6. Concin N, Zeillinger C, Tong D, *et al.* Comparison of *p53* mutational status with mRNA and protein expression in a panel of 24 human breast carcinoma cell lines. *Breast Cancer Res Treat* 2003;**79**:37–46.
7. Lynch LC, Milner J. Loss of one *p53* allele results in four-fold reduction of *p53* mRNA and protein: a basis for *p53* haplo-insufficiency. *Oncogene* 2006;**25**:3463–70.
8. Russo A, Migliavacca M, Zanna I, *et al.* *p53* Mutations in L3-loop zinc-binding domain, DNA-ploidy, and S phase fraction are independent prognosis indicators in colorectal cancer: a prospective study with a five-year follow-up. *Cancer Epidemiol Biomarkers Prev* 2002;**11**:1322–31.
9. Anensen N, Haaland I, D'Santos C, Van Belle W, Gjertsen BT. Proteomics of p53 in diagnostics and therapy of acute myeloid leukemia. *Curr Pharm Biotechnol* 2006;**7**:199–207.
10. Renneville A, Roumier C, Biggio V, Nibourel O, Boissel N, Fenaux P, Preudhomme C. Cooperating gene mutations in acute myeloid leukemia: a review of the literature. *Leukemia* 2008;**22**:915–31.
11. Tawara M, Hogerzeil SJ, Yamada Y, *et al.* Impact of *p53* aberration on the progression of adult T-cell leukemia/lymphoma. *Cancer Lett* 2006;**234**:249–55.
12. Vassilev LT, Vu BT, Graves B, *et al.* In vivo activation of the p53 pathway b small-molecule antagonists of MDM2. *Science* 2004;**303**:844–8.
13. Yamada Y, Sugawara K, Hata T, *et al.* Interleukin-15 (IL-15) can replace the IL-2 signal in IL-2-dependent adult T-cell leukemia (ATL) cell lines: expression of IL-15 receptor alpha on ATL cells. *Blood* 1998;**91**:4265–72.
14. Untergasser A, Nijveen H, Rao X, Bisseling T, Geurts R, Leunissen LKM. Primer3Plus, an enhanced web interface to Primer3. *Nucleic Acids Res* 2007;**35**:71–74. web service issue, doi:10.1093/nar/gkm306
15. Hasegawa H, Yamada Y, Komiyama K, *et al.* Dihydroflavonol BB-1, an extract of natural plant Blumea balsamifera, abrogates TRAIL resistance in leukemia cells. *Blood* 2006;**107**:679–88.
16. Wada M, Bartram CR, Nakamura H, *et al.* Analysis of *p53* mutations in a large series of lymphoid hematologic malignancies of childhood. *Blood* 1993;**82**:3163–9.
17. Hollstein M, Rice K, Greenblatt MS, Soussi T, Fuchs R, Sorlie T, Hovig E, Smith-Sorensen B, Montesano B, Harris CC. Database of p53 gene somatic mutations in human tumors and cell lines. *Nucleic Acids Res* 1994;**22**:3551–5.
18. Prokocimer M, Unger R, Rennert HS, Rotter V, Rennert G. Pooled analysis of *p53* mutations in hematological malignancies. *Hum Mutat* 1998;**12**:4–18.
19. Nelson HH, Wilkojmen M, Marsit CJ, Kelsey KT. TP53 mutation, allelism and survival in non-small cell lung cancer. *Carcinogenesis* 2005;**26**:1770–3.
20. Yatabe Y, Konishi H, Mitsudome T, Nakamura S, Takahashi T. Topographical distributions of allelic loss in individual non-small cell lung cancers. *Am J Pathol* 2000;**157**:985–93.
21. Baumbusch LO, Myhre S, Langerod A, Bergamaschi A, Geisler SB, Lonning PE, Deppert W, Dornreiter I, Borresen-Dale AL. Expression of full-length p53 and its isoform delta-p53 in breast carcinomas in relation to mutation status and clinical parameters. *Mol Cancer* 2006;**5**:47 http://www.molecular-cancer.com/content/5/1/47.
22. Yins Y, Charles W, Stephenst M, Lucianis G, Fahraeuss R. *p53* stability and activity is regulated by Mdm2-mediated induction of alternative p53 translation products. *Nat Cell Biol* 2002;**4**:462–7.
23. Mills AA. *p53*: link to the past, bridge to the future. *Genes Dev* 2005;**19**:2091–9.
24. Walker DR, Bond JP, Tarone RE, Harris CC, Makalowski W, Boguski MS, Greenblatt MS. Evolutionary conservation and somatic mutation hotspot maps of *p53*: correlation with p53 protein structural and functional features. *Oncogene* 1999;**19**:211–8.

Leukemia (2009) 23, 2090–2101

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ORIGINAL ARTICLE

Activation of p53 by Nutlin-3a, an antagonist of MDM2, induces apoptosis and cellular senescence in adult T-cell leukemia cells

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It has been reported that the induction of cellular senescence through p53 activation is an effective strategy in tumor regression. Unfortunately, however, tumors including adult T-cell leukemia/lymphoma (ATL) have disadvantages such as p53 mutations and a lack of $p16^{INK4a}$ and/or $p14^{ARF}$. In this study we characterized Nutlin-3a-induced cell death in 16 leukemia/lymphoma cell lines. Eight cell lines, including six ATL-related cell lines, had wild-type p53 and Nutlin-3a-activated p53, and the cell lines underwent apoptosis or cell-cycle arrest, whereas eight cell lines with mutated p53 were resistant. Interestingly, senescence-associated-β-galactosidase (SA-β-gal) staining revealed that only ATL-related cell lines with wild-type p53 showed cellular senescence, although they lack both $p16^{INK4a}$ and $p14^{ARF}$. These results indicate that cellular senescence is an important event in p53-dependent cell death in ATL cells and is inducible without $p16^{INK4a}$ and $p14^{ARF}$. Furthermore, knock-down of Tp53-induced glycolysis and apoptosis regulator (TIGAR), a novel target gene of p53, by small interfering RNA(siRNA) indicated its important role in the induction of cellular senescence. As many patients with ATL carry wild-type p53, our study suggests that p53 activation by Nutlin-3a is a promising strategy in ATL. We also found synergism with a combination of Nutlin-3a and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), suggesting the application of Nutlin-3a-based therapy to be broader than expected.
Leukemia (2009) **23**, 2090–2101; doi:10.1038/leu.2009.171; published online 27 August 2009
Keywords: p53; senescence; apoptosis; $p14^{ARF}$; adult T-cell leukemia; Nutlin

Introduction

DNA damage activates the tumor-suppressor protein, p53, as part of the surveillance mechanism.[1] Through the transcriptional activation or inactivation of target genes, p53 executes the appropriate physiological response, such as apoptosis, cell-cycle arrest or senescence.[2] To date, a number of target genes involved in p53-induced apoptosis have been identified, such as *BAX, NOXA, PUMA, PIG3* and *DR5*.[3,4] p53 can inhibit cell-cycle progression by initiating arrest at the G1, S or G2 phases. An inhibitor of cyclin-dependent kinase $p21^{WAF1/CIP1}$ (p21), GADD45α and 14-3-3σ have been implicated as major mediators of p53-induced growth arrest.[5] In addition, the recent discovery of a new target gene, C12orf5, also known as TP53-induced glycolysis, and an apoptosis regulator, Tp53-induced glycolysis and apoptosis regulator (TIGAR), revealed an unexpected function of p53 that regulates glucose metabolism and apoptosis.[6] Substantial evidence supports the idea that p53 can drive either apoptosis or cell-cycle arrest depending on which target genes it chooses to activate.[1,4] Meanwhile, a recent study showed that hematopoietic zinc-finger (Hzf) is induced by p53 and binds to its DNA-binding domain, resulting in the preferential transactivation of pro-arrest p53 target genes over its pro-apoptotic target genes.[7,8] These findings suggest that Hzf has an important role in regulating cell-fate decisions in response to genotoxic stress.

