THE HEPATIC PHARMACOKINETICS OF DOXORUBICIN AND LIPOSOMAL DOXORUBICIN

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ABSTRACT:

To determine the role of the hepatic sinusoidal endothelium in the hepatic disposition of liposomal doxorubicin, we compared the hepatic pharmacokinetics of doxorubicin hydrochloride and the pegylated, liposomal formulation of doxorubicin (Caelyx). The multiple indicator-dilution technique and electron microscopy were used to study the disposition of doxorubicin and liposomal doxorubicin in the rat liver. Doxorubicin had a volume of distribution 1.56 ± 0.46 times greater than that of the extracellular marker, sucrose, whereas liposomal doxorubicin had a volume of distribution 0.56 ± 0.30 times smaller than that of sucrose (P < 0.001). The recovery of doxorubicin was less than that of liposomal doxorubicin (70 ± 24% versus 94 ± 17%, P < 0.05). The disposition of liposomal doxorubicin was found to be flow-limited, whereas a permeability-limited sequestration model fitted doxorubicin. The transfer of doxorubicin across the hepatocyte membrane was symmetrical (permeability—surface area product for influx 0.02 ± 0.01 ml/s/g versus 0.03 ± 0.02 ml/s/g for efflux) and consistent with diffusion. Electron microscopy confirmed that liposomes were restricted entirely to the sinusoidal lumen and none were seen in the extracellular space of Disse. Liposomal doxorubicin is restricted to the sinusoidal lumen, presumably secondary to steric exclusion by fenestrations in the sinusoidal endothelium. This provides the mechanism for the longer half-life and reduced hepatic extraction of liposomal doxorubicin compared with doxorubicin. The sinusoidal endothelium and fenestrations within the sinusoidal endothelium have an important role in hepatic pharmacology and are important considerations when designing liposomal preparations.

Doxorubicin is a chemotherapeutic agent whose mode of action includes intercalation between adjacent base pairs of the DNA double helix, binding DNA-associated enzymes such as topoisomerase, and effects on membranes (Bodley et al., 1989). To reduce the toxicity of doxorubicin and improve delivery to tumor sites, a liposomal formulation of doxorubicin has been developed (Gabizon et al., 1998, 2003). The pegylated, liposomal formulation of doxorubicin hydrochloride (Caelyx) contains doxorubicin encapsulated in liposomes with surface-bound methoxy polyethylene glycol groups. Pegylation is thought to provide protection from uptake by the mononuclear phagocyte system, thus increasing blood circulation time and promoting opportunity for tumor uptake (Gabizon et al., 1998, 2003). The pharmacokinetics of doxorubicin are profoundly influenced by administration as a pegylated, liposomal formulation. In humans, the area under the curve (AUC) of liposomal doxorubicin compared with the free drug is increased 300-fold, the clearance decreased 250-fold, and the volume of distribution reduced 60-fold (Gabizon et al., 2003).

Doxorubicin is metabolized predominantly by the liver to the major metabolite, doxorubicinol, and several cytotoxic aglycone metabolites (Ballet et al., 1987; Dodion et al., 1987). To undergo hepatic metabolism, drugs must first cross the hepatic sinusoidal endothelium to enter the space of Disse and gain access to the hepatocytes (Le Couteur et al., 2004b). Liver sinusoidal endothelial cells are highly specialized cells, lining the wall of the hepatic sinusoid, that separate the sinusoidal blood, derived primarily from the portal vein, from hepatocytes. Liver sinusoidal endothelial cells are perforated by fenestrations. Fenestrations are pores approximately 100 to 200 nm in diameter and grouped together in clusters known as liver sieve plates. Unlike other capillaries, fenestrations in these cells are true discontinuities in the endothelium, lacking either a diaphragm or underlying basal lamina. The cytoplasmic extensions of sinusoidal endothelial cells are extremely attenuated, with widths in the order of 100 to 300 nm (Braet and Wisse, 2002; Le Couteur et al., 2002). These morphological features facilitate the transfer of drugs and other xenobiotics from portal blood into hepatocytes (Le Couteur et al., 2004b). However, for larger substrates such as liposomes (Poste et al., 1982; Scherphof and Kamps, 2001) and lipoproteins (Le Couteur et al., 2002), the sinusoidal endothelium may act as a low-pressure ultrafiltration system, excluding the passage of larger particles on the basis of size and, in some cases, electrical charge. Thus, it has been suggested that the fenestrated endothelium influences the passage of

