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Magnetically Driven Bioreactors as new Tools in Drug Delivery

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Abstract

The pharmacological properties of many drugs can be improved by drug delivery systems able to drive therapeutic agents to target regions. The use of carriers, in fact, may reduce possible cytotoxic effects of drugs and increase their bioavailability at the site of action, thus improving the efficacy and the safety of treatments. Therefore, we have developed an erythrocyte-based drug delivery system (erythro-magneto-HA virosome), which has the potential to be magnetically guided to specific sites and to fuse with target cells. These engineered erythrocytes have demonstrated in previous work a very high *in vitro* capability to release anticancer drugs directly inside target cells. Because the erythro-magneto-HA virosomes (EMHVs) proved to be promising carriers, we decided to investigate in more details the effectiveness and safety of this erythrocyte-based drug delivery system.

We evaluated the ability of the EMHVs to be specifically localized *in vivo* to desired sites by means of an external magnetic field and to protect an anticancer drug such as 5-Aza-2'-deoxycytidine from degradation. Additionally we have assessed the ability of the EMHVs to act as bioreactors and to convert the pro-drug 5-Aza-2'-deoxycytidine into an active drug. Finally, we have studied the interaction of the EMHVs with the host immune system.

The pro-drug 5-Aza-2'-deoxycytidine has short half-life when systemically injected and needs to be phosphorylated to become an active drug. We found that when inside the engineered erythrocytes it is protected by degradation and is transformed in its active form thus becoming readily available for uptake by the targeted cells. Moreover, we have observed that the EMHVs used didn't cause either a cell-mediated or a humoral immune response in host mice having the same haplotype of the donors.

These findings suggest that erythro-magneto-HA virosomes are a safe and useful drug delivery system that may offer numerous advantages for several clinical applications.

Keywords: Erythrocyte-based drug delivery system;Bioreactor;5-aza-2'-deoxycytidine;Targeted therapy

Introduction

A large number of pharmacological compounds are administered systemically, however this method of treatment can often offer some limitations. Some of the compounds used in therapy can be unstable in physiological fluids due to their short half-life or may become unstable as a result of modification by endogenous enzymes. Moreover, many pharmaceutical drugs are unspecific and when diffuse into healthy tissues can cause severe side effects, thus limiting the efficacy of treatments. On the contrary, other molecules although specific can have limited access to the target sites and consequently large quantities of the pharmaceutical have to be used in order to achieve therapeutic effects.

For all of the above reasons, many efforts are made to develop new drug delivery systems capable of improving the delivery of therapeutic agents to target tissues. [1-5]. Engineered peptides [6,7], nanoparticles [8,9], and polymers [10,11] have all been proposed as potential carriers for bioactive compounds. However, these delivery systems may have different factors limiting their application including the reduced amount and/or dimensions of molecules that can be delivered, the restricted carrying capacity, and the possibility of altering the drugs release [1, 12].

In the last years, cell-based carriers including erythrocytes, leukocytes, platelets, hepatocytes, and fibroblasts have been suggested as possible vehicles for therapeutic compounds [13]. Cellular carriers constitute attractive possibilities especially to deliver those compounds, which are expensive to synthesize, have reduced half-lives, or are rapidly inactivated *in vivo* following administration [14]. In particular, the use of erythrocytes has gained remarkable interest and several types of erythrocytes have been engineered to deliver various therapeutic agents. Some of these systems include erythrocytes from mice, rats, rabbits, chicken, cattle, pigs, dogs, sheep, goats, and monkeys. There are several advantages to use red blood cells as vehicles for therapeutic compounds. In fact, because the erythrocytes can be endogenous, the possibility of acquiring a severe immunologic reaction is greatly reduced. [15,16]. Moreover, erythrocytes provide an intracellular environment, which can protect the loaded drug from degradation [16,17]. Consequently, the erythrocytes used as cell-based carriers can improve the pharmacokinetics and pharmacodynamics of drugs used in treatments [15], and at the same time can reduce potential side effects. Furthermore, the relatively simple but stable metabolic system of the red blood cells offers also an additional advantage. Pro-drugs, which have to be metabolically converted into active compounds, can be activated by endogenous enzymes following their encapsulation in the erythrocytes. Therefore, when the activated compounds reach

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the target site, they are readily available for uptake by the cells to be treated [18,19]. Because of all of the above, erythrocytes can act as either passive carriers or bioreactors that can play an active role in the pharmacokinetics of the drug. Furthermore, due to their physiological role in nature, erythrocytes have the ability to reach all tissues of the body.

In previous work we have explored the potential abilities of red blood cells to be cellular carrier and have developed a new drug delivery system with the aim of improving the release of therapeutic compounds within specific target cells [20]. By encapsulating superparamagnetic nanoparticles within erythrocytes and by loading into their membrane the viral fusogenic protein hemagglutinin (HA), we have produced erythro-magneto-HA virosomes (EMHVs), which have the potential to be magnetically concentrated to specific sites and also to fuse with target cells. To complete our delivery system we have encapsulated the demethylating agent 5-Aza-2'-deoxycytidine (5-Aza-dC) into the EMHVs, and have demonstrated that, due to the capacity of this novel delivery system to release the compound directly inside the targeted cells, very low doses of drug may be sufficient for an effective therapy [18]. Our results suggested that this system may reduce cytotoxic effects of drugs by increasing its bioavailability at the site of action, thus improving its pharmacokinetics. These features make our engineered erythrocytes an appealing candidate to be used in the treatment of cancer and other diseases that require the delivery of therapeutics to specific sites.

