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Pegylated-liposomes increase the efficacy of Idelalisib in lymphoma B-cells

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A R T I C L E I N F O

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A B S T R A C T

New drugs and technologies are continuously developed to improve the efficacy and minimize the critical side effects of cancer treatments. The present investigation focuses on the development of a liposomal formulation for Idelalisib, a small-molecule kinase inhibitor approved for the treatment of lymphoid malignancies. Idelalisib is a potent and selective antitumor agent, but it is not indicated nor recommended for first-line treatment due to fatal and serious toxicities. Herein, liposomes are proposed as a delivery tool to improve the therapeutic profile of Idelalisib. Specifically, PEGylated liposomes were prepared, and their physicochemical and technological features were investigated. Light-scattering spectroscopy and cryo-transmission electron microscopy revealed nanosized unilamellar vesicles, which were proved to be stable in storage and in simulated biological fluids.

The cytotoxicity of the liposome formulation was investigated in a human non-Hodgkin's lymphoma B cell line. Idelalisib was able to induce death of tumor cells if delivered by the nanocarrier system at increased efficacy. These findings suggest that combining Idelalisib and nanotechnologies may be a powerful strategy to increase the antitumor efficacy of the drug.

1. Introduction

Idelalisib is an inhibitor of phosphatidylinositol 3-kinase p110δ (PI3Kδ), an enzyme often hyperactivated in B-cell malignancies. PI3Kδ is central to multiple signaling pathways that drive proliferation, survival, homing, angiogenesis, and maintenance of malignant cells in lymphoid tissues and bone marrow (Keating, 2015; Martini et al., 2014). By blocking PI3Kδ, Idelalisib induces apoptosis and inhibits proliferation in cell lines derived from malignant B-cells and in primary tumor cells (Raedler, 2015).

Idelalisib is approved by EMA (European Medicine Agency) for the treatment of cancers affecting B lymphocytes: relapsed chronic lymphocytic leukemia (oral Idelalisib in combination with intravenous rituximab) and relapsed follicular lymphoma (oral Idelalisib as single agent) (Yang et al., 2019). However, Idelalisib is associated with a range of relevant side effects that include infections, hepatotoxicity, neutropenia, lymphocytosis, diarrhea/colitis, and intestinal perforation (Cuneo et al., 2019). Such difficulties trigger the need to develop novel technologies and formulations that can improve efficacy and minimize the critical side effects of Idelalisib. Nanotechnologies have gained wide attention in oncology research. Liposomes are the first nanotechnology-based therapy approved for the treatment of cancer, and they are still the best-investigated platform in preclinical research and clinical trials (Jensen and Hodgson, 2020; Nisini et al., 2018). Since 1995, when Doxil® (a liposomal formulation of doxorubicin) was approved, several nanomedicines have been clinically approved, and there has been a rapid expansion in clinically-promising nanoscale systems for drug delivery, such as liposomes (Hartshorn et al., 2018). Liposomes represent a

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leading strategy to address the challenges of cancer treatments: they offer crucial advantages over therapy with free drugs, such as increased solubility and bioavailability of the loaded drug, controlled drug release, drug protection, and selectivity against targets (Olusanya et al., 2018). These features overall result in increased efficacy and reduced dosages, thus decreasing systemic toxicity and side effects (Alavi and Hamidi, 2019; Daraee et al., 2016; Lee, 2020). Furthermore, liposomes are characterized by high versatility: they can be customized as a function of the drug and the therapeutic purpose.

Here, a liposomal formulation was developed for Idelalisib by a simple and rapid procedure that led to the production of vesicles in the nanoscale range. Since the formulation was intended for parenteral administration, liposomes were prepared with PEGylated phospholipids to prolong circulation time in the bloodstream (Yadav and Dewangan, 2021). Idelalisib PEG-liposomes were characterized to assess their main physicochemical and technological properties, such as size, charge, morphology, entrapment efficiency, stability in storage and in biological fluid. Additionally, both cytotoxicity and ability to induce apoptosis of the human B cell lymphoma cell line DOHH-2 were investigated, with the aim to assess whether liposomal Idelalisib had higher efficacy compared to its free form.

2. Materials and methods

2.1. Materials

Phospholipon90G (>94 % phosphatidylcholine; P90G), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), and [N-(carbonyl-methoxypolyethylene glycol-5000)-1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine, sodium salt] (MPEG-5000-DPPE) were purchased from Lipoid GmbH (Ludwigshafen, Germany). Idelalisib ((S)-2-(1-((9H-Purin-6-yl)amino)propyl)-5-fluoro-3-phenylquinazolin-4(3H)-one; IDE) was obtained from abcr GmbH (Karlsruhe, Germany).

