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Synthetic phosphoethanolamine has *in vitro* and *in vivo* anti-leukemia effects

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Background: We recently showed that synthetic phosphoethanolamine reduces tumour growth and inhibits lung metastasis *in vivo*. Here, we investigated its anti-leukaemia effects using acute promyelocytic leukaemia (APL) as a model.

Methods: Cytotoxic effects of Pho-s on leukaemia cells were evaluated by MTT assay. Leukaemic cells obtained from hCG-PML-RARa transgenic mice were transplanted to NOD/SCID mice. After the animals were diagnosed as leukaemic, treatment started with Pho-s using all-trans retinoid acid or daunorubicin as positive control or and saline control. Cell morphology and immunophenotyping were used to detect the undifferentiated blast cells in the spleen, liver and bone marrow. The induction of apoptosis *in vitro* and in malignant leukaemic clones was evaluated.

Results: Synthetic phosphoethanolamine is cytotoxic and induces apoptosis through the mitochondrial pathway *in vitro* to leukaemia cell lines. *In vivo* Pho-s exhibits anti-proliferative effects in APL model reducing the number of CD117⁺ and Gr-1⁺ immature myeloid cells in the BM, spleen and liver. Synthetic phosphoethanolamine impairs the expansion of malignant clones CD34⁺/CD117⁺, CD34⁺ and Gr-1⁺ in the BM. In addition, Pho-s induces apoptosis of immature cells in the spleen and liver, a notable effect.

Conclusion: Synthetic phosphoethanolamine has anti-leukaemic effects in an APL model by inhibiting malignant clone expansion, suggesting that it is an interesting compound for leukaemia treatment.

The primary amine phosphoethanolamine is a central precursor in the biosynthesis of membrane phospholipids. It is a precursor of phosphatidylcholine and phosphatidylethanolamine, which are both involved in the turnover of cell membranes (Ellison *et al*, 1987). These phospholipids take part in the lipid signalling pathways and may induce its effects either by direct stimulation of membrane receptors or through the generation of second messengers (Eyster, 2007). We recently reported that Pho-s has potent effects on a wide variety of solid tumour cell lines. Interestingly, Pho-s presents anti-proliferative and pro-apoptotic effects on melanoma cells by inducing an arrest of the cell cycle in G₂/M with the loss of mitochondrial potential and increased caspase-3 activity (Ferreira *et al*, 2011). In addition, Pho-s showed antitumor effects *in vivo* on the Ehrlich ascites carcinoma and melanoma models. Most importantly, the

results showed that Pho-s inhibits tumour growth and increases the lifespan of animals without causing liver or haematological toxicity (Ferreira *et al*, 2012).

Recently, the putative therapeutic potential of Pho-s was validated in a renal carcinoma model, on which the notable *in vivo* results showed that Pho-s potentially inhibits lung metastasis in nude mice. Indeed, Pho-s also inhibited endothelial cell proliferation, migration and tube formation by inducing a cell cycle arrest at the G₂/M phase. It caused a decrease in cyclin D1 mRNA, VEGFR1 gene transcription and VEGFR1 receptor expression. Interestingly, using cyclosporin A, a specific inhibitor of cyclophilin and Z-VAD-fmk, a pan-caspase inhibitor, Pho-s was confirmed to induce apoptosis through the mitochondrial-dependent pathway (Ferreira *et al*, 2013a,b).

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The effect of Pho-s on leukaemic cells has not been established. Based on the fact that leukaemic cells are more sensitive than their normal counterparts to molecules that impair energy generation by mitochondria, we hypothesised that Pho-s may have anti-leukaemia effects. The sensitivity to drugs that target mitochondria (also known as mitocans) is particularly well characterised in acute promyelocytic leukaemia (APL), a subtype of acute myeloid leukaemia associated with the t(15;17)-PML/RAR α gene rearrangement (Rohlena *et al*, 2011; dos Santos *et al*, 2012). Moreover, APL cells are sensitive to the differentiating effect of the all-*trans* retinoic acid (ATRA) and therefore constitute a good model to determine if Pho-s has differentiating action. Finally, APL cells are resistant to several pro-apoptotic stimuli and *in vitro* and *in vivo* data suggest that APL resistance to apoptosis is mediated by the fusion protein PML/RAR α , which is degraded by arsenic trioxide treatment through a mechanism involving mitochondria-dependant generation of reactive oxygen species (Freitas *et al*, 2009). Therefore, in the present work we investigated the *in vitro* and *in vivo* cytotoxic activity of Pho-s against leukaemia, using APL as a model.

MATERIALS AND METHODS

Chemical. Synthetic phosphoethanolamine was prepared according to Outhouse (1936) with purity over 99% analysed by high-performance liquid chromatography. The 1 M stock solution was diluted in water and monoethanolamine to adjust the pH to 7.2. It was stored at room temperature and diluted in phosphate buffered saline (PBS-vehicle) for the *in vitro* and *in vivo* tests.

Cell culture. The KG-1 (human myeloid-ATCC CCL-246), K562 (human erythromyeloblastoid leukaemia-ATCC CCL-243) and Jurkat (human T-Cell leukaemia—Jurkat, Clone E6-1 ATCC TIB-152) cell lines were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). All cell lines were routinely cultured in Modified Dulbecco's Medium or RPMI supplemented with 2 mM L-glutamine and 5% (v/v) FCS and maintained at 37 °C in 95% humidified atmosphere, containing 5% CO₂.

MTT colorimetric assay. Cells were plated in 96-well at a concentration of 1×10^4 cells per well. The cells were allowed to grow for 24 h then treated with Pho-s at concentrations ranging from 0.39 to 100 nM, in six replicates. After 24 h of treatment, cell viability was determined by MTT (3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma, St Louis, MO, USA). Briefly, 20 μ l of MTT solution were added to each well (final concentration, 0.5 mg ml⁻¹) and the plate was incubated for an additional 4 h. Finally, 200 μ l of DMSO were added to each well and the absorbance was measured in a plate reader (Thermoplate TP Reader, Gendoji-cho, Fujinomiya-shi Shizuoka-ken, Japan) at 540 nm. The 50% inhibitory concentration (IC₅₀) values were calculated using a proper analysis (nonlinear regression-curve fit).

Experimental animals. Leukaemic cells obtained from hCG-PML-RAR α transgenic mice were maintained at -80 °C. Before transplantation, the cells were thawed and suspended in RPMI 1640 with 10% FCS. After 12 h of sublethal cobalt irradiation with 250 cGy, 2×10^6 cells were intravenously injected in the ocular plexus of 10- to 12-week-old NOD/SCID mice (The Jackson Laboratory, Bar Harbour, ME, USA). These mice were bred and maintained under in pathogen-free conditions at the Animal Facility of the Fundação Hemocentro, Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo. In order to verify the successful transplantation, genotyping was performed by PCR analysis of genomic DNA using the pair of primers C1 and D as described elsewhere (van Dongen *et al*, 1999). To monitor the peripheral blood (PB) counts, mice were bled from the tail 3 weeks post-transplantation with hCG-PML/RAR α .

The transgenic mouse models harbouring the PML/RAR α fusion were kindly provided by Professor Pier Paolo Pandolfi (Beth Israel Deaconess Medical Centre, Harvard Stem Cell Institute, Boston, MA, USA) and their generation has been described elsewhere (He *et al*, 1997).

Ethics statement and therapeutic protocol. The mice were randomly allocated to groups of five animals. After the animals were diagnosed as leukaemic, characterised with leukocytosis, anaemia and thrombocytopenia the treatment was started with the intraperitoneal administration of one of the following preparations: 40 mg kg⁻¹ Pho-s, 80 mg kg⁻¹ Pho-s and 1 mg kg⁻¹ ATRA, 10 mg kg⁻¹ daunorubicin (DA) (positive controls) and continued daily for 15 days. In addition, mice were treated with 100 μ l PBS (vehicle) to induce the same stress conditions of treatment to in the control animals. All animal experimentation was conducted under a protocol in agreement with ethical principles in animal research and approved by the ethics committee for animal research of the Butantan Institute (process number 566/09).

