# ITF2357 Interferes with Apoptosis and Inflammatory Pathways in the HL-60 Model: A Gene Expression Study

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**Abstract.** *Background: Cytotoxic and pro-apoptotic effects exerted by the histone deacetylase inhibitor ITF2357 have been reported in acute myeloid leukemia HL-60 cells. In the current study, its mechanism of action was investigated at the molecular level. Materials and Methods: Cell proliferation was evaluated by methyl thiazol tetrazolium bromide reduction; apoptosis by annexin V, mitochondrial transmembrane potential by tetramethylrhodamine ethyl ester. Functional experiments and gene expression evaluations were performed by flow cytometry, microarray, and quantitative polymerase chain reaction. Results: Significant cell growth inhibition and increased apoptosis were observed. ITF2357 reduced protein levels of BCL-2, MCL-1, and BCL-X, and increased levels of BAK. Exposure to ITF2357 did not abrogate NF-κB DNA binding. After microarray analysis, interleukin-10, interleukin-6, epidermal growth factor, peroxisome proliferator-activated receptor (PPAR), transforming growth factor β, P38 mitogen-activated protein kinase, aryl hydrocarbon receptor, xenobiotic metabolism, PPAR/retinoic acid receptor, NF-κB, apoptosis, lipopolysaccharide/interleukin-1, G-protein receptor, T-cell receptor, and platelet-derived growth factor were the de-regulated pathways. Conclusion: This study shows that ITF2357 influences both proliferation and inflammatory pathways in HL-60 cells; this observation could have possible applications in clinical practice.*

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Histone acetylation and deacetylation are reversible events involved in gene transcription regulation. Disturbance of the dynamic between histone acetyl-transferases (HATs) and deacetylases (HDACs) can result in several hematological malignancies, such as acute leukemia (1, 2), and diffuse large B-cell lymphoma (3). Consequently, histone deacetylase inhibitors (HDACIs) have been recently developed as promising anticancer drugs. They are able to block proliferation, induce differentiation and apoptosis in a wide variety of transformed cell in culture, to increase expression of the cyclin-dependent kinase inhibitor $p21^{waf1}$, reduce levels of cyclin A and D, and inhibit the activity of thymidylate synthase. Moreover, HDACIs up-regulate the expression of TNF-related apoptosis-inducing ligand (*TRAIL*), *FAS, BAK, BAX* and down-regulate the expression of X-linked inhibitor of apoptosis protein (*XIAP*), *BCL-2* and *MCL-1* genes in the context of apoptosis (4).

Moreover, HDACIs are also promising anti-inflammatory compounds: they reduce nitric oxide production, circulating levels of tumor necrosis factor α (TNFα), interleukin-1β (IL-1β), interleukin-6 (IL-6), interferon-γ (IFNγ), interleukin-12 (IL-12), and interleukin-2 (IL-2) transcription in T-lymphocytes (5). Moreover, HDACIs inhibit NF-κB transcriptional activity in several models (6, 7), whereas, in other cases, NF-κB activation has been reported (8).

ITF2357, a novel member of the hydroxamic acid family, has been shown to prevent inflammation in lipopolysaccharide-induced shock and concanavalin A-induced hepatitis in mice, (9) and is able to attenuate intestinal inflammation and inflammation-associated tumor growth in a murine model of colitis-associated cancer (10).

A potent cytotoxic and pro-apoptotic effect exerted by this HDACI has also been reported in the acute myeloid leukemia HL-60 cell line model, where ITF2357 up-regulated the expression of p21, and down-modulated *BCL-2* and *MCL-1* gene expression. Moreover, this HDACI was highly cytotoxic towards freshly isolated myeloma and acute leukemia samples *in vitro*, even when they were stimulated by co-culture with bone marrow-derived mesenchymal stromal cells (11).

On these bases, we decided to re-evaluate the effects of ITF2357 on the acute myeloid leukemia model HL-60 from the transcriptional point of view. With this purpose, we performed real-time RT-PCR and microarray assays; our intent was to use these molecular methods to better identify the pathways de-regulated by this HDACI, with the possibility of identifying new targets for therapy and new fields of application.

## Materials and Methods

*Cell line*. HL-60 cell line, a cell line originating from a patient with acute myeloid leukemia, firstly diagnosed as acute promyelocytic leukemia (FAB class M3), but re-evaluated as FAB class M2 (acute myeloblastic leukemia with differentiation, PML/RARα-negative) (12), purchased from DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany, was grown in RPMI-1640 medium (Gibco-Life Technologies, Carlsbad, CA, USA), supplemented with 10% fetal bovine serum (FBS), 2 mmol/l L-glutamine and 1% gentamicin at 37˚C, in a humidified atmosphere containing 5% $CO_2$. All experiments were performed using logarithmically growing cells. ITF2357 was supplied by Italfarmaco (Milan, Italy). It was dissolved in dymethylsulfoxide (DMSO) as a 20 mM stock solution, stored at –20˚C and diluted to the required concentrations with serum-free culture medium, just before use. Cell suspensions were placed in sterile Falcon flasks and treated with different concentrations of ITF2357 (0.1-1 μM) for different time intervals, according to experimental targets (2-48 h).

*Reagents*. Caspase-8 inhibitor Z-IETD-FMK was purchased from Promega, (Milan, Italy); caspase-9 inhibitor Z-LEHD-FMK was obtained from Calbiochem (La Jolla, CA, USA). For flow cytometric analyses, monoclonal mouse fluorescein isothiocyanate (FITC)-anti-CD95, human annexin-V, and isotype control monoclonal antibodies were purchased from Becton Dickinson Biosciences (San Jose, CA, USA). Phycoerythrin (PE)-anti-human DR4 (clone DJR1) and PE-anti-human DR5 (clone DJR2-4) were obtained from BioLegend (San Diego, CA, USA). For immunocytochemical evaluation of NF-κB, goat FITC-IgG anti-NF-κB p65 subunit antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Probes for electrophoretic mobility shift assay were obtained from Primm (Milan, Italy). Cell culture supernatants for the cytokine assay were frozen and stored at -20˚C until analysis. Human tumor necrosis factor-α, intercellular adhesion molecule-1 (ICAM1), interleukin-1β, interleukin-6, interleukin-10, and tumor growth factor-β ELISA Kits were used (Bender Medsystem, Burlingame, CA, USA).

*Cell survival assays*. Cell viability was determined by trypan blue exclusion assay, and proliferative responses assayed by a colorimetric test based on methyl thiazol tetrazolium bromide reduction (MTT), as reported elsewhere (13).

