Uroguanylin-decorated Nanosystems Containing Etoposide, a Potential Targeted Combination Therapy for Colorectal Cancer

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Research

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Abstract

Colorectal cancer is the third most frequently diagnosed cancer malignancy and the second leading cause of cancer-related deaths worldwide. Guanylyl Cyclase C (GCC), a membrane receptor that is expressed in 95% of primary and metastatic colorectal cancer tumors is a promising target for the design of new drug delivery strategies. In this work we propose the use of Uroguanylin (UroG), a natural ligand for GCC, for the preparation of targeted sphingomyelin-based nanosystems (UroG-SNs). The results from in vitro cell culture studies showed that UroG-SNs have a specific interaction with the GCC receptor, resulting in an antiproliferative response. Subsequently, the anti-cancer drug etoposide (Etp) was encapsulated into the UroG-SNs. The evaluation of the resulting system showed a synergistic effect of the two drugs, thus highlighting the therapeutic potential of this strategy for the treatment of colorectal cancer.

Background

According to World Health Organization (WHO), cancer causes more deaths than all heart diseases or strokes [1]. Colorectal cancer (CRC) is the third most frequently diagnosed cancer malignancy and the second leading cause of cancer-related deaths in the world, causing approximately 10% of deaths with an increase of over 20 million new cancer cases expected annually by 2025 [2]. Moreover, the presence of local or distant metastasis remains the leading cause of death among cancer patients, with an overall mortality above 50%. These facts emphasize an unmet clinical need for the effective targeting of colorectal cancer metastasis [3]. Guanylyl Cyclase C receptor (commonly referred as GCC or GUCY2C) is expressed at the apical membrane of enterocytes from duodenum to distal rectum and also by primary and metastatic colorectal cancer cells, however, it is not expressed in healthy extraintestinal tissue such as liver and lungs, where colorectal cancer cells usually metastasize [4–6]. GCC is activated upon binding to the paracrine hormones Guanylin (Gn) and Uroguanylin (UroG) as well as to the enterotoxigenic *Escherichia coli* heat stable enterotoxin (ST) [7]. GCC-paracrine hormones axis is considered a key regulator of several cellular processes such as differentiation, apoptosis, proliferation and migration [7, 8]. Numerous works have reported that the levels of mRNA of both guanylin and uroguanylin hormones were markedly downregulated in adenocarcinomas even at first stages, but high levels of the GCC receptor continue to be expressing [9–11]. With respect to its use in therapy, there are several studies in T84, HT29 and CaCo2 colorectal cancer cell lines showing that both Gn, UroG and the ST enterotoxin can inhibit cell proliferation based on GCC activation [5, 12]. This clinical evidence prompted several GCC agonists already approved by the FDA (Linaclotide, Plecanatide and Dolcanatide) as potential candidates for oral cancer chemoprevention [13]. In line with these discoveries, some authors have reported the development of radiotracers, based on chemical modification of the endogenous agonists (UroG, Gn and ST), and exploiting their ability to target GCC for PET and SPECT molecular diagnosis [14–16]. To the best of our knowledge, nanosystems targeted to GCC to deliver anticancer therapies to colorectal cancer have not been reported to date. The use of nanosystems for the development of anticancer therapeutics has several advantages, such as an increase in the therapeutic effectiveness, diminishing the

administered dose, and/or decreasing the secondary effects by means of targeting strategies (active/passive) that increase drug accumulation into the tumors [17]. On the other hand, nanosystems offer the possibility to include a variety of anticancer drugs in a single carrier for the development of combination therapies [18].

The objective of this work was the design, development and characterization of an actively targeted therapy consisting of drug-loaded sphingomyelin nanosystems (SNs) functionalized with Uroguanylin (UroG). For this, the first step was the preparation of a derivative of the natural hormone UroG by conjugation to a PEG-lipid moiety (UroGm) for its facile incorporation into SNs [19]. In a second step, the anticancer drug etoposide [20] was encapsulated into SNs, and the potential of this combination therapy for interfering with colorectal cancer cells proliferation was investigated.

