-10) (ELISA). Finally the mutagenic activity was assessed by the Ames test using the *Salmonella typhimurium* strain TA 98. Cisplatin was used as a standard. The IC₅₀ ranges for the growth inhibition of EAT cells and PECs were found to be (72.8 $\pm$ 3.23) μ M and (137.65 $\pm$ 0.22) μ M for **1** and (39.7 $\pm$ 0.30) μ M and (146.51 $\pm$ 2.67) μ M for **2**, respectively. The production of NO, IL-12, and TNF- α , but not IL-10, was induced by both complexes and cisplatin. The complexes showed no mutagenicity *in vitro*, unlike cisplatin, which was mutagenic in the strain. These results indicate that the complexes are not mutagenic and have potential immunological and antitumour activities. These properties make them promising alternatives to cisplatin.

Key words: Macrophages, Ehrlich Tumour, Organometallic, Mutagenicity

Introduction

The use of metal complexes in medicine is widespread, especially in cancer therapies. *cis*-Diamminedichloroplatinum(II) (CDDP) is used to treat various types of cancer. This complex was the first member of a class of platinum-containing anticancer drugs that now includes carboplatin and oxaliplatin (Pampillo *et al.*, 2008; Leyva *et al.*, 2007). *In vivo*, these platinum complexes bind to and crosslink DNA, ultimately triggering apoptosis, but in humans, these complexes have many side effects such as nausea, nephrotoxicity, and neurotoxicity (Rocha *et al.*, 2007; Abu-Surrah and Kettunen 2006). Thus palladium(II) derivatives have been researched as anticancer agents; there is great interest in their ability to form complexes that are sufficiently stable to al-

low the drug to act effectively in the body at very low concentrations. These complexes were chosen due to their structural analogy to complexes containing Pt(II) (Abu-Surrah and Kettunen 2006; Casas *et al.*, 2008).

Ehrlich tumour is a species-specific, transplantable neoplasia derived from the malignant epithelium of a mouse mammary adenocarcinoma, and this type of tumour has been used as a transplantable tumour model to investigate the antineoplastic effects of many substances and compounds (Ehrlich and Apolant, 1905).

The effector activities of compounds can trigger the immune system. In tumours, the activation of oncogenes can orchestrate the production and/or recruitment of inflammatory cells (Mantovani, 2009). In this context, the immune response is of fundamental importance in the development and

eradication of tumours. During inflammation, macrophages are characterized by an increased phagocytic capacity and an increased ability to generate reactive oxygen (ROIs) and nitrogen (RNIs) intermediates and cytokines. Evidence suggests that these activated macrophages can destroy tumour cells (Eberhardt, 2001; Janeway, 2006).

Nitric oxide (NO) and several other immune mediators are involved in inflammatory processes. The cytokines interleukin-12 (IL-12) and tumour necrosis factor- α (TNF- α) are known to have pro-inflammatory roles and to participate in a variety of biological activities related to acute and chronic inflammatory diseases (Eigler *et al.*, 1997; Rigby *et al.*, 2007). Conversely, interleukin-10 (IL-10) is produced by both macrophages and lymphocytes and has anti-inflammatory properties that are mediated by reducing the amplification of inflammatory processes (Clemons *et al.*, 2000; Naundorf *et al.*, 2009).

The effects of anticancer substances on DNA are very important, given that mutations are directly related to the development of many degenerative diseases such as cancer and atherosclerosis (De Flora, 1998; Seo *et al.*, 2000). The discovery of new compounds capable of reducing the rate of mutations may reduce the incidence of cancer and degenerative diseases, provided that the exposure of the public to these products is increased (Hayatsu *et al.*, 1988; Ferguson *et al.*, 2004).

Because palladium(II) complexes have potential antitumour activity, this study assessed the effects of two compounds, [Pd(dmmba)(Cl)tu] (**1**) and [Pd(dmmba)(N₃)tu] (**2**) (dmmba = *N,N*-dimethylbenzylamine and tu = thiourea), and cisplatin. The cytotoxicity, immune effects, and mutagenic effects on Ehrlich tumour cells and macrophages from mice bearing solid Ehrlich tumour were assessed *in vitro*.