*Apoptosis assays*. After drug exposure, apoptosis was evaluated by annexin V-FITC/propidium iodide (PI) staining and flow cytometric analysis, as recommended by the manufacturer (Becton Dickinson, Franklin Lake, NJ, USA). Briefly, $10^6$ cells were incubated with the drug for 24 and 48 h. After washing in 1× PBS, cells were re-suspended in 100 μl binding buffer (10 mM HEPES/NaOH (pH 7.4), 140 mM NaCl, 2.5mM $CaCl_2$) and then consecutively stained with 5 μl annexin V and 10 μl PI at room temperature, in the dark, for 15 minutes. Samples were analyzed by flow cytometry to determine the percentage of cells displaying annexin V-positive /PI-negative (early apoptosis) or annexin V-positive/PI-positive staining (late apoptosis and cell death).

To evaluate mitochondrial transmembrane potential ($\Delta\Psi_m$) changes, cells were stained for 30 min at 37˚C with 25 nM tetramethylrhodamine ethyl ester (TMRE) fluorescent dye (Sigma-Aldrich, St. Louis, MO, USA) and then analyzed by flow cytometry.

For detecting FAS and TRAIL receptor expression, HL-60 cells were incubated for 30 min at 4˚C with monoclonal mouse FITC-anti-human CD95, PE-anti-human DR4 (clone DJR1), and PE-anti-human DR5 (clone DJR2-4). Isotype control monoclonal antibodies were used to estimate the non-specific binding of target antibodies to cell surface antigens. Analysis was performed immediately using a BD FACScan flow cytometer equipped by Cell Quest Software (Becton Dickinson).

BCL-2 Family Antibody Sampler Kit was purchased from Epitomics (Burlingame, CA, USA); expression of BCL-2, BCL-X, BAK, and MCL-1 proteins was determined by flow cytometry. Briefly, $10^6$ cells were fixed in 2% paraformaldehyde, permeabilized with 1× FACS permeabilizing solution (Becton Dickinson) and incubated with blocking buffer (0.5% BSA in 1× PBS) for 30 min. Cells were then incubated with different monoclonal rabbit primary antibodies for 30 min at room temperature, followed by incubation with fluorescently-conjugated secondary antibody for 30 min at room temperature.

*Immunocytochemistry for NF-κB p65 subunit localization*. Briefly, cells exposed to ITF2357 for 2 h were plated on a glass slide by centrifugation, air dried at room temperature and fixed with 4% paraformaldehyde for 15 min. For antigen retrieval, cytospins were heated in a microwave oven for 5 min at 600 W in 10 mM sodium citrate and further exposed to 1% Triton X-100 solution for 10 min. Slides were then blocked with 10% FBS for 20 min at 37˚C and then incubated with goat FITC-IgG anti-NF-κB p65 subunit (Santa Cruz Biotechnology) diluted 1:20 with 1% Triton X-100, for 1 h at 37˚C. After 4'-6-diamino-2-phenylindole (DAPI) nuclear counterstaining, slides were mounted and visualized under a Nikon Eclipse TE-2000U fluorescence microscope equipped with a Nikon D-Eclipse C1 spectral confocal laser. Pictures were captured using EZ-C1 software, version. 3.40 (Nikon, Milan, Italy).

*Electrophoretic mobility shift assay*. For electrophoretic mobility shift assay (EMSA), nuclear extracts from HL-60 cells were prepared using the methods of Andrews and Faller (14), with some modifications. Briefly, the cell pellet was washed with washing buffer (10 mM HEPES pH 7.9, 2 mM $MgCl_2$, 200 μM EDTA), centrifuged at 1,000 ×g for 2 min and lysed in buffer A (10 mM HEPES pH 7.9, 2 mM $MgCl_2$, 10 mM KCl, 200 μM EDTA) with 1% Triton X-100, 4 μg/ml leupeptin and 1 μg/ml aprotinin, on ice for 30 min. The cell suspension was then centrifuged at 3,000 ×g at 4˚C for 10 min. The supernatant of centrifugation (cytoplasmic

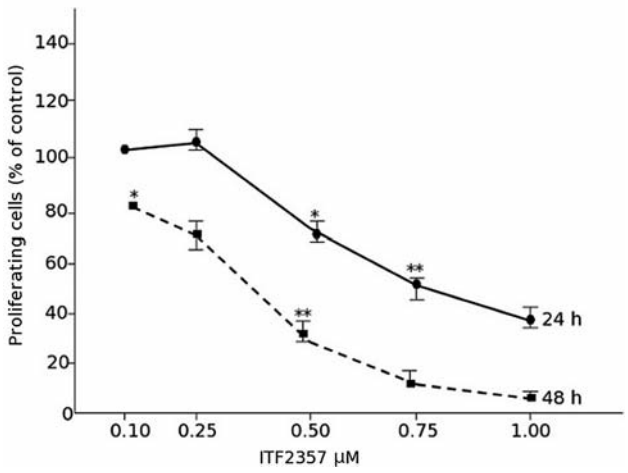


Figure 1. *Effects of ITF2357 on HL-60 proliferation. Proliferation of HL-60 cells incubated with increasing doses (0.1-1 μM) of ITF2357 for 24 and 48 h (*p<0.05; **p<0.001); the number of proliferating cells was determined using an MTT assay.*

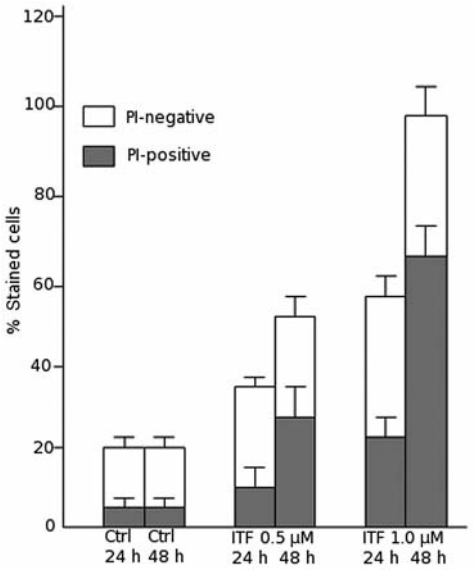


Figure 2. *Induction of apoptosis in HL-60 cells by ITF2357. HL-60 cells were exposed to 0.5 μM and 1 μM ITF2357 for 24 and 48 h, after which apoptosis was determined by annexinV/PI and flow cytometry, as described in the Materials and Methods. Data are the means of three separate experiments±SD.*

extract) and the pellet (nuclear fraction) were stored at –20˚C until used for Western blot experiments. EMSA assay was performed as previously reported by our group in the same cell model (15).

