

Effect of the Isocoumarin Paepalantine on the Luminol and Lucigenin Amplified Chemiluminescence of Rat Neutrophils

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Paepalantine (9,10-dihydroxy-5,7-dimethoxy-1H-naphtho(2,3c)pyran-1-one), a natural isocoumarin isolated from the capitula of *Paepalanthus bromelioides* (Eriocaulaceae), was assessed for its effect on the respiratory burst (zymosan-stimulated luminol-enhanced chemiluminescence and PMA-stimulated lucigenin-enhanced chemiluminescence) of polymorphonuclear neutrophils *in vitro*. Special attention was devoted to establishing the IC₅₀ for neutrophils. Paepalantine was able to decrease luminol and lucigenin chemiluminescence, reflecting an inhibitory effect on the respiratory burst, with an ED₅₀ of 0.44±0.05 and 0.84±0.15 µg/ml, respectively. A cell-free system was performed with paepalantine on myeloperoxidase/H₂O₂ and myeloperoxidase/H₂O₂/Cl⁻ systems. Paepalantine inhibited luminol oxidation in both systems. This inhibition was related to the interaction of paepalantine-myeloperoxidase and its scavenger effect on HOCl.

Key words antioxidant activity; paepalantine; isocoumarin; chemiluminescence

The respiratory burst of phagocytes involves a striking increase in oxygen consumption, accompanied by activation of the hexose monophosphate shunt, which generates reactive oxygen species (ROS) including superoxide, hydrogen peroxide, hydroxy radicals and hypochlorous acid. The respiratory burst is triggered by the NADPH oxidase system, which is activated following the perturbation of the plasma membrane during phagocytosis or following the interaction between the cell surface and a variety of agents.¹⁾

NADPH oxidase, a membrane multienzyme complex, catalyzes the univalent reduction of molecular oxygen by NADPH and generates O₂⁻. Subsequently, H₂O₂, which is also generated during the respiratory burst, is utilized by myeloperoxidase (MPO), an enzyme located in azurophil granules. MPO in the presence of a suitable halide ion such as Cl⁻ produces hypochlorous acid (HOCl).²⁾ Neutrophil-derived oxidants (in particular HOCl) react with a variety of different biological targets and can cause tissue damage. The ability of HOCl to damage proteins, amino acids, lipids and nucleic acids is thought to contribute to bacterial killing.³⁾

Neutrophils can defend themselves against the oxidant they produce through a variety of potent antioxidant systems. However, when produced in excess in the extracellular medium, oxidative species can damage host tissue.⁴⁾ These reactive species are implicated in many diseases, including atherosclerosis, respiratory tract disorders, neurodegenerative diseases and cancer.^{5,6)} Therefore, controlling the respiratory burst may be useful for therapeutic intervention in many human diseases.⁷⁾

Understanding of the biological activity of plant compounds has emerged during the last decade. Isocoumarins, secondary metabolites derived from the acetate pathway, have a wide range of activity, including: antitumoral, antileucemic, antiviral and antimicrobial.^{8–10)} We have reported on the activities of the isocoumarin paepalantine (9,10-dihydroxy-5,7-dimethoxy-1H-naphtho(2,3c)pyran-1-one) (Fig. 1), including its cytotoxic effect,¹¹⁾ mutagenic property¹²⁾ and potential antimicrobial activity.¹³⁾ In continuation of our

study on the biological activities of paepalantine, we evaluated its antioxidant potency by its interference in the respiratory burst of neutrophils and the MPO/H₂O₂ system.

RESULTS AND DISCUSSION

A large number of techniques has been developed to monitor the generation and reduction of ROS by stimulated phagocytes during respiratory burst activity. As yet, no single technique is sufficient to determine the effects of different pharmacological agents affecting cellular responses. Chemiluminescence has been widely used as a sensitive and accurate method for assessing the capacity of neutrophils to produce ROS.^{14,15)} It is generally admitted that luminol is a relatively small molecule which is able to enter the cell and, therefore, reflects both extra and intracellular events.¹⁶⁾ Luminol is oxidized by ROS generated during the respiratory burst, and luminol-enhanced chemiluminescence is dependent upon an MPO-mediated reaction, *via* peroxidative and chlorinating activity (generation of HOCl).¹⁷⁾ The lucigenin chemiluminescence is generally attributed to extracellular O₂⁻. Being a larger molecule unable to enter in cells, lucigenin only reflects extracellular events.¹⁶⁾

