Chem. Pharm. Bull.

Regular Articles

Enhancing Anticancer Potency of a 13-Substituted Berberine Derivative with Cationic Liposomes
Nuttapon Apiratikul, a Kanlayanee Sriklung, b Kulvadee Dolsophon, a Pattamaporn Thamvapee, b Ramida Watanapokasin, b Boonek Yingyongnarongkul, c Nattisa Niyomtham, d John B. Bremner, e Petcharat Watanavetch, a and Siritron Samosorn*, a

a Department of Chemistry and Center of Excellence for Innovation in Chemistry, Faculty of Science, Srinakharinwirot University, Bangkok 10110, Thailand:
b Department of Biochemistry, Faculty of Medicine, Srinakharinwirot University, Bangkok 10110, Thailand:
c Department of Chemistry and Center of Excellence for Innovation in Chemistry, Faculty of Science, Ramkhamhaeng University, Bangkok 10240, Thailand:
d International College of Dentistry, Walailak University, Nakhon Si Thammarat 80161, Thailand:

Corresponding author: Siritron Samosorn*
Mailing address: Department of Chemistry and Center of Excellence for Innovation in Chemistry, Faculty of Science, Srinakharinwirot University, Bangkok 10110, Thailand
E-mail: siritron@g.swu.ac.th
Summary

Cationic liposomal formulations of the telomeric G-quadruplex stabilizing ligand, 13-(2-naphthylmethoxy)berberine bromide (1), have been developed with the purpose of delivering 1 into the nucleus of cancer cells for potential telomere targeting. Berberine derivative 1 was encapsulated in various cationic lipids 2-4 by the thin film evaporation method; these lipids are cationic after amine protonation. The most appropriate liposomal berberine formulation was that of 1 and the cholesterol derived cationic lipid 4 in a weight ratio of 1:20 with 76.5% encapsulation efficiency of 1. Cellular uptake studies in the HeLa and HT-29 cancer cells line showed that the liposomal berberine derivative uptake in the cells was higher and more stable than for berberine derivative 1 alone while free 1 was completely decomposed in the cells within 60 min exposure to the cells. Anticancer activity of the liposomal berberine derivative 1 based on 4 was greater than that for the free berberine derivative 1 in the MCF-7, HeLa and HT-29 cell line by 2.3-, 4.9- and 5.3-fold, respectively, and also, interestingly, superior to the anticancer drug doxorubicin against the HT29 cancer cell line.

Keywords berberine derivatives; cancer; cationic lipid; encapsulation; telomeres
Introduction

Telomeres are pieces of DNA that are thought to cap the end of chromosomes and protect them from damage.¹ In most cells, successive rounds of DNA replication cause telomeres to be shortened since replication at the ends of short pieces of single-stranded (ss) DNA is inefficient.² When telomeres reach a minimum size it is a signal for the cell to enter senescence and it is targeted for cell death. This is part of the normal aging process of somatic cells.³ However, in many, though not all cancer cells, telomere length is maintained, either by the enzyme telomerase (~85% of cancers) or by another mechanism called Alternative Lengthening of Telomeres (ALT, ~15% of cancers).⁴ After these occurrences, cancer cell growth is uncontrolled. Telomerase can interact with guanine-rich sequences in telomeres to extend the telomere length. Guanine (G)-rich sequences are found at the ends of telomeres and in the promoter regions of proto-oncogenes. These sequences have the ability to form G-quadruplex structures preventing the interaction with telomerase or changing the regulation of cancer genes and causing the death of cancer cells.⁷⁻⁹ This suggests that compounds that stabilize G-quadruplex DNA structures may interrupt key biological processes leading to cancer cell death, eventually. It should be noted however that the situation with regard to telomerase inhibition by G-quadruplex DNA ligands has been re-evaluated and many may not be simple telomerase inhibitors.¹⁰⁻¹¹