For this reason, in the current work we analyzed more details the effectiveness of this drug delivery system to better characterize it for its use during *in vivo* applications. In particular, we studied the quality and the amount of the 5-Aza-dC encapsulated in the EMHVs. Additionally, we have evaluated the ability of the EMHVs to localize to target sites under the application of a magnetic field and have investigated the immune response following the injections of EMHVs containing 5-Aza-dC in syngeneic animals and have demonstrated that the EMHVs represent a safe and valid drug delivery system that may offer numerous advantages for several clinical applications.

Materials and Methods

Preparation of EMHVs

Human and murine erythrocytes were obtained from blood of healthy donors by centrifugation at 400 g for 30 min and then washed twice in 1X PBS (1.37 M NaCl, 57 mM KCl, 54 mM Na_2HPO_4, 45 mM KH_2PO_4 pH 7.4). $2x10^9$ erythrocytes were lysed in 250 µl lysis buffer (10 mM TRIS, 0.1 mM EDTA, 1 mM $MgCl_2$ at pH 7.2) for 60 min at 0°C. The isotonicity was then restored by adding 130 µl of resealing buffer (65 µl of 10 X PBS pH 7.4 and 65 µl of 15 mM $MgCl_2$), supplemented with 0.1 mg of 100 nm red-labelled superparamagnetic nanoparticles (nano-screenMAG, Chemicell, Berlin, Germany), 1 µg of hemagglutinin (HA) influenza virus glycoprotein, obtained as previously described [20], and 75 µg of 5-Aza-dC (Sigma-Aldrich, Milan, Italy). The suspension was incubated for 45 min at 37°C under mild agitation. EMHVs were then collected by centrifugation at 8,000 g for 15 min at 4°C. Successively they were washed twice with 1X PBS by centrifugation at 8,000 g for 15 min at 4°C, resuspended in 1X PBS and conserved at 4°C.

Samples preparation for HPLC-MS analysis

$2x10^9$ of freshly prepared 5-Aza-dC loaded EMHVs were seeded in six-wells plates containing 3 ml of RPMI medium, and left at 37°C in an atmosphere of 5% CO_2 for 24h. Successively, 5-Aza-dC loaded EMHVs were collected, lysed and the supernatant containing 5-Aza-dC was used for HPLC-MS analysis.

HPLC-MS analysis of 5-Aza-dC

HPLC grade acetonitrile, N,N-dimethylhexylamine (DMHA), 2'-deoxyuridine (dU) and cytidine-5'-triphosphate disodium salt (CTP) were purchased from Sigma-Aldrich. Methanol and ammonium acetate were obtained from Carlo Erba (Milan, Italy).

$2x10^9$ EMHVs containing 5-Aza-dC were lysed in 200 µl of lysis buffer for 60 min at 0°C and then centrifuged at 10,000 g for 15 min at 4°C. The supernatant was transferred into a Microcon centrifugal filter device with MWCO 10,000 Da (Amicon YM-10, Millipore, Vimodrone MI, Italy) and centrifuged at 14,000 g for 2 hours at 4°C. Filtered samples were kept at -80°C until analysis. 170 ng of dU or 400 ng of CTP were added to 200 µl of the samples, as internal standards for the analysis of the degradation products and of the phosphorylated forms of 5-Aza-dC, respectively.

The HPLC system used was a Dionex 3000 Ultimate series LC (Sunnyvale, CA, USA) connected to a linear ion trap LTQ-Orbitrap mass spectrometer (Thermo Fisher Scientific, USA), equipped with an electrospray ion source. Data were acquired and processed with Excalibur 2.1 software.

Compounds were separated on a Mediterranea Sea18 reverse-phase column (150 mm, 2.1 mm I.D., and 5 µm particle size) from Teknokroma (Analytical Technology S.r.l., Brugherio MI, Italy). For all the experiments the column was set at a flow rate of 0.25 ml/min, at a temperature of 36°C, and sample volumes of 15 µl were injected.

To analyze 5-Aza-dC and its degradation products, the mobile phase consisted of 5 mM ammonium acetate (solvent A) and acetonitrile (solvent B). The first 13 minutes were an isocratic run with solvent A; between 13 and 20 minutes the percentage of mobile phase B was increased to 35%, maintained for 2 minutes and then the initial the mobile phase was re-established within 2 minutes. The mass spectrometer was operated in positive electrospray mode and the collision energy was 35 eV.

To identify the different compounds, the transitions monitored were *m/z* 229--->113 for dU; *m/z* 229--->113 for 5-Aza-dC; *m/z* 219-->103 for guanylurea derivatives; m/z 247--->131 for formylated derivatives. All the transitions were attributable to the cleavage of the bond between the nucleobase and the sugar.

5-Aza-dC standard solutions used for the calibration curve were 2, 4, 6, 8, 10 and 15 µM, all of them containing dU 8 µM. The peak area ratio of sample/dU was used for quantization.

