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In vivo models for endocrine-dependent breast carcinomas: special considerations of clinical relevance

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Abstract

Tumours in hormone-regulated organs such as the breast, prostate or ovaries are among the most frequent malignancies. Because of their endocrine-dependent development and growth, they offer a unique opportunity for antihormonal treatment either single or long-term or in combination with radio- or chemotherapy. A prominent example is breast carcinoma, for which the anti-oestrogen tamoxifen has been used successfully for several years. Unfortunately, a substantial number of tumours are intrinsically tamoxifen-resistant, despite oestrogen-receptor positivity, and, eventually, almost all breast carcinomas acquire resistance towards tamoxifen. The recently developed pure anti-oestrogen Faslodex™ and the third-generation aromatase inhibitors (Letrozol™, anastrozole (Arimidex™)) offer the possibility of alternative therapies. Preclinical models are needed, as most of the mechanisms of hormonal tumour dependence and the causes of the appearance of antihormone resistance are not yet fully understood. This review focuses on the development and characterisation of breast cancer xenografts derived directly from surgical resections. With their help, a deeper insight into the mechanisms of hormone regulation and anti-oestrogen resistance can be gained. The xenograft models have already been used in differential gene-expression analysis on DNA microarrays and for the evaluation of approaches to overcoming tamoxifen resistance.

Keywords: Breast carcinomas; Oestrogen receptor; Anti-oestrogen resistance; Tamoxifen; Microarray studies

1. Introduction

Tumours in hormone-responsive organs such as the breast, ovaries, prostate, adrenal and thyroid glands may respond to endocrine therapy. Such treatments play a large part clinically in breast and prostate cancer. For the development of novel therapeutic approaches, preclinical models are required that on the one hand maintain hormonal dependency for progressive growth and on the other are susceptible to an (anti-)hormonal intervention.

For the preclinical pharmacologist, the established models of hormonally-dependent cancers create a challenge in handling to maintain their properties stably during successive transplantations. Well-characterised *in vivo* models offer a unique chance of investigating regulatory mechanisms of interest, e.g. ligand–receptor interactions, the selection of molecular targets or signalling pathways in relation to time or any exogenous influences.

As our group has longstanding experience in the field of breast carcinoma, this article will focus on this tumour and will refer to the following:

- the establishment of xenografts from patients' samples
- the characterisation of hormonal and molecular targets
- chemo- and (anti-)hormone sensitivity
- mechanisms of anti-oestrogen resistance:
  - the development of tamoxifen (Tam)-resistant xenografts
  - the regulation of steroid hormone receptor expression

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- the molecular structure of the oestrogen receptor-α (ERα)
- studies on gene-expression profiles
- approaches to overcoming Tam resistance.

1.1. Establishment of xenografts from patients

As, due to their low degree of differentiation, we felt dissatisfied with xenografts derived from *in vitro* cell lines, we began a programme to establish patient-derived preclinical models. Fresh surgical samples of breast cancers that had been evaluated by a pathologist for malignancy were transplanted subcutaneously (s.c.) or into the mammary fat pad of nude mice. From almost 100 samples, nine successively transplantable tumours were eventually obtained (Table 1), confirming the relatively low take of breast tumours compared with other carcinomas in immunodeficient mice [1]. In our experience, the success rate can be increased by pre- and co-supplementation with oestrogens, and by the transplantation of relatively large tumour fragments in order to maintain the microenvironment that is apparently essential for tumour growth.

1.2. Characterisation of hormonal and molecular targets

Among the panel of serially transplantable xenograft lines designated by four-digit numbers (Table 1) are breast carcinomas derived from pre- and post-menopausal women, with tubular, medullary or ductal features. The histology and architecture of the xenografts were similar to those of the original sample, but the proportion of malignant cells had increased after relatively few passages in the nude mice. One xenograft was clearly ERα-positive (3366), while others (4151, 4134, 4586) were positive for ERα at the mRNA, but not the protein level. From one Tam-sensitive tumour (3366), a resistant subline (3366/Tam) was generated by treating nude mice with the anti-oestrogen during successive passaging. None of the xenograft tumours expressed ERβ, whereas one model (4586) was classified as aromatase sensitive as it showed androstenedione-dependent growth. The tumour doubling times in nude mice were between 10 and 30 days.

