Polycationic peptide R\(^7\)-G-A\(\beta\)\(_{25-35}\) selectively induces cell death in leukemia Jurkat T cells through speedy mitochondrial depolarization, and CASPASE-3-independent mechanism

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**ABSTRACT**

**Background:** Acute lymphoblastic leukemia (ALL) is still incurable hematologic neoplasia in an important percentage of patients. Therefore, new therapeutic approaches need to be developed.

**Methods:** To evaluate the cellular effect of cell-penetrating peptides (C-PP) on leukemia cells, Jurkat cells—a model of ALL were exposed to increasing concentration (50-500 \(\mu\)M) Aβ\(_{25-35}\), R\(^7\)-G-Aβ\(_{25-35}\) and Aβ\(_{25-35}\)-G-R\(^7\) peptide for 24 h. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, flow cytometry (FC), and fluorescent microscopy (FM) analysis were used to assess metabolic viability, cell cycle and proliferative cells.

**Results:** We report for the first time that the R\(^7\)-G-Aβ\(_{25-35}\) but not Aβ\(_{25-35}\) peptide, induced selective cell death in Jurkat cells more efficiently than the Aβ\(_{25-35}\)-G-R\(^7\) peptide. Indeed, R\(^7\)-G-Aβ\(_{25-35}\) (200 \(\mu\)M) altered the metabolic activity (~25%), arrested the cell cycle in the G2/M-phase (15%), and induced a significant reduction of cellular proliferation (i.e., ~74% reduction of Ki-67 nuclei reactivity). Moreover, R\(^7\)-G-Aβ\(_{25-35}\) induced the dissipation of mitochondrial membrane potential (ΔΨ\(_m\), 51%) and produced an important amount of reactive oxygen species (ROS, 75% at 8 h) in Jurkat cells. The exposure of cells to antioxidant/cytoprotectant N-acetylcysteine (NAC) did not prevent R\(^7\)-G-Aβ\(_{25-35}\) from a loss of ΔΨ\(_m\) in Jurkat cells. The peptide was also unable to activate the executor CASPASE-3, thereby preserving the integrity of the cellular DNA corroborated by the fact that the caspase-3 inhibitor NSCI was unable to protect cells from R\(^7\)-G-Aβ\(_{25-35}\)-induced cell damage. Further analysis showed that the R\(^7\)-G-Aβ\(_{25-35}\) peptide is specifically localized at the outer mitochondria membrane (OMM) according to colocalization with the protein translocase TOMM20. Additionally, the cytotoxic effect of the poly-R\(^7\) peptide resembles the toxic action of the uncoupler FCCP, mitocan oligomycin, and rotenone in Jurkat cells. Importantly, the R\(^7\)-G-Aβ\(_{25-35}\) peptide was innocuous to menstrual mesenchymal stromal cells (MenSC)–normal non-leukemia proliferative cells.

**Conclusion:** Our findings demonstrated that the cationic Aβ peptide possesses specific anti-leukemia activity against Jurkat cells through oxidative stress (OS)- and CASPASE-3-independent mechanism but fast mitochondrial depolarization.

**1. Introduction**

Acute lymphoblastic leukemia (ALL) is a malignant transformation and proliferation of either B-cell or T-cell lymphoid progenitor cells in the bone marrow, blood, and extramedullary sites affecting children, young adolescents, and adults [1] around the world [2]. According to the American Cancer Society, there are approximately 5690 new cases of ALL, and about 1580 deaths in 2021. While childhood ALL studies have shown improved 5-year overall survival (OS) rates exceeding 90% [3], only 30–40% of adult patients with ALL will achieve long-term remission [4]. Although conventional cytotoxic chemotherapy [5,6] or in combination with monoclonal antibodies (e.g., Ref. [7]) used to treat ALL results in high cure rates, treatment is suboptimal in an important percentage of pediatric and adult patients with relapsed and refractory...
ALL. Therefore, new treatment options are needed for the treatment of resistance ALL. Indeed, leukemia cells avoid apoptosis—a regulated form of cell death [8] contributing to their pathological features. Since apoptosis can be initiated through the intrinsic pathway that depends on mitochondrial outer membrane permeabilization or the extrinsic pathway that depends on external signals via cell receptors [9], a reasonable strategy would be to trigger apoptosis by agents that directly or indirectly target mitochondria or by external signals to boost cell death for anti-leukemic therapy.

