The potential anticancer agent PK11195 induces apoptosis irrespective of p53 and ATM status in chronic lymphocytic leukemia cells

Antonio F. Santidrián, Ana M. Cosialls, Llorenç Coll-Mulet, Daniel Iglesias-Serret, Mercè de Frias, Diana M. González-Gironès, Clara Campàs, Alicia Domingo, Gabriel Pons, Joan Gil

From the Departament de Ciències Fisiològiques II, IDIBELL-Universitat de Barcelona, L'Hospitalet de Llobregat, Spain (AFS, AMC, LCM, DIS, MdeF, DMGG, CC, GP, JG); Servei d'Hematologia, IDIBELL-Hospital de Bellvitge, L'Hospitalet de Llobregat, Spain (AD).

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Correspondence:
Joan Gil, Ph.D., Departament de Ciències Fisiològiques II, IDIBELL-Universitat de Barcelona, Campus de Bellvitge, Pavelló de Govern, 4ª planta, E-08907 L'Hospitalet de Llobregat, Barcelona, Spain.
E-mail: jgil@ub.edu

ABSTRACT

Background and Objectives

The potential anticancer agent 1-(2-chlorophenyl-N-methylpropyl)-3-isoquinolinecarboxamide (PK11195), a translocator protein (18KDa) (TSPO) ligand, facilitates the induction of cell death by a variety of cytotoxic and chemotherapeutic agents. Primary chronic lymphocytic leukemia (CLL) cells overexpress TSPO. The aim of this study was to examine the effects of PK11195 on CLL cells.

Design and Methods

Using cytometric analysis, we studied the cytotoxic effects of PK11195 on peripheral B and T lymphocytes from patients with CLL and from healthy donors. Western blot and cytometric analyses were used to study the mitochondrial effects of PK11195 on CLL cells. Moreover, we analyzed the cytotoxic effect of PK11195 in patients' cells with mutated p53 or ATM.

Results

PK11195 induces apoptosis and had additive effects with chemotherapeutic drugs in primary CLL cells. Other TSPO ligands such as RO 5-4864 and FGIN-1-27 also induce apoptosis in CLL cells. PK11195 induces mitochondrial depolarization and cytochrome c release upstream of caspase activation, and dithiocyana-tostilbene-2,2-disulfonic acid (DIDS), a voltage-dependent anion channel (VDAC) inhibitor, inhibits PK11195-induced apoptosis, demonstrating a direct involvement of mitochondria. CLL cells and normal B cells are more sensitive than T cells to PK11195-induced apoptosis. Interestingly, PK11195 induced apoptosis in CLL cells irrespective of their p53 or ATM status.

Interpretation and Conclusions

These results suggest that PK11195 alone or in combination with chemotherapeutic drugs might be a new therapeutic option for the treatment of CLL.

Key words: PK11195, apoptosis, chronic lymphocytic leukemia, chemotherapy.

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Chronic lymphocytic leukemia (CLL) is characterized by the accumulation of mature malignant $CD5^+$ B lymphocytes.[1] Although many drugs have been used in the therapy of CLL, at present there is no curative therapy, and the search for new candidate drugs for future treatment of CLL is an active area of research. Most drugs currently used in CLL therapy induce apoptosis of the leukemic cells, at least partially, through activation of the p53 pathway.[2-7] The mechanisms of resistance to such drugs include inactivation of p53, which is mutated in 5-10% of CLL cases at diagnosis, but in nearly 30% of chemotherapy-resistant cases of CLL.[2-4,8] Hence, new therapies that overcome these defects by acting independently of p53 are of great interest.[9]

The potential anticancer agent 1-(2-chlorophenyl-N-methylpropyl)-3-isoquinolinecarboxamide (PK11195) facilitates the induction of cell death by a variety of agents including Fas ligand (FasL) and chemotherapeutic drugs.[10-16] Furthermore, in some cell types, PK11195 alone is able to induce apoptosis.[16-21] Importantly, PK11195 chemosensitizes primary human acute myeloid leukemia and multiple myeloma cells.[22-24] PK11195 was ini-

Table 1. Characteristics of the patients with chronic lymphocytic leukemia.