Experimental

Organometallic complexes

The organometallic complexes [Pd(dmmba)(Cl)tu] and [Pd(dmmba)(N₃)tu] were synthesized according to the literature (Lucca Neto *et al.*, 1998; De Almeida *et al.*, 2005), at the Laboratory of Organometallic and Coordination Chemistry, Department of General and Inorganic Chemistry, UNESP, Araraquara, Brazil. Cisplatin (FH Fauld-

ing & Co. Ltd., Adelaide, Australia) was used as standard drug.

For the biological assays, the complexes were dissolved in dimethyl sulfoxide (DMSO) and diluted with RPMI-1640 culture medium (Sigma, São Paulo, Brazil) immediately before use.

Animals

Swiss mice (6–8 weeks old, weighing 18–25 g) were maintained in a polycarbonate box [(23 ± 1) °C, (55 ± 5)% humidity, 10–18 circulations/h, and a 12-h light/12-h dark cycle], with free access to water and chow (Purina, Mantena, Brazil). All animals were maintained and handled according to the International Ethical Guidelines for the Care of Laboratory Animals (Faculty Ethics Committee # 21/2007).

Tumour cell line

The Ehrlich ascites tumour (EAT) cells were kindly provided by Dr. Denise Fechhio from the Department of Pathology, UNESP, Botucatu, Brazil and were maintained in the peritoneal cavities of mice by injecting 0.1 mL of ascitic fluid every 7 d. Ascites tumour cell counts were performed using a Neubauer hemocytometer (Baeco, Hamburg, Germany) with the trypan blue dye exclusion method. The cell viability was always found to be 95% or higher. Tumour cell suspensions were prepared in phosphate-buffered saline (PBS). All experiments were performed in triplicate.

Ehrlich solid tumour

Tumour cells (1 · 10⁷ tumour cells/mL) were subcutaneously (s.c.) injected into the right hind limb (thigh) of all animals. Approximately 30 d later, the mice bearing solid Ehrlich tumour were sacrificed, and peritoneal exudate cells (PECs) were suspended in RPMI-1640 culture medium to perform the assays.

Peritoneal exudate cells

Thioglycollate-elicited PECs were harvested from mice bearing solid Ehrlich tumour in 5.0 mL of PBS (pH 7.4). The cells were washed three times by centrifugation at 200 × g for 5 min at 4 °C with 3.0 mL of PBS. The cells were then resuspended in 1.0 mL of RPMI-1640 culture medium containing 20 µM β-mercaptoethanol (Sigma), 100 U/mL penicillin (Sigma), 100 µg/mL

streptomycin (Sigma), and 5% fetal bovine serum (FBS; Sigma). This medium was designated complete RPMI-1640 culture medium (complete RPMI-1640) and was used for cell counts in a Neubauer chamber. For the proposed tests, the cell suspension was adjusted to a concentration of $5 \cdot 10^6$ cells/mL.

MTT assays

PECs from mice bearing solid Ehrlich tumour ($5 \cdot 10^6$ cells/mL) and EAT cells ($1 \cdot 10^6$ cells/mL) were resuspended in complete RPMI-1640 as described above. The cells ($100 \mu\text{L}$) were incubated in $100 \mu\text{L}$ of complex **1**, complex **2**, and cisplatin at different concentrations for 24 h. Each condition was tested in quadruplicate. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay was performed, and the absorbance at 540 nm was determined with an ELISA reader (Multiskan Ascent Labsystems, Helsinki, Finland) (Mosman, 1983). The results represent the mean of four independent experiments and are expressed as the IC_{50} , *i.e.* the concentration that reduced the optical density of the treated cells with respect to untreated controls by 50%.