ABSTRACTIONS: AUC, area under the curve; MTT, mean transit time; V, volume of distribution; Q, portal flow rate; tC, catheter and nonexchanging vessel transit time; t1, time following injection corrected for tC; 1 + γ, ratio of the volumes of distribution of sucrose to doxorubicin; k1, rate constant for influx; k2, rate constants for efflux; k3, rate constant for sequestration; PS, permeability-surface area product.
liposomes including those carrying doxorubicin (Romero et al., 1999; Scherphof and Kamps, 2001).

Here, we investigated whether the fenestrated sinusoidal endothelium impairs the hepatic metabolism of liposomal doxorubicin by preventing the transfer of liposomes from the sinusoid into the extracellular space of Disse. This would provide a mechanism for the pharmacokinetic profile of liposomal doxorubicin and emphasize the importance of the sinusoidal endothelium in hepatic pharmacology more generally. We used the multiple indicator-dilution technique and electron microscopy because these are the most appropriate methods to study transport across the hepatic endothelium (Reichen, 1999).

Materials and Methods

Animals. Wistar male rats (aged 15–19 weeks, weight 435–480 g) were obtained from Animal Research Centre (Perth, Australia). The animals were allowed free access to water and commercial rat pellets. The study was approved by the Central Sydney Area Health Service Animal Ethics Committee.

Materials. 3H-H2O (1 mCi/ml), 14C-sucrose (0.1 mCi/ml), and bovine serum albumin were obtained from Sigma Chemical (Sydney, Australia). Pentobarbitone sodium (60 mg/ml) was obtained from Merial (Parramatta, Australia). Carbenog (95% O2/5% CO2) gas was obtained from BOC Gases (North Ryde, Australia). Doxorubicin hydrochloride injection (10 mg/5 ml) was obtained from Mayne Pharma Pty. Ltd. (Parkville, Australia). Liposomal doxorubicin hydrochloride (Caelyx) (20 mg/10 ml) was obtained from Schering-Plough Pty. Ltd. (Baulkham Hills, Australia).

Multiple Indicator-Dilution Experiments. Liver perfusions and multiple indicator-dilution experiments were performed as described previously (Le Couteur et al., 1993; Yang et al., 2001). Rats were anesthetized with pentobarbitone sodium (60 mg/kg i.p.). Through a midline laparotomy incision, the portal vein was cannulated with an 18 gauge intravenous cannula (Johnson and Johnson, Sydney, Australia), and the thoracic inferior vena cava was cannulated with a 10-cm length of polyethylene tubing (i.d. 1.4 mm, o.d. 1.9 mm). The perfusate was Krebs-Henselet bicarbonate buffer (10 mM glucose, pH 7.4, saturated with 95% O2/5% CO2, 1% bovine serum albumin, 37°C). The perfusate flow rate was maintained at approximately 1 ml/min/g of liver using a cartridge pump (Extech Equipment, Boronia, Australia) in a nonrecirculating system. The flow rate in milliliters per minute was determined volumetrically and converted to ml/min/g of liver once the experiment was completed and liver weight was established. Viability was confirmed by macroscopic appearance, oxygen consumption, portal venous pressure, and electron microscopic assessment of endothelial integrity and hepatocyte glycogen stores, and of mitochondrial morphology.