Patient No.	Age/ sex	WBC	Lymphocytes (%)	CD38 (%)	ZAP70 (%)	Genomic* alterations	Binet/ Rai‡	PK50† (%)	F/T (%)
1	79/M	30	91	<20	<20	del 13q°	B/2	70	F
2	76/F	38	79	<20	48	del 13q	C/4	71/75	F/T
3	63/M	84	91	<20	43	del 13q°	A/0	83	F
4	65/M	19	77	64	53	tris 12°	B/2	54	F
5	70/M	70	79	24	86	del 11q, del 13q	C/4	41	T
6	49/F	31	ND	<20	<20	ND	A/1	48	F
7	75/F	91	ND	<20	<20	del 13q°	B/1	8	F
8	78/F	41	97	83	44	del 11q, del 13q	B/1	84	T
9	77/M	23	81	ND	50	ND	A/0	11	F
10	54/F	23	79	71	<20	normal	C/4	64	T
11	73/M	41	89	<20	22	del 13q	B/2	77	F
12	71/M	97	97	51	48	del 11q, del 13q	A/0	80	F
13	66/M	30	ND	66	ND	normal°	C/4	64	F
14	69/F	53	95	<20	28	ND	C/4	72	F
15	59/F	46	ND	<20	ND	del 13q	B/1	78	T
16	70/M	110	ND	<20	26	normal#	C/3	42	T
17	70/M	83	98	63	82	del 13q°	C/3	61	F
18	69/M	81	90	<20	<20	del 13q°	A/0	24	F
19	71/F	45	69	ND	nd	ND	A/0	51	F
20	67/F	110	93	41	42	del 11q, tris 12	C/4	39	T
21	84/F	91	88	71	<20	ND	C/3	60	F
22	84/F	69	94	<20	<20	ND	A/0	51	F
23	67/M	50	94	<20	ND	ND	A/0	8	F
24	70/M	118	99	93	39	del 13q, del 17p	C/4	56/48	F/T
25	82/M	40	87	ND	<20	del 13q	A/0	63	F
26	76/M	24	81	<20	<20	ND	A/1	84	F
27	80/M	22	50	>20	<20	tris 12#	C/3	17	F
28	79/M	27	86	<20	<20	del 13q#	A/1	70	F
29	65/M	27	81	<20	<20	del 13q	A/0	47	F
30	40/M	81	90	<20	46	del 13q	B/2	61	F
31	74/M	31	88	92	<20	ND	C/4	45	T
32	69/M	25	85	<20	<20	ND	C/4	38	T
33	80/F	66	96	<20	<20	del 13q	C/4	51	T
34	81/M	53	81	<20	<20	normal#	A/0	47	T
35	62/F	46	ND	<20	79	normal	C/4	59	T
36	51/M	128	100	<20	<20	ND	C/4	64	F
37	65/M	61	90	ND	<20	del11q°	B/1	39	T
38	73/M	32	82	<20	<20	del11q#	C/4	54	T
39	82/M	28	81	57	<20	del 11q°	A/1	60	T
40	47/M	36	93	30	<20	del 11q 13q#	B/2	34	T

WBC indicates white blood cell count (10^9/L); F: female; M: male; del: deletion; and ND: not determined; F: fresh cells; T: frozen-thawed cells. ZAP-70 was determined by flow cytometry in fixed cells with a conjugated antibody (Alexa-Fluor 488) (Caltag Laboratories, Burlingame, CA, USA). A sample was considered negative when the expression was less than 20%.
**Genomic alterations were determined by fluorescent in situ hybridization (FISH) except for °determined by MLPA or #determined by MLPA and FISH. ‡According to Binet/Rai's classification. †Cell viability after 50 μM PK11195 treatment for 24 hours. Control viability was normalized to 100%.*

tially described as a ligand for peripheral benzodiazepine receptor (PBR),[25] whose new proposed name is translocator protein (18 KDa) (TSPO).[26] TSPO is a transmembrane protein that is located mainly in the outer mitochondrial membrane, but is also expressed in other subcellular compartments. TSPO is associated with the regulation of cholesterol transport, the synthesis of steroid hormones, porphyrin transport, heme synthesis, apoptosis and cell proliferation.[26] Other chemically unrelated TSPO ligands induce apoptosis in different cell types,[15,17,19–21] indicating a TSPO-dependent mechanism; however, TSPO-independent mechanisms have been proposed to inhibit cell proliferation or sensitize cells to apoptosis.[24,27,28] It has been reported that PK11195 induces apoptosis by altering the mitochondrial permeability transition.[10,11] Interestingly, in a variety of systems, PK11195 can reduce or abrogate the anti-apoptotic effect of BCL-2-like proteins, including BCL-2 and BCL-X_L.[22,29] Since primary CLL cells overexpress TSPO[30] and BCL-2,[31,32] we decided to examine the effects of PK11195 on CLL cells.

Design and Methods

Samples, patients with CLL, healthy donors and cell isolation

Samples from patients with CLL (Table 1) or healthy donors were studied. Most of the patients had not been treated previously (patients number 1, 13, 17, 23, 24, 27, 28 and 33 were, however, treated). The patient number in Table 1 corresponds to the patient number in the figures. CLL was diagnosed according to standard clinical and laboratory criteria. Blood samples were obtained from the Hospital de Bellvitge, Barcelona, Spain. Written informed consent was obtained from all patients in accordance with the Hospital de Bellvitge Ethical Committee. Mononuclear cells from peripheral blood samples were isolated by centrifugation on a Ficoll-Hypaque (Seromed, Berlin, Germany) gradient and cryopreserved in liquid nitrogen in the presence of 10% dimethyl sulfoxide (DMSO).

