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Inhibition of breast cancer metastasis by dual liposomes to disturb complex formation

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ABSTRACT

The interaction between tumour cells and blood components, mainly platelets, plays an important role in metastasis. In this study, the anti-metastatic effect of vesicles containing the cytotoxic drug perifosine (OPP) and the haemostatic inhibitor dipyridamole (DIP) was tested. These dual liposomes (DIP/OPP-L) encapsulating up to 400 μg DIP/ml and 6 mM OPP were prepared by extrusion technique. *In vitro*, DIP/OPP-L significantly inhibited the aggregation of platelets and reduced their adhesion to immobilized MT3 cells by up to 60% and 24.7%, respectively. Complex formation between platelets and tumour cells *in vitro* was completely prevented by DIP/OPP-vesicles. These combinatory liposomes also inhibited the metastatic capacity of circulating tumour cells by reducing the complex formation with platelets. Formation of lung and extrapulmonary metastases after intravenous administration of MT3 breast cancer cells was significantly reduced when mice were treated with a single intravenous dose of DIP/OPP-L containing 100 nmol lipid 6 h before tumour cell inoculation.

1. Introduction

Metastasis is the major cause of mortality in cancer patients. Therefore, the search for new therapeutic targets and the development of inhibitors of tumour cell resettlement and metastatic growth is an ongoing challenge. Tumour dissemination is a complex multistep process involving inter- and intracellular signalling, activation and adhesion processes. Tumour cell arrest at distant sites requires an interplay between circulating tumour cells and endothelial cells and/or blood components such as platelets and leucocytes (Trousseau, 1865; Crissman et al., 1988; Borsig et al., 2002; Im et al., 2004).

Clear evidence exists for the participation of platelets in metastasis. In 1865, Trousseau already reported that cancer was frequently associated with thromboembolic diseases (Trousseau, 1865), and coagulation activation has been shown to facilitate tumour cell spreading (Nierodzik et al., 1991; Amirkhosravi and Francis, 1995). Platelets seem to support tumour cell colonization at remote sites and are tightly associated with tumour cells following vascular arrest, especially in the lung (Sindelar et al., 1975; Crissman et al., 1988; Borsig et al., 2002; Im et al., 2004; Gao et al., 2008).

Among the strategies to prevent or to reduce metastasis, inhibiting the interaction between circulating tumour cells and endothelial cells or platelets is a focus of intense investigations. The approaches studied included the disturbance of microemboli development (Borsig et al., 2001; Bobek and Kovarik, 2004; Mancuso and Sternberg, 2006) and the inhibition of adhesion processes (Miyake et al., 1996; Wahrenbrock et al., 2003; Zeisig et al., 2004). Anti-coagulatory drugs are known to have anti-metastatic effects by influencing cell motility and cancer cell adhesion (Hejna et al., 1999; Bobek and Kovarik, 2004; Kragh and Loechel, 2005). In addition, the alteration of platelet functionality in general was shown to inhibit metastasis (Amirkhosravi et al., 2003).

Previous investigations using sialyl Lewis^X-bearing liposomes to specifically block E-selectin to prevent tumour cell adhesion to the endothelium as a precondition to develop metastases revealed that liposomes can facilitate the formation of tumour cell–platelet aggregates and accumulated within such complexes *in vitro* (Keil et al., 2005). We hypothesized that these tumour cell–platelet aggregates could function as micro-thrombi and support the settlement of tumour cells in small blood capillaries. To prevent or disturb this aggregate formation and development could be a new approach to inhibit metastasis.

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Therefore we developed liposomes incorporating two pharmacologically active agents, attacking both tumour cells and platelets simultaneously. The encapsulation of two drugs within one vesicle has been already used to combine anticancer drugs and targeted agents (Kwak et al., 2007) because of the clear advantages of shared pharmacokinetics and distribution.

To our knowledge, no liposomal combination of a cytotoxic substance and a haemostatic inhibitor has been investigated before. We hypothesized that such a dual approach would disturb the interaction between circulating tumour cells and platelets and/or the endothelial membrane during vascular tumour cell aggregation and resettlement.

The new liposomes contained the cytotoxic alkylphospholipid perifosine (D21266, OPP) and the haemostatic inhibitor dipyridamole (DIP) (Rivey et al., 1984) and were investigated for their effect on tumour cell–platelet interaction and on adhesion processes *in vitro*. Further on, we were interested if these vesicles could influence metastasis in a breast cancer model *in vivo*.

2. Materials and methods

2.1. Material

DIP was obtained from Aldrich (Steinheim, Germany). Cholesterol and dicetylphosphate (DCP) were received from Serva (Heidelberg, Germany). N-methyl-(polyethyleneglycyl)$_{2000}$-1,2-distearyl-s,n-glycero-3-phosphatidylethanolamine (PEG-PE) was purchased from Lipomed (Arlesheim, Switzerland), and thrombin was from Sigma–Aldrich (Taufkirchen, Germany). OPP was a generous gift from Dr. Hilgard, ASTA Medica Frankfurt and egg phosphatidylcholine (PC-E) from Lipoid (Ludwigshafen). Calcein-AM was a product of Molecular Probes (Eugene, USA) and bisbenzimide fluorochrome Hoechst 33258 was obtained from Calbiochem (San Diego, USA). Solvents and high performance thin layer chromatography (HPTLC) plates were purchased from Merck (Darmstadt, Germany). Membrane tubing and weighted closures for dialysis of liposomes were purchased from Spectrum Laboratories (Rancho Dominguenz, USA).

