

Enhancement of TRAIL cytotoxicity by AG-490 in human ALL cells is characterized by downregulation of cIAP-1 and cIAP-2 through inhibition of Jak2/Stat3

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The ability of death-inducing tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) to selectively kill a variety of cancer cells has been largely described, but one of the major concerns with the treatment is the occurrence of drug resistance and possible toxic side effects. Here, we report that TRAIL induces apoptosis in Jurkat and SUPT1 T cell lines and in human T-ALL blasts but not in healthy subject-derived peripheral blood mononuclear cells. In parallel, the treatment with TRAIL and Tyrphostin (AG-490), a selective Janus kinase 2 inhibitor, produces an evident enhancement of cytotoxicity, characterized by a significant inhibition of Stat3 phosphorylation compared to controls or to TRAIL alone-treated samples, and associated with a dramatic decrease of both cIAP-1 and cIAP-2 mRNA levels. Downregulation of cIAP-1 and cIAP-2 by specific small interference RNAs significantly amplifies TRAIL-reduced cytotoxicity. All together, these findings strongly indicate that cIAP-1 and cIAP-2 downregulation is a fundamental step in the signaling pathways mediating the combinatorial effect of TRAIL and AG-490 on T cell leukemia. These findings may help to open new routes for the development of less toxic pharmacological strategies in the treatment of patients affected by TRAIL-sensitive leukemias.

Keywords: ALL, AG-490, TRAIL, Stat3, IAPs, apoptosis

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Introduction

Acute lymphoblastic leukemia (ALL) is a malignant disorder originating from a single B- or T-lymphocyte progenitor. Leukemic cell proliferation and accumulation give rise to the suppression of healthy haematopoiesis and involve different organs including the liver, lymph nodes, thymus, spleen, gonads and meninges. Recent reports indicate that in children and adolescents with ALL,

an overall cure rate of 90% may be achieved [1] but that many patients still develop serious acute and late complications owing to the side effects of treatments. Cure rates of T-ALL in adults are considerably lower than in the younger population and relapsed T-ALL continues to confer a sad prognosis as evidenced by the 5-year survival rates in adults (32% to 46%) [2]. The search for more effective and safer anti-leukemia therapies continues to identify agents and combinations of agents with specific activity against the disease with the aim to devise new treatment approaches to improve both the cure rate and the life quality of T-ALL patients.

One promising approach that is of interest in the treatment of human T leukemia is the use of death-inducing tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL). TRAIL has attracted interest because of

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its selective apoptotic activity on a number of tumor cell types without displaying toxicity for normal cells [3, 4]. TRAIL interacts with a complex receptor system, which includes at least two agonistic receptors (death receptors) and three antagonistic receptors. TRAIL-R1 and TRAIL-R2 (agonistic receptors) contain a cytoplasmic death domain, eliciting apoptotic signals upon TRAIL binding. TRAIL-R3, TRAIL-R4 and Osteoprogenin (antagonistic receptors) do not display a functional death domain and may act as decoy receptors [5], possibly protecting normal cells and TRAIL-resistant tumor cells from apoptosis. The regulation and the function of TRAIL are complex, as results from different studies suggest no relationship between TRAIL sensitivity and the expression of antagonistic receptors [6]. Therefore, the mechanism related to the tumor-selective activity of TRAIL requires further investigations. The signaling system responsible for the apoptotic cell death is activated by a TRAIL-dependent clustering of TRAIL-R1 and TRAIL-R2, which induces in turn the activation of the initiator and the effector procaspases, resulting in cell death induction. TRAIL resistance of certain tumors and the scarce knowledge about the consequence of TRAIL on normal cells, present a potential concern about this type of therapy. In this context, the combination of TRAIL with other chemotherapeutic drugs or with ionizing radiations has already been attempted in order to obtain a synergistic effect against tumor cells [7, 8]. Tyrphostin (AG-490) has been reported to be a molecule with promising clinical potential as an immunotherapeutic drug due to its inhibitory effects on T cell-signaling pathways [9-12]. AG-490 is a tyrosine kinase inhibitor able to selectively block Janus kinase 2 (Jak2) activity, suppressing, in turn, Jak/Stat (signal transducers and activators of transcription) signaling. The Jak/Stat cascade modulates the proliferation of different cancer cell lines [13, 14], including adult T cell leukemia/lymphoma cells [15]. Studies in animals have indicated that AG-490 administration prolonged rat allograft survival and inhibited IL-2-induced activation and translocation of Stat5a/b into the nucleus of lymphocytes infiltrating the irradiated spleen allotransplant [16]. The potential clinical relevance of AG-490 has been concluded from the observation that systemic administration of AG-490 suppresses the growth of acute lymphoid leukemia cell lines injected into SCID mice [17]. Very recently, it has been demonstrated that AG-490 efficiently blocks even Jak3 activity in T cells [12, 18]. Jak proteins are cytosolic tyrosine kinases critical for mediating proliferation, differentiation and survival signals [19, 20]. Recent findings revealed that Jak2/Stat3 activity confers resistance to apoptosis to lymphoid tumor cells [21-23] by means of upregulation of inhibitor of apoptosis pro-

teins (IAPs) [24] and Fas-associated death-domain-like interleukin-1 β -converting enzyme (FLICE) inhibitory protein (cFLIP) [25]. IAPs represent a family of proteins expressed in various cell types, including haematopoietic cells. These proteins have been shown to suppress apoptosis by inhibiting active caspase-3, caspase-7 and procaspase-9 [26-28]. Since we have previously reported a synergistic influence of AG-490 on TNF-mediated apoptosis, in this study we investigated the biological effects of the combination of AG-490 and scalar doses of TRAIL on human lymphoblastic leukemia cells and examined the associated signaling events.

