Zalypsis has *in vitro* activity in acute myeloid blasts and leukemic progenitor cells through the induction of a DNA damage response

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The online version of this article has a Supplementary Appendix.

ABSTRACT

Background
Although the majority of patients with acute myeloid leukemia initially respond to conventional chemotherapy, relapse is still the leading cause of death, probably because of the presence of leukemic stem cells that are insensitive to current therapies. We investigated the antileukemic activity and mechanism of action of zalypsis, a novel alkaloid of marine origin.

Design and Methods
The activity of zalypsis was studied in four acute myeloid leukemia cell lines and in freshly isolated blasts taken from patients with acute myeloid leukemia before they started therapy. Zalypsis-induced apoptosis of both malignant and normal cells was measured using flow cytometry techniques. Gene expression profiling and western blot studies were performed to assess the mechanism of action of the alkaloid.

Results
Zalypsis showed a very potent antileukemic activity in all the cell lines tested and potentiated the effect of conventional antileukemic drugs such as cytarabine, fludarabine and daunorubicin. Interestingly, zalypsis showed remarkable *ex vivo* potency, including activity against the most immature blast cells ($CD34^+$ $CD38^-$ Lin^-) which include leukemic stem cells. Zalypsis-induced apoptosis was the result of an important deregulation of genes involved in the recognition of double-strand DNA breaks, such as Fanconi anemia genes and *BRCA1*, but also genes implicated in the repair of double-strand DNA breaks, such as *RAD51* and *RAD54*. These gene findings were confirmed by an increase in several proteins involved in the pathway (pCHK1, pCHK2 and pH2AX).

Conclusions
The potent and selective antileukemic effect of zalypsis on DNA damage response mechanisms observed in acute myeloid leukemia cell lines and in patients' samples provides the rationale for the investigation of this compound in clinical trials.

Key words: zalypsis, acute myeloid leukemia, DNA damage response, antileukemic activity.

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Introduction

Acute myeloid leukemia (AML) is a heterogeneous clonal disorder of hematopoietic progenitor cells that lose their ability to differentiate normally and to respond to normal regulators of proliferation.[1-3] Treatment of AML has traditionally relied on the combination of the pyrimidine analog cytarabine (araC) with anthracycline antibiotics such as daunorubicin, and has not evolved for more than 30 years.[1,4] Although the majority of patients initially respond, relapse is the leading cause of death in AML, probably due, among other reasons, to the presence of leukemic stem cells, which are relatively insensitive to current therapies.[5,6] The problem of relapse is especially notorious in patients with complex karyotypes and secondary AML, who have a dismal prognosis.[3,7] Several cytogenetic abnormalities such as *TP53* mutations are particularly frequent in this subset of patients with a bad prognosis.[8-14] In this regard, the presence of a functional p53 is necessary for the induction of cell death caused by most antitumoral agents, whereas a non-functional p53 is clearly associated with the development of drug resistance.[15]

One of the sources of drugs which has attracted interest in recent years is the marine environment.[16] The antitumor activity of many compounds obtained from tunicates and other sea organisms is being tested in preclinical and clinical studies, and some of them, such as Yondelis®, have already been approved for the treatment of advanced soft tissue sarcomas and ovarian cancer.[17] Zalypsis (PM00104) is a new synthetic alkaloid related to jorumycin, renieramycins, safracins and saframycins. Zalypsis has demonstrated significant *in vitro* and *in vivo* activity against human solid and hematologic neoplasms via p53-dependent and p53-independent mechanisms,[18,19] and is currently being evaluated in early phase clinical trials on solid organ neoplasms and multiple myeloma.

In this study we investigated the activity and mechanism of action of zalypsis in AML cell lines and patients' samples with different biological and cytogenetic characteristics.

Design and Methods

Reagents and immunochemicals

Cell culture media, serum and penicillin-streptomycin were from Invitrogen Corporation (Gaithersburg, MD, USA). Zalypsis was provided by PharmaMar S.A.U., (Madrid, Spain and Cambridge, MA, USA). Daunorubicin, cytarabine and fludarabine were purchased from Sigma-Aldrich (Madrid, Spain). Annexin V-fluorescein isothiocyanate (FITC) was obtained from BD Biosciences (San Diego, CA. USA). Z-VAD-FMK was purchased from Calbiochem (San Diego, CA, USA). Other generic chemicals were purchased from Sigma-Aldrich, Roche Biochemicals (Mannheim, Germany), or Merck (Darmstadt, Germany).

The providers of the different monoclonal antibodies employed in the western blotting analyses were as follows: anti-GADD45B, anti-p21, anti-MDM2, anti-PUMA, anti-Bcl-2 and anti-Mcl-1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA); anti-AIF, anti-pH2AX; anti-Bax, anti-pCHK1, anti-pCHK2, anti-p53, anti-Bcl-XL, anti-PARP, anti-caspase-3, anti-caspase-7, anti-caspase-8 and anti-caspase-9 (Cell Signaling, Boston, MA, USA); anti-cytochrome c (BD Biosciences); anti-tubulin (Calbiochem, San Diego, USA); and anti-endo G (Serotec, Raleigh, USA). The horse radish peroxidase-conjugated secondary antibodies were from Amersham (Buckinghamshire, UK).

