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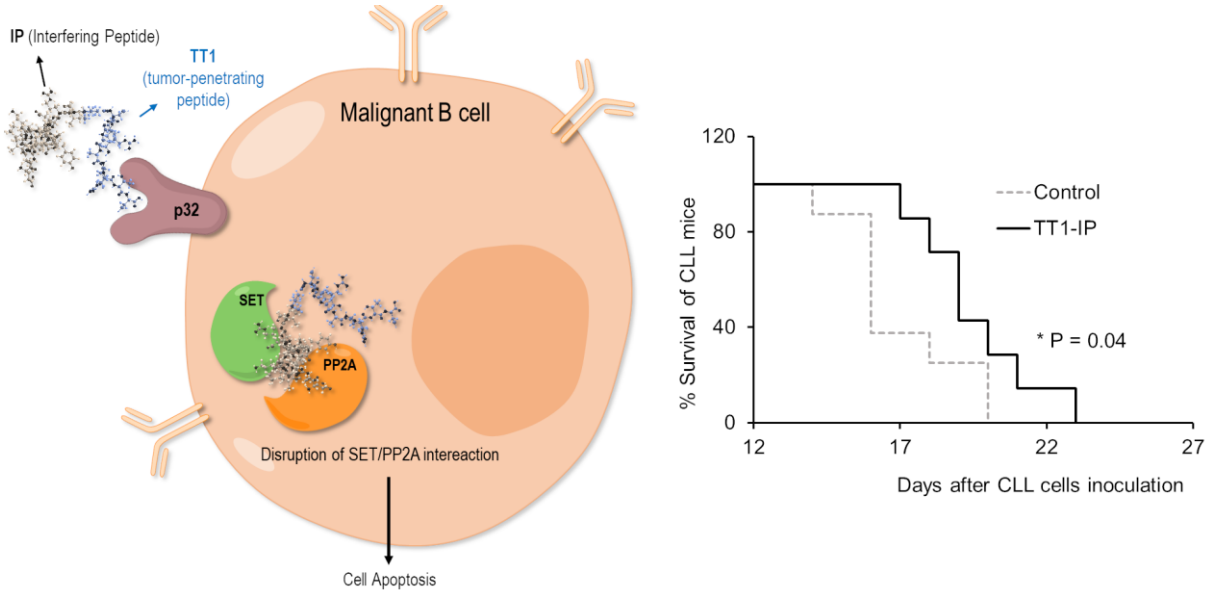
GRAPHICAL ABSTRACT



ABSTRACT

Chronic lymphocytic leukemia (CLL) is the most common form of leukemia in adults. ~~This pathology~~ The disease is characterized by the accumulation of tumoral B cells resulting from a defect of apoptosis. We have *in vitro* and *in vivo* preclinically validated a tumor-penetrating peptide (named TT1) coupled to an interfering peptide (IP) that dissociates the interaction between the serine/threonine protein phosphatase 2A (PP2A) from its physiological inhibitor, the oncoprotein SET. ~~P2A~~ ~~PP2A/SET~~ This TT1-IP peptide has anti-tumoral effect on CLL, as shown by the increased survival of mice bearing xenograft models of CLL, compared to control mice. The peptide did not show toxicity, as indicated by the mouse body weight and the biochemical parameters, such as renal and hepatic enzymes. In addition, the peptide induced apoptosis *in vitro* of primary tumoral B cells isolated from CLL patients but not of those isolated from healthy patients. Finally, the peptide had ~~around~~ approximately 5 hours half-life in human serum and showed pharmacokinetic parameters compatible with clinical development as therapeutic peptide against CLL.

Key words: chronic lymphocytic leukemia/ xenograft models/ tumor-penetrating and interfering peptides

INTRODUCTION

Chronic Lymphocytic Leukemia (CLL), the most common form of leukemia in adults [1], is defined by an accumulation of monoclonal neoplastic B cells in hematopoietic organs resulting from a defect in programmed cell death. Clinical outcome and the prognosis of CLL patients depends on factors such as cellular and genetic markers, as well as the age and health profile of the patients. For young CLL patients, the standard chemoimmunotherapy combining chemotherapy (cytostatic agents such as fludarabine and cyclophosphamide) and immunotherapy (anti-CD20 antibodies, Rituximab) is well tolerated and effective in controlling the disease [2]. In contrast, in ~~older~~ patients ~~or~~ with underlying health conditions, this frontline treatment can cause severe adverse effects [3]. Moreover, the patient cohort with a deletion in chromosome 17 (del17), or mutation in the p53 protein show resistance to chemotherapy that cannot be overcome by treatment with anti-CD20 antibodies. These patients, as well as patients with IGHC unmutated status [4], have rapid disease progression and lower survival. In these cases, the use of specific inhibitors interrupting pathways for CLL cell survival (such as inhibitors of the Bruton kinase and anti-apoptotic Bcl-2 ~~Ibrutinib and Idelalisib~~) has shown promising results [3]. However, some patients relapse due to acquired mutations (e.g. in the gene encoding for Bruton tyrosine kinase), rendering the disease resistant to the proliferation inhibitors. Therefore, it is important to develop new CLL therapies for patients in which the current treatments are not effective or have severe side effects.