Multiplex ligation-dependent probe amplification (MLPA) for genomic alterations and reverse transcriptase (RT)-MLPA

DNA was isolated and analyzed by MLPA using SALSA MLPA kits P037 and P038 from MRC-Holland (Amsterdam, The Netherlands). These kits were used to determine the loss of p53 (17p13; 8 probes), the RB1/DLEU/MIR15-16 region on 13q14 (12 probes) and the ATM gene on 11q23 (7 probes) in DNA samples obtained from CLL cells. RNA was isolated from cultured cells by the RNeasy Micro kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer's protocol. RNA was analyzed by RT-MLPA using the SALSA MLPA kit R011 Apoptosis mRNA from MRC-Holland (Amsterdam, The Netherlands) for the simultaneous detection of 38 mRNA molecules.[7,33]

Analysis of apoptosis by flow cytometry

Apoptosis was assessed by exposure of phosphatidylserine and membrane integrity. This was determined by annexin V-fluorescein isothiocyanate (FITC), propidium iodide (PI) double staining, and flow cytometric analysis using FACSCalibur and CellQuest software (Becton Dickinson, Mountain View, CA, USA), as described previously.[34] Cell viability was measured as the percentage of annexin V and PI double-negative cells. To

analyze apoptosis in T cells and B cells from the samples, 5×10^5 cells were incubated for 24 or 48 hours with the indicated factors. Cells were then washed in phosphate-buffered saline (PBS), and incubated in 50 µL annexin-binding buffer with allophycocyanin (APC)–conjugated anti-CD3 and phycoerythrin (PE)–conjugated anti-CD19 from Becton Dickinson (Franklin Lakes, NJ, USA) for 10 minutes in the dark. Cells were then diluted with annexin-binding buffer to a volume of 150 µL and incubated with 1 µL annexin V–FITC for 15 minutes in the dark. Cells were analyzed using the FACScalibur and CellQuest software.

Cytochrome c release measurements

Release of cytochrome c from mitochondria into the cytosol was measured by western blot as previously described[35] with some modifications. Cells (25×10^6) were harvested, washed once with ice-cold PBS and gently lysed for 30 seconds in 80 µL ice-cold lysis buffer (250 mM sucrose, 1 mM EDTA, 0.05% digitonin, 25 mM Tris, pH 6.8, 1 mM dithiothreitol, 1 µg/mL leupeptin, 1 µg/mL pepstatin, 1 µg/mL aprotinin, 1 mM benzamidine, and 0.1 mM phenylmethylsulfonyl fluoride). Lysates were centrifuged at 12,000×g at 4°C for 3 minutes to obtain the supernatants (cytosolic extracts free of mitochondria) and the pellets (the fraction containing the mitochondria). Supernatants (50 µg) were electrophoresed on a 15% polyacrylamide gel and then analyzed by western blot using anti-cytochrome c antibody (7H8.2C12, from Pharmingen, San Diego, CA, USA) and an electrochemiluminescence system, as described in online supplementary data.

Statistical analysis

Results are shown as the mean ± standard deviation (SD) of values obtained in independent experiments. The paired Student's *t* test was used to compare differences between paired samples. Data were analyzed using the SPSS 11.5 software package (Chicago, IL, USA). Additive and synergistic effects were analyzed using the fractional product method.[36]

Results

PK11195 and other TSPO ligands induce apoptosis in CLL cells

As FasL does not induce apoptosis in CLL cells[37] we first examined whether PK11195 reverses this resistance in CLL cells, but PK11195 was not able to do so (Figure 1A). However, when combined with the chemotherapeutic drugs, dexamethasone, doxorubicin, chlorambucil, fludarabine and mafosfamide, PK11195 had an additive effect in inducing apoptosis in CLL cells (Figures 1B, 1C and 1D). It is interesting to note that 50 µM PK11195 alone induced apoptosis in most of the samples analyzed in Figure 1. Thus, we studied the effect of several doses of PK11195 on the viability of CLL cells. Although there

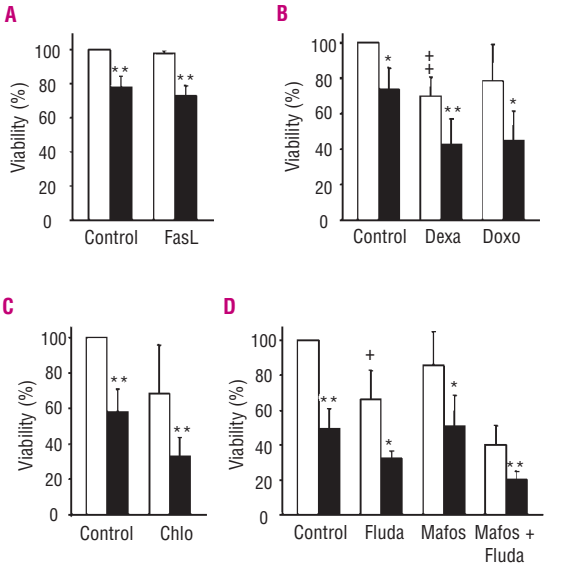

Figure 1. Additive effect of PK11195 and chemotherapeutic drugs. Cells from four different CLL patients were treated without (open bars) or with (filled bars) 50 µM PK11195 and with 100 ng/mL FasL (A), or with 10 µM dexamethasone (Dexa) or 0.4 µM doxorubicin (Doxo) for 24 hours (B), or with 10 µM chlorambucil (Chlo) for 48 hours (C), or with 3 µM fludarabine (Fluda) or 5 µM mafosfamide (Mafos) or both for 48 hours (D). Viability was measured by analysis of phosphatidylserine exposure and PI uptake as described in the *Design and Methods* section, and is expressed as the mean value ± SD of the percentage of nonapoptotic cells. $^{**}p<0.05$, $^{*}p<0.1$, PK11195 treated cells versus PK11195 untreated cells. $^{\ddagger}p< 0.05$, dexamethasone or mafosfamide plus fludarabine treated cells versus control cells. $^{\dagger}p<0.1$, fludarabine treated cells versus control cells.