The Annexin V-FITC Apoptosis Detection Kit was purchased from eBioscience™ (catalog # BMS500FI-300). Trypan Blue, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and fetal bovine serum (FBS) were purchased from Sigma-Aldrich (St. Louis, MO, US). Roswell Park Memorial Institute (RPMI) 1640 medium was from Sigma-Aldrich (catalog #R8758-500ML). Penicillin/streptomycin and ethanol 96 % were from Sigma/Merck (Milan, Italy).

2.2. Preparation and characterization of Idelalisib PEG-liposomes

Idelalisib PEG-liposomes were produced by dispersing phospholipids (P90G, DPPC, MPEG-5000-DPPE, lipid molar ratio 64.8:34.7:0.5) and drug (IDE, 2 mg/ml) in ultrapure water + 5 % *v/v* ethanol 96 % (Table 1). The resulting dispersion was sonicated with a Soniprep 150 plus disintegrator (MSE Crowley, London, UK) using the following parameters: 8 cycles of 5 sec on /2 sec off. Empty PEG-liposomes were prepared following the same procedure, in absence of Idelalisib (Table 1). Similarly, non-PEGylated liposomes, both empty and loaded with Idelalisib, were prepared without PEGylated phospholipid MPEG-5000-DPPE (Table 1).

Table 1
Composition of non-PEGylated and PEGylated liposomes.

	P90G	DPPC	MPEG-5000-DPPE	IDE	EtOH	H_2O
Empty liposomes	40 mg	20 mg	––	––	50 μl	950 μl
IDE liposomes	40 mg	20 mg	––	2 mg	50 μl	950 μl
Empty PEG-liposomes	40 mg	20 mg	2 mg	––	50 μl	950 μl
IDE PEG-liposomes	40 mg	20 mg	2 mg	2 mg	50 μl	950 μl

P90G, Phospholipon90G; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; MPEG-5000-DPPE, [N-(carbonyl-methoxypolyethylene glycol-5000)-1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine, sodium salt]; IDE, Idelalisib; EtOH, ethanol 96 %.

The morphology of Idelalisib PEG-liposomes was examined using cryogenic-transmission electron microscopy (cryo-TEM). The vesicle dispersion (5 μl) was placed on a glow-discharged holey carbon grid and blotted against filter paper. The resulting thin film spanning the grid holes was vitrified by plunging the grid (100 % humidity, at room temperature) into ethane (maintained at its melting point with liquid nitrogen) using a Vitrobot (FEI Company, Eindhoven, The Netherlands). The vitreous film was transferred to a Tecnai F20 transmission electron microscope (FEI Company) using a Gatan cryo-transfer (Gatan, Pleasanton, CA, US) and images were acquired at 200 kV and at –170/–175 °C, using low-dose imaging conditions not exceeding 20 $e^{-}/Å^2$, with a 4096 × 4096 pixel CCD Eagle camera (FEI Company).

The mean diameter, polydispersity index, and zeta potential of the vesicles were determined at 25 °C via dynamic and electrophoretic light scattering using a Zetasizer nano-ZS (Malvern Panalytical, Worcestershire, UK), after diluting them in water (1:100, *v/v*).

Idelalisib PEG-liposomes were purified and the non-incorporated Idelalisib excluded by dialysis. The dispersion (1 ml; $n = 4$) was loaded into Spectra/Por® tubing (12–14 kDa MW cut-off; Spectrum Laboratories Inc., Breda, The Netherlands) and dialyzed against water (2 l) to allow for the removal of the non-incorporated drug. After 2 h, both unpurified and purified vesicles were disrupted with methanol, and Idelalisib content was quantified by high performance liquid chromatography (Alliance 2695, Waters, Milan, Italy). The entrapment efficiency was calculated as the percentage of the amount of Idelalisib detected in purified *vs.* unpurified samples. Idelalisib was detected at 268 nm while running a mobile phase constituted by acetonitrile, water and acetic acid (93:6.77:0.23, *v/v*) through a SunFire C_{18} column, (3.5 μm, 4.6 × 150 mm, Waters) at a flow rate of 1 ml/min.

2.3. Stability in storage and in simulated biological fluid

The storage stability of PEG-liposomes was evaluated by monitoring their mean diameter (a parameter that affects stability, drug loading and biodistribution), polydispersity (a measure of particle homogeneity), and zeta potential (a measure of surface charge) for three months at 4 °C. The behavior in biological fluids was evaluated in Hank's balanced salt solution (pH 7.4) (Caddeo et al., 2018) by measuring the above-mentioned parameters immediately after dilution (1:100, *v/v*) and after 24 h of incubation at 37 °C.

