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Equilibrium Binding of Anthracycline Cytostatics to Serum Albumin and Small Unilamellar Phospholipid Vesicles as Measured by Gel Filtration

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ABSTRACT. A Sephadex G-200 gel filtration method was used to measure directly the equilibrium binding of five important anthracycline analogs to serum albumin. The order of the overall binding constant (K) in a 150 mM NaCl, 20 mM Hepes buffer (pH 7.45) was doxorubicin < daunorubicin < 4-demethoxydaunorubicin ≈ 13-dihydro-4′-deoxy-4′-iododoxorubicin < 4′-deoxy-4′-iododoxorubicin for human serum albumin (K = 2.67 ± 0.07 mM^{-1} to 24.5 ± 3.1 mM^{-1}) and bovine serum albumin (K = 1.36 ± 0.25 mM^{-1} to 48.4 ± 5.2 mM^{-1}). Data were given on the pH-dependence of K. The anthracycline-albumin association reaction was compared with measurements of drug partitioning into unilamellar phospholipid membranes and octanol. The results provide important new data required for a systematic kinetic analysis of anthracycline transport in tumor cells under serum conditions in a biological system. BIOCHEM PHARMACOL 55;1:27–32, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. anthracyclines; lipophilicity; association constants; serum albumin (human, bovine); membrane phospholipid

The reversible binding of anthracycline antitumor drugs to serum components is known to affect the diffusional passage and distribution of drug molecules across cell membranes [1–4]. Serum albumin, which contains several domains capable of binding hydrophobic ligands, is probably the major binding protein [5, 6]. For this reason, any detailed *in vitro* kinetic study on anthracycline transport in tumor cells aimed at predicting anthracycline pharmacokinetics and therapeutic antitumor activity must take the ligand binding properties of serum albumin into account. First of all, accurate estimates of drug association constants are needed. Early binding studies with doxorubicin (**1**) (Fig. 1) and daunorubicin (**2**) were carried out by equilibrium dialysis [5] and in a system with red blood cells [2]. Interactions of 4-demethoxydaunorubicin (**3**), 4′-deoxy-4′-iododoxorubicin (**4**) and *N*-acylated doxorubicin derivatives with serum albumin were characterized by spectrophotometric titrations [7, 8]. Very recently, serum albumin binding of a series of anthracycline derivatives was measured by means of an ultrafiltration method [6]. Lipophilic anthracyclines bind strongly to dialysis and ultrafiltration membranes, and in the work summarized in this report, we have examined the use of gel filtration as an alternative method for direct determination of equilibrium binding of anthracyclines to serum albumin. Overall association constants for five derivatives (**1–5**) to human serum albumin (HSA)§ and BSA are reported and compared with measurements of drug lipophilicity and binding to phospholipid in small unilamellar membrane vesicles.

MATERIALS AND METHODS

Chemicals

Hydrochlorides of anthracyclines were obtained from Sigma (**1, 2**) and Farmitalia (**3, 4, 5**). Stock solutions (1–3 mM) were prepared in water or methanol and stored at −20°. Concentrations were estimated spectrophotometrically after dilution of samples into methanol-1 M aqueous HCl (100:1, by vol.) to give a final concentration of approximately 10 μM. The molar extinctioncoefficients **2**, ϵ_{478} = 13.1 mM^{-1} cm^{-1} and **3**, ϵ_{480} = 9.97 mM^{-1} cm^{-1} were determined in this solvent in agreement with values reported in the literature: **2**, ϵ_{478} = 12.1 mM^{-1} cm^{-1} [9] and **3**, ϵ_{480} = 9.90 mM^{-1} cm^{-1} [10]. The ϵ_{478} of **1**, **4** and **5** was assumed to be equal to that of **2**. Fatty acid free HSA was obtained from Sigma and fatty acid free BSA from Boehringer. Sephadex G-200 (fine) was obtained from

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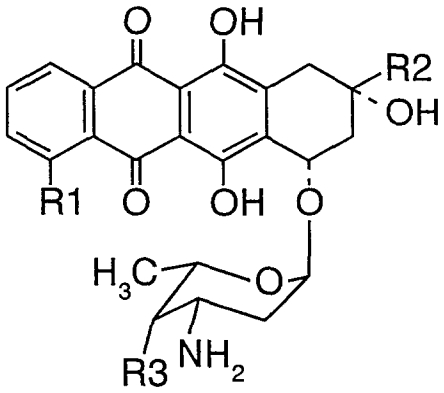
§ *Abbreviations:* HSA, human serum albumin; K, overall binding constant; SUV, small unilamellar vesicles; v, average number of anthracycline molecules bound per molecule of acceptor.



