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Development of transferrin-bearing vesicles encapsulating aspirin for cancer therapy

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Originally developed for the treatment of inflammatory disorders, the non-steroidal anti-inflammatory drug aspirin was shown to have a preventive effect against cancer in the past decades. Most importantly, recent studies suggested that it might also provide a therapeutic benefit in the treatment of cancer *in vitro*. However, this drug failed to specifically reach tumors at a therapeutic concentration following intravenous administration, thus resulting in lack of efficacy on tumors.

In this work, we demonstrated that aspirin could be formulated in transferrin-bearing vesicles and that this tumor-targeted formulation could lead to an increase in the anti-proliferative efficacy of the drug in three cancer cell lines *in vitro*. The *in vitro* therapeutic efficacy of aspirin was significantly improved when formulated in transferrin-bearing vesicles, by about 2-fold compared to that of drug solution. These results are promising and support the optimization of this delivery system to further improve its potential as a therapeutic tool in combination with other anti-cancer therapies.

Keywords: Aspirin; transferrin; tumor targeting; drug delivery; cancer therapy

Introduction

The non-steroidal anti-inflammatory drug aspirin is arguably the most commonly used synthetic drug in human history. It was originally developed for the treatment of inflammatory disorders, but was shown to have a preventive effect against cancer in the past decades (Alfonso *et al.* 2014; Rothwell *et al.* 2011). Most importantly, recent studies suggested that aspirin might provide a therapeutic benefit in the treatment of cancer *in vitro*. Several anti-cancer mechanisms of aspirin were ascribed to inhibition of the

Epidermal growth factor receptor (EGFR), down-regulation of Nuclear factor-κ B (NF-κB) activity and Protein kinase B (PKB/Akt) activation and modulation of B-cell lymphoma 2 (Bcl-2) family proteins in a wide range of cancer cells (Kutuk and Basaga 2003; Park *et al.* 2010; Stark *et al.* 2007; Xiang *et al*. 2010). Even in the same cancer (such as colorectal cancer), aspirin exerts antineoplastic actions against cancer cells with and without cyclooxygenase-2 (COX-2) expression via different mechanisms (Ashktorab *et al.* 2005; Voutsadakis *et al.* 2010). The mechanisms whereby aspirin exhibits anti-proliferative activity are therefore very diverse, not entirely elucidated and still the object of intense investigation.

However, despite its antineoplastic potential, aspirin failed to specifically reach tumors after intravenous administration, thus resulting in a lack of efficacy on tumors and possible secondary effects on healthy tissues (increased risk of bleeding, particularly intracranial bleeding) (Rose *et al.* 2011). Furthermore, as a result of its rapid metabolism and elimination, aspirin has a short half-life in the whole blood of 22.2 ± 3.9 min (Costello and Green 1982), leading to plasma aspirin concentrations much lower than those necessary to exert effective anti-cancer activity. Given the potential anti-proliferative properties of aspirin, it is highly important to target the delivery of this drug specifically to its site of action, at a therapeutic dose.

On the basis that iron is essential for tumor cell growth and can be effectively carried to tumors by using transferrin receptors overexpressed on cancer cells (Calzolari *et al.* 2007; Daniels *et al.* 2006), we propose to encapsulate aspirin in transferrin-bearing vesicles to overcome this limitation. The combination of active targeting, resulting from the conjugation of transferrin (Tf) ligands to the vesicles, with the passive accumulation of delivery systems in tumors due to enhanced permeability and retention (Maeda 1992), has been widely used in tumor-targeted strategies (Fu *et al.* 2009; Fu *et al.* 2011; Karim

et al. 2017; Lemarié *et al*. 2012; Qian *et al.* 2002). We have previously prepared Tf-bearing Solulan-based unilamellar vesicles and tocopheryl-based multilamellar vesicles encapsulating the green tea compound epigallocatechin gallate and palm oil compound tocotrienol, which presented the same anti-cancer therapeutic potential and delivery issues as aspirin. We demonstrated that these targeted formulations significantly increased the cellular uptake of the drugs in comparison with control vesicles and drug solution for all the tested cancer cell lines. In addition, the intravenous administration of these Tf-bearing nanomedicines resulted in a rapid and sustained tumor regression over one month on mice bearing subcutaneous tumors, with disappearance of 40% of the tested A431 epidermoid carcinoma and B16-F10 melanoma for both drugs (Fu *et al*. 2009; Fu *et al.* 2011; Lemarié *et al.* 2012).

Building on these highly promising vesicles, we now would like to develop a novel Tf-bearing nanomedicine ultimately able to specifically deliver aspirin to tumors after intravenous administration, thus leading to an improved anti-cancer therapeutic effect without secondary effects to normal tissues. To date, no formulation of aspirin encapsulated in a tumor-targeted delivery system has been reported.

The objectives of this study were therefore 1) to develop and characterize novel transferrin-bearing vesicles encapsulating aspirin and 2) to evaluate their *in vitro* therapeutic efficacy on various cancer cell lines.

Materials and Methods

Cell lines and reagents

Aspirin (Acetylsalicylic acid), human holo-transferrin (Tf), hydrogenated phosphatidylcholine (HPC), cholesterol and all other chemicals that are not specifically mentioned below were purchased from Sigma Aldrich (Poole, UK). 1,2-distearoyl-sn-

glycero-3-phosphoethanolamine-N-(Carbonyl-methoxypolyethyleneglycol 2000, sodium salt (DSPE-PEG2000) came from NOF Corporation (Tokyo, Japan). Cholesterol-PEG5000-maleimide was obtained from Nanocs (New York, NY). A431 human epidermoid carcinoma and T98G glioblastoma were purchased from the European Collection of Cell Cultures (Salisbury, UK), while Bioware® B16-F10-luc-G5 mouse melanoma that expresses the firefly luciferase was obtained from Caliper Life Sciences (Hopkinton, MA). Dulbecco's Modified Eagle Medium (DMEM) and Roswell Park Memorial Institute (RPMI)-1640 cell culture media, fetal bovine serum, L-glutamine and penicillin-streptomycin came from Invitrogen (Paisley, UK).