Cell cultures, cell proliferation, cell cycle, and apoptosis analyses and western blotting and subcellular fractionation procedures

The source and the culture conditions of the AML cell lines as well as the detailed methodology for analyzing proliferation of AML cells using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, cell cycle profiles, cytometric evaluation of apoptosis in AML cell lines using annexin-V-FITC, mitochondrial membrane potential ($\Delta\Psi$m), subcellular fractionation and western blotting have been described previously.[20]

For combination experiments, we used suboptimal doses of zalypsis, daunorubicin, cytarabine, fludarabine and bortezomib for 24 and 48 h, and analyzed the cytotoxicity of the combination by MTT absorbance assays as previously described.[21,22]

Ex vivo experiments in freshly isolated acute myeloid leukemia cells

The use of clinical samples for investigation was approved by the Ethical Committee of the University Hospital of Salamanca. The cytometric analyses of apoptosis have been described elsewhere.[20] Briefly, bone marrow cells were obtained from ten patients with AML at diagnosis before any treatment. The biological characteristics of these patients are summarized in Table 1. Bone marrow cells (1×10^6) were incubated in six-well plates with zalypsis (10 nM) or in the absence of drug (control) for 18 h at 37 °C in a humidified atmosphere in the presence of 5% CO_2-95% air. Using quadruple staining, we were able to identify the blast cell population ($CD34^{-/+}$, $CD33^{-/+}$, $CD45^{dim}$)[23] and the population of the most immature blasts in which leukemic stem cells are found ($CD34^+$, $CD38^-$, Lin^-), and accurately quantify the number of apoptotic cells in each cell population.[24-26] In addition, the toxicity to normal hematopoietic progenitor cells ($CD34^+$) was analyzed in bone marrow samples from four non-leukemic patients. In all cases, the percentage of apoptotic events was corrected according to the proportion of apoptotic cells in the control (untreated) cells. The monoclonal antibodies used for these experiments were: anti-CD33-PE; anti-CD45-PerCP; anti-CD34-APC; anti-CD64-PE; anti-CD19-PerCP-Cy5.5; anti-CD45-AmCyan and anti-CD38-Alexa-Fluor-700 (BD Biosciences, San Diego, CA, USA).

Microarray RNA analyses

HEL and HL60 cells were treated with zalypsis (10 nM) for 16 h in order to obtain 20% apoptosis, as assessed by annexin-V stain-

Table 1. Baseline characteristics of the AML patients' samples.

	Cytogenetics	*FLT3* mutation	*NPM1* mutation	CD34 expression
AML 1*	No mitosis	WT	WT	+
AML 2	Normal	NE	NE	-
AML 3	NE	NE	NE	+
AML 4	46,XY [12]/46,XY,+8 [5]/ 48,idem,+mar [4]	WT	WT	+
AML 5	Normal	ITD	Mut	-
AML 6	46,XY [8]/47,XY,+8 [5]/ 47,idem,-14,+mar [3]	WT	WT	+
AML 7	45,XX, -22 [17]	WT	WT	+
AML 8 †	46,XX [5]/47,XX,+11 [11]	WT	WT	+
AML 9	46,XX [4]/46,XX,-10,-13,+2mar [4]	WT	NE	+
AML 10	Normal	ITD	WT	-

FLT3: fms-like tyrosine kinase 3; ITD: internal tamdem duplication; NPM1*: nucleophosmin; NE: not evaluated; Mut: mutated; +: positive; -: negative. *FISH studies demonstrated a loss of 5q in 84% of cells; †molecular studies demonstrated a* JAK2 *mutation.*

ing.[21] At this time point, the total RNA was extracted using Trizol reagent (Life Technologies, MD, USA) and purified with an RNAeasy Mini Kit (Qiagen, CA, USA). HEL and HL60 cells cultured in the same conditions in the absence of drug were used as controls. RNA integrity was verified with the Agilent 2100 Bioanalyzer (Agilent, CA, USA). Double-strand cDNA and biotinylated cRNA were synthesized with T7-polyT primer and the BioArray RNA labeling kit (Enzo, NY, USA), respectively. The labeled RNA was then fragmented and hybridized to HG-U133 Plus 2.0 oligonucleotide arrays (Affymetrix, CA, USA), which were scanned in a Gene Array Scanner and analyzed using DNA-Chip Analyzer software (DChip) and Ingenuity Pathway Analysis (Ingenuity Systems, California, USA). Greater than two-fold changes in gene expression were considered significant.

Real-time quantitative polymerase chain reaction

The results obtained from microarrays were confirmed by real-time quantitative polymerase chain reaction (PCR). The sequences of the primers used are shown in *Online Supplementary Table S1*. The reactions were performed using iQTM SYBR Green Supermix (Bio-Rad Laboratories, Madrid). A standard curve was made using serial dilutions (20 ng, 2 ng and 0.2 ng) of control cDNA. The PCR conditions were: (step 1) 95°C for 10 min; (step 2 , x40) 95°C for 15 s and 58°C for 1 min; (step 3) 95°C for 1 min; (step 4) 65°C for 1 min; (step 5, x60) 65°C for 10 s; (step 6) 4°C. Quantification was based on increased fluorescence, which was measured and recorded using Bio-Rad iQ5 software; the results were normalized to those of the reference gene, *GAPDH*.

Fluorescent microscopy

Cells were collected, washed with phosphate-buffered saline, fixed with 4% formaldehyde (1 h) and then permeabilized with 0.1% Triton X-100 (3 s). After this, the cells were washed again with phosphate-buffered saline and incubated with blocking solution (10% bovine serum albumin in phosphate-buffered saline for 30 s). After washing with phosphate-buffered saline, the cells were incubated with rabbit antibody against pH2AX (Ser139) (Cell Signaling Technology, Danvers, MA, USA) overnight at 4°C and then with AlexaFluor488 goat anti-rabbit antibody (Invitrogen, Eugene, OR, USA) for 30 s at room temperature. Cells were incubated with DAPI (0.5 μg/mL), placed onto slides by cytospin and then mounted using Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA). A Leica confocal microscope was used to obtain confocal images. At least six images were taken for each condition, and the percentage of cells with positive pH2AX nuclear foci with respect to the total number of cells (total DAPI-stained nuclei excluding apoptotic cells) was calculated using Scion Image software.