Compound	R1	R2	R3
1	OCH_3	$COCH_2OH$	OH
2	OCH_3	$COCH_3$	OH
3	H	$COCH_3$	OH
4	OCH_3	$COCH_2OH$	I
5	OCH_3	$CH(OH)CH_2OH$	I

FIG. 1. Chemical structure of the anthracycline compounds examined. 1, doxorubicin; 2, daunorubicin; 3, 4-demethoxy-daunorubicin; 4, 4′-deoxy-4′-iododoxorubicin; 5, 13-dihydro-4′-deoxy-4′-iododoxorubicin.

Pharmacia and 1,2-di[1-^{14}C]palmitoyl-L-3-phosphatidylcholine (113 mCi/mmol) from Amersham International. All other chemicals and organic solvents were of analytical grade.

Buffer and Solvent Systems

The following buffer system was used: (buffer 1), 150 mM NaCl, 20 mM Hepes adjusted with NaOH to pH 7.45. Lipids and anthracyclines were analyzed by TLC on 0.25 mm silica gel 60 plates (Merck) with the solvent systems: (A) $CHCl_3$-methanol-H_2O (32:12:2, by vol.) and (B) hexane-diethylether-acetic acid (35:15:0.5, by vol.). Lipid compounds were visualized in iodine vapour and by a $CuSO_4$–H_3PO_4 char reagent at 180°.

Octanol-Buffer Partitioning

Partitioning was measured by adding 50 μL of anthracycline stock solution (1 mM) to 2.5 mL of 1-octanol (saturated with buffer 1) and 2.5 mL of buffer 1 (saturated with 1-octanol). After mixing by rotation of the samples in closed glass tubes at 120 rpm for 2 hr at room temperature, the phases were separated by centrifugation at 2000 × *g* for 10 min. For determination of the partition coefficient, 1 mL samples were removed and diluted with 1 mL of methanol-1 M aqueous HCl (100:2, by vol.) before analysis by means of spectrofluorometry (excitation 480 nm, emission 590 nm). 1.00 μM standards of each compound were prepared in both the buffer (saturated with 1-octanol) and in 1-octanol (saturated with buffer). Calibration curves could be well approximated with a linear function within the reproducibility of the assay.

Extraction of Tumor Cell Phospholipids

Total lipid extracts of Ehrlich ascites tumor cells (EHR2/DNR+) [11] were prepared by $CHCl_3$-methanol extraction according to the Folch procedure [12]. The phospholipid fraction was isolated on a silica gel column (2 × 10 cm, 230–400 mesh) washed with $CHCl_3$ (100 mL) and eluted with methanol (50 mL). The eluate was evaporated under vacuum and the dry phospholipid was dissolved in $CHCl_3$ (5 mg/mL) and stored under nitrogen at −20°. The yield was *ca.* 40 mg/10^9 cells. Phospholipid concentrations were determined using the phosphorus assay of Bartlett [13].

Lipid Vesicle Preparation

Small unilamellar vesicles (SUV) were prepared from tumor cell phospholipid by probe sonication [14]. In a typical procedure, an aliquot corresponding to 5 mg of lipid together with a trace of [^{14}C]phosphatidylcholine was evaporated to dryness under a stream of nitrogen and placed under vacuum overnight. The lipid was then hydrated in 5 mL of buffer 1 and sonicated for 10 min at 20–30°. Large multilamellar vesicles were removed by centrifugation at 120,000 × *g* for 2 hr. The SUV preparation was kept at 4° and used for gel filtration experiments within one week of preparation to avoid problems connected with lipid peroxidation.

