Abstract. Background: Farnesyltransferase inhibitors have the ability to interfere with various intracellular pathways, reducing cell survival and proliferation. They have become an attractive tool for cancer therapy, namely acute leukemias. In this work, we have studied the efficacy of α-hydroxyfarnesylphosphonic acid (α-HFPA) in CEM (acute T-cell lymphoblastic leukemia) in culture. Materials and Methods: CEM cells were incubated with α-HFPA at different concentrations; viability and proliferation studies were performed using the trypan blue exclusion assay and cell morphological analysis. Expression of lamin A/C, cyclin D1 and BAD were analyzed by flow cytometry. Results: Our results show that α-HFPA significantly decreases Farnesyltransferase activity, reduces cell proliferation and induces cell death through apoptosis in CEM cells, which is correlated with a reduction of cyclin D1 levels. Conclusion: This study suggests that α-HFPA blocks the cell cycle and induces cell death through apoptosis in CEM cells and may be a therapeutic approach in ALL.

Acute lymphoblastic leukemia (ALL) is the most prevalent malignancy under the age of 18 years; however, a poor prognosis is associated with ageing. In fact, the best clinical series report that the rate of sustained complete remissions in adults is about 40%, much lower than the 80% cure rate in children (1). Due to the inability to sustain complete remissions and to the high rate of resistance to conventional therapy, new and effective approaches to ALL have been under investigation for the past years (2).

Individual variation in the response to therapy has been observed. As several genes associated with the response to vincristine, duanorubicine, prednisolone and asparaginase have been identified (3), it is becoming evident that therapeutic success in ALL requires a rational approach, using molecules that target specific proteins whose function is essential to the leukemogenic process. On the other hand, as cancer is a multifactorial process and, in order to obtain a complete therapeutic success, it may be necessary to search for optimal associations between drugs acting via several molecular mechanisms, namely in the signal transduction pathways involved in carcinogenesis/leukemogenesis (3).

The farnesyltransferase inhibitors (FTIs) are a heterogeneous group of compounds capable of inactivating RAS protein, with therapeutic potential demonstrated both in vitro and in vivo (1, 4). These molecules are able to inhibit farnesyltransferase, an intracellular enzyme that catalyzes the farnesylation of several proteins, including RAS. The three RAS genes code four 21 kDa proteins (H-RAS, K-RAS4A, K-RAS4B and N-RAS). Each of these proteins suffers a sequence of post-translational modifications, the first of which is the transfer of an isoprenoid farnesyl group, catalyzed preferentially by Farnesyltransferase. The addition of the farnesyl group is made at the carboxylic terminal, next to the CAAX amino acid sequence (C, cysteine;
a, any aliphatic aminoacid; X, any other aminoacid). This step allows the interaction of the protein with the hydrophobic layer of the cell membrane and is critical to the conversion of RAS into the membrane-associated and biologically active form (Figure 1)(5-7). These modifications are essential to the interaction between these proteins and the inner face of the cellular membrane, where they will perform their function by binding and hydrolyzing GTP, acting as a molecular switch, i.e., they form an interface between membrane receptors and intracellular signaling pathways (8, 9). The mutations in the RAS genes are among the most frequently associated with cancer. About 30% of human cancer cases are linked to some form of mutation of these genes (90% of pancreas, 50% of colon, 30% of lung cancer cases, as many as 30% of the cases of infantile and juvenile forms of chronic myelogenous leukemia; also ALL (10%), chronic myelomonocytic leukemia (65%) and myelodysplastic syndromes (6, 10-13). Most mutations with oncogenic potential are responsible for the loss of the GTPase capacity. Under this condition, membrane-associated RAS remains constitutively active, continuously stimulating cell proliferation. Although this mechanism remains the same throughout these malignancies, there are subtle differences concerning the underlying mutation: while in hematological malignancies the most frequently mutated isoform is N-RAS, in the case of cancer of the lung, colon and pancreas, it is K-RAS (6, 11, 14, 15). Basic and clinical investigation has shown that the presence of a mutated RAS gene, although important to carcinogenesis, is not determinant to the action of FTIs. Two pre-clinical studies testing the effects of FTIs on cell lines derived from malignancies of the skin, lung, breast, pancreas, ovary, prostate, bladder, uterus, and colon have shown that 70% of these are sensitive to FTIs independently of the presence of a mutation in the RAS gene (4). This fact widens the range of carcinomas over which FTIs might be effective; however, the concentrations needed to achieve a therapeutic effect are 100 to 1000 times greater than those for cases with the mutation (6, 12). The range of biological activities of FTIs may also be larger than initially expected (11). Given the role of RAS in the resistance to radiotherapy, FTIs may act as radio-sensitizers and reverse resistance to this kind of therapy. FTIs may also play a role as inhibitors of angiogenesis, as suggested by the reduction of vascular endothelium-derived growth factor (VEGF) in the presence of tipifarnib (4, 6, 11, 16, 17).