Statistics

Statistical significance was calculated by Student's t-test, the Mann-Whitney U or Wilcoxon signed rank test as appropriate, with Prism software (GraphPad). All *P* values less than 0.05 are considered statistically significant and are referred to as such in the text. Unless otherwise specified, all studies for which data are presented are representative of at least two similar studies.

Results

Zalypsis displays potent antileukemic activity and potentiates the effects of conventional antileukemic agents

To test the antiproliferative/cytotoxic effect of zalypsis against AML cells, four cell lines were treated with increasing concentrations of the compound (0.1-10 nM) for 24 and 48 h, and the viability of the cells was analyzed by an MTT assay (Figure 1A). Zalypsis showed a very potent antileukemic activity in the four AML cell lines tested, with IC_{50} values at 48 h below 1 nM. When compared with the *in vitro* activity of conventional antileukemic agents, the potency of zalypsis was found to be 10 to 100 times higher than that of cytarabine, daunorubicin or fludarabine (*data not shown*).

To determine whether zalypsis could increase the activity of standard-of-care drugs for AML, two different cell lines, HEL and HL60 cells originally derived from patients with French-American-British M2 AML and erythroid leukemia, respectively, were treated with several combinations of zalypsis and some conventional antileukemic agents (cytarabine, daunorubicin, fludarabine) and the novel drug bortezomib. Zalypsis increased the effect of all these anti-AML drugs, with the most potent combination in both cell lines being the one that included the anthracycline, daunorubicin. Zalypsis slightly increased the effect of cytarabine and fludarabine, whereas only a modest effect was observed in combination with bortezomib (Figure 1B). The greater potentiation in the case of HL-60 cells was observed at earlier time points (24 h), probably due to the higher potency of zalypsis in this cell line, whereas in HEL cells, 48 h were required for a similar activity.

Zalypsis targets both mature and immature blast cells

We next analyzed the activity of zalypsis in freshly isolated blast cells from ten AML patients by using multiparametric flow cytometry which allows the identification of blast cells, and, more interestingly, discrimination of the more mature leukemic cell populations ($CD34^{-/+}$, $CD33^{-/+}$, $CD45^{dim}$) from the most immature population ($CD34^{+}$, $CD38^{-}$, Lin^{-}).[6,27] At concentrations that can be clinically achieved in the plasma of treated patients, zalypsis induced significant cell death of the blast population in all cases analyzed (Figure 1C) which, interestingly, included patients with bad cytogenetic prognosis based on karyotypic abnormalities or molecular mutations (Table 1). In four of these patients we were also able to analyze the efficacy of zalypsis in the most immature blast population, which is thought to include the leukemic stem cells; in all cases, this population was also sensitive to zalypsis (Figure 1C and D). Finally, we studied the activity of this drug in non-tumoral $CD34^{+}$ bone marrow progenitors obtained from four non-AML samples. In these samples, the $CD34^{+}$ progenitors were preserved after treatment with zalypsis (Figure 1C), in particular, the $CD34^{+}CD38^{-}Lin^{-}$ population which represents the compartment of the non-lineage-committed hematopoietic progenitors (*data not shown*). These findings suggest that although zalypsis may target the leukemic stem cell compartment, the cytopenias caused by the treatment would be reversible, since the normal immature hematopoietic precursor cells are preserved.

Zalypsis induces apoptosis in acute myeloid leukemia cells

To assess whether the decrease of MTT uptake induced by zalypsis was due to cell cycle blockade, an increase in cytotoxicity, or both, HEL and HL60 cells were treated with zalypsis (10 nM) for different periods, and, after-

wards, changes in cell cycle profiles and induction of apoptosis were analyzed by flow cytometry. Propidium iodide staining did not show significant changes in the cell cycle phases (*data not shown*). By contrast, a significant, time-dependent induction of annexin V-positive cells was observed after treatment with zalypsis (Figure 2A) and, accordingly, zalypsis induced DNA laddering in both HL-60 and HEL cell lines (Figure 2B). We next evaluated the biochemical parameters that are affected by apoptotic cell death. Zalypsis provoked PARP, caspase-3, caspase-7, caspase-8 and caspase-9 cleavage, with generation of active cleaved fragments in both cell lines (Figure 2C). This indicates that zalypsis activates both the intrinsic and the extrinsic apoptotic pathways.[28] Interestingly, and concordant with the results of the annexin V studies, the cleavage of these proteins occurred earlier in HL60 cells than in HEL ones: thus, while the processing of PARP, effector caspases 3 and 7, and caspase 8 required 24 h of treatment with zalypsis in the HEL cell line, it was already observed at 12 h in HL60 cells. Cleavage of caspase 9 was the first biochemical process observed, after 6 h in HL60 cells and after 12 h in HEL ones.

Zalypsis deregulates mitochondrial permeability in acute myeloid leukemia cells

As cleavage of caspase 9 is a very early event in zalypsis-induced apoptosis, and this change indicates activation of the intrinsic pathway of apoptosis, we next studied the effect of zalypsis on mitochondria, which are critically involved in the triggering of apoptotic cell death through this pathway. For this purpose we first used DioC6(3) dye to explore whether zalypsis was able to induce changes in the mitochondrial membrane potential ($\Delta\Psi$m), a situation that results in an increase in the mithocondrial outer membrane permeability.[29] A decrease in $\Delta\Psi$m after treatment with zalypsis was observed in both cell lines, with this change occurring earlier in HL60 cells (observed after 6 h) than in HEL cells (24 h) (Figure 2D).