Polycationic peptides are a large class of short amino acid sequences (5–30 residues) composed mainly of multiple lysines (K) and/or arginine (R) residues usually used as anti-microbial agents (e.g., Refs. [10–14]). It is the cationic properties that promote the preferential binding of peptides to the negatively charged bacterial cytoplasmic membrane instead of the zwitterionic membrane of mammalian cells [15]. Interestingly, not only they can impair microbial cell walls and plasma membrane phospholipids with the zwitterionic membrane of mammalian cells [15]. Indeed, they can impair microbial cell walls and plasma membrane phospholipids [15]. Interestingly, not only resistance ALL. Indeed, leukemia cells avoid apoptosis—a regulated form of cell death [8] probably through membrane multilamellar and subsequently enter via formation of a fusion pore [22], thereby enhancing their capacity to directly permeabilize mitochondria (e.g., Ref. [23]). Indeed, synthetic cell-penetrating peptides (CPP) poly-R (n > 6) have currently been used as the delivery vector [24,25]. For instance, the peptide r7-klα (D-hepta-arginine linked to D-forms of KLA) induced apoptosis (48%) in leukemia Jurkat cells—a T-cell ALL line [23]. Moreover, it has been shown that cationic peptides such as P7-4 (R²-G-YLTALAKWLG) and P7-5 (YLYTALAKWLG-GG-R²) significantly induced demide (29% and 31%, respectively) of leukemia Jurkat cells [17]. Taken together these data suggest that some poly-R²-spacer-cargo peptides provoke apoptosis in leukemia cells mainly through targeting the mitochondrial membrane.

The Aβ(25-35) with sequence GSNKAIIGLM is an eleven-residue peptide (the synthesis method described [29]). For arginine, an additional coupling step was performed. Deprotection and coupling were performed as described [29]. The crude peptide was precipitated by cold diethyl ether, centrifuged, washed with cold Et2O five times, dried, dissolved in ultrapure water, frozen, and lyophilized. Peptide R²-G-Aβ(25-35) was synthesized by Dr. S Estrada-Gomez and CF Salinas-Restrepo (Oftidism/Escorpionism Program, Universidad de Antioquia). The peptide was chemically synthesized through the solid phase using Fmoc/Bu as the orthogonal protection strategy in the Rink AM resin using the simultaneous synthesis strategy described in Ref. [30]. The (Benzotriazole-1-yl) tetramethylammonium hexafluorophosphate (BHTU), (Benzotriazoly) tetramethylammonium tetrafluoroborate (TBTU), DIC, and O-(6-Chloro-1-hydrobenzotriazol-1-yl)-1,3,3-tetramethylammonium tetrafluoroborate (TCTU) were used as activators, and the deprotection was carried away with piperidine 20% in DMF. For the cleavage a 92.5% TFA, 2.5% TIS, 2.5% 2-mercaptoethanol and 2.5% water solution was used as described in Ref. [29]. The crude and folded peptide was solubilized in 200 μL of solution A (0.1% TFA in water) and centrifuged at 1,000 for 3 min. The supernatant was applied to a reverse-phase CNW Athena C18-WP (CNW Technologies, Düsseldorf, Germany) column (4.6 × 100 mm, 5 μm, 100 Å), and separated by RP-HPLC on a Shimadzu Prominence chromatograph (Kyoto, Japan). The crude peptide was precipitated by cold diethyl ether, centrifuged, washed with cold Et2O three times, dried, dissolved in ultrapure water, frozen, and lyophilized. All reagents were from Merck Millipore (Merck Millipore, Billerica, MA, USA). Peptide Aβ(25-35) was acquired from Sigma-Aldrich (Cat# A4559). For culture experiments, peptides were resuspended in Roswell Park Memorial Institute (RPMI)-1640 culture medium with glucose (11 mM; Gibco/Invitrogen, Grand Island, New York, USA).

2.2. Cell line and reagents

Jurkat clone E6-1 cells (Catalog no. TIB-152; American Type Culture Collection (ATCC), Manassas, Virginia, USA) were cultured according to the supplier’s indications. The cell suspension (1x10⁶ cells/well in 1 mL final volume) was exposed to increasing concentrations of synthetic peptides (0–500 μM) freshly prepared in RPMI-1640 medium with glucose (11 mM; Gibco/Invitrogen, Grand Island, New York, USA) in the absence or presence of different products of interest (e.g., antioxidant, inhibitors) for 24 h at 37 °C. All other reagents were from Sigma-Aldrich (St Louis, Missouri, USA).

2.3. Isolation of mesenchymal stromal cells (MSCs)

Isolation of MenSCs was performed according to previous reports [31]. Briefly, a menstrual blood sample (tissue bank code (TBC # 69308) was delivered to the laboratory and mixed with an equal volume of phosphate buffer saline (PBS) containing 1 mM ethylenediamine tetra-acetic acid (EDTA), with 100 U/mL penicillin/streptomycin 0.25 mg/mL amphotericin B, and standard Ficoll procedures. After centrifugation, the cells were suspended in a Buffy coat and were transferred into a new tube, washed in PBS twice, and resuspended in a growth medium (low-glucose Dulbecco’s modified Eagle’s medium (DMEM) medium supplemented with 10% fetal bovine serum (FBS, Gibco, USA),
100 U/mL penicillin/streptomycin, and 0.25 mg/mL amphotericin B), and seeded into 25-cm² plastic cell culture flasks at 37 °C with 5% humidified CO₂.