Results

Synthesis and characterization of an UroG derivative

The conjugation of UroG to an amphiphilic molecule, poly(ethylene glycol) (PEG) with a hydrophobic stearic acid derivative (C₁₈), was explored in order to promote a successful insertion/anchoring of the peptide in SNs while exposing UroG linked to the PEG section in the outer part, thus making it accessible for receptor recognition. The primary amino group of UroG was linked to the terminal carboxylic group of the amphiphilic surfactant COOH-PEG₁₂-C₁₈. Activation of the carboxyl group through DMTMM is a simple reaction where DMTMM chloride generates the activated ester releasing 4-methylmorpholine in the first step. An amide bond is then formed between the activated ester and the amine present (Fig. 1A). Uroguanylin reaction progress was monitored by HPLC, evidenced by a decreasing intensity of the original peptide peak along reaction time and its shifting from $t_R(\text{UroG}) = 13.8$ min to $t_R(\text{UroGm}) = 14$ min (Fig. 1B). Once the purification of the product was done, HPLC analysis indicated a 75.45% conjugation yield. In order to certify the conjugation and the identity of the conjugate, MALDI-TOF analyses were subsequently carried out (Fig. 1C). These data provided the molecular weight (MW) of the conjugate, approximately 2560 Da, which corresponds to the formation of a 1:1 conjugate. Next, this data was further corroborated by NMR analysis through: ¹H-NMR (**Figure S1A**) and TOCSY (**Figure S1B**) experiments. The diffusion coefficients (D) of the single reagents (UroG and PEG₁₂-C₁₈) and the reaction product (UroG-PEG₁₂-C₁₈ conjugate, UroGm) were also determined by diffusion NMR (DOSY-NMR) experiments (Fig. 1D). This parameter is directly related with many intrinsic properties of the molecule such as MW, size, shape and charge[21, 22]. In the present study, a lower D value of the conjugate was obtained in comparison with non-modified peptide ($D_{\text{UroG}} = 3.7 \times 10^{-10} \text{ m}^2\text{s}^{-1}$, $D_{\text{UroGm}} = 0.45 \times 10^{-10} \text{ m}^2\text{s}^{-1}$, $D_{\text{C18-PEG12}} = 0.46 \times 10^{-10} \text{ m}^2\text{s}^{-1}$) as expected since lower diffusion coefficients correspond to higher MW species. Although it was not possible to calculate the coefficient through the peptide peaks in the UroGm sample, due to a deficient signal-to-noise ratio, the PEG-lipid signals were highly robust to allow the calculation of the coefficient in a consistent manner. Finally, TOCSY analysis proved the presence of UroG in the conjugate sample (**Figure S2**). Conjugation is also proved due to the observation of broader peaks

in the ^1H -NMR spectra, a well-established characteristic after polymer conjugation, as well as the shifting of NH-signals of the UroG in comparison with the pure peptide. Overall, the results of the three characterization techniques led us to conclude that conjugation prompted a structurally well-defined peptide-PEG-lipid (UroG-PEG₁₂-C₁₈), certifying that UroG peptide was successfully conjugated through an amide bond.

Development Of A Targeted Nanosystem

Sphingomyelin-based nanosystems (SNs) composed by an oleic acid core and stabilized by sphingomyelin were prepared by spontaneous emulsification using ethanol injection method [23]. UroGm was incorporated into the aqueous phase, followed by the addition of the lipids dissolved in ethanol. The physicochemical properties of the obtained UroGm-SNs were exhaustively studied using a wide panel of analytical techniques. UroGm-SNs showed a nanometric size below 150 nm and a negative surface charge (Table 1). A decrease in the mean particle size was observed for the decorated UroGm-SNs (131 ± 12 nm) with respect to plain SNs (149 ± 10 nm). This variation could be associated to the ability of the PEG₁₂-C₁₈ to act as a capping agent due to the O-H bond present at the end of the molecule, which rises the hydrophilic character of the UroGm-SNs. The conjugated peptide could then behave as a new surfactant molecule due to its amphiphilic character[24]. Variation in charge towards more negative values was also observed indicating an efficient association of the negative conjugate to the nanosystem surface. Monodisperse populations were obtained for both SNs and UroGm-SNs, according to the homogeneity values, polydispersity index < 0.2 and SPAN value < 1. Particle concentration measurements established an average of 5×10^{11} particles/mL, irrespective of the presence of peptide. Morphological examination was subsequently performed by Field Emission Scanning Electron Microscopy (FESEM). The images in Fig. 2A showed a defined spherical shape which corroborated the same size values obtained by the other technics, thus highlighting the robustness of the system. Interestingly, the morphology of the UroGm-SNs was found to be more irregular than non-decorated SNs, which may be due to the presence of a more hydrophilic part (corresponding with the PEG chain and the UroG peptide moieties) decorating the surface of the nanosystems. Stability of SNs and UroGm-SNs was next assessed at 37°C for 4 hours (Fig. 2B). The results show a good colloidal stability in suspension at these conditions for both nanosystems (SNs and UroGm-SNs). Nevertheless, significant differences were found when incubated with supplemented and non-supplemented cell culture medium (DMEM). SNs suffered a major increase in size during the first hour of incubation in these media, thus suggesting a strong interaction with serum proteins (FBS). However, the size of the decorated nanosystems (UroGm-SNs) remained constant at the same tested conditions, indicating an improved stability due to the presence of the amphiphilic UroGm at the interface. Additional evidence of the incorporation of UroGm to SNs was obtained by NMR analysis. Figure 2C shows the appearance of a signal from the PEG₁₂ peak (δ 3.69–3.71) in the spectra of the UroGm, which is also observed in spectra of the functionalized UroGm-SNs, but not in that of non-functionalized SNs. For an accurate integration of UroGm, NMR signals corresponding to PEG₁₂ peak were normalized to an internal control (TSP). Calculation of the precise amount of UroGm