A promising berberine-derived anticancer agent, 13-(2-naphthylmethoxy)berberine bromide (1), was developed by us¹² and has been reported to have selective interaction with model G-quadruplex (G4) DNA structures over duplex DNA analogues,¹¹ as well as partially inhibiting telomerase unfolding and extension of an intramolecular parallel G4.⁶ These selectivities are greater than that for the anticancer agent berberine itself.¹³⁻¹⁵ Interestingly, compound 1, which causes cell cycle arrest, is selectively toxic to cancer cells over normal (Vero) cells, being essentially non-toxic to the latter. It can go into the cell nucleus where telomeres are located if telomerase or ALT
inhibitory activities were responsible for the anti-cancer cell activity. Unfortunately, compound 1 was completely decomposed after uptake into the cell nucleus within 1 hour. Additionally, other issues associated with the derivative 1 are that it is poorly soluble in aqueous solution while it is soluble in other organic solvents and decomposition has been shown to occur. In dichloromethane (DCM) 1 is completely decomposed within 12 h at room temperature and air exposure to give the non-anticancer compound 13-hydroxyberberine \{Table 1; MCF-7 cell line \textit{in vitro}; no antitumor activity (sarcoma-180 ascites) \textit{in vivo} in mice\}\cite{16} and 2-naphthaldehyde, while in DMSO it is partially decomposed (28%) within the same time. Therefore, the efficacy of compound 1 is most likely compromised by difficulties in effective delivery because of aqueous insolubility and stability issues amongst other factors. In order to address these issues, a study was undertaken to assess whether incorporation of 1 in suitable liposomes could increase cancer cell cytotoxicity potencies while still maintaining the selectivity profile.

Liposomes are lipid vesicles, which are formed when lipids are hydrated in water and have spherical bilayer structures that can entrap small molecule drugs.\cite{17} The advantage of liposomes as drug delivery systems in cancer treatment is that newly formed blood vessels are fragile and highly permeable compared with pre-existing vessels, thus macromolecules such as liposomes are known to passively accumulate in cancer tissue as a reflection of this feature. This results in an enhanced anticancer effect of liposomally delivered cancer agents compared to free drug; applications of this with anti-cancer compounds include, for example, the alkaloid berberine itself encapsulated in liposomes from non-cationic neutral lipids \cite{18-23} or in combination with other drugs\cite{19} or in liposomes with, for instance, Tariquidar, an inhibitor of the permeability glycoprotein (P-gp) drug efflux pump, and enabling good potency \textit{in vitro} against a doxorubicin-resistant cell line (K562).\cite{24} The small molecule telomerase inhibitor, rapamycin, has also been delivered in neutral liposomes for enhancement of the effect of the chemotherapeutic anti-cancer agent 5-fluorouracil.\cite{25} Other advantages of liposomes include increased stability of drugs via encapsulation, biocompatibility,
biodegradability, and non-immunogenicity for systemic and non-systemic administration.\textsuperscript{26}

Cationic liposomes are known to be efficient cellular drug delivery entities as a result of electrostatic forces between positive charges of the liposomes (from protonation of amino groups in the lipids) and negative charges of cell membranes and cell surface proteoglycans facilitating cellular uptake.\textsuperscript{27} Cancer cell surfaces are more negatively charged than those of normal cells as a result of the overexpression of phosphatidylcholines and sialoglycolipids in the outer leaflet of the lipid bilayer in the former.\textsuperscript{28-29} Novel hybrid cationic liposomes (which include phospholipid and polyethyleneglycol surfactants as well as cationic lipid) target negatively charged cell membranes in pancreatic cancer cells.\textsuperscript{30} Liposomes from polyamines are also of interest as a part of systems for gene therapy,\textsuperscript{31} while lipid nanoparticles based on ionizable cationic lipids offer further opportunities for the efficient delivery of macromolecules.\textsuperscript{32} Cationic liposomes have been revealed to selectively accumulate in tumor endothelial cells, and have been used to encapsulate the anticancer drug, paclitaxel, to overcome its aqueous insolubility and also to improve anticancer efficacy.\textsuperscript{33-34} In addition, liposomes modified with cationic polymethacrylate have been shown to markedly improve the delivery of doxorubicin as well as it’s antitumor activity.\textsuperscript{35} Cationic liposomes have also been reported as drug carriers for bioactive natural products, such as curcumin, to increase cellular uptake in cancer cells.\textsuperscript{36} Therefore, cationic liposome encapsulation is a promising tool to improve bioavailability and to selectively deliver drugs to tumor cells.\textsuperscript{37-38} However, as far as can be ascertained from the literature, no previous work has been undertaken on berberine or substituted berberine derivatives encapsulated in cationic liposomes with regard to enhancing delivery and anti-cancer activity characteristics.

In the present study, the novel non-toxic spermine-based and cholesterol-based (with a part-spermine related polar sidechain) cationic lipid precursors \textbf{2-4} (structures are shown in Fig. 1 together with their acronyms from two of the synthesis papers)\textsuperscript{36, 39-40} were used for the encapsulation of our berberine derivative \textbf{1} and the results with regard to anti-cancer cell potency are
Results and discussion

Cytotoxicities of liposomal berberine derivative 1, and empty cationic liposomes derived from 2-4.

