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p53 and cell cycle independent dysregulation of autophagy in chronic lymphocytic leukaemia

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Background: Activation of wild-type p53 with the small molecule sirtuin inhibitor Tenovin-6 (Tnv-6) induces p53-dependent apoptosis in many malignant cells. In contrast, Tnv-6 reduces chronic lymphocytic leukaemia (CLL) cell viability with dysregulation of autophagy, without increasing p53-pathway activity.

Methods: Here, we have investigated whether a quiescent phenotype (unique to CLL) determines the Tnv-6 response, by comparing the effects of Tnv-6 on activated and proliferating CLL. We further studied if these responses are p53-dependent.

Results: Unlike quiescent cells, cell death in activated cultures treated with Tnv-6 was consistently associated with p53 upregulation. However, p53 acetylation remained unchanged, without caspase-3 cleavage or apoptosis on electron microscopy. Instead, cellular ultrastructure and protein profiles indicated autophagy inhibition, with reduced ubiquitin–proteasome activity. In specimens with mutant *TP53* cultured with Tnv-6, changes in the autophagy-associated protein LC3 occurred independently of p53. Cells treated with Tnv-6 analogues lacking sirtuin inhibitory activity had attenuated LC3 lipidation compared with Tnv-6 ($P \leqslant 0.01$), suggesting that autophagy dysregulation occurs predominantly through an effect on sirtuins.

Conclusion: These cell cycle and p53-independent anti-leukaemic mechanisms potentially offer novel therapeutic approaches to target leukaemia-sustaining cells in CLL, including in disease with p53-pathway dysfunction. Whether targets in addition to sirtuins contribute to autophagy dysregulation by Tnv-6, requires further investigation.

Tenovin-6 (Tnv-6) is a small molecule that inhibits members of the sirtuin family of histone deacetylases (Lain *et al*, 2008). As non-histone proteins are also deacetylated by sirtuins, including the tumour suppressor p53, Tnv-6 is a non-genotoxic p53 activator. In cell lines derived from several solid organ malignancies, and chronic myeloid leukaemia, Tnv-6 increases levels of acetylated p53 and stimulates p53-dependent apoptosis (Lain *et al*, 2008; Li *et al*, 2012; Yuan *et al*, 2012). These properties make Tnv-6 potentially relevant to the management of most patients with symptomatic chronic lymphocytic leukaemia (CLL), a disease in which p53 activity is a known determinant of outcomes after genotoxic chemotherapy (Pettitt *et al*, 2001; Rosenwald *et al*, 2004;

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Zenz *et al*, 2008). However, when the effects of Tnv-6 were studied in primary human CLL cells (MacCallum *et al*, 2013), unexpectedly, no consistent increase in the expression of p53 or proteins associated with the p53 pathway was detected, despite evidence for dose-dependent cytotoxicity. Further studies indicated that death in CLL cells was non-apoptotic and associated with the dysregulation of autophagy.

Reasons for the differences between the effects of Tnv-6 on CLL cells and other types of neoplasia (Lain *et al*, 2008; Li *et al*, 2012; Yuan *et al*, 2012) require investigation. As the effects of sirtuin inhibitors can be tissue or context-specific (Cea *et al*, 2011), the autophagy-dysregulatory properties of Tnv-6 could be unique to CLL cells. In contrast to the highly proliferative cells examined in previous studies on Tnv-6 (Lain *et al*, 2008), CLL cells are relatively quiescent in culture. Although these non-dividing cells constitute the bulk of the leukaemic compartment *in vivo*, these have extensive proliferative histories (Lin *et al*, 2008). In addition, there is increasing evidence for the existence of sub-populations of cells with enhanced replicative potential that maintain the disease and are responsible for progression (Messmer *et al*, 2005; Calissano *et al*, 2009).

We hypothesised that the cell cycle and activation status of CLL cells may influence the effects of Tnv-6, with p53-dependent apoptosis being a feature of dividing cells and autophagy dysregulation being characteristic of non-cycling cells. Indeed, in CLL cells stimulated to divide *in vitro*, treatment with the sirtuin inhibitor nicotinamide has been shown to increase p53-pathway function and apoptotic cell death (Audrito *et al*, 2011). If these cellular responses were to be reproduced in studies with Tnv-6, then its cell cycle dependent effects would suggest the ability to target biologically heterogeneous leukaemic cell compartments through different mechanisms, as described with protein kinase inhibition (Natoni *et al*, 2011). In addition, the ability to demonstrate p53-dependent apoptosis would provide the rationale for the inclusion of Tnv-6 in therapeutic strategies for previously untreated patients with progressive CLL, most of whom have normal p53-pathway activity, but not in disease characterized by mutations in, or downstream of *TP53* (Zenz *et al*, 2008; Lin *et al*, 2012).

In the present studies, we have analysed the responses of metabolically active and proliferating CLL cells to treatment with Tnv-6. In addition, we have investigated whether the cellular response to Tnv-6 is altered in the presence of *TP53* dysfunction.

MATERIALS AND METHODS

Specimen selection. Blood specimen collection from consenting CLL patients was approved by the Tayside Committee on Medical Research Ethics. All studies were performed following approval from Tayside Tissue Bank. Cells from a total of 21 patients (median age 70 years, range 58–86) were studied. Nine patients had received previous therapy, but no patient was on treatment at the time of blood collection. Enriched populations of CLL cells were isolated from blood as described previously (MacCallum *et al*, 2013). Prior to studies with Tnv-6, the integrity of p53 induction in these cells was confirmed in 24 h cultures with the genotoxic drug fludarabine (3 mM, Sigma, Dorset, UK).

CLL cell activation. Chronic lymphocytic leukaemia cells were cultured at a final concentration of 2×10^6 cells ml^{-1} using 10% fetal calf serum in RPMI (culture medium) in a 5% CO_2 humidified incubator at 37 °C. To activate cells, cultures were treated with 100 units ml^{-1} interleukin-2 (IL-2, Novartis, Surrey, UK) and 1 μM ml^{-1} CpG oligonucleotides (CpG ODN, DSP30, TIB MolBiol GmbH, Berlin, Germany) (Decker *et al*, 2000). Controls consisted of cells incubated in culture medium. Cells recovered at 24, 48, 72 and 96 h from IL-2/CpG ODN containing and control cultures were assayed for activation and proliferation, through morphological assessment, spectrophotometric measurement of intracellular ATP content and flow cytometric analysis of the expression of Ki-67 and DNA content using propidium iodide (PI) labelling (Supplementary Methods). The effects of Tnv-6 were then investigated by treating optimally activated cultures with 10 μM Tnv-6 for a further 4 or 24 h.

