# Reduction of tamoxifen resistance in human breast carcinomas by tamoxifen-containing liposomes *in vivo*

Reiner Zeisig[a], Dominik Rückerl[b] and Iduna Fichtner[a]

**We investigated whether it is possible to reduce anti-estrogen resistance using liposomally encapsulated tamoxifen *in vivo*. Small liposomal vesicles containing up to 5.1 mg tamoxifen/ml liposomal suspension, together with an alkylphospholipid to enhance the cellular uptake, were prepared and characterized. Mice transplanted with different tumor models were treated with tamoxifen liposomes administered i.p. or orally as a bolus dose of 50 mg/kg once a week or as a daily dose of 10 mg/kg/day, both during a 4-week period. After orally administered tamoxifen liposomes, tumor growth was significantly reduced for the 3366/tamoxifen (acquired resistance) and for the MCF-7 (inherent resistance) models to 47 and 16%, respectively (treated to control value of relative tumor volume). Intraperitoneal treatment with tamoxifen liposomes revealed similar results. Investigation of biodistribution revealed especially an accumulation of liposomal tamoxifen in MCF-7 tumors and livers of the treated mice. These liposomes had uterotrophic properties comparable to the dissolved compound. This study demonstrates for the first time that a liposomal formulation of tamoxifen was able to induce pharmacological effects and to improve the therapeutic efficacy in several anti-estrogen-resistant xenografts. *Anti-Cancer Drugs* 15:707–714 © 2004 Lippincott Williams & Wilkins.**

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[a]Max-Delbrück-Center for Molecular Medicine, Department of Experimental Pharmacology, Berlin, Germany and [b]Technical University of Berlin, Faculty III, Institute of Biotechnology, Berlin, Germany.

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Correspondence to R. Zeisig, Max-Delbrück-Center for Molecular Medicine, Department of Experimental Pharmacology, POB 74 02 38, 13092 Berlin-Buch, Germany.
Tel: +49 30 9406 3289; fax: +49 30 9406 3686;
e-mail: rzeisig@mdc-berlin.de

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## Introduction

In early breast cancer, tamoxifen produces a 25% reduction in relapse rates and a 17% reduction in mortality rates. The most serious problem with this therapy is the development of resistance during the treatment that was observed in nearly all of the patients treated with tamoxifen for several weeks or months.

Attempts to overcome acquired tamoxifen resistance mainly apply specific estrogen receptor modulators (SERM) [1] or new non-steroidal anti-estrogens like Faslodex (ICI182780), Raloxifene [2], Toremifene [3], Droloxifene [3], ERA-923 [4] or the prodrug TAT-59 [5]. An alternative possibility is based on the knowledge that the transport of the anti-estrogen to, into and within the tumor cell seems to be a very crucial factor for the development of tamoxifen resistance [6]. An encapsulation of a drug into nanospheres or into liposomes changes the pharmacokinetic properties of an encapsulated compound clearly, as has been shown for several other drugs [7,8], and may also improve circulation time and distribution of tamoxifen (or of derivatives of this hormone antagonist) in a beneficial way.

In addition, liposomes have proven their potential to reduce the toxic side-effects of anti-cancer agents and to maintain or to enhance their therapeutic effects, e.g. by modified traffic through inter- and intracellular barriers [9].

This study shows for the first time that it is possible to prepare tamoxifen-containing liposomes that are sufficiently active to reduce significantly tamoxifen resistance in several human breast cancer xenografts *in vivo*.

## Materials and methods

Tamoxifen (free base), tamoxifen citrate and dioleyl-*sn*-glycero-3-phosphoethanolamine (DOPE) were purchased from Sigma-Aldrich (Steinheim, Germany). Octadecyl-(1,1-dimethyl-piperidino-4-yl)-phosphate (OPP) was a generous gift from Dr Hilgard (ASTA Medica, Frankfurt, Germany). Lecithin (PC E) was obtained from Lipoid (Ludwigshafen, Germany). Dicetylphosphate (DCP) was a product of Serva (Heidelberg, Germany), whereas Sephadex G50 was purchased from Pharmacia (Uppsala, Sweden). Phosphate-buffered saline (PBS, pH 7.5) was obtained from Gibco/Life Technologies (Eggersstein, Germany).