We have recently developed bifunctional peptides composed of a tumor-penetrating peptide (TPP), which selectively internalizes in malignant B cells and tumoral hepatocytes [5], in tandem with an interfering peptide (IP), which blocks the interaction between ~~2~~ two cytoplasmic proteins, PP2A and SET [6, 7]. SET is an oncoprotein overexpressed in several cancers including CLL. In CLL, SET is involved in the disease initiation and progression as

well as in the development of therapeutic resistance[7, 8]. The IP blocks the interaction of SET and PPA2, resulting in cancer cell death [7]. The dissociation of PP2A from SET allows the activation of PP2A. To guide and deliver the IP peptide to the tumoral ~~B cells of~~ CLL cells, we previously tested four different TPPs: RPARPAR, iRGD, TT1, and LinTT1. TPPs bind to and internalize into tumor cells and are used to deliver therapeutic and imaging cargoes into tumors. The TPPs containing the sequence R/KXXR/K at the C-terminal (CendR peptides) bind to neuropilin-1 (NRP-1), a receptor overexpressed in different types of tumors and malignant cells, including B cells from CLL patients [9]. The CendR peptide RPARPAR binds NRP-1 [10] and has been used to deliver nanoparticles into cancer cells [11, 12]. iRGD peptide (internalizing RGD) binds to αvβ3 integrin in the tumor vasculature and after being processed by tumor-related proteases engages NRP-1 to activate penetration into the tumor tissue [13-15,16]. The receptor of the cyclic TT1 peptide (CKRGARSTC) and its linear version LinTT1 (AKRGARSTA) is the p32/gC1qR/C1QBP/HABP1 protein (p32) that is aberrantly expressed on the surface of malignant and tumor-~~associated~~environment cells (tumor-associated lymphatic vessels and tumor-associated macrophages) [17]. TT1 and LinTT1 are first recruited to p32, followed by proteolytic cleavage to allow NRP-1 engagement [18]. LinTT1 has been used for precision therapy and imaging of different solid tumor models [19-23]. We previously found that the B cells from CLL patients express ~~the~~ receptors (integrin αvβ3, NRP-1, and p32) of the ~~four~~ three four TPPs here mentioned, and that the TPPs in tandem with the IP (TPP-IP) specifically internalize in and kill malignant B cells from CLL patients *in vitro*[6]. Importantly, we have shown that the treatment with iRGD-IP significantly increased the survival of CLL mice [6]. In the current report, we describe the *in vitro* and *in vivo* validation in a xenograft model of CLL of the ~~other~~ three bifunctional tumor-penetrating and interfering peptides (RPARPAR-IP, TT1-IP, and LinTT1-IP) as well as the biodistribution and

pharmacokinetic parameters of the TT1-IP, the peptide that showed the highest increase in the survival of CLL mice.

MATERIALS AND METHODS

Peptide synthesis and sequence

Peptides were synthesized in an automated multiple peptide synthesizer with solid phase procedure and standard Fmoc chemistry by GL Biochem with a purity of 98% (Shanghai, China). The purity and composition of the peptides were confirmed by reverse phase high performance liquid chromatography (HPLC) using an increasing CH_3CN gradient and by mass spectrometry (MS). Unlabelled peptides were used for the apoptosis assays and experimental treatments. For live imaging experiments, TT1-IP was synthesized with the fluorochrome Cy7 at the N-terminus. The sequences of all peptides are shown in Table I.

Peptide ID	Sequences
TT1	**C**KRGARST**C**-$CONH_2$ (**C-C** disulfide bond)
iRGD-IP	ETVTLLVALKVRYRERIT-Ahx-**C**RGDKGPD**C**-$CONH_2$ (**C-C** disulfide bond)
RPARPAR-IP	ETVTLLVALKVRYRERIT-Ahx-RPARPAR-OH
LinTT1-IP	ETVTLLVALKVRYRERIT-Ahx-AKRGARSTA-$CONH_2$
TT1-IP	Cy7-Ahx-ETVTLLVALKVRYRERIT-Ahx-**C**KRGARST**C**-$CONH_2$ (**C-C** disulfide bond)
	ETVTLLVALKVRYRERIT-Ahx-**C**KRGARST**C**-$CONH_2$ (**C-C** disulfide bond)

Table 1: Sequence of the peptides used in the study. Ahx = aminohexanoic acid

CLL xenograft models and experimental treatments

The 6-8 weeks old female CB-17 SCID mice were purchased from Envigo (Gannat, France). All mice were maintained under conditions and protocols in accordance with the directives of the Council of European Animal Welfare. The animal experiments were done following the protocols approved by the Estonian Ministry of Agriculture, Committee of Animal Experimentation (project #196) and all the experiments were conducted following the guidelines of the mentioned committee.

CLL Jok 1.3 cells (2×10^6) in 100 µL of phosphate saline buffer were injected into the tail vein. We have previously used the same CLL xenograft model[6] and observed that 100% of the tumor-induced mice developed CLL; therefore, no evaluation of the transplantation was performed before starting the treatment. Mice were intraperitoneally injected with saline (control group) or with the peptides three days after the inoculation. One experimental treatment was performed with four different groups (control, TT1-IP, LinTT1-IP, and RPARPAR-IP) and another treatment was performed with the control and TT1 groups. In both treatments, seven mice per group were used. The peptide dose was 2.6 µmols/Kg (corresponding to 5 mg/Kg of TT1-IP peptide). Mice were monitored daily for the presence of hind leg paralysis, which is indicative of infiltration of leukemic cells in the central nervous system, and in that case, sacrificed.

Fluorescence live imaging

The 6–8 weeks old female CB-17 SCID mice were purchased from Charles River Laboratories (L'Arbresle, France). All mice were maintained under conditions and protocols in accordance with the Directive 2010/63/UE of the Council of Europe on Animal Welfare. The study (number authorization 27656) was approved by the French Ethics Committee for animal experimentation number 74 and all experiments were conducted following the guidelines of the aforementioned committee.

Animals were sedated with 2% isoflurane in air. CLL Jok5.3 cells (2×10^6) in 100µL of NaCl 0.9% were injected into the retro-orbital vein of SCID mice. Three days after cell transplantation, mice were intravenously (IV) injected with saline or 2.6 µmols/kg (5 mg/kg) of Cy7-TT1-IP peptide followed by fluorescence imaging. Acquisitions were performed 15 min after IV injection, then regularly, up to 6 h post-administration. Before the acquisition, the animal was anesthetized by isoflurane inhalation (2% air-isoflurane blend). Then, the mouse was placed inside the acquisition chamber of an *in vivo* imaging system equipped with

a cooled slow-scan CCD camera and driven with Indigo software (Berthold Technologies, Bad Wildbad, Germany). Images were captured at 1 × 1 binning with exposure times ranging from 0.1 to 1.0 s, depending on the fluorescence intensity.