Measurement of cell viability. Viability of CLL cells was studied by flow cytometric analysis of cells stained with 7-aminoactinomycin D (7-AAD) (Tauro *et al*, 2002; Zembruski *et al*, 2012). Data from 10 000 cells was acquired on a FACScan (Becton-Dickinson, Oxford, UK) within 2 h of fixation and the proportion of live and dead cells was analysed using Cell Quest software (Becton-Dickinson).

Intracellular protein expression. Changes in intracellular proteins following culture with Tnv-6 were analysed by western blotting as described previously (MacCallum *et al,* 2013). The following antibodies were used during the study: DO-1 anti-p53 (1 : 1000) or polyclonal anti-p53 (1 : 3000, SAPU, Karluke, UK), p21^{waf1} (1 : 1000, Cell Signalling, Hertfordshire, UK), anti-p53 acetylated at Lysine position 320 (K-320), at K-373 (both 1 : 1000, Upstate Biotechnology, USA) or at K-382 (1 : 500, Bio-Legend, San Diego, CA, USA), anti- microtubule-associated proteins 1 A/1B light chain 3 A (LC3, 1 : 1000, Sigma), p62/Sequestosome 1 (1 : 5000, Abnova, Taipei, Taiwan), anti-cleaved caspase-3 (1 : 1000, Cell Signaling Technology, Danvers, MA, USA) and anti-ubiquitin (1 : 2000, Abcam, Cambridge, UK). The protocol was modified slightly for the analysis of ubiquitinated proteins (Dayal *et al*, 2009). In brief, equal amounts of proteins for each sample were migrated in 4–12% SDS-PAGE in MES SDS Running Buffer (Life Technologies, Paisley, UK) and blotted onto nitrocellulose membranes. After washing in PBS, the membranes were boiled in deionized water for 30 min to expose ubiquitin epitopes before blocking and incubation with the primary anti-ubiquitin antibody.

To control for protein loading, each membrane was re-probed with a mouse monoclonal antibody to β-actin (1:1000, Santa Cruz Biotechnology, Dallas, TX, USA). Changes in protein levels were quantified through densitometric analysis with ImageJ software.

Cellular ultrastructure. To study sub-cellular changes, 10×10^6 cells cultured with Tnv-6 for 4 h were fixed in 2.5% glutaraldehyde, 4% paraformaldehyde in 0.1 M cacodylate buffer, processed and imaged using a JEOL 1200 EX transmission electron microscope.

Single-cell analysis for LC3 expression and *TP53*. In Tnv-6-treated cells from three patients with monoallelic loss of *TP53*, combined fluorescence-based assessment of the autophagy-associated protein LC3 and *in situ* hybridisation (FISH) for *TP53* was undertaken to investigate changes in autophagy relative to *TP53* status. In two patients, the burden of cells with *TP53* loss exceeded 80% of the leukaemic cells. In the third patient, *TP53* was deleted in 57% of cells. Mutational analysis by *TP53* sequencing in two patients identified the missense mutations c.817C > A (exon 8, with resulting p.R273S) in one and c.613T > G (exon 6, causing p.Y205D) in the other, both associated with loss of p53 transcriptional activity (International Agency for Research in Cancer, *TP53* Database, http://p53.iarc.fr).

For combined single-cell analysis, cells from cultures with or without Tnv-6 were fixed, permeabilised and stained with anti-LC3 (1:500, MBL, St Woods Hole, MA, USA) followed by Alexa Fluor 488 goat anti-mouse secondary antibody (Life Technologies). Slides were then stained with DAPI II (Abbot Molecular, Berkshire, UK) to enable localisation of nuclei and viewed on a Nikon Eclipse Ti fluorescence microscope. Fluorescence from LC3 puncta from a total of 50 cells was captured using an Olympus BX61 (Hamburg, Germany) fluorescence microscope with X-Cite fluorescence

system and CytoVision software to create Z-stacked images. Following image capture, slides were washed before being permeabilised in sodium thiocyanate (Abbott, Abbott Park, IL, USA) at 80 °C for 15 min. Following further washes in distilled water and sodium citrate buffer (pH 7.0), slides were treated with protease solution (Abbott) for 10 min at 37 °C after which *in situ* hybridisation with the CEP17/*TP53* (17p13.1) (Abbott) probes was performed as described previously (Groves *et al*, 2012). Fluorescence was captured from cells in the same microscopy field-settings as for LC3 determination, enabling simultaneous analysis of LC3 fluorescence and *TP53* signals in 50 individual cells. The difference in the mean corrected total cell fluorescence between Tnv-6-treated and control cultures was compared using ImageJ analysis.

Cellular response to Tnv-6 analogues. To investigate whether the effects of Tnv-6 in CLL cells are mediated through the targeted inhibition of sirtuins (Lain *et al*, 2008), activated cultures were treated with structural analogues that have reduced activity against recombinant human SirT1 and SirT2. Several reports on the preparation and characterisation of Tnv-6 analogues have been reported to date (Lain *et al*, 2008; McCarthy *et al*, 2012). Tenovins-30q and −37 were prepared as described previously in the literature (McCarthy *et al*, 2012; Pirrie *et al*, 2012) whereas the novel analogue Tenovin-45 was prepared as described in the Supplementary Material. All three analogues lack activity against SirT1 and SirT2 *in vitro*, but their water solubility and ability to cross biological membranes are likely to be the same as Tnv-6. Changes in p53 and LC3 expression in optimally activated CLL cells were examined after 4 h of culture with 10 μM of each analogue and compared with Tnv-6.

Statistical analysis. The arithmetic mean was used to measure the central tendency of data. The dispersion of values around the mean was expressed as the standard deviation (s.d.) in analysis of raw data, or as standard error of the mean (s.e.m.) when mean values were being compared. The significance of difference between the means of untreated control and corresponding Tnv-6-treated cells was tested using the paired Student's *t*-test. All *P*-values were two-tailed and statistical significance was set at the level of $P < 0.05$.