### Liposome preparation

Vesicles were obtained from appropriate stock solutions of the individual lipids and drugs used in the range

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**Table 1 Characterization of tamoxifen/OPP liposomes**

| | Tafmoxifen | PC | OPP | DCP | DOPE |
|---|---|---|---|---|---|
| *Composition* | | | | | |
| Preparation range (mg/ml)[a] | 6.25 | 2–20 | 1.25 | 0.1–0.5 | 0–6.25 |
| Optimized vesicles (mg/ml)[b] | 6.25 | 17.0 | 1.25 | 0.5 | 3.0 |
| Mass ratio | 10 | 27 | 2 | 1 | 5 |
| Molar ratio | 10 | 19 | 3 | 1 | 4 |
| *Properties* | | | | | |
| Content[c] | | | | | |
| after extrusion | 5.1 ± 0.3 (81.1%) | 15.8 ± 1.1 (93.1%) | | | |
| after gel chromatography | 3.9 ± 0.3 (62.5%) | 13.7 ± 0.3 (80.3%) | | | |
| Size[d] (nm) | 126 ± 14 | | | | |
| Polydispersity index | 0.12 ± 0.05 | | | | |

[a]Liposomes were prepared with individual components in the indicated range of amounts.
[b]Best liposome preparation with regard to highest amount of tamoxifen.
[c]Amount in mg/ml, in parenthesis in percent in comparison to starting material.
[d]Unimodal results.

**Table 2 Characterization of tumor models used and treatment modalities**

| Xenograft | | Original histology | Response to tamoxifen | Reference |
|---|---|---|---|---|
| Breast | 3366 | invasive ductal carcinoma | sensitive | [36] |
| Breast | 3366/Tam | invasive ductal carcinoma | acquired resistant | [37] |
| Breast | MCF-7 | pleural effusion | resistant | [38] |
| Breast | 4134 | invasive ductal carcinoma | resistant | [28] |
| Breast | 4586 | invasive lobular carcinoma | sensitive | [28] |
| Ovarian | HOC-2 | serous ascites | sensitive | [39] |
| Ovarian | OVCAR-3 | serous ascites | sensitive | [40] |

indicated in Table 1 by lipid film hydration with PBS, pH 7.4, followed by shaking overnight to obtain multilamellar vesicles (MLV). Repeated extrusion of MLV through two stacked polycarbonate filters (100 nm pore size or 200 nm pore size for therapeutic formulations) using a LiposoFast Basic System (Avestin, Ottawa, Canada) generated small vesicles (SV) as described previously [10].

The composition of the liposomes was optimized to obtain vesicles with a high amount of tamoxifen (in relation to PC and in absolute amount/ml) to keep the necessary volume of the formulation as low as possible for animal dosing. Best tamoxifen SV could finally be produced containing 5.1 mg tamoxifen/ml using the composition given in Table 1.

The amount of liposomal incorporated/bound tamoxifen was determined by HPTLC after size exclusion chromatography using Sephadex G50.

### Determination of drug and lipid concentrations

Tamoxifen, OPP and phosphatidylcholine (PC) concentrations were determined by high-performance thin-layer chromatography (HPTLC) using dichloromethane:-methanol:acetic acid:water [50:15:1:1 (Tam) and 50:30:8:4 volume parts (OPP, PC)] as the mobile phase on HPTLC plates (Merck, Darmstadt, Germany). The plates were dried at 140°C for 10 min. Plates for OPP and PC determinations were then dipped into a solution of 0.4 g $MnCl_2 \cdot 2H_2O$ in 60 ml $H_2O$/60 ml MeOH with 4 ml $H_2SO_4$ for 5 s and after that kept at 140°C for 7 min to visualize the lipid spots. Tamoxifen determination was done without chemical modification on HPTLC $F_{254}$ plates. Quantification was performed using the Camag Cats 3 program (Camag Muttenz, Switzerland) after scanning at 254 nm (tamoxifen) or 564 nm (OPP, PC) with a Camag scanner II and by calculating the concentration in comparison to a standard curve of tamoxifen, OPP or PC applied in appropriate amounts to the same plate.

### Liposome size

Size was measured by dynamic light scattering using a Coulter Counter N4 MD (Coulter Electronics, Hialeah, FL).