*Gene expression assays*. After exposure to 0.3 μM ITF2357, RNA from HL-60 cells was isolated using RNeasy Mini Kit (QIAGEN, Valencia, CA, USA); RNA from untreated cells were used as reference in the gene expression assays. Hybridization was performed on Whole Human Genome Microarray platforms (Agilent, Milan, Italy). Obtained data were imported into GeneSpring 6.1 software for analysis (GeneSpring 6.1; Silicon Genetics, Redwood City, CA, USA). The fold changes were analyzed by filtering the dataset using *p*-values <0.01 and a signal-to-noise ratio >2 for use in ANOVA statistical analysis. An additional filtering (minimum 2-fold change) was applied to extract the most significant of these genes which were further analyzed using Ingenuity Pathways Analysis (IPA) Software (Ingenuity Systems, Redwood City, CA, USA). Those genes with known symbols (HUGO) and their corresponding expression values were uploaded into the software. Each gene symbol was mapped to its corresponding gene object in the Ingenuity Pathways Knowledge Base. Networks of these genes were algorithmically generated based on their connectivity. Canonical pathways analysis identified the pathways from the IPA library of canonical pathways which were most significant to the input data set. The significance of the association between the data set and the canonical pathway was determined based on two parameters: (i) a ratio of the number of genes from the data set that map to the pathway divided by the total number of genes that map to the canonical pathway and (ii) a probability (*p*) value calculated using Fischer's exact test, determining the probability that the association between the genes in the data set and the canonical pathway is due to chance alone.

RNAs used for microarray experiments were also used for the real-time PCR quantitative assays, performed by RT² Profiler™ PCR Arrays Human NF-κB Signaling Pathway (PAHS-025) (SABioscience Corporation, Frederick, MD, USA). This PCR array profiles the expression of 84 key genes related to NF-κB-mediated signal transduction. The array includes genes that encode members of the Reticuloendotheliosis viral oncogene homolog (*REL*), *NF-κB,* and *IκB* families, NF-κB-responsive genes, extracellular ligands and receptors that activate the pathway, and kinases and transcription factors that propagate the signal. Moreover, in order to assess expression of genes involved in apoptosis, we employed the TaqMan® Low Density Array Human Apoptosis Panel (Applied Biosystems, Foster City, CA, USA) that contains assays for 93 genes involved in the apoptotic and inflammatory pathways. Plates were analyzed by an AB 7900HT apparatus.

*Statistical analysis*. All the experiments were repeated at least three times. Reported values represent the means±SD. The significance of differences between experimental conditions was determined using a two-tailed Student's *t*-test or by Kolmogorov-Smirnov test, where appropriate. The level of significance was set at $p<0.05$.

## Results

*ITF2357 inhibits proliferation and induces apoptosis of the HL-60 cells through both intrinsic and extrinsic pathways*. HL-60 cells were exposed to an increasing concentration of ITF2357 (from 0.1 μM to 1 μM) for 24 h and 48 h, respectively. As shown in Figure 1, a dose- and time-dependent inhibition of cell proliferation was observed, with an $IC_{50}$ of 0.8±0.02 μM after 24 h and 0.4±0.02 μM after 48 h. Trypan blue exclusion tests confirmed these results: cell viability after 48 h-incubation with ITF2357 at 0.5 μM was 50±10% ($p<0.05$) and decreased to 29±11% after 72 h (untreated cells=100%).

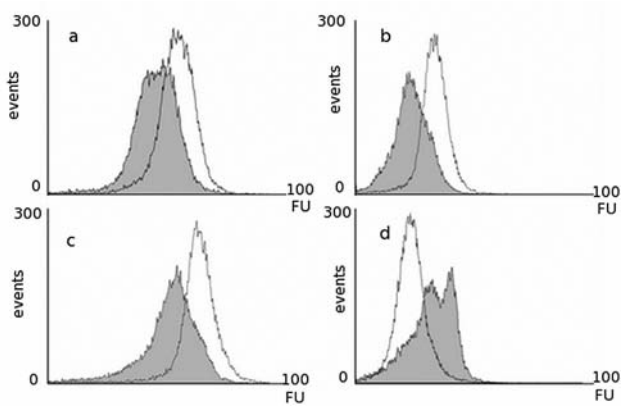


Figure 3. *Flow cytometric analysis of BCL-2 family protein levels. Cells were labelled with goat anti-rabbit FITC-conjugated secondary antibody. Unfilled histograms represent untreated cells; filled histograms represent results after 24 h exposure to ITF2357 0.5 μM. a: Down-regulation of BCL-2; b: Down-regulation of BCL-X; c: Down-regulation of MCL-1; d: up-regulation of BAK.*

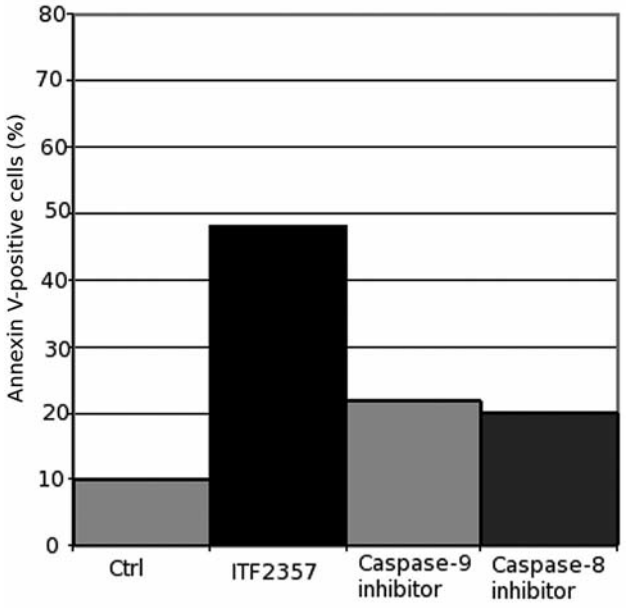


Figure 4. *Role of caspases in ITF2357-mediated apoptosis. Cells were pre-treated for 1 h with 25 μM caspase-8 and -9 inhibitors, after which the percentage of cells exhibiting positive staining for annexin V-FITC was determined by flow cytometry. Values represent the means±SD for three separate experiments.*

To determine whether this anti-proliferative activity was associated with the induction of apoptosis, we evaluated the extent of apoptosis by annexinV/PI staining. As shown in Figure 2, ITF2357 significantly increased apoptosis percentage in a dose- and time-dependent manner.

Apoptosis induction was confirmed by a dose-dependent loss of $\Delta\Psi_m$: after 48 h incubation at 0.5 μM, the percentage of cells expressing low TMRE levels (sign of damage of mitochondrial membrane) was 23%±5% *versus* 8%±1% of the untreated cells ($p<0.05$).

Moreover, treatment of HL-60 cells with ITF2357 reduced levels of BCL-2, BCL-X, and MCL-1, proteins, whereas increased levels of the BAK protein (Figure 3).

To investigate the role of caspases, two inhibitors, Z-IETD-FMK (for caspase-8), and Z-LEHD-FMK (for caspase-9) were tested. As shown in Figure 4, when both caspase-8 and -9 inhibitors were added at non toxic concentrations, apoptosis was reduced by 56% and 52%, respectively. Finally, we performed FACS analysis to assess whether ITF2357 would increase expression of pro-apoptotic FAS receptor (FASR) and TRAIL receptors (DR4 and DR5) and/or of their ligands (FAS and TRAIL). A 23-fold increase of DR5 was noted ($p<0.001$); no differences in FASR, DR4, FASL and TRAIL were found before and after ITF2357 treatment (Figure 5).