RESULTS

p53 pathway function is intact in CLL specimens. Presence of intact p53-pathway function in CLL cells was confirmed by quantifying changes in intracellular p53 in cells treated with the genotoxic drug fludarabine. After 24 h, increased expression of p53 was detected in cells from all patients compared with untreated cultures (Supplementary Figure S1). By densitometric quantification, a mean 2.0-fold increase in p53 induction was present (range: 1.3–3.2; $P = 0.0038$). In addition, it was possible to identify cleaved caspase-3 in all treated specimens (Supplementary Figure S1), indicating the integrity of p53-pathway function and the suitability of the specimens for analysis of p53 responses to Tnv-6.

IL-2/CpG ODN optimally activates CLL cells between 36 and 48 h. In replicate cultures from each specimen ($n = 9$), changes in cellular morphology, ATP levels, Ki-67 expression and DNA content following culture in IL-2/CpG ODN for 24, 48, 72 and 96 h identified the period of optimal CLL cell activation as being between 36 and 48 h (Supplementary Table S1). To study the effects of Tnv-6, cultures activated with IL-2/CpG ODN for 42 h were incubated for a further 4 h (total culture time of 46 h) or 24 h (total culture time of 66 h) with 10 μM Tnv-6. Cell responses were compared with those of quiescent cells cultured in growth medium for 42 h followed by incubation with Tnv-6 (10 μM) for an additional 4 or 24 h.

Effects of Tnv-6 on CLL cells

Tnv-6 causes equivalent cytotoxicity in activated and quiescent cultures. Cellular viability following incubation with Tnv-6 was assessed by flow cytometric analysis of 7-AAD-stained cells from nine sets of quiescent and activated cultures. Levels of cell death varied amongst control and Tnv-6 containing cultures at 24 and 4 h, indicating the biological heterogeneity of CLL. Analysis of raw data using the Student's *t*-test for paired samples indicated a higher proportion of 7-AAD$^{int/high}$ (non-viable) cells in the presence of Tnv-6 in quiescent cultures at 24 ($P = 0.0008$) and 4 h ($P = 0.0025$), as well as in activated cultures at these time-points ($P = 0.0002$ and 0.017, respectively). These differences are illustrated in Figure 1; for simplicity of presentation, the mean result from Tnv-6-treated cultures has been normalised to the mean for controls. A representative FACS profile of 7-AAD labelling without (control) and in the presence of Tnv-6 is shown in Supplementary Figure S2A–D.

Tnv-6 increases p53 without induction of apoptosis. Changes in intracellular proteins in CLL cells following Tnv-6 treatment were investigated in 4 h cultures (Figure 2). Treatment of activated cultures with Tnv-6 associated with consistent increases in p53 expression. The mean increase of 11-fold (range 5–23, $P = 0.0007$, $n = 9$) contrasted with the lack of significant p53 expression observed under culture conditions where CLL cells are quiescent. In addition, there was a 1.4 ± 0.23-fold increase in expression of p21^{waf1} ($P = 0.005$). The functional consequences of p53 induction for apoptosis in activated cells were investigated further through an analysis of changes in caspase-3 (Fuchs *et al*, 1997). However, there was no increase in cleaved caspase-3 in either quiescent or activated cells despite elevated levels of p53 (Figure 2). Furthermore, p53 acetylation at Lysine residues 320, 373 or 382 (targeted by sirtuins) (Knights *et al*, 2006) remained unchanged (Supplementary Figure S3), despite the increases in total p53.

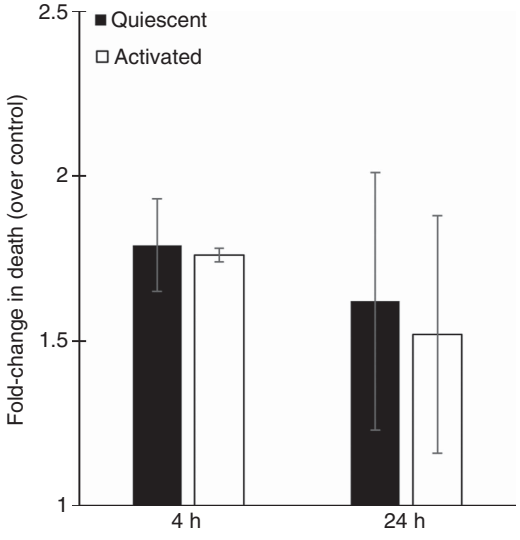

Figure 1. Cytotoxicity of Tnv-6 to quiescent and activated CLL cells. The proportion of 7-AAD$^{int/high}$ cells in cultures was used to measure cytotoxicity with Tnv-6 and expressed relative to untreated control cultures. Significant levels of cytotoxicity were observed with Tnv-6 at both 4 and 24 h in cultures conditions favouring quiescence as well as activation.