### Mice and tumor models

Female NMRI:*nu*/*nu* mice were obtained from M & B (Ry, Denmark), the NOD/SCID mice from Jackson Laboratories (Bar Harbor, ME). The animals were held under pathogen-free conditions in filter cages under standardized environmental conditions (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*. Characteristics of the tumor xenografts used can be taken from Table 2. Two breast (3366 and 4586) and two ovarian xenografts (OCVCAR-3 and HOC-22) are tamoxifen sensitive, while two other breast models (MCF-7 and 4134) have to be considered as being

**Table 3 Efficacy of orally administered tamoxifen, dissolved or in liposomal form in breast and ovarian cancer xenografts**

| Xenograft | Treatment | Dose (mg/kg/injection) | Tamoxifen dissolved | | Tam-OPP liposomes[a] | | OPP liposomes[b] | |
|---|---|---|---|---|---|---|---|---|
| | | | T/C (%) | BWC (%) | T/C (%) | BWC (%) | T/C (%) | BWC (%) |
| 3366 | intermittent[c] | 50 | 26[e] | 2 | 25[e] | 37 | 77 | 38 |
| 3366/Tam | intermittent | 50 | 74 | −1 | 47 | 2 | 84 | 2 |
| | daily[d] | 10 | 51[e] | −1 | 50[e] | 0 | 70 | 4 |
| MCF-7 | intermittent | 50 | 92 | −1 | 44 | −1 | 99 | −1 |
| | daily | 10 | 36[e] | −5 | 16[e,f] | −4 | 56 | −1 |
| 4134 | intermittent | 50 | 89 | −1 | 89 | −5 | 104 | −6 |
| 4586 | intermittent | 50 | 42[e] | −5 | 46[e] | −2 | 73 | 1 |
| HOC-22 | daily | 10 | 25[e] | 2 | 44[e] | 6 | 58 | 3 |
| OVCAR-3 | daily | 10 | 57[e] | −3 | 57[e] | −2 | 79 | 2 |

Mean values of six to eight nude mice per group. T/C = treated to control value of relative tumor volume, BWC = body weight change.
[a]Molar composition: tamoxifen:PC:OPP:DCP:DOPE (10:19:3:1:4).
[b]Molar composition: cholesterol:PC:OPP:DCP:DOPE (10:19:3:1:4).
[c]Once per week for 4 weeks.
[d]Daily for 4 weeks.
[e]Significant ($p < 0.05$) to corresponding solvent group.
[f]Significant ($p < 0.05$) to corresponding tamoxifen group.

intrinsically anti-hormone resistant for the doses and schedules used. From the relatively tamoxifen-sensitive model 3366, a subline with acquired resistance (3366/Tam) was generated within 2 years by treating tumor-bearing nude mice with that anti-estrogen and successive passaging of tumors.

Breast tumors from routine passage in nude mice were cut into pieces and fragments of $3 \times 4$ mm were transplanted s.c. into left flank of nude mice under anesthesia (Radenarkon, Etomidat; ASTA Medica; 40 mg/kg i.p.). Ovarian carcinoma cells were taken from ascites of routine passage; $10^7$ cells per mouse in 0.2 ml PBS were transplanted i.p. into NOD/SCID mice. Mice were randomized immediately after tumor transplantation.

It was known from previous experiments that the breast xenografts responded to estradiol supplementation with enhanced growth. Therefore, breast tumor-bearing mice were treated once per week during the total experimental period with the estradiol valeriate (0.5 mg/kg i.m.) to mimic physiological conditions of humans.

Treatment started in the s.c. breast cancer experiments when the tumors reached a palpable size (4–5 mm), in the i.p. ovarian cancer experiments 5 days after tumor cell inoculation.

Tamoxifen in both free and liposomal forms was tested in parallel. Tamoxifen liposomes were used without separation of the unencapsulated drug; tamoxifen was dissolved in benzylbenzoate and diluted with Ol. arachidis immediately before use as a 'free' drug. The administration volume was 0.2 ml/20 g body weight. Doses and schedules are given in Table 3.

The tumor size of s.c. growing breast tumors was measured twice weekly with a caliper-like instrument. Tumor volumes (TV) from each group at each measurement day were calculated using Excel software according to $TV = (\text{width}^2 \times \text{length})/2$ and related to the first value (RTV = relative tumor volume) determined on the first treatment day. The RTV was used for the calculation of treated-to-control (T/C) values as a percentage.