Cellular senescence is also an important mechanism in tumor suppression, which can be triggered by DNA damage or oncogene activation.[9] Cells entering cellular senescence are characterized by persistent cell-cycle arrest, a large flattened morphology, failure to replicate their DNA and enzymatic activity senescence-associated-β-galactosidase (SA-β-gal). The tumor suppressors $p16^{INK4a}$ (p16) and $p14^{ARF}$ (p14) have long been recognized as mediators of senescence, and research on oncogene-induced senescence has progressed rapidly; however, many questions remain regarding the programs and signals of cellular senescence.[10,11]

Adult T-cell leukemia/lymphoma (ATL) is a neoplasm of T-lymphocyte origin etiologically associated with human T-cell lymphotropic virus type 1(HTLV-1), and is known to be resistant to standard anticancer therapies.[12–14] HTLV-1 Tax has been shown to interfere with most DNA repair mechanisms, prevent cell-cycle arrest and apoptosis and contribute to the stepwise leukemic process leading to ATL.[15,16] Earlier studies using a p53-responsive reporter plasmid pG13-Luc and/or γ-irradiation have shown that Tax can repress the transcriptional activity of p53 in various transformed cell lines.[17–19] To date, several different mechanisms by which Tax inactivates p53 have been proposed, although the effects are indirect and complex.[20,21]

Mutations in the *p53* gene are found in about 50% of human cancers, and they abrogate DNA binding and the transactivation of p53.[22] Wild-type p53 protein is inactivated through binding to a specific E3 ubiquitin ligase, MDM2, which mediates the degradation of p53. Recently, a small antagonist of MDM2, Nutlin-3a, has been developed. Nutlin-3a binds MDM2 in the p53-binding pocket, activates the p53 pathway in human cancer cells with wild-type p53 and has shown promising results in an animal tumor model.[23,24] As mutated p53 proteins have been found in less than one-fourth of ATL cases and Tax expression is frequently lost in primary ATL cells, the activation of p53 by therapeutic drugs may become a promising approach to ATL therapy.[13,25]

In this study we analyze the potential therapeutic utility of Nutlin-3a in a number of ATL-related cell lines. Our experiments also provide new insights into the mechanism of cellular

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Received 12 May 2009; revised 20 July 2009; accepted 23 July 2009; published online 27 August 2009

senescence and the possibility of expanding Nutlin-3a-based cancer therapy.

Materials and methods

Cell lines

The ATL-derived cell lines, ST1, KOB, LM-Y1, KK1, SO4 and OMT, were established in our laboratory from the respective ATL patients.[26] These cell lines were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum and 0.5 U/ml of interleukin (IL-2) (kindly provided by Takeda Pharmaceutical Company, Ltd., Osaka, Japan). We also used the ATL-derived cell line MT1, HTLV-1-infected T-cell lines MT2[27] and HuT102,[28] human T-cell leukemia cell lines Jurkat and MOLT-4, human B lymphoblastoid cell line SKW6.4, Burkitt lymphoma cell line Ramos, transformed follicular lymphoma cell line SUDHL-4, acute myeloid leukemia cell line HL-60, monocytic leukemia cell lines THP-1 and U937 and erythromyeloblastoid cell line K562. These cell lines were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum.

Mutation analysis of p53

Total RNA from cell lines was isolated using ISOGEN (Wako, Osaka, Japan). After contaminating DNA was removed (Message Clean kit; GenHunter, Nashville, TN, USA), complementary DNA was constructed using the Thermoscript PCR with reverse transcription System (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Reverse transcriptase-PCR was performed to amplify the sequence targeting the open reading frame of p53 (GenBank accession number NM_000546) with the following primers: (forward) 5′-TCCGGGGACACTTTG CGTT-3′ and (reverse) 5′-AGGTGTGCGTCAGAAGCACC-3′. PCR products were then sequenced and analyzed using the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA) and an ABI-PRISM model 310 Genetic Analyzer (Applied Biosystems). All mutations were confirmed by sequencing in both directions. Additional primers for sequencing were as follows: (forward) 5′-TGCATTCTGGGA-CAGCCAAGT-3′ and 5′-CATCACACTGGAAGACTC-3′, and (reverse) 5′-CCAAGTCTGTGACTTGCACGTACT-3′ and 5′-GA GGAAGAGAATCTCCGCAAGAAAG-3′.

Deletion analysis of p14 *and* p16 *and quantitative PCR for human T-cell lymphotropic virus type 1 (HTLV-I) Tax*

As *p16* and *p14* share *exon 2* but have different open reading frames, loss of DNA in this region means the deletion of both proteins. Deletions of *p16* and *p14* were analyzed using *p16 exon 1-α* and *p14 exon 1-β* respectively. We designed specific sets of primers and TaqMan probes as follows: for *p16 exon 1-α*: 5′-GAGCAGCATGGAGCCTTC-3′ and 5′-CGTAACTATTCGGT GCGTTG-3′, TaqMan probe: 6FAM-CCGCACCTCTACCCGAC CC-TAMRA, for *p14 exon 1-β*: 5′-GCGCAGGTTCTTGGTGAC-3′ and 5′-CCTGAGTAGCATCAGCACGA-3′, TaqMan probe: 6FAM-TGTGAACCACGAAAACCCTCACTCG-TAMRA, for *INK4a/ARF exon 2*: 5′-ATTGAAAGAACCAGAGAGGC-3′ and 5′-ACGTTAA AAGGCAGGACATT-3′, TaqMan probe: 6FAM-ACCGAAGGTC CTACAGGGCCACAACT-TAMRA and for *β-globin*: 5′-CTTG AGGTTGTCCAGGTG-3′ and 5′-TGCTGGTGGTCTACCCTT-3′, TaqMan probe: 6FAM-GCCATGAGCCTTCACCTTAGGGTT G-TAMRA. Genomic DNA from cell lines was isolated using a QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) and subjected to a real-time quantitative PCR method based on TaqMan chemistry. PCRs were performed using Roche LC480 (Roche Diagnostics, Basel, Switzerland) with LightCycler 480 Probes Master mix (Roche Diagnostics) according to the manufacturer's directions. All data were normalized to the *β-globin* gene measured in the same samples. Samples with levels below the detection limit were considered to have homozygous deletions. Real-time quantitative PCR for HTLV-I Tax was performed as previously described.[29]

Chemicals and cell proliferation assay

Nutlin-3a (Alexis, San Diego, CA, USA), recombinant human soluble-TRAIL (Biomol Research Laboratories, Plymouth Meeting, PA, USA) and sodium butylate (Merck, Darmstadt, Germany) were used in this study. The cell proliferation assay (MTS assay) was performed using Cell Titer 96 AQueos Cell Proliferation Assay kit (Promega, Madison, WI, USA) in accordance with the manufacturer's instructions. Determination of the synergistic effect of combined treatment with Nutlin-3a and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) was achieved using isobolographic analysis, as described earlier.[30]

Flow cytometric detection of apoptosis, cell-cycle analysis and death receptors

Apoptosis and the cell cycle were examined using flow cytometry. To evaluate apoptotic changes, cells were stained simultaneously with Annexin-V and propidium iodide (Bender Medsystems, Vienna, Austria). Cell-cycle measurements based on DNA content were performed using a CycleTEST PLUS DNA reagent kit (BD Biosciences, San Jose, CA, USA). Cells were harvested after treatment and analyzed using a FACSCalibur flow cytometer and Cellquest software (BD Biosciences). The cell-surface expression of death receptors was examined by flow cytometer using DR5 (Alexis) or CD95 (BD Biosciences) monoclonal antibodies. Mouse IgG1 (DAKO, Kyoto, Japan) was used as a negative control.

Cellular senescence assay

For SA-β-gal staining, cells were harvested after treatment, and then placed on glass slides by cytospin centrifugation at 700 *g* for 5 min. Fixation of cells and staining for SA-β-gal in a humidified box kept at 37 °C overnight at pH 6.0 were performed using the Senescent Cells Staining kit (Sigma Chemicals, St Louis, MO, USA). The slides were washed three times in phosphate buffer solution and evaluated. May–Grünwald–Giemsa staining was also performed to distinguish between nuclear material and the cytoplasm. Cells were considered positive when the cytoplasm was stained with SA-β-gal.

