ABSTRACT:

Cosalane is a potent inhibitor of HIV replication with a broad range of activity. In this study, the hepatic disposition of cosalane was investigated with a noncirculating isolated perfused rat liver technique. When 6 μM cosalane was infused into livers from untreated rats, the drug was highly extracted by the liver (only 2.5% of influent cosalane concentration appeared in the effluent perfusate). Pretreatment of rats with various inducers of cytochrome P-450 before perfusion neither altered the effluent cosalane concentration nor resulted in the appearance of detectable metabolites in the effluent perfusate or liver homogenates. Hepatic uptake of cosalane was negligible when the drug was infused in the presence of BSA, and infusion of albumin after cosalane resulted in a significant displacement of the drug into the effluent perfusate. Furthermore, permeabilization of perfused livers with digitonin significantly diminished effluent cosalane concentration while enhancing cosalane uptake by the liver. Based on our data, it appears that a significant proportion of cosalane does not penetrate the hepatocyte membrane and may accumulate in the lipid bilayer of the cell membrane. This finding supports the proposed mechanism explaining the antiviral effect of cosalane which stipulates that this compound appears to imbed perpendicularly in the lipid bilayer of the cell membrane and the viral envelope. Also, cosalane does not seem to be metabolized by the liver as evidenced by the lack of detectable metabolites in the effluent perfusate, liver homogenates, and liver microsomal incubations.

DISPOSITION OF COSALANE, A NOVEL ANTI-HIV AGENT, IN ISOLATED PERFUSED RAT LIVERS

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Materials and Methods

Chemicals. Cosalane disodium salt [1,1-di(3’-sodium carboxy-5’-chloro-4’-hydroxyphenyl)-4(3β-cholestanyl-1-butenyl) and dihydrocosalane were kindly supplied by Professor Mark Cushman, Purdue University (West Lafayette, IN). 3-Methylcholanthrene, clofibrate, digoxin, glucose 6-phosphate, glucose 6-phosphate dehydrogenase, and NADP were obtained from Sigma Chemical Co. (St. Louis, MO). HPLC-grade solvents used for HPLC analysis were obtained from Fisher Scientific Co. (Pittsburgh, PA). All other chemicals were of analytical grade and were used as received from Sigma.

Animals. Adult male Sprague-Dawley rats (Sasco, Inc., Boston, MA) weighing 180 to 225 g were used in all experiments. Rats had access to regular laboratory chow, and water was allowed ad libitum. Where indicated, animals were treated with 3-methylcholanthrene (80 mg/kg i.p.) or clofibrate (250 mg/kg intragastrically) 3 or 2 days, respectively, before the experiment. Other
animals received phenobarbital (1 mg/ml) in drinking water for 7 days or dihydrocosalane (10 mg/kg/day i.p.) for 3 days before experiments.

**Liver Perfusion.** Animals were anesthetized with pentobarbital (60 mg/kg i.p.), and the portal vein and vena cava were cannulated for inflow and outflow of the perfusate, respectively. Livers were then perfused in a noncirculating system using Krebs-Henseleit bicarbonate buffer (pH 7.4) maintained at 37°C and saturated with an oxygen-carbon dioxide mixture (95:5). After an equilibrium period of 10 to 15 min with buffer, cosalane (3–24 μM) was infused (15–40 min) in Krebs-Henseleit buffer alone or with BSA as indicated in the figure legends. In some experiments, BSA was infused for 15 min after the termination of the cosalane infusion. In other experiments, livers were permeabilized by brief infusion (2 min) of digitonin in Krebs-Henseleit buffer (0.3 mg/ml) before the infusion of cosalane. Samples of effluent perfusate were collected for analysis of cosalane and possible metabolites. At the end of the perfusion experiment, the liver was blotted dry, weighed, and stored at −80°C for the determination of cosalane liver content and possible metabolites in the liver.

**Microsomal Incubations.** Male Sprague-Dawley rat liver microsomes were obtained from Xenotech (Kansas City, KS). Microsomes (1 mg/ml) were incubated at 37°C in 5-ml (final volume) incubation mixture containing potassium phosphate buffer (50 mM, pH 7.4), MgCl2 (3 mM), EDTA (1 mM), NADP (1 mM), glucose 6-phosphate (5 mM), glucose 6-phosphate dehydrogenase (1 U/ml), and cosalane (10–100 μM). Reactions were initiated by the addition of NADPH-generating system. Aliquots (100 μl) were withdrawn at predetermined time intervals and the reaction was stopped by the addition of 100 μl of a mixture of chilled methanol and acetonitrile (5:4). The precipitated protein was separated by centrifugation at 5000g for 5 min at 4°C. The supernatant was stored at −80°C for analysis of cosalane and possible metabolites by HPLC.