Ovarian carcinoma-bearing mice were sacrificed in a moribund stage. Uterus, ovarian and ascites weights were determined, and used as read outs for the calculation of T/C values.

At the end of some experiments the weights of uterus and ovaries were also determined as parameters for uterotrophic activity.

Body weight was determined twice per week; mean values per group were related to initial values (BWC = body weight change).

### Pharmacological distribution

Three MCF-7 bearing nude mice were treated once orally with 200 mg/kg tamoxifen in solution or in liposomes. Serum was taken after 1, 6 and 24 h; tumor, uterus and liver after 24 h. Samples were frozen at −20°C until tamoxifen quantification.

Tamoxifen was extracted from homogenates of serum (82–225 µl) and of tumor and organs (if possible, divided into pieces of 90–250 mg) by a modified Blight Dyer extraction as described recently for pharmacokinetic analysis of hexadecylphosphocholine [11]. Following this procedure, 85.3 ± 3.1% of tamoxifen can be recovered as control experiments revealed using mouse liver for extraction after addition of defined amounts of tamoxifen (data not shown). The quantification of the anti-estrogen was finally performed similarly to tamoxifen quantification of liposomes by HPTLC as described above. The

detection limit obtained with this method was 10 ng/spot on the HPLTC plate. The data represent the mean of three organs, each sample determined at least 2 times.

All animal experiments were performed according to the German Animal Protection Law and with a permit from the responsible authorities.

### Statistics

Statistical evaluation was performed with the non-parametric *U*-test of Mann and Whitney at a significance level of $p \leq 0.05$. The calculations were performed with the software Statistica 5.1 for Windows.

## Results

### Tamoxifen liposomes

SV were optimized to contain a high absolute amount of tamoxifen per volume and in relation to PC. Therefore the amounts of PC, DOPE, DCP and OPP were modified as described in Table 1, while tamoxifen and OPP were kept constant with 6.25 and 1.25 mg/ml, respectively. The composition with the highest amount of tamoxifen was found to consist of tamoxifen:PC:OPP:DCP:DOPE in a mass ratio of 10:27:2:1:5, which corresponds to a molar ratio of 10:19:3:1:4, respectively (Table 1).

The extrusion of MLV to obtain SV was accompanied with a loss of 18.9% tamoxifen and 6.9% PC. Best liposomes obtained in this way contained 5.1 mg tamoxifen/ml. The vesicles had a mean diameter of $126 \pm 14$ nm with a polydispersity index of $0.12 \pm 0.05$ (Table 1), which corresponds to a diameter of $101.5 \pm 7.8$ nm if weight result calculations were used.

Gel chromatography of SV revealed that about 3.9 mg of the drug was incorporated into these liposomes (63% of the initial drug amount), whereas the rest was unencapsulated or only slightly associated with the vesicles and could be separated from the liposomes (Table 1).

Tamoxifen liposomes for animal experiments were composed corresponding to the optimized composition given in Table 1, but were only extruded using a filter with a pore size of 200 nm.These liposomes had a final diameter of $180 \pm 14$ nm with a polydispersity index of $0.19 \pm 0.13$ (data not shown). Vesicles were used for *in vivo* experiments without separation of non-encapsulated drug to keep the tamoxifen concentration as high as possible to have a sufficient amount of the drug in a small volume for animal treatment.

Tamoxifen vesicles were stable in PBS without a significant change in size. The diameter increased only by 2% following a storage period of 6 weeks/4°C as it was followed by PCS measurements (data not shown).