Preparation and characterization of transferrin-bearing vesicles entrapping aspirin

HPC (19.2 mg), DSPE-PEG2000 (6.4 mg), cholesterol (5.3 mg) and cholesterol-PEG5000-maleimide (1.1 mg), at molar ratios of 60: 6: 34: 0.5, were shaken in 1.96 mL PBS (pH 7.4) at 60 °C for 1 hour. Aspirin solution (40 µL, 20 mg, measured from a stock solution of 500 mg/mL prepared in dimethylsulfoxide) was then added to the mixture, followed by probe sonication using Sonics Vibracell® VCX 500 (Sonics®, Newtown, CT) for 5 x 2 minutes.

Transferrin was then thiolated to be able to react with the thiol-reactive maleimide group of cholesterol-PEG-maleimide (Figure 1). To do so, 10 mg of transferrin were dissolved in 1 mL of 50 mM sodium phosphate and 150 mM sodium chloride buffer (pH 8) and were then shaken with 85 µL of 10-fold molar excess of 2-iminothiolane (Traut's reagent, 2 mg/mL in distilled water) at 25 °C for 1 hour. The thiolated transferrin was then isolated from unreacted Traut's reagent using Vivaspin® 4 centrifuge tubes with a molecular weight cut-off of 5 000 Daltons (Sartorius Ltd., Epsom, UK), after

centrifugation at 9 500 rpm (10 500 g) for 15 min at 20 oC (Hermle® Z323K centrifuge, Wehingen, Germany).

The control vesicles were immediately conjugated with the thiolated Tf by incubation with continuous stirring at 25 °C for 2 hours. Free aspirin and/or free Tf were removed from both Tf-bearing and control vesicles using Vivaspin® 6 centrifuge tubes with a molecular weight cut-off of 100 000 Daltons (Sartorius Ltd., Epsom, UK) by centrifugation at 7 500 rpm (6 600 g) for 15 min at 20 °C.

Vesicles were visualized by transmission electron microscopy (TEM), using a FEI Tecnai TF20 (ThermoFisher Scientific, Waltham, MA) with a Gatan 794 MultiScan® camera (Gatan, Pleasanton, CA), as previously described (Aldawsari *et al.* 2011).

The entrapment of aspirin in the vesicles was quantified by spectrofluorimetry using an Agilent Varian Cary Eclipse® spectrofluorometer (Agilent Technologies, Santa Clara, CA), following disruption of the vesicles with isopropanol. Fluorescence intensity was measured at the aspirin excitation wavelength (λ_{exc}: 298 nm) and emission wavelength (λ_{em}: 406 nm), with linearity range of 0.125 to 1.5 µg/mL.

The amount of transferrin conjugated to the vesicles was measured by Lowry assay (Fu *et al.* 2009). Briefly, 1 mL of sodium potassium tartrate solution (2% w/v in distilled water) and 1 mL of cupric sulphate solution (1% w/v in distilled water) were added dropwise (under continuous stirring to avoid precipitation) into 25 mL of sodium carbonate anhydrous solution (2% w/v in 0.1 M NaOH) to make up Solution A. The bovine serum albumin (BSA) was prepared as a standard protein solution (concentration ranging from 0 to 500 µg/mL). One hundred microliters of Tf-bearing or control liposomes (diluted 1:20 in PBS) or BSA standard solution, was mixed with 1 mL of Solution A and incubated at 25 °C for 10 min. Subsequently, 100 µL of 1N Folin-Ciocalteu's phenol reagent was added to these samples (with immediate vortexing),

followed by incubation at 25 °C for 30 min (protected from light). The absorbance of each sample was determined at a wavelength of 750 nm using an Agilent Varian Cary® 50 UV-Vis Spectrophotometer (Agilent Technologies, Santa Clara, CA). Blank liposomes were used as the reference cell to set zero. The experiment was done in quadruplicates. The amount of Tf was calculated by correlating the absorbance of each sample with the standard curve of BSA. The results were expressed as percentage of Tf conjugated to nanoparticles compared to the initial amount of Tf added.

Size and zeta potential of the vesicles were respectively determined by photon correlation spectroscopy and laser Doppler electrophoresis using a Zetasizer Nano-ZS® (Malvern Instruments, Malvern, UK).

Drug release from the vesicles

To confirm that aspirin could be released from the vesicles, the release profile of the drug was determined by dialysis at pH 5.5, 6.5 and 7.4, respectively mimicking the subcellular endosome, the tumor extracellular environment and the physiological pH in normal tissue and blood. Each vesicular formulation (100 µg/mL in phosphate buffer) was placed into a SnakeSkin® dialysis tube with a molecular weight cut-off of 3 500 Daltons (ThermoFisher Scientific, Waltham, MA) and was dialyzed against 200 mL of phosphate buffer (pH 5.5, 6.5 and 7.4) at 37°C under stirring at 100 rpm. At specific time points (15, 30, 45 minutes, then every hour for the first six hours (2, 3, 4, 5 and 6 h), then every 2 hours for the next 6 hours (8, 10 and 12 h), and every 24 hours for 3 days), 1 mL sample of the dialysate was withdrawn in quadruplicates and replaced with an equal volume of fresh media. The amount of aspirin in the samples was quantified by spectrofluorimetry and reported as a percentage cumulative release.

