

Reversion of apoptotic resistance of *TP53*-mutated Burkitt lymphoma B-cells to spindle poisons by exogenous activation of JNK and p38 MAP kinases

M Farhat¹, A Poissonnier¹, A Hamze², C Ouk-Martin¹, J-D Brion², M Alami², J Feuillard^{1,3} and C Jayat-Vignoles^{*1}

Defects in apoptosis are frequently the cause of cancer emergence, as well as cellular resistance to chemotherapy. These phenotypes may be due to mutations of the tumor suppressor *TP53* gene. In this study, we examined the effect of various mitotic spindle poisons, including the new *isocombretastatin* derivative *isoNH₂CA-4* (a tubulin-destabilizing molecule, considered to bind to the colchicine site by analogy with *combretastatin A-4*), on BL (Burkitt lymphoma) cells. We found that resistance to spindle poison-induced apoptosis could be reverted in tumor protein p53 (*TP53*)-mutated cells by EBV (Epstein Barr virus) infection. This reversion was due to restoration of the intrinsic apoptotic pathway, as assessed by relocation of the pro-apoptotic molecule Bax to mitochondria, loss of mitochondrial integrity and activation of the caspase cascade with PARP (poly ADP ribose polymerase) cleavage. EBV sensitized *TP53*-mutated BL cells to all spindle poisons tested, including vincristine and taxol, an effect that was systematically downmodulated by pretreatment of cells with inhibitors of p38 and c-Jun N-terminal kinase (JNK) mitogen-activated protein kinases. Exogenous activation of p38 and JNK pathways by dihydrosphingosine reverted resistance of *TP53*-mutated BL cells to spindle poisons. Dihydrosphingosine treatment of *TP53*-deficient Jurkat and K562 cell lines was also able to induce cell death. We conclude that activation of p38 and JNK pathways may revert resistance of *TP53*-mutated cells to spindle poisons. This opens new perspectives for developing alternative therapeutic strategies when the *TP53* gene is inactivated.

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Subject Category: Cancer

Among underlying causes of cancer is accelerated or deregulated proliferation, with alteration of key cell cycle regulators and perturbation of proliferative negative retro-controls such as cell cycle checkpoints or DNA damage response. Another driving force of cell transformation is aberrant cell survival, a consequence of the inability to undergo apoptosis. Many cancer treatments stop tumor progression by inducing DNA damage and/or by perturbation of mitosis, then inducing apoptosis by activation of stress cell signals.¹

Apoptosis induced by cellular stress is often mediated by the intrinsic mitochondria-initiated cell death pathway.² This pathway, regulated by the members of the Bcl2 family, converges on Bax and Bak activation by BH3-only subgroup of Bcl2-related proteins (such as Bim, Bmf, Bid, p53 upregulated modulator of apoptosis (PUMA) or Noxa). Activation, relocalization to the mitochondria and polymerization of Bax and Bak, as well as their association with the anti-apoptotic

molecules (Bcl2, Bcl-xl or myeloid cell leukemia sequence 1 (Mcl1)), induce outer mitochondrial membrane permeabilization and release of apoptogenic factors, such as cytochrome c, apoptosis-inducing factor, Smac/DIABLO (second mitochondria-derived activator of caspase/direct inhibitor of apoptosis protein (IAP)-binding protein with low pI) or Omi/HtrA2 (Omi/high temperature requirement protein A).³ In the cytosol, the apoptosome complex (cytochrome c/Apaf1/caspase 9) triggers activation of caspase 9 and therefore the caspase cascade.³ Smac/DIABLO and Omi/HtrA2 promote caspase activation by binding to IAP.^{3,4}

By blocking mitosis at the M-phase, spindle poisons are stress signal inducers. These molecules, which are anti-neoplastic drugs, exert their activity through activation of the spindle assembly checkpoint, thereby arresting cells in mitosis. Prolonged mitotic arrest leads to activation of the protein kinase p38, c-Jun N-terminal kinase (JNK) and casein kinase II, which, in turn, result in phosphorylation

¹Univ Limoges, Faculté de Médecine, CNRS UMR 7276, Laboratoire CRIBL, Limoges, France; ²Univ Paris Sud, Faculté de Pharmacie, CNRS UMR 8076, Laboratoire BioCIS, Châtenay Malabry, France and ³CHU Limoges, Hôpital Dupuytren, Service d'hématologie, Limoges, France

*Corresponding author: C Jayat-Vignoles, Faculté de Médecine, UMR CNRS 7276, 2, rue du Dr Marcland, Limoges 87000, France. Tel: +33 55 5435 867; Fax: +33 55 5435 897; E-mail: chantal.vignoles@unilim.fr

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Abbreviations: ACVBP, adriamycin, cyclophosphamide, vindesine, bleomycin, prednisone; BL, Burkitt lymphoma; CA-4, combretastatin A-4; CHOP, cyclophosphamide, hydroxydaunorubicin (adriamycin), oncovin (vincristine), prednisone; CLL, chronic lymphocytic leukemia; DAPI, 4',6-diamidino-2-phenylindole; DIABLO, direct inhibitor of apoptosis protein-binding protein with low pI; DiOC6(3), 3,3'-dihexyloxycarbocyanine iodide; DMSO, dimethyl sulfoxide; EBV, Epstein Barr virus; FLICA, fluorochrome-labeled inhibitors of caspases; H2AX, H2A histone member X; HtrA2, high temperature requirement protein A; JNK, c-Jun N-terminal kinase; LMP, latent membrane protein; MAP kinase, mitogen-activated protein kinase; Mcl1, myeloid cell leukemia sequence 1; MDM2, mouse double minute 2 homolog; MTS, (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt; PARP, poly ADP ribose polymerase; PUMA, p53 upregulated modulator of apoptosis; PRIMA, 2,2-bis(hydroxymethyl)-1-aza-bicyclo[2.2.2]octan-3-one; PTPC, permeability transition pore complex; RITA, 5,5'-(2,5-furandiyl)bis-2-thiophenemethanol; Smac, second mitochondria-derived activator of caspase; TP53, tumor protein p53

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and degradation of the anti-apoptotic protein Mcl1 and prevent Bax and Bak polymerization at the mitochondria membrane.⁵ Long-term mitotic arrest also induces inhibition of Bcl2, also mediated by JNK phosphorylation.⁶

Spindle poisons are classified into two categories, those that stabilize the microtubules, like taxanes, and those that bind to the colchicine domain (such as colchicine) or to vinca domain (vinca alkaloids such as vincristine, vinblastine or vindesine) and destabilize tubulin microtubules.^{7,8} Binding of these molecules to β -tubulin disturbs microtubule dynamics when the mitotic spindle is formed and disassembles the cytoskeleton during cell division. Classical chemotherapy protocols very often include spindle poisons, such as vincristine or vindesine for hematological disorders (polychemotherapy CHOP: cyclophosphamide, adriamycin, vincristine, prednisone or ACVBP: adriamycin, cyclophosphamide, vindesine, bleomycin, prednisone),⁹ or taxanes for solid tumors.¹⁰ However, synthesis of these antimetabolic agents is complex and in addition they have numerous toxic side effects like peripheral neuropathy, neutropenia and diarrhea¹¹ and emergence of multidrug-resistant phenotypes can be observed.^{12,13}

