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Research paper

In vivo biodistribution of erythrocytes and polyethyleneglycol-phosphatidylethanolamine micelles carrying the antitumour agent dequalinium

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Abstract

Dequalinium (DQA), a lipophilic drug with anti-cancer activity has been incorporated into mouse red blood cells (DQA-RBCs) and polyethylene glycol phosphatidylethanolamine micelles (DQA-PEG-PE-micelles) in order to overcome the drug's solubility problems and to make it suitable for in vivo applications. The incorporation of DQA into erythrocytes, the release of DQA from RBCs in the presence of autologous plasma and the biodistribution of ^{51}Cr-DQA-RBCs and ^{111}In-DQA-PEG-PE micelles in mice has been studied. Under optimal conditions, up to 84.9% of 0.2 mM dequalinium can be incorporated into erythrocytes. The incubation of DQA-RBC with serum leads to the release of DQA over a period of 24 h. Since ^{51}Cr-DQA-RBCs were found to have a long circulation half-life (5–6 days), the use of RBCs as a sustained release system for DQA can be suggested. In contrast to DQA containing erythrocytes, however, DQA loaded ^{111}In-PEG-PE micelles displayed a shorter half-life (4 h) due to their quick uptake by the liver. The further exploration of PEG-PE micelles as a fast acting release system for DQA appears warranted.

Keywords: Drug delivery system; Drug carriers; Micelles; Red blood cells; Dequalinium; Antitumour agent

1. Introduction

Lipophilic cations with a delocalised positive charge centre (DLCs), such as dequalinium (DQA), accumulate preferentially in mitochondria of carcinoma cells in response to both the elevated cell and mitochondrial membrane potential in comparison to untransformed cells. This phenomenon provides an attractive basis for selective tumour cell killing [1,2]. DQA was shown to display anticarcinoma activity in a variety of cell lines as well as in experimental animal models [1]. The mechanism of action of DQA toxicity is not well understood, the inhibition of both, mitochondrial respiration and mitochondrial DNA synthesis has been suggested [1–3].

A severe drawback of these selective anticarcinoma agents is their limited solubility in aqueous solutions making the development of suitable drug delivery systems necessary. Recently, we have succeeded in preparing micelles made from polyethylene glycol derivatives of phosphatidylethanolamine (PEG-PE), which are able to incorporate up to 1.65 mM DQA [4].

As alternative drug carrier systems, red blood cells (RBC) are being explored. It has been demonstrated that bioactive molecules can be encapsulated into RBCs without changing their morphology and function. RBCs show unique advantages as drug carriers such as deformability, wide biodistribution, long circulatory life span, the possible use of autologous RBCs and their large inner volume for drugs encapsulation. In vitro and in vivo studies have demonstrated the usefulness of RBCs as a slow release system for antitumour agents [5].

In this communication we report the incorporation of DQA into mouse erythrocytes utilizing two methods, the incubation of RBCs with DQA and electroporation. We

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present pharmacokinetic data for DQA-RBCs and for DQA-PEG-PE micelles in mice and data about the release of DQA from DQA-RBCs in the presence of autologous mouse plasma. Our results make the further exploration of RBCs and PEG-PE micelles as drug carrier systems for dequalinium and other DLCs with anticarcinoma activity warranted.

2. Materials and methods

2.1. DQA incorporation to mouse RBCs

Whole blood was drawn from male Swiss CD1 mice (25–35 g) by cardiac punction with a heparinised (10U/ml) syringe. RBCs were collected by centrifugation (800 × *g*, 10 min, 4 °C), washed and resuspended to 50% haematocrit in 0.15 M NaCl. To prepare DQA-RBCs, erythrocytes were electroporated (Electro Square Porator T820; BTX Inc., San Diego, CA) in 0.2 mM DQA (diluted from a 10 mM DQA stock solution in 5 mM HEPES, pH 7.4), by applying two pulses of 420 V for 1 ms, every 15 min and further resealing for 1 h at 37 °C [6,7]. The incorporation of DQA into RBCs was also achieved by direct incubation of erythrocytes in 0.2 mM DQA for 1 h at 37 °C. DQA-RBCs were washed with 0.15 M NaCl to remove non-incorporated DQA. Haematological parameters of control- and DQA-RBCs were analysed (Haematology Analyser System 9000, Serono-Baker Diagnostic, Allentown, PA, USA). Cell recovery was expressed as a percentage of the initial RBC counts. The stability of DQA-RBCs was studied by dialysis against HBS (5 mM Hepes, 0.14 M NaCl, pH 7.4) for 2 days (with four changes of buffer). RBC membranes were isolated (20,000 × *g* for 20 min) after freeze–thawing to determine bound-DQA.