was heterogeneity in the sensitivity of CLL cells, PK11195 induced apoptosis in all the samples analyzed in a dose-dependent manner (Figure 2A) and the IC_{50} was 60±21 µM (n=29) at 24 hours. The viability after incubation for 24 hours with 50 µM PK11195 decreased from 66±18% to 38±21% (n=33), and with 100 µM PK11195 it decreased from 71±16% to 16±13% (n=19). Fresh or frozen-thawed CLL cells showed the same sensitivity to PK11195 (Table 1). The average of the effect of 50 µM PK11195 was 54±21% for fresh cells and 53±15% for thawed cells. Furthermore, PK11195 had the same effect on viability of fresh or frozen-thawed CLL cells from the same sample (patients 2 and 24, Table 1). PK11195 induced apoptosis independently of sex, ZAP70 or CD38 status (Table 1). Next, we analyzed whether other compounds described as TSPO ligands could induce apoptosis in CLL cells. Thus, CLL cells were incubated for 24 hours with PK11195 and the TSPO ligands RO 5-4864 and FGIN-1-27[26] in a range of concentrations (12.5-100 µM). Similarly to PK11195, both RO 5-4864 and FGIN-1-27 induced apoptosis in a dose-dependent manner and their IC_{50} were 83±18 µM and 88±31 µM, respectively (n=9) (Figure 2B). Moreover, PK11195 had a synergistic effect with regards to induction of apoptosis in CLL cells when combined with the other TSPO ligands, RO 5-4864 and FGIN-1-27 (Figure 2C).

Characterization of PK11195-induced apoptosis in CLL cells: loss of mitochondrial membrane potential and cytochrome c release precede caspase activation

The effect of PK11195 on the loss of mitochondrial membrane potential ($\Delta\psi_m$) and cytochrome c release was analyzed in CLL cells. First, we analyzed the effect of 50 μM PK11195 on $\Delta\psi_m$ using the JC-1 dye. PK11195 induced apoptosis (Figure 3A), and decreased $\Delta\psi_m$ (Figure 3B) at 6 hours. The caspase inhibitor Z-VAD.fmk did not inhibit loss of $\Delta\psi_m$ but did inhibit PK11195-induced apoptosis, indicating that early loss of $\Delta\psi_m$ is caspase-independent. Furthermore, to analyze the involvement of cytochrome c release in PK11195-induced apoptosis, cytosolic fractions were obtained and the presence of cytochrome c was analyzed by western blotting. Treatment with 50 μM PK11195 for 6 hours produced an increase in cytochrome c in the cytosolic fraction of CLL cells from two different representative patients (Figure 3C), demonstrating that PK11195 induced cytochrome c release. Z-VAD.fmk did not inhibit PK11195-induced cytochrome c accumulation in the cytosol. Furthermore, PK11195 induced cleavage and activation of caspase-9 at 9 hours (Figure 3D). These results demonstrate that the mitochondrial effects of PK11195 are caspase-independent and precede the activation of caspases.

To study the mechanism involved in PK11195-induced cytochrome c release, we used RT-MLPA to analyze changes in the expression of genes involved in the control of apoptosis.[7,33] PK11195 treatment for 3 and 6 hours did not modify the expression of any of the genes of the BCL-2 family analyzed (*data not shown*). Furthermore, treatment with PK11195 for 3 or 6 hours neither increased the levels of the pro-apoptotic proteins BIM, PUMA, and BMF, nor decreased the levels of the anti-apoptotic proteins MCL-1, BCL-X_L and BCL-2 (Figure 4A). Furthermore, treatment with PK11195 did not change the levels of the pro-apop-