Results

Cytotoxic effect of TRAIL alone or in combination with AG-490 in Jurkat and SUPT1 T cell lines

It has been shown that 50 μ M AG-490 blocks hyperactive forms of Jak2 in ALL [17]. We evaluated the cytotoxic effect of TRAIL alone or in combination with AG-490 (50 μ M) by using different approaches: (a) 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide (MTT) assay, (b) count of viable cells by trypan blue exclusion assay and (c) flow cytometric analysis of apoptosis by simultaneously staining the cells for phosphatidylserine surface exposure and propidium iodide (PI) incorporation. Results from MTT assay indicated that after the addition of TRAIL alone, at different concentrations (ranging from 5 to 300 ng/ml), Jurkat cells showed a distinct pattern of responses: a modest sensitivity to apoptosis in presence of 5 or 10 ng/ml TRAIL, and an increased sensitivity within the range of 25-300 ng/ml but without any significant difference among the concentrations included in this range (Figure 1A and 1B). We then investigated the cytotoxicity when AG-490 (50 μ M) was combined with different concentrations of TRAIL. As summarized in Figure 1A and 1B, by combining both of the compounds together, a dramatic increase of the cytotoxic effects with respect to the use of TRAIL alone was found. Maximal cytotoxic effects were achieved when AG-490 (50 μ M) was combined with TRAIL used at concentrations ranging between 25 and 300 ng/ml. Therefore, with the aim of using a minimum dose of TRAIL without impairing its specific apoptotic effects, we used 25 ng/ml TRAIL for further experiments, either alone or in combination with AG-490. Similar results have been obtained with SUPT1 cells. Trypan blue exclusion test and flow cytometry analysis of apoptosis, performed in Jurkat (Figure 1C and 1D) and SUPT1 (Figure 1E and 1F) cell lines, largely confirmed the data reported in Figure 1A and 1B.

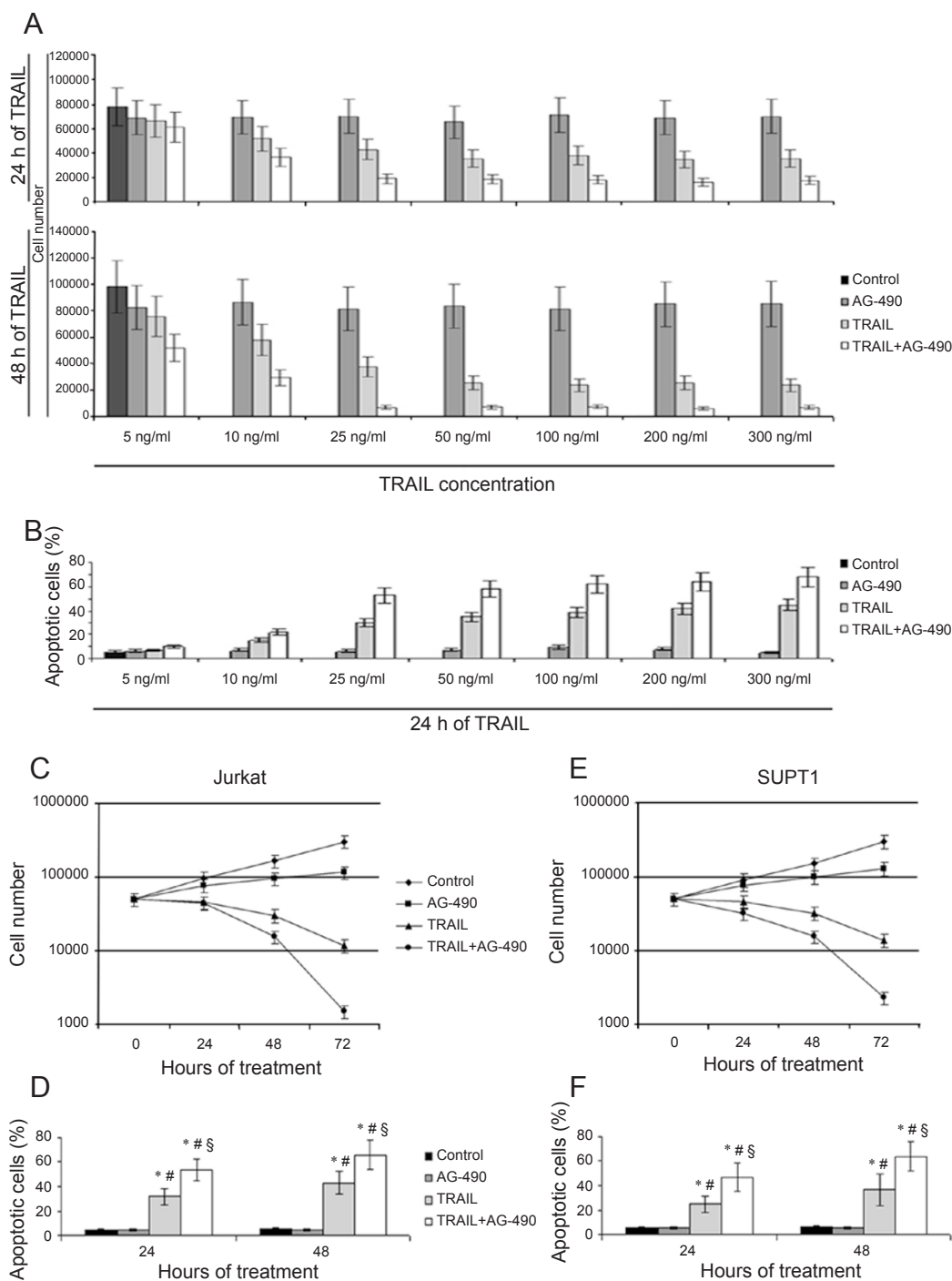


Figure 1 Susceptibility of Jurkat and SUPT1 cells to the cytotoxic activity of TRAIL or AG-490 used alone or in combination. Cytotoxicity evaluation (MTT assay) **(A)** and flow cytometry analysis of apoptosis **(B)** in Jurkat cells treated with AG-490 (50 μ M) or with different concentrations of TRAIL alone, or with both of the agonists combined together. Data are the mean of three separate experiments \pm SD. Evaluation of Jurkat **(C)** and SUPT1 **(E)** cell growth after the treatment with AG-490 (50 μ M), TRAIL (25 ng/ml) or of both of the agonists combined together. Cell number over 3 days of culture was evaluated by trypan blue exclusion test. Data are the mean of three separate experiments \pm SD. Flow cytometry analysis of apoptosis in Jurkat **(D)** and SUPT1 **(F)** cells treated for 24 or 48 h by AG-490 (50 μ M) or TRAIL (25 ng/ml) or both of the agonists combined together. Apoptosis was assessed by evaluating phosphatidylerine surface expression. Data are the mean of three separate experiments \pm SD; *, # ($P < 0.01$) indicates statistically significant values with respect to control samples; § ($P < 0.01$) indicates statistically significant values with respect to samples treated by TRAIL alone. In this figure, all samples treated with AG-490 were pre-treated for 12 h.

