MDM2 Inhibitor Nutlin-3a Induces Apoptosis and Senescence in Cutaneous T-Cell Lymphoma: Role of p53

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P53 is rarely mutated in cutaneous T-cell lymphoma (CTCL) and is therefore a promising target for innovative therapeutic approaches. Nutlin-3a is an inhibitor of MDM2 (human homolog of murine double minute 2), which disrupts its interaction with p53, leading to the stabilization and activation of p53. To investigate the potential therapeutic use of nutlin-3a in CTCL, we screened CTCL lines Hut-78, SeAx, MyLa2000, Mac1, and Mac2a by measuring p53 levels after nutlin-3a treatment. In MyLa2000, Mac1, and Mac2a, we observed the increase in p53, indicating the fully functional p53. In the remaining cell lines, P53 mutation analysis identified a homozygous nonsense mutation (R196Stop in Hut-78) and a homozygous missense mutation (G245S in SeAx). In MyLa2000, Mac1, and Mac2a carrying wild-type P53, nutlin-3a induced apoptosis and senescence demonstrated by permanent G0/G1 cell-cycle block and expression of the senescence-associated b-galactosidase. This effect was abolished in cells in which p53 was silenced by small interfering RNA. Sézary cells lack functional p53 and were resistant to nutlin-3a. However, nutlin-3a potentiated the efficacy of conventional chemotherapeutics not only in cells with intact p53 but also in Hut-78, SeAx, and Sézary cells. Thus, targeting p53 by nutlin-3a may constitute a therapeutic approach in CTCL because of increased apoptosis and senescence of tumor cells.

INTRODUCTION

Cutaneous T-cell lymphoma (CTCL) is a heterogeneous group of T-cell lymphomas, of which mycosis fungoides (MF), Sézary syndrome (SS), and CD30⁺ lymphoproliferative diseases are the most common types. Whereas MF in the early stage has a good prognosis, progression into erythroderma and tumors is associated with aggressive clinical course and shortened survival (Mao et al., 2002; Willemze et al., 2005; Agar et al., 2010). Primary cutaneous CD30⁺ lymphoproliferative disorders comprise ~30% of all primary CTCLs and include lymphomatoid papulosis and primary cutaneous anaplastic large-cell lymphoma. Advanced CTCL is a therapeutic challenge. Traditional therapies comprising radiotherapy and anthracycline- or nucleoside analog-based regimens provide only short-lived responses (Zinzani et al., 2000; Wollina et al., 2003). Current research efforts are therefore concentrated on a better understanding of chemotherapy resistance in advanced CTCL and on identification of new pharmacological targets (Kamstrup et al., 2010a, 2010b; Manfé et al., 2010).

p53 is a tumor-suppressor protein that is activated in response to stress signals and restricts cell proliferation by inducing apoptosis, cell-cycle arrest, or senescence (Vogelstein et al., 2000; Harris and Levine, 2005; Vousden and Prives, 2009; Nardella et al., 2011). However, p53 can also, paradoxically, contribute to cell survival, suggesting that p53 response depends on the type of tissue, the nature of the stress signal, and the cellular environment (Vousden and Prives, 2009). p53 serves as a transcriptional activator of numerous target genes (e.g., BAX, NOXA, PUMA, and p21WAF1CIP1), but no p53 target gene knockout mouse has been shown to recapitulate the cancer predisposition of p53-null mice (Lozano and Zambetti, 2005). Moreover, in some tumor cell lines, specific inhibition of p53 target gene transcription did not affect p53-dependent apoptosis (Caëelles et al., 1994; Wickremasinghe et al., 2011). Thus, an alternative p53 pathway may cause cellular responses independent of de novo gene transcription (Wickremasinghe et al., 2011). This nontranscriptional mechanism of p53 was described in leukemia and predominantly involves the core apoptotic
Nutlin-3a in Cutaneous T-Cell Lymphoma