2.2. In vitro release of DQA to plasma from DQA-RBCs

DQA-RBCs were resuspended in autologous plasma (25% haematocrit) for 24 h at 37 °C. To avoid saturation, plasma was replaced every 2 h. DQA released into plasma or associated to RBCs was determined at different times. The binding of released DQA to plasma proteins was studied by protein precipitation (40%, v/v trichloroacetic acid). As a control, DQA (65 μM) was incubated in BSA (30 g/l) for 1 h.

2.3. ^{51}Cr-DQA-RBCs and ^{111}In-DQA-PEG-PE micelles biodistribution

Control- and DQA-RBCs were labelled with ^{51}Cr [7]. DQA-PEG-PE micelles, prepared as described previously [4], were labelled with ^{111}In-citrate [8]. Either ^{51}Cr-RBCs or ^{51}Cr-DQA-RBCs (1 mg DQA/kg) and ^{111}In-DQA-PEG-PE micelles (0.6 mg DQA/kg) were intravenously injected via the caudal tail vein into Swiss CD1 male mice (30–35 g). At different times, blood samples were drawn with heparinised Pasteur pipettes by retro-orbital puncture and radioactivity measured. Results were expressed as a percentage of the radioactivity remaining in circulation at each time relative to that found 10 min after the i.v. injection (practically 100% radioactivity, time zero). Data were fitted to a biexponential model [Eq. (1)] using non-linear regression (SigmaPlot scientific graphic software, SPSS Inc.) [7]:

$$C = Ae^{-\alpha t} + Be^{-\beta t} \quad (1)$$

where C = % radioactivity at time t, α and β = apparent rate constants for fast and slow decay components, and A and B are the percent radioactivity of both components, extrapolated to time zero. Half-life constants of the two different phases ($t_{1/2\alpha}$ and $t_{1/2\beta}$) were calculated from α and β. The area under the curve was calculated as:

$$\mathrm{AUC} = A/a + B/b \quad (2)$$

and mean clearance as:

$$\mathrm{CL} = (\text{total initial dose i.v. injected/AUC}) \quad (3)$$

Student's *t*-test was used to compare time courses of control- and DQA-RBCs. For biodistribution studies, groups of five male Swiss CD1 mice were i.v. injected with labelled RBC and micelles as described above. At different times, blood was drawn, animals killed by cervical dislocation and organs (liver, kidneys, lungs, and spleen) collected. The radioactivity in blood and organs was measured and expressed as a percentage of the total injected radioactivity.

2.4. DQA determination

DQA concentration in the different samples was measured by fluorimetry (Perkin-Elmer LS-50B Spectrophotometer, excitation 335 nm, emission 360 nm) after methanol extraction.

3. Results and discussion

3.1. DQA incorporation into mouse RBCs

The incorporation of DQA into mouse RBCs was studied by two different methods: electroporation and direct incubation. RBCs were electroporated in the presence of 0.2 mM DQA under similar conditions to those previously applied for enzyme encapsulation [6,7]. The highest DQA incorporation yield (89%) and cell recovery (86.40%) were achieved by applying two pulses of 420 V for 1 ms every 15 min, followed by resealing for 1 h at 37 °C.

