LGP1, A Histone Deacetylase Inhibitor Analogue of FR235222, Sensitizes Promyelocytic Leukaemia U937 Cells to TRAIL-mediated Apoptosis

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Abstract. *Background: It has been shown that chemotherapeutic agents, such as histone deacetylase inhibitors (HDACi), are able to increase TRAIL-induced apoptosis in many types of cancer. In the present study, we investigated the effects of the novel HDACi LGP1, a new simplified analogue of FR235222, in human leukaemia U937 cells resistant to TRAIL-induced apoptosis. Materials and Methods: U937 cells were incubated with TRAIL/LGP1 for 24 h and apoptosis was evaluated using flow cytometric assay and cleavage of caspase-3 and (PARP) by Western blot. Western blot analysis was also used to detect the expression of p21, p27, (NF-κB), Bcl-2 and the levels of H4 histone acetylation. Finally, flow cytometry was used to monitor the enhancement of TRAIL-receptor levels. Results: Treatment with LGP1 caused accumulation of acetylated histone H4 and G_1 cycle arrest accompanied by increase of p21. The compound was also able to sensitize U937 cells to TRAIL-induced apoptosis through multiple mechanisms: (i) activation of caspase-3 and cleavage of PARP; (ii) induction of p21 and p27; (iii) cleavage of NF-κB and down-regulation of Bcl-2. Finally, LGP1 induced up-regulation of TRAIL-R1 receptor expression. Conclusion: These results demonstrate that U937 cells can be effectively killed by a combination treatment of subtoxic doses of LGP1 and TRAIL.*

Tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) is a member of the tumour necrosis factor (TNF) family of proteins including FasL, and TNF-α. Since TRAIL is able to induce apoptosis in many transformed and malignant cells, it is being developed as an anticancer agent (1). TRAIL interacts with two membrane-bound death receptors, DR4 (TRAIL-R1) and DR5 (TRAIL-R2), two putative membrane-bound decoy receptors, TRAIL-R3 and TRAIL-R4, and the soluble receptor osteoprotegrin (2). Receptor triggering results in recruitment of the adaptor molecule (FADD/MORT1), followed by both activation of caspase-8 and formation of the death-inducing signaling complex (DISC) (3). Active caspase-8, the apical caspase in death receptor-induced apoptosis, can then activate other caspases, such as caspase-3, which in turn cleaves many cellular substrates resulting in the biochemical and morphological features characteristic of apoptosis (4).

However, several reports have demonstrated that many tumour cells acquire resistance to the apoptotic effects of TRAIL and this may limit the efficiency of TRAIL in cancer therapy (5-8). The mechanisms of TRAIL resistance in cancer cells are not completely understood and lack of expression of functional receptors or overexpression of inhibitory molecules have been proposed. To overcome the resistance of cancer cells to TRAIL, the combination of TRAIL and chemotherapy has been used. This conbination gave enhanced effects (9, 10). Given that human leukaemia cells have also shown resistance to TRAIL-induced apoptosis (11, 12), the study of the mechanisms that control TRAIL resistance of leukaemia cells might enhance our knowledge of DR-mediated signalling and help to develop TRAIL-based approaches in combination with other chemotherapies for the treatment of human leukaemia.

Recently, accumulating evidences has suggested that (HDAC) inhibitors (HDACi) can be used as anticancer drugs due to their selective toxicity and synergistic activity with chemotherapeutic drugs (13, 14). Several structural classes of HDACi have been identified, including derivatives of short-chain fatty acids, hydroxamic acids, cyclic tetrapeptides, cyclic peptides, and benzamides. The acetylation status of histones which alters chromatin

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structure, thereby modulating gene expression, is governed by the relative activities of histone acetyltransferases and HDACs. Altered histone acetyltransferase and HDAC has been associated with many types of cancer, including haematological malignancies (13, 14). HDACi exert their antitumor effects due to their ability to induce growth arrest, differentiation and apoptosis (15). In human leukaemia cells, these effects are mediated in part by selective alteration in gene regulation, such as induction of *p21* and *p27* expression, which are associated with cell cycle arrest and apoptosis (16). The cyclopeptide FR235222 has been recently isolated from the fermentation broth of *Acremoniumsp sp*. (17). The compound is a selective inhibitor of T-cell proliferation and lymphokine production and is a potent inhibitor of class I HDACi (18). We previously demonstrated that FR235222 caused accumulation of acetylated histone H4 and inhibition of cell proliferation in human promyelocytic leukaemia U937 cells. The antiproliferative effect was related to the ability of FR235222 to cause growth inhibition at the G_0/G_1 phase transition of the cell cycle, accompanied by increase of p21 (19). Simplified FR235222 (now called LGP1) was synthesized with the aim of producing active compounds that are easier to obtain maintaining the essential structural aspects of the HDACi enzyme interactions (18). Comprehensive studies on structure-activity relationships were carried out and results obtained for LGP1 indicate that decreasing in steric bulk on D-Me-Pro by way of removal of a methyl group, does not significantly change the 50% inhibitory concentration IC_{50} value (IC_{50}=50 nM) relative to that of FR235222 (IC_{50}≅60 nM), indicating that the methyl group on the proline residue does not contribute to inhibitory potency.

