Plasma and Tissue Pharmacokinetics of Epirubicin and Paclitaxel in Patients Receiving Neoadjuvant Chemotherapy for Locally Advanced Primary Breast Cancer

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The objective of the study was to assess individual distribution of antineoplastic drugs into the tumor. Twelve advanced-stage primary breast cancer patients with neoadjuvant epirubicin + paclitaxel chemotherapy were studied. Plasma concentrations of epirubicin and paclitaxel were monitored for 24 h. Epirubicin concentrations in subcutaneous and tumor tissues were measured using microdialysis up to 12 h postdose. Epirubicin concentrations were described by a compartmental population pharmacokinetic model (NONMEM). Noncompartmental analysis was used for paclitaxel. Plasma pharmacokinetics corresponded to published data. Mean epirubicin exposure in the tumor and in subcutaneous tissue was very similar, but tissue C_{max} and area under the curve values reached only (means) 1% and 11%, respectively, of plasma values. Epirubicin doses were significantly correlated to tumor exposure irrespective of body surface area. There is no specific barrier for epirubicin to reach primary breast cancer tumors.

It is obvious that achieving adequate concentrations at the site of action is a prerequisite for therapeutic efficacy of any drugs including antineoplastic agents. Therefore, although tumor resistance at a molecular level has been recognized as a key component in therapeutic failure, current attention is also focused on local drug transport and drug delivery. Among various distribution processes, drug transfer from the central compartment into the interstitial space is considered a critical step in clinical resistance of tumors.[1,2]

Solid tumors have several potential barriers to drug delivery such as alterations in the distribution of blood vessels, blood flow, capillary permeability, interstitial pressure, and lymphatic drainage that may limit drug distribution from the blood vessel and provide inherent mechanisms of resistance. These factors may vary considerably between tumors.[1,3] A method that allows the quantification of local drug concentrations *in vivo* is the microdialysis technique, which is based on the diffusion of analytes from the interstitial compartment through a semipermeable membrane.[4] In 1996, an initial pilot study in cancer patients reported the feasibility of microdialysis for assessing intratumoral carboplatin concentrations.[5] Thus far, clinical microdialysis has been employed for the characterization of intratumoral drug concentrations of carboplatin,[5] cisplatin,[6] 5-fluorouracil,[7] methotrexate,[8,9] dacarbacine,[10] capecitabine,[11] and melphalan[12] in various types of cancer, such as breast cancer,[7,8,11] malignant melanoma,[5,10,12] osteosarcoma,[12,13] Merkel cell tumor,[12] and cancer of the oral cavity.[6] As a potential caveat for the use of microdialysis in cancer patients, puncturing solid tumors have been suspected to induce metastases, raising ethical concerns. However, clinical studies following fine-needle biopsy of tumors reported an incidence of such events in the range of 0.003–0.005%.[14] There was no evidence that puncture of tumor lesions

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affected either the course or prognosis of the underlying disease.[14]

The combination of epirubicin and paclitaxel is one of several regimens used in neoadjuvant treatment of breast cancer (*e.g.*, ref. 15). There is no information on tissue distribution of these two drugs, with the exception of epirubicin concentrations reported in tumor tissue after surgery[16] and paclitaxel concentrations in ascites fluid in a single patient.[17] The objective of this study was to assess individual drug distribution into the tumor and, as a reference site, into the abdominal subcutaneous adipose tissue in breast cancer patients, assuming that the interstitial tumor dose intensity, which is defined as the product of concentration at the effect site and time of exposure,[5] would be more predictive for clinical tumor response than plasma concentrations.

RESULTS

Evaluation of the microdialysis method

Epirubicin. In the *in vitro* tests, a negligible adsorption of epirubicin to the equipment was found. Already in the first 30 min microdialysate sample, an equilibrium was reached (**Figure 1**). After transfer of the membrane into a solution without epirubicin, it took less than 60 min until the concentration was below the quantification limit of 2 μg/l. The *in vitro* recovery and delivery of epirubicin was constant over a wide concentration range of 14.2–650 μg/l and reached a mean $\pm$ SD value of 52.8 $\pm$ 1.4%. The *in vivo* microdialysis calibration by retrodialysis conducted as a proof of principle in patients 11 and 12, gave a recovery of 48.5% and 56.7% in tumor and of 47.8% and 48.5% in subcutaneous adipose tissue, respectively, confirming the *in vitro* results. Thus, the mean *in vitro* recovery was used as a correction factor (*i.e.*, 0.528) for all microdialysis concentrations *in vivo* to calculate tissue concentrations from measured concentrations.

Paclitaxel. Paclitaxel showed pronounced adsorption to the microdialysis membrane as well as to the outlet tubes. The amount bound to the equipment exceeded that recovered in the perfusate. Therefore, even with high albumin concentrations in the perfusion fluid in order to bind the drug, it took approximately 2 h to reach an equilibrium between external and perfusate paclicaxel concentrations, with a pronounced variation. Owing to this result, we decided to exclude paclitaxel from further *in vitro* and *in vivo* microdialysis experiments.

Outcome of treatment

Of the 12 patients, two responded completely to the initial chemotherapy before surgery, five responded partially, and five had stable disease. Local relapse of the disease after surgery, which was monitored for a median postoperative observation period of 68 (range, 55–75) months, occurred in six of the patients, including disease-related death in two patients.

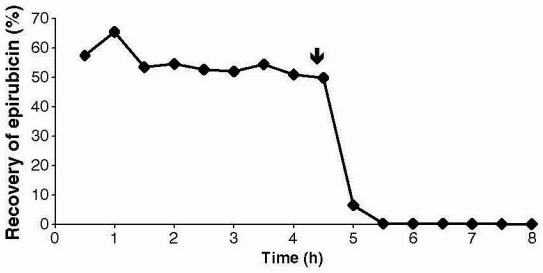

Figure 1 *In vitro* recovery of epirubicin. In this experiment, a microdialysis membrane was placed for 300 min in artificial interstitial fluid (Ringer's solution with 2.7% human serum albumin) containing 635 μg/l of epirubicin; thereafter, the membrane was placed in the same medium without epirubicin (↓).