In order to determine an appropriate type of liposome for encapsulation of the berberine derivative 1, cytotoxicities of 1 encapsulated in liposomes derived from the precursor lipids 2-4 (plus the neutral lipid dioleoyl-L-α-phosphatidylethanolamine\textsuperscript{41} – DOPE), and the empty cationic liposomes from 2-4 were tested against the breast cancer cell line MCF-7. The concentration of 1 was 8.59 μM (IC\textsubscript{50} of berberine derivative 1 against the MCF-7 cell line) in each liposomal formulation, and 0.5% DMSO in water (v/v) was used as a control. Values represent the means ± SDs of three independent experiments. Concentration ratios of 1 to each lipid at 1:10, 1:20, 1:30, and 1:40 wt/wt were prepared. Empty liposomes were also tested for cytotoxicity comparison. The concentration of empty liposomes was the same as in each liposomal berberine derivative 1 formulation. Results are shown in Fig. 2. The high concentration ratio at 1:20 of the 1/lipid 4 formulation was the most toxic to the tested cell while empty liposome from 4 at the same concentration was non-toxic to the tested cell. Lipid 4 was thus used for the subsequent encapsulated product testing.

Berberine derivative 1 encapsulation efficiency

In this study, four different weight ratios of berberine derivative 1 to lipid 4 were made, and their encapsulation efficiencies were determined using a standard plot (UV/VIS) of berberine derivative 1 as a reference (y = 0.0319x - 0.0152, R\textsuperscript{2} = 1). The amounts of berberine derivative 1 in the respective liposomal berberine derivative 1 formulations at the ratios of 1:10, 1:20, 1:30, and 1:40 were found to be 40.8, 76.5, 51.0, and 29.9 % wt/wt, respectively. The formulation containing a
1:20 weight ratio of berberine derivative 1 showed the highest encapsulation efficiency. Consequently, this ratio was selected for use in the cellular uptake and cytotoxic activity testing.

**Stability study**

Stability tests of berberine derivative 1 and liposomal berberine derivative 1 from lipid precursor 4 were undertaken using high performance liquid chromatography-mass spectrometry (HPLC-MS) based on the peak area, which showed that the solution of berberine derivative 1 in PBS was degraded more than 50 percent within 1 h and up to 99 percent at 3 h. This indicates that berberine derivative 1 was unstable in PBS buffer at 37°C, whereas liposome encapsulation completely protected compound 1 in the lipid bilayer for the 24 h period. After 24 h, compound 1 was gradually degraded under prolonged exposure in PBS to 70 percent at 168 h (Fig 3).

**Cytotoxic activity of berberine derivative 1.**

The cytotoxic activity of free berberine derivative 1, empty liposome derived from lipid precursor 4, and liposomal berberine derivative 1 from lipid precursor 4 were evaluated against three human cancer cell lines: MCF-7, HeLa, and HT-29; in all these cell types telomerase is expressed or upregulated. Since free berberine derivative 1 and empty liposome 4 were non-toxic to normal cells (Vero) it was assumed the cytotoxicity of liposomal berberine derivative 1 might be non-toxic as well. Consequently, the cytotoxicity assessment of liposomal berberine derivative 1 in the Vero cell line was not conducted.

The cytotoxic activity results are shown in Fig. 4 and summarized in Table 1. IC₅₀ values of the empty liposome from 4 were over 85.25 μM for all tested cell lines, indicating it might be suitable and safe for use as a drug delivery system. Cytotoxic activity of liposomal berberine derivative 1 was up to 2.3-5.3 fold more potent than free berberine derivative 1 against MCF-7, HT29, and HeLa cells with IC₅₀ values of 3.79 ± 0.06, 0.87 ± 0.001 and 2.17 ± 0.49 μM, respectively. The enhanced
cytotoxic activity of liposomal berberine derivative 1 was likely associated with the capability of the liposome from 4 to increase cellular uptake and stability of the berberine derivative 1 in the cells. Moreover, the liposomal berberine derivative 1 was 6.3-fold more active than the anticancer drug, doxorubicin, against the HT-29 cell line.

**Cellular uptake of berberine derivative 1 in HeLa cells and HT-29**

The liposomal berberine derivative 1 and berberine derivative 1 itself were able to enter the cells after 15 min and 30 min exposure respectively. The fluorescence intensity of liposomal berberine derivative 1 increased upon increasing exposure time, whereas that of 1 itself disappeared after 60 min (Fig. 5). This was probably due to metabolic (or possibly non-enzymatic) transformation of the free berberine derivative 1 in the cells, together with some precipitation of 1 or its metabolites in the cells due to poor solubility in aqueous solution. A precipitate could be seen under a fluorescence microscope. It was seen that the cationic liposome from 4 was efficient in delivering berberine derivative 1 to the tested cells by enhancing cellular uptake, and protecting 1 from degradation, as well as gradually releasing 1 in the cells.