Gel Filtration

All gel filtration experiments were performed with a 0.9 × 28 cm Sephadex G-200 gel column in the dark at 22 ± 1°. The column was connected to a peristaltic pump with capillary polyethylene tubings and equilibrated at a flow rate of 15–20 mL/hr with buffer 1 containing anthracyclines **1–5** (4–10 μM). Sample volumes were 400–600 μL and elutions were performed at a flow rate of 5–6 mL/hr. Concentrations of anthracycline, albumin and phospholipid in separate experiments are given in the legends to Tables 1 and 2. A representative run with HSA was as follows: the column was equilibrated with 40 mL of buffer 1 containing 8.06 μM **2** (flow rate 17.1 mL/hr). HSA (28.2 mg) was dissolved in 1.40 mL of the equilibration buffer and after 10 min, a 514 μL sample was applied at the top of the column. Elution was performed with 30 mL of equilibration buffer (flow rate 5.2 mL/hr) and fractions were collected every 6 min into disposable 4 mL polystyrene tubes with 10 μL of acetic acid. Between each new run, the column was washed with 100 mL of buffer 1 containing 0.1% NaN_3 (flow rate 17 mL/hr). In the case of **4** and **5**, the top 2 cm column material containing aggregated anthracycline ma-

TABLE 1. Overall binding constants (K) of anthracycline analogs for serum albumin

Compound[a]	K (mM^{-1})[b] HSA	BSA
1	2.67 ± 0.07	1.36 ± 0.25
2	3.82 ± 0.57	2.21 ± 0.62
3	12.8 ± 2.32	15.1 ± 3.3
4	24.5 ± 3.1	48.4 ± 5.2
5	13.6 ± 1.9	9.94 ± 1.04

[a] Defined in Fig. 1.

[b] Binding constants were calculated on the basis of one binding site per albumin molecule. Data are the means ± SD from analysis of 10–15 top fractions in 2–3 separate Sephadex G-200 filtration experiments. Sample concentrations of albumin were: 30–50 mg/mL (1, 2); 20 mg/mL (3, 5) and 5–10 mg/mL (4). Equilibrium concentrations of drug (C_{eq}) were: 8.5–8.8 μM (1, 2); 6.9–9.5 μM (3); 3.8–6.4 μM (4) and 4.7–5.8 (5).

terial was replaced by new gel. The column could be reused for more than 50 runs.

Anthracycline, Protein and Lipid Analysis

Concentrations of protein and anthracycline in serial column fractions were determined spectrophotometrically. The following molar extinction coefficients were determined in buffer with a composition similar to the collected column fractions (buffer 1 with 2% [by vol.] acetic acid): BSA, ϵ_{280} = 42.9 mM^{-1} cm^{-1}; HSA, ϵ_{280} = 35.3 mM^{-1} cm^{-1}; **1**, **2**, **4**, **5**, ϵ_{485} = 11.9 mM^{-1} cm^{-1}, and **3**, ϵ_{480} = 9.63 mM^{-1} cm^{-1}. Corrections for anthracycline absorbance at 280 were made using: **1**, **2**, **4**, **5**, ϵ_{280} = 8.3 mM^{-1} cm^{-1} and **3**, ϵ_{280} = 7.9 mM^{-1} cm^{-1}. For all anthracycline compounds, the concentration dependence of the extinction coefficient because of dimerization was <2% in the acidic (pH 3.5) buffer, which is within the error of our measurements on individual column fractions. One problem in the exact spectrophotometric determination of anthracycline concentrations is a pronounced change in the extinction coefficient of the anthracycline when it

TABLE 2. Anthracycline octanol/buffer partition coefficients (P_{ow}) and binding constants (K) for small unilamellar phospholipid vesicles

Compound*	P_{ow}†	K (mM^{-1})‡
1	2.10 ± 0.14	0.62 ± 0.09
2	8.94 ± 0.16	1.89 ± 0.15
3	17.8 ± 1.0	6.30 ± 0.71
4	477 ± 65	3.08 ± 0.43
5	174 ± 26	2.11 ± 0.19

* Defined in Fig. 1.

† 1-octanol-buffer (pH 7.45) partition coefficient. Values are means ± SD (n = 3).

‡ Overall binding constants were calculated on the basis of one binding site per phospholipid molecule. Data are the means ± SD from analysis of 8–10 top fractions in 2 separate Sephadex G-200 filtration experiments. Sample concentration of phospholipid was 0.78 mg/mL. Equilibrium concentrations of drug (C_{eq}) were: 8.5–10.1 μM (1, 2); 7.9 μM (3); 5.3 μM (4) and 6.2 μM (5). Small unilamellar phospholipid vesicles were prepared from total Ehrlich ascites tumor cell phospholipid.

