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Effects of lipophilicity and protein binding on the hepatocellular uptake and hepatic disposition of two anthracyclines, doxorubicin and iododoxorubicin

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Abstract The anthracyclines, in particular doxorubicin (DOX), have been used for the intra-arterial locoregional therapy of liver tumours for over two decades. However, the results obtained with this form of therapy have been disappointing. It is widely recognised that DOX has a slow and limited tissue uptake, and we hypothesised that lipophilic analogues could be more suitable for locoregional administration. Using rat hepatocyte suspensions and the isolated rat liver, we examined the effects of lipophilicity, as determined from the octanol: buffer partition coefficient ($K_{oct:buf}$), and protein binding of several anthracyclines on hepatocellular uptake. In particular, we compared DOX with 4′-iodo-4′-deoxy-doxorubicin (IDX), which differs only in the substitution of the daunosamine hydroxyl by an iodine molecule. Using a direct spectrofluorimetric method to evaluate cell uptake, we found that the influx rates correlated with the logarithm of $K_{oct:buf}$ and that IDX had the highest rate. However, the addition of bovine serum albumin (BSA) to the medium reduced the hepatocellular uptake of IDX more extensively than that of DOX such that the DOX uptake exceeded that of IDX with 4% BSA. Experiments in the isolated perfused rat liver confirmed these findings. We suggest that a trade-off of cellular uptake for reduced protein binding is desirable in the selection of drugs for intrahepatic administration. This may be accomplished by choosing anthracyclines with intermediate lipophilicity.

Key words Lipophilicity · Anthracyclines · Hepatocyte Protein binding

Abbreviations *IDX* 4′-Iodo-4′-deoxy-doxorubicin · *IDXOL* 4′-iodo-4′-deoxy-doxorubicinol · *DOX* doxorubicin · *DOXOL* doxorubicinol · *IDA* idarubicin (demethoxy-daunorubicin) · *EGTA* ethylene glycol-*bis*-(β-amino-ethylether)-*N*,*N*,*N*′,*N*′-tetra-acetic acid · *HPLC* high-performance liquid chromatography · *LDH* lactate dehydrogenase · *DNase* deoxyribonuclease · *BSA* bovine serum albumin

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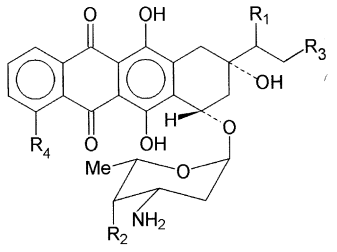
Introduction

Malignant tumours of the liver are important causes of morbidity and mortality worldwide [14]. For either primary or secondary resectable tumours, surgery remains the most effective therapy. However, successful resection can be performed in only a minority of cases and, as an alternative, regional delivery of chemotherapy is being increasingly utilised [14]. For this technique, the drug is usually delivered via the hepatic artery because it is the principal blood supply of most larger tumours (>5 mm diameter). In general, the cytotoxicity of anticancer drugs towards tumour cells in culture follows steep dose-response curves. Therefore, the principal purported advantage of regional chemotherapy is delivery of the cytotoxic drug to the tumour at the highest possible concentration whilst the non-target tissues are exposed to lower concentrations as a result of systemic and local clearance.

The anthracyclines have been used for the intra-arterial chemotherapy of hepatic tumours since the mid-1970s [4, 14], doxorubicin (DOX, Fig. 1) being the one chosen most frequently. On the whole, however, the efficacy of intra-hepatic chemotherapy with DOX has been disappointing [4, 14]. In retrospect, failure of this drug to fulfil many of the necessary criteria for the success of this form of therapy could explain the observed lack of efficacy. For example, it is known that anthracyclines enter cells principally by the diffusion of the neutral species across the bi-lipid layer of plasma membranes [8, 9]. Doxorubicin, however, is a relatively



	R_1	R_2	R_3	R_4
DOX	=O	OH	OH	OMe
DOXOL	—OH	OH	OH	OMe
IDX	=O	I	OH	OMe
IDXOL	—OH	I	OH	OMe
IDA	=O	OH	H	H

Fig. 1 Structures of DOX, DOXOL, IDX, IDXOL and IDA

water-soluble molecule with a macromolecular p*K*a of 8.5 [1]. Thus, most DOX would be ionised at physiological pH and have poor overall membrane permeability [8]. This translates into a poor penetration of DOX into tumour spheroids [15], and when given i.p., DOX has been shown to accumulate appreciably only in the outermost cellular layers of murine ovarian tumours [18].

