

A novel aminohydroxy sulfonamide formulated in PEGylated liposomes with potential antitumor activity

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ARTICLE INFO

Keywords:

Aminohydroxy sulfonamide
PEGylated liposomes
Parenteral delivery
Hepatic cancer cells
Anticancer formulation

ABSTRACT

Cancer ranks as a leading cause of death worldwide, with liver cancer being one of the most commonly diagnosed. Currently, several molecules are being studied in order to find new therapeutic options that can reduce cancer recurrence rate and increase patient survival. This study proposes PEGylated liposomes for the delivery of a newly synthesized aminohydroxy sulfonamide, BupM-NH₂, which has shown dose-dependent cytotoxicity towards hepatic tumor cells. The prepared PEG-liposomes were nanosized, spherical, and unilamellar, as shown by light scattering data and cryo-TEM micrographs. The physical stability of the PEG-liposomes was preserved when tested in simulated body fluids. Similar results were found for the storage stability evaluation. Furthermore, the PEG-liposomes efficiently entrapped BupM-NH₂ and modulated its release. The antitumor activity of BupM-NH₂ in PEG-liposomes was assessed *in vitro* in hepatocarcinoma cells. The viability assay showed that PEG-liposomes were able to control the release of BupM-NH₂ over time and induce the death of HepG2 cells at a concentration about two-times lower than that required by free BupM-NH₂ (IC₅₀: 33.31 vs. 57.05 μM). Furthermore, the liposomal formulation showed a less cytotoxic effect against non-cancerous cell line (IHH) compared to the free molecule (IC₅₀ > 200 vs. 106.9 μM), encouraging further investigation to confirm its effective and safe use.

1. Introduction

Cancer ranks as a leading cause of death in the world. Its incidence and mortality are growing due to aging of the population and increase in risk factors [1]. Liver cancer is one of the most common cancer [2] and hepatocellular carcinoma is the most common type of liver cancer [1,3]. Systemic therapies based on tyrosine kinase inhibitors, immune-checkpoint inhibitors, and monoclonal antibodies are currently being used to treat hepatocellular carcinoma, resulting in a marked increase in patients' overall survival and quality of life [4]. However, the

effectiveness of chemotherapy can be limited by drug rapid metabolism, toxic side effects, and the development of resistance [5]. Hence, there still is a growing need for novel formulation approaches and technologies to develop safe and effective therapies for a continued improvement in overall survival in the front-line setting. In this scenario, nanotechnologies are having a major impact on human health for the treatment of tumors. Liposomes were the first type of nanoparticle-based drug delivery systems to be approved for clinical use, and still are the most used platform for marketed pharmaceuticals, and the best-studied platform in academic research and clinical trials [6–9]. Most of the liposomal

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<https://doi.org/10.1016/j.jddst.2025.106739>

Received 29 October 2024; Received in revised form 18 February 2025; Accepted 19 February 2025

Available online 20 February 2025

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commercial products are approved for cancer treatment, upon parenteral administration (intravenous or intramuscular) [10]. Since 1995, when U.S. Food & Drug Administration approved the first liposomal doxorubicin formulation, Doxil®, liposomes garnered significant attention as chemotherapeutic agents and various strategies, such as surface modification, loading of radionuclides, genes and proteins, combination with other therapeutic regimens like radiotherapy and immunotherapy, and integration with other emerging technologies like gene editing and biosensors, have been employed to facilitate their translation from bench to bedside [11]. The favorable outcomes of nanoincorporation of drugs in liposomes include increased drug solubility and bioavailability, controlled release of drug, protection of drug, specificity to cancer target versus host and reduced drug dose, which minimize systemic toxicity and increase efficacy of therapy [12,13]. Liposomes are primarily composed of phospholipids, which are generally recognized as safe (GRAS), as well as biocompatible, biodegradable, and non-immunogenic [14]. One limitation of liposomes is their preparation on a large industrial scale with reproducible properties [15]. The stability of liposomes represents another limitation, and lyophilizing the produced vesicle dispersion is one of the proposed solutions to overcome this limitation [16]. To develop a scalable, reproducible, and stable product, the liposomal formulation should be not too complex and/or laborious, yet effective, and customized as a function of both drug and therapeutic purpose. Therefore, in this study, we produced a liposomal formulation by a facile, solvent-free method that led to the formation of nanosized vesicles. Since the formulation is intended for parenteral administration, PEGylated liposomes were produced. The PEG chains surround the liposomes' surface, shielding against opsonin recognition and consequent phagocytosis, thus extending circulation time [17–20].