*ITF2357 does not inactivate the NF-κB transcription factor.* Considering the fundamental role that the transcriptional factor NF-κB plays both in the apoptotic and in the inflammatory pathways, we performed an EMSA assay to evaluate the effect of ITF2357 on DNA-binding activity of the NF-κB p65 subunit in HL-60 cells. Cells were incubated for 2 h with medium (negative control) or 0.3 μM ITF2357. Untreated HL-60 cells showed a partial constitutive activation of NF-κB (Figure 6, lane 4); exposure to ITF2357 did not abrogate the NF-κB DNA binding (Figure 6, lane 6). We also investigated the effect of ITF2357 on the localization of the NF-κB p65 subunit by immunocytochemical staining (Figure 7). In the untreated HL-60 cells, the partial constitutive activation of NF-κB observed by EMSA was confirmed by the localization (even if limited) of the p65 subunit in both the cytoplasm and in the nucleus (Figure 7a). After exposure to ITF2357, the active p65 subunit further moved into the nucleus (Figure 7b).

*ITF2357 interferes with the apoptotic and inflammatory pathways: gene expression evaluation.* We have shown above that ITF2357 induced significant cytotoxicity and apoptosis; to understand what occurs at the mRNA expression level, we performed real-time RT-PCR and microarray assays. These tests could also be useful for better understanding the finding regarding NF-κB, considering that neither EMSA nor immunocytochemistry have a quantitative value. As reported in Table I, we quantified the mRNAs of 4 NF-κB inhibitors (*CHUK, NFKBIA, NFKBIB, NFKBIE*), 3 activators (*IKBKB, IRAK1, IRAK2*), and of the *NFκB1* and *NFκB2* subunits. ITF2357 significantly down-regulated *IRAK1, NFκB1*, and

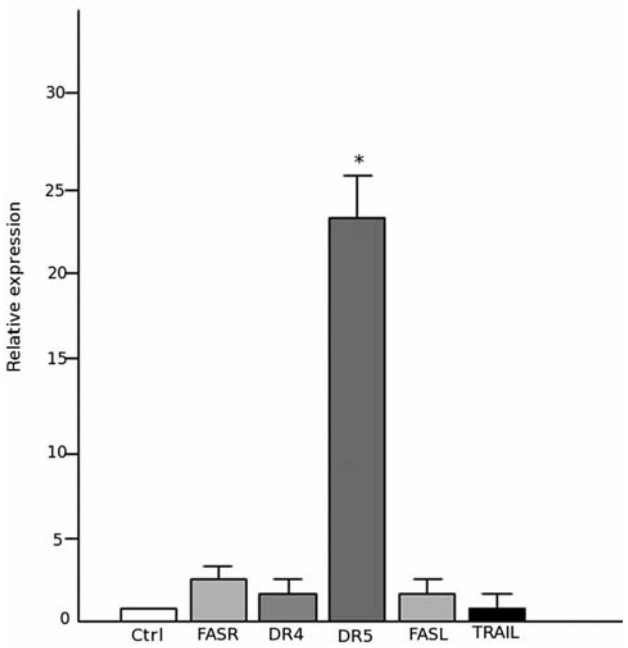


Figure 5. *Expression of FASL, FASR, TRAIL, DR4 and DR5 after treatment with ITF2357 0.5 μM. HL-60 cells were incubated for 30 min at 4˚C with monoclonal mouse FITC-anti-human CD95, FASL, TRAIL, PE-anti-human DR4 (clone DJR1), and PE-anti-human DR5 (clone DJR2-4). Values represent the means±SD for three separate experiments (*p<0.05 with respect to control, ITF2357 alone).*

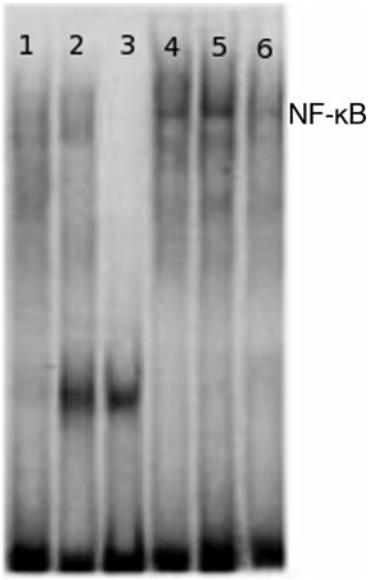


Figure 6. *EMSA of HL-60 cells nuclear extracts. Lane 1, Supershift assay: nuclear extract of TNFα-stimulated cells (20 ng/ml, 45 min) was preincubated with antibodies against NF-κB (p65); lane 2, competition experiment: nuclear extract of TNFα-stimulated cells was preincubated with a 200-fold molar excess of unlabeled probe; lane 3, biotinilated probe was incubated in the absence of HL-60 cell nuclear extracts; lane 4, electrophoretic mobility shift assay showing constitutive NF-κB binding activity in HL-60 cells; lane 5, NF-κB binding activity in TNFα (20 ng/ml 45 min)-stimulated cells; lane 6, HL-60 cells treated with ITF2357 (0.3 μM for 2 h).*

*NFκB2*, whereas it up-regulated *IRAK-2*. The expression of other analysed genes was not significantly changed.

The results of the analysis of expression of some genes involved in apoptosis are also listed in Table I. ITF2357 reduced *BCL-2* mRNA level; on the contrary, expression of *BAD, BAK1, BCL2L1 (BCL-X), MCL1,* and *BCL-3* did not change. As reported above, expression of BCL2 protein also decreased after exposure to ITF2357. On the contrary, while *BCL-X* and *MCL1* mRNAs did not change, the respective proteins decreased after HDACI treatment, probably as an effect on post-transcription events. The same hypothesis could be made for BAK1 whose protein increased after exposure to ITF2357.

Among pro-apoptotic genes (*CARD9, CASPASE 2, CASPASE 3, CASPASE 8, CASPASE 9, DEDD, DIABLO, FADD, FAS, FASL, HRK, HIP1, LRDD, NALP1, TNFRSF1B, TNFRSF21,* and *TRAIL*), ITF2357 significantly up-regulated *DEDD, DIABLO, HIP1, HRK*, and *NALP1*. Moreover, ITF2357 increased levels of *BIRC1*, an anti-apoptotic gene. In line with data about protein levels reported above, no changes in the expression of FAS receptor (*FASR*) and *FASL* were observed.