Measurement of toxicity of TT1-IP peptide

Eight weeks old female BALB/c mice bred at the Institute of Biomedicine and Translational Medicine (Tartu, Estonia) were intraperitoneally injected with 2.6 µmols/Kg (5mg/Kg) of TT1-IP or saline (control) and after 24 h, mice were anesthetized and 500 µL of blood were collected by retro-orbital bleeding into lithium heparin tubes (BD Vacutainer, BD-Plymouth, UK). The samples were centrifuged for 10 min at 1800 *g* at 4 °C and the plasma was analyzed for the concentration of glucose, creatinine, and activity of alanine aminotransferase (ALAT), using a Cobas 6000 IT-MW (Roche Diagnostics GmbH, Mannheim, Germany) machine and reagents for creatinine CREP2 and for alanine aminotransferase (ALAT) ALTLP.

Detection of apoptosis by Annexin-V staining

The apoptosis induction of TT1-IP was analyzed by flow cytometry on cells labelled with annexin V-FITC staining (Biosciences, Fisher Scientific, France). Primary cells were treated with 25 µM of peptides for 12 h. After the treatment, cells were harvested, washed and treated according to the manufacturer's protocol. The level of apoptosis was measured by flow cytometry (FACS Canto II, BD Biosciences, New Jersey, USA).

Analysis of peptide stability in human serum

The peptide TT1-IP was incubated at 37^{o} C in 250 µL of human serum (Gibco, Villebon-sur-Yvette, France) for different periods of time (0, 1, 6, and 24 h). Samples were collected and peptide degradation stopped by freezing at -20^{o}C. The peptide was extracted from samples using the Proteo Miner Protein Enrichment System (Bio-Rad, France). The percentage of intact peptide was estimated by mass spectrometry (MS) using Matrix-Assisted Laser Desorption/Ionization – Time of Flight (MALDI-TOFF) using the protocol previously

described [24] (Bruker Autoflex II, France) according to the manufacturer's instructions. Measurements were performed in triplicate. MS data were analysed using the software Cliprot tools, Flex analysis, Bruker (Palaiseau, France).

Liquid chromatography/mass spectrometry (LC/MS)-based analysis of pharmacokinetic of TT1-IP peptide

BALB/c mice (Janvier, France) were injected ~~whit~~ with 2.6 µmols/Kg (5 mg/kg) of TT1-IP peptide and plasma collected at 10, 20, 30, 45, 60, 120 and 360 min.

Plasma samples (0.05 mL) of injected mice were transferred to 1.5 mL polypropylene tubes. A total of 0.08 mL of acetonitrile/water (10/90 volume/volume, v/v) containing 2.5 % of ammonia was added. Samples were mixed and then transferred to Oasis HLB elution plates (Waters Corporation, Guyancourt, France) preconditioned with 0.2 mL of methanol and 0.2 mL of water. The plates were washed with 0.2 mL of acetonitrile/water (10/90, v/v). Plates were dried and the analytes were eluted three times with 0.1 mL of acetonitrile/water (75/20, v/v) containing 1% of formic acid. The elution fractions were dried under nitrogen at 40° C. The residue was reconstituted by 30 µL of a mixture of acetonitrile/water (75/25 v/v) containing 1% formic acid. The plate was vortexed for 2 minutes and then 0.12 mL of water were added to each well. The plate was gently shaken for 30 seconds. Aliquots of 20 µL were injected onto the HPLC system. The specifications of the LC-MS/MS instrumentation were Autosampler SIL-20AC XR maintained at approximately 10°C, Shimadzu (Noisiel, France). Pumps UFLC: LC-20AD XR, Shimadzu (Noisiel, France). Flow: 0.3 mL/min. Degasser: DGU-20A5, Shimadzu (Noisiel, France). Column oven: CTO-20AC set at 60°C, Shimadzu (Noisiel, France). The mobile phase was a linear gradient consisting of acetonitrile containing 0.25% formic acid and an aqueous solution of 0.25% formic acid.

Statistical analysis

For *in vivo* data, statistical comparisons between groups were performed using Gehan-Breslow-Wilcoxon test. For the *in vitro* apoptosis assay, one-way analysis of variance ANOVA and Fisher's least significant difference (LSD) test was used.

RESULTS

Antitumoral effect of TPP-IPs on CLL xenograft models

~~We have previously shown that the TPP-IP bi-functional peptides specifically penetrate and induce apoptosis of primary malignant B cells and hepatocytes, without any effect on healthy primary cells [6].~~ The antitumoral effect of TT1-IP, LinTT1-IP, and RPARPAR-IP was evaluated in a mouse model of CLL generated using the cell line Jok 1.3. The mice were treated with the peptides using a dose of 2.6 µmols/kg (corresponding to 5 mg/kg of TT1-IP), 5 days per week, or with saline (control group) until paralysis of the hind legs ~~(indicative of infiltration of leukemic cells in the CNS)~~ was observed. As shown in Figure 1A and B, treatment of CLL-bearing mice with TT1-IP and LinTT1-IP had a therapeutic effect, reflected by an increase in the median survival. The average survival for TT1-IP and LinTT1-IP treated groups was 19 days (the survival range for TT1-IP group was 17-23 days and for LinTT1-IP group was 13-25 days) and for the control group 16 days (range of 14-20 days). In contrast, treatment with RPARPAR-IP did not increase the median survival (16 days, range of 14-19 days, Fig. 1C). When compared with the control group, TT1-IP showed significantly higher survival rate, and therefore TT1-IP was chosen to further study its *in vivo* toxicity, biodistribution, stability in serum, and pharmacokinetic (PK) parameters.

To confirm that the therapeutic effect of the TT1-IP was due to the cargo (IP) associated to the TPP, the CLL xenografted mice were treated with the shuttle TT1 alone. Figure 1D shows that TT1 did not increase the median survival compared with the control group, confirming that the anticancer effect resulted from the activity of the therapeutic interfering peptide.