Herein, PEGylated liposomes were used for the loading and delivery of *N*-((2R,3S)-3-amino-2-hydroxy-4-phenylbutyl)-*N*-isobutyl-4-methoxybenzenesulfonamide, named BupM-NH₂ (Fig. 1), a newly synthesized molecule, which has been previously found to exert a remarkable anti-tumor activity against hepatocellular carcinoma [21]. BupM-NH₂ is characterized by poor solubility in water, while being soluble in organic solvents such as dimethyl sulfoxide and acetonitrile. The liposomal formulation is expected to increase BupM-NH₂ solubility, bioavailability, and consequently biological activity. Key physico-chemical and technological features (i.e. size, charge, morphology, stability in storage and in biological milieu, drug release) of the BupM-NH₂ PEG-liposomes were studied. The cytotoxicity of the BupM-NH₂ PEG-liposomes was investigated in a hepatoma cell line (HepG2) and a healthy hepatocyte cell line (IHH) to assess whether the liposomal formulation could increase the efficacy of the drug in comparison to what was previously found for the free drug [21].

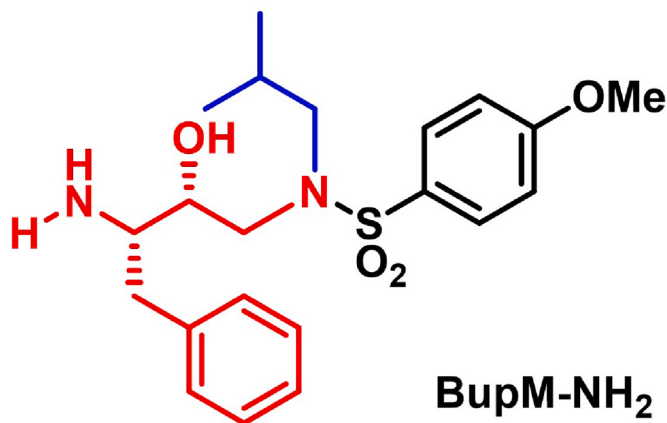


Fig. 1. Chemical structure of aminohydroxy sulfonamide BupM-NH₂. MW: 406.54, Log P: 2.97 ± 0.47 (calculated according to Crippen's fragmentation [41]).

2. Materials and methods

2.1. Materials

The aminohydroxy sulfonamide (*N*-((2R,3S)-3-amino-2-hydroxy-4-phenylbutyl)-*N*-isobutyl-4-methoxybenzenesulfonamide, BupM-NH₂, was synthesized as previously reported [21,22].

[N-(carbonyl-methoxypolyethylenglycol-2000)-1,2-distearoyl-sn-glycero-3-phosphoethanolamine, sodium salt] (MPEG-2000-DSPE) and 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) were from Lipoid GmbH (Ludwigshafen, Germany); propylene glycol (PG) was from Galeno (Carmignano, Prato, Italy); phosphate buffered saline (PBS; pH 7.0) was from Carlo Erba (Cornaredo, Milan).

Dulbecco's Modified Eagle's Medium (DMEM) was from Corning (Glendale, AZ, US). Dulbecco's phosphate buffered saline, fetal bovine serum, penicillin-streptomycin solution, and *L*-glutamine were from EuroClone (Milan, Italy). Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham (DMEM/F-12), trypsin-EDTA solution, dimethyl sulfoxide (DMSO), Thiazolyl Blue Tetrazolium Bromide (MTT), isopropanol and Triton X-100 were from Merck (Saint Louis, MO, US).

2.2. Vesicles' preparation and characterisation

To produce BupM-NH₂ PEG-liposomes, DPPC and MPEG-2000-DSPE (60 mg/ml - 81.7 mM and 1 mg/ml - 0.35 mM, respectively; lipid molar ratio 99.5:0.5), BupM-NH₂ (2 mg/ml - 5 mM), PG (6 mg/ml) were dispersed in PBS and the dispersion was sonicated at 9 cycles of 5 s on and 2 s off with an ultrasound disintegrator (Soniprep 150 plus; MSE Crowley, London, UK) [23,24].

For comparative purposes, empty PEG-liposomes were prepared according to the above composition, in absence of BupM-NH₂. Liposomes without MPEG-2000-DSPE, empty and loaded with BupM-NH₂, were prepared to assess the impact of the PEGylated phospholipid on the vesicles' features.

The morphology of BupM-NH₂ PEG-liposomes was studied using cryo-TEM. A thin film of the liposome dispersion deposited on a grid was vitrified in ethane using a Vitrobot (FEI Company, Eindhoven, The Netherlands). The vitrified film was observed using a Tecnai F20 TEM (FEI Company), and micrographs were acquired at 200 kV, using low-dose imaging conditions.