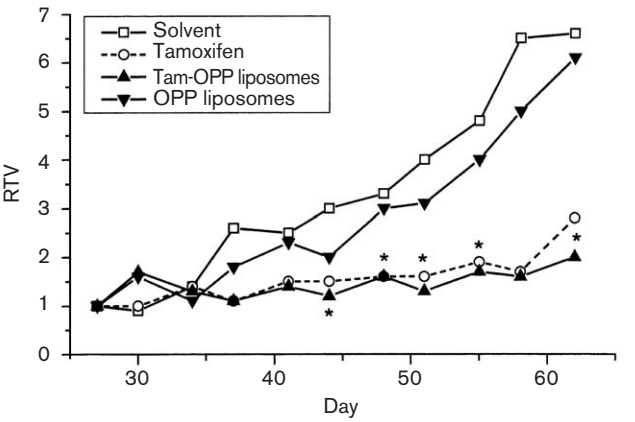
### Therapeutic experiments

In a first set of experiments, mice bearing 3366 and 3366/Tam were treated i.p. once per week for 4 weeks with a bolus injection of 50 mg/kg tamoxifen (intermittently) either dissolved or as a liposomal preparation. The 3366 tumors responded equally well to both tamoxifen formulations, while 'empty' OPP-containing liposomes were inefficient (Fig. 1). Conversely, the 3366/Tam tumor was resistant to dissolved tamoxifen, while the drug-containing liposomes resulted in a significant inhibition of tumor growth (Fig. 2). A similar result was obtained when the MCF-7 tumor model with inherent resistance was treated orally once a week intermittently (Fig. 3).

In a second set of experiments tamoxifen was administered orally by gavage in order to mimic the clinical method of administration. For that purpose, mice were treated once a week intermittently with 50 mg/kg or daily for 4 weeks with 10 mg/kg/day. Dissolved tamoxifen, tamoxifen liposomes or 'empty' OPP liposomes were used in the same schedules and doses. The results are summarized in Table 3.

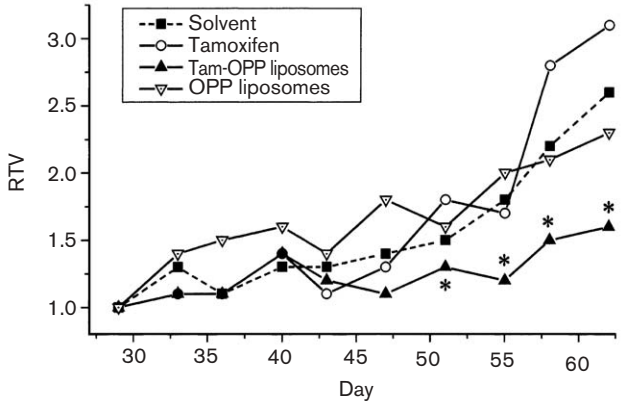
In the xenografts 3366/Tam and MCF-7, orally intermittently administered tamoxifen liposomes led to significant inhibition of tumor growth, while equal doses of dissolved tamoxifen were inefficient. In another experiment using an MCF-7 xenograft, tamoxifen was administered daily. Using this schedule, again a significantly better growth inhibition of MCF-7 tumor growth was obtained with tamoxifen liposomes compared to free tamoxifen (Table 3).

**Fig. 1**



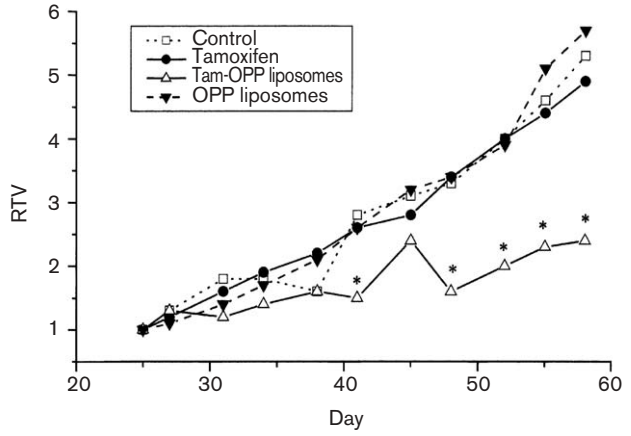

Growth curves of breast carcinoma xenografts 3366. Six to eight nude mice per group received s.c. fragments of the tumor at day 0. Mice were supplemented with estradiol (once/week 0.5 mg/kg i.m.) and treated with tamoxifen or tamoxifen liposomes (50 mg/kg once/week i.p. for 4 weeks) starting at day 26. Separate groups of mice received the solvent or OPP liposomes in equal volumes and schedules. *Significantly different to tumor volume of control group.

**Fig. 2**




Growth curves of breast carcinoma 3366/Tam. Six to eight nude mice per group received s.c. fragments of the tumor at day 0. Mice were supplemented with estradiol (once/week 0.5 mg/kg i.m.) and treated with tamoxifen or tamoxifen liposomes (50 mg/kg once/week i.p. for 4 weeks) starting at day 29. Separate groups of mice received the solvent or OPP liposomes in equal volumes and schedules. *Significantly different to tumor volume of control group.