The results of analysis of the expression of some genes involved in the inflammatory pathway are shown in Table II. Among the 30 pro-inflammatory genes analyzed, ITF2357 significantly down-regulated *ATF1, CFB, ICAM1, IL8, IRAK1, LTBR, RIPK1, TLR4*, and *TNFA*. On the other hand, ITF2357 increased levels of *IL1A, IL1B, IRAK2, LTA, TLR1, TLR6, TLR7,* and *TLR9*. The remaining 13 tested genes were unaffected by the treatment. Among the anti-inflammatory genes, the exposure of HL-60 cells to ITF2357 induced increased expression of *HMOX1* and *NLRP12*; no changes of expression were measured for the *IL10, PPM1A,* and *TNFAIP3* genes.

The results described above show that the expression of genes involved in apoptotic and inflammatory pathways were significantly affected by ITF2357. To confirm and eventually extend these data, we performed microarray assays, by using the same RNAs extracted for real-time PCR (12 h exposure to 0.3 μM ITF2357). Figure 8 represents how many and which principal pathways are de-regulated by ITF2357. Major functions of de-regulated genes were related to signaling of *IL-10* (9% of genes comprised in this pathway were de-regulated), *IL-6* (8% of genes de-regulated), EGF (6% of genes de-regulated), *PPAR* (5% of genes de-regulated), *TGFβ, P38MAPK,* aryl hydrocarbon receptor, xenobiotic metabolism, collagen metabolism, *NF-κB,* apoptosis, *LPS/IL1*, G-protein receptor, T-cell receptor, and *PDGFRB* (3% of genes de-regulated).

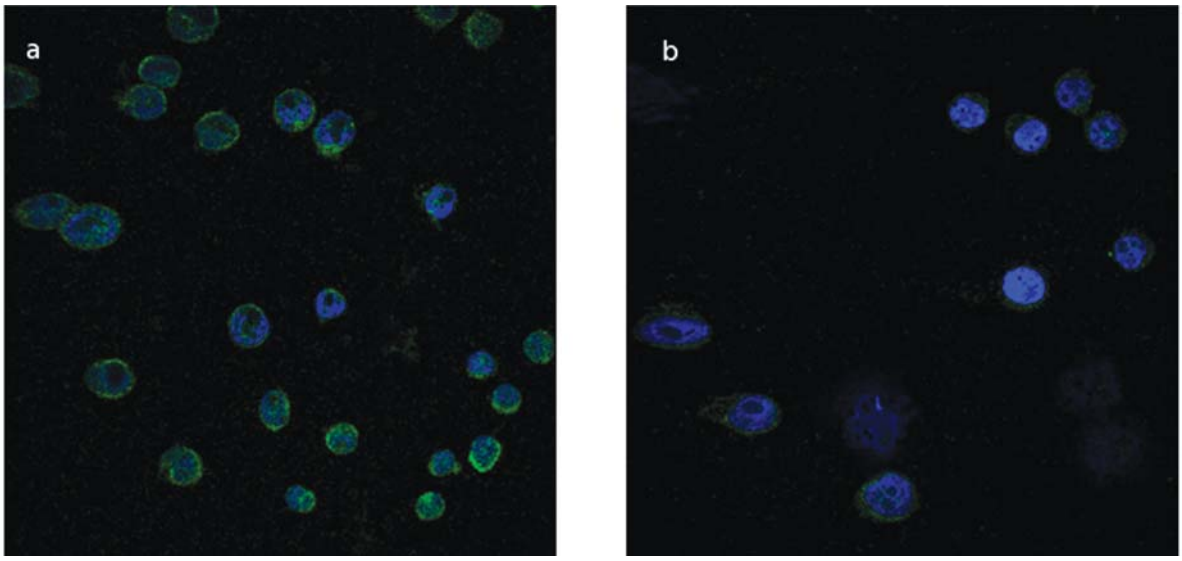


Figure 7. *Effects of ITF2357 on localization of the p65 NF-κB subunit. Analysis at room temperature, in the dark, by confocal immunofluorescence microscopy. HL-60 cells (a) treated with ITF2357 0.5 μM for 2 h and untreated cells (b) were subjected to immunostaining of p65 (green fluorescence) and nuclear counterstaining (blue), as detailed in the Materials and Methods. As depicted in the figures, ITF2357 increases the nuclear localization of the p65 NF-κB subunit. The results of a representative study are shown; two additional experiments yielded similar results (magnification, ×40).*

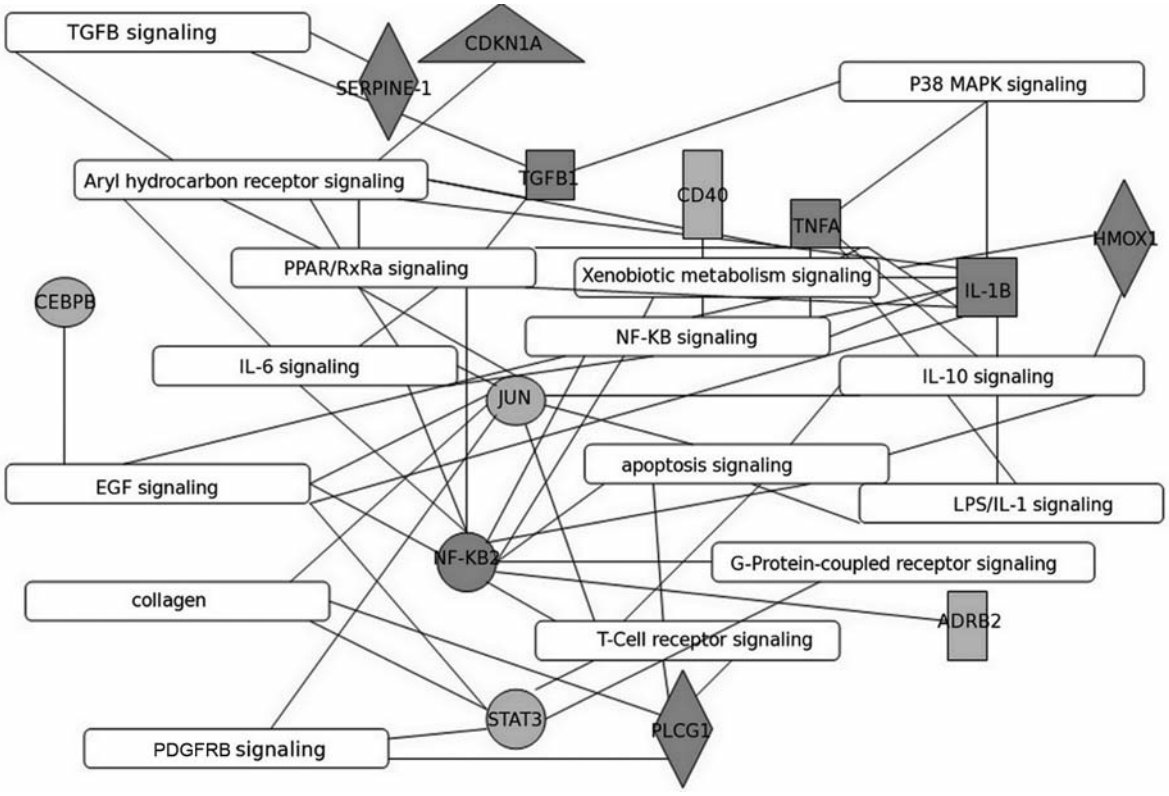


Figure 8. *Microarray analysis: main pathways de-regulated by ITF2357. Most significant pathways de-regulated in HL-60 treated versus untreated cells. Node (gene) and edge (gene relationship). In the figure the most significantly down-regulated genes are shown; the intensity of the node color (grey) indicates the degree of down-regulation. Shapes denote enzymes (◇), kinases (△), transcription factors (□), cytokines and growth factors (▯), and receptors (○).*