Given that the resistance to TRAIL-induced apoptosis might be an important therapeutic problem, the effects of LGP1 in combination with TRAIL in human promyelocytic leukaemia U937 cells were investigated here.

Materials and Methods

Drugs. Simplified FR235222 (LGP1) was synthesized at the University of Siena according to published procedures (18). Soluble human recombinant SuperKiller TRAIL was purchased from Alexis Corporation (San Diego, CA, USA). The general caspase inhibitor Z-VAD-FMK was obtained from BD Pharmingen (BD Bioscience, Bedford, USA).

Cell cultures. Human promyelocytic leukaemia U937 cells were provided by the American Type Culture Collection (Manassas, VA, USA).

Analysis of apoptosis. Hypodiploid DNA was analysed using the method of propidium iodide (PI) staining and flow cytometry as described elsewhere (20). Briefly, cells were washed in phosphate-buffered saline (PBS) and resuspended in 500 μl of a solution containing 0.1% sodium citrate, 0.1% Triton X-100 and 50 μg/ml PI (Sigma- Aldrich Co, St Louis, MO, USA). After incubation at 4˚C for 30 minutes in the dark, cell nuclei were analyzed with Becton Dickinson FACScan flow cytometer (Becton Dickinson, CA. USA) using the Cell Quest program. Cellular debris was excluded from analysis by raising the forward scatter threshold, and the DNA content of the nuclei was registered on logarithmic scale. The percentage of the cells in the hypodiploid region was calculated

Cell cycle analysis. Cells were plated and exposed to LGP1 50 nM. After incubation at 4˚C for 30 minutes in the dark, cells were harvested and fixed in cold 70% ethanol at –20˚C. Cell cycle profiles were evaluated by DNA staining with 2.5 mg/ml PI in PBS supplemented with 100 U/ml ribonuclease A for 30 min at room temperature. Samples were analysed with a FACScan flow cytometer (Becton Dickinson) using Cell Quest evaluation program. The distribution of cells in distinct cell cycle phases was determinate using ModFit LT cell cycle analysis software.

Total proteins extraction. Total intracellular proteins were extracted from the cells by freeze/thawing in lysis buffer of 50 mM Tris-HCl containing protease and phosphatase inhibitors (1 mM (PMSF), 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 μg/ml aprotinin, 1 μM Na_3VO_4, 1 μM NaF. Protein content was estimated according to Biorad protein assay, and the samples either analysed immediately or stored at –80˚C. For cytosolic extracts, U937 cells were plated in 10 cm plates and incubated with or without LGP1 50 nM, and TRAIL 10 ng/ml, for 24 hours. Total extracts were then analysed by Western blotting.

Western blotting analysis. Samples (50 μg protein) were loaded onto 10-12% acrylamide gels and separated by (SDS-PAGE) in denaturating conditions at 50 V. The separated proteins were then transferred electrophoretically to nitrocellulose paper soaked in transfer buffer (25 mM Tris, 192 mM glycine) and 20% methanol v/v. Non-specific binding was blocked by incubation of the blots in 5% non-fat dry milk powder in TBS/0.1% Tween (25 mM Tris; 150 mM NaCl; 0.1% Tween v/v) for 60 min. After washing, the blots were incubated overnight at 4˚C with the following primary antibodies: rabbit polyclonal anti-p65, rabbit polyclonal anti-caspase-3, rabbit polyclonal anti-p21, rabbit polyclonal anti-p27, mouse monoclonal anti-(PARP), mouse monoclonal Bcl-2 (all from Santa-Cruz Biotechnology, D.B.A. ITALIA s.r.l, Milan, Italy), rabbit polyclonal anti-histone H4 and rabbit polyclonal anti-histone acetyl-H4 (Upstate Biotechnology, Lake Placid, NY, USA), and mouse monoclonal anti-tubulin (Sigma-Aldrich Co, St Louis, MO, USA). After incubation with the primary antibodies and washing in TBS/0.1% Tween, the appropriate secondary antibody, either anti-mouse, or anti-rabbit (both from Sigma-Aldrich, Italy) was added for 1 h at room temperature. Immunoreactive protein bands were detected by chemiluminescence using enhanced chemiluminescence reagents (ECL) and exposed to Hyperfilm. The blots were then scanned and analysed (Gel-Doc; 2000, BIO-RAD).