2.4. MTT assay

The proliferation of Jurkat cells was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay by determining the conversion of the water-soluble MTT to an insoluble formazan [32]. Briefly, cells were pelleted and were incubated under increasing (0–500 μM) concentrations of synthetic peptides for 24 h. Thereafter, the medium was removed and replaced with fresh medium without phenol red. Cells were incubated with 1.2 mM MTT at 37 °C for 4 h. Thereafter, the medium was removed and replaced with fresh medium with MTT. Thereafter, the medium was removed and replaced with fresh medium without phenol red. Cells were incubated with 1.2 mM MTT at 37 °C for 3 h and after incubated with dimethyl sulfoxide (DMSO). Finally, samples were mixed, and the absorbance was read at 570 nm using a Stat Fax 3200 microplate spectrophotometer (Awareness Technology, Palm City, Florida, USA). MTT absorbance was assessed 3 times in independent experiments.

2.5. Ki-67 immunofluorescence

To evaluate the effect of synthetic peptides on Jurkat cells proliferation, we evaluated the percentage of Ki-67 positive nuclei according to Ref. [33]. Briefly, the treated cells were fixed with cold ethanol (–20 °C) for 20 min followed by 10% bovine serum albumin (BSA) blockage. Thereafter, cells were incubated overnight with anti-Ki-67 monoclonal mouse antibody conjugated with fluorescein isothiocyanate isomer 1 (FITC, Cat #F0788, Dako) 1:200. The nuclei were stained with 1 μM Hoechst 33342 (life technologies). Ki-67 reactivity was quantified in a Zeiss Axioskop fluorescence microscope equipped with a Zeiss AxioCam Cam1 (Zeiss Wöhlk-Contact-Linsen, Gmb Schönkirchen, Germany) by assessing the labeling percentage from the ratio of the number of nuclei-stained Ki-67 to the total number of nuclei counted per section. At least 10 different randomly selected areas were counted.

2.6. Determination of DNA fragmentation and cell cycle by flow cytometry

DNA fragmentation and cell cycle were determined by using a hypotonic solution of propidium iodide (PI, [34]). After treatments, cells (1x10⁶) were washed with PBS (pH 7.2) and stored in 95% ethanol overnight at –20 °C. Thereafter, cells were washed and incubated in a 400 μL solution containing PI (50 μg/mL), RNase A (100 μg/mL), EDTA (50 mM), triton X-100 (0.2%), and propidium iodide (0.5 μM) for 30 min at 37 °C. The cell suspension was analyzed for PI fluorescence by using a BD LSRSorter II flow cytometer (BD Biosciences). Quantitative data and figures were obtained using FlowJo 7.6.2 Data Analysis Software. For cell cycle analysis, the sub-G1 population was subtracted from the total acquired events, and the Dean Jett Fox analysis was applied (root mean square (RMS) < 10). The experiment was conducted three times, and 10,000 events were acquired for analysis.

2.7. Evaluation of intracellular hydrogen peroxide (H₂O₂) by flow cytometry

H₂O₂ was determined with 2′,7′-dichlorofluorescein diacetate (0.5 μM, DCFH₂-DA) according to Ref. [35]. Briefly, after cell treatment with compounds of interest, cells (1 x 10⁷) were incubated with DCFH₂-DA reagent for 30 min at 37 °C in the dark. Cells were washed and dichlorofluorescein (DCF) fluorescence was determined using a BD LSRSorter II flow cytometer (BD Biosciences). The experiment was conducted three times, and 10,000 events were acquired for analysis. Quantitative data and figures were obtained using FlowJo 7.6.2 Data Analysis Software.

2.8. Analysis of mitochondrial membrane potential (ΔΨm) by flow cytometry

Assessment of the ΔΨm was performed according to Ref. [36]. We incubated cells (1x10⁶) for 20 min at r. t. in the dark with a deep red mitotracker (20 nM final concentration) compound (Thermo Scientific, cat# M22426). Cells were analyzed by using a BD LSRSorter II flow cytometer (BD Biosciences). The experiment was conducted three times, and 10,000 events were acquired for analysis. Quantitative data and figures were obtained using FlowJo 7.6.2 Data Analysis Software.

2.9. Assessment of cell death by fluorescent microscopy

MenSC and Jurkat cells were incubated under increasing (0–500 μM) concentrations of synthetic peptides for 24 h. Nuclei were stained with 0.5 μM Hoechst 33342 (0.5 μM, deep red mitotracker (20 nM), and DCFH₂-DA, (0.5 μM). Thereafter, cells were deposited in a microscope slide and covered with a coverslip. Fluorescence microscopy analysis was performed with a Zeiss Axioscope 100 fluorescence microscope equipped with a Zeiss AxioCam Cm1 (Zeiss Wöhlk-Contact-Linsen, Gmb Schönkirchen, Germany). The adjustment of the images obtained was provided with the software provided by the manufacturer (ZEN 2 Core).

