

Antineoplastic activity of rinvanil and phenylacetylirinvanil in leukaemia cell lines

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Abstract. In the search for novel chemotherapeutic agents for cancer treatment, capsaicin has been shown to inhibit proliferation and induce apoptosis in various types of cancer cell line, including leukaemia cell lines. The capsaicin analogues, rinvanil and phenylacetylirinvanil (PhAR), share a binding affinity for vanilloid receptors and may have biological activities similar to capsaicin; however, their anticancer potential has not yet been reported. This study analyses the antineoplastic activities of rinvanil and PhAR in leukaemia versus normal cells. P388, J774 and WEHI-3 leukaemia cell lines, as well as mouse bone marrow mononuclear cells, were cultured with varying concentrations of rinvanil and PhAR. Following this, proliferation and apoptosis were determined by the sulforhodamine B (SRB) assay and DNA ladder. Cultured leukaemia cell lines and mouse bone marrow mononuclear cells demonstrated a dose-dependent inhibition of proliferation, while non-diseased cells were less sensitive to the cytotoxic effect of capsaicin, rinvanil and PhAR. Rinvanil and PhAR also induced apoptosis in leukaemia cell lines but not in bone marrow. Given the lower IC₅₀ values for apoptosis induction in leukaemia cells compared with that of normal cells, PhAR is a promising selective anticancer agent.

Introduction

A number of advances in the understanding of cancer on a biochemical, molecular, cellular and physiological level have been made in recent years and used in clinical diagnosis and

the implementation of novel therapies. Despite this, there has been an increase in the number of new clinical cases of cancer, which is spurring the need for new medicines and treatment alternatives to tackle what is now a leading cause of mortality worldwide (1).

Capsaicin (N-[(4-hydroxy-3-methoxyphenyl)methyl]-8-methyl-(6E)-6-nonenamide; Fig. 1) is a naturally occurring organic compound and the main member of the capsaicinoid family, a group of compounds that give a characteristic pungency to the fruit of the chili pepper plant (*Capsicum spp.*). This effect is due to the agonist activity of the capsaicin receptor, transient receptor potential vanilloid subfamily 1 (TRPV1). Expressed in sensory neurons, TRPV1 is a nonselective cation channel that is activated by heat ($\geq 43^{\circ}\text{C}$) or protons, and also plays an important role in the transmission of pain impulses (2). Prolonged treatment with capsaicin desensitises neurons and thus generates an analgesic effect (3). Consequently, numerous studies have been investigating the role of capsaicin and its interaction with TRPV1 receptors in the treatment of a variety of pathologies that present with hyperalgesia, including rheumatoid arthritis (4,5), chronic neuropathic pain (6), diabetic neuropathy (7,8) and other neuralgias (9). In the search to identify new chemotherapeutic agents for cancer treatment, it has been revealed that capsaicin has inhibitory effects on cell proliferation and apoptosis induction in various types of cancer cell lines, including HepG2 (human hepatoma); AGS (gastric cancer); PC-3 (prostate cancer); MCF-7 (breast cancer); U373, U87, FC1 and FLS (glioma); NB4, UF-1, Kasumi-1, HL-60, K562, KU812 and U937 (leukaemia); and HT-29 cells (colon cancer) among others (10-16). Furthermore, several studies have demonstrated that capsaicin may have chemoprotective properties against certain carcinogenic and mutagenic agents (17,18) as well as the ability to induce terminal differentiation in A172 human glioblastoma cells (19). The evidence from these studies indicates the strong antitumour potential of capsaicin. The methods of obtaining capsaicin from natural sources are often inefficient and expensive, due to its low content in the fruits of the genus *Capsicum* and the presence of other compounds with similar polarity. In addition, due to its high pungency, it is difficult to manage its production by synthetic methods (20), which is an obstacle to its development as a potential chemotherapeutic

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agent. Two capsaicin analogues, rinvanil and phenylacetylirinvanil (PhAR) have been synthesised (Fig. 1). The latter is the most potent TRPV1 receptor agonist synthesised to date and is devoid of pungency (21,22); in fact, it is 1,000x more potent than capsaicin in its affinity for TRPV1 receptors (23).