was done as well by NMR, revealing an actual concentration of UroGm in the formulation of $2.08 \pm 0.14 \mu\text{g/mL}$. Following Eq. 1A and B (as detailed in methods section), we estimated a density of 0.012 UroGm molecules/nm².

Table 1
Physicochemical characterization of SNs and UroGm-SNs.

Formulation	Zetasizer (DLS and LDA)			Nanosight (NTA)					
	Size (nm)	Pdl	ZP (mV)	Size (nm)	D ₁₀	D ₅₀	D ₉₀	SPAN	Conc. (particles/mL)
SNs	149 ± 10	0.2	-23 ± 5	151 ± 3	107 ± 1	139 ± 3	208 ± 9	0.73	$4.9 \times 10^{11} \pm 3.7 \times 10^{10}$
UroGm-SNs	131 ± 12	0.2	-44 ± 4	110 ± 2	72 ± 1	95 ± 1	152 ± 4	0.84	$5.6 \times 10^{11} \pm 2.8 \times 10^{10}$
nm: nanometer, Pdl: polydispersity index, ZP: zeta potential in millivolts (mV), UroG: Uroguanylin									

Evaluation Of The Targeting And Therapeutic Capacities Of Urog

The next experiments were intended to determine the therapeutic potential of the UroGm amphiphilic derivative and the targeting capacity of UroGm-decorated SNs. All experiments were carried out in a metastatic colorectal cancer cell line constitutively expressing the GCC receptor, SW620 [25] (expression was confirmed by immunofluorescence and western blot assays, as presented in supplementary methods and **Figure S3**). Firstly, the successful functionalization and the effective targeting capacity of UroGm-SNs were studied. The results showed that, after 1 h of incubation, the green fluorescence intensity (associated with TopFluor®-sphingomyelin) was higher for cells incubated with UroGm-SNs than for the ones incubated with the non-decorated nanosystems (Fig. 3). However, after 4 h of incubation, both nanosystems reached similar levels of green fluorescence intensity. These results suggest that despite the chemical modification of UroG, the new conjugate preserves the GCC targeting capacity, resulting in a faster initial internalization of the surface-decorated UroG-SNs.

Secondly, a tumor colony forming assay (CFA) was used to quantitatively evaluate the capacity of UroGm to impede the ability of a single cell to grow into a large colony through clonal expansion [26, 27]. After addition of increasing concentrations of UroGm in solution, from 50 nm to 1 μM , it was observed that the number of colonies significantly decreased following a concentration dependent pattern (**Figure S4**). Subsequent experiments were carried out to determine the potential of UroGm-SNs for the development of anticancer therapeutics to colorectal cancer. Results from CFA manifest an augmented antiproliferative activity of UroGm when it is loaded into SNs, something that is not seen with the free

molecule (Fig. 4A), and that confirms the relevance of including this macromolecule in a delivery system for improved therapeutic effects. Additionally, results from a cell viability study (MTT) show a markedly decrease in cell viability of SW620 cells treated with UroGm-SNs at a particle concentration of 0.1 mg/ml in comparison with control cells (Fig. 4B). A dose-response assay with blank SNs exhibit no unspecific effects due to the nanosystems forming components at the same tested concentration (0.1 mg/ml) (Figure S5).