Western blot analysis and antibodies

Whole cell lysates (20–40 μg) were prepared and western blotting was performed as described earlier.[31] Analysis was performed using antibodies to p53 (DO-1), MDM2 (Ab-1), PUMA, NOXA and PIG3 (Merck); caspase-3, BAX, Bcl-xL and p21 (Cell Signaling Technology, Beverly, MA, USA); TIGAR and Hzf (Abcam, Cambridge, MA, USA); GADD45α and p27 (Santa Cruz Biotechnology, Santa Cruz, CA, USA); Bcl-2 and 14-3-3σ (Upstate Biotechnology, Waltham, MA, USA); c-FLIP (Dave-2) (Alexis); survivin (R&D Systems Inc., Minneapolis, MN, USA); DEC-1 (Novus Biologicals, Littleton, CO, USA); DcR2 (Cayman

Chemical, Ann Arbor, MI, USA); and β-actin and α-tubulin (Sigma Chemicals). HTLV-1 Tax was detected by anti-Tax monoclonal antibodies from the National Institutes of Health AIDS Research Reference Reagent Program.[32]

Luminex protein analysis

Protein expression of total p53 and phospho-p53^{ser15} was measured using a bead multiplex system (BioSource International, Camarillo, CA, USA). Samples were incubated for 2 h at room temperature with anti-p53 or anti-phospho-p53^{ser15} beads in a 96-well plate. Detector antibodies were added to each well and incubated for 1 h, and the plate was read using Luminex100 IS instrument (Luminex, Austin, TX, USA). The concentrations of p53 and phospho-p53^{ser15} were determined using a standard curve assayed at the same time with known amounts of recombinant p53 and phospho-p53^{ser15}. This method permits a quantitative analysis of p53 protein or phospho-p53^{ser15}, which is considered difficult in western blot analysis.

p53-DNA binding enzyme-linked immunosorbent assay

The TransAM p53 Transcription Factor Assay kit (Active Motif, Carlsbad, CA, USA) was used following the manufacturer's protocol. Nuclear extracts from cells were prepared using a nuclear/cytosol fractionation kit (BioVision, San Diego, CA, USA) according to the manufacturer's protocol. Samples were diluted to 10 μg total protein with lysis buffer and applied to plates containing an immobilized oligonucleotide containing the p53 consensus binding site. After 1 h at room temperature, plates were washed and incubated with p53 antibody for another hour. After incubation with the secondary antibody, the developing solution was added, incubation was continued to allow color development and absorbance was read at 450 nm with a reference wavelength of 650 nm.

Transfection, luciferase assay and small interfering RNA (siRNA)

Transfection was performed with a Cell Line Nucleofector kit V or kit T and the Nucleofector system (Amaxa Biosystems, Cologne, Germany). The transfection program for ST1 (O-17), HuT102 (O-16), KOB (T-20) and KK1 cells (T-20) was determined so that high levels of transfection efficiency and cell viability could be achieved (data not shown). Cells were transfected with the luciferase reporter plasmid containing 13 copies of a p53 consensus binding site (pG13-Luc) and incubated with or without Nutlin-3a for 24 h.[20] Luciferase activity in 10 μg cell lysate was measured using luciferase assay reagents (Promega) according to the manufacturer's instructions in a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA, USA). Each experiment was carried out in triplicate. siRNA of p21 (Silencer Validated siRNA no. 1621), TIGAR (Silencer Select siRNA no. 32679) and control siRNA (Silencer Negative Control no. 1) were purchased from Applied Biosystems. Each siRNA was transfected at a final concentration of 20 nM. At 24 h after transfection, cells were used for experimentation.

Results

Mutations of p53 *and deletions of* p14 *and* p16

We first analyzed the p53 status of 16 hematological cell lines, including 9 ATL-related cell lines, and found that 8 had wild-type p53 and 8 had mutated p53 (Table 1). ST1, KOB and OMT had single nucleotide polymorphisms at codon 72, which is the most extensively studied polymorphism in p53.[22] Consistent with previous reports, there was no mutation in p53 of HuT102, MT2, SUDHL-4 and MOLT-4 cells.[33–35] Mutations detected in Ramos and Jurkat, deletions in HL60 and an insertion in K562 cells were all in accordance with earlier reports[34,36–38] or the data from the International Agency for Research on Cancer (http://www-p53.iarc.fr/). Accordingly, six of nine ATL-related

Table 1 Mutations of p53 and deletions of *p14* and/or *p16*

Cell line	*Origin*	*Exon*	*Codon*	*WT codon*	*Mutant codon*	*Effect*	*p53 Status*	p16^{INK4a}/p14^{ARF}	*HTLV-I-Tax mRNA*
ST1	ATL	4	72	CCC	CGC	Missense (SNP)	WT	del/del	0.4
KOB	ATL	4	72	CCC	CGC	Missense (SNP)	WT	del/del	542
LM-Y1	ATL	4	36	CCG	CCA	Silent	WT	del/del	1741
MT1	ATL	5	176	TCG	TAC	Missense	MUT	del/del	17.5
SO4	ATL	6	223	CCT	CAT	Missense	MUT	del/del	0.2
KK1	ATL	3	31	GTT	ATT	Missense	MUT	del/del	0.03
		5	152	CCG	CTG	Missense			
OMT	HTLV-I-infected T-cell	4	72	CCC	CGC	Missense (SNP)	WT	del/del	971
HuT102	HTLV-I-infected T-cell						WT		2381
MT2	HTLV-I-infected T-cell						WT		9331
MOLT-4	T-cell leukemia						WT	del/del	ND
Jurkat	T-cell leukemia	4	125	ACG	ACA	Silent	MUT	del/del	0
		6	196	CGA	TGA	Nonsense			
SUDHL-4	B-cell lymphoma						WT	del/	ND
Ramos	B-cell lymphoma	7	254	ATC	GAC	Missense	MUT		ND
U937	Monocytic leukemia	4	105	GGC	AGC	Missense	MUT	del/del	ND
		4	125	ACG	ACA	Silent			
		6	196	CGA	TGA	Nonsense			
K562	Erythroblastic leukemia	4	72	CCC	CGC	Missense (SNP)	MUT	del/del	ND
		5	136			Ins 1			
HL60	Myeloid leukemia	Gross deletion					MUT		ND

Abbreviations: ATL, adult T-cell leukemia/lymphoma; blank column, intact; del, deletion; HTLV-1, human T-cell lymphotropic virus type 1; ins, insertion; MUT, mutation; ND, not determined; SNP, single nucleotide polymorphism; WT, wild type.
The p53 status was determined using direct sequence targeting of the open reading frame, after RNA isolation, complementary DNA synthesis and reverse transcriptase-PCR amplification (GenBank accession number NM_000546). Deletions of *p14* and *p16* were determined using genomic DNA by the real-time quantitative PCR method based on TaqMan chemistry (GenBank accession number AF527803). Quantitative analysis of HTLV-1-Tax mRNA expression was also performed.