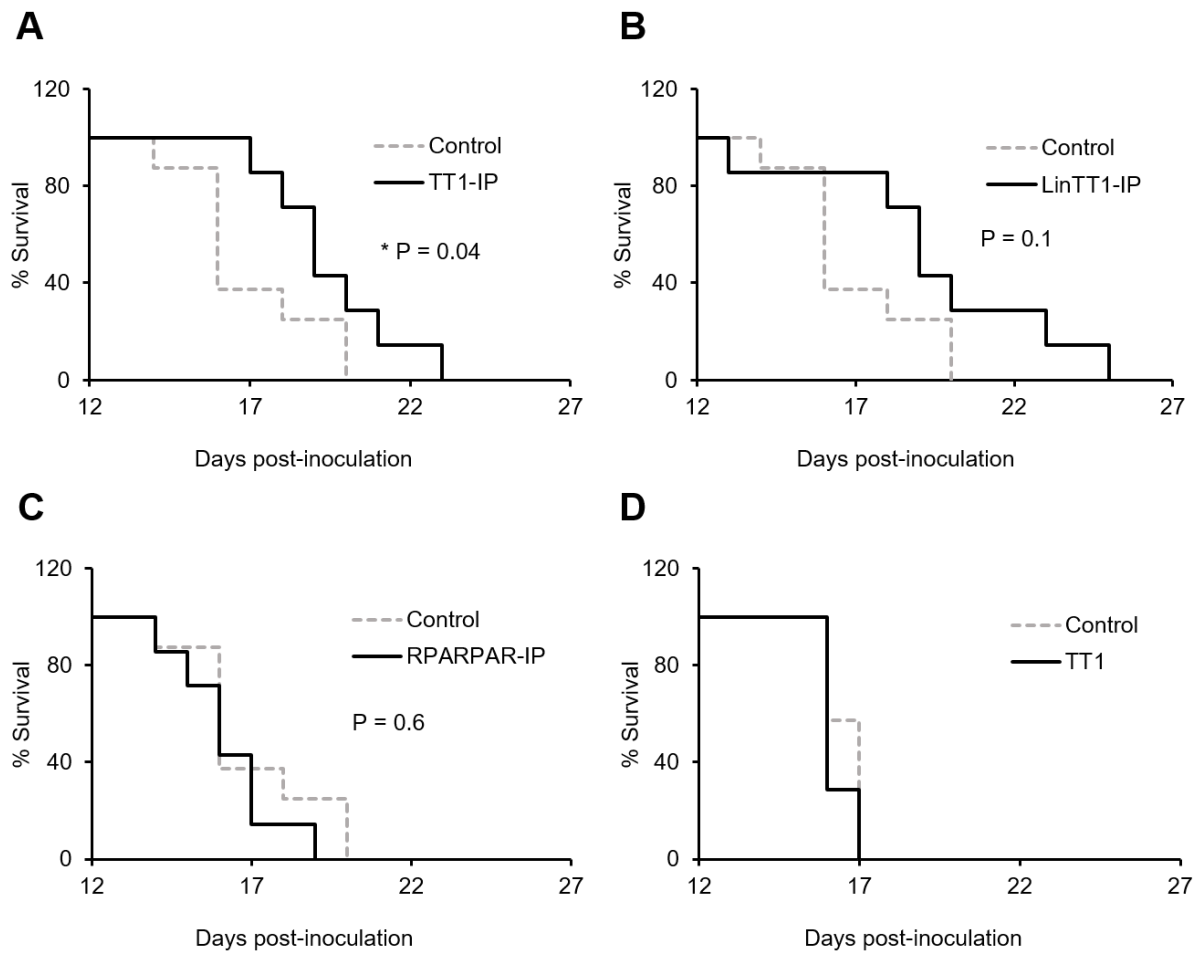


Figure 1. TPP-IP peptide increased the survival of CLL mice. Survival curves of mice treated with TT1-IP (**A**), LinTT1-IP (**B**), RPARPAR-IP (**C**), or TT1 (**D**). Panel **A-C** represent the graphs from the same treatment. SCID mice were intravenously inoculated with Jok 1.3 cells and treated 3 days after injection with 2.6 μmols/kg of peptides or saline (control), 5 days per week. The survival was monitored over time. Gray dashed line: control group, black line: group treated with the corresponding peptide. Statistical comparisons were performed using Gehan-Breslow-Wilcoxon test. (N = 7 per group).

Systemic TPP-IP treatment does not cause toxicity in mice

To assess potential toxicity of the TPP-IPs, we first compared the dynamics of body weight of the mice in different treatment groups. As shown in Figure 2A and B, the mice started to lose weight between day 10 and 14. However, no significant reduction of the body weight was observed in the treated mice compared with the control group, suggesting that the weight loss was due to the disease development but not to the peptide toxicity. Although the mice treated

with TT1-IP and LinTT1-IP showed higher survival rate, the mice in these groups also showed a weight loss due to the progression of the disease. To confirm the absence of toxicity of TT1-IP, immunocompetent BALB/c mice were injected with 2.6 µmols/kg (5 mg/kg) of the peptide and after 24 h, the blood was collected to evaluate the liver and renal toxicity by quantification of the hepatic and renal enzymes ALAT and creatinine. The level of glucose was also quantified as an indicative of the correct metabolic and endocrine functions. Figure 2C shows that the peptide did not produce any significant change in the level of glucose, creatinine, and ALAT when comparing to control mice, suggesting that the peptide did not produce hepatic and renal toxicity or glucose-related metabolic dysfunction, at the used dose.