2.4. Cell culture

The human B cell lymphoma cell line DOHH-2 (ACC 47, DSMZ, Brauschweig, Germany) was grown in RPMI 1640 medium supplemented with 10 % FBS, 1 % penicillin/streptomycin, and maintained at 37 °C in a humidified 5 % CO_2 incubator.

2.5. Cell viability and cytotoxicity assays

To assess the viability of the DOHH-2 line, the cells were stained with Trypan Blue. DOHH-2 cells were seeded in 48-well plates at 0.5×10^6 cells/ml and incubated with Idelalisib ethanol-based solution, Idelalisib PEG-liposomes, and empty PEG-liposomes, at 25, 50 and 100 μM, for 18 and 24 h. At the end of the incubation time, 20 μl of the cell suspensions were added to 20 μl of 0.4 % Trypan Blue and counted. Data are expressed as the percentage of viable cells relative to untreated control cells.

To quantitatively determine cytotoxicity, we performed an MTT assay (Gerlier and Thomasset, 1986). Briefly, DOHH-2 cells were plated in 96-well plates at 0.5×10^6 cells/ml and exposed to Idelalisib solution, Idelalisib PEG-liposomes and empty PEG-liposomes, at 25, 50 and 100

μM, for 18 and 24 h. Subsequently, 10 μl of the MTT reagent (5 mg/ml) were added to each well and kept in the dark for 2 h at 37 °C in 5 % CO_2. After incubation, the plate was centrifuged at 3000 rpm for 10 min, the cells were rinsed with PBS, and the formazan crystals (exclusively produced by the active mitochondria of viable cells) precipitated at the bottom of the wells were dissolved in 100 μl of 10 % DMSO-90 % isopropanol for further measurement. The plate was read at 540 nm (reference wavelength: 620 nm) using a VictorTM X3 Multilabel Plate Reader (Waltham, MA, US) to quantify viability by calculating the percentage of the absorbance of the treated cells relative to the absorbance of the untreated control cells.

2.6. Apoptosis assay

The eBioscience™ Annexin V-FITC Apoptosis Detection Kit was used for the apoptosis assay according to the manufacturer's instructions. DOHH-2 cells were seeded in 24-well plates at 0.5×10^6 cells/ml and incubated with Idelalisib solution, Idelalisib PEG-liposomes, and empty PEG-liposomes, at 50 and 100 μM. After 18 and 24 h of treatment, the cells were harvested, washed in PBS and resuspended in 195 μl Binding Buffer (1 ×). Subsequently, Annexin V-FITC (5 μl) was added and incubated for 10 min at room temperature. The cells were then washed and resuspended in 190 μl Binding Buffer (1 ×) supplemented with 10 μl PI. Fluorescence was detected by flow cytometry (CytoFLEX, Beckman, Indianapolis, IN, US).

2.7. Statistical analysis

Results are reported as means ± standard deviation (SD). Statistical analysis was performed using GraphPad Prism 10 software. p values < 0.05 were considered statistically significant.

3. Results and discussion

3.1. Characterization of liposomes' features

Key parameters that influence the functionality of Idelalisib PEG-liposomes, such as size, polydispersity, zeta potential, and stability were studied. In parallel, empty PEG-liposomes and non-PEGylated liposomes, with or without Idelalisib, were prepared to assess how drug incorporation and PEGylation affected the vesicles' features. The size, polydispersity index, and zeta potential of the vesicles were measured by light scattering spectroscopy (Table 2). The results showed that the empty PEG-liposomes were small in diameter (93 ± 4 nm), monodispersed (0.26 ± 0.02 of polydispersity index), and negatively charged (zeta potential of –28 ± 2.9 mV). Incorporating Idelalisib did not change the mean diameter of the vesicles (95 ± 4.2 nm) or their polydispersity (0.26 ± 0.02), but it slightly reduced their negative charge (–25 ± 4.1 mV, $p < 0.01$). This is probably due to a re-arrangement of the phospholipids upon accommodating the drug.

The PEG-liposomes were compared with non-PEGylated liposomes, both empty and loaded with Idelalisib, to evaluate the effect of PEGylation on the liposomes' features (Table 2). The results showed that PEGylation induced an enlargement of the vesicles, which suggests the presence of the hydrophilic PEG chains on the surface of the vesicles. The non-PEGylated liposomes were significantly smaller (≤90 nm, $p < 0.05$) than the corresponding PEG-liposomes. PEGylation also made the liposomes more uniform, reducing the polydispersity index from ca. 0.3 to 0.26 ($p < 0.05$; Table 2). Moreover, PEGylation increased the negative surface charge of the vesicles, from ~ –8 mV to ≥ –25 mV ($p < 0.001$), which is more favorable to avoid vesicle aggregation, and thus physical instability, by electrostatic repulsion.