However, the mechanisms of action have been under intense debate and remain, mostly, uncharacterized. Although most evidence points to the fact that the anticarcinogenic effect is mainly related to RAS (both in cells with and without wild-type RAS gene), RHO-related GTP-binding protein (RHOB), centromere protein (CENP) or other proteins may be relevant alternative targets (10, 18). Adding to this, some of the promising results obtained in vitro did not have the expected correspondence in phase II and III clinical trials, showing how important it is to thoroughly describe the mechanisms of action of these drugs. In this way, rational applications and associations may be addressed in future trials (4, 19). In this context, our goal was to evaluate and characterize the therapeutic effect of FTIs, alone and in combination with conventional anticarcinogenic agents, in an ALL cell line. Materials and Methods

Reagents and cell line culture conditions. The FTI α-hydroxyfarnesylphosphonic acid (α-HFPA) was acquired from Biomol Research Laboratories (Plymouth, PA, USA). Stock solutions were prepared by dilution in water and frozen at –20°C. For flow cytometry, anti-cyclin D1 and anti-lamin A/C antibodies labeled with phycoerythrin (PE), provided by Santa Cruz Biotechnology (Santa Cruz, CA, USA); PE-labeled anti-BCL-2-associated death promoter (BAD) antibodies provided by Dako (Glostrup, Denmark). Monoclonal mouse antibody isotype IgG1/FITC and antibody isotype IgG2b/PE provided by Dako (Glostrup, Denmark). Antibodies against phospho-p44/p42 mitogen-activated protein kinase (MAPK) (ERK1/ERK2), phospho-p38 MAPK, and phospho-v- AKT murine thymoma viral oncogene (AKT) (Ser473) were from Cell Signaling Technology (Danvers, MA, USA). Pan anti-ERK, anti-p38 and anti-AKT were from Cell Signaling Technology. Anti-actin antibody was purchased from Millipore Corporation (Billerica, MA, USA). One ALL-T cell line cell (CEM, ATCC® CRL-2265™: American Type Culture Collection, Manassas, VA, USA), passaged in our laboratory for fewer than 2 months after regeneration, was used in this study. During assays, cells were maintained in RPMI-1640 culture medium with 10% Fetal bovine serum (FBS), at a temperature of 37°C and in a humidified atmosphere with 5% CO2.
Cell viability, cell density and morphological analysis. Cells were incubated in the absence and presence of α-HFPA at concentrations of 10 nM to 100 μM. Cell density was evaluated 6, 12, 24 and 48 hours after the initial incubation, by cell counting in a Neubauer chamber. Cell viability was estimated by trypan blue exclusion assay and cell morphology was evaluated by light microscopy examination of May-Grünwald-Giemsa stained cells using a Leitz Dialux 20 microscope fitted out with a photographic chamber.