Over the last decade or so, many new anthracyclines have been developed that are less cardiotoxic and have increased activity against pleiotropic multi-drug-resistant cells in experimental systems. Many of these newer anthracyclines (e.g. idarubicin, pirarubicin and iododoxorubicin) are lipophilic and display more rapid uptake into cultured tumour cells even when the latter are selected for resistance to DOX [7]. This appears to translate into retained or only marginally reduced cytotoxic activity against such resistant cell lines [6]. In addition, the total body clearance of these drugs is high [21]. Therefore, these new drugs could be better candidates than DOX for loco-regional therapy.

Using suspensions of rat hepatocytes and the isolated perfused rat liver, we examined in the present study whether lipophilic analogues of DOX display more rapid and extensive hepatic uptake than DOX and identified factors of importance in the hepatic disposition of these drugs. In particular, we chose to compare DOX with iododoxorubicin (IDX, Fig. 1), which differs from DOX only in the substitution of the daunosamine hydroxyl by an iodine group. This change modifies the ionisation (p*K*a) of the nearby amine residue to 6.4, such that IDX is mostly neutral at physiological pH, and results in a more rapid and extensive uptake into a variety of cell lines, higher intranuclear drug concentrations and improved efficacy relative to DOX [6, 7, 13].

Both DOX and IDX are metabolised to lipophilic 13-dihydrometabolites [21], doxorubicinol (DOXOL) and 4′-iodo-4′-deoxy-doxorubicinol (IDXOL). Therefore, we examined the hepatocellular uptake of these metabolites and the relationship between the uptake and the octanol: buffer partition coefficient of each compound. Finally, we examined the relative kinetics of DOX and IDX in the perfused rat liver.

Materials and methods

Chemicals and reagents

The hydrochloride salts of DOX, DOXOL, IDX, IDXOL and idarubicin (IDA, demethoxy-daunorubicin) were donated by Farmitalia Carlo Erba (Melbourne, Australia). Trypan blue and 1-octanol were supplied by ICN Biochemicals (Costa, Mesa, Calif., USA). High-molecular-weight DNA from calf thymus was obtained from Boehringer Mannheim (Mannheim, Germany); bovine serum albumin (BSA), fraction V, from Sigma (St. Louis, Mo.); deoxyribonuclease type I from bovine pancreas (E.C. 3.1.21.1; DNase), from Calbiochem (San Diego, Calif.); and Percoll, from Pharmacia (Uppsala, Sweden). Collagenase class IV (30–40 mg/100 ml) was obtained from Worthington (Freehold, N.J.). All other reagents were of the highest grade available. Glassware was sialinised to minimize the adsorption of anthracyclines.

Octanol: buffer partition coefficient

The partition coefficient ($K_{Oct:buf}$) between octanol and buffer (120 m*M* NaCl, 25 m*M* Na_2HPO_4, 4.7 m*M* KCl, 1.2 m*M* KH_2PO_4, 1.2 m*M* $MgSO_4$, 12 m*M* glucose, pH 7.4) containing drug (3 μ*M*) was studied at 37°C for DOX, DOXOL, IDX, IDXOL and IDA. The phases were agitated in an orbital incubator (200 rpm, 37°C, Labline Instruments Inc., Ill.) for 20 min and analysed for drug content by high-performance liquid chromatography (HPLC). The coefficients were calculated according to conventional mass-balance principles.

Animals

Inbred female Wistar-Wag rats (150–200 g) from the Central Animal Breeding House of the University of Queensland were used. The study was approved by the university's Animal Ethics Committee.

Isolation of hepatocytes

Hepatocytes were isolated using a modification of the collagenase perfusion method of Berry and Friend [5]. Debris and non-viable cells were removed by Percoll-gradient centrifugation. Viable cells were washed three times in Krebs-Henseleit. Cell viability, determined by Trypan blue exclusion and release of lactate dehydrogenase (LDH) at the beginning and end of each experiment, always exceeded 90%.