Bcl-2 family members act as important regulators of mitochondrial outer membrane permeability. Western

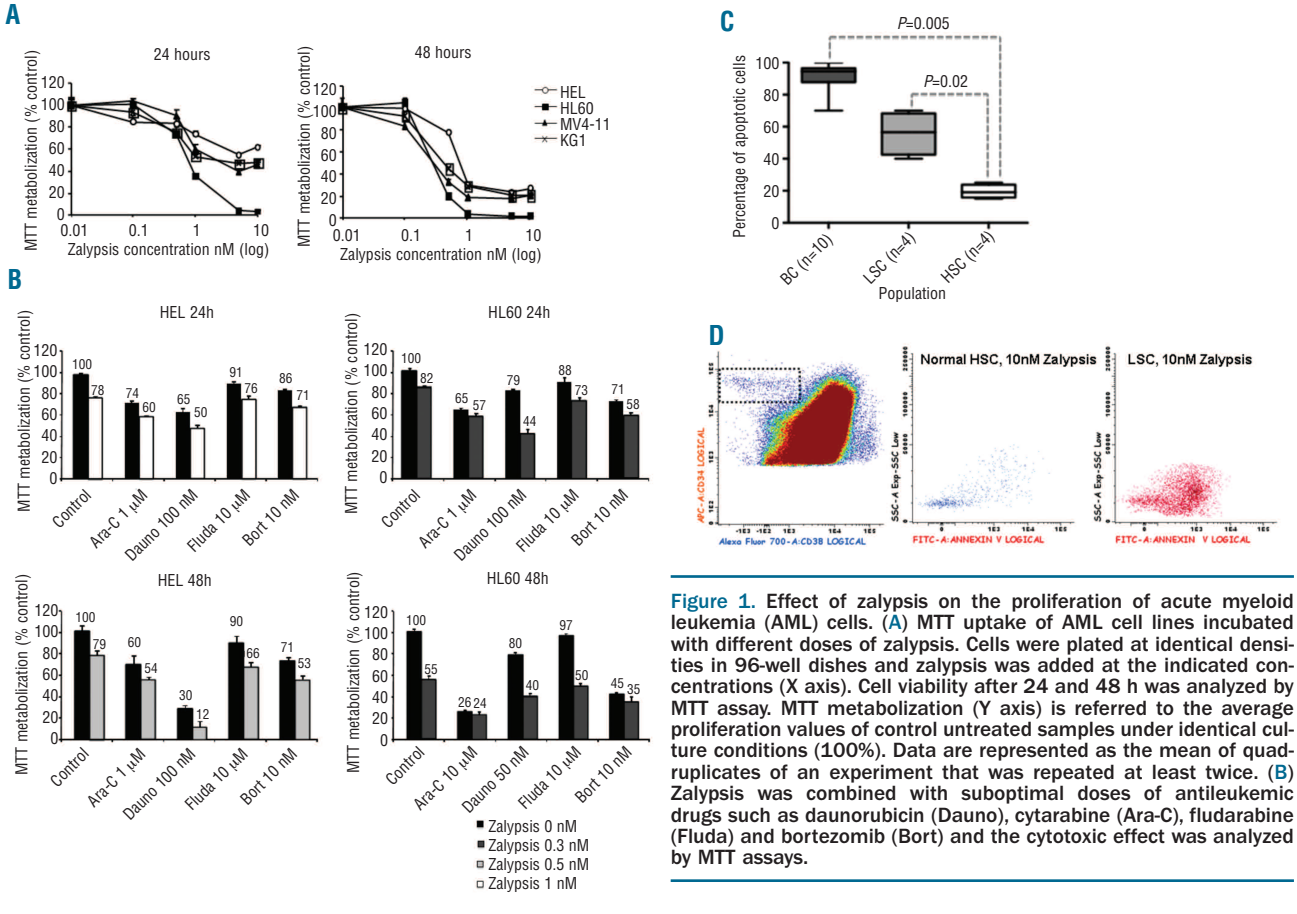

Figure 1. Effect of zalypsis on the proliferation of acute myeloid leukemia (AML) cells. (A) MTT uptake of AML cell lines incubated with different doses of zalypsis. Cells were plated at identical densities in 96-well dishes and zalypsis was added at the indicated concentrations (X axis). Cell viability after 24 and 48 h was analyzed by MTT assay. MTT metabolization (Y axis) is referred to the average proliferation values of control untreated samples under identical culture conditions (100%). Data are represented as the mean of quadruplicates of an experiment that was repeated at least twice. (B) Zalypsis was combined with suboptimal doses of antileukemic drugs such as daunorubicin (Dauno), cytarabine (Ara-C), fludarabine (Fluda) and bortezomib (Bort) and the cytotoxic effect was analyzed by MTT assays. (C) Zalypsis has antileukemic activity against both mature and inmature AML cells. Freshly isolated bone marrow cells obtained from ten AML patients were plated in six-well plates, and treated *ex vivo* with zalypsis (10 nM) or without drug (control) for 18 h. After the incubation period, cells were stained with anti-annexin V-FITC and a combination of monoclonal antibodies which allowed the discrimination of blast cells (BC). In samples from four AML patients we separately analyzed apoptosis induction in the immature blast cell compartment or leukemic stem cells (LSC) ($CD34^+$, $CD38^-$, Lin^-) and compared it to that of the same population obtained from four patients with non-tumoral $CD34^+$ bone marrow progenitor cells (HSC). The number of annexin V-positive events in this population was measured and normalized to the viable cell fraction in the control, untreated cells, as described in the *Design and Methods* section. (D) Representative example of the apoptosis study on the immature blast cell compartment. $CD34^+$ cells were gated. The first graph allows the identification of the immature populations with the two monoclonal antibodies CD34 and CD38; the second plot shows the quantification of apoptosis induction (by annexin-V staining) in each compartment; normal HSC are shown as blue dots and LSC are shown as red dots.