Caspase-3 activity assay. Caspase-3 activity was evaluated using Caspase-3 Fluorometric Detection kit (Assay Designs, Ann Arbor, MI, USA). Whole lysate of U937 all treated with or without LGP1 50 nM and TRAIL 10 ng/ml was added to the specific caspase-3 substrate and incubated for 30 minutes at 37˚C. After incubation the emission of fluorescence was measured. Results are expressed as % caspase-3 activity in excess of that of control cells.

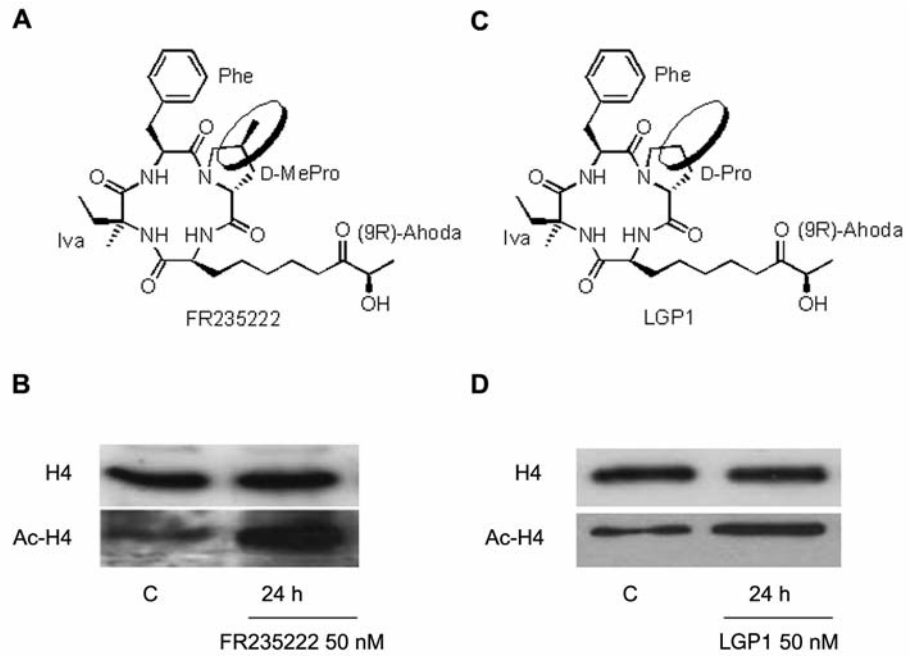

Figure 1. *Structure of FR235222 and LGP1 and their effects on histone acetylation in U937 cells. FR235222 (A) and LGP1 (C) were synthesized as described elsewhere (18). U937 cells were treated with 50 nM FR235222 (B) and 50 nM LGP1 (D) for 24 hours, after which histone H4 and acetylhistone H4 were measured by Western blotting.*

Flow cytometry of TRAIL receptors. U937 cells were plated in 6 cm wells and then treated with LGP1 50 nM and TRAIL 10 ng/ml, as co-treatment for 24 h. Cells were washed with PBS and collected in 1 mM PBS-EDTA. Pellets were incubated with 100 µl PBS with monoclonal anti-TRAIL-1, anti-TRAIL-2 antibodies, (Alexis Corporation, Vinci-Biochem, Italy) on ice for 1 hour. After incubation cells were washed twice and incubated in 100 µl PBS with secondary fluorescein isothiocyanate-conjugated monoclonal antibody (Alexis Corporation) on ice for 1 h. After washing with PBS, the expression of death receptors was analysed by flow cytometry.

Statistical analysis. All results are the mean±SEM of 3 experiments performed in triplicate. The optical density of the protein bands detected by Western blotting was normalized against tubulin levels. Statistical comparisons between groups were made using Bonferroni parametric test. Differences were considered significant if $p<0.05$.

Results

LGP1 induces accumulation of acetylated histones. We have previously reported that treatment with 50 nM FR235222 (Figure 1A) caused accumulation of acetylated histone H4 (Figure 1B) in human promyelocitic leukaemia U937 cells (19). In order to verify whether LGP1 (Figure 1C), the simplified analogue of FR235222, is able to induce hyperacetylation of histone H4, U937 cells were incubated with LGP1 50 nM for 24 h and protein extracts were used for Western blot analysis. Data in Figure 1D show that LGP1 promoted a significant increase in acetylation compared with control, showing similar HDAC inhibitory activity to the natural cyclopeptide.

