Nutlin-3 down-regulates the expression of the oncogene TCL1 in primary B chronic lymphocytic leukemic (B-CLL) cells

Running title: p53-dependent down-regulation of TCL1

Rebecca Voltan1§, Maria Grazia di Iasio1§, Raffaella Bosco1, Nicola Valeri2, Yuri Pekarski2, Mario Tiribelli3, Paola Secchiero1, Giorgio Zauli4

§The first two Authors equally contributed to this work.

1Department of Morphology and Embryology and LTTA Centre, University of Ferrara, Ferrara, Italy; 2Human Cancer Genetics Program and Department of Molecular Virology, Immunology and Medical Genetics, Ohio State University, Columbus, USA; 3Department of Medical and Morphological Research, Division of Hematology and Bone Marrow Transplantation, University Hospital, Udine, Italy; 4Institute for Maternal and Child Health, IRCCS Burlo Garofolo, Trieste, Italy.

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Corresponding author: Rebecca Voltan, Department of Morphology and Embryology and LTTA Centre, University of Ferrara, Via Fossato di Mortara 66, 44100 Ferrara, Italy. Tel: 39-0532-455572; Fax: 39-0532-207351; e-mail: rebecca.voltan@unife.it

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Statement of Translational Relevance: The oncogene TCL1 has been shown to play a key role in promoting leukemic transformation in B chronic lymphocytic leukemia (B-CLL), but whether TCL1 can be modulated by pharmacological treatments is currently unknown. By analyzing the effect of Nutlin-3, a non-genotoxic activator of the p53 pathway, on primary B-CLL patient cells and in a panel of B leukemic cell lines with different p53 status, the authors demonstrated for the first time that Nutlin-3 induces the down-regulation of endogenous TCL1 in a p53-dependent manner. Given the lack of toxic effects of Nutlin-3 in initial human testing, the results of this study provide insight for further clinical evaluation of Nutlin-3 in B-CLL.
Abstract

**Purpose**: The oncogene TCL1 plays a key role in the development of B-chronic lymphocytic leukemia (B-CLL), but it is not known whether TCL1 could be modulated by therapeutic approaches.

**Experimental Design**: B-CLL patient samples (n=35) and B leukemic cell lines (EHEB, JVM2, JVM3, MEC1, MEC2 and BJAB) with different p53 status were exposed to Nutlin-3, a small molecule inhibitor of the MDM2/p53 interaction. Modulations of the steady-state mRNA levels of TCL1 were analyzed by quantitative RT-PCR and Western blot in both primary B-CLL samples and leukemic cell lines. In addition, transfection experiments with either p53 siRNA or with a TCL1-expression plasmid were carried out in the EHEB B-CLL cell line.

**Results**: Upon *ex-vivo* treatment with Nutlin-3, TCL1 was significantly (p<0.05) decreased in 23 out of 28 B-CLL p53^wild-type_. The functionality of the p53 pathway in the same leukemic cell samples was underscored by the concomitant ability of Nutlin-3 to significantly (p<0.05) up-regulate the p53 target gene MDM2 in the p53^wild-type_ leukemic cells. The dependence of TCL1 down-regulation by a functional p53 pathway was confirmed in a panel of B lymphoblastoid cell lines and by p53 knock-down experiments with p53 siRNA. The importance of TCL1 in promoting leukemic cell survival was underscored in transfection experiments, in which TCL1 overexpression significantly counteracted the Nutlin-3-mediated induction of apoptosis in EHEB.

**Conclusions**: Our data indicate that the Nutlin-3 down-regulates TCL1 mRNA and protein, which likely represents an important molecular determinant in the pro-apoptotic activity of Nutlin-3.
Introduction

The T cell leukemia/lymphoma 1 (TCL1) oncogene was discovered as a target of chromosomal translocations and inversions at 14q31.2 in T cell prolymphocytic leukemias (1). The B-cell transforming potential of TCL1 has been subsequently shown in IgVH/µ-TCL1 transgenic mice, which exhibit emergence of clonal CD5⁺/IgM⁺ cells expansion resembling the course and phenotype of IgVH-unmutated human B chronic lymphocytic leukemia (B-CLL) (2-4). It has been previously described that TCL1 shows a differential and regulated expression pattern in B-CLL (5). Different groups of investigators have observed association of high protein levels of TCL1 with features of aggressive disease in B-CLL (6, 7). These results indicate that deregulation of TCL1 is critically important in the pathogenesis of the aggressive form of B-CLL (3). Although the mechanism(s) through which TCL1 mediates its oncogenic effects are incompletely understood, it has been proposed that the pro-oncogenic role of TCL1 could be mediated by hyperactivation of the Akt pathway (8-10).