Cell culture material was obtained from Life Technologies (Karlsruhe, Germany) or Biochrom (Berlin, Germany). Microtiter plates from Nunc GmbH & Co. KG (Wiesbaden, Germany) were used for adhesion experiments (FluoroNunc™ MaxiSorp Surface) and for fluorescence measurements (F16 Black Maxisorp).

2.2. Methods

2.2.1. Preparation and characterization of vesicles

Liposomal vesicle composition and properties are summarized in Table 1. Liposomes were prepared using a initial concentration of 32 mM from stock solutions of lipids by the lipid film/hydration method in combination with extrusion through polycarbonate filters of 400 nm pore size as previously described (Zeisig et al., 2001).

Non-encapsulated material was removed by size exclusion chromatography using Sephadex-G50. Vesicle size was determined by photon correlation spectroscopy using a Coulter Counter N5 (Coulter Electronics Inc., Hialeah, USA). The final concentrations of liposomal phosphatidylcholine (PC), OPP and DIP were determined by HPTLC as described (Zeisig et al., 2001) using dichlormethane/methanol/water/acidic acid (60/30/8/4; all volume parts) or dichlormethane/methanol/aqueous ammonia solution (80/20/5), respectively, as the mobile phase. DIP was scanned after drying using F_{Ex} 436 nm and a 460-nm edge filter.

Table 1
Liposome composition[a].

Code	Total lipid[b] (mM)	Components (molar ratio)				
		PC[c]	OPP	CH	DCP	PEG-PE
Con-L[d]	31.9	20.0		10	0.8	1.1
OPP-L	32.0	15.6	6	6	3	1.4
DIP-L	32.0	21.6		6	3	1.4
DIP/OPP-L	32.0	15.6	6	6	3	1.4

[a] Initial composition of the lipid film used for the preparation of liposomes as described in Section 2.
[b] Start concentration of all lipid components.
[c] PC: phosphatidylcholine, CH: cholesterol, DCP: dicetylphosphate, PEG-PE: polyethylene$_{2000}$-phosphatidylethanolamine.
[d] Con-L: control liposomes, OPP-L: OPP-liposomes, DIP-L: DIP-liposomes, DIP/OPP-L: liposomes containing DIP and OPP.

2.2.2. Drug release from the DIP/OPP-L in the presence of PBS

500 μl DIP/OPP-L with a total lipid amount of 9.9 μmol were injected into a Spectra/Por® dialysis membrane with a molecular weight cut off of 6000–8000. The membrane tube was sealed by Spectra/Por® closures on both ends. The liposomes were dialysed against 500 ml PBS under stirring. For each time point between 1 h and 7 days an individual sample was used and analysed for the remaining incorporated drugs.

Amounts of OPP, DIP and PC (used as internal standard lipid) were determined by HPTLC as described above (Zeisig et al., 2001).

2.2.3. Cell culture

Human MT3 breast cancer cells (Naundorf et al., 1992) were cultured to subconfluence in RPMI media supplemented with 10% fetal bovine serum (FBS) and 2 mol/ml L-glutamine, at 37 °C, 90% humidity and 5% CO_2. The cells were detached by trypsin digestion (0.25% trypsin, 0.53 mmol EDTA/L phosphate buffered saline (PBS)).

2.2.4. MTT assay

The influence of liposomal formulations on the proliferation of MT3 tumour cells was analysed with a MTT assay as described previously (Zeisig et al., 1998), except that 1×10^5 MT3 cells were incubated for 24 h with DIP/OPP-L, the liposomes containing only one or none of the active drugs as well as the free substances DIP and OPP, all serially diluted with medium to concentrations between 200 and 12.5 μM.

2.2.5. Platelet isolation

Human platelets were isolated from platelet aphaeresis concentrates provided by Haema, Berlin as follows: platelets and plasma were separated by centrifugation at 1000 × g for 15 min at 22 °C. The platelets were washed three times using citrate buffer (pH 6.5) (Keil et al., 2005) followed by centrifugation at 1000 × g for 15 min at 22 °C after each washing step. Finally, the platelets were resuspended in plasma or Tyrode-Hepes-2 buffer (pH 7.4) (Keil et al., 2005) to the appropriate number.

2.2.6. Labeling platelets and tumour cells

Platelets: platelets in citrate buffer (2.5×10^8 cells/ml) were incubated with 25 nmol of calcein-AM-solution (0.5 μmol/ml dimethyl sulfoxide) for 90 min at 37 °C. Calcein-labelled platelets were washed with citrate buffer and, after centrifugation (1000 × g for 15 min), finally resuspended to an appropriate number in TH-buffer. Fluorescence was measured at F_{Ex}: 490 nm and F_{Em}: 530 nm.

MT3 cells: tumour cells cultured in a 25-cm^2 cell culture flask were labelled after media removal by incubation in 2 ml of Hoechst 33258 (50 μM in PBS) for 30 min at 37 °C and harvested by trypsin digestion and resuspended in PBS containing 2 mM Ca^{2+}. Fluorescence was measured at F_{Ex}: 355 nm and F_{Em}: 460 nm.