Evaluation of anti-leukaemia effects. For monitoring PB, differential counts were performed using Leishman–Wright–Giemsa stain. The mice were considered leukaemic according to the following criteria: presence of blasts and promyelocytes in the PB; leukocytosis $> 30 \times 10^3$ per μ l, haemoglobin < 10 g dl⁻¹) and platelets $< 500 \times 10^3$ per μ l. Automated counts of these cells were performed using a T-890 Coulter cell counter (Coulter Corporation, Hialeah, FL, USA), and differential counts were obtained from Leishman–Wright–Giemsa-stained smears. After the end of the treatments, leukaemic cells from bone marrow (BM), liver and spleen were obtained by flushing with RPMI 1640 (Gibco, Grand Island, NY, USA). The splenic and hepatic cell suspensions were obtained by mechanical disruption. Then, cells were washed once and resuspended in PBS at a concentration of 10⁶ per ml. Cytospin slides were prepared in a Cytospin 3 Cell Preparation System (Shandon, Pittsburgh, PA, USA) and stained with Leishman–Wright–Giemsa. For morphological analysis, a minimum of 200 cells were counted and myeloid cells were classified as immature, intermediate or mature according to the Bethesda Proposals for Classification of non-lymphoid hematopoietic neoplasms in mice (Kogan *et al*, 2002).

Flow cytometry analysis. Cell suspensions of BM, spleen and liver were obtained as described above. Cells were first fixed and permeabilised with Triton X-100 0.02%, then stained with fluorescein isothiocyanate (FITC)-conjugated, allophycocyanin (APC), peridin chlorophyll protein (Per-CP) and phycoerythrin-conjugated monoclonal antibodies (mAb) and their respective isotype-matched controls (Southern Biotechnology Associates, Birmingham, AL, USA). The antibodies employed were CD117-APC/Alexa Fluor750 (immature myeloid cells); CD34-FITC (hematopoietic stem cells); CD45-Per-CP (pan leukocyte marker) and Gr-1-APC/Alexa Fluor750 (immature myeloid cells) (Biosciences Pharmingen, San Diego, CA, USA). For the exclusion of erythrocytes, cells were lysed immediately after labelling by incubation with 2 ml of FACS lysing solution (Becton Dickinson San Jose, CA, USA) as recommended by the manufacturer. The immunophenotype of the cells was determined by flow cytometry with FACScalibur (Becton Dickinson). A total of 10 000 cells per sample were acquired and the percentage of cells was recorder using FlowJo software.

Measurement of mitochondrial membrane potential. Leukaemia cells at density of 10⁶ cells were seeded in 6-well plates and incubated overnight, and next day treated with Pho-s. After 12 h of treatment, the cells were washed once with PBS and stained with 100 nM Mytotracker Red for 15 min at 37 °C. Next, the cells were washed once with PBS and kept at 4 °C during measurements. The mitochondrial membrane potential ($\Delta\Psi$) was measured by

flow cytometry with FACScalibur (Becton Dickinson). A total of 10 000 cells per sample were acquired for each sample in three independent experiments.

Apoptosis assay. Following the FACS analyses described above, gate regions for CD117⁺ and Gr-1⁺ cells, infiltrated in the spleen and liver, and were previously determined in the FACS for further analysis of apoptosis induced by the treatments. The cells were then centrifuged and the cell pellet was suspended at the concentration of 10⁶ cell per ml with binding buffer (100 μ l). *In vitro* apoptotic effects of Pho-s were evaluated on KG-1, K562 and Jurkat cell lines, the cells were plated in six-well culture plates, grown overnight and treated with IC50 values measured. The apoptosis was measured every 2 h during a total of 8 h of treatment. Cells were then stained with Dead Cell Apoptosis Kit with Annexin V Alexa Fluor 488 and propidium iodide (PI) (BD Bioscience, San Jose, CA, USA) and incubated for 15 min at room temperature in the dark. After incubation, 400 μ l of binding buffer were added and cells were analysed by flow cytometry with FACScalibur (Becton Dickinson) and data were processed using FlowJo software, determining the percentage of apoptotic cells. A minimum of 10 000 events were acquired for each sample analysed.

Detection of caspase-3 activity. To further evaluate the apoptosis mechanism, the caspase-3 activity involved in the early stage of apoptosis was evaluated. The leukaemia cells previously incubated overnight were treated with increasing concentrations of Pho-s from IC50 values for 8 h. Next, cells were lysed in 100 ml of 1 \times lysis buffer. The lysates were collected and stored at -70 $^{\circ}$ C until analysed. Briefly, 50 ml of 1 \times reaction buffer were added to each well used in the assay and incubated at 37 $^{\circ}$ C for 5 min. The caspase activity was measured using a caspase-3 fluorometric assay kit from Biovision. The plate was read in a CytoFluor II PerSeptive Biosystems (Farmington, MA, USA) fluorometric plate reader with excitation at 360 nm and emission at 460 nm.

Statistical analysis. All values were expressed as mean \pm s.d. Each value is the mean of at least three independent experiments in each group. For significance analysis Student's *t*-tests and one-way analysis of variance were performed using GraphPad Prism 4.0 (San Diego, CA, USA). *P*-values *P* < 0.05, 0.01 and 0.001 were considered significant.

RESULTS

Cytotoxicity of Pho-s on leukaemia cell lines. To evaluate the cytotoxic effects of Pho-s on leukaemic cell lines, an MTT assay was performed after 24 h of treatment. Pho-s exhibited a potent dose-dependent cytotoxic effect on KG-1, K562 and Jurkat cells with IC50 values of 9; 6 and 12 mM, respectively. Of interest, among the screened cells, K562 was the most sensitive to Pho-s as demonstrated by the IC50 value (Figure 1).

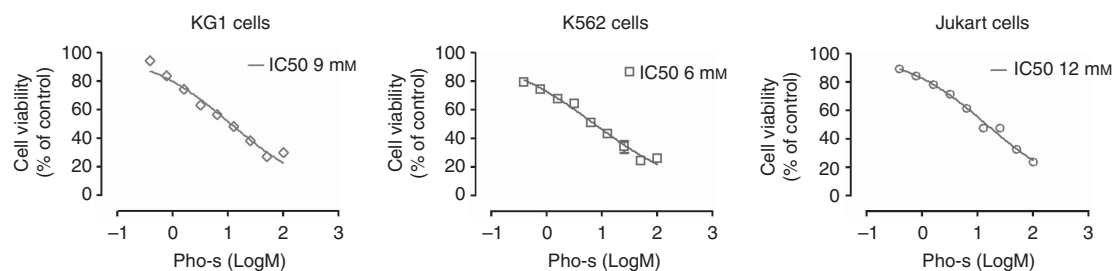


Figure 1. Effects of Pho-s on the cell viability of KG-1, K562 and Jurkat cells. Cells were seeded into 96-well plates at 10⁴ cells per well and treated with Pho-s for 24 h. Cell viability was assessed by MTT assay and is expressed as the percentage of cells comparing the optical density (540 nm) of the treated cells with the optical density of the untreated cells. The data are representative of three independent experiments performed in triplicate.