Nutlin-3 is a small molecule inhibitor of the p53-MDM2 interaction, which leads to increased levels of p53 protein and, subsequently, promotion of induction of cell cycle arrest and apoptosis in a variety of tumor cells (11). The use of Nutlin-3 has been suggested for innovative therapeutic purposes (11). Interestingly, unlike solid tumors, mutations and/or deletions of p53 in hematological malignancies have been detected in less than 20% of patients at diagnosis, mostly in patients with 17p monosomy, which is often associated to p53 mutation of the second allele (12). Of note, recent studies have demonstrated that Nutlin-3, used alone or in combination with chemotherapeutic drugs, effectively increases the degree of apoptosis in different hematopoietic malignancies (13-15), including B-CLL (16-19). In spite of these studies, the potential mechanisms of action of Nutlin-3 are still not completely
understood. In particular, both transcriptional-dependent and transcriptional-independent mechanisms have been proposed to explain the cytotoxic activity of Nutlin-3 (20-24).

On these bases, the aim of this study was to investigate the effect of Nutlin-3 on the expression levels of TCL1 in primary B-CLL patient samples, as well as in a panel of B lymphoblastoid cell lines.
Materials and Methods

Primary B-CLL patient cells and continuous cell lines

For experiments with primary cells, peripheral blood samples were collected in heparin-coated tubes from 35 B-CLL patients following informed consent, in accordance with the Declaration of Helsinki and with approval obtained from the institutional review board of the University-Hospital of Udine (Udine, Italy). The diagnosis of B-CLL was made by peripheral blood morphology and immunophenotyping. Rai stage and doubling time (DT) were abstracted from clinical records. B-CLL samples were characterized for ZAP70 levels, p53 mutational status and chromosomal abnormalities by interphase fluorescence in situ hybridization (FISH), as previously described (17). All patients had untreated CLL without history of relapse. Peripheral blood mononuclear cells (PBMC) were isolated and cultured as described in Supplementary Methods.

The p53^{wild-type} (EHEB, JVM-2, JVM-3) and p53^{deleted/mutated} (MEC1, MEC2, BJAB) B leukemic cell lines were purchased from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany) and cultured as described in Supplementary Methods.

Culture treatments and assessment of cell viability and apoptosis

Both primary B-CLL cells and lymphoblastoid cell lines were seeded at a density of 1x10^6 cells/ml before treatment with Nutlin-3 (10 µM; Cayman Chemical, Ann Arbor, MI). Cell viability was examined at 24h after treatment by Trypan blue dye exclusion (25) and confirmed by MTT (3-(4,5-dimethilthiazol-2yl)-2,5-diphenyl tetrazolium bromide) colorimetric assay (Roche Diagnostics Corporation, Indianapolis, IN). The induction of apoptosis was quantified by Annexin V-FITC/propidium iodide (PI) staining (Immunotech,
Marseille, France) followed by flow cytometry analysis, as previously detailed (26).

RNA analyses

Total RNA was extracted from cells by using the Qiagen RNeasy Plus mini kit (Qiagen, Hilden, Germany) according to the supplier’s instructions and as previously described (27). For detailed description of procedures please refer to Supplementary Methods.

Western blot analyses

Cells were lysed in Ripa buffer (50 mM Tris pH 7.5, 150 mM NaCl, 0.1% SDS, 1% Nonidet P-40, 0.25% sodium deoxycholate) supplemented with protease inhibitors (Roche; Germany), and processed as previously described (28). Equal amounts of protein for each sample were migrated in acrylamide gels and blotted onto nitrocellulose filters. The following mAb were used in our experiments: anti-ZAP70 (Millipore, Billerica, MA), anti-MDM2 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-TCL1 (Cell Signaling Technology, Danvers, MA) and anti-tubulin (Sigma-Aldrich, St Louis, MO). After incubation with peroxidase-conjugated anti mouse IgG, specific reactions were revealed with the ECL detection kit (Amersham Pharmacia Biotech).