LGP1 induces cell cycle arrest in the G_1 phase but has no effect on apoptosis in U937 cells. To determine the effects of LGP1 on the cell cycle, U937 cells were exposed to 50 nM LGP1 for 24 h and subjected to cell cycle analysis by FACS. Data in Figure 2A indicate that 31.9% of the untreated cells were in the G_0/G_1 phase and 55.3% in the S phase. Treatment with LGP1 caused a time-dependent increase of the percentage of cells in the G_0/G_1 phase, with a peak of 83.6% at 24 h, as well as a reduction of cells in the S phase (9.18% at 24 h). These data are consistent with a cell cycle arrest at the G_0/G_1 phase. We next investigated the effects of LGP1 on U937 cell apoptosis. The cells were exposed to 50 nM LGP1 for different times. Apoptosis was measured by cytofluorimetric analysis of PI staining of hypodiploid nuclei. The results indicate that 50 nM LGP1 had no effect on apoptosis in U937 cells up to 96 h (Figure 2B). The effects on the cell cycle and the lack of effect on apoptosis by LGP1 are similar to those already reported for 50 nM FR235222 (19).

LGP1 sensitizes U937 cells to TRAIL-induced apoptosis. It has been shown that TRAIL at low concentrations is not able to efficiently kill U937 cells (21, 22). On the other hand HDACi potentiated TRAIL-induced apoptosis in various cell types, including Jurkat, U937, K562, and freshly isolated (CLL) cells (23). Moreover, recent reports indicate that some cancer cell lines are rendered more susceptible to TRAIL-induced apoptosis when arrested in the G_0/G_1 phase, and that

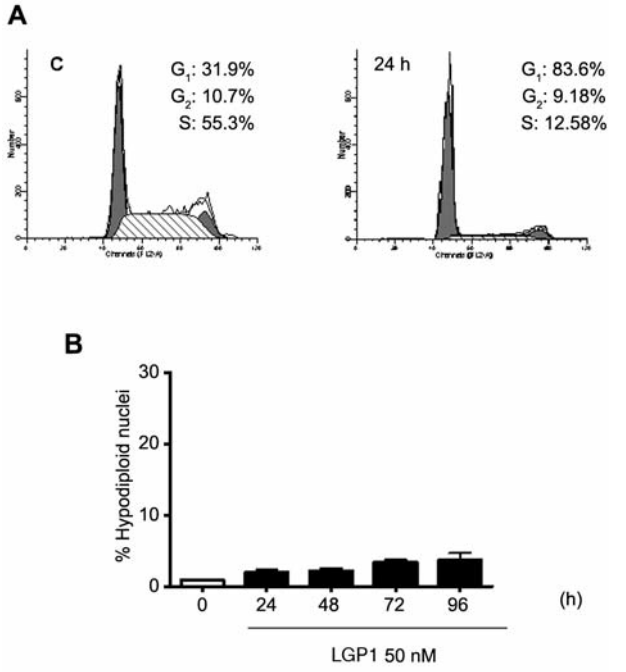

Figure 2. *Effects of LGP1 on cell cycle and apoptosis in U937 cells. A: Cell cycle analysis of U937 cells after exposure to LGP1 (50 nM) for 0 (Control) and 24 h. The U937 cells were incubated with PI and subjected to cell cycle analysis using a Becton Dickinson FACScan flow cytometer and ModFit software as described in the Materials and Methods. B: U937 cells were treated with 50 nM LGP1 for different times (0-96 h) and apoptosis was assessed by PI staining of hypodiploid nuclei by flow cytometry. Results are shown as the mean±SEM of 3 experiments performed in triplicate.*