Evaluation of lamin A/C, cyclin D1 and apoptotic protein expressions by flow cytometry. To evaluate the effect of α-HFPA on farnesylation, CEM cells were incubated with α-HFPA at a range concentrations of 10 to 100 nM. After 48 hours, 1×10⁶ cells were centrifuged and incubated with an anti-lamin A/C, anti-BAD, anti-BCL-2 or anti-cyclin D1 monoclonal antibody according to the manufacturer’s protocols. The levels of cellular fluorescence, proportional to the concentration of protein in each cell, were measured by flow cytometry and results were plotted using normalized arbitrary units of mean fluorescence intensity (MFI). This value represents the medium fluorescence intensity detected in the cells, which is proportional to the number of molecules labeled by the antibody. For all the assays, negative controls were established with isotype immunoglobulin G (IgG), IgG1 and IgG2b, and submitted to the same procedures.

Evaluation of p38, AKT and ERK protein expressions by western blot analysis. To evaluate the effect of α-HFPA on farnesylation, CEM cells were incubated for 48 h with α-HFPA at a range concentrations of 10 nM to 100 nM. Subsequently, 50 μg of protein from cells extracts was electrophoretically separated on a 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel, and transferred to a polyvinylidene difluoride membrane (Millipore Corporation, Bedford, MA, USA). The membranes were blocked with 5% (w/v) fat-free dry milk in Tris-buffered saline containing 0.1% (v/v) Tween 20 (TBS-T) for 1 hour at room temperature. Then the membranes were incubated overnight at 4°C with the primary antibodies against the different proteins: phospho-ERK1/ERK2, phospho-p38 MAPK and phospho-AKT. The membranes were then washed for 25 min with TBS-T and incubated for 1 hour at room temperature with alkaline phosphatase-conjugated anti-rabbit or anti-mouse antibodies (GE Healthcare, Chalfont St. Giles, UK). To test whether similar amounts of protein for each sample were loaded, the membranes were stripped and re-probed with antibodies to total ERK1/2, p38 MAPK, and AKT or with an anti-actin antibody, and blots were developed with alkaline phosphatase-conjugated secondary antibodies and visualized by enhanced chemifluorescence.

Statistical analysis. Statistical analysis was carried out using GraphPad Prism software, version 5.00 for Windows (GraphPad Software, San Diego, CA, USA). Comparison of groups was performed by ANOVA and Tukey test. Statistical significance was considered for differences with \( p<0.05 \).

Results

Analysis of cell viability. Starting with a cell density of 0.25×10⁶ cells/ml, it was observed that α-HFPA led to a decrease in cell viability in a way that was dependent on the concentration of the drug and on the time of incubation (Figure 2). In fact, when cells were incubated with 100 nM of FTI for 48 h, there was a reduction in cell viability by 50% and this effect correlated well with concentration until 500 nM. At higher concentrations, the effects are similar.

Analysis of the effect of α-HFPA on farnesylation. To analyze the effect of α-HFPA on farnesylation, CEM cells were incubated with selected concentrations of α-HFPA, below the half maximal inhibitory concentration (IC₅₀) found at 48 h in viability studies. The results, as shown in Figure 3, demonstrate a statistically significant decrease, beyond 50%, of the levels of lamin A/C (Figure 3B). This effect is similar in all tested conditions and no dose-response curve could be
inferred. As noted, the decrease of the levels of lamin A/C reflects a similar decrease of the activity of FT. The number of cells that expressed detectable levels of lamin A/C increase for concentrations above 10 nM but no statistical significance was found (Figure 3A).

Evaluation of expression of survival and apoptotic protein.
In order to determine if the antiproliferative effect was accompanied by an interruption in the MAPK pathway, we analyzed the expression of cyclin D1 using the conditions described above for lamins. As depicted in Figure 4, a significant reduction of the intracellular concentration of cyclin D1, was observed for all tested conditions (Figure 4A). However, the number of treated cells expressing cyclin D1 is similar to the one of control cells. To further characterize the effects of α-HFPA in cell signaling, namely in apoptosis, we analyzed the expression of BAD. Results shown in Figure 5 demonstrate that despite the intracellular levels of BAD remaining statistically unchanged (Figure 5B), there is a significant increase of the number of cells expressing this pro-apoptotic molecule (Figure 5A), which may reflect a general tendency towards cell death through apoptosis. This result is α-HFPA dose-dependent. Our results also show that phosphorylation of p38, AKT and ERK proteins remained approximately the same under all tested conditions (data not shown).