2.2.7. Platelet aggregation in vitro

Platelet aggregation was quantified by following the change in electrical impedance using a Chrono Log aggregometer. Human

platelets (2.25×10^8 in 900 μl plasma) were co-incubated with liposomes containing 32 μg DIP and/or 0.166 μg OPP, or equal amounts of free drugs. Platelets were activated by adding 2 μg/ml collagen and impedance was measured for 20 min at 37 °C with continuous stirring until a steady state was reached. Each value presents the mean maximal amplitude [$\Omega \pm$ S.D.] for five independent measurements, each done in duplicates.

2.2.8. Aggregate formation between platelets and MT3 breast carcinoma cells in vitro

Platelets (2.5×10^6) and fluorescently labelled MT3 cells (1×10^4) were mixed with 10 nmol liposomes in 100 μl TH-buffer, 0.01 U thrombin was added and the suspension was shaken for 20 min at room temperature. The formation of tumour cell aggregates was analysed by microscopic quantification as described recently (Keil et al., 2005).

The results present the mean number of MT3 cell complexes with at least five cells counted in two representative areas with a total of 80–160 tumour cells from each area. Each experiment was performed in duplicate for three different platelet populations.

To prepare micrographs of tumour cell–platelet aggregates, calcein-AM-labelled platelets were mixed with MT3 cells (Hoechst 33258-labeled) and liposomes, and the above procedure was repeated. Single photographs of different fluorescence channels were used to prepare the final overlay.

2.2.9. Adhesion of platelets to immobilized tumour cells in vitro

MT3 tumour cells (1×10^5 cells/well) were immobilized in a 96-well microtiter plate (test plate) over night. Tumour cells were incubated in 1% BSA at ambient temperature to prevent unspecific binding. The cells were washed, and 2.5×10^7 platelets in 100 μl were added to each well followed by 0.05 U thrombin/well for activation. The cells were finally incubated for 30 min at 37 °C with EDTA (10 μmol), DIP/OPP-L (100 nmol total lipid with 17.3 nmol OPP and 1.46 μg DIP), DIP-liposomes (1.46 μg DIP), OPP-liposomes (17.3 nmol OPP), or control liposomes (100 nmol total lipid).

After washing with PBS, 50 μl lysis buffer was added to each well. The plates were shaken for 3 min and contents of each well were transferred to a black Maxi-Sorp plate (well-to-well) followed by fluorescence measurement with a Fluostar spectrophotometer at λ_{Ex} = 490 nm and λ_{Em} = 530 nm.

Tumour cell-bound platelets were quantified using fluorescence-labelled platelets, which were seeded in defined numbers, serially diluted, into a separate black Maxi-Sorp plate to obtain a standard curve for quantification. Each test was performed in triplicate, and at least four independent experiments were performed.

2.2.10. Experimental metastasis in vivo

All animal experiments were performed according to the German Animal Protection Law and with approval of the local responsible authorities.

Female NMRI:nu/nu mice were obtained from Taconic Europe, Denmark. The animals were housed under pathogen-free conditions in individually ventilated cages (22 °C room temperature, 50 ± 10% relative humidity, 12 h light–dark rhythm). They received autoclaved food and bedding (Sniff, Soest, Germany) and acidified (pH 4.0) drinking water *ad libitum*.

2.2.11. Platelet functionality in vivo

Four female NMRI:nu/nu mice per group received intravenously (i.v.) 100 μl liposome suspension containing 100 nmol total lipid of DIP/OPP-L. At different time points mice were sacrificed and blood was taken from tail vain and collected in citrate buffer containing vials. Blood was immediately used for aggregation measurements as described above.

Each of 4–6 female NMRI:nu/nu mice per experimental group received i.v. 5×10^6 human MT3-breast carcinoma cells. Mice were treated i.v. with 100 μl liposome suspension containing 100 nmol total lipid of DIP/OPP-L 6 h before tumour cell inoculation. Control mice were treated with PBS. In additional control groups, mice were injected with 100 μl of liposomes containing one single drug, or with the free drugs, all in drug concentration corresponding to that in DIP/OPP-L, as indicated in Fig. 4B. Mice were sacrificed after 29–33 days and the number and location of the metastases were determined visually.

2.2.12. Statistical evaluation

Statistical comparisons of data were performed with the unpaired Student's *t*-test for two populations. Differences were considered to be significant at $p < 0.05$.

3. Results

We prepared liposomal vesicles containing both OPP and DIP (DIP/OPP-L). The composition of the liposomes and control formulations are shown in Table 1. These liposomes were prepared for the first time by the lipid film hydration/extrusion method. The incorporation of a sufficient amount of both drugs into the membrane was possible if 32 μmol of basic lipid (phosphatidylcholine and cholesterol) was used. In addition, a negatively charged lipid (DCP) and PEG_{2000}-phosphatidylethanolamine were incorporated into the vesicle membrane to prevent macrophage uptake.

DIP/OPP-L and DIP-liposomes were stable at 4 °C for at least 119 days (storage stability), whereas OPP-liposomes and control liposomes exhibited earlier size increases, indicated by changes in the shape of the liposomes (Table 2).