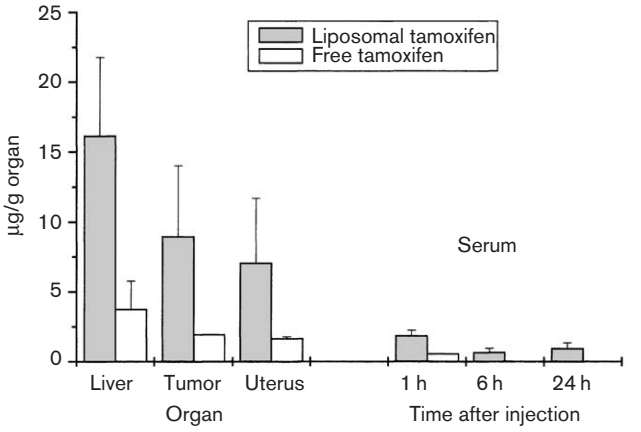
**Fig. 3**




Growth curves of MCF-7 breast carcinoma xenografts. Six to eight nude mice per group received $10^7$ carcinoma cells per mouse in 0.2 ml PBS i.p. at day 0. Mice were supplemented with estradiol (once/week 0.5 mg/kg i.m.) and treated with tamoxifen or tamoxifen liposomes (50 mg/kg once/week orally for 4 weeks) starting at day 28. Separate groups of mice received the solvent or OPP liposomes in equal volumes and schedules. *Significantly different to tumor volume of all other groups.

In one other breast and two ovarian models the liposomes were similarly efficient (4568, OVCAR-3, HOC-22) or inefficient (4134) as the corresponding dissolved tamoxifen (Table 3). Empty OPP liposomes were tested in each model and had no significant effect on tumor growth compared to solvent-treated controls. All treatment modalities were well tolerated and did not significantly influence the body weight of mice.

**Fig. 4**




For the pharmacological distribution study, three MCF-7 bearing nude mice each were treated once orally with 200 mg/kg tamoxifen in solution or as liposomal formulation. After 1, 6 and 24 h, serum was collected, and after 24 h, tumor, uterus and liver was taken. Tamoxifen concentration in homogenized samples was extracted by a Blight–Dyer extraction followed by a quantification by HPTLC technique as described in Material and methods. The data represents the mean content of tamoxifen per gram organ ± SD of two or three determinations, each done in duplicate.

### Biodistribution of tamoxifen

The organ distribution of tamoxifen in MCF-7 bearing mice after treatment with free and liposomal tamoxifen (Fig. 4) clearly revealed a delayed elimination from the blood for the liposomal formulation at all time points investigated, while the concentration of the free drug in serum was already after 1 h below the detection level of 10 ng/spot (corresponding to 650 ng/ml). Further, a preferred accumulation in liver, tumor and uterus tissue was observed for liposomal tamoxifen after 24 h. The ratio of tamoxifen determined in tumor compared to that found in liver was 0.08 for the dissolved and 0.16 for the liposomal drug.

### Anti-uterotropic effect

The anti-uterotropic effect of the various treatment modalities was determined by measuring the uterine and ovary wet weights of tumor-bearing mice at the end of a 4-week treatment period (Table 4). The results showed a significant decrease in uterus weight induced both by 'free' and liposomal tamoxifen to about the same degree. Tamoxifen-free OPP liposomes also led to a moderate, but not significant, decrease. Ovary weight remained uninfluenced by all treatment modalities (data not shown).

## Discussion

Anti-estrogens, especially tamoxifen, are the agents of choice for the treatment of all stages of breast cancer [3,12–14]. Tamoxifen as a classical anti-estrogen and its

**Table 4 Uterus weight after tamoxifen treatment**

| Xenograft | Treatment | Formulation | | | |
|---|---|---|---|---|---|
| | | Solvent | Tamoxifen dissolved | Tamoxifen liposomes[a] | OPP liposomes[b] |
| MCF-7 | intermittent | 292 ± 44 | 178 ± 30[c] | 169 ± 31[c] | NT |
| MCF-7 | daily | 342 ± 32 | 149 ± 36[c] | 112 ± 28[c,d] | 256 ± 43[c] |
| 3366/Tam | daily | 320 ± 90 | 150 ± 30[c] | 160 ± 20[c] | 250 ± 20 |