As the p53 activation is inhibited by the binding to the E3 ubiquitin ligase, human homolog of the murine double minute 2 (MDM2) (Haupt and Haupt, 2004), a small antagonist of MDM2, nutlin-3a, has been highlighted as a promising tool to stabilize and activate p53 (Vassilev et al., 2004; Lau et al., 2008). In this regard, nutlin-3a has been shown to trigger apoptosis or senescence in several hematological malignancies including Hodgkin’s lymphomas, mantle cell lymphomas, Kaposi’s sarcoma-associated herpesvirus lymphomas, and anaplastic large-cell lymphomas (Saha et al., 2010), and to synergize with chemotherapies (Coll-Mulet et al., 2006; Secchiero et al., 2007, 2009; Barbarotto et al., 2008; Carter et al., 2008; Kojima et al., 2008; Zhu et al., 2008; Drakos et al., 2009; Voltan et al., 2010) and radiation (Long et al., 2010). Recently, it was demonstrated that nutlin-induced activation of the p53 pathway results in p53-dependent apoptosis in cells harboring P53 wild type (wt), as well as mutated (mut) but partially functional P53 (Drakos et al., 2009). In cells lacking a functional p53, nutlin-3a can instead induce the activation of other partners of MDM2, including p73 or E2F1, and trigger apoptosis in a p53-independent mechanism (Lau et al., 2008).

P53 mutations occur rarely in MF and CD30 + lymphoproliferative diseases and are not necessarily associated with disease progression, as demonstrated by recent studies that found mutation rates of 2% and 5%, respectively (Kapur et al., 2001, 2005; Dereure et al., 2002). A focal deletion at 17p13.3–17p13.1, leading to the loss of P53, has been detected in 75–80% of patients with SS (Marrogi et al., 1999; Vermeer et al., 2008; Steininger et al., 2011). Here we investigated the potential therapeutic utility of nutlin-3a either alone or in combination with other pro-apoptotic drugs in CTCL cell lines and in primary leukemic Sézary cells. We showed that nutlin-3a could induce apoptosis and senescence in CTCL cells harboring P53wt, but it was unable to induce apoptosis in cells with P53mut, and in purified primary Sézary cells. We demonstrated that nutlin-induced apoptosis in CTCL cells depends on the presence of a functional p53 and at least in part occurs via a p53 nontranscriptional mechanism. The p53 transcriptional function limits induction of apoptosis probably through the transcriptional upregulation of anti-apoptotic proteins. Finally, we found that nutlin-3a potentiates the effect of several pro-apoptotic agents including γ-secretase inhibitor-1, bortezomib, and doxorubicin in cells with P53mut and P53wt, and in primary leukemic Sézary cells.

RESULTS

Expression and mutation status of P53 in CTCL cells

The functionality of the p53 pathway in primary leukemic cells from SS and in the CTCL cell lines was probed with nutlin-3a (Figure 1a). Cells with functionally intact p53 are expected to respond to nutlin-3a with an increase in cellular p53 content and its downstream target, p21WAF1/CIP1 (Vassilev et al., 2004). As a consequence of focal deletion at 17p13.3–17p13.1 of P53 (Vermeer et al., 2008; Steininger et al., 2011), Sézary cells showed no p53 expression (Figure 1a) and lack of a functional p53. Similarly, Hut-78 cells lacked p53 expression, consistent with a possible deletion or nonsense mutation. SeAx, MyLa2000, Mac1, and Mac2a showed an accumulation of p53 and MDM2 proteins after 24 hours of exposure to nutlin-3a. p21 increased as expected in MyLa2000, Mac1, and Mac2a cells, but not in SeAx, suggesting that the transcriptional activity of p53 was impaired in the latter cell line. These findings implied that p53 is fully functional in MyLa2000, Mac1, and Mac2a, but not in Hut-78 and SeAx. To further explore these findings, we sequenced the entire open reading frame of P53 in Hut-78 and SeAx. We verified the previously reported homozygous nonsense P53 mutation at codon 196 in Hut-78 (R196Stop) (Ri et al., 2009) and identified a homozygous missense mutation at codon 245 (G245S) of exon 7 in SeAx cells at the DNA-binding domain (Table 1). The G245S is a nonfunctional mutation located in the mutational hot spot region (exons 5–9) of P53 and is observed in several cancers including hematological malignancies (Hu et al., 1992; Wada et al., 1993; Kurosawa et al., 1995; Villuendas et al., 1997; Wong et al., 1999; Leroy et al., 2002; Wojcik et al., 2005). Although the G245S mutation disrupts the DNA-binding surface, the overall scaffold is retained when the protein is folded (Wong et al., 1999), explaining the observed stabilization of p53 after nutlin-3a.