Pho-s induces *in vitro* apoptosis on leukaemic cell lines through the mitochondrial pathway. We evaluated whether the apoptosis is a mechanism resulting from the cytotoxic effects induced by Pho-s on leukaemic cell lines. For this purpose, we first evaluated whether Pho-s affect mitochondrial permeability transition (MPT). Our findings show that the treatment with 9 mM Pho-s to KG-1; 6 mM Pho-s to K562 and 12 mM Pho-s to Jurkat induces mitochondrial depolarisation leading to $\Delta\Psi$ collapse (Figure 2A). To confirm whether the apoptotic effects of Pho-s were associated with MPT, the apoptosis was observed on leukaemic cells treated with Pho-s for different periods of time using the IC50 values calculated to each cell lines. As shown in Figure 2B after treatment with Pho-s for 8 h, a significant increase (**P* < 0.05) in apoptotic cells in all cell lines tested was detected with longer incubation times. Our results show that after 8 h of treatment there is an increase in apoptotic cells in response to Pho-s. This incubation time was then selected to better evaluation of caspase-3 activity. Accordingly, through the Annexin V/PI assay, we found that Pho-s causes a significant increase (**P* < 0.05) in caspase-3 activation in leukaemic cells after 8 h of treatment with both concentrations tested (Figure 2C). Importantly, in this study we have shown that Pho-s induces apoptosis through the mitochondrial-dependent pathway.

Evaluation of the APL transplant model. To monitor the progression of the disease, transplanted mice were bled from the tail 15 days after transplantation. The haematological analysis reveals that the transplant was successful as evidenced by the leukocytosis (12.8 \pm 0.73%) anaemia (6.2 \pm 0.8%) and thrombocytopenia (488 \pm 45.9%) observed in the PB of the animals (Figure 3A). In addition, four animals were euthanised and leukaemia cells infiltrated in the BM, spleen and liver were analysed. Consistent with these haematological changes in PB, there was an increase in the number of blast cells in the spleen (17.5 \pm 3.5%), liver (14.0 \pm 2.8%) and BM (68.5 \pm 2.1%) (Figure 3B).

Pho-s induces a haematological response in PML-RARa leukaemic mice. We performed a cyto-morphological analysis to evaluate the antitumor effects of Pho-s in the transgenic model. An important effect observed during the leukaemic phase was an increase in the number of white blood cells of the NOD/SCID control mice, which was accompanied by an increase in the number of immature cells in the BM, PB, spleen and liver. Of note, the immature cells observed in the leukaemia model are a typical characteristic of human promyelocytes. The treatment of the mice with either Pho-s, at 40 or 80 mg kg⁻¹, 1 mg kg⁻¹ ATRA or 10 mg kg⁻¹ DA (positive controls) reduced the percentage of white blood and immature cells. It is interesting to note that Pho-s and ATRA were both more efficient than DA in normalising the number of mature cells in the PB, BM spleen and liver (Table 1).

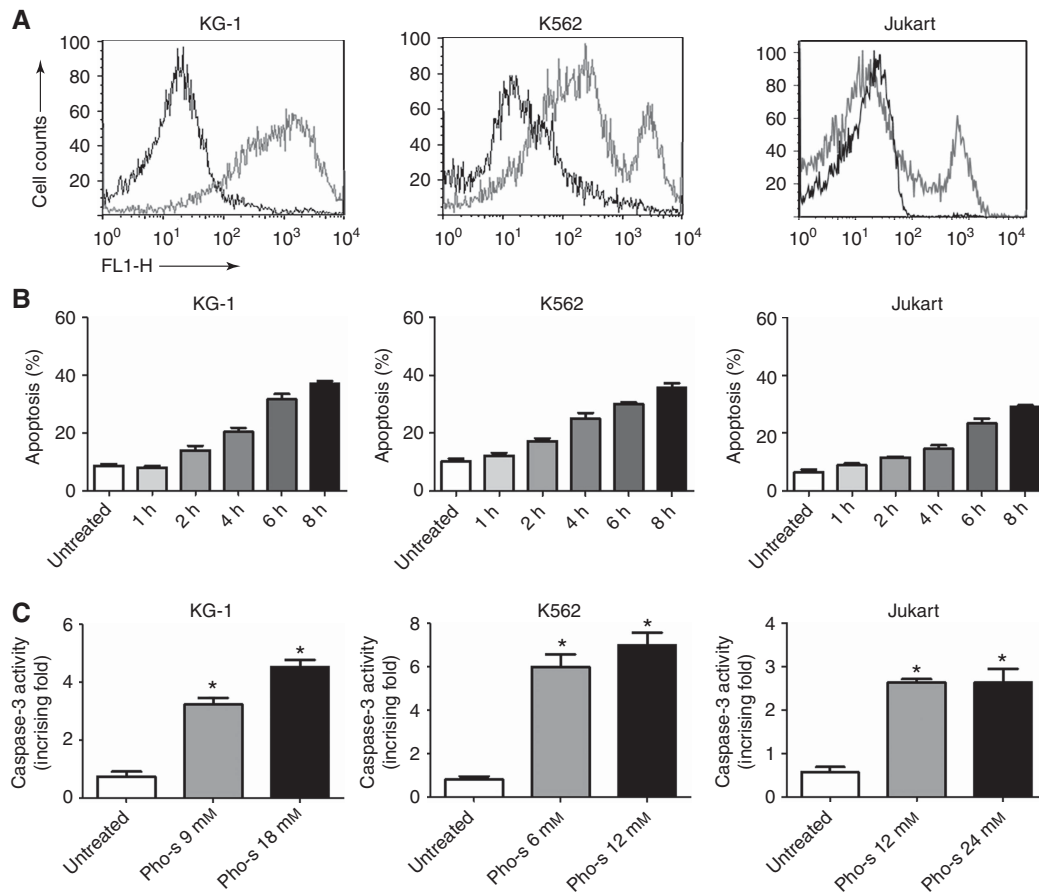


Figure 2. Pho-s induces mitochondrial membrane depolarisation and caspase-3 activity in leukemic cell lines. **(A)** Representative histograms of cells stained with Mitotracker red are shown. There is a substantial reduction in fluorescence emission, indicating a decrease of $\Delta m\Psi$ in the leukaemic cells treated with Pho-s (black line) as compared with untreated cells (red line). **(B)** A substantial increase in apoptotic cells was observed after 8 h of treatment with Pho-s. **(C)** The increase in caspase-3 activity correlates with the increase in the frequency of apoptotic cells evaluated after the treatment. The data are representative of three independent experiments performed in triplicate. Significant differences are indicated as: * $P < 0.05$ statistically different between Pho-s versus untreated.

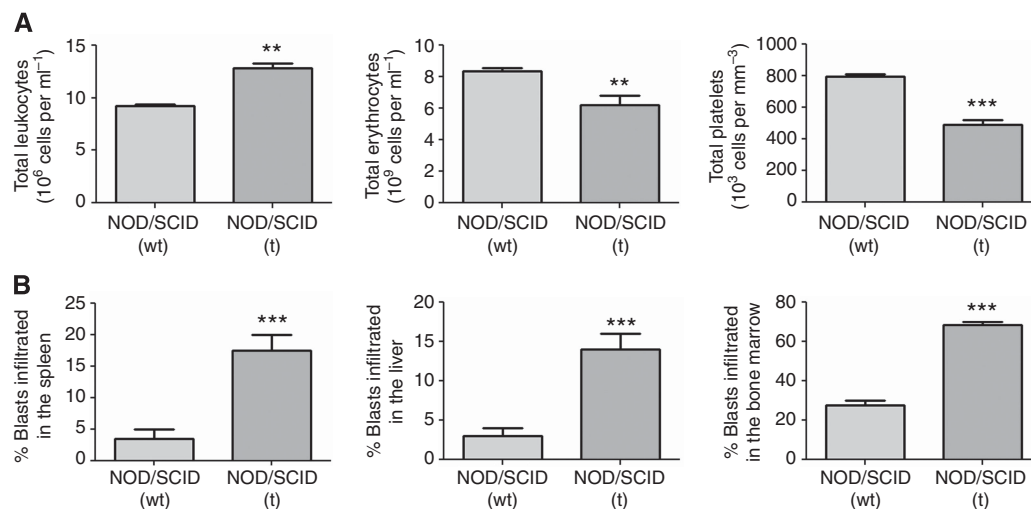


Figure 3. Haematological and cytological analysis of leukaemic cells. To confirm the success of the transplant, the mice were bled from the tail 15 days after transplantation. **(A)** The increases in the total number of leukocytes, as well as thrombocytopenia and anaemia, are evidences of the progression of the disease. **(B)** An increase in the number of undifferentiated cells, characteristic of blast cells, infiltrated in the bone marrow, spleen and liver was observed.