Looking for new and easier ways to synthesize molecules that are less toxic and have different spectrums of action is still valid. Among new recent molecules are combretastatins, particularly combretastatinA-4 (CA-4), which bind to the colchicine domain, exhibit a strong inhibitory effect on tubulin assembly, possess antivasular properties and inhibit cell proliferation at nanomolar concentrations.¹⁴ A soluble prodrug CA-4P (phosphate disodic CA-4) has been developed for use in clinical trials.¹⁵ But CA-4, characterized by a Z-stilbene structure, is subject to isomerization, with a strong loss of antitumoral effect for the E-isomer compared with the Z-isomer.¹⁶ A stable analog, *iso*NH₂CA-4, which is not prone to double-bond isomerization even during storage and administration, has then been synthesized.¹⁷ Other antimetabolic molecules are also in development and in clinical trials and include microtubule-stabilizing or microtubule-destabilizing agents.^{7,8,18}

In this study, we wanted to determine the effect of the new combretastatin derivative *iso*NH₂CA-4 on proliferation of transformed B cells. Comparing the effect of this new molecule with that of other mitotic spindle poisons, we found that Epstein Barr virus (EBV) infection of tumor protein 53 (*TP53*)-mutated BL (Burkitt lymphoma) cells reverted resistance to all mitotic spindle poisons. Spindle poisons induced Bax relocalization to mitochondria and caspase cascade activation with PARP (poly ADP ribose polymerase) cleavage in *TP53*-mutated EBV-infected BL cells. We showed that this fortuitous effect of EBV was related to activation of the JNK/p38 activation pathways by EBV. Exogenous activation of JNK/p38 pathways by dihydrosphingosine also rendered *TP53*-mutated EBV-negative BL cells sensitive to spindle poisons, opening new perspectives in bypassing the resistance to these drugs when the *TP53* gene was inactivated.

Results

Spindle poison effects on cell proliferation and apoptosis induction. To test the effect of the *isocombretastatin* derivative *iso*NH₂CA-4, we used unmutated (BL2) or mutated

(BL41) BL cell lines for *TP53* and their EBV-positive counterparts (BL2B95.8 and BL41B95.8). As control molecules, we used colchicine as the reference for spindle poisons belonging to the colchicine domain binding destabilizing-tubulin molecules and CA-4 as the combretastatin family reference. *iso*NH₂CA-4 was the most effective molecule, regardless of cell type. Independently of the drug used, 50% inhibition of proliferation was obtained with about twofold less drug for EBV-infected counterparts (Table 1).

We then analyzed the effect of *iso*NH₂CA-4 on apoptosis, as compared with colchicine and CA-4. Except for BL41, the three drugs induced apoptosis (Figure 1a). Cell cycle analyses were then performed by flow cytometry to estimate the percentage of cells in G2M and sub-G1 phases after 8, 24 or 48 h treatment by colchicine, CA-4 or *iso*NH₂CA-4. As expected, accumulation of both BL41 and BL41B95.8 cells in G2M was seen, being earlier for BL41B95.8 cells (Figure 1b). Accumulation of cells in G2M phases without apoptosis was seen for BL41. Meanwhile, BL41B95.8 cells initially accumulated in G2M and then in sub-G1 phase (Figure 1b). Similar results to those obtained for BL41B95.8 (accumulation in G2M and then in sub-G1) were observed for BL2 and BL2B95.8 cell lines (data not shown). These results were confirmed by epifluorescence microscopy, after staining of nuclei with propidium iodide. Results are shown for *iso*NH₂CA-4 in Figure 1c. BL41 cells clearly accumulated in the M-phase without signs of apoptosis, whereas for BL41B95.8 cells, mitotic accumulation was first observed after 8 h drug treatment followed by fragmentation of nuclei, a feature of apoptotic terminal events, at 24 h and later. Similar observations were seen for all spindle poisons (data not shown). Therefore, it appeared that BL41 cells were resistant to apoptosis induction, but not to cell cycle arrest by spindle poisons. Indeed, washing *TP53*-mutated BL41 cells exposed spindle poisons that allow them to regrow in a new medium (data not shown).

Apoptosis pathway induced by spindle poisons in EBV-infected TP53-mutated BL41 cells.

Complementary analyses were performed to characterize apoptosis induced by spindle poisons in BL41B5.8 cells. Treatment of BL41 cells with colchicine, CA-4 or *iso*NH₂CA-4 did not induce any caspase 3 and PARP cleavage, whereas both proteins were cleaved in BL41B95.8 cells, suggesting a caspase-dependent apoptotic process (Figure 2a). Similar results to those obtained for BL41B95.8 (caspase 3 and PARP cleavage) were observed for BL2 and BL2B95.8 cell lines (data

Table 1 IC50 values for cytotoxic assays (nM)

Cell line	BL2	BL2B95.8	BL41	BL41B95.8
Colchicine	30	20	30	20
CA-4	20	10	20	10
<i>iso</i> NH ₂ CA-4	10	5	10	5

Abbreviation: IC50, half-maximal inhibitory concentration
Spindle poison effects on cell proliferation. IC50 values of MTS cell proliferation assays on BL2- and *TP53*-mutated BL41 cell lines and their EBV-infected counterparts (BL2B95.8 and BL41B95.8) treated with colchicine, CA-4 or *iso*NH₂CA-4 for 24 h at concentrations ranging from 5 to 100 nM. IC50 values were calculated as means of three values