Morphological studies. Optical microscopy was used to further characterize the cytotoxic effects observed by flow cytometry. Figure 6 shows the morphology of cell smears stained with May-Grünwald-Giemsa before (Figure 6A) and after (Figure 6B) treatment with α-HFPA. After exposure to the drug, cells show morphological characteristics typical of cell death by apoptosis, such as cell contraction, nuclear fragmentation, blebbing and apoptotic bodies (Figure 6B).

Discussion
The viability of CEM cell populations was reduced after incubation with α-HFPA in a concentration- and time-dependent manner. The IC50 was found to be near 100 nM at 48 h and the maximal effect was observed for cells treated with 500 nM. No significant increase in biological effects of α-HFPA was observed above this concentration. Other studies have shown that despite the promising results obtained in cell lines, α-HFPA, a first-generation FTI, did not have the same success in animal models (11). As shown in this and other articles, α-HFPA induces inhibition of FT and has a cytotoxic effect on ALL cells, inducing cell death mainly through apoptosis. The results obtained suggest that the efficacy of this FTI is dependent on the cell type, concentration and time of incubation and may be affected by the dynamics of complex biological systems, as described above. We believe that at the molecular level, the mechanism of action of α-HFPA can offer some clues to the general mechanism of action of other FTIs and that primary cell cultures represent the ideal tool to study these events.

There are four known types of FTIs (11). The first to be described were the competitive inhibitors of farnesylpyrophosphate (FPP) (the donor of the farnesyl group during the process of farnesylation), such as manumycin and α-HFPA. However, due to the high intracellular concentrations of FPP –
for which FT has a high affinity – the efficacy of these drugs in several cell lines is very low. Moreover, as FPP is necessary for a multitude of other important intracellular processes, even in normal cells, these compounds have been shown to be very toxic. The research for molecules that could overcome these limitations gave rise to a second generation of FTIs – the peptidomimetic inhibitors, designed rationally after the CAAX recognition terminal of FT-targeted proteins. Initially, poor intracellular penetration and rapid degradation by proteases made these compounds very ineffective. The later development of pro-drugs, in the form of esters, increased their capacity to pass the plasma membrane. One such molecule was L-778,123, whose phase I clinical trial was interrupted due to serious deleterious side-effects. Plasmatic esterases, however, would still pose a problem because they degrade these pro-drugs and render them susceptible to the action of proteases.

Two other families of FTIs were recently described: the non-peptidomimetic bi-substrate inhibitors (capable of inhibiting

Figure 4. Analysis of cyclin D1 expression in CEM cells treated with α-hydroxyfarnesylphosphonic acid (α-HFPA). A: The percentage of cells that expressed cyclin D1; B: the levels of expression as mean intensity of fluorescence (MIF), represented in relation to that of the control. The number of cells where cyclin D1 was detectable remained stable. A statistically significant reduction of the intracellular concentration of cyclin D1 was observed for all tested conditions. The results were obtained after 24h of incubation with α-HFPA and represent the mean±SD of three independent experiments. *p<0.05.

Figure 5. BCL-2-associated death promoter (BAD) expression in cells treated with α-hydroxyfarnesylphosphonic acid (α-HFPA). A: The percentage of cells that expressed BAD; B: Levels of BAD expression as mean of intensity of fluorescence (MIF), represented in relation to that of the control. The number of cells where BAD was detectable increased in a statistically significant manner above 10 nM. Intracellular levels of BAD had a mild tendency to increase but did not reach statistical significance. The results were obtained after 24h of incubation with α-HFPA and represent the mean±SD of three independent experiments. *p<0.05.
the interaction of FT with both the FPP and the CAAX terminal of target proteins) and the non-peptidomimetic CAAX analogs (non-PCA). Due to their good intracellular availability and high efficacy in inhibiting FT, drugs belonging to this last category are the most promising from the clinical point of view (15). Tipifarnib and lonafarnib have been tested in several solid and non-solid malignancies and are now in clinical test and use. Association with conventional drugs has allowed the use of lower concentrations without loss of efficacy, constituting one of the most promising applications of such drugs.