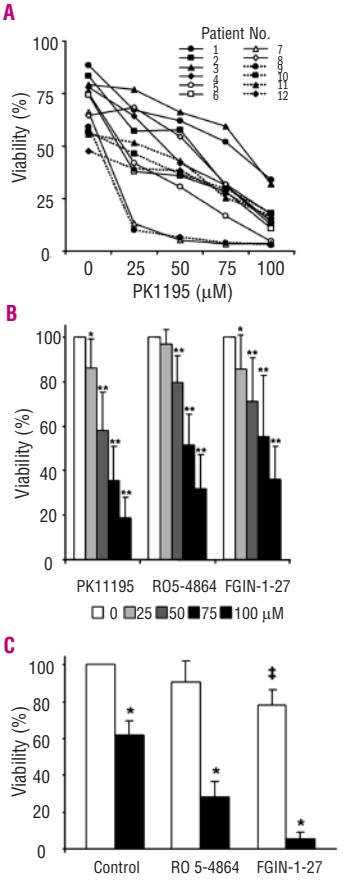

Figure 2. Cytotoxic effect of PK11195 and other TSPO ligands on CLL cells. (A) Cells from 12 CLL patients were incubated with the indicated doses of PK11195 for 24 hours. (B) Dose-response of the cytotoxic effect of PK11195, RO 5-4864 or FGIN-1-27. Cells from nine CLL patients were incubated with several doses of the drugs for 24 hours. $^{**}p<0.01$; $^{*}p<0.05$ treated cells versus control cells. (C) Cells from five CLL patients were treated without (open bars) or with (filled bars) 50 μM PK11195 and with 50 μM RO 5-4864 or with 50 μM FGIN-1-27.$^{*}p<0.01$, PK11195 treated cells versus PK11195 untreated cells. $^{\ddagger}p<0.01$ FGIN-1-27 treated cells versus control cells. Data are shown as the mean value ± SD. Viability was measured by analysis of phosphatidylserine exposure and PI uptake as described in the *Design and Methods* section and it is expressed as the percentage of non-apoptotic cells.

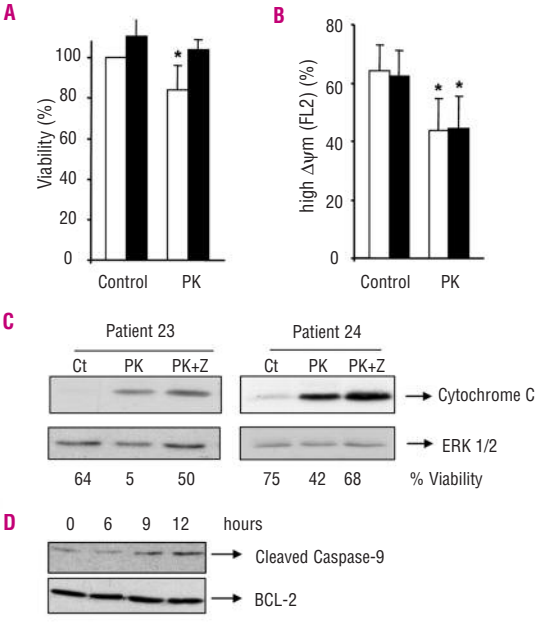

Figure 3. Caspase-independent mitochondrial effects of PK11195 on CLL cells. Cells from CLL patients were incubated without (Control) or with (PK) 50 μM PK11195 and in the presence (filled bars) or in the absence (open bars) of 200 μM Z-VAD.fmk for 6 hours. (A) Cell viability was quantified by annexin V binding at 6 hours and is expressed as the percentage of non-apoptotic cells ± SD (n=5). Control viability was normalized to 100%. $^{*}p<0.05$ treated cells versus untreated cells. (B) Changes in $\Delta\psi_m$ were measured by staining with JC-1. The loss in $\Delta\psi_m$ is seen as a shift to lower JC-1 red fluorescence (FL-2) and is expressed as the percentage of high FL-2 cells. Data are shown as the mean value ± SD of five CLL samples. (C) Effect of Z-VAD.fmk on PK11195-induced cytochrome c release into the cytosol. Cells were pre-incubated without or with 200 μM Z-VAD.fmk for 30 minutes and then treated with 50 μM PK11195 for 6 hours. Cytochrome c and ERK 1/2, which was used as a control of protein loading in cytosolic cell extracts, were analyzed by western blot as described in the *Design and Methods* section. Cell viability at 24 hours is also shown. (D) CLL cells were treated with 50 μM PK11195 for 0, 6, 9 and 12 hours. Cells were lysed and cleaved caspase 9 and BCL-2 were analyzed by western blot as described in the *Design and Methods* section. Results from a representative patient are shown.

totic protein BAX and induced an increase in the levels of MCL-1 protein in most of the samples analyzed (Figure 4A and 4B). Moreover, inhibition of cAMP-dependent protein kinase (PKA) or JNK with the specific inhibitors (H89 or SP600125, respectively) did not affect PK11195-induced apoptosis. Interestingly, DIDS, a voltage-dependent anion channel (VDAC) inhibitor,[38] reduced the pro-apoptotic activity of PK11195 in CLL cells (Figure 4C). As a control, DIDS did not inhibit apoptosis induced by fludarabine or doxorubicin (*data not shown*).

Differential effect of PK11195 on B and T cells from patients with CLL and healthy donor

Next, we analyzed the sensitivity of normal B and T cells to apoptosis induced by PK11195. The number of apoptotic $CD3^+$ T cells was measured in blood samples from four CLL patients and four healthy donors exposed to several doses of PK11195, ranging from 12.5 μM to 100 μM, for 24 hours (Figure 5A). Treatment with 50 μM PK11195 reduced the percentage of viable CLL $CD19^+$ B cells to 46±7% and the IC_{50} was 47±2 μM. In contrast, under the same treatment the percentage of viable T cells from the same samples was 95±4% with an IC_{50} of 87±8 μM. Similar results were obtained with cells obtained from healthy donors such that 50 μM PK11195 reduced the viability of B cells to 42±3%, with an IC_{50} of 49±4 μM, while the viability of T cells was 98±6% with an IC_{50} of 82±8 μM. At doses of PK11195 higher than 50 μM, T cells from CLL patients and healthy donors showed sensitivity to apoptosis although to a lesser extent than B cells. These dose-response experiments show that B cells are more sensitive than T cells to PK11195-induced apoptosis. Similarly, T cells are more resistant than B cells from CLL samples to RO 5-4864- and FGIN-1-27-induced apoptosis (Figure 5B).