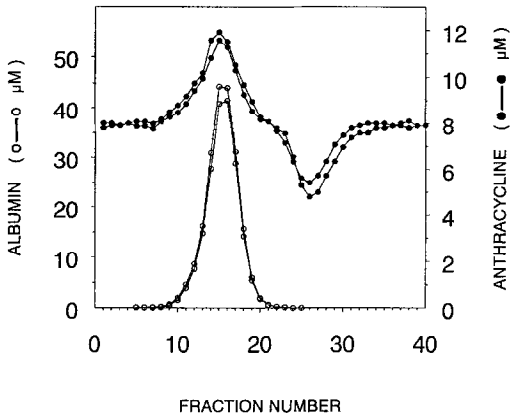


FIG. 2. Elution profiles for HSA on a Sephadex G-200 column equilibrated with 3 in 150 mM NaCl, 20 mM HEPES buffer (pH 7.45). Two samples (514 μL and 517 μL) containing HSA (20.00 mg/mL) and 3 (8.20 μM) were analyzed in separate runs. The equilibrium concentrations of 3 (C_{eq}) in the column were measured to 7.87 μM and 8.08 μM. Fractions collected every 6 min were analyzed for protein (○——○) and anthracycline (●——●) as described in "Materials and Methods."

associates to serum albumin [7, 8]. Here the presence of acetic acid in the column fractions has the additional advantage that the albumin binding of anthracycline is reduced at acidic pH [7, 8]. Control experiments confirmed that BSA in the concentration range investigated (0–100 μM) had no significant effect (<5%) on the extinction coefficient of compounds **3** and **4** with the highest BSA binding affinity. In filtration experiments with SUV, 0.5% (v/v) Triton X-100 was added to all samples to remove liposome turbidity. Total phospholipid was determined from the [^{14}C]phosphatidylcholine content of the fractions as determined by liquid scintillation counting.

Data Analysis

Overall binding constants (K) were calculated on the assumption of one binding site per albumin molecule [7, 8] and by means of the equation

$$K = (C_{out} - C_{eq})/((C_{alb} - C_{out} + C_{eq})C_{eq})$$

where C_{out} is the total (free plus bound) concentration of anthracycline in the column effluent, C_{eq} the equilibrium concentration of anthracycline determined before the albumin peak and C_{alb} the concentration of albumin in the effluent (Fig. 2). K values were calculated for 5–6 fractions collected around the top of the albumin peak. C_{eq} (4–10 μM) and C_{alb} (20–100 μM) were adjusted to ensure an average number of anthracycline molecules bound per molecule of albumin (v) of <0.2 in all runs. K values for anthracycline binding to SUV were calculated in a similar manner with C_{eq} = 6–11 μM, peak concentrations of phospholipid 300–800 μM and $v < 0.025$.

RESULTS

Sephadex G-200 Filtration Method

Equilibrium binding studies with anthracyclines (**1–5**, Fig. 1) were carried out using the Sephadex gel filtration method initially developed by Hummel and Dryer [15] and modified by Fairclough and Fruton in a study on tryptophan interaction with BSA [16]. The anthracycline compounds were observed to be retarded on the Sephadex G-25 gel material used in the original method which, in an effort to avoid this problem, was replaced by Sephadex G-200, which binds anthracycline compounds less strongly. Full experimental details are given in "Materials and Methods." Using the Sephadex G-200 filtration method, several important factors must be noted. 1) In the case of derivatives **1**, **2** and **3**, drug concentrations in the effluent (C_{eq}) approached the concentration in the eluent (C_{in}) within 95–98% during equilibration of the column. In contrast, C_{eq} attained a constant level at only 70–80% of C_{in} with derivatives **4** and **5**, because they were subject to significant binding in the Sephadex gel material. Therefore, a gradient from C_{in} to C_{eq} is present down the column. However, most of the anthracycline retained in the column was tightly bound in the top 1–2 cm column material and could not be removed in subsequent washing steps. The gradient of free anthracycline available for binding to albumin in the remaining 26 cm column was well below 10% and the calculated K values were not corrected for this small gradient. 2) Using fluorometric detection methods we carried out some gel filtration experiments with low (0.01–0.1 μM) anthracycline concentrations. However, our experience shows that problems associated with unspecific drug binding to the capillary tubings and the column gel material make precise determination of protein binding parameters very difficult under these conditions. 3) Even though Sephadex G-200 only partially excluded serum albumin, it was possible to reach equilibrium binding conditions as confirmed by the return of C_{out} to the equilibrium concentration of anthracycline (C_{eq}) between the albumin peak and the subsequent negative drug peak. This is illustrated in Fig. 2, which gives two elution profiles obtained with HSA (10.3 mg) on a column equilibrated with **3** (C_{eq} = 7.9–8.1 μM). 4) To avoid a decrease in light absorbance and fluorescence of the anthracycline compounds because of photodecomposition during passage through the gel column, all filtrations had to be carried out in the dark. 5) A problem associated with drug decomposition from the time of sample collection to the time of analysis could be minimized by collection of samples into tubes containing acetic acid.