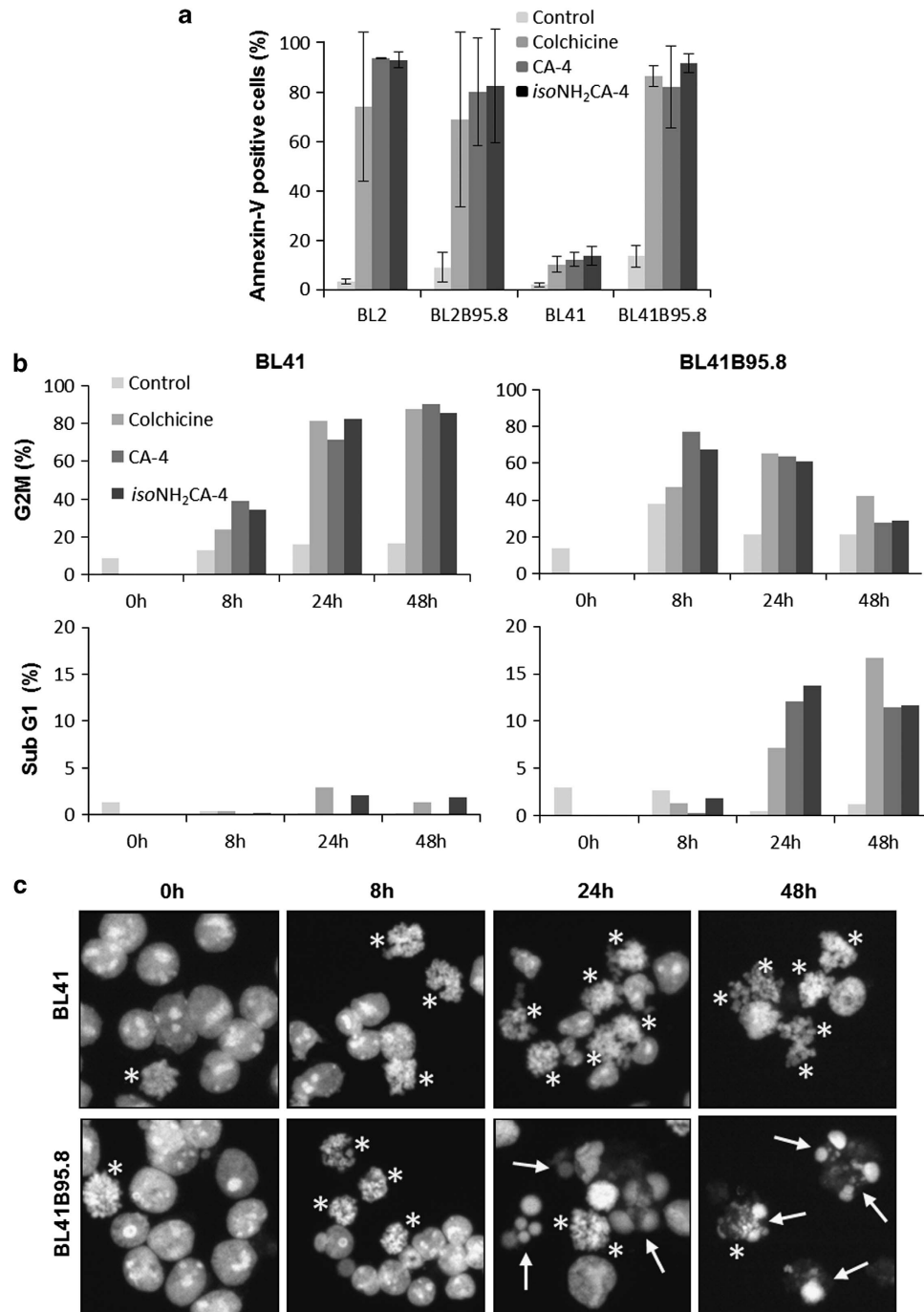


Figure 1 EBV renders TP53-mutated cells permissive to spindle poison-induced apoptosis. (a) Cells were treated with colchicine (20 nM), CA-4 (10 nM) or isoNH₂CA-4 (5 nM) for 72 h (a and b). (a) Apoptosis induction was evaluated by flow cytometry after labeling of external phosphatidyl serine residues (Annexin-V-positive cells) on BL2- and TP53-mutated BL41 cell lines as well as their EBV-infected counterparts (BL2B95.8 and BL41B95.8). (b) Percentage of cells in G2M (M, mitosis) and sub-G1 phases was evaluated by flow cytometry cell cycle analysis (propidium iodide staining of total DNA content). (c) The same isoNH₂CA-4-treated cells were also analyzed by epifluorescence microscopy. Cells in M are indicated by *. Cells with fragmented nuclei are indicated by →. Results are representative of three independent experiments

not shown). By flow cytometric fluorochrome-labeled inhibitors of caspases (FLICA) assay, no activity was observed for caspase 3, 8 and 9 in BL41 cells treated with isoNH₂CA-4 (Figure 2b). In contrast, all three caspases were activated in isoNH₂CA-4-treated BL41B95.8 cells. Similar results were observed in BL2 and BL2B95.8 cell lines (data not shown). Short-term kinetic experiments showed that caspase 8 and 9

were simultaneously activated (data not shown). No increase in Fas or Fas ligand expression levels could be detected (data not shown).

To further dissect the apoptotic pathways induced in TP53-mutated EBV-infected BL41 cells, mitochondrial potential was studied by flow cytometry, using 3,3'-dihexyloxycarbocyanine iodide (DiOC6(3)) as a selective probe for intact mitochondria.

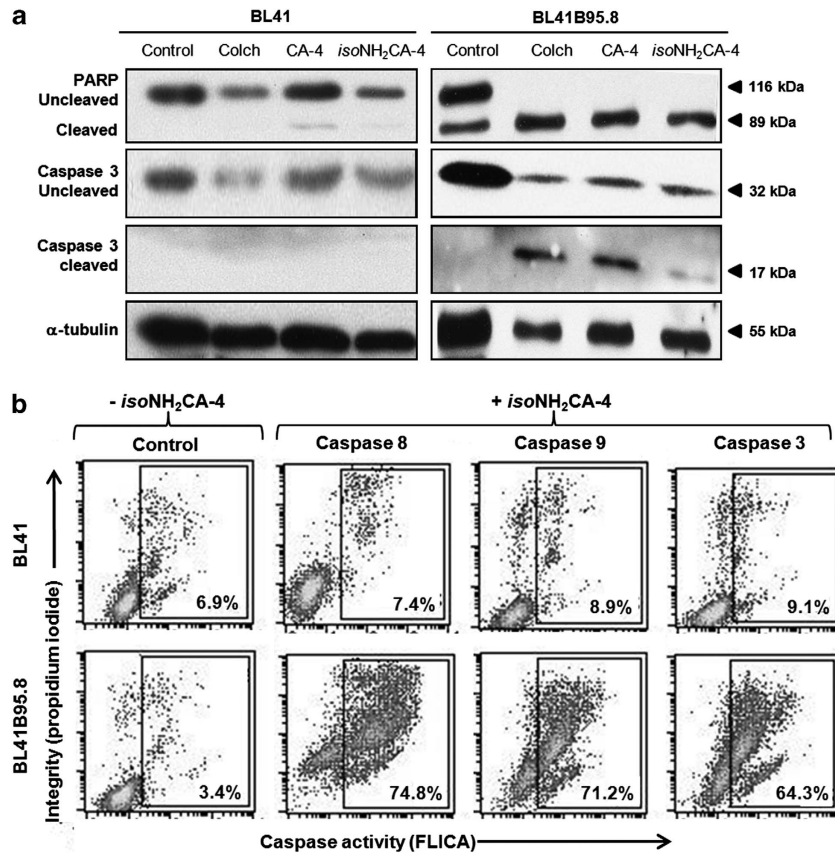


Figure 2 Apoptosis induced by spindle poisons in EBV-infected BL41B95.8 cells is caspase 3, 8 and 9 dependent. BL41 and BL41B95.8 cells were treated with colchicine (20 nM), CA-4 (10 nM) or *isoNH₂CA-4* (5 nM) for 24 h. (a) PARP and caspase 3 cleavage as well as α -tubulin (loading control) were analyzed by western blot. (b) Detection of caspase 8, 9 and 3 activation was evaluated by flow cytometry using the caspase-specific FLICA assay. Results are representative of three independent experiments

Results are given for *IsoNH₂CA-4* (Figure 3a). Different concentrations of *isoNH₂CA-4* (5, 7.5 and 10 nM) were used. Percentages of cells with weak staining of DiOC6(3) (due to mitochondrial transmembrane potential loss) were increased for all cell lines, except for BL41. At 5 nM *isoNH₂CA-4*, EBV promoted the loss of mitochondrial transmembrane potential in both infected BL cell lines when compared with their EBV-negative counterparts. Similar results were obtained for colchicine and CA-4 (data not shown). Colocalization of Bax with the mitochondrial marker TOM20 by confocal microscopy (relocalization of Bax to mitochondria) was observed only in *isoNH₂CA-4*-treated BL41B95.8 cells (Figure 3b). Similar results (Bax relocalization with *isoNH₂CA-4* treatment) were observed for BL2 and BL2B95.8 cell lines (data not shown).