The xenografts from early passages (maximum 10) were cryoconserved and all further experiments were performed with tumours originating from this stock in order to maintain close similarity with the original tumour. In this way, a panel of preclinical models of breast carcinoma was established that encompasses the various features of clinical breast tumours. These xenograft models are a valuable tool for the preclinical development of novel diagnostic or therapeutic approaches.

1.3. Chemo- and (anti-)hormone sensitivity

The newly established xenografts were characterised for their responses to a panel of clinically used cytotoxic drugs. In this part of the study, further tumour lines such as the ERα-negative MaTu [2] and MDA-MB 435, and the ERα-positive MCF-7 (originating from the National Cancer Institute (NCI) cell-line panel) were included. When the tumours were palpable, six to eight mice per group were given the maximum tolerated dose of the respective drug in order to mimic clinical treatment schedules. Summarised results (Table 2) reveal a

Table 1
Breast cancer xenografts derived from patients

Breast cancer xenograft	Oestrogen receptor-α[a]	Histology	Response to		c-erbB2[c]	EGF-receptor[c]	Reference
			Oestradiol	Tamoxifen			
3366	+	Postmenopausal, ductal invasive carcinoma	*	+		–	[21]
3366/Tam	+	Tamoxifen-resistant subline of carcinoma 3366	*	–		–	[5]
4000	–	Postmenopausal, ductal invasive carcinoma	*	+	–	–	[19]
4134	–/+[b]	Premenopausal, ductal invasive carcinoma	–	–			[20]
4151	–/+[b]	Postmenopausal, ductal invasive carcinoma	–	–	–	–	[19]
4296	–	Premenopausal, squamous epithelial carcinoma	–	–	–	–	[18]
4586	–/+[b]	Premenopausal, relapsed invasive lobular carcinoma	*	–	–	n.t.	n.p.
4898	–	Relapsed invasive ductal carcinoma	–	–	+	n.t.	n.p.
MT-1	–	Undifferentiated medullary growth	–	–	–	n.t.	[22]
MT-3	–	Well-differentiated tubular adenocarcinoma	–	–	–	n.t.	[22]

*, Growth stimulation; +, Growth inhibition; –, no effect on growth; n.p., not published; n.t., not tested; EGF, epidermal growth factor.
[a] Determined with enzyme immunoassay (EIA) (Abbott, ER-EIA Monoclonal Abbott GmbH, Wiesbaden, Germany).
[b] EIA-negative, reverse transcriptase-polymerase chain reaction-positive.
[c] Determined immunohistologically.

Table 2
In vivo sensitivity of breast carcinomas towards cytotoxic drugs

	Xenograft tumour											
Substance	3366	4000	4134	4151	4296	4586	MaTu	MCF−7	MDA−MB−435	MT−1	MT−3	Efficacy (%)
Doxorubicin	+	+ + +	+ + +	+ + +	+ + +	(+)	+ +	+	+ +	+ + +	+ + +	11/11 (100)
Daunorubicin	n.t.	−	+	n.t.	−	(+)	+ +	−	n.t.	+ + +	+ + +	5/8 (63)
Mitoxantrone	+ +	−	+ + + +	+ + +	−	−	+	+ + +	+ +	+ +	+ +	8/11 (73)
Cyclophosphamide	+	+ + + +	+	+ + +	−	−	−	−	+ +	+ + + +	−	5/11 (45)
Methotrexate	−	n.t.	−	−	+ + +	−	+ +	n.t.	n.t.	−	−	2/8 (25)
Vincristine	+ +	+	−	+ + +	−	−	+ +	−	n.t.	+	−	5/10 (50)
5-Fluorouracil	−	n.t.	−	−	+ +	−	−	−	n.t.	−	−	1/9 (11)
Carboplatin	−	n.t.	−	n.t.	n.t.	−	−	(+)	n.t.	−	+ +	2/7 (29)
Cisplatin	n.t.	n.t.	−	n.t.	−	+	−	n.t.	n.t.	n.t.	+ +	2/5 (40)
Miltefosine	−	−	−	+	−	−	+ + +	−	+	+ + + +	+ +	5/13 (38)
Paclitaxel	−	−	n.t.	−	n.t.	−	+ +	−	n.t.	+	+	2/8 (25)
Sensitivity (%)	4/9 (44)	3/7 (43)	4/10 (40)	5/8 (63)	3/9 (33)	1/11 (9)	7/11 (64)	1/9 (11)	4/4 (100)	7/10 (70)	7/11 (64)	