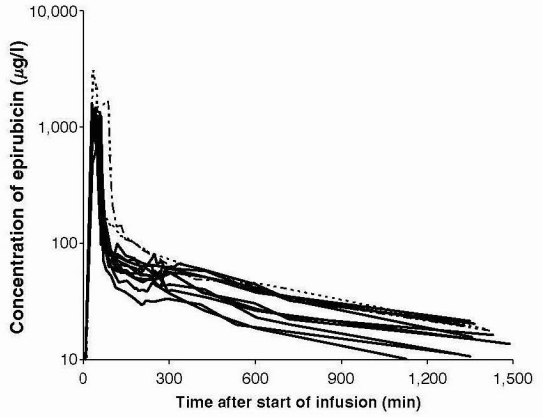

Figure 2 Individual concentration time profiles of epirubicin in plasma after a 1 h infusion of 90 mg/m^2 (protocol A, black) or 150 mg/m^2 (protocol B, dotted lines).

Plasma, tumor, and subcutaneous tissue pharmacokinetics of epirubicin

The individual time vs (total) plasma concentration profiles of epirubicin are shown in **Figure 2**. As expected, a rapid decay of concentrations occurred after the end of the infusion, followed by a slower elimination period. Evaluation of pharmacokinetic parameters in plasma was most appropriate using a three-compartment model (**Figure 3**, see Methods section). The obtained tissue concentrations were much lower than (total) plasma concentrations (**Figure 4**). In the three-compartment model initially developed from plasma concentrations only, the predicted concentration vs time profiles and pharmacokinetic parameters for compartment no. 3 were very similar to those measured in subcutaneous tissue corrected by a scaling factor reflecting binding differences of epirubicin between plasma (where total concentrations were measured) and interstitial fluid obtained by microdialysis. Thus, the subcutaneous concentrations were allocated to the existing compartment no. 3 from the analysis of plasma concentrations and included into the model (**Table 1**). This model provided plausible results with narrow confidence intervals for the various characteristics (**Table 1**). Age and body mass index (BMI) had an

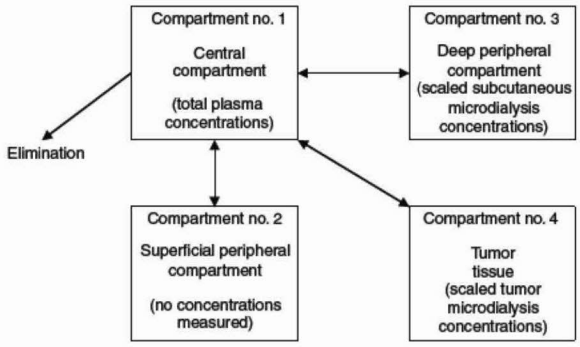

Figure 3 Compartmental pharmacokinetic model used for epirubicin. A three compartment model was fitted using plasma and subcutaneous microdialysis concentrations. To account for binding differences between measured plasma and microdialysis concentrations, a scaling factor for microdialysis data was included in the model. After obtaining pharmacokinetic parameters for the three compartment model, all parameters were fixed for estimation of the parameters for the tumor compartment.

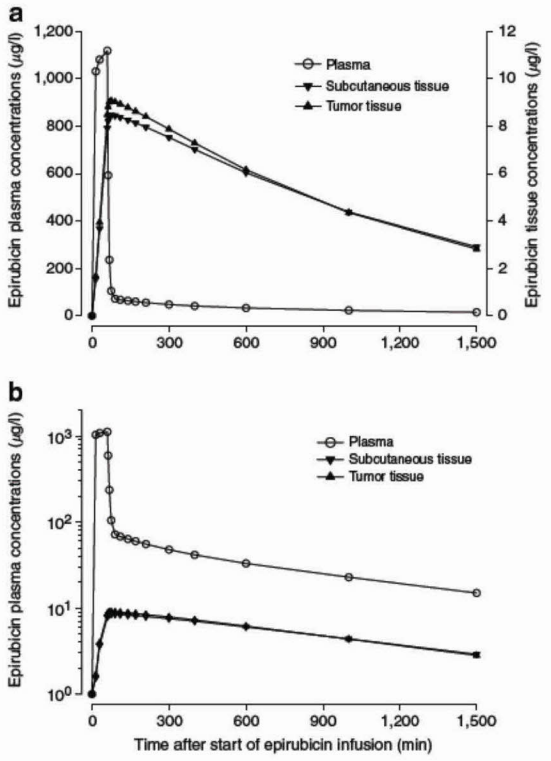

Figure 4 Population mean concentration time profiles of epirubicin in plasma, subcutaneous tissue, and tumor following a 1 h infusion of 90 mg/m^2 BSA dose in a patient with mean demographic data (170 cm body height, 79.5 kg body weight, dose 171.9 mg). (**a**) Linear scale; (**b**) log scale. Please note the 100 fold difference in scales between plasma and tissue concentrations in **a** only.

independent significant effect on the volume of distribution in compartment no. 2 (**Table 1**).

Finally, the incorporation of tumor concentrations into the pharmacokinetic model was successful as assessed by the criteria described in the Methods section, using the same scaling factor. (We also tried to estimate a separate scaling factor; this was almost identical to the factor found for subcutaneous fluid, but could not be estimated reliably.) The corresponding goodness-of-fit plots are shown in **Figure 5**. The procedure allowed the estimation of concentration vs time profiles in tumor beyond the observation period despite the higher random noise inherent to microdialysis data (**Figure 6**). After the distribution processes have reached the equilibrium, the lowest rate constant, in this case redistribution from compartment no. 3 to the central compartment (**Table 1**), governs the decay of concentrations in all tissues. The corresponding population estimate for the respective half-life, which may be calculated as ln (2) × clearance ^{1} × volume of distribution, was 466 min or roughly 8 h.