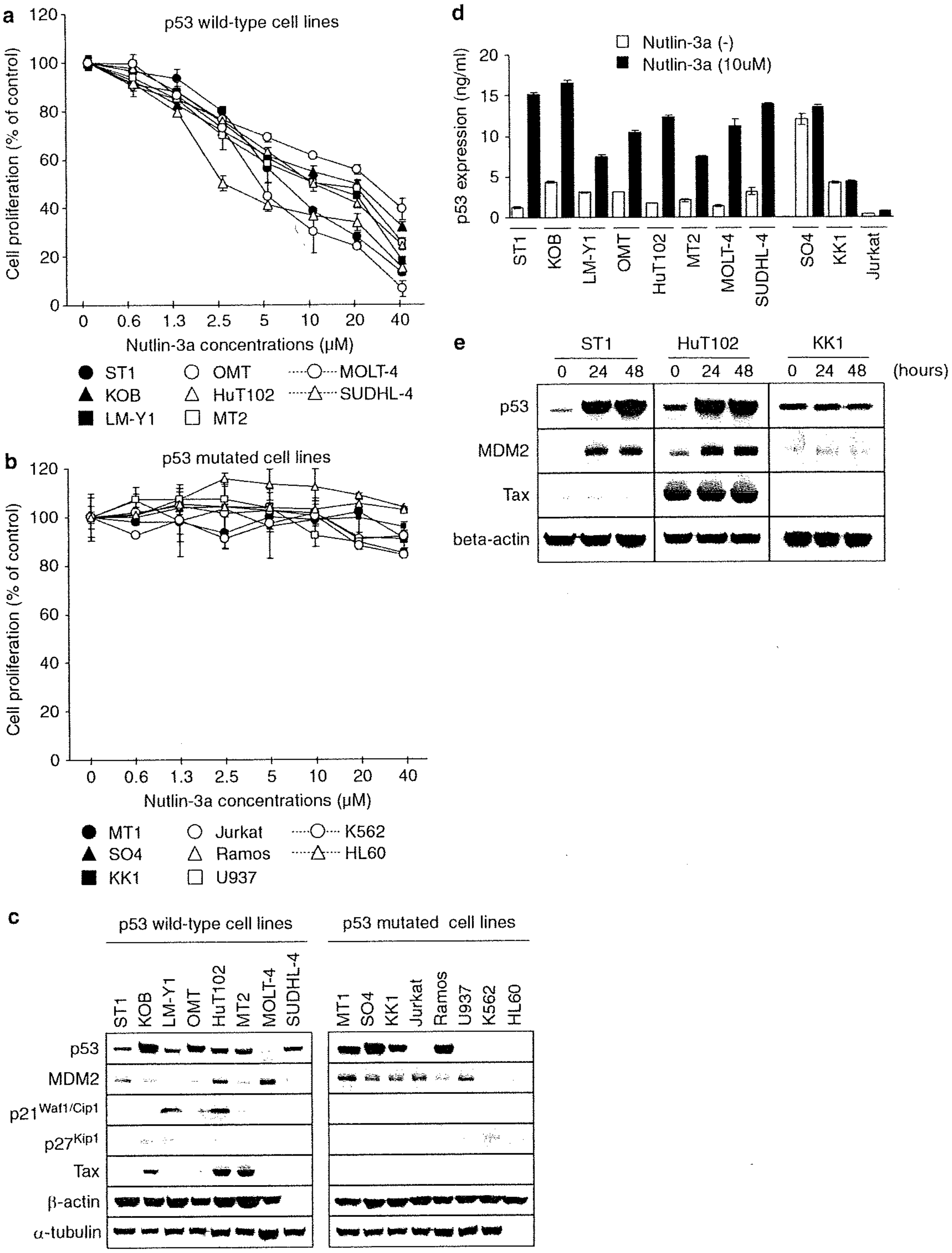


Figure 1 Sensitivity of hematological cell lines to Nutlin-3a and accumulation of p53 protein. (**a**, **b**) Cells (5–7 × 10^5/ml) were cultured for 48 h with the indicated concentrations of Nutlin-3a, and cell proliferation (percentage against control cells) was evaluated using MTS assay. All experiments were performed in triplicate and the results are expressed as mean ± s.d. (**c**) Whole cell lysate (40 μg) was prepared and the basal expression levels of p53-related proteins were detected using western blot analysis. (**d**, **e**) Cells were cultured with or without 10 μM Nutlin-3a for 24 h or the indicated period. (**d**) About 10 μg cell lysate was prepared and Luminex analysis was performed to evaluate the accumulation of p53 protein. Experiments were performed in duplicate and the results are expressed as mean ± s.d. (**e**) Whole cell lysate (20 μg) was prepared and the expression levels of p53-related proteins were detected using western blot analysis.

cell lines carried wild-type p53 in our analysis. The results of deletion analysis of *p16* and *p14* genes are summarized in Table 1. Deletions of both *p16* and *p14* occurred in all ATL-derived cell lines, and 11 cell lines lacked both, irrespective of their p53 status.

Nutlin-3a induces cell-growth inhibition and accumulation of p53

We next examined the effect of Nutlin-3a on the growth of the 16 cell lines. All eight cell lines with wild-type p53 showed apparent growth inhibition following treatment with Nutlin-3a, but the eight cell lines with mutated p53 were resistant (Figures 1a and b). As Nutlin-3a is expected to stabilize and activate p53 protein, we first examined the basal expression levels of p53-related proteins by western blot analysis (Figure 1c). Among p53 wild-type cell lines, ATL-related cell lines had elevated basal levels of p53 and some cells also had detectable levels of p21, in agreement with earlier reports.[33,39,40] Four p53-mutated cell lines showed strong expression of p53, probably because of mutations, and the others showed almost no band, consistent with the fact that they have deletions or nonsense mutations (Table 1). More important, these results were in accordance with those of Luminex analysis (Figure 1d). Although KOB, LM-Y1 and K562 showed a faint expression of p27, other cell lines did not express detectable levels of p27. HTLV-1 Tax protein was detected in HuT102, MT2, OMT and KOB, which is coincident with the results of mRNA expression (Table 1). In Luminex analysis, p53 wild-type cell lines showed a 4- to 12-fold increase in p53 expression after 24-h exposure to Nutlin-3a as a result of the inhibition of p53 degradation, which was not observed in p53-mutated cell lines (Figure 1d). Furthermore, time-dependent accumulation of p53 and MDM2 was clearly detected using western blot analysis (Figure 1e and data not shown). Meanwhile, there was no change in the expression of HTLV-1 Tax by Nutlin-3a treatment (Figure 1e).

Activation of p53 by Nutlin-3a in adult T-cell leukemia/lymphoma (ATL)-related cell lines

In addition to the accumulation of p53 protein, we further evaluated transcriptional activities of p53 from various perspectives. We first analyzed the level of DNA-binding activity of p53 using enzyme-linked immunosorbent assay. When cells were treated with Nutlin-3a, all p53 wild-type cells showed higher activities than positive control cells (Figure 2a). Next, we transfected ST1, HuT102, KOB and KK1 cells with a luciferase reporter plasmid, pG13-Luc, treated with or without Nutlin-3a, and performed a luciferase assay. As a result, fold inductions of luciferase activities were observed in a dose-dependent manner and showed maximum activities with 5 μM Nutlin-3a in ST1 and HuT102 cells, whereas KK1 cells with mutated p53 showed no change (Figure 2b). Luminex analysis revealed that Nutlin-3a caused obvious phosphorylation of p53, a 4- to 20-fold increase, in all p53 wild-type cell lines, which had not occurred in KK1 cells (Figure 2c). These results indicate that Nutlin-3a causes the accumulation, increase of transcriptional activity and phosphorylation of p53 in ATL-related cell lines with endogenous wild-type p53.

Nutlin-3a causes apoptosis in p53 wild-type cells

To clarify the details of Nutlin-3a-induced cell death, we performed Annexin-V/propidium iodide staining in p53