−, Treated to control values (T/C) >50%; + +, T/C 21–35%; + + + +, T/C 0-5%; n.t., not tested; (+), borderline activity; +, T/C 36–50%; + + +, T/C 6–20%.

differential sensitivity of the xenograft tumour lines to the cytotoxic agents. The most sensitive line was the MDA-MB-435 and the least responsive were the MCF-7 and 4296. Concerning the efficacy of the different drugs, doxorubicin can be considered the most active, followed by mitoxantrone, daunorubicin, vincristine and cyclophosphamide. These are the agents that also induce the highest response rates clinically.

Concerning the oestrogen and Tam response of the different xenografts, it became obvious that oestradiol supplementation stimulated growth in four out of the nine xenografts tested and that among those was one tumour (4000) with an ERα below the detection limit of 15 fmol/mg protein (Table 1). The same tumour was also moderately growth inhibited by Tam. This suggests a residual hormone responsiveness even in tumours whose ERα protein is below the detection sensitivity of the ER-enzyme immunoassay (EIA) and this could explain the observation that clinically 15% of tumours diagnosed as ERα-negative also respond to Tam. Another interesting xenograft, 4586, reveals intrinsic Tam resistance despite being ERα-positive, a phenomenon that is also observed clinically in approximately one third of the breast tumours diagnosed as being positive for ERα.

The panel of tumour xenografts available reflects the differential hormonal dependencies registered in the clinic as well. Therefore, these models are suggested as suitable for the testing of novel endocrine therapies.

1.4. Mechanism of anti-oestrogen resistance

1.4.1. Development of a tamoxifen-resistant xenograft

In the clinic, an inherent anti-oestrogen resistance is observed in approximately 30% of ERα-positive breast carcinomas, and acquired resistance develops in almost all Tam-treated women after long-term medication [3]. The mechanisms of Tam resistance are not yet fully understood. Disturbances in ER signalling, differential interaction with co-repressors or co-activators, and changes in the transport or metabolism of Tam are possibilities [4]. We decided to develop a Tam-resistant xenograft line by treating 3366 tumour-bearing nude mice with the anti-oestrogen during successive passaging. It took more than two years for resistance to appear, a period similar to the duration of clinical therapy until relapse. The final result was the Tam-resistant subline, 3366/Tam, which was used for further investigations [5]. Fig. 1 shows the histology of the Tam-sensitive 3366 and-resistant 3366/Tam line in the presence or absence of oestradiol supplementation (0.5 mg/kg per week for 4 weeks). The 3366 xenograft was originally derived from a postmenopausal woman and was initially classified as ductal invasive carcinoma with moderate differentiation. After 4 weeks' treatment of the xenografts with oestradiol in physiological doses, the histological appearance changed to that of a duct-forming, more differentiated growth [5]. In contrast, the 3366/Tam subline that was histologically identical with the original line was not influenced in histological terms by long-term hormonal treatment. This finding indicates that, due to the Tam-resistant phenotype, inherent regulatory mechanisms must be disturbed, leading also to a different morphology.