Combination of TRAIL and AG-490 increases the induction of apoptosis with respect to TRAIL alone in Jurkat cells and in human T-ALL primary blasts but not in healthy subject-derived PBMCs

The degree of apoptosis following independent treatments with TRAIL, AG-490 or both compounds together, was investigated by flow cytometric analyses in Jurkat cells and T-ALL-derived blasts, compared to healthy donor-derived peripheral blood mononuclear cells (PBMCs). The results indicated that the treatment with TRAIL alone produced a distinct degree of apoptosis in both Jurkat cells and in T-ALL-derived blasts but not in healthy donor-derived PBMCs (Figure 2A). No significant effect was noticed following AG-490 treatment of Jurkat cells, T-ALL blasts and healthy PBMCs. Of note, compared to the treatment with TRAIL alone, the combination of the two compounds gave rise to a distinct increase of the number of apoptotic events, both in Jurkat cells and T-ALL-derived blasts, without affecting healthy PBMCs. In order to investigate to what extent such synergic activity was reproducible, blasts derived from five T-ALL patients were analyzed by flow cytometry. Results, summarized in Figure 2B, showed that the combination of the two compounds produced higher cytotoxicity than TRAIL alone even though the amplitude of increase varied among the patients.

Surface expression of TRAIL receptors is not influenced by the treatment with AG-490

It has been previously demonstrated that toxic agents are able to modulate TRAIL receptors in different cell types [4] through different molecular pathways. Therefore, we investigated whether AG-490 treatment was able to modify the surface expression of the different TRAIL receptors on Jurkat and SUPT1 cells. Flow cytometric analysis indicated that, with respect to controls, AG-490 did not modulate the surface expression pattern of TRAIL receptors, demonstrating that the increase of the cytotoxic effect, following the addition of AG-490 to TRAIL, is not related to changes of the surface expression of TRAIL receptors (Supplementary information, Figure S1).

AG-490-dependent P-Stat3 downregulation is associated with the reduction of cIAP-1 and cIAP-2 mRNA and protein levels

AG-490 is a well-known Jak2 inhibitor, able to effectively block Stat3 activation in different cancer cell lines [13]. Activation of Stat3, on the other hand, has been associated with increased expression of different apoptosis inhibitors [23]. We therefore sought to determine whether AG-490 was effective in inhibiting Stat3 phosphorylation

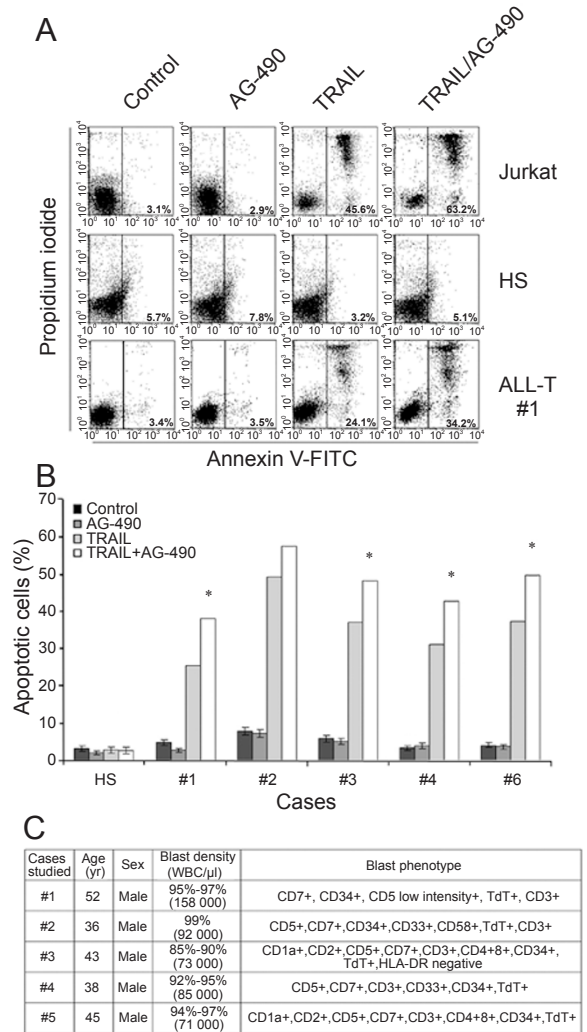


Figure 2 Flow cytometry analysis of apoptosis in Jurkat cells, PBMCs derived from healthy subjects and human T-ALL blasts after the treatment with AG-490, TRAIL or with both agonists combined together. **(A)** Flow cytometry analysis of apoptosis in Jurkat cells, in PBMCs from healthy subjects and blasts from T-ALL patients, after 24 h treatment with AG-490 (50 μ M) or TRAIL (25 ng/ml) or the combination of both of the agonists *in vitro*. Samples treated by AG-490 were always pre-treated for 12 h. Dot plots simultaneously compare the surface levels of phosphatidylserine staining (annexin-V-FITC) and propidium iodide (PI) incorporation. Data are representative of three separate experiments. **(B)** Flow cytometry analysis of apoptosis of blasts from five T-ALL patients after the treatment with AG-490 (50 μ M) or TRAIL (25 ng/ml) or with both of the agonists combined together *in vitro*. Samples treated by AG-490 were always pre-treated for 12 h. The combination of both compounds always induced an increase in the cytotoxicity with respect to treatment with TRAIL alone, which was statistically significant ($P < 0.01$) for four out of five patients. Data are the means of one experiment in triplicate \pm SD. * ($P < 0.01$) indicates statistically significant values with respect to treatment with TRAIL alone. **(C)** Case study. Age, sex, surface antigen expression and blast density characterizing ALL-T patients.