EBV restored spindle poison-induced apoptosis in TP53-inactivated cells. Altogether, data presented in Figures 1–3 indicate that EBV rendered the TP53-mutated BL41 cell line sensitive to cytotoxic effects of spindle poisons. We thus hypothesized that EBV could bypass resistance to spindle poison apoptosis induction of TP53-mutated BL41 cells. To test this hypothesis, we transfected wild-type TP53 BL2 and BL2B95.8 cells with small interfering RNAs (siRNAs) against the TP53 mRNA and treated them or not with *IsoNH₂CA-4*. Apoptosis of treated BL2 cells was significantly decreased in presence of TP53 siRNAs

(Figure 4a), showing the functional importance of this protein in apoptosis induction by mitotic spindle poisons. Apoptotic BL2B95.8 cells were markedly increased after treatment with *IsoNH₂CA-4* and were not significantly decreased in presence of TP53 siRNAs (Figure 4b). This result suggests that EBV was able to sensitize cells to apoptosis induced by *IsoNH₂CA-4* independently from TP53.

Inhibitors of P38 and JNK mitogen-activated protein kinases decreased apoptosis induced by EBV in TP53-mutated cells treated with *isoNH₂CA-4*. Bax relocation to mitochondrial membranes can be obtained not only after TP53 activation but also after activation of both p38 and JNK mitogen-activated protein (MAP) kinases, and these kinases can be activated by EBV (through latent membrane protein 1 (LMP1) and LMP2A). We thus treated BL41B95.8 cells for 24 or 48 h with 10 nM *isoNH₂CA-4*, after 1 h of pretreatment or not with p38 inhibitor (SB203580), JNK inhibitor (SP600125) or both together. Pretreatment with inhibitors decreased percentages of annexin-V-positive BL41 cells, an effect that was additive (Figures 5a and c). Percentages of cells with weak mitochondrial potential (DiOC(6)3 staining) decreased when cells were pretreated with p38, JNK or both inhibitors (Figure 5b). Similar annexin-V and DiOC(6)3 results were obtained for BL2B95.8 cells (Figure 5c and data not shown). Altogether, these results suggest that p38 and JNK were

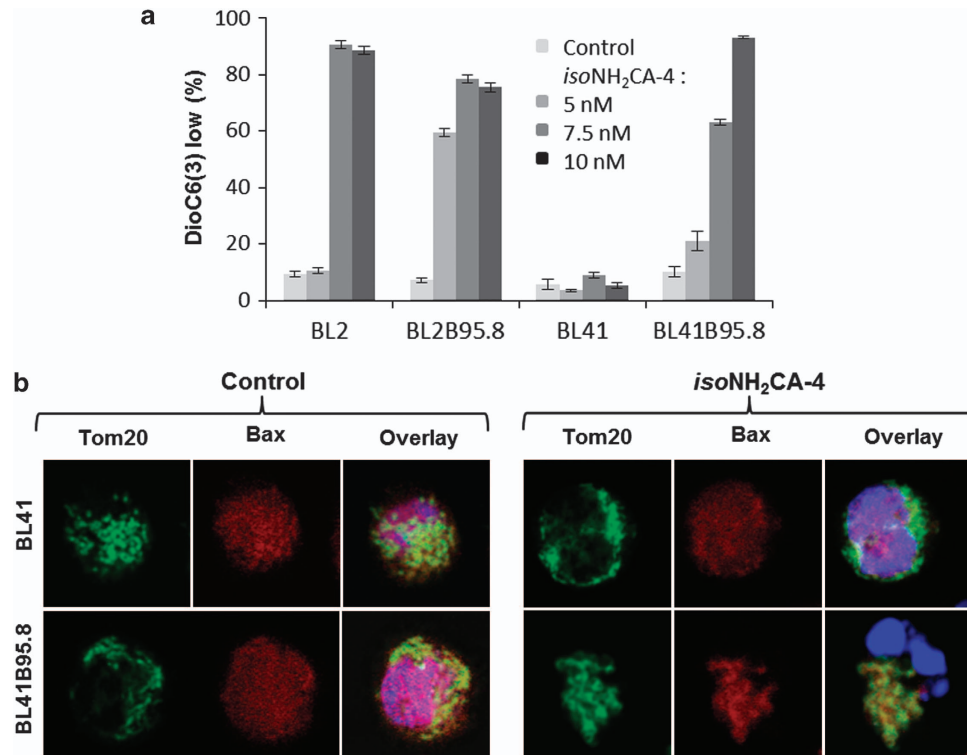


Figure 3 Mitochondria have a pivotal role in apoptosis induced by *isoNH₂CA-4* in EBV-infected BL41B95.8 cells. Cells were treated with 5, 7.5 or 10 nM *isoNH₂CA-4* for 24 h. (a) Mitochondrial integrity was evaluated by flow cytometry using DiOC6(3) staining of BL2- and TP53-mutated BL41 cell lines and their EBV-infected counterparts (BL2B95.8 and BL41B95.8). (b) For BL41 and BL41B95.8 cells treated with 10 nM *isoNH₂CA-4* for 24 h, colocalization of Bax with the mitochondrial marker Tom20 was studied by confocal microscopy (original magnification $\times 630$). Results are the mean of three independent experiments

involved in spindle poison-induced apoptosis of EBV-infected cells.

EBV sensitized TP53-mutated BL41 cells to apoptosis induction by any class of spindle poisons, an effect partially reversed by P38 and JNK MAP kinase inhibitors.

We examined whether inhibition of p38 and JNK MAP kinases also decreased apoptosis sensitization when cells were treated with other classes of spindle poisons. We used taxol (taxane-binding site), a representative actin-stabilizing agent. For destabilizing actin drugs, analyses were performed with reference molecules for colchicine-binding domains (colchicine and CA-4) and vinca-binding domains (vinblastine and vincristine). Similar to colchicine, CA-4 or *isoNH₂CA-4*, no apoptosis was observed for BL41 cells treated with taxol, vinblastine or vincristine, according to annexin-V/propidium iodide and DiOC6(3)/TOPRO-3 tests (Figure 6). In contrast, percentages of annexin-V-positive or DiOC6(3) weak BL41B95.8 cells were increased after treatment with any spindle poison. Treatment with p38 and JNK inhibitors systematically decreased the percentage of apoptotic cells (Figure 6). These results suggest that EBV-infected cells render TP53-mutated BL cells sensitive to apoptosis induction by all classes of spindle poisons tested, an effect likely to be caused by activation of p38 and JNK MAP kinases.