The average diameter, polydispersity index, and zeta potential of the vesicles were measured via dynamic and electrophoretic light scattering (Zetasizer nano-ZS; Malvern Panalytical, Worcestershire, UK). For size/polydispersity index measurements, samples were analysed after dilution (1:100 v/v) with PBS. For zeta potential measurements, samples were analysed without dilution.

The liposome dispersions (1 ml; $n = 4$) were dialysed against water (2 l) to allow purification from the non-incorporated BupM-NH₂ by loading into 12–14 kDa MW cut-off tubing (Spectrum Laboratories Inc., Breda, The Netherlands). After 2 h under gentle stirring, both unpurified and purified dispersions were diluted (1:100 v/v) with methanol to disrupt the vesicles and free BupM-NH₂. The latter was quantified by HPLC (Alliance 2695, Waters, Milan, Italy), and the entrapment efficiency (E) was calculated as the percentage of BupM-NH₂ in purified vs. unpurified samples [23,24]. BupM-NH₂ was assayed using a fluorescence detector (λ_{exc} 238 nm and λ_{em} 295 nm), an X-Terra column (Waters), and a mobile phase of 99.5 acetonitrile:0.48 water, 0.02 acetic acid (v/v) run at 0.7 ml/min. A stock standard solution of BupM-NH₂ was prepared in acetonitrile and working standard solutions in methanol, and a linear regression of the peak areas vs. analyte concentration was calculated. The limits of detection (LoD) and quantification (LoQ) were 1 ng and 10 ng, respectively.

2.3. Stability in storage and in simulated biological fluids

The stability of the PEG-liposomes was studied by measuring their

size, homogeneity, and charge for 60 days of storage at $4 \pm 2^\circ\text{C}$.

The stability of the PEG-liposomes in simulated biological fluids was studied by measuring their size, homogeneity, and charge via incubation with Hank's balanced solution and modified-Simulated Body Fluid (m-SBF), both at $\text{pH } 7.4 \pm 0.1$. The former was prepared according to Yang et al. [25], and the latter according to Ascencio et al. [26]. Both solutions contain inorganic salts and organic compounds (e.g., glucose, HEPES) that create an osmotic pressure similar to that of intercellular fluids [23]. The above parameters were measured after dilution (1:100 v:v) of PEG-liposomes with the simulated fluids, and after incubation at $37 \pm 0.1^\circ\text{C}$ for 24 h.

2.4. Drug release

Release studies were performed using a USP/EP compliant dissolution tester (DT126, Erweka, Heusenstamm, Germany). The BupM-NH₂ PEG-liposomes were loaded into dialysis tubing floating in saline (250 ml) kept at $37 \pm 0.1^\circ\text{C}$ and stirred at 100 rpm. One ml of the dissolution medium was withdrawn at regular time intervals and replenished with fresh saline to maintain sink conditions. BupM-NH₂ was quantified by HPLC (Section 2.2).

2.5. Cell culture and treatments

The hepatocellular carcinoma cell line HepG2 (ATCC #HB-8065; Manassas, VA, US) was cultured in DMEM properly supplemented with 100 IU/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine, and 10 % fetal bovine serum. The immortalized human hepatocytes cell line IHH from healthy primary hepatocytes stably transfected with the recombinant plasmid SV40 (kindly gifted by Prof. Tiribelli at Italian Liver Foundation, Trieste, Italy), was cultured in DMEM/F-12 properly supplemented with 100 IU/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine, 1 µM dexamethasone, 1.2×10^{-7} M insulin from bovine pancreas, and 10 % fetal bovine serum. Both cell lines were grown under standard conditions (5 % CO₂, 37°C). BupM-NH₂ PEG-liposomes (BupM-NH₂-L) and empty PEG-liposomes (EL) were diluted in complete growth medium to reach the working concentrations (25, 37.5, 50, 75, 100, 150 and 200 µM).

2.6. Evaluation of cell viability

The effect of nanoformulated BupM-NH₂ on the viability of HepG2 and IHH cells was studied via the MTT assay. The cells (1.5×10^4 cells/well) were seeded into 96-well plates and treated with the proper dilutions of PEG-liposomes and incubated. After 24 and 48 h, the spent medium was removed, and MTT was added to each well (100 µl, 0.75 mg/ml in complete growth medium). After 4 h, the MTT was discarded and the formed formazan was dissolved in isopropanol:DMSO (1:1) + 1 % Triton X-100. The absorbance was measured at 570 nm. The optical density in the control cells (untreated cells) was considered as 100 % viability. Data were expressed as the mean percentage of cell viability vs. control, and the half-maximal inhibitory concentration (IC₅₀) was calculated by nonlinear regression analysis using Prism software v. 8.4.2 (GraphPad, Boston, MA, US).

