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Differential Effects of Chemotherapeutic Drugs Versus the MDM-2 Antagonist Nutlin-3 on Cell Cycle Progression and Induction of Apoptosis in SKW6.4 Lymphoblastoid B-Cells

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Abstract We have compared the cytotoxic/cytostatic responses of the SKW6.4 lymphoblastoid B-cells to the alkylating agent chlorambucil, the purine analog fludarabine, the non-genotoxic activator of the p53 pathway, Nutlin-3, used alone or in association with the death-inducing ligand recombinant TRAIL. Exposure to chlorambucil, fludarabine, and Nutlin-3 induced p53 accumulation and variably affected cell cycle progression in SKW6.4 lymphoblastoid cells. In particular, chlorambucil induced cell cycle accumulation at the G2/M checkpoint; Nutlin-3 induced early cell cycle arrest at the G1/S checkpoint, while fludarabine showed an intermediate behavior. On the other hand, recombinant TRAIL alone did not affect cell cycle progression but induced a rapid increase of apoptosis. Analysis of the gene expression profile of the p53-transcriptional targets showed distinct features between chlorambucil, Nutlin-3 and fludarabine, which likely account for their differential effect on cell cycle in SKW6.4 cells. In particular, chlorambucil upregulated the steady-state mRNA expression of SFN/14-3-3σ, a gene involved in G2/M cell cycle arrest. Of note, all agonists upregulated TRAIL-R2 expression in SKW6.4 cells both at the mRNA and protein levels. Consistently, pretreatment with chlorambucil, fludarabine and Nutlin-3 enhanced SKW6.4 sensitivity to TRAIL-mediated apoptosis. J. Cell. Biochem. 104: 595–605, 2008. © 2007 Wiley-Liss, Inc.

Key words: TRAIL; p53; chemotherapeutic drugs; cell cycle

The conventional chemotherapeutic regimens used in the treatment of indolent B-cell malignancies, are based on alkylating agents, such as chlorambucil or on purine analogs, such as fludarabine [Zinzani, 2003]. These therapeutic compounds are thought to induce cytotoxic response at least in part by activating the p53 pathway [Pettitt et al., 1999, 2001; Sturm et al., 2003; Rosenwald et al., 2004]. In this respect, it is noteworthy that deletions and/or mutations of the p53 gene are relatively rare (10–20%) in hematological malignancies with respect to solid tumors, and usually they become more frequent as the disease progresses [Pettitt et al., 1999, 2001; Sturm et al., 2003; Rosenwald et al., 2004]. The activation of p53 is tightly regulated by murine double minute 2 (MDM2) gene, whose expression is regulated in part by a p53-responsive promoter. In turn, MDM2, which is an E3 ubiquitin ligase for p53 and itself, controls p53 half-life via ubiquitin-dependent degradation. In response to cellular stresses, the interaction between p53 and MDM2 is disrupted and p53 undergoes post-translational

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modifications on multiple sites. In particular, phosphorylation plays an important role in the stabilization and activation of p53 [Vousden and Lu, 2002]. Recently, potent and selective small molecules, inhibitors of p53-MDM2 interaction, the Nutlins, have been discovered [Vassilev et al., 2004]. These non-genotoxic compounds bind MDM2 in the p53 binding pocket with high selectivity and can release p53 from negative control, leading to effective stabilization of p53 and activation of the p53 pathway [Vassilev et al., 2004].

The death-inducing ligand tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is a member of the TNF family of cytokine, which appears to induce apoptosis preferentially in tumor cells versus normal cells [Koschny et al., 2007]. The TRAIL-induced apoptotic response is mediated by binding of TRAIL to TRAIL-R1 and TRAIL-R2, which contain a functional death domain and transduce either apoptotic or non-apoptotic signals through the adaptor protein FADD [Zauli and Secchiero, 2006]. In contrast, TRAIL-R3 and TRAIL-R4 contain the external TRAIL-binding region, as well as a region that anchors the receptors to the membrane, but lack the intracellular tail required for interacting with FADD. Of note, a study performed in $TRAIL^{-/-}$ null mice showed that the incidence of spontaneous lymphoid malignancies was increased by 25% with respect to control $TRAIL^{+/+}$ animals [Zerafa et al., 2005], strongly suggesting that TRAIL plays a crucial role in the immune-surveillance against lymphoid malignancies.

On these bases, this study was designed to analyze the effects on cell cycle and/or apoptosis induced by the chemotherapeutic drugs chlorambucil and fludarabine in comparison to the MDM2 antagonist Nutlin-3, used alone or in combination with TRAIL. The SKW6.4 B-lymphoblastoid was used as a model system of indolent B-cell malignancies.

MATERIALS AND METHODS

Cell Cultures and Reagents

SKW6.4 lymphoblastoid cell line was purchased from American Type Culture Collection (ATCC, Manassas, VA) and cultured in RPMI-1640 containing 10% fetal bovine serum (FBS; Gibco BRL, Gaithersburg, MD). Fludarabine (fludarabine des-phosphate) and chlorambucil were purchased from Sigma-Aldrich (St. Louis, MO), Nutlin-3 was purchased from Cayman (Cayman Chemical, Ann Arbor, MI). Recombinant TRAIL was prepared as previously described [Secchiero et al., 2002; Zauli et al., 2003].