Uterus wet weights (mg) was determined after a 4-week treatment period as described in Materials and methods. Data represent the mean weight for eight mice ± SD. All mice received an additional estradiol supplementation. NT = not tested.
[a]Molar composition: tamoxifen:PC:OPP:DCP:DOPE (10:19:3:1:4).
[b]Molar composition: cholesterol:PC:OPP:DCP:DOPE (10:19:3:1:4).
[c]Significant to solvent.
[d]Significant to dissolved tamoxifen.

derivatives (SERMs) are regarded as competitive inhibitors at the level of ER protein. As net results, gene transcription, DNA synthesis and cellular proliferation are lowered or prevented [15,16].

Despite the tremendous therapeutic and commercial success of tamoxifen during the last 15 years, intrinsic or acquired resistance in almost all patients has to be faced during prolonged treatment.

The reasons for tamoxifen resistance have not yet been fully elucidated, but several possibilities are discussed. These include metabolic changes during transport to and into the tumor (cell), disturbance of binding to the estrogen receptor or during receptor dimerization, modifications in the binding process of the homodimer/estrogen complex to DNA and/or modulation in the activation of the transcription cascade [17,18].

Possibilities to overcome (acquired) tamoxifen resistance arose from the use of newly developed anti-estrogens [19], progestins [19] or aromatase inhibitors [20]. However, none of these agents could completely replace tamoxifen so far from first-line clinical use [18,21].

Liposomes have shown their advantage for the improvement of drug formulation barriers [9]. Liposomes are able to reduce toxic side-effects of anti-cancer agents while maintaining or enhancing their therapeutic effects [7,9,18,22,23], e.g. by improvement of traffic through inter- and intracellular barriers [24–26].

To date, tamoxifen has not been used as a liposomal formulation in cancer therapy, but a potential benefit may be expected from an encapsulation of the poorly water-soluble drug into vesicles. A recent study showed that it is possible to encapsulate tamoxifen into sterically stabilized nanospheres prepared from poly(MePEG-cyanoacrylate-*co*-hexadecyl-cyanoacrylate) 1:4 poly(PEG-CA-*co*-HDCA) copolymers, but the advantage of these nanospheres was rather low concerning their biological effect *in vitro*, because of a substantial and immediate drug release [6].

We report in our present study for the first time on the preparation of anti-estrogen-containing liposomes and on the resulting therapeutic effects in different breast cancer models *in vivo*.

The liposomes consisted of PC as the basic lipid for membrane formation, DCP to introduce a negative charge, OPP as a membrane active lipid to change the properties of the target tumor cell membrane and, finally, DOPE. This unsaturated lipid is a helper lipid known to have a stabilizing effect on the liposomal membrane because of its chain flexibility.

The preparation and extrusion could be carried out without any problem and allows the production of homogeneous small vesicles with a diameter of 120 nm in a reproducible way. The loss of tamoxifen by extrusion was about 19%.

The amount of encapsulated tamoxifen could be increased up to 5.1 mg/ml, which was sufficient for an application of 50 mg/ml/kg at 200 µl/mouse. This is a clear advantage of the liposomes over nanospheres, which had only about 25 µg tamoxifen/ml encapsulated as reported recently [6]. Additional investigation on the liposomes using gel chromatography to separate unbound material revealed that in the suspension of extruded liposomes about 76% of tamoxifen (62% of starting drug) was encapsulated into, or tightly bound to, the vesicles. It can be expected from its lipophilic character and the molecular similarities to cholesterol that it is incorporated into the liposomal bilayer.

Formulations for animal treatment were used without separation of this reversibly bound portion to prevent an additional loss of tamoxifen that would increase the necessary volume of injection and accepting that only 76% of the applied drug was encapsulated.

The tamoxifen liposomes were tested in parallel to the dissolved drug and the drug-free OPP liposomes in five breast and two ovarian carcinoma xenografts. In two models, the sensitive 3366 tumor (Fig. 1) and the 3366/Tam (Fig. 2) with acquired resistance, the liposomal

formulation induced a significant tumor growth inhibition when given i.p. on a weekly schedule.