Nutlin-3a promotes apoptosis in P53mut cells independent of the transcriptional activity of p53

Having established the functionality of p53 in the CTCL cell lines, we tested the ability of nutlin-3a to induce apoptosis. As shown in Figure 1b, nutlin-3a potently induced apoptosis in Mac1 cells, whereas the effect in MyLa2000 and Mac2a was weaker. The viability of P53mut Hut-78 and SeAx and Sézary cells (Figure 1c) was not altered by nutlin-3a, underscoring the assumption that nutlin-induced apoptosis is p53 dependent. This is further confirmed by the observation that the specific attenuation of P53 using small interfering RNA (siRNA; Figure 1d) abolished nutlin-induced apoptosis in Mac1 (Figure 1e). The p53 protein accumulation after nutlin-3a was effectively downregulated in p53 siRNA-transfected groups compared with the scrambled siRNA-transfected ones (Figure 1d). We observed no correlation between the different responses to nutlin-induced apoptosis in CTCL cell lines and the cytotoxic effect of two pro-apoptotic agents, doxorubicin and etoposide (Supplementary Figure S1a and b online). Thus, we suggest that the apoptotic response induced by doxorubicin or etoposide in CTCL cells is modulated by factors other than P53 status.

An interesting insight in the role of the transcriptional versus the nontranscriptional mechanism of p53 action in apoptosis was gained using a pharmacological inhibitor of the p53-mediated transcription, pifithrin-α (PFT-α). Preincubation
with 25 μM PFT-α substantially inhibited the nutlin-3a induction of the p53 transcriptional target, p21, without affecting the p53 accumulation in Mac1 cells (Figure 2a). PFT-α alone did not show any apoptotic effect, but increased the cytotoxicity of nutlin-3a (Figure 2b and c) in Mac1. PFT-α had no effect on the P53mut cells, SeAx, or Hut-78 (data not shown). These data indicate that the p53 transcriptional activity is dispensable for apoptosis and may even inhibit the nutlin-induced apoptosis in CTCL cell lines.

We then investigated whether PFT-α can also affect the apoptosis induced by doxorubicin and etoposide. PFT-α did not affect doxorubicin or etoposide cytotoxicity in both P53wt

![Figure 1](https://example.com/figure1.png)
and P53mut CTCL cells (Supplementary Figure S1c and d online), suggesting that the chemotherapeutics-dependent apoptosis mainly occurs independent of the transcriptional activity of p53.

**Nutlin-3a induces senescence followed by cell-cycle block**

Exposure to nutlin induced a complete inhibition of BrdU incorporation in MyLa2000, Mac1, and Mac2a within 24 hours (Figure 3a). Flow cytometric cell-cycle profiling further demonstrated an 80% reduction in the percentage of cells in the S phase, with a concomitant increase in the G0/G1 population (Figure 3b). Mac1 showed a rapid and pronounced apoptosis that made analyzing of the cell cycle difficult. The proliferation of CTCL cells lacking a functional p53 was essentially unaffected even after a long period of exposure to nutlin (up to 1 week, data not shown). The arrest in cell proliferation in Mac2a and Myla2000 was irreversible as it did not resume even up to 40 hours after nutlin-3a removal (Figure 3c).