Table 2
Mean diameter (MD), polydispersity index (PI), and zeta potential (ZP) of non-PEGylated and PEGylated liposomes. Mean values ± standard deviations (SD) are reported ($n > 10$). § $p < 0.05$ *vs.* the corresponding PEG-liposomes, §§ $p < 0.001$ *vs.* the corresponding PEG-liposomes, ** $p < 0.01$ *vs.* empty PEG-liposomes.

	MD nm ± SD	PI ± SD	ZP mV ± SD
Empty liposomes	§85 ± 3.4	§0.29 ± 0.02	§§–9 ± 1.1
IDE liposomes	§90 ± 2.9	§0.33 ± 0.04	§§–8 ± 1.0
Empty PEG-liposomes	93 ± 4.0	0.26 ± 0.02	–28 ± 2.9
IDE PEG-liposomes	95 ± 4.2	0.26 ± 0.02	**–25 ± 4.1

The entrapment efficiency of the Idelalisib PEG-liposomes after purifying them by dialysis was high (83 % ± 2.2), indicating a good drug-loading capacity of these liposomes.

The morphology of the Idelalisib PEG-liposomes was observed by cryo-TEM (Fig. 1), which revealed that the vesicles were spherical, unilamellar, and around 100 nm, a diameter consistent with the light-scattering spectroscopy data (Table 2).

The stability of Idelalisib PEG-liposomes was tested under two conditions: storage and simulated biological fluid. The storage stability was evaluated by visually observing the vesicle dispersions and monitoring their mean diameter, polydispersity index, and zeta potential of the vesicles for three months. At the end of the storage period, no macroscopic signs of physical alteration were observed, and the three parameters did not change significantly ($p > 0.05$) from the initial values (Table 2), which indicated good stability of the nanoformulation. Namely, after three months, the mean diameter was 94 nm ± 3.8, the polydispersity index was 0.26 ± 0.02, and the zeta potential was –23 mV ± 3.0.

The stability of Idelalisib PEG-liposomes was evaluated by diluting and incubating them for 24 h in Hank's solution, which simulates the body fluids, as it contains inorganic salts and glucose that confer an osmotic pressure similar to that of intercellular fluids (Caddeo et al., 2018). The vesicles did not change in size and polydispersity, remaining at 87 ± 2.8 and 0.2 ± 0.03 at the initial values (t_0) and 87 ± 1.1 nm and 0.2 ± 0.02 at 24 h (t_{24}) (Table 3). Overall, these data demonstrate that the structure of Idelalisib PEG-liposomes was maintained despite the osmotic stress/pressure impinged by high ion concentrations in the medium. The zeta potential values instead varied greatly, as compared to the value measured upon preparation (–25 mV; Table 2), approaching neutrality already at t_0 (–2 ± 0.3 mV; Table 3), due to the ions from the Hank's solution adsorbing on the vesicles' surface.

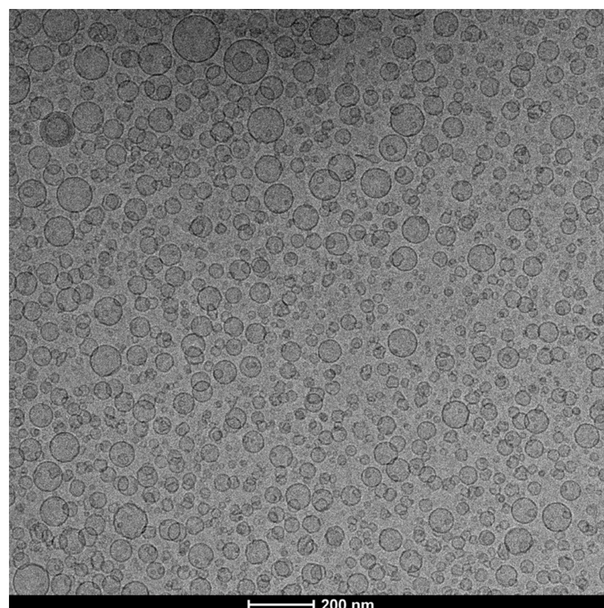

Fig. 1. Cryo-TEM image of Idelalisib PEGylated-liposomes.

Table 3
Mean diameter (MD), polydispersity index (PI), and zeta potential (ZP) of Idelalisib PEGylated liposomes after dilution (t_0) with Hank's solution and after 24 h (t_{24}) of incubation at 37 °C. Mean values ± standard deviations (SD) are reported ($n = 4$).