The responsiveness of the 3366 and 3366/Tam xenografts is shown in Fig. 2. The growth of 3366 xenografts (Fig. 2a) was clearly stimulated by oestradiol supplementation. Treating the mice with Tam (50 mg/kg intramuscularly (i.m.) twice a week) for several weeks led to a significant prevention of tumour development, regardless of whether the anti-oestrogen was administered as a single agent or combined with oestradiol. The

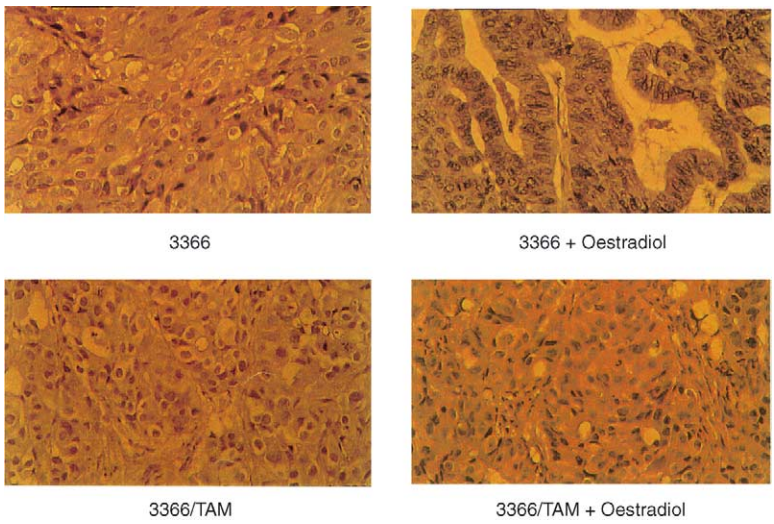

Fig. 1. Histology of breast cancer xenografts 3366 or 3366/Tam either untreated or after treatment with oestradiol valeriate (0.5 mg/kg per week intramuscularly (i.m.)) for 4 weeks; magnification ×150.

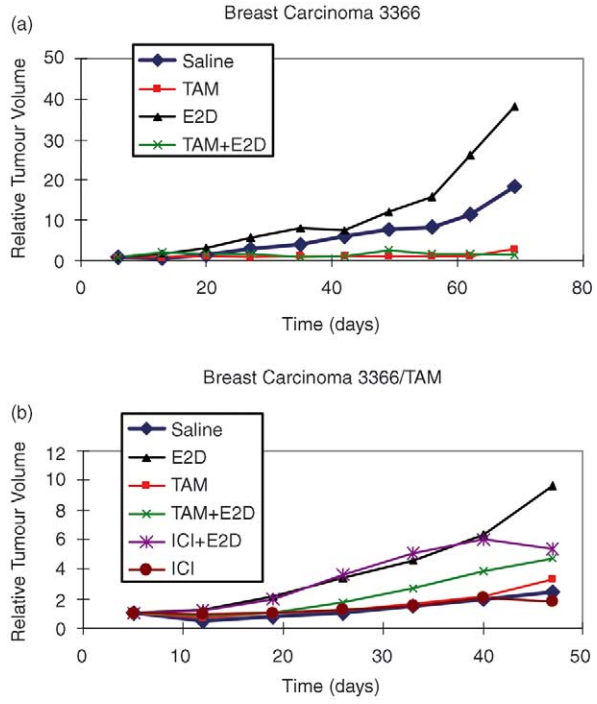

Fig. 2. Growth curves of 3366 (a) or 3366/Tam (b) xenografts treated in nude mice (6 per group) with saline, oestradiol valeriate [E2D, 0.5 mg/kg per week i.m.], Tam (TAM, 50 mg/kg twice per week i.m.) or ICI 182780 (25 mg/kg twice per week i.m.). Treatment was initiated 1 week after tumour transplantation and continued until the end of the experiment.

growth of xenografts of the 3366/Tam tumour was also stimulated by supplementation with oestradiol valeriate, proving that the hormone response was maintained (Fig. 2b). However, neither Tam nor ICI 182780 (25 mg/kg i.m. twice a week) significantly influenced oestradiol-stimulated tumour growth. This result suggests some cross-resistance between the two anti-oestrogens,

Table 3
Oestrogen receptor-α (ERα) and progesterone receptor (PR) expression of tamoxifen-sensitive and -resistant breast carcinoma (fmol/mg protein)

		Treated with		
	Tumour	Saline	Tamoxifen[b]	Oestradiol[c]
ERα content	3366	101±42	90±17	274±121[a]
	3366/Tam	82±31	94±32	50±23
PR content	3366	9±8	84±2[a]	287 ±104[a]
	3366/Tam	15±16	77±26[a]	419±358[a]

[a] Significant difference from the saline-treated group ($P \leqslant 0.05$)
[b] 50 mg/kg twice a week for 4 weeks.
[c] 0.5 mg/kg once a week for 4 weeks.