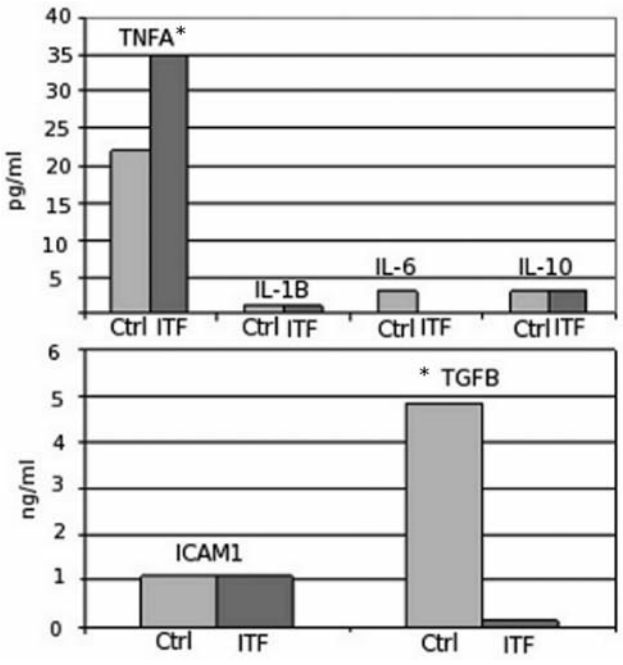


Figure 9. *Measure of TNFa, IL-1b, IL-6, IL-10, ICAM-1, and TGFβ levels in HL-60 cell culture medium. Levels of cytokines before (grey) and after (black) treatment with ITF2357 (*p<0.05 with respect to ITF2357 alone).*

The major proportion of these genes were down-regulated; thus, microarray analysis revealed other genes to be down-regulated, in addition to those already found by real-time PCR: *CCL2, CD40, CD86, CDKN1A, CEBPB, COL1A1, F5, IL-1E, JUN, PLCG1, PTX3, TGFB1, SERPINE1, SPP1, STAT3*, and *WT1*. On the contrary, up-regulated genes included *ADRB2, EDN1* and *KLF2*.

*ITF2357 reduces levels of IL-6 and TGFβ in the supernatant of HL-60 cell culture medium*. Finally, we measured levels of some cytokines in the supernatant of the HL-60 cell line after 24 h of culture in the presence of 0.3 μM ITF2357. As reported in Figure 9, levels of ICAM1, IL1-β, and IL-10 did not change, whereas IL-6 and TGFβ levels significantly decreased. Differing from that observed for the *TNFA* gene whose expression was reduced by ITF2357, TNFα protein levels in the HL-60 culture medium were increased.

## Discussion

The purpose of our study was to further investigate the effects exerted by ITF2357 on HL-60 cells, adding data obtained by gene expression assays to those already available from literature.

The first debated item concerns how ITF2357 induces apoptosis in HL-60 acute leukemia cells. Indeed, apoptosis seems to depend both on the cell type adopted and on the specific type of the HDAI. After treatment with valproic acid, increased expression of *TRAIL, DR5*, and *FASL* genes and correspondent proteins has been reported in cell lines expressing the PML-RARα or AML1-ETO oncoproteins and in all responsive cases, whereas no differences was noted in all resistant patients (16). On the other hand, in lymphoma and chronic myeloid leukemia cell lines, other authors showed that other HDACIs (LBH589 and SAHA) induced apotposis by the intrinsic apoptotic pathway (17). In KG-1 cells (an AML-M1 cell line), Golay *et al*. showed that ITF2357 activated the intrinsic pathway, with no pro-caspase-8 cleavage detectable in this and another myeloma cell line (11).

In our study, we showed that both intrinsic and extrinsic pathways are involved in the apoptotic process. Indeed, ITF2357 caused mitochondrial membrane depolarization, up-regulation of BAK, and reduced expression of BCL-2, MCL-1, and BCL-X proteins. ITF2357 did not change levels of *BAD, BAK1, BCL-X, MCL-1,* and *BCL-3* genes. Nevertheless, *BCL2L10* was down-regulated: the protein encoded by this gene can interact with other members of BCL-2 protein family including BCL-2, BCL-XL, and BAX. Reduced expression of *BAX* gene has been shown to induce cell apoptosis, possibly favouring cytochrome-*c* release from the mitochondria, and thus activating caspase-3 activation. Moreover, DR5 expression increased, with a possible involvement also of the extrinsic way, as supported by the reduction of apoptosis measured after treatment with inhibitors of caspase-8 and -9.

Reduced BCL-2 expression would have relevant clinical implications in acute leukemia; it has been reported that low BAX/BCL-2 ratio determined by flow cytometry significantly correlated with higher CD34 and CD117 levels, poor karyotype and worse survival (18). Thus, treatment with ITF2357 would be probably useful in improving the prognosis of AML patients.

Another interesting issue lies in the action of ITF2357 on the *NF-κB* gene. This is a debated and still unresolved question: in acute myeloid leukemia, lung, squamous cell and renal carcinoma, SAHA potentiated the apoptosis induced by TNFα by suppressing NF-κB activation, IκK activation, IκBα phosphorylation, IκBα ubiquitination, and p65 nuclear translocation, without any effect on direct binding of NF-κB to DNA (19). On the other hand, in myeloma cell lines, sodium butyrate and SAHA resulted in a modest increase in NF-κB DNA binding; only co-treatment with the proteasome inhibitor bortezomib diminished NF-κB activity (20). Finally, in non-small cell lung cancer, trichostatin A and SAHA attenuated NF-κB nuclear translocation and DNA binding (21).