Additionally, the release of the two drugs DIP and OPP from DIP/OPP-L in PBS was determined by dialysis. Fig. 1 shows, that the concentrations of the basic lipid PC and of the cytotoxic drug OPP remained constant over 7 days, indicating that the DIP/OPP-L are stable in PBS under *in vitro* conditions. On the other hand, a significant release of the haemostatic inhibitor DIP was determined after 24 h of dialysis. After 7 days of dialysis only 42% of the DIP

Table 2
Drug content and size of liposomes used.

Liposomes code	Total lipid[a] (mM)	DIP (μg/ml)	OPP (mM)	Diameter[b] (nm)	PI[c]	Stable until day[d]
Con-L	31.0 ± 1.1			188 ± 13	0.18 ± 0.04	38
OPP-L	31.2 ± 4.3		3.6 ± 0.7	213 ± 20	0.20 ± 0.02	38
DIP-L	25.7 ± 0.1	111.3 ± 12.9		134 ± 9	0.20 ± 0.04	193
DIP/OPP-L	25.3 ± 3.4	148.7 ± 24.0	4.3 ± 0.5	137 ± 27	0.12 ± 0.06	119

[a] Final total lipid was calculated from PC content as determined by HPTLC and the molar composition (Table 1).
[b] Mean diameter ± standard deviation based on unimodal analysis determined by PCS as described in Section 2.
[c] Polydispersity index, indicating a size distribution varying between monodisperse (PI = 0) and polydisperse (PI = 1).
[d] Liposomes were considered to be stable in buffer at 4 °C (storage stability) if the size of vesicles was constant until the indicated day when an increase in diameter greater than 5% was recognized.

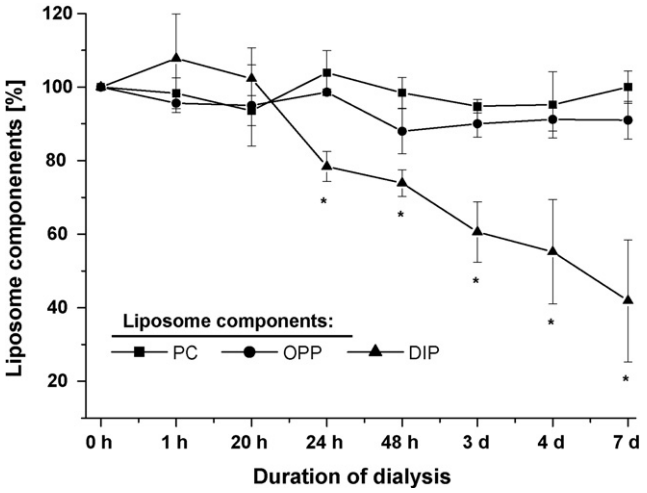

Fig. 1. Drug retention in DIP/OPP-L in the presence of PBS *in vitro*. The release of the liposomal incorporated drugs OPP and DIP in the presence of PBS was measured in comparison to the lipid component phosphatidylcholine (PC). The concentration of PC, OPP and DIP was detected via HPTLC measurements as described in Section 2. Given is the remaining concentration of the liposomal components PC, OPP and DIP in percent ± S.D. after dialysis in relation to the start concentration ($t = 0$). Results were obtained from two independent experiments. *Significantly different to the starting point $t = 0$ ($p < 0.05$).

amount in relation to the start concentration ($t = 0$) was found in the membrane.

As expected, the MTT assays showed, that OPP, OPP-liposomes and DIP/OPP-liposomes had an inhibitory effect on MT3 tumour cell proliferation with an IC_{50} of 13.8 ± 4.2, 27.9 ± 5.9 and, 70.9 ± 30.6 μM, respectively (data not shown), which agrees with earlier findings (Stekar et al., 1993; Zeisig et al., 2001).

The effect of liposomes on platelet aggregation in plasma was characterized by co-incubation of platelets with the vesicles and measurement of the electrical impedance. Collagen activation of the platelets produced an impedance value of 24 Ω, whereas the incubation of non-activated platelets over 20 min did not result in an impedance value. The results (Fig. 2A) demonstrate a significant inhibition of platelet aggregation by all liposomes except drug free control liposomes with a reduction between 60% and 15%.

These results are consistent with data obtained for the binding of platelets to tumour cells in the presence of DIP/OPP-L by a static adhesion assay using immobilized MT3 tumour cells. Non-activated platelets adhered to tumour cells only in a very low number (2.7%). An activation with thrombin enhanced this binding by 81%. Both 100 nmol DIP/OPP-liposomes or DIP-liposomes significantly reduced the adhesion of activated platelets by about 25%. OPP-liposomes and control liposomes did not affect adhesion, whereas EDTA strongly prevented any platelet binding (Fig. 2B).

Fig. 3 presents micrographs for the co-incubation of MT3 breast cancer cells and thrombin-activated platelets without and in the presence of liposomes. For the control non-activated platelets were incubated with tumour cells, where no aggregate formation between platelets and tumour cells could be observed (Fig. 3A). Platelets were found in large numbers close to or between aggregated tumour cells when the platelets were activated with thrombin (Fig. 3B). Addition of DIP/OPP-L significantly reduced both the number and size of aggregates to the level of MT3 cells in the presence of non-activated platelets (Fig. 3C).