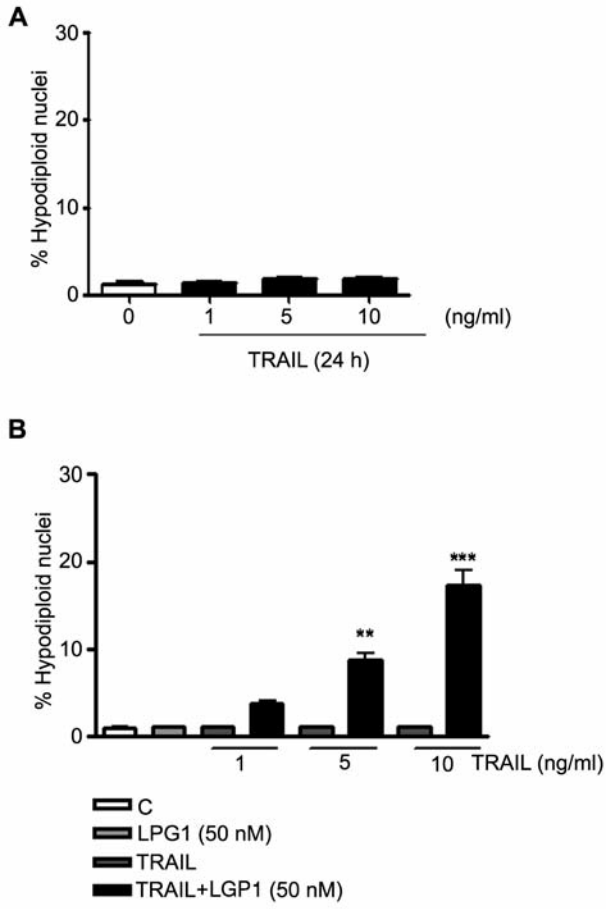

Figure 3. *Effects of TRAIL alone and in combination with LGP1 on apoptosis of U937 cells. A: U937 cells were treated for 24 h with TRAIL at different concentrations (1-10 ng/ml) and apoptosis was assessed by propidium iodide (PI) staining of hypodiploid nuclei by flow cytometry. Results are shown as the mean±SEM of 3 experiments performed in triplicate. B: U937 cells were treated with LGP1 (50 nM) alone or in combination with TRAIL (1-10 ng/ml) for 24 h. Analysis of apoptosis was performed by PI staining using flow cytometry. Results are shown as the mean±SEM of 3 experiments performed in triplicate. **p<0.01 ***p<0.001 vs. control cells.*

TRAIL-related induced apoptosis appears to be associated with an up-regulation of *p21* (24). Thus, we examined the effect of TRAIL on U937 cells and whether co-treatment of U937 cells with LGP1 would increase their susceptibility to TRAIL-induced apoptosis. The cells were incubated with TRAIL at different concentrations and apoptosis was measured by PI staining of hypodiploid nuclei as described in the Material and Methods. Figure 3A shows that TRAIL was unable to induce significant apoptotic effects in U937 cells stimulated at 3 different concentrations (1, 5 and 10 ng/ml) after 24 hours. In different experiments U937 cells were exposed to different concentrations of TRAIL alone or with LGP1 (50 nM) and apoptosis analyzed by PI staining. Figure 3B shows that the simultaneous incubation of cell with LGP1 and TRAIL induced a synergistic apoptotic dose-response effect in U937 cells.

Combined treatment with LGP1 and TRAIL induces caspase activation in U937 cells. In order to verify whether LGP1 is able to sensitize the U937 cells to TRAIL-induced apoptosis through a caspase-dependent mechanism, we evaluated caspase-3 expression by Western blotting. U937 cells were treated with LGP1 (50 nM) and TRAIL (10 ng/ml) alone or in combination for 24 h. Figure 4A shows that apoptosis induced by the co-treatment with LGP1 and TRAIL are associated with activation of caspase-3, and cleavage of PARP, a substrate of caspase-3 in U937 cells. The involvement of caspase-3 in this mechanism is also confirmed by caspase-3 activity measured as by fluorimetric analysis. Data in Figure 4B indicate that the synergistic effect between TRAIL and LGP1 led to a two-fold increase of caspase-3 activity after 24 h of treatment. These data clearly indicate that the cleavage of PARP matched the increased activation of caspase-3 following incubation with LGP1 plus TRAIL. Finally, U937 cells were incubated with the caspase inhibitor