In our study, we showed that ITF2357 down-regulated expression of *IRAK1, NFκB1*, and *NFκB2*; nevertheless, these

Table I. *Quantitative real-time PCR for NF-κB and apoptotic pathway genes. Genes studied after 0.3 μM ITF2357 versus no treatment (control) are listed.*

| Gene symbol | Gene name | ENTREZ Gene ID | Gene function | Effect of ITF2357 |
|---|---|---|---|---|
| *BAD* | BCL2-associated agonist of cell death | 572 | Pro-apoptotic | No change |
| *BAK1* | BCL2-antagonist killer 1 | 578 | Pro-apoptotic | No change |
| *BCL2* | B-cell CLL/lymphoma 2 | 596 | Anti-apoptotic | Down-regulation |
| *BCL2L1* | B-cell lymphoma like X (BCLX) | 598 | Anti-apoptotic | No change |
| *BCL2L10* | BCL2-like 10 (apoptosis facilitator) | 10017 | Pro-apoptotic | Down-regulation |
| *BCL3* | B-cell CLL/lymphoma 3 | 602 | Anti-apoptotic | No change |
| *BIRC1* | NLR family, apoptosis inhibitory protein | 4671 | Anti-apoptotic | Up-regulation |
| *CARD9* | Caspase recruitment domain family, 9 | 64170 | Pro-apoptotic | No change |
| *CASPASE2* | Caspase 2 | 835 | Pro-apoptotic | No change |
| *CASPASE3* | Caspase 3 | 836 | Pro-apoptotic | No change |
| *CASPASE8* | Caspase 8 | 841 | Pro-apoptotic | No change |
| *CASPASE9* | Caspase 9 | 842 | Pro-apoptotic | No change |
| *CHUK* | Conserved helix-loop-helix ubiquitous kinase | 1147 | NF-κB Inhibitor | No change |
| *DEDD* | Death effector domain containing | 9191 | Pro-apoptotic | Up-regulation |
| *DIABLO* | Diablo homolog | 56616 | Pro-apoptotic | Up-regulation |
| *FADD* | Fas (TNFRSF6)-associated via death domain | 8772 | Pro-apoptotic | No change |
| *FASR* | TNF receptor superfamily, member 6 | 355 | Pro-apoptotic | No change |
| *FASL* | TNF receptor superfamily, member 6 ligand | 356 | Pro-apoptotic | No change |
| *HIP1* | Huntingtin interacting protein 1 | 3092 | Pro-apoptotic | Up-regulation |
| *HRK* | Harakiri, BCL2 interacting protein | 8739 | Pro-apoptotic | Up-regulation |
| *IκBKB* | Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta | 9641 | NF-κB Activator | No change |
| *IRAK1* | Interleukin-1 receptor-associated kinase 1 | 3654 | Pro-inflammatory | Down-regulation |
| *IRAK2* | Interleukin-1 receptor-associated kinase 2 | 3656 | Pro-inflammatory | Up-regulation |
| *LRDD* | Leucine-rich repeats and death domain containing | 55367 | Pro-apoptotic | No change |
| *MCL1* | Myeloid cell leukaemia sequence 1 (BCL2-related) | 4170 | Anti-apoptotic | No change |
| *NALP1* | NLR family, pyrin domain containing 1 | 22861 | Pro-apoptotic | Up-regulation |
| *NFκBIA* | Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha | 4792 | NF-κB Inhibitor | No change |
| *NFκBIB* | Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, beta | 4793 | NF-κB Inhibitor | No change |
| *NFκBIE* | Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, epsilon | 4794 | NF-κB Inhibitor | No change |
| *TNFRSF1B* | Tumor necrosis factor receptor superfamily, member 1B | 7133 | Pro-apoptotic | No change |
| *TNFRSF21* | Tumor necrosis factor receptor superfamily, member 21 | 27242 | Pro-apoptotic | No change |
| *TRAIL* | Tumor necrosis factor (ligand) superfamily, member 10 | 8743 | Pro-apoptotic | No change |
| *NFκB1* | Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 | 4790 | NF-κB | Down-regulation |
| *NFκB2* | Nuclear factor of kappa light polypeptide gene enhancer in B-cells 2 | 4791 | NF-κB | Down-regulation |

promising effects did not result in reduction of DNA binding activity, or in the cytoplasm re-localization of the p65 subunit. All these observations would confirm the hypothesis that the effect of HDACIs on NF-κB activity would be cell type-dependent, with the consequent perspective of combining HDACIs with other drugs able to inhibit NF-κB, such as proteasome inhibitors (15) or arsenic trioxide (22).

The third interesting novelty deriving from our study concerns the possibility that ITF2357 may exert both an anti-tumor and an anti-inflammatory effect in the same cell model. Our data showed that ITF2357 increased expression of *HMOX1, EGR1,* and *NLRP12*, decreased levels of *ICAM1, IL1E, MCP1, PLCG1, LTBR1, SPP1, CCL2, CEBPB, PTX3,* and of some adhesion molecules, such as *CD40* and *CD86*. *EGR1* can mediate apoptosis in endothelial cells; its reduction might enforce the anti-angiogenetic effect already reported for ITF2357 (11). MCP1 protein is an important factor for monocytes chemotaxis, and its reduction may sustain the anti-inflammatory effect attributed to this HDACI. When stimulated by IL-1, *CEBPB* inhibits production of IL-6, and this may contribute to the reduction of IL-6 levels that we measured in the supernatant of cell cultures.

Moreover, the PPAR pathway was one of the most down-regulated pathways. PPARs are nuclear receptors that control many cellular and metabolic processes, affecting energy homoeostasis and inflammatory responses.

Among toll-like receptors (*TLRs*), *TLR4* was the gene most significantly down-regulated; LPS signalling is mediated by *TLR4*, resulting in activation of NF-κB, MAPK and JAK/STAT pathways and subsequent expression of IL-1, TNFα, IL-6, and IFNγ. In our study, *STAT3* mRNA was diminished on treatment with ITF2357; also in HEL and

Table II. *Quantitative real-time PCR for inflammatory pathway genes. Genes studied after 0.3 μM ITF2357 versus no treatment (control) are listed.*