In the tumor of one patient and in the abdominal subcutaneous adipose tissue of four patients, epirubicin concentrations in the perfusate were always below the limit of quantification. We did not take these concentrations into account for the development of the model (see Discussion section).

The expected significant positive correlations were found in the correlation matrix: dose was correlated to body surface area (BSA) and related parameters such as body weight, AUC, and C_{max} for respective sites that were correlated, the AUC in tumor tissue calculated by the NONMEM model was correlated to the scaling factor, age and BMI were negatively correlated to volume of distribution in compartment no. 2 (V2) (as also seen in the evaluation of covariates of the population pharmacokinetic analysis, **Table 1**), etc. Despite the similar mean concentrations obtained in tumor and subcutaneous tissue (**Figure 4**), individual C_{max} and AUC values showed no significant correlation between the two sites. Interestingly, tumor C_{max} and $AUC_{0\text{–}503\ min}$ were positively correlated to the absolute dose (Spearman's rank correlation coefficient $r_S = 0.752$, $P = 0.008$ and $r_S = 0.633$, $P = 0.036$) and related parameters (body weight, BSA, BMI) and had a negative correlation to V2 ($r_S = -0.655$, $P = 0.029$ and $r_S = -0.582$, $P = 0.060$, NS).

In **Table 2**, selected pharmacokinetic parameters of epirubicin in tumor, s.c. tissue, and plasma are sorted according to response and relapse of the disease. Despite mean differences, there was no statistically significant difference between groups for any pharmacokinetic parameter in the Kruskal–Wallis test (including those not shown in **Table 2**). Also, none of the demographic characteristics were related to efficacy of treatment. However, the two patients with the highest tumor epirubicin concentrations, *i.e.*, patients no. 1 and 3, had at least partial response and no relapse, whereas those two patients with the lowest concentrations, *i.e.*, patients no. 8 and 12, showed no histological response and also had relapse of cancer, and patient no. 8 died from the disease (**Figure 6**).

Plasma pharmacokinetics of paclitaxel

The individual concentration vs time profiles of paclitaxel in 11 patients are shown in **Figure 7**; plasma pharmacokinetic parameters obtained by noncompartmental methods are

Table 1 Pharmacokinetic parameters of epirubicin in plasma, tumor, and subcutaneous tissue

Pharmacokinetic parameter of epirubicin[a] (unit)	Lower limit of 95% CI	Point estimate	Upper limit of 95% CI	Interindividual CV (%)
Absolute dose (mg)	157	188	219	NA
Maximal concentration in compartment no. 1 (plasma) taken from raw data (μg/l), n=12	1108	1264	1421	NA
Maximal concentration in compartment no. 3 taken from raw data (μg/l), n=8	6.6	11.2	15.8	NA
Ratio of maximal concentration in compartment no. 3 over compartment no. 1 (%), n=8	0.44	0.97	1.49	NA
Maximal concentration in compartment no. 4 (tumor) taken from raw data (μg/l), n=11	9.1	13.3	17.6	NA
Ratio of maximal concentration in compartment no. 4 over compartment no. 1 (%), n=11	0.81	1.06	1.31	NA
AUC in compartment no. 1 (mg/l $\times$ min)	116	126	136	NA
AUC from predose to 503 min after start of infusion in compartment no. 3 calculated by noncompartmental methods (mg/l $\times$ min), n=8	3.1	4.5	5.9	NA
AUC from predose to 503 min after start of infusion in compartment no. 4 calculated by noncompartmental methods (mg/l $\times$ min), n=11	3.1	4.6	6.0	NA
AUC extrapolated to infinity in compartment no. 4 (tumor) calculated numerically, (mg/l $\times$ min)	12.2	14.7	17.2	NA
Ratio of AUC extrapolated to infinity in compartment no. 4 over compartment no. 1 (%), n=11	10.1	11.6	13.0	NA
Elimination clearance (L/min)	1.27	1.33	1.39	14.1
Clearance for exchange between compartments no. 1 and no. 2 (L/min)	0.245	0.290	0.335	NS
Clearance for exchange between compartments no. 1 and no. 3 (L/min)	0.799	0.876	0.953	28.0
Clearance for exchange between compartments no. 1 and no. 4 (L/min)	0.148	0.222	0.296	56.9
Volume of distribution in compartment no. 1 (plasma) (L)	10.8	11.7	12.6	NS
Volume of distribution in compartment no. 2 (L)	34.9	47.9	60.9	NS
Volume of distribution in compartment no. 3 (L)	537	573	609	NS
Volume of distribution in compartment no. 4 (tumor) (L)	99	135	171	NS
Scaling factor accounting for lower protein binding in tissue (no unit)	0.0793	0.0894	0.0995	32.9
Effect of BMI on volume of distribution in compartment no. 2 (% change of volume of distribution per kg/m^2 of BMI above mean BMI)	3.67	2.99	2.32	NA
Effect of age on volume of distribution in compartment no. 2 (% change of volume of distribution per year of age above mean age)	3.99	3.02	2.05	NA

AUC, area under the curve; BMI, body mass index; CI, confidence interval; CV, coefficient of variation; NA, not applicable; NS, inclusion of this element of intraindividual variation did not improve the model significantly. AUC and C_{max} values are normalized to an epirubicin dose of 90 mg/m^2 BSA. Two patients received 150 mg/m^2, all other patients received 90 mg/m^2. [a]Results of compartmental population pharmacokinetic evaluation if not indicated otherwise.

presented in **Table 3**. (In one patient, plasma volumes were not sufficient to measure paclitaxel concentrations.) Much of the apparent variability in the concentration vs time curves was caused by the highly variable duration of infusion (range 87–224 min). Tissue concentrations of paclitaxel could not be determined (see above).