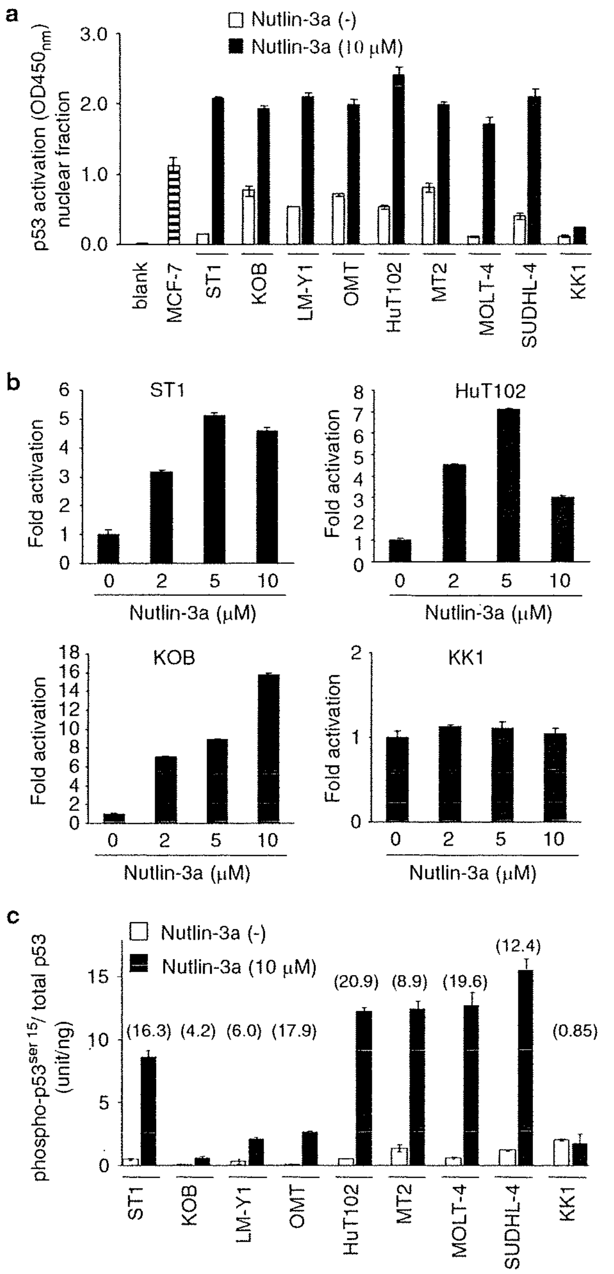


Figure 2 Transcriptional activation of p53 by Nutlin-3a treatment. Cells were treated with or without the indicated concentrations of Nutlin-3a for 24 h. After cells were harvested, enzyme-linked immunosorbent assay (ELISA), luciferase assay and Luminex analysis were performed. KK1 (p53 mutant) cells were used as an accumulation-negative control. (**a**) Nuclear extract from H_2O_2-treated MCF-7 cells contained in the kit was used as a positive control. About 10 μg nuclear extract was used, experiments were performed in duplicate and results are expressed as mean ± s.d. (**b**) For the luciferase assay, cells were co-transfected with 0.1 μg pG13-Luc, incubated for 12 h and treated with or without Nutlin-3a for 24 h. Experiments were performed in triplicate and results are expressed as the mean ± s.d. The level of activation (fold induction) was obtained by setting the value without Nutlin-3a as 1.0. (**c**) About 10 μg whole cell lysate was prepared, experiments were performed in duplicate and results are expressed as mean ± s.d. Fold induction was also obtained by setting the value without Nutlin-3a as 1.0 and is indicated on the graph.

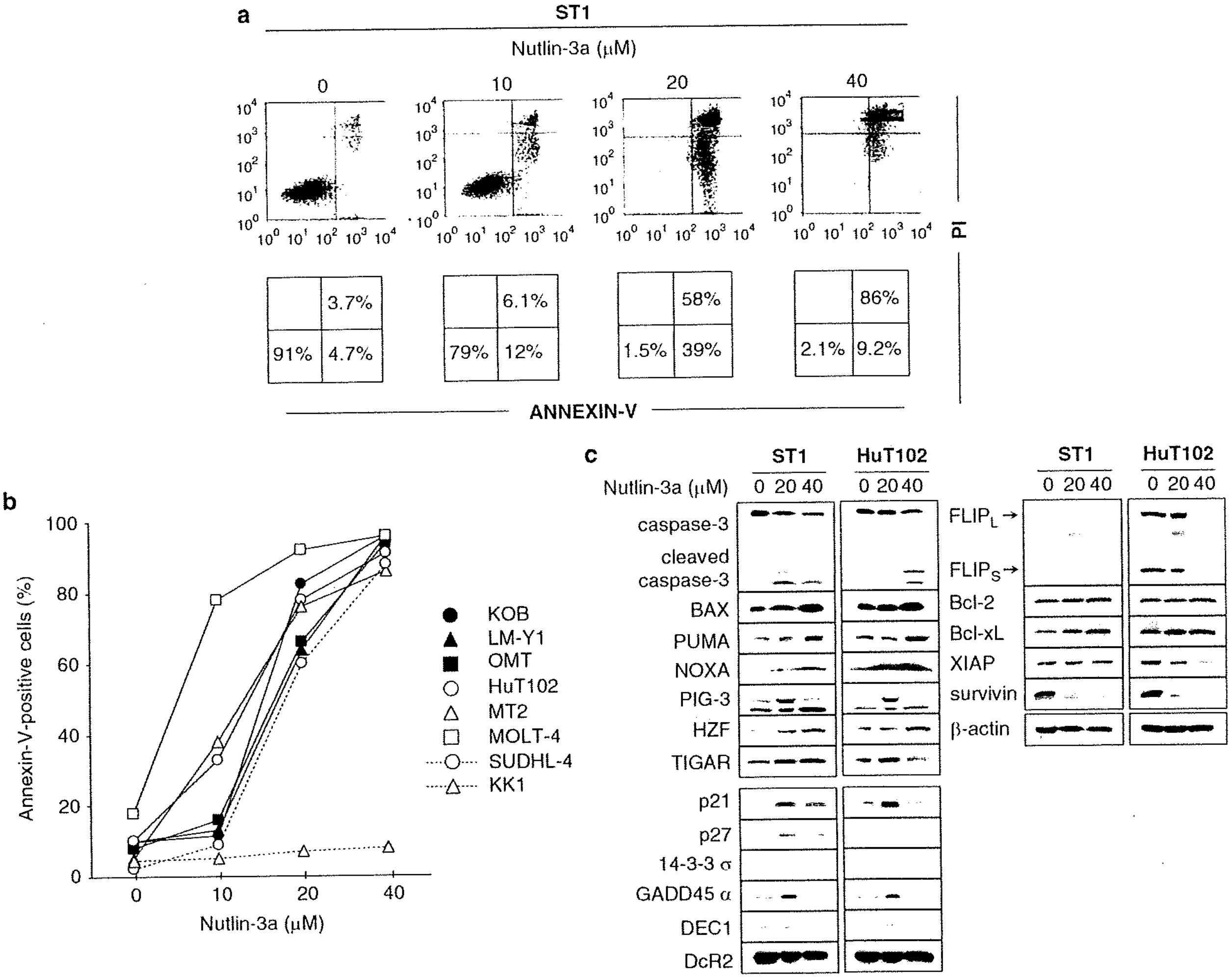


Figure 3 Analysis of molecules in Nutlin-3a-induced apoptosis. Cells were treated with or without the indicated concentrations of Nutlin-3a for 24 h. After cells were harvested, flow cytometric analyses by Annexin-V/ propidium iodide (PI) staining (**a**, **b**) and western blot analysis (**c**) were performed.

wild-type cell lines. We found weak apoptotic changes with 10 μM Nutlin-3a and less than 30% of cells became positive for Annexin-V, except MOLT-4 cells, which showed apparent apoptosis (Figures 3a and b). In contrast, with more than 20 μM Nutlin-3a, all p53 wild-type cell lines showed rapid apoptosis and almost no living cell fraction was observed by Annexin-V/propidium iodide staining. These results suggest that Nutlin-3a eventually causes apoptosis in p53 wild-type cell lines in a dose-dependent manner. We then performed western blot analysis of key molecules of the p53 pathway. In addition to cleaved forms of caspase-3, we found dose-dependent increase of BAX, PUMA, NOXA and Hzf, no change of Bcl-2 and dose-dependent decrease of XIAP and survivin (Figure 3c).

Nutlin-3a induces cellular senescence followed by cell-cycle arrest in p53 wild-type cells

Despite weak apoptotic changes, cell growth of p53 wild-type cell lines was significantly depressed by Nutlin-3a at a concentration of 10 μM. To clarify this discordance, we performed cell cycle analysis. When cells were treated with 10 μM Nutlin-3a for 24 h, these cells showed significant cell-cycle arrest in the G1 phase instead of apoptosis. The G1 cell population increased from 63 to 78% in ST1 and from 68 to 92% in HuT102 (Figure 4a). Similar results were observed in the other p53 wild-type cell lines (Figure 4b). Although it has been reported that p53 has a central role in the induction of cellular senescence, Nutlin-3a-induced cellular senescence has not been reported in leukemia cells.[41,42] We thus performed SA-β-gal staining as a marker of cellular senescence after incubation with 10 μM Nutlin-3a for 72 h. Five of eight p53 wild-type cell lines (ST1, HuT102, MT2, OMT and KOB) were clearly stained by SA-β-gal and all were ATL-related cell lines (Figure 4c and data not shown). No non-ATL cell lines became positive for SA-β-gal staining. In contrast, after treatment with 1 mM sodium butylate, a well-known senescence inducer, all cell lines examined, including those with mutant p53 (SO4 and K562), became positive for SA-β-gal staining (data not shown). Among five SA-β-gal-positive cell lines, three lacked both p16 and p14 (Table 1), suggesting that these cells underwent p53-dependent cellular senescence without the participation of p16 and/or p14. We further analyzed whether removal of Nutlin-3a alters cell fate, senescence or cell proliferation. To this purpose, p53 wild-type ATL cell lines were cultured with Nutlin-3a for 72 h to