**HPLC Analyses.** The liver extracts, and effluent perfusate samples were analyzed for cosalane and possible metabolites according to a published method (Venkatesh et al., 1996), with a slight modification. A fluorescence detector (Schoeffel model 970, McPherson, Chelmsford, MA) (λem 450 nm; λex 230 nm; exc 230 nm; exc 230 nm; exc 230 nm) set at 254 nm was also connected in series to detect any cosalane metabolites that may not possess fluorescence properties. The mobile phase [methanol-tetrahydrofuran-phosphoric acid (74:25:1, v/v/v)] was pumped at a flow rate of 1 ml/min through a reversed phase column packed with C18 (Luna, 5 μm; 4.6 mm i.d. × 25 cm; Phenomenex, Torrance, CA) at room temperature.

To 100-μg samples of thoroughly minced livers, 900 μl of a mixture of methanol + acetonitrile (5:4) were added. Samples were then homogenized (Tissue Tearer; Biospec, Bartlesville, OK), vortexed, and centrifuged (TL 100, Beckman) at 16,000g for 15 min at 4°C. The supernatant was diluted appropriately and 20 μl was injected onto HPLC for the analysis of cosalane and/or metabolites. Samples (100 μl) of the effluent perfusate were directly injected into the HPLC column.

**Determination of Lactate Dehydrogenase (LDH)** in Effluent Perfusate. LDH released into the effluent perfusate was quantitated as described previously (Belinsky et al., 1986). Briefly, samples of the effluent perfusate were added to a 50 mM triethanolamine buffer (pH 8.0) containing pyruvic acid (1 mM) and NADH (140 μM). The disappearance of NADH was monitored spectrophotometrically at 340 nm. One unit of enzyme activity equals 1 μmol of NADH oxidized per minute.

**Statistical Analysis.** The Student’s t test was applied to evaluate the significance of differences observed.

**Results**

**Effect of Metabolizing Enzyme Inducers on the Disposition of Cosalane.** When 6 μM cosalane was infused into livers of untreated rats, the effluent cosalane concentration reached steady-state levels within 5 to 10 min (Fig. 2). No cosalane metabolites were detected in the effluent perfusate. Pretreatment of rats with various inducers of cytochrome P-450 did not alter effluent cosalane concentrations, nor did it result in the appearance of any detectable metabolites either in the effluent or in the liver homogenates. In livers from rats pretreated with dihydrocosalane, a higher steady-state cosalane concentration (0.25 μM) was detected in the effluent perfusate (Fig. 2). Again, no cosalane metabolites were detected in the effluent perfusate or homogenates of livers from dihydrocosalane-treated rats. Furthermore, no detectable metabolites were found when cosalane was incubated with rat liver microsomes (results not shown).

**Influence of Hepatocyte Permeation on Liver Cosalane Uptake.** Cosalane was not detected in the effluent perfusate when a 3 μM concentration of the drug was infused (Fig. 3A). Step-wise increases in the influent cosalane concentration resulted in corresponding increases in the drug concentrations appearing in the effluent perfusate (Fig. 3A). However, no cosalane metabolites were detected in the perfusate at any infused cosalane concentration. Brief pretreatment of perfused livers with digitonin resulted in hepatocyte permeation, as evidenced by the release of LDH into the effluent perfusate (Fig. 3B). In permeabilized livers, cosalane virtually disappeared from the effluent perfusate at all levels of influent cosalane concentrations (Fig. 3A). Concomitantly, permeabilized livers retained significantly higher cosalane concentrations compared with control livers (Fig. 3A, inset).

**Role of Protein Binding in Hepatic Handling of Cosalane.** The presence of BSA with cosalane in the influent perfusate totally inhibited cosalane uptake by the liver, resulting in the appearance of nearly 100% of the infused cosalane in the effluent perfusate (Fig. 4A). This effect was mirrored by the virtual absence of cosalane in homogenates of livers perfused with the drug in the presence of BSA (Fig. 4B).

When 6 μM cosalane was infused into livers from untreated rats, steady-state levels of 0.15 μM effluent cosalane concentrations were attained within 5 min (Fig. 5). Subsequent infusion of BSA resulted in a remarkable, immediate increase (7-fold) in the effluent cosalane concentration, which remained significantly elevated for the entire
duration of the BSA infusion, but gradually declined upon its termination (Fig. 5).

Discussion

In a concerted and intensified effort to prevent and treat AIDS, various vaccines and novel antiviral agents are currently at different stages of development. Among these novel compounds is cosalane (Fig. 1), which is obtained by attaching a cholestane moiety to a dichlorodisalicylmethane by a three-carbon linker chain (Cushman et al., 1994).

Data available in the literature on the disposition of cosalane are very limited. A report shows that cosalane has a poor renal excretion in mice (Bigelow et al., 1994). Similarly, both renal and biliary excretion of cosalane are very low in rats, and this drug accumulates to a significantly high level in the liver (C.U. and A.K.M., unpublished data, 1998). Consequently, this study was designed to investigate hepatic handling of cosalane by using the noncirculating perfused rat liver technique.