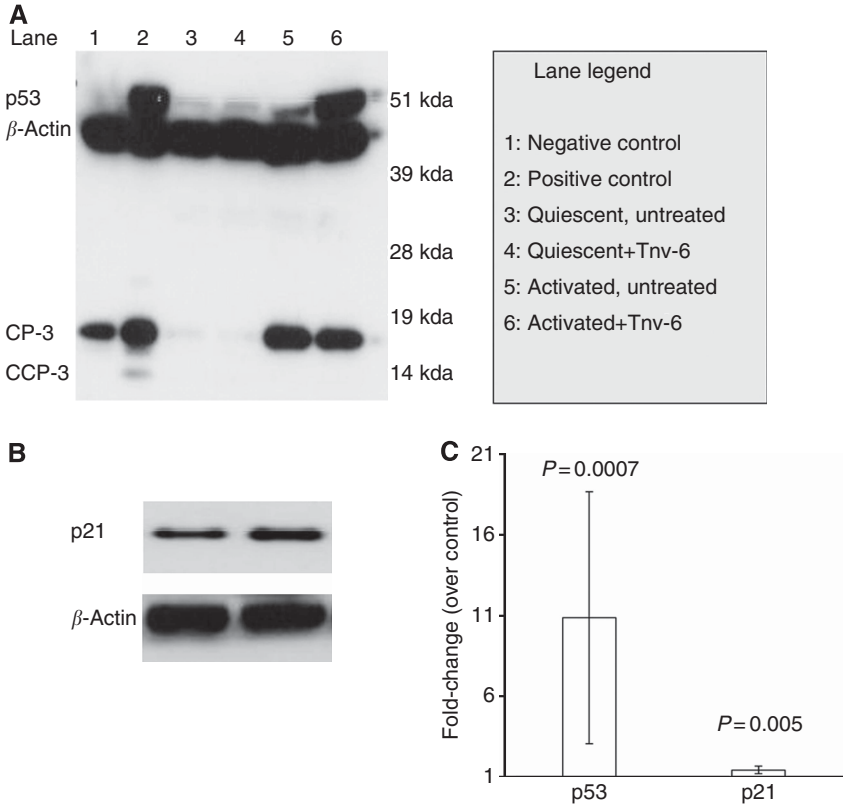

Figure 2. p53 expression in activated CLL cells treated with Tnv-6. Representative western blot gel images demonstrate increased p53 in cells from Tnv-6-treated activated CLL cultures (Tnv-6) compared with untreated cultures and quiescent cultures without (untreated) or with Tnv-6 (**A**). However, total caspase-3 (CP-3) remained unchanged with no detectable cleaved caspase-3 (CCP-3) in cells from cultures with Tnv-6. The validity of the assay is confirmed by the detection of CCP-3 in the positive control consisting of protein from CLL cells cultured with fludarabine.
An increase in $p21^{waf1}$ expression also occurred following Tnv-6 treatment of activated cells (right lane in **B**) compared to cells from control cultures (left lane in **B**); however, the overall magnitude of the change compared with controls (1.4-fold; $P = 0.005$) was lower than the 11-fold increase ($P = 0.0007$) observed with p53 (**C**).

Tnv-6 inhibits autophagy. Transmission electron microscopy (TEM), performed to clarify the mode of cell death following Tnv-6 treatment, showed no ultrastructural cellular changes to suggest apoptosis (Taatjes *et al*, 2008) ($n = 3$). Instead, an alteration in autophagosomes was evident from the increase in intracytoplasmic double-membraned vacuoles containing cytoplasmic components (Figure 3). Number of autophagosomes per cell (from a total count of 50 cell sections per culture) were 12-fold higher (range 9–18) at 4 h in Tnv-6 containing activated cultures than in corresponding untreated controls ($P = 0.01$). Similarly, and consistent with our previous results (MacCallum *et al*, 2013), a significant increase in autophagosome numbers was also observed in cells from quiescent cultures treated with Tnv-6, but the magnitude of the change was less than in activated cells (data not shown).

The dysregulation of autophagy was further investigated in protein expression studies. The expression profile of the autophagy-associated proteins LC3 and p62 in quiescent cells treated with Tnv-6 was identical to that described previously (MacCallum *et al*, 2013), and therefore, only results with activated cells will be described hereafter. Culture with Tnv-6 resulted in a mean 2.7-fold (range 1.8–4.2) increase in the lipidated form of LC3 (LC3 II) ($P = 0.0003$, $n = 9$, Figure 4). Together, the TEM features and accumulation of lipidated LC3 indicated a change in autophagy in Tnv-6-treated CLL cells. As similar increases in autophagosomes can occur during autophagy induction (Hussain *et al*, 2008) or inhibition (Barth *et al*, 2010; Klionsky *et al*, 2012), further studies were undertaken to characterise the nature of autophagy dysregulation with Tnv-6.

The expression of p62/Sequestosome 1, an adaptor protein that regulates the clearance of select ubiquitinated substrates (Korolchuk *et al*, 2009) was studied, as it accumulates during the inhibition of autophagy (Bjørkøy *et al*, 2005). A significant increase in p62/Sequestosome 1 (sixfold, range 2–12, $P = 0.003$) was detected in cells from Tnv-6-treated cultures suggesting the inhibition of autophagy in the presence of Tnv-6 (Figure 4B). However, p62/Sequestosome 1 increases can also associate with the induction of autophagy (Klionsky *et al*, 2012), and therefore, further studies through the concomitant use of agents that stimulate or block autophagy were undertaken in two specimens, to characterise the autophagy-flux in Tnv-6-treated cells (MacCallum *et al*, 2013). Lysosomal degradation was blocked by treating cultures with 10nM bafilomycin-A1 (that targets vacuolar-type H^+-ATPase), such that stimulation of autophagy following Tnv-6 treatment would result in a synergistic accumulation of LC3 II. However, although an increase in LC3 II expression was evident with bafilomycin-A1 compared with untreated cells, this change was similar to the LC3 II increase in Tnv-6-treated cells, and the addition of bafilomycin-A1 to Tnv-6 cultures caused no further increase in LC3 II (Figure 4C). This was in contrast to combining bafilomycin-A1 with the mTOR inhibitor Ku-0063794 (4 μM) (García-Martínez *et al*, 2009), known to induce autophagy. These studies thus indicate a role for Tnv-6 in inhibiting the later stages of autophagy in activated CLL cells.

Tnv-6 delays ubiquitin–proteasome signalling. As the inhibition of autophagy associates with the delayed clearance of substrates through the ubiquitin–proteasome pathway (Korolchuk *et al*, 2009), we hypothesised that Tnv-6 treatment of CLL cells