For the multiple indicator-dilution experiments, two different injectates were used: 1) doxorubicin (100 µl), 3H-H2O (2 µl), 14C-sucrose (2 µl); and 2) liposomal doxorubicin (100 µl), 3H-H2O (2 µl), 14C-sucrose (2 µl). Sucrose is used as a marker of the extracellular space and water is a marker of the perfused cellular and extracellular space. After administration of each injectate, 30 outflow samples were collected using a Universal Fraction Collector (Extech Equipment) at 2–5 intervals. Outflow samples were analyzed for 14C and 3H specific activity (PerkinElmer Liquid Scintillation Counter; PerkinElmer Life and Analytical Sciences, Sydney, Australia). Doxorubicin absorbance was determined using a UV-visible spectrophotometer (UV-1601; Shimadzu, Rydalnere, Australia) at 490 nm.

Dose-normalized outflow time-activity curves were constructed. The AUC and the area under the first moment of the curve were determined using the rectangular rule because the outflow was collected for the entire time period rather than at discrete time points. Monoeponential extrapolation was not used because the terminal activities were at or below the limits of quantification. The mean transit time (MTT) was estimated from the ratio of the area under the first moment of the curve and AUC. MTT was corrected for the catheter and nonexchanging vessel transit time (t0), estimated from the time of the first appearance of activity above background levels. The volume of distribution (V) was determined from the product of the MTT and the portal flow rate (Q). The volumes of distribution and recoveries of doxorubicin and liposomal doxorubicin were expressed as a fraction of the corresponding sucrose values in each experiment.

The distributed models developed by Goresky and colleagues (Goresky, 1963, 1984; Goresky et al., 1973) were used to model the outflow curves as described previously (Le Couteur et al., 1993; Yang et al., 2001). The sucrose curves ([C(sucrose)/(t)]) were regressed onto the liposomal doxorubicin curves ([C(liposomal-doxorubicin)/(t)]) where t′ is the time following injection corrected for t0, according to the equation for the Goresky model of flow-limited exchange (Goresky, 1963):

\[ C_{\text{sucrose}}(t) = \frac{1}{1 + \gamma} C_{\text{liposomal-doxorubicin}}(t') \]

to determine goodness of fit and the ratio of the volumes of distribution of sucrose to liposomal doxorubicin (1+γ).

The doxorubicin curves ([C(doxorubicin)/(t)]) were regressed onto the sucrose curves ([C(sucrose)/(t)]) according to a modification of the equation for the permeability-limited sequestration model developed by Goresky and colleagues (Goresky et al., 1973; Le Couteur et al., 1993):

\[ C_{\text{doxorubicin}}(t) = e^{-kt_{1}} C_{\text{sucrose}}(t) + e^{-kt_{2}} \int_{0}^{\infty} e^{-(t-t')} \cdot C_{\text{sucrose}}(t') \cdot \frac{k_{2}}{t_{2}} \cdot e^{-kt_{2}} \cdot t' \cdot e^{-t'} dt' \]

The values estimated by the curve-fitting process were k1 and k2, the rate constants for the influx and efflux of doxorubicin, respectively, and A, the rate constant for sequestration. The other parameters in this equation are: t′, the time following injection corrected for t0, which was estimated from the time of the first appearance of indicators; A, a dummy variable of integration; and \( t_{2} \), a modified first-order Bessel function. The regression equations were written in QBASIC by Dr. Laurent Rivory (Johnson and Johnson Research, Sydney, Australia) as described previously (Le Couteur et al., 1993) (see Acknowledgments). The permeability-surface area product (PS) for the influx (PS\(_{\text{influx}}\)) of doxorubicin across the hepatocyte membrane from the space of Disse into the hepatocyte was determined from the product of the observed extracellular volume (V\(_{\text{extracellular}}\)) and k1. The PS for efflux (PS\(_{\text{efflux}}\)) was determined from the product of k2 and the observed intracellular volume (V\(_{\text{water}/V_{\text{succrose}}}\)).