Activation of P38 and JNK MAP kinases sensitized EBV-negative TP53-mutated cells to apoptosis. Having shown that p38 and JNK inhibitors decreased apoptosis

induced by spindle poisons in TP53-mutated EBV-infected cells, we treated or not BL41 cells with *isoNH₂CA-4* and sphingosine, a p38/JNK activator. Results, presented in Figure 7, show that sphingosine sensitized TP53-mutated BL41 cells to *isoNH₂CA-4*-induced apoptosis (Figure 7). To check the specificity of the sphingosine effect on p38 and JNK activation, the same experiment was repeated in the presence of p38 and JNK inhibitors. Sphingosine treatment no longer sensitized TP53-mutated BL41 cells to *isoNH₂CA-4*-induced apoptosis (Figure 7b). This confirms that p38 and JNK were indeed activated by dihydrosphingosine and responsible for BL41 cell apoptosis after *isoNH₂CA-4* treatment. In a next step, we assayed two other TP53-deficient K562 (myeloid lineage) and Jurkat (T-cell lineage) cell lines. Results show that dihydrosphingosine treatment was able to induce cell death on its own (Figures 7c and d). This indicates that according to cell type, p38 and JNK MAP kinase activation may be sufficient to induce the death of TP53-deficient tumor cells, without help from spindle poisons.

Discussion

Inactivation of TP53 may contribute to apoptosis resistance in most types of cancers. Bypassing resistance to apoptosis caused by TP53 inactivation is an important issue. We discovered that, when cells were infected with EBV, apoptosis can be induced by spindle poisons in TP53-mutated BL cell lines. We showed that p38 and JNK apoptotic MAP kinases were responsible for this reversion.

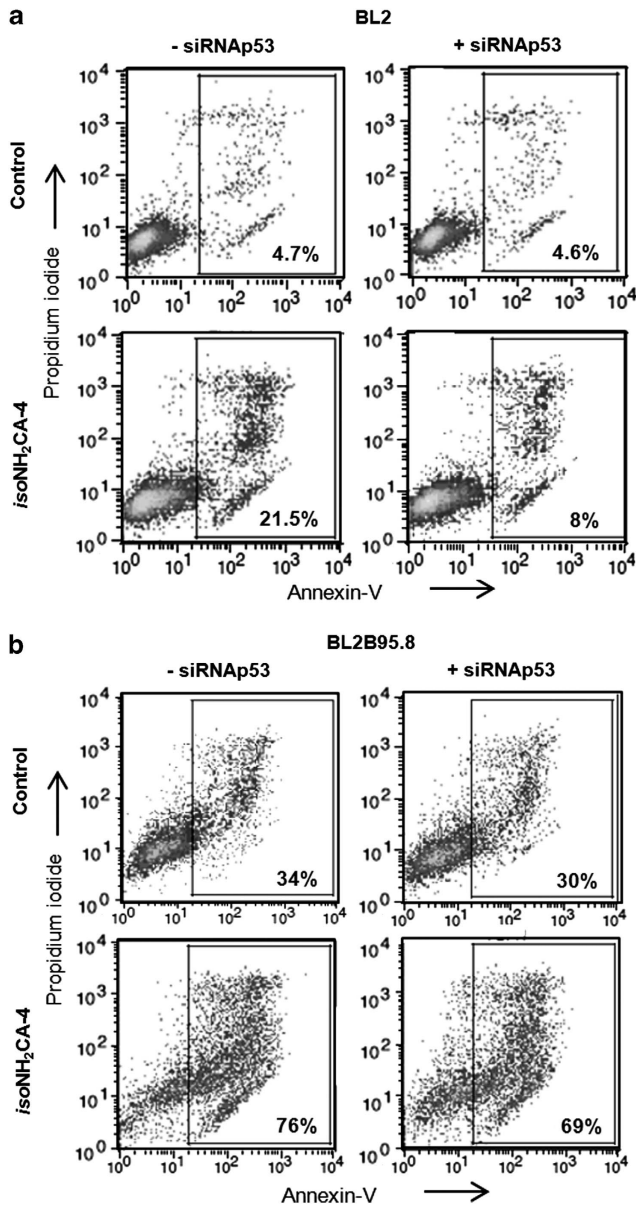


Figure 4 Apoptosis is induced in EBV-infected BL2 cells treated with *isoNH₂CA-4* after TP53 downregulation. Downregulation of TP53 expression was obtained (+ siRNAp53) or not (- siRNAp53) by transfection of BL2 (a) and BL2B95.8 (b) cells with either small interfering RNA (siRNA) against TP53 mRNA or scramble siRNA, respectively. Apoptosis induction as evaluated by flow cytometry (Annexin-V labeling). Cells were treated with 10 nM of *isoNH₂CA-4*. Results are representative of three independent experiments

The TP53 protein is a well-characterized tumor suppressor protein, encoded by the *TP53* gene, located on the short arm of chromosome 17 (17p13.1).²⁰ It has a key role in cell cycle regulation, development, differentiation, gene amplification, DNA recombination, chromosome segregation and cell senescence.^{21,22} Following DNA damage, TP53 upregulates expression of genes involved in cell cycle arrest and DNA repair. If damage cannot be resolved, TP53 can induce intrinsic apoptosis by upregulation of pro-apoptotic genes, such as BH3-only proteins (PUMA, Noxa or Bid), Bax and Apaf-1.^{23–25} TP53 is also stabilized by phosphorylation

following JNK pathway activation by cell stress-inducing signals, such as mitotic inhibitors.

Inactivation of *TP53* is either due to genetic alterations (mutation or deletion) or due to functional inhibition, by dysregulation of the p19/ARF-mouse double minute 2 homolog (MDM2) axis. TP53 inactivation has been reported in more than 60% of human primary cancers. Hematological malignancies and leukemia exhibit a lower incidence (<20%), but prognosis of this secondary genetic event is systematically unfavorable.²⁶ Among B lymphomas, the incidence of *TP53* mutation is highest for chronic lymphocytic leukemia (CLL) (15%), Richter's syndrome (CLL complication, 40%), B high upgrade lymphomas (30%) and BL (40%).²⁷

Loss of TP53 activity is responsible for cancer progression and resistance to chemotoxic drugs that induce apoptosis, including spindle poisons.²⁸ Different strategies such as gene therapy (introduction of a wild-type *TP53*), immunotherapy (TP53 vaccines) and drug therapy (disruption of TP53 interaction with its inhibitor MDM2 by small molecules such as nutilin-3b, 5,5'-(2,5-furandiyl)bis-2-thiophenemethanol (RITA), 2,2-bishydroxymethyl-1-aza-bicyclo[2.2.2]octan-3-one (PRIMA-1) or 9-ethyl-*N*-methyl-9*H*-carbazole-3-methanamine hydrochloride (Phikan083), have been examined to restore TP53-mediated apoptosis.^{29–31}

Another way to restore apoptosis is via induction of a TP53-independent pathway in TP53-mutated cells. As reported in some studies, apoptosis has been induced in malignant B and T lymphocytes with arsenic trioxide by mitochondrial membrane potential collapse.³² PRIMA-1 has been shown to induce apoptosis in colorectal cell line through the JNK pathway.³³ The BH3-only protein PUMA (a JNK substrate) can kill cells by apoptosis with or without TP53.³⁴ Fenretinide, a synthetic retinoid derivative, increases intracellular levels of ceramide (a p38 and JNK inducer), which is metabolized to GD3, leading to oxidative stress via induction of the transcription factor Gadd153, Bak and intrinsic apoptosis.³⁵ Moreover, it has been shown that overexpression of the eukaryotic translation initiation factor 5A1 in lung cancer cells is associated with p38 and JNK signaling, which in turn could induce apoptosis independently of TP53 activity.³⁶

