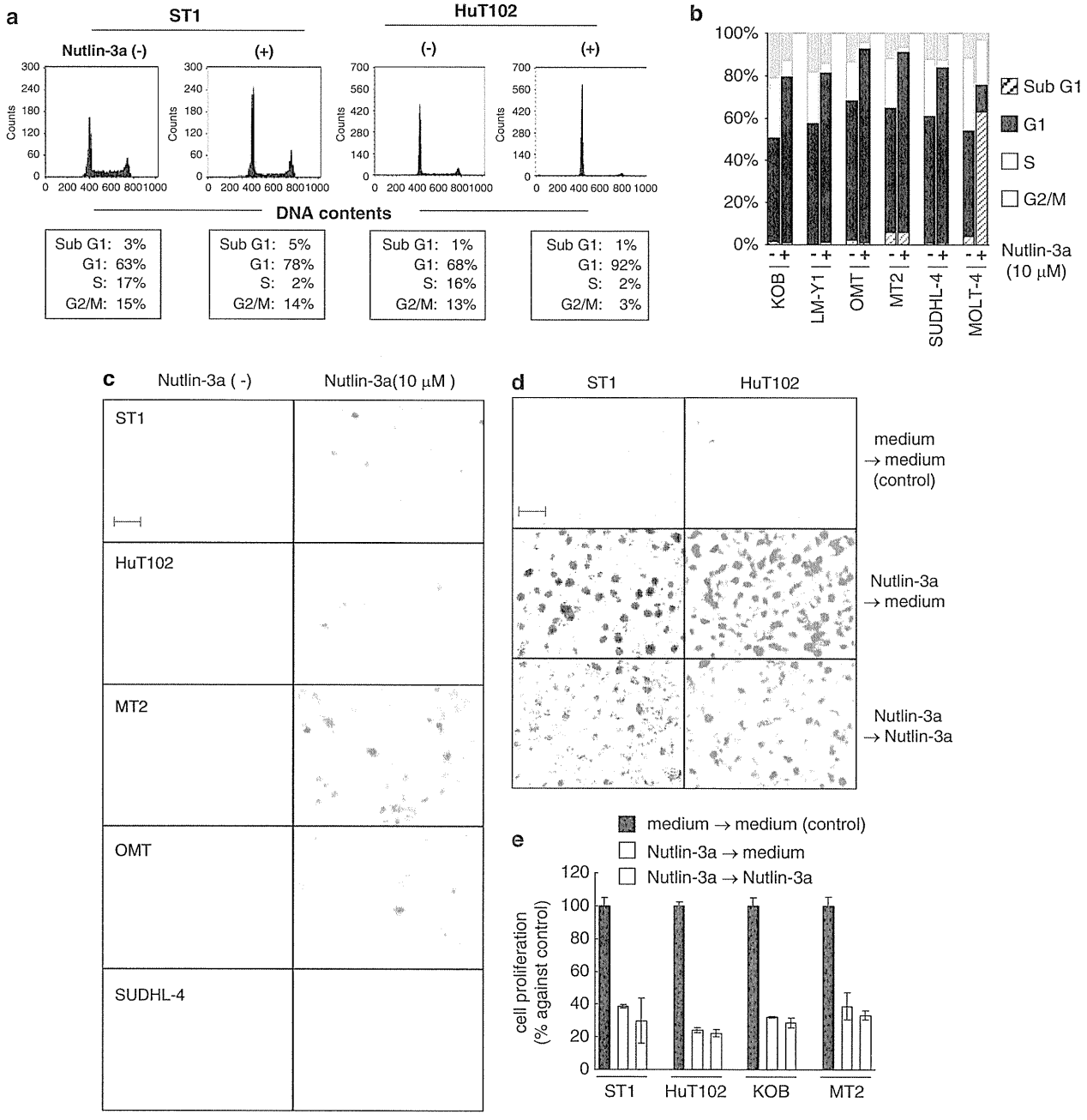


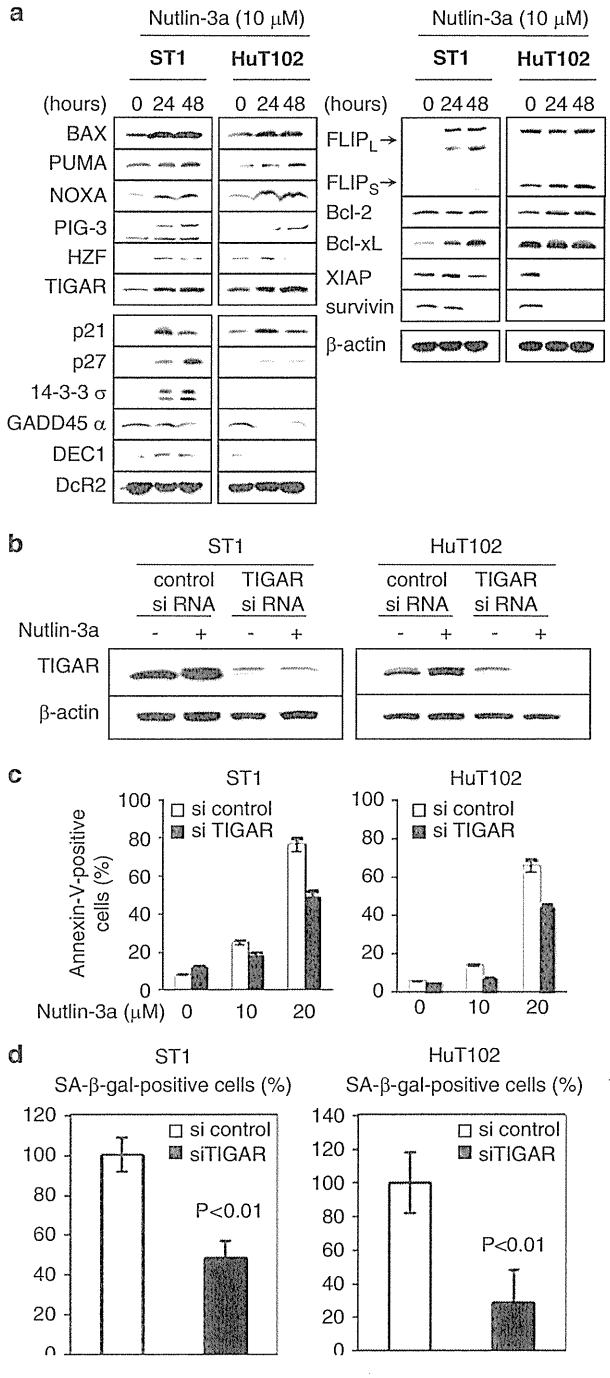
Figure 4 Nutlin-3a induces cellular senescence followed by cell-cycle arrest. (**a**, **b**) Cells were treated with or without the indicated concentrations of Nutlin-3a for 24 h. After cells were harvested, cell-cycle analysis was performed using flow cytometer. (**c**) Similarly, after 72 h, cells were harvested and senescence-associated-β-galactosidase (SA-β-gal) staining was performed and examined microscopically. Bar: 20 μm. (**d, e**) Washout experiments. p53 wild-type cell lines were cultured with Nutlin-3a for 72 h to induce cellular senescence. Then, cells were washed and incubated with or without Nutlin-3a for another 48 h. SA-β-gal staining (**d**) and MTS assay were performed (**e**).

induce cellular senescence. Then, cells were harvested, washed and incubated with or without Nutlin-3a for another 48 h. Both Nulin-3a re-treated cells and washed-out cells continuously showed G1 cell-cycle arrest and were persistently positive for SA-β-gal staining (Figure 4d and data not shown). In addition, cell proliferation assay revealed that 'senescent' cells were not proliferated even after washout of Nutlin-3a (Figure 4e). These results indicate that Nutlin-3a-induced cellular senescence, as a result of continuous growth arrest, is an irreversible change.

Analysis of molecules involved in p53-dependent cellular senescence

Little is known about the key molecules or markers of cellular senescence. To explore such molecules, we performed western blot analysis comparing the cellular senescence setting with the apoptotic setting (Figures 5a and 3c). As expected, p21 expressions increased in the senescence setting (Figure 5a) but rather decreased in the rapid apoptotic setting (Figure 3c). Most of the typical effectors of apoptosis, BAX, PUMA and NOXA,

tended to increase and antiapoptotic factors, such as XIAP and survivin, decreased even when cells were induced to cell-cycle arrest and senescence (Figure 5a). The expressions of p27, 14-3-3σ and $FLIP_L$ were increased in ST1 cells but not in HuT102 cells (Figure 5a). It should be noted that PIG3 and TIGAR were increased in a time-dependent manner in both ST1 and HuT102 cells (Figure 5a), which was not observed in the apoptotic setting (Figure 3b). Collectively, these results suggest that the activations of p21, PIG3 and TIGAR are important in the induction of cellular senescence. Although we expected that Hzf would have a key role in determining either progression to cell-cycle arrest with senescence or apoptosis, we did not find any significant differences between the two settings.