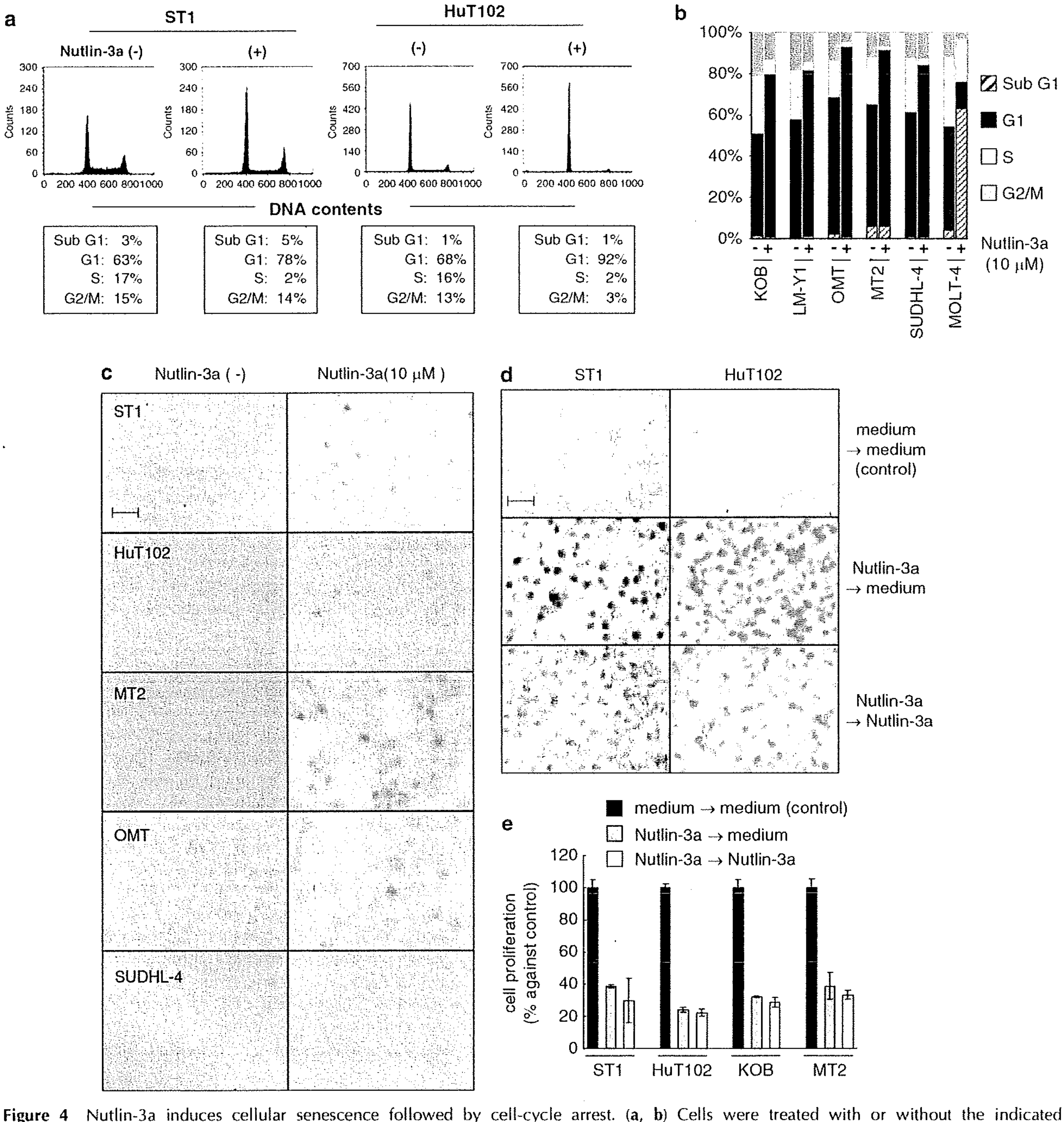
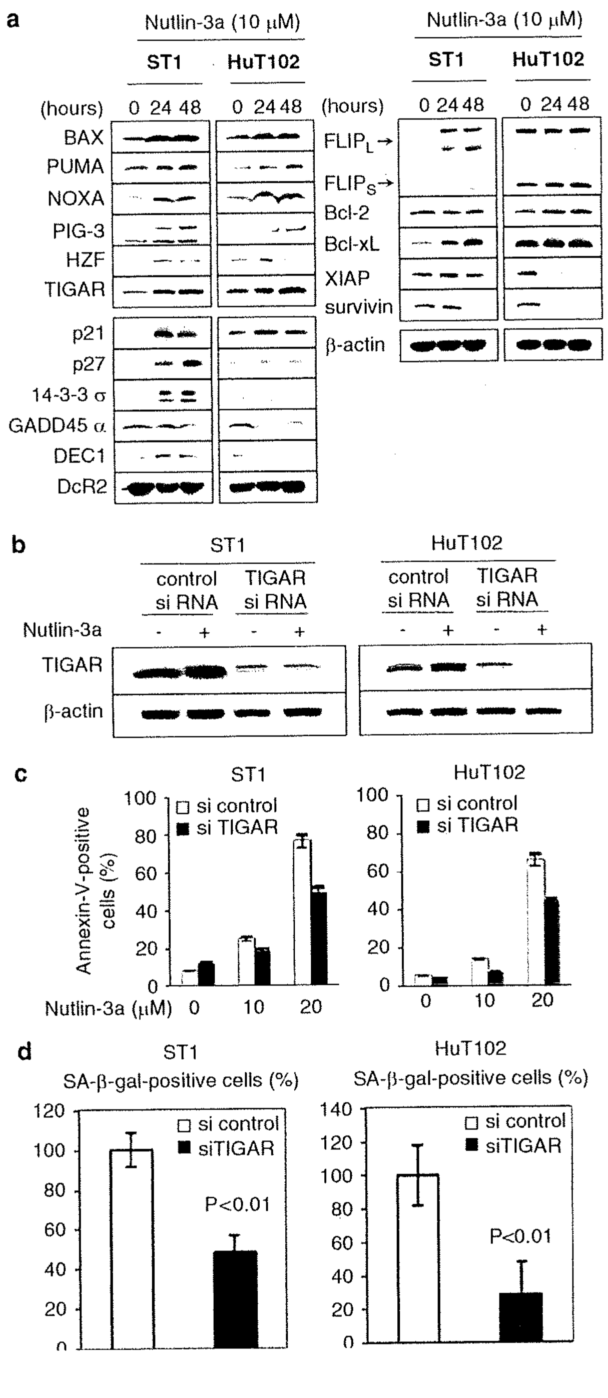


Figure 4 Nutlin-3a induces cellular senescence followed by cell-cycle arrest. (**a**, **b**) Cells were treated with or without the indicated concentrations of Nutlin-3a for 24 h. After cells were harvested, cell-cycle analysis was performed using flow cytometer. (**c**) Similarly, after 72 h, cells 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.