	Time (h)	MD (nm ± SD)	PI ± SD	ZP (mV ± SD)
IDE PEG-liposomes	t_0	87 ± 2.8	0.20 ± 0.03	−2 ± 0.3
	t_{24}	87 ± 1.1	0.20 ± 0.02	−2 ± 0.4

3.2. Idelalisib PEG-liposomes reduced the viability of the DOHH-2B lymphoma cell line

Trypan Blue and MTT assays were used to assess how Idelalisib in solution or nanoincorporated in PEG-liposomes inhibited the viability of DOHH-2 cells. DOHH-2 cells were exposed to different concentrations of Idelalisib solution, Idelalisib PEG-liposomes, and empty PEG-liposomes (25, 50 and 100 μM) for 18 and 24 h. Fig. 2 shows the percentage of viable cells at two different time points post treatment (18 h and 24 h). After 18 h (Fig. 2**A**), only the highest concentration of Idelalisib solution (100 μM) significantly decreased the percentage of viable cells, as compared to the control treatment with ethanol (77.12 ± 3.59; p = 0.0029). When Idelalisib was incorporated in PEG-liposomes, a similar effect was detected at the concentration of 100 μM, resulting in a viability of 72.86 % ± 5.22 ($p < 0.0001$), compared with cells treated with empty PEG-liposomes. After 24 h (Fig. 2**B**), the reduction in viability of cells treated with 100 μM of Idelalisib solution was minimal (75.28 % ± 1.70, p = 0.0186), whereas Idelalisib PEG-liposomes significantly reduced the viability in a dose-dependent fashion, starting at 50 μM, and reaching the maximum effect at the highest concentration tested, resulting in a 2.18-fold decrease (79.58 % ± 9.34 at 25 μM, ns; 69.56 % ± 9.16, p = 0.0317 at 50 μM; and 39.95 % ± 1.68, $p <$ 0.0001 at 100 μM). Taken together, these data suggest that Idelalisib delivered by PEG-liposomes has a higher efficacy after 24 h of treatment as compared to Idelalisib in its free form ($p < 0.0001$ at 100 μM). Importantly, empty PEG-liposomes at 25, 50 and 100 μM did not induce any significant decrease in cell viability, excluding any cytotoxic effects of the nanocarrier and implying that the reduction in cell viability is specifically caused by the nanoformulated Idelalisib.

The anticancer effect of Idelalisib was assessed using the MTT assay (McCauley et al., 2013). MTT is reduced by enzymes in the endoplasmic reticulum to formazan, which reflects the number of metabolically active cells (Twarużek et al., 2018). DOHH-2 cells were exposed to different concentrations of Idelalisib solution, Idelalisib PEG-liposomes and empty PEG-liposomes (as a control) at 25, 50, and 100 μM for 18 h (Fig. 2**C**) and 24 h (Fig. 2**D**). In agreement with Trypan Blue data, after 18 h of incubation, the 100 μM Idelalisib solution showed a significant reduction in cell viability (76.41 %, p = 0.0053), as compared to the vehicle control (EtOH = 95.89 %), whereas Idelalisib PEG-liposomes induced a higher decrease in cell viability at both 50 μM (76.17 %, p = 0.0130) and 100 μM (60.93 %, $p < 0.0001$), as compared to the empty PEG-liposomes. After 24 h of treatment with the Idelalisib PEG-liposomes, a significant dose-dependent cytotoxic effect was shown already at the lowest concentration tested. In particular, cells displayed a ~ 25 % (68.53 ± 9.50 *vs.* 92.74 ± 9.33), ~50 % (39.63 ± 10.60 *vs.* 86.60 ± 3.67), and ~ 75 % (21.77 ± 12.03 *vs.* 85.15 ± 5.34) reduction at 25 μM ($p < 0.0001$), 50 μM ($p < 0.0001$), and 100 μM ($p < 0.0001$), respectively, as compared to their empty PEG-liposomes counterparts. Importantly, Idelalisib PEG-liposomes at both 50 and 100 μM displayed a significant effect as compared to Idelalisib solo treatment after 24 h (p < 0.0001 for both concentrations). Hence, taken together, our evidence strongly supports the hypothesis that the nanoformulated Idelalisib enhances loss of cell viability.