In vitro biological characterization

Cell culture

A431, T98G and B16-F10-luc-G5 cell lines overexpressing transferrin receptors were grown in either DMEM (for A431 and T98G cells) or RPMI-1640 medium (for B16-F10-luc-G5 cells) supplemented with 10% (v/v) fetal bovine serum, 1% (v/v) L-glutamine and 0.5% (v/v) penicillin-streptomycin. Cells were cultured at 37ºC in a humid atmosphere of 5% CO_2.

Cellular uptake of aspirin

Quantitative analysis of cellular uptake was performed by spectrofluorimetry. Cells were seeded in 6-well plates at a density of 2×10^5 cells/well and grown for 72 hours before being treated with aspirin (200 µg/well), either entrapped in transferrin-bearing vesicles, control vesicles or in solution. After 24 hours' treatment, the cells were washed three times with 2 mL cold phosphate buffer saline (PBS) before addition of trypsin (500 µL/well) and incubation for 10 min at 37 ºC to detach the cells. Cells were then lysed with 10% Triton-X (500 µL/well) and incubated for another 24 hours at 37 ºC. Cell lysates were then centrifuged at 10 000 rpm (9 300 g) for 15 min using an IEC Micromax® centrifuge (ThermoFisher Scientific, Waltham, MA). The amount of aspirin in the supernatant was quantified by spectrofluorimetry (λ_{exc}: 298 nm, λ_{em}: 406 nm), using a FlexStation 3® multi-mode microplate reader (Molecular Devices, Sunnyvale, CA). Untreated cells were used as blanks to remove any possible interferences of the cells and medium with the measurements.

In vitro anti-proliferative activity

A431, T98G and B16-F10 cells were seeded at a density of 2 x 10^3 cells per well in 96-well plates and grown for 72 hours before being treated with aspirin entrapped in transferrin-bearing vesicles, control vesicles, or in solution (final drug concentrations of 100 to 500 µg aspirin/mL). Following 72 hours' treatment, anti-proliferative activity of the formulations was evaluated by measurement of the growth inhibitory concentration for 50% of the cell population (IC_{50}) in a standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay (n=15). Briefly, 50 µl of MTT solution (0.5% w/v in PBS) was added to each well and incubated for 4 hours at 37 ºC, protected from light. The solution was then replaced with DMSO (200 µL per well) to solubilize the precipitated formazan product. The absorbance of the solution was measured at 570 nm using a Multiskan Ascent microplate reader (Thermo Labsystems, Beverly, MA). Dose-response curves were fitted to percentage absorbance values to obtain IC_{50} values (three independent experiments with n=5 for each concentration level).

Statistical Analysis

Results were expressed as mean ± standard error of the mean (S.E.M). Statistical significance was assessed by one-way analysis of variance (ANOVA) and Tukey multiple comparison post-test, using Minitab® 17.1.0 software (Minitab Ltd., State College, PE). Differences were considered statistically significant for P values lower than 0.05.

Results

Preparation and characterization of transferrin-bearing vesicles entrapping aspirin

Transferrin-bearing and control spherical unilamellar vesicles entrapping aspirin have been successfully prepared, as confirmed by TEM imaging (Figure 2). The percentage of drug loading within these vesicles was respectively 97.36 ± 0.02% (9.74 ± 0.01 mg/mL, 54.04 ± 0.01 mM) for transferrin-bearing vesicles and 98.97 ± 0.01% (9.90 ± 0.01 mg/mL, 54.94 ± 0.01 mM) for control vesicles. Holo-transferrin was conjugated to the vesicles at a level of 5.14 ± 0.11 mg (51.37 ± 1.06% of the initial transferrin added, 2.57 ± 0.06 mg Tf per mL liposomes, 32.70 ± 0.80 µM or 160.60 ± 3.40 µg Tf per mg of liposomes). As expected, the conjugation of transferrin to the surface of vesicles resulted in a larger mean vesicle size of 208.40 ± 3.86 nm (polydispersity: 0.36 ± 0.02) than that of control vesicles (106.60 ± 1.30 nm, polydispersity: 0.20 ± 0.01). These vesicle sizes were also confirmed by TEM imaging. Additionally, the conjugation of transferrin also increased their zeta potential compared to that observed for control vesicles (18.40 ± 0.46 mV and -15.50 ± 0.95 mV respectively for transferrin-bearing and control vesicles).

Drug release from the vesicles

Transferrin-bearing and control vesicles exhibited an initial fast release of aspirin (about 20 to 30 %) in the first hour, followed by a sustained release of the drug in a pH-dependent manner over 72 hours (Figure 3). In addition, the conjugation of a targeting ligand to the surface of the vesicles had an impact on the release profile of the drug. Specifically, at pH 7.4, 99.04 ± 0.41% of the drug was released from transferrin-bearing vesicles over a 72-hour period, while 83.16 ± 0.25% and 61.56 ± 0.10% of the drug was released from these vesicles respectively at pH 6.5 and 5.5 during the same period. The drug release from the control vesicles followed a similar pH-dependent profile, but was slower than

for the targeted vesicles (cumulative drug release of 72.53 ± 0.37% at pH 7.4, 69.90 ± 0.27% at pH 6.5, 59.96 ± 0.23% at pH 5.5 over 72 hours).