For flow cytometry analysis, anti-human TRAIL-R1, TRAIL-R2, TRAIL-R3, and TRAIL-R4 monoclonal antibodies (MoAb, all from R&D Systems, Minneapolis, MN) were used as previously described [Secchiero et al., 2004a; Zauli et al., 2004]. Release of GDF15/MIC-1 in supernatants of SKW6.4 cell cultures was measured by using a commercially available ELISA kit purchased from R&D Systems, according to the manufacturer's instructions. Results were read at an optical density of 450 nm using an Anthos 2010 ELISA reader (Anthos Labtec Instruments Ges.m.b.H, Wals Salzburg, Austria).

Viability, Apoptosis, and Cell Cycle Assays

SKW6.4 cells were seeded at a density of 1×10^6 cells/ml and either left untreated or treated with various concentrations of chlorambucil, fludarabine, Nutlin-3, or TRAIL for 24 or 48 h. Cell viability was examined by Trypan blue dye exclusion and flow cytometry. For flow cytometric analysis of apoptosis, 5×10^5 cells were double stained with annexin V-fluorescein isothiocyanate (FITC, Immunotech, Marseille, France) and propidium iodide (PI, Sigma-Aldrich) in binding buffer followed by FACS analysis using a FACScan flow cytometer (Becton-Dickinson, San Jose, CA), as described [Secchiero et al., 2001]. To avoid non-specific fluorescence from dead cells, live cells were gated tightly using forward and side scatter. Cell cycle profile was analyzed by staining 3×10^5 cells in 200 μl of PBS with BrdU (Sigma-Aldrich), followed by anti-BrdU MoAb and counterstained with PI [Milani et al., 1993].

Western Blot Analysis

Cells were lysed in buffer containing 50 mM Tris-HCl, pH 8.0, 250 mM NaCl, 0.5% NP40, 2 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 2 μg/ml of pepstatin, as described [Zauli et al., 1996; Gibellini et al., 1998]. For Western blot analysis, 50 μg of protein were resolved on 10% SDS–polyacrylamide gels and transferred to nitrocellulose membranes. Blots were incubated with the following antibodies (Abs): anti-p53, PARP, MDM2, p21, SFN/14-3-3σ (all from

Santa Cruz Biotechnology, Santa Cruz, CA), PIG3 (Exalpha Biologicals Inc., Watertown, MA) and actin (Sigma-Aldrich) as loading control. Blots were incubated with goat anti-mouse or goat anti-rabbit horseradish peroxidase-conjugated secondary Ab (Calbiochem, La Jolla, CA) and detection was performed using the ECL detection kit (Amersham Pharmacia Biotech, Buckinghamshire, UK). Densitometry values were estimated by the ImageQuant TL software (Amersham). Multiple film exposures were used to verify the linearity of the samples analyzed and to avoid saturation of the film.

cDNA Microarray

Total RNA was extracted from SKW6.4 cells by using the Qiagen RNeasy mini-kit (Qiagen, Hilden, Germany) according to the supplier's instructions. The quality of the total RNA preparation was verified by agarose gel and, when necessary, further purification was performed with the RNeasy cleanup system (Qiagen) to remove chromatin DNA.

Three microgram of total RNA were transcribed into cDNA using GEArray AmpoLabeling-LPR Kit (Superarray Bioscience Corporation, Frederick, MD). An in vitro linear polymerase reaction was then performed to generate biotinylated cDNA. Labeled cDNA was hybridized with customized cDNA microarrays, containing arrays of genes representative of several different pathways frequently altered during the progression of cancer together with housekeeping genes (the list of the genes is available at: SuperArray Bioscience Corporation. Gene list: OHS-802; http://www.superarray.com/gene_array_product/html/ohs-802.html and HS-603; http://www.superarray.com/gene_array-_product/html/hs-603.html). Hybridization was revealed by alkaline phosphatase-conjugated streptavidin, using a chemiluminescent detection kit (Superarray Bioscience Corporation). Signal intensity was measured for each microarray, the minimal intensity was used for background subtraction and the values were normalized to the median signal value for each array. Expression levels were compared in SKW6.4 cells left untreated or treated for 24 h with TRAIL (1 μg/ml), Nutlin-3, fludarabine, or chlorambucil (10 μM, each). Data were filtered for the genes whose expression levels increased or decreased by at least twofold, that is, filtering the ratio for values ≥ 2.0 or ≤ 0.5.

Assessment of the Effect of Combination Treatment

The effects of combining TRAIL with fludarabine, chlorambucil, and Nutlin-3 in SKW6.4 cells were investigated. SKW6.4 cells were treated in vitro with serial dilutions of TRAIL (range: 0.5–50 nM) or Nutlin-3 (range: 0.1–10 μM) or the active moiety of fludarabine (range: 0.1–10 μM) or chlorambucil (range: 0.1–10 μM) individually or in combination using a constant ratio (TRAIL/Nutlin-3, TRAIL/fludarabine, or TRAIL/chlorambucil) for 48 h. Results were analyzed using CalcuSyn software program (Biosoft, Cambridge, UK), which uses the method of Chou and Talalay [1984] to determine whether combined treatment yields greater effects than expected from summation alone. A combination index (CI) of 1 indicates an additive effect, a CI above 1 indicates an antagonistic effect, and a CI below 1 indicates a synergistic effect.