Development Of A Combination Nanotherapy

After proving the targeting and therapeutic capacity of UroGm-SNs, we decided to explore this bifunctional nanosystem for the development of a combination therapy. For this purpose, we selected the Topoisomerase II inhibitor etoposide (Etp), indicated for the treatment of metastatic colorectal cancer [28]. Given its hydrophobic nature, Etp could easily be incorporated in our lipid SNs. The resulting formulation (UroGm-Etp-SNs) as displayed in Table 2, showed a homogeneous distribution of particles with a similar size to UroGm-SNs and a negative zeta potential. Effective incorporation of UroGm and Etp into the nanosystem was studied by NMR and HPLC respectively. Results show similar peptide concentration (2.30 ± 0.12 vs 2.08 ± 0.14 $\mu\text{g/mL}$) and ligand density (0.010 vs 0.012 molecules/ nm^2) in UroGm-Etp-SNs and UroG-SNs. Etoposide concentration in UroGm-Etp-SNs was 40.51 ± 5 $\mu\text{g/mL}$. Therefore, it is possible to conclude that etoposide was successfully included in the formulation, without modifying substantially the properties or the ligand density of UroGm-SNs. The potential of this combination nanotherapy was assessed for the treatment of metastatic colorectal cancer using the same cell line as in previous assays. CFA was performed to evaluate the exact concentration range at which a combination effect of both drugs is obtained. We observed that by combining separated drugs at subtherapeutic concentrations (as previously determined, ≤ 500 nM of etoposide and ≤ 50 nM of UroGm, **Figures S4 and S6**), a potentiated effect was achieved (**Figure S7**). As shown in Fig. 5, a concentration of 50 nM / 500 nM of UroGm / Etp is able to reduce colony formation down to 4 times lower than the control. These results expose the great potential of this targeted therapeutic alternative for metastatic colorectal cancer treatment.

Table 2
Physicochemical characterization of UroGm-Etp-SNs.

Formulation	Zetasizer (DLS and LDA)			Nanosight (NTA)					
	Size (nm)	Pdl	ZP (mV)	Size (nm)	D ₁₀	D ₅₀	D ₉₀	SPAN	Conc. (particles/mL)
UroGm-Etp-SNs	126 ± 14	0.2	- 45 ± 5	122 ± 2	81 ± 1	108 ± 2	173 ± 3	0.85	$5.6 \times 10^{11} \pm 1.8 \times 10^{10}$
<i>nm: nanometer, Pdl: polydispersity index, ZP: zeta potential in millivolts (mV), UroG: Uroguanylin</i>									

Discussion

Nanosystems intended for cancer treatment have been mostly designed relying on the intrinsic capacity of these small particles to enhance their circulation times and eventually undergo passive accumulation into the tumor tissues due to the enhanced permeability and retention effect (EPR effect) [29]. Recent studies highlighted the need for targeted therapies that could enhance the accumulation of nanoparticles in the tumor [30, 31]. By means of active targeting, nanoparticles can theoretically achieve higher levels of drug concentration in tumour tissues via receptor-mediated endocytosis [17]. In fact, the use of targeting ligands has been shown to reduce side effects and improve the therapeutic output [32–34]. Typically, well-known receptors involved in tumour progression, such as HER2, folate receptor, CD44, and EGFR, have been exploited for that purpose [35]. One of the problems associated to these strategies relies on the fact that most of these receptors are non-specific for cancer cells but ubiquitously expressed in the body. In addition, competition with endogenous ligands may frequently hamper the potential of this approach. Therefore, the identification of selective markers on tumor cells becomes critical to enable selectivity for tumor tissue over normal cells [36]. Considering this, we selected the Guanylyl Cyclase C (GCC) receptor for targeting, since is only expressed at the apical membrane of enterocytes, as well as by primary and metastatic colorectal cancer cells, but not by healthy extraintestinal tissue [4–6, 9].

Based on this previous knowledge, the objective of this work was to design a targeted therapy that could be used in combination with an anti-cancer drug in order to achieve a synergistic therapeutic effect. In a first step, we selected the natural hormone uroguanylin (UroG) since it is a ligand of the GCC receptor expressed on colorectal cancer cells [7], and specifically inhibits cell proliferation [5, 12]. We synthesized a new uroguanylin derivative (UroGm) in order to provide a firmer association of the hormone to SNs, and evaluated the activity of the resulting surface-decorated formulation (UroGm-SNs). In addition, the anticancer drug etoposide was encapsulated in the SNs to develop a combination therapy approach (UroG-Etp-SNs) that could provide improved antitumoral effects in colorectal cancer cells. SNs were produced using a very mild and simple technique (ethanol injection method), an optimized low energy manufacturing technique traditionally used for liposome preparation [19, 37]. In addition, without changing this production methodology, the inclusion of the targeting molecule (UroGm) into the nanosystem was expected to happen through the C₁₈ lipophilic segment of the conjugate [38, 39]. In that way, the hydrophilic targeting moiety would be oriented towards the external aqueous phase.