While the structural and physiological associations between rinvanil, phenylacetylirinvanil (PhAR) and capsaicin are known, their antineoplastic effects have not yet been described. This study evaluates the antiproliferative and pro-apoptotic effects of rinvanil and PhAR on P388 mouse leukaemia cell lines, J774 and WEHI-3 leukaemia cell lines, and cultures of mononuclear cells from normal mouse bone marrow.

Materials and methods

Capsaicin and capsaicin analogues. Capsaicin was purchased from Sigma-Aldrich (St. Louis, MO, USA). Rinvanil and PhAR were synthesised and characterised as described in the study by Castillo *et al.* (24).

Animals. In this study, 12-week-old female BALB/c were used and maintained in pathogen-free conditions. Experiments were performed in the animal facility of FES-Zaragoza, National Autonomous University of Mexico (Iztapalapa, Mexico), in accordance with institutional guidelines. The mice were provided with autoclaved water and fed a standard powdered rodent diet *ad libitum*. All experimental protocols were approved by the ethics committee of FES-Zaragoza, National Autonomous University of Mexico in accordance with national regulations for the care and use of experimental animals.

Cell culture. Total bone marrow cells of the mice were obtained from the femur by flushing with Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% FBS. Mononuclear cells (MNCs) were obtained from the total cells via gradient separation with Ficoll-Paque (Amersham Biosciences AB, Uppsala, Sweden) at a density of 1.077 g/ml and washed twice with phosphate-buffered saline (PBS). MNCs were cultured for 120 h in IMDM (Gibco-BRL, Carlsbad, CA, USA) supplemented with 15% (v/v) FBS, 5% (v/v) horse serum (Gibco-BRL) and 5 ng/ml recombinant mouse interleukin-3 (rmIL-3) (R&D System, Minneapolis, MN, USA). The cells were maintained in a humidified atmosphere containing 5% CO₂ at 37°C and were maintained in a culture for a maximum of 120 h. P388, J774, and WEHI-3 mouse myeloid leukaemia cells were obtained from American Type Culture Collection (Manassas, VA, USA) and cultured in Iscove's Modified Dulbecco's Medium (Gibco-BRL) supplemented with 10% v/v foetal calf serum (Gibco-BRL) previously heat inactivated and kept at 37°C with 5% CO₂ and saturating humidity.

Cell proliferation assays. For proliferation assays, cells were grown in 96-well plates (Corning, Tewksbury, MA, USA) in the presence or absence of capsaicin, rinvanil and PhAR for 72 h under the culture conditions described above; proliferation was assessed with the sulforhodamine B (SRB) assay (Sigma-Aldrich) (25). Briefly, the cultured cells were fixed by adding 50 µl/well of cold trichloroacetic acid (TCA; Sigma-Aldrich) and incubated at 4°C for 1 h, followed by several washings to remove the TCA before the cell culture

plate was allowed to dry at room temperature. The cells were then added to 100 µl/well of 0.4% SRB diluted in 1% acetic acid. After remaining under the stain for 20 min, several washes were performed with 1% acetic acid and the plate was allowed to dry at room temperature. The dye was solubilised with 50 µl/well of 10 mM Tris base (pH 10.4) and the plate was read on a spectrophotometer (Tecan Spectra A-5082; Tecan Austria GmbH, Grödig, Austria) at 570 nm.

Cell viability. P388, J774 and WEHI-3 cell lines and normal mouse bone marrow cells were cultured in 96-well plates at cell densities of 3x10⁴, 5x10³, 1.3x10⁴ and 1x10⁵, respectively. They were then treated with or without IC₅₀ concentrations of capsaicin, PhAR and rinvanil (Table I) and incubated for 72 h (leukaemia lines) or 120 h (mononuclear cells from normal mouse bone marrow). Finally, the cell suspensions were mixed with an equal volume of Trypan blue dye (Sigma-Aldrich) and counted directly under a light microscope (Axio Vert.A1, Primo Star Carl Zeiss, Göttingen, Germany). Unstained cells were considered as viable and stained cells nonviable (26).

Apoptotic bodies. To assess the percentage of apoptotic bodies, the P388, J774 and WEHI-3 cell lines and mouse bone marrow cells, plated at a density of 1x10⁵ cells/ml, were treated with or without an IC₅₀ concentration of capsaicin, rinvanil and PhAR. After 24 h of incubation, a sample of cells was fixed with methanol, stained with Giemsa dye and observed by light microscopy using a 100X objective.