Statistical Analysis

The results were evaluated by using analysis of variance with subsequent comparisons by Student's t-test and with the Mann–Whitney rank-sum test. Statistical significance was defined as $P < 0.05$.

RESULTS

Surface Expression of TRAIL-R2 and TRAIL-R4 on SKW6.4 Cells and Accumulation of p53 Protein in Response to Either Chemotherapeutic Drugs or Nutlin-3

In order to analyze the differential ability of chemotherapeutic agents, Nutlin-3 and recombinant TRAIL to modulate cell cycle progression and to induce apoptosis in a relevant model of indolent B-cell malignancies, we have chosen the lymphoblastoid SKW6.4 B-cell line, taking into account of its pattern of expression of TRAIL receptors (clear-cut expression of TRAIL-R2 and TRAIL-R4, Fig. 1A) and of its p53 wild-type status. These characteristics have been previously reported for primary B-cell malignancies, such as indolent non-Hodgkin's B-cell lymphoma and B-chronic lymphocytic leukemia (B-CLL) [Secchiero et al., 2004b; Secchiero et al., 2005] and thus render SKW6.4 cells a suitable model for the purpose of this study.

In line with the p53 wild-type status, the p53 basal level in SKW6.4 cell lysates was very low

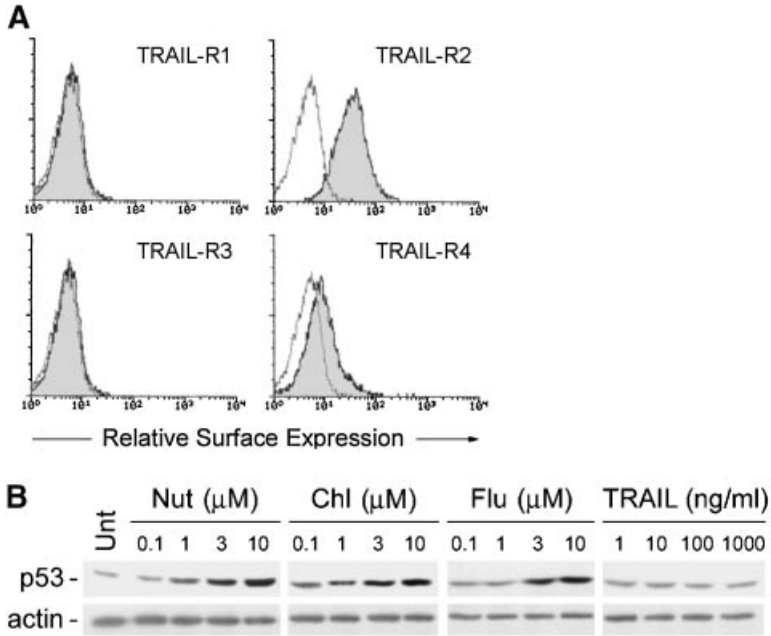

Fig. 1. Expression of TRAIL-Rs and p53 modulation in SKW6.4 cells. **A**: Surface expression of TRAIL-Rs antigens was determined in SKW6.4 B-cell line by flow cytometry using PE-conjugated anti-TRAIL-Rs monoclonal antibodies (mAbs). Non-specific staining was determined using isotype-matched irrilevant mAbs. Representative phenotypes are shown. **B**: Dose-dependent induction of p53 in SKW6.4 B-cell line. After 24 h of incubation with increasing doses of Nutlin-3, chlorambucil, fludarabine, and TRAIL, p53 protein levels were analyzed by Western blot analysis. Comparable loading of protein in each lane was confirmed by staining with the antibody to actin. Representative examples out of three independent experiments are shown.

under normal unstressed culture conditions, while exposure to the potent non-genotoxic activator of the p53 pathway Nutlin-3 induced a dose-dependent accumulation of p53 protein (Fig. 1B) after 24 h of treatment. Also the chemotherapeutic agents, chlorambucil and fludarabine, two drugs commonly used in the treatment of indolent B-cell malignancies [Zinzani, 2003], induced a comparable accumulation of p53 at equimolar concentrations after 24 h of treatment (Fig. 1B). On the other hand, recombinant TRAIL did not modulate the basal levels of p53 at any concentration examined after 24 h of treatment (Fig. 1B).