PK11195 induces apoptosis irrespective of p53 and ATM status in CLL cells

To study the role of p53 in PK11195-induced apoptosis we analyzed the effect of this compound on p53 protein levels (Figure 6A). The patients with mutated p53 (patients 24 and 33) or altered expression were described previously.[7] Patient 24 has a frame-shift mutation in one allele (nucleotide deletion in codon 272) and a 17p deletion in the other allele in 86% of cells from peripheral blood leukocyte (PBL) samples, and patient 33 has the M246V muta-

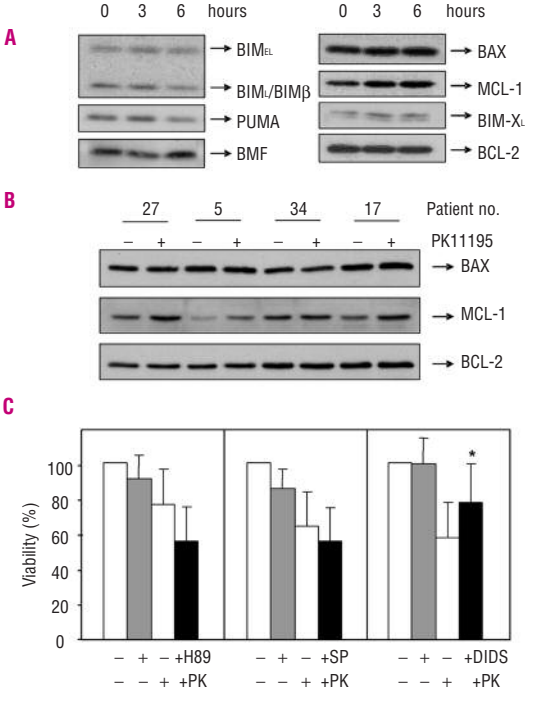

Figure 4. Characterization of PK11195-induced apoptosis in CLL cells. (A) Cells from a representative patient were treated with 50 μM PK11195 for 0, 3, and 6 hours. Cells were lysed and analyzed by western blot as described in the *Design and Methods* section. Total levels of BIM, PUMA, BAX, MCL-1, BCL-XL, and BCL-2 are shown. (B) Cells from four different patients were treated with 50 μM PK11195 for 6 hours. Cells were lysed and analyzed by western blot as described in the *Design and Methods* section. Total levels of BAX, MCL-1, and BCL-2 are shown. (C) Cells from several CLL patients were preincubated with 10 μM H89 (n=4), or with 10 μM SP600125 (SP) (n = 3), or with 200 μM DIDS (n=5) for 30 minutes prior to exposure to 50 μM PK11195 (PK) for 24 hours. Cell viability was quantified by annexin V binding and is expressed as the percentage of non-apoptotic cells ± SD. Viability of untreated cells was normalized to 100%. *p<0.05 treated cells versus untreated cells

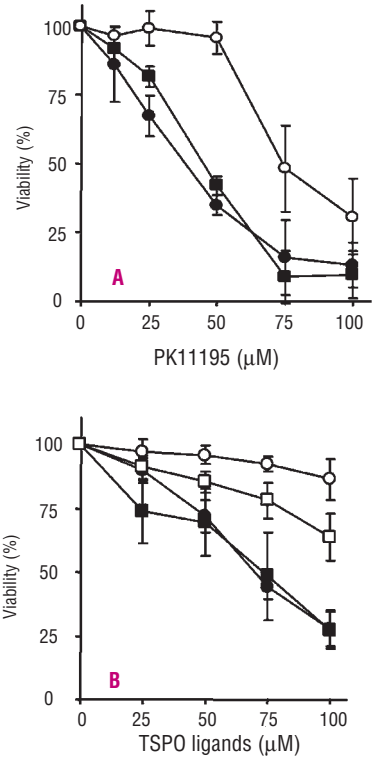

Figure 5. Differential effect of TSPO ligands on B and T cells. (A) Dose-response of the cytotoxic effect of PK11195 on CD3-/CD19+ B cells from four CLL patients (●), $CD3^-/CD19^+$ B cells from three healthy donors (■) and $CD3^+/CD19^-$ T cells from four CLL and three healthy donors (○). Cells were incubated with the indicated doses of PK11195 for 24 hours. (B) Cells from three CLL patients were incubated with the indicated doses of RO 5-4864 (●, ○) or FGIN-1-27 (■, □) for 24 hours. Viability was measured as nonapoptotic $CD3^+/CD19^-$ T cells (●,■) or $CD3^-/CD19^+$ B cells (○, □) as described in the *Design and Methods* section and expressed as the percentage of the viability of non-treated cells. Data are shown as the mean value ± SD.

tion, which has been described to interfere with wild type p53.[39] When CLL cells were incubated with 50 μM PK11195 there was no change in p53 levels (Figure 6A). As a control of p53 activation we used doxorubicin (0.8 μM), which induced apoptosis and p53 protein accumulation in wild type p53 cells (from patients 16 and 34) but not in mutated p53 cells (from patients 24 and 33). In patient 24, doxorubicin induced a low accumulation of mutated p53. Interestingly, PK11195 induced apoptosis in CLL cells with mutated p53 (Figure 6A). Furthermore, chemotherapeutic drugs that activate p53 did not increase the apoptotic effect of PK11195 in p53-mutated samples (*data not shown*).