Anti-leukaemic effects of Pho-s correlate with the reduction of CD34⁺ CD117⁺ cells in the *in vivo* model of APL. We next evaluated the phenotype of cells infiltrated in the parenchyma. As

expected, there was an increase of CD117⁺ and Gr-1⁺ cells infiltrated in the spleen and liver of the mice treated with vehicle and compared with wild-type mice (WT). Interestingly, we found

Table 1. Counting and classification of cells with in accordance with criteria establish by Bethesda for leukemia in mice

(%) Cells	Control	ATRA 1 mg kg ⁻¹	DA 10 mg kg ⁻¹	Pho-s 40 g kg ⁻¹	Pho-s 80 mg kg ⁻¹
Peripheral blood					
Immature	19 ± 4.8	0.00 ± 0.00*	9.0 ± 4.7*	0.00 ± 0.00*	0.00 ± 0.00*
Intermediate	16.7 ± 8.4	1.00 ± 0.02*	5.3 ± 0.78*	8.00 ± 0.13*	5.4 ± 0.65*
Mature	42.1 ± 12.6	16.7 ± 2.8*	32.2 ± 12.8	18.7 ± 3.1*	14.2 ± 4.9*
Lymphocytes	29 ± 9.4	82.3 ± 17.2	53.5 ± 20.0	73.3 ± 14.8	80.3 ± 14.8
Bone marrow					
Immature	50.6 ± 20.4	18.9 ± 12*	32.5 ± 17.4*	13.9 ± 2.1*	10.1 ± 1.1*
Intermediate	28.7 ± 12.6	25.2 ± 8.7	23.2 ± 9.7	19 ± 12.7*	18 ± 7.2*
Mature	18.5 ± 8.4	38.3 ± 2.8*	19.5 ± 4.8	38.6 ± 5.5*	36.6 ± 4.5*
Lymphocytes	4.1 ± 0.98	16.1 ± 4.6*	24.1 ± 4.8*	28.1 ± 4.6*	35 ± 4.9*
Liver					
Immature	41.2 ± 10.7	18.1 ± 7.7*	24 ± 4.2*	16.8 ± 2.3*	20 ± 7.5*
Intermediate	23.8 ± 5.5	9.1 ± 0.51*	16.7 ± 2.8*	18.1 ± 0.45*	15.6 ± 2.5*
Mature	14 ± 1.7	20.4 ± 9.1*	32.4 ± 7.5*	10.1 ± 0.57	18.4 ± 9.0*
Lymphocytes	20 ± 3.4	52.4 ± 4.9*	27.8 ± 12.2	55 ± 21.2*	47.1 ± 3.8*
Spleen					
Immature	43.6 ± 11.4	13.4 ± 2.5*	15 ± 1.8*	4.7 ± 0.5*	2.3 ± 0.25*
Intermediate	23.9 ± 7.5	3.1 ± 0.18*	20.1 ± 0.98	2.1 ± 0.18*	3.2 ± 0.22*
Mature	11 ± 0.21	14.4 ± 8.9	26.4 ± 12.3*	12.4 ± 3.7	16.1 ± 0.21
Lymphocytes	27.1 ± 5.1	70 ± 13.0*	38.6 ± 6.7*	81.2 ± 18.2*	78.4 ± 18.2*

Abbreviations: ATRA = all-trans retinoid acid; DA = daunorubicin; Pho-s = synthetic phosphoethanolamine. The data are shown as means ± s.d. of three mice per group of treatment. The statistic values were obtained using one-way ANOVA followed by Tukey-Kramer post-hoc test (**P* < 0.05).

that Pho-s at both 40 and 80 mg kg⁻¹ (15 ± 2.8%), (19.5 ± 2.1%), respectively, induces a decrease in the number of CD117⁺ cells present in the spleen. In the liver, the treatment with 40 and 80 mg kg⁻¹ of Pho-s (36 ± 5.6%), (39 ± 3.5%) and 1 mg kg⁻¹ ATRA (53 ± 1.4%) induced a reduction of CD117⁺ cells (***P* < 0.01) Figure 4A. Both CD117⁺ and Gr-1⁺ cells are frequently found in the BM, spleen, liver and lymphoid organs of patients with myelodysplastic syndromes and in tumour-bearing animals. We also observed a decrease of immature myeloid Gr-1⁺ cells in the spleen of mice treated with 40 and 80 mg kg⁻¹ of Pho-s (48.5 ± 2.0%), (47 ± 4.2%) and 1 mg kg⁻¹ ATRA (47.3 ± 1.6%). In addition, the reduction of immature myeloid Gr-1⁺ cells in the liver of mice treated with 40 and 80 mg kg⁻¹ of Pho-s (22.5 ± 2.1%), (30 ± 2.8%) and 1 mg kg⁻¹ ATRA (19.5 ± 3.5%) Figure 4B.

We then investigated whether Pho-s has effects against the malignant clones in the principal hematopoietic compartment, the BM. Cells were selected as the percentage of CD45⁺ cells to exclude mature erythrocytes, and cell subsets were generated and evaluated for the expression of CD117, CD34 markers and also for the asynchronous CD117/34 co-expression. We did not observe changes in CD117⁺ and CD45⁺/CD117⁺ expression as a result of any of the treatments when compared with vehicle and WT. However, the treatment with 40 mg kg⁻¹ Pho-s (23.1 ± 2.4%) or 10 mg kg⁻¹ DA (20.7 ± 2.5%) increased the percentage of CD45⁺ cells (Figure 5A). The treatment with 1 mg kg⁻¹ ATRA or Pho-s 40 mg kg⁻¹ (2.9 ± 0.7%), (5.1 ± 0.9%), respectively, induced a reduction in the number of CD34⁺ hematopoietic progenitor cells as well as of CD34⁺/45⁺ cells (43.5 ± 2.2% to 1 mg kg⁻¹ ATRA) and (38.2 ± 1.4% to Pho-s 40 mg kg⁻¹) (Figure 5B). The oncogene CD117 encodes a receptor protein present on CD34⁺ cells and constitutes a specific phenotype observed in acute myelogenous leukaemia. Therefore, the reduction of these two asynchronous antigens, CD34⁺/CD117⁺ in response to treatment with 40 or 80 mg kg⁻¹ of Pho-s (40.4 ± 2.8%), (69 ± 1.4%), or

1 mg kg⁻¹ ATRA (14.6 ± 2.5), respectively constitute a notable antitumor effect (Figure 6A).