Cytotoxic Effects of Chlorambucil, Fludarabine, Nutlin-3, and TRAIL on SKW6.4 Lymphoblastoid B-Cells

In the following group of experiments, SKW6.4 cells were treated with increasing concentrations of chlorambucil, fludarabine, Nutlin-3 (range: 0.1–10 μM), and recombinant TRAIL (range: 1–1,000 ng/ml). After 24 and 48 h of culture, the total number of viable cells was scored by Trypan blue dye exclusion. As shown in Fig. 2A, cell viability showed a dose- and time-dependent decline in cultures treated with chlorambucil, fludarabine, and Nutlin-3 with a cell viability between 50 and 60% at 48 h of treatment with 10 μM of each agent. On the other hand, SKW6.4 viability never dropped under 70% at any TRAIL concentration employed, with no significant differences between 100 and 1,000 ng/ml (Fig. 2A). Moreover, while the cytotoxicity of both chemotherapeutic agents and Nutlin-3 increased progressively with the culture time (Fig. 2A,B), TRAIL cytotoxicity was already maximal after 24 h and it showed a plateau thereafter (Fig. 2A,B).

Effects of Chlorambucil, Fludarabine, Nutlin-3, and TRAIL on Cell Cycle and Apoptosis in SKW6.4 Lymphoblastoid B-Cells

Activated p53 induces both cell cycle arrest at the G1/S and G2/M borders and apoptosis [Vogelstein et al., 2000]. In order to disclose the events underlining the decrease of cell viability induced by chlorambucil, fludarabine, Nutlin-3 and TRAIL (Fig. 2A,B), we next analyzed by BrdU/PI staining and flow cytometry analysis the cell cycle profile as well as the levels of apoptosis in SKW6.4 cells after 24 h of each treatment. As shown in Fig. 3A, TRAIL did not substantially change the cell cycle profile of