Spectrofluorimetric measurement of the uptake and efflux of the anthracyclines and their metabolites

The fluorescence of anthracyclines is quenched when they complex with DNA. Any measured fluorescence in a suspension of cells is due

almost exclusively to that of extracellular drug, and fluorescence decay represents cellular uptake [19]. This method enables the quantitation of initial uptake rates, even when these are rapid. Cellular uptake of the drugs and their metabolites from protein-free medium was measured in disposable acrylic cuvettes (Sarsdedt, Germany) by recording of the time course of the fluorescence in a Perkin-Elmer LS-50 fluorimeter (Perkin-Elmer, Australia). The excitation and emission wavelengths were 473 and 557 nm, respectively. The cell compartment was maintained at 37°C and the contents of the cuvettes were stirred magnetically. For 5 min before the addition of drug, the suspensions of hepatocytes (3 ml; 1×10^6 cells/ml) were exposed to DNase (0.3 mg/ml at 37°C) to remove extracellular DNA and reduce clumping. Then, cellular autofluorescence was measured, following which 60 µl of the designated drug solution (150 µ*M*) was added to yield a final concentration of 2.88µ*M*. Fluorescence data were acquired over 200 s.

Analysis of fluorimetric data

The loss of fluorescence from the cuvettes was analysed using bi-exponential equations and corrected for the loss due to absorption on the cuvette walls. Because experimental data collected in the absence of hepatocytes revealed that the rate of desorption from the cuvette was greater than the rate of adsorption, the equation describing the three-compartment system (cuvette, buffer and hepatocytes) could be simplified to:

$$c(t) = Ae^{(-k_1+k'_1)t} + Be^{-k_z t} + \frac{k_1}{k_1 + k'_1}, \quad (1)$$

where A and B are constants and k_z is the rate constant for desorption from the cuvette. Equation 1 was used to obtain the rate constants for hepatocellular uptake (k_1) and efflux (k'_1). Non-linear, least-squares regression of the data according to Eq. 1 was performed using the fitting procedure of Yamaoka et al. [23].

Effect of albumin on the hepatocellular accumulation of DOX and IDX

Formation of complexes between lipophilic anthracyclines and albumin causes a dramatic reduction in drug fluorescence [20]. Therefore, the effect of albumin on the initial hepatocellular rate of uptake of the anthracyclines could not be measured by the fluorimetric method described above. Instead, the effect of protein binding on the extent of uptake was studied by measurement of intracellular concentrations of drug over a 30-min time course as follows. Suspensions of hepatocytes (3 ml; 1×10^6 cells/ml) in Krebs-Henseleit buffer containing 0, 1% or 4% (w/v) BSA (pH 7.4) were incubated at 37°C in borosilicate tubes for 5 min in the presence of DNase I (0.3 mg/ml). Then, 60 µl of the designated drug solution (150 µ*M*) was added to yield a final concentration of 2.88 µ*M*. Samples (600 µl) of the suspension were withdrawn at 2, 5, 10 and 30 min, placed immediately on ice and centrifuged at 4°C to pellet the cells; 400 µl of the supernatant was transferred to an Eppendorf tube. The cell pellet was washed in 600 µl of ice-cold buffer twice and both supernatant and cell-pellet samples were stored at − 20°C until analysis.

Supernatants (400 µl) were extracted as per Formelli et al. [10] using IDA as the internal standard (IS). Pellets were extracted by a similar procedure following the addition of 50 µl 1M $LaCl_3$ and vigorous vortex mixing to precipitate proteins, DNA and nucleotides [2]. The addition of La resulted in extraction recoveries similar to those of Formelli et al., but these extracts contained less interference from endogenous fluorescent compounds.