2.10. Antioxidant and pharmacological inhibitor protection experiments

Jurkat cell suspension (1x10⁶/well in 1 ml final volume) was left untreated or treated with R²-G-Ab₃₃₃₅ synthetic peptide (200 μM) alone or in combination with either antioxidant N-acetyl-l-cysteine (NAC, 1 mM) or the caspase-3 inhibitor 1-(4-Methoxybenzyl)-5-[2-(pyridin-3-yloxymethyl)pyrrolidine-1-sulfonyl]-1H-indole-2,3-dione (NSCI, 10 μM, Sigma-Aldrich, catalog N1413) at 37 °C for 24 h. The cells were evaluated for ΔΨm by flow cytometry. The assessment was repeated three times in independent experiments.

2.11. Flow cytometry analysis for CASPASE-3

Flow cytometry acquisition was used to determine the percentage of Caspase-3 positive cells according to Ref. [37]. Jurkat cells were treated according to the above-mentioned procedures. The fixated Jurkat cells were stained with rabbit anti-Caspase-3 (Millipore, cat# AB3623) primary antibody (1:200) at 4 °C overnight. Cell suspensions were washed and incubated with DyLight 488 donkey anti-rabbit antibody (1:500). Finally, cells were washed and re-suspended in PBS for analysis on a BD LSRSorter II flow cytometer (BD Biosciences). Twenty thousand events were acquired, and the acquisition analysis was performed using FlowJo 7.6.2 Data Analysis Software.

2.12. Analysis of mitochondrial membrane potential by fluorescent microscopy

The ΔΨm was evaluated according to Ref. [38]. Briefly, we incubated cells (1 x 10⁵) for 20 min at 37 °C in the dark with cationic and lipophilic 3,3′-dihexyloxacarbocyanine iodide (DiO(0)), 20 nM final concentration compound (Calbiochem, Darmstadt, Germany; cat # D-273). Cells were incubated with mitochondrial-targeted drugs (i.e., carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone, FCCP, 10 μM), oligomycin (5 μg/mL), rotenone (100 μM) or R²-G-Ab₃₃₃₅ peptide (200 μM) for 0, 30, 60, 120 and 240 s. Fluorescence microscopy analysis was performed with a Zeiss Axioscope fc microscope equipped with a Zeiss AxioCam Cm1 (Zeiss Wöhlk-Contact-Linsen, Gmb Schönkirchen, Germany). The adjustment of the images and the spectral images were obtained with the software provided by the manufacturer (ZEN 2 Core). The experiment was conducted three times.
2.13. Immunofluorescence colocalization analysis of the \( \beta \)-amyloid peptide and cluster of differentiation (CD)45 or translocase of the outer mitochondrial membrane (TOMM)20

The immunofluorescent staining procedure was according to the standard procedure [39]. Briefly, untreated, and treated cells (0 or 200 \( \mu \)M, respectively) with synthetic peptides (A\( _{25-35} \)-G-\( \beta \)-M, respectively) with synthetic peptides (A\( _{25-35} \)-G-\( \beta \)-M) synthetic peptides. Figures represent 1 out of 3 independent experiments. The numbers represent the mean percentage of three independent experiments. *\( p<0.05 \), **\( p<0.01 \), and ***\( p<0.001 \). Image magnification 20x.

2.14. Photomicroscopy and image analysis

The fluorescent microscopy photographs were taken using a Zeiss Axiostart 100 Fluorescence Microscope equipped with a Zeiss AxioCam Cm1 (Zeiss Wöhlk-Contact-Linsen, GmbH Schönkirchen, Germany). Images were analyzed by ImageJ software [40]. The figures were transformed into 8-bit images and the background was subtracted. The cellular measurement regions of interest (ROI) were drawn over cell structures (i.e., membrane or mitochondria) and the fluorescence intensity was subsequently determined by applying the same threshold for controls and treatments.

2.15. Statistical analysis

Statistical analyses were performed using the GraphPad Prism 6 scientific software (GraphPad, Software, Inc. La Jolla, CA, USA). Data are expressed as the mean ± S.D. of a minimum of three independent experiments. One-way ANOVA with a Tukey post hoc test was used to compare the differences between the experimental groups. A \( P \)-value <0.05 (*), <0.01 (**) and <0.001 (***)) were statistically significant.