As cellular senescence is the condition commonly associated with the irreversible G0 cell-cycle arrest, we performed senescence-associated β-galactosidase (SA-β-gal) staining to investigate the possible induction of senescence by nutlin-3a. As shown in Figure 3d, MyLa2000 and Mac2a cells were clearly SA-β-gal positive after 72 hours of exposure to nutlin-3a, whereas P53mut cells (SeAx, Hut-78; Figure 3d) and Sézary cells (data not shown) remained unstained. PFT-α-pretreated cells retained the ability to enter cell-cycle block and senescence after nutlin-3a (Figure 4b and c) even in the absence of p21, which is a known inducer of senescence. This effect of PFT-α was not observed in SeAx and Hut-78 cells (data not shown). Thus, p53 induces senescence in MyLa2000 and Mac2a cells, independently of its transcriptional activation of p21.

### Table 1. CTCL cell lines with P53 mutations

<table>
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<tr>
<th>Cell</th>
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<th>End pos</th>
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<td>R</td>
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<td>196</td>
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</table>

Abbreviations: AA, amino acid; chr17, chromosome 17; freq, frequency; nuc, nucleotide; Ref accno, accession number of the reference sequence; SNP, single-nucleotide polymorphism ID; Start or End pos, start or end position within the reference sequence.

### Figure 2. Inhibition of the p53 transcriptional activities by pifithrin-α (PFT-α) increased nutlin-induced apoptosis.

(a) The p53 and p21 immunoblots of Mac1 lysates pretreated with 25 μM PFT-α for 4 hours, followed by 5 μM nutlin-3a for additional 20 hours. β-Actin was used as the housekeeping gene, and relative protein expression is reported as in Figure 1a. (b, c) Increase in apoptotic, annexin V-positive cells in Mac1 cells pretreated with PFT-α and exposed to nutlin (1.25, 2.5; 24 hours) compared with those not exposed to PFT-α. Flow cytometry was performed as described in Figure 1e. Representative example of (b) three independent experiments and (c) quantification. Columns indicate mean (n = 3) and bars indicate SEM. *P-value <0.05 compared with cells not exposed to PFT-α.
Nutlin-3a sensitized P53wt and P53mut CTCL cells to chemotherapeutic agents

We compared the effect of nutlin-3a on chemotherapy-induced apoptosis in CTCL cells harboring a functional p53 (MyLa2000) or P53mut (Hut-78). We considered three chemotherapeutic agents, bortezomib, doxorubicin, and γ-secretase inhibitor-I (inhibitor of Notch-1), that have previously shown to be powerful pro-apoptotic agents in CTCL cells (Kamstrup et al., 2010a, 2010b). As shown in Figure 5, the apoptotic effect of the three chemotherapeutic agents was significantly enhanced by nutlin-3a in both MyLa2000 and Hut-78. Importantly, nutlin-3a potently increases the apoptosis induced by doxorubicin and bortezomib in primary leukemic Sézary cells (Figure 5c). As Hut-78 cells and Sézary cells lack a functional p53, we concluded that nutlin-3a enhanced the effect of chemotherapy in a p53-independent manner. Moreover, we suggest that other pathways, including the p53-related proteins p73 and E2F1 (Ambrosini et al., 2007), may compensate for p53 function and thus contribute to the increased apoptotic effect in P53mut CTCL cells.
**DISCUSSION**