To search for possible phosphorylated forms of 5-Aza-dC, the mobile phase consisted of 20 mM DMHA pH 7 (solvent A) and methanol-water 80:20 (solvent B). The first 12 minutes were an isocratic run with 10% solvent B; between 12 and 15 minutes the percentage of mobile phase B was increased to 80%, maintained for 1 minute and then the initial the mobile phase was re-established within 2 minutes. The mass spectrometer was operated in negative electrospray mode and the collision energy was 15 eV.

The transitions monitored were *m/z* 482--->384 for CTP; *m/z* 467--->369 for 5-Aza-dC triphosphate; *m/z* 387--->289 for 5-Aza-dC diphosphate; *m/z* 307--->208 for 5-Aza-dC monophosphate

corresponding to the elimination of a neutral molecule of H_3PO_4.

For the calibration curve, CTP standard solutions 0.1, 0.25, 0.5, 1, 3 and 6 μM were used. The peak area ratio of sample/CTP was used for quantization.

Animals

All the experiments were performed at the Animal Care Facility of Marshall University and at the animal facility of the "Toscana Life Sciences" foundation. Both Animal Facilities comply with all necessary institutional animal care and biosafety regulations.

Regarding the animal study conducted at Marshall University, they were performed in accordance with NIH recommendations and the approval of the institutional animal research committee (MU approval #458/2010). Animal care and humane use and treatment of mice were in strict compliance with (1) institutional guidelines, (2) the Guide for the Care and Use of Laboratory Animals (National Academy of Sciences, Washington, DC, 1996), and (3) the Association for Assessment and Accreditation of Laboratory Animal Care International (Rockville, MD, 1997). All the animals used in the studies conducted at Marshall University were 8- to 12-week-old female/male congenitally athymic BALB/c nude mice, homozygous for the *nu/nu* allele, bred in house. The colony of the mice was developed from breeding stock obtained from Charles Rivers Laboratories, Wilmington, MA.

Regarding the animal study conducted at the animal facility of the "Toscana Life Sciences" foundation, the Italian Minister of Health have approved the animal study proposal before becoming an active protocol (ISS Protocol approved number CNR-270111 exp 1/prot 3). All research involving animals was conducted in full agreement with EU legislation and directives (Directive 86/609/EEC of 1986 and Directive 2010/63/UE) and following the recommendations of local animal ethics committees. The experimental design and the experimental procedures were customized to avoid or at least to minimize pain, distress and other suffering for the animals' sake (3Rs). Measures to minimize the number of animals used were also taken into consideration.

Animals used in the "Toscana Life Sciences" facility were athymic nude-*foxn1*^{nu} and inbred C57BL/6 female mice, 6 weeks of age, which were purchased from Harlan Laboratories (Udine, Italy). Animals were housed in micro-isolators in the experimental animal facility in autoclaved cages with polyester fiber filter covers, under germ-free conditions. All food, water, and bedding were sterilized and the animals were maintained in an ambient temperature of 23 ± 2°C in rooms having a 12 hour light/dark cycle.

X-ray analysis

Athymic Nude-*Foxn1*^{nu} mice were injected with EMHVs prepared from human donors in the presence or absence of static magnetic field. Following tail vein injection of 1.5x10^8 EMHVs, dissolved in 150 μl of PBS, two earth magnets were placed on the lower lateral abdominal region for 30 minutes while mice were kept under 2% isofluorane anaesthesia. Magnets were made of neodinium (round shaped 5.0 mm) and their coercive force was 1000 KOersted, approximately. A control group of mice was tail vein injected with EMHVs as previously described but without the application of the magnetic field. At the end of the treatment course, mice were sacrificed by CO_2 asphyxiation and the accumulation of ferrous beads was assessed by X-ray imaging. X-ray imaging was performed using a Philips DigitalDiagnost direct digital radiography system with flat detector technology (Philips, Hamburg, Germany) with a dose of 60 kVp at 5 mAs.

Animal treatment for immunological studies

Eight inbred C57BL/6 mice were used as donors of erythrocytes for immunological studies. Murine blood was collected from donor mice by retro-orbital venous sinus bleeding, while they were under isofluorane anesthesia. The donor mice were then sacrificed by CO_2 asphyxiation. Murine erythrocytes were engineered following the procedure used for human EMHVs. 5x10^8 EMHVs dissolved in 150 μl of PBS were tail vein injected in five C57BL/6 host mice, while other five C57BL/6 untreated mice were used as controls. After 15 days from the injection, mice were sacrificed and their blood and spleens were harvested. The experiment was repeated three times with similar results.

Isolation of lymphocytes from spleens

Spleen were harvested from mice, immersed in 2 ml of RPMI medium in a 3 cm Petri dish and disrupted by scratching them through a 70 μm nylon mesh cell strainer (BD Biosciences, Buccinasco, MI) with the rubber end of a plunger from a 1 ml syringe. The grid was washed with 2 ml of RPMI medium and the cell suspension was then transferred in a tube and centrifuged at 300g for 10 min. The pellet was resuspended in 500μl RBC Lysis buffer (NH_4Cl 155 mM, $KHCO_3$ 10 mM, EDTA 0.1 mM, pH 7.2), gently mixed and then let sit for five minutes. The volume was brought up to 4 ml with PBS 1X and left to rest at room temperature to decant the membranes for a few minutes. The cell suspension was then centrifuged at 300g for 10 min and the pellet of cells were used for FACS analysis.