The cellular uptake of liposomal (from the lipid precursor 4) and free berberine derivative 1 into HeLa cells was observed under a fluorescence microscope and the results are shown in Fig. 5. The cationic liposome demonstrated enhanced cellular uptake of berberine derivative 1 into the tested cells. This might be due to the cationic charge of the liposome from the protonation of the basic groups at physiological pH, which can induce favorable electrostatic interactions with the negatively charged cell membrane and facilitate cellular uptake.\(^{29,45}\) Enhanced cellular uptake of a double-stranded RNA (dsRNA) with a dsRNA/cationic liposome complex with the liposome derived from 4, has also been seen in shrimp cells.\(^{40}\)

To support the facilitated uptake rationale, the zeta potentials and particle sizes of the cationic liposome 4 and cationic liposome encapsulated compound 1 with the lipid 4 were investigated. The dynamic light scattering assay gave an average particle size for the cationic liposome 4 itself of 291.9
± 5.1 nm, while with the berberine derivative 1 encapsulated (weight ratio of 20:1) the average particle size was found to be approximately 352.1 ± 22.0 nm. This increase in size is consistent with encapsulation but still in a range suitable for drug delivery.\(^{38}\) The zeta potential is a measure of the electrical field of cationic liposomes in an aqueous environment. It was found that the zeta potential of the liposome from 4 was 53.9 mV. The zeta potential of the cationic liposome berberine derivative 1 complex was found to be 54.4 mV. This highly positive value in the complex would facilitate strong electrostatic interactions with the usually negatively charged cell membrane and enhance cellular uptake.

Further studies were also undertaken on translocation of the liposomal berberine derivative complex from the cytoplasm to the nucleus in HT-29 cells, a cell line in which the complex displayed particularly potent cytotoxicity (Table 1). These studies involved fluorescence microscopy and selective DNA staining with Hoechst 33342 and the resultant images are shown in Fig. 6. From these images significantly greater fluorescence intensity could be seen after incubation for 15 min. with the liposomal berberine derivative 1 compared with the berberine derivative 1 alone, whereas no fluorescence was seen for the empty cationic liposome 4 and the control. It was not possible to assign any specific interaction of 1 with G-quadruplex motifs \textit{in vivo} and further detailed study would be needed to probe this, perhaps via the promising fluorescent probe technology reported by Zhang et al.\(^{46}\)

While MCF-7 cells are reported to also have high telomerase activity,\(^{47-49}\) unfortunately their cell morphology did not show any contrast of the fluorescence signal and this cell line was not studied further. HeLa cells could be used for the study of cellular uptake with fluorescence microscopy to distinguish time exposure in the cell, but unfortunately translocation of the complex with 1 from the cytoplasm to the nucleus could not be as clearly seen as in the HT-29 cells.
Conclusion

From the studies reported in this work, it is established that cationic liposomal encapsulated berberine derivative 1 from lipid precursor 4 affords the possibility for increasing stability, cellular uptake, nucleus permeability, and anticancer activity of 1. Very good cytotoxic potency was seen against the HT-29 cancer cell line in vitro. This encapsulated complex should also be useful in future studies to assess whether the ability of berberine derivative 1 to selectively interact with the G-quadruplex DNA structure is related to telomerase inhibition or whether some other inhibition mechanism of telomere lengthening is involved.

Experimental

Materials

Merrifield resin was obtained from ChemPep, Inc (Miami, FL, USA). Berberine (chloride salt) and reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA). All chemicals were used as supplied. Berberine derivative 1 and lipids 2-4 were synthesized according to previous methods.\(^{12,36,39,50}\) The absorbance of the berberine derivative 1 was measured at 349.5 nm using a Shimadzu UV-2450 UV-Visible spectrophotometer (Japan) for a standard curve. The efficiency of cellular uptake was observed using an inverted fluorescence microscope (IX73 P1F, Olympus, Essex, United Kingdom).