Transmission Electron Microscopy. After completion of the multiple indicator-dilution experiments, liver specimens were fixed for light microscopy in 4% buffered paraformaldehyde and for electron microscopy with 2% glutaraldehyde/3% paraformaldehyde in 0.1 M sodium cacodylate buffer (0.1 M sucrose, 2 mM CaCl\(_{2}\)).

Randomly selected specimens were embedded with Spurr’s resin. Ultrathin (70–90 nm) sections were cut, placed on copper grids, and stained with uranyl acetate and lead citrate. Sections were examined at magnification 10000 to 80000X using a Philips CM 12 and a CM 120 transmission microscope. In each of the sections, representative fields showing sinusoids and perfused particles were photographed. Image analysis was performed using ScanPro (SPSS Inc., Chicago, IL).

Statistics. The results are expressed as mean ± standard deviation. Comparisons of the pharmacokinetic data for preparations of doxorubicin hydrochloride in 4% buffered paraformaldehyde and for electron microscopy with 2% glutaraldehyde/3% paraformaldehyde in 0.1 M sodium cacodylate buffer (0.1 M sucrose, 2 mM CaCl\(_{2}\)).

Results

Recovery and Volume of Distribution. Figure 1 shows the outflow curves from experiments with liposomal doxorubicin (Fig. 1A) and doxorubicin (Fig. 1B) performed in the same perfused liver. In both experiments the marker of the extracellular space is 14C-sucrose. The two doxorubicin curves are very different. The liposomal doxorubicin curve precedes that of the sucrose, indicating that it has distributed into a smaller volume of distribution than the extracellular space. The AUC is similar to that of sucrose, indicating that there has not been substantial extraction. On the other hand, the outflow curve for free doxorubicin is delayed compared with sucrose, indicating that doxorubicin has distributed into the cellular space. The AUC is less...
than that of sucrose, indicating that there has been sequestration of doxorubicin during a single pass through the liver.

Table 1 shows the volumes of distribution of each formulation of doxorubicin as a fraction of the extracellular sucrose space, and of the total cellular and extracellular water space. Doxorubicin had a volume of distribution between that of sucrose and water, whereas the liposomal doxorubicin had a volume of distribution smaller than that of sucrose and water. The volume of distribution of liposomal doxorubicin was $0.56 \pm 0.30$ times smaller than the sucrose volume compared with that of doxorubicin, which was $1.56 \pm 0.45$ times larger than the sucrose volume ($P < 0.001$). The ratio of the mean fractional recovery of doxorubicin was $70 \pm 24\%$ and was significantly smaller than that of liposomal doxorubicin, which was $94 \pm 17\%$ ($P > 0.05$).

**Physiological Modeling.** The flow-limited model fitted the sucrose-liposomal doxorubicin curves very well (Fig. 2A) and returned a fitted value of $1 + \gamma$ of $1.33 \pm 0.16$ ($n = 6$), with a sum of the squares value of $0.02 \pm 0.01$. The curve-fitting failed for one of the seven replicate experiments.

The permeability-limited sequestration model fitted the doxorubicin curves with a sum of the squares value of $3 \pm 1$ ($n = 4$). The values of $k_1$, $k_2$, and $k_3$ were $0.24 \pm 0.06$ s, $0.40 \pm 0.16$ s, and $0.08 \pm 0.14$ s, respectively. The estimated values for the $PS_{influx}$ and $PS_{efflux}$ of doxorubicin across the hepatocellular membrane were $0.02 \pm 0.01$ ml/s/g and $0.03 \pm 0.02$ ml/s/g, respectively.

**Transmission Electron Microscopy.** Transmission electron microscopy was performed on random samples from four rat livers that had been perfused with both formulations of doxorubicin. The fenestrated sinusoidal endothelium was clearly apparent, perforated with fenestrations approximately 100 nm in diameter (Fig. 3). Liposomes ($n = 290$) were found only in the sinusoidal lumen and none were seen in the space of Disse (Fig. 3). Interaction of the liposomes with Kupffer cells was observed but was rare (Fig. 4).