DNA fragmentation. To confirm the induction of apoptosis, DNA fragmentation was analysed. Briefly, P388, J774 and WEHI-3 cell lines (1x10⁶ cells/5 ml) were cultured in the presence or absence of IC₅₀ concentrations of capsaicin, rinvanil or PhAR for 24 h. Cells were collected by centrifugation and washed twice with PBS. The cell pellet was resuspended in 0.3 ml lysis buffer [100 mM NaCl, 10 mM Tris-HCl (pH 8.0), 25 mM EDTA (pH 8.0), 0.5% SDS, 100 µg/ml proteinase K; Promega, Madison, WI, USA] and incubated while shaken at 37°C for 4 h. The lysate was treated with 100 µg/ml RNase (Promega) for 1 h at 37°C, followed by extraction with phenol-chloroform-isoamyl alcohol (25:24:1); the upper phase was collected and precipitated with absolute ethanol overnight at -20°C. DNA was collected by centrifugation (14,000 x g; 4°C; 20 min) and washed with 75% ethanol. The DNA pellet was resuspended in 100 µl TE buffer [1 mM EDTA (pH 8.0), 10 mM Tris-HCl (pH 8.0)] and incubated at 65°C for 1 h to facilitate solubilisation. Finally, the DNA in 0.5 mg/ml ethidium bromide was subjected to electrophoresis using a 2% agarose gel (Invitrogen Life Technologies, Carlsbad, CA, USA) at 60 V for 2.5 h and visualised under UV light (27).

The results are shown as the mean ± SD of three independent experiments with three replicates. Statistical significance was determined using one-way analysis of variance followed by Dunnett's contrast. P<0.05 was considered to indicate a statistically significant difference.

Results

Rinvanil and PhAR inhibit the proliferation of leukaemia cell lines and mouse bone marrow cells. P388, J774 and WEHI-3

Table I. IC₅₀ values for the different leukaemia cell lines and mononuclear cells of normal mouse bone marrow.

Treatment	IC ₅₀ (μg/ml) ^a			
	P388	J774	WEHI-3	Bone marrow
Capsaicin	72.1±1.8	32.5±1.7	47.7±2.5	53.5±3.1
Rinvanil	49.2±2.2	10.2±3.0	31.2±3.6	72.6±2.4
PhAR	9.0±2.0	8.0±3.7	3.0±3.1	40.7±2.4

^aNecessary concentration to inhibit cell proliferation by 50%. PhAR, phenylacetylirinvanil.

Table II. Cell viability (%) of the leukaemia cell lines and mononuclear cells of normal mouse bone marrow treated with the IC₅₀ concentrations of capsaicin, rinvanil and PhAR.

Treatment	Cell viability (%)		
	Capsaicin	Rinvanil	PhAR
(A)			
Vehicle	97±2.8	97±2.8	97±2.8
IC ₅₀	80±9.0 ^a	93±6.2 ^a	93±3.8 ^a
(B)			
Vehicle	98±1.9	98±1.9	98±1.9
IC ₅₀	55±6.2 ^a	76±6.9 ^a	60±8.0 ^a
(C)			
Vehicle	98±4.2	98±4.2	98±4.2
IC ₅₀	86±5.0 ^a	91±7.4	91±7.7
(D)			
Vehicle	97±3.3	99±3.3	99±3.3
IC ₅₀	94±2.2	80±6.3 ^a	91±3.7

Percent viability of (A) P388, (B) J774, (C) WEHI-3 leukaemia cell lines and (D) bone marrow mononuclear cells of normal mice treated with IC₅₀ concentrations of capsaicin, PhAR and rinvanil. Cell cultures were stimulated at time zero and after 3 days of incubation the viability was assessed by trypan blue exclusion. The results are shown as the mean ± SD for three independent experiments with six replicates. ^aP<0.05 compared with vehicle (0 mg/ml), according to analysis of variance with Dunnett's test. PhAR, phenylacetylirinvanil.

leukaemia cell lines and normal mouse mononuclear bone marrow cells were cultured in 96-well plates at cell densities of 3×10⁴, 5×10³, 1.3×10⁴ and 1×10⁵, respectively, to demonstrate that rinvanil, PhAR and capsaicin (as a control), inhibit the proliferation of leukaemia cell lines, as well as mouse bone marrow cells, in a dose-dependent manner (Fig. 2). Based on the values of the average doses of inhibition (IC₅₀), the leukaemia cell lines, except for P388 cells treated with capsaicin, were more sensitive than the normal bone marrow cells (Table I). Even the IC₅₀ value of PhAR in P388 cells, 9.0 μg/ml, was increased to 40.7 μg/ml in bone marrow, providing evidence that leukaemia cells are 4.5-fold more sensitive compared with normal cells.