Preparation of liposomal berberine derivative 1

Liposomes were prepared in an equivalent weight ratio of cationic lipid 2 (3 or 4) and dioleoyl-L-α-phosphatidylethanolamine (DOPE); different total lipid/berberine derivative 1 ratios were used. A solution of berberine derivative 1 in EtOH (1 μg/μL) and lipid in DCM (1 μg/μL) were mixed in an Eppendorf tube, and then the organic solvents were evaporated under a stream of nitrogen and the residue was dried under high vacuum for 2 h. The resulting thin film was hydrated with phosphate
buffer saline (PBS, pH 7.4, 20 μL) at room temperature. The mixture was then sonicated for 30 min; the concentration of the berberine derivative 1 in each case was the same. The resulting liposomal berberine derivative 1 was used immediately. A solution of free berberine derivative 1 was prepared by dissolving in DMSO, then diluting with distilled water to a final concentration of DMSO of 0.5% (v/v).

**Zeta potential and size measurements**

Malvern Zetasizer Nano ZS (Malvern Instruments Ltd., Malvern, U.K.) was used to determine the particle size and surface charge of the cationic liposomes and cationic liposome encapsulated berberine derivative 1. The empty cationic liposome from 4 and cationic liposome encapsulated berberine derivative 1 at a weight ratio of 20:1 were diluted with distilled water that had been filtered through a 0.22 μm membrane filter to meet instrumental sensitivity requirements. All measurements were performed in triplicate and carried out at room temperature.

**Berberine derivative 1 encapsulation efficiency**

The berberine derivative 1 content encapsulated in the cationic liposome from 4 was determined using the absorbance standard curve and with 1 as a standard. The thin film of berberine derivative 1 and cationic lipid 4 in the weight ratios of 1:10, 1:20, 1:30, and 1:40 were hydrated with PBS at room temperature. The mixture was sonicated for 30 min. The supernatant containing the liposomal berberine derivative 1 complex was separated from insoluble unencapsulated berberine derivative 1 by centrifugation at 3000 rpm for 5 min on an Eppendorf miniSpin. The absorbance of the supernatant was measured immediately at 349.5 nm using a Shimadzu UV-2450 UV-Visible spectrophotometer.
Stability study

The stability of berberine derivative 1 and of liposomal berberine derivative 1 from lipid precursor 4 (both in PBS buffer solution pH 7.4 at 37 °C) were measured for 7 days by HPLC-MS (Thermo Scientific Dione Ultimate 3000 Ultra High Performance Liquid Chromatography-Bruker micrOTOF II). The HPLC column was a C18 (3 μm particle, 2.1 mm ×150 mm) and acetonitrile/water/0.1 % formic acid was used as the mobile phase. Gradient elution at a flow rate of 0.2 ml/min was used and sample injection volumes were 2 μL. Sample solutions were prepared at a final concentration of 1 mg/mL for 1, and 1 : lipid 4 in a ratio of 1:20 mg/mL for liposomal 1; solutions were injected for HPLC-MS without further dilution. Experiments were done in triplicate.51)

Cell culture

The cancer cell lines used in this study were: Breast adenocarcinoma (MCF-7), Cervical epithelial adenocarcinoma (HeLa), and Colon Adenocarcinoma (HT-29) all being obtained from the American Type Culture Collection (ATCC, Manassas, VA). Cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO2.

Cytotoxicity assays

MCF-7, HeLa, and HT-29 cancer cell lines were used in this study. To determine cell cytotoxicity/viability, the cells were plated at a density of 1 × 10⁵ cells per well. Cytotoxicities of the derivative 1, cationic liposomes from 2-4, liposomal berberine derivative 1, and 13-hydroxyberberine were assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay as described previously.52) The experiment was performed in 96-well plates. The amount of cationic liposome was varied from 0.5 to 5.0 μg/well. After 24 h incubation, the medium was removed and replaced with a phenol red-free medium (90 μL). MTT (3
mg/mL) was added (10 µL/well) to the cells, followed by MTT solubilisation solution (Sigma) (100 µL) to dissolve the resulting crystals, and the absorbance was measured at 520 nm on a microplate reader. The change in metabolic activity was calculated as \( A_{520} \) with compound/\( A_{520} \) without compound. Doxorubicin and 0.5% DMSO in water (v/v) were used as a positive and negative control, respectively. Experiments were done in triplicate.

Normal cell line cytotoxicities were assessed using the African green monkey kidney cell line (Vero). The Green Fluorescent Protein (GFP)-based assay was carried out by adding 45 µL of Vero cell suspension at 3.3 x 10^4 cells/mL to each well of 96-well plates containing 5 µL of test compound solution previously diluted in 0.5% DMSO in water (v/v), and then incubating for 4 days in a 37 °C incubator with 5% CO_2. Positive and negative controls were 0.83 µg/mL ellipticine and 0.5% DMSO/H_2O, respectively. Fluorescence signals were measured by using a SpectraMax M5 microplate reader (Molecular Devices, USA) in the bottom reading mode with excitation and emission wavelengths of 485 and 535 nm.