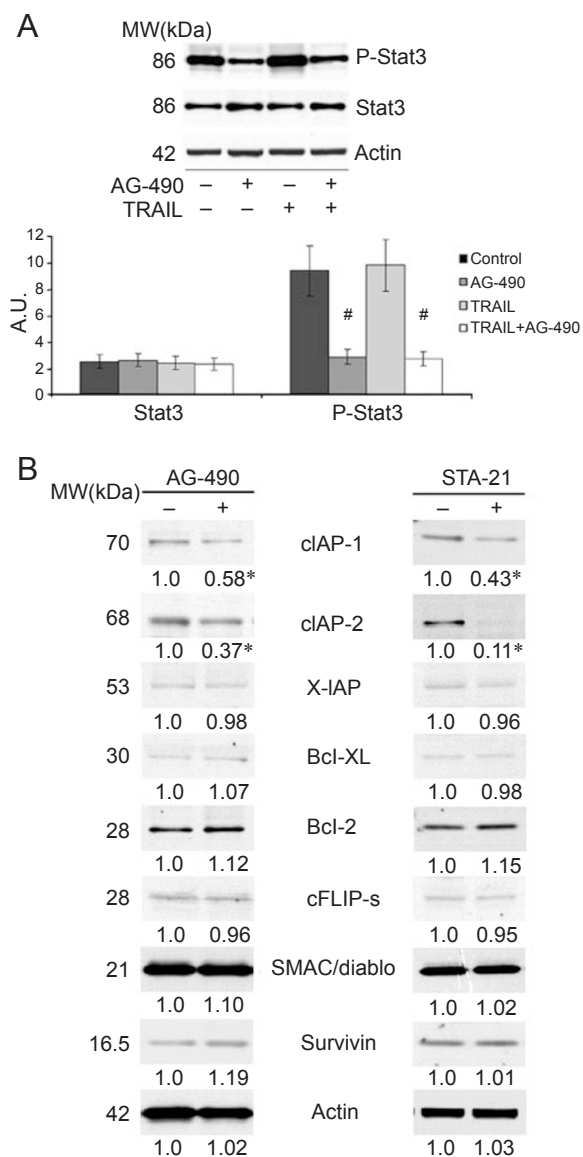


Figure 3 Analysis of Stat3 activation and expression of several apoptosis-related proteins in Jurkat cells treated with AG-490 or STA-21. **(A)** The intensity of immunoreactivity bands (10 μ g of protein) of Stat3 and P-Stat3 was measured by densitometry analysis and expressed as arbitrary units and normalized with respect to the corresponding β -actin bands (bottom histograms) in Jurkat cells after 24 h of treatment with AG-490 (50 μ M) or TRAIL (25 ng/ml) or the combination of both agonists. Samples treated by AG-490 were always pre-treated for 12 h. β -actin bands were used as internal control. Data are representative of three separate experiments. # ($P < 0.01$) indicates statistically significant values with respect to control. **(B)** Jurkat cells were incubated for 12 h with AG-490 (50 μ M) or STA-21 (30 μ M) or their vehicle (0.1% DMSO) alone as control, and the expression of cIAP-1, cIAP-2, X-IAP, Bcl-XL, Bcl-2, cFLIP-s, SMAC/Diablo, Survivin and Actin was analyzed by western blotting by using the appropriate antibodies. Data are representative of four separate experiments. β -actin bands were used as internal control. * ($P < 0.01$) indicates statistically significant values with respect to control.

in Jurkat cells and whether this effect could modulate the expression of some of the well-known apoptosis inhibitors. Figure 3A shows that, while Stat3 was unaffected, P-Stat3 was significantly ($P < 0.01$) downregulated with respect to controls and TRAIL alone-treated samples. Such a downregulation was paralleled by an evident decrease of cIAP-1 and cIAP-2 protein expression (Figure 3B, left panel). Similar results were achieved using STA-21 (Figure 3B, right panel), a novel Stat3-selective inhibitor [29] that is able to bind SH2 domain of Stat3 and block the dimerization of Stat3 and its DNA binding as well as Stat3-dependent luciferase reporter activity [30, 31].

Flow cytometric analysis of apoptosis confirmed that Stat3 inhibition leads to a significant increase of TRAIL cytotoxicity, giving further strength to the hypothesis of the involvement of Jak2/Stat3 signaling in the modulation of cIAP-1 and cIAP-2 expression (Supplementary information, Figure S2). The analysis of cIAP-1 and cIAP-2 mRNA and protein levels is summarized in Figure 4A and 4B, respectively, showing a significant ($P < 0.01$) decrease of both cIAP-1 and cIAP-2 in AG-490-treated samples. The downregulation of cIAP-1 and cIAP-2 observed upon the addition of AG-490 was maximal at 48 h of treatment. It is worth noting that no significant modulation of cFLIPs/l mRNA and protein expression was found in the above reported experiments (Supplementary information, Figure S3). These results supported therefore the hypothesis that P-Stat3 downregulation and inhibition of cIAP-1 and cIAP-2 expression might be the molecular events involved in the increase of TRAIL-mediated cytotoxicity when it is used in combination with AG-490 (Supplementary information, Figure S4).

cIAP-1 and cIAP-2 knockdown increases TRAIL cytotoxic effects

In order to investigate the possibility that reduction of cIAP-1 and cIAP-2 expression might be responsible for the increased TRAIL sensitivity, we performed RNAi-mediated knockdown using si-cIAP-1 and/or si-cIAP-2. First, we verified the efficiency of transfection and the effectiveness of silencing. After 24 h, the percentage of transfected cells was about 80% of living cells. The efficacy of si-cIAP-1 and/or si-cIAP-2 in inhibiting cIAP-1 and cIAP-2 mRNA and protein expression is reported in Figure 5A and 5B. Then, we sought to determine the cytotoxicity of TRAIL in transfected cells. Transfection of si-cIAP-1 and/or si-cIAP-2 in the absence of TRAIL did not lead to significant cell apoptosis (not shown). However, addition of TRAIL in cIAP-1 or cIAP-2 knockdown cells amplified TRAIL-induced cytotoxicity, and the maximum effect was observed when both small interference RNAs (siRNAs) were co-transfected (Figure 5C).