Pho-s induces antitumor effects by induction of apoptosis of CD117⁺ and Gr-1⁺ cells infiltrates in the spleen and liver. To further examine the antitumor effects of Pho-s, we evaluated whether apoptosis is involved in the process. For this purpose, the proportion of dead cells in the liver and spleen was assessed by Annexin V/PI double staining. Populations of CD117⁺ and Gr-1⁺ cells selected at the dot plot gates were evaluated. The apoptotic values were estimated as a percentage of the total number of CD117⁺ and Gr-1⁺ cells for each treatment. On the other hand, the fact that the number of cells labelled according to their respective antigens can vary in response to each different treatment, the percentage of apoptotic cells is shown relative to the number of CD117⁺ and Gr-1⁺ previously selected. In accordance with the reduction of CD117⁺ cells in the spleen, 40 mg kg⁻¹ of Pho-s induced apoptosis of these cells (Figure 7A and B). In addition, a significant number of CD117⁺ cells died by apoptosis in the liver in response to treatment with Pho-s at the concentrations of the 40 and 80 mg kg⁻¹ (Figure 8A and B). Of note, Pho-s was more potent than ATRA e DA in the induction of apoptosis of CD117⁺ cells infiltrated in the spleen and liver. Apoptosis induced by Pho-s was also evaluated in Gr-1⁺ cells from the same parenchyma. The treatments with 40 or 80 mg kg⁻¹ of Pho-s, 1 mg kg⁻¹ ATRA or 10 mg kg⁻¹ DA induce apoptosis of immature myeloid Gr-1⁺ cells present in the spleen (Figure 9A and B) and liver (Figure 10A and B).

DISCUSSION

In this study, we demonstrate for the first time that Pho-s has antitumor activity against leukaemia cells. Thus, the Pho-s shows antitumor activity not only against solid tumours but also against

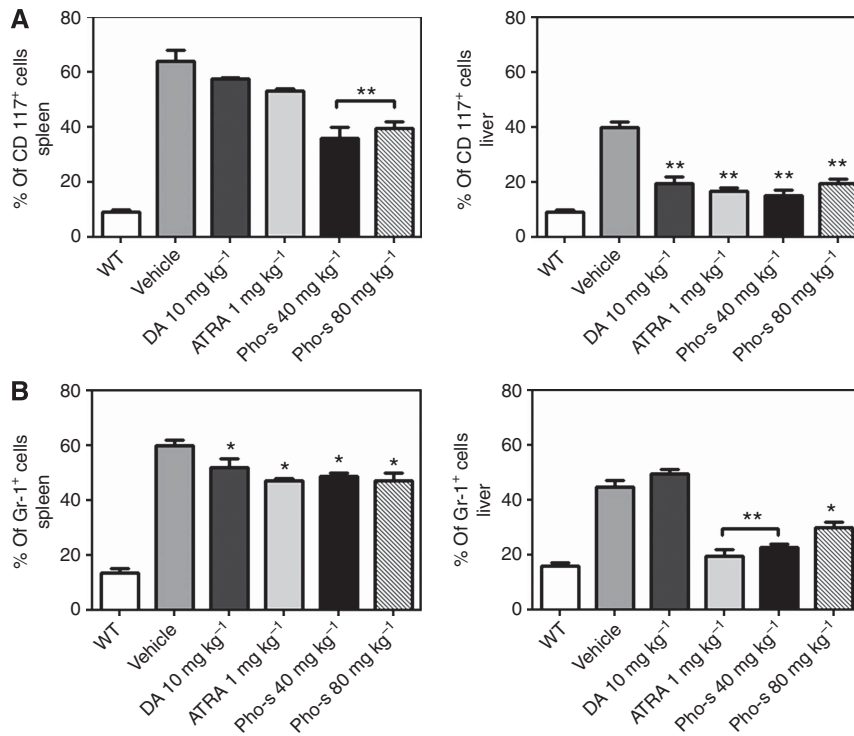


Figure 4. Immunophenotyping by flow cytometry of CD117⁺ and Gr-1⁺ cells infiltrated in the spleen (A) and liver (B) of NOD/SCID leukaemic mice. The bar diagram shows the percentage of CD117⁺ and Gr-1⁺ cells in the parenchyma from untreated mice and from mice treated with 40 mg kg⁻¹ Pho-s, 80 mg kg⁻¹ Pho-s, 1 mg kg⁻¹ ATRA or 10 mg kg⁻¹ DA (the last two, positive controls) and WT mice. The data are expressed as mean \pm s.d. from five mice. * P <0.05; ** P <0.01 and *** P <0.001 versus untreated mice, One-way analysis of variance (ANOVA) with Tukey–Kramer *post-hoc* test.

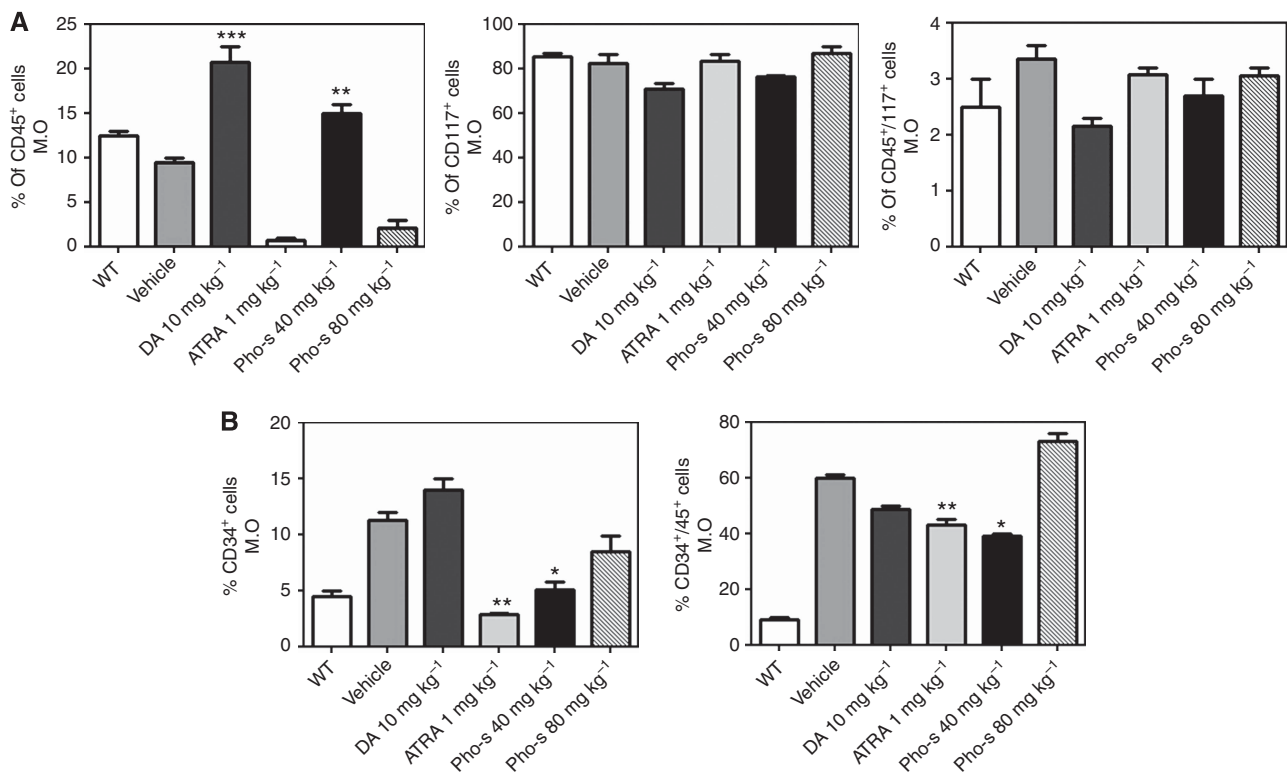


Figure 5. Immunophenotyping by flow cytometry of CD45⁺, CD117⁺ and CD34⁺ cells infiltrated in the BM of NOD/SCID leukaemic mice. The bar diagram shows CD45 and CD117 (A) and CD34 (B) expression in the BM from untreated mice and from mice treated with 40 mg kg⁻¹ Pho-s, 80 mg kg⁻¹ Pho-s, 1 mg kg⁻¹ ATRA or 10 mg kg⁻¹ DA (the last two, positive controls). The data are expressed as mean \pm s.d. from five mice. * P <0.05; ** P <0.01 and *** P <0.001 versus untreated mice, one-way analysis of variance (ANOVA) with Tukey–Kramer *post-hoc* test.

haematological malignancies. It is interesting to note that Pho-s produced cytotoxic effects on these cells independently of specific characteristics, such as resistance to apoptosis and chemotherapy. As we demonstrated that Pho-s is cytotoxic to leukaemic cell lines, we investigated the underlying mechanisms. Here, we show that Pho-s triggers cell death by apoptosis through the mitochondrial-dependent pathway. Interestingly, our results indicate that the

apoptotic effects of Pho-s most likely involve the disruption of $\Delta\Psi$ combined with an increase of MPT and the release of pro-apoptotic members from the mitochondria, which induces an increase in caspase-3 activity leading to phosphatidylserine externalisation. It is in agreement with our recent report that showed that Pho-s induces apoptosis via the intrinsic pathway in murine renal carcinoma (Renca cells) and in human breast cancer (MCF-7 cells) (Ferreira *et al*, 2013a,b). Taken together, our results show that Pho-s has *in vitro* pro-apoptotic activity against leukaemic cell lines.