Aggregate formation of tumour cells in the presence of platelets was also quantified after spinning down the mixture onto glass slides to obtain micrographs (Table 3). When non-activated platelets were mixed with tumour cells, an average of only 2.3 complexes per evaluated area of these micrographs was determined, these complexes bound 18.1% of the tumour cells found in the investigated area. Platelet activation by thrombin significantly increased the number of aggregates to 5.9 per evaluated area compromising 31.3% of all tumour cells counted in the area. This aggregation could be completely prevented by a co-incubation with DIP/OPP-L, which reduced the number of complexes to 1.8, enclosing 16.9% of the tumour cells. Whereas DIP-liposomes still weakly but significantly inhibited the number of aggregates, no effect was observed for OPP-liposomes or drug free control liposomes.

DIP/OPP-L were further on investigated in the human MT3 breast cancer xenograft model in nude mice. In this metastasis model, a single i.v. bolus injection of 5×10^6 human MT3 breast carcinoma cells produces lung and extrapulmonary metastases.

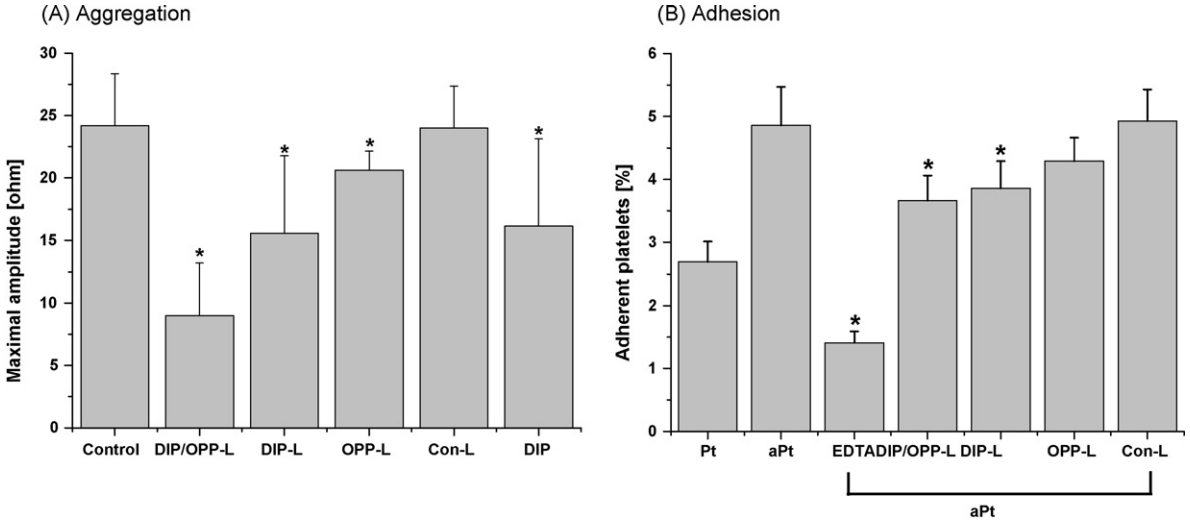

Fig. 2. Platelet aggregation and adhesion *in vitro*. (A) Aggregation of platelets in the presence of liposomes *in vitro*. Platelet aggregation in the presence of liposomes was determined by impedance measurements as described in Section 2. Given is the maximal amplitude (mean Ω ± S.D.), determined after 20 min for five independently performed experiments, each done in duplicate. *Significantly different to control ($p < 0.05$). (B) Adhesion of platelets to MT3 tumour cells in the presence of liposomes *in vitro*. Binding of 2.5×10^7 activated, fluorescently labelled platelets (Pt) to immobilized MT3 tumour cells at 37 °C in the presence of buffer (control), liposomes (100 nmol total lipid), or EDTA (10 μmol) was determined after 30 min incubation by fluorescence measurements of platelets. Displayed is the mean number of bound platelets ± S.D. in percent of added platelets for four independently performed experiments, each done in triplicate. *Significantly different to control ($p < 0.05$).