Owing to the central role of p53 in preventing inappropriate cell proliferation and maintaining genomic integrity following genotoxic stress, there is an interest in the development of pharmacological strategies aimed to manipulate p53 and maximize the selectivity and efficiency of cancer cell eradication. These strategies include gene therapy approaches that intend to restore *de novo* p53 expression and compounds, such as nutilin-3a that relies on induction of p53 activity by relieving p53 from the inhibition of MDM2 (Haupt and Hauert, 2004; Bossi and Sacchi, 2007). In this study, we provided rationale for the future development of nutilin-3a for the therapy of CTCL. We showed that nutilin-3a induces apoptosis and senescence in CTCL cell lines. This effect is dependent on intact p53 as cells with mutated *P53* (Hut-78, SeAx, and primary leukemic Sézary cells) did not respond to nutilin-3a, and downregulation of p53 by siRNA attenuated the apoptotic effect in cells with *P53*wt. However, pifithrin-α, the potent inhibitor of the transcriptional activity of p53, enhanced, rather than attenuated, apoptosis and did not have any influence on cell-cycle arrest after nutilin-3a, strongly indicating that the effect of p53 is transcription independent.

Our data are supported by previous studies that emphasized the complex relationship between the transcriptional and nontranscriptional signaling pathways (Steele et al., 2008; Vaseva et al., 2009). The biological p53 functions may just in part be related to the p53 transcriptional activity, and biochemical actions at the mitochondrial outer membrane can trigger apoptosis in specific settings as tumor suppression (Brady et al., 2011; Wickremasinghe et al., 2011). The p53 transcriptional functions, instead, can paradoxically result in a feedback anti-apoptotic response and, thus, reduce the apoptosis induced by agents that elevate cellular levels of p53. Thus, identifying a strategy to selectively inhibit some p53 functions that impair its therapeutic effect would be valuable for chemotherapy in therapy-resistant CTCL.

It has been suggested that p53-induced senescence is an important mechanism of tumor regression induced by standard genotoxic chemotherapy (Roninson et al., 2001, 2003), and has recently been shown to be a key determinant of tumor response to nutlin-3a (Eleyan et al., 2007; Hasegawa et al., 2009; Villalonga-Planells et al., 2011). Although there was skepticism to the clinical use of pro-senescent therapy, it is now clear that cellular senescence is a robust physiological antitumor response and that senescent cells are cleared by the immune system in vivo (Nardella et al., 2011). Here, we showed that senescence is a major response to agents that induce p53 activity in CTCL. Moreover, we considered the combination of the therapeutic induction of senescence with already established therapies (e.g., doxorubicin and bortezomib) as a possible implementation in the clinical treatment of CTCL. p21 is a common senescence-associated effector, and its persistent expression after nutlin-3a treatment has been associated with senescence in leukemia (Drakos et al., 2009; Hasegawa et al., 2009; Shen and Maki, 2010; Nardella et al., 2011). Surprisingly, we reported that attenuation of p21 by pifithrin-α enhanced rather than attenuated nutilin-induced senescence in CTCL cells. Our data are in agreement with a previous finding that suggested opposing roles of p21 depending on cell type and context (Gartel, 2006). Usually, p21 functions as a tumor suppressor (Philipp et al., 1999; Martin-Caballero et al., 2001), but in some cases may display pro-cancer and anti-apoptotic activities (De la Cueva et al., 2006).
Taking into account the absolute requirement for p53 in nutlin-induced apoptosis and senescence, the finding that nutlin-3a enhanced the effects of chemotherapeutics even in P53mut cells and Sézary cells was surprising. The exact nutlin-3a mechanism in P53mut CTCL cell lines remains unknown, although it has been suggested that other partners of MDM2, including p73 or E2F1, are capable of compensating for p53 function (Ambrosini et al., 2007). The finding that the enhancing efficacy of nutlin-3a on chemotherapy-induced cytotoxicity is preserved in the absence of p53 has consequences for drug development. Preclinical trials combining nutlin-3a with tumor necrosis factor–related apoptosis-inducing ligand or bortezomib are currently being performed for leukemia (Hasegawa et al., 2009; Jin et al., 2010). Although nutlin-3a treatment alone represents a promising therapeutic treatment for MF and CD30+ lymphoproliferative diseases, the lack of a functional p53 impairs the nutlin-3a application in SS. We thus suggest that a nutlin-3a combined treatment could offer a promising treatment of SS.