Nitric oxide assay

PECs ($5 \cdot 10^6$ cells/mL) were resuspended in RPMI-1640 culture medium containing 5% heat-inactivated FBS, 100 IU/mL penicillin, $100 \mu\text{g}/\text{mL}$ streptomycin, and $20 \mu\text{M}$ β -mercaptoethanol. Aliquots of $100 \mu\text{L}$ of PECs in the presence of the same volume of complex **1**, complex **2**, and cisplatin were incubated for 24 h. *Escherichia coli* O26:B6 lipopolysaccharide (LPS) was used as a positive control. After incubation, $50\text{-}\mu\text{L}$ aliquots of the supernatants from each well were mixed with equal volumes of Griess reagent (1% w/v sulfanilamide, 0.1% w/v naphthylethylenediamine, and 3% H_3PO_4) and incubated at room temperature for 10 min in the dark. The extent of the resultant colorimetric reaction was determined using an ELISA reader as described above. The supernatants from quadruplicate cultures were assayed in four experiments, and the results are reported as mmol NO per $5 \cdot 10^5$ cells. Solutions containing known concentrations of NaNO_2 were used for calibration (Green *et al.*, 1982).

Quantification of IL-12, IL-10, and TNF- α

The amounts of cytokines released by PECs ($5 \cdot 10^6$ cells/mL) during a 24-h culture period at 37°C under 5% CO_2 in RPMI-1640 culture medium, with or without LPS (positive control), cisplatin or the test complexes **1** and **2** were measured using a commercially available ELISA kit (Kit DuoSet[®]; R & D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions. The results of the colorimetric reaction were read at 450 nm with wavelength correction at 570 nm using an ELISA plate reader (Multiskan Ascent Labsystems).

Salmonella mutagenic assay

Mutagenic activity was assessed using the *Salmonella*/microsome assay, with the *Salmonella typhimurium* tester strain TA 98, which was kindly provided by B. N. Ames (Berkeley, CA, USA). This assay was performed, with and without metabolic activation using the pre-incubation method (Maron and Ames, 1983). Bacteria from frozen cultures were grown overnight for 12–14 h in Oxoid nutrient broth No. 2 (Thermo Fisher Scientific, Toronto, Canada). The metabolic activation mixture (S9) was freshly prepared before each test. Five different doses of test compounds were assayed; all of them were diluted in DMSO. The concentrations were selected based on the results of a preliminary toxicity test. In all subsequent assays, the upper limit of the tested dose range was either the highest non-toxic dose or the lowest toxic dose identified in this preliminary assay. Reductions in the number of His⁺ revertants and alterations in the auxotrophic background, *i.e.* background lawn, were considered indicative of toxicity. The various concentrations of the complexes and cisplatin to be tested were added to 0.5 mL of 0.2 M phosphate buffer (pH 7.4) or 0.5 mL of 4% S9 mixture and 0.1 mL of bacterial culture and then incubated at 37°C for 20–30 min. After this incubation, 2 mL of top agar were added to the mixture which was then poured onto a plate containing minimal agar. The plates were incubated at 37°C for 48 h, and the revertant colonies were counted manually. All experiments were analysed in triplicate. The results were analysed with the Salanal statistical software package (US Environmental Protection Agency, Monitoring Systems Laboratory, Las Vegas, NV, USA; version 1.0 from Research Triangle Insti-

tute, RTP, Durham, NC, USA) (Myers *et al.*, 1991), adopting the model of Bernstein *et al.* (1982). The data (revertants/plate) were assessed by analysis of variance (ANOVA), followed by linear regression. The mutagenic index (MI) was also calculated for each concentration tested. This value is the average number of revertants per plate treated with the test compound divided by the average number of revertants per plate treated with the negative (solvent) control. A sample was considered mutagenic when a dose-response relationship was detected and a two-fold increase in the number of mutants ($MI \geq 2$) was observed for at least one concentration (Santos *et al.*, 2006). When only one of these criteria was met, the sample was considered to present signs of mutagenicity, in agreement with the guidelines of McGeorge *et al.* (1985). The standard mutagens used as positive controls were 4-nitro-*o*-phenylenediamine ($10 \mu\text{g}/\text{plate}$) in the experiments without the S9 mix, and 2-anthramine ($1.25 \mu\text{g}/\text{plate}$) in the experiments with metabolic activation. DMSO served as the negative (solvent) control ($75 \mu\text{L}/\text{plate}$).