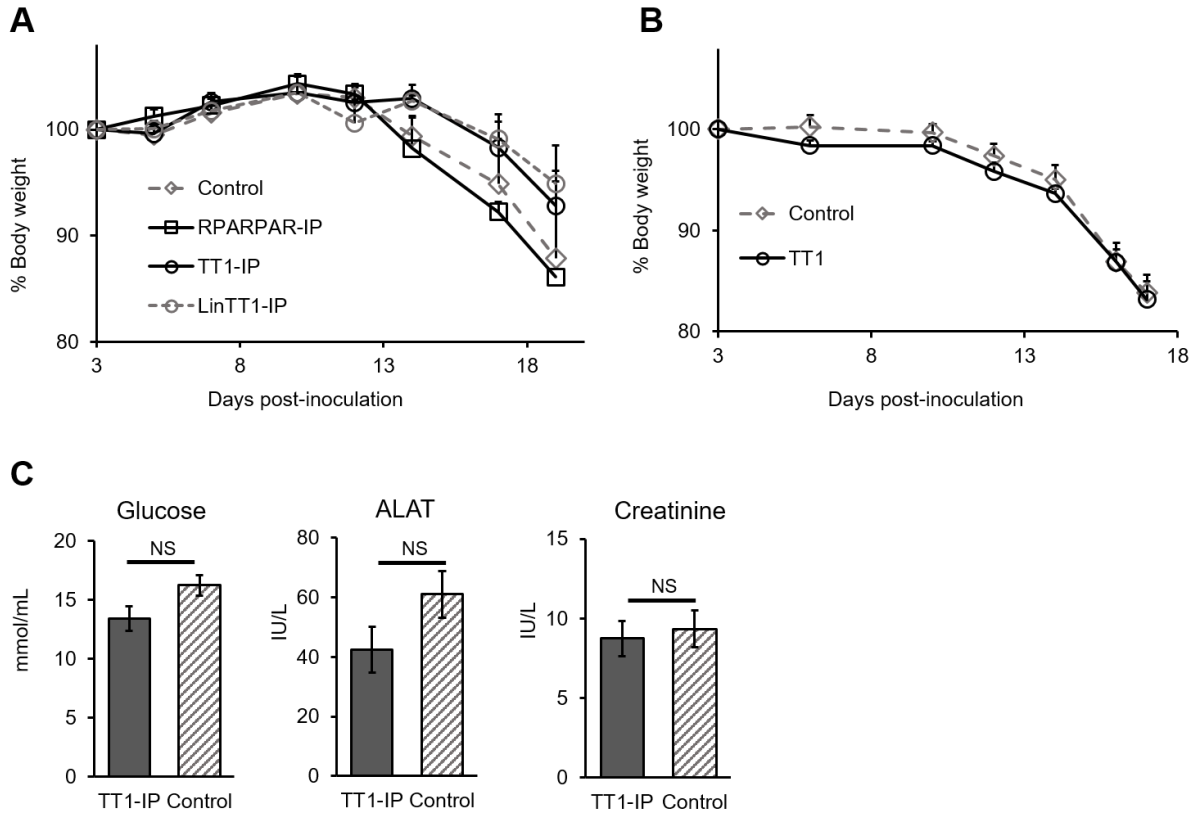


Figure 2. Effect of TPP-IPs on the body weight and plasma biochemical parameters. A) Percent of the initial body weight of CLL-bearing mice treated with RPARPAR-IP, TT1-IP, LinTT1-IP or saline (control). **B)** Percent of the initial body weight of CLL-bearing mice treated with TT1 or saline (control). The weight of the mice was monitored every three days. Gray dashed line with square markers: control group, gray dashed line with round markers: LinTT1-IP, black line with square markers: RPARPAR-IP, black line with round markers: TT1-IP or TT1. N = 7; Bars = ±SEM (standard error of the mean). **C)** Effect of TT1-IP

peptide on the level of hepatic and renal enzymes. Immunocompetent BALB/c mice were injected with TT1-IP peptide (2.6 µmol/kg, equivalent to 5 mg/kg) and the glucose, creatinine, and ALAT in plasma was quantified after 24 h of injection. Bars with solid gray fill: TT1-IP, bars with gray pattern fill: control. N = 3; Bars = ±SEM.

TT1-IP peptide treatment causes apoptosis on primary B cells from CLL

Disrupting the SET/PP2A interaction reactivates PP2A activity and produces cancer cell apoptosis[25]. We tested the apoptotic effect of TT1-IP, RPARPAR-IP and LinTT1-IP and studied whether the TT1 or IP alone has any apoptotic effect. B cells from healthy donors and CLL patients were treated with the peptides and the apoptotic effect was estimated by annexin staining. Figure 3 shows that TT1-IP, RPARPAR-IP and LinTT1-IP strongly induced apoptosis of primary CLL cells but not of healthy B cells, while TT1 alone and IP alone did not have apoptotic effect on any of the primary B cells. These results confirm that the apoptotic effect of TT1-IP, RPARPAR-IP and LinTT1-IP is specific for malignant B cells and it is mediated by the PP2A/SET-interfering peptide.

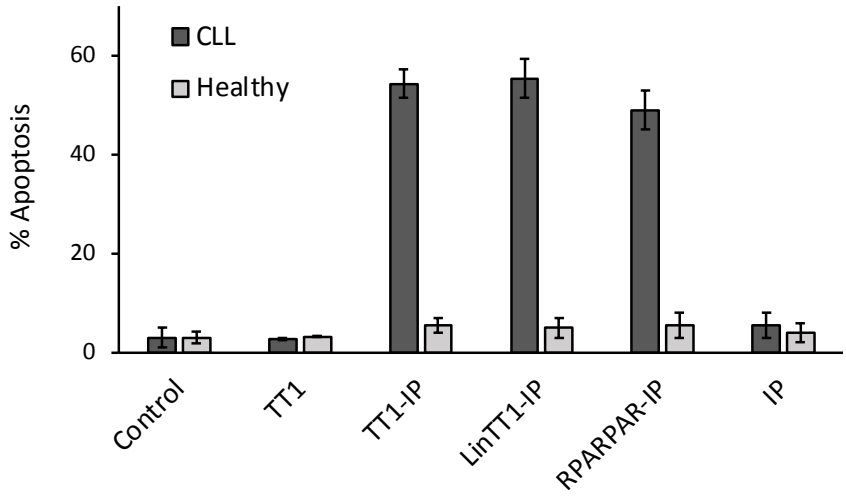


Figure 3. TT1-IP and not TT1 induced apoptosis of B cells from CLL patients. Healthy B cells from donors or B cells from CLL patients were isolated, treated with 25 µM of TT1, TT1-IP, LinTT1-IP, RPARPAR-IP, or IP peptides for 12 h, and apoptosis measured by

annexin-FITC staining. Untreated cells were used as control. Dark gray bars: B cells from CLL patients, light gray bars: B cells from healthy donors. N = 2-3. Bars = ±SEM.