3.3. Apoptosis increased upon Idelalisib PEG-liposomes treatment

To understand how Idelalisib PEG-liposomes affect DOHH-2 cell viability, the apoptotic process was investigated at the two highest concentrations screened in the viability assays: 50 μM and 100 μM. Apoptosis was evaluated at two time points: 18 and 24 h (Fig. 3**A-H and suppl** Fig. 1**A-H**). Fluorescence-assisted cell sorting (FACS) analysis revealed that 50 μM Idelalisib solution did not affect the cells, as shown by the representative FACS plots (Fig. 3**A**), as compared to the vehicle (EtOH)-treated cells (**suppl** Fig. 1**A**). At both time points, the number of viable (81.83 % ± 5.87 and 77.77 % ± 13.05, respectively), early- (7.54 % ± 2.10 and 11.26 % ± 6.11, respectively) and late-apoptotic cells (5.34 % ± 2.57 and 4.19 % ±1.55, respectively) did not show any statistically significant change, as compared to their vehicle (ethanol) controls (**suppl** Fig. 1 **A-B**). Then, cells were incubated with Idelalisib PEG-liposomes at 50 μM and measured their viability was determined at 18 and 24 h. As shown by the FACS plot (Fig. 3**B**), most cells were viable at both time points, with cell survivals of 84.13 % ± 3.31 at 18 h and 75.33 % ± 1.7 at 24 h, with most cells in the lower left quadrant of the FACS plot (double negative quadrant). The effect of Idelalisib solution and Idelalisib PEG-liposomes was also compared at 50 μM after 18 h (Fig. 3**E**) and 24 h (Fig. 3**F**). The percentages of early and late apoptotic cells did not differ significantly between the two treatments at either time point. In particular, samples treated with Idelalisib PEG-liposomes displayed early apoptotic cells in a range of 2.69 % ± 0.27 and 2.79 % ± 0.33 at 18 h and 24 h, respectively. Late apoptotic cells were 4.63 % ± 0.48 and 7.05 % ± 0.15 at 18 and 24 h, respectively. The majority of the cells were in the lower left quadrant of the FACS plot (double negative quadrant), indicating that no apoptosis occurred (Fig. 3**A-B**). A similar result was obtained in cells incubated with 100 μM Idelalisib solution at 18 h and 24 h, as shown by the FACS plots in Fig. 3**C**. **Supplementary** Fig. 1**A-C** shows that no significant changes in viable (73.50 % ± 9.91 at 18 h and 73.60 ± 10.88 at 24 h), early (9.81 % ± 5.10 at 18 h and 15.13 % ± 7.86 at 24 h) and late (10.09 % ± 0.77 at 18 h and 5.95 % ± 2.22 at 24 h) apoptotic cell numbers were reported, as compared to the vehicle (EtOH)-treated cells.

Cells incubated with Idelalisib solution and Idelalisib PEG-liposomes (100 μM) for 18 h did not display any difference (Fig. 3**G**). However, remarkably, by incubating the cells with the highest concentration of Idelalisib PEG-liposomes (100 μM) for 24 h, the percentage of viable cells went down to 48.20 % ± 2.75 and the number of late apoptotic cells increased (24.47 % ± 1.21) (Fig. 3**D and 3H**). Such effect is not present at 18 h post Idelalisib PEG-liposomes (100 μM) treatment (Fig. 3G). Compared to cells treated with 100 μM Idelalisib solution for 24 h, this represents a 0.65-fold decrease of viability cells and a 4.13-fold increase of late apoptotic cells.

To validate the relevance of Idelalisib nanoincorporation, the effect/s of empty PEG-liposomes on the cells were checked, as a control. No apoptosis occurred at 50 μM and 100 μM after 18 h (**suppl** Fig. 1**D-G**) and 24 h (**suppl** Fig. 1**D-F and H**) incubation. No statistically significant differences were highlighted by comparing the 18 h and 24 h time points at both concentrations (**suppl** Fig. 1 **G-H**), confirming that empty PEG-liposomes did not have any effects on the cells.

4. Conclusions

We formulated Idelalisib, a kinase inhibitor used to treat B-cell malignancies, in PEGylated liposomes to enhance its bioactivity. We characterized the vesicles for key parameters that affect their functionality: nanometric size, high entrapment efficiency and physical stability. PEG-liposomes increased the antitumor activity of Idelalisib in DOHH-2 lymphoma B cells, as measured by the apoptotic effect. These promising findings confirm the potential of nanotechnologies for delivering antitumor drugs and prompt further investigation of the therapeutic potential of the Idelalisib PEG-liposomes formulation.