For the present study, nonopsonized zymosan and PMA were selected from among the panel of existing activators, because our priority was to determine the active dose of paepalantine with good precision in a one-well standardized system. A significant inhibition of the intensity of luminol

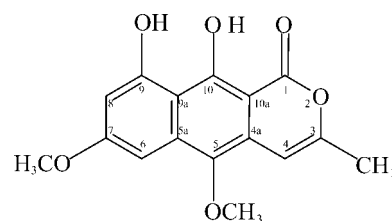


Fig. 1. Chemical Structure of Paepalantine

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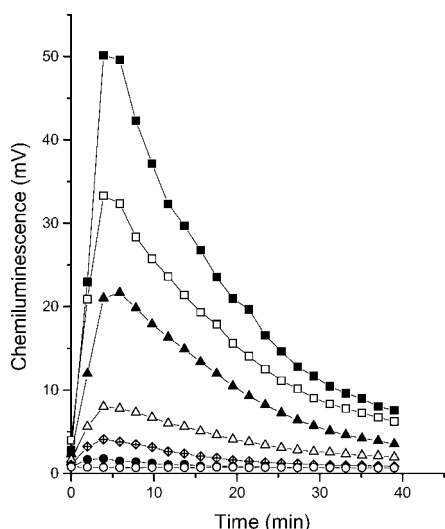


Fig. 2. Influence of Different Doses of Paepalantine on Non-Opsonized Zymosan-Induced Chemiluminescence of Polymorphonuclear Neutrophils

ROS production in neutrophils (1.0×10^6 cells/ml) is kinetically represented as millivolt (mV) values detected by luminol enhanced chemiluminescence assay. ROS production in the cells treated with nonopsonized zymosan only, as the control (solid rectangle), or PBS-D only as the background (open circle) was measured during a period of 0 to 40 min. The cells were treated with paepalantine at 0.12 $\mu\text{g/ml}$ (open rectangle), 0.48 $\mu\text{g/ml}$ (solid triangle),* 0.98 $\mu\text{g/ml}$ (open triangle),* 1.98 $\mu\text{g/ml}$ (cross diamond)* and 3.9 and 7.8 $\mu\text{g/ml}$ (solid circle).* * $p < 0.05$.

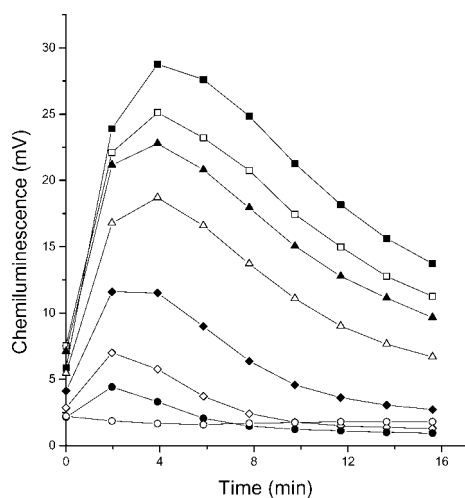


Fig. 3. Effect of Paepalantine on PMA-Stimulated Lucigenin-Enhanced Chemiluminescence of Polymorphonuclear Neutrophils

Superoxide production in the cells treated with PMA only, as the control (solid rectangle), or PBS-D only as the background (open circle) was measured during a period of 0 to 15 min. The cells were treated with paepalantine at 0.12 $\mu\text{g/ml}$ (open rectangle), 0.48 $\mu\text{g/ml}$ (solid triangle),* 0.98 $\mu\text{g/ml}$ (open triangle),* 1.98 $\mu\text{g/ml}$ (solid diamond),* 3.9 (open diamond)* and 7.8 $\mu\text{g/ml}$ (solid circle).* * $p < 0.05$.

and lucigenin-enhanced chemiluminescence in stimulated neutrophils was observed (Figs. 2, 3). Our results showed that paepalantine is able to modulate the respiratory burst of phagocytes in a dose-dependent manner with ED_{50} of 0.44 ± 0.05 $\mu\text{g/ml}$ and 0.84 ± 0.15 $\mu\text{g/ml}$ for luminol and lucigenin-enhanced chemiluminescence, respectively. This decrease in the luminol and lucigenin chemiluminescence is probably due to an inhibitory effect on some reactive oxygen generating systems, like the $\text{MPO}/\text{H}_2\text{O}_2/\text{Cl}^-$ and/or the NADPH oxidase complex. Myeloperoxidase catalytic activity is complex and involves two distinct pathways (Fig. 4). Besides its regu-