Transfection experiments

EHEB cells (7x10^6) were resuspended into 0.1 ml of Nucleofector\textsuperscript{TM} solution of human nucleofector kit V (Amaxa, Cologne, Germany). Two µg of plasmid DNA (GFP-construct) were mixed with the 0.1 ml of cell suspension, transferred into a 2.0 mm electroporation cuvette, and nucleofected using an Amaxa Nucleofector II apparatus (program U-013), following the manufacturer guidelines. Transfection efficiency was estimated in each experiment by scoring the number of GFP-positive cells by flow cytometry analysis. In other
experiments, cells were transfected with the mammalian vector pcDNA3TCL1fl expressing the full-length TCL1 (29), or with the control empty plasmid pcDNA3. For siRNA transfection description, please refer to Supplementary Methods.

Statistical analysis

Data are calculated and shown as mean±SD or as median and Interquartile Range (IQR), according to the distribution. The results were evaluated by using analysis of variance with subsequent comparisons by Student’s t-test and with the Mann-Whitney rank-sum test. Statistical significance was defined as p<0.05.
Results

Differential baseline levels of TCL1 in primary B-CLL samples and B lymphoblastoid cell lines with ZAP70\textsuperscript{high} versus ZAP70\textsuperscript{low/absent}

B-CLL patient samples enrolled in this study (n=35) were characterized for ZAP70 expression levels, chromosomal aberrations and presence of p53 deletion and/or mutations, as summarized in Table 1. Baseline levels of TCL1 mRNA evaluated by RT-PCR (Figure 1A) were significantly (p<0.01) higher in those patient samples with higher ZAP70 expression, evaluated by Western blot analysis as exemplified in Figure 1B. Similar findings were observed when TCL1 mRNA was analyzed in a panel of B lymphoblastoid cell lines with different ZAP70 levels (Figure 1B). Also in this case, EHEB, MEC1 and MEC2 cell lines, characterized by ZAP70\textsuperscript{high} expression, showed significantly (p<0.05) higher levels of TCL1 mRNA with respect to BJAB, JVM2 and JVM3 cell lines, characterized by ZAP70\textsuperscript{low/absent} expression (Figure 1A-B).

The non-genotoxic activation of the p53 pathway down-regulates TCL1 in p53\textsuperscript{wild-type} but not in p53\textsuperscript{mutated} B primary B-CLL cells and B lymphoblastoid cell lines

Among the 35 B-CLL patients involved in our study, 12 showed a 17p- deletion at FISH analysis (patients #1, 2, 6, 11, 22, 23, 28, 29, 30, 31, 33, 34 of Table 1). Six patients with 17p- deletion also presented a mutation in the remaining TP53 allele, consisting mainly of amino-acid substitutions (patients #28, 29, 30, 33, 34) or deletion (patient #31) in exons involved in DNA binding function. An additional patient showed p53 mutation resulting in an amino-acid substitution, in the absence of 17p- deletion (patient #32 of Table 1). These parameters are relevant taking into consideration that the mechanism of action of Nutlin-3 requires an intact p53 pathway for optimal activity (11).
After treatment with Nutlin-3 for 24 hours, a significant (p<0.05) down-modulation of TCL1 mRNA with respect to untreated samples was observed in 22 out of 28 B-CLL with p53 wild-type, including 6 samples carrying 17p- and only in 1 out of 7 B-CLL with p53 mutated. On the other hand, TCL1 mRNA levels were completely unaffected in 5 B-CLL samples carrying 17p- and p53 mutated and in 1 p53 mutated B-CLL without 17p- (Figure 2A). Consistently, Nutlin-3 down-regulated TCL1 mRNA levels also in p53 wild-type but not in p53 mutated B lymphoblastoid cell lines (Figure 2A). The functionality of the p53 pathway in these leukemic cell samples was underscored by the concomitant ability of Nutlin-3 to significantly (p<0.05) up-regulate the steady-state mRNA levels of the p53 target gene MDM2 (11) in p53 wild-type but not in the p53 mutated cell lines (Figure 2B). The opposite modulation of TCL1 and MDM2 in p53 wild-type, but not in p53 mutated, leukemic cells in response to Nutlin-3 treatment was confirmed also at protein levels (Figure 2C).

The knock-down of p53 counteracts the Nutlin-3- down regulation of TCL1

The data illustrated above suggested, but did not prove, that the Nutlin-3-mediated down-regulation of TCL1 requires a functional p53 pathway. In this respect, a direct role for p53 in regulating/repressing the TCL1 levels was further suggested by the observation that computational analysis (as predicted by SABiosciences' Text Mining Application and the UCSC Genome Browser) on TCL1 promoter demonstrated the presence of some p53 consensus DNA binding sites (Figure 3A). In order to clarify the p53-dependence of the observed gene modulations, we have analyzed the effect of Nutlin-3 in EHEB cells transiently transfected with p53 siRNA (Figure 3B). Since in EHEB cells transfection efficiency is approximately 30-40%, p53 is not completely silenced but yet significantly knocked-down, as evaluated by quantitative RT-PCR assay (data not shown). The ability of Nutlin-3 to decrease TCL1 RNA levels was significantly (p<0.05) attenuated with respect to cells transfected with
control scramble siRNA (Figure 3B).