Isolation of lymphocytes and plasma from blood

Bloods collected in heparinized tubes were centrifuged at 400g for 10 min at 4°C. Each plasma was transferred in a new tube and kept at -20°C until analysis. The cell pellet were resuspended in 6 ml of RPMI medium and Ficoll density centrifuged at 400g for 30 min. The lymphocytes were recovered, washed twice with PBS 1X and then used for FACS analysis.

FACS analysis of lymphocytes and Antibodies anti-EMHVs

Subpopulations of peripheral blood and spleen lymphocytes were calculated using flow cytometry (Becton-Dikinson BD FACS CantoII). The surface markers on lymphocytes were detected by incubation with specific monoclonal antibodies against -CD4 (FITC-conjugated), -CD8 (APC-conjugated), -CD19 (FITC-conjugated) and -CD154 (PE-conjugated) (Miltenyi Biotech, Bergisch Gladbach, Germany).

Lymphocyte samples from both bloods and spleens were divided into two groups and used for two different analyses. One group of sample was double-stained with anti-CD4 and anti-CD8 antibodies; the other group of sample was double-stained with anti-CD19 and anti-CD154, following manufacturer's instructions (Miltenyi Biotech, Bergisch Gladbach, Germany). Double-stained samples were then analyzed by flow cytometry.

Serum levels of immunoglobulins were detected as follow: plasma from each mouse, previously treated with EMHVs, was diluted 1:5, 1:10, 1:100 and 1:1000 with 1X PBS. 1x10^8 EMHVs were resuspended in 500μl of each dilution and incubated for 1 hour at 4°C. EMHVs were washed twice in 1X PBS by centrifugation at 300 g for 5 min at 4°C and

resuspended in 100 µl of 1X PBS. Samples were then incubated with FITC-conjugated chicken anti-mouse secondary antibody (Abnova, Taiwan) at 1:500 dilution, for 30 min at 4°C. EMHVs were washed twice in 1X PBS by centrifugation at 300 g for 5 min at 4°C, resuspended in 100 µl of 1X PBS and analyzed by flow cytometry.

Results and Discussion

In vivo localization of EMHVs

Magnetic erythrocytes, resulting from the encapsulation of some ferrofluids such as cobalt-ferrite and magnetite, have been described able to localize to the desired sites under the application of a magnetic field [21,22]. To verify if EMHVs can be effectively driven to target regions *in vivo*, athymic nude-Foxn1n mice were treated with human EMHVs and exposed or not to static magnetic field. To expose mice to the magnetic field, two magnets were applied for 30 minutes in their lower lateral abdominal region. We estimated that 30 minutes were sufficient to assure repeated passages of the EMHVs near the site of interest. After the treatment, we imaged the mice by X-ray to examine the localization of the EMHVs. Figure 1 shows the radiographs of two mice treated with EMHVs in the presence or absence of static magnetic field. In the mice treated with EMHVs and magnets, it is interesting to notice a well-defined localized bright spot in the abdomen, which corresponds to the site of magnetic application (Figure 1A). On the contrary, in the mice treated with EMHVs without the application of magnets there is a rather diffuse and homogeneous radiographic evidence of metal particles throughout all the body (Figure 1B). These findings demonstrates that EMHVs can be driven and held at the target organ/tissue by means of an external magnetic field and suggest that the distribution of this drug delivery system could be controlled because of the magnetic properties of nanoparticles encapsulated inside the red blood cells.

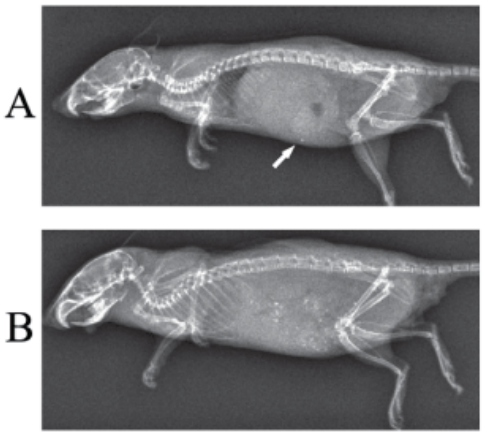

Figure 1: X-ray image of athymic nude-Foxn1nu mice injected in the tail vein with EMHVs in the presence (A) or absence (B) of a static magnetic field. The arrow indicates the accumulation of magnetic nanoparticles in a concentrated area.

Characterization of 5-Aza-dC encapsulated inside the EMHVs

It is known that 5-Aza-dC is a quite unstable compound and tends to decompose at physiological temperature and pH if free in the blood [23-25]. The main mechanism by which this molecule works is the inhibition of the enzyme methyltransferase [26]. However, its degradation products are thought to have pharmacological effects that may account for the other properties of this drug, such as its toxicity [27], its capability to induce the DNA-repair mechanism [28], and its suggested mutagenicity [29].