Disposition of the anthracyclines in the isolated perfused rat liver

The isolated perfused rat liver was prepared as described previously [16] and perfused in situ with Krebs-Henseleit bicarbonate buffer (pH 7.4) containing 4% (w/v) BSA, equilibrated with 95% O_2 - CO_2 to an oxygen pressure (PO_2) of 450 mmHg and a carbon dioxide pressure (PCO_2) of 35 mmHg. The perfusion path was kept as short as possible to minimise drug adsorption to the tubing. A peristaltic pump (Cole Parmer, Chicago, Ill.) delivered the perfusate at a flow rate of 20 ml/min in a non-recirculating manner for 20 min.Then, the perfusion was changed to the recirculating mode through a 100-ml reservoir that was stirred continuously and contained DOX or IDX at an initial concentration of 1 µ*M*. Samples (100 µl) were collected from the reservoir every 5 min for 60 min. Viability of the liver was judged by macroscopic appearance, LDH release, bile flow, oxygen consumption and portal venous pressure [16]. After the 60-min perfusion the liver was removed, blotted dry and frozen at − 70°C until analysis. Bile was collected during the 20-min equilibration period and for the duration of the recirculation. Samples were stored at − 20°C until analysis. Perfusion experiments performed in the absence of a liver and with 4% BSA in the perfusate showed that there was negligible adsorption of drug onto the tubing and the reservoir. However, removal of the BSA led to an extensive loss of drug in similar experiments, precluding the use of this model for protein-free liver perfusions.

Tissue analysis for anthracyclines

Frozen liver was thawed and homogenised in 4 vol. of 0.1 *M* NaCl. Following centrifugation of aliquots (400 µl) in Eppendorf tubes at 15,800 *g* for 15 min, sample supernatants and pellets were analysed separately as per the hepatocyte incubation experiments. The liver drug content was estimated from the sum of the supernatant and pellet contents and the liver weight and was expressed as a percentage of the initial dose.

HPLC analysis

The HPLC system (Millipore, Waters Chromatography Division, Australia) consisted of a model 510 pump, a 710B autoinjector, a 470 fluorescence detector, and Maxima 820 data-reduction software. Separation of the peaks of interest was carried out as per Belvedere et al. [3], and these were detected using excitation and emission wavelengths of 473 and 557 nm, respectively. Standard curves were prepared daily and were linear over the concentration range investigated.

Determination of the unbound fraction of DOX

Binding of DOX to 4% BSA was determined by equilibrium dialysis in 1 ml Teflon chambers separated by a dialysis membrane. The buffer (130 m*M* Na_2PO_4/$NaHPO_4$, 85 m*M* NaCl, pH 7.4) had sufficient capacity to minimise the fluctuation in pH to $< 0.10 \pm 0.02$ U throughout the period of incubation (6 h at 37°C). Prior to dialysis, DOX (0.1–1.0 µM) was added to the albumin solution. The concentrations of DOX in both sides of the chamber were determined by HPLC and the equilibrium unbound fraction of DOX was calculated as the ratio of the concentration of DOX in the buffer to that in the BSA solution.

The protein binding of IDX cannot be determined by equilibrium dialysis under physiological conditions [12]. Instead, the unbound fraction (α) was calculated from:

$$\alpha = \frac{1}{1 + K_a C_a}, \quad (2)$$

where K_a is the equilibrium association constant for the BSA: IDX interaction and C_a is the concentration of BSA. The value of K_a at pH 7.4 has been determined previously [20].

Analysis of pharmacokinetic data

The profiles of the perfusate concentration versus time data were fitted to multi-exponential equations with the required number of exponential terms determined from the Aikaike's Criterion Index value calculated by the Yamaoka program [23]. The area under the concentration-time curve (AUC, $0 \rightarrow \infty$), the total and unbound clearance, the volume of distribution at steady state and the mean residence time were calculated from the appropriate moments using the fitted coefficients and constants [22]. The steady-state extraction was taken as the clearance divided by the flow of perfusate through the liver.

Statistical analysis

The significance of the regression of the uptake rate and the logarithm of $K_{oct:buf}$ was tested using Student's t-test. A P value of ≤ 0.05 was considered to be significant.

Results

Octanol: buffer partition coefficients and protein binding

The octanol: buffer partition coefficients $K_{oct:buf}$ for the five tested compounds were as follows DOX 2.42 ± 0.08, DOXOL 1.19 ± 0.06, IDX 624 ± 133, IDXOL 318 ± 21, and IDA 42.8 ± 2.4 (mean $\pm$ SD; $n = 4$). The unbound fraction of DOX in the presence of 4% BSA was found to be 0.356 ± 0.025 and to be independent of DOX concentration over the range 0.1–1 μM. The unbound fraction of IDX was calculated to be 0.030.