3. Results

3.1. \( R^2 \)-G-A\( _{25-35} \) and A\( _{25-35} \)-G-\( \beta \)-M, but not A\( _{25-35} \)-G-\( \beta \)-peptide, induce cell cycle arrest, reduce cellular proliferation, and diminish cellular metabolic activity in Jurkat cells

We first wanted to evaluate the effect of A\( _{25-35} \)-G-\( \beta \)-M, \( R^2 \)-G-A\( _{25-35} \), and A\( _{25-35} \)-G-\( \beta \)-M peptides on cellular metabolic activity, cell proliferation, and cell cycle in Jurkat cells. To this aim, cells were left untreated or treated with an increasing concentration of the peptides (0–500 \( \mu \)M) for 24 h. As shown in Fig. 1 A, Jurkat cells exposed to A\( _{25-35} \)-G-\( \beta \)-peptide remain unaffected according to cellular metabolic activity assay when compared to untreated cells. However, leukemia cells showed a progressive and significant concentration-dependent viability loss by both \( R^2 \)-G-A\( _{25-35} \) (e.g., –25, –48% reduction at 200–500 \( \mu \)M, respectively).
and \( \text{Aβ}_{25-35} \)-\( \text{G-R}^7 \) (e.g., -15 -33%) peptides. Importantly, \( \text{R} \text{G}-\text{Aβ}_{25-35} \) significantly reduced cellular metabolic activity compared to the \( \text{Aβ}_{25-35} \)-\( \text{G} \)-\( \text{R}^7 \) peptide. Cell cycle analysis revealed that \( \text{Aβ}_{25-35} \)-\( \text{R} \text{G} \) did not affect the Jurkat cell cycle (Fig. 1B and E), whereas \( \text{Aβ}_{25-35} \)-\( \text{G-R}^7 \) (Fig. 1C and F) and \( \text{R} \text{G}-\text{Aβ}_{25-35} \)-\( \text{R}^7 \) (Fig. 1D and G) induced a significant \( G_1 \)-\( M \)-phase cell arrest in a concentration-dependent manner, albeit \( \text{R} \text{G}-\text{Aβ}_{25-35} \) (200 \( \mu \text{M} \)) was more effective altering the Jurkat cell cycle than the \( \text{Aβ}_{25-35} \)-\( \text{G-R}^7 \) peptide. Similarly, we found that \( \text{Aβ}_{25-35} \) (e.g., 200 \( \mu \text{M} \)) did not affect proliferative-associated Ki-67 protein expression (Fig. 1H and I), whereas \( \text{R} \text{G}-\text{Aβ}_{25-35} \) peptide (e.g., 200 \( \mu \text{M} \)) and \( \text{Aβ}_{25-35} \)-\( \text{G-R}^7 \) induced a significant reduction (~74% and -23%) of Ki-67 nuclear activity, respectively (Fig. 1I). Because the \( \text{R} \text{G}-\text{Aβ}_{25-35} \) peptide altered cellular metabolic activity, cell proliferation, and cell cycle more efficiently than \( \text{Aβ}_{25-35} \)-\( \text{G-R}^7 \) in Jurkat cells, the former peptide was selected for further experiments. The \( \text{Aβ}_{25-35} \) peptide was included for comparative purposes.

3.2. \( \text{R} \text{G}-\text{Aβ}_{25-35} \), but not \( \text{Aβ}_{25-35} \) peptide, induces dissipation of mitochondrial membrane potential (\( ΔΨ_m \)) and produced reactive oxygen species (ROS) preserving cellular DNA integrity in Jurkat cells

Next, we wanted to determine whether the synthetic peptides induce \( ΔΨ_m \), ROS production, and DNA fragmentation in leukemic cells. Therefore, Jurkat cells were left untreated or treated with increasing concentrations (0–500 \( \mu \text{M} \)) of \( \text{Aβ}_{25-35} \) or \( \text{R} \text{G}-\text{Aβ}_{25-35} \) for 24 h at 37 °C. As shown in Fig. 2, \( \text{Aβ}_{25-35} \) neither alter the \( ΔΨ_m \) (Fig. 2A and C) nor influenced ROS production (Fig. 2D) nor induced DNA fragmentation (Fig. 2F), whereas \( \text{R} \text{G}-\text{Aβ}_{25-35} \) peptide not only significantly induced a concentration-dependent loss of \( ΔΨ_m \) (Fig. 2B and C) but also induced ROS production (Fig. 2E) according to flow cytometry analysis. These observations were confirmed by fluorescent microscopy (Fig. 2H and I). Analysis of the content of cellular DNA indicated that \( \text{Sub}_G_1 \) remained unaffected when exposed to either peptide (Fig. 2F and G).

3.3. The toxic effect of the \( \text{R} \text{G}-\text{Aβ}_{25-35} \) peptide is independent of ROS production and \( \text{CASPA} \) activation in Jurkat cells

Since ROS plays an important role as a signaling molecule [41], we evaluated whether the generation of ROS by \( \text{R} \text{G}-\text{Aβ}_{25-35} \) peptide-induced activation of \( \text{CASPA} \)-3 over time. As shown in Fig. 2A, \( \text{R} \text{G}-\text{Aβ}_{25-35} \) induced a significant reduction in ROS production up to 8 h (i.e., ~73% increase) followed by a significant reduction (e.g., ~71% reduction) after 24 h according to \( \text{DCF} \)-\( \text{DA} \)–\( \text{DCF} \) fluorescence assay (Fig. 3A). \( \text{CASPA} \)-3 activity remained unaffected during observational time points (Fig. 3B). To further corroborate these observations, we left Jurkat cells untreated or treated with \( \text{R} \text{G}-\text{Aβ}_{25-35} \) peptide alone or in combination with NAC – a well-known cytoprotective and antioxidant agent [42, 43], or NSCI -a caspase-3 inhibitor. Fig. 3 shows that neither NAC (5 \( \mu \text{M} \)) nor NSCI (10 \( \mu \text{M} \)) protected Jurkat cells against \( \text{R} \text{G}-\text{Aβ}_{25-35} \) peptide-induced loss of \( ΔΨ_m \) (Fig. 3C and D).