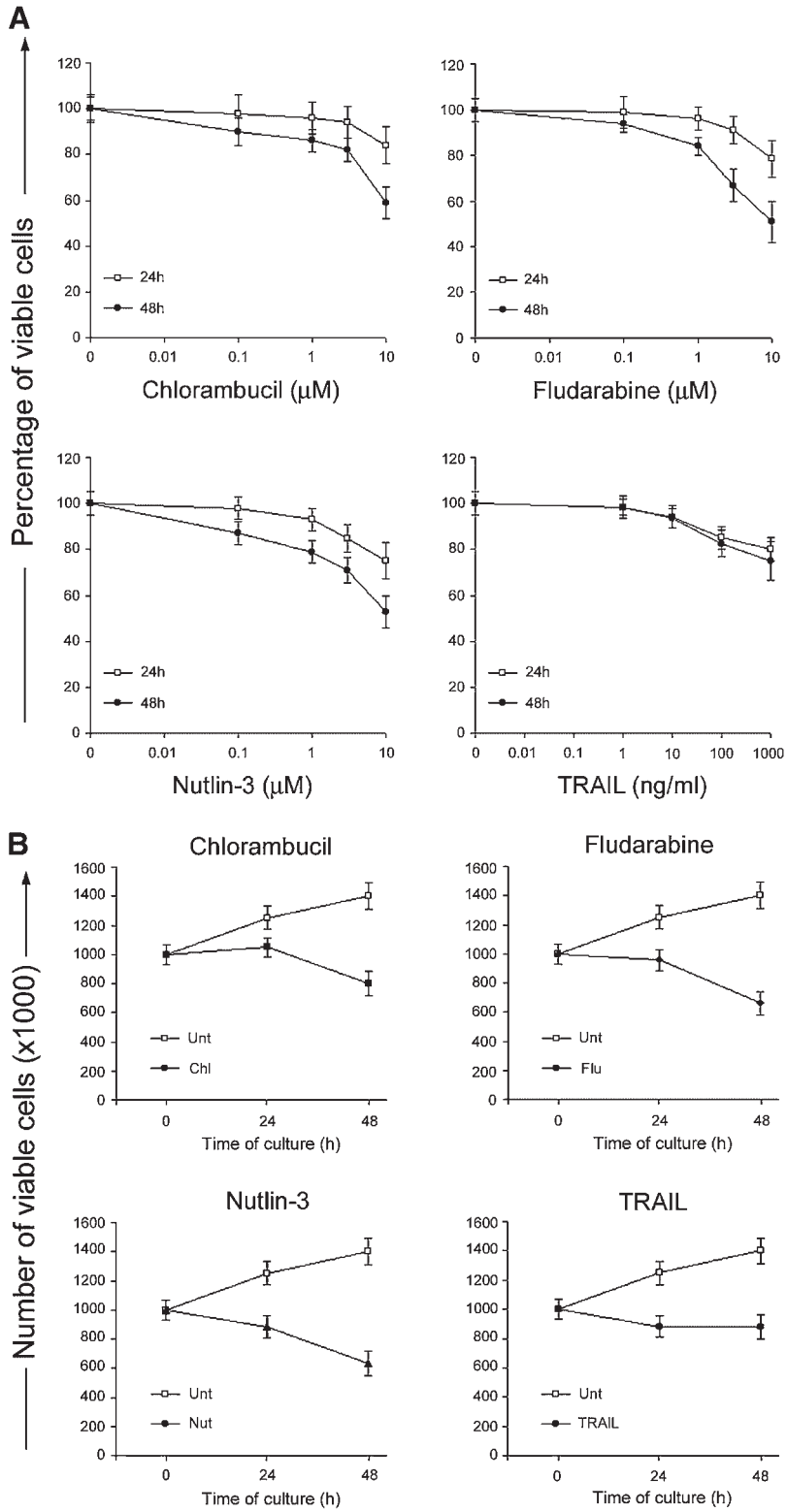

Fig. 2. Effect of Nutlin-3, chlorambucil, fludarabine and TRAIL on the viability of SKW6.4 cells. SKW6.4 B-cells were incubated with different concentrations of Nutlin-3, chlorambucil, fludarabine, and TRAIL and analyzed for cell viability by Trypan blue dye exclusion. **A**: Dose-dependent effect on cell viability was assessed at different time points by scoring the total number of viable cells. **B**: Cells were treated with Nutlin-3, chlorambucil, fludarabine (each at 10 μM), and TRAIL (1 μg/ml). Time-dependent effect was assessed by scoring the total number of viable cells after 24 and 48 h of treatment. A,B: Data are expressed as means + SD of three independent experiments each performed in triplicate.

SKW6.4 cells with respect to untreated control. On the other hand, both Nutlin-3 and chemotherapeutic drugs profoundly affected cell cycle progression. In particular, Nutlin-3 induced a significant ($P < 0.05$) and progressive accumulation of cells in the G1 phase of the cell cycle, while chlorambucil showed a marked ($P < 0.05$) accumulation of cells in the G2 phase of the cell cycle (Fig. 3A,B). Otherwise, fludarabine showed an intermediate behavior (Fig. 3A,B).

In parallel, all the agonists were investigated for their ability to induce apoptotic cell death of SKW6.4 cells by annexin V/PI double staining (Fig. 3C) as well as by analyzing PARP cleavage (Fig. 3D). Recombinant TRAIL significantly ($P < 0.05$) increased apoptosis already after 24 h of culture without inducing further increase at 48 h (Fig. 3C,D). On the contrary, chemotherapeutic drugs and Nutlin-3 induced a significant increase of apoptosis with a delayed kinetics, starting to be significant ($P < 0.05$) only from 48 h of culture onwards (Fig. 3C,D).

Taken together with the data illustrated above, these results indicate that chlorambucil, fludarabine, and Nutlin-3 reduced the total number of viable SKW6.4 cells (Fig. 2A,B) by a combination of cell cycle arrest (Fig. 3A,B) and apoptosis (Fig. 3C,D), while recombinant TRAIL only induced apoptosis of SKW6.4 cells without affecting the cell cycle profile.

Effects of Chlorambucil, Fludarabine, and Nutlin-3 in Combination With TRAIL on SKW6.4 Cells

To investigate the molecular bases underlining the ability of chemotherapeutic drugs