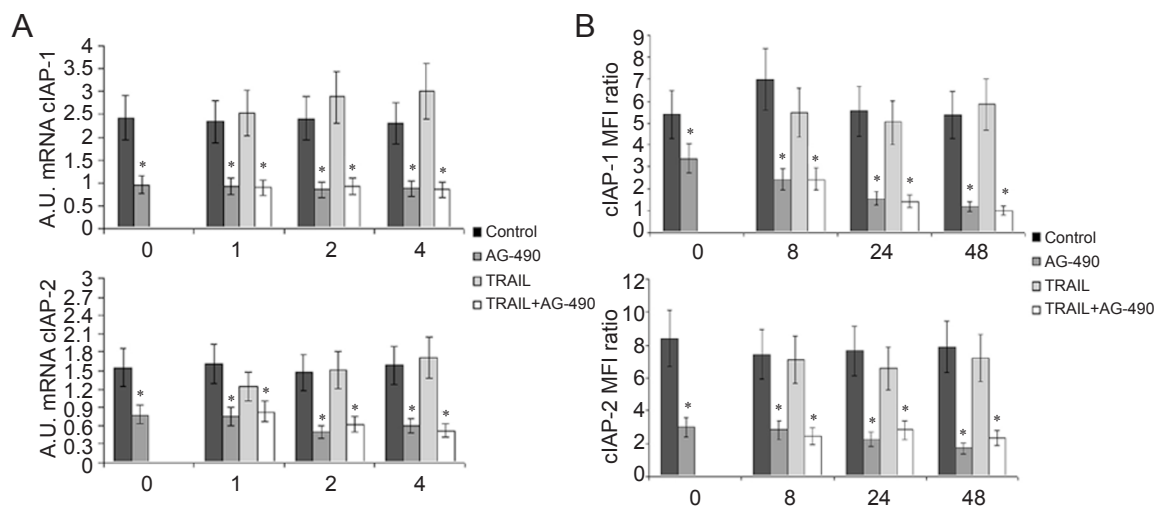


Figure 4 ciAP-1 and ciAP-2 mRNA transcription and protein levels. **(A)** ciAP-1 and ciAP-2 mRNA transcription levels at different time points (0-1-2-4 h) in Jurkat cells treated by AG-490 or TRAIL (25 ng/ml) or the combination of both agonists. The intensity of ciAP-1 and ciAP-2 and bands was measured, expressed as arbitrary units (A.U.) and normalized to the corresponding GAPDH bands (internal control). Data are the mean of three separate experiments \pm SD. **(B)** Flow cytometry analysis of intracellular ciAP-1 and ciAP-2 protein expression levels at different time points (0-8-24-48 h) in Jurkat cells treated by AG-490 or TRAIL (25 ng/ml) or the combination of both agonists. The expression of ciAP-1 and ciAP-2 was measured and expressed as mean fluorescence intensity (MFI) ratio. Data are the mean of three separate experiments \pm SD. * ($P < 0.01$) indicates statistically significant values with respect to control samples. In this figure, all samples treated by AG-490 were pre-treated for 12 h.

Discussion

Important breakthroughs in leukemia therapy include clinical applications of agonistic antibodies and small inhibitory molecules [26, 32, 33]. Potential therapeutic effects have also been recently reported for physiological pro-apoptotic proteins such as galectin-1 or TRAIL [34]. Although nowadays most children with T-ALL are cured [35], the outcome for adults with leukemia remains less promising, since adults with T-ALL frequently relapse after conventional therapies [36]. Thus, the development of drug resistance remains a looming problem. One goal is to create therapeutic strategies that minimize chemotherapy doses, without impairing the specific apoptotic effect on tumor cells; therefore, limiting the development of drug-resistance. Apart from the use of the above reported compounds as mono therapy, combinations of traditional chemotherapeutics are particularly interesting in the light of possible enhancement of the effect of mono therapies. In this study, we have demonstrated that in human T cell lines and in T-ALL primary cells, the combination of AG-490 and TRAIL results in a synergistic apoptotic response without inducing toxic effects in normal cells. It has been reported that TRAIL can mediate the biological activity of chemotherapeutic agents [37] and the use of TRAIL as anticancer treatment *in vivo* has

been proposed on the basis of a preferential sensitivity of cancer cells rather than normal cells to TRAIL-induced apoptosis. Nevertheless, the problem of TRAIL-resistance development and some degree of cytotoxicity on normal tissues and cells including hematopoietic cells [38, 39] remain a major issue. In this context, our results indicating that, when combined with AG-490, TRAIL doses could be significantly reduced without affecting, or even enhancing, its cytotoxic effects on cancer cells, provide a new clue that deserves further investigations. Indeed, the possibility of administering lower doses with very minimal or no toxic effects on normal cells could open new therapeutic perspectives.

We have also addressed the mechanism underlying the synergistic effect of AG-490 and TRAIL on Jurkat cells. Other investigations have highlighted that the combination of drugs such as proteasome inhibitors [40] or ionizing radiation [41] with TRAIL can enhance the apoptotic effect of the latter by modulating the surface expression of TRAIL-R1 and TRAIL-R2 and/or decreasing the levels of the anti-apoptotic protein c-FLIP. In this respect, the lack of influence on the pattern of surface expression of TRAIL receptors following the treatment with AG-490, along with the finding of no consistent modulation of c-FLIP and other apoptosis-related molecules, strongly suggests that there are other possible pathways