It could be additionally shown for the first time that an oral administration of tamoxifen-loaded liposomes induced pharmacological (Table 3) and pharmacokinetic effects (Fig. 4) different from the dissolved drug. In five other xenografts (three breast and two ovarian), the tumor response to tamoxifen was unchanged by liposomal formulation. This is plausible if one takes into account that the breast cancer xenografts 3366 and 4586 were already highly sensitive to tamoxifen, whereas the breast cancer (4143) as well as the ovarian cancer xenografts (HOC-22 and OVCAR-3) were intrinsically resistant. An enhancement of the therapeutic effect was not possible in both cases because of these borderline situations. In contrast to this, an improvement could be obtained in models with acquired resistance (3366/Tam and MCF-7), especially if the liposomal formulation was administered as a bolus once a week. Only under these conditions can the advantage of liposomes of being a slow-release depot for tamoxifen come to fruition.

Tamoxifen liposomes had comparable uterotrophic properties to the dissolved compound, although the accumulation in the uterus was slightly enhanced. That suggests a comparable profile of side-effects between dissolved and liposomal tamoxifen.

As an additional active component, the alkylphospholipid OPP was included into the liposomes with the aim to enhance the transport of tamoxifen into the tumor cell. We assume that the alkylphospholipid could work as a helper lipid that changes the integrity of the tumor cell membrane because of its amphiphilic, detergent-like properties [27]. OPP liposomes, used as control for the effect of liposomal alkylphospholipid, lacked a significant inhibitory ability in all models. This was expected, because we used in the present study exclusively preclinical models with estrogen responsiveness, which are known to be insensitive to alkylphospholipids [28]. In contrast to this, the combination of tamoxifen and OPP improved the therapeutic effect in most models used in this study.

In order to understand the reason for the therapeutic advantage of liposomal tamoxifen, the concentrations of tamoxifen in serum, in relevant organs and in the tumors of MCF-7-bearing mice were determined using the HPTLC technique 24 h after administering a single large dose of 200 mg/kg.

Data for biodistribution were obtained using the HPTLC technique after performing a Blight–Dyer extraction. This data offers only a very preliminary characterization of tamoxifen biodistribution after administering a single large dose of the drug. HPTLC is not as sensitive as the modified HPLC technique, which allows discrimination between the parent anti-hormone and metabolites formed during circulation [29].

Nevertheless, we found two main effects of liposomal encapsulation. First, a clearly higher serum level was found at all investigated time points, which is probably due to the slow-release properties of the liposomes. As could be expected, the highest absolute amount of tamoxifen was found in the liver, where especially liposomes will be taken up by Kupffer cells [30]. On the other hand, the level of liposomal tamoxifen in the tumor was more than twice as high as for the free drug, explaining possibly the increased efficacy in some xenografts.

In addition to findings on differences between free tamoxifen and the liposomal formulation at the cellular level (publication in preparation), a strong impact can be expected from different extravasation profiles of both formulations. In particular, liposomes with a rigid bilayer or sterically stabilized vesicles circulate longer in the blood [31]. This can also be assumed for the tamoxifen liposomes used in our experiments. Tamoxifen has a high structural homology with cholesterol [32]. The presence of cholesterol increases membrane rigidity and results in a reduced macrophage uptake [33–35]. It could be assumed that the rigid tamoxifen liposomes will also passively accumulate in the tumor vasculature and transport in this way a higher amount of the drug to the tumor side compared to the solution of the free drug.

Finally, it can be suggested that tamoxifen liposomes, once they are accumulated in the tumor interstitium, act similarly to a slow-release device as shown for other liposomes [26]. This is also supported by the observation that an improvement by liposomes was obtained if the drug was administered intermittently, whereas a daily treatment resulted in a similar (3366/Tam and OVCAR) or lower effect (HOC-22) of liposomal tamoxifen in comparison to the free drug. This indicates the necessity of a relatively constant level of the drug in the blood, which could be provided by circulating liposomes with a slow-release function.

In summary, our experiments showed that a liposomal formulation of tamoxifen could have some clinical advantage compared to the free drug, especially when given as a bolus treatment. In that case, breast tumors resistant to the drug in this schedule became responsive. For the first time, it could be shown that an orally administered liposomal formulation of an anti-estrogen was able to induce pharmacological effects.

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