Next, we analyzed the effect of PK11195 on CLL cells with ATM alterations. ATM expression was analyzed by western blotting in several patients with and without 11q deletion (Figure 6B). We found loss of ATM expression in three patients with an 11q deletion (patients 5, 8 and 39). Patients 5 and 8 have the 11q deletion in one allele in 90% and 86% of cells from PBL samples, respectively. The low expression of ATM likely corresponds to the percentage of contaminating normal cells. PK11195 induced apoptosis in CLL cells with 11q deletion irrespectively of the expression of ATM protein (Figure 6C).

Discussion

In this work we show that the potential anticancer agent PK11195 induces apoptosis in primary CLL cells. Several targets for the induction of apoptosis by PK11195 have been suggested. The best known target of PK11195 is TSPO,[25] which is overexpressed in CLL cells.[30] Other TSPO ligands such as RO 5-4864 and FGIN-1-27 also induce apoptosis in primary CLL cells. However, the role of TSPO in PK11195-induced apoptosis is controversial. PK11195 binds to TSPO *in vivo* at nanomolar concentrations,[25] although PK11195 induces apoptosis at much higher concentrations (50-100 μM). Furthermore, siRNA experiments demonstrate that TSPO is not necessary to inhibit cell proliferation[27] or sensitize cells to apoptosis.[28] Multidrug resistance (MDR) has been proposed as a PK11195 target to sensitize cells to apoptosis and could explain the synergism between PK11195 and other chemotherapeutic drugs that are transported by MDR proteins.[24] PK11195 inhibits MDR in CLL cells (*data not shown*) and this could contribute to the additive effect of PK11195 and chemotherapeutic drugs. However, PK11195 was able to induce apoptosis alone in CLL, whereas it is not clear how inhibition of MDR could induce apoptosis in the absence of other drugs. Furthermore, cyclosporine A inhibits MDR without affecting the viability of CLL cells (*data not shown*). Thus, there is no correlation between inhibition of MDR and induction of apoptosis by PK11195 alone. Taken together, these results indicate that the apoptotic activity of PK11195 cannot be attributed to its inhibitory effect on MDR.

Moreover, PK11195 was described as a phosphodiesterase (PDE) inhibitor *in vitro*.[40] PDE inhibitors induce cyclic AMP (cAMP) levels and have been described as apoptotic inducers in CLL cells.[41] However, H89, a cAMP-dependent protein kinase inhibitor did not inhibit PK11195-induced apoptosis, indicating that PK11195 induces apoptosis in CLL cells through a different mechanism. JNK has also been involved in the induction of apoptosis by the combination of PK11195 and bortezomib,[23] but in CLL cells inhibition of JNK did not protect from PK11195-induced apoptosis.

We have shown that PK11195 induces caspase-dependent apoptosis in CLL cells. Furthermore, PK11195 has