Cellular uptake of aspirin

The cellular uptake of aspirin following treatment with transferrin-bearing vesicles was significantly increased in comparison with control vesicles for the 3 cell lines, respectively by 1.2-fold in B16-F10 cells, 1.9-fold in A431 cells and 2-fold in T98G cells (Figure 4). Among these 3 cancer cell lines, the highest cellular uptake of aspirin was observed in B16-F10 cells, with 6.19 ± 0.42 µg, 4.87 ± 0.38 µg and 6.51 ± 0.38 µg aspirin in the cells following treatment with transferrin-bearing vesicles, control vesicles and the drug solution respectively. However, the cellular uptake following treatment with free aspirin was similar to that observed in Tf- bearing vesicles in all three cell lines, which might occur by passive diffusion.

In vitro anti-proliferative activity

The anti-proliferative efficacy of aspirin was significantly enhanced when formulated in vesicles, from 1.5- to 1.8-fold depending on the cell lines (Table 1, Figure 5). The conjugation of transferrin to the vesicles further improved this efficacy, by 2.1-fold for B16-F10 cells, 2.6-fold for A431 cells and at least 2.2-fold for T98G cells, compared to that of aspirin solution. The highest anti-proliferative efficacy was observed on B16-F10 cells following treatment with aspirin loaded in transferrin-bearing vesicles (IC_{50}: 197.97 ± 15.91 µg/mL). This result correlates well with the increased cellular accumulation of the drug encapsulated in the targeted vesicles, which was the highest for B16-F10 cells amongst the tested cell lines. The treatment of A431 and T98G cells by transferrin-bearing vesicles encapsulating aspirin led to higher anti-proliferative efficacy compared

to control vesicles and free solution at most concentrations (with IC_{50} of 343.13 ± 19.24 µg/mL and 227.22 ± 8.92 µg/mL respectively on A431 and T98G cells). The cell viability following treatment with the empty carriers was higher than 90% for the 3 cell lines.

Discussion

Aspirin has been reported to show a potentially preventive and therapeutic effect in many cancers. However, the possibility of using aspirin for cancer treatment is limited by the inability of the compound to specifically reach tumors at a therapeutic concentration once administration due to its short biological half-life, rapid metabolism and elimination. To overcome this issue, we hypothesize that loading aspirin into a tumor-targeted delivery system would enhance specific delivery of aspirin to cancer cells, resulting in an increase therapeutic efficacy and reduce secondary effects to healthy tissues.

In this study, PEGylated liposomes were selected as carriers of aspirin as they demonstrated long blood circulation half- life achieved by sterically stabilized liposomes with PEG using DSPE-PEG2000 (Čeh *et al.* 1997). Liposomes also offer many advantageous properties as delivery systems, including biocompatibility, protection of the entrapped drugs from physiological environment and ability to carry large payloads of hydrophilic and hydrophobic drugs (Sercombe *et al*., 2015).

Holo-transferrin has 10 to 100-times higher affinity for the transferrin receptor than that of iron-free transferrin at physiological pH (Richardson and Ponka 1997) and was therefore preferred as a transferrin receptor ligand in our study. We successfully prepared the vesicles entrapping aspirin with high drug loading. The high entrapment of the drug into the lipid bilayer of the liposomes was a direct result of the high lipophilicity of the drug (log P 1.19). Transferrin was conjugated to the vesicles using the thiol–maleimide ‘click’ reaction, which showed similar conjugation level compared to our previous report

(around 50%) when using dimethylsuberimidate as a crosslinking (Dufès *et al.* 2000; Dufès *et al.* 2004).

Transferrin-bearing vesicles entrapping aspirin displayed the required sizes for extravasation which has been found to be 400-600 nm for most tumors (Yuan *et al.* 1995), enabling them to be taken up by the transferrin receptor-expressing cancer cells. Zeta potential experiments demonstrated that transferrin-bearing vesicles entrapping aspirin were bearing a positive surface charge, unlike the negatively charged control vesicles.

This zeta potential increase might be due to the release of ferric iron Fe^{3+} from transferrin at acidic pH. A study by Lamb and Leake (1994) demonstrated that transferrin (which is 30% iron-saturated) released a negligible quantity of ferric iron at pHs 7.4 and 7.0. However, at acidic pH 5.5, around 4 µM ferric iron Fe^{3+} were released from Tf, which represents around 10% of the total iron bound to transferrin. As aspirin is an acidic compound, it facilitates the release of the positively charged ferric iron, thus resulting in an increase in zeta potential of Tf-bearing liposomes. This increased zeta potential (between 15 and 30 mV) is associated with higher stability of the vesicles (Das *et al.* 2005). This combination of active targeting (based on the use of transferrin ligands) and passive targeting (based on the accumulation of delivery systems due to the enhanced permeability and retention) should provide tumor-selective targeting to the cancer cells.

Drug release experiment indicated that aspirin could be efficiently released from its targeted vesicular formulation in a sustained manner, within 72 hours, which might further contribute to its therapeutic efficacy. Although a large percentage of the drug was released at pH 6.5 (mimicking the tumor extracellular environment) and 5.5 (mimicking the subcellular endosome), the highest percentage of drug release was obtained at pH 7.4, the physiological pH in normal tissue and blood. This formulation should therefore be further optimized to accomplish pH-triggered drug release only extracellularly in the