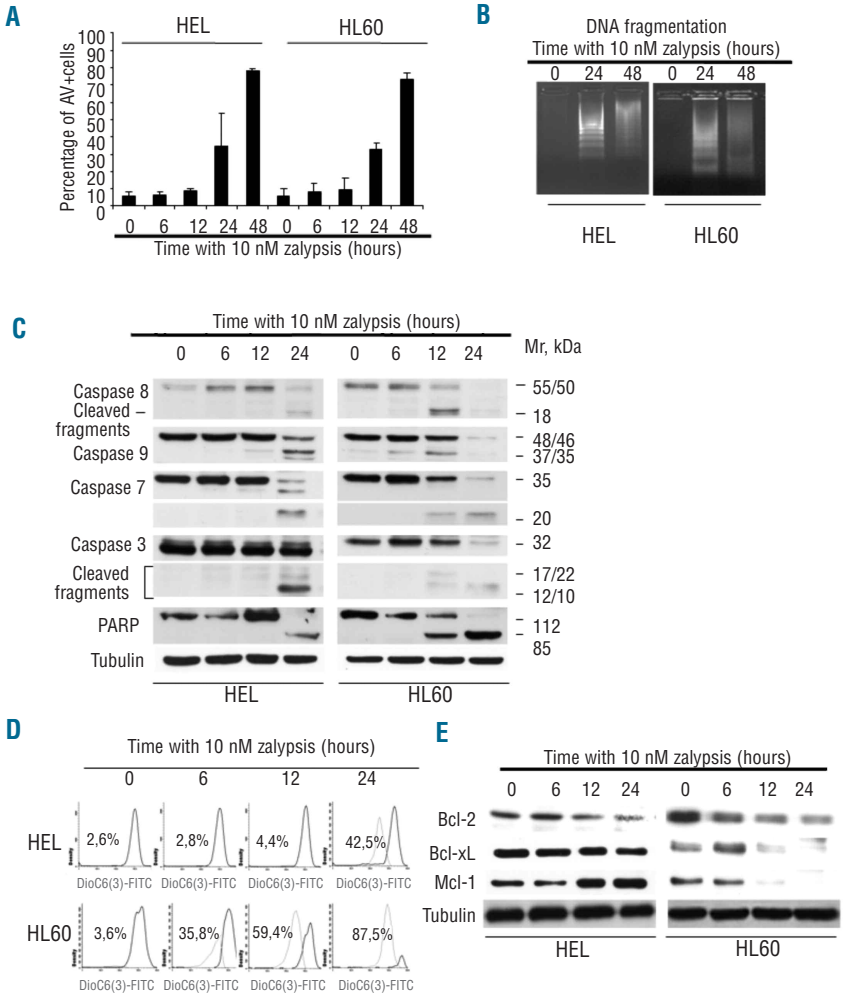

Figure 2. Zalypsis causes apoptotic cell death in acute myeloid leukemia cell lines. (A) Time-course of the effect of zalypsis on HEL and HL60 cells. Cells were plated in six-well plates, treated for different times with zalypsis (10 nM). Cells were stained with annexin V-FITC and propidium iodide (B) Zalypsis provokes internucleosomal DNA fragmentation, HEL and HL60 cells were treated with zalypsis for the indicated times and DNA was isolated and analyzed by agarose gel electrophoresis. (C) HEL and HL60 cells were treated with zalypsis (10 nM) for the indicated times, and the expression of PARP, caspase-3, caspase 7, caspase-8 and caspase-9 proteins analyzed by western blotting. Tubulin was used as a loading control. (D) In order to assess the loss of $\Delta\Psi m$, HEL and HL60 cells were treated with zalypsis (10 nM) for the indicated times, propidium iodide-negative cells were gated and the expression of DiOC6(3) was analyzed by flow cytometry. The percentage of DiOC6(3)^{low} cells is indicated. (E) HEL and HL60 cells were treated with zalypsis (10 nM) for the indicated times, and cell extracts were used for western blotting with anti-Bcl-2, anti-Bcl-X or anti-MCL-1 antibodies.

blot analyses indicated that zalypsis down-regulated Bcl-2 in both cell lines, as shown in Figure 2E. Other Bcl-2 family members studied, such as MCL-1 and BLC-XL, were processed only in HL60 cells after treatment with zalypsis, and their level was stable or even increased in the HEL cell line, probably acting as paradoxical survival signals in order to counterbalance the cytotoxic activity of zalypsis.

Zalypsis induces cell death through caspase-dependent and caspase-independent pathways

The increase in mitochondrial outer membrane permeability also favors the release of mediators of the intrinsic pathway of apoptosis such as cytochrome C which, together with APAF-1 and caspase 9, forms the apoptosome. Subcellular fractionation of HEL and HL60 cells treated for 18 h with zalypsis showed that this drug caused a translocation of cytochrome C from the mitochondrial to the cytosolic fraction after treatment (Figure 3A), confirming the activation of the intrinsic pathway of apoptosis. To further investigate the importance of caspases in the anti-leukemic action of zalypsis, the ability of the pan-caspase inhibitor Z-VAD-FMK to rescue cells from zalypsis-induced cell death was evaluated.[30] Cells were preincubated for 60 min with Z-VAD-FMK, then zalypsis was added, and the incubation continued for 24 h. As shown in Figure 3B, preincubation with the pan-caspase inhibitor did not abrogate cell death in the HEL cell line and only a mild effect was observed in HL60 cells. In line with these results, AIF, one of the proteins involved in caspase-independent apoptosis, was also released into the cytosol (Figure 3A) after treatment with zalypsis. These results indicate that both caspase-dependent and caspase-independent mechanisms are involved in zalypsis-induced apoptosis.