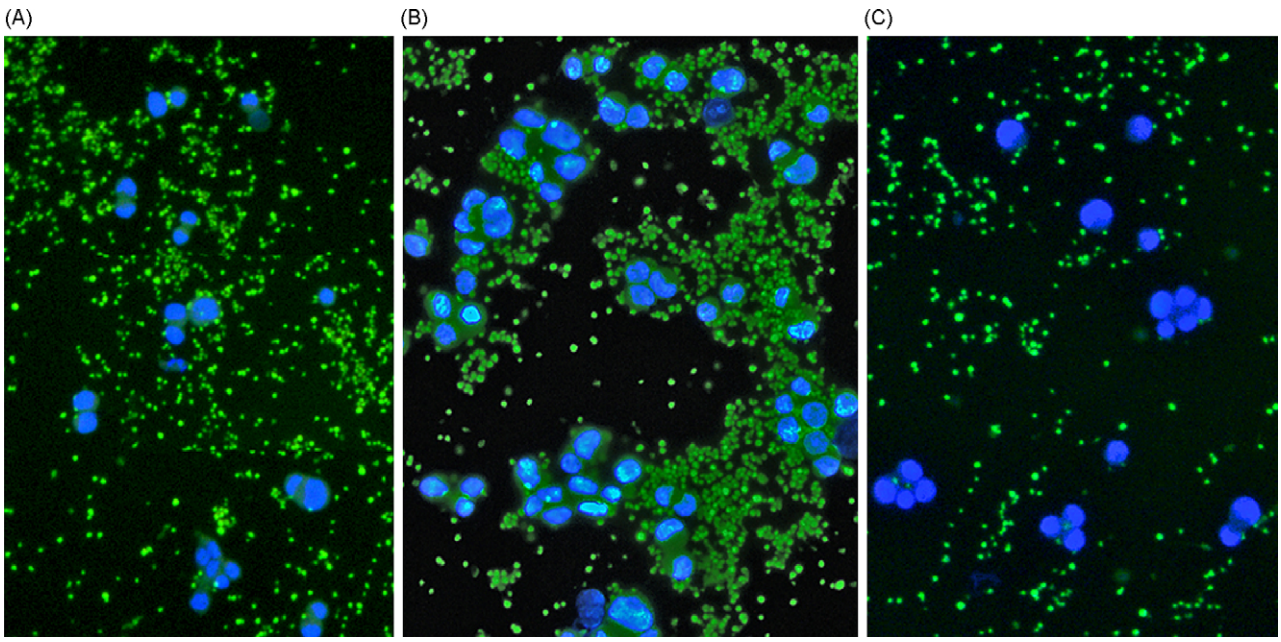

Fig. 3. Complex formation of MT3 tumour cells in the presence of platelets and liposomes *in vitro*. Tumour cells (blue) and fluorescently labelled platelets (green) were incubated with liposomes and micrographs (magnification: 20×) were prepared as described in Section 2. (A) Platelets (without activation) and MT3 breast cancer cells, (B) activated platelets and MT3 breast cancer cells and (C) activated platelets and MT3 cells co-incubated with DIP/OPP-liposomes.

Table 3
Influence of liposomes on aggregate formation *in vitro*.

MT3 cells in the presence of[a]	MT3 cells[b]	Aggregates[c]	MT3 cells in aggregates[d] (%)
+Pt	81 ± 35	2.3 ± 2.0*	18.1 ± 13.0*
+aPT	140 ± 65	5.9 ± 3.2	31.3 ± 13.9
+aPt + Con-L	157 ± 56	6.9 ± 2.9	35.6 ± 11.0
+aPt + OPP-L	140 ± 48	5.1 ± 2.1	29.1 ± 10.0
+aPt + DIP-L	103 ± 61	3.4 ± 3.0*	23.7 ± 13.4
+aPt + DIP/OPP-L	82 ± 34	1.8 ± 1.5*	16.9 ± 11.7*

[a] MT3 cells were mixed with the indicated components (Abbr.: Pt: platelets; aPt: activated platelets; Con-L: control liposomes; OPP-L, DIP-L and DIP/OPP-L: liposomes with entrapped dipyridamole, OPP or both drugs, respectively) as described in Section 2.
[b] Number of MT3 cells in the evaluated micrograph field.
[c] Number of aggregates (five or more MT3 cells) per evaluated micrograph field.
[d] Mean percentage of MT3 cells in aggregates related to total number of tumour cells in the evaluated micrograph field.
All data are given as mean values ± S.D. for 3 different platelet preparations, each determined in duplicate.
* Significantly different to "MT3 cells + aPt" (Student's *t*-test, $p < 0.05$).

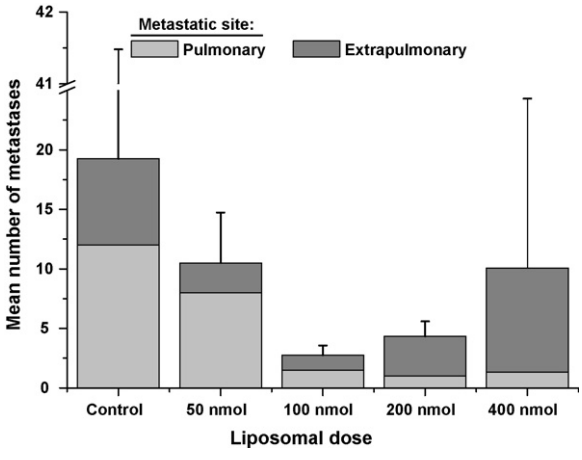

Fig. 4. Influence of liposomal dose on metastasis. Mice were treated i.v. with the indicated doses of DIP/OPP-L 6 h before i.v. injection of 5×10^6 MT3 tumour cells to 4 NMRI:nu/nu mice per group. After 30 days the mice were sacrificed to determine the number of metastases. Given is the mean number of metastases ± S.D. for one experiment.

First, the optimal dose was determined to be 100 nmol of total lipid for DIP/OPP-L (Fig. 4). An analysis of platelets in mice blood after liposome treatment revealed that an injection 6 h prior tumour cell inoculation resulted in the strongest impairment of platelets aggregation (Fig. 5). Thus, this time point was chosen for all experiments.