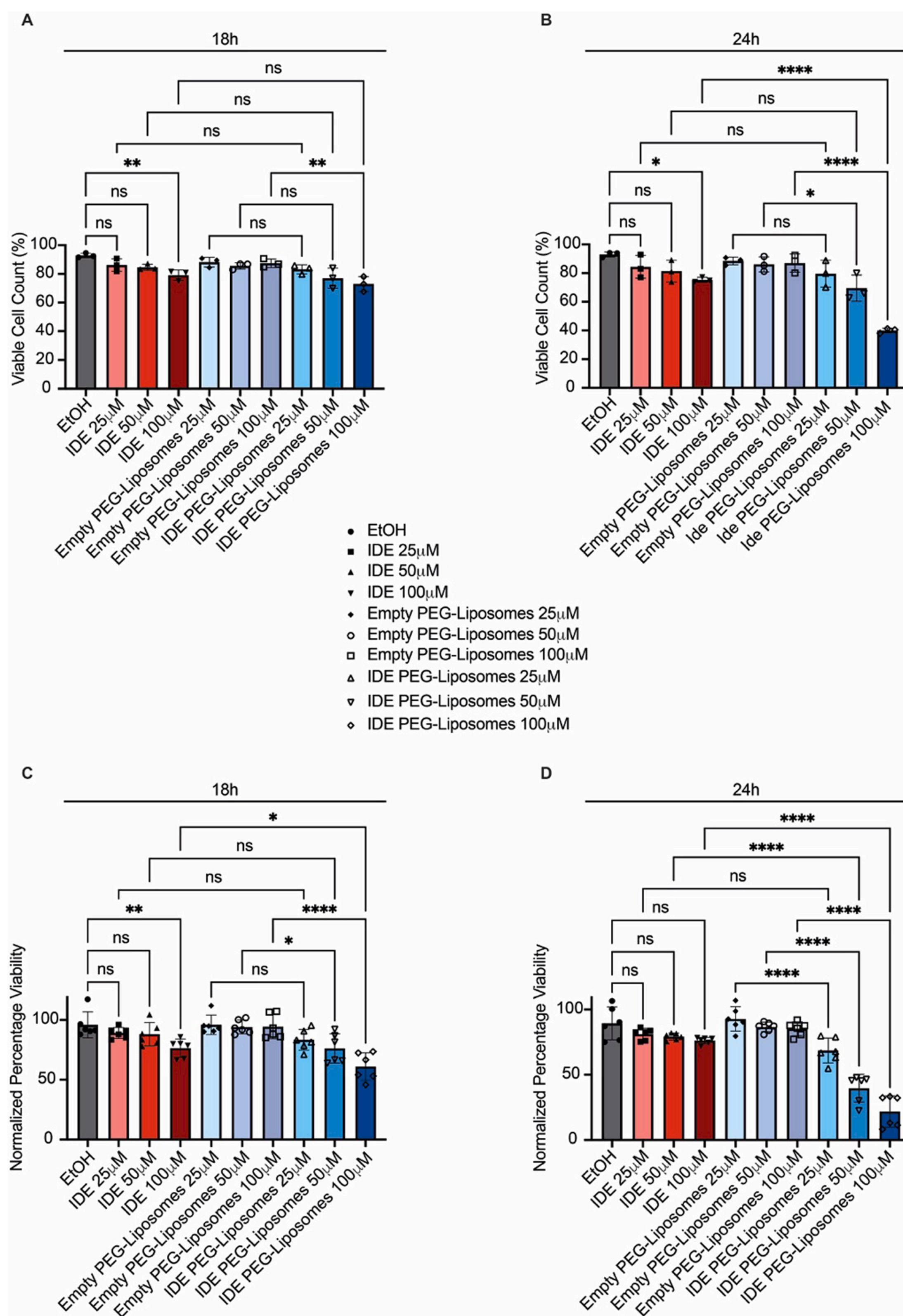

Fig. 2. Cell viability assays. (A, B) Trypan Blue assay in DOHH-2 cells exposed to different concentrations (25, 50 and 100 μM) of Idelalisib solution, Idelalisib PEG-liposomes and empty PEG-liposomes after A) 18 h and B) 24 h. (C, D) MTT assay in DOHH-2 cells exposed to different concentrations (25, 50 and 100 μM) of Idelalisib solution, Idelalisib PEG-liposomes and empty PEG-liposomes after C) 18 h and D) 24 h. Data are represented as percentage of viable cells. One-way ANOVA for multiple comparisons was applied. * $p < 0.0032$, ** $p < 0.0021$, *** $p < 0.0002$, **** $p < 0.0001$.