Serum Albumin

Samples of HSA and BSA were subjected to gel filtration in buffer 1 containing anthracyclines **1–5**. As shown in Table 1, which summarizes the binding data, the K values vary over a near 35-fold range from 1.36 mM^{-1} for **1** up to 48.4 mM^{-1} in the case of **4**. The affinities for HSA and BSA were found to follow the same order. The pH-dependence of anthracycline binding to serum albumin in buffer 1 adjusted with HCl or NaOH to various pH values in the range from 6.88 to 8.27 is shown in Fig. 3, where K values on a log-scale are plotted against pH. Binding of **4** increased 40-fold (from 8.24 ± 0.84 mM^{-1} to 337.5 ± 55.4 mM^{-1}) and **3** increased 11-fold (from 4.73 ± 0.85 mM^{-1} to 51.9 ± 9.7 mM^{-1}) as the pH was increased over this range. By contrast, in the case of **2**, only a 3-fold increase was measured (from 1.28 ± 0.08 mM^{-1} to 3.72 ± 0.65 mM^{-1}).

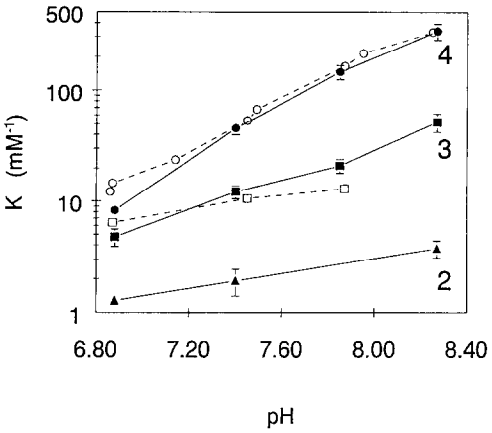


FIG. 3. pH-dependence of anthracycline association to BSA as measured by Sephadex G-200 gel filtration. Experimental conditions as defined in Table 1, except that the pH of the equilibration buffer was adjusted to 6.88, 7.40, 7.85 and 8.27. Overall binding constants (K) were calculated on the basis of one binding site per albumin molecule. Derivatives 2 (▲), 3 (■) and 4 (●) as in Fig. 1. Data are the means ± SD from analysis of 10–15 top fractions in 2–3 separate filtration experiments. Values of K obtained by Rivory et al. [7] with 3 (□---□) and 4 (○---○) are included in the figure.

Phospholipid

In a further application of the gel filtration method, we investigated the binding of anthracyclines **1–5** to SUV prepared from total Ehrlich ascites tumor cell phospholipid. Samples of SUV eluted in the void volume of the column with a recovery of phospholipid >90% (data not shown). The method allowed us to measure directly the association of anthracycline to phospholipid bilayers. Calculated K values are reported in Table 2 and vary over a near 10-fold range from 0.62 mM^{-1} for **1** up to 6.30 mM^{-1} for **3**. We found no clear correlation between K values and overall drug lipophilicity as measured by the octanol-buffer phase partitioning coefficient (P_{ow}).

DISCUSSION

This study reports conditions under which a gel filtration method is applicable for direct determination of anthracycline binding to serum albumin as well as liposomal phospholipid. Important new data required for a kinetic

analysis of anthracycline transport in tumor cells were obtained.