Tam and ICI 182780 (clinically Faslodex™ or Fulvestrant). Cross-resistance of 3366/Tam towards another steroidal anti-oestrogen, RU 58668, was also noticed (data not shown). The xenograft line 3366/Tam is one of the very few *in vivo* models for studying the anti-oestrogen resistance of breast carcinomas. That it was established directly from a patient-derived tumour and during a procedure involving long-term Tam treatment similar to the clinical time-frame for the appearance of acquired Tam resistance makes it an invaluable pre-clinical model for investigations on the underlying regulatory and molecular mechanisms of Tam resistance.

1.4.2. Regulation of hormone receptor expression

Both the Tam-sensitive 3366 and the -resistant xenograft line 3366/Tam expressed ERα protein as determined by Abbott EIA (Table 3). Long-term treatment of the 3366 xenografts with oestradiol at physiological concentrations for 4 weeks led to a significant, more than 2-fold increase in the ERα protein, while in the resistant subline no change was observed after hormo-

nal or antihormonal treatment. The remarkable upregulation of the progesterone receptor by either oestradiol or Tam in both lines indicates that the ER-dependent transcription machinery was intact. This was confirmed by the observation that the expression of other well-known ER-regulated genes such as pS2 and cathepsin D was also changed by oestradiol or Tam treatment, but the direction and the fold-regulation differed in the sensitive and the resistant xenografts [5].

1.4.3. Molecular structure of the oestrogen receptor

Several reports suggest that ERα mutations and splice variants could be responsible for an anti-oestrogen-resistant phenotype. Therefore, we sequenced the hormone-binding domain of the ERα isolated from the different breast cancer xenografts. Fig. 3 shows part of the sequence alignment as derived from entries for the ERα in databases or from the sequencing of our samples. No differences were found in relation to the responsiveness of xenografts to Tam: the breast tumours with intrinsic (4586, 4134) and acquired (3366/Tam) resistance all revealed a nucleotide sequence identical to that of the Tam-sensitive 3366 tumours. Amino acid replacements in helix 12 as reported by Brzozowski and colleagues [6] and Maalouf and colleagues [7], or in the C-terminal transcription activation function-2 (TAF-2) as reported by Montana and colleagues [8], were not found in the ERα of our samples. A difference in position 1559 between all of our sequences and the published sequences leads to a replacement of valine with glycine, but this exchange does not correlate with anti-oestrogen responsiveness. In addition, the exon-7 splice variant that was discussed as possibly involved in anti-oestrogen resistance was encompassed by our primers and showed no difference with respect to hormone sensitivity or resistance.

These sequence analyses confirm reports by Tonetti and Jordan [9] and Yao and colleagues [10], who found no correlation between ERα mutations and Tam resistance in clinical or xenografted breast cancers, and a report by Madsen and colleagues [11], who found no correlation between ERα splice variants and anti-oestrogen resistance.

1.4.4. Gene-expression profile studies

As a ligand-inducible transcription factor, ERα itself is closely involved in the cellular transcription machinery. If Tam resistance is attributable to changes in the concentration or activity of co-regulators, does the ERα-Tam complex more effectively recruit certain co-activators or fail to recruit co-repressors [12]? Several other ER-dependent and-independent mechanisms of Tam resistance have been proposed [13], yet a single responsible mechanism has not been and will most probably never be identified, because resistance seems to be multicausal. Growth factors, their receptors, extracellular proteins, immediate early genes, transcription factors, cell-cycle regulators and signal-transduction molecules have been identified as being potentially involved in Tam resistance [13,14]. Thus, Tam resistance seems to be the consequence of changes in complex molecular mechanisms resulting from disturbed interactions and differential expression or modification of proteins.