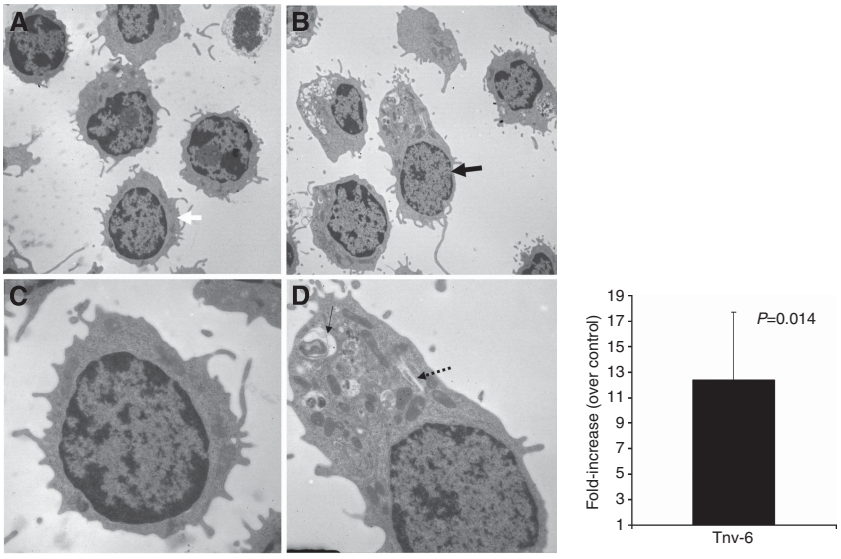

Figure 3. Changes in cellular ultrastructure following Tnv-6 treatment of activated CLL cells. The representative TEM images (× 10K magnification) from one (out of 3) CLL specimens analysed by transmission electron microscopy shows sub-cellular differences between cells cultured without (**A**) or in the presence of Tnv-6 (**B**). To visualize these differences in greater detail, images at higher magnification (× 30K) are shown of a cell each from control cultures (**C**) and Tnv-6 containing cultures (**D**). The cell cultured with Tnv-6 (highlighted by the black arrow in (**B**)) contains double-membrane bound cytoplasmic vacuoles (interrupted black arrow) and larger vacuoles with cytoplasmic contents (continuous black arrow) suggestive of early and late autophagosomes respectively (**D**). The mean numbers of autophagosomes per cell in 50 cell sections per culture ($n = 3$) was 12-fold higher (range 9–18) with Tnv-6 than controls not cultured with Tnv-6 ($P = 0.01$) (**E**).

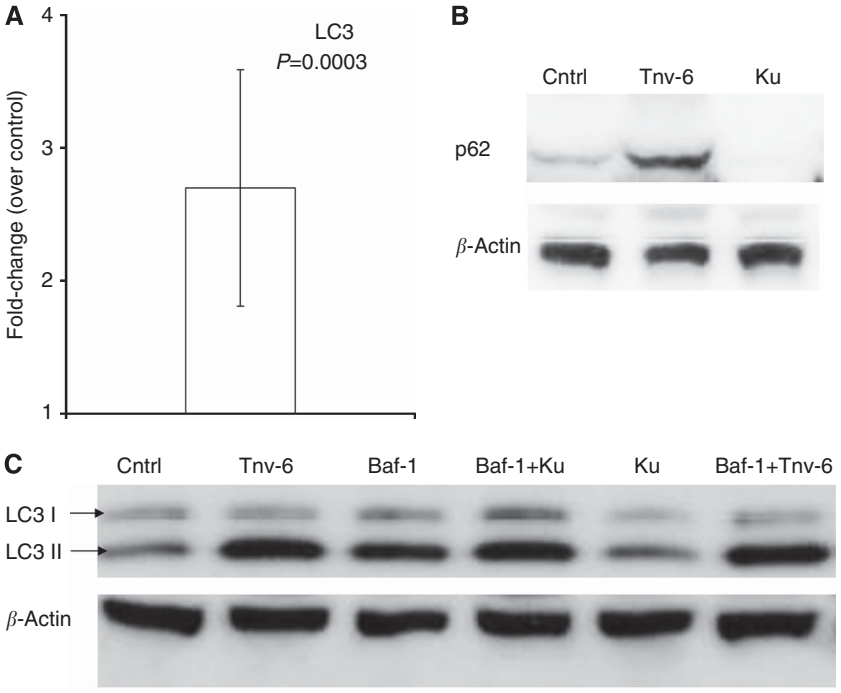

Figure 4. Changes in autophagy-associated protein expression following Tnv-6 treatment of activated CLL cells. Following Tnv-6 treatment, a mean 2.7-fold increase in lipidated LC3 (LC3 II) was observed in activated CLL cells by densitometric analysis ($P = 0.0003$) (**A**). In addition, the representative western blot image (**B**) shows accumulation of p62/Sequestosome 1 in the presence of Tnv-6 suggesting inhibition of autophagy. The concurrent analysis of protein from cells treated with the autophagy-inducer Ku-0063794 (Ku) in this panel (**B**) demonstrates the expected reduction in p62 to validate the assay. In **C**, the western blot image demonstrates changes in autophagy-flux in CLL cells from one (of two patients) patient with single-agent or combination treatment with Tnv-6, Bafilomycin-A1 (Baf-1) and Ku. There is absence of a synergistic increase in lipidated LC3 (LC3 II) in cells simultaneously treated with Baf-1 and Tnv-6 (unlike that observed with Baf-1 and Ku), confirming the inhibition of autophagy-flux by Tnv-6.

could affect ubiquitin–proteasome function and cause the accumulation of ubiquitinated proteins, including p53, that undergo physiological degradation through this pathway. By western blotting of total protein lysates from activated cultures, heterogeneity in the levels of ubiquitinated proteins was detected in cells from different patients; following treatment with Tnv-6, the

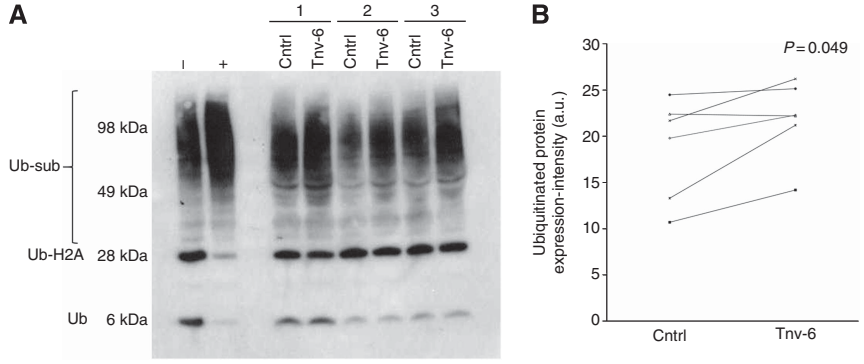

Figure 5. Changes in ubiquitin–proteasome pathway activity following Tnv-6-treatement of activated CLL cells. (**A**) Western blot images from three patient specimens (1, 2 and 3) cultured with Tnv-6 show an increase in ubiquitinated substrates (Ub-sub) compared with corresponding controls (Cntrl). Protein extracts from A375 melanoma cells cultured without (−) or in the presence (+) of the proteasome inhibitor Bortezomib were used as controls. A corresponding decrease in ubiquitinated nucleosomal histone H2A (Ub-H2A) (described with proteasome inhibitors in tumour cells, Mimnaugh *et al*, 1997) is also evident in the presence of Tnv-6. There is no difference in free ubiquitin (Ub) between cells from Tnv-6-treated and Cntrl cultures. Despite heterogeneity in responses, Tnv-6-treated CLL cells showed accumulation of ubiquitinated proteins (expressed in arbitrary units, a.u. after standardisation to β-actin) compared with corresponding untreated controls (**B**).

levels of ubiquitinated substrates in cells (standardised to actins) increased to a mean of 21.9 ± 4.2 arbitrary units (a.u.) from 18.7 ± 5.4 a.u. in control cultures ($P = 0.049$, $n = 6$, Figure 5). However, no increase in the amount of ubiquitinated p53 was detected in cells recovered from Tnv-6-treated cultures (data not shown).