**Cellular uptake studies**

HeLa cells and HT-29 cells were seeded in 24 well plates at a seeding density of 1 x 10^5 cells per well in 500 µL of growth medium and were allowed to attach for 24 h. For the studies of concentration-dependent uptake, cells were treated with free form berberine derivative 1, liposome and liposomal berberine derivative 1, and each were incubated for 0, 15, 30, 45 and 60 min. At each time point, the medium was removed and the cells were washed three times with PBS. Cells were fixed in 4 % formaldehyde for 15 min at room temperature and permeated with methanol at 20 °C for 10 min. The cells were stained with Hoechst 33342 (for 10-15 min in the dark) and then washed with PBS. Slides were mounted and counter-stained using Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA). Slides were visualized under a standard Compound Microscope System (IX73 P1F, Olympus, Essex, United Kingdom) with excitation/emission 361/497
nm for Hoechst and excitation/emission 420/535 nm for berberine derivative 1.

**Statistical Analysis**

All values are presented as mean ± standard deviation differences between mean values of normally distributed data as assessed by one-way Analysis of Variance (ANOVA) using GraphPad Prism version 9.10 (221) (GraphPad Software, San Diego, CA, USA). The criterion for differences was considered significant at p < 0.05.

**Acknowledgements**

We wish to thank Srinakharinwirot University, Ramkhamhaeng University and Srinakharinwirot University Research Grant No. 039/2557, Thailand for supporting this work. Support through the University of Wollongong is also gratefully acknowledged.