EBV is a member of the gamma herpes virus family. It is one of the most common human viruses, as it infects as many as 95% of adults.³⁷ After primary infection, classically during infancy, the latent viral infection is maintained throughout life in resting memory B cells. Even if EBV immortalizes B cells both *in vitro* and *in vivo*, its persistence is due to the host/virus equilibrium that keeps the infected cells under the continuous cytotoxic pressure of the host immune system.^{38,39} Rupture of this equilibrium may lead to EBV-associated lymphoproliferative syndromes. EBV is also associated with various cancers (nasopharyngeal carcinomas, BLs, Hodgkin's lymphomas, T-cell lymphomas and immunodeficiency-related B-cell lymphomas).⁴⁰ *In vitro*, EBV immortalization of primary B cells is due to the proliferating program (so-called latency III), which corresponds to the expression of the full range of viral latent proteins. Among them, LMP1 (the major viral oncoprotein) and LMP2A possess conflicting functions, as they constitutively activate cell proliferation and survival pathways, as well as pro-apoptotic pathways.^{41–44} Indeed, we previously

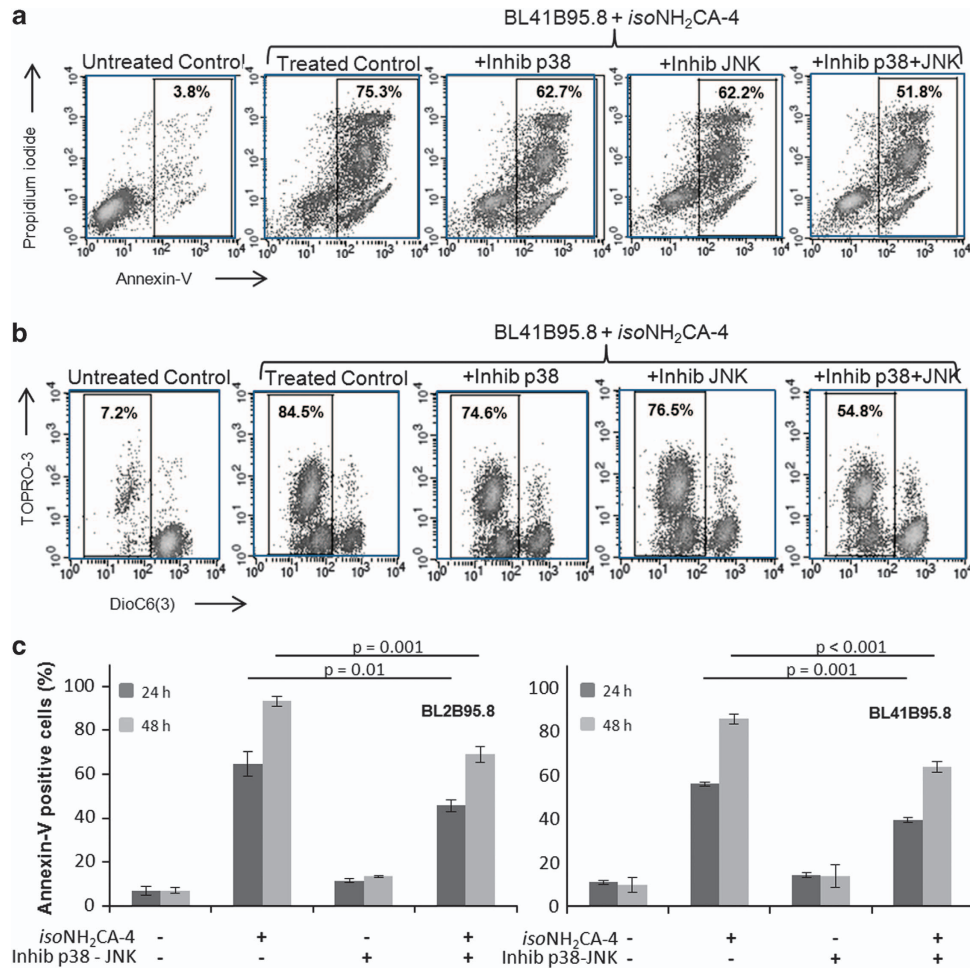


Figure 5 p38 and JNK MAP kinase inhibition decreases apoptosis induced by *isoNH₂CA-4* in EBV-infected BL41B95.8 cells. Cells were treated with 10 nM *isoNH₂CA-4* with or without 1 h pretreatment with the p38 inhibitor SB203580 (25 μ M), the JNK inhibitor SP600125 (7.5 nM) or both. (a and b) An example of apoptosis induction and mitochondrial loss of integrity in BL41B95.8 cells as assessed on flow cytometry biparametric histogram after annexin-V and propidium iodide (a) or DiOC6(3) and propidium iodide (b) labeling. Percentages of apoptotic cells are indicated in each graph. (c) Percentages of BL2B95.8 (left panel) and BL41B95.8 (right panel) annexin-V-positive cells in presence (+) or absence (-) of *isoNH₂CA-4* and/or both p38 and JNK inhibitors at 24 and 48 h. Results are representative of three independent experiments

demonstrated that, depending on the cell context, the EBV latency III program may also promote B-cell apoptosis as well interactions between the infected B cells and killing T lymphocytes.^{45,46} As demonstrated in our study, EBV also potentiated spindle poison-induced apoptosis in a TP53-independent manner. EBV induced reversion of apoptotic resistance to mitotic spindle poison treatments in TP53-mutated B cells by activation of the MAPK JNK and p38 pathways.

p38 and JNK pathways have an essential role in intrinsic apoptosis induction particularly via molecules of the Bcl2 family. These pathways contribute to activation of the pro-apoptotic BH3-only molecules Bim and Bmf,^{47,48} responsible for Bax activation and thereafter permeabilization of the mitochondrial membrane. P38 and JNK pathways also inactivate the anti-apoptotic Bcl2 and Bcl-xl proteins by phosphorylation.^{6,49-51} Other pro-apoptotic molecules are also targets for JNK-mediated phosphorylation, such as the histone guardian of the genome H2AX.⁵² This suggests that JNK and p38 pathways can directly induce intrinsic apoptosis. Sphingoid bases (sphingosine and sphinganine)

can potentiate apoptosis in breast⁵³ and colon cancer cells⁵⁴ in a TP53-independent manner. Our results showed that chemical activation of p38 and JNK pathways with sphingosine could bypass apoptosis resistance to spindle poisons in TP53-mutated B cells.