Tnv-6 dysregulates autophagy independently of p53. Whether the increase in p53 in response to Tnv-6 is functionally relevant to the dysregulation of autophagy was investigated in cells from three patients with monoallelic deletions of *TP53*. As shown in Figure 6, the protein expression profiles indicate the presence of mutant non-functional protein in two patients, suggesting the existence of a type-A p53 pathway defect in these cells (Pettitt *et al*, 2001). Following culture with Tnv-6, no change in p53 expression was evident in specimens from patients 1 and 2 (Figure 6). Although a slight increase in p53 levels was observed in cells from patient 3, overall, the differences were not statistically significant. An increase in $p21^{waf1}$ expression was also observed (Figure 6), but unlike Tnv-6-treated cells with intact p53 pathway activity described previously, the mean increase in p21waf1 (1.8-fold, range 1.7–2.3) in *TP53* mutant CLL was not statistically significant compared with controls ($P = 0.18$).

When the expression of lipidated LC3 (LC3 II) was compared by western blotting, increased levels were noted in cells cultured with Tnv-6 compared with controls (Figure 6), $P = 0.04$, $n = 3$. By fluorescence microscopic analysis of single cells stained for LC3 and del(17p) (Figure 7), an increase in LC3 'puncta' was observed in cells from all three specimens cultured with Tnv-6, indicating altered autophagy despite *TP53* loss. In 50 cells with monoallelic *TP53* loss per specimen, the mean corrected total cellular fluorescence for LC3 was twofold higher (range 1.5–3.0) in cells from Tnv-6 containing activated cultures than corresponding control cultures (Figure 7), $n = 3$, $P = 0.04$.

Effects of Tnv-6 analogues on p53 and autophagy. As the cellular response to Tnv-6 in our studies contrasts with the results reported in other neoplastic cells treated with Tnv-6 (Lain *et al*, 2008; Li *et al*, 2012; Yuan *et al*, 2012), we investigated whether autophagy dysregulation in CLL occurs potentially through alternative targets of this sirtuin inhibitor. The ability of three Tnv-6 analogues lacking *in vitro* activity against SirT1 and SirT2, to alter p53 expression and LC3 lipidation in cells from six activated cultures was compared with Tnv-6. The structures and a

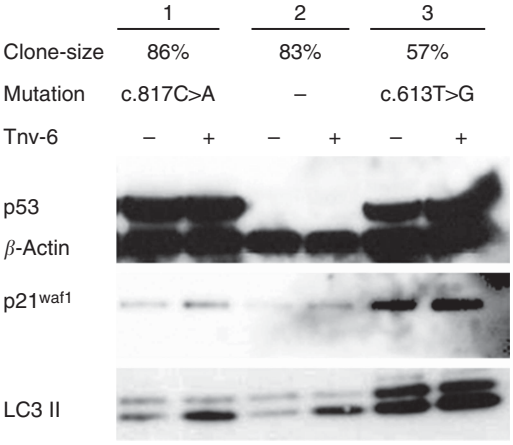

Figure 6. Protein expression in activated *TP53*-deleted CLL cells cultured with Tnv-6. Basal p53 expression profiles in patients 1 and 3 with *TP53* mutant clones comprising 86 and 57% of cells suggest a type-A p53-pathway defect. The *TP53* gene in patient 2 was not sequenced, but the complete absence of p53 makes a nonsense mutation likely. Following Tnv-6 treatment, there is no change in p53 expression in patients 1 and 2, although levels increase over basal levels in patient 3, probably reflecting changes in cells without *TP53* loss. There is a slight increase in the expression of $p21^{waf1}$ in each specimen treated with Tnv-6 compared with untreated controls. Despite the presence of *TP53* deletions, there is significant accumulation of lipidated LC3.

comparison of the biological activity and physical properties of the analogues used are described in Supplementary Table S2 and Supplementary Figure S4. By visual examination, no increases in p53 or LC3 lipidation were evident in CLL cells treated with Tnv-6 analogues compared with Tnv-6 (Figure 8A and B). To control for potential differences in protein loading, quantitative densitometry of p53 and LC3 II expression compared with β-actin was undertaken and confirmed the difference in expression levels between cells treated with Tnv-6 and its analogues ($P \leqslant 0.01$ (p53) and $P < 0.0004$ (LC3 II)). These results suggest that the Tnv-6 effects were related to sirtuin inhibition. However, densitometric analysis also indicated that LC3 II expression with Tnv-6 analogues was slightly higher in the presence of Tnv-45 ($P = 0.015$) and