Tolerability of microdialysis

Microdialysis experiments were well tolerated by all patients. No patient required administration of local anesthetics before insertion of the microdialysis syringes. No other adverse events occurred including hemorrhage or excessive pain at the site of probe insertion for both tumor and subcutaneous tissue, respectively. All patients could easily tolerate the pharmacokinetic measurement after having received neoadjuvant chemotherapy. Most importantly, no local metastases were observed at the site of probe insertion during the postoperative observation period.

DISCUSSION

In this study, beyond plasma pharmacokinetics of epirubicin and paclitaxel, we assessed drug transfer of epirubicin into the interstitial space of tumors and subcutaneous tissue in breast cancer patients, assuming that local exposure would be more predictive for treatment efficacy than plasma concentrations.

Epirubicin plasma pharmacokinetics were similar to published data. This applies to both the three-compartment model and the quantitative results obtained here.[18–21] It has been reported that paclitaxel may increase the AUC of epirubicin when given consecutively.[22] However, as only two patients in our study received epirubicin alone, such an effect could not be substantiated in the present small study.

Interestingly, it was possible to allocate a compartment from the plasma model to a real site. The scaling factor introduced in the model, *i.e.*, 0.09 (**Table 1**), is lower than the unbound fraction of epirubicin in plasma.[23] However,

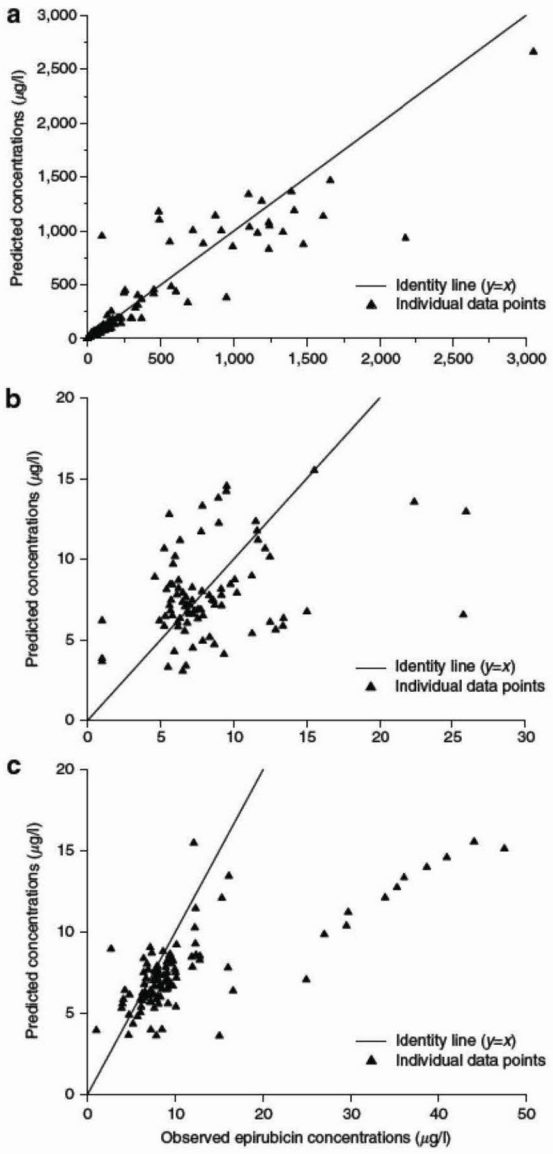

Figure 5 Goodness of fit plots for epirubicin concentrations in (**a**) plasma (compartment no. 1), (**b**) subcutaneous tissue (compartment no. 3), and (**c**) tumor (compartment no. 4). Predicted concentrations were calculated by the pharmacokinetic model as described in the Methods and Results sections, using the population estimates for pharmacokinetic parameters (see **Table 1**). The concentrations grossly deviating from the population estimates on the right side of **c** originate from patient no. 3, but were properly estimated with individual parameters (see **Figure 6**).

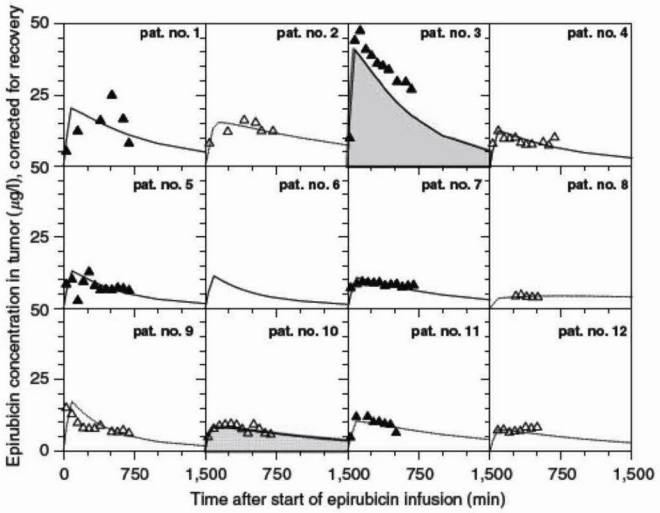

Figure 6 Individual concentration time profiles of epirubicin in tumor tissue. Triangles represent the raw tumor concentration data (only those above the lower limit of quantification, *i.e.*, 2 μg/l, are shown), lines represent the individual estimates obtained by population pharmacokinetics (see Methods section). Lines also indicate histological response (dotted: no change, continuous: partial response, continuous with hatched areas: complete response). Open symbols were used for patients with local relapse of disease, closed symbols for patients with no relapse during the period of observation (patient no. 6 had no concentrations above the limit of quantification, therefore the population mean was used for this patient who had no relapse). The dose was 90 mg/m^2 BSA (protocol A), for all patients except nos. 2 and 3 who obtained 150 mg/m^2 BSA (protocol B) (see the text). Patients nos. 2 and 8 have died from progressive disease 13 and 30 months after surgery, respectively.

we used an artificial interstitial fluid for microdialysis both for *in vitro* validation and for perfusion of the system, which should provide total interstitial concentrations rather than unbound concentrations. The complex interaction of epirubicin with blood components including proteins and erythrocytes,[24] the indirect access microdialysis provides to tissue concentrations, and the differences in binding of epirubicin at the different sites make it difficult to provide a physiological explanation for the magnitude of the observed scaling factor. It was, however, useful to model the concentration vs time curves, both in the subcutaneous tissue and the tumor, and thus to obtain information on the concentration vs time profile in the tissue beyond the observation period.