Due to the highly targeted mode of action of non-PCA FTIs, and despite the ubiquity of the process of farnesylation, the level of toxicity induced by therapeutic doses is low, as shown in animal and human studies. The explanation to this observation may lie in the action of geranylgeranyl-transferase inhibitor (GGT-I), which is able to farnesylate several substrates of FT. On the other hand, malignant cells may be more dependent on survival pathways involving RAS (14). To these facts we could add the negative dominant effect of non-farnesylated forms of H-RAS and RHOB (6, 11, 15): it has been described that non-farnesylated mutant RAS may exert a dominant negative effect on membrane-associated RAS. This effect was not observed for non-mutant RAS, which makes it specific to malignant cells with RAS mutation, aiding to explain the specificity of FTIs (14). These results have been challenged by Chen and collaborators, who showed that the relation between cell death and the increase in the action of GGT on RHOB (as would be achieved with the inhibition of FT), can also be seen with the increase of the activity of FT on RHOB. Such results support the idea that other relevant molecular targets of FTIs are yet to be described (15, 21).

In our study the activity of FT was evaluated following the general principles described by Adjei et al. (22), according to which, among the group of specific molecular markers of farnesylation, prelamin A and heat shock protein 40 (HSP40 or HDJ-2) present similar sensibility as a marker of FTI efficacy. It is even stated that as both molecules are ubiquitous, they may be used as markers of farnesylation in clinical studies, as shown by Adjei and collaborators in a phase I trial of lonafarnib (14, 15, 22). FT is essential for the processing of prelamin A into mature lamin A (23) and the use of monoclonal antibodies to measure the intracellular levels of both these molecules may serve as marker of the activity of FT. Lamins A, B and C are important elements of the nuclear envelope. Lamin B (but not lamin C) is also a target of FT (24). However, it is not affected by the use of FTIs to the same extent as is lamin A (22). The processing of prelamin A is independent of the cell cycle or apoptosis. As expected, the use of an FTI led to the decrease of the level of mature lamin A. This result shows that α-HFPA is able to inhibit FT, even at concentrations as low as 10 nM. By comparing Figure 2 and 3, it becomes obvious that the inhibition of FT by α-HFPA is not immediately correlated to the decrease of cell viability. This effect is similar to what was observed for other FTIs, where the concentrations necessary to obtain biological effects were consistently superior to the concentrations needed to inhibit farnesylation (15, 25). It has been hypothesized that malignant cells may activate alternative molecular mechanisms that reduce their dependence on the activation of RAS (14) and allow them to proliferate in a relative absence of functional RAS. This may explain the poor relation between the inhibition of FT and the decrease of cell viability. It was interesting to observe a plateau of maximal inhibition, beyond which an increase of the concentration of α-HFPA does not improve its biological effect. This fact may have clinical consequences as it may indicate that the dose escalade may not yield any therapeutic benefit, contributing only to enhance possible secondary effects (26).

Having confirmed the antiproliferative efficacy of α-HFPA, we have assessed how this observation correlates with the