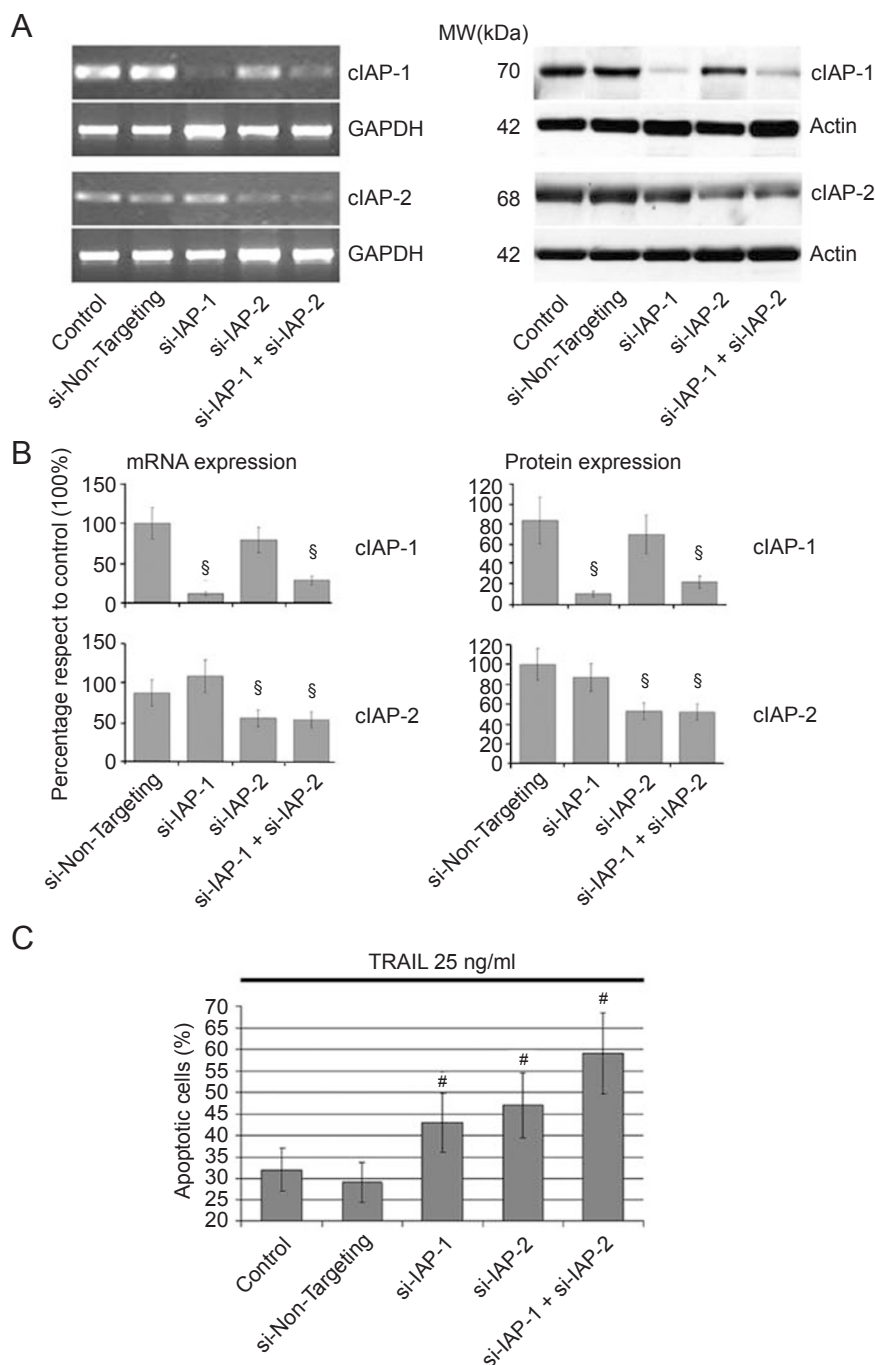


Figure 5 Post-transcriptional gene silencing in Jurkat cells after si-cIAP-1 and/or si-cIAP-2 nucleofection. **(A)** mRNA transcription and protein expression levels as evaluated 24 h after the nucleofection of cIAP-1 and cIAP-2 in Jurkat cells. GAPDH bands were used as internal control for cIAP-1 and cIAP-2 mRNA transcription levels, while β -actin bands were used as internal control for cIAP-1 and cIAP-2 protein expression levels. Data are representative of three separate experiments. **(B)** Densitometric analysis of mRNA transcription and protein expression levels of cIAP-1 and cIAP-2 in Jurkat cells after 24 h of nucleofection with si-Non-Targeting, si-cIAP-1, si-cIAP-2 and both silencers combined together. The values, expressed as arbitrary units, have been normalized with respect to GAPDH or to β -actin bands for mRNA transcription and protein levels, respectively. Bars represent the percentage with respect to control (100%). Data are the mean of three separate experiments \pm SD. § ($P < 0.01$) indicates statistically significant values with respect to control. **(C)** Flow cytometry analysis of apoptosis in Jurkat cells induced by TRAIL (25 ng/ml) for 24 h, after 1 day of si-cIAP-1 and/or si-cIAP-2 nucleofection. Bars represent the percentage of apoptotic cells evaluated by surface phosphatidylserine staining. Data are the mean of three separate experiments \pm SD. # ($P < 0.01$) indicates statistically significant values with respect to control.

responsible for the synergistic apoptotic activity. AG-490 is a member of the tyrphostin family of tyrosine kinase inhibitors able to block Jak2/Stat3 activation in T cells [12]. Of interest, while many kinase inhibitors are often promiscuous in the enzymes they target, AG-490 is unique in that it does not inhibit other lymphocytic tyrosine kinases such as Lyn, Btk, Lck, Sic, Src, Jak1 or Tyk2 [9-12]. The IAP family is a relatively new group of apoptosis-regulating proteins, which consists of a number of proteins that can bind to and inhibit caspases [24]. There is growing evidence that these proteins are expressed in various types of cells including hematopoietic cells and different cancers [42]. It has been demonstrated that the Jak2/Stat3 pathway is constitutively active in T cell leukemia [43, 44] and that its activation upregulates the expression of IAPs [24], thus promoting the anti-apoptotic action [45]. Therefore, inhibition of the Jak2/Stat3 pathway by the addition of AG-490 to TRAIL-treated T leukemia cells and the consequent downregulation of cIAP-1 and cIAP-2 appear to contribute to the increase of TRAIL apoptotic effect. The use of STA-21, a novel Stat3-selective inhibitor [30], strongly supports the hypothesis that Jak2/Stat3 signaling is responsible for the expression of cIAP-1 and cIAP-2. Of note, cIAP-1 and cIAP-2 have been reported to be overexpressed in T leukemia cells [46-48]. Their involvement in the signal machinery targeted by the combination of AG-490 and TRAIL (Supplementary information, Figure S4) is suggested by the knockdown experiments performed in TRAIL-treated cells, as the results evidently indicated a relationship between the downregulation of these proteins and the increase of TRAIL-induced cytotoxicity.