TCL1 over-expression counteracts the Nutlin-3-induced apoptosis in EHEB cells

Considering previous studies suggesting that TCL1 expression has been linked to an aggressive behavior of B-CLL (5, 6), in the last group of experiments we sought to investigate the role played by TCL1 in modulating the response to Nutlin-3. For this purpose, upon transfection of the p53<sup>wild-type</sup> EHEB cell line with the TCL1 expressing plasmid (Figure 4A), we could observe that the over-expression of TCL1 efficiently (p<0.05) counteracted the cytotoxicity induced by Nutlin-3, as indicated by the recovery of the cell viability and by the reduction of the percentage of apoptotic cells (Figure 4B). These data support the idea for a critical role of TCL1 in modulating the anti-leukemic activity of Nutlin-3.
Discussion

Previous studies have shown that the pattern of response to B-cell receptor (BCR) engagement in B-CLL is highly correlated with cellular levels of the lymphoid oncogene TCL1 and with the formation of activation complexes at the BCR that include TCL1, AKT, and membrane-proximal tyrosine kinases such as ZAP70. The CLL cases with high TCL1 showed also more aggressive growth features in vivo, including advanced clinical stage, higher white blood cell counts, and shorter lymphocyte doubling time, poor response to all therapy types, with TCL1 levels as an independent predictor of outcome in multivariate models (5, 6).

While little doubt exists on the link between Nutlin-3-mediated transcriptional activity of p53 and cell cycle arrest mediated by p21, some recent studies have provided evidence for a transcription-independent induction of apoptosis by Nutlin-3 (20-22, 30, 31). In particular, these studies have shown that the transcription-independent mitochondrial p53 program plays an important role in Nutlin-3-induced p53-mediated tumor cell apoptosis. In fact, aside from nuclear stabilization, Nutlin-3a causes cytoplasmic p53 accumulation and translocation to mitochondria (20, 30). However, previous studies from our group have shown that the ability of Nutlin-3 to induce a characteristic gene expression signature directly correlates with the cytotoxic activity of Nutlin-3 (24). Starting from these bases, in this study we have demonstrated for the first time that Nutlin-3 down-regulated the oncogene TCL1 in the majority of primary B-CLL samples carrying wild-type p53 (22 out of 28), including 6 samples with the unfavorable chromosomal abnormality 17p-, while B-CLL samples carrying mutated p53 did not show any decrease of TCL1. Nutlin-3 significantly down-regulated TCL1 mRNA levels also in p53\textsuperscript{wild-type} but not in p53\textsuperscript{mutated} B lymphoblastoid cell lines. The p53 dependence of TCL1 down-regulation was demonstrated by experiments performed with
siRNA specific for p53. In fact, knock-down of p53 counteracted the ability of Nutlin-3 to down-regulates TCL1 in leukemic cells. Although relatively little is known about the regulation of TCL1 expression, and previous data have shown that TCL1 can be regulated by selective miRNA (32), two potential p53 binding sites are present in the TCL1 promoter, suggesting that p53 might directly induce the transcriptional repression of TCL1.

Another important conclusion of our study is that TCL1 over-expression counteracts Nutlin-3-mediated apoptosis in lymphoblastoid B cells, substantiating an important role of TCL1 in promoting leukemogenesis. Thus, although it has been argued that transcriptional independent mechanisms play a key role in mediating the cytotoxic activity of p53 (20-22, 30, 31), perhaps due to the presence of negative feed-back loops (33), our data demonstrate for the first time that the transcriptional down-regulation of the oncogene TCL1 likely represents an important mechanism of action by which Nutlin-3 promotes apoptosis in leukemic cells.

In conclusion, we have demonstrated for the first time that activation of p53 represses the transcription of TCL1 in primary B-CLL cells and p53\textsuperscript{wild-type} lymphoblastoid cells and we propose that such down-regulation is an important mediator of Nutlin-3 cytotoxic activity. Therapeutic strategies able to down-regulate TCL1 should be further explored in order to improve the anti-leukemic activity of Nutlin-3.
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References


Table 1. Clinical and laboratory features of untreated CLL patients

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CLL indicates chronic lymphocytic leukemia; DT, doubling time; na, not available.