Statistical analysis

The results are representative of three independent experiments and are presented as average values $\pm$ standard deviations (SDs) from quadruplicate observations. The data were statistically analysed using ANOVA and the Tukey-Kramer post-test, using a significance level of $p < 0.05$, in InStat Graph PadTM.

The results of the mutagenicity assay were analysed with the Salanal statistical software package (Myers *et al.*, 1991), adopting the model of Bernstein *et al.* (1982).

Results and Discussion

A variety of cyclopalladated compounds have been used successfully as cytotoxic agents to treat tumour cell lines resistant to cisplatin. The antitumour activities of the compounds $[\text{Pd}(\text{C}^2, N\text{-dmmba})(\mu\text{-X})_2]$ and $[\text{Pd}(\text{C}^2, N\text{-dmmba})(\text{X})\text{tu}]$ (dmmba = *N,N*-dimethylbenzylamine; X = Cl, Br; tu = thiourea) against LM3 (breast tumour) and LP07 (lung tumour) cells demonstrated that these compounds have cytotoxic potential *in vitro* (Moro *et al.*, 2009). Reports on the cytotoxicity of palladium-derived compounds in the two leukemic cell lines HL60 and K562, as assessed by

the MTT and trypan blue exclusion assays, suggest that the toxic effects of these compounds are dose-dependent (Rocha *et al.*, 2003).

The cytotoxic effect was examined and the viability of the cells was measured by the MTT assay after their treatment with either complex **1**, complex **2** (Fig. 1), or cisplatin for 24 h. The cytotoxic activities of the palladium(II) complexes and cisplatin were tested using peritoneal macrophages from mice bearing solid Ehrlich tumour. We found that cisplatin was the most cytotoxic [$\text{IC}_{50} = (113.21 \pm 0.28) \mu\text{M}$], closely followed by compounds **1** and **2** [$\text{IC}_{50} = (137.65 \pm 0.22) \mu\text{M}$ and $(146.51 \pm 2.67) \mu\text{M}$, respectively], but none of these differences were statistically significant (Fig. 2).

Previous studies have shown that thiourea-containing complexes exhibit cytotoxicity against tumour cell lines (Navarro-Ranninger *et al.*, 1996; Quiroga *et al.*, 1998, 1999). In the present study, EAT cells were susceptible to the action of the complexes, with complex **2** [$\text{IC}_{50} = (39.70 \pm 0.30) \mu\text{M}$] exhibiting similar cytotoxic potential as cisplatin [$\text{IC}_{50} = (40.28 \pm 2.97) \mu\text{M}$] while complex **1** was less cytotoxic [$\text{IC}_{50} = (72.80 \pm 3.23) \mu\text{M}$] (Fig. 3).

Recent research has demonstrated that organopalladated compounds containing diphenylphosphine ligands can activate macrophages *in vitro* (De Almeida *et al.*, 2005) via immunological activation. This effect was demonstrated by assessing the amount of H_2O_2 released in cultures of peritoneal macrophages from Swiss mice in the presence of organopalladated compounds of the type $[\text{Pd}(\text{dmmba})(\text{X})(\text{dppp})]$ (dmmba = *N,N*-dimethylbenzylamine; dppp = 1,3-bis(diphenylphosphine)propane; X = Cl, N₃, NCO, NCS). An excellent activation of macrophages by $[\text{Pd}(\text{dmmba})(\text{X})(\text{dppp})]$ compounds was observed, and the influence of the ligand X on the immune response was verified. Activated macrophages secrete several sub-

Fig. 1. Chemical structures of the complexes $[\text{Pd}(\text{dmmba})(\text{X})\text{tu}]$ [X = Cl (**1**) or N₃ (**2**)].

Fig. 2. Dose-response curves for the effect of the complexes **1** and **2** and cisplatin (CIS) on the viability of PECs from mice bearing solid Ehrlich tumour after 24 h of exposure.

Fig. 3. Dose-response curve for the effect of the complexes **1** and **2** and cisplatin (CIS) on EAT tumour cells after 24 h of exposure.

stances that are directly involved in tumour cell killing, *i.e.* tumour necrosis factor (TNF) and nitric oxide (NO). TNF and NO are considered as the most important mediators directly involved in tumour cell killing (Klimp *et al.*, 2002).