Our *in vivo* study was carried out on a well-established transgenic APL model which develops leukaemia 14 days after transplantation (de Figueiredo-Pontes *et al*, 2011). Our results are in accordance with this well-established transgenic model, which in the leukaemic phase is characterised by a marked increase in white blood cells and thrombocytopenia. After the animals were diagnosed in the acute phase, the anti-leukaemia effects of Pho-s were evaluated. Exploring the antitumor effects, we first demonstrated that Pho-s induces a decrease in blast cells and leukocytes in the PB. This finding, while preliminary, suggests that Pho-s exhibits anti-proliferative effects in APL blocking the dissemination of malignant clones. In accordance with these results, 40 and 80 mg kg⁻¹ of Pho-s reduced the leukaemic progenitors (immature cells). To the best of our knowledge and based on the literature, this is strong evidence that Pho-s has anti-leukaemia effects. These results consistently indicate that Pho-s blocks the capacity of malignant stem cells to acquire self-renewal capacity and consequently act as malignant stem cells (Stuart *et al*, 2009). Indeed, Pho-s inhibits malignant clone expansions, which could subsequently originate undifferentiated blast cells, contributing to the maintenance of the disease (Minami *et al*, 2012).

To further evaluate how Pho-s inhibits the progression of leukemogenesis, we performed an immunophenotypic analysis.

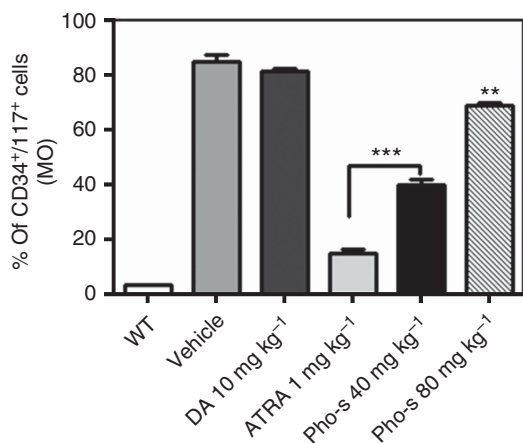


Figure 6. Immunophenotyping by flow cytometry of CD117⁺ and CD34⁺ cells infiltrated in BM of NOD/SCID leukaemic mice. The bar diagram shows CD117 and CD34 expression in the BM of untreated mice and from mice treated with 40 mg kg⁻¹ Pho-s, 80 mg kg⁻¹ Pho-s, 1 mg kg⁻¹ ATRA or 10 mg kg⁻¹ DA (the last two, positive controls). The data are expressed as mean \pm s.d. from five mice. ** $P < 0.01$ and *** $P < 0.001$ versus untreated mice, one-way analysis of variance (ANOVA) with Tukey–Kramer *post-hoc* test.

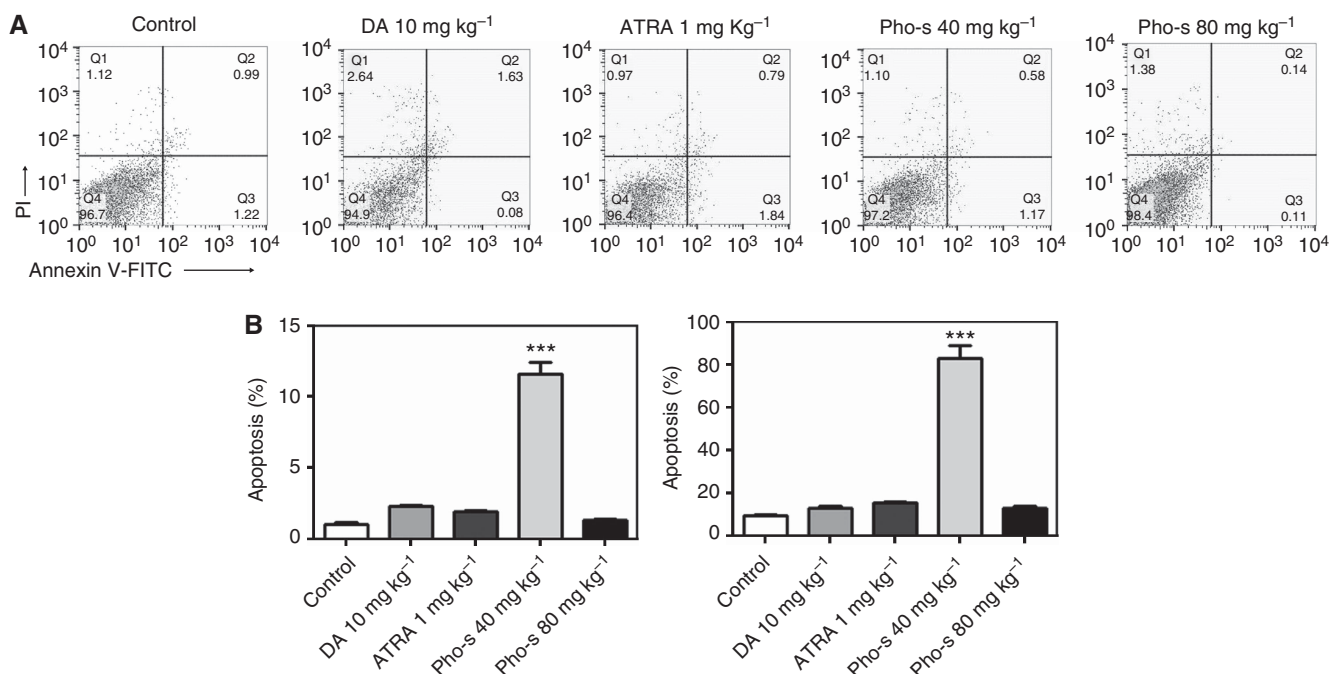


Figure 7. Evaluation of cell death by apoptosis using annexin V-FITC/PI staining. (A) Dot plot profile of cells from untreated mice and from mice treated with 40 mg kg⁻¹ Pho-s, 80 mg kg⁻¹ Pho-s, 1 mg kg⁻¹ ATRA or 10 mg kg⁻¹ DA (the last two, positive controls). The apoptotic cells selected at the gate to CD117⁺ infiltrates in the spleen were stained with V-FITC/PI and analysed by flow cytometry. (B) Left panel: The percentage of apoptotic CD117⁺ cells is shown in the bar diagram as mean \pm s.d. from five mice. Right panel: The percentage of apoptotic cells is shown in relation to the total number of CD117⁺ infiltrates in the spleen. The values were *** $P < 0.001$ versus untreated mice, one-way analysis of variance (ANOVA) with Tukey–Kramer *post-hoc* test.