The direct incubation of RBCs with DQA was performed under different experimental conditions. The incorporation efficiency of DQA into RBCs was dependent on the haematocrit, the incubation time and the temperature. The highest DQA incorporation of 84.9% and cell recovery of about 96% were achieved by incubating RBCs at 50%

haematocrit with 0.2 mM DQA for 1 h at 37 °C. About 72% of DQA incorporated was found to be strongly associated with the erythrocyte membrane. Studies on the stability of DQA associated with RBCs showed that after dialysis against HBS for 2 days, only 5% of incorporated DQA was released. The insolubility of DQA in physiological solutions [4] may account for its low release rate into HBS media and its strong association with the erythrocyte membrane. Finally, haematological parameters of DQA-RBCs prepared by direct incubation were similar to that of control-RBCs (Table 1).

Our results indicate that the yield of DQA incorporation was similar in both methods. However, the direct incubation method appears to be milder than electroporation allowing the highest cell recovery without RBCs alterations.

3.2. In vitro DQA release from DQA-RBCs to plasma

An essential feature for the use of RBCs as a drug delivery system is their capability to release the drug associated with the erythrocyte membrane. The time course of DQA release from DQA-RBCs in autologous plasma, at 37 °C, is shown in Fig. 1. It is apparent that the concentration of DQA in plasma increases continuously while the amount of DQA remaining incorporated in RBCs decreases to the same extent. After 24 h, all DQA has practically been released from the carrier. We conclude that RBCs can act indeed as a delivery system for DQA, which is in agreement with previous studies describing the release of recombinant human erythropoietin from human and mouse RBCs [9] and the release of alcohol dehydrogenase and aldehyde dehydrogenase coencapsulated into mouse RBCs [7].

Because of the limited solubility of DQA in physiological solutions, the possible association of released DQA with plasma proteins was examined. After the in vitro incubation of mouse plasma with DQA, around 45.62 ± 7.17% DQA was found associated to plasma proteins (mainly albumin, 40.95 ± 8.50%). Therefore, it can be assumed that DQA most likely will circulate

Table 1
Cell recovery and haematological parameters of control-RBCs and DQA-RBCs. DQA-RBCs were obtained by direct incubation of RBCs (50% hematocrit) with DQA for 1 h, at 37 °C

	Control-RBC	DQA-RBCs
DQA in whole cells (%)		84.90 ± 4.82
DQA in membrane (%)		71.84 ± 3.89
Cell recovery (%)	98.20 ± 0.30	96.32 ± 0.93
MCV (fl)	52.05 ± 0.51	53.23 ± 0.55
MCH (pg)	17.48 ± 0.44	17.49 ± 0.27
MCHC (g/dl)	33.55 ± 0.73	32.86 ± 0.40

MCV, mean cell volume; MCH, mean cell haemoglobin and MCHC, mean cell haemoglobin concentration. Values are the mean ± S.E.M. ($n = 12$).

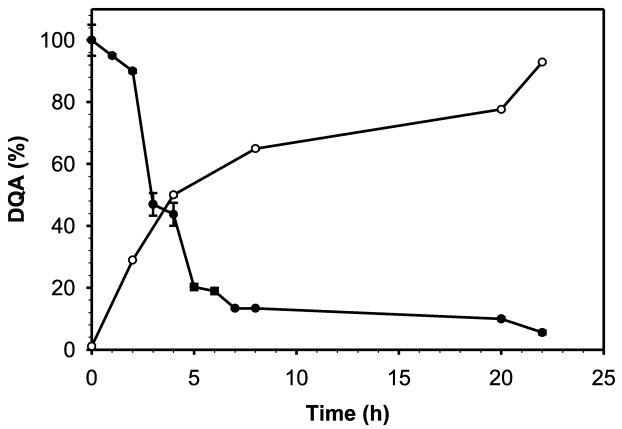

Fig. 1. In vitro DQA release from DQA-RBCs to plasma. DQA-RBCs were incubated in autologous plasma at 37 °C during 24 h. Plasma was replaced every 2 h. DQA bound to RBCs (●), DQA delivered to plasma (○). Data are the mean ± S.E.M. ($n = 5$).

associated with plasma proteins after its release from DQA-RBCs.