**Discussion**

The formulation of doxorubicin in pegylated liposomes has a dramatic effect on systemic clearance. In rats, for example, the systemic clearance of free doxorubicin was 121 ml/h/kg and was reduced to 2.0 ml/h/kg after administration of doxorubicin in the pegylated liposomal formulation at a similar dose (Working and Dayan, 1996). Free doxorubicin is metabolized in the liver to doxorubicinol and aglycones and is excreted extensively into the bile by various transporters including P-glycoprotein (Ballet et al., 1987; Booth et al., 1998), whereas liposomal doxorubicin is thought to be protected from hepatic metabolism and instead is taken up by reticuloendothelial cells (Gabizon et al., 2003).

The hepatic disposition of liposomes has been studied and is of considerable significance for the pharmacokinetics of pharmacotherapies delivered using liposomes (Romero et al., 1999; Scherphof et al., 1999).
Phosphatidylserine was not found to alter fenestrations. It was concluded that but were taken up by isolated hepatocytes in vitro. Furthermore, phosphatidylserine-riched with phosphatidylserine), large solid liposomes (298 nm, injected intravenously with large fluid liposomes (337 nm, en-

Large solid liposomes were not taken up by hepatocytes in vivo, deformed. S, sinusoidal lumen; E, sinusoidal endothelium.

We found that the hepatic extraction of liposomal doxorubicin was accounted for some of the absorbance. However, studies on the hepatic disposition of doxorubicin in the isolated perfused rat liver performed over 120 min found that only 15% of the doxorubicin was metabolized to doxorubicinol (Booth et al., 1998); therefore, in our very short duration experiments, the absorbance reflects only the parent compound. It was found that the liposomal doxorubicin formulation had a dramatically reduced volume of distribution in the liver compared with free doxorubicin and was essentially restricted to the vascular space. The hepatic volume of distribution of liposomal doxorubicin was only 56 ± 30% of that of the extracellular marker, sucrose, and on electron microscopy, liposomes were only seen in the vascular space. On the other hand, the hepatic volume of distribution of doxorubicin was 156 ± 45% of that of the extracellular marker and 73 ± 15% of that of water, indicating that doxorubicin enters the cellular space. Likewise, the volume of distribution in the whole body is reduced and the reported effect is dramatic. In the rat, the volume of distribution of free doxorubicin was 5070 ml/kg, whereas the volume of distribution of liposomal doxorubicin was only 65 ml/kg (Working and Dayan, 1996). In the liver, it would be expected that the restriction of the liposomal doxorubicin to the vascular space should impair hepatic metabolism because the doxorubicin is unable to gain access to the hepatocytes.

Accordingly, we found that the recovery of doxorubicin was lower for free doxorubicin (70 ± 24%) compared with liposomal doxorubicin (90 ± 17%, P < 0.05), indicating that hepatic sequestration is impaired by the liposomal formulation. The value for the recovery of free doxorubicin that we calculated is similar to that described in a previous perfused rat liver study of about 0.76 (Ballet et al., 1987). The major route of sequestration of free doxorubicin in the perfused rat liver is biliary excretion via P-glycoprotein, with a small fraction metabolized to doxorubicinol (Ballet et al., 1987; Booth et al., 1998). We found that the hepatic extraction of liposomal doxorubicin was small. Doxorubicin is tightly bound to liposomes, and any metabolism...
is thought to be mediated primarily by reticuloendothelial cells within the vascular space (Wisse et al., 1976; Gabizon et al., 2003). We studied pegylated liposomes, and pegylation reduces uptake of liposomes by Kupffer cells and other macrophages. Kupffer cells play a major role in clearing particulate materials, including those with a size range similar to that of liposomal doxorubicin, from the circulation (Ogawara et al., 1999). Interaction of Kupffer cells with liposomes was observed on electron microscopy in our experiments. Thus, the minor extraction of liposomal doxorubicin that we detected may be secondary to uptake by Kupffer cells.