Nanosystems coated with both proteins and PEG chains are well known to avoid aggregation and to be stable in cellular media [40]. PEGylated lipids, and particularly PEG-DSPE (phosphatidylethanolamine), have been widely used in nanoformulations as surface stabilizers, mainly to improve their circulation half-life [41]. In the last decade, the bifunctionality of PEG has allowed further exploitation for the conjugation of bioactive molecules such as antibodies or peptides through a great variety of linkers, constructing cell-specific targeting nanocarriers [42]. Furthermore, peptide-PEG-lipid conjugates can be exhaustively characterized, a fact that positively contributes to address later regulatory processes and clinical translation [43]. The selection of an amide bond as the linkage functional group was based on its simplicity, non-immunogenicity and was consistent with reported studies considering the importance of

the carboxy-terminal conserved domain (cysteine domain) for the proper folding and bioactivity of the hormone [15, 44]. Besides, the amide group provides a stable union between the peptide and the PEGylated lipid, avoiding a prompt release of the targeting moiety from the nanosystem under multiple biological scenarios, enhancing its half-life and subsequent accumulation via EPR effect [45]. The *in vitro* cell culture studies showed that the hydrophobized derivative (UroGm) preserves the native UroG ability to efficiently target the GCC receptor and to decrease cell tumorigenicity for concentrations in the same order of magnitude as published by other authors [46, 47]. This fact confirms that the biological function of the hormone remains intact despite the chemical modification. Although many reports in the literature have shown diverse ways to conjugate ligands to the nanosystems surface, the majority of them do not specify the ligand density obtained [30, 44, 48–50]. The values of ligand density observed in this work are in line with those reported for different nanosystems, oscillating from 0.225 to 0.005 molecules/nm² [30, 44]. The use of combination therapies involving the incorporation of two anticancer agents into a nanosystem may provide benefits in the sense that different drugs may attack cancer cells at varying stages of their growth cycle [51]. Considering this, combination nanomedicine should be designed in such a way that it targets multiple signaling pathways with limited toxicity [36]. In this work, the decoration of SNs with UroGm combined with the encapsulation of the anticancer drug etoposide was selected to promote a synergistic effect of both drugs. This synergy could be justified due to the joint activation of cGMP-AKT axis by the UroGm and the DNA damage response triggered by Etp [52, 53], and to an improved uptake of Etp due to its encapsulation into a nanosystem [54, 55].

Conclusion

In this work we have shown the potential of UroG to be used in new targeted therapeutics for colorectal cancer. UroGm-SNs can interact with GCC-expressing colorectal cancer cells in an efficient way, due to the exposure of the UroG ligand on the nanoparticle surface, while simultaneously inducing a therapeutic response. Moreover, the developed UroGm-Etp-SNs could mediate the delivery of a second drug for the development of combination therapies. From our knowledge there are no studies using the UroG hormone either alone or in combination with other drugs for the development of targeted nanomedicines. Therefore, this strategy might represent a useful approach for the development of a new treatment for metastatic colorectal cancer.

Methods

Materials

C₁₈-PEG₁₂-COOH (MW 825 g/mol) was obtained from Creative PEGWorks (Winston Salem, NC, USA). 4-(4,6-dimethoxy[1, 3, 5]triazin-2-yl)-4-methylmorpholinium chloride salt (DMTMM·Cl, MW 276.72 g/mol) was purchased from Sigma Aldrich (Madrid, Spain). Uroguanylin (UroG, MW 1667.9 Da; NDDCELCVNACTGCL) was purchased from Bachem (King of Prussia, PA, USA). Oleic Acid was acquired from Sigma Aldrich (Madrid, Spain). Sphingomyelin (Lipoid E SM) was kindly provided by Lipoid GmbH

(Ludwigshafen, Germany). Etoposide (purity $\geq$ 98%) was purchased from Cayman Chemical Company (Ann Arbor, MI, USA). MiniDyalisis Kit, 1 kDa cut-off was obtained from GE Healthcare (GE Healthcare Bio-Science Corp., NJ, USA). HPLC grade Acetonitrile (ACN) and Ethanol (EtOH) were obtained from Fisher Chemicals (Thermo Fisher Scientific, USA) and Trifluoroacetic acid (TFA) was provided by Sigma-Aldrich (Madrid, Spain). Dimethyl sulfoxide (DMSO, 99.8% D) was purchased from (Cortecnet Inc., Paris, France). All other chemicals used were HPLC or UPLC purity grade.