3.3. ^{51}Cr-RBCs and ^{51}Cr-DQA-RBCs in vivo biodistribution

To determine the half-life of dequalinium-carrying erythrocytes, ^{51}Cr-DQA-RBCs and 'for control' ^{51}Cr-RBCs were injected i.v. in mice followed by determining the blood radioactivity over time. As can be seen in Fig. 2, the decrease of blood radioactivity obeys a biphasic pattern. The initial phase, during which labelled RBCs are being removed rapidly from circulation, is followed by a prolonged phase characterized by the slow decline of radioactivity, i.e. slow elimination of labelled RBCs. The half-life constants for the two different phases ($t_{1/2\alpha}$ and $t_{1/2\beta}$) are shown in Table 2. The short $t_{1/2\alpha}$ (4 h) for the initial phase indicates that probably the most damaged

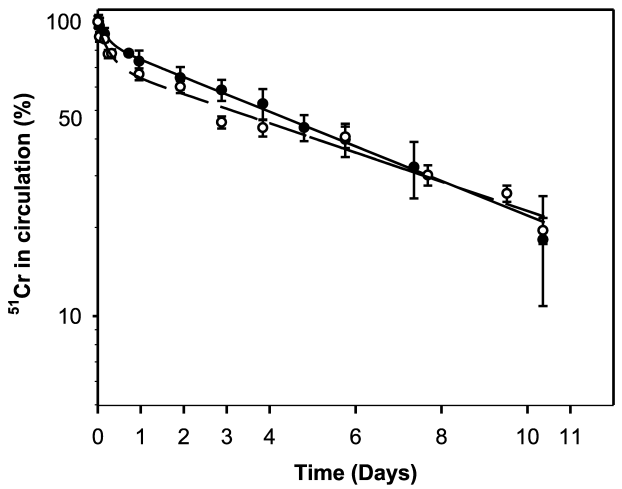

Fig. 2. ^{51}Cr-RBCs half-life in blood. In vivo kinetic in blood of control ^{51}Cr-RBCs (●) and ^{51}Cr-DQA-RBCs (○) i.v. injected into mice. Results are expressed as percentage of the radioactivity remaining in circulation at different times with respect to that measured at time zero. Data are the mean ± S.E.M. ($n = 7$).

Table 2
Mean kinetic parameters of control ^{51}Cr-RBCs, ^{51}Cr-DQA-RBCs and ^{111}In-DQA-PEG-PE micelles, calculated from the fit of data in Figs. 2 and 3 to a biexponential model using non-linear regression

	$t_{1/2\alpha}$ (h)	$t_{1/2\beta}$ (days)	AUC (% radioactivity% × day)	CL (% radioactivity/day)
^{51}Cr-RBCs	3.89	5.30	658	0.1512
^{51}Cr-DQA-RBCs	4.85	6.30	658	0.1512
^{111}In-DQA-PEG-PE	0.36	0.18	9	12

AUC, area under the curve; CL, mean clearance (see Materials and methods for details).

RBCs are quickly removed from circulation, whereas less damaged cells circulate in blood for a longer time ($t_{1/2\beta} = 5–6$ days). A similar kinetic behaviour has been observed previously for mouse RBC subpopulations enriched in young or old cells. The oldest RBCs showed shorter half-lives and were more quickly eliminated from circulation than the youngest cells, which kept circulating for longer times [10].

No significant differences ($P > 0.05$) between control- and DQA-RBCs half-life constants ($t_{1/2\alpha}$ and $t_{1/2\beta}$) could be found (Table 2). Also, the kinetic parameters, area under the curve, and clearance values were similar for both, control- and DQA-RBCs. Hence, DQA incorporation into RBCs does not seem to alter the cells' circulation in blood.

Although during the first hours, a small portion of the DQA-RBCs is quickly eliminated from the bloodstream, the majority of them circulate for at least 5–6 days thus demonstrating their potential as a sustained release system for DQA.