Novel evidence that TIGAR has a role in cellular senescence

Previous studies reported that PIG3 has a critical role in p53-dependent apoptosis and that p21 can contribute to both cellular senescence and cell-cycle arrest.[2,4] We first performed a knockdown experiment of p21 with siRNA in ST1 cells. When control-siRNA cells and p21-siRNA cells were treated with Nutlin-3a, a 56% reduction of SA-β-gal-positive cells was observed in p21-siRNA cells when compared with control-siRNA cells (data not shown). Next, we focused on TIGAR and performed siRNA experiments using ST1 and HuT102 cells because the study of TIGAR has started only recently and its role in p53-depedent cell death remains unclear.[6] We confirmed that control-siRNA cells showed upregulated TIGAR expression after Nutlin-3a treatment, which was effectively suppressed by TIGAR-siRNA (Figure 5b). Although TIGAR is reported to be an inhibitor of apoptosis, si-TIGAR did not enhance apoptosis but rather reduced Annexin-V-positive cells (Figure 5c). In a cellular senescence setting, the proportion of SA-β-gal-positive cells was significantly reduced in TIGAR-siRNA cells (reduced to 48 and 29% in ST1 cells and HuT102 cells respectively) (Figure 5d). Similar results were also obtained in KOB cells (data not shown). These results suggest that TIGAR has an important role in the induction of cellular senescence.

Synergistic effect of Nutlin-3a and TRAIL

As some death receptors (DRs) are known as downstream genes of p53, we analyzed the cell-surface expression of DRs using flow cytometer.[4] As a result, most p53 wild-type cell lines showed the upregulation of Fas (DR for Fas-ligand) and DR5 (DR for TRAIL) expressions after Nutlin-3a treatment (Figure 6a). Our previous studies showed that although ATL cells are resistant to TRAIL, upregulation of DR5 expression can overcome TRAIL resistance.[26,43] In fact, ST1 and KOB cells were resistant to TRAIL and the half-maximal inhibitory concentration of each cell line was very high, 13.5 and 17.1 μg/ml, respectively. It should be noted that the combination of Nutlin-3a and TRAIL significantly decreased cell proliferation, and the synergistic effects were confirmed by isobolographic analysis (Figure 6b). As shown in Figure 6c, although the half maximal inhibitory concentration of ST1 and KOB cells for Nutlin-3a was 6.85 and 11.35 μM, respectively, a combination of 2.5 μM Nutlin-3a and only 0.6 μg/ml TRAIL markedly increased the proportion of

Figure 5 Analysis of molecules in Nutlin-3a-induced cellular senescence. (**a**) Cells were treated with 10 μM Nutlin-3a for the indicated period and western blot analysis was performed using the same antibodies as in Figure 3c. (**b**) Effects of Tp53-induced glycolysis and apoptosis regulator/small interfering RNA (TIGAR-siRNA). At 24 h after transfection, cells were incubated for 24 h with or without 10 μM Nutlin-3a and western blot analysis was performed. (**c**) Twenty-four hours after transfection, cells were incubated for 24 h with or without 20 μM Nutlin-3a, and Annexin-V/propidium iodide (PI) staining was performed using flow cytometer (FCM). Experiments were performed in triplicate, Annexin-V-positive cells were counted and results are expressed as mean ± s.d. (**d**) Twenty-four hours after transfection, cells were incubated for 72 h with or without 5–10 μM Nutlin-3a and senescence-associated-β-galactosidase (SA-β-gal) staining was performed. SA-β-gal-positive cells among 500 cells were counted both in si-control cells and si-TIGAR cells and the percentage against si-control cells was calculated. Results are expressed as mean ± s.d. of three independent studies and were also analyzed using Student's *t*-test.