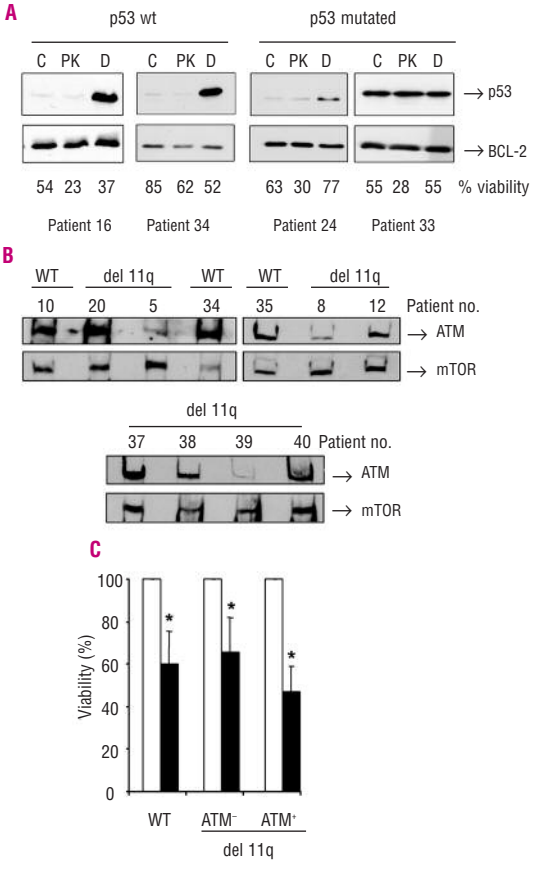

Figure 6. PK11195 induces apoptosis independently of p53 and ATM in CLL cells. (A) PK11195 does not induce p53 stabilization and accumulation in CLL cells. Cells from two CLL patients with wild-type p53 (patients 16 and 34) and two with mutant p53 (patients 24 and 33) were untreated (C), or treated with 50 μM PK11195 (PK), or 0.8 μM doxorubicin (D) for 24 hours. Cells were lysed and analyzed by western blot as described in the *Design and Methods* section. Total levels of p53, and BCL-2 were analyzed. Viability was measured by analysis of phosphatidylserine exposure and PI uptake as described in the *Design and Methods* section and is expressed as the percentage of non-apoptotic cells. (B) Analysis of ATM expression in cells from CLL patients without genomic alterations (WT) (patients 10, 34 and 35), or with 11q deletion (del 11q) (patients 5, 8, 20, 37, 38, 39 and 40). mTOR was used as a control of protein loading. (C) Cells without genomic alteration and with ATM expression (WT) (patients 10, 34 and 35), or cells with 11q deletion (del 11q) and without ATM expression (ATM^- (patients 5, 8,and 39), or with ATM expression (ATM^+) (patients 20, 37, 38 and 40) were incubated with (filled bars) or without (open bars) 50 μM PK11195 for 24 hours. Viability was measured by analysis of phosphatidylserine exposure and PI uptake as described in the *Design and Methods* section, and is expressed as the mean value ± SD of the percentage of non-apoptotic cells. Viability of untreated cells was normalized to 100%. *p<0.05 treated cells versus untreated cells.

effects in mitochondria upstream of caspase activation, as a caspase inhibitor was not able to inhibit the mitochondrial effects induced by PK11195, such as depolarization and cytochrome c release. However, the levels of the pro-apoptotic proteins BIM, PUMA, BMF and BAX did not increase, and the levels of the anti-apoptotic proteins MCL-1, BCL-X_L and BCL-2 did not decrease. It should be pointed out that DIDS, a VDAC inhibitor, inhibited PK11195-induced apoptosis, suggesting an involvement of VDAC. Significantly, VDAC has been proposed as one of the components of the permeability transition pore complex (PTPC) that mediates cytochrome c release.[40] Additionally, VDAC interacts and modulates or is modulated by BCL-2 family members such as BCL-2, BCL-X_L, BAX, BAK, and BIM.[42-44] BAX and BAK may co-operate with the PTPC to form a channel, but whether cytochrome *c* release is mainly mediated by the PTPC and/or the pore-forming function of BAX/BAK is still open to debate.[42,43] Thus, the mechanism by which PK11195 induces cytochrome *c* release requires further investigation using cells lines in which the candidate proteins could be down-regulated efficiently.

Our experiments indicate that CLL cells and normal B cells are more sensitive to PK11195-, RO 5-4864-, and FGIN-1-27-induced apoptosis than are T cells from the same samples. Chemotherapeutic drugs including fludarabine, chlorambucil, and doxorubicin induce apoptosis equally in both B and T cells[5,45] leading to immunosuppression.[46] Thus, the differential effect of PK11195 in B and T lymphocytes is of interest. Most drugs currently used in the therapy of CLL act, at least partially, through activation of the p53 pathway.[2,5-7] p53 is mutated in 5-10% of CLL cases at diagnosis, but in nearly 30% of chemotherapy-resistant tumors.[2-4,8] Furthermore, ATM is inactivated in 10-20% of CLL cases thus providing an alternative way for disabling p53 function.[47,48] Tumors with alterations upstream of p53 would not respond adequately to genotoxic chemotherapeutics that act through the p53 pathway, for example, alkylating agents (chlorambucil, cyclophosphamide), purine nucleosides (fludarabine, cladribine) or topoisomerase inhibitors (doxorubicin, mitoxantrone). Genetic alterations in *P53* and *ATM* are among the worst prognostic factors in CLL patients,[3,4,46-49] and p53 alterations confer resistance to conventional chemotherapy.[3,4,7] Thus, new approaches to induce apoptosis in cells with mutated p53 or ATM are needed. Here, we demonstrate that PK11195 induces apoptosis in CLL cells irrespectively of their p53 or ATM status.

From a therapeutic perspective, it is interesting to note that the doses of PK11195 that induce apoptosis in CLL samples *in vitro* are achievable *in vivo*, as previously demonstrated by their potent antitumor effect in mouse models of human cancer.[11,21,50] PK11195 has been administered safely to patients.[51,52] Furthermore, *PK11195* has additive effects with chemotherapeutic drugs in CLL. This suggests that it could be possible to lower the doses of chemotherapeutic drugs used in the treatment of CLL and thus reduce cytotoxicity to normal T cells. In conclusion, the results presented here suggest that PK11195 alone or in combination with chemotherapeutic drugs might be a new therapeutic option for the treatment of CLL.

Authors' Contributions

AFS performed the research, analyzed the data and wrote the paper. AMC, LC-M, DI-S, MdF, DMG-G performed research; CC contributed with analytical tools; AD contributed with patients samples and data; GP designed the research and analyzed the data; JG designed and supervised the research, analyzed the data and wrote the paper. All authors revised the manuscript critically and approved the final version to be published.

Conflict of Interest

The authors reported no potential conflicts of interest.

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