Figure 6. Morphological analysis of CEM cells after incubation with α-hydroxyfarnesylphosphonic acid (α-HFPA). Control cells are shown on the left panel (A) and cells treated with α-HFPA on the right (B). There can be seen is morphological evidence of cell death by apoptosis, such as cellular contraction, nuclear fragmentation, blebbing and formation of apoptotic bodies. Cell smears were stained with May-Grünwald-Giemsa. Scale bar=10 μm.
RAS/MAPK pathway. For that purpose, we analyzed the expression of cyclin 1, a protein involved in cell cycle progression from G1 to S phases. The decrease in cyclin D1 was significant even for concentrations of α-HFPA as low as 10 nM, ten times inferior to the IC50. As in the case of lamins, the molecular effect also precedes the biological effect, suggesting that at low concentrations of FTI cells may activate compensatory mechanisms through alternative pathways, allowing them to maintain their proliferative capacity. The study of these processes may open doors to rational design of future therapeutic associations. Other FTIs have a different effect on the cell cycle: as shown by Song and collaborators in pancreatic adenocarcinoma cells, the FTI L-744,832 led to an increase in the number of tetraploid cells by stopping progression of the cell cycle through G2/M, increasing the concentration of cyclin B1 and inducing apoptosis (16).

The mechanisms that lead to the effects of FTIs on cyclins are not fully described but the leading hypothesis points to a disturbance in the transport of these molecules through the nuclear membrane as being a key factor (27). In this context, the effect of FTIs over the metabolism of lamins cannot be overlooked as a contributing factor. As observed in this study, the decrease of cyclin D1 correlates with the inhibition of FT by α-HFPA, suggesting a relation between both processes. These results are in accordance with other articles pointing out that the MAPK pathway is inhibited by FTIs, as would be predicted by its dependence on the action of RAS (28).

Adding to the antiproliferative effect, we observed a cytotoxic effect. As shown in Figure 6, cell death induced by α-HFPA shows characteristics of apoptosis. In order to assess the mechanisms involved at the molecular level, we analyzed the expression of key molecules in apoptosis, such as BAD. Other studies have failed to show a relation between the use of FTIs and the activation of such cell death mechanisms (29). Our results were not conclusive concerning BAX and BCL-2 (data not shown). However, BAD, whose activation is dependent on the phosphatidyl-3-inositol kinase signaling pathway, was shown to be affected by the use of α-HFPA, and may constitute a marker of the tendency towards apoptosis among the studied cell populations, as we confirmed by morphological studies. We hope to address this subject in further depth in future experiments.

This work supports the idea that FTIs may be useful even in the absence of RAS mutation and provides further evidence as to the involvement of other molecules (such as CENP and RHOB), which may prove to be suitable targets of future experiments and trials. FTIs have complex mechanisms of action that must be addressed in future studies in order to provide a logical frame of evidence that may explain the multitude of different results described in the literature. It has been pointed out that the main factor contributing to the relative lack of success of FTIs as single agents in clinical trials may be due to the genetic diversity and genomic instability of malignant cells. FTIs act on a specific pathway involving RAS and, although this enzyme may play a primordial role in the initial phases of malignant transformations, as in ovarian and colon cancer, it may lose its relative weight to other mechanisms that impose themselves on the process of maintenance, growth and spread of the cancer mass (12, 15). On the other hand, some of the targets of FT (including RAS) maintain their affinity towards GGT, which may be sufficient to maintain farnesylation and, consequently, produce the malignant phenotype (6, 30).

At least one aspect seems to be certain: future therapies including FTIs must be undertaken on a long-term program, as the cessation of such drugs leads to the resurgence of the malignant phenotype (6). The present evidence also shows that RAS may be only one among many other important proteins affected by FTIs, even acquiring a secondary role in the absence of mutation (11). The global target may be composed of a group of proteins, involved in several intracellular key processes that, in turn, may be compensated by accessory pathways. The use of markers for each possible path may allow the monitoring and assessment of the efficacy of FTIs (12, 15, 31). However, the scenario where an to date uncharacterized target may play an important role is still possible. The relative lack of efficacy of FTIs in solid tumors is counterbalanced by some promising results in the area of hemato-oncology, where these drugs have proven to be effective in selected cases (15, 20, 32, 33). Ongoing clinical trials may further extend our knowledge on which subpopulations may benefit from this therapy, and also allow the scientific community to describe associations with existing drugs proven to be synergistic with FTIs. We believe that the understanding of the key molecular events underlying such observations may provide a rational framework to support present and future therapeutic trials in hematological malignancies, namely acute leukemia.

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**References**