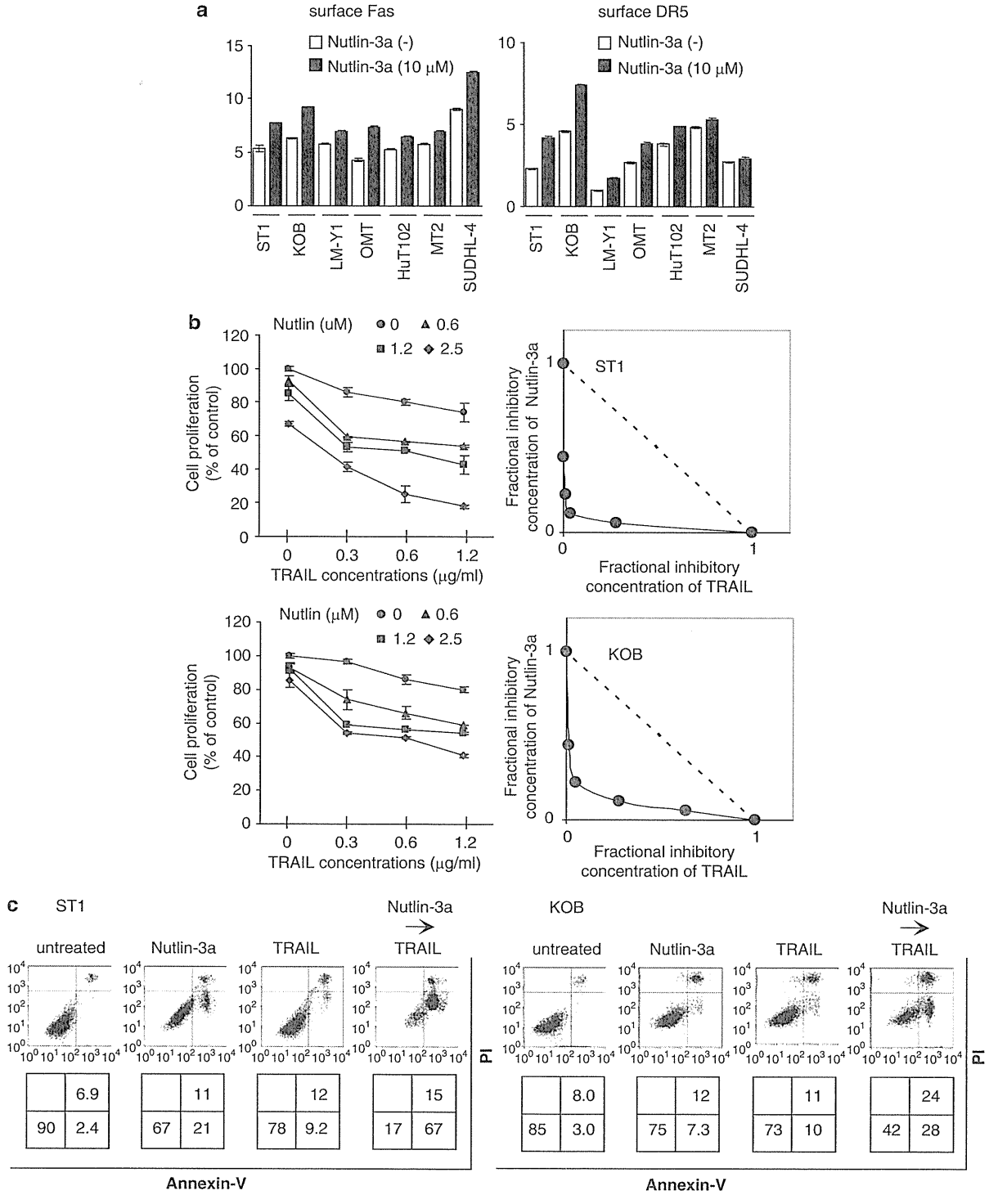


Figure 6 Combination of Nutlin-3a and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced synergistic effects. (**a**) Cells were treated with or without the indicated concentrations of Nutlin-3a. Cell-surface expression of death receptors was examined using flow cytometer (FCM) and evaluated using relative fluorescence intensity (RFI; ratio of mean fluorescence intensity for specific staining to that for control staining) and the results are expressed as mean ± s.d. (**b**) ST1 and KOB cells were treated with the indicated concentration of Nutlin-3a for 24 h, TRAIL was then added at the indicated concentration and cell proliferation (percentage against control cells) was evaluated after another 24 h by MTS assay. All experiments were performed in triplicate and the results are expressed as mean ± s.d. In isobolographic analysis, fractional inhibitory concentrations were determined using the half-maximal inhibitory concentration of either agent alone or in combination. Sums of 1 indicate synergy, additivity and antagonism respectively. Four experimental points were found to be significantly below the theoretical additive line (dotted line), indicating a synergistic effect. (**c**) Combination of 2.5 μM Nutlin-3a and 0.6 μg/ml TRAIL. Annexin-V/propidium iodide (PI) staining was performed and the percentages of intact cells and early and late apoptotic cells are indicated in the lower panels.

Annexin-V-positive cells compared with cells treated with either agent alone (from 32.0 and 21.2 to 82% in ST1 cells and from 9.0 and 19.3 to 52% in KOB cells). These results indicate that, with the combined use of TRAIL, the dose of Nutlin-3a could be successfully reduced.