The individual different responses of the five ALL patients to the combination of the two compounds remain, anyway, to be explained. Apparently, the efficacy of the combinatorial treatment was inversely related to the extent of the cytotoxic effect when TRAIL was used alone. This observation might be related to the discrete expression of cIAP-1 and cIAP-2 following the treatment with TRAIL alone. It has been suggested that in some cell lines the expression of IAP family proteins might be responsible for the development of the resistance to TRAIL [49]. Therefore, it is possible that different individual expression of cIAP-1 and cIAP-2 in ALL primary cells may differently antagonize the cytotoxicity of TRAIL alone, producing, in turn, a different response upon the addition of AG-490. Taken together, these findings shed light on a novel biological effect produced by the chemotherapeutic drug AG-490, which is able to selectively sensitize T-ALL primary cells and T leukemia cell lines, but not normal T lymphocytes, to TRAIL-induced apoptosis, thus possibly providing new perspectives for the characterization of combined therapies for adult ALLs.

Materials and Methods

Antibodies

Anti-TRAIL-R1, anti-TRAIL-R2, anti-TRAIL-R3 and anti-TRAIL-R4 antibodies were obtained from R&D Systems (Minneapolis, MN, USA). Anti-cIAP-1, anti-cIAP-2, anti-FLIP_{s/l}, anti-Bcl-2, anti-Bcl-XL, survivin and anti- β -actin antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-Stat3, anti-Phospho-Stat3 (Tyr705), anti-XIAP and anti-SMAC/diablo were obtained from Cell Signaling Technology (Danvers, MA, USA). Secondary appropriate Fluorescein Isothiocyanate (FITC)-conjugated antibodies were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA, USA), while Horseradish Peroxidase (HRP)-conjugated secondary antibodies were obtained from Santa Cruz Biotechnology.

Cells and human subjects

Jurkat and SUPT1 cell lines (human leukemia T cells) were purchased from the American Type Culture Collection (Rockville, MD, USA). Subjects with T-ALL were recruited from the Haematology Unit, Pescara hospital, Italy. Five male subjects (aged 36-52) with blast density between 71 000-158 000 white blood cells/ μ l (WBC/ μ l) were characterized as T-ALL patients by using a surface antigen panel, including the characterization of CD14, CD45, CD1a, CD2, CD3, CD4, CD5, CD7, CD8, CD10, CD19, CD22, CD20, HLA-DR, TCR α/β , TCR γ/δ , CD13, CD11b, CD33, CD34, CD15, CD117, CD61, CD103, TdT, CD79a, MPO (Beckman Coulter, Fullerton, CA, USA). Results of diagnosis exams were reported in Figure 2C. Peripheral blood from ALL-T patients and healthy individuals was collected according to guidelines established by the local ethical committee for human subject studies.

Cell separation

PBMC were separated from freshly drawn heparinized whole blood by centrifugation (400 \times g for 30 min) over Ficoll-Hypaque (Amersham-Pharmacia Biotech, Uppsala, Sweden).

Cell culture

Jurkat and SUPT1 cell lines were grown in RPMI-1640 medium (Gibco, Carlsbad, CA, USA) supplemented with 100 U/ml penicillin, 0.1 mg/ml streptomycin (Invitrogen, Carlsbad, CA, USA) and 10% fetal bovine serum (Gibco), at 37 °C in an atmosphere of 5% CO₂ in air. When required, cells were pre-treated for 12 h and, then treated with tyrphostin AG-490 (Calbiochem, San Diego, CA, USA) at a final concentration of 50 μ M [13] for 24 or 48 h, or with different concentrations (5-300 ng/ml) of TRAIL (Alexis Biochemical, San Diego, CA, USA) in the absence or presence of 50 μ M AG-490, which was added 12 h before TRAIL. Cell growth and viability were quantified by trypan blue exclusion test. When required, cells were treated for 24 and 48 h with a Stat3 highly selective inhibitor (STA-21 30 μ M) (Biomol International, Plymouth Meeting, PA, USA) [30, 31] alone, or with 25 ng/ml of TRAIL alone or with the combination of both the molecules. Treatments with STA-21 always started 12 h before the onset of the experiments.

MTT assay

A total of 5 \times 10⁵ cells stimulated with 50 μ M AG-490, 25 ng/ml TRAIL, or both for 24 and 48 h were incubated with 5 mg/ml MTT (Sigma-Aldrich, St Louis, MO, USA) for 3 h at 37 °C in trip-

licate. After a wash, cells were solubilized with DMSO (Sigma-Aldrich) and quantified by measuring the absorbance at 570 nm in a micro plate reader Spectra Max 190 (Molecular Devices, Sunnyvale, CA, USA). Data analysis was performed with a SOFTmax PRO 4.0 software (Molecular Devices).

Reverse transcriptase-PCR analysis

Total RNA was isolated from Jurkat cells with SV Total RNA Isolation System (Promega Corporation, Madison, WI, USA). To generate cDNA, 1 µg of total RNA in reaction mixture with Oligo (dT) primer and MMLV reverse transcriptase (100 U/µl) (Celbio, Milan, Italy), was used. Semi quantitative RT-PCR analysis was performed using PCR system 9700 (Applied Biosystems, Foster City, CA, USA). PCR reaction mixture contained cDNA, dNTPs (10 mM), Taq polymerase (5 U/µl) (Biotools B&M Labs, Madrid, Spain) and MgCl₂ (1.5 mM). The conditions for PCR amplification were as follows: 2 min at 95 °C for one cycle, 30 cycles of 30 s at 95 °C followed by 30 s at 56 °C and 30 s at 72 °C for cIAP-1 and cIAP-2 primers; 2 min at 95 °C for one cycle, 30 cycles of 45 s at 95 °C followed by 45 s at 55 °C and 45 s at 72 °C for cFLIPs/l primers. cDNA was amplified using the following primers: cIAP-1 5'-GCT GTT GTC AAC TTC AGA TAC CAC T-3' (sense), 5'-TGT TTC ACC AGG TCT CTA TTA AAG CC-3' (antisense); cIAP-2 5'-ACT TGA ACA GCT GCT ATC CAC ATC-3' (sense), 5'-GTT GCT AGG ATT TTT CTC TGA ACT GTC-3' (antisense); cFLIPs/l 5'-TGT TGC TAT AGA TGT GG-3' (sense), 5'-AAG GAT CCT TGA GAC TCT-3' (antisense) [50-52]. As internal control, GAPDH amplified from sense and antisense primers (5'-GAA GGT GAA GGT CGG AGT CA-3' and 5'-TTC ACA CCC ATG ACG AAC AT-3') was used. The PCR products were run on a 2% agarose gel containing ethidium bromide and visualized by Gel Doc (Bio Rad, Hercules, CA, USA). Densitometry analysis of PCR products was performed using Quantity ONE 4.3.1 (Bio Rad).