Zalypsis induces a profound gene deregulation

To further investigate the mechanism of action of zalypsis, we analyzed treatment-induced changes in the gene expression profile of two AML cell lines. In order to analyze changes specifically associated with the effect of zalypsis and not with late induction of apoptosis, we collected cells at the very beginning of apoptosis; for this purpose, HEL and HL60 cells were treated with zalypsis (10 nM) for 16 h in order to obtain 20% apoptosis, as assessed by annexin-V staining. Based on a fold-change of two or more compared to the untreated cell line, a total of 1685 genes were found to be deregulated in HEL cells after treatment with zalypsis, whereas in the HL60 cell line a more profound deregulation of gene expression was observed, with 5576 altered genes. Most of these genes were down-regulated after treatment with the drug. The most significantly deregulated functional categories were cell cycle (568 and 606 genes, respectively), DNA replication, recombination and repair (333 and 424 genes, respec-

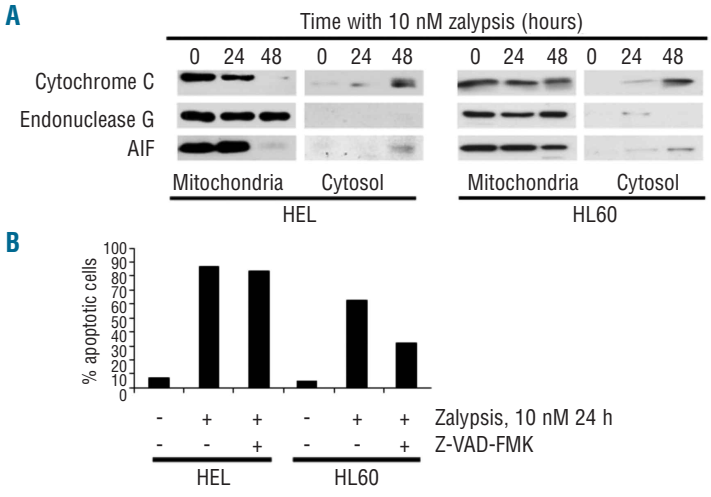

Figure 3. Zalypsis causes cell death through caspase-independent mechanisms. (A) HL60 and HEL cells were treated for the indicated times with zalypsis (10 nM), and the subcellular distribution of AIF, endonuclease G and cytochrome C in mitochondrial and cytosolic fractions was analyzed by western blotting. (B) Effect of the pancaspase inhibitor, Z-VAD-FMK, on zalypsis-induced cell death. HEL and HL60 cells were plated and pre-treated, where indicated, with Z-VAD-FMK (20 μM) for 60 min. Zalypsis (10 nM) was added to the samples, and the experiment continued for 24 h. Flow cytometry was carried out as described in the *Design and Methods* section.

tively), RNA post-transcriptional modification (96 and 173 genes, respectively) and cell death (823 and 967 genes, respectively).

Interestingly, 934 genes were deregulated in both cell lines (Figure 4A). When we focused on this group of genes, an important number of DNA damage response genes were found to be deregulated (Table 2). The deregulated genes also included some implicated in the recognition of double-strand breaks, such as Fanconi anemia genes (including *BRCA2*) and *BRCA1*, and in the repair of double-strand breaks, such as *RAD51* and *RAD54*. A possible mechanism of action of zalypsis is, therefore, the induction of DNA double-strand breaks. The MRN complex is composed of Mre11, Rad50 and Nbs1; it acts as a sensor of double-stand breaks and recruits the ATM kinase to sites of broken DNA. This complex therefore plays a key role in the immediate early response to DNA damage.[31-33] Under these circumstances, ATM is activated and phosphorylates substrates such as histone *H2AX* and *CHK2*.[34,35] These results were confirmed using western blotting, since treatment of HEL and HL60 cells with zalypsis induced histone *H2AX* and *CHK2* phosphorylation (Figure 4B). In fact, using immunocytochemistry we observed a time-dependent intranuclear localization of pH2AX in the form of nuclear foci after treatment with daunorubicin and, even more markedly, with daunorubicin plus zalypsis (Figure 4C). Of note, genes involved in mismatch repair, such as *MLH1* and *MSH6*, were also highly deregulated. In addition, there was a significant increase in *GADD45B* levels after treatment with zalypsis, reinforcing the belief that the DNA damage pathway is an important mechanism for the cytotoxic effect induced by this drug (Figure 4B).

To further confirm the effects of zalypsis, changes in the RNA expression of genes found to be deregulated in the gene expression profiling experiments were also analyzed by quantitative PCR. We focused on genes related to DNA damage response as this was the most deregulated pathway. As shown in *Online Supplementary Figure S1*, we confirmed that in both HEL and HL60 cells exposed to the drug there was up-regulation of *GADD45B* and down-regulation of other genes involved in DNA damage detection and repair pathways such as Fanconi anemia genes (*BRCA2*, *FANCG*, *FANCL*), *BRCA1*, *RAD51*, *RAD54*, *CHEK2*, *ATR*, *MSH6* and *MLH1*.