NO is formed in activated phagocytes as a product of the conversion of L-arginine into L-citrulline by an inducible isoform of nitric oxide

synthase. This reaction, which occurs during the oxidative burst, involves O₂ uptake. Nitrite (NO₂⁻) and nitrate (NO₃⁻) are generally believed to be the end products of the RNI-generating system in macrophages. Although many other agents are cytotoxic to target cells, NO is often found to be the primary mediator of macrophage-induced cytotoxicity (Tamir and Tannenbaum, 1996).

With regard to NO production by PECs from mice bearing solid Ehrlich tumour, complex **2** ($p < 0.001$) was the most potent NO inducer followed by cisplatin ($p < 0.001$) compared with the negative control (Fig. 4). NO and TNF- α have important roles in the pathogenesis of various diseases during the acute inflammation stage (Nathan, 1987).

Fig. 4. Nitric oxide production by PECs from mice bearing solid Ehrlich tumour in the presence of either complex **1**, complex **2**, and cisplatin (CIS). Adherent cells ($5 \cdot 10^6$ cells/mL) were incubated for 24 h with 100 μ L of a complex or cisplatin. The cell-free supernatant was mixed with Griess reagent. LPS was used as a positive control (C+), cells in the plain medium (RPMI-1640) were used as a negative control (C-). Each bar represents the mean $\pm$ SD of five experiments. *** $p < 0.001$ significantly different from the negative control.

Moreover, TNF- α affects tumour progression by modulating the cell motility or by increasing the expression of adhesion molecules in target organs (Gelin *et al.*, 1991; Kundu and Surh, 2008). For PECs from mice bearing solid Ehrlich tumour, complex **2** induced the highest level of cytokine production ($p < 0.001$), followed by complex **1** and cisplatin ($p < 0.05$) compared with the negative control (Fig. 5). IL-12 is a multifunctional cytokine that stimulates both innate and adaptive immunity through the Th1 response, which is necessary for tumour growth inhibition. The immunomodulatory and antiangiogenic functions of IL-12 justify its use as an anticancer agent (Barnes, 2003; Berraondo *et al.*, 2009). In this study, IL-12 production was most strongly induced by complex **2**, followed by complex **1** and cisplatin ($p < 0.01$ compared with the negative control) (Fig. 5).

Opposing the functions of NO and other cytokines, IL-10 has anti-inflammatory properties mediated by the inhibition of macrophage activation thereby limiting inflammatory processes (Clemons *et al.*, 2000; Naundorf *et al.*, 2009). Fig. 5 demonstrates that the production of this cytokine was not detected in 24-h cultures of PECs from mice bearing Ehrlich tumour.

In addition to their immunomodulatory activities, another important feature of anticancer sub-

Fig. 5. Cytokine production induced by PECs from mice bearing solid Ehrlich tumour in the presence of complex **1**, complex **2**, and cisplatin (CIS). Cells incubated with LPS were used as a positive control (C+), and cells incubated in plain medium (RPMI-1640) were used as a negative control (C-). Each bar represents the mean $\pm$ SD of five experiments. *** $p < 0.001$ significantly different from the negative control, ** $p < 0.01$ and * $p < 0.05$ significantly different from the negative control.

stances that requires analysis is their effects on DNA. The conversion of normal cells into cancer cells usually involves several steps, but one of the earlier stages of this process usually involves the action of a genotoxic carcinogen (Weisburger, 2000). The test used to assess mutagenicity was the reverse gene mutation assay in *Salmonella typhimurium* (Ames test), which is a rapid test designed to identify a wide variety of chemicals capable of inducing extensive DNA damage that leads to the emergence of mutations (Mortelmans and Zeiger, 2000).

Table I shows the mean number of revertants/plate (M), the standard deviation (SD), and the mutagenic index (MI) after the treatment of *S. typhimurium* strain TA 98 cells with either complex **1**, complex **2** or cisplatin, in the presence (+S9) and absence (–S9) of metabolic activation. In the presence and absence of external metabolic activation using the S9 mix, cisplatin was the only compound with a mutagenic index higher than 2.0. Cisplatin's mutagenicity in strain TA 98 increased in a dose-dependent manner, with a mutagenic index higher than 3.12 at 25.00 μM .