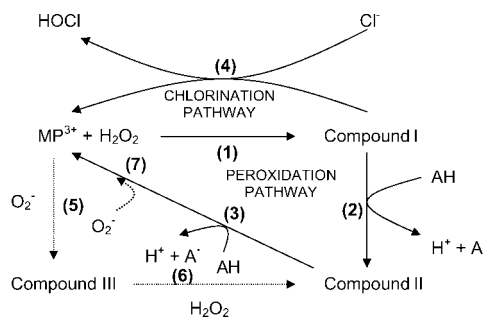


Fig. 4. Representation of Myeloperoxidase Catalytic Activity Pathways

MP^{3+} : native enzyme; Compound I: $\text{MP}^{3+} \text{H}_2\text{O}_2$; Compound II: $\text{MP}^{2+} \text{H}_2\text{O}_2$; Compound III: $\text{MP}^{3+} \text{O}_2^-$. AH: reducing substrate; O_2^- : superoxide anion. The broken lines mean that the reaction is hypothetical under *in vivo* conditions (adapted from Nève *et al.*, 2001).

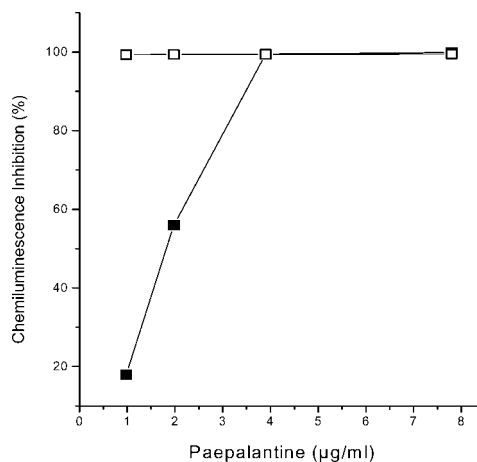


Fig. 5. Influence of Different Doses of Paepalantine on Luminol Amplified Chemiluminescence in a Cell-Free System

Chemiluminescence inhibition (%) was calculated after incubation of paepalantine with $\text{MPO}/\text{H}_2\text{O}_2$ (open rectangle) and $\text{MPO}/\text{H}_2\text{O}_2/\text{Cl}^-$ (solid rectangle) systems in relation to the control. The addition of luminol on both systems without paepalantine (control) resulted in an immediate chemiluminescence response.

lar peroxidative activity (Fig. 4; 1—3), myeloperoxidase has HOCl generation activity (Fig. 4; 1, 4).¹⁸⁾ We investigated the effect of paepalantine on $\text{MPO}/\text{H}_2\text{O}_2$ (peroxidative activity) and $\text{MPO}/\text{H}_2\text{O}_2/\text{Cl}^-$ (HOCl generating activity). In both cell-free systems, paepalantine inhibited luminol oxidation (Fig. 5). The reduction of chemiluminescence by paepalantine is due to myeloperoxidase inhibition and a scavenging effect on HOCl. It seems that the inhibitory activity of the paepalantine on luminol-enhanced chemiluminescence from myeloperoxidase is a consequence of its interference with the enzymatic system rather than of scavenging activity. The scavenger effect of paepalantine on HOCl was confirmed by its ability to oxidize TNB test. The ability of HOCl to oxidize TNB resulted in a loss of absorbance at 412 nm. Any HOCl scavenger present in the system will compete with TNB for HOCl and decrease the change in absorbance at 412 nm.¹⁹⁾ Incubation of HOCl with paepalantine before the addition of TNB diminishes the amount of TNB to be oxidized, indicating that paepalantine reacts with HOCl (Table 1).

Although there is a wealth of data on the importance of antioxidants in conferring stability towards or protection from oxidation, the correlation between antioxidant activity and chemical structure is far from clear.²⁰⁾ It is already well

Table 1. Effect of Paepalantine on HOCl-Dependent TNB Oxidation

Additions	A412 ^{a)}
No addition	0.440
Plus HOCl	0.160
HOCl and paepalantine	
1.98 $\mu\text{g/ml}$	0.388
3.9 $\mu\text{g/ml}$	0.404
7.8 $\mu\text{g/ml}$	0.408

a) Values are the means from two independent measurements that varied by 5%.