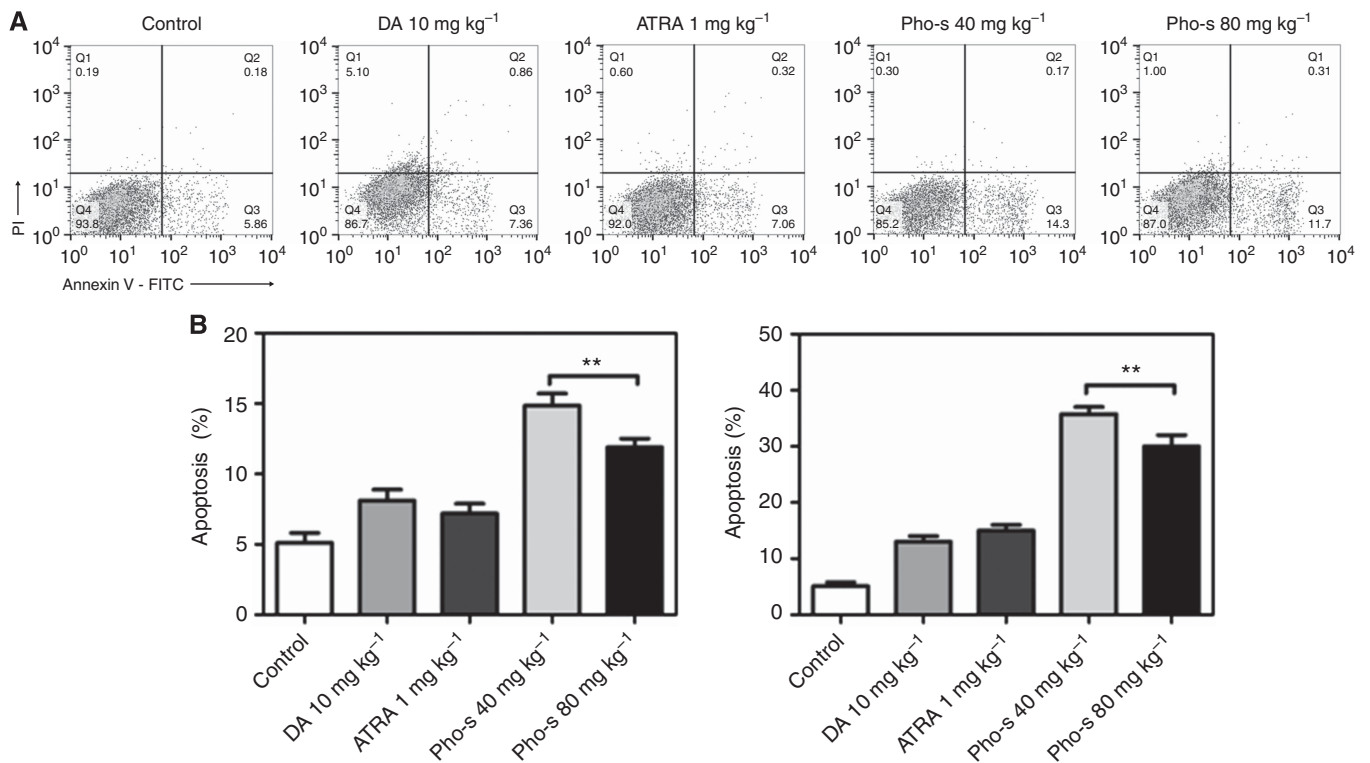


Figure 8. Evaluation of cell death by apoptosis using annexin V-FITC/PI staining. (A) Dot plot profile of cells from untreated mice and from mice treated with 40 mg kg⁻¹ Pho-s, 80 mg kg⁻¹ Pho-s, 1 mg kg⁻¹ ATRA or 10 mg kg⁻¹ DA (the last two, positive controls). Apoptotic cells selected at the gate for CD117⁺ infiltrates in the liver were stained with V-FITC/PI and analysed by flow cytometry. (B) Left panel: The percentage of apoptotic CD117⁺ cells is shown in the bar diagram as mean ± s.d. from five mice. Right panel: The percentage of apoptotic cells is shown in relation to the total number of CD117⁺ infiltrates in the liver. The values were **P<0.001 versus untreated mice, one-way analysis of variance (ANOVA) with Tukey–Kramer post-hoc test.

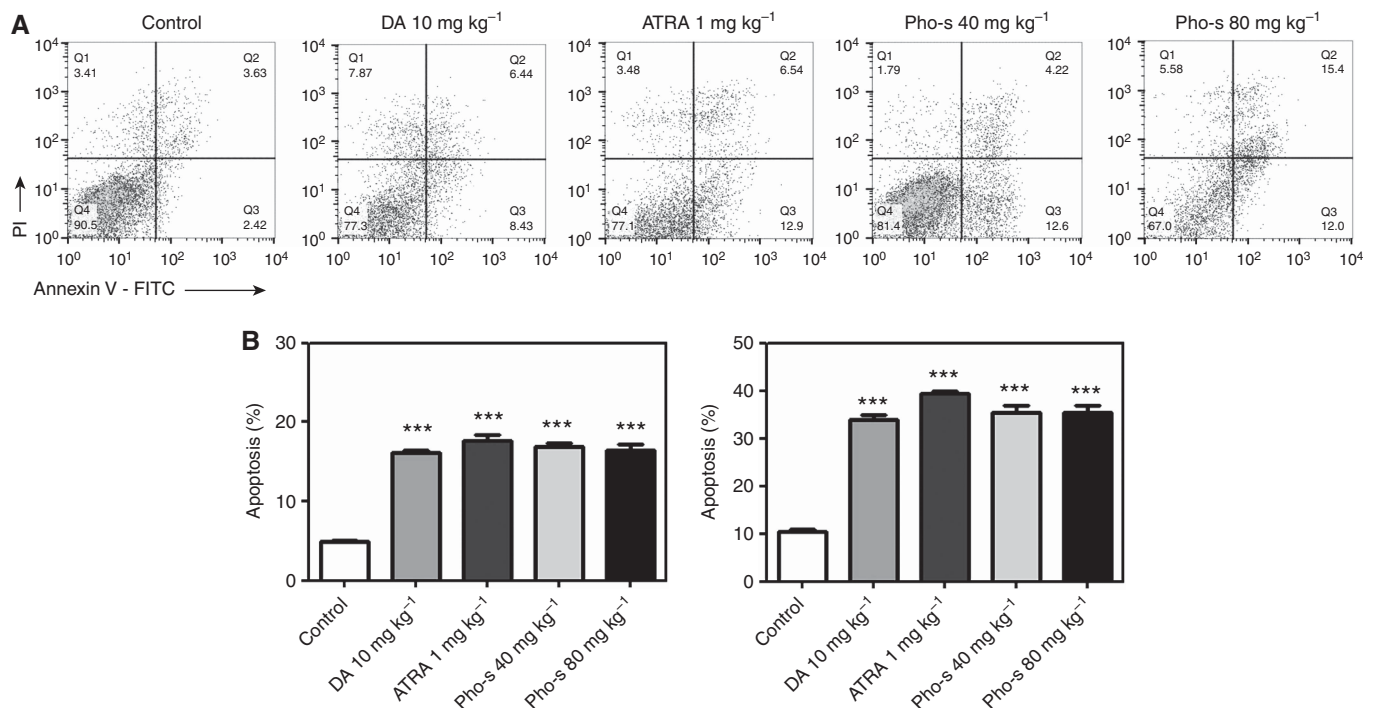


Figure 9. Evaluation of cell death by apoptosis using annexin V-FITC/PI staining. (A) Dot plot profile of cells from untreated mice and from mice treated with 40 mg kg⁻¹ Pho-s, 80 mg kg⁻¹ Pho-s, 1 mg kg⁻¹ ATRA or 10 mg kg⁻¹ DA (the last two, positive controls). Apoptotic cells selected at the gate for Gr-1⁺ infiltrates in the spleen were stained with V-FITC/PI and analysed by flow cytometry. (B) Left panel: The percentage of apoptotic Gr-1⁺ cells is shown in the bar diagram as mean ± s.d. from five mice. Right panel: The percentage of apoptotic cells is shown in relation to the total number of Gr-1⁺ infiltrates in the spleen. The values were ***P<0.001 versus untreated mice, one-way analysis of variance (ANOVA) with Tukey–Kramer post-hoc test.