The individual measured epirubicin plasma concentrations were not predictive of interstitial concentrations in tumor or subcutaneous adipose tissue. The lack of correlation between plasma and tumor concentrations (and derived pharmacokinetic parameters) has also been described in other microdialysis studies[5,7,8] and may be explained by typical characteristics of tumor biology and vascular architecture.[7,8] However, mean values in tumor and in subcutaneous tissue were almost identical, as was interindividual variation (**Table 2**). Furthermore, individual tissue concentration vs time profiles showed some irregularities (**Figure 6**) and could not be assessed in the tumor in one patient and in the subcutaneous tissue in four patients. We attributed these findings to technical problems of the microdialysis method, such as (intermittent) placement in areas with insufficient interstitial fluid to allow (more complete) exchange of concentrations. Finally, the method we used to estimate *in vivo* recovery is a compromise between feasibility and accuracy (for an extensive discussion, see ref. 25). Thus, it cannot be excluded that the lack of correlation between tissue and plasma concentrations is a methodological problem rather than a true phenomenon. The same mean concentrations in both tissues tested suggests

Table 2 Parameters directly related to exposure to epirubicin in relationship to histopathological response to the neoadjuvant chemotherapy and to relapse of disease

	Response			Relapse	
Pharmacokinetic parameter of epirubicin (unit)	No change (n=5)[a]	Partial response (n=5)[a]	Complete response (n=2)[a]	Yes (n=6)[a]	No (n=6)[a]
Absolute dose (mg)	190 (31)	173 (4)	146; 294	182 (26)	193 (20)
Maximal concentration in compartment no. 1 (plasma) taken from raw data (μg/l)	1247 (113)	1259 (124)	1474; 3051	1285 (99)	1558 (315)
Maximal concentration in compartment no. 3 taken from raw data (μg/l)	17.1 (5.0)	8.6 (1.4)	9.5	19.4 (6.3)	9.1 (0.9)
Maximal concentration in compartment no. 4 (tumor) taken from raw data (μg/l)	11.2 (2.1)	14.9 (3.4)	9.3; 47.7	11.0 (1.8)	21.3 (7.1)
AUC in compartment no. 1 (mg/l $\times$ min)	130 (21)	133 (8)	136; 249	133 (17)	151 (21)
AUC from predose to 503 min after start of infusion in compartment no. 3 calculated by noncompartmental methods (mg/l $\times$ min)	5.5 (1.3)	3.5 (0.6)	3.7	6.0 (1.7)	3.7 (0.4)
AUC from predose to 503 min after start of infusion in compartment no. 4 calculated by noncompartmental methods (mg/l $\times$ min)	3.8 (0.8)	4.8 (0.7)	3.7; 18.0	3.7 (0.7)	7.5 (2.7)
AUC extrapolated to infinity in compartment no. 4 (tumor) calculated numerically, (mg/l $\times$ min)	16.8 (3.2)	13.7 (2.6)	14.7; 34.1	16.1 (2.7)	17.5 (3.9)

Abbreviation: AUC, area under curve. Pharmacokinetic parameters are not dose normalized. Means (SEM; individual values for $n<3$) are shown. Compartment no. 3 represents subcutaneous adipose tissue data, compartment no. 4 represents data obtained from breast cancer tumor microdialysis. [a]Numbers for individual groups of data may be lower because tissue concentrations could not be quantified in all cases (see **Table 1**).

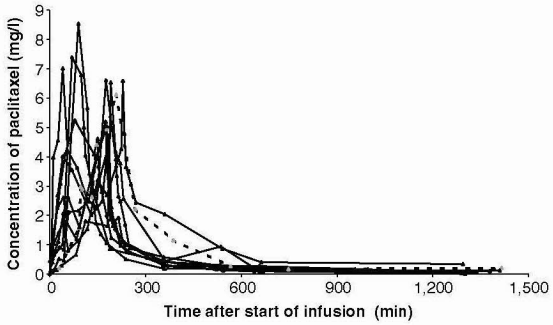

Figure 7 Concentration time profiles of paclitaxel in plasma after a 3 h infusion of 175 mg/m^2 (protocol A, black) and 250 mg/m^2 (protocol B, dotted lines).

that distribution of epirubicin into the relatively small solid tumors examined here is not a general limiting factor for tumor exposure and thus for antineoplastic efficacy.

Epirubicin AUC in plasma is closely related to the effect on white blood cell counts.[26] In an attempt to achieve uniform AUC values, the drug usually is administered at doses normalized by BSA, as has been done in the underlying phase III study. However, it has been shown that BSA is neither a significant covariate for plasma concentrations nor for neutropenia.[27] A more recent study on epirubicin pharmacokinetics identified aspartate aminotransferase, but not BSA, as a significant covariate of systemic epirubicin exposure.[20] Here, we found that tumor exposure is related to absolute rather than to BSA-normalized doses, probably caused by a reduction in the volume of distribution in compartment no. 2 in individuals with higher BMI. As increasing age had the same independent effect on V2, this compartment may be related to total body water, and it is conceivable that a reduction in total body water would result in higher concentrations of epirubicin at other sites.