3.4. The \( \text{R} \text{G}-\text{Aβ}_{25-35} \) peptide dramatically decreases \( ΔΨ_m \) by its mitochondrial accumulation in Jurkat cells

To further characterized the effect of the \( \text{R} \text{G}-\text{Aβ}_{25-35} \) peptide on mitochondria, we wanted to specifically localize the cellular site of action of the peptide. To this aim, we left Jurkat cells untreated or treated with \( \text{Aβ}_{25-35} \) or \( \text{R} \text{G}-\text{Aβ}_{25-35} \) peptides for 24 h. Thereafter, we used the plasma membrane marker CD45, or outer mitochondrial membrane receptor TOMM20 together with antibody \( \text{Aβ}_{25-35} \) for co-localization purposes. As shown in Fig. 4, \( \text{Aβ}_{25-35} \) localizes neither at the plasma membrane (Fig. 4B, i.e., positive CD45 /negative \( \text{Aβ}_{25-35} \) fluorescence) nor at mitochondria (Fig. 4E, i.e., positive TOMM20/negative \( \text{Aβ}_{25-35} \) fluorescence) compared to untreated cells (Fig. 4A and D), whereas \( \text{R} \text{G}-\text{Aβ}_{25-35} \) peptide was positively detected at mitochondria (Fig. 4C, 4F–H).

3.5. The \( \text{R} \text{G}-\text{Aβ}_{25-35} \) peptide induces a rapid loss of \( ΔΨ_m \) by affecting mitochondria oxidative phosphorylation and uncoupling mitochondrial potential in Jurkat cells

The above observations prompted us to evaluate the effect \( \text{R} \text{G}-\text{Aβ}_{25-35} \) peptide on the mitochondria in Jurkat cells over time. Flow cytometry analysis indicates that \( \text{R} \text{G}-\text{Aβ}_{25-35} \) peptide significantly reduced the \( ΔΨ_m \) as early as 4 h in Jurkat cells (Fig. 5A, 50% ~ 37% early (green circle, Q4) plus 13% late (orange circle, Q1) mitochondrial.
damage), and this effect was stable over time up to 24 h (∼50%, Fig. 5B) followed by a stepwise progressive cell membrane permeabilization according to propidium iodide-stained cells (Fig. 5A). We wanted to determine whether the R7-G-Aβ25-35 peptide was linked to the impairment of mitochondria oxidative phosphorylation constituents. To this aim, we compared the effect of R7-G-Aβ25-35 peptide with other well-known mitochondrial-targeted drugs such as mitochondria Complex I (NADH:ubiquinone oxidoreductase) inhibitor rotenone (class 5 mitocan), complex V (ATP synthase) inhibitor oligomycin (class 5 mitocan), and classical uncoupler of oxidative phosphorylation FCCP (carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone) reflected as $\Delta\Psi_m$ changes on Jurkat cells. As expected, in about 240 s, FCCP (Fig. 5C), oligomycin (Fig. 5D), and rotenone (Fig. 5E) induced a rapid and progressive loss of $\Delta\Psi_m$ according to DiOC6(3) fluorescence change analysis (Fig. 5G). Strikingly, R7-G-Aβ25-35 peptide-a class 6 mitocan had a similar effect on the $\Delta\Psi_m$ when compared to mitochondrial-targeted drugs (Fig. 5F and G).

Fig. 3. Synthetic peptide R7-G-Aβ25-35 induces cell death in a CASPASE-3 (CASP-3)- and reactive oxygen species-independent mechanism in Jurkat cells
(A) Representative histograms show the DCF+ mean percentage in Jurkat cells treated with R7-G-Aβ25-35 peptide (200 μM) for 0, 4, 8, and 24 h. (B) Representative histograms show the CASP-3+ mean percentage of Jurkat cells treated with R7-G-Aβ25-35 (200 μM) peptide for 0, 4, 8, and 24 h. (C) Representative histograms show the DiOC6(3)low mean percentage of Jurkat cells untreated, or with NSCI (10 μM) or NAC (5 mM) only for 24 h. (D) Representative histograms show the DiOC6(3)low mean percentage of Jurkat cells treated with R7-G-Aβ25-35 peptide (200 μM) only or with NSCI (10 μM) or with NAC (5 mM) for 24 h. The numbers represent the mean percentage of three independent experiments. *p<0.05; **p<0.01; ***p<0.001.