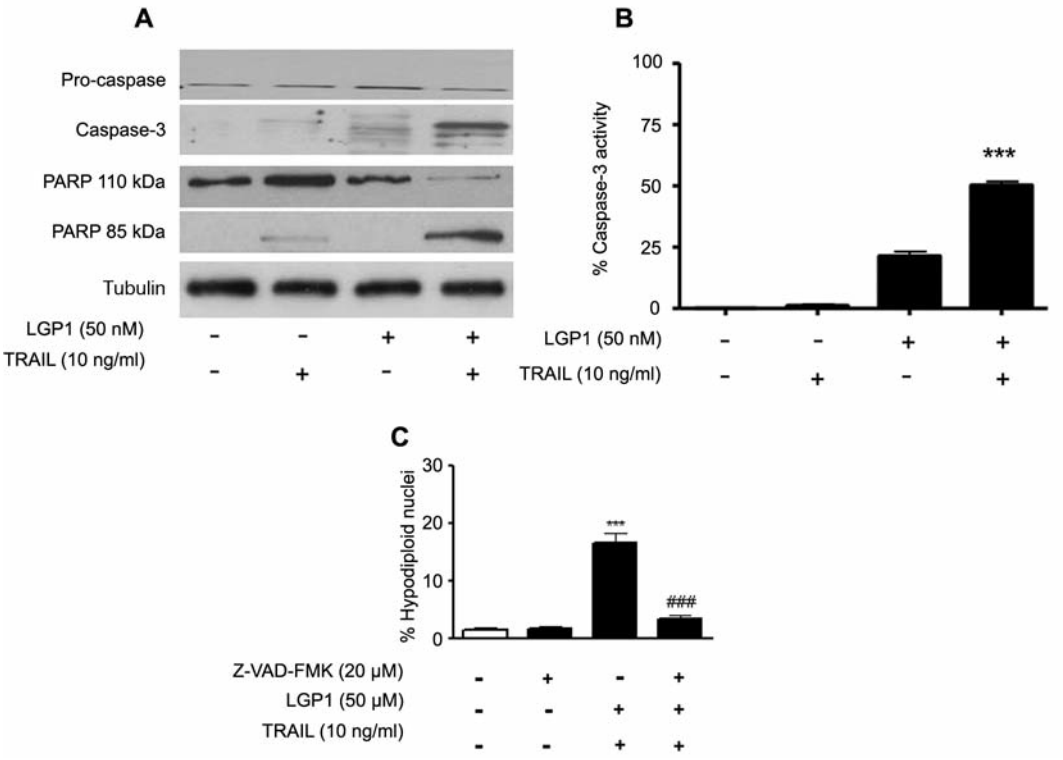

Figure 4. *Effects of the combined treatment with LGP1 and TRAIL on caspase-3 activity in U937 cells. A: Western blotting analysis of caspase-3 and PARP expressions after 24 h incubation with LGP1 alone or in combination with 10 ng/ml TRAIL. Blots are representative of 3 different experiments. B: Caspase-3 activity was assessed using caspase-3 fluorimetric detection kit. Lysate of U937 cells treated with LGP1 (50 nM) alone or in combination with TRAIL (10 ng/ml) for 24 h was added to the specific caspase-3 substrate and incubated for 30 minutes at 37°C. After incubation, the emission of fluorescence was measured as % caspase-3 activity in excess of that of control cells. Results are shown as the mean±SEM of 3 experiments performed in triplicate. ***p<0.001 vs. control cells. C: Effects of caspase inhibitor Z-VAD-FMK on LGP1 and TRAIL-induced apoptosis. Z-VAD-FMK (20 μM) was administered 30 min before incubation of cells with LGP1 (50 nM) and TRAIL (10 ng/ml) for 24 h. The percentage of apoptotic cells was evaluated by flow cytometry. Results are shown as the mean±SEM of 3 experiments performed in triplicate. ***p<0.001 vs. control cells, ###p<0.001 vs. LGP1 and TRAIL.*

Z-VAD-FMK (20 μM) for 30 min before adding TRAIL and LGP1 for a further 24 h. Z-VAD-FMK efficiently inhibited apoptosis induced by the combination of LGP1 and TRAIL (Figure 4C).

The synergistic effect between LGP1 and TRAIL involves several pathways. HDACi enhance the apoptosis-inducing effect of TRAIL in leukaemia cells through multiple synergistic mechanisms such as the activation of caspase-3, the induction of *p21* and *p27* and the down-regulation of (IAPs), (cFLIP) and Bcl-2. In particular, it has been reported that cell cycle arrest in several cell lines is related to the enhancement of sensitization to TRAIL (25). Thus, we investigated the effects of LGP1-induced sensitization to TRAIL-induced apoptosis in U937 cells on cell cycle-regulatory proteins as p21 and p27. Protein extracts were prepared from cells treated for 24 hours and analyzed by Western blotting. Figure 5 shows that stimulation with LGP1 at 50nM induced a strong increase of p27, confirming previous results with FR235222 (19), and an even stronger increase in p21 expression. Surprisingly, we also found that during co-treatment with LGP1 and TRAIL, the increase in full-length p21 level was associated with the appearance of a cleaved p21 band in the cytosol. Moreover, a marked up-regulation of of p27 during co-treatment with TRAIL (10 ng/ml) and LGP1 (50 nM) was observed, as demonstrated by Western blot analysis (Figure 5). Since FR235222 has been recently described to be an NF-κB inhibitor (26), it is likely that NF-κB inactivation may sensitize U937 cells to death by TRAIL. Thus, the effect of LGP1 on NF-κB was investigated. Figure 5 shows that no changes in levels of subunit p65 of NF-κB were detected in U937 cells treated for 24 h with TRAIL or LGP1 alone. Interestingly, the smaller band of p65 was recognized in U937 cells treated with LGP1 and TRAIL. The truncated p65 is assumed to be a cleavage product generated during the apoptotic process. Finally, we analysed the expression levels of Bcl-2 to evaluate the role of the mitochondria and the