To better characterize 5-Aza-dC encapsulated inside the EMHVs, we performed HPLC-MS studies by evaluating the native and intact structure of this molecule, but also its principal degradation products. In a recent study, the 5-Aza-dC decomposition has been investigated by different techniques and the main degradation products were identified as formylated and guanylurea derivatives [23]. As a consequence, in addition to 5-Aza-dC, we searched also these compounds in our EMHVs. To perform this study, 5-Aza-loaded EMHVs were freshly prepared and kept at 37°C for 24 hours before HPLC-MS analysis.

Figure 2A shows the results of the HPLC-MS analysis. The HPLC peaks shown represent all the 5-Aza-dC structures found inside freshly prepared EMHVs. The four peaks in the top diagram of figure 2A identify the guanylurea derivative compounds, which are the α-pentose (RT: 2.9), β-pentose (RT: 3.9), α-hexose (RT: 4.5) and β-hexose (RT: 6.5) isomers. In the diagram in the middle of the same figure are evident the peaks of the 5-Aza-dCα- and β-anomers with RT: 5.8 and 7.4 respectively, and the peak of the internal standard dU (RT: 11.3). The peak in the bottom diagram of figure 2A represents the formylated derivatives of 5-Aza-dC (RT: 9.5).Figure 2C shows the calibration curve we used to quantify all these compounds (see materials and methods for more details).

To better understand the fate of 5-Aza-dC loaded inside the EMHVs, we analyzed their content following incubation in culture medium at the physiological temperature of 37°C for 24-hours. In these conditions, we could not find either the α- and β-anomers of 5-Aza-dC, or their formylated derivatives. The only compounds detected by HPLC-MS analysis were all four of the guanylurea derivatives of 5-Aza-dC. On the other hand, when we analyzed the free drug, we detected the presence of all the structures even after many hours of incubation at 37°C (data not shown). To understand the fate of the remaining forms of 5-Aza-dC, we performed further analysis.

Since it is known that erythrocytes are complete cells with a simple metabolic system, able to act as bioreactors [30-32], we also investigated the possibility that enzymes inside the EMHVs could phosphorylate the loaded 5-Aza-dC. This molecule, in fact is a pro-drug which requires to be activated by a phosphorylation reaction to yield the active compound [33]. Because 5-Aza-dC is a nucleoside analogue, it needs to be converted to the corresponding 5'-triphosphate nucleotide by the cell, before to be incorporated into replicating DNA. For this reason, we performed HPLC-MS analysis to verify the presence of 5'-mono, -di and -triphosphate forms of 5-Aza-dC (5-Aza-dCMP, 5-Aza-dCDP and 5-Aza-dCTP). Also in this case, we prepared 5-Aza-loaded EMHVs and left them at 37°C for 24 hours before proceeding with the analysis.

Figure 2B shows the chromatograms obtained from this study. Figure 2B on top shows the chromatogram extracted at m/z = 307, where we expected to find the monophosphate form of 5-Aza-dC. However, in the most part of the samples, we did not observe a detectable amount of this compound. On the other hand, in the second diagram from the top of figure 2B, we can see one peak around 16.6 minutes, which reveals the presence of 5-Aza-dCDP. In the third diagram from the top