Discussion

Cellular senescence is emerging as an important *in vivo* anticancer response elicited by multiple stresses. Recent reports have shown that cancers in mice can be eliminated through the activation of a single gene *p53* and that cellular senescence is a primary mechanism of tumor regression.[44–46] Nutlin-3a was reported to induce apoptosis in various cancer cells with functional p53; however, the induction of cellular senescence in leukemia cells has not been reported.[41,42,47,48] In this study, we showed for the first time that Nutlin-3a induces cellular senescence in a number of ATL-related cell lines with wild-type p53.

Although the relative importance of *p16*, *p14* and *p53* in cellular senescence is still unclear and the mechanism is thought to be different in humans versus mice or by cell type, there is evidence to suggest that p14ARF is closely associated with the induction of cellular senescence in mice.[10,11,49] Consistent with this scenario, cells without both *p16* and *p14* have not been shown to undergo cellular senescence, as far as we know. In this study, however, although most ATL-related cell lines examined lacked both *p16* and *p14*, Nutlin-3a did induce cellular senescence in these cells, suggesting that p53-dependent cellular senescence was induced without the participation of *p16* and *p14*. Markers of cellular senescence may be useful as diagnostic or prognostic tools and may help to monitor treatment response. In addition to SA-β-gal, *p16* and *p14*, Collado *et al.* identified *de novo* markers of cellular senescence using oncogene-induced senescent cells.[50] These markers were $p15^{INK4b}$ BHLHB2 (Dec1) and TNFRSF10D (DcR2). In this study we did not find any change in the level of protein expression of Dec1 and DcR2 in cells that underwent cellular senescence. Instead, we found apparent upregulation of p27, 14-3-3σ and $FLIP_L$ in ST1 cells and these changes were not observed in apoptotic cells. More important, the expression of TIGAR was increased in both ST1 and HuT102 cells during cellular senescence. In addition, using TIGAR-siRNA, we showed that TIGAR has an important role in the induction of cellular senescence. Our results suggest that TIGAR is a novel maker of cellular senescence. In this aspect, Bensaad *et al.* proposed that TIGAR might modulate the apoptotic response, allowing cells to survive following stress signals.[6] As the inhibition of apoptosis is one of the important processes of cellular senescence, knockdown of TIGAR may result in the inhibition of cellular senescence. Another important point is that all cells that underwent senescence were ATL-related cell lines. ATL cells are known to be resistant to various apoptotic signals, the mechanism of which may lead ATL cells toward cellular senescence. A previous report pointed out that an antiviral drug, zidovudine, can activate p53 in ATL cells and that cellular senescence contributes to zidovudine-induced cell death.[51] Supporting their theory, our study clearly indicated that cellular senescence is an important pathway of p53-dependent cell death in ATL cells.

Although it has been thought that the p53 pathway is disturbed in ATL cells by HTLV-1 Tax,[15,21] other chemicals alternatively activate p53 through inhibition of the nuclear factor κB or phosphatidylinositol 3-OH kinase (PI3K)/AKT pathway and induce apparent cell-cycle arrest and/or apoptosis.[52–54] In our study Nutlin-3a caused rapid apoptosis in a number of ATL-related cell lines with wild-type p53, and typical targets of p53, such as BAX, NOXA, PUMA, DR5 and survivin, actually responded to Nutlin-3a treatment. These results indicate that Nutlin-3a can overcome Tax-induced p53 impairment even in cells with high Tax expression. Meanwhile, a recent report showed that Tax binds the anaphase promoting complex, stabilizes the expression of p21 and p27 independently of p53 and induces rapid senescence.[55] It was suggested that evading senescence through a loss of p27 is critical for cell transformation and the development of ATL. This scenario, an early event in the development of ATL, might not be reflected by the ATL-related cell lines used in this study; however, upregulation of p27 in ST1 cells when they underwent senescence is quite suggestive.

Previous studies have shown that the use of Nutlin-3a in combination with genotoxic drugs is more effective in leukemia cells than each agent alone.[41,42] On the other hand, a recent report pointed out that rapid MDM2 reduction or profound p53 activation in mice results in an unfavorable outcome.[56] In our study, TRAIL successfully reduced the dose of Nutlin-3a and showed synergism. TRAIL-related drugs (soluble-TRAIL or antibodies to DR4 or DR5) are now in clinical trials and combination therapy with other antineoplastic agents is now becoming important.[57,58] As p53-induced proapoptotic molecules, including DR5, were upregulated and antiapoptotic molecules were decreased by Nutlin-3a treatment, the combinatory use of TRAIL-related drugs may be one of the most rational choices for Nutlin-3a-based cancer therapy.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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