2.7. Evaluation of cell morphology

Both liver cell lines (2×10^5 cells/well) were seeded into 12-well plates and exposed to BupM-NH₂ PEG-liposomes (BupM-NH₂-L; 25, 37.5, 50 and 100 µM) for 24 h. The cell morphology was observed under an Eclipse TS100 inverted microscope (Nikon, Tokyo, Japan; 40 × magnification). Untreated cells were used as negative control.

2.8. Evaluation of cell intake via LC-MS/MS

HepG2 cells (2.5×10^5 cells/well) were seeded into 12-well plates

and treated with 37.5 µM BupM-NH₂ (in solution or in PEG-liposomes). After 3, 6, 12, 24, 36 h, the cells were pelleted. To extract BupM-NH₂, 40 % methanol with 0.1 % formic acid was added to the cells, which were lysed by sonication. Lysates were centrifuged and supernatants were collected and concentrated [27]. The residues were dissolved in methanol and injected (10 µl) in an LC-MS apparatus for BupM-NH₂ quantification. An Ultimate 3000 UPLC coupled to a Q Exactive Orbitrap high-resolution mass spectrometry (Thermo Fisher Scientific, Milan, Italy) was used. A Luna reverse phase C₁₈ column (Phenomenex, Torrance, CA, US) and a 0.1 % formic acid/acetonitrile mobile phase, with a gradient from 30 % to 100 % of acetonitrile in 15 min, was used.

Full MS data were acquired in positive ion mode. A precursor ion at 476.5 *m/z*, corresponding to the protonated ion of BupM-NH₂ [M+H]⁺, was used for quantification. Data were processed with Xcalibur software v. 2.2 (Thermo Fisher Scientific).

2.9. Statistical analysis

Data are presented as means ± Standard Deviation or Standard Error of Measurement (SEM). Statistically significant differences ($p < 0.05$) were determined by one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test or by unpaired, two-tailed *t*-test, using Prism software v. 8.4.2 (GraphPad, Boston, MA, US).

3. Results and discussion

3.1. Vesicles' production and characterization

This study aimed to develop an efficient formulation for a novel synthetic drug, BupM-NH₂, that exhibited toxicity in liver cancer cells. To this end, PEGylated liposomes were produced, and a thorough characterization was carried out to establish key characteristics of the vesicles and their efficacy in enhancing the bioactivity of the drug *in vitro*. To assess the effect of BupM-NH₂ on the vesicles' characteristics, empty PEG-liposomes were produced for a proper comparison. Table 1 summarizes light scattering results. Empty PEG-liposomes were characterized by small size (77 nm), high homogeneity (0.22 of polydispersity index), and slightly positive zeta potential (+3 mV). The loading of BupM-NH₂ induced a statistically significant increase in size (82 nm; $p < 0.05$) and zeta potential (+4 mV; $p < 0.01$). These findings suggest a modification of the arrangement of the phospholipids to accommodate BupM-NH₂, which did not negatively affect the polydispersity index, leading to equally homogenous systems.

The impact of PEGylation on the liposomes was assessed by comparison with non-PEGylated liposomes, empty or loaded with BupM-NH₂. Data in Table 1 show that the non-PEGylated liposomes, regardless of the loading of BupM-NH₂, were smaller (~70 nm; $p < 0.05$) than PEG-liposomes. Hence, the PEGylation induced a statistically significant enlargement of the liposomes, which would suggest the arrangement of the PEG chains on the vesicles' surface. On the other hand, the effect of

Table 1

Characteristics of empty and BupM-NH₂ PEG-liposomes and non-PEGylated liposomes: mean diameter (MD), polydispersity index (PI), and zeta potential (ZP), and entrapment efficiency (E). Each value represents the mean ± SD ($n > 10$). * and **: values statistically different ($p < 0.05$ and $p < 0.01$, respectively) from empty PEG-liposomes; ° and °: values statistically different ($p < 0.05$ and $p < 0.01$, respectively) from BupM-NH₂ PEG-liposomes and empty PEG-liposomes, respectively.

	MD nm ± SD	PI ± SD	ZP mV ± SD	E% ± SD
Empty PEG-liposomes	77 ± 5.9	0.22 ± 0.02	+3 ± 0.2	
BupM-NH ₂ PEG-liposomes	*82 ± 6.9	0.24 ± 0.02	**+4 ± 0.8	83 ± 5.8
Empty liposomes	°67 ± 2.8	0.30 ± 0.05	+3 ± 0.2	
BupM-NH ₂ liposomes	°72 ± 3.6	0.26 ± 0.01	+4 ± 0.6	77 ± 5.3

PEGylation on the other two examined parameters (i.e., polydispersity index and zeta potential) was negligible ($p > 0.05$).