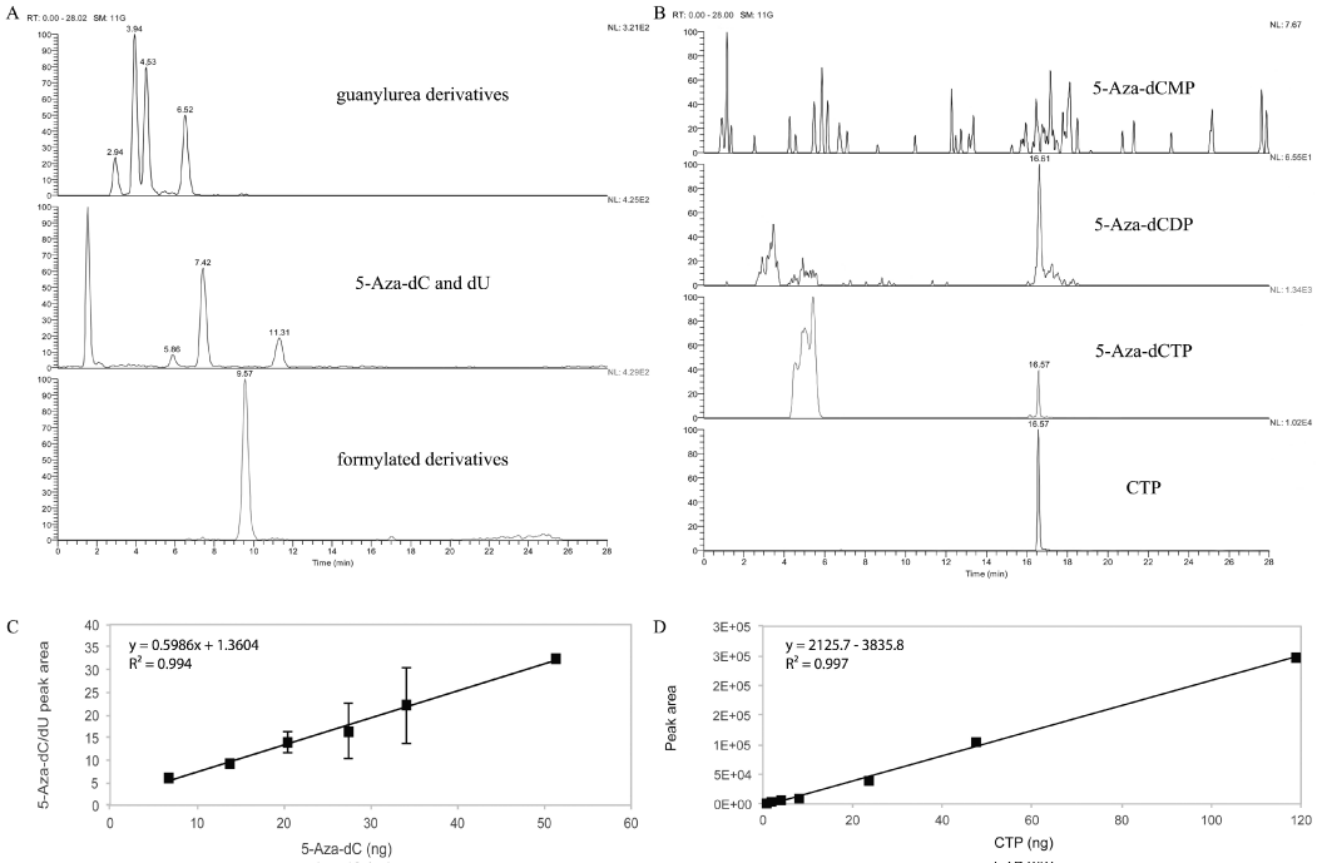

Figure 2: (**A**) HPLC-MS chromatograms of 5-Aza-dC and its degradation products. The upper diagram shows the guanylurea derivatives α-pentose (RT: 2.9), β-pentose (RT: 3.9), α-hexose (RT: 4.5) and β-hexose (RT: 6.5). The diagram in the middle displays theα-and β–anomers of 5-Aza-dC (RT: 5.8 and 7.4, respectively) and the internal standard dU (RT: 11.3). The lower diagram shows the formylated derivatives (RT: 9.5). (**B**) HPLC-MS chromatograms of the phosphorylated forms of 5-Aza-dC. From top to bottom are displayed the chromatograms for 5-Aza-dC monophosphate (5-Aza-dCMP), diphosphate (5-Aza-dCDP) (RT: 16.6), triphosphate (5-Aza-dCTP) (RT: 16.5) and the internal standard CTP (RT: 16.5). (**C**) Calibration curve reporting the correlation of the peak area ratio between 5-Aza-dC/dU and nanograms of 5-Aza-dC. (**D**) Calibration curve reporting the correlation between the peak area and nanograms of CTP.

is shown that 24 hours from loading the EMHVs, there is the presence of triphosphate 5-Aza-dC, as revealed by the appearance of a peak around 16.5 minutes. The bottom diagram of figure 2B shows the peak generated by the internal standard CTP (RT: 16.5), that we added to our samples as a control. To quantify the amount of the phosphorylated forms of 5-Aza-dC, we considered the ratio of sample/CTP. The calibration curve has been used to extract this data (Figure 2D). We found that 2x109 EMHVs contained about 60 ng of diphosphate 5-Aza-dC (5-Aza-dCDP) and 85 ng of triphosphate form (5-Aza-dCTP), thus confirming that EMHVs can also behave as a bioreactor and activate the 5-Aza-dC pro-drug.

The observation of the presence of phosphorylated forms of 5-Aza-dC within EMHVs after 24 hours can explain the absence of its α- and β-anomers, and of its formylated derivatives. We can suppose that deoxycytidine kinases inside EMHVs use both α- and β-5-Aza-dC as substrates for phosphorylation [34]. As a consequence, if the reaction proceeds long enough, the enzyme activity will cause complete depletion of these substrates. Furthermore, as the phosphorylation takes place, part of 5-Aza-dC starts to decompose giving rise to the above mentioned degradation products. However, since the formylated forms of 5-Aza-dC are intermediates, which are irreversibly transformed into guanylurea derivatives [23], if enough time is available for the reaction to take place, then 5-Aza-dC is no more available and all the degradation products will be guanylurea derivatives. This can explain why, after 24 hours of incubation of the loaded EMHVs at 37 °C, we do not find any more α-, β-5-Aza-dC and their formylated derivatives.

The conversion of 5-Aza-dC to phosphorylated 5-Aza-dC (5-Aza-dCTP) can account for the very high response to the drug that we observe when we treat tumor cells with 5-Aza-dC loaded EMHVs *in vitro* [20]. The rapidity of action and efficacy of the drug, in fact, are not only due to the ability of EMHVs to release the therapeutic compound directly inside the cells, but also to their capacity to phosphorylate the compound, so that the released drug is already activated at the time of delivery. As a consequence of this, the cells are more responsive to the delivered 5-Aza-dC in comparison to cells which are treated with free drug, even if at higher doses [20].

If we consider the 5-Aza-dC and all its derivative compounds that we found inside the EMHVs (degradation products and phosphorylated forms), we estimated that $2x10^9$ EMHVs accommodated about 120ng of total drug. Since during the preparation of the EMHVs we added 75 μg of 5-Aza-dC, it means that the efficiency of incorporation was approximately 3.5%.