Mice treatment with a single dose of 100 nmol total lipid DIP/OPP-L 6 h before tumour cell injection, resulted in a significant reduction in both lung and extrapulmonary metastases as summarized in Fig. 6A for three independent experiments. The liposomal combination was superior to the simultaneous application of both non-encapsulated drugs in corresponding doses.

Finally, the efficiency of DIP/OPP-L was compared to additional controls of corresponding drug concentration (Fig. 6B). The most efficient reduction in the number of metastases was obtained with DIP/OPP-L, whereas OPP-L and both free drugs had only a week inhibitory effect. Extrapulmonary metastases were mainly found in the extremities of the mice, sometimes also in the neck/head, but often associated with lymph nodes. To characterize these metastatic localizations, detailed investigations are ongoing.

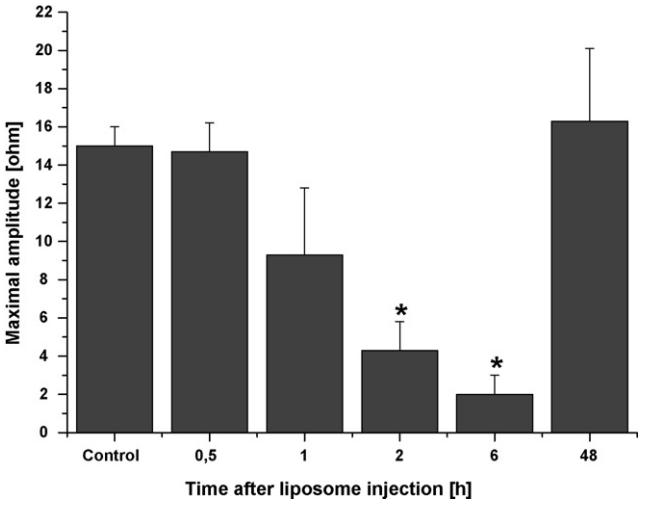

Fig. 5. Effect of *in vivo* liposome administration on platelet aggregation *ex vivo*. Blood was taken from of nude mice after i.v. injection of 100 nmol total lipid of DIP/OPP-L at the indicated time points and maximal platelet aggregation was determined with an aggregometer. The results present the maximal amplitude (mean $\Omega \pm$ S.D.) after 20 min in a typical experiment performed with four mice per group. *Significantly different to control ($p < 0.05$).

4. Discussion

Complex and dynamic processes are necessary for the formation of blood-borne metastases involving numerous interactions between tumour cells and host tissue or blood cells.

A liposome-based delivery system can be an interesting tool to interfere with interactions which are necessary for the formation of blood-borne metastases.

Such vesicular systems have been used to inhibit tumour cell adhesion (Bloemen et al., 1995; Saiki et al., 1996; Mastrobattista et al., 1999; Kessner et al., 2001; Zeisig et al., 2004; Gosk et al., 2005; Maeda et al., 2006), angiogenesis (Ichikawa et al., 2005; Fidler, 2006) and invasion (Asai and Oku, 2005). In addition, circulating tumour cells (Raffaghello et al., 2003; Rivera, 2003), platelets (Kim et al., 2003), and macrophages (Derksen et al., 1988; Fidler, 1992; Anderson, 2006) have been targeted by liposomes to prevent adhesion of tumour cells to endothelial cells, to avoid platelet aggregation, and to prevent activation of macrophages, respectively.

The crucial role of platelets in metastasis was recently described more in detail (Hejna et al., 1999; Borsig et al., 2002; Im et al., 2004). Considering that the coagulation system is intimately involved in metastasis (Amirkhosravi and Francis, 1995; Hejna et al., 1999; Al-Jamal and Kostarelos, 2007) and taking into account our own data showing the effect of sialyl Lewis^X-bearing liposomes on complex formation between tumour cells and platelets (Stahn et al., 2001; Zeisig et al., 2004; Keil et al., 2005), we decided to target the aggregates formed by tumour cells and platelets in the blood circulation using a liposomal formulation that encapsulated two drugs to attack platelets and tumour cells simultaneously. Thus, we hoped to either prevent aggregate formation by entrapment of a haemostatic inhibitor, or to inhibit the proliferation of the tumour cell(s) in the aggregates with a cytotoxic drug, once they had developed. Therefore, we selected the haemostatic inhibitor DIP (Rivey et al., 1984) and the cytotoxic alkylphospholipid OPP (Stekar et al., 1993) to formulate these dually active liposomes.

DIP-liposomes have already been used to treat acute respiratory distress syndrome (Ji et al., 2006), but not yet to prevent aggregation during metastasis. To our knowledge, a combinatory liposomal formulation encapsulating an anticancer drug and an anticoagulant for inhibiting metastasis has not been previously described. The advantage of such vesicular formulation is that both drugs have the same pharmacokinetics and distribution (Mayer et al., 2006; Al-Jamal and Kostarelos, 2007).

The dual DIP/OPP-L formulation described herein had a high potential *in vitro* to (a) inhibit platelet aggregation, (b) reduce the adhesion of platelets to tumour cells, and (c) prevent complex formation between platelets and tumour cells. Each of these effects appear to contribute to the inhibition of metastasis that was demonstrated *in vivo* using a human MT3 breast cancer xenograft model.