Spectrofluorimetric measurement of the uptake and efflux of the anthracyclines

The loss of fluorescence from the cuvette observed in the absence of hepatocytes was not extensive in comparison with that seen in the presence of cells for IDX, IDXOL and IDA, but it represented most of the loss observed for DOX and DOXOL soon after their introduction into the cuvette (Fig. 2). Addition of DNA to the cuvette at the end of the 200-s period resulted in almost complete quenching of the remaining fluorescence for all compounds, confirming that the fluorescence measured was indeed due to extracellular drug.

The rate constants obtained for influx (k_1) and efflux ($k_{1'}$) from the fitting of Eq. 1 are presented in Table 1. There was a significant correlation between the logarithm of the octanol/buffer partition coefficient and both k_1 ($r^2 = 0.88$, $P < 0.001$) and $k_{1'}$ ($r^2 = 0.85$, $P < 0.001$) as shown in Fig. 3.

Hepatocellular accumulation in the presence of albumin

These data were consistent with those obtained with spectrofluorimetry, although the initial rate of uptake of IDX was too rapid to be quantitated

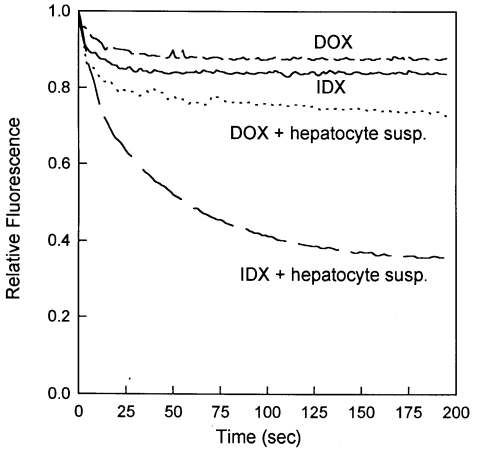


Fig. 2 Representative decay curves generated for the fluorescence of DOX and IDX in the presence and absence of a suspension (*susp.*) of hepatocytes

Table 1 Kinetic parameters of the hepatocellular uptake of selected anthracyclines measured by spectrofluorimetry[a]

Drug	k_1 (s^{-1})	k'_1 (s^{-1})
DOX	0.7	0.9
	(0.3)	(1.4)
DOXOL	0.2	0.1
	(0.1)	(0.1)
IDA	9.1	8.8
	(2.9)	(2.7)
IDX	17.3	8.2
	(2.6)	(1.5)
IDXOL	9.1	9.3
	(2.7)	(2.4)

[a] Data represent mean values ($\pm$ SD in parentheses; $n = 3$ or 4)

with this technique. When there was no BSA in the incubation fluid, the extent of accumulation of IDX exceeded that of DOX (Fig. 4). On the other hand, in the presence of either 1% or 4% BSA, the amount of DOX taken up after 30 min exceeded that of IDX. In the presence of 4% BSA the cellular uptake of IDX was 7.9-fold (at the peak) and 13.2-fold (at 30 min) lower than that seen in the absence of BSA. At the corresponding time points, 4% BSA reduced the cellular incorporation of DOX by a factor of 2.3 and 2.4, respectively. No fluorescent metabolite of DOX or IDX was detected in either the cellular fraction or the supernatant after 30 min.

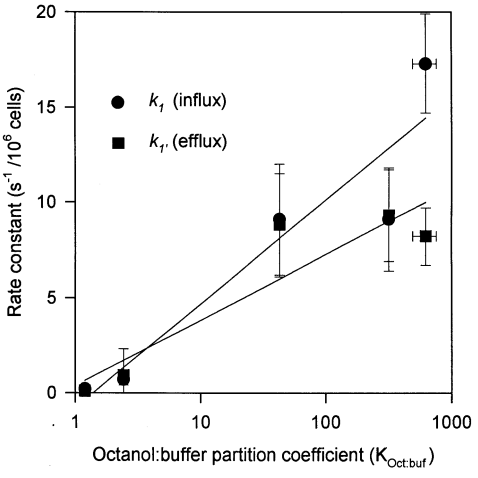


Fig. 3 Relationship between the octanol: buffer partition coefficient ($K_{oct:buf}$) and the influx and efflux of anthracyclines measured using suspensions of hepatocytes