The effect of p38/JNK MAP kinase activation on tumor cell death may depend on the cell lineage. Indeed, dihydro-sphingosine treatment of BL41 TP53-mutated cells marginally induced cell death, whereas it strongly decreased cell viability of K562 and Jurkat cells. As expected, EBV activation of p38/JNK MAP kinase did not promote BL cell death on its own. In that case, apoptotic potential of p38/JNK MAP kinase activation was revealed by spindle poison treatment. On one hand, it can be put forward that apoptotic potential of p38/JNK MAP kinase activation by EBV was masked by other latency proteins that induce anti-apoptotic pathways such as NF- κ B (nuclear factor kappa-light-chain-enhancer of activated B cells) activation or Bcl2 overexpression. On the other hand, these differences between cell lines could be due to differences in energetic metabolism and aerobic glycolysis.

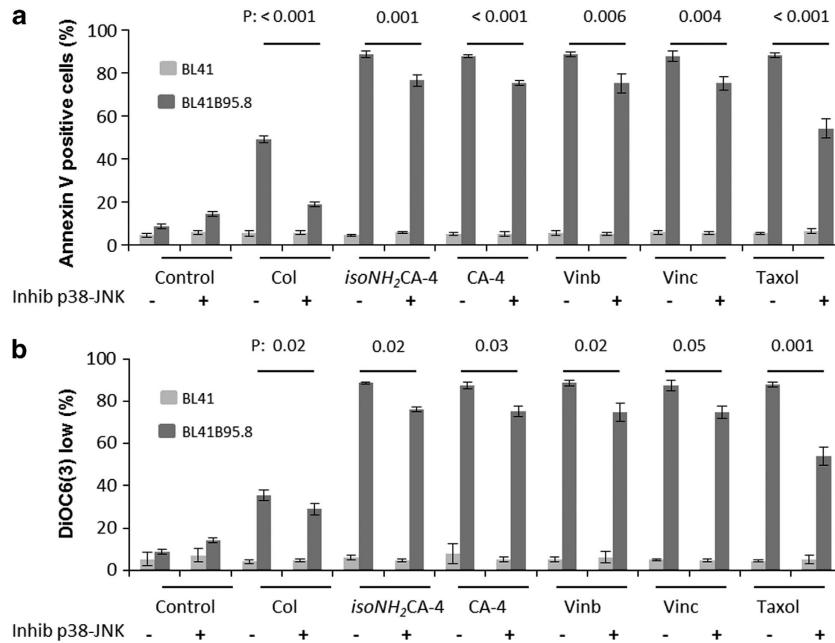


Figure 6 p38 and JNK inhibitors counteract apoptosis induced by any class of spindle poisons in EBV-positive BL41B95.8 cells. BL41 and BL41B95.8 cells were treated with actin-stabilizing (Taxol) or with actin-destabilizing drugs specific for the colchicine site (colchicine: 30 nM Col; 10 nM (*iso*NH₂CA-4, *iso*CA-4); 20 nM CA-4) or the vinca site (vinblastine: 25 nM Vinb; vincristine: 25 nM Vinc) for 24 h. Cells were additionally pretreated (+) or not (-) for 1 h with the p38 inhibitor SB203580 (25 μ M) and the JNK inhibitor SP600125 (7.5 nM). (a) Percentages of BL41 and BL41B95.8 annexin-V-positive cells assessed by flow cytometry. (b) Percentages of DiOC6(3) negative cells, assessed by flow cytometry. These results correspond to at least three independent experiments

Permeability transition pore complex (PTPC) opening and closing not only involves molecules of the Bcl2 family, such as Bax or Bcl2, but also mitochondrial bioenergetic.⁵⁵ A hallmark of tumor cells is their increased consumption of glucose and their switch of energy supply from oxydative phosphorylation to aerobic glycolysis (the Warburg effect), which is essential for tumor biomass increase. Hexokinase II, a mitochondrial protein that catalyses the first step of glycolysis, inhibits PTPC opening. Moreover, aerobic glycolysis leads to intracellular pH increase, favouring caspase inactivation. Overexpression of glycolytic genes is due to oncogene activation (such as c-Myc or RAS), functional loss of tumor suppressor (such as TP53 or phosphatase and tensin homolog), even in the presence of oxygen, or hypoxia.⁵⁶ The Warburg effect has been found in most, if not all, types of malignant tumors, including hematological malignancies, with a possible prognostic value.⁵⁷ Aerobic glycolysis level combined with TP53 status could thus influence sensitivity to apoptotic cell death induced by p38 and JNK MAP kinase activation. In that sense it would be interesting to test the effect of p38/JNK MAP kinase activation on other cancer models such as epithelial tumor cells, as these cells are characterized by extensive cytoplasmic modifications that contribute to resistance to apoptosis.

In conclusion, we showed that resistance to spindle poison-induced apoptosis of TP53-mutated tumor cells can be bypassed by EBV or by chemical agents such as sphingosine derivatives, which activate JNK and p38 pathways that in turn induce the intrinsic mitochondrial apoptosis pathway. These results may lead to new combinatorial therapeutic strategies for patients with tumors carrying deletions of parts of the short arm of chromosome 17 (del17p).

Materials and Methods

Cell lines, drugs and chemical treatments. K562 (myeloid tumor cell line), Jurkat (lymphoma T-cell line), BL2 and BL41 (EBV-negative BL B-cell lines) and their EBV-infected counterparts (infected with EBV wild-type strain B 95.8), BL2.B95.8 and BL41 B95.8 were cultured in RPMI 1640 medium (Lonza, Verviers, Belgium), supplemented with 100 U/ml penicillin, 100 μ g/ml streptomycin, 1 mM sodium pyruvate, 1% nonessential amino acids, 2 mM L-glutamine (All from Gibco BRL, Fisher, Illkirch, France) and 10% fetal calf serum (Lonza) and maintained in a 5% CO₂ incubator at 37 °C. Cells were treated with antimetabolic tubulin poisons: colchicine, CA-4 and *iso*-amino CA-4 (*iso*NH₂CA-4) from CNRS, BioCIS UMR 8076, Therapeutic Chemistry Laboratory, Faculty of Pharmacy, Chatenay-Malabry, France, as well as with vinblastine (Alloga, Marseille, France), vincristine and taxol (the two from Hospira, Meudon la Forêt, France). Treatment duration and drug concentrations varied and are indicated in legends. Cells were pretreated or not with the p38 inhibitor SB203580 (25 μ M) (Calbiochem, VWR, Strasbourg, France), the JNK inhibitor SP600125 (7.5 nM) (Sigma-Aldrich, Saint Quentin Fallavier, France) or D-erythro-dihydroshingosine (7.5 μ M) (Sigma-Aldrich) for 1 h. All drugs were reconstituted in dimethyl sulfoxide (DMSO), used as control treatment.

Cytotoxicity and viability assays. MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt) cell proliferation assay, labeling of annexin-V, propidium iodide staining for cellular DNA content and caspase activity (FLICA tests) were performed as previously described.^{19,46} Assessment of mitochondrial potential with DiOC6(3) was performed as follows: 1 μ l DiOC6(3) solution (0.5 μ M in DMSO, Invitrogen, Fisher) was added to 500 μ l of cell suspension (2.5 \times 10⁵ cells) and incubated for 20 min at room temperature. Before flow cytometric analysis (on a FACS Calibur, Becton Dickinson, Franklin Lakes, NJ, USA), 1 μ l TOPRO-3 (1 mM, Invitrogen) was added to each sample. DiOC6(3) was excited with a 488-nm argon laser and TOPRO-3 with a 633-nm helium-neon laser. Fluorescence were collected with a 530 \pm 15-nm band-pass filter for DiOC6(3) and a 650-nm long-pass filter for TOPRO-3.