Synthesis And Characterization Of Uroguanylin Derivative (urogm)

Uroguanylin (UroG) was covalently linked to C₁₈-PEG₁₂-COOH through an amide linker. As carboxyl activating agent, DMTMM was used [56]. Firstly, stock solutions of all single reagents were prepared: C₁₈-PEG₁₂-COOH and DMTMM were dissolved at 40 mg/mL in MilliQ water and UroG was dissolved at 1 mg/mL in HEPES 300 mM buffer (pH = 8) [15]. DMTMM (120 eq, 276.72 g/mol) was added over C₁₈-PEG₁₂-COOH solution (100 eq, 825 g/mol) under magnetic stirring and left for 10 minutes at room temperature (RT) to promote the activation of the carboxylic groups. Then, 200 μ L of UroG stock solution (1 eq, 1667,9 Da) were added dropwise to the previous mixture. HEPES buffer was used to adjust the pH to 7.6 with a final buffer concentration of 150 mM. The reaction was allowed to proceed for 8 h at RT. For purification, the reaction volume was dialyzed against deionized water 3 times for 20 h by using a MiniDyalisis Kit (MWCO 1 kDa) and then analyzed by HPLC, NMR and MALDI-TOF techniques (detailed information in supplementary materials).

Preparation Of Urogm-sns

Sphingomyelin nanosystems incorporating the modified Uroguanylin (UroGm) were prepared by ethanol injection technique. Briefly, UroGm was dissolved in water at a concentration of 0.5 mg/mL. On the other hand, oleic acid and sphingomyelin were dissolved in ethanol at a concentration of 200 mg/mL and 40 mg/mL respectively. Subsequently, 50 μ L of the oily phase (composed by 2.5 mg of oil and 0.5 mg of surfactant) were injected into 450 μ L of ultrapure water (containing the appropriate quantity of UroGm) under continuous magnetic stirring and nanosystems were spontaneously formed. Increasing amounts of UroGm were added to the formulation in order to explore the maximum loading capacity (data not shown) establishing a final amount of 10 μ g of UroGm per formulation as the best condition. Formulations were then isolated by centrifugation (20000 RFC for 45 minutes at 15°C) using an Eppendorf 5417R centrifuge (Eppendorf, Germany) to purify the nanosystem.

Physicochemical Characterization

Particle size and polydispersity index (Pdl) were determined by Dynamic Light Scattering (DLS), and Z-potential values by Laser Doppler Anemometry (LDA), using a Zetasizer NanoZS® (Malvern Instruments, UK). Measurements were performed at 25 °C with a detection angle of 173° upon 1/10 dilution with ultrapure water (MilliQ®). Nanosystems were additionally characterized by Nanoparticle Tracking Analysis (NTA), a method to measure particle size based on imaging of individual nanosystems. Experiments were conducted with a NanoSight NS3000 System (laser operating at $\lambda = 488$ nm) (Malvern Instruments, UK). Briefly, nanosystems were injected in the sample chamber at a 1000-fold dilution in ultrapure water. Five captures, with a camera level of 14, were used to determine several parameters such as average size, homogeneity and particle concentration. Colloidal stability of the nanosystems was determined after being stored at 4 and 37°C, as well as after incubation in biological media (DMEM high glucose, Sigma Aldrich) supplemented or not with 1% v/v fetal bovine serum (FBS, Gibco).

Morphological Examination

Morphological examination of the formulation was performed by Field Emission Scanning Electron Microscopy (FESEM) Ultra Plus (Zeiss, Germany) configured with InLens and STEM modes and operating at 20 kV. For the preparation of FESEM samples, 20 μ L of the nanosystem suspension were mixed with 20 μ L of 2% (w/v) phosphotungstic acid and stained for 6 hours. The mixture was placed onto a copper grid with a formvar-carbon film, washed with 500 μ L of ultrapure water and dried overnight in a desiccator under vacuum.