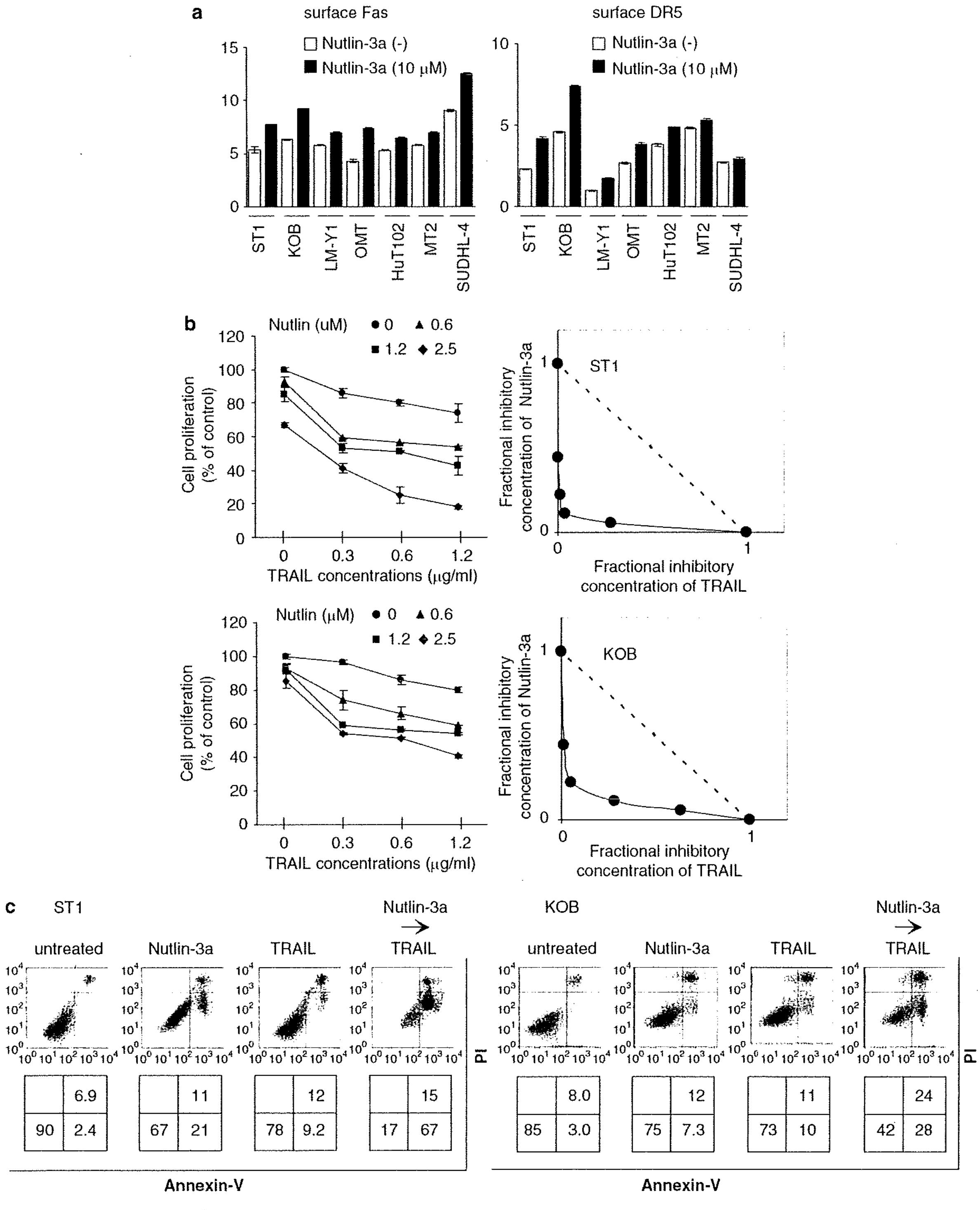


Figure 6 Combination of Nutlin-3a and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced synergistic effects. (**a**) Cells were treated with or without the indicated concentrations of Nutlin-3a. Cell-surface expression of death receptors was examined using flow cytometer (FCM) and 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, knock-down 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

We are grateful to Dr Norman E. Sharpless, Departments of Medicine and Genetics, The Lineberger Comprehensive Cancer Center, The University of North Carolina, for his critical review and constructive suggestions on this article. This study was supported in part by a Grant-in-aid for Scientific Research (18590510) from the Japan Society for the Promotion of Science.

References

1 Horn HF, Vousden KH. Coping with stress: multiple ways to activate p53. *Oncogene* 2007; **26**: 1306–1316.
2 Helton ES, Chen X. p53 modulation of the DNA damage response. *J Cell Biochem* 2007; **100**: 883–896.
3 Chipuk JE, Green DR. Dissecting p53-dependent apoptosis. *Cell Death Differ* 2006; **13**: 994–1002.
4 Levine AJ, Hu W, Feng Z. The P53 pathway: what questions remain to be explored? *Cell Death Differ* 2006; **13**: 1027–1036.
5 Yu J, Zhang L. The transcriptional targets of p53 in apoptosis control. *Biochem Biophys Res Commun* 2005; **331**: 851–858.
6 Bensaad K, Tsuruta A, Selak MA, Vidal MN, Nakano K, Bartrons R *et al.* TIGAR, a p53-inducible regulator of glycolysis and apoptosis. *Cell* 2006; **126**: 107–120.
7 Sugimoto M, Gromley A, Sherr CJ. Hzf, a p53-responsive gene, regulates maintenance of the G2 phase checkpoint induced by DNA damage. *Mol Cell Biol* 2006; **26**: 502–512.
8 Das S, Raj L, Zhao B, Kimura Y, Bernstein A, Aaronson SA *et al.* Hzf determines cell survival upon genotoxic stress by modulating p53 transactivation. *Cell* 2007; **130**: 624–637.
9 Collado M, Blasco MA, Serrano M. Cellular senescence in cancer and aging. *Cell* 2007; **130**: 223–233.