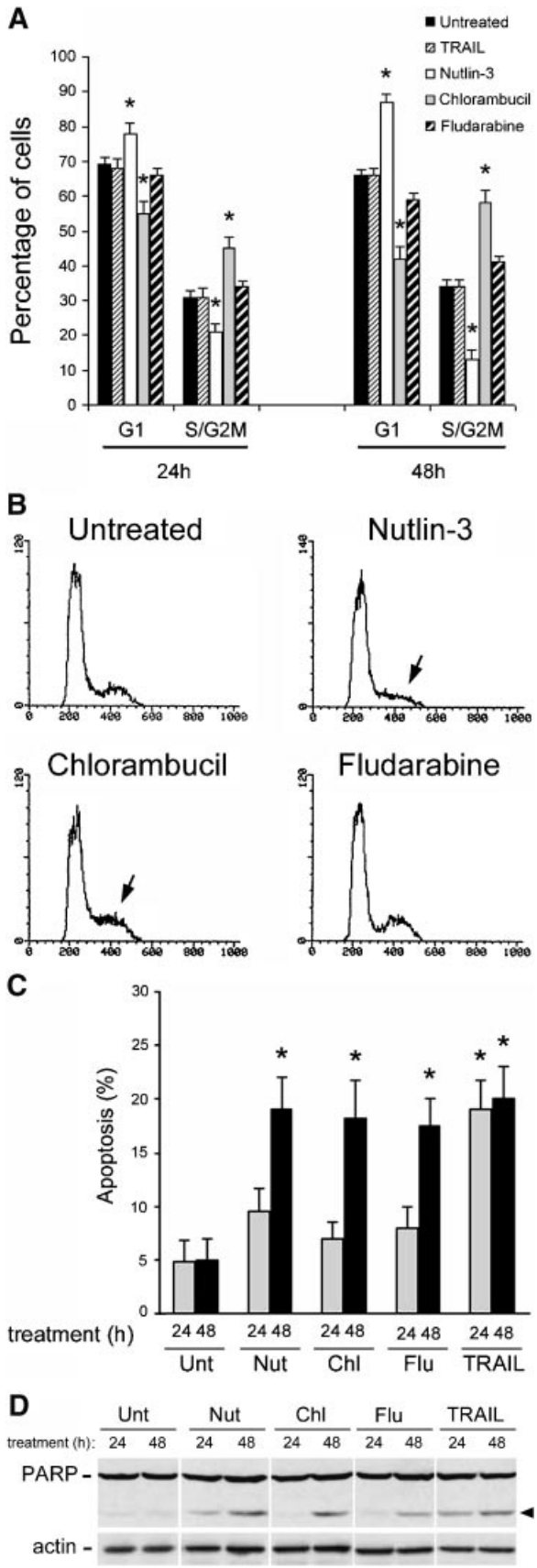

Fig. 3. Effect of Nutlin-3, chlorambucil, fludarabine, and TRAIL on cell cycle profile and apoptosis in SKW6.4 cells. SKW6.4 cells were treated with Nutlin-3, chlorambucil, fludarabine (10 μM each), and TRAIL (1 μg/ml). After 24 and 48 h of treatments, the cells were analyzed for the cell cycle profile and apoptosis. **A**: Cell cycle profile was assessed by BrdU/PI staining and flow cytometry. Cell-cycle distribution was calculated as means + SD from at least four independent experiments. Asterisks: (*) significance at $P < 0.05$. **B**: SKW6.4 cells were treated as indicated and then stained with PI before the analysis by flow cytometry. Representative panels of the cell cycle profile are shown. Arrows indicate cells in S/G2M phase. **C**, **D**: The induction of apoptosis was analyzed by flow cytometry, after staining with annexin V/PI, and by Western blot on cell lysates. In C, data are expressed as means + SD of three independent experiments each performed in triplicate. Asterisks: (*) significance at $P < 0.05$. In D, representative examples of Western blot analysis of PARP in cell lysates obtained from SKW6.4 cultures, treated as indicated, are shown. The proform of PARP (115 kDa) and the cleaved form (80 kDa; arrowhead) are shown. C, D: Unt. = untreated cells.

and Nutlin-3 to affect the cell cycle progression and to induce apoptosis of SKW6.4 cells, we have compared the modulation of the steady-state mRNA levels of several genes involved in the p53 pathway [El-Deiry, 1998]. Interestingly, chlorambucil, fludarabine, and Nutlin-3 showed a wide overlapping in their ability to upregulate the steady-state mRNA levels of p53 targets CDKN1A/p21, GDF15, PIG3, and TRAIL-R2 (Fig. 4). It should be noticed, however, that Nutlin-3 was more potent than fludarabine and chlorambucil in upregulating the mRNA levels of these genes, and was the only agent able to markedly induce MDM2 (Fig. 4A). Of note, chlorambucil showed the distinct feature to significantly upregulate SFN/14-3-3σ (Fig. 4A), a gene that has been previously involved in blocking the cell cycle progression at G2/M border [Hermeking et al., 1997]. On the other hand, TRAIL alone did not affect the levels of p53 and of its transcriptional targets (data not shown), as previously described in a fibroblast cell model [Yang et al., 2005]. These differences in terms of activation of p53-target genes likely account for the differential effect of Nutlin-3 versus chemotherapeutic drugs in cell cycle-induced modifications. To validate the mRNA data at the protein level, CDKN1A/p21, PIG3 and MDM2 and SFN/14-3-3σ, were analyzed by Western blot (Fig. 4B) and GDF15 was measured by ELISA assay (Fig. 4C). Among the different genes activated by chemotherapeutic agents and Nutlin-3, the upregulation of TRAIL-R2 mRNA by all agonists,

