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APOPTOTIC ACTIVITY OF ISOESPINTANOL DERIVATIVES IN HUMAN POLYMORPHONUCLEAR CELLS

ACTIVIDAD APOPTÓTICA DE DERIVADOS SEMISINTÉTICOS DEL ISOPENTANOL EN CÉLULAS POLIMORFONUCLEARES HUMANAS

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ABSTRACT

Background: Inflammation is a complex physiopathologic response to different stimuli. Recently, some pharmacological strategies have been proposed that could be used for resolution of inflammation by enhancing apoptosis of inflammatory cells. **Objectives:** To study in vitro apoptotic activity of isoespintanol [ISO] and of two semi-synthetic derivatives, bromide isoespintanol [BrI] and demethylated isoespintanol [DMI], in human polymorphonuclear (PMN) cells. **Methods:** PMN were exposed to the different concentrations of ISO, BrI and DMI for 30 min in phosphate-buffered saline pH 7.4 containing 1 mg/mL glucose, 0.4 mM Mg^{2+}, and 1.20 mM Ca^{2+}. Viability was assessed by dimethylthiazol diphenyl tetrazolium bromide (MTT). To distinguish between the two modes of cell death, apoptosis and necrosis, we examined differences in morphological and biochemical changes of cells stained with annexin V- FITC (An) and/or propidium iodide (PI) using two different assays based on flow cytometry **Results:** The MTT assay revealed the ability of cells to reduce MTT salt to formazan. In the presence of BrI and DMI a significant concentration-dependent decrease of cell viability was observed. The annexin V- FITC binding assay showed a high proportion of apoptotic cells for those treated with BrI (An^+/ PI^-: 62.3 ± 8.2% vs. 2.1 ± 0.5% of control, $P<0.05$). The population of PMN treated with DMI produced the highest percentage (An^+/IP^+: 43.4 ± 5.2 % vs. 0.4 ± 0.3 % of control, $P<0.05$) of necrotic cells. Apoptotic nuclei were analyzed by PI staining. The cell population in the sub G_0/G_1 region represents cells with hypodiploidal DNA, an indicator of apoptosis. When cells were incubated with 50 and 100 μM of BrI, the cell population in the sub G_0/G_1 region increased, suggesting a dose-dependent increase in the population of apoptotic cells. The presence of the pan-inhibitor of caspases (Z-VAD-fmk) showed a significant reduction in cell population in the sub G_0/G_1 region, indicating less degradation of DNA. **Conclusions:** Bromide isoespintanol [BrI] induces an apoptotic process in PMN, mediated –at least in part– by activation of caspases, although this compound may probably act through other caspase-independent mechanisms as well.