In previous studies of multiple myeloma performed by our group,[18] we had observed that although zalypsis was able to induce cell death in all myeloma cell lines, those cells with low basal levels of p53 by western-blot (indicating wild-type p53) were more sensitive to the drug. This situation was also confirmed in AML as all cell lines were extremely sensitive to zalypsis irrespective of their p53 status. In fact, western blot studies showed that some of them displayed high basal levels of this protein, corresponding to mutated p53 (HEL and MV4-11),[36] whereas p53 expression in the two other cell lines was low (Figure 4D). Interestingly, the more sensitive cell line (HL-60) displayed the lowest levels of p53, whereas the less sensitive line (HEL) had high levels of this protein. In HL-60 cells, exposure to zalypsis stabilized or slightly increased the levels of this protein (Figure 4E). In contrast, the HEL cell line, which has high basal levels of p53, showed no increase in the expression of the protein. The increase in p53 levels in HL60 cells is likely due to stabilization of the protein, as no evident changes in mRNA levels were observed in the microarray data analysis. Interestingly, 60 genes in the p53 canonical pathway were deregulated in the HL60 cell line, whereas only 15 genes were deregulated in the HEL cell line.

Discussion

Nearly 80% of patients with AML achieve complete remission with induction chemotherapy. However, a high proportion relapse and eventually die of their disease.[1,2] Recent studies have shown that zalypsis may represent a valuable novel anticancer therapy, with strong preclinical antineoplastic efficacy and preliminary evidence of activity and a good safety profile in phase I clinical studies. In the present study, this compound was found to have a strong antileukemic activity in cell lines as well as in fresh leukemic cells from AML patients. Furthermore, zalypsis potentiated the effect of drugs conventionally used in AML treatment, supporting the future development of clinical trials using this drug in combination with daunorubicin or cytarabine among others.

Any successful therapeutic strategy in AML patients should target not only the mature blast cells, but also the leukemic stem cell compartment that is responsible for the

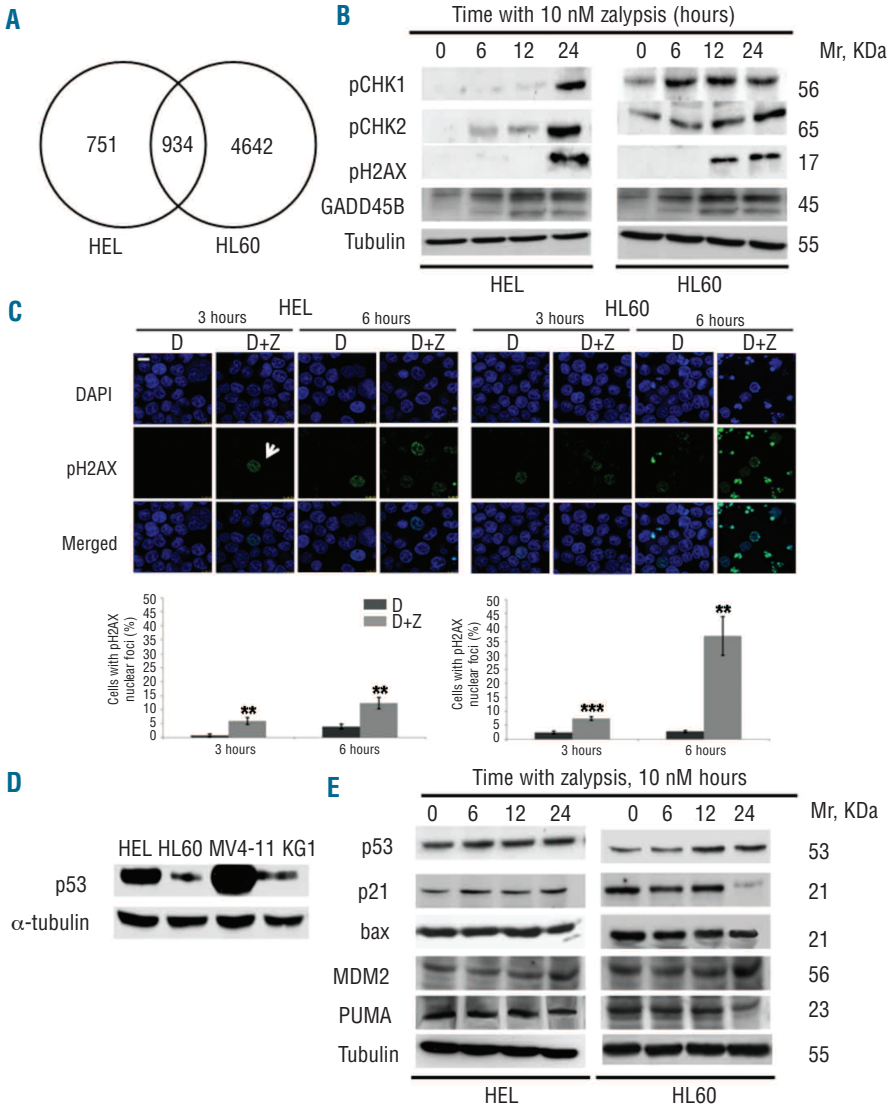

Figure 4. Zalypsis stimulates a DNA damage response through the induction of double-strand breaks. (A) Venn diagram depicting the overlap of deregulated genes in HEL and HL60 cells after zalypsis treatment. (B) HEL and HL60 cells were treated with zalypsis 10 nM for the indicated time points, cell protein extracts were obtained from each condition and the levels of pH2AX, pCHK1 and pCHK2 and *GADD45B* were analyzed by western blotting. (C) Fluorescent microscopy of pH2AX in HEL and HL60. Cells were treated with 500 nM daunorubicin (D) or 500 nM daunorubicin plus 10 nM zalypsis (D+Z) for 3 or 6 h. The percentage of cells with pH2AX nuclear foci was calculated as described in the *Design and Methods* section. The number of cells with pH2AX nuclear foci in the absence of drug was not significantly different (*data not shown*). The figure shows, for each condition, representative images for DAPI and pH2AX stain and merged. A representative example of a cell with pH2AX nuclear foci is indicated with an arrow. Statistical differences with respect to daunorubicin treatment are shown as ***$P<0.001$ and **$P<0.01$ (Student's t-test; n=6). Scale bar = 10 μM. (D) Western blot showing the basal protein levels of p53 protein in the four AML cell lines tested. (E) HEL and HL60 cells were treated with zalypsis 10 nM for the indicated time points, cell protein extracts were obtained from each condition and p53 and p53-dependent proteins levels were assessed at the same times. Equal loading was confirmed with an anti-tubulin antibody.