Stability of TT1-IP peptide in serum

Proteolytic degradation of peptide-based drugs is considered as a major drawback, often limiting systemic therapeutic applications[26]. We analyzed the stability of TT1-IP peptide upon incubation at 37° C in human serum by MS. Figure 4 shows that approximately 40% of the peptide was recovered after 6 h incubation and approximately 20% after 24 h.

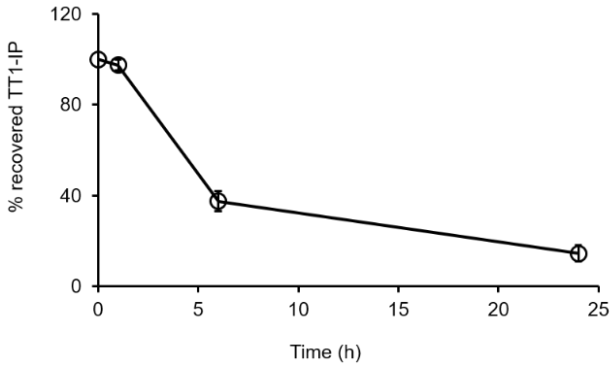


Figure 4. Stability of TT1-IP peptide on human serum. TT1-IP peptide was incubated in human serum at 37° C for different periods of time. The integrity of the peptide was analyzed by MS. The percentage of the recovered peptide with time is represented. Every measurement was performed in triplicate. Bars represent ± SEM.

Fluorophore-labeled TT1-IP peptide accumulates in CLL xenograft

We evaluated by intravital imaging whether the targeting of TT1-IP to malignant B cells translated to a higher accumulation of the peptide in CLL mice than in non-grafted mice. Mice were intravenously injected with 2.6 µmols/kg (5 mg/kg) of Cy7-labelled TT1-IP (Cy7-TT1-IP) and imaged at different times points post-injection. The fluorescence in CLL xenografted and healthy mice was observed after 15 min post-injection (Figure 5A) and the intensity increased reaching a plateau at 1 h post-injection. A decrease of fluorescence intensity was observed after 3 h, showing only traces of fluorescence after 6 h of injection. Interestingly, the intensity of fluorescence observed in CLL mice was higher than in healthy

mice and the intensity ratio between CLL and healthy mice increased with time, reaching a ratio of four after three hours of peptide injection (Figure 5B). This is consistent with the idea that Cy7-TT1-IP is specifically internalized by circulating malignant B cells of CLL in the xenograft model.