Keywords: Isoespintanol, inflammation, neutrophils, apoptosis

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RESUMEN

Antecedentes: La inflamación es una respuesta fisiopatológica compleja generada por diferentes estímulos. Recientemente, se han propuesto nuevas estrategias farmacológicas que podrían ser utilizadas para conducir a la resolución de la inflamación mediante el aumento de la apoptosis de células inflamatorias. **Objetivo**: Estudiar la actividad apoptótica in vitro del isopentanol [ISO] y dos derivados semisintéticos –bromuro de isoespintanol [BrI] e isoespintanol desmetilado [DMI] – en células polimorfonucleares humanas (PMN). **Métodos:** Las PMN fueron expuestas a diferentes concentraciones de los compuestos durante 30 min en una disolución salina tamponada con fosfato (pH 7,4). La viabilidad celular se evaluó utilizando el ensayo de 3-[4,5-dimetil-tiazol-2-il]-2,5-difenil tetrazolio (MTT). Para distinguir entre los dos modos de muerte celular, la apoptosis y la necrosis, se examinaron las diferencias en los cambios morfológicos y bioquímicos de las células teñidas con anexina V (An) y/o yoduro de propidio (PI) usando dos técnicas de citometría de flujo. **Resultados:** Mediante el ensayo con MTT, se demostró que los compuestos BrI y DMI disminuyeron significativamente y de manera concentración-dependiente la viabilidad celular. El ensayo de unión con la anexina V-FITC mostró una alta proporción de células apoptóticas en las células tratadas con BrI (An^+/PI^-: 62,3 ± 8,2% versus 2,1 ± 0,5% del control, $P < 0,05$). La población de células tratadas con DMI mostraron el porcentaje más alto de células necróticas (A^+/PI^+: 43,4 ± 5,2% versus 0,4 ± 0,3% del control, $P < 0,05$). El análisis de núcleos apoptóticos se llevó a cabo a través de tinción con PI. La población de células en la región sub G_0/G_1 representa células con ADN hipodiploidal, que es un indicador de apoptosis. Cuando las células se incubaron con BrI, la población de células en la región sub G_0/G_1 aumentó, confirmando su mecanismo citotóxico. En presencia de un inhibidor de caspasas (Z-VAD-FMK), se observó una reducción significativa en la población celular en la región sub G_0/G_1, indicando una menor degradación del ADN. **Conclusiones:** El bromuro de isoespintanol [BrI], induce un proceso apoptótico en PMN que está mediado –al menos en parte– por la activación de las caspasas, aunque este compuesto probablemente podría actuar también a través de otros mecanismos independientes de las caspasas.

Palabras clave: Isoespintanol, inflamación, neutrófilos, apoptosis

INTRODUCTION

Mature human neutrophils have the shortest life span of all leukocytes; their spontaneous apoptosis is therefore fundamental for maintaining a normal level of circulating neutrophils and ensuring rapid resolution of inflammatory responses (1). Not surprisingly, a reduction in neutrophil apoptosis has already been linked to several inflammatory conditions including rheumatoid arthritis and acute respiratory distress syndrome (2, 3).

The occurrence of isoespintanol (2-isopropyl-3,6-dimethoxy-5-methylphenol) was reported in the chloroform extract of aerial parts of *Eupatorium saltense* (4) and has also been synthesized as part of the structure elucidation of the natural product espintanol (5). Recently, we demonstrated that isoespintanol, isolated from the leaves of *Oxandra cf. xylopioides*, reduces paw edema induced by carrageenan. Moreover, it reduces IL-1b production and decreases IL-1b mRNA synthesis in murine macrophages RAW264.7, both mechanisms possibly responsible for its anti-inflammatory action (6). We also studied and demonstrated its antioxidant activity in both theoretical and experimental protocols (7, 8).

In this work, we study *in vitro* apoptotic activity of isoespintanol [ISO] and two semisynthetic derivatives, bromide isoespintanol [BrI] and demethylated isoespintanol [DMI] Figure 1, on human polymorphonuclear (PMN) cells.

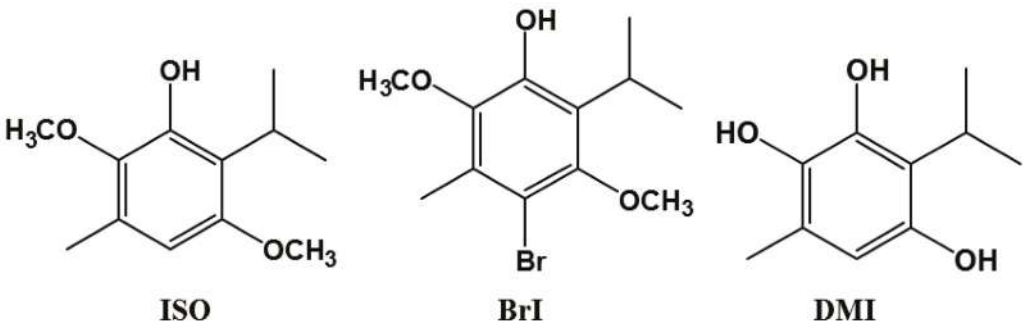

Figure 1. Chemical structures of isoespintanol (2-isopropyl-3,6-dimethoxy-5-methylphenol, ISO), isoespintanol 4-bromide (BrI) and 3,6-demethylated isoespintanol (DMI).

MATERIALS AND METHODS

Test compounds and chemicals

Isoespintanol [ISO, purity >99%, ^{1}H-NMR] was previously isolated from leaves of *Oxandra cf. xylopioides*. BrI and DMI (purity of both >99%, ^{1}H-NMR) were obtained from isoespintanol as previously reported by Galeano et al. (9). All other chemicals were the highest analytic grade available and were purchased from Sigma-Aldrich (USA) or from Merck (Germany).

Cell isolation and culturing

Neutrophils were isolated from fresh anti-coagulated peripheral blood obtained from healthy volunteers, then purified using a standard protocol. Briefly, erythrocytes were sedimented by adding an equal volume of dextran/saline solution (3% dextran T-500 in 0.9% NaCl) at room temperature for 30 min. The leukocyte-rich upper layer of the suspension was then collected and centrifuged on a density gradient with Histopaque®-1077 (Sigma-Aldrich) according to manufacturer's instructions.

Residual erythrocytes were removed by hypotonic lysis (cold water). Neutrophils were washed twice in phosphate-buffered saline (PBS) pH 7.4, then resuspended in the same buffer containing 1 mg/mL glucose, 0.4 mM Mg^{2+}, and 1.20 mM Ca^{2+}. Cell viability was determined by the trypan blue dye exclusion method.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

The MTT assay (10) was used to determine the cytotoxicity of the compounds. Briefly, human PMN cells (2.5×10^6) were incubated at 37 °C for 3 h with PBS (pH 7.4) containing the compounds (1 – 100 μM). Controls were treated with the vehicle and correspond to 100% viability. Formazan deposits produced by the cells in each tube were dissolved in a solution of 10% sodium dodecyl sulfate (SDS) in 0.01 M HCl and tubes were incubated overnight. The soluble product was quantified, measured at 570 nm (with background subtraction at 630 nm) in a Beckman DU 640.

Analysis of apoptotic (hypodiploid) nuclei

Apoptotic nuclei were analyzed by propidium iodide staining, based on the protocol described by Nicoletti et al. (11) with slight modifications. Briefly, cells were incubated under the same conditions previously described, then centrifuged (500×*g*). The resulting pellet was suspended in ice-cold ethanol (70%) and stored at –20 °C for at least 30 min. Cells were then washed twice in PBS at 4 °C and resuspended in 500 mL of DNA staining solution (20 mg/mL PI plus 0.2 mg/mL RNAse A in PBS). Resuspended cells were incubated in the dark at room temperature for 30 min, after which fluorescence of individual nuclei was measured with a Becton Dickinson FACSScan flow cytometer (USA). A minimum of 20,000 events were counted per sample. The percentage of apoptotic cell nuclei (hypodiploid DNA peak) was then calculated. Benzyloxycarbonyl-Val-Ala-Asp (OMe) fluoromethylketone (Z-VAD-fmk), a pan-inhibitor of caspases, was added to the cell suspension just before application of each compound.

Annexin V-fluorescein isothiocyanate (FITC) in apoptotic cells

Phosphatidylserine exposure was measured by annexin V-FITC binding (12). After appropriate incubations with the compounds (100 mM), cells

(2.5×10^6) were washed twice with cold PBS and resuspended in a binding buffer solution (10 mM Hepes/NaOH pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) at a concentration of 1×10^6 cells/mL. Then, 1×10^5 cells were transferred to a 5 mL culture tube to which 5 mL of Annexin V-FITC and 10 mL of propidium iodide solution (50 mg/mL in PBS) were added. Tubes were then incubated for 15 min at room temperature in the dark. After incubation, 400 mL of binding buffer were added to each tube and flow cytometry analyses were run.

Statistical analysis

Results are expressed as means ± SD for the indicated number of observations or illustrated by an observation representative of results from several different experiments. Statistical analysis used ANOVA followed by Dunnett's *t*-test for multiple comparisons using the Prism analysis program (GraphPad). Differences were considered significant when $P<0.05$.

RESULTS

Our research included an assessment of the cytotoxicity of the extracts on human PMN cells. The MTT assay revealed that the ability of cells to reduce the MTT salt to formazan decreased significantly in the presence of BrI and DMI (Figure 2). The results were expressed as a percentage of the control (cells treated with 0.5% DMSO). No cytotoxic activity was detected in selected concentrations of ISO.

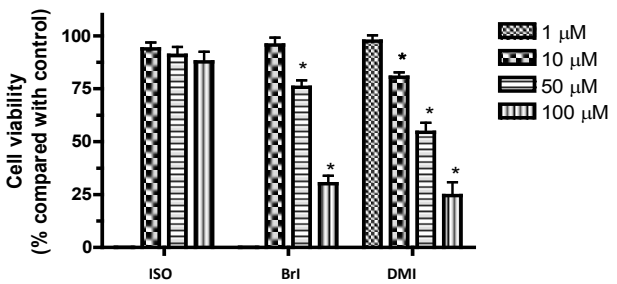

Figure 2. Effects of isoespintanol [**ISO**] and related compounds [**BrI** and **DMI**] in four concentrations (1, 10, 50 and 100 mM) on cell viability of PMN cells. Values represent percentages of live cells compared to the control group treated with vehicle only (culture medium with 0.5% dimethyl sulfoxide). Data represent the mean and standard deviation of five different experiments. The symbol ⋆ indicates the significant difference (*P<0.05*).

The nature of the cytotoxicity was evaluated with different flow cytometry methods, including the exposure of membrane phosphatidylserine through Annexin V-FITC binding and the development of hypodiploid nuclei by propidium iodide.

While only a small proportion of PMN untreated or treated with ISO underwent apoptosis [Annexin V-FITC positive, propidium iodide negative (An^+/IP^-)], the proportion was significantly greater for the cells treated with BrI (An^+/PI^-: 62.3 ± 8.2% vs. 2.1 ± 0.5% of control, $P<0.05$). The population of PMN cells treated with DMI produced the highest percentage (A^+/IP^+: 43.4 ± 5.2 % vs. 0.4 ± 0.3 % of control, $P<0.05$) with a typical distribution indicative of the necrotic process (Figure 3).

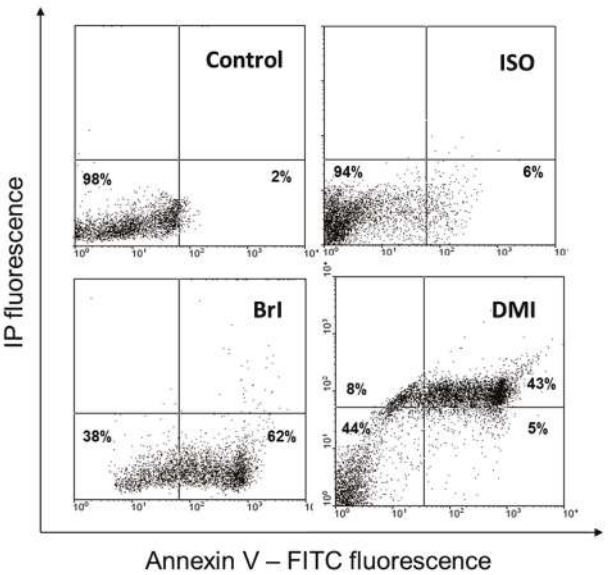

Figure 3. Promotion of apoptosis in neutrophils by isoespintanol and related compounds. Representative dot plots of annexin V-FITC (An) /propidium iodide (PI) staining. The lower left quadrant shows the vital (double negative) population. The lower right quadrant shows the apoptotic (An^+/PI^-) population. Finally, cells in the top right quadrant (An^+/PI^+) are in later stages of apoptosis and necrosis. Graph shows the experiment of the compounds at 100 μM. Data are representative of three experiments.

The relative distribution of DNA in the cells incubated with 50 and 100 μM of BrI is shown in Figure 4A and 4B. The line in the sub G_0/G_1 region of the histogram indicates the range of cells with hypodiploidal DNA, an indicator of apoptosis. The results showed that BrI-induced concentration-dependent changes in the percentage population of cells with hypodiploidal DNA.

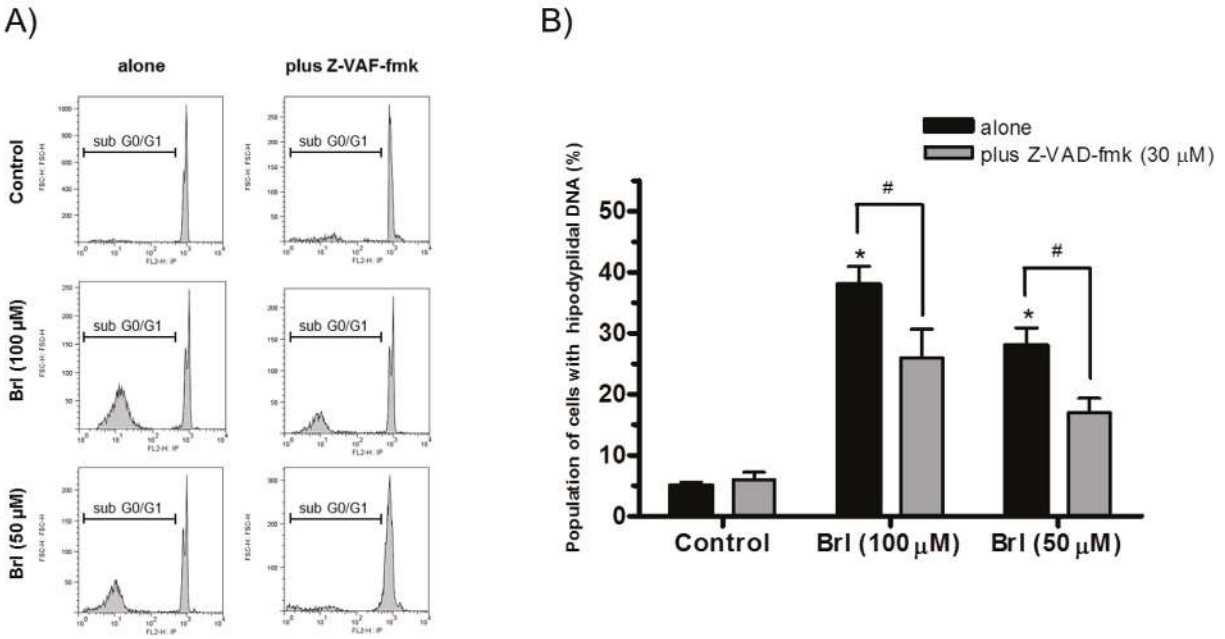

Figure 4 (A and B). Effects of Benzyloxycarbonyl-Val-Ala-Asp (OMe) fluoromethylketone (Z-VAD-fmk) on BrI-induced changes in the percentage population of cells with hypodiploidal DNA. A) The histogram of propidium iodide fluorescence monitored from 20,000 cells. The line in the sub G_0/G_1 region of the histogram indicates the range of cells with hypodiploidal DNA; B) BrI-induced concentration-dependent changes in the percentage population of cells with hypodiploidal DNA. The column and bar indicate the mean and standard deviation of 4–6 experiments. The symbol ⋆ indicates the significant difference ($P<0.05$) between the control group and the BrI-treated group. The symbol # indicates a significant difference ($P<0.05$) between groups with and without 30 μM Z-VAD-fmk treatment.

In order to investigate involvement of caspase activation in BrI-induced cell death, the effect of the pan-inhibitor of caspases (Z-VAD-fmk) was examined (Figura 4A and 4B). The increase in the population of cells with hypodiploidal DNA induced by BrI was suppressed by 30 μM Z-VAD-fmk, showed a significant reduction in the cell population in the sub G_0/G_1 region (Figura 4B).

DISCUSSION

Inflammation is a complex physiopathologic response to different stimuli. It can be treated and resolved by acting on different mediators, enzymes, and pathways involved in the process. This may include influencing arachidonate metabolism by inhibiting either certain transcription factors or production and/or scavenging of free radicals produced during the process, and by acting on cells involved in the process. New cellular and molecular circuits involved in resolution of inflammation are being found at a rapid pace. In some experimental models, a causal relationship has been detected between neutrophil apoptosis and the outcome of acute tissue injury (13). Recently, several publications have proposed pharmacological strategies that could be used for resolution of inflammation by enhancing apoptosis of inflammatory cells (1, 14). Consequently, our research included assessment of the cytotoxicity of monoterpene isoespintanol [ISO] and two related compounds, BrI and DMI, on human PMN cells.

In the presence of BrI and DMI a significant and concentration-dependent decrease of cell viability was observed. No cytotoxic activity was detected in the assay of compound ISO (Figure 2).

To distinguish between the two modes of cell death, apoptosis and necrosis, we examined differences in morphological and biochemical changes of dying cells by two different assays based on flow cytometry techniques.

As shown by the annexin V- FITC binding assay (Figure 3), the proportion of apoptotic cells was much greater for those treated with BrI (An^+/PI^-: 62.3 ± 8.2% vs. 2.1 ± 0.5% of control, $P<0.05$). The population of PMN cells treated with DMI produced a typical distribution indicative of the

necrotic process (Figure 3), for this reason, it was not selected for the following studies.

The relative distribution of DNA in the cells incubated with BrI is shown in Figure 4A and 4B. The cell population in the sub G_0/G_1 region as shown in Figure 4A represents cells with hypodiploidal DNA, an indicator of apoptosis. When cells were incubated with 50 and 100 μM of BrI, the cell population in the sub G_0/G_1 region increased, suggesting that BrI induced a dose-dependent increase in the population of apoptotic cells Figure 4A.

Apoptosis of granulocytes is a sensitive, responsive and highly regulated process. Various signaling pathways confer these features. Each pathway is responsive to specific endogenous stimuli and mediates pro- or anti-apoptotic effects; some are dependent on the intervention of caspases and other independents (1, 13-17).

Caspases are a family of cysteine proteases that play essential roles in apoptosis, necrosis, and inflammation (18). These proteases are essential in cells for apoptosis, or programmed cell death, and their role in the mechanism of active compounds can be investigated using Z-VAD-fmk, a pan-inhibitor of caspases (19). For studying the possible effects of our compounds on this way of programed cell death, we applied the inhibitor to cell culture in order to know the possible inhibition of capases can be related with the mechanism of isoespintanol and its derivatives. The presence of Z-VAD-fmk showed a significant reduction in the cell population in the sub G_0/G_1 region, indicating less DNA degradation Figure 4B, whereas the presence of the inhibitor suppressed the increase in the cell population with hypodiploidal DNA induced by BrI.

In this paper, we intend to contribute to evidence of the ability of some compounds to induce apoptosis in PMN. We conclude that BrI induce an apoptotic process in human PMN cells partially mediated by activation of caspases, although this compound may also probably act through other caspase-independent mechanisms.

We agree that extrapolation in vivo of results obtained in cultured cells with a compound is problematic and that is why commentaries on such potential extrapolation in the text of the manuscript are very cautious. Further studies should focus on studying possible mechanisms of action of this compound, which contributes to neutrophil apoptosis, as well as its potential anti-inflammatory activity in *in vivo* models.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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