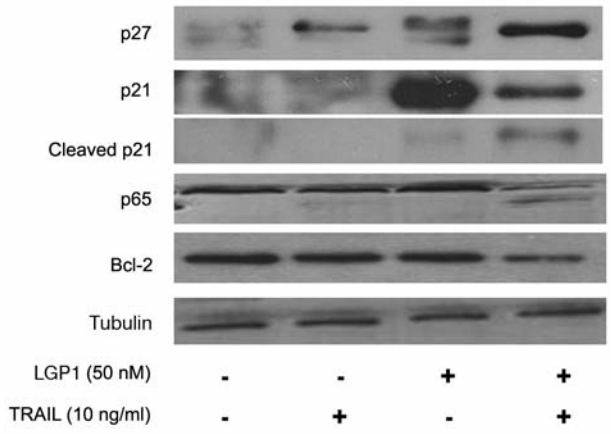

Figure 5. *Effects of combined treatment with LGP1 and TRAIL on the expression of proteins involved in cell cycle and apoptosis of U937 cells. A: U937 cells were treated with LGP1 (50 nM) and TRAIL (10 ng/ml) alone or in combination for 24 h. Expression of p21, p27, NF-κB and Bcl-2 was then evaluated by Western blotting analysis. Results are representative of 3 experiments.*

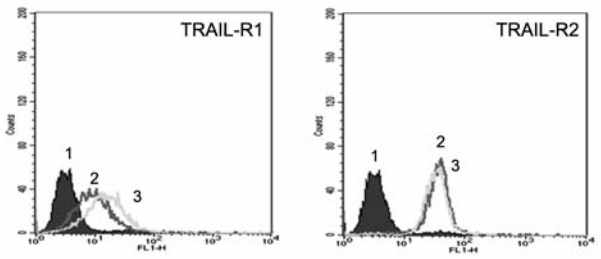

Figure 6. *Effects of LGP1 on TRAIL receptor expression in U937 cells. U937 cells were treated with LGP1 (50 nM) for 24 h. Immunofluorescence of TRAIL-R1 and TRAIL-R2 was measured by flow cytometric analysis as described in the Material and Methods. Curve 1: Control cells. Curve 2: TRAIL-R expression in control cells. Curve 3: TRAIL-R expression in 24 h LGP1 (50 nM) treated cells. Results are representative of 3 experiments.*

intrinsic pathway of the apoptosis induced by the co-treatment. Twenty-four hours after exposure of U937 cells to LGP1 and TRAIL, a decrease in Bcl-2 expression levels was found.

Involvement of TRAIL-R1 but not TRAIL-R2 in enhancement of TRAIL-induced apoptosis through LGP1. Several studies of HDACi-mediated sensitization to TRAIL have proposed that an important mechanism occurs through up-regulation of TRAIL receptors. We investigated the expression of TRAIL (10 ng/ml) receptors after LGP1 (50 nM) treatment for different times by immunofluorescence flow cytometric analysis. The results in Figure 6 show up-regulation of TRAIL-R1 expression after 24 h LGP1 treatment. No significant changes in TRAIL-R2 (Figure 6) or decoy TRAIL receptor expression (data not shown) were observed after treatment with LGP1.

Discussion

Induction of apoptosis in cancer cells by TRAIL is a promising therapeutic principle in oncology, although toxicity and resistance to TRAIL are limiting factors. Indeed, many tumours remain resistant to TRAIL-induced apoptosis, which is related to the dominance of anti-apoptotic signals. Several reports have demonstrated that chemotherapeutic agents, such as HDACi, are able to increase TRAIL-induced apoptosis in many types of cancer (27, 28). Cells from patients with chronic lymphocytic leukaemia (CLL), which are inherently resistant to TRAIL, are also sensitized by prior treatment with HDACi by facilitating increased formation of the TRAIL DISC (16, 23). Although only a few studies have explored the sensitivity of acute myeloid leukaemia (AML) cells to TRAIL, they have also shown a very low sensitivity of AML blasts to the apoptotic effects of TRAIL (29). Moreover, it is known that some leukaemia cell lines, such as human chronic myelogenous leukaemia K562, were observed to be resistant to TRAIL (30), while human promyelocytic leukaemia U937 cells exhibited only modest cell killing in response to low concentrations of TRAIL (21, 22).