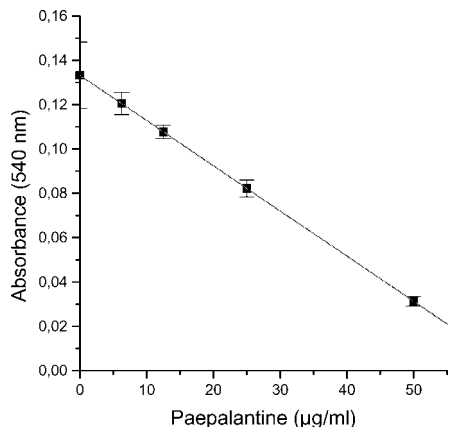


Fig. 6. Dose-Effect of Paepalantine on Neutrophils Performed by MTT Assay

Cells were seeded to 96-well microtitre plates at a concentration of 6×10^6 cells/ml and treated with paepalantine, at different doses, for 24 h. MTT was added to all wells, and plates were incubated at 37 °C. After 3 h, the absorbance of the formazan was measured at 540 nm with 620 nm as a reference. Each point shows the mean \pm standard deviation of four experimental points.

established that flavonoids act as scavengers of various oxidizing species. The importance of the adjacency of the two hydroxyl groups (catechol) in the *ortho*-diphenolic arrangement of the B-ring of quercetin to its antioxidant activity is reported.²¹⁾ The presence of OH-9 and OH-10 groups in paepalantine provides a catecholic-like system, and may allow the antioxidant activity of this isocoumarin as detected on MPO/H₂O₂/Cl⁻ system.

Many studies have been undertaken in lipophilic systems to establish the structural criteria for the activity of polyhydroxy phenolics in enhancing the stability of fatty acid dispersions, lipids and low density lipoproteins towards oxidation.²⁰⁾ The specific mode of inhibition of oxidation by the individual polyphenols is not clear. The carbonyl at position 1 and the hydroxyl groups at positions 9 and 10 of paepalantine allow the formation of two strong intramolecular hydrogen bridges, making this molecule lipophilic, as has been previously verified by its high solubility in chloroform.¹³⁾

The cytotoxic midpoint (IC₅₀) of paepalantine against neutrophils using the MTT technique was $32.78 \pm 1.27 \mu\text{g/ml}$ (Fig. 6). However, there is a large difference in the dose dependency of neutrophil protective effects of paepalantine compared to its cytotoxic index. This raises an interesting issue when assessing antioxidant activities. A significant dose-dependent inhibition of the luminol-enhanced chemiluminescence was observed with paepalantine at almost an 80-fold lower concentration than that obtained for IC₅₀. Due to the difference in the toxic amount dose, consequences of biological effects on neutrophils may have a significant bearing

on its therapeutic efficacy. It is clear from the foregoing that ROS produced from phagocytes are important in the pathogenesis of many conditions. The development of drugs to antagonize these oxidizing species may thus prevent at least some of the damage inflicted on tissues by these very reactive agents.

EXPERIMENTAL

Animal and Chemicals Male rats (*Rattus albinus novgicus*) with 190–200 g body weight and the following reagents were used: luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) sodium salt (A4685), lucigenin (M8010), zymosan A (Z4250), PMA (P8139), glycogen type II from oyster (G8751), DMSO (D8779), RPMI (R7755) and MTT-tetrazolium (M2128), all from Sigma Chemical Co. (U.S.A.); Ficoll-Paque Plus d 1077 (17-1440-02) from Pharmacia Biotech (Sweden). Paepalantine was obtained according to the procedure previously reported by Vilegas *et al.* (1990), and the stock solution was prepared at 10 mg/ml in DMSO. The highest absorption of paepalantine was at 265 nm.