The biodistribution study of ^{51}Cr-RBCs shows that labelled erythrocytes are mainly taken up by the liver. The percentage of ^{51}Cr-RBCs found in the liver, decreased from 9% after the first hour to 5% after 4 h and 2.5% after 11 days. During the same time period, kidney, lungs, and spleen accumulated less than 3% of the total radioactivity. These results are in perfect agreement with the long half-lives ($t_{1/2\beta}$) for labelled RBCs with and without DQA (Table 2). Evidently, DQA-loaded ^{51}Cr–RBCs are taken up poorly by the organs of the mononuclear phagocytic system, thereby meeting an essential pre-requisite for a long circulating slow-release system.

3.4. In vivo biodistribution of ^{111}In-DQA-PEG-PE micelles

A potential drawback of micelles for use as a drug delivery system is their possible disintegration upon dilution in the blood. However, it was shown in previous studies that DQA-PEG-PE micelles, able to bind up to 1.65 mM DQA, have an extremely low critical micellar concentration (140 nM), which makes their use as a drug carrier system in blood feasible [4].

To determine their half-life, ^{111}In-DQA-PEG-PE micelles were injected i.v. into mice and the decrease of radioactivity in the blood circulation was measured over time. Our results, shown in Fig. 3 and Table 2, reveal a biphasic pattern: An initial phase characterized by the fast disappearance of micelles from circulation ($t_{1/2\alpha} = 0.36$ h) is followed by a slow phase of elimination from blood ($t_{1/2\beta} = 0.18$ days). These data demonstrate that DQA-PEG-PE micelles have a relatively short circulation time in blood. About 50% of injected micelles were removed from circulation within the first hour and the remaining 50% were completely eliminated from blood within 24 h after the injection. Consequently, PEG-PE micelles may potentially be considered as a faster acting delivery system for DQA in comparison to RBCs.

The biodistribution study indicates that ^{111}In-DQA-PEG-PE micelles are mainly taken up by the liver. One hour post injection, 12% of the total amount of radioactivity was found in the liver. This amount increased further to 30% after 4 h and remained elevated at around 20% even after 24 h. In contrast, kidney, lungs and spleen accumulated over the same period of time less than 2% of the total radioactivity. A similar distribution pattern has been found for PEG-PE micelles with incorporated protein [8] suggesting that the incorporation of DQA into PEG-PE micelles does not seem to significantly alter the biodistribution of PEG-PE micelles.

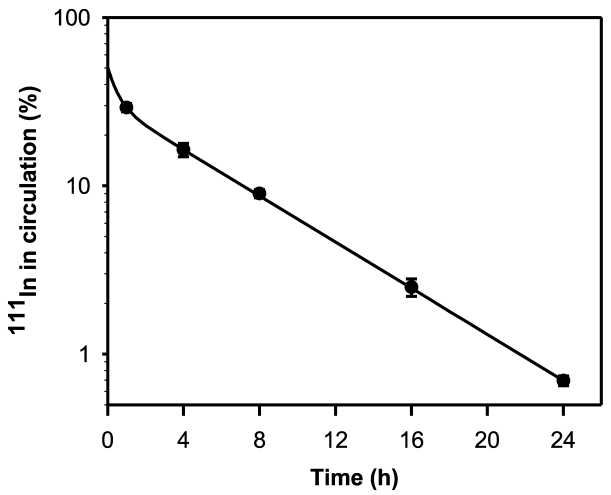

Fig. 3. ^{111}In-DQA-PEG-PE micelles half-life in blood. In vivo kinetic in blood of ^{111}In-DQA-PEG-PE micelles i.v. injected into mice. Results are expressed as a percentage of the radioactivity remaining in circulation at different times with respect to that measured at time zero. Data are the mean ± S.E.M. ($n = 5$).

In summary, we have shown that both DQA-RBCs and DQA-PEG-PE micelles remain in blood circulation for an extended period of time. DQA-RBCs circulate for several days, while DQA-loaded micelles remain detectable in circulation for up to 24 h. Radioactively labelled PEG-PE micelles are taken up by the liver quicker and at a higher proportion than labelled RBCs. Based on our results, we suggest the further exploration of RBCs as slow-acting and of PEG-PE micelles as fast-acting delivery systems for DQA and similar sparingly soluble anti-cancer drugs.

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