In the present study, we evaluated the effect of the novel HDAC inhibitor LGP1, a new simplified analogue of FR235222, in human leukaemia U937 cells resistant to TRAIL. It has been shown that LGP1 exhibits the same HDAC inhibitor activity as FR235222 (18). We here report that in U937 cells, treatment with LGP1 caused accumulation of acetylated histone H4. Moreover, LGP1 caused growth inhibition at the G_0/G_1 phase transition of cell cycle, while it had no effect on apoptosis. These findings are very similar to those previously obtained with FR235222 in U937 cells (19). As previously stated, U937 cells exhibited only modest apoptosis in response to subtoxic doses of TRAIL. Hence, we decided to investigate the effects of the combined treatment with LGP1 and TRAIL on apoptosis of U937 cells. We found that LGP1 was able to sensitize U937 cell to TRAIL-induced apoptosis. The combined treatment induced activation of caspase-3, and cleavage of PARP. Indeed, apoptosis was almost abolished by incubation of U937 cells with the caspase inhibitor Z-VAD-FMK.

It has been demonstrated that colon and lung cells arrested in G_0/G_1 phase of the cell cycle are more susceptible to TRAIL-induced apoptosis (25). We have previously shown that FR235222 is able to induce cell cycle arrest in G_0/G_1 phase with the simultaneous augmentation of p21 levels (19). According to these data we show that the G_1 cycle arrest by LGP1 was accompanied by an increase of p21, also suggesting that LGP1 is a potent inhibitor of cell cycle

progression. p21 is a well-characterized cyclin-dependent kinase inhibitor and its overexpression is strongly related in G_0/G_1 cell cycle arrest. Interestingly, the apoptotic effect mediated of LGP1 in combination with TRAIL was also accompanied by marked cleavage of p21. Several studies have suggested that cleavage of p21 may be implicated in induction of apoptosis by various agents, including HDACi and TRAIL (31). Consequently, it is tempting to speculate that the cleavage of p21 in TRAIL/LGP1-treated cells contributed to enhanced apoptosis. Moreover, we also show that apoptosis induced by cotreatment with LGP1 and TRAIL is associated with increased expression of p27. Overexpression of p27 protein is usually involved in arresting the cells in G_0/G_1 and often induces apoptosis (32).

FR235222 has been recently described as an NF-κB inactivator (33). Therefore, we reasoned that NF-κB inactivation might also sensitize U937 cells to death by TRAIL. Thus, the effect of LGP1 on NF-κB was investigated. We found that the treatment with LGP1 alone did not regulate the levels of subunit p65 of NF-κB while the exposure of U937 cells to both TRAIL and LGP1 led to cleavage of p65. In several studies, it has been shown that p65 is cleaved by caspases, and truncated p65 leads to the inhibition of transcriptional activity of NF-κB (34). Given that the cleavage of NF-κB by caspases inhibits its activity during apoptosis (34), we may conclude that cleavage of p65, suppressing the NF-κB survival pathway, contribute to the synergistic effect of LGP1 and TRAIL.

In several types of cancer cell, NF-κB protects against cell death by up-regulating anti-apoptotic factors such as Bcl-2 (35). Accordingly, we show that in U937 cells, LGP1 in combination with TRAIL reduced the expression of Bcl-2, indicating the involvement of the mitochondria in this apoptotic event.

Different studies have demonstrated that HDACi enhance expression of cell surface TRAIL-R1 and/or TRAIL-R2, giving rise to the hypothesis that this is a key event in enhanced TRAIL-mediated apoptosis after the addition of HDACi. We showed that LGP1 caused up-regulation of the expression of TRAIL-R1, whereas no changes in the expression of TRAIL-R2 were noticed. These finding are in agreement with previous reports that have shown that HDACi-mediated sensitization of CLL cells occurs almost exclusively through TRAIL-R1, not TRAIL-R2 (36). Moreover, these findings in accordance with other studies, demonstrate that class I, and not class II HDACs, are involved in the sensitization to TRAIL (38).

In summary, our results show that U937 cells, which are usually resistant to low concentrations of TRAIL, can be effectively killed by a combination treatment of subtoxic doses of LGP1 in combination with TRAIL. LGP1 sensitization of TRAIL-induced apoptosis appears to be mediated through up-regulation of TRAIL-R1 receptor. Additional mechanisms including the arrest of the cell cycle, the involvement of NF-κB inactivation and reduction of Bcl-2 were observed.

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