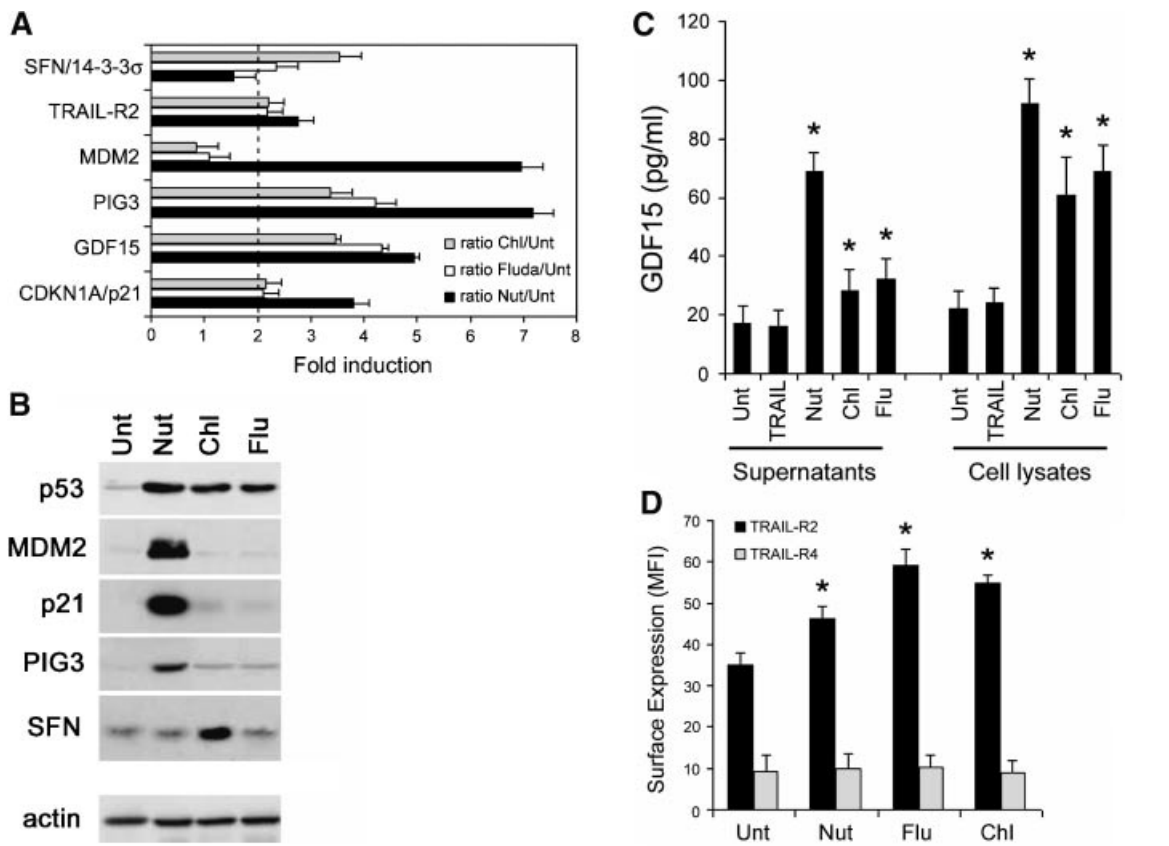

Fig. 4. p53-dependent target gene activation in response to Nutlin-3, chlorambucil, and fludarabine. SKW6.4 B-cell line was treated with Nutlin-3, chlorambucil, and fludarabine (10 μM each). After 24 h of treatment, the cells were analyzed for p53-related gene expression. **A**: Gene expression profile for molecules related to p53 target genes was assessed by cDNA microarray analysis. Ratios represent Nutlin-3, chlorambucil, or fludarabine values divided by untreated values. The genes significantly up-modulated by the treatments (above the cut-off of twofold of induction) are shown. Data are expressed as means + SD of three independent experiments each performed in triplicate. **B**: The mRNA data were validated by Western blot analysis. A representative of four experiments is shown. **C**: The activation of GDF15 p53-target gene was assessed by ELISA on culture supernatants and cell lysates. Data are expressed as means + SD of results from three-to-five independent experiments each performed in duplicate. $^*P < 0.05$. **D**: Surface expression of TRAIL-R2 and TRAIL-R4 was analyzed by flow cytometry. Results are reported as mean fluorescence intensity (MFI) and are expressed as means + SD of three independent analyses. Asterisk (*) indicates significance at $P < 0.05$. C, D: Unt. = untreated cells.

confirmed by flow cytometry analysis (Fig. 4D), was particularly noteworthy for the purpose of this study. Indeed, on the basis of these observations, we next analyzed the effect of combined treatment of chlorambucil, fludarabine, or Nutlin-3 with TRAIL in inducing cytotoxicity of SKW6.4 cells. Both chlorambucil and fludarabine significantly enhanced the TRAIL-mediated cytotoxicity on SKW6.4 cells ($CI < 1$) (Table I). Remarkably, also the non-genotoxic agent Nutlin-3 showed a synergistic cytotoxic activity when combined to TRAIL ($CI < 1$) (Table I).

DISCUSSION

Indolent B-cell neoplasia are the most common type of hematological malignancies in the western world, accounting for more than 25% of all hematological malignancies [Rozman and Montserrat, 1995]. Treatment of indolent B-cell malignancies is reserved for symptomatic patients or patients in advanced clinical stage. Despite improvements in response rates using chemoimmunotherapy combinations, CLL remains incurable, and patients refractory to fludarabine or patients that have suffered multiple disease relapses have a poor prognosis [Byrd et al., 2005; Keating et al., 2005; Wierda et al., 2005]. Thus, continued pre-clinical studies on innovative therapeutic strategies are warranted. The identification of new agents that suppress the survival of indolent B-cell neoplasia by interfering with their cell cycle progression and/or promoting apoptosis is one critical approach to improving therapeutic outcome. In this respect, it has been already established that the overall cytotoxic response to recombinant TRAIL used alone is variable but usually not satisfactory in indolent B-cell neoplasia [Olsson et al., 2001; MacFarlane et al., 2002, 2005; Johnston et al., 2003; Secchiero et al., 2004b, 2005]. Similarly to the trend observed in primary B-cell neoplasia, also the SKW6.4 lymphoblastoid cell line used in this study showed a low-moderate cytotoxic response when treated with recombinant TRAIL alone, in agreement with the findings of previous authors obtained on this cell line [Luciano et al., 2002; Guo et al., 2004]. On the other hand, a number of recent studies have shown that Nutlin-3 significantly induces in vitro apoptotic cell death in multiple myeloma and B-CLL cells, harboring a p53 wild-type phenotype [Stuhmer et al., 2005; Coll-Mulet et al., 2006; Kojima et al., 2006; Secchiero et al., 2006], suggesting that Nutlin-3 might be of potential therapeutic value in B-cell malignancies.