DNA microarrays provide the opportunity of taking a genome-wide look at the patterns of molecular events leading to the phenotype of Tam resistance. Therefore, we undertook a comprehensive gene-expression study of the Tam-sensitive xenograft line 3366 and its resistant subline 3366/Tam using high-density oligonucleotide arrays from Affymetrix, in order to find key molecules causally involved in Tam resistance, and to determine

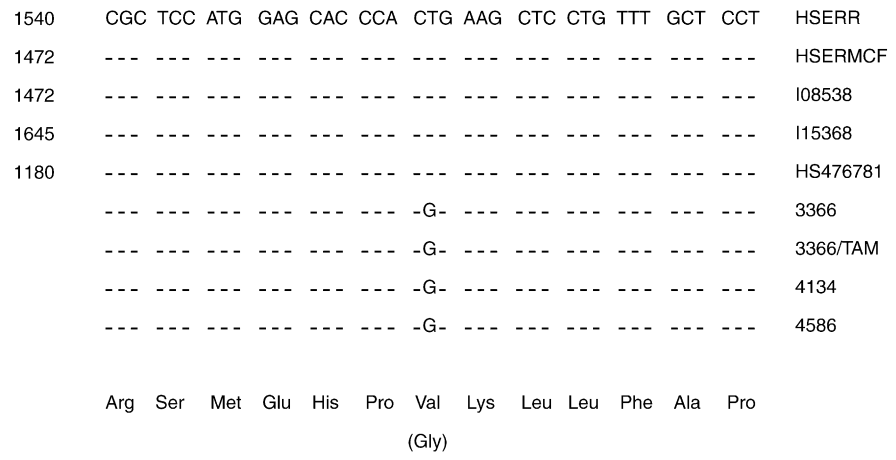

Fig. 3. Sequence alignment of the ligand-binding domain of the oestrogen receptor-α as derived from mRNA extracted from our own breast cancer xenografts with corresponding reference sequences from public database entries (GeneBank Accession Numbers X03635, M12674, I08538, I15368, U47678).

the expression profiles for sets of genes that might be of diagnostic value.

For comparative analysis, fragments of both tumour lines were transplanted subcutaneously into female nude mice, which were then supplemented with oestradiol to support tumour growth. Total RNA was isolated from the tumour tissues, pooled per treatment group and hybridised to Affymetrix HuGeneFL chips [interrogating roughly 6800 genes and expressed sequence tags (EST)] [15]. To confirm the reproducibility of the data, the whole tumour-growth experiment was repeated once, whereat the RNA samples were hybridised to a later generation of Affymetrix arrays (Hu95Av2) containing more probe sets interrogating 12 000 genes and ESTs. Unsupervised and supervised comparisons and clustering algorithms were used to identify differentially expressed genes and patterns of gene expression, respectively. For unsupervised analysis, two-dimensional hierarchical clustering as well as '*k*-means' clustering algorithms were applied and genes were clustered into different groups showing a distinct expression pattern. Following the supervised approach, genes that were differentially expressed between sensitive and resistant xenograft tumours were identified using three independently conducted *t*-tests resulting in two sets of up- and downregulated genes.

As revealed by unsupervised hierarchical cluster analysis, the gene-expression patterns of the Tam-sensitive and-resistant breast carcinoma xenografts clearly differed. Following supervised analysis, two sets of 56 interesting genes each were identified that were either up- or downregulated in the Tam-resistant xenograft line in comparison to the-sensitive line. These sets contained genes coding for proteins involved in transcription regulation, signal transduction, cell growth, cell-cell adhesion and immune response. Some of them are even known to be involved in breast cancer. In addition, among the genes most strongly upregulated in the Tam-resistant xenografts was a set of nine genes that are annotated as being interferon-inducible [15].

The data provide the possibility of deriving markers for predicting Tam resistance, either in the form of distinct genes or sets of genes. Genes whose expression differs significantly between both lines will be further evaluated as potential targets for diagnostic or therapeutic approaches. A clinical validation of potential markers by real-time reverse transcriptase-polymerase chain reaction, immunohistochemistry and *in situ* hybridisation in breast sections from patients with Tam-resistant breast cancer is currently under investigation.