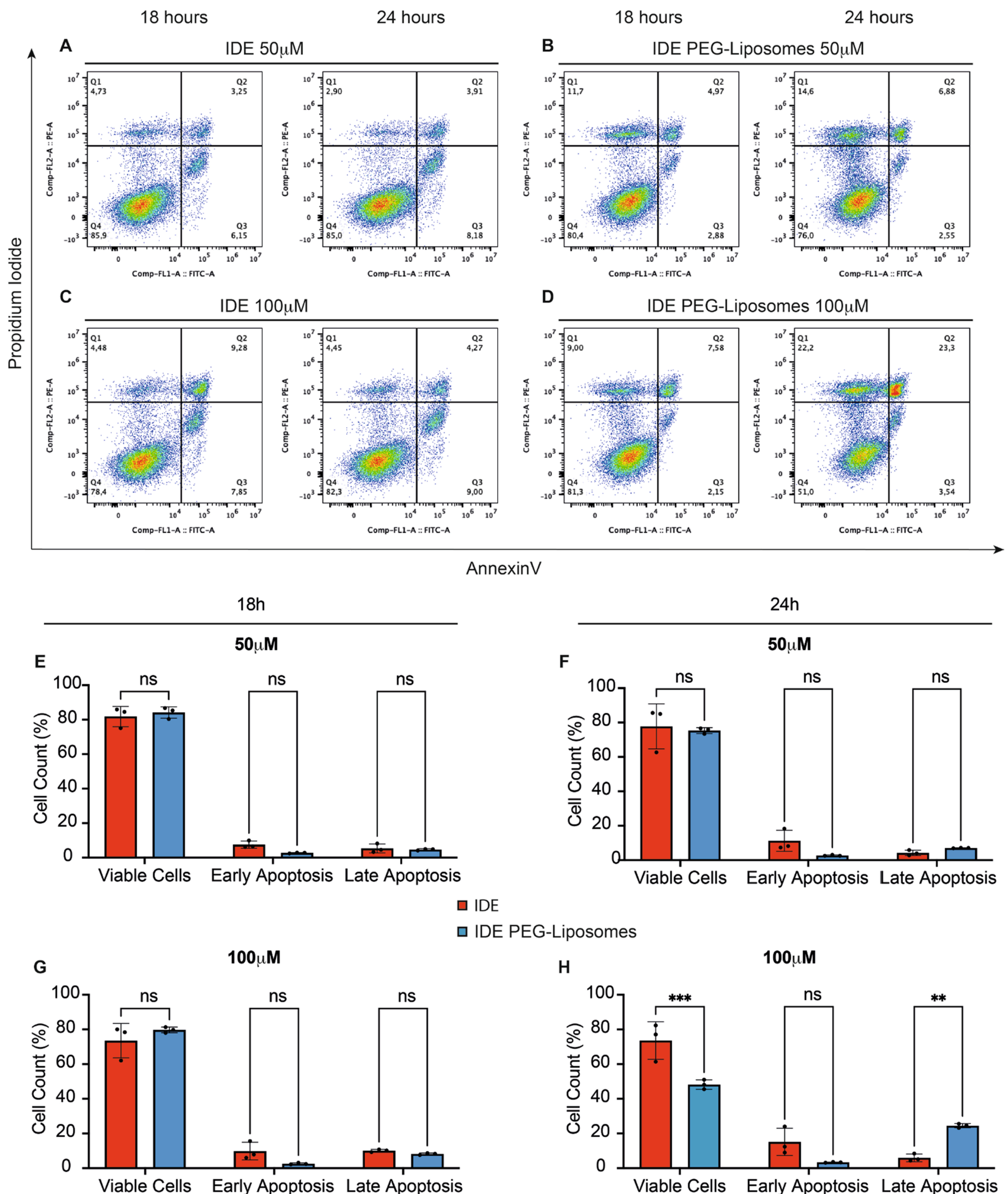

Fig. 3. (A-D) FACS plots representing apoptosis assay on DOHH-2 cells at 18 h and 24 h upon exposure to 50 μM of A) Idelalisib solution, B) Idelalisib PEG-liposomes, and 100 μM of C) Idelalisib solution, and D) Idelalisib PEG-liposomes. Bar charts (E-H) indicate the percentage of viable cells and cells in early, and late apoptosis, comparing Idelalisib solution- (red bars) and Idelalisib PEG-liposomes-treated cells (blue bars) at 50 μM after 18 h (E) and 24 h (F), and 100 μM after 18 h (G) and 24 h (H). 2-way ANOVA for multiple comparisons was applied. * $p < 0.0032$, ** $p < 0.0021$, *** $p < 0.0002$, **** $p < 0.0001$.

CRediT authorship contribution statement

Giorgia Maroni: Writing – review & editing, Writing – original draft, Visualization, Investigation, Formal analysis, Data curation. **Elena Tomassi:** Writing – review & editing, Supervision, Funding acquisition, Data curation. **Donatella Valenti:** Investigation. **Xavier Fernàndez-Busquets:** Writing – review & editing, Investigation. **Laura Pucci:** Writing – review & editing, Validation. **Elena Levantini:** Writing – review & editing, Supervision, Funding acquisition, Data curation. **Carla Caddeo:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpharm.2024.124144.

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