mildly acidic tumor tissues and intracellularly in the more acidic endosome following transferrin receptor-mediated endocytosis. The more pronounced drug release observed at pH 7.4 may be due to the intrinsic properties of the drug. The crossing of the lipid bilayer of the vesicles would occur when aspirin (pKa: 3.5) is in its conjugate base form. The ratios of aspirin conjugate base to protonated aspirin are respectively 7943, 1000 and 100 at pH 7.4, 6.5 and 5.5. At a higher pH of 7.4, more aspirin is in the conjugate base form than at pH 6.5 and 5.5 and therefore available for crossing the lipid bilayer of the vesicles. The release of aspirin from transferrin-bearing vesicles was faster than from control vesicles. This might be explained by a possible membrane destabilizing effect of transferrin upon conjugation to liposomes, as described by Paszko and colleagues (2013) regarding another liposomal formulation. The release of aspirin from transferrin-bearing vesicles was similar to that previously described from solid lipid nanoparticles in combination with curcumin and sulforaphane (Sutaria *et al.* 2012). Albumin nanoparticles were also able to release the drug in a sustained and prolonged manner, with 90% of the drug released within 72 h (Das *et al.* 2005), similarly to our results. Other publications described a much faster release of aspirin than in our experiments. For example, 63% of aspirin was released from poly(4-vinylpyridine)-coated liposomes within 20 min at 37 °C, pH 7, followed by a slow, constant rate up to 140 min (Dong and Rogers 1993). In another study, 75% of aspirin was released from acacia-gelatin microencapsulated dipalmitoylphosphatidylcholine-based liposomes within 30 min, followed by a slow, constant release (Dong and Rogers 1993). Thus, not pH-optimal yet, the controlled release observed in our experiments could have significant implications in future experiments, as it not only limits a “burst effect” of the drug, which occurs with a simple drug solution with a peak concentration after 30 min (Das *et al.* 2005), but also allows for a prolonged release of the drug. The rapid release of the drug observed within

1 h could be explained by the fact that positively charged liposomes display drug transfer rates faster than negatively charged liposomes, leading to the rapid release of drug payloads (Hefesha *et al*. 2011). The prolonged release of the drug observed within 72 h would result in a decrease of frequency of treatment injections, and maintenance of therapeutic coverage for prolonged duration.

Cellular uptake studies demonstrated that the conjugation of transferrin to the vesicles significantly enhanced aspirin uptake in comparison with control vesicles on the three tested cell lines. These improvements were similar to that previously observed when using tocotrienol and epigallocatechin gallate as the carried drugs in transferrin-bearing Solulan-based vesicles (Fu *et al*. 2009; Lemarié *et al*. 2012). In addition, the highest uptake observed in B16-F10 cells might be a consequence of the fast growth rate of this cell line compared to the other tested cell lines. Liposomal uptake has been shown to be correlated with the growth of tumors, with fast-growing tumors having a higher drug uptake, although the causes of this correlation have not been elucidated yet (Bolkestein *et al.* 2016). The administration of aspirin as a solution resulted in a cellular uptake comparable to that observed with the transferrin-bearing vesicles. This might be explained by the fact that cellular uptake pathway of vesicles is different from that of free drug solutions: transferrin-bearing vesicles are specifically taken up by the cells overexpressing transferrin receptors via receptor-mediated (caveolae-mediated) endocytosis, control liposomes are mainly taken up by clathrin-mediated endocytosis, which is the main mechanism of nanomedicine internalization (Alshehri et al., 2018), while aspirin, which is a small molecule with a molecular weight of 188.18 Da, can be internalized by non-specific passive diffusion. We could not find any other studies reporting the cellular uptake of aspirin loaded in delivery systems to compare with our results.

In vitro treatment of the cells with transferrin-bearing vesicles and, to a lesser extent, control vesicles entrapping aspirin, resulted in an enhanced anti-proliferative activity on the three tested cell lines. Aspirin solution, however, only exerted a limited anti-proliferative effect on these cells, even though the cellular uptake was similar to that of transferrin-bearing vesicles for these cell lines. This might be explained by the fact that vesicles bearing transferrin ligand and control vesicles facilitated the internalization of the drug respectively by caveolae-mediated endocytosis and by clathrin-mediated endocytosis. It is hypothesized that the uptake of the transferrin-bearing and control vesicles via endocytosis would result in a high accumulation of aspirin in the endosome, then in the cell nucleus, which would be essential for its anti-proliferative activity, unlike the dispersed diffusional accumulation observed with the free drug. Only one previous study with aspirin entrapped in a delivery system has reported anti-proliferative efficacy of this system (Zhou *et al.* 2015), but with experimental parameters that would not allow a comparison with our current results (co-delivery of aspirin with curcumin, encapsulation in PLGA nanoparticles, anti-cancer efficacy tested on ES-2 and SKOV3 ovarian cancer cell lines).

Aspirin has previously been encapsulated in various delivery systems, such as polymer-silica composites (Kierys 2014), stearylamine-based liposomes (Kierys 2014), albumin nanoparticles (Das *et al.* 2005), acacia-gelatin microencapsulated dipalmitoylphosphatidylcholine-based liposomes (Dong and Rogers 1993) and poly(4-vinylpyridine)-coated liposomes (Dong and Rogers 1992). It has also been co-encapsulated in solid lipid nanoparticles with curcumin and sulforaphane (Sutaria *et al.* 2012). However, to our knowledge, our present study describes for the first time a formulation of aspirin encapsulated in a tumor-targeted delivery system. The resulting

targeted delivery of the drug would allow a specific release of the drug to its site of action, in order to obtain an enhanced therapeutic effect against the cancer cells.

This study demonstrated that aspirin has the ability to kill cancer cells *in vitro*, provided it is efficiently targeted to the cancer cells. However, the IC_{50} obtained with this targeted drug were still very low compared to the anti-cancer drugs commercially available, which limits its efficacy as a therapeutic molecule administered alone but encourages its use in combinatorial therapy.

Conclusion

We have demonstrated for the first time that aspirin could be formulated in tumor-targeted vesicles and that this targeted formulation could lead to an increase in the anti-proliferative efficacy of the drug in three cancer cell lines *in vitro*.

Although the cellular uptake was similar for transferrin-bearing vesicles and the free drug solution, the *in vitro* therapeutic efficacy of aspirin was significantly improved when formulated in transferrin-bearing vesicles, by about 2-fold compared to that of drug solution.