**Conflict of Interest**

The authors declare no conflict of interest.
References

1) Xu Y., Goldkorn A., Genes, 7, 22 (2016).
2) Watson J. D., Nat. New Biol., 239, 197-201 (1972).
3) Allsopp R. C., Harley C. B., Exp. Cell Res., 219, 130-136 (1995).
4) Dagg R. A., Pickett H. A., Neumann A. A., Napier C. E., Henson J. D., Teber E. T., Arthur J. W., Reynolds C. P., Murray J., Haber M., Sobinoff A. P., Lau L. M. S., Reddel R. R., Cell Rep., 19, 2544-2556 (2017).
5) Bryan T. M., Englezou A., Dalla-Pozza L., Dunham M. A., Reddel R. R., Nat. Med., 3, 1271-1274 (1997).
6) Paudel B. P., Moye A. L., Abou Assi H., El-Khoury R., Cohen S. B., Holien J. K., Birrento M. L., Bryan T. M., Gornall K. C., Samosorn S., Tannahill D., Pedley R. B., Evans T. R. J., Wilson W. D., Balasubramanian S., Neidle S., J. Med. Chem., 61, 2500-2517 (2018).
7) Rodriguez R., Miller K. M., Forment J. V., Bradshaw C. R., Nikan M., Britton S., Oelschlaegel T., Xu Y., Goldkorn A., Chen Y., Vanti G., Coronnello M., Henson J. D., Teber E. T., Arthur J. W., Reynolds C. P., Balasubramanian S., Neidle S., J. Med. Chem., 61, 2500-2517 (2018).
8) De Cian A., Cristofari G., Reichenbach P., Lemos E., Monchaud D., Teulade-Fichou M.-P., Shin-yak L., Lacroix L., Lingner J., Mergny J.-L., PNAS, 104, 17347-17352 (2007).
9) Birrento M. L., Bryan T. M., Samsorn S., Beck J. L., J. Am. Soc. Mass Spectrom., 26, 1165-1173 (2015).
10) Samsorn S., Tanvirat B., Muhamad N., Casadei G., Tomkiewicz D., Lewis K., Suksamrarn A., Prammanan T., Bremner J. B., Biomed. Res. Int., 2013, 1-10 (2013).
11) Gornall K. C., Samsorn S., Tanvirat B., Suksamrarn A., Bremner J. B., Kelso M. J., Beck J. L., ChemComm, 46, 6602-6604 (2010).
12) Sun Y., Xun K., Wang Y., Chen X., Anticancer Drugs, 20, 757-769 (2009).
13) Hoshi A., Ikekawa T., Ikeda Y., Shirakawa S., Iigo M., Gan, 46, 321-325 (1976).
14) Olusanya T. O. B., Haj Ahmad R. R., Ibegrhu D. M., Smith J. R., Elkardy A. A., Molecules, 23, 907 (2018).
15) Lin Y. C., Kuo J. Y., Hsu C. C., Tsai W. C., Li W. C., Yu M. C., Wen H. W., Int. J. Pharm., 441, 381-388 (2013).
16) Habtemariam S., Molecules, 25, 1426 (2020).
17) Zheng X., Wu F., Lin X., Shen L., Feng Y., Drug Deliv., 25, 398-416 (2018).
18) Haghir Alsadat B. F., Amirpour Z., Nafisi B., Iran J. Ped. Hematol. Oncol., 10, 221-229 (2020).
19) Vanti G., Coronello M., Bani D., Mannini A., Bergonzi M. C., Bilac A. R., Pharmaceutica, 13, 306 (2021).
20) Chen Y., Zhu W. T., Lin C. Y., Yuan Z. W., Li Z. H., Yan P. K., Int. J. Nanomedicine, 16, 269-281 (2021).
26) Rommazi F., Esfandiari N., *Nanoscale Res. Lett.*, **16**, 95-114 (2021).
27) Chono S., Tanino T., Seki T., Morimoto K., *J. Pharm. Pharmacol.*, **59**, 75-80 (2007).
28) Liu X., Li Y., Li Z., Lan X., Leung P. H.-m., Li J., Yang M., Ko F., Qin L., *J. Fiber Bioeng. Inform.*, **8**, 25-36 (2015).
29) Singh M., Kumar V., Sikka K., Thakur R., Harioudh M. K., Mishra D. P., Ghosh J. K., Siddiqi M. I., *J. Chem. Inf. Model.*, **60**, 332-341 (2020).
30) Motomura M., Ichihara H., Matsumoto Y., *Bioorganic Med. Chem. Lett.*, **28**, 1161-1165 (2018).
31) Puchkov P. A., Maslov M. A., *Pharmaceutics*, **13**, 920 (2021).
32) Sato Y., *Chem. Pharm. Bull.*, **69**, 1141-1159 (2021).
33) Schmitt-Sody M., Strieth S., Krasnici S., Sauer B., Schulze B., Teifel M., Michaelis U., Naujoks K., Dellian M., *Clin. Cancer Res.*, **9**, 2335-2341 (2003).
34) Thurston G., McLean J. W., Rizen M., Baluk P., Haskell A., Murphy T. J., Hanahan D., McDonald D. M., *J. Clin. Invest.*, **101**, 1401-1413 (1998).
35) Wang W., Shao A., Zhang N., Fang J., Ruan J. J., Ruan B. H., *Sci. Rep.*, **7**, 43036 (2017).
36) Apiratikul N., Penglong T., Suksen K., Svasti S., Chairoungdua A., Yingyongnarongkula B., *Russ. J. Bioorganic Chem.*, **39**, 444-450 (2013).
37) Ternullo S., Basnet P., Holsaeter A. M., Flaten G. E., de Weerd L., Škalko-Basnet N., *Eur. J. Pharm. Sci.*, **125**, 163-171 (2018).
38) Liu C., Zhang L., Zhu W., Guo R., Sun H., Chen X., Deng N., *Mol. Ther. Methods Clin. Dev.*, **18**, 751-764 (2020).
39) Niyomtham N., Apiratikul N., Suksen K., Opanasopit P., Yingyongnarongkul B. E., *Bioorg Med Chem Lett.*, **25**, 496-503 (2015).
40) Apiratikul N., Yingyongnarongkul B., Assavalapsakul W., *Aquac. Res.*, **45**, 106-112 (2014).
41) Du Z., Munye M. M., Tagalakis A. D., Manunta M. D. I., Hart S. L., *Sci. Rep.*, **4**, 7107 (2014).
42) Ivanković M., Ćukušić A., Gotić I., Škrobot N., Matijašić M., Polančec D., Rubelj I., *Biogerontology*, **8**, 163-172 (2007).
43) Jafari M. A., Ansari S. A., Alqahtani M. H., Shay J. W., *Genome Medicine.*, **8**, 69 (2016).
44) Brown T., Sigurdson E., Rogatko A., Broccoli D., *Ann. Surg. Oncol.*, **10**, 910-915 (2003).
45) Laouini A., Jaafar-Maalej C., Limayem-Blouza I., Sfar S., Charcosset C., Fessi H., *J. Colloid Sci. Biotechnol.*, **1**, 147-168 (2012).
46) Zhang, S., Sun, H., Yang, D., Liu, Y., Zhang, X., Chen, H., Li, Q., Guan, A., Tang, Y., *Anal. Chim. Acta.*, **X 2**, 100017 (2019).
47) Raymond E., Sun D., Izbicka E., Mangold G., Silvas E., Windle B., Sharma S., Soda H., Laurence R., Davidson K., Hoff D. D. V., *Br. J. Cancer.*, **80**, 1332-1341 (1999).
48) Awada Z., Nasr R., Akika R., Ghantous A., Hou L., Zgeib N. K., *Mol. Biol. Rep.*, **47**, 3541-3549 (2020).
49) Gurung R. L., Lim S. N., Low G. K., Hande M. P., *J. Nutrigenet. Nutrigenomics*, **7**, 283-298 (2014).
50) Samosorn S., Brenmer J. B., Ball A., Lewis K., *Bioorg. Med. Chem.*, **14**, 857-865 (2006).
51) Bhuket P. R. N., Wichitnithad W., Sudtanon O., Rojsitthisak P., *Heliyon*, **6**, e04561 (2020).
52) Denizot F., Lang R., *J. Immunol. Methods.*, **89**, 271-277 (1986).
53) Hunt L., Jordan M., De Jesus M., Wurm F. M., *Biotechnol. and Bioeng.*, **65**, 201-205 (1999).
Table 1 Cytotoxicity of berberine derivative 1, empty liposome from 4, liposomal berberine derivative 1 from lipid precursor 4, 13-hydroxyberberine, and anticancer drug doxorubicin against a normal cell line (Vero), and cancer cell lines: MCF-7, HT29, and HeLa.