Western blotting

A total of 1×10^6 cells were harvested by centrifugation and solubilized in a lysis buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% IGEPAL CA-630 (Sigma-Aldrich), 0.1 % sodium dodecyl sulfate (Sigma-Aldrich), 0.5 % deoxycholic acid sodium salt added with 1 mM Na₂VO₄, 1 µg/ml leupeptin, 1 µg/ml aprotinin and 0.5 mM phenyl-methanesulfonyl fluoride. Total lysates (10 µg of protein) were separated on NuPAGE 4-12% Bis-Tris Gel (Invitrogen) and blotted onto nitrocellulose membrane (Amersham). After saturation with 5% skim milk powder (Sigma-Aldrich) in tris buffer saline pH 7.4 and 0.05 % tween20 (Sigma-Aldrich), the membranes were incubated with 0.2 µg/ml anti-cIAP-1, anti-cIAP-2, anti-FLIP_{s/l}, anti-Bcl-2, anti-Bcl-XL, survivin, anti-Stat3, anti-Phospho-Stat3 (Tyr705), anti-XIAP and anti-SMAC/diablo and anti-β-actin (internal control) antibodies followed by the appropriate HRP-conjugated secondary antibodies (0.27 µg/ml). Immunoreactive bands were detected by using the ECL system (Santa Cruz Biotechnology), according to the manufacturer's instructions. Chemiluminescence was detected by Gel Doc (Bio Rad) and analyzed by Quantity ONE 4.3.1 (Bio Rad) [53].

Detection of apoptosis by flow cytometry

Human annexin V-FITC kit (Bender MedSystems, Vienna, Austria) was used to detect phosphatidylserine expression on cell membranes, in order to discriminate apoptotic cells, counterstained

by PI. Each sample (5×10^5 cells) was treated according to the manufacturer's instructions. After staining procedures, samples were analyzed by flow cytometry using a FACSCalibur cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). Finally, data were analyzed using CELLQuest 3.2.1.fl software (Becton Dickinson).

Analysis of TRAIL receptor expression by flow cytometry

For each staining 5×10^5 cells were incubated with 20 µg/ml anti-TRAIL-R1, anti-TRAIL-R2, anti-TRAIL-R3 or anti-TRAIL-R4 in PBS containing 0.5% albumin from bovine serum (BSA, Sigma-Aldrich) for 30 min, followed by 15 µg/ml FITC-conjugated appropriate secondary antibody in PBS containing 0.5% BSA for 30 min. Secondary antibody-matched controls were used to assess unspecific fluorescence in the FL-1 channel. After staining procedures, samples were analyzed by flow cytometry using a FACSCalibur cytometer (Becton Dickinson). Finally, data were analyzed using CELLQuest 3.2.1.fl software (Becton Dickinson).

Flow cytometry analysis of intracellular cIAP-1 and cIAP-2

A total of 5×10^5 cells were fixed with 2% paraformaldehyde for 5 min at 25 °C and treated with 150 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl pH 7.4, 0.05% IGEPAL CA-630 (Sigma-Aldrich), 0.25% lambda-carrageenan (Sigma-Aldrich) and 0.02% NaN₃ (Sigma-Aldrich) (NET Gel). For each staining, 2 µg/ml anti-cIAP-1 or anti-cIAP-2 antibody in NET Gel, was reacted with the sample for 30 min, followed by incubation with 15 µg/ml FITC-conjugated appropriate secondary antibodies in NET Gel for 30 min. Secondary antibody-matched controls were used to assess unspecific fluorescence in FL-1. After staining procedures, samples were analyzed by flow cytometry using a FACSCalibur cytometer (Becton Dickinson). Finally, data were analyzed using CELLQuest 3.2.1.fl software (Becton Dickinson).

Nucleofection

All transient transfections were performed by using a nucleofector device associated to its corresponding kits (Amaxa, Inc., Cologne, Germany). A total of 3×10^6 Jurkat cells per sample were resuspended in 100 µl of the nucleofector solution containing 100 nM siRNA and nucleofected according to Amaxa's protocol. Cells were seeded at a density of 1.5×10^5 per coverslip in a 24-well plate. First, the transfection program for Jurkat cells was optimized. Briefly, 3×10^6 Jurkat cells were transfected with the nucleofector solution V, using different nucleofector programs (C-16, I-10 and S-18); the I-10 program provided the highest transfection efficiency associated with the lowest cell mortality and was identified as the best program (90% live cells). A Green Fluorescent Protein (GFP) expression vector pEGFP-C1 (Clontech, Mountain, California, USA) was co-transfected with the respective GFP siRNA sequences. After 24 h, a specific flow cytometric analysis indicated that the percentage of GFP-positive cells was around 80% of the living cells. Successively, Jurkat cells were nucleofected with the following siRNA: non-targeting siRNA pool, siGENOME SMART pool BIRC2 (cIAP-1) and siGENOME SMART pool BIRC3 (cIAP-2) (Dharmacon, Lafayette, CO, USA).

Statistical analysis

Data were analyzed by ANOVA and Mann-Whitney rank-sum tests. Data are shown as mean ± SD unless otherwise specified. Statistical significance was defined as $P < 0.01$.

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