These results are promising and support the optimization of this tumor-targeted formulation of aspirin to further improve its potential as a therapeutic tool in combination with other anti-cancer therapies.

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Declaration of interest statement

The authors report no conflict of interest.

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Table with caption

Table 1. Anti-proliferative efficacy of aspirin, either encapsulated in transferrin-bearing vesicles, control vesicles or free in solution, expressed as IC_{50} values, in B16-F10, A431 and T98G cell lines (n=15).

Cell lines	IC_{50} (µg/mL) (mean ± S.E.M.)		
	Tf-bearing vesicles	Control vesicles	Aspirin solution
B16-F10	197.97 ± 15.91	244.46 ± 9.23	431.04 ± 10.91
A431	343.13 ± 19.24	> 500	893.82 ± 60.84
T98G	227.22 ± 8.92	332.24 ± 8.69	> 500

Figures

Figure 1

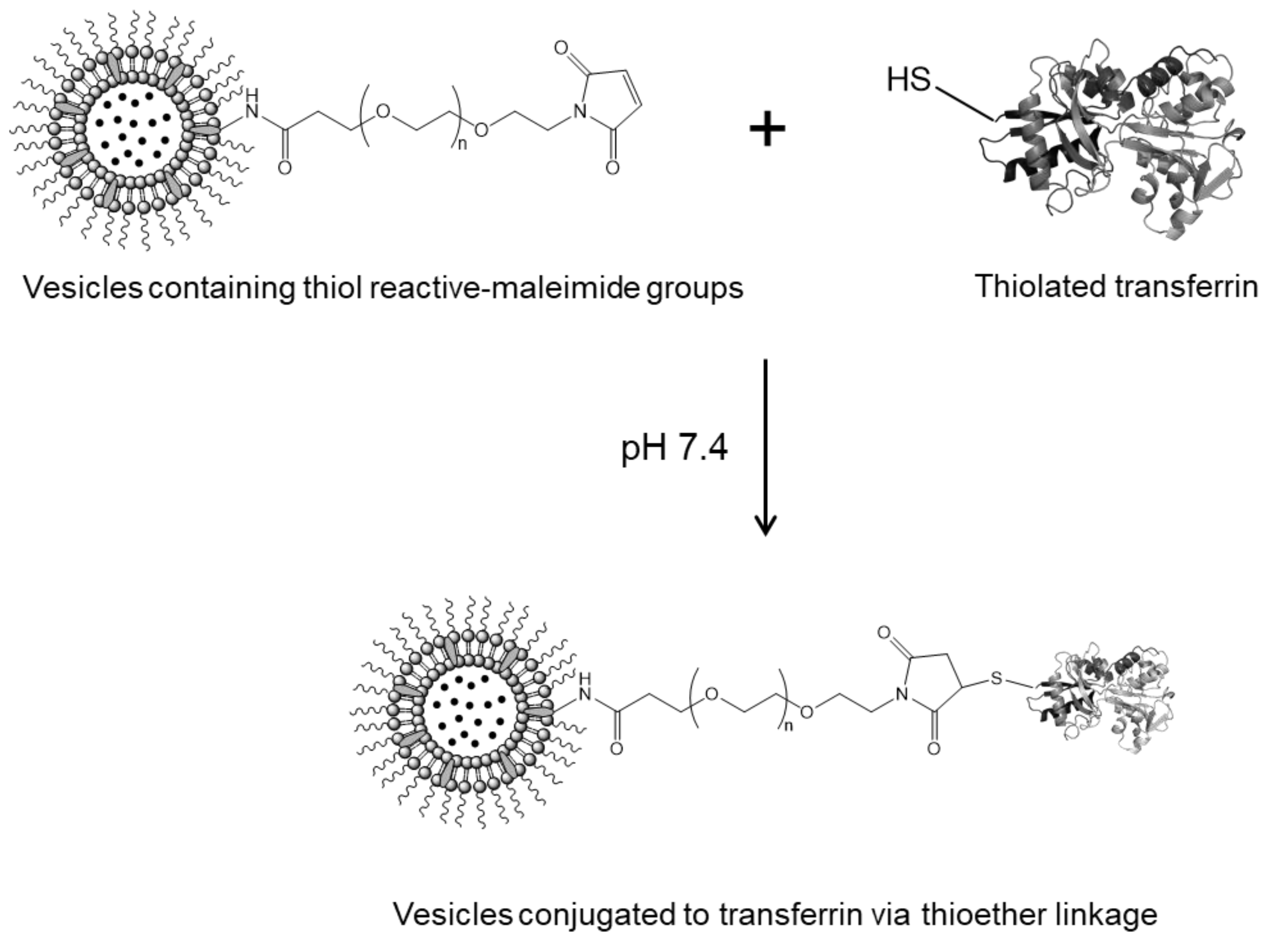
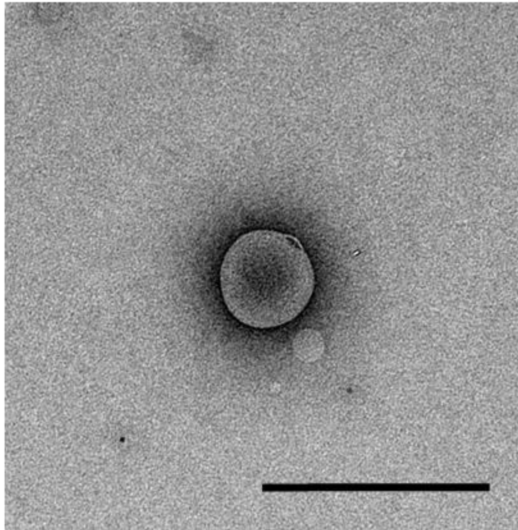