Ligand Density Calculation

Efficient incorporation of UroGm into the nanosystems surface was determined by NMR. To achieve an accurate quantification, a fraction of the non-isolated UroGm-SNs were collected to quantify the precise total amount of UroGm presented in the formulation. After isolating the nanosystem, both the supernatant (where the decorated UroGm-SNs are located) and the undernatant (containing the free compounds in solution) were collected for further analysis. These three fractions (i.e. total, supernatant and undernatant) were freeze dried to remove traces of ethanol that were found to interfere with the analysis (peaks of ethanol overlap with the peak of the PEG in the ^1H -NMR spectrum), and eventually dissolved in 500 μ L of deuterated DMSO (99.8% D). NMR experiments were conducted at 25 °C on a Bruker NEO 17.6 T spectrometer (proton resonance 750 MHz) (Bruker, US), equipped with a $^1\text{H}/^{13}\text{C}/^{15}\text{N}$ triple resonance probe and shielded PFG z-gradient. All the spectra were processed with MestreNova software v12.0 (Mestrelab Research Inc., Spain). The chemical shifts were referenced automatically with respect to the deuterium lock. Samples were prepared in 5 mm thin wall NMR tubes. A 1D proton spectra (^1H) was acquired for each sample using the pulse-acquisition sequence. The spectrum was acquired under quantitative conditions by using a low excitation tilt pulse angle of only 30 degrees, an inter-scan delay (d_1) of 6 s and an acquisition time (aq) of 2.75 s. The proton spectrum was processed with Fourier transformation and the phase and baseline were carefully corrected. For control, plain SNs were also

prepared and characterized following the same methodologies, without the addition of UroGm in the aqueous phase. Surface density of UroGm molecules was subsequently calculated as the number of molecules per surface unit of nanosystem (nm^2). Firstly, the number of UroGm particles were calculated with **Eq. 1A** from the previously NMR determined concentration. On the other side, nanosystems surface area were calculated using **Eq. 1B**, considering SNs morphology as perfect spheres, and with the concentration and radius parameters obtained from NTA measurements.

Equation 1

Formulas for calculation of number of particles (A) and surface area of a sphere (B).

$$\text{A)} \quad N_{\text{UroGm}} = \frac{\text{Mass} \times N_A}{\text{MW}} \quad \text{B)} \quad SA_{\text{SNs}} = 4 \pi r^2$$

N_{UroGm} : number of UroGm molecules; N_A : Avogadro Constant; MW : molecular weight; SA_{SNs} : SNs Surface Area; r^2 : radius squared.

Preparation Of Dual-loaded Sns

UroGm-functionalized sphingomyelin nanosystems (UroGm-SNs) were additionally loaded with the chemotherapeutic drug etoposide (UroGm-Etp-SNs). In this case, up to 250 μg of etoposide (40 mg/mL in DMSO) were placed into the organic phase within the 50 μL of ethanol and injected into the 450 μL of ultrapure water containing UroGm. Nanosystems were isolated using the same conditions as previously described. Encapsulation efficiency was determined by direct quantification of etoposide in the nanosystem using an isocratic HPLC method optimized from the literature [57]. Analyses were performed in an HPLC system 1260 Infinity II Agilent (Agilent Technologies, US) equipped with a pump G7111A, an autosampler G7129A and an UV-Vis detector G7114A set at 254 nm. Separation was achieved on an InfinityLab Poroshell 120 EC-C18 (100 mm x 4.6 mm, 4 μm pore size) Agilent column. The mobile phases are composed of water and acetonitrile ($\text{H}_2\text{O}:\text{ACN}$, 70:30 v/v) at a flow rate of 1 mL/min . Standard calibration curves were linear in the range of 1 to 15 $\mu\text{g/mL}$ ($R^2 = 0.9999$) (Limit of quantification, LOQ = 1 ppm).

In vitro studies

Cell Viability Studies

Cell toxicity analyses were performed to determine the viability of metastatic colorectal cancer cells SW620 (ATCC® CCL-227™) upon exposure to increasing concentrations of SNs (from 0.01 to 10 mg/mL) in a final volume of 150 μL (25 μL corresponding to the nanosystem and 125 μL to complete medium).

Etp-SNs were also tested to evaluate the effect of encapsulating of the cytostatic drug. Cells were seeded at a density of 10.000 cells/well in 96-well plates 24 h before the experiment. After 48 hours of incubation with SNs and Etp-SNs, medium was removed and 100 µL of tetrazolium dye (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, MTT) solution (5 mg/mL in PBS, MTT Alfa Aesar, Germany) were added to each well. After 3 hours of incubation this solution was also removed and formazan crystals were solubilized with 100 µL of DMSO and maintained at 37°C for 15 minutes protected from light. Results were obtained by measuring absorbance at 570 nm in a microplate spectrophotometer (Multiskan EX, Thermo Labsystems). Cell viability in percentage (%) was calculated in comparison with control wells containing untreated cells.