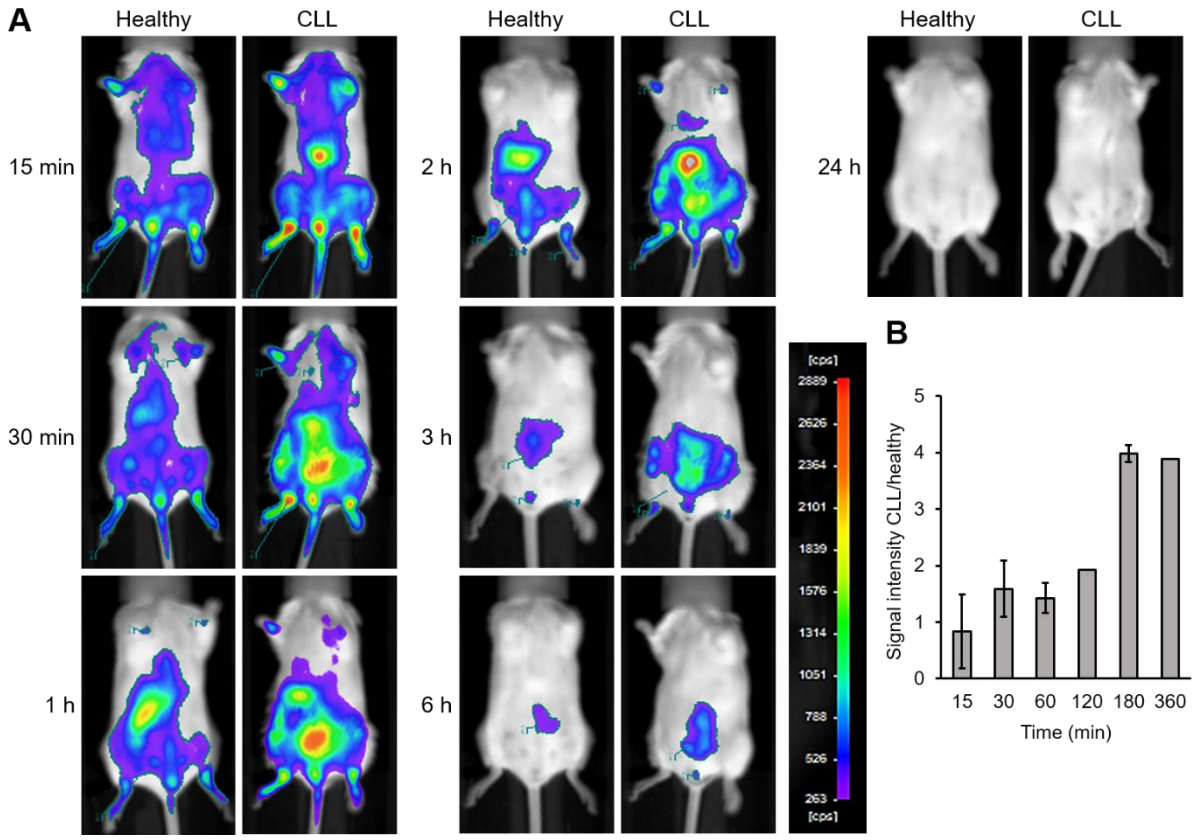


Figure 5. A) Biodistribution of Cy7-TT1-IP peptide in CLL and control mice. SCID mice were grafted with Jok 1.3 cell line and 3 days after injected with 2.6 µmols/kg (5 mg/kg) of Cy7-labelled TT1-IP peptide (Cy7-TT1-IP). Healthy mice injected with the same dose of peptide were used as control. Mice were imaged at different times upon injection. Representative images from N = 2. The scale bar on the right shows the intensity of the fluorescence. Red represents higher intensity and purple lower intensity. **B)** Ratio of the peptide intensity signal in CLL mice/ peptide intensity signal in healthy mice. The intensity was quantified as overall counts per second (cps) using the Indigo software (Berthold

Technologies, Bad Wildbad, Germany). N = 2. Bars = ±SEM. The signal in one of the healthy mice at 120 and 360 min could not be detected.

Pharmacokinetic (PK) parameters of TT1-IP peptide

We then analyzed the PK parameters of TT1-IP to study whether this peptide could be developed as candidate for the treatment ~~its compatibility with a preclinical development as therapeutic peptide~~ against CLL. PK parameters were assessed by monitoring variations in the concentration of the peptide in plasma after IV injection, thus giving an overall indication of the behavior of the peptide in the body. The analyzed parameters, shown in Table 2, were: T_{max} (time to reach maximum concentration), C_{max} (maximum concentration), T_{last} (time of the last observed concentration), C_{last} (concentration of the time point with measurable concentration), AUC_{Total} (total area under the curve), AUC_{last} (area under the curve in the last point), $T_{1/2}$ (terminal half-life), CL (total clearance), V_{ss} (volume of distribution at steady state), and C_0 (initial concentration). The detected C_0 was 2976 ng/mL and the V_{ss} 2700 mL/kg, indicating a good distribution capacity. The time to reach maximum concentration was 10 min. The $T_{1/2}$ was 28 min. The concentration of TT1-IP in plasma decreased with time, indicating the peptide clearance from the body. The clearance was 96 mL/min/Kg and the exposure (AUC_{Total}) 1003 ng/mL/min.

T_{max}	C_{max}	T_{last}	C_{last}	AUC_{Total}	AUC_{last}	$T_{1/2}$	CL	V_{ss}	C_0
(min)	(ng/mL)	(min)	(ng/mL)	(ng/mL×h)	(ng/mL×h)	(min)	(mL/min/Kg)	(mL/Kg)	(ng/mL)
10	1173	120	49	1003	963	28	96	2700	2976

Table 2. PK parameters of TT1-IP. TT1-IP was IV administered in a single dose of 2.6 µmols/kg (5mg/Kg). The peptide was extracted from the serum, quantified by HPLC/MS and the PK parameters were calculated, T_{max} = time to reach maximum concentration; C_{max} = maximum concentration; T_{last} = time of the last observed concentration; C_{last} = concentration of the time point with measurable concentration; AUC_{Total} = total area under the curve;

AUC_{last} = area under the curve in the last point; $T_{1/2}$ = half-life; CL = total clearance; V_{ss} = volume of distribution; C_0 = initial concentration.

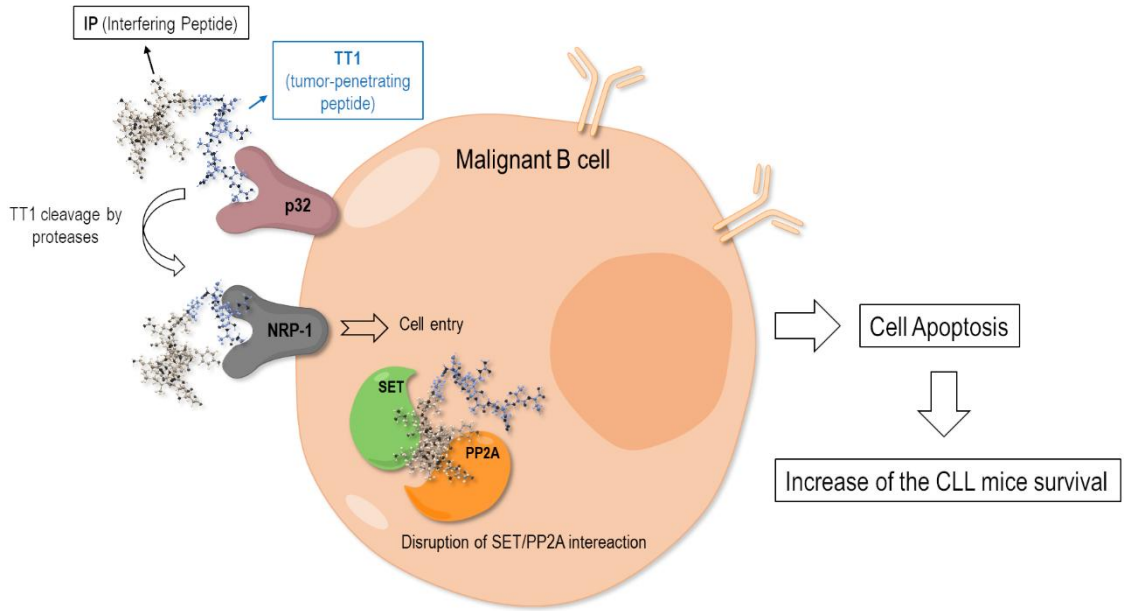


Figure 6. Cartoon summarizing the results of this manuscript. The TT1-IP internalize in the tumoral B cells via specific receptors. First, TT1 peptide binds to p32 receptor overexpressed on the surface of malignant B cells. TT1 can then be cleaved by proteases exposing the CendR motif that binds to NRP-1, triggering the peptide cell internalization. Once inside the cell, the IP dissociates the interaction PP2A/SET and, as a consequence, there is induction of apoptosis and death of the tumoral cell.