Table 3 Main pharmacokinetic parameters of paclitaxel in plasma

Pharmacokinetic parameter of paclitaxel (unit)	Lower limit of 95% CI	Point estimate	Upper limit of 95% CI
Maximal concentration taken from raw data (mg/l)	5.08	5.90	6.73
AUC extrapolated to infinity (mg/l $\times$ h)	13.3	15.7	18.1
Apparent terminal elimination half life (h)	6.2	8.2	10.2
Clearance (L/h/m^2 BSA)	10.2	11.7	13.1

AUC, area under curve; BSA, body surface area. Results are normalized to a dose of 175 mg/m^2 BSA. Data are given for 11 patients (no plasma available for patient no. 3).

Paclitaxel pharmacokinetic parameters in plasma were similar to published data,[19,28,29] taking different administration routes or duration of infusions into account. With respect to microdialysis of the highly lipophilic and poorly water-soluble drug paclitaxel,[30] many difficulties have been reported for microdialysis of lipophilic drugs. High protein binding (binding to the membrane and outlet tubes) is among the most important problems.[31,32] Even the addition of albumin to the perfusion fluid, which is recommended to decrease the binding of paclitaxel to plastics and glass,[33] did not prevent the binding of paclitaxel to membrane and tubes. As a constant recovery was not attainable, we were unable to study paclitaxel tissue pharmacokinetics using the microdialysis technique.

The merely descriptive evaluation of the relationship between epirubicin tumor exposure and therapeutic efficacy in this small study gave no clear results. The apparent trend to higher tumor exposure in patients with better efficacy as opposed to the other sites examined corresponded to the expectation but was not statistically significant. However, taking interindividual differences in the chemotherapy protocol, doses and paclitaxel plasma pharmacokinetics into account, in addition to methodological problems (see above), the power of this study to identify epirubicin tumor exposure as a major source of variation in therapeutic efficacy was low. Published data suffer from the same limitation. Müller *et al.*[7] reported a positive relationship between efficacy and 5-fluorouracil exposure in tumor, but not at other sites, in patients with primary breast cancer undergoing neoadjuvant chemotherapy. In contrast, no methothrexate pharmacokinetic parameter was related to clinical responses in a microdialysis study conducted in nine breast cancer patients.[8] One potential problem is highlighted in a preclinical study with melphalan in limb malignancies where a significant correlation between tumor response and melphalan concentration in subcutaneous tissue, but not in tumor was seen. The authors discussed that in this case, microdialysis probes may have been located in necrotic tumor regions which would result in microdialysis drug concentrations not representative for other regions of the tumor with living malignant cells.[12]

In summary, this study clearly shows that there is no specific barrier for epirubicin to reach primary breast cancer tumors. It appears that total, rather than BSA-adjusted doses, are related to tumor exposure, and that low total body water increases tumor exposure. These assumptions, however, need to be addressed in further studies.

METHODS

Patients. Twelve advanced stage patients with biopsy proven primary breast cancer (tumor size ≥ 3 cm) showing no evidence of overt metastatic disease and thus qualified for neoadjuvant chemotherapy were enrolled. All patients were treated within a multicenter randomized controlled phase III trial for neoadjuvant chemotherapy of locally advanced or inflammatory primary breast cancer initiated by the Arbeitsgemeinschaft Gynakologische Onkologie Breast Study Group. This trial compared a standard dose combination chemotherapy with four cycles of intravenous epirubicin at 90 mg/m^2 and intravenous paclitaxel at 175 mg/m^2 given every 3 weeks (protocol A) with a biweekly dose dense protocol comprising three cycles of epirubicin at 150 mg/m^2 followed by three cycles of paclitaxel at 250 mg/m^2 (protocol B). Protocol B was obligatory supported by the administration of filgrastim (Neupogen®, Amgen, Munich, Germany) given on days 3 10 after every chemotherapy course. All patients with hormone responsive disease received tamoxifen after completing chemotherapy. Local radiotherapy was performed according to the institutional guidelines. Patients had a mean age of 52 ± 6 years (mean $\pm$ SD) and a mean BMI of 29.2 ± 5.4 kg/m^2. Ten patients received protocol A, two patients (nos. 2 and 3) received protocol B. The actual mean ($\pm$SD) duration of the intravenous infusion of epirubicin and paclitaxel was 59 ± 12 min and 160 ± 44 min, respectively.

Study procedure. The study protocol, which was closely synchronized with the underlying phase III trials, was approved by the Ethics Committee of the Medical Faculty of the University of Cologne, Germany, and conducted according to the updated Declaration of Helsinki (published online: http://www.wma.net/e/policy/b3.htm) and the Good Clinical Practice Guidelines of the European Commission. All patients gave their written informed consent to participate in both the Arbeitsgemeinschaft Gynakologische Onkologie trial and the pharmacokinetic study reported here. On the day of the first epirubicin and paclitaxel administration, plasma concentrations of epirubicin and paclitaxel were monitored up to 24 h postdose. Additionally, the concentrations of epirubicin in the tumor as well as in abdominal subcutaneous adipose tissue were measured in 60 min intervals up to 12 h postdose by microdialysis. Definitive surgery was performed 2 4 weeks after cessation of protocol B and 3 5 weeks after completing protocol A, respectively.

Assessment of tumor response. Clinical assessment of response to neoadjuvant chemotherapy was based on both tumor imaging performed before and after treatment (mammography, ultrasound examination, and magnetic resonance tomography) and histological examination of the tissue removed during surgery using standard Eastern Cooperative Oncology Group criteria: complete response was defined as complete disappearance of all measurable tumor for at least 4 weeks; partial response was defined as a reduction of the tumor size by 50% or more measured by two perpendicular diameters; progressive disease was defined as any increase of tumor size by more than 25% or occurrence of new lesions. Any case other than CR, PR, or PD was classified as having stable disease. Categorization of pathological complete response required the microscopical absence of any invasive tumor lesion at the time of definitive surgery.