Table III. Percentage of apoptotic bodies in different leukaemia cell lines and mononuclear cells of normal mouse bone marrow.

Treatment	Apoptotic bodies (%)			
	P388	J774	WEHI-3	Bone marrow
Vehicle	0	2±1	0	5±3
Capsaicin	41±2 ^a	31±3 ^a	17±3 ^a	6±4
Rinvanil	41±3 ^a	30±3 ^a	15±2 ^a	7±2
PhAR	42±5 ^a	37±2 ^a	16±2 ^a	6±4

The results are shown as the mean ± SD of three independent experiments with three replicates. ^aP<0.05 compared with vehicle (0 mg/ml), according to analysis of variance with Dunnett's test. PhAR, phenylacetylirinvanil.

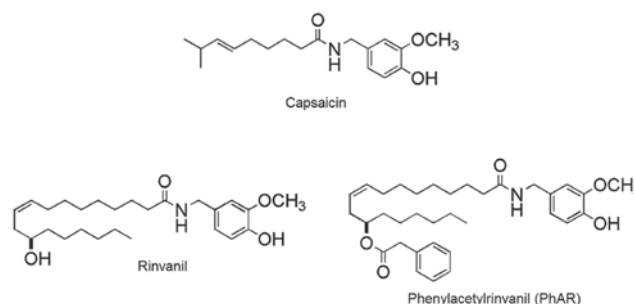


Figure 1. Chemical structures of capsaicin, rinvanil and phenylacetylirinvanil.

PhAR is selectively cytotoxic to P388, J774 and WEHI-3 leukaemia cells. To determine if the decrease in proliferation was due to cell death induced by capsaicin, rinvanil and PhAR, cell viability was determined by evaluating membrane integrity with trypan blue dye exclusion. The P388, J774 and WEHI-3 leukaemia cells and normal mouse bone marrow cells were cultured with IC₅₀ concentrations of capsaicin, PhAR and rinvanil, and incubated for 72 h (leukaemia lines) or 120 h (mononuclear mouse bone marrow). Our results show that in the case of J774 and P388 leukaemia lines, the three compounds significantly reduced cell viability with respect to the vehicle, although J774 cells were more sensitive, with viability percentages of only 55, 76 and 60% for cells treated with capsaicin, PhAR and rinvanil, respectively. In the case of WEHI-3 cells, capsaicin was the only agent to significantly reduce viability compared with cells treated with vehicle alone. Cells from normal mouse bone marrow had viabilities of 94 and 91% when treated with capsaicin and PhAR, respectively, without significant difference from vehicle, while rinvanil reduced viability to 80% (Table II). These results confirm that PhAR is selectively cytotoxic to leukaemia and similar to capsaicin (28,29).

Rinvanil and PhAR induce cell death by apoptosis in P388, J774 and WEHI-3 cell lines, but not in mouse bone marrow cells. One important characteristic of cancer cells is their resistance to death by apoptosis (programmed cell death); apoptosis induction is an important parameter in determining the therapeutic antitumour potential of a compound (30).