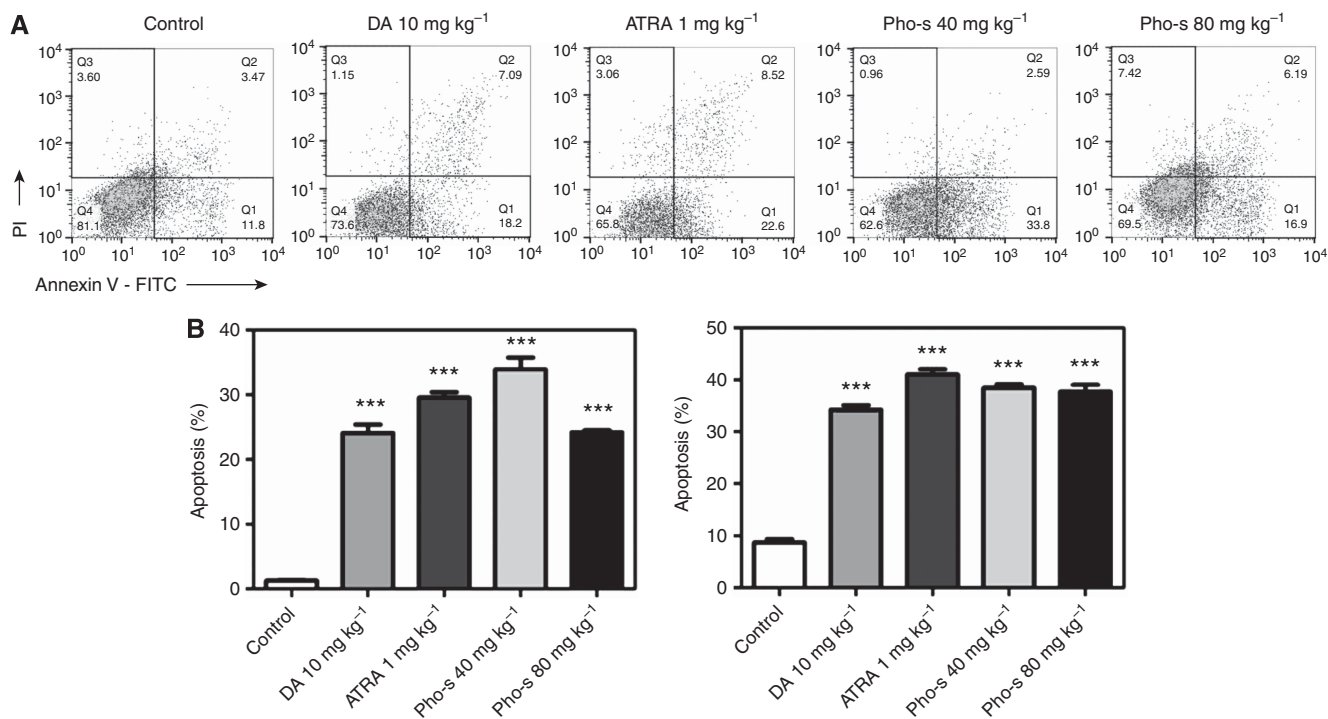


Figure 10. Evaluation of cell death by apoptosis using annexin V-FITC/PI staining. **(A)** Dot plot profile of cells from untreated mice and from mice treated with 40 mg kg^{-1} Pho-s, 80 mg kg^{-1} Pho-s, 1 mg kg^{-1} ATRA or 10 mg kg^{-1} DA (the last two, positive controls). The percentage of apoptotic cells selected into the gate to Gr-1⁺ infiltrates in the liver were stained with V-FITC/PI and analysed by flow cytometry. **(B)** Left panel: The percentage of apoptotic Gr-1⁺ cells is shown in the bar diagram as mean \pm s.d. from five mice. Right panel: The percentage of apoptotic cells is shown in relation to the total number of Gr-1⁺ infiltrates in the liver. The values were *** $P < 0.001$ versus untreated mice, one-way analysis of variance (ANOVA) with Tukey–Kramer *post-hoc* test.

One unanticipated finding was the decrease, in the spleen, CD117⁺ cells. CD117 is a c-kit proto-oncogene that encodes a transmembrane tyrosine kinase receptor involved in the proliferation of leukaemic cells (Rossi *et al*, 2013). Interestingly, this reduction was observed only upon treatment with 40 and 80 mg kg^{-1} of Pho-s. It suggests in turn that Pho-s has a good biodistribution profile, allowing it to act as an antitumor compound against tumour cell infiltrates in different organs. Notably, Pho-s, 1 mg kg^{-1} ATRA and 10 mg kg^{-1} DA also reduce the population of CD117⁺ in the liver, which can result in a normal progression and differentiation of the myeloid lineage. Importantly, Gr-1⁺ immature myeloid cells, generally present in patients and animals with tumours (Li *et al*, 2004) were suppressed by treatment with Pho-s, ATRA and DA. Thus far, these results suggest that Pho-s impaired the malignant clone expansion and proliferation. We then investigated whether apoptosis is a mechanism involved in the elimination of the malignant clones. Our results clearly show that the anti-leukaemia effects of Pho-s can be attributed to the induction of apoptosis especially of cells CD117⁺ an antigen present in the early stages of myeloid differentiation and Gr-1⁺ immature myeloid cells. In this regard, we recently demonstrated that Pho-s induces apoptosis in carcinoma renal murine in a mitochondrial-dependent manner (Ferreira *et al*, 2013a,b).

The expression of the CD34⁺ and CD117⁺ antigens on the t(15; 17) constitute the phenotype used as a marker for the diagnosis and prognosis of APL. It is also interesting to note that Pho-s causes the reduction in the BM of asynchronous antigen expression CD34⁺/CD117⁺ and it is characteristically observed in acute myelogenous leukaemia (Santana *et al*, 2006). In addition, Pho-s inhibits the increase of malignant clones in the BM compartment by causing a reduction of CD34⁺ and Gr-1⁺

immature myeloid cells (Li *et al*, 2012). The major importance of these results is that Pho-s inhibits the growth, differentiation and clonal expansion of malignant cells. In this study, the therapeutic effects of Pho-s are equivalent to those of ATRA, a potent granulocytes differentiator, which induces high rates of complete remission, even in cases resistant to chemotherapy (Gausdal *et al*, 2013).

In summary, we found that Pho-s is cytotoxic *in vitro* to leukaemia cell lines. Most importantly, we demonstrate several anti-leukaemic effects *in vivo*: Pho-s exhibits anti-proliferative effects in APL by blocking the dissemination of malignant clones; it reduces the number of CD117⁺ cells and Gr-1⁺ immature myeloid cells in the BM, spleen and liver. Pho-s impairs the expansion of malignant clones CD34⁺/CD117⁺ as well as CD34⁺ and Gr-1⁺ in the BM. In addition, Pho-s induces apoptosis of immature cells in the spleen and liver, which is a remarkable effect. Overall, Pho-s is a compound that shows anti-leukaemic effects in an APL model by inhibiting malignant clone expansion, suggesting that it is an interesting compound for an anti-leukaemia treatment.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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