MATERIALS AND METHODS

Cell cultures and reagents

Five CTCL cell lines were used: MyLa2000, derived from a plaque biopsy of a patient with MF (Gootenberg et al., 1981), SeAx (Kaltoft et al., 1987) and Hut-78 (Gazdar et al., 1980), derived from peripheral blood of patients with SS, and Mac1 and Mac2a, derived from different clinical specimens of a patient showing progression from lymphomatoid papulosis to anaplastic large-cell lymphoma (Davis et al., 1992). Cells were maintained as described (Kamstrup et al., 2008, 2010b). MyLa2000 and SeAx cells were cultured in Gibco...
Glutamax (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum at 37 °C under 5% CO₂, H ut-78, Mac2a, and Mac1 cells were cultured in RPMI-1640 containing 2 mm L-glutamine and 10% fetal bovine serum. N utlin-3a was obtained from Cayman Chemical (Ann Arbor, MI), and γ-secretase inhibitor-1 (Z-Leu-Leu-Nle-CHO) from Merck Calbiochem (Darmstadt, Germany). Bortezomib (Z-Leu-Leu-CHO) was purchased from Selleck (West Paterson, NJ), and doxorubicin hydrochloride from Sigma-Aldrich (St Louis, MO).

Isolation of CD7⁻ CD4⁺ Sézary cells
Peripheral blood mononuclear cells were isolated, after written informed consent, from blood of three patients diagnosed with SS, as described previously (Kamstrup et al., 2010b). CD7⁻ CD4⁺ cells were isolated using the Anti-Biotin Multisort Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) together with an anti-CD7 biotin-conjugated antibody (eBioscience, San Diego, CA) and anti-CD4 micro beads (Miltenyi Biotec) according to the manufacturer’s instructions. Sézary cells were maintained in RPMI-1640 containing 2 mm L-glutamine, 10% fetal bovine serum, and 1% penicillin/streptomycin. The study was approved by the Ethics Committee of Copenhagen and Frederiksborg and was conducted in accordance with the Declaration of Helsinki Principles.

Detection of P53 mutation
Genomic DNA was extracted from 5 x 10⁶ cells using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. Elution was performed in 200 μl AE buffer (Qiagen). The entire open reading frame of the P53 gene was amplified using Platinum Taq DNA Polymerase High Fidelity (Invitrogen) and a specific set of primers (Supplementary Table S1 online). The PCR program included initial DNA denaturation at 94 °C (2 minutes), followed by 35 cycles of 94 °C (20 seconds), 62 °C (20 seconds), and 68 °C (8.5 minutes), and finally extension at 68 °C (5 minutes). The PCR products were separated by electrophoresis on a 1.2% FlasGel DNA cassette (Lonza, Rockland, ME) using the FlasGel system (Lonza). The remaining PCR product was purified by QiaQuick (Qiagen) spin columns to remove leftovers from the PCR. Elution volume was 30 μl of buffer EB (Qiagen). The concentration of each fragment was determined using a fluorescent DNA-binding dye (QuantFlour dsDNA System, Promega, Madison, WI) according to the manufacturer’s protocol. The fragments were then pooled in equimolar ratios. The cell line PCR products were sequenced in multiplex on the GS Junior sequencing platform (454 Sequencing, Branford, CT). Specific bar-code sequences were ligated onto the PCR products of each cell line during the library preparation. Sample preparation and sequencing were done using the manufacturer’s protocol. The obtained sequences were sorted based on the ligated bar-code sequence. The sequence of each cell line was mapped to the DNA sequence of chromosomes 17 downloaded from the University of California, Santa Cruz (UCSC) genome browser (http://genome.ucsc.edu/) using the GS Reference Mapper software (454 Sequencing). Default settings were used for sequence filtering and quality control. The missense mutation of the SeAx cell line was identified in 100% of the sequences and had a total coverage of 81. The nonsense mutation of the Hut-78 cell line was identified in 100% of the sequences and had a total coverage of 163. The missense mutation of the Hut-78 cell line was identified in 97% of the sequences and had a total coverage of 380.