eventual recurrence of the disease. Moreover it is also important to spare normal hematopoietic stem cells, otherwise severe toxicities could develop, especially at a hematologic level. To address these issues, we performed multiparametric flow cytometry experiments in both malignant and normal cells. Zalypsis showed strong antileukemic activity in all the studied AML samples, including $CD34^{+}CD38^{-}$ blast cells which had a similar sensitivity to zalypsis as that of the more mature blast cell populations. These observations suggest that zalypsis targets not only the mature but also the immature blast cell populations, in which leukemic stem cells are thought to be found. Interestingly, normal hematopoietic stem cells were almost unaffected under the same conditions, which indicates that hematologic toxicity would be reversible, due to the specific action of zalypsis on the leukemic cells.

The mechanism of action of zalypsis in AML was found to be multifactorial, and involved a strong apoptotic response. The apoptosis was detected by both annexin/propidium iodide staining and DNA laddering and indirectly by the loss of mithocondrial membrane potential observed in AML cells. Apoptosis was caspase-dependent as cleavage of pro-caspases 8, 9 and 3 as well as PARP was detected, but caspase-independent pathways such as the release of AIF from mitochondria could also play a role in zalypsis-induced cell death.

Microarray data interpretation led to the identification of DNA damage as a potential important mechanism of action of zalypsis. Gene expression profiling identified several genes implicated in double-strand break repair and *BRCA1* pathways, Fanconi anemia genes [*FANCG*, *FANCL*, *FANCF*, *BRCA2* (*FANCD2*), *ATR*, *RAD51*, *RAD54L*, *BRCA1*, and *BARD1*] whose levels were down-regulated, in addition to other genes also implicated in DNA mismatch detection and repair such as *MSH6* and *MLH1*. Western blotting studies confirmed this mechanism as zalypsis up-regulated the levels of pH2AX, a surrogate marker of double-strand breaks as well as pCHK2, both substrates of ATM. The levels of *GADD45B*, another marker of DNA damage, were also higher. A large number of genes implicated in fundamental cell processes such as transcription or DNA replication were also deregulated, indicating that zalypsis provokes DNA damage. DNA damage and error-prone repair mechanisms, causing

Table 2. Genes involved in the DNA damage pathway deregulated by zalypsis in HEL and HL60 cell lines.

Probe set	Gene name	HEL fold change	HL60 fold change	Comments
207574_s_at	*GADD45B*	39.10	5.08	growth arrest and DNA-damage-inducible, beta
1565716_at	*FUS*	6.52	2.53	fusion (involved in t(12;16) in malignant liposarcoma)
203564_at	*FANCG*	-2.22	-5.75	Fanconi anemia, complementation group G
202520_s_at	*MLH1*	-2.45	-4.68	mutL homolog
201663_s_at	*SMC4*	-2.95	-5.37	structural maintenance of chromosomes 4
201423_s_at	*CUL4A*	-3.39	-8.22	cullin 4A
210416_s_at	*CHEK2*	-3.50	-5.39	CHK2 checkpoint homolog (S. pombe)
218397_at	*FANCL*	-3.77	-5.32	Fanconi anemia, complementatio group L
209849_at_at	*RAD51*	-3.93	-3.45	RAD51Homolog(S. cerevisiae)
204531_s_at	*BRCA1*	-4.05	-4.01	breast cancer 1, early onset
204558_at	*RAD54L*	-4.35	-14.99	RAD54-like (S. cerevisiae)
208368_s_at	*BRCA2*	-6.45	-24.51	breast cancer 2, early onset
209259_s_at	*SMC3*	-7.52	-10.19	structural maintenance of chromosomes 3
205345_at	*BARD1*	-7.80	-4.54	BRCA1 associated RING domain 1
201202_at	*PCNA*	-10.89	-4.50	proliferating cell nuclear antigen
209902_at	*ATR* (includes *EG:545*)	-12.55	-19.72	ataxia telangiectasia and Rad3 related
218689_at	*FANCF*	-14.12	-6.23	Fanconi anemia, complementation group F
202911_at	*MSH6*	-21.99	-10.44	mutS homolog
212997_s_at	*TLK2*	-101.25	-15.53	tousled-like kinase 2

genomic instability, have been implicated in leukemia[37-39] and can, therefore, be targeted by drugs inducing additional DNA damage or inhibiting DNA repair pathways.[40] Importantly, one of the principal routes involved in the DNA damage response is the p53 pathway. In cells with high p53 basal levels and, therefore, non-functional p53, zalypsis was still able to trigger the DNA damage response, as indicated by its action on pH2AX and pCHK2. Consequently, it seems that p53-independent routes can be activated by zalypsis in AML. Taken together, these findings suggest that zalypsis provokes a DNA damage response, probably double strand breaks in the DNA, and this may be the leading mechanism of action of this drug.

In conclusion, the potent and specific activity of zalypsis in mature and immature AML blast cells, through the induction of DNA damage, supports the investigation of this novel compound in the treatment of this disease in *ad hoc* clinical trials, alone or in combination with other agents.

Authorship and Disclosures

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