†FISH defects were found using a B-CLL FISH panel.

‡ZAP70 expression was determined by Western Blot analysis.
Figure Legends

Figure 1. Correlation between ZAP70 expression and high baseline levels of TCL1 mRNA in B-CCL cells. Baseline TCL1 expression was comparatively analyzed by quantitative RT-PCR in primary B-CLL patient samples (Table 1) and in B-CLL lines (EHEB, MEC1, MEC2, JVM2, JVM3 and BJAB). Levels of TCL1 mRNA were analyzed in relationship to the ZAP70 (low versus high) expression. Horizontal bars are median, upper and lower edges of box are 75th and 25th percentiles, lines extending from box are 10th and 90th percentiles. Asterisk: p<0.05. In B, ZAP70 protein levels, analyzed by Western blot, are shown for representative B-CLL patient (Pt.) samples and for the B-CLL cell lines. Tubulin staining is shown as loading control.

Figure 2. Transcriptional down-modulation of TCL1 by Nutlin-3 in p53"wild-type" B-CLL cells. B-CLL leukemic patient samples (Table 1) and cell lines (EHEB, MEC1, MEC2, JVM2, JVM3 and BJAB) were either left untreated or exposed to Nutlin-3 (10 μM). After 24 hours of treatment, mRNA levels of TCL1 and MDM2 (analyzed as internal control, for comparison) were assessed by quantitative RT-PCR. Results were expressed as fold of either TCL1 (A) or MDM2 (B) modulation in Nutlin-3 treated-cultures with respect to the control untreated-cultures, which were set to 1 (white bars). TCL1 and MDM2 mRNA modulation was compared between the p53"wild-type" (p53"wt") and the p53"mutated" (p53"mut") leukemic patient cell samples or cell lines. In A and B, data are expressed as means±SD. Asterisk: p<0.05. In C, TCL1 and MDM2 protein modulation by Nutlin-3, analyzed by Western blot, are shown for representative B-CLL patient (Pt.) samples and B-CLL cell lines after 24 hours of treatment. Tubulin staining is shown as loading control.
Figure 3. Role of p53 in TCL1 transcriptional down-modulation by Nutlin-3. A, Regulatory p53 transcription factor binding sites in the TCL1 gene sequence. B, TCL1 mRNA modulation by Nutlin-3 was analyzed after 24 hours of treatment in EHEB cells either not transfected, or transiently transfected with p53 siRNA or with control scramble (scr.) siRNA. Data are reported as means±SD of results from at least three experiments, each performed in triplicate. Asterisks, p<0.05.

Figure 4. TCL1 overexpression counteracts Nutlin-3 cytotoxicity. EHEB cells were transiently transfected with pTCL1-expressing vector or with control empty vector (A). EHEB cell culture, either left not transfected, or transfected as indicated, were exposed to Nutlin-3 (10 μM) and analyzed for apoptosis induction (B), after 48 hours of treatment. Data are reported as means±SD of results from at least three experiments, each performed in triplicate. Asterisks, p<0.05.
Figure 1

A

B

TCL1 mRNA baseline levels (R.U.)

ZAP70^{High}  ZAP70^{Low}  ZAP70^{High}  ZAP70^{Low}

B-CLL patients  B-cell lines

p<0.01  p<0.05

ZAP70

Tubulin

7AP70

Tubulin

EHEB  MEC2  JVM2  JVM3  BJAB  MEC1

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Figure 2

A

B

C

TCL1 mRNA modulation (Nutlin-3/untreated)

Untreated
Nutlin-3

p53wt
p53mut

B-CLL patients
B-cell lines

MDM2 mRNA modulation (Nutlin-3/untreated)

Untreated
Nutlin-3

p53wt
p53mut

B-CLL patients
B-cell lines

Nutlin-3

CLlCD
MEG2
JVM2
JVM3
Pt.2
Pt.29
Pt.10

TCL1
MDM2
Tubulin

- - - - - + - -

* * *
Figure 3

A

B

TCL1 mRNA modulation (Nutlin-3/untreated)

Untr.
not transf.
scr. siRNA
p53 siRNA

transfected

Nutlin-3
Figure 4

A

B

Transf. empty pTCL1

TCL1- Tubulin -


eempty pTCL1

Apoptosis (%)
Nutlin-3 down-regulates the expression of the oncogene TCL1 in primary B chronic lymphocytic leukemic (B-CLL) cells

Rebecca Voltan, Maria Grazia di Iasio, Raffaella Bosco, et al.

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