| Compound                          | Vero   | MCF-7  | HT29   | HeLa   |
|-----------------------------------|--------|--------|--------|--------|
| Berberine derivative 1            | >87.55 | 8.59 ± 0.05 | 4.58 ± 0.53 | 10.54 ± 2.73 |
| Liposome from 4                   | >85.25 | >85.25 | >85.25 | >85.25 |
| Liposomal berberine derivative 1  | NT     | 3.79 ± 0.06 | 0.87 ± 0.001 | 2.17 ± 0.49 |
| (ratio 1:20)                      |        |        |        |        |
| 13-Hydroxyberberine               | NT     | >140.02 | NT     | NT     |
| Doxorubicin                       | NT     | 1.27 ± 0.04 | 5.50 ± 1.47 | 0.91 ± 0.06 |

NT = not tested. The highest concentration of all compounds tested was 50 µg/mL.
Figure 1. Structures of 13-(2-naphthylmethoxy)berberine bromide (1) and cationic lipid precursors: Cy (2), Dx (3) and AmGly-Chol (4).
Figure 2. Cytotoxicity of berberine derivative 1 in various liposomes at a ratio of 1:10 to 1:40, and empty liposomes from 2, 3 and 4 against the MCF-7 cell line.
Figure 3. Stability of free berberine derivative 1 (blue) and liposomal berberine derivative 1 from lipid precursor 4 (red) presented as the percentage of berberine derivative 1 remaining at each time point during 7 days incubation in PBS pH 7.4 at 37°C. Mean ± SD values (n = 3) are presented.
Figure 4. Inhibition concentration (IC$_{50}$) of berberine derivative 1, liposomal berberine derivative 1 from lipid precursor 4 and doxorubicin with MCF-7 (3A), HeLa (3B) and HT-29 (3C) cancer cell lines. Values represent the means ± SDs of three independent experiments. This figure has been generated using GraphPad, the following annotations relate to the p value when comparing between doxorubicin and liposomal berberine derivative 1: **** denotes a p value <0.0001, *** denotes a p value 0.0001 to 0.001, ** denotes a p value 0.001 to 0.01, * denotes a p value 0.01 to 0.05 and no annotation denotes a p value = >0.05.
Figure 5. Fluorescence microscopy images of HeLa cells incubated with Berberine derivative 1, Liposomal berberine derivative 1 from lipid precursor 4 (ratio 1:20) compared with Control (0.5% DMSO in culture media (v/v)) for 0, 15, 30, 45 and 60 min at 37°C. The fluorescent signal from berberine derivative 1 was visualized by fluorescence confocal microscopy (100x).
Figure 6. Fluorescence microscopy images of HT-29 cells incubated with empty Liposome 4, Berberine derivative 1 alone, and Liposomal berberine derivative 1 from lipid precursor 4 compared with Control (0.5% DMSO in culture media (v/v)) for 15 min at 37°C. The fluorescent signal from berberine derivative 1 (green color) and Hoechst 33342 was used for nuclear staining (blue color) visualized by fluorescence confocal microscopy (200x).