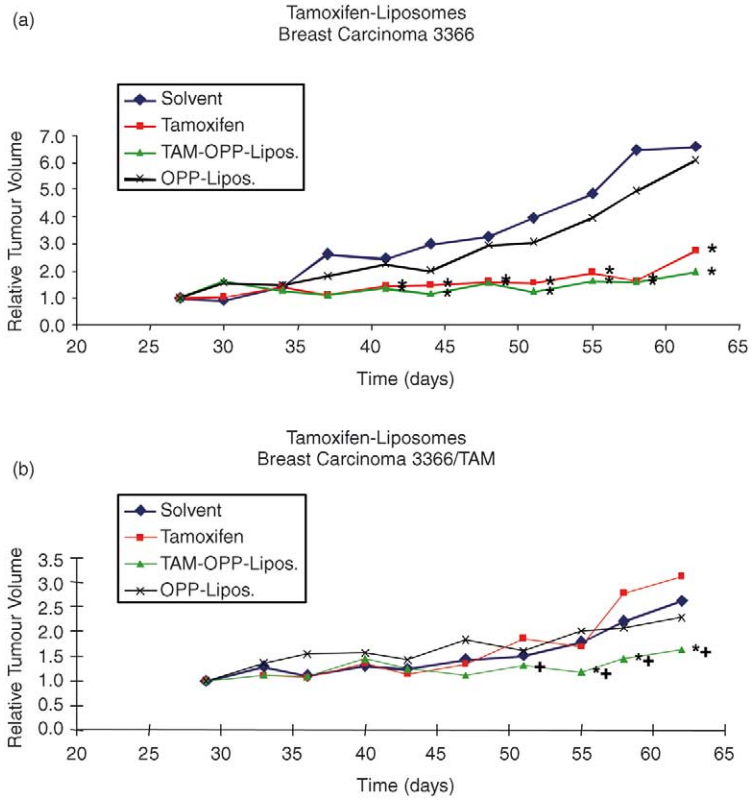

Fig. 4. Growth curves of breast carcinoma xenografts 3366 (a) and 3366/Tam (b). 6–8 nude mice per group received subcutaneous (s.c.) fragments of the tumour at day 0. Mice were supplemented with oestradiol (once per week 0.5 mg/kg i.m.) and treated with tamoxifen (Tam) or Tam-containing liposomes (50 mg/kg once per week intraperitoneally (i.p.) for 4 weeks) starting at day 26. Control groups of mice received the solvent or Tam-free OPP liposomes in equal volumes and schedules. *Significantly different to tumour volume of the control group. + Significantly different to tumour volume of Tam-treated group.

1.4.5. Approaches to overcoming Tam resistance

Based on the fact that Tam acts intracellularly by competing with the natural ligand for binding to the ERα and that membrane trafficking can be compromised by the overexpression of P-glycoprotein for which Tam is a substrate [16], we developed a way of overcoming this potential obstacle to efficient transportation. We designed a stable liposomal formulation containing a cancerostatic alkylphospholipid [17] (octadecyl-(1,1-dimethyl-4-piperidino-4-yl)-phosphate; OPP) as a special membrane constituent. Tumour-bearing nude mice were treated once per week intraperitoneally (50 mg/kg) for 4 weeks with Tam-containing or drug-free liposomes. As can be seen in Fig. 4a, Tam in both the standard oil formulation and in liposomes significantly inhibited the growth of 3366 breast xenografts. In contrast, the 3366/Tam xenografts were only significantly inhibited by the liposomal formulation, while the tumours did not respond to the standard drug formulation. Tam-free OPP liposomes had no effect in both lines. One explanation for the higher antitumour efficacy of liposomal Tam could be the prolonged presence of Tam not only in the serum and the tumour, but also in the liver and uterus of the xenograft-bearing nude mice (R. Zeisig, personal communication). These results warrant further investigation of the mechanisms leading to the enhanced accumulation of the Tam liposomes and are an example of how the established xenograft models of breast tumours can be used to address clinically relevant problems.

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