The BupM-NH₂ PEG-liposomes demonstrated a good loading ability, as indicated by the high entrapment efficiency ($83 \% \pm 5.8$), which was not statistically different from the value found for non-PEGylated liposomes (77 ± 5.3 ; $p > 0.05$).

Cryo-TEM micrographs of BupM-NH₂ PEG-liposomes (Fig. 2) displayed spherical, unilamellar structures below 100 nm in size, in alignment with light scattering data (Table 1).

The stability of liposomal formulations, which depends on both composition and manufacturing method, is essential for a safe and effective use. Hence, the PEG-liposomes were stored for 60 days and their size, homogeneity, and charge were measured. The PEG-liposomes showed no evidence of relevant physical modification, as indicated by the values of the above parameters, which changed slightly after 60 days of storage (mean diameter $96 \text{ nm} \pm 0.6$, 0.25 ± 0.01 of polydispersity index, zeta potential $+4 \text{ mV} \pm 0.8$).

The stability of the BupM-NH₂ PEG-liposomes was also assessed in simulated body fluids. The vesicles remained unaltered upon incubation with both Hank's solution and m-SBF. The average diameter and polydispersity index did not vary from the starting t_0 values ($\sim 82 \text{ nm}$ and ~ 0.24 , respectively; Table 2), and from the values reported in Table 1. This indicates that the vesicles' structure was preserved despite the osmotic stress caused by the high ions' concentration in the simulated media. The zeta potential values approached neutrality in Hank's solution (Table 2), while they were unchanged in m-SBF. This is due to the salt composition of the fluids, whose ions are adsorbed on the vesicles' surface.

Finally, the ability of the PEG-liposomes to release BupM-NH₂ was assessed in saline over 48 h. The release profile presented in Fig. 3 shows a faster trend in the first 2 h, leading to a percentage of $\sim 37 \%$ of drug released, then progressing at a slower rate that led to a final percentage of $\sim 65 \%$ of drug released. Hence, the PEG-liposomes modulated the release of BupM-NH₂ providing an initial burst release, followed by a sustained release.

3.2. Cell viability assessment

A dose-dependent cytotoxic effect of BupM-NH₂ was previously

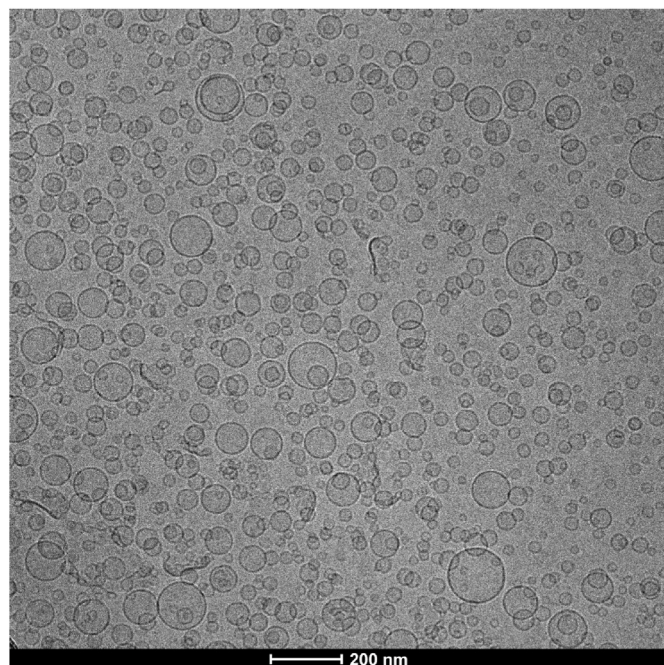


Fig. 2. Cryo-TEM micrograph of BupM-NH₂ PEG-liposomes.

Table 2

Mean diameter (MD), polydispersity index (PI) and zeta potential (ZP) of BupM-NH₂ PEG-liposomes diluted and incubated with simulated body fluids (Hank's solution and m-SBF, pH 7.4) for 24 h, at 37°C . The measurements were carried out immediately after dilution (t_0) and after 24 h (t_{24}) of incubation. Mean values $\pm$ SD are reported ($n = 6$).