Cellular Internalization Studies

Internalization studies in SW620 metastatic cancer cells were performed by confocal microscopy (Leica SP8, Germany). Fluorescent UroGm-SNs were prepared by adding the modified lipid TopFluor®-Sphingomyelin in their composition (0.5 µg/nanosystem). To evaluate cellular uptake, 200.000 cells were seeded on a 24-well plate over a glass coverslip. After 24 h, the cells were washed with PBS and then incubated for up to 4 h with Etp-SNs, UroGm-SNs and UroGm-Etp-SNs at a concentration of 0.13 mg/mL per well (added onto 500 µL of cell culture medium). After this period, the medium was removed and cells were washed twice with PBS. Then, they were fixed with paraformaldehyde (4% w/v) for 15 minutes and then washed with PBS. Cellular nuclei were stained with Hoescht 33342 (Invitrogen, US) for 5 minutes and then cells were washed three times with PBS. Finally, the coverslips were mounted over microscope slides using 8 µL of Mowiol mounting medium (Calbiochem, US). Coverslips were dried in the dark overnight (at room temperature) before visualization.

Colony Forming Assays

SW620 colorectal cancer cells were plated in triplicate at a density of 600 cells/well in 12-well plates and cultured in a humidified incubator (37 °C, atmosphere of 5% CO₂ and 95% RH). Drug treatments were maintained in contact with cells for the complete duration of the experiment (15 days). After this period cells were stained with an MTT solution (5 mg/mL) for 3 to 4 hours and subsequently dried and scanned. Obtained images were analyzed using ImageJ software. *In vitro* differences were statistically determined by one-way ANOVA (GraphPad PRISM, version 6.0, GraphPad Software, Inc., US)

List Of Abbreviations

SNs, sphingomyelin nanosystems

UroG, uroguanylin

UroGm, uroguanylin derivative

Etp, etoposide

GCC, guanylyl cyclase C receptor

Declarations

Ethics approval and consent to participate.

Not applicable.

Consent for publication.

Not applicable

Availability of data and material.

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests.

The authors declare no competing interest.

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Author's contribution.

B.L.B. performed experimental design, conducted the experiments, prepared the figures and wrote the manuscript. S.L., R.J. and I.C.S contributed to experimental design, conducted experiments and helped with the formal analysis. S.A performed cell experiments. All authors discussed the results presented on the manuscript. M.J.A., I.C.S and M.F. supervised the project. M.F. design and administered the project. All authors read and approved the final manuscript.

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Figures

Figure 1

A) Schematic representation of the chemical synthesis of the UroG-PEG12-C18 conjugate (UroGm) through DMTMM-carboxyl activation. B) HPLC chromatograms for: (i) the reaction medium, COOH-PEG12-C18 and DMTMM·Cl dissolved in HEPES 150mM (blue line), (ii) the standard unmodified peptide UroG (yellow line) and (iii) the purified compounds UroG + UroGm conjugate (turquoise line). C) MALDI-TOF signals of COOH-PEG12-C18 (blue area), UroG unmodified peptide (green area) and purified conjugate UroGm (red

area).D) DOSY analysis of the parent peptide UroG (left) and its conjugate UroGm (right) (* = UroG signals, ↓=PEG12-C18 signals).

C)

Figure 2

A) Scanning transmission electron microscope images of SNs and UroGm-decorated SNs using InLens (immersion lens) mode. Scale bar = 200nm. B)Colloidal stability of non-decorated (SNs) and decorated (UroGm-SNs) nanosystems assessed at 37°C in different media (supplemented or not with FBS). DMEM:

Dulbecco's Modified Eagle Medium; FBS: Fetal Bovine Serum.C) Comparison between the 1H-NMR spectrum of functionalized nanosystems (UroG-SNs), UroG-PEG12-C18 spectrum and the sphingomyelin nanosystems(SNs).

Figure 3

Confocal microscope images showing the internalization of SNs and UroGm-SNs in SW620 cells after 15 minutes, 1 hour and 4 hours of incubation. Green channel: TopFluor®-sphingomyelin (TopFluor®-SM) labelled nanosystems. Blue channel: nuclei stained with Hoechst.

Figure 4

A) Colony forming assay testing the capacity of UroGm-SNs to inhibit cellular proliferation (dose 50 nM). P value ** $p < 0.01$. B) MTT results showing the antiproliferative effect of nanosystems containing UroGm at a concentration of 0.1 mg/mL of SNs. P value **** $p < 0.0001$.

Figure 5

Graphical representation of the treatments evaluated in this work (UroGm-SNs, Etp-SNs and the combination UroGm-Etp-SNs) all at the same concentration of the active drugs (500 nM Etp and 50 nM UroGm). P value: **** $p < 0.001$ and ** $p < 0.01$.

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