Figure 2



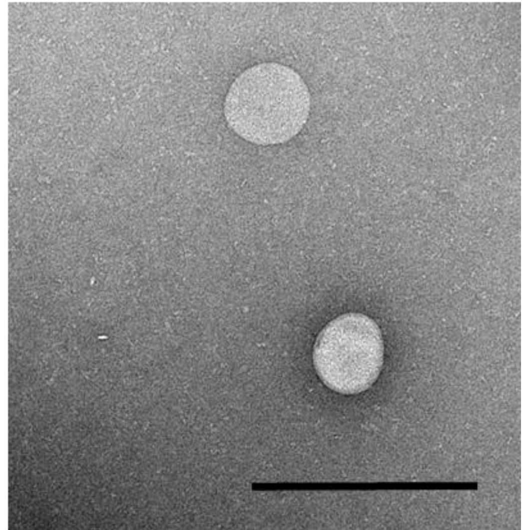


Figure 3

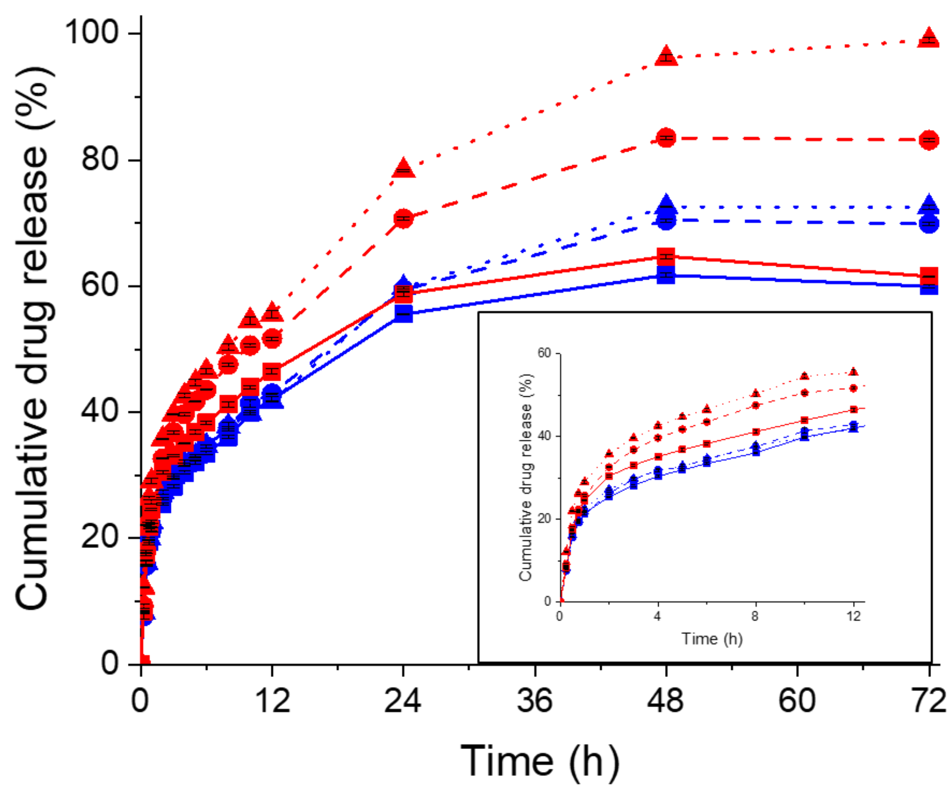


Figure 4

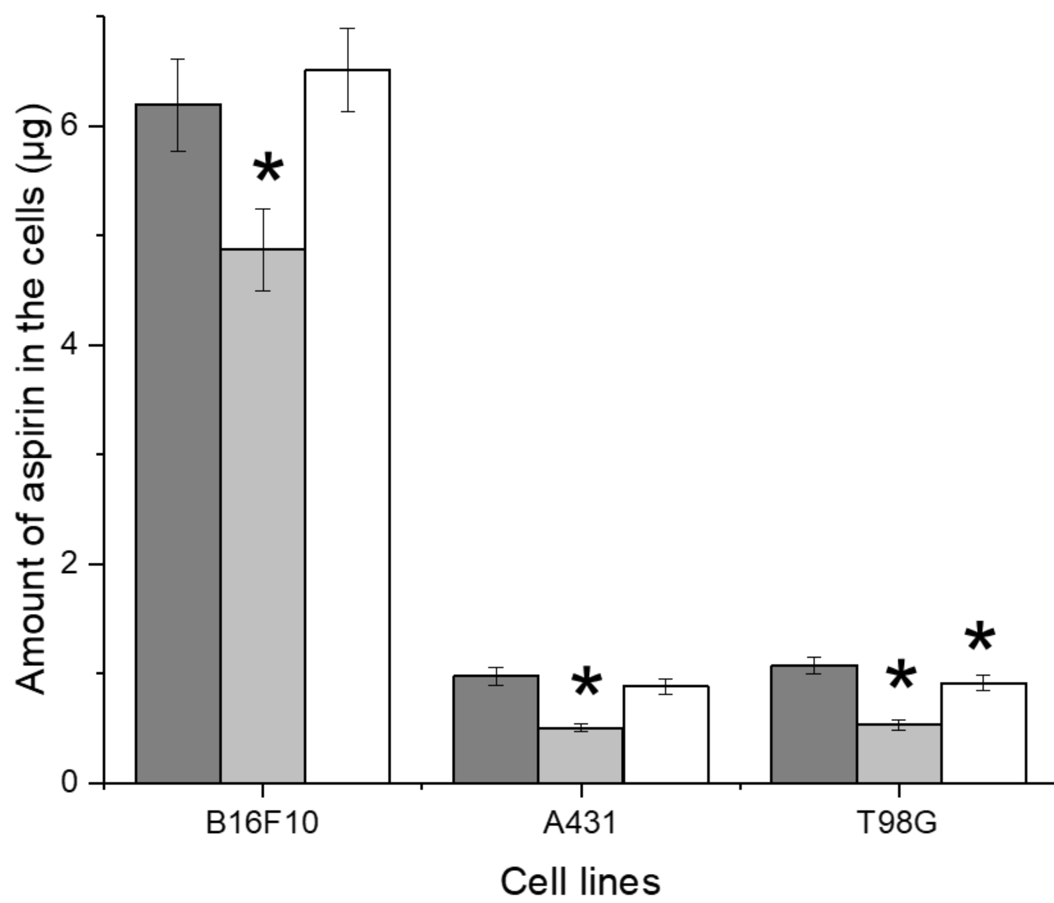


Figure 5

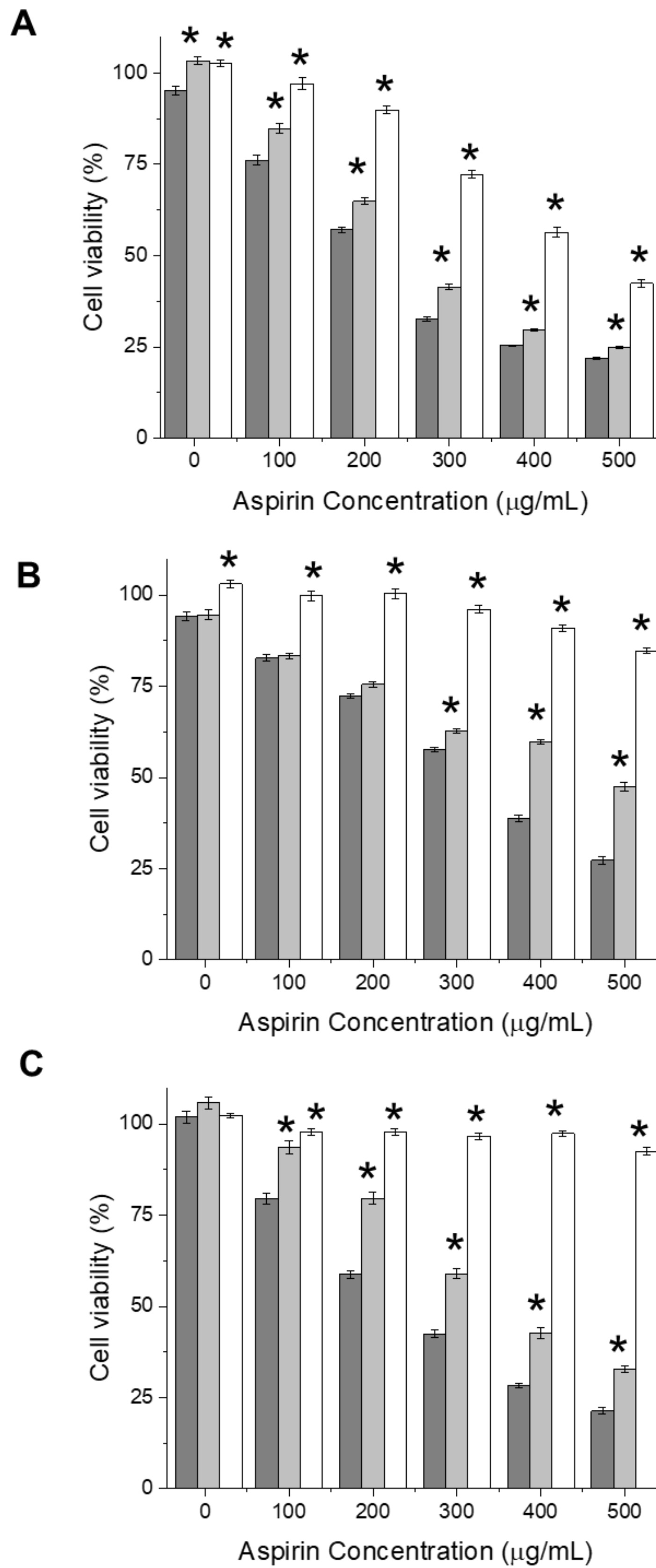


Figure captions

Figure 1. Conjugation of transferrin to the vesicles via thioether linkage

Figure 2. Transmission electron micrograph images of transferrin-bearing (A) and control vesicles (B) entrapping aspirin (Bar: 500 nm).

Figure 3. *In vitro* release of aspirin from transferrin-bearing (red) and control vesicles (blue) upon dialysis against PBS (pH 5.5 (■), 6.5 (●) and 7.4 (▲)) at 37ºC for 72 h (n=4) (insert: *in vitro* release of aspirin from transferrin-bearing (red) and control vesicles for 12h).

Figure 4. Spectrofluorimetric quantification of the cellular uptake of aspirin (200 µg/well), either encapsulated in transferrin-bearing vesicles (dark grey), control vesicles (light grey) or free in solution (white), in B16-F10, A431 and T98G cell lines (n=6) (*: $p<0.05$ vs transferrin-bearing vesicles treatment group).

Figure 5. Anti-proliferative efficacy of aspirin, either encapsulated in transferrin-bearing vesicles (dark grey), control vesicles (light grey) or free in solution (white), in B16-F10 (A), A431 (B) and T98G (C) cell lines (n=15) (*: $p<0.05$ vs transferrin-bearing vesicles treatment group).