| Gene symbol | Gene name | ENTREZ gene ID | Gene function | Effect of ITF2357 |
|---|---|---|---|---|
| *AGT* | Angiotensinogen | 183 | Pro-inflammatory | No change |
| *ATF1* | Activating transcription factor 1 | 466 | Pro-inflammatory | Down-regulation |
| *CD27* | CD27 | 939 | Pro-inflammatory | No change |
| *CFB* | Complement factor B | 629 | Pro-inflammatory | Down-regulation |
| *HMOX1* | Heme oxygenase 1 | 3162 | Anti-inflammatory | Up-regulation |
| *ICAM1* | Intercellular adhesion molecule 1 | 3383 | Pro-inflammatory | Down-regulation |
| *IFNA1* | Interferon alpha 1 | 3439 | Pro-inflammatory | No change |
| *IFNA2* | Interferon alpha 2 | 3440 | Pro-inflammatory | No change |
| *IFNG* | Interferon gamma | 3458 | Pro-inflammatory | No change |
| *IL10* | Interleukin 10 | 3586 | Anti-inflammatory | No change |
| *IL1A* | Interleukin 1 alpha | 3552 | Pro-inflammatory | Up-regulation |
| *IL1B* | Interleukin 1 beta | 3553 | Pro-inflammatory | Up-regulation |
| *IL1R1* | Interleukin 1 receptor 1 | 3554 | Pro-inflammatory | No change |
| *IL6* | Interleukin 6 | 3569 | Pro-inflammatory | No change |
| *IL8* | Interleukin 8 | 3576 | Pro-inflammatory | Down-regulation |
| *IRAK1* | Interleukin-1 receptor-associated kinase 1 | 3654 | Pro-inflammatory | Down-regulation |
| *IRAK2* | Interleukin-1 receptor-associated kinase 2 | 3656 | Pro-inflammatory | Up-regulation |
| *LTA* | Lymphotoxin alpha | 4046 | Pro-inflammatory | Up-regulation |
| *LTBR* | Lymphotoxin beta receptor | 4055 | Pro-inflammatory | Down-regulation |
| *NLRP12* | NLR family, pyrin domain containing 12 | 91662 | Anti-inflammatory | Up-regulation |
| *PPM1A* | Protein phosphatase 1 alpha | 5494 | Anti-inflammatory | No change |
| *RIPK1* | Receptor (TNFRSF)-interacting STK1 | 8737 | Pro-inflammatory | Down-regulation |
| *STAT1* | Signal transducer and activator of transcription 1 | 6772 | Pro-inflammatory | No change |
| *TICAM1* | Toll-like receptor adaptor molecule 1 | 148022 | Pro-inflammatory | No change |
| *TICAM2* | Toll-like receptor adaptor molecule 2 | 353376 | Pro-inflammatory | No change |
| *TLR1* | Toll-like receptor 1 | 7096 | Pro-inflammatory | Up-regulation |
| *TLR2* | Toll-like receptor 2 | 7097 | Pro-inflammatory | No change |
| *TLR3* | Toll-like receptor 3 | 7098 | Pro-inflammatory | No change |
| *TLR4* | Toll-like receptor 4 | 7099 | Pro-inflammatory | Down-regulation |
| *TLR6* | Toll-like receptor 6 | 10333 | Pro-inflammatory | Up-regulation |
| *TLR7* | Toll-like receptor 7 | 51284 | Pro-inflammatory | Up-regulation |
| *TLR8* | Toll-like receptor 8 | 51311 | Pro-inflammatory | No change |
| *TLR9* | Toll-like receptor 9 | 54106 | Pro-inflammatory | Up-regulation |
| *TNFA* | Tumor necrosis factor alpha | 7124 | Pro-inflammatory | Down-regulation |
| *TNFAIP3* | Tumor necrosis factor, alpha-induced protein 3 | 7128 | Anti-inflammatory | No change |

K562 cells, ITF2357 has been reported to lead to the disappearance of phosphorylated JAK2V617F, as well as pSTAT5 and pSTAT3, supporting the possibility of employing this drug in patients affected by chronic myeloproliferative disorders (23). Another interesting pathway down-regulated by ITF2357 is the p38 MAPK pathway; its inhibition could prevent transcription of TNFα and other pro-inflammatory cytokines. These data differ from those reported by other authors in LPS-stimulated cultured peripheral blood mononuclear cells (9); however, these two models are different and our results derive from microarray gene expression assays.

In effect, our study is the first report in literature describing what occurs at the gene expression level after exposure of HL-60 cells to ITF2357. In 2003, Chambers *et al*. evaluated the profile of gene expression in HL-60 and a T-lymphoblastoid cell line after treatment with trichostatin; down-regulation of *MYC, MYB, FLI1* and up-regulation of *HOXB6* was reported (24). The expression of 9% of the 12000 genes present on the gene chip they used changed after HDACI exposure; in our case, we evaluated 41,000 genes and 48 pathways were de-regulated, with percentages ranging from 9 to 1% of genes included in each pathway. This difference could depend either upon different technologies used, or a more potent effect of ITF2357 *versus* trichostatin A.

When levels of ICAM1, IL-1β, IL-10, TNFα, IL-6, and TGFβ were measured in the supernatant of the cell cultures before and after exposure to ITF2357, we showed that TNFα levels increased, whereas these of IL-6 and TGFβ decreased. Elevated levels of TNFα may be associated with the significant apoptotic rate. Reduction of IL-6 production has

been already reported (11, 25) and it is relevant, considering that IL-6 is a well-known growth factor for cell growth, migration and drug-resistance of myeloma cells (26). This would support the clinical use of ITF2357 in multiple myeloma patients, as proposed in the clinical trial now recruiting in the U.S.A. (code: NCT00792506).

Moreover, in LPS-stimulated cultured human peripheral blood mononuclear cells, ITF2357 reduced release of TNFα, IL-1α, IL-1β, and IFNγ. TNFα and IFNγ diminished also in mice treated by oral administration of 1-10 mg/kg ITF2357 (9). In that study, the authors proposed that IL-12 and IL-18 induced IFNγ *via* a signalling pathway sensitive to inhibition by ITF2357, whereas the pathway for IFNγ *via* the T-cell receptor (TCR) was not affected by this HDACI. Our results on the HL-60 cell line are different because 3% of genes involved in the TCR pathway were de-regulated by ITF2357. In particular, the decrease of *CD86*, normally expressed on antigen-presenting cells that provide co-stimulatory signals necessary for T-cell activation and survival, and of *CD40*, essential in mediating a broad variety of immune and inflammatory responses, appear particularly interesting.

On these bases, there is a possibility for employing ITF2357 in the allogenic transplant setting; in mice with graft-*versus*-host disease (GVHD), SAHA treatment reduced mortality, cytokine production, and histological markers of disease severity, without impairing graft-*versus*-tumor responses (27). Because GVHD results from tissue damage attributable to conditioning regimens, followed by the donor T-cell activation and migration in the target organs, processes in which IL-6 and TGFβ are particularly relevant, the reduction of these cytokines would sustain the use of ITF2357 in the treatment or prevention of GVHD.

Finally, ITF2357 significantly reduced expression of the Wilm's tumor gene (*WT1*) which plays a significant role in differentiation and leukemogenesis. *WT1* is overexpressed in about 70% of patients affected by acute myeloid leukemia at diagnosis, and high expression of *WT1* was significantly associated with a worse long-term outcome, especially in young patients (28). Increased *WT1* expression during the follow-up of acute leukemia is predictive of an impending hematological relapse, even after allogenic transplantation (29). Previously, our group reported that high levels of WT1 were significantly correlated with high levels of *MDR1* gene. The co-expression of these genes did not significantly influence the rate of complete response to induction therapy (30), but the inhibition of *WT1* would represent an interesting target in order to reduce chemoresistance.

In summary, by gene expression assays we showed how many molecular pathways are modified by ITF2357; these data support the possible clinical utility of this HDAI for leukemia therapy.

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