	Simulated body fluid	Time	MD (nm $\pm$ SD)	PI $\pm$ SD	ZP (mV $\pm$ SD)
BupM-NH ₂ PEG-liposomes	Hank's solution	t_0	83 ± 9.9	0.24 ± 0.02	-0.1 ± 0.8
		t_{24}	90 ± 1.1	0.24 ± 0.01	-0.9 ± 0.7
BupM-NH ₂ PEG-liposomes	m-SBF	t_0	82 ± 2.7	0.22 ± 0.02	$+3 \pm 0.9$
		t_{24}	81 ± 2.5	0.23 ± 0.01	$+4 \pm 0.6$

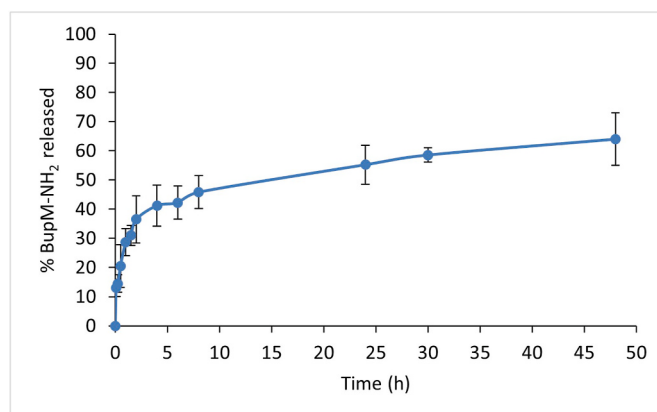


Fig. 3. BupM-NH₂ released from PEG-liposomes during 48 h in saline. Error bars represent standard deviations, $n = 4$.

demonstrated in three liver cancer cell lines (i.e., HepG2, HuH7 and JHH6), while an attenuated effect was observed in non-cancerous IHH cell line [21]. This highlights the selective nature of BupM-NH₂'s action on cancer cells, a desirable feature for reducing side effects in clinical settings. In the present study, the cytotoxicity of a liposomal formulation of BupM-NH₂ was investigated in HepG2 (cancerous) and IHH (non-cancerous) cells, aiming to evaluate the potential enhancement of the drug's pharmacological properties through nanoformulation. As shown in Fig. 4, BupM-NH₂ PEG-liposomes exhibited dose-dependent toxicity in HepG2 cells, with a significantly stronger effect compared to IHH cells. In contrast, empty PEG-liposomes showed minimal toxicity in both cell lines, with IC_{50} values above $200 \mu\text{M}$, indicating that the liposomal formulation itself did not induce significant cytotoxicity. This observation is consistent with previous studies on liposomal formulations, which often show low toxicity when liposomes do not contain active ingredients [28,29]. Specifically, the greater toxicity of nanoformulated BupM-NH₂ compared to the free molecule is evident from the IC_{50} values. The IC_{50} value for the liposomal formulation (BupM-NH₂-L) in HepG2 was $33.31 \mu\text{M}$, while it was $57.05 \mu\text{M}$ for the free drug (BupM-NH₂) [21], which validates the hypothesized superior efficacy of the nanoformulated drug. This increased cytotoxicity is likely due to the improved pharmacokinetic and pharmacodynamic properties of the drug when administered via a liposomal carrier. Liposomes can enhance intracellular drug uptake through endocytosis, thereby increasing the drug's bioavailability at the site of action [10,30].

Moreover, the IC_{50} values measured after 48 h of exposure were consistent with those observed after 24 h. The IC_{50} values were $29.45 \mu\text{M}$ for the nanoformulated drug and $44.25 \mu\text{M}$ for the free drug, not significantly different from the values obtained at 24 h, suggesting that the liposomal formulation provides sustained release and prolonged