In this study, we demonstrated that chlorambucil, fludarabine, and Nutlin-3 were all able to induce the accumulation of p53 in the SKW6.4 cell line, used as a model system of indolent B-cell malignancies for its p53 wild-type status. However, the potency of p53 induction, evaluated in terms of folds induction of p53 transcriptional targets and effective concentration $(EC)_{50}$ cytotoxicity was Nutlin-3 > chlorambucil > fludarabine. It is also noteworthy that while Nutlin-3 induced a progressive accumulation of the cells in G1, chlorambucil blocked SKW6.4 cells in G2/M, and fludarabine showed an intermediate behavior. These remarkable differences in cell cycle block correlated with the ability of chlorambucil and to a lesser extent of fludarabine, but not Nutlin-3, to upregulate the expression level of SFN/14-3-3σ, a gene previously involved in blocking the cell cycle progression in G2/M [Hermeking et al., 1997].

In spite of these differences in inducing cell cycle arrest, the combination of either chemotherapeutic agonists or Nutlin-3 with recombinant TRAIL resulted in synergistic cytotoxic effects on SKW6.4 cell line. Previous studies have reported conflicting results on the preferential sensitization to TRAIL cytotoxicity of

TABLE I. Combination Index Values for Effects of TRAIL Plus Nutlin-3, Chlorambucil, or Fludarabine on Cell Viability

Cells	ED50	ED75	ED90	Averaged CI*
Nut + TRAIL (24 h)	0.34	0.29	0.24	0.29
Nut + TRAIL (48 h)	0.42	0.17	0.07	0.22
Flu + TRAIL (24 h)	0.39	0.25	0.16	0.27
Flu + TRAIL (48 h)	0.29	0.28	0.28	0.28
Chl + TRAIL (24 h)	0.07	0.25	0.90	0.41
Chl + TRAIL (48 h)	0.05	0.20	0.78	0.34

*The averaged combination index (CI) values were calculated from the ED50, ED75, and ED90.

malignant cells blocked in G1/S or G2/M [Jin et al., 2002; Liu et al., 2003; Ray et al., 2007]. In the SKW6.4 cells, no striking correlations between the ability to block in G1/S or G2/M phases of the cell cycle and sensitization to apoptosis were noticed. A possible molecular explanation for the synergistic activity of chemotherapeutic drugs or Nutlin-3 in combination with TRAIL is the ability of all these agonists to upregulate the expression of TRAIL-R2. However, a recent study proposed that engagement of TRAIL-R2 by recombinant TRAIL is unable to induce apoptotic cell death in B-CLL cells [MacFarlane et al., 2005]. This does not exclude the possibility that the levels of surface TRAIL-R2 in freshly isolated B-CLL cells are insufficient to mediate the apoptotic signal by TRAIL while greater surface levels of TRAIL-R2, induced by chemotherapeutic agents or Nutlin-3, are able to transduce the cytotoxic effect of TRAIL. In this respect, although we have not firmly established whether the upregulation of TRAIL-R2 observed in SKW6.4 lymphoblastoid B-cells in response to chemotherapeutic drugs or Nutlin-3 is due to p53 upregulation, it is noteworthy that the p53-dependent transcriptional induction of TRAIL-R2 is restricted to cells undergoing apoptosis and not cells undergoing exclusively p53-dependent G1 arrest [Wu et al., 1999; Burns et al., 2001].

Although the elucidation of the molecular mechanism(s) mediating the cytotoxic effect of chemotherapeutic drugs or Nutlin-3 in combination with TRAIL on the SKW6.4 lymphoblastoid cell line deserves further investigation, the potential clinical relevance of our findings should be underlined. In fact, current treatment regimens for indolent B-cell malignancies employ alkylating agents, purine analogs, monoclonal antibodies, or combinations thereof [Zinzani, 2003]. Such combination therapies result in greater response rates than those seen with single agent-based therapy alone, but none of these therapies is curative. In fact, there is now increasing evidence that drug combinations targeting different pathways simultaneously are a better approach for cancer therapy [Zimmerman et al., 2007]. In this respect, the data illustrated in Table I strongly suggest that a sequential treatment of malignant B-cells with chemotherapeutic drugs or Nutlin-3 followed by recombinant TRAIL might represent an important therapeutic option. The ability of Nutlin-3 to sensitize SKW6.4 lymphoblastoid cells to TRAIL cytotoxicity is particularly promising. In fact, when considering the potential therapeutic application of the combination of chemotherapeutic agents plus recombinant TRAIL in the treatment of indolent B-cell neoplasia, it should be emphasized that the therapeutic activity of any of the currently used cytotoxic drugs for that subset of patients requiring treatment is a trade-off between efficacy and adverse effects, namely unspecific genotoxic collateral damage, which may induce genetic instability and selection of drug-resistant tumor subclones. Thus, the ability of the non-genotoxic small molecule Nutlin-3 to synergize with TRAIL is particularly remarkable since it should avoid the side effects of chemotherapy.

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