Fig. 4. Synthetic peptide R7-G-Aβ25-35 co-localizes with mitochondria but not with the cell membrane in Jurkat cells.
(A-C) Representative colocalization images (A-C) and merge images (A’-C’) show Aβ25-35 epitope sequence (red; A’-C’) and CD45 (green; A’-C’) of untreated (A), treated with Aβ25-35 (B) or with R7-G-Aβ25-35 peptide (C) in Jurkat cells for 24 h. (D-F) Representative colocalization images (D-F) and merge images (D’-F’) show Aβ25-35 epitope sequence (red; D’-F’) and TOMM20 (green; D’’-F’’) of untreated (D), treated with Aβ25-35 (E) or with R7-G-Aβ25-35 peptide (F) in Jurkat cells for 24 h. (G) Quantitative data show the mean percentage of membrane or mitochondria area co-localized with β25-35 epitope sequence in Jurkat cells treated with R7-G-Aβ25-35 (200 μM) peptide. (H) Representative images show the measurement of total and co-localized area. The numbers represent the mean percentage of three independent experiments. *p<0.05; **p<0.01; ***p<0.001. Image magnification 100x. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.6. The R7-G-Aβ25-35 peptide is innocuous to non-leukemic proliferative stromal cells
To test the selective effect of R7-G-Aβ25-35 peptide on normal non-leukemic proliferative cells, we left mesenchymal stromal cells (MSC) untreated or incubated with Aβ25-35 or R7-G-Aβ25-35 peptides (200 μM) for 24 h, and evaluated the $\Delta\Psi_m$, ROS production and cell cycle analysis by flow cytometry and fluorescent microscopy (FM). Flow cytometry and FM analysis reveal that, like untreated cells (Fig. 6A and D, 6E'), neither Aβ25-35 (Fig. 6B, D, 6F') nor R7-G-Aβ25-35 (Fig. 6C, D, 6G')
peptides affected ΔΨm (Fig. 6A–G). Further, the peptides neither induced ROS production (Fig. 6F and 6G) nor affected the cell cycle progression in MSCs (Fig. 6I–K) compared to untreated cells (Fig. 6H, K, and 6E).

4. Discussion

Cell-penetrating peptides (C-PP) are promising anti-cancer agents [18,25]. Here, we report for the first time that the synthetic cationic peptide R²-G-Aβ25-35 and Aβ25-35-G-R², but not Aβ25-35 peptide, induced cell death in Jurkat cells, albeit with different strengths. Effectively, the R²-G-Aβ25-35 reduced cellular metabolic activity (e.g., 25 to ~48% reduction), induced G2/M-phase cell arrest (15–20%), and reduced cell proliferation (~74% reduction). Why is R²-G-Aβ25-35 more cytotoxic than Aβ25-35-G-R²? One possible explanation is that the poly-R² located at the amino-terminal of the Aβ25-35 might provide a better cell-penetrating capability of the peptide through a passive translocation mechanism [22] than when the poly-R⁻ is located at the carboxyl-terminal position of the Aβ25-35. Interestingly, we found that the Aβ25-35 peptide was innocuous to Jurkat cells. This observation might be the result of significant differences in the net charge (at pH 7.0) between the Aβ25-35 fragment (~+1.00) and R²-G-Aβ25-35 (~+8.00). Indeed, the high net charge of the poly-R-peptide might favor its faster interaction with the highly negative charge of the cellular plasma membrane (~−60 mV), thereby facilitating its cell entrance. Together these observations suggest that the poly-R⁻ attached to the amino position is critical for efficient cell-penetrating peptide. However, further chemical structural studies are needed (e.g., circular dichroism spectroscopy) to fully understand the biochemical behavior of the R²-G-Aβ25-35 (Aβ25-35-G-R²) peptide. Whatever the mechanism of interaction between poly-R⁻ peptides and plasma membrane, we found that once inside the cell, the R²-G-Aβ25-35 targets mitochondria.
Mounting evidence has shown that mitochondria play a central role in the regulation of cellular metabolism, bioenergetics, and cell life/death decision during carcinogenesis [44]. Therefore, therapeutic interventions (e.g., mitocans) for the targeting of mitochondria exhibit enormous potential for future leukemia therapeutic strategies [20]. In line with this view, we show that R²-G-Aβ25-35 induced a rapid depolarization of the ΔΨm as early as 240s post-exposure in Jurkat cells. Remarkably, the R²-G-Aβ25-35-induced loss of ΔΨm resembled the rapid depolarization of the mitocan 5 rotenein, which specifically blocks mitochondrial complex I, and of the mitocan 5 oligomycin, which specifically blocks F0 of the ATP synthase [19]. These observations suggest that R²-G-Aβ25-35 might work as mitocan 5. However, whether R²-G-Aβ25-35 directly interacts with mitochondrial complex I and/or ATP synthase, or if the mitochondrial depolarization is the consequence of the interaction of the poly-R² peptide with the outer mitochondria membrane requires further investigation. However, since R²-G-Aβ25-35 -induced mitochondrial depolarization also resembled the effect of the uncoupler FCCP, our observations favor the view that loss of ΔΨm might be the result of a complex interaction of the peptide with the inner mitochondrial membrane leading to uncoupling mitochondria [45], thereby inhibiting the complex I and ATP synthase, and generating ROS. This assumption is supported by 3 observations. First, R²-G-Aβ25-35 accumulated at the outer mitochondria membrane according to colocalization with protein TOMM20. However, whether TOMM20 - a mitochondrial import receptor translocase [46] participates in the import of R²-G-Aβ25-35 to the mitochondrial matrix needs further investigation. Second, R²-G-Aβ25-35 induced an important production of ROS concomitant with mitochondrial depolarization, an effect that was not reversed by the antioxidant/cytoprotectant NAC [42,43]. Third, it is well established that a drastic loss of ΔΨm generates ROS [47]. Taken together these observations suggest that R²-G-Aβ25-35 induces a deleterious domino-like phenomenon in Jurkat cells, involving its accumulation at and ensuing depolarization of mitochondria through uncoupling mechanism, subsequent inhibition of Complex I, generation of ROS, and dysfunction of ATP synthase.