DISCUSSION

Despite improvement in the overall survival of CLL patients, in some clinical situations (e.g., in the case of relapsed or refractory CLL) new therapeutic approaches are needed [27]. The most recent approaches for transforming a combination therapy into a targeted chemotherapy (antibody-drug conjugated, ADC) [28] are unfortunately not applicable to CLL. In order for the ADC strategy to be effective, the receptor targeted by the antibody needs to be internalized after binding to transport the drug inside the cells, but monoclonal antibodies evaluated for CLL treatment bind to non-internalizing targets (CD20). Alternative targeted therapies for CLL are the use of B cell receptors inhibitors, as well as the use of PI3K and Bcl-2 inhibitors or cellular therapy. However, these treatments can cause adverse effects

The use of peptides as targeting ligands in cancer have been widely explored [29, 30] as they offer several advantages over antibodies, such as superior penetration in solid tumors due to their lower molecular weight and lower affinity [30], and lower production cost. We show here that the use of tumor-penetrating peptides targeting p32 can be an alternative strategy to selectively deliver a bioactive IP into malignant B cells.

We previously showed that malignant B cells from CLL patients but not healthy B cells expressed the p32 and NRP-1 receptors [6]. CendR or cryptic CendR peptides in tandem with the IP disrupting the PP2A/SET interaction [7] were internalized by the tumoral B cells, producing apoptosis. Herein, we showed that the TT1-IP significantly increased the survival of mice in a CLL xenograft model and that it caused apoptosis of the malignant B cells from CLL patients. Importantly, as we previously showed with the iRGD-IP bifunctional peptide [6], the TT1-IP did not cause body weight loss in the treated mice and did not modify the levels of glucose, ALAT, and creatinine, suggesting the absence of liver and kidney toxicity. These findings suggest a potential advantage of the TPP-IPs over current CLL therapies based on chemotherapy which are not tolerated by patients due to the severe side effects [3].

The cell surface-expressed form of p32 is a marker of tumor cells and tumor-associated macrophages/myeloid cells, being a good target in tumor diagnosis and therapy [17]. TT1 and LinTT1 are proteolytically cleaved by tumor-related enzymes after binding to p32 [18]. The cleavage activates the CendR motif to become able to interact with NRP-1 to trigger internalization of the peptide along with cargo in tumor cells. This multistep mechanism makes TT1 highly tumor-specific, as opposed to RPARPAR peptide, which directly binds to NRP-1.

Functionalization of nanocarriers with LinTT1 peptide increased the nanocarrier homing to different solid tumors [19-23]. When analyzing the biodistribution of Cy7-labeled TT1-IP, a higher fluorescence intensity in xenograft models of CLL was observed compared to healthy mice, suggesting a preferential targeting to malignant B cells and expanding its applicability in the treatment of leukemia.

The serine/threonine phosphatase is known to be frequently deregulated in hematological cancers [31-33] , being an interesting target for novel anti-cancer therapies. The oncoprotein SET, the physiological inhibitor of PP2A, associates to the catalytic subunit of PP2A. The disruption of the PP2A/SET interaction restores the PP2A function inducing cell death; therefore, many efforts have been made to restore PP2A activity by dissociating PP2A/SET interaction[8, 34-37].

We previously identified the binding site between PP2A and SET and designed a peptide that blocks this interaction [7]. We showed that the IP alone did not internalize in neither B cells from CLL patients nor in healthy B cells and that the IP coupled to a cell-penetrating peptide internalized in both cancer and healthy B cells [6, 7]. Here we show that TT1 promotes the specific internalization of the IP in malignant B cells but not healthy cells, causing the cell apoptosis.

Poor PK parameters are major reason for the lack of therapeutic activity of some drug candidates. PK parameters are assessed by monitoring variations on the concentration of the drug and/or its metabolites in physiological fluids that are easy to access, such as plasma and urine. PK parameters give an overall indication of the behavior of the drug in the body. Here we show that the PK parameters of TT1-IP are compatible with its preclinical development as therapeutic peptide. The $T_{1/2}$ of the intravenous administered peptide was 28 min, similar to the half-life found for other peptides in preclinical or clinical stages [38]. ~~This half-life is similar to that of the cell penetrating and interfering peptide PEP-010 (clinical trial Phase I).~~ For example, the $T_{1/2}$ of some Bombesin analog was around 30 min, and for the peptide in clinical development Ep-100 (LHRH coupled to a cationic peptide) was 16 min [38]. The half-life of aspirin is around 20 min and for the antibiotics ampicillin and clavulanic acid, the half-life rang from 40 to 60 min.

Immune dysfunction in CLL contributes to tumor immune scape promoting cancer development. Recent findings showed that the expression of Programmed Death 1 Ligand (PDL-1) is higher in CLL patients that in control ones [39] and that disrupting the PD-1/PDL-1 interaction prevents immune dysfunction and leukemia development in a mouse model of CLL [40]. Therefore, the combination of our TPP-IPs with immune check inhibitors (such as PD-1) could be an interesting strategy to develop a more potent CLL therapy with lower side effects.

Our results present a peptide with anti-cancer activity in CLL that could be a potential candidate for the clinical treatment of this disease ~~compatible with a clinical development~~.

CONCLUSION

The tumor penetrating and interfering peptide TT1-IP increased the survival of mice with CLL and had an apoptotic effect in malignant B cells from CLL patients. Moreover, the PK parameters of TT1-IP suggest that it could be a potential candidate for the clinical treatment of this disease. (He cambiado aqui lo de “clinical development” que no le gustaba al referee 1)

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Notes

The authors declare no competing financial interest

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