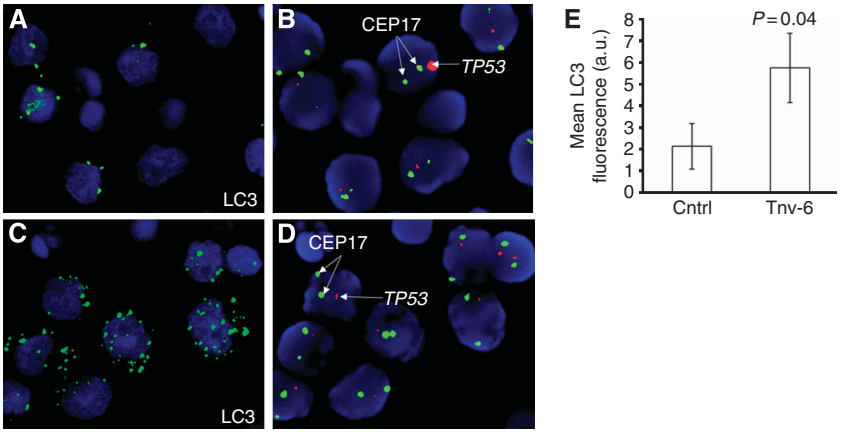

Figure 7. Dysregulation of autophagy by Tnv-6 in activated CLL cells with monoallelic loss of *TP53*. Basal LC3 expression by immunofluorescence (green signals) in untreated (control) cells (**A**). FISH staining for *TP53* (red signal) and the chromosome enumeration probe, CEP for chromosome 17 (green signal) identifies cells with monoallelic deletion of *TP53* (hence one red signal) in this field (**B**). Following Tnv-6 treatment, LC3 puncta are increased (**C**) in these cells, evident in the FISH image for *TP53* and CEP17 corresponding to the same field (**D**). The graphical representation of results following analysis of two additional specimens indicates the mean corrected cellular LC3 fluorescence to be significantly increased in *TP53*-deleted cells cultured with Tnv-6, indicating p53-independent autophagy dysregulation (**E**).

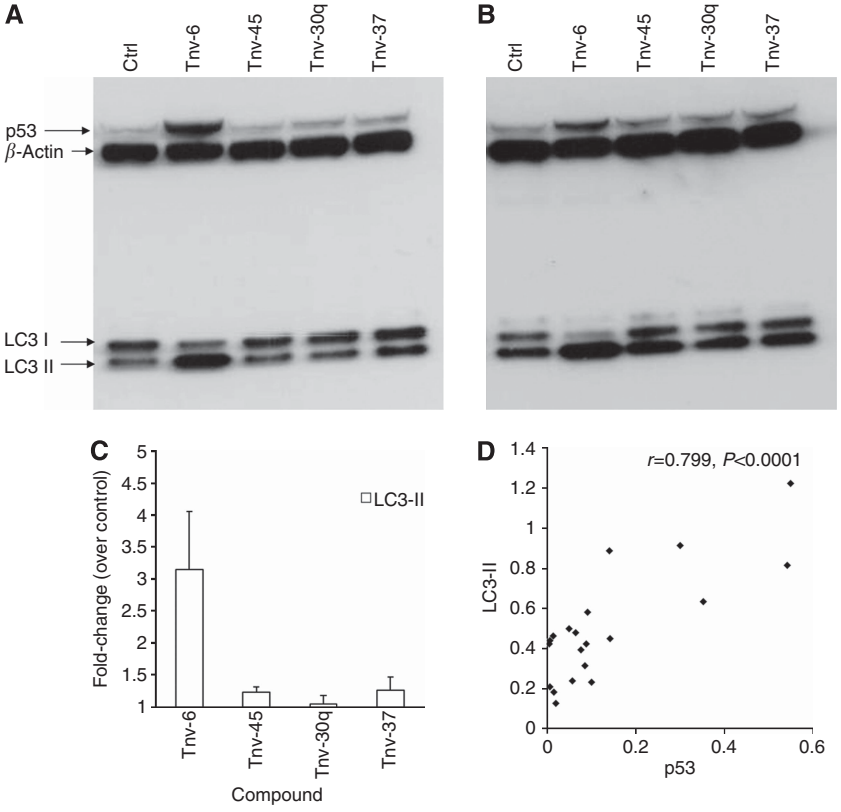

Figure 8. Changes in p53 and LC3 expression following treatment of activated CLL cells with Tnv-6 analogues. Panels **A** and **B** show protein expression from two patient specimens, each cultured with Tnv-6 and analogues (Tnv-45, Tnv-30q and Tnv-37) compared with untreated control (Cntrl) cultures. The increase in p53 and lipidated LC3 (LC3 II) seen in cells in culture with Tnv-6 is significantly attenuated in the presence of the analogues (**B**). However, by densitometric quantification of LC3 II expression standardised to β-actin, Tnv-45 and -37 retain slight, but significant ability to upregulate LC3 II expression compared with Cntrl cultures (**C**). Overall, a strong positive correlation existed between the expression intensities of LC3 II and p53 in CLL cells treated with Tnv-6 or analogues (**D**).

Tnv-37 ($P=0.012$) than in untreated control cells (Figure 8C). Comparison of the LC3 II response to Tnv-6, with that observed in cultures with the analogues (Figure 8C) suggests that $\geqslant 90\%$ of the Tnv-6 effect occurs through structural moieties required to inhibit sirtuins. We also observed that the expression intensities of LC3 II and p53 correlated strongly in cells treated with Tnv-6 or its analogues ($r=0.79$; $P<0.0001$, Figure 8D).

DISCUSSION

Previously, in quiescent CLL cells with intact genotoxic p53 responses treated with Tnv-6, we identified the preferential dysregulation of autophagy, in the absence of p53 activity (MacCallum *et al*, 2013). As these observations were at variance

with the p53-dependent apoptotic effects of Tnv-6 in other malignancies (Lain *et al*, 2008; Li *et al*, 2012; Yuan *et al*, 2012), here we have investigated whether the biological responses to Tnv-6 in CLL are unique to the disease, consequent to the quiescent cellular phenotype, and are p53-independent. Through the analysis of changes in cellular ultrastructure and protein expression, we have been able to demonstrate that the cytotoxic effects of Tnv-6 in CLL cells associates with the inhibition of autophagy even in conditions that favour cellular activation and proliferation. As Tnv-6 is non-toxic to normal haemopoietic cells (Li *et al*, 2012; Yuan *et al*, 2012; MacCallum *et al*, 2013), the cell cycle-independent dysregulation of autophagy in CLL suggests its clinical potential to effectively target leukaemic sub-populations, including disease-sustaining cells with proliferative capability.

As with quiescent cells, death is not associated with apoptosis. 7-AAD-based flow cytometry was used to measure cytotoxicity with Tnv-6, as the dye permeates cells with loss of membrane integrity during cell death. Despite its frequent use as an assay to quantify 'apoptosis' (Zembruski *et al*, 2012), 7-AAD labelling does not specify apoptosis (Fujisawa *et al*, 2007) and therefore electron microscopy, considered the 'gold standard' for detecting apoptosis (Taatjes *et al*, 2008) was used to confirm the absence of ultrastructural changes indicative of apoptotic death. The absence of apoptotic cell death was surprising, as a striking increase in p53 was identified following Tnv-6 treatment in all activated CLL cultures. This p53 response contrasted with that in quiescent cells here, and in previous studies (MacCallum *et al*, 2013), where no increase in p53 expression is detected. The activation and proliferation status of CLL cells thus appears to be a key determinant of the Tnv-6-associated increment in p53.