10 Kim WY, Sharpless NE. The regulation of INK4/ARF in cancer and aging. *Cell* 2006; **127**: 265–275.
11 Satyanarayana A, Rudolph KL. p16 and ARF: activation of teenage proteins in old age. *J Clin Invest* 2004; **114**: 1237–1240.
12 Yoshida M. Discovery of HTLV-1, the first human retrovirus, its unique regulatory mechanisms, and insights into pathogenesis. *Oncogene* 2005; **24**: 5931–5937.
13 Taylor GP, Matsuoka M. Natural history of adult T-cell leukemia/lymphoma and approaches to therapy. *Oncogene* 2005; **24**: 6047–6057.
14 Yamada Y, Tomonaga M. The current status of therapy for adult T-cell leukaemia-lymphoma in Japan. *Leuk Lymphoma* 2003; **44**: 611–618.
15 Grassmann R, Aboud M, Jeang KT. Molecular mechanisms of cellular transformation by HTLV-1 Tax. *Oncogene* 2005; **24**: 5976–5985.
16 Marriott SJ, Semmes OJ. Impact of HTLV-I Tax on cell cycle progression and the cellular DNA damage repair response. *Oncogene* 2005; **24**: 5986–5995.
17 Akagi T, Ono H, Tsuchida N, Shimotohno K. Aberrant expression and function of p53 in T-cells immortalized by HTLV-I Tax1. *FEBS Lett* 1997; **406**: 263–266.
18 Pise-Masison CA, Choi KS, Radonovich M, Dittmer J, Kim SJ, Brady JN. Inhibition of p53 transactivation function by the human T-cell lymphotropic virus type 1 Tax protein. *J Virol* 1998; **72**: 1165–1170.
19 Mulloy JC, Kislyakova T, Cereseto A, Casareto L, LoMonico A, Fullen J *et al.* Human T-cell lymphotropic/leukemia virus type 1 Tax abrogates p53-induced cell cycle arrest and apoptosis through its CREB/ATF functional domain. *J Virol* 1998; **72**: 8852–8860.
20 Miyazato A, Sheleg S, Iha H, Li Y, Jeang KT. Evidence for NF-kappaB- and CBP-independent repression of p53's transcriptional activity by human T-cell leukemia virus type 1 Tax in mouse embryo and primary human fibroblasts. *J Virol* 2005; **79**: 9346–9350.
21 Pise-Masison CA, Jeong SJ, Brady JN. Human T cell leukemia virus type 1: the role of Tax in leukemogenesis. *Arch Immunol Ther Exp (Warsz)* 2005; **53**: 283–296.
22 Soussi T, Wiman KG. Shaping genetic alterations in human cancer: the p53 mutation paradigm. *Cancer Cell* 2007; **12**: 303–312.
23 Vassilev LT, Vu BT, Graves B, Carvajal D, Podlaski F, Filipovic Z *et al. In vivo* activation of the p53 pathway by small-molecule antagonists of MDM2. *Science* 2004; **303**: 844–848.
24 Vassilev LT. MDM2 inhibitors for cancer therapy. *Trends Mol Med* 2007; **13**: 23–31.
25 Tawara M, Hogerzeil SJ, Yamada Y, Takasaki Y, Soda H, Hasegawa H *et al.* Impact of p53 aberration on the progression of adult T-cell leukemia/lymphoma. *Cancer Lett* 2006; **234**: 249–255.
26 Hasegawa H, Yamada Y, Harasawa H, Tsuji T, Murata K, Sugahara K *et al.* Sensitivity of adult T-cell leukaemia lymphoma cells to tumour necrosis factor-related apoptosis-inducing ligand. *Br J Haematol* 2005; **128**: 253–265.
27 Yoshida M, Miyoshi I, Hinuma Y. Isolation and characterization of retrovirus from cell lines of human adult T-cell leukemia and its implication in the disease. *Proc Natl Acad Sci USA* 1982; **79**: 2031–2035.
28 Posner LE, Robert-Guroff M, Kalyanaraman VS, Poiesz BJ, Ruscetti FW, Fossieck B *et al.* Natural antibodies to the human T cell lymphoma virus in patients with cutaneous T cell lymphomas. *J Exp Med* 1981; **154**: 333–346.
29 Usui T, Yanagihara K, Tsukasaki K, Murata K, Hasegawa H, Yamada Y *et al.* Characteristic expression of HTLV-1 basic zipper factor (HBZ) transcripts in HTLV-1 provirus-positive cells. *Retrovirology* 2008; **5**: 34.
30 Berenbaum MC. A method for testing for synergy with any number of agents. *J Infect Dis* 1978; **137**: 122–130.
31 Hasegawa H, Yamada Y, Komiyama K, Hayashi M, Ishibashi M, Sunazuka T *et al.* A novel natural compound, a cycloanthranilylproline derivative (Fuligocandin B), sensitizes leukemia cells to apoptosis induced by tumor necrosis factor related apoptosis-inducing ligand (TRAIL) through 15-deoxy-Delta 12, 14 prostaglandin J2 production. *Blood* 2007; **110**: 1664–1674.
32 Iha H, Kibler KV, Yedavalli VR, Peloponese JM, Haller K, Miyazato A *et al.* Segregation of NF-kappaB activation through NEMO/IKKgamma by Tax and TNFalpha: implications for stimulus-specific interruption of oncogenic signaling. *Oncogene* 2003; **22**: 8912–8923.
33 Reid RL, Lindholm PF, Mireskandari A, Dittmer J, Brady JN. Stabilization of wild-type p53 in human T-lymphocytes transformed by HTLV-I. *Oncogene* 1993; **8**: 3029–3036.
34 Cheng J, Haas M. Frequent mutations in the p53 tumor suppressor gene in human leukemia T-cell lines. *Mol Cell Biol* 1990; **10**: 5502–5509.
35 Sugito S, Yamato K, Sameshima Y, Yokota J, Yano S, Miyoshi I. Adult T-cell leukemia: structures and expression of the p53 gene. *Int J Cancer* 1991; **49**: 880–885.
36 Gaidano G, Ballerini P, Gong JZ, Inghirami G, Neri A, Newcomb EW *et al.* p53 mutations in human lymphoid malignancies: association with Burkitt lymphoma and chronic lymphocytic leukemia. *Proc Natl Acad Sci USA* 1991; **88**: 5413–5417.
37 Sugimoto K, Toyoshima H, Sakai R, Miyagawa K, Hagiwara K, Ishikawa F *et al.* Frequent mutations in the p53 gene in human myeloid leukemia cell lines. *blood* 1992; **79**: 2378–2383.
38 Neubauer A, He M, Schmidt CA, Huhn D, Liu ET. Genetic alterations in the p53 gene in the blast crisis of chronic myelogenous leukemia: analysis by polymerase chain reaction based techniques. *Leukemia* 1993; **7**: 593–600.
39 Takemoto S, Trovato R, Cereseto A, Nicot C, Kislyakova T, Casareto L *et al.* p53 stabilization and functional impairment in the absence of genetic mutation or the alteration of the p14(ARF)-MDM2 loop in *ex vivo* and cultured adult T-cell leukemia/lymphoma cells. *Blood* 2000; **95**: 3939–3944.
40 Cereseto A, Diella F, Mulloy JC, Cara A, Michieli P, Grassmann R *et al.* p53 functional impairment and high p21waf1/cip1 expression in human T-cell lymphotropic/leukemia virus type I-transformed T cells. *Blood* 1996; **88**: 1551–1560.
41 Kojima K, Konopleva M, Samudio IJ, Shikami M, Cabreira-Hansen M, McQueen T *et al.* MDM2 antagonists induce p53-dependent apoptosis in AML: implications for leukemia therapy. *Blood* 2005; **106**: 3150–3159.
42 Coll-Mulet L, Iglesias-Serret D, Santidrián AF, Cosialls AM, de Frias M, Castaño E *et al.* MDM2 antagonists activate p53 and synergize with genotoxic drugs in B-cell chronic lymphocytic leukemia cells. *Blood* 2006; **107**: 4109–4114.
43 Hasegawa H, Yamada Y, Komiyama K, Hayashi M, Ishibashi M, Yoshida T *et al.* Dihydroflavonol BB-1, an extract of natural plant Blumea balsamifera, abrogates TRAIL resistance in leukemia cells. *Blood* 2006; **107**: 679–688.
44 Martins CP, Brown-Swigart L, Evan GI. Modeling the therapeutic efficacy of p53 restoration in tumors. *Cell* 2006; **127**: 1323–1334.
45 Xue W, Zender L, Miething C, Dickins RA, Hernando E, Krizhanovsky V *et al.* Senescence and tumour clearance is triggered by p53 restoration in murine liver carcinomas. *Nature* 2007; **445**: 656–660.
46 Ventura A, Kirsch DG, McLaughlin ME, Tuveson DA, Grimm J, Lintault L *et al.* Restoration of p53 function leads to tumour regression *in vivo*. *Nature* 2007; **445**: 661–665.
47 Tovar C, Rosinski J, Filipovic Z, Higgins B, Kolinsky K, Hilton H *et al.* Small-molecule MDM2 antagonists reveal aberrant p53 signaling in cancer: implications for therapy. *Proc Natl Acad Sci USA* 2006; **103**: 1888–1893.
48 Van Maerken T, Speleman F, Vermeulen J, Lambertz I, De Clercq S, De Smet E *et al.* Small-molecule MDM2 antagonists as a new therapy concept for neuroblastoma. *Cancer Res* 2006; **66**: 9646–9655.
49 Sherr CJ. Divorcing ARF and p53: an unsettled case. *Nat Rev Cancer* 2006; **6**: 663–673.
50 Collado M, Gil J, Efeyan A, Guerra C, Schuhmacher AJ, Barradas M *et al.* Tumour biology: senescence in premalignant tumours. *Nature* 2005; **436**: 642.
51 Datta A, Bellon M, Sinha-Datta U, Bazarbachi A, Lepelletier Y, Canioni D *et al.* Persistent inhibition of telomerase reprograms adult T-cell leukemia to p53-dependent senescence. *Blood* 2006; **108**: 1021–1029.
52 Dasgupta A, Jung KJ, Jeong SJ, Brady JN. Inhibition of methyltransferases results in induction of G2/M checkpoint and programmed cell death in human T-lymphotropic virus type 1-transformed cells. *J Virol* 2008; **82**: 49–59.

53 Jeong SJ, Dasgupta A, Jung KJ, Um JH, Burke A, Park HU *et al.* PI3K/AKT inhibition induces caspase-dependent apoptosis in HTLV-1-transformed cells. *Virology* 2008; **370**: 264–272.
54 Jung KJ, Dasgupta A, Huang K, Jeong SJ, Pise-Masison C, Gurova KV *et al.* Small-molecule inhibitor which reactivates p53 in human T-cell leukemia virus type 1-transformed cells. *J Virol* 2008; **82**: 8537–8547.
55 Kuo YL, Giam CZ. Activation of the anaphase promoting complex by HTLV-1 tax leads to senescence. *EMBO J* 2006; **25**: 1741–1752.
56 Ringshausen I, O'Shea CC, Finch AJ, Swigart LB, Evan GI. Mdm2 is critically and continuously required to suppress lethal p53 activity *in vivo*. *Cancer Cell* 2006; **10**: 501–514.
57 Duiker EW, Mom CH, de Jong S, Willemse PH, Gietema JA, van der Zee AG *et al.* The clinical trail of TRAIL. *Eur J Cancer* 2006; **42**: 2233–2240.
58 Schaefer U, Voloshanenko O, Willen D, Walczak H. TRAIL: a multifunctional cytokine. *Front Biosci* 2007; **12**: 3813–3824.