Peritoneal Neutrophils Collection Rats peritoneal neutrophils were obtained by the intraperitoneal injection of 10 ml of oyster glycogen solution 0.5% (w/v). Peritoneal exudate was collected 12 h later with Dulbecco's phosphate-buffered saline without calcium (PBS-D), containing 10 UI heparin/ml. The cells were washed twice and carefully layered onto 5 ml of Ficoll-Paque, and centrifuged for 30 min at 700 g. The neutrophils were washed twice in Dulbecco's phosphate-buffered saline (PBS) and adjusted to a concentration of 1.0×10^6 cells/ml. This procedure yields a preparation containing 95% neutrophils, and the control of their viability showed 98% of the cells, as judged by MTT-tetrazolium assay.^{22,23)}

Effect of Paepalantine on Viability of Neutrophils One hundred microlitres of RPMI containing approximately 6×10^6 cells/ml were seeded into 96-well tissue-culture plates and incubated at 37 °C. After 1 h, the RPMI was removed and the cells were treated with paepalantine, in different doses, using PBS-D as a solvent. After 24 h, the medium was removed and the plates were prepared for the MTT-tetrazolium assay.^{14,15)} After brief agitation, the plates were transferred to a microplate reader (Spectra & Rainbow (Shell) Readers–Tecan, Austria), and the optical density of each well was measured using a 540 nm filter and a 620 nm reference wavelength. All experiments were performed at least four times, using three wells for each concentration of the chemical tested. The cytotoxicity data was standardized by determining absorbance and calculating the correspondent chemical concentrations. Linear regression analysis with 95% confidence limits was used to define dose–response curve and to compute the cytotoxic midpoint (IC₅₀), the concentration required to obtain a 50% decrease in the spectrophotometric absorbance as compared to the control.²⁴⁾

Chemiluminescence Assay Chemiluminescence was measured on a BioOrbit model 1251 luminometer (Turku, Finland) using polypropylene tubes with a 1.0 ml reaction mixture. Briefly, 1.0×10^6 cells/ml of neutrophils, and 2×10^{-5} M luminol or 2×10^{-5} M lucigenin were added in tubes containing PBS-D. After incubation for 10 min at 37 °C, 0.7 mg/ml nonopsonized zymosan or 3×10^{-7} M PMA and

PBSD with paepalantine at non-cytotoxic concentrations (0.12, 0.48, 0.98, 1.98, 3.9, 7.8 $\mu\text{g/ml}$) were mixed thoroughly. The background was measured without stimuli and paepalantine. The luminol and lucigenin-enhanced chemiluminescence was calculated by the software Multiuse 2.0. The chemiluminescence response was quantitated as an integrated area (IA) below the resulting chemiluminescence curve during a period of 0 to 40 min for luminol chemiluminescence and 0 to 15 min for lucigenin chemiluminescence. The values were compared to those of the control curve containing no paepalantine, *i.e.*, cells/luminol or lucigenin/stimuli. The ED_{50} , *i.e.*, dose of paepalantine producing 50% inhibition of control chemiluminescence, was calculated using a log-plot transformation of the data.

Effect of the Paepalantine on $\text{MPO}/\text{H}_2\text{O}_2$ and $\text{MPO}/\text{H}_2\text{O}_2/\text{Cl}^-$ Reaction mixtures, including PBS– MgSO_4 (chloride-ion-free) or PBSD pH 7.4, 2×10^{-5} M luminol (final concentration), 75 mU MPO and paepalantine at concentrations of 0.98, 1.98, 3.9 and 7.8 $\mu\text{g/ml}$, were mixed thoroughly. The assay was initiated by the addition of H_2O_2 (5×10^{-5} M), and chemiluminescence was recorded for 10 min. The values were compared with the control curve (systems without paepalantine), which was taken as 100% chemiluminescence.

Reactions with Hypochlorous Acid Reactions of HOCl were studied using the method of Ching *et al.*,²⁵ based on the oxidation of 5-thio-2-nitrobenzoic acid (TNB). TNB was obtained by reducing a 1 mM solution of 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) in a 50 mM KH_2PO_4 –KOH buffer, pH 6.6, containing 5 mM EDTA, and 20 mM sodium borohydride. For the assay, 25 μM HOCl was incubated with 50 μM TNB for 5 min in the presence or absence of paepalantine in a final volume of 1 ml. The ability of the paepalantine to scavenge HOCl was determined by measuring the oxidation of TNB to DTNB at 412 nm, preincubating the paepalantine with HOCl, and then adding TNB.

Statistical Analysis The parameters were expressed as mean \pm standard deviation. Statistical analysis was performed with analysis of variance (ANOVA). Differences were considered significant at $p < 0.05$.

Acknowledgements This study was supported by a

grant of CNPq to RRR, a fellowship to WV, and financial assistance from the PADC-UNESP.

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