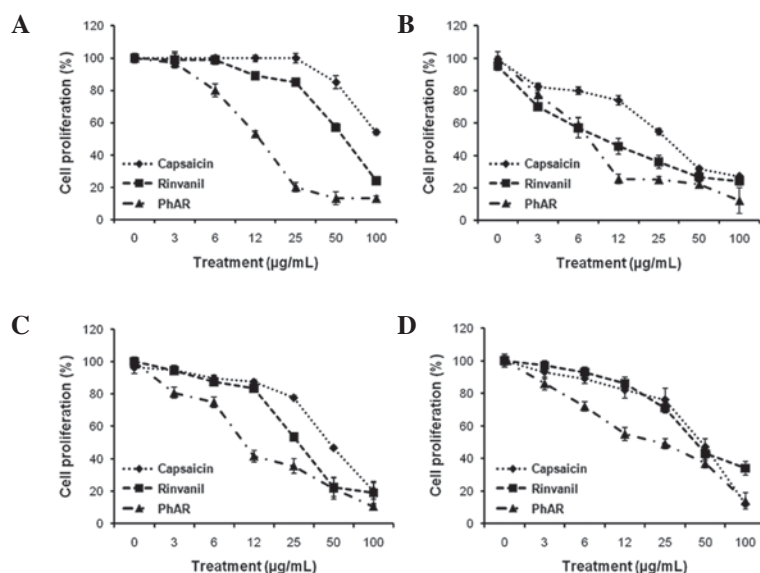


Figure 2. Antiproliferative effect of capsaicin, PhAR and rinvanil in (A) P388, (B) J774 and (C) WEHI-3 leukaemia cell lines and (D) mononuclear cells of normal mouse bone marrow. The cells were grown in 96-well plates with cell densities of 3×10^4 , 5×10^3 , 1.3×10^4 and 1×10^5 cells/ml, respectively, and treated with increasing concentrations of the compounds tested (0, 3, 6, 12, 25, 50 and 100 mg/ml). Cell proliferation was determined with the sulforhodamine B assay after 72 h incubation for leukaemia cell lines and 120 h for bone marrow cells. The results are shown as the mean \pm SD of three independent experiments with three replicates each. PhAR, phenylacetylirinvanil.

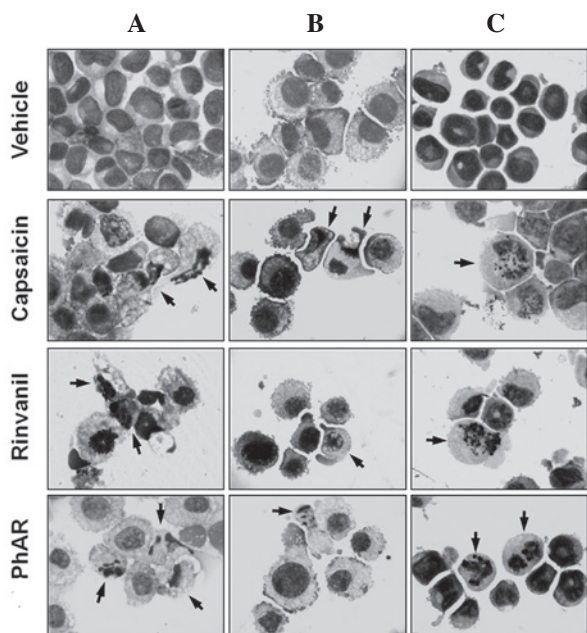


Figure 3. Morphology of apoptotic bodies of (A) J774, (B) P388 and (C) WEHI-3 cells. Leukaemia cell lines were cultured at a density of 1×10^5 cells/ml and treated with or without IC_{50} concentrations of capsaicin, PhAR and rinvanil for 24 h. After treatment, cells were resuspended and applied to slides by cytospinning, fixed with methanol, stained with Giemsa and observed under light microscopy using a 100X objective. PhAR, phenylacetylirinvanil.

To assess whether PhAR and rinvanil induce cell death by apoptosis, P388, J774 and WEHI-3 cell lines and mouse bone marrow cells were cultured at a density of 1×10^5 cells/ml with or without IC_{50} concentrations of PhAR and rinvanil, using capsaicin as a positive control for the induction of apoptosis. After 24 h of incubation for leukaemia cell lines or 120 h for