Microdialysis procedure. The principles of microdialysis have been described previously.[1,4,31]

A commercially available microdialysis probe (CMA 60, CMA, Solna, Sweden) with a molecular cutoff of 20 kDa, an outer diameter of 600 μm, and a membrane length of 30 mm was used. The probe was constantly perfused at a flow rate of 2.00 μl/min by a CMA 107 microdialysis pump (CMA, Solna, Sweden). Owing to technical problems when inserting the membrane into the dense breast tumor tissue,[34] we used a special introducer for the implantation in the tumor manufactured by CMA (Solna, Sweden). The perfusion fluid was Ringer's solution with human serum albumin in a final concentration of 2.7% in order to mimic *in vivo* interstitial conditions[35,36] and to prevent fluid loss into the tissue.[36]

The *in vivo* microdialysis experiments could only be performed for epirubicin because of extensive adsorption of paclitaxel to the material of the microdialysis equipment.

The microdialysis probes were inserted without local anesthesia into the primary tumor and into abdominal subcutaneous adipose

tissue, respectively. In order to implant the microdialysis membrane completely in the tumor tissue, only patients with a tumor size $\geqslant 3$ cm were enrolled. The correct position of the probe in the tumor was established by two dimensional ultrasound scanning. Microdialysates were collected in special microvials (CMA, Solna, Sweden) and were stored at −80°C until analysis. After a 60 min run in sampling period, the epirubicin infusion and the sampling of the microdialysates were started in 10 patients. In the last two patients, an additional *in vivo* calibration was performed by retrodialysis with epirubicin as described below. Microdialysis pumps were fixed to the skin of the patients with adhesive strips so that they had no mobility restrictions.

Assessment of microdialysis probe recovery. To estimate true concentrations of an analyte in interstitial fluid from the concentration in the dialysate, it is obligatory to determine the recovery of the procedure.[31,37]

In vitro experiments: For *in vitro* recovery assessment, the microdialysis probes were placed in polypropylene tubes containing Ringer's solution with 2.7% human serum albumin and different drug concentrations at 37°C. Concentrations measured in the dialysate were expressed as a percentage of the concentration in the surrounding medium (recovery *in vitro*). Probes were also placed in the artificial interstitial fluid containing a fixed concentration of the drugs in order to check for adsorption of the analytes to the membrane and the tubes. After monitoring the concentration in the dialysate over several hours, the membrane was transferred into the same medium, but without drugs to observe the decline of drug concentrations in the dialysate.

In vivo experiments: Because the diffusion process is equal in both directions through the semipermeable membrane, *in vivo* recovery of epirubicin can be assessed using the retrodialysis method.[10,31] To this end, epirubicin was added to the perfusion medium ("perfusate") and the disappearance rate (delivery) through the membrane was calculated and taken as a measure of *in vivo* recovery. Thus, the *in vivo* recovery was calculated as:

$$\text{recovery}\ (\%) = 100 \quad (100 \times \text{epirubicin}_{\text{dialysate}} / \text{epirubicin}_{\text{perfusate}})$$

where $\text{epirubicin}_{\text{dialysate}}$ is the epirubicin concentration in the dialysate and $\text{epirubicin}_{\text{perfusate}}$ is the epirubicin concentration in the perfusate.

In vivo recovery in the tumor and in the subcutaneous adipose tissue was assessed for the last two patients only with a perfusion fluid containing 10 μg/l epirubicin as the mean value of two periods of 30 min. This was done to check whether recovery determined *in vitro* provides a reasonable estimate of the *in vivo* situation and therefore may be used to calculate tissue concentrations of microdialysate results. Before the therapeutic administration of epirubicin, the perfusion fluid was changed again to Ringer's solution with 2.7% human serum albumin and the system was flushed for 30 min. Then, epirubicin was administered as above and sampling was continued at 60 min intervals for another 9 h in these two patients.

Epirubicin analysis. Plasma and microdialysate samples containing epirubicin were analyzed by high performance liquid chromatography. For stock solutions, we used polypropylene tubes in order to prevent loss owing to adsorption to glass,[9] and we stored the solutions at −80°C.

Extraction procedure of microdialysate samples: Microdialysate (50 μl) was mixed with 50 μl of daunorubicin solution 750 ng/ml (= internal standard) in 0.05 M ammonium formate buffer, pH 4.0. The solution was filtered through a solid phase extraction pipette tip (SPEC PLUS™ C18, DRG Instruments GmbH, Marburg, Germany), which was pretreated with acetonitrile (1 × 100 μl) and water (2 × 200 μl). The pipette tip was washed with water (2 × 250 μl) and 5% acetonitrile (500 μl). The analytes were eluted with 50 μl ammonium formate buffer 0.05 M, pH 4.0/acetonitrile (17.5:82.5 v/v). The resulting solution was injected (45 μl) into a high performance liquid chromatography system.

Extraction procedure of plasma samples: Plasma samples (400 μl) were mixed with the same volume of daunorubicin solution (see above). The plasma solutions were filtered through a solid phase extraction column (Isolute C18 100 mg, ICT Handels GmbH, Bad Homburg, Germany) which was pretreated with acetonitrile (1 × 500 μl) and water (2 × 500 μl). The column was washed with water (2 × 250 μl) and 5% acetonitrile (500 μl). The analytes were eluted twice with 200 μl ammonium formiate buffer 0.05 M, pH 4.0/acetonitrile (17.5:82.5 v/v). The resulting solution (100 μl) was injected into a high performance liquid chromatography system.

High performance liquid chromatography system: Chromatographic separation was carried out with a Waters 2690 Separations Module (Waters, Milford, MA) using a column temperature of 50°C and an isocratic flow of 1.0 ml/min. The mobile phase consisted of 0.05 M ammonium formate buffer (pH 4.0) with acetonitrile (63:37 v/v) containing 0.06% (v/v) triethylamine, and a C18 reversed phase column (Spheri GROM ODS 2, 50 μm, 250 × 4 mm, Grom, Herrenberg, Germany) was used. Run time was 10 min. The analytes were determined by a Jasco fluorescence detector (FP 920, Jasco Groβ Umstadt, Germany) operating with 470 nm excitation and 580 nm emission wavelengths, respectively.