The superior effect of this combination approach, as compared to liposomes containing only one drug or to the individual free drugs, is only convincing if simultaneous contribution of both drugs is taken into account. Both free and liposomal DIP restricted platelet functionality, but to a lower degree than that obtained by com-

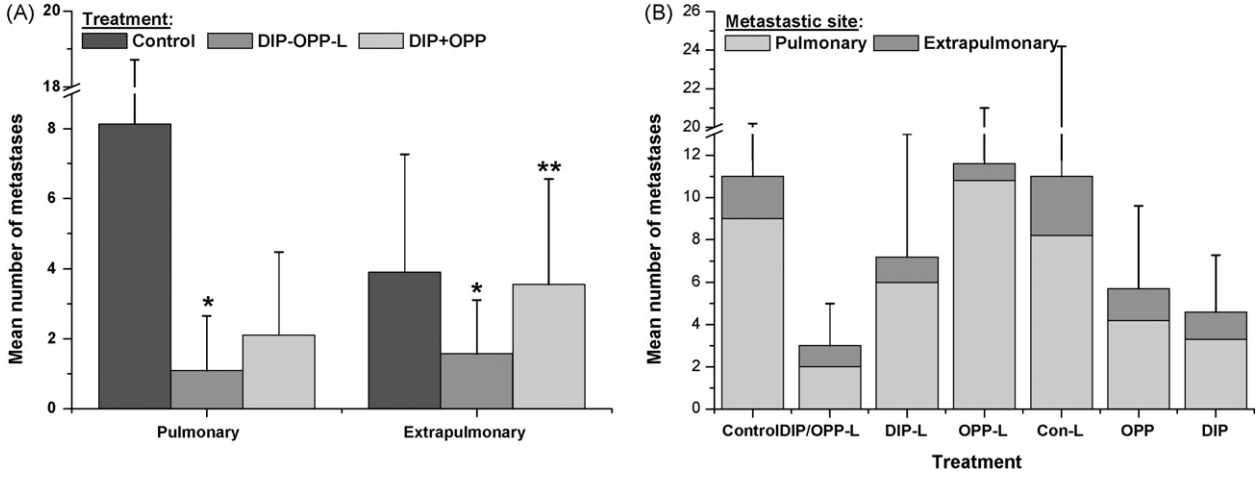

Fig. 6. Effect of liposomes on experimental metastasis *in vivo*. (A) Influence of DIP/OPP-liposomes on development of metastases. Mice were treated with 100 nmol total lipid/mouse of DIP/OPP-liposomes or the corresponding amounts of the free drugs DIP and OPP (DIP + OPP) 6 h before i.v. MT3 tumour cell (5×10^6 MT3) inoculation. Animals were sacrificed between days 29 and 33, and the number and location of metastases were determined. Given is the mean number of metastases ± S.D. ($n = 13$ or 14, three independently performed experiments for control and DIP/OPP-L; $n = 9$ in one experiment for free DIP + OPP). *Significantly different to control ($p < 0.05$). **Significantly different to DIP/OPP-L treatment ($p < 0.05$). (B) Effect of DIP/OPP-liposomes in comparison to control formulations. The experiment was performed as described in Fig. 4A. Mice were treated once with 100 nmol DIP/OPP-L, drug free control liposomes (Con-L), liposomes containing one of the drugs (DIP-L; OPP-L) or with the free drugs (DIP; OPP) in corresponding amounts to the DIP/OPP-L 6 h before tumour cell inoculation. The results present the mean number of metastases ± S.D. of one experiment with $n = 5$ or 6 mice/group.

bination liposomes. In micrographs, liposomes have been shown to be associated with platelets (Keil et al., 2005), suggesting that these liposomes inhibited mainly (or firstly) the functionality of platelets thereby reducing complex formation with tumour cells. On the other hand, a formation of complexes involving platelets, tumour cells, and liposomes cannot be completely excluded. We know that OPP additionally alters particularly properties of the tumour cell membrane. It was recently shown that changes in tumour cell membrane rigidity affect their ability to adhere and to metastasize (Zeisig et al., 2007). The transfer of alkylphospholipids from liposomes into the membrane of closely associated tumour cells enhances the cytotoxic effect of OPP and would additionally inhibit tumour cell proliferation. This would further promote the inhibition of metastasis.

Our animal experiments support the *in vitro* findings. We observed a clear reduction in the number of metastases in the lung and at extrapulmonary sites after treatment with a single dose of 100 nmol total lipid of DIP/OPP-liposomes, which corresponds to 17 nmol OPP (7.80 μg) and 1.17 nmol DIP (0.59 ng), 6 h before tumour cell inoculation.

It is obvious from our results, that a single liposomal treatment prior to tumour cell injection is efficiently preventing metastasis, but more research is necessary to adapt this system to treatment modalities in a more clinically relevant situation. Having this in mind, we are currently optimizing the treatment schedule to further improve the therapeutic effect of DIP/OPP-L. Future studies will also focus on increasing our understanding of the cellular mechanism(s) of aggregate formation *in vivo*. This novel dual drug liposome formulation extends the spectrum of approaches for anti-metastasis therapy.

Conflict of interest

All authors disclose any actual or potential conflict of interest including any financial, personal or other relationships with other people or organizations within that could inappropriately influence (bias) their work.

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