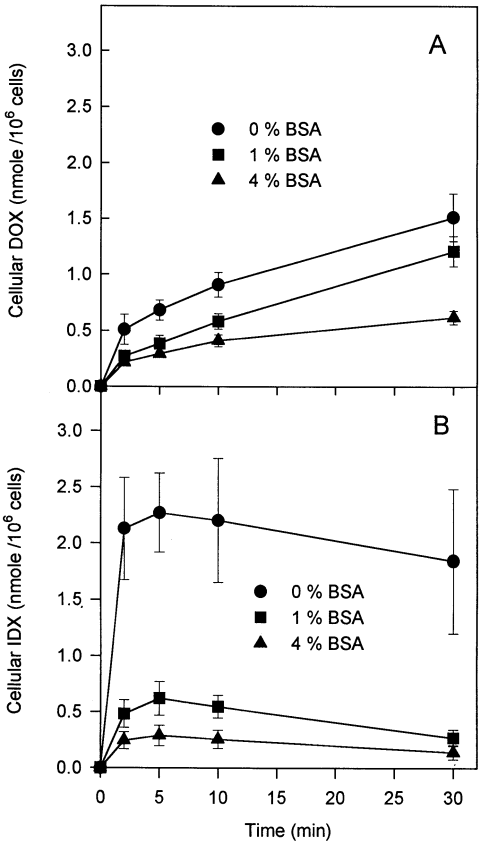


Fig. 4 **A** Time course of the hepatocellular accumulation of DOX over a 30-min period of incubation in the presence of three concentrations of BSA. **B** Time course of the hepatocellular accumulation of IDX over a 30-min period of incubation in the presence of three concentrations of BSA. Data represent mean values ± SD ($n = 4$)

Disposition of the anthracyclines in the isolated perfused rat liver

The concentrations of DOX and IDX in the perfusate entering the liver as a function of time are shown in Fig. 5. The total clearance and extraction of DOX were higher and the half-life and mean residence time, shorter (Table 2). On the other hand, the steady-state volumes of distribution were similar for the two drugs. The unbound clearance of DOX was much lower than that of IDX.

The recovery of unchanged drug from the liver at the end of the perfusion was 37.8 ± 12.5% and 23.5 ± 1.6% of the dose for DOX and IDX, respectively. The dihydrometabolites represented only 2–6% of the delivered dose. There appeared to be some conversion to other fluorescent metabolites, presumably aglycones, as they had identical retention times during the HPLC analysis of liver samples from the IDX and DOX perfusions (Fig. 6).

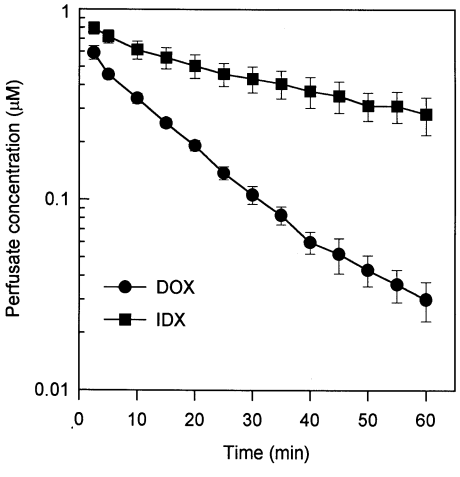


Fig. 5 Concentration-time curves generated for DOX and IDX in red-cell-free buffer containing 4% BSA over 60 min of perfusion in the intact perfused rat liver. Data represent mean values ± SD ($n = 6$)

Table 2 Kinetic parameters of DOX and IDX in the isolated perfused rat liver[a]

Drug	Liver weight (g)	Total clearance (ml min^{-1})	Half-life (min)	Extraction	Volume of distribution (ml)	Mean residence time (min)	Fraction unbound	Unbound clearance (l min^{-1})
DOX	4.59 (0.42)	8.7 (1.3)	13.2 (3.3)	0.43 (0.06)	160.6 (30.8)	17.9 (4.2)	0.356 (0.025)	24.4 (3.7)
IDX	4.49 (0.15)	2.5 (1.1)	43.8 (13.8)	0.13 (0.05)	142.9 (35.0)	62.2 (20.4)	0.030	83.3 (36.7)

[a] Data represent mean values ($\pm$ SD in parentheses, $n = 6$)