Quality control of epirubicin analysis: Linearity of the method was obtained in the concentration range of 1.1 μg/l (*i.e.*, lower limit of quantification) to 3,340 μg/l in plasma and of 2.1 1,114 μg/l in microdialysates, respectively. Mean recovery of epirubicin was 89% for plasma and 52% for microdialysate. For both plasma and microdialysate samples spiked with three different concentrations (2,700, 130, 2.6 μg/l), intra and interassay precision ($n = 5$ and 6, respectively) were always less than 6%, accuracy (relative error) was better than 5%.

Paclitaxel analysis. *Sample preparation*: Plasma (50 μl) or the microdialyates from the preliminary *in vitro* tests were added to 20 μl of internal standard solution (docetaxel 10 mg/l in acetonitrile), 130 μl of 0.9% NaCl and 1 ml of tert butylmethylether. The mixture was shaken for 2 min and stored at −18°C until the bottom layer was frozen. The organic phase was decanted, evaporated and the residue was dissolved in 12 μl of the sample buffer. To prevent evaporation, paraffin was added to the sample.

Capillary electrophoresis: The analysis of paclitaxel concentrations in plasma and microdialysates from *in vitro* experiments were performed by capillary electrophoresis on a Beckman P/ACE 5510 CE system with ultraviolet detection (230 nm) (Beckman Coulter, Fullerton, CA) as described previously[38] with slight modifications. A 57 cm long capillary with an inner diameter of 500 μm, with an extended light path at the detection window (bubble cell, Hewlett Packard, Langen, Germany), was used. Samples were introduced into a capillary by pressure injection with 0.5 p.s.i. for 30 s. The electrophoresis buffer was 25 mM TRIS adjusted to pH 8.5 with 100 mM phosphoric acid, 100 mM sodium dodecyl sulfate, 10 mM hydroxypropyl β cyclodextrin and 35% acetonitrile. The sample buffer was similar with 10 mM sodium dodecyl sulfate without hydroxypropyl β cyclodextrin. After each run, the capillary was rinsed for 2 min with water, 2 min with the sodium hydroxide, and 2 min with the electrophoresis buffer. The separation was carried out with a voltage of 491 V/cm (28 kV). The lower limit of quantification was 50 ng/ml, intra and interassay precision were always less than 17.5%, accuracy (relative error) was better than 8%.

Data analysis. All data are presented as means and 95% confidence intervals. Statistical evaluations were performed using Microsoft Excel® 97 (Microsoft, Seattle, WA). Interstitial tissue concentrations were calculated using the *in vitro* recovery by the

following formula:

$$\text{Absolute tissue concentration} = 100 \times \text{concentration}_{\text{dialysate}} \times \textit{in vitro}\ \text{recovery}^{-1}$$

Pharmacokinetic parameters of paclitaxel in plasma were calculated by standard noncompartmental analysis using the WinNonlin™ Professional software Version 2.1 (Pharsight, Palo Alto, CA). The AUC was calculated by log linear trapezoidal rule with extrapolation to infinity.

Pharmacokinetics of epirubicin was best described by compartmental models. Although a two compartment open model could be fitted to individual plasma concentrations of all individuals, but (based on diagnostic plots) apparently did not describe concentration time profiles correctly in most individuals, a three compartment model could be fitted to the data only in four of the patients. Furthermore, because the duration of microdialysis was limited to up to 12 h for tolerability reasons and concentrations did not allow fitting of a log linear concentration decay at the end of this period in several individuals, it was not possible to assess the AUC extrapolated to infinity in the tissue by noncompartmental methods. Thus, an integrated population pharmacokinetic model was used, including plasma and tissue concentrations (NONMEM V software version 1.1, NONMEM Project Group, University of California at San Francisco, 1998). Microdialysate data were entered in the analysis assuming that the measured concentrations equal the concentration in the middle of the respective collection interval. In the first step, only plasma concentrations were evaluated. An exponential error model was chosen for the interindividual variability, whereas a proportional error model was used for the residual variability. Fitting was performed with the "first order conditional estimates" algorithm, taking interactions between the parameters into account. The most simple model, *i.e.*, a one compartment model with first order absorption and no interindividual variation, was stepwise expanded to more complex models if these were superior as assessed by the plausibility of the parameter estimates and their 95% confidence intervals (which were not allowed to include zero and/or unity), by goodness of fit plots, and by a significant ($P<0.05$) reduction of the objective function provided by NONMEM. Covariates tested included body weight, body height, BMI, and age. After completion of the model in the last step, tumor concentrations allocated to a new compartment were incorporated into the model. Before this step, all model parameters not related to the tumor compartment were fixed because it is not expected that the exchange of epirubicin with the tiny tumor compartment would change systemic pharmacokinetics.[3]

Plasma AUC was calculated as dose divided by clearance. As a measure of tumor exposure, AUC values for tumor tissue were calculated by two different methods: noncompartmental methods were used to calculate $AUC_{0-503\,min}$ in the tumor (and also for concentrations in subcutaneous tissue) in all subjects. This was the minimum duration of sampling and enabled a comparison among individuals. If microdialysis samples were available for a longer period, the respective 503 min data point was obtained by log linear extrapolation. The second method was numerical calculation of $AVC_{0-\infty}$ based on the pharmacokinetic parameters obtained in the population model. In the single patient with no tumor concentration above the limit of quantification (patient no. 6), $AUC_{0-\infty}$ was set to the population mean assuming technical failure of microdialysis.

A descriptive Spearman's rank correlation matrix of pharmacokinetic and demographic data were calculated to see which parameter might predict tumor exposure. A descriptive analysis was also performed to compare exposure among response groups by using the Kruskal Wallis test. These analyses were performed with SPSS (SPSS, Chicago, IL; version 11).

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CONFLICT OF INTEREST

The authors state no conflict of interest.

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