Immunological analysis

Drug delivery systems are currently being used for many therapeutic applications. However, some immunological risks could be associated with their administration, and with serious clinical implications. Our engineered erythrocytes carry a potential immunogenic molecule, the HA protein inserted in their membranes, which could potentially cause some undesired immune reactions. To gain a deeper understanding into the safety of our drug delivery system, we investigated the possible immune response induced by the EMHV drug delivery system in mice.

With this aim in mind, erythrocytes from inbred C57BL/6 mice (donors) were engineered as described in material and methods, in order to obtain murine EMHVs. Based on the fact that mice have about $9x10^9$ erythrocytes per ml of blood and approximately 1.2 ml of total blood, $5x10^8$ murine EMHVs were intravenously injected via the tail vein into C57BL/6 recipient mice. This amount of engineered erythrocytes would be high enough to mount an immune reaction in an immunocompetent host and, at the same time, low enough to avoid unnecessary pain to the animals caused by an excessive increase of their hematocrit. Moreover, the use of inbred C57BL/6 mice both as donors of erythrocytes and as recipient of EMHVs allowed us to reveal possible immune reactions due exclusively to the HA of the vehicle itself. C57BL/6 mice, in fact, share the same H-2b haplotype and consequently no immune response should result from a simple red blood cells transfusion between these animals.

After 15 days from the injection, we analyzed the lymphocytes subpopulation from both treated and untreated mice in order to assess if there were changes in the levels of T and B cells in treated mice, in comparison to untreated ones, because of an immune response induced by EMHVs infusion. Additionally, we tested for the presence of antibodies produced against our delivery system in the plasma of the same mice.

The presence of specific receptors on the surface of lymphocytes allows distinguishing the different cell types. T helper cells, in fact, which assist other white blood cells in different processes, expose the CD4 protein on their surface [35]. Cytotoxic T cells, which defend the body against various infections and are responsible for reaction to transplants, are also known as CD8+ cells because of the characteristic glycoprotein they expose on the surface [35]. When CD4+ T cells become activated by specific antigens, they also expose the CD154 glycoprotein that thus can be used as molecular marker to identify them [36]. On the other hand, B lymphocytes, which produce antibodies against antigens they get in contact with, are instead characterized by the presence of the CD19 protein on their surface [37].

Therefore, using the Fluorescence Activated Cell Sorting (FACS)

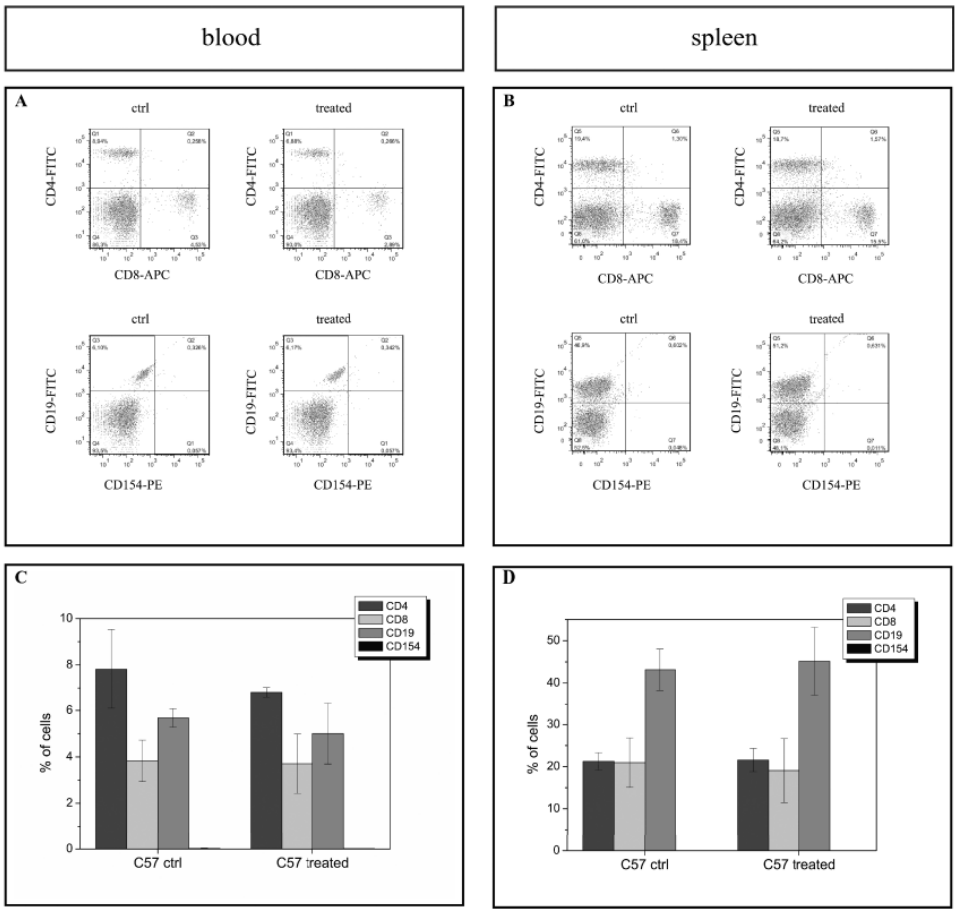

Figure 3: (**A**)Dot plots diagram showing the CD4+ vs. CD8+ and the CD19+ vs. CD154+ profiles of lymphocytes from a representative blood (A) and spleen (B) sample of a C57BL/6 mouse which was treated or untreated (ctrl) with EMHVs. Histograms reporting the different lymphocyte populations from bloods (C) and from spleens (D) of untreated (ctrl) and treated C57BL/6 mice.

method, we studied the status of CD4+, CD8+, CD19+ and CD154+ lymphocytes in treated and untreated C57BL/6 mice.