The transport of the doxorubicin hydrochloride across the hepatocyte membrane was symmetrical and rapid, which is consistent with diffusion. The rate constants for the hepatocellular membrane influx and efflux derived using the physiological model of Goresky and colleagues (Goresky, 1984) were 0.24 ± 0.06 s and 0.40 ± 0.16 s, respectively. The PS products calculated from these rate constants for influx and efflux were similar (0.02 ± 0.01 and 0.03 ± 0.02 ml s/g), indicating symmetrical transfer, and consistent with diffusion. The transfer of doxorubicin across erythrocyte membranes has been demonstrated to occur via simple diffusion with a permeability coefficient of 2.4 × 10⁻⁵ cm/s (Dalmark and Storm, 1981). If it is assumed that the sinusoidal surface area is 5900 cm²/g (Blouin et al., 1977), then the permeability coefficient for doxorubicin transfer across the hepatocyte membrane is likely to be in the order of 10⁻⁴ cm/s in the intact liver from our results. However, transfer is highly dependent on pH, temperature, and concentration, which are likely to differ substantially between these experimental models (Dalmark and Storm, 1981). In a recirculating perfused liver model using a compartmental analysis, Booth et al. (1998) reported that the first-order rate constant for the sinusoidal uptake of doxorubicin was 7.22 min⁻¹. More recently, Rivory et al. (1996) have reported the uptake of doxorubicin in isolated rat hepatocytes. In these experiments, the rate constant for the uptake of doxorubicin was 0.7 ± 0.3 s⁻¹, which is similar to our result in the intact liver.

Liposomal doxorubicin was restricted to the vascular space. The mostly likely mechanism is that the liposomes are unable to pass through the fenestrations in the sinusoidal endothelium, presumably on the basis of their size, charge, and deformability. This is supported by the absence of any liposomes seen within the space of Disse on transmission electron microscopy. The stated diameter of liposomal doxorubicin (Caelyx) is around 100 nm, although on transmission electron microscopy, we saw many liposomes that were significantly larger. The mean diameter of fenestrations in the hepatic sinusoidal endothelium is around 100 to 200 nm in rats, humans, and nonhuman primates (Le Couteur et al., 2001; Cogger et al., 2003; McLean et al., 2003); therefore, the liposomes would be unable to pass on the basis of their size. Furthermore, the composition of pegylated liposomal doxorubicin (hydrogenated soy phosphatidylcholine, cholesterol, and distearoyl-phosphatidylethanolamine conjugated to polyethylene glycol) (Gabizon et al., 2003) does not facilitate crossing the sinusoidal fenestrations because saturated lipids, such as the distearoyl-phosphatidylethanolamine, are rigid and difficult to deform (Poste et al., 1982; Scherphof and Kamps, 2001). In general, it has been observed that passage of particles through pores in membranes ceases when the radius of the particle exceeds 30% of the radius of the pore (Le Couteur et al., 2004a), which, if applicable to the sinusoidal endothelium, indicates that even much smaller liposomes would be excluded from the space of Disse for subsequent hepatic metabolism.

Liposomal formulations of pharmaceutical agents have been developed and introduced to clinical practice, particularly in cancer chemotherapy, where liposomal doxorubicin is widely administered. Our results indicate that free doxorubicin transfers symmetrically and rapidly across the hepatocellular membrane, whereas liposomal doxorubicin is restricted to the vascular space, thus impairing opportunity for hepatic metabolism. Liposomes are unable to pass through the fenestrations within the hepatic sinusoidal endothelium, emphasizing the importance of the endothelial barrier for hepatic metabolism. The characteristics of the hepatic endothelial fenestrations are an important aspect in understanding the pharmacology of, and hence the formulation of, liposomal medications.

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