By use of equilibrium dialysis, Eksborg et al. [5] reported low affinity association of **1** and **2** to HSA. The extent of binding was estimated to be 62–63% for both compounds at 1 μg/mL drug and 45 g/liter albumin, which gives K = 2.4 mM^{-1}. While our data on **1** are very close to this value, we find a 1.6 times higher value for **2**. More recently, Rivory et al. [7] reported binding experiments based on the quenching of anthracycline fluorescence obtained upon drug association with the albumin molecule. Binding of the drug monomer to BSA was characterized in terms of a single binding site with K = 10.6 mM^{-1} and 53.8 mM^{-1} in the case of **3** and **4**, respectively. This spectroscopic approach will, of course, not be valid if there are additional anthracycline binding sites on the protein surface which do not affect or even increase the drug fluorescence intensity. A very reasonable agreement of our K values for **3** and **4** with those reported in [7] is therefore a most important finding. It can be seen from Fig. 3 that the two independent methods give similar results over a range of pH values. Thus, our data confirm the reliability of fluorescence quenching as a method to measure the extent of anthracycline binding to serum albumin. Because of dimerization of **4** at >1 μM, the K value determined in our experiments may reflect the binding of the dimer to some extent. Using the dimerization constant of **4** (21.1 mM^{-1}, pH 7.49) reported in [7], approximately 15% is calculated to be present as a dimer at C_{eq} = 5 μM. However, the agreement of our K value with the value of the monomer [7] would suggest that the extent of dimer binding is relatively low. While this paper was being written, important anthracycline binding data with HSA were published [6]. Using ultrafiltration, Chassany et al. measured K values of 2.6 mM^{-1}, 2.3 mM^{-1} and 24.3 mM^{-1} for **1**, **2** and **4**, respectively, which are very similar to our gel filtration results.

Considering hydrophobic ligand binding domains with high conformational flexibility in the albumin molecule [17], it would seem reasonable to expect a correlation between anthracycline binding affinity and overall lipophilicity. Comparison of the binding data in Table 1 with the measured P_{ow} values given in Table 2 shows that this is true for derivatives **1**–**4**, where the more lipophilic have the highest affinity. However, **5** is found to bind with a K value close to that of **3**, even though it is much more lipophilic. Thus, even limited to the five anthracyclines selected in this study, there are indications of a binding domain on the albumin that is able to interact more specifically with substituents on the anthracycline molecule. Previous spectrophotometric studies [7, 8] on the 1:1 binding of anthracyclines to BSA indicate a location of the drug chromophore close to a water-protein interface region with positively charged amino acid residues. This would indicate that polar as well as hydrophobic interactions contribute to the anthracycline-albumin association reaction. The order of K values are the same for HSA and BSA, indicating that the two proteins behave similarly in respect to anthracycline binding.

Binding of derivatives **4** and **5** to SUV phospholipid bilayers is found to be very much weaker than predicted from their P_{ow}. This result is consistent with the binding experiments of Praet et al. [18], indicating a reduced ability of **4** to enter into liposomal phospholid vesicles. Thus, while the ligand binding sites of serum albumin possess sufficient flexibility to accommodate the bulky iodine atom at the *c4'* position on the anthracycline molecule, complex formation with the structured lipid bilayer appears to be strongly inhibited by the presence of this substituent. Burke and Tritton [10], using fluorescence anisotropy, also observed a reduced lipid bilayer affinity for the daunorubicin derivative rubidazone which contains a bulky benzoylhydrazone substituent at the *c13* position. It is notable that our K values determined for binding of **1**, **2** and **3** to SUV composed of tumor cell phospholipids are comparable with the values in [10], where SUV with an entirely different lipid composition (dimyristoyl-phosphatidylcholine) are used. They also compare well with the recent binding study of Gallois et al. [19] using large unilamellar vesicles containing egg phosphatidylcholine with various amounts of phosphatidic acid and cholesterol.

Derivatives **3** and **4** are characterized by a high plasma membrane permeability [20, 21] and are of particular interest in that they are able to bypass the *P*-glycoprotein-related drug efflux mechanism in MDR tumor cells [22–25]. However, up to now most of the structure-activity data have been obtained in model test systems with MDR cells, without consideration of the interactions of these lipophilic derivatives with potential extracellular drug binding components such as serum albumin. Therefore, one must question whether the available *in vitro* data are adequate for predicting drug pharmacokinetics and clinical activity against MDR cells in the growing tumor. For example, in one recent study [4] it is demonstrated that the presence of serum albumin (40 g/liter) leads to an 8- to 13-fold reduced cellular uptake of **4**. We recently developed a mathematical model for kinetic analysis of anthracycline transport in MDR tumor cells [26]. With the data in the present study, it will be possible to refine this model and to carry out theoretical considerations of the relationship between such factors as drug lipophilicity, membrane permeability and cell killing activity in MDR tumor cells under serum conditions (Demant EJF and Friche E, manuscript in preparation).

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