Perfused livers from untreated rats extracted more than 97% of infused cosalane, with steady-state effluent cosalane concentrations attained within 5 to 10 min after the commencement of the cosalane infusion (Fig. 2). This was not surprising due to the known extreme lipophilicity (log octanol/buffer coefficient $8.8 \times 10^6$) of this compound (Venkatesh et al., 1996). Because no metabolites were detected in either the effluent perfusate or liver homogenates of control rats, various hepatic cytochrome P-450 isozymes, which may potentially metabolize cosalane, were induced before liver perfusion experiments. Phenobarbital, 3-methylcholanthrene, and clofibrate, which are known inducers of cytochrome P-450 3A4, 1A1, and 4A1, respectively, also failed to yield any detectable traces of cosalane metabolites in the effluent perfusate or liver homogenates. To examine the possibility that cosalane might induce its own metabolism, cosalane was infused into livers from rats pretreated with dihydrocosalane (Fig. 2). Dihydrocosalane is a cosalane congener in which the double bond attached to the central carbon of dichlorodisalicylmethane fragment of cosalane is saturated. Dihydrocosalane was chosen over cosalane itself as a possible inducer of cosalane metabolism to avoid potential leakage of liver-accumulated drug into the effluent perfusate, thus
artificially contributing to the effluent cosalane concentration. Again, no metabolites were detected in the effluent perfusate or in liver homogenates from dihydrocosalane-pretreated rats. Interestingly, the steady-state cosalane effluent concentration was higher in livers from dihydrocosalane-pretreated rats compared with all other groups (Fig. 2). This may be due to partial saturation of cosalane binding sites by dihydrocosalane. Incubations of effluent perfusate and liver homogenates with β-glucuronidase or sulfatase, or under mild and strong alkaline conditions, did not reveal the existence of any cosalane conjugates (not shown).

It has been proposed that the hydrophobic steroid moiety of cosalane may imbed perpendicularly in the lipid bilayer of the cell membrane and the viral envelope, with the dichlorodisalicylmethane fragment pointing outward in an obstructive mode (Cushman et al., 1995). Such an interaction with the cell membrane and viral envelope lipid bilayers may inhibit the fusion of the viral envelope with the cell membrane, contributing to the antiviral effect of cosalane (Cushman et al., 1994). Because this interaction would be expected to interfere with the ability of the drug to cross the hepatocyte membrane, we evaluated the impact of hepatocyte permeabilization on hepatic uptake of cosalane. Very low concentrations of digitonin are known to permeabilize hepatocytes without causing cell death (Boon and Zaman, 1988). This technique has been very valuable in obtaining measurements of transport parameters in intracellular organelles while they are present in their physiological milieu (Fiskum and Lehninger, 1980). It also facilitates electron microscopic visualization of certain intracellular structures, particularly the cytoskeletal network of filaments (Fiskum et al., 1980). Permeabilizing liver cells with digitonin resulted in a significant enhancement in hepatic cosalane uptake (Fig. 3A, inset) and significantly lowered effluent cosalane concentrations (Fig. 3A), suggesting that intact hepatocyte cell membrane may indeed act as a barrier preventing cosalane from crossing into the cell interior. Fractionation of homogenates of cosalane-infused rat livers revealed the localization of this drug in the cell membrane fraction, giving additional support for this conclusion. Despite enhanced hepatic cosalane uptake in permeabilized livers, no metabolites were detected in the effluent perfuse or liver homogenates in these experiments. The absence of detectable cosalane metabolites upon incubating the drug with rat liver microsomes in vitro suggests that cosalane is not readily metabolized by the rat liver. Binding cosalane to BSA virtually abolished hepatic uptake of this compound (Fig. 4). This may be due to very tight binding of cosalane to albumin, which in turn hinders its release for cellular uptake. Furthermore, infusion of BSA into livers after administration of cosalane extracted the compound from its “binding sites” in the liver extracellular space (Fig. 5).

Because most drugs enter the hepatocyte by passive diffusion, hepatic uptake is expected to depend on the lipophilicity of the compound of interest (Rowland and Tozer, 1989). Although increasing lipophilicity of drugs is known to enhance their cellular uptake, compounds that are extremely lipophilic (log octanol/buffer coefficient > 5) have a diminished capacity to cross biological membranes (Wils et al., 1994). This finding may explain the apparent inability of cosalane, which has a log octanol/buffer coefficient of 8.8 × 10^{-6}, to gain access to the hepatocyte interior. This conclusion is further supported by the inability of cosalane to inhibit viral protease and reverse transcriptase in infected cells, suggesting that cosalane fails to permeate the cell membrane and enter cells (Cushman et al., 1994).

In conclusion, cosalane represents a new class of anti-HIV compounds with a long plasma half-life and clear resistance to metabolism. Although cosalane may have a limited potential to treat intracellularly sequestered HIV, this compound may be a valuable candidate in the defense against new waves of cell invasion by HIV.