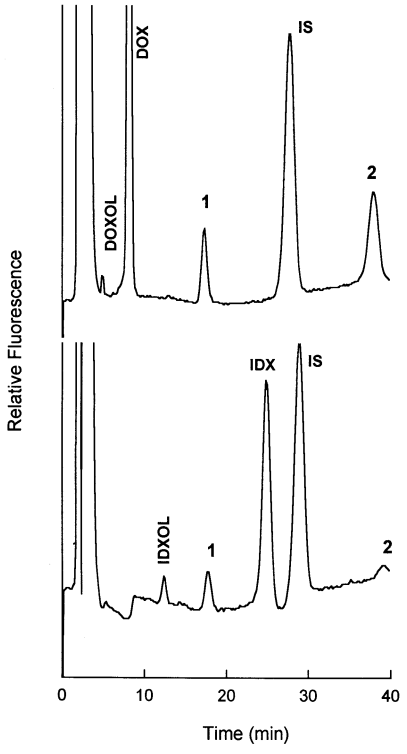


Fig. 6 Chromatograms of extracts of liver tissue collected after the 60 min of isolated perfusion with DOX (*top*) and IDX (*bottom*). Two unidentified metabolites absent in control experiments are likely to correspond to the aglycones of DOX and DOXOL, which can be generated from both DOX and IDX

Discussion

Because factors such as the ionisation and self-association of anthracyclines are known to affect their uptake into cells [8, 9], we estimated drug lipophilicity from $K_{oct:buf}$ under conditions of relevance to the hepatocellular experiments, namely, low drug concentrations and physiological pH and ionic strength.

The fluorimetric method of determining the cellular disposition kinetics of the anthracyclines is well established [11, 19], but we modified the analysis of the kinetics by allowing for the component of fluorescence lost by adherence of the drug to the surfaces of the cuvette. This propensity of the anthracyclines to adsorb onto many different surfaces confounds the results obtained for the hydrophilic anthracyclines in particular. In the current study this produced an additional "rapid" uptake phase that was evident for DOX and DOXOL in particular. This would have caused a major overestimation of the uptake rates if tangent methods had been used instead [11].

The uptake rate constants correlated well with the lipophilicity of the drugs. This is consistent with the evidence that the permeability of cells to the anthracyclines is mediated via the lipid domain of the plasma membrane as shown for DOX [8, 9]. The studies of the hepatocellular uptake of anthracyclines carried out using cellular extraction and HPLC analysis enabled us to determine the effect of protein binding. In the absence of protein, we confirmed the fluorimetric data in that the uptake of IDX was observed to be more rapid relative to that of DOX. DOX uptake persisted after 30 min of incubation, whereas IDX uptake appeared largely complete after 2 min. The slight decreases in cellular concentrations of IDX that were observed at later times were accompanied by depletion of the extracellular concentrations (data not shown) and are likely to have been the result of metabolism of IDX, although no fluorescent metabolite was observed. For DOX and IDX the inclusion of 4% BSA in the incubation medium reduced the uptake. This effect was much more marked for IDX, with a 7-fold reduction in peak cellular IDX content being observed in the 4%-BSA experiments as compared with the no-BSA experiments.

The experiments carried out with the isolated perfused rat liver confirmed the results obtained with the hepatocyte suspensions. In the presence of physiological concentrations of albumin the clearance of DOX was greater than that of IDX. However, the unbound clearance of IDX was considerably greater than that of DOX, which is strongly suggestive that the major limitation of IDX uptake is protein-binding, whereas DOX uptake is limited by its membrane permeability.

In conclusion, although IDX, by virtue of its higher lipophilicity, has a greater hepatocellular permeability, the presence of plasma proteins greatly reduces the extent of uptake into hepatocytes. Nevertheless, the

approach of using lipophilic anthracyclines to augment hepatic uptake appears well founded as demonstrated recently by Munck et al. [17].

It is noteworthy that the influx rate constant was correlated to the *logarithm* of $K_{oct:buf}$ and that IDA, although $\sim$15 times less lipophilic than IDX, had an influx rate constant that was more than half that of IDX. This indicates that extreme lipophilicity is not required for substantial membrane permeability. Furthermore, the association constant for the interaction of BSA with IDA is one-fifth that for IDX at pH 7.4 [20]. Hence, a trade-off of a slight loss in membrane permeability for a significant reduction in protein binding appears both possible and desirable in the selection of anthracyclines for intrahepatic chemotherapy.

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