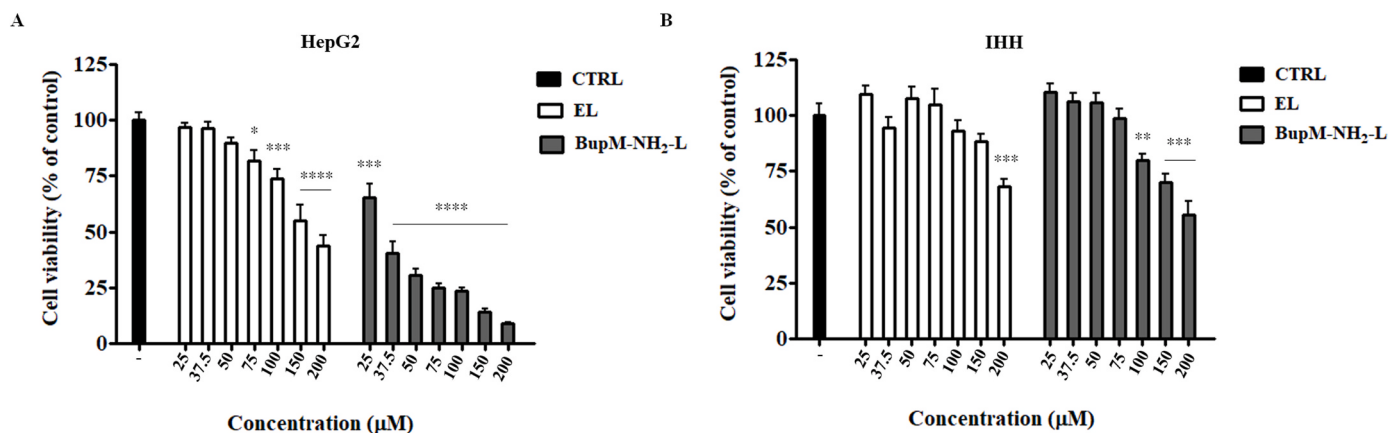


Fig. 4. Cytotoxic activity of BupM-NH₂ PEG-liposomes (BupM-NH₂-L) evaluated by the MTT assay. (A) Hepatoma cells (HepG2) and (B) normal hepatocytes (IHH) were exposed to BupM-NH₂ PEG-liposomes (BupM-NH₂-L) or empty PEG-liposomes (EL) for 24 h. Data are shown as means $\pm$ SEM of three experiments, each performed in triplicate. * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001 as determined by ANOVA followed by Dunnett's post hoc test.

action of the drug within the cells. This is in alignment with the intracellular intake of BupM-NH₂ discussed in Section 3.4. These results are consistent with other studies showing that liposomal formulations can improve drug action duration, maintaining or enhancing their effectiveness [31].

In non-cancerous IHH cells, the IC₅₀ value was >200 μ M for the nanoformulated BupM-NH₂, while it was 106.9 μ M for the free drug. These values were markedly higher than those found in HepG2 cancerous cells, which suggests that BupM-NH₂ may have selective toxicity, preferentially targeting cancer cells while sparing normal cells. This selectivity is a highly desired feature in cancer therapies, as it reduces side effects commonly associated with conventional chemotherapy drugs [11,32,33].

The results of this study validate the hypothesis that a liposomal formulation of BupM-NH₂ is more pharmacologically active than the free drug, offering a promising strategy to increase the therapeutic index of this novel compound. The greater cytotoxicity observed in HepG2 cells, along with the reduced toxicity in non-cancerous IHH cells, suggests that liposomal incorporation not only enhances the drug's potency, but also contributes to its selective action against cancer cells.

3.3. Cell morphology analysis

HepG2 and IHH cells were exposed to BupM-NH₂ PEG-liposomes (25, 37.5, 50 and 100 μ M) for 24 h and cell morphology was studied. As shown in Fig. 5, several dose-dependent alterations were detected in

HepG2-treated cells, including cell size decrease, cell shrinkage detachment, rounded morphology, and intracellular vacuolation. In contrast, the nanoformulation did not compromise IHH cell morphology, in alignment with cell viability data.

3.4. Intracellular intake of BupM-NH₂

To evaluate the cellular intake of BupM-NH₂, LC-MS analyses were performed. HepG2 cells were incubated with 37.5 μ M BupM-NH₂ in its free form (i.e., solution) or in the liposomal formulation for 36 h. The BupM-NH₂ intake increased in a time-dependent manner, especially when delivered by the PEG-liposomes. Apparently, the PEG-liposomes facilitated the transport of BupM-NH₂ across the cell membrane, as demonstrated by a 2-fold higher amount detected in the cytoplasm at 24 h (Fig. 6). It is noteworthy that the BupM-NH₂ intake did not increase after 36 h of exposure of cells to its liposomal formulation. On the other hand, a time-dependent increase in BupM-NH₂ intake was detected upon exposure to its free form, yet significantly lower than that obtained with its liposomal formulation (Fig. 6). This controlled and enhanced delivery and consequent intracellular accumulation of BupM-NH₂ can reasonably be held responsible for the greater cytotoxicity observed upon exposure of HepG2 cells to the nanoformulations as compared to the free drug in solution. These results make plausible the idea that, the prepared PEG-liposomes can efficiently carry BupM-NH₂ and potentiate its antitumor activity in hepatocarcinoma cells. This aligns with previous findings concluding that liposomes can significantly enhance the cytotoxic