Unexpectedly, we found that the R²-G-Aβ25-35 peptide did not induce CASPASE-3 activity, thus preserving nucleus integrity [48,49]. We speculate that this phenomenon might be due to the rapid depolarization of mitochondria, which in turn might trap effector cytochrome C, and pro-CASPASE-9 between the inner and outer mitochondria membrane space akin to "Venus’s flytrap" mechanism, and depletion of ATP content, thus disabling the apoptosis complex to process pro-CASPASE-3 into active CASPASE-3 [50]. Of note, the specific inhibitor caspase-3 NCSI was unable to protect Jurkat cells against R²-G-Aβ25-35-induced toxicity. These results imply that R²-G-Aβ25-35 can kill leukemia cells independently of CASPASE-3 [51]. In contrast to the above observations, R²-G-Aβ25-35 was innocuous to MenSC -normal non-leukemic proliferative cells. These data suggest that R²-G-Aβ25-35 specifically deletes leukemia cells.

5. Conclusion

We demonstrate that R²-G-Aβ25-35 (Aβ25-35-G-R²) specifically induces cell death in Jurkat cells through a direct and fast disruption of the mitochondria membrane potential, and CASPASE-3 independent mechanism. In agreement with our observations, others have reported that C-PP (e.g., r7-kla; RRRRRRR-GG-IYLATALAKWALKQGF) were able to kill cancer cell lines, including Jurkat cells through permeabilization of the mitochondrial inner membrane, and apoptosis [17,23]. These observations comply with the notion that some C-PP affect mitochondria membranes rather than other cellular membranes. Accordingly, the unmodified Aβ25-35 (Fig. 7, step 1) is unable to pass the cellular plasma membrane. Therefore, functional mitochondria (s2) and intact nuclei are observed in Jurkat cells (s3). In contrast, when cells are exposed to R²-G-Aβ25-35 peptide (s4), it translocates passively to the cell cytoplasm (s5). Due to its high net charge (+8.0), the peptide is attracted by the high negative mitochondrial potential (−140 to −180 mV [52]), leading to extensive accumulation of poly-R² cation within mitochondria (s6) probably with the assistance of protein TOMM20. These actions can disrupt mitochondria membrane integrity, respiration, and ATP synthesis (s7), thereby generating a high amount of ROS (s8). As a result of those mitochondria alterations, depolarized mitochondria instantly trap pro-apoptogenic proteins pro-caspase-9 and cytochrome C (s9) avoiding activation of CASPASE-3 (s10). Therefore, despite mitochondria dysfunction, no nuclei damage is appreciably detected (s11). Taken together these observations suggest that the cationic Aβ peptide might be a potential anti-leukemia agent against ALL.

**Ethical approval and consent to participate**

Not applicable.

**Consent for publication**

Not applicable.

**Availability of supporting data**

All relevant data and materials are within the paper.
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Authors’ contributions

Conceptualization, C.V.-P., and M.J.-Del-R.; methodology, C.V.-P.; validation, M.M.-P.; formal analysis, M.M.-P., and C.V.-P.; investigation, M.M.-P.; resources; C.V.-P. and M.J.-Del-R.; data curation, C.V.-P.; writing—original draft preparation, C.V.-P., and M.J.-Del-R.; writing—review and editing, M.M.-P.; C.V.-P. and M.J.-Del-R.; supervision, C.V.-P., and M.J.-Del-R.; project administration, M.J.-Del-R.; funding acquisition, C.V.-P. All authors have read and agreed to the published version of the manuscript.

Declaration of competing interest

All the authors have no competing interests to declare. The funders of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report.

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