The increase in p53 expression in activated cells is concordant with that observed in other neoplastic cell types treated with Tnv-6 (Lain *et al*, 2008; Li *et al*, 2012; Yuan *et al*, 2012). However, unlike other malignancies, there is no p53 pathway-dependent apoptotic signalling in CLL cells, as the expected downstream effect on caspase-3 (Audrito *et al*, 2011) was not observed and ultrastructural features of apoptosis (to suggest alternative pro-apoptotic mechanisms) are absent. That the p53, despite its increase, is non-functional is suggested by the lack of an increase in p53-activating acetylation at commonly modified Lysine residues (Knights *et al*, 2006). In particular, there was absence of acetylation at residue 320, which is known to preferentially promote cell cycle arrest over apoptotic death (Cheng *et al*, 2003), and could have clarified reasons for the absence of apoptosis in our experiments. To rationalise the discrepancy between p53 expression and activity, we examined the possibility that the rise in cellular p53 levels in Tnv-6-treated cells occurs due to ubiquitin–proteasome (UP) pathway dysfunction during autophagy inhibition. The delayed degradation of UP-dependent client proteins (that include p53) is known to occur during inhibition of autophagy through the accumulation of p62/Sequestosome 1 (Korolchuk *et al*, 2009). As rapid increases in p62/Sequestosome 1 were detected following Tnv-6 treatment, we expected to detect a coincident increase in ubiquitinated proteins and therefore, non-functional p53. In support of our hypothesis, an increase in ubiquitinated proteins was seen in Tnv-6-treated CLL cells, confirming delayed UP-transit. However, no change in ubiquitinated p53 was detected, suggesting the complexity of p53 regulation in CLL cells treated with Tnv-6.

We therefore decided to clarify whether p53-pathway activity is important for autophagy dysregulation (Kenzelmann Broz *et al*, 2013; Napoli and Flores, 2013) by analysing LC3 responses to Tnv-6 in cells with deletions of *TP53*, a critical determinant of impaired p53-pathway responses (Zenz *et al*, 2008; Mohr *et al*, 2011). Dysfunction in p53-dependent responses in these cells was indicated by the 'loss of function' mutations identified through sequencing of the retained *TP53* allele and the high basal level of p53 protein expression in two specimens (Pettitt *et al*, 2001). Although CLL patients with a *TP53* mutant clone size of $>20\%$ are classed as '*TP53* mutant CLL' (Catovsky *et al*, 2007), here, by selecting specimens with a higher burden of *TP53*-deleted clones (86%, 83% and 57%, respectively) we were able to avoid the potentially confounding effects of protein expression from sub-clones with wild-type *TP53* (Mohr *et al*, 2011) and demonstrate the accumulation of LC3 II following Tnv-6 treatment.

In addition, we undertook more definitive analysis of single cells, by combining FISH and immunofluorescence studies to demonstrate the alteration of LC3 expression in cells with monoallelic *TP53* deletions and mutant *TP53*, confirming the redundancy of p53-dependent signalling in the dysregulation of autophagy. Despite p53-pathway dysfunction in these cells, there was a non-significant increase in $p21^{waf1}$ expression. It is likely that this change in $p21^{waf1}$, described earlier in cells with intact p53 responses to DNA damage, and at the transcriptional level (MacCallum *et al*, 2013) occurs independently of p53, similar to mechanisms in breast cancer cells with p53 dysfunction (McCarthy *et al*, 2013). Arguably, p53-dependent regulation of $p21^{waf1}$ expression in the *TP53* mutant cases studies here could occur in leukaemic cells without *TP53* loss (14%, 17% and 43%, respectively), but we have no evidence that the increase in total p53 following Tnv-6 treatment associates with enhanced p53-pathway function, making the increase in $p21^{waf1}$ a likely p53-independent effect. Further studies on sub-populations of CLL cells (based on *TP53* status) would help confirm this hypothesis, but the loss of cell viability during separation of clones will preclude further functional studies.

The p53-independent anti-leukaemic mechanisms of Tnv-6 identified through our studies could be particularly relevant to CLL patients with sub-types of p53-pathway dysfunction associated with poor outcomes to current therapy (Lin *et al*, 2012). Whether these mechanisms, seemingly unique to CLL cells, are initiated through sirtuin inhibition, or represent off-target effects of Tnv-6 was investigated. Cell-transfection studies (as those performed previously (Lain *et al*, 2008; Sunami *et al*, 2013) towards identifying Tnv-6 targets are challenging in CLL due to the fragile nature of these cells, and therefore the effects of Tnv-6 analogues (lacking the capacity to inhibit SirT1 and SirT2) on LC3 responses were studied here. The fact that over 90% of the Tnv-6 effect on LC3 II was reduced with the use of analogues suggests that the effects of Tnv-6 in CLL occur predominantly through sirtuin inhibition. However, LC3 lipidation was not completely abolished in the presence of the analogues. These results could reflect residual anti-SirT1/SirT2 activity of Tnv-6 analogues *in vivo* or the effects of Tnv-6 on an alternative cellular target. The residual, attenuated LC3 II response with the analogues also provided an opportunity to investigate a possible association between the expression of lipidated LC3 II and p53. Based on the strength of correlation between the expression of these proteins, and the redundancy of p53 networks in lipidation of LC3, we suggest that the accumulation of non-functional p53 in Tnv-6-treated cells reflects the magnitude of autophagy inhibition.

It would be of interest to investigate whether the changes in autophagy parallel p53-dependent signalling in 'non-CLL' cells treated with Tnv-6 (Lain *et al*, 2008; Yuan *et al*, 2012), with possible differences in the Tnv-6 target or autophagy-flux resulting in an apoptotic cellular response. An evaluation of the relationship between autophagy and apoptosis in neoplastic cells studied previously (Lain *et al*, 2008; Li *et al*, 2012; Yuan *et al*, 2012) is required before the Tnv-6- effect in CLL can be considered to be tissue-specific. Our observations on mechanisms associated with the cytotoxicity of Tnv-6 in proliferating and *TP53* mutant CLL could then find wider application in cancer therapeutics.

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