Immunofluorescence double staining for confocal microscopy and nuclei staining for epifluorescence microscopy

Immunostaining. Cells (5 \times 10⁵) were fixed on slides with 4% paraformaldehyde for 10 min at room temperature, washed with phosphate buffered saline

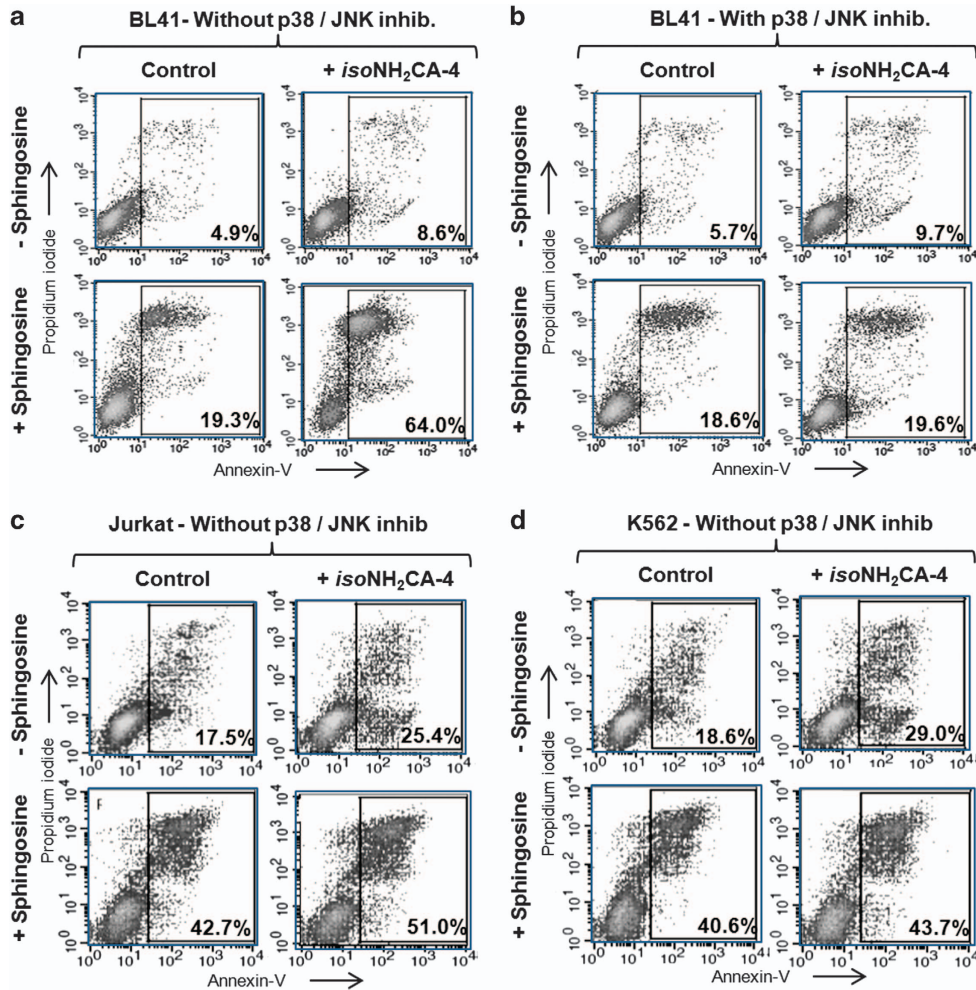


Figure 7 Dihydrospingosine renders TP53-mutated and EBV-negative BL41 cells permissive to apoptosis. BL41 (a and b), Jurkat (c) and K562 (d) cells were pretreated or not for 1 h with dihydrospingosine (7.5 μ M) and then treated or not with 10 nM isoNH₂CA-4 for 24 h. Apoptosis induction was evaluated by flow cytometry, after annexin-V and propidium iodide staining. Cells were pretreated with dihydrospingosine in absence (a, c and d) or in presence (b) of both p38 and JNK inhibitors. Percentages of apoptotic cells are indicated in each graph. Results are representative of three independent experiments

(PBS), permeabilized with 100 μ l 0.1% Triton X-100 for 8 min at room temperature, washed with PBS and incubated for 20 min at room temperature with 2 μ l of primary Abs: anti-Bax IgG2b mAb (sc-7480) or its isotypic control (IgG2b mouse, sc-3879) and anti-Tom20 polyclonal Ab(FL-145) (sc-11415) or its isotypic control (IgG rabbit, sc-3888), all from Santa Cruz Clinisience, Nanterre, France. Then, slides were washed with PBS and incubated with the secondary Abs: 1 μ l of Alexa Fluor 488 donkey anti-rabbit IgG and 1 μ l of Alexa Fluor 633 F(ab')₂ goat anti-mouse IgG (both from Invitrogen) for 20 min at room temperature. Slides were washed with PBS, and nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI) (1 mg/mL). Images were collected using a Zeiss LSM-510 laser scanning confocal microscope (Zeiss, Marly-le-Roi, France). DAPI, Alexa Fluor 488 and Alexa Fluor 633 were excited with a 405-nm laser diode, a 488-nm argon laser and a 633-nm helium-neon laser, respectively. Fluorescence of DAPI, Alexa Fluor 488 and Alexa Fluor 633 were collected with a 420 \pm 30-nm band-pass filter, 530 \pm 15-nm band-pass filter and 650-nm long-pass filter, respectively. For epifluorescence microscopy (DMI 6000 B, Leica Microsystems, Nanterre, France), nuclei were stained with propidium iodide (0.5 mg/ml), after permeabilization of cells with 0.1% Triton X-100 diluted in PBS.

Western blotting. Total protein extracts were obtained as described.⁵⁸ For western blots, primary antibodies were anti PARP (Rabbit polyclonal 9542, Cell signaling, at 1/1000), anti caspase 3 (Rabbit mAb 8G10, Cell signaling, St-Quentin-en-Yvelines, France at 1/1000), anti-cleaved caspase 3 (Rabbit polyclonal D175, Cell signaling, at 1/1000) and anti α -tubulin (sc-23948, Santa Cruz at 1/200). Secondary

horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit immunoglobulins (Bio-Rad, Marnes-la-Coquette, France) were used at 1/5000 for 1 h.

siRNA transfection. Double-stranded RNAs (21 nt) against TP53wt was chemically synthesized by Eurogentec (Angers, France) according to the previously published sequence:⁵⁹ 5'-GCAGAACCGGAGGCCCAUdT-3'. A universal control (CU) consisting in a scrambled sequence was purchased from Eurogentec. Transfections were done using the Amaxa cell optimization kit V (Lonza, Verviers, Belgium), following the Amaxa guidelines. Briefly, cells were re-suspended in Nucleofector V solution. siRNA (2 μ g) were added to cell suspensions (7 \times 10⁶ in 100 μ l), transferred into chambers and nucleofected using the R.013 program (Amaxa, Lonza).

Conflict of Interest

The authors declare no conflict of interest.

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