siRNA transfection
Transfection was carried out using 4 μl TurboFect in vitro Transfection Reagent (Fermentas, Burlington, Canada). A volume of 50 nM p53 siRNA or scrambled siRNA from Dharmacon (Thermo Scientific, Chicago, IL) was mixed with 200 μl GIBCO Opti-MEM 1 (Invitrogen) and incubated for 20 minutes before application to 500,000 cells per ml. The efficiency and specificity of knockdown was documented at the protein level by western blot analysis.

Flow cytometry analysis
Measurement of cell-cycle phase distribution and proliferation rate was performed using BrdU/DNA analysis using the Cell Lab Quanta SC MPL flow cytometer (Beckman Coulter, Fullerton, CA). Cells were incubated with 10 μM BrdU. Following cell fixation in ice-cold 70% ethanol overnight, cells were incubated in 2 μM HCl for 30 minutes, washed in neutralizing buffer (0.2 μM Na₂B₄O₇, pH 8.5), and resuspended in dilution buffer (0.5% Tween-20 and 0.5% albumin bovine serum in phosphate-buffered saline (PBS)) before the addition of anti-BrdU mouse antibodies (Becton Dickinson, Franklin Lakes, NJ). Goat anti-mouse antibodies labeled with Alexa-Fluor 488 (1:1,000; Invitrogen) were applied as secondary antibodies. Subsequently, the DNA was counterstained with 7-aminoactinomycin D (Becton Dickinson).

Cell viability was determined using propidium iodide (5 μg/ml) exclusion assay and flow cytometry analysis. Apoptosis was determined by flow cytometry using the FITC-annexin V/propidium iodide protocol of the manufacturer (Beckman Coulter) as previously described (Kamstrup et al., 2010b).

Cellular senescence assay
The SA-β-gal staining was performed as a marker of cellular senescence. The cells were exposed to nutlin-3a for 72 hours and then placed on glass slides by cytospin centrifugation at 13,000 r.p.m. for 8 minutes. Following two washings in PBS (pH 7), samples were fixed in 0.5% glutaraldehyde in PBS (pH 7) for 15 minutes, washed twice with 20 μl 1 mM MgCl₂ in PBS (pH 6), and stained with the SA-β-gal staining solution containing 1 mg/ml of 5-bromo-4-chloro-3-indolyl β-galactopyranosid (0.04 ml dimethylformamide per ml), 0.0012 mM potassium ferrocyanide, and 1 mM MgCl₂ in PBS (pH 6) overnight at 37 °C. Cells were considered positive when the cytoplasm was stained with SA-β-gal.

Cell extracts and western blotting
Whole-cell extracts were prepared as described previously (Kamstrup et al., 2010b). Equal amounts of protein were separated by 12% Bis-Tris gel electrophoresis at 200V, followed by electrophoretic transfer to a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA). p53, MDM2, and p21 from Cell Signalling (Beverly, MA) and anti-β-actin antibody from Sigma-Aldrich were used. Immunoreactivity was detected and quantified with the infrared Odyssey Imaging System (Li-Cor, Lincoln, NE).

Statistics
Continuous data are reported as means with SD. The differences were evaluated using Student’s t-test. Statistical analysis was performed using GraphPad Prism Version 4.03 (GraphPad Software, San Diego, CA) or Excel (Microsoft, Redmond, WA). A P-value of <0.05 was considered significant.
CONFLICT OF INTEREST
The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL
Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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V Manfè et al.
Nutlin-3a in Cutaneous T-Cell Lymphoma

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