Due to several disadvantages associated with the use of cisplatin, there is an expanding search for new cytotoxic and cytostatic drugs with antitumour activities that are more selective and cause less systemic toxicity.

In conclusion, the peritoneal macrophages from mice bearing solid Ehrlich tumour, as well as Ehrlich tumour cells themselves, are susceptible to the toxicity of the tested Pd complexes. The tested complexes were able to stimulate the production of nitric oxide and pro-inflammatory cytokines, but not the production of the anti-inflammatory cytokine IL-10. Complex **2** exhibited a greater pro-inflammatory potential than complex **1**. The mutagenicity test demonstrated that these complexes are not mutagenic *in vitro*, unlike cisplatin. The promising results obtained for these complexes open up new avenues for further study and provide a better understanding of mouse mammary adenocarcinomas.

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Table I. Mutagenic activity expressed as the mean $\pm$ SD of the number of revertants/plate (and the mutagenic index) in *Salmonella typhimurium* strain TA 98 cells treated with [Pd(dmmba)(Cl)tu] (**1**), [Pd(dmmba)(N₃)tu] (**2**), or cisplatin, at various doses, with (+S9) or without (–S9) metabolic activation.

Treatment [μM]	Cisplatin		[Pd(dmmba)(Cl)tu]		[Pd(dmmba)(N ₃)tu]	
	–S9	+S9	–S9	+S9	–S9	+S9
DMSO ^a	41.00 $\pm$ 7.21	22.33 $\pm$ 4.04	41.00 $\pm$ 7.21	22.33 $\pm$ 4.04	41.00 $\pm$ 7.21	22.33 $\pm$ 4.04
3.12	112.00 $\pm$ 16.09 ** (2.66)	51.67 $\pm$ 6.03** (2.31)	48.33 $\pm$ 4.16 (1.15)	29.67 $\pm$ 8.96 (1.32)	62.67 $\pm$ 8.50 (1.49)	27.33 $\pm$ 5.51 (1.22)
6.25	111.00 $\pm$ 20.78 * (2.64)	34.33 $\pm$ 7.51 (2.53)	53.67 $\pm$ 4.93 (1.27)	24.67 $\pm$ 5.69 (1.10)	68.33 $\pm$ 11.93 (1.62)	27.33 $\pm$ 5.51 (1.22)
12.50	104.33 $\pm$ 6.66 ** (2.47)	57.67 $\pm$ 24.42 (2.58)	52.67 $\pm$ 8.50 (1.25)	26.33 $\pm$ 9.29 (1.17)	57.33 $\pm$ 12.01 (1.36)	27.67 $\pm$ 4.16 (1.23)
18.75	91.33 $\pm$ 1.15** (2.17)	54.00 $\pm$ 6.56 ** (2.41)	58.67 $\pm$ 15.50 (1.39)	25.33 $\pm$ 12.10 (1.13)	31.33 $\pm$ 9.02 (0.74)	27.67 $\pm$ 3.21 (1.23)
25.00	88.00 $\pm$ 14.53* (2.09)	49.33 $\pm$ 10.60* (2.20)	36.00 $\pm$ 4.58 (0.85)	30.00 $\pm$ 1.00 (1.34)	19.00 $\pm$ 2.65 (0.45)	19.33 $\pm$ 6.43 (0.86)
Control ^b +	445.33 $\pm$ 10	867 $\pm$ 40	445.33 $\pm$ 10	867 $\pm$ 40	445.33 $\pm$ 10	867 $\pm$ 40

* $P < 0.05$ (ANOVA); ** $P < 0.01$ (ANOVA).

^a Negative control, DMSO (75 μL /plate).

^b Positive controls (Control +), 4-nitro-*o*-phenylenediamine (NPD – 10.0 μg /plate – TA 98), without S9, and 2-aminothranine* (1.25 μg /plate – TA 98), with S9.

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