Figure 3 depicts the representative dot plots showing CD4 vs. CD8 and CD19 vs. CD154 profiles of the whole lymphocytic populations, obtained from the blood (Figure 3A) and the spleens (Figure 3B) of treated and untreated C57BL/6 mice. Moreover, the histograms in Figure 3C (blood) and 3D (spleen) represent the lymphocytic populations found in the blood and spleen of treated and untreated C57BL/6 mice, respectively. We observed that the percentages of the four different lymphocytic subpopulations were comparable between control and treated animals. Furthermore, neither in the controls nor in the treated samples we found CD154+ cells, suggesting that no animals had activated T lymphocytes and no cell-mediated immune response was induced by EMHVs in C57BL/6 mice (Figure 3C and D).

To assess if EMHVs induce early immune response and therefore the production of antibodies against our drug delivery system, we also analyzed the plasma from treated mice and compared it to control mice. EMHVs were incubated with plasma samples at different dilutions. In this way, if there were antibodies against our vehicle in the plasma of treated mice, they should bind to the EMHVs. Anti-mouse secondary antibodies FITC-conjugated were used to detect possible antibodies bound to EMHVs by flow cytometry. Figure 4 shows representative histograms of the fluorescent signal recorded from these samples. More specifically, Figure 4A displays the background fluorescence, characteristic of our vehicle, obtained by measuring the signal of EMHVs not incubated with plasma and secondary antibodies. On the other hand, Figure 4B shows the fluorescence of EMHVs incubated with the different plasma samples and secondary FITC-conjugated antibody. No changes in the fluorescence intensity has been observed in EMHVs exposed (Figure 4B) to plasma of EMHVs injected mice respect to control (Figure 4A) suggesting that no antibodies against EMHVs were produced by the C57BL/6 treated mice.

Taken together, the results of these preliminary immunological studies indicate that neither a cell-mediated nor a humoral immune response was induced by our engineered erythrocytes in C57BL/6 mice. The results suggest that EMHVs are seen as normal homologous erythrocytes by the immune system, and consequently, when injected in mice having the same haplotype of the donors, they do not cause any immune reactions. Our findings suggest that the use of EMHVs having the same surface antigens of the red blood cells of the recipient (autologous erythrocytes or erythrocytes from individuals having the same blood type) could be sufficient to ensure the safety of this drug delivery system.

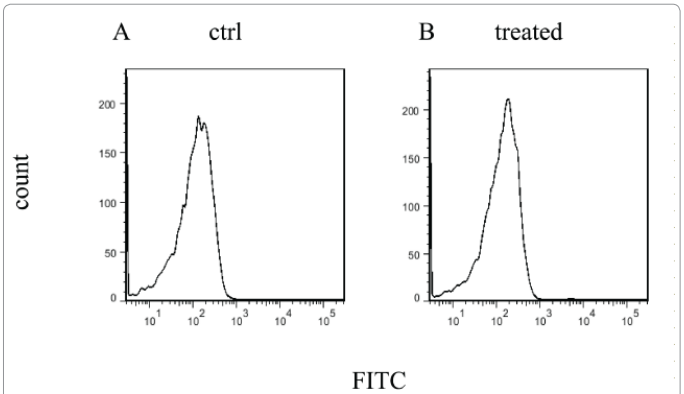

Figure 4: Histograms are showing the comparison of antibodies present in the plasma of treated vs. control mice binding to the EMHVs as detected by flow cytometry, following incubation with anti-mouse FITC-conjugated secondary antibodies. Histograms are representative of results obtained with plasma of C57BL/6 mice that were not injected [untreated (**A**) (ctrl)] or injected with murine EMHVs [treated (**B**)].

Conclusions

In this work we investigated the effectiveness and safety of EMHVs, to gain more knowledge about their use for *in vivo* applications. We observed that these engineered erythrocytes, loaded with magnetic nanoparticles are successfully driven at the target region by means of an external magnetic field. We also detected that EMHVs can metabolize the pro-drug 5-Aza-dC into the active phosphorylated 5-Aza-dC (5-Aza-dCTP) suggesting that this drug delivery system is able to deliver activated therapeutic molecules to the target cells, which can readily use them without the need for further modifications. In addition, we observed neither a cell-mediated nor a humoral immune response in mice treated with homologous EMHVs, suggesting that although these vehicles contain an immunogenic protein (HA) these vehicles have the benefit not to elicit an immune response in immunocompetent animals. All together these results highlight that EMHVs have many advantages that make them potential candidate to be used in the treatment of several diseases that require the delivery of therapeutic agents to target sites.

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