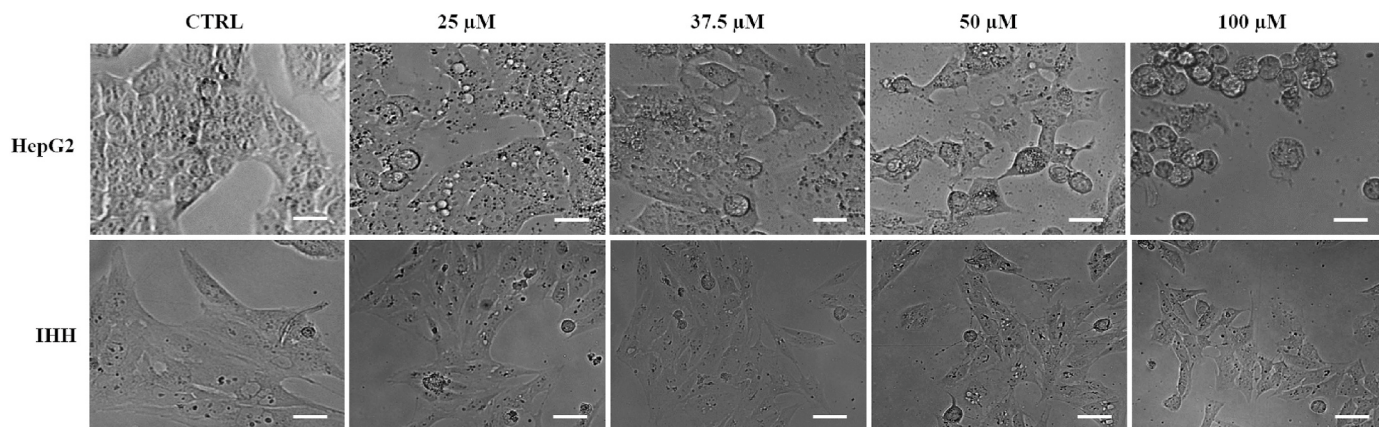


Fig. 5. Representative images of HepG2 and IHH cells following exposure to increasing concentrations of BupM-NH₂ PEG-liposomes for 24 h. Magnification: 40 $\times$; scale bar: 100 μ m.

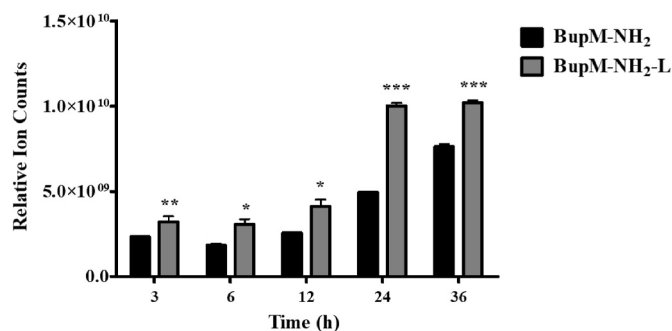


Fig. 6. Intracellular intake of BupM-NH₂. HepG2 cells were treated with 37.5 μ M BupM-NH₂, in solution or in PEG-liposomes (BupM-NH₂-L) for 3, 6, 12, 24 and 36 h. The amounts of BupM-NH₂ accumulated in the cytoplasm are presented as areas of the chromatographic peak (means of three replicates $\pm$ standard deviation). Significant differences between the amount of BupM-NH₂ within cells upon treatment with its solution or its liposomal formulation, measured for each tested time, were evaluated by unpaired, two-tailed *t*-test (**p* < 0.05, ***p* < 0.01, ****p* < 0.001).

activity of conventional chemotherapeutic agents (e.g., docetaxel, doxorubicin, and 5-fluorouracil) [34–36] or phytochemicals (e.g., catechin and resveratrol) [37,38], alone or in combination with microwave ablation or radiotherapy [39,40].

4. Conclusions

PEG-liposomes were demonstrated to be an efficient platform for the incorporation and delivery of a novel aminohydroxy sulfonamide, BupM-NH₂. The BupM-NH₂ PEG-liposomes were small in size, stable in storage and in biological milieus, and capable of facilitating the interaction with liver cancer cells, modulating the release and enhancing the bioavailability of BupM-NH₂, thus potentiating its cytotoxicity.

Therefore, the proposed nanoformulation might responds to the need of high quality, safe and effective alternative treatment options for liver cancer. Surely, this nanotechnological approach needs further investigation to corroborate the *in vitro* potential, for instance by conducting *in vivo* tests to assess the biodistribution of the BupM-NH₂ PEG-liposomes and the targeting of the tumor.

CRedit authorship contribution statement

Carla Caddeo: Writing – review & editing, Writing – original draft, Investigation, Conceptualization. **Ilaria Nigro:** Writing – review & editing, Writing – original draft, Investigation. **Lucia Chiummiento:** Investigation. **Maria Funicello:** Investigation. **Paolo Lupattelli:** Investigation. **Alessandro Santarsiere:** Investigation. **Xavier Fernández-Busquets:** Investigation. **Donatella Valenti:** Investigation. **Emanuele Rosa:** Investigation. **Rocchina Miglionico:** Writing – review & editing, Writing – original draft, Investigation. **Maria Francesca Armentano:** Writing – review & editing, Writing – original draft, Conceptualization. **Antonio Vassallo:** Validation, Investigation.

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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