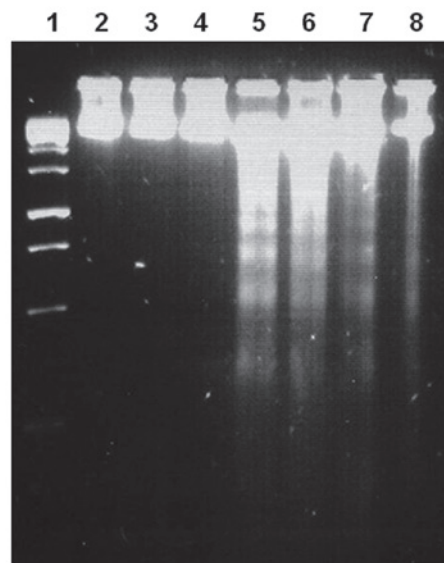


Figure 4. DNA fragmentation induced by PhAR in P388, J774 and WEHI-3 leukaemia cell lines, or by capsaicin in P388 cells. Lane 1, molecular weight marker; lanes 2-4, P388, J774 and WEHI-3 cells, respectively, treated with vehicle alone; lanes 5-7, P388, J774 and WEHI-3 cells, respectively, treated with IC_{50} concentrations of PhAR; lane 8, P388 cells treated with an IC_{50} concentration of capsaicin. P388, J774 and WEHI-3 cell lines (1×10^6 cells/5 ml) were cultured for 24 h in the presence or absence of IC_{50} concentrations of PhAR, and P388 cells were treated with an IC_{50} concentration of capsaicin. At the end time of stimulation, DNA was extracted and visualised on an 2% agarose gel under UV light. PhAR, phenylacetylirinvanil.

cultured bone marrow cells, cells were stained with Giemsa and examined by light microscopy. The cell lines demonstrated characteristics of apoptosis (31), including an increase in cell volume, cytoplasmic modifications, change in nuclear size, nuclear chromatin condensation and nuclear fragmentation, as well as an intact cell membrane with the formation of apoptotic

bodies (Fig. 3). Cultured mouse bone marrow mononuclear cells treated with these compounds showed no significant changes in cell morphology with respect to the vehicle. A quantification of the percentage of apoptotic bodies confirms that the leukaemia cell lines undergo apoptosis in response to PhAR and rinvanil treatment, while normal bone marrow cells do not (Table III).

As PhAR was demonstrated to have a lower IC₅₀ for apoptosis induction, as observed by the formation of apoptotic bodies in cell lines, this molecule was used to confirm the induction of apoptosis by DNA fragmentation analysis.

For this, P388, J774 and WEHI-3 cells (1x10⁶ cells/5 ml) were cultured in the presence or absence of IC₅₀ concentrations of PhAR for a period of 24 h; as a control, P388 cells were treated with an IC₅₀ concentration of capsaicin. DNA was then extracted and visualised on an agarose gel under UV light (Fig. 4). These results show a typical pattern of internucleosomal DNA fragmentation of 180-200 bp in leukaemia cells treated with PhAR, as does capsaicin in P388 cells.

Discussion

It is known that capsaicin can inhibit the proliferation of several human and mouse tumour cell lines without affecting normal cell proliferation, and that this antiproliferative effect is due to the induction of apoptosis (28,29). As rinvanil and PhAR are synthetic capsaicinoids, we evaluated the effect of these compounds on cell proliferation using capsaicin as a control. Based on the values of IC₅₀, the leukaemia cell lines were more sensitive than the normal bone marrow cells (except for P388 treated with capsaicin). As the high potential for inhibition of proliferation observed with PhAR for normal and leukaemia cells correlates with its high affinity for vanilloid receptors, it would be of interest in the future to assess whether the difference in proliferative response is due to the density of receptors expressed in each cell type.

To determine whether the decrease in proliferation was due to cell death induced by capsaicin, rinvanil and PhAR, cell viability was determined by evaluating membrane integrity with trypan blue dye exclusion. The results confirm that PhAR is selectively cytotoxic to leukaemia, similar to capsaicin (28,29).

Capsaicin, the pungent component present in chili pepper, has potential anti-inflammatory, antioxidant, antiproliferative and anticancer properties; it also has chemopreventive effects against chronic inflammatory diseases, including cancer (31). Rinvanil and PhAR are synthetic capsaicinoids that have powerful cytotoxic and apoptotic effects on leukaemia cells and as they lack pungency, they may act as improved antineoplastic agents over the naturally occurring capsaicin compound. PhAR, in particular, stands out for its greater cytotoxic activity, which inhibits the proliferation of leukaemia cells by promoting apoptosis. The majority of drugs currently used for cancer chemotherapy, produce side effects such as immunosuppression and anaemia which are in many cases permanent or irreversible and lethal (33,34), at this time the effect of PhAR *in vivo* is unknown, in the future these events will be analyzed.

In conclusion, PhAR and rinvanil inhibit proliferation of leukaemia lines and bone marrow mononuclear cells in a dose-dependent manner, although the latter were less sensitive to the cytotoxic effect of rinvanil and PhAR, as well

as capsaicin. Inhibition of cell proliferation was due to the induction of cell death by apoptosis in leukaemia lines but not in normal cells. The characteristics of low pungency and selective toxicity toward leukaemia cells suggests that both